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Waste Remediation and Value Creation: the Recovery of Nutrients from Dairy Industry Wastewater Using the Aquatic Plant *Lemna minor*

Thesis presented by

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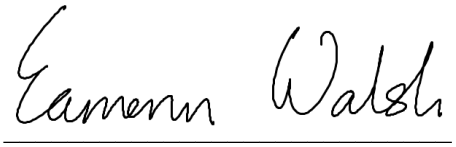
Digital signature of the candidate: 

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Abstract

The production of food is a major source of carbon emissions and environmental damage worldwide. The sector is also coming under significant stress from decreasing resource availability, climate change and soil erosion, among other things. In order to increase the sustainability of the sector action is required to maintain resources within the system. The circular economy (CE) is one economic model which can improve the recycling of resources within a system, particularly through the treatment of waste as a resource.

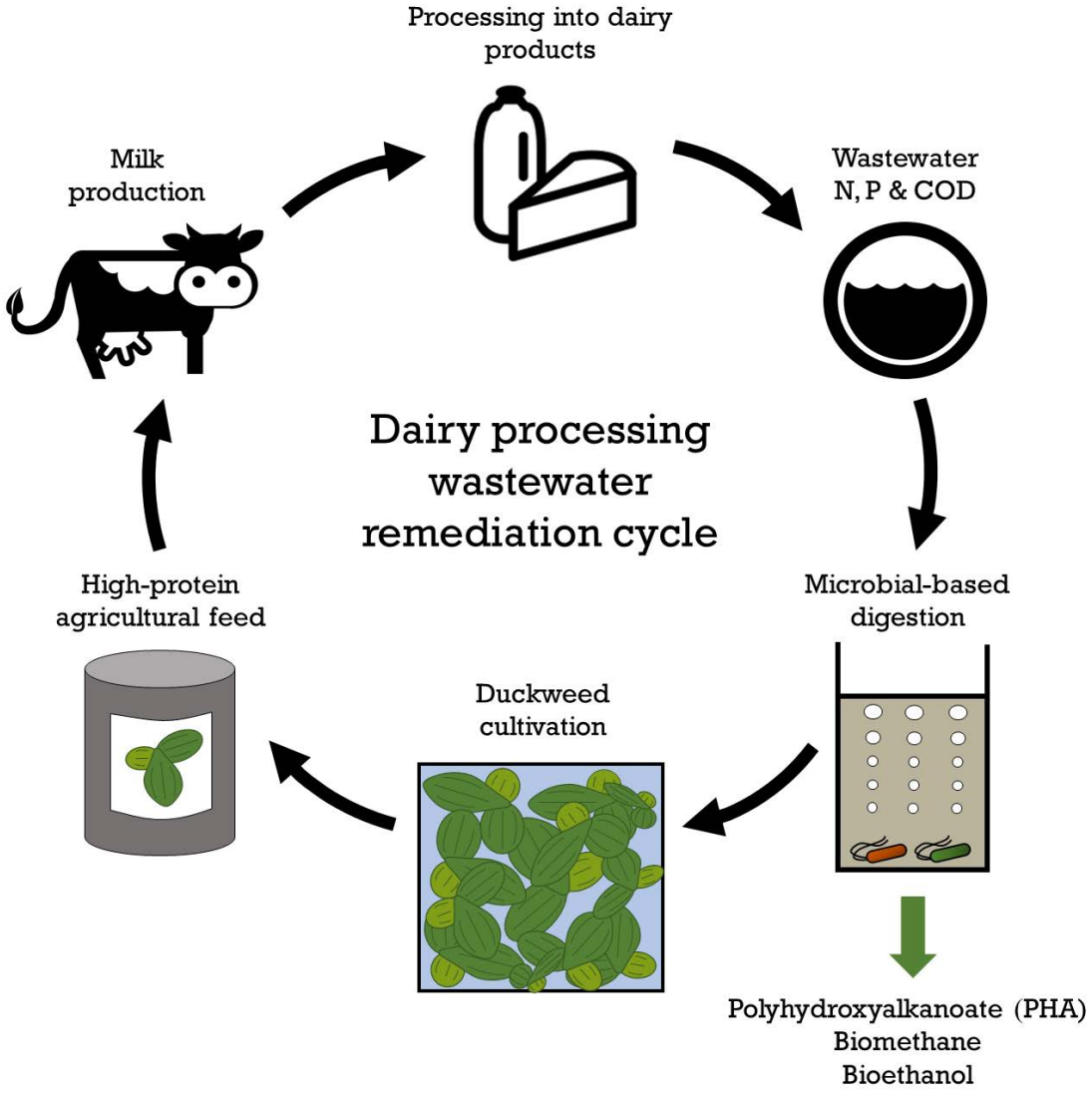
The dairy industry is one significant component of the worldwide food production industry. As part of dairy processing, large amounts of wastewater are generated, in some cases up to ten times the volume of the processed milk. This large volume of wastewater must be treated for appropriate disposal. Usually this involves removal and disposal of the polluting components, such as organic compounds, N and P. However, there is a wealth of compounds contained within dairy processing wastewater which could be reused. This reuse is called valorisation, which can be part of a CE approach to waste treatment. There are different ways of valorising waste but a particularly interesting way in the context of dairy processing wastewater is phytoremediation, which was assessed in this thesis using aquatic Lemnaceae, i.e. duckweed. Accordingly, environmentally polluting compounds, such as N and P, are removed from the wastewater by plant action and in duckweed a valuable biomass is created which can be used as a feed or biofuel.

In general, it was found that dairy processing wastewater is suitable for Lemnaceae cultivation. However, this research highlights how some adjustments to the wastewater are necessary for Lemnaceae survival, while other adjustments made to the growing conditions can optimise cultivation and remediation. Overall, high removal of N and P compounds was achieved and duckweed biomass with a high protein content was produced. Furthermore, the combination of

duckweed cultivation with microbial digestion led to superior remediation of the dairy processing wastewater, particularly for organic components.

These results will facilitate the integration of duckweed into the large-scale industrial remediation of dairy processing wastewater, which would improve the sustainability of the food production sector while also producing a versatile biomass that can be utilised in multiple ways.

Graphical Abstract



Outline

This is a Publication-Based Philosophiae Doctor (PhD) Thesis. I am the first author and the key contributor to this work. Individual author contributions are specified for each chapter. There are seven chapters in total. Chapter 1 is a general introduction to the topic of sustainability, wastewater remediation and Lemnaceae. Chapters 2-6 are the research chapters. In Chapter 2 the suitability of synthetic dairy wastewater for duckweed cultivation is assessed, with a focus on the Ca:Mg ratio. This chapter has been published as a research paper in *The International Journal of Phytoremediation*. Chapter 3 presents the effects of light intensity on Lemnaceae growth and remediation of synthetic dairy wastewater. This chapter has been published as a research paper in *Environmental Science and Pollution Research*. Chapter 4 presents the impacts of density condition on Lemnaceae growth and remediation of synthetic wastewater. This chapter is published as a research paper in *MDPI Plants*. Chapter 5 concerns the comparison of 13 species and clones of Lemnaceae in terms of growth and remediation ability. This chapter is being prepared for submission. Chapter 6 presents the integration of microbial remediation systems with duckweed remediation for remediation of wastewater from a dairy processing facility. This chapter is being prepared for submission. Chapter 7 is a concluding chapter which brings together all of the research and presents conclusions with a future outlook. Published research chapters have been changed to a consistent format for the thesis.

