Use of high-rate filamentous algal ponds for primary municipal wastewater treatment

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This thesis is dedicated to my Koro, Reginald Joseph O'Brien.

May you forever be by the sea.

Abstract

High-rate filamentous algal pond (HRFAP) systems offer a promising alternative to conventional municipal wastewater treatment. Research on selecting filamentous algal species for municipal wastewater bioremediation is currently limited. Chapter 2 introduces a screening protocol aimed at identifying robust cultivars suitable for HRFAP monoculture systems. Evaluating eleven cultivars under local seasonal ambient and extreme conditions played a crucial role in cultivar selection. Based on their consistent biomass productivity and bioremediation performance across ambient and extreme conditions, *Klebsormidium* sp. *(KLEB B)*, *Stigeoclonium* sp. *(STIG A)* and *Ulothrix* sp. were identified as target cultivars for nutrient bioremediation of primary municipal wastewater.

The identification of target cultivars has previously been based on laboratory conditions, which are insufficient for practical applications. Chapter 3 assessed the biomass productivity and nutrient bioremediation performance of three cultivars - *Klebsormidium flaccidum*, *Oedogonium calcareum*, and *Oedogonium* sp. – in outdoor HRFAP mesocosms. *K. flaccidum* had the highest biomass productivity and bioremediation performance, while *O. calcareum* had complete die-off. Competition experiments at varying stocking densities highlighted *K. flaccidum* dominance at lower densities (0.25 and 0.5 g FW L^{-1}), positioning it as the preferred cultivar for nutrient bioremediation in primary municipal wastewater within HRFAP systems.

Effective management of operational parameters is crucial for optimising wastewater treatment in HRFAP systems. Therefore, in Chapter 4 the effects of hydraulic retention time (HRT), stocking density, and harvest frequency on the growth and nutrient bioremediation performance of *K. flaccidum* in primary municipal wastewater in outdoor HRFAPs were examined during summer and winter. Seasonal conditions impacted biomass productivity, which was 48.3% higher in summer compared to winter. A HRT of 4 days was optimal for both seasons based on bioremediation of total ammoniacal-nitrogen (TAN). Lower stocking densities of 0.25 and 0.5 g FW L^{-1} demonstrated enhanced bioremediation efficiency, while higher densities were preferable during slower growth periods to mitigate potential toxicity risks from primary wastewater. Harvest frequencies of two, four and six days did not significantly affect nutrient removal rates across different treatments and seasons. These

results highlight the importance of seasonal optimisation of HRFAP systems to maximise biomass production and nutrient bioremediation.

Wastewater treatment plants (WWTPs) are major sources of per- and polyfluoroalkyl substances (PFAS) pollution entering the environment. Certain algal species have demonstrated the ability to bioaccumulate PFAS compounds, indicating their potential for removing PFAS from wastewater. Therefore, in Chapter 5 a laboratory study was conducted to assess the ability of *K. flaccidum* to reduce concentrations of PFAS and PFAS precursors in primary municipal wastewater under two HRTs. *K. flaccidum* maintained stable productivity in the wastewater. Removal rates of PFAS and PFAS precursors, however, varied considerably. Specifically, reductions were observed in three individual PFAS and in all measured PFAS precursors present in the wastewater. Despite these reductions, PFAS was not detected in the algal biomass of *K. flaccidum*, making it suitable for a range of biomass applications provided it remains free of other contaminants.

Overall, this thesis has demonstrated that HRFAP systems are an effective alternative treatment for nutrient reduction in primary municipal wastewater. Application of the screening protocol to select target species, and seasonal optimisation of HRFAP operating parameters will enable more consistent and effective year-round nutrient bioremediation and algal biomass productivity to be achieved.

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Chapter 2

Indira N. Novak: Methodology, Investigation, Data curation, Formal analysis, Writing – original draft.

Rebecca J. Lawton: Supervision, Conceptualization, Writing – review and editing, funding.

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List of abbreviations

Abbreviation	Full description
6:2 FTS	1H,1H,2H,2H-Perfluorooctane sulfonate
ATS	Algal Turf Scrubbers
BOD	Biochemical Oxygen Demand
C	Carbon
CO_2	Carbon dioxide
CO_3^{2-}	Carbonate
DRP	Dissolved reactive phosphorus, the readily available fraction that can stimulate problem growths of algae and water plants
DW	Dry Weight
E. coli	*Escherichia coli*
F	Fluorine
FW	Fresh Weight
HCO_3^-	Bicarbonate
HRAP	High-rate Algal Pond
HRFAP	High-rate Filamentous Algal Pond
HRT	Hydraulic retention time- the average time of water flowing through or retained within a treatment system
N	Nitrogen
NH_3	Ammonia
NH_4	Ammonium
NIWA	National Institute of Water and Atmospheric Research
NO_2-N	Nitrite
NO_3-N	Nitrate
O_2	Oxygen
P	Phosphorus
PFAS	Perfluoroalkyl and polyfluoroalkyl substances
PFBA	Perfluorobutanoic acid
PFBS	Perfluorobutane sulfonate
PFHpA	Perfluoroheptanoic acid
PFHpS	Perfluoroheptane sulfonate
PFHxA	Perfluorohexanoic acid

PFHxS	Perfluorohexane sulfonate
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
PFPeA	Perfluoropentanoic acid
PFPeS	Perfluoropentanesulfonic acid
TAN	Total Ammoniacal-Nitrogen
TN	Total Nitrogen
TOPA	Total Oxidizable Precursor Assay
TP	Total Phosphorus
UV	Ultraviolet
WWTP	Wastewater Treatment Plant

Chapter 1 - General introduction

1.1 Nutrient enrichment of freshwater and marine environments

Eutrophication resulting from anthropogenic nitrogen (N) and phosphorus (P) inputs is one of the greatest stressors to freshwater and marine ecosystems worldwide (Häder et al., 2020; Malone & Newton, 2020; Li et al., 2021a). Eutrophication of rivers, lakes, and oceans, can result in algal blooms which deplete oxygen within the water column when they decay, creating hypoxic or dead zones ultimately leading to a decline in ecosystem health (Damania et al., 2019; Richardson et al., 2019; Wurtsbaugh et al., 2019). Globally the occurrence of dead zones (hypoxic and anoxic) in coastal areas is estimated to have increased from < 5 prior to 1940 to ~ 700 today (Vaquer-Sunyer & Duarte, 2008; Altieri & Diaz, 2019). Eutrophication of lakes and streams is estimated to cost US$2.4 billion annually in the United States and eutrophication of coastal waters is estimated to cost US$1 billion annually in Europe (Davidson et al., 2014). These economic costs are due to a decline in coastal property values, tourism and recreational sectors, food security, human health and the advanced treatment of drinking water required to eliminate excess N levels, and the presence of harmful algal blooms (Wurtsbaugh et al., 2019; Gobler, 2020).

Sources of nutrients, primarily N and P, entering aquatic environments include agriculture, forestry, urbanisation, industrial activities, the draining of wetlands, coastal aquaculture, and the increased burning of fossil fuels (Wurtsbaugh et al., 2019; Bonsdorff, 2021). As the global population continues to grow and cities expand, the threat of nutrients leaching from underground septic tanks, stormwater runoff, human sewage and wastewater discharges is increasing (Peters & Meybeck, 2000; Damania et al., 2019). Furthermore, the global production of food has led to agriculture becoming a primary contributor to eutrophication due to the increased use of inorganic fertilisers and disposal of manure (Mueller et al., 2012; Mateo-Sagasta et al., 2018; Ward et al., 2018; Huang et al., 2020). Nutrient inputs enter freshwater and marine waters as non-point (diffuse) sources or point sources (Malone & Newton, 2020). Non-point sources such as nutrients from agricultural activities are difficult to mitigate as the source is not directly treatable. However, point sources such as municipal wastewater treatment plant (WWTP) effluent are confined and

therefore adequate treatment of these discharges can provide an effective approach towards reducing eutrophication (van Puijenbroek et al., 2019; Wurtsbaugh et al., 2019).

1.2 Municipal wastewater treatment plants

Municipal wastewater treatment processes were established in the early 20th century however, industrialisation, emerging contaminants and population growth have meant that current processes are now outdated (Kehrein et al., 2020). Conventional municipal wastewater treatment consists of a preliminary treatment, primary settling where solid materials are removed, sludge processing, secondary treatment utilising aerobic microbial organisms to further reduce dissolved organic substances, secondary clarification, tertiary treatment (nutrient removal), and disinfection with the aim to reduce pathogenic bacteria and viruses in the final discharged effluent (Neveux et al., 2016; Quach-Cu et al., 2018; Kim et al., 2019). Despite these treatment processes, pollutants such as nutrients (van Puijenbroek et al., 2019), pathogens (Cai & Zhang, 2013; Jäger et al., 2018), heavy metals and emerging contaminants (e.g. endocrine disruptors, personal care products, pharmaceuticals, microplastics) (Schwarzenbach et al., 2006; Tran et al., 2018; Meng et al., 2020) remain within the discharged effluent and enter into the environment (Pagilla et al., 2006; Ben et al., 2018; van Puijenbroek et al., 2019).

Municipal WWTP effluent is considered a major point source contributor of nutrients entering the aquatic environment (Carey & Migliaccio, 2009; Martí et al., 2009; Hu et al., 2012; van Puijenbroek et al., 2019). Insufficient removal of nutrients from WWTP effluents combined with an increasing global population have contributed to the increase in nutrients entering the aquatic environment (van Puijenbroek et al., 2019). This increased nutrient load is expected to affect the biological integrity of freshwater and marine ecosystems and the services they provide (Smith, 2003; Valero-Rodriguez et al., 2020; El-Sheekh et al., 2021). There is also concern regarding the concentrations of pathogenic bacteria in municipal wastewater effluents as WWTPs have been identified as a significant source of waterborne pathogens in natural environments (Wen et al., 2009; Tyagi et al., 2011; Ajonina et al., 2015; Al-Gheethi et al., 2018). Contaminants held within WWTP effluent not only enter into the environment as a result of direct discharges to freshwater and marine surface waters but also through the reuse of effluent by application to land (Chakravarthy et al., 2019; Adhikari &

Fedler, 2020; Baawain et al., 2020). Consequently, there is a need to treat the pathogens present within WWTP effluents to prevent the spread of waterborne illness and disease (Zanetti et al., 2010; Li et al., 2013; Zhi et al., 2020).

1.2.1 New Zealand wastewater treatment plants

Municipal wastewater management for many New Zealand communities is under review as The National Policy Statement for Freshwater Management 2020 (Freshwater-NPS) has reduced the concentration of nutrients permitted to enter freshwater environments (New Zealand Government, 2020). Changes in the environmental regulatory framework to reduce WWTP nutrient discharges is motivated by greater public awareness of water quality issues and an increasing demand on water use and recreation (Lowe et al., 2013; Kirk et al., 2020). It is expected that 35% of all WWTPs in New Zealand will undertake a consent renewal process in the next 10 years (required for their continued operation and discharge of effluent to aquatic receiving environments) and reduced nutrient standards will apply. This will require WWTPs to revise their current treatment technologies to adhere to the revised acceptable minimum nutrient concentrations of the Freshwater-NPS (GHD & Boffa Miskell, 2019).

Wastewater treatment in New Zealand was originally influenced by the need to protect public health while environmental effects (e.g. nutrient inputs to aquatic systems) were of lesser concern (Ministry for the Environment, 2020). Consequently, oxidation ponds formed the basis of municipal wastewater treatment as a simple cost-effective solution (Ministry for the Environment, 2020). Oxidation pond systems were constructed throughout New Zealand from the 1960s through to the 1980s and remain the most common form of treatment in rural New Zealand (Ministry for the Environment, 2020) but provide little nutrient removal. Mechanical treatment plants in other locations only incorporate primary and/or secondary treatment without nutrient removal (Ministry for the Environment, 2020). Consent renewal will result in stricter nutrient discharge limits which many oxidation ponds fail to meet, requiring a fundamentally different treatment process (GHD & Boffa Miskell, 2019; Ministry for the Environment, 2020). However, upgrading WWTP infrastructure is likely to be difficult in many cases as the vast majority of WWTPs in New Zealand only service a small portion of the total population (Ministry for the Environment, 2020). As a result, there is limited funding for WWTP services by local councils, restricting plant design

and the infrastructure upgrades required for adequate treatment of municipal wastewater (Curtis, 2014).

In some cases, the impact of WWTP effluent entering surface waters may seem insignificant in volume and nutrient load. However, longer retention times within a freshwater reservoir/lake could mean WWTP nutrient discharges could have a cumulative effect over time. Consequently, improvements to municipal wastewater treatment will assist in achieving Freshwater-NPS standards (Ministry for the Environment, 2020). Local industry and domestic inputs determine the flow, volume and variability of the influent entering the WWTP, meaning that each treatment plant may require differing treatment upgrades to meet environmental performance standards (Cass & Lowe, 2016). For example, a quarter of the wastewater entering Morrisville WWTP is from industrial inputs including a diary processing facility and a large abattoir which represent 21% and 5% of total chemical oxygen demand loads, and 13% and 27% of total N loads respectively (Ministry for the Environment, 2020). Treatment of these industrial loads may require specialised treatment processes to meet consent discharge limits (Ministry for the Environment, 2020). As the treatment technology increases in complexity, and consent limits become more stringent, the costs of treatment increase (Ministry for the Environment, 2020). Treated municipal wastewater is considered a waste stream in New Zealand, however treatment and reuse of wastewater effluent can produce valuable resources (Asano et al., 2007; Ge & Champagne, 2017; Arashiro et al., 2018). One emerging wastewater treatment technology demonstrating this resource potential is algal bioremediation, both as a source of sustainable biomass and as a tool to recover waste nutrients on a commercial scale (Cole et al., 2015; Neveux et al., 2015).

1.3 Algal bioremediation

Algal bioremediation or “phycoremediation” is a promising technology using macro- or microalgae for the treatment of water (Mulbry et al., 2008; Olguín & Sánchez-Galván, 2012; Phang et al., 2015). Algal bioremediation technologies have proven to be very effective for the treatment of various types of urban, industrial, agricultural and aquaculture wastewater; reducing nutrients, pathogens and emerging contaminants (Bostock et al., 2010; Phang et al., 2015; Ge et al., 2018; Mawi et al., 2020). To treat municipal wastewater using algal bioremediation, algae is grown directly in the wastewater assimilating N and P and other contaminants during growth, consuming nutrients as a food source to convert into

biomass (Fig. 1.1; Farahdiba et al., 2020; Liu et al., 2020). Nutrient and contaminant removal efficiency is quantified by the amount contained within the algal tissue on harvest (Neori et al., 2004; Wu et al., 2017). The harvested biomass can be further used as a feedstock to produce biofuels, biofertilisers, animal feed, and other valuable compounds (Arashiro et al., 2018).

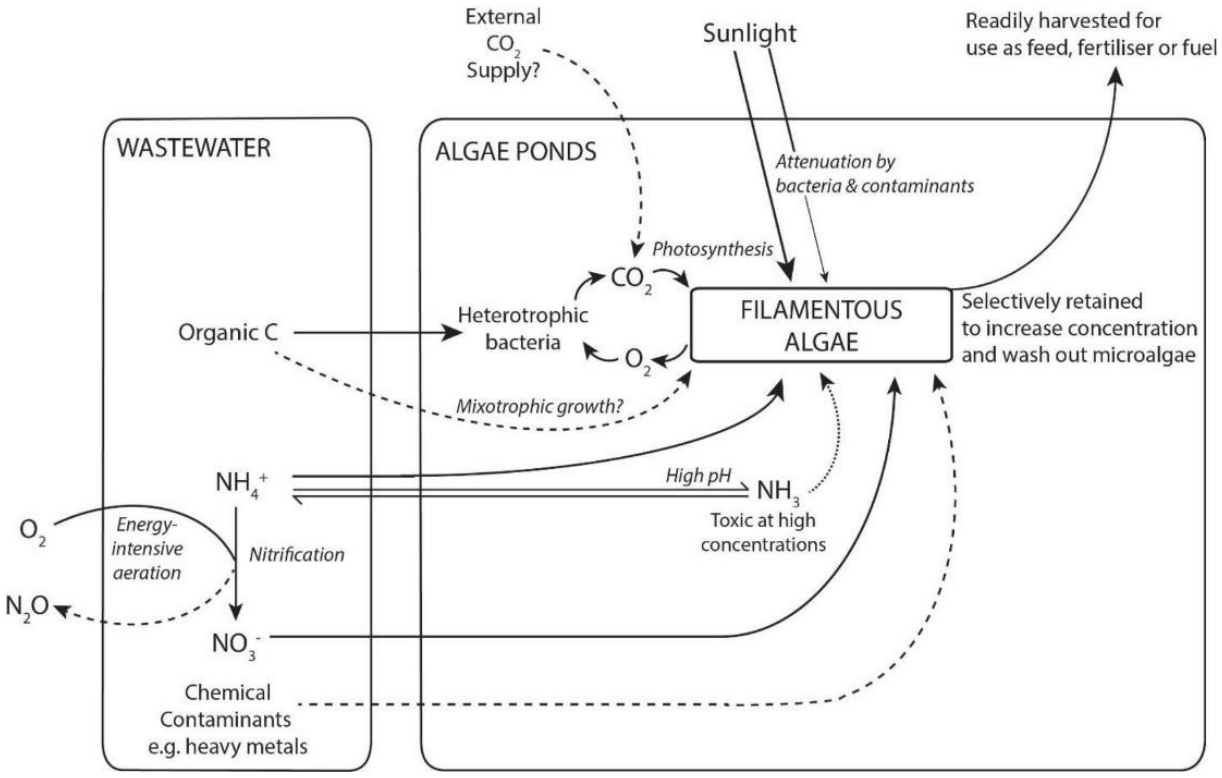

Figure 1.1 Schematic diagram of some aspects of algal bioremediation using filamentous macroalgae (Liu et al., 2020).

1.3.1 Algal bioremediation for the treatment of wastewater

Local governments have increasingly installed WWTPs based on costly engineered solutions causing infrastructure upgrades to become progressively more difficult to implement (Grigg, 2012; Phang et al., 2015; Valero-Rodriguez et al., 2020). Although economic constraints do exist for the treatment of wastewater in rural communities, algal bioremediation holds the potential to help alleviate economic pressures through wastewater reuse and resource recovery (Guest et al., 2009; Ma et al., 2013; Breach & Simonovic, 2018). There is an increasing demand for greener infrastructure and sustainable cities, where

wastewater treatment is not perceived as a human health and environmental concern but as an ecological solution from which water, nutrients, and other valuable resources could be recovered (Puchongkawarin et al., 2015; Kehrein et al., 2020). Annually, less than 13% of treated municipal wastewater is reused worldwide, with the majority discharged into the environment (Carey & Migliaccio, 2009; Sato et al., 2013). However, if treated adequately, municipal wastewater could become a reclaimed freshwater source for public re-use, providing a resource that could potentially offset supply issues in periods of drought (National Research Council, 2012). Additionally, recovery of N and P from municipal wastewater through algal bioremediation can offset operational and maintenance costs, with the potential for treatment plants to become profitable (McCarty et al., 2011; van Loosdrecht & Brdjanovic, 2014).

1.3.2 Algal bioremediation systems

Macroalgae can be cultivated through a wide range of systems including high-rate algal ponds (HRAPs), artificial ponds, tanks, algal turf scrubbers, ropes, and offshore structures (nets and rafts) (Neori et al., 2004; Lawton et al., 2017a). Large-scale algal bioremediation using macroalgae is typically achieved by algal turf scrubber (ATS) systems or open ponds (Lawton et al., 2017b). The type of algal culture selected for bioremediation will determine the cultivation system most appropriate for wastewater treatment. In general, ATS systems are suitable for a mixed algal community while HRAPs maintain a macroalgal monoculture (Lawton et al., 2017b). HRAPs are a common open pond system that are gaining popularity for the treatment of municipal wastewater (Garfí et al., 2017).

1.4 High-rate algal ponds

HRAPs are shallow, paddlewheel mixed raceway ponds where algae grow free floating and unattached. HRAPs aim to maximise wastewater treatment by creating optimal conditions for algal growth and oxygen production – the key processes which assimilate nutrients and degrade organic matter (Fig. 1.2; Fig. 1.3; Craggs et al., 2014; Park et al., 2011; Young et al., 2017). The potential for HRAPs to provide improved wastewater treatment has gained increased attention (Garfí et al., 2017) for the treatment of a variety of wastes including domestic, (Chen et al., 2003), tannery (Rose et al., 1996), dairy (Craggs et al.,

2003b) and piggery (Fallowfield & Garrett, 1985). Since their initial development in the 1950s, HRAPs have operated in many countries including Israel (Shelef & Azov, 1987), France (Picot et al., 1991), the United Kingdom (Fallowfield & Garrett, 1985), Morocco (El Hamouri, 2009), Spain (García et al., 2008), Australia (Young et al., 2016), China (Chen et al., 2003) and New Zealand (Craggs et al., 2003a).

Figure 1.2 Demonstration of HRAPs with an algal harvester in Christchurch, New Zealand (Craggs et al., 2012).

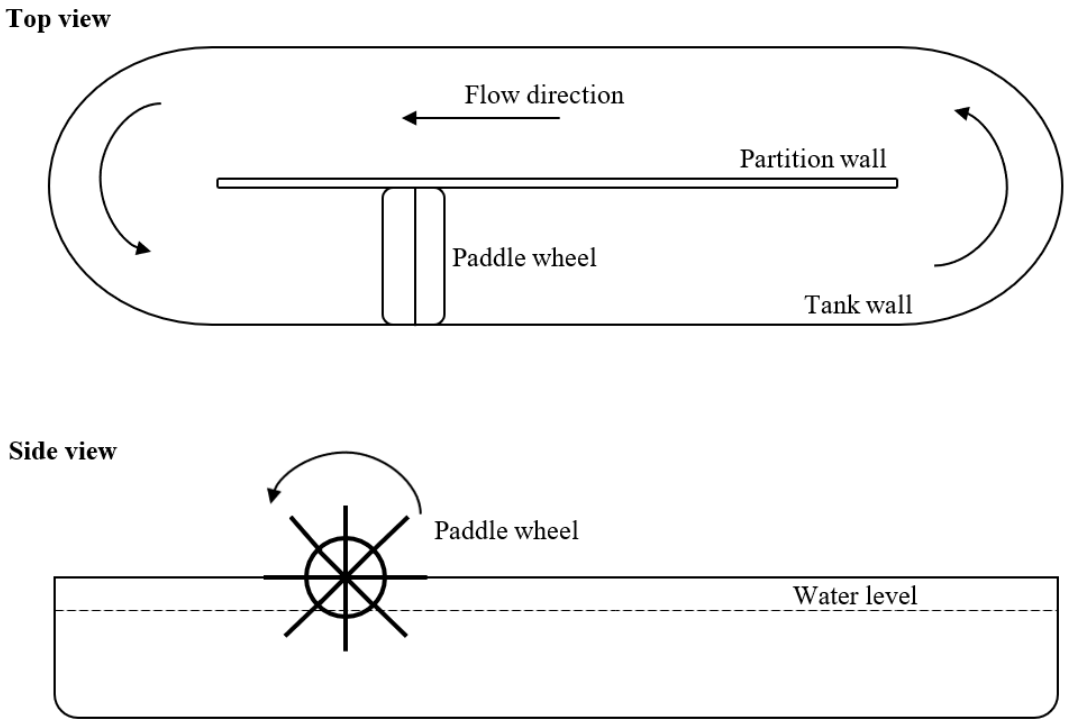

Figure 1.3 Schematic diagram of the top and side view of the HRAP.

HRAPs have been considered as a suitable replacement for oxidation ponds for the treatment of municipal wastewater (Young et al., 2017). To determine the feasibility of HRAPs as a viable alternative to oxidation pond systems it is necessary to compare the costs and benefits associated with both treatment systems. A cost analysis comparing a HRAP system and a five-cell oxidation pond system (commonly used in rural South Australia) found that the cost of constructing the HRAP system was 39.2% of the cost of constructing the oxidation pond system when operated at a depth of 0.32 m and 47.5% when operated at a depth of 0.43 m (Buchanan, 2014). This analysis found that the wastewater could be treated in the HRAP system within 20% of the time of other pond/lagoon systems while using 50% less surface area (Buchanan, 2014). Furthermore, compared to HRAPs, oxidation ponds can experience thermal stratification and hydraulic short-circuiting leading to poor treatment outcomes, and require periodic desludging which is costly (Fallowfield & Garrett, 1985; Cromar et al., 1996; Chambonniere et al., 2021). Therefore, HRAPs are ideal for WWTPs servicing rural, peri-urban and remote communities as they are effective in the treatment of wastewater and have lower power, land and on-site management requirements (Garfí et al., 2017; Young et al., 2017). HRAP systems can be altered in depth, mixing rate, and hydraulic retention time to maximise algal growth and bioremediation of wastewaters (Garcia et al., 2000; Cole et al., 2014a; Craggs et al., 2014).

Previous research has primarily focused on the use of microalgae in HRAPs for the treatment of wastewater (Craggs et al., 2003a; Craggs et al., 2012; Craggs et al., 2014; Sutherland et al., 2014b; Craggs et al., 2015; Young et al., 2017) with minimal information available in the literature on the use of macroalgae in HRAP systems. Although the existing literature is less applicable to the current project, which focuses on macroalgae, it is still a comparative basis by which the potential of HRAPs in the treatment of WWTP effluent could be reviewed. Nutrient removal mechanisms within the HRAP system for dissolved inorganic N, e.g. NO_3^- and NH_4^+ are considered to be mainly through algal biomass assimilation and pH-dependent ammonia volatilisation (Cromar et al., 1996; Garcia et al., 2000; Craggs et al., 2003a). Reported removal efficiencies from WWTP effluent within a HRAP for ammoniacal-N range between 16.3 – 42 mg L^{-1} (Picot et al., 1991; El Hafiane & El Hamouri, 2005). Nutrient removal mechanisms within the HRAP system for P are considered to be mainly through algal biomass assimilation and pH-dependent precipitation (Young et al., 2017). Reports of orthophosphate (PO_4^{3-}) removal from WWTP effluent range between 2.4 – 5.2 mg L^{-1} (Picot et al., 1991; El Hafiane & El Hamouri, 2005). Pathogen removal from wastewater in HRAP systems occurs mainly through exposure to solar radiation, high pH toxicity, and toxic algae metabolites, all of which increase pathogen decay (Chambonniere et al., 2021). Compared to oxidation ponds, HRAPs have demonstrated equal performance in the removal of pathogens and enhanced performance in the removal of heavy metals (Picot et al., 1992; Toumi et al., 2000; El Hamouri et al., 2003; Buchanan, 2014). Heavy metals in wastewater are primarily removed in HRAP systems through adsorption to algal and microbial biomass (Rose et al., 1998; Toumi et al., 2000). HRAPs have achieved 1.3 times greater removal of zinc, ten times greater removal of copper and two times greater removal of lead compared to oxidation ponds (Toumi et al., 2000).

1.4.1 High-rate algal pond parameters affecting algal production

i. Depth

The depth of a HRAP system is crucial for modifying exposure to solar radiation and a determining factor in maximising algal biomass productivity (Azov & Shelef, 1982; Grobbelaar, 2009; Rawat et al., 2011). Operational depths of HRAPs range between 0.2 and 1.0 m with the most common being around 0.3 m (Craggs et al., 2003a; Park & Craggs, 2011;

Park et al., 2011b; Kiran et al., 2017). Turbidity of the wastewater entering into the HRAP system may be an important determinant of depth as high turbidity can reduce the light available for growth (Craggs et al., 2014; Messyasz et al., 2018). Pathogen removal (e.g. *Escherichia coli* (*E. coli*)) in HRAPs has been suggested to mainly depend on solar irradiance (Craggs et al., 2004), pond depth and pH (Buchanan et al., 2011) or all three factors combined (Fallowfield et al., 1996). HRAP depth influences the rate of exposure to solar radiation, and pH is influenced by algal photosynthesis which in turn is driven by solar radiation, thereby suggesting that depth is the main factor influencing *E. coli* removal within a HRAP system (Young et al., 2017).

The influence of depth and light attenuation on biomass productivity and bioremediation performance within a HRAP system has been considered. For example, a comparison of three pilot-scale HRAPs operated at depths of 0.2, 0.3, and 0.4 m found that greatest removal of ammonia and highest algal productivity occurred in the system with a depth of 0.4 m (Sutherland et al., 2014b). Similarly, performance of a full-scale (30 m x 5 m) HRAP operated at depths of 0.32, 0.43, and 0.55 m for the treatment of septic tank wastewater was compared (Buchanan, 2014). Highest ammonia removal rates were found for the HRAP operated at 0.43 m depth (Buchanan, 2014). Although these studies are not comparable due to the effluent quality and variations in the HRAP systems used, these findings suggest that the optimal depth is ~ 0.4 m for a HRAP as a secondary wastewater treatment system (Young et al., 2017).

ii. Mixing

HRAP mixing and pond circulation is predominantly carried out by a paddlewheel which circulates the water horizontally around the system, with minimal energy lost through turbulence at surface water velocities between 0.15 and 0.3 m s^{-1} (Sutherland et al., 2015b). The paddlewheel rotation speed is an important parameter to provide horizontal flow and vertical mixing to circulate the algal culture and prevent settlement (Whitton et al., 2015). Increasing vertical mixing increases intermittent light exposure of algal cells, promoting growth and therefore bioremediation performance (Kusmayadi et al., 2020). Paddlewheel speed can regulate the potential for gas exchange and the passive diffusion of atmospheric CO_2 into the culture water by fluctuating the air/water surface boundary layer (Van Den

Hende et al., 2012). Therefore, high rotation paddlewheel speed creates an increase in flow and potentially better CO_2 dissolution, enhancing algal growth (Kusmayadi et al., 2020). However, increased paddlewheel speed also increases risk of algal cell damage due to the sensitivity of certain cultivars to sheer stress (Rueda Villegas et al., 2017). Paddlewheel mixing also aids in the removal of pathogens by increasing the frequency of pathogen exposure to high light intensity near the ponds surface (Chambonniere et al., 2021). Mixing within a HRAP increases the transfer of nutrients to algae cells by reducing the cell boundary layer (Grobbelaar et al., 1992; Borowitzka, 1999), further reducing the hydraulic retention time required for the same level of treatment compared to an unmixed oxidation pond, thereby increasing bioremediation efficiency (Green et al., 1996; Buchanan, 2014).

iii. Hydraulic retention time

Effective removal of nutrients and pathogens can be achieved by determining the optimal hydraulic retention time (HRT, the mean residence time of the wastewater held within the system) (Harrison & Hurd, 2001; Yun et al., 2015; Ge et al., 2018). The optimal HRT in HRAP systems may vary seasonally e.g. 3 – 4 days in summer and 7 – 9 days in winter (Craggs et al., 2014). The optimal HRT in HRAP systems will also vary with nutrient concentration. Wastewater with higher nutrient concentrations, such as primary municipal wastewater, will require an increased HRT to allow for adequate nutrient removal through algal uptake and growth, compared to wastewater with lower nutrient concentrations (e.g., tertiary treated municipal wastewater) (Mulholland et al., 1991; Neveux et al., 2016). However, long HRTs can rapidly exhaust the supply of dissolved inorganic carbon (DIC) in the wastewater, thus reducing algal growth (Mata et al., 2007; Cole et al., 2014b) and limiting nutrient removal rates (Cole et al., 2014a), but increasing *E. coli* removal (Chambonniere et al., 2021). However, each HRAP has different design parameters and therefore HRTs would need to account for pond size and depth to achieve the site-specific targeted nutrient and pathogen removal rates that are required for adequate treatment.

1.4.2 Optimisation of high-rate algal ponds

i. Additional CO_2

Macroalgae, like all plants, use CO_2 for photosynthesis (Lise Middelboe & Juel Hansen, 2007). Growth of freshwater macroalgae in HRAPs is often constrained by carbon limitation due to elevated daytime pH resulting from assimilation of DIC for photosynthesis (Craggs et al., 2012; Roberts et al., 2013; Cole et al., 2014b). However, CO_2 availability for algal growth is dependent on pH (Yen et al., 2019). DIC is available in the form of CO_2, bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), with the proportion of each being dependent upon the pH (Fig. 1.4; Lobban et al., 1994). The influence of pH on the pool of DIC is important to consider as although all algae can use CO_2, only some can use HCO_3^-, and none are able to use CO_3^{2-} as a source of carbon for photosynthesis (Lobban et al., 1994; Giordano et al., 2005). For example, in freshwater at a pH of 8 the concentration of dissolved CO_2 is minimal (<1.5%), with HCO_3^- making up 95% of the dissolved inorganic carbon, while at a pH of 10.5, CO_3^{2-} makes up approximately 65% with the remainder as HCO_3^- (Fig. 1.4). Consequently, the algal species grown in large-scale HRAP systems will need to assimilate DIC in the form of HCO_3^- for photosynthesis to maintain high productivity at elevated pH (Cole et al., 2017).

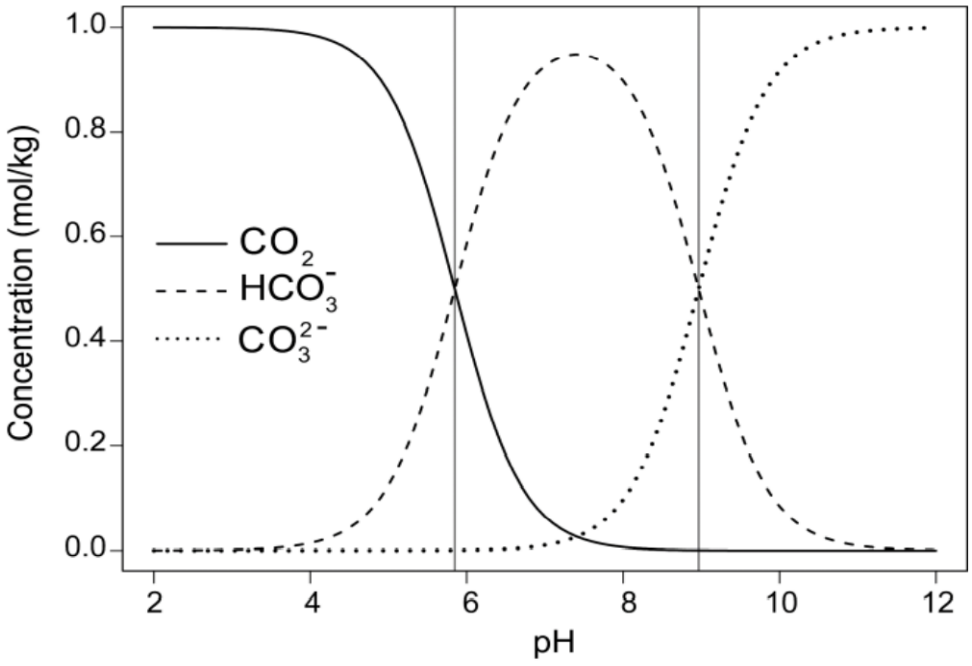

Figure 1.4 Effect of pH on concentrations of inorganic carbon CO_2, HCO_3^- and CO_3^{2-} in freshwater (Pimenta & Grear, 2018).

Provision of additional carbon to algal cultivation systems and the subsequent lowering of pH is typically achieved through the diffusion of CO_2 gas into wastewater

(Chisti, 2016). However, this technique has several restrictions in HRAPs, including the high cost of the pressurized CO_2 and the ability for CO_2 to diffuse into the wastewater media (Rickman et al., 2013; Chisti, 2016). Moreover, the amount of additional CO_2 added to the wastewater is disproportionate to the amount of CO_2 which is subsequently incorporated into the algal biomass (7 – 35%) (Ketheesan & Nirmalakhandan, 2012; Cole et al., 2014b). To address this issue, molasses, the waste residue from the sugar industry, has been proposed as a possible alternative option to provide supplemental carbon to lower pH and increase algal growth (Cole et al., 2017). However, there are some limitations to this option as molasses may only be available in sufficiently large quantities in regions where sugarcane is processed. There is also the potential for CO_2 recovery from biogas generated through wastewater anaerobic digestion (Young et al., 2019). More research is required on how to alleviate carbon limitation in algal cultures in a cost-effective manner. One way to achieve this would be to select species for cultivation which are capable of using HCO_3^- for photosynthesis.

ii. Control of grazers and parasites

Establishment of zooplankton grazers, parasitic fungi, infective bacteria and viruses are typical in outdoor HRAP systems (Ibekwe et al., 2017). Effective management of these grazers and parasites is required to achieve high productivity within a HRAP system (Park et al., 2011b) Zooplankton grazers commonly found in HRAPs include ciliates, rotifers, cladocerans, copepods and ostracods (Montemezzani et al., 2017). Zooplankton grazers and parasites enter HRAPs from the surrounding environment by wind dispersal (Cáceres & Soluk, 2002), bird-mediated transport (Cohen & Shurin, 2003), or attached to equipment (Waterkeyn et al., 2010). There are limited studies on the effect of zooplankton within farmed macroalgae systems (Wood et al., 2017). However, zooplankton may occur frequently as macroalgae HRAP systems offer favourable habitat conditions for zooplankton in terms of shelter and food availability (Hobson & Chess, 1976; Hammer, 1981; Pakhomov et al., 2002).

Once established, herbivorous zooplankton, fungal parasites and viral infections can rapidly reduce algal biomass concentrations, leading to a decline in bioremediation performance (Nurdogan & Oswald, 1995; Kagami et al., 2007; Montemezzani et al., 2016; Kiran et al., 2017). There are various chemical, physical, and biological methods for the control of zooplankton in HRAPs (Montemezzani et al., 2015; Kiran et al., 2017). Physical

methods include changes in temperature, cavitation, shear stress and filtration (Sawant et al., 2008; Tunowski, 2009). Chemical methods include variations in pH (high or low) and ammoniacal-N effects (Ivanova & Kazantseva, 2006; Kiran et al., 2017). For example, municipal wastewater can contain high levels of ammonia (30 mg/L) resulting in a high pH which has been found to be toxic to some zooplankton (Kiran et al., 2017). Biological methods include the introduction of predatory zooplankton or fish (Kiran et al., 2017; Montemezzani et al., 2017). Additionally, there are commercially available chemical agents, enzymes and infochemicals (chemicals that carry information between two individuals, and induce a physiological or behavioural response in the receiver) which can be used to reduce zooplankton grazers (Kiran et al., 2017). However, zooplankton control and disinfection needs to be operationally cost-effective and easy to implement (Sutherland & Ralph, 2020). For many treatment options, the techniques are expensive and time consuming, and are yet to be demonstrated beyond laboratory-scale (Day et al., 2012).

iii. Biomass harvest

Previous research has focused specifically on alternative methods to improve microalgae harvesting (Park et al., 2011a; Tiron et al., 2017; Singh & Patidar, 2018; Markeb et al., 2019) as performing efficient harvesting and dewatering of microalgae at full-scale remains one of the major challenges that needs to be overcome for cost-effective biomass recovery (Sharma et al., 2013; Sutherland & Ralph, 2020). Harvesting of freshwater macroalgae has competitive advantages compared to microalgae (Liu et al., 2020). Harvesting and dewatering of microalgae require specialised equipment, which has been estimated to account for 25 – 60% of the total production costs, limiting commercial viability (Molina Grima et al., 2003). In contrast, harvesting costs for freshwater macroalgae are lower due to the macroscopic size of filaments which enables biomass to be easily and economically harvested using mesh, nets, and ropes (Grayburn et al., 2013; Yun et al., 2014; Nwoba et al., 2016; Neveux et al., 2018; Liu et al., 2020). In addition, the ability to physically retain filamentous algae while washing out bacteria, diatoms and microalgae assists in maintaining unfouled, unialgal cultures of a desired cultivar (Liu et al., 2020). Furthermore, the harvested freshwater macroalgal biomass can be processed into commercially viable products such as animal feed (Nwoba et al., 2016; Vucko et al., 2017), slow-release fertiliser (Mulbry et al., 2005), and biofuel (Park et al., 2011b; Yun et al., 2015; Neveux et al., 2016).

1.5 Filamentous algae species for bioremediation

1.5.1 Selecting a target species

Selection of a target species for cultivation is critical to achieve optimal bioremediation performance in a HRAP system (Lawton et al., 2013b; Neveux et al., 2018). Performance of a particular species or cultivar is dependent on both genetic variability and the environment in which it is cultivated (G x E effects) (Robinson et al., 2013). Therefore, understanding the genetic and environmental drivers of performance is essential for increasing biomass production and bioremediation efficiency (Robinson et al., 2013). Local cultivars, and cultivars which are pre-exposed to municipal wastewater or high nutrient loading may possess traits that provide superior performance compared to other species (Kawecki & Ebert, 2004; Cheregi et al., 2019; Pankratz et al., 2019). Consequently, selecting local cultivars is often very important to the optimisation of large-scale monocultures (Fig. 1.5; Borowitzka & Moheimani, 2012). Target species should be selected based on four key criteria: adaptability to varying conditions, competitive dominance, high areal productivity, and consistent biochemical composition (Lawton et al., 2013a). The most important trait required of a species is to possess high bioremediation performance, ensuring adequate treatment of municipal wastewater.

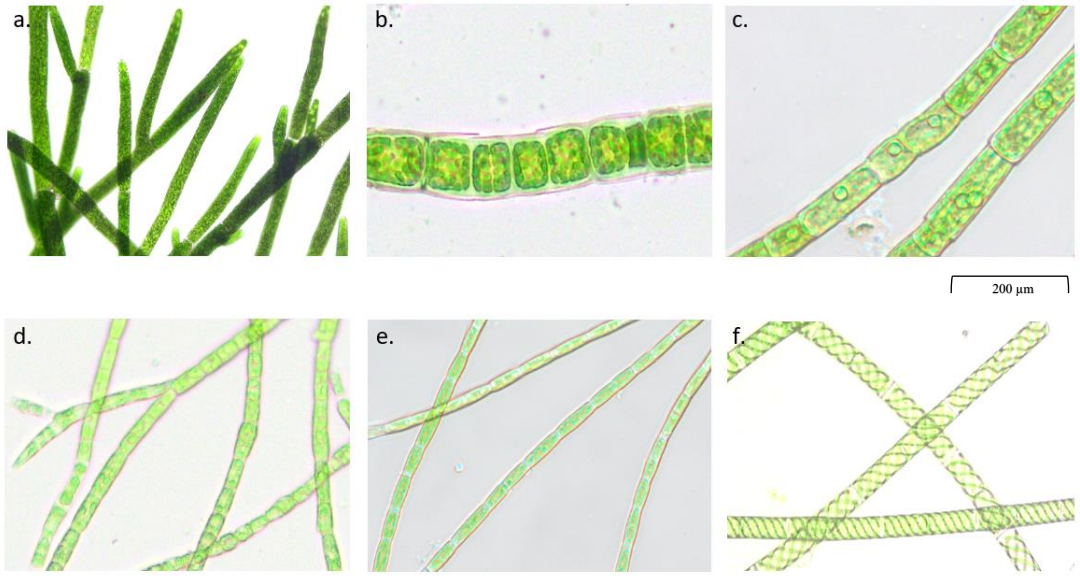

Figure 1.5 Examples of common filamentous algae genera a. *Cladophora,* b. *Ulothrix,* c. *Oedogonium,* d. *Stigeoclonium,* e. *Tribonema,* f. *Spirogyra* (photos taken using microscope: Olympus - CKX53).

The use of freshwater filamentous macroalgae for wastewater treatment in monoculture HRAPs is a developing area of research (Liu et al., 2020). Monocultures are desirable compared to bi-cultures and poly-cultures as monocultures produce biomass of more consistent quality overtime, thereby producing a dependable resource while ensuring continuous bioremediation at appropriate scale (Valero-Rodriguez et al., 2020). Careful species selection also enables increased productivity, allowing for specialised production while providing an efficient and low-maintenance culture. Monoculture control within a HRAP is dependent on environmental, operational, and biological parameters such as temperature, nutrient concentration and composition, HRT, pH, light exposure, grazers and parasites, etc. (Kiran et al., 2017). For a HRAP to be effective it must provide the appropriate conditions for the selected species to adequately treat the municipal wastewater while simultaneously producing biomass (Kiran et al., 2017). Although the selected species should be locally adapted it is also a requirement that treatment and biomass production occur across a wide range of conditions (de Paula Silva et al., 2012), with the aim to meet treatment standards. Monoculture species must also achieve higher productivity compared to undesired species (Liu et al., 2020) and demonstrate resilience to grazer pressure to increase the quality of the biomass by remaining unialgal and unfouled (Sutherland & Ralph, 2020). Operational conditions, such as altering HRT, recycling a proportion of harvested algal biomass or manipulating pond conditions (e.g. CO_2 addition), can also assist in maintaining dominance

of target species (Weissman & Benemann, 1979; Sheehan et al., 1998). Finding a species that is capable of meeting the necessary criteria can be an extensive process, however, it is essential for maintaining an effective and long-lasting HRAP monoculture (Shukla et al., 2018).

1.5.2 Approaches to species and cultivar selection

Cultivar selection is a fundamental step in achieving algal bioremediation within a HRAP monoculture system. Successful cultivation has previously been achieved with intensive and time-consuming research into physiology, life cycle, fouling prevention, and cultivar selection (Hafting et al., 2015). Therefore, a rapid screening protocol is required to identify suitable cultivars of freshwater macroalgae for large-scale cultivation in high-rate filamentous algal pond (HRFAP) systems across a range of locations. As outlined above, target species need to have consistent high nutrient assimilation and growth rates, favourable biochemical composition, and competitive ability. Therefore, development of a cultivar selection protocol to measure these parameters will ensure optimal cultivar selection. Cultivar selection of freshwater macroalgae has been investigated for various applications in a range of studies, however, no set protocol has been identified to select optimal macroalgal cultivars for bioremediation within HRAPs to date.

Typically, screening methods have focused on cultivar biomass productivity to achieve a specific objective, such as bioremediation performance (Valero-Rodriguez et al., 2020) and increased yields for end-products e.g. *Gracilaria* for agar production (Oliveira et al., 2000). These approaches typically determine biomass productivity of several cultivars under a range of light and temperature treatments to determine seasonal growth capabilities (Kim et al., 2007; Lawton et al., 2013b; Piotrowski et al., 2020; Valero-Rodriguez et al., 2020). Competitive dominance has also been tested by analysing cultivar performance in monoculture, bi-culture and poly-culture systems (Lawton et al., 2013a; Valero-Rodriguez et al., 2020). Experiments have also been conducted to assess bioremediation performance and biomass productivity between species in the removal of metals (Mustafa et al., 2012; Roberts et al., 2015) and nutrients at different concentrations (Liu & Vyverman, 2015; Nwoba et al., 2016; Liu et al., 2020). Although growth capabilities under various conditions have been used to identify suitable cultivars, progress is needed in the development of a macroalgal cultivar screening technique (Yang et al., 2020). This will involve a more robust selection process

which incorporates efficient techniques and provides a standardised method to identify suitable cultivars isolated from naturally occurring macroalgal populations.

