Sustainable aquaculture production systems through nutrient recycling from fish farming effluents using duckweed

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Declaration

The candidate confirms that the work submitted is his/her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Shortage of water, food and mineral resources, along with the increase in waste generation, are among the main challenges that humanity will face in the coming years. Changes in consumption habits induced by the continuous increase of global population and raise of household income are creating pressures on water security and food supply systems, particularly regarding the production and supply of animal protein. As a result, the entire aquaculture sector has become a focus of attention as it provides healthier options to meat products at affordable prices. However, its higher-than-average growth in the past decades is creating negative impacts to aquatic ecosystems – i.e., untreated effluents from fish farming containing large loads of nutrients can cause eutrophication and hypoxia in receiving water bodies. Sustainable solutions like the use of aquatic plants (e.g., duckweed) for nutrient control and recovery from fish farm effluents have been studied over years, but the mechanisms governing nutrient uptake and accumulation are still unknown.

Preliminary work conducted by the BioResource Systems Research Group at Leeds on the use of duckweed for phosphorus control from wastewater effluents, has demonstrated the possibility to uncouple biomass growth from luxury phosphorus uptake even under winter conditions in the UK, with the potential to meet stringent discharge consents (< 1 mg P L^{-1}). This outcome, however, has not been tested under tropical climate conditions, nor integrated to current aquaculture systems. This work presents the opportunities and hurdles to fully develop sustainable aquaculture production systems through nutrient control using duckweed ponds and recycling by the valorisation of enriched harvested duckweed biomass. Moreover, it highlights current research gaps to efficiently control nitrogen and phosphorus discharges from fish farms effluents. At the end, the proposed research seeks to apply lab-based outcomes on nutrient recovery by duckweed to the design of plant-based ponds for the treatment of aquaculture effluents in Indonesia.

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4PL	FOUR-PARAMETER LOGISTIC REGRESSION
AMP	ASSET MANAGEMENT PERIOD
ATP	ADENOSINE-TRI-PHOSPHATE
ATP	ADENOSINE-TRI-PHOSPHATE
BCA	BICINCHONINIC ACID
BOD	BIOLOGICAL OXYGEN DEMAND
BP	BIOMASS PRODUCTIVITY
BSA	BOVINE SERUM ALBUMIN
CHON	CARBON, HYDROGEN, OXYGEN, NITROGEN
COD	CHEMICAL OXYGEN DEMAND
CSTR	CONTINUOUS STIRRED TANK REACTOR
CTAB	CETYL-METHYLAMMONIUM-BROMIDE
DLI	DAILY LIGHT INTEGRAL
DO	DISSOLVED OXYGEN
DW	DUCKWEED
DW	DRY WEIGHT
DWT	DUCKWEED-BASED WASTEWATER TREATMENT
EE	ETHER EXTRACT
FAME	FATTY ACID METHYL ESTER
FP	FISH POND
FW	FRESH WEIGHT
GDP	GROSS DOMESTIC PRODUCT
HM	HOAGLAND'S MEDIA
HRT	HYDRAULIC RETENTION TIME
MUFA	MONOUNSATURATED FATTY ACID
NBS	NATURE BASED SOLUTIONS
NFE	NITROGEN FREE EXTRACT
NH4-N	AMMONIUM NITROGEN

NLRS	SURFACE NITROGEN LOADING RATE
NO2-N	NITRITE NITROGEN
NO3-N	NITRATE NITROGEN
NRR	NITROGEN REMOVAL RATE
PAR	PHOTOSYNTHETICALLY ACIVE RADIATION
PCR	PLYMARASE CHAIN REACTION
PHT	PHOSPHORUS TRANSPORTER PROTEIN
PLRS	SURFACE PHOSPHORUS LOADING RATE
PO4-P	ORTHOPHOSPHATE PHOSPHORUS
PRR	PHOSPHORUS REMOVAL RATE
PUFA	POLYUNSATURATED FATTY ACIDS
RAS	RECIRCULATING AQUACULTURE SYSTEM
RGR	RELATIVE GROWTH RATE
RSM	RESPONSE SURFACE METHODOLOGY
SDG	SUSTAINABLE DEVELOPMENT GOAL
SFA	SATURATED FATTY ACID
SS	SUSPENDED SOLIDS
SS	SETTEABLE SOLIDS
TDS	TOTAL DISSOLVED SOLIDS
TN	TOTAL NITROGEN
TP	TOTAL PHOSPHORUS
TPC	THERMAL PERFORMANCE CURVE
TS	TOTAL SOLIDS
TSS	TOTAL SUSPENDED SOLIDS
UWWT	URBAN WASTEWATER TREATMENT
UWWTR	URBAN WASTEWATER TREATMENT REGULATIONS
WP	WORK PACKAGE
WWTS	WASTEWATER TREATMENT SYSTEM
Y_{PX}	PHOSPHORUS-TO-BIOMASS YIELD COEFFICIENT

Chapter 1 Introduction

1.1 Overview

Humanity has always found ways to take advantage of what the planet offers, whether it be gathering fruits and hunting wild animals for food, or the exploitation and supply of fossil energy derivatives from 200 years ago. This exploitation has raised concerns about the environmental and societal impacts of economic activities. Current production systems have supported industrialization and population growth but have done so at the expense of the environment. The sustainability of these systems is now in question as ecosystems suffer from severe degradation due to soil erosion, agrochemical usage, biodiversity loss, and water contamination (Mallin and Cahoon 2003; Mgbemene 2017). Furthermore, as economic activities, individual consumption, and manufacturing processes have grown more evident and intense, sustainable approaches to these issues have been developed.

Indeed, the establishment of the concept of sustainability marks one of humanity's most significant and pivotal achievements in recent decades. This concept encompasses all major challenges facing humanity today, including extreme poverty, inequality, climate change, and water and food security. These challenges are addressed through the paradigm of 'sustainable development' as defined by the United Nations in 1987. Consequently, countries worldwide have endorsed the Sustainable Development Goals (SDGs) established by the United Nations (United Nations 1987).

Specifically, SDG 2 (Zero hunger), SDG 6 (Clean water and sanitation), SDG 12 (Responsible consumption and production), and SDG 14 (Life below water) integrate the framework of the present study and support the research objectives. These goals focus on water management and nutrient recycling and recovery, highlighting the interconnectedness of these objectives with health, education, economic growth, and environmental sustainability. Achieving these SDGs by 2030 is crucial for fostering a sustainable future (Bhaduri *et al.* 2016; Stevens and Kanie 2016).

When considering water management, it is essential that all human activities involving water undergo waste treatment processes to guarantee the

continuity of the resource for future generations (Abu-Zeid 1998; Ahmad, Ahmad, and Alam 2016). Aquaculture, recognized as the fastest-growing sector of food production (FAO 2009a), is no exception to this rule. The need for effective wastewater treatment systems (WWTS) is imperative, particularly because only about 20% of global wastewater is currently treated before being released back into ecosystems (United Nations 2016), and most existing WWTS only achieve secondary treatment, primarily removing the carbon load from effluents (Kalavrouziotis *et al.* 2013).

This insufficient treatment contributes directly to the increasing eutrophication of water bodies, exacerbated by inadequate recovery of nutrients such as nitrogen (N) and phosphorus (P). Thus, the discharged effluents must be of high quality to prevent water pollution and other environmental impacts (Akpor and Muchie 2011). Ecological methods, like the cultivation of aquatic macrophytes, are particularly effective for nutrient removal and recovery from wastewater and for restoring eutrophic water bodies (Dhote and Dixit 2009; Li *et al.* 2009; Liu, Dai, and Sun 2017). Duckweeds (DW), among these plants, are noted for their efficiency in removing nutrients, organic matter, and toxic substances from wastewater. Their use in treatment systems is favoured due to their versatility, rapid growth, low maintenance, and suitability for both urban and rural settings, which is attributed to their low energy requirements and practicality (Brix 1994). Additionally, one of the most significant advantages of using duckweeds is that they do not compete with traditional agricultural land used for food production.

Duckweeds have a high growth rate, demanding substantial amounts of carbon, nitrogen, and phosphorus (Dhote and Dixit 2009). These nutrients are transformed within the plant into valuable biomolecules: carbon is stored as starch and lipids; nitrogen is converted into amino acids and proteins; and phosphorus is used in nucleic acids, phospholipids, and high-energy molecules like nucleoside triphosphates (Hillman and Culley 1978). These compounds make duckweed a nutritionally rich food source, playing a crucial role in the food system (Appenroth *et al.* 2018; Hassan and Edwards 1992; Hillman and Culley 1978; Oron, de-Vegt, and Porath 1988).

Coming back to the subject of aquaculture, the high nutrient concentrations in the effluents of these systems pose significant environmental concerns today (Boyd 2003; Read and Fernandes 2003). Furthermore, the large use of water and feed for fish rearing is putting the sustainability of the process into question. However, the widespread recognition of duckweed as an effective fish feed suggests potential synergies between these two fields, potentially

integrating aquaculture and plant-based water treatment methods (El-Shafai *et al.* 2004; Hassan and Edwards 1992; Sogbesan and Onoja 2015).

Duckweeds have a variety of applications beyond human and animal food; they can be used as fertilizers, as raw materials for biogas production, or even converted into biofuels such as bioethanol and biodiesel, according to recent studies (Cheng and Stomp 2009; Costa *et al.* 2016)[JPP[1]]. This versatility underlines the importance of researching duckweed ponds at mesocosm and pilot scales to understand the dynamics of nutrient removal and their potential as biological systems for mitigating contamination from aquaculture effluents. Additionally, analysing the composition of harvested biomass would provide insights into its nutritional value and inform about the behavior of duckweeds under controlled culture conditions.

Further scientific research into how these plants respond to different nutrient loads and environmental conditions could lead to engineered solutions that optimize biomass quality while achieving nutrient reduction in aquaculture effluent. This is crucial as it allows for the production of high-quality biomass that can be used for fish feed, thereby enhancing water quality. Ultimately, these advancements could contribute to more sustainable aquaculture systems that provide affordable, healthier, and safer sources of animal protein. Such systems would promote socially and environmentally responsible fish production, aligning with global sustainability goals.

1.2 State of aquaculture

According to the FAO (1997), aquaculture is the professional art of breeding and raising animal or plant species, from continental or oceanic waters, under controlled conditions. This technique involves some form of intervention in the breeding process to increase production, such as sowing, feeding, and protecting against predators, etc. Depending on the cultured species it is classified as fish farming (fishes), shrimp farming (crustaceans), oyster farming (molluscs) and algaculture (algae).

Aquaculture dates back to 1500 B.C. in China and India with simple carp farming, alongside similar practices in the Mediterranean by the Etruscans and Romans. Initially based on empirical observations, significant advances didn't occur until the 19th century when pollution impacts on wild fish populations prompted research into artificial breeding and pond farming (Nash 2011). By the 20th century, improvements in materials and feeding practices further enhanced fish farming techniques. Today, aquaculture is recognized globally as a

sustainable solution to food and water security challenges, highlighting its historical evolution and increasing importance (Daniel 2009; Nash 2011; The World Bank 2013; Willett *et al.* 2019).

Fisheries and aquaculture are vital for global food security and nutrition, especially as the world's population grows. Marine resources are increasingly recognized for their significant role in providing essential protein and supporting livelihoods in many developing countries (Godfray *et al.* 2010; HLPE and FAO 2014). Aquaculture is seen as sustainable due to its efficient water use compared to terrestrial livestock and the benefits of water recycling and treatment processes (Verdegem, Bosma, and Verreth 2006).

Between 1960 and 2020, global demand for seafood grew annually by over 3.1%, with per capita consumption rising from 10 kg to 20 kg (FAO 2022). This surge has diversified and enriched global diets. Fish, providing 20% of the world's animal protein intake in 2015, is a rich source of high-quality protein and essential nutrients such as fatty acids, vitamins, and minerals. These nutritional benefits not only enhance diet quality but also aid in combating obesity (FAO 2018; Sampels 2014; Tacon and Metian 2013).

The fishing and aquaculture sectors not only support approximately 820 million people economically but also employ around 59.6 million people worldwide (Allison, Delaporte, and Hellebrandt de Silva 2013). Introducing nutrient recovery technologies in aquaculture effluents could further boost community incomes and support social development (FAO 2018).

Since the 1950s, seafood production from fishing saw a substantial rise, climbing from 18 million tonnes to 90 million tonnes by 1988 before stabilizing with minor fluctuations (Figure 1-1). From the 1980s onward, while wild fishery production has remained relatively stable, aquaculture's contribution to fish supply for human consumption has significantly increased. In 1974, aquaculture accounted for only 7% of fish supply, but by 2020, it had risen to 48%, matching wild fisheries.

Aquaculture production reached 87.5 million tonnes in 2022, growing at an average annual rate of 5.1% between 2000 and 2022, driven by advancements in intensive production technologies and stricter fishing regulations (FAO 2018, 2022). This sector now cultivates a diverse array of species across all continents, with 652 aquatic species farmed globally by 2020, 313 of which are fish species (FAO 2022). In 35 countries, aquaculture yields exceed wild fish catches. By 2030, it is expected that aquaculture output will outpace wild fishery production by 10%.

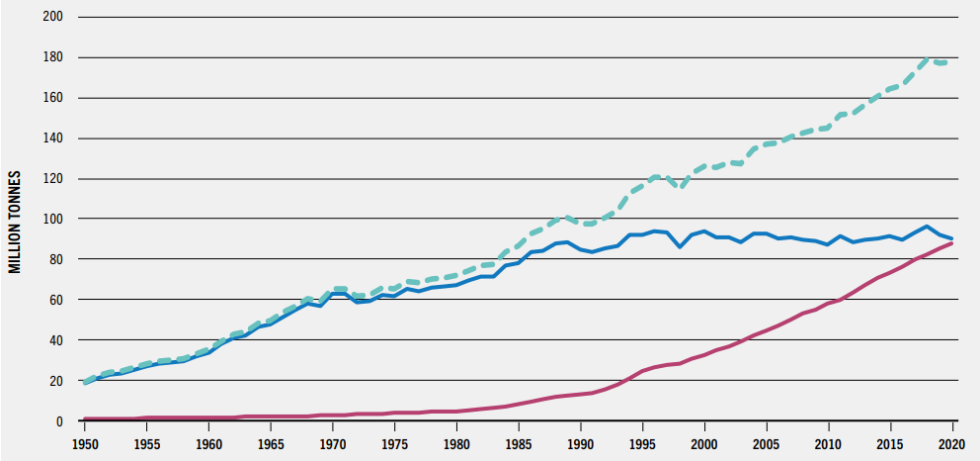

Figure 1-1 World capture fisheries and aquaculture production

Production expressed as million tonnes per year (excluding algae). The red line represent aquaculture production, the blue line capture fisheries production and the discontinuous line total seafood production **(FAO 2022)**.

Geographically, Asia dominated global aquaculture in 2020, producing 88% of the world's output, followed by the Americas at 5%. Over the past 25 years, production in regions like Latin America and North Africa has significantly increased, while in more developed areas like Europe and North America, aquaculture production has halved (FAO 2022).

Asia hosts the top producing countries, with China alone accounting for over 64% of global production, and countries like Indonesia, India, Vietnam, and Bangladesh together contributing another 27%. Favourable geographic and climatic conditions have long supported extensive, small-scale aquaculture practices in these regions, where aquaculture is deeply integrated into society and has been a means of food self-sufficiency for centuries. Aquaculture is notably significant in Asia, contributing over 2% to the region's GDP and standing as a more developed and crucial industry compared to other global regions (Little, Newton, and Beveridge 2016).

However, the growth in aquaculture has led to an increase in effluents, placing environmental pressures that can threaten local ecosystems. As a result, there is an urgent need to address these challenges by valuing and recovering nutrients from these effluents in alignment with sustainable development goals. Implementing strategies for nutrient recovery not only mitigates environmental impact but also enhances the sustainability of this expanding sector. Such efforts are essential to balance economic growth with ecological health and ensure the long-term viability of aquaculture globally.

1.3 Effluents from fish farming

Wastewater is produced by most human activities involving water. The more water is required for production processes or for securing human requirements, the more wastewater is produced, and the environmental impact linked to pollution is worse. Consequently, the perspective on wastewater is shifting from seeing it merely as waste to be disposed of, to viewing it as a valuable resource for recovering energy, water, nutrients, organic matter, and other useful by-products (Remondis 2017). Currently, industries account for 22% of the global water demand and are a significant source of wastewater. It is anticipated that industrial water demand could increase by up to five times in the coming years, potentially constituting 20 – 60% of global water use by 2030 (UNESCO 2003).

As aquaculture intensive systems have become more important today to face the current demand of fish food for healthy and sustainable diets, waste generation has increased and now it is compromising the sustainability of fish farming systems (Buschmann *et al.* 2006; Martins *et al.* 2010). In a fish farming pond, the quality of the water is the result of the source that supplies it, the characteristics of the soil it passes through, added food rations, the density of organisms, and various physical, chemical, and biological interactions. These factors are also shaped by the pond's geomorphological and climatic conditions. Key water quality parameters monitored in aquaculture include turbidity, pH, alkalinity, dissolved oxygen, electrical conductivity, temperature, nutrients (nitrogen and phosphorus), and chlorophyll (Martins *et al.* 2010).

Feed is the primary source of waste in aquaculture, with the digestibility and nutrient content of the feed being crucial for effective ration formulation (Martins *et al.* 2010). Poor-quality feed or improper feeding management can increase faecal waste and unused food, degrading water quality. Issues such as low-quality feed, inadequate or excessive feeding amounts, inappropriate feed for the growth stage or species, and unbalanced rations can compromise water quality, leading to increased phytoplankton, increased turbidity, and lower dissolved oxygen levels (Miller and Semmens 2002). Efficient feeding management is essential as it directly affects water quality and the health of the aquatic organisms; only about 15-20% of the nitrogen and phosphorus in feed and fertilizers are utilized for biomass formation (as detailed in Figure 1-2), with the remainder settling in pond sediment or being discharged in effluents (Boyd 2011; D'Orbcastel and Blancheton 2006; Hakanson *et al.* 1988; Pillay 2004).

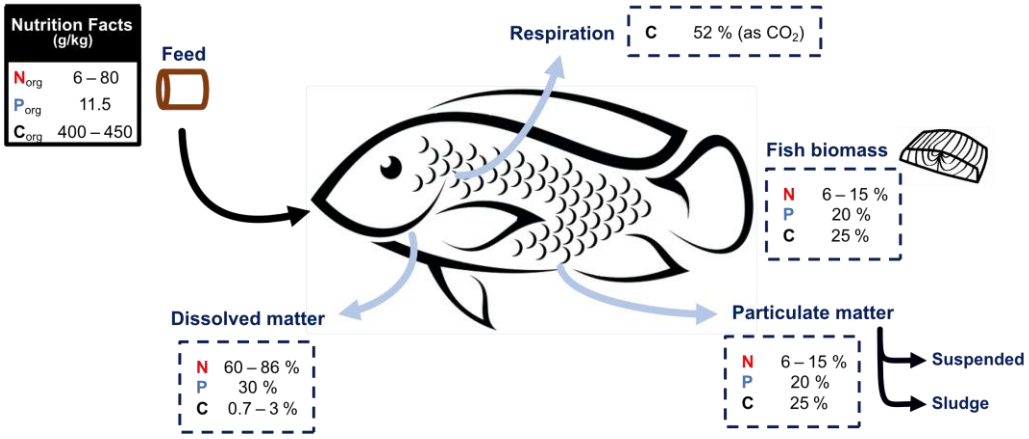

Figure 1-2 General mass balance of feed components on fish breeding processes

The figure presents a mass balance based on the average nitrogen (N), phosphorus (P) and carbon composition of fish feed. It details the distribution and fate of these nutrients within the system, expressed as a percentage of their total amount introduced through the feed. Data from **(D'Orbcastel and Blancheton 2006)**.

1.3.1 Wastewater composition

Effluents from fish farming primarily consist of dissolved and particulate solid wastes, resulting from fish metabolism and the decomposition of uneaten feed. These solid wastes, categorized into suspended and settled solids, pose significant risks as they can clog fish gills and increase the concentration of dissolved organic matter and nitrogen compounds when they break down (Akinwole, Dauda, and Olodae 2016). Efficient feed management can reduce waste generation, potentially lowering the feed-to-waste ratio to about 30% (Miller and Semmens 2002). This also benefits water quality by reducing the depletion of dissolved oxygen due to lower chemical and biochemical oxygen demand, achieved through good feed manufacturing practices (Timmons and Losordo 1994).

Dissolved wastes, mainly comprising phosphorus and nitrogen from the metabolic breakdown of proteins and other macromolecules, are excreted through fish gills and kidneys. These nutrients, existing as rich-phosphate particulates and ammonia, respectively, can harm aquatic life if released in high concentrations into water bodies (Ansah 2010; Stephens and Farris 2004). A summary of the main characteristics found in fish farming effluent is presented in Table 1-1.

Ammonia stands out as the second most hazardous pollutant in fish farming after solid wastes, posing a risk of acute toxicity that varies with fish species and growth stages (Levit 2010). It is recommended to keep ammonia

levels below 1 mg L^{-1} in fish farms to safeguard the fish, although effluents containing up to 5 mg L^{-1} are considered environmentally safe (Ajani, Akinwole, and Ayodele 2011; Boyd 2003). Ammonia can transform into nitrite, which is toxic, or into less harmful nitrate, tolerable by fish in concentrations up to 200 mg L^{-1} (Dauda and Akinwole 2015). However, both nitrates and phosphates are known to drive eutrophication when discharged into aquatic ecosystems.

Table 1-1 Physicochemical and biological characteristics of effluents resulting from farming of different species of fish

Reference	(Akinwole *et al.* 2016)	(Ansah 2010)		(Stephens and Farris 2004)	(Effendi, Wahyuningsih, and Wardiatno 2017)	(Schulz, Gelbrecht, and Rennert 2003)	(Dumas *et al.* 1998)	(Chin, Ong, and Foo 1993)			(Henry-Silva and Camargo 2006)
Fish species	Clarias	Oreo	Clarias	Clarias	Tilapia	Trout	Trout	Tilapia	Bass	Bass	Tilapia
Physical characteristics											
Temperature (ºC)	27.0	27.0	28.0	20.0	29.4	14.6	13.5	28.0	28.0	28.0	-
Conductivity (µS cm^{-1})	-	102.2	132	408	-	677	-	2100	4800	4240	-
Salinity (%)	-	0.15	0.06	-	-	-	-	-	-	-	-
TS (mg L^{-1})	-	-	79.6	-	-	-	-	1220	2670	2480	-
TDS (mg L^{-1})	2275	51.2	0.47	-	-	-	-	78	72	41	-
TSS (mg L^{-1})	1125	86.2	66.0	106	-	14.1	-	152	110	78	-
SS (mg L^{-1})	-	0.31	8.5	-	-	-	-	-	-	-	-
Chemical characteristics											
pH	7.2	7.2	7.1	8.1	8.15	7.75	7.0	7.6	7.6	7.3	-
DO (mg L^{-1})	4.90	1.9	1.5	5.7	5.73	6.23	-	-	-	-	-
COD (mg L^{-1})	-	-	-	-	-	41.0	21	110	70	95	-
TP (mg L^{-1})	-	-	-	-	-	0.35	-	-	-	-	0.08
PO_4-P (mg L^{-1})	-	0.60	0.64	5.37	-	0.12	0.06	0.8	1.3	1	0.05
TN (mg L^{-1})	-	1.96	2.99	-	-	2.40	-	-	-	-	0.67
NH_4-N (mg L^{-1})	3.0	-	-	0.73	3.57	0.61	0.5	2	3	1	0.10
NO_3-N (mg L^{-1})	5.0	-	-	0.06	0.95	0.70	2.2	1.5	3	1.5	0.48
NO_2-N (mg L^{-1})	0.25	-	-	0.03	0.03	-	-	1.2	-	-	0.09
Biological characteristics											
BOD_5 (mg L^{-1})	-	5.8	-	-	-	-	-	12.0	8.0	6.0	-
Coliforms (count 100 mL)	-	241	682	897	225	-	-	-	-	-	-
Streptoc. (count 100 mL)	-	2800	3032	-	-	-	-	-	-	-	-

TS: total solids, TDS: Total dissolved solids, TSS: Total suspended solids, SS: Settleable solids

Phosphorus, mainly present in effluents as orthophosphate (PO_4^{3-}), constitutes only 20% of the phosphorus from feed and poses no toxicity to fish. Yet, elevated phosphate levels in effluents can also lead to eutrophication. Phosphorus is typically excreted as faecal matter, but species such as Tilapias may release 60-62% of consumed phosphorus in a dissolved form, complicating the management of effluents (Piedrahita 2003).

In addition to nutrient waste, aquaculture systems also generate waste from chemicals and pathogens. Common chemicals used include medications, disinfectants, and antifoulants (Read and Fernandes 2003). Pathogens, on the other hand, are responsible for significant economic losses, estimated at USD 9.58 billion annually, due to diseases affecting aquaculture (Tavares-Dias and Martins 2017). The use of antibiotics, vaccines, and salts in disease management poses further environmental risks due to the proliferation of emerging contaminants (Boyd and McNevin 2015; Sapkota *et al.* 2008). Organic fertilizers, particularly those containing animal manure, introduce high levels of faecal coliforms into water bodies, exacerbating stress on aquatic organisms and increasing environmental pollution (Goldburg and Triplett 1997; Sapkota *et al.* 2008).

1.4 Treatment of fish farm effluents

A variety of physical, chemical, and biological methods are employed to treat effluents from aquaculture systems. These methods range from basic sedimentation to remove solids to more complex filtration techniques involving artificial water bodies, plants, algae, and molluscs. The latter are based on biotransformation processes such as the oxidation of organic matter, nitrification, denitrification, and phosphate uptake. These biotransformation techniques not only provide ecological and social benefits but also allow for the production of additional biomass without incurring extra input costs (Troell *et al.* 2005).

Three main methodologies are utilized to reduce the contaminant load and volume of effluents: sedimentation, biological waste exploitation through biofilters and artificial water bodies, and water reuse via recirculation systems.

1.4.1 Sedimentation

Sedimentation ponds are designed to retain water long enough for solids to settle. However, discharging large volumes of water from a fish pond at harvest time can make this method somewhat impractical. Its efficiency can be improved

if only the final portion of the effluent, which is more concentrated in nutrients and solids, is processed through these ponds, typically involving 5 – 20% of the pond volume (Green *et al.* 1996). A common practice is to allow the final 25% of the pond effluent to settle in sedimentation before discharge, provided suspended solids are below 30 mg L^{-1}.

The residence time in the sedimentation ponds is a critical factor that determines the size and number of ponds required. Studies have shown that while sedimentation effectively removes suspended solids, it is less effective at eliminating dissolved nitrogen and phosphorus, making it just one stage of the effluent treatment process (Teichert-Coddington *et al.* 1999).

1.4.2 Biofiltration

The effectiveness of biofiltration in reducing pollutants depends on the species used as the biological filter. Bivalve molluscs, for example, are highly efficient, capable of significantly reducing phytoplankton, nutrient levels, and suspended solids in the water column (Soto and Mena 1999).

Most research in this area has focused on saltwater molluscs, such as the mangrove sururu (*Mytella guyanensis*) and the native oyster (*Crassostrea Rhizophora*) used in the bioremediation of shrimp farm effluents in Brazil (Olivera and Brito 2005). Positioned in trays within the pond near the water outlet, these molluscs help reduce the load of phosphates, nitrites, and nitrates in the effluents discharged into the environment while also generating additional food products during the filtration process, thereby enhancing the quality of the farm's effluent.

1.4.3 Artificial water bodies

Artificial water bodies, also known as constructed wetlands, can be engineered or optimised naturally occurring environments like marshes. These systems typically utilize aquatic plants (macrophytes) rooted in substrates such as sand, gravel, or broken pebbles to promote the growth of diverse microorganisms. This setup facilitates the treatment of effluents through biological, chemical, and physical processes (Redding, Todd, and Midlen 1997).

Aquatic macrophytes are considered highly productive ecosystems, offering surfaces for epiphytes and nitrifying bacteria that aid in organic matter decomposition. The combination of floating and submerged macrophytes is

particularly effective for nitrogen removal, enhancing the potential for nutrient recycling in aquaculture integration (Brix and Schierup 1989).

Studies have shown that wetlands can remove substantial percentages of suspended solids, BOD, ammonium, and nitrate from prawn culture effluents (Lin *et al.* 2005). Extended water retention times in these systems have also been found to improve water quality for channel catfish production by retaining and assimilating nutrients (Posadas 2009).

1.4.4 Water recirculation systems

Recirculating Aquaculture Systems (RAS) enable increased production through efficient water use. These closed systems are gaining global popularity as they allow producers to control environmental conditions year-round, minimizing effluent release and water exchange. Water in RAS is reused after undergoing mechanical (e.g., pebbles, expanded clay) or biological (bacterial) treatment, offering a sustainable alternative by reducing water and energy use and nutrient emissions (Aich *et al.* 2020; Martins *et al.* 2010).

Despite requiring significantly less water than open systems, less than 100 littres per kilogram of product compared to several cubic meters, the complexity of closed systems brings challenges, such as high construction and operational costs and the need for skilled operators (Aich *et al.* 2020). High animal densities in tanks demand meticulous management, particularly in feeding practices and nitrite control. While RAS offers numerous advantages, managing ammonia and nitrite levels remains a critical challenge.

1.5 Use of duckweed for nutrient control and recovery

Despite their negative environmental impact, nutrients in aquaculture effluents are often discharged with minimal efforts toward effective control, recovery, and reuse. This is particularly true in rural areas, where less technologically advanced fish farming systems are common, and centralized water treatment facilities are scarce. However, the nutrients present in these effluents could be utilized to cultivate floating macrophytes like duckweeds, which thrive in constructed wetlands or water bodies with high concentrations of nutrients.

1.5.1 Duckweeds (or water lentils)

The *Lemna*ceae family, commonly known as duckweed, is recognized for its potential in water treatment and as a feed source in aquaculture, particularly in low-cost tropical systems. It is edible to various fish and crustaceans and offers a significant source of high-quality protein (El-Shafai *et al.* 2004; Hassan and Edwards 1992).

Duckweeds are small, simple monocot vascular plants that float on water surfaces and form dense, uniform layers in still water bodies like ponds, lakes, and lagoons (Figure 1-3) (Bonomo, Pastorelli, and Zambon 1997; Hillman and Culley 1978; Rusoff, Blakeney, and Culley 1980; Zuberer 1982). They belong to five genera, *Landoltia*, *Lemna*, *Spirodela*, *Wolffia*, and *Wolffiela*, encompassing 38 species (Les *et al.* 2002; Les and Crawford 1999). As the smallest flowering plants (Rothwell *et al.* 2004), duckweeds lack a true stem or leaves, consisting instead of one or more flat, ovoid fronds, typically under 12 mm in length. Some species may not develop roots, while others, like those in the *Lemna* and *Spirodela* genera, grow one or more roots depending on nutrient availability (Cole and Voskuil 1996; Hillman and Culley 1978; Lemon and Posluszny 2000).

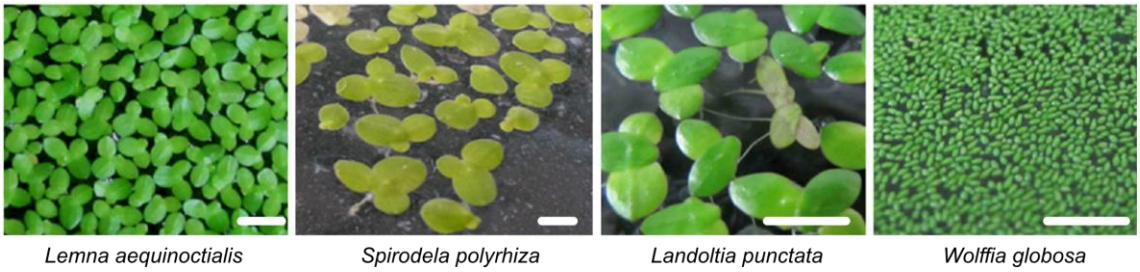

Figure 1-3 Pictures of some duckweed species found in nature

Each picture belongs to different duckweed genera. Scale bars represent 5 mm **(Xu *et al.* 2015)**

Duckweeds are notable for their rapid growth and efficient nutrient removal, outperforming other aquatic macrophytes (Oron *et al.* 1988). In ideal conditions (adequate nutrients, light, and water temperature) duckweeds can double their biomass every two to three days, quickly covering water surfaces, particularly in nutrient-rich conditions (Portielje and Roijackers 1995; Rusoff *et al.* 1980). They can achieve a protein content ranging from 15% to 45% dry weight by assimilating large amounts of nitrogen, both ammoniacal and nitrated forms, and phosphorus as orthophosphate (Caicedo *et al.* 2000; Cheng, Landesman, *et al.* 2002).

Duckweed species vary in their nitrogen preference, which is influenced by factors like pH, temperature, specific strain, and the forms of nitrogen present in the water (Y. Y. Fang *et al.* 2007). When used in sewage treatment, duckweed produces high-quality biomass, converting nitrogen to protein and carbon to starch, which are then useful as animal feed or agricultural fertilizer.

1.5.2 Duckweed cultivation

Like other photosynthetic organisms, duckweeds require a supply of macronutrients (carbon, nitrogen, and phosphorus) and trace nutrients for optimal growth. Carbon is vital for synthesizing organic matter such as starch and other carbohydrates during photosynthesis, which can then be utilised for energy or used to synthesise other C containing macromolecules. Phosphorus plays a crucial role in cellular structures as a component of phospholipids, DNA, and RNA, and it also functions as an energy carrier in ATP. Nitrogen is essential for the synthesis of amino acids (the building blocks of proteins), chlorophyll, and nucleic acids (DNA and RNA) (Maathuis 2009). These macronutrients are critical for plant tissue growth, making them key performance factors. Duckweeds are particularly effective for nutrient recovery in aquaculture systems due to their ability to assimilate both mineral (NO_3^-, NH_4^+, PO_4^{3-}) and organic forms of nutrients found in fish farming discharges.

In addition to nutrient concentration, factors such as temperature, pH, initial plant density, photoperiod, and light intensity significantly influence duckweed growth and nutrient uptake. The optimal ranges for these parameters for the most common duckweed species are summarized in Figure 1-4. Detailed information about the main environmental conditions tested in duckweed cultivation for wastewater treatment is synthesised and analysed in a meta-analysis study presented in Chapter 4 of this thesis.

Driever *et al.* (2005) observed that at low population densities, the Relative Growth Rate (RGR) of duckweeds increases. However, as density increases, RGR declines, possibly due to fronds stacking upon each other, restricting nutrient access for the upper layers and light or CO_2 for the lower ones, thereby impeding growth. Research on duckweed's productivity varies widely due to different experimental conditions, from lab settings to outdoor pilot projects, and uses various genera within the family. Despite this variability, relative growth rates are generally high, ranging from 0.10 to 0.35 day^{-1}, with annual yields of about 55 tons of dry weight per hectare, assuming two to three harvests weekly (Oron 1994). Multiple studies have reported optimal culture densities and significant dry

matter production rates for species such as S. *polyrhiza* and *L. minor* (Cheng, Bergmann, *et al.* 2002; Cheng, Landesman, *et al.* 2002; Hassan and Edwards 1992; Reddy and DeBusk 1987)[JPP[2]].

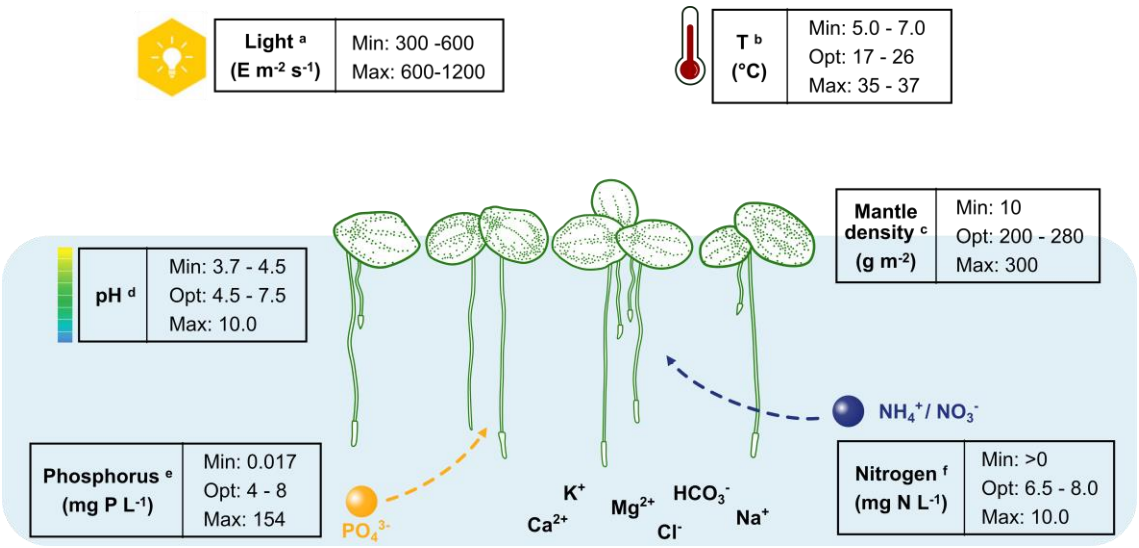

Figure 1-4 General growth conditions for optimal duckweed cultivation

Minimum (min), optimal (opt), and maximum (max) ranges for the cultivation of diverse duckweed species is presented. References: a **(Filbin and Anton 2010; Wedge and Burris 1982)**, b **(Buddhavarapu and Hancock 1991; Oron, Porath, and Jansen 1987; Skillicorn, Spira, and Journey 1993)**, c **(Anh and Preston 1997; Driever *et al.* 2005)**, d **(McLay 1976; Zirschky and Reed 1988a)**, e **(FAO 2009b)**, f **(FAO 2009b)**.

*Lemna*ceae have the ability to assimilate organic molecules like carbohydrates and amino acids directly from the environment through both the fronds and roots (Giovanelli, Mudd, and Datko 1985; Porath and Pollock 1982). Nutrient absorption primarily occurs from the underside of the frond as they float above the water (Ice and Couch 1987; Oron 1994). Contrary to this, some studies have shown that species like *L. minor* and *L. punctata* can absorb significant amounts of inorganic nitrogen through their roots (Cedergreen and Vindbadk 2002; Y. Y. Fang *et al.* 2007).

The preference for ammonium over nitrate as a nitrogen source within the *Lemna*ceae family is still debated. Generally, duckweed prefers ammonium due to the lower energy requirement for assimilation into amino acids and proteins (Oron 1994; Porath and Pollock 1982). However, ammonium assimilation is sensitive to both temperature and occurs optimally at pH values between 6 and 8 (Caicedo *et al.* 2000). When both ammonium and nitrate are present, duckweeds will prioritize ammonium but can utilize nitrate if it is the only available

source (Fang *et al.* 2007). When considering a wider pH range, some species show a preference for nitrate, particularly when it enhances the uptake of other macronutrients like phosphorus (Paterson 2017).

1.5.3 Nutritional value of duckweed biomass

Duckweeds have a high nutritional value because the entire plant consists of metabolically active non-structural tissue (Wolverton and Mcdonald 1981). The main constituents of duckweed biomass are carbohydrates (mainly in the form of starch), proteins and fibre (Figure 1-5).

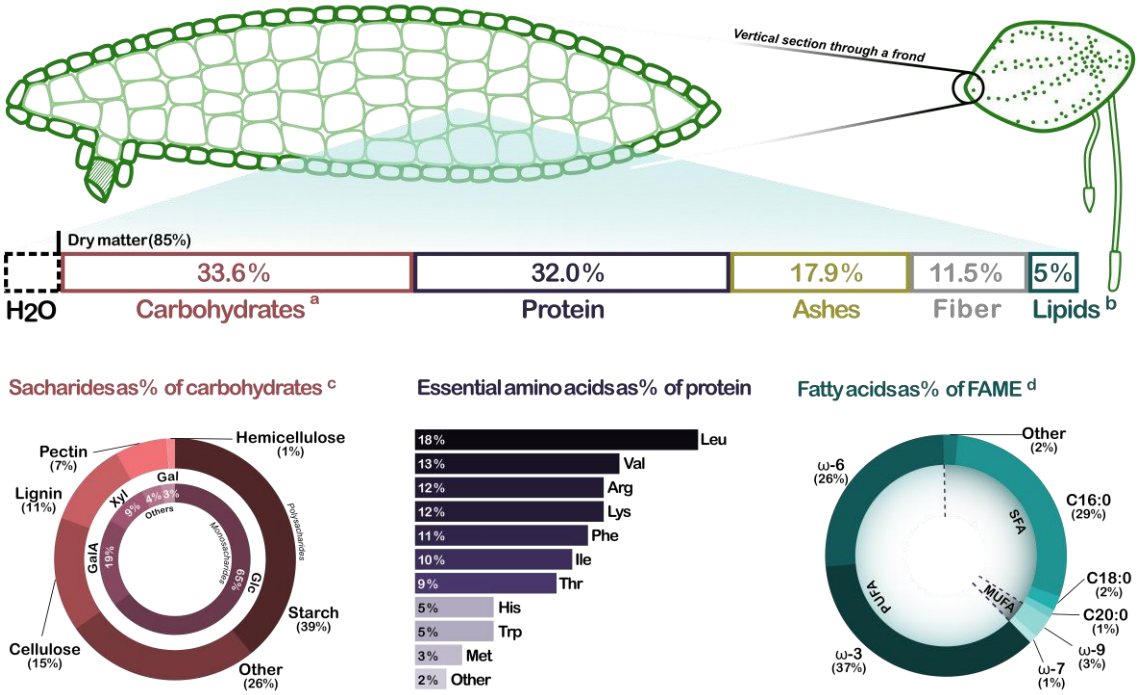

Figure 1-5 Average chemical composition of duckweed biomass.

Content of principal saccharides, amino acids and FAME is highlighted. Notes: [a] Carbohydrates measured as Nitrogen Free Extract (NFE), [b] Lipids quantified as Ether Extract (EE), [c] FAME = Fatty Acid Methyl Ester, PUFA = Poly unsaturated fatty acids, SFA = Saturated Fatty acids, MUFA = Monounsaturated fatty acids. References: Duckweed composition **(Appenroth *et al.* 2018; Leng, Stambolie, and Bell 2005; Tavares *et al.* 2008)**, amino acids **(Appenroth *et al.* 2018)**, saccharides **(Pagliuso *et al.* 2018)**, and FAME **(Appenroth *et al.* 2018)**.

Proteins in duckweed can range from 13% to as high as 41% of dry weight, depending on the species and environmental conditions such as water quality and available nutrients. This variability is influenced by factors like nitrogen concentration in the water, retention time, and the rate of organic matter degradation (Caicedo *et al.* 2000). For instance, studies have shown that under nutrient-rich conditions, *Lemna* species can achieve protein levels comparable to

those of soybeans, making them a highly promising source of protein for both human consumption and animal feed (Cheng, Landesman, *et al.* 2002). It offers an amino acid profile superior to that of other plant proteins, closely resembling animal protein, making it an excellent source of arginine, a crucial amino acid for fish farming (Hillman and Culley 1978).

Carbohydrates and starch are other significant components of duckweed, contributing to its nutritional and energy value. Duckweeds are rich in carbohydrates, primarily in the form of starch, which typically constitutes about 30% to 40% of the dry weight (Appenroth, Ziegler, and Sree 2021). This high starch content, especially in species like *Lemna* and *Wolffia*, is beneficial for use in bioethanol production as well as a feed ingredient in aquaculture and livestock diets (Cheng and Stomp 2009). The ability of duckweed to efficiently convert atmospheric CO_2 into biomass rich in carbohydrates makes it an excellent candidate for carbon sequestration and sustainable biomass production.

Fibre content in duckweed also plays a crucial role in its application as animal feed. Generally, fibre percentages range between 5% and 15% of the dry weight, with variations depending on the specific duckweed species and growing conditions (Appenroth *et al.* 2017, 2018; Yahaya *et al.* 2022). For example, the genus *Spirodela* tends to have higher fibre content, which can affect the digestibility when used as feed (Hassan and Edwards 1992). Despite this, the overall low to moderate fibre content helps to maintain a balanced diet for livestock and aquaculture, providing essential roughage and aiding in digestive health (Davies 1985). This composition makes duckweed a versatile component in feed formulations, supporting gut health while supplying a range of necessary nutrients.

Despite some deficiencies in histidine and methionine, duckweed serves as an excellent source of essential amino acids in complete grain diets, complementing the amino acid profiles of other protein sources. However, duckweed also contains anti-nutritional factors such as tannins and phytic acid, which can interfere with nutrient utilization and affect animal health (Goopy and Murray 2003). For instance, fresh *Spirodela polyrhiza* contains 1% tannins and 1.23% phytic acid, though these levels can be reduced significantly through Bacillus fermentation (Bairagi *et al.* 2002). Other studies report the presence of trypsin inhibitors, calcium oxalate, and cyanide in different species, highlighting the variability in toxin levels, which can often be mitigated through heat treatment (Kalita, Mukhopadhyay, and Mukherjee 2007).

1.5.4 Use of duckweed for wastewater treatment

Worldwide, natural wastewater treatment systems are becoming popular, due to the low cost of construction, which is related to the need for large areas, in addition to its easy handling and operation. Stabilization ponds based on macrophytes, principally duckweeds and water hyacinths, are implemented the most for secondary and tertiary wastewater treatment in wide areas where land is not an impossibility for its integration (Bal Krishna and Polprasert 2008; Teles *et al.* 2017).

*Lemna*ceae lagoons or ponds are polishing ponds, or tertiary treatment, which play a very important role in the removal of nutrients by various mechanisms (Buddhavarapu and Hancock 1991; Van Der Steen, Brenner, and Oron 1998). Duckweeds form an important substrate for the aerobic layer in stabilization ponds, which is essential for nitrification and oxidation of organic matter (Van Der Steen *et al.* 1998). The high growth rate (the highest among angiosperms) is another important characteristic of duckweed that, together with its high nitrogen and phosphorus demand, results in exemplary removal and accumulation of nutrients; in addition, the ease of harvesting ends in low operational costs and gives it a good economic resource potential (Gijzen and Khondker 1997; Skillicorn *et al.* 1993).

Due to the physical barrier that forms on the surface of the pond, duckweeds reduce the action of wind, allowing a better sedimentation of solids, preventing the reproduction of insects and blocking the passage of light though the water column, thus preventing the growth of algae (Vieira 2013). Aquatic systems with floating macrophytes significantly reduce the passage of sunlight and restrict the transfer of gases between the atmosphere and water (Van Der Steen *et al.* 1998). Duckweed ponds used in the treatment of domestic effluents perform the function of converting nutrients into rich biomass, which acquires great importance, since this technology addresses the treatment of effluents and the generation of aggregate value through the valorisation of biomass as source for energy or food production (Skillicorn *et al.* 1993).

For the implementation of duckweed pond systems, factors such as environmental temperature, effluent pH, concentration of nutrients and their bioavailability, salinity, toxicity, and climatic conditions must be evaluated (Mohedano *et al.* 2012). The best species for polishing effluents are *Landoltia punctata*, *Lemna gibba* and *Lemna minor*, but the adaptation of the species to the environment and to the composition of the effluent to which they will be exposed must also be assessed (Bergmann *et al.* 2000). The economic potential that each species of macrophyte possesses for the treatment of liquid effluents

depends to a great extent on its growth rate and efficiency in the elimination of nutrients in different environmental conditions, as well as the ease and cost of maintenance of the treatment system, and subsequent application for added value products (Brix and Schierup 1989; Redding *et al.* 1997).

Undoubtedly *Lemna*ceae are a fundamental component in aquatic systems and provide numerous benefits when used for water treatment, although they also cause some environmental problems that should be addressed properly to guarantee the sustainability of the technology (Table 1-2).

Table 1-2 Advantages and challenges associated to the use of duckweed for wastewater treatment

Advantages	Challenges
• Duckweed aerates the root system and provide oxygen to the microorganisms that live in the rhizosphere. • Atmospheric CO_2 fixation. • Nutrient absorption and recycling (nitrogen and phosphorus). • Filtration of solids through the root system. • Sedimentation of solid particles in suspension is facilitated. • Elimination of other pollutants assimilating them directly into the plant tissue. • Potential for nutrient recovery by harvesting and use of biomass as source for animal feed or fertilizer production. • Low operational cost and maintenance of ponds. Low energy requirements. • Low proliferation of insects and microalgae. • Odour release and water evaporation is minimized.	• Relatively high area requirement. • Lack of oxygen in the water column at large mantle densities. • Treatment system blockages due to small size of plants. • Occurrence of pathogenic organisms in the rhizosphere. • Variability in the performance of the treatment system under temperate climate conditions.

In the last decade, several research works have highlighted the potential of duckweeds for diverse wastewater remediation, as well as the environmental and economic benefits that can obtained from them (Cheng, Landesman, *et al.* 2002; Mohedano *et al.* 2012; Nahar and Sunny 2020; Rusoff *et al.* 1980; Stadtlander *et al.* 2019; Su *et al.* 2014; Toyama *et al.* 2018). All agree that the

implementation of these plants, for nitrogen and phosphorus uptake, generates enriched biomass giving opportunities for nutrients recycling while quality of effluents is improved prior final discharged. Interestingly, previous researchers at the University of Leeds have demonstrated that it is possible to uncouple growth from phosphorus uptake when duckweed is grown under temperate climate conditions (Paterson, Camargo-Valero, and Baker 2020). This suggests that, even if there is not active growth of the plant, it is possible to recover nutrients at low temperatures and short photoperiods. Therefore, duckweed-based technologies can be constantly applied for the treatment of wastewater and the uptake and recycling of nutrients in big urban and small rural communities.

1.5.5 Nutrient recovery by duckweed

To effectively recover nutrients from fish farming effluents using duckweeds, it's crucial to recognize that not all duckweeds are equally capable of nutrient recovery and valuable biomass production. The efficiency of these processes depends on the specific strain of duckweed used and the cultivation conditions (Bergmann *et al.* 2000; Cheng and Stomp 2009). Therefore, selecting the right strain is critical and should consider factors such as native species in the field, environmental conditions, nutrient sources, and the intended use of the biomass. In tropical environments, chosen duckweed strains must tolerate mild temperatures year-round, seasonal variations, local pests, and high humidity (Sengupta, Medda, and Dewanji 2010).

Nitrogen and phosphorus removal rates by duckweeds, particularly for species from the *Lemna* and *Spirodela* genera, vary widely (Table 1-3). Cheng *et al.* (2002b) reported N removal rates of 2.11 g m^{-2} d^{-1} and P removal rates of 0.59 g m^{-2} d^{-1} using *L. minor*. Reddy and De Busk (1987) noted annual N and P consumption rates ranging from 350 - 1200 kg ha^{-1} and 116 - 400 kg ha^{-1}, respectively. As nutrient levels decrease, the roots of duckweed lengthen and start absorbing other substances in the medium, potentially leading to sediment accumulation of toxins and heavy metals (Clark *et al.* 1981).

Duckweed indirectly contributes to nutrient removal through the nitrification-denitrification processes carried out by bacteria and algae within the plant mantle, accounting for 35-46% of N loss and 31-71% of P loss. Direct removal by duckweed itself ranges from 16-47% for N and 9-61% for P (Alaerts, Rahman Mahbubar, and Kelderman 1996; Körner and Vermaat 1998). Islam *et al.* (2004) found that duckweed could significantly reduce faecal coliforms in

sewage, suggesting that biomass from these ponds could safely be used as fish feed, minimizing the risk of enteric pathogens.

Table 1-3 Nitrogen and phosphorus removal by diverse duckweed species

Region	Species	Daily removal (g m^{-2} d^{-1})		Reference
		N	P	
Louisiana	Duckweed	0.47	0.16	(Hillman and Culley 1978)
USA	*Lemna* sp	1.67	0.22	(Zirschky and Reed 1988b)
Bangladesh	*Spirodela polyrhiza*	0.26	0.05	(Alaerts *et al.* 1996)
China	*Spirodela polyrhiza*	0.36 – 0.69	0.037	(Sun *et al.* 2020)
Pakistan	*Lemna minor*	0.11 – 0.16	0.09 – 0.11	(Iqbal, Javed, and Baig 2019)
UK	*Lemna minor*	-	0.02 – 1.83	(Paterson *et al.* 2020)
UK	*Lemna minor*	0.11 – 0.45	0.04 – 0.15	(Van Echelpoel, Boets, and Goethals 2016)
Belgium	*Lemna* minuta	0.16 – 0.34	0.04 – 0.20	(Van Echelpoel *et al.* 2016)
Ireland	*Lemna minor*	0.18 – 0.74	0.03 – 0.12	(Walsh, Kuehnhold, *et al.* 2021)
Malaysia	*Lemna minor*	-	0.07 -0.09	(Nesan, Selvabala, and Chan Juinn Chieh 2020)
Brazil	*Landoltia punctata*	0.26 – 0.73	0.06 – 0.22	(Garcia, Albertin, and Matsumoto 2017)
Yamen	*Lemna minor*	0.05 – 0.2	0.01 – 0.05	(Al-Nozaily and Alaerts 2002)
China	*Spirodela polyrhiza*	0.49 – 0.60	0.06	(Yin *et al.* 2015)
China	*Spirodela polyrhiza*	0.10 – 0.59	0.03 – 0.31	(Wang *et al.* 2015)

Additionally, duckweeds are known to accumulate heavy metals such as cadmium, selenium, lead, zinc, nickel, boron, silver, chromium, and copper (Davis, Shokouhian, and Ni 2001; Glandon and Mcnabb 1978; Srivastava and Appenroth 1995; Staves and Knaus 1985; Wahaab, Lubberding, and Alaerts 1995; Zayed, Gowthaman, and Terry 1998). Research into their growth rates and nutrient absorption capabilities has been pivotal in developing duckweed as a feasible solution for treating wastewater containing these metals.

Ammonia, a major component of wastewater, typically ranges from 10 to 50 mg of ammoniacal nitrogen per litre but can reach as high as 200 mg L^{-1} in industrial and fish farming effluents (Korner, Vermaat, and Veenstra 2003). Duckweed thrives in environments with high nutrient levels, such as those found in domestic sewage and livestock farm runoff. It supports a microbial community including diazotrophic cyanobacteria and heterotrophic bacteria that can fix nitrogen in both dark and light conditions, providing at least 15-20% of the nitrogen duckweed needs for growth (Zuberer 1982).

In terms of carbon sequestration, duckweeds not only fix atmospheric carbon via photosynthesis but also utilize dissolved CO_2 in water. The carbon flux in duckweed ponds involves CO_2 emissions from organic matter decomposition and CO_2 fixation through photosynthesis, with the latter process being nearly three times more significant (Costa *et al.* 2016). This demonstrates that duckweeds not only remove phosphorus and nitrogen from wastewater efficiently but also act as a valuable carbon sink, proving to be more effective and easier to harvest than other vascular plants and microalgae (Dai *et al.* 2015).

1.6 Duckweed ponds and aquaculture

Duckweed is increasingly recognized as an effective natural solution for the recapture of carbon, nitrogen, and phosphorus from aquaculture effluents. This plant's ability to thrive under various nutrient loads are the base for developing engineered solutions tailored for decentralised wastewater treatment. Studying how duckweed respond to different nutrient levels will help building effective and eco-friendly wastewater management systems.. Additionally, by precisely defining design parameters for scaled-up production systems, there is potential to enhance the economic efficiency of these systems through the recycling of nutrients. This process involves harvesting nutrient-enriched biomass, thereby completing the production cycle and contributing to more sustainable practices.

Figure 1-6 illustrates the comprehensive steps and processes involved in using duckweed for nutrient control and recovery in fish farms. It also delineates the scope of the current project, which focuses on the study of environmental and operational parameters that affect duckweed’s efficiency for biomass production and nutrient recovery. This research is aimed at supporting the design, construction, and initial setup of a pilot-scale treatment system for the treating effluents from fish farms. The ultimate goal is to develop a system that not only

mitigates environmental impact but also enhances the quality and safety of the biomass produced.

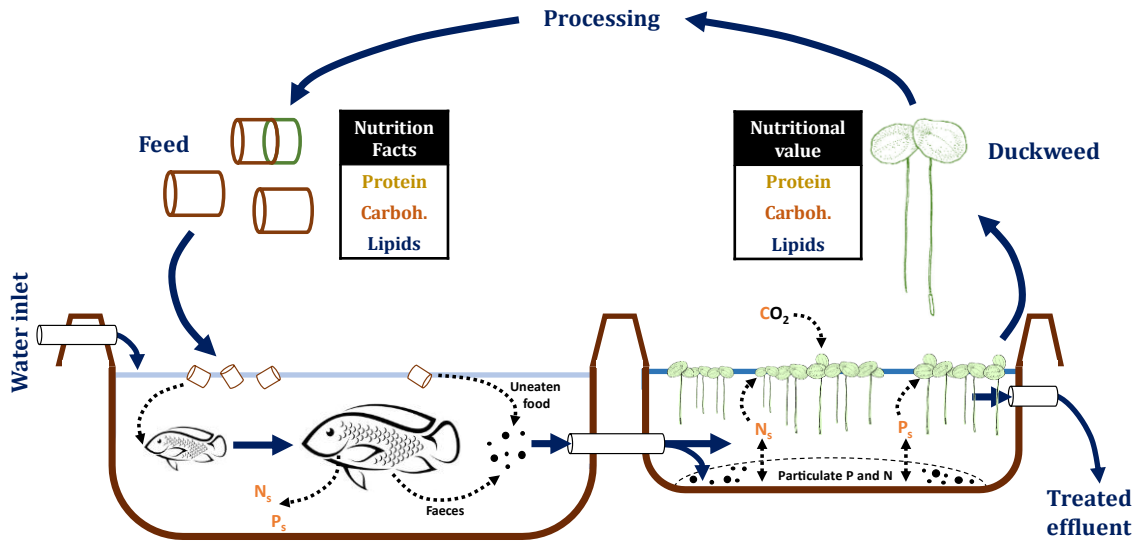

Figure 1-6 Aquaculture production system through nutrient recycling using duckweed ponds

Ultimately, employing duckweed in aquaculture production systems can lead to the generation of affordable, healthier, and safer animal protein sources. By integrating duckweed-based remediation methods, these systems can become more sustainable, supporting environmentally and socially responsible fish production. This approach aligns with global efforts to develop aquaculture practices that are both economically viable and ecologically sound, with an emphasis on tropical climate regions, ensuring long-term sustainability and food security.

1.7 Research Question and Hypothesis

1.7.1 Research question

Based on the existing knowledge of biological processes for nutrient recovery from wastewater and reviews on using duckweed in remediation and feeding in aquaculture, this research seeks to explore how sufficient nutritionally valuable duckweed biomass can be obtained from the adequate treatment of aquaculture effluents in tropical climate regions. The study aims to determine how

environmental changes in duckweed cultivation affect its growth and nutrient absorption. It also seeks to define the design and operational conditions necessary for effective nutrient recovery in a continuous flow system and to assess the feasibility of using duckweed-based technology for treating water and recovering nutrients from fish farming effluents under real tropical conditions.

To address the research question, it is crucial to first examine how environmental changes affect the plants' ability to grow and absorb nutrients from water, to then determine the necessary operational conditions for effective nutrient recovery in a continuous flow treatment system. Finally, it is important to assess whether duckweed-based technology for water treatment and nutrient recovery can be effectively integrated with aquaculture systems in real-world tropical climates scenarios.

1.7.2 Hypothesis

The hypothesis driving this project is that carefully designed duckweed ponds can effectively remove nitrogen and phosphorus from fish farming effluents while producing high-quality biomass for nutrient reuse. Specifically, it is anticipated that under controlled tropical conditions and at a mesocosm scale, managing nutrient levels, temperature, and stock density will enable modelling of duckweed treatment systems to efficiently remove nutrients and support the growth of nutritionally valuable biomass. Additionally, it is hypothesized that duckweed will thrive on fish farming effluent in ponds designed based on outputs from small-scale studies and modelling of nutrient uptake by plants, making this integrated system both feasible and sustainable.

1.8 Project Aims and objectives

1.8.1 Aim

The primary aim of this project is to determine experimentally how environmental and operational parameters affect the performance of a duckweed-based system for effluent treatment and the production of enriched biomass in fish farms. The ultimate goal is to develop sustainable aquaculture production systems that provide affordable, healthy, and safe animal protein sources, supporting socially and environmentally responsible fish production

through an integrated wastewater and nutrient recovery system based on duckweed.

In order to achieve practical results, the research will involve: (a) laboratory-based activities to assess the nutritional value of duckweed biomass upon modification of culture conditions and treatment systems configurations, (b) modelling of continuous flow treatment systems based on duckweed to inform the design and operation of large scale pilot systems, and (c) field work activities in Indonesia, to test a pilot scale wastewater treatment and nutrient recovery systems using native duckweed species

1.8.2 Objectives

The specific research objectives include:

- Estimating the impact of environmental conditions on duckweed's potential for maximum nutrient uptake and the production of nutrient-rich biomass under simulated tropical conditions using synthetic growth media.
- Evaluating the influence of operational parameters in continuous flow systems on nutrient control and recovery, by means of in silico simulations, for the design and operation of scaled-up treatment systems using duckweed.
- Assessing the application and integration of pilot-scale systems based on duckweed for treating real fish farm effluents

1.9 Thesis structure

The thesis comprises a total of eight chapters. The first chapter serves as an introduction to the thesis topic, summarising the current state of the art regarding the need for effluent treatment in aquaculture and the use of duckweeds for wastewater treatment and nutrient removal. Chapter two details the materials and methodologies employed for the experimental and computational work conducted in this project. Chapter three expands on the literature review concerning the impact of abiotic environmental conditions on duckweed growth through a meta-analysis study.

Chapter four presents preliminary experimental considerations that must be taken into account when using duckweeds for wastewater treatment. Chapter five reports the results obtained at lab scale during the kinetic study in batch culture systems of duckweed, as well as the development of the corresponding

mathematical model. Chapter six expands the mathematical model to continuous treatment systems and presents the results of simulations for different operational conditions of these systems. Chapter seven includes testing of various configurations for cultivating duckweed in continuous systems as well as the development and application of a duckweed-based pilot system at a fish farm in Indonesia. Finally, Chapter eight wraps up the thesis with a general discussion that aims to consolidate and summarize the key findings from Chapters 3 to 7, evaluating how the project's objectives were achieved and suggesting directions for future research.

Chapter 2 : Materials and methods

2.1 Summary

The research conducted in this thesis was organized into five work packages (WP 1-5), each focusing on different aspects of duckweed growth and application.

WP1 involved a systematic review of the main environmental factors influencing duckweed growth. Various studies were analysed using a meta-analysis approach to identify the optimal growing conditions for different duckweed species. The findings from this WP are detailed in Chapter 3.

Reported in Chapter 4, WP2 focused on screening different duckweed species under controlled culture conditions. The goal was to identify the species with the best performance in terms of nutrient uptake and biomass growth. This work package also explored preliminary cultural considerations necessary for efficient duckweed cultivation.

WP3 comprised controlled, small-scale laboratory experiments that manipulated temperature and nutrient availability to study their effects on growth and nutrient uptake by *Wolffia angusta*. The results from these experiments were used to develop, validate, and calibrate a kinetic model for batch cultivation of duckweeds, as discussed in Chapter 5.

In WP4, the mathematical model was expanded to include operational parameters for a continuous flow treatment system utilizing duckweed. This package involved performing experiments to validate the model and conducting in silico simulations to establish optimal operational conditions. Design criteria of pilot scale treatment systems were obtained from the analysis of the results. The comprehensive details of these activities are presented in Chapter 6.

Finally, WP5, described in Chapter 7, included testing under simulated conditions of a continuous flow treatment system and evaluating different system configurations. Additionally, a trial of a large-scale pilot system was conducted in a real environment at a fish farm in Indonesia. This work package aimed to apply the research in a practical setting and assess the feasibility and effectiveness of the developed systems.

Each work package required specific experimental and analytical methods for proper execution, which are thoroughly detailed in this chapter.

2.2 Duckweed strains and stock maintenance of stock cultures

Duckweed strains used in the preliminary experiments (Chapter 4) were outsourced from three different places. One strain was collected and isolated from a water beck in Leeds, UK. Upon molecular identification, it was stablished that the duckweed species was *Lemna minuta*. Two more isolates were obtained from the Landolt Duckweed Culture Collection, ETH Zurich (http://www.duckweed.ch), corresponding to clones of *Wolffia angusta* (strain 8878), and *Wolffia globosa* (strain 8356). Finally, 3 more duckweed isolates were purchased to the Rutgers Duckweed Stock Cooperative, University of Jena (http://www.ruduckweed.org/). In this case, all were clones of *Landoltia punctata* (strain 9348, 9939, 0011), originally isolated from tropical climate countries.

Stocks cultures of all strains were maintained in sterile conditions in plastic containers containing Hoagland's growth solution, in a Sanyo MLR-351H growth chamber at constant temperature 20˚C, with an 8hr light cycle, and light intensity of 100umol m^{-2} s^{-1}. The growth solution was replaced every two weeks, and every month a dead plants on the surface and bottom of the plastic container were removed.

In the case of the duckweed species collected in the UK, pure cultures of *Lemna* minuta were prepared using sterile methods. The roots of the duckweed were removed, and the fronds were submerged in a solution of 10% bleach mixed with water for two minutes. This was done to eliminate any bacteria or microalgae on the plants. After the bleach treatment, individual fronds were placed in 100 mL of sterile Hoagland's nutrient solution inside clean plastic containers. To maintain health and axenic conditions, fronds showing no signs of algae or contamination were transferred every two days to new containers filled with fresh nutrient solution. This process was repeated until a sufficient number of clean fronds were grown, showing no visible signs of algae contamination.

2.3 Molecular identification of duckweed species

The identification of duckweed species collected in the UK was performed using two methods. Firstly, a dichotomous guide, as described by Bog *et al.* (2020), was used to classify the species based on their morphological features. Secondly, molecular identification techniques were applied to further verify the

species. This involved DNA extraction and PCR techniques to amplify and analyse the atpF-atpH non-coding region of the chloroplast DNA, a method previously outlined by Wang et al (2010).

2.3.1 DNA extraction

To extract DNA from duckweed samples, the following detailed procedure was employed:

Firstly, the duckweed samples were thoroughly rinsed in deionized water (dH_2O) and dried using paper towels. 0.2 gram fresh weight was placed in 2 mL Eppendorf tubes that contained steel ball bearings. These samples were then quickly frozen in liquid nitrogen and homogenized for 60 seconds using a tissue lyser (Qiagen, UK). After homogenization, 800 µL of extraction buffer was added to each tube, and the samples were homogenized again for an additional two minutes.

Following the second homogenization, the tubes were incubated at 60°C for 30 minutes and subsequently mixed thoroughly using a vortex. Then, 500 µL of a 24:1 mixture of chloroform:isoamyl alcohol solution was added. The tubes were centrifuged at 13,000 g for two minutes, and the clear upper portion was carefully transferred to new 1 mL Eppendorf tubes. The residue in the original tubes was centrifuged again under the same conditions to extract more supernatant.

The combined supernatants were then precipitated by adding two-thirds of their volume in isopropanol, up to 400 µL, and centrifuged at 13,000 g for 30 minutes. After removing the supernatant, the DNA pellet was washed with 200 µL of 70% ethanol and gently mixed, followed by another round of centrifugation for two minutes at 13,000 g. This step was repeated to ensure thorough washing. After discarding the final wash, the DNA pellet was left to air dry briefly to evaporate any remaining ethanol. It was then re-suspended in 50 µL dH_2O and 0.5 µL RNase. The concentration of the extracted DNA was then measured using a Nanodrop spectrophotometer, setting a blank with dH_2O and RNase.

The DNA extraction buffer used in this process was prepared by dissolving 2 g of Cetyl methylammonium bromide (CTAB) in 30 mL dH_2O with a magnetic stirrer in a 100 mL volumetric flask. To this solution, 28 mL of 5M NaCl, 4 mL of 0.5 M EDTA pH 8, and 10 mL of 1 M Tris HCl were added sequentially. After all components were fully dissolved, the volume was adjusted to 100 mL with dH_2O.

Then, 10 mL of this buffer was transferred to a falcon tube, and 100 µL of 2-mercaptoethanol was added before inverting the tube to mix.

2.3.2 PCR and product purification

To identify specific regions within the chloroplast genome of duckweed, primers designed for the atpF-atpH non-coding spacer region (ranging from 579-622 bp) were used, following the protocol recommended by Wang *et al.* (2010). The polymerase chain reaction (PCR) was performed using a thermal cycler. The process began with an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing at 50°C for 15 seconds, and extension at 72°C for 40 seconds, concluding with a final extension at 72°C for 5 minutes.

The PCR reaction mixture included the following components:

- 17.75 µL of autoclaved deionized water (dH_2O)
- 1 µL of DNA
- 1 µL each of forward and reverse primers, at a concentration of 5 pmol
- 0.5 µL of dNTP mix at 10 mM
- 2.5 µL of Red Taq PCR buffer
- 1.25 µL of Red Taq DNA polymerase

The specific primer sequences used were:

- Forward: 5'-ACTCGCACACACTCCCTTTCC-3'
- Reverse: 5'-GCTTTTATGGAAGCTTTAACAAT-3'

To verify the correct amplification of the target DNA sequence, 5 µL of the PCR product was run on a 1.2% agarose gel using 1x TAE buffer at 100 volts for approximately 40 minutes. A molecular ruler with known sizes was used as a reference.

After gel electrophoresis, the PCR products were diluted and purified using ExoSap-IT (USB Corporation) in preparation for sequencing. The samples were then sent to Genewiz ®, UK, for sequencing. Upon receipt of the sequenced data, consensus sequences were generated using Clustal Omega software. These

sequences were further analysed for matches with other species using the BLAST database program.

2.4 Growth solutions preparation

All the experiments were conducted using Hoagland's growth solution, prepared from six autoclaved stock solutions (Paterson *et al.* 2020). The specific concentrations of nutrients in each solution and the volumes required to prepare 1 litter of media are provided in Table 2-1.

For all experiments, unless specified otherwise, the pH of the culture media was adjusted to 7 using 1M NaOH, without the use of a buffer.

In experiments that involved varying phosphorus levels, the volume of the KH_2PO_4 stock solution was adjusted to achieve the desired phosphorus concentration. Any resultant potassium deficit was compensated by adding 1M KCl to maintain nutrient balance.

Table 2-1 Hoagland's media components and preparation

Reagent	Stock solution	Use (mL stock / L media)	Final concentration in media	
			mg L^{-1}	µM
$Ca(NO_3)_2 * 4H_2O$	23.6 g / 100 mL	2.3	543	2299
$MgSO_4 * 7H_2O$	24.6 g / 100 mL	1.0	246	998
KH_2PO_4	13.6 g / 100 mL	0.5	68.0	500
KNO_3	10.1 g / 100 mL	2.5	253	2498
Micronutrients		0.5		
H_3BO_3	2.86 g/L		1.430	23.1
$NaMoO_4 * 2H_2O$	0.09 g/L		0.045	0.19
$MnSO_4 * 5H_2O$ *	2.22 g/L		1.110	4.60
* If MnCl2 * 4 H_2O	1.82 g/L		0.910	
$ZnSO_4 * 7\ H_2O$	0.22 g/L		0.110	0.13
$CuSO_4 * 5\ H_2O$	0.09 g/L		0.045	0.18
EDTA-Fe solution		20		
FeCl3 * 6 H2O	0.121 g / 250 mL		9.68	35.8
EDTA	0.375 g / 250 mL		30.0	103

Similarly, when adjusting the total nitrogen concentration in the media, the volumes of the $Ca(NO_3)_2$ and KNO_3 stock solutions were varied proportionally. Any imbalances in potassium and calcium levels due to these adjustments were corrected by adding 1M KCl and 1M $CaCl_2$, respectively.

2.5 Analysis of water samples

Throughout the project, water samples were taken from cultivation tanks to measure the levels of phosphorus and nitrogen species. All samples were filtered through a 0.45 µm pore size membrane (Fisher Scientific, UK) prior analysis. If immediate analysis was not feasible the samples were stored in a freezer at -5°C.

2.5.1 Phosphorus analysis in solution

The concentration of orthophosphate in the solutions was determined using two methods:

Method 1: Vanadate-Molybdate Method

This method was routinely used in the lab to quantify both total phosphorus and orthophosphate in water (Holman 1943). The procedure involves the formation of a blue-coloured antimony-phospho-molybdate complex in an acidic environment, indicating the presence of inorganic phosphorus (P_i). The intensity of the blue colour is proportional to the P_i concentration. The procedure required the preparation of a combined reagent including:

- Sulfuric Acid (2.5 M): Add 70 mL of concentrated H_2SO_4 to 400 mL of deionized water (dH_2O), cool down, and dilute to 500 m*L.*
- Ammonium Molybdate Solution: Dissolve 20 g of $(NH_4)_6Mo_7O_{24}•4H_2O$ in 500 mL of dH_2O and store at 4°C.
- Ascorbic Acid (0.1 M): Dissolve 1.76 g of ascorbic acid in 100 mL of dH_2O. This solution is stable for one week if stored in an opaque bottle at 4°C.
- Potassium Antimonyl Tartrate Solution: Dissolve 1.3715 g of $K(SbO)C_4H_4O_4•0.5\ H_2O$ in 400 mL of dH_2O, make up to 500 mL, and store in an opaque bottle.

To prepare the combined reagent, the following components were mixed at room temperature: 50 mL of sulfuric acid solution, 15 mL of ammonium molybdate solution, 30 mL of ascorbic acid solution, and 5 mL of potassium antimonyl tartrate solution. Each component was added sequentially and thoroughly mixed. Once prepared, this reagent remained stable for up to 8 hours when stored in an opaque bottle.

For the analysis, 2 mL of a filtered water sample was added to phosphate-free test tubes, followed by 0.32 mL of the combined reagent. The contents were mixed and allowed to stand at room temperature for 15 minutes. The absorbance of the solution was then measured at 880 nm, using a combined reagent and deionized water blank for calibration. The absorbance results were compared against a previously prepared calibration curve that utilized phosphate standard solutions of known concentrations.

To prepare the combined reagent, at room temperature, 50 mL of the sulfuric acid solution, 15 mL of the ammonium molybdate solution, 30 mL of the ascorbic acid solution, and 5 mL of the potassium antimonyl tartrate solution were well mixed one by one. This reagent remains stable for up to 8 hours when stored in an opaque bottle.

For the analysis, add 2 mL of the filtered water sample into phosphate-free test tubes, followed by 0.32 mL of the combined reagent. Mix the contents and let them stand at room temperature for 15 minutes. Measure the absorbance of the solution at 880 nm, using a reagent and deionized water blank for calibration. The absorbance results are compared against a previously prepared calibration curve that uses phosphate standard solutions of known concentrations. This method is capable of quantifying phosphorus concentrations ranging from 0.1 to 1 mg PO_4-P L^{-1}.

Method 2: Phosphate Cuvette Test Kits (HACH LANGE, UK, LCK349)

This method was employed for cases requiring higher sensitivity or to double-check results obtained from the first method. The HACH test kits allowed rapid quantification of phosphorus within a range of 0.05-1.5 mg PO_4-P L^{-1}.

2.5.2 Nitrogen analysis in solution

For nitrogen analysis, water samples were collected and also passed through a 0.45 µm filter. The concentrations of two nitrogen species in the water, ammonium and nitrate, were quantified using cuvette test kits. Specifically, the LCK339 kit was used for nitrate, allowing for measurements within a range of 0.23 to 13.5 mg NO_3-N L^{-1}. For ammonium, the LCK304 kit was employed, which provides a measurement range between 0.015 and 2.0 mg NH_4-N L^{-1}.

2.6 Analysis of biomass samples

The analysis of dry duckweed biomass samples involved the characterisation of their elemental composition (CHON+P) as well as its protein and starch content. Plant samples were dried in an oven set at 70°C for 48 hours.

2.6.1 Protein quantification

To quantify the protein content in duckweed biomass, a Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Catalogue number: 23225) was used, which is based on the bicinchoninic acid (BCA) method (Walker 2009). This method involves a biuret reaction where proteins reduce Cu^{2+} to Cu^{+} in an alkaline environment, with the detection of monovalent copper ions being concentration-dependent. The bicinchoninic acid acts as a chromogenic reagent that chelates the reduced copper, forming a purple complex that absorbs strongly at 562 nm.

In the procedure, approximately 10 mg of dry duckweed biomass was homogenized in 1 mL of Tris-SDS extraction buffer for one minute. Post-homogenization, a 20 µL aliquot of this mixture was transferred into a new tube containing 80 µL of the extraction buffer, creating a 1:5 dilution. Next, 25 µL of this diluted extract was pipetted into a well of a 96-well microplate, followed by the addition of 200 µL of the combined reagent from the kit. The plate was then mixed thoroughly using a plate shaker for 30 seconds. It was covered and incubated at 37°C for 30 minutes, after which it was allowed to return to room temperature. The absorbance of the solution was measured at 562 nm against a blank consisting of extraction buffer and the combined reagent. A calibration curve was prepared using various dilutions of a bovine serum albumin (BSA) standard to calculate the protein concentration, which the assay can measure between 25 and 2000 µg mL^{-1}.

To prepare the Tris-SDS buffer (50 mM Tris at pH 7.5 and 1% (w/v) SDS), the following procedure was followed. Initially, 5 mL of 1M Tris pH 7.5 was added to 80 mL of dH_2O in a suitable container. Subsequently, 10 mL of a 10% SDS solution was incorporated. After combining these ingredients, the total volume was adjusted to 100 mL with additional dH_2O to complete the buffer solution.