Chapter 1

Introduction: duckweed-based remediation of dairy processing wastewater along the principles of the circular economy

1.1. Sustainability, the circular economy and waste

1.1.1 Sustainability

The World Commission on Environment and Development (WCED) provided a general, and commonly cited, definition of sustainability and sustainable development in a 1987 report; namely, sustainability is meeting the needs of the present populace without compromising the needs of future generations (Brundtland 1987). Fitting with such a broad definition, sustainability covers a broad variety of ideas and topics that have context-dependent applications. For example, in an ecological context the use of sustainability can be applied to the conservation of the biosphere (Chapin et al. 1996), which entails the balancing of the exploitation of the environment with regeneration to maintain the provision of 'eco-system services' (Chapin et al. 2010; Rufí-Salís et al. 2020). In a business context sustainability can indicate the survivability and profitability of a business in the long-term (Bryson and Lombardi 2009; Lo 2010). Sustainability can also be applied to socio-economic conditions, which goes to the core of the sustainability of the ways in which people live (Biermann et al. 2017), for example, the sustainability of rural living (Copus and Crabtree 1996) or the sustainability of urban water supplies (Krueger et al. 2020). These abovementioned contexts are not independent from one another, in fact, it has been argued that the study of sustainability should integrate ecological, societal and economic concerns (Chapin et al. 2010). It is useful in this context to think of a diagram of three circles in which the smallest, the economy, is nested inside a middle circle, society, which is itself nested inside the largest circle, the environment (Daly 1991). An exclusive focus on economic sustainability misses how the economy is tightly integrated with the ecosystem (Chapin et al. 2010). A prominent example would be the agricultural sector, in which goods (food, timber, fabrics) are derived from the ecosystem; the long-term sustainability of agriculture is dependent on the sustainability of the ecosystem which provides the raw material for the produced goods (DeClerck et al. 2016; Weiner 2017).

This integrated approach to sustainability is gaining greater appreciation in different sectors, for example, in the business community (Albino et al. 2009; Bansal and DesJardine 2014) and in the farming community (Zeweld et al. 2017). In addition, research is being produced that focuses on improving sustainability in a broad range of sectors of the economy (Leal Filho et al. 2018; Nidheesh and Kumar 2019). Furthermore, sustainability is being embraced and discussed by nearly all countries and regions worldwide, with prominent examples in China (Su et al. 2013), Europe (De Besi and McCormick 2015) and Brazil (Martinelli et al. 2010). In the past decade the European Union (EU) has published several strategies for sustainable industrial growth (European Commission 2011, 2012; De Besi and McCormick 2015). Within the 'Europe 2020' strategy there was a focus on creating sustainable economic growth through resource efficiency (European Commission 2011). Furthermore, the European Commissions' 'Innovating for Sustainable Growth' report provides a basic framework for better resource efficiency and a roadmap towards a low-carbon economy (European Commission 2012). In the long-term the EU envisions that growth in the economy or in particular industries will be achieved sustainably (McCormick and Kautto 2013).

1.1.2 The circular economy (CE)

The circular economy (CE) is an economic model that is nested within concept of sustainability (Andersen 2007), and builds on ideas from the field of environmental economics (Ghisellini et al. 2016). Researchers working in this field of environmental economics started to place an emphasis on ecology and the environment in economic theories and models (Pearce and Turner 1989; Erkman 1997). As understood today, CE is generally accepted to denote closed-loop production patterns, long-term value retention, reduction of the use of raw resources and reduction in waste production, among other things (Ghisellini et al. 2016; Morseletto 2020). These practices can have a positive impact on the sustainability of the ecosystem as well as the economy (Rufí-Salís et al. 2020). However, that is not the whole picture as CE is interpreted

in a variety of ways, sometimes wrongly, by different sectors and groups as shown in research by Kirchherr, Reike, and Hekkert (2017). They showed that a common error is equating CE with just recycling and waste management. They also noted that businesses primarily saw CE as a vehicle for increasing economic prosperity, while academics tended to include environmental considerations (and less commonly, social considerations) (Kirchherr et al. 2017). Some researchers also argue that research on CE is vague and contains 'semi-scientific' ideas (Korhonen et al. 2018a). While other researchers argue that current CE research is too focused on practical economic elements of the model to the detriment of a more comprehensive change in the entire socio-economic system (Ghisellini et al. 2016). Disagreements over the definition of the term stem, on the one hand, from the fact that it is a new concept still being intensely studied (Kirchherr et al. 2017; Korhonen et al. 2018b), while on the other hand, disagreements are due to differences in worldview. As such, a business will focus on economic elements, while an environmental researcher will naturally include ecological considerations.

There are very pressing economic and environmental concerns that are fuelling an interest in closed-loop approaches, from the near-depleted supply of phosphorous (derived from non-renewable phosphate rock) (Cordell et al. 2009; Elser and Bennett 2011), to the finite supply of fossil fuels (Shafiee and Topal 2009), and to the unsustainability of food production (Jurgilevich et al. 2016). Many industrial economies are creating development strategies that incorporate circular economy ideas (Yong 2007; Su et al. 2013), such as China and their 'Circular Economy Promotion Law of the People's Republic of China' (Lieder and Rashid 2016). The circular economy has also been promoted throughout Europe through recent initiatives by the EU, such as the 'Circular Economy Action Plan' in 2014 and a subsequent implementation report in 2017 (European Commission 2015, 2017).

1.1.3. Waste, valorisation and legislation

Waste comprises a broad spectrum of outputs ranging from small-scale household waste to large-scale industrial waste (Karnchanawong and Suriyanon 2011; Pirani and Arafat 2014; Ahmad et al. 2019). In many countries there exists a legal definition of waste which usually covers materials that are intended for disposal (Bontoux and Leone 1997). For example, in the EU waste is "any substance or object which the holder discards, intends to discard, or is required to discard" (European Commission 2008). This legal definition sits inside the broader framework of waste regulations and policies (van Ewijk and Stegemann 2020). A definition of waste is important for laws and legislation, however, the definition is broad to reflect the myriad forms that waste can take (Cheyne and Purdue 1995).

Most industrial processes create some form of waste product (in gas, liquid or solid form), which will have a distinct composition that depends on the process in question. As a result, it is not possible to design a general formula for the composition of waste. However, waste can be broken up into three broad categories based on handling: waste that is recycled or reused (Ragaert et al. 2017), waste that is disposed (Powell et al. 2016), and waste that is currently disposed but could be reused (Pappu et al. 2007). This last category of waste contains potential 'value' that is currently uncaptured i.e. waste that can be valorised (Yang et al. 2017; Zacho et al. 2018). One example of valorisation is the faecal waste of fish being used as a fertiliser to promote plant growth (Blidariu and Grozea 2011). In this example, nutrients, such as nitrogen, are not lost from the system but move between components within it. Indeed, the use of animal faeces as a fertiliser has a long history (Motavalli and Miles 2002). Other ways of reusing animal faeces include its use as a fuel or building material (Pappu et al. 2007). Another example of valorisation is the cultivation of microalgae on wastewater whereby the algal biomass can be used for a range of outputs including bioethanol, animal feed or natural dyes (Shahid et al. 2020). Further examples include the generation of energy-rich biogas from industrial waste

(Dalpaz et al. 2020) or the recovery of copper from electronic waste (Sinha et al. 2018). It is clear valorisation can include diverse waste types as well as diverse outputs. Another example, which is the subject of this thesis, of a wastewater from which there is much value to be captured is dairy processing wastewater (Ahmad et al. 2019).