1.6 Per- polyfluoroalkyl substances in WWTP effluents

Per- and polyfluoroalkyl substances (PFAS) are a diverse group of synthetic chemicals used extensively since the 1940's for their hydrophobic and lipophobic properties (Gao et al., 2020; Sinclair et al., 2020). PFAS chemicals are used in numerous products, including non-stick cookware, fire-fighting foams, stain-resistant fabrics, and various food packing materials (Espartero et al., 2022). Due to their unique molecular structure, characterised by strong carbon-fluorine bonds, PFAS are highly resistant to degradation (Gao et al., 2020). PFAS contamination has become a significant environmental concern because these substances persist in soil and water and can bioaccumulate in wildlife and humans, potentially causing adverse health effects (Niu et al., 2019; Brown et al., 2020). PFAS enter WWTPs through multiple pathways (Lenka et al., 2021; Nguyen et al., 2022a). Industrial processes and the manufacturing or use of PFAS-containing products can discharge these chemicals into the wastewater system and PFAS are therefore present in WWTP influent at varying concentrations (Drage et al., 2023; Dias et al., 2024). The removal of PFAS from WWTPs poses a significant challenge due to their chemical stability (Zhou et al., 2024). During primary and secondary treatment stages, PFAS can be partially removed using conventional treatment (Kim et al., 2022). The wastewater sludge can contain absorbed PFAS, so sludge removal will reduce the concentration of long-chain PFAS in the treated effluent, however overall removal can be minimal as much of the PFAS remains in solution (Kim et al., 2022). PFAS concentrations can fluctuate throughout various stages of a WWTP depending on the type of treatment used and the types of PFAS present, including PFAS precursors (Nguyen et al., 2019). For instance, certain advanced oxidation processes (photocatalysis, electrochemical oxidation) might degrade and transform PFAS precursors into more stable forms of PFAS, increasing the overall concentration of PFAS within the wastewater effluent (Kim et al., 2022). Assessing these precursors using techniques such as a total oxidisable precursors (TOP) assay is essential for a comprehensive evaluation of PFAS contamination (Al Amin et al., 2020). Current conventional treatment methods such as activated carbon adsorption, ion exchange resins, and high-pressure membrane filtration (i.e., reverse osmosis), have been implemented to reduce PFAS concentrations in wastewater (Gao et al., 2021b). However, these current methods are energy intensive, costly, and produce

secondary waste-streams that require further management (Meegoda et al., 2022). Furthermore, none of these methods can provide complete removal of PFAS (Meegoda et al., 2022). Therefore, there is a growing interest in exploring more sustainable and cost-effective treatment options (Arslan & Gamal El-Din, 2021). Algal bioremediation may be a promising alternative, as algae utilise natural metabolic processes to absorb and potentially degrade contaminants, including PFAS (Marchetto et al., 2021). Algae can be cultivated in various wastewaters, making them a versatile and adaptable option for bioremediation (Liu et al., 2020). Research into algal bioremediation for PFAS removal is limited, however studies have shown that certain species have the capability to uptake PFAS from contaminated wastewaters (Marchetto et al., 2021). Investigating algal bioremediation as an alternative solution could lead to the development of innovative, eco-friendly, and sustainable PFAS treatment practices.

1.7 Thesis structure and objectives

The primary objective of this thesis was to investigate the bioremediation performance of filamentous freshwater macroalgae in primary municipal wastewater. This thesis is structured into 4 experimental Chapters, each demonstrating the feasibility of cultivating macroalgae for this purpose. Chapter 2 develops a laboratory screening protocol to identify robust cultivars for cultivation in primary municipal wastewater under seasonal conditions. Chapter 3 assesses the growth and competitive dominance of three target cultivars identified using the screening protocol in outdoor HRFAPs. Chapter 4 focuses on optimising operational conditions in monoculture HRFAPs to enhance bioremediation performance, using the top performing cultivar identified in previous experiments. Chapter 5 explores the potential for PFAS bioremediation from primary wastewater using the same top performing cultivar under laboratory conditions. Each research Chapter has specific aims detailed below and is written as a stand-alone study suitable for individual publication, while sequentially building on earlier findings.

1.7.1 Chapter 2: Screening protocol for freshwater filamentous macroalgae bioremediation of primary municipal wastewater

The aim of this Chapter was to create a screening protocol for selecting cultivars of filamentous freshwater macroalgae specifically for nutrient bioremediation in primary municipal wastewater. The specific aims were: (i) to develop a screening protocol to identify and isolate cultivars from wild collected algal samples that are competitively dominant and exhibit rapid growth in primary municipal wastewater; and (ii) to compare the nutrient bioremediation efficiency and biomass productivity of these isolated cultivars when grown in primary municipal wastewater under varying local seasonal and extreme conditions.

1.7.2 Chapter 3: Productivity and competitive dominance of freshwater filamentous macroalgae cultivars for nutrient bioremediation of primary municipal wastewater

Building on the previous protocol, this Chapter focused on three freshwater filamentous cultivars (*Klebsormidium flaccidum*, *Oedogonium calcareum*, and *Oedogonium* sp.) selected in Chapter 2 as potential candidates for primary municipal wastewater treatment in HRFAP monocultures. The study's objective was to further evaluate the growth, bioremediation performance, and competitive dominance of these cultivars when grown in primary wastewater in outdoor HRFAP mesocosm systems. The specific aims were: (i) to compare the growth and nutrient removal rates of these cultivars when grown in outdoor HRFAP monocultures, and (ii) to assess the dominance of these cultivars when grown in outdoor HRFAP bi-cultures.

1.7.3 Chapter 4: Optimisation of high-rate filamentous algal pond operating parameters for nutrient bioremediation of primary municipal wastewater

This Chapter investigated how operational parameters of HRFAPs influence the performance of filamentous freshwater macroalgal monocultures in outdoor systems under seasonal temperate conditions. The study's specific aims were to: (i) determine the effects of hydraulic retention time (HRT), stocking density, and harvest frequency on the growth and bioremediation performance of a selected cultivar when grown in primary municipal wastewater in HRFAPs, and (ii) to compare how these parameters (HRT, stocking density, and harvest frequency) impacted growth and bioremediation performance across different seasons (summer and winter).

1.7.4 Chapter 5: Freshwater filamentous macroalgal bioremediation of per- and polyfluoroalkyl substances (PFAS) from primary municipal wastewater

This Chapter assessed the feasibility of using freshwater filamentous algal monocultures to treat PFAS in primary municipal wastewater. The specific objectives were: (i) to quantify the growth of *Klebsormidium flaccidum* and evaluate its ability to bioremediate PFAS and PFAS precursors, and (ii) to determine if the harvested algal biomass was contaminated with PFAS when cultivated in primary municipal wastewater under two different hydraulic retention times under laboratory summer conditions.

Chapter 2 - Screening protocol for freshwater filamentous macroalgae bioremediation of primary municipal wastewater

This chapter has been published in Journal of Applied Phycology as:
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2.1 Abstract

A screening protocol was developed and applied to isolate and select cultivars of freshwater filamentous macroalgae for year-round monoculture cultivation and nutrient bioremediation of primary municipal wastewater. The screening protocol is a step-by-step guide to identify robust cultivars which possess key attributes of competitive dominance, high biomass productivity and bioremediation performance under local seasonal and extreme conditions. Forty-four mixed samples of freshwater filamentous macroalgae were collected during summer and winter from a range of local aquatic environments. Eleven isolated cultivars were grown in primary treated municipal wastewater and their biomass productivity and bioremediation performance under local ambient (summer and winter), and extreme summer (max. summer) and winter (min. winter) conditions were assessed. Extreme conditions proved to be an important determining factor for cultivar selection as biomass productivity and bioremediation performance significantly declined under min. winter conditions. However, biomass productivity was not directly related to bioremediation performance, as cultivars with low growth rates maintained high nutrient removal rates under min. winter conditions. Top performing cultivars were *Klebsormidium* sp. *(KLEB B)* which reduced total ammoniacal-N concentrations by 99.9% to 0.01 mg L^{-1} (± 0.01 SE), *Oedogonium* sp. *(OEDO D)* which reduced nitrate-N concentrations by 90.2% to 0.08 mg L^{-1} (± 0.7 SE) and *Rhizoclonium* sp. which reduced dissolved reactive phosphorous concentrations by 98.7% to 0.02 mg L^{-1} (± 0.01 SE). Based on overall biomass productivity and bioremediation performance across seasonal and extreme conditions *Klebsormidium* sp. *(KLEB B)*, *Stigeoclonium* sp. *(STIG A)* and *Ulothrix* sp. were identified as top performing cultivars suitable for the nutrient bioremediation of primary municipal wastewater.

2.2 Introduction

Wastewater treatment plants (WWTPs) are point-sources for nutrient discharges entering into the aquatic environment (Carey & Migliaccio, 2009; Arzate et al., 2019; van Puijenbroek et al., 2019). Consequently, there is an increasing need to upgrade existing WWTP infrastructure to achieve treatment standards, improve water quality of the receiving aquatic environment, and utilise primary municipal wastewater as a sustainable resource (De La Cueva Bueno et al., 2017; Salgot & Folch, 2018; Chrispim et al., 2019). Filamentous algal bioremediation - the use of algae for the absorption and degradation of organic pollutants in aquatic environments (Baghour, 2019) - can be a practical solution to upgrade municipal WWTP infrastructure (Cole et al., 2016a; Lavrinovičs & Juhna, 2017; Bhatt et al., 2021). Primary municipal wastewater is nutrient-rich (Simha & Ganesapillai, 2017), providing a suitable medium to cultivate filamentous algae and thereby enable nutrient recovery through algal bioremediation (Renuka et al., 2013; Chrispim et al., 2019; Leong et al., 2021). A high rate filamentous algal pond (HRFAP) system is low cost and simple to operate (Craggs et al., 2014; Garfí et al., 2017; Young et al., 2017), providing the added benefit of a consistent source of biomass for resource recovery and subsequent value-added products (Cole et al., 2016a; Lawton et al., 2017b; Leong et al., 2021). Therefore, algal bioremediation using HRFAPs could provide a circular economy approach to primary municipal wastewater treatment, converting a major waste stream into a commercially viable industry (Wreford et al., 2019; Kehrein et al., 2020; Catone et al., 2021; Škufca et al., 2021).

Monocultures are preferable for the effective treatment of primary municipal wastewater within a HRFAP to ensure bioremediation performance (Kebede-Westhead et al., 2006; Liu & Vyverman, 2015; Liu et al., 2016; Lawton et al., 2021a) and consistent biomass composition for end-use product applications (Lawton et al., 2013a; Neveux et al., 2016; Lawton et al., 2017b; Liu et al., 2020). Therefore, cultivar (a plant which has been cultivated by selective breeding) selection is a fundamental process in the initial production phase of any monoculture system (Borowitzka, 2013). Cultivar selection should target native cultivars from within the local aquatic catchment or within the selected municipal WWTP (Kube et al., 2018; Bao et al., 2022) as the performance of a species or cultivar is dependent on genetic variability and the environment in which it grows naturally (Robinson et al., 2013). Consequently, cultivars which have adapted to nutrient-rich waters at municipal WWTPs may possess performance-enhancing traits which enable high biomass productivity and

nutrient bioremediation performance (Kawecki & Ebert, 2004; Cheregi et al., 2019; Pankratz et al., 2019). Targeting a native cultivar also avoids any potential biosecurity impacts incurred by the introduction of a foreign species as introducing cultivars to new locations can have undesirable effects on local species and ecological processes (Ricciardi & Simberloff, 2009; Champion, 2018; Reid et al., 2019). Therefore, the WWTP was identified as a key site for sample collection.

Currently there is no standardised approach to screen local cultivars for a HRFAP monoculture system. To date, cultivars for filamentous algal bioremediation of municipal wastewater have been selected through laboratory-scale competition experiments under different seasonal conditions (Valero-Rodriguez et al., 2020), growth experiments within a variety of treated wastewaters (Ge et al., 2018), and assessment of biomass productivity and nutrient bioremediation performance in outdoor cultures in municipal wastewater (Lawton et al., 2021a). However, in all these studies cultivars were first pre-selected based on survival and scalability in standardised growth media under laboratory conditions. This cultivar pre-selection criterion may be appropriate for selecting cultivars for tertiary treated municipal wastewater, however, it is not effective when targeting primary municipal wastewater. This is because primary municipal wastewater contains nitrogen in the form of ammonia, which can be toxic to many algae when present at a high concentration (Wang et al., 2013; Ge et al., 2018). Ammonia concentrations in primary wastewater are also much higher than those found in standardised growth media and aquatic environments where algae typically grow (Ge & Champagne, 2017; Karri et al., 2018). Consequently, only some cultivars can survive and be cultivated within primary municipal wastewater (Lu et al., 2018; Divya et al., 2023). Therefore, a standardised screening protocol must be specifically formulated to accurately identify local cultivars suitable for bioremediation of primary municipal wastewater.

An effective screening protocol must assess a range of attributes to determine the suitability of cultivars for nutrient bioremediation of primary municipal wastewater (Rani et al., 2021; Sameena et al., 2022). The screening protocol must identify cultivars which are competitively dominant to sustain monoculture production (Liu et al., 2020; Lawton et al., 2021b), thereby maintaining the quality of the biomass by remaining unialgal and uncontaminated (Sutherland & Ralph, 2020). Primary municipal wastewater composition is nutrient-rich and highly variable depending on location and the type of wastewater influent entering the WWTP (Zhou et al., 2019). Thus, the screening protocol must ensure that

selected cultivars can survive and maintain high biomass productivity when cultivated within primary wastewater from the targeted WWTP. Finally, bioremediation performance and biomass production can vary between seasons (Valero-Rodriguez et al., 2020). Therefore, a screening protocol needs to assess performance over a wide range of seasonal conditions (de Paula Silva et al., 2012; Lam & Lee, 2014) to ensure water quality standards are met and production is maintained year-round (Stachowicz et al., 2008; Lawton et al., 2017b).

The overall objective of this study was therefore to develop a screening protocol to select target cultivars of filamentous freshwater macroalgae for nutrient bioremediation of primary municipal wastewater. The specific aims were (i) to develop a screening protocol to identify and isolate cultivars from wild collected algal samples that are competitively dominant and fast-growing within the targeted medium; and (ii) compare nutrient bioremediation performance and biomass productivities of isolated cultivars grown in primary treated municipal wastewater under local seasonal conditions. It was hypothesised that cultivars collected from the WWTP, particularly from within primary wastewater clarifiers, would achieve high biomass productivity during acclimation and have high nutrient bioremediation performance during growth trials. Overall, this screening protocol was designed to select high performing cultivars which can then undergo further on-site pilot-scale trials to identify a single target cultivar suitable for year-round cultivation and nutrient bioremediation of primary municipal wastewater in a HRFAP monoculture.

2.3 Methods

2.3.1 Isolation and screening protocol

An isolation and screening protocol was developed to identify cultivars of naturally occurring filamentous freshwater macroalgae that are suitable for primary municipal wastewater bioremediation. This protocol included four key steps: sample collection, acclimation, growth trials, and bioremediation trials (Figure 2.1). Each step is outlined in detail below. Briefly, mixed samples of filamentous algae were collected from local environments to target species which were acclimated to local climatic conditions. Samples were then maintained in culture under nutrient concentrations similar to primary wastewater for four weeks (acclimation). Based on acclimation performance, cultivars were selected for

experiments which measured survival rate in primary wastewater (growth trials). Biomass productivity and bioremediation performance of surviving cultivars in primary wastewater in response to seasonal variation in temperature and light was then assessed (bioremediation performance trials).

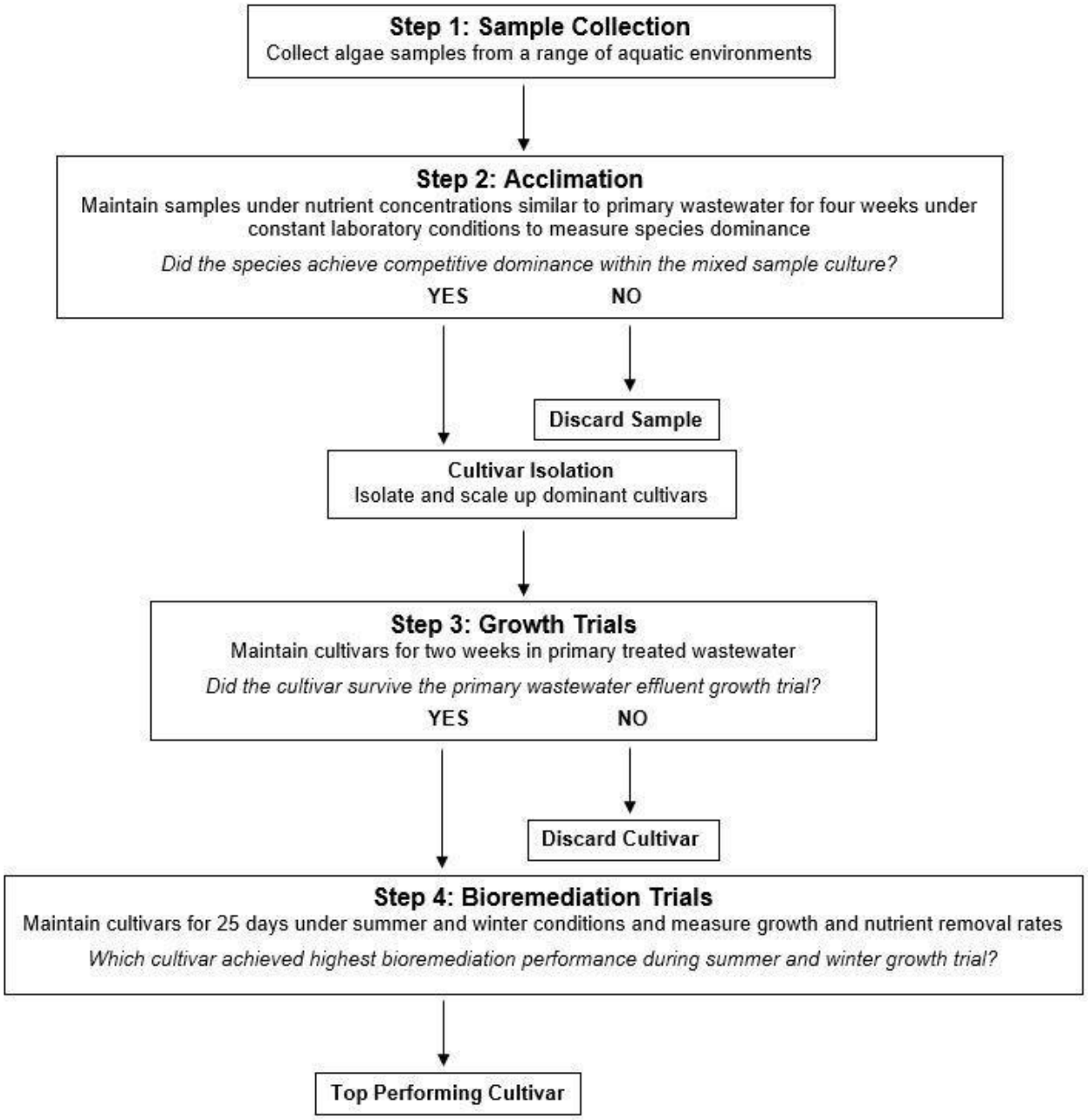

Figure 2.1 Flow chart detailing each step of the screening protocol developed for the selection of filamentous freshwater macroalgal cultivars for nutrient bioremediation of municipal wastewater

2.3.2 Sample collection

Forty-four mixed samples of freshwater filamentous macroalgae were collected from the Te Puke WWTP, Aotearoa New Zealand, and aquatic environments in the vicinity of the plant including streams, agricultural drains, wetlands, and ephemeral water bodies (Appendix 2.7 Table 2A.1). Samples were collected during summer and winter to ensure the screening protocol included a range of species. Collected samples of < 2 g fresh weight (FW) were placed in 250 mL plastic containers in water from the collection site and stored in an insulated container and transported to the University of Waikato Coastal Marine Field Station, Tauranga, Aotearoa New Zealand. Any large debris were then manually removed, and the entire sample was poured through a 100 µm filter to remove excess suspended solids. The algal biomass was then divided into three replicate subsamples. Algal subsamples containing sufficient biomass were stocked at a rate of 1 g FW L^{-1} into separate 250 mL clear plastic containers with lids (LabServ™) filled to 150 mL with filtered dechlorinated freshwater (tap water) enriched with nutrients at a pH of 5.6 at 19.1 °C. Nutrient concentrations were based on assumed dilutions of primary wastewater concentrations in a HRAP of 5 mg NH_4^-N L^{-1} (NH_4Cl), 1.3 mg PO_4^-P L^{-1} ($NaH_2PO_4 \cdot 2H_2O$) and trace metal concentrations ($FeCl_3 \cdot 6H_2O$, $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$, ZnSO4 · $7H_2O$, $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $Na_2MoO_4 \cdot 2H_2O$) as per F/2 artificial growth medium (Ryther & Guillard, 1962; Guillard, 1975). All growth media components were analytical grade and measured at a pH of 2.2 prior to dilution. Algal samples containing smaller quantities of biomass were separated into three large petri dishes (Nest, 60 – 90 mm ø) until biomass had grown sufficiently to stock into 250 mL plastic containers at 1 g FW L^{-1}. The acclimation step started immediately following this step.

2.3.3 Acclimation

The three replicate subsamples of each sample were maintained for four weeks under constant laboratory conditions (18 °C, 12:12 light:dark cycle at 60 – 100 µmol m^{-2} sec^{-1} using cool fluorescent lights). Cultures were grown in the primary wastewater nutrient medium (as above). Cultures in 250 mL plastic containers were maintained in suspension by bubbling a constant gentle stream of filtered (Filtrec – FS134B8TI25 and Whatman™ Uniflo syringe filters, 0.22µm) air into each container. Cultures in petri dishes were not aerated until

biomass was upscaled into 250 mL plastic containers. Replicates were arranged on shelves in a randomized block design and were rotated daily within each block to minimise any edge effects and variation in light intensity. Biomass in each replicate container was harvested, and culture medium was exchanged once every seven days over the four-week acclimation period. Biomass was harvested by mixing the contents of each container thoroughly to ensure that no algae had settled on the bottom of the container and then tipping the entire contents of each container (culture water and algae) into a fine mesh bag. Once excess water had drained from the bag, it was placed in a centrifugal spin dryer (Spindle NZ, SPL-265) and spun for approximately three minutes to remove any remaining water. The algae were then removed from the bag and weighed to determine the FW. Following harvesting, stocking density was reset to 1 g FW L^{-1} by restocking 0.15 g of the harvested biomass into each 150 mL replicate culture. Species within each sample were identified by morphological characteristics under a microscope (Olympus model CKX53) using freshwater algal identification guidebooks (Bellinger et al., 2010; John et al., 2021). Species composition in each replicate was estimated at the start and the end of the acclimation period by taking a small sample of the biomass from each replicate container and photographing ten sub-samples under a dissecting microscope (Olympus model CKX53) at 20 x magnification. Proportional species composition of each sub sample was estimated by placing a 100-point grid over each photo and summing the number of grid points directly overlying each species.

2.3.4 Cultivar isolation

Sixteen cultivars were selected for isolation and scale up based on their dominance in samples at the end of the acclimation period (Table 2.2). Approximately 20 – 30 individual filaments of each target cultivar were isolated from the mixed samples and placed into a sterile petri dish filled with filtered dechlorinated freshwater, enriched with F/4 artificial growth medium at a pH of 5.7 at 17.4 °C (6.15 mg L^{-1} nitrate (NO_3^-), 0.56 mg L^{-1} phosphorus (PO_4^-), Rhyther and Guillard 1862; Guillard 1975). This process was repeated six times to establish six replicate petri dishes for each individual cultivar. Petri dishes were maintained under constant laboratory conditions (18 °C, 12:12 light:dark cycle at 50 µmol m^{-2} sec^{-1} using cool fluorescent light). When the total biomass of each cultivar reached approximately 0.1 g FW, cultures were maintained with growth medium containing 5 mg NH_4^-N L^{-1} (NH_4Cl), 1.3 mg PO_4^-P L^{-1} ($NaH_2PO_4 \cdot 2H_2O$) as described above and trace metals corresponding to rates found in F/2 artificial growth media (Ryther & Guillard, 1962; Guillard, 1975). Nitrate (NO_3^-

) was selected for upscaling isolated filaments, as previous attempts using ammonium (NH_4^- N) had caused algae to die-off. Culture media were replaced weekly, and cultivars were upscaled into larger petri dishes as biomass increased, then into 250 mL clear plastic containers, and then into 2 L clear plastic containers. Seven of the 16 cultivars selected for isolation could not be scaled up due to slow growth and repeated issues with biomass cross-contamination of other cultivars, and fungal infections.

2.3.5 Growth trials

Growth trials were conducted to determine whether the nine isolated cultivars that were successfully scaled up could survive in diluted primary treated wastewater. Three additional cultivars that have been recently identified as targets for wastewater nutrient bioremediation were also included in the growth trials to provide a comparative measure of the performance of isolated cultivars. The three additional cultivars were an *Oedogonium calcareum (OEDO A)* cultivar from the University of Waikato Coastal Marine Field Station, Tauranga, Aotearoa New Zealand (Lawton et al., 2021a) and an *Oedogonium* sp. *(OEDO B)* and a *Rhizoclonium* sp. cultivar from the National Institute of Water and Atmospheric Research (NIWA), Hamilton, New Zealand (Hariz et al., 2022). Five replicate cultures of each cultivar (n = 60 cultures) were maintained in a temperature-controlled plant growth cabinet (Panasonic MLR-352, 18 °C, 12:12 light:dark cycle at 100 – 200 µmol m^{-2} sec^{-1}).

Experiments were conducted using free-floating (suspension) cultures of each cultivar in 250 mL plastic containers (LabServ™) filled to 150 mL at a stocking density of 1g FW L^{-1}. Cultures were maintained in suspension by bubbling a constant gentle stream of filtered (Resun LP-40 air pump and Whatman™ Uniflo syringe filters, 0.22 µm) air into each container. Cultures were grown in primary treated wastewater collected from the Te Puke WWTP during daily peak inflow. Wastewater was settled for four hours in 20 L plastic buckets on collection. Physico-chemical parameters of the primary wastewater were measured at a pH of 7.5, temperature of 22.4°C, biological oxygen demand (BOD5) of 310 mg L^{-1} and chemical oxygen demand (COD) of 750 mg L^{-1}. These analyses were conducted following standard methodology by Tauranga City Council Laboratory, Tauranga, New Zealand. Supernatant primary wastewater was then removed from the top of the bucket and diluted at a ratio of 1:3 with filtered dechlorinated freshwater (i.e., 25% wastewater).

Wastewater was diluted to simulate wastewater concentrations within a HRAP where there would be a constant low flow of wastewater entering the HRAP, mixing with pre-existing partially treated wastewater. At each water collection, three additional 500 mL water samples were collected post-dilution and physico-chemical parameters were measured as total suspended solids (TSS) at 64 – 116 mg L^{-1}, total Kjeldahl nitrogen (TKN) at 14.6 – 15.5 mg L^{-1}, total ammoniacal-N (TAN) at 9.7 – 11.4 mg L^{-1}, nitrate-N + nitrite-N at 0.50 – 0.75 mg L^{-1}, dissolved reactive phosphorus at 0.96 – 1.31 mg L^{-1} and total phosphorus at 2.5 – 3.1 mg L^{-1}. These analyses were conducted following standard methodology by Hill Laboratories in Hamilton, New Zealand. Cultures were arranged within the plant growth cabinet using a randomized block design where one replicate of each cultivar was placed on each shelf of the cabinet and replicates within a shelf were rotated daily to minimise any edge effects.

The experiment was run for 14 days and biomass in each replicate container was harvested on day 7 and day 14 as described above. Total suspended solids and bacterial loads declined within the first 24-hour period, as no bacterial flocs were present. Once harvested, the culture medium in each container was replaced with new diluted primary wastewater from the Te Puke WWTP and stocking density was reset to 1 g FW L^{-1} by restocking 0.15 g of the harvested biomass back into each 150 mL replicate culture. Excess biomass not restocked back into containers on day 7 from each replicate culture, and all biomass on day 14 from each replicate culture was dried in an oven at 60 °C for 48 hours and reweighed to determine the fresh weight to dry weight (FW:DW) ratio for each replicate. FW:DW ratios were used to convert the initial biomass and the harvested biomass for each replicate, which were both measured in FW, into DW. Biomass productivity (g DW m^{-2} day^{-1}) was calculated for each replicate for each harvest using the equation $P = (B_f - B_i)/ A / T$, where B_f and B_i are the final and initial algal biomasses (g DW), A is the area (m^{-2}) of culture container and T is the culture period (seven days).

2.3.6 Bioremediation trials

To identify top performing cultivars for year-round cultivation, cultivars which survived cultivation in primary wastewater effluent (n = 8) and the three additional cultivars were then maintained under temperatures and light intensities representative of local summer and winter conditions. Four experiments were conducted to represent ambient summer, ambient winter, maximum summer (max. summer) and minimum winter (min. winter)

conditions. Methods were identical for each experiment, with the exception of temperature and light settings. Conditions were based on the National Climate Database weather recording station located in Te Puke (-37.82455, 176.32048, data available from www.cliflo.niwa.co.nz). Temperature profiles were based on the average high and average low temperature from the previous January for summer ambient conditions, and from the previous July for winter ambient conditions (Table 2.1, Figure 2.2a). Temperature profiles for max. summer conditions were based on the day of the previous year with the highest recorded summer temperature, using the average highest temperature recorded over a six-hour period of that day and the average temperature of the remaining 18-hour period to provide a maximum and minimum temperature respectively (Table 2.1, Figure 2.2a). Temperature profiles for min. winter conditions were based on the day of the previous year with the lowest recorded winter temperature, using the average lowest temperature recorded over a six-hour period of that day and the average temperature of the remaining 18-hour period to provide a minimum and maximum temperature respectively (Table 2.1, Figure 2.2a). The same light profiles were used for both the ambient and maximum/minimum experiments. These were based on the average daily light data recorded from the previous January for summer conditions, and from the previous July for winter conditions (Table 2.1, Figure 2.2b).

Table 2.1 Ambient and maximum/minimum temperature and light conditions used in bioremediation performance trials.

Seasonal condition	**Temperature (°C)**		**Light (µmol m^{-2} sec^{-1})**	
	Min	**Max**	**Min**	**Max**
Summer	14.3	24.9	150	800
Max. summer	20.6	29.6	150	800
Winter	4.9	14.6	50	200
Min. winter	1.4	7.7	50	200

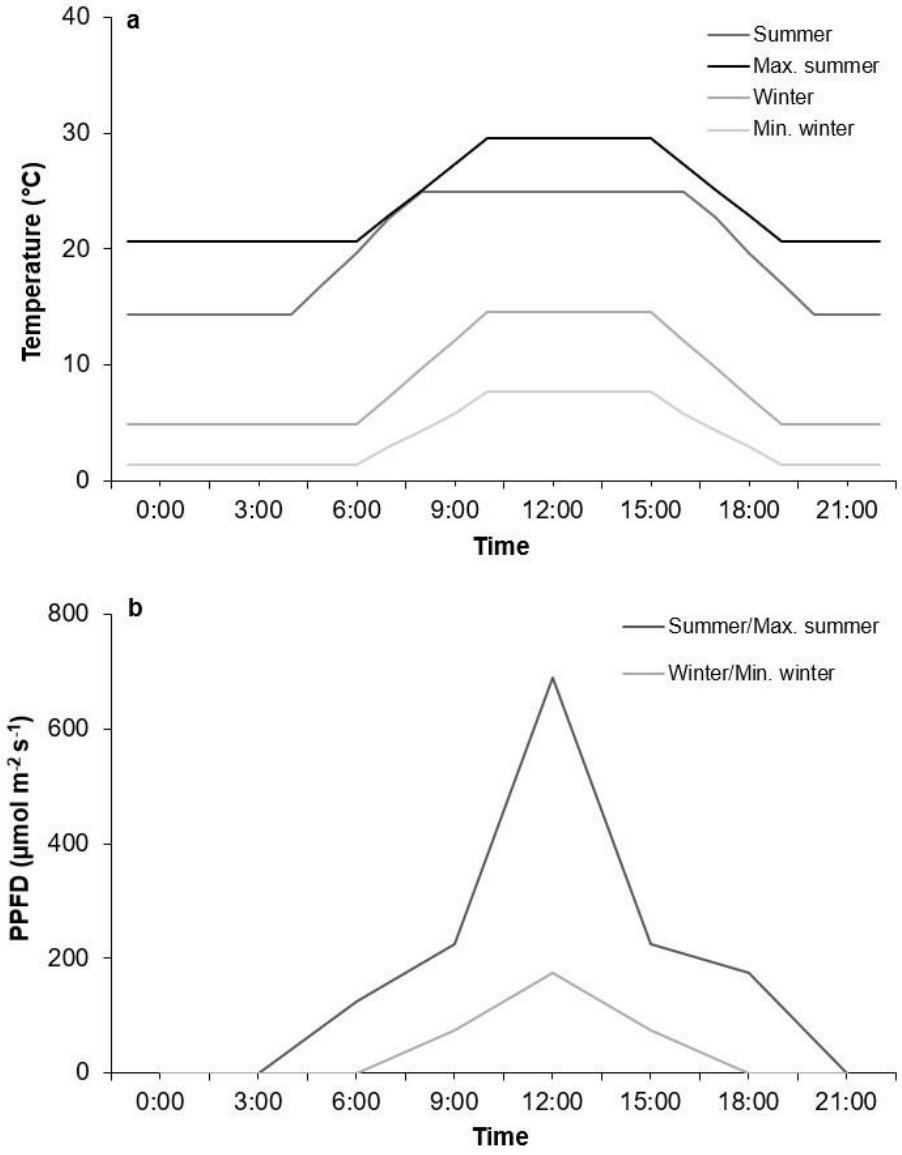

Figure 2.2 Daily temperature (°C) and light profiles (photosynthetic photon flux density (µmol m^{-2} sec^{-1}) used for ambient and maximum/minimum conditions in bioremediation performance trials.

Five replicate cultures of each cultivar (n = 55 cultures in total) were maintained in a temperature-controlled plant growth cabinet (Panasonic MLR-352) for each experiment. Cultures were maintained for 21 days for each of the summer and winter experiments, with a harvest every seven days. Cultures were then maintained in the cabinets for a further four days under max. summer conditions immediately following the summer experiment, and under min. winter conditions immediately following the winter experiment. Experiments

were conducted using the same methodology described above in the primary wastewater effluent growth trial, with the exception of temperature and light conditions.

A pulse amplitude-modulated (PAM) fluorometer (Junior-PAM, Heinz Walz GmbH, Effeltrich, Germany) was used to measure the maximal quantum yield (F_v/F_m) in each replicate at each harvest. F_v/F_m was measured as an estimate of the maximal photochemical PSII efficiency and to indicate the presence of stress in the algal cultures (Kromkamp et al., 2008; Figueroa et al., 2013). Measurements were taken immediately before each harvest (i.e. once every seven days) at approximately the same time for each experiment on biomass samples that had been dark-adapted for 15 minutes (Stirbet, 2011).

The bioremediation capabilities of cultivars were quantified by measuring concentrations of total ammoniacal-N (TAN), nitrate-N, and dissolved reactive phosphorous (DRP) in the initial and final wastewater of the final seven-day cultivation cycle for the summer and winter experiments, and for the max. summer and min. winter experiments. A 150 mL sample of diluted filtered primary wastewater was collected at the initial restock of each cultivation cycle on day 14 and day 21, and from each replicate at harvest on day 21 and day 25. All wastewater samples were stored in individual 250 mL clear plastic containers with lids (LabServ™). Total ammoniacal-N (sum of both molecular ammonia NH_3 and ionic ammonium NH_4^+), nitrate-N and DRP concentrations in each sample were measured using a HACH 900 (HACH, Loveland, CO, USA) spectrophotometer using the USEPA Nessler method (HACH method 8038), the nitrate-N cadmium reduction method (HACH method 8039), and the ascorbic acid method (HACH method 8048), respectively.

2.3.7 Statistical analysis

Biomass productivity and optimal quantum yield (F_v/F_m) measurements for summer and winter experiments were analysed using two factor repeated-measures analyses of variance (ANOVA) with cultivars and harvests as fixed factors. Biomass productivity and optimal quantum yield (F_v/F_m) measurements for max. summer and min. winter experiments were analysed using a one-way ANOVA with cultivars as a fixed factor. Nutrient concentrations for summer/max. summer experiments and winter/min. winter experiments were analysed using a one-way ANOVA with cultivars as a fixed factor. Data for each experiment were analysed separately. Normality was assessed using the Shapiro-Wilks

normality test. All analyses were conducted in SPSS Statistics (version 29). All data are reported as means ± S.E.

2.4 Results

2.4.1 Species collection, acclimation, cultivar isolation and growth trials

Of the 44 samples collected, 18 samples completed the acclimation step, providing a total of 66 species from 12 genera (Table 2.2). A greater number of samples were collected in summer (30 samples) compared to winter (14 samples). However, the range of species present across all samples collected within a season did not vary between seasons and an equal number of species from both seasons were successfully upscaled following acclimation. Summer species were primarily collected from the WWTP, while winter species were generally collected from wetlands. Most species that were found in river habitats had low survival rates. The most common genus identified within acclimated samples from summer and winter was *Oedogonium* with 12 isolates identified in samples collected from a range of habitats including drainage channels, wetlands, and within the WWTP. *Stigeoclonium* was also commonly found, with 11 isolates collected from a range of habitats. During acclimation, *Klebsormidium, Oedogonium, Stigeoclonium,* and *Ulothrix* were competitively dominant within the mixed sample cultures (Table 2.2). Following acclimation, 19 species were isolated and of these, nine species from six genera were further upscaled (Table 2.2). Cultivars of *Klebsormidium* sp. and *Cladophora* sp. collected in the summer had the highest growth rate during the isolation and upscale process. Eight of the nine cultivars that were successfully upscaled survived cultivation in the primary municipal wastewater (Table 2.2).

Table 2.2 Species composition (%) of freshwater filamentous macroalgal samples collected in summer (sample codes beginning with S) and winter (sample codes beginning with W) before and after acclimation. Cultivars which were successfully isolated, scaled up, and survived growth trials in primary municipal wastewater are indicated by x.

Sample	Cultivar	Species composition (%)		Species isolated	Species scaled up	Survived growth trials
		Day 0	Day 28			
S11	*Oedogonium* sp.	4	0			
	Rhizoclonium sp.	1	0			
	Stigeoclonium sp.	27	13			
	Ulothrix sp.	11	0			
	Klebsormidium sp. *(KLEB A)*	0	27	x	x	x
	Tribonema sp.	5	0			
	Microspora sp.	12	0			
	Vaucheria sp.	12	0			
	Spirogyra sp.	28	60	x		x
S12	*Stigeoclonium* sp.	25	39			
	Compsopogon sp.	75	61			
S13	*Oedogonium* sp.	30	15			
	Stigeoclonium sp.	36	0			
	Tribonema sp.	21	56			
	Microspora sp.	13	8			
	Vaucheria sp.	0	17			
	Spirogyra sp.	0	4			
S14	*Spirogyra* sp.	100	100	x		
S17	*Cladophora* sp.	94	96			
	Stigeoclonium sp.	6	4			
S21	*Cladophora* sp. *(CLAD)*	97	65	x	x	x
	Stigeoclonium sp.	0	13			
	Ulothrix sp.	0	22	x		
	Microspora sp.	3	0			

Sample	Cultivar	Species composition (%)	Species isolated	Species scaled up	Survived growth trials	Sample
S22	*Cladophora* sp.	100	50			
	Stigeoclonium sp.	0	28			
	Ulothrix sp.	0	17			
	Vaucheria sp.	0	5			
S24	*Oedogonium* sp.	0	27			
	Cladophora sp.	98	45			
	Ulothrix sp. *(ULOT)*	0	28	x	x	x
	Spirogyra sp.	2	0			
S25	*Oedogonium* sp.	12	69			
	Rhizoclonium sp.	88	17			
	Stigeoclonium sp.	0	14			
S27	*Oedogonium* sp.	74	100			
	Rhizoclonium sp.	26	0			
S28	*Oedogonium* sp.	0	23			
	Stigeoclonium sp.	0	4			
	Klebsormidium sp. *(KLEB B)*	0	44	x	x	x
	Tribonema sp.	0	5			
	Vaucheria sp.	99	21			
	Spirogyra sp.	1	3			
W36	*Oedogonium* sp. *(OEDO C)*	60	75	x	x	x
	Oedogonium sp.	40	25	x		
W38	*Oedogonium* sp. *(OEDO D)*	44	70	x	x	x
	Cladophora sp.	46	0			
	Klebsormidium sp.	10	30			
W44	*Oedogonium* sp.	0	5			
	Spirogyra sp. *(SPIR)*	70	41	x	x	
	Zygnema sp.	30	44			
	Stigeoclonium sp.	0	10	x		

Sample	Cultivar	Species composition (%)	Species isolated	Species scaled up	Survived growth trials	Sample
W47	*Zygnema* sp.	100	100	x		
W50	*Oedogonium* sp.	22	31	x		
	Rhizoclonium sp.	31	36	x		
	Ulothrix sp.	0	4			
	Klebsormidium sp.	0	7			
	Microspora sp.	13	0			
	Merismopedia sp.	34	22	x		
W54	*Stigeoclonium* sp. *(STIG A)*	50	60	x	x	x
	Vaucheria sp.	50	40	x		
W56	*Oedogonium* sp.	0	16			
	Rhizoclonium sp.	0	22			
	Stigeoclonium sp. *(STIG B)*	0	51	x	x	x
	Microspora sp.	0	11			
	Compsopogon sp.	100	0			

2.4.2 Biomass productivity and photosynthetic efficiency

Biomass productivity varied significantly among cultivars and harvests under both summer and winter conditions, however, the cultivar with the highest productivity varied between harvests (Two-way ANOVA: summer: cultivar x harvest, $F_{20,88} = 30.1$, $p = <0.001$, winter: cultivar x harvest, $F_{20,88} = 5.2$, $p = <0.001$). Biomass productivity also varied significantly among cultivars under both max. summer (One-way ANOVA: $F_{9,40} = 64.1$, $p = <0.001$) and min. winter conditions (One-way ANOVA: $F_{10,44} = 7.3$, $p = <0.001$). Biomass productivity was 233 % higher on average across all cultivars under summer conditions compared to winter conditions, followed by max. summer, winter and min. winter conditions (Figure 2.3a). *Oedogonium* sp. *(OEDO A)* had the highest productivity under summer conditions at 6.85 g m^{-2} DW day^{-1} (± 0.36) and *Oedogonium* sp. *(OEDO C)* had the highest productivity under max. summer conditions at 7.29 g m^{-2} DW day^{-1} (± 0.28). *Klebsormidium* sp. *(KLEB B)* had the highest productivity under winter conditions at 2.01 g m^{-2} DW day^{-1} (± 0.36) and *Stigeoclonium* sp. *(STIG A)* had the highest productivities under min. winter

conditions at 1.65 g m^{-2} DW day^{-1} (± 0.32). Average biomass productivity across all cultivars declined under max. summer conditions by 12% compared to summer conditions. All cultivars except *Klebsormidium* sp. (*KLEB A*), *Oedogonium* sp. *(OEDO A, OEDO C, OEDO D)* and *Ulothrix* sp. declined in biomass productivity under max. summer conditions compared to summer conditions. Similarly, average biomass productivity across all cultivars declined under min. winter conditions by 92% compared to winter conditions. All cultivars except for *Klebsormidium* sp. (*KLEB B*), *Oedogonium* sp. *(OEDO B), Stigeoclonium* sp. *(STIG A, STIG B)* and *Ulothrix* sp. (Table 2.3) had biomass die-off under min. winter conditions.

Optimal quantum yields varied significantly among harvests; however, this variation was not consistent among cultivars under both summer and winter conditions (Two-way ANOVA: summer: cultivar x harvest, $F_{30, 132} = 3.5$, $p = <0.001$, winter: cultivar x harvest, $F_{30, 132} = 5.8$, $p = <0.001$). Optimal quantum yields also varied significantly among cultivars under max. summer and min. winter conditions (One-way ANOVA: max. summer: cultivar, $F_{9,40} = 7.5$, $p = <0.001$, min. winter: cultivar, $F_{10,44} = 59.7$, $p = <0.001$) (Figure 2.3b). Across all cultivars, optimal quantum yields were the highest on average under summer conditions (0.7) and lowest on average under min. winter conditions (0.6).