The 10% Sodium Dodecyl Sulphate (SDS) solution was prepared by weighing out 5 grams of SDS under a fume hood to avoid inhalation of the powder. The SDS was then added to 40 mL of dH_2O in a beaker equipped with a stirrer bar. The beaker was covered with parafilm to prevent evaporation and contamination. The mixture was stirred, applying gentle heat if necessary to help

dissolve the SDS completely. Once dissolved, the volume was adjusted to 50 mL while still under the fume hood.

The 1M Tris solution was prepared by adding 12.1 grams of Tris-base to 80 mL of dH_2O and dissolved thoroughly. The pH was adjusted to 7.5 using concentrated HCl. Finally, the solution was topped up to a total volume of 100 mL with additional dH_2O.

2.6.2 Starch content

The starch content in duckweed samples was quantified using a colorimetric assay kit from Cell Biolabs Inc. (Catalogue number MET-5025). The assay works by initially breaking down the starch into glucose monomers using amyloglucosidase. Subsequently, these glucose monomers are oxidized into D-gluconic acid and hydrogen peroxide by glucose oxidase. The resulting hydrogen peroxide is then detected with a colorimetric probe that is highly specific. Horseradish peroxidase catalyses the reaction between the probe and hydrogen peroxide, forming a pink-coloured complex in a 1:1 ratio. The intensity of the pink colour, which correlates with the starch content, can be measured by absorbance at 560 nm (Rasmussen and Henry 1990).

The procedure began by weighing 10 mg of dry duckweed biomass into a 2 mL screw-cap Eppendorf tube. To this, 400 µL of 2N NaOH were added, and the mixture was homogenized. The tubes were then incubated at 95°C for 30 minutes in a hot water bath. After cooling to room temperature, the tubes were centrifuged at 120,000 g for 2 minutes. The supernatant was transferred to a new tube, and the extraction process was repeated. The supernatants from both extractions were pooled together, and the total volume was measured. An equal volume of 2N HCl was added to neutralize the mixture, followed by an equal volume of 1M Tris Base pH 6 buffer, and the mixture was thoroughly mixed.

For the starch assay, 50 µL of the sample was placed in a well of a 96-well microplate, followed by the addition of 10 µL of amyloglucosidase. The plate was incubated at 37°C for 30 minutes. Afterwards, 50 µL of the reaction mix containing the colorimetric probe and glucose oxidase was added to each well. The plate was mixed thoroughly and incubated for an additional 45 minutes at 37°C, protected from light. Once at room temperature, the absorbance was measured at 540 nm against a calibration curve prepared with dilutions of a starch standard included in the kit.

2.6.3 Total P in plant

The determination of total phosphorus in plant tissues was conducted using a method modified from Ames (1966), as described below.

Plant samples were first dried in an oven set at 70°C for 48 hours inside a clean jar. After cooling, the samples were thoroughly homogenized using a spatula and then transferred into 2 mL Eppendorf tubes. These samples were flash-frozen in liquid nitrogen and further homogenized using a tissue lyser with a ball bearing. From this, 10 mg of the powdered plant material was placed into new 1.5 mL Eppendorf tubes containing 1 mL of extraction buffer, then homogenized again for three minutes.

To prepare for analysis, 20 µL of the homogenized sample was mixed with 980 µL of 1% glacial acetic acid in new 1.5 mL Eppendorf tubes. Similarly, for control measurements, 20 µL of extraction buffer was added to 980 µL of the same buffer to act as a blank. These samples were then incubated at 42°C for 30 minutes. After this incubation, 100 µL of the mixture was transferred to glass test tubes, to which 30 µL of 10% magnesium nitrate ($Mg(NO_3)_2$) in 95% ethanol was added. The tubes were then flamed to ash twice using a Bunsen burner, cooled, and 300 µL of 0.5 M HC) was added for digestion at 65°C for 30 minutes.

Upon cooling, 700 µL of a combined reagent, made from 1 part 10% ascorbic acid solution and 6 parts of 0.42% ammonium molybdate in 1N sulfuric acid, was added to each tube. The tubes were then incubated at 37°C for one hour. The contents were subsequently transferred to plastic cuvettes, and the absorbance was measured at 820 nm against the reagent blank. A calibration curve was produced using phosphate standard solutions to quantify the phosphorus content.

For the extraction buffer, 0.61 g Tris-base, 0.19 g EDTA, 2.92 g NaCl, 0.09 g PMSF (dissolved in ethanol), and 0.5 mL 2-mercaptoethanol were sequentially added to 500 mL of dH_2O under continuous stirring and gentle heat.

2.6.4 Elemental analysis of plant tissue

The analysis of the elemental composition (carbon, hydrogen, oxygen, and nitrogen) of duckweed samples was carried out by Adrian Cunliffe at the School of Chemical and Process Engineering, University of Leeds, UK. For this analysis, each duckweed sample was prepared in triplicate to ensure reliability and accuracy. Each of these triplicate samples consisted of 2.5 mg of dry biomass.

2.7 Experimental set-up of batch cultures

Prior to each experiment, healthy plants from the stock were selected, rinsed with deionized water, and pre-cultivated under the specific conditions required for each experiment. The pre-cultures were kept for a week in 1-liter plastic containers with full Hoagland's media, which was replenished every 2 days.

Before commencing each experiment, the pre-cultivated fronds were thoroughly washed with deionized water and then placed into a separate container containing Hoagland's medium deficient in phosphorus. These fronds were subjected to phosphorus starvation for a minimum of 5 days to maximize the phosphorus uptake response during the experiments. To ensure nutrient balance was maintained, any potassium deficiency was made up by adding KCl.

Batch experiments were conducted in a Fitotron® growth chamber (model SG066 CHX-F), in 150 mL plastic pots (6.25 cm diameter) containing 100 mL sterile growth solution. All the experiments were performed under non-sterile conditions and media pH was fixed to 7 at the start but not buffered. In all the experiments light was constant and provided by fluorescent tubing at 100 µmol photon m^{-2} s^{-1}, in 12h light cycles. Experimental pots were placed into a cardboard box to avoid incidence of lateral light and avoid algae proliferation. Experiments were conducted in triplicate using 100 mg initial fresh mass of duckweed per pot.

2.7.1 Preliminary experiments

Before proceeding with the main experiments of the project, it was necessary to establish which species of duckweed would be used, as well as the initial cultivation conditions (amount of inoculum, pot size and source of nitrogen) that were to be employed (Chapter 4).

Initially, a screening experiment was carried out all species of duckweed under stable environmental conditions — 25°C temperature, 100 µmol photon m^{-2} s^{-1} light intensity, and 12-hour light cycles. Each duckweed species was replicated in three pots, where 50 mg of fresh duckweed sample was introduced into Hoagland's medium. The cultures were then allowed to grow over a 12-day period. Following this, the residual phosphorus levels in the cultivation medium were determined, along with the biomass each species produced. The harvested duckweed biomass was subsequently dried in an oven for two days, for protein quantification. Comparative assessments were made between the duckweed species focusing on their relative growth rates, protein contents, phosphorus

removal percentages, and specific phosphorus removal rates. These comparisons were standardized using Z-scores to provide a clear measure of how each species performed relative to others. The Z-scores were calculated based on Eq. (2-1), where x_i represents the response value (such as growth rate or protein content) for a specific duckweed species, μ is the calculated mean value of the responses for all duckweed species was calculated, and σ represents the standard deviation of these responses.

(2-1) $$Z - Score_i = \frac{x_i - \mu}{\sigma}$$

Using the calculated Z-scores, the most effective duckweed species was chosen for further experimentation.

In the subsequent experiment, varying amounts of the selected duckweed species, ranging from 50 to 1000 mg of fresh weight, were inoculated into pots containing 100 mL of standard Hoagland's medium under the same cultivation conditions as the initial experiment. At the conclusion of this experiment, the grown biomass was harvested and its productivity calculated. Additionally, the phosphorus concentration remaining in the medium was measured to assess phosphorus removal rates. This experiment helped determine the optimal initial biomass amount required for future experiments.

To establish the appropriate pot size for future experiments, 100 mg of fresh weight duckweed was inoculated into pots of different diameters (4.65 and 6.25 cm), providing varying surface areas for plant growth. Both pot sizes contained the same volume of Hoagland's medium, 100 m*L.* The experiment was observed over 12 days, with measurements taken on biomass growth and phosphorus removal.

Lastly, the preferred nitrogen source for the selected duckweed species was evaluated. In this experiment, 100 mg of fresh weight duckweed was inoculated into standard Hoagland's media containing 100 mg N L^{-1}, available either as nitrate or ammonium. The experiment was conducted under the same cultivation conditions and monitored over 12 days. The outcomes were then compared based on the growth of the duckweed and phosphorus removal, enabling the selection of the most suitable nitrogen source for duckweed cultivation in the next experiments.

2.7.2 Temperature experiments

To evaluate the impact of temperature on the growth and nutrient uptake of *W. angusta* (Chapter 5), three different temperatures were studied (20°C, 25°C, and 30°C). During the pre-culture phase, containers were maintained at each temperature to acclimate the plants and prevent any lag-phase in growth during the experimental trials. Each experiment lasted for 8 days, during which water samples and top-view pictures from each pot were taken every second day. To mitigate evapotranspiration, the experimental pots daily were replenished with HM lacking both nitrogen and phosphorus.

2.7.3 Phosphorus and nitrogen experiments

Nutrient-dependent growth kinetics were measured with short-term batch cultures of *W. angusta* by varying the initial concentration of nutrients in media (Chapter 5). At the beginning of each experiment series, 100 mg duckweed fresh biomass were inoculated into plastic pots containing 100 mL of modified culture medium.

Phosphate concentration in HM ranged from 0.2 – 8.0 mg PO_4-P L^{-1}, while the nitrogen concentration varied between 0.5 – 2 mg NO_3-N L^{-1}. In each case, nitrogen or phosphorus was kept constant at the standard HM level (100 mg NO_3-N L^{-1} and 15 mg PO_4-P L^{-1}, respectively) to ensure single-nutrient limitation effect. When necessary, medium was supplemented with KCl and $CaCl_2$ to address any deficiencies in potassium and calcium.

To counter evapotranspiration experimental pots were topped-up daily with HM that lacked either phosphorus or nitrogen, depending on the specific experiment. The experiments were conducted at 25°C for 8 days, with sampling conducted every 2 days.

2.7.4 Response surface experiments

W. angusta was cultivated in HM with varying concentrations of phosphate and nitrate under three different temperatures (Chapter 5). Duckweed performance for biomass production and nutrient uptake was evaluated considering relative growth rate, P and N removal rates, and biomass composition as responses. A Box-Behnken design was employed to obtain the response surfaces using Minitab Statistical Software.

The experimental design included two nutrient factors (phosphorus as PO_4-P concentration and nitrogen as NO_3-N concentration) as well as temperature. All other cultivation parameters were maintained the same as for the stock cultures. All experiments last 8 days, and were conducted in 150 mL plastic pots, each using 100 mg fresh duckweed as inoculum. At the end of each experiment, duckweed biomass was harvested, oven dried and characterise (elemental analysis, P in plant, protein and starch).

Table 2-2 provides an overview of the three levels of analysis for the selected factors. In the experimental design, twelve experiments with varying temperature, P, and N concentration were conducted in triplicates, in addition to six replicates at the central point. The effect of independent factors on the various responses was fitted to a quadratic model Eq. (2-2):

(2-2)

$$Y = a_0 + a_1A + a_2B + a_3C + a_{11}A^2 + a_{22}B^2 + a_{33}C^2 + a_{12}AB + a_{13}AC + a_{23}BC$$

where Y is the response, a_0 is the offset term; a_1, a_2 and a_3 are the linear coefficients; a_{11}, a_{22} and a_{33} are the squared term coefficients; a_{12}, a_{13} and a_{23} are the interaction coefficients. A, B and C are temperature, phosphorus and nitrogen concentration respectively.

Table 2-2 Factors and levels for experimental design of *W. angusta* performance for biomass production and nutrient uptake.

Factors	A – Temperature (°C)	B – P concentration (mg PO_4-P L^{-1})	C – N concentration (mg NO_3-N L^{-1})
Level 1	-1 (20)	-1 (0.2)	-1 (0.5)
Level 2	0 (25)	0 (2.0)	0 (5.0)
Level 3	1 (30)	1 (4.0)	1 (10.0)

The responses from the response surface methodology included relative growth rate, biomass productivity, protein and starch content in biomass, total phosphorus and nitrogen in biomass, nutrient removal rates, nutrient-to-biomass yields, and any derived productivity from biomass components.

2.7.5 Batch model formulation and experimental validation

The development of a mathematical model for cultivating duckweed biomass in batch systems is detailed and presented as a result in Section 5.4.5. To experimentally validate this model, *Wolffia angusta* was grown in duplicate in 1.5-litter rectangular tanks measuring 26 x 11 x 5 cm (length x width x height), each filled with 1 litre of standard Hoagland's media. The conditions were consistently maintained at 25°C with a light intensity of 100 µmol m^{-2} s^{-1} and a 12-hour daylight cycle. The tanks were covered with foil to prevent lateral light exposure. Duckweed was pre-cultured under these conditions until sufficient biomass was generated to cover 30% of the tank's surface area (286 cm^2). The experiment lasted for eight days, during which water samples and serial photographs of the tank's surface were taken daily.

Throughout the experiment, whenever the duckweed biomass fully covered the tank surface, a portion was harvested to approximately reset coverage to 30%. This management ensured continuous growth without overcrowding. The concentrations of residual phosphate and nitrate in the water column were measured and compared against the predictions from the mathematical model. Results for biomass growth, monitored by measuring increases in the surface area covered by the duckweed, were also contrasted with outcomes from the model.

2.8 Experimental set-up of continuous flow treatment systems

Two distinct continuous flow treatment systems were established for different purposes. The first system was set up in a greenhouse at the Plant Growth Suite of the Faculty of Biological Sciences, University of Leeds, UK. This setup aimed to validate the application of a mathematical model to continuous flow treatment systems and to experimentally test various tank configurations for wastewater treatment. The second pilot system was constructed and installed at a fish farm in Malang, Indonesia. The objective of this system was to evaluate the feasibility of using duckweed to treat effluents from a fish farm in a real-world scenario.

2.8.1 Setup of a small-scale pilot system in Leeds

This pilot system, as discussed in Sections 6.3.2, 7.4.1, and 7.4.2, consisted of black open top rectangular tanks (Kingspan, UK) each with a 30 L capacity (40 x 25 x 30 cm, L, W, H, respectively). It was housed in a greenhouse that simulated the average climate conditions of Indonesia, maintaining an

ambient air temperature of 25°C, light intensity ranging from 100 – 200 µmol m^{-2} s^{-1} at the water surface, and 12-hour daylight cycles using LED lighting bulbs (Phytolux Attis 7, 3W Epistar).

The tanks were configured either in parallel or in series, based on the specific experiment being conducted. They were supplied with modified standard Hoagland's media, containing 4 mg P $L^{-}1$ and 20 mg N L^{-1}, delivered from a separate 30L storage tank (Ryman, UK) using a peristaltic pump (Model 205S, Watson Marlow, UK). In the parallel setup, independent channels of the peristaltic pump ensured consistent flow among tanks. For the series setup, a single channel fed the first tank, and the tanks were interconnected using 1-inch silicon tubing. In all tanks, both the inlet and outlet ports were positioned 12 cm from the bottom, maintaining a working volume of 12 L per tank. The ports were baffled to prevent the washout of surface-growing plants.

Operational flow rates were adjusted based on the configuration: in parallel, a flow rate of 1.71 L d^{-1} per tank was used, resulting in a residence time of approximately 7 days per tank. For series configuration with two tanks, the flow rate was increased to 3.42 L d^{-1}, keeping the combined retention time constant at 7 days.

Throughout the experiments, top-view pictures were taken to quantify duckweed growth when possible, and water samples from the inlet and outlet of the tanks were collected to analyse phosphorus and nitrogen concentrations. Temperature in the greenhouse was continuously monitored using a data logger (SenseAnywhere, UK).

At the start of each experiment (both parallel and series configurations), sufficient biomass was pre-cultivated separately to cover 25% of each tank's total area. Whenever the biomass covered the entire surface, 75% was harvested, leaving 25% to continue treatment. In a sub-experiment within the series configuration, when the biomass covered both tanks' surfaces, all biomass from the first tank was harvested, and 25% of the 75% harvested from the second tank was recirculated.

2.8.2 Setup of large-scale pilot system in Indonesia

A larger pilot system was installed near a fish farm in Indonesia, designed for integrated wastewater treatment and duckweed cultivation. The system included several components to ensure efficient operation (Figure 2-1).

A centrifugal pump was used to draw water from the fish pond, equipped with a screen filter to prevent large solids and fish from entering the system. A check valve was installed to prevent backflow due to gravity. The extracted water was then pumped into a 250-liter heading tank situated 1.5 meters above the ground. An automatic floating valve at the tank's inlet prevented overflow and maintained a consistent water level.

The flow rate within the system was controlled by a gate valve located at the outlet of the heading tank. Water flowed by gravity from this tank to the treatment pond.

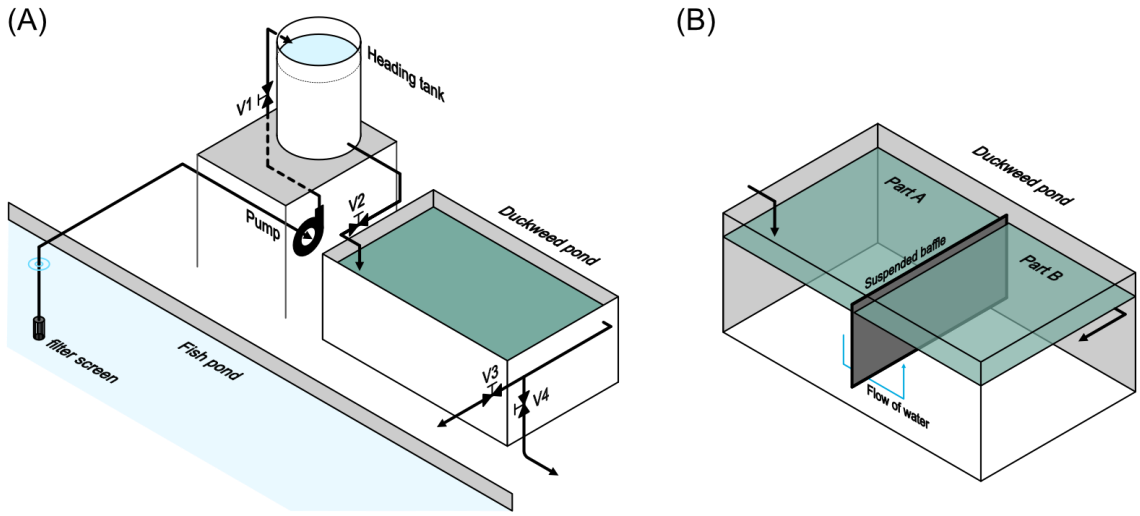

Figure 2-1 Hydraulic setup pilot system in Indonesia

(A) Detail of the components of the pilot system based- on duckweed for the treatment of fish farm effluents in Indonesia. V1: Valve to control water extraction flow from the fish pond, V2, Valve to control flow of the treatment pond, V3: Valve for water sampling, V4: Valve to discharge to drainage. **(B)** Detail of the treatment pond and the flow of water when floating baffle was installed.

The treatment pond, measuring 2.5 meters in length, 1.5 meters in width, and 1 meter in depth, had a total capacity of 3.75 cubic meters. It was constructed from PVC liner and supported by an iron frame. To maintain an operational volume of 3 m^3, the outlet port was positioned 80 cm from the bottom of the tank. A 500 µm plastic mesh covered the outlet port to prevent the loss of duckweed. Two valves were installed at the outlet: one for continuous flow back into the fishery's drainage system and another for water sampling.

When necessary, a plastic baffle could be installed within the pond to enhance water circulation and distribution of nutrients. The baffle, measuring 2.0 meters by 0.8 meters, was suspended midway along the pond's length by a metal pole. It was secured to one long side of the pond, with heavy weights anchoring

it on the other side. The baffle was designed to allow water recirculation between two compartments under it. The short sides of the baffle were sealed to the pond's internal walls with duct tape.

2.9 Data analysis and calculations

2.9.1 Biomass growth and productivity

The growth and productivity of duckweed were assessed using two methods: weight measurements and image analysis.

For biomass productivity, fresh duckweed fronds were blotted on paper towels to remove excess water and then weighed using an analytical balance. To determine dry weight and moisture content, the fronds were dried in an oven at 70°C for two days within glass jars and weighed again. This procedure was carried out at both the start (using a subsample of the inoculum) and the end of each experiment (using the total biomass produced). Biomass productivity was then calculated using Eq. (2-3):

(2-3) $$\boldsymbol{BP = \frac{DW_t - FW_0 * (1 - w0)}{t}}$$

Where BP represents the net biomass productivity (mg day^{-1}), DW_t the biomass dry weights at the end of the experiment, FW_0 and w_0 the biomass fresh weight and moisture content at the beginning of the experiment, respectively.

To estimate the relative growth rate (RGR), top-view photographs of the cultivation pots were taken from a consistent height of 20 cm above the water surface using a smartphone. These images were then processed using ImageJ® software to calculate the total surface area covered by the duckweed fronds. The diameter or length of the treatment ponds in each image served as a reference scale to convert pixel measurements into surface area (cm²). The relative growth rate was determined by fitting the change in total frond area over time to an exponential growth model, as shown in Eq. (2-4):

(2-4) $$\boldsymbol{X = X_0 * exp^{(RGR * t)}}$$

where RGR is the relative increase in total frond area per unit of time, t is the time of sampling, X_0 is the total frond area at the start of the exponential phase, and X the total frond area at any given time. This method provided a visual and quantitative assessment of duckweed growth over time.

2.9.2 Biomass derived productivities

To estimate the productivities of phosphorus, protein, and starch derived from biomass in each experiment, the formula presented in Eq. (2-5) was applied:

(2-5) $$\boldsymbol{BP_i = BP * x_i}$$

Where BP_i represents the productivity of a specific component within the biomass, measured in terms of mass per day, and BP es the net biomass productivity, and x_i is the proportion of the component being assessed within the biomass. The component can be protein or starch, which are expressed as a percentage of the dry weight mass, or phosphorus, which is expressed in milligrams of phosphorus per gram of dry biomass.

2.9.3 Maximum nutrient removal rate and specific nutrient uptake

In each experiment, the change of nutrients concentration over time was fitted to four parameter logistic curves Eq. (2-6) in order to establish the maximum rate at which each nutrient was being removed.

(2-6) $$\boldsymbol{S = d + \frac{a-d}{1+\left(\frac{t}{c}\right)^b}}$$

where a is the minimum substrate concentration that can be reached ≈ 0; b is the slope of the curve at the inflection point, also known as the Hill coefficient; c is the time at which the curve inflects; d is the maximum nutrient concentration (= S_0, substrate concentration at the beginning of the experiment); t is the time of sampling. The maximum phosphorus removal rate (PRR_{max}) and the maximum nitrogen removal rate (NRR_{max}) are reached at the time the inflection point is reached. The value of both rates can be obtained by deriving Eq. (2-7) at t = c.

The negative sign in Eq. (2-7) corresponds to the change from high to low nutrient concentration.

(2-7) $$\frac{dS}{dt} = -\frac{b(a-d)\left(\frac{t}{c}\right)^b}{t\left(1+\left(\frac{t}{c}\right)^b\right)^2} \quad \rightarrow \quad \left.\frac{dS}{dt}\right|_{t=c} = -\frac{b(a-d)}{4c}$$

The specific uptake of substrates per biomass unit, Y_{sx}, was calculated as:

(2-8) $$P_0 - P_t = Y_{Px} * (X_t - X_0)$$

(2-9) $$N_0 - N_t = Y_{Nx} * (X_t - X_0)$$

Where X_t is the total fronds area at each sampling time, m^2; X_0 is the total fronds area at the beginning of the experiment, m^2; P_0 and N_0 are the initial phosphorus and nitrogen concentration respectively, mg/L; P_t and N_t are the phosphorus and nitrogen concentration at each sampling time. Slope of plot between (P_0 - P_t) at (X_t – X_0) resulted in the average yield coefficient for P uptake, Y_{PX} (mg P/L-cm^2). Same procedure was applied for nitrogen.

2.9.4 Data analysis

All the data were subjected to analysis of variance with ANOVA + Tukey's test. Differences obtained at a level of $P<0.05$ were significant. Fitting of experimental data to the different models and response surface analysis were performed in Minitab. 3D surface plots were graphed in Matlab® (MathWorks Inc., USA). Image analysis of duckweed pots to determine growth parameters was done in ImageJ.

Chapter 3 : Unravelling the impact of light, temperature and nutrient dynamics on duckweed growth: A meta-analysis study

3.1 Introduction

Nature-based solutions (NBS) harnessing the possibility of growing photosynthetic organisms in wastewater are being thoroughly investigated as a cost-effective method for decentralized wastewater treatment. Among the NBS, treatment systems based on aquatic plants – e.g., macrophytes – have been widely used to remove pollutants from water, as a tool for proper wastewater management and disposal. For more than forty years, these systems have been implemented in Europe and North America for nutrient control and recovery from wastewater at low loading rates – i.e., wastewater treatment units for polishing final effluents (Brix 1994; Donde *et al.* 2018). Today, macrophytes are increasingly being used worldwide to treat different types of effluent, including municipal and industrial wastewaters, acid mine drainage, agricultural and livestock wastes, and leachate from landfills, among others. In rural areas and developing countries, macrophyte-based systems play a vital role in the treatment of municipal wastewater from small and decentralised systems, where energy intensive treatment units are not suitable due to technical or economic constrains (Upadhyay, Bankoti, and Rai 2016).

Aquatic macrophytes act as a biological filter, taking up nutrients from polluted waters to support biomass production, while fixing atmospheric carbon dioxide. The great diversity of macrophytes has resulted in a wide variety of systems being used for wastewater treatment, ranging from systems using large aquatic plants like water hyacinth, to very small plants like duckweed. The latter have proven to be efficient in removing nutrients, organic matter, and toxic substances from water. The success of such treatment systems is based on their adaptability and fast-growing capacity. Furthermore, wastewater treatment systems using duckweeds have proven their ability to perform well in both urban and rural settings, and strong environmental credentials due to their low energy consumption and operational costs (Brix 1994).

Duckweeds have been tested for a wide range of wastewater treatment conditions (El-Shafai *et al.* 2004; Hassan and Edwards 1992). These plants grow

very rapidly and remove nutrients at a higher rate than other aquatic macrophytes (Oron *et al.* 1988). Under optimal growth conditions, including nutrient bioavailability, light intensity and water temperature, they can double their weight every 2 or 3 days (Rusoff *et al.* 1980). This reproduction rate is greater than that of any other higher plant, resulting in the formation of dense mantles over the surface of water bodies, especially when the concentrations of nitrogen and phosphorous in the water column correspond to mesotrophic/eutrophic environments (Portielje and Roijackers 1995).

Despite the multiple benefits of duckweed-based systems for wastewater treatment, some limitations associated with their engineering design and operation persist. For instance, the efficiency of treatment processes is seasonal, in response to changing environmental conditions and free surface area available to support biomass growth and photosynthesis. These conditions have a direct effect on the ability of duckweed to take up and metabolise nutrients, which ultimately affect the quality of the final effluent. For this reason, it is necessary to firstly appraise the performance of duckweed-based systems for wastewater treatment under a range of culture conditions, typical to the corresponding application (i.e., nutrient loading rates, flow rates, retention times, climate conditions, etc.,)

As other photosynthetic organisms, duckweeds require a supply of macronutrients (carbon, nitrogen and phosphorus) and trace nutrients to grow. These nutrients are all present in wastewaters, either in mineral or organic form, hence the potential for using wastewater as a medium to support duckweed biomass growth. Apart from the concentration of nutrients in the growth medium (i.e., wastewaters), culture conditions such as temperature, pH, initial mantle density, surface area availability, photoperiod and light intensity, have a significant influence on duckweed growth and nutrient uptake.

Moreover, to successfully improve the quality of wastewater effluents, we need to be aware that not all duckweed species are equally effective at taking up nutrients and hence, biomass productivity and composition vary. For that reason, process performance is highly dependent on duckweed strains which may be well or poorly adapted to specific operation and/or environmental conditions (Bergmann *et al.* 2000; Cheng and Stomp 2009). In this sense, appropriate selection of duckweed strains to work with must be undertaken.

Overall, reported outcomes on how environmental and operational conditions impact growth and nutrient uptake by duckweeds are highly variable in published literature. Therefore, it is very difficult to extract meaningful comparisons for such diverse studies which use different duckweed species,

different growth media or effluents, different culture setups and controlled or naturally varying photoperiod and temperature. To try to synthesise this information and draw meaningful conclusions a systematic review was undertaken, and a meta-analysis applied to the data from the retrieved publications. This approach has its origins in medical studies where there are often small sample sizes and confounding variables, but the methodology is much more generally applicable (Page *et al.* 2021). By drawing on a large number of studies, patterns or trends emerge which are not visible in individual studies.

This meta-analysis study focuses on establishing the influence that temperature, light and Nitrogen and Phosphorus have on duckweed biomass growth and nutrient uptake, considering tested natural and engineered environments, that will support the importance of selecting suitable duckweed isolates and species for process development studies and engineering applications. By comparing different outcomes under a standardized methodology, it is possible to plan and design more reliable, robust, and resilient duckweed-based systems for wastewater treatment and nutrient recovery. The ultimate goal is to offer an integrated analysis of the dynamics involved in nutrient reclamation and biomass production by duckweeds.

3.2 Methodology

3.2.1 Literature search

The data for the present meta-analysis study was put together from three different peer-reviewed literature databases (PubMed, Web of Science and Scopus) following the Prisma guidelines (http://www.prisma-statement.org/) (Figure 3-1). All scientific articles published prior to June 2021 were retrieved using the advanced search tool from each database. Different keywords and synonyms were grouped into five topics to be searched using the following Boolean operation: TITLE-ABSTRACT-KEYWORDS - (growth OR composition) AND (duckweed) AND (nutrient OR reclamation).

3.2.2 Inclusion and exclusion criteria

Article titles and abstracts were manually screened to exclude studies not related to the topic. Only studies in wastewater treatment and nutrient recovery using different species of duckweed were included in the analysis. In a further

step, relevant articles were examined to determine fit to the eligibility criteria of this review. The exclusion criteria included the following:

(1) Toxicological studies using duckweeds: Studies assessing the potential of plants for emerging contaminants remediation or the ecotoxicological effect of pollutants on duckweed growth. These studies were excluded as the use of standard culture conditions for the cultivation of duckweeds was limited to the control experiment.
(2) Review papers: Publications collecting and reviewing data from other authors already included within the database or papers presenting the state of art of duckweeds in wastewater treatment.
(3) Not enough data of interest: Papers in which either the relative growth rate of plants or the data/plots required for its calculation is not presented.
(4) Different research question: Scientific reports whose objective was other than assessing the effect of temperature, light, and nutrient availability on the growth of duckweeds.
(5) Non-retrieved papers: Papers that cannot be found using selected databases or without any response from contacted authors.
(6) Language: Papers published in a language other than English or without any English translation available.

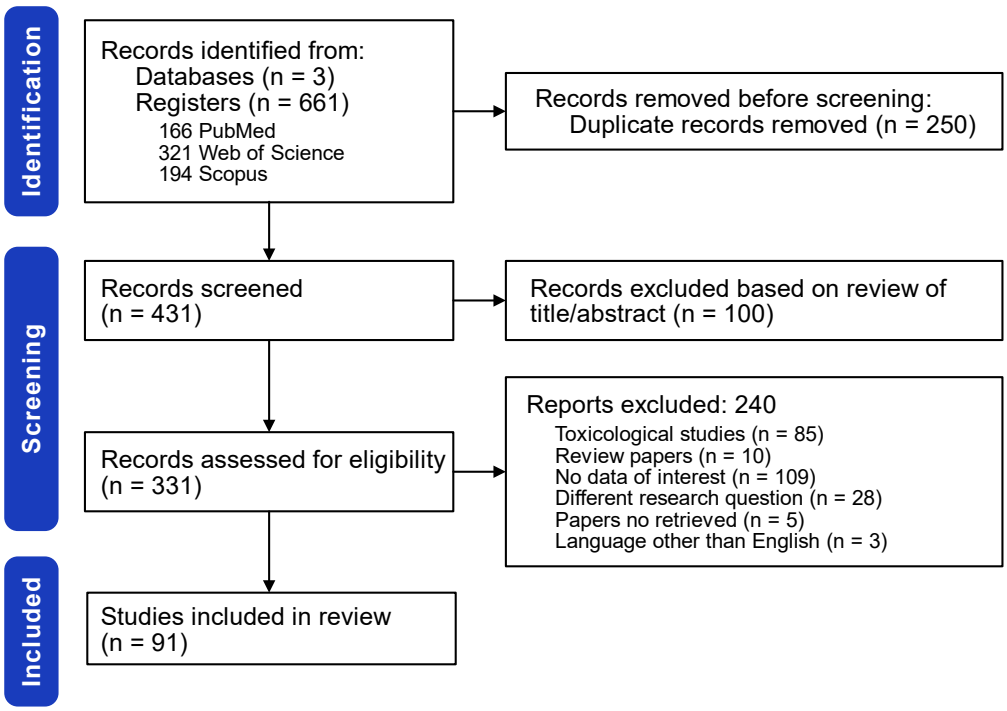

Figure 3-1 Identification of studies via databases and registers

This PRISMA flow diagram **(Moher *et al.* 2010)** shows the literature search results, highlighting the main exclusion criteria used in the screening stage, of peer-reviewed papers published in English prior June 2021.

3.2.3 Data extraction

All data retrieved from the studies included in the review is available in Appendix 1. From each study, the following data was extracted: (1) authors and year of publication, (2) test species, (3) culture conditions, (4) culture media characteristics, and (5) observed response in the treatments. A summary of extracted variables and their respective units is presented in Table 3-1.

Whenever provided, data on the characteristics of duckweed studied, such as genus, species, collection reference number and country of origin, were included. Culture conditions tested in each of the studies were collected and classified either as environmental or simulated culture conditions. When provided, the volume and total surface area of cultivation, initial stocking density, temperature, photoperiod, and light intensity were noted as in the original publication. In some cases, surface area was calculated upon the dimensions of the containers in which the experiments were done. The initial stocking density, or mat density, was calculated as the amount plant material, in fresh or dry basis, per unit of surface area at the beginning of the experiment. Where experiments were conducted under ambient/outdoor culture conditions, and data on temperature, photoperiod and light intensity were not reported, these data were retrieved from the Photovoltaic Geographical Information System from the European Commission (https://re.jrc.ec.europa.eu/pvg_tools/en/) for the particular location. Normal direct irradiance values were converted to Photosynthetically Active Radiation (PAR) using a conversion factor of 4.6 (Langhans and Tibbitts 1997).

Table 3-1 Variables and reported units extracted from independent experiments in reviewed reports

No. Reports:	91	**No. Experiments:**	220	**No. Datapoints:**	920
Duckweed	**Culture conditions (units)**		**Culture media (units)**		**Responses (units)**
Genera	Real / Simulated	-	Real / Synthetic	-	RGR	(d^{-1})
Species	Stocking density	($mg\ m^{-2}$)	Nitrogen source	-	BC	(% dw)
Clone	Coverage	(%)	Total N	($mg\ N\ L^{-1}$)	EC	(% dw)
Origin	Temperature	(°C)	Ammonium	($mg\ NH_4\text{-}N\ L^{-1}$)	N removal rate	($mg\ N\ L^{-1}\ d^{-1}$)
	Photoperiod	(Light hours)	Nitrate	($mg\ NO_3\text{-}N\ L^{-1}$)	P removal rate	($mg\ P\ L^{-1}\ d^{-1}$)
	Light intensity	($\mu mol\ m^{-2}\ s^{-1}$)	Total P	($mg\ P\ L^{-1}$)		
			Orthophosphate	($mg\ PO_4\text{-}P\ L^{-1}$)		

RGR = Relative growth rate, BC = Biochemical composition (protein, lipid, starch), EC = Elemental composition (C, H, O, N)

In addition to this, some characteristics of the culture media in which plants were grown were recorded. Medium was classified as synthetic or real, based on the methods described by the authors. Total Nitrogen (TN) was noted along with the initial concentration of both ammonium and nitrate in the media, all expressed as mg N L^{-1}. Total Phosphorus (TP) and phosphate concentrations are reported as mg P L^{-1}.

Finally, duckweed growth parameters like biomass productivity, relative growth rate (RGR) and doubling time were taken out from the screened literature. When data was not provided, RGR was calculated as RGR = Ln (X_f / X_i) / t, with X_f and X_i either the dry biomass, wet biomass, number of fronds or total fronds area at the end and start of the experiment respectively, and t the cultivation time in days. In cases where biomass growth was presented in time course plots, the corresponding RGR was calculated by fitting growth curves data to the differential form of the equation dX/dt = RGR x t.

When possible, data were extracted from tables and text of the publication; however, when results were presented only on graphs, they were retrieved by reversing data visualizations using the software WebPlotDigitizer (https://automeris.io/WebPlotDigitizer/). To facilitate the analysis, all data collected for each variable were converted to the same units (as per Table 3-1) using relevant conversion factors.

3.2.4 Data analysis

Data obtained from the literature search was catalogued and curated using Microsoft Excel software; data analysis and visualisation was conducted using R software. Statistical analysis of RGR values included one-way ANOVA with Tukey's multiple comparisons test. Statistical significance criterium was defined as p value < 0.05. Z-scores were used to standardize the size effect of culture conditions on response variables, to a same scale, in order to make them comparable. For each independent experiment, Z-values for any response variable were obtained as $Z = (x - \mu)/\sigma$, where x corresponds to the value of the response variable at any given culture condition within the experiment; μ is the mean response, and σ the standard deviation. A further transformation was performed on the data in order to avoid negative values at the time of obtaining regression curves. For this, Z-values were adjusted to have a mean of 100 and a standard deviation of 15. The new values, so called IQ-scores, were calculated as $IQ = Z * 15 + 100$.

3.3 Results and discussion

3.3.1 Study sample and experimental design characteristics

This review identified 661 studies that met the inclusion criteria, see Figure 3-1 for the PRISMA flow diagram summarizing the study selection process. Out of these studies, 91 provided sufficient information to be included in the final quantitative analysis.

Lemna seems to be the most studied duckweed genus as 62% of the studies included in the review had this genus as a study subject (Figure 3-2 A). *L. minor* and *L. gibba* were the species for which most experimental data was available, from 39 and 23 publications respectively. The fact that *L. minor*, also known as common duckweed, is the most widespread duckweed species and broadly used in toxicity testing (Moody and Miller 2005; OECD 2002) makes it the most extensively studied of all duckweed species (Ceschin *et al.*, 2016; Wang, 1986). Of the 14 species discovered for this genus, data were available for 8 of them. The next most studied duckweed genera were *Spirodela* and *Landoltia*, of which the same number of studies (16) was available for the species S. polyrrhiza and *L. punctata*. Finally, *Wolffia* and Wolffiela are the least studied duckweed genera. Recognised as rootless duckweed, both genera contribute only 11% of the papers selected for analysis. Being studied in 6 of the selected publications, *W.* arrhiza is the species with the highest representation of this group. Overall, our database has a good representation of the different duckweed species (19 out of 36 species discovered so far) for each of the genera and, the variability of climates from which each species is representative (61% from temperate climate locations – including China, 24% from tropical climate regions, and 15% from subtropical climate areas).

In terms of culture conditions, data related to temperature, photoperiod and light intensity was collected, and classified according to the degree of control over the experiments (Figure 3-2 B). As per the density plots, 50% of the selected experiments were carried out at temperatures between 23 and 26°C, with a median value of 24°C, under controlled cultured conditions. The temperature range increased when cultures were carried out under ambient/outdoor conditions (from 19 to 27°C) due to seasonality in temperate climate regions. In the latter case, the distribution of data is multimodal, with peaks at 20, 26 and 39°C.

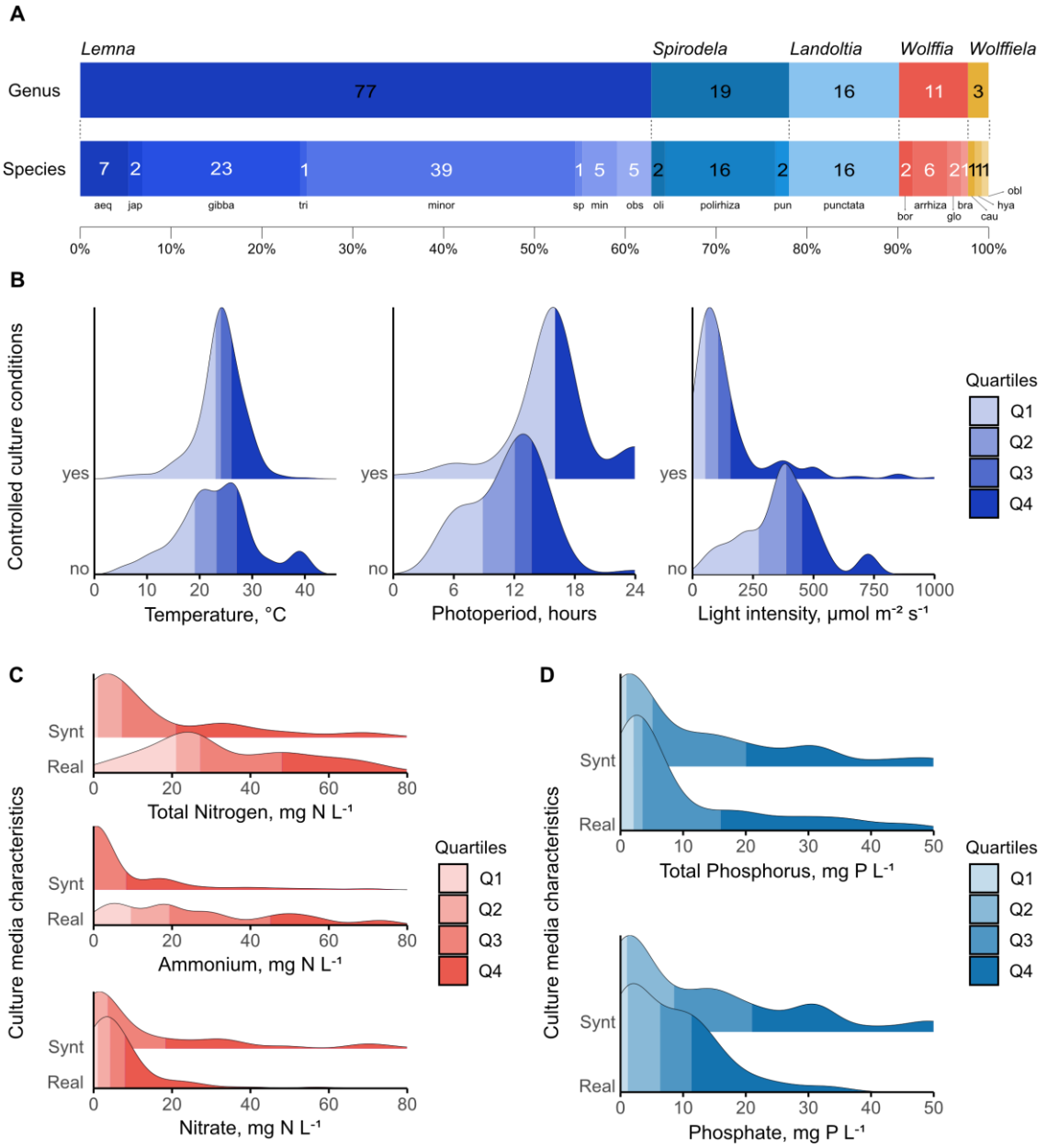

Figure 3-2 Summary of selected dataset descriptors grouped by variable category

(A) Number of experiments per duckweed Genus and species included in the review, **(B)** Density plots showing the data distribution of environmental factors studied across different studies performed under controlled and uncontrolled culture conditions, **(C)** Density plots showing the data distribution for Total Nitrogen and nitrogen species concentration from papers using real and synthetic wastewater as culture media, **(D)** Density plots showing the data distribution for Total Phosphorus and Phosphate concentration from papers using real and synthetic wastewater as culture media.

Regarding photoperiod, under controlled culture conditions, the preference was to carry out trials under simulated long daylight conditions (16h of light for 50% of the data), while under ambient conditions, most of the studies

were carried out under natural light, with photoperiods varying between 8.5 and 14 hours of light per day, with a median of 12h of light per day. Perhaps the biggest difference between data collected at ambient and controlled growing conditions concerns light intensity. At the former condition, several authors used sunlight as a source of energy radiation. Light intensity values were normally distributed, with most of the tests carried out between 270 and 450 µmol m^{-2} s^{-1}, consistent with average values for solar PAR radiation in countries with climates ranging from temperate to subtropical (Global Solar Atlas, n.d.; Wang *et al.*, 2013). In contrast, data from experiments performed in controlled environments present a right-skewed distribution, and only 25% of the data exceeds 150 µmol m^{-2} s^{-1}.

When considering the characteristics of the culture medium, whether synthetic or real wastewater, there are differences in the composition of the different phosphorus and nitrogen species. As far as nitrogen is concerned, there are two main species that contribute to the total nitrogen content of the culture medium, nitrate and ammonium. Figure 3-2 C shows the number of publications studying the effect of either nitrogen species or total nitrogen on duckweed growth. In works using urban wastewater, the major input of total nitrogen comes from ammonium resulting from the decomposition of organically bound nitrogen. In this sense, the plots show how the number of studies on ammonium significantly influences the results that can be found when total nitrogen is the variable of study. The nitrogen concentration ranges used in the studies on ammonium and total nitrogen removal/uptake were distinct from each other. Half of the research on ammonium utilized concentrations ranging from 11 to 51 mg N L^{-1}. Meanwhile, around 50% of the studies on total nitrogen employed concentrations between 25 to 70 mg N L^{-1}. These ranges are similar to what is typically observed in urban wastewater (Ma *et al.* 2016; Metcalf, Eddy, and Tchobanoglous 2004). Higher ammonia concentrations tested by authors correspond to the use of wastewater from sources other than urban areas. In contrast, studies on ammonium carried out with synthetic media barely exceeded 8 mg L^{-1} (just 25% of the data).

In general, experiments carried out using nitrate as the sole nitrogen source in both synthetic and real wastewater showed that the concentrations of nitrate were usually lower than the total nitrogen concentrations tested in other studies. Whereas nitrate concentration varied between 0 – 8 mg N L^{-1} in 75% of cases, higher concentrations were used only in synthetic media, where the nitrogen source was adjusted in such a way as to match the total nitrogen values normally found in wastewater (25% of the test run in synthetic media had a nitrate concentration over 18 mg N L^{-1}).

Finally, data distribution regarding total phosphorus (TP) and phosphate (PO_4-P) concentration in synthetic and real wastewater is reported in (Figure 3-2 D). For both types of culture media (real and synthetic media), there is a correlation between the phosphate concentration and the total phosphorus values tested. Regardless of the type of culture media, half of the experiments tested phosphorus concentrations below 6 mg P L^{-1}, which was reported either as phosphate or total phosphorus. Moreover, authors using synthetic media in their studies tended to cover a wider range of phosphate concentration to assess scenarios mimicking repleted and depleted nutrient conditions.

3.3.2 Effect of environmental factors on plant growth

In recent years an increasing number of researchers have focused on understanding how global climate is changing and the corresponding impacts on life on earth. It is undeniable that any change in environmental conditions has direct repercussions on living organisms, consequently influencing their metabolism (e.g., growth rates) and performance in engineering applications. For biomass growth, duckweeds use light and nutrients to carry out photosynthesis. While growing, these aquatic plants also produce and accumulate metabolic products, of which relative amounts in biomass depend upon the specific species studied, and environmental conditions tested (i.e., Light intensity and photoperiod, temperature, and availability of nutrients). To have a comprehensive understanding on the effect of these parameters on the growth of duckweeds, it is necessary to critically assess the existing literature.

3.3.2.1 Temperature

Temperature is probably the most important environmental factor regulating duckweed growth, composition, and nutrient uptake. As for other aquatic organisms, water temperature controls the rate at which biochemical reactions take place, including duckweed's photosynthesis, metabolism and catabolism. These plants can grow in a broad range of temperatures, subject to species and isolate, acclimation, and seasonal ambient conditions. The relationship between temperature and duckweed growth can be described by the Arrhenius equations, previously used in kinetic models for other photosynthetic organisms (Feng *et al.*, 1990; Goldman & Carpenter, 1974). It is assumed that duckweed growth rate continuously increases with temperature increments up to a point in which growth rate decreases, i.e., optimal temperature. Therefore, in

order to have a more accurate representation of the relationship between growth rate and temperature data, the thermal performance model curve (TPC), described by the Hinshelwood equation was employed (Hinshelwood 1947). The Hinshelwood thermal model assumes that the rate of biomass growth is proportional to the overall enzyme activity and the kinetic growth rate constant. It also assumes that changes in the kinetic growth rate constant as a function of temperature can be described by the Arrhenius equation. The model predicts a unimodal relationship between biomass growth rate and temperature, with an optimal temperature at which the rate is at its maximum.

Based on the data obtained from published literature (Figure 3-3), the tested ambient air temperatures ranged from 5 to 40°C, within which the actual relative growth rate (RGR) varied between 0.0 and 0.41 d^{-1}; the highest RGR value correspond to *Lemna minor* cultured in synthetic media under controlled culture conditions (Lasfar *et al.* 2007).

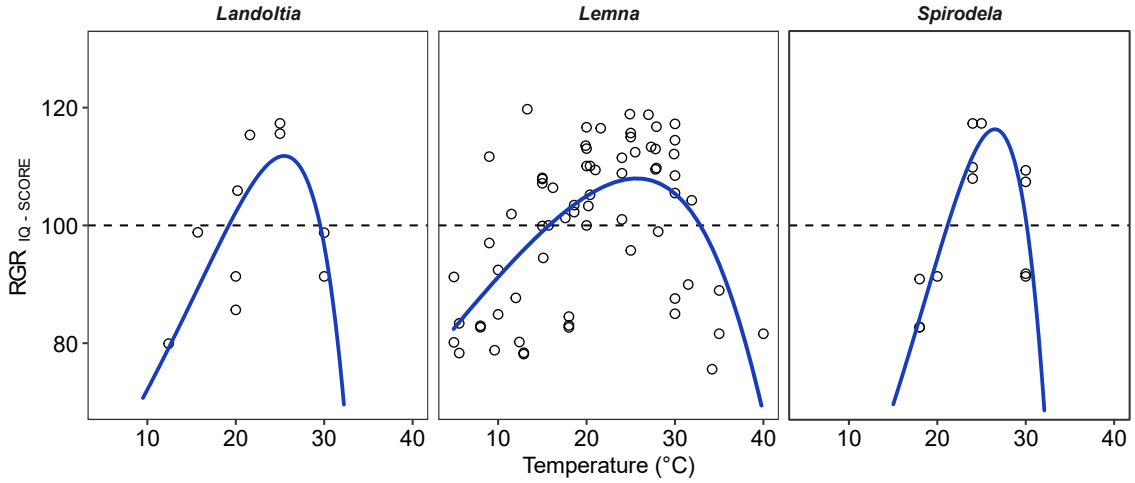

Figure 3-3 Effect of temperature on the relative growth rate (RGR) of different duckweed genera.

Thermal performance curves for *Landoltia*, *Lemna* and *Spirodela* were fitted to datasets from 3, 15 and 4 independent experiments respectively, using the Hinshelwood model. In all cases, RGR is expressed as the IQ scores from each independent experiment. Dashed lines represent the $RGR_{IQ\text{-}score}$ baseline (= 100). Coefficients and standard errors for the fitted curves are given in Appendix 2.

In general, it is found that temperature affects duckweed biomass growth in similar ways in the different studies analysed The growth rate increases as the temperature rises from 5°C and reaches a maximum at around 25ºC. Above this temperature, plants get stressed and reduce their growth rate. This behaviour follows well known fundamental principles of plant growth and experimental

results from other species of aquatic plants and microalgae used for wastewater treatment (Carr, Duthie, and Taylor 1997; Ras, Steyer, and Bernard 2013). Outside the optimal temperature range (20-30°C), or even at extreme temperature values, the plants do not grow as fast or simply die. This fact explains why *L. minor* and *L.* minuta do not survive over winter in uncontrolled outdoor experiments (Paolacci, Jansen, and Harrison 2018). Duckweeds sense environmental conditions and when these are not favourable, most of them can enter a dormant state by turion formation (Appenroth 2002; Kuehdorf and Appenroth 2012). This ability allows these plants to survive in environments with seasonal climatic variability.

When analysing the variation of RGR IQ-scores with respect to temperature, a region is revealed, above the baseline (IQ-score = 100), where plants growth is better than the average from all the experiments included in the review (average growth rate = 0.119 d^{-1}). Based on the thermal performance curves resulting from the datasets for different genera of duckweed (Figure 3-3), we found that the temperatures range within which the area of optimal growth is contained varied between genera. On one hand, we found that *Lemna* species can cope better with extreme temperatures and exhibit a good growth performance in a wide range of temperatures (11.4 to 38.1°C). On the other hand, the range of temperatures for optimal growth was narrower for *Landoltia* and *Spirodela* species (18.1 to 32.3°C, and 19.0 to 29.2°C, respectively). In the case of *Lemna*, plants that are grown outside the optimal temperature range end up having a RGR 55% lower than the average RGR above the IQ-scores baseline. These results highlight the importance of choosing the most suitable duckweed species according to local temperature conditions.

The temperature range for RGR IQ-scores higher than 100% is of great importance for the development of duckweed-based processes for wastewater treatment. If temperature alone is considered, a treatment system operated at ambient conditions will be reliable as long as the selected duckweed species perform well within the local temperature variations. Thus, the implementation of duckweed-based systems for wastewater treatment in regions with tropical or subtropical climates is favoured due to narrow temperature variations from optimal duckweed growth conditions throughout the year. In cool/cold temperature regions actions need to be taken to engineered wastewater treatment systems to avoid temperature falling below the optimal range.

3.3.2.2 Light

Light is an essential factor for plant growth as it is the energy source for photosynthesis, which is vital for plants to fix atmospheric inorganic carbon and turn it into organic compounds. This process is driven by two main photosystems, Photosystem I (PSI) and Photosystem II (PSII), which work sequentially to convert light energy into chemical energy (Nelson and Yocum 2006). PSII absorbs light and uses the energy to extract electrons from water molecules, releasing oxygen as a byproduct. The energized electrons are then passed down an electron transport chain to PSI, where they are re-energized by light absorption. PSI uses these high-energy electrons to reduce NADP+ to NADPH. Along with ATP, which is produced by the electron transport chain, NADPH provides the necessary energy and reducing power for the Calvin cycle, where carbon dioxide is fixed into glucose (Nelson and Yocum 2006).

When referring to light during the cultivation of duckweeds, three factors must be considered: light intensity, light/dark cycles or photoperiod, and light spectral composition. All factors affect duckweed biomass growth through their impact on photosynthesis. In terms of light intensity, it has been found that the growth rate of aquatic plants and microalgae increases with increasing light intensity, up to a maximum RGR value when light saturation conditions are reached (Madsen and Sand-Jensen 1994; Sorokin and Krauss 1958). Further light intensity increments above this point reduce plant growth rates and may even inhibit photosynthesis (photo-inhibition). However, results may vary depending on the species and isolates studied, as well as on photoadaptation processes that improve the photosynthetic efficiency of the organisms. This includes changes in chlorophyll content and ratios, number of chloroplasts and respiration patterns (Lichtenthaler *et al.* 1981).

Although light intensity plays a fundamental role in photosynthesis, the time during which the radiation is incident on the plants must also be considered. At low light intensities the RGR of duckweeds increases with longer day conditions, but at high light intensities longer photoperiods negatively impact plants growth rate (Lasfar *et al.* 2007; Yin *et al.* 2015). In this sense, it is necessary to consider the total amount of radiation that reaches the plants while they are exposed to the light (e.g., daily light integral – DLI) in order to avoid photosystem inhibition and damage, so that photosynthesis can continue (Sundby, McCaffery, and Anderson 1993).

Light intensity

The relative growth rate of different duckweed species increases with increasing light intensity, reaching maximum biomass growth at around 200 µmol m^{-2} s^{-1} (Figure 3-4); further increases in light intensity do not significantly affect plant growth (even up to 800 µmol m^{-2} s^{-1}, not shown in the figure). The change in IQ-scores of the selected duckweed species with respect to light intensity was fitted to a Monod-like model widely used for microalgae (Béchet, Shilton, and Guieysse 2013). From the results, it can be established that overall, for all duckweed species, light saturation is reached at around 100 µmol m^{-2} s^{-1}. One exception is *L. aequinoctialis* grown in continuous light, which is saturated by light at an intensity of 50 µmol m^{-2} s^{-1}. Furthermore, within the range of reported light intensities (0 – 800 µmol m^{-2} s^{-1}), no evidence of photoinhibition can be seen. Similar results were reported by Wedge et. al (1982), who found that, depending on the temperature, light saturation in *Lemna minor* plants occurs between 300 – 600 µmol m^{-2} s^{-1} and that there is no photoinhibition unless the light intensity is greater than 1200 µmol m^{-2} s^{-1}.

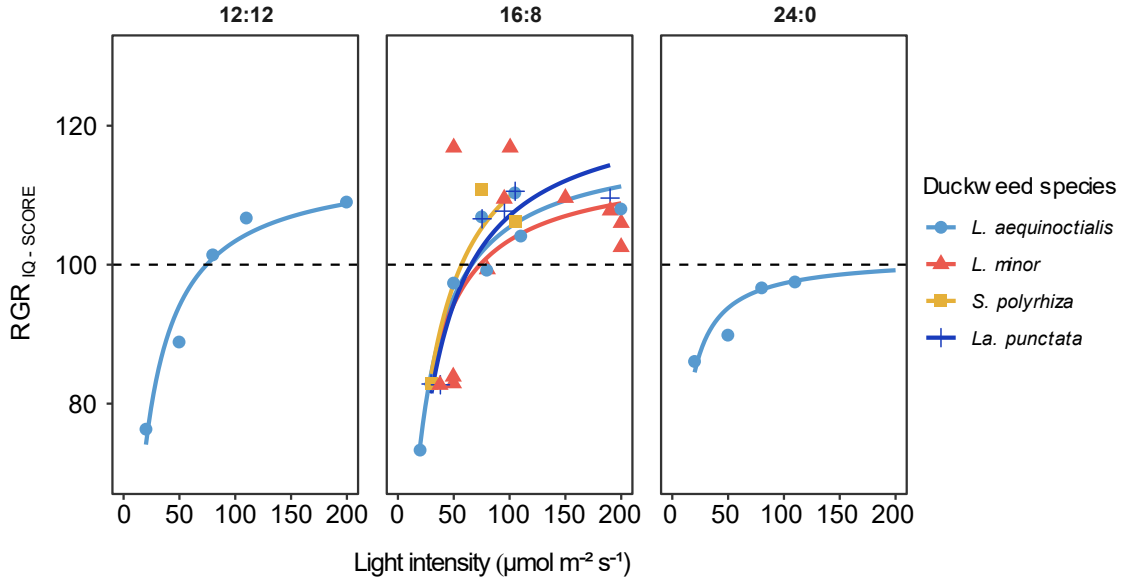

Figure 3-4 Effect of light intensity on the relative growth rate (RGR) of duckweeds cultivated under different photoperiods (light hours : dark hours).

Curves represent the general trend of the data for different duckweed species upon parametric fitting of datasets to Monod-like equations. The numbers above the boxes represent the number of hours light: dark per day. In all cases, RGR is expressed as the IQ scores from each independent experiment. Dashed line represents the $RGR_{IQ\text{-}score}$ baseline (= 100). Data for *L. aequinoctialis*, *L. minor*, S. polyrrhiza and La. *Punctata* was retrieved from 4, 4, 1 and 2 independent experiments respectively.

Although the overall effect of light intensity on the RGR of the plants is the same (direct increment until saturation), the magnitude of the effect varies according to the photoperiod and duckweed species in question. On the one hand, the positive effect of increasing light intensity on RGR is compromised as the length of light hours increases. In the case of *L. aequinoctialis*, there is no significant effect of increasing the photoperiod from 12 to 16 hours, but an additional increase of 8 hours reduces the RGR by 24% (Yin *et al.* 2015). On the other hand, when grown at the same day length (16 h) and below light saturation condition, the RGR of different duckweeds species improves differently for each unit by which the light intensity is increased. As an example, an increment of 50 µmol m^{-2} s^{-1} improves the RGR of *L. minor*, *L. aequinoctialis*, *La. punctata* and *S. polyrrhiza* by 23.1, 31.9, 31.2 and 33.3% respectively (Li *et al.* 2016; Walsh, Kuehnhold, *et al.* 2021; Z. Zhao *et al.* 2014).

Photoperiod

When it comes to photoperiod, two different trends are discernible when analysing the growth of *L. minor* under constant light intensity (Figure 3-5 A). When the light intensity at which duckweeds grow is higher than 300 µmol m^{-2} s^{-1}, the RGR increases from 0.01 to 0.43 day^{-1} when the light exposure is increased from 0 to 12 hours a day. Longer photoperiods reduce the rate at which the plants grow. In this case there is a region above the IQ-scores baseline, between 7 and 18 hours of day length, in which the RGR of duckweeds is greater than the average RGR of all the retrieved data. This range can be defined as the optimal photoperiod range for duckweed growth. At low light intensities (e.g., 156 µmol m^{-2} s^{-1}) the effect of photoperiod on the RGR of *L. minor* is the same as at high intensities, however, the optimal range in which plants can grow is extended by 7 hours. In this case, we found that the effect of photoperiod on the RGR was the same despite the difference in culture temperature between the experiments (Lasfar *et al.* 2007; Paterson *et al.* 2020).

A particular case is that of *L. aequinoctialis*, whose RGR increases with longer day length, reaching a maximum under continuous light independently of the light intensity (ranging from 20 to 400 µmol m^{-2} s^{-1}, data not shown) (Yin *et al.* 2015). The difference between both *Lemna* species highlights the importance of species selection in the design of wastewater treatment systems. *L. minor* copes better with daylength changes in open air treatment systems, while *L. aequinoctialis* can be used in engineered indoors systems with a continuous supply of light. When analysing the effect of the photoperiod on the RGR of plants that were grown outdoors under variable light intensity conditions (Figure 3-5 B),

it is observed that the data follows trends similar to those of plants cultivated under constant light intensities, but in a narrower range. For light intensities varying between 100 and 300 µmol m^{-2} s^{-1} the optimal photoperiod range for three different duckweed species is reduced to 7 hours only, between 9 and 15h of day length on average.

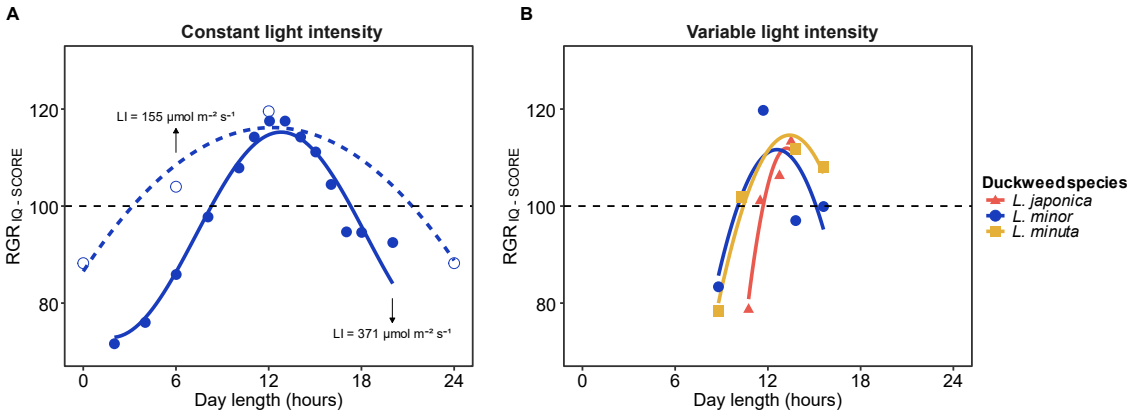

Figure 3-5 Effect of photoperiod on the relative growth rate (RGR) of duckweeds under natural and controlled culture conditions.

(A) Duckweed cultivated in controlled environments at two different constant light intensities; **(B)** Duckweed cultivated in real environments with varying photoperiod and light intensities (ranging between 100 – 400 µmol m-2 s-1). Curves represent the general trend of the data upon non-parametric fitting of datasets. In all cases, RGR is expressed as the IQ scores from each independent experiment. Dashed line represents the RGR IQ-score baseline (= 100). Data for *L. minor*, *L.* japonica and *L.* minuta was retrieved from 3, 1 and 1 independent experiments respectively.

A particular case is that of *L. aequinoctialis*, whose RGR increases with longer day length, reaching a maximum under continuous light independently of the light intensity (ranging from 20 to 400 µmol m^{-2} s^{-1}, data not shown) (Yin *et al.* 2015). The difference between both *Lemna* species highlights the importance of species selection in the design of wastewater treatment systems. *L. minor* copes better with daylength changes in open air treatment systems, while *L. aequinoctialis* can be used in engineered indoors systems with a continuous supply of light. When analysing the effect of the photoperiod on the RGR of plants that were grown outdoors under variable light intensity conditions (Figure 3-5 B), it is observed that the data follows trends similar to those of plants cultivated under constant light intensities, but in a narrower range. For light intensities varying between 100 and 300 µmol m^{-2} s^{-1} the optimal photoperiod range for three

different duckweed species is reduced to 7 hours only, between 9 and 15h of day length on average.

Although the results do not establish a direct relationship between photoperiod and other duckweed genera and species (not enough data from data collection process), they do lead to the conclusion that photoperiod and light intensity should be considered together when analysing the effect of light on plant growth.

Combined effect of light intensity and photoperiod – The daily light integral concept

By integrating the light intensity at which the plants are grown together with the time at which they are exposed to light, it is possible to analyse the combined effect of those two variables on the relative growth rate of duckweeds (Figure 3-6). This combined variable is named as daily light integral (DLI), which describes the number of photosynthetically active radiation (PAR) measured as photons (individual particles of light in the 400-700 nm range) that are delivered to a specific area over a period of time (mol m^{-2}). When the two effects of light intensity and exposure are integrated into the DLI variable, it was found that a biomass growth rate increases with DLI until reaching a maximum at 15 mol m^{-2}, corresponding to the light saturation value. Below this value, plant growth declines rapidly because they do not receive enough energy to efficiently carry out photosynthesis and therefore there is no cell reproduction. On the other hand, when a DLI of 24 mol m^{-2} is reached, the effect of photosystem inhibition becomes significant and the RGR falls below the IQ-scores baseline.

In this regard, further studies testing the turnover of D1 protein of photosystem II in varying DLI values need to be addressed to confirm the extent to which the damage in the photosystem affect the RGR in duckweeds (Aro, Virgin, and Andersson 1993). In the past, it has been proven that DLI not only affects plant growth but many other plant traits (Poorter *et al.* 2019). In general, it was found in the literature that plant growth is limited below a DLI of 5 mol m^{-2}, whereas saturation of most traits occurs beyond 20 mol m^{-2}. The fact that the reported data fell within this range supports the idea that there is little difference in plasticity with respect to DLI between different plant species.

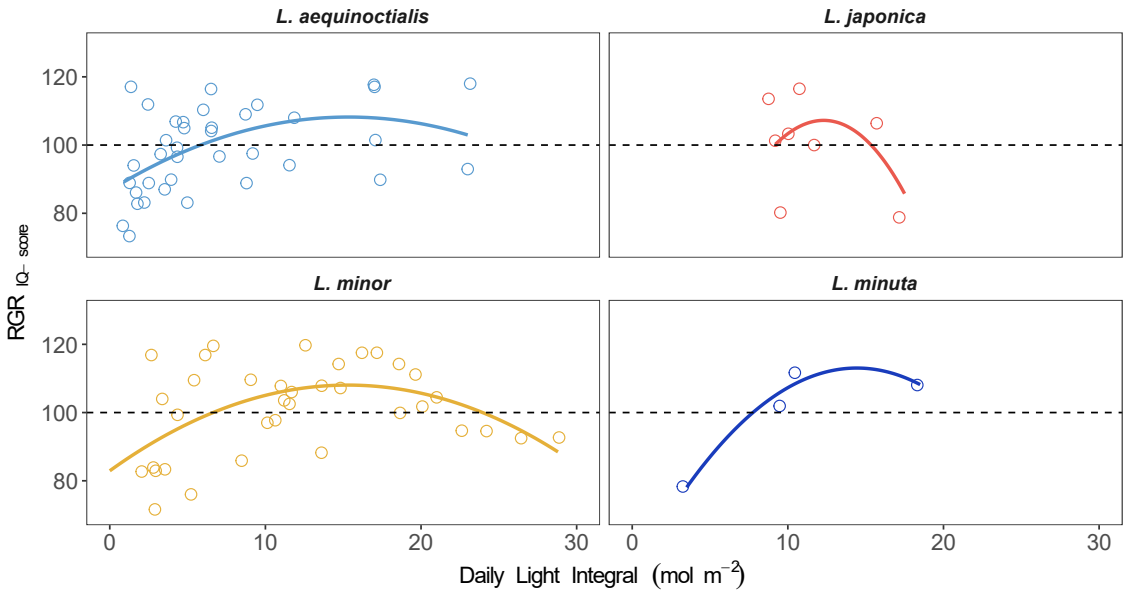

Figure 3-6 Daily light integral (DLI) as a parameter to assess the effect of light on the relative growth rate (RGR) of *Lemna* species.

Curves represent the general trend of the data upon non-parametric fitting of datasets. In all cases, RGR is expressed as the IQ scores from each independent experiment. Dashed line represents the RGR IQ-score baseline (= 100). Data for *L. aequinoctialis*, *L.* japonica, *L. minor* and *L.* minuta was retrieved from 10, 2, 7 and 1 independent experiments respectively.

The analysis of the DLI as a control variable, suggest that the effect of light on RGR of duckweed is independent of the duckweed species (different *Lemna* species in this case) being tested. The finding is useful for the design of engineered treatment systems based on duckweed biomass and using either natural or artificial light. In the former case, the DLI supports the potential use of solar energy as a source of radiation, thus reducing energy costs and dependence on fossil fuels. Furthermore, DLI monitoring would allow prediction of biomass growth in environments where the intensity and amount of light is not constant during the system operation period, making it possible to get more reliable systems for engineering applications (e.g., wastewater treatment). Moreover, the analysis of the data reveals that after exceeding a threshold value in DLI (7.5 mol m^{-2}), there is no major gain in terms of RGR so that energy savings can be considered during the design of the treatment process. For instance, by doubling the DLI from 7.5 to 15 mol m^{-2}, the energy cost doubles in a system of constant area while the RGR of the plants improves by only 5.8%.

The spectral composition of light, or the specific wavelengths of light that are present, is another parameter that can affect the growth of duckweed. Different pigments in the plant absorb different wavelengths of light, which can

stimulate or inhibit growth. Studies have concluded that red and blue light are the most effective in promoting growth and increasing biomass production. Duckweed grown in either blue or red light resulted in 10% and 31% increase in dry weight, respectively, in comparison to cultures under cool white light (Li *et al.* 2022). Moreover, the combination of red and blue light at different ratios does not significantly impact duckweed growth but has an effect on the accumulation of starch (Li *et al.* 2022; Petersen *et al.* 2022). These findings have important implications for optimizing duckweed cultivation for applications such as wastewater treatment with resource recovery (i.e., production of starch-rich duckweed biomass for animal feed).

3.3.3 Effect of nutrient supply on duckweed growth

In addition to light, plants need the right combination of nutrients to live, grow and reproduce. Both excess and deficiency of nutrients can cause problems to plant growth. Among the elements that plants need in relatively high amounts, macronutrients like phosphorus and nitrogen are of most interest due to their low bioavailability in aquatic environments (Vitousek *et al.* 2010). Although both nutrients are abundant in agricultural runoff and wastewater discharges, their presence in aquatic ecosystems is undesirable due to the potential development of anoxia and eutrophication in surface waters. As we well know, nitrogen and phosphorus fertilizers are usually added to soils to ensure that plants have adequate access to these essential nutrients. In plants, both nutrients are present either as ionic species or as constituents of biomolecules of great importance for the plant (Maathuis 2009). Like terrestrial plants, duckweeds can acquire significant amounts of inorganic nutrients through their root system (Cedergreen and Vindbadk 2002; Y. Fang *et al.* 2007). However, due to their aquatic nature and the fact that the fronds float directly on the surface of the water, nutrient absorption is mostly carried out from the underside of the frond (Ice and Couch 1987; Oron 1994). The extent to which duckweeds growth is affected by nitrogen and phosphorus supply is reviewed in the context of their use for wastewater treatment.

3.3.3.1 Nitrogen

Nitrogen (N) is involved in the synthesis of amino acids (the building blocks of proteins), chlorophyll and nucleic acids (DNA, RNA). It promotes the photosynthetic capacity and the growth of plant tissue, making it an important

performance factor (Barker and Bryson 2006; Novoa and Loomis 1981). N is present in wastewater in mineral ($N\text{-}NO_3$ and $N\text{-}NH_4$) or organic forms, but it is mainly absorbed by duckweeds in mineral form like ammonium and/or nitrate (Ding *et al.* 2018; Joy 1969). Both nitrogen deficiency and excess affect plant growth, but the extent to which it is affected depends on the species of nitrogen used for cultivation.

Nitrogen species

The forms of nitrogen in wastewater vary depending on the type of wastewater, pH and temperature (Caicedo *et al.* 2000). As a result of different biological and chemical processes, the main nitrogen compounds in wastewater are ammonium, nitrate and nitrite. Among them, ammonium is the main chemical specie, as it originates from the decomposition of organic matter. However, significant amounts of nitrate can be found in wastewaters and runoff resulting from industrial or farming activities requiring significant amounts of nitrate-based chemicals or fertilisers.

In the *Lemna*ceae family, the preference for ammonium over nitrate is still a subject of discussion. Most of authors have stated that ammonium is adopted as the first source of nitrogen by duckweed, because it is important for the synthesis of amino acids and proteins, and there is an associated saving of energy for the assimilation process (Oron 1994; Porath and Pollock 1982). However, ammonium assimilation is temperature sensitive and occurs only at pH values between 6 and 8 (Caicedo *et al.* 2000). It has also been pointed out that ammonium is a limited source of nitrogen due to its toxicity to plants (Joy 1969). When both nitrogen sources are available in the medium, the plant prefers to absorb ammonium, but can take nitrate when it is the only nitrogen source (Y. Fang *et al.* 2007). When a wider range of pH values is considered, some duckweeds species have shown predilection for nitrate over ammonium while the absorption of other macronutrients (P) was enhanced (Paterson *et al.* 2020).

The possibility of using both ionic species as a source of nitrogen to grow duckweeds is reflected in the number of publications studying the effect of different ammonium to nitrate ratios on the RGR of plants. In the case of *Lemna* species, when considering the sole effect of the nitrogen source on duckweeds RGR, it was found that there is no statistically significant difference ($p > 0.05$) between mean RGR values when using ammonia, nitrate or both nitrogen species in the culture medium (Figure 3-7). The differences between the culture conditions employed in studies considered in the analysis reveal that the preferred nitrogen source for each duckweed species is species-dependent and

may be determined by the acclimatisation of plants to the growing conditions, the nitrogen concentration and the N:P ratio.

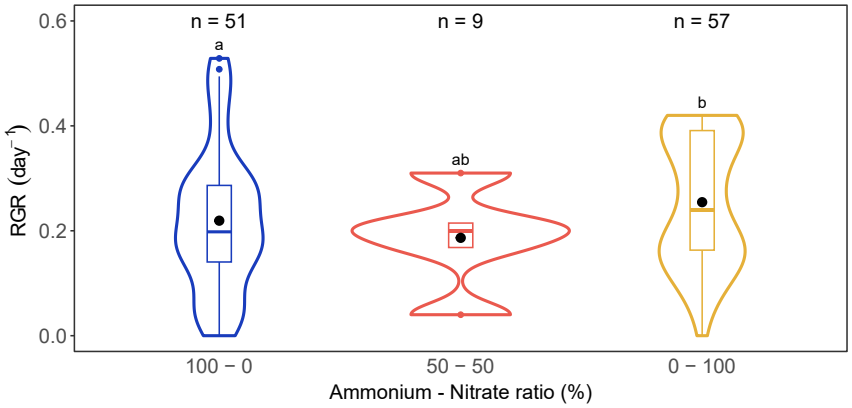

Figure 3-7 Differences in the relative growth rate (RGR) of duckweeds grown at different ammonium to nitrate ratios.

Violin plots represent the distribution of datapoints, and box plots represent the median, the 25th and 75th percentiles, minimum, maximum and outlying points. Black points mark the average RGR value for each NH4 - NO3 ratio. Only data for different species of the genus *Lemna* are presented. The number of observations per group (n) is presented on top of each plot. Lower case letters represent statistical significance ($p < 0.5$).

Nitrogen concentration

Total nitrogen concentration in domestic wastewater varies between 20 and 80 mg N L^{-1}. Ammonia is the major contributor to total nitrogen (~ 60%), followed organic nitrogen and nitrate (Henze *et al.* 2002). In some cases, total nitrogen concentration can be as high as 200 mg N L^{-1}, especially in wastewater from industries like aquaculture, and run-off water from agriculture (Korner *et al.* 2003). If duckweeds are intended to remediate wastewater from aquaculture, it is necessary not only to understand the effect of the nitrogen species present in the effluent of this industry, but also the effect of the concentration of nitrogen on the potential of these plants to grow.