While much research to develop valorisation technologies is being conducted (Banerjee et al. 2018; Garcia-Garcia et al. 2019), the take-up of valorisation in the wider economy is lacking in many countries (see Marino & Pariso (2020) for an EU comparison). Legislation can create an environment in which waste must be treated and discharged in a legally-defined manner. Many countries legislate for waste disposal to minimise environmental damage, for example, the EU's IPPC directive on industrial emissions in 2008 (The European Parliament 2008). Legislation dealing with valorisation, however, is less common, although there has been progress in that direction, as mentioned in the previous section, with the publication of action plans and legislation for the promotion of CE (European Commission 2015; Lieder and Rashid 2016). Some countries have legislated for valorisation in specific sectors, such as Japan's e-waste legislation (Patil and Ramakrishna 2020). The creation of legislation, however, does not necessarily remove many of the barriers to proper valorisation of waste, such as high start-up costs, issues of quality and a lack of awareness about CE (Kirchherr et al. 2018; Jaeger and Upadhyay 2020). There is also the fact that waste disposal can be very cheap and easy to carry out, as seen for plastic waste in which it is estimated only 9% of plastic made from 1950 to 2015 was recycled into something else (Geyer et al. 2017).

1.2. The dairy industry

1.2.1 The dairy industry and waste production

The production and trading of dairy products, i.e. the dairy industry, is a significant component of the global economy, which is important for food, employment and trade (OECD-FAO 2020).

Raw milk, the vast majority at 80% being cow's milk (OECD-FAO 2020), is processed into a range of foodstuffs like dried milk powder, cheese and yoghurt (Gösta 2015). Milk production was estimated to be 852 million tonnes (Mt) per year in 2019 with predictions that it will increase to 997 Mt by 2029. India is the largest milk producer in the world at 192 Mt per year (OECD-FAO 2020). In the EU region a recent increase in milk production was due, in part, to the removal of milk quotas in 2015 (Madau et al. 2017), which had been in force as part of the Common Agricultural Policy since 1984 (Keane and O'Connor 2016). In the case of some countries and regions, such as the EU and New Zealand (Hoerl and Hess 2017), a large amount of the produced milk is exported to other countries, with China being one of the biggest importers (Bai et al. 2018).

An increase in milk production, as seen in the last number of years, is a good thing economically for many countries but it also creates more environmentally damaging waste to treat and dispose of (Finnegan et al. 2017; Chen et al. 2018). Indeed, the dairy industry produces a substantial amount of waste from its production processes. Over the course of a year a dairy processing plant can process between 200-550 million litres of milk (Baskaran et al. 2003). Estimates of the amount of wastewater produced per litre of milk processed vary, which is perhaps due to dairy plants employing different processes for production, cleaning and wastewater treatment (Wang and Serventi 2019). Nevertheless, it is estimated that with every litre of milk processed between 0.2-11 litres of wastewater can be generated (Baskaran et al. 2003; Gösta 2015; Wang and Serventi 2019). The creation of large amounts of wastewater poses a significant problem for the industry. The disposal of wastewater is costly because of the facilities, chemicals and energy that are required to bring it to the acceptable level for disposal into surface waters (Ahmad et al. 2019).

Wastewater from dairy production facilities, i.e. dairy processing wastewater, is a complex mixture of organic and non-organic components. There is usually a high concentration of

organic compounds, primarily fatty acids and sugars (Carvalho et al. 2013), which are mostly measured as chemical oxygen demand (COD): 2000-70150 mg L^{-1} (Ghaly and Singh 1989; Malaspina et al. 1996; Ince 1998; Saddoud et al. 2007). Cheese whey wastewater displays particularly high COD levels as, due to its production process, it retains a lot of sugars and protein e.g. lactose (Malaspina et al. 1996; Carvalho et al. 2013). Additionally, inorganic micronutrients such as ammonia, nitrate and phosphate are usually abundantly present: 4.6-19.3 mM NH_3-N (64-270 mg L^{-1}), 0.64-2.1 mM NO_3-N (9-30 mg L^{-1}), 0.65-11.5 mM PO_4-P (20-356 mg L^{-1}) (Ghaly and Singh 1989; Malaspina et al. 1996; Ince 1998). Furthermore, dairy processing wastewater contains many metals and ions e.g. Ca, Mg, K, Fe, Na, Cl. Different types of salts are also often heavily present in dairy processing wastewater: sodium chloride (NaCl), potassium chloride (KCl) and calcium chloride ($CaCl_2$) (Demirel and Yenigun 2004; Carvalho et al. 2013). The combination of salts can lead to high concentrations of Na and Cl: 10.9-42.9 mM Na (250-986 mg L^{-1}) and 14.8-38.1 mM Cl (525-1349 mg L^{-1}) (Demirel and Yenigun 2004; Goyal and Gandhi 2009). Further to this, there are proteins, vitamins and detergents present (Carvalho et al. 2013; Santos et al. 2017; Menchik et al. 2019; Foroutan et al. 2019). The composition of the wastewater is quite changeable, varying significantly between production facilities (Slavov 2017), but also from day to day and season to season within the same facility , due to differences in the products being made (Baskaran et al. 2003; Ryan and Walsh 2016).

1.2.2 Treatment of dairy processing wastewater

Dairy processing plants usually have on-site facilities to treat and dispose of the generated wastewater (Chen et al. 2018; Ahmad et al. 2019). This is in order to reduce the pollutants in the wastewater to below the allowed maximal concentration for disposal to surface waters, as determined by national legislation (Li et al. 2012; Schellenberg et al. 2020). In the EU, IPPC regulations set out for each industry the emission limits of various compounds from industrial

sources (The European Parliament 2008). Dairy processing, for example, is included in the food production industry regulations (European Commission 2019). In the context of dairy processing wastewater, environmentally-damaging compounds present in high concentrations such as organic compounds (COD), ammonia, nitrate and phosphate are governed by these regulations and are, therefore, closely monitored (European Commission 2019).

In dairy processing plants, wastewater treatment facilities generally handle a combination of wastewaters from the processing plant, which besides wastewater from dairy processing, can often include water used to clean machinery and water coming in from storm drains (Baskaran et al. 2003; Slavov 2017). Dairy processing wastewater treatment facilities typically employ a number of sequential techniques to reduce the concentrations of environmentally damaging pollutants (Ahmad et al. 2019; Wang and Serventi 2019). These techniques are broadly broken into physico-chemical and biological techniques.

Physico-chemical techniques are often employed first (but not exclusively) and use physical or chemical processes to remove pollutants from wastewater. These physico-chemical techniques generally remove colloids (suspended solids) of fats, oils and grease from wastewater i.e. much of the suspended organic matter, but also N and P compounds. These techniques include the use of settling tanks (Ryan and Walsh 2016), dissolved air flotation (Ryder et al. 2018) and flocculation through electrolytic treatment or the addition of chemicals (Tchamango et al. 2010; Dela Justina et al. 2018). Phosphorous in particular usually requires further specific chemical treatment; a common treatment is the addition of metal salts, such as aluminium chloride, which precipitate phosphorous from the wastewater (Bunce et al. 2018). The resulting solids are removed by filtering or settling. This precipitate sludge, however, is another waste product that needs to be disposed of or used. It can be used as a fertiliser in land spreading to debateable positive effect (López-Mosquera et al. 2000).