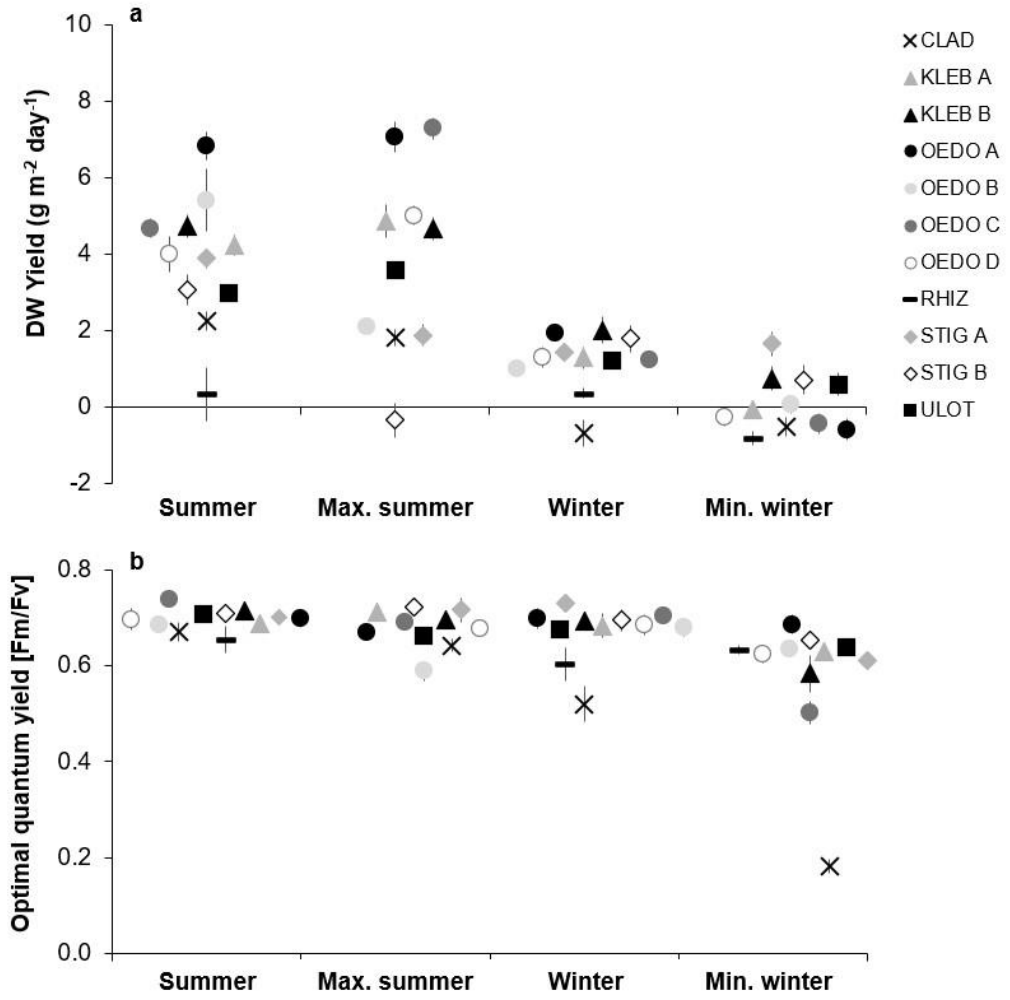

Figure 2.3 Mean (±S.E.) biomass productivity (a) and optimal quantum yield (Fm/Fv) of chlorophyll *a* fluorescence (b) of 11 cultivars of freshwater filamentous macroalgae during the four seasonal light and temperature treatments. Data are averages of all three harvests for summer and winter experiments, and one harvest for max summer and min winter experiments. N = 5.

2.4.3 Bioremediation performance

Total ammoniacal-N concentrations within the diluted primary wastewater (ratio of 1*:3* primary wastewater:freshwater) across all seasons ranged between 10.0 – 11.1 mg L^{-1}. Total ammoniacal-N concentrations in the primary wastewater post-harvest varied significantly among cultivars under all seasonal conditions (One-way ANOVA: summer: $F_{10,44} = 12.2$, $p = <0.001$, max. Summer: $F_{10,44} = 6.4$ $p = <0.001$, winter: $F_{10,44} = 12.2$, $p = <0.001$, min. Winter: $F_{10,44} = 85.8$, $p = <0.001$, Figure 2.4a, Table 2.3). Across all cultivars,

reductions in TAN were higher on average under summer conditions (92.4%) and max. summer conditions (99.1%), compared to colder conditions where there was a decline in TAN of 71.2% on average across cultivars under winter conditions and 45.1% under min. winter conditions. *Cladophora* sp. achieved the highest bioremediation performance under summer and max. summer conditions, reducing TAN concentrations by 94.7% to 0.56 mg L^{-1} (± 0.05) and 99.8% to 0.02 mg L^{-1} (± 0.01), respectively. *Klebsormidium* sp. *(KLEB B, KLEB A)* achieved the highest bioremediation performance under winter and min. winter conditions, reducing TAN concentrations by 99.9% to 0.01 mg L^{-1} (± 0.01) and 93.1% to 0.72 mg L^{-1} (± 0.06), respectively. Across all seasons, *Klebsormidium* sp. *(KLEB B)* had the highest bioremediation performance on average, reducing TAN by 94.8% to 0.6 mg L^{-1} (± 0.35).

Nitrate-N concentrations within the diluted primary wastewater (ratio of 1:3 primary wastewater:freshwater), across all seasons ranged between 0.8 – 1.1 mg L^{-1}. Nitrate-N concentrations in the primary wastewater post-harvest varied significantly among cultivars under all seasonal conditions (One-way ANOVA: summer: $F_{10,44} = 6.2$, $p = <0.001$, max. summer: $F_{10,44} = 3.6$, $p = 0.001$, winter: $F_{10,44} = 6.2$, $p = <0.001$, min. winter: $F_{10,44} = 4.1$, $p = 0.001$, Figure 2.4d, Table 2.3). Across all cultivars, reductions in nitrate-N were higher on average under summer conditions (64.8%) and max. summer conditions (52.8%) compared to colder conditions where there was an increase in nitrate-N concentrations of 35.8% on average across cultivars under winter conditions and 177.5% under min. winter conditions. *Oedogonium* sp. *(OEDO A)* achieved the highest bioremediation performance under summer conditions, reducing nitrate-N concentrations by 89.8% to 0.11 mg L^{-1} (± 0.05) and *Oedogonium* sp. *(OEDO D)* reduced nitrate-N concentrations under max. summer conditions by 90.2% to 0.08 mg L^{-1} (± 0.07). *Klebsormidium* sp. *(KLEB B)* achieved the highest reduction in nitrate-N concentrations under winter conditions by 63.9% to 0.34 mg L^{-1} (± 0.10). All cultivars had an increase in nitrate-N under min. winter conditions except *Cladophora* sp. which reduced nitrate-N concentrations by 27.3% to 0.54 mg L^{-1} (± 0.21). However, all cultivars under min. winter conditions showed a reduction in combined concentrations of TAN and nitrate-N except for *Rhizoclonium* sp. and *Oedogonium* sp. *(OEDO A, OEDO B)* (Figure 2.4b). Across all seasons, *Klebsormidium* sp. *(KLEB B)* had the highest bioremediation performance on average, reducing nitrate-N by 34.0% to 0.59 mg L^{-1} (± 0.27).

Dissolved reactive phosphorous concentrations within the diluted primary wastewater (ratio of 1:3 primary wastewater:freshwater), across all seasons ranged between 1.17 – 1.41 mg L^{-1}. Dissolved reactive phosphorous concentrations in the primary wastewater post-harvest varied significantly among cultivars under all seasonal conditions (One-way ANOVA: summer: $F_{10,44} = 3.5$, $p = <0.001$, max. summer: $F_{10,44} = 73.3$, $p = <0.001$, winter: $F_{10,44} = 3.5$, $p = <0.001$, min. winter: $F_{10,44} = 40.6$, $p = <0.001$, Figure 2.4c, Table 2.3). Across all cultivars, reductions in DRP were highest on average under summer conditions (95.9%), comparable under max. summer (87.0%) and winter conditions (86.8%) and lowest under min. winter conditions (60.2%). *Rhizoclonium* sp. *(RHIZ)* achieved the highest reduction in DRP concentrations under summer conditions by 98.7% to 0.02 mg L^{-1} (± 0.01). *Cladophora* sp. achieved the highest reduction in DRP concentrations under max. summer conditions by 98.2% to 0.02 mg L^{-1} (± 0.01). *Oedogonium* sp. *(OEDO C)* achieved the highest reduction in DRP concentrations under winter conditions by 93.6% to 0.06 mg L^{-1} (± 0.03) and *Oedogonium* sp. *(OEDO B)* achieved the highest reduction in DRP concentrations under min. winter conditions by 94.9% to 0.09 mg L^{-1} (± 0.03). Across all seasons, *Oedogonium* sp. *(OEDO B)* had the highest bioremediation performance on average, reducing DRP by 94.3% to 0.07 mg L^{-1} (± 0.03).

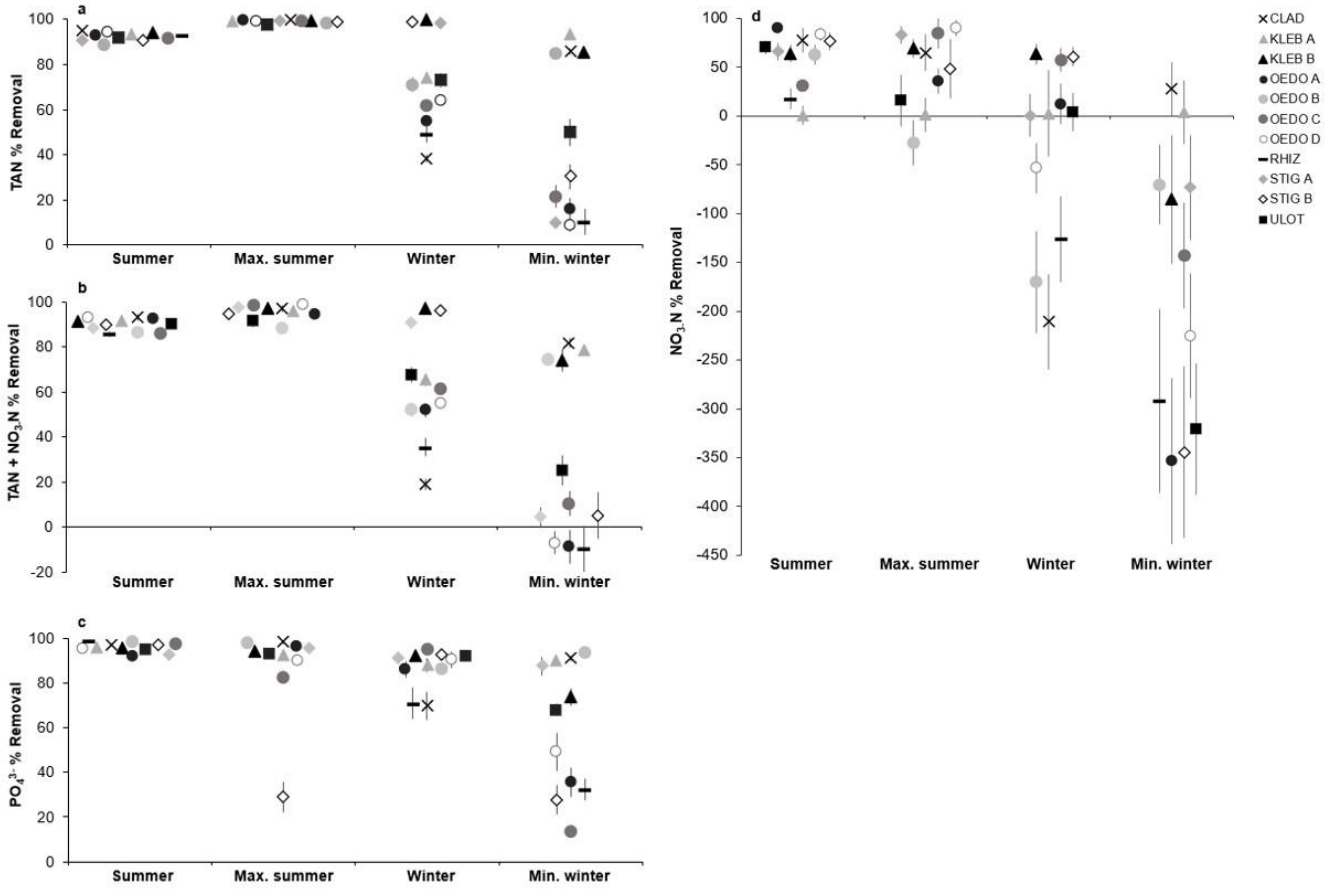

Figure 2.4 Mean (±S.E.) total ammoniacal-N (TAN % Removal) (a), total ammoniacal-N + nitrate-N (TAN + NO_3-N % Removal) (b), and dissolved reactive phosphorus (DRP % Removal) (c), nitrate-N (NO_3-N % Removal) (d) percentage of removal in the culture water of 11 cultivars of freshwater filamentous macroalgae when grown under four seasonal light and temperature treatments. N = 5.

Table 2.3 Summary of key biomass productivity (g m^{-2} day^{-1}) and nutrient concentration (mg L^{-1}) parameters for 11 cultivars of freshwater filamentous macroalgae grown under four seasonal light and temperature treatments. Summer and winter biomass data are averages of the 3 harvests for the summer and winter experiments. Summer and winter nutrient concentrations were measured at final harvest (21 days). Max. summer and min. winter biomass productivity and nutrient concentrations were measured at final harvest (25 days). Data are means ± standard error, N = 5.

Cultivar	**Biomass productivity (g m^{-2} day^{-1})**	**Nutrient concentration (mg L^{-1})**		
		TAN	**Nitrate-N**	**DRP**
Cladophora sp.				
Summer	2.25 ± 0.25	0.06 ± 0.05	0.24 ± 0.13	0.04 ± 0.01
Max. summer	1.81 ± 0.21	0.02 ± 0.01	0.30 ± 0.16	0.02 ± 0.01
Winter	-0.70 ± 0.35	6.86 ± 0.19	2.95 ± 0.46	0.35 ± 0.08
Min. winter	-0.53 ± 0.25	1.49 ± 0.13	0.54 ± 0.21	0.13 ± 0.01
Klebsormidium sp. *(KLEB A)*				
Summer	4.24 ± 0.26	0.71 ± 0.05	0.29 ± 0.10	0.05 ± 0.01
Max. summer	4.88 ± 0.43	0.09 ± 0.02	0.31 ± 0.15	0.10 ± 0.01
Winter	1.28 ± 0.27	2.89 ± 0.14	1.26 ± 0.42	0.14 ± 0.04
Min. winter	-0.08 ± 0.17	0.72 ± 0.06	1.67 ± 0.24	0.14 ± 0.02
Klebsormidium sp. *(KLEB B)*				
Summer	4.74 ± 0.31	0.63 ± 0.07	0.38 ± 0.10	0.05 ± 0.00
Max. summer	4.66 ± 0.31	0.04 ± 0.01	0.26 ± 0.09	0.08 ± 0.01
Winter	2.01 ± 0.36	0.01 ± 0.01	0.34 ± 0.10	0.09 ± 0.01
Min. winter	0.74 ± 0.32	1.53 ± 0.12	1.39 ± 0.49	0.37 ± 0.05
Oedogonium sp. *(OEDO A)*				
Summer	6.85 ± 0.36	0.77 ± 0.07	0.11 ± 0.05	0.10 ± 0.03
Max. summer	7.07 ± 0.40	0.04 ± 0.01	0.55 ± 0.11	0.05 ± 0.01
Winter	1.92 ± 0.18	4.98 ± 0.24	0.83 ± 0.20	0.16 ± 0.05
Min. winter	-0.60 ± 0.28	8.79 ± 0.49	3.03 ± 0.87	0.91 ± 0.09

Cultivar	Biomass productivity (g m^{-2} day^{-1})	Nutrient concentration (mg L^{-1})	Cultivar	Biomass productivity (g m^{-2} day^{-1})
Oedogonium sp. *(OEDO B)*				
Summer	5.42 ± 0.82	1.19 ± 0.03	0.39 ± 0.11	0.02 ± 0.01
Max. summer	2.10 ± 0.11	0.17 ± 0.01	1.08 ± 0.20	0.03 ± 0.01
Winter	1.01 ± 0.11	3.22 ± 0.33	2.57 ± 0.50	0.16 ± 0.03
Min. winter	0.06 ± 0.22	1.55 ± 0.08	1.28 ± 0.30	0.09 ± 0.03
Oedogonium sp. *(OEDO C)*				
Summer	4.68 ± 0.26	0.89 ± 0.06	0.73 ± 0.03	0.03 ± 0.01
Max. summer	7.29 ± 0.28	0.03 ± 0.01	0.13 ± 0.13	0.23 ± 0.04
Winter	1.22 ± 0.18	4.26 ± 0.23	0.40 ± 0.12	0.06 ± 0.01
Min. winter	-0.43 ± 0.27	8.20 ± 0.52	1.82 ± 0.41	1.22 ± 0.05
Oedogonium sp. *(OEDO D)*				
Summer	4.00 ± 0.47	0.60 ± 0.05	0.17 ± 0.07	0.06 ± 0.01
Max. summer	5.01 ± 0.25	0.07 ± 0.00	0.08 ± 0.07	0.13 ± 0.02
Winter	1.30 ± 0.27	4.00 ± 0.20	1.46 ± 0.24	0.11 ± 0.04
Min. winter	-0.27 ± 0.25	9.52 ± 0.29	2.44 ± 0.48	0.72 ± 0.12
Rhizoclonium sp.				
Summer	0.33 ± 0.70	0.79 ± 0.04	0.87 ± 0.11	0.02 ± 0.01
Max. summer				
Winter	0.35 ± 0.14	5.65 ± 0.39	2.15 ± 0.42	0.34 ± 0.08
Min. winter	-0.82 ± 0.19	9.36 ± 0.61	2.94 ± 0.71	0.95 ± 0.07
Stigeoclonium sp. *(STIG A)*				
Summer	3.88 ± 0.26	0.98 ± 0.04	0.36 ± 0.10	0.09 ± 0.02
Max. summer	1.87 ± 0.28	0.09 ± 0.01	0.14 ± 0.08	0.06 ± 0.01
Winter	1.42 ± 0.18	0.16 ± 0.06	0.94 ± 0.21	0.10 ± 0.01
Min. winter	1.65 ± 0.27	9.41 ± 0.19	1.30 ± 0.40	0.17 ± 0.06
Stigeoclonium sp. *(STIG B)*				
Summer	3.07 ± 0.39	0.96 ± 0.04	0.25 ± 0.10	0.04 ± 0.01
Max. summer	-0.36 ± 0.46	0.11 ± 0.01	0.44 ± 0.08	0.97 ± 0.09

Cultivar	Biomass productivity (g m^{-2} day^{-1})	Nutrient concentration (mg L^{-1})	Cultivar	Biomass productivity (g m^{-2} day^{-1})
Winter	1.78 ± 0.34	0.12 ± 0.06	0.37 ± 0.21	0.08 ± 0.01
Min. winter	0.70 ± 0.38	7.29 ± 0.19	3.33 ± 0.40	1.02 ± 0.09
Ulothrix sp.				
Summer	2.97 ± 0.13	0.84 ± 0.04	0.31 ± 0.07	0.06 ± 0.01
Max. summer	3.59 ± 0.08	0.23 ± 0.06	0.71 ± 0.23	0.09 ± 0.02
Winter	1.21 ± 0.21	2.99 ± 0.35	0.91 ± 0.19	0.10 ± 0.01
Min. winter	0.59 ± 0.31	5.23 ± 0.62	3.15 ± 0.51	0.45 ± 0.04

2.5 Discussion

A screening protocol was developed as a step-by-step guide and successfully applied to select freshwater filamentous macroalgal cultivars for nutrient bioremediation of primary municipal wastewater. As cultivar performance is dependent on genetic variability and the environment in which it grows naturally (Robinson et al., 2013), the WWTP was identified as a key habitat for sample collection. However, contrary to expectations, the cultivars which exhibited high biomass productivities during acclimation were collected from a range of habitats (e.g., agricultural drains, wetlands) as well as from the WWTP. Moreover, only four of the 11 cultivars collected from the WWTP progressed through to cultivar isolation and growth trials (*Cladophora* sp.*, Klebsormidium* sp. *(KLEB A), Stigeoclonium* sp. *(STIG A)* and *Ulothrix* sp.), and only two of those cultivars demonstrated high productivity and nutrient bioremediation performance across all seasons (*Stigeoclonium* sp. *(STIG A)* and *Ulothrix* sp.). This poorer than expected performance of cultivars collected from the WWTP could be due to the change in cultivation conditions between the WWTP and the acclimation step. The samples from the WWTP were collected from areas of low-flow wastewater where they were growing attached to a substrate. In contrast, during the acclimation samples were cultivated in artificial growth media in free floating (suspension) cultures under laboratory conditions. It is possible therefore that substrate and physiochemical conditions could have reduced the number of cultivars which were able to grow during the acclimation period (Pikosz et al., 2017). However, the acclimation may have provided the opportunity for cultivars from various aquatic environments (e.g., wetlands) to dominate as increased nutrient

concentrations generally stimulate growth of opportunistic algae (Krause-Jensen et al., 2007). The results of this study demonstrate that nutrient-rich aquatic environments such as WWTP's should not be assumed to produce cultivars which are suitable for nutrient removal of primary wastewater. Instead, various local aquatic environments beyond WWTP's, such as agricultural drains and wetlands, should also be sampled and surveyed to identify cultivars which exhibit superior biomass productivity and nutrient bioremediation performance in primary wastewater.

Optimum light and temperature conditions for algal growth are species-specific (Yun et al., 2014; Vadeboncoeur et al., 2021). Nutrient bioremediation performance of individual cultivars may also vary from season to season (Nguyen et al., 2022b) and previous research has recommended that bi-cultures (Li et al., 2021c) or monocultures with seasonal rotation of dominant cultivars is required to maximise stable biomass production for year-round cultivation (Valero-Rodriguez et al., 2020). Therefore, sample collection was undertaken during summer and winter to ensure a wide range of cultivars were collected that could grow under varying light and temperature conditions and increase the potential of identifying dominant cultivars for year-round nutrient bioremediation of primary wastewater. However, the same key genera were present in samples during summer and winter collections, with an equal number of cultivars from both seasons further selected for cultivar isolation and growth trials. Cultivars which demonstrated high nutrient bioremediation performance and biomass productivity were also collected during both seasons. However, in some instances, cultivars did not perform better under the seasonal conditions that corresponded to the season in which they were collected. For example, during the bioremediation performance trial under winter conditions, *Klebsormidium* sp. and *Ulothrix* sp. collected in summer outperformed cultivars collected during winter in both biomass productivity and bioremediation performance. Biomass productivity throughout bioremediation performance trials was highest under summer conditions across all cultivars regardless of which season samples were collected. In combination, these findings suggest that single season sampling may be sufficient to capture a diverse range of cultivars with the added benefit of saving on time and resources. However, if possible, and particularly in cases where diversity is low, it is important to sample during summer and winter to increase the number of samples collected and subsequently increase the probability of identifying a superior cultivar.

Monocultures require a cultivar to be competitively dominant, with high tolerance and adaptability to varying environmental conditions (Borowitzka, 2013; Liu et al., 2020). Therefore, the acclimation step was designed to identify cultivars that are competitively dominant when grown in a nutrient medium with high concentrations of ammonia-N comparable to a constant low flow of primary municipal wastewater. Notably, this medium differs significantly from the environment in which most cultivars would naturally occur. Patterns of cultivar dominance and response to the growth medium varied during the acclimation step. Some cultivars remained dominant from the time of collection through to the end of acclimation. Other cultivars which were not detected in the initial samples became abundant during acclimation and, in some cases, dominant upon completion of this phase. This occurred in a total of 10 samples collected across various sampling sites and was most common in cultivars from the genera *Oedogonium*, *Stigeoclonium*, and *Ulothrix*. This response is known to occur in natural environments where species lay dormant until favourable conditions arise (Brawley & Johnson, 1992). These findings indicate that cultivars which demonstrate competitive dominance in their natural habitat or upon sample collection may not remain dominant once acclimated within a nutrient medium with high concentrations of ammonia-N. Therefore, an acclimation period using a nutrient medium with high concentrations of ammonia-N is critical to identify cultivars that will be competitively dominant in nutrient-rich primary wastewater.

Nutrient bioremediation of TAN is a key indicator of algal bioremediation performance in primary wastewater where nitrogen exists mainly in the form of ammonium (NH_{4+}) along with low levels of nitrate (NO_3^-) and nitrite (NO_2^-) (Tchobanoglous et al., 2003; Ge & Champagne, 2017). Algae can utilize different forms of nitrogen; however, the preferred form of nitrogen differs between species (Silkina et al., 2017; Salbitani & Carfagna, 2021). Consequently, cultivar uptake rates of TAN and nitrate-N may vary, as was found during bioremediation performance trials with significant differences in uptake rates between cultivars in each season. Cultivar biomass productivity will also affect nitrogen assimilation across seasons. Estimations of nitrogen assimilation by *Klebsormidium* and *Oedogonium* cultivars were determined based on an average dry weight nitrogen content of 6.3% for *Klebsormidium* and 6.7% for *Oedogonium* (Lawton et al., 2021a). Estimations show that average nitrogen assimilation rates of *Klebsormidium* (summer: 78%, winter: 32%) and *Oedogonium* (summer: 94% and winter: 27%) declined by 59% and 71%, retrospectively

between summer and winter. Consequently, the lower TAN and nitrate-N removal rates recorded for most cultivars under winter conditions compared to summer conditions were most likely due to lower algal assimilation as a result of low growth and survivorship (Figure 2.5). An unexpected result during winter bioremediation performance trials was the higher concentration of nitrate-N measured in the diluted primary wastewater at the time of harvest compared to the beginning of the trial. Since assimilation of nutrients by algae declines as rates of photosynthesis reduce under colder and lower light conditions in winter, nitrate-N assimilation was likely slower than the rate of nitrate-N generation by bacterial nitrification, contributing to the higher nitrate-N concentrations at the time of harvest. These results were further exacerbated under min. winter conditions, as a further reduction in photosynthesis further reduced the assimilation of nutrients. Nitrate-N concentrations may have also increased under winter and min. winter conditions due to the aeration supplied to the culture vessels, further promoting nitrification (Lehtovirta-Morley, 2018; Cruz et al., 2019). However, across seasons biomass productivity did not directly relate to bioremediation performance as cultivars with low growth rates maintained high nutrient removal rates under min. winter conditions. These findings highlight the need to measure biomass productivity and nutrient bioremediation performance under a range of seasonal conditions to manage expectations around minimum bioremediation performance and allow design of management options for extreme weather conditions. Higher nutrient removal under summer conditions was most likely due to algal assimilation and ammonia volatilisation (Figure 2.5), which occurs when algal photosynthetic activity elevates daytime pH above 9.0 (Park & Craggs, 2011; Craggs et al., 2014). During summer an additional consideration is the reduced activity of nitrifying bacteria. These are highly sensitive to pH and are inhibited at pH > 9.0 (Sutherland et al., 2015a). Consequently, nitrification can become inhibited under summer conditions due to high daytime culture pH, leading to relatively higher concentrations of ammonia-N than nitrate-N in summer compared to winter. This highlights the need to measure both TAN and nitrate-N/nitrite-N during seasonal trials to ensure that correct estimates of the total amount of dissolved inorganic nitrogen bioremediation are obtained.

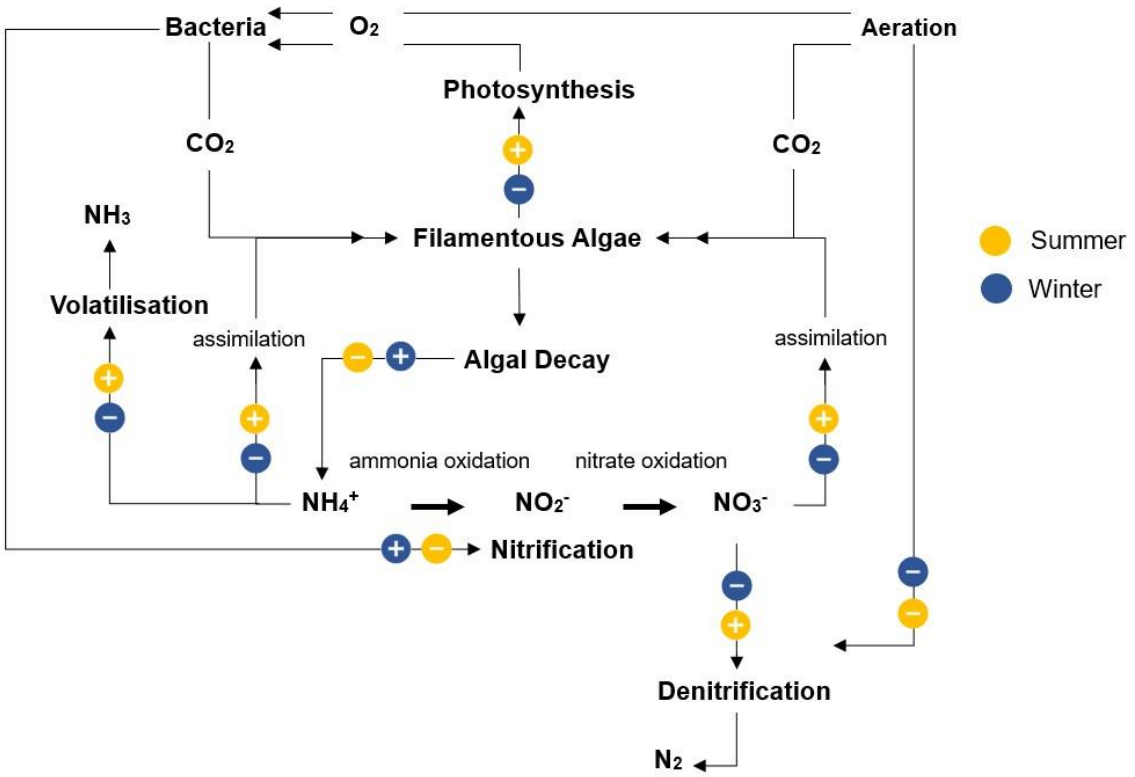

Figure 2.5 Schematic diagram of the nitrogen (N) cycle in algal cultures, depicting which processes increase (+) and decrease (-) under summer and winter conditions.

Previous studies assessing bioremediation performance of common macroalgal genera have identified *Oedogonium* as a target genus for nutrient bioremediation of municipal wastewater (Lawton et al., 2013a; Lawton et al., 2021a). Two cultivars of *Oedogonium (OEDO A, OEDO C)* achieved the highest biomass productivity under summer and max. summer conditions. Two cultivars of *Oedogonium* (*OEDO A, ODEO D)* also achieved the highest bioremediation of nitrate-N under summer and max. summer conditions. However, nutrient bioremediation and growth of all *Oedogonium* sp. declined under winter and min. winter conditions (1.92 to -0.60 g m^{-2} DW day^{-1}) compared to summer and max. summer conditions (7.29 to 2.10 g m^{-2} DW day^{-1}). These findings correspond to previous studies which have identified *Oedogonium* sp. as a dominant summer cultivar with high biomass productivity (Hariz et al., 2022, 2023) and high removal of nitrate-N in wastewater effluent under summer conditions (Lawton et al., 2021a), and variable growth and biomass die off during colder conditions (< 10°C) (Lawton et al., 2014; Cole et al., 2018). *Klebsormidium* sp. *(KLEB B)* and *Stigeoclonium* sp. *(STIG A)* achieved the highest biomass productivity under winter and min. winter conditions, respectively. *Klebsormidium* sp. *(KLEB B, KLEB A)* also

achieved the highest bioremediation of TAN under both winter and min. winter conditions in addition to the highest nitrate-N removal under winter conditions. *Klebsormidium* sp. and *Stigeoclonium* sp. are known to be highly tolerant of colder climates and often outperform other cultivars during winter and min. winter conditions (Borchhardt & Gründling-Pfaff, 2020). *Cladophora* sp. achieved the highest removal of TAN under summer and max. summer conditions and was the only cultivar that reduced nitrate-N concentrations under min. winter conditions. Previous research has recognised *Cladophora* sp. for year-round growth and ability to uptake different forms of nitrogen from wastewater (Ross et al., 2018). Overall, *Klebsormidium* sp. *(KLEB B)*, *Stigeoclonium* sp. *(STIG A)* and *Ulothrix* sp. were identified as top performing cultivars suitable for the year-round nutrient bioremediation of primary municipal wastewater based on their ability to maintain high growth rates and high nutrient removal rates across all seasons.

This is the first screening protocol to be developed to select target cultivars of filamentous freshwater macroalgae for bioremediation of primary municipal wastewater. The screening protocol ensures applicability and consistency from the point of sample collection to completion of growth trials by providing a step-by-step guide to identify target cultivars. The simple methodology outlined within the protocol allows for ease of replication. Future protocols could include testing effluents from common pre-treatments, allowing for broad application of the screening protocol without the need for individual testing at each WWTP. Each phase of the protocol uses cost effective and easily accessible experimental equipment. Sampling locations are the decision of the individual conducting the protocol rather than a feature of the protocol itself and thus the protocol is therefore inclusive of all local habitats. The protocol ensures that the target cultivar is robust and possesses key attributes e.g., competitive dominance, high biomass productivity and bioremediation performance under local, seasonal, and extreme conditions. Overall, this screening protocol has been proven successful through application to identify target cultivars which can maintain a monoculture for year-round nutrient bioremediation of primary municipal wastewater.

2.6 Conclusion

The screening protocol outlined in this study successfully identified filamentous freshwater macroalgal cultivars for nutrient bioremediation of primary municipal wastewater.

The screening protocol identified the need to sample from a range of habitats, to undertake growth trials under extreme seasonal conditions and measure TAN and nitrate-N concentrations when analysing bioremediation performance. In general, biomass productivity and bioremediation performance were highest across all cultivars under summer and max. summer conditions. However, biomass productivity was not directly related to bioremediation performance. Based on overall growth and nutrient bioremediation performance under all seasonal and extreme conditions, the screening protocol successfully identified *Klebsormidium* sp. *(KLEB B), Stigeoclonium* sp. *(STIG A)* and *Ulothrix* sp. as potential candidates for year-round monoculture cultivation for nutrient bioremediation of primary municipal wastewater. However, HRFAP environments are highly-variable and cultivar performance under laboratory conditions is not always indicative of cultivar performance within outdoor systems. Therefore, the next step to confirm the suitability of these cultivars is to conduct competition experiments in outdoor pond trials in primary municipal wastewater to ensure that cultivars can maintain monocultures within a HRFAP system. Once implemented at scale, optimal operational parameters can be determined to further increase biomass productivities and bioremediation performance of target cultivars.

2.7 Appendix

Table 2A.1: Freshwater filamentous macroalgal sample collection data

Sample	Collection date	Season	Site	Latitude	Longitude	Habitat description
1	18/02/2021	Summer	Te Puke WWTP	37° 78' 26" S	176° 33' 72" E	Clarifier 1
2	18/02/2021	Summer	Te Puke WWTP	37° 78' 26" S	176° 33' 72" E	Clarifier 1
3	18/02/2021	Summer	Te Puke WWTP	37° 78' 24" S	176° 33' 78" E	Attached to wall of the UV outlet
4	18/02/2021	Summer	Te Puke WWTP	37° 78' 21" S	176° 33' 72" E	Clarifier 3
5	18/02/2021	Summer	Drainage channel	37° 78' 15" S	176° 33' 68" E	Open channel growing on submerged vegetation
6	18/02/2021	Summer	Te Puke WWTP	37° 78' 01" S	176° 33' 77" E	Open channel growing on submerged vegetation
7	18/02/2021	Summer	Kaituna River, Rangiuru	37° 78' 24" S	176° 36' 30" E	Free floating in the water
8	18/02/2021	Summer	Te Puke WWTP	37° 78' 26" S	176° 33' 72" E	Clarifier 1
9	18/02/2021	Summer	Te Puke WWTP	37° 78' 21" S	176° 33' 72" E	Clarifier 3
10	18/02/2021	Summer	Te Puke WWTP	37° 78' 21" S	176° 33' 72" E	Clarifier 3
11	18/02/2021	Summer	Te Puke WWTP	37° 78' 24" S	176° 33' 72" E	Clarifier 2
12	18/02/2021	Summer	Kaituna River, Rangiuru	37° 79' 29" S	176° 36' 30" E	Free floating in the water and growing on submerged vegetation

Sample	Collection date	Season	Site	Latitude	Longitude	Habitat description
13	18/02/2021	Summer	Te Puke WWTP	37° 78' 22" S	176° 33' 89" E	WTTP discharge point, under long grass
14	18/02/2021	Summer	Te Puke WWTP	37° 78' 24" S	176° 33' 72" E	Clarifier 2
15	18/02/2021	Summer	Te Puke WWTP	37° 78' 24" S	176° 33' 72" E	Clarifier 2
16	18/02/2021	Summer	Te Puke WWTP	37° 78' 21" S	176° 33' 72" E	Clarifier 3
17	18/02/2021	Summer	Te Puke WWTP	37° 78' 24" S	176° 33' 78" E	Attached to wall of the UV outlet
18	18/02/2021	Summer	Te Puke WWTP	37° 78' 24" S	176° 33' 78" E	Attached to wall of the UV outlet
19	18/02/2021	Summer	Kaituna River, Rangiuru	37° 79' 29" S	176° 36' 30" E	Free floating in the water
20	18/02/2021	Summer	Te Puke WWTP	37° 78' 22" S	176° 33' 89" E	WTTP discharge point, under long grass
21	25/02/2021	Summer	Te Puke WWTP	37° 78' 24" S	176° 33' 78" E	Attached to wall of the UV outlet
22	25/02/2021	Summer	Te Puke WWTP	37° 78' 24" S	176° 33' 78" E	Attached to wall of the UV outlet
23	25/02/2021	Summer	Kaituna River, Rangiuru	37° 79' 29" S	176° 36' 30" E	Free floating in the water
24	25/02/2021	Summer	Te Puke WWTP	37° 78' 24" S	176° 33' 78" E	Attached to wall of the UV outlet
25	25/02/2021	Summer	Stormwater Wetland	37° 73' 71" S	176° 35' 20" E	Stormwater pond, free-floating among vegetation and debris on the side of the pond.
26	25/02/2021	Summer	Kaituna River, Rangiuru	37° 79' 29" S	176° 36' 30" E	Free floating in the water

Sample	Collection date	Season	Site	Latitude	Longitude	Habitat description
27	25/02/2021	Summer	Stormwater Wetland	37° 73' 71" S	176° 35' 20" E	Stormwater pond, free-floating among vegetation and debris on the side of the pond.
28	25/02/2021	Summer	Drainage channel	37° 84' 66" S	176° 35' 81" E	Open channel, free floating in the water
29	25/02/2021	Summer	Kaituna River, Rangiuru	37° 74' 40" S	176° 35' 96" E	Free floating in the water and growing on submerged vegetation
30	25/02/2021	Summer	Kaituna River, Rangiuru	37° 79' 29" S	176° 36' 30" E	Free floating in the water and growing on submerged vegetation
32	29/06/2021	Winter	Kaituna River, Rangiuru	37° 79' 29" S	176° 36' 30" E	Free floating in the water
34	29/06/2021	Winter	Te Puke WWTP	37° 78' 24" S	176° 33' 78" E	Attached to wall of the UV outlet
35	29/06/2021	Winter	Te Puke WWTP	37° 78' 24" S	176° 33' 72" E	Clarifier 2
36	29/06/2021	Winter	Drainage channel	37° 84' 66" S	176° 35' 81" E	Open channel, free floating in the water
38	20/07/2021	Winter	Kaituna Wetland	37° 76' 72" S	176° 37' 42" E	Wetland pond, free floating/growing on submerged vegetation
39	20/07/2021	Winter	Kaituna Wetland	37° 75' 81" S	176° 37' 38" E	Attached to rock along a pond
43	20/07/2021	Winter	Drainage channel	37° 76' 34" S	176° 41' 30" E	Open drainage channel, free floating in the water
44	20/07/2021	Winter	Kaituna Wetland	37° 75' 16" S	176° 37' 42" E	Growing on grass as a mat

Sample	Collection date	Season	Site	Latitude	Longitude	Habitat description
45	20/07/2021	Winter	Kaituna Wetland	37° 75' 17" S	176° 37' 41" E	Wetland pond, free floating in the water and growing on submerged vegetation
47	20/07/2021	Winter	Kaituna Wetland	37° 75' 17" S	176° 37' 41" E	Wetland pond, free floating in the water
50	20/07/2021	Winter	Kaituna Wetland	37° 75' 68" S	176° 37' 42" E	Growing on grass as a mat
54	20/07/2021	Winter	Drainage channel	37° 84' 66" S	176° 35' 81" E	Open channel, free floating in the water
55	20/07/2021	Winter	Drainage channel	37° 76' 38" S	176° 41' 54" E	Open drainage channel, free floating in the water
56	20/07/2021	Winter	Drainage channel	37° 76' 38" S	176° 41' 55" E	Open drainage channel, free floating in the water

Chapter 3 - Productivity and competitive dominance of freshwater filamentous macroalgal cultivars for nutrient bioremediation of primary municipal wastewater

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3.1 Abstract

Cultivar selection is fundamental to the performance of any high-rate algal pond monoculture system. Therefore, this study compared the biomass productivity and bioremediation performance of the three freshwater filamentous cultivars *Klebsormidium flaccidum*, *Oedogonium calcareum*, and *Oedogonium* sp. in primary municipal wastewater in outdoor high-rate filamentous algal pond mesocosms. *Klebsormidium flaccidum* had the highest biomass productivity (3.09 g dry weight m^{-2} day^{-1} ± 0.20 SE across all harvests), followed by *Oedogonium* sp. (0.99 g dry weight m^{-2} day^{-1} ± 0.29 SE across all harvests), while the complete die-off of *O. calcareum* occurred by day 8. Bioremediation performance was highest on average by *K. flaccidum*, reducing TAN concentrations by 51% to 14.8 mg L^{-1} (± 0.81 SE), nitrate-N concentrations by 59% to 0.30 mg L^{-1} (± 0.02 SE) and DRP concentrations by 15% to 3.52 mg L^{-1} (± 0.07 SE). Water quality variables also showed improvement, with *K. flaccidum* achieving the greatest reductions in total suspended solids (54%), carbonaceous biochemical oxygen demand (93%) and chemical oxygen demand (74%), while *K. flaccidum* and *Oedogonium* sp. both reduced *Escherichia coli* concentrations by 1.7 and 1.48 log_{10}, respectively. Competitive dominance of *K. flaccidum* and *Oedogonium* sp. was subsequently assessed in bi-cultures initially stocked at equal proportions of both cultivars at three stocking densities. By day 12, the proportion of *K. flaccidum* had increased to 64% (± 6.1 SE) and 73% (± 5.0 SE) at a stocking density of 0.25 g and 0.5 g FW L^{-1}, respectively. Based on superior biomass productivity, bioremediation performance and competitive dominance, *K. flaccidum* was identified as a key target cultivar for bioremediation of primary municipal wastewater.

3.2 Introduction

High-rate algal ponds (HRAPs) are common outdoor pond systems utilised to improve municipal wastewater treatment (Craggs et al., 2014; Leong et al., 2021). These shallow, mixed raceway-based systems are designed to maximise algal biomass growth, microbial activity, and wastewater treatment efficiencies (Young et al., 2017; Saravanan et al., 2021; Oruganti et al., 2022). Algal-bacterial symbiosis occurs in HRAPs as algae utilise CO_2 for photosynthesis, assimilate nutrients into biomass, and release oxygen which is utilised by bacteria for oxidising organic matter and ammonia (Oruganti et al., 2022). HRAPs were originally designed for microalgal wastewater treatment (Nurdogan & Oswald, 1995). However, a major operational issue with HRAPs is the challenge and cost associated with harvesting microalgal biomass (Singh & Patidar, 2018; Lane, 2022). While much research has focused on ways to improve microalgal harvestability (Park et al., 2013b), the cultivation of filamentous freshwater macroalgae in these systems has been proposed as an alternative solution (Liu et al., 2020).

The use of freshwater filamentous macroalgal monocultures for wastewater treatment in HRAPs is a developing area of research (Liu et al., 2020). Monocultures in HRAPs are often preferred to bi-cultures and poly-cultures as they may offer greater ability to control and predict treatment performance through more consistent nutrient removal rates and biomass yields with more uniform biochemical composition (Liu et al., 2020; Sutherland & Ralph, 2020). However, full-scale implementation of algal-based monoculture wastewater treatment systems can be challenging as large, outdoor pond systems are susceptible to contamination by wild algal strains (Newby et al., 2016). Therefore, target cultivars must achieve higher productivity compared to undesired species (Liu et al., 2020) to remain unialgal and thereby maintain consistent nutrient removal rates and prevent variation in biomass composition (Sutherland & Ralph, 2020). Furthermore, municipal wastewater is highly variable as unknown pollutants within the influent can potentially lead to cultivar die-off (Petrie, 2015). Therefore, target cultivars must be highly tolerant and able to withstand diurnal fluctuations in municipal wastewater composition (Gao et al., 2023). Hence, cultivar resilience is critical to the successful operation and stability of full-scale algal-based monoculture systems. Cultivar selection processes therefore must include trials in open outdoor pond mesocosms to identify cultivars that remain productive and are competitively dominant when exposed to invasion pressure and outdoor environmental conditions (Nalley et al., 2014).

To date, two studies have measured the competitive dominance of filamentous algae. *Oedogonium* was highly dominant compared to *Cladophora* and *Spirogyra* when grown in outdoor free-floating (tumble) bi-cultures and mixed polycultures (Lawton et al., 2013a). Specific growth rates of *Oedogonium* were also higher in mixed cultures compared to monoculture, demonstrating that while fast growth rates are expected to provide a competitive advantage (Borowitzka, 1992), monoculture growth rates are not an accurate measure of a target cultivar's competitive ability in the presence of other cultivars (Lawton et al., 2013a). *Oedogonium* (tropical strain), and *Stigeoclonium,* and *Hyalotheca* (both temperate strains) were also compared in competition experiments under typical seasonal conditions in the laboratory (Valero-Rodriguez et al., 2020). Growth of cultivars was highest under seasonal conditions corresponding to environments where cultivars originated, with *Oedogonium* dominant under warmer conditions, while *Stigeoclonium* was dominant under cooler conditions (Valero-Rodriguez et al., 2020). Based on these results, it was concluded that stable large open culture systems could be maintained through a seasonal rotation of monocultures, or continuous cultivation of bi-cultures of the most dominant cultivars (Valero-Rodriguez et al., 2020). However, establishing a dominant monoculture might be possible by selecting local cultivars which have been well-adapted to local climatic conditions (de Paula Silva et al., 2012; Bao et al., 2022). Although these studies have highlighted the importance of conducting competition experiments in the preliminary stages of cultivar selection for monoculture cultivation, they were conducted on a small scale (1 L bottles – 20 L buckets) and algae were grown in dechlorinated tap water enriched with nutrient media. Therefore, cultivar growth and response to competition may vary when cultivated in municipal wastewater within a large-scale, open outdoor pond system such as a HRAP. Unlike most laboratory settings, HRAPs are constantly varying environments with limited control over environmental factors and constant exposure to potential contamination by wild algal strains, which can enter by wind or through the inflow of wastewater (Borowitzka & Moheimani, 2013). Therefore, measuring target cultivar dominance and performance within an outdoor system is essential for maintaining an effective and long-lasting HRAP monoculture (Shukla et al., 2018).