Studies have shown that duckweeds have a wide range of tolerance to nitrogen concentrations, and the optimum concentration may vary depending on the species, growing conditions and nitrogen source (Figure 3-8).

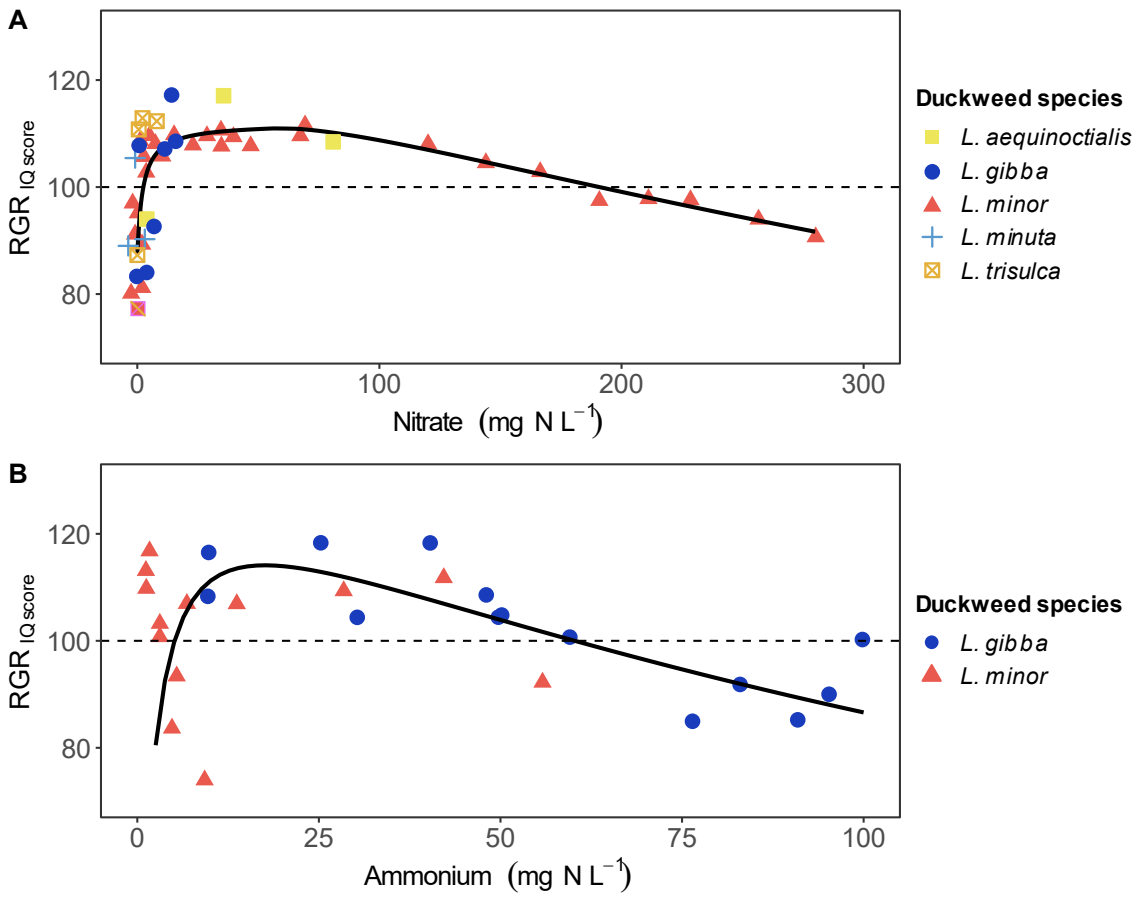

Figure 3-8 Effect of the supply of different nitrogen species on the relative growth rate (RGR) of duckweeds.

(A) Duckweeds grown in media with nitrate as only source of nitrogen; **(B)** duckweeds grown in media with ammonium as only source of nitrogen. Curves represent the general trend of the data upon parametric fitting of datasets to a substrate-inhibition kinetic mode **(Haldane 1965)**. In all cases, RGR is expressed as the IQ scores from each independent experiment. Dashed line represents the $RGR_{IQ\text{-}score}$ baseline (= 100). Data for *L. aequinoctialis*, *L. gibba*, *L. minor*, *L.* minuta and *L.* trisulca was retrieved from 1, 2, 3, 1 and 1 independent experiments respectively.

In general, when nitrate is the only nitrogen source, duckweed growth is supported at moderate concentrations (between 2-70 mg N L^{-1}, Figure 3-8 A). In presence of ammonium duckweed growth is supported at lower concentrations (between 5-15 mg N L^{-1}, Figure 3-8 B). However, higher nitrogen concentrations beyond RGR maxima are detrimental to duckweed growth in both cases. In addition to the above, when comparing the kinetic curves obtained with respect to N concentrations and the $RGR_{IQ\text{-}score}$ baseline, the RGR of the *Lemna* species is higher than the average RGR value in cultures grown with nitrate (2 – 195 mg N L^{-1}) than those grown in ammonium (5 – 60 mg N L^{-1}).

The fact that the RGR response curves to different nitrogen concentrations follow the same trend for nitrate and ammonium suggests that duckweed does

not have a particular preference for a specific nitrogen source, since, under certain conditions, both nitrogen sources benefit plant growth. What the results suggest is that to some extent ammonium has greater inhibitory effects on the growth of *L. gibba* and *L. minor* than nitrate. This can be explained due to potential ammonium toxicity.

There are two main mechanisms by which ammonium is toxic to plants. The first derives from the ease at which ammonium is transported across the cell membrane and the second from changes in pH as a result of ammonium uptake (Britto and Kronzucker 2002). Both ammonium (NH_4^+)and its non-ionised form, ammonia (NH_3), are transported into the membrane by low affinity transporters, which activity is upregulated at high external nitrogen concentrations, resulting in increased influx of nitrogen (Cerezo *et al.* 2001; Wang *et al.* 1993). As the ammonium uptake rate of the plant exceeds the assimilation rate or the storage capacity, the plant will actively transport ammonium back to the exterior (Hecht and Mohr 1990; Husted *et al.* 2000). As a result, the energy demand for this process (Britto *et al.* 2001), together with a reduced influx of other cations (e.g., K^+, Mg^{+2}, Ca^{+2}) and increased uptake of anions (Cl^-, $SO_4^=$) may limit overall plant growth (Gerendás *et al.* 1997; Roosta and Schjoerring 2007; Van Beusichem, Kirkby, and Baas 1988). A recent study on *Landoltia punctata* has shown that the coordination of carbon and nitrogen metabolism in duckweeds may act as ammonium detoxification mechanism, making duckweeds more tolerant to ammonium than other higher plants (Tian *et al.* 2021).

The second proposed mechanism by which ammonium is toxic to plants relates to external and internal pH changes (McQueen and Bailey 1990; Schubert and Yan 1997). Ammonium uptake by higher plants is linked to a cation counter-phase, in order to compensate for the charges on the cell membrane potential. This effect occasionally leads to the acidification of the culture medium in which the plant is growing (Brix, Dyhr-Jensen, and Lorenzen 2002; Ruan *et al.* 2007; Schubert and Yan 1997). Moreover, nitrate reduction in plants is considered a sink for excess NADPH production by photosynthesis. When an already reduced source of nitrogen is supplied, like ammonium, the accumulation of NADPH can indirectly affect the internal cell pH by altering the reactive oxygen species and enzymes involved in maintaining the pH balance (Guo *et al.* 2007). In duckweeds, it has been found that the optimum pH value for growth is around 7 (Caicedo *et al.* 2000; Jones *et al.* 2023; McLay 1976), so that, in cases where ammonium is the only available source of nitrogen, there is a double stress factor that reduces plant growth.

3.3.3.2 Phosphorus

Phosphorus (P) is a cellular constituent and an energy carrier. It is a component of the phospholipids that make up cell membrane and DNA, RNA, and ATP molecules (Maathuis 2009). As a cellular constituent, P supports plant growth, particularly in the development of roots that have a number of adaptive responses to acquire P from the soil and aquatic environments. P also promotes flowering, fruit setting and seed formation (Maathuis 2009). In wastewaters, phosphorus can be found in mineral form, mainly as orthophosphates (PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$,) and, in a smaller amount, in organic form. The form at which mineral phosphorus can be found strongly depends on water temperature and pH. Furthermore, phosphorus is a non-renewable resource, that is unevenly distributed in the world, hence the importance of its recovery and reuse from waste streams (Slocombe *et al.* 2020).

Phosphorus concentration

The occurrence of phosphorus in wastewater is closely related to the sources of phosphorus. Industrial, agricultural and household activities have the greatest impact on the amount of phosphorus found in wastewater. As such, phosphorus concentration can be relatively low, as in domestic wastewater (0.2 – 20 mg P L^{-1}) or high, as in effluents from intensive crop and livestock production (12 – 780 mg P L^{-1}) (Carrillo *et al.* 2020). A particular case is that of aquaculture where the large volumes of water used for fish production dilute the phosphorus concentration to values below 1 mg P L^{-1}. In aquatic environments (fresh waters), phosphorus is usually considered as the limiting nutrient controlling growth of photosynthetic organisms. Therefore, the effect of low phosphorus concentrations on duckweed growth needs to be assessed.

The results show that increasing phosphorus concentration of the culture medium improves duckweed relative growth rate, however, how this occurs depends on the nitrogen source used for the culture (Figure 3-9). On one hand, when ammonium is used as the sole source of nitrogen, *L.* minuta reaches a maximum growth rate at a phosphorus concentration of 1.5 mg P L^{-1}. Thereafter, higher P concentrations reduce the rate at which the plant grows (Figure 3-9 A). On the other hand, in the presence of nitrate, the RGR of different duckweed species reaches a maximum at a phosphorus concentration of 1 mg P L^{-1}. In this case, the growth rate is not affected by further increases in phosphorus supply, remaining always above the $RGR_{IQ\text{-}score}$ baseline (Figure 3-9 B).

In higher plants, phosphorus uptake and relocation are carried out by phosphorus transporter proteins (PHT) (Młodzińska and Zboińska 2016). There is evidence that PHT proteins can be induced either at low (high-affinity) or high (low-affinity) external phosphorus concentration (Bayle *et al.* 2011). In a recent study, 73 PHT highly conserved genes have been identified in different duckweed species (Zhao *et al.* 2021). Within these, 21 belong to the PHT1 subfamily, responsible for P acquisition from the environment, suggesting that P uptake by duckweed follows similar mechanisms to those previously reported in terrestrial plants.

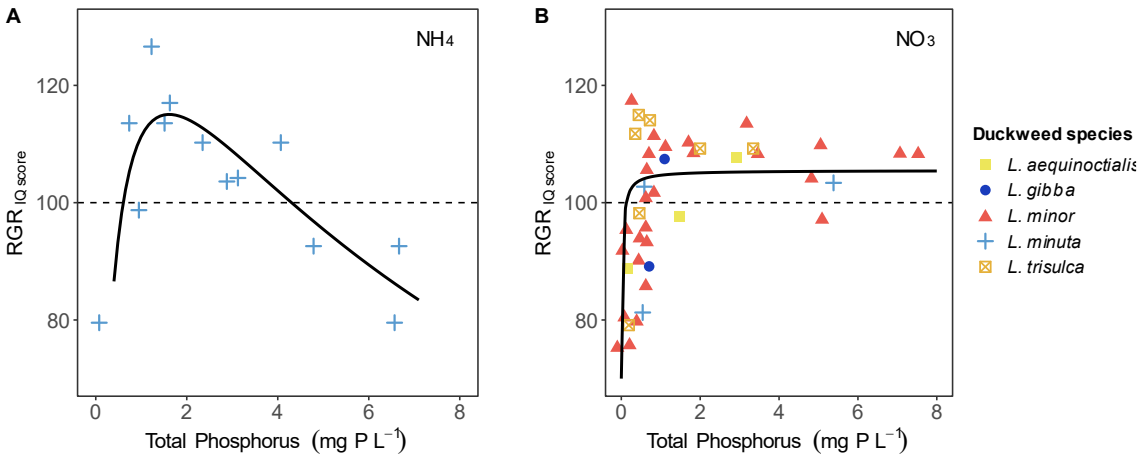

Figure 3-9 Effect of phosphorus supply on the relative growth rate (RGR) of duckweed in media with different nitrogen source.

(A) Duckweeds grown in media with ammonium as only source of nitrogen (median N:P ratio = 7.0); **(B)** duckweeds grown in media with nitrate as only source of nitrogen (median N:P ratio = 6.5). Curves represent the general trend of the data upon non-parametric fitting of datasets. In all cases, RGR is expressed as the IQ scores from each independent experiment. Dashed line represents the RGR IQ-score baseline (= 100). Data for *L. aequinoctialis*, *L.* japonica, *L. minor* and *L.* minuta was retrieved from 10, 2, 7 and 1 independent experiments respectively.

In general, an excess supply of phosphorus does not negatively affect plant growth, unless the concentration of phosphorus in the plant tissues exceeds 1% of the plant dry weight, a phenomenon known as P_i toxicity (Marschner 1996; Takagi *et al.* 2020). The estimated Michaelis-Menten constant (K_m) for low-affinity and high-affinity PHT transporters suggest that saturation condition is reached at external P concentration of 0.1 and 1.5 mg P L^{-1} respectively (Nussaume *et al.* 2011). Also, there is a close link between nitrogen and phosphorus uptake, in which PHT proteins interact with nitrogen transport proteins to maintain nutrient balance in the plant (Feng *et al.* 2017). As a result, high external phosphorus concentrations induce higher P uptake and consequently higher N uptake,

meaning that plant growth would not be affected by phosphorus but by the concentration and species of nitrogen being taken up. When ammonium is the only nitrogen source (as in Figure 3-9-A), at constant N:P ratios, the concentration of nitrogen can be such that duckweed growth is inhibited, as explained in the previous section.

Nitrogen and phosphorus supply balance

The nitrogen to phosphorus supply ratio (N:P ratio) is important for plant nutrition as it is a parameter that indicates the availability of phosphorus and nitrogen for plant growth. The assessment of the N:P ratio allows the condition in which plant growth can be limited by low availability of a nutrient, or the appropriate proportion of nutrients for biomass production to be stablished. The optimal N:P ratio for plant growth can vary depending on the plant species and the environmental conditions.

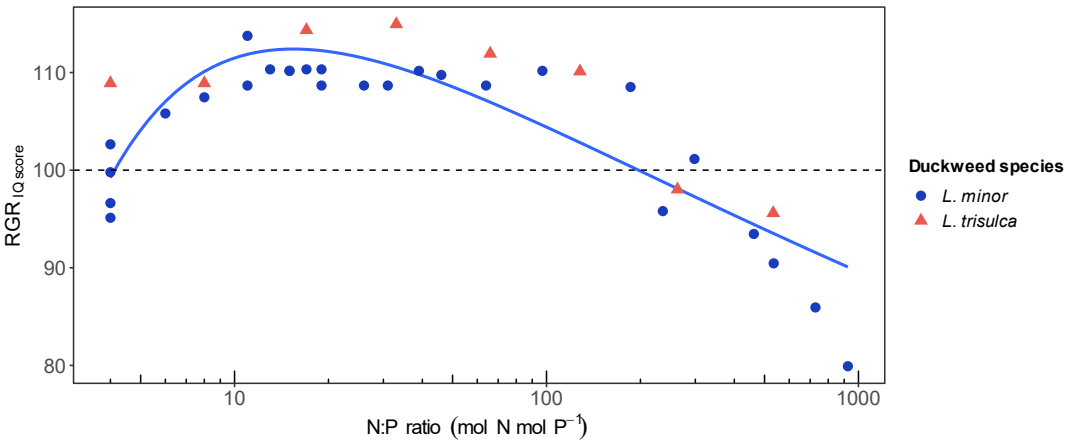

Figure 3-10 Nitrogen and phosphorus balance affect the relative growth rate (RGR) of duckweeds.

Nitrogen to phosphorus ratio (N:P ratio) was calculated only for those experiments carried out between 25 – 27°C using nitrate as the sole source of nitrogen. The curve represents the general trend of the data upon non-parametric fitting of datasets. RGR is expressed as the IQ scores from each independent experiment. Dashed line represents the RGR IQ-score baseline (= 100). Data for *L. minor* and *L.* trisulca was retrieved from 2 and 1 independent experiments, respectively.

In the case of two different *Lemna* species, we found that the optimal N:P supply ratio that maximises plant growth is 15:1 (Figure 3-10). At lower N:P ratios (the nutrient imbalance causes plants to undergrow due to lack of nitrogen, and at higher ratios the lack of phosphorus and excess nitrogen cause plant growth to be limited or inhibited. It has previously been reported that the optimal N:P molar ratio for plant growth is 15:1 (7:1 mass ratio) (Koerselman and Meuleman

1996) which is consistent with that found for *Lemna* species. Similar values were also found for grain legumes (Sadras 2006) and microalgae (Liu, Li, and Jia 2011). In wastewater, the molar N:P ratio varies on the type of wastewater and usually fluctuates between 11:1 and 22:1 (5:1 – 10:1, mass ratio) (de Godos *et al.* 2016; Wang *et al.* 2010), suggesting that wastewater can be used for duckweed cultivation without the need for additional nutrient supply. In conventional wastewater treatment nutrient balance is also an important parameter as it influences microbial activity responsible for the removal of organic matter and oxygen consumption.

3.4 Limitations of the study

The limitations of this study are noteworthy, particularly concerning the absence of data on additional factors influencing duckweed growth beyond temperature, light, and nutrient availability. Firstly, there is not enough studies addressing variables such as pH, plant mat density, interactions with other microorganisms, and others, making it challenging to draw meaningful comparisons and conclusions. Secondly, the difficulty in extracting quantitative data from existing literature can be attributed to a lack of standardization in result presentation, complicating the calculation of comparison indicators for a more comprehensive analysis. Lastly, the focus of the study may have been deliberately narrowed to ensure a more in-depth analysis of the specified variables, sacrificing a broader understanding of the multifaceted aspects affecting duckweed growth. Consequently, these limitations emphasize the need for future research to explore the interplay of a wider array of factors to enhance the comprehensiveness of findings in the field of duckweed cultivation and its applicability for wastewater treatment.

3.5 Conclusions

Duckweed-based systems for wastewater treatment and nutrient recovery have the potential to provide sustainable and cost-effective solutions for water pollution and nutrient management. However, careful consideration must be given to various factors that affect the growth and nutrient uptake of duckweeds, such as temperature, light, and nutrient supply. These factors can be controlled

and optimized through proper design, construction and operation of duckweed-based systems.

Temperature is a critical factor that affects the growth and development of duckweeds, and the selection of the appropriate duckweed species for the local climate is essential. Overall, optimal duckweed growth is achieved between 11.4 and 32.3°C. While temperature controls the rate of chemical reactions and influences the growth rate of duckweeds, light is the primary energy source for photosynthesis. Light intensity and photoperiod are crucial in regulating the total amount of radiation that reaches the plants and understanding the effect of these factors on duckweed growth can help optimize cultivation conditions and inform new technology developments, particularly for indoor cultivation using artificial light. Net amounts of light below 5 mol m^{-2} limit duckweed growth, whereas amounts 20 mol m^{-2} saturate plant's photosystems.

Nutrient supply, especially nitrogen and phosphorus, significantly affects duckweed growth and nutrient uptake. Nitrogen plays a crucial role in the growth and development of duckweeds, while phosphorus is an essential component of cellular structure and an important energy carrier in plants. In duckweeds, growth is limited only when nitrogen and phosphorus concentrations fall below 5 mg N L^{-1} and 1 mg P L^{-1}, respectively. The maximum concentration of nitrogen that duckweeds can tolerate is dictated by the nitrogen source. The concentration of both, phosphorus and nitrogen in wastewater can vary depending on the source of the wastewater; careful control of nutrient supply is essential for optimal duckweed growth, but typical N:P ratios (above 15:1, N:P) in wastewater are sufficient to support duckweed growth. The recovery of nitrogen and phosphorus from wastewater is particularly crucial due to its non-renewable nature and P uneven distribution in the world.

Duckweed-based systems can provide a sustainable solution for nutrient recovery from wastewater and overall nutrient management in catchments if use as an alternative for wastewater remediation. With the right design, construction and operation, duckweed-based systems can offer a cost-effective and sustainable alternative to conventional wastewater treatment methods. Overall, the implementation of duckweed-based systems for wastewater treatment and nutrient recovery requires a comprehensive understanding of the various factors that affect duckweed growth and nutrient uptake. By considering temperature, light, and nutrient supply in the planning and design of these systems, sustainable and cost-effective solutions can be developed for water pollution and nutrient management.

Chapter 4 : Preliminary considerations for duckweed cultivation

4.1 Introduction

Duckweed, a small floating aquatic plant, has garnered attention for its rapid growth rate and potential applications in wastewater treatment, sustainable biofuel production, and as a nutrient-rich feed in aquaculture and livestock farming (Baek, Saeed, and Choi 2021; Oláh, Appenroth, and Sree 2023; Vu *et al.* 2020). Their ability to thrive in a variety of environmental conditions and types of wastewater makes them an excellent candidate for ecological projects aimed at phytoremediation, where they can effectively remove pollutants from aquatic environments (Ekperusi, Sikoki, and Nwachukwu 2019; Liu *et al.* 2021). However, successful cultivation of duckweed depends on understanding several key factors that can influence its growth and productivity.

While environmental conditions such as temperature, light intensity, and nutrient levels in the water are crucial and can be adjusted to enhance growth (as discussed in Chapter 3), there are additional practical considerations that significantly affect the performance of duckweed cultivation systems (Coughlan, Walsh, Bolger, *et al.* 2022). These include selecting the appropriate duckweed species, managing the space available for growth to prevent overcrowding, and optimizing conditions based on the species' specific nitrogen preferences.

Moreover, although this study focuses on the primary cultivation and application considerations, it's important to recognize that other variables like water pH, salinity, and the presence of competitive microorganisms also influence duckweed growth . These factors, while not the primary focus here, have been studied in previous research and contribute to a comprehensive understanding of duckweed cultivation dynamics (Caicedo *et al.* 2000; Ishizawa *et al.* 2019; Roijackers, Szabó, and Scheffer 2004; Ullah *et al.* 2021).

Selecting the right duckweed species is a crucial first step in successful cultivation and application. Duckweed species differ greatly in terms of growth rates, nutrient needs, and tolerance to various environmental conditions (Sree, Sudakaran, and Appenroth 2015; Z. Zhao *et al.* 2014). For instance, certain species may flourish in cooler climates, while others are better adapted to warmer environments (El-Shafai *et al.* 2007; Papadopoulos and Tsihrintzis 2011; Y.

Zhao, Fang, Jin, Huang, Bao, Fu, *et al.* 2014). Additionally, some duckweed species excel in nutrient uptake and bioaccumulation, making them particularly effective for phytoremediation projects (Goopy and Murray 2003; Xiao *et al.* 2013; Xu and Shen 2011). This variation is not only seen across different species and genera but also within isolates and clones of the same species (Bog *et al.* 2022; Friedjung Yosef *et al.* 2022; Xue *et al.* 2012). The geographical origin of each duckweed species, along with the environmental conditions and water quality of their growth areas, significantly influences their morphological features and metabolic reactions to variable stimuli. Therefore, a detailed understanding of the unique characteristics and requirements of duckweed species, both at the isolate and clone level, is essential. This knowledge enables the development of customized cultivation strategies that optimize both yield and the specific functional benefits desired from the duckweed.

Area availability and the management of plant density also play pivotal roles in optimizing duckweed cultivation (Driever *et al.* 2005; Kufel, Strzałek, and Przetakiewicz 2018; Rejmánková, Rejmánek, and Kvet 1990). As duckweeds are floating plants, they cover the water surface, and their growth dynamics are largely dependent on the amount of available surface area. Overcrowding can easily occur when the surface area is insufficient relative to the amount of biomass. This leads to reduced light penetration, as the leaves overlap, blocking sunlight which is essential for photosynthesis (Driever *et al.* 2005; Petersen *et al.* 2022). Additionally, overcrowded conditions restrict access to essential nutrients, as more plants compete for the same resources (Kufel *et al.* 2018). These conditions can significantly reduce growth rates and decrease overall biomass productivity. Effective management of these factors is crucial to maintaining a healthy duckweed population and maximizing yield.

Finally, the choice of nitrogen source is crucial for promoting healthy and robust duckweed growth. Duckweed can utilize various forms of nitrogen, including ammonium, nitrate, and urea (Goopy and Murray 2003; Holst and Yopp 1979). The type and concentration of nitrogen available can significantly affect growth rates, protein content, and the overall health of the plants (Petersen *et al.* 2022). Moreover, the nitrogen source can impact the water chemistry, influencing other water quality parameters such as pH, critical for duckweed survival and growth (Caicedo *et al.* 2000). Furthermore, the choice of nitrogen source also affects phosphorus uptake. Opting for the right nitrogen type for a specific duckweed species can enhance the plant's efficiency in absorbing and utilizing phosphorus from the water. Since nitrogen and phosphorus are often absorbed concurrently, the nitrogen source can significantly impact the plant's effectiveness in nutrient remediation processes.

Selecting the right duckweed species, along with determining the optimal pot sizes, initial duckweed densities, and nitrogen source, is crucial when considering the assessment of additional environmental factors in future research. This careful selection facilitates the isolation of the effects of specific variables, simplifying the study of the fundamental mechanisms that govern duckweed growth and nutrient uptake. These preliminary considerations are essential for developing effective duckweed cultivation strategies, as they establish the foundation for thorough and efficient research and application.

4.2 Aims

The primary aim of this chapter is to evaluate various duckweed species for their efficacy in nutrient uptake and the production of nutrient-rich biomass, alongside exploring practical aspects of their cultivation. Specifically:

1) To identify and select the most effective duckweed species for phosphorus recovery from wastewater
2) To determine the optimal culture conditions by examining the impact of different surface areas and duckweed densities on growth and nutrient uptake.
3) To assess the impact of different nitrogen sources on the growth dynamics and phosphorus uptake efficiency of the chosen duckweed species

4.3 Methodology

Three distinct experiments were carried out in plastic pots filled with 100 mL of Hoagland's culture media under non-sterile conditions. The setup maintained a constant temperature of 25°C, with lighting provided by fluorescent tubes delivering 100 µmol photons m^{-2} s^{-1} across light-dark cycles of 12 hours each. All experiments were conducted in triplicate and lasted between 7 to 12 days. Throughout each experiment, the growth of the duckweed was closely monitored, and water quality parameters were measured at the start and conclusion of each trial. Detailed information about the experiments is described in Section 2.7.

In brief, the first experiment focused on observing the growth of six different duckweed isolates under the specified culture conditions. In the second experiment, the effects of water surface area on the growth and nutrient uptake of *Wolffia angusta* were examined by adjusting the initial stocking density

(modifying the initial biomass amount) and altering the available surface area (changing the net surface area accessible for plant growth). The third experiment assessed the impact of altering the nitrogen source on the nutrient reclamation behavior of *W. angusta*. Here, the standard nitrate in Hoagland's media was entirely replaced with ammonia. The outcomes, specifically regarding biomass growth and nutrient uptake, were compared against results obtained from the standard Hoagland's media setup. Duckweed growth rates were quantified daily by measuring changes in the total area of the fronds.

4.4 Results and discussion

4.4.1 Importance of duckweed selection

Considering that the primary objectives of the project are the removal of nutrients from wastewater and the production of nutritious duckweed biomass, a screening of multiple duckweed species was conducted to identify the best candidates for further experimentation.

In total, six duckweed species were tested, with most originally isolated from countries with tropical climates, each displaying distinct morphological features. The species included *Wolffia angusta* 8878, *Wolffia globosa* 8356, and four different clones of *Landoltia punctata*, specifically strains 9348, 9939, 0011, and KJA001 (Figure 4-1).

All plants were grown under the same conditions in standard Hoagland's media for 12 days, during which daily photographs were taken to monitor growth rates. Figure 4-2 illustrates both the increase in total frond area over the course of the experiment and the percentage of pot surface area covered by the plants over time.

Initially, each pot was inoculated with 50 mg fresh weight of each duckweed species. However, there were variations in the initial total frond area between the *Landoltia* isolates and *Wolffia* species. All *Landoltia* species exhibited similar growth patterns, starting with a total frond area of 4.0 cm^2 and expanding to cover the entire surface of the pots by the experiment's end, reaching 29.5 cm^2. In contrast, both *Wolffia* species began with a frond area of 1.4 cm^2 but showed different growth outcomes by the experiment's conclusion. *Wolffia globosa* ended with a total frond area of 12.0 cm^2, covering 39% of the pot's surface, while the other *Wolffia* species reached a total frond area of 16.5 cm^2, covering only 52% of the surface.

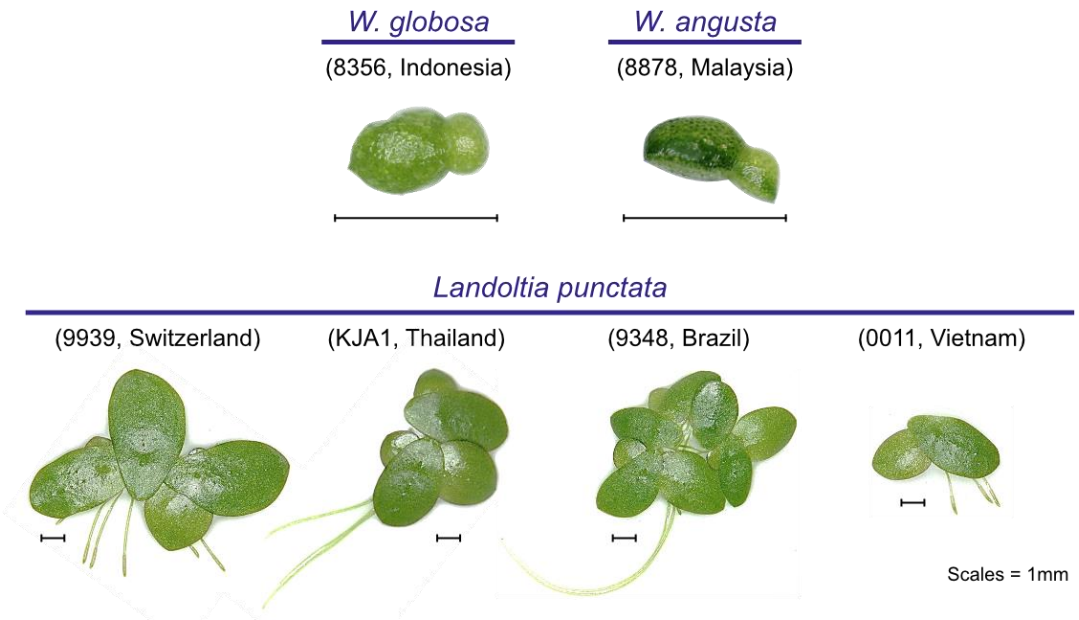

Figure 4-1 Comparative morphology of screened duckweed species and isolates

On the top left, *Wolffia globosa*, characterized by its round shape and small size. Adjacent to it on the top right is *Wolffia angusta*, noted for its elongated form. Below, the species *Landoltia punctata* is displayed, showcasing its distinct, broader leaf structure and multiple fronds. Scale bars indicate size comparison. Numbers and countries in brackets correspond to ID reference number in the duckweed repository and the country of origin of the isolate.

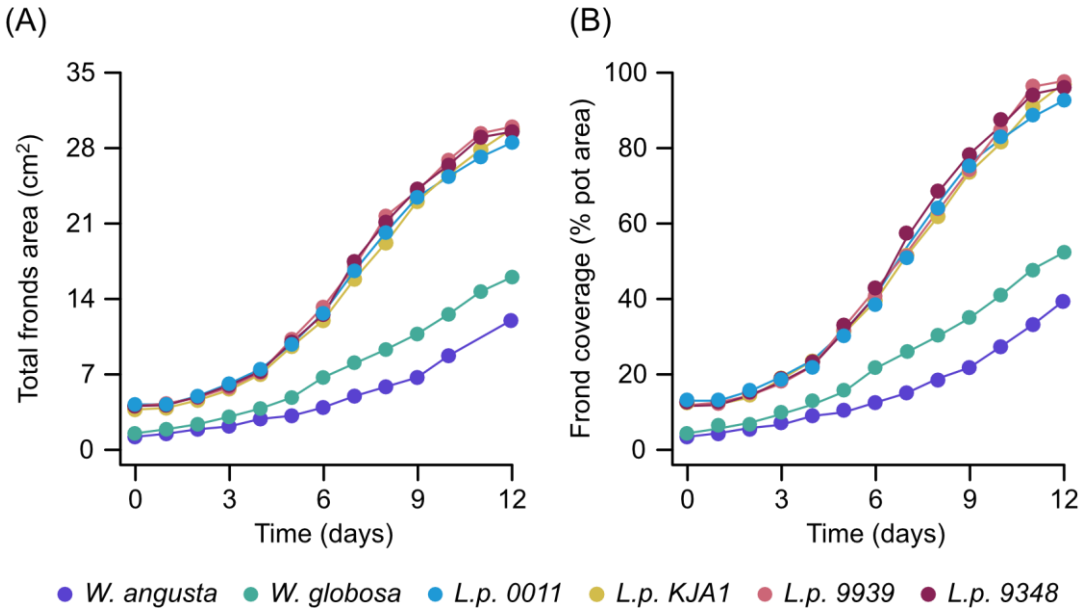

Figure 4-2 Comparative growth curves for different duckweed species

(A) Increase in total frond area, **(B)** Increase of frond coverage of the surface of water. Plants were cultivated in plastic pots with a diameter of 6.25 cm, each containing 100 mL of standard Hoagland's solution. *L.p.* refers to *Landoltia punctata*.

The relative growth rates of the plants, recorded between 0.190 and 0.253 per day (Table 4-1), showed that all clones of *Landoltia punctata* grew at a consistent rate. Interestingly, despite variations in total frond area, there was no statistical difference in the relative growth rates between *Landoltia* clones and *Wolffia angusta*. *Wolffia globosa*, however, exhibited the lowest growth rate among the species studied.

Upon completion of the experiment, the biomass was harvested, dried, and its protein content analysed (Table 4-1). The results indicated no statistical difference in protein content among isolates of *Landoltia punctata*, with an average protein content of 25.2% dry weight. The protein content of *Wolffia globosa* was similar, but *Wolffia angusta* showed a significantly higher protein content at 28.8% dry weight.

Table 4-1 Main results from the screening of different duckweed species

Parameter	Duckweed species (strain number)					
	L. punctata				*W. globosa*	*W. angusta*
	(9939)	(KJA1)	(9348)	(0011)	(8356)	(8878)
Relative growth rate, d^{-1}	0.228 [a]	0.228 [a]	0.239 [a]	0.230 [a]	0.190 [b]	0.253 [a]
Protein content, % dw	25.9 [a]	24.9 [a]	24.4 [a]	25.6 [a]	24.5 [a]	28.8 [b]
Final P concentration, mg L^{-1}	1.87	2.13	1.73	1.88	5.61	1.25
(% P removal)	(88.0) [a]	(86.3) [a]	(88.9) [a]	(88.0) [a]	(64.1) [b]	(91.9) [c]
Specific P uptake rate, mg g dw^{-1} d^{-1}	2.42 [a]	2.53 [a]	2.18 [a]	2.44 [a]	5.84 [b]	4.18 [c]

Values with the same letter are not statistically different (ANOVA + Tukey post-hoc test, $p < 0.05$)

The residual concentration of phosphorus in media was also measured at the end of the experiment (Table 4-1). The pots containing *Wolffia angusta* had the lowest phosphorus concentration in the medium, averaging 1.25 mg P L^{-1}. The removal of phosphorus was significantly higher than other species. *Landoltia* clones followed, reducing the phosphorus concentration in the medium to 1.90 mg P L^{-1}. *Wolffia globosa* showed the least phosphorus removal, only removing 64% of the initial phosphorus in the medium.

Despite having the lowest phosphorus removal, *Wolffia globosa* was the most efficient at phosphorus uptake per unit of biomass mass per day. With a

specific phosphorus uptake rate of 5.84 mg P g^{-1} d^{-1}, *Wolffia globosa* outperformed both *Wolffia angusta* and *Landoltia punctata* clones, the latter showing the least phosphorus removed per unit of biomass produced.

The results concerning relative growth rates align with findings from other studies, indicating that RGR varies not so much by genus or species but rather reflects the adaptation of individual isolates to specific local conditions (P Ziegler *et al.* 2015). This is particularly evident in the case of *Wolffia* species. Although the *Wolffia* family is known for the fastest growth rates among the *Lemna*ceae, the contrasting trends observed in *W. angusta* and *W. globosa* in this study — where one exhibited the highest RGR and the other the lowest among all tested duckweed species — highlight the significant variability within this group (Sree *et al.* 2015). Additionally, all clones of *Landoltia punctata* demonstrated uniform growth rates, suggesting genetic consistency and stability. This uniformity is particularly noteworthy given that these clones were originally isolated from diverse geographical locations and climate conditions, which might typically influence growth variations. This consistency under the experimental conditions that were employed indicates a robust genetic adaptation of these isolates.

Regarding protein content, all duckweed species tested had an average protein content of 26% by the end of the experiment, which is within the previously reported range of 10–37% for duckweed (Appenroth *et al.* 2017). Typically, *Wolffia* species are known to have protein contents about 10% higher than other duckweed species (Appenroth *et al.* 2018); however, the current results did not show significant differences, likely due to the abundant nitrogen availability in the Hoagland's media used.

In terms of phosphorus removal, the results emphasized the proficiency of *W. angusta* in removing phosphorus quickly compared to other species, although it was not the most efficient on a per-biomass basis. Instead, *W. globosa* excelled at removing more phosphorus per unit of biomass, which might be attributed to physiological adaptations that enhance phosphorus removal, storage, and recycling within the plant (Chaiprapat *et al.* 2005; Gérard and Triest 2014). Depending on the specific needs of a treatment system—speed or efficiency—*W. angusta* could be preferable for faster treatment outcomes, whereas *W. globosa* might be better suited for situations where space is limited and efficiency per biomass is critical.

To determine the most suitable duckweed species for further experimentation, all collected data was analysed by standardizing responses to Z-scores. This approach allowed the comparison of the deviation of each species' responses from the mean response across all tested species. As shown in Figure

4-3, achieving a balance between high biomass growth, robust protein content, and effective nutrient removal proved challenging for nearly all tested duckweed species. This trade-off between high growth and nutrient removal is a common phenomenon observed in other biological systems used for wastewater treatment (Paterson *et al.* 2020).

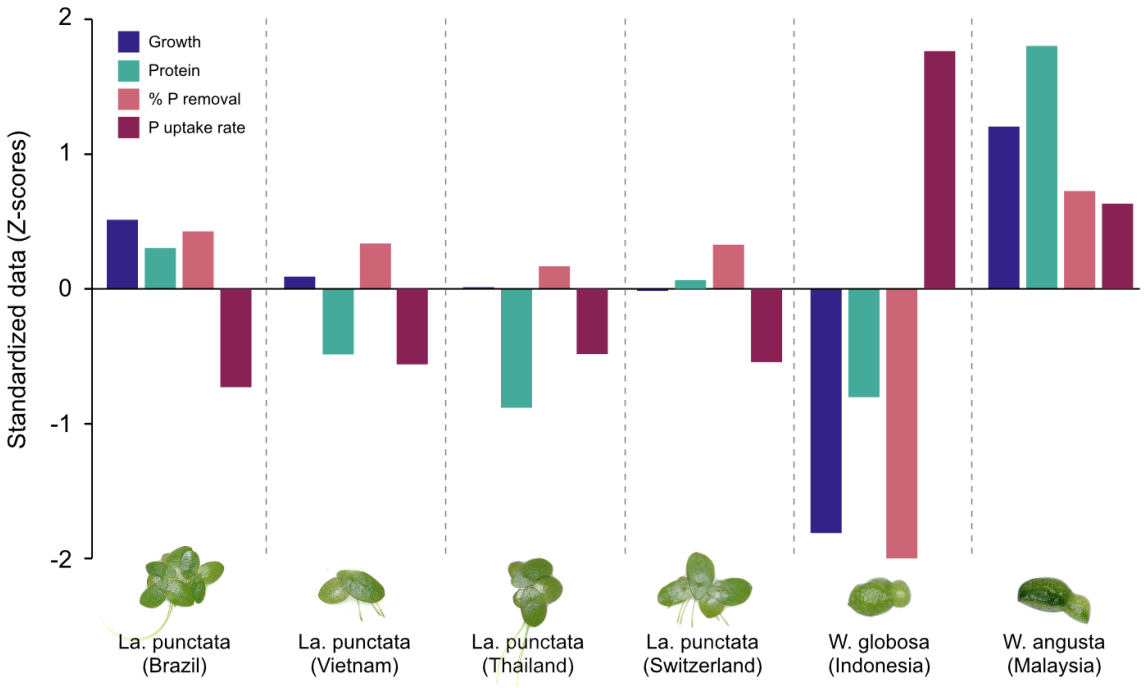

Figure 4-3 Standardised Z-scores for the main responses from the comparative analysis of different duckweed species

Z-scores were calculated for each tested duckweed species and isolate, focusing on four main responses: relative growth rate, protein content in the plants, percentage of phosphorus (P) removal from the media, and specific P uptake rate. A positive Z-score indicates that the response exceeds the average, whereas a negative Z-score suggests that the response is below the average of the responses for all duckweed species

Despite its efficiency in phosphorus uptake, *Wolffia globosa* performed poorly in other measured parameters, emerging as the least effective among the duckweeds tested. Among the *Landoltia* clones, the one originally isolated in Brazil showed the best overall balance in terms of growth, protein content, and nutrient removal. However, it was outperformed in all categories by *W. angusta*. This species not only excelled in biomass production but also demonstrated significant potential for phosphorus removal from water. The fact that all of *W. angusta*'s parameters were above the average for the screened species indicates a potential to uncouple growth from nutrient uptake (Paterson *et al.* 2020). This characteristic makes *W. angusta* 8878 a promising candidate for further research.

4.4.2 Consideration of surface area

In duckweed cultivation, the availability of surface area is crucial because these plants grow exclusively on water surfaces. To determine how this factor impacts growth, two studies were carried out using *Wolffia angusta*.

4.4.2.1 Varying stocking density

The first experiment introduced varying amounts of fresh duckweed biomass, ranging from 50 to 1000 mg, into pots with a fixed area of 17 cm^2. These pots were kept under standard growth conditions for 7 days. Growth was evaluated by comparing the dry weight of the duckweed at both the beginning and end of the experiment. Initially, when the biomass was between 50 and 200 mg, the duckweed fronds did not fully cover the pot surfaces. However, at 500 mg and above, the duckweed started to overlap and form multiple layers, as shown in Figure 4-4.

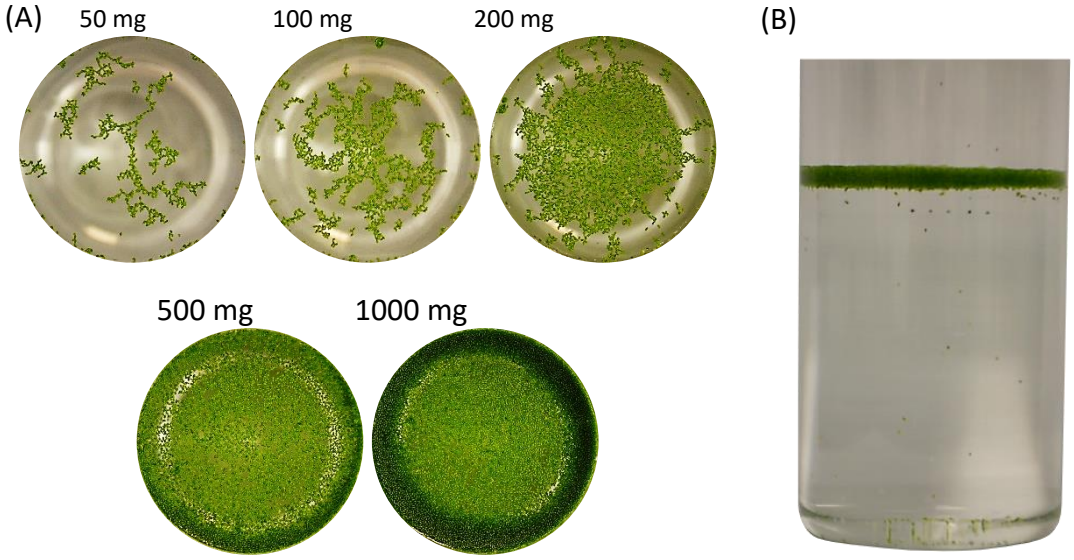

Figure 4-4 Initial state of cultivation pots with varied amounts of fresh *Wolffia angusta* biomass

(A) Top-View Images of the pots displaying varying degrees of water surface coverage at different initial amounts of fresh duckweed biomass. **(B)** Front-View Image of the pot inoculated with 1000 mg of fresh duckweed, illustrating how the fronds overlap from the outset of the experiment.

The findings revealed that the relative growth rate of *W. angusta* declined as the initial biomass increased, dropping from 0.272 d^{-1} at 50 mg to 0.100 d^{-1} at 1000 mg (Figure 4-5 A). This decline in growth rate did not align directly with the

increase in initial biomass. As for biomass productivity (Figure 4-5 B), it increased with higher initial biomass levels, ranging from 1.20 to 4.24 g dry weight per square meter per day. The lowest productivity occurred at the 50 mg initial duckweed biomass, and the highest at 1000 mg, with the changes showing a non-linear pattern across the biomass ranges tested.

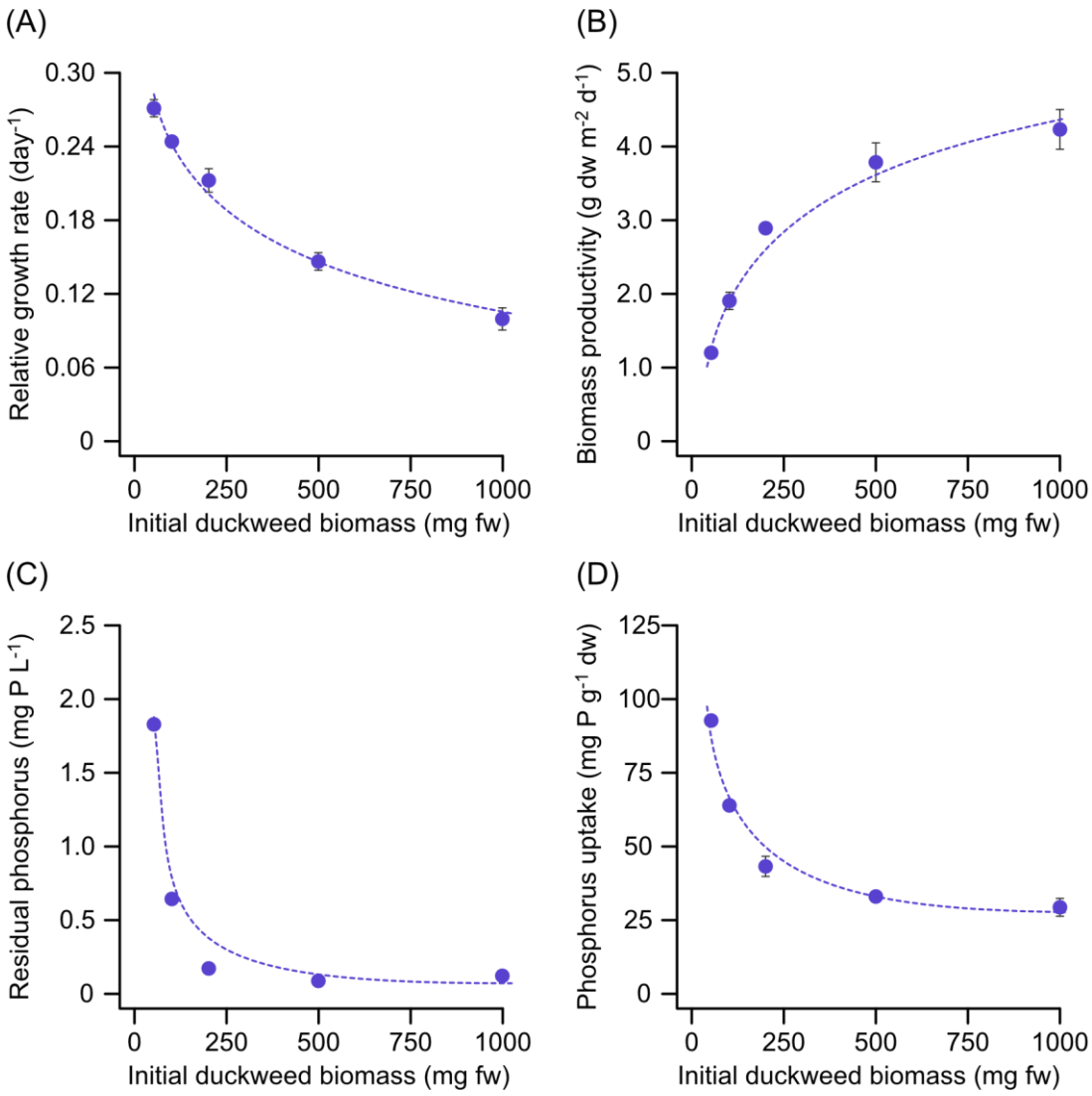

Figure 4-5 Impact of different initial quantities of *Wolffia angusta* in fixed-area cultivation pots

(A) Relative growth rate, **(B)** Dry biomass productivity, **(C)** Residual phosphorous concentration in the media at the end of the experiment, **(D)** Specific phosphorus uptake. The plants were grown in standard Hoagland's media in pots with a surface area of 17 cm².

In terms of phosphorus removal, the final concentration of phosphorus in the water was consistently below 1.8 mg L^{-1} in all experiments, achieving removal efficiencies above 87.9% (Figure 4-5 C). The least effective removal was seen with a 50 mg biomass, followed by 100 mg, which achieved 95.7% removal. At

200 mg of biomass and higher, the removal rates were statistically similar, averaging 99.1%. The pattern of specific phosphorus removal mirrored these trends, where higher initial amounts of duckweed resulted in less efficient nutrient removal, with rates varying from 29.8 to 93.0 mg phosphorus per gram of dry weight duckweed (Figure 4-5 D).

The analysis of the data reveals two distinct trends regarding the impact of increasing initial biomass on relative growth and biomass productivity. Initially, there is a marked decrease in growth rate as biomass increases, a phenomenon known as the crowding effect. This effect has also been observed in *Lemna minor* when pond surface coverage increases from 10% to 100% (Verma and Suthar 2015; Walsh, Coughlan, *et al.* 2021). More specifically, at higher biomass densities (e.g., above 118 g m^{-2}, corresponding to the scenario where 200 mg of fresh duckweed was added), *W. angusta* starts to stack up, forming multiple layers. This multilayer formation likely limits both light and carbon dioxide access for the lower layers and reduces nutrient availability (particularly phosphorus and nitrogen) for the upper layers, which in turn negatively impacts overall growth (Kufel *et al.* 2018). Previous research has indicated that the optimal biomass density for cultivating *Lemna minor* effectively is around 180 g m^{-2} (Driever *et al.* 2005; Lasfar *et al.* 2007). It's important to note that the ideal limit for biomass density varies between duckweed species, influenced by specific culture conditions as well as differences in duckweed morphology and size.

Conversely, the production of biomass shows a nonlinear increase, which can be attributed to a more effective utilization of space and resources by a larger initial population, despite the reduced relative growth rates at higher biomass levels. For instance, increasing the initial biomass fivefold, from 100 mg to 500 mg, led to a 40% reduction in relative growth rate, but a 99% increase in biomass productivity. This pattern suggests that although the growth rate of individual plants may slow due to higher crowding, the total biomass accumulation continues to rise. This increase is largely due to the exponential growth characteristics of duckweed when there is ample availability of nutrients (Zhang *et al.* 2014). Essentially, the larger initial mass provides a broader base for rapid expansion, overcoming the slower growth rates per individual plant by leveraging the overall enhanced capacity for nutrient removal.

Furthermore, a higher initial biomass of duckweed also increases the surface area available for phosphorus uptake, leading to lower phosphorus concentrations at the end of the experiment. The reduction in phosphorus concentration is proportional to the increase in initial duckweed biomass (Kufel *et al.* 2018; Verma and Suthar 2015; Walsh, Coughlan, *et al.* 2021). Specifically, for

Wolffia angusta, once the biomass exceeds 200 mg in the presence of an initial phosphorus concentration of 15 mg L^{-1}, as typically found in Hoagland's media, any additional biomass results in almost complete removal of phosphorus from the medium. This leads to a stabilization of biomass production and phosphorus concentration in the medium, explaining why specific phosphorus uptake also stabilizes at high biomass levels. Lower specific phosphorus removal rates at higher biomass levels suggest a limitation effect where the initial biomass exceeds the available phosphorus in the medium (Al-Nozaily, Alaerts, and Veenstra 2000; Paterson *et al.* 2020).

Selecting an appropriate initial amount of biomass is crucial for future experiments, as it significantly impacts both biomass growth and phosphorus removal. Initial biomass levels below 200 mg fresh weight are recommended. Particularly, an initial amount of 100 mg is advantageous, as it allows for sustained high relative growth rates (above 0.2 d^{-1}) and high phosphorus uptake (above 40 mg P g^{-1}), without depleting phosphorus in the medium over a 12-day period. This setting helps isolate the effects of other cultivation conditions from nutrient limitations.

4.4.2.2 Varying pot size

In the second experiment, the variable adjusted was the size of the pot, while the initial biomass of duckweed remained constant. Initially, 50 mg of fresh weight *Wolffia angusta* were introduced into pots with surface areas of 17 cm^2 and 30 cm^2. The experiment ran for 12 days, during which daily photos of the pot surfaces were taken, and water samples for phosphorus characterization were collected only at the start and end of the experiment.

The analysis of the pictures revealed different growth patterns for *Wolffia angusta* based on the pot size (Figure 4-6 A). In both pots, the total frond area at the start was 1.8 cm^2. Until day 9, the growth rate in both pots was statistically similar. However, from day 9 onward, the plants in the smaller pots grew more slowly, ending with a total frond area of 10.4 cm^2 compared to 16.1 cm^2 in the larger pot. This resulted in relative growth rates of 0.170 and 0.206 d^{-1}, respectively. In terms of surface coverage (Figure 4-6 B), initially, plants in the smaller pot covered 9.3% of the surface, while those in the larger pot covered 5.0%. By the end of the experiment, coverage increased to 67.6% in the smaller pots and 52.4% in the larger pots. On day 9, the coverage in the smaller pot was already 50.1%.

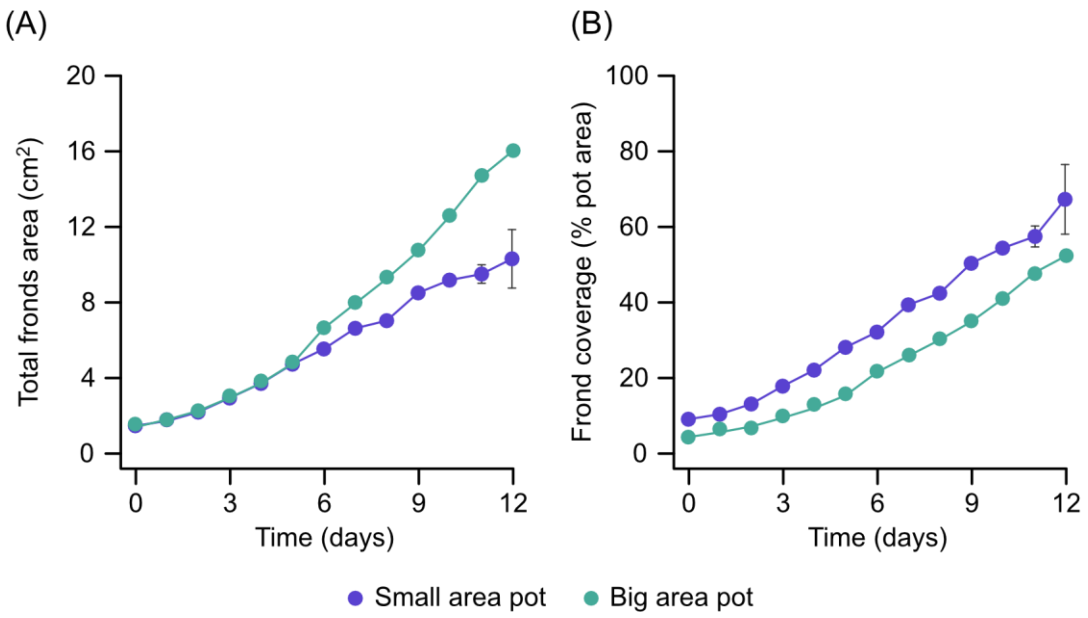

Figure 4-6 Effect of different cultivation pot surface areas on *Wolffia angusta* growth

(A) Increase in total frond area, **(B)** Increase in the plant's coverage of the water surface. A consistent starting weight of 50 mg of fresh duckweed was grown in standard Hoagland's media in pots with surface areas of 17 cm² (small pot) and 30 cm² (big pot).

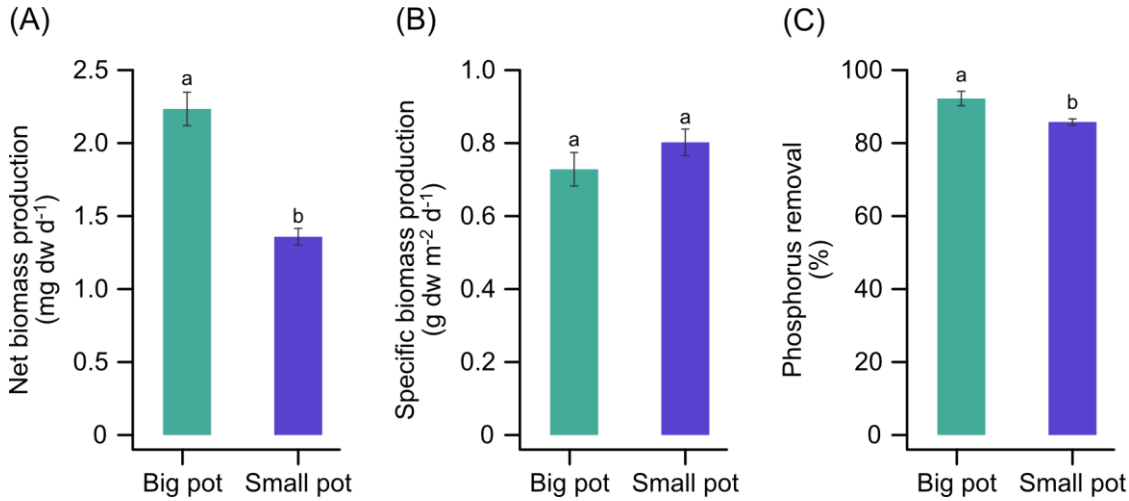

Figure 4-7 Production of biomass and removal of phosphorus by *Wolffia angusta* cultivated in pots of different surface area

(A) Net biomass production, **(B)** Specific biomass production, normalized to the available surface area for plant growth in each pot, **(C)** Phosphorus removal. A consistent starting weight of 50 mg of fresh duckweed was grown in standard Hoagland's media in pots with surface areas of 17 cm² (small pot) and 30 cm² (big pot). Bars with the same letter are not statistically different from each other ($p < 0.05$, ANOVA + Tukey HSD test).

Regarding net biomass productivity, pots with a larger surface area produced 65% more dry biomass per day than those with a smaller surface area (Figure 4-7 A). However, when considering the available area for plant growth, no significant differences in specific biomass productivity were found, which averaged 0.763 g m^{-1} d^{-1} (Figure 4-7 B). Additionally, phosphorus removal was more efficient in pots with a larger surface area, achieving 91.3% removal compared to 85.9% in smaller pots (Figure 4-7 C).

The results of the experiment suggest that larger pot sizes create a more conducive environment for the growth of *Wolffia angusta*, evidenced by higher relative growth rates in these conditions. This enhancement in growth rates can likely be attributed to reduced competition for space and resources, allowing the plants to expand more freely as they increase in size (Frédéric *et al.* 2006; Verma and Suthar 2015). The significance of this finding becomes particularly relevant when considering the scalability of biomass production systems. Larger cultivation areas could lead to enhanced growth efficiency and productivity, which is crucial in applications requiring high yields, such as biofuel or duckweed-based feed production.

However, while larger pots facilitate increased biomass production (65% more than in smaller pots), the experiment also demonstrated that once adjusted for pot surface area, the biomass productivity per unit area (0.763 g m^{-2} d^{-1}) did not show significant differences between small and large pots. This outcome suggests that while larger areas allow for more total biomass accumulation, the efficiency of biomass production per unit area levels out after a certain point. This indicates a diminishing return on space expansion, which implies that simply increasing pot size may enhance total biomass but not necessarily improve area-specific productivity. This finding is essential for optimizing space usage and requirement in duckweed cultivation.

Moreover, the experiment demonstrated that larger pots are more effective for phosphorus removal, as the increased biomass production offers more surface area for nutrient uptake. This finding is particularly important for phytoremediation projects, where the primary goal is to maximize nutrient removal from wastewater. Consequently, the results support scaling up pot sizes to enhance both plant growth and nutrient removal efficiency. However, careful management of these larger systems is crucial to prevent issues such as overcrowding, which could diminish the effectiveness of the cultivation space. Further experimentation using *Wolffia angusta* will be conducted in larger pots. These larger pots not only facilitate greater duckweed growth and more efficient phosphorus removal but also help manage plant density more effectively. This

setup allows for extended monitoring periods before the water surface becomes fully covered by plants, thereby providing better insights into the dynamics of duckweed growth.

4.4.3 Nitrogen source

In the final experiment, *Wolffia angusta* was cultivated using two different nitrogen sources: nitrate and ammonium. The plants were maintained under standard culture conditions for 7 days using standard Hoagland's media, which contained 110 mg L^{-1} of nitrogen in the form of nitrate, and a modified version of the media containing only ammonium as the nitrogen source. Throughout the experiment, the growth of the duckweed was closely monitored, and at its conclusion, the residual nutrient concentrations were analysed to determine the percentage of nutrient removal.

Results indicated a clear difference in growth rates depending on the nitrogen source. Plants grown in nitrate-containing media exhibited a significantly faster growth rate, with a relative growth rate of 0.22 d^{-1}, compared to those grown in ammonium-based media, which had an RGR of 0.16 d^{-1} (Figure 4-8 A). In terms of nutrient removal, there was also a notable difference in phosphorus removal efficiencies: the media containing nitrate achieved a 55.6% removal rate of phosphorus after 7 days, which was significantly higher than the 36.5% removal rate observed in the ammonium-only media, as shown in Figure 4-8 B. However, the removal of nitrogen from both types of media was relatively similar, averaging around 30%, indicating that the type of nitrogen did not significantly impact the overall nitrogen removal efficiency (Figure 4-8 C).

The experimental findings reveal that the source of nitrogen significantly impacts the growth and nutrient removal effectiveness of *Wolffia angusta*. Plants cultivated in nitrate-rich media demonstrated a notably higher relative growth rate than those grown in media containing ammonium. This observation suggests that *Wolffia angusta* is more efficient at utilizing nitrate, which suggests that this species is potentially better adapted or more efficient at utilizing nitrate compared to ammonium. Although this preference for nitrate is seen in other duckweed species (Mohedano *et al.* 2012; Smith *et al.* 2020), there is currently no consensus on a preferred nitrogen source across all Genus and species (Y. Fang *et al.* 2007; Schubert and Yan 1997).

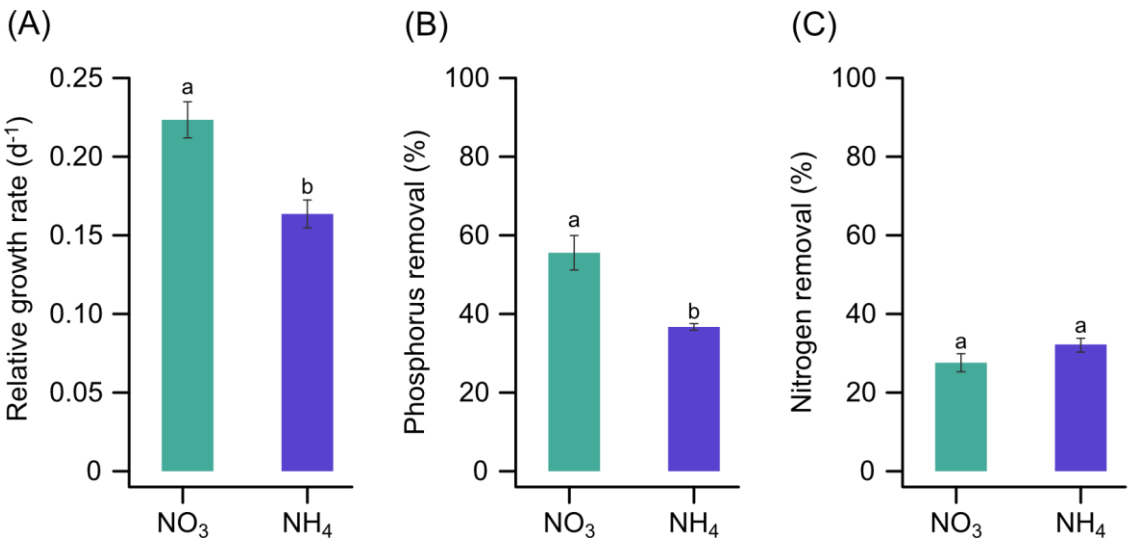

Figure 4-8 Effect of nitrogen source on the relative growth rate and nutrient removal efficiencies of *Wolffia angusta*

(A) Relative growth rate, **(B)** Phosphorus removal, and **(C)** Nitrogen removal of *Wolffia angusta* cultivated in standard Hoagland's media containing 110 mg N L^{-1}, either as nitrate (NO_3) or ammonium (NH_4). At the beginning of the experiment 100 mg of fresh duckweed biomass were inoculated in pots with surface area of 30 cm2, containing 100 mL of culture media. Bars with the same letter are not statistically different from each other ($p < 0.05$, ANOVA + Tukey HSD test).

Additionally, ammonium has been identified as more toxic to duckweeds than nitrate, potentially inhibiting growth by disrupting cellular functions, hindering photosynthesis, and altering the pH of the culture media (Körner *et al.* 2001). In scenarios where ammonium is the sole nitrogen source, duckweeds often release H^+ ions to balance the depolarization of the membrane potential, leading to a drop in media pH and creating acidic conditions that further restrict plant growth and nutrient absorption (Caicedo *et al.* 2000; Von wiren *et al.* 2001). To explore these dynamics further, additional experiments involving various ammonium concentrations and careful pH monitoring are needed.

Moreover, the experiments demonstrated a significantly higher phosphorus removal efficiency in nitrate-enriched media compared to those containing ammonium, underscoring nitrate's role in enhancing overall nutrient removal. This improved efficiency is likely due to the more robust growth of plants in nitrate-rich conditions, facilitating greater nutrient uptake. This relationship between rapid growth and effective nutrient absorption is important in wastewater treatment.

However, the similar rates of nitrogen removal between nitrate and ammonium sources, despite the disparity in growth rates, suggest an interesting dynamic. A possible explanation for this could be that ammonium, being a smaller and more mobile molecule, is absorbed more efficiently per unit surface area of

the duckweed (Y. Fang *et al.* 2007). This enhanced uptake efficiency might compensate the slower growth rates experienced under ammonium-rich conditions, leading to comparable nitrogen removal rates across both types of media.

4.5 Conclusion

Among the various factors influencing wastewater treatment (WWT) using duckweeds, critical parameters such as species and isolates, surface area availability, and nitrogen source must be prioritized in the design of treatment systems. Specifically, *W. angusta* 8878 exhibits significant potential for biomass productivity due to its adaptability to tropical conditions and its capacity to thrive at varying initial mat densities. This particular strain achieved a maximal relative growth rate of 0.275 d^{-1} at low stocking densities, although further assessment is needed regarding its nutrient removal efficiency. Notably, *W. angusta* demonstrated a distinct preference for nitrate over ammonium as a nitrogen source, achieving not only higher RGR but also enhanced phosphorus removal capabilities. This preference underscores the importance of selecting the appropriate nitrogen source to optimize both growth and nutrient uptake. Understanding these key variables is essential for effectively studying duckweed growth and its capacity for nutrient recovery, which in turn is pivotal for developing decentralized wastewater treatment systems that employ native duckweed species.

Chapter 5 : Unravelling the relationship between environmental conditions and duckweed cultivation to model batch nutrient reclamation systems

5.1 Introduction

In the search for sustainable aquaculture practices, understanding the intricate relationship between environmental factors and the growth performance of duckweed becomes of importance, especially in the context of nutrient recovery from fish farm effluents. The need to assess the effects of various environmental parameters on duckweed growth is underlined by its potential applications in nutrient cycling, wastewater treatment, and biomass production. Moreover, the end-use of duckweed biomass stands out as a promising sustainable solution to produce biofuels, biofertilizers and animal feed.

Duckweed species variability, cultivation density, and the presence of other microorganisms are perhaps the most studied biotic factors in duckweed cultivation systems. Comparative studies have been conducted to establish inter- and intra-genus as well as intra-species variability of duckweed under standard cultivation conditions (Körner and Vermaat 1998; Paolacci *et al.* 2021; Sembada and Faizal 2019; P. Ziegler *et al.* 2015). Similarly, the inhibitory effect of plant cultivation density (Demirezen, Aksoy, and Uruç 2007; Driever *et al.* 2005; Monette *et al.* 2006; Njambuya, Stiers, and Triest 2011), and the beneficial effect of the microbiota attached to duckweed roots on its own growth and the removal of organic matter have been investigated (Ishizawa *et al.* 2017; Ma *et al.* 2023; Yamakawa, Jog, and Morikawa 2018). In most of the cases, the results provide insights into the effects of each of these variables on duckweed growth but do not allow the establishment of models predicting their effects on other responses of duckweed-based systems, and far less their interaction with other cultivation parameters.

The abiotic factors that have been most investigated are temperature, light-related parameters, salinity, and nutrient concentration. Numerous studies have assessed the impact of these variables on the growth performance of duckweed, examining both their individual and combined effects (Iqbal *et al.*

2019; Lasfar *et al.* 2007; McCann 2016; Nesan *et al.* 2020; Yin *et al.* 2015). Efforts have also been made to model the responses of cultivation systems and derived kinetic parameters (Bal Krishna and Polprasert 2008; Calicioglu *et al.* 2021; Peng *et al.* 2007; Smith and Moelyowati 2001, 2001). However, most of the results have been focused on a single goal, whether it be biomass production, the nutritional value of the biomass, or water remediation.

Despite the historical utilization of various duckweed species and cultivation systems for wastewater treatment, the literature is deficient in reporting kinetic data for biomass growth and nutrient uptake. There is still a need for a comprehensive and integrated analysis on how variations in culture conditions, particularly temperature and nutrient availability, impact the outcomes of duckweed-based wastewater treatment (DWT) systems. Addressing this gap is essential to unravel the trade-offs involved in DWT systems, specifically in terms of nutrient-rich biomass production and nutrient removal from wastewater. Furthermore, the species-dependent nature of kinetics necessitates a thoughtful and empirical approach when evaluating new species.

5.2 Aims

This chapter addresses existing gaps in the literature by the specific examination of the duckweed species *Wolffia angusta*.

The main aim is to develop of a kinetic model tailored for the batch cultivation of duckweed under simulated tropical climate conditions, specifically:

1) To characterise the individual and combined effect of temperature and nutrient supply (P and N) on duckweed biomass growth and composition, and nutrient uptake and removal.
2) To estimate the optimal culture conditions for nutrient-rich biomass production and nutrient control to inform operation and design of DWT systems
3) To develop a kinetic model for the batch cultivation of *Wolffia angusta*

5.3 Methodology

To address the aims set out above experiments were conducted to estimate the effect of environmental culture conditions on the potential of duckweed for maximal nutrient uptake and the production of nutrient-rich biomass. *Wolffia angusta* 8878 (Landolt Duckweed Culture Collection, www.duckweed.ch) was

selected as model plant because in screening of different duckweed species originally isolated from tropical climate countries, this species gave the best results in terms of growth rate and phosphorus removal under standard cultivation conditions (for more details see Section 4.4.1). The plants were maintained on Hoagland's media (HM) in an incubator simulating average tropical climate conditions (25°C, 100 µmol m^{-2} s^{-1}, 12h light cycles).

The study primarily focused on the influence of temperature and nutrient availability. Light was not considered because due to its relatively little variability in tropical climate regions. Three separate experiments were conducted in triplicate to evaluate the individual impacts of temperature, phosphorus concentration, and nitrogen concentration on the growth of duckweed in batch cultivation, as outlined in Chapter 2.

Additionally, to further explore the interplay between environmental factors and their specific incidence on the growth and nutrient uptake of *W. angusta*, an additional set of experiments was conducted using a Box-Behnken composite experimental design. The levels at which factor was systematically examined are further described in Chapter 2. The results of these experiments were analysed using the Response Surface Methodology (RSM) with the objective of not only understanding but also optimizing the effects of the factors on the growth and performance of *W. angusta* for nutrient uptake and removal, based on different end-uses for duckweed biomass.

Finally, kinetic parameters for biomass production and nutrient removal were calculated upon experimental results and were used to generate a mathematical model for the batch cultivation of duckweed. At the end, the model was validated against the results of a new experiment, ensuring that the model accurately captured real dynamics. Calibration was performed by fine-tuning parameters to optimize model's predictions. Aligning them closely with observed outcomes.

5.4 Results and discussion

5.4.1 The impact of temperature on duckweed growth rate influences its capacity for nutrient removal

5.4.1.1 Higher temperature promotes duckweed growth

To assess the potential of *W. angusta* for biomass production and resource recovery from wastewater, temperature experiments were conducted for eight days in the range of 20 to 30°C, with a fixed photoperiod of 12 hours and

light intensity of 100 µmol cm^{-2} s^{-1}. Pictures of the cultivation pots from a top-view perspective were taken every other day to monitor the growth of the plants using image analysis (Figure 5-1 A).

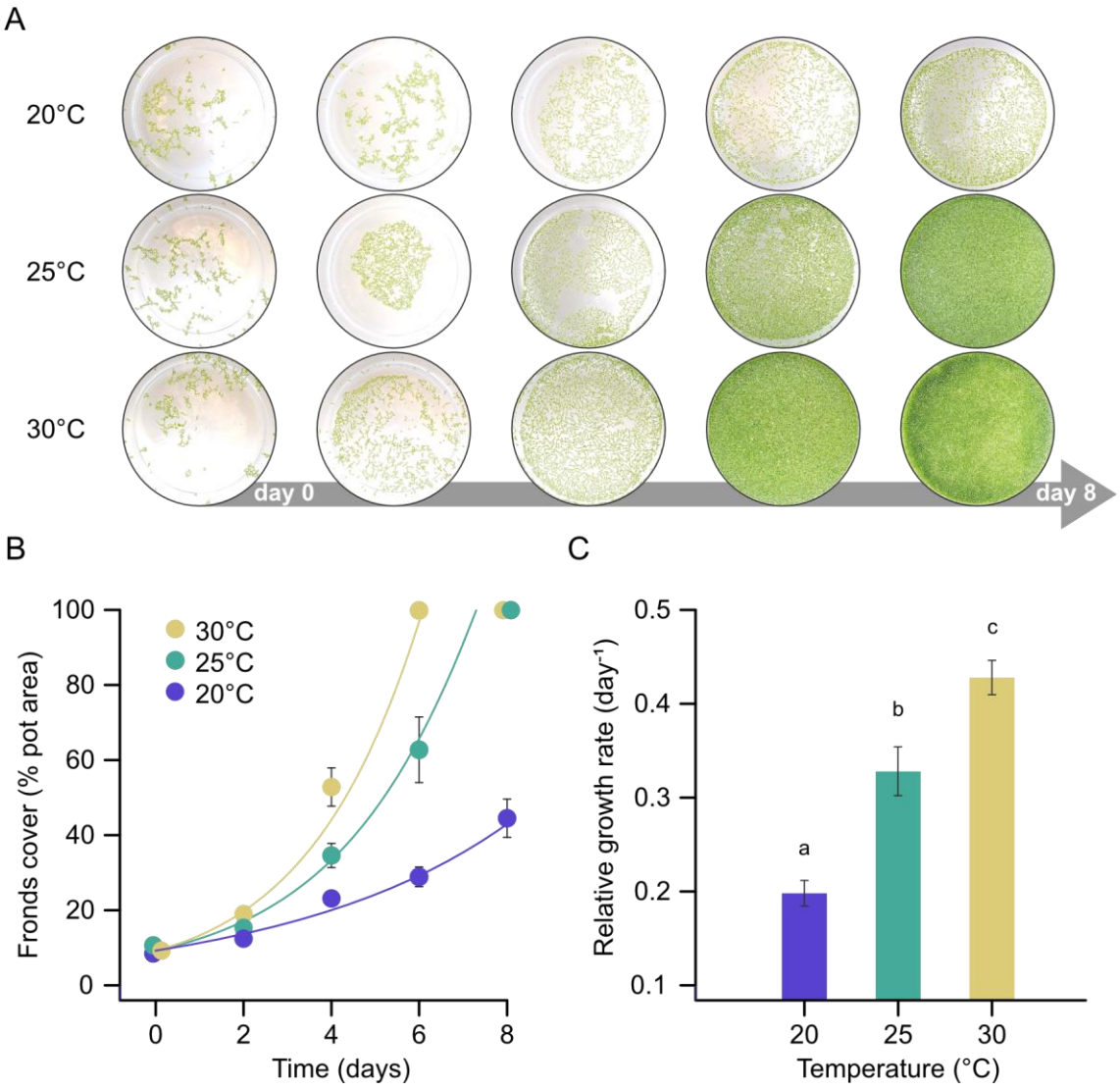

Figure 5-1 Effect of temperature on the *Wolffia angusta* growth in standard Hoagland's culture media.