Subsequently, biological treatments are commonly employed to further 'clean-up' wastewater (Ahmad et al. 2019). These biological techniques include those that rely on aerobic processes operating through, for example, activated sludge (Emerald et al. 2012) or sequential batch reactor (SBR) (Kushwaha et al. 2013), and those that rely on anaerobic processes, operating through, for example. anaerobic filters (Ince et al. 2000) or upflow anaerobic sludge blanket (UASB) (Latif et al. 2011). These techniques make use of microbial organisms to remove high amounts of dissolved organic and inorganic compounds from wastewater (Emerald et al. 2012). The microbial organisms that are present in these systems include *Bacteria*, *Archaea* and protozoa (Narihiro and Sekiguchi 2007; Priya et al. 2007). This community of microorganisms uses organic compounds, such as polysaccharides, protein and lipids, as energy and nutrient sources (Amani et al. 2010). In anaerobic systems, these compounds are metabolised through a series of steps, the first of which is the hydrolysation of large molecules into smaller soluble polymers, such as the breakdown of large polysaccharides into sugars or protein into amino acids (Gujer and Zehnder 1983; Noike et al. 1985). These compounds are converted into a number of intermediary organic acids (acidogenesis), which in turn are converted into acetic acid (acetogenesis) (Gujer and Zehnder 1983). The hydrolysis, acidogenesis and acetogenesis steps are carried out by different bacterial species (Lim et al. 2013). Then, the process culminates in the production of carbon dioxide and methane from acetic acid and hydrogen, a reaction catalysed by methanogenic archaea (Gujer and Zehnder 1983; Lee et al. 2009). At the end of the process, environmentally damaging organic compounds have been removed from the wastewater through metabolisation by microorganisms and converted into gas. In aerobic systems a similar process takes place but without the production of methane (Seviour and Nielsen 2010).

Highly common inorganic nitrogen compounds, such as ammonia and nitrate are removed through different processes during biological treatments. In aerobic systems, nitrification-

denitrification is the most prevalent process (Obaja et al. 2003). In this cycle ammonia is biologically oxidised to nitrate, then this nitrate is biologically reduced to nitrogen gas (Farazaki and Gikas 2019). As with the process of breaking down organic compounds a distinct group of microorganisms performs each reaction (Geets et al. 2007). In anaerobic systems a common means of nitrogen removal is the CANON process in which anammox bacteria convert ammonia to dinitrogen gas in anoxic conditions (Third et al. 2001), with a similar end result to nitrification-denitrification. Phosphorous is taken up in biological treatments through the normal metabolic activities of microorganisms (Yeoman et al. 1988).

A general overview of a typical dairy wastewater treatment process is given in Ryan and Walsh (2016). Wastewater is first screened for large debris items, after which it goes into a balancing tank to create a homogeneous mixture. Dissolved air floatation and/or settling is applied to the mixture. Then, wastewater is treated aerobically, mostly with SBR and bio-towers, and anaerobically, with an anaerobic digester, in sequence. Subsequent treatment mostly involves the removal of phosphorous through the addition of chemicals like aluminium chloride. The wastewater then goes through a settling stage again to remove the precipitated sludge. Some wastewater plants then also employ constructed wetlands. At the end of this whole process wastewater can be disposed into the environment by funnelling it into bodies of surface water, commonly a river.

1.2.3 Valorisation of dairy processing wastewater

Most of the above-mentioned techniques treat wastewater primarily to achieve low enough pollutant levels to enable disposal i.e. as an end of life product, with extracting value a secondary concern if present at all. For dairy processing wastewater, currently employed exceptions to this are the extraction and use of biogas for energy creation (Latif et al. 2011) and sludge for land spreading (Ashekuzzaman et al. 2019). There are, however, a host of other techniques for extracting value from dairy processing wastewater, which are currently less used

in commercial settings but produce valuable outputs. One promising example of dairy wastewater valorisation is the production of organic acids (such as succinic, lactic and citric acid), that have useful industrial applications as preservatives, acidifiers and flavour enhancers (Ahmad et al. 2019). Organic acids can be produced through the fermentation by microorganisms of sugars (glucose, fructose, lactose) present in dairy wastewater (Wan et al. 2008). Another interesting example is the production of bioplastics derived from polyhydroxyalkanoates (PHAs), which are produced by wastewater-grown bacteria (Bosco and Chiampo 2010; Dinesh et al. 2020). Bacteria produce PHAs through the fermentation of organic compounds such as sugars and lipids (Lu et al. 2009). Overall, a desired reduction in COD in the wastewater is achieved while valuable polymers, PHAs, are also produced.

Valorisation of dairy wastewater can also be achieved through its use as a cultivation medium for plants and algae (Hemalatha et al. 2019; Akansha et al. 2020). Feedstocks to extract protein, fine-chemicals and biofuel, among other things, can be derived from these wastewater-cultivated plants and algae (Ahmad et al. 2019; Hemalatha et al. 2019). The main topic of this thesis is one particular method of dairy wastewater valorisation. Namely, the cultivation of Lemnaceae, commonly called duckweed, on dairy processing wastewater. Lemnaceae can extract value from the wastewater through its uptake of nutrients, primarily nitrogen and phosphorous, and the production of protein-rich biomass for further use.

1.3. Phytoremediation of dairy processing wastewater

1.3.1 Why Lemnaceae for wastewater remediation?

The use of wastewater-grown Lemnaceae as a food source for animals in small-scale farms is an established practice in many parts of the world, particularly in Asia (Leng 1999). An example of this practice is the use of swine waste to raise Lemnaceae for fowl feeding (Leng 1999). More recently, there has been a significant increase in interest in this practice of

Lemnaceae-grown wastewater from researchers, companies and governments throughout the world (Appenroth et al. 2015; Dinh et al. 2020; Hu et al. 2020). From a governmental and industrial perspective, an overarching reason for this interest is the encouragement and creation of sustainable closed-loop systems within the economy (Morseletto 2020), of which wastewater reuse is a central part (Rufí-Salís et al. 2020). As part of a CE approach, Lemnaceae cultivated on wastewater can remediate the wastewater and then be used as a sustainable feedstock for animals (Anderson et al. 2011), a biofuel (Ge et al. 2012) or as a fertiliser (Ahmad et al. 1990). The reason for the interest in using Lemnaceae for wastewater remediation is its rapid growth rate (Bergmann et al. 2000; Ziegler et al. 2015), its efficient removal of nitrogen and phosphorous (Zhao et al. 2014b) (which is linked to its growth rate, see Cheng et al. (2002b)) and its tolerance of sub-standard or poor growing media (Landolt 1986) such as wastewater. Overall, the Lemnaceae-based remediation of wastewater can reduce the environmental impacts of waste, extract useful material and promote sustainable practice.

1.3.2 Lemnaceae as feed

Soybean is a widely and commonly used feed and feed supplement in animal husbandry because of its good nutritional content in comparison with other feeds (Hymowitz et al. 1972; Culley Jr and Epps 1973; Cheng and Stomp 2009). However, the import of soybean, for example into the EU, is not considered a sustainable practice (Bertheau and Davison 2011; de Visser et al. 2014). It contributes to ‘food miles’ and habitat destruction while also reducing food security (Karlsson et al. 2021). Furthermore, there is concern over contamination of conventional soybean with GM soybean leading to problems of labelling and traceability (Rostoks et al. 2019). The use of locally produced ‘novel’ high protein ingredients as feed, such as Lemnaceae biomass, is an alternative to soybean that can be more sustainable (Tallentire et al. 2018). In addition, the use of wastewater-cultivated Lemnaceae biomass adds a CE aspect to the feed supply, further improving sustainability (Cheng and Stomp 2009).

