Filamentous Algae as a Nutrient Scrubber for Agricultural Drainage Treatment

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"It's our responsibility to do everything within our power to create a planet that provides a home not just for us, but for all life on Earth."

– Sir David Attenborough

Preface

The main body of this thesis comprises six chapters. Chapters 2, 3, 4, and 5 of this thesis have been published in peer-reviewed scientific journals. I was responsible for the field work, laboratory work, data analysis and research writing, unless otherwise referenced. The information in this thesis was produced from my own ideas, and all work presented was carried out under the guidance and supervision of Dr Rebecca Lawton from the University of Waikato and Dr Rupert Craggs from the National Institute of Water and Atmospheric Research Ltd. (NIWA).

Chapter 2 has been published in Journal of Applied Phycology:
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Chapter 5 has been published in Agricultural Water Management:
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Abstract

Filamentous algae nutrient scrubbers (FANS) have potential as a novel on-farm treatment system to remove and recover diffuse nutrients from agricultural drainage water. FANS use attached filamentous algae that grow and assimilate nutrients from the water which are removed when algal biomass is harvested for beneficial use (e.g. biofertilizer, animal feed supplement). This thesis investigated the attachment abilities, growth rates, biomass productivity and nutrient removal rates of four locally isolated filamentous algae species with the potential for use on FANS to bioremediate nutrients in agricultural drainage water.

To date, a standardised method to assess the productivity and nutrient removal of attached filamentous algae and identify target species for FANS cultivation has not been developed. Therefore, a reproducible bioassay was developed to rapidly assess the ability of four filamentous algae species to attach and grow, remove nutrients, and produce harvestable algal biomass using microscale FANS (µFANS) under controlled indoor conditions. This bioassay included the development of a method for rapid seeding by 'hooking" algal filaments onto the FANS liner to provide initial physical attachment. Within 14 days, a "lawn" of the seeded algae had established and the "hooked" biomass had attached biologically onto the FANS liner. *Oedogonium* sp. Was identified as the best performing species overall, with the strongest holdfast attachment, high biomass productivity and high nutrient removal rates.

As the growth and bioremediation performance of filamentous algae can vary with seasonal changes in environmental conditions, the performance of four filamentous algae species was assessed under summer and winter ambient outdoor conditions on mesocosm-scale FANS. *Oedogonium* sp. Had the highest biomass productivity and nitrate removal rates under both seasons, confirming it as the best performing species. In addition, *Oedogonium* sp. FANS had the lowest contamination in percentage cover of non-target species compared to the other three species under both summer and winter conditions. These results demonstrate that *Oedogonium* sp. Has a higher tolerance than the other three species to summer and winter ambient temperature and light variation, enabling *Oedogonium* sp. To maintain dominance on the floway for a longer period. For these reasons, *Oedogonium* sp. Was identified as a promising target for year-round cultivation on FANS.

FANS operating parameters can strongly influence algal biomass productivity and nutrient removal. Therefore, the effects of the influent flow rate, harvesting frequency and initial standing crop on unialgal *Oedogonium* sp. FANS biomass productivity and nutrient removal performance were assessed under controlled environmental conditions to remove any variability in performance due to variations in ambient conditions. Results suggested that an initial standing crop of 70-80 g DW m^{-2}, harvesting frequency of four days and influent flow rate of 1 L min^{-1} (16.7 L min^{-1} .m width) were optimal for *Oedogonium* sp. Cultivated on FANS to maximize biomass productivity and nutrient removal under controlled laboratory conditions. These results contribute to understanding the impacts of operating parameters on optimizing unialgal *Oedogonium* sp. FANS biomass production and nutrient removal performance.

Despite their potential influence on performance, the effects of FANS seeding methods and species composition have received little attention. Therefore, the effects of seeding method (controlled seeding vs. natural establishment) and seeded species composition (single species vs. mixed species algal assemblages) were investigated in FANS treating agricultural drainage on a dairy farm over seven months. FANS seeded using controlled seeding established biomass five times faster (10 days) than FANS left to establish naturally (7 weeks). Overall, the seeding method and species composition of algae seeded on FANS did not significantly affect biomass productivity and nutrient removal performance. However, FANS seeded with a single species (*Oedogonium* sp.) had a lower contamination rate in terms of percentage coverage of non-target species than FANS seeded with a mixed species assemblage. These results demonstrate that controlled seeding and cultivation of a single target filamentous algae species can help to maintain a higher abundance of a target species on FANS over a longer period of time, enabling the recovery of high-quality biomass with low variation in algae species composition.

This thesis has demonstrated that FANS can be successfully used to treat nutrients in agricultural drainage water, and therefore could be implemented as a novel tool to assist in mitigating diffuse pollution in New Zealand. The findings can be used to provide farmers and land managers with a clearer understanding of how FANS can be used to remove nutrients from agricultural drainage water to meet water quality objectives while minimizing the impacts on farming activities.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

Word/Acronym/ Abbreviation	Full description/translation
ACT	Algae Contact Time- the average time of algae in contact with the flowing water
ADP	Adenosine diphosphate is an important organic compound in metabolism and is essential to the flow of energy in living cells
Agricultural Drainage Water	A non-point (diffuse) source of polluted water that comes from surface ditches and subsurface drain pipes (tiles) consisting of high quantities of nutrients, sediments, organic particulates, and legumes
ANOVA	Analysis of variance- Statistical models and their associated estimation procedures used to analyze the differences among means
ATP	Adenosine triphosphate - known in biochemistry as the "molecular currency" of intracellular energy transfer. ATP can store and transport chemical energy within cells
ATS	Algal Turf Scrubbers
Bioremediation	A process used to treat contaminated media, including water, soil and subsurface material, by altering environmental conditions to stimulate the growth of microorganisms and degrade the target pollutants
BOD	Biochemical Oxygen Demand
CO_2	Carbon dioxide
Denitrification	A process where nitrate-N is converted to inert N_2 gas by microbes
Denitrifying-Bioreactor	A saturated permeable bed of carbonaceous solids which water passes to promote microbial nitrate removal
Diffuse Nutrients	Nonpoint source nutrient pollution is transferred to water bodies through various diffuse processes
DIN	Dissolved inorganic nitrogen- the readily available fraction that can stimulate problem growths of algae and water plants
DO	Dissolved Oxygen

DRP	Dissolved reactive phosphorus- the readily available fraction that can stimulate problem growths of algae and water plants
DW	Dried Weight
Eutrophication	The body of water becomes overly enriched with nutrients and minerals, which induce excessive growth of algae
FANS	Filamentous Algae Nutrient Scrubbers
FW	Fresh Weight
HDPE	High density polyethylene
HRT	Hydraulic retention time- the average time of water flowing through or retained within a treatment system
NH_3	Ammonia
NH_4	Ammonium
Nitrification	A process where ammonium-N is converted to nitrite-N and nitrate-N by microbes
NIWA	National Institute of Water and Atmospheric Research
NO_2-N	Nitrite
NO_3-N	Nitrate
NPS-FM	National Policy Statement for Freshwater Management
Periphyton	Collective term for the algae, cyanobacteria, heterotrophic microbes, and detritus that grows attached to submerged surfaces or the base of streams and rivers
RMA	Resource Management Act 1991 - the New Zealand law that governs all management of land and water resources
TN	Total Nitrogen
TP	Total Phosphorus
UNEP	United Nations Environment Programme
UV	Ultraviolet

Chapter 1
General Introduction

1.1 Agricultural Drainage

1.1.1 Water Quality Degradation

Water quality degradation affects marine and freshwater ecosystems worldwide (Dudgeon et al., 2006; Smith et al., 2006; Diggles, 2013; Duprey et al., 2016). Worsening water quality leads to a decline in ecosystem health (Rapport et al., 1998; Smith et al., 2006; Brooks et al., 2016), loss of biodiversity (Dudgeon et al., 2006; Duprey et al., 2016), and a reduction in the aesthetic quality and recreational value of rivers and streams (Marsh & Mkwara, 2013). A range of factors cause water quality degradation, including urban development (Yin et al., 2005; Xian et al., 2007), deforestation (Kuisma & Haanperä; Jawan & Sumin, 2012), agricultural activities (Ripa et al., 2006; Mateo-Sagasta & Burke, 2010; Schoumans et al., 2014; Scarsbrook & Melland, 2015a; Duncan, 2017), marine dumping (Wang et al., 2011; Yang et al., 2012), industrial discharges (Rajaram & Das, 2008; Kanu & Achi, 2011), and radioactive waste discharges (Durham & Joshi, 1980).

Water quality degradation is also affecting New Zealand's freshwater ecosystems. Nutrients, sediments, and pathogens are the three water contaminants of most concern in New Zealand (Wright, 2012). However, nutrient pollution, specifically excessive amounts of nitrogen and phosphorus, is the primary driver of degrading freshwater quality (Davies-Colley, 2009; Howard-Williams et al., 2010; Abell et al., 2011; Davies-Colley, 2013; Ballantine & Davies-Colley, 2014). Over the last three decades, increasing nitrogen and phosphorus loads have impacted New Zealand's lakes, rivers, and streams (White, 1983; Abell et al., 2011). Between 2013 and 2017, 58 % and 59 % of river length in New Zealand had modelled concentrations of total nitrogen (TN) and total phosphorus (TP), respectively that exceeded guideline values, and 49 % and 32 % of river length in New Zealand had modelled concentrations of nitrate (NO_3-N) and ammonium (NH_4-N), respectively that exceeded guideline values (Ministry for the Environment, 2019). Similarly, between 2009 and 2013, 36%

(24/67 sites) of monitored lake sites in New Zealand were categorized as eutrophic or super eutrophic (Ministry for the Environment, 2019).

Agriculture, horticulture, forestry, urban, and transportation land uses are associated with increased nutrient loads in New Zealand freshwater ecosystems (Abell et al., 2011; Davies-Colley, 2013). Pastoral agriculture contributes the most TN and TP to freshwater bodies (Quinn et al., 2009; Abell et al., 2011; Davies-Colley, 2013; McDowell et al., 2013; Ballantine & Davies-Colley, 2014; Scarsbrook & Melland, 2015a), and about 70% of the TN load to the coast originates from pastoral land uses (Elliott et al., 2005). Pastoral agriculture contributes nutrients through runoff and soil erosion that mobilizes TN and TP from the deposition of livestock manure and urine (Howard-Williams et al., 2010; Davies-Colley, 2013). Dairy farming is the most significant contributor of diffuse nutrients compared to other pastoral agricultural land uses, with TN losses from dairy farmland being four times higher than losses from other types of pastoral agriculture (Quinn et al., 2009). Horticulture is the second-highest contributor of nutrient pollutants after pastoral agriculture, followed by forestry, urban and transport land uses, which all release relatively small loads of TN and TP to water bodies (Davies-Colley, 2013). New Zealand horticulture involves vegetable and fruit cultivation (De Silva & Forbes, 2016) and can contribute to increasing TN and TP loads when fertilizer is applied in excess of plant requirements.

1.1.2 Agricultural Drainage Water

In the agriculture sector, drainage systems are typically used to manage farm surface runoff and subsurface water (Randall & Goss, 2001). Agricultural drainage water is a non-point (diffuse) source of polluted water that comes from surface ditches and subsurface drain pipes (tiles) consisting of high quantities of nutrients, sediments and organic particulates (Smith, 1992; Nguyen & Sukias, 2002). Dissolved inorganic nitrogen (DIN), organic nitrogen, dissolved reactive phosphate (DRP), and particulate phosphate are the main nutrients in agricultural drainage (Anderson & Cabana, 2005; Heathwaite et al., 2005a). These nutrients are transported through surface runoff or soil erosion into agricultural drainage, eventually reaching the rivers and streams (Heathwaite & Dils, 2000a; Anderson & Cabana, 2005; Heathwaite et al., 2005a). Intensive livestock production can result in a high runoff of dissolved inorganic nitrate (NO_3-N), nitrite (NO_2-N), and ammonium (NH_4-N) originating from livestock wastes (Randall & Goss, 2001; Heathwaite et al., 2005a; Tanner & Kadlec, 2013). Typically,

NO_3-N is the primary form of nitrogen transported to receiving water bodies (Randall & Goss, 2001). Livestock manure and over application of fertilizer contributes to the increased concentration of phosphorus in receiving water bodies through soil erosion. Particulate phosphorus is a common component in soils and sediments that binds strongly to soil particles. Once the capacity of the soil to store phosphorus is exceeded, it will leach with surface runoff to receiving water bodies in the form of dissolved reactive phosphate (DRP or orthophosphate) (Nguyen & Sukias, 2002).

Diffuse nutrients from agricultural drainage have a range of negative impacts on freshwater ecosystems. High concentrations of dissolved nitrogen and phosphorus in rivers and streams can lead to eutrophication and promote nuisance plant growth and algal blooms (Davies-Colley, 2013). Under eutrophic conditions, dissolved oxygen (DO) in rivers or streams becomes depleted due to high rates of oxygen consumption by decomposing bacteria following the die off of algal blooms (Sallenave, 2012; Paerl & Otten, 2013). This may lead to hypoxia and harm the aquatic ecosystem (Anderson et al., 2008; Abell et al., 2011; Paerl & Otten, 2013). Deterioration of water quality is also a threat to humans when water becomes unsafe for consumption or recreational activities (UNEP, 2016). For example, water with nitrate concentrations above 10 mg L^{-1} is considered unsafe for human consumption and may lead to methemoglobinemia, which is also known as a blue-baby syndrome (Avery, 1999). There is also recent evidence that other health impacts occur with much lower nitrate concentration in drinking water, including the risk of bowel cancer at nitrate concentration of 0.87 mg L^{-1} and above (Schullehner et al., 2018; Richards et al., 2022), thyroid disease, neural tube defects, and risk of premature birth was found doubled at nitrate concentration of 5 mg L^{-1} and above (Sherris et al., 2021).

1.1.3 Management and Treatment of Nutrients in Agricultural Drainage Water

Two main pieces of legislation govern nutrients in agricultural drainage water. The Resource Management Act (RMA) is the main New Zealand government legislation relating to the management of land, water, air, and coastal environments (New Zealand Government, 2022). Input of diffuse nutrients from agricultural drainage to surface waters is identified in the RMA as one of the key water quality concerns in New Zealand. Diffuse pollution is more difficult to control than point source pollution because it originates from many dispersed

origins and generally results from land runoff, precipitation, atmospheric deposition and drainage (Ma et al., 2011). The National Policy Statement for Freshwater Management (NPS-FM) took effect on 1st July 2011. The main aim of the NPS-FM is to set out objectives and policies that direct local governments to manage water in an integrated and sustainable manner. Underlying NPS-FM 2014 policies, all unitary and regional councils must set objectives around the water quality of rivers and lakes for surface and groundwater in their region by the end of the year 2030 and establish an effective range of standards that can be used to determine whether objectives are being met (Ministry for the Environment, 2017). Among the compulsory standards that all councils must monitor are the concentration of nutrients (primarily TN and TP) and periphyton biomass in water bodies (Ministry for the Environment, 2017). Implementing the NPS-FM means that diffuse nutrient input to freshwaters from agricultural drainage will need to be managed and reduced to meet required standards.

Agricultural drainage can be managed on farms through lateral and/or in-stream methods. The lateral drainage management involves the construction of riparian buffer zones across farm areas (Vymazal & Kröpfelová, 2009; Mateo-Sagasta et al., 2017). In-stream methods, such as wetlands and denitrification bioreactors, utilise nutrient uptake by plants and microbes to reduce nutrient concentrations (Collins et al., 2009; Vymazal & Březinová, 2015). Wetlands promote nitrification and denitrification (Vymazal & Kröpfelová, 2009; Nivala et al., 2013) and can significantly reduce nutrient concentrations in water. For example, a constructed horizontal-flow wetland reduced TN by 49-58% from an inflow TN concentration of 42 g N m^{-3} and TP by 36-65% from an inflow TP concentration of 5 g P m^{-3} with denitrification rates of 3-12 g N m^{-3} d^{-1} (Tanner et al., 2012). Denitrification bioreactors use carbonaceous solids (e.g. woodchips, compost, tree mulch, manure, coconut husk and sawdust) to promote reduction of NO_3-N to gaseous N_2 by denitrifying bacteria, which permanently removes nitrogen from water (Robertson & Merkley, 2009; Schipper et al., 2010). For instance, denitrification bioreactors had a denitrification rate of 2-22 g N m^{-3} d^{-1} and reduced nitrate concentrations by up to 60% from an inflow nitrate concentration of 53 g N m^{-3} (Schipper et al., 2010). In New Zealand, riparian buffer zones, wetlands and denitrification bioreactors have all been used to manage and treat agricultural drainage (Howard-Williams et al., 2010; Tanner & Kadlec, 2013; Goeller et al., 2019; Rivas et al., 2019), however, these methods are not yet widely employed by farms in New Zealand as treatment of diffuse nutrients is considered minor and economically unimportant compared to treatment of point source discharges which are more easily regulated and cleaned up (McDowell, 2009). In addition, methods such as

wetlands and bioreactors are not always practical to implement as they require a large land areas to operate effectively (Muñoz et al., 2018).

Filamentous algae nutrient scrubbers (FANS) are an alternative method to existing on-farm treatment systems for reducing nutrient loads in agricultural drainage. Research indicates that filamentous algae treatment systems would require half the land area compared to that required for wetland treatment systems to achieve the same nitrogen removal rate (Sutherland & Craggs, 2017). Moreover, filamentous algae treatment systems not only remove the diffuse nutrients from the agricultural drainage, but also store these nutrients in algal biomass. The algal biomass can then be regularly harvested and converted into products, thereby reusing the nutrients (Smith, 2014; Lawton et al., 2017). Recovering and reusing nutrients from waste is crucial for a sustainable nutrient cycle (Jhansi & Mishra, 2013; Carey et al., 2016). Nutrient recovery can help to reduce dependence on limited resources and the high energy processes required to produce fertilizers (Cordell et al., 2011; Dawson & Hilton, 2011; Udert & Wächter, 2012). In the case of agricultural nutrient dependency, nutrient recovery and reuse can reduce or eliminate the use of imported nutrients (Gourley et al., 2007; Smit et al., 2009; Cordell et al., 2011; Dawson & Hilton, 2011), and create a revenue source from recovered nutrients to offset the costs of nutrient removal (Cordell et al., 2011; Drechsel et al., 2011). Additionally, nutrient recovery can provide a sustainable way to meet global nutrient demand, which is increasing due to an expanding human population (Childers et al., 2011; Metson et al., 2012).

1.2 Filamentous Algae Systems for Nutrient Treatment

Filamentous algae can be cultivated in a range of systems, including free-floating in suspension (Cole et al., 2014; Lawton et al., 2021), attached to a solid substrate as applied in algal turf scrubbers (ATS) (Craggs, 2001; Kebede-Westhead et al., 2006; Adey et al., 2013; Kangas & Mulbry, 2014; D'Aiuto et al., 2015) or within net-bags in suspension (Osterling & Pihl, 2001). Filamentous algae systems can remove significant quantities of nutrients from water bodies. In Australia, free-floating filamentous algae in suspension systems have been successfully used to treat nutrients from municipal wastewater (Cole et al., 2016; Neveux et al., 2016) and fish farm effluent (Cole et al., 2015). Algal turf scrubbers (ATS) are the most common attached filamentous algae systems. These systems have been widely studied and applied at varying scales to treat nutrients in the United States for a range of surface waters,

including a marine algae system in St. Croix in the Caribbean (Figure 1.1) (Adey et al., 2011), a mesohaline algae system in the Chesapeake Bay region (Figure 1.2) (Adey et al., 2013) and freshwater algal systems in California (Figure 1.3) (Craggs et al., 1996a), Maryland (Mulbry et al., 2008a; Mulbry et al., 2008b; Blersch et al., 2013), and Florida (Figure 1.4) (Hydromentia, 2005). Nutrient removal rates and biomass productivities of attached filamentous algal systems vary considerably among studies. For example, an attached filamentous algae system reduced nitrogen and phosphorus concentrations in tertiary sewage at a rate of 1.0 g N m^{-2} d^{-1} and 0.7 g P. m^{-2} d^{-1}, respectively (Craggs et al., 1996a), while a pilot-scale attached filamentous algae agricultural drainage treatment system reported nitrogen and phosphorus removal rates at 0.10 g N m^{-2} d^{-1}, 0.03 g P m^{-2} d^{-1} (Kangas & Mulbry, 2014), and a pilot-scale attached filamentous algae system removed about 16 % phosphate from 0.03 mg PO_4-P L^{-1} and 49 % nitrate from 0.40 mg NO_3-N L^{-1} from citrus farm runoff (D'Aiuto et al., 2015).

Figure 1.1: An early-stage of algal turf growth in coral reef environments in the eastern Caribbean Sea during the 1970s – 1980s (Adey et al., 2011)

Figure 1.2: Fiberglass trough ATS floways on the Great Wicomico River off the Central Chesapeake Bay. The left floway is 0.61 m wide x 15.2 m long with a 1% slope; the right floway is 0.61 m wide x 24.4 m long with a 2% slope (after Adey et al., 2013)

Figure 1.3: The large-scale ATS at Patterson, California, for secondary sewage treatment from 1994-1996 (after Adey et al., 2011)

Figure 1.4: A large-scale ATS treating up to 1 million gallons per day of agricultural drainage water operated by HydroMentia, Inc near Lake Okeechobee, in central Florida (after Adey et al., 2011)

1.2.1 Algae Turf Scrubbers (ATS)

ATS are an ecologically engineered, artificial stream that grow attached filamentous algae and associated periphyton to treat nutrients in water (Craggs et al., 1996a; Craggs et al., 1996b; Adey et al., 2011; Adey et al., 2013). ATS systems consist of a shallow, gently sloped basin or trough (floway) with an overlying screen to which the algal " turf " attaches (Figure 1.5) (Craggs, 2003; Adey et al., 2011). Nutrient-rich water is pumped onto the top of the floway and runs down its length. Nutrients are mainly removed by assimilation into algal biomass, producing dissolved oxygen through photosynthesis to drive aerobic bacterial breakdown of organic matter (Adey et al., 2011).

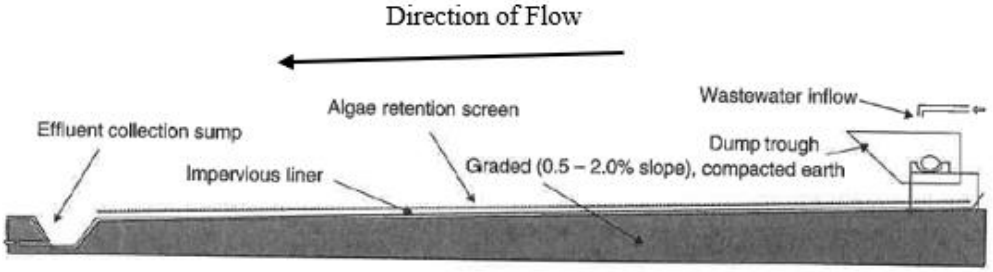

Figure 1.5: Schematic diagram of filamentous algae treatment system (after Craggs, 2003)

ATS technology was first developed in the early 1980s by Adey and associates at the Smithsonian Institution, Washington DC (Adey et al., 2013). The first ATS systems were built to control water quality in low nutrient (300 mg N m^{-3} and 30 mg P m^{-3}), low light (1500 µmol m^{-2} s^{-1}) marine coral reef microcosm and mesocosm ecosystem simulation models (Adey, 1983; Adey & Loveland, 2011; Adey et al., 2013). High algal biomass productivity was reported in these systems, ranging from 5 to 20 g m^{-2} d^{-1} (dry weight) (Adey et al., 2011; Adey & Loveland, 2011). In the late 1980s, ATS systems were trialled to treat raw sewage and chicken manure in terms of nitrogen, phosphorus, and biological oxygen demand (BOD) removal (Adey & Loveland, 2007). From the early 1990s, the ATS system was scaled up to treat wastewater and finfish aquaculture effluent (Adey et al., 2011). In 1991, a pilot-scale ATS (15 meters long and 0.8 meters wide) was constructed at a Florida Everglades sugar cane farm to treat drainage water and was tested over six months for nutrient removal (Adey et al., 1993). Biomass productivity ranged from 33 to 39 g m^{-2} d^{-1} (dry weight) with corresponding phosphorus removal of 0.10 to 0.14 g m^{-2} d^{-1} (Adey et al., 2011). In the mid-1990s, a large-scale (0.1-

hectare) ATS floway was built at Patterson, California to treat secondary sewage effluent (Craggs et al., 1996a). This ATS system could treat a wastewater flow of up to 950 m^3 d^{-1} and had annual mean biomass productivity of 35 g m^{-2} d^{-1} with corresponding nitrogen and phosphorus removal of 1.1 ± 0.5 and 0.7 ± 0.2 g m^{-2} d^{-1} respectively (Craggs et al., 1996a; Adey et al., 2011). In the 2000s, ATS technology was further scaled-up by HydroMentia Inc. as a nutrient pollution treatment technology (Adey et al., 2013). A large-scale 1-hectare ATS system was constructed at Lake Okeechobee in Florida to treat up to 3786 m^3 d^{-1} of agricultural drainage water, primarily from cattle production (Adey et al., 2011).

So far, the use of ATS systems has been successfully demonstrated in the United States. There is no comparable attached filamentous algae system that has been used for nutrient removal in New Zealand. However, based on the successful treatment of nutrients by ATS systems in the United States, attached filamentous algae treatment systems could be used to treat agricultural drainage water in New Zealand.

1.2.2 Algae Bioremediation and Nutrient Removal Mechanisms

Filamentous algae treatment systems remove nutrients through a combination of algal assimilation (including luxury uptake), adsorption, filtration, volatilization, and precipitation (Figure 1.6) (Craggs et al., 1996b; Craggs, 2001; Sandefur et al., 2011a; Sutherland & Craggs, 2017). Nutrient removal through algal assimilation is achieved by a light-driven biochemical pathway (Powell et al., 2008). Assimilation of nutrients happens when higher nutrient concentrations are found outside algal cells. Nutrient ions are transferred from the outside to the inside of the algal cell and stored as biomass (Aksnes & Egge, 1991). Assimilated nitrogen is converted to nucleic acids and protein stored in the form of algal biomass (Powell et al., 2008; Cai et al., 2013). Ammonium (NH_4) is the most preferred form of nitrogen for transport through the algal cells as it requires less energy for the assimilation process, followed by nitrite (NO_2-N), nitrate (NO_3-N), and finally, the organic forms of nitrogen (Cai et al., 2013). In the case of phosphorus, algae will assimilate dissolved reactive phosphate (DRP) (Cai et al., 2013). The DRP is transported into algal cell nucleotides to synthesise ribosomal RNA (Cai et al., 2013). Sufficient assimilation of nitrogen by algae will enable the synthesis of protein that allows phosphorus assimilation (Cai et al., 2013). Both nitrogen and phosphorus are assimilated

as part of normal algal growth. However, under certain conditions, algae can take up additional nutrients and store them in the biomass, a process known as luxury nutrient uptake (Powell et al., 2008; Powell et al., 2009).

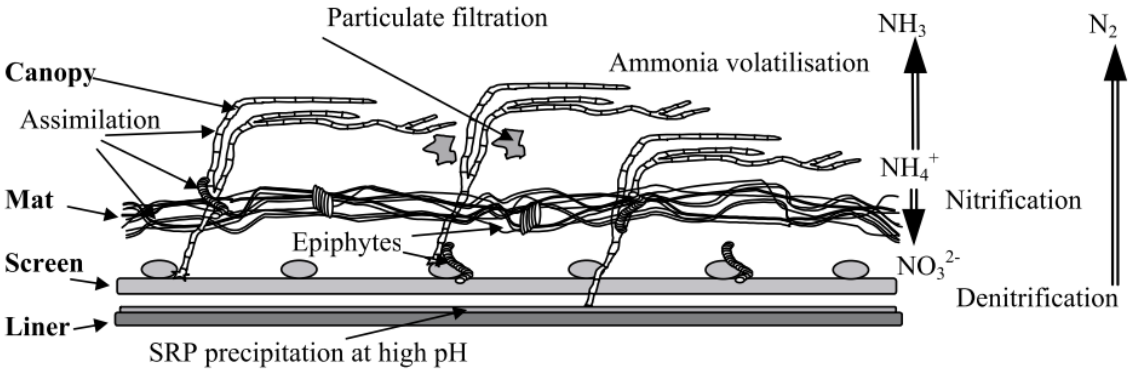

Figure 1.6: Schematic diagram of nutrient removal mechanisms by attached filamentous algae treatment system (after Craggs, 2003)

Filamentous algae systems are also capable of removing nutrients through particulate filtration. Significant amounts of nitrogen, phosphorus and suspended solids (SS) associated with particulate matter or solids will be physically filtered or adsorbed to the attached filamentous algae (Craggs, 2001). These adsorbed nutrients and solids will accumulate together with algal biomass and are removed when the algae biomass is harvested.

Ammonia volatilization and phosphorus precipitation are other nutrient removal mechanisms that occur in filamentous algal treatment systems. Typically, ammonia volatilization or stripping occurs when the pH of the water exceeds 9 (Delgadillo-Mirquez et al., 2016) and the kinetic equilibrium of ammoniacal-N shifts between NH_4^+ to ammonia (NH_3), leading to NH_3 gas release (Martınez et al., 2000; Zimmo et al., 2003). DRP precipitation occurs when the water pH increases above 8.9 (Craggs et al., 1996b; Sutherland & Craggs, 2017). Most phosphorus is removed in filamentous algae treatment systems through algal assimilation or precipitation of DRP (Craggs, 2001).

1.3 Filamentous Algae Species and Characteristics

Filamentous algae are categorized as visible chains, threads or filaments of algae cells that can rapidly regenerate from their basal cells (Adey & Hackney, 1989). Filamentous algae either grow floating on the water surface, attached to the bottom of shallow waterbodies, or attached to objects (rocks or aquatic plants) in the water or at the water's edge (Jensen, 1997; Kiirikki & Lehvo, 1997; Pihl et al., 1999).

The dominance and distribution of specific algal species over other species in a particular habitat are strongly dependent on algal ecological preferences such as nutrient availability (Cambra & Aboal, 1992; Pihl et al., 1999; McManus & Polsenberg, 2004; de Paula Silva et al., 2008; Sutherland & Craggs, 2017), hydrology (Johansson, 1982; Cambra & Aboal, 1992; Pihl et al., 1999; Ahn et al., 2013), water pH and salinity (Cambra & Aboal, 1992; de Paula Silva et al., 2008; Griffiths & Harrison, 2009; Lawton et al., 2015a), local climate (temperature, light, and humidity) (Griffiths & Harrison, 2009; Lawton et al., 2013b; Lawton et al., 2014; Singh & Singh, 2015; Phillips et al., 2016; Mata et al., 2017), season (Pihl et al., 1999; Craggs, 2001; Sutherland & Craggs, 2017), and algal species competition or grazing pressure from invertebrates (McManus & Polsenberg, 2004). Even so, there are common species with global distributions and some locally rare species requiring special conditions for active growth (Cambra & Aboal, 1992). *Oedogonium*, *Cladophora*, *Rhizoclonium*, *Stigeoclonium*, *Spirogyra*, and *Ulothrix*. (Figure 1.7) are some examples of common filamentous algae genera that have a worldwide distribution (Johansson, 1982; Francke & Den Oude, 1983; Vymazal, 1988; Cambra & Aboal, 1992; Simons, 1994) which tends to be highly correlated with the nutrient content in water bodies (Pikosz & Messyasz, 2016). Nutrient enrichment is generally considered one of the most important factors affecting algal species dominance and distribution in water bodies (Littler & Littler, 1984; Pihl et al., 1999; Thacker et al., 2001; Ahn et al., 2013). However, under conditions without nutrient limitation, algal species dominance and distribution are primarily influenced by water velocity (Pihl et al., 1999; Ahn et al., 2013). Most filamentous algae are found in slow-flowing or stagnant water as only a few species are specialized to grow in fast-flowing waters (Johansson, 1982; Skinner & Entwisle, 2004). Filamentous algae usually bloom and have rapid growth during warm summer conditions when

low rainfall (low water flow rates) and more sunlight (high light intensities) create an environment where they can thrive (Pihl et al., 1999; Craggs, 2001; Phlips et al., 2007).

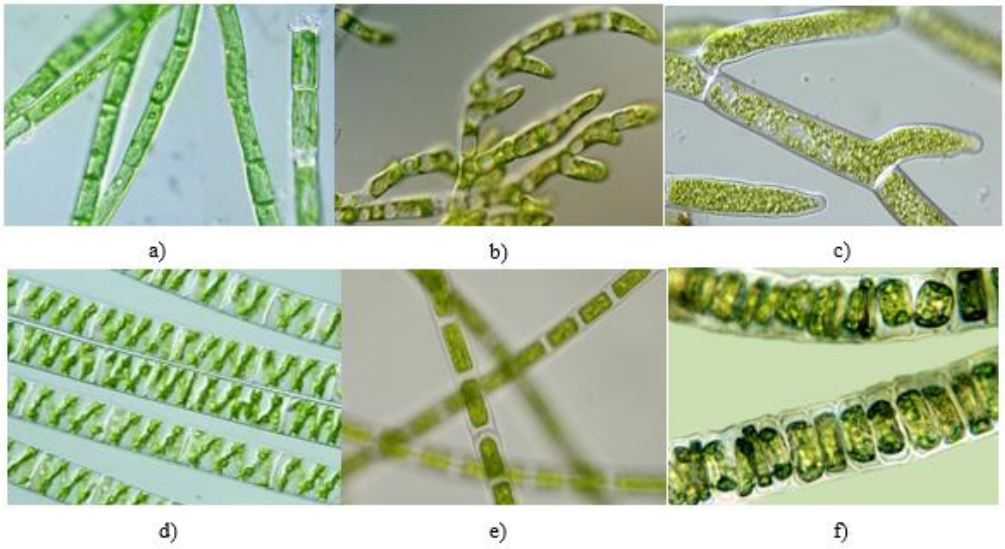

Figure 1.7: Examples of common filamentous algae genera with worldwide distribution (a) Oedogonium, (b) Stigeoclonium, (c) Cladophora, (d) Spirogyra, (e) Rhizoclonium and (f) Ulothrix (Source: AlgaeBase)

1.4 Selection of Filamentous Algae Species for Bioremediation

Freshwater filamentous algae have been widely used for bioremediation to treat a broad range of polluted waters. Common species found in or used for bioremediation systems include *Oedogonium* sp. (Sládečková et al., 1983b; Cole et al., 2015); *Rhizoclonium* sp. (Sládečková et al., 1983b; Mulbry et al., 2008a); *Cladophora* sp. (Craggs et al., 1996a; de Paula Silva et al., 2008; D'Aiuto et al., 2015); *Spirogyra* sp. (Vymazal, 1988; Kangas & Mulbry, 2014); *Stigeoclonium* sp. (Sládečková et al., 1983b; Craggs, 2001); *Ulotrix* sp. (Sládečková et al., 1983b; Vymazal, 1988; Craggs et al., 1996a; Craggs, 2001; Kangas & Mulbry, 2014) and *Microspora* sp. (Craggs et al., 1996a; Kangas & Mulbry, 2014). These filamentous algae species have been cultivated in either suspension systems or attached to a solid substrate to treat various waste streams that include river water (Vymazal, 1988), sewage (Sládečková et al., 1983b; Craggs et al., 1996a), agricultural drainage (Morgan et al., 2006b; Kangas & Mulbry, 2014; D'Aiuto et al., 2015), dairy manure effluent (Kebede-Westhead et al., 2004;

Mulbry et al., 2008a), swine manure effluent (Kebede-Westhead et al., 2006; Mulbry et al., 2008a) and fish farm wastewater (Cole et al., 2015).

Understanding algal species growth and nutrient uptake is essential for improving the performance of an algal-based bioremediation system (de Paula Silva et al., 2008; Lawton et al., 2013b; Singh & Singh, 2015). Algal growth rates and tolerance to environmental conditions are key characteristics for selecting suitable filamentous algae species for bioremediation (Griffiths & Harrison, 2009). Knowledge of species optimum conditions and growth requirements assists in promoting algal growth and nutrient uptake for bioremediation (de Paula Silva et al., 2008; Singh & Singh, 2015). Using common native algae species in a treatment system is beneficial since these algae are already adapted to local conditions and will be naturally seeded in the system (Simons, 1994; Grobbelaar, 2010; Wilkie et al., 2011).

Filamentous algae species that are widely distributed and abundant in New Zealand freshwaters and have potential for cultivation in agricultural drainage treatment systems include *Oedogonium* sp., *Cladophora* sp., *Rhizoclonium* sp., *Spirogyra* sp., *Microspora* sp., *Tribonema* sp., *Ulothrix* sp., and *Zygnema* sp. (Towns, 1981; Biggs, 1990; Winterbourn et al., 2000). Furthermore, *Cladophora* sp. (Morgan et al., 2006b), *Spirogyra* sp., *Microspora* sp., and *Ulothrix* sp. (Kangas & Mulbry, 2014) are species that have already been used to treat agricultural drainage water in other countries. These widely distributed species can tolerate a wide range of cultivation conditions, including nutrient load and temperature (Simons, 1994; Griffiths & Harrison, 2009; Lawton et al., 2017) and therefore are likely to be suitable candidates to target for cultivation in attached treatment systems such as FANS.

1.4.1 Factors Affecting Algae Biomass Productivity and Nutrient Removal

An understanding of factors affecting growth and biomass productivity is important because these factors are correlated to the efficacy of bioremediation as higher algal growth typically results in higher nutrient removal (Kebede-Westhead et al., 2006; Mulbry et al., 2008a; Cole et al., 2014). In outdoor cultivation systems, a combination of light, temperature, nutrients and turbulence can all affect algal nutrient uptake, growth and biomass productivity. Temperature, photoperiod, and solar irradiance are uncontrollable factors as outdoor operation depends on local climate, season, and day/night cycles (Craggs, 2001; Sandefur et al., 2011b). However, it is possible to enhance light exposure by optimizing parameters such as algae initial

standing crop, influent flow rate, water depth and harvesting frequency which affect the established standing crop of algae on the floway. Nutrient load and water turbulence are factors that can be controlled directly by manipulating operational parameters such as water flow rate (Blersch et al., 2013).

i. Light

Light is an important factor that drives photosynthesis (Bouterfas et al., 2002; Sutherland et al., 2015b). Under non-limiting nutrient conditions, the rate of photosynthesis increases with increasing light intensity until the maximum growth rate is achieved at the light saturation point (Bouterfas et al., 2002). In outdoor operation, light availability for algal photosynthesis can be enhanced by having an optimal water depth (Vymazal, 1988; Craggs et al., 1996a; Sutherland et al., 2020a). For example, algal growth in an outside algal floway dominated by *Cladophora* was higher at a shallow depth (0.06 m compared to 0.64 m) (Vymazal, 1988). A shallow water depth increases light penetration as less distance is needed for light to travel through the water to reach the algae (Kim et al., 2018). However, light penetration is closely related to water turbidity and higher turbidity reduces the amount of light for algae photosynthesis (Brown, 1984). A lower water depth also promotes rapid gas exchange, which enables higher algal photosynthetic rates (Vymazal, 1988; Geider, 2013). Besides water depth, light exposure can be increased through harvesting as this reduces the height of the standing algal crop, and thereby reduces self-shading (Craggs, 2001; D'Aiuto et al., 2015).

ii. Temperature

Temperature can also influence algae biomass productivity and nutrient uptake rates. At temperatures above a species' optimal range, the photosynthetic enzyme RubisCO may become less efficient at differentiating oxygen from carbon dioxide, thus reducing photosynthetic rates (Osborne & Beerling, 2006; Yamori et al., 2014). In some cases, extremely high temperatures can cause heat stress resulting in membrane injury and alterations to algal metabolic fluxes (Shin et al., 2016; Barati et al., 2019). Lower temperatures may not necessarily inhibit growth, but could reduce membrane fluidity (Inaba et al., 2003), limit electron transport and photon capture (Lobban & Harrison, 1994) and deteriorate photosynthetic pigment-proteins (Eggert, 2012). Continuous long-term operation of outdoor algal treatment systems can be challenging due to high variability in prevailing environmental conditions, including seasonal temperatures

(Leong et al., 2021). Excessive heat in summer and extreme cold temperatures in winter may negatively affect algae photosynthetic rates, reducing algal growth and nutrient uptake rates (Lavery et al., 1991; Borja et al., 2013). Consequently, selecting high performing species with broad temperature and irradiance tolerance for cultivation would enable continuous year-round biomass production and bioremediation performance.

iii. Nutrients

The concentration of nutrients that algae are cultivated in determines biomass productivity and nutrient uptake rates (Kangas & Mulbry, 2014; Liu et al., 2020). Nutrients (mainly nitrogen and phosphorus) are important for cellular energetics (ATP, ADP) and membrane structures (phospholipids) that support algal growth and biomass production (Lapointe, 1987). The ratio of nitrogen to phosphorus (N:P) in water is one of the most important factors affecting growth and nutrient removal efficiency. An imbalanced N:P ratio could result in essential nutrient exhaustion for algae growth requirements. Very few studies have investigated the optimal N:P ratio for filamentous algae, and this can vary between species (Liu and Vyverman, 2015). Additionally, even within a species, the optimal N:P ratio can vary with variation in external factors such as ambient temperature and light (Rhee & Gotham, 1980). The effect of the N:P ratio on filamentous algae growth could be further investigated to aid the selection of a target species for FANS operation. The concentration of nutrients on FANS can be manipulated by controlling the influent flow rate. Increasing nutrient loads typically leads to increased assimilation by the algae resulting in higher algal growth rates and biomass production (Kangas & Mulbry, 2014). However, high ammonia concentrations (> 400 mg L^{-1}) in water can be toxic and inhibit algal growth (Tam & Wong, 1996; Lu et al., 2018). This should not be a problem for agricultural drainage water as nitrate is the main form of nitrogen present which has much lower toxicity than ammonia.

iv. Turbulence

Turbulence affects algae growth and biomass productivity by improving algae gas exchange and nutrient assimilation (Adey & Loveland, 2011; Blersch et al., 2013). Turbulence in algae suspension systems can be provided through aeration or mixing. For FANS, turbulence can be controlled by adjusting the influent flow rate to provide sufficient frictional losses as

the water flows over and through the attached algae on floway. Increasing the flow rate will increase turbulence, which improves nutrient transport across the boundary layer around the algal cells. This can enhance algal assimilation and promote higher nutrient removal and biomass productivity (Busse et al., 2006; Blersch et al., 2013; Stocking et al., 2016).

1.5 Thesis Structure and Objectives

The key aim of this thesis was to investigate the ability of attached filamentous algae to grow and bioremediate agricultural drainage water using FANS. The thesis is comprised of a series of experiments conducted at increasingly larger scale and complexity, starting with the laboratory-scale, and progressively increasing through mesocosm-scale to pilot-scale experiments. Chapters 2 and 3 selected the best performing species to be cultivated on FANS based on their attachment capability, biomass productivity and nutrient removal rates at laboratory and mesocosm scale. Chapter 4 investigated the effects of FANS operating parameters such as initial standing crop, harvesting interval and influent flow rate on algae biomass productivity and nutrient removal rates. Chapter 5 assessed the long term performance of on-farm FANS treating agricultural drainage water under ambient environmental conditions. The specific aims of each research chapter are described below. Each chapter has been written as a stand-alone study suitable for individual publication.

1.5.1 Chapter 2: Novel Assay for Attached Filamentous Algae Productivity and Nutrient Removal

This chapter aimed to investigate the attachment abilities, growth rates, and nutrient removal rates of four locally isolated species of filamentous algae under controlled indoor conditions in small-scale laboratory experiments. This study developed a reproducible bioassay to rapidly assess the suitability of different species of filamentous algae for FANS based on their ability to attach and grow, remove nutrients and produce harvestable algal biomass. The specific aims were to (i) determine if a single filamentous algae species can be rapidly seeded onto microscale filamentous algae nutrient scrubbers (μFANS) to develop a stable monoculture of uniform biomass composition and (ii) to compare the biomass productivity and nutrient

removal rates of µFANS monocultures of four common filamentous freshwater algae species (*Cladophora* sp., *Oedogonium* sp., *Rhizoclonium* sp. and *Spirogyra* sp).

1.5.2 Chapter 3: Nutrient Removal and Productivity of Filamentous Algae on Mesocosm-Scale FANS Under Ambient Summer and Winter Conditions

The overall aim of this chapter was to compare the performance of monocultures of four common filamentous freshwater algae species (*Cladophora* sp., *Oedogonium* sp., *Rhizoclonium* sp. and *Spirogyra* sp.) on mesocosm-scale FANS under both ambient outdoor summer and winter conditions to select the best species to be cultivated on FANS. The specific aims were (i) to compare the establishment of species on FANS, (ii) to compare biomass productivity and nutrient removal rates of species on FANS, (iii) to compare the dominance of species on FANS, and (iv) to provide a realistic test of how monospecies FANS are likely to perform under outdoor conditions.

1.5.3 Chapter 4: Effects of Operational Parameters on the Performance of Unialgal *Oedogonium* sp. Filamentous Algae Nutrient Scrubbers under Controlled Environmental Conditions

The overall aim of this chapter was to investigate how FANS operating parameters affect productivity and nutrient removal performance of the best performing species identified in Chapters 2 and 3. The specific aim was to compare biomass productivity and nutrient removal rate under varying influent flow rates, initial standing crops and harvest frequencies. Experiments were conducted under controlled environmental conditions to remove any performance variability due to light and temperature variation that occur under ambient conditions.

1.5.4 Chapter 5: Effect of Seeding Method and Single versus Mixed Species Assemblages on the Performance of Filamentous Algae Nutrient Scrubbers (FANS) for the Treatment of Agricultural Drainage

The overall aim of this chapter was to compare the effects of seeding method and seeded species composition (single species vs. mixed species algal assemblages) on the performance of pilot-scale on-farm FANS for the treatment of agricultural drainage. The specific aims were (i) to compare the time taken to establish a uniform algal standing crop, biomass productivity, nutrient removal and dominance of species on FANS seeded using natural establishment and FANS seeded through controlled seeding of a single target species; and (ii) to compare the time taken to establish a uniform algal standing crop, biomass productivity, nutrient removal and dominance of species on FANS seeded through controlled seeding of a single target species and FANS seeded through controlled seeding of an adapted mixed species algal assemblage.

Chapter 2

Novel Assay for Attached Filamentous Algae Productivity and Nutrient Removal

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

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2.1 Abstract

Filamentous algae nutrient scrubbers (FANS) have demonstrated potential for cost-effective and sustainable nutrient bioremediation of a wide range of wastewaters. Typically, FANS are seeded with a mixed assemblage of algae species, however, growing a monoculture of one species on FANS could facilitate biomass use by providing a more consistent and high-quality substrate for end-product applications. To date, a standardised bioassay to assess the productivity and nutrient removal of filamentous algae attached to a bottom substrate (that could help identify promising species for FANS monoculture) has not been developed. Therefore, we developed a microscale filamentous algae nutrient scrubber (µFANS) and a protocol to establish monocultures of freshwater filamentous algae to compare performance in terms of attachment capability, nutrient removal and biomass production. Four common filamentous algae species (*Cladophora* sp., *Oedogonium* sp., *Rhizoclonium* sp. and *Spirogyra* sp.) were seeded by evenly distributing and rubbing the biomass onto µFANS textured liner to "hook" algal filaments, providing initial physical attachment. Within 14 days, a "lawn" of the seeded algae had established and the "hooked" biomass had attached biologically. Depending on species, biological attachment resulted from either holdfast development from filaments that grew from settled zoospores, growth of rhizoids or adhesion of filament fragments to mucilage. Biomass productivity of each species ranged from 2.2 to 5.3 g DW m^{-2} d^{-1} while nutrient removal rates ranged from 8.8 to 28.4 mg NO_3-N g^{-1} DW d^{-1} and 2.2 to 8.1 mg PO_4-P g^{-1} DW d^{-1}. *Oedogonium* sp. was the best performing species overall, with the strongest holdfast attachment, high biomass productivity (mean 4.2 g DW m^{-2} d^{-1}) and high nutrient removal

rates (mean 21.8 mg NO_3-N g^{-1} DW d^{-1} ; 5.6 mg PO_4-P g^{-1} DW d^{-1}). These attributes demonstrate that *Oedogonium* sp. is a promising species for FANS cultivation, but further larger-scale testing under outdoor conditions is required to confirm this.

2.2 Introduction

Filamentous Algae Nutrient Scrubbers (FANS) and similar algae turf scrubbers (ATS) are ecologically engineered, artificial streams that grow attached filamentous algae and associated periphyton to treat polluted water (Craggs, 2001; Adey et al., 2011; Adey et al., 2013; Sutherland & Craggs, 2017). FANS consist of a shallow, gently sloped floway with a liner and sometimes an overlying screen to which the algal "turf" attaches. This macroalgae-based treatment system has been successfully used for cost-effective and sustainable nutrient bioremediation of a wide range of wastewater types, including sewage (Sládečková et al., 1983a; Craggs et al., 1996a), horticultural wastewater (Liu et al., 2016a), agricultural drainage (Morgan et al., 2006a; Kangas & Mulbry, 2014), dairy manure effluent (Kebede-Westhead et al., 2006; Mulbry et al., 2008b; Mulbry et al., 2009), swine manure effluent (Kebede-Westhead et al., 2006; Mulbry et al., 2008a; Mulbry et al., 2008b), citrus farm runoff (D'Aiuto et al., 2015) and anaerobically digested food-waste centrate (Sutherland et al., 2020b).

FANS typically grow a mixed-species assemblage of filamentous algae (Mulbry et al., 2008a; Adey et al., 2013; D'Aiuto et al., 2015; Sutherland et al., 2020b). The algae are established by either letting them naturally colonise the floway or seeding the floway surface with mixed algal species collected from nearby water bodies. The resulting uncontrolled species composition on the floway can lead to variability in algal productivity and the composition and characteristics of the harvested algal biomass. An alternative approach is to seed a single filamentous algal species onto the floway. While the growth of a single algal species may not be required for effective nutrient removal (Hannon et al., 2010), it can provide a consistent source of high-quality biomass with low variation in biochemical composition for use in a range of end-product applications (Lawton et al., 2013a). Such approach has worked well in suspension (free-floating) algal systems (Cole et al., 2016; Mata et al., 2016) and therefore may work in attached algal systems such as FANS. However, maintaining a pure single species can be challenging, especially when operating in an open system like FANS due to naturally occurring undesired algae species or other organisms that may establish. In addition,

it can also be challenging to maintain a single species system under outdoor ambient conditions across seasons as algae may have different tolerance to temperature and irradiance (de Paula Silva et al., 2008; Singh and Singh, 2015).

As FANS are an attached algae treatment system, it is essential to select a target filamentous algae species for seeding onto the floway that naturally grows attached to a substrate (Sutherland & Craggs, 2017). Strong algae attachment enables the algae to tolerate higher water turbulence (Dodds, 1991), making the algae more resilient to breaking and sloughing off the floways during increased horizontal water velocity resulting from runoff during heavy rainfall events (Craggs et al., 1996a; Adey et al., 2011; Sutherland & Craggs, 2017). Filamentous algae can initially be attached onto floways during seeding by physically hooking the filaments onto the textured bottom liner or mesh. Biological attachment by a holdfast can occur through two mechanisms, rhizoid formation and zoospore formation and settling.

Algal filament fragments that become physically hooked onto substrates can subsequently develop anchoring rhizoids that become holdfasts (Etherington, 1964; Fletcher & Callow, 1992). As the filaments grow, attachment is provided by the growth of rhizoids, increasing the surface contact between the algae and the substrate, thereby forming a stronger attachment (Fletcher & Callow, 1992). The development of rhizoids is highly dependent on the substrate topography and light source and could also be associated with environmental conditions such as temperature and water pH (Nagata, 1973, 1977; Fletcher & Callow, 1992). In general, rhizoid growth is promoted by increased surface roughness, moderate temperature (~20°C), and neutral pH (Nagata, 1973, 1977; Fletcher & Callow, 1992). High or low pH can affect rhizoid formation and an extremely low temperature can inhibit rhizoid growth, although this is dependent on the algal species (Nagata, 1973, 1977; Fletcher & Callow, 1992).

Zoospores are released by the asexual reproduction of cells of the parent filament, these subsequently settle and attach to a substrate by developing a holdfast from which a new filament grows (Rosemarin, 1985; De Wreede & Klinger, 1988; Rindi, 2010). Depending on species, induction of zoospore production is primarily associated with environmental factors such as photoperiod, light irradiance, temperature, and to a lesser degree nutrient concentration or biotic interactions (Maggs & Callow, 2003). Zoospore settlement on a surface is dependent on several factors, including the roughness and topography of the substratum, biofilm chemical

signals, and attractive surface properties mediated by adsorbed macromolecules, microbial communities and hydrodynamics (Amsler et al., 1999; Callow & Callow, 2000; Maggs & Callow, 2003). Roughness and topography of a surface appear to play a critical role in zoospore attachment, leading to the general view that filaments develop more easily on roughened surfaces as these enhance survival of the newly attached spores from dislodgement by water currents (Callow et al., 2000; Maggs & Callow, 2003).

In order to select target macroalgal species for use in FANS, a bioassay is required to provide a rapid and rigorous comparison of species performance in terms of attachment capability, biomass productivity and nutrient removal rate. Bioassays using filamentous algae have typically been conducted in suspension cultures in a similar way to studies with microalgae. For example, Lawton et al. (2013a) compared biomass productivity and biochemical composition of three species of freshwater filamentous algae in 20 litre semi-continuous cultures under a range of conditions, including aeration rate and CO_2 addition. Lawton et al. (2014) also used suspension culture assays to compare the growth of isolates of *Oedogonium* collected from multiple geographic locations under a range of temperature treatments. Similarly, Valero-Rodriguez et al. (2020) compared the productivity and competitive ability of three filamentous macroalgae species in 1 litre semi-continuous cultures with continuous aeration under seasonal light and temperature conditions. However, to provide a realistic indication of species performance and suitability for use in FANS, a bioassay must compare the performance of algae growing attached to a bottom substrate under conditions comparable to those on a FANS. To date, few studies have compared the performance of species of filamentous algae when grown attached to a substrate. De Vries et al. (1983) developed a microscope slide bioassay to test for nitrogen and phosphorus limitation in eleven strains of filamentous algae genus *Stigeoclonium* Kutz. However, this bioassay is unsuitable for use as a FANS bioassay as the cultivation conditions are different from those that the algae would experience when grown on a FANS. Although the algae were grown attached, they were maintained in still water with periodic water exchanges (e.g., static batch cultures). In contrast, a FANS bioassay must have continuous flow of nutrient-enriched water over the attached algae on the floway. Additionally, the De Vries et al. (1983) bioassay involved the induction and settlement of spores, followed by a 3-week growth period before the algal filaments had reached sufficient size for testing in the bioassay. Consequently, the use of this assay would require the development of protocols to induce zoospore release for each algal species and a considerable time lag from seeding until the start of the bioassay.

Therefore, the overall aim of this study was to develop a reproducible bioassay to rapidly assess the suitability of different species of filamentous algae for FANS based on their ability to attach and grow, remove nutrients and produce harvestable algal biomass. The specific aims were to (i) determine if a single filamentous algae species can be rapidly seeded onto microscale filamentous algae nutrient scrubbers (µFANS) to develop a stable monoculture of uniform biomass composition and (ii) to compare the biomass productivity and nutrient removal rates of µFANS monocultures of four common filamentous freshwater algae species (*Cladophora* sp., *Oedogonium* sp., *Rhizoclonium* sp. and *Spirogyra* sp).