(A) Temporal top-view photos of duckweed cultivation pots maintained at different temperatures. **(B)** Increase in duckweed frond coverage at 20, 25, and 30°C over time. The continuous lines represent the fitting of the data to an exponential growth model. **(C)** Relative growth rate of *W. angusta* at varying temperatures. Bars with the same letter are not statistically different from each other ($p < 0.01$, ANOVA + Tukey HSD test).

Duckweed growth, expressed as the relative increase in total frond area with respect to the cultivation pot area, also referred to as fronds cover percentage, is presented in Figure 5-1 B. The figure shows that plants grew at all temperatures tested with no lag-phase. Plants maintained at 20°C displayed the

least growth, reaching only 45% coverage of the pot area at the end of the experiment. In contrast, plants cultivated at 25°C and 30°C displayed more significant growth, completely covering the entire surface of the pots by the 7th and 6th days of cultivation, respectively. When the plants covered 100% of the surface, the duckweed fronds accumulated on top of each other, making it impossible to quantify their growth beyond this point through image analysis.

The relative growth rate of the plants, RGR, was obtained by fitting an exponential growth model ($y = a * exp^{(-RGR*x)}$) to the data collected before the plants completely covered the surface of the pots. Temperature was found to have a significant effect on plants RGR ($p < 0.01$). An increase in temperature within the investigated range resulted in an improvement of the RGR at which *W. angusta* grew, going from 0.197 day^{-1} at 20°C to 0.409 day^{-1} at 30°C (Figure 5-1 C).

Like any other photosynthetic organism, there are certain culture conditions at which duckweed growth is optimal. The correlation of relative growth rate and temperature was similar to other reports in the literature. For example, Wedge & Burris (1982) reported that duckweeds had a photosynthetic optimum temperature range, 30 to 35°C, at which responses for both growth and CO_2 uptake are maximum. Similarly, Lasfar *et al.* (2007) proposed an optimum range for duckweed growth between 23 and 28°C. In both cases, changes in temperature on either side of the optimal value significantly reduce the RGR.

Differences in the optimal temperature for duckweed growth arise, among other factors, from the acclimation of duckweed to culture conditions, and the disparities between duckweed species and isolates (P. Ziegler *et al.* 2015). The RGR of *W. angusta* 8878 falls within the range found for other *Wolffia* species and clones (0.255 - 0.457 day^{-1}) (Ziegler *et al.*, 2015), but further studies should be carried out to establish whether 30°C is the optimum culture temperature.

5.4.1.2 High temperatures boost nutrient removal by duckweed without affecting nutrient uptake

Media samples were collected from the pots every second day to measure orthophosphate (PO_4-P) and nitrate (NO_3-N) concentrations. The variation in nutrient concentrations throughout the experiments is presented in Figure 5-2.

It was observed that phosphorus depletion occurred faster as temperature increased from 20 to 30°C (Figure 5-2 A). By the end of the experiment, there was almost no phosphorus left in the media of pots maintained at 25°C and 30°C, while 37% of the initial phosphorus supply remained in the pots kept at 20°C.

There was still nitrate present in media at the conclusion of the experiments, but the amount remaining decreased as the cultivation temperature increased (Figure 5-2 B). Nitrogen removal percentages were 29%, 52%, and 67% at 20°C, 25°C, and 30°C, respectively. To account for the variation in P and N concentration over time, a four-parameter logistic (4PL) regression model was employed (fit data parameters in Appendix 3).

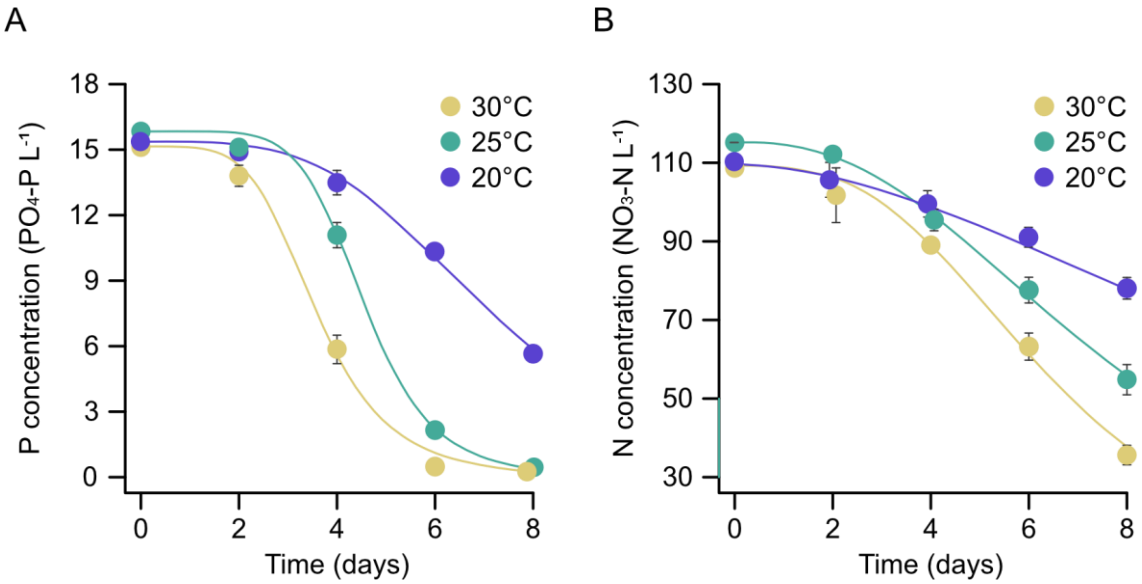

Figure 5-2 Nutrient depletion in Hoagland's media during the cultivation of *W. angusta* across different temperature conditions

(A) Change in orthophosphate concentration, expressed as mg PO_4-P L^{-1}. **(B)** Change in nitrate concentration, expressed as mg NO_3-N L^{-1}. The solid lines correspond to the fit of the data to a four-parameter logistic regression.

When considering the average nutrient removal rates, two different trends emerged. For phosphorus, the average phosphorus removal rate (PPR) was minimum at 20°C (1.21 mg P L^{-1} day^{-1}) and maximum at 25°C (1.91 mg P L^{-1} day^{-1}). Deviations above the latter temperature did not improve the rate at which P was removed ($p < 0.01$ ANOVA + Tukey HSD test). On the other hand, the average nitrogen removal rate (NRR) was found to increase with rising temperatures from 20 to 30°C, ranging from 3.93 to 9.14 mg N L^{-1} day^{-1} ($p < 0.05$, ANOVA + Tukey HSD test).

For both nutrients, the instantaneous removal rate gradually increased over time, reaching a peak value before starting to decline once again. In the case of phosphorus (Figure 5-3 A), temperature had a significant effect on the maximum phosphorus removal rate (PRR_{max}). Specifically, when the plants were cultivated at 25°C instead of 20°C, the PRR_{max} improved by 160%, with further

increases in temperature not significantly affecting PRR_{max}. Temperature also influenced the time at which PRR_{max} was achieved, occurring earlier as the temperature increased within the studied range.

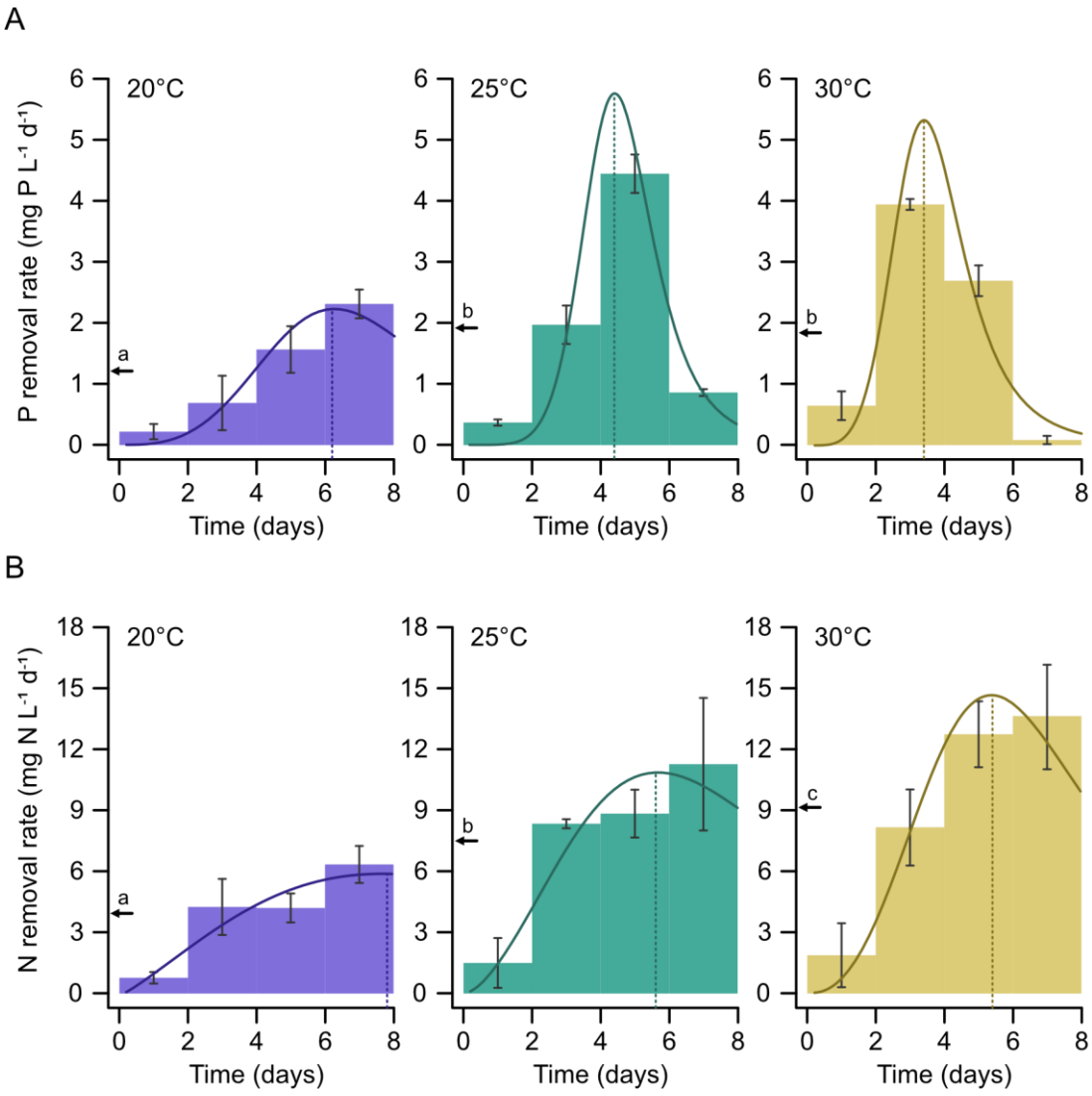

Figure 5-3 Nutrient removal rates during the cultivation of *Wolffia angusta* in Hoagland's media at different temperatures

(A) Phosphorus removal rates. **(B)** Nitrogen removal rates. The bars in the graph illustrate the daily experimental nutrient removal rate between 2 consecutive sampling days, while the continuous lines represent the instantaneous nutrient removal rate at any given time, derived from the four-parameter logistic equation used to model nutrient depletion. Dotted lines indicate the time at which nutrient removal rate was maximal. Average nutrient removal rates at each temperature are indicated by arrows on the left axis. Arrows sharing the same letter are not statistically different from each other ($p < 0.01$, ANOVA + Tukey HSD test).

The maximal nitrogen removal rate (NRR_{max}) consistently increased as the temperature went up, going from 5.9 to 14.7 mg N L^{-1} day^{-1} (Figure 5-3 B). Furthermore, higher temperatures also had an impact on how quickly NRR_{max}

was reached. At a temperature of 20°C, NRR_{max} was attained by day 8. However, at 25°C and 30°C, the peak removal rate was reached within a span of 6 days.

Overall, the nutrient removal process was predominantly mediated by plants, as evidenced by the absence of changes in phosphorus and nitrogen concentrations in control pots without plants (data not shown). Consequently, two primary factors are likely to influence nutrient removal: the quantity of actively growing plants at any given time and the impact of temperature on the metabolic activity of the plants. Since temperature also affects the relative growth rate of plants, the higher rates of nutrient removal observed at elevated temperatures may be attributed to the increased production of plants actively removing nutrients from the growth media.

To assess the magnitude of this impact, it is necessary to consider the specific nutrient uptake, i.e., the amount of nutrients removed per unit of biomass increase. By plotting the total frond area against the residual nutrient content in the pots at different time points (Figure 5-4 A,C), it is possible to calculate the specific nutrient uptake as the slope of the regression. At all temperatures, the reduction in total P and N in the pots was proportional to the increase in plants' total frond area, suggesting that the specific uptake of both nutrients remained consistent over time (linear regression, $R^2 > 0.95$). The results indicate that while the specific uptake of phosphorus remains largely unaffected by temperature, there is a detrimental effect on the specific uptake of nitrogen, as illustrated in Figure 5-4 B,D.

The Q_{10} temperature coefficients for uptake of both nutrients were 0.84 and 0.58, respectively. The term Q_{10} denotes the increase in metabolism accompanying a 10°C temperature rise (Mundim *et al.* 2020). A Q_{10} value below 1 signifies a decline in nutrient uptake as temperature increases. In our context, the Q_{10} values imply greater uptake of nutrients at lower temperatures, and that nitrate uptake by duckweed is more impacted by temperature than phosphorus uptake. This may be attributed to factors such as changes in the activity of transport proteins, alterations in the metabolic processes linked to nitrate and phosphate uptake, or other temperature-dependent cellular mechanisms.

In summary, our findings suggest that, at the tested concentration of nutrients, temperature primarily influences nutrient removal through its impact on the relative growth rate of plants rather than on their metabolic activity.

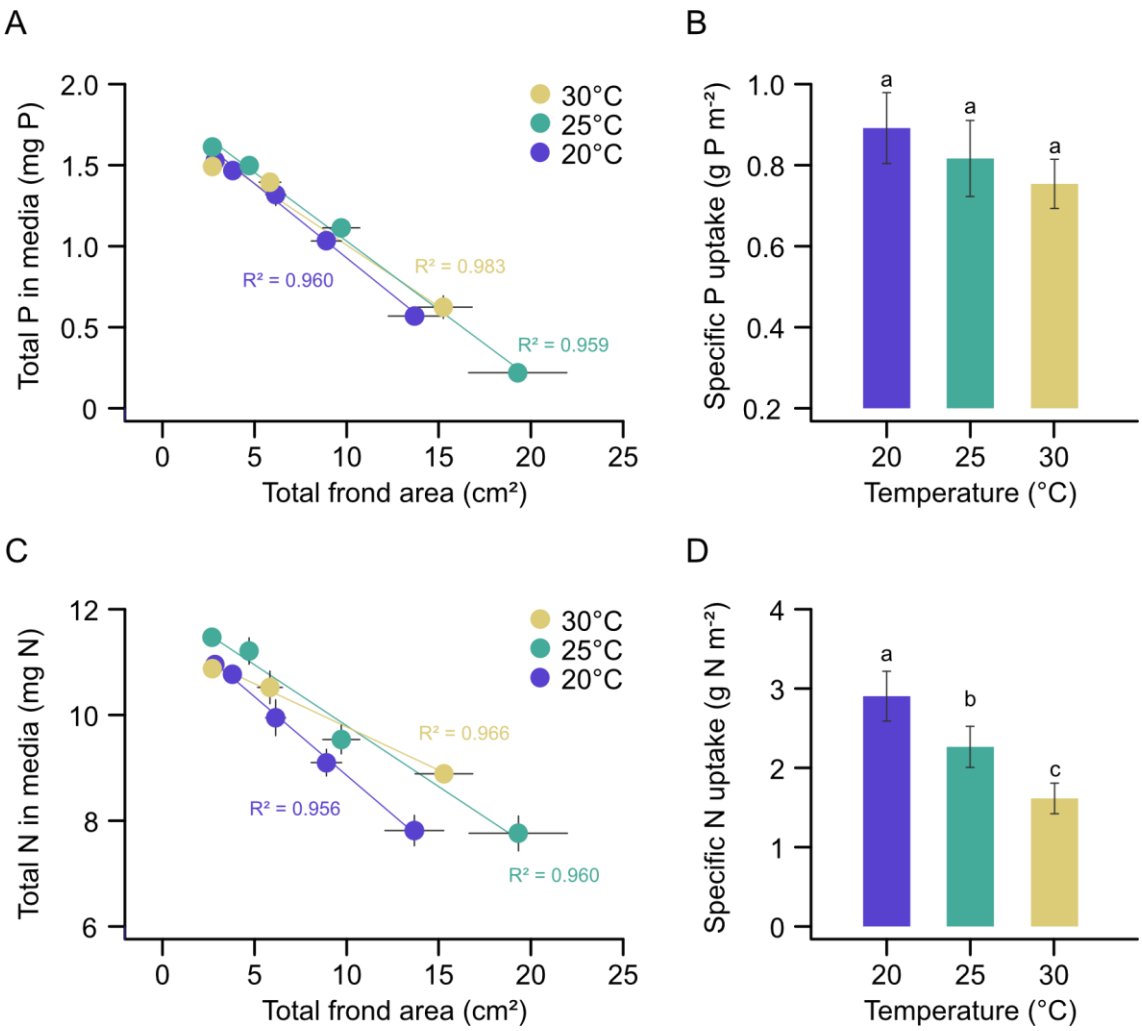

Figure 5-4 Nutrient uptake per unit area of *Wolffia angusta* grown at different temperatures.

(A, C) Correlation between duckweed total frond area and residual total phosphorus and nitrogen in pots throughout the experiments performed at 20, 25 and 30°C. **(B, D)** Specific phosphorus and nitrogen uptake by *W. angusta* at varying temperatures. Bars with the same letter are not statistically different from each other ($p < 0.05$, ANOVA + Tukey HSD test).

5.4.2 Nutrient supply has a higher impact on nutrient removal than on duckweed growth

5.4.2.1 Duckweed growth is solely impacted under low nutrient supply

To assess how nutrient availability affects the growth and nutrient uptake of *Wolffia angusta*, two experiments were carried out at a constant temperature of 25°C, over an 8-day period, maintaining a photoperiod of 12 hours and a light intensity of 100 µmol cm^{-2} s^{-1}.

In the first experiment, the impact of phosphorus supply alone was tested by adjusting the P concentration in the standard Hoagland's medium in the range of 0.2 to 8 mg P L^{-1}. The nitrate concentration remained constant at 100 mg N L^{-1}. Throughout the experiment, the experimental pots were monitored, and top-view images were taken every 2 days to quantify biomass growth through image analysis (Figure 5-5 A). In the pictures, there is no observable visual distinction among the plants cultivated at 4, 6, and 8 mg P L^{-1}.

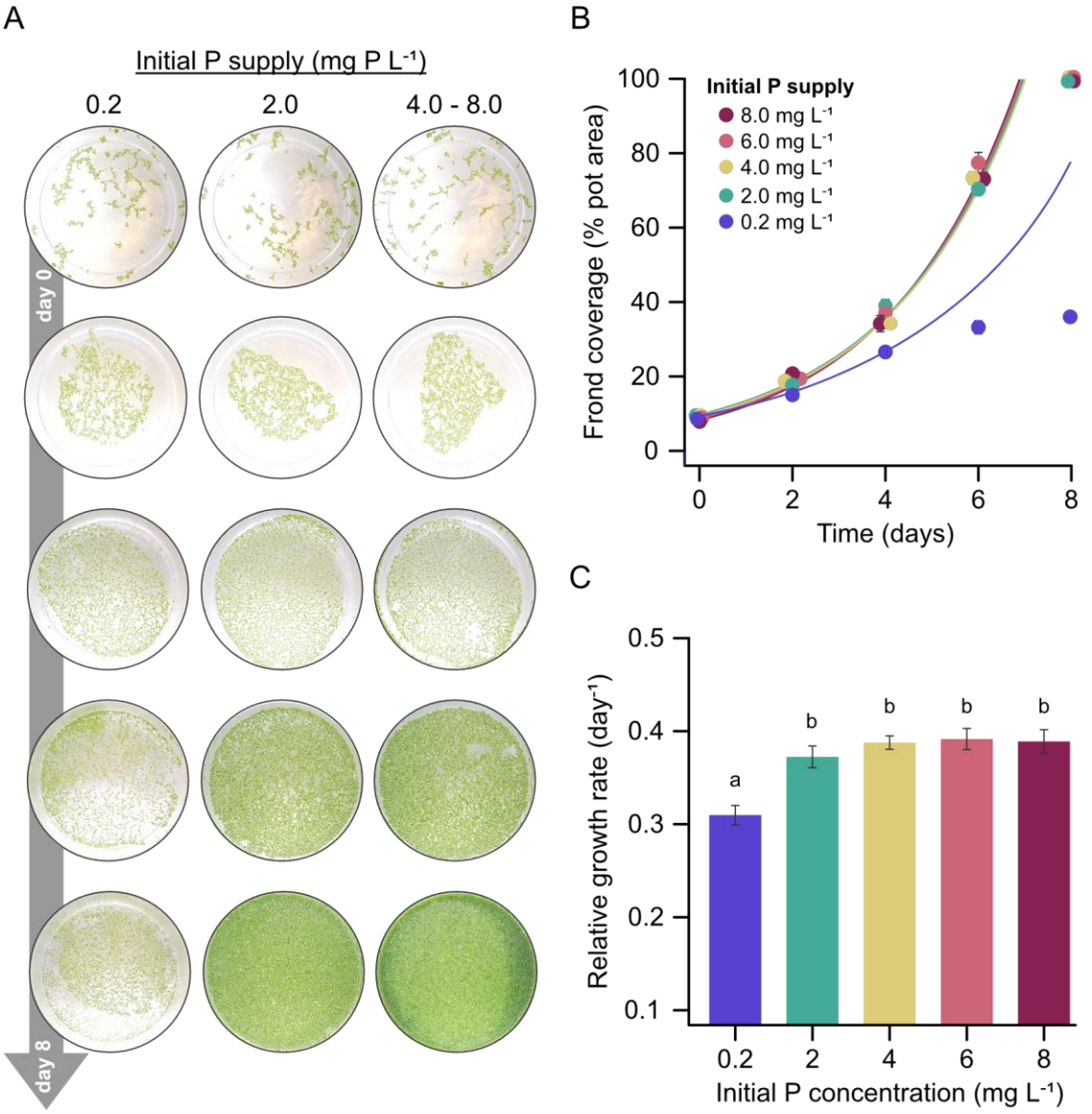

Figure 5-5 Effect of phosphorus supply on *Wolffia angusta* growth at 25°C

(A) Temporal top-view photos of duckweed cultivation pots maintained at different initial concentrations of phosphorus. **(B)** Increase in duckweed frond coverage at different initial concentrations of phosphorus, ranging from 0.2 to 8.0 mg P L^{-1}. The continuous lines represent the fitting of the data to an exponential growth model. **(C)** Relative growth rate of *W. angusta* at varying initial phosphorus concentration. Bars with the same letter are not statistically different from each other ($p < 0.05$, ANOVA + Tukey HSD test).

Wolffia angusta showed strong growth across all tested P concentrations, except for pots starting with 0.2 mg P L^{-1}. Under this condition, duckweed fronds began to bleach and settled at the bottom of the pots from day 6 onwards. By the end of the experiment, duckweed in these pots covered only 36% of the water surface, while at the other tested P concentrations, the plants completely covered it (Figure 5-5 B). Similar results to those obtained at higher P concentrations were obtained when plants were cultivated at 25°C, using standard Hoagland's media containing 15.5 mg P L^{-1} (as previously shown in Figure 5-1).

The relative growth rate of duckweed at each culture condition was determined by fitting and exponential growth model to the data collected during the plant's exponential growth phase and before they covered the entire pot surface. Except for plants cultivated at the lowest initial phosphorus supply, the duckweed's RGR was not influenced by the initial concentration of phosphorus ($p<0.05$, ANOVA + Tukey HSD test). Under these conditions, the RGR maintained an average value of 0.35 day^{-1}, whereas at the lowest P concentration, the RGR decreased by 26% (Figure 5-5 C).

In the second experiment, the sole influence of nitrogen was investigated by varying nitrate concentration in Hoagland's media from 0.5 to 20 mg N L^{-1}, while keeping phosphorus concentration at 15.5 mg P L^{-1}. In this case, top-view images of the pots revealed that duckweed growth was limited at nitrogen concentrations below 10 mg N L^{-1} (Figure 5-6 A).

At the lowest initial nitrogen concentration, a significant number of fronds settled at the bottom of the pots from day 4, resulting in a final frond cover of only 24%. As the nitrogen concentration was increased to 5 mg N L^{-1}, fronds still settled down from day 6, but the final frond cover increased to 43%. Further increments in the initial nitrogen supply led to a complete coverage of the pot surfaces (Figure 5-6 B), even when plants were cultivated with the standard nitrogen concentration of Hoagland's media (100 mg N L^{-1}, as previously shown in Figure 5-1).

Concerning nutrient concentration, the relative growth rate of the plants followed the same trend as in the phosphorus experiment. When the initial nitrogen concentration was 0.5 mg N L^{-1}, the RGR was 0.25 day^{-1}. However, when the nitrogen concentration was higher, the RGR value increased and remained constant, averaging 0.34 day^{-1} ($p<0.05$, ANOVA + Tukey HSD test, Figure 5-6 C).

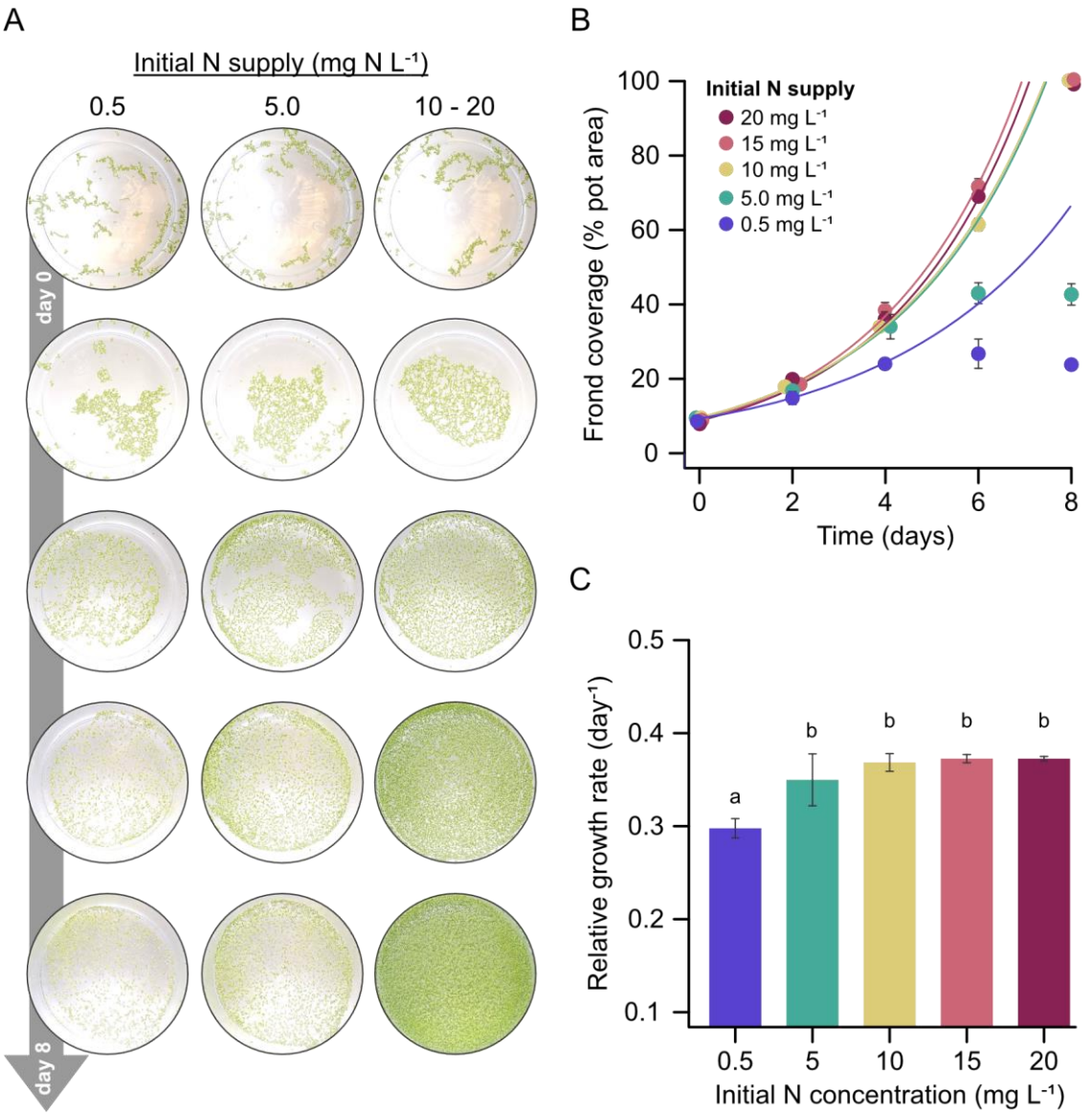

Figure 5-6 Effect of nitrogen supply on *Wolffia angusta* growth at 25°C

(A) Temporal top-view photos of duckweed cultivation pots maintained at different initial concentrations of nitrogen. There is no observable visual distinction among the plants cultivated at 10, 15, and 20 mg N L^{-1}. **(B)** Increase in duckweed frond coverage at different initial concentrations of nitrogen, ranging from 0.5 to 20 mg N L^{-1}. The continuous lines represent the fitting of the data to an exponential growth model. **(C)** Relative growth rate of *W. angusta* at varying initial phosphorus concentration. Bars with the same letter are not statistically different from each other ($p < 0.05$, ANOVA + Tukey HSD test).

Nutrient availability plays a crucial role in regulating plant growth and activity. In the case of duckweeds, they acquire nutrients directly from the water column in which they grow. Therefore, it is essential to evaluate how plants respond to nutrient availability, particularly under conditions of limited nutrient access. Phosphorus is the least available nutrient in fish farm effluents, with concentrations ranging from 0.05 to 1.0 mg P L^{-1} (see Table 1-1). Thus,

understanding how low concentrations of this nutrient affect duckweed growth is of importance.

Wolffia angusta growth was impacted only when the initial phosphorus concentration in the medium dropped below 2 mg P L^{-1}. Similar findings were reported by Lasfar *et al.* (2007) for *Lemna minor* cultivated at 27°C. However, the ability to grow at low P concentrations depends on various factors, including duckweed species, the plant's internal phosphorus reserves, and acclimation to the environment (Chaiprapat *et al.* 2005; Paterson *et al.* 2020). Even at the lowest P concentration tested, *W. angusta* managed to grow, suggesting that, plants used their internal phosphorus reserves to sustain growth. However, by the sixth day of cultivation, the plants started to die and settle at the bottom of the containers due to plant’s nutrient unbalance.

Nitrogen is found in fish farm effluents in two primary forms, ammonium and nitrate. The former is the main waste product in aquaculture systems, typically resulting from excretion process by fish and the decomposition of leftover feed. The latter, results from the use of oxidizing columns in recirculation aquaculture systems, that facilitate the conversion of ammonium into nitrate, a less toxic nitrogen species for fish. Due to water being recirculated to fish tanks, nitrate ends up being the predominant nitrogen species in final effluents. *W. angusta* prefers nitrate over ammonium as nitrogen source (see Section 4.4.3), hence the effect of nitrate concentration alone on duckweed growth was tested in the lab. The results indicate that *W. angusta* growth becomes limited when nitrate concentration falls below 10 mg N L^{-1}. This outcome aligns with previous findings for *Lemna minor* (Lasfar *et al.* 2007). The greater limitation imposed by nitrogen as opposed to phosphorus can be attributed to the higher demand for nitrogen in plants to support protein synthesis, chlorophyll production and reproduction.

Understanding the threshold concentrations of phosphorus and nitrogen that limit duckweed growth is crucial, as plant growth mediates nutrient removal within DWT systems. Inadequate plant growth can have a detrimental impact on the overall performance of systems designed for nutrient recovery from wastewater. The knowledge of the limiting P and N concentrations not only sheds light on the dynamics of duckweed growth but also enables the implementation of practical strategies. These may include optimizing biomass harvesting and adjusting hydraulic residence time in treatment systems, thus enhancing the effectiveness of nutrient recovery processes.

5.4.2.2 Nutrient removal is influenced by initial nutrient supply

In addition to duckweed growth, the removal of phosphorus and nitrogen at varying initial concentrations of both nutrients was also assessed. Water samples were collected every second day to measure residual phosphorus and nitrogen concentrations in the media. Phosphorus was quantified only in pots with varying initial phosphorus concentrations (Figure 5-7), while nitrogen was measured solely in pots with varying initial nitrogen concentrations (Figure 5-8). The complementary nutrient in each experiment was added continuously to ensure duckweed growth limitation by either phosphorus or nitrogen, respectively.

The variation in phosphorus concentration over the course of the experiment is presented in Figure 5-7 A. It was observed that phosphorus reached a minimum value more rapidly when the initial phosphorus supply was lower. Complete removal of phosphorus from the media only occurred when the initial phosphorus supply was 0.2 mg P L^{-1}. In all other cases, phosphorus removal ranged from 96% to 98% (Figure 5-7 B). To account for the fluctuation in phosphorus concentration over time, a four-parameter logistic regression model was applied to each dataset (fit data parameters in Appendix 3).

Both the average and maximum phosphorus removal rates (PRR) increased with each increment in the initial phosphorus concentration in the media. The PRR increased from 0.24 to 0.99 mg P L^{-1} d^{-1}, while the PPR_{max} jumped from 0.79 to 1.98 mg P L^{-1} d^{-1} when the initial phosphorus concentration increased from 2 to 8 mg P L^{-1} (Figure 5-7 C). In all cases, the PPR_{max} value was, on average, 2.2 times the PRR value. Additionally, the time it took to reach PRR_{max} was shorter when lower phosphorus amounts were supplied at the beginning of the experiment.

Temporal changes in nitrogen concentration, in pots containing different initial concentrations of nitrogen, revealed that duckweed could remove all nitrogen under all tested conditions by the end of the experiment (Figure 5-8 A). When the initial nitrogen concentration was below 15 mg N L^{-1}, nitrogen was completely removed by day 4 of cultivation. Otherwise, complete removal was achieved by day 6 (Figure 5-8 B).

The analysis of the regression models for nitrogen removal revealed a direct correlation between the initial nitrogen supply and both the average and maximum nitrogen removal rates (Figure 5-8 C, fit data parameters in Appendix 3). At an initial nitrogen concentration of 5 mg N L^{-1}, the NRR and NRR_{max} were 0.96 and 1.87 mg N L^{-1} d^{-1}, respectively. Conversely, at an initial nitrogen supply of 20 mg N L^{-1}, both removal rates peaked at 3.12 and 7.09 mg N L^{-1} d^{-1},

respectively. Even though no significant difference was observed when changing the initial nitrogen concentration from 10 to 15 mg N L^{-1}, the time required to reach NRR_{max} increased with each increment in nitrogen supply.

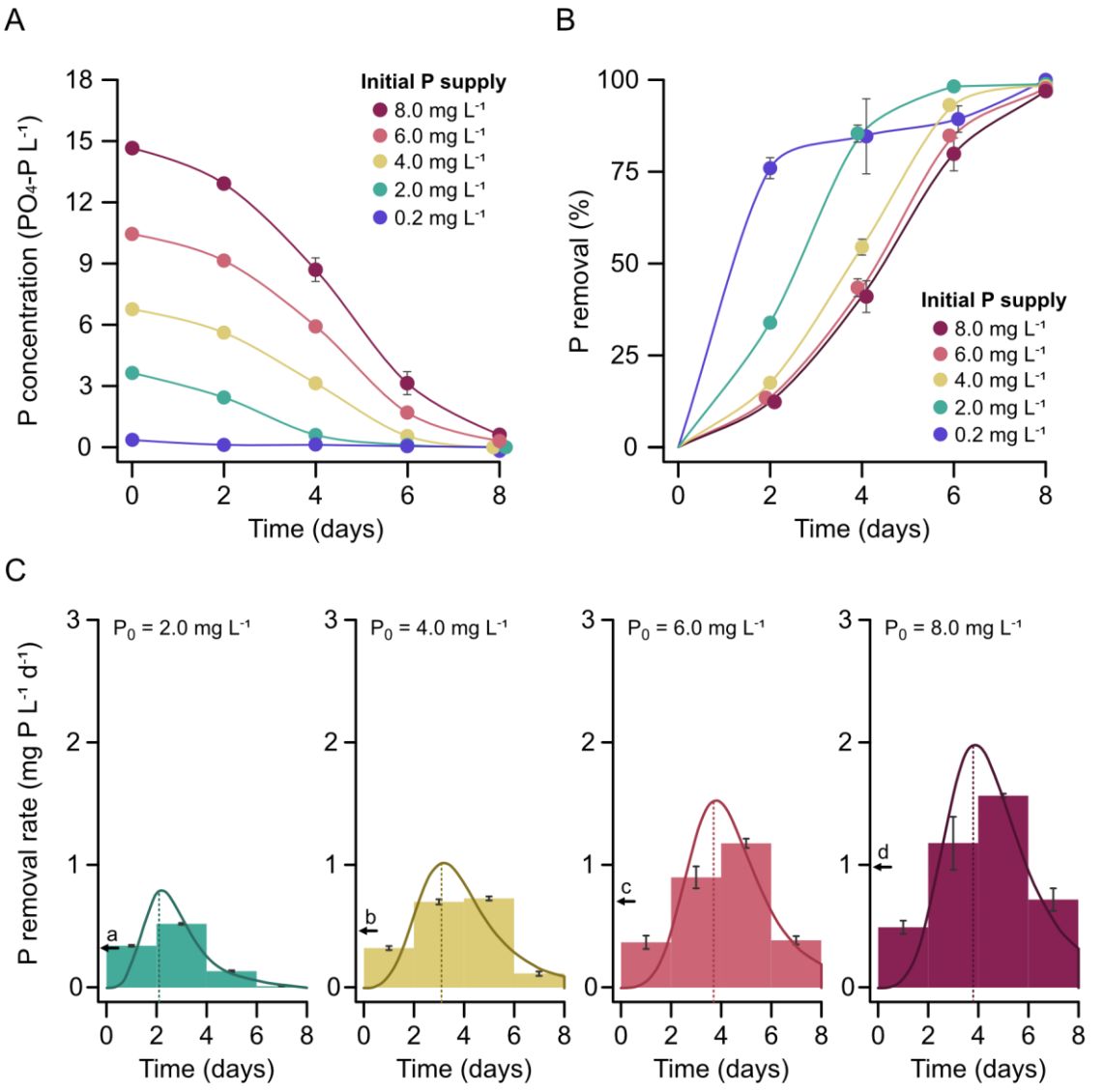

Figure 5-7 Phosphorus removal by *Wolffia angusta* cultivated at 25°C under different initial concentrations of phosphorus.

(A) Change in orthophosphate concentration, expressed as mg PO_4-P L^{-1}. The solid lines correspond to the fit of the data to a four-parameter logistic regression. **(B)** Percentage of phosphorus removed at each initial phosphorus concentration. **(C)** Phosphorus removal rates at varying initial phosphorus concentrations. The bars in the graph illustrate the daily experimental nutrient removal rate between 2 consecutive sampling days, while the continuous lines represent the instantaneous nutrient removal rate at any given time, derived from the four-parameter logistic equation used to model nutrient depletion. Dotted lines indicate the time at which nutrient removal rate was maximal. Average nutrient removal rates at each temperature are indicated by arrows. Arrows sharing the same letter are not statistically different from each other ($p < 0.01$, ANOVA + Tukey HSD test).

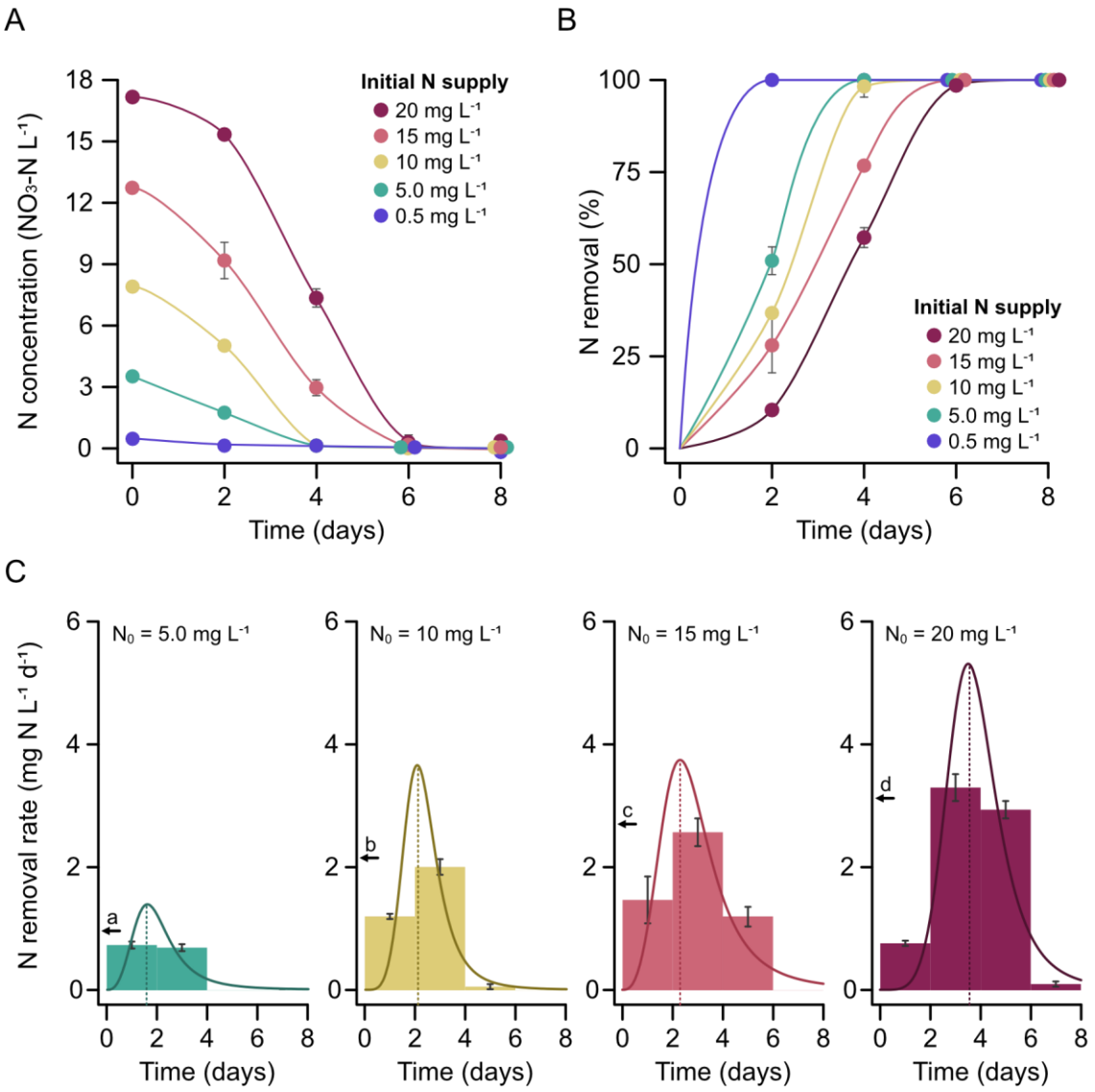

Figure 5-8 Nitrogen removal by *Wolffia angusta* cultivated at 25°C under different initial concentrations of nitrogen.

(A) Change in nitrate concentration, expressed as mg NO_3-N L^{-1}. The solid lines correspond to the fit of the data to a four-parameter logistic regression. **(B)** Percentage of phosphorus removed at each initial phosphorus concentration. **(C)** Nitrogen removal rates at varying initial nitrogen concentrations. The bars in the graph illustrate the daily experimental nutrient removal rate between 2 consecutive sampling days, while the continuous lines represent the instantaneous nutrient removal rate at any given time, derived from the four-parameter logistic equation used to model nutrient depletion. Dotted lines indicate the time at which nutrient removal rate was maximal. Average nutrient removal rates at each temperature are indicated by arrows. Arrows sharing the same letter are not statistically different from each other ($p < 0.01$, ANOVA + Tukey HSD test).

The removal of both phosphorus and nitrogen is affected by the initial concentration of each nutrient during the cultivation of *Wolffia angusta*. The removal rate of both nutrients increased with every single increment on the initial supply of nutrients. The maximal rates for P removal and N removal were reached

at the highest concentration tested, 8 mg P L^{-1} and 20 mg N L^{-1}, respectively. The results imply that the saturation point for either nutrient was not reached and that further increments in the initial supply of nutrients may further improve their removal rate.

The findings in the present study are similar to those previously reported for *Landoltia punctata* 7776 (Cheng, Bergmann, *et al.* 2002). When the initial concentration of both phosphorus and nitrogen was varied in the cultivation system, the highest PRR and NRR values were obtained at the highest initial supply of each nutrient (3.10 mg P L^{-1} d^{-1} at P_0 = 31 mg PO_4-P L^{-1}, and 22.9 mg N L^{-1} d^{-1} at N_0 = 240 mg NH_4-N L^{-1}). The values reported in the study are higher than those presented here for *W. angusta* due to several reasons. Firstly, the duckweed species differs. Secondly, the concentration of both nutrients is higher than that presented in this chapter. Thirdly, in the specific case of nitrogen, the nitrogen source used for the cultivation of duckweed was ammonium rather than nitrate.

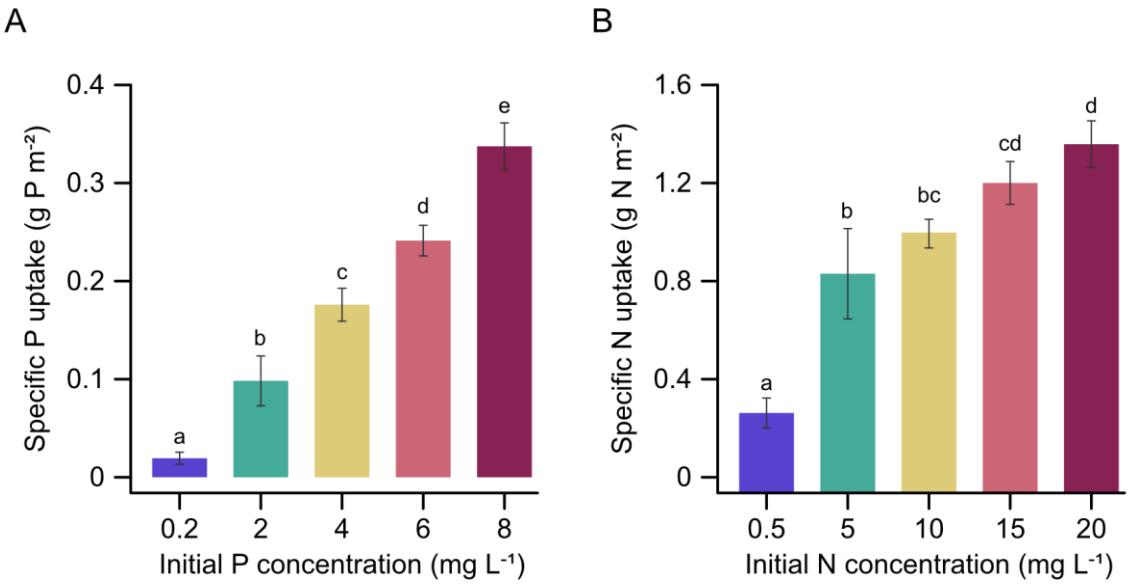

Figure 5-9 Nutrient uptake per unit area of *Wolffia angusta* grown at 25°C under different initial concentrations of phosphorus and nitrogen.

(A) Specific phosphorus uptake at varying initial concentrations of phosphorus (Constant nitrogen concentration, 110 mg N L^{-1}). **(B)** Specific nitrogen uptake by *W. angusta* at varying initial concentrations of nitrogen (Constant phosphorus concentration, 15.5 mg P L^{-1}). Bars with the same letter are not statistically different from each other ($p < 0.05$, ANOVA + Tukey HSD test).

Apart from the lowest initial concentration of phosphorus and nitrogen tested, biomass productivity was constant across all cases. However, nutrient removal rates varied depending on the initial concentration of each nutrient. The

overall disparity in nutrient removal among concentrations may arise from differences in how much phosphorus or nitrogen was removed per unit area of biomass (Figure 5-9). The uptake of both nutrients increased proportionally with their respective initial concentrations, suggesting again that saturation conditions for nutrient removal were not reached within the studied concentration range. It is assumed that duckweed reallocates nitrogen for biomass increase rather than biomass enrichment, as observed by Körner and Vermaat (1998). On the contrary, phosphorus is accumulated as a precaution against potential future depletion scenarios, as noted by Gérard and Triest (2014). Consequently, higher nitrogen concentration induces increased uptake for more biomass production, while higher phosphorus concentration leads to similar biomass production but with varying levels of phosphorus content.

These findings highlight the ability of duckweed to adapt to fluctuations in nutrient availability within a water treatment system. Notably, *W. angusta* exhibits higher tolerance to phosphorus stress compared to nitrogen stress. Additionally, it is important to acknowledge that while both nitrogen and phosphorus are essential for biomass production, the demand for nitrogen in plant maintenance and reproduction is higher. Moreover, phosphorus must be considered for the production of phosphorus-rich biomass. In conclusion, maintaining an adequate nutrient supply is crucial for supporting healthy plant growth and optimizing nutrient removal and uptake efficiency.

5.4.3 Combined effect of temperature and nutrient supply

As shown in previous sections, the production of *Wolffia angusta* biomass, along with nutrient uptake and removal, are influenced by temperature, phosphorus and nitrogen availability. The extent of each effect under simultaneous variations in growing conditions remains unclear. Moreover, previous experiments exclusively focused on assessing the impact of the initial concentration of each nutrient on its individual removal and uptake from media, neglecting the effects on the removal and uptake of the complementary nutrient. Consequently, an experimental design was implemented to enable a comprehensive analysis of all variables at the same time and their potential impact on diverse responses within a *Wolffia angusta* cultivation system.

5.4.3.1 Duckweed biomass production, nutrient uptake and removal are influenced differentially by culture conditions

In the experimental design, three study levels were selected for each variable. The culture temperature varied from 20 to 30°C, the initial phosphorus concentration varied from 0.2 to 4 mg P L^{-1}, and the initial nitrogen concentration ranged from 0.5 to 10 mg N L^{-1}. The reason for these variations in nutrient concentrations is that, as previously explained, the performance of *W. angusta* for nutrient recovery from wastewater is notably influenced when nutrient availability is low. Moreover, these concentration ranges align better with typical levels of these elements in fish farm effluents.

Throughout the study, all trials were monitored for 5 days. During this period, daily water samples were collected, and plant growth was documented through top-view photographs. The photoperiod was consistently maintained at 12 hours of light with an intensity of 100 µmol m^{-2} s^{-1}. The responses derived from the experimental design can be categorized into two main groups. The first group encompasses responses related to the production of high-quality biomass, including parameters such as RGR, biomass productivity, protein and starch content in biomass, as well as nitrogen and phosphorus content in plant tissue. The second group is focused on responses associated with nutrient removal, including maximum phosphorus and nitrogen removal rates, along with the ratios at which each substrate is converted into biomass (i.e., phosphorus-to-biomass yield and nitrogen-to-biomass yields). Additionally, the daily productivity of each biomass-derived constituent was also considered.

Through a comprehensive response surface analysis, which considered linear and quadratic parameters as well as the interactions between variables, it became feasible to quantify the relative impact of each variable on the aforementioned responses (as represented in Figure 5-10). Notably, the selected responses can be categorized according to the environmental conditions that exert the most significant influence on each response.

Firstly, there are responses most influenced by temperature, including parameters related to the growth of *W. angusta*, its starch content (and derived productivity), and nitrogen-to-biomass yield. As observed in previous experiments, temperature has a significant effect on the RGR of *W. angusta*, increasing by 2-fold times when the temperature increases by 10 degrees. RGR and biomass dry weight productivity are closely interconnected, and as a result, temperature also wields a substantial impact on the latter parameter. However, unlike results for RGR, it is important to note that the availability of phosphorus and nitrogen also have a relatively high effect on biomass productivity. The

discrepancy arises because RGR is calculated only during the exponential growth phase, while biomass productivity accounts for the total duration that the biomass was kept in the cultivation pots (5 days) even if either one or both nutrients were already depleted from the culture medium affecting plant's growth.

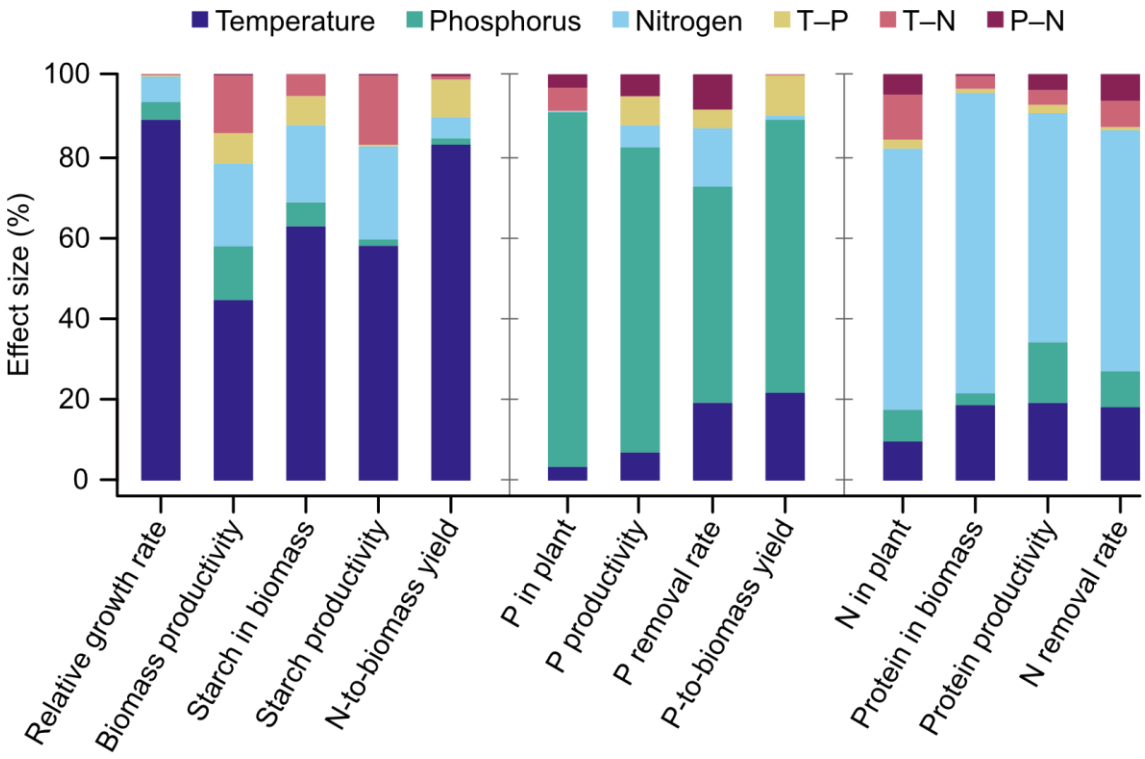

Figure 5-10 Relative contribution of temperature, phosphorus and nitrogen on key response variables during the cultivation of *Wolffia angusta* for nutrient reclamation and the production of nutrient-rich biomass.

The bars are organized based on the primary factor driving the response: temperature exerts the greatest impact on the left group, phosphorus in the middle group, and nitrogen on the right group. The effect size of temperature, phosphorus, and nitrogen accounts for both their linear and quadratic contributions. The interactions between temperature and phosphorus (T-P), temperature and nitrogen (T-N), as well as phosphorus and nitrogen (P-N) are also presented.

In the second group are the responses most affected by the initial phosphorus concentration. As expected, this group includes the parameters related to the phosphorus content in the biomass, the phosphorus-to-biomass yield, as well as the removal rate of this element from the culture medium. Finally, the responses in the last group are primarily influenced by the initial supply of nitrogen. Like phosphorus, this category includes responses related to the nitrogen content in the biomass, including protein content, and responses associated to nitrogen removal from the culture medium.

Furthermore, interactions between variables are also included in the analysis. Interactions between temperature and nutrients carry more significance in the responses related to biomass production, whereas interactions between nutrients are more relevant to responses associated with the nutrient content of the biomass and the removal of nutrients from the culture medium.

5.4.3.2 Influence of culture conditions on the production of nutritious and nutrient-rich duckweed biomass

Cultivation conditions play a crucial role in determining both the quantity and quality of the produced duckweed biomass. Regarding quality, two critical attributes come to the forefront when considering duckweed biomass for use in food or fertilizer production: its nutritious value in terms protein and starch composition, and its nutrient content in relation to elemental phosphorus and nitrogen. Additionally, the selection of optimal culture conditions should take into account the net productivity of each biomass component, considering trade-offs resulting from varying effects of culture conditions.

- **The production of duckweed biomass increases with higher temperatures and nutrient supply**

Figure 5-11 illustrates how biomass productivity responds to variations in culture conditions. *Wolffia angusta* biomass productivity ranged from 2 to 8 mg dry weight per day (mg dw day^{-1}), reaching a peak value at 30°C, 2 mg P L^{-1} and 10 mg N L^{-1}. As in previous experiments, a rise in temperature and initial concentrations of both phosphorus and nitrogen led to an increased biomass production. Notably, the impact of temperature on biomass productivity becomes more pronounced at higher nutrient concentrations, and similarly, nutrient levels exhibit a more significant effect at elevated temperatures.

Concerning the ratio between both nutrients (Figure 5-11 C), it is observed that at 25°C, biomass productivity reaches a local maximum value of 6 mg dw day^{-1} when the P concentration is 3 mg L^{-1}, and the N concentration is 8 mg L^{-1}. This peak productivity value is only altered if the temperature is changed and aligns with a molar N:P ratio of 5.5:1, which research has shown to be close to the optimal nutrient ratio for proper plant development (Güsewell 2004). Interestingly, the temperature begins to have a significant effect on the biomass productivity of *W. angusta* only when the N:P ratio of 5.5:1 is reached, as observed in Figure 5-11 A, B. This demonstrates the need to consider the

combined effect of all cultivation variables, as biomass productivity improvements of up to 130% can only be achieved with a proper balance of phosphorus and nitrogen.

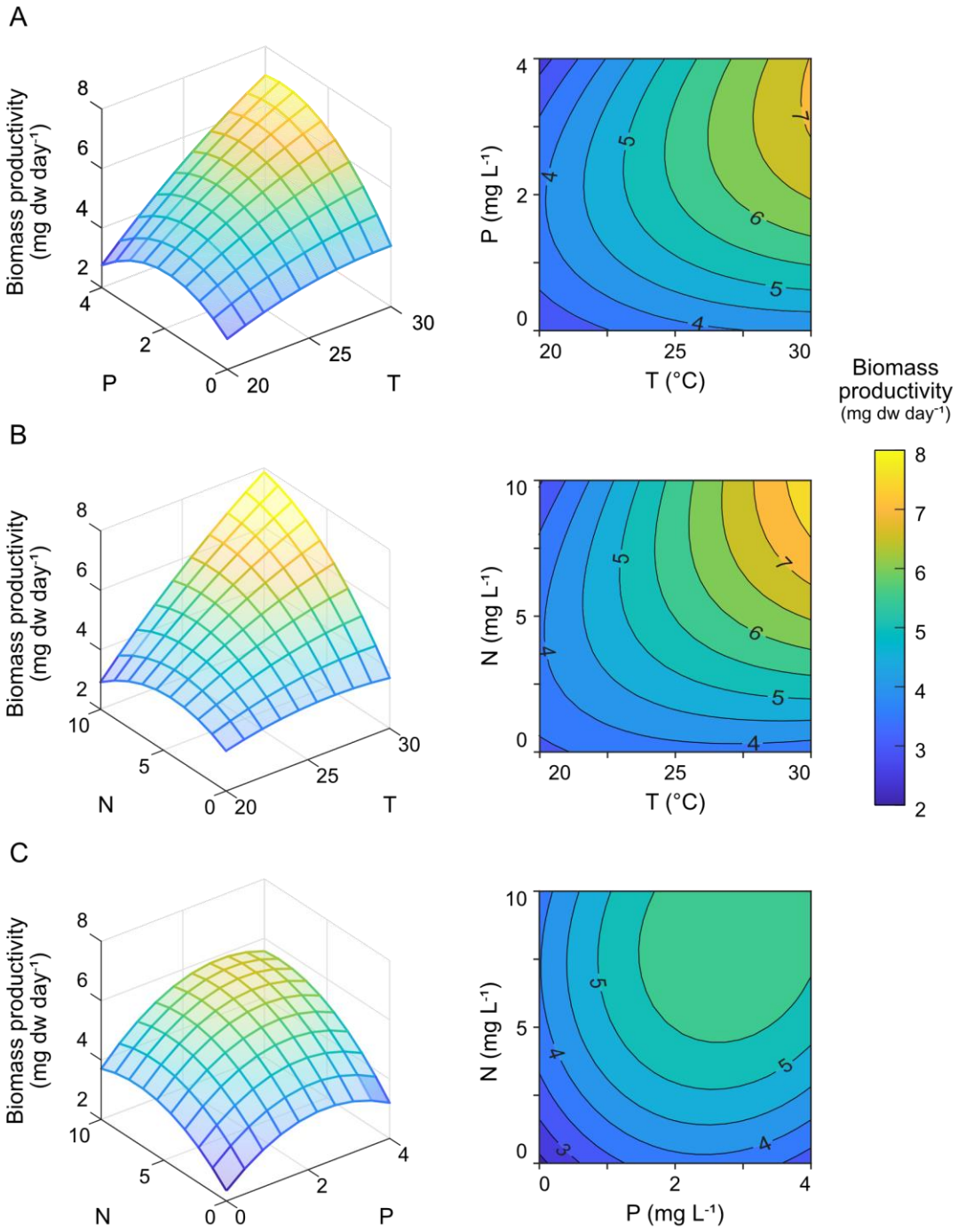

Figure 5-11 Response surface (3D) and contour plots (2D) of the two-way interaction of factors affecting the production of *Wolffia angusta* biomass.

Biomass productivity is shown as a function of **(A)** temperature vs. phosphorus concentration at a fixed nitrogen concentration of 5 mg N L^{-1}, **(B)** temperature vs. nitrogen concentration at a fixed phosphorus concentration of 2 mg P L^{-1}, **(C)** phosphorus concentration vs. nitrogen concentration at a fixed temperature of 25°C. T, temperature; P, phosphorus concentration; N, nitrogen concentration.

– **Protein-rich duckweed biomass can be obtained at low temperatures and with high nitrogen concentrations**

Contrasting trends emerge when assessing the influence of cultivation conditions on protein and starch content within the biomass. Protein content in the biomass can vary between 14% and 29% of dry weight, with higher values occurring at lower cultivation temperatures and elevated nutrient concentration (Figure 5-12 A).

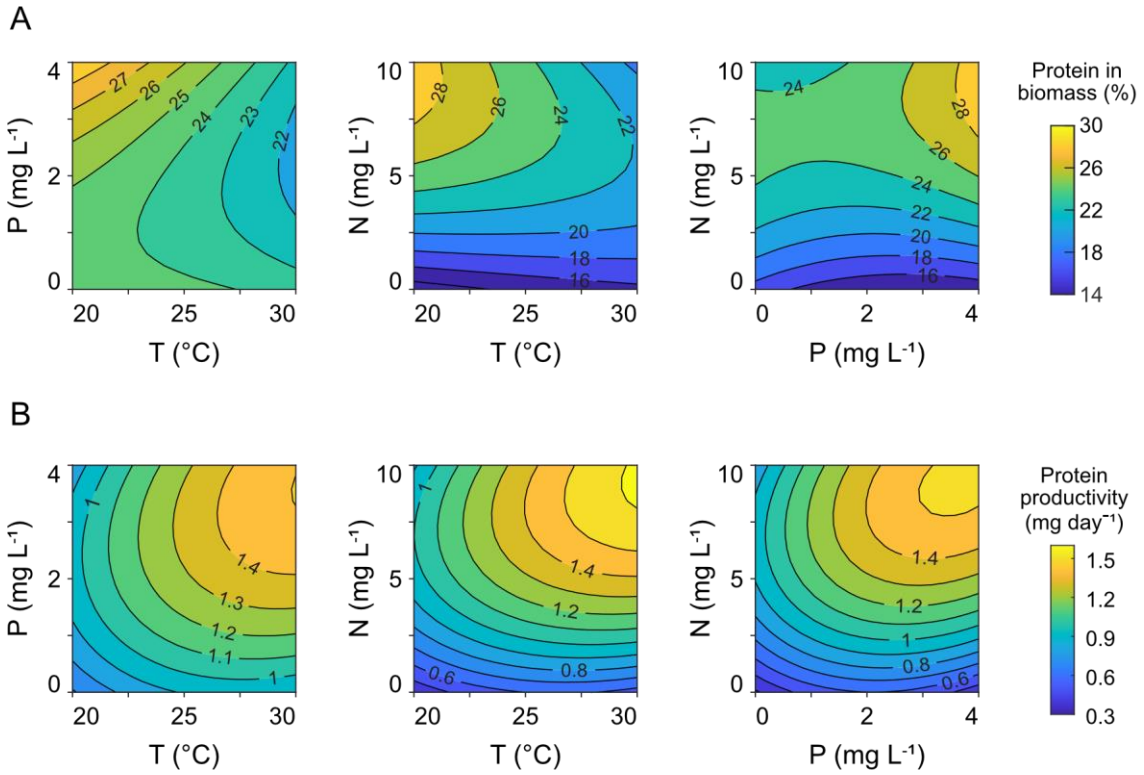

Figure 5-12 Protein levels and productivity derived from *Wolffia angusta* biomass in response to varying temperature, phosphorus supply, and nitrogen supply.

(A) Contour plots for protein content in biomass as percentage of dry weight. **(B)** Contour plots for duckweed-derived protein productivity (mg protein day^{-1}). Both responses are shown by plot colours, and are also indicated by exact values along contour lines. Hold values for TP, TN and PN interaction plots were 5 mg N L^{-1}, 2 mg P L^{-1} and 25°C, respectively.

In general, increments in temperature tend to reduce the protein content in the biomass while increments in P and N supply improve it. Moreover, at any given temperature the initial phosphorus concentration has a less significant effect on duckweed protein content compared to the initial nitrogen concentration. For example, at 25°C, protein content can increase significantly (from 15% to 24% dry weight) when transitioning from a medium without nitrogen to one containing 10 mg N L^{-1}. On the other hand, at the same temperature, an

increment of just 2% in protein content occurs when adding 4 mg P L^{-1} to a P-free medium.

When examining the combined effect of nutrients, it is found that, under nitrogen-limiting conditions, phosphorus has little impact on protein content. However, when nitrogen concentration exceeds 5 mg N L^{-1}, two effects are observed: at low phosphorus concentrations, protein content remains relatively stable at 25% dry weight, but as phosphorus concentration increases, protein content can reach 29% dry weight.

The decrease in protein content with rising temperature can be attributed to the accelerated metabolic activity of duckweed at higher temperatures, primarily driven by reproductive processes. In such instances, mechanisms favouring reproduction-related proteins and enzymes are promoted, while proteins and enzymes required for plant maintenance are limited and distributed among the offspring (Cookson *et al.* 2010). Furthermore, it is worth noting that nitrogen is the primary component of proteins, so higher nitrogen concentrations induce greater protein production by plants (Novoa and Loomis 1981).

When combining biomass productivity and protein content, the daily protein productivity from duckweed is obtained (Figure 5-12 B). Protein productivity is at its lowest (0.3 mg protein per day) under the most adverse conditions tested, while it reaches its maximum value at the highest conditions within the range (1.5 mg protein per day). In this case, even though the effect of temperature on protein content opposes its effect on biomass productivity, the latter exerts a more substantial influence on net protein productivity. A mere 10°C change diminishes protein content by 28% in the most adverse scenario while augmenting biomass productivity by 50%.

- **Higher temperatures and low phosphorus supply lead to increased starch accumulation by duckweed**

Elevated temperature resulted in an increased starch content in biomass, with the impact of both phosphorus and nitrogen becoming significant only when temperature exceeded 25°C (Figure 5-13 A). The lowest starch levels, less than 3.5% dw, were obtained at 20°C, while the highest values were observed at 30°C when there was either no phosphorus or abundant nitrogen in the culture media (7.8% dw). Overall, the starch content of *W. angusta* was found to be similar to that reported for other *Lemna*ceae and *Wolffia* species (Appenroth *et al.* 2017, 2018). At each temperature, a minimum local value for starch content was obtained when varying the concentration of P and N simultaneously. At 25°C, the

minimum starch content in duckweed biomass was achieved at a molar N:P ratio of 2:1. Higher ratios, obtained by either increasing N supply or reducing P supply, are shown to improve starch content.

The rise in starch levels in duckweed tissue may be attributed to two phenomena. On one hand, starch functions as a storage form of energy in plants that is primarily driven by photosynthesis (Pfister and Zeeman 2016), a process that is generally favoured by higher temperatures, up to a certain threshold. As a result, increased temperatures enhance the enzymatic reactions involved in photosynthesis, leading to an increased sugar production and subsequent high starch content.

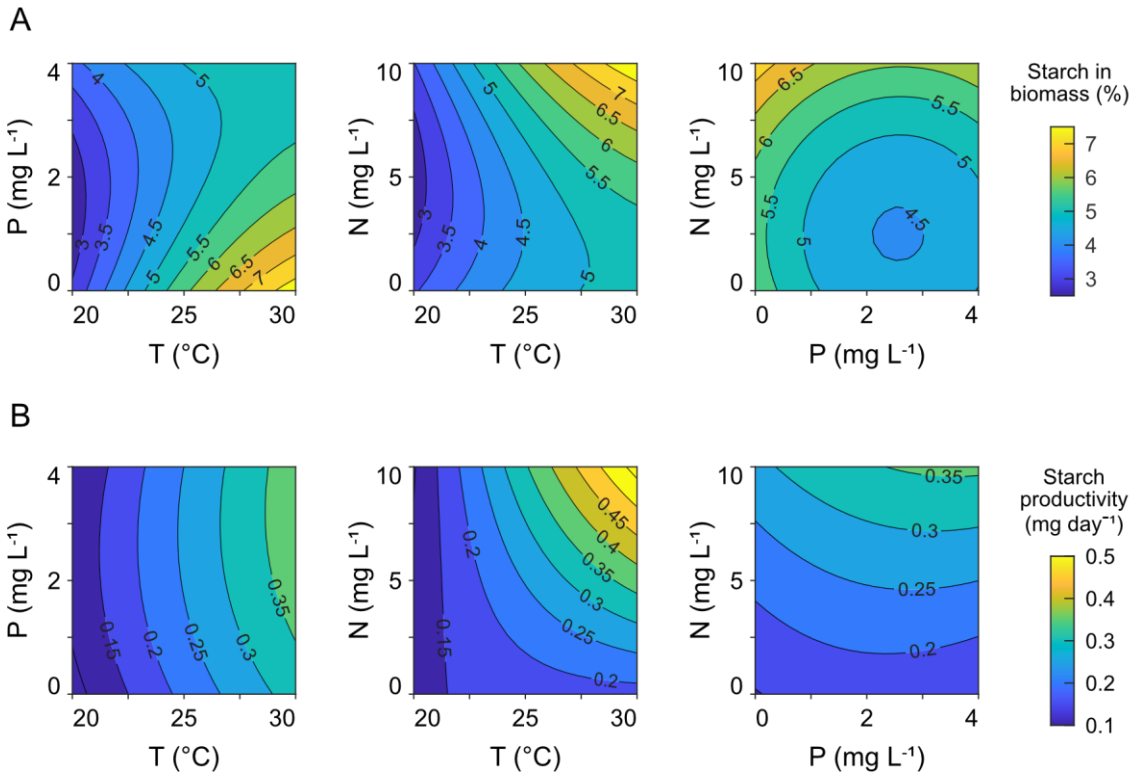

Figure 5-13 Starch levels and productivity derived from *Wolffia angusta* biomass in response to varying temperature, phosphorus supply, and nitrogen supply.

(A) Contour plots for **s**tarch content in biomass as percentage of dry weight. **(B)** Contour plots for duckweed-derived starch productivity (mg starch day^{-1}). Both responses are shown by plot colours and are also indicated by exact values along contour lines. Hold values for TP, TN and PN interaction plots were 5 mg N L^{-1}, 2 mg P L^{-1} and 25°C, respectively.

On the other hand, exposure to limiting conditions, including lack of nutrients and low temperature, can trigger a stress response in plants. This response involves the redirection of resources, including the conversion of sugars into starch, as a survival strategy (Appenroth *et al.* 2021). Specifically, phosphate

starvation has been demonstrated to induce starch accumulation in duckweeds (Li *et al.* 2021). Our findings suggest that this mechanism for starch accumulation is more pronounced at high temperature, when plant growth is elevated, consequently leading to lower P content within plant tissues.

Just as in the case of protein production from duckweed, starch productivity is more influenced by the impact of cultivation variables on plant growth than on the starch content of the plants. However, in contrast to protein production, the two effects in this case are additive. Thus, the highest starch productivity is achieved under the highest cultivation conditions that were tested (Figure 5-13 B). Under these conditions, both the starch content and plant growth are maximized, resulting in a starch productivity of up to 0.57 mg day^{-1}.

In summary, it is possible to produce biomass with a high protein or starch content by controlling the cultivation conditions of *W. angusta*. A high protein content can be achieved at low cultivation temperatures while maintaining an optimal N:P ratio, whereas high starch contents are easily obtained at high temperatures and nutrient-limiting conditions. The prioritization of the biomass component depends on its ultimate end use. Generally, the protein content was much higher than the starch content (29% versus 8% dry weight at the best culture conditions, respectively), making *W. angusta* biomass a suitable candidate as a food supplement for both humans and animals. However, the net protein productivity should be considered, as high biomass production implies a trade-off in its protein content.

- **High levels of phosphorus and nitrogen in duckweed can be achieved by ensuring a sufficient supply of nutrients at low temperatures**

In relation to the phosphorus and nitrogen levels found in the biomass, the study revealed that the initial concentration of each respective nutrient in the growth medium has the most significant impact (Figure 5-14 A,B). With a constant nitrogen concentration, the phosphorus content in the biomass can increase up to four times when transitioning from a medium without phosphorus to one containing 4 mg P L^{-1}, regardless of the cultivation temperature. Conversely, with a constant phosphorus concentration, the nitrogen content in the biomass can increase by an average of 2.5 times when shifting from a nitrogen-free medium to one with 10 mg N L^{-1}. Detailed information about the elemental composition of the duckweed biomass grown at the different culture conditions is presented in Appendix 5.

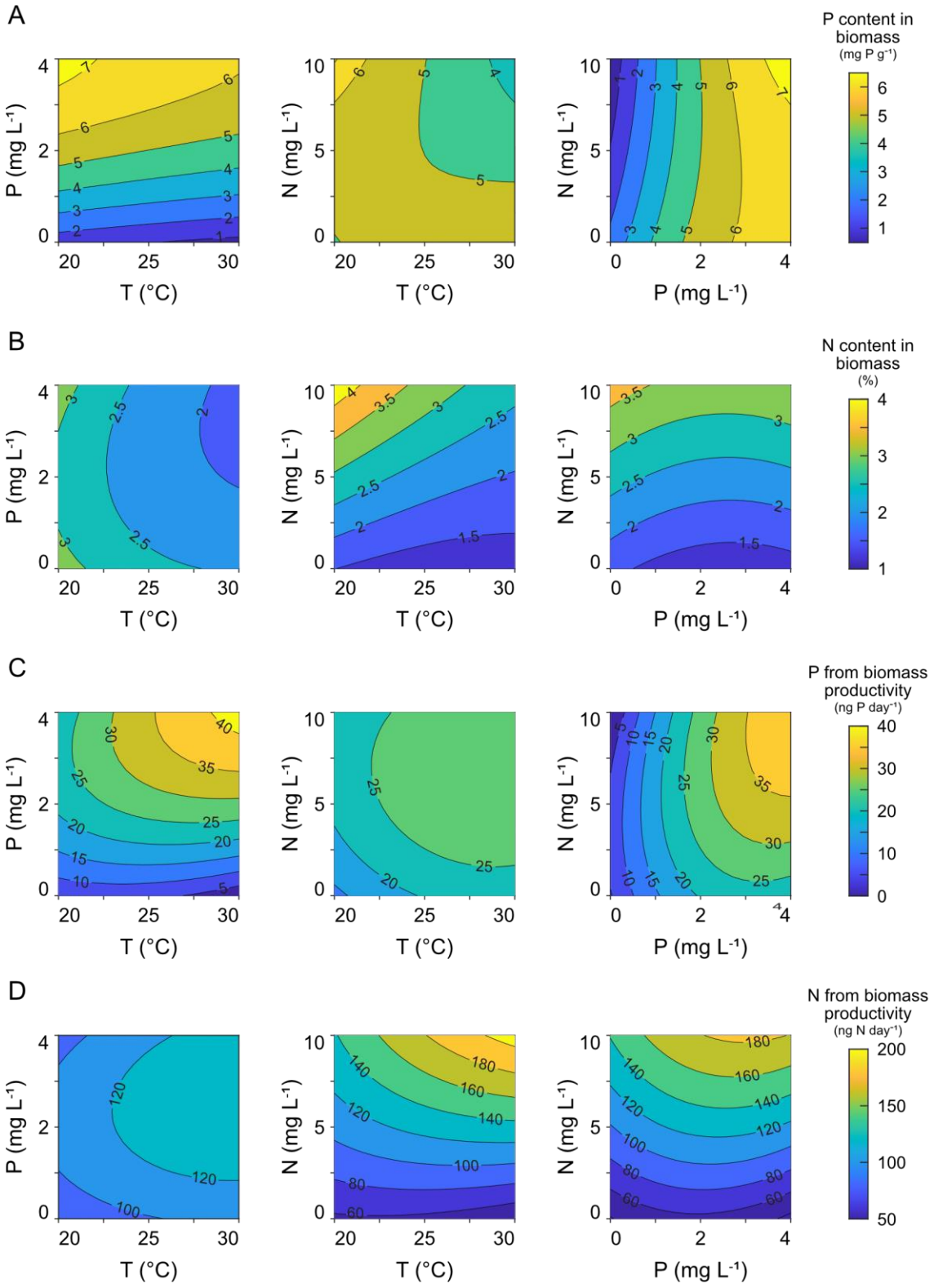

Figure 5-14 Contour plots (2D) of the two-way interaction of factors affecting the elemental P and N composition of *Wolffia angusta*.