The focus of thesis is on the use of Lemnaceae biomass as a source of high-quality protein, which can be used as a feed supplement for agricultural animals. For use as a feed supplement, to replace soybean and others, two important aspects of plant biomass are energy content and protein quantity and quality (Beski et al. 2015). Protein quantity is simply the amount of protein per biomass; Lemnaceae plants can contain protein on a comparable level with soybean (up to 45% and 33–45%, respectively) (Hymowitz et al. 1972; Landolt and Kandeler 1987). Protein quality is the amino acid make-up of the protein, with the amount of essential amino acids (EAA) being particularly important (Kaplan et al. 2019). In this aspect Lemnaceae can provide EAAs on par with other feeds (Cheng and Stomp 2009).

There have been numerous studies published that show Lemnaceae being used as a feed source for a range of animals: cattle (Huque et al. 1996), goats (Reid Jr 2004), fish (Hassan and Edwards 1992; Islam et al. 2004), poultry (Haustein et al. 1994; Anderson et al. 2011). Generally, in these studies Lemnaceae was not used as a sole feed source but rather as a feed supplement. Nevertheless, the cited studies in general did not show adverse effects of using Lemnaceae as a feed supplement, on the contrary, Lemnaceae can be used as part of a healthy diet for a variety of animals. There are less studies that show Lemnaceae being used as a feed supplement for ruminants and this needs to be explored further to determine whether it is a suitable feed supplement for these types of animals (Van der Spiegel et al. 2013).

Lemnaceae have been grown not only for animal but also for human consumption, mostly by small farm holders in parts of Asia, for generations (Bhanthumnavin and McGarry 1971). Recently, there has been a renewed interest in using Lemnaceae for human consumption (Appenroth et al. 2017). Some researchers have even determined the perception among the general populace (within Europe) of consuming Lemnaceae (de Beukelaar et al. 2019). This avenue is likely to develop further, however, in the EU there is a barrier to selling Lemnaceae for human consumption. Namely, Lemnaceae is considered in the EU a ‘novel’ food, i.e. a food

that was not consumed to a significant degree before 1997. Accordingly, it has to be approved and authorised before it can be brought to market (Jones 2012; Van der Spiegel et al. 2013). A company 'Rubisco Foods' is currently following this application process for a Lemnaceae dried powder that it wants to bring to market (see https://rubiscofoods.com/). In other jurisdictions a processed duckweed powder called 'Mankai' is available (Yaskolka Meir et al. 2019). However, it must be said that for human consumption wastewater generally is not favoured as a medium for Lemnaceae cultivation.

1.4. Introduction to Lemnaceae

1.4.1 Taxonomy and morphology

It is important to delve a bit deeper into Lemnaceae in order to understand how these species can be used for remediation; the environmental conditions they prefer, their composition, their removal of nutrients from media etc.

Lemnaceae, i.e. duckweeds, are a family of 36 species of free-floating aquatic monocotyledonous angiosperms (Bog et al. 2020). The family of Lemnaceae consists of five genera: *Lemna*, *Spirodela*, *Landoltia*, *Wolffia* and *Wolfiella* (Les et al. 2002), with *Landoltia* being the most recently added genus (Les and Crawford 1999). The Lemnaceae species of interest for this project are *Lemna minor*, and to a lesser extent, *Lemna minuta*. *L. minor* is the most wide-spread native species of Lemnaceae that occurs in Ireland (see National Biodiversity Data Centre for records of Lemnaceae species in Ireland, https://www.biodiversityireland.ie). *L. minuta* is considered an invasive species in Europe and was introduced into Ireland from the early 1990's onwards in multiple waves (Ceschin et al. 2018).

Lemnaceae are considered a morphologically reduced family of higher plants; their morphological characteristics are more basic than of most other aquatic and land-based higher plants (Les et al. 1997). Lemnaceae consist primarily of a stem-leaf structure, called a frond,

sometimes attached to a single root or multiple hair-like roots (Landolt 1986). For many species multiple fronds group together to form a colony. The frond is a basic structure comprising characteristics of both stem and leaf, for example, being engaged in both nutrient uptake and photosynthesis (Landolt 1986). For *Lemna*, *Spirodela* and *Landoltia* species the frond is disc shaped and of variable size (two to several mm in length) and thickness (Landolt 1986). These three genera also have a root or roots that drop down into the water from the fronds. The fronds of *Wolffia* and *Wolfiella* species are smaller (often just two mm across or less), tend more towards a rectangular shape and have no root-like structures (Landolt 1986). For *L. minor* fronds of 3-5 mm length form colonies that can be made up of 1–15 fronds (although 3–5 fronds appears to be the most common size), to which a single root is attached (Landolt 1986; Cheng and Stomp 2009). Roots measure from less than 1 to 3 cm, depending on the strain, as well as environmental conditions (Porath et al. 1980).

Lemnaceae generally reproduce vegetatively with new fronds budding from 'parent' fronds and growing from within the same colony, only on rare occasions do they flower and reproduce sexually (Hillman 1961). Notwithstanding predominantly vegetative growth, the Lemnaceae family displays morphological and genetic diversity (Xu et al. 2015; Paolacci et al. 2021). A measure of this diversity can be seen in a study by Xu *et al.*, 2015, in which the species and clonal level diversity were studied on Hainan island in the South China Sea. They found 220 distinct clones across four different Lemnaceae species (from four different genera) on the island.

1.4.2. Geographic distribution and environmental conditions

Lemnaceae are widely distributed throughout the world in freshwater environments with a particularly prominent presence in North America, Europe and Asia (Landolt 1986). They are rarely found in very dry regions, such as deserts, or very wet regions, such as rainforest (Landolt 1986). Lemnaceae are most commonly found on still water such as pools, ponds and

lakes, as their dense mat-like growth is broken up in fast-moving water bodies such as streams and rivers (Landolt 1986). Lemnaceae grow on the surface or near the surface of water, so they are sensitive to movements of the surface caused by currents or wind (Bonomo et al. 1997). The depth of water bodies in which Lemnaceae are found in nature can depend on the climatic conditions. In high temperature areas evaporation can cause shallow water bodies to dry, while in areas with milder climates a shallow pool is not as much of a limiting factor for Lemnaceae growth (Landolt 1986).

Lemnaceae can live in areas with a broad range of temperature conditions from as low as 5 up to 35 °C (Wilkinson 1963; Li et al. 2020), with an optimum from 20–30 °C (Wedge and Burris 1982; Zirschky and Reed 1988). Temperature conditions can significantly affect Lemnaceae light utilisation. Temperatures up to 29 °C can increase Lemnaceae photosynthesis and growth, but beyond 30 °C, however, a reduction in light utilisation is found (Wedge and Burris 1982; Landolt and Kandeler 1987). In nature they generally do not occupy water bodies with temperatures below 10 °C during the three warmest months of the year (Landolt 1986). This mostly excludes regions of high latitude, such as Arctic and Antarctic regions, and areas of high altitude (above 4000 m) from Lemnaceae growth.