2.3 Methods

2.3.1 Microscale Filamentous Algae Nutrient Scrubber (µFANS)

We developed µFANS to provide a small-scale approximation of how filamentous algae grow on large-scale FANS (e.g., attached to a bottom substrate floway with nutrient-enriched water continuously flowing over and through the attached algae) to enable the performance of different algal species to be tested under relevant cultivation conditions. The µFANS system consisted of a rectangular polypropylene (PP) plastic container (size: 15 x 24 x 5.6 cm) with high density polyethylene (HDPE) textured liner (size: 19.5 x 11 cm or 0.0215 m^2). This liner was chosen as studies have shown that textured substratum or surfaces with variable topography can enhance algal biomass attachment (Maggs & Callow, 2003; Blersch et al., 2017). The textured liner was permanently attached to the base of the container using a multi-purpose permanent elastic sealant/adhesive glue (Bostik Simson ISR 70-03) (Figure 2.1 a-b). The lined µFANS rested on the top of a water storage container that provided a sump with two litres of total working volume. A submersible water pump continuously circulated nutrient-enriched water from the sump to a weir at the top of the µFANS and treated water drained back into the sump through holes at the bottom end of the µFANS. The pump tubing included an adjustable plastic valve to control the water flow rate.

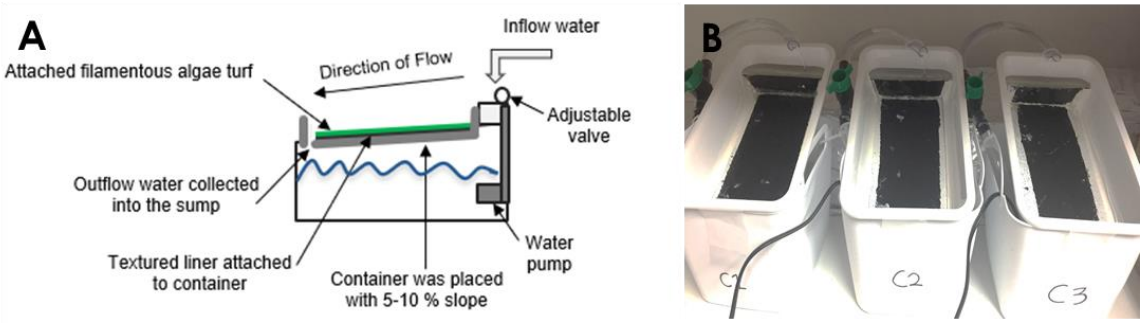

Figure 2.1: (a) Schematic diagram of microscale filamentous algae nutrient scrubber (µFANS), (b) Photo of three µFANS

2.3.2 µFANS Seeding

Four locally occurring filamentous algae species (*Cladophora* sp., *Rhizoclonium* sp., *Oedogonium* sp., and *Spirogyra* sp.; Figure 2.2 a-d) were compared in this study. These species were selected for cultivation on FANS as they are widely distributed in New Zealand and worldwide and have previously shown promise for nutrient bioremediation (Saunders et al., 2012; Khataee et al., 2013; Lawton et al., 2017; Liu et al., 2020; Valero-Rodriguez et al., 2020). In addition, using common locally occurring algae species in a treatment system is beneficial since these algae are already adapted to local conditions and will be naturally seeded in the system (Grobbelaar, 2010; Wilkie et al., 2011). These four filamentous algae species were collected from natural water bodies, ponds, and agricultural drainage systems close to Ruakura, Hamilton, Aotearoa, New Zealand. Algae samples were transported back to the National Institute of Water and Atmospheric Research (NIWA) research facility at Ruakura, where they were cleaned and identified using a compound microscope. Samples were maintained in nutrient-enriched (2 g m^{-3} KNO_3-N and 0.2 g m^{-3} K_2HPO_4-P soluble reactive phosphorus; EMSURE ®) dechlorinated water in a temperature and light controlled laboratory (20-21 ºC, 12:12 light:dark cycle, 250-300 µmol m^{-2} s^{-1}). Single species stock cultures were created by isolating individual filaments from samples, scaling these up in sterile petri dishes, and transferring cultures into a consecutively larger culture vessels.

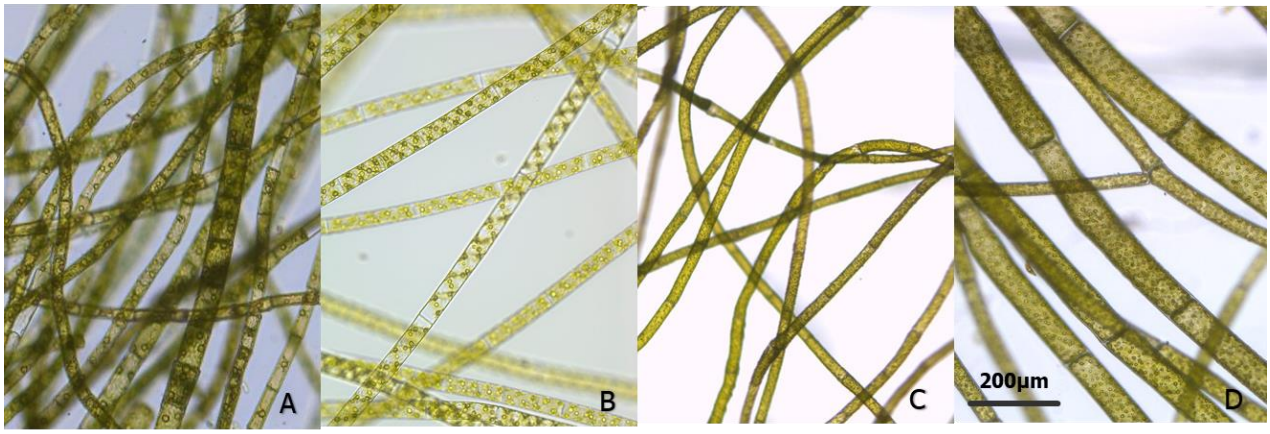

Figure 2.2: Microscopic images of filamentous algae species assessed in this study a. *Oedogonium* sp., b. *Spirogyra* sp., c. *Rhizoclonium* sp., and d. *Cladophora* sp. The scale bar applies to all pictures

Five replicate µFANS of each species were seeded by evenly distributing 1.2 g fresh weight (FW) (~56 g m^{-2}) of dewatered algae by rubbing it down the µFANS textured bottom liner to hook algal filaments, providing initial physical attachment. The seeded biomass rate was enough to cover the whole surface area of the µFANS bottom liner. This seeding method was selected based on pilot trials that compared seeding using algal zoospores and seeding by rubbing biomass onto the liner surface. The rubbing method resulted in a denser and more even biomass coverage over the liner surface in a shorter time than seeding using algal zoospores. Over time, secondary biological attachment occurred through the development of holdfasts following the growth of rhizoids from the hooked basal regions of the filaments and/or the subsequent release and settlement of zoospores on the liner surface.

The seeded µFANS were operated in batch culture using the same nutrient-enriched dechlorinated filtered freshwater that was used to maintain the algae stock cultures, but with higher nutrient concentrations (10 g m^{-3} KNO_3-N and 2 g m^{-3} K_2HPO_4-P soluble reactive phosphorus; EMSURE®). These nutrient concentrations were selected based on preliminary trials, which showed these concentrations provided sufficient nutrient supply for algal growth over a six-day growth period. The nutrient water was pumped continuously at ~0.5 L min^{-1} to the top of the µFANS floway. This flow rate was used for the seeding stage as preliminary trials indicated that it was high enough to cover the whole surface area of the floway in water, yet low enough to avoid washing the biomass off from the liner. The experiment was conducted under constant laboratory conditions (light intensity of 250-300 µmol m^{-2} s^{-1}using full-

spectrum LED plant growth lights, 12:12 light and dark cycle, 20-21°C) at the NIWA facility at Ruakura, Hamilton.

This seeding phase lasted for three six-day batch cycles, and the nutrient-enriched water was replaced at the start of each new batch cycle. Daily reseeding was performed in the first batch cycle by respreading any clumped algal biomass over the liner surface to promote a more rapid uniform establishment of a dense algal turf on each µFANS. The amount of biomass needed for reseeding was determined according to the biomass coverage on the liner of each µFANS.

2.3.3 FANS Operation

At the end of the seeding phase, the algal biomass on each µFANS was harvested by drawing a metal scraper down the length of the liner to cut longer filaments and remove any detached biomass to leave a uniform coverage of attached algal filaments of <1 cm length on the liner as a standing crop. As much as possible, µFANS were harvested to provide a similar standing crop between replicates and species.

Following harvesting and standing crop biomass measurement, each µFANS was reassembled, and 2 L of fresh nutrient-enriched dechlorinated water was added. The µFANS were then cultured under the same conditions described in Section 2.3.2 above for the next three-day batch cycle. At the end of each batch cycle, the pump on each µFANS was turned off to allow the water to drain from the liner for 2 minutes before biomass measurement, biomass harvest and standing crop measurement. This process was repeated five times to give six consecutive three-day growth cycles.

2.3.4 Biomass Measurement

Attached algal biomass FW growing on each replicate µFANS was measured directly by draining and then spinning each replicate µFANS lined plastic floway (placed vertically with the algae facing inwards) at 2800 rpm for 30 seconds in a spin dryer (Spindel SPL265, 6.5 kg capacity) to remove excess water before weighing. The weight of each replicate µFANS lined plastic floway (that had been measured before algal seeding) was then subtracted from this

weight to calculate the spun fresh weight (g FW) of the attached algae. This method was used to measure the weight of the standing crop (biomass remaining following harvest) and the final biomass (biomass on µFANS at the end of each three-day cycle). Biomass growth (g FW) for each µFANS was then calculated as the final biomass (g FW) – initial standing crop (g FW). Subsamples of harvested biomass from each replicate µFANS were dried overnight in an oven at 65ºC to determine the dry weight and the dry weight to fresh weight (DW:FW) ratio of algal biomass for each replicate µFANS. This specific DW:FW ratios were used to convert the measured FW biomass of the standing crop and the final algal biomass to dry weight (DW) for each replicate µFANS.

2.3.5 Biomass Composition

Following weighing, a small amount of the attached algal biomass was sampled from each replicate µFANS and examined under a compound microscope to check the condition and purity (e.g., species composition) of the biomass.

2.3.6 Nutrient Removal Analysis

Water samples were collected daily from each replicate µFANS to determine the amount of nitrate (NO_3-N) and dissolved reactive phosphate (DRP) removed by the algae. Immediately before water sample collection, filtered dechlorinated water was mixed into the sump of each µFANS to replace any water that had evaporated over the three-day growth cycle (approximately 5-7% of the total water volume, equating to an average of 100-140 ml every 24 hours). A 12 ml water sample was collected from each µFANS replicate, filtered using 0.7 µm glass microfibre filter (GF/F) and the concentration of nitrate and DRP in each water sample was determined using the nitrate cadmium reduction method (Hach Method 8039) and the ascorbic acid method (Standard Method Phosphorus 4500-P) respectively. As nutrient adsorption by the µFANS and other processes such as denitrification and evaporation may have contributed to nutrient reduction, one control µFANS for each species was operated throughout the experiment. These were maintained under the same conditions and treated the same way as all other replicate µFANS except that they were not seeded with any algae. Concentrations of nitrate and DRP were determined in water samples taken daily from each control µFANS as described above. The nutrients removed by each replicate µFANS were calculated as the

difference in nutrient concentrations between the day zero and day three water samples. The difference in nutrient concentrations between the day zero and day three water samples from the control µFANS was then subtracted from this value to estimate the total amount of nitrate and DRP removed by the algae over the three-day experimental period. Nitrate and DRP removal rates were calculated as the amount (mg) of nitrate and DRP respectively reduced per g DW of biomass produced in a day.

2.3.7 Statistical analyses

Differences in standing crop, DW:FW ratio, biomass productivity, total nitrate removal, total DRP removal, nitrate removal rate and DRP removal rate between species were tested using two-way analyses of variance (ANOVA) with species and batch cycle as fixed factors, or a Kruskal-Wallis test for variables that failed normality and/or homogeneity of variance tests. Linear regression analysis was used to test for a relationship between nitrate removal rate and specific growth rate, and phosphate removal rate and specific growth rate. Data were analysed separately for each species. All statistical analyses were carried out using SigmaPlot software (Systat Software Inc., Point Richmond, CA, USA). All data are reported as means ±S.D.

2.4 Results

2.4.1 Biomass Seeding and Attachment

All species took about 10-14 days to establish uniformly on the liner of the µFANS (Table 2.1, Figure A.1-6). *Oedogonium* sp. displayed the fastest and strongest biomass attachment on the liner. Based on light microscopy observations, the strong attachment of *Oedogonium* sp. appeared to be mainly a result of asexual zoospore production (Fig. A.2) with a subsequent settlement of the zoospores and holdfast attachment of the new filaments to the µFANS liner (Fig. A.3). There was evidence of filament fragmentation in *Spirogyra* sp. (Fig. A.4) and some fragments adhered to mucilage. However, microscopic observations showed a large proportion of *Spirogyra* sp. filament fragments were washed off the µFANS instead of attaching to the liner. Filaments of both *Rhizoclonium* sp. and *Cladophora* sp. remained firmly hooked to the liner surface and developed rhizoids for biological attachment (Fig. A.5-6).

Table 2.1: Attachment of filamentous algae species at the end of the seeding phase

Filamentous algae species	Time taken to establish uniform biomass attachment on liner (day)	Observed mechanism of biological attachment	Filament characteristics
Oedogonium sp.	10-12	Spore production, settling and holdfast attachment of new filament	Most filaments strongly attached
Rhizoclonium sp.	12-14	Developed rhizoids	Filaments attached and entangled but not easily fragmented
Cladophora sp.	12-14	Developed rhizoids	Filaments attached and entangled but not easily fragmented
Spirogyra sp.	10-14	Adhered to mucilage	Filaments attached but easily fragment during operation

2.4.2 Biomass Productivity

Overall, *Oedogonium* sp. and *Rhizoclonium* sp. had the highest standing crop (average of all cycles ~0.8 g DW ± 0.2 for both species), except in cycle 4 where *Cladophora* sp. had the highest standing crop. Standing crop varied significantly among species and cycles (Table 2.2, Figure 2.3). The standing crop of *Cladophora* sp. (average 0.7 g DW ± 0.1) was more consistent among batch cycles and replicates than *Oedogonium* sp. and *Rhizoclonium* sp. *Spirogyra* sp. had the lowest average standing crop (0.5 g DW ± 0.04) (Table 2.3), and this was more consistent among batch cycles (0.47 - 0.57 g DW) than for the other three species (Figure 2.3).

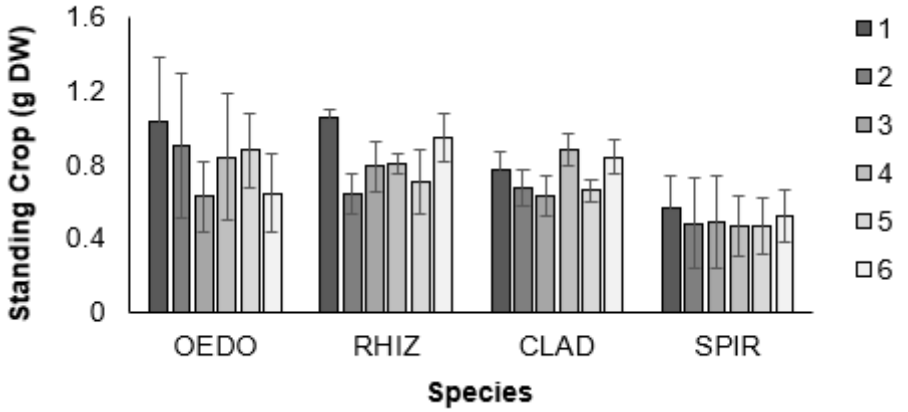

Figure 2.3: Average (± S.D.) standing crop (g DW) on µFANS of four species of filamentous algae over six batch growth cycles. OEDO: *Oedogonium* sp., RHIZ: *Rhizoclonium* sp., CLAD: *Cladophora* sp., SPIR: *Spirogyra* sp. N = 5

The DW:FW ratio was different for each algal species and varied between cycles (Table 2.2, Figure 2.4). *Cladophora* sp. had the highest DW:FW ratio (0.1 ± 0.01), while *Spirogyra* sp. had the lowest DW:FW ratio (0.05 ± 0.01, Table 2.3). The DW: FW ratio of *Oedogonium* sp. and *Rhizoclonium* sp. were similar at 0.07 ± 0.01 and 0.08 ± 0.01, respectively.

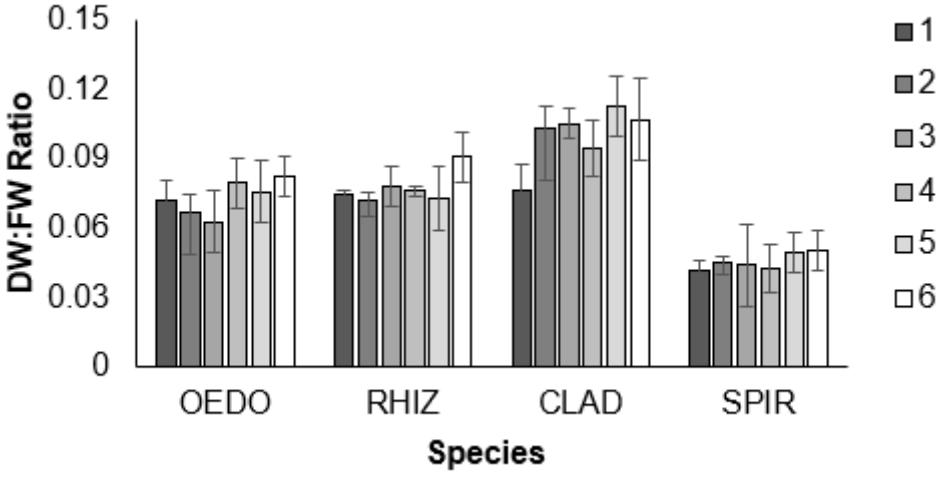

Figure 2.4: Average (± S.D.) DW:FW ratio of four species of filamentous algae over six batch growth cycles. OEDO: *Oedogonium* sp., RHIZ: *Rhizoclonium* sp., CLAD: *Cladophora* sp., SPIR: *Spirogyra* sp. N = 5

Biomass productivity varied significantly among species, however, the species with the highest biomass productivity was not consistent among cycles, as evidenced by a significant cycle x species interaction effect (Table 2.2, Figure 2.5). *Rhizoclonium* sp. had the highest

biomass productivity of all species in all cycles except cycle 1 where *Cladophora* sp. had the highest biomass productivity, while in all cycles *Spirogyra* sp. had the lowest biomass productivity. Across all cycles, biomass productivity of *Rhizoclonium* sp., *Oedogonium* sp., and *Cladophora* sp. (5.3 g DW biomass m^{-2} d^{-1} ± 1.1, 4.2 g DW biomass m^{-2} d^{-1} ± 1.1, and 4.2 g DW biomass m^{-2} d^{-1} ± 1.0, respectively) was nearly double that of *Spirogyra* sp. (2.2 g DW biomass m^{-2} d^{-1} ± 0.1) (Table 2.3). Biomass productivity was reasonably consistent between cycles for *Spirogyra* sp. (2.1 - 2.3 g m^{-2} d^{-1}) but more variable between cycles for the other three species.

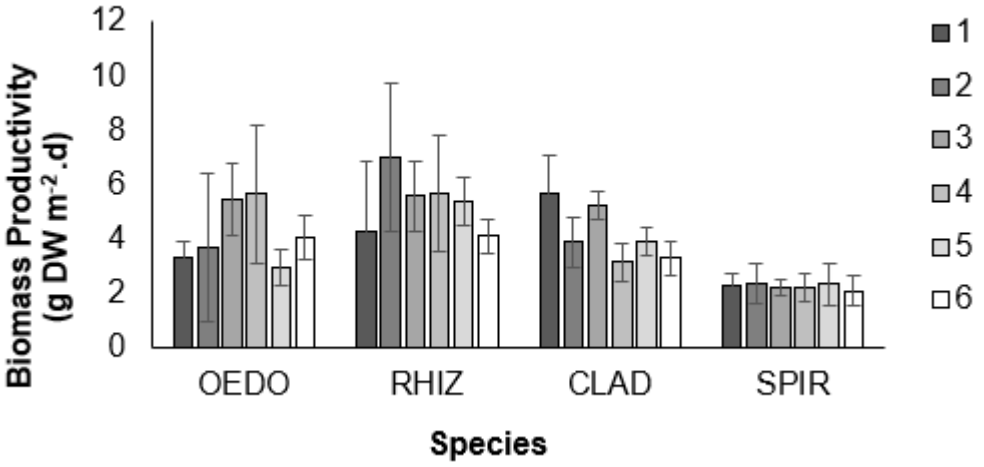

Figure 2.5: Average (± S.D.) biomass productivity (g DW biomass m^{-2}. d) of four species of filamentous algae over six batch growth cycles. OEDO: *Oedogonium* sp., RHIZ: *Rhizoclonium* sp., CLAD: *Cladophora* sp., SPIR: *Spirogyra* sp. N = 5

2.4.3 Nutrient Removal

Some nutrient removal (<20% nitrate and <12% DRP) was observed in the control µFANS without algae over the three-day cycle period. The total amount of nitrate removed over the three-day batch growth cycle by each species was significantly different (Table 2.2), but the species with the highest removal rate was not consistent between cycles, as evidenced by a significant cycle x species interaction effect (Table 2.2, Figure 2.6). Across all cycles, *Oedogonium* sp. had the highest average nitrate removal (5.2 g m^{-3} ± 1.3), followed by *Rhizoclonium* sp. (4.9 g m^{-3} ± 0.2) and *Spirogyra* sp. (4.2 g m^{-3} ± 0.7), while *Cladophora* sp. had the lowest nitrate removal (2.5 g m^{-3} ± 1.0) (Table 2.3). Nitrate removal was reasonably consistent between cycles for *Rhizoclonium* sp. (4.7-5.2 g m^{-3}), but more variable between cycles for the other three species.

Nitrate removal rates based on the biomass produced (e.g. mg N g^{-1} DW biomass d^{-1}) varied significantly between species and were not consistent among cycles for each species, as evidenced by a significant cycle x species interaction effect (Table 2.2, Figure 2.6). Across all cycles, mean nitrate removal rates per biomass produced were highest for *Spirogyra* sp. (28.4 ± 5.5 mg N g^{-1} DW biomass d^{-1}) followed by *Oedogonium* sp. (21.8 ± 7.0 mg N g^{-1} DW biomass d^{-1}), *Rhizoclonium* sp. (16.2 ± 4.5 mg N g^{-1} DW biomass d^{-1}), and then *Cladophora* sp. (8.8 ± 3.0 mg N g^{-1} DW biomass d^{-1}) (Table 2.3).

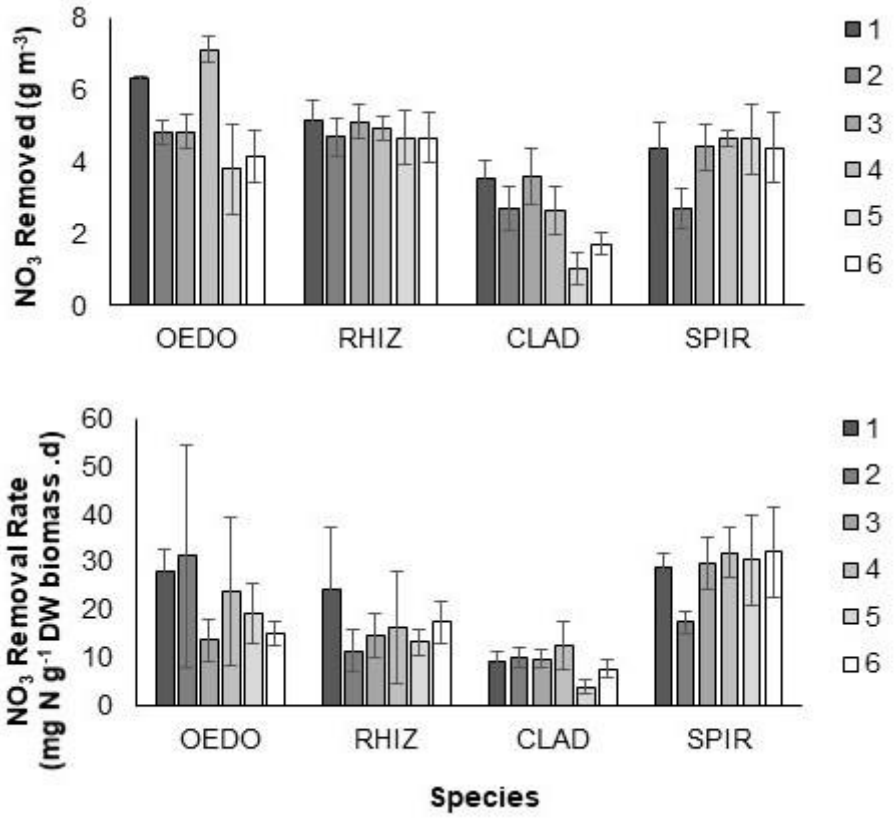

Figure 2.6: Average (± S.D.) NO_3-N removal (g m^{-3}, upper panel) and NO_3-N removal rate (mg N g^{-1} DW biomass. d, lower panel) over three days by four species of filamentous algae over six batch growth cycles. OEDO: *Oedogonium* sp., RHIZ: *Rhizoclonium* sp., CLAD: *Cladophora* sp., SPIR: *Spirogyra* sp. N = 5

Across all cycles, *Oedogonium* sp., *Rhizoclonium* sp. and *Spirogyra* sp. had the highest mean DRP removal (1.3 g P m^{-3} ± 0.2, 1.3 g P m^{-3} ± 0.1 and 1.2 g P m^{-3} ± 0.1, respectively), while that of *Cladophora* sp. was at least 50% lower (0.6 g P m^{-3} ± 0.2, Table 2.3). Phosphate removal over the three-day growth cycle varied significantly between species and cycles, but was not consistent, as evidenced by a significant cycle x species interaction effect (Table 2.2,

Figure 2.7). DRP removal rates based on the biomass produced significantly varied between species and cycles, but not in a consistent way as evidenced by a significant cycle x species interaction effect (Table 2.2, Figure 2.7). The mean DRP removal rate across all cycles was highest for *Spirogyra* sp. (8.1 ± 1.0 mg P g^{-1} DW biomass d^{-1}) followed by *Oedogonium* sp. (5.6 ± 2.1 mg P g^{-1} DW biomass d^{-1}), *Rhizoclonium* sp. (4.2 ± 0.7 mg P g^{-1} DW biomass d^{-1}) and the lowest was for *Cladophora* sp. (2.2 ± 1.0 mg P g^{-1} DW biomass d^{-1}). The DRP removal rate significantly varied between species and for each species between cycles, as evidenced by a significant cycle x species interaction effect (Table 2.2, Figure 2.7).

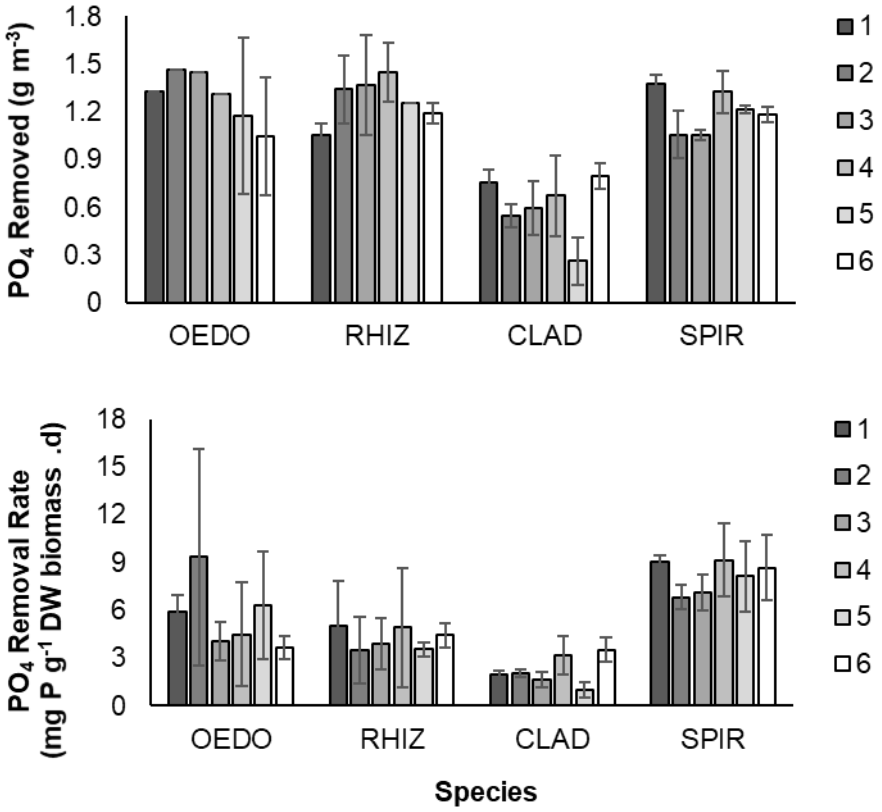

Figure 2.7: Average (± S.D.) PO_4-P removal (g m^{-3}, upper panel) and PO_4-P removal rate (mg P g^{-1} DW biomass d^{-1}, lower panel) over three days by four species of filamentous algae over six batch growth cycles. OEDO: *Oedogonium* sp., RHIZ: *Rhizoclonium* sp., CLAD: *Cladophora* sp., SPIR: *Spirogyra* sp. N = 5

Table 2.2: Results of statistical analysis on the effects of the treatment cycle and species on standing crop, DW:FW ratio, biomass productivity and nutrient removal

Variable	Effect	df	F	P
Standing Crop (g DW) [1]	Species	3	20.8	<0.001
	Cycle	5	3.7	0.005
	Species x Cycle	15	1.1	0.371
	Residual	96		
DW:FW Ratio	Species	3	101.9	< 0.001
	Cycle	5	4.4	0.001
	Species x Cycle	15	1.7	0.074
	Residual	96		
Biomass Productivity (g DW biomass m^{-2} d^{-1}) [1]	Species	3	26.3	<0.001
	Cycle	5	2.0	0.079
	Species x Cycle	15	2.4	0.006
	Residual	96		
Total Nitrate removed (g N m^{-3})	Species	3	103.3	<0.001
	Cycle	5	18.1	<0.001
	Species x Cycle	15	7.6	<0.001
	Residual	96		
Nitrate removal rate (g N g^{-1} DW biomass d^{-1}) [1]	Species	3	32.4	<0.001
	Cycle	5	1.9	0.108
	Species x Cycle	15	2.2	0.010
	Residual	96		

Total Phosphate removed (g P m^{-3}) [1]	Species	3	104.2	<0.001
	Cycle	5	3.3	0.008
	Species x Cycle	15	4.3	<0.001
	Residual	96		
Phosphate removal rate (g P g^{-1} DW biomass d^{-1}) [1]	Species	3	37.3	<0.001
	Cycle	5	1.0	0.404
	Species x Cycle	15	1.9	0.033
	Residual	96		

[1] Variables were tested using the Kruskal-Wallis test as they did not satisfy ANOVA assumptions

Table 2.3: Summary of key biomass productivity and nutrient removal parameters for four filamentous algae species on μFANS over six three-day treatment cycles. Data are means ± standard deviations, N = 6, mean values for each cycle were averaged to provide a global mean for each species

Species	*Oedogonium* sp.	*Rhizoclonium* sp.	*Cladophora* sp.	*Spirogyra* sp.
Biomass Productivity				
Standing Crop (g DW)	0.8 ± 0.2	0.8 ± 0.2	0.7 ± 0.1	0.5 ± 0.04
DW:FW Ratio	0.07 ± 0.01	0.08 ± 0.01	0.1 ± 0.01	0.05 ± 0.01
Biomass Productivity (g DW biomass m^{-2} d^{-1})	4.2± 1.1	5.3 ± 1.1	4.2 ± 1.0	2.2 ± 0.1
Nutrient Removal				
Total Nitrate removal in three-day treatment (g N m^{-3})	5.2 ± 1.3	4.9 ± 0.2	2.5 ± 1.0	4.2 ± 0.7
Nitrate removal rate (mg N g^{-1} DW biomass d^{-1})	21.8 ± 7.0	16.2 ± 4.5	8.8 ± 3.0	28.4 ± 5.5
Total Phosphate removal in three-day treatment (g P m^{-3})	1.3 ± 0.2	1.3 ± 0.1	0.6 ± 0.2	1.2 ± 0.1
Phosphate removal rate (mg P g^{-1} DW biomass d^{-1})	5.6 ± 2.1	4.2 ± 0.7	2.2 ± 1.0	8.1 ± 1.0

Linear regression analyses showed that specific growth rate (% day^{-1}) was a significant predictor of nitrate removal rate (g N m^{-3} d^{-1}) for *Cladophora* sp. ($F_{1,28}$ = 13.1, P = 0.001), but not for *Oedogonium* sp. ($F_{1,28}$ = 1.5, P = 0.22), *Rhizoclonium* sp. ($F_{1,28}$ = 1.9, P = 0.17), or *Spirogyra* sp. ($F_{1,28}$ = 0.21, P = 0.65) (Figure 2.8). However, the coefficient of determination (R^2) showed a weak linear relationship between these two variables for all species (*Oedogonium* sp.: R^2=0.051, *Rhizoclonium* sp.: R^2=0.064, *Cladophora* sp.: R^2=0.318, *Spirogyra* sp.: R^2=0.007, Figure 2.8). Linear regression analyses showed that specific growth rate (% d^{-1}) was also a significant predictor of DRP removal rate (g P m^{-3} d^{-1}) rate for *Cladophora* sp. ($F_{1,28}$ = 9.05, P = 0.005), but not for *Oedogonium* sp. ($F_{1,28}$ = 1.54, P = 0.23), *Rhizoclonium* sp. ($F_{1,28}$ = 0.74, P = 0.39), or *Spirogyra* sp. ($F_{1,28}$ = 0.37, P = 0.54, Figure 2.8). However, the coefficient of determination (R^2) showed a weak linear relationship between

these two variables for all species (*Oedogonium* sp.: R^2=0.052, *Rhizoclonium* sp.: R^2=0.026, *Cladophora* sp.: R^2=0.244, *Spirogyra* sp.: R^2=0.013).

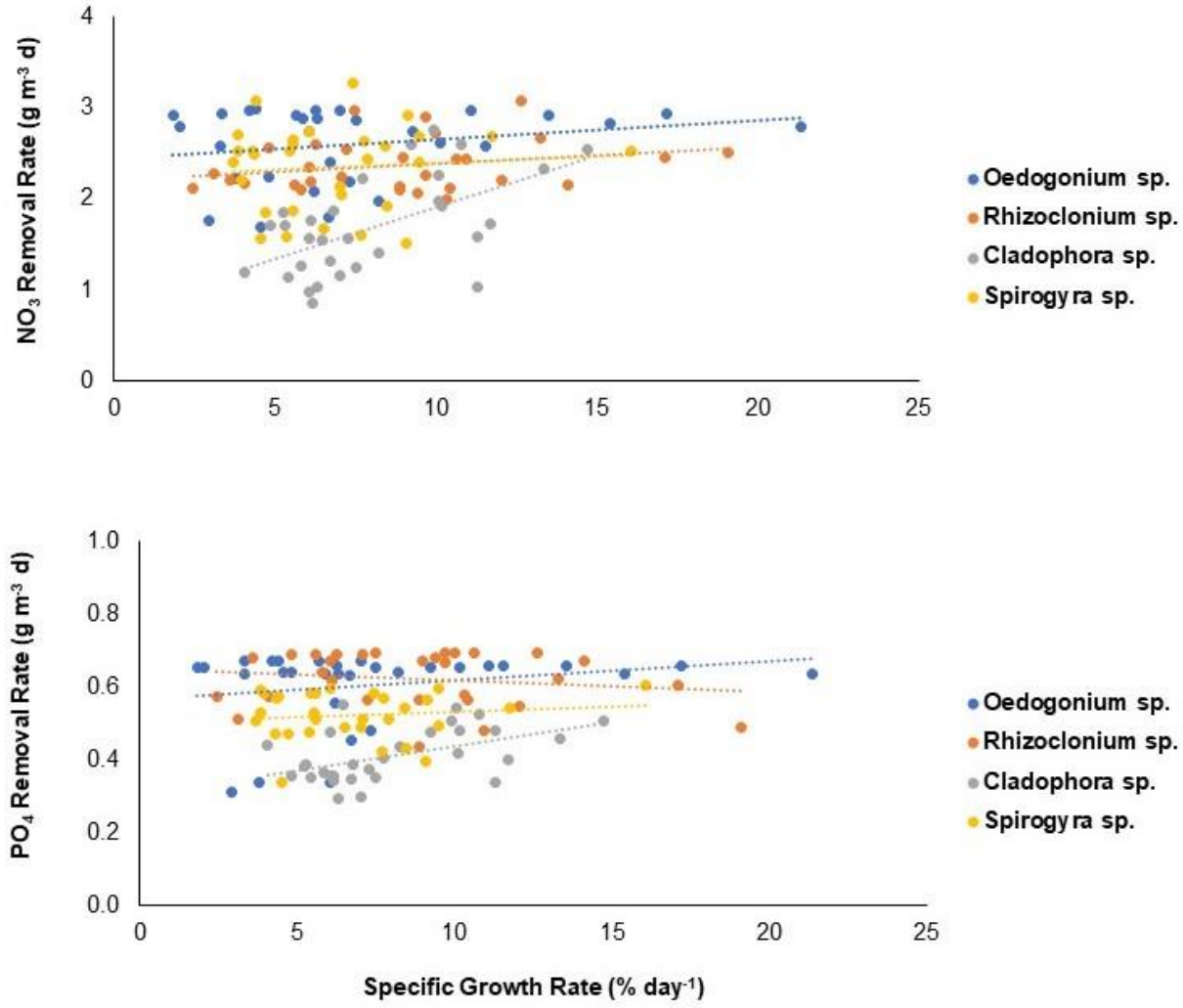

Figure 2.8: Relationship between NO_3-N and PO_4-P removal rate (g m^{-3} d^{-1}) and specific growth rate (% day^{-1}) for four species of algae over six growth cycles. Values for each replicate µFANS for each species and cycle are plotted.

2.5 Discussion

We developed a novel attached filamentous algae bioassay (µFANS) to assess the performance and suitability of filamentous freshwater algal species for cultivation on large-scale FANS. Our µFANS bioassay provides a comparative assessment of algal productivity among species under controlled growth conditions and nutrient supply. The µFANS bioassay was effective as it enabled rapid uniform establishment of attached filamentous algae species,

with simple operation and maintenance for laboratory comparison of performance under growth conditions representative of those that would be experienced on a large-scale FANS. Moreover, the biomass productivity rates we recorded on our µFANS (~4 to 5 g m^{-2} d^{-1}) were comparable to those reported for pilot scale (30 m^2) outdoor FANS with continuous flow (2.5 g m^{-2} d^{-1}, (Mulbry et al., 2008b)), suggesting that our µFANS bioassay provides a good indication of how species will perform on FANS operated in an outdoor setting with continuous flow. The small-scale of µFANS makes the experimental set-up portable, space-efficient (allowing multiple replicates to be tested simultaneously), and manageable to operate for the cultivation of several macroalgae species in the laboratory. In addition, the µFANS were economical to construct (~NZ$40) and were assembled easily from readily available materials. Moreover, the µFANS can be operated in batch culture with recirculating nutrient-enriched water, enabling this approach to be implemented where a constant supply of flow through nutrient-rich water is not available.

The attachment capability of a species is the most important criterion when selecting species for use on FANS. Stronger algae attachment could significantly reduce biomass sloughing off the floway (Gross et al., 2016), especially during heavy rainfall events (Sandefur et al., 2011a; Sutherland & Craggs, 2017). We found apparent differences in the relative abilities of species to attach to the liner surface, most likely resulting from differences in the mechanisms of attachment. *Oedogonium* sp. formed biological attachment primarily through the release of numerous zoospores which subsequently settled and developed new filaments with holdfasts anchoring them to the liner surface. These findings agree with previous studies which show that asexual reproduction in *Oedogonium* sp. takes place by the formation of young filaments developed from zoospores that attach to substratum with the help of a basal cell holdfast (Hoffman, 1965; Hoffman, 1967; Arora & Sahoo, 2015). In contrast, both *Rhizoclonium* sp. and *Cladophora* sp. became firmly anchored onto the liner surface probably through the development of rhizoids. This type of attachment has been observed in previous life history studies of these species (Nienhuis, 1974; Parodi & Cáceres, 1993). Both *Rhizoclonium* sp. and *Cladophora* sp. can also produce zoospores through asexual reproduction (Bellis & McLarty, 1967; Parodi & Cáceres, 1993; Aroca et al., 2020), however spore production was not observed in these two species in the present study. *Spirogyra* sp. filaments did not readily hook onto the textured liner and easily fragmented, with fragment attachment probably occurring by adhering to mucilage or by rhizoid formation. Mucilage secretion and adherence has been observed in fragmented filaments of *Spirogyra* sp. (Nagata, 1977) and

rhizoid formation has been induced in cut *Spirogyra* sp. fragments under laboratory conditions (Nagata, 1973). However, not all developed rhizoids will anchor to a substratum, and some may remain floating in still water (Ikegaya et al., 2008). There is also evidence that anchored *Spirogyra* sp. filaments can detach themselves due to changes in surface tension (Nagata, 1973). *Spirogyra* sp. spore production was observed in this study, however spores did not settle on the liner surface. This highlights the importance of considering not just the mechanisms of physical attachment (hooking) and biological attachment (zoospore production, settling and holdfast development; mucilage secretion, and; rhizoid formation), but also how easily filaments fragment in the absence of zoospore formation when assessing the suitability of species for FANS cultivation. Overall, *Oedogonium* sp. had the fastest biomass establishment with the strongest biological attachment of all four species. It had the most attachment points (holdfasts), and filaments were not easily fragmented or detached.

Of the four species we tested, *Rhizoclonium* sp. had the highest biomass productivity, while *Spirogyra* sp. had the lowest. *Spirogyra* sp. also had a consistently lower standing crop and took at least 50% longer to reach the same standing crop biomass compared to the other three species. It was challenging to get a similar standing crop for all species to start each batch cycle as 1) biomass moisture content of each species was different, which affected the DW:FW ratio and the standing crop estimation of all species based on the standardized DW; 2) mechanisms of attachment and texture of biomass were different for all species and therefore each species required a different approach to be taken to harvest the attached biomass; and 3) they grew at a different rate which affected the biomass establishment on the liner. In general, the standing crop in attached algae cultivation systems like FANS is equivalent to the stocking densities of macroalgae in suspension culture, where a higher stocking density can result in higher biomass productivity (Pereira et al., 2006). Therefore, a higher standing crop may have contributed to the higher biomass productivity of *Rhizoclonium* sp., *Oedogonium* sp. and *Cladophora* sp. compared to *Spirogyra* sp. in the current study. However, standing crop may not be the dominant factor affecting algae biomass productivity in FANS. The tolerance of algal species to the particular growth conditions (including light, temperature, pH, and type of cultivation system) can also impact biomass productivity (Singh & Singh, 2015). The low biomass productivity of *Spirogyra* sp. in the current study compared to the other three species may have been caused by elevated pH of the recirculated nutrient-enriched water, as high pH can limit algal growth (Grobbelaar, 2010; Park & Craggs, 2011a). *Spirogyra* sp. appears to be less tolerant to high pH than the other algae species tested as it is only been found in streams

with a pH below 8.6 (Khanum, 1982; Pikosz & Messyasz, 2015). Furthermore, *Spirogyra* sp. had higher growth rates in suspension culture when CO_2 was added, resulting in a reduction in pH (Lawton et al., 2013a). In the current study, pH was consistently recorded between 8.5-9.5, which could be too high for optimum growth of *Spirogyra* sp.

The high biomass productivity of *Rhizoclonium sp.* in the current study relative to the other three species demonstrates its suitability as a target species for FANS cultivation. This finding is supported by previous studies, which have found that *Rhizoclonium sp.* is common in mixed-species assemblages in attached algal cultivation systems and often outperforms other species of filamentous algae. For example, *Rhizoclonium* sp. was consistently the most abundant species on self-seeded FANS floways operated year round to treat dairy manure effluent (Mulbry et al., 2008b). Similarly, *Rhizoclonium* sp. was one of the most abundant species on self-seeded FANS treating drainage water from a citrus farm (D'Aiuto et al., 2015). *Rhizoclonium* was also one of only two species of filamentous macroalgae that were successfully isolated from industrial wastewater and maintained indoors under a controlled conditions for more than a month (Nwoba et al., 2017). Further supporting the selection of this species as a target for cultivation on FANS, *Rhizoclonium* has a wide tolerance to factors such as temperature, salinity, photoperiod and nutrient concentration (Aroca et al., 2020). Both *Oedogonium* sp. and *Cladophora sp.* also had relatively high biomass productivity in the current study and as such would be suitable targets for FANS cultivation. Supporting this conclusion, these species are often dominant components of the algal community on self-seeded FANS (Mulbry et al., 2008a; Mulbry et al., 2008b; D'Aiuto et al., 2015) and have comparatively high biomass productivities to other species in suspension monocultures (Lawton et al., 2013a; Liu & Vyverman, 2015; Lawton et al., 2021).

Nutrient removal rates were calculated based on differences in nitrate and phosphate concentrations between the FANS inflow and outflow. A combination of several removal mechanisms could cause this concentration difference, including algae assimilation (for growth and biomass production) as the main removal mechanism, algae luxury uptake (nutrient uptake not associated with biomass production), denitrification by denitrifying bacteria, and phosphate precipitation. As a result, the estimation of total nutrient removal reported in the current study may exceed the actual nutrient removed through algae assimilation. As such, these values represent comparative measures of nutrient removal across species rather than absolute estimates. However, assuming all replicate FANS had a similar rate of nutrient removal through

mechanisms other than algae assimilation, the nutrient removal rates reported here can be used to assess the relative bioremediation performance of each species. Nutrient removal rates were highest for *Spirogyra* sp. and lowest for *Cladophora* sp.. The variation in nutrient removal rates among algae species tested may have been caused by variability in nutrient assimilation efficiency and nutrient storage capacity (Fujita, 1985; Ohtake et al., 2021). Nutrient assimilation efficiency of algal species is affected by cell wall permeability and mechanisms of active transport across the cell wall (Kuffner & Paul, 2001; Vermeij et al., 2010). For example, *Spirogyra sp.* has been found to have higher cell wall permeability than *Cladophora sp.* (Osterhout, 1913; Bergman, 1949). This may explain the higher nutrient removal rates of *Spirogyra* sp. in the current study compared to the other three species. In addition, the nutrient removal rate of algae could also be associated with the surface area to volume ratio (SA:V) of algal cells due to the effect of algae cell thickness on nutrient transport distances (Rosenberg & Ramus, 1984; Hein et al., 1995). Algae with greater SA:V have been found to have higher nutrient uptake rates (den Haan et al., 2016). In the current study, higher nutrient removal rates were observed in species with thinner cells, such *Spirogyra* sp., *Oedogonium* sp. and *Rhizoclonium* sp. compared to *Cladophora* sp.

Although *Spirogyra* sp. was the best performing species in terms of nutrient removal per gram of biomass production; *Oedogonium* sp. had the highest overall nutrient removal when considering the total concentration of nutrients in the water before and after FANS treatment. This was despite the fact that *Oedogonium* sp. had 20-30% lower nutrient removal rates per gram of biomass production (mean 21.8 mg NO_3-N g^{-1} DW d^{-1} ; 5.6 mg PO_4-P g^{-1} DW d^{-1}) compared to *Spirogyra* sp. These differences were due to the much higher biomass productivity of *Oedogonium* sp. (mean 4.2 g DW biomass m^{-2} d^{-1}) compared to *Spirogyra* sp. (mean 2.2 ± 0.1 g DW biomass m^{-2} d^{-1}). The higher biomass productivity recorded for *Oedogonium* sp. in addition to its high overall nutrient removal make it a better target for combined bioremediation and biomass production than *Spirogyra sp*. More generally, these results highlight the importance of considering multiple performance metrics when selecting target species for cultivation on FANS. Overall, our results suggest that macroalgae nutrient removal rate is not directly correlated with growth rate as linear regression analyses showed a weak relationship between these two variables for all species. This could be because the assimilated nutrients are not always associated with biomass yield since different macroalgae species may have different mechanisms of nutrient uptake and storage and, to varying degrees, for example, luxury consumption (Gerloff & Krombholz, 1966; Reef et al., 2012).

We have several recommendations and insights for future studies using a µFANS bioassay. The optimum duration of a three-day growth period for each cycle was selected to minimise microalgal contamination of the culture water that could affect macroalgae growth and nutrient removal results. Thus, the recirculating nutrient-enriched media needs to be replaced every three days and we advise cleaning the sump at the same time. We found four to six cycles of a three-day growth period was sufficient to compare the performance and suitability of macroalgae species on FANS as this provides more than a month (including the seeding stage duration) for the algae to adapt to and thrive in the µFANS set up. The time for algae establishment could be reduced from the 14 days observed in the current study, and algae attachment could be sped up by employing daily biomass reseeding and redistribution on the liner surface. This approach could reduce the seeding duration to one week based on preliminary tests. However, seeding too much biomass (more than 56 g m^{-2}) onto the liner could limit light and therefore growth. Additionally, redistributing the biomass too frequently on the liner (< 24-hour interval) can disturb any attachment that has partially formed. Estimating the amount of FW algae biomass that needs to be harvested from each µFANS to provide for a standardised standing crop in DW across all replicates was challenging as each species' DW:FW ratio varied. For this step, we therefore recommend that estimation of how much FW biomass needs to be harvested for each µFANS should be based on the DW:FW ratio of a biomass subsample collected a day before the actual harvesting day.

2.6 Conclusion

We developed a novel, standardised, reproducible bioassay to assess the suitability of different filamentous algae species for use on FANS based on their ability to attach and grow, remove nutrients and produce harvestable algal biomass. The developed microscale FANS bioassay is practicable and effective and provides an initial comparative assessment of algal productivity and nutrient removal among species under controlled growth conditions. This study has also proved that a single target algae species could achieve nutrient mitigation and biomass production on a small-scale FANS. Generally, attached algal systems are self-seeded with mixed-species assemblages. However, our results demonstrate that single target species can be maintained on FANS and achieve nutrient mitigation and biomass productivity at least over month timescales. *Oedogonium* sp. was the best performing algal species in terms of most

uniform biomass distribution and attachment, high productivity and high nutrient removal rates. These criteria make *Oedogonium* sp. an ideal species for cultivation on large-scale FANS, however further larger-scale testing under outdoor conditions is required to confirm this. As the results from this laboratory-scale study may not fully reflect the complex interaction between abiotic and biotic factors in large-scale outdoor FANS, we suggest additional studies are conducted that compare monocultures of filamentous algae species on FANS over an extended period under ambient outdoor conditions to provide a realistic test of how monospecies FANS are likely to perform in the field. Our findings provide a new bioassay to grow and assess filamentous algae species performance on laboratory-scale FANS based on attachment capability, biomass productivity and nutrient removal. This has helped select algal species for future studies of filamentous algae species performance on outdoor larger-scale FANS.