(A,B) Contour plots for phosphorus and nitrogen content in dry duckweed biomass. **(C,D)** Contour plots for duckweed-derived phosphorus and nitrogen productivity. Both responses are shown by plot colours and are also indicated by exact values along contour lines. Hold values for TP, TN and PN interaction plots were 5 mg N L^{-1}, 2 mg P L^{-1} and 25°C, respectively.

The cultivation temperature, in turn, affects the content of each element in the biomass based on the availability of both nutrients in the growth medium. Concerning phosphorus content (Figure 5-14 A), the findings indicate an absence of interaction between temperature and phosphorus supply but reveal an interaction between temperature and nitrogen supply. When nitrogen concentrations are below 5 mg L^{-1}, the impact of temperature is negligible since plant growth is limited by nitrogen. Consequently, the P content in the plants remains consistently between 5 and 6 mg P g^{-1} dw. However, beyond this concentration, temperature starts to adversely affect phosphorus levels in plants as its distribution among a greater number of plants produced at higher temperatures.

As for nitrogen content (Figure 5-14 B), there is a slight interaction between temperature and the initial supply of each nutrient in the growth medium. Overall, lower temperatures generally result in higher nitrogen content in the biomass. This effect is more pronounced with higher initial supplies of both nutrients. For instance, in a media containing 2 mg P L^{-1} and 5 mg N L^{-1}, the nitrogen content in the plants increased from 2 to 3% dry weight when transitioning from 30°C to 20°C. In contrast, when the initial nitrogen concentration was elevated to 10 mg L^{-1}, the nitrogen content in the plants exceeded 4% dry weight within the same temperature range. The results also suggest that the interaction between the initial concentration of both nutrients does not significantly affect the content of each nutrient in the duckweed biomass.

Just like the protein and starch productivity, the productivity of phosphorus and nitrogen obtained from duckweed biomass is primarily determined by the overall productivity of the duckweed itself, rather than the specific content of each element in the biomass (Figure 5-14 C, D). In general, the phosphorus productivity derived from duckweed biomass can fluctuate between 5 and 40 ng P day^{-1}, while the duckweed-derived nitrogen productivity may fall within the range of 50 and 200 ng N day^{-1}.

It is important to emphasize that the outcomes regarding protein content are closely associated with the findings for nitrogen content in plants, given that nitrogen is its primary constituent. A common assumption is that proteins typically consist of around 16% nitrogen, leading to a protein-to-nitrogen ratio of 6.25 (Moore *et al.* 2010). Experimental findings for *Wolffia angusta* revealed that the actual protein content in all instances is lower than what is computed from the nitrogen content of the plants. The disparity between these values varies from 5 to 50%, with a more significant gap observed at higher temperatures and lower initial nitrogen concentrations. This discrepancy may indicate reduced efficiency

in protein conversion, potentially attributable to genetic factors inherent to the studied duckweed species, nitrogen limitation circumstances, environmental stress induced by elevated temperatures, or accelerated plant growth.

5.4.3.3 Influence of culture conditions on nutrient uptake and removal by *Wolffia angusta*

In addition to influencing the biomass production and quality of duckweed, cultivation conditions also have a significant effect on nutrient removal in wastewater treatment systems.

5.4.3.3.1 Culture conditions for a high production of duckweed biomass improve the net removal rates of phosphorus but temperature hinder its uptake by plants

The rate at which phosphorus is removed from the cultivation medium increases proportionally with temperature and nutrient supply (Figure 5-15 A). The highest phosphorus removal rates (> 0.8 mg P L^{-1} d^{-1}) were achieved at 30°C with the highest initial concentrations of phosphorus and nitrogen. Conversely, PRR was low or even negligible when plants were kept in P-free media, or the cultivation temperature was low.

Two different effects are observed when adjusting the initial concentration of phosphorus. Below 2 mg P L^{-1}, the phosphorus removal rate is directly proportional and primarily governed by the initial phosphorus concentration. Changes in temperature and nitrogen supply do not impact the PRR. This effect could be attributed to the limited growth of *W. angusta* due to phosphorus concentration falling below saturation conditions (as previously shown in Figure 5-5). When the initial P concentration exceeds 2 mg L^{-1}, saturation conditions are attained, and duckweed growth is no longer limited by phosphorus. Consequently, temperature and nitrogen availability exert a more pronounced influence on PPR. Under these circumstances, elevated temperatures induce higher activity of phosphate transporters, resulting in higher PPR. Similarly, higher nitrogen concentrations enhance PPR up to the point when optimal N:P ratio for duckweed growth is reached.

The analysis of the specific phosphorus uptake reveals that this response is closely linked to the initial phosphorus concentration (Figure 5-15 B). Furthermore, it indicates a negative impact of temperature, with negligible effects from nitrogen concentration. Notably, the influence of the initial phosphorus

concentration is more pronounced at lower temperatures than at higher temperatures. This may be attributed to the plant's stress response to low temperatures, leading to higher phosphorus accumulation in anticipation to future adverse conditions. In contrast, at high temperatures, the plant actively removes only what is necessary for its reproduction and growth.

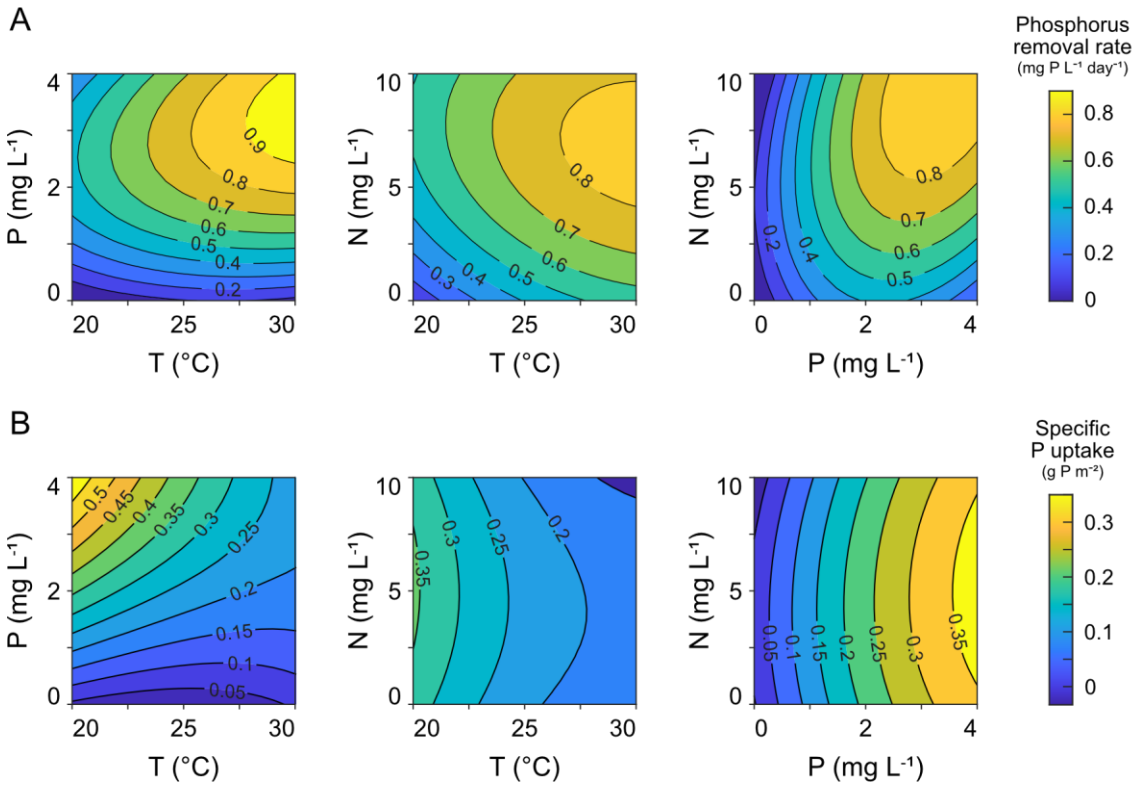

Figure 5-15 Contour plots (2D) of the two-way interaction of factors affecting the phosphorus removal by *Wolffia angusta*.

(A) Contour plots for phosphorus (PO_4-P) removal rate. **(B)** Contour plots for specific phosphorus (PO_4-P) uptake. Responses are shown by plot colours and are also indicated by exact values along contour lines. Hold values for TP, TN and PN interaction plots are 5 mg N L^{-1}, 2 mg P L^{-1} and 25°C, respectively.

The fact that temperature affects specific phosphorus uptake in a manner opposite to its impact on the PPR confirms that the increased growth experienced by plants at high temperatures is the primary factor influencing net phosphorus removal. For instance, when *W. angusta* cultures were conducted at 30°C instead of 20°C in a medium containing 4 mg N L^{-1} and 2 mg P L^{-1}, there was a 180% increase in biomass production, along with a 150% increase in the phosphorus removal rate, while the area specific phosphorus removal was reduced by 60%.

5.4.3.3.2 Culture conditions for a high production of duckweed biomass improve the net removal rates of nitrogen but temperature hinder its uptake by plants

The results indicate that the nitrogen removal rate is influenced by the three variables under study (Figure 5-16 A). Like phosphorus, higher removal rates are achieved at elevated temperatures and in plentiful nutrient availability, while lower values result from the most adverse cultivation conditions. In this case, the initial supply of nitrogen is the variable with the most significant impact on the NRR. Below the saturation level, the increase in NRR is proportional to the rise in nitrogen concentration. When the saturation threshold is reached, both phosphorus and temperature begin to have significant effects. This is because, firstly, nutrient availability rises, enhancing the N:P ratio, and secondly, plants exhibit accelerated growth at high temperatures.

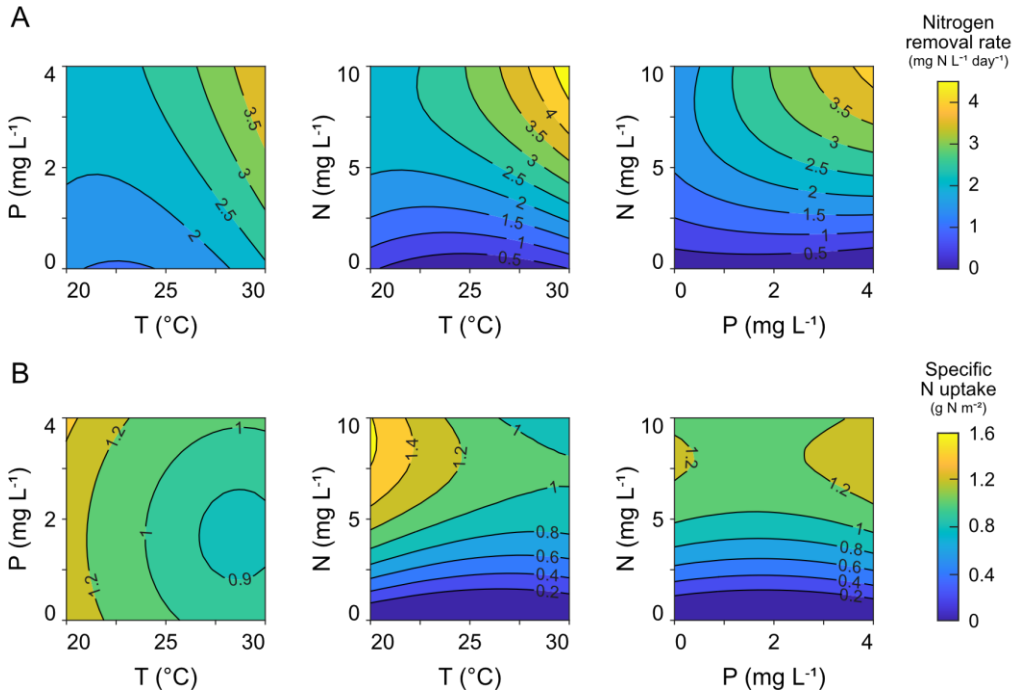

Figure 5-16 Contour plots (2D) of the two-way interaction of factors affecting the nitrogen removal by *Wolffia angusta*.

(A) Contour plots for nitrogen (NO_3-P) removal rate. **(B)** Contour plots for specific nitrogen (NO_3-P) uptake. Responses are shown by plot colours, and are also indicated by exact values along contour lines. Hold values for TP, TN and PN interaction plots are 5 mg N L^{-1}, 2 mg P L^{-1} and 25°C, respectively.

The initial nitrogen concentration is the primary determinant of the specific uptake of this element, while the availability of phosphorus seems to have no impact (Figure 5-16 B). Moreover, the specific nitrogen uptake is adversely influenced by temperature. In experiments conducted with an initial nitrogen concentration of 5 mg L^{-1}, the specific N removal diminishes by 36% when shifting

from 20°C to 30°C. Despite fluctuations in cultivation temperature, the specific nitrogen removal remains significant and never drops below 0.9 g N m^{-2}. Lower values are only observed in cases of insufficient nitrogen supply during cultivation. The influence of temperature diminishes when the nitrogen concentration falls below the saturation level (5 mg N L^{-1}). Under these circumstances, nitrogen uptake is strictly proportional to nitrogen supply, ranging between 0 and 1.0 g N m^{-2}.

In general, despite its negative effect on the specific uptake of nutrients, there is an observed preference for higher temperatures and elevated concentration of nutrients in the cultivation medium, leading to increased net removal of phosphorus and nitrogen. Nutrient removal rates were mostly driven by duckweed growth while the specific uptake of both phosphorus and nitrogen was predominantly affected by the initial concentration of each nutrient. Additionally, it was determined that the presence of phosphorus and nitrogen does not impact the specific removal of the complementary nutrient.

5.4.4 Optimal conditions for duckweed cultivation in wastewater treatment systems may vary depending on final biomass use

As observed earlier, the growth responses of *Wolffia angusta* are variably affected by cultivation conditions. At times, the effects are additive, but in many instances, these effects oppose each other. Consequently, an optimization process through a composite desirability analysis was proposed to address these dynamics. The optimization process focused on two specific biomass end-uses, considering the application of duckweed for nutrient recovery in wastewater treatment systems.

5.4.4.1 Optimal conditions for nutrient removal and recovery by *Wolffia angusta*

The primary goal of DWT systems is the removal of nutrients from wastewater. Hence, it is essential to identify optimal conditions to maximize nutrient removal. The key parameters associated with the removal of phosphorus and nitrogen from wastewater include the removal rate for each nutrient and their specific absorption by the plants. When aiming to maximize both outcomes, the identified optimal cultivation conditions involve a temperature of 22.5°C, with an initial supply of phosphorus and nitrogen at 4.0 and 9.7 mg L^{-1}, respectively (Figure 5-17 A). The expected nitrogen and phosphorus removal rates, along with

the specific removal of each element, are 3.5 mg N L^{-1} d^{-1}, 0.70 mg P L^{-1} d^{-1}, 1.51 g N m^{-2} duckweed, and 0.45 g P m^{-2} duckweed, respectively. Biomass production is not considered in this optimization process, as it is inherently accounted for in the nutrient removal rates, as noted earlier.

As mentioned previously, nutrient removal is closely tied to the initial nutrient supply, making it logical for removal to be maximized at higher initial concentrations of P and N considered in the study. However, the composite desirability is less than one due to the conflicting effects of temperature on nutrient removal rates and specific nutrient uptake. Lower temperatures enhance nitrogen and phosphorus uptake per unit area of duckweed but decrease the rate at which both elements are removed from the water. Despite both variables having equal weight, the optimization process favours specific nutrient uptake over nutrient removal rates because, as on average, the former has a higher desirability value.

In practice, high values in specific nutrient removal offer advantages in DWT treatment systems by allowing the removal of more nutrients with less biomass, resulting in a smaller treatment surface area. Consequently, equipment with smaller footprint can be used enabling its use in areas with limited space, while saving costs in system construction. However, at the optimal culture conditions, low nutrient removal rates imply a longer operating time to achieve treatment goals. Ultimately, this results in an extended residence time of wastewater, leading to either the treatment of lower quantities of water or larger volume equipment. With a constant generation of wastewater, both options involve higher construction costs for the wastewater treatment system.

The obtained optimal conditions, however, can only be sporadically achieved due to variability in environmental conditions and wastewater strength. The optimal initial nutrient concentrations obtained approach the upper limit of what can be found in aquaculture effluents. To achieve a maximal nutrient removal, the batch treatment of this type of wastewater would require nutrient addition, or in continuously operating systems, a higher surface loading rate. Similarly, controlling the optimal temperature is costly in outdoor-operated systems. As a result, nutrient removal rates and specific uptake are dynamic responses during treatment. Anticipating optimal conditions allows for establishing operational criteria based on the average treatment temperature. To maintain consistent treatment effectiveness, variations in nutrient uptake and removal due to temperature can be compensated for by adjusting flow rate or the initial coverage of duckweed. These crucial aspects for the functioning of DWT systems are examined in more detail in the following chapter.

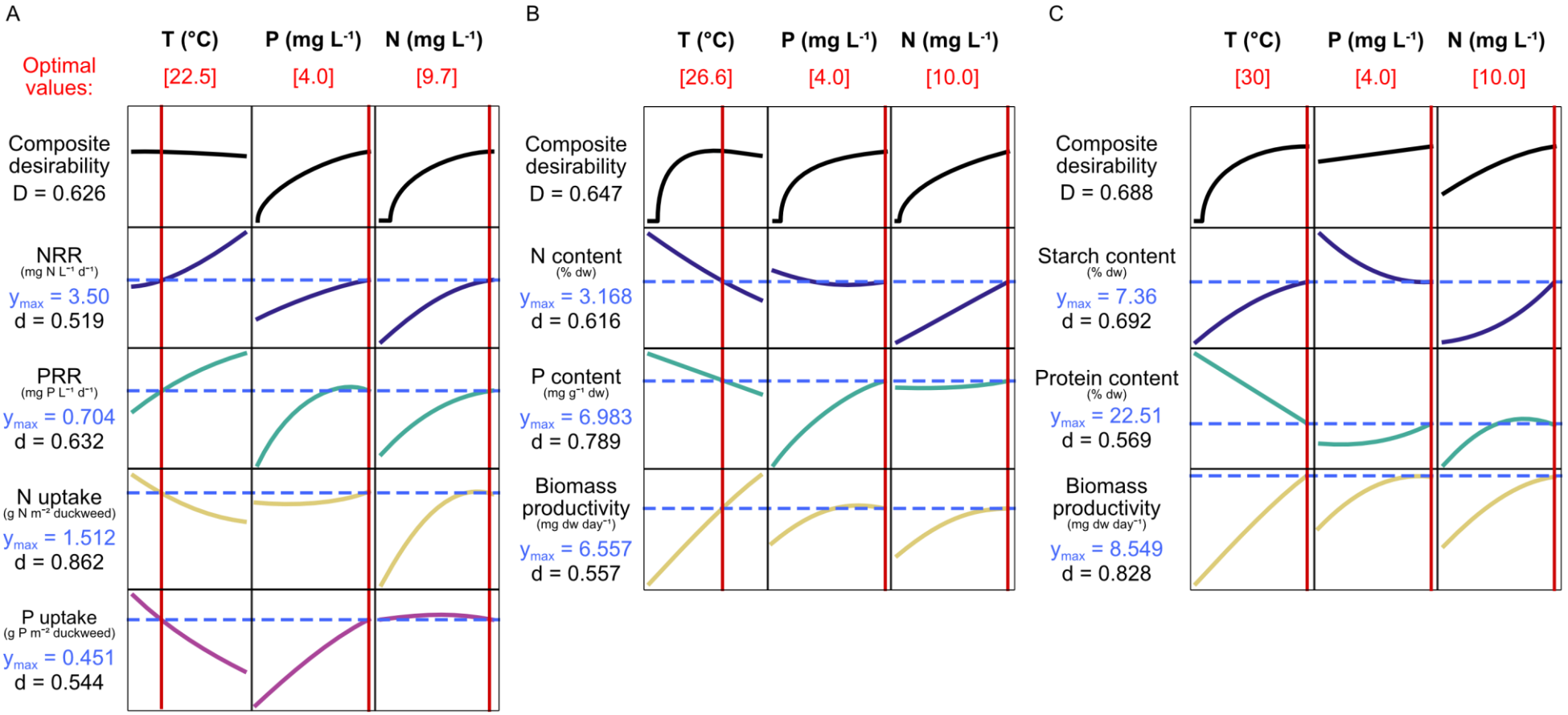

Figure 5-17 Prediction profile plots for nutrient uptake and removal, and production of *Wolffia angusta* biomass

Optimal culture conditions for **(A)** nutrient uptake and removal, **(B)** the production of nutrient-rich biomass, and **(C)** the production of nutritious duckweed biomass, are highlighted in red. The figure displays maximum values for specific nutrient uptake and removal rates, biomass productivity, and quality (P, N, starch and protein content) in blue. All the responses were maximized with the same weight. D, composite desirability; d, individual desirability, NRR, nitrogen removal rate; PRR, phosphorus removal rate.

5.4.4.2 Optimization of *Wolffia angusta* biomass production for biofertilizer production

Considering the utilization of duckweed biomass for biofertilizer production, three parameters must be taken into account: the quantity of biomass produced and its nitrogen and phosphorus content. The cultivation conditions that maximize all three responses are a temperature of 26.6°C, an initial phosphorus concentration of 4 mg L^{-1}, and an initial nitrogen concentration of 10 mg L^{-1} (Figure 5-17 B). Under these conditions, a productivity of 6.56 mg dry weight per day of *Wolffia angusta* biomass with a nitrogen content of 3.17% dw and a phosphorus content of 6.98 mg P g^{-1} dw is expected. In this case, the composite desirability is 0.647, again due to opposing effects of temperature on the responses. Even though at the optimal temperature, the nitrogen and phosphorus content in the biomass is not the highest, the desirability values for these parameters are higher than those obtained for biomass productivity. This indicates that the effect of temperature is more significant in the latter variable and, therefore, is prioritized during optimization.

Previous studies have demonstrated the potential to obtain biofertilizer from duckweed through biomass composting (Dissanayaka *et al.* 2023; Gusain and Suthar 2020; Kreider *et al.* 2019). For instance, composting *Spirodela polyrhiza* biomass for one month, with an initial nitrogen and phosphorus content of 2.75% and 0.22%, respectively, yields compost with an additional 2% of each element (Gusain and Suthar 2020). In the present study, the content of both elements in *W. angusta* biomass is higher than that reported for S. *polyrhiza*, indicating an even greater potential for biofertilizer production. Furthermore, the content of both elements is comparable to that previously reported for various microalgae species, which are presented as another potential source for fertilizer production (Cao *et al.* 2023).

The higher temperature required to produce nutrient-rich duckweed biomass would negatively impact the specific nutrient removal from wastewater but would increase the net rate at which nutrients are removed. In optimization, by setting the temperature at 26.6°C, the desirability of nitrogen and phosphorus removal rates improves by 34%, while the desirability of specific P and N uptake decreases on average by 28%. This implies that with shorter retention times and a higher coverage of duckweed biomass in the treatment system, the maximum amount of nutrients can be removed and recovered through the production of biomass with a suitable N and P content for biofertilizer production.

5.4.4.3 Optimizing the production of *Wolffia angusta* biomass to produce fish feeding

When considering the use of duckweed biomass for animal feed production, the optimization of variables should encompass not only the quantity of produced biomass but also its nutritional value, particularly in terms of protein and starch content. Cultivation conditions maximizing these responses include a temperature of 30°C and a culture medium with 4 mg P L^{-1} and 10 mg N L^{-1} (Figure 5-17 C). Under these conditions, it is possible to obtain 8.55 mg of duckweed biomass per day, with a starch content of 7.36% dw and a protein content of 22.5% dw.

The composite desirability is 0.688, attributed to opposing effects of both temperature and initial phosphorus concentration on the responses. Higher phosphorus concentrations induce increased biomass growth and higher protein accumulation but lower starch production. On the contrary, temperature induces higher starch content at the expense of lower protein content due to accelerated plant growth. Despite these effects, the optimization process prioritizes biomass production over its nutritional content (high desirability value: 0.828).

In typical fish farm effluents, phosphorus concentrations are generally below the optimal values identified in the optimization process. At lower phosphorus concentrations, there is an improvement in starch content within the biomass, but this comes at the cost of reduced protein content and biomass quantity. This situation could be advantageous in multi-stage DWT systems, where varying phosphorus supplies are implemented (Coughlan, Walsh, Bolger, *et al.* 2022; Paolacci *et al.* 2022). When plants experience phosphorus deprivation, their growth is restricted, but they accumulate more starch and utilize their internal phosphorus reserves. This phenomenon leads to an increased phosphorus uptake when plants are transferred to a medium with higher phosphorus content (Li *et al.* 2021; Liu *et al.* 2018). Consequently, this approach makes it feasible not only to produce biomass with high starch content but also with elevated phosphorus content while concurrently treating wastewater. As for the optimal temperature, it facilitates the production of a substantial biomass quantity with a balanced protein and starch content. Additionally, it allows for more efficient wastewater treatment within shorter periods of time due to higher net removal rates of phosphorus and nitrogen.

The nutritional composition derived from *Wolffia angusta* endorses the production of animal feed, particularly fish feed (Cheng and Stomp 2009; Goopy and Murray 2003). The distribution of essential amino acids in duckweed protein closely resembles that of animal protein, making it a viable candidate for

substituting fish meal in the formulation of fish feed (Hillman and Culley 1978). Therefore, as in a previous study on *Lemna minor* (Aslam and Zuberi 2017), *W. angusta* biomass could be utilized independently or in conjunction with other components for the cultivation of herbivorous fish. As a result, *Wolffia angusta* can serve not only in the treatment of wastewater generated by fish farming but also as a method for capturing and recycling nutrients within fish farming systems. This approach not only yields economic advantages but also enhances the sustainability of aquaculture practices.

5.4.5 Development of a mathematical model for the batch cultivation of *Wolffia angusta*

5.4.5.1 Mathematical model formulation

The mathematical model aims to predict the growth of *Wolffia angusta*, as well as its performance in nutrient uptake and recovery from culture media in batch culture systems.

To formulate the model, certain assumptions were made. Initially, it was assumed that the cultivation pots were uniformly mixed. Secondly, the growth of duckweed was presumed to be correlated with the concentrations of nitrogen and phosphorus in the cultivation medium. Moreover, it was assumed that there was no impact from other microorganisms, the cultivation temperature remained constant, and there were no limitations on plant growth due to light. Additionally, it was considered that there was no water evaporation from the culture pots, the sole source of carbon was environmental CO_2, and finally, that there were no mass transfer limitations.

Based on these assumptions and depending on the availability of nutrients in the culture media, the biomass growth rate can be described by an exponential growth model (Eq. 5-1). Here, r_X or dX/dt represents the plant growth rate expressed as the increase in the surface coverage of the pot ($cm^2\ cm^{-2}\ d^{-1}$) or the total area of duckweed fronds over time ($cm^2\ d^{-1}$); μ denotes the relative growth rate, formerly abbreviated as RGR; X stands for the coverage ($cm^2\ cm^{-2}$) or the total area of duckweed fronds (cm^2) at any given time; and L_i is the carrying capacity of the system in the absence of the nutrient i (expressed either as total area of duckweed fronds or duckweed coverage).

(5-1) $$\boldsymbol{r_X = \frac{dX}{dt} = \mu * X}$$

The kinetic model proposed by Jacques Monod (Monod 1949) was used to estimate the influence of the external concentration of limiting nutrients on duckweed growth rate, according to Eq. 5-2. In the model, μ_{max} is the maximum relative growth rate; P and N represent the phosphorus and nitrogen concentrations in media at any given time (mg PO_4-P L^{-1}, and mg NO_3-N L^{-1}, respectively). K_P and K_N are substrate half saturation constants corresponding to the concentrations of nutrients at which μ is half μ_{max} (mg PO_4-P L^{-1}, and mg NO_3-N L^{-1}, respectively).

(5-2) $$\boldsymbol{\mu = \mu_{max} * \frac{P}{K_P+P} * \frac{N}{K_N+N}}$$

Under conditions of nutrient excess and constant light intensity and photoperiod, μ will correspond to μ_{max} and can be described solely as a function of temperature following an Arrhenius equation (Eq. 5-3). In the formula, A_1 is a constant, day^{-1}; E_1 is the activation energy, J mol^{-1}; R is the universal gas constant, J mol^{-1} K^{-1}; and T is the temperature in Kelvin scale.

(5-3) $$\boldsymbol{\mu_{max} = A_1 * exp^{\left(\frac{-E_1}{R*T}\right)}}$$

The removal of phosphorus and nitrogen from media is mediated by plant growth, as previously noted. Therefore, the rates of substrate utilization can be described as follows:

(5-4) $$\boldsymbol{r_P = \frac{r_X}{Y_{XP}}}$$

(5-5) $$\boldsymbol{r_N = \frac{r_X}{Y_{XN}}}$$

Where r_P and r_N is the rate of substrate utilization, or removal, mg substrate L^{-1} d^{-1}; and Y_{XP} and Y_{XN} are the biomass yield coefficients related to each nutrient in culture media, cm^2 / mg^{-1} substrate. The inverse of the biomass yield coefficients corresponds to the specific uptake of substrates introduced in earlier sections.

The biomass yield for both nutrients is not constant during the treatment but varies depending on the concentration of each nutrient in the cultivation medium (as depicted in Figure 5-9). The variation of Y_{XP} and Y_{XN} with the respective residual P and N concentration was adjusted to a logistic function (Eq. 5-6, 5-7). The equation explains the constant value for both yields (equal to $\boldsymbol{Y_{XP}}^{\boldsymbol{min}}$ and $\boldsymbol{Y_{XN}}^{\boldsymbol{min}}$**)** seen at high concentrations of nutrients, and the ${Y_{XP}}^{max}$ and ${Y_{XN}}^{max}$ obtained in absence of external phosphorus or nitrogen supply, respectively. $\boldsymbol{K'_P}$ and $\boldsymbol{K'_N}$ are constants for the rate of change of each biomass yield (g^2 L^{-1} m^{-2}). Additionally, a modified Arrhenius equation was employed to compensate the previous parameters for temperature changes, using values obtained at 25°C as reference (Eq. 5-8).

(5-6) $$\boldsymbol{Y_{XP}} = \frac{\boldsymbol{K'_P} * \boldsymbol{Y_{XP}}^{\boldsymbol{max}} + \boldsymbol{P}}{\boldsymbol{K'_P} + (\boldsymbol{1/Y_{XP}}^{\boldsymbol{min}}) * \boldsymbol{P}}$$

(5-7) $$\boldsymbol{Y_{XN}} = \frac{\boldsymbol{K'_N} * \boldsymbol{Y_{XN}}^{\boldsymbol{max}} + \boldsymbol{N}}{\boldsymbol{K'_N} + (\boldsymbol{1/Y_{XN}}^{\boldsymbol{min}}) * \boldsymbol{N}}$$

(5-8) $$\boldsymbol{Value_T} = \boldsymbol{Value_{25°C}} * \boldsymbol{\theta}^{(\boldsymbol{T-25})}$$

The kinetic parameters presented in equations 5-1 to 5-5 were calculated from the experimental data provided in sections 5.4.1 and 5.4.2. The compiled values are summarized in Table 5-1.

Table 5-1 Kinetic constants for the growth model of *Wolffia angusta*

Parameter	Description	Value (s.d.)	Unit
${\mu_{max}}^{25°C}$	Maximum RGR at 25°C	0.302 (0.01)	day^{-1}
$Ln(A_1)$	N-log Arrhenius pre-exponential factor	18.59 (3.90)	day^{-1}
E_1	Growth activation energy	48.97 (9.68)	kJ mol^{-1}
K_P	Half saturation constant for phosphorus	0.048 (0.012)	mg P L^{-1}
K_N	Half saturation constant for nitrogen	0.244 (0.030)	mg N L^{-1}
$Y_{XP_{25°C}}^{max}$	Maximum biomass-to-phosphorus yield at 25°C	113.6 (37.1)	cm^2 mg^{-1}
$Y_{XP_{25°C}}^{min}$	Minimum biomass-to-phosphorus yield at 25°C	18.9 (2.52))	cm^2 mg^{-1}
$\boldsymbol{K'_{P_{25°C}}}$	Biomass-to-phosphorus yield rate constant	0.055 (0.014)	mg^2 L^{-1} cm^{-2}
$Y_{XN_{25°C}}^{max}$	Maximum biomass-to-nitrogen yield at 25°C	24.7 (6.6)	cm^2 mg^{-1}
$Y_{XN_{25°C}}^{min}$	Minimum biomass-to-nitrogen yield at 25°C	4.94 (0.48)	cm^2 mg^{-1}
$\boldsymbol{K'_{P_{25°C}}}$	Biomass-to-phosphorus yield rate constant	0.566 (0.356)	mg^2 L^{-1} cm^{-2}
$\boldsymbol{\theta}$	Temperature correction factor	1.054 (0.012)	-

The value obtained for the maximum relative growth rate of *W. angusta* at 25°C is in accordance with the values found in the literature for other *Wolffia* species (P. Ziegler *et al.* 2015). Half saturation values for phosphorus and nitrogen regarding *W. angusta* growth have not been reported in the literature so far. However, the estimated value for K_P and K_N are significantly higher than those previously reported for *Lemna minor* (1.89 ug P L^{-1}, 0.02 mg N L^{-1}) and *Spirodela polyrhiza* (0.87 mg P L^{-1}, 0.02 mg N L^{-1}) (Kufel, Strzalek, and Wysokinska 2012).

5.4.5.2 Model calibration

The formulated mathematical model was calibrated and improved using the experimental data from the experiments presented in section 5.4.3. The comparison of some experimental data obtained at different temperatures and initial concentrations of phosphorus and nitrogen against the results obtained by the mathematical model is presented in Figure 5-18.

The correlation between the experimental data and the model-predicted values was less than 0.85 in all cases, with the highest correlation observed for phosphorus concentration data (r^2 = 0.834), followed by nitrogen concentration data (r^2 = 0.762), and finally biomass production data (r^2 = 0.652) (correlation figures can be observed in Appendix 6).

The correlation value was higher for all responses (r^2 > 0.787) when only experiments with good nutrient availability at the start of the experiments were considered, and none of these were completely depleted from the culture medium by the end of the experimental period, as in the case of the experiment conducted at 20°C, with a medium containing 2 mg P L^{-1} and 10 mg N L^{-1}. However, the correlation for the responses was lower when any of the nutrients was not added to the culture medium or was completely consumed before the end of the experimental period, as in the experiments conducted at 30°C shown in Figure 5-18.

The significant differences between the experimental and predicted results were attributed to the Monod kinetic equation used to model plant growth. As this is an unstructured kinetic model, it does not consider the role of internal concentrations of phosphorus and nitrogen on plant growth; it only considers the external supply of these elements. Thus, as soon as one of the nutrients is removed from the culture medium, the mathematical model assumes that the plants cannot grow further, and therefore, no more nutrient removal occurs. In practice, however, this is not the case, as in nutrient scarcity situations, plants can use their own reserves of nitrogen and phosphorus to continue growing.

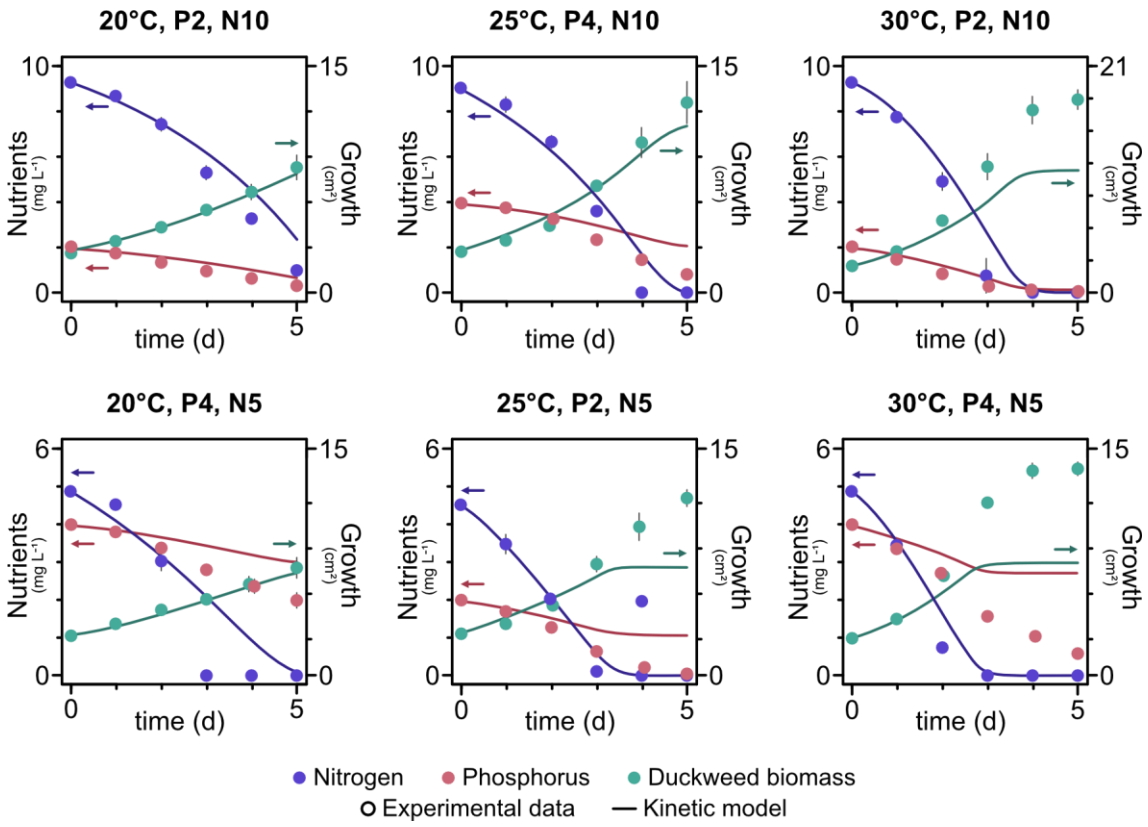

Figure 5-18 Experimental and modelled outcomes for the cultivation of *W. angusta* at varying culture conditions

Dots represent experimental data while continuous line the predicted values from the kinetic model. Data for nitrogen and phosphorus concentration should read against left axis while duckweed growth data (expressed as increased in total fronds area) should be read against the right axis, as marked by the arrows. Culture conditions for each experiment are shown at the top of each box. The initial concentration of phosphorus (P) and nitrogen (N) is represented as the number next to the letters, in mg PO_4-P L^{-1} and mg NO_3-N L^{-1}, respectively.

For this reason, the kinetic model was adjusted to explain a finite growth rate in the absence of one or both nutrients. Experimental data from pots in which plants were grown at different temperatures in phosphorus and/or nitrogen-free media were used for this calibration (data not shown). Under these conditions, although the plants grew, their development was limited until reaching a maximum point, presumably the point at which the internal reserves of the nutrient that was not present in the culture medium were depleted.

Hence, duckweed growth was fitted to a logistic growth model instead of an exponential model (Eq. 5-9). Two new parameters were obtained from the equation: a specific growth rate $\boldsymbol{\mu}$, which becomes $\boldsymbol{\mu_{P_0}}$, $\boldsymbol{\mu_{N_0}}$, or $\boldsymbol{\mu_{P_0,N_0}}$ in the absence of phosphorus, nitrogen, or both nutrients in the culture medium, respectively, and a parameter $\boldsymbol{L_i}$, representing the carrying capacity of the cultivation system in the absence of the nutrient $\boldsymbol{i}$ (expressed either as total area of duckweed fronds or duckweed coverage).

(5-9) $$r_X = \frac{dX}{dt} = \begin{cases} \mu * X, & [P], [N] > 0 \\ \mu * X * \left(\frac{L_i - X}{L_i}\right), & [P], [N] \leq 0 \end{cases}$$

Similarly, the expression for the relative growth rate of *Wolffia angusta*, based on the Monod equation, was adjusted according to Eq. 5-10 to integrate duckweed growth in the absence of external nutrients.

(5-10) $$\mu = \mu_1 + \mu_2 + \mu_3 + \mu_4 \begin{cases} \mu_1 = \mu_{max} * \frac{P}{K_P + P} * \frac{N}{K_N + N} \\ \mu_2 = \mu_{P_0} * \frac{K_P}{K_P + P} * \frac{N}{K_N + N} \\ \mu_3 = \mu_{N_0} * \frac{K_N}{K_N + N} * \frac{P}{K_P + P} \\ \mu_4 = \mu_{P_0,N_0} * \frac{K_P}{K_P + P} * \frac{K_N}{K_N + N} \end{cases}$$

When comparing the experimental results again with those of the calibrated model, the accuracy of the predicted data for all variables significantly improved. The new correlation coefficients are now r^2 = 0.908 for biomass production, r^2 = 0.971 for phosphorus concentration, and r^2 =0.959 for nitrogen concentration (correlation figures can be observed in Appendix 6). At the end, the calibrated model allow for the prediction of outcomes from duckweed-based batch cultivation systems within a 90% confidence interval.

5.4.5.3 Model validation

Finally, the fine-tuned kinetic model was validated through an experiment performed in duplicate. *Wolffia angusta* was cultured at 25°C in rectangular tanks containing 1L of the standard Hoagland's growth medium. The tank was inoculated with enough duckweed fronds to cover 30% of the surface, and the cultivation was sustained for 8 days. Throughout this period, daily samples were extracted from the water column to measure the residual concentrations of phosphorus and nitrogen.

Additionally, aerial photographs of the tank were captured to assess plant growth. Upon complete coverage of the tank's surface by the plants, a portion of the biomass was harvested, while another section was retained in the tank

(sufficient to cover 30% of the tank's surface) for continued treatment. The outcomes of the experiment are depicted as data points in Figure 5-19.

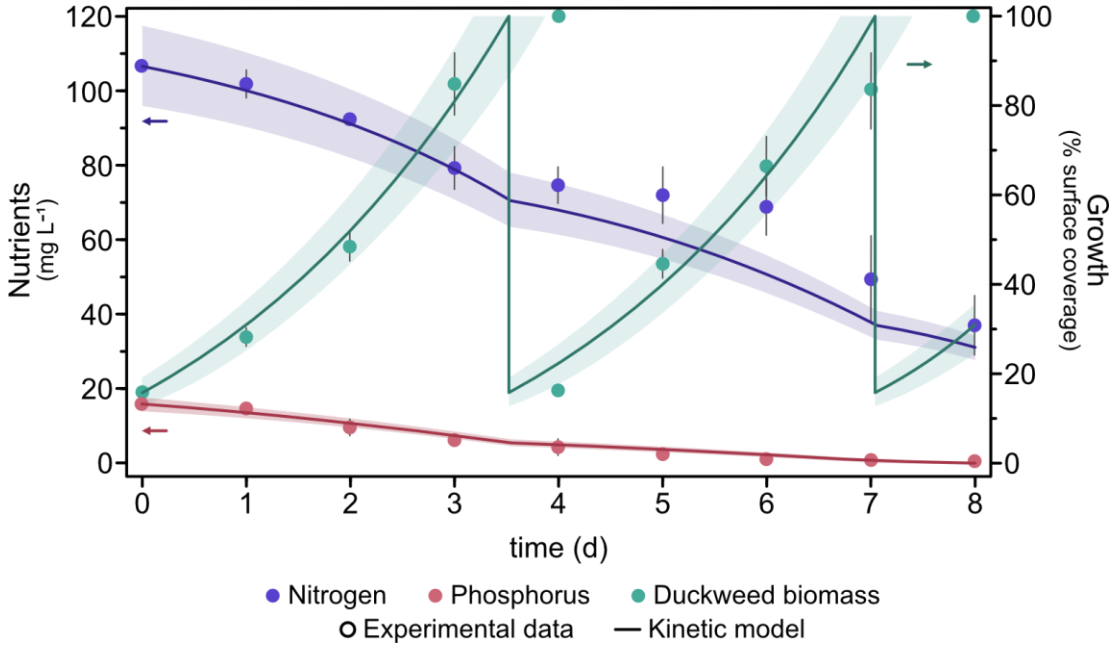

Figure 5-19 Experimental validation of the calibrated model for the batch cultivation of *Wolffia angusta*

Dots represent experimental data while continuous line the predicted values from the calibrated kinetic model. Data for nitrogen and phosphorus concentration should read against left axis while duckweed growth data (expressed as the percentage of total area coverage) should be read against the right axis, as marked by the arrows. The experiment was conducted at constant temperature, light intensity and photoperiod (25°C 100 µmol cm^{-2} $s^{-}1$, 12h light, respectively). Plants were cultivated in standard Hoagland's media (P_0 = 15 mg PO_4-P L^{-1}, N_0 = 110 mg NO_3-N L^{-1}). Colour bands represent a 90% confidence interval for the predicted responses. The error bars correspond to the standard deviation (n=2).

Concurrently, the experimental input parameters (P_0 = 15 mg L^{-1}, N_0 = 110 mg L^{-1}, Initial coverage = 30%, maximal coverage = 100%, T = 25°C) were used to execute the previously calibrated kinetic model. The anticipated results of the model are also illustrated in Figure 5-19.

The model predicts that by the end of the treatment period there is complete removal of phosphorus and a 71% removal of nitrogen. Moreover, the model anticipates two biomass harvests during the treatment duration. In practice, biomass had to be harvested twice, on the fourth and eighth days of treatment, resulting in removal rates of 96% for phosphorus and 65% for nitrogen.

Overall, the model forecasts data for biomass growth and phosphorus removal with an error below 10%, while for nitrogen removal, the error stays below 20%. The latter error might be elevated due to experimental and model time lag regarding biomass harvest. The model predicts that the tank's entire surface will be covered by plants after 3.5 days, marking the point when the plant growth model resets to its initial 30% coverage, allowing for the simulation of other variables. However, in practice, biomass was harvested only after the completion of the fourth day of cultivation, introducing a 0.5-day lag that could influence the predicted nitrogen concentration values in the subsequent growth cycle. Despite this time lag and the model's temporal overestimation of nitrogen removal, the variance between the overall treatment results and the simulated outcomes does not exceed 10%.

5.5 Conclusion

In conclusion, this thesis chapter elucidates the intricate dynamics governing the relationship between temperature, nutrient supply, and duckweed-based treatment systems, focusing specifically on *Wolffia angusta*. The research highlights the pivotal role of temperature in shaping both biomass growth and nutrient removal rates, with elevated temperatures proving advantageous for enhanced duckweed growth. Interestingly, the specific nutrient uptake remains unaffected, suggesting a nuanced relationship between temperature and nutrient-related processes. Conversely, nutrient supply emerges as a key determinant, exerting a more substantial influence on nutrient removal rates and specific nutrient uptake than on the overall growth of the plant. The simultaneous manipulation of temperature, nitrogen supply, and phosphorus supply underscores temperature as the primary factor influencing the responses of the duckweed-based treatment system, revealing limited interaction between temperature and nutrient supply effects.

Furthermore, this chapter delves into the contrasting impacts of culture conditions on biomass productivity and quality, emphasizing the need for a holistic approach in optimizing duckweed-based treatment systems. The findings suggest that a batch treatment system thrives when abundant nutrients are available, while the optimal temperature for plant cultivation depends on the intended application of the biomass. A significant contribution lies in the development of a robust mathematical kinetic model, derived from experimental data, which accurately predicts outcomes in terms of biomass productivity,

phosphorus and nitrogen removal, even in scenarios where one or both nutrients are deficient in the culture media. This calibrated model represents a valuable tool for comprehensively assessing the effects of temperature and nutrient supply on *Wolffia angusta* and provides a means for predicting and optimizing the performance of duckweed-based treatment systems.

Chapter 6 : Towards the Design of Continuous Flow Wastewater Treatment Systems Based on *Wolffia angusta* for Nutrient Reclamation Using Mathematical modelling

6.1 Introduction

The optimization of duckweed cultivation systems for biomass production and nutrient removal represents a critical facet of sustainable wastewater treatment strategies. To achieve this optimization, a holistic approach is essential, considering both abiotic factors such as temperature, pH, and nutrient concentrations, as well as biotic factors including species-specific growth characteristics and interactions within the ecosystem. Moreover, the control and assessment of operational conditions, including nutrient levels, hydraulic loading rates, and biomass harvesting strategies, become crucial in attaining desired treatment goals.

The growing interest in duckweed-based wastewater treatment systems (DWT) has led to experimental studies looking for the formulation of mathematical models, each attempting to capture the intricate relationships among the multiple variables at play. However, a considerable gap exists in the literature, as most of these models predominantly focus on the effect of environmental conditions on duckweed growth in batch systems, through the development of kinetic models (Monette *et al.* 2006; Van Dyck *et al.* 2021; Zhang *et al.* 2014). This leaves a significant gap in the understanding of continuous flow treatment systems, especially those based on *Wolffia angusta*, a species that has been relatively understudied in this regard. Only a few studies have focused on modelling continuous flow systems (Adhikari, Harrigan, and Reinhold 2015; Bal Krishna and Polprasert 2008; Calicioglu *et al.* 2021). Nonetheless, the existing research has primarily concentrated on evaluating established treatment systems and determining model parameters, rather than assessing the impact of variable operational conditions on the performance of the DWT system.

In wastewater treatment, in silico studies have gained prominence as powerful tools for assessing designs and operational strategies (Duarte *et al.* 2023). Computational simulations enable the exploration of various scenarios, offering insights into the potential performance of treatment systems without the

need for extensive and resource-intensive experimental work. The role of simulation studies in DWT systems becomes increasingly apparent, aiding not only in system evaluation but also in unravelling the complex interdependencies between operational variables and cultivation conditions.

This chapter addresses the gap in the existing literature by delving into three fundamental considerations for the operation of continuous flow DWT systems: flow rates, surface loading rates, and biomass retention time. Flow rates, closely tied to hydraulic retention times, play a pivotal role in the effective treatment of wastewater. By examining the effect of operational flow rates and retention times, it is possible to elucidate optimal conditions for nutrient uptake and biomass production and also provide information about the size of the pond required for the treatment. Surface loading rates, related to nutrient concentrations in influent and tanks, form another critical parameter influencing the efficiency of nutrient removal. A nuanced exploration of these rates will contribute to the development of strategies that maximize nutrient uptake while minimizing the risk of nutrient limitation for plants growth. Biomass retention time, intricately linked to harvesting regimes and surface coverage, represents a delicate balance that influences nutrient removal efficiency.

In essence, this chapter not only contributes to the broader understanding of duckweed-based treatment systems but also serves as a foundational step towards designing adaptable and resilient systems capable of addressing the complexities posed by diverse environmental variables. Through a combination of simulation studies and sensitivity analyses, this chapter seeks to give insights for more effective and sustainable wastewater treatment solutions for the aquaculture sector leveraging the potential of duckweed.

6.2 Aims

The primary objective of this chapter is to employ a mathematical model to provide insights into the design and operation of continuous flow duckweed-based wastewater treatment systems. Specifically, the goals are:

1) To formulate and validate a kinetic model for the continuous cultivation of *Wolffia angusta*.
2) To estimate the impact of hydraulic retention time, surface nutrient loading rates and initial duckweed coverage on the performance of the treatment

systems regarding nutrient removal and biomass production using in silico simulations.

3) To establish guidelines for the design of operation of treatment systems based on duckweed

6.3 Methodology

6.3.1 Continuous flow model formulation from batch kinetics

Based on the kinetic model presented in Section 5.4.5 for the cultivation of *W. angusta* in batch systems, it is possible to expand the model to account for the inflow and outflow of water from the treatment tank, in other words, to model a continuous treatment system.

Firstly, in addition to the previous considerations for modelling biomass growth and nutrient removal, it was assumed that the treatment tank was perfectly mixed. This implies that the temperature and nutrient concentration at any point in the tank are homogeneous, and there are no changes in liquid density during treatment. Additionally, it was assumed that there is no constant biomass outflow from the treatment tank, and it is only removed when the fronds completely cover the tank's surface.

With these assumptions, the treatment system was modelled as a continuous stirred tank reactor (CSTR). From the design equation of this type of reactor and the biomass material balance, the amount of biomass accumulated during wastewater treatment ($\mathrm{dN_X/dt}$) corresponds to the biomass produced, expressed as the product of the biomass growth rate ($\mathrm{r_X}$) and the area in which it is cultivated (A). Unlike a CSTR, only the surface area of the reactor is considered, not its entire volume, as the plants float, and the growth of biomass and nutrient removal occur only at the surface of water. By dividing both terms of Eq. 6-1 by the cultivation tank area, Eq. 6-2 is obtained, where the accumulation of plants in the system is now expressed as the relative coverage of plants ($\mathrm{dX/dt}$).

(6-1) $$\frac{\mathbf{dN_X}}{\mathbf{dt}} = \mathbf{r_X} * \mathbf{A}$$

(6-2) $$\frac{\mathbf{dX}}{\mathbf{dt}} = \mathbf{r_X}$$

In Equation 1-2, the expression for r_X is the same as for the batch duckweed cultivation considered in the previous chapter (Eq. 5-9) and uses the relative coverage of duckweed fronds as the plant growth parameter.

Regarding nutrients, the change in the quantity of nutrient S (either phosphorus or nitrogen) over time in the treatment tank (dN_S/dt) depends on of the rate at which the nutrient is removed from the system by duckweed, r_S, and the total cultivation area, A. It also depends on the amount of nutrient fed (F_{S0}) and removed (F_S) from the treatment tank, according to Eq. 6-3.

(6-3) $$\frac{dN_S}{dt} = -r_S * A + F_{S0} - F_S$$

If it is considered that the liquid density is constant over time, and the evaporation of water from the treatment system is negligible, the difference between the amount of nutrients entering and leaving the system can be expressed as the difference in nutrient concentration at each point (S_0 at the inlet, and S at the outlet), multiplied by the treatment flow rate, Q.

(6-4) $$\frac{dN_S}{dt} = -r_S * A + Q * (S_0 - S)$$

Additionally, if all terms are divided by the reactor volume, V, Eq. 6-5 is obtained, in which the net change in nutrients within the reactor. dS/dt, is expressed as the change in their concentration. Here, d represents the depth of the treatment tank, and τ the hydraulic retention time (HRT), resulting from dividing the treatment tank volume by the operating flow rate. The expression for calculating r_S was presented earlier in Chapter 5 (Eq. 6-4, 6-5).

(6-5) $$\frac{dS}{dt} = -\frac{r_S}{d} + \frac{(S_0 - S)}{\tau}$$

Thus, by utilizing equations 6-1 and 6-5, it is possible to model the continuous treatment of wastewater using duckweed and assess the impact that operational variables have on treatment efficiency. Additionally, the model outputs allow for informing on the design of treatment tanks.

6.3.2 Experimental validation of the mathematical model

To validate the expanded mathematical model, a new experiment was conducted in triplicate. On this occasion, the cultivation of *Wolffia angusta* took place in rectangular tanks with an operational volume of 12 L, through which Hoagland's growth medium was circulated at a constant flow rate of 1.71 L d^{-1}. The medium was initially adjusted to phosphorus and nitrogen concentrations representing the highest expected levels in fish farm effluents (4 mg P L^{-1} and 20 mg N L^{-1}). At the beginning, sufficient duckweed biomass was inoculated at the beginning of the experiment to cover 25% of the tanks' surface area. The treatment was monitored for 30 days, during which 75% of the biomass was harvested each time it covered 100% of the tank surface. Daily water samples from the influent and effluent of the treatment tanks were taken to monitor changes in pH, phosphorus, and nitrogen concentrations. Likewise, aerial photographs were captured to quantify the growth of the plants. More details about the setup are described in Chapter 3.

The experiment took place in a greenhouse with temperature control set at 25°C. However, the control system proved inefficient, resulting in variable ambient temperatures throughout the experiment (Figure 6-1). The experimental results were compared with those resulting from the mathematical model using both a constant temperature of 25°C and the real ambient air temperature.

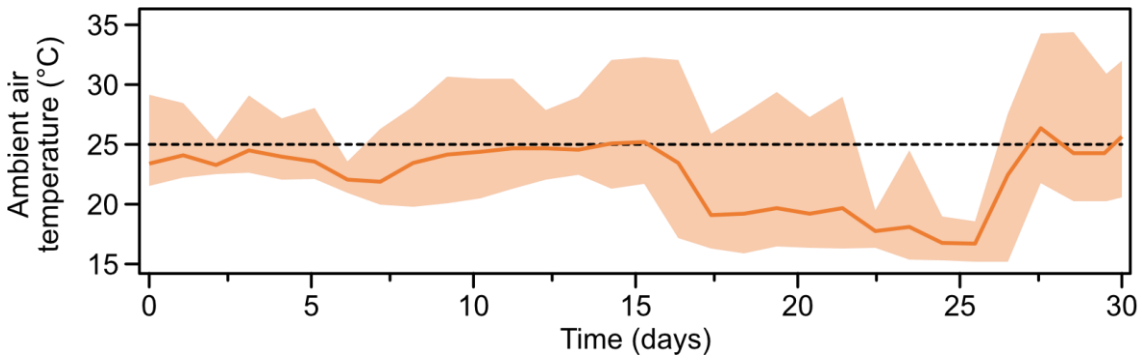

Figure 6-1 Daily ambient air temperature during the experimental validation of the mathematical model for a continuous flow treatment system.

The average daily air temperature in the greenhouse, where the experiment was conducted, is represented by the continuous line. The colour band marks the maximum and minimum daily air temperature. The dash line indicates the temperature set in the greenhouse control system (25°C).

6.3.3 Model application

With the validated mathematical model, the effect of some operational parameters (hydraulic retention time, nutrient loading rate, and initial duckweed coverage) on the performance of a continuous flow treatment system based on duckweed was evaluated by means of in silico simulations. The performance of the treatment system was assessed in terms of the resulting quality of the final effluent and the biomass productivity once it was estimated the treatment system reached steady state. At the end, based on the outcomes of this study, some insights regarding the design and operation of DWT systems are presented.

6.4 Results and Discussion

6.4.1 Modelling continuous flow wastewater treatment systems based on *Wolffia angusta*

Over the 30-day treatment period, plant growth and changes in the concentration of nutrients in the influent and effluent from the cultivation tanks were monitored. Simultaneously, the experiment inputs were utilized to run the mathematical model, producing two different results: one with a fixed temperature of 25°C, and another with the temperature set to the actual ambient air temperature during the experiment (Figure 6-2).

The in silico results at 25°C indicate that biomass covers the entire surface of the treatment tanks within 4.8 days whenever the initial plant cover is set back to 25%, leading to biomass harvesting six times in a 30-day treatment period (Figure 6-2 A). In contrast, considering the actual ambient temperature results in a variable harvesting frequency due to daily temperature fluctuations. In this case, the model suggests that duckweed should only be harvested five times, with each cultivation cycle taking between 4.6 and 6.3 days. The longest duration corresponds to the fourth cultivation cycle, during which the greenhouse temperatures were the lowest recorded.

Experimentally, biomass was harvested only four times, partly due to the mismatch between the actual and expected harvest days, and the prolonged duration of the last growth cycle, where the plants took 12 days to cover the surface of the treatment tank. When comparing the two simulations, it is clear that the results from the one considering the actual greenhouse air temperature align more to the experimental results, despite the anomaly in the last cultivation cycle.

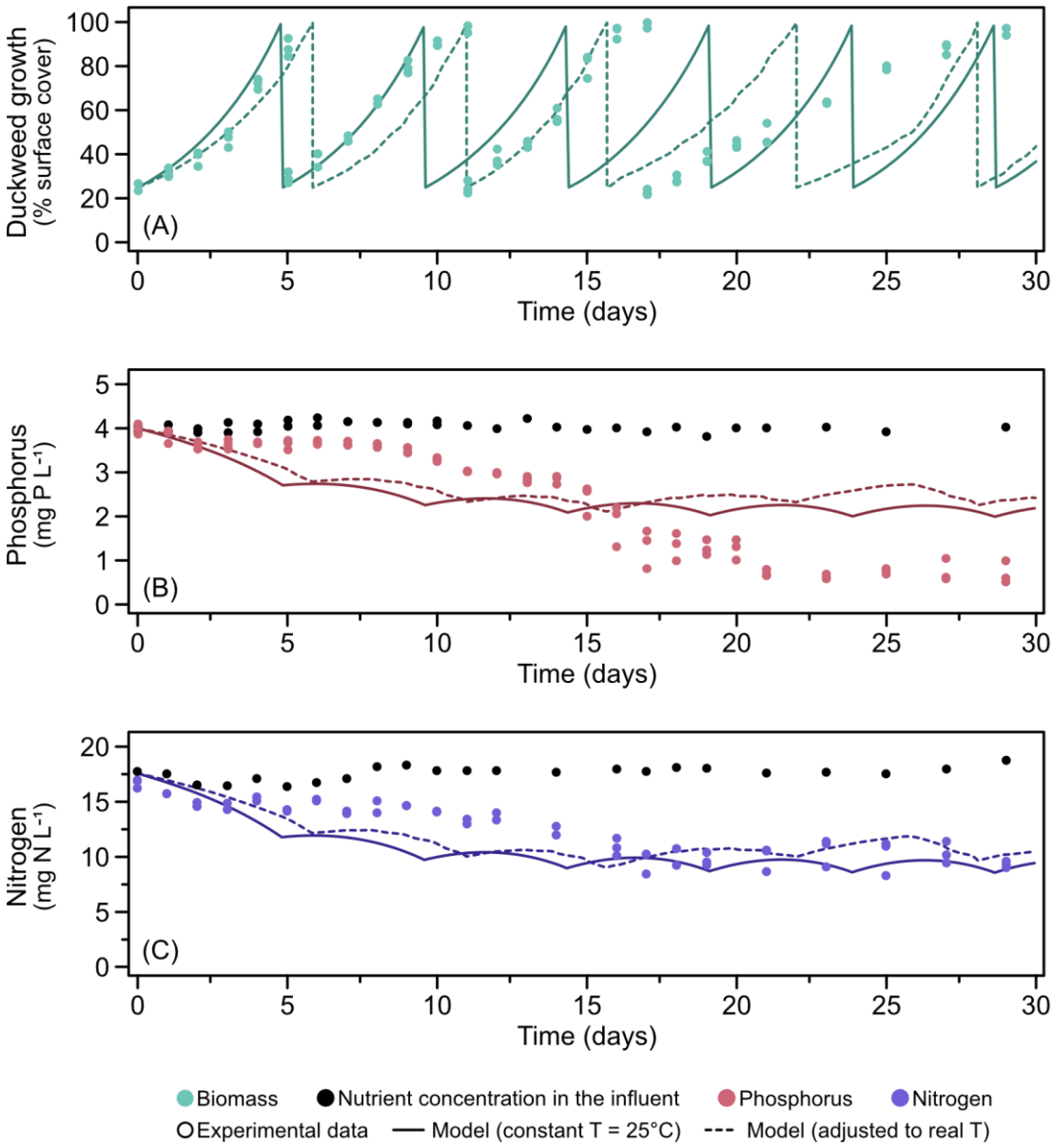

Figure 6-2 Experimental validation of the mathematical model for the cultivation of *Wolffia angusta* in a continuous flow treatment system.

To validate the mathematical model, experimental data for biomass growth **(A)**, influent and effluent phosphorus **(B)**, and nitrogen **(C)** concentration were collected in triplicate (dots) and plotted alongside the expected outcomes from the mathematical model (lines). The model was run using an average air temperature of 25°C (continuous lines), and the actual air temperature in the greenhouse where the treatment tanks where kept (dashed lines). The experiment was conducted at constant light intensity and photoperiod (100 µmol cm^{-2} s^{-1}, 12h light, respectively). Modified Hoagland's media (pH = 7, P_0 = 4 mg PO_4-P L^{-1}, N_0 = 20 mg NO_3-N L^{-1}) was pumped at a flow rate of 1.71 L d^{-1} through treatment tanks with an operational volume of 12 L each.

Regarding the removal of phosphorus and nitrogen (Figure 6-2 B, C), the simulations differ primarily in the time it takes to achieve a steady state. At a constant temperature of 25°C, the simulated results indicate that steady-state is reached around the 10th day of treatment, whereas considering the actual temperature extends the duration to 16 days. Concerning the final concentration

of nutrients in the effluent, after reaching steady-state, both simulations diverge only by 10%, with the final concentration being higher in the simulation that considers the actual treatment temperature (2.31 mg P L^{-1} and 9.52 mg N L^{-1})

Experimental findings for phosphorus (Figure 6-2 B) indicate that there was a reduction in phosphorus levels in the cultivation medium, stabilizing at an effluent concentration of 0.71 mg P L^{-1} after 20 days of treatment. This value was below the model's expected outcome. By the end of the treatment period, 82% of the phosphorus was removed from the media. Results from the experiment regarding nitrogen (Figure 6-2 C) closely align with those anticipated by the mathematical model. An steady concentration of 10 mg N L^{-1} in the effluent was achieved after 17 days of treatment.

Concerning the distribution of nutrients within the various components of the treatment system, the material balance indicates that both phosphorus and nitrogen were removed from the water as the treatment progressed (Figure 6-3). Up to day 11 of treatment, the reduction of both nutrients in the water corresponds to their increase in duckweed biomass. From day 11 onward, the proportion of phosphorus in the duckweed biomass remains practically unchanged, despite a greater reduction of phosphorus in the water. At the end of the treatment, only a small fraction (3% out of 71%) of the phosphorus removed from the water was transformed into duckweed biomass, with the rest remaining unaccounted for.

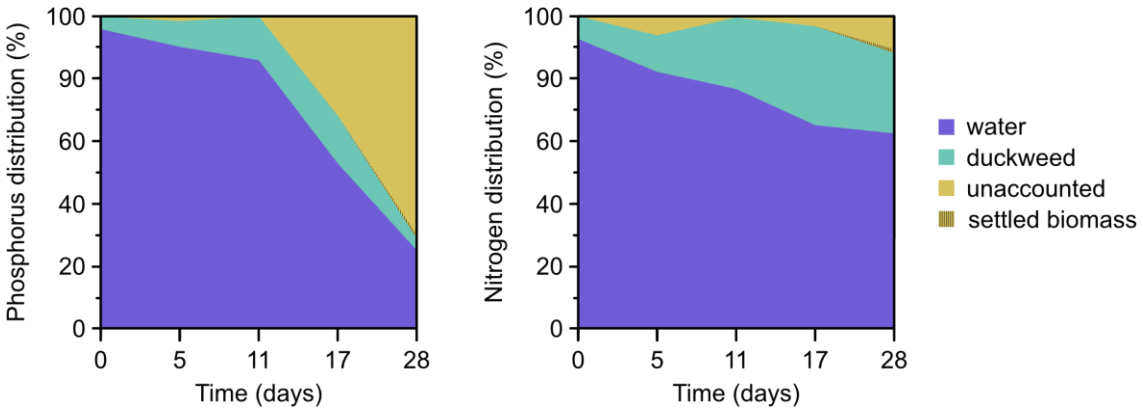

Figure 6-3 Elemental distribution of phosphorus and nitrogen in a continuous flow treatment system based on *Wolffia angusta*.

The elemental balance represent the distribution of both nutrients at each time the duckweed biomass was harvested. The share of phosphorus and nitrogen in the settled biomass was only measured at the end of the treatment period.

As for nitrogen, the results show that after 17 days of treatment, almost all the nitrogen removed from the water was converted into duckweed biomass.

However, during the last treatment cycle, there was a slight reduction in the share of nitrogen in both the water and duckweed biomass, resulting in 20% of the nitrogen being unaccounted for. Three percent of the unaccounted nitrogen was nitrogen contained in dead biomass found at the bottom of the treatment tanks at the end of the experiment.

During the experiment, biomass was harvested whenever it completely covered the surface of the treatment tank. This was done to prevent the formation of multiple layers of duckweed on the surface, thereby limiting the growth and performance of the plants for nutrient removal (Said *et al.* 1979; Verma and Suthar 2015). The decision on the harvest timing in each cycle relied on visual inspection of the tanks rather than on image analysis results. This resulted in instances where the harvest occurred earlier than anticipated, like in the first growth cycle, and others where it took longer, like in the third cycle. Except for the fourth growth cycle, the deviation in plant harvest time compared to the model's expectations was at most one day.

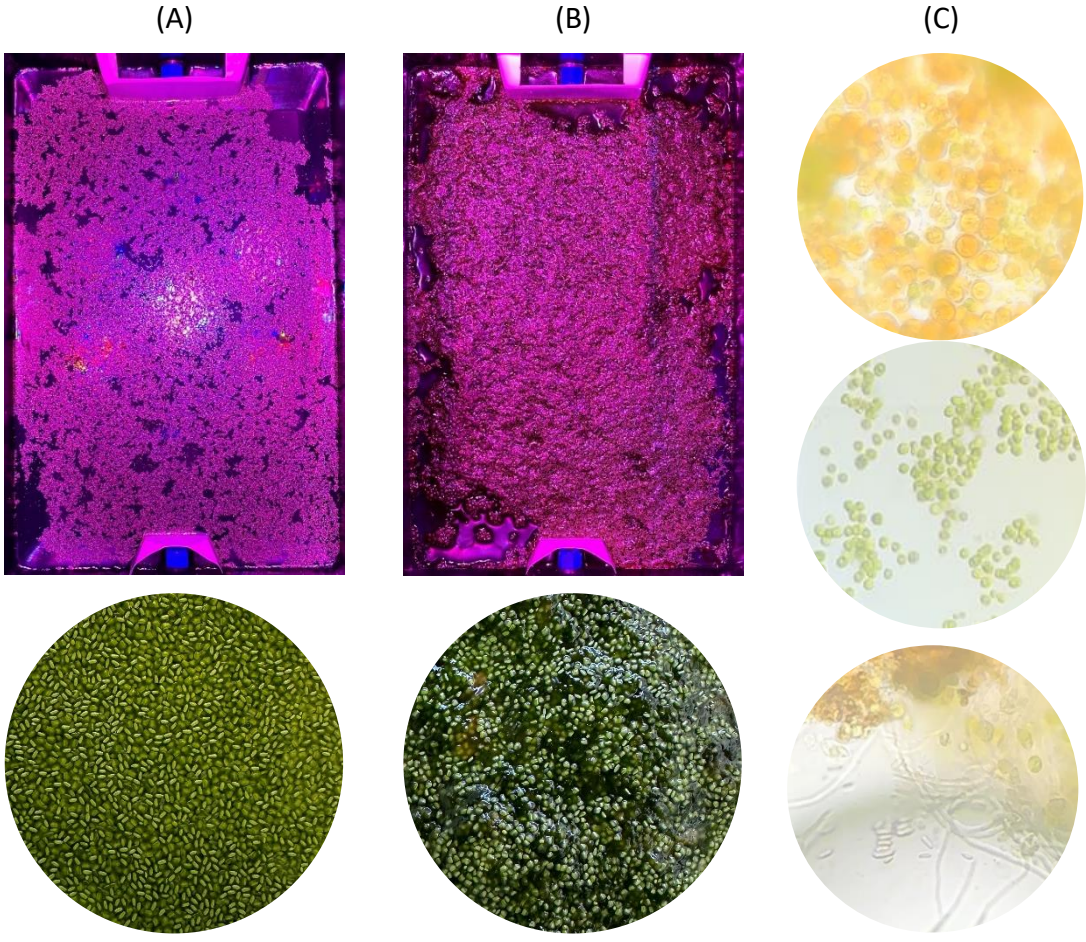

Figure 6-4 Surface and detail of the treatment tanks during the last treatment cycles of the experiment validating the mathematical model for continuous cultivation systems of *Wolffia angusta*

Figure 6-4 (continuation)

Photographs depicting the surface of the treatment tank and the detail of the harvested biomass at the end of the third **(A)** and fourth **(B)** cultivation cycles of *Wolffia angusta*. At the end of the third cultivation cycle, no algae presence is observed on the surface of the treatment tank or in the harvested biomass. However, a microalgae film developed on the surface of the tank at the end of the fourth cultivation cycle. The film is clearly visible in the detail picture of the harvested biomass. Further examination of the biofilm under an optical microscope **(C)** reveals the presence of green, brown, and filamentous algae.

The results indicate that the increased removal of phosphorus from water is not primarily attributed to phosphorus uptake by microalgae, as there is no concurrent increase in nitrogen removal. Szabó *et al.* (1999) emphasized in their study on *Lemna* and microalgae competition that the presence of algae in the cultivation system should lead to the simultaneous removal of phosphorus and nitrogen, with both nutrients being assimilated into the algal biomass. Even if we assume that all unaccounted phosphorus and nitrogen in the material balance corresponds to P and N in the algal biomass (Figure 6-3), the N:P ratio (1 : 3.5) differs from typical microalgae ratios (Geider and Roche 2002). Therefore, the primary factor for the enhanced phosphorus removal appears to be the pH level. Indeed, the water pH reached 10 during the final treatment cycle (Figure 6-5), a pH threshold at which phosphorus tends to precipitate as calcium phosphate (Wood 2020).

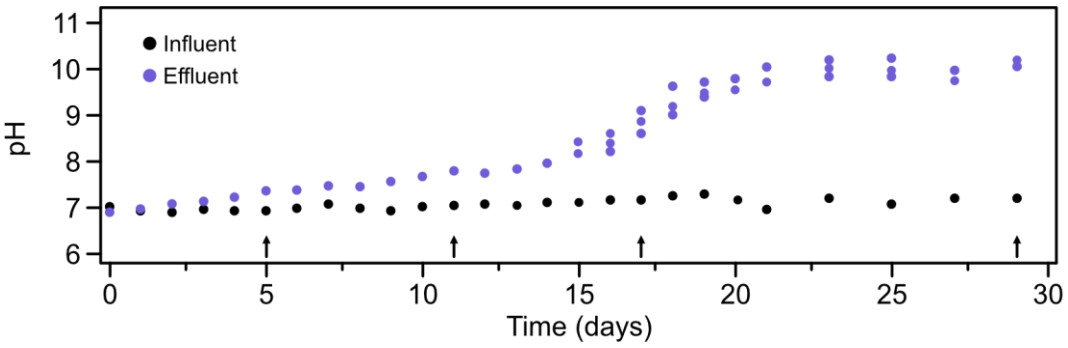

Figure 6-5 Variation in the effluent pH during continuous treatment with *Wolffia angusta*

The influent medium for experimental validation had an unbuffered pH, consistently adjusted to 7 throughout the experiment. The arrows indicate the points in time when biomass was harvested.

When analysing the nutrient data trends before reaching a stable state, it is evident that the experimental concentrations of phosphorus and nitrogen were consistently higher than the model-predicted concentrations. This is attributed to the plants needing to acclimate to the new cultivation conditions, which include continuous nutrient injection into the system and the source and type of light

present in the greenhouse. Despite this, and considering temperature fluctuations, which have a greater impact on biomass growth than nutrient removal, as concluded in Chapter 5, the steady state is reached within a period of 17 days, not deviating significantly from the time predicted by the model.

Ultimately, the comparison of the predicted values for duckweed coverage, and the concentrations of phosphorus and nitrogen in the effluent, calculated with the model adjusted to the real ambient temperature, with the data obtained during experimental validation, yields the results presented in Figure 6-6. When considering all the experimental data, it is noted that the formulated model does not correlate satisfactorily with the experimental data (R^2 values ranging between 0.10 and 0.66). The model predicts biomass growth, phosphorus removal, and nitrogen removal with respective mean absolute errors of 23.6%, 68.3%, and 12.6%. The significant errors in biomass production and phosphorus removal are due to the presence of algae during the fourth cultivation cycle, as previously discussed.

If we overlook variations in harvest time and the increased phosphorus removal due to elevated pH (data depicted by empty dots in Figure 6-6), the model's predictions exhibit improved correlation with the experimental data (R^2 values ranging from 0.62 to 0.85), and the errors for biomass production and phosphorus removal decrease to 12.6% and 16.7%, respectively. Ultimately, the model can predict the performance of a DWT system based on *Wolffia angusta* with an average error of 15%, underestimating all responses. A substantial portion of the error is attributed to the data variability during the transient state of treatment. If only results from the steady state were considered, the error would be significantly lower.