Lemnaceae mostly grow under autotrophic conditions, using light energy as their primary source of energy (Yin et al. 2015). The growth saturation point that lies anywhere between 250–750 µmol m^{-2} s^{-1}, depending on species and clone (Wedge and Burris 1982; Landolt and Kandeler 1987; Paolacci et al. 2018a). Plants can continue to absorb light beyond 750 µmol m^{-2} s^{-1} but it will not be reflected in higher growth rates (Landolt and Kandeler 1987). However, Lemnaceae can also grow well (or even better) in heterotrophic conditions where no light is available but organic compounds such as sugars are (Sun et al. 2020).

Lemnaceae grow optimally in pH conditions around pH 5–8 (Landolt and Kandeler 1987; Zirschky and Reed 1988), but tolerate a wider range from pH 3-10 at reduced growth rates (McLay 1976). The pH of a medium can affect the bioavailability and absorption by Lemnaceae of many nutrients and metals (Frick 1985; Khellaf and Zerdaoui 2013; Verma and Suthar 2015). The pH can also change the ionisation of some compounds or elements, leading to effects on Lemnaceae growth. For example, the equilibrium between ammonia (NH_3) and ammonium (NH_4^+) favours ammonia at higher pH. Ammonia is toxic to Lemnaceae at low concentrations (Caicedo et al. 2000; Körner et al. 2001).

Two important nutrients that Lemnaceae require are nitrogen (N) and phosphorous (P), of which indicative minimal concentrations can be taken from the literature. Lemnaceae species require at least 0.005 mM (0.07 mg L^{-1}) N, with optimal concentrations between 0.2-25 mM (2.8-350 mg L^{-1}) and a maximum tolerated of around 150 mM (2101 mg L^{-1}) (Landolt and Kandeler 1987). N is mostly taken up in the form of ammonia and nitrate. Lower concentrations of P than N are required; at least 0.0001 mM (0.003 mg L^{-1}), an optimal range from 0.01–1.75 mM (0.3–54.2 mg L^{-1}) and a maximum tolerated at around 10 mM (310 mg L^{-1}) (Landolt and Kandeler 1987; Paolacci et al. 2016). P is primarily taken up in the form of phosphate.

1.4.3. Growth

Fronds of *L. minor* float on the surface of the water and are usually drawn together in large groups, forming dense mats that can expand quickly across the water surface (Landolt 1986). This dense mat form of expansion can improve the efficiency of surface area use (Kufel et al. 2018), mitigate the effects of wind and current (Bonomo et al. 1997), and lessen the competition from algae (Parr et al. 2002; Roijackers et al. 2004). A high plant density will, however, restrict Lemnaceae's own growth as well (Debusk et al. 1981; Driever et al. 2005).

A major advantage of Lemnaceae, compared to other plant species, in the context of wastewater treatment, is the very fast growth rate and high biomass accumulation (Bergmann et al. 2000; Ziegler et al. 2015). Lemnaceae growth rates are amongst the highest in the plant kingdom (Ziegler et al. 2015), and growth can be expressed as doubling times. These doubling times vary from 1.3 to 4.5 days (Ziegler et al. 2015). Lemnaceae generally follow a log growth curve over time; after an acclimation period, or lag phase, Lemnaceae grow at a linear rate against time, which plateaus as nutrient availability is reduced (Zhang et al. 2014). However, under replenishing nutrient conditions Lemnaceae can maintain linear growth (Dinh et al. 2020). Under optimal conditions, this kind of growth can lead to >70 tonnes of dry matter being produced per hectare per year (tDW ha^{-1} y^{-1}) (Landolt and Kandeler 1987). However, under more realistic field conditions yields can vary considerably from 27.3 tDW ha^{-1} y^{-1} (Xu et al. 2012) to 68 tDW ha^{-1} $year^{-1}$ (Mohedano et al. 2012). Notwithstanding such variation, these numbers compare favourably to growth rates of major agronomic crops (Hillman and Culley 1978).

Maximum attainable growth rates often vary as much between different clones as between different species (Bergmann et al. 2000; Ziegler et al. 2015). Growth rates can also vary significantly due to environmental conditions (Körner et al. 2001), nutrient availability (Paolacci et al. 2016) and competition from other organisms (Cheng and Stomp 2009). Lemnaceae reach their highest growth rate when kept in sterile, optimised conditions in a laboratory setting (such as in Ziegler et al. (2015)). Outdoor conditions will often have a negative effect on Lemnaceae growth rate, as it is often a more challenging and stressful environment for the plants, in which Lemnaceae have to deal with temperature fluctuations, low light conditions and competition with other organisms (Rejmánková 1973; Szabó et al. 2005). This means there is often a distinct difference between the growth of Lemnaceae in laboratory conditions and those in nature.

1.4.4. Nutritional composition

The nutritional composition of Lemnaceae varies on a species and clonal level (Cheng and Stomp 2009; Appenroth et al. 2017). Composition is also influenced to some extent by environmental conditions. For example the starch content of Lemnaceae plants can be manipulated by adjusting pH, nutrient supply and/or light conditions (Xiao et al. 2013; Cui and Cheng 2015). Therefore, the most common organic components of Lemnaceae are present in a wide range of quantities. In terms of percentage of dry weight, typical values range for protein content from 4–45 %, for carbohydrate content from 14–50 %, for starch from 4–50 %, for fibre from 6–16 % and for lipid and fatty acids from 2–9 % (Landolt and Kandeler 1987; Cheng and Stomp 2009; Appenroth et al. 2017).

In the context of this project where the aim is to use plant biomass as a food source, protein and amino acid content (protein quality) are important considerations (Sá et al. 2020). Protein content of dry Lemnaceae biomass can vary widely, 4–45 %, when the high and low extremes are taken into account (Landolt and Kandeler 1987). More commonly reported values are found to be between 15–35 % (Mohedano et al. 2012; Appenroth et al. 2017; Sun et al. 2020). The quality of the protein, i.e. the amino acid profile, also varies on a species and clonal level (Rusoff et al. 1980; Cheng and Stomp 2009; Appenroth et al. 2017). Lemnaceae contain relatively high levels of many of the essential amino acids (EAAs) when compared to other plants (Cheng and Stomp 2009). Most importantly, this includes amino acids that cannot be synthesised *de novo* by animals such as methionine, leucine, lysine, threonine and valine. Typical value ranges for some essential amino acid in Lemnaceae (as percentages of total protein) are methionine 0.8–2 %, leucine 4.1–8 % and lysine 2.7–5.8 %, threonine 1.9-4.7 % and valine 2.7-4.9 % (values summarised from (Cheng and Stomp 2009; Appenroth et al. 2017; Chakrabarti et al. 2018)).

In summary, Lemnaceae can tolerate a wide range of environmental conditions, in addition to having an excellent, but variable, nutritional content. This makes Lemnaceae a good candidate for growth on variable wastewaters. In the next sections we will see how Lemnaceae has been successfully grown on different wastewater types and what might be expected from cultivation on dairy processing wastewater.