2.7 Appendix

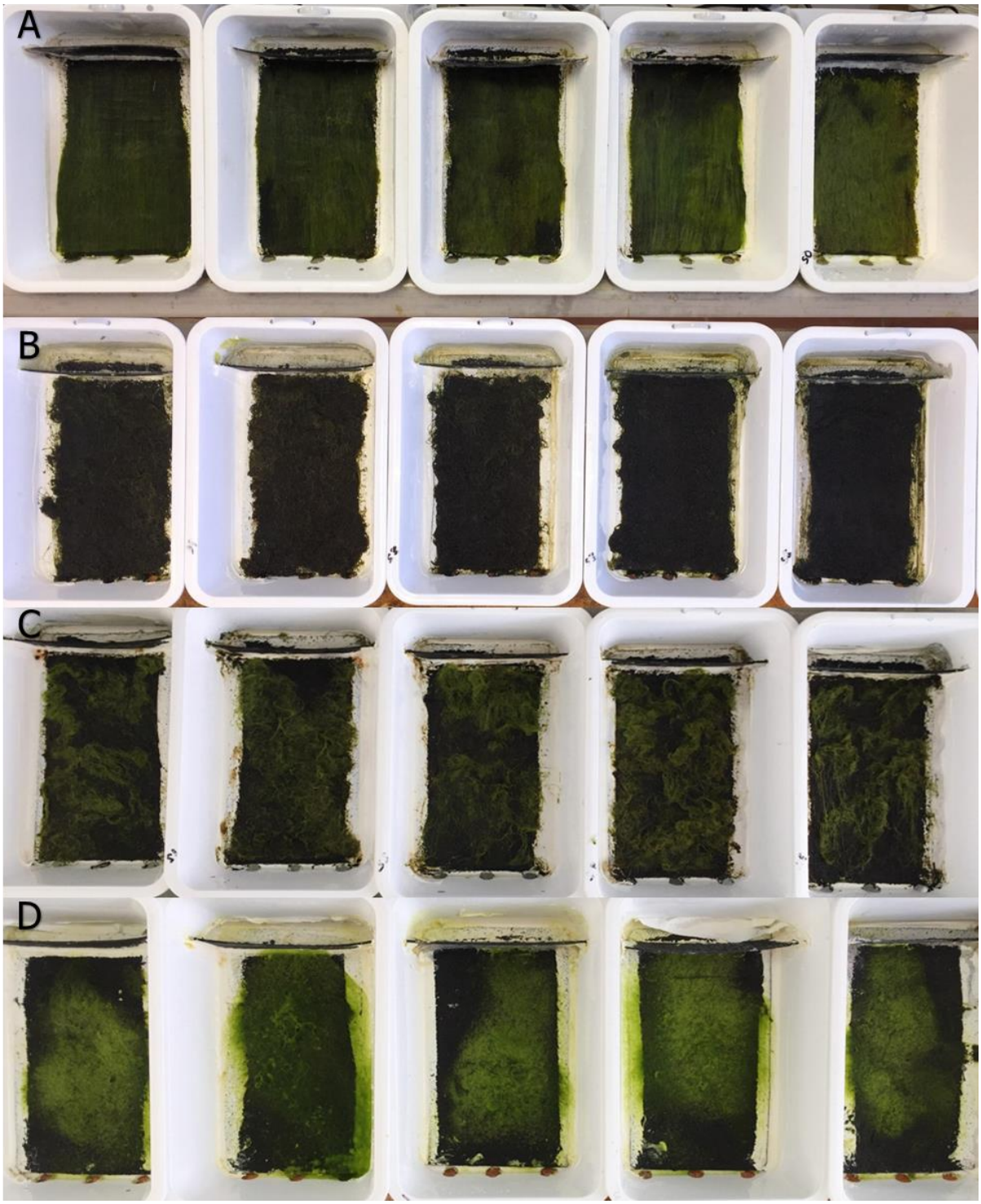

Figure 2A.1: The top view of established a. *Oedogonium* sp., b. *Rhizoclonium* sp., c. *Cladophora* sp. and d. *Spirogyra* sp. standing crop on five replicate µFANS

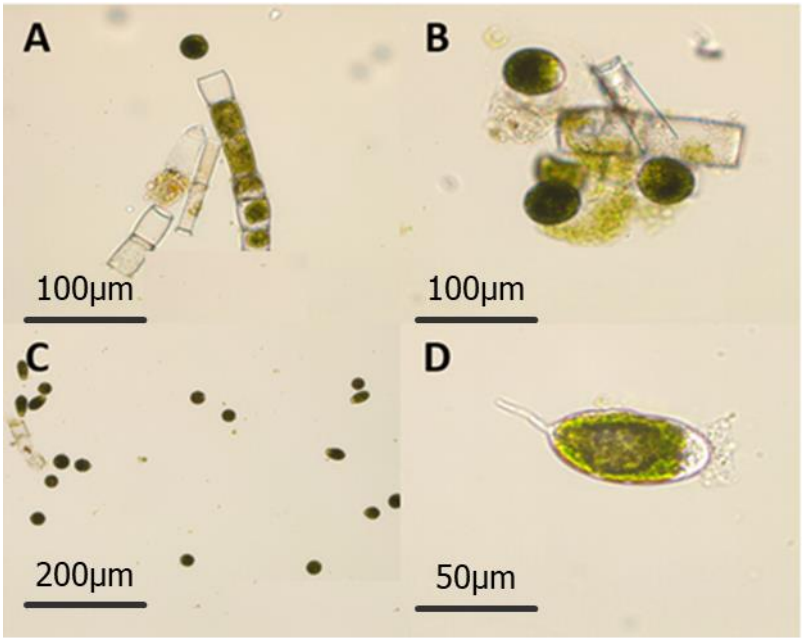

Figure 2A.2: Light microscopy images of *Oedogonium* sp. reproduction. a. spore released from fragmented *Oedogonium* sp. filaments, b. Several non-motile spores stuck to algae biofilm; c. motile and non-motile spores found in suspension and d. Motile zoospores with flagella

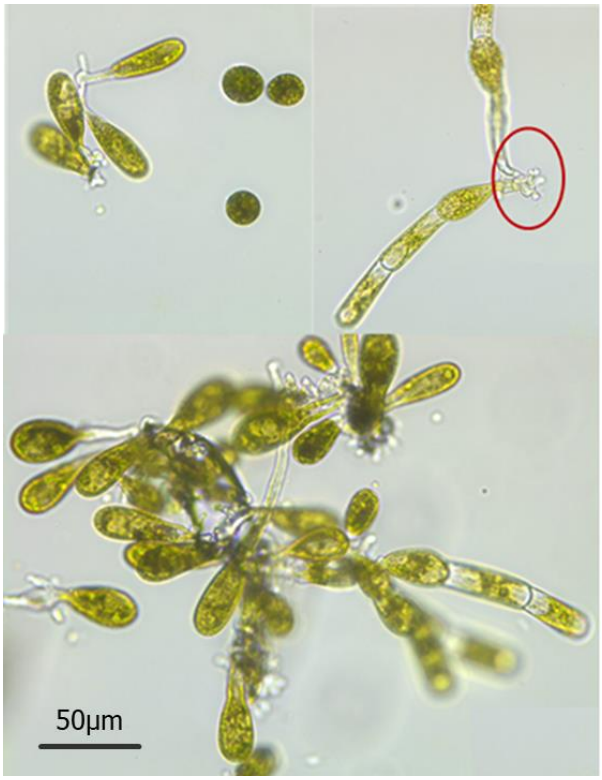

Figure 2A.3: Light microscopy images of juvenile *Oedogonium* sp. filaments with the root-like structure of holdfast (indicated in the circle)

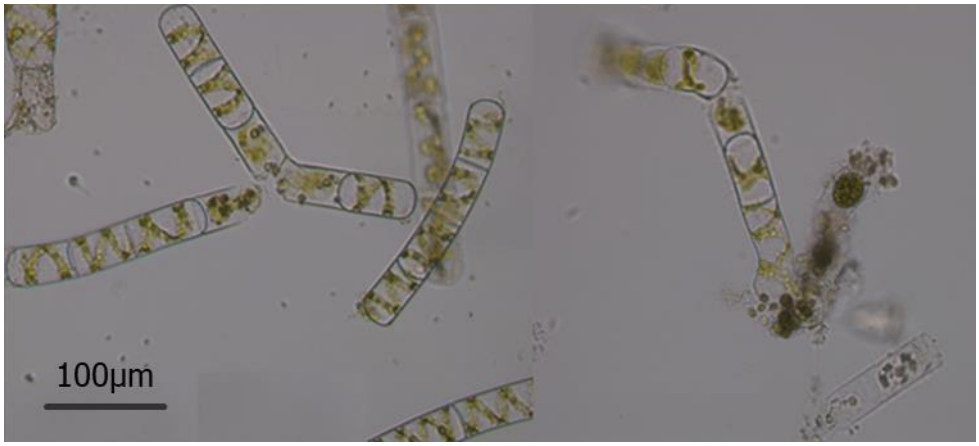

Figure 2A.4: Light microscopy images of fragmented *Spirogyra* sp. filaments that had been growing on the µFANS

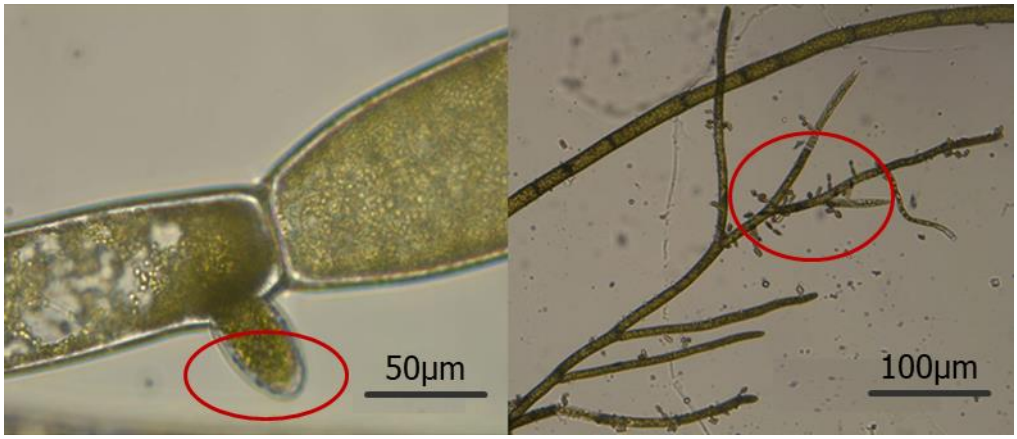

Figure 2A.5: Light microscopy images of rhizoidal branches formed on hooked filaments of *Cladophora* sp.

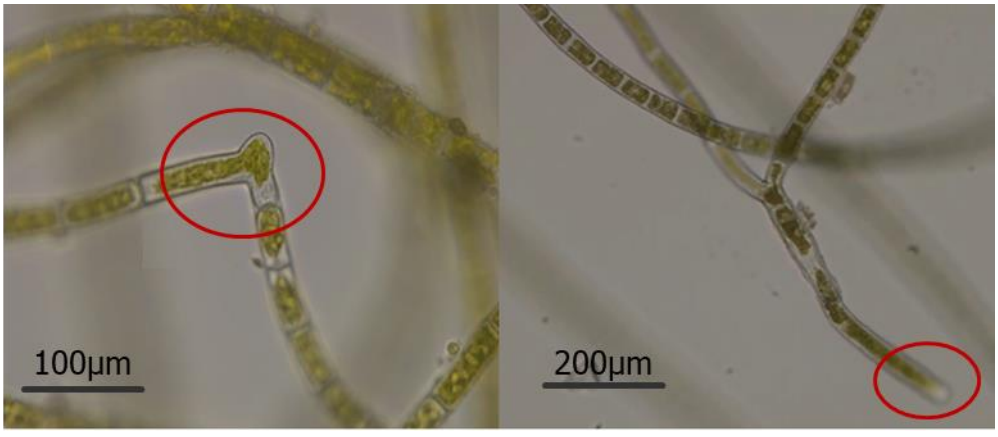

Figure 2A.6: Light microscopy images of rhizoidal branches (indicated in the circle) formed on hooked filaments of *Rhizoclonium* sp.

Chapter 3

Nutrient Removal and Productivity of Filamentous Algae on Mesocosm-Scale FANS Under Ambient Summer and Winter Conditions

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3.1 Abstract

Filamentous algae nutrient scrubbers (FANS) have demonstrated potential for cost-effective and sustainable nutrient bioremediation of a wide range of polluted waters. Their performance depends on the rate at which the algae can assimilate nutrients from the water and produce biomass. This study investigated the growth rates and nutrient removal rates of four locally isolated filamentous algae species (*Cladophora* sp., *Oedogonium* sp., *Rhizoclonium* sp., and *Spirogyra* sp.) under ambient summer and winter conditions to identify target species for use on FANS. Variation in environmental conditions had a large effect on the performance of each species. Biomass productivity of the four species ranged from 1.2 to 4.5 g DW m^{-2} d^{-1} in summer and 0.7 to 1.6 g DW m^{-2} d^{-1} in winter. Nutrient removal rates ranged from 0.39 to 0.51 g NO_3-N m^{-2} d^{-1} and 0.16 to 0.18 g PO_4-P m^{-2} d^{-1} in summer and 0.32 to 0.51 g NO_3-N m^{-2} d^{-1} and 0.08 to 0.09 g PO_4-P m^{-2} d^{-1} in winter. Overall, *Oedogonium* sp. was the best performer of the four species tested, with the highest biomass productivity (mean 4.5 g DW m^{-2} d^{-1} in summer; 1.6 g DW m^{-2} d^{-1} in winter) and the highest nitrate removal rates under summer (mean 0.51 g NO_3-N m^{-2} d^{-1}) and winter (mean 0.51 g NO_3-N m^{-2} d^{-1}) conditions. Furthermore, *Oedogonium* sp. FANS had the lowest contamination in terms of percentage cover of non-target species compared to the other three species under both summer and winter conditions. These results demonstrate that *Oedogonium* sp. has higher tolerance than the other three species to summer and winter ambient temperature and light variation, allowing *Oedogonium* sp. to

maintain dominance on the floway for a longer period. For these reasons, *Oedogonium* sp. is identified as a promising target for year-round cultivation on FANS.

3.2 Introduction

FANS, also known as algal turf scrubbers (ATS), are artificial streams that are ecologically designed to cultivate attached filamentous algae and associated periphyton to reduce nutrient concentrations in polluted water through algal photosynthesis (Craggs, 2001; Adey et al., 2011; Sutherland & Craggs, 2017). This attached filamentous algae treatment system has been successfully used for bioremediation of a wide range of polluted water (Craggs et al., 1996a; Kebede-Westhead et al., 2006; Kangas & Mulbry, 2014; D'Aiuto et al., 2015; Liu et al., 2016a; Sutherland et al., 2020a). However, the continuous long-term operation of large-scale FANS can be challenging due to high variability in prevailing environmental conditions such as irradiance, temperature and rainfall (Leong et al., 2021). Growth of the attached algae may be limited during summer when exposed to excessive irradiance and heat in the day (Shin et al., 2016). While during winter, cold temperatures may negatively affect growth and reproduction (Lavery et al., 1991; Borja et al., 2013) and shorter diurnal irradiation may reduce photosynthetic rates reducing both algal growth and nutrient uptake rates (Lee et al., 2015). In addition, heavy rainfall and the resulting increased water flow can cause algae to slough off the FANS (Adey et al., 2011; Borja et al., 2013). As the growth and nutrient uptake of all filamentous algae species is strongly influenced by ambient environmental conditions, in particular irradiance and temperature (Lee et al., 2016; Xu et al., 2021), seeding a single, high performing species that can tolerate seasonal changes in environmental conditions would enable effective year-round FANS operation. Furthermore, selecting a single target species could allow the optimization of FANS operational parameters to maximise the performance of target species for a specific aim (e.g. bioremediation, biomass composition or both).

Several characteristics should be considered when selecting a target species for outdoor FANS cultivation. These include the ability to rapidly establish a dense algal turf on the floway upon seeding , a strong biomass attachment (Adey et al. 2011, Sutherland and Craggs 2017), high rates of nutrient uptake, high biomass productivities, and the ability to maintain dominance over other undesired species under varying outdoor conditions (Lawton et al., 2013a; Liu et al., 2020). Typically, most attached algal treatment systems are seeded with a

mixed-species assemblage of filamentous algae (Mulbry et al., 2008a; Sandefur et al., 2011a; Adey et al., 2013; D'Aiuto et al., 2015). However, our indoor study using microFANS under constant environmental conditions recently demonstrated that a single target species could be maintained on FANS and achieve nutrient mitigation and biomass productivity over at least a one-month timescale (Hariz et al., 2022). As high performance in indoor systems may not translate to high performance in outdoor systems (Lawton et al., 2015b), the next step to select a single target species for cultivation on FANS is to compare the performance of potential target species under ambient outdoor conditions using larger mesocosm scale FANS. Importantly, performance must be compared across multiple seasons as species may have different tolerance towards extreme temperature and irradiance, especially during peak summer and winter (Liu et al., 2020). Targeting the best performing species to grow in a wide range of conditions will reduce the impact of environmental variation on biomass productivity and nutrient removal efficiency for long-term FANS operation.

Several freshwater filamentous algae have been proven effective at treating wastewater in attached algal systems and could be suitable target species for FANS operation, including *Spirogyra* sp. (Mulbry et al., 2010; Kangas et al., 2017; Marella et al., 2019), *Oedogonium* sp. (Mulbry & Wilkie, 2001; Kebede-westhead et al., 2003), *Rhizoclonium* sp. (Kebede-westhead et al., 2003; Mulbry et al., 2008b), *Cladophora* sp. (Mulbry et al., 2010; Ray et al., 2015), *Stigeoclonium* sp. (De Vries & Hotting, 1985), *Ulothrix* sp. (Mulbry et al., 2010), *Microspora* sp. (Kebede-westhead et al., 2003) and *Klebsormidium* sp. (Liu et al., 2016b). But although these species have been successfully cultivated on attached algal systems to treat wastewater, they were grown as part of mixed algal assemblages and only our previous study has compared the performance of individual species seeded as a monoculture on FANS. Hariz et al. (2022) compared the performance of *Oedogonium* sp., *Rhizoclonium sp.*, *Spirogyra* sp. and *Cladophora* sp. on small scale FANS under controlled laboratory conditions and found that *Oedogonium* sp. had the highest nutrient uptake rates and reasonably high biomass productivity compared to the other three species. *Oedogonium* sp. also had the fastest overall biomass establishment with the strongest biological attachment of all four species. Similarly, *Oedogonium* sp. was one of the most abundant species on self-seeded FANS treating nutrients from dairy manure effluent (Pizarro et al., 2002) and anaerobically digested food-waste centrate (Sutherland et al., 2020a). *Oedogonium* sp. can grow under a wide range of temperatures and is tolerant to various environmental conditions (Lawton et al., 2017) (Table 3A.1). In combination, these factors make it an ideal species to target for outdoor FANS

cultivation. However, several other species of filamentous algae may also be suitable targets for outdoor FANS cultivation. *Rhizoclonium* sp. is often one the dominant species found on self-seeded FANS (Mulbry et al., 2008b; D'Aiuto et al., 2015) and has wide tolerance to temperature, salinity and photoperiod (Aroca et al., 2020). *Cladophora* sp. also has broad tolerance to temperature, irradiance, salinity and nutrient concentration (Taylor et al., 2001). *Spirogyra* sp. has been abundant on FANS in several studies (Mulbry et al., 2010; Kangas et al., 2017), and can maintain biomass productivity under both summer and winter conditions (Marella et al., 2019). Hence, comparing the performance of these species under the same experimental conditions during summer and winter ambient conditions is necessary to identify the best performing species for use on large-scale outdoor FANS.

Therefore, the overall aim of this study was to compare the performance of monocultures of four common filamentous freshwater algae species (*Cladophora* sp., *Oedogonium* sp., *Rhizoclonium* sp. and *Spirogyra* sp.) on mesocosm-scale FANS under ambient outdoor conditions to select the best summer and winter species to be cultivated on FANS. The specific aims were (i) to compare establishment of species on FANS under summer and winter conditions, (ii) to compare biomass productivity and nutrient removal rates of species on FANS under summer and winter conditions, (iii) to compare the dominance of species on FANS under summer and winter conditions, and (iv) to provide a realistic test of how monospecies FANS are likely to perform under outdoor conditions.

3.3 Methods

3.3.1 Experimental design

Algae were grown outdoors under ambient conditions on 12 individual mesocosm-scale FANS systems at the National Institute of Water and Atmospheric Research (NIWA) facility at Ruakura, Hamilton, New Zealand. Each FANS consisted of 6 m long aluminium I-beam (with 0.12 m channel width and 0.05 m maximum channel depth, Figure 3.1) with the bottom of the channel covered with a high-density polyethylene (HDPE) textured liner (total surface area of 0.72 m^2). This liner was chosen as studies have shown that textured substratum or surfaces with topographic features can enhance algal biomass attachment (Maggs & Callow, 2003; Blersch et al., 2017). The textured liner was permanently attached to the base of the I-beam using a multi-purpose permanent elastic sealant/adhesive glue (brand: Bostik ISR 70-03).

Each FANS had a 2 % slope to allow water to flow through gravity down the length of the FANS. The slope was adjusted by changing the height of the metal frame crossbar at the top (inflow) end of the I-beam.

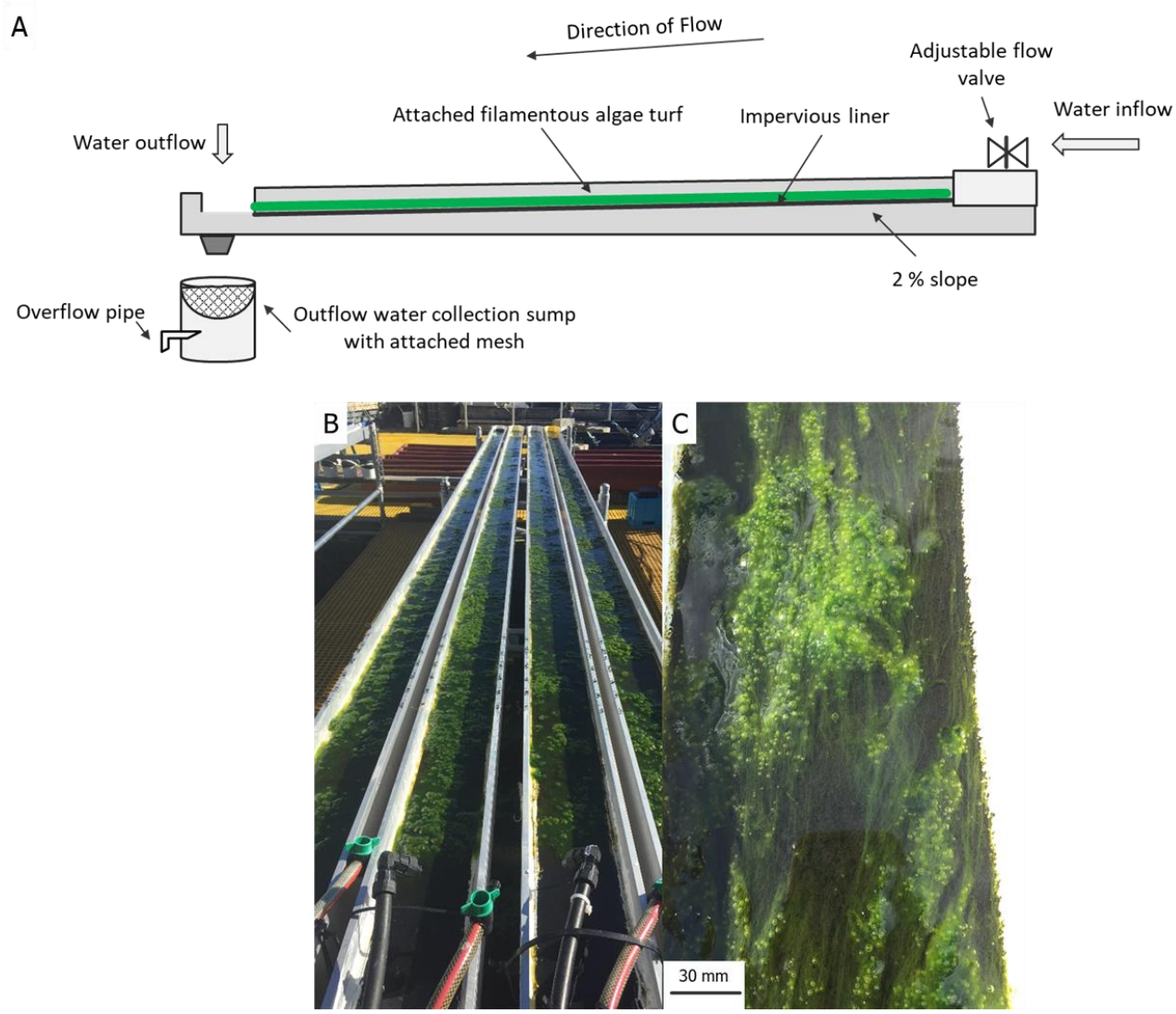

Figure 3.1: Outdoor mesocosm-scale FANS used in this study (a) Schematic diagram of FANS, (b) Four adjacent FANS, (c) Close-up of filamentous algae attached to FANS liner

Nutrient enriched water was pumped to the top of all FANS from a water storage tank. A new batch of concentrated nutrient stock solution was prepared each week using hydroponic grade NO_3-N (YaraLiva) and di-potassium hydrogen phosphate, K_2HPO_4 (EMSURE®). This nutrient stock solution was added to the water storage tank using a peristaltic dosing pump to dilute it with filtered water inside the storage tank to produce an inflow concentration of 2 mg L^{-1} of NO_3-N and 0.2 mg L^{-1} of PO_4-P. These concentrations were selected based on preliminary trials investigating nutrient removal rates of all four species in a continuous flow FANS system which showed that concentrations of nitrate and phosphate used in this study

ensured nutrients were up to 50% in excess of requirements for growth over the five-minute hydraulic retention time of the FANS. The flow rate of nutrient enriched water to each FANS was controlled by an adjustable valve at the top of each FANS floway. Water continuously flowed from the top (inflow) end to the bottom (outflow) end of each FANS floway and then drained into a separate collection sump. Water was not recirculated. A 200 µm mesh bag was attached to the outlet water collection sump at the bottom end of the FANS to collect any algal biomass that sloughed off the floway.

The performance of the four algal species was assessed in two separate experiments conducted in summer and winter under exactly the same experimental setup unless otherwise specified. The summer experiment was conducted for six five-day growth cycles after a ten-day seeding stage (January to February 2021, total of 40 days). The winter experiment was conducted for two 14-day growth cycles after a 24-day seeding stage (July to August 2021, total of 52 days). Light intensity, ambient temperature and FANS outflow water temperature were continuously recorded during each experiment using three loggers (HOBO Pendant MX2200 water temperature data logger, HOBO Pendant MX2200 light data logger and HOBO MX100 ambient temperature data logger). The water temperature data logger was placed inside one of the FANS outflow sumps. The light data logger was secured next to the FANS on a leveled horizontal surface. The ambient temperature data logger was positioned close to the FANS and shaded from direct sunlight. In the summer experiment, the sunlight intensity ranged from 500-2500 µmol m^{-2} s^{-1} (average of 14:10 light and dark cycle), the ambient temperature ranged from 10-33°C, and the FANS outflow water temperature ranged from 14-33°C (Figures 3A.1-3). In the winter experiment, the sunlight intensity ranged from 200-1300 µmol m^{-2} s^{-1} (average of 10:14 light and dark cycle), the ambient temperature ranged from -1-20°C, and the FANS outflow water temperature ranged from 1-21°C (Figures 3A.1-3).

3.3.2 Seeding phase

Four locally isolated filamentous algae species - *Oedogonium* sp., *Spirogyra* sp., *Rhizoclonium* sp., and *Cladophora* sp. - were used in this study . All FANS were placed parallel to each other in three blocks, with four FANS in a single block. One replicate FANS of each species was allocated to each block. Each species was placed in a different position within each block to minimise any environmental/edge effects. Three replicate FANS were seeded with each filamentous algae species. Each FANS was seeded by evenly distributing algal biomass

(equivalent to a standardized amount of 5 g dry weight (DW) or loading rate of 7 g DW m^{-2}) by rubbing it down the textured liner to hook algal filaments onto the liner, providing initial physical attachment (hooking). Over time, secondary biological attachment on the liner occurred through zoospore production, settling and holdfast development; mucilage secretion; and rhizoid formation from the hooked basal regions of the filaments. This seeding method was selected based on lab-scale trials that showed that rubbing biomass on the liner surface rapidly established a dense algal turf and resulted in denser and more even biomass coverage than seeding using algae zoospores (Hariz et al., 2022).

The nutrient-enriched water was continuously pumped over the surface of the flow-way at a rate of 0.5 L min^{-1} (4 L min^{-1}. m width of floway) during the seeding phase. Preliminary trials indicated that this flow rate was sufficient to cover the entire surface area of the floway and keep the newly seeded algae biomass submerged under water but was low enough to avoid washing the biomass off from the floway. Once the biomass had uniformly established on the liner, the flow rate was increased to 1 L min^{-1} (8 L min^{-1}. m width of flow-way). This flow rate was maintained for the rest of experiment.

The seeding phase lasted for ten days in the summer experiment and 24 days in the winter experiment. Any algae that had washed off the floway was reseeded each day for the first five days of the summer experiment and the first 14-days of the winter experiment by adding, rubbing, and spreading the algae biomass over the liner surface to rapidly establish a dense algal turf on each FANS. All FANS were covered with 50% UV shade cloth mesh for the first five days of the seeding stage in the summer experiment to facilitate acclimation of the algal biomass to the high summer light intensity and temperature. The same shade cloth mesh was used for the first seven days in the winter experiment to minimise the impacts of heavy rainfall on the floway surface, which could disrupt the initial algae attachment and wash biomass off the floway. All four species took about five to seven days in summer to establish on the FANS liner and 15-20 days in winter (Figure 3A.4, Table 3.1).

Table 3.1: Time taken (days) to establish uniform biomass of four filamentous algae species on FANS floways during the seeding stage of summer and winter experiments and a biomass re-establishment experiment

Species	Summer experiment	Winter experiment	Biomass re-establishment experiment
Oedogonium sp.	5	15	3
Spirogyra sp.	6	18	4
Rhizoclonium sp.	7	20	3
Cladophora sp.	7	20	3

3.3.3 Biomass productivity

After the seeding phase, algal biomass on each FANS was harvested by drawing a metal scraper along the length of the liner to leave a uniform coverage of attached algal biomass as an initial standing crop to start the experiment. A different scraper was used for each species to avoid cross-contamination. A standardized initial standing crop of 70-80 g dry weight (DW) m^2 (approximately 0.5-1 cm algal turf height) was targeted for all species at the start of each growth cycle to establish a comparable productivity and nutrient removal performance among species. A small subsample of attached biomass (~ 2 g FW) was collected from each replicate FANS on the day before harvest day. The biomass subsamples were dewatered by compressing the FW biomass over 200 µm filter mesh until no more water droplets were produced, weighed to determine the fresh weight (FW), and dried overnight in an oven at 65ºC. Samples were reweighed the following morning to determine the DW and enable the calculation of an individual DW:FW ratio for each replicate FANS. This DW:FW ratio was used to estimate the amount of biomass that needed to be harvested from each replicate to achieve the target standardized initial standing crop.

A strip biomass monitoring method was used to quantify the attached algal standing crop in g DW on each replicate FANS. The attached biomass was completely scraped off from a horizontal strip (0.12 m x 0.025 m) at three points (top, middle and bottom) along the length of each FANS. The scraped biomass samples were dewatered as described above and weighed to determine the FW. The DW standing crop of the entire FANS was then estimated using the

DW:FW ratio obtained from the biomass subsampling undertaken on the day before harvesting using the equation:

Standing crop (g DW) = [(FW Top + FW Middle + FW Bottom) / 3] x [DW:FW ratio] x 240

where 240 is the ratio of the total FANS area to the horizontal strip biomass monitoring area.

The biomass on each FANS was then harvested to achieve the target standardized initial standing crop. Following harvesting, strip biomass monitoring was repeated for each replicate FANS following the methods described above to quantify the initial standing crop (standing crop at the start of each growth cycle). All strip biomass monitoring points were marked for future reference to ensure the same sampling point was avoided in subsequent strip monitoring and observe the time taken for biomass to regrow.

Strip biomass monitoring was repeated after five days for the summer experiment and 14 days for the winter experiment following the methods described above to determine the DW final standing crop for each replicate FANS. FANS were then harvested as described above, and the initial standing crop, final standing crop and biomass productivity for each FANS were calculated as follows:

Initial Standing Crop (g DW) = [(DW Top + DW Middle + DW Bottom)/3] X 240

Final Standing Crop (g DW) = [(DW Top + DW Middle + DW Bottom)/3] X 240

where 240 is the ratio of the total FANS area to the horizontal strip biomass monitoring area.

Biomass Productivity (g DW m^{-2} d^{-1}) = [(DW Final Standing Crop – DW Initial Standing Crop) / FANS area] / total number of days of growth

This protocol was repeated six times during the summer experiment (six consecutive harvests and growth cycles), and two times during the winter experiment (two consecutive harvests and growth cycles). In the winter experiment, strip biomass monitoring was also conducted after seven days to provide an interim estimate of biomass productivity between

each harvest. Biomass that sloughed off from the FANS between harvest dates was collected in a 200 µm mesh bag attached to the outflow water collection sump at the bottom end of the FANS. This biomass was not reseeded onto FANS, but the weight of the sloughed biomass was added to the weight of the harvested biomass and thereby included in the biomass productivity quantification.

3.3.4 Nutrient removal analysis

Water samples were collected on day one, three and five in summer and on day two, four and seven in winter from the inflow and outflow of each individual FANS to determine the amount of nitrate (NO_3-N) and dissolved reactive phosphate (DRP) removed by each FANS. The concentration of nitrate and phosphate in each water sample was determined using the ultraviolet spectrophotometric method (Standard Method Nitrate 4500-NO3−B) and the ascorbic acid method (Standard Method Phosphorus 4500-P) on a microplate reader (SPECTROstar Nano, BMG LABTECH). The amount of nutrients removed by each replicate FANS for each growth cycle was calculated as the average difference in nutrient concentrations between the inflow and outflow samples for the three water samples.

3.3.5 Relative species abundance

At the end of every growth cycle, a small sample (~ 2 g FW) of harvested biomass from each FANS was collected prior to dewatering to quantify the relative proportion of target algae species and other contamination in the harvested biomass under the microscope. Samples were preserved in a 15 ml vial with a few drops of Lugol's solution. All samples were analysed within five days of collection. Immediately before analysis, each sample was filtered using a 200 µm sieve and then washed with 10 ml of filtered water which was collected for analysis (filtrate). This step separated epiphytic diatoms, midges, and any other visible organisms attached to the algal filaments to make it easier to see cellular detail and identify algae species under the microscope. The retained algal biomass and 10 ml filtrate were analysed under an inverted microscope (Leica DMi1). The combined biomass and filtrate were proportionately divided onto each of ten microscopy slides for each sample. Then a photograph was taken under 100 X magnification from each slide for a total of ten photos for each sample. In each photo, the percentage composition of each of the four study species and broad classes of algae and

other contaminants (other green filamentous algae, diatoms, and cyanobacterial) was estimated based on the surface area covered. The average composition across ten photographs was quantified to get an overall percent composition for each sample.

3.3.6 Re-establishment experiment

All FANS had become dominated by non-target species of microalgae and cyanobacteria by the end of growth cycle 6 of the summer experiment (see Results, Section 3.4.3). Therefore, to investigate how quickly each species was able to re-establish and regain dominance, each FANS was reseeded with algae biomass from stock cultures of each species on top of existing attached biomass and the summer experiment was continued for a further three five-day growth cycles. During this re-establishment experiment, the sunlight intensity ranged from 600-2000 µmol m^{-2} s^{-1} (average of 14:10 light and dark cycle), the ambient temperature ranged from 10-33°C, and FANS water temperature ranged from 18-31°C (Figure 3A.5). Before reseeding, all FANS were harvested to leave a standardized standing of crop 60-70 g DW m^{-2}. About 2 g DW (~ 3 g DW m^{-2}) biomass was then added to each FANS by rubbing the biomass across the surface of the liner to re-establish the algae for the subsequent growth cycle. Biomass was reseeded only on a single instance at the start of the re-establishment experiment (at the beginning of growth cycle seven). This re-establishment experiment followed the same methods described above for the summer experiment. However, the initial standing crop for growth cycle seven was calculated as the sum of the total amount of biomass added and the standing crop before reseeding. A re-establishment experiment was not conducted at the end of the winter experiment.

3.3.7 Statistical analyses

Differences in the initial standing crop, DW:FW ratio, biomass productivity, and nitrate and phosphate removal between species were tested using two-way analyses of variance (ANOVA) with species and growth cycle as fixed factors, or a Kruskal-Wallis test for variables that failed normality and/or homogeneity of variance tests. Linear regression analysis was used to test for a relationship between nitrate removal rate and specific growth rate, and phosphate removal rate and specific growth rate. Data for summer and winter experiments were analysed

separately. All statistical analyses were carried out using SigmaPlot software (Systat Software Inc., Point Richmond, CA, USA). All data are reported as means ±S.D.

The specific growth rate (% d^{-1}) was calculated for each replicate FANS using the formula:

Specific Growth Rate (% d^{-1}) = [(Ln DW Final Standing Crop – Ln DW Initial Standing Crop) / total number of days of growth] x 100%

3.4 Results

3.4.1 Biomass productivity

The initial standing crop varied significantly among species and growth cycles in the summer experiment, ranging from 77-81 g DW m^{-2} across all four species. *Oedogonium* sp. and *Spirogyra* sp. had the highest initial standing crop on average across all growth cycles (80.5 g DW m^{-2} ± 3.4 and 80.4 g DW m^{-2} ± 3.0, respectively) and *Rhizoclonium* sp. and *Cladophora* sp. had the lowest initial standing crop on average across all growth cycles (77.7 g DW m^{-2} ± 2.9 and 76.9 g DW m^{-2} ± 2.3, respectively) (Table 3.2, Figure 3.2). However, the species with the highest initial standing crop varied among growth cycles, as evidenced by a significant growth cycle x species interaction effect (Table 3.3). The initial standing crop in the winter experiment was slightly lower compared to the summer experiment for each species, but also varied significantly among species and growth cycles, ranging from 70-76 g DW m^{-2} across all four species. *Oedogonium* sp. had the highest initial standing crop on average across all growth cycles (75.9 g DW m^{-2} ± 1.9) and *Spirogyra sp.* had the lowest initial standing crop on average across all growth cycles (69.9 g DW m^{-2} ± 1.7) (Table 3.2, Figure 3.2). However, the species with the highest initial standing crop was variable among growth cycles, as evidenced by a significant growth cycle x species interaction effect (Table 3.4).

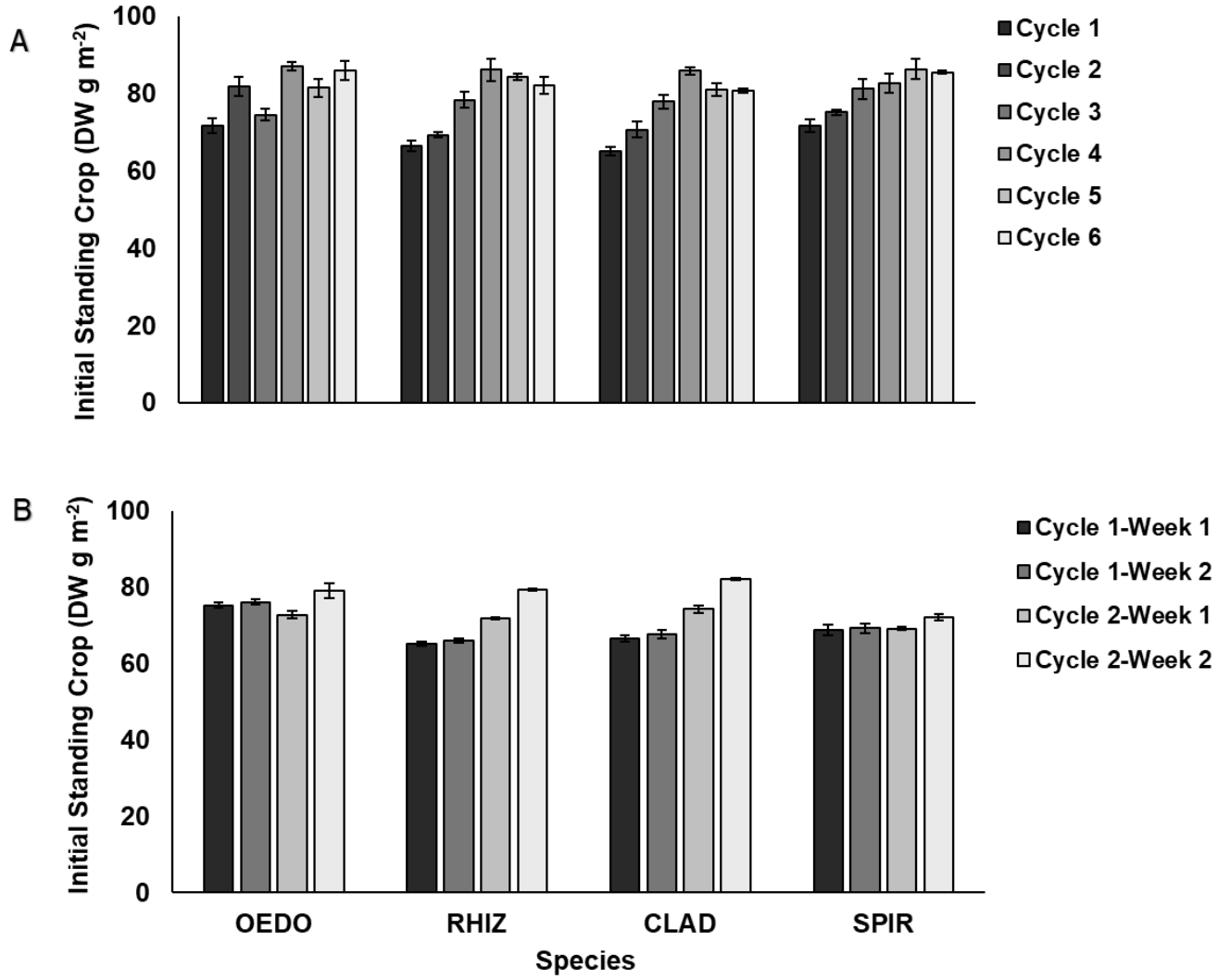

Figure 3.2*:* Average (±S.D.) initial standing crop (g DW m^{-2}) of four species of filamentous algae over (a) six five-day growth cycles during summer and (b) two 14-day growth cycles during winter. OEDO: *Oedogonium* sp., RHIZ: *Rhizoclonium* sp., CLAD: *Cladophora* sp., SPIR: *Spirogyra* sp. N = 3

DW:FW ratios varied significantly among species in the summer experiment, but this variation was inconsistent among growth cycles, as evidenced by a significant growth cycle x species interaction effect (Table 3.3, Figure 3.3). *Rhizoclonium* sp. had the highest DW:FW ratio on average across all growth cycles (0.36 ± 0.03), while *Spirogyra* sp. had the lowest DW:FW ratio (0.19 ± 0.02, Table 3.3). The average DW: FW ratio of *Oedogonium* sp. and *Cladophora* sp. across all growth cycles was 0.31 ± 0.03 and 0.30 ± 0.03, respectively. Similarly, DW:FW ratios varied significantly among species in the winter experiment, but this variation was inconsistent among growth cycles, as evidenced by a significant growth cycle x species interaction effect (Table 3.4, Figure 3.3). *Cladophora* sp. had the highest DW:FW ratio

on average across all growth cycles (0.31 ± 0.02), while *Spirogyra* sp. had the lowest DW:FW ratio (0.24 ± 0.03, Table 3.3). The average DW: FW ratio of *Rhizoclonium* sp. and *Oedogonium* sp. across all growth cycles was 0.29 ± 0.03 and 0.26 ± 0.03, respectively. The DW:FW ratios for each species were comparable between summer and winter experiments. There was a trend of decreasing DW:FW ratios through both the summer and winter experiments, potentially due to the increased contamination of the other non-target species of microalgae and cyanobacteria (see Results, Section 3.4.3) that affected the biomass quality of the initially seeded unialgal species.

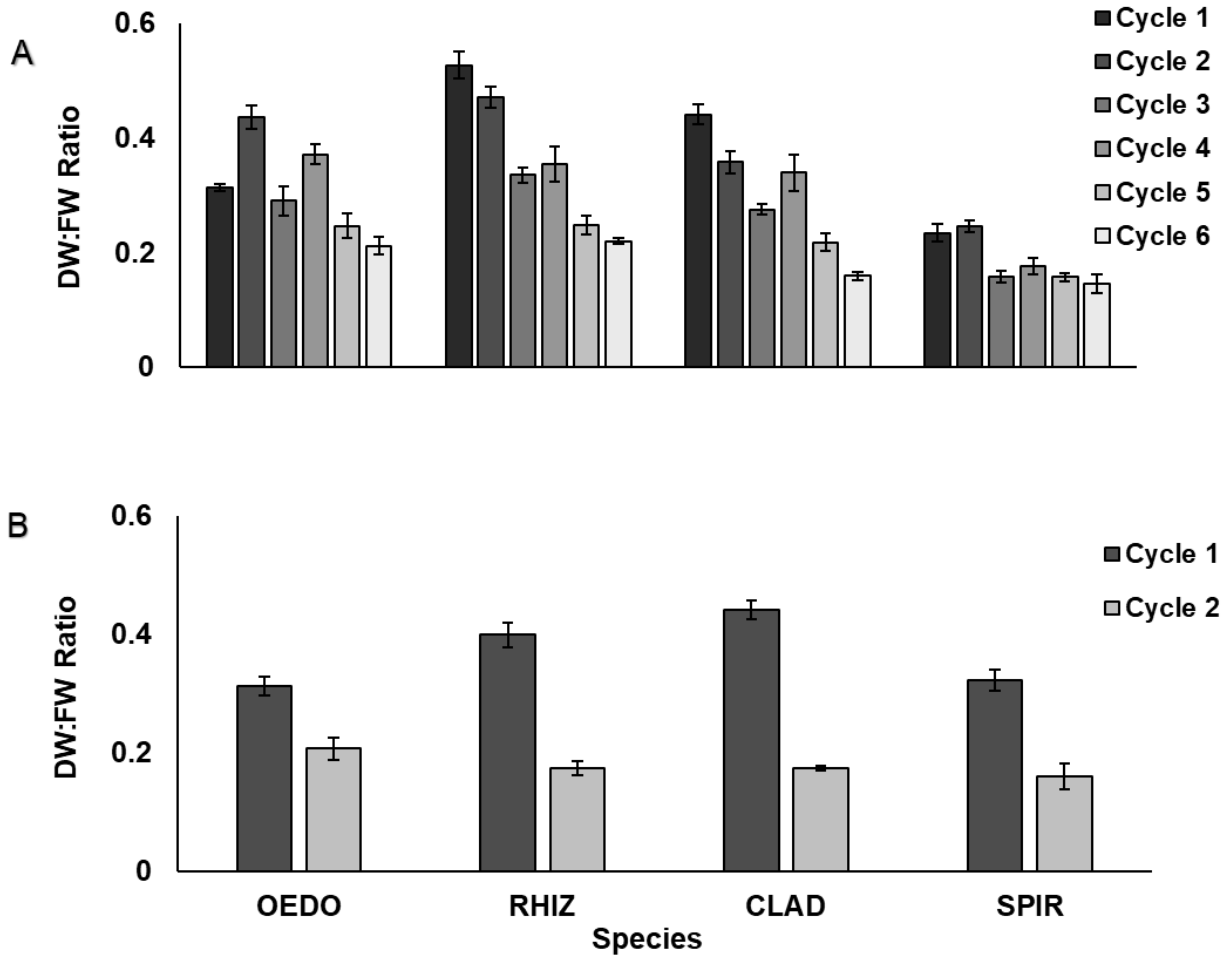

Figure 3.3: Average (± S.D.) DW: FW ratio of four species of filamentous algae over a) six five-day growth cycles during summer and b) two 14-day growth cycles during winter. OEDO: *Oedogonium* sp., RHIZ: *Rhizoclonium* sp., CLAD: *Cladophora* sp., SPIR: *Spirogyra* sp. N = 3

Biomass productivity varied significantly among species and growth cycles in the summer experiment. However, the species with the highest biomass productivity was not consistent among growth cycles, as evidenced by a significant growth cycle x species interaction effect (Table 3.3, Figure 3.4). *Oedogonium* sp. had the highest biomass productivity of all species in all growth cycles except in growth cycle six, where *Rhizoclonium* sp. had the highest biomass productivity. While in all growth cycles, *Spirogyra* sp. had the lowest biomass productivity. All species had their lowest biomass productivity in growth cycle six. Across all growth cycles, average biomass productivity of *Oedogonium* sp. (4.5 g DW m^{-2} d^{-1} ± 0.5), *Rhizoclonium* sp. (3.5 g DW m^{-2} d^{-1} ± 0.7), and *Cladophora* sp. (2.5 g DW m^{-2} d^{-1} ± 0.8) was at least two-fold higher than *Spirogyra* sp. (1.2 g DW m^{-2} d^{-1} ± 0.4) (Table 3.2). The global average biomass productivity of each species in the summer experiment was more than one and a half times that measured in the winter experiment. Biomass productivity varied significantly among species and among growth cycles for each species in the winter experiment (Table 3.4, Figure 3.4). *Oedogonium* sp. had the highest biomass productivity of all species in all growth cycles except in growth cycle two, where *Rhizoclonium* sp. had the highest biomass productivity, *Spirogyra* sp. had the lowest biomass productivity in all growth cycles. Across all growth cycles, average biomass productivity of *Oedogonium* sp. (1.6 g DW m^{-2} d^{-1} ± 0.3), *Rhizoclonium* sp. (1.4 g DW m^{-2} d^{-1} ± 0.1), and *Cladophora* sp. (1.4 g DW m^{-2} d^{-1} ± 0.2) was at least double that of *Spirogyra* sp. (0.7 g DW m^{-2} d^{-1} ± 0.1) (Table 3.2).

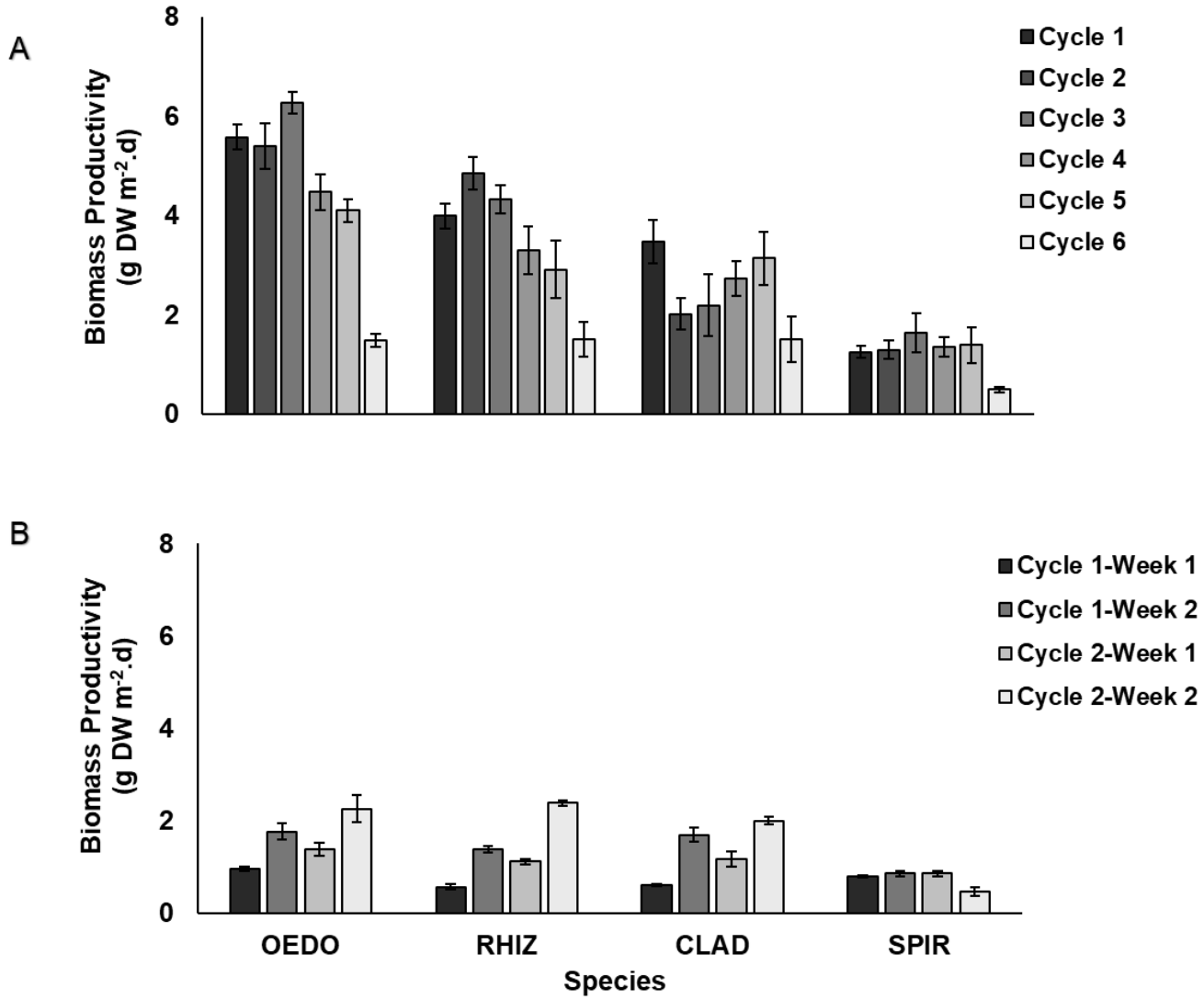

Figure 3.4: Average (± S.D.) biomass productivity (g DW m^{-2} d^{-1}) of four species of filamentous algae over a) six five-day growth cycles during summer and b) two 14-day growth cycles during winter. OEDO: *Oedogonium* sp., RHIZ: *Rhizoclonium* sp., CLAD: *Cladophora* sp., SPIR: *Spirogyra* sp. N = 3

3.4.2 Nutrient removal

Nitrate removal rates did not vary significantly with species or growth cycles in the summer experiment (Table 3.3). *Oedogonium* sp. had the highest average nitrate removal rate (0.51 g N m^{-2} d^{-1} ± 0.07) across all growth cycles, followed by *Rhizoclonium* sp. (0.47 g N m^{-2} d^{-1} ± 0.08), *Cladophora* sp. (0.46 g N m^{-2} d^{-1} ± 0.09) and *Spirogyra* sp. (0.39 g N m^{-2} d^{-1} ± 0.06) (Table 3.2, Figure 3.5). Nitrate removal rates for each species were comparable between summer and winter experiments. However, in contrast to the summer experiment, nitrate

removal rates varied significantly among species in the winter experiment, but the relative performance of species was not consistent among growth cycles, as evidenced by a significant species x growth cycle interaction effect (Table 3.4). Across all growth cycles in the winter experiment, the nitrate removal rate was highest for *Oedogonium* sp. (0.51 g N m^{-2} d^{-1} ± 0.06) followed by *Rhizoclonium* sp. and *Cladophora* sp. (0.50 g N m^{-2} d^{-1} ± 0.05 and 0.50 g N m^{-2} d^{-1} ± 0.08 respectively) and *Spirogyra* sp. (0.32 g N m^{-2} d^{-1} ± 0.07). (Table 3.2, Figure 3.5).

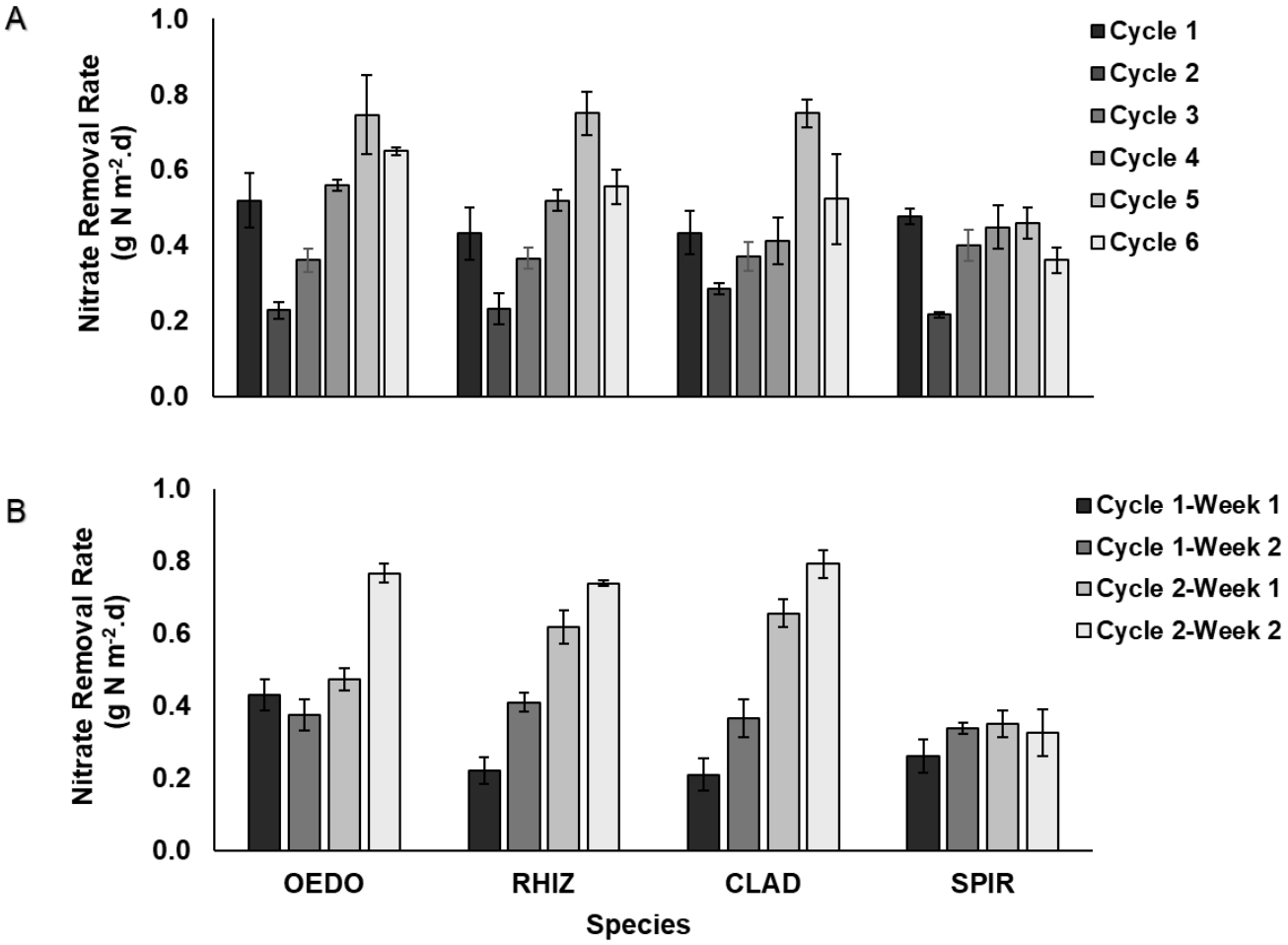

Figure 3.5: Average (± S.D.) nitrate removal rate (g N m^{-2} d^{-1}) of four species of filamentous algae over a) six five-day growth cycles during summer and b) two 14-day growth cycles during winter. OEDO: *Oedogonium* sp., RHIZ: *Rhizoclonium* sp., CLAD: *Cladophora* sp., SPIR: *Spirogyra* sp. N = 3

Phosphate removal rates did not vary significantly among species in the summer experiment, and across all growth cycles ranged from 0.16 - 0.18 g P m^{-2} d^{-1} (Tables 3.2 and 3, Figure 3.6). However, phosphate removal rates varied significantly among growth cycles, and

across all species were highest in growth cycle five (0.29 g P m^{-2} d^{-1} ± 0.05) and lowest in growth cycle one (0.08 g P m^{-2} d^{-1} ± 0.01) (Table 3.3). The global average phosphate removal rate in the summer experiment was more than one and a half times that measured in the winter experiment for each species (Table 3.2, Figure 3.6). In the winter experiment, phosphate removal rate did not vary significantly between species or growth cycles, and across all growth cycles ranged from 0.08 - 0.09 g P m^{-2} d^{-1} (Tables 3.2 and 3.4, Figure 3.6).