Cultivar selection is essential for optimising algal bioremediation performance in HRAP systems, making it necessary to understand how different cultivars perform within these pond systems. While existing studies suggest that monocultures can provide consistent

nutrient removal (Valero-Rodriguez et al., 2020; Novak et al., 2024), there is limited research on their effectiveness in open outdoor HRAP systems, particularly concerning how target cultivars tolerate fluctuating conditions when cultivated in primary wastewater. This study aims to address these gaps by assessing the competitive dominance and resilience of various algal cultivars in outdoor high-rate filamentous algal pond (HRFAP) monocultures, with a focus on their ability to maintain effective nutrient removal under variable conditions. This study builds on recent research that developed a screening protocol to select cultivars of freshwater filamentous macroalgae for nutrient bioremediation of primary municipal wastewater (Novak et al., 2024). Based on that protocol, three freshwater filamentous cultivars – *Klebsormidium flaccidum*, *Oedogonium calcareum*, and *Oedogonium* sp. - were identified as potential target cultivars for primary municipal wastewater treatment in HRFAP monocultures due to their competitive dominance, high biomass productivity and bioremediation performance under local seasonal and extreme conditions (Novak et al., 2024). The objective of this study was to further quantify the growth, bioremediation performance, and competitive dominance of these target cultivars treating primary wastewater in outdoor HRFAP systems. The specific aims of this study were (i) to compare cultivar growth and nutrient removal rates when cultivated in outdoor HRFAP monocultures, and (ii) to compare the dominance of cultivars when cultivated in outdoor HRFAP bi-cultures.

3.3 Methods

Three freshwater filamentous macroalgal cultivars - *Klebsormidium flaccidum*, *Oedogonium calcareum*, and *Oedogonium* sp. (Figure 3.1) were selected for these experiments due to their superior nutrient removal capabilities and greater growth potential compared to other cultivars (Novak et al., 2024). DNA barcoding was used to identify *Klebsormidium flaccidum* (Appendix 3.7, Section 3.7.1) and *Oedogonium calcareum* (Lawton et al., 2021a). *Oedogonium* sp. was identified to genus level using morphological characters, but it was not possible to identify this cultivar to species level as target DNA barcoding regions could not be successfully amplified (Appendix 3.7, Section 3.7.2). Prior to experiments, cultivars were grown in a nutrient medium made from filtered dechlorinated tap water in outdoor 1,000 L tanks for at least three months at the Facility for Aquaculture Research of Macroalgae, University of Waikato Coastal Marine Field Station, Tauranga, New Zealand. Cultures were stocked at 0.5 g fresh weight (FW) L^{-1} and grown in batch culture

with weekly water changes. The concentrations of nutrients in the medium were based on diluted primary wastewater concentrations of 5 mg NH_4^-N L^{-1} (NH_4Cl), 1.3 mg PO_4^-P L^{-1} ($NaH_2PO_4 \cdot 2H_2O$) and trace metal concentrations ($FeCl_3 \cdot 6H_2O$, $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $Na_2MoO_4 \cdot 2H_2O$) using analytical grade chemicals as per F/2 growth medium (Ryther & Guillard, 1962; Guillard, 1975). The nutrient medium had a pH of 5.6 after dilution.

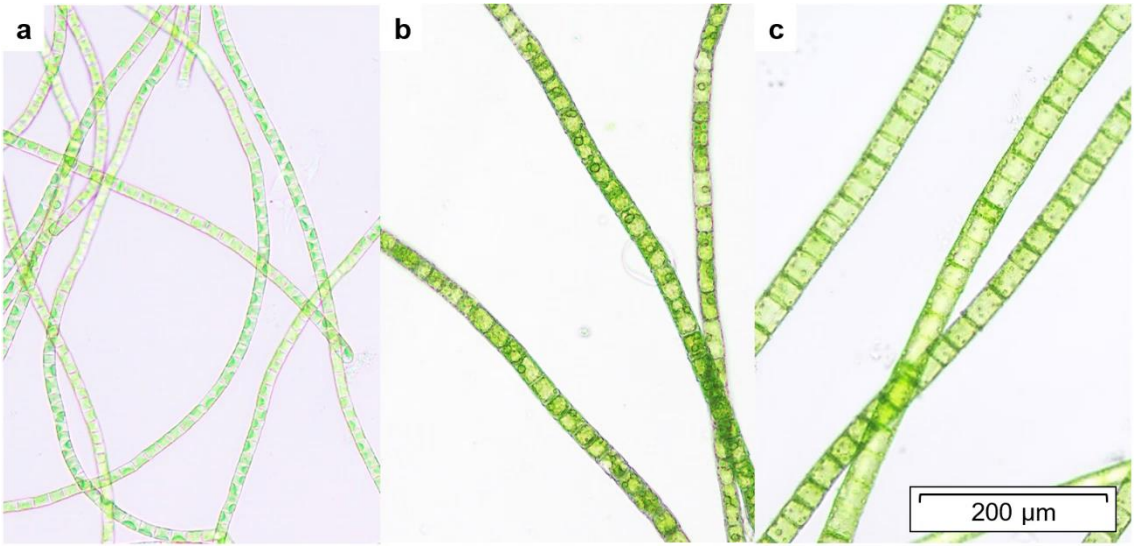

Figure 3.1 Microscopic images of freshwater filamentous algae cultivars used in this study - (a) *K. flaccidum*, (b) *O. calcareum*, and (c) *Oedogonium* sp.

3.3.1 Study site

Experiments were conducted at the Te Puke municipal wastewater treatment plant (WWTP), located in the Bay of Plenty Region of New Zealand. The Te Puke municipal WWTP currently services a population of approximately 8,100 people and treats an annual average daily flow of 1,800 m^3 day^{-1}. The municipal WWTP primary wastewater used throughout this experiment was taken after solids sedimentation. Average water quality variables of the primary treated wastewater were: 106 mg L^{-1} total suspended solids (TSS), 177 mg L^{-1} carbonaceous biological oxygen demand ($cBOD_5$), 43.0 mg L^{-1} total ammoniacal-N (TAN), 1.1 mg L^{-1} nitrate-N (NO_3^-N), and 4.5 mg L^{-1} dissolved reactive phosphorus (DRP). Prior to experiments, all cultivars were acclimated to primary wastewater by initially growing them at the WWTP for four days in primary wastewater that had been diluted 1:1 with dechlorinated tap water and then for a further four days in 100% primary wastewater.

3.3.2 High-rate filamentous algal pond (HRFAP) mesocosms

Experiments were conducted in nine HRFAP mesocosms (Figure 3.2). Each HRFAP consisted of a plastic 113 L trough (Base: 80 cm L x 54 cm W, Top: 88 cm L x 62 cm W, Height: 31 cm) filled with 70 L (approximate water depth 24 cm) of primary wastewater. HRFAPs were maintained with a hydraulic retention time of four days, where half (35 L) of the culture water was removed every two days and refilled to 70 L with primary wastewater from the WWTP. The primary wastewater was continuously circulated within each HRFAP by a 3-bladed stainless steel paddle wheel rotating at a speed of 8 rpm and standpipes were fitted into each HRFAP to maintain pond water volume by enabling overflow of accumulated rainfall.

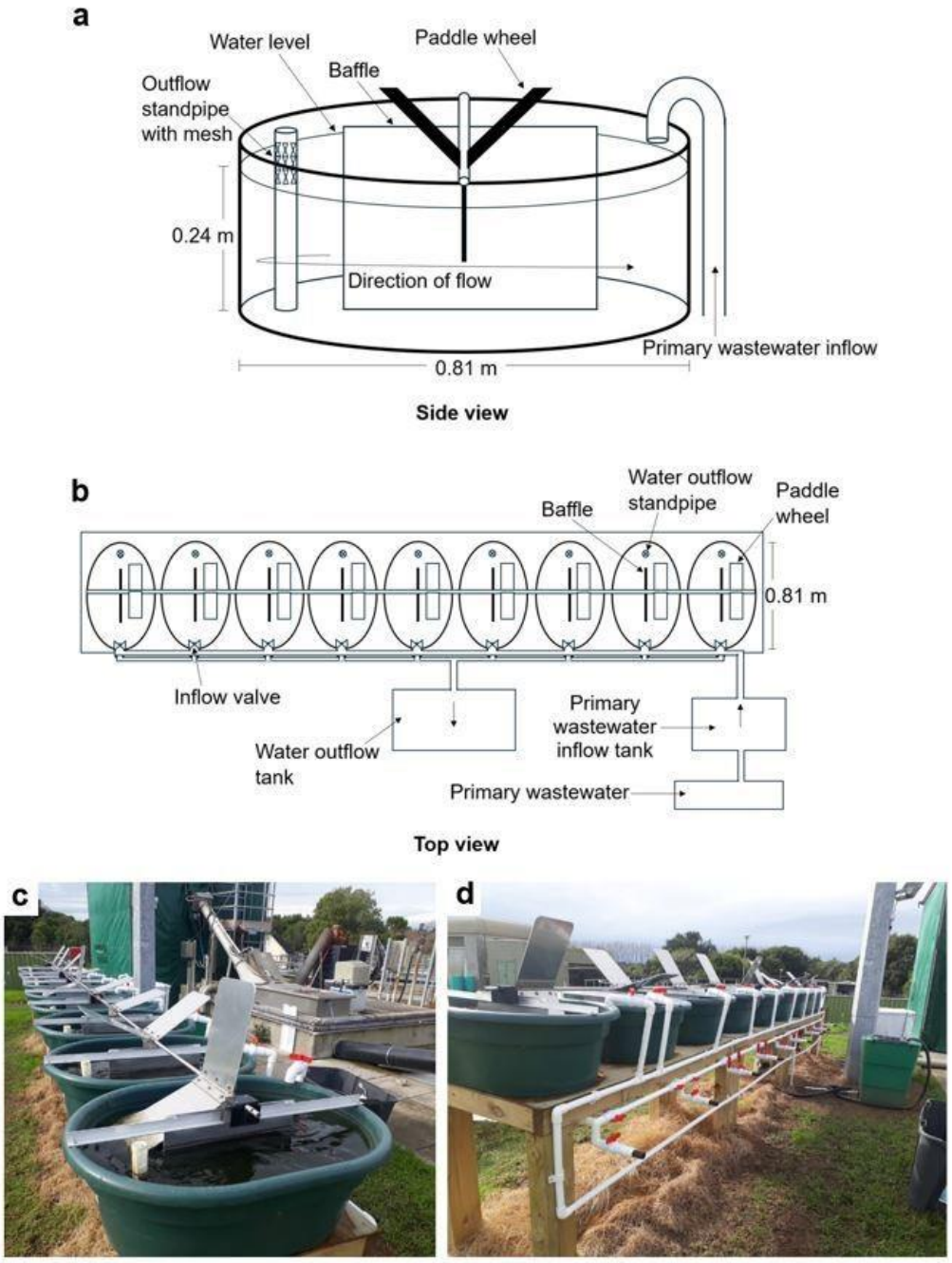

Figure 3.2 HRFAP side view diagram (a), HRFAPs system top view diagram (b), the nine outdoor HRFAPs used in this study set up on site at the WWTP (c, d).

3.3.3 Water quality variable monitoring

Pond water temperature and light intensity were measured continuously from the bottom of three of the HRFAPs using HOBO Pendant MX Temp/Light MX2202 water light and temperature loggers (Onset). Dissolved oxygen and pH were measured every two days

(at approximately 10 am) within each replicate at mid-HRFAP depth (12 cm) using an OxyGuard Handy Polaris 2 Dissolved Oxygen Meter and an OxyGuard Handy pH Meter. Temperature and precipitation data were obtained from the National Climate Database weather recording station located in Te Puke (-37.82455, 176.32048, data available from www.cliflo.niwa.co.nz). Experiments were conducted for a total of 24 days during spring (November) to avoid extreme seasonal effects. The maximum light intensity at the bottom of the HRFAPs ranged from 16.5 to 117.9 µmol m^{-2} s^{-1}, culture water temperature ranged from 8°C to 26°C, ambient air temperature ranged from 7°C to 25°C and a total rainfall of 89.6 mm occurred across 9 days during the experiment (Appendix 3.7, Figures 3A.1 & 3A.2). Three 500 mL water samples were collected from each HRFAP monoculture immediately before the final harvest during the biomass productivity and bioremediation performance experiments. These samples were used to measure concentrations of TSS, $cBOD_5$, chemical oxygen demand (COD) and *Escherichia coli (E. coli)*. These analyses were conducted by Hill Laboratories in Hamilton, New Zealand, using the following methodologies: TSS APHA 2540 D (modified) 23rd ed. 2017, $cBOD_5$ APHA 5210 B (modified) 23rd ed. 2017, COD APHA 5220 D 23rd ed. 2017 and *E. coli* APHA 9222 I 23rd ed. 2017.

3.3.4 Biomass productivity and bioremediation performance

Growth experiments were conducted to compare biomass productivity and bioremediation performance of the three cultivars. Three replicate cultures of each cultivar were grown in HRFAPs at a stocking density of 0.105 g dry weight (DW) L^{-1} (equivalent to 0.5 - 0.7 g FW L^{-1}). Biomass was harvested from each HRFAP once every four days for three consecutive harvest cycles (12 days total duration). Biomass was harvested by straining the entire contents of each HRFAP (culture water and algae) through a fine mesh bag. Culture water was retained in a separate container. Once excess water had drained from the bag, it was placed in a centrifugal spin dryer (Spindle NZ, SPL-265) and spun at 2800 rpm for four minutes to remove any remaining water. The algae were then removed from the bag and weighed to determine the FW. There were no algae present on the paddlewheels or surfaces. Each HRFAP was cleaned by scrubbing the surface of the paddlewheel and the HRFAP with a brush and then each HRFAP was refilled at a 1:1 ratio of strained culture water to fresh primary wastewater from the WWTP as described in section 3.3.2. Stocking density was reset to 0.105 g DW L^{-1} by restocking the FW equivalent of 7.35 g DW of the harvested biomass

back into each replicate HRFAP. Biomass not restocked back in the HRFAP was dried in an oven at 60 °C for 48 hours and reweighed to confirm the fresh weight to dry weight (FW:DW) ratio for each replicate. FW:DW ratios were used to convert the initial biomass and the harvested biomass for each replicate, which were both measured in FW, into DW. Biomass productivity (g DW m^{-2} day^{-1}) was calculated for each replicate for each harvest using the equation $P = (DW_f - DW_i) / A / T$, where DW_f is the final algal biomass (g DW), DW_i is the initial biomass (g DW), A is the HRFAP surface area (m^{-2}), and T is the number of days in culture. Growth rate (% day^{-1}) was calculated for each replicate for each harvest using the equation $GR = ((FW_f / FW_i)^{1/t} - 1) * 100$, where FW_f is the final algal biomass (g FW), and FW_i is initial algal biomass (g FW), and t is the number of days in culture.

A pulse amplitude-modulated (PAM) fluorometer (Junior-PAM, Heinz Walz GmbH, Effeltrich, Germany) was used to measure the effective quantum yield (*Y(II)*) and optimal quantum yield (F_v/F_m) of each replicate at each harvest. *Y(II)* was used to measure algal cell stress, by assessing changes in the chlorophyll fluorescence yield of photosystem II (PS II) (Heinz Walz GmbH, 2017). F_v/F_m was measured in biomass samples that were dark-adapted for 15 minutes prior to analysis (Stirbet, 2011). F_v/F_m was then used to estimate the maximal photochemical PSII efficiency as an indicator of photosynthetic performance (Schreiber et al., 1995; Kromkamp et al., 2008; Figueroa et al., 2013). Measurements were taken at approximately the same time of day immediately before each harvest (i.e., once every four days).

The bioremediation performance of cultivars was quantified by measuring concentrations of TAN, nitrate-N, and DRP in the primary wastewater and HRFAP water samples. Water samples (30 mL) of primary wastewater and water from each replicate HRFAP were taken every two days. Primary wastewater samples were taken in the morning at the time of peak inflow to the WWTP and HRFAP water samples were taken immediately prior to water changes. Water samples were filtered upon collection into individual sterile 50 mL clear plastic test tubes (LabServ™) using a vacuum filtration system (Whatman™ GF/C™, 0.22 μm) and immediately frozen. Samples were analysed within a week of collection after defrosting. Concentrations of TAN, nitrate-N, and DRP in each water sample were measured using a spectrophotometer (HACH DR 900, HACH, Loveland, CO, USA) following the USEPA Nessler method (HACH method 8038), the nitrate cadmium reduction method (HACH method 8039) and the ascorbic acid method (HACH method 8048) respectively. Nutrient removal rate (NR, % day^{-1}) was calculated at each water change using

the equation $NR = CW_f / ((CW_i + PW_i) / E) * 100$, where CW_f and CW_i are the final and initial nutrient concentrations of the culture water, PW_i is the initial nutrient concentration of the primary wastewater and E is the proportion of water exchanged.

3.3.5 Competition experiments

The competitive dominance of the cultivars, *K. flaccidum* and *Oedogonium* sp. were assessed following completion of the biomass productivity and bioremediation performance experiment. The cultivar *O. calcareum* was excluded from these bi-culture experiments as it did not survive through to the end of the growth experiments (see Results, Section 3.4.1). The competitive dominance of *K. flaccidum* and *Oedogonium* sp. was measured by growing bi-cultures with a cultivar ratio of 1:1 at three stocking densities in HRFAPs for three consecutive harvests (12 days total). Three replicate cultures (total N= 9) were established with a combined total stocking density of both cultivars at 0.25, 0.5, and 1 g FW L^{-1} (equivalent to DW stocking densities of 0.052, 0.105, and 0.210 g DW L^{-1}) to ensure all cultures started with an equal DW biomass composition of each cultivar. Experimental protocols followed the same methodology for water changes, harvesting, biomass processing, and biomass productivity analysis as described in Section 3.3.4. At each harvest, excess biomass was removed to reset stocking density, however, cultivar composition was not reset back to a 1:1 ratio to enable changes in cultivar composition, and therefore competitive dominance, to be quantified over the duration of the experiment.

Biomass samples of 0.5 g FW were collected from each replicate HRFAP on the first day of the experiment, at each harvest, and on the final day of the experiment. The cultivar composition of each biomass sample was analysed on the day of collection by photographing ten sub-samples of each biomass sample using a dissecting microscope (Olympus model CKX53) at 20 x magnification. The proportional composition of each cultivar was estimated by placing a 100-point grid over each photograph and summing the number of grid points directly overlying each species. The average proportional composition across all ten photographs was then used to determine the final cultivar composition within each replicate HRFAP.

3.3.6 Statistical analysis

Biomass productivity, growth rate, cultivar composition, effective quantum yield (*Y(II)*) and optimal quantum yield (F_v/F_m) measurements were analysed using two factor repeated-measures analyses of variance (ANOVA) with cultivars and harvests as fixed factors. Nutrient concentrations were analysed using two factor repeated-measures analyses of variance (ANOVA) with cultivars and days as fixed factors. Data for each experiment were analysed separately. All analyses were conducted in SPSS Statistics (version 29). A significance level of 0.05 was used for all tests, and F-values were calculated to assess differences among groups. All data are reported as means ± S.E.

3.4 Results

3.4.1 Biomass productivity and photosynthetic efficiency

Biomass productivity varied significantly among cultivars and harvests (ANOVA: cultivar x harvest $F_{4,12} = 20.12$, $p = <0.001$, Figure 3.3). The highest average biomass productivity across all harvests was achieved by *K. flaccidum* at 3.09 g DW m^{-2} day^{-1} (± 0.20), followed by *Oedogonium* sp. at 0.99 g DW m^{-2} day^{-1} (± 0.29) and *O. calcareum* at 0.72 g DW m^{-2} day^{-1} (± 0.08). *Klebsormidium flaccidum* biomass productivity declined from 5.36 g DW m^{-2} day^{-1} (± 0.17) at day 4 to 1.48 g DW m^{-2} day^{-1} (± 0.19) at day 8, but then increased to 2.44 g DW m^{-2} day^{-1} (± 0.24) at day 12. *Oedogonium* sp. biomass productivity remained consistent across all three harvests, ranging from 0.92 - 1.07 g DW m^{-2} day^{-1} (± 0.07 - 0.46). Biomass productivity of *O. calcareum* was 2.16 g DW m^{-2} day^{-1} (± 0.25) at day 4; however, complete die-off occurred by day 8.

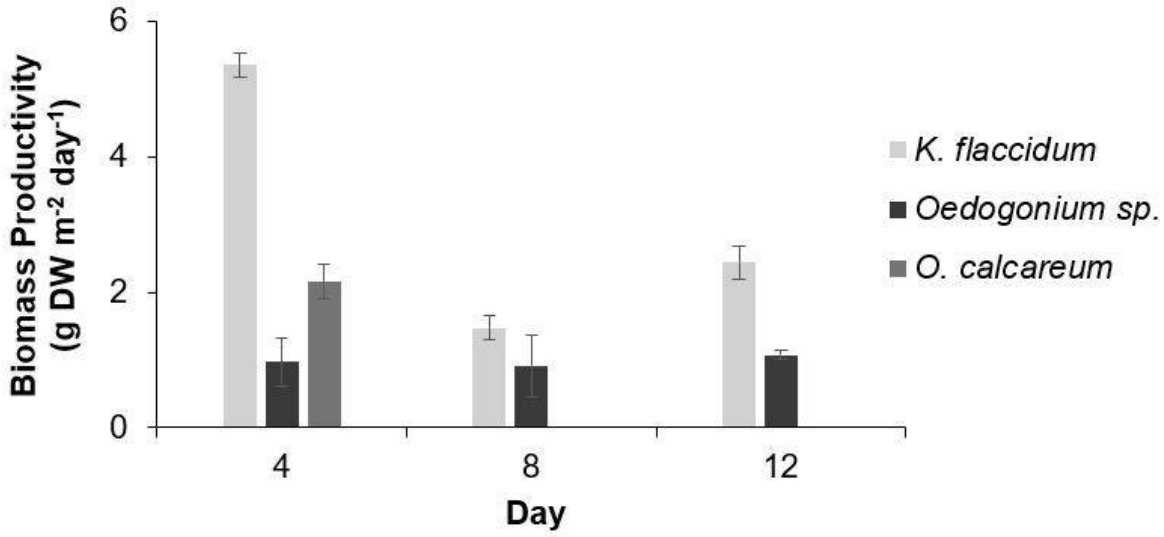

Figure 3.3 Mean (± S.E.) biomass productivity (g DW m^{-2} day^{-1}) of *K. flaccidum, O. calcareum* and *Oedogonium* sp. over three consecutive four-day harvest cycles. N = 3.

Maximal quantum yields and optimal quantum yields varied significantly among cultivars and harvests (ANOVA: YII: cultivar x harvest, $F_{4,12} = 7.34$, $p = 0.003$, Fv/Fm: cultivar x harvest, $F_{4,12} = 53.41$, $p = <0.001$, Figure 3.4). *Klebsormidium flaccidum* had the highest maximal quantum yield and optimal quantum yield across all harvests on average (0.60 ± 0.05 and 0.60 ± 0.02, respectively). However, the optimal quantum yield of *K. flaccidum* declined from 0.70 (± 0.01) at day 0 to 0.40 (± 0.03) at day 4, before returning to the previous level of 0.70 (± 0.03) at day 8. Similarly, maximal quantum yields, and optimal quantum yields for *Oedogonium* sp. declined from 0.3 (± 0.02) and 0.5 (± 0.03), respectively, at day 0 to 0.20 (± 0.11) and 0.00 (± 0.01), respectively, at day 4. However, yields returned to previous levels of 0.50 (± 0.02) and 0.30 (± 0.01) at day 12. Maximal quantum yields and optimal quantum yields of *O. calcareum* also declined from 0.30 (± 0.03) and 0.40 (± 0.03), respectively, at day 0 to 0.01 (± 0.00) and 0.01 (± 0.00), respectively, at day 4. As noted above, complete die-off of this cultivar had occurred by day 8.

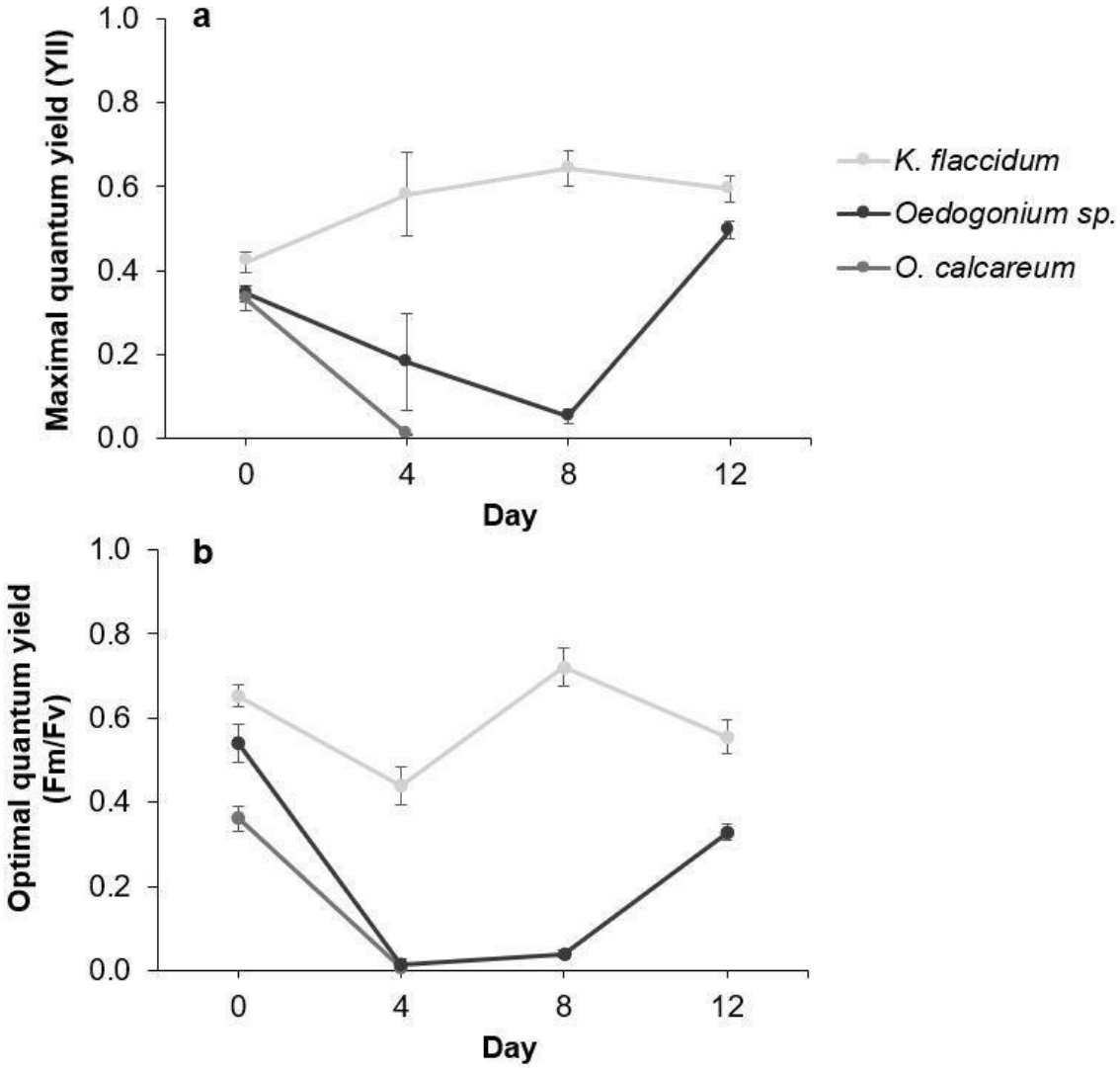

Figure 3.4 Mean (± S.E.) maximal quantum yield (YII) (a) and optimal quantum yield (Fm/Fv) (b) of chlorophyll *a* fluorescence of *K. flaccidum, O. calcareum* and *Oedogonium* sp. over three consecutive four-day harvest cycles. N = 3.

3.4.2 Bioremediation performance

TAN concentrations in primary wastewater influent ranged from 40.39 to 45.49 mg L^{-1}. TAN concentrations in culture water varied significantly among cultivars and days (ANOVA: cultivar x day, $F_{10,30} = 14.01$, $p = <0.001$; Figure 3.5a). Across all days, the highest reductions in TAN on average were achieved by *K. flaccidum*, which reduced concentrations by 50.8% day^{-1} (± 2.8) from 31.29 mg L^{-1} (± 0.33) to 14.81 mg L^{-1} (± 0.81), followed by *Oedogonium* sp., which reduced concentrations by 38.4% day^{-1} (± 4.4) from

33.97 mg L^{-1} (± 0.51) to 19.64 mg L^{-1} (± 1.31) and *O. calcareum*, which reduced concentrations by 17.4% day^{-1} (± 5.2) from 34.10 mg L^{-1} (± 0.71) to 24.18 mg L^{-1} (± 1.59). TAN removal rates by *K. flaccidum* showed an initial decline from 76.8% day^{-1} (± 2.7) on day 2 to a low of 28.1% day^{-1} (± 3.9) on day 4. However, removal rates increased thereafter to a maximum of 59.1 % day^{-1} (± 3.4) on day 12, following a slight decrease in removal rates from day 8 to day 10. TAN removal rates by *Oedogonium* sp. declined slightly from 38.2% day^{-1} (± 3.6) on day 2 to 30.2% day^{-1} (± 3.3) on day 6. Removal rates fluctuated thereafter and reached a maximum of 53.1% day^{-1} (± 5.7) on day 12. TAN removal rates by *O. calcareum* showed a sharp decline from 72.3% day^{-1} (± 4.7) on day 2 to 14.0% day^{-1} (± 7.6) on day 4 and continued to decline to reach a low of 6.7% day^{-1} (± 2.6) on day 8.

Nitrate-N concentrations in primary wastewater influent ranged between 0.95 to 1.31 mg L^{-1}. Nitrate-N concentrations in culture water varied significantly among cultivars and days (ANOVA: cultivar x day, $F_{10,30} = 13.74$, $p = <0.001$; Figure 3.5b). Across all days, the highest reductions in nitrate-N on average were achieved by *K. flaccidum,* which reduced concentrations by 58.7% day^{-1} (± 3.3) from 0.80 mg L^{-1} (± 0.01) to 0.30 mg L^{-1} (± 0.02), followed by *Oedogonium* sp., which reduced concentrations by 41.1% day^{-1} (± 4.8) from 0.87 mg L^{-1} (± 0.02) to 0.49 mg L^{-1} (± 0.04) and *O. calcareum,* which reduced concentrations by 31.8% day^{-1} (± 3.4) from 0.87 mg L^{-1} (± 0.01) to 0.56 mg L^{-1} (± 0.03). Nitrate-N removal rates by *K. flaccidum* showed an initial decline from 89.3% day^{-1} (± 2.2) on day 2 to a low of 10.2% day^{-1} (± 1.9) on day 6. However, removal rates increased thereafter to 70.4% day^{-1} (± 4.2) from 0.64 mg L^{-1} (± 0.03) to 0.19 mg L^{-1} (± 0.02) on day 12. Nitrate-N removal rates by *Oedogonium* sp. showed a decline from 87.1% day^{-1} (± 5.8) on day 2 to a low of 6.8% day^{-1} (± 3.4) on day 6. However, removal rates increased thereafter to 49.1% day^{-1} (± 3.1) on day 12. Nitrate-N removal rates by *O. calcareum* showed a sharp decline from 89.3% day^{-1} (± 5.8) on day 2 to a low of 3.4% day^{-1} (± 1.7) on day 6, followed by a slight increase to 4.8% day^{-1} (± 1.1) on day 8.

DRP concentrations in primary wastewater influent ranged between 3.53 mg L^{-1} to 5.45 mg L^{-1}. DRP concentrations in culture water varied significantly among cultivars and days (ANOVA: cultivar x day, $F_{10,30} = 2.87$, $p = 0.012$; Figure 3.5c). Across all days, the highest reductions in DRP on average were achieved by *K. flaccidum*, which reduced concentrations by 15.2% day^{-1} (± 2.1) from 4.20 mg L^{-1} (± 0.03) to 3.52 mg L^{-1} (± 0.07), followed by *Oedogonium* sp., which reduced concentrations by 8.5% day^{-1} (± 1.7) from 4.44

mg L^{-1} (± 0.04) to 4.04 mg L^{-1} (± 0.09) and *O. calcareum,* which reduced concentrations by 7.7% day^{-1} (± 1.1) from 4.75 mg L^{-1} (± 0.02) to 4.35 mg L^{-1} (± 0.05). DRP removal rates by *K. flaccidum* showed an initial decline from 32.0% day^{-1} (± 2.9) on day 2 to a low of 5.3% day^{-1} (± 2.5) on day 4. However, removal rates increased thereafter to 17.4% day^{-1} (± 1.8) on day 6, followed by 12.2% day^{-1} (± 1.1) on day 12. DRP removal rates by *Oedogonium* sp. declined from 20.9% day^{-1} (± 3.4) on day 2 to a low of 2.0% day^{-1} (± 1.0) on day 4. Removal rates increased thereafter to 10.0% day^{-1} (± 1.3) on day 12. DRP removal rates by *O. calcareum* showed a sharp decline from 23.8% day^{-1} (± 1.9) on day 2 to 2.5% day^{-1} (± 1.0) on day 4. Removal rates continued to decline to a low of 1.3% day^{-1} (± 0.6) on day 8.

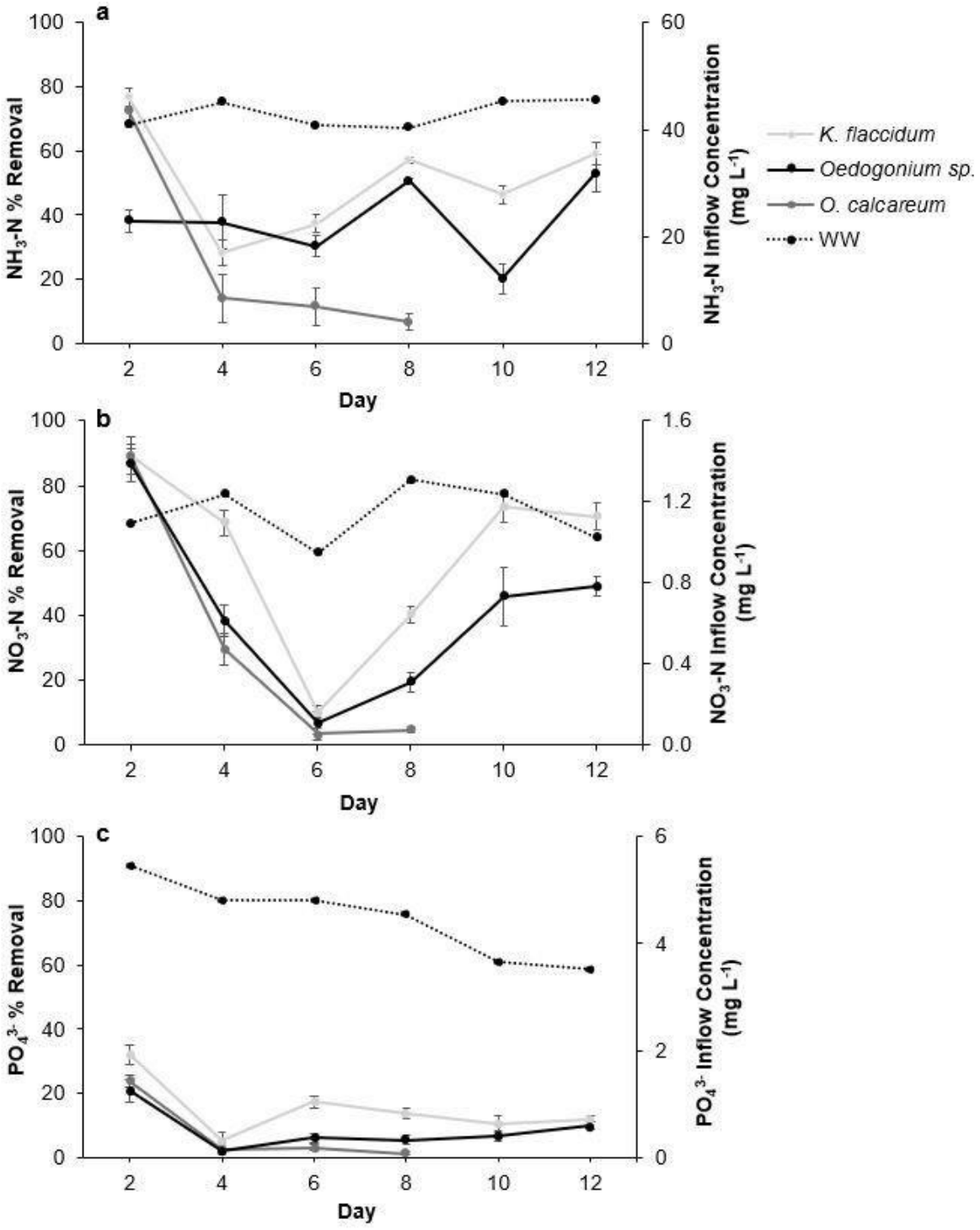

Figure 3.5 Mean (±S.E.) TAN % removal and TAN inflow concentration (mg L^{-1}) in wastewater (WW) (a), NO_3-N % removal and NO_3-N inflow concentration (mg L^{-1}) in wastewater (b) and DRP % removal and DRP inflow concentration (mg L^{-1}) in wastewater (c) of *K. flaccidum, O. calcareum* and *Oedogonium* sp. cultures over three consecutive four-day harvest cycles. N = 3.

Water quality variables of the HRFAP culture water varied between cultivars (Table 3.1). The concentration of total suspended solids (TSS) in *K. flaccidum* culture water was 53.8% lower than in the primary wastewater, whereas in *Oedogonium* sp. culture water, it was 9.4% higher. Carbonaceous biochemical oxygen demand ($cBOD_5$) of the *K. flaccidum* and *Oedogonium* sp. cultures were 92.7% and 74.6% lower respectively compared to the primary wastewater. Chemical oxygen demand (COD) of the *K. flaccidum* and *Oedogonium* sp. cultures were 73.9% and 43.3% lower respectively compared to the primary wastewater. The concentration of *E. coli* in the *K. flaccidum* and *Oedogonium* sp. culture water were both 1 log lower than that of the primary wastewater. Water quality variables were not measured for *O. calcareum* as complete die-off had occurred during experiments.

Table 3.1 Water quality variables of primary wastewater, and *K. flaccidum* and *Oedogonium* sp. culture water at the final harvest (day 12). DO, dissolved oxygen; TSS, total suspended solids; $cBOD_5$, carbonaceous biochemical oxygen demand; COD, chemical oxygen demand; *E. coli*, *Escherichia coli*.

	Primary wastewater	***K. flaccidum***	***Oedogonium* sp.**
pH	7.5	8.80	8.56
DO (mg L^{-1})		8.57	8.64
TSS (mg L^{-1})	106	49	116
$cBOD_5$ (mg L^{-1})	177	13	45
COD (mg L^{-1})	460	106	230
E. coli (colony forming units per 100 mL)	6 x 10^6	1.2 x 10^5	2 x 10^5

3.4.3 Bi-culture biomass productivity and growth

Biomass productivity of *K. flaccidum* and *Oedogonium* sp. bi-cultures varied significantly among stocking densities and harvests (ANOVA: stocking density x harvest $F_{4,12} = 22.23$, $p = <0.001$, Figure 3.6a). Across all harvests, biomass productivity was highest at a stocking density of 1 g FW L^{-1} at 3.94 g DW m^{-2} day^{-1} (± 0.17), followed by a stocking density of 0.25 g FW L^{-1} at 3.31 g DW m^{-2} day^{-1} (± 0.10), while a stocking density of 0.5 g FW L^{-1} had the lowest biomass productivity (3.16 g DW m^{-2} day^{-1} ± 0.15). Similarly, the

growth rate of bi-cultures varied significantly among stocking densities and harvests (ANOVA: stocking density x harvest, $F_{4,12} = 35.81$, $p = <0.001$, Figure 3.6b). Contrary to biomass productivity, the growth rate was highest at a stocking density of 0.25 g FW L^{-1} across all harvests reaching 66.1% day^{-1} (± 1.4), followed by a stocking density of 0.5 g FW L^{-1} at 45.1% day^{-1} (± 0.8), while a stocking density of 1 g FW L^{-1} had the lowest growth rate at only 37.7% day^{-1} (± 0.5). Overall, both biomass productivity and growth rate increased throughout the experiment and were highest across all stocking densities at day 12.

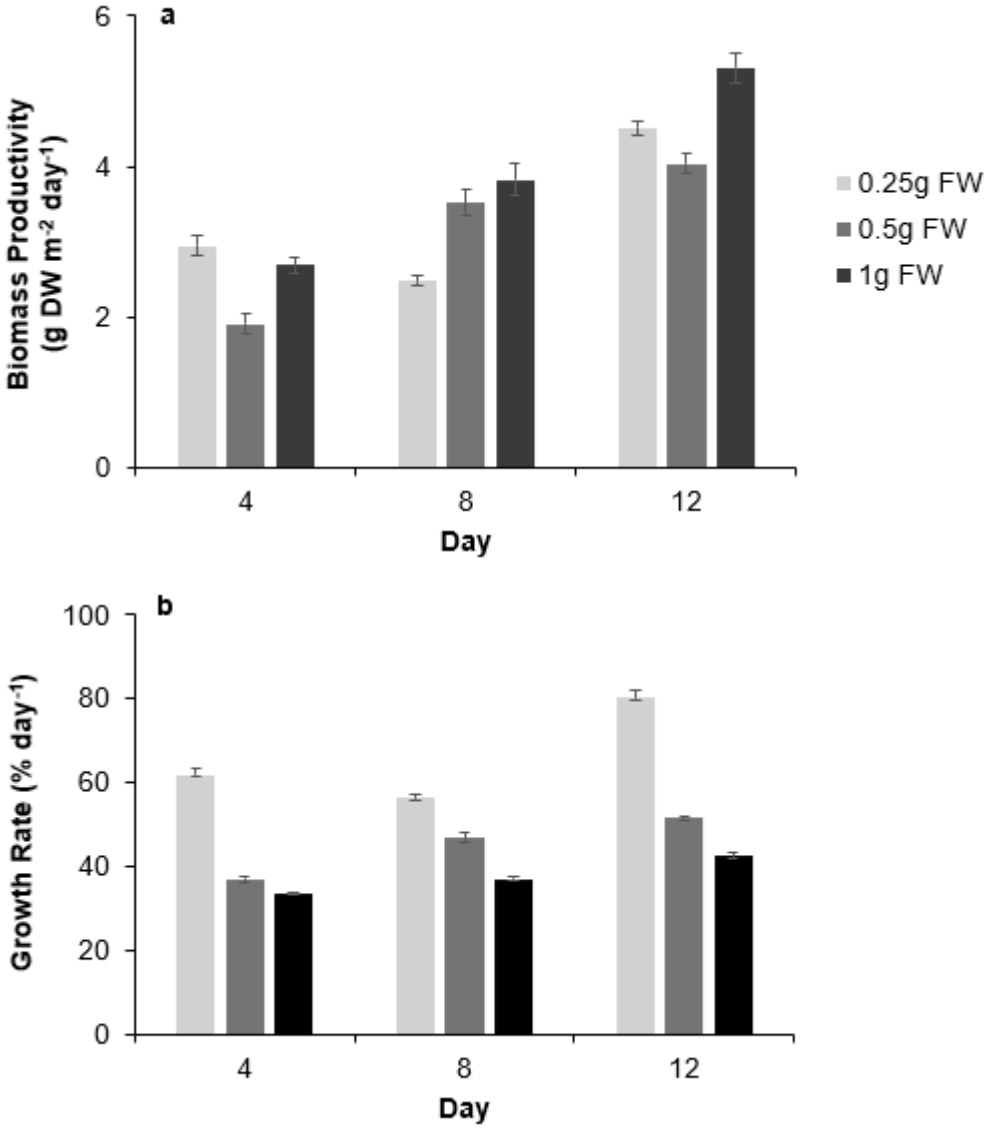

Figure 3.6 Mean (±S.E.) biomass productivity (g DW m^{-2} day^{-1}) (a) and growth rate (% day^{-1}) (b) of bi-cultures of *K. flaccidum* and *Oedogonium* sp. grown under three stocking densities (g FW L^{-1}) over three consecutive four-day harvest cycles. N = 3.

3.4.4 Proportional composition

Proportional composition of bi-cultures varied significantly among harvests; however, the dominant cultivar at each harvest varied between the stocking density treatments (ANOVA, stocking density x harvest, $F_{4,174} = 2.08$, $p = 0.085$, Figure 3.7). Over time, the proportional composition of *K. flaccidum* increased to 64.0% (± 6.1) and 73.0% (± 5.0) on day 12 at stocking densities of 0.25 g FW L^{-1} and 0.5 g FW L^{-1}, respectively. In contrast, at the higher stocking density of 1 g FW L^{-1}, *K. flaccidum* showed an initial increase in proportional composition on day 4 (57.0% ± 5.9), but then decreased thereafter and by day 12, proportional composition of both cultivars was similar (*K. flaccidum*: 46.0% ± 7.1; *Oedogonium* sp.*:* 54.0% (± 7.1).

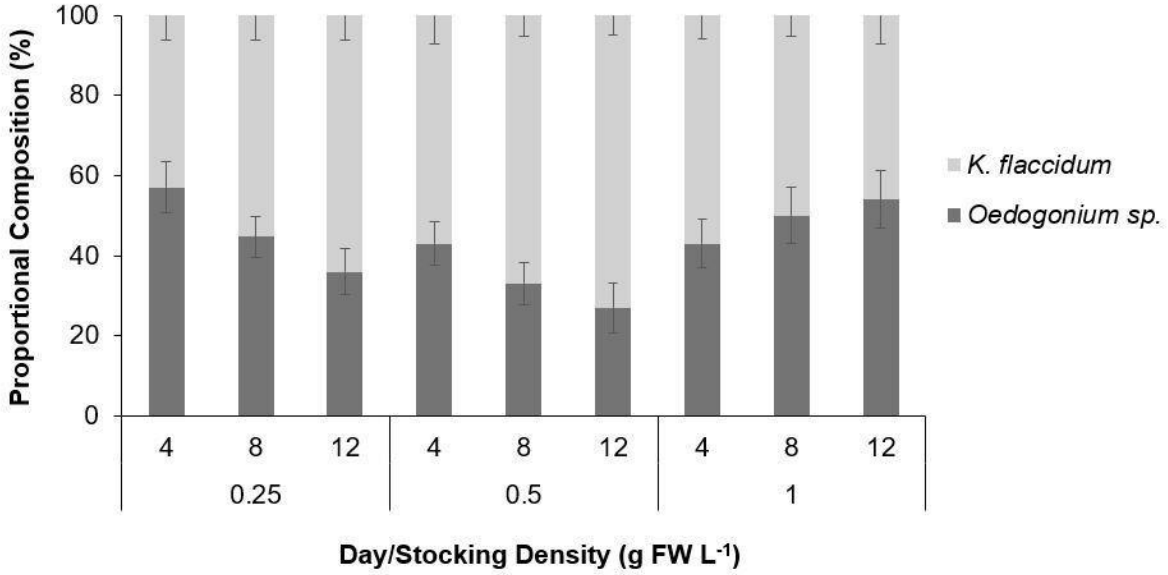

Figure 3.7 Mean (±S.E.) proportional composition (%) of *K. flaccidum* and *Oedogonium* sp. grown in bi-cultures under three stocking densities (0.25, 0.5 and 1 g FW L^{-1}) over three consecutive four-day harvest cycles. N = 3.