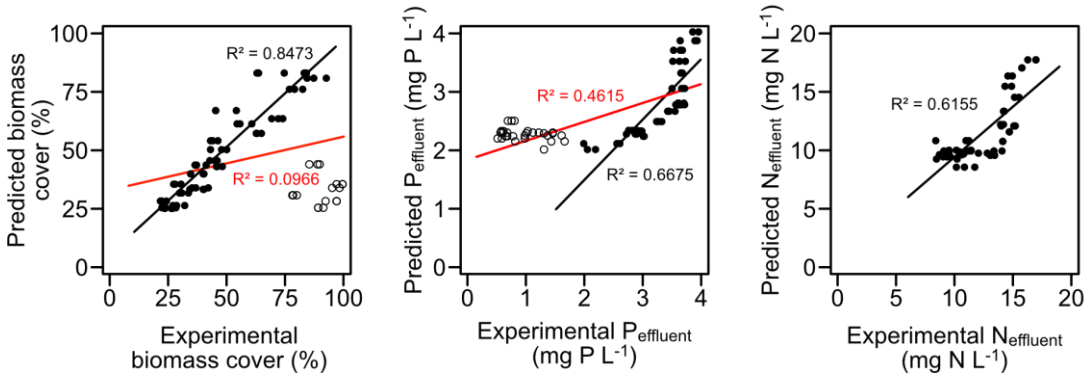

Figure 6-6 Correlation plots between experimental and predicted data during wastewater treatment using *Wolffia angusta*

The correlation plots depict data for biomass coverage, and the concentration of phosphorus and nitrogen in the effluent, during and before reaching the steady state. Void circles represent experimental data for the fourth cultivation cycle. Two correlation lines are presented, one considering all experimental data (red line) and another excluding data from the fourth cultivation cycle (black line).

The errors in the predictions of the formulated model in this chapter closely align with those calculated by Adhikeri *et al.* (2015) when assessing first-order kinetic models originally designed for wetland applications (Kadlec, Bastiaens, and Urban 1993; Kadlec and Wallace 2009). The mean standard error of the predicted variables in those models ranges between 6.7% and 15.8%. Similar magnitudes of errors were encountered during the validation of the duckweed-based wastewater treatment (DUBWAT) model (Bal Krishna and Polprasert 2008). The error assessments in these studies was based on the comparison of treatment system responses once the steady state was achieved. In all instances, reduced errors could be attained if the model incorporated the effects and interactions of other biotic and abiotic factors influencing the performance of a DWT system.

6.4.2 Effect of operational parameters on the performance of duckweed-based wastewater treatment systems

Within the operational parameters of duckweed-based wastewater treatment systems, the hydraulic retention time (HRT), nutrient surface load, and initial plant coverage were selected as the variables with the most significant impact on the treatment system's performance. The following outlines the effect of these variables on nutrient removal and biomass production.

6.4.2.1 Long hydraulic retention times improve effluent quality but limit duckweed growth

In silico results for the treatment of a wastewater containing 4 mg P L^{-1} y 20 mg N L^{-1} reveal that, at a constant initial duckweed coverage, increasing the hydraulic retention time results in (1) lower concentrations of phosphorus and nitrogen in the final effluent from a DWT system, and (2) a generally unaffected production of duckweed biomass (Figure 6-7).

The first effect is more prominent at higher initial duckweed coverage values. Across all scenarios, phosphorus removal was nearly complete (Figure 6-7 A), reaching a minimum effluent phosphorus concentration of 0.02 mg P L^{-1}. Greater initial duckweed coverage allows this phosphorus concentration to be achieved at shorter hydraulic retention times. For instance, with an initial coverage of 90%, only a 10-day HRT is required, whereas a 22-day HRT is needed for an initial coverage of 10%. Similar trends are noted when examining

nitrogen results (Figure 6-7 B), although the maximum removal achievable for nitrogen is 88.5%.

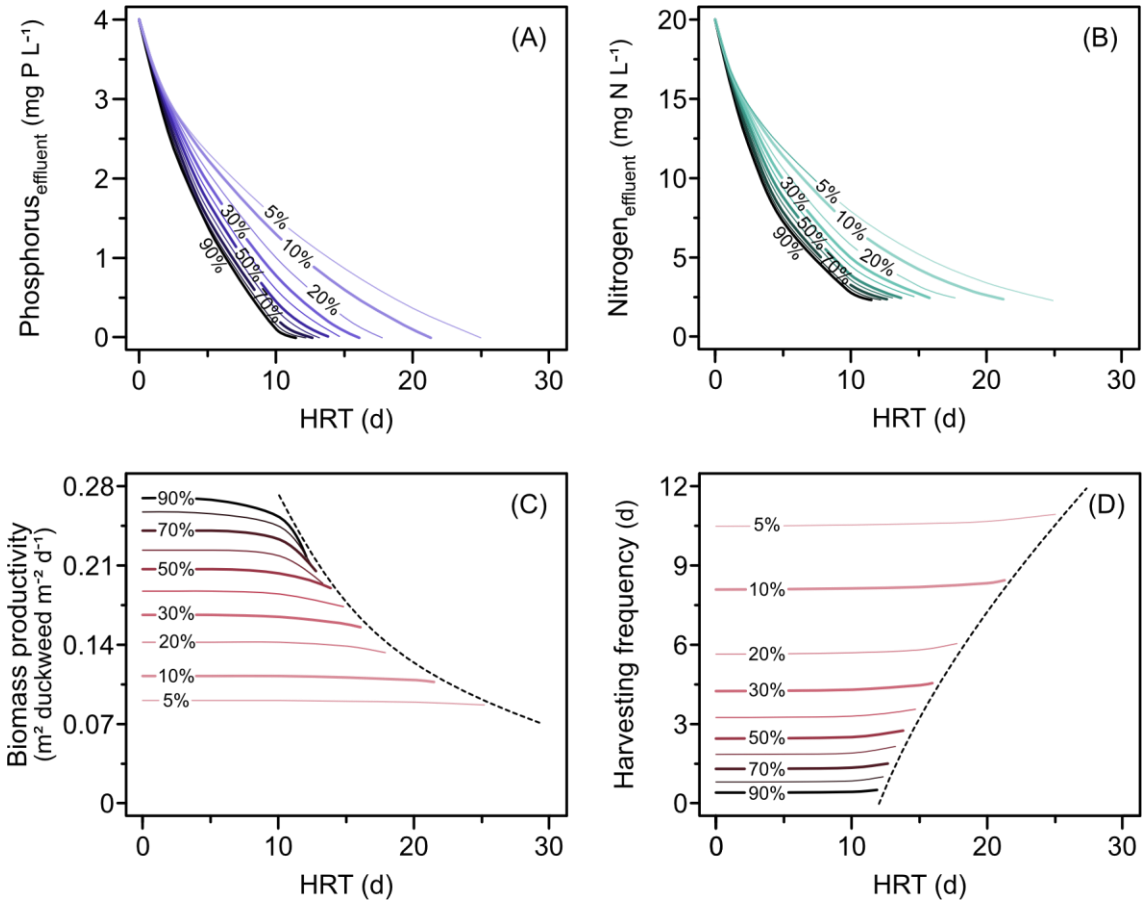

Figure 6-7 Influence of the hydraulic retention time (HRT), at varying percentages of initial duckweed coverage, on the performance of a continuous flow wastewater treatment system based on *Wolffia angusta*.

(A) Effect on the final effluent phosphorus concentration, **(B)** Effect on the final effluent nitrogen concentration, **(C)** Effect on daily biomass productivity, and **(D)** Effect on the frequency at which biomass needs to be harvested. The results were obtained under steady state conditions. The continuous lines, accompanied by their respective numerical values, depict various initial coverage percentages of duckweed on the surface of the treatment tank at the start of each cultivation cycle. In the simulations, the biomass was harvested every time it covered the entire surface of the treatment tank. The dashed lines indicate the maximum hydraulic retention time (HRT) permissible in the system, given an initial duckweed coverage. Influent characteristics: 4 mg P L^{-1}, 20 mg N L^{-1}, 25°C.

The findings also reveal that, for constant duckweed coverages, there exists a range where the HRT has no impact on biomass production and harvesting frequency (Figure 6-7 C,D). This range diminishes as the initial duckweed coverage increases. As the HRT approaches the maximum operating value for a given plant coverage, there is a significant decline in biomass productivity due to phosphorus limitation, particularly pronounced at initial duckweed coverages exceeding 25%.

In general, the findings indicate that an increase in hydraulic retention times leads to a higher removal of nutrients at any given initial duckweed coverage. This effect of the HRT has been observed in previous studies involving other macrophytes and *Lemna*ceae used for wastewater treatment (Alahmady, Stevens, and Atkinson 2013; Kadlec *et al.* 1993; Y. Zhao, Fang, Jin, Huang, Bao, He, *et al.* 2014). With the HRT increase, the water in the treatment tank is not renewed as frequently, allowing the plants to take up nutrients more rapidly than they are introduced into the system.

For systems utilizing *Wolffia angusta*, doubling the HRT with constant initial duckweed coverages results, on average, in a 25% higher removal of both phosphorus and nitrogen. The beneficial effect of doubling the HRT is greater than the 3-8% improvement reported previously for *Lemna* minuta (Alahmady *et al.* 2013) and *Lemna aequinoctialis* (Y. Zhao, Fang, Jin, Huang, Bao, He, *et al.* 2014). This difference could be attributed, in part, to the way the HRT was modified in the other studies. The adjustment of the depth of the treatment tanks instead of the flow rate, can potentially result in poor mixing conditions and concentration gradients of nutrients in the water column. Furthermore, *W. angusta*'s higher specific growth rate compared to *Lemna* (P. Ziegler *et al.* 2015) may also contribute to the observed differences.

The analysis of initial duckweed coverage suggests that at low duckweed coverages, the extended time required for plants to cover the entire treatment tank surface can be compensated by a longer hydraulic retention time. This is mainly due to a simultaneous lower nutrient load to the system. Thus, in a treatment tank of fixed dimensions, this would ensure a consistent level of treatment over time. Although higher HRTs contribute to lower phosphorus concentrations in the effluent, complete removal is never achieved, as the balance between nutrient input and removal must always be positive to sustain plant growth. The minimum balance is achieved when the phosphorus concentration reaches the half-saturation constant (0.048 mg P L^{-1} in the case of *W. angusta*). This was previously demonstrated by varying the operation time of a wastewater treatment reactor based on *Lemna aequinoctialis* (Alahmady *et al.* 2013). It was found that there is an operation time at which biomass productivity is maximum, corresponding to the point where the removal of the limiting nutrient is nearly complete. Prolonged operation times resulted in the failure of the treatment system due to the lack of nutrients necessary for plant growth.

The findings also suggest that there is a maximum HRT value, for a given initial duckweed coverage, at which a DWT system can reliably be operated. The maximum HRT is also influenced by the phosphorus concentration in the influent.

Specifically, at an influent phosphorus concentration of 4 mg P L^{-1}, the maximum HRT for the treatment system varies between 11.8 and 25 days (Figure 6-8). This maximum retention time tends to increase as the initial plant coverage decreases, with more significant variations observed when the initial duckweed coverage is below 30%. For coverages exceeding 30%, the average maximum HRT stabilizes around 13 days.

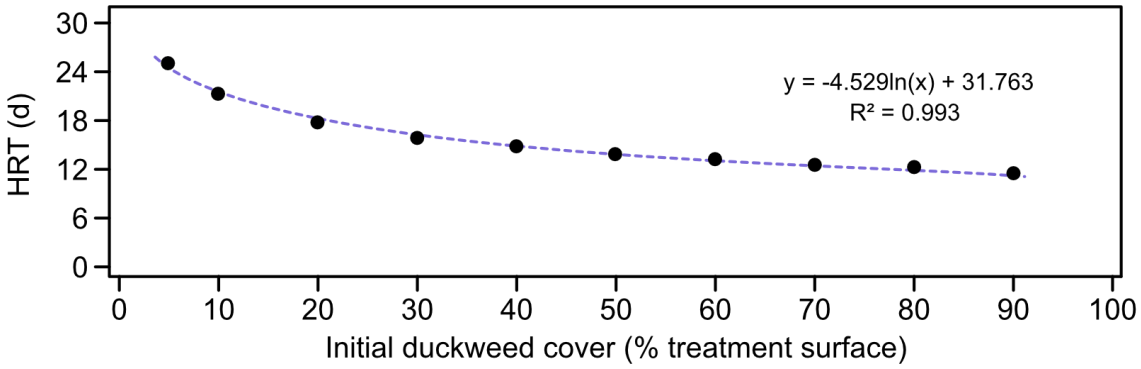

Figure 6-8 Maximum hydraulic retention time at which a continuous wastewater treatment system based on *Wolffia angusta* can operate, depending on the initial surface coverage of the plants

The dashed line represents the trend of the data. HRT, hydraulic retention time.

The analysis of the results suggests that, in practical terms, maintaining an operational hydraulic retention time of 12 days ensures the consistent functioning of treatment systems relying on *W. angusta*. This is valid regardless of the initial plant coverage, and can guide the determination of the minimum size for a DWT system based on the expected effluent flow rates from a specific fish farm. It is anticipated that this HRT value will rise with higher concentrations of phosphorus in the wastewater to be treated and will decrease with elevated ambient temperatures.

6.4.2.2 The quality of the final effluent improves with a reduction in the surface loading of nutrients

Assessing the effect of the surface loading rate of nutrients in the system yields contrasting results compared to the observed effects of hydraulic retention time. These opposing outcomes are anticipated due to the fixed dimensions of the treatment tank used in the in silico simulation and the constant influent wastewater concentrations of both phosphorus and nitrogen. The adjustment of the hydraulic retention time leads to changes in the operational flow rate,

subsequently altering the nutrient loading rates. The following analysis focuses on the surface load of nutrients, rather than the volumetric load, as it is at the surface of the pond where duckweed nutrient uptake occurs. Figure 6-9 illustrates the impact of surface phosphorus loading rate (PLR_S) and surface nitrogen loading rate (NLR_S) on effluent quality during duckweed treatment, as well as on biomass productivity.

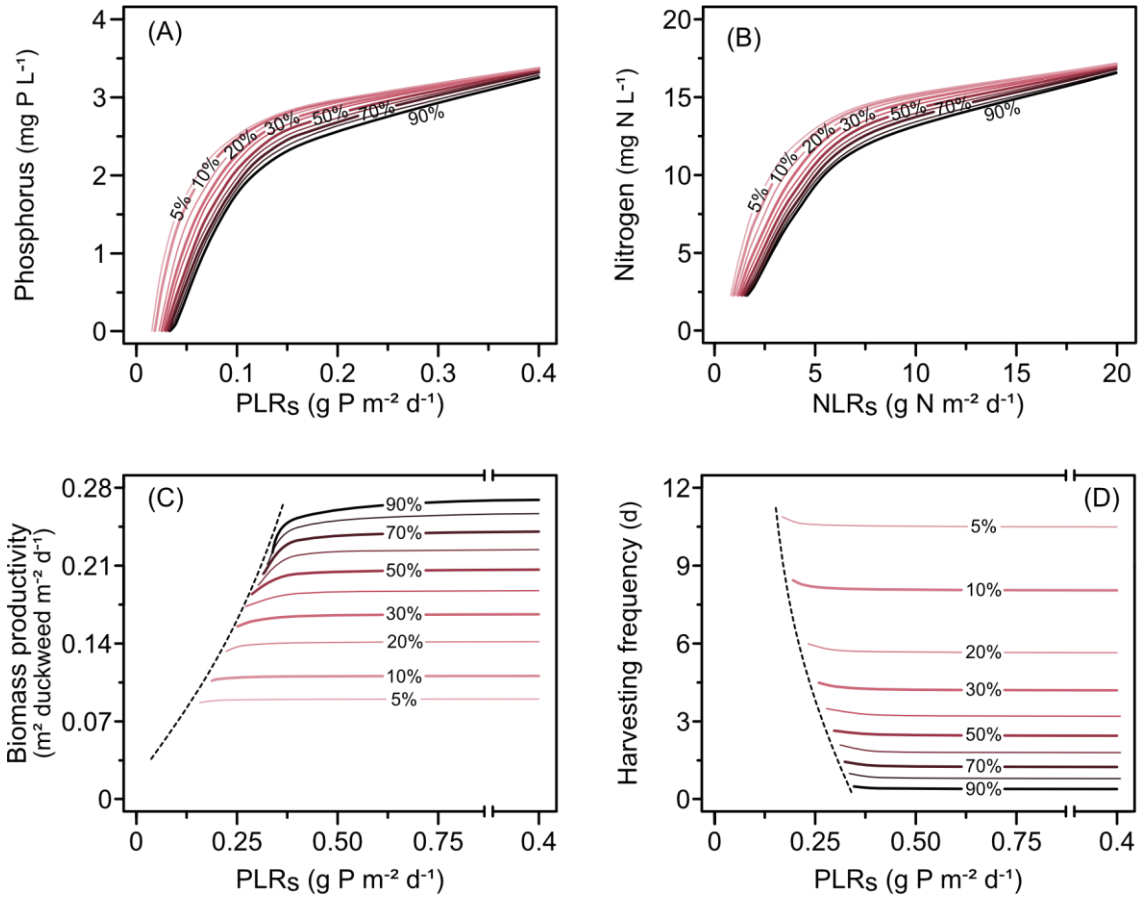

Figure 6-9 Influence of the surface phosphorus loading rate (PLR_S) and surface nitrogen loading rate (NLR_S), at varying percentages of initial duckweed coverage, on the performance of a continuous flow wastewater treatment system based on *Wolffia angusta*.

(A) Effect of PLR_S on the final effluent phosphorus concentration, **(B)** Effect of NLR_S on the final effluent nitrogen concentration, **(C)** Effect of PLR_S on daily biomass productivity, and **(D)** Effect of PLR_S on the frequency at which biomass needs to be harvested. The results were obtained under steady state conditions. The continuous lines, accompanied by their respective numerical values, depict various initial coverage percentages of duckweed on the surface of the treatment tank at the start of each cultivation cycle. In the simulations, the biomass was harvested every time it covered the entire surface of the treatment tank. The dashed lines indicate the minimum surface phosphorus loading rate (PLR_S) necessary for system operation, given an initial duckweed coverage. Influent characteristics: 4 mg P L^{-1}, 20 mg N L^{-1}, 25°C.

In general, the findings indicate that, regardless of the initial duckweed coverage, reducing the surface loads of phosphorus and nitrogen leads to

decreased levels of both elements in the effluent (Figure 6-9 A, B). This effect becomes more pronounced when both loading rates fall below 0.15 g P m^{-2} d^{-1} and 7.5 g N m^{-2} d^{-1}, respectively. Below these values, at a constant plant coverage, a 20% reduction in PLR_S and NLR_S results, on average, in a 60% and 49% decrease in the effluent P and N concentration, respectively. Conversely, above the threshold values, a 20% reduction in both loading rates leads to only a 4.8% and 5% reduction in phosphorus and nitrogen concentrations in the effluent, respectively.

Similar to HRT, the nutrient surface loading rate has minimal impact on biomass production and harvesting frequency, regardless of the initial plant coverage employed (Figure 6-9 C, D). A substantial decline in biomass production is only observed when the loading rate of the limiting nutrient approaches the minimum value required for plant growth. The results reveal that the minimum PLR_S varies with the initial plant coverage, being lower at lower plant coverage values, and ranging between 0.16 and 0.38 g P m^{-2} d^{-1}. Below these phosphorus loads values the treatment systems fails. Similarly, for nitrogen, there is a minimum NLR_S for each initial plant coverage. However, these values are not evident in the simulation results as phosphorus was the limiting nutrient.

Similar to HRT, the nutrient surface loading rate has minimal impact on biomass production and harvesting frequency, regardless of the initial plant coverage employed (Figure 6-9 C, D). A substantial decline in biomass production is only observed when the loading rate of the limiting nutrient approaches the minimum value required for plant growth. The results reveal that the minimum PLR_S varies with the initial plant coverage, being lower at lower plant coverage values, and ranging between 0.16 and 0.38 g P m^{-2} d^{-1}. Below these phosphorus loads values the treatment systems fails. Similarly, for nitrogen, there is a minimum NLR_S for each initial plant coverage. However, these values are not evident in the simulation results as phosphorus was the limiting nutrient.

A low surface phosphorus loading rate is observed to exert a detrimental effect on duckweed biomass productivity. This phenomenon can be attributed to the essential role of phosphorus in plant growth and metabolism, where inadequate phosphorus availability limits the physiological processes crucial for biomass production. Several studies support this observation, including research on *Lemna aequinoctialis*, where increased PLR_S corresponded to enhanced biomass production (Alahmady *et al.* 2013). Additionally, investigations on different duckweed species, such as *Lemna minor*, *Spirodela polyrhiza* and *Wolffia* brasiliensis, have indicated a positive correlation between phosphorus availability and biomass productivity (McCann 2016). Comparison values across

the studies emphasize the significance of phosphorus in promoting optimal growth conditions for duckweed, as evidenced by substantial reductions in biomass productivity when PLR_S fall below the previously mentioned critical thresholds. The findings highlight the fundamental role of maintaining an adequate surface phosphorus loading rate to support robust duckweed biomass productivity and efficient DWT systems.

6.4.2.3 Greater plant cover enhances biomass productivity alongside an increase in harvest frequency

The outcomes of the initial duckweed coverage's impact on effluent quality and biomass production are presented in Figure 6-10. Regarding nutrient concentration in the effluent, the initial plant coverage has little effect at low hydraulic retention times but exerts a greater impact at higher HRT (Figure 6-10 A, B). When the HRT is less than 5 days, there is no substantial reduction in N or P concentration in the effluent when changing the initial duckweed coverage from 5% to 90%. However, removal of both nutrients improves with HRTs exceeding 5 days. At these retention times, increases in the initial duckweed coverage result in lower concentrations of nutrients in the effluent. For example, at an HRT of 10 days, phosphorus removal increases from 62%, with a 5% initial duckweed coverage, to 98%, with a 90% initial coverage. With HRTs exceeding 10 days, there is a maximum initial plant coverage value that can be employed. This coverage value decreases with higher HRTs.

Concerning biomass productivity and harvest frequency, it is apparent that the primary factor influencing these factors is the initial duckweed coverage (Figure 6-10 C,D). Increased plant coverage results in higher biomass productivity but entails a higher harvest frequency. Biomass productivity can vary from 0.07 to 0.27 m^2 duckweed m^{-2} d^{-1}, with harvesting frequencies ranging between 0.4 and 13 days, when adjusting the initial duckweed coverage between 2.5% and 90%. However, the extent of these responses is contingent on the hydraulic retention time during treatment. Longer HRT reduces the maximum biomass productivity and increases the minimum frequency for biomass harvesting. Hydraulic retention times below 10 days permit a maximum aerial biomass productivity of 0.27 m^2 duckweed m^{-2} d^{-1}, with biomass being harvested every 0.4 days, for initial duckweed coverages up to 90%. Conversely, at HRTs surpassing 10 days, the maximum biomass is adversely affected as there exists an upper limit value to the initial duckweed cover.

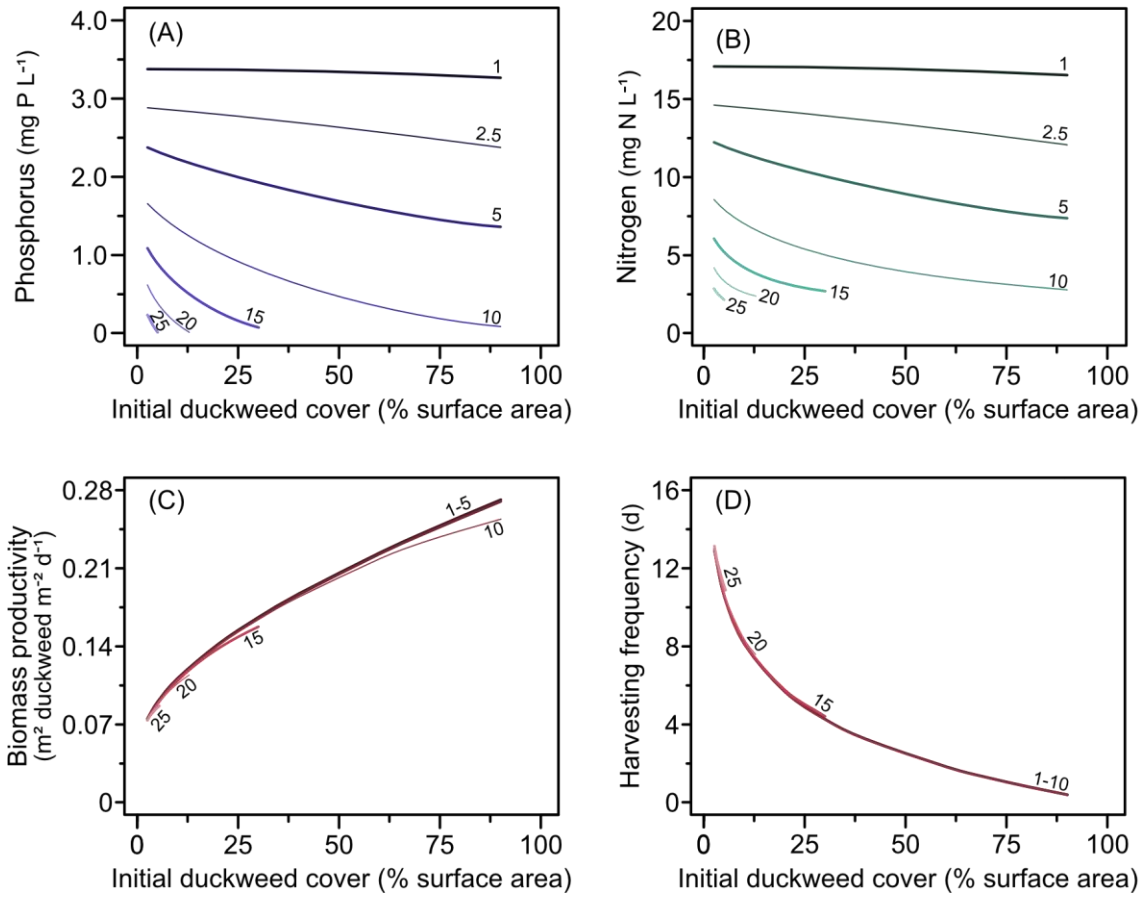

Figure 6-10 Influence of the initial duckweed coverage, at varying hydraulic retention times, on the performance of a continuous flow wastewater treatment system based on *Wolffia angusta*.

(A) Effect on the final effluent phosphorus concentration, **(B)** Effect on the final effluent nitrogen concentration, **(C)** Effect on daily biomass productivity, and **(D)** Effect on the frequency at which biomass needs to be harvested. The results were obtained under steady state conditions. The continuous lines, accompanied by their respective numerical values, depict various hydraulic retention times, in days. In the simulations, the biomass was harvested every time it covered the entire surface of the treatment tank. Influent characteristics: 4 mg P L^{-1}, 20 mg N L^{-1}, 25°C.

Additional duckweed studies corroborate the conclusions regarding the impact of the initial plant coverage on the performance of DWT systems. In a batch cultivation system with abundant nutrient availability, elevating the initial coverage of *Lemna minor* from 20% to 80% resulted in a 68%, 75%, and 37% increase in phosphorus and nitrogen removal, as well as biomass production, respectively (Walsh, Coughlan, *et al.* 2021). The significance of these enhancements was more pronounced when the coverage varied between 20% and 50% compared to the range of 50% to 80%. Despite dealing with different duckweed species and cultivation systems, the outcomes align with those observed for *W. angusta* at hydraulic retention times around 10 days.

The quantity of plants introduced into the treatment system can also be expressed as their mass per unit of treatment area, known as stocking density. Investigations manipulating the stocking density of various duckweed species also demonstrate the positive impact of increasing plant numbers on both biomass productivity and the final effluent's quality (Debusk *et al.* 1981; Monette *et al.* 2006; Said *et al.* 1979; Verma and Suthar 2015). Nonetheless, in relation to the treatment area, high stocking density values usually imply equivalent coverages values surpassing 100%. This leads to the development of multilayer culture systems hindering plant growth and consequently affecting the overall performance of the treatment system (Färber, Königshofer, and Kandeler 1986; Kufel *et al.* 2018). The mathematical model used for the simulations is limited in that sense. Expanding the model to incorporate virtual coverage values exceeding 100%, along with the inhibitory effects of overcrowding and mortality of plants, can enable a more thorough analysis of potential failure points in the treatment system, similar to the comprehensive analyses reported by Frederic *et al.* (2006).

Upon close examination of the results, it is evident that the maximum initial plant coverage that can be utilized in a DWT system depends on the hydraulic retention time at which the system operates and the initial nutrient concentration in the wastewater.

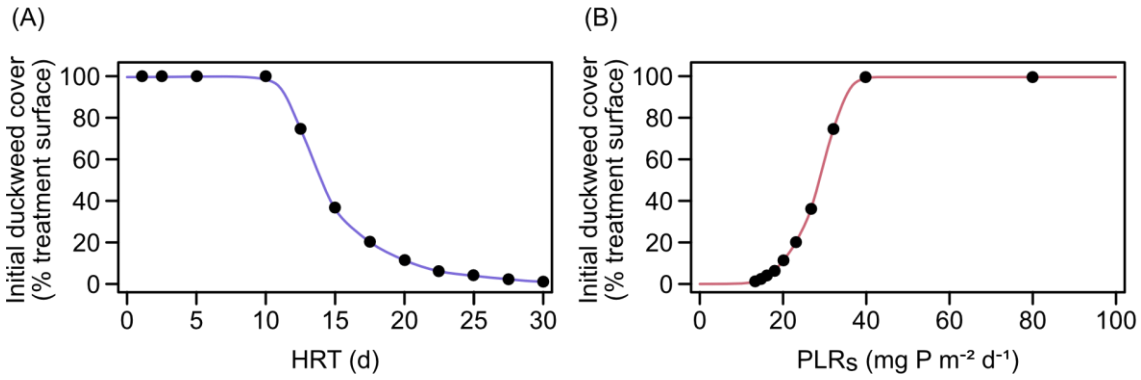

Figure 6-11 Maximum initial duckweed coverage at which a continuous wastewater treatment system based on *Wolffia angusta* can operate

The maximum initial duckweed cover can vary depending on the hydraulic retention time **(A)** or the loading rate of the limiting nutrient **(B)**. In the simulations, phosphorus was set as the limiting nutrient. HRT, hydraulic retention time; PLRs, surface phosphorus loading rate.

Increases in the HRT, above 10 days, lead to a reduced operational initial coverage of duckweed (Figure 6-11 A). If the coverage remains constant, the system would fail because the loaded nutrients are insufficient to support the

growth of a large plant population. Considering phosphorus as the limiting nutrient, this implies that the initial coverage of duckweed is actually determined by the surface load of this nutrient in the treatment system. In fact, it is when the PLR_S is below 40 mg P m^{-2} d^{-1} that the initial coverage of duckweed must be reduced to ensure effective wastewater treatment (Figure 6-11 B).

While the highest biomass production and phosphorus removal from wastewater are achieved when the initial plant coverage is 90% and the PLR_S is 40 mg P m^{-2} d^{-1}, the costs and human labour involved in harvesting the biomass every 5 hours make these conditions operationally impractical.

6.4.3 Towards the design and operation of robust and reliable treatment systems

The primary goal of duckweed-based ponds for wastewater treatment is nutrient removal, functioning analogously to maturation lagoons in conventional wastewater treatment systems (Tanner *et al.* 2005). In this regard, the design and sizing of the equipment must consider three main factors: firstly, the environmental conditions of the treatment location; secondly, the characteristics of the influent to be treated; and lastly, the desired treatment level. This last parameter is usually dictated by local legislation regarding discharge limits of urban wastewater treatment works (UWWT) into surface water bodies. In the case of the United Kingdom, the Environmental Agency ensures that wastewater discharge complies with the Urban Wastewater Treatment Regulations (UWWTR), which in turn depend on the population equivalent (PE) served by a wastewater treatment plant (Environment Agency 2019). For highly populated sites with a PE greater than 100,000, the current regulation stipulates that the phosphorus concentration limit in the discharge should be 1 mg P L^{-1}, with a minimum removal of 80% concerning the nutrient load in the influent. Regarding nitrogen, the discharge concentration limit should be 10 mg N L^{-1}, with a minimum removal between 70% and 80% concerning the load in the influent. For UK water companies, the current Asset Management Period (AMP7) within the framework of the Water Industry National Environment Programme (WINEP) establishes a new technically achievable limit for phosphorus of 0.25 mg P L^{-1} (Environment Agency 2021).

The predictive mathematical model developed earlier in this chapter can inform the design and operation of a duckweed -based pilot-scale system for nutrient recovery from wastewater. By considering the current legislation in the

UK and using a flow rate of 10 L day^{-1} as the basis for calculation, strategies for sizing and operating a duckweed-based water treatment system can be established.

6.4.3.1 Pond sizing

To determine the dimensions of the treatment tank, the scenario of effluent from a fish farm containing 1.25 mg P L^{-1} is considered. This phosphorus concentration is chosen since, considering regulations for wastewater discharge, the required treatment level to achieve 0.25 mg P L^{-1} in the final effluent, first treatment objective, corresponds to an 80% phosphorus removal, the second treatment objective.

Under these conditions and by varying the depth of the treatment tank, it is possible to obtain the required area for plant cultivation at different temperatures with a constant initial duckweed coverage of 25% (Figure 6-12).

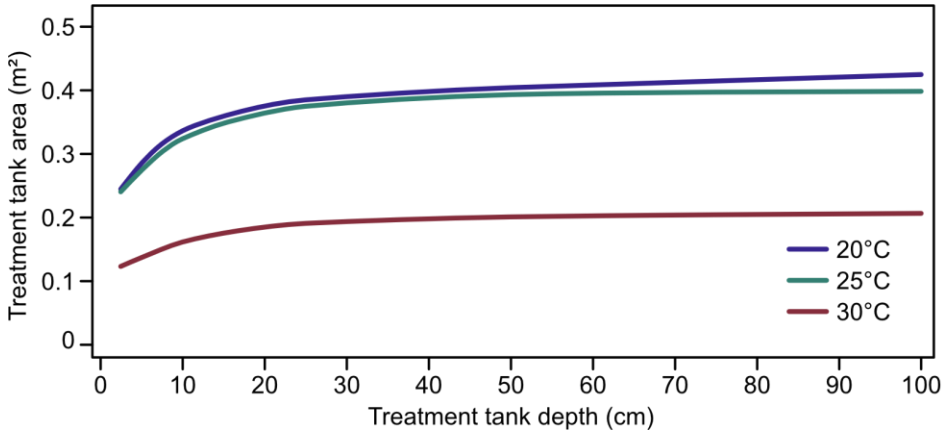

Figure 6-12 Dimensions required for wastewater treatment tanks based on *Wolffia angusta* to ensure compliance with wastewater discharge limits according to UK regulations

The required depth and area of the tank to ensure a phosphorus concentration in the effluent of 0.25 mg P L^{-1} and a minimum removal of this nutrient by 80%, relative to the influent load, were obtained at three different ambient temperatures, considering an initial duckweed coverage of 25% of the treatment area. The curve calculations were based on an operating flow rate of 10 L min^{-1} and an influent phosphorus concentration of 1.25 mg L^{-1}.

Two observations can be made from the figure. Firstly, for constant treatment temperatures, the required area for wastewater treatment increases with deeper tanks until reaching a point where the required area remains constant

despite increasing depth. If rectangular treatment tanks are considered, this implies that three types of tanks can be considered: tanks with all dimensions identical (cubic tank), tanks with much greater surface area than depth (shallow ponds), and tanks with much greater depth than surface area (deep ponds). At 20°C, the depth required for a cubic tank is 64.3 cm, which is 2% more than the required depth at 25°C and 1.5 times larger than the depth at 30°C.

Another observation from the figure is the effect of temperature on the required treatment area at a constant tank depth. It is found that lower temperatures require a larger treatment area to meet treatment objectives. At 20°C and with a depth of 100 cm, for instance, a tank with an area of 0.43 m^2 is required. When the temperature increases by 5 degrees, the area reduces to 0.40 m^2, but if the temperature rises to 30°C, the required area decreases considerably to 0.21 m^2, which is less than half of the area required at 20°C. This reduction in the required area at higher temperatures is due to the increased plant growth. Considering the temperature effect on tank sizing, it would be recommended to use the lowest ambient temperature at the treatment location as the design temperature. This ensures that the equipment is always oversized and can continue to meet treatment objectives even if the ambient temperature rises. The mean temperature of the coldest month is widely used for the design of waste stabilisation and maturation ponds in tropical climate countries (Mara 2004).

Taking the above into consideration, the decision regarding the depth and required area for treatment is primarily based on the curve obtained at 20°C. Generally, deep tanks are not recommended due to poor mixing, given the larger water column (Shammas, Wang, and Wu 2009). This results in a concentration gradient of nutrients in the tank, impacting plant growth and, consequently, treatment system efficiency. On the other hand, shallow tanks ensure better nutrient homogeneity across the treatment surface but are more susceptible to water loss through evapotranspiration (Alaerts *et al.* 1996). For this reason, it is advisable to limit the maximum depth of the treatment tank to that obtained for a cubic tank (approximately 60 cm), which serves as an intermediate option. A tank of this depth should then have a minimum area of 0.41 m^2 to treat 10 L day^{-1} of wastewater containing 1.25 mg P L^{-1}. Higher operational flows or phosphorus concentrations would necessitate a proportional increase in the area, as the nutrient surface load to the treatment system would rise. A water depth of 60 cm is the minimum depth that has been previously recommended for large-scale duckweed-based wastewater treatment systems, typically falling within the range of 0.6 m to 1.5 m (Skillicorn *et al.* 1993; Smith and Moelyowati 2001).

6.4.3.2 Pond operation

The main operational parameters of a duckweed-based wastewater treatment system include the water flow rate and the desired initial and final plant coverage levels during treatment. In addition to serving as an operational variable, the flow rate of the wastewater to be treated functions as a crucial design parameter.

- Operational flow rate

Previous findings (Section 6.4.3.1) determined the dimensions of a treatment tank required to process 10 L d^{-1} of wastewater containing 1.25 mg P L^{-1}. In cases where the phosphorus concentration in the influent or the flow rate of the water being treated vary, the treatment area must be adjusted to account for changes in the phosphorus surface loading rate.

Figure 6-13 illustrates the treatment area required to achieve the previously outlined treatment objectives when variations occur in phosphorus concentration in the wastewater. Under a constant operational flow, increases in the influent phosphorus concentration result in a proportionate increase in the treatment area. However, the proportionality constant is not uniform across the entire range of tested P concentrations, it is higher when the P concentration in the influent exceeds 1.25 mg P L^{-1} (Table 6-1).

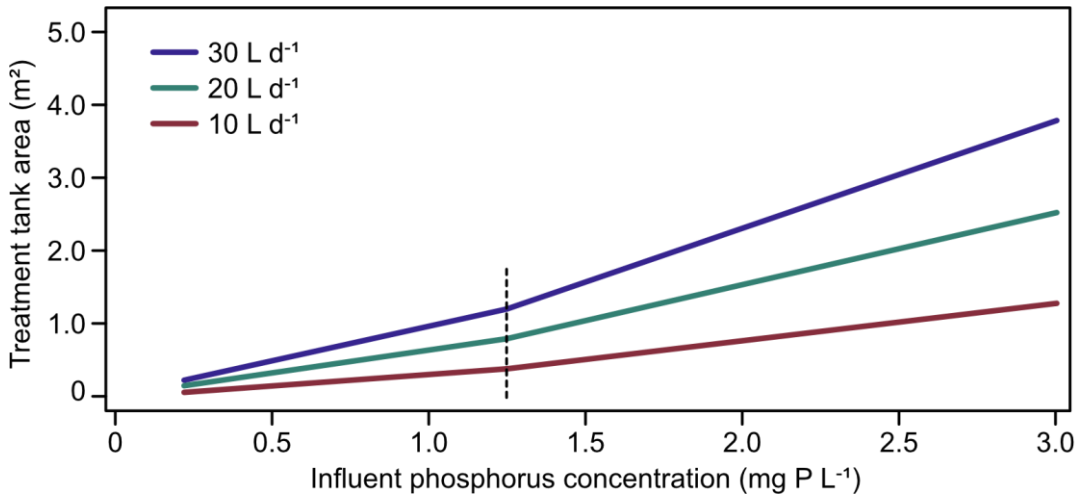

Figure 6-13 Required area for wastewater treatment using *Wolffia angusta* as a function of influent phosphorus concentration at varying flow rates

The required area of the tank to ensure a phosphorus concentration in the effluent of 0.25 mg P L^{-1} and a minimum removal of this nutrient by 80%, relative to the influent load, were obtained at three different flow rates, considering an initial duckweed coverage of 25% of the treatment area. The curve calculations were based on a treatment tank with a depth of 60 cm and an ambient air temperature of 20°C. The dashed line marks an influent phosphorus concentration of 1.25 mg P L^{-1}, for which but treatment goals are simultaneously met.

When the phosphorus concentration is constant, the area required for treatment, when adjusting the operational flow, is proportional to the change in flow rate, irrespective of the phosphorus concentration. For instance, doubling the operational flow when the P concentration in the wastewater is 1.0 mg P L^{-1} implies having a tank with twice the surface area, assuming the depth remains constant.

Table 6-1 Proportionality constant (K) for adjustments in the treatment area of WWT systems based on *Wolffia angusta* at varying flow rates and influent phosphorus concentrations

		Influent P concentration	
		< 1.25 mg P L^{-1}	> 1.25 mg P L^{-1}
Flow rate	10 L d^{-1}	0.318	0.499
	20 L d^{-1}	0.637	0.998
	30 L d^{-1}	0.955	1.498

Under a constant flow rate, fluctuations in the phosphorus concentration within the wastewater dictate the treatment objective that should be emphasized during the treatment process. When the phosphorus concentration in the wastewater is below 1.25 mg P L^{-1}, the treatment aim is to remove 80% of the phosphorus load from the influent. By achieving this level of phosphorus removal under such conditions, the phosphorus concentration in the final effluent will consistently remain below 0.25 mg P L^{-1}. Conversely, if the influent exhibits a phosphorus concentration exceeding 1.25 mg P L^{-1}, the treatment objective shifts to maintaining a nutrient concentration of 0.25 mg L^{-1} in the effluent. Successful attainment of this goal ensures phosphorus removal consistently exceeding 80%. The shift in treatment priorities is the factor influencing the change in the proportionality constant when the influent phosphorus concentration reaches 1.25 mg P L^{-1}.

The results also suggest that adjustments in the operational flow have a comparatively less significant impact on the necessary treatment area for wastewater treatment in contrast to fluctuations in the phosphorus concentration in the influent, especially when it surpasses 1.25 mg P L^{-1}. For example, increasing the initial phosphorus concentration from 1.25 to 2.5 mg L^{-1}, at any given flow rate, leads to a 135% expansion in the treatment area, while doubling

the treatment flow, at any given phosphorus concentration, results in a 100% larger treatment area.

In operational terms, Figure 6-13 can provide insights into the flow rate at which a treatment system based on *Wolffia angusta*, with a fixed area and an initial plant coverage of 25%, should operate when there are fluctuations in the phosphorus concentration in the effluent to be treated. For instance, a treatment tank of 2 m^2 must be operated at a lower flow rate (26 L d^{-1} instead of 62 L d^{-1}) when the phosphorus concentration in the wastewater increases from 1 to 2 mg P L^{-1}. Flows exceeding the established would compromise the attainment of treatment goals. Lower operational flows might be permissible, as long as the surface phosphorus loading rate does not fall below the minimum value required for plant development. The operational flows can be determined through interpolation, considering the proportionality constants presented in Table 6-1.

- Operational duckweed coverage

When fluctuations occur in the phosphorus concentration in wastewater, adjustments to the treatment flow are not the only operational considerations, the quantity of plants at the start of each cultivation cycle can also be modified. Figure 6-14 illustrates how varying the phosphorus concentration in the influent affects the treatment tank area needed to meet treatment goals, considering different initial duckweed coverages.

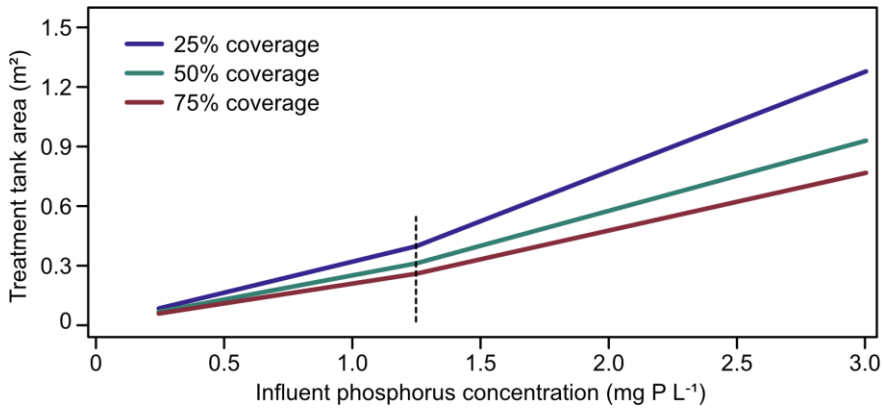

Figure 6-14 Required area for wastewater treatment using *Wolffia angusta* as a function of influent phosphorus concentration at varying initial duckweed cover values

The required area of the tank to ensure a phosphorus concentration in the effluent of 0.25 mg P L^{-1} and a minimum removal of this nutrient by 80%, relative to the influent load, were obtained at three different duckweed cover percentages, considering an operational flow rate of 10 L d^{-1}. The curve calculations were based on a treatment tank with a depth of 60 cm and an ambient air temperature of 20°C. The dashed line marks an influent phosphorus concentration of 1.25 mg P L^{-1}, for which but treatment goals are simultaneously met.

Maintaining a constant duckweed coverage requires a larger treatment area with increased influent phosphorus concentration, while a constant phosphorus concentration involves a smaller area with higher coverage. Similar to the operational flow results, the graph indicates a pivotal shift in treatment goals at a phosphorus concentration of 1.25 mg P L^{-1}.

Increasing plant coverage reduces the required treatment area due to a higher net nutrient removal rate, allowing for a shorter hydraulic retention time. Despite the decreasing area requirement with higher initial duckweed coverage, the rate of reduction diminishes at high coverage values. Coverages beyond 75% have minimal impact on the required treatment area but significantly affect the frequency of plant harvesting, as discussed in section 6.4.2.3.

The proportionality constants obtained when varying the initial plant coverage (Table 6-2) are lower than those found for operational flow adjustments. This suggests that changes in coverage have a less pronounced effect on the required treatment area than changes in flow rate. Nevertheless, Figure 6-14 serves as a guide for determining the necessary initial duckweed cover when treating wastewater with a fixed treatment area and operational flow. The results imply that when the treatment area is significantly constrained, and the influent phosphorus concentration is excessively high, achieving treatment goals becomes challenging, despite the treatment tank surface consistently being covered by plants. This difficulty arises from the exceptionally high surface phosphorus loading. In such cases, it is advisable to explore adjustments to the treatment flow.

Table 6-2 Proportionality constant (K) for adjustments in the treatment area of WWT systems based on *Wolffia angusta* at varying cover values and influent phosphorus concentrations

		Influent P concentration	
		< 1.25 mg P L^{-1}	> 1.25 mg P L^{-1}
Coverage	25%	0.318	0.499
	50%	0.239	0.353
	75%	0.199	0.289

6.5 Conclusion

Mathematical modelling of wastewater treatment systems based on *Wolffia angusta* is achievable with an impressive 85% precision. However, the current model exhibits limitations, and there is potential for enhancement by incorporating additional biotic and abiotic factors. The model's accuracy could be further refined to provide a more comprehensive understanding of the dynamics governing *Wolffia angusta*-based treatment systems.

The in silico evaluation of continuous treatment system, led to the finding that the ultimate quality of the effluent is predominantly influenced by the hydraulic retention time and the surface loading of nutrients, while biomass productivity and harvesting frequency are more significantly impacted by the initial plant coverage. The interplay of HRT and nutrient loading rates reveals both maximum (HRT), and minimum (loading rate), thresholds that must be carefully considered to facilitate plant growth and effective treatment. These findings underscore the importance of fine-tuning these operational parameters to guarantee the continuous treatment of wastewater.

Taking into account local wastewater discharge regulations is imperative for the design and operation of *Wolffia angusta*-based systems. The study highlights key considerations, such as designing systems for the lowest ambient temperature possible, maintaining a treatment depth of 60cm for optimal system functionality, and tailoring the treatment area based on the concentration of the limiting nutrient in wastewater, along with the operational flow rate and the initial cover of plants employed. These variables not only contribute to effective system design but also offer insights for the operation of existing cultivation systems.

In conclusion, this chapter not only contributes valuable insights into the mathematical modelling and operational dynamics of treatment systems based on *Wolffia angusta* but also underscores the necessity of aligning system design with local regulatory frameworks for sustainable and effective wastewater treatment.

Chapter 7 : Operational efficiencies and applicability of duckweed-based wastewater treatment systems for nutrient recovery in Indonesia

7.1 Introduction

Duckweed-based systems for wastewater treatment and nutrient recovery have shown significant promise due to their efficiency and effectiveness. Characterized by their robustness and adaptability, these systems can adjust to changing environmental conditions and varying wastewater qualities, ensuring a consistent quality of treated effluent (Coughlan, Walsh, Bolger, *et al.* 2022; Landesman, Fedler, and Duan 2011). The effectiveness of duckweed in wastewater treatment is supported by research highlighting its capacity for rapid growth and nutrient uptake under a range of environmental conditions (Cheng and Stomp 2009; Xu *et al.* 2015). Despite these advantages, the requirement for substantial treatment areas is a limitation, especially as demand increases with higher wastewater volumes and nutrient concentrations.

In contexts where spatial limitations prevail, the implementation of strategies that utilize multiple tank operations is critical. Such strategies, encompassing both parallel and sequential tank configurations, offer tailored solutions to space constraints. Parallel tank operations distribute the nutrient load evenly across all tanks, facilitating reduced treatment durations and uniform conditions that promote consistent biomass productivity and quality across the system (Korner *et al.* 2003).

In contrast, sequential (or series) tank operations introduce a gradient of nutrient loads across the tanks. This gradient introduces variations in biomass production and quality due to the distinct acclimatization responses of the plants to the differential conditions they grow (Demmig-Adams *et al.* 2022; Landesman *et al.* 2011). Although this variability exists, sequential configurations have been observed to enhance the quality of the effluent more effectively than parallel setups.

The practice of routinely harvesting biomass and reincorporating a portion of it back into the treatment tanks is crucial for maintaining the efficacy of the treatment process (Calicioglu *et al.* 2021; Rejmánková *et al.* 1990). This recycling

of biomass not only fosters new plant growth but also facilitates further nutrient removal. The complex adaptation mechanisms of the plants to specific cultivation conditions, especially regarding nutrient availability, add another layer of complexity to the treatment process. Sequential treatment setups, which involve the recirculation of biomass from stages characterized by low nutrient availability to those with higher nutrient concentrations, present an opportunity for potential optimization. This strategy, reminiscent of nutrient deprivation-repletion dynamics observed in other photosynthetic organisms, suggests that plants subjected to nutrient starvation may exhibit enhanced nutrient removal capabilities when relocated to nutrient-rich environments (Slocombe *et al.* 2020; Solovchenko *et al.* 2019).

This chapter delves into the operational efficiencies of *Wolffia angusta*-based wastewater treatment systems arranged in either series or parallel configurations. It seeks to bridge the knowledge gap regarding the impact of biomass recirculation in sequential tank setups on nutrient removal efficiency. By drawing comparisons between data from controlled pilot systems and field observations from a *Lemna*-based installation in an Indonesian fishery, the discussion aims to shed light on the practical scalability and applicability of duckweed-based wastewater treatment methodologies in real-world settings, offering a comprehensive understanding of these innovative strategies.

7.2 Aims

The primary aim of this chapter is to critically assess the operational performance and scalability of duckweed-based wastewater treatment configurations and its application in real tropical climate scenarios. This involves:

1) Evaluating the operational efficiencies of *Wolffia angusta*-based systems operating under series and parallel configurations.
2) Investigating the impact of biomass recirculation in a sequential tank configuration
3) Assessing the performance and applicability of a duckweed-based pilot scale system for the treatment of real fish farm effluents in Indonesia

7.3 Methodology

7.3.1 Duckweed cultivation at pilot-scale under simulated tropical climate conditions

For the initial segment of this chapter, trials involving *Wolffia angusta* were conducted within a greenhouse, with the aim of sustaining an average ambient temperature of 25°C. The plants were grown in rectangular tanks, each with a working volume of 12 litres, through which a Hoagland's nutrient solution, adjusted to the highest concentrations of phosphorus and nitrogen expected for fish farm effluents (4 mg P L^{-1} and 20 mg N L^{-1}), was fed under continuous flow conditions. At the beginning of each experiment, 8.9 g of fresh duckweed, adequate to cover 25% of the water surface, was introduced into each cultivation tank. Three distinct tank configurations were evaluated, as depicted in Figure 7-1.

For tanks arranged in parallel (Figure 7-1 A), the nutrient solution was pumped into each tank at a flow rate of 1.71 L d^{-1}, resulting in a hydraulic retention time per tank of 7 days. The system was monitored for 30 days. Whenever the duckweed entirely covered the tank surface, 75% of the biomass was harvested, while the remaining 25% was retained in each tank for ongoing treatment.

In the case of tanks arranged in series, two different experiments regarding biomass harvesting were established. In both trials, two tanks were connected in series, and the nutrient solution was circulated at a rate of 3.42 L d^{-1} to ensure that the combined hydraulic retention time matched that of a parallel tank (7 days). The tanks were monitored for 40 days. In the first experiment, whenever the surface was completely covered by plants, 75% of the biomass from each tank was harvested, while the remaining 25% was left in its respective tank for further treatment, similar to parallel-connected tanks (Figure 7-1 B). In the second experiment, whenever the plants completely covered the water surface, 100% and 50% of the biomass from the first and second tanks in series, respectively, were harvested. Of the remaining 50% from the second tank, 25% was recirculated to the first tank, and the other 25% was retained in the second tank (Figure 7-1 B).

In all experiments, during each harvest, the quantity of biomass produced was measured, and the protein and phosphorus content in *Wolffia angusta* biomass were analysed. Daily samples of effluent from each tank, whether in parallel or in series, were collected to quantify phosphorus and nitrogen removal.

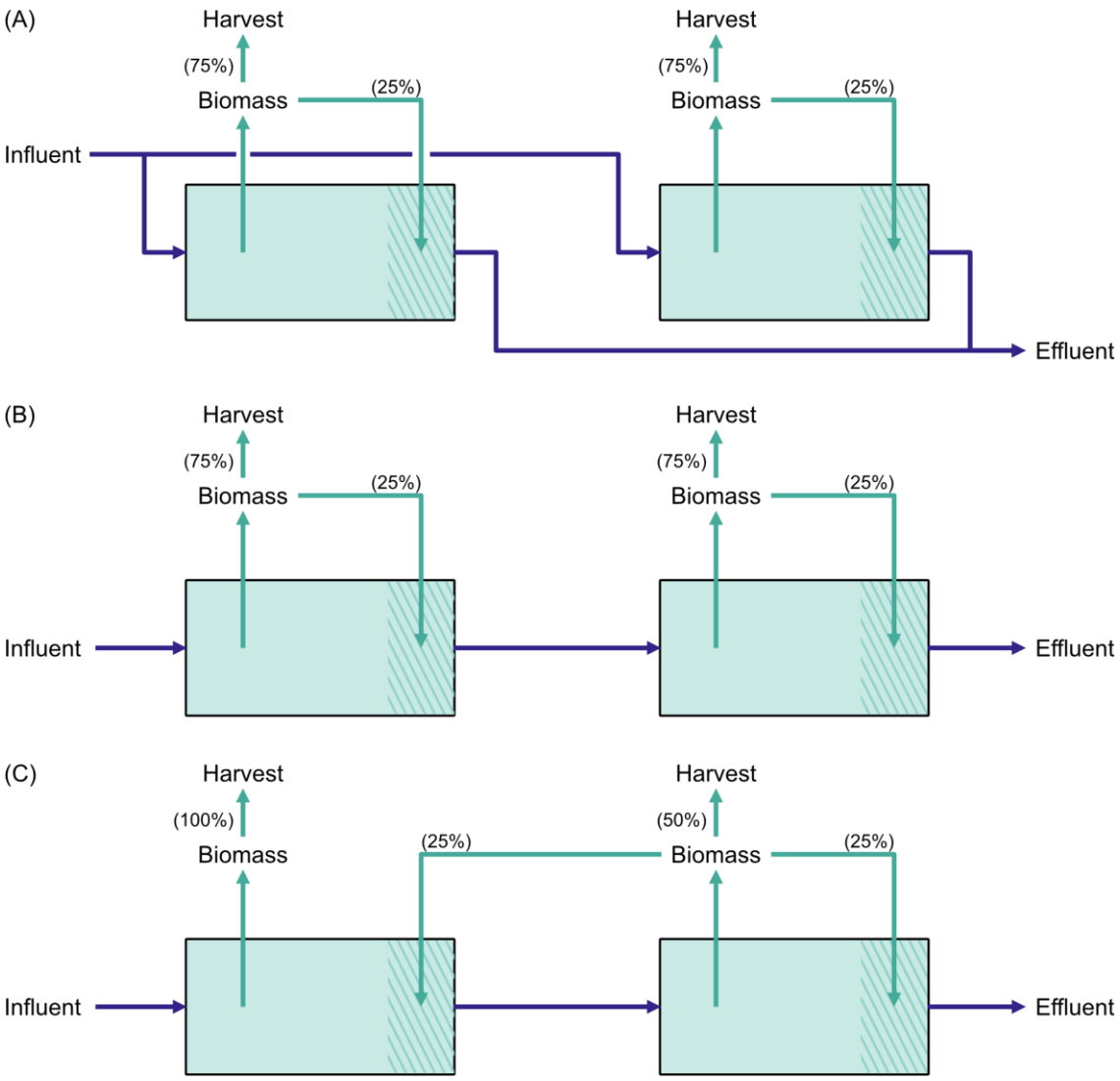

Figure 7-1 Diagram of the different configurations tested for continuous treatment systems using *Wolffia angusta*

(A) Treatment tanks arranged in parallel. This configuration involves multiple tanks operating simultaneously, with each tank treating wastewater independently from the others. **(B)** Sequential tanks without biomass recirculation: In this setup, tanks are connected in series, allowing wastewater to flow from one tank to the next. However, there is no recirculation of biomass between the tanks. **(C)** Sequential tanks with biomass recirculation. this configuration also features tanks connected in series, but with an added process of recirculating a portion of the biomass from the second tank back to the first tank.

7.3.2 Duckweed cultivation at pilot-scale in Malang, Indonesia

The results obtained from laboratory experiments on *Wolffia angusta*, conducted at both bench scale and pilot scale, focusing on the influence of temperature, nutrient availability, and operational variables in continuous treatment systems for the efficacy of duckweed in wastewater treatment and nutrient removal, served as the foundation for setting up and evaluating a pilot

system outdoors near a fish farm in Malang, Indonesia. The detailed information about the treatment system set-up is presented in Chapter 2.

In brief, the treatment system comprised a rectangular pond with a working volume of 3 m^3, through which water sourced from one of the fish farm ponds (where Tilapia and Catfish were reared) was circulated. The water from the fish pond was pumped to a header tank, enabling control over the water flow for treatment. In the treatment tank, *Lemna minor*, a duckweed species isolated from the city's flower market, was cultivated. Two system configurations were tested, maintaining a constant influent flow of 4.4 L day^{-1}: (1) a single tank without baffles, and (2) a compartmentalized tank with baffles.

In the first configuration, the system underwent monitoring for a month and the entire surface area of the treatment tank, 3.75 m^2, was used to grow duckweed. Weekly samples of both influent and effluent from the treatment tank were collected, and the water quality was assessed. During the treatment process, whenever the plants covered the entire water surface, the biomass was harvested, weighed, and then reintroduced into the system in the required quantity to cover 50% of the surface. The remaining biomass was sun-dried for three days and shipped to the UK to analyse its biochemical composition.

In the second configuration, a floating baffle was incorporated into the treatment system, facilitating water movement from one compartment to another at the tank's bottom while preventing plant displacement on the surface. This arrangement effectively operated as two tanks connected in series. In this setup, the initial biomass in each compartment was sufficient to cover 50% of the surface. Upon reaching full coverage (100%) of each compartment, the biomass from the first compartment was completely harvested, with half of the second compartment's biomass recirculated to the first. The remaining 50% of biomass stayed in the second compartment. The system was monitored for one month, with weekly collection of water and biomass samples.

7.4 Results and discussion

7.4.1 Efficiency of parallel and series configurations of systems for *Wolffia angusta* cultivation

The analysis of effluent samples from tanks operating in parallel and in series demonstrates the successful removal of phosphorus and nitrogen in both

configurations, with steady-state conditions achieved within approximately 20 days, as illustrated in Figure 7-2.

In the case of tanks connected in parallel, a net removal of 82% for phosphorus and 40% for nitrogen is achieved (Figure 7-2 A, C). The average concentrations of nutrients in the final effluent stood at 0.71 mg P L^{-1} and 10.1 mg N L^{-1}. Conversely, tanks connected in series exhibited superior nutrient removal capabilities. In this setup, the overall efficiency of phosphorus removal achieved 95%, while for nitrogen, it reached 91%. As a result, the final effluent presented average concentrations of 0.70 mg P L^{-1} and 1.51 mg N L^{-1}. (Figure 7-2 B, D).

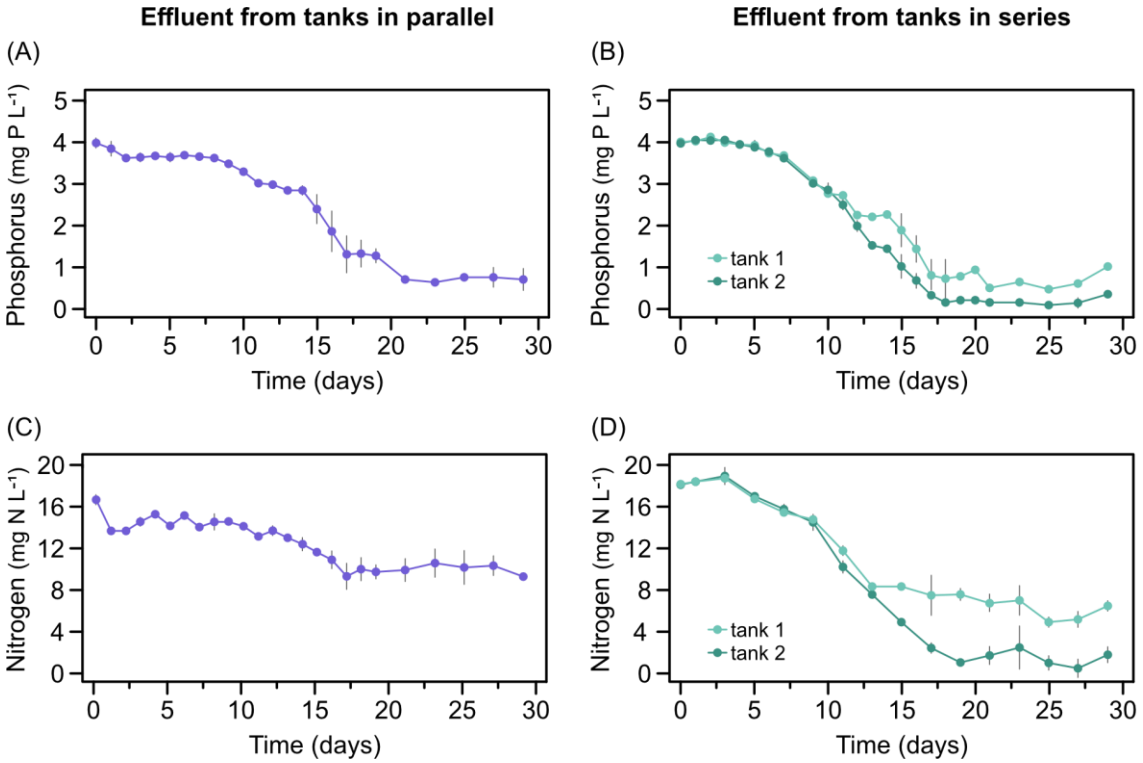

Figure 7-2 Evolution of effluent quality over time for parallel and sequential treatment systems based on *Wolffia angusta*

(A-C) Concentrations of phosphorus and nitrogen in the effluent for tanks arranged in parallel. **(B-D)** Phosphorus and nitrogen concentrations at the outlet of each tank within a sequential configuration. Error bars indicate the standard deviation (n=3).

Notably, the first tank in the series configuration contributed significantly to nutrient removal, accounting for 86% and 69% of the net phosphorus and nitrogen removal, respectively. Regarding phosphorus, this contribution is not statistically different from tanks operated in parallel, underscoring the potential and need for additional treatment before the final effluent disposal. Incorporating

a second tank in series remarkably enhanced the system's efficiency, enabling it to remove over 90% of nutrients present in water.

In terms of biomass production, results for per-tank biomass productivity show distinctive patterns based on tank configuration (Figure 7-3). When operating in parallel, there was a consistent rise in biomass productivity with each successive harvest, culminating in a peak productivity of 352 mg dw d^{-1} when biomass was harvested for the third time. However, this upward trend was offset by a notable decline once steady state conditions were achieved.

On the other hand, when tanks were interconnected in series, the results show that there was no statistically significant difference between the amounts of biomass produced in the first and second tanks in the series. However, as time progressed, the biomass productivity of both tanks exhibited a downward trend, starting at an average 286 mg dw d^{-1} during the initial harvest and gradually decreasing to 67 mg dw d^{-1} in the final harvest (Figure 7-3 A).

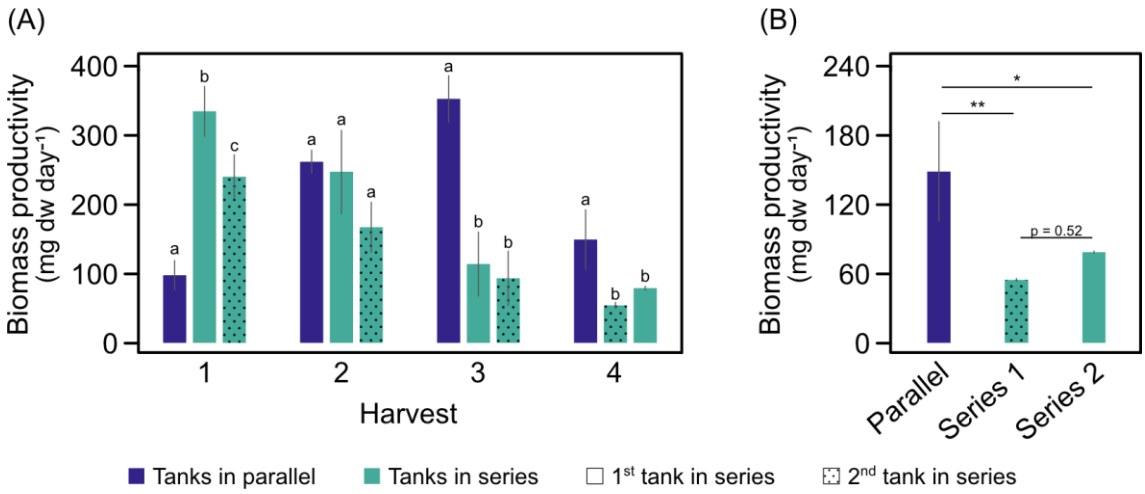

Figure 7-3 Changes in duckweed biomass productivity over time until steady state in parallel and sequential *Wolffia angusta*-based treatment systems

(A) Biomass productivity per treatment tank at each harvesting time point, with blue bars indicating productivity from the parallel configuration and green bars representing sequential tanks. Bars annotated with the same letters indicate no statistically significant difference in productivity ($p<0.05$, ANOVA – Tukey ad hoc test, 95% confidence level). **(B)** Biomass productivity per tank at steady state conditions for both parallel and series configurations, with significance levels marked as (*) for $p = 0.05$ and (**) for $p = 0.01$, analysed using the Tukey-Kramer HSD Post-Hoc Test at a 95% confidence level. Biomass productivity is expressed in mg dry weight per day, and error bars correspond to the standard deviation (n=3).

The findings suggest that, at steady state, the amount of biomass that is produced in the sequential configuration is not statistically significant between

both tanks connected in series (Figure 7-3 B). The average per-tank biomass productivity of this configuration was found to be 45% of that observed in a tank operating in parallel.

The analysis of the biomass composition of *Wolffia angusta* revealed that its protein content remained relatively stable throughout the treatment period, averaging 23.4% dw. This stability is illustrated in Figure 7-4 A, where no significant variation in protein content was observed in either parallel or sequential configurations. When examining the amount of protein that is produced per tank at steady state in both scenarios, a distinct difference emerged (Figure 7-4 B). Tanks arranged in parallel yielded a higher protein output, producing an average of 29.7 mg of protein per day, in contrast to the 14.3 mg day^{-1} observed in each tank configured in series. Statistical analysis confirmed that there was no significant difference between the protein yields of the first and second tanks in series.

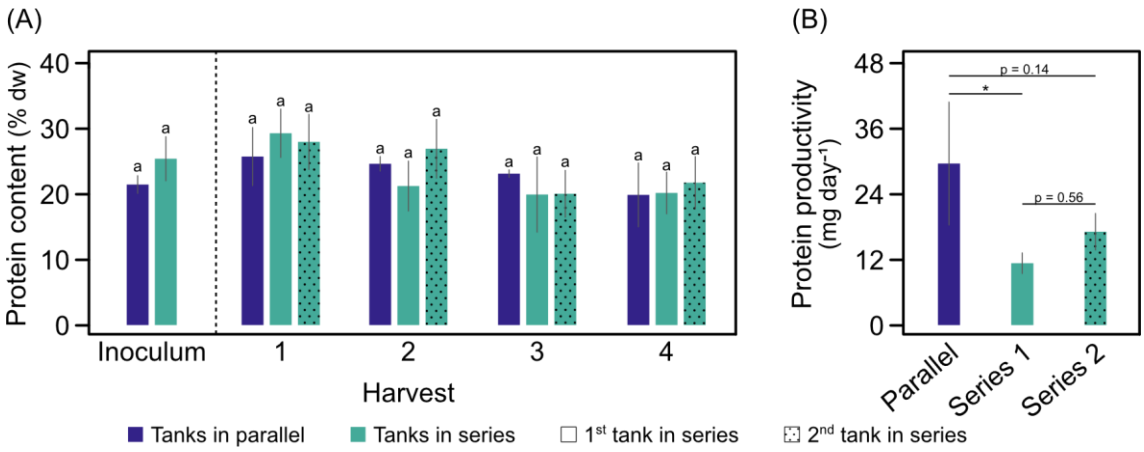

Figure 7-4 Protein content and productivity in *Wolffia angusta* biomass in parallel and sequential treatment systems

(A) Percentage of protein in the dry duckweed biomass from each treatment tank at various harvest points, including the protein content in the initial biomass used for inoculation in each experiment. Bars with the same letters indicate no significant differences among treatments and tanks ($p<0.05$, ANOVA – Tukey ad hoc test, 95% confidence level). **(B)** Protein production at steady state , comparing outputs from tanks in parallel and series configurations. Statistical significance is denoted by (*) for $p = 0.05$, determined through the Tukey-Kramer HSD Post-Hoc test with a confidence level of 95%.

The phosphorus content of the biomass presented a more complex pattern, as depicted in Figure 7-5 A. Initially, the inoculum used for the tanks in series exhibited a P content three times higher than that of the parallel tanks, likely due to variability during the preculture stage. In parallel configurations, a significant increase in biomass phosphorus content was recorded at the first

harvest, reaching up to 10.4 mg P g^{-1} dw. Subsequently, the P content began to decline, stabilizing at 2.6 mg P per gram of dry biomass weight by the final harvest. Conversely, in the series configuration, a continuous reduction in P content was noted with each harvest. Notably, from the second harvest onward, the P content in the biomass from the second tank in the series was significantly lower than that from the first tank.

Under steady-state conditions, the P content stabilized at 3.8 mg P g^{-1} dw and 1.6 mg P g^{-1} dw for the first and second tanks in series, respectively. These observations resulted in a differential productivity of biomass-derived phosphorus between the parallel and series tank configurations, with overall P productivity being higher in the parallel arrangement (Figure 7-5 B). Tanks connected in parallel allow for a production of biomass-derived phosphorus of 0.39 mg P d^{-1}, while sequential tanks produced in average 0.17 mg P d^{-1}.

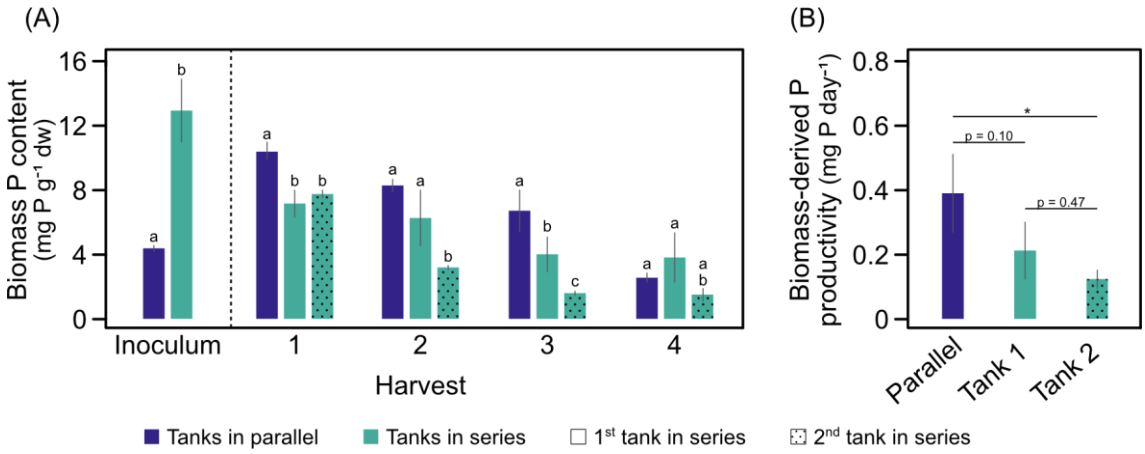

Figure 7-5 Phosphorus content and biomass-derived phosphorus productivity in parallel and sequential treatment systems based on *Wolffia angusta*

(A) Phosphorus content in the dry duckweed biomass from each treatment tank at various harvest points, including the protein content in the initial biomass used for inoculation in each experiment. Bars with the same letters indicate no significant differences among treatments and tanks ($p<0.05$, ANOVA – Tukey ad hoc test, 95% confidence level). **(B)** Biomass-derived phosphorus production at steady state, comparing outputs from tanks in parallel and series configurations. Statistical significance is denoted by (*) for $p = 0.05$, determined through the Tukey-Kramer HSD Post-Hoc test with a confidence level of 95%.

The comparative analysis of effluent samples from parallel and series tank configurations offers deep understanding into the effectiveness of these systems for nutrient removal. Achieving steady-state conditions within approximately 20 days for both configurations (Figure 7-2) implies a rapid adaptation of the biomass to the treatment conditions. The acclimation of duckweed to new environmental

conditions or culture media is an important step for an optimal duckweed growth, as reported earlier (Cheng, Bergmann, *et al.* 2002). The distinction in nutrient removal efficiencies, with parallel tanks achieving 82% phosphorus and 40% nitrogen removal and series tanks outperforming at 95% and 91% respectively, underscores the improved performance of series configurations in treating wastewater. These results align with findings from similar studies, such as those by Steiner & Freeman (1989), who highlighted the superior performance of series configurations for nutrient uptake in constructed wetlands due to prolonged exposure and sequential nutrient absorption opportunities.

The greater effectiveness of the series configuration compared to parallel tanks is also evidenced when comparing the steady-state nutrient removal rates with respect to the loading rates (Table 7-1). In parallel configurations, lower nitrogen and phosphorus removal rates are achieved, primarily due to the low nutrient loading rate in the system, which limits their absorption by the plants. The removal efficiencies are 41% and 82% for nitrogen and phosphorus, respectively. These efficiencies are low when compared to those obtained for the first tank in series, even more so when considering that the rate at which nutrients are applied to this tank is double that of the parallel tanks.

Table 7-1 Nutrient rates (loading and removal) in the duckweed tanks connected in parallel or series

Tanks	Loading rate (g m^{-2} d^{-1})		Removal rate (g m^{-2} d^{-1})		Efficiency (%)	
	NO_3-N	PO_4-P	NO_3-N	PO_4-P	NO_3-N	PO_4-P
Parallel	0.291	0.068	0.121	0.056	41.6	82.4
Series 1	0.616	0.137	0.399	0.113	64.8	82.5
Series 2	0.215	0.024	0.166	0.017	77.2	70.8

The highest nitrogen removal rate obtained, 0.40 g m^{-2} d^{-1}, is below the range reported for other duckweed species (0.54 – 4.4 g N m^{-2} d^{-1}) (Benjawan and Koottatep 2007; Cheng, Landesman, *et al.* 2002; Körner, Lyatuu, and Vermaat 1998; Mohedano *et al.* 2012). This is partly due to variability among duckweed species and the fact that other studies, which report total nitrogen removal without specifying nitrogen species, use media or wastewater with higher ammonium than nitrate content, a form that can be more easily absorbed by

plants. Regarding phosphorus removal rate, the value found for *W. angusta* is lower than that previously reported for *Lemna minor*, 0.59 g m^{-2} d^{-1} (Cheng, Landesman, *et al.* 2002), because concentrations of phosphorus four times lower were used in the present study.

The role of the first tank in a series configuration is particularly noteworthy, as it significantly contributes to overall removal of nutrients (86% for phosphorus and 69% for nitrogen). This efficiency aligns with observations by Dinh *et al.* (2020), who identified the initial stages of series setups employing *Lemna minor* as crucial for bulk nutrient reduction. However, this efficacy is contingent upon the nutrient concentration on the wastewater being treated, which in turn, can inhibit plant growth in the first tanks of the series due to the high loading rates. Despite the commendable performance of the parallel configuration, the comparative analysis suggests a need for additional or enhanced treatment mechanisms before final effluent disposal. This emphasises the importance of serial duckweed tanks for wastewater polishing, to ensure compliance with local discharge consent regulations (Kumar *et al.* 2022).