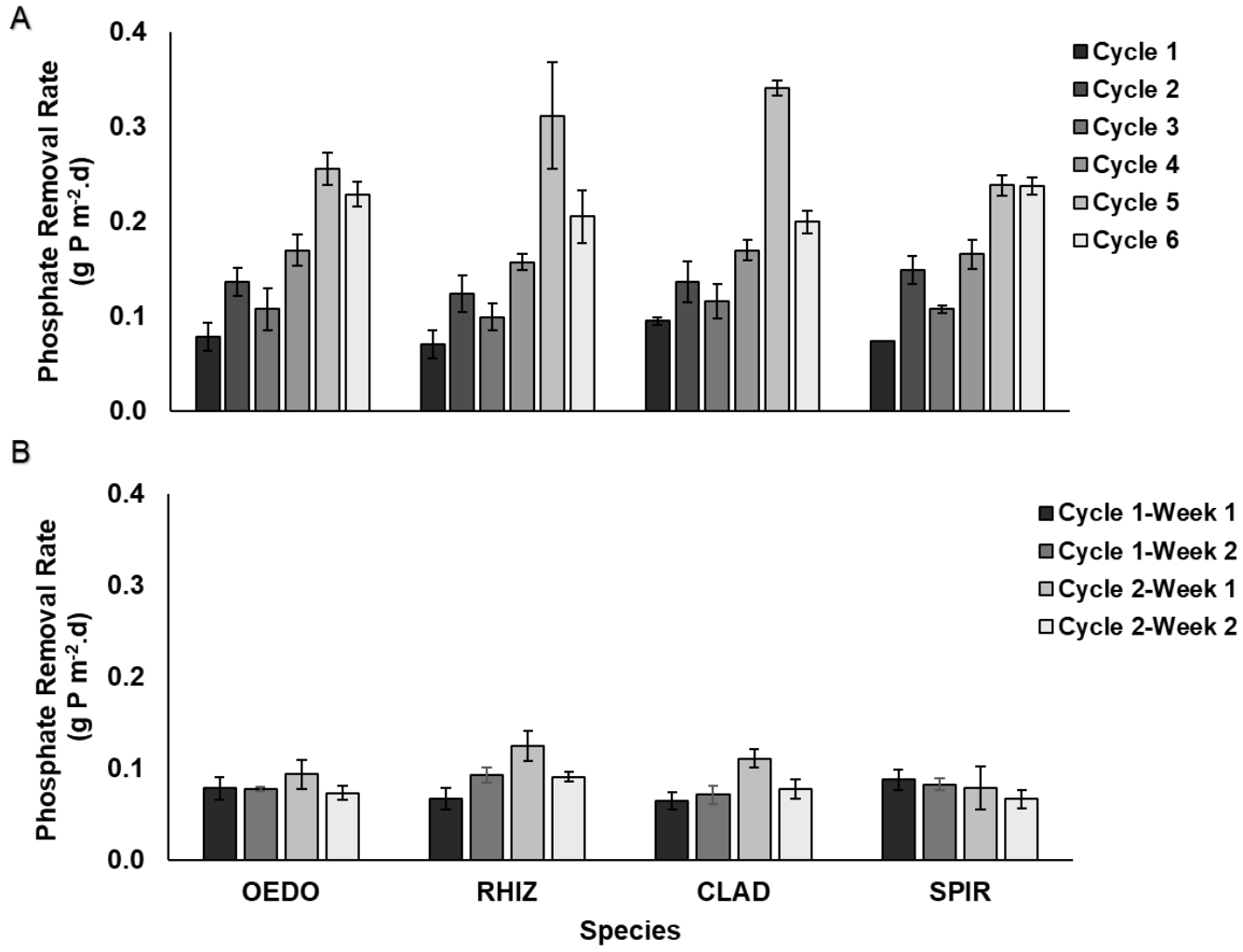

Figure 3.6: Average (± S.D.) phosphate removal rate (g P m^{-2} d^{-1}) of four species of filamentous algae over a) six five-day growth cycles during summer and b) two 14-day growth cycles during winter. OEDO: *Oedogonium* sp., RHIZ: *Rhizoclonium* sp., CLAD: *Cladophora* sp., SPIR: *Spirogyra* sp. N = 3

Table 3.2: Summary of key biomass productivity and nutrient removal parameters for four filamentous algae species on FANS under summer conditions over six five-day growth cycles and winter conditions over two 14-day growth cycles. Data are means ± standard deviations. Mean values for each growth cycle were averaged to provide a global mean for each species

Parameter	*Oedogonium* sp.	*Rhizoclonium* sp.	*Cladophora* sp.	*Spirogyra* sp.
		Summer		
Biomass Productivity				
Initial Standing Crop (g DW m^{-2})	80.5 ± 3.4	77.7 ± 2.9	76.9 ± 2.3	80.4 ± 3.0
DW:FW Ratio	0.31 ± 0.03	0.36 ± 0.03	0.30 ± 0.03	0.19 ± 0.02
Biomass Productivity (g DW biomass m^{-2} d^{-1})	4.5± 0.5	3.5 ± 0.7	2.5 ± 0.8	1.2 ± 0.4
Nutrient Removal				
Nitrate removal rate (g N m^{-2} d^{-1})	0.51 ± 0.07	0.47 ± 0.08	0.46 ± 0.09	0.39 ± 0.06
Phosphate removal rate (g P m^{-2} d^{-1})	0.16 ± 0.03	0.16 ± 0.04	0.18 ± 0.02	0.16 ± 0.02
		Winter		
Biomass Productivity				
Initial Standing Crop (g DW m^{-2})	75.9 ± 1.9	70.7 ± 0.7	72.7 ± 1.4	69.9 ± 1.7
DW:FW Ratio	0.26 ± 0.03	0.29 ± 0.03	0.31 ± 0.02	0.24 ± 0.03
Biomass Productivity (g DW biomass m^{-2} d^{-1})	1.6± 0.3	1.4 ± 0.1	1.4 ± 0.2	0.7 ± 0.1
Nutrient Removal				
Nitrate removal rate (g N m^{-2} d^{-1})	0.51 ± 0.06	0.50 ± 0.05	0.50 ± 0.08	0.32 ± 0.07
Phosphate removal rate (g P m^{-2} d^{-1})	0.08 ± 0.02	0.09 ± 0.02	0.08 ± 0.02	0.08 ± 0.02

Table 3.3: Results of statistical analyses testing the effects of growth cycles and species on the initial standing crop, DW:FW ratio, biomass productivity, and nutrient removal rate of four filamentous algae species in the summer experiment

Variable	Effect	df	F	P
Initial Standing Crop (g DW m^{-2})	Species	3	6.0	0.001
	Cycle	5	49.8	<0.001
	Species x Cycle	15	2.8	0.003
	Residual	48		
DW:FW Ratio	Species	3	99.8	< 0.001
	Cycle	5	80.8	< 0.001
	Species x Cycle	15	7.5	< 0.001
	Residual	48		
Biomass Productivity (g DW biomass m^{-2} d^{-1})	Species	3	91.3	<0.001
	Cycle	5	24.1	<0.001
	Species x Cycle	15	4.7	<0.001
	Residual	48		
Nitrate Removal Rate (g N m^{-2} d^{-1}) [1]	Species	3	1.6	0.207
	Cycle	5	0.5	0.803
	Species x Cycle	15	0.9	0.552
	Residual	48		
Phosphate Removal Rate (g P m^{-2} d^{-1})[1]	Species	3	1.0	0.419
	Cycle	5	68.5	<0.001
	Species x Cycle	15	1.5	0.132

	Residual	48		

[1] Variables were analysed using a Kruskal-Wallis test as they did not satisfy ANOVA assumptions

Table 3.4: Results of statistical analyses testing the effects of growth cycles and species on the initial standing crop, DW:FW ratio, biomass productivity, and nutrient removal of four filamentous algae species in the winter experiment

Variable	Effect	df	F	P
Initial Standing Crop (g DW m^{-2}) [1]	Species	3	10.1	<0.001
	Cycle	1	46.9	<0.001
	Species x Cycle	3	11.3	<0.001
	Residual	40		
DW:FW Ratio	Species	3	5.5	0.009
	Cycle	1	243.9	<0.001
	Species x Cycle	3	8.3	0.001
	Residual	16		
Biomass Productivity (g DW biomass m^{-2} d^{-1}) [1]	Species	3	6.5	0.001
	Cycle	1	6.9	0.012
	Species x Cycle	3	1.8	0.156
	Residual	40		
Nitrate Removal Rate (g N m^{-2} d^{-1})	Species	3	9.6	<0.001
	Cycle	1	75.9	<0.001
	Species x Cycle	3	8.4	<0.001
	Residual	40		

Phosphate Removal Rate (g P m^{-2} d^{-1})	Species	3	1.2	0.311
	Cycle	1	3.5	0.067
	Species x Cycle	3	2.4	0.084
	Residual	40		

[1] Variables were analysed using a Kruskal-Wallis test as they did not satisfy ANOVA assumptions

Linear regression analyses showed that specific growth rate (% day^{-1}) was a significant predictor of nitrate removal rate (g N m^{-2} d^{-1}) rate for *Oedogonium* sp. ($F_{1,16}$ = 5.4, P = 0.03) and *Rhizoclonium* sp. ($F_{1,16}$ = 14.8, P = 0.001) in the summer experiment, but not for *Cladophora* sp. ($F_{1,16}$ = 0.22, P = 0.65) and *Spirogyra* sp. ($F_{1,16}$ = 0.37, P = 0.55) (Figure 3.7). However, the coefficient of determination (R^2) showed a weak linear relationship between these two variables for *Oedogonium* sp. (R^2=0.251) and *Rhizoclonium* sp. (R^2=0.481). The specific growth rate was a significant predictor of nitrate removal rate for all species in the winter experiment (*Oedogonium* sp.: $F_{1,10}$ = 15.1, P = 0.003; *Rhizoclonium* sp.: $F_{1,10}$ = 76.3, P < 0.0001; *Cladophora* sp.: $F_{1,10}$ = 16.6, P = 0.002; and *Spirogyra* sp.: $F_{1,10}$ = 10.2, P = 0.01; Figure 3.8). The coefficient of determination (R^2) showed a moderate linear relationship between these two variables for *Oedogonium* sp. (R^2=0.602), *Cladophora* sp. (R^2=0.624), and *Spirogyra* sp. (R^2=0.505) and a strong linear relationship for *Rhizoclonium* sp. (R^2=0.884). Linear regression analyses showed that specific growth rate (% day^{-1}) was a significant predictor of phosphate removal rate (g P m^{-2} d^{-1}) rate for all species in the summer experiment (*Oedogonium* sp.: $F_{1,16}$ = 20.3, P = 0.0004; *Rhizoclonium* sp.: $F_{1,16}$ = 6.8, P = 0.02 and *Spirogyra* sp.: $F_{1,16}$ = 4.5, P = 0.05) except *Cladophora* sp. ($F_{1,16}$ = 0.25, P = 0.62) (Figure 3.7). However, the coefficient of determination (R^2) showed a moderate linear relationship between these two variables for *Oedogonium* sp. (R^2=0.560), and a weak linear relationship for *Rhizoclonium* sp. (R^2=0.298), and *Spirogyra* sp. (R^2=0.218). In contrast, linear regression analyses showed that specific growth rate (% day^{-1}) was not a significant predictor of phosphate removal rate for all species (*Oedogonium* sp.: $F_{1,10}$ = 0.02, P = 0.90, *Rhizoclonium* sp. $F_{1,10}$ = 2.21, P = 0.17, *Cladophora* sp. $F_{1,10}$ = 0.84, P = 0.38, and *Spirogyra* sp. $F_{1,10}$ = 0.77, P = 0.40) in the winter experiment (Figure 3.8).

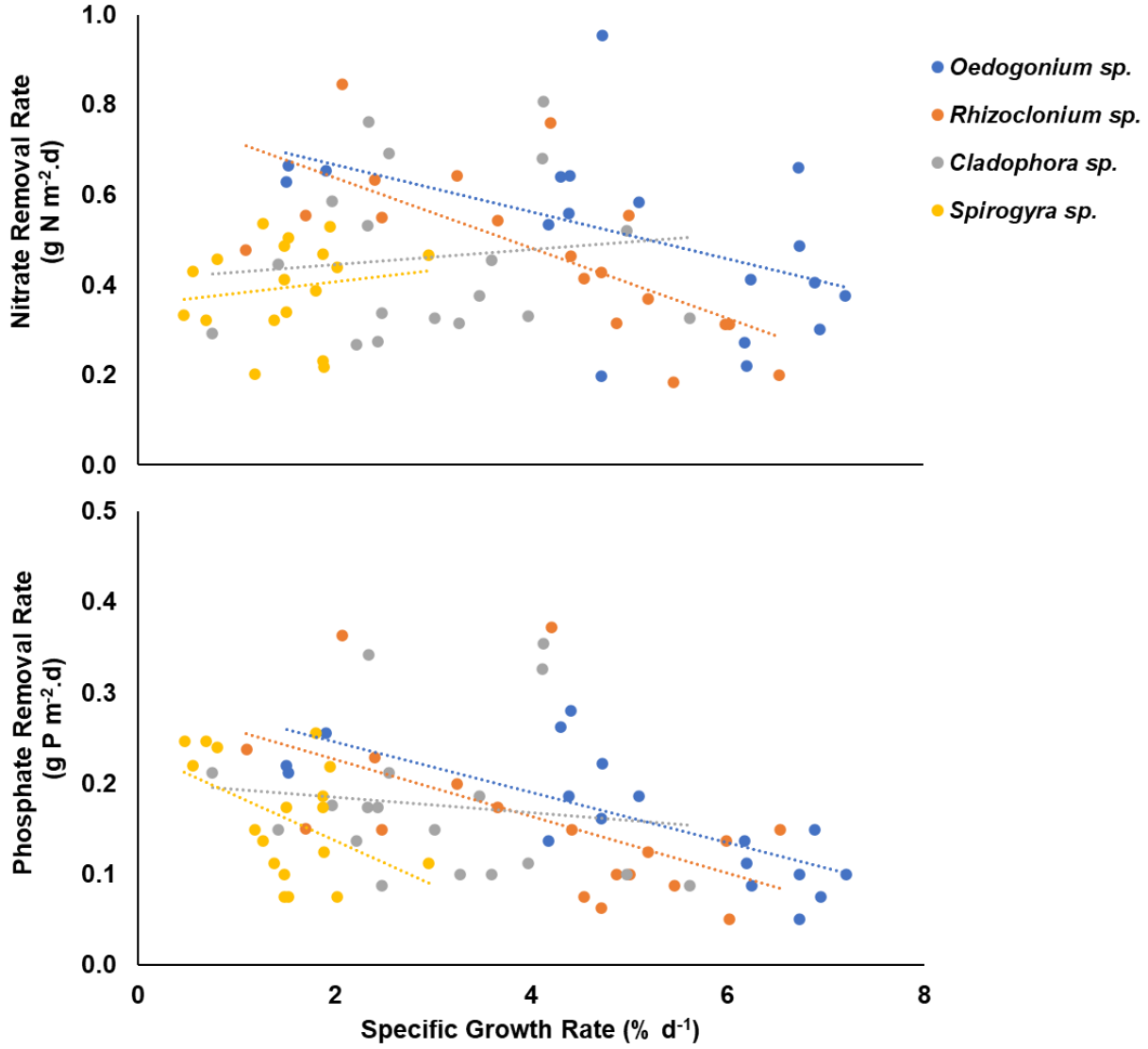

Figure 3.7: Relationship between nitrate removal rate (g N m^{-3} d^{-1}, upper panel) and phosphate removal rate (g P m^{-3} d^{-1}, lower panel) and specific growth rate (% day^{-1}) for four species of algae over six growth cycles in the summer experiment. Values for each replicate FANS for each species and growth cycle are plotted

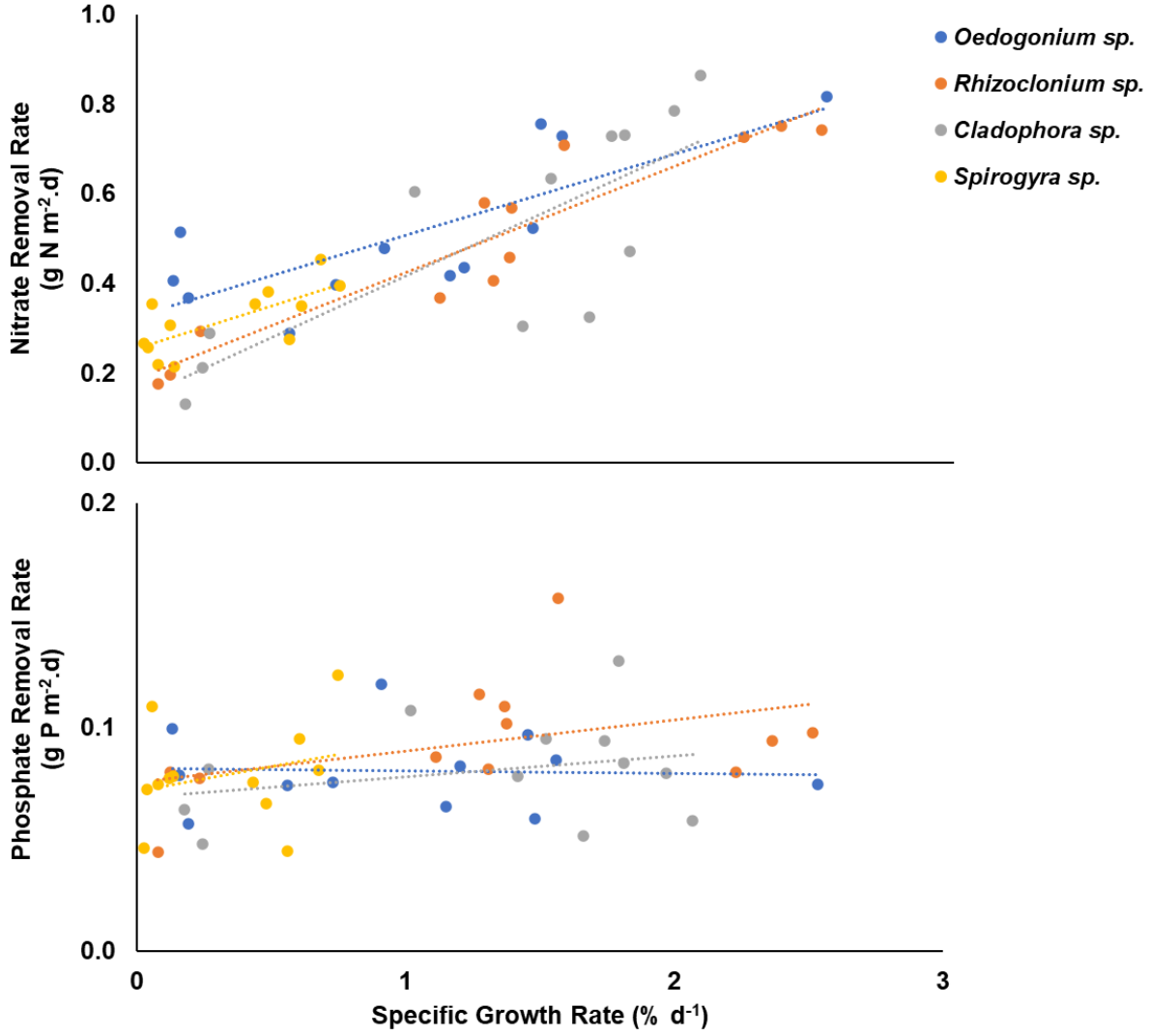

Figure 3.8: Relationship between nitrate removal rate (g N m^{-3} d^{-1}, upper panel) and phosphate removal rate (g P m^{-3} d^{-1}, lower panel) and specific growth rate (% day^{-1}) for four species of algae over two growth cycles in the winter experiment. Values for each replicate FANS for each species and growth cycle are plotted

3.4.3 Relative species abundance

All FANS were initially seeded with biomass from unialgal stock cultures (~ 99% monoculture of target filamentous algae species). However, the relative abundance of each target species that was initially seeded on FANS decreased substantially as the experiment progressed due to the growth of other non-target algae species, including free-floating diatoms (*Cymbella* sp. and *Nitzschia* sp.) and filamentous cyanobacteria (*Oscillatoria* sp.) on the floways. In the summer experiment, the target species initially seeded onto the FANS

comprised more than 50% of the harvested biomass for the first 30 days after seeding. At the end of the summer experiment, we found most FANS had been dominated by non-target species of microalgae and cyanobacteria and a significant quantity of midges and cocoons were present on all replicate FANS. *Oedogonium* sp. had the highest relative abundance in harvested biomass (nearly 60% at the end of the summer experiment, 43 days after seeding) compared to the other three species (Figure 3.9). The relative abundance of *Rhizoclonium* sp. and *Cladophora* sp. in harvested biomass was around 20-25% at the end of the summer experiment, while *Spirogyra* sp. had the lowest relative abundance at 10%.

The relative abundance of initially seeded target species increased for five days after reseeding was performed (on day 44 onwards) at the start of the re-establishment experiment, but thereafter slowly decreased towards the end of the experiment for all species. The highest relative abundance of filamentous cyanobacteria in harvested biomass was observed in *Spirogyra* sp. FANS (up to 75% at the end of the summer experiment), and less than ten days after reseeding for the re-establishment experiment filamentous cyanobacteria dominated nearly 80% of the *Spirogyra* sp FANS surface. A high relative abundance of epiphytic and free-floating diatoms (up to 60% at the end of the summer experiment) were observed for both *Cladophora* sp. and *Rhizoclonium* sp. FANS. Across all FANS, at least 5% of other filamentous algae growth was found, including *Klebsormidium* sp. and *Oedogonium* sp.

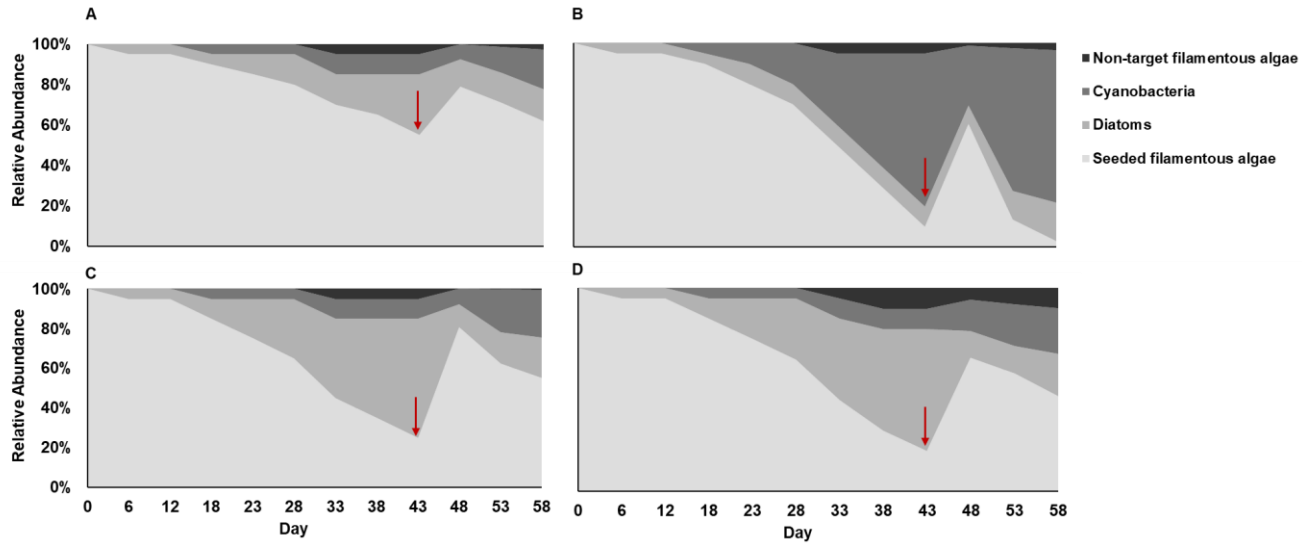

Figure 3.9: Average relative abundance of representative classes of algae on the floway surface of three replicate FANS seeded with unialgal stock cultures of a) *Oedogonium* sp., b) *Spirogyra* sp., c) *Rhizoclonium* sp. and d) *Cladophora* sp. during the summer experiment. Red arrow indicates the point that the re-establishment experiment started and FANS were reseeded with algal biomass of target species

In comparison to the summer experiment, initially seeded species were able to maintain a higher abundance for a longer time, the proportional abundance of filamentous cyanobacteria was nearly four-fold lower and the proportional abundance of diatoms was at least two-fold lower in the winter experiment. The target species that were initially seeded onto the FANS in the winter experiment comprised more than 75% of the harvested biomass for the first 40 days after seeding (Figure 3.10). Proportional abundance of epiphytic and free-floating diatoms in harvested biomass ranged from 33-45 % on *Oedogonium* sp., *Cladophora* sp. and *Rhizoclonium* sp. FANS, but was less than 30% on the *Spirogyra* sp. FANS.

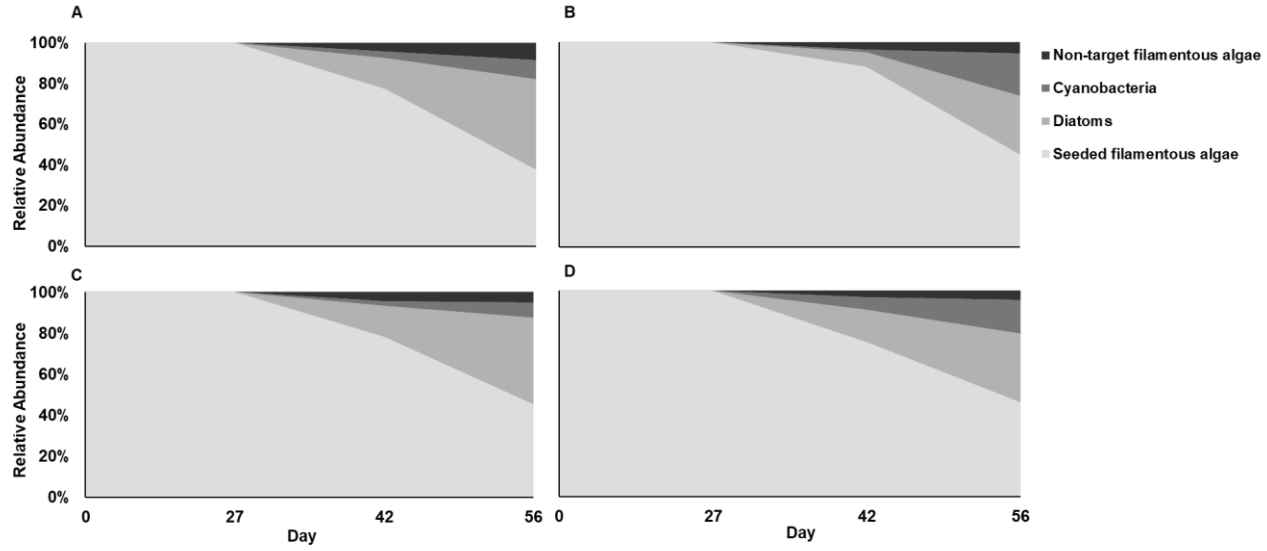

Figure 3.10: Average relative abundance of representative classes of algae on the floway surface on three replicate FANS seeded with unialgal stock cultures of a) *Oedogonium* sp., b) *Spirogyra* sp., c) *Rhizoclonium* sp. and d) *Cladophora* sp. during the winter experiment

3.5 Discussion

We investigated the performance of freshwater filamentous algal species on outdoor mesocosm-scale FANS in both summer and winter to provide a comparative assessment of algal productivity and nutrient removal among species under ambient conditions. The ability of species to form a dense uniform coverage on the liner is a key criteria when selecting the best species to grow on FANS as this can significantly promote higher biomass productivity and nutrient removal. We found apparent differences in species' abilities to establish uniformly on the liner, most likely resulting from differences in attachment mechanisms. Generally, all algae species established faster in summer (up to seven days) compared to winter (~20 days). *Oedogonium* sp. had the most rapid biomass establishment, and the highest number of filaments attached to the liner surface in the shortest seeding period - as quick as five days in summer and 15 days in winter. The rapid establishment of *Oedogonium* sp. on FANS is most likely to due to its ability to produce numerous zoospores (Hoffman, 1965; Hoffman, 1967; Arora & Sahoo, 2015) and establish secondary attachment through holdfast development .

Oedogonium sp. had the highest biomass productivity of the four species tested under both summer and winter conditions. Ambient temperature affects algal productivity more than irradiance and nutrient availability (Richmond, 1992). Therefore, the high biomass

productivity of *Oedogonium* sp. suggests that it was more tolerant to summer and winter conditions and less likely to be affected by temperature variations than the other three species. This conclusion is supported by laboratory studies which have assessed the performance of *Oedogonium* sp. under controlled conditions and shown that growth is maintained under a wide range of temperatures. For example, 11 isolates of *Oedogonium* originating from tropical and temperate locations in Eastern Australia had high growth rates under temperatures ranging from 8-27 ºC (Lawton et al., 2014). Similarly, a tropical species of *Oedogonium* was able to maintain growth rates under temperatures ranging from 15-40 ºC, and was able to survive in temperatures as low as 5 ºC (Cole et al., 2018). In addition to having a wide range of optimal growth temperatures, the high biomass productivity of *Oedogonium* sp. may also be driven by a physiological ability to endure and rapidly adapt to temperatures beyond their defined optimal range. Such an ability has been reported for other species of algae (Ras et al., 2013), but is yet to be investigated for *Oedogonium* sp.. Regardless, the high biomass productivity of *Oedogonium sp.* under both summer and winter conditions compared to three other species demonstrates its suitability as a target species for cultivation on FANS across multiple seasons. In contrast, *Spirogyra* sp. had the lowest biomass productivity across both summer and winter, suggesting that it is unlikely to do well under extreme high and low temperatures and therefore should not be targeted for cultivation on outdoor FANS. This conclusion is supported by a two-year field study of four species of *Spirogyra* which showed that under conditions of no to low light (0-60 μmol m^{-2} s^{-1}) and/or at high temperatures (> 35ºC) photosynthetic efficiency of *Spirogyra* sp. was reduced and cohesiveness of the attached algal filaments was significantly weakened (Berry & Lembi, 2000).

Nitrate removal rates were highest for *Oedogonium* sp. and lowest for *Spirogyra* sp. across both summer and winter. Although the estimation of total nutrient removal reported in the current study may exceed the actual nutrient removed through algae assimilation due to processes such as algae luxury uptake, denitrification by denitrifying bacteria, and phosphate precipitation also contributing to total nutrient removal, these estimates provide a comparative measure that can be used to assess nutrient removal performance across species. Assuming all FANS of all species experienced a similar rate of nutrient removal through mechanisms other than algae assimilation, variation in nutrient removal efficiency among the algae species tested may have been caused by extreme temperature and light conditions significantly affecting their photosynthetic efficiency and thereby their growth (Yamori et al., 2014). This is in agreement with linear regression analyses which showed that growth was a significant predictor of nutrient

removal rates for most species under both summer and winter conditions, with the exception of phosphate removal rates in winter. The ability of *Oedogonium* sp. to maintain high photosynthetic efficiency and growth under extreme temperature and light conditions (Cole et al., 2018) may facilitate higher nutrient removal rates compared to other species. In contrast, *Spirogyra* sp. is unable to maintain photosynthesis and growth under high temperatures and/or low light conditions (Berry & Lembi, 2000). Therefore, the low nutrient removal rates observed here for *Spirogyra* sp. across both seasons may have been caused by the unfavourable effects of high temperatures in summer and low irradiances in winter on photosynthetic efficiency. In addition, alteration of metabolic fluxes under high temperatures (Shin et al., 2016; Barati et al., 2019) and the limitation of photosynthetic electron transport under low irradiances and temperatures (Lobban & Harrison, 1994) may also have affected the photosynthetic efficiency of *Spirogyra* sp. The high nutrient removal rates of *Oedogonium* sp. further support its selection as a target species for outdoor FANS cultivation.

The ability of a target species to maintain dominance over other undesired species under varying environmental conditions is a key characteristic to consider when selecting the best species to grow in mass culture (Lawton et al., 2013a). One month after seeding in the summer experiment, *Oedogonium* sp. had the highest (50%) relative abundance on the floway surface of FANS that it had been seeded onto, while *Spirogyra* sp. had the lowest relative (10%) abundance of all species tested. The high dominance of *Oedogonium* sp. on floways for an extended period of FANS operation is most likely due to its ability to tolerate higher temperatures and irradiances and therefore maintain higher rates of biomass productivity under summer conditions compared to the other three species, despite the establishment of the non-target species (diatoms and cyanobacteria) on FANS. Our findings are similar to those reported for a tropical strain of *Oedogonium*, which was competitively dominant over *Cladophora* sp. and *Spirogyra* sp. within three weeks under a relatively high temperature (30°C) and light intensity (35.5 µmol m^{-2} s^{-1}) (Lawton et al., 2013a). Likewise, two temperate strains of *Oedogonium* grown in tertiary treated municipal wastewater during summer remained free of fouling in two separate two week experiments, while two other strains of the filamentous algae *Klebsormidium* were fouled by other non-target algal species (Lawton et al., 2021). These results suggest that competitive dominance may be a widespread trait within species of *Oedogonium*, further highlighting its suitability for monospecies cultivation on outdoor FANS. We found higher relative abundance of filamentous cyanobacteria (*Oscillatoria* sp.) in *Spirogyra* sp. FANS compared to the other species, and this observation could be related to the

allelopathic activity of *Spirogyra* sp. that stimulates *Oscillatoria* sp. growth, especially in summer (Mohamed, 2002). We also found that *Spirogyra* sp. FANS had lower relative abundance of epiphytic diatoms (*Cymbella* sp. and *Nitzschia* sp.) compared to the other species tested. This may be a result of *Spirogyra* sp. constantly generating mucilage that prevents adherence of algal epiphytes (Weber & Schagerl, 2007). In contrast, these epiphytic diatoms were highest on *Cladophora* sp. and *Rhizoclonium* sp. FANS, potentially due to their more coarse-textured filaments which may have enabled a high number of diatoms to adhere to them.

We found considerable differences in the overall performance of FANS between the summer and winter experiments. Global biomass productivity of each species in summer was more than one and a half times that recorded in winter, and *Oedogonium* sp. productivity was almost three-fold higher in summer compared to winter. Higher algal biomass productivity in summer compared to winter has also been reported in other studies. For example, a pilot-scale FANS treating citrus farm drainage water had approximately four-fold higher biomass productivity during summer months (4-16 g m^{-2} d^{-1}) compared to winter months (1-4 g m^{-2} d^{-1}) (D'Aiuto et al., 2015). Similarly, biomass productivity was almost 15-fold higher in summer (maximum of 60 g m^{-2} d^{-1}) compared to winter (4 g m^{-2} d^{-1}) on a field-scale FANS treating tertiary sewage (Craggs et al., 1996a). Differences in productivity between summer and winter are most likely due to differences in temperature and irradiance between seasons. Higher irradiances and warmer temperatures in the summer months are likely to promote higher algal growth rates as an increase in temperature and light intensity usually stimulates algal metabolic rates and increases photosynthesis (Davison, 1991; Savitch et al., 1996). However, higher temperatures can also decrease algae productivity. At temperatures above a species' optimal range, the photosynthetic enzyme RubisCO may become less efficient at differentiating oxygen from carbon dioxide, thus reducing photosynthetic rates (Osborne & Beerling, 2006; Yamori et al., 2014). Moreover, in some cases, extremely high temperatures can cause heat stress resulting in membrane injury and alterations to algal metabolic fluxes (Shin et al., 2016; Barati et al., 2019). Lower temperatures in winter may not necessarily inhibit growth, but could reduce membrane fluidity (Inaba et al., 2003), limit electron transport and photon capture (Lobban & Harrison, 1994) and deteriorate photosynthetic pigment-proteins (Eggert, 2012). All of these may have significant negative effects on growth and nutrient removal efficiency. Consequently, selecting species with broad temperature and irradiance tolerance for cultivation on FANS would enable continuous year-round biomass production and bioremediation performance.

A further difference in FANS performance between seasons was the dominance of seeded species on the floway. We found that the dominance of seeded species was higher in winter compared to summer, with seeded species having a relative abundance of more than 75% in the winter experiment compared to only 10 – 50% in the summer experiment one month after seeding. This decrease in the relative abundance of seeded species on the FANS in summer was accompanied by an increase in diatom and cyanobacteria relative abundance. The relative abundance of diatoms and cyanobacteria was about two- and three-fold higher respectively in summer compared to winter. Similar shifts in species composition toward cyanobacterial dominance have been reported for outdoor FANS under higher summer temperatures (Adey et al., 1993; O'Neil et al., 2012). Our study suggests selecting a target species that can maintain its dominance over cyanobacteria, diatoms and other non-target filamentous species growth, especially during high temperatures and irradiances would be ideal for all year-round FANS operation.

3.6 Conclusion

This study demonstrated that environmental variations in ambient light and temperature impact the biomass yield and nutrient removal rates of filamentous algae grown on outdoor FANS. Overall, *Oedogonium* sp. was the best performing algal species in terms of biomass productivity, nutrient removal rates, and its dominance over other non-target species on the floway. Our findings suggest *Oedogonium* sp. is the ideal species to be cultivated on FANS for combined bioremediation and biomass production throughout the year, across all seasons. Frequent (two to three week intervals) reseeding of FANS with target filamentous algae biomass during summer may help to maintain target species dominance and minimise the growth of non-target species. The approach used in this study can be applied in other locations and used as a template to identify suitable target filamentous algal species for continuous year-round cultivation on outdoor FANS.

3.7 Appendix

Table 3A.1: Reported range of environmental conditions that *Oedogonium* sp., *Rhizoclonium* sp., *Cladophora* sp. and *Spirogyra* sp. have been cultivated under

Parameters	*Oedogonium sp.*	*Rhizoclonium sp.*	*Cladophora sp.*	*Spirogyra sp.*
Temperature (ºC)	5 -32	10-20	10-30	18-35
Irradiance (µmol m^{-2} s^{-1})	49-700	30-35	0-175	710-1241
Salinity (psu)	0-3	5-30	0-34	-
Photoperiod (light cycle, hours)	-	8, 12 and 16	16	-
Nitrate Concentration (mg NO_3-N L^{-1})	0.6-23	25-50	0-180	46-75
Phosphate Concentration (mg PO_4-P L^{-1})	0.2-4	-	0-36	3-5
References	(Lawton et al., 2017)	(Aroca et al., 2020)	(Taylor et al., 2001)	(Marella et al., 2019)

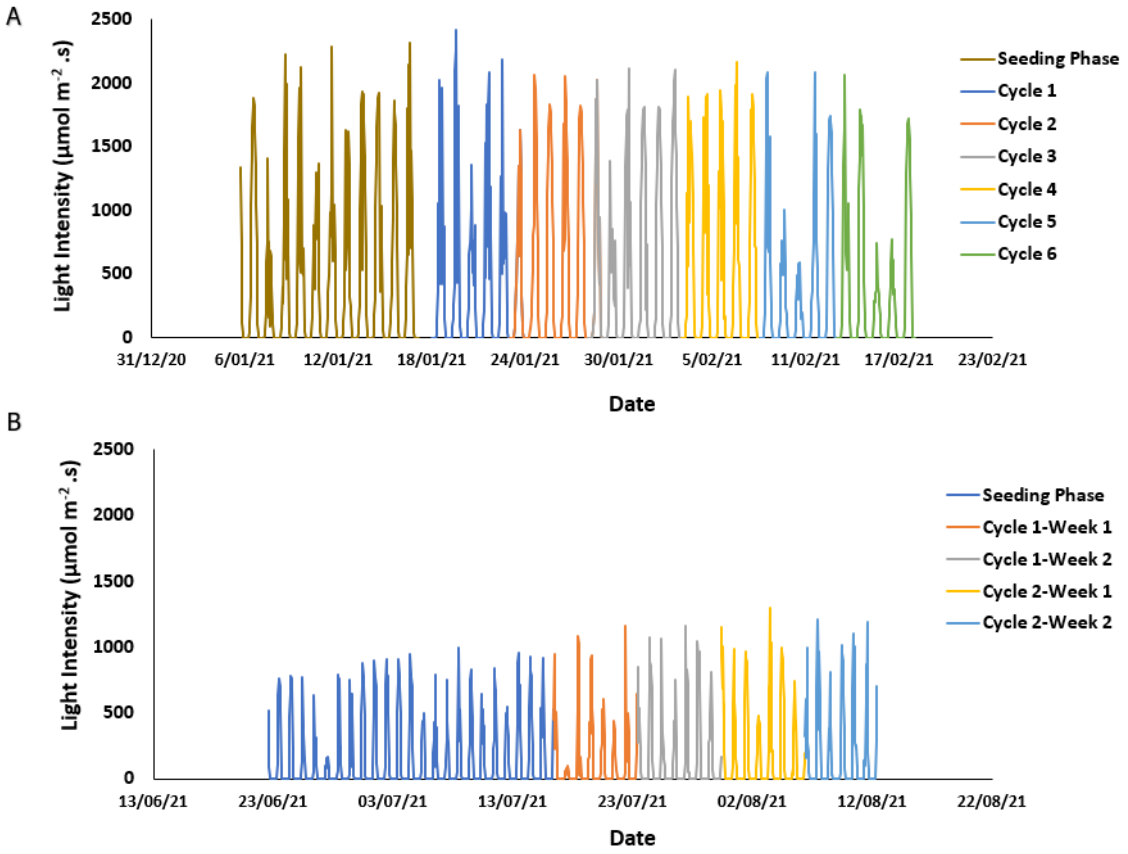

Figure 3A.1: Recorded light intensity during the (a) summer and (b) winter experiments. Data encompass the seeding phase through to the end of growth cycle 6 in summer and through to the end of growth cycle 2 in winter

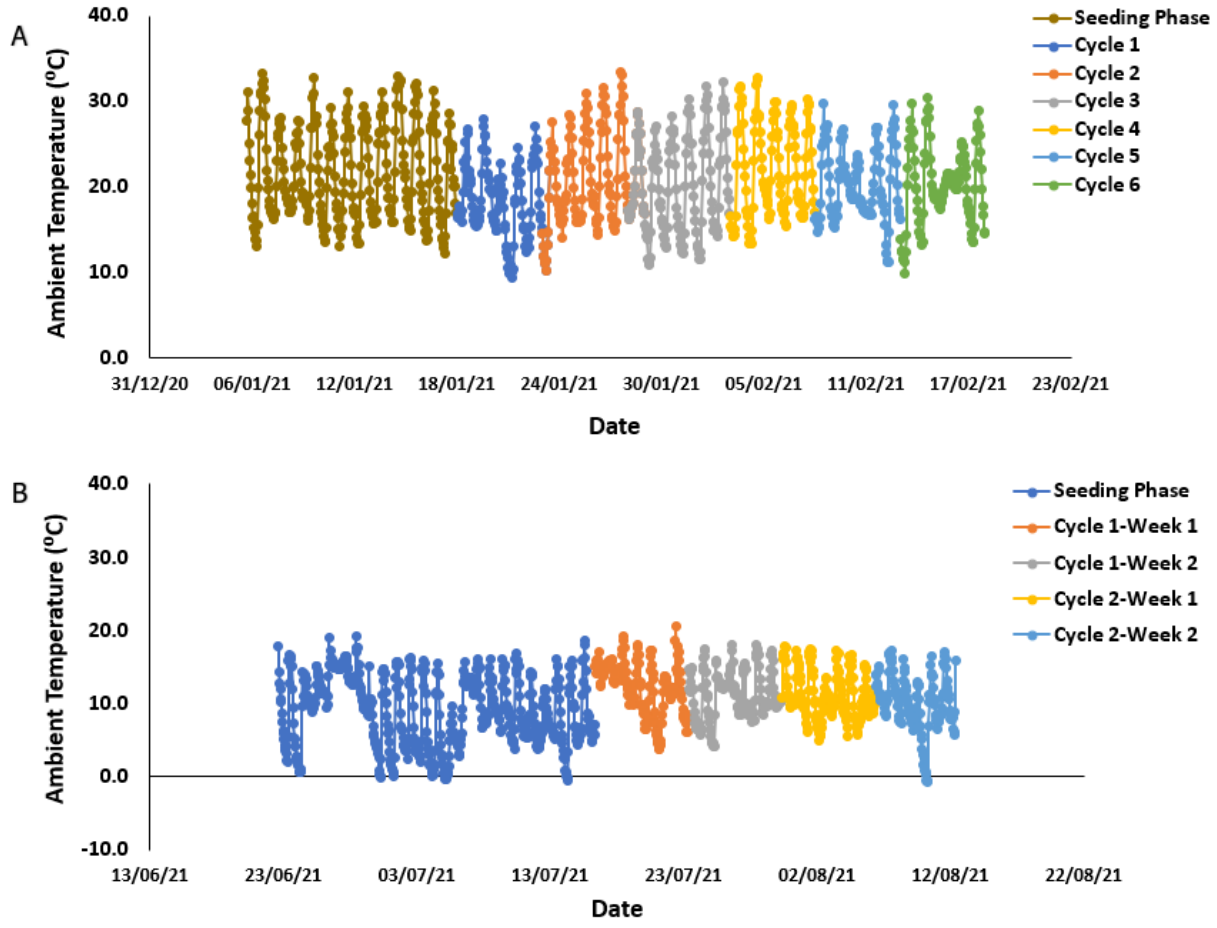

Figure 3A.2: Recorded ambient temperature during the (a) summer and (b) winter experiments. Data encompass the seeding phase through to the end of growth cycle 6 in summer and through to the end of growth cycle 2 in winter

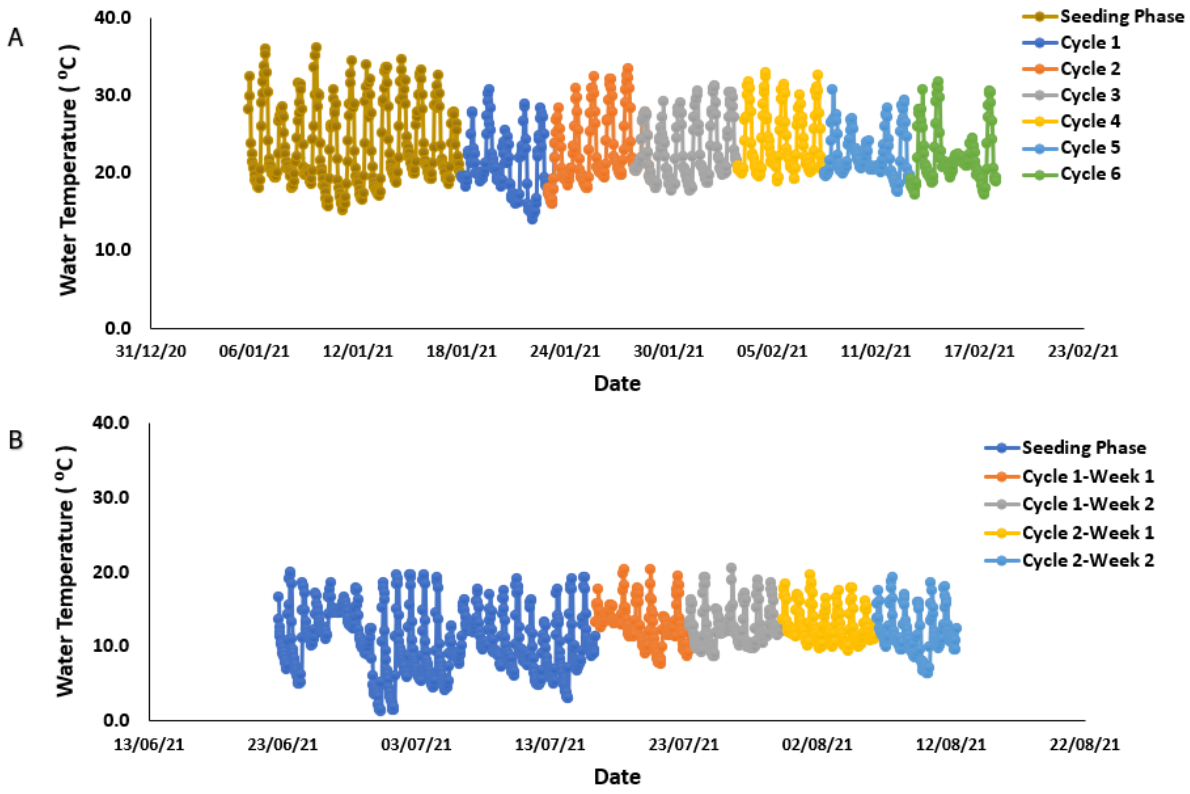

Figure 3A.3: Recorded outflow water temperature during the (a) summer and (b) winter experiments. Data encompass the seeding phase through to the end of growth cycle 6 in summer and through to the end of growth cycle 2 in winter

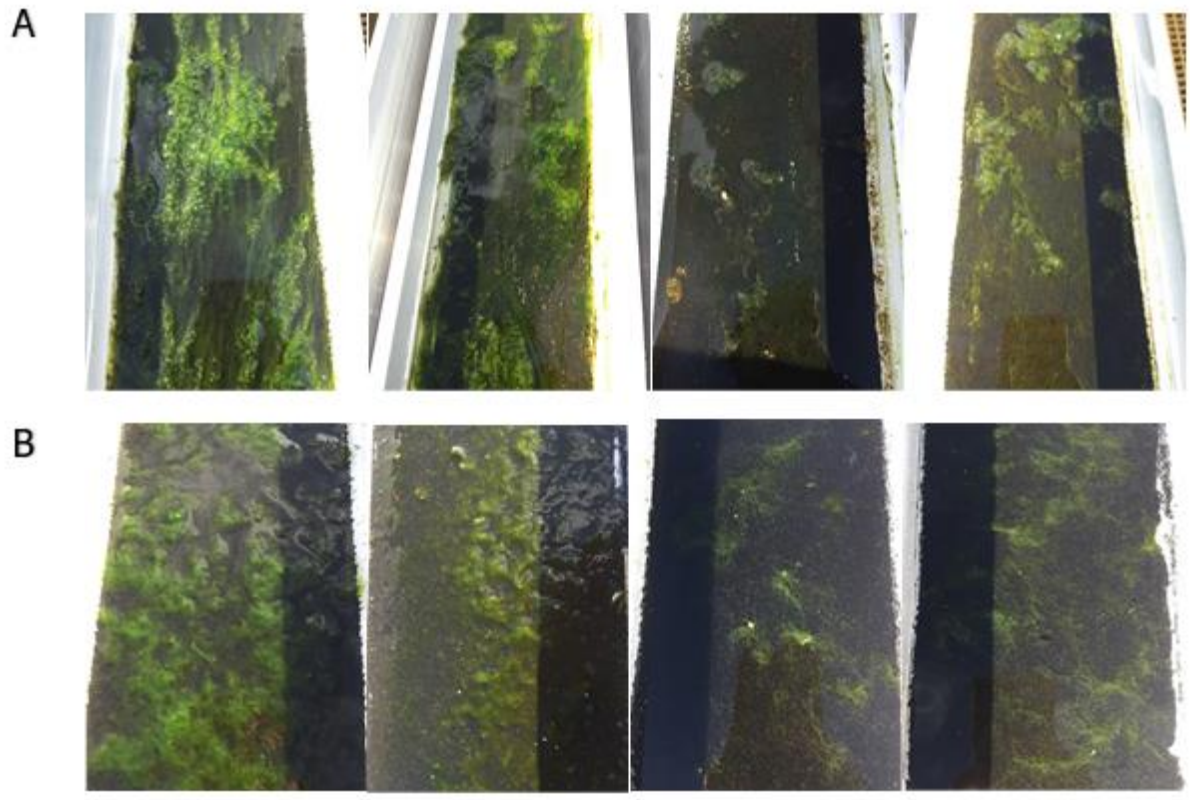

Figure 3A.4: Attached biomass on FANS after (a) the five day seeding phase during summer and (b) the 17 day seeding phase during winter. From left: *Oedogonium* sp., *Spirogyra* sp., *Rhizoclonium* sp. and *Cladophora* sp.

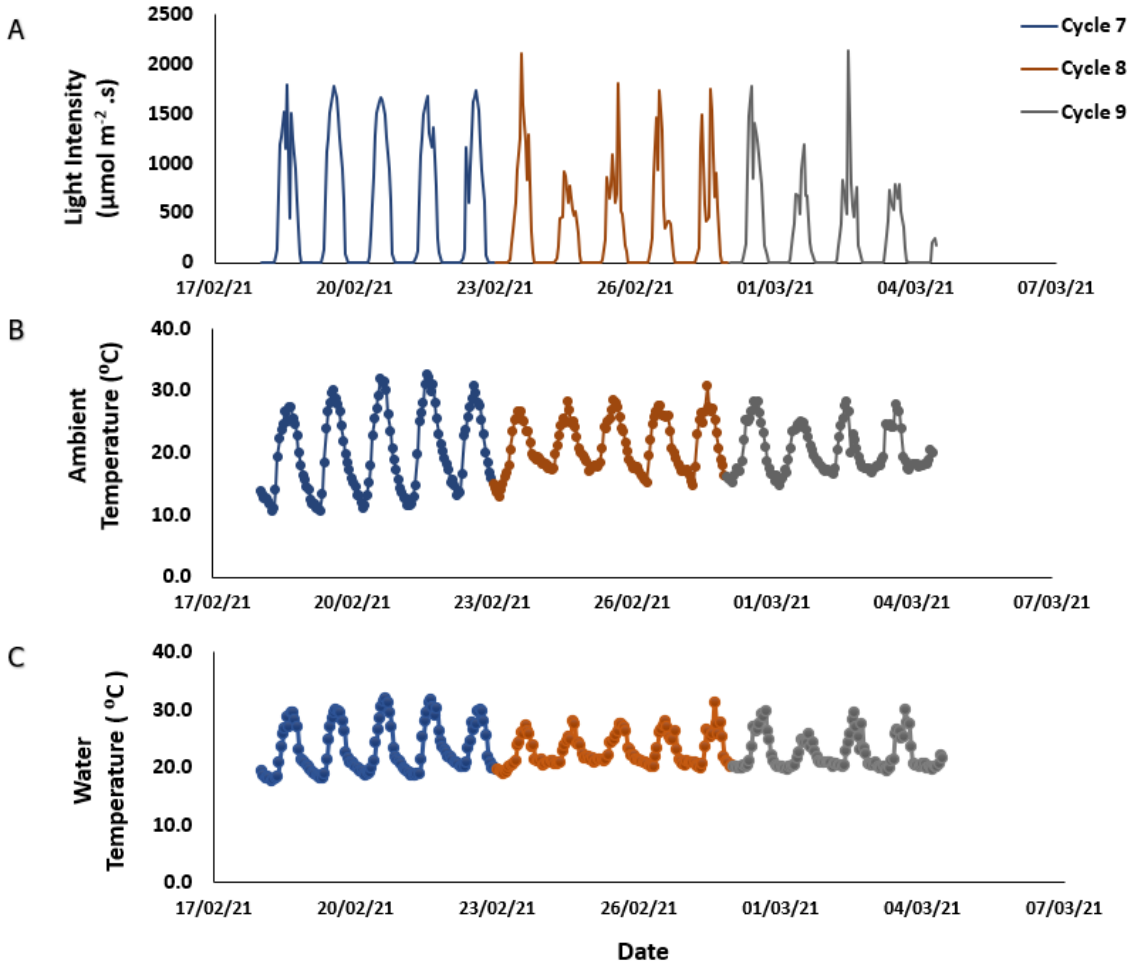

Figure 3A.5: Recorded (a) light intensity, (b) ambient temperature and (c) outflow water temperature during the re-establishment experiment (Data from growth cycle 7-9 of the summer experiment)

Chapter 4

Effects of Operational Parameters on the Performance of Unialgal *Oedogonium* sp. Filamentous Algae Nutrient Scrubbers under Controlled Environmental Conditions

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4.1 Abstract

Filamentous algae nutrient scrubbers (FANS) operating parameters can strongly influence algal biomass productivity and nutrient removal. However, few studies to date have investigated the effects of FANS operating parameters such as initial standing crop, harvesting frequency and influent flow rate on biomass productivity and nutrient removal performance, especially for FANS that cultivate a single species of algae. Therefore, the overall aim of this study was to investigate how operating parameters affect biomass productivity and nutrient removal performance of *Oedogonium* sp. - a promising species for unialgal FANS. Three experiments were performed indoors under controlled irradiance and temperature to test the effects of initial standing crop (60-110 g DW m^{-2}), harvesting frequency (intervals of one to five days) and influent flow rate (1-8 L min^{-1}) on pilot scale FANS operated with constant inflow nutrient concentrations (2 mg L^{-1} nitrate and 0.2 mg L^{-1} phosphate). Initial standing crop had a significant effect on biomass productivity, with productivities being highest (8.6 ± 0.5 g DW biomass m^{-2} d^{-1}) when the initial standing crop was 60-70 g DW m^{-2}. However, the daily nutrient removal rate was highest (0.47 ± 0.06 g N m^{-2} d^{-1} and 1.24 ± 0.13 g P m^{-2} d^{-1}) at the highest initial standing crop (100-110 DW m^{-2}). Biomass productivity was highest with a three-day growth period, regardless of size of the initial standing crop: 60-70 g DW m^{-2} (32.7 ± 0.4

g DW biomass m^{-2} d^{-1}), 100-110 g DW m^{-2} (21.9 ± 1.5 g DW biomass m^{-2} d^{-1}) and 160-170 g DW m^{-2} (11.4 ± 1.0 g DW biomass m^{-2} d^{-1}). Therefore, a four-day harvesting interval was selected as the optimal harvesting regime to promote exponential growth and the highest biomass production. Influent flow rate had a significant effect on biomass productivity, which was highest (9.3 ± 1.7 g DW m^{-2} d^{-1}) for the 1 L min^{-1} flow rate and also gave the highest instantaneous nutrient removal rate (0.05 ± 0.02 g N m^{-3} and 0.14 ± 0.05 g P m^{-3}). Our results suggest that an optimum initial standing crop of 70-80 g DW m^{-2}, harvesting frequency of four days and influent flow rate of 1 L min^{-1} (16.7 L min^{-1} .m width) were optimal for *Oedogonium* sp. cultivated on FANS to maximize their biomass production and nutrient removal under controlled laboratory conditions. These results contribute to understanding the impacts of operating parameters on optimizing unialgal *Oedogonium* sp. FANS biomass production and nutrient removal performance.