3.5 Discussion

We assessed the productivity and bioremediation performance of three freshwater filamentous macroalgal cultivars (*K. flaccidum*, *O. calcareum* and *Oedogonium* sp.) grown in outdoor HRFAP mesocosms treating primary municipal wastewater. *Klebsormidium flaccidum* was identified as the most suitable cultivar for bioremediation of primary

municipal wastewater due to its superior biomass productivity and nutrient removal rates, and competitive dominance at low stocking densities. Previous research has compared biomass productivity and bioremediation performance of *Klebsormidium* sp. and *Oedogonium* sp. within tertiary treated municipal effluent (Lawton et al., 2021a). *Oedogonium* sp. demonstrated greater biomass productivity and bioremediation performance compared to *Klebsormidium* sp., thereby identifying *Oedogonium* sp. as a target cultivar for bioremediation of tertiary treated WWTP effluent (Lawton et al., 2021a). However, compared to primary municipal wastewater, tertiary treated WWTP effluent has low turbidity, permitting high penetration of light, which may have contributed to the superior performance of *Oedogonium* sp. Conversely, our study measured cultivar productivity in highly turbid (TSS 106 g m^{-3}) primary municipal wastewater. This high turbidity reduced light availability within the HRFAPs (maximum light intensity 16.5 - 117.8 µmol m^{-2} s^{-1}) and may have led to the lower biomass productivity of *Oedogonium* sp. as this species exhibits optimal growth under moderate to high light conditions (Cole et al., 2018). In contrast, *Klebsormidium* sp. demonstrated high productivity in primary municipal wastewater despite reduced light availability, further demonstrating that this genus has low light requirements for growth and photosynthesis (Karsten & Rindi, 2010), making it a suitable candidate for cultivation in wastewaters with high turbidity.

Across all three nutrients analysed (TAN, nitrate-N, and DRP), *K. flaccidum* consistently exhibited higher bioremediation performance compared to *O. calcareum* and *Oedogonium* sp. Nutrient removal rates by *K. flaccidum* compared with *Oedogonium* sp. and *O. calcareum* were 32% and 94% greater, respectively for TAN, were 43% and 85% greater respectively, for nitrate-N, and 78% and 98% greater respectively for DRP. These findings align with previous laboratory-scale research identifying *Klebsormidium* sp. as a key target cultivar for bioremediation of primary municipal wastewater (Novak et al., 2024). *Klebsormidium* sp. has previously demonstrated reductions in nutrient concentrations, when cultivated in primary municipal wastewater under summer conditions, with TAN reduced by 94% from 10.7 mg L^{-1} to 0.6 mg L^{-1} (± 0.07), nitrate-N by 64% from 1.1 mg L^{-1} to 0.4 mg L^{-1} (± 0.10) and phosphate by 91% from 1.3 mg L^{-1} to 0.1 mg L^{-1} (± 0.00) (Novak et al., 2024). Similarly, *Klebsormidium* sp. cultivated in urban wastewaters with varying nutrient concentrations (total nitrogen (TN): 10 mg L^{-1}- 50.7 mg L^{-1}, TP: 3.2 mg L^{-1} - 10.7 mg L^{-1}), achieved nutrient removal rates of 63 - 96% for TN and 69 - 74% for TP (La Bella et al., 2023). *Klebsormidium flaccidum* has also demonstrated high nutrient removal rates of TAN

(28 mg L^{-1}) and phosphate (15 mg L^{-1}) from synthetic municipal wastewater (Umetani et al., 2023). However, previous studies have not extensively reported improvements in water quality variables of wastewater resulting from filamentous algae cultivation. In this current study, monocultures of *K. flaccidum* and *Oedogonium* sp. were found to have large effects on water quality variables, resulting in improved water quality. However, removal rates varied between cultivars, with cultivation of *K. flaccidum* resulting in greater reductions in TSS, $cBOD_5$ and COD, while both *K. flaccidum* and *Oedogonium* sp. produced comparable reductions in *E. coli*. The effectiveness of different species of filamentous algae in improving water quality variables in wastewater is yet to be thoroughly compared, and therefore, cultivar-specific mechanisms and physico-chemical bioremediation performance are not well understood. Regardless, these results further support the selection of *K. flaccidum* as a target cultivar for bioremediation of primary municipal wastewater.

In addition to demonstrating superior biomass productivity and nutrient removal performance, *K. flaccidum* was also competitively dominant, however this dominance was not consistent across all three stocking densities. *Klebsormidium flaccidum* was most dominant under lower stocking densities of 0.25 and 0.5 g FW L^{-1}, however, no cultivar was dominant after 12 days at the stocking density of 1 g FW L^{-1}. Contrary to previous studies, monoculture biomass productivity was a reliable predictor of bi-culture performance. For example, monocultures of *Oedogonium* sp. at a stocking density of 0.5 g FW L^{-1} and bi-cultures at a stocking density of 0.5 g FW L^{-1} had similar biomass productivities of 1.07 DW m^{-2} day^{-1} and 1.09 g DW m^{-2} day^{-1}, respectively, on day 12. Stocking density also had a significant effect on productivity, as bi-culture biomass productivities of *Oedogonium* sp. were 34.4% and 62.7% higher on day 12 at a stocking density of 0.25 and 1 g FW L^{-1}, respectively, compared to the monocultures at a stocking density of 0.5 g FW L^{-1}. However, the proportional composition at various stocking densities may not have reached equilibrium. Therefore, future assessments of competitive dominance should be undertaken for longer durations to increase the reliability of findings. Experiments should also be conducted across multiple seasons to provide insight into cultivar dominance under varying light and temperature conditions as the biomass productivities of individual cultivars may vary from season to season (Ranjan et al., 2019; Novak et al., 2024).

Primary municipal wastewaters often contain a wide variety of micropollutants from domestic and industrial inputs, including pesticides, personal care products, pharmaceuticals,

polycyclic hydrocarbons (PAHs), plasticizers, and surfactants (Rout et al., 2021), which may be toxic to certain algal species (Rydh Stenström et al., 2021; Othman et al., 2023). It is likely that a micropollutant was present in the wastewater during the initial experiment investigating biomass productivity and bioremediation performance of monocultures as frothy water occurred in the HRFAPs on day 8, indicating the likely presence of a surfactant within the culture water. At this time, all replicate cultures of *O. calcareum* had completely died off, highlighting the potential toxic effect of primary wastewater on cultivar growth and cell health. In contrast, while biomass productivity of *K. flaccidum* had declined by day 8 relative to day 4, maximal quantum yields were maintained throughout the experiment. Biomass productivity of *Oedogonium* sp. remained consistent throughout the experiment, but maximal and optimal quantum yields declined at day 8 relative to day 4 measurements, before returning to optimal levels at day 12. These results demonstrate the robustness of *K. flaccidum* and *Oedogonium* sp. and their capacity for growth under highly unpredictable and potentially toxic conditions. Prior research has often measured nutrient bioremediation performance of filamentous algae when cultivated in nutrient-rich synthetic wastewater under small-scale laboratory conditions (Liu & Vyverman, 2015; Umetani et al., 2023). However, the current results show that previous assessments of cultivar performance within synthetic wastewaters and treated wastewater effluents are not applicable when selecting target cultivars for primary wastewater treatment. Instead, nutrient bioremediation performance of cultivars should be assessed using the actual wastewater in outdoor HRFAPs, with long-term exposure to detect tolerance to stochastically occurring micropollutants (Sabatte et al., 2024).

To date, few studies have demonstrated the potential of filamentous algae for primary municipal wastewater treatment (Neveux et al., 2016; Ge et al., 2018; Kube et al., 2022). Among these studies, two were conducted under controlled laboratory conditions utilising photobioreactors (Kube et al., 2022) and flat-plate aquariums (Ge et al., 2018), while one study utilised 20 L outdoor cylindrical tanks and pilot-scale aerated open pond systems (with a total volume of 10 m^3) (Neveux et al., 2016). While these studies successfully demonstrated the potential of integrating monocultures of freshwater filamentous algae into wastewater treatment operations, it is important to note that the choice of cultivation system significantly influences biomass yields and bioremediation performance (Ge et al., 2018; Sabatte et al., 2024). Factors such as depth (Sutherland et al., 2014b), surface area (Sutherland et al., 2020b), mixing/turbulence (Grobbelaar, 2010), and grazer pressure (Smith & Mcbride, 2015) all play pivotal roles in determining algal production rates. Furthermore, outdoor climatic

conditions are critical for algal cultivation, as water bodies are highly responsive to environmental change (Meerhoff et al., 2012). Shallow ponds (with operating depths of 15 – 30 cm) commonly used in algal cultivation are particularly sensitive to changes in light and temperature (Smith & Mcbride, 2015). Further research is required before filamentous HRAP systems can be widely implemented into mainstream municipal wastewater treatment operations (Liu et al., 2020; Sabatte et al., 2024). Hence, future studies should prioritise evaluating cultivar performance within outdoor HRAPs at large-scale to confirm their feasibility as a treatment system.

3.6 Conclusion

This study confirms *K. flaccidum* as a target cultivar for nutrient bioremediation of primary municipal wastewater based on superior biomass productivity, high nutrient bioremediation performance, improvements in selected water quality variables, and competitive dominance. *Klebsormidium flaccidum* outperformed the other cultivars by significantly reducing TAN, nitrate-N and DRP concentrations as well as achieving notable reductions in TSS, $cBOD_5$, COD, while also reducing *E. coli* concentrations by 1.7 $\log_{10}$. Additionally, *K. flaccidum* demonstrated increased competitive dominance over time in bi-cultures, establishing itself as a key target cultivar for primary municipal wastewater bioremediation. The results of this study support the use of our previously developed screening protocol which identified *K. flaccidum* as a key target cultivar, therefore this current study has confirmed the screening protocol as an accurate tool for selecting suitable target cultivars for the bioremediation of primary municipal wastewater. Our experimental design effectively assessed cultivar productivity and nutrient bioremediation in primary wastewater over a short period. Future research now needs to focus on assessing competitive dominance and seasonal variability in cultivar performance over annual time scales. Future research should also identify optimal operational parameters, which are necessary for improving biomass productivity and bioremediation efficiency in larger systems.

3.7 Appendix

3.7.1 Cultivar identification of Klebsormidium flaccidum and Oedogonium sp. using DNA barcoding.

3.7.2 Methods

DNA was extracted from cultivars using Chelex 100 following the manufacturer's protocol (BioRad) and two regions of the ITS1-5.8S-ITS2 rRNA sequence were amplified using primers 9F and 7R and CladoITS-9F and CladoITS-7R for *Klebsormidium* sample, and primers ITS4+ITS1 for the *Oedogonium* sample (Hayakawa et al., 2012). The LSU region of the *Oedogonium* sample was also amplified using primers C'1+D2 (Hayakawa et al. 2012). Polymerase chain reaction (PCR) amplifications were performed in a 25 µL reaction mixture containing 5 µL of 5x reaction buffer, 25 pmol of both the forward and reverse primer, dNTPs to a final concentration of 0.2 mM each, 1–3 µL of template DNA and 0.5 U Kapa 2G Robust Hotstart DNA polymerase (Sigma-Aldrich, St Louis, MO). ITS amplifications were performed on a Biometra TOne 96G thermal cycler with a touchdown PCR cycling profile (3 min at 94°C, 10 cycles of 15 s denaturing at 95°C, 20 s annealing at 48°C, 30 s extension at 72°C with the annealing temperature decreasing by 0.5°C each cycle, followed by 20 cycles of 15 s denaturing at 95°C, 20 s annealing at 48°C, 30 s extension at 72°C, and a final extension of 2 min at 72°C).The results of PCR were assessed via agarose gel electrophoresis and successful reactions were purified using ExoSAP-IT (Applied Biosystems) prior to submission to a commercial facility for Sanger DNA sequencing (Macrogen Inc.) using the same primers. Resulting sequence chromatograms were assessed by eye and forward and reverse sequences were assembled using Geneious Prime v2021.1.1 (Biomatters Ltd.). The assembled contigs were submitted to the 'nt' nucleotide database of NCBI-GenBank using a *blastn* query and sequences with the highest similarities were selected for consideration as potential taxonomic matches.

3.7.3 Results

DNA sequencing of the *Klebsormidium* sample resulted in a 386bp contig for primers 9F+7R and 782bp for primers CladoITS-9F+CladoITS-7R. The 9F+7R contig matched an existing sequence of *K. flaccidum* (KM197123) with 99.5% similarity (a 1bp difference) and the three next-best matches were also congeners. The CladoITS-9F+CladoITS-7R ITS sequence matched *Chlamydomonas* for its five best-hits (similarities of 99.3-99.4%; a 7bp difference), which was taken as evidence of an environmental contaminant and this sequence

was disregarded. Based on these results, this cultivar was identified as the species *Klebsormidium flaccidum.*

DNA sequencing of the *Oedogonium* sample resulted in contigs for both sets of primers that only matched to sequences for other species. As such, these were taken as evidence of environmental contaminants and were disregarded. Further species identification using DNA barcoding was not attempted for this cultivar.

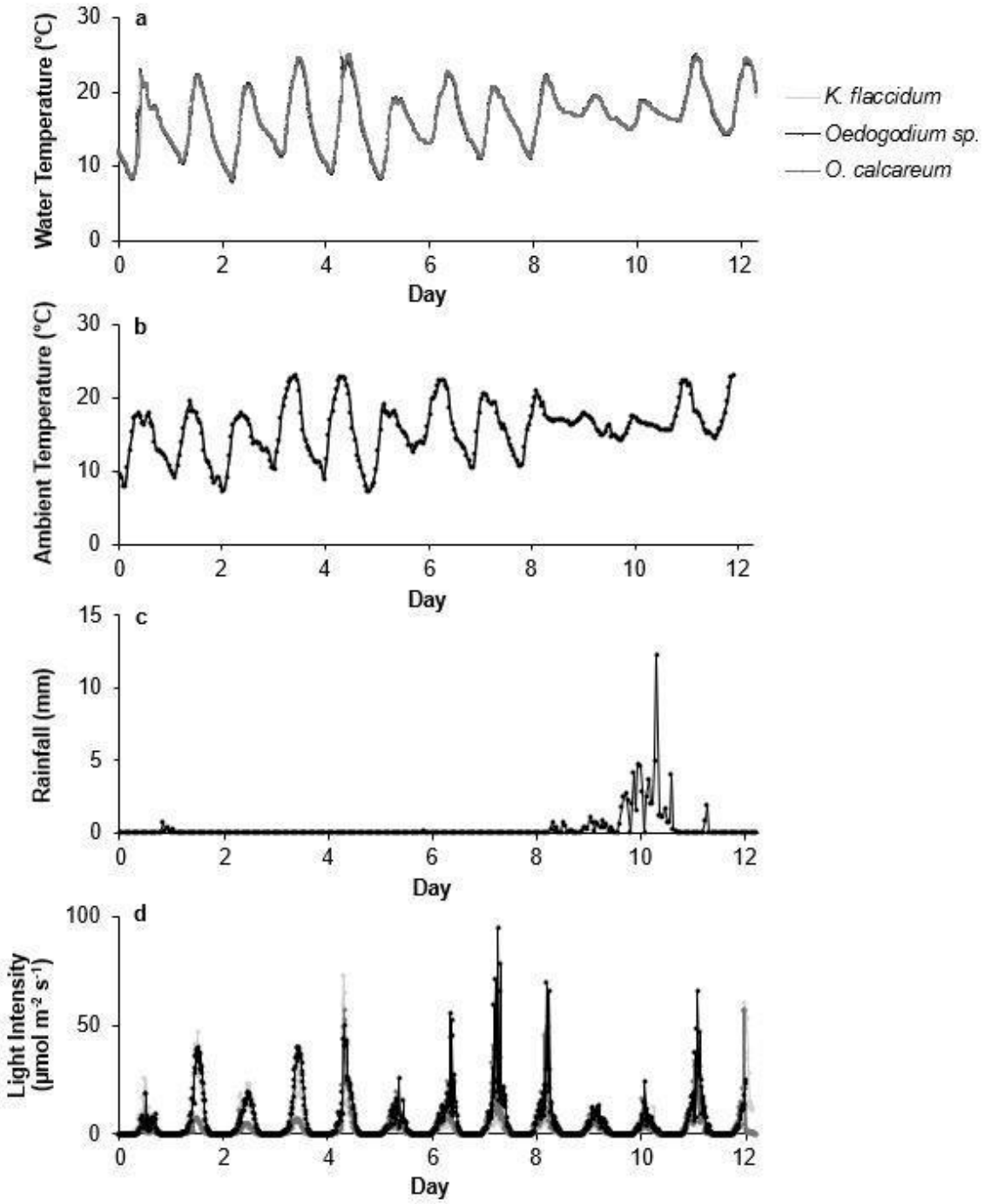

Figure 3A.1 Culture water temperature (a), ambient air temperature (b), rainfall (c) and culture light intensity (d) recorded in *K. flaccidum, O. calcareum* and *Oedogonium* sp. culture water over three consecutive four-day harvest cycles during the biomass productivity and bioremediation performance experiment.

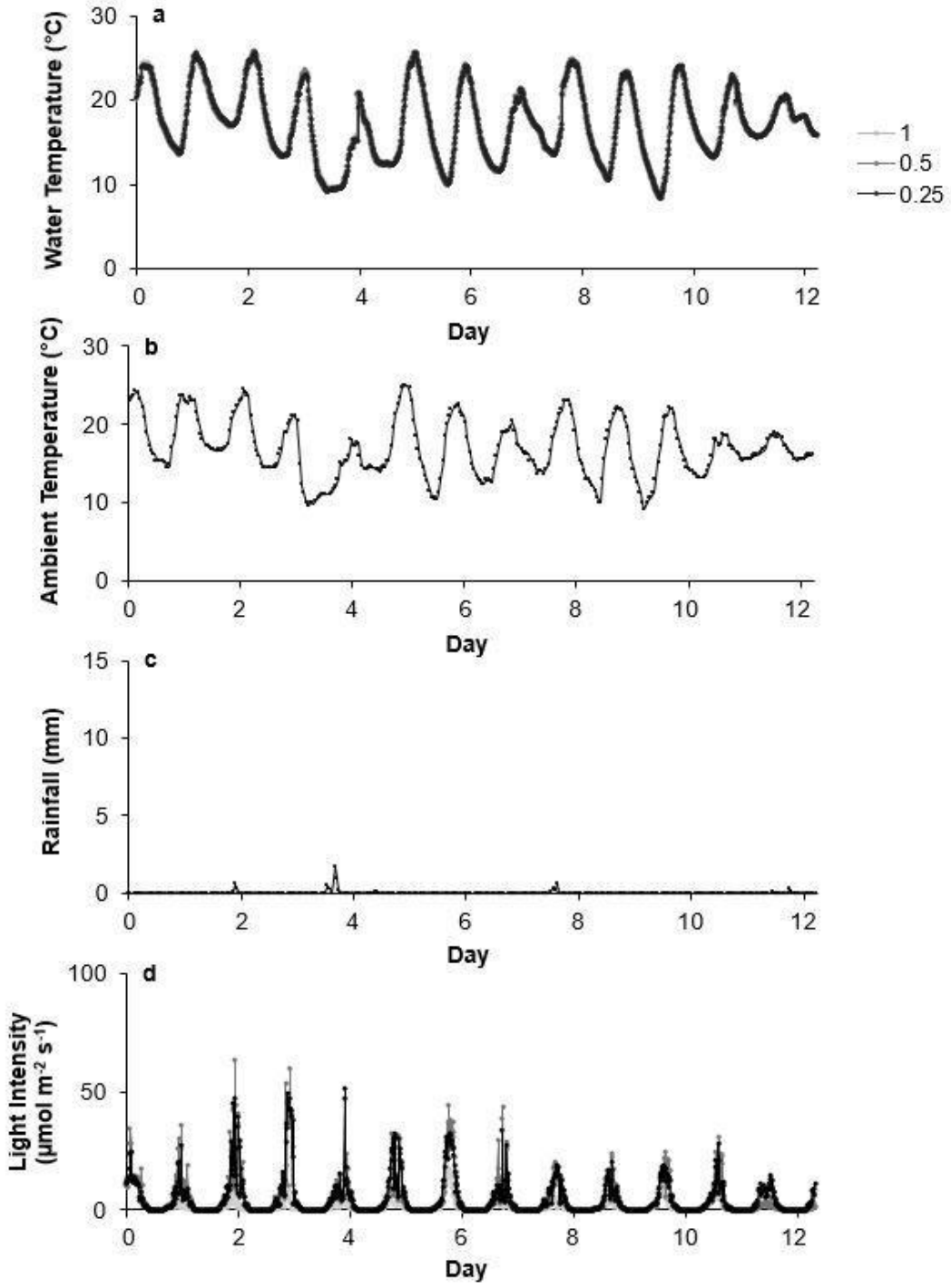

Figure 3A.2 Culture water temperature (a), ambient air temperature (b), rainfall (c) and culture light intensity (d) recorded in culture water of three stocking densities over three consecutive four-day harvest cycles during bi-culture composition experiment.

Chapter 4 - Optimisation of high-rate filamentous algal pond operating parameters for nutrient bioremediation of primary municipal wastewater

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4.1 Abstract

Effective management of operational parameters is crucial for optimising wastewater treatment in high-rate filamentous algal pond (HRFAP) systems. This study examined the influence of three key operational parameters - hydraulic retention time (HRT), stocking density, and harvest frequency - on the growth and nutrient bioremediation efficiency of *Klebsormidium flaccidum* cultivated in primary municipal wastewater in outdoor HRFAPs in summer and winter. Seasonal conditions significantly influenced biomass productivity, with productivity being 48.3% higher in summer compared to winter across all experiments. The optimal HRT was 4 days for both seasons as it resulted in the highest average reductions in total ammoniacal-nitrogen (TAN) concentration (64.6% day^{-1} ± 1.8 SE in summer, 32.3% day^{-1} ± 1.8 SE in winter) and acceptable reductions in nitrate-N (66.6% day^{-1} ± 3.5 SE in summer, 42.6% day^{-1} ± 9.0 SE in winter) and dissolved reactive phosphorous (DRP, 19.8% day^{-1} ± 1.0 SE in summer, 15% day^{-1} ± 2.0 SE in winter). A stocking density of 0.25 g FW L^{-1} was optimal in summer as it resulted in the highest reductions in TAN (75.9% day^{-1} ± 2.5 SE), nitrate-N (43.8% day^{-1} ± 3.3 SE), and DRP (21.6% day^{-1} ± 1.4 SE). In winter, a stocking density of 0.5 g FW L^{-1} was optimal to mitigate the risk of primary wastewater toxicity during slower growth periods. Harvest frequency did not significantly affect nutrient removal rates across treatments and seasons; however, biomass productivity was significantly higher in summer with a 4-day harvest frequency (7.83 g DW m^{-2} day^{-1}). Longer HRTs improved water quality variables, with the highest *Escherichia coli (E. coli)* reduction (1.92 log_{10}) observed with a stocking density of 0.25 g FW L^{-1} in winter. This study highlights the importance of seasonal optimisation of HRFAP operation to maximise biomass production and nutrient bioremediation for effective treatment of primary municipal wastewater.

4.2 Introduction

High-rate algal ponds (HRAP) are shallow, mixed raceway-based outdoor systems that utilise algae to assimilate nutrients from wastewater (Nurdogan & Oswald, 1995; Park & Craggs, 2011; Craggs et al., 2013). The cultivation of monocultures of freshwater filamentous macroalgae in HRAP systems is emerging as an effective alternative to traditional treatment of municipal wastewater (Liu et al., 2020; Sabatte et al., 2024). Maintaining consistent bioremediation performance, biomass productivity and resource recovery in HRAP systems is critical for effective wastewater treatment (Cole et al., 2016b; Arashiro et al., 2018). Operational parameters, including hydraulic retention time (HRT), stocking density, and harvest frequency have been identified as key factors influencing HRAP performance (Sutherland et al., 2020a; Sutherland & Ralph, 2021). These operational parameters can be refined based on the preferences of the target cultivar to maximise the cost-effectiveness and energy-efficiency of HRAP systems (Sutherland & Ralph, 2021). Furthermore, understanding the interaction between operational parameters and environmental conditions, such as ambient light and temperature, can inform pond system management practices across varying seasonal conditions (Ranjan et al., 2019; Liu et al., 2020). While previous research has investigated the effect of individual parameters on HRAP performance (Siville & Boeing, 2020; Ishika et al., 2021; Sutherland & Ralph, 2021), to date, the role of modifying operational parameters, both individually and in combination, within an open outdoor pond system under varying seasonal conditions has not been investigated (Sabatte et al., 2024). This is a critical knowledge gap that needs to be addressed to ensure that HRAP systems can provide consistent year-round treatment of municipal wastewater (Ranjan et al., 2019).

The HRT – the average time a given volume of water is retained within a system - is the metric used to determine the total time (days) required for effective removal of nutrients from wastewater within HRAP systems (Yun et al., 2015; Ge et al., 2018). Typically, HRAPs operate with shorter HRTs in summer when algal productivity is at its highest and nutrient removal is correspondingly high, and longer HRTs in winter when productivity and nutrient removal are lowest, to ensure adequate nutrient removal to meet effluent discharge standards (Park et al., 2013a; Arcila & Buitrón, 2016; Leong et al., 2021). For example, HRAP systems stocked with microalgae treating wastewater utilised HRTs of 3 - 4 days in summer and 7 - 9 days in winter (Craggs et al., 2014). The HRT required to achieve adequate nutrient removal

is also affected by the initial nutrient concentration in the wastewater and the bioremediation efficiency of the target cultivar (Sutherland et al., 2020c). Wastewater with higher nutrient concentrations, such as primary treated municipal wastewater, may require longer HRTs to facilitate adequate nutrient removal through algal uptake, compared to wastewater with lower nutrient concentrations (Mulholland et al., 1991; Neveux et al., 2016). Furthermore, the high turbidity of primary municipal wastewater can limit light penetration into the water column thereby reducing photosynthetic production. Consequently, effective nutrient removal in turbid wastewater may require extended HRTs compared to wastewater with higher clarity (Sutherland & Ralph, 2020). However, prolonged HRTs may deplete the supply of dissolved inorganic carbon (DIC) within the wastewater, thereby diminishing algal growth (Mata et al., 2007; Cole et al., 2014b) and nutrient removal rates (Cole et al., 2014a). As HRT is one of the main operational parameters within a HRAP that can be modified, optimal HRTs must be determined across seasons for specific wastewater types and target cultivars for the effective management of HRAP systems (Sutherland et al., 2020c).

Stocking density - the weight of algal biomass in a given volume of water - is a critical factor that influences algal biomass production and nutrient removal (Jabłońska-Trypuć et al., 2023). In open outdoor pond systems such as HRAPs, lower stocking densities are typically preferred to mitigate self-shading, which can hinder photosynthetic activity and growth (Pereira et al., 2006; Ranjan et al., 2019). Additionally, primary municipal wastewater can contain elevated concentrations of suspended solids which limit light penetration into the water column, potentially restricting photosynthesis, and algal growth (Craggs et al., 2014; Messyasz et al., 2018; Ullah et al., 2023). Consequently, lower stocking densities can allow more light to penetrate into the water column, enhancing photosynthetic activity and therefore biomass productivity and nutrient removal within the culture (Dasgupta et al., 2019). However, higher stocking densities can also offer advantages, particularly for the treatment of primary wastewater, which may contain toxic compounds that can lead to algal die-off (Ahmed et al., 2021; Chapter 3). Higher stocking densities can enhance tolerance to toxic compounds, ensuring a sustainable culture capable of maintaining continued growth and nutrient removal in wastewater containing stochastically occurring micropollutants (Chapter 3). Additionally, higher stocking densities can promote the dominance of the target cultivar over wild algal strains, ensuring consistent bioremediation performance (Liu et al., 2020). Given the critical role of stocking density in HRAP management, identifying the optimal

stocking density of the target cultivar across seasons is imperative to facilitate efficient nutrient removal rates.

The optimum harvest frequency – the rate by which algal biomass is removed from the pond system – is a function of the growth rate of the target cultivar and the desired stocking density of algal biomass within the pond system. While many long-term demonstrations of wastewater treatment systems with filamentous algae have employed a weekly harvesting regime across multiple seasons, the frequency can vary from monthly for slow-growing algae in winter to bi-weekly for fast-growing algae in summer (Cole et al., 2016b; Neveux et al., 2016). Increased harvest frequencies can improve biomass yields and nutrient removal rates (Sutherland et al., 2020a). Additionally, increased harvesting can mitigate self-shading effects, control grazer populations, and maintain algal biomass densities by preferential removal of large, mature, slow-growing filaments (Sutherland et al., 2015b; Chen et al., 2016). However, higher harvesting frequencies may result in elevated operational costs, making low harvest frequencies advantageous for the economic sustainability of algal based wastewater treatment systems (Diao et al., 2024; Uzoejinwa & Asoiro, 2024). Therefore, establishing the optimal harvest regime across seasons is critical for effective pond system management and achieving consistent bioremediation outcomes.

Despite the recognised importance of operational parameters for achieving consistent nutrient bioremediation performance, biomass productivity and resource recovery, prior research on freshwater filamentous algae has primarily focused on species/strain selection (Lawton et al., 2013a; Valero-Rodriguez et al., 2020; Lawton et al., 2021a), with limited studies investigating operational parameters (Cole et al., 2014a; Cole et al., 2018). Furthermore, these studies were conducted in tropical locations where seasonal variation in environmental conditions is low, and the algae were cultivated in aerated circular or rectangular tanks in which the algae may perform differently compared to HRAP systems. Therefore, the objective of this study is to investigate the effects of HRAP operational parameters on the performance of filamentous freshwater macroalgal monocultures in outdoor systems across seasonal temperate conditions. Specifically, this study focuses on the freshwater filamentous cultivar *Klebsormidium flaccidum* which was previously identified as a target cultivar for primary municipal wastewater treatment based on its growth, nutrient bioremediation performance, and competitive dominance when cultivated in primary municipal wastewater in high-rate filamentous algal ponds (HRFAP) (Chapter 3). The

specific aims of this study were to (i) determine the effects of HRT, stocking density, and harvest frequency on the growth and bioremediation performance of *K. flaccidum* when cultivated in primary municipal wastewater in HRFAPs, and (ii) compare the effects of HRT, stocking density, and harvest frequency on growth and bioremediation performance across seasons (summer and winter). Understanding the interaction between HRFAP operational parameters and environmental conditions will facilitate effective management of HRFAP systems to maximise bioremediation performance.

4.3 Methods

The freshwater filamentous macroalgal cultivar *K. flaccidum* was originally collected from a culvert in Rangiuru, Te Puke (37° 84' S 176° 35' E), proximate to the tributary into which the Te Puke wastewater treatment plant (WWTP) discharges and was identified using DNA barcoding (Chapter 3). Prior to experiments, this cultivar was scaled up and maintained in outdoor 1,000 L tanks for at least three months at the Facility for Aquaculture Research of Macroalgae, University of Waikato Coastal Marine Field Station, Tauranga, New Zealand. Cultures were stocked at 0.5 g fresh weight (FW) L^{-1} and maintained with weekly water exchanges of filtered dechlorinated freshwater (town water supply) with nutrient concentrations which were based on primary wastewater concentrations of 5 mg NH_4-N L^{-1} (NH_4Cl), 1.3 mg PO_4-P L^{-1} ($NaH_2PO_4 \cdot 2H_2O$) and trace metal concentrations ($FeCl_3 \cdot 6H_2O$, $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $Na_2MoO_4 \cdot 2H_2O$) as per F/2 artificial growth medium (Ryther & Guillard, 1962; Guillard, 1975). All components of the nutrient media were analytical grade.

4.3.1 Study site

Experiments were conducted at the Te Puke municipal WWTP, Western Bay of Plenty, NZ. The WWTP treats an annual average flow of 1,800 m^3 per day, servicing about 8,100 residents. The primary wastewater used for experiments was collected after the solids were removed and before the primary wastewater discharged into the clarifier (Figure 4.1). During summer experiments the primary treated wastewater contained an average total ammoniacal-N (TAN) concentration of 36.4 mg L^{-1}(± 0.94 SE), an average nitrate-N (NO_3-N) concentration of 0.9 mg L^{-1} (± 0.07 SE) and an average dissolved reactive phosphorous

(DRP) concentration of 4.7 mg L^{-1} (± 0.34 SE). During winter experiments the primary treated wastewater contained an average TAN concentration of 41.9 mg L^{-1} (± 1.19 SE), an average nitrate-N concentration of 1.0 mg L^{-1} (± 0.05 SE) and an average DRP concentration of 4.4 mg L^{-1} (± 0.27 SE). Prior to experiments, *K. flaccidum* was acclimated at the WWTP as described previously (Chapter 3)

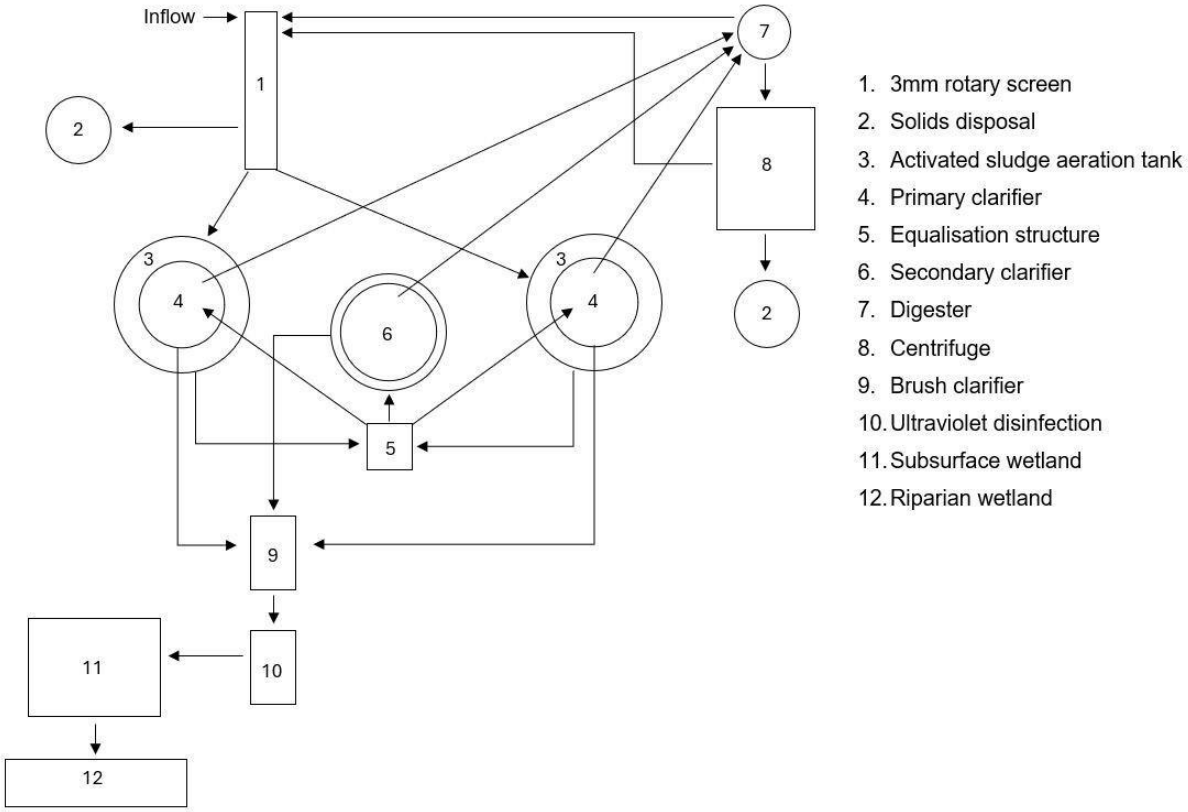

Figure 4.1 Te Puke wastewater treatment plant process layout. Primary wastewater collected prior to entering the activated sludge aeration tanks.

4.3.2 High-rate filamentous algal pond mesocosms

Nine HRFAP mesocosms were set up for experimentation, each consisting of a plastic 113 L^{-1} trough (H 31 cm H x 88 cm L x 62 cm W) filled with 70 L of primary wastewater (Figure 4.2). The approximate water depth was 24 cm. A stainless-steel paddle wheel, rotating at 8 rpm, continuously circulated the primary wastewater within each HRFAP. To manage water volume, standpipes with mesh panels allowed overflow of accumulated rainfall. Partial water exchanges occurred every two days to maintain desired HRTs, with drained water equivalent to the daily total exchanged volume for each HRT treatment if the

HRFAPs operated on flow-through conditions. This drained water was replaced with primary wastewater from the Te Puke municipal WWTP.

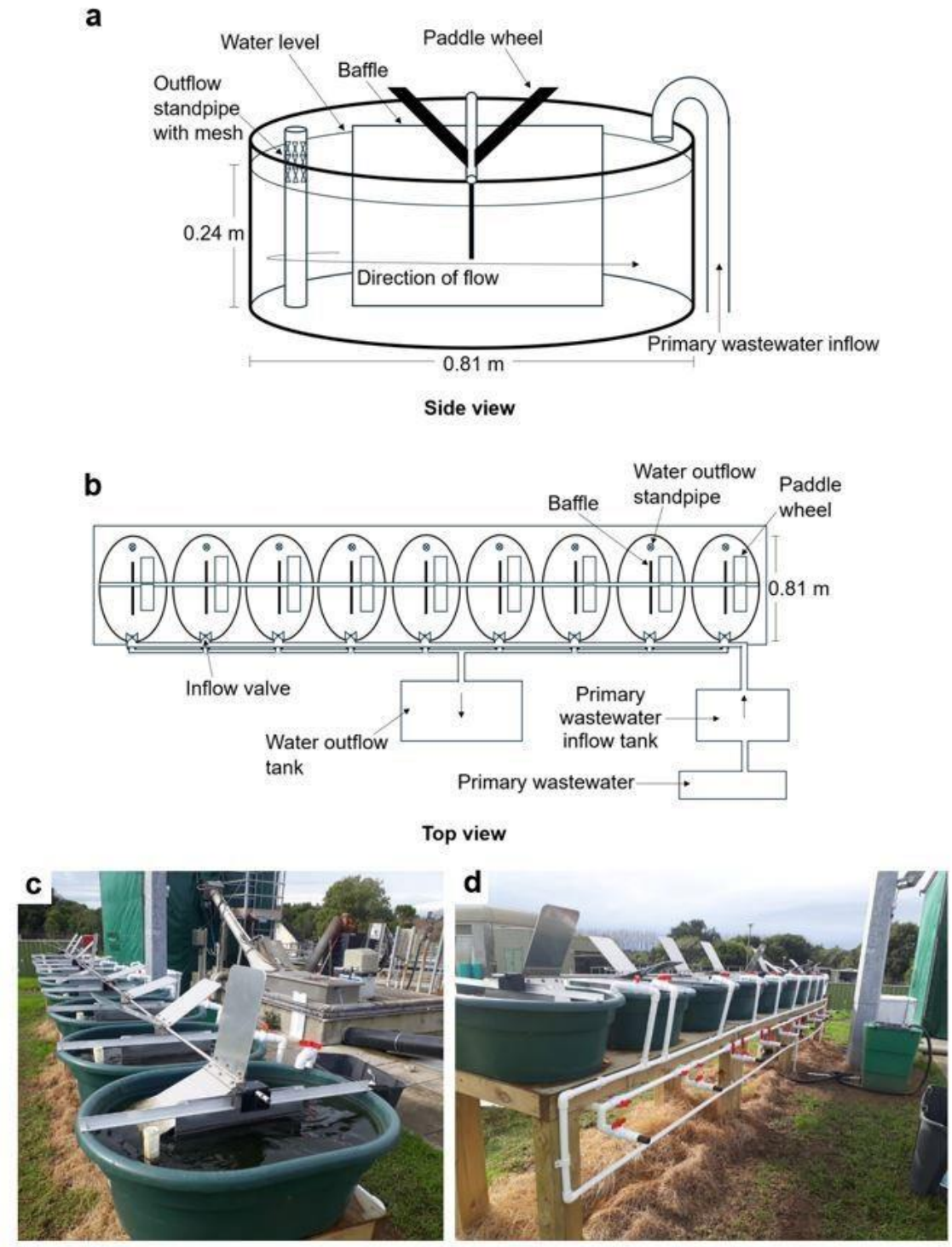

Figure 4.2 HRFAP side view diagram (a), HRFAPs system top view diagram (b), the nine outdoor HRFAPs used in this study set up on site, at the WWTP (c, d).

4.3.3 Physico-chemical monitoring

Culture water light and temperature was measured continuously at the bottom of three of the HRFAPs using HOBO MX2201 water temperature loggers (Onset). Dissolved oxygen and pH were measured at approximately 10 am every two days within each HRFAP at a mid-HRFAP depth of 12 cm using an OxyGuard Handy Polaris 2 Dissolved Oxygen Meter and an OxyGuard Handy pH Meter. Air temperature and precipitation data were obtained from the National Climate Database weather recording station located in Te Puke (-37.82455, 176.32048, data available from www.cliflo.niwa.co.nz). The maximum light intensity within the HRFAPs ranged from 3.07 to 184 μmol m^{-2} s^{-1} in summer and from 6.08 to 125 μmol m^{-2} s^{-1} in winter (Appendix 4.7 Figure 4A.1 - 4A.3). Culture water temperature ranged from 9.5°C to 32.9°C in summer and from 5.1°C to 30.9°C in winter, ambient temperature ranged from 8.8°C to 29.1°C in summer and from 4.4°C to 24.7°C in winter (Appendix 4.7 Figure 4A.1 - 4A.3). A total rainfall of 93 mm and 325 mm was recorded during experiments in summer and winter, respectively (Appendix 4.7 Figure 4A.1 - 4A.3). Three 500 mL water samples were collected from each HRFAP immediately before the final harvest of each of the three experiments described below to measure total suspended solids (TSS), biological oxygen demand (BOD), chemical oxygen demand ($cBOD_5$) and abundance of *Escherichia coli (E. coli)*. These analyses were conducted by Hill Laboratories in Hamilton, New Zealand, using the following APHA 23rd ed. 2017 methodologies: TSS 2540 D (modified), $cBOD_5$ 5210 B (modified), COD 5220 D, and *E. coli* 9222 I.

4.3.4 Experimental methodology

Three consecutive experiments were conducted to assess the effects of hydraulic retention time, stocking density, and harvest frequency on biomass productivity and nutrient removal. Experiments were conducted in summer and repeated in winter to determine the optimal seasonal operational parameters for *K. flaccidum* to achieve maximal nutrient bioremediation performance. The three experiments were conducted consecutively for a total of 48 days during each season. Identical methods were used in each season for each experiment unless specified below.

4.3.5 Hydraulic retention time experiment

Klebsormidium flaccidum was grown in HRFAP mesocosms with HRTs of two days, four days, and six days, with three replicate HRFAPs for each HRT (total n=9 ponds). *Klebsormidium flaccidum* was grown in each HRFAP at a stocking density of 1 g fresh weight (FW) L^{-1}. Biomass was harvested from each HRFAP once every four days for a total of six harvest cycles, equating to a total experimental duration of 24 days per season. Biomass was harvested by straining the entire contents of each HRFAP (culture water and algae) through a fine mesh bag. Culture water was retained in a separate container. Once excess water had drained from the bag, it was placed in a centrifugal spin dryer (Spindle NZ, SPL-265) and spun for three minutes to remove any remaining water. The algal biomass was then removed from the bag and weighed to determine the FW. There were no algae present on the paddlewheels or surfaces. Each HRFAP was cleaned by scrubbing the surface of the paddlewheel and the HRFAP with a brush. HRFAPs were then refilled with the culture water which was drained from them during harvesting. Stocking density was reset to 1 g FW L^{-1} by restocking 70 g FW of the harvested biomass back into each replicate HRFAP. Biomass not restocked back in the HRFAP was dried in an oven at 60 °C for 48 hours and reweighed to determine the fresh weight to dry weight (FW:DW) ratio for each replicate HRFAP. FW:DW ratios were used to convert the initial biomass and the harvested biomass for each replicate, which were both measured in FW, into DW. Biomass productivity (g DW m^{-2} day^{-1}) was calculated for each replicate for each harvest using the equation $P = (DW_f - DW_i) / A / T$, where DW_f is the final algal biomass and DW_i is the initial algal biomass (g DW), A is the surface area (m^2) of the HRFAP, and T is the number of days in culture.

The bioremediation efficacy of cultivars was assessed by measuring the concentrations of total ammoniacal-N (TAN), nitrate-N (NO_3-N), and dissolved reactive phosphorous (DRP) in both the primary wastewater and the HRFAP culture water. Every two days, a 30 mL sample of primary wastewater and culture water was collected from each replicate HRFAP. Primary wastewater samples were obtained in the morning during peak inflow to the WWTP, while HRFAP water samples were collected immediately before water changes. Upon collection, water samples were filtered into individual sterile 50 mL clear plastic test tubes (LabServ™) using a vacuum filtration system (Whatman™ GF/C™, 0.22µm) and immediately frozen. Samples were thawed and analysed within a week of collection. TAN, nitrate-N, and DRP concentrations in each sample were determined using a spectrophotometer (HACH DR 900, Loveland, CO, USA) following the USEPA Nessler method (HACH method 8038), the nitrate cadmium reduction method (HACH method 8039)

and the ascorbic acid method (HACH method 8048) respectively. Nutrient removal rate (% day^{-1}) was calculated at each water change using the equation $NR = CW_f / ((CW_i + PW_i) / E) * 100$, where CW_f and CW_i are the final and initial nutrient concentrations of the culture water, PW_i is the initial nutrient concentration of the primary wastewater and E is the proportion of water exchanged.

4.3.6 Stocking density experiment

Klebsormidium flaccidum was grown in HRFAP mesocosms at fresh weight stocking densities of 0.25 g L^{-1}, 0.5 g L^{-1} and 1 g L^{-1}, with three replicate HRFAPs for each stocking density (total n=9 ponds). A HRT of four days was maintained in both summer and winter based on the results of the experiment above. Biomass was harvested from each HRFAP once every four days for a total of three harvest cycles, equating to a total experimental duration of 12 days per season. Experimental protocols followed the same methodology for water changes, harvesting, biomass processing, biomass productivity analysis, and bioremediation performance analysis as described above.