Biomass productivity analysis further outlines the operational differences between parallel and series configurations. The initial increase in biomass productivity in parallel configurations, peaking at 352 mg dw d^{-1}, before declining at steady state, aligns with patterns observed in dynamic growth systems where initial nutrient availability boosts biomass yield, as discussed by Verma & Suthar (2014). Conversely, the steady decrease in biomass productivity in series configurations, culminating in a 45% lower yield at steady state compared to parallel tanks, highlights the impact of nutrient depletion over time on plant's growth, a factor well-documented in sequential cultivation systems of *Spirodela polyrhiza* (Caicedo-Bejarano 2005). It's worth noting that, although the higher nutrient load to the first tank in series, compared to the parallel tank, results in higher removal rates, the same trend is not observed with the amount of biomass produced (Figure 7-3). The greater presence of nutrients at the start of the series system leads to increased activity of phosphorus and nitrogen transporters, resulting in higher uptake (Smith 2002). However, there is a risk of nutrient overload, particularly with nitrogen, which can adversely affect plant growth. Nitrogen's relatively higher toxicity compared to phosphorus may inhibit duckweed development (Körner *et al.* 2001; Wang 1991).

The stability of protein content in *Wolffia angusta* observed in our study (averaging 23.4% across treatment time) aligns with findings by Petersen et. al (2022), who reported that environmental conditions such as light intensity and nutrient availability have minimal impact on the protein levels of *L. minor* and *W.*

hyalina within certain thresholds. This consistency underscores the potential of *Wolffia angusta* as a reliable source of plant-based protein, supporting its proposed use in sustainable aquaculture systems (Fiordelmondo *et al.* 2022; Sońta, Rekiel, and Batorska 2019). The variance in protein production between tank configurations, with parallel systems yielding nearly double the protein compared to series setups, underscores the significant influence of biomass production on the net protein productivity of the treatment system.

Lastly, the differential phosphorus content observed in biomass from parallel and series configurations illustrates the nuanced impact of cultivation conditions on nutrient assimilation and storage within *Wolffia angusta* biomass. The initial high phosphorus uptake in parallel configurations and its subsequent decline contrast sharply with the steady reduction observed in series configurations. This behavior may be attributed to variations in available nutrient concentrations and the plants' adaptive mechanisms to optimize nutrient use efficiency (Liu *et al.* 2021; Paterson *et al.* 2020). Overall, these observations highlight the complex interplay between tank configuration, nutrient loading rate, biomass productivity, and nutrient dynamics, offering valuable insights for optimizing wastewater treatment and biomass production systems.

7.4.2 Effect of recirculating duckweed biomass within sequential treatment systems using *Wolffia angusta*

In duckweed cultivation systems configured in series, there are fundamentally two strategies for harvesting and managing biomass. One method involves the separate harvesting and handling of a portion of the biomass from each tank, while the other strategy entails harvesting the entirety of the biomass from the first tank, followed by recirculating a portion of the biomass from the second tank to the first one.

The results reveal that steady-state conditions are achieved within 20 days across both strategies, with biomass recirculation significantly enhancing phosphorus removal from wastewater, as detailed in Figure 7-6.

Specifically, non-recirculation systems achieved a 94% phosphorus removal efficiency, while recirculation systems showed a slightly higher efficiency at 97%. This improvement is largely attributed to the first tank in the series (Figure 7-6 A), where the average phosphorus concentration was lower (0.39 mg P L^{-1}) in recirculation systems compared to those without recirculation (0.7 mg P L-1), indicating a 9% increase in efficiency with biomass recirculation ($p<0.05$, ANOVA-Tukey 95%). The second tank showed a modest 3% improvement in

phosphorus concentration reduction in system where biomass was recirculated (Figure 7-6 B). Across both scenarios, the first tank was crucial for most of the phosphorus removed, accounting for up to 86% and 97% of total P removal in recirculating and non-recirculating systems, respectively.

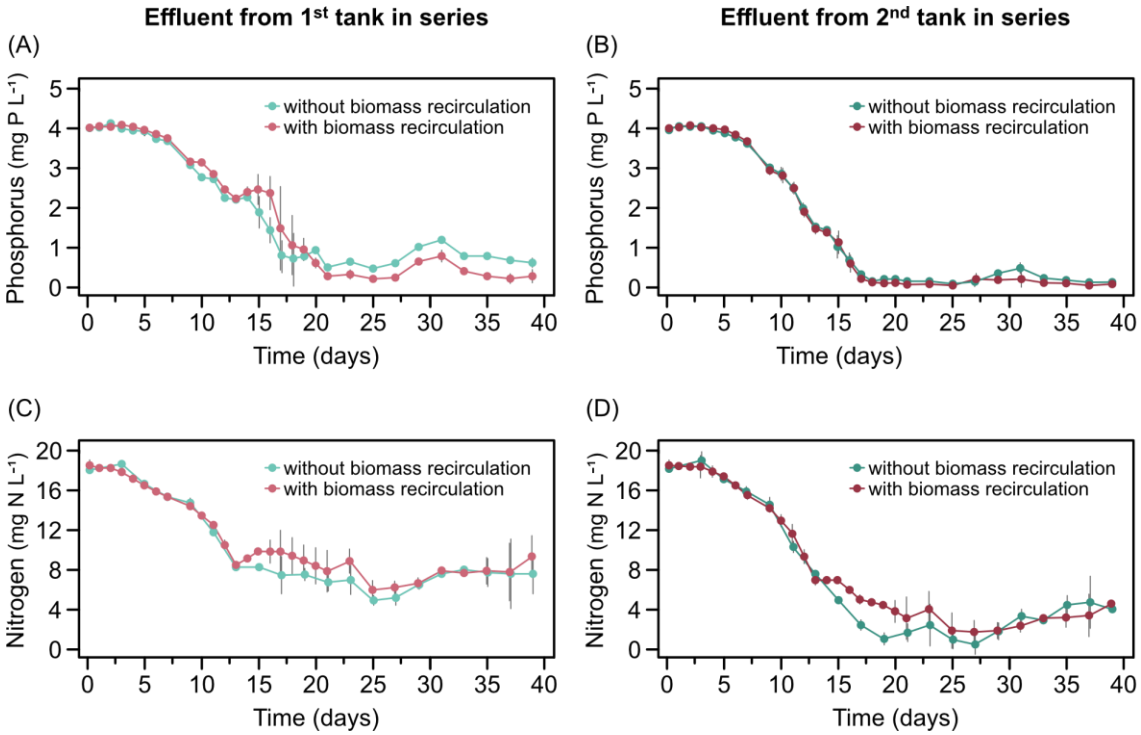

Figure 7-6 Evolution of effluent quality over time in sequential treatment systems with and without *Wolffia angusta* biomass recirculation

(A-C) Concentrations of phosphorus and nitrogen in the effluent from the first tank in the series. **(B-D)** Concentrations of phosphorus and nitrogen in the effluent from the second tank in the series Error bars indicate the standard deviation (n=3).

Nitrogen removal efficiency averaged 84% for both biomass management strategies, with 60% of total nitrogen being removed in the first tank, leading to intermediate nitrogen concentrations of 7.27 mg N L^{-1} and final effluent concentrations of 2.84 mg N L^{-1} across both scenarios (Figure 7-6 C, D).

Biomass production per tank decreased over time in both scenarios, stabilizing by the fourth harvest with a notable decline in productivity, particularly in recirculated systems where it dropped from 250 to 30 mg dw d^{-1} (Figure 7-7 A). At the fourth harvest, productivity in the second tank without recirculation slightly surpassed that of the first tank and was significantly higher than in tanks with recirculated biomass. In steady state conditions, biomass recirculating

systems produced 23mg of dry biomass per day per tank, while non-recirculating tanks produce in average 79 mg dw d^{-1} (Figure 7-7 B).

The study also revealed that protein content in *Wolffia angusta* remained constant at 25% dry weight until the fourth harvest when steady state was reached (Figure 7-8 A). At this point, only in the recirculation scenario the protein content in the biomass harvested from second tank significantly drop to 10%. Protein productivity was substantially higher in tanks without biomass recirculation (Figure 7-8 B). In these systems, the protein productivity of both tanks in series did not differ significantly, with an average production of 14.3 mg of protein per day. However, the recirculation of biomass notably affected the second tank's protein productivity, reducing it to half of what was observed in the first tank, which recorded a productivity of 4.4 mg protein d^{-1}.

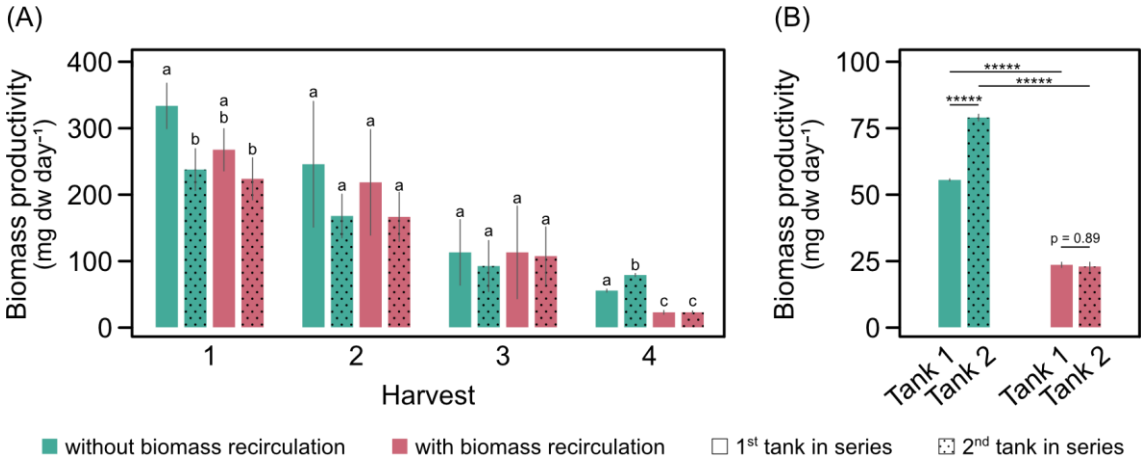

Figure 7-7 Changes in duckweed biomass productivity over time until steady state in sequential *Wolffia angusta*-based treatment systems with and without biomass recirculation

(A) Biomass productivity per treatment tank at each harvesting time point, with green and red bars indicating productivity from the sequential system without and with biomass recirculation, respectively. Bars annotated with the same letters indicate no statistically significant difference in productivity ($p<0.05$, ANOVA – Tukey ad hoc test, 95% confidence level). **(B)** Biomass productivity per tank at steady state conditions, with significance levels marked as (*****) for p = 0.00001, analysed using the Tukey-Kramer HSD Post-Hoc Test at a 95% confidence level. Biomass productivity is expressed in mg dry weight per day, and error bars correspond to the standard deviation (n=3).

The study also revealed that protein content in *Wolffia angusta* remained constant at 25% dry weight until the fourth harvest when steady state was reached (Figure 7-8 A). At this point, only in the recirculation scenario the protein content in the biomass harvested from second tank significantly drop to 10%.

Protein productivity was substantially higher in tanks without biomass recirculation (Figure 7-8 B). In these systems, the protein productivity of both tanks in series did not differ significantly, with an average production of 14.3 mg of protein per day. However, the recirculation of biomass notably affected the second tank's protein productivity, reducing it to half of what was observed in the first tank, which recorded a productivity of 4.4 mg protein d^{-1}.

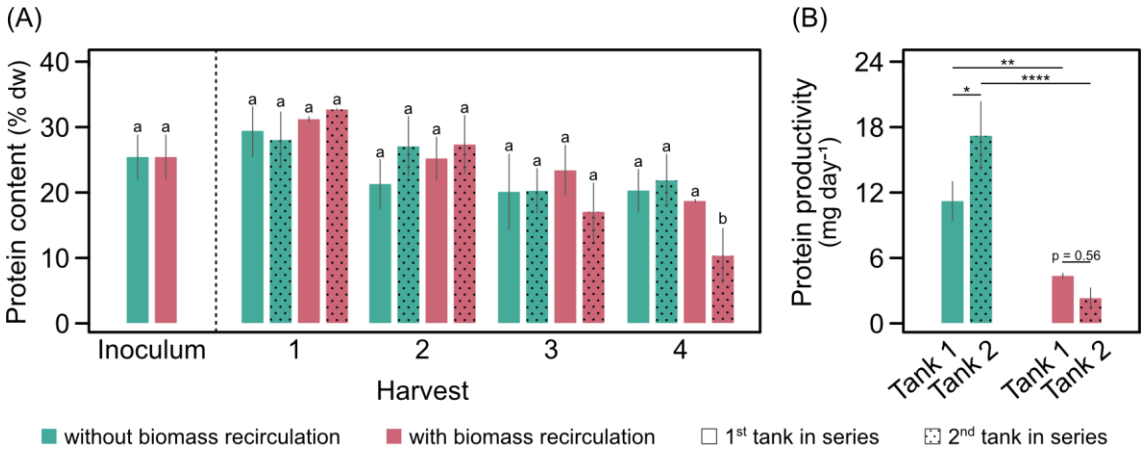

Figure 7-8 Protein content and productivity in *Wolffia angusta* biomass in sequential treatment systems with and without biomass recirculation

(A) Percentage of protein in the dry duckweed biomass from each treatment tank in series at various harvest points, including the protein content in the initial biomass used for inoculation in the experiment. Bars with the same letters indicate no significant differences among treatments and tanks ($p<0.05$, ANOVA – Tukey ad hoc test, 95% confidence level). **(B)** Protein production at steady state , comparing outputs from tanks in series without and with biomass recirculation. Statistical significance is denoted by (*) for $p = 0.05$, (**) for $p = 0.01$, and (****) for $p = 0.0001$, determined through the Tukey-Kramer HSD Post-Hoc test with a confidence level of 95%.

Figure 7-9 A shows that phosphorus content in the biomass decreased from an initial 13 mg P g^{-1} dw in both scenarios, with the second tank consistently showing significantly lower P content. Notably, between the third and fourth harvest, P content in the first tank where biomass was not recirculated remained constant at 3.9 mg P g^{-1}, whereas recirculated systems saw a 36% increase in P content in the harvested biomass from the first tank (jumping from 4.6 to 6.3 mg P g^{-1} dw).

In terms of phosphorus productivity derived from biomass, systems without recirculation exhibited significantly higher productivity than those with recirculation (Figure 7-9 B). Specifically, productivity in non-recirculated systems was 0.21 and 0.13 mg P d^{-1} for the first and second tanks, respectively, compared to 0.15 and 0.03 mg P d^{-1} in recirculated systems. There was no significant difference in productivity between the first tanks of both experiments; however,

the productivity from the second tank in recirculated systems was notably lower, being four time lower than that in non-recirculated systems.

The analysis of duckweed cultivation systems configured in series, focusing on two distinct biomass management strategies, separate harvesting and biomass recirculation, reveals significant implications for wastewater treatment efficiency and biomass productivity. The enhanced phosphorus removal efficiency observed with biomass recirculation (97%) compared to non-recirculation (94%) underscores the critical role of biomass management in optimizing nutrient uptake. This supports the hypothesis that recirculation strategies enhance nutrient removal capacity by manipulating internal P reserves in the plants.

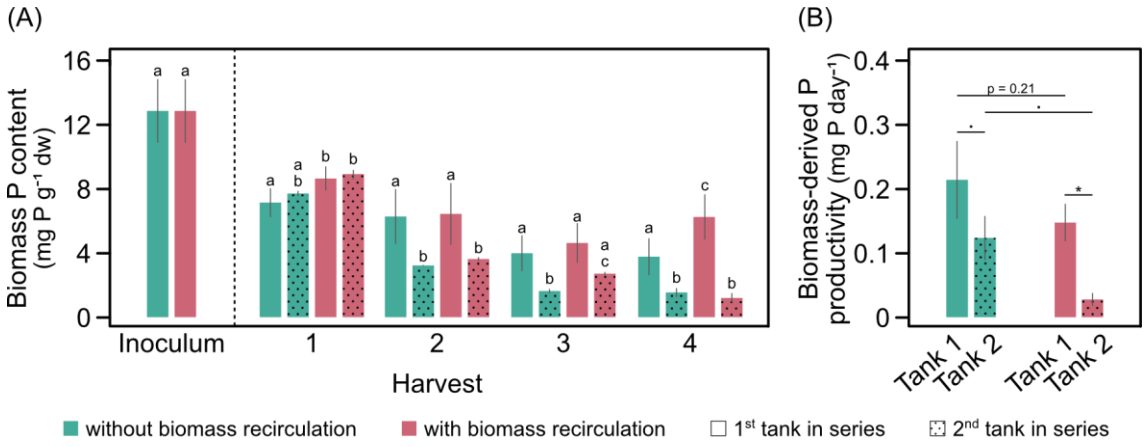

Figure 7-9 Phosphorus content and biomass-derived phosphorus productivity in sequential treatment systems based on *Wolffia angusta* with and without biomass recirculation

(A) Phosphorus content in the dry duckweed biomass from each treatment tank at various harvest points, including the protein content in the initial biomass used for inoculation in each experiment. Bars with the same letters indicate no significant differences among treatments and tanks ($p<0.05$, ANOVA – Tukey ad hoc test, 95% confidence level). **(B)** Biomass-derived phosphorus production at steady state , comparing outputs from tanks in series without and with biomass recirculation. Statistical significance is denoted by (.) for p = 0.1, and (*) for p = 0.05, determined through the Tukey-Kramer HSD Post-Hoc test with a confidence level of 95%.P content in biomass series no/yes biomass recirculation

This effect already observed in microalgae, demonstrates how nutrient deficiency leads to a reduction in internal phosphorus (P) reserves, which, in turn, triggers a mechanism for increased phosphorus accumulation when these organisms are later exposed to environments rich in phosphorus (Hernandez, de-Bashan, and Bashan 2006; Slocombe *et al.* 2020; Solovchenko *et al.* 2019). This effect, however, has been less explored in duckweed. Available research, particularly on *Lemna minor*, indicates that after acclimatization to phosphorus-

deficient growth mediums, and subsequent exposure to environments containing 15 mg P L^{-1}, these plants demonstrate a 30% increase in phosphorus removal efficiency, doing so more swiftly than those not previously acclimatized (Nakamura 2013; Paterson *et al.* 2020; Powell *et al.* 2009). This suggests that, despite not storing phosphorus as polyphosphate like microalgae (Sanz-Luque, Bhaya, and Grossman 2020; Zhao *et al.* 2021), similar physiological mechanisms of nutrient adaptation might be present in duckweed, although further research is necessary to fully understand and utilize these adaptations in wastewater treatment applications.

In duckweed-based continuous treatment systems configured with two sequential tanks, a scenario is created where nutrient concentration is high in the first tank and substantially less in the second. This setup, at steady state, revealed that when duckweed biomass was not recirculated, there was an 82% discrepancy in phosphorus load between the two tanks. Managing the biomass separately for each tank led to plants acclimating to both nutrient-rich and nutrient-poor environments. Despite these differences in nutrient availability, the amount of biomass produced in each tank remained similar (Figure 7-7). However, there was a notable difference in the phosphorus removal efficiency and the phosphorus concentration within the biomass itself (Figure 7-9). This phenomenon, where growth continues at the cost of reduced nutrient assimilation, has been observed in various duckweed species as well (Paterson *et al.* 2020; Zhao *et al.* 2015). Such varied acclimation to nutrient levels across the tanks not only affects the quality of the biomass produced but also necessitates extended treatment durations in subsequent tanks to make up for the decreased nutrient absorption by the duckweed.

When biomass is recirculated, the initial biomass production from the tanks is not affected, but both the amount of phosphorus removed and the phosphorus content in the plants in the first tank are enhanced. This implies that recirculating biomass not only maintains or improves biomass yield but also significantly boosts the efficiency of phosphorus removal. Paterson *et al.* (2020) have already shown that in *Lemna minor*, growth and phosphorus uptake can be decoupled by pre-adapting the plants to phosphorus scarcity conditions. Such a strategy could potentially align with environmental goals by ensuring that effluent discharged into aquatic ecosystems meets stricter regulatory standards, thereby minimizing the risk of eutrophication. However, this strategy must be carefully planned as enhanced phosphorus removal could lead to nutrient levels that are insufficient for the proper growth of plants. Consequently, this could result in lower biomass productivity and protein content once equilibrium is reached, compared to systems where biomass is not recirculated (Figure 7-7 B). This approach is most

suitable for wastewater systems with high nutrient loads, where the nutritional quality of the biomass is not a critical factor.

7.4.3 Scaling up lab-based outcomes to a duckweed-based pilot-scale system in Indonesia

7.4.3.1 Setup and startup of the pilot system

A pilot duckweed-based treatment system was implemented adjacent to a fish hatchery in Malang, Indonesia (8°2' 31.54"S 112°39'8.84"E) (Figure 7-10 A).This initiative aimed to evaluate the efficacy of duckweed in improving water quality in a real-world aquaculture setting.

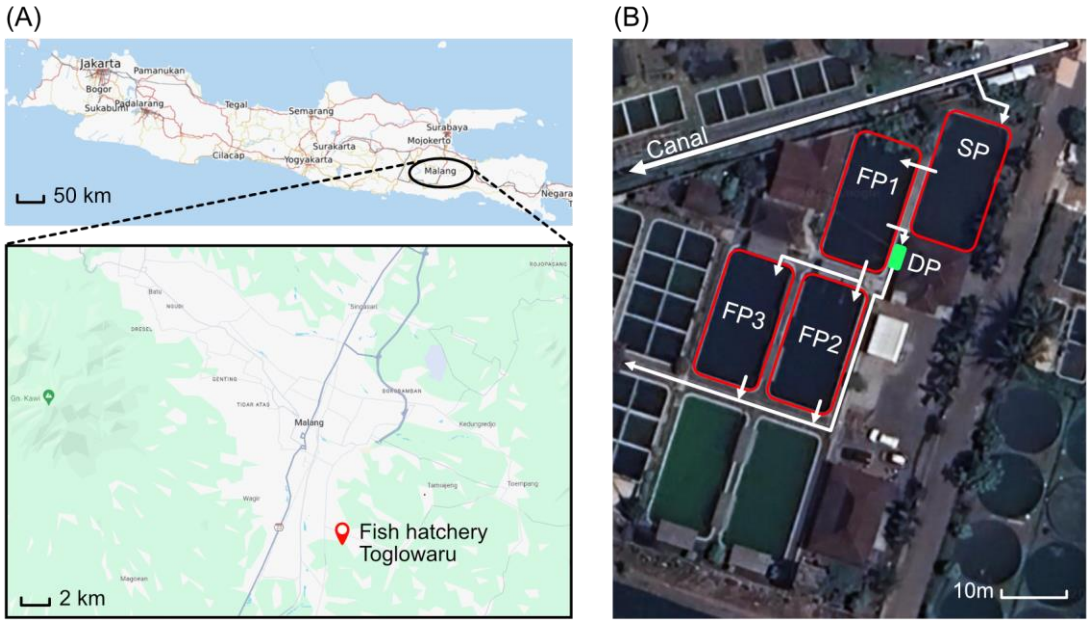

Figure 7-10 Location of the fishery and the duckweed-based wastewater treatment pilot System.

(A) Geographical location of the fishery in Malang, Indonesia, where the pilot system was installed. **(B)** Configuration of the Tlogowaru fish hatchery in Malang, showing the point at which water is drawn for the operation of the fishery and the various ponds it comprises. SP: Settling Pond, FP: Fish Ponds, DP: Duckweed Pond for Wastewater Treatment.

The hatchery comprised five 200 m² ponds, each stocked with a mix of Gourami and Tilapia fish at a 50:50 ratio, maintaining a density of 60 fish per pond. The fish were raised to their juvenile stage before being sold to other farms for further growth to commercial size. Daily, 4 kg of fish feed were prepared and distributed evenly across all ponds in two feeding sessions.

Water for the ponds was sourced from an upstream rice fields irrigation channel. Prior to entering the fish ponds, the water was passed through a settling pond, facilitating the removal of solids through sedimentation. This process ensured cleaner water was introduced to the first pond and subsequently distributed among the remaining ponds. Water flow through the ponds was continuous, with effluents discharged into a downstream water channel. Pond maintenance, including emptying and cleaning, occurred quarterly.

The duckweed treatment system was strategically placed between the first fish pond and the hatchery's management building for easy access to power sources. Water from the first pond was pumped into the duckweed treatment tank, with a heading tank used to regulate the treatment system's flow rate. The treatment tank had a 3 m^3 capacity and a 3.75 m^2 surface area. Treated water was then discharged into the hatchery's drainage system (Figure 7-11). A detailed hydraulic setup is presented in Chapter 2.

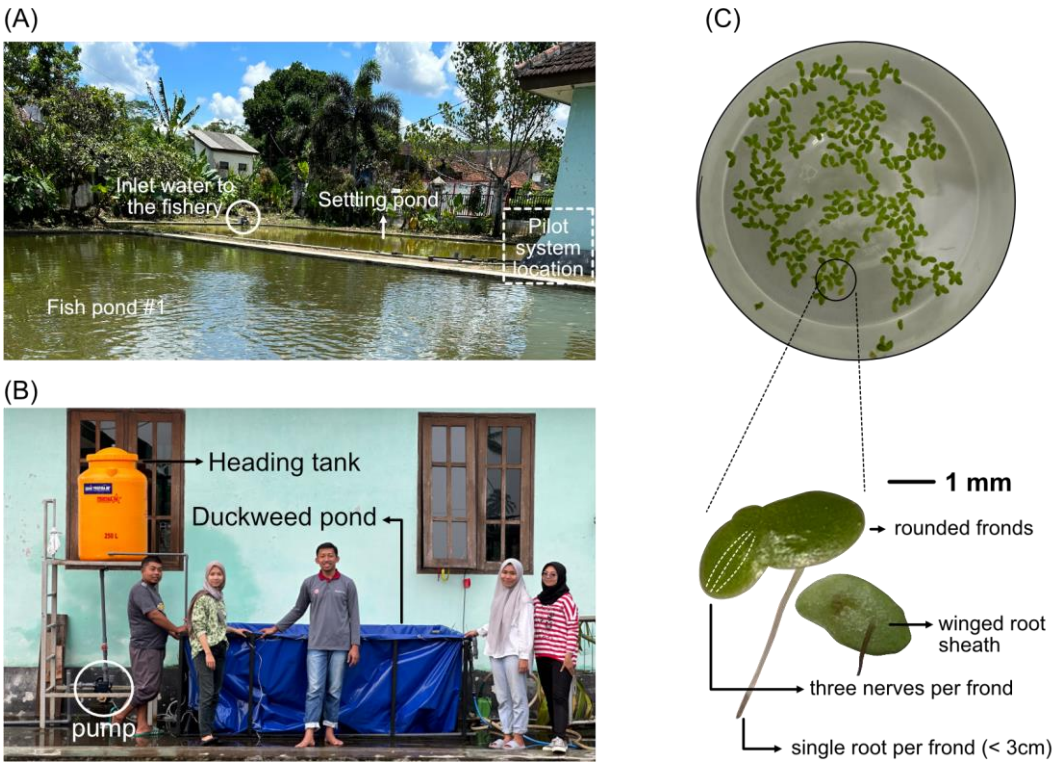

Figure 7-11 Layout of the pilot system and details on duckweed species used in field experiments

(A) Overview of the first two ponds at the Tlogowaru fish hatchery in Malang, Indonesia, illustrating the pilot system's location for wastewater treatment. **(B)** Image displaying the key components of the pilot system. The photo includes a fish farmer (on the left) and final-year Civil Engineering students from the Technical University of Malang, who contributed to the setup and ongoing monitoring of the pilot system. **(C)** Image and specifics of the duckweed species collected at Malang's flower market. The details include key morphological characteristics used for identification and the determination that the species is *Lemna aequinoctialis*, based on a dichotomous guide **(Bog *et al.* 2020)**.

Two treatment configurations were tested: one utilized the entire treatment area for growing duckweed, while the other introduced a baffle mid-tank, creating two compartments (A and B). the baffle impeded the movements of plants between compartments but allowed water flow between them. In this scenario, a portion of the biomass from compartment B was recirculated back to A, mirroring laboratory experiments described in Section 7.4.2.

Before commencing operations, the duckweed species collected from a local flower market was identified as *Lemna aequinoctialis* based on its morphological characteristics (Figure 7-11 C). In preparation for the trials, stock cultures of this plant were grown in the laboratory using standard Hoagland's media. To acclimate the plants to the pilot system conditions, they were gradually exposed to increasing concentrations of fish farm effluent mixed with Hoagland's media (25%, 50%, and 100% fish farm effluent). Once the biomass reached sufficient levels to cover at least 25% of the pilot system's surface area, the plants were transferred there. Preliminary trials involved adjusting operational flow rates to determine the minimum rate that would sustain duckweed growth. Despite the development of a mathematical model intended to guide the operation of duckweed-based pilot systems (Section 5.4.5), the model was not entirely reliable for application in the Malang pilot system due to its specific limitations related to the duckweed species used.

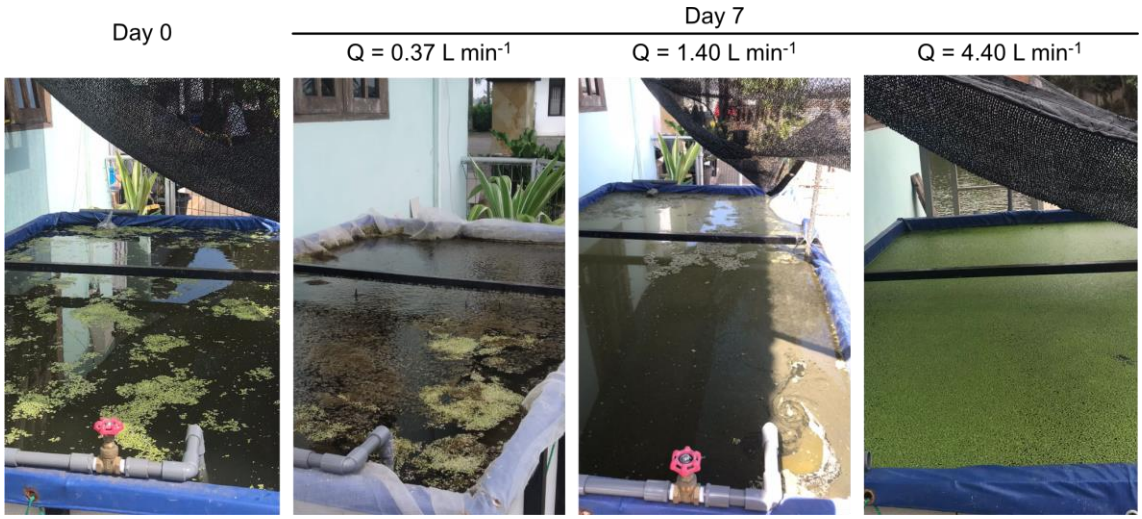

Figure 7-12 Initial trials of the duckweed-based pilot wastewater treatment system in Indonesia.

The images display the initial conditions of the pilot system when operations commenced at three distinct flow rates, and also show the state of the plants after a week of treatment for each flow rate.

At the onset of the pilot system, three distinct flow rates (0.37, 1.40, and 4.40 L min^{-1}) were tested to evaluate the viability of duckweed in the system. Each adjustment of the flow rate involved replenishing the treatment pond with fresh wastewater from the neighbouring fish pond, while keeping the initial duckweed coverage at 25% of the pond's surface area. Within a week of treatment at each flow rate, varied results were observed (Figure 7-12). At the lowest flow rate, the majority of the duckweed biomass turned brown, perished, and remained floating, with an algae and moss biofilm forming on it. Bubbles on the water's surface were also observed, indicating possible anaerobic conditions. At the medium flow rate of 1.40 L min^{-1}, the duckweed did not die but showed minimal growth, and excessive algae proliferation turned the pond water green. Conversely, at the highest flow rate, the pond was fully covered with healthy duckweed fronds after seven days.

During the operation of the treatment system, at the three selected flow rates, the average concentrations of total phosphorus and total nitrogen from the fish farm effluent were 0.24 mg P L^{-1} and 1.70 mg N L^{-1}, respectively. The failure of the treatment system at the lowest flow rate could have been due to a lack of nutrients to sustain the biomass, competition for nutrients with microalgae present in the fish farm effluent, lack of acclimation of the plants to the effluent being treated, or an overload of organic matter. Although bubbles indicated potential anaerobic conditions, the biological oxygen demand (BOD) analysis revealed that the actual organic load (25.5 kg ha^{-1} d^{-1}) was significantly below the recommended 262.5 kg ha^{-1} d^{-1} needed to maintain aerobic conditions in a maturation pond under Malang's ambient conditions (Mara 2004). Thus, oxygen deficiency was unlikely the failure cause.

The low nutrient availability coupled with competition from microalgae likely led to the decline in both algae and duckweed biomass. The phosphorus loading rate was only 0.034 g P m^{-2} d^{-1}, ten times lower than the minimum needed for effective operation with *Wolffia angusta* at 25% coverage, suggesting nutrient scarcity as a major constraint (as concluded in Section 6.4.2.2). The loading rate of phosphorus was also lower than loads previously reported for other species of duckweed species and microalgae (Zimmo, van der Steen, and Gijzen 2005). At the end, the decay of both biomasses may have contributed to the amount of organic matter in the system and the possible subsequent development of anoxic conditions, although this could not be confirmed as the BOD concentration in the effluent from the treatment system was not measured at these times.

When the flow was increased to 1.4 L min^{-1}, the phosphorus load rose to 0.12 g P m^{-2} d^{-1}, still below optimal levels for *W. angusta* and other duckweed

species but adequate to sustain the growth of microalgae naturally occurring in the effluent. This is likely why the treatment water turned green after one week of operation. A further increase in flow rate (4.4 L min^{-1}) ensured the organic load remained within acceptable limits for aerobic activity and provided enough phosphorus for plant growth, making this setting optimal for extended monitoring in subsequent trials. This strategic adjustment highlights the balance required between nutrient loading and flow rates to optimize growth conditions and wastewater treatment efficacy.

7.4.3.2 Monitoring of the pilot treatment system

Initially, the treatment system was operated without any internal divisions (baffles) at a flow rate of 4.4 L min^{-1}, which resulted in a hydraulic retention time of seven days. The full surface area of the treatment tank was used to grow *Lemna aequinoctialis*, starting with 50% surface coverage. Approximately every 8 days, the surface of the tank was completely covered by the plants, and 50% of the total biomass was harvested, allowing the remaining 50% to stay in the tank for continued treatment. The system underwent monitoring for a total duration of four weeks.

Fluctuations in nutrient levels between the inflow and outflow of the treatment system were recorded, alongside the percentage removal of each nutrient, as shown in Figure 7-13. Throughout the monitoring period, the concentration of orthophosphate in the influent remained relatively stable at an average of 0.15 mg PO_4-P L^{-1}. In contrast, the concentration of total phosphorus exhibited significant variability, ranging between 0.15 and 0.6 mg P L^{-1} (Figure 7-13 A). Analysis of the effluent samples taken on each sampling date revealed that concentrations of both orthophosphate and total phosphorus were consistently lower than those in the influent. The system proved equally efficient at removing both orthophosphate and total phosphorus, with removal efficiencies fluctuating by no more than 10%. Moreover, removal rates improved significantly over time, increasing from an average of 20% on the eighth day to 70% by the 28th day of treatment (Figure 7-13 B).

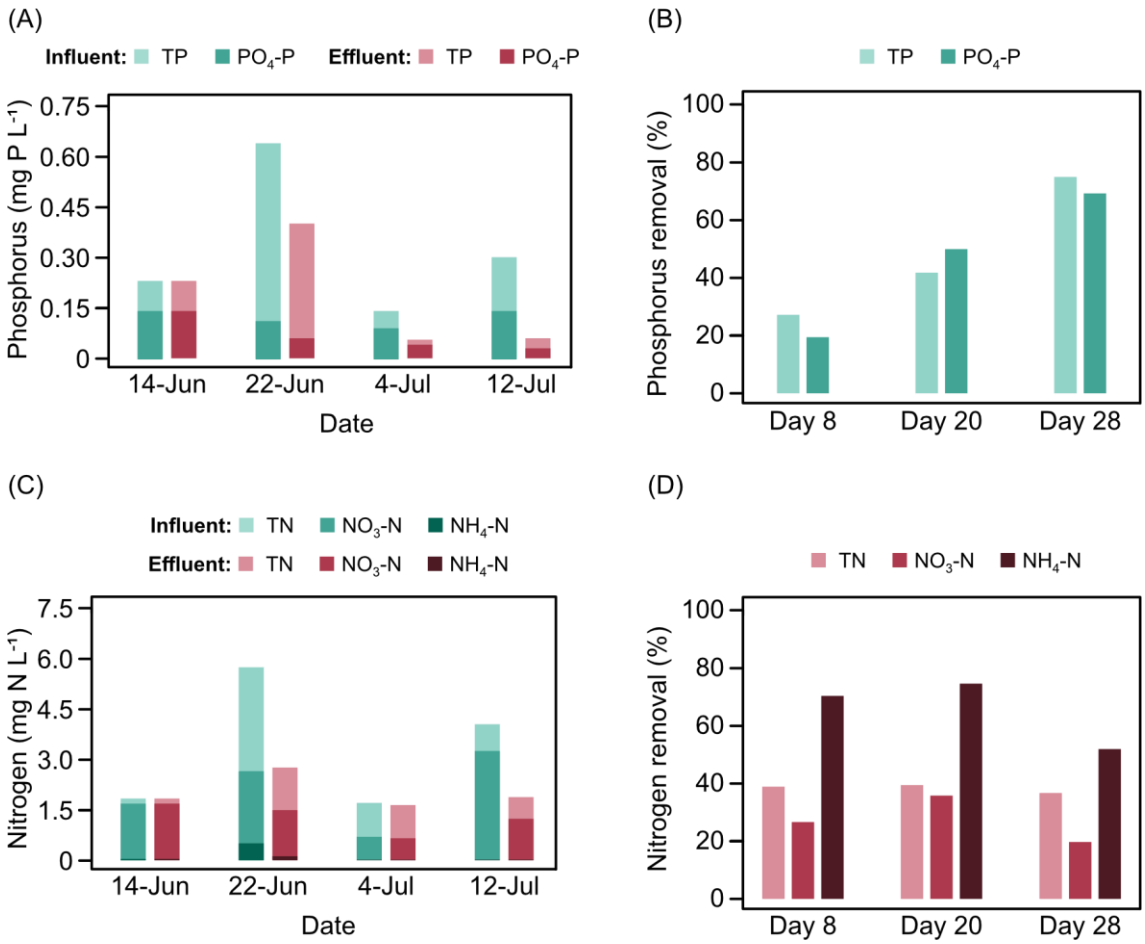

Figure 7-13 Variations in nutrient content and their removal during the operation of the non-compartmentalized pilot treatment system.

(A) Changes in the levels of total phosphorus (TP) and orthophosphate (PO_4-P) in the influent and effluent of the treatment system. **(B)** Percentage removal of phosphorus. **(C)** Changes in the levels of total nitrogen (TN), nitrate (NO_3-N), and ammonium (NH_4-N) in the influent and effluent of the treatment system. **(D)** Percentage of nitrogen removal.

In terms of nitrogen, the concentrations of nitrate, ammonium, and total nitrogen in both the influent and effluent were monitored (Figure 7-13 C). Over the course of the treatment month, total nitrogen levels in the effluent varied from 1.6 to 5.7 mg N L^{-1}. The nitrogen species distribution in the influent was predominantly nitrate, accounting for 43 to 91% of the total nitrogen, followed by organic nitrogen at 6 to 56%, and ammonium at 0.3 to 8.8%. The nitrogen removal by *L. aequinoctialis* remained relatively constant throughout the monitoring period (Figure 7-13 C). On average, 66% of the ammonium nitrogen was removed, followed by 39% of the total nitrogen and 28% of the nitrate initially present in the fish farm effluent.

The production of duckweed biomass varied throughout the treatment, with daily yields ranging from 181 to 304 grams of fresh weight (Figure 7-14). The peak productivity was observed at the end of the third week, coinciding with high

nutrient concentration peaks in the influent the week before. At this operational conditions, the dry duckweed biomass had an average protein, starch, fat and phosphorus content of 23.6%, 4.7%, 3.2%, and 0.61 mg P g^{-1}, respectively. More detail information about the biomass composition in Appendix 7.

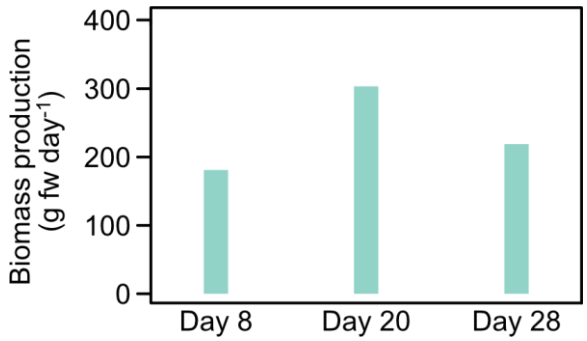

Figure 7-14 Biomass yield from the non-compartmentalized pilot treatment system at each biomass harvesting day.

After operating for one month, a baffle was introduced midway through the treatment tank to split it into two sections, thus creating distinct treatment zones with varying nutrient concentrations. This setup was analogous to connecting two tanks in series. The intention behind this arrangement was to explore whether greater nutrient removal could be achieved by exposing the plants to conditions of nutrient depletion initially. The flow rate was consistently maintained at 4.4 L min^{-1}, resulting in a combined hydraulic retention time of seven days, and the system continued to be monitored over an additional month.

During the study period, the quality of the fish farm effluent showed considerable variability. The total phosphorus concentration fluctuated between 0.25 to 0.99 mg P L^{-1}, while orthophosphate levels ranged from 0.07 to 0.71 mg P L^{-1}, as depicted in Figure 7-15 A. Initially, orthophosphate constituted over 60% of the total phosphorus content in the influent for the first three weeks. However, by the final week, this proportion had dropped to no more than 39%.

Despite one unusual reading on September 22nd, where the treated effluent displayed a higher total phosphorus concentration than the influent, likely due to a spike in influent phosphorus levels three days earlier, the phosphorus levels in the effluent were generally lower than those in the influent throughout the study. The concentrations of total phosphorus and orthophosphate in the treated effluent were recorded between 0.13 to 0.35 mg P L^{-1} and 0.06 to 0.31 mg PO_4-P L^{-1}, respectively. The phosphorus removal rates remained relatively constant, maintaining around 40% for total phosphorus and 38% for

orthophosphate, except on the 21st day of treatment when removal efficiencies exceeded 60% for both parameters, as shown in Figure 7-15 C.

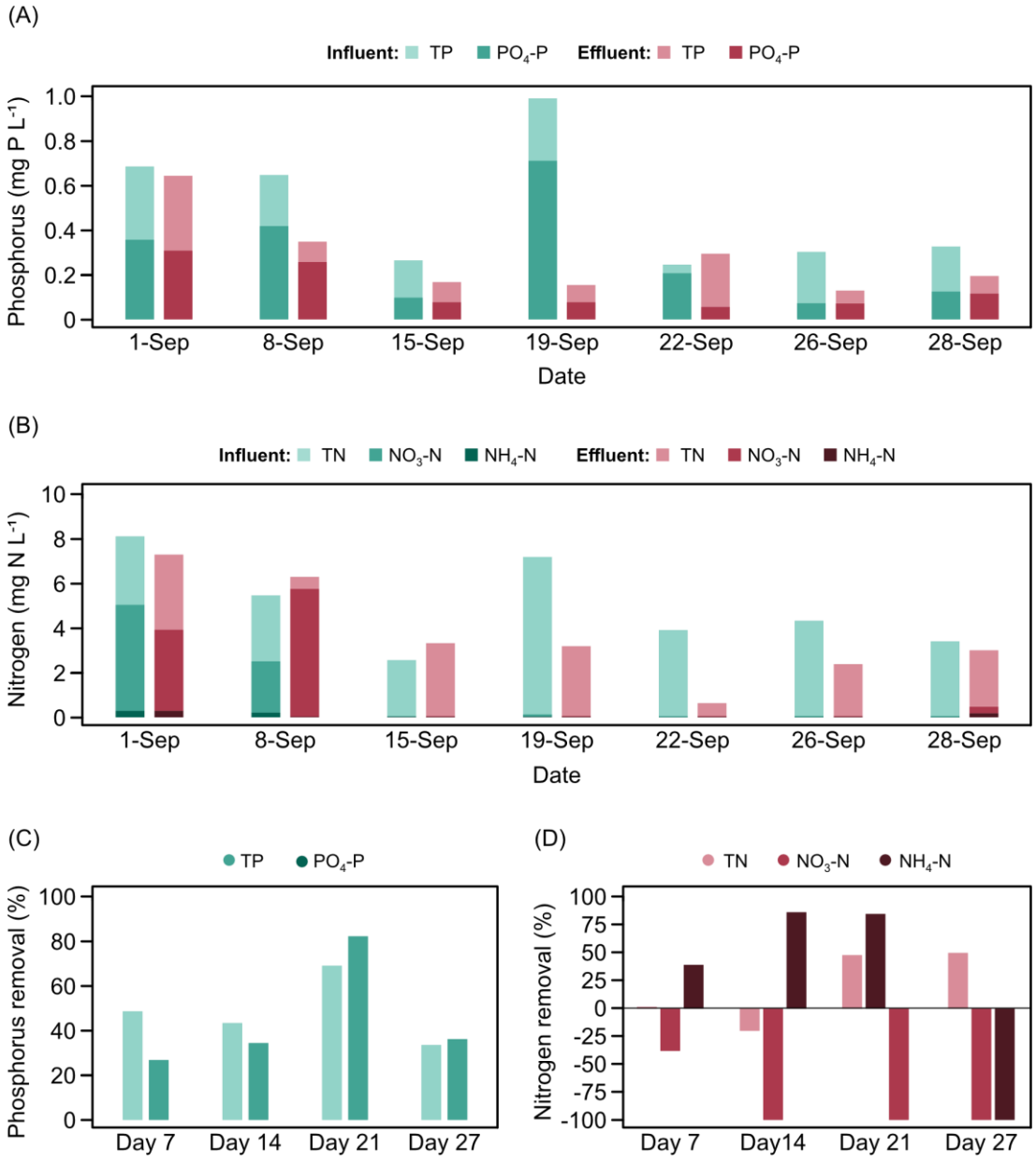

Figure 7-15 Variations in nutrient content and their removal during the operation of the compartmentalized pilot treatment system.

(A) Changes in the levels of total phosphorus (TP) and orthophosphate (PO_4-P) in the influent and effluent of the treatment system. **(B)** Percentage removal of phosphorus. **(C)** Changes in the levels of total nitrogen (TN), nitrate (NO_3-N), and ammonium (NH_4-N) in the influent and effluent of the treatment system. **(D)** Percentage of nitrogen removal.

Similarly, variations in total nitrogen, nitrate, and ammonium levels were monitored in the influent and effluent (Figure 7-15 B). Total nitrogen in the influent fluctuated between 2.5 and 8.1 mg N L^{-1}, nitrate ranged from 0.01 to 5.7 mg N L^{-}

^{1}, and ammonium varied from 0.01 to 0.28 mg N L^{-1}. After the ninth day, the amounts of ammonium and nitrate in both the influent and effluent were negligible. Throughout the treatment, the concentration of total nitrogen in the effluent was consistently lower than in the influent, with levels ranging from 0.64 to 7.23 mg N L^{-1}. Initially, total nitrogen removal was minimal but increased to about 65% after the second week of treatment (Figure 7-15 D). The removal of ammonium was notably high at 75% during the initial stages of treatment, while nitrate levels actually increased, suggesting negative removal rates. However, these findings should be approached with caution, as the methods used to measure ammonium and nitrate concentrations were of low sensitivity, with average concentrations below 0.02 mg N L^{-1}.

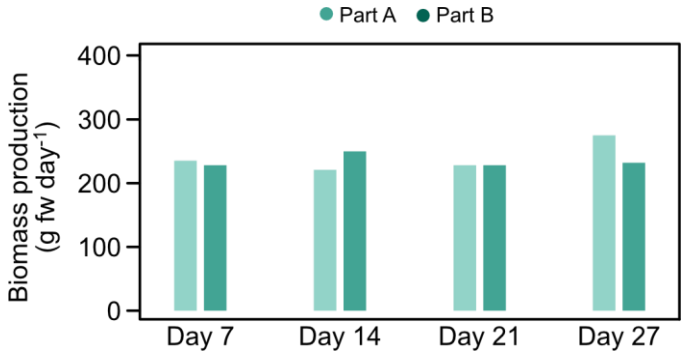

Figure 7-16 Biomass yield from the compartmentalized pilot treatment system at each biomass harvesting day.

Light bars represent biomass production from the first compartment, and dark bars biomass production from the second compartment.

No significant differences were observed in biomass production between the two compartments of the system (Figure 7-16). On average, the first compartment produced 240 grams of fresh weight per day, and the second compartment produced slightly less at 235 grams per day during the month of monitoring. The final duckweed biomass was dried and its composition analysed, resulting in protein, starch, fat and phosphorus content of 24.5%, 1.3%, 3.4%, and 0.68 mg P g^{-1}, respectively. More detail information about the biomass composition in Appendix 7.

Despite fluctuations in the quality of the fish farm effluent, the pilot treatment system using *L. aequinoctialis* proved effective in removing both phosphorus and nitrogen from fish farm effluents in Indonesia while also producing nutritious duckweed biomass. Acknowledging and managing the variability in wastewater quality is crucial, as sudden spikes in nutrients can harm the plants, and periods with insufficient nutrients might not sustain plant survival.

These fluctuations are often influenced by a range of factors including precipitation, changes in fish pond management practices such as feeding routines, fish population density and growth stages, as well as pond cleanliness and the quality of the source water used in the fishery. This water quality is often affected by upstream activities such as other wastewater discharges or agricultural runoff. Properly addressing these factors is key to maintaining the treatment system's effectiveness and efficiency.

In both trials, after the first half of the treatment period, the system consistently removed total phosphorus and orthophosphate with efficiencies ranging from 40 to 70%. The average removal rate for total phosphorus was 0.22 mg P m^{-2} d^{-1}, and for orthophosphate, it was 0.08 mg PO_4-P m^{-2} d^{-1}. These rates are significantly lower than those previously reported for *Lemna minor* (Adhikari *et al.* 2015) and *Wolffia angusta* (Section 5.4), likely due to variations in duckweed species, ambient temperature and nutrient concentrations in the wastewater. The fish farm effluent had a much lower phosphorus concentration compared to those previous studies, which negatively impacted the P removal efficiency, as concluded in Chapter 5. Nonetheless, the phosphorus removal rates for *L. aequinoctialis* are similar to those found for *Lemna minor* in continuous flow hydroponic systems (Coughlan, Walsh, Ahern, *et al.* 2022) and exceed those for *Wolffia* arrhiza (Soda *et al.* 2013). A more comprehensive understanding on the fate of phosphorus in the pilot system is essential, as the net phosphorus removal rates can be affected by the presence of other organisms such as bacteria and algae, as well as the balance between decaying biomass and the biotransformation of phosphorus species, including organic phosphates, within the treatment setup (Al-Nozaily and Alaerts 2002; Reddy 1983).

The nitrogen removal results from the treatment system were complex. The system effectively removed a significant portion of ammonium, a form of nitrogen that can be easily absorbed by duckweed (Caicedo *et al.* 2000; Petersen *et al.* 2021). However, an increase in nitrate concentrations, indicated by negative removal rates, raises concerns. This increase could be attributed to the sensitivity of the measuring technique or the nitrification process, where ammonium is converted into nitrate by nitrifying bacteria within the system (Zimmo 2003). While this conversion is part of the natural nitrogen cycle, it requires careful management to prevent excess nitrate buildup, which could lead to eutrophication if discharged into natural water bodies.

Despite nitrate accounting for more than 50% of the total nitrogen introduced to the treatment system, its removal was less effective compared to ammonium (70% removal) and other forms of nitrogen like organic nitrogen and

nitrite (40% removal). This indicate that *L. aequinoctialis*, like other *Lemna* species, may prefer ammonium over nitrate as nitrogen source (Caicedo *et al.* 2000; Körner *et al.* 2001; Mohedano *et al.* 2012). Another aspect to consider is the decomposition of organic matter containing nitrogen by bacteria, a process that generates more ammonium which duckweed can then quickly absorb. The decomposition and absorption processes are faster than nitrate uptake, explaining why more organic nitrogen is removed than nitrate, both by the duckweed and any associated bacteria or microalgae (Joy 1969; Peng *et al.* 2007).

Overall, the rate of nitrogen removal in the pilot system was 3.0 mg N m^{-2} d^{-1}. This rate is lower than that previously recorded for *Wolffia angusta*, due to the lower nitrogen concentration in the fish farm effluent compared to the modified Hoagland's media used in earlier studies (see Section 2.4), but it is higher than rates reported for other *Lemna* species (Coughlan, Walsh, Ahern, *et al.* 2022; Soda *et al.* 2013). The dynamics of nitrogen removal highlight the need to account for microbial populations within duckweed systems to effectively balance the nitrification and denitrification processes with plant uptake, a factor not previously considered in the modelling of *Wolffia angusta* (Section 6.4.1).

The introduction of a baffle after one month of operation significantly enhanced the phosphorus removal efficiency, although it did not substantially change the concentration of phosphorus in the final treated effluent. Without the baffle, the effluent had an average orthophosphate concentration of 0.043 mg P L^{-1}, with a removal efficiency of 61%. After installing the baffle, the orthophosphate concentration in the treated effluent slightly increased to 0.082 mg P L^{-1}, but the removal efficiency also rose to 66%. The fish farm effluent had a higher phosphorus concentration during the time the baffle was installed in the pilot treatment system. The improved phosphorus removal can be attributed to the recirculation of biomass between compartments, which allows for better adaptation and acclimatization of the duckweed to varying nutrient levels. As previously detailed in Section 7.4.2, pre-acclimatising plants to lower phosphorus concentrations leads to enhanced removal capabilities when they are later exposed to nutrient-rich environments. This effect is further supported by the phosphorus content in the harvested biomass, which was 11.5% higher when the baffle was installed compared to the biomass grown without the baffle.

In terms of nitrogen removal, the introduction of the baffle did not lead to significant improvements. The average total nitrogen concentration in the effluent was recorded at 2.3 mg N L^{-1}, with an overall TN removal efficiency of 42%. This indicates that while the baffle improved phosphorus removal through biomass

management, it did not have the same effect on nitrogen, possibly due to the different mechanisms involved in nitrogen uptake and transformation within the system. Unlike phosphorus, nitrogen dynamics can be influenced by a broader range of biological processes such as nitrification and denitrification, which may not have been as effectively managed by the simple physical intervention of a baffle. Additionally, the treatment conditions did not induce nitrogen starvation, which could potentially enhance subsequent nitrogen uptake (Kufel *et al.* 2012).

Higher nutrient loading rates were recorded in the pilot system with the baffle due to increased concentrations of nutrients in the wastewater at the time of operation. This resulted in enhanced production of duckweed biomass. Despite the changes in nutrient loading, the protein and fat content within the duckweed biomass remained relatively consistent, averaging 24.1% and 3.3% dry weight, respectively, irrespective of whether the baffle was installed or not. Interestingly, the starch content in the biomass was significantly higher in the absence of the baffle, being 3.6 times greater than when the baffle was in use. This marked increase in starch could be related to the lower nitrogen levels present in the system without the baffle. In aquatic plants like duckweed, nitrogen limitation is often linked to increased carbohydrate accumulation as the plant shifts its metabolic processes. When nitrogen is scarce, duckweed may divert resources typically used for protein synthesis (which requires nitrogen) towards carbohydrate (starch) storage (Guo *et al.* 2020; Yu *et al.* 2017). This metabolic shift serves as an adaptive strategy allowing the plant to store energy in the form of carbohydrates, which can be mobilized when growth conditions improve or when nitrogen becomes available again.

The analysis of the results reveals that the duckweed biomass contained 10% less protein, 20% less starch, and 21% fewer carbohydrates, but 16% more fibre than the feed currently used in the fishery (as detailed in Appendix 7). These variations highlight the potential of using the produced duckweed biomass as a supplementary ingredient or a partial replacement in fish feed formulations (Leng *et al.* 2005; Minich and Michael 2024). However, enhancing the nutritional value of the duckweed biomass through further processing could be beneficial. Specifically, pre-treatments aimed at increasing its protein content and reducing fibre could make the biomass more suitable for fish nutrition (Ifie *et al.* 2021; Minich and Michael 2024).

Based on the protein content observed in the duckweed and the standard feed used at the fishery in Malang, combined with the specific feeding regime and the productivity of the duckweed from the compartmentalized system, it was calculated that only 0.37% of the total protein required for fish rearing could be

sourced from duckweed biomass. This low substitution rate suggests that significant enhancements are required either through expanding the treatment areas to boost biomass production or by optimizing the nutritional content of the duckweed. Improving protein levels in *L. aequinoctialis*, for instance, could be pursued by adjusting cultivation conditions such as nutrient availability or by employing genetic or agronomic techniques to enhance protein synthesis (Vu *et al.* 2020; Yamamoto *et al.* 2001).

Additionally, considering the local ecosystem and the specific nutritional requirements of the fish, exploring other duckweed species better adapted to the local conditions might also provide more effective solutions. Screening for species with inherently higher protein content, like those belonging to the Genus *Wolffia*, or better growth rates could potentially meet the nutritional demands more efficiently (Appenroth *et al.* 2018). This approach would not only improve the sustainability of the aquaculture system but also enhance the economic viability of using duckweed as a feed ingredient.

The pilot-scale system has shown promising results, especially in the effective removal of phosphorus, demonstrating duckweed's adaptability to various nutrient loading rates and its potential for high biomass production rich in protein. This makes duckweed an excellent candidate for integration into aquaculture systems. However, challenges remain in managing nitrogen cycling. In Indonesia, the current legislative framework under Regulation number 68 of 2016 sets a maximum limit for ammonium concentration at 10 mg L^{-1} in domestic wastewater yet it does not impose limits on phosphorus and lacks specific regulations for fisheries (Indonesian Minister of Environment and Forestry 2016). Consequently, effluent from the Tlogowaru fish farm is not mandated to undergo treatment before being discharged into the environment, unlike in more stringent regulatory environments such as the UK.

This regulatory scenario might be particularly relevant to the chosen fishery, where the pilot system was installed. The continuous water flow in the fish ponds naturally helps dilute and remove waste, thereby minimizing the potential environmental impact. Furthermore, the fishery's operational focus on producing fingerlings—which require minimal feed and consequently generate less waste—also helps reduce overall waste outputs (Campanati *et al.* 2022; Dauda *et al.* 2019). Despite these factors, the pilot system has proven its capability to operate effectively within the existing phosphorus concentration limits, successfully recovering nutrients and producing biomass of commercial value. It is important to note that due to the very low strength of the wastewater produced by the fishery, the use of a compartmentalized tank or sequential tanks

offers no additional benefit. This suggests that simpler systems might be equally effective under these specific operational conditions, providing a more cost-effective solution while still achieving desired environmental and production outcomes.

Expanding on this, the integration of duckweed into aquaculture systems could provide a dual benefit: enhancing water quality through nutrient uptake and contributing to the circular economy by generating valuable biomass from waste nutrients. The ability of duckweed to thrive in varying nutrient concentrations and its fast growth rate make it a sustainable option for nutrient recovery in aquaculture effluents. Moreover, considering the ecological role of duckweed, where it naturally filters and cleans water bodies, its use in controlled aquaculture systems could also improve the water quality of effluents before they are released back into the environment, aligning with global sustainability goals. However, the success of such systems heavily relies on careful management of nutrient ratios and ensuring the system's design can handle fluctuations in nutrient levels to maintain efficient operation and biomass production.

7.5 Conclusion

The comparative analysis of duckweed cultivation in parallel and series tank configurations reveals distinct differences in nutrient removal efficacy. Achieving steady-state conditions within approximately 20 days, the series configuration outperformed the parallel setup by achieving up to 95% phosphorus and 91% nitrogen removal, compared to 82% and 40% in parallel tanks, respectively. This enhanced performance is attributed to the prolonged nutrient exposure and sequential absorption opportunities in series configurations, improving effluent treatment quality. Despite the high effectiveness of series systems in handling high nutrient loads, the study suggests that parallel configurations might require additional enhancements to comply with discharge regulations, emphasizing the importance of tank design and operational management in maximizing the efficiency of duckweed-based wastewater treatment systems for sustainable aquaculture.

Additionally, exploring different biomass management strategies, such as separate harvesting versus biomass recirculation, highlights the critical role of biomass management in optimizing nutrient uptake. Biomass recirculation notably increased phosphorus removal efficiency to 97% from 94%, illustrating the critical role of strategic biomass management in enhancing nutrient uptake

by leveraging internal phosphorus reserves—a mechanism also observed in microalgae. This adaptation, which mimics natural nutrient cycling by depleting and replenishing phosphorus stores within the plants, has proven to boost phosphorus removal efficiency significantly, as demonstrated in *Lemna minor* (Paterson *et al.* 2020), and suggests potential adaptability across different aquatic plant species. Additionally, while this strategy promises to align with stringent environmental standards by optimizing phosphorus removal and reducing eutrophication risks, it requires careful balancing to avoid nutrient deficiencies that could compromise plant growth and biomass quality, particularly in high-nutrient load scenarios where pollutant removal is prioritized over biomass production.

The pilot-scale study of duckweed cultivation for nutrient removal from fish farm effluents in Indonesia demonstrated promising outcomes, particularly in phosphorus removal. This highlights duckweed's adaptability to varying nutrient loads and its potential to produce high-protein biomass suitable for integrated aquaculture systems. The system's effectiveness was influenced by fluctuations in effluent quality. Despite these variables, the system effectively removed significant amounts of phosphorus and nitrogen, with improved phosphorus removal efficiency noted upon the introduction of a baffle. This modification facilitated enhanced biomass recirculation, optimizing nutrient uptake and confirming the importance of system design and management in maximizing treatment efficiency.

The integration of duckweed-based treatment tanks to aquaculture systems underscores the potential of duckweed not only to treat wastewater but also to support sustainable aquaculture practices by providing an additional source of biomass for fish feed. This dual benefit of environmental management and resource recovery offers significant insights into scaling up these technologies for real-world applications in tropical climates, where climate conditions are optimal to sustain duckweed growth, and nutrient-rich aquaculture effluents present both challenges and opportunities for sustainable management practices.

Chapter 8 General discussion and conclusions

8.1 Overview of research aims and gaps

The central hypothesis of this thesis is that it is possible to design and operate wastewater treatment systems using duckweed that can both recover nutrients and generate high-quality biomass from fish farm effluents, supporting sustainable aquaculture systems. The second objective of this research was to experimentally investigate how various environmental and operational conditions influence nutrient removal by duckweed and the production of nutritious biomass for use in nutrient recirculation within tropical aquaculture systems. This study integrated multiple concepts that, while individually researched in the past or applied in different contexts, have not been collectively explored.

Duckweed-based systems for wastewater treatment have been demonstrated to be effective across various studies (J. Cheng, Landesman, *et al.* 2002a; Mohedano *et al.* 2012; Paolacci *et al.* 2022). These systems benefit from the rapid growth and high nutrient uptake rates of duckweed, which make it an ideal candidate for bioremediation and recovery of nutrients from effluents. Traditionally, such systems have focused on the removal of organic matter and nutrients, optimizing conditions to maximize these aspects without necessarily considering the secondary benefits of biomass production.

While the effectiveness of duckweed in nutrient removal is well-documented, practical applications of these systems at a larger scale remain limited. Most existing models and pilot studies have been conducted under temperate climate conditions (El-Shafai *et al.* 2007; Paolacci *et al.* 2022; Paterson 2017; Zimmo *et al.* 2002), which do not directly translate to the complexities and unique environmental factors encountered in tropical regions. Moreover, the practical application of theoretical mathematical models into scalable solutions has often been absent or limited due to a lack of comprehensive real-world testing.

The impact of both biotic and abiotic factors on the performance of duckweed has been extensively studied, providing a substantial foundation for understanding how these plants adapt to and thrive in varied environmental conditions (Ishizawa *et al.* 2017; Liu *et al.* 2018; Wedge and Burris 1982; Z. Zhao

et al. 2014). However, there is limitation regarding the duckweed species that have been studied with studies focusing more on *Lemna minor*. Moreover, the application of these insights to enhance both water treatment efficiency and biomass quality simultaneously has not been fully explored. Often, the conditions ideal for nutrient removal from wastewater are not conducive to producing high-quality biomass, presenting a significant challenge in system design, balancing the trade-offs between treatment efficacy and biomass utility.

Additionally, although pilot-scale systems have provided valuable insights, their reliability is limited because many are based merely on extrapolations from lab results (Coughlan, Walsh, Bolger, *et al.* 2022; Paolacci *et al.* 2022; Zirschky and Reed 1988b). This approach has led to uncertainties when attempting to scale up these systems for commercial or industrial applications. The nutritional value of the plants grown in wastewater, another critical factor, has been explored to a lesser extent. While the potential of duckweed as a feed supplement in aquaculture is recognized due to its high protein and low lignin content (Leng *et al.* 2005), varying nutrient conditions can significantly alter its nutritional profile.

This thesis aimed to bridge these gaps by providing a comprehensive analysis of duckweed-based wastewater treatment systems designed specifically for tropical fish farms. By examining the interactions between system design, operational management, and environmental factors, it seeks to demonstrate that these systems can not only effectively manage wastewater but also contribute positively to sustainable aquaculture practices. Through rigorous testing and adaptation to local conditions, the goal was to optimize both the treatment process and the quality of the resulting biomass, ensuring that the systems are economically viable and environmentally sustainable.

Initially, a bench-scale study was carried out to identify the most effective duckweed species for nutrient removal and biomass production. This led to an investigation of the effects of environmental conditions on the growth and nutrient removal capacity of the duckweed species *Wolffia angusta* through a kinetic study. Key parameters from this study were used to develop a mathematical model that can predict outcomes in both batch and continuous wastewater treatment and cultivation systems.

This model was then applied to evaluate the impact of operational parameters on the treatment system's performance using in silico simulations. It also guided the operation of a pilot system under simulated tropical climate conditions, where various tank configurations were tested. Ultimately, this comprehensive approach culminated in the design and installation of a large-scale pilot system for treating real fish farm effluents in Indonesia. The insights

gained from this pilot system, along with results gathered throughout the project, provided valuable considerations that must be addressed for the integration of duckweed and aquaculture to be both viable and successful. This research underscored the potential of duckweed-based systems to make aquaculture systems more sustainable and resource efficient in tropical regions.

8.2 Key outcomes and insights from the project

8.2.1 Meta-analysis as a tool for comparing duckweed outcomes

Meta-analysis serves as a crucial tool for synthesizing diverse study results in environmental science, particularly in the study of duckweeds used for biomass production and nutrient uptake under varying environmental conditions. The wide variability in results and reporting units across different studies, which involve various duckweed species, growth media, and environmental settings, makes it challenging to draw direct comparisons and meaningful conclusions. Meta-analysis addresses these challenges by aggregating findings from unrelated studies to reveal underlying patterns or trends that individual studies may not discern (Moher *et al.* 2010; Strube and Hartmann 1983). Originating from medical research, the approach is ideal for environmental science due to its ability to handle variations and small sample sizes across studies. It allows for the effective generalization of results across different studies by systematically analysing the collective data (Haidich 2010; Sutton and Higgins 2008).

In the present project, the meta-analysis specifically aimed to assess the impact of abiotic environmental factors such as temperature, light, and nutrient levels (notably nitrogen and phosphorus) on the growth of duckweeds, as detailed in Chapter 3. By implementing a uniform methodological framework to evaluate data from various sources, the meta-analysis established a solid foundation for identifying the optimal experimental conditions that could be tested in the laboratory. These insights are instrumental in the design and improvement of duckweed-based systems for practical applications like wastewater treatment and nutrient recovery (Korner *et al.* 2003; Smith and Moelyowati 2001).

A significant methodological advancement introduced in this meta-analysis is the use of IQ-scores to compare outcomes across various duckweed studies. This approach enabled the synthesis of data independent of the diverse reporting units and methods used in individual studies. Utilizing IQ-scores allowed for direct comparisons of different duckweed growth measurements—such as fresh or dry weight, number of fronds, or total frond area—regardless of

the specific measurement techniques employed. This is crucial because there is no universally accepted method for estimating the relative growth rate (RGR) of duckweed (Iqbal *et al.* 2019; McCann 2016; Pagliuso *et al.* 2018; Sońta *et al.* 2020). The focus on growth trends through IQ-scores rather than exact RGR values, which can vary with the estimation method, provided methodological consistency and enhanced comparability across different duckweed species and experimental conditions.

Additionally, the use of IQ-scores in the meta-analysis facilitated the establishment of optimal ranges for environmental variables that promote duckweed growth, moving away from the reliance on single optimal values often reported in other studies (Wedge and Burris 1982; Yang *et al.* 2022). This approach provides significant benefits; by identifying a range of favourable conditions, researchers can more flexibly and effectively tailor duckweed cultivation environments to meet specific operational requirements. This adaptability is particularly valuable in applied settings such as outdoor wastewater treatment, where environmental conditions can be challenging and highly variable (Alisawi 2020). This systematic approach not only helps define the necessary environmental parameters for effective duckweed cultivation but also supports the development of robust systems that can adapt to varying conditions while maintaining efficiency in nutrient recovery and biomass production.

8.2.2 Importance of duckweed species selection and considerations for duckweed cultivation

Selecting the appropriate duckweed species is a critical factor in the successful implementation of duckweed-based systems for wastewater treatment. Each species of duckweed possesses unique traits that can significantly affect its suitability for specific environmental conditions and treatment goals. For example, local native species are often preferred for such applications because they are already adapted to the local climate and ecological conditions, which can enhance survival and performance (Coughlan, Walsh, Bolger, *et al.* 2022; Gupta and Prakash 2013). Using native species also minimizes risks associated with the introduction of non-native species, such as potential invasiveness or disruption of local biodiversity (Ceschin *et al.* 2019).

The choice of duckweed species should align with the specific characteristics of the wastewater to be treated, taking into consideration factors such as growth rate, nutrient uptake capacity, and the quality of the final biomass. Section 4.4.1 of this thesis highlighted the variability in response among different

duckweed genera and even within isolates of the same species originally from tropical climates (McCann 2016; Paolacci *et al.* 2021). This variability underscores the necessity of conducting preliminary screening experiments to identify the most suitable species for a given application.

Such screening experiments can reveal significant differences among species; some may excel in biomass production, while others might be more efficient at nutrient removal. Occasionally, a species such as *Wolffia angusta* excels in both areas, making it particularly valuable for projects with dual goals of biomass production and nutrient recovery. This possibility of uncoupling growth from nutrient uptake, two responses that typically counteract each other, has been previously reported for other duckweed species (Paterson *et al.* 2020).

In addition to species selection, the design of duckweed cultivation systems must be tailored to the specific needs and conditions of the local environment. This requires considering both biological and engineering factors, such as the configuration of the cultivation area and the management of inputs and outputs. The area required for duckweed cultivation directly depends on the intended scale of water treatment and the biomass productivity of the chosen duckweed species (Bonomo *et al.* 1997; Smith and Moelyowati 2001). It is essential to design systems that not only optimize space but also prevent growth limitations due to overcrowding (Kufel *et al.* 2012), ensuring enough area is provided for optimal plant development (as discussed in Section 4.4.2). Effective management of initial duckweed densities is also crucial; it allows for the maintenance of optimal growth rates and maximizes nutrient uptake efficiency, especially in settings where space is limited (Lasfar *et al.* 2007).

The source of nitrogen available in wastewater is another crucial factor in duckweed cultivation. Duckweeds are capable of utilizing various forms of nitrogen commonly found in wastewater (Goopy and Murray 2003; Holst and Yopp 1979). However, different species have varying preferences and efficiencies for these nitrogen sources, which can affect their growth and nutrient removal performance. Understanding these preferences is essential for optimizing nutrient removal from wastewater. For instance, some species might thrive better in conditions rich in nitrate, commonly found in agricultural runoff and certain industrial effluents , while others might prefer ammonium, more prevalent in urban and livestock wastewater (Ritter 2002). Results for *Wolffia angusta* showed that under standard culture conditions, this duckweed species has a preference for nitrate over ammonium (Section 4.4.3).

Overall, the successful application of duckweed-based systems for wastewater treatment requires a well-considered integration of species selection,

system design, and understanding of nutrient dynamics. By carefully selecting the appropriate local duckweed species and designing adapted cultivation systems, it is possible to create effective and sustainable solutions for nutrient management and water treatment. These systems not only help in reducing pollution but also contribute to the recovery of valuable nutrients, promoting circular economy.

8.2.3 Optimal temperature and nutrient conditions for growth and nutrient uptake in *Wolffia angusta*

Understanding how environmental conditions affect duckweed growth is crucial for optimizing wastewater treatment in aquaculture systems. Temperature plays a pivotal role both in duckweed growth rate and its ability to serve as a mechanism for nutrient removal from wastewater, as studies show that duckweeds thrive within a specific temperature range optimal for photosynthesis (Wedge and Burris 1982). The optimal temperature for *Wolffia angusta*, as suggested by the results in Section 5.4.1, is around 30°C. At this temperature, photosynthetic activity is likely to be high, which not only promotes robust growth but also enhances the plant's capacity to absorb carbon, effectively making duckweed a significant carbon sink. This is particularly relevant in the context of global carbon management strategies, where biological solutions are sought to mitigate atmospheric CO_2 levels (Khalid *et al.* 2022).

However, while higher temperatures led to increased biomass production due to enhanced metabolic rates, they adversely affected the specific nitrogen uptake capacities of duckweed. This is possibly due to the increase in biomass production despite nitrogen transporters operating at capacity at elevated temperatures (Forde and Clarkson 1999; Glass 2003). Further research is needed to draw definitive conclusions on this aspect. This observation is important because it indicates that although duckweed may grow more abundantly in warmer conditions, potentially increasing the total nutrient removal, its efficiency in removing nutrients such as nitrogen per unit of biomass may be reduced.

The implications of these results are particularly pertinent for tropical climates, where temperatures are relatively stable but are expected to rise due to global warming. The increase in temperature could push the operational conditions beyond the current optimal range for duckweed, challenging the resilience of the plants, and the stability and efficiency of existing wastewater treatment setups (O'Brien *et al.* 2020; Ziegler, Appenroth, and Sree 2023). In

regions where aquaculture is a major industry, and where effective wastewater management is crucial for both environmental sustainability and compliance with regulations, being able to predict and adjust to these changes is essential.

Phosphorus and nitrogen availability are other critical factors impacting duckweed performance in wastewater treatment systems. Phosphorus availability, in particular, is a common constraint in fish farm effluents, where concentrations often fall below levels that support optimal duckweed growth (refer to Table 1-1). However, the results indicate that *Wolffia angusta*'s growth is hindered only when nitrogen and phosphorus concentrations drop below their respective half-saturation constants (0.244 mg N L^{-1} and 0.048 mg P L^{-1}, as detailed in Section 5.4.2). This relatively low threshold for nutrient requirements is advantageous in environments where nutrient levels in effluents are typically low and variable.

The implications of nutrient availability extend beyond plant growth limitation. Higher nutrient levels in wastewater corresponded to increased rates of nutrient removal and uptake by the plants. This is a critical consideration for wastewater treatment, as it suggests that ensuring adequate nutrient levels can directly enhance the system's efficiency to treat fish farm effluents. Moreover, the composition of the biomass itself is influenced by the availability of nutrients (Mohedano *et al.* 2012; Y. Zhao, Fang, Jin, Huang, Bao, Fu, *et al.* 2014). The results on *W. angusta* showed that increased nitrogen availability boosts nitrogen and protein content in the biomass (as detailed in Section 5.4.3). Conversely, high phosphorus availability not only leads to an increase in phosphorus reserves within the plants, potentially enhancing their long-term nutrient storage capacity and resilience in fluctuating environments (Chaiprapat *et al.* 2005), but also reduces starch accumulation, which is more pronounced under conditions of phosphorus scarcity (Li *et al.* 2021). Phytate can be one metabolite used for P storage in plants (Feng *et al.* 2018), however its accumulation in duckweed tissue pose a challenge for fish feed production due to most fishes lacking endogenous enzymes to break it down (Prabu *et al.* 2017)

Despite the significant effects of individual factors like temperature and nutrient availability, the interaction between these variables appears minimal. This observation is particularly noteworthy as it suggests that duckweed's response to nutrient availability is robust across a range of thermal conditions, provided that nutrient concentrations do not drop to growth-limiting levels or reach inhibitory highs. Again, this demonstrates duckweed's resilience and adaptability to varying environmental conditions, which is crucial for ensuring consistent efficacy in wastewater treatment.

8.2.4 Modelling of duckweed-based wastewater treatment systems

The use of duckweed in wastewater treatment has gained increasing interest due to its efficient nutrient removal capabilities and potential for biomass production. To optimize these systems, kinetic studies play a pivotal role in understanding the dynamics of biomass growth and nutrient removal. These studies focus not only on characterizing biological systems but also on developing models that can predict and enhance the performance of wastewater treatment technologies. Various modelling approaches are employed, ranging from those based on external nutrient conditions to those considering internal nutrient supplies (Kufel *et al.* 2012), and they can be complex as many abiotic and biotic factors that influence duckweed performance are addressed.

In the case of *Wolffia angusta*, the initial model described in Section 5.4.5 was specifically designed to assess the effects of temperature and external nutrient supply, variables that fluctuate considerably in tropical environments. This model was later expanded in Section 6.3.1 to include factors like flow rates and harvesting regimes pertinent to continuous flow systems. The modelling strategy adopted focused primarily on external nutrient inputs, enabling quick data collection and straightforward model calibration. This approach, while simplifying some aspects of system dynamics, allows for immediate feedback and adjustments in operational strategies.