4.2 Introduction

Filamentous algae nutrient scrubbers (FANS) are artificial streams that cultivate assemblages of attached filamentous algae to remove nutrients from polluted water (Adey et al., 2011; Sutherland & Craggs, 2017). FANS consist of a gently sloped floway with an attached textured liner to provide a medium for the filamentous algae to attach to. Polluted water is pumped onto the FANS and is treated as it runs down the floway through the attached algal matrix, promoting nutrient assimilation and algal growth. Algal growth and nutrient assimilation performance of FANS are strongly affected by seasonal changes in ambient temperature and irradiance, with both parameters showing a marked decline from summer to winter (Hariz et al., 2023c). However, growth and nutrient assimilation performance are also influenced by FANS operational parameters (Liu et al., 2020; Sutherland et al., 2020b, 2020a; Park et al., 2022). Consequently, there is potential to enhance FANS biomass productivity and nutrient removal independently of seasonal variation by optimizing these operational parameters.

Several operational parameters can be optimized to maximize algae productivity and nutrient removal on FANS, including the initial algal standing crop, harvesting frequency and influent flow rate. The initial standing crop plays a vital role in productivity and nutrient removal. A higher initial standing crop provides a higher reproduction rate due to high rates of

spore release (Grote, 2019) and, in the absence of other limiting factors, produces a higher final standing crop of attached algae that has captured nutrients from the flowing water (Pereira et al., 2006). However, if the initial standing crop is too thick the amount of light reaching the lower layers of attached algae is reduced due to self-shading (Sutherland et al., 2020b). The greater the thickness of the attached algal mat, the greater the light reduction. A thinner algal mat promotes greater light availability, gas exchange and water distribution to more of the algae biomass, promoting photosynthesis and thereby increasing algal growth and nutrient assimilation (Berry & Lembi, 2000; Sutherland et al., 2020b).

Harvesting frequency may also play a role in maximizing productivity and nutrient removal. Long intervals between harvesting could cause a thick algal mat to develop between harvests, limiting light and nutrients reaching the bottom layer of the algal mat and thereby reducing growth and nutrient assimilation (Krause-Jensen et al., 1996; Hillebrand, 2005; Busse et al., 2006). Besides reducing light and nutrient distribution, a thicker algal mat is more likely to cause lower gas exchange efficiency, especially at the bottom layer of the algal mat. Oxygen bubbles generated through photosynthesis or respiration are often trapped under the lower layer of the algal mat, causing the top layer to float above water level and dry when exposed to air (Berry & Lembi, 2000; Sutherland et al., 2020b). An optimal harvesting regime helps maintain an optimal standing crop range on FANS that reduces the likelihood of low rates of photosynthesis due to light limitation and poor nutrient distribution, thereby maintaining maximum algal productivity. The optimal harvesting frequency is best selected based on the growth performance of algae, where algal biomass should be maintained in the exponential phase of growth to enhance biomass production and nutrient removal (Craggs et al., 1996a; Adey et al., 2011). Considering the seasonal effects of ambient temperature and irradiance on algal growth rate , the optimal harvesting regime could be implemented according to seasonal growth performance. For instance, a weekly harvesting regime was used during peak biomass production in the summer months on a large scale FANS (152 m long and 6.7 m wide) treating municipal sewage, while up to a month harvesting regime was used in winter months when biomass production was lowest .

Influent flow rate may significantly impact FANS productivity and nutrient removal. Influent flow affects both the algal contact time on a floway of a set length but also the horizontal flow velocity (Park et al., 2022). Higher horizontal flow velocities increase turbulence and mixing to improve nutrient transport across the boundary layer around the algal

cells which enhances algal assimilation, thus promoting higher nutrient removal (Busse et al., 2006; Blersch et al., 2013; Stocking et al., 2016; Yan et al., 2018). For example, higher nitrate removal was observed when the flow rate rose up to 125 L min^{-1}.m width of floway compared to 64 L min^{-1}.m width and 95 L min^{-1}.m width on floways treating agricultural drainage water (Kangas & Mulbry, 2014). However, a higher flow rate reduces algae contact time (ACT) with water, thus reducing the time for nutrient assimilation by the algal biomass (Park et al., 2022). Excessive flow rates could also cause biomass to wash off the floway due to turbulence that shears and detaches the attached algal filaments from the FANS liner (Ahn et al., 2013; Blersch et al., 2013; Sutherland & Craggs, 2017; Marella et al., 2019). Additionally, excessive shear may damage the algal cells, affecting biomass productivity and nutrient removal of FANS.

Cultivating a single species of algae may not be required for effective FANS bioremediation (Hannon et al., 2010) however, it can provide a more consistent source of high-quality biomass with low variation in biochemical composition for use in a range of end-product applications (Lawton et al., 2013a). In addition, cultivating a single species of algae on FANS could reduce performance variability and facilitate the optimization of performance for a specific aim (e.g. bioremediation, biomass production or both). In the present study, *Oedogonium* sp. was selected as the target species to be cultivated on FANS based on its performance across a wide range of temperature and light conditions (Hariz et al., 2023c). Characteristics which make *Oedogonium* sp. a good filamentous algae species to grow on FANS include its robust reproduction and strong attachment that may prevent significant quantities of biomass from being washed off the FANS, especially during high rainfall events (Hariz et al., 2022), and its high productivity and nutrient removal performance compared to other filamentous algae species such as *Cladophora* sp., *Rhizoclonium* sp. and *Spirogyra* sp. (Hariz et al., 2023c).

There is still limited understanding of how FANS operating parameters such as initial standing crop, harvesting frequency and influent flow rate affect biomass productivity and nutrient removal. Therefore, the overall aim of this study was to investigate how these operating parameters affect productivity and nutrient removal performance of unialgal *Oedogonium* sp. FANS. The specific aim was to compare biomass productivity and nutrient removal rate under varying influent flow rates, initial standing crops and harvesting frequencies. Experiments were conducted under controlled environmental conditions to remove any

variability in performance due to variations in light and temperature which occur under ambient conditions.

4.3 Methods

4.3.1 Pilot-scale Filamentous Algae Nutrient Scrubber

The pilot-scale FANS system used in this study consisted of two sets of seven floways (size: 3 m length x 0.06 m width x 0.05 m depth) constructed from two single sheets of stainless-steel roofing (Figure 4.1) with a high-density polyethylene (HDPE) textured liner (size: 3 m x 0.06 m or 0.18 m^2). This liner was chosen as studies have shown that textured substratum or surfaces with variable topography enhance algal biomass attachment (Maggs & Callow, 2003; Blersch et al., 2017). The textured liner was permanently attached to the base of the floway using a multi-purpose permanent elastic sealant/adhesive glue (brand: Bostik ISR 70-03).

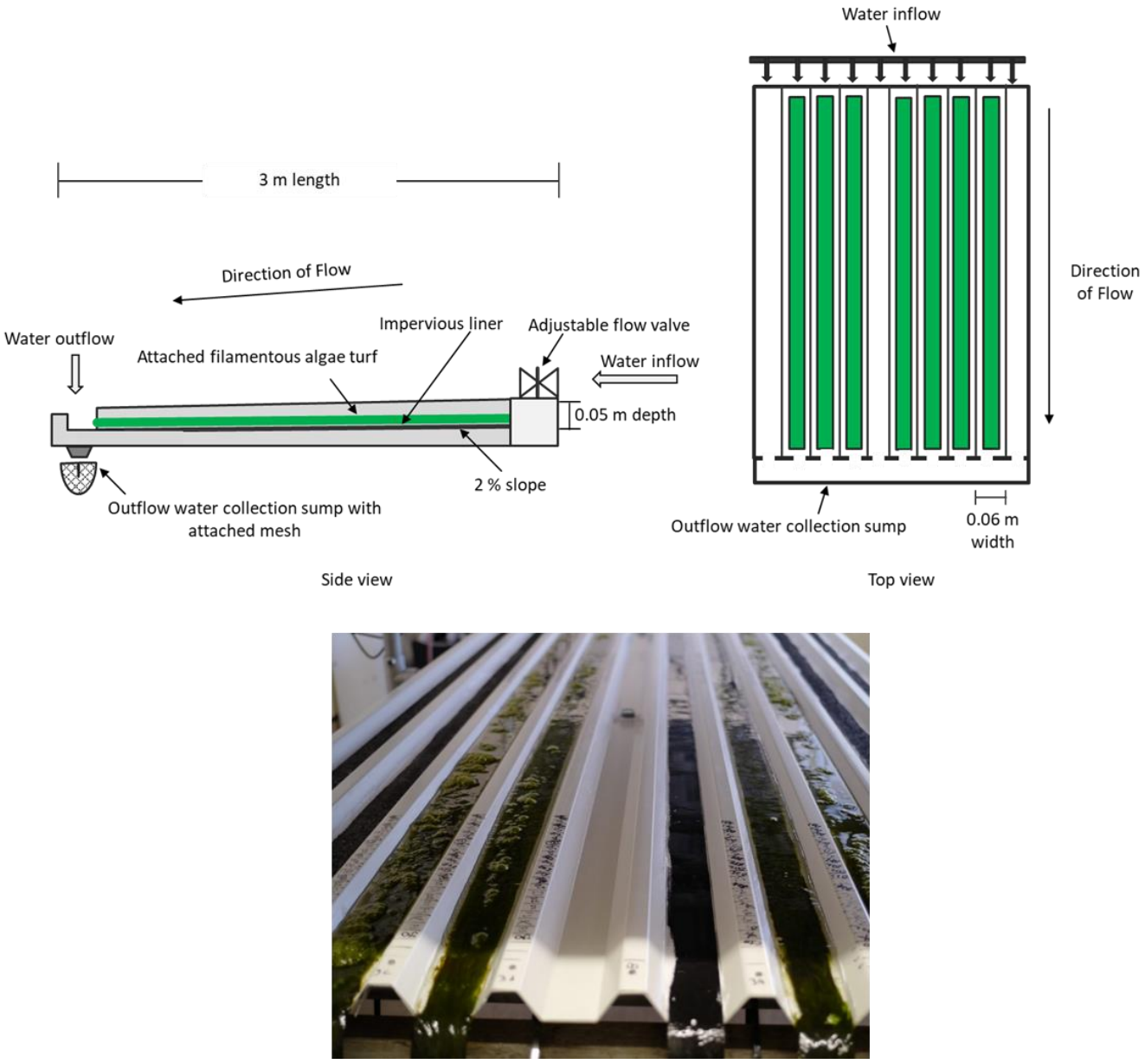

Figure 4.1: Schematic diagram of indoor pilot scale FANS used in this study from the side (top left) and above (top right) and an overview photograph showing FANS seeded with algae

Nutrient enriched water was continuously pumped to the top of each floway through pipes from a 5000 L stock solution tank. The influent flow rate of each FANS was controlled by an adjustable valve that allowed accurate and consistent water flow. The flow rate on each valve was measured using a digital flow rate meter (DigiFlow 6710M, Vyair). The nutrient stock solution was prepared from hydroponic grade NO_3-N (YaraLiva) and di-potassium hydrogen phosphate, K_2HPO_4 (EMSURE ®). A nutrient concentration of 2 mg L^{-1} of NO_3-N and 0.2 mg L^{-1} of PO_4-P was used as preliminary trials showed this provided sufficient nutrient supply for algal growth on a continuous flow-through (no water recirculation) FANS system.

4.3.2 Seeding phase

This study used the filamentous algae species, *Oedogonium* sp. Each FANS was seeded by evenly distributing 7 g fresh weight (FW) algal biomass, which was equivalent to 1.2 g dry weight (DW) or a loading rate of 6.7 g DW m^{-2}, by rubbing it down the textured liner to hook algae filaments onto the liner and provide initial physical attachment. The amount of seeded biomass used was enough to cover the whole surface area of the FANS textured liner. Over time, secondary biological attachment occurred through the growth of holdfasts from the settlement of *Oedogonium* sp. zoospores onto the liner. The nutrient-enriched water was pumped continuously over the surface of each floway at ~0.5 L min^{-1} (8 L min^{-1}.m width of floway) during the seeding phase. This flow rate was used for the seeding stage as preliminary trials indicated that it was high enough to cover the whole surface area of floway in water, yet low enough to avoid detaching the algae and washing it off from the liner. The experiment was conducted indoors under controlled conditions (light intensity of 600-650 µmol m^{-2} s^{-1}, 14:10 light and dark cycle, room temperature set at 22°C) at the National Institute of Water and Atmospheric Research (NIWA) facility at Ruakura, Hamilton, Aotearoa New Zealand. The seeding phase lasted for six days and daily reseeding was performed in the first three days by spreading any biomass that was washed off and collected from the outflow mesh back over the liner surface of each FANS. After four days, *Oedogonium* sp. had established a dense algal turf on the liner surface of each FANS. At the end of the seeding phase, biomass on each FANS was harvested by drawing a metal scraper down the surface of the liner, cutting the algal filaments to leave a uniform coverage of attached algal biomass of approximately 0.5-1 cm height with the required initial standing crop to start the experiment.

4.3.3 Experiments

The effects of operational parameters on biomass productivity and nutrient removal were investigated in three separate laboratory experiments. The first experiment investigated the effects of initial standing crop and harvesting frequency on biomass productivity. The second experiment investigated the effects of the initial standing crop on biomass productivity and nutrient removal rate. The third experiment investigated the effects of influent flow rate on biomass productivity and nutrient removal rate.

4.3.3.1 Effect of initial standing crop and harvesting frequency on biomass productivity

This experiment investigated the effect of the initial standing crop on biomass productivity and aimed to select an optimum harvesting frequency based on the biomass productivity of attached algae. Duplicate FANS were each operated with three different initial standing crops – low (60 – 70 g DW m^{-2}), medium (100 – 110 g DW m^{-2}) and high (160 – 170 g DW m^{-2}). These initial standing crop ranges were selected based on the minimum uniform standing crop left on FANS following harvest and the maximum standing crop that could grow attached on FANS based on the outdoor mesocosm FANS trials . The experiment was conducted over five days, with a constant influent flow rate of 1 L min^{-1} (16 L min^{-1}.m width of floway).

Strip biomass monitoring was conducted to estimate the amount of FW biomass that needed to be removed during harvesting to achieve the target initial standing crops in dry weight (DW) to start the experiment. The attached algal biomass was scraped entirely off the liner in a horizontal strip area (0.06 m x 0.02 m) at 3 locations down the length of the FANS (top, middle and bottom) to estimate the FW of the attached standing crop before harvest. The scraped biomass was dewatered by compressing the FW biomass over 200 µm filter mesh until no more water droplets were produced and then weighed for the fresh biomass weight (FW). These FW measurements were then converted into DW using an *Oedogonium* sp. specific DW:FW ratio of 0.16, which was based on the average DW:FW ratio calculated for five replicate samples of the *Oedogonium* sp. biomass used to seed the FANS. The DW initial standing crop was then estimated as follows:

Entire FANS area: 0.06 m x 3 m = 0.18 m^2

Strip monitoring area: 0.06 m x 0.02 m = 0.0012 m^2

Ratio of strip monitoring area to total FANS area: 0.18 m^2/0.0012 m^2 = 150

Estimated DW of entire FANS (g DW)
= [(FW Top + FW Middle + FW Bottom)/3] x 0.16 x 150

The entire FANS was then harvested using a metal scraper as described above leaving a uniform coverage of attached algal biomass of 0.5-1 cm height depending on the required initial standing crop. Immediately after harvesting, the strip biomass monitoring steps were repeated (in a slightly different location) to determine the initial standing crop (standing crop following harvest). The scraped biomass samples were dried overnight in an oven at 65 ºC and then reweighed to determine the actual DW of initial standing crop on each individual FANS. Strip biomass monitoring was then repeated daily (in a slightly different location on each occasion) over a five-day experimental period. The scraped biomass samples collected on each strip monitoring occasion were dried overnight at 65 ºC, weighed and used to calculate the standing crop on each FANS for each day of the experiment as follows:

FANS Standing Crop Day n (g DW) = [(DW Top + DW Middle + DW Bottom)/3] x *150*

The daily biomass productivity of each FANS was calculated in g of DW biomass per m^2 per day using the formula:

Daily Biomass Productivity (g DW m^{-2} d^{-1}*) = (FANS DW Standing Crop Day n – FANS DW Standing Crop Day n-1) / 0.18* m^2,

where 0.18 m^2 is the surface area of the FANS. This metric provided a measure of the total amount of biomass grown on each day of the five-day growth period.

Biomass productivity of each FANS was calculated in g of DW biomass per m^2 per day for each day of the five-day growth period using the formula:

Biomass Productivity (g DW m^{-2} d^{-1}*) = (FANS DW Standing Crop Day n – FANS DW Standing Crop Day 0) / 0.18* m^2*/N,*

where *N* is the number of days of growth period. This metric provided a standardized measure of the average amount of biomass produced per day over growth periods ranging in duration from one to five days, thereby demonstrating the effect of different harvest frequencies ranging from one to five days.

4.3.3.2 The effect of initial standing crop on biomass productivity and nutrient removal rate

This experiment investigated the effect of the initial standing crop on *Oedogonium* sp. biomass productivity and nutrient removal rate. Four initial standing crops were tested: 60-69, 70-79, 80-90 and 100-110 g DW m^{-2} with three replicate FANS for each standing crop. These initial standing crops were selected based on the range of initial standing crops that gave the highest biomass productivity in the first experiment (Section 4.3.3.1) in order to identify the optimal initial standing crop for *Oedogonium* sp. on FANS. The experiment was conducted over three four-day growth cycles, with a constant influent flow rate of 1 L min^{-1} (16 L min^{-1}.m width of floway). The four-day harvesting interval was selected as the first experiment showed that biomass productivity of *Oedogonium sp*. increased from day zero until day three, when the maximum productivity was reached, with little growth from day four onwards. Strip biomass monitoring as described in Section 4.3.3.1 was conducted to determine the initial standing crop on each replicate FANS at the start of the experiment and then repeated daily for the duration of the experiment. FANS were harvested at the end of each four-day growth cycle as described above. The biomass productivity (DW g m^{-2} d^{-1}) of each replicate FANS was calculated for each of the three four-day growth cycles using the formula:

Biomass Productivity (g DW m^{-2} d^{-1}) = [(FANS DW Standing Crop Day 4 – FANS DW Standing Crop Day 0) / 0.18 m^2] / 4 days.

A 20 ml water sample was collected daily (around the same time at 10 am) from the inflow and outflow of each replicate FANS. The sample was filtered using a 0.7 µm glass microfiber filter (Brand: Whatman GF/F) and the concentration of nitrate (NO_3-N) and dissolved reactive phosphate (DRP) in each water sample was determined using the ultraviolet spectrophotometric method (Standard Method Nitrate 4500-NO_3−B) and the ascorbic acid method (Standard Method Phosphorus 4500-P) on a microplate reader (SPECTROstar Nano, BMG LABTECH). The nutrient reduction of each FANS replicate was quantified based on the difference in nutrient concentrations between the inflow and outflow samples.

The daily nutrient removal rate for each replicate FANS was calculated for NO_3-N and DRP using the formula:

Daily Nutrient Removal Rate (g NO_3-N m^{-2} d^{-1} *or g* DRP m^{-2} d^{-1}*) = (FANS inflow nutrient concentration (g* m^{-3}*) – FANS outflow concentration (g* m^{-3}*))* x *FANS Working Volume (*m^3 d^{-1}*) / 0.18* m^2

4.3.3.3 The effect of influent flow rate on biomass productivity and nutrient removal rate

This experiment investigated the effect of influent flow rate on growth, biomass productivity and nutrient removal. Influent flow rates of 1, 3 and 8 L min^{-1} (equivalent to 16, 50 and 133 L min^{-1}.m width, respectively) were tested. These flow rates were selected based on the minimum and the maximum flow rates used in published studies of attached algal treatment systems which ranged from 25 to 131 L min^{-1}.m width (Mulbry et al., 2008b; Kangas & Mulbry, 2014; D'Aiuto et al., 2015; Sutherland et al., 2020b). Due to limitations on water flow for our pilot system, it was only possible to have one replicate FANS operating for each flow rate at a time. Therefore, the four-day growth cycle experiment was repeated five times to provide replication for each flow rate.

To start the experiment, FANS were harvested to a constant initial standing crop of 70-80 g DW m^{-2}. This optimal initial standing crop was chosen as it provided the highest biomass productivity and nutrient removal rates in Experiment 2 (Section 4.3.3.2). Strip biomass monitoring, as described in Section 4.3.3.1, was conducted to determine the actual initial standing crop on each FANS. FANS were harvested at the end of each four-day cycle, and biomass productivity (DW g m^{-2} d^{-1}) was calculated for each FANS as described above. A 20 ml water sample was collected once every two days from the FANS inflow and outflow to determine the daily nitrate and DRP removal rate as described in Section 4.3.3.2. The instantaneous nutrient reduction rate for each replicate FANS was calculated for NO_3-N and DRP using the formula:

Instantaneous Nutrient Reduction Rate (g NO_3-N m^{-3} *or g* DRP m^{-3}*) = FANS inflow nutrient concentration (g* m^{-3}*) – FANS outflow nutrient concentration (g* m^{-3}*)*

4.3.4 Statistical analyses

Differences in biomass productivity, and nitrate and DRP removal rates among FANS were tested using one-way or two-way analyses of variance (ANOVA) with the initial standing crop, day, growth cycle duration and flow rate included as fixed factors as appropriate. A Kruskal-Wallis test was used for variables that failed normality and/or homogeneity of variance tests. All statistical analyses were carried out using SigmaPlot software (Systat Software Inc., Point Richmond, CA, USA). All data are reported as mean ± S.D.

4.4 Results

4.4.1 The Effect of Initial Standing Crop and Harvesting Frequency on biomass productivity

Across all initial standing crop treatments, the standing crop increased exponentially from day zero to three, then slowed down and reached a stationary point from day four to five (Figure 4.2). FANS with the low initial standing crop had the highest biomass increase from day zero (67.5 g DW m^{-2}) to day three (165.7 g DW m^{-2}), while FANS with the high initial standing crop had the lowest biomass increase from day zero (163.2 g DW m^{-2}) to day three (197.3 g DW m^{-2}).

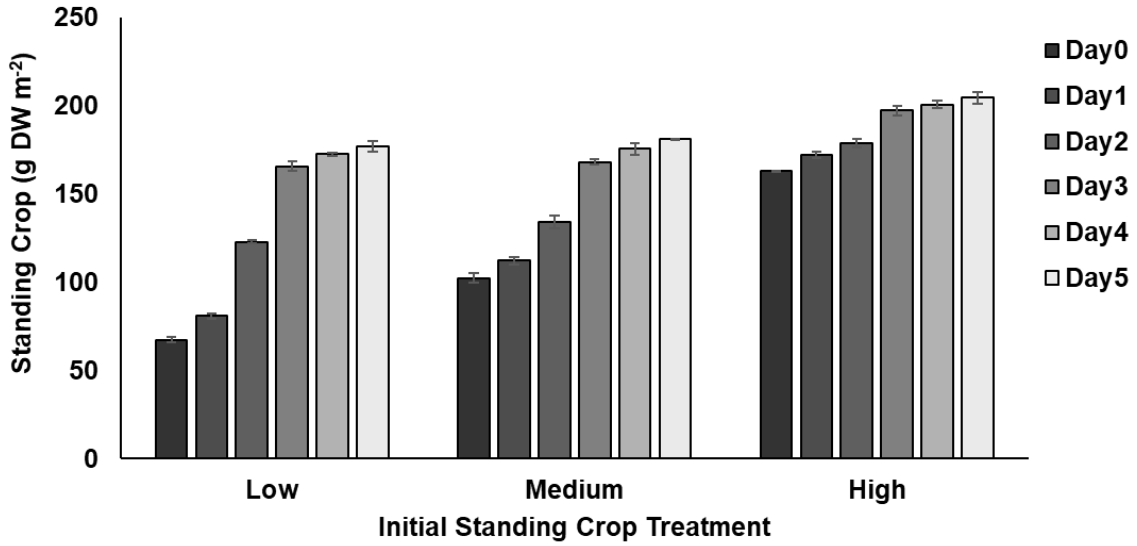

Figure 4.2: Average (±S.D.) standing crop (g DW m^{-2}) over a consecutive five-day period on duplicate FANS with a low (60-70 g DW m^{-2}), medium (100-110 g DW m^{-2}) and high (160-170 g DW m^{-2}) initial standing crop. N = 2

The initial standing crop had a significant effect on daily biomass productivity, however this effect was not consistent over the five-day growth period (ANOVA, initial standing crop x day interaction; $F_{8,15}$=11.4; P <0.001, Figure 4.3). Daily biomass productivity was highest for FANS with the low initial standing crop on day one to three of the experiment (13.5 – 42.7 g DW m^{-2} d^{-1}) but on day four to five, FANS with the medium initial standing crop had the highest biomass productivity (5.6 – 7.4 g DW m^{-2} d^{-1}). Daily biomass productivity was highest on day three across all initial standing crop FANS and decreased toward day five.

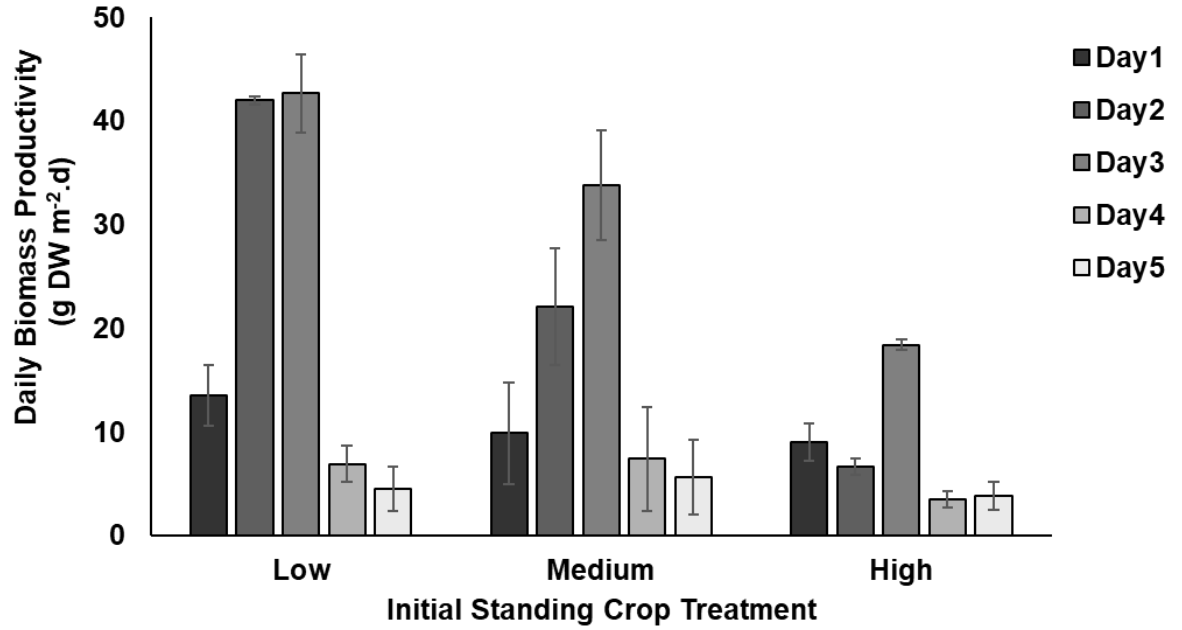

Figure 4.3: Average (± S.D.) daily biomass productivity (g DW m^{-2} d^{-1}) over a consecutive five-day period on duplicate FANS with a low (60-70 g DW m^{-2}), medium (100-110 g DW m^{-2}) and high (160-170 g DW m^{-2}) initial standing crop. N = 2

The initial standing crop had a significant effect on biomass productivity, however this effect was not consistent among the growth cycle durations (ANOVA, initial standing crop x day interaction; $F_{8,15}$=7.714; P <0.001, Figure 4.4). The lowest initial standing crop FANS (60-70 g DW m^{-2}) had the highest productivity for all growth cycle durations (13.5 – 32.7 g DW m^{-2}). In contrast, the highest initial standing crop FANS (160-170 g DW m^{-2}) had the lowest productivity for all growth cycle durations (7.8 – 11.4 g DW m^{-2}). Biomass productivity was highest for a three-day growth cycle across all initial standing crops; 60-70 g DW m^{-2} (32.7 ± 0.4 g DW biomass m^{-2} d^{-1}), 100-110 g DW m^{-2} (21.9 ± 1.5 g DW biomass m^{-2} d^{-1}) and 160-170 g DW m^{-2} (11.4 ± 1.0 g DW biomass m^{-2} d^{-1}), and was lowest for a one-day growth cycle for the low (13.5 ± 2.9 g DW biomass m^{-2} d^{-1}) and medium (9.9 ± 4.9 g DW biomass m^{-2} d^{-1}) initial standing crops and for a two-day growth cycle for the high (7.8 ± 1.3 g DW biomass m^{-2} d^{-1}) initial standing crop.

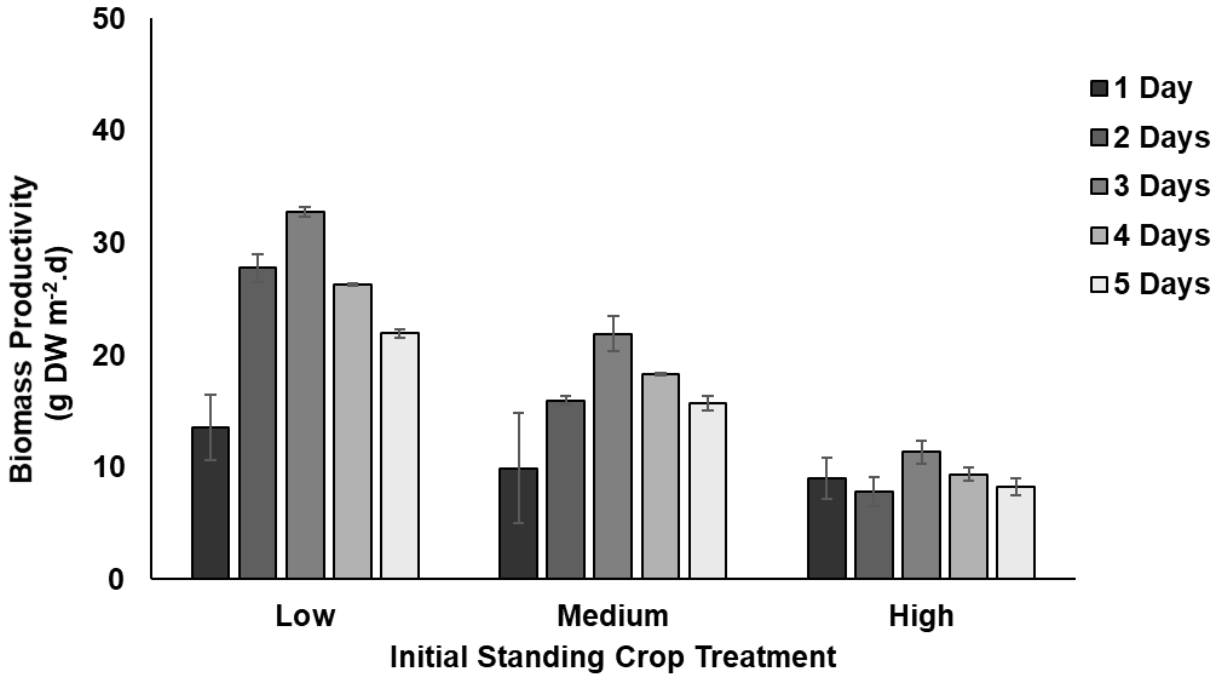

Figure 4.4: Average (± S.D.) biomass productivity (g DW m^{-2} d^{-1}) over growth cycles ranging in duration from one to five days on duplicate FANS with a low (60-70 g DW m^{-2}), medium (100-110 g DW m^{-2}) and high (160-170 g DW m^{-2}) initial standing crop N = 2

4.4.2 The effect of initial standing crop on biomass productivity and nutrient removal rate

Biomass productivity varied significantly among initial standing crops and growth cycles (ANOVA, initial standing crop x growth cycle interaction, $F_{6,24}$=2.1; P = 0.098, Figure 4.5). Across initial standing crops, biomass productivity was highest in the low (60-69 g DW) initial standing crop (8.6 $\pm$ 0.5 g DW m^{-2} d^{-1}) and lowest in the high (100-110 g DW) initial standing crop (5.9 $\pm$ 0.6 g DW m^{-2} d^{-1}). The low initial standing crop FANS had the highest biomass productivity in cycle 1 (9.7 $\pm$ 0.6 g DW m^{-2} d^{-1}) and cycle 3 (8.5 $\pm$ 0.3 g DW m^{-2} d^{-1}), however the medium low initial standing crop FANS had the highest productivity in cycle 2 (8.7 $\pm$ 0.9 g DW m^{-2} d^{-1}). Across all initial standing crops, biomass productivity was highest in cycle 1 (8.3 $\pm$ 1.3 g DW m^{-2} d^{-1}) and lowest in cycle 2 (6.7 $\pm$ 1.8 g DW m^{-2} d^{-1}).

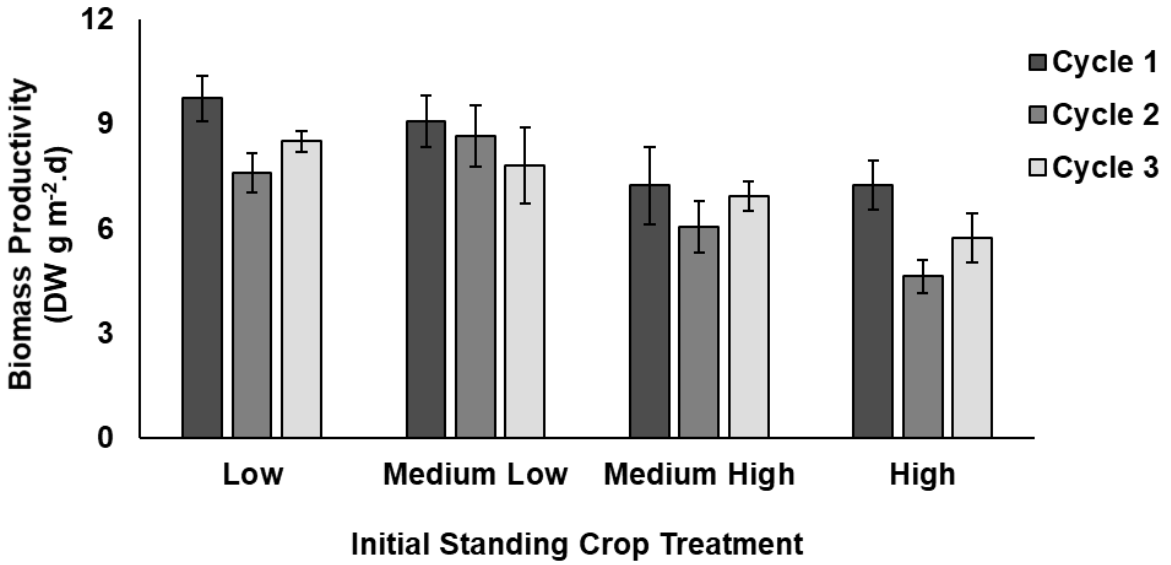

Figure 4.5: Average (± S.D.) biomass productivity (g DW m^{-2} d^{-1}) on triplicate FANS with a low (60-69 g DW m^{-2}), medium low (70-79 g DW m^{-2}), medium high (80-90 g DW m^{-2}) and high (100-110 g DW m^{-2}) initial standing crop over three four-day growth cycles. N = 3

Daily nitrate removal rate varied significantly among initial standing crops (ANOVA, $F_{3,24}$=6.397; P = 0.002), but not among growth cycles (ANOVA, $F_{2,24}$=1.774; P = 0.191, Figure 4.6). Across all growth cycles, the high initial standing crop FANS had the highest mean daily nitrate removal rate (0.47 g N m^{-2} d^{-1} ± 0.06), and the low initial standing crop FANS had the lowest mean daily nitrate removal rate (0.36 g N m^{-2} d^{-1} ± 0.04, Table 4.1). In contrast to nitrate removal rate, the daily phosphate removal rate varied significantly among growth cycles (ANOVA, $F_{2,24}$=6.933; P = 0.004), but not among initial standing crops (ANOVA, $F_{3,24}$=0.623; P = 0.607, Figure 4.6). Across all initial standing crops, the daily phosphate removal rate was lowest in cycle 1 (mean of 1.1 ± 0.06 g P m^{-2} d^{-1}) and highest in cycle 3 (mean 1.3 ± 0.04 g P m^{-2} d^{-1}).

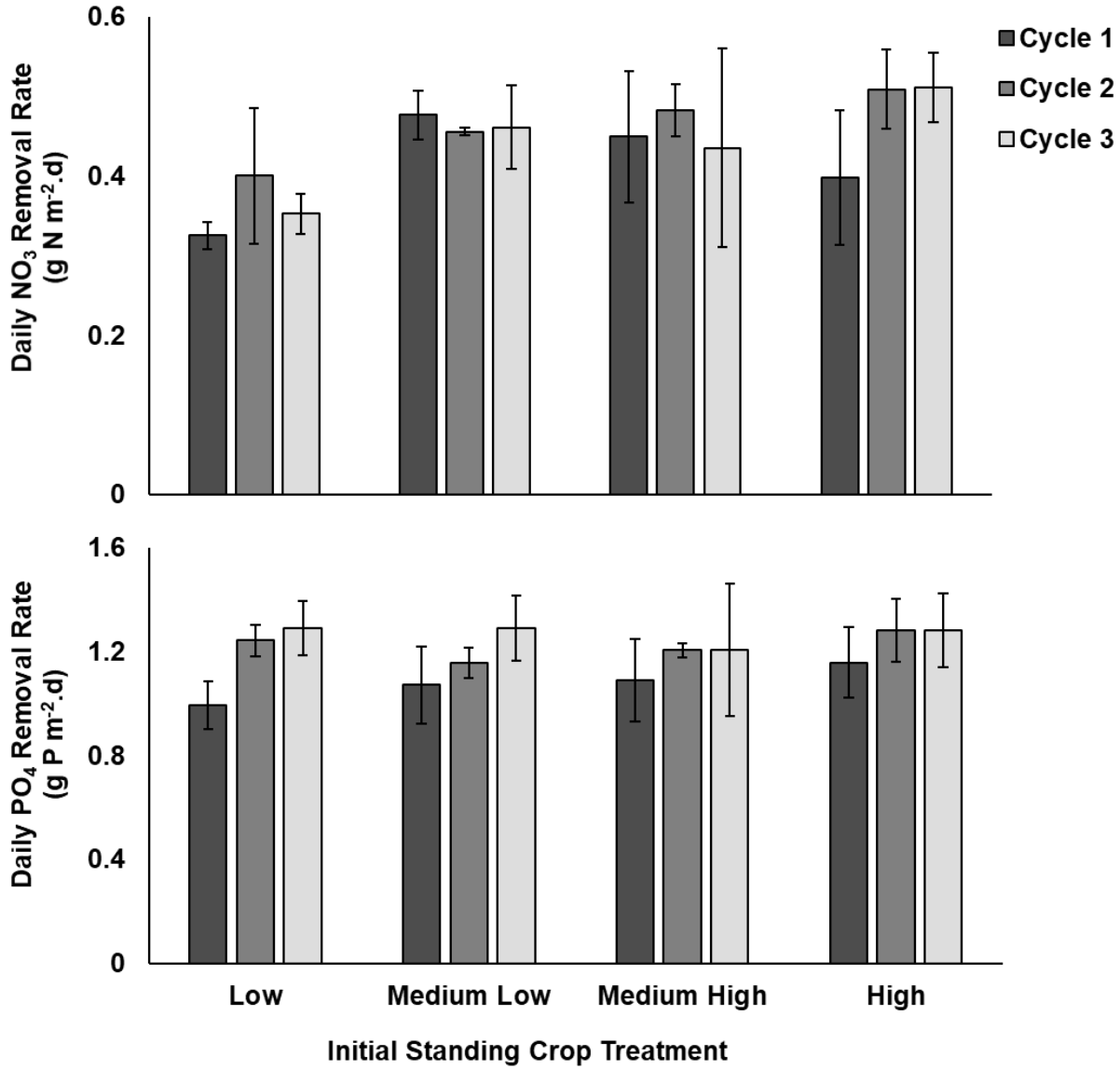

Figure 4.6: Average (± S.D.) daily NO_3-N removal rate (g N m^{-2} d^{-1}, upper panel) and daily PO_4-P removal rate (g P m^{-2} d^{-1}, lower panel) on triplicate FANS with a low (60-69 g DW m^{-2}), medium low (70-79 g DW m^{-2}), medium high (80-90 g DW m^{-2}) and high (100-110 g DW m^{-2}) initial standing crop over three four-day growth cycles. N = 3

4.4.3 The effect of influent flow rate on biomass productivity and nutrient removal rate

Influent flow rate had a significant effect on biomass productivity (ANOVA, $F_{2,12}$=17.7; P < 0.001, Figure 4.7). The FANS with the lowest flow rate (1 L min^{-1}) had the highest biomass productivity (9.3 ± 1.7 g DW m^{-2} d^{-1}), while the FANS with the highest flow rate (8 L min^{-1}) had the lowest biomass productivity (4.1 ± 1.6 g DW m^{-2} d^{-1} , Table 4.2). Influent flow rate also had considerable effects on the amount of biomass washed off the floways. On average 2%, 5%, and 10% of the total biomass produced in the 1 L min^{-1}, 3 L min^{-1} and 8 L min^{-1} flow rates respectively washed off the floways.

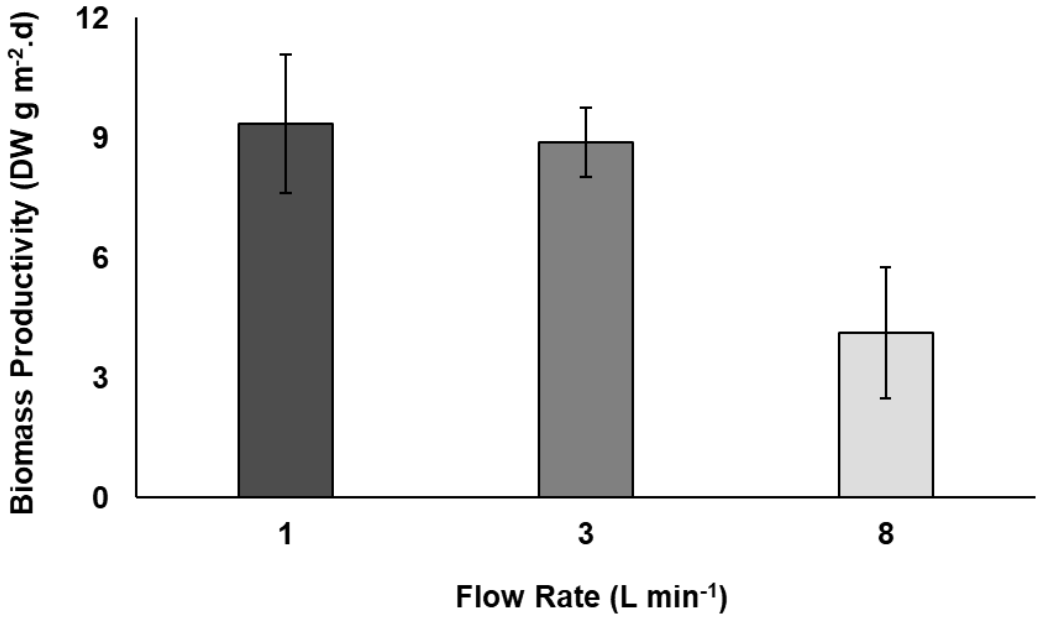

Figure 4.7: Average (± S.D.) biomass productivity (g DW m^{-2} d^{-1}) of FANS with influent flow rates of 1 L min^{-1}, 3 L min^{-1} and 8 L min^{-1} N = 5

The instantaneous nitrate reduction rate did not vary significantly among the three influent flow rates (ANOVA, $F_{2,12}$= 0.07; P = 0.924, Figure 4.8). Instantaneous nitrate reduction rate ranged from 0.047 ± 0.05 g N m^{-3} for the medium (3 L min^{-1}) flow rate FANS to 0.052 ± 0.02 g N m^{-3} for the low (1 L min^{-1}) flow rate FANS (Table 4.2). However, when nitrate removal rates were calculated based on the volume of water treated per day (e.g. g N m^{-2} d^{-1}), there were significant differences among flow rates (Kruskal Wallis test, H_2 = 8.4; P = 0.015, Figure 4.8). The FANS with the highest flow rate (8 L min^{-1}) had the highest daily nitrate

removal rate (3.28 ± 1.9 g N m^{-2} d^{-1}), while the FANS with the lowest flow rate (1 L min^{-1}) had the lowest daily nitrate removal rate (0.42 ± 0.2 g N m^{-2} d^{-1}, Table 4.2).

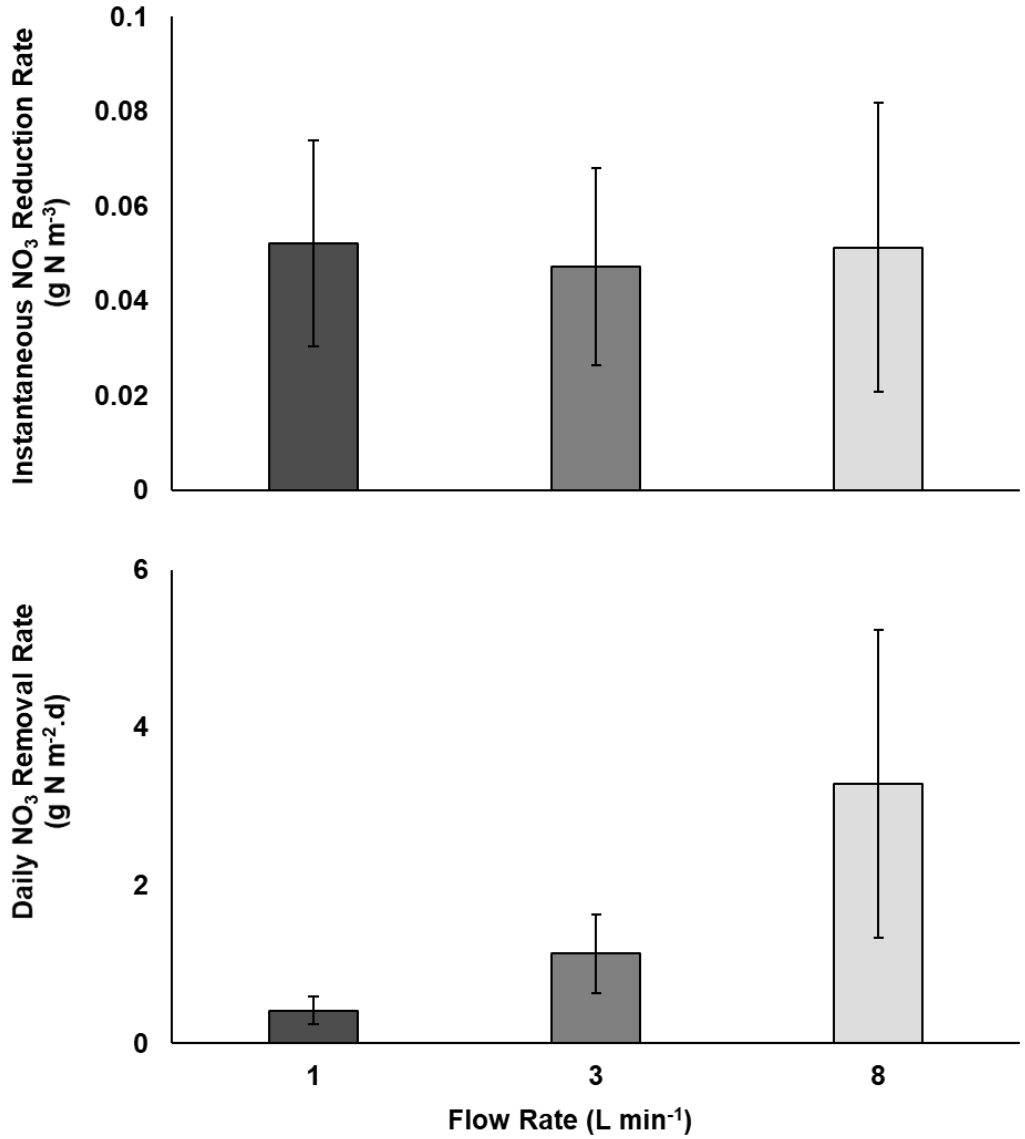

Figure 4.8: Average (± S.D.) instantaneous NO_3-N reduction rate (g N m^{-3}, upper panel) and daily NO_3-N removal rate (g N m^{-2} d^{-1}, lower panel) of FANS with influent flow rates of 1 L min^{-1}, 3 L $min^{-1}$and 8 L min^{-1} N = 5

Similarly, the instantaneous phosphate reduction rate did not vary significantly among flow rates (ANOVA, $F_{2,12}$=1.0; P = 0.387, Figure 4.9). Instantaneous phosphate reduction rates ranged from 0.102 ± 0.06 g P m^{-3} for the medium (3 L min^{-1}) and high (8 L min^{-1}) flow rates to 0.144 ± 0.05 P m^{-3} for the low (1 L min^{-1}) flow rate (Table 4.2). However, when phosphate reduction rates were calculated based on the volume of water treated per day (e.g. g P m^{-2} d^{-1}),

there were significant differences among flow rates (Kruskal Wallis test, H_2=8.4; P = 0.015, Figure 4.9). The FANS with the highest flow rate (8 L min^{-1}) had the highest daily phosphate removal rate (mean 6.56 ± 3.7 g P m^{-2} d^{-1}), while the FANS with the lowest flow rate (1 L min^{-1}) had the lowest daily phosphate removal rate (mean 1.15 ± 0.4 g P m^{-2} d^{-1} , Table 4.2).