4.3.7 Harvest frequency experiment

Klebsormidium flaccidum was grown in HRFAP mesocosms with a harvest frequency of two days, four days, and six days, with three replicate cultures per harvest frequency (total n=9 ponds). The experiment was run for a total of 12 days for each season, involving six, three and two consecutive harvests for the two-day, four day and six-day harvest treatments respectively. *Klebsormidium flaccidum* was grown in each HRFAP at a stocking density of 0.25 g FW L^{-1} in summer and 0.5 g FW L^{-1} in winter, and a HRT of four days in summer and winter based on the results of experiments described above. Experimental protocols followed the same methodology for water changes, harvesting, biomass processing and biomass productivity analysis as described above.

4.3.8 Statistical analysis

Two-way repeated-measures analyses of variance (ANOVA) were used to analyse differences in biomass productivity and nutrient removal with HRT (experiment 1), stocking

density (experiment 2), harvest frequency (experiment 3) and days as fixed factors. In cases where variables did not meet normality or homogeneity of variance assumptions, a Kruskal-Wallis test was utilised. Data for each experiment and each season were analysed separately. All analyses were conducted in SPSS Statistics (version 29). All data are reported as means ± S.E.

4.4 Results

4.4.1 Hydraulic retention time experiment

Biomass productivity in summer did not vary significantly among HRT treatments, and across all days was comparable between the 2-, 4- and 6-day HRT treatments (2-day HRT: 4.62 g DW m^{-2} day^{-1} ± 0.20; 4-day HRT: 5.07 g DW m^{-2} day^{-1} ± 0.18; 6-day HRT: 4.86 g DW m^{-2} day^{-1} ± 0.11, Figure 4.3, Table 4.1). However, across all HRT treatments, biomass productivity varied significantly among days, showing an overall decrease through time (Figure 4.3, Table 4.1). Conversely, in winter, biomass productivity varied significantly with HRT treatment and days (Table 4.1). Productivity across all days was highest in the 2-day HRT treatment (4.24 g DW m^{-2} day^{-1} ± 0.53) and there was an overall increase in productivity through time across all HRT treatments. Nutrient removal rates in both seasons varied significantly among days and HRT treatments (Figure 4.4, Table 4.1). Both the 4-day and 6-day HRT treatments consistently reduced TAN concentrations and nitrate-N concentrations throughout the experiment at comparable rates of 64.6% day^{-1} (± 1.8) and 66.6% day^{-1} (± 3.5) respectively for a 4-day HRT, and 63.6% day^{-1} (± 2.9) and 66.2% day^{-1} (± 7.0) respectively for a 6-day HRT. Additionally, both HRT treatments reduced TAN concentrations to zero by day 24, and the 6-day HRT treatment also reduced nitrate-N concentrations to zero by day 24 (Figure 4.4). Similarly, in winter, the 4-day and 6-day HRT treatments reduced TAN concentrations at comparable rates of 32.3% day^{-1} (± 3.6) and 39.2% day^{-1} (± 3.7) respectively, with the 6-day HRT treatment reducing TAN concentrations to zero by day 24. In contrast, patterns for nitrate-N concentrations were variable, with concentrations reduced to zero in all HRT treatments by day 6, followed by smaller reductions in nitrate-N concentrations for the 2-day and 4-day HRT treatments from day 14 onwards, and a large increase nitrate-N concentrations for the 6-day HRT treatment from day 14 throughout the remainder of the experiment. DRP concentrations consistently decreased across all treatments

in summer, with reduction rates ranging between 17.3% day^{-1} (± 0.5) and 19.8% day^{-1} (± 1.0). In contrast, DRP concentrations varied in winter, and the 2-day HRT had the highest reduction rate across all days (20.2% day^{-1} ± 1.4).

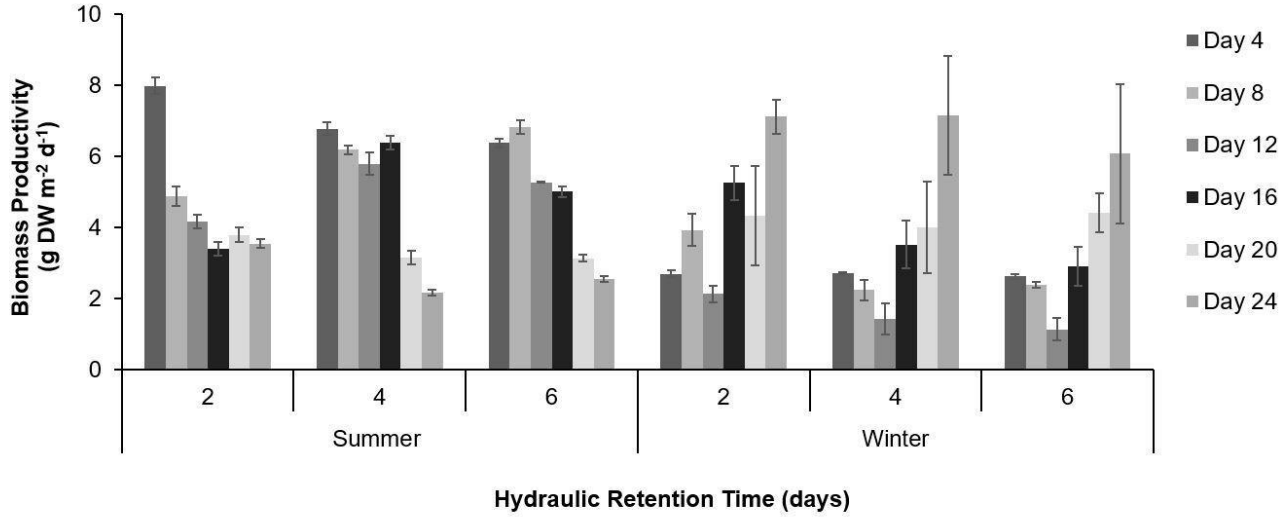

Figure 4.3 Mean (± S.E.) biomass productivity (g DW m^{-2} day^{-1}) of *Klebsormidium flaccidum* cultures grown in HRFAP mesocosms with a hydraulic retention time (HRT) of two, four or six days over a 24-day period during summer and winter. N = 3.

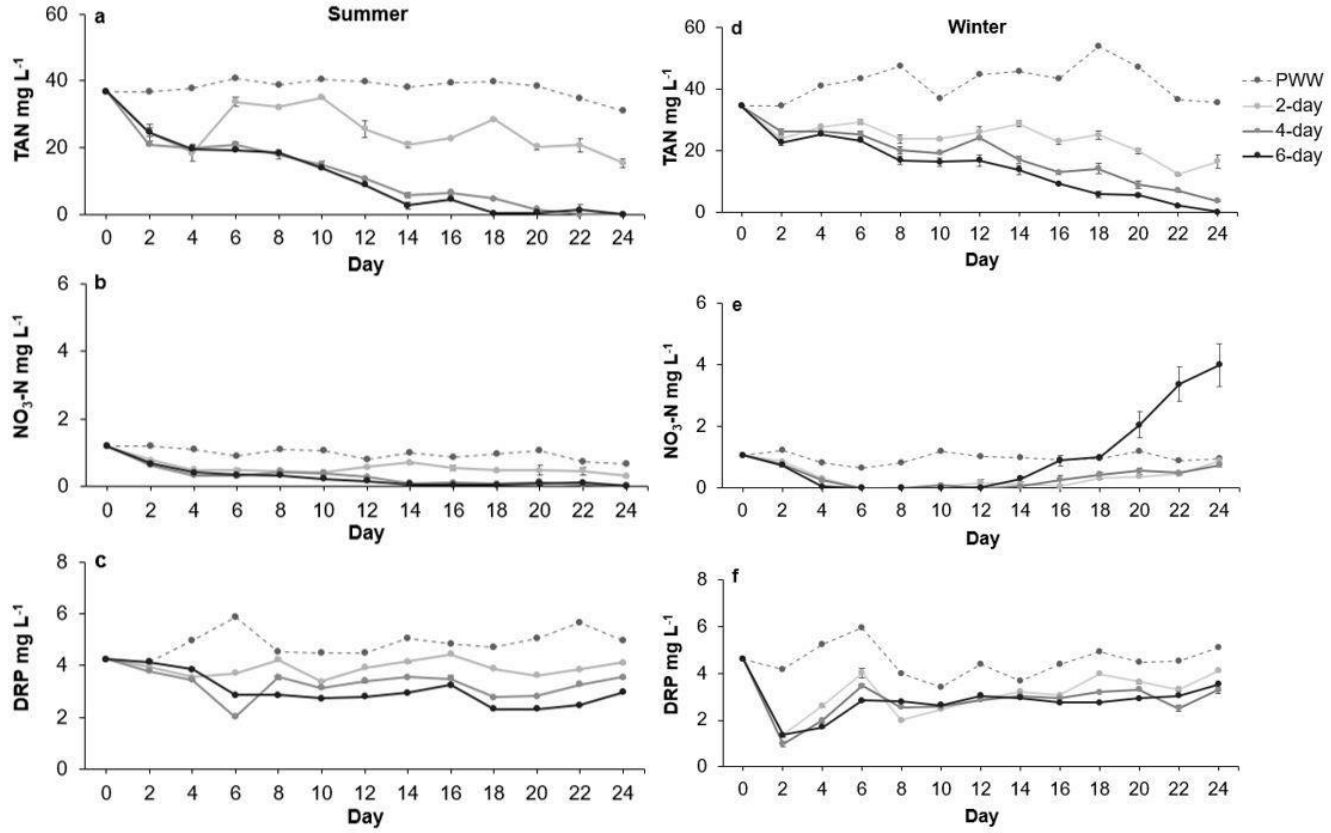

Figure 4.4 Mean (± S.E.) concentrations of TAN (mg L^{-1}) (a, d), nitrate-N (mg L^{-1}), and (b, e) DRP (mg L^{-1}) (c, f) in primary wastewater (PWW) inflows and *Klebsormidium flaccidum* cultures grown in HRFAP mesocosms with a hydraulic retention time (HRT) of two, four, or six days over a 24-day period during summer and winter. N = 3.

Table 4.1 Results of two-way repeated-measures ANOVA testing the effects of days and HRT, stocking density and harvest frequency on the biomass productivity, and concentrations of TAN, nitrate-N and DRP in culture water during winter and summer experiments.

Variable	Effect	df	F	P
HRT				
Summer				
Biomass Productivity	HRT	2	4.5	0.065
	Day	1	1312.4	<0.001
	HRT x Day	2	4.0	0.079
	Residual	6		
TAN	HRT	2	251.6	<0.001
	Day	11	98.0	<0.001
	HRT x Day	22	14.3	<0.001
	Residual	66		
Nitrate-N	HRT	2	19.9	<0.001
	Day	11	7.3	<0.001
	HRT x Day	22	4.1	<0.001
	Residual	66		
DRP	HRT	2	84.6	<0.001
	Day	11	386.7	<0.001
	HRT x Day	22	84.7	<0.001
	Residual	66		
Winter				
Biomass Productivity	HRT	2	25.3	0.001
	Day	1	0.007	0.938
	HRT x Day	2	20.4	0.002
	Residual	6		
TAN	HRT	2	6.5	0.031
	Day	11	67.9	<0.001
	HRT x Day	22	9.5	<0.001
	Residual	66		

Variable	Effect	df	F	P
Nitrate-N	HRT	2	115.4	<0.001
	Day	11	10.7	<0.001
	HRT x Day	22	4.5	<0.001
	Residual	66		
DRP	HRT	2	107.2	<0.001
	Day	11	33.9	<0.001
	HRT x Day	22	9.0	<0.001
	Residual	66		
Stocking Density (SD)				
Summer				
Biomass Productivity	SD	2	244.2	<0.001
	Day	2	16.0	<0.001
	SD x Day	4	7.5	0.003
	Residual	12		
TAN	SD	2	14.4	0.005
	Day	5	21.8	<0.001
	SD x Day	10	4.0	0.001
	Residual	30		
Nitrate-N	SD	2	1797.3	<0.001
	Day	5	240.5	<0.001
	SD x Day	10	4.7	<0.001
	Residual	30		
DRP	SD	2	47.4	<0.001
	Day	5	50.4	<0.001
	SD x Day	10	11.1	<0.001
	Residual	30		
Winter				
Biomass Productivity	SD	2	9.6	0.014

Variable	Effect	df	F	P
	Day	2	155.09	<0.001
	SD x Day	4	27.4	<0.001
	Residual	12		
TAN	SD	2	81.7	<0.001
	Day	5	17.9	<0.001
	SD x Day	10	4.9	<0.001
	Residual	30		
Nitrate-N	SD	2	4.4	0.066
	Day	5	9.3	<0.001
	SD x Day	10	3.2	0.006
	Residual	30		
DRP	SD	2	20.4	0.002
	Day	5	107.6	<0.001
	SD x Day	10	11.4	<0.001
	Residual	30		
Harvest Frequency (HF)				
Summer				
Biomass Productivity	HF	2	58.8	<0.001
	Day	1	2.8	0.145
	HF x Day	2	19.4	0.002
	Residual	6		
TAN	HF	2	3.4	0.104
	Day	5	10.9	<0.001
	HF x Day	10	3.9	0.002
	Residual	30		
Nitrate-N	HF	2	2.5	0.164
	Day	5	4.9	0.002
	HF x Day	10	7.1	<0.001
	Residual	30		

Variable	Effect	df	F	P
DRP	HF	2	234.3	<0.001
	Day	5	730.5	<0.001
	HF x Day	10	8.0	<0.001
	Residual	30		
Winter				
Biomass Productivity	HF	2	25.3	0.001
	Day	1	0.007	0.938
	HF x Day	2	20.4	0.002
	Residual	6		
TAN	HF	2	17.7	0.003
	Day	5	128.2	<0.001
	HF x Day	10	6.7	<0.001
	Residual	30		
Nitrate-N	HF	2	23.2	0.002
	Day	5	20.4	<0.001
	HF x Day	10	2.4	0.028
	Residual	30		
DRP	HF	2	708.1	<0.001
	Day	5	47.6	<0.001
	HF x Day	10	21.1	<0.001
	Residual	30		

4.4.2 Stocking density experiment

Biomass productivity in summer and winter varied significantly among stocking density treatments and days (Figure 4.5, Table 4.1). In summer, productivity was highest at stocking densities of 0.25 g FW L^{-1} and 0.5 g FW L^{-1} (5.18 g DW m^{-2} day^{-1} ± 0.11 and 5.02 g DW m^{-2} day^{-1} ± 0.18, respectively across all days) compared to a stocking density of 1 g FW L^{-1} (3.41 g DW m^{-2} day^{-1} ± 0.11, across all days, Figure 4.5, Table 4.1). In winter, however, biomass productivity was highest in the 1 g FW L^{-1} stocking density

(2.45 g DW m^{-2} day^{-1} ± 0.09 across all days). Nutrient removal rates in both seasons varied significantly among days and HRT treatments (Figure 4.6, Table 4.1). During both summer and winter, all stocking density treatments reduced TAN concentrations throughout the experiment at rates of 70.6% day^{-1} (± 1.5) to 75.9% day^{-1} (± 2.5) in summer and 38.8% day^{-1} (± 2.6) to 45.2% day^{-1} (± 1.9) in winter. In contrast, removal rates of nitrate-N were variable throughout the experiment, ranging from 29.0% day^{-1} (± 6.3) to 43.8% day^{-1} (± 3.3) in summer, and 33.5% day^{-1} (± 8.1) to 46.6% day^{-1} (± 11.6) in winter. DRP concentrations consistently decreased across all stocking density treatments in both seasons, but reduction rates varied through time, ranging from 14.6% day^{-1} (± 1.0) to 21.6% day^{-1} (± 1.4) in summer and from 22.6% day^{-1} (± 0.9) to 26.6% day^{-1} (± 2.0) in winter.

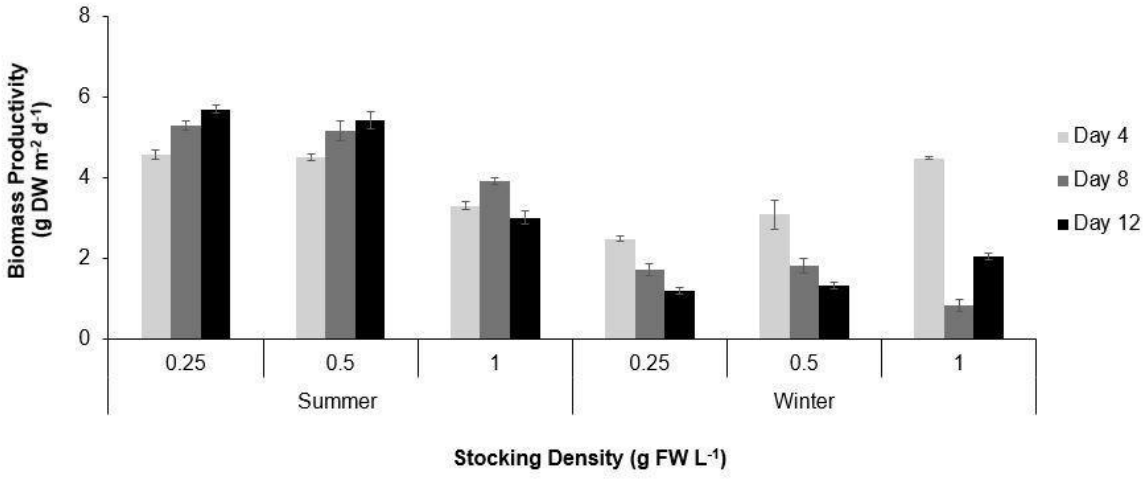

Figure 4.5 Mean (± S.E.) biomass productivity (g DW m^{-2} day^{-1}) of *Klebsormidium flaccidum* cultures grown in HRFAP mesocosms with a stocking density of 0.25 g FW L^{-1}, 0.5 g FW L^{-1} or 1 g FW L^{-1} over a 12-day period during summer and winter. N = 3.

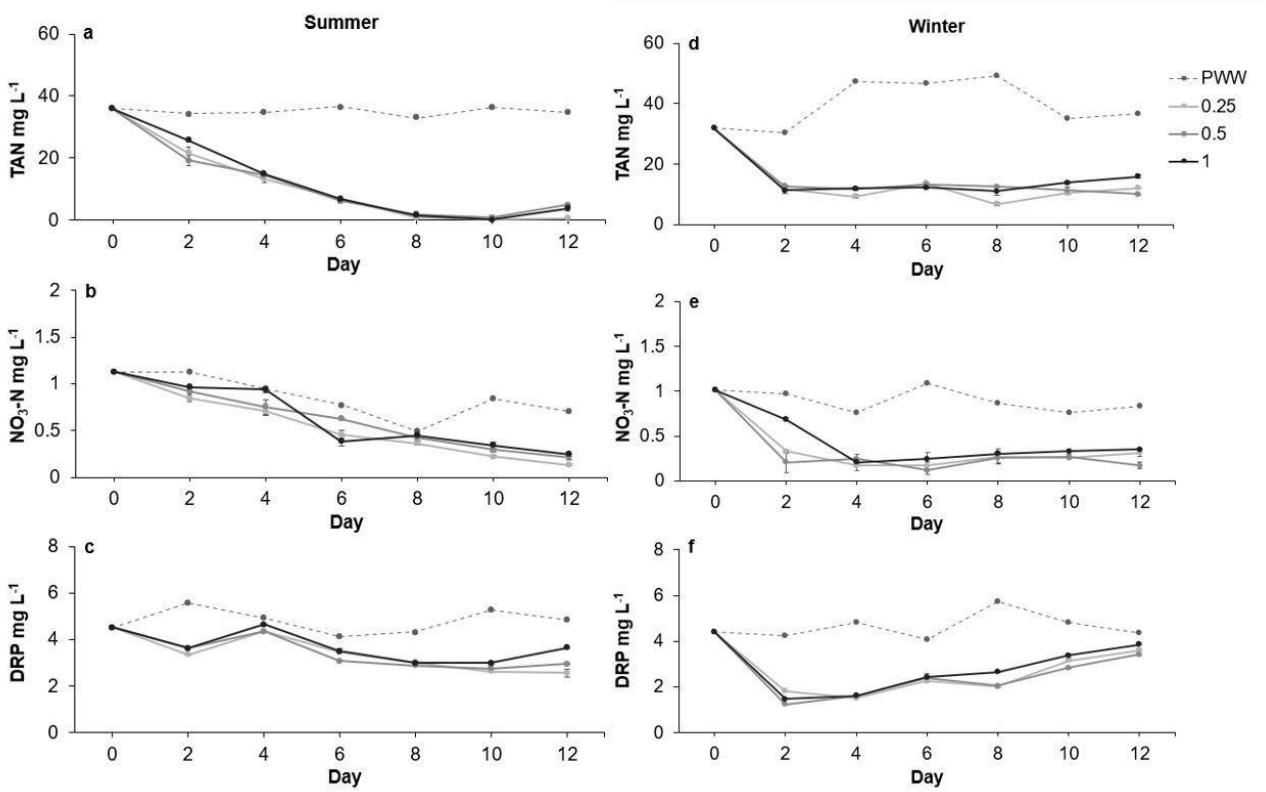

Figure 4.6 Mean (± S.E.) concentrations of TAN (mg L^{-1}) (a, d), nitrate-N (mg L^{-1}) (b, e), and DRP (mg L^{-1}) (c, f) in primary wastewater (PWW) inflows and *Klebsormidium flaccidum* cultures grown in HRFAP mesocosms with a stocking density of 0.25 g FW L^{-1}, 0.5 g FW L^{-1} or 1 g FW L^{-1} over a 12-day period during summer and winter. N = 3.

4.4.3 Harvest frequency experiment

Biomass productivity in summer and winter varied significantly among harvest frequency treatments and days (Figure 4.7, Table 4.1). In summer, productivity was highest with a 4-day harvest frequency (7.83 g DW m^{-2} day^{-1} ± 0.23 across all days) followed by a 6- and 2-day harvest frequency (5.09 g DW m^{-2} day^{-1} ± 0.09 and 3.62 g DW m^{-2} day^{-1} ± 0.29 respectively across all days). In winter, however, biomass productivity was comparable at a 4- and 6-day harvest frequency (2.18 g DW m^{-2} day^{-1} ± 0.18 and 2.15 g DW m^{-2} day^{-1} ± 0.10 respectively across all days) and approximately 31.0% higher than a 2-day harvest frequency (1.49 g DW m^{-2} day^{-1} ± 0.16, across all days, Figure 4.7, Table 4.1). Nutrient removal rates in both seasons varied significantly among days and harvest frequency treatments (Figure 4.8, Table 4.1). All harvest frequency treatments reduced TAN concentrations throughout the experiment at rates of 73.2% day^{-1} (± 2.0) to 78.4% day^{-1} (± 2.4) in summer and 34.2% day^{-1}

(± 1.2) to 43.3% day^{-1} (± 1.2) in winter. Similarly, in summer, all harvest frequency treatments reduced nitrate-N concentrations at rates of 29.0% day^{-1} (± 2.4) to 37.3% day^{-1} (± 4.2), reductions in nitrate-N removal rates were higher in the second half of the experiment (day 6 onwards) compared to days 0 – 4. In winter, both 4- and 6-day harvest frequency treatments consistently reduced nitrate-N concentrations throughout the experiment at comparable rates of 41.5% day^{-1} (± 5.3) and 43.7% day^{-1} (± 4.5) respectively across all days, whereas nutrient reduction rates were 33.0% lower for a 2-day harvest frequency at 28.1% day^{-1} (± 4.7). Nutrient reduction rates for DRP were variable across all harvest frequency treatments in both seasons, ranging from 12.5% day^{-1} (± 0.5) to 20.1% day^{-1} (± 0.6) in summer and from 8.4% day^{-1} (± 1.7) to 17.2% day^{-1} (± 1.1) in winter.

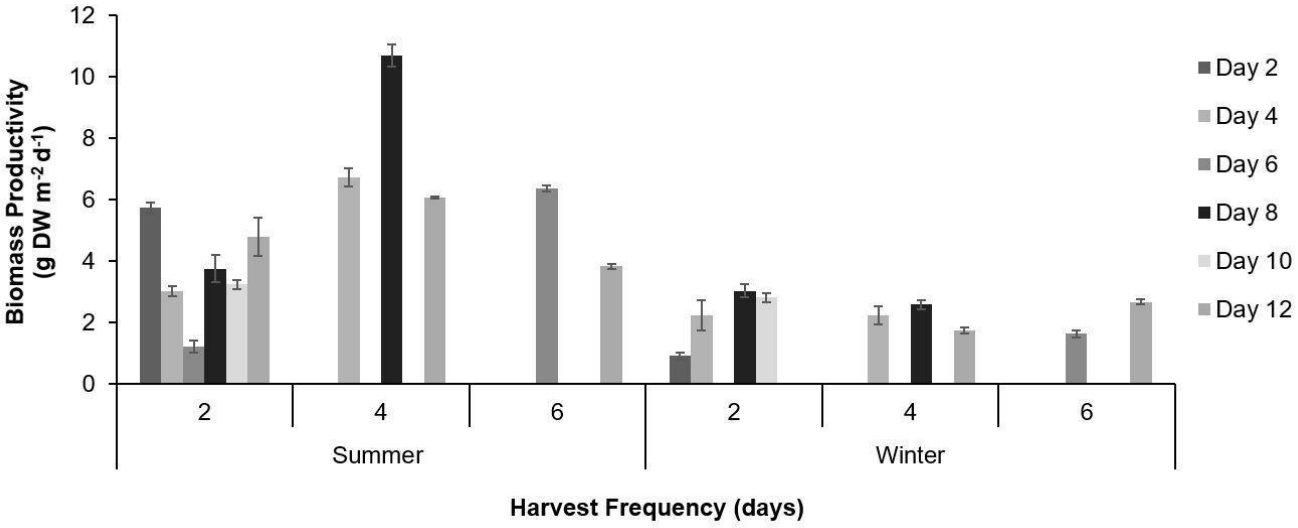

*Figure **4.7*** Mean (± S.E.) biomass productivity (g DW m^{-2} day^{-1}) of *Klebsormidium flaccidum* cultures grown in HRFAP mesocosms with a harvest frequency of two, four or six days over a 12-day period during summer and winter. N = 3.

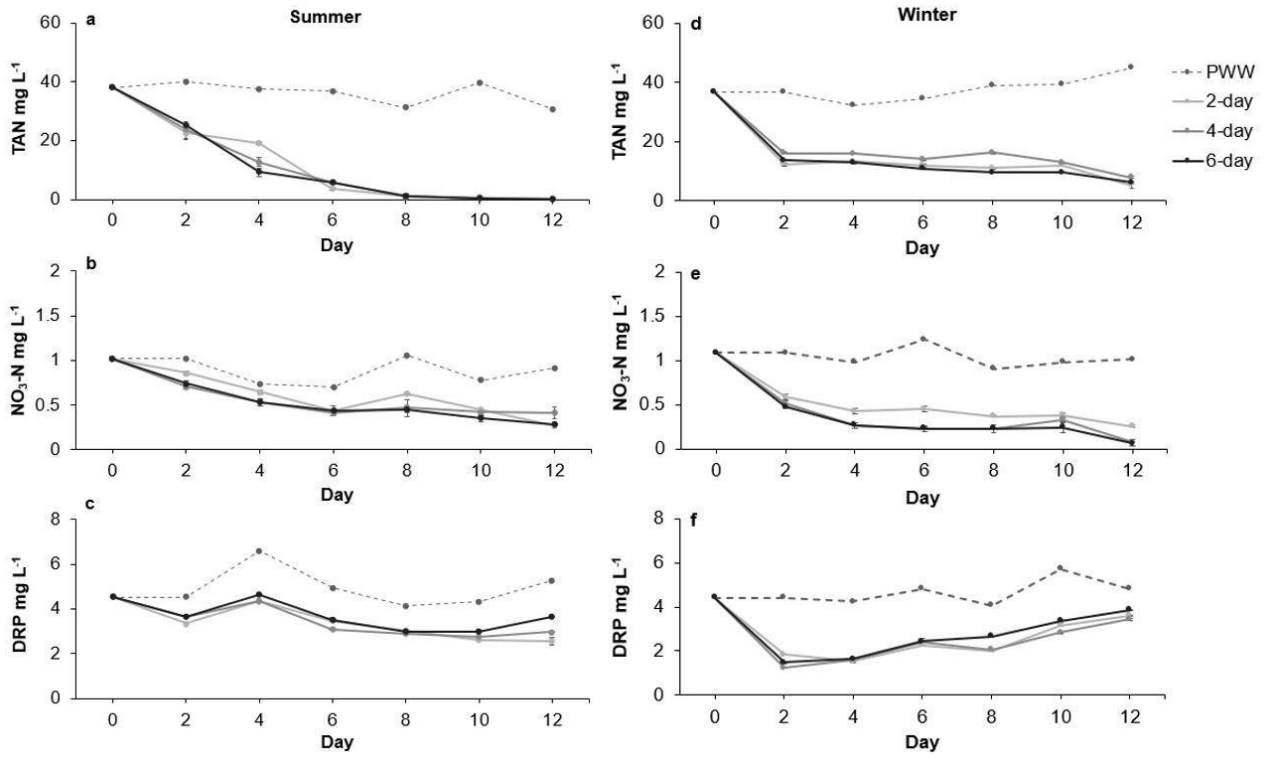

Figure 4.8 Mean (± S.E.) concentrations of TAN (mg L^{-1}) (a, d), nitrate-N (mg L^{-1}) (b, e), and DRP (mg L^{-1}) (c, f) in primary wastewater (PWW) inflows and *Klebsormidium flaccidum* cultures grown in HRFAP mesocosms with a harvest frequency of two, four or six days over a 12-day period during summer and winter. N = 3.

4.4.4 Water quality variables

The cultivation of *K. flaccidum* led to considerable reductions in concentrations of TSS, *E. coli,* $cBOD_5$, and COD in the culture water compared to concentrations in the primary wastewater inflow (Table 4.2). In the HRT experiment in summer, a 4-day HRT achieved the highest reductions in TSS and *E. coli* concentrations (76.7% and 2.42 log_{10}, respectively), while a 6-day HRT achieved the highest the highest reductions in $cBOD_5$ and COD (52.4% and 84.0%, respectively) compared to primary wastewater. In contrast, in winter, the 6-day HRT achieved the highest reductions for all water quality variables except *E. coli*, where a 4-day HRT was more effective. In the stocking density experiment in summer, a stocking density of 0.25 g FW L^{-1} resulted in the highest reduction in *E. coli* concentration (1.92 log_{10}), while a stocking density of 1 g FW L^{-1} achieved the highest reduction in TSS concentration (73.5%), $cBOD_5$ (91.0%) and COD (75.0%) compared to primary wastewater. In winter, a stocking density of 1 g FW L^{-1} resulted in the highest

reduction for all water quality variables compared to primary wastewater, with all treatments achieving comparable reductions in TSS concentration. In the harvest frequency experiment, reductions in water quality variables were generally comparable across treatments in both summer and winter. However, in summer, a 2-day harvest frequency achieved the highest reductions in TSS and *E. coli* concentrations (96.3% and 2.19 log_{10}, respectively), while a 6-day harvest frequency resulted in the highest reductions in $cBOD_5$, and COD (97.8 and 85.9%, respectively) compared to primary wastewater. In winter, a 2-day harvest frequency achieved the highest reductions for all water quality variables except for $cBOD_5$, which was highest with a 6-day harvest frequency.

Table 4.2 Water quality variables of primary wastewater and culture water of *Klebsormidium flaccidum* cultures in HRFAP mesocosms grown under various hydraulic retention time (HRT), stocking density and harvest frequency treatments in three experiments under summer and winter conditions. Variables were measured at the final harvest of each experiment. DO - dissolved oxygen; TSS- total suspended solids; $cBOD_5$ - carbonaceous biochemical oxygen demand; COD - chemical oxygen demand; *E. coli* - *Escherichia coli*.

	pH	DO ($mg\ L^{-1}$)	TSS ($mg\ L^{-1}$)	$cBOD_5$ ($mg\ L^{-1}$)	COD ($mg\ O_2\ L^{-1}$)	*E. coli* (cfu per 100 mL^{-1})
HRT						
Summer						
Primary wastewater			150	240	550	1×10^7
2-day	8.2	11.47	71	21	140	23×10^5
4-day	8.2	11.37	35	11	131	38×10^3
6-day	8.3	11.84	54	10	88	42×10^3
Winter						
Primary wastewater			280	230	600	12×10^6
2-day	7.9	12.03	114	24	310	43×10^3
4-day	7.9	12.08	94	14	174	35×10^3
6-day	7.9	11.98	61	11	106	4×10^4
Stocking density						
Summer						
Primary wastewater			162	178	480	26×10^6
0.25 g FW L^{-1}	8.1	12.19	99	23	178	32×10^3

	pH	DO ($mg\ L^{-1}$)	TSS ($mg\ L^{-1}$)	$cBOD_5$ ($mg\ L^{-1}$)	COD ($mg\ O_2\ L^{-1}$)	*E. coli* (cfu per 100 mL^{-1})
0.5 g FW L^{-1}	8.0	12.51	120	23	184	12×10^4
1 g FW L^{-1}	8.2	12.64	43	16	120	24×10^4
Winter						
Primary wastewater			310	230	500	34×10^6
0.25 g FW L^{-1}	8.2	12.50	67	20	110	41×10^4
0.5 g FW L^{-1}	8.1	12.88	75	30	156	17×10^5
1 g FW L^{-1}	8.1	12.78	65	34	210	13×10^5
Harvest frequency						
Summer						
Primary wastewater			300	182	680	14×10^6
2-day	8.2	12.33	11	6	104	9×10^4
4-day	8.2	12.23	15	6	108	11×10^4
6-day	8.1	12.31	12	4	96	11×10^4
Winter						
Primary wastewater			210	180	460	16×10^6
2-day	8.3	12.54	42	27	110	11×10^5
4-day	8.3	12.20	55	31	140	12×10^5
6-day	8.3	12.92	54	25	126	14×10^5

4.5 Discussion

This study investigated how HRT, stocking density, and harvest frequency can influence biomass productivity and nutrient removal efficiency in outdoor HRFAP systems across seasons. HRT was the factor with the largest influence on nutrient removal rates. A 4-day HRT was optimal in summer as it resulted in the highest overall reductions in TAN, nitrate-N, DRP, TSS, and *E. coli*, as well as the highest biomass productivity. In contrast, there was no HRT in winter that had the best performance across all metrics (e.g. biomass productivity, nutrient removal, water quality). Typically, longer HRTs are preferred in winter when algal growth is slower and light availability is reduced to ensure adequate nutrient removal to meet discharge requirements (Lee et al., 2015; Leong et al., 2021; Mohsenpour et al., 2021). However, for *K. flaccidum*, a 6-day HRT in winter did not consistently result in improved nutrient removal compared to a 2- or 4-day HRT, indicating that longer HRTs may

not be required for *K. flaccidum* to achieve consistent reductions in nutrient concentrations in winter. This is most likely because the cultivar is tolerant of colder conditions, as reported for other species of *Klebsormidium* (Borchhardt & Gründling-Pfaff, 2020). This tolerance enables the cultivar to maintain high growth and nutrient removal rates in winter. Contrary to expected improved nutrient removal under longer HRTs (Lee et al., 2015; Mohsenpour et al., 2021) concentrations of nitrate-N continually increased from day 12 in the 6-day HRT treatment, and by day 18 had surpassed influent levels. This rise in nitrate-N concentrations was likely due to increased nitrification activity (Gonzalo Ibrahim et al., 2023) that resulted from a reduction in algal biomass productivity and, consequently, the uptake of inorganic carbon and ammonium (NH_4^+), thereby increasing the availability of these substrates for nitrifiers (Delgadillo-Mirquez et al., 2016). The 6-day HRT is also likely to have increased the concentration of nitrate-N by facilitating the development of bacterial biomass to accumulate, thereby further enhancing the rate of nitrification (Park & Craggs, 2011; Ding et al., 2016). However, longer HRTs can result in improved water quality, as was seen in both summer and winter where reductions in concentrations of TSS and *E. coli*, and in $cBOD_5$ and COD relative to primary wastewater increased with increasing HRT. Longer HRTs can also be beneficial to reduce the potential impact of toxic pollutants in the primary wastewater influent on algal biomass. Wastewater toxicity likely affected the initial phase of the HRT summer experiment, indicated by a decrease in biomass productivity in the 2-day HRT treatment starting on day 4 and a decline in TAN removal rates from day 6. Longer HRTs can mitigate the risk of wastewater toxicity by reducing the inflow rate, thereby lowering the concentrations of toxic pollutants in the culture water and minimizing exposure to toxic pollutants which can inhibit algal growth (Andreas & Maria, 2017; Xin et al., 2021).Therefore, a 4-day HRT is recommended for winter to achieve the greatest improvement in water quality while minimising the potential impact of toxic pollutants and reducing the occurrence of nitrification.

In summer the optimal stocking density was 0.25 g FW L^{-1} as this resulted in the highest overall reductions in TAN, nitrate-N, DRP, *E. coli*, and the highest biomass productivity. In contrast, in winter, stocking densities of 0.25 g FW L^{-1} and 0.5 g FW L^{-1} were both optimal, as 0.25 g FW L^{-1} resulted in the highest overall reductions in TAN and *E. coli*, and 0.5 g FW L^{-1} resulted in the highest overall reductions in nitrate-N and DRP. Stocking density had contrasting effects on productivity in summer and winter due to its impact on light penetration. In winter, limited ambient light led to low growth rates.

However, the highest stocking density proved most productive because the greater initial biomass resulted in larger cumulative growth. Conversely, in summer, biomass productivity was highest at lower stocking densities. These findings align with previous studies showing that lower stocking densities in summer enhance algal growth (Pereira et al., 2006; Piñosa & Apines-Amar, 2023), and are likely to due to higher light availability at lower stocking densities as self-shading at higher densities can significantly reduce light availability (Sutherland et al., 2015c; Krichen et al., 2021). However, if stocking densities are too low during summer, photosynthetic efficiency can be reduced by photoinhibition (Borowitzka, 1998). This is unlikely to be an issue in outdoor pond systems for primary wastewater treatment though, as the relatively high bacterial biomass and the presence of light-absorbing dissolved matter in the wastewater means it is often turbid. While higher productivity is often a preferred trait of freshwater cultivars (Cole et al., 2018; Ge et al., 2018), it does not always ensure high bioremediation performance (Chapter 2). In this study, biomass productivity in winter was highest at the stocking density of 1 g FW L^{-1}; however, removal rates were higher at lower stocking densities of 0.25 g FW L^{-1} to 0.5 g FW L^{-1}, with nutrient reduction rates decreasing as stocking density increased. Moreover, in contrast to biomass productivity, stocking density had no effect on bioremediation performance across seasons. Instead, seasonal differences in TAN removal rates were due to differences in the inflow concentrations of TAN in the primary wastewater, which were 9.3% higher in winter than in summer. As stocking density did not significantly affect nutrient removal rates between treatments, this is less critical to optimize compared to HRT. This finding suggests that stocking density could be adjusted to enhance other parameters of interest (e.g. productivity, biomass composition), with minimal impact on nutrient removal.

A 6-day harvest frequency was optimal in both summer and winter as it resulted in the highest overall reductions in TAN and nitrate-N, and comparable biomass productivity and improvements in water quality to other harvest frequency treatments. Nutrient removal of TAN and nitrate-N was highest at a 6-day harvest frequency. In contrast, more frequent harvesting (e.g. 2- or 4-day harvest frequency) resulted in a greater reduction of *E. coli*, specifically through reducing stocking density more frequently thereby increasing light availability and penetration in the water, and consequently UV-driven inactivation (Bolton et al., 2010; Beardall & Raven, 2013). Increased harvest frequency also increased biomass productivity, with productivity being twice as high in summer with a 2-day harvest frequency

compared to a 6-day harvest frequency. This finding is consistent with previous research showing that increased harvest frequency can improve biomass productivity (Sutherland et al., 2020a). Higher biomass productivity can lead to increased product yields when wastewater treatment is coupled with use of the cultivated algal biomass for product applications such as biofuels, bioplastics, fertilisers, or pigments (Sutherland et al., 2020a; Kumar et al., 2021; Patel et al., 2022). Therefore, optimising both wastewater treatment and biomass production is crucial for the economic viability of the system (Sutherland et al., 2020a; Alazaiza et al., 2022). However, while increased harvest frequency improves biomass productivity and wastewater treatment, it may result in higher operation costs. Therefore, future research should focus on offsetting these costs through automation of harvest systems (Fasaei et al., 2018; Lu et al., 2022).

Understanding the influence of seasonal conditions on biomass productivity and nutrient bioremediation performance in HRFAPs is essential for optimising their performance. Seasonal conditions significantly influenced biomass productivity across all experiments, with lower overall productivity observed in winter compared to summer. The primary factor affecting biomass productivity between summer and winter was likely light availability, as increased light and longer daylight hours in summer can lead to higher biomass productivity (Pereira et al., 2006; Cole et al., 2016b; Gonzalo Ibrahim et al., 2023). Temperature played a less significant role, as the culture water temperature remained relatively stable across seasons (average 20.2°C in summer and 15.9°C in winter), despite substantial variation in ambient temperature. The average culture water temperature was primarily influenced by the consistent temperature of the primary wastewater influent (19.6°C). Nutrient limitation varied seasonally, with higher degree of limitation observed in the summer, and this was a critical factor impacting biomass productivity. In summer, biomass productivity declined towards the end of the experiment. This decline was likely due to nutrient limitation, as nutrient concentrations declined to zero by the end of the experiment Specifically, TAN and nitrate-N concentrations reached zero at a 4-day HRT by days 22 and 24, respectively, and at a 6-day HRT by days 18 and 16, respectively. While carbon or light limitations might have contributed to the reduced productivity, this is unlikely because culture pH levels remained below pH 8.3 during the summer and were similar to winter levels, and light availability is higher under summer conditions. These findings highlight the importance of investigating seasonal differences in operational parameters, as performance outcomes and system management strategies may differ significantly between seasons.

4.6 Conclusion

Our findings demonstrate that HRT, stocking density, and harvest frequency significantly influence biomass productivity and nutrient bioremediation performance in HRFAPs during summer and winter seasons. The optimal HRT was identified as 4-days in both seasons. For stocking density, the optimal amount was 0.25 g FW L^{-1} in summer while in winter, both 0.25 g FW L^{-1} and 0.5 g FW L^{-1} were effective. A 6-day harvest frequency was optimal across both seasons. Seasonal variations impacted biomass productivity, with lower productivity observed in winter primarily due to reduced light availability. This study highlights the opportunity to strategically manage HRT, stocking density, and harvest frequency to optimise year-round HRFAP performance. HRT was the most influential parameter and should be the primary focus for improving nutrient removal rates. Stocking density and harvest frequency can be adjusted to meet other objectives, such as maximising biomass production and improving water quality (e.g. reductions in the content of TSS or *E. coli*). As the target cultivar was pre-selected based on a screening protocol to identify robust species for primary wastewater treatment, it is important to note that different species may yield varying results, necessitating species-specific parameter testing. Additionally, while this study was conducted under temperate conditions, optimising by seasons would be less critical in tropical climates. By balancing these parameters, high biomass yields, and efficient nutrient bioremediation can be achieved, ensuring more consistent year-round performance of HRFAP systems.

4.7 Appendix

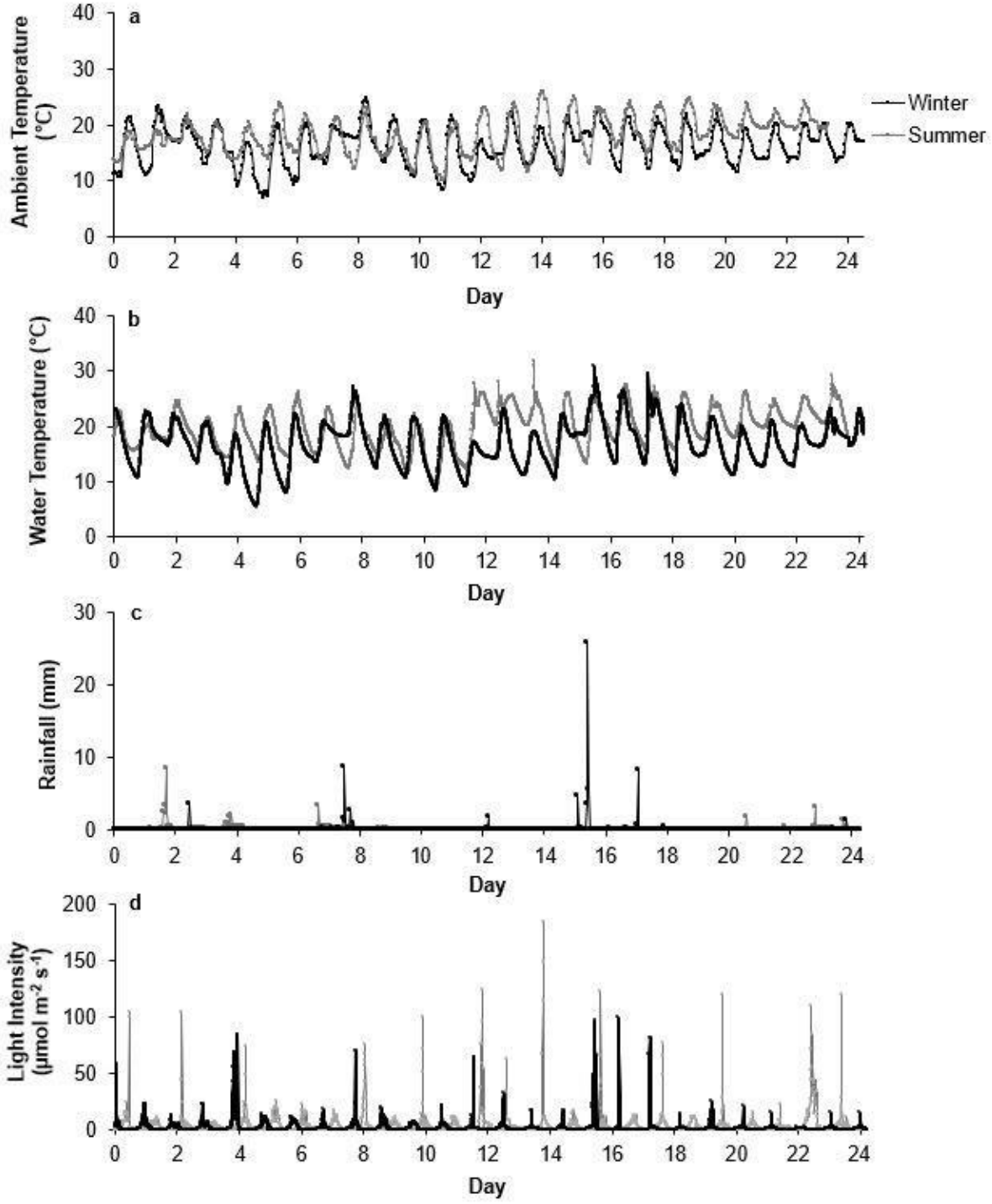

Figure 4A.1 Recorded ambient air temperature (a), culture water temperature (b), rainfall (c) and light intensity (d) recorded in *Klebsormidium flaccidum* HFRAP cultures over six consecutive four-day harvest cycles during the HRT experiments during summer and winter.