1.5. Lemnaceae-based wastewater remediation

1.5.1. Lemnaceae and different types of wastewater

Lemnaceae have a high capacity for remediation of different forms of wastewater. Best documented are studies of growth and remediation on swine effluent (Bergmann et al. 2000; Xu and Shen 2011; Mohedano et al. 2012; Toyama et al. 2018), municipal and domestic wastewater (Alaerts et al. 1996; Ozengin and Elmaci 2007; Iatrou et al. 2015) and industrial wastewater (Sekomo et al. 2012; Teixeira et al. 2014). In general, a variety of species and clones are used in these studies. Indeed, it is unlikely that one species or clone of Lemnaceae would be suitable for remediation of different kinds of wastewater, the composition of which can vary significantly. Studies have shown that different species and clones of Lemnaceae differ in their tolerance to wastewaters. For example, Zhao et al. (2015) showed significant differences in growth rates, nutrient recovery rates and protein content for a few Lemnaceae species (*Wolffia globosa*, *Lemna japonica*, *Landoltia punctata*, *Spirodela polyrrhiza*) grown on the same wastewater source. Bergmann et al. (2000) showed that species and clones differ markedly in their growth rates on swine lagoon waste. Indeed, the researchers found clones of the same species often differ as much as clones from different species. Thus, different types of waste are compositionally different, and in each case a specific strategy for remediation by Lemnaceae needs to be developed.

1.5.2. Lemnaceae growth and yield on wastewater

It can be difficult to compare Lemnaceae growth rates across multiple studies as researchers often employ different means of measuring and calculating the growth of Lemnaceae. Relative growth rate (RGR) is a commonly employed calculation, which is calculated from measurements of starting and final fresh or dry Lemnaceae biomass (g) or frond number (Connolly and Wayne 1996). Another frequently employed parameter is the yield per surface area per day (i.e. g m^{-2} d^{-1}). As well as these standardised calculations, some studies use unique measures that relate to their specific experiment, such as Lemnaceae fresh weight gain over the course of an experiment (as seen in Bergmann et al. (2000)). This raises problems when assessing, from literature sources, the capability of any particular species of Lemnaceae to grow well in different wastewaters. Notwithstanding these issues, numerous studies have shown that various Lemnaceae species have good growth rates on a variety of wastewater types (Mohedano et al. 2012; Zhao et al. 2015; Toyama et al. 2018). However, Lemnaceae often grow at a slower rate on wastewater media (see Iatrou et al. 2015) compared to optimised media (see Ziegler et al. 2015), due to the frequently non-optimal composition of wastewaters. Typical values of RGR for Lemnaceae grown indoors on wastewater range from 0.15-0.3 d^{-1} (Körner and Vermaat 1998; Caicedo et al. 2000; Iatrou et al. 2015), but rates can also go as low as 0.07 d^{-1} depending on conditions such as wastewater dilution and pH (Dinh et al. 2020). Studies that present growth as the g m^{-2} d^{-1} yield of Lemnaceae give a typical range of 23-38 g m^{-2} d^{-1} (dry weight) (Cheng et al. 2002a; Verma and Suthar 2014).

At a laboratory scale, Lemnaceae are usually grown in small vessels that hold 50-250 mL of wastewater, under favourable conditions in plant growth facilities in which the environment is controlled, e.g. light is provided with lamps, and the temperature is kept stable (Bergmann et al. 2000; Caicedo et al. 2000). Whereas in so-called pilot-scale studies, that are conducted on a larger scale, tanks with up to hundreds of litres are often placed in outdoor settings due to

their size (Mohedano et al. 2012; Xu et al. 2012; Zhao et al. 2015). These pilot-scale studies provide conditions that are more approximate to conditions for potential industrial applications. However, an outdoor setting can bring additional complexity. An outdoor pilot-scale Lemnaceae reactor is exposed to variability in weather and climate conditions, which can affect Lemnaceae growth and remediation (see Cheng et al. (2002b) for differences between spring and autumn). As well as this, animals that feed on Lemnaceae may be present (Jacobs 1947), and Lemnaceae will come under strong competition from other organisms such as algae (Xu and Shen 2011) and aquatic macrophytes like *Azolla* spp. (Paolacci et al. 2018b). For these reasons, Lemnaceae growth rates attained in laboratory settings, as given above, may not reflect the growth rates and yields attainable in outdoor, large-scale facilities e.g. 18 g m^{-2} d^{-1} in an outdoor pilot-scale facility in Mohedano et al. (2012). In order to avoid reduced growth and yield from using outdoor pilot-scale Lemnaceae remediation systems, some researchers and companies are looking at indoor stacked systems as an alternative (Goldsmith et al. 2020), even in the context of space travel (Escobar and Escobar 2017). Indoor stacked systems optimise the use of space, eliminate negative climate effects and reduce establishment of other organisms (Januszkiewicz and Jarmusz 2017; Park et al. 2019). They are especially important in colder climates where outdoor Lemnaceae growth is slow or non-existent during winter months (Landolt 1986). Overall, indoor systems can ensure Lemnaceae growth and yield is maintained at optimal levels on a large scale.

1.5.3. Lemnaceae-mediated nitrogen and phosphorous removal from wastewater

The N and P component of dairy processing wastewater is usually relatively high and contributes to its negative environmental impact (Liu et al. 2008; Lewis Jr et al. 2011). Therefore, it is important that the concentrations of these components are reduced before wastewater is released into the environment. Yet, while N and P are potential causes of

environmental damage, they are also the components of the wastewater that are essential for Lemnaceae growth (Landolt 1986). Much of the research into Lemnaceae-mediated wastewater remediation concerns removing these components, which is often expressed as the removal rate or the removal efficiency (Körner et al. 2003; Xu and Shen 2011).

Nitrogen is mainly taken up by Lemnaceae in the form of nitrate, ammonia or urea (Landolt and Kandeler 1987). Studying the different dairy processing wastewaters used for Lemnaceae cultivation in the literature shows that N is mainly available to Lemnaceae in the forms of ammonia and nitrate (Malaspina et al. 1996; Carvalho et al. 2013). Some studies do not measure ammonia directly but instead measure total Kjeldahl nitrogen (TKN), which includes ammonia, as an indication of available N (Ince 1998; Demirel and Yenigun 2004). Ammonia comes in two forms, unionised ammonia (NH_3) and ionised ammonium (NH_4^+), which Lemnaceae tolerate differently. The un-ionised NH_3 form can cause toxicity to Lemnaceae at concentrations greater than 1 mg L^{-1} (manifested as slower growth), with a maximum tolerance of around 8 mg L^{-1} (Körner et al. 2001). Lemnaceae can tolerate the ionised form NH_4^+ to a much greater extent; optimally up to concentrations of around 70 mM (1000 mg L^{-1}) at pH 4.5 (Paolacci et al. 2016). The ratio of NH_3 to NH_4^+ is determined by pH and a solution of low pH will favour the presence of NH_4^+ (Emerson et al. 1975). If a wastewater stream contains a high concentration of ammonia then a lower pH is more suitable for Lemnaceae growth as this will keep much of the ammonia in an ionised form, thus avoiding toxicity (Caicedo et al. 2000; Körner et al. 2001).

Phosphorous is mainly present in dairy processing wastewater in the form of orthophosphate (Malaspina et al. 1996; Carvalho et al. 2013). Total phosphorous (TP) and orthophosphate concentrations are frequently similar in value because of this.