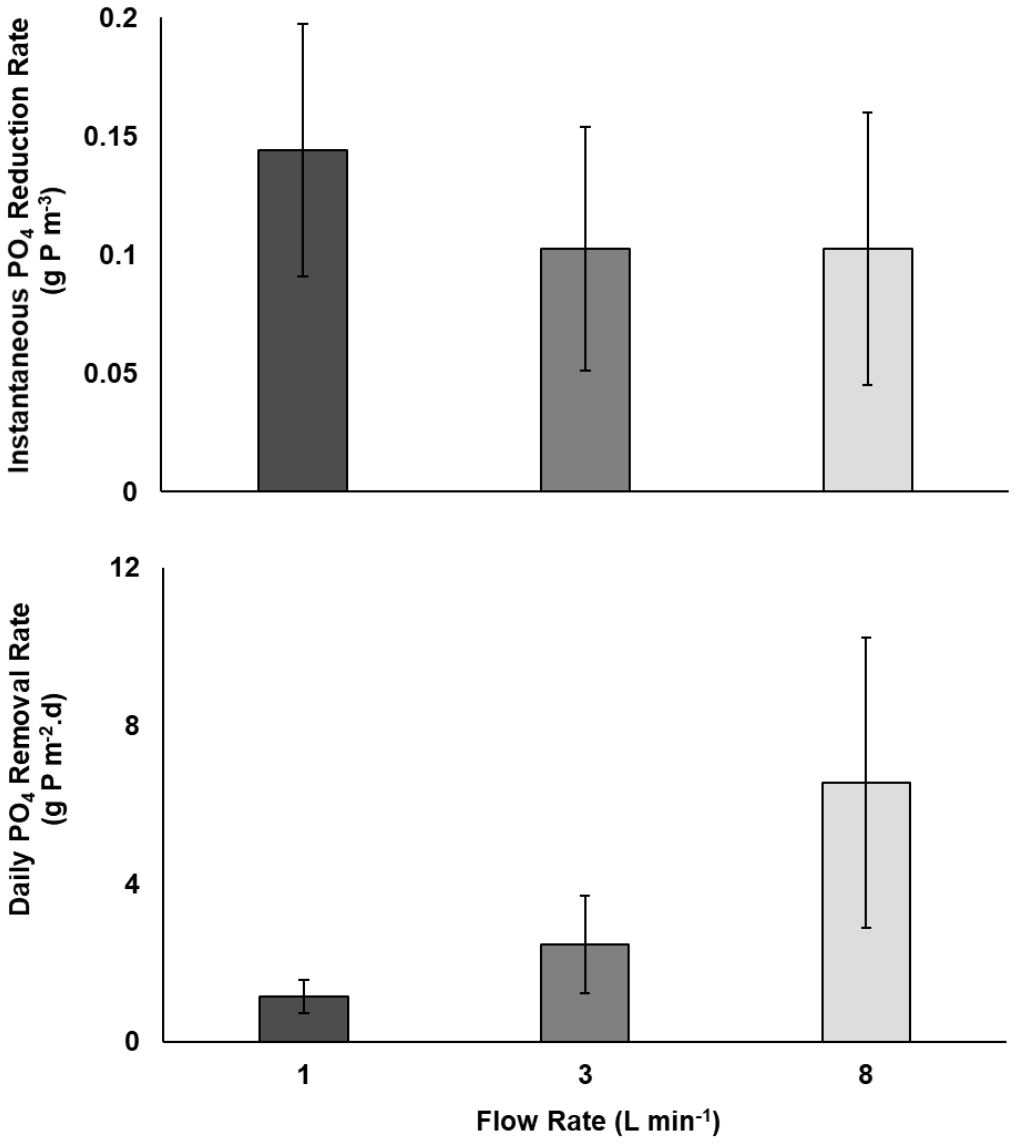

Figure 4.9: Average (± S.D.) instantaneous PO_4-P reduction rate (g P m^{-3}, upper panel) and daily PO_4-P removal rate (g P m^{-2} d^{-1}, lower panel) of FANS with influent flow rates of 1 L min^{-1}, 3 L min^{-1} and 8 L min^{-1} N = 5

Table 4.1: Key biomass productivity and nutrient removal parameters for *Oedogonium* sp. on FANS with different initial standing crops (60 – 110 g DW m^{-2}) over three four-day growth cycles. Data are means ± standard deviations, N = 3, mean values for each growth cycle were averaged to provide a global mean for each initial standing crop

Initial Standing Crop (g DW m^{-2})	60-69	70-79	80-90	100-110
Biomass Productivity				
Measured Initial Standing Crop (g DW m^{-2})	63.5 ± 1.2	73.1 ± 1.8	87.9 ± 1.6	106.4 ± 2.6
Biomass Productivity (g DW biomass m^{-2} d^{-1})	8.6± 0.5	8.5 ± 0.9	6.7 ± 0.8	5.9 ± 0.6
Nutrient Removal				
Nitrate removal rate (g N m^{-2} d^{-1})	0.36 ± 0.04	0.46 ± 0.03	0.45 ± 0.08	0.47 ± 0.06
Phosphate removal rate (g P m^{-2} d^{-1})	1.18 ± 0.09	1.17 ± 0.11	1.17 ± 0.15	1.24 ± 0.13

Table 4.2: Key biomass productivity and nutrient removal parameters for *Oedogonium sp.* on FANS with different influent flow rates (1 – 8 L min^{-1}) over five four-day growth cycles. Data are means ± standard deviations, N = 5

Influent Flow Rate (L min^{-1})	1	3	8
Biomass Productivity			
Initial Standing Crop (g DW m^{-2})	76.3 ± 3.0	76.8 ± 2.7	75.4 ± 3.3
Biomass Productivity (g DW biomass m^{-2} d^{-1})	9.3± 1.7	8.9 ± 0.9	4.1 ± 1.6
Nutrient Removal			
Nitrate Removal Rate (g N m^{-2} d^{-1})	0.42 ± 0.2	1.13 ± 0.5	3.28 ± 1.9
Instantaneous Nitrate Reduction (g N m^{-3})	0.052 ± 0.02	0.047 ± 0.05	0.051 ± 0.05
Phosphate Removal Rate (g P m^{-2} d^{-1})	1.15 ± 0.4	2.46 ± 1.2	6.56 ± 3.7
Instantaneous Phosphate Reduction (g P m^{-3})	0.144 ± 0.05	0.102 ± 0.05	0.102 ± 0.06

4.5 Discussion

We investigated the performance of *Oedogonium* sp. on pilot-scale indoor FANS under controlled irradiance and temperature to provide a comparative assessment of algal productivity and nutrient removal under variable operating parameters. The initial standing crop had a significant impact on algal biomass productivity. In the first experiment, the FANS with the lowest initial standing crop (60-70 g DW m^{-2}) had the highest total biomass increase across the five-day growth cycle and the highest daily biomass productivity on day one, two and three of the five-day growth cycle. Likewise, in the second experiment we found the highest biomass productivity for FANS with the lowest initial standing crop (60-70 g DW m^{-2}) throughout the four-day growth cycles. Lower productivity with a higher initial standing crop and thicker attached algal mat was expected as the rate of photosynthesis and biomass

production are reduced due to factors including increased self-shading of light; lower nutrient mass transfer and lower photosynthetic gas exchange (Larned et al., 2004; Wang et al., 2015; Sutherland et al., 2020b). Based on the results of this study, an initial standing crop of 60-70 g DW m^{-2} of *Oedogonium* sp. on FANS is optimal as it had the highest biomass productivity of all initial standing crops tested. However, FANS with initial standing crops lower than 60 g DW m^{-2} could potentially result in higher productivity than we have reported and therefore should be explored. As algal biomass productivity is dependent on ambient conditions (Hariz et al., 2023c), the optimal initial standing crop may also vary seasonally, especially when operating FANS under outdoor conditions. A higher initial standing crop may be optimal in summer as the higher light intensity may allow light to penetrate further into the algal mat. Conversely, a lower initial standing crop may be required in winter when light intensity is lower.

In contrast to results for biomass productivity where a lower initial standing crop was optimal, we found that FANS with the highest initial standing crop (100-110 g DW m^{-2}) had the highest nitrate and phosphate removal rates compared to FANS with lower initial standing crops (< 100 g DW m^{-2}), although differences in nutrient removal rates among standing crops were not large. As long as the light distribution and nutrient transfer rates are not limiting, a higher standing crop of algae on FANS facilitates a higher rate of nutrient removal. In agreement with our results, several studies have reported an increase in nitrate and phosphate removal from wastewater in algal treatment systems as the standing crop size increases (Pereira et al., 2006; Choi & Lee, 2012; Bohutskyi et al., 2016). However, other studies have reported a decrease in nutrient removal rates when the standing crop exceeds the optimal amount due to reduced photosynthesis caused by light limitation from algal self-shading (Ruiz-Marin et al., 2010; Park & Craggs, 2011b). These contrasting results suggest that the optimal initial standing crop for FANS is likely to be system and species specific. For *Oedogonium* sp. on FANS, the present study identified 70-80 g DW m^{-2} as the optimum initial standing crop for combined effective nutrient removal (mean 0.46 ± 0.03 g N m^{-2} d^{-1} and 1.17 ± 0.11 g P m^{-2} d^{-1}) and biomass productivity (mean 8.5 ± 0.9 g DW biomass m^{-2} d^{-1}).

Harvesting frequency was another critical factor that impacted biomass productivity on FANS. Harvesting at an optimal frequency prevents light limitation due to self-shading of the algae and promotes greater nutrient and carbon dioxide transfer rates, thereby maintaining high growth rates (Adey et al., 2011). In addition, when attached algae on FANS are not frequently

harvested, the bottom layer of attached algae may become light and nutrient-deprived which causes filaments to lose their viability and easily detach and wash off the FANS (Schnurr & Allen, 2015). Under the controlled irradiance and temperature conditions in the current study, daily biomass productivity increased rapidly in the first three days of the growth period across all initial standing crops, but then slowed down towards day five. Furthermore, when biomass productivity was calculated for growth cycles ranging in duration from one to five days, it was highest for a three-day growth cycle, regardless of the initial standing crop. To date, the effects of harvesting frequency on FANS have only been investigated in a single study. Sutherland et al. (2020b) tested harvesting frequencies ranging from seven to fourteen days outdoors during summer and found that the electron transport rate was significantly higher in the lower layer of the algal mat at a shorter harvesting interval, suggesting that the lower layer of a thinner algal mat on FANS receives more light compared to the lower layer of a thicker algal mat that results from a longer harvesting interval. Similar to our findings, biomass productivity was highest two days following harvesting, regardless of harvesting frequency (Sutherland et al., 2020b). In combination, these results suggest that a shorter harvesting interval results in higher biomass productivity on FANS than a longer harvesting interval. However, the optimal harvesting frequency may be longer for FANS with lower initial standing crops that those tested here. Regardless, our results demonstrate that biomass productivity peaks on day three and we therefore recommended harvesting the following day (on day four) would be ideal to ensure that *Oedogonium* sp. on FANS maintain high growth rates to maximize biomass production and are not limited by self-shading or other factors such as low carbon dioxide and nutrient mass transfer rates.

Influent flow rate is another essential determinant of algal biomass productivity on FANS, with different species of algae being more suited to certain flow conditions (Liu et al., 2016b). Most filamentous green algae, including *Oedogonium* sp. prefer low water velocities (Whitford & Schumacher, 1964; Ghosh & Gaur, 1998; Labiod et al., 2007). This is reflected by communities of long filamentous green algae in natural environments typically occurring at low water velocities, with *Oedogonium* sp. dominating communities at horizontal water velocities less than 0.1 m s^{-1} (Biggs et al., 1998). These findings align with our results, where biomass productivity was highest on FANS with an influent flow rate of 1 L min^{-1} (equivalent horizontal water velocity of 0.04 m s^{-1}) compared to FANS with an influent flow rate of 3 L min^{-1} (equivalent horizontal water velocity of 0.12 m s^{-1}) and above. In contrast, Park et al. (2022) found a decrease in algal biomass productivity when the horizontal flow velocity

decreased from 0.16 to 0.04 m s^{-1} on FANS treating water from the Waikato River in Hamilton, New Zealand that were dominated by filamentous diatoms. In addition to the difference in algal species composition compared to our study, other factors such as increased pH and temperature may have reduced productivity at the lower influent flow rate measured by Park et al. (2022). Their FANS were at least double the length of our FANS, resulting in longer algae contact time (ACT) of up to 10 min compared to 1.3 min ACT at 1 L min^{-1} on our FANS. This longer ACT resulted in an increase in water pH of up to 10.7 along the FANS (maximum pH was 8.9 on our FANS) and an increase in daytime water temperature to above 30°C (maximum water temperature was 22°C on our FANS). The high biomass productivity under low influent flow rates found in the present study is most likely due to lower turbulence levels compared to those generated by higher influent rates, as higher turbulence can cause higher shear stress and drag that can lead to biomass dislodgment and sloughing, subsequently reducing biomass productivity (Ahn et al., 2013; Blersch et al., 2013). Supporting this, we found that influent flow rates of 3 L min^{-1} (equivalent horizontal water velocity of 0.12 m s^{-1}) and higher caused much greater rates of biomass sloughing compared to influent flow rates of 1 L min^{-1} (equivalent horizontal water velocity of 0.04 m s^{-1}). While it is difficult to quantify the amount of shear that algae cells can endure, and this will vary depending on the species, comparing the growth performance of a monoculture species on FANS under different influent flow rates may help to determine their ability to persist under varying rates of turbulence.

In addition to influencing algal biomass productivity, influent flow rate can also significantly affect the nutrient removal performance of FANS. A lower influent flow rate creates a longer hydraulic retention time (HRT) for polluted water in the FANS, providing greater potential for the attached algae to assimilate more nutrients and subsequently reduce the nutrient concentration at the outflow (Craggs et al., 1996a; Park et al., 2022). We found that there were no significant differences in nitrate and phosphate removal rates when these were calculated as instantaneous rates (e.g., differences in nutrient concentrations between FANS inflow and outflow), but there were significant differences when rates were calculated based on the volume of water treated per day (e.g., areal daily nutrient removal rate). Although comparing FANS instantaneous nutrient reduction among flow rates is essential, the areal daily nutrient removal rate comparison is more relevant to a large-scale FANS operation where abundant polluted water requires treatment. We found that higher influent flow rates lead to higher daily nutrient removal rates for both nitrate and phosphate. This result is an important finding in the context of large-scale FANS bioremediation as it demonstrates that operating

FANS with a higher influent flow rate is likely to result in the removal of a higher total load of nutrients per day.

4.6 Conclusion

Overall, our results suggest that an initial standing crop of 70-80 g DW m^{-2}, harvesting interval of four days and influent flow rate of 1 L min^{-1} (16.7 L min^{-1}.m width; equivalent horizontal water velocity of 0.04 m s^{-1}) were optimal for unialgal *Oedogonium* sp. FANS to maximize biomass productivity and nutrient removal under controlled irradiance and temperature. Importantly, we found that optimal settings were a trade-off between nutrient removal and productivity, and we chose the operating parameter that gave the best overall result. There is still limited understanding of the influence of FANS operating parameters such as initial standing crop, harvesting frequency and influent flow rate on biomass productivity and nutrient removal rates. This study provides insights into these factors and our findings are likely to apply to other filamentous algae species with similar physical and ecological features to *Oedogonium* sp. which can be cultivated on FANS (e.g., *Cladophora* sp., *Rhizoclonium* sp., and *Spirogyra* sp.). While the optimal operating parameters identified in the present study for *Oedogonium* sp. may vary somewhat for other species, they provide a good starting point for future optimization studies and performance testing.

Chapter 5

Effects of Seeding Method and Single versus Mixed Species Assemblages on the Performance of Filamentous Algae Nutrient Scrubbers (FANS) for the Treatment of Agricultural Drainage

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5.1 Abstract

FANS have potential for on-farm treatment to remove and recover nutrients from agricultural drainage. The performance of FANS can be influenced by the seeding method and the species composition of the algal biomass. This study compared the effects of seeding approach (controlled seeded vs. natural establishment, experiment 1) and single species (*Oedogonium* sp.) vs. mixed species algal assemblages (experiment 2) on the biomass productivity and nutrient removal rates of FANS treating agricultural drainage in two on-farm experiments, each conducted over a three-month period. In the first experiment, biomass established five times faster on controlled seeded FANS (10 days) compared to FANS left to naturally establish (7 weeks). In the second experiment, both *Oedogonium* sp. and mixed species algal assemblages seeded through controlled seeding established uniformly on FANS within two weeks. Overall, the seeding method and species composition of algae seeded on FANS did not significantly affect biomass productivity and nutrient removal performance. However, the amount of biomass washed off from *Oedogonium* sp. FANS was four and a half times lower than that washed off the mixed species FANS, resulting in a higher standing crop on *Oedogonium* sp. FANS and a subsequently higher nutrient removal rate due to algal

removal. Furthermore, FANS seeded with *Oedogonium* sp. had a lower contamination rate in terms of percentage coverage of non-target species than mixed species FANS. These results demonstrate that controlled seeding and cultivation of a single target filamentous algae species will help maintain a higher abundance of a target species over a longer period enabling the recovery of high-quality biomass with low variation in algae species composition.

5.2 Introduction

Rapid growth and intensification of agriculture in New Zealand have negatively impacted freshwater quality, especially in the form of diffuse nutrients (Ripa et al., 2006; Howard-Williams et al., 2010; Duncan, 2017). Agricultural drainage is a major source of diffuse nutrients to freshwaters, causing eutrophication that promotes nuisance growth of filamentous algae (Davies-Colley, 2013). Eutrophic conditions in water bodies affect the survival of aquatic animals (e.g. deoxygenation, toxic algal blooms) and can result in the degradation of ecosystems (Anderson et al., 2008; Abell et al., 2011; Paerl & Otten, 2013). Diffuse nutrients in agricultural drainage, mainly nitrogen and phosphorus, result from the use of fertiliser and the deposition of faeces and urine by grazing livestock which are transported into waterways through surface runoff and shallow groundwater through subsurface drainage (Heathwaite & Dils, 2000b; Nguyen & Sukias, 2002; Heathwaite et al., 2005b). Reducing nutrient concentrations in agricultural drainage is therefore critical to significantly reduce the eutrophication of New Zealand's waterways (Ballantine & Davies-Colley, 2014; Scarsbrook & Melland, 2015b).

Filamentous algae nutrient scrubbers (FANS) could provide a natural, effective and sustainable approach to reduce nutrient concentrations in agricultural drainage. FANS are ecologically engineered floways that grow attached filamentous algae and associated periphyton to assimilate dissolved nutrients through algal photosynthesis (Craggs, 2001; Adey et al., 2011; Sutherland & Craggs, 2017). FANS have been successfully operated for extended periods of time under varying outdoor conditions and across multiple seasons to bioremediate a wide range of polluted water (Craggs et al., 1996a; Mulbry et al., 2008b; Mulbry et al., 2010; Sandefur et al., 2011b; Adey et al., 2013; Kangas & Mulbry, 2014; D'Aiuto et al., 2015; Kangas et al., 2017; Marella et al., 2019). FANS have the potential to not only bioremediate the diffuse nutrients in agricultural drainage, but also to store these nutrients in the form of algal biomass that can be regularly harvested, thereby reusing the nutrients as a feedstock for the production

of value-added products (Smith, 2014; Lawton et al., 2017). In addition, FANS may only require half of the land area to achieve the same nitrogen removal compared to other approaches such as constructed wetlands that are commonly used to treat agricultural drainage (Sutherland & Craggs, 2017; Park et al., 2022).

Filamentous algae naturally grow attached to a substrate, aided by several biological mechanisms including algal biofilm establishment, microbial succession, and symbiotic relationships with bacteria (Riding, 2000; Mieszkin et al., 2013). Therefore, FANS are typically seeded by allowing algae from nearby waterways to establish naturally on the FANS liner (Wilkie & Mulbry, 2002; Mulbry et al., 2008b; Adey et al., 2013; D'Aiuto et al., 2015). However, filamentous algal attachment can also be achieved through the novel approach of controlled seeding by rubbing algal biomass onto the FANS liner . This provides initial attachment through physically "hooking" algal filaments onto the FANS liner. Secondary attachment then occurs through the subsequent development of holdfasts through two biological attachment mechanisms - rhizoid formation and zoospore formation and settling. There are several ways that the seeding approach (e.g., natural establishment vs. controlled seeded) may affect overall FANS performance. Natural establishment FANS take longer to establish a uniform attached biomass (three weeks to two months (Mulbry et al., 2008b) compared to controlled seeded FANS (as little as four days) (Hariz et al., 2023a). This could be due to an uncontrolled or insufficient amount of algal seed introduced as inoculum when using a natural establishment seeding approach as the amount of algal seed is highly dependent on the abundance of algae in the waterway used for seeding (Phang et al., 2004; Carl et al., 2014). In contrast, a large amount of algal seed can be added to the FANS liner at the start of seeding through the controlled seeding method, subsequently promoting a higher rate of settlement on FANS liner by algal spores (Hariz et al., 2022). In addition, faster and more uniform biomass establishment across the entire FANS surface can be achieved through controlled seeding by adding more biomass or conducting daily reseeding on selected areas that are lacking coverage. Aside from differences between seeding methods in the time taken to establish a uniform biomass attachment and coverage, the dominance of algal species that establish on natural establishment FANS may be difficult to control and can vary widely through time, with potential significant effects on overall FANS performance (Craggs et al., 1996b; Craggs, 2001).

FANS are typically seeded with mixed algal assemblages (Sandefur et al., 2011a; Liu et al., 2016a; Park et al., 2022). However, it is also possible to establish and maintain a single selected filamentous algae species on FANS (Hariz et al., 2022). While the cultivation of a single species on FANS may not be required for effective nutrient removal, targeting a single high performing species that can grow under a broad range of conditions could reduce the impact of environmental variation on biomass productivity and nutrient removal efficiency (Hariz et al., 2023c). Furthermore, selecting a single target species could provide a consistent source of high-quality biomass with low variation in algae species composition (Lawton et al., 2013a; Neveux et al., 2016) and facilitate the optimization of operational parameters for a specific aim (e.g. bioremediation, biomass production or both) (Hariz et al., 2023a, 2023c). However, maintaining a single species on outdoor FANS treating polluted water could be challenging if the target species has been previously maintained under controlled laboratory conditions as it may be poorly adapted to varying ambient environmental conditions and water quality (Liu et al., 2020). Seeding FANS with a mixed species assemblage collected from a nearby waterway may help the algae establish faster as it is likely be adapted to ambient conditions. However, the algal biomass may contain a significant amount of non-target competing species such as diatoms and cyanobacteria that could grow faster than the green filamentous algae and quickly come to dominate the biomass on the FANS (Hariz et al., 2023c) (Lawton et al., 2013a; Cole et al., 2015; Hariz et al., 2023c). This could significantly affect the overall FANS performance in terms of biomass productivity and nutrient removal rates and result in a harvested biomass of low quality (Hariz et al., 2023c). Despite the potential large influence of using a single vs. mixed species assemblage on the performance of FANS, this issue has only been addressed in two studies using suspension (non-attached) algal cultivation systems (Lawton et al., 2013a; Valero-Rodriguez et al., 2020). Thus far, the effects of single vs. mixed species assemblages on growth, nutrient removal rates and algal composition dynamics in attached algal cultivation systems such as FANS have not been investigated and remain a critical knowledge gap (Liu et al., 2020; Karimi et al., 2022).

The overall aim of this study was to compare the effects of seeding approach, and single species vs. mixed species algal assemblages on the performance of pilot-scale FANS for the in-situ treatment of agricultural drainage on a dairy farm. The specific aims were (i) to compare the time taken to establish a uniform algal standing crop, biomass productivity, nutrient removal and species dominance on FANS seeded by either natural establishment or by controlled seeding of a single target species; and (ii) to compare the time taken to establish a

uniform algal standing crop, biomass productivity, nutrient removal and dominance of species on FANS seeded through controlled seeding of a single target species and FANS seeded through controlled seeding of an adapted mixed species algal assemblage. This study was conducted in two separate experiments. The first experiment compared the performance of FANS seeded with the target species *Oedogonium* sp. and FANS naturally seeded with filamentous algae originating from an on-farm drainage stream. The second experiment compared the performance of FANS seeded using controlled seeding with the target species *Oedogonium* sp. and FANS seeded using controlled seeding with a mixed algal assemblage collected from a nearby drainage stream on the farm. *Oedogonium* sp. was selected as the target species to be seeded on FANS as it had up to 4-fold higher biomass productivity and the highest nutrient removal rate compared to *Cladophora* sp., *Rhizoclonium* sp. and *Spirogyra* sp. when cultivated on outdoor pilot-scale FANS under ambient summer and winter conditions (Hariz et al., 2023c). In addition, *Oedogonium* sp. had the highest zoospore production and strongest secondary attachment capability of these four species in small scale microFANS cultivation systems (Hariz et al., 2022).

5.3 Methods

5.3.1 Pilot-scale Filamentous Algae Nutrient Scrubber

The pilot-scale FANS system used in this study consisted of six floways made from aluminium I-beams (size: 6 m length x 0.12 m width x 0.05 m depth) with a high-density polyethylene (HDPE) textured liner (size: 6 m x 0.12 m or 0.72 m^2). The textured liner was permanently attached to the base of the floways using a multi-purpose permanent elastic sealant/adhesive glue (brand: Bostik ISR 70-03). This liner was chosen as studies have shown that textured substratum or surfaces with variable topography can enhance algal attachment (Maggs & Callow, 2003; Blersch et al., 2017).

5.3.2 Study Site

The study was conducted by an agricultural drainage stream on a 94-hectare low land dairy farm near Morrisville, New Zealand (37°41'41.0"S, 175°34'19.2"E). The farm drainage ultimately discharges into the Piako River which drains into the Firth of Thames on the North

Island of New Zealand (Figure 5A.1). The farm produces milk from a herd of approximately 420 cows which are pasture fed. The pasture is predominantly covered by ryegrass. The farm drainage stream receives water from surface runoff and subsurface runoff from the paddocks through subsurface drain pipes (tiles). The stream flows between paddocks, with electric fences installed along the stream embankment to exclude cows. The first experiment was conducted next to a small stream that was 1 m wide and 1.5 m deep (Site 1, Figure 5A.1). The stream continually flowed all year but had minimal flow during the autumn (April to June). The second experiment was located approximately 200 m downstream from the location of the first experiment, next to a small river (10 m wide and 3 m deep; Site 2, Figure 5A.1). This river flowed year-round as it received water from multiple streams and drainage ditches on the farm, including the stream used in the first experiment.

5.3.3 Controlled seeded single species FANS vs. natural establishment mixed species FANS

The on-farm performance of FANS seeded with the single species *Oedogonium* sp. (controlled seeded) and FANS seeded through the natural establishment of mixed species algal assemblages to treat agricultural drainage was compared in a three-month study. Six FANS floways were installed on three horizontal lengths of scaffolding steel tubes approximately 50 cm above the stream water level on the embankment next to the stream. The floways were placed to flow in the same direction as the stream (Figure 5.1). The horizontal scaffolding tubes were adjusted so that the floways had a slope of approximately 2 % to provide gravity flow from the top (inflow) to the bottom (outflow). During the day water from the stream was pumped into a 200 L black water HDPE header tank (58 cm diameter x 98 cm high) using a solar powered pump. A 200 W solar panel provided power to the pump through two 7 amp/hour 12 V batteries. The 24 V DC submersible pump (3 phase) was installed underwater at approximately ~30 cm above the stream bottom and pumped water through a 32 mm pressure PVC water pipe into the header tank which was placed on the stream embankment. The submersible water pump intake was covered with a 1 mm mesh to filter out sediment particles (sand, silt and clay) and plant debris. Accumulated sediment was regularly removed (at least once a week) by cleaning and backwashing the mesh. An additional 25 mm PVC water pipe was installed approximately ~10 cm from the top of the water tank to channel water back into the stream to prevent water from overflowing when the tank was full. Water collected in the

water tank was channeled to an inflow water distribution sump (100 L x 25 cm width x 21 cm height, UV resistant plastic) through a 32 mm pressure PVC water pipe. Additional water was channeled into the same inflow water distribution sump through a separate water pipe (32 mm diameter x 25 m length UV resistant, flexible pool hose) that collected water from upstream through gravity flow (intake point approximately 25 m upstream from where the FANS were installed). The gravity flow intake water hose was covered with a 1 mm mesh and was regularly cleaned as described above. Flow from the water collection tank and the gravity flow water hose to the inflow water distribution sump was controlled by two 32 mm ball valves installed inside the inflow water distribution sump. These ball valves were adjusted to deliver 6 L min^{-1} from the 200 L black water collection tank, and 3 L min^{-1} from the gravity flow into the inflow distribution sump. The inflow water distribution sump was placed at the top of all FANS and had six inflow pipes, each with adjustable valves (25 mm brass gate valve), installed to channel water to the top of each FANS. The inflow flow rate to each FANS was adjusted to 1 L min^{-1} (equivalent to 8 L min^{-1}.m width of floway) using a digital flow rate meter (DigiFlow 6710M, Vyair). Each floway received 1 L min^{-1} of flow during the day (average of 10 hours through a combination of water pumped through solar power and the gravity flow) and approximately 0.4 L min^{-1} (equivalent to 3 L min^{-1}.m width of floway) of flow during the night (where the system was completely dependent on the gravity flow). Flow rates were maintained throughout the experimental period and checked volumetrically twice a week. The outflow water from each FANS was collected and channeled back into the stream using a 3 m length of PVC spouting. The outflow of each floway was equipped with 0.5 mm mesh to collect any algal biomass that washed off.

Figure 5.1: The farm stream and the 6 replicate FANS installed along the stream used in Experiment 1. Pumped stream water was distributed between the FANS using a sump with six adjustable valves

Three replicate FANS were seeded with a single species of *Oedogonium* sp. at the National Institute of Water and Atmospheric Research (NIWA) facility at Ruakura, Hamilton, New Zealand. The FANS were seeded by evenly distributing and rubbing 30 g fresh weight (FW) *Oedogonium* sp. biomass (equivalent to 5 g dry weight (DW) or a loading rate of 7 g DW m^{-2}) onto the textured liner to hook algae filaments and provide initial physical attachment (controlled seeding) (Hariz et al., 2023c). A continuous influent flow rate of 0.5 L min^{-1} (equivalent to 4 L min^{-1}.m width of floway) was used for the first seven days after seeding. Thereafter, an influent flow rate of 1 L min^{-1} (equivalent to 8 L min^{-1}.m width of floway) was used throughout the seeding phase. Over time, secondary biological attachment occurred through the growth of holdfasts from the settlement of *Oedogonium* sp. zoospores onto the liner. It took ten days (from 10th to 20th December 2021) to establish a dense algal turf on the liner surface. The three *Oedogonium* sp. seeded FANS were then transported to the farm and installed along the stream on the 21st December 2021. Each FANS received a stream water inflow of 1 L min^{-1} for 10 hours during the day and then only 0.4 L min^{-1} during the night (as described above). The three *Oedogonium* sp. seeded FANS were harvested once every seven days by drawing a metal scraper down the surface of the liner to maintain a target initial algal standing crop of approximately 0.5-1 cm height. On the 13th December 2021, the natural establishment FANS were installed unseeded and operated to allow algae from the stream to

begin to naturally establish. The natural establishment FANS took approximately seven weeks (until 1st February 2022) to form a uniform algal turf on all replicate FANS.

Weekly biomass monitoring and water sampling was conducted from 4th of January 2022, providing time for the controlled seeded *Oedogonium* sp. biomass to adjust to the new growing conditions and farm drainage water, and for the natural establishment FANS algal turf to establish on the liner. At the start of this monitoring period, the biomass on the *Oedogonium* sp. seeded FANS was harvested by drawing a metal scraper down the surface of the liner to leave a comparable initial standing crop on all replicates of approximately 65 g DW m^2 (algal turf of approximately 0.5-1 cm height). However, the natural establishment FANS had uneven algal biomass coverage on the liner (~50 % surface area covered) and an initial standing crop of only approximately 16 g DW m^2 (algal turf of approximately < 0.5 cm height) and so were left unharvested. Weekly biomass monitoring and water sampling were then conducted over the following 91 days as described below, resulting in 13 consecutive growth cycles.

Loggers were installed to continually record light intensity (LI-COR LI-1500 light sensor data logger), ambient temperature (HOBO Pendant® temperature data logger), rainfall (HOBO Pendant® event data logger) and water temperature (HOBO Pendant® MX2200 water temperature data logger). The water temperature data logger was placed inside the inflow water distribution sump and the outflow FANS drainage gutter. The light data logger was secured next to the FANS on a levelled horizontal surface. The ambient temperature data logger was placed inside a solar radiation shield to avoid direct solar radiation. The study was conducted during the summer months from mid-December 2021 to early April 2022. The maximum sunlight intensity of each day ranged from 194-2072 µmol m^{-2} s^{-1}, the ambient air temperature ranged from 7-35°C, the FANS inflow water temperature ranged from 9-28 °C and outflow water temperature ranged from 10-30 °C (Figures 5A.2-6).

A strip biomass monitoring method was used to quantify the initial standing crop and the biomass productivity (DW g m^{-2} d^{-1}) of each replicate FANS once per week for the duration of the experiment. The initial standing crop was determined by entirely scraping the attached algal biomass off the floway in a horizontal strip area (0.12 m x 0.025 m) at 3 points along the length of the FANS (top, middle and bottom). A different metal scraper was used for *Oedogonium* sp.

seeded FANS and natural establishment FANS to avoid cross-contamination. The scraped biomass samples were dried overnight in an oven at 65 ºC, subsequently weighed and used to calculate the DW initial standing crop. The total FANS initial standing crop was then estimated as follows:

Total FANS area: 0.12 m x 6 m = 0.72 m^2

Strip monitoring area: 0.12 m x 0.025 m = 0.003 m^2

Strip monitoring to total FANS area ratio: 0.72 m^2/0.003 m^2 = 240

FANS Initial Standing Crop Day 0 (g DW) = [(DW Top + DW Middle + DW Bottom)/3] x 240

Strip biomass monitoring was repeated after seven days for each replicate FANS as described above to determine the DW final standing crop (Day 7, standing crop before harvest). The final standing crop was then calculated as follows:

FANS Final Standing Crop Day 7 (g DW) = [(DW Top + DW Middle + DW Bottom)/3] x 240

Any algal biomass that had washed off each floway was collected from the outflow mesh, dried overnight at 65 ºC and weighed, and was included in the biomass productivity measurement for each FANS. The biomass productivity of each replicate FANS was then calculated in g of DW biomass per m^2 per day (DW g m^{-2}. d) using the equation:

Biomass Productivity (DW g m^{-2}. d) = [(FANS DW Standing Crop Day 7 – FANS DW Standing Crop Day 0) + Washed Off Biomass (g DW) / 0.72 m^2] / 7 days.

where 0.72 m^2 is the surface area of the FANS.

The percentage of biomass washed off each replicate FANS each week was calculated based on total g of DW of washed off biomass that was collected from the outflow mesh per g of DW biomass as final standing crop (%) using the equation:

Washed Off Biomass (%) = Washed Off Biomass (g DW) / [FANS Final Standing Crop Day 7 + Washed Off Biomass (g DW)] X 100 %

Immediately after strip monitoring was conducted to determine the final standing crop, the entire surface of each FANS was harvested using a metal scraper, leaving a uniform cover of attached algal biomass with 0.5-1 cm height. The natural establishment FANS were only harvested from week five of the experiment onwards as a dense uniform algal turf did not form until four weeks after the biomass monitoring and water sampling had started. Immediately after harvesting, the strip biomass monitoring steps were repeated as described above to determine the DW initial standing crop (standing crop following harvest) for each replicate FANS for the next weekly growth cycle.

Each week, a 50 ml water sample was collected from the inflow and outflow of each replicate FANS immediately before harvest and 20 min following flow being restored following harvest. The sample was filtered using a 0.7 µm glass microfiber filter (Brand: Whatman GF/F) and the concentration of nitrate (NO_3-N) and dissolved reactive phosphate (DRP) in each water sample was determined on a microplate reader using the ultraviolet spectrophotometric method (Standard Method Nitrate 4500-NO3−B) and the ascorbic acid method (Standard Method Phosphorus 4500-P) respectively. The ammonia concentration of the inflow measured was very low ranged from 0.07 - 0.12 g N m^{-3}. Through this experiment, the nitrate and phosphate concentrations of the inflow ranged from 2.1 - 5.7 g N m^{-3} and 0.04 - 0.87 g P m^{-3} (Figure 5A.7). The nutrient removal rate for each replicate FANS was calculated using the formula:

Nutrient Removal Rate (g m^{-2} d^{-1}) = (FANS Inflow Nutrient Concentration (g m^{-3}) – FANS Outflow Nutrient Concentration (g m^{-3})) x FANS Working Volume (m^3 d^{-1}) / 0.72 m^2

Inflow water samples were analysed for total volatile solids (VSS) and ash content once every four weeks according to standard methods (APHA, 2005) (Figure 5A.8).

Every fortnight immediately before harvesting, a small subsample of attached algal biomass was collected from the strip monitoring area (0.12 m x 0.025 m or 0.003m^2) on each replicate FANS and used to microscopically quantify the relative abundance of algae species and other contamination. Samples were preserved in a 15 ml vial with a few drops of Lugol's

solution and were analyzed within five days of collection. Immediately before analysis, each sample was filtered using a 200 µm sieve and then washed with 10 ml of filtered water, which was collected for analysis (filtrate). This step separated epiphytic diatoms, midges, and any other visible organisms attached to algal filaments to make it easier to see cellular detail and identify algae species under the microscope. The retained algal biomass and 10 ml filtrate were analysed using an inverted microscope (Leica DMi1). The combined biomass and filtrate were proportionately divided onto each of ten microscope slides for each sample. Then a photograph of each slide was taken under 100 x magnification giving a total of ten photos for each sample. In each photo, the percentage composition of green filamentous algae genera and other contaminants (diatoms and cyanobacteria) was estimated based on the surface area covered. The percentage compositions were then averaged to provide a measure of the relative abundance of each genera of green filamentous algae and other contaminants for each replicate FANS.

The ash content of the harvested algal biomass on each replicate FANS was analysed once every four weeks (Figure 5A.9). Total harvested biomass was blended using a handheld blender (Living & Co SRO8310 600W Stick Stainless Steel Mixer) and a 100 ml subsample of blended biomass was dried overnight in an oven at 65°C. The dried subsample was weighed as total dried biomass (g DW) and further dried at 500°C in a furnace for 5 hours. The dried biomass was then weighed as total ash (g). The ash content in the harvested algal biomass was then calculated as percentage ash (%) using the equation:

Ash Content (%) = Total Ash (g)/Total Dried Biomass (g DW) x 100%

5.3.4 Single species FANS vs. adapted mixed species assemblage FANS

The on-farm performance of FANS seeded with *Oedogonium* sp. and FANS seeded with a mixed species assemblage of farm-adapted filamentous algae was compared in a second three-month study. Three replicate FANS were seeded with *Oedogonium* sp. and three replicate FANS were seeded with a mixed species assemblage of filamentous algae that was collected from the Experiment 1 farm drainage stream. The mixed species assemblage was comprised of 35% of *Oedogonium* sp., 35% of *Spirogyra* sp., 10% of *Cladophora* sp., 10% of filamentous

diatoms (*Melosira* sp.), 5% of filamentous cyanobacteria (*Oscillatoria* sp.) and 5% of other green filamentous algal (*Klebsormidium* sp. *Ulothrix* sp. and *Stigeoclonium* sp.).

All six FANS floways were installed on horizontal scaffolding tubes approximately 20 cm above the ground on the embankment next to the river. The floways were placed so that they flowed in the same direction as the river (Figure 5.2). The scaffolding tubes were adjusted so that the floways had a slope of approximately 2 % to provide gravity flow from the top (inflow) to the end (outflow). A solar powered pump was used to transfer river water to a water storage system consisting of eight 1000 L black IBC water tanks. A solar power system was installed at the site to only pump water during daylight hours. It consisted of six 455 W solar panels directly connected to six 220 amp/hour 12V batteries with an inverter connected to two submersible pumps (Xylem Lowara DOC 3 submersible pump). The submersible water pump was installed underwater at approximately ~30 cm above the river bottom and pumped water through a 32 mm pressure PVC water pipe into the eight 1000 L water tanks which were placed on the stream embankment. Two tanks were used for intermediate water storage during the day and the remaining six tanks stored water to enable continuous flow during the night. Water collected in the tanks was channeled to an inflow water distribution sump (100 L x 25 cm width x 21 cm height, UV resistant plastic) through a 32 mm pressure PVC water pipe. Water flow from the day and night water tanks were controlled by two 32 mm ball valves installed inside the inflow water distribution sump. These ball valves were adjusted to deliver 6 L min^{-1} from the day flow tank and 4 L min^{-1} from the night flow tank into the inflow distribution sump. The daytime inflow flow rate to each FANS was adjusted to 1 L min^{-1} (equivalent to 8L min^{-1}.m width of floway) pumped through solar power. At night (when the solar pump had stopped pumping and water was sourced from the collected water stored in the tanks) the flow reduced to approximately 0.5 L min^{-1} (equivalent to 4 L min^{-1}.m width of floway) of flow). These flow rates were maintained throughout the experimental period and checked volumetrically twice a week.

Figure 5.2: The farm river and the 6 replicate FANS installed along the river used in Experiment 2. Pumped river water was distributed between the FANS using a sump with six adjustable valves

All replicate FANS were seeded on the farm using controlled seeding as described in Section 5.3.3 with 30 g fresh weight (FW) biomass for each FANS, equivalent to 5 g dry weight (DW) or a loading rate of 7 g DW m^{-2}. A continuous influent flow rate of 0.5 L min^{-1} (equivalent to 4 L min^{-1}.m width of floway) was used for the first seven days after seeding. Thereafter, the influent flow rate was adjusted to 1 L min^{-1} (equivalent to 8 L min^{-1} .m width of floway) for the day flow (approximately for 10 hours duration) and 0.5 L min^{-1} (equivalent to 4 L min^{-1} .m width of floway) for the night flow (approximately for 14 hours duration). All replicate FANS took about two weeks (from 26th April 2022 to 10st May 2022) to establish a comparable standing crop of approximately > 30 g DW m^{-2} on the liner surface (algal turf of approximately 0.5-0.8 cm height). Weekly biomass monitoring and water sampling were started seven days after seeding (on 3rd May 2022), and were conducted for the following 84 days, resulting in 12 consecutive growth cycles. All experimental methods were identical to those described above in Section 5.3.3. In brief, strip biomass monitoring was conducted and biomass on each FANS was harvested at the start of every growth cycle to leave a standardized initial standing crop of > 30 g DW m^{-2} on the liner surface. Strip biomass monitoring was then repeated weekly to quantify the biomass productivity (DW g m^{-2} d^{-1}) of each replicate FANS for the duration of

the experiment. Algal biomass washed off each floway was collected from the outflow mesh, dried and weighed and was included in productivity calculations. A 50 ml water sample was collected before and after harvest from the inflow and outflow of each replicate. The water sample was filtered, and the nutrient concentration was analyzed. The ammonia concentration of the inflow was undetected. Through this experiment, the nitrate and phosphate concentrations of the inflow ranged from 0.67 – 6.72 g N m^{-3} and 0.04 - 0.23 g P m^{-3} (Figure 5A.10). The inflow water samples were analysed for total volatile solids (VSS) and ash content once every four weeks according to standard methods (APHA, 2005) (Figure 5A.11). The relative abundance of filamentous algae and other contaminants in the biomass on each replicate floway was quantified every fortnight using a microscope. The ash content in harvested algal biomass on each replicate FANS was analysed once every four weeks (Figure 5A.12).

Loggers were installed as described in Section 5.3.3 to record light intensity, ambient temperature, rainfall and water temperature. The study was conducted from mid-autumn in early May through mid-winter end of July 2022. During the experiment, the maximum sunlight intensity of each day ranged from 95-970 µmol m^{-2} s^{-1}, the ambient air temperature ranged from -2-23° C, FANS inflow water temperature ranged from -2-35 °C and outflow water temperature ranged from -5-33 °C (Figures 5A.13-17).

5.3.5 Statistical analyses

Differences in the initial standing crop, final standing crop, biomass productivity, instantaneous nitrate and phosphate reduction, and nitrate and phosphate removal rates between seeding methods (Experiment 1, fixed factor) or seeded species (Experiment 2, fixed factor) and growth cycles (fixed factor) were tested using two-way analyses of variance (ANOVA), or a Kruskal-Wallis test for variables that failed normality and/or homogeneity of variance tests. Data for each experiment was analysed separately. All statistical analyses were carried out using SigmaPlot software (Systat Software Inc., Point Richmond, CA, USA). All data are reported as means ± S.D.

5.4 Results

5.4.1 Controlled seeded single species FANS vs. natural establishment mixed species FANS

The initial standing crop on all FANS ranged from 17-83 g DW m^{-2} and varied significantly between the two seeding methods, however this variation was not consistent among growth cycles (ANOVA, seeding method x growth cycle interaction, $F_{12,52}$=6.2; P = <0.001). Controlled seeded *Oedogonium* sp. FANS had a higher initial standing crop on average across all growth cycles (62.2 ± 13.1 g DW m^{-2}) compared to the natural establishment mixed species FANS (51.1 ± 18.0 g DW m^{-2}) (Table 5.1, Figure 5.3). However, the natural establishment mixed species FANS had a higher initial standing crop than the controlled seeded *Oedogonium* sp. FANS in three of the 13 growth cycles. Similarly, the final standing crop ranged from 41-122 g DW m^{-2} across all FANS and varied significantly among seeding methods, however, this variation was not consistent among growth cycles (ANOVA, seeding method x growth cycle interaction, $F_{12,52}$=2.8; P = 0.005). Controlled seeded *Oedogonium* sp. FANS had a higher final standing crop on average across all growth cycles (83.9 ± 21.2 g DW m^{-2}) compared to the natural establishment mixed species FANS (72.7 ± 25.1 g DW m^{-2}) (Table 5.1, Figure 5.3). However, the natural establishment mixed species FANS had a higher final standing crop than the controlled seeded *Oedogonium* sp. FANS in four of the 13 growth cycles.

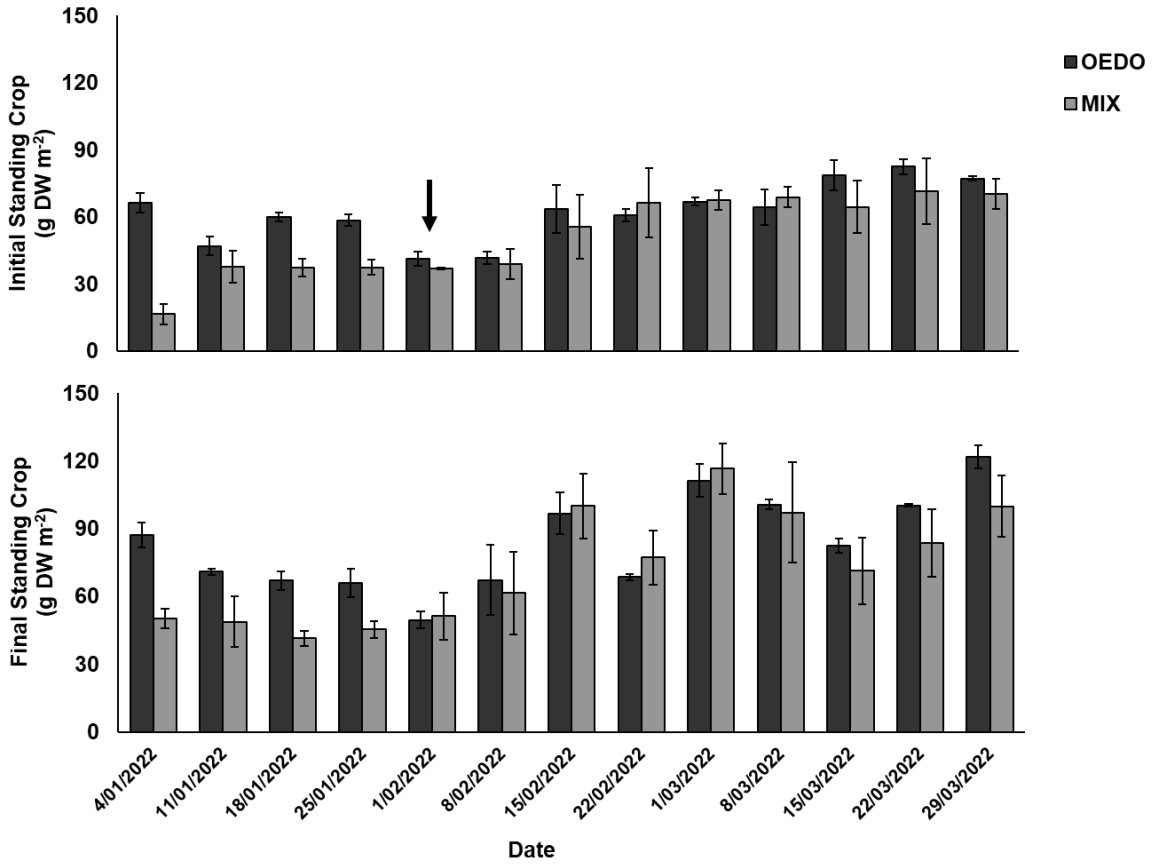

Figure 5.3: Average (± S.D.) initial standing crop (g DW m^{-2}, upper panel) and final standing crop (g DW m^{-2}, lower panel) over 13 consecutive seven-day growth cycles of controlled seeded *Oedogonium* sp. FANS (OEDO) and natural establishment mixed species FANS (MIX). N = 3. The arrow indicates the point that the natural establishment FANS reached uniform biomass establishment over the entire FANS

Biomass productivity ranged from 0.6-7.0 g DW m^{-2} d^{-1} across all FANS and varied significantly among growth cycles (ANOVA, $F_{12,52}$=16.6; P <0.001), but not between the seeding methods (ANOVA, $F_{1,52}$=0.05; P = 0.823, Figure 5.4). For both seeding methods, biomass productivity was lowest in week 3 (18th January 2022, 0.8 ± 0.3 g DW m^{-2} d^{-1}) and highest in week 9 (1st March 2022, 6.7 ± 0.4 g DW m^{-2} d^{-1}). Throughout the experiment, no biomass was washed off from any of the replicate FANS.

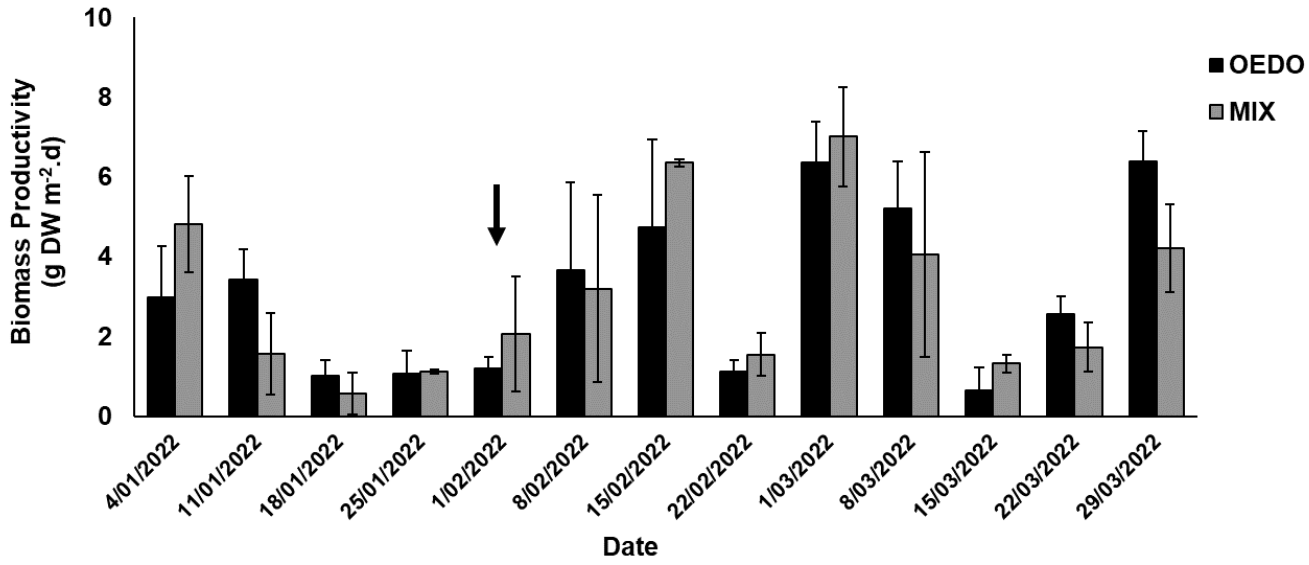

Figure 5.4: Average (± S.D.) biomass productivity (g DW m^{-2} d^{-1}) over 13 consecutive seven-day growth cycles of controlled seeded Oedogonium sp. FANS (OEDO) and natural establishment mixed species FANS (MIX). N = 3. The arrow indicates the point that the natural establishment FANS reached uniform biomass establishment over the entire FANS

Nitrate removal rate varied significantly between the two seeding methods, however this variation was not consistent among growth cycles (ANOVA, seeding method x growth cycle interaction, $F_{12,52}$=3.2; P = 0.002). Across all growth cycles, the natural establishment mixed species FANS had a higher mean nitrate removal rate (0.4 ± 0.3 g N m^{-2} d^{-1}) compared to the controlled seeded *Oedogonium* sp. FANS (0.3 ± 0.2 g N m^{-2} d^{-1}) (Table 5.1, Figure 5.5). However, the controlled seeded *Oedogonium* sp. FANS had a higher nitrate removal rate than the natural establishment mixed species FANS in five of the 13 growth cycles. For both seeding methods, the nitrate removal rate was lowest in week 1 (4th January 2022, 0.1 ± 0.0 g N m^{-2} d^{-1}) and highest in week 7 (15th February 2022, 0.9 ± 0.3 g N m^{-2} d^{-1}).

Similar to the nitrate removal rate, the phosphate removal rate varied significantly between the two seeding methods, but this variation was not consistent among growth cycles (ANOVA, seeding method x growth cycle interaction, $F_{12,52}$=2.1; P = 0.032). Across all growth cycles, the natural establishment mixed species FANS had a higher mean phosphate removal rate (0.07 ± 0.06 g P m^{-2} d^{-1}) compared to the controlled seeded *Oedogonium* sp. FANS (0.05 ± 0.04 g P m^{-2} d^{-1}) (Table 5.1, Figure 5.5). However, the controlled seeded *Oedogonium* sp. FANS had a higher phosphate removal rate than the natural establishment mixed species FANS in five of the 13 growth cycles. For both seeding methods, the phosphate removal rate was

lowest in week 3 (18th January 2022, 0.02 ± 0.004 g P m^{-2} d^{-1}) and highest in week 7 (15th February 2022, 0.18 ± 0.06 g N m^{-2} d^{-1}).

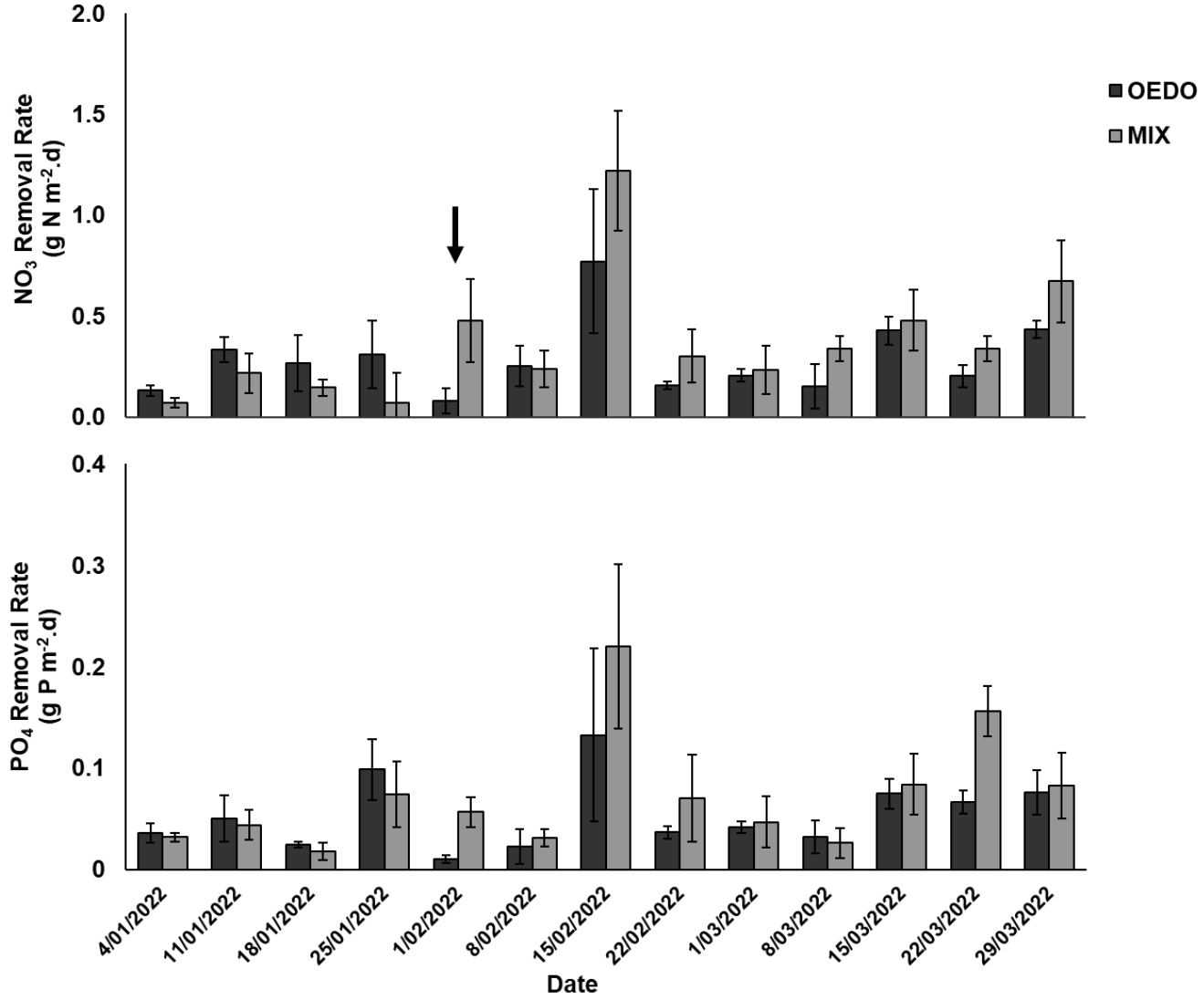

Figure 5.5: Average (± S.D.) NO_3-N removal rate (g N m^{-2} d^{-1}, upper panel) and PO_4-P removal rate (g P m^{-2} d^{-1}, lower panel) over 13 consecutive seven-day growth cycles of controlled seeded *Oedogonium* sp. FANS (OEDO) and natural establishment mixed species FANS (MIX). N = 3. The arrow indicates the point that the natural establishment FANS reached a uniform biomass establishment over the entire FANS

Table 5.1: Key biomass productivity and nutrient removal parameters for controlled seeded *Oedogonium* sp. FANS (OEDO) and natural establishment mixed species FANS (MIX) over 13 seven-day growth cycles in experiment 1. Data are means ± standard deviations, N = 3, mean values for each growth cycle were averaged to provide a global mean for each seeding method

Seeding Method	Controlled seeded with *Oedogonium* sp. FANS (OEDO)	Natural establishment mixed species FANS (MIX)
Biomass Productivity		
Initial Standing Crop (g DW m^{-2})	62.2 ± 13.1	51.5 ± 18.0
Final Standing Crop (g DW m^{-2})	83.9 ± 21.2	72.7 ± 25.1
Biomass Productivity (g DW biomass m^{-2} d^{-1})	3.1 ± 2.2	3.0 ± 2.2
Nutrient Removal		
Nitrate removal rate (g N m^{-2} d^{-1})	0.29 ± 0.2	0.37 ± 0.3
Phosphate removal rate (g P m^{-2} d^{-1})	0.05 ± 0.04	0.07 ± 0.06

The proportion of *Oedogonium* sp. on FANS seeded with *Oedogonium* sp. biomass from unialgal stock cultures decreased substantially as the experiment progressed due to the growth of naturally occurring algae species on the floways, including filamentous diatoms (*Melosira* sp.) and filamentous cyanobacteria (*Oscillatoria* sp.) (Figure 5.6). The relative abundance of *Oedogonium* sp. remained at more than 50% of the biomass on the floway during the first 40 days following seeding, and thereafter fluctuated between 20-45% for the rest of the experiment. The relative abundance of filamentous diatoms increased to 55% after 42 days and thereafter fluctuated between 20-45% for the rest of the experiment. The relative abundance of filamentous cyanobacteria increased to 12% throughout the experiment. Across all replicates, the combined relative abundance of all other filamentous algae including *Spirogyra* sp., *Rhizoclonium* sp., *Stigeoclonium* sp., *Klebsormidium* sp. and *Ulothrix* sp. was up to 30%, with no single species dominating.