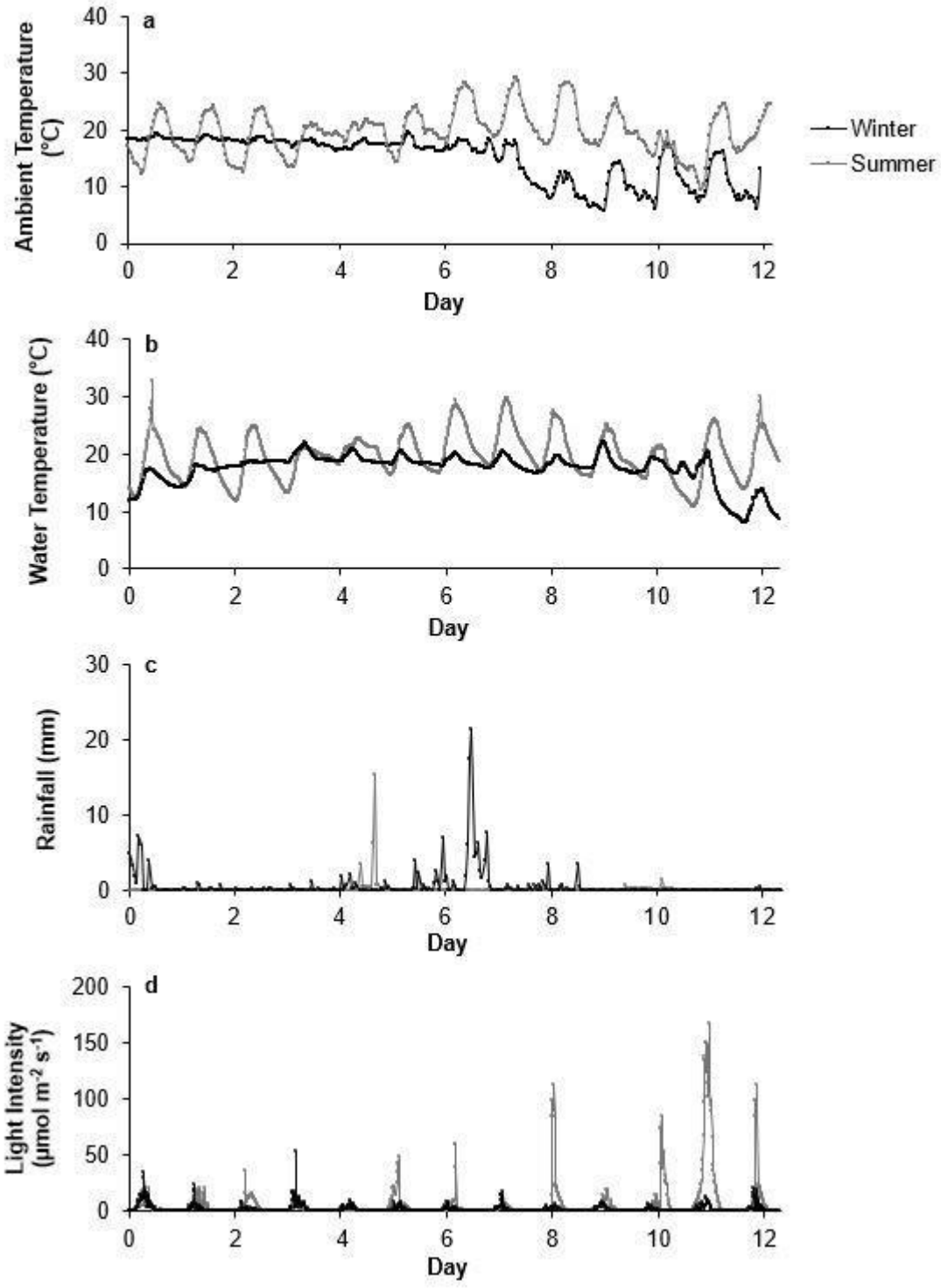

Figure 4A.2 Recorded ambient air temperature (a), culture water temperature (b), rainfall (c) and light intensity (d) recorded in *Klebsormidium flaccidum* HFRAP cultures over three consecutive four-day harvest cycles during the stocking density experiments during summer and winter.

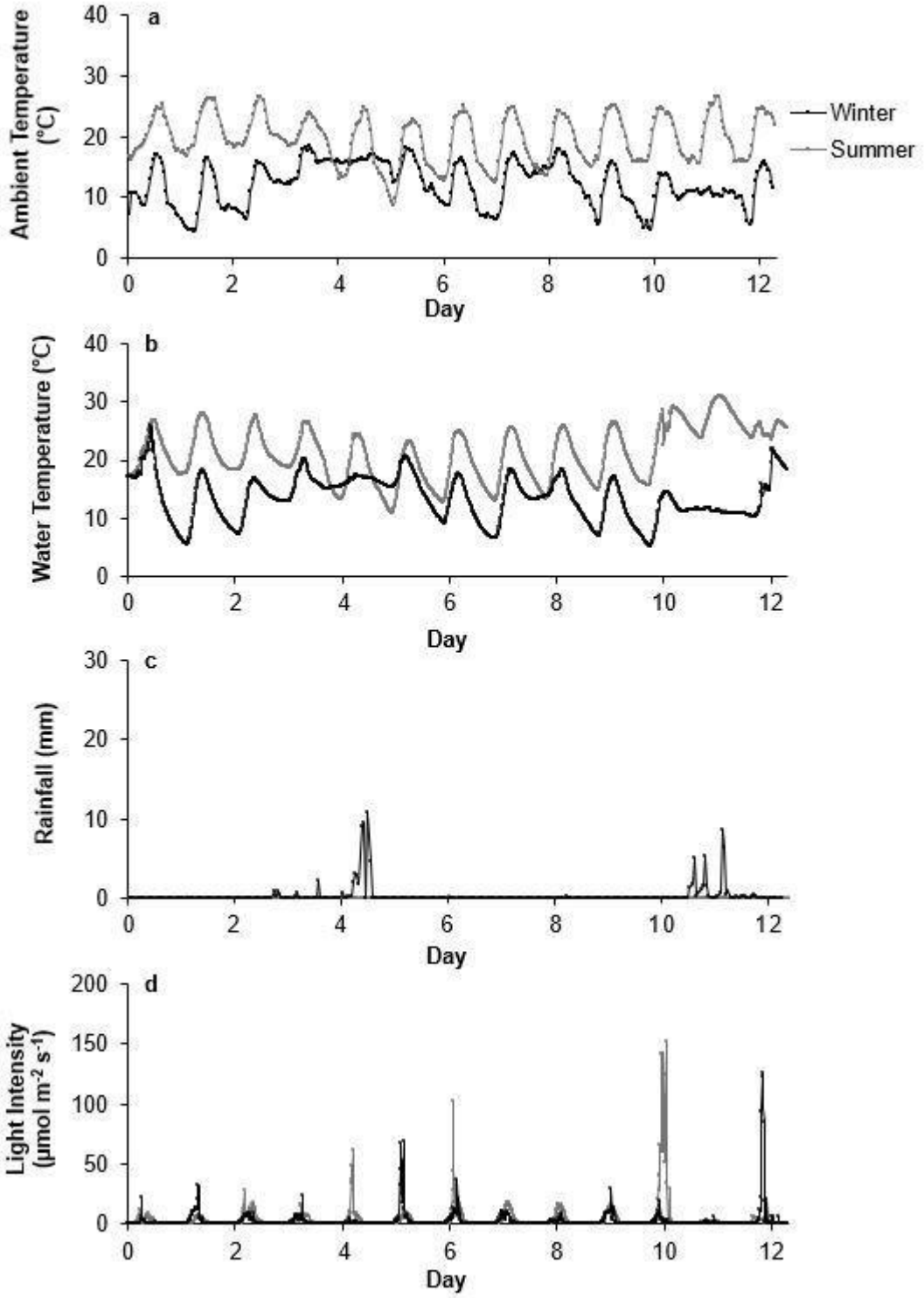

Figure 6A.3 Recorded ambient air temperature (a), culture water temperature (b), rainfall (c) and light intensity (d) recorded in *Klebsormidium flaccidum* HFRAP cultures over consecutive harvest cycles during the harvest frequency experiments during summer and winter.

Chapter 5 - Freshwater filamentous macroalgal bioremediation of per- and polyfluoroalkyl substances (PFAS) from primary municipal wastewater

5.1 Abstract

Perfluoroalkyl and polyfluoroalkyl substances are a diverse class of synthetic chemicals used in numerous consumer products and industrial processes. PFAS are highly persistent in the environment, raising significant health and ecological concerns. Wastewater treatment plants are a major point source of PFAS, significantly contributing to their release into the environment. This study examined the capacity of the freshwater filamentous macroalga *Klebsormidium flaccidum* to bioremediate PFAS from primary municipal wastewater under summer conditions. Algae were cultured in primary wastewater under laboratory-controlled conditions using hydraulic retention times (HRTs) of 3- and 6-days. PFAS concentrations in the water and PFAS uptake by algae were measured. Eleven types of PFAS were identified in primary wastewater samples, with Perfluorohexanoic acid (PFHxA), Perfluorooctanoic acid (PFOA), and Perfluorobutane sulfonate (PFBS) consistently detected. PFAS precursors were also detected throughout the experiment, sometimes at higher concentrations than PFAS, highlighting the need to analyse these PFAS precursors to accurately represent PFAS concentrations entering the environment. PFAS concentrations in the primary wastewater varied significantly throughout the experiment, ranging from 0.002 – 0.040 μg L^{-1}. However, the highest PFAS concentration in the primary wastewater (PFOS: 2.7 μg L^{-1}) was recorded in baseline samples taken prior to the experiment. *Klebsormidium flaccidum* maintained stable biomass productivity for both 3- and 6-day HRTs (average of 4.35 g DW m^{-2} day^{-1} ± 0.15 S.E. and 4.37 g DW m^{-2} day^{-1} ± 0.17 S.E., respectively). PFAS and PFAS precursor removal rates varied, with reductions observed in three individual PFAS: PFOA (30.0 – 77.8%), PFHxS (44.4 – 77.0%), and PFBS (3.0%), as well as in all PFAS precursors (5.0 – 75.0%). Despite these reductions, PFAS was not detected in the biomass of *K. flaccidum*, making it suitable for a variety of biotechnological applications, provided it remains free of other contaminants. Future research should evaluate the survival of *K. flaccidum* and its efficacy in removing PFAS under higher concentrations and across different seasons to determine the species effectiveness in PFAS remediation.

5.2 Introduction

Perfluoroalkyl and polyfluoroalkyl substances (collectively referred to as PFAS) represent a large and diverse group of synthetic chemicals extensively used in a wide range of consumer products and industrial applications (Espartero et al., 2022). PFAS are often referred to as “forever chemicals” due to their persistence in the environment and their tendency to accumulate over time (Dias et al., 2024). The strong carbon-fluorine (C-F) bonds in the molecular structures of PFAS increase thermal stability and resistance to degradation (Zhou et al., 2024). Perfluoroalkyl substances are fully fluorinated, rendering them highly stable and resistant to both chemical and biological degradation (Arslan & Gamal El-Din, 2021). In contrast, polyfluoroalkyl substances are not completely fluorinated, making them more susceptible to biotic and/or abiotic degradation (Shahsavari et al., 2021). As a result of the widespread use of PFAS and their persistence and accumulation in the environment, there are significant environmental and health concerns associated with their use (Brunn et al., 2023). The continual release of PFAS into the environment poses serious risks to aquatic life as these substances can bioaccumulate in aquatic organisms leading to toxic effects and reproductive and developmental issues in fish and other wildlife at higher trophic levels (Blaine et al., 2014; Niu et al., 2019; Brown et al., 2020). Furthermore, continual human consumption of PFAS contaminated water or aquatic organisms can lead to serious health problems, including various cancers (such as kidney and testicular cancer), liver damage, thyroid disease, decreased fertility and endocrine disruption (Calvert et al., 2022; Sebe et al., 2023). Additionally, PFAS exposure has been associated with developmental issues in children, such as lower birth weights, developmental delays, and decreased vaccine response (Rappazzo et al., 2017; Blake & Fenton, 2020).

In addition to PFAS, PFAS precursors are also a significant issue. These are oxidizable substances that, under certain conditions, can convert into various PFAS compounds, thereby increasing the amount of PFAS entering the environment (Al Amin et al., 2020). Environmental factors such as photolysis (Taniyasu et al., 2013), microbial activity (Berhanu et al., 2023; Yan et al., 2024), and chemical oxidants (López-Vázquez et al., 2024) in water treatment processes can facilitate this conversion. Furthermore, some PFAS precursors may exhibit toxic properties, contributing to ecological and human health risks even before converting into more stable forms of PFAS (Wang et al., 2015). Therefore, considering PFAS precursors that can degrade into more persistent and toxic PFAS forms, as

well as PFAS, is vital to enable a complete understanding of the total load of PFAS present in an environment (Tavasoli et al., 2021).

Wastewater treatment plants (WWTPs) are increasingly recognised as significant sources of PFAS pollution (Helmer et al., 2022). Concentration and composition of PFAS and PFAS precursors in WWTP influents can vary substantially, ranging from tens to thousands of ng L^{-1} (Nguyen et al., 2022a). This large variability suggests contributions from multiple domestic sources, including household consumer products containing PFAS (Kotthoff et al., 2015; Namazkar et al., 2024), PFAS-contaminated drinking water (Guelfo et al., 2018; Khanal & Elbakidze, 2024; Levin et al., 2024) and catchment-specific sources from commercial and industrial discharges (Espartero et al., 2022; Nguyen et al., 2022a). Industries such as textiles, aviation, and manufacturing heavily rely on PFAS, resulting in elevated concentrations of these substances in discharges from these industries (Anderson et al., 2023; Drage et al., 2023; Dias et al., 2024). The frequent detection of PFAS and PFAS precursors in WWTP effluents and biosolids highlights the inability of conventional treatment processes to remove these persistent chemicals (Vestergren & Cousins, 2009; Eriksson et al., 2017; Behnami et al., 2024). As these effluents are discharged into natural water bodies and the biosolids are applied onto land for agricultural use (Gewurtz et al., 2024), WWTPs inadvertently become major point sources of PFAS, amplifying their presence in downstream ecosystems and contributing to widespread environmental pollution (Arvaniti & Stasinakis, 2015; Wang et al., 2020).

Current methods for removing PFAS and PFAS precursors from wastewater employ various advanced filtration techniques, including activated carbon adsorption, ion exchange, and advanced oxidation processes (Ahmed et al., 2020; Gao et al., 2021a). Activated carbon absorption is widely used due to its high surface area, which can effectively capture various PFAS compounds (Lei et al., 2023). However, this method requires frequent replacement or regeneration of the carbon, resulting in significant costs (Gao et al., 2021a). Ion exchange resins provide a highly selective means of PFAS removal by efficiently targeting specific PFAS compounds (Dixit et al., 2021). However, due to their selectivity, these resins often struggle with the diversity of PFAS present in wastewater, leading to incomplete removal (Gao et al., 2021a). Advanced oxidation processes, which involve the generation of reactive oxygen species to degrade contaminants, can be effective against certain PFAS but are energy-intensive and may not completely mineralise the most resistant PFAS compounds,

leaving behind potentially harmful byproducts (Ahmed et al., 2020; Mojiri et al., 2023b). Phytoremediation, which uses plants to remove PFAS from wastewater, has recently emerged as a promising alternative to traditional methods. Certain plants, particularly those used in constructed wetlands, such as *Eriophorum angustifolium* and *Carex rostrata*, can absorb PFAS through their root systems (Greger & Landberg, 2024). Previous research on PFAS removal from contaminated water found that 0.03% to 24% of the removed PFAS accumulated in plant tissue and the remaining PFAS were likely degraded into metabolites in the water or taken up by the plants and degraded in the plant tissue (Greger & Landberg, 2024). The presence of plants also enhanced microbial activity, resulting in more effective PFAS removal compared to plant-free systems (Greger & Landberg, 2024). This approach utilises the natural ability of plants to absorb and sequester contaminants, offering a cost-effective and sustainable solution (Colares et al., 2020). However, the efficiency of phytoremediation can vary significantly depending on the plant species, the specific PFAS compounds, and environmental conditions such as soil type and climate (Arslan & Gamal El-Din, 2021).

The use of freshwater filamentous algae to bioremediate PFAS and PFAS precursors from primary municipal wastewater presents an innovative approach to mitigating the persistence of these harmful chemicals in the environment. Algal-bacterial symbiosis, particularly with filamentous algae, has proven effective in various wastewater treatment processes, demonstrating the ability to remove a wide range of complex contaminants, including heavy metals (Lee & Chang, 2011), pharmaceuticals (Wang et al., 2017), and organic pollutants (Flores-Morales et al., 2020). Algae bioremediate these contaminants through mechanisms such as biosorption, bioaccumulation, and biodegradation (Liu et al., 2020; Mustafa et al., 2021). The unique structure of filamentous algae, which has a larger surface area relative to volume, increases their capacity to absorb and assimilate pollutants from water, leading to a greater potential ability for PFAS bioaccumulation compared to other organisms (Liu et al., 2018; Liu et al., 2020). This characteristic makes them a promising candidate for efficient PFAS remediation. Furthermore, integrating the cultivation of filamentous algae into existing wastewater treatment infrastructure could potentially lower the overall costs associated with PFAS removal. This approach aligns with sustainable and circular economy principles (Bhatt et al., 2021; Koul et al., 2022), as the biomass (low in PFAS) produced from algal treatment systems can be harvested and potentially used for a range of bioproduct applications such as biofuel and biostimulants, offering an additional

economic incentive (Craggs et al., 2011; Lawton et al., 2017b; Jayasooriya et al., 2024). However, to date, the use of freshwater filamentous algae for the bioremediation of PFAS and PFAS precursors from primary municipal wastewater has not been investigated. Therefore, the objective of this study was to quantify the growth and PFAS bioremediation performance of *Klebsormidium flaccidum* when cultivated in primary municipal wastewater. By assessing the growth and removal rate of PFAS and PFAS precursors, we aim to determine the feasibility of using freshwater filamentous algae for the treatment of PFAS and PFAS precursors in municipal wastewater.

5.3 Methods

5.3.1 Algal cultivar and wastewater

A cultivar of the freshwater filamentous macroalga *K. flaccidum* was originally collected from a culvert in Rangiuru, Te Puke, Aotearoa New Zealand (37° 84' S 176° 35' E) and was identified using DNA barcoding (Chapter 3). Prior to experiments, this cultivar was scaled up into stock cultures and maintained as described in (Chapter 4). The primary wastewater used within this study was sourced from a WWTP situated in the Bay of Plenty Region of New Zealand. The municipal WWTP currently services a population of approximately 35,000 people, including a large industrial zone, with an average daily inflow of 20,000 m^3. The primary treated wastewater contained an average total ammoniacal-N (TAN) concentration of 45.6 mg L^{-1} (± 1.0 S.E.), an average nitrate-N (NO_3^-N) concentration of 0.8 mg L^{-1} (± 0.0 S.E.), and an average dissolved reactive phosphorus (DRP) concentration of 3.4 mg L^{-1} (± 0.3 S.E.).

5.3.2 Biomass productivity and PFAS bioremediation experiment

Ten replicate cultures of *K. flaccidum* (n = 10) were maintained in 250 mL polypropylene containers (LabServ™), with a stocking density of 0.5 g FW L^{-1} and hydraulic retention times (HRT) of 3 days or 6 days (five replicates per HRT). These HRTs were selected to provide a range of potential days required for effective PFAS removal. Algal biomass in cultures was maintained in suspension by a continuous, gentle stream of filtered air (Resun LP-40 air pump and Whatman™ Uniflo syringe filters, 0.22 µm) bubbled into each container. Algal biomass in each culture was cultivated in 150 mL primary treated

wastewater collected every six days at midday during peak inflow from the primary clarifier at the WWTP. Wastewater was collected at midday as pre-experiment data indicated that contaminant concentrations were highest at this time of day. Samples were collected more than three days after rainfall to minimise dilution effects. Wastewater was settled for 1 hour in 20 L plastic buckets immediately following collection, and then the supernatant primary wastewater was transferred to the experimental containers. An additional ten replicate containers were filled with primary wastewater only (i.e., without algal biomass) and maintained with a 3-day or 6-day HRT (five replicates per HRT) under identical conditions as the algal cultures as experimental controls (n = 10). Cultures were arranged in a temperature-controlled plant growth cabinet (Panasonic MLR-352) and arranged in a randomised block design. Each culture and control replicate for each HRT treatment was placed on a different shelf, and replicates were rotated daily within each block to minimise edge effects and variations in light intensity. The cultures were maintained for 24 days under summer conditions based on the National Climate Database weather recording station located in Tauranga (-37.67478, 176.19236, data available from www.cliflo.niwa.co.nz). Summer ambient temperature profiles were based on the average high and low temperatures from the previous January (15.4 - 25.3°C, Figure 5A.1). Summer conditions were selected to ensure continuous high growth and prevent productivity from being a limiting factor in the bioremediation experiment.

The water in each culture was replaced with new primary wastewater collected from the WWTP as described above every three or six days according to the designated HRT of each culture. Immediately prior to each water change, the entire contents of each container (culture water and algae) were thoroughly mixed to prevent the algae from settling at the bottom of the container. The mixture was then poured into a fine mesh bag to separate the biomass from the water. After excess water had drained from the bag, the algae were returned to the container with fresh primary wastewater. Biomass in each replicate container was harvested every six days for a total of four consecutive harvests over 24 days. The biomass was harvested by pouring the algae and culture water through a mesh bag as described above, which was then placed in a centrifugal spin dryer (Spindle NZ, SPL-265) for approximately three minutes to remove any residual water. The algae were then removed from the bag and weighed to determine the FW and then 0.075 g of the harvested biomass was restocked back into each replicate culture to reset the stocking density to 0.5 g FW L^{-1} and the container was refilled with 150 mL fresh primary wastewater. Excess biomass not restocked back into

containers was dried in an oven at 60 °C for 48 hours and reweighed to determine the fresh weight to dry weight (FW:DW) ratio for each replicate. This FW:DW ratio was used to convert both the initial and the harvested biomass for each replicate, which were both measured in FW, into DW. Biomass productivity (g DW m^{-2} day^{-1}) was calculated for each replicate for each harvest using the equation $P = (B_f - B_i)/ A / T$, where B_f and B_i are the final and initial algal biomasses (g DW), A is the surface area (m^{-2}) of culture container and T is the culture period (six days).

5.3.3 PFAS and PFAS precursor analysis

Prior to the experiments, three 150 mL wastewater samples were collected simultaneously from the WWTP at three different times on the same day (9am, 12pm and 3pm, n=9). This was done to determine baseline concentrations of PFAS and PFAS precursors in the primary wastewater. Additionally, three tap water samples were collected from the University of Waikato Coastal Marine Field Station where the experiment was conducted to determine whether PFAS or PFAS precursors were present in the town water supply used for cleaning equipment throughout the experiment. PFAS was not detected within these three town water supply samples. To establish potential baseline concentrations of PFAS and PFAS precursor concentrations in the algal biomass prior to the experiment, two fresh weight biomass samples (0.5 g FW each) from stock cultures were collected. To determine final concentrations of PFAS and PFAS precursors in culture water and algal biomass, a 150 mL sample of culture water was collected from three replicate algal cultures and three replicate controls of each HRT treatment immediately prior to harvesting on the last day of the experiment (n=12) and algal biomass samples (0.25 g FW each) were collected from three replicate cultures of each HRT treatment immediately following harvesting (n=6). All samples were analysed by SGS Australia. The concentrations of PFAS and PFAS precursors in water samples were analysed following Per- and Polyfluoroalkyl Substances (PFAS) in Aqueous Samples – MA-1523 methodology (PFAS) and Perfluorinated Surfactants in Water – TOPA - MA-1523_TOPA methodology (PFAS precursors). The concentrations of PFAS and PFAS precursors in biomass samples were analysed using the following Per- and Polyfluoroalkyl Substances (PFAS) in Solid Samples – MA-1523 methodology (PFAS) and Perfluorinated Surfactants in Soil – TOPA - MA-1523_TOPA methodology (PFAS precursors). These analyses measured the concentrations of thirty

common PFAS and PFAS precursor compounds (Table 5.1), which were then summed to provide a total PFAS and total PFAS precursor concentration for each sample. SGS provided the sample containers and blanks for the analysis. Throughout the experiment, strict protocols and guidelines were followed to prevent leaching of PFAS from equipment and cross-contamination during the collection, processing, and handling of PFAS samples (Massachusetts Department of Environmental Protection, 2021). Total PFAS and PFAS precursor removal rates (PR, % day^{-1}) and individual PFAS and PFAS precursor removal rates were calculated at the final harvest using the equation $PR = (CW- ACW / CW) *100$, where ACW is the PFAS or PFAS precursor concentration in the algal culture water and CW is the PFAS or PFAS precursor concentration in the control water.

Table 5.1 List of short-chain and long-chain per- and polyfluoroalkyl substances (PFAS) and PFAS precursors analysed in water and algal biomass samples in this study.

Abbreviation	Name
Short-Chain	
PFBA	Perfluorobutanoic acid
PFPeA	Perfluoropentanoic acid
PFHxA	Perfluorohexanoic acid
PFBS	Perfluorobutane sulfonate
PFPeS	Perfluoropentane sulfonate
4:2 FTS	1H,1H,2H,2H-Perfluorohexane sulfonate
6:2 FTS	1H,1H,2H,2H-Perfluorooctane sulfonate
Long-Chain	
PFHpA	Perfluoroheptanoic acid
PFOA	Perfluorooctanoic acid
PFNA	Perfluorononanoic acid
PFDA	Perfluorodecanoic acid
PFUnA	Perfluoroundecanoic acid
PFDoA	Perfluorododecanoic acid
PFTrDA	Perfluorotridecanoic acid

PFTeDA	Perfluorotetradecanoic acid
PFHxDA	Perfluorohexadecanoic acid
PFHxS	Perfluorohexane sulfonate
PFHpS	Perfluoroheptane sulfonate
PFOS	Perfluorooctane sulfonate
PFNS	Perfluorononane sulfonate
PFDS	Perfluorodecane sulfonate
PFDoS	Perfluorododecane sulfonate
8:2 FTS	1H,1H,2H,2H-Perfluorohexane sulfonate
PFOSA	Perfluoroctane sulfonamide
N-MeFOSA	N-Methylperfluoroctane sulfonamide
N-EtFOSA	N-Ethylperfluoroctane sulfonamide
N-MeFOSE	2-(N-Methylperfluorooctane sulfonamido)-ethanol
N-EtFOSE	2-(N-Ethylperfluorooctane sulfonamido)-ethanol
N_MeFOSAA	N-Methylperfluorooctanesulfonamidoacetic acid
N-EtFOSAA	N-Ethylperfluorooctanesulfonamidoacetic Acid

5.3.4 Statistical analysis

Differences in biomass productivity among HRT treatments and harvests (fixed factors) were analysed using two-way repeated-measures analyses of variance (ANOVA). Overall variation in the concentration of all individual PFAS and PFAS precursor types in primary wastewater baseline samples across different collection times (fixed factor) were analysed using multivariate analysis of variance (MANOVA). Differences in total PFAS and PFAS precursor concentrations and the concentration of individual PFAS and PFAS precursor types in primary wastewater baseline samples across different collection times (fixed factor) were then analysed using one-way ANOVAs. Overall variation in the concentration of all PFAS and PFAS precursor types in water samples collected at the end of the experiment among HRTs and treatments (algal vs. control cultures, both fixed factors) were analysed using MANOVA. Differences in total PFAS and PFAS precursor concentrations, and the concentration of individual PFAS and PFAS precursor types in water samples collected at the end of the experiment among HRTs and treatments (algal vs. control

cultures, both fixed factors) were then analysed using two-way ANOVAs. Differences in removal rates of total PFAS and PFAS precursors, and individual PFAS and PFAS precursor types in algal culture water samples among HRTs (fixed factor) were analysed using one-way ANOVA. Normality was assessed using the Shapiro-Wilk test. Pillai's trace statistic was used to determine the significance of MANOVA results. All analyses were conducted in SPSS Statistics (version 29). All data are reported as means ± S.E.

5.4 Results

5.4.1 Biomass productivity

Biomass productivity did not vary significantly among HRT treatments (two-way ANOVA: $F_{3,32} = 0.01$, $p = 0.910$). Across all harvests average productivity was 4.35 g DW m^{-2} day^{-1} ± 0.15 for the 3-day HRT treatment and 4.37 g DW m^{-2} day^{-1} ± 0.17 for the 6-day HRT treatment (Figure 5.1). In contrast, biomass productivity did vary significantly among harvests (two-way ANOVA: $F_{1,32} = 5.11$, $p = 0.005$), ranging from 4.50 DW m^{-2} day^{-1} ± 0.03 to 4.91 g DW m^{-2} day^{-1} ± 0.03 on day 6 to 4.03 g DW m^{-2} day^{-1} ± 0.35 to 3.88 g DW m^{-2} day^{-1} ± 0.25 on day 24 for the 3- and 6-day HRT treatments, respectively (Figure 5.1). There was no significant interaction between these factors (Two-way ANOVA: $F_{3,32} = 1.11$, $p = 0.359$).

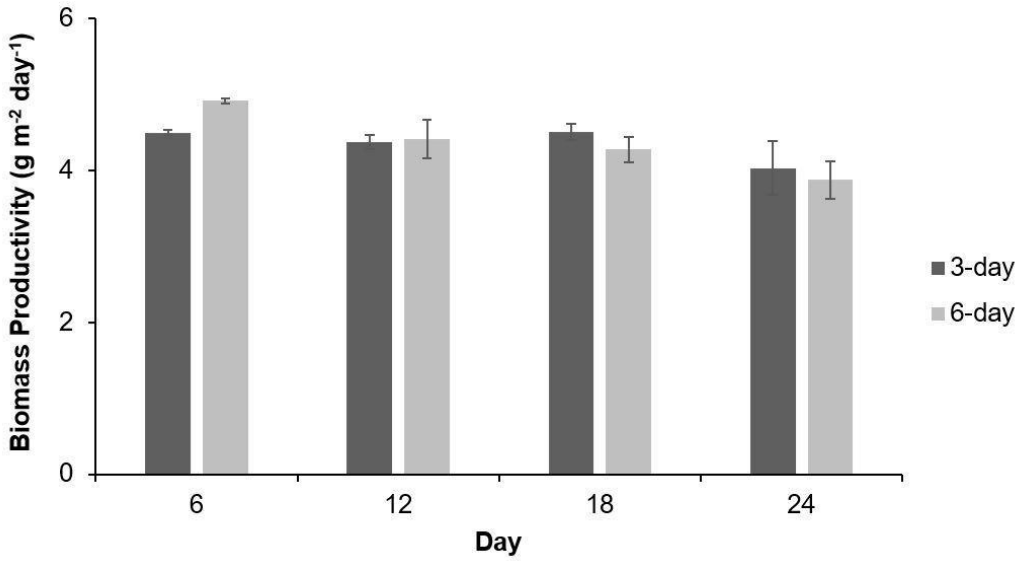

Figure 5.1 Mean (± S.E.) biomass productivity (g DW m^{-2} day^{-1}) of *Klebsormidium flaccidum* cultures with a hydraulic retention time (HRT) of three days and six days over a 24-day period under summer conditions. N = 5.

5.4.2 PFAS and PFAS precursor concentrations

A total of 10 PFAS and 7 PFAS precursor types were detected in wastewater samples (Table 5A.1). The total concentrations of PFAS and PFAS precursors in baseline (pre-experiment) primary wastewater varied significantly among sample collection times (One-way ANOVA, PFAS: $F_{2,81} = 5.53$, $p = 0.006$; PFAS precursors: $F_{2,60} = 12.70$, $p = <0.001$; Figure 5.2a, Table A.1). Average total PFAS concentrations were highest at 12pm with a total concentration of 3.877 μg L^{-1} ± 0.200, which was 1 to 2 orders of magnitude higher than the concentrations observed in the 9am and 3pm samples (0.039 μg L^{-1} ± 0.004 and 0.297 μg L^{-1} ± 0.017, respectively, Figure 5.2). Average total PFAS precursor concentrations (0.355 μg L^{-1} ± 0.007) were 1 order of magnitude lower than those of PFAS (4.212 μg L^{-1} ± 0.222) and varied throughout the day. Average total PFAS precursor concentrations at 9am (0.019 μg L^{-1} ± 0.001) were 8 times lower than the concentrations at midday, while average total PFAS precursor concentrations at 12pm and 3pm were comparable, measuring 0.162 μg L^{-1} ± 0.004 and 0.174 μg L^{-1} ± 0.002, respectively. The time of sample collection significantly affected the overall composition of PFAS types detected in primary wastewater but did not have a significant effect on PFAS precursor types (MANOVA, PFAS: $V = 1.99$, $F_{12,4} = 112.15$, $p = <0.001$, PFAS precursors: $V = 1.00$, $F_{14,2} = 0.14$, $p = <0.992$). The concentration of all individual PFAS and PFAS precursor types in baseline (pre-experiment) primary wastewater varied significantly among sample collection times, with the exception of the PFAS type PFHpA (Table 5.2). Concentrations of all individual PFAS types and three of the seven PFAS precursor types were highest at 12pm, while the remaining PFAS precursors were highest at 3pm (Figure 5.2b and 5.2c). Concentrations of all individual PFAS and PFAS precursor types were lowest at 9am. PFOS had the highest concentration of all the PFAS types (2.730 μg L^{-1} ± 0.129 at 12pm), while PFHxA had the highest concentration of all the PFAS precursor types (0.065 μg L^{-1} ± 0.129 at 3pm). Equal numbers of short-chain and long-chain PFAS types were detected. However, long-chain PFAS types were dominant in terms of concentration, accounting for 87.5% of the total PFAS concentration.

Table 5.2 Results of ANOVAs testing for differences in the concentration of individual types of per- and polyfluoroalkyl substances (PFAS) and PFAS precursor in baseline (pre-experiment) primary wastewater samples collected from the wastewater treatment plant (WWTP) at 9am, 12pm and 3pm on a single day. PFBA, Perfluorobutanoic acid; PFPeA, Perfluoropentanoic acid; PFHxA, Perfluorohexanoic acid; PFHpA, Perfluoroheptanoic acid; PFPeS, Perfluoropentane sulfonate; PFOA, Perfluorooctanoic acid; PFBS, Perfluorobutane sulfonate; PFHxS, Perfluorohexane sulfonate; PFHpS, Perfluoroheptane sulfonate; 6:2 FTS, 1H,1H,2H,2H-Perfluorooctane sulfonate; PFOS, Perfluorooctane sulfonate.

	df	F	P
PFAS			
PFBA	2	35.9	<0.001
PFPeA	2	1784.7	<0.001
PFHxA	2	13.3	0.006
PFHpA	2	4.1	0.075
PFPeS	2	37.4	<0.001
PFOA	2	34.73	<0.001
PFBS	2	226.3	<0.001
PFHxS	2	519.1	<0.001
PFHpS	2	186.8	<0.001
PFOS	2	406.4	<0.001
PFAS Precursors			
PFBA	2	242.5	<0.001
PFPeA	2	719.4	<0.001
PFHxA	2	1197.8	<0.001
PFHpA	2	384.5	<0.001
PFOA	2	2764.5	<0.001
PFBS	2	9.57	<0.001
PFHxS	2	150.9	<0.001

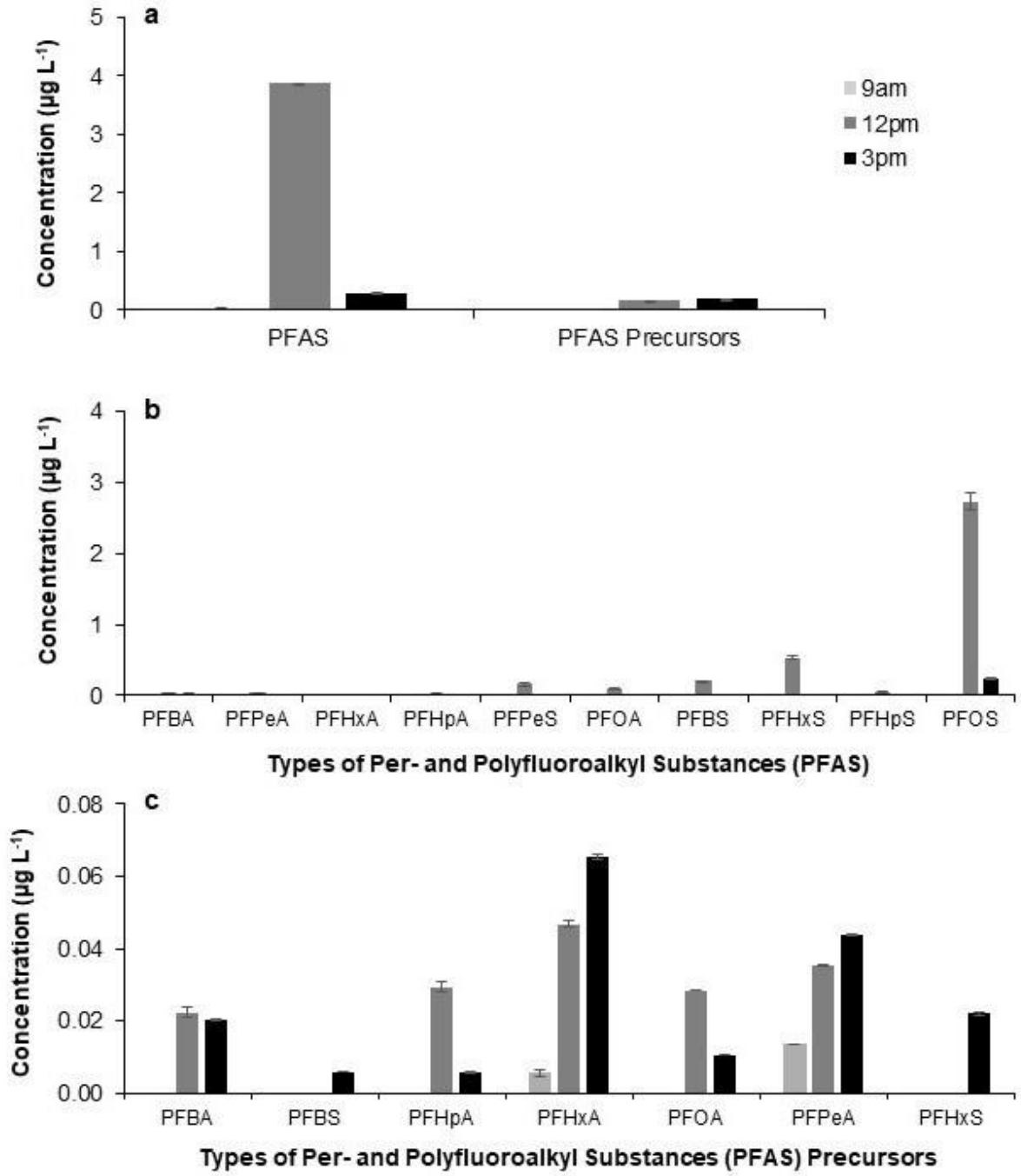

Figure 5.2 Mean (± S.E.) total concentrations (µg L^{-1}) of per- and polyfluoroalkyl substances (PFAS) and PFAS precursors (a), individual types of per- and polyfluoroalkyl substances (PFAS) precursors (b), and individual types of per- and polyfluoroalkyl substances (PFAS) (c) in primary wastewater samples collected from the wastewater treatment plant (WWTP) at 9am, 12pm and 3pm on a single day.

A total of 10 PFAS and 7 PFAS precursor types were detected in both the control and algal culture water treatments at the end of the experiment (Figure 5.3, Table 5.3). The HRT

and the type of treatment (algal culture vs. control) did not significantly affect the total concentration of PFAS in the water samples at the end of the experiment (Two-way ANOVA: HRT: $F_{1, 128} = 1.124$, $p = 0.291$; Treatment: $F_{1,128} = 0.125$, $p = 0.724$; Figure 5.3), and there was no significant interaction effect (Two-way ANOVA: HRT x Treatment: $F_{1,128} = 0.159$, $p = 0.691$). Similarly, the HRT and the type of treatment (algal culture vs. control) did not significantly affect the total concentration of PFAS precursors in the water samples at the end of the experiment (Two-way ANOVA: HRT: $F_{1,56} = 0.02$, $p = 0.882$; Treatment: $F_{1, 56} = 2.20$, $p = 0.144$; Figure 5.3), and there was no significant interaction effect (Two-way ANOVA: HRT x Treatment: $F_{1,56} = 1.15$, $p = 0.287$). Notably, total PFAS concentrations across all samples were 7.5% higher than total PFAS precursor concentrations (0.240 μg $L^{-1} \pm 0.013$ and 0.222 μg $L^{-1} \pm 0.030$, respectively). The overall composition of PFAS types varied significantly between treatments (algal culture vs. control), but this effect was not consistent among HRTs (MANOVA: HRT x Treatment: $V = 0.992$, $F_{7,2} = 37.18$, $p = 0.026$). In contrast, the overall composition of PFAS precursor types did not vary significantly among HRT or treatment (algal culture vs control) (MANOVA: HRT: $V = 0.642$, $F_{5,4} = 1.44$, $p = 0.374$; Treatment: $V = 0.741$, $F_{5,4} = 2.29$, $p = 0.222$) and there was no significant interaction effect (MANOVA: HRT x Treatment: $V = 0.717$, $F_{5,4} = 2.03$, $p = 0.256$). The PFAS types PFHxA, PFOA, PFBS, PFHxS and 6:2 FTS were consistently detected in algal culture water and control water samples across both HRTs, while PFPeA was only detected in algal culture water for both HRTs and PFHpS was only detected in algal culture water for the 6-day HRT, and PFBA was not present in any samples. The PFAS precursor types PFBA, PFPeA, PFHxA, PFOA and PFBS, were consistently detected in algal culture water and control water samples across both HRTs, and the types PFHxS, PFHpS and 6:2 FTS were not present in any samples. Concentrations of individual PFAS types varied significantly between treatments (algal culture vs. control) but not between HRTs, with the exception of the PFAS types PFBS, PFHxA and PFHpS (Table 5.4). In contrast, the concentrations of individual PFAS precursor types did not vary significantly between treatments (algal culture vs. control) or HRTs, with the exception of PFHxA (Table 5.4). Under the 3-day HRT, the concentrations of the PFAS types PFOA and PFHxS were significantly lower in algal culture water compared to the control water. Conversely, the concentrations of PFPeA, PFHxA, and 6:2 FTS were significantly higher in algal culture water compared to the control water. Under the 6-day HRT, only the concentration of PFHxS was significantly lower in the algal culture water compared to the control water. PFBS had the highest concentration of all PFAS types in both the algal culture water and control water for the 3-day HRT (0.021 and 0.020 μg $L^{-1} \pm$

0.001 and 0.001 respectively), and the 6-day HRT (0.022 µg L^{-1} ± 0.001, for both treatments), while PFPeA had the highest concentration of all PFAS precursor types in both the algal culture water and control water for the 3-day HRT (0.023 and 0.027 µg L^{-1} ± 0.005 and 0.000, respectively), and the 6-day HRT (0.026 and 0.028 µg L^{-1} ± 0.001 and 0.002, respectively). Among all detected PFAS types, 53.3% were short-chain, and 46.7% were long-chain. However, short-chain PFAS comprised of 71.3% of the total PFAS concentration, while long-chain PFAS accounted for only 28.7%.

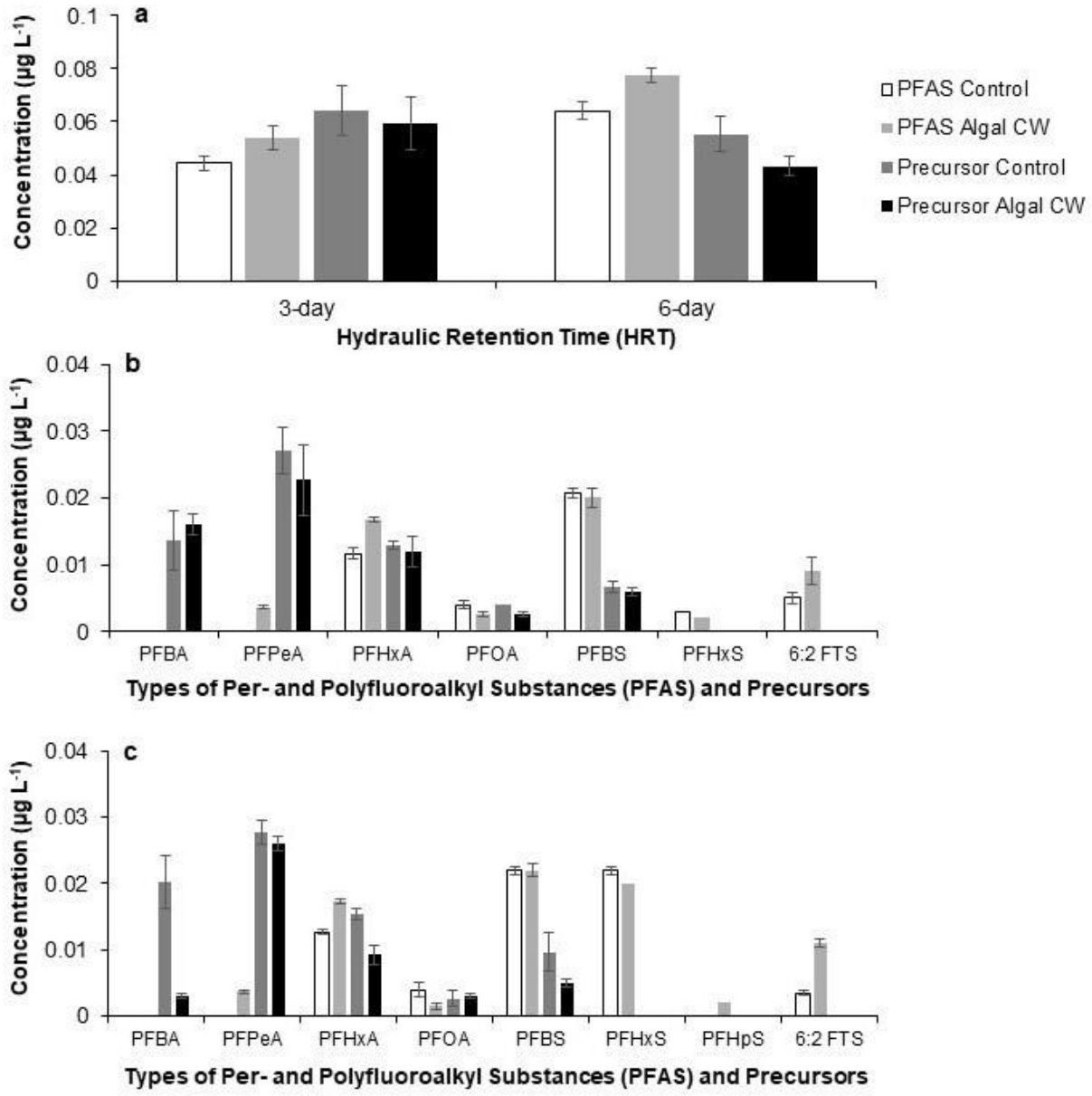

Figure 5.3 Mean (± S.E.) total concentrations (µg L^{-1}) of per- and polyfluoroalkyl substances (PFAS) and PFAS precursors in control and algal culture water (CW) samples with a three-

and six-day HRT (a), concentrations of individual types of PFAS and PFAS precursors in control and algal culture water (CW) samples with a three-day HRT (b) and concentrations of individual types of PFAS and PFAS precursors in control and algal culture water (CW) samples with a six-day HRT (c). N = 3.