In batch experiments, where conditions were meticulously controlled, the model demonstrated high accuracy with prediction errors under 10% (Section 5.4.5). This precision confirms that the model's assumptions were valid and that it is well-suited for environments where the effects of other external variables are limited. However, the situation changed significantly in continuous flow systems, where the model's prediction errors increased to as much as 20% (Section 6.4.1). This reduced accuracy can be attributed to these systems operating under conditions that more closely mirror real-world environments. Initially, the model's predictions closely matched actual outcomes. However, as treatment progressed, discrepancies emerged, particularly due to the presence of algae. Algae compete with duckweed for both nutrients and light, which significantly alters the nutrient dynamics within the system (Roijackers *et al.* 2004; Szabó 1999). This competition not only affects nutrient removal efficiencies but also influences the overall productivity and quality of the duckweed biomass, introducing additional complexities to managing and optimizing the treatment process.

Despite these challenges, the model remains a valuable tool for predicting nutrient concentrations in treated effluents, removal efficiencies, biomass productivity, and operational parameters such as the time to reach steady state and harvesting frequency. Although the model may not fully capture the impacts of other external influences like algal or co-existing duckweed competition, precipitation, presence of bacteria, among others, they still provide a solid foundation for system design and management.

The model provided substantial insights but also highlighted the need for a more comprehensive approach that incorporates a wider range of biotic interactions and abiotic factors. Enhancing the model to include these elements could significantly improve its predictive accuracy and reliability, making it more applicable in real tropical climates. Additionally, understanding these interactions is crucial for effectively scaling up the duckweed-based system to ensure its sustainability and efficacy in practical scenarios. Although the model was specifically tailored for *Wolffia angusta*, a key outcome of this project is the development of a methodology for conducting kinetic studies and creating models, which can be adapted to other duckweed species.

8.2.5 Operation, design and strategies for effective nutrient removal

The study of duckweed-based wastewater treatment systems through in silico simulations and pilot-scale experiments under simulated tropical conditions has provided valuable insights into the operational dynamics and necessary design considerations for effective implementation. This critical analysis synthesizes the results, emphasizing the nuanced impacts of hydraulic retention time, nutrient loading rates, and plant coverage on system performance, and delineates design strategies for scalable application.

8.2.5.1 Effect of hydraulic retention time and duckweed coverage

The study demonstrated that extending hydraulic retention times and reducing nutrient loading rates primarily enhanced the quality of the final effluent, showcasing a direct relationship between the duration of nutrient exposure and treatment efficacy (as detailed in Sections 6.4.2.1 and 6.4.2.2). These parameters influence not only the amount of nutrients entering the system per unit area per day but also the overall health and productivity of the duckweed. While prolonged retention times facilitate greater nutrient absorption, leading to cleaner effluent , they may also reduce nutrient loading rates to levels that might

not support optimal duckweed growth, reducing the system's overall efficiency and biomass yield (Alahmady *et al.* 2013; Said *et al.* 1979). Conversely, high loading rates can lead to nutrient overload, potentially harming the duckweed and resulting in poor water quality outcomes, such as algal proliferation from unabsorbed nutrients (Roijackers *et al.* 2004; Szabó and Scheffer 2003). This dichotomy underscores the need for a balanced system design that ensures both robust nutrient removal and sustainable biomass production.

Adjustments in plant coverage within the system significantly impacted biomass production and the necessity for more frequent harvesting as detailed in Section 6.4.2.3. Higher plant coverage increases biomass output but also demands intensified labour due to increased harvesting frequency. This introduces a crucial trade-off between operational costs and productivity gains (Oron 1990). The economic balance is influenced by the methods used for biomass collection, manual or mechanical, and the economic value of the harvested biomass, which varies based on its intended final use (Cao, Fourounjian, and Wang 2018). These factors play a significant role in determining the economic feasibility of integrating duckweed-based treatment systems with aquaculture operations. Despite the operational challenges posed by increased plant coverage, this variable did not significantly alter the quality of the final effluent.

Plant coverage has a multifaceted impact on the treatment system, affecting not just nutrient processing capacity and biomass productivity but also gas exchange and evaporation rates (Abtew 1996; Rabaey and Cotner 2022). Increased plant coverage expands the photosynthetic area, potentially enhancing nutrient uptake and biomass output. However, this benefit necessitates more frequent harvesting to prevent overcrowding, ensuring that each plant has adequate light and space to thrive (Driever *et al.* 2005; Kufel *et al.* 2018). Conversely, insufficient plant coverage may not provide enough biomass to effectively remove nutrients, thus compromising effluent quality.

A key finding from the study was that duckweed can thrive at phosphorus loading rates exceeding 40 mg P m^{-2} d^{-1}, regardless of plant coverage. This suggests that maximal biomass productivity of *Wolffia angusta* is achievable when the treatment system is supplied with at least this loading rate and maintains nearly full plant coverage throughout. Conversely, when phosphorus loading rates fall below this threshold, the treatment system's capacity to support healthy plant growth declines sharply.

8.2.5.2 Comments on treatment systems design

The design of large-scale pilot systems requires careful consideration of local environmental conditions and wastewater discharge regulations. A critical factor in these designs is the local climate, especially the ambient air temperatures, as detailed in Section 6.4.3. Systems must be designed at the lowest average temperatures experienced in the region because cooler temperatures can slow duckweed's metabolic rate, reducing its growth and nutrient uptake efficiency (Lasfar *et al.* 2007). Designing the system to function optimally at these lower temperatures means that the treatment area might be larger than necessary during warmer periods. This approach ensures that the system remains effective year-round, accommodating fluctuations in temperature without compromising its efficiency.

Another critical factor in the design of duckweed-based treatment systems is the depth of the treatment tanks. The surface area of the treatment tank is essential to provide sufficient surface space for duckweed coverage and effective water treatment, but the depth of the water column also plays a crucial role by influencing the hydraulic retention time. Excessively deep tanks can lead to issues with mixing, such as poor light penetration and uneven distribution of nutrients, which can impede duckweed growth and reduce the efficiency of nutrient uptake (Maguire *et al.* 2024; Shammas *et al.* 2009). To avoid these issues, a recommended depth range of 60 cm to 1.5 meters was suggested. This range helps balance the volume of water with effective mixing dynamics, ensuring optimal conditions for duckweed performance and system efficacy.

The proportionality constants derived for *Wolffia angusta*-based treatment systems offer a pioneering approach to establishing the necessary treatment area based on the phosphorus concentration in wastewater (Section 6.4.3.2). This method allows for precise scaling of the treatment area to ensure that the effluent meets stringent consent limits of 0.25 mg P L-1 (like in the UK). Adjusting the treatment area based on the initial phosphorus load and duckweed coverage can optimize any given system’s efficiency. Similar suggestions or inputs into the design of duckweed-based wastewater treatment tanks have not been reported or published to date at the time of writing.

8.2.5.3 Operational strategies of duckweed-based pilot systems

The use of multiple tanks arranged in series for wastewater treatment with duckweed is a strategic approach that offers numerous benefits over single-tank systems connected in parallel. Serial tanks provide a staged environment where

each tank can be tailored to specific nutrient removal requirements, allowing duckweed to acclimate to gradually changing nutrient conditions. This staged acclimation generally results in more effective nutrient uptake as the biomass becomes progressively adapted to the nutrient concentration gradient present through the series (Demmig-Adams *et al.* 2022; Paterson *et al.* 2020). These advantages, however, bring complexities that require careful management.

One such complexity in serial configurations is maintaining adequate phosphorus levels, especially in the later stages of the series. It is essential to ensure that phosphorus concentrations do not fall below the minimum required for duckweed growth. If phosphorus levels become too low, it could severely limit the growth and nutrient uptake capabilities of the duckweed, compromising the overall effectiveness of the treatment system. This could lead to inefficient use of space and resources, as well as increased operational costs without corresponding treatment benefits.

Additionally, the strategy of biomass recirculation within sequential tanks presents a promising option for enhancing phosphorus uptake. By acclimating biomass in phosphorus-depleted conditions and then transferring it to phosphorus-rich environments, duckweed can potentially increase its phosphorus uptake efficiency as proven for other photosynthetic organisms (Hernandez *et al.* 2006; Slocombe *et al.* 2020; Solovchenko *et al.* 2019). This approach, which mimics natural adaptive responses of plants to fluctuating environmental conditions, has been explored in batch cultivation systems (Paterson *et al.* 2020) but adapting it to continuous flow systems is a novelty of this thesis and offers new challenges. Transitioning this approach from a controlled batch setting to a continuous flow system introduces variables that can affect consistency and reliability. Continuous systems are dynamic, with fluctuating inflow water quality and variable nutrient loads that can alter the effectiveness of acclimated biomass.

8.2.6 Complexities and challenges of full scale pilot systems

The installation of the pilot duckweed-based wastewater treatment system in a fish farm in Indonesia has provided valuable insights into its practical implementation, alongside highlighting various challenges and limitations. The quality of wastewater proved to be highly variable, influenced by activities within the fish farm, changes in upstream water quality, and environmental factors like evaporation and rainfall. These fluctuations impacted the effectiveness of nutrient removal and the production of biomass. Despite these challenges, duckweed has

demonstrated a strong adaptability, maintaining performance under varying conditions.

A major challenge identified in the pilot study was the high organic matter content in the wastewater, that at increased flow rates, led to elevated loading rates of both nutrients and organic matter into the wastewater treatment system. This excess organic matter can hinder the efficient uptake of nutrients and biomass production in duckweed-based systems. The decomposition of organic matter depletes oxygen levels, which is crucial for duckweed's health and growth, while also encouraging the growth of competing microorganisms that consume nutrients needed by the duckweed (Körner *et al.* 1998; Mohedano *et al.* 2014). Additionally, the accumulation of decomposing organic matter, including duckweed, and algae, can further consume dissolved oxygen and clog the system, diminishing even more its operational efficacy. Implementing pre-treatment steps to remove suspended solids from the water before it enters the treatment tanks, such as sedimentation or mechanical filtration, could help mitigate the impact of organic matter on the system's performance (Cripps and Bergheim 2000).

Moreover, the presence of microorganisms in the system posed both benefits and challenges. Beneficial bacteria within the system can enhance nutrient bioavailability through biotransformation processes, aiding the duckweed's nutrient uptake (Ishizawa *et al.* 2017, 2019). However, algae present a significant challenge as they compete with duckweed for the same nutrients, particularly under conditions of high light and nutrient availability. This competition can potentially lead to algae dominance, overshadowing duckweed growth and reducing the system's overall efficiency for nutrient removal and biomass production. The pilot study highlighted the crucial need for careful management of nutrient loads and duckweed coverage to maintain an optimal balance. Low plant coverage often led to ineffective treatment and proliferation of algae, while very high coverage necessitated frequent, labour-intensive biomass harvesting but effectively suppressed algae growth (Hancock and Buddhavarapu 1993; Roijackers *et al.* 2004). Operational observations from the pilot system indicated that maintaining an initial plant coverage of about 50% was ideal for ensuring efficient nutrient removal while preventing excessive light penetration that could favour algae growth, thus maintaining a stable and productive system.

The presence of pathogens, antibiotics, pests, and other contaminants in fish farm effluents is an important consideration for duckweed-based treatment systems, as these substances can adhere to or be absorbed by the duckweed (Markou *et al.* 2018; Reinhold *et al.* 2010). Although these contaminants typically

do not interfere with the system's ability to uptake nutrients or its overall operation, they can significantly impact the safety and utility of the harvested biomass. For example, if pathogens or antibiotics are present in the duckweed biomass, they could pose serious health risks if the biomass is used as fish feed (Markou *et al.* 2018). This risk necessitates rigorous management practices, including the potential need for additional processing or treatment of the biomass before it is used, to ensure it is safe for aquaculture and does not introduce harmful substances into the food chain. Addressing these concerns is crucial for maintaining the quality and safety of aquaculture products and for the successful implementation of duckweed-based wastewater treatment systems.

Finally, the pilot system demonstrated that the compartmentalization of the surface area to create two distinct zones of nutrient availability for duckweed growth might not always be necessary, especially in scenarios where nutrient concentrations in the wastewater are low. The deployment of multiple tanks or segregated sections within a system can inadvertently increase operational and installation costs without yielding corresponding improvements in nutrient removal.

8.3 Conclusion

The primary aim of this project was to explore how environmental and operational parameters influence the performance of duckweed-based systems for effluent treatment and biomass production in fish farms, with the broader goal of developing sustainable aquaculture systems. This thesis has demonstrated that adjusting the cultivation and operational conditions can significantly improve system efficacy. It has been demonstrated that different species or strains of duckweed may perform better under specific environmental conditions, highlighting the need to tailor system designs to local contexts for a safe nutrient management.

The research highlighted the vital role of temperature in regulating the metabolic activities and growth rates of duckweed, directly impacting nutrient removal efficiency and the quality of the biomass produced. This is critical for optimizing wastewater treatment protocols to ensure they remain effective under varying climatic conditions. The findings also confirm that nutrient availability is a more crucial factor than temperature in determining the efficiency of nutrient uptake and biomass quality. These insights are essential for designing and

operating aquaculture wastewater treatment systems that are both effective in pollutant removal and capable of generating economically valuable biomass.

Moreover, this study has highlighted the importance of developing reliable models to simulate and predict the performance of duckweed-based systems under different operational and environmental conditions. Such models can save time and resources in experimental setups and help refine system designs to enhance efficiency and adaptability to changing environmental conditions.

The implementation of serial tank configurations was shown to enhance nutrient removal efficiency, especially for phosphorus, by allowing duckweed to adapt progressively to changing nutrient concentrations. The strategy of biomass recirculation within these configurations has proven effective in maximizing phosphorus uptake by pre-adapting duckweed in phosphorus-depleted conditions before exposing it to phosphorus-rich environments. This method, while adding complexity, offers a promising approach to optimizing nutrient removal in continuous flow systems.

The successful deployment of a pilot duckweed-based wastewater treatment system in Indonesia underlines the practical application of theoretical research in real-world settings. This initiative has proven the dual benefits of improving water quality and generating valuable biomass for aquaculture feed. Insights from operating in tropical conditions have refined the understanding of system design and operational strategies, helping to bridge the gap between lab studies and industrial applications.

In conclusion, the comprehensive approach adopted in this thesis not only advances our understanding of the operational dynamics of duckweed-based treatment systems but also contributes significantly to the field of sustainable aquaculture. By integrating innovative design strategies with practical operational insights, this research paves the way for developing efficient, scalable, and environmentally friendly wastewater treatment solutions that support the aquaculture industry's sustainability goals. The successful application of these systems in real-world settings underscores the potential of integrating scientifically informed strategies with industry practices to enhance both economic viability and environmental sustainability.

8.4 Future perspectives

The research presented in this thesis opens several avenues for future exploration, each of which could significantly expand the understanding on

duckweed mechanisms for nutrient uptake and its practical applications. One key area for further study is the assessment of additional environmental factors such as salinity, pH, light intensity, and photoperiod on the growth and nutrient uptake of *W. angusta*. These factors are crucial for optimizing cultivation conditions and maximizing the efficiency of nutrient recovery.

Another promising direction involves exploring different nitrogen sources in the growth media, such as ammonium or combinations of ammonium and nitrate, which are commonly found in fish farm effluents. This could provide insights into how *W. angusta* adapts to different nitrogen forms and how these adaptations can be leveraged in aquaculture wastewater management.

Biotic interactions also present grounds for research. Studying the effects of bacteria and competition with algae on duckweed growth and nutrient uptake is essential, especially since these interactions are inevitable in natural and engineered ecosystems. Understanding these relationships can help improve duckweed cultivation strategies to enhance organic matter and nutrient removal efficiency.

Integrating these variables into a more generalized mathematical model could also enhance the applicability of the findings to broader contexts. Improving the model to predict not only duckweed growth but also its composition under varying culture conditions could be invaluable.

Besides, conducting further trials at a pilot scale over extended periods could help assess the seasonal variability of climate in outdoor setups, providing a comprehensive overview of the capabilities and limits of duckweed systems for wastewater treatment in real-world conditions.

Finally, assessing the risks associated with using duckweed biomass, particularly as fish feed, is critical. Since duckweed can remove nutrients and pathogens from water and accumulate micropollutants, it is essential to evaluate the safety and potential risks of reintroducing such biomass into the food chain, especially concerning fish and ultimately human consumption. This aspect of research is crucial for ensuring the safety and sustainability of using duckweed in integrated aquaculture systems.

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Appendix 1

Database for meta-analysis study

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^2]	[g m^{-2}]	(°C)	(days)	(h light)	[µmol m^{-2} s^{-1}]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
1	Sun	2020	Spirodela	polyrhiza	yes	600	133	377 (100)	25	25	16	65	Synthetic	440	0	440	21	21	0.0963
											16	65							0.0386
											0	0							0.0443
2	Kruger	2020	Landoltia	punctata	yes	200	116	52 (25)	21	24	16	39	Real	266			54		0.0671
									23					114			21		0.0856
									24					57			10		0.0636
3	Miranda	2020	Landoltia	punctata	yes	500	79	56 dw (100)	23	5	16	50	Synthetic	70	60	10	1200	1200	0.0811
														35	30	5	600	600	0.1560
4	Iqbal	2019	Lemna	minor	no	250	26	23	39	10	14	385	Synthetic	47	24	22	43	16	0.1371
																			0.1392
																			0.1364
													Real	50	26	23	37	14	0.1010
																			0.1129
																			0.0983
5	stewart	2020	Lemna	*gibba*	yes	1000	177	1132 fn	25	4	24	100	Synthetic						0.4955
												200							0.5529
												500							0.5862
												700							0.6174
6	Pagiluso	2018	Spirodela	polyrhiza	yes	100			25	7	16	20	Synthetic	192	18	173	31	31	0.2414
			Landoltia	punctata															0.2262
			Lemna	*gibba*															0.1426
			Wolffiela	caudata															0.1027
			Wolffia	borealis															0.1369
			Spirodela	polyrhiza								500							0.3642
			Landoltia	punctata															0.3264
			Lemna	*gibba*															0.3189
			Wolffiela	caudata															0.3000
			Wolffia	borealis															0.4208
7	Li	2016	Lemna	aequinoctialis	yes		252	12 (70)	25	12	16	105	Synthetic	175	0	175	77.5	77.5	0.1900
			Landoltia	punctata															0.1900
			Spirodela	polyrhiza															0.1800
			Lemna	aequinoctialis					20										0.1800
			Landoltia	punctata															0.1700
			Spirodela	polyrhiza															0.1600
			Lemna	aequinoctialis					30										0.1700
			Landoltia	punctata															0.1700
			Spirodela	polyrhiza															0.1600

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^2]	[g m^{-2}]	(°C)	(days)	(h light)	[µmol m^{-2} s^{-1}]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
7	Li	2016	Lemna	aequinoctialis	yes		252	12 (70)	25	12	16	30	Synthetic	175	0	175	77.5	77.5	0.1100
			Landoltia	punctata															0.1100
			Spirodela	polyrhiza															0.1000
			Lemna	aequinoctialis								75							0.1800
			Landoltia	punctata															0.1700
			Spirodela	polyrhiza															0.1600
			Lemna	aequinoctialis								105							0.1900
			Landoltia	punctata															0.1800
			Spirodela	polyrhiza															0.1500
			Lemna	aequinoctialis					25	12	16	105		35	0	35	15	15	0.1900
			Landoltia	punctata															0.1900
			Spirodela	polyrhiza															0.1800
			Lemna	aequinoctialis										4	0	4	2	2	0.1000
			Landoltia	punctata															0.0900
			Spirodela	polyrhiza															0.0900
			Lemna	aequinoctialis										0	0	0	0	0	0.0800
			Landoltia	punctata															0.0700
			Spirodela	polyrhiza															0.0600
8	Paterson	2020	Lemna	minor	yes	100	17	1767 (100)	15	4	0	155	Synthetic	100	0	100	15	15	-0.2007
											6								0.0323
											12								0.0642
											24								-0.0023
									8	4	6	155							0.0074
									15										0.0354
									21										0.0371
									15	20	6	155		15	15	0	5	5	-0.0170
															0	15			0.0484
														50	50	0	15	15	0.0149
															0	50			0.0626
9	Chikuvire	2018	Wolffia	arrhiza	yes	2725	545	150 (100)	26	7	12	117	Real	29			2		0.2059
										14									0.2344
										7									0.1076
																			0.2478
														59			4		0.2432
														88			6		0.2144
10	Ceschin	2020	Lemna	minuta	no	70000	4301	69 ^{dw} (100)	18	28	15	438	Synthetic	1.4	0	1.4	2.7	2.7	0.0830
								201 ^{dw} (100)											-0.0390
11	Mohedano	2012	Landoltia	punctata	no		14700	220 (100)	24	365	12	379	Real	832	636		92		0.8227
							9000							44	28		10		0.3773
12	Zhang	2014	Lemna	minor	yes	1000	95	53	23	72	16	39	Synthetic	1	1	0	31	31	0.3200
														3	3				0.3100
														5	5				0.2900
							133	38											0.2500
							21	238											0.1000

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^2]	[$g\ m^{-2}$]	(°C)	(days)	(h light)	[$\mu mol\ m^{-2}\ s^{-1}$]		[$mg\ N\ L^{-1}$]	[$mg\ N\ L^{-1}$]	[$mg\ N\ L^{-1}$]	[$mg\ P\ L^{-1}$]	[$mg\ P\ L^{-1}$]	[day^{-1}]
13	Van Echelpoel	2016	Lemna	minor	yes	250	50	99	23	4	16	52	Synthetic	69.3	0	69.3	21.0	21.0	0.6330
														33.0		33.0	10.7	10.7	0.5210
														16.2		16.2	5.4	5.4	0.5170
														8.8		8.8	2.6	2.6	0.5750
														4.2		4.2	1.3	1.3	0.6250
				minuta										69.3		69.3	21.0	21.0	0.4500
														33.0		33.0	10.7	10.7	0.5000
														16.2		16.2	5.4	5.4	0.4000
														8.8		8.8	2.6	2.6	0.3650
														4.2		4.2	1.3	1.3	0.5080
14	Zhao	2015	Lemna	japonica	no		170000	0.41 (150)	12	90	6	465	Real	19.3	18.7	0.7	3.7		-0.0002
									16			520		21.0	19.2	1.6	3.6		0.0054
									22			495		25.3	17.1	7.9	3.1		0.0103
									20			452		27.1	17.5	8.4	3.4		0.0063
			Landoltia	punctata	no			0.38 (150)	12			465		19.3	18.7	0.7	3.7		-0.0087
									16			520		21.0	19.2	1.6	3.6		-0.0004
									22			495		25.3	17.1	7.9	3.1		0.0065
									20			452		27.1	17.5	8.4	3.4		0.0024
			Spirodela	polyrhiza	no			0.48 (150)	22			495		25.3	17.1	7.9	3.1		0.0027
									20			452		27.1	17.5	8.4	3.4		-0.0064
			Wolffia	*globosa*	no			0.45 (150)	22			495		25.3	17.1	7.9	3.1		0.0030
									20			452		27.1	17.5	8.4	3.4		-0.0068
15	Yu	2014	Lemna	aequinoctialis	yes		2400	125 (100)	23	24	16	110	Synthetic	36	36	0	140	140	-0.3762
										18			Real	50	50		20	20	-0.5386
16	Wang	2014	Lemna	minor	yes				23	7	16	55	Synthetic	99	0	99	23	23	0.2730
														2	2	0			0.2680
														7	7				0.2870
														28	28				0.3100
														84	84				0.2860
														280	280				0.2650
														840	840				0.1950
17	Mkandawire	2006	Lemna	*gibba*	yes	700			20	21	16	105	Synthetic	7	3.5	3.5	7.2	7.2	0.2207
																	0.003	0.003	0.1536
																	0.46	0.46	0.2420
																	3.59	3.59	0.2222
18	Zhao	2014	Lemna	aequinoctialis	no	2400	120000	412 (150)	16	84	11	177	Real	25	18	4.8	3.3		0.0294
						3600													0.0313
						4800													0.0320
						6000													0.0337
						6000		206 (75)											0.0367
								412 (150)											0.0337
								618 (225)											0.0295
								882 (300)											0.0245

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^2]	[g m^{-2}]	(°C)	(days)	(h light)	[µmol m^{-2} s^{-1}]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
19	Fujita	1999.0	Wolffia	arrhiza	yes	3000	707	0.01 [dw]	30	7	16	88	Synthetic	13.3	3.6	9.7	7.1	7.1	0.1343
								30000 [fn]	28	15	24	44		6.5	1.8	4.8	4.6	4.6	0.0978
								520000 [fn]											0.0049
								130000 [fn]	28	15	24	22		13.0	3.5	9.5	9.1	9.1	0.0885
												72							0.1103
											16	44		2.6	0.7	1.9	1.8	1.8	0.0924
											24								0.1312
20	Walsh	2021	Lemna	minor	yes	100	42	6509 [fn]	18	5	16	10	Synthetic		43.78	0	10.9	10.9	0.1120
												50							0.1910
												80							0.1620
												100							0.1910
												150							0.1790
												200							0.1690
												250							0.1750
												350							0.1660
												500							0.1510
												850							0.1350
												50							0.3120
												200							0.2800
												850							0.3190
												50		65.2	17.5	47.7	45.5	45.5	0.2450
												200							0.3600
												850							0.4280
21	Ben-shalom	2014	Lemna	*gibba*	no	35000	2000	46899 [fn]	19	30	13	729	Real		28			6.3	0.2231
									20						28			6.3	0.1881
									24						45			8.6	0.2119
									24						45			8.6	0.2183
									27						57			11	0.0000
									27						57			11	0.2084
									25						52			11	0.0000
									25						52			11	0.1805
									20						28			6.3	0.2108
									24						45			8.6	0.2151
									27						57			11	0.0000
									25						52			11	0.1002
									20						28			6.3	0.2183
									25						45			8.6	0.2209
									28						57			11	0.0000
									25						52			11	0.1917
22	Mohapatra	2012	Lemna	*gibba*	no		1800	0.02 [fn]	27	20	12	400	Real		6.0	0.66		1.24	0.1070
									26						6.0	0.73		0.78	0.1060
									28						5.6	0.99		0.26	0.1030
									28						5.6	0.91		0.15	0.0910
									32						5.2	0.61		0.14	0.0810

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^{-2}]	[g m^{-2}]	(°C)	(days)	(h light)	[µmol m^{-2} s^{-1}]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
									34						5.5	0.81		0.16	0.0650
23	Frederic	2006	Lemna	minor	yes		0.09	3.9 dw	20	7	13	342	Synthetic	100	0	100	31	31	0.2804
								3.7 dw											0.2589
								5.4 dw											0.2327
								8.4 dw											0.2619
								9.4 dw											0.2619
								7.7 dw											0.2496
								9.2 dw											0.2496
								12 dw											0.2465
								13 dw											0.2265
								15 dw											0.2250
								16 dw											0.2265
								18 dw											0.2034
								22 dw											0.2003
								23 dw											0.1880
								26 dw											0.1849
								25 dw											0.1803
								30 dw											0.1726
								31 dw											0.1787
								43 dw											0.1541
								43 dw											0.1387
								46 dw											0.1448
								48 dw											0.1448
								54 dw											0.1325
								60 dw											0.1217
								70 dw											0.1063
								86 dw											0.0786
								93 dw											0.0724
								128 dw											0.0324
24	Bieleski	1968	Spirodela	oligorrhiza	yes	20	20	6 dw	24	7	24	38	Synthetic	112	112	0	31	31	0.3311
																	0	0	0.1762
25	Sonta	2020	Lemna	minuta	yes	8500	360	556 fn	24	15	16	126	Real	184	143		20	13	-0.0611
														138	107		15	9.8	-0.0611
														92	71		10	6.5	-0.0611
														64	45		7.1	4.6	0.0149
														46	36		5.1	3.3	0.0149
														23	18		2.5	1.6	0.0392
														14	11		1.6	1.0	0.0611
														11	8.6		1.2	0.8	0.0611
														7.2	5.6		0.8	0.5	0.0899
														55	48		6.4	3.4	0.1172
														35	31		4.1	2.2	0.1390
														27	24		3.2	1.7	0.1343
														21	19		2.5	1.3	0.1390

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^{2}]	[g m^{-2}]	(°C)	(days)	(h light)	[µmol m^{-2} s^{-1}]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
														14	12		1.6	0.8	0.1438
														7.0	6.2		0.8	0.4	0.1316
														3.5	3.1		0.4	0.2	0.1172
26	Dinh	2020	Lemna	minor	no	300	13	239	22.5	6	12	248	Real	26			1.6		0.0521
														600	105	290	1000		0.0463
						3400	249	68		3				27.2			1.75		0.0946
														15			1.09		0.1323
														14			0.90		0.1359
														12			0.67		0.1350
														8.9			0.55		0.0575
27	Nesan	2020	Lemna	minor	yes	300	79	293 (100)	25	7	16	21	Synthetic	35	17	19	6.6	6.6	0.0550
																			0.0420
																			0.3750
																			0.0200
																			-0.0300
28	Iatrou	2019	Lemna	minor	no	5000	1000	130	20	16	24	44	Real	41	0.3	6.3	1.2		0.0998
				gibba				130						38	1.7	5.5	1.2		0.1320
				minor				262						70	32	5.8	1.3		0.1408
				gibba				302						69	28	5.9	1.4		0.1773
29	Stadtlander	2019	Landoltia	punctata	yes	1200	440	91	25	25	12.0	127	Real		6.2	1.5		4.8	0.1168
															11	3.7		14	0.1344
			Spirodela	polyrhiza											6.2	1.5		4.8	0.1292
															11	3.7		14	0.1403
30	Sudiarto	2019	Lemna	sp.	no	10000	1000	75 (50)	31	21	14	133	Real	152	19	120	27		0.0500
													Synthetic	126	14	76	20	20	0.1400
31	Paolacci	2018	Lemna	minor	no		1963	(0.25)	6		9	113	Real			4.1		0.03	0.0167
									9		14	206							0.0337
									15		16	330							0.0373
									12		10	254							0.0620
				minuta					6		9	113							0.0000
									9		14	206							0.0527
									15		16	330							0.0470
									12		10	254							0.0373
32	Basiglini	2018	Lemna	minor	no	900	314	115 (80)	33	7	15	729	Synthetic	10	0	10	3.1	3.1	0.0910
									19		9	347							0.0000
									33		15	729	Real	12			2.9		0.0680
									19		9	347		109			4.1		-0.0320
33	Gerard	2018	Lemna	minor	no		64		23	20	15	78	Real				0.1		0.1250
				minuta															0.1400
				minor	yes	250			25		16	67.5	Synthetic	100	0	100	0.03	0.03	0.1530
				minuta															0.1520
34	Chen	2018	Spirodela	polyrhiza	no		120000	488 (150)	23	16	14	712	Real	6.1	6.1	0.03	0.58	0.41	0.0844
			Lemna	minor				412 (150)											0.1028
			Landoltia	punctata				375 (150)											0.0990

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^2]	[g m^{-2}]	(°C)	(days)	(h light)	[µmol m^{-2} s^{-1}]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
35	Pena	2017	Lemna	minor	yes		560	215 (80)	24	12	10	98	Real	48	43.6		8.49		0.0544
													Synthetic	100	0	100	31	31	0.0590
36	Iqbal	2017	Lemna	minor	no	250	26	23	39	22	14	438	Real	95	55		78	18	0.0738
														74	40		64	14	0.0808
														59	35		45	10	0.0843
														37	20		28	8	0.0869
														21	12		18	5	0.0888
									27	22	12	350		102	58		82	32	0.0345
														82	46		66	26	0.0611
														61	32		47	22	0.0633
														40	17		33	12	0.0664
														23	10		19	9	0.0693
37	Garcia	2017	Landoltia	punctata	no	440000	400	40 [dw] (100)	22	7	11	330	Synthetic	63			12	12	0.0656
								48 [dw] (100)											0.0025
								40 [dw] (100)						43			19	19	0.0610
								51 [dw] (100)						32			10	10	0.0279
								40 [dw] (100)						37			14	14	0.0759
														52			22	22	0.0836
														54			18	18	0.0500
38	Paolacci	2016	Lemna	minuta	yes	100	50	0.92	20	7	16	40	Synthetic	0	0	0	93	93	0.1730
														0.003		0.003			0.2510
														0.03		0.03			0.2580
														0.3		0.3			0.2220
														0.7		0.7			0.1730
														1.0		1.0			0.1600
				minor				2.25						0		0			0.1310
														0.0030		0.003			0.1650
														0.03		0.03			0..175
														0.3		0.3			0.1190
														0.7		0.7			0.0980
														1.0		1.0			0.7200
				minuta				0.92						0	0	0			0.1740
														0.003	0.003				0.2240
														0.03	0.03				0.1890
														0.3	0.3				0.2440
														0.7	0.7				0.1980
														1.0	1.0	0			0.2230
				minor				2.25						0	0				0.1310
														0.003	0.003				0.1580
														0.03	0.03				0.1320
														0.3	0.3				0.1740
														0.7	0.7				0.1490
														1.0	1.0				0.1570
																	0	0	0.0460

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^{2}]	[$g\ m^{-2}$]	(°C)	(days)	(h light)	[$\mu mol\ m^{-2}\ s^{-1}$]		[$mg\ N\ L^{-1}$]	[$mg\ N\ L^{-1}$]	[$mg\ N\ L^{-1}$]	[$mg\ P\ L^{-1}$]	[$mg\ P\ L^{-1}$]	[day^{-1}]
				minuta				0.92									0.05	0.05	0.0490
																	0.1	0.1	0.1480
																	0.5	0.5	0.1420
																	5.0	5.0	0.1510
																	50	50	0.1680
														0.01	0	0.01	93	93	0.2440
				minor				2.25									0	0	0.0720
																	0.05	0.05	0.1210
																	0.1	0.1	0.1410
																	0.5	0.5	0.1290
																	5.0	5.0	0.1480
																	50	50	0.1380
																	93	93	0.2050
39	Peeters	2016	Lemna	minor	yes	350	7	311 (100)	20		12	230	Synthetic	4.2			1.55	1.55	0.0643
								334 (100)									0.03	0.03	0.0070
																	3.1	3.1	0.3062
														16	0	16	0.8	0.8	0.2861
																	0.2	0.2	0.2161
																	0	0	0.1402
40	Horemans	2016	Lemna	minor	yes	100	53	5681 [fn]	24	7	24	102	Synthetic				115	115	0.1995
																	11	11	0.2984
														123	0	123	1.1	1.1	0.2815
																	0.1	0.1	0.3022
																	0	0	0.2424
								6352 (20)											0.0868
								14091 (40)											0.0681
41	Verma	2015	Lemna	*gibba*	yes	4000	707	21192 (60)	25	21	16	8	Real		22	57	50		0.0592
								28209 (80)											0.0528
								34420 (100)											0.0378
														1.8	1.4		0.1		0.1911
														8.9	7.0		0.5		0.2109
														36	28		2.1		0.1770
														63	49		3.6		0.1082
42	Wang	2015	Spirodela	polyrhiza	no	16000	1602	62	22	7	13	66	Real	89	70		5.2		0.0477
														1.7	1.4		0.1		0.1809
														8.4	7		0.5		0.1987
														34	28		2.0		0.1467
														59	49		3.5		0.0803
														84	70		5.1		0.0292
									20										0.1644
			Landoltia	punctata					25										0.1765
									30		12	16	133						0.1697
									20										0.1710
			Lemna	minor					25										0.1623

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^2]	[g m^{-2}]	(°C)	(days)	(h light)	[µmol m^{-2} s^{-1}]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
43	Zhao	2014			yes		216	139 (100)	30				Synthetic	34		34	15	15	0.1589
			Landoltia	punctata					25	12	16	38							0.0988
												95							0.1533
												190							0.1574
			Lemna	minor								38							0.0667
												95							0.1396
												190							0.1350
			Landoltia	punctata					25	12	16	133		345		345	150	150	0.1816
														35		35	15	15	0.1812
														3.5		3.5	1.5	1.5	0.1751
														0.35		0.35	0.15	0.15	0.1667
														0		0	0	0	0.1483
			Lemna	minor										345		345	150	150	0.1538
														35		35	15	15	0.1548
														3.5		3.5	1.5	1.5	0.1535
														0.35		0.35	0.15	0.15	0.1467
														0		0	0	0	0.1363
			Landoltia	punctata									Real		51			12	0.1405
			Lemna	minor															0.1263
			Landoltia	punctata											32			2.4	0.1883
			Lemna	minor															0.1735
44	Huang	2013	Lemna	minor	yes		1750		23	14	16	42	Synthetic	0	0	0	16	16	0.0320
														7	7.0				0.0460
														14	14				0.0460
														28	28				0.0470
														42	42				0.0480
														56	56				0.0400
45	Kittiwong	2013	Lemna	minor	yes	40	13	11937 [fn]	24	4	16	50	Synthetic	841	289	552	39	39	0.2773
			Landoltia	punctata										210	0	210	31	31	0.3014
														841	289	552	39	39	0.2666
														210	0	210	31	31	0.3014
46	Ge	2012	Lemna	minor	yes	10		5.00	23	18	12		Real		56	0.8		16	0.0350
										27			Synthetic	531	36	495	85	85	0.1640
47	Njambuya	2011	Lemna	minuta	yes	250	64	1.73 [dw]	25	20	16	68	Synthetic	340	0	340	150	150	0.1600
														110	0	110	50	50	0.1520
														30	0	30	10	10	0.0990
								3.46 [dw]						340	0	340	150	150	0.1260
														110	0	110	50	50	0.1130
														30	0	30	10	10	0.1040
				minor				2.36 [dw]						340	0	340	150	150	0.1340
														110	0	110	50	50	0.1500
														30	0	30	10	10	0.1090
								4.72 [dw]						340	0.0	340	150	150	0.1250
														110	0	110	50	50	0.1110

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^{-2}]	[g m^{-2}]	(°C)	(days)	(h light)	[µmol m^{-2} s^{-1}]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
														30	0	30	10	10	0.1080
48	Xu	2011	Spirodela	oligorrhiza	no	200		11 (100)	24	7	12	146	Real	117	87		31		-0.0536
														101	103		26		0.0307
														80	70		20		0.1274
														58	52		16		0.1365
														40	35		12		0.1245
														22	19		5.5		0.1120
						9490	949	211 (80)		3				59	50		16		0.0744
								158 (60)		6									0.0851
								105 (40)		16									0.0573
								53 (20)		30									0.0536
49	Fulton	2010	Lemna	*gibba*	yes	1000		(70)	25	21	24	88	Synthetic	0.14	0	0.14	85	85	0.2290
														1.4		1.4			0.3930
														14		14			0.4020
50	Fulton	2009	Lemna	*gibba*	yes	100	55	1450 [fn]	25	7	24	88	Synthetic	127	0	127	0.1	0.1	0.3744
																	0.3	0.3	0.4026
																	1.5	1.5	0.4432
																	12	12	0.4606
																	16	16	0.4533
																	93	93	0.4262
51	Song	2009	Lemna	minor	yes	300	50	398 (100)	27	15	16	36	Synthetic	31	0	31	93	93	0.4047
														99	0	99	0.01	0.01	0.2614
																	0.2	0.2	0.3005
																	0.5	0.5	0.2910
																	5.0	5.0	0.2683
																	50	50	0.2413
			Spirodela	polyrhiza	yes	300	50	398 (100)	27	15	16	36	Synthetic	99	0	99	0.01	0.01	0.2162
																	0.2	0.2	0.2307
																	0.5	0.5	0.2199
																	5.0	5.0	0.2157
																	50	50	0.1867
									27	3	15	371		92	0	92	0	0	0.0000
																	0.09	0.09	0.1410
																	0.22	0.22	0.2090
																	0.28	0.28	0.2490
																	0.38	0.38	0.2790
																	0.44	0.44	0.2990
																	0.68	0.68	0.3500
																	1.1	1.1	0.3990
																	2.1	2.1	0.4100
																	3.2	3.2	0.4000
																	5.2	5.2	0.4100
																	6.7	6.7	0.4000
																	8.0	8.0	0.4000

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^{-2}]	[$g\ m^{-2}$]	(°C)	(days)	(h light)	[$\mu mol\ m^{-2}\ s^{-1}$]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
																	9.7	9.7	0.4000
																	10	10	0.4110
																	11	11	0.4110
																	14	14	0.4100
																	14	14	0.4100
																	16	16	0.4110
																	20	20	0.4000
																	26	26	0.3920
																	27	27	0.3810
																	42	42	0.3600
																	45	45	0.3410
																	48	48	0.3200
																	54	54	0.3100
														0		0			0.0000
														1.1		1.1			0.2390
														2.0		2.0			0.2900
														2.8		2.8			0.3790
														3.2		3.2			0.3910
														5.3		5.3			0.4000
														7.5		7.5			0.4000
														9.1		9.1			0.4000
														15		15			0.4110
														23		23			0.4000
														29		29			0.4100
52	Lasfar	2007	Lemna	minor	yes		900	750					Synthetic	35		35			0.3990
									27	3	15	371		39	0	39	14	14	0.4090
														47		47			0.3980
														67		67			0.4100
														70		70			0.4200
														120		120			0.4000
														144		144			0.3800
														166		166			0.3700
														191		191			0.3390
														211		211			0.3410
														229		229			0.3400
														257		257			0.3200
														280		280			0.3010
														320		320			0.3010
														343		343			0.3010
									5										0.0110
									8										0.0400
									10										0.0600
									12										0.0890
									15										0.1590

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^{-2}]	[g m^{-2}]	(°C)	(days)	(h light)	[µmol m^{-2} s^{-1}]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
									20	3	15	371		90	0	90	15	15	0.3200
									25										0.4110
									27										0.4100
									28										0.3890
									30										0.3410
									32										0.2600
									35										0.1020
									27	3	2	371		90	0	90	16	16	0.0100
											4								0.0500
											6								0.1400
											8								0.2480
											10								0.3400
											11								0.3980
											12								0.4280
											13								0.4280
											14								0.3980
											15								0.3700
											16								0.3090
											17								0.2200
											18								0.2190
											20								0.2000
53	Demirezen	2007	Lemna	*gibba*	yes		206	0.01 dw	23	10	14	350	Synthetic	15	3.4	12	2.5	2.5	0.4047
								0.03 dw											0.2834
								0.07 dw											0.2528
								0.11 dw											0.0600
								0.20 dw											0.0011
								0.32 dw											0.0011
54	Driever	2005	Lemna	minor	yes	2000	27	5.5 dw	23	7	14	180	Synthetic	15	3.4	12	2.5	2.5	0.2300
								10 dw											0.3000
								90 dw											0.0200
								180 dw											-0.0200
								915 dw											-0.0800
55	Cedergreen	2004	Lemna	minor	yes	10000	2100	100	18	14	16	300	Synthetic	7	0	7	20	20	0.3200
														0.14		0.14			0.1300
												30		7.0	0	7.0			0.2100
														0.14		0.14			0.1200
						5000	450	200				300		0.14	0.07	0.07			0.0400
														1.4	0.7	0.7			0.2000
														7	3.5	3.5			0.3100
56	Al-Nozaily	2002	Lemna	*gibba*	no		500	240	20	5	8	554	Real		25				0.1700
															40				0.1700
															50				0.1090
															60				0.0900
															83				0.0500

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^{-2}]	[g m^{-2}]	(°C)	(days)	(h light)	[µmol m^{-2} s^{-1}]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
															91				0.0200
															100				0.0000
57	Forni	2001	Lemna	minor	no	10000	1064	376 (80)	21	14	14	300	Synthetic	28	0	28	16	16	0.1740
														83	0	83			0.1615
														157	157	0			0.0625
														210	105	105			0.0873
58	Caicedo	2000	Spirodela	polyrrhiza	yes				25	14	16	93	Synthetic	7	3.5	3.5	7.1	7.1	0.2910
														40	20	20			0.2730
														100	50	50			0.2180
														200	100	100			0.1110
59	Classen	2000	Lemna	*gibba*	no	1E6	11582	1727 (100)	20	48	12	390	Real	113			44		0.0384
														45			18		0.0337
									13	108	12	390		69			27		0.0164
														48			19		0.0154
									19	56	12	390		129			36		0.0330
														46			12		0.0325
									28	63	12	390		96			32		0.0380
														81			27		0.0390
60	Hammouda	1995	Lemna	*gibba*	no		160000	0.002 [dw]	21	16	13	554	Real		0.4	0.3	0.3		0.1195
															0.7	8.0	0.6		0.1253
															5.0	20	0.9		0.1366
															10	21	1.3		0.1319
															9	29	1.5		0.1334
61	Vaughan	1994	Lemna	*gibba*	yes	40	43	3.5	20	21	18	98	Synthetic	210	0	210			0.1637
														210	70	140			0.1698
														21	0	21			0.1751
														0	0	0			0.1809
				minor										210	0	210			0.1668
														210	70	140			0.1734
														21	0	21			0.1754
														0	0	0			0.0660
62	Smernoff	1993	Lemna	*gibba*	yes	4524	754	3.0 [dw]	34	4	24	650	Synthetic	126	42	84	93	93	0.5701
									22										0.6555
									26										0.7367
									30										0.7716
63	Huebert	1991	Lemna	trisulca	yes	750	127	237 [fn]	25	14	24	400	Synthetic	7	0	7	3.7	3.7	0.1430
																	1.9	1.9	0.1430
																	0.9	0.9	0.1520
																	0.5	0.5	0.1530
																	0.2	0.2	0.1480
																	0.1	0.1	0.1450
																	0.06	0.06	0.1250
																	0.03	0.03	0.1210
																	0.015	0.015	0.1040

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^{-2}]	[g m^{-2}]	(°C)	(days)	(h light)	[µmol m^{-2} s^{-1}]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
																	0.007	0.007	0.0940
																	0	0	0.0830
														7		7			0.1300
														1.8		1.8			0.1290
														0.4	0	0.4	0.2	0.2	0.1270
														0.1		0.1			0.1000
														0.03		0.03			0.0810
														0		0			0.0600
								3183 [fn]						0	0				0.4943
								2829 [fn]						1.2	1.2				0.5079
64	Wang	1991	Lemna	minor	yes	15	28	2476 [fn]	27	4	24	86	Synthetic	1.9	1.9	0	1.9	1.9	0.5286
														3.2	3.2				0.4813
														5.3	5.3				0.4596
								1768 [fn]						8.9	8.9				0.4024
65	Devlamynck	2021	Lemna	minor	no	967000		0.05 [dw] (100)	17	175	9	365	Real	16	3	14	4.6		0.1720
														46	4	40	37		0.1790
													Synthetic	124	0.7	122	4.2		0.1760
66	Ishizawa	2020	Lemna	minor	no	1000	200	3.5 [dw]	16	7	6	219	Real		3.0	4.8		1.0	0.3057
															6.0	8.6		1.8	0.3987
															1.8	4.5		0.5	0.2953
			Spirodela	polyrhiza											3.9	4.1			0.5342
															75	2.3			0.5846
															30	3.3			0.5197
			Lemna	minor											3.9	4.1			0.1681
															75	2.3			0.2475
67	Toyama	2018			yes	1000	200	15 (50)	28	4	16	80	Real		30	3.3			0.2420
				gibba											3.9	4.1			0.2147
															75	2.3			0.2616
															30	3.3			0.1917
			Landoltia	punctata											3.9	4.1			0.3944
															75	2.3			0.5024
															30	3.3			0.4819
			Lemna	minor										0.5			0.08		0.0720
														5			0.8		0.1620
														10			1.6		0.1540
			Spirodela	polyrhiza					18					0.5			0.08		0.0880
														5			0.8		0.0182
														10			1.6		0.2000
			Wolffia	brasiliensis										0.5			0.08		0.0400
														5			0.8		0.1390
														10			1.6		0.1640
			Lemna	minor										0.5			0.08		0.0910
														5			0.8		0.1890
														10			1.6		0.2220

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^2]	[$g\ m^{-2}$]	(°C)	(days)	(h light)	[$\mu mol\ m^{-2}\ s^{-1}$]		[$mg\ N\ L^{-1}$]	[$mg\ N\ L^{-1}$]	[$mg\ N\ L^{-1}$]	[$mg\ P\ L^{-1}$]	[$mg\ P\ L^{-1}$]	[day^{-1}]
68	McCann	2016	Spirodela	polyrhiza	yes	4	4		24	12	14	140	Synthetic	0.5			0.08		0.0175
														5			0.8		0.1810
														10			1.6		0.2110
			Wolffia	brasiliensis										0.5			0.08		0.0260
														5			0.8		0.1510
														10			1.6		0.1950
			Lemna	minor					30					0.5			0.08		0.0870
														5			0.8		0.2110
														10			1.6		0.2210
			Spirodela	polyrhiza										0.5			0.08		0.0910
														5			0.8		0.1900
														10			1.6		0.2100
			Wolffia	brasiliensis										0.5			0.08		0.0170
														5			0.8		0.1510
														10			1.6		0.1980
69	Soda	2015	Lemna	minor					28	7	16	98	Synthetic	6000	1050	2900	10000		0.2200
														3000	525	1450	5000		0.2610
														600	105	290	1000		0.1590
			Spirodela	polyrhiza										6000	1050	2900	10000		0.2810
														3000	525	1450	5000		0.2420
														600	105	290	1000		0.1660
			Wolffia	*globosa*										6000	1050	2900	10000		0.2980
														3000	525	1450	5000		0.2600
														600	105	290	1000		0.1120
				arrhiza										6000	1050	2900	10000		0.2890
														3000	525	1450	5000		0.2410
														600	105	290	1000		0.1190
70	Yin	2015	Lemna	aequinoctialis	yes	500	156	128 (100)	23	39	12	400	Synthetic	192	18	173	31	31	0.0928
												200							0.0836
												110							0.0866
												80							0.0879
												50							0.0765
												20							0.0694
											16	400							0.0974
												200							0.0863
												110							0.0915
												80							0.0892
												50							0.0852
												20							0.0710
											24	400							0.1330
												200							0.0981
												110							0.0930
												80							0.0919
												50							0.0831

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^2]	[g m^{-2}]	(°C)	(days)	(h light)	[µmol m^{-2} s^{-1}]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
												20							0.0782
71	Verma	2014	Lemna	*gibba*	yes	2000	707	99	27	21	16	8	Real			14	13		0.1527
																11	10		0.1473
																7.2	6.3		0.1390
																3.6	3.2		0.1339
72	Tu	2012	Lemna	minor	no	60000	4000	400	27	14	13	291	Real	0					0.0871
														24					0.0944
														48					0.1037
														72					0.1101
														96					0.1085
														120					0.1013
73	Cheng	2002	Lemna	minor	yes	150	26	0.01 (100)	23	22	16	40	Synthetic	336	336	0	24		0.1719
														252	252	0	18		0.1679
														168	168	0	12		0.1665
														84	84	0	6.0		0.1555
74	Rapparini	2002	Lemna	*gibba*	yes	50			5	38	24	25	Synthetic	350	0	350	155	155	0.0955
									10										0.1073
									15	18									0.2530
									20										0.3113
									25										0.3375
									30										0.3529
									35	4									0.0000
									40	2									0.0000
75	Korner	2001	Lemna	*gibba*	yes	250	64	3125 [fn]	24	12	16	160	Real	18	10		0.9	0.5	0.3000
														48	30		2	1.2	0.2800
														68	50				0.2800
														118	100				0.2590
														168	150				0.3980
														218	199				0.1990
														268	249				0.1790
														318	299				0.1680
														18	10		0.9	0.5	0.2790
														66	48		2	1.15	0.2490
														95	76				0.1600
														114	95				0.1790
														15	7.6		0.7	0.4	0.2290
														41	23		2	1.2	0.1480
76	Monselise	1993	Wolffia	arrhiza	yes		53	189	24	8	24	21	Synthetic	14	0	14	93	93	0.0270
														15	1.4				0.0272
														28	14				0.0280
														154	140				0.0292
			Lemna	*gibba*										14	0				0.0220
														15	1.4				0.0230
														28	14				0.0235

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^{2}]	[g m^{-2}]	(°C)	(days)	(h light)	[µmol m^{-2} s^{-1}]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
														154	140				0.0193
77	Kadir	2020	Lemna	minor	no	2000	491	41	26	10	12	350	Real		2.9	1.0		1.3	0.1130
															2.4	0.70		1.2	0.1170
															2.3	0.66		0.8	0.2290
															1.7	0.48		0.7	0.2590
78	Sembada	2019	Landoltia	punctata	no		1276	12	29	12	16	292	Synthetic	78	0	78	3.1	3.1	0.2235
			Lemna	aequinoctialis															0.2116
			Wolffia	arrhiza															0.1766
79	Vermaat	1998	Lemna	*gibba*	yes	250	64	400	20	18	16	160	Synthetic	14	14	0	7.1	7.1	0.1600
				minor															0.0800
				trisulca															0.0300
			Spirodela	polyrhiza															0.1600
			Wolffia	arrhiza															0.0000
			Lemna	*gibba*						14									0.0000
				minor															0.0000
			Spirodela	polyrhiza															0.0000
			Wolffia	arrhiza															0.0000
			Lemna	*gibba*						12			Real	52			8.7		0.2800
				minor															0.1500
			Spirodela	polyrhiza															0.1600
			Wolffia	arrhiza															0.1700
			Lemna	*gibba*						18			Synthetic	14	14	0	7.1	7.1	0.1700
				minor															0.2000
				trisulca															-0.0200
			Spirodela	polyrhiza															0.1700
			Wolffia	arrhiza															0.0600
			Lemna	*gibba*						14									0.2300
				minor															0.2800
			Spirodela	polyrhiza															0.2200
			Wolffia	arrhiza															0.1300
			Lemna	*gibba*						12									0.2700
				minor															0.3000
			Spirodela	polyrhiza															0.1500
			Wolffia	arrhiza															0.0000
			Spirodela	polyrrhiza				1989 [fn]											0.1680
																			0.3060
																			0.3860
																			0.3280
																			0.3020
																			0.2640
																			0.3040
				intermedia															0.3120
																			0.3580
																			0.3120

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^2]	[g m^{-2}]	(°C)	(days)	(h light)	[µmol m^{-2} s^{-1}]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
80	Ziegler	2014	Landoltia	punctata	yes	300	50		25	7	24	100	Synthetic	192	18	174	40	40	0.3880
																			0.3800
																			0.3650
																			0.3990
			Lemna	aequinoctialis				3979 [fn]											0.4760
																			0.4780
																			0.4730
				disperma															0.2090
																			0.3870
																			0.3500
				gibba															0.4180
																			0.3730
																			0.5030
				minor															0.4260
																			0.3740
																			0.4200
				obscura															0.1530
																			0.4330
																			0.4740
			Wolffia	arrhiza															0.4260
																			0.3440
				columbiana															0.2550
																			0.2490
				globosa															0.3280
																			0.4570
																			0.3860
																			0.3690
			Wolffiela	hyalina				9947 [fn]											0.5190
				lingulata															0.2600
81	Bergmann	2000	Lemna	aequinoctialis	yes		28	(35)	23	11	16	40	Synthetic	376	375	0.2	98	98	0.1956
				gibba															0.1716
				minor															0.1729
			Spirodela	polyrrhiza															0.1987
			Wolffiela	oblonga															0.1902
			Spirodela	punctata															0.2027
			Lemna	*gibba*															0.1782
				minor															0.1867
			Spirodela	punctata															0.2017
			Lemna	obscura															0.2120
				aequinoctialis															0.2339
82	Bergmann	2000	Lemna	*gibba*	yes	36000	2880	(100)	25	12	14	397	Real	176	115	0.43	59	34	0.1428
														131	86	0.32	44	25	0.1555
														86	57	0.21	29	17	0.1459
														66	43	0.16	22	13	0.1460

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^{-2}]	[g m^{-2}]	(°C)	(days)	(h light)	[µmol m^{-2} s^{-1}]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
														52	34	0.13	18	10	0.1429
				minor										98	65	0.24	33	19	0.1509
			Spirodela	punctata															0.1340
																			0.1079
83	Szabo	2003	Lemna	*gibba*	yes	2000	104	1540 fn (75)	25	10	16	180	Synthetic	5	5	0	0.5	0.5	0.3510
														10	10		1	1	0.3630
														5	5				0.3500
														10	10				0.3580
														5	1.1	3.9	0.8	0.8	0.2300
														10	3.4	12	2.5	2.5	0.2640
84	Lemon	2001	Spirodela	polyrrhiza	yes				24	60	12	200	Synthetic	2.3	0.52	1.8	2.4	2.4	0.0058
			Lemna	minor															0.2849
			Wolffia	borealis															0.4137
85	Paolacci	2021	Lemna	minuta	yes				20	7	16	40	Synthetic	134	0	134	93	93	0.1360
																			0.1380
																			0.1360
																			0.1310
																			0.1170
																			0.0940
																			0.1290
																			0.1460
																			0.1800
				minor															0.1780
																			0.1490
																			0.1830
																			0.1070
																			0.1760
																			0.1730
																			0.1190
																			0.1910
																			0.2100
86	Li	2021	Landoltia	punctata	yes		0.05	13 dw	25	15	16	110	Synthetic	70		70	31	31	0.1599
																	0	0	0.1506
87	Walsh	2021	Lemna	minor	yes	100	42	21 (10)	22	7	16	50	Synthetic	134	0	134	93	93	0.1890
								42 (20)											0.1690
								63 (30)											0.1580
								84 (40)											0.0970
								105 (50)											0.1070
								126 (60)											0.0740
								147 (70)											0.0860
								168 (80)											0.0630
88	Paolacci	2021	Lemna	minor	yes	100	36	25 (20)	20	4	16	300	Synthetic	33	8	25	2	2	0.0841
								50 (40)											0.0841
								75 (60)											0.0807

Paper information			Duckweed species		Culture conditions								Culture media						Response
ID	Author	Year	Genus	Species	CCC	V	A	Mat density (% coverage)	T	t	PP	LI	Origin	TN	NH4-N	NO3-N	TP	PO4-P	RGR
						[mL]	[cm^2]	[$g\ m^{-2}$]	(°C)	(days)	(h light)	[$\mu mol\ m^{-2}\ s^{-1}$]		[mg N L^{-1}]	[mg N L^{-1}]	[mg N L^{-1}]	[mg P L^{-1}]	[mg P L^{-1}]	[day^{-1}]
								100 (80)											0.0763
89	Stewart	2021	Lemna	*gibba*	yes	1000	177	1132 [fn]	25	4	24	50	Synthetic	383	37	347	81	81	0.4900
												1000							0.5000
90	Tu	2021	Lemna	japonica	no		120000	413 (100)	10	4	11	452	Real	25	18		2.5		0.1520
									16		13	350		28	18		2.5		0.2338
									20		14	189		18	14		2.4		0.2550
									18		12	224		18	18		2.2		0.2186
91	Tian	2021	Landoltia	punctata	yes	500	54		25	10	16	110	Synthetic	70	0	70	31	31	0.1570
														470	400	70			0.1430
														400	400	0			0.1220

Appendix 2

Summary statistics for the thermal models

The effect of temperature to the relative growth rate of duckweed was adjusted to Hinshelwood's thermal model, described by the following equation:

$$\mathrm{RGR}_{\mathrm{IQ-Score}} = \mathrm{a} * \exp\left(-\frac{\mathrm{E}}{\mathrm{RT}}\right) - \mathrm{b} * \exp\left(-\frac{\mathrm{E_h}}{\mathrm{RT}}\right)$$

Parameter	Estimate	Std. Error	t value	P
Landoltia				
a	6.810e+06	9.053e+07	0.075	0.942
E	2.793e-01	3.270e-01	0.854	0.426
b	3.981e+32	5.513e+34	0.007	0.994
E_h	1.855e+00	3.671e+00	0.505	0.631
Residual standard error: 10.44 on 6 degrees of freedom				
Lemna				
a	6.810e+06	9.053e+07	0.075	0.942
E	2.793e-01	3.270e-01	0.854	0.426
b	3.981e+32	5.513e+34	0.007	0.994
E_h	1.855e+00	3.671e+00	0.505	0.631
Residual standard error: 10.44 on 6 degrees of freedom				
Spirodela				
a	6.810e+06	9.053e+07	0.075	0.942
E	2.793e-01	3.270e-01	0.854	0.426
b	3.981e+32	5.513e+34	0.007	0.994
E_h	1.855e+00	3.671e+00	0.505	0.631
Residual standard error: 10.44 on 6 degrees of freedom				

Appendix 3

Parameters estimates for the 4-Parameters Logistic Model

The 4-parameter logistic model was used to model the change of phosphorus and nitrogen concentration in culture media over time, at varying cultivation temperatures and initial supply of phosphorus and nitrogen. The model followed the equation:

$$Response = d + \frac{(a-d)}{1 + \left(\frac{t}{c}\right)^{b}}$$

Where, d is the maximum value of the response (the concentration of P or N at time 0), a is the minimum expected concentration of nutrients during cultivation (≈ 0), *c* is the time at which the concentration of nutrient in media is half the concentration at the beginning of the experiment, and *b* is a parameter related to the maximal slope of the curve.

- Parameter estimates for P removal at varying temperatures

	20°C		25°C		30°C	
Parameter	**Estimate**	**SE Estimate**	**Estimate**	**SE Estimate**	**Estimate**	**SE Estimate**
a = min	0	-	0	-	0	-
d = max	15.35	-	15.82	-	15.11	-
c = t50	7.070	0.102	4.552	0.049	3.590	0.078
b = slope	-3.836	0.265	-6.505	0.373	-4.858	0.472

- Parameter estimates for N removal at varying temperatures

	20°C		25°C		30°C	
Parameter	**Estimate**	**SE Estimate**	**Estimate**	**SE Estimate**	**Estimate**	**SE Estimate**
a = min	0	-	0	-	0	-
d = max	109.6	-	115	-	109	-
c = t50	12.354	0.773	7.816	0.172	6.500	0.085
b = slope	-2.121	0.209	-2.503	0.173	-3.170	0.166

- Parameter estimates for P removal at varying initial supply of phosphorus

	0.2 mg P L^{-1}		2.0 mg P L^{-1}		4.0 mg P L^{-1}	
Parameter	**Estimate**	**SE Estimate**	**Estimate**	**SE Estimate**	**Estimate**	**SE Estimate**
a = min	0	-	0	-	0	-
d = max	0.185	-	1.998	-	3.750	-
c = t50	0.816	0.281	2.435	0.018	3.569	0.129
b = slope	-1.191	0.309	-3.538	0.081	-3.585	0.388

	6.0 mg P L^{-1}		8.0 mg P L^{-1}		15.0 mg P L^{-1}	
Parameter	**Estimate**	**SE Estimate**	**Estimate**	**SE Estimate**	**Estimate**	**SE Estimate**
a = min	0	-	0	-	0	-
d = max	5.785	-	8.136	-	15.820	-
c = t50	4.127	0.108	4.286	0.115	4.552	0.049
b = slope	-4.098	0.423	-3.909	0.392	-6.505	0.373

- Parameter estimates for N removal at varying initial supply of nitrogen

	0.5 mg N L^{-1}		5.0 mg N L^{-1}		10.0 mg N L^{-1}	
Parameter	**Estimate**	**SE Estimate**	**Estimate**	**SE Estimate**	**Estimate**	**SE Estimate**
a = min	0	-	0	-	0	-
d = max	0.51	-	3.840	-	8.740	-
c = t50	-	-	1.865	0.064	2.204	0.064
b = slope	-	-	-3.313	0.358	-4.708	0.755

	15.0 mg N L^{-1}		20.0 mg N L^{-1}		100.0 mg N L^{-1}	
Parameter	**Estimate**	**SE Estimate**	**Estimate**	**SE Estimate**	**Estimate**	**SE Estimate**
a = min	0	-	0	-	0	-
d = max	14.04	-	19.00	-	115.0	-
c = t50	2.664	0.088	3.710	0.102	7.699	0.141
b = slope	-3.484	0.306	5.347	0.843	2.114	0.150

Appendix 4

Response surface methodology results

During the assessment of how temperature, phosphorus, and nitrogen supply affect growth, the response surfaces created incorporated both the linear and quadratic effects of these variables, as well as the interactions between them. The results include the predicted coefficients for each response variable's surface and an Analysis of Variance (ANOVA) that evaluates the significance of these effects. The details of these findings are presented below.

- Response surface for relative growth rate

Coded Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.32323	0.00832	38.84	0	
T	0.12588	0.00417	30.21	0	1
P	0.02278	0.00447	5.1	0	1.04
N	0.03563	0.00447	7.98	0	1.04
T*T	0.0199	0.00658	3.03	0.005	1.07
P*P	-0.04303	0.00731	-5.88	0	1.11
N*N	-0.0241	0.00731	-3.29	0.002	1.11
T*P	0.00978	0.00619	1.58	0.124	1
T*N	0.01122	0.00619	1.81	0.079	1
P*N	0.0071	0.00651	1.09	0.284	1

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.020384	97.08%	96.26%	95.02%

- Response surface for starch content in biomass

Coded Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	4.662	0.391	11.91	0	
T	1.521	0.196	7.76	0	1
P	-0.433	0.21	-2.06	0.048	1.04
N	0.718	0.21	3.42	0.002	1.04
T*T	-0.428	0.31	-1.38	0.176	1.07
P*P	0.741	0.344	2.15	0.039	1.11
N*N	0.711	0.344	2.07	0.047	1.11
T*P	-0.807	0.291	-2.77	0.009	1
T*N	0.695	0.291	2.39	0.023	1
P*N	-0.022	0.306	-0.07	0.943	1

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.958955	76.42%	69.78%	59.89%

- Response surface for protein content in biomass

Coded Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	23.541	0.901	26.12	0	
T	-1.786	0.451	-3.96	0	1
P	0.901	0.484	1.86	0.072	1.04
N	5.02	0.484	10.37	0	1.04
T*T	-0.225	0.713	-0.32	0.754	1.07
P*P	1.504	0.792	1.9	0.067	1.11
N*N	-4.218	0.792	-5.33	0	1.11
T*P	-1.325	0.67	-1.98	0.057	1
T*N	-3.085	0.67	-4.6	0	1
P*N	2.156	0.705	3.06	0.004	1

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.20767	85.45%	81.36%	74.47%

- Response surface for phosphorus content in biomass

Coded Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	4.989	0.247	20.21	0	
T	-0.48	0.124	-3.88	0	1
P	2.792	0.132	21.08	0	1.04
N	-0.204	0.132	-1.54	0.133	1.04
T*T	0.049	0.195	0.25	0.805	1.07
P*P	-1.139	0.217	-5.25	0	1.11
N*N	0.245	0.217	1.13	0.267	1.11
T*P	-0.115	0.184	-0.63	0.534	1
T*N	-0.995	0.184	-5.42	0	1
P*N	0.794	0.193	4.11	0	1

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.604516	94.23%	92.60%	89.82%

- Response surface for specific phosphorus uptake

Coded Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.2353	0.0268	8.77	0	
T	-0.0875	0.0134	-6.52	0	1
P	0.1797	0.0144	12.48	0	1.04
N	-0.0072	0.0144	-0.5	0.622	1.04
T*T	0.0357	0.0212	1.68	0.102	1.07
P*P	-0.0433	0.0236	-1.84	0.076	1.11
N*N	-0.0319	0.0236	-1.35	0.186	1.11
T*P	-0.0964	0.0199	-4.83	0	1
T*N	-0.009	0.0199	-0.45	0.653	1
P*N	0.0078	0.021	0.37	0.711	1

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.065673	87.90%	84.50%	78.54%

- Response surface for specific nitrogen uptake

Coded Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.9549	0.0387	24.67	0	
T	-0.205	0.0194	-10.58	0	1
P	0.0393	0.0208	1.89	0.068	1.04
N	0.6948	0.0208	33.44	0	1.04
T*T	0.1389	0.0306	4.54	0	1.07
P*P	0.1084	0.034	3.19	0.003	1.11
N*N	-0.5538	0.034	-16.28	0	1.11
T*P	-0.0085	0.0288	-0.3	0.769	1
T*N	-0.1715	0.0288	-5.96	0	1
P*N	0.0226	0.0303	0.74	0.462	1

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.094825	97.86%	97.26%	96.25%

- Response surface for phosphorus removal rate

Coded Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.6005	0.0249	24.14	0	
T	0.198	0.0175	11.29	0	1.61
P	0.2007	0.0187	10.75	0	1.65
N	0.1558	0.0154	10.1	0	4.5
T*T	-0.0742	0.0218	-3.39	0.002	1.07
P*P	-0.3262	0.0243	-13.43	0	1.11
N*N	-0.0396	0.00607	-6.52	0	4.58
T*P	0.1318	0.0206	6.41	0	1
T*N	-0.0161	0.0103	-1.57	0.127	1.61
P*N	0.0954	0.0108	8.82	0	1.61

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.06769	96.61%	95.66%	93.97%

- Response surface for nitrogen removal rate

Coded Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	1.349	0.173	7.79	0	
T	0.307	0.122	2.51	0.017	1.61
P	0.202	0.13	1.55	0.131	1.65
N	1.081	0.107	10.06	0	4.5
T*T	0.448	0.152	2.95	0.006	1.07
P*P	-0.153	0.169	-0.91	0.372	1.11
N*N	-0.1708	0.0423	-4.04	0	4.58
T*P	0.23	0.143	1.6	0.118	1
T*N	0.3417	0.0716	4.77	0	1.61
P*N	0.3664	0.0753	4.87	0	1.61

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.471382	91.74%	89.42%	85.62%

- Response surface for biomass productivity

Coded Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	5.504	0.311	17.68	0	
T	1.321	0.156	8.47	0	1
P	0.679	0.167	4.06	0	1.04
N	0.933	0.167	5.58	0	1.04
T*T	-0.279	0.246	-1.13	0.266	1.07
P*P	-1.023	0.274	-3.74	0.001	1.11
N*N	-0.786	0.274	-2.87	0.007	1.11
T*P	0.814	0.231	3.52	0.001	1
T*N	1.152	0.231	4.98	0	1
P*N	0.249	0.243	1.02	0.314	1

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.762304	84.39%	80.00%	72.27%

Appendix 5

Elemental composition of duckweed biomass

The data presented in the table below corresponds to the elemental composition of the duckweed biomass harvested at the end of experiments varying ambient temperature, and the initial supply of phosphorus and nitrogen.

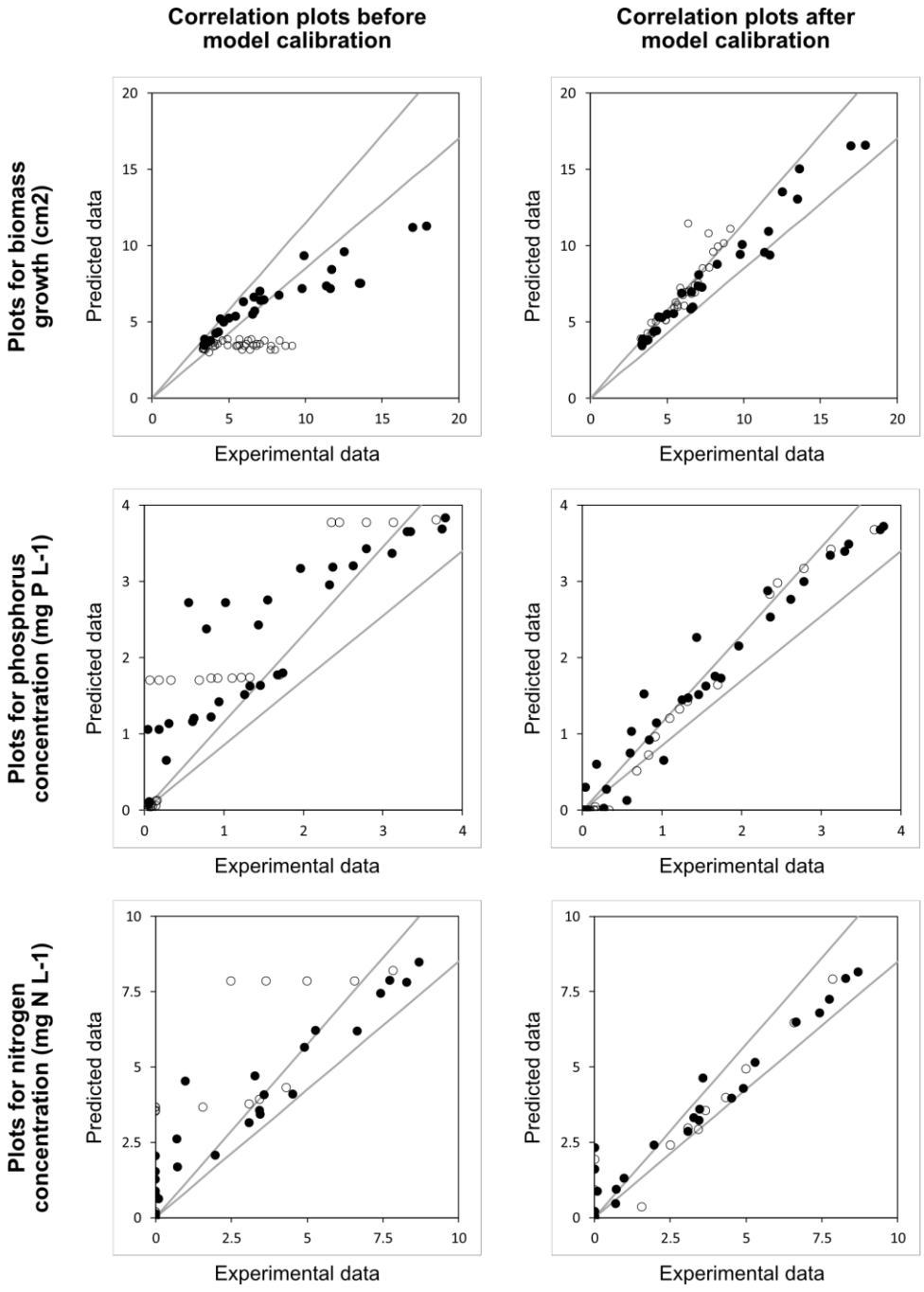
Temperature (°C)	Phosphorus (mg P L^{-1})	Nitrogen (mg N L^{-1})	C (% dw)	H (% dw)	N (% dw)	S (% dw)
20	4.0	5.0	36.6 (0.66)	5.19 (0.15)	3.13 (0.21)	0.26 (0.03)
	2.0	10.0	37.4 (0.23)	5.32 (0.07)	4.26 (0.05)	0.26 (0.03)
	2.0	0.5	37.5 (0.38)	5.50 (0.08)	1.53 (0.02)	0.16 (0.01)
	0.2	5.0	38.1 (0.93)	5.48 (0.16)	3.38 (0.11)	0.19 (0.03)
25	0.2	0.5	36.2 (0.06)	4.89 (0.00)	1.58 (0.02)	0.20 (0.02)
	0.2	10.0	36.3 (0.95)	4.97 (0.30)	3.49 (0.09)	0.25 (0.02)
	2.0	5.0	35.9 (0.46)	4.78 (0.07)	2.31 (0.15)	0.18 (0.14)
	4.0	0.5	35.8 (0.47)	4.88 (0.10)	1.61 (0.04)	0.00 (0.00)
	4.0	10.0	36.0 (1.02)	4.79 (0.28)	3.59 (0.44)	0.00 (0.00)
30	0.2	5.0	36.0 (0.28)	5.04 (0.13	2.55 (0.19)	0.15 (0.15)
	2.0	10.0	35.8 (0.37)	4.99 (0.30)	2.79 (0.52)	0.09 (0.16)
	2.0	0.5	34.3 (0.37)	3.87 (0.20)	1.36 (0.01)	0.00 (0.00)
	4.0	5.0	34.4 (0.48)	4.94 (0.21)	1.63 (0.04)	0.00 (0.00)

Average values for three replicates. Values in brackets correspond to the standard deviation

Appendix 6

Correlation plots between experimental data and data predicted by uncalibrated and calibrated kinetic batch models

Continuous lines mark a 90% CI. Black points are for experiments performed with plenty availability of nutrients, while white dots represent experiments performed in P-free or N-free culture medium.



Appendix 7

Composition of fish feeding and duckweed biomass produced at the pilot system in Indonesia

In the fishery, two different types of feed are utilized: Feeding A (Hi-PRO-VITE 781, CP Prima) and Feeding B (Prima Feed, PF800). These are administered at a ratio of 3 kg of Feeding A to 1 kg of Feeding B per day. Samples of both types of feed were sent for bromatological analysis to characterize their composition. Additionally, samples of duckweed biomass harvested from both the uncompartmentalized pilot system (Duckweed 1) and the pilot system with a baffle (Duckweed 2) were also analysed.

Parameters	Units	Feeding A	Feeding B	Duckweed 1	Duckweed 2
Energy	kcal/100g	344	323	204	203
Fat	g/100g	9.4	3.1	3.2	3.4
Saturates	g/100g	3.2	1.2	1.7	1.8
Mono-unsaturated	g/100g	3.2	0.9	0.6	0.4
Polyunsaturated	g/100g	2.6	0.9	0.8	0.9
Carbohydrate	g/100g	24.2	28.1	4.7	1.3
Total sugars	g/100g	2.2	2.0	< 0.1	< 0.1
Starch	g/100g	22.0	26.1	4.7	1.3
Dietary fibre	g/100g	14.7	9.2	31.1	34.8
Protein (N x 6.25)	g/100g	33.3	41.1	23.6	24.5
Salt	g/100g	1.18	1.35	0.55	0.95
Ash	g/100g	9.5	10.6	26.8	27.1
Moisture	g/100g	8.9	7.9	10.6	9.0
Phosphorus	mg/100g	1377.50	1451.13	606.28	683.08
Potassium	mg/100g	1137.06	961.26	5819.32	5109.71