The establishment of filamentous algal biomass was visible on the natural establishment mixed species FANS 22 days after the FANS were initially installed. The relative abundance of *Oedogonium* sp. originating from the on-farm stream slowly increased up to 50% after 64 days, but thereafter declined to 27% by the end of the experiment. The relative abundance of filamentous diatoms increased up to 54% after 36 days and thereafter fluctuated between 26-39% for the rest of the experiment. The relative abundance of filamentous cyanobacteria was less than 10% throughout the experiment. Across all replicates, the combined relative abundance of all other filamentous algae growth including *Spirogyra* sp., *Rhizoclonium* sp., *Stigeoclonium* sp., *Klebsormidium* sp. and *Ulothrix* sp. was up to 26%, with no single species dominating.

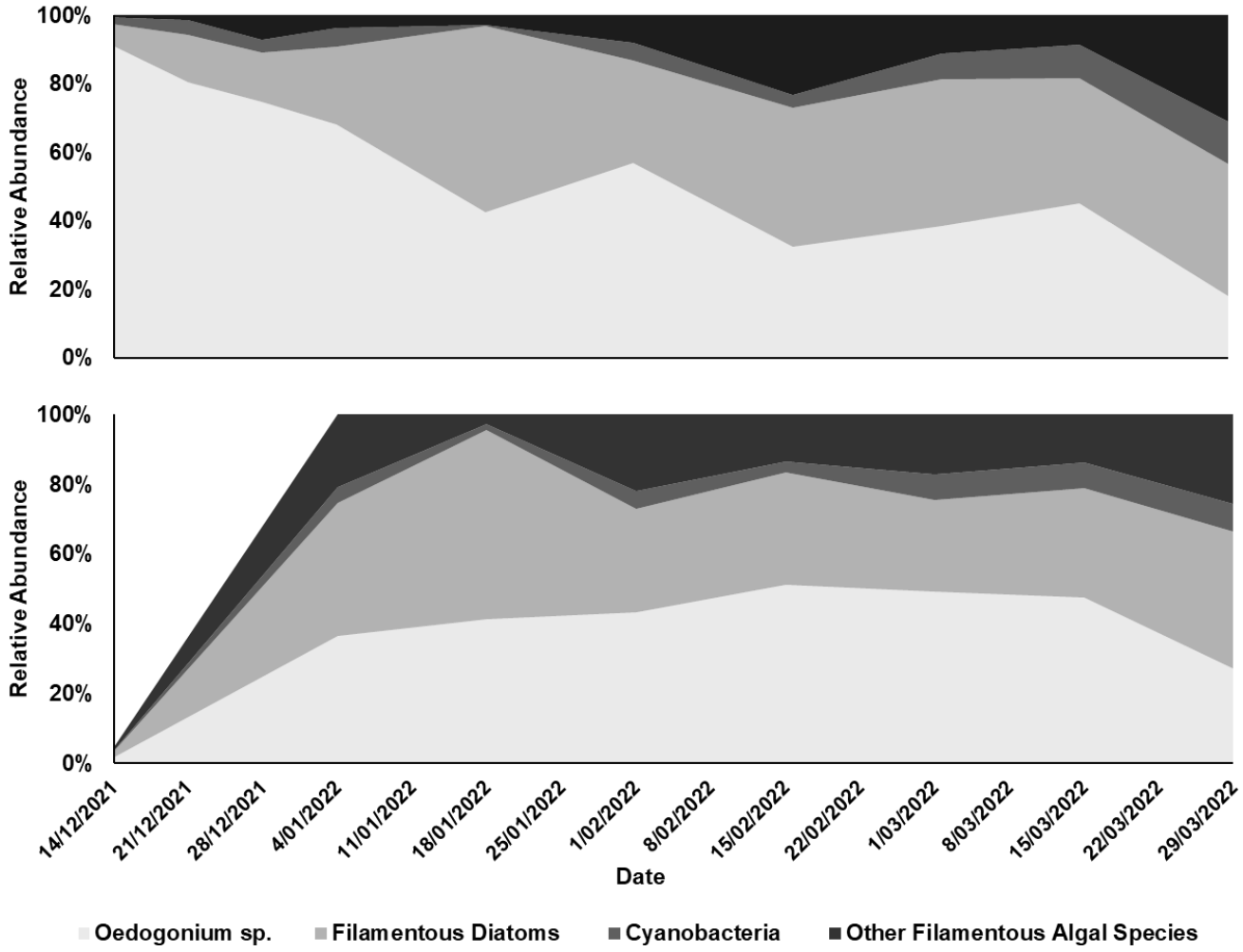

Figure 5.6: Average relative abundance of representative classes of algae on triplicate controlled seeded *Oedogonium* sp. FANS (upper panel) and natural establishment mixed species FANS (lower panel). Data encompass the biomass establishment period through to the end of the experiment (growth cycle 13). N=3. Error bars omitted for visual clarity

5.4.2 Single species FANS vs. adapted mixed species assemblage FANS

The initial standing crop across all FANS ranged from 33-92 g DW m^{-2} and varied significantly among seeded species, however this variation was not consistent among growth cycles (ANOVA, seeded species x growth cycle interaction, $F_{11,48}$=16.7; P = <0.001). *Oedogonium* sp. seeded FANS had a higher initial standing crop on average across all growth cycles (67.2 ± 15.1 g DW m^{-2}) compared to the mixed species FANS (56.0 ± 19.3 g DW m^{-2}) (Table 5.2, Figure 5.7). However, the mixed species FANS had a higher initial standing crop than the *Oedogonium* sp. FANS in one of the 12 growth cycles. Similarly, the final standing

crop across all FANS ranged from 33-111 g DW m^{-2} and varied significantly among seeded species, however, this variation was not consistent among growth cycles (ANOVA, seeded species x growth cycle interaction, $F_{11,48}$=12.1; P = <0.001). *Oedogonium* sp. seeded FANS had a higher final standing crop on average across all growth cycles (76.0 ± 18.6 g DW m^{-2}) compared to the mixed species FANS (65.2 ± 25.3 g DW m^{-2}) (Table 5.2, Figure 5.7). However, the mixed species FANS had a higher final standing crop than the *Oedogonium* sp. FANS in two of the 12 growth cycles.

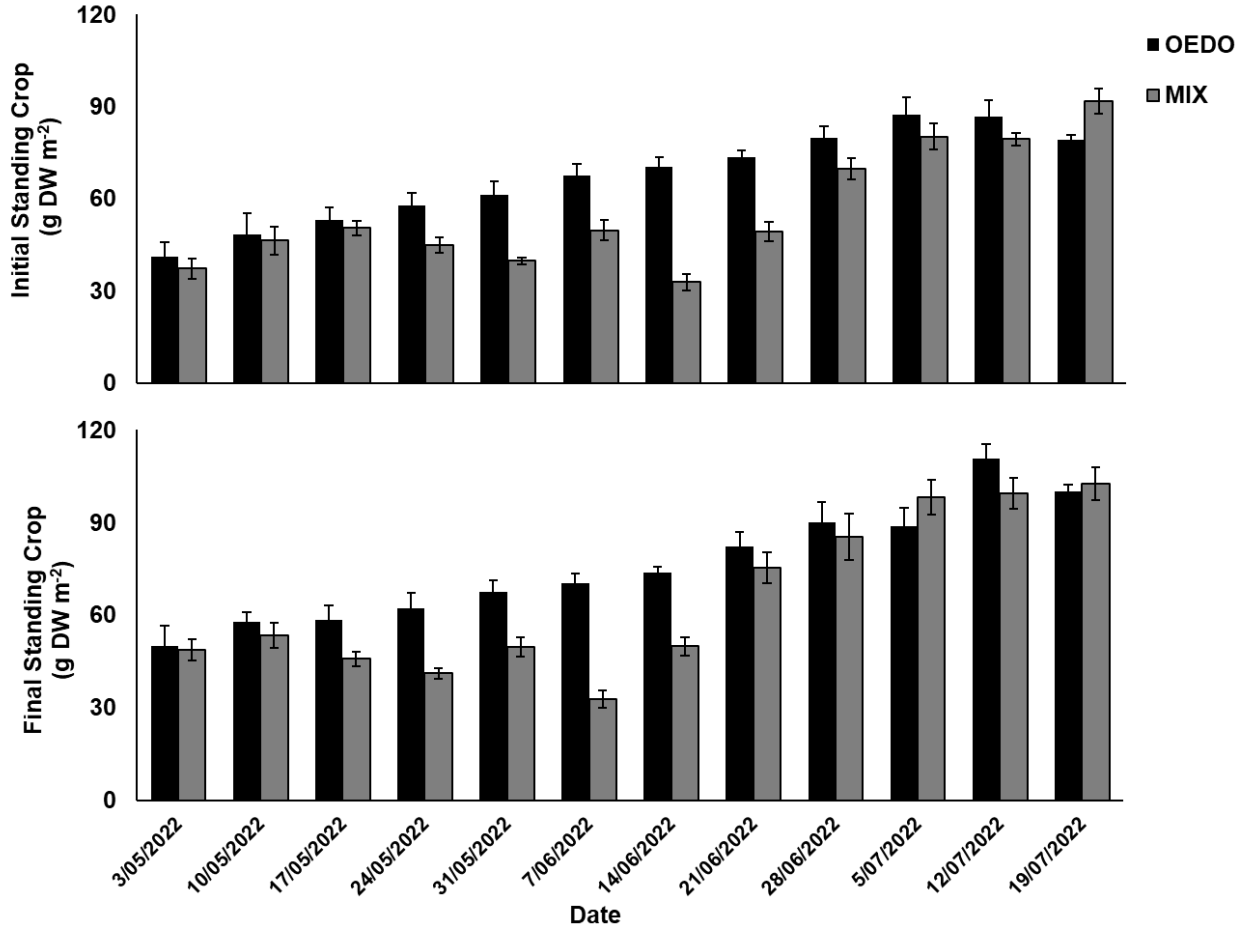

Figure 5.7: Average (± S.D.) initial standing crop (g DW m^{-2}, upper panel) and final standing crop (g DW m^{-2}, lower panel) over 12 consecutive seven-day growth cycles of *Oedogonium* sp. FANS (OEDO) and mixed species FANS (MIX). N = 3

Biomass productivity ranged from 0.6-3.8 g DW m^{-2} d^{-1} across all FANS and varied significantly among seeded species, however this variation was not consistent among growth cycles (ANOVA, seeded species x growth cycle interaction, $F_{11,48}$=5.8; P = <0.001). Mixed species FANS had a higher biomass productivity on average across all growth cycles (2.1 ± 1.1 g DW m^{-2} d^{-1}) compared to *Oedogonium* sp. FANS (1.6 ± 0.9 g DW m^{-2} d^{-1}) (Table 5.2, Figure 5.8). However, the *Oedogonium* sp. seeded FANS had a higher productivity than the mixed species FANS in four of 12 growth cycles. Biomass productivity of all FANS was lowest in week 3 (17th May 2022, 0.8 ± 0.1 g DW m^{-2} d^{-1}) and highest in week 11 (12th July 2022, 3.7 ± 0.2 g DW m^{-2} d^{-1}).

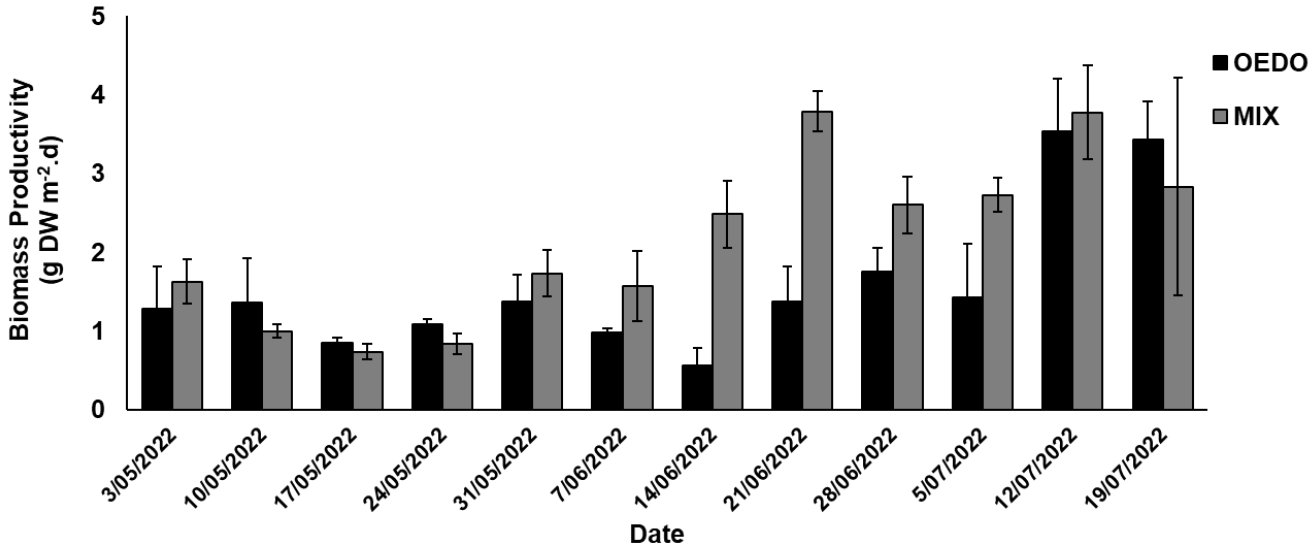

Figure 5.8: Average (± S.D.) biomass productivity (g DW m^{-2} d^{-1}) over 12 consecutive seven-day growth cycles of *Oedogonium* sp. FANS (OEDO) and mixed species FANS (MIX). N = 3

The amount of biomass washed off the floways ranged from 0 - 85 % of the final standing crop across all FANS and varied significantly among seeded species, however this variation was not consistent among growth cycles (ANOVA, seeded species x growth cycle interaction, $F_{11,48}$=110.8; P = <0.001). Mixed species FANS had a higher percentage of washed-off biomass on average across all growth cycles (mean 12.9 ± 23.6 %) compared to *Oedogonium* sp. FANS (mean 2.8 ± 3.3 %) (Table 5.2, Figure 5.9). The standing crop on mixed species FANS was observed to be easily fragmented compared to *Oedogonium* sp. FANS, especially when there was a high cumulative rainfall (Figure 5A.17). Across both seeded species, the

percentage of washed off biomass was lowest in the first two weeks of the experiment (3rd to 16th May 2022, 0 ± 0 %) and highest in week 6 (7th June 2022, 45.5 ± 56.4 %).

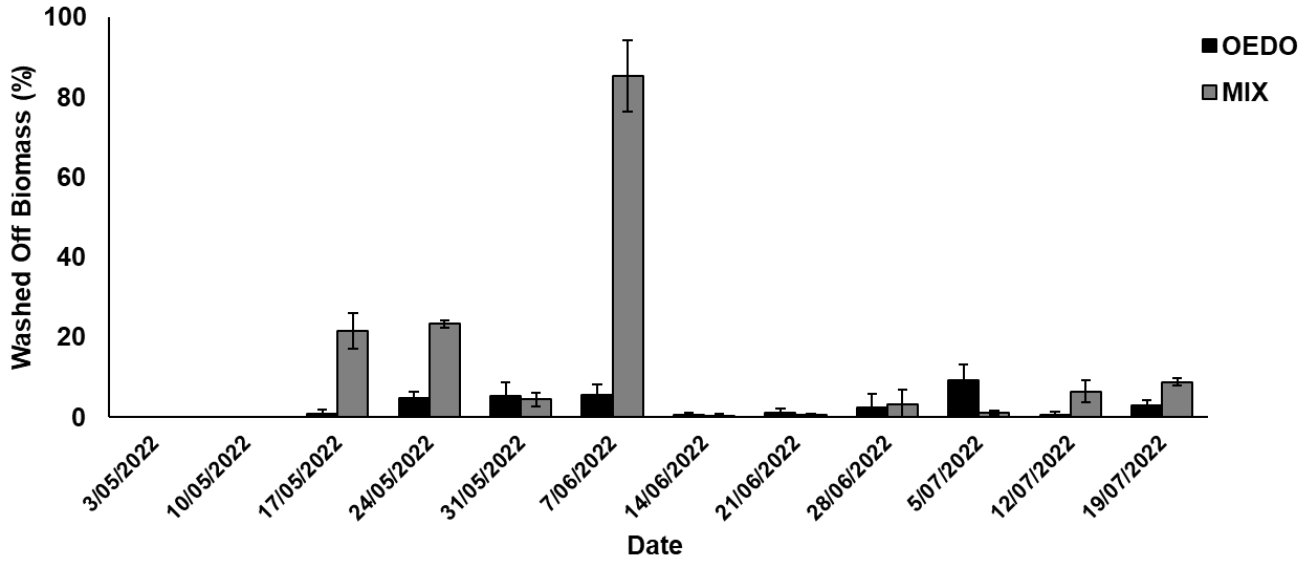

Figure 5.9: Average (± S.D.) biomass washed off (% of final standing crop) floways of *Oedogonium* sp. FANS (OEDO) and mixed species FANS (MIX) over 12 consecutive seven-day growth cycles. N = 3

Nitrate removal rate varied significantly between the seeded species, however this variation was not consistent among growth cycles (ANOVA, seeded species x growth cycle interaction, $F_{11,48}$=2.8; P = 0.007). Across all growth cycles, the *Oedogonium* sp. FANS and the mixed species FANS had a comparable mean nitrate removal rate (0.17 ± 0.05 and 0.17 ± 0.08 g N m^{-2} d^{-1}, respectively) (Table 5.2, Figure 5.10). However, the *Oedogonium* sp. seeded FANS had a higher nitrate removal rate than the mixed species FANS in eight of the 12 growth cycles. For both seeded species, the nitrate removal rate was lowest in week 2 (10th May 2022, 0.09 ± 0.03 g N m^{-2} d^{-1}) and highest in week 1 (3rd May 2022, 0.29 ± 0.09 g N m^{-2} d^{-1}).

Similar to the nitrate removal rate, the phosphate removal rate varied significantly among seeded species, however this variation was not consistent among growth cycles (ANOVA, seeded species x growth cycle interaction, $F_{11,48}$=4.9; P = <0.001). Across all growth cycles, the *Oedogonium* sp. FANS and the mixed species FANS had a comparable mean phosphate removal rate (0.04 ± 0.01 g P m^{-2} d^{-1}) (Table 5.2, Figure 5.10). However, the *Oedogonium* sp. seeded FANS had a higher phosphate removal rate than the mixed species FANS in seven of

the 12 growth cycles. For both seeded species, the phosphate removal rate was lowest in week 4 (24th May 2022, 0.027 ± 0.011 g P m^{-2} d^{-1}) and highest in week 9 (28th June 2022, 0.049 ± 0.000 g P m^{-2} d^{-1}).

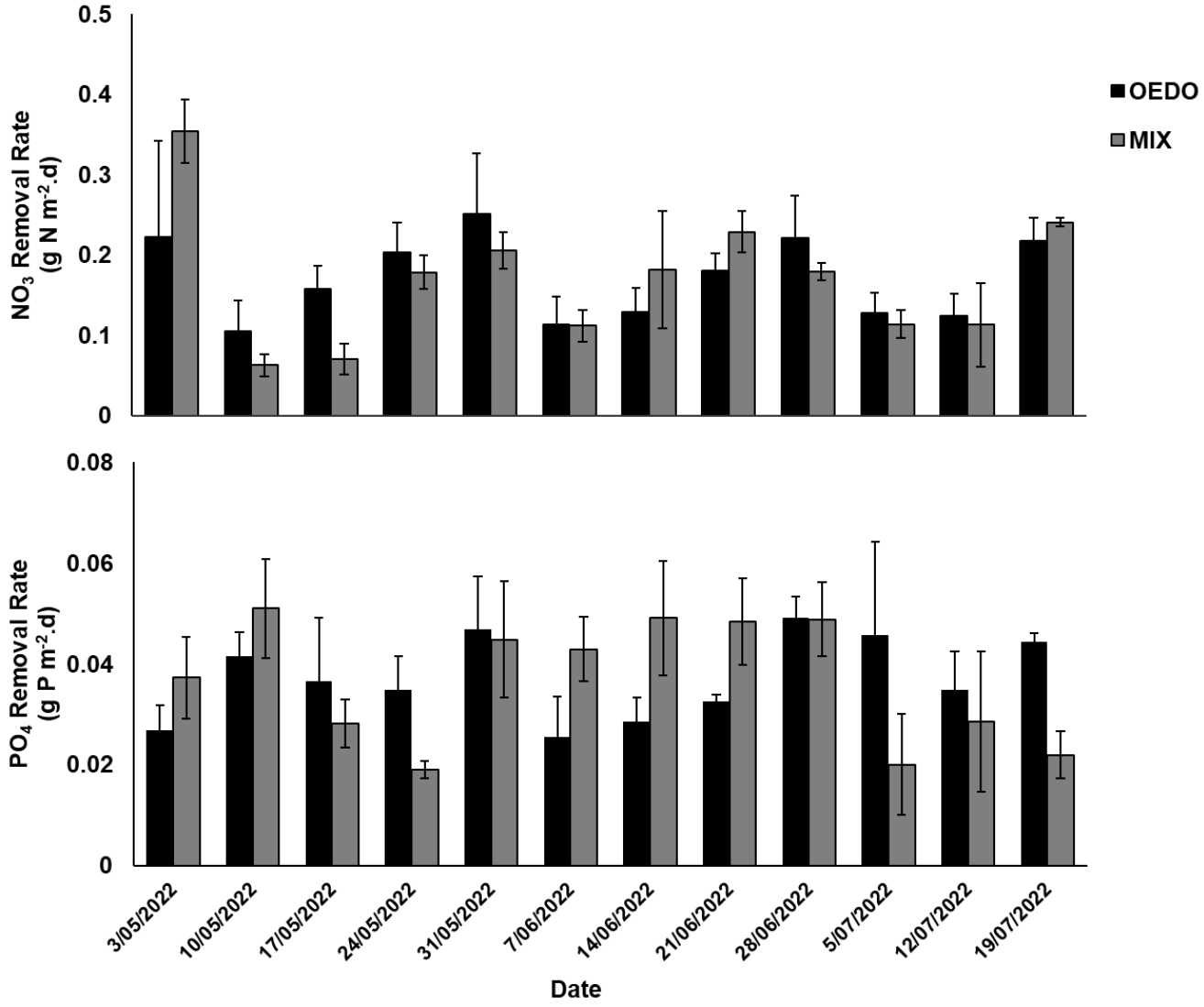

Figure 5.10: Average (± S.D.) NO_3-N removal rate (g N m^{-2} d^{-1}, upper panel) and PO_4-P removal rate (g P m^{-2} d^{-1}, lower panel) over 12 consecutive seven-day growth cycles of *Oedogonium* sp. FANS (OEDO) and mixed species FANS (MIX). N = 3

Table 5.2: Key biomass productivity and nutrient removal parameters for FANS seeded with *Oedogonium* sp. (OEDO) and FANS seeded with mixed species (MIX) over 12 seven-day growth cycles in experiment 2. Data are means ± standard deviations, N = 3, mean values for each growth cycle were averaged to provide a global mean for each seeded species

Seeded Species	*Oedogonium* sp. (OEDO)	Mixed species (MIX)
Biomass Productivity		
Initial Standing Crop (g DW m^{-2})	67.2 ± 15.1	56.0 ± 19.3
Final Standing Crop (g DW m^{-2})	76.0 ± 18.6	65.2 ± 25.3
Biomass Productivity (g DW biomass m^{-2} d^{-1})	1.6 ± 0.9	2.1 ± 1.1
Biomass Washed off (%)	2.8 ± 3.3	12.9 ± 23.6
Nutrient Removal		
Nitrate removal rate (g N m^{-2} d^{-1})	0.17 ± 0.05	0.17 ± 0.08
Phosphate removal rate (g P m^{-2} d^{-1})	0.04 ± 0.01	0.04 ± 0.01

The proportion of *Oedogonium* sp. on FANS seeded with *Oedogonium* sp. biomass from unialgal stock cultures decreased substantially as the experiment progressed due to the growth of naturally occurring algae species, including filamentous diatoms (*Melosira* sp.) and filamentous cyanobacteria (*Oscillatoria* sp.) on the floways (Figure 5.11). The relative abundance of *Oedogonium* sp. remained at more than 50% of the established biomass for the first 60 days after seeding, and thereafter *Oedogonium* sp. relative abundance fluctuated between 20-30 % until the end of the experiment. The relative abundance of filamentous diatoms increased gradually between weeks 1 and 7, and then rapidly increased between weeks 7 and 9 and then continued to increase slightly until the end of the experiment. The relative abundance of filamentous cyanobacteria was low but increased gradually throughout the experiment, reaching up to 13% by the end of the experiment. The combined relative

abundance of all other filamentous algae including *Spirogyra* sp., *Rhizoclonium* sp., *Stigeoclonium* sp., *Klebsormidium* sp. and *Ulothrix* sp. was up to 7%.

The algal biomass (30 g FW for each FANS) collected from the nearby stream that was used to seed the mixed species FANS was comprised of around 35% *Oedogonium* sp., 35% *Spirogyra* sp., 10% *Cladophora* sp., 10% filamentous diatoms (*Melosira* sp.), 5% filamentous cyanobacteria (*Oscillatoria* sp.) and 5% other green filamentous algae (*Klebsormidium* sp. *Ulothrix* sp. and *Stigeoclonium* sp.). On the mixed species FANS, the relative abundance of *Oedogonium* sp. fluctuated from 10-40% for the first 50 days after seeding, and thereafter decreased to less than 5% by the end of the experiment. The relative abundance of *Spirogyra* sp. decreased rapidly in the first week and then remained low for the rest of the experiment. The relative abundance of *Cladophora* sp. fluctuated from 10-20% for the first 20 days after seeding and thereafter reduced to less than 5% for the rest of the experiment. The relative abundance of *Rhizoclonium* sp. remained below 5% throughout the experiment. The relative abundance of filamentous diatoms increased rapidly in the first week of the experiment and continued to increase to 80% by the end of the experiment. The relative abundance of filamentous cyanobacteria remained relatively steady throughout the experiment, fluctuating from 5-13%. Across all replicates, the combined relative abundance of other filamentous algal species growth including *Stigeoclonium* sp., *Klebsormidium* sp. and *Ulothrix* sp. increased to up to 17% for the first 50 days after seeding and then reduced to less than 5% towards the end of the experiment.

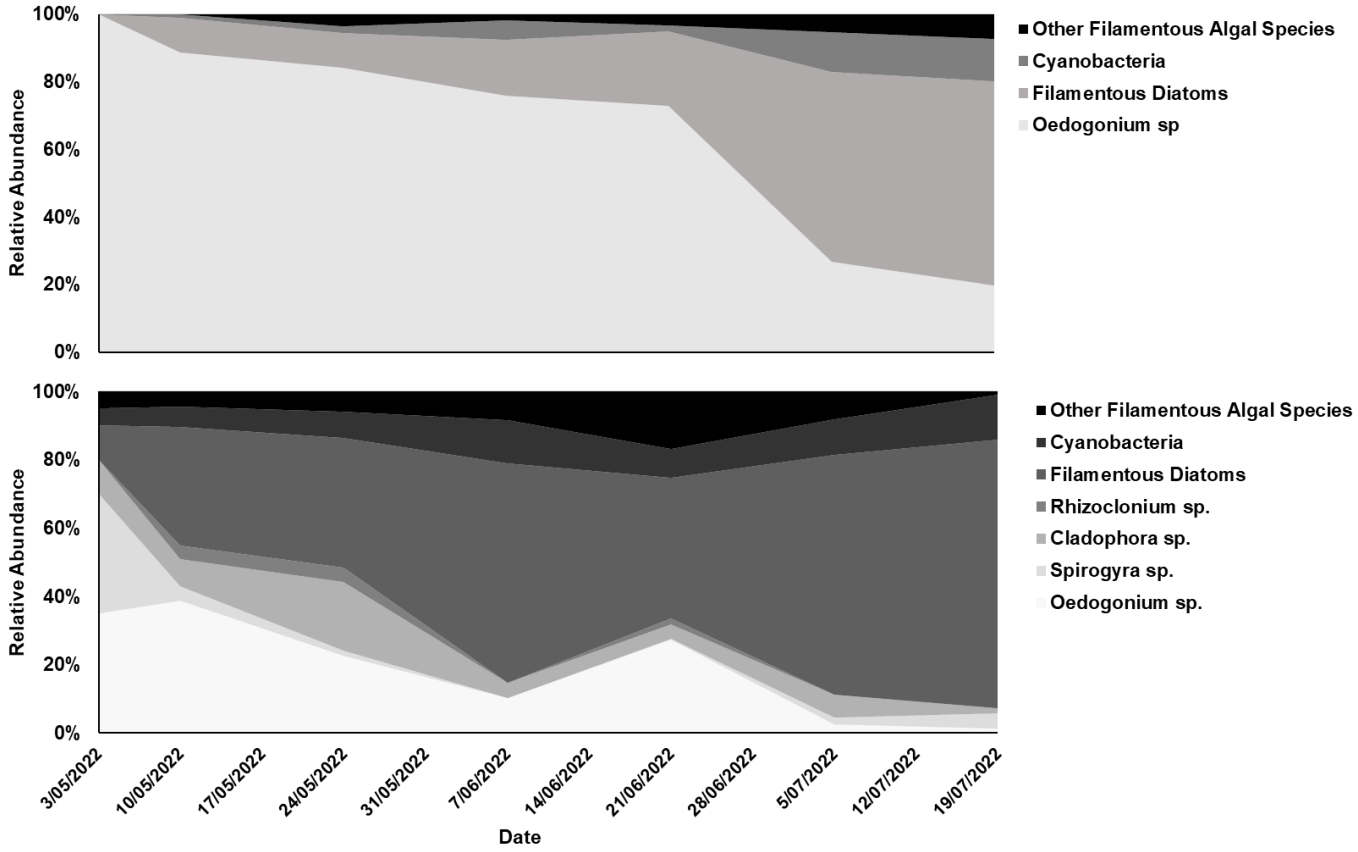

Figure 5.11: Average relative abundance of representative classes of algae on triplicate *Oedogonium* sp. FANS (upper panel) and FANS mixed species FANS (lower panel). Data encompass the biomass establishment period through to the end of the experiment (growth cycle 12). N=3. Error bars omitted for visual clarity

5.5 Discussion

Controlled seeding vs. natural establishment

The ability of filamentous algae to quickly establish and form a dense uniform cover on the FANS liner is desirable when operating FANS. This can significantly speed up the seeding stage and establishment of a target initial standing crop. In the first experiment, we found considerable differences in the time taken to establish a dense uniform biomass cover on the liner between seeding methods. The controlled seeded FANS established a uniform algal turf about five times faster (10 days) than the natural establishment FANS (~50 days). The rapid establishment of *Oedogonium* sp. biomass on controlled seeded FANS is most likely due to the large amount of algal biomass added on a single occasion at the start of seeding which promoted

the rapid development of secondary algal attachment through the growth of rhizoids and holdfasts from spores (Hariz et al., 2022). In contrast, the natural establishment FANS were gradually seeded over several weeks by algal spores and fragments from the stream water that were caught on and subsequently attached to the liner. This natural seeding could have been faster if there had been greater algal biomass growing in the stream at the time. The low abundance of filamentous algae in the stream may have been due to a range of factors including time of year (sub-optimal light and temperature), lack of attachment substrate, unfavourable stream current, high water turbidity, low nutrient availability and grazing by stream invertebrates which made conditions in the stream suboptimal for growth (Ghosh & Gaur, 1998; Álvarez & Peckarsky, 2005; Dzialowski et al., 2008; McCall et al., 2017). In particular, seasonal variation in temperature and irradiance are likely to have large influence on the abundance of algae in the stream as algae are typically naturally abundant from spring through summer until early autumn when temperatures are warm, and have lower abundance during cooler temperatures in winter (De Vries & Hillebrand, 1986; Berry & Lembi, 2000; Salovius & Bonsdorff, 2004; Sousa-Dias & Melo, 2008). However, we observed a higher abundance of algae in the stream in the late summer/early autumn compared to when we first installed the natural establishment FANS in early summer (December 2021) when algal biomass in the stream was hardly seen. In addition, we observed the water flow in the stream was higher when we first started the experiment and thereafter the water flow gradually decreased until the end of the experiment. A higher water flow increases physical disturbance to naturally occurring algae in the stream, leading to higher rate of algal biomass washing off and a subsequent lower algal abundance. Our results show that the success of the natural establishment method is highly dependent on the season and stream current when seeding occurs. In particular, the establishment of a dense biomass coverage on FANS will be faster when natural algal abundance is high and is likely to take longer during periods when natural algal abundance is low.

Throughout the three-month experiment, the average biomass productivity was 3.1 ± 2.2 g DW m^{-2} d^{-1} for controlled seeding FANS and 3.0 ± 2.2 g DW m^{-2} d^{-1} for natural establishment FANS. These productivities are comparable to the average biomass productivity reported for FANS treating agricultural drainage (4.9 ± 1.6 g DW m^{-2} d^{-1} , Kangas and Mulbry, 2014, 5.5 ± 3.5 g DW m^{-2} d^{-1} , D'Aiuto et al., 2015), horticultural wastewater (1.4 ± 0.6 g DW m^{-2} d^{-1} , Liu et al., 2016), and anaerobically digested and undigested dairy manure (5.4 ± 0.1 g DW m^{-2} d^{-1},

Wilkie and Mulbry, 2002). Our results show there was no significant difference in biomass productivity between seeding methods, however there was a significant effect of growth cycle suggesting that environmental conditions potentially had a stronger effect on biomass productivity than seeding method. Several environmental conditions are known to contribute to changes in algal biomass productivity, including inflow nutrient concentration, temperature, and solar irradiance (Sand-Jensen, 1989; Sutherland et al., 2013; Cole et al., 2016). However, biomass productivity did not appear to vary predictably with changes in any one of these factors. Instead, variation in biomass productivity between growth cycles may have been driven by interactive effects between environmental factors, most likely irradiance and temperature. Such a result has been demonstrated previously for *Oedogonium* sp., where biomass productivity increased under lower temperatures (below optimal range of 20-35°C) when irradiance was high but decreased under higher temperatures (>35°C) when irradiance was high (Cole et al., 2018). Temperatures and irradiances fluctuated considerably throughout the experiment and were not always correlated with each other or biomass productivity. For example, our results show that while productivity appeared to increase or decrease with corresponding increases or decreases in temperature in some growth cycles, in five of the 13 growth cycles there appeared to be an inverse relationship where productivity decreased with an increase in temperature (e.g., cycles 2 and 8), or increased with a decrease in temperature (e.g. cycles 5, 9 and 12, Figure 5A.18 and Table 5A.1). Similarly, productivity appeared to increase or decrease with corresponding increases or decreases in light intensity in only four of the 13 cycles (cycles 3, 5, 11 and 13). These results suggest a similar interactive effect between temperature and irradiance on biomass productivity could have been occurring.

Nitrate and phosphate removal rates varied significantly between seeding methods however the best performing seeding method varied throughout the experiment. This variation in nitrate and phosphate removal rates appeared to be driven by the concentration of nitrate and phosphate in the FANS inflow which varied markedly throughout the experiment (2.1 g N m^{-3} to 5.7 g N m^{-3} and 0.04 g P m^{-3} to 0.87 g P m^{-3}), with higher nutrient concentrations resulting in higher nutrient removal rates (Figure 5A.19). This finding of nutrient concentrations having a stronger effect on nutrient removal than seeding method is another example of environmental variation driving performance rather than seeding method, as was found for biomass productivity. Notably, our results provide no clear indication whether the increase in nutrient loading enhanced the nutrient removal efficiency of the algae as the uptake of nutrients per unit

of algal biomass was not determined for the harvested biomass of each growth cycle. But as recent studies have reported increases in algal nutrient removal efficiency with an increase in nitrate and phosphate loading (Mhatre-Naik et al., 2021; Sutherland & Ralph, 2021), we expect that a similar effect was occurring here.

The potential of the target species to maintain dominance on a floway over other non-target species (e.g. diatoms and cyanobacteria) under varying environmental conditions for an extended period is a desirable characteristic to consider when selecting the best seeding method for FANS. A high relative abundance of a single green filamentous algal species on FANS provides a higher consistency of biomass composition, and therefore higher quality of biomass than a mixed species assemblage which can have a variable species abundance, and therefore a variable biomass composition through time. The controlled seeded FANS maintained a high relative abundance (>50%) of *Oedogonium* sp. over 40 days after seeding. In contrast, the natural establishment FANS took over 60 days to establish at least a 50% relative abundance of green filamentous algae (e.g., *Oedogonium* sp.) that originated from the stream. This result suggests that controlled seeding allows a higher relative abundance of a target filamentous algae species to be maintained for a longer duration on FANS than the natural establishment method. This may be because there were fewer empty spaces on the controlled seeded FANS compared to the natural establishment FANS, which resulted in a lower rate of colonization of non-target species such as competitively dominant diatoms. Filamentous diatoms are highly competitive against other green algae (Biggs, 1990), which probably enabled them to grow and establish rapidly on FANS, especially when space was available following harvest. Consequently, non-target species such as filamentous diatoms can establish rapidly on FANS that start with an empty liner (e.g. natural establishment FANS) compared to FANS that have already been seeded with a dominant species (e.g. *Oedogonium* sp. controlled seeded FANS).

Single species vs mixed species assemblages

We found reasonable similarity in the time taken to establish a dense uniform algal turf on FANS between single species of *Oedogonium* sp. and mixed species when controlled seeding was used for all FANS in the second experiment. Both FANS took approximately two weeks to establish a uniform turf on the liner. However, there was a considerable difference in the ability of the seeded algae to maintain the established standing crop on the FANS. In most

growth cycles, *Oedogonium* sp. FANS had a higher standing crop than the mixed species FANS, resulting from a lower rate of biomass washing off the floway. In contrast to *Oedogonium* sp. FANS, the standing crop established on mixed species FANS was easily fragmented, especially when there was a high current and rainfall. We found that filamentous diatoms established twice as quickly on the mixed species FANS (>60% relative abundance in 35 days) than on the single species *Oedogonium* sp. seeded FANS (56% relative abundance in 63 days). Therefore, a higher rate of biomass wash off may be a consequence of the higher relative abundance of filamentous diatoms (e.g. *Melosira* sp.) established on mixed species FANS as filamentous diatoms are known to fragment easily compared to green filamentous algae (Tapolczai et al., 2016; Park et al., 2022). Although the filament characteristics and attachment strength may vary depending on the algal species, this finding suggests that maintaining a single target species that does not fragment easily could minimize the rate of biomass wash off from the floways due to filament fragmentation.

Biomass productivity varied significantly between *Oedogonium* sp. FANS and mixed species FANS, however, this variation was not consistent throughout the experiment. As we found for experiment 1, environmental conditions, in particular rainfall and temperature, appeared to have a stronger effect on biomass productivity than the seeded species. We observed a notable decline in biomass productivity in growth cycle 3 (from 17th to 24th of May 2022) which coincided with a significant period of high rainfall (sum of rainfall for this period 59.2 mm, Figure 5.8 and Figure 5A.17). In contrast, biomass productivity was highest in growth cycle 11 (12th to 19th of July 2022) when the rainfall was two and half times lower (sum of rainfall for this growth cycle 23.8 mm) compared to cycle 3. Similarly, we observed a decrease in biomass productivity from growth cycle 1 to 3 (5th to 24th May 2022) that coincided with a substantial drop in inlet water temperature to as low as -2 ºC, but overall biomass productivity subsequently started to increase when water temperature rose above 0 ºC after 29th May 2022. Rainfall can result in increased biomass fragmentation that leads to increased rates of biomass washing off the floway due to higher flow disturbance. Furthermore, rainfall can result in increased siltation in the influent, causing a negative impact on algal photosynthesis and growth due to light attenuation and smothering of the algal biomass by sediments (Chen et al., 2015). While lower water temperature reduces membrane fluidity, limits electron exchange and decreases photosynthetic efficiency (Cardinale et al., 2013; Smith & Crews, 2014; Newby et al., 2016), which could significantly affect biomass production. Notably, the maximum

biomass productivity in the second experiment was about one and a half times lower than in the first experiment. This lower overall biomass productivity of both seeded species may have been due to the lower water temperature, lower irradiance, shorter daylight period, lower ambient temperature (including the occasional night frost), and higher cumulative rainfall in the second experiment compared to the first experiment, which resulted in suboptimal growth conditions. A strong seasonal effect on FANS biomass productivity has been reported previously, with *Oedogonium* sp. productivity on FANS under outdoor ambient conditions being almost three-fold higher in summer compared to winter due to the differences in water and ambient temperatures and solar irradiance between seasons (Hariz et al., 2023c). These results further highlight the strong influence of environmental conditions on FANS performance.

Nitrate and phosphate removal rates varied significantly between species, however, as for biomass productivity, this variation was not consistent throughout the experiment. The nitrate removal rate increased with increasing nitrate concentration in the FANS inflow. However, this relationship was not evident for phosphate removal and phosphate concentration in the FANS inflow. The lack of an apparent trend for phosphate removal rate relative to inflow phosphate concentration may be a result of the variation in the concentration of phosphate in the FANS inflow (range: 0.02 g P m^{-3} and 0.45 g P m^{-3}) being considerably lower than the variation in the concentration of nitrate in the FANS inflow (range: 0.67 g N m^{-3} and 6.72 g N m^{-3}). We found a considerable difference in overall nutrient removal rates between the first and second experiments. The removal rates of nitrate and phosphate were at least 1.7-fold and 1.3-fold respectively lower in the second experiment compared to the first experiment, most likely as a result of lower biomass productivities in the second experiment due to lower ambient temperature and irradiance and higher rainfall. These findings suggest that there is a strong positive relationship between nutrient removal and biomass productivity for FANS, where high nutrient removal rates are correlated with high biomass productivity.

A further difference in FANS performance between seeded species was their ability to maintain a higher relative abundance of the seeded species on the FANS floway. FANS seeded with the single species *Oedogonium* sp. maintained a relative abundance of *Oedogonium* sp. of more than 50% over 60 days after seeding. In contrast, the relative abundance of each green filamentous algal species seeded on mixed species FANS varied greatly throughout the

experiment and none of the seeded mixed green filamentous algae species dominated the FANS (all had <50% relative abundance). The larger amount of pure *Oedogonium* sp. added to the single species FANS during seeding may have resulted in their higher relative abundance over other non-target species for an extended period. In contrast, the total amount of each green filamentous algae species that was added onto the mixed species FANS during seeding was lower, potentially making it difficult for any one species to gain dominance. These results suggest that differences in the total amount of each species added upon seeding, in particular whether a single species or multiple species are seeded, can influence their long-term abundance on FANS. This conclusion is supported by a recent study assessing the performance of pilot scale FANS treating river water over a three-month period (Park et al., 2022). FANS were initially seeded using controlled seeding with a mixed assemblage of green filamentous algae comprised of equal proportions of *Oedogonium* sp., *Cladophora* sp., *Rhizoclonium* sp., and *Spirogyra* sp. However, despite the higher initial loading rates of algal biomass on the floway during seeding (~40 g DW m^{-2} compared to 7 g DW m^{-2} in the current study), no single seeded species was able to dominate and non-target diatoms eventually accounted for more than 80% of the total biomass (Park et al., 2022). In comparison to the first experiment, mixed species seeded through controlled seeding in the second experiment maintained the initially seeded abundance of filamentous algae longer than mixed species seeded through natural establishment in the first experiment. In particular, the mixed species FANS seeded through controlled seeding maintained an abundance of *Oedogonium* sp. of up to 40% for the first 50 days compared to natural establishment FANS where *Oedogonium* sp. took 60 days to establish 50% relative abundance but was unable to maintain this abundance for longer than two weeks, and showed a marked decline towards the end of the experiment. In combination, these results demonstrate that controlled seeding provides faster, higher and longer control of FANS species composition than natural establishment.

5.6 Conclusion

This study demonstrated that controlled seeding promotes faster biomass establishment on FANS than the natural establishment method. However, environmental conditions had a stronger effect on biomass productivity and nutrient removal than seeding method and seeded species (single species vs mixed species). Seeded species did influence the standing crop that was able to be maintained on FANS, with single *Oedogonium* sp. FANS having a lower rate

of biomass wash-off compared to filamentous diatom dominated mixed species FANS. Moreover, these results clearly demonstrate that FANS seeded with a single target species have the potential to be operated with a higher relative abundance for a longer period compared to FANS seeded with a mixed species assemblage. Our findings provide insights into how controlled seeding and targeting single filamentous algae species can influence nutrient removal and the production of high-quality biomass with low variation in algae species composition on FANS. The cultivation approach used in this study can be applied in other locations to promote year-round FANS operation and treat various types of polluted water.

5.7 Appendix

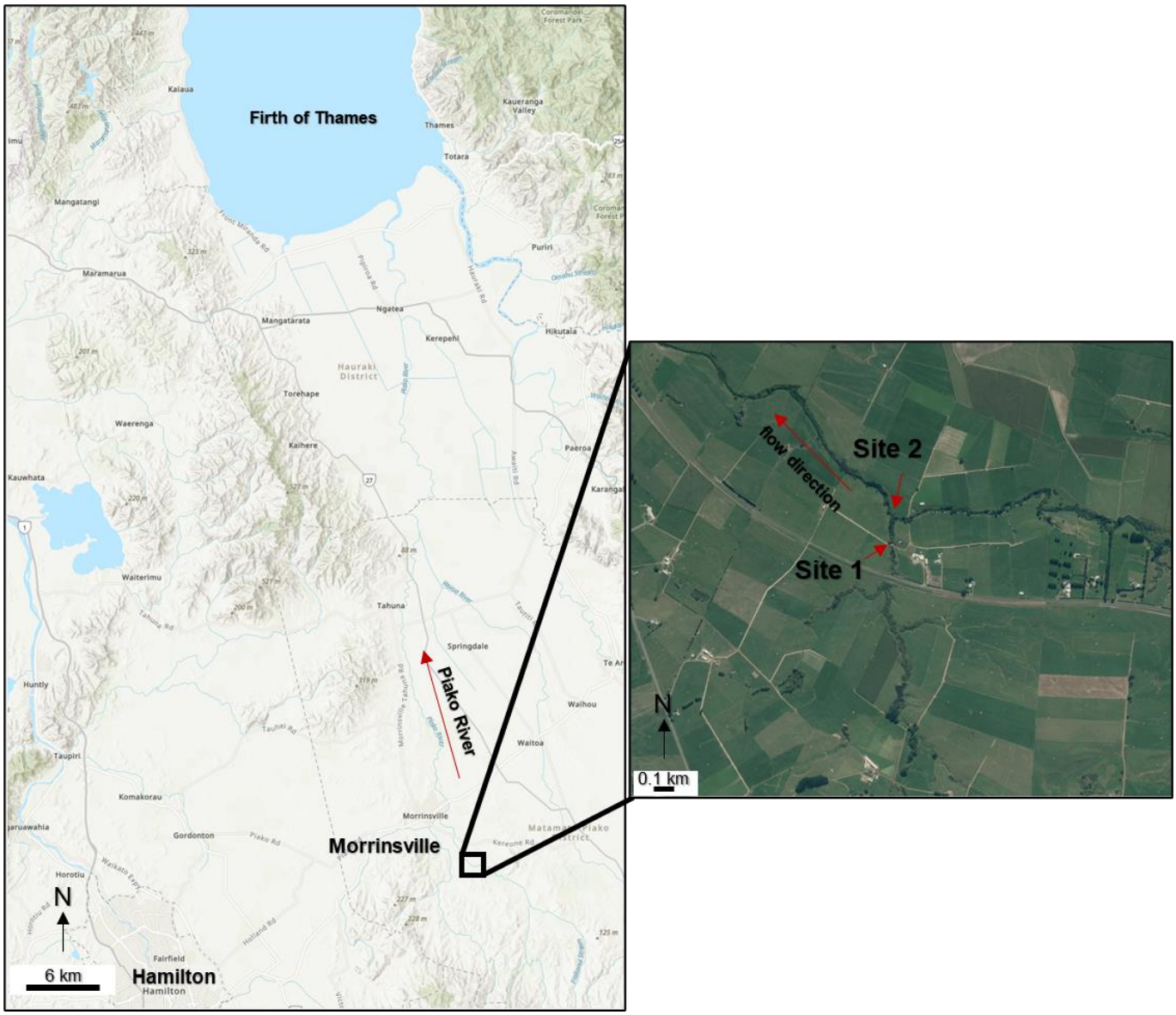

Figure 5A.1: Map of the river network and the study location of experiment 1 (site 1) and experiment 2 (site 2) (source: Eagle Technology, LINZ)

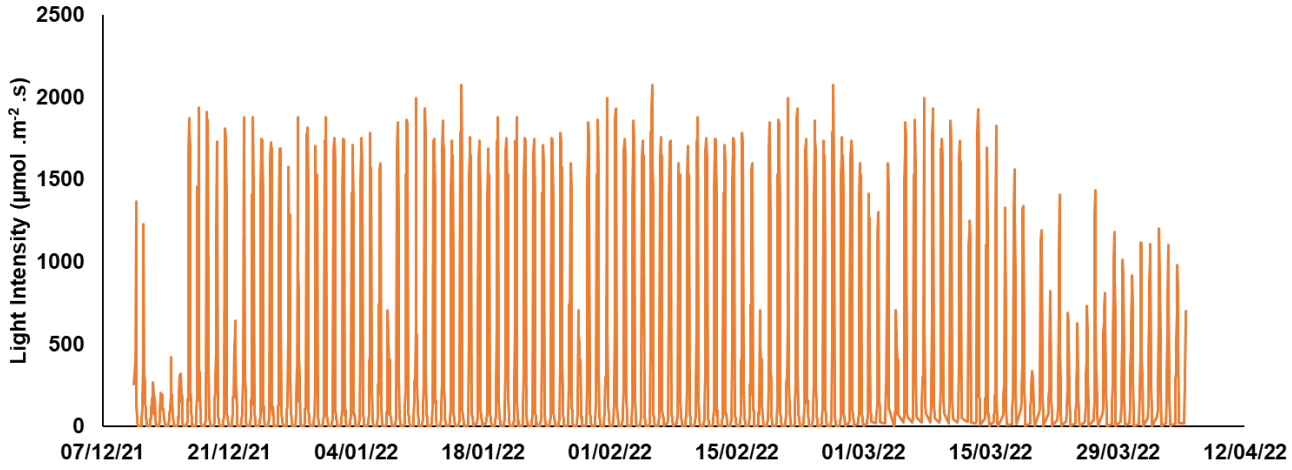

Figure 5A.2: Recorded light intensity during experiment 1. Data encompass the on-farm biomass establishment phase through to the end of the week 13 of the experiment (from 13th December 2021 to 5th April 2022)

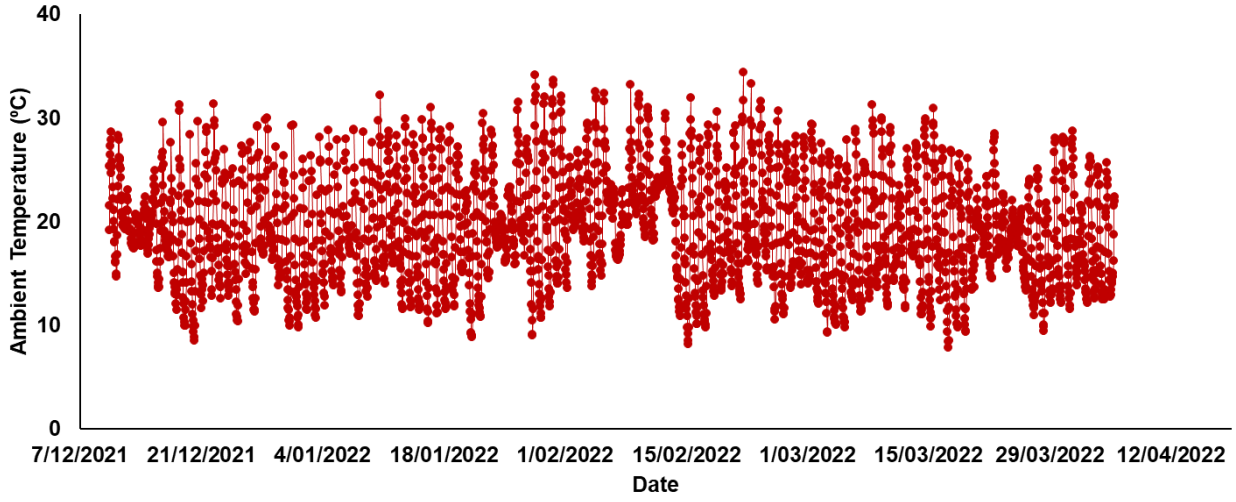

Figure 5A.3: Recorded ambient temperature during experiment 1. Data encompass the on-farm biomass establishment phase through to the end of the week 13 of the experiment (from 13th December 2021 to 5th April 2022)