Table 5.3 Concentrations (µg L^{-1}) of individual types of per- and polyfluoroalkyl substances (PFAS) and PFAS precursors in control water, algal culture water and algal biomass of *K. flaccidum* at the final harvest (day 24). Cultures were maintained with a 3-day or 6-day HRT. PFBA, Perfluorobutanoic acid; PFPeA, Perfluoropentanoic acid; PFHxA, Perfluorohexanoic acid; PFOA, Perfluorooctanoic acid; PFBS, Perfluorobutane sulfonate; PFHxS, Perfluorohexane sulfonate; PFHpS, Perfluoroheptane sulfonate; 6:2 FTS, 1H,1H,2H,2H-Perfluorooctane sulfonate. <LOD, below levels of detection. Data are means ± standard error, N = 3.

	PFBA	PFPeA	PFHxA	PFOA	PFBS	PFHxS	PFHpS	6:2 FTS	Total
3-day HRT									
PFAS									
Control water	<LOD	<LOD	0.012 ± 0.001	0.004 ± 0.001	0.021 ± 0.001	0.003 ± 0.001	<LOD	0.005 ± 0.003	0.044 ± 0.003
Algal culture water	<LOD	0.004 ± 0.000	0.017 ± 0.000	0.003 ± 0.000	0.020 ± 0.000	0.002 ± 0.000	<LOD	0.009 ± 0.002	0.054 ± 0.005
Algal biomass	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	-
PFAS Precursors									
Control water	0.014 ± 0.000	0.027 ± 0.000	0.013 ± 0.000	0.004 ± 0.000	0.007 ± 0.000	<LOD	<LOD	<LOD	0.064 ± 0.001
Algal culture water	0.016 ± 0.002	0.023 ± 0.005	0.012 ± 0.002	0.003 ± 0.000	0.006 ± 0.001	<LOD	<LOD	<LOD	0.059 ± 0.010
Algal biomass	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	-
6-day HRT									
PFAS									
Control water	<LOD	<LOD	0.013 ± 0.001	0.004 ± 0.001	0.022 ± 0.001	0.022 ± 0.000	<LOD	0.004 ± 0.001	0.064 ± 0.003
Algal culture water	<LOD	0.004 ± 0.000	0.017 ± 0.000	0.002 ± 0.000	0.022 ± 0.001	0.020 ± 0.000	0.002 ± 0.000	0.011 ± 0.000	0.078 ± 0.003

	PFBA	PFPeA	PFHxA	PFOA	PFBS	PFHxS	PFHpS	6:2 FTS	Total
Algal biomass	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	-
PFAS Precursors									
Control water	0.014 ± 0.000	0.027 ± 0.000	0.013 ± 0.000	0.004 ± 0.000	0.007 ± 0.000	<LOD	<LOD	<LOD	0.055 ± 0.007
Algal culture water	0.003 ± 0.000	0.026 ± 0.001	0.009 ± 0.001	0.003 ± 0.000	0.005 ± 0.001	<LOD	<LOD	<LOD	0.043 ± 0.004
Algal biomass	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	-

Table 5.4 Results of ANOVAs testing for differences in the concentration of individual types of per- and polyfluoroalkyl substances (PFAS) and PFAS precursor in water samples under different treatments (algal culture vs. control) with three- and six-day HRTs. PFBA, Perfluorobutanoic acid; PFPeA, Perfluoropentanoic acid; PFHxA, Perfluorohexanoic acid; PFOA, Perfluorooctanoic acid; PFBS, Perfluorobutane sulfonate; PFHxS, Perfluorohexane sulfonate; PFHpS, Perfluoroheptane sulfonate; 6:2 FTS, 1H,1H,2H,2H-Perfluorooctane sulfonate.

	Effect	df	F	P
PFAS				
PFPeA	HRT	1	0.000	1.000
	Treatment	1	242.00	<0.001
	HRT x Treatment	1	0.000	1.000
	Residual	8		
PFHxA	HRT	1	2.500	0.153
	Treatment	1	84.100	<0.001
	HRT x Treatment	1	0.100	0.760
	Residual	8		
PFOA	HRT	1	1.32	0.284
	Treatment	1	8.90	0.018
	HRT x Treatment	1	0.28	0.284
	Residual	8		

	Effect	df	F	P
PFBS	HRT	1	6.25	0.037
	Treatment	1	0.25	0.631
	HRT x Treatment	1	0.25	0.631
	Residual	8		
PFHxS	HRT	1	14.63	0.005
	Treatment	1	6.01	0.040
	HRT x Treatment	1	4.24	0.073
	Residual	8		
PFHpS	HRT	1	1.00	0.347
	Treatment	1	1.00	0.347
	HRT x Treatment	1	1.00	0.347
	Residual	8		
6:2 FTS	HRT	1	0.108	0.750
	Treatment	1	22.28	0.002
	HRT x Treatment	1	0.976	0.352
	Residual	8		
PFAS Precursors				
PFBA	HRT	1	0.67	0.437
	Treatment	1	2.79	0.133
	HRT x Treatment	1	4.53	0.066
	Residual	8		
PFPeA	HRT	1	0.351	0.570
	Treatment	1	0.790	0.400
	HRT x Treatment	1	0.156	0.703
	Residual	8		
PFHxA	HRT	1	0.01	0.002
	Treatment	1	5.73	0.417
	HRT x Treatment	1	2.92	0.268

	Effect	df	F	P
	Residual	8		
PFOA	HRT	1	0.64	0.446
	Treatment	1	0.64	0.446
	HRT x Treatment	1	1.79	0.218
	Residual	8		
PFBS	HRT	1	0.40	0.543
	Treatment	1	2.88	0.128
	HRT x Treatment	1	1.62	0.239
	Residual	8		

The total concentration of PFAS in the algal culture water increased by 11.3% ± 6.5 and 8.8% ± 5.1 compared to control treatments in the 3- and 6-day HRT respectively, while the total concentration of PFAS precursors decreased by 4.0% ± 20.8 and 17.6% ± 25.9 in the 3- and 6-day HRTs respectively. However, variation among replicates was high, and consequently total PFAS and PFAS precursor removal rates did not vary significantly between HRTs (One-way ANOVA, PFAS: $F_{1,81} = 0.01$, $p = 0.940$; PFAS precursors: $F_{1,28} = 0.40$, $p = <0.531$; Figure 5.4a). Removal rates for individual PFAS and PFAS precursor types were highly variable, ranging from -155.6% to 77.8% and -70.0% to 75.0%, respectively, and did not vary significantly between HRTs (Table 5). Among all individual PFAS types, removal rates were highest for PFOA (33.3% ± 8.3) under the 3-day HRT and PFBA (75.0% ± 25.0) under the 6-day HRT. Among all individual PFAS precursor types, removal rates were highest for PFHxS (44.4% ± 25.7) under the 3-day HRT, and for PFOA (77.8% ± 44.9) under the 6-day HRT. However, there were instances where removal did not occur, and in some cases, concentrations increased. Among all individual PFAS types, the concentrations of PFHxA increased by 44.9% (± 25.9), 6:2 FTS by 88.9% (± 51.3) and PFBS by 0.3% (± 0.2) under the 3-day HRTs. Similarly, concentrations of PFHxA increased by 37.0% (± 21.3) and 6:2 FTS by 155.6% (± 89.8) under 6-day HRTs. Among all individual PFAS precursor types, the concentrations of PFBA increased by 37.1% (± 33.4) under the 3-day HRT, and PFOA increased by 70.0% (± 77.8) under the 6-day HRT. Notably, all PFAS and PFAS precursor concentrations were below the limits of detection within the algal biomass of *K. flaccidum* (Table 5.3).

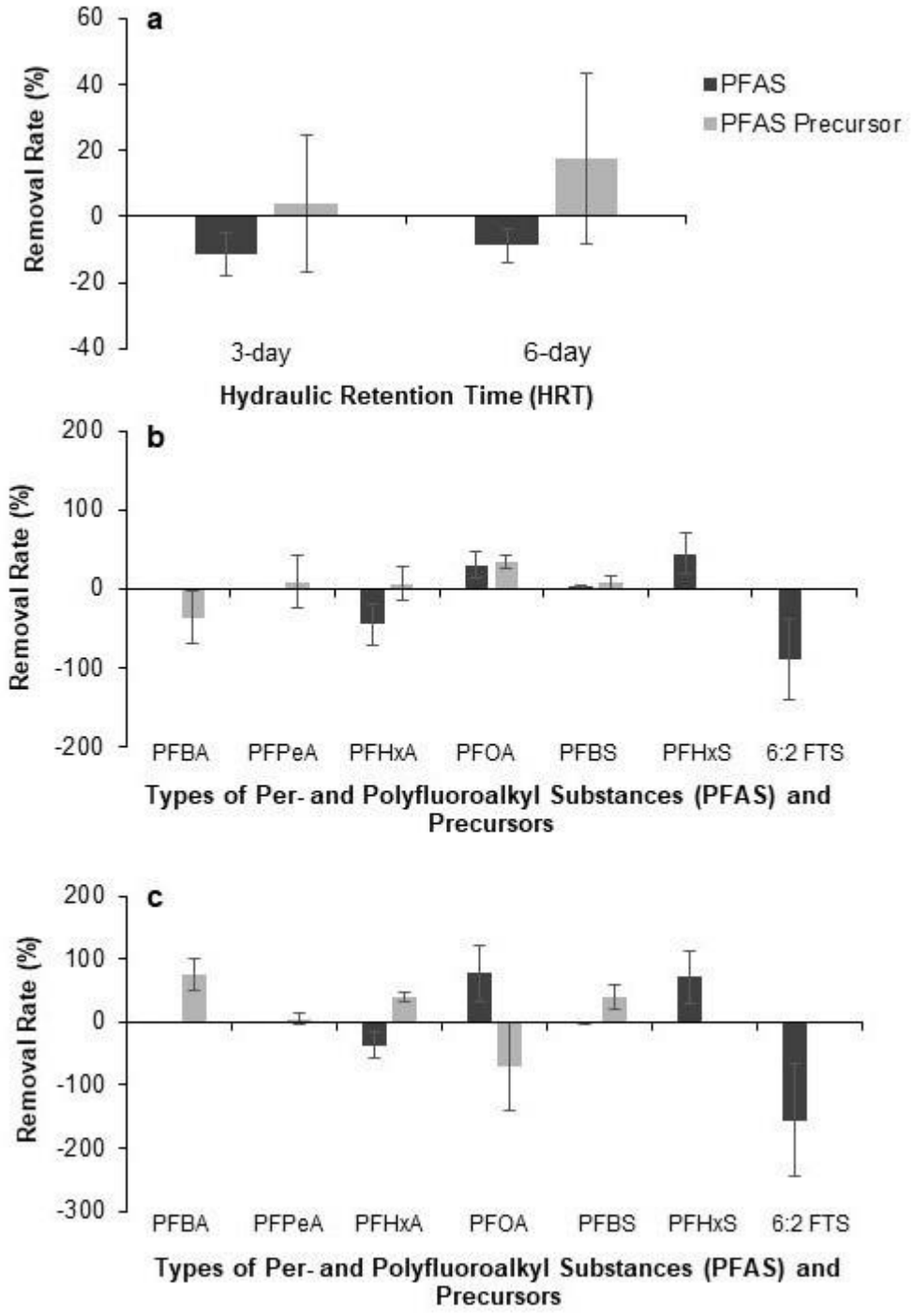

Figure 5.4 Mean (± S.E.) total removal rates (%) of all per- and polyfluoroalkyl substances (PFAS) and PFAS precursors in algal culture water samples with a three- and six-day HRT (a), removal rates (%) of individual types of PFAS and PFAS precursors in algal culture water samples with a three-day HRT (b) and removal rates (%) of individual types of PFAS and PFAS precursors in algal culture water samples with a six-day HRT (c). N = 3.

Table 5.5 Results of ANOVAs testing for differences in the removal rate of individual types of per- and polyfluoroalkyl substances (PFAS) and PFAS precursor in algal culture water samples with three- and six-day HRTs. PFBA, Perfluorobutanoic acid; PFPeA, Perfluoropentanoic acid; PFHxA, Perfluorohexanoic acid; PFOA, Perfluorooctanoic acid; PFBS, Perfluorobutane sulfonate; PFHxS, Perfluorohexane sulfonate; 6:2 FTS, 1H,1H,2H,2H-Perfluorooctane sulfonate.

	df	F	P
PFAS			
PFHxA	1	0.3	0.602
PFOA	1	5.1	0.087
PFBS	1	0.2	0.675
PFHxS	1	0.4	0.555
6:2 FTS	1	0.5	0.539
PFAS Precursors			
PFBA	1	7.2	0.055
PFPeA	1	0.0	0.915
PFHxA	1	2.2	0.213
PFOA	1	2.1	0.217
PFBS	1	2.1	0.224

5.5 Discussion

This study investigated the potential for the freshwater filamentous macroalga *K. flaccidum* to remove PFAS from primary municipal wastewater. Previous research has demonstrated that PFAS can bioaccumulate in freshwater algae when it is cultivated in contaminated water (Liu et al., 2018; Penland et al., 2020), with algae showing a higher capacity to bioaccumulate PFAS compared to other biota, likely due to their comparatively larger surface area to volume ratio (Liu et al., 2018). In this study, concentrations of the PFAS types PFOA, PFBS and PFHxS were reduced within the algal culture water samples compared to control water samples, suggesting that the cultivation of *K. flaccidum* reduced the concentration of these compounds. Although research on the mechanisms of PFAS

removal by algae is limited, existing studies suggest that individual PFAS types are reduced through different processes. For example, PFOS has been shown to be removed from synthetic wastewater primarily through bioaccumulation, as some was internalised within the algal biomass of *Synechocystis* sp. (Marchetto et al., 2021). In contrast, PFOA may be actively metabolised through enzymatic breakdown (Marchetto et al., 2021), converting these compounds into non-toxic forms or smaller molecules (Zhou et al., 2022). In this study, *K. flaccidum* did not show effective direct removal of PFAS compounds through bioaccumulation, as PFAS and PFAS precursors were not detected within the biomass of *K. flaccidum* following 24 days of cultivation in primary wastewater containing these compounds. However, as the wastewater PFAS concentrations in this experiment were very low, the content of bioaccumulated PFAS in the algal biomass could be diluted by new biomass growth, potentially leading to undetectable levels. This highlights the need for further research to explore the PFAS removal capabilities and mechanisms of various algal species to identify those most effective for wastewater treatment applications. Nevertheless, algal biomass free of PFAS compounds is highly desirable for various industries, including food, pharmaceuticals, cosmetics, and bioplastics (Harris et al., 2022). PFAS-free algal products are critical for complying with stringent regulatory requirements and enhancing consumer confidence in the safety of these products (Ng et al., 2021; Kemper et al., 2024). Therefore, the absence of PFAS in *K. flaccidum* biomass samples show that cultivation of this species in wastewater containing PFAS can produce biomass that is suitable for a wide range of product applications, provided that the algal biomass is free from contamination by other pollutants, including heavy metals, organic compounds (including pesticides and estrogenic substances), and microbial pathogens.

Research on the potential of algal bioremediation for PFAS removal has been limited and has primarily focused on microalgae, yielding varied results. For example, the cyanobacteria *Synechocystis* sp. achieved estimated removal rates of approximately 7 and 18 mg m^{-2} day^{-1} for PFOA and PFOS, respectively, from synthetic wastewaters (Marchetto et al., 2021). This removal was considered to be achieved by cell absorption and enzymatic degradation (Marchetto et al., 2021). In contrast, the microalga *Desmodesmus subspicatus* showed no removal of three perfluoroalkyl substances (PFHxPA, PFOPA and PFDPA) from wastewater effluent (Llorca et al., 2018). In the current study, using primary municipal wastewater, overall PFAS removal was inconsistent. Significant removal of PFOA (30.0% to 77.8%) and PFHxS (44.4% to 71.0%) was observed under 3-day and 6-day HRTs,

respectively. Given that PFOA has been previously considered to be enzymatically digested by algae, it is plausible that a similar degradation process occurred in this instance. Conversely, concentrations of PFHxA and 6:2 FTS in algal culture water increased by more than 88.9% and 44.9%, respectively, relative to controls under both HRTs. Specific PFAS types may have increased in algal culture water compared to control water due to the algae's ability to adsorb these compounds, which were then subsequently released back into the culture water (Mantripragada et al., 2023). Additionally, conditions within the algal culture, such as changes in pH and microbial activity, can result in lower or higher PFAS concentrations in the algal culture water (Tavasoli et al., 2021; Zhang et al., 2022). The differential removal of various PFAS types may be partially explained by their chemical structure, specifically whether they are short-chain or long-chain compounds. Among all the individual types of PFAS analysed, PFOA and PFHxS were the only long-chain PFAS detected in the control water. These compounds also exhibited the highest PFAS removal rates in the algal cultures, demonstrating that long-chain PFAS are more readily removed (30.0 – 77.8% removal rate) compared to short-chain PFAS (3.0% removal rate). This could be due to the longer carbon-fluorine chains and increased hydrophobicity of long-chain PFAS, which may enhance their affinity for the algal cells, leading to more effective adsorption, bioaccumulation and removal (Wu et al., 2022; Mao et al., 2023; Smaili & Ng, 2023). This observation aligns with previous findings indicating that short-chain PFAS are more persistent in the environment due to their low adsorption potential in aqueous solutions, due to their shorter carbon-fluorine chains making them less hydrophobic (Valencia et al., 2023). Most research to date has focused on the removal of individual PFAS, such as PFOA and PFOS (Marchetto et al., 2021; Mao et al., 2023). However, it is important to concurrently measure the removal of multiple PFAS types as municipal WWTP influents can contain a wide range of PFAS compounds and the removal rates of individual PFAS types may vary depending on the composition of the wastewater and the presence of other PFAS types (Barisci & Suri, 2021). WWTP influents also contain a complex mixture of organic and inorganic compounds, nutrients, and microorganisms that may affect PFAS removal rates (Abdel-Raouf et al., 2012). These components can interact with PFAS and PFAS precursors, potentially affecting algal growth and bioremediation outcomes (Yan et al., 2024). As a result, municipal wastewater rather than synthetic wastewater should be used in experiments to ensure that results more accurately reflect the conditions encountered in operational settings. This is crucial for providing a realistic assessment of the algae's capacity to remediate PFAS and for developing effective and scalable algal treatment systems.

The ability of algae to bioremediate PFAS from primary municipal wastewater can be significantly influenced by the concentration of PFAS present in the wastewater. Concentrations of specific types of PFAS as low as 0.01 μg L^{-1} have been shown to inhibit algal growth and cause toxic effects (Niu et al., 2019). This inhibitory effect has been attributed to several factors, including cellular toxicity (Niu et al., 2019), disruption of cellular processes, decreased chlorophyll production, reduced cell viability, and impaired metabolic functions (Hu et al., 2023; Mojiri et al., 2023a). PFAS exposure can also lead to the downregulation of essential genes (Li et al., 2021b) and inhibit enzymatic activities crucial for photosynthesis and respiration (Li et al., 2022). These toxic effects are dependent on the concentration and composition of PFAS and can vary across different algal species (Niu et al., 2019). In the current study, *K. flaccidum* maintained consistent growth (3.88–4.91 g DW m^{-2} day^{-1}) throughout the experiment, despite the presence of PFAS in the culture water at total concentrations of 0.054 – 0.078 μg L^{-1}. However, these PFAS concentrations are on the lower end of those that have caused toxic effects and growth inhibition in algae in previous studies (0.01 μg L^{-1} – 31 mg L^{-1}) (Niu et al., 2019; Pietropoli et al., 2024). It is possible therefore that if PFAS concentrations were higher, similar effects might be observed for *K. flaccidum*. As levels of PFAS in municipal wastewater can fluctuate considerably, it is crucial to assess both the bioremediation potential and growth of algae across the typical range of PFAS concentrations and compositions found in municipal wastewater. This comprehensive approach will help to manage acute toxicity risks, as PFAS levels in municipal wastewater can occasionally spike to higher concentrations (Chen et al., 2018). Additionally, investigating how PFAS removal rates might vary in winter is important, as this study was conducted in summer when algal productivity is typically high. Reduced growth rates in colder conditions could lead to higher detectable PFAS concentrations in the algal biomass if bioaccumulation rates remain constant. A thorough understanding of these dynamics will better inform strategies for effective PFAS treatment.

PFAS concentrations in wastewater can fluctuate dramatically over short periods due to factors such as varying industrial discharges, household product use and rainfall events (Nguyen et al., 2019), making it challenging to measure and predict their presence. In the current study, total PFAS concentrations in primary wastewater ranged from 0.034 to 3.9 μg L^{-1}. These values are typical of the PFAS concentrations reported in municipal WWTP effluents, which have been reported to range from 0.05 ng L^{-1} to 11 μg L^{-1} (Dauchy et al.,

2017; Houtz et al., 2018; Coggan et al., 2019; Chiriac et al., 2023). Additionally, the types of PFAS identified were consistent with those commonly found in WWTPs (Barisci & Suri, 2021). Similar to previous studies (Lenka et al., 2021), short-chain PFAS were predominant, accounting for 71.3% of the total PFAS load in the wastewater. One potential factor contributing to the high concentration of short-chain PFAS is the increased use of the short-chain PFAS PFHxA as a replacement for the long-chain PFAS PFOA (Shan et al., 2014). This was evident in the current study, as average PFHxA concentrations were more than three times greater than those of PFOA. Short-chain PFAS were developed to replace long-chain PFAS to mitigate long-term ecological and health concerns associated with their persistence in the environment (Li et al., 2020). However, short-chain PFAS have proven to be equally persistent and widespread, highlighting the challenges associated with managing newer PFAS alternatives (Grgas et al., 2023). PFAS precursors were also detected throughout the experiment, with total concentrations ranging from 0.043 – 0.064 μg L^{-1}. In some cases, precursors were measured at higher concentrations than the corresponding PFAS. These findings demonstrate the importance of assessing both PFAS and their PFAS precursors to accurately measure the concentrations being discharged into the environment (Lenka et al., 2021). Conducting these measurements is crucial for developing effective strategies to address the persistent and variable nature of PFAS in municipal wastewater.

5.6 Conclusion

This study investigated the potential of algal bioremediation to remove PFAS and PFAS precursors from primary municipal wastewater. Although *K. flaccidum* cultures exhibited promising growth and achieved reductions in various PFAS and PFAS precursors, the significant variation in treatment effectiveness resulted in *K. flaccidum* being ineffective in completely removing PFAS. The effectiveness of PFAS bioremediation is likely to be algal species-specific and also to vary depending on the type of PFAS. The variability of PFAS concentrations in primary municipal wastewater presents challenges for accurate measurement and prediction of PFAS loads. Therefore, using real municipal wastewater in experiments is essential to obtain accurate and applicable results. Additionally, analysing PFAS precursors is crucial to understand the broader environmental impact of these contaminants. This study has demonstrated that *K. flaccidum* is a robust cultivar that can tolerate PFAS at relatively low concentrations (< 3.88 μg L^{-1}). The absence of PFAS and

PFAS precursors in *K. flaccidum* biomass could enable it to be used for a wide range of product applications that require PFAS-free biomass as long as it is also free of other contaminants. To develop effective and scalable algal-based solutions for PFAS contamination, future studies should also explore other algal species that may exhibit high PFAS removal capabilities.

5.7 Appendix

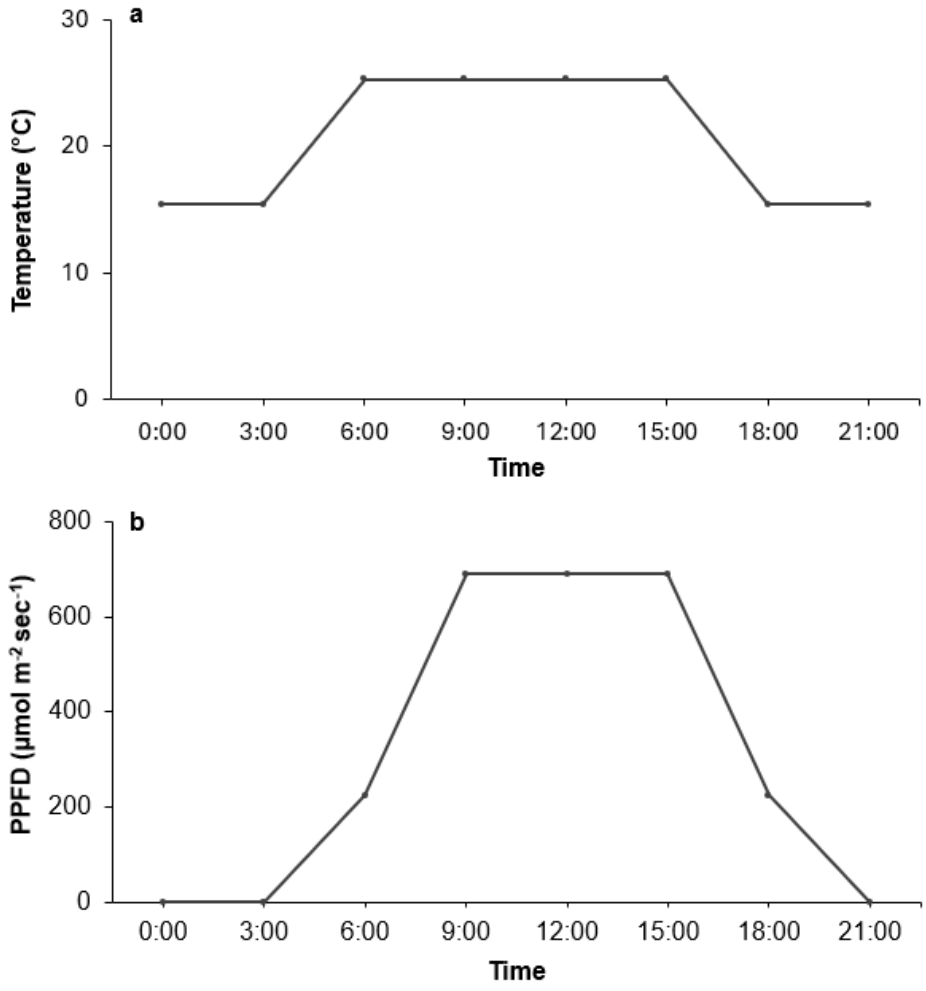

Figure 5A.1 Summer temperature and light (photosynthetic photon flux density) profiles used in the hydraulic retention time experiment.

Table 5A.1 Concentrations of per- and polyfluoroalkyl substances (PFAS) and PFAS precursors (µg L^{-1}) in in primary wastewater samples collected from the wastewater treatment plant (WWTP) at 9am, 12pm and 3pm on a single day. PFBA, Perfluorobutanoic acid; PFPeA, Perfluoropentanoic acid; PFHxA, Perfluorohexanoic acid; PFHpA, Perfluoroheptanoic acid; PFOA, Perfluorooctanoic acid; PFBS, Perfluorobutane sulfonate; PFPeS, Perfluoropentane sulfonate; PFHxS, Perfluorohexane sulfonate; PFHpS, Perfluoroheptane sulfonate; PFOS, Perfluorooctane sulfonate; <LOD, below levels of detection. Data are means ± standard error, N = 3.

	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFBS	PFPeS	PFHxS	PFHpS	PFOS	Total
9am											
PFAS	0.018 ± 0.000	<LOD	0.004 ± 0.002	<LOD	<LOD	0.006 ± 0.001	0.006 ± 0.000	<LOD	<LOD	0.005 ± 0.001	0.039 ± 0.004
Precursor	<LOD	<LOD	<LOD	0.006 ± 0.001	<LOD	<LOD	0.014 ± 0.001	<LOD	<LOD	<LOD	0.019 ± 0.002
12pm											
PFAS	0.036 ± 0.003	0.039 ± 0.001	0.013 ± 0.001	0.026 ± 0.002	0.093 ± 0.006	0.202 ± 0.006	0.160 ± 0.026	0.530 ± 0.023	0.048 ± 0.004	2.730 ± 0.129	3.877 ± 0.200
Precursor	0.022 ± 0.001	<LOD	0.029 ± 0.001	0.047 ± 0.001	<LOD	0.028 ± 0.000	0.035 ± 0.000	<LOD	<LOD	<LOD	0.162 ± 0.004
3pm											
PFAS	0.022 ± 0.001	0.003 ± 0.000	0.004 ± 0.000	0.002 ± 0.000	0.005 ± 0.001	0.006 ± 0.000	<LOD	0.018 ± 0.001	<LOD	0.237 ± 0.015	0.297 ± 0.017
Precursor	0.020 ± 0.000	0.006 ± 0.000	0.006 ± 0.000	0.065 ± 0.001	0.022 ± 0.000	0.011 ± 0.000	0.044 ± 0.000	0.022 ± 0.000	<LOD	<LOD	0.196 ± 0.002

Chapter 6 - General discussion

6.1 Research outcomes

Species selection is essential for optimising the bioremediation performance of algal monoculture systems. Optimising operational parameters such as HRT, stocking density and harvest frequency can significantly impact the growth and bioremediation efficiency of the target cultivar. This thesis systematically investigated all these factors to explore the potential of freshwater filamentous macroalgae for treating primary municipal wastewater within HRFAP systems.

Chapter 2 established a screening protocol to assess the bioremediation capabilities of freshwater filamentous algae. Using this protocol, target cultivars were isolated from forty-four mixed samples of freshwater filamentous macroalgae collected across various local aquatic environments during summer and winter. Eleven selected cultivars were cultivated in primary treated municipal wastewater under controlled laboratory conditions, evaluating their biomass productivity and bioremediation performance under local ambient (summer and winter) and extreme (maximum summer and minimum winter) conditions. The key finding in this study was that extreme conditions played a crucial role in determining cultivar performance, with biomass productivity and bioremediation efficiency notably decreasing under minimum winter conditions. Interestingly, biomass productivity did not directly correlate with bioremediation performance as some cultivars with slower growth rates exhibited high nutrient removal rates under winter conditions. *Klebsormidium* sp. *(KLEB B)*, *Stigeoclonium* sp. *(STIG A)* and *Ulothrix* sp. were identified as top performing cultivars due to their competitive dominance, growth, and nutrient bioremediation performance across both summer and winter conditions. Overall, this Chapter successfully developed a screening protocol to identify promising cultivars for year-round nutrient bioremediation in primary municipal wastewater. Additionally, this protocol can be adapted to various locations and target wastewaters.

Chapter 3 built upon the outcomes of the screening protocol by examining the biomass productivity and bioremediation performance of three freshwater filamentous cultivars - *Klebsormidium flaccidum*, *Oedogonium calcareum*, and *Oedogonium* sp. –

cultivated in primary municipal wastewater within outdoor HRFAP mesocosms. During monoculture growth experiments, *O. calcareum* experienced complete die-off which was likely due to the presence of a micropollutant. The main finding from this Chapter was that the day-to-day variability in the concentrations of contaminants in primary municipal wastewaters mean that the assessment of cultivar performance using synthetic wastewaters or treated effluents is inadequate for selecting target cultivars for the treatment of more concentrated and variable primary wastewater. Instead, nutrient bioremediation performance of cultivars should be evaluated using actual wastewater in outdoor HRFAPs, allowing for long-term exposure to detect tolerance to stochastically occurring micropollutants. Chapter 3 also assessed the competitive dynamics between *K. flaccidum* and *Oedogonium* sp. in bi-cultures initially stocked with equal proportions of both cultivars at three different stocking densities. *Klebsormidium flaccidum* demonstrated greater competitive dominance at lower stocking densities. However, additional research is needed to ascertain its dominance at higher stocking densities.

Based on the findings from Chapters 2 and 3, *K. flaccidum* was identified as a target cultivar for treating primary municipal wastewater due to its high growth rates, effective nutrient bioremediation performance, and competitive dominance when cultivated in HRFAPs. Chapter 4 then investigated how three key operational parameters – HRT, stocking density, and harvest frequency – affected the growth and nutrient bioremediation efficiency of *K. flaccidum* in outdoor HRFAPs during summer and winter conditions. The study revealed that seasonal variations significantly influenced biomass productivity, with lower stocking densities proving optimal for increasing yields. Notably, harvest frequency showed minimal impact on nutrient removal rates compared to its effects on biomass productivity across different treatments and seasons. This study highlighted the importance of HRT when optimising HRFAP operation to maximise both biomass production and nutrient bioremediation efficiency. These findings further validate the viability of HRFAP systems for the effective treatment of primary municipal wastewater.

Chapter 5 investigated the effectiveness of the freshwater filamentous macroalga *K. flaccidum* in removing per- and polyfluoroalkyl substances (PFAS) and PFAS precursors from primary municipal wastewater under laboratory conditions using HRTs of 3- and 6-days. The study identified eleven types of PFAS and ten types of PFAS precursors in the primary wastewater used for this experiment, as collected from a local treatment plant,

highlighting the diverse range of chemicals present in municipal wastewater. *K. flaccidum* reduced the concentrations of three individual PFAS and all measured PFAS precursors in the primary wastewater. Additionally, neither PFAS nor PFAS precursors were detected in the algal biomass. These results suggest that *K. flaccidum* is not effective for achieving complete PFAS removal from primary wastewater. However, its consistent biomass productivity in primary municipal wastewater containing a range of PFAS and PFAS precursors confirms the suitability of this cultivar for nutrient bioremediation in primary wastewaters. Notably, *K. flaccidum* showed resilience against PFAS toxicity at the lower concentrations observed in the primary wastewater. Furthermore, the absence of PFAS in *K. flaccidum* biomass suggests potential for its use in various applications requiring PFAS-free algal biomass, such as biostimulants and agricultural feed.

6.2 Implications and contributions

This thesis highlights the potential of freshwater filamentous macroalgae as a promising option for treating primary municipal wastewater, offering a more sustainable alternative to traditional wastewater treatment systems. Overall, this thesis demonstrated that through species selection and optimisation of operational parameters, freshwater filamentous algae can efficiently treat primary municipal wastewater, resulting in substantial reductions in nutrient concentrations and improvements in water quality. Based on the findings of this study, potential strategies to maximise the performance of HRFAPs include:

i. Species selection and screening protocol: The screening protocol introduced in Chapter 2 represents a significant advancement as the first step-by-step guide to identify target cultivars of freshwater filamentous macroalgae for bioremediation in primary municipal wastewater. This protocol ensures consistency and applicability throughout the process, from sample collection to completion of bioremediation performance trials. Its straightforward methodology facilitates ease of replication and ensures that the selected target cultivars exhibit essential traits such as competitive dominance, high biomass productivity, and effective bioremediation performance under varying local, seasonal, and extreme conditions. Therefore, application of this screening protocol should be an essential first step for any new ventures employing algal bioremediation for municipal wastewater treatment.

ii. Winter tolerant cultivars: Selecting cultivars that exhibit robust growth and efficient bioremediation during winter conditions is crucial. Winter poses challenges such as slower growth rates and the need for longer HRTs due to reduced light and colder temperatures in HRFAP systems. While certain cultivars may thrive throughout both winter and summer, not all cultivars that excel in warmer months can maintain high performance under the cooler months of winter. Therefore, identifying winter-tolerant cultivars ensures consistent and effective wastewater treatment throughout the year. These winter-tolerant cultivars can be identified as part of the screening protocol.

iii. Growth trials under extreme seasonal conditions: It is crucial to conduct growth trials under extreme seasonal conditions to thoroughly evaluate algal performance, particularly during winter with longer HRTs exceeding 4-days. These trials should incorporate measurements of both TAN and nitrate-N concentrations to accurately assess bioremediation efficiency, considering the impact of nitrification processes. If time, logistics, funding, or other constraints preclude undertaking the full screening protocol, assessing cultivar performance under extreme seasonal conditions can provide a rapid indication of robust cultivars and should be considered as the minimum assessment. Performance of cultivars under extreme seasonal conditions can be used as a rapid and effective indicator to identify robust cultivars capable of maintaining high nutrient removal rates despite challenging environmental conditions. These trials provide valuable insight into cultivar resilience and suitability for more consistent performance of HRFAP systems.

iv. Use of municipal wastewater: Evaluating the performance of algal bioremediation using wastewater sourced directly from target WWTPs is essential for conducting accurate and relevant experiments. Findings from Chapter's 2, 3 and 5 highlight the limitations of previous research that has assessed cultivar performance using synthetic wastewaters. Synthetic wastewaters fail to replicate the complex composition of organic and inorganic compounds found in municipal wastewater. Therefore, to accurately select cultivars for primary wastewater treatment, it is imperative to assess their nutrient bioremediation performance in outdoor HRFAPs using the actual wastewater. Long-term exposure is necessary to determine how cultivars perform under the diverse and fluctuating conditions typical of municipal wastewater

environments. This approach ensures that the cultivars have been selected to be robust and effective under real-world conditions.

v. Optimisation of HRFAP operational parameters: In this study, of the parameters measured, HRT was identified as the most critical parameter significantly influencing bioremediation outcomes. Therefore, if resources are limited, prioritising optimisation of HRT should be the primary focus, given its higher impact on performance. To further enhance growth and bioremediation performance, operational parameters of HRFAPs – including stocking density and harvest frequency – should be tailored for the selected target cultivar. Each cultivar may respond differently to these parameters, particularly across different seasons as illustrated in Chapter 4, which provides insights into managing optimal operational parameters and their various bioremediation outcomes across seasons.

vi. PFAS-Free algal products: Testing for PFAS in algal biomass is crucial to assess its suitability for various product applications, thereby impacting economic outcomes associated with algal remediation operations. PFAS are persistent organic pollutants which are prevalent in the environment and pose risks to human health. Testing for PFAS in algal biomass is essential to ensure that the biomass meets regulatory standards for safe product applications such as bioplastics and animal feed supplements. Therefore, PFAS testing determines the economic viability and value of algal biomass in remediation operations.

vii. Practical operational strategies for HRFAPs in rural communities: The findings from this research highlight the potential of HRFAPs as a viable and sustainable wastewater treatment technology for rural communities. These systems offer relatively simple operation and low maintenance, making them an affordable solution in areas with limited infrastructure. Key operational strategies, such as controlling stocking densities and HRTs, can be optimised to meet the specific needs of rural communities. A major challenge, however, is the inability to adjust HRT by varying influent flow rates, as the flow may be constant in rural settings. Instead, adjusting the pond depth provides a practical and flexible control mechanism, as it simultaneously changes HRT while maintaining a consistent daily flow rate. This adaptability allows the system to better accommodate variable influent nutrient loads, making it scalable for rural areas. Identifying an optimal combination of HRT, stocking density and harvest frequencies that ensures compliant wastewater treatment across seasons, with only minor adjustments, would further simplify the operation of the system. While further research is

needed to address challenges such as variability in treatment effectiveness and system scaling, overcoming these obstacles could lead to widespread adoption of HRFAPs, significantly improving wastewater treatment in rural communities and contributing to better environmental and public health outcomes.

6.3 Future research

Continued research into the bioremediation potential of various algal species remains important for advancing this field and developing robust, adaptable, and high-performing wastewater treatment solutions. While key genera such as *Oedogonium* sp. and *Klebsormidium* sp. show promise, there is a need to explore a wider range of species for their bioremediation capabilities. The screening protocol developed within this thesis can be utilised to evaluate the bioremediation potential of different cultivars across diverse seasonal conditions. This approach is critical as species that perform well under specific light and temperature profiles can potentially be applied to other locations with similar environmental conditions. Given the variability in performance among cultivars, conducting the screening protocol in new locations is recommended to increase the number of species identified with superior traits and enhance the probability of identifying an even more effective cultivar. Additionally, the composition of the wastewater, especially industrial inputs, significantly influences bioremediation efficacy. This highlights the importance of applying the screening protocol in new locations to improve the selection of algal species based on the specific contaminants present in the wastewater to optimise treatment outcomes.

The optimisation of operational parameters such as HRT, stocking density, and harvest frequency has proven highly effective in enhancing bioremediation performance and increasing biomass productivity in HRFAPs. These findings highlight the importance of systematically optimising operational parameters in future research, particularly across different seasons, as environmental conditions can significantly impact bioremediation outcomes. Among the parameters assessed, HRT was determined to be the most influential, primarily due to its direct impact on nutrient availability within the pond system. Given the dynamic environmental conditions throughout the year, seasonal adjustments to HRT are essential to consistently achieve maximum bioremediation outcomes. Therefore, ongoing research should prioritise the refinement of operational parameters, with a specific focus on optimising HRT, to enhance the overall performance of HRFAP systems.

To advance the use of HRFAPs, researchers should aim to assess the feasibility of HRFAPs as an alternative to oxidation pond systems. Seasonal bioremediation performance and productivity data can inform crude modelling of year-round viability, helping to understand HRFAP efficiency and sustainability under different seasonal conditions. Estimating costs is crucial for large-scale implementation, which can be based on operational parameters outlined in Chapter 4 or through undertaking operational parameter trials. Identifying the optimal HRT and stocking density will determine pond size requirements across seasons, aiding accurate scaling and cost estimation. This includes factors such as nutrient removal and growth rates, which influence water quality and biomass generation. With this data, it is possible to predict the overall productivity and economic viability of HRFAPs as a large-scale alternative to traditional oxidation ponds.

Future research should focus on conducting year-long studies to measure the effectiveness of algal bioremediation for treating primary municipal wastewater in large-scale HRFAPs. While short-term and seasonal experiments provide valuable insights into algal system performance under specific conditions, they may not capture the full range of environmental variables and operational challenges encountered throughout an entire year (Valero-Rodriguez et al., 2020). Seasonal fluctuations in temperature, light availability, and nutrient inputs can significantly impact the growth and bioremediation efficiency of algae (Sutherland et al., 2014a). Longer experimental durations are crucial as they enable a more thorough evaluation of species performance (Cole et al., 2016b), and therefore more accurate estimates of expected biomass productivities and nutrient removal rates. A limitation observed in Chapter 3 was the incomplete determination of cultivar dominance due to the relatively short experimental period. Therefore, continuous long-term studies are essential to understand how algal cultivars perform across various seasons and environmental conditions. Furthermore, transitioning from small-scale experimental systems to large-scale HRFAPs is critical for assessing the practical feasibility of algal bioremediation technologies (Sutherland et al., 2018). Small-scale studies are useful for initial screenings and controlled investigations of specific variables (e.g. light, temperature) (Valero-Rodriguez et al., 2020). However, large-scale studies are necessary to identify system challenges, including variations in cultivar performance, wastewater composition, operational stability, and system maintenance. Therefore, conducting long-term and large-scale experiments is crucial to demonstrate the

feasibility and potential integration of HRFAPs into municipal and industrial applications (Young et al., 2017; Arashiro et al., 2019).

Exploring the use of algal biomass for various product applications presents a promising area for future research. By integrating product development with biomass quality analyses, researchers can ensure that the end products meet safety standards. For example, by focusing on specific product applications such as cellulose or bio-stimulants (Vucko et al., 2021; Jayasooriya et al., 2024), experiments could be conducted using real wastewater supplemented with priority contaminants. This approach allows for the quantification of contaminant accumulation in the biomass and their potential transfer into the final products (Salehipour-Bavarsad et al., 2024). Such data would enable accurate modelling of product safety based on specific water quality and algal biomass characteristics, ensuring the safe and effective use of algal derived products.

The effectiveness of PFAS removal is species-specific, with certain algal species demonstrating notable capabilities in PFAS uptake and degradation (Marchetto et al., 2021). However, as demonstrated in Chapter 5, the removal rates of PFAS and PFAS precursors using *K. flaccidum* varied. Future studies could enhance the screening protocol by integrating PFAS bioremediation as an additional step to systematically measure the capacity of algal species in PFAS removal. Evaluating the resilience and robustness of algal cultivars in wastewater contaminated with PFAS is crucial for assessing their ability to thrive under such conditions. It is essential to measure both productivity and survival rates across a range of wastewater samples collected at different times and/or from various WWTPs. This approach ensures a comprehensive evaluation of the tolerance and growth potential of cultivars in environments with varying PFAS concentrations. While algal bioremediation traditionally focuses on oxygen supply and nutrient removal, demonstrating effective PFAS removal capability would strengthen the applicability of algal-based technologies in municipal wastewater treatment.

6.4 Concluding remarks

Despite significant progress in filamentous algae bioremediation, several knowledge gaps persist, particularly regarding the identification and performance of algal cultivars in real-world conditions. This thesis has contributed to addressing these knowledge gaps. The

screening protocol introduced in Chapter 2 represents a novel method for identifying robust algal cultivars and comparing their performance. The findings highlight that not all cultivars are suitable for primary wastewater treatment in outdoor HRFAP systems, as evidenced by significant variations in growth and bioremediation performance under ambient and extreme seasonal conditions. Furthermore, monoculture experiments and assessment of competitive dominance in outdoor HRFAPs have identified specific cultivars capable of effectively treating nutrients in primary municipal wastewater. Optimising operational parameters such as HRT and stocking density can further enhance bioremediation performance. Overall, this thesis identified *K. flaccidum* as a promising cultivar capable of bioremediating primary wastewater under varying seasonal conditions. While *K. flaccidum* only achieved partial reductions in PFAS within primary wastewater, its biomass remained free of PFAS, making it highly suitable for product applications.

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