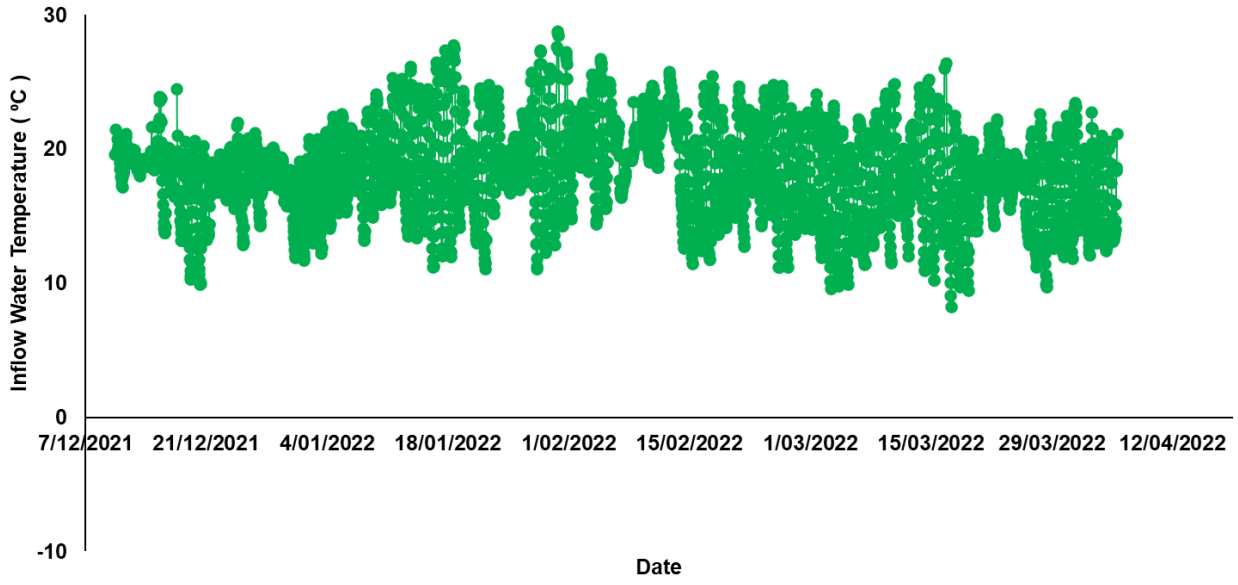

Figure 5A.4: Recorded inflow water temperature during experiment 1. Data encompass the on-farm biomass establishment phase through to the end of the week 13 of the experiment (from 13th December 2021 to 5th April 2022)

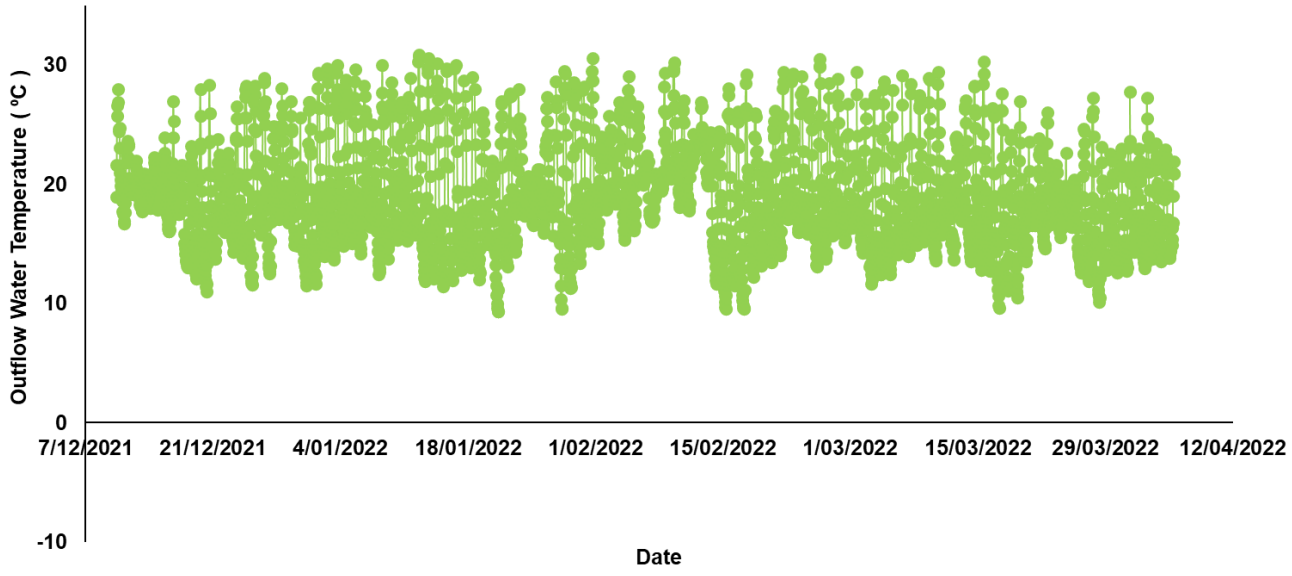

Figure 5A.5: Recorded outflow water temperature during experiment 1. Data encompass the on-farm biomass establishment phase through to the end of the week 13 of the experiment (from 13th December 2021 to 5th April 2022)

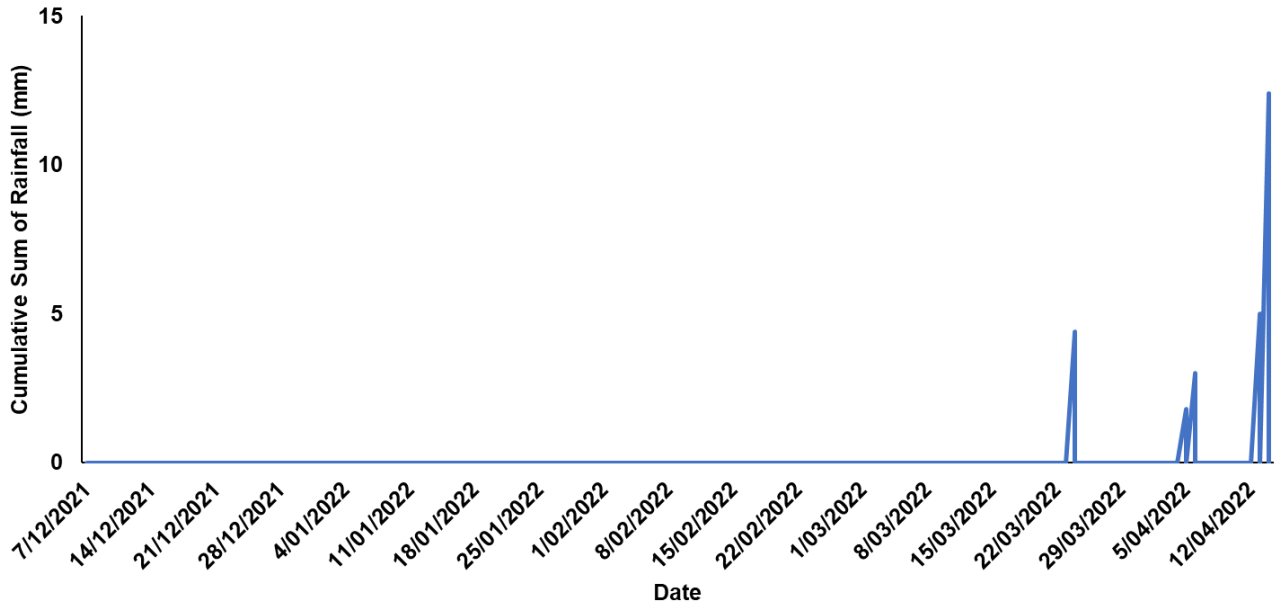

Figure 5A.6: Recorded rainfall events during experiment 1. Data encompass the on-farm biomass establishment phase through to the end of the week 13 of the experiment (from 13th December 2021 to 5th April 2022)

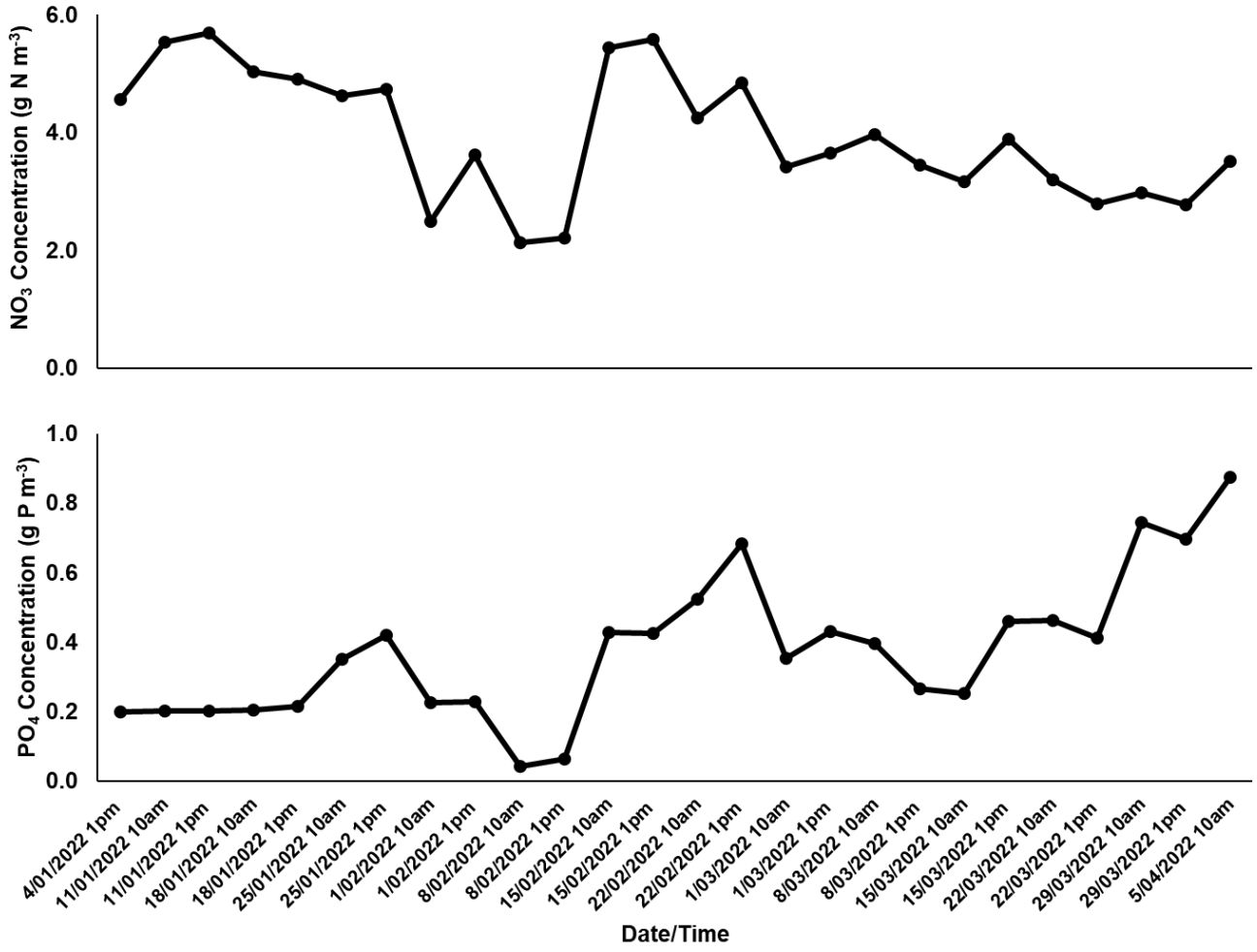

Figure 5A.7: NO_3-N concentration (g N m^{-3}, upper panel) and PO_4-P concentration (g P m^{-3}, lower panel) of FANS inflow over 13 consecutive seven-day growth cycles in experiment 1

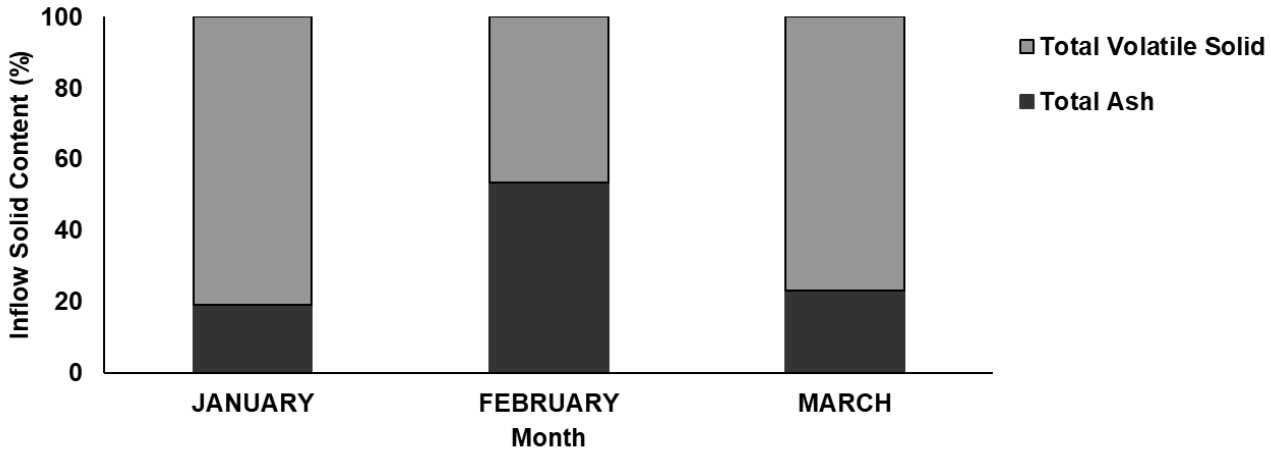

Figure 5A.8: Total volatile solid and ash content (%) in the inflow in experiment 1 over three consecutive months (from January to March 2022) where inflow water samples collected once every four weeks from the inflow sump

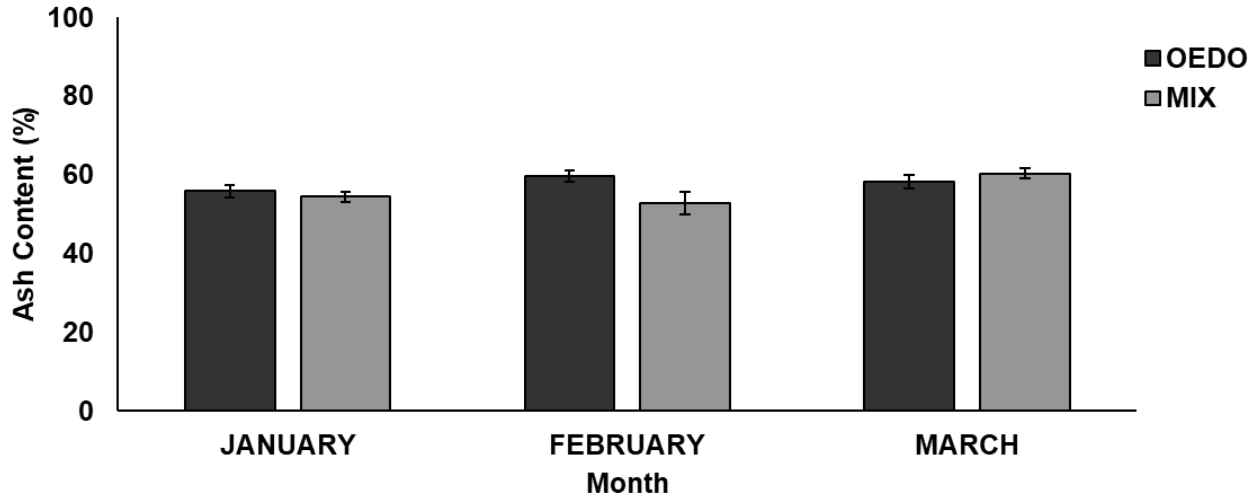

Figure 5A.9: Average (± S.D.) ash content (%) in the harvested biomass in experiment 1 over three consecutive months (from January to March 2022) where samples collected once every four weeks from all replicate FANS seeded with single species of *Oedogonium* sp. (OEDO) and the natural establishment mixed species FANS (MIX). N = 3

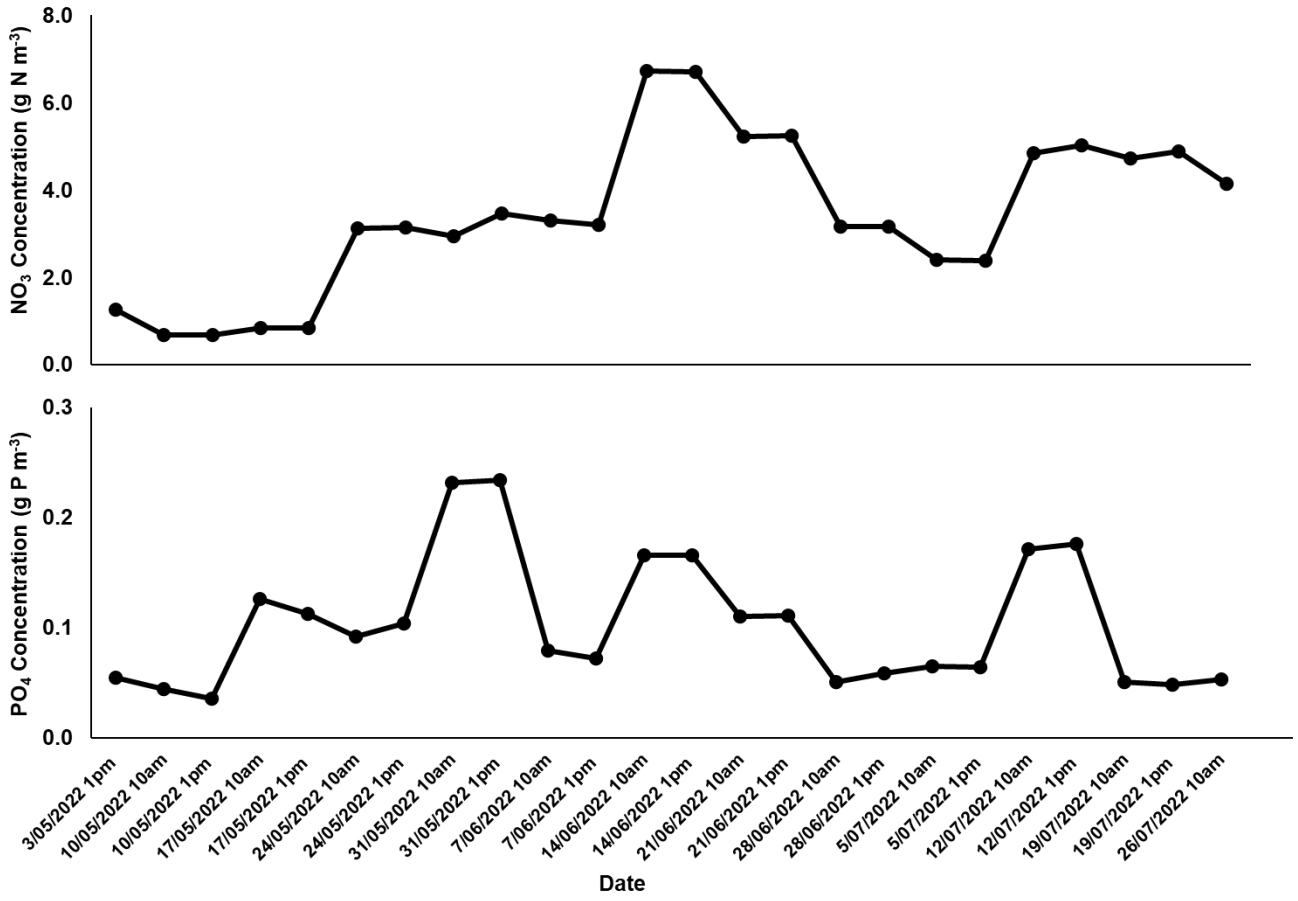

Figure 5A.10: NO_3-N concentration (g N m^{-3}, upper panel) and PO_4-P concentration (g P m^{-3}, lower panel) of FANS inflow over 12 consecutive seven-day growth cycles in experiment 2

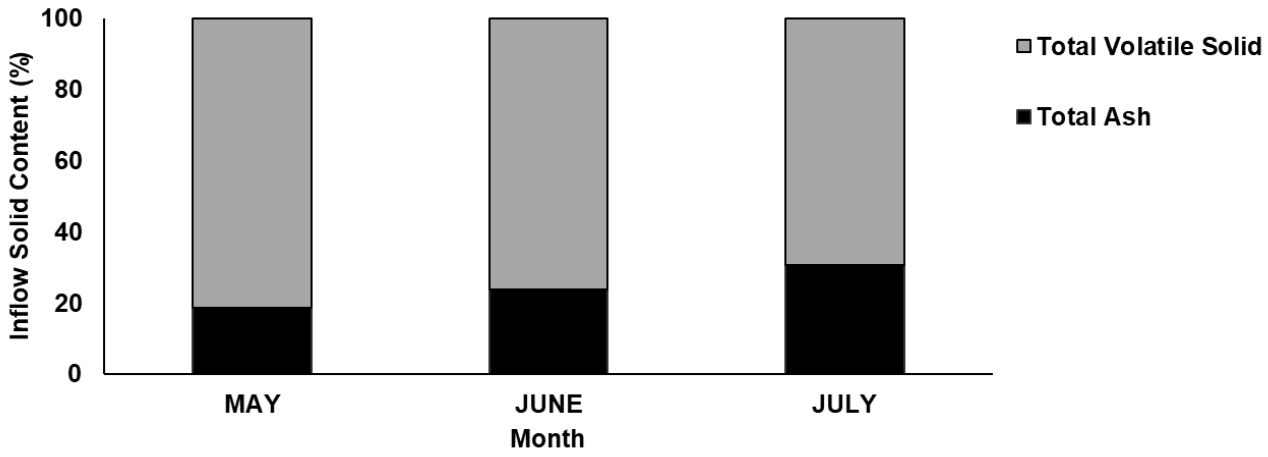

Figure 5A.11: Total volatile solid and ash content (%) in the inflow in experiment 2 over three consecutive months (from May to July 2022) where inflow water samples collected once every four weeks from the inflow sump

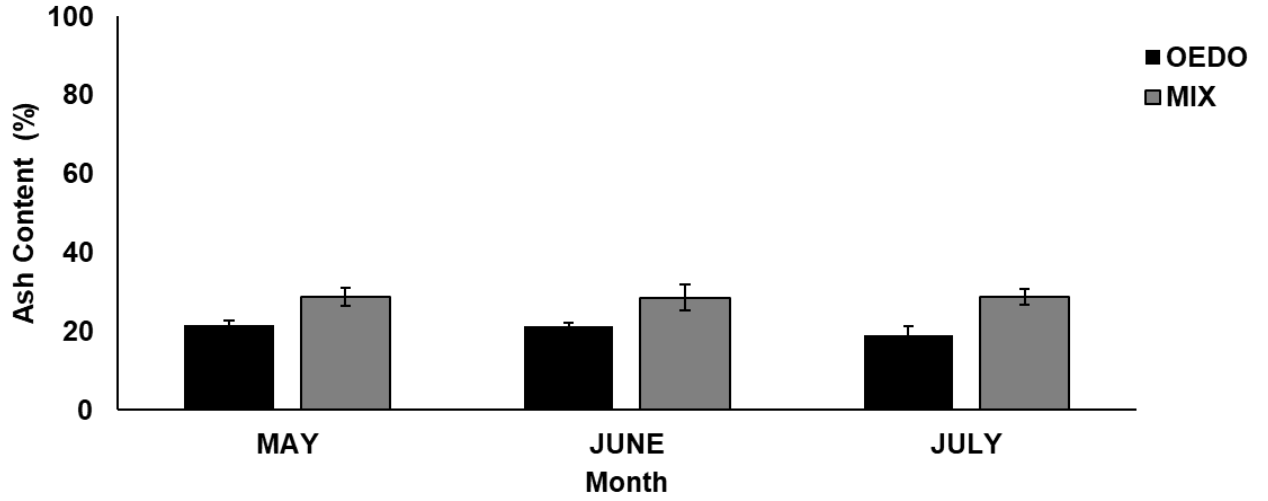

Figure 5A.12: Average (± S.D.) ash content (%) in the harvested biomass in experiment 2 over three consecutive months (from May to July 2022) where samples collected once every four weeks from all replicate FANS seeded with *Oedogonium* sp. (OEDO) and mixed species FANS (MIX). N = 3

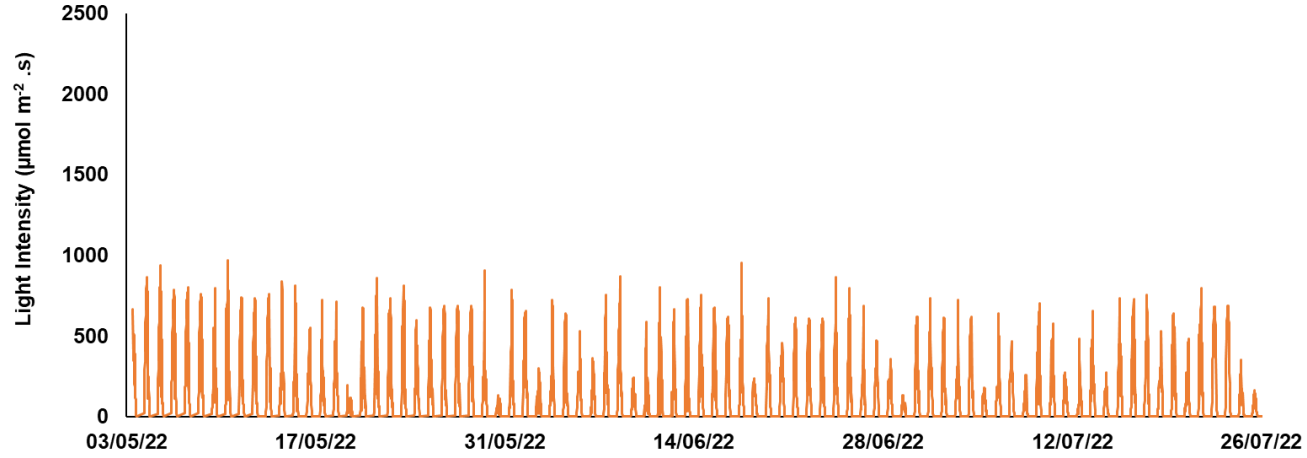

Figure 5A.13: Recorded light intensity during experiment 2. Data encompass the on-farm biomass establishment phase through to the end of the week 12 of the experiment (from 3rd May 2022 to 26th July 2022)

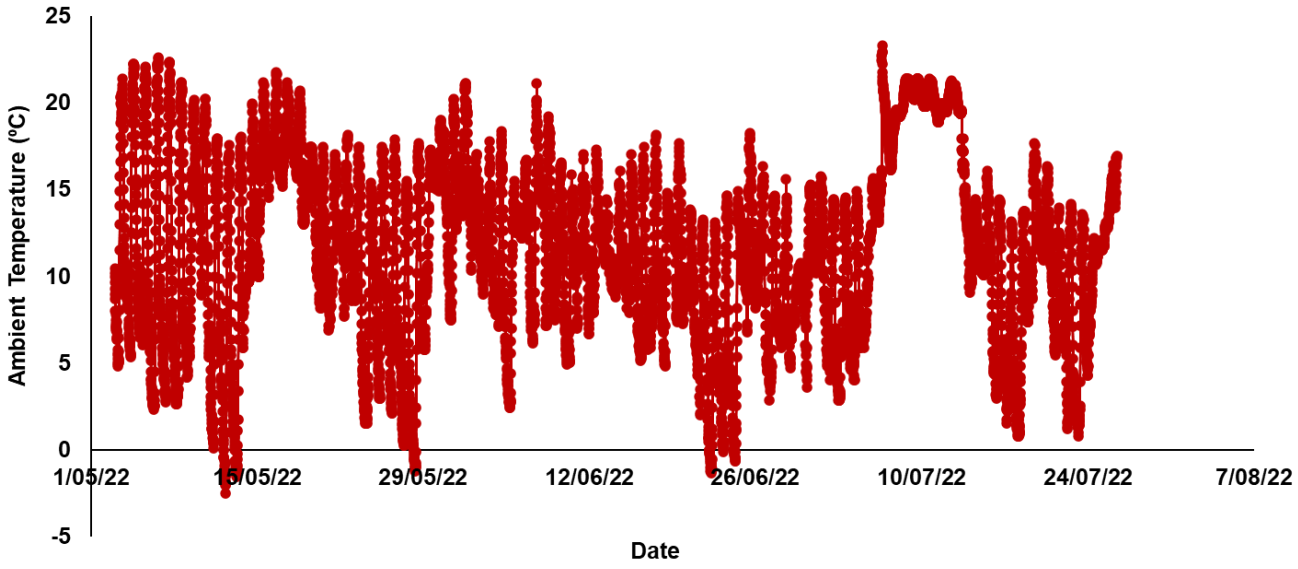

Figure 5A.14: Recorded ambient temperature during experiment 2. Data encompass the on-farm biomass establishment phase through to the end of the week 12 of the experiment (from 3rd May 2022 to 26th July 2022)

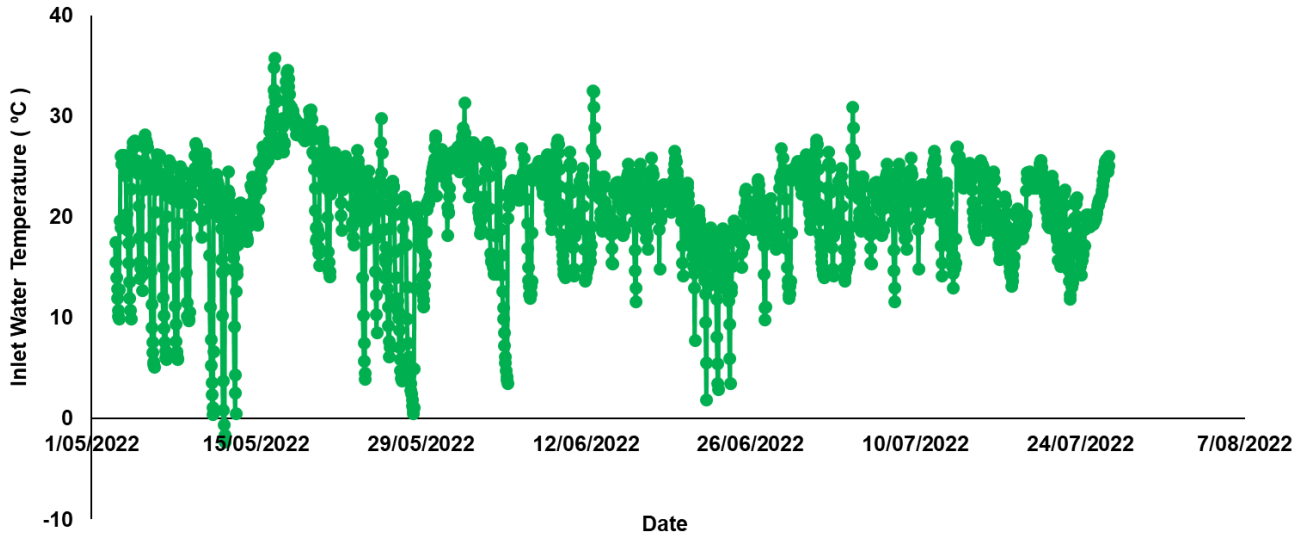

Figure 5A.15: Recorded inflow water temperature during experiment 2. Data encompass the on-farm biomass establishment phase through to the end of the week 12 of the experiment (from 3rd May 2022 to 26th July 2022)

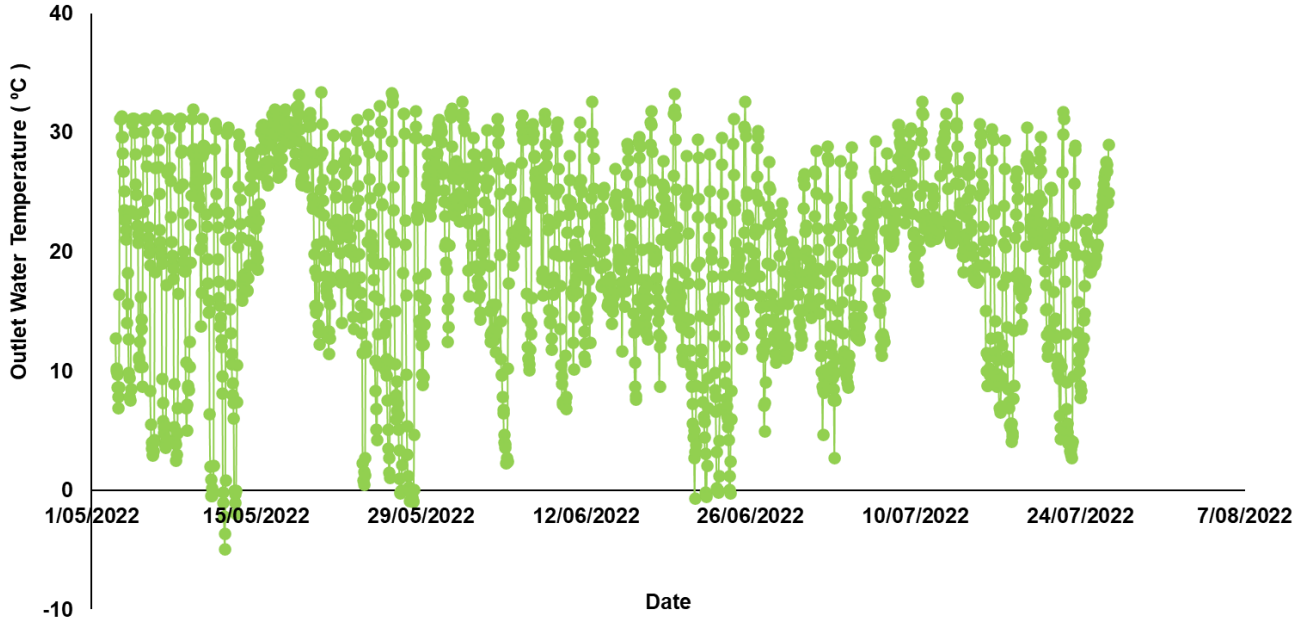

Figure 5A.16: Recorded outflow water temperature during experiment 2. Data encompass the on-farm biomass establishment phase through to the end of the week 12 of the experiment (from 3rd May 2022 to 26th July 2022)

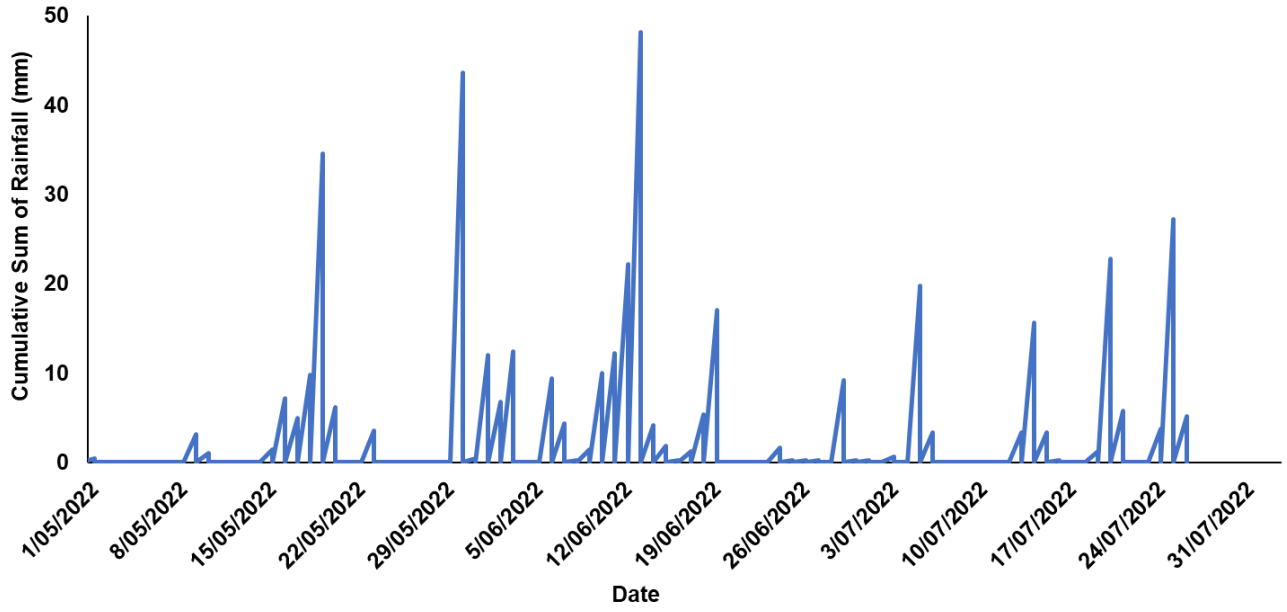

Figure 5A.17: Recorded rainfall events during experiment 2. Data encompass the on-farm biomass establishment phase through to the end of the week 12 of the experiment (from 3rd May 2022 to 26th July 2022)

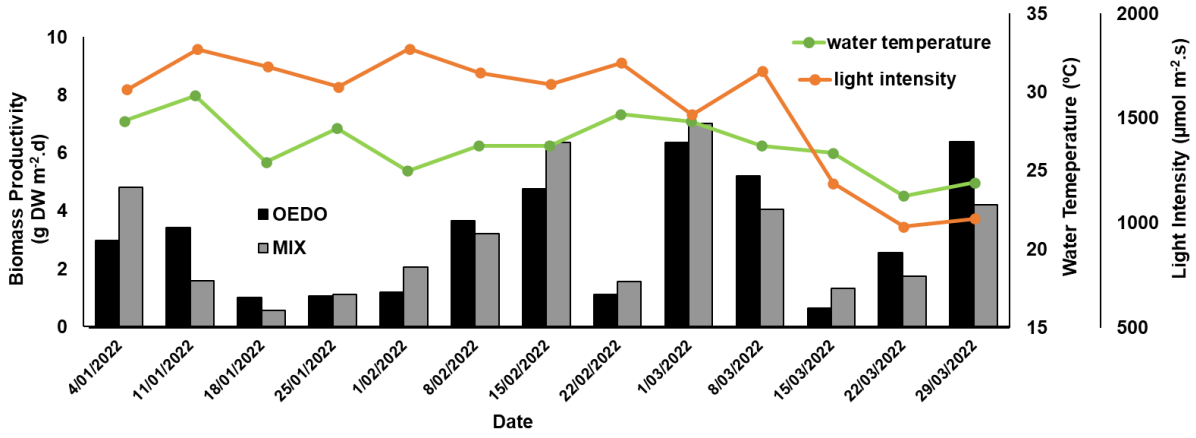

Figure 5A.18: Biomass productivity (g DW m^{-2} d^{-1}), water temperature (ºC) and light intensity (µmol m^{-2} s^{-1}) throughout the experiment over 13 consecutive seven-day growth cycles of controlled seeded *Oedogonium* sp. FANS (OEDO) and natural establishment mixed species FANS (MIX). The temperature and light intensity values was calculated based on the average maximum daily water temperature and light intensity for every growth cycle

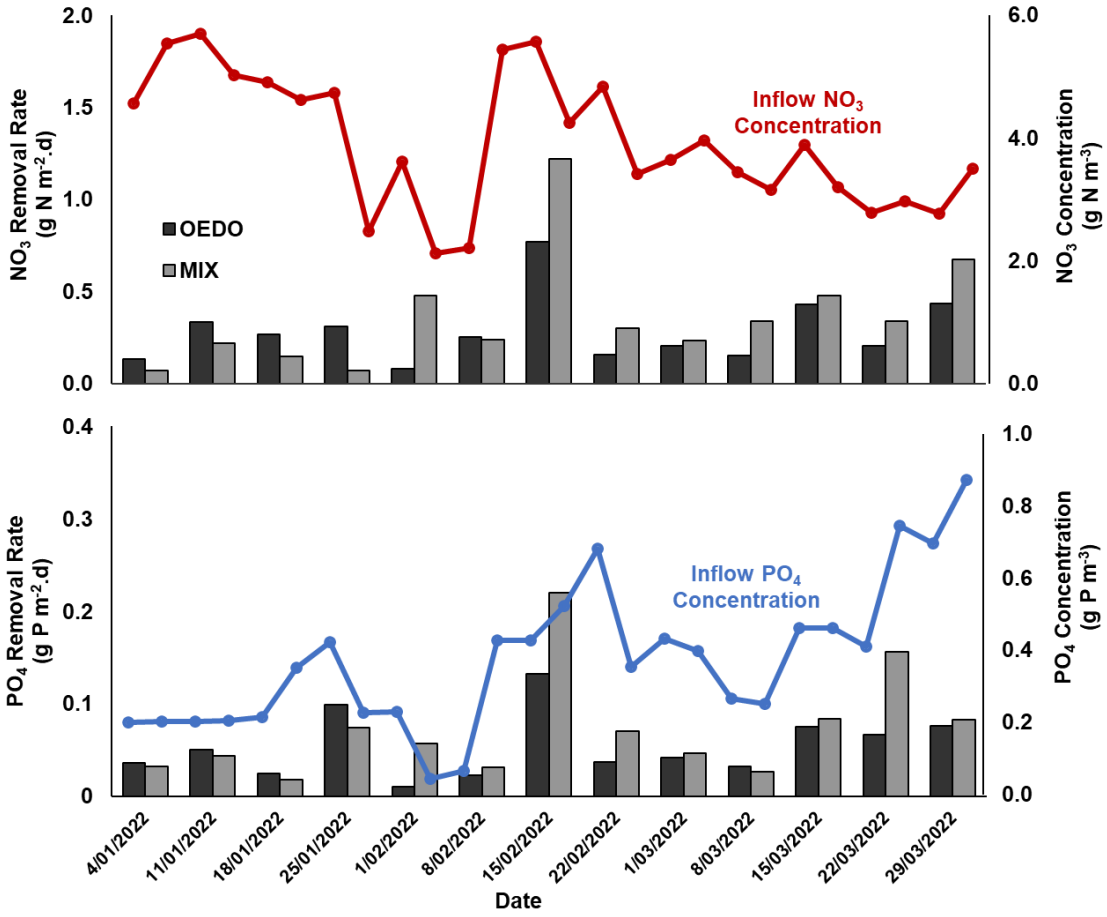

Figure 5A.19: NO_3-N removal rate (g N m^{-2} d^{-1}) and inflow NO_3-N concentration (g N m^{-3}) throughout the experiment (upper panel) and PO_4-P removal rate (g P m^{-2} d^{-1}) and inflow PO_4-P concentration (g P m^{-3}) throughout the experiment (lower panel) over 13 consecutive seven-day growth cycles of controlled seeded *Oedogonium* sp. FANS (OEDO) and natural establishment mixed species FANS (MIX)

Table 5A.1: Recorded trend in water temperature, light intensity, and biomass productivity throughout the experiment over 13 consecutive seven-day growth cycles of controlled seeded *Oedogonium* sp. FANS and natural establishment mixed species FANS

Growth Cycle	Biomass Productivity	Water Temperature	Light Intensity
1			
2	Decrease	Increase	Increase
3	Decrease	Decrease	Decrease
4	Increase	Increase	Decrease
5	Increase	Decrease	Increase
6	Increase	Increase	Decrease
7	Increase	Stable	Decrease
8	Decrease	Increase	Increase
9	Increase	Decrease	Decrease
10	Decrease	Decrease	Increase
11	Decrease	Decrease	Decrease
12	Increase	Decrease	Decrease
13	Increase	Increase	Increase

Chapter 6
General Discussion

6.1 Summary of Research Findings

The potential influence of seasonal ambient conditions, FANS operating parameters, seeding methods and species composition on algae growth and bioremediation performance may have important significance for establishing an effective FANS treatment. This thesis investigated these factors and compared filamentous algae species' performance on FANS.

In Chapter 2, the ability of local species of filamentous algae to attach, grow and remove nutrients on FANS was investigated. The performance of monocultures of filamentous algae species was assessed under controlled indoor conditions using a novel microscale FANS bioassay. Attached algal systems are typically self-seeded with mixed-species assemblages. However, this chapter demonstrated that a single target species can be maintained on a small-scale FANS and achieve nutrient mitigation and biomass productivity at least over a month timescale. *Oedogonium* sp. was the best performing species as it had the most uniform biomass distribution and attachment, high productivity, and high nutrient removal rates. These criteria make *Oedogonium* sp. an ideal target species for cultivation on large-scale FANS. An important output of this chapter was the development of a novel microscale FANS bioassay. For the first time, this bioassay provides a reproducible method to concurrently and easily assess the suitability of multiple species of filamentous algae for FANS based on their ability to attach and grow, remove nutrients and produce harvestable algal biomass

As Chapter 2 assessed species performance on small scale FANS under controlled indoor conditions, results from this laboratory-scale study may not fully reflect the complex interaction between abiotic and biotic factors in large-scale outdoor FANS. Therefore, to confirm the findings of Chapter 2, Chapter 3 compared the performance of species on mesocosm-scale FANS under ambient outdoor conditions across summer and winter. This

study demonstrated that environmental variations in ambient light and temperature impacted the biomass yield and nutrient removal rates of filamentous algae grown on outdoor FANS. Overall, as was found in Chapter 2, *Oedogonium* sp. was the best performing algal species across summer and winter conditions in terms of biomass productivity, nutrient removal rates, and its dominance over other non-target species on the floway. These findings suggest *Oedogonium* sp. is the ideal species to be cultivated on FANS for combined bioremediation and biomass production throughout the year. Importantly, this chapter showed that selecting a target species that can maintain its dominance over cyanobacteria, diatoms, and other non-target filamentous species, especially during summer high temperatures and irradiances, is critical to enable year-round FANS operation with a single species. Moreover, the approach used in this study can be applied in other locations and used as a template to identify suitable target filamentous algal species for continuous year-round cultivation on outdoor FANS.

There is still limited understanding of the influence of FANS operating parameters such as initial standing crop, harvesting frequency and influent flow rate on biomass productivity and nutrient removal rates. Therefore, Chapter 4 investigated the effects of FANS controlled operating parameters on the performance of *Oedogonium* sp., which was selected as a target species based on the results of the previous two chapters. Overall results suggest that an initial standing crop of 70-80 g DW m^{-2}, a four-day harvesting interval, and an influent flow rate of 1 L min^{-1} (16.7 L min^{-1}.m floway width; equivalent horizontal water velocity of 0.04 m s^{-1} down the floway) were optimal for unialgal *Oedogonium* sp. FANS to maximize biomass productivity and nutrient removal under controlled irradiance and temperature. These findings can be used to optimize the operation of FANS to maximise biomass productivity and nutrient removal performance. Notably, although this study focused on *Oedogonium* sp., the findings are likely to apply to other filamentous algae species with similar physical and ecological features (e.g., *Cladophora* sp., *Rhizoclonium* sp., and *Spirogyra* sp.).

Chapter 5 assessed on-farm performance of pilot-scale FANS over seven months, comparing the effects of seeding methods (controlled seeding vs. natural establishment) and seeded species (single target species vs. mixed algae assemblage) in two separate experiments. Controlled seeding promoted faster biomass establishment on FANS than the natural establishment method. However, environmental conditions had a stronger effect on biomass productivity and nutrient removal than the difference in seeding method and seeded species (single species vs. mixed species). Crucially, these experiments demonstrated that FANS

seeded with a single target species have the potential to be operated with a higher relative abundance on the FANS for a longer period of time compared to FANS seeded with a mixed species assemblage, enabling the recovery of high-quality biomass with low variation in algae species composition for on-farm FANS.

6.2 Implication of Research Findings

This thesis highlights the relationships of various factors and their effects on FANS performance relative to the aims of FANS for bioremediation, biomass production or both. Overall, this study suggests that selecting the best performing species, understanding, and optimizing the operational parameters and improving the seeding approach could maximize the year-round performance of on-farm FANS. Based on the results of this study, possible ways to maximize FANS performance and lessen the impact of seasonal and environmental variations on performance include:

i) Selecting filamentous algae species that can strongly attach to the liner and have high biomass productivity and high nutrient removal rates. This can be achieved by comparing the performance of a range of locally isolated species under controlled laboratory conditions using the μFANS bioassay developed in Chapter 2. This bioassay allows a high number of replicates of each species to be compared simultaneously, providing more reliable results.

ii) Selecting filamentous algae species that can tolerate both summer high temperature and irradiance and/or winter low temperature and irradiance. A different best performing species may be cultivated on FANS for each season, or a single species that performed well under both seasons may be selected as the target species year-round. Importantly, Chapters 4 and 5 showed that the amount of biomass washed off FANS can be substantial, particularly during periods of high water flow due to rainfall. It is therefore essential to include the quantification of biomass washed off in biomass productivity calculations to get an accurate estimate of productivity when comparing performance between species.

iii) Optimizing FANS operation parameters (e.g. initial standing crop, harvest frequency and influent flow rate) for the selected target species. Particular species may be affected differently by these operational parameters, so studying how they affect the performance of the target species is crucial. The current study provides a standardized methodology to conduct strip biomass monitoring, enabling the biomass productivity of algae on large scale FANS to be easily assessed.

iv) Using controlled seeding to provide faster biomass establishment, and a greater level of control of FANS species composition for a longer period than using the natural establishment method for seeding.

6.3 Future Research Needs

FANS operational parameters can have large impacts on FANS performance (Liu et al., 2020; Sutherland et al., 2020b; Park et al., 2022). Chapter 4 assessed the effects of operational parameters under controlled conditions, but Chapter 3 showed that biomass productivity and nutrient removal performance on FANS varies between seasons. This means that optimal operational parameters may also vary between seasons. Therefore, optimal operational parameters could be developed according to season to reduce the variability in performance of ambient outdoor FANS. For example, increasing the water depth on FANS in summer may help to reduce any heating effects while at the same time reducing potential light stress to the attached algal biomass from high irradiances (Park et al., 2022). However, a higher water depth may not be necessary for FANS operation during winter as irradiances are generally much lower than in summer (Sutherland et al., 2020a). Similarly, controlling the algal biomass standing crop by either altering the harvest interval or the amount of biomass remaining on the floway according to the season may also help to reduce the variation in FANS biomass productivity and nutrient removal performance between summer and winter operations.

Current research on FANS is focused on understanding factors that impact biomass productivity and nutrient removal performance. However, for FANS to be a successful method for the treatment of agricultural drainage water, viable biomass applications will need to be identified for the harvested algal biomass (Lawton et al., 2017). Hence, future research should investigate the biochemical composition of the harvested algae biomass from FANS treating

agricultural drainage water and assess its suitability for a range of biomass applications. For example, algae cultivated in nutrient rich agricultural drainage water with a high nitrogen concentration could produce harvested biomass with a high protein content. As such, the biomass would be suitable for use in animal feed application, either as a whole ingredient or as derived protein extracted from the biomass to use as an ingredient in compound animal feeds (Cole et al., 2016; Lawton et al., 2017).

This thesis assessed FANS biomass productivity and nitrate and phosphate removal performance. However, other contaminants that may be in agricultural drainage water such as trace metals, emerging organic contaminants (EOCs), pesticides, herbicides, and hormones have been detected in New Zealand freshwater (Greig et al., 2010; Bernot et al., 2019). Some of these contaminants are likely to be originating from agricultural drainage (Veach & Bernot, 2011). Several studies have demonstrated that microalgae can effectively remove EOCs from wastewater (de Godos et al., 2012; Matamoros et al., 2015). The potential for macroalgae to bioremediate EOCs has not been investigated, but given their strong ability to bioremediate metals, nutrients, and other contaminants (Lee & Chang, 2011; Anacleto et al., 2017; Lawton et al., 2021), they are likely to be successfully able to bioremediate a wide range of EOCs. Therefore, future studies should investigate capability of macroalgae to bioremediate EOCs and other contaminants in agricultural drainage water using FANS.

Chapter 5 demonstrated that FANS could successfully treat agricultural drainage water on a dairy farm. Future studies should investigate the ability of FANS to treat drainage water on other types of farms (e.g. sheep, beef and horticulture) as the composition of the drainage water might differ between farming types and could potentially influence the overall FANS biomass productivity and nutrient removal performance. Based on the FANS performance in Chapter 5 and that reported in previous studies conducted on other farm types (Kangas & Mulbry, 2014; D'Aiuto et al., 2015; Liu et al., 2016a; Hariz et al., 2023b), nutrient concentration and turbidity appear to be the key factors that vary between agricultural drainage water from different sources. As these factors are likely to affect the relative dominance of algal species, and algal growth and nutrient removal, the performance of FANS may differ between farm types. As such, biomass productivity and nutrient removal performance on a horticulture farm may be lower than on a dairy farm if there were comparatively lower nutrient levels in the horticulture farm drainage water. Hence, the results of FANS performance on one farm type may not indicate performance on another farm. Furthermore, the top performing

target species for one farm may not be the top performing species in a different farm with a different type of drainage water. Consequently, target species selection may need to be conducted for each farm type if single species FANS cultivation is targeted.

FANS technology is an effective treatment for bioremediation in comparison to other drainage treatment technologies (e.g. wetlands) as it requires less land area to attain a similar nutrient removal efficiency (Sutherland and Craggs, 2017). In addition, studies comparing the nutrient removal performance of FANS and wetlands have shown the average total nitrogen removal of FANS system (88 g m^{-2} $year^{-1}$) was more than double that of typical agricultural drainage wetland treatment (40 g m^{-2} $year^{-1}$), while total phosphorus removal of FANS (12 g m^{-2} $year^{-1}$) was four times higher than typical agricultural drainage wetland treatment (3 g m^{-2} $year^{-1}$) (Tanner & Sukias, 2011; Karpuzcu, 2012; Tanner et al., 2012; Hariz et al., 2023b). FANS has a faster implementation time due to the higher growth rate of algae compared to wetland plants, it has a higher rate of nutrient assimilation in the algal biomass, and the nutrients can be captured in the form of harvestable biomass that can be recycled for beneficial uses such as biofertilizer, biofuel and production of biomaterials (Wilkie & Mulbry, 2002; Sutherland et al., 2015a; Tsarpali et al., 2021). However, frequent harvesting is required for FANS technology compared to other treatment technologies (Sutherland & Craggs, 2017), which means that FANS have higher operation costs. Therefore, future research should focus on maximizing or at least maintaining performance with minimal harvest. This is likely to require the development of a technique that can reduce harvest frequency or the time taken to harvest the FANS. One technique that could be explored is self-harvesting FANS. Since a higher water current can cause the attached biomass to wash off the floway, a semi-automated hydraulic surge system could be developed to wash a certain portion of the biomass off the floway into an outflow biomass collection sump. Moreover, a curve dewatering wedge wire screen could be modified and connected to the FANS outflow and the biomass collection sump to allow water to drain away and wet biomass to roll into the sump. For long-term FANS operation, labour may only be needed to collect the strained biomass periodically. Such a self-harvesting FANS may assist in solving the issue of labour-intensive operation and potentially result in more cost-effective FANS technology.

One potential issue with FANS operation is the establishment of midges on floways during summer (Craggs et al., 1996a; Kangas & Mulbry, 2014). There was a high rate of midge establishment during summer (approximately >1100 midge counts per square metre during

summer in the final growth cycle) at the end of the mesocosm scale FANS experiment at the Ruakura Facility (Chapter 3) that considerably reduced biomass productivity. In contrast, the on-farm FANS experiment (Chapter 5) had a lower rate of midge establishment (approximately <20 midge counts per square metre across summer). The lack of midges at the farm site may be due to lack of suitable habitat in the stream. Further research should be conducted to provide a greater understanding of the factors affecting midge establishment, potentially relative to changes in water quality, nutrient concentrations, and local ambient conditions. This information could then be used to develop methods to control midge establishment without affecting the established algae on FANS.

6.4 Concluding remarks

Prior to this research being undertaken, there was limited information available on the factors that influence monoculture algae FANS bioremediation of agricultural drainage water. Most available filamentous algae studies were conducted in suspension or in attached systems seeded with uncontrolled species composition. Moreover, the details of filamentous algae biomass attachment and establishment on the FANS system were rarely discussed. This thesis has provided crucial information to fill these knowledge gaps. The microscale FANS bioassay developed in Chapter 2 is a novel method to rapidly establish an attached monoculture of filamentous algae to compare performance. The significant differences in attachment capability between species of filamentous algae reported here demonstrate that not all species are suitable for FANS cultivation. Experiments in this study have shown that monoculture FANS have the potential to generate biomass with a lower contamination rate than mixed species FANS. However, the on-farm study in Chapter 5 showed that neither monoculture nor mixed species composition significantly affected FANS biomass productivity and nutrient removal performance. Therefore, single species FANS may not be needed in a farm setting, and FANS could be easily seeded with the naturally available mixed algae biomass obtained from near-by streams. However, if the harvested biomass of a specific algae such as *Oedogonium* sp. can easily be beneficially used, the higher value of the harvested biomass may offset the increased operation costs for isolating the algae from a local waterbody and more regular seeding onto the FANS.

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