The uptake of N and P (as well as nutrients generally) by Lemnaceae from wastewater is measured in two main ways. One common method is to measure the removal of the nutrient from the wastewater over a period of time, in which the difference between the nutrient concentration at the start of the experiment and that at the end represents the amount removed (Cheng et al. 2002b; Xu and Shen 2011; Verma and Suthar 2014). This removal can be basically expressed as mg removed or the percentage removal (of total) over the course of the experiment (Marín and Oron 2007; Mohedano et al. 2012; Verma and Suthar 2014). This is a crude measure of removal as it is not standardised against the amount of plant material, the volume of wastewater, nor the length time in which the plants grew in the wastewater. To account for this, removal values can be standardised to facilitate comparisons with other studies. Different methods of standardisation are found in literature sources, for example g L^{-1}, g m^{-2} d^{-1} or g g^{-1} d^{-1} (Cheng et al. 2002b; Xu and Shen 2011; Tang et al. 2017). In the two latter examples, nutrient removal values (g) are standardised by the amount of Lemnaceae biomass (fresh or dry mass in g) or surface area (m^2), as well as by time (d) (Körner and Vermaat 1998; Cheng et al. 2002b; Dinh et al. 2020), making these calculations reliable for comparative purposes. One drawback, however, is that these calculations do not take account of the fact that Lemnaceae grow throughout the course of the experiment, with mass and surface area increasing over time. Usually the initial or final mass of Lemnaceae must be taken for the calculation but it is not always clear which one is taken (Cheng et al. 2002b). Sometimes Lemnaceae is managed to maintain it at a constant surface area or mass (Zhao et al. 2014a), which makes the removal rate calculation more reliable.

A second method of measuring N and P uptake is by measuring the content of the nutrient in the plant biomass (Körner and Vermaat 1998; Zayed et al. 1998). In this context, Lemnaceae uptake rates can be expressed as g or mM of the compound removed per g of Lemnaceae (fresh or dry) per hour or day (Kwan and Smith 1991; Cedergreen and Madsen 2002). In

ecotoxicology, in particular, a bioconcentration factor (BCF) is used, which is calculated as the ratio between the concentration of a nutrient in the plant and the concentration in the surrounding medium (Marín and Oron 2007).

Bearing the abovementioned issues in mind, literature sources can be used to get a general idea of the removal rate of N and P by Lemnaceae. If we take studies that use the widely used standardised removal rate g m^{-2} d^{-1}, quite a wide range of removal rates are found. For example, the following N removal rates are found: 0.124 g N m^{-2} d^{-1} (Körner and Vermaat 1998), 2.73 g N m^{-2} d^{-1} (Dinh et al. 2020), 3.4 g N m^{-2} d^{-1} (Cheng et al. 2002b), 4.4 g N m^{-2} d^{-1} (Mohedano et al. 2012). For outdoor remediation systems, the removal of N from wastewater is lower in colder months compared to warmer: 0.39 g N m^{-2} d^{-1} compared to 0.93 g N m^{-2} d^{-1} in Zhao et al. (2014a) and 1.24 g TKN m^{-2} d^{-1} compared to 2.11 g TKN m^{-2} d^{-1} in Cheng et al. (2002b).

The removal rate of P also exhibits quite a range of values: 0.014 g P m^{-2} d^{-1} (Körner and Vermaat 1998), 0.167 g P m^{-2} d^{-1} (Dinh et al. 2020), 0.47 g P m^{-2} d^{-1} (Mohedano et al. 2012), 0.59 g P m^{-2} d^{-1} (Cheng et al. 2002b). The same seasonal pattern is also found for P removal: 0.071 g P m^{-2} d^{-1} in colder months compared to 0.105 g P m^{-2} d^{-1} in warmer months in Zhao et al. (2014a) and 0.26 g P m^{-2} d^{-1} compared to 0.59 g P m^{-2} d^{-1} in Cheng et al. (2002b).

The majority of the cited studies were performed under non-axenic conditions as they were conducted with wastewater. This means other organisms would have present in the cultivation system, primarily microorganisms. Körner and Vermaat (1998) showed that in such a non-axenic system Lemnaceae may only account for one third of N removal (of a total of 0.2 g m^{-2} d^{-1} in the study), with direct uptake by the biofilm attached to the Lemnaceae or the container accounting for another third, and a final third lost to biofilm-mediated nitrification-denitrification. For P, between 20-50 % of removal was attributable to Lemnaceae with the rest removed by biofilms of microorganisms (Körner and Vermaat 1998). However, in axenic

systems the removal rate of Lemnaceae is not necessarily lower than that of plants in non-axenic systems. Cheng et al. (2002a) reported removal rates of 1.33 g m^{-2} d^{-1} for NH_4^+-N and 0.18 g m^{-2} d^{-1} for PO_4-P in an axenic culture of Lemnaceae grown on a synthetic swine wastewater.

It is clear that experimental factors such as, the type of wastewater, the Lemnaceae species or clone used, environmental conditions and the axenic nature of the system, among many others can account for differences in N and P removal rates between studies (Körner et al. 2003; Mohedano et al. 2012; Xu et al. 2012; Zhao et al. 2015). Nevertheless, high N and P removal rates can be achieved even in challenging growing conditions.

1.5.4. Salinity

Wastewater from the dairy processing industry can be very saline, containing high concentrations of sodium chloride (NaCl), potassium chloride (KCl) and calcium chloride ($CaCl_2$) (Demirel and Yenigun 2004; Carvalho et al. 2013).The concentrations of sodium and chloride, two elements that can cause problems for Lemnaceae growth, are often particularly high: 986 mg L^{-1} Na and 525 mg L^{-1} Cl in Demirel and Yenigun (2004), and 250-350 mg L^{-1} Na and 1349-1167 mg L^{-1} Cl in Goyal and Gandhi (2009)). Lemnaceae require very little sodium and chloride for growth and can tolerate a maximum concentration around 200 mM (4600 mg L^{-1}) for Na and 100 mM (3545 mg L^{-1}) for Cl (Landolt and Kandeler 1987). Although this level of tolerance varies for species and clones, negative effects can be found at much lower concentrations: 20-30 mM NaCl (460-690 mg L^{-1} Na and 710-1065 mg L^{-1} Cl) (Sree et al. 2015; Liu et al. 2017). Furthermore, Hounkpe et al. (2013) found more generally that wastewater conductance values above 1400 µS cm^{-2} have a negative impact on *Spirodela polyrrhiza*. Considering that dairy wastewater contains sodium and chloride in concentrations considered non-optimal, the concentrations of them should be monitored to ensure they do not reach the level at which they negatively affect Lemnaceae growth.

1.5.5. Organic matter

A major component of dairy processing wastewater is organic matter, which includes a broad range of carbon-based compounds such as carbohydrates, fats, proteins, amino acids, vitamins and detergents (Jungclaus et al. 1978; Santos et al. 2017; Menchik et al. 2019). Different organic compounds will have different effects on Lemnaceae. Lemnaceae can take up carbohydrates from the surrounding medium and have a preference for some (glucose, fructose) over others (lactose, acetate) (Landolt and Kandeler 1987). Detergents, which end up in the wastewater as they are used to clean machinery (Santos et al. 2017), can be toxic to Lemnaceae at higher concentrations (Dirilgen and İnce 1995). Vitamins and amino acids may be taken up by Lemnaceae, but are unlikely to have much of an effect on growth in well-balanced media (Landolt and Kandeler 1987).

The amount of organic matter in a body of water is usually measured using two parameters, chemical oxygen demand (COD) and biochemical oxygen demand (BOD). COD measures all the oxidisable compounds in a solution while BOD only measures the compounds that are oxidisable biologically (Bourgeois et al. 2001). As shown earlier, the organic component of dairy processing wastewater is variable but constitutes a major portion of the wastewater: 783-1058 mg L^{-1} COD (Cheng et al. 2002b); 260-290 mg L^{-1} COD (Dinh et al. 2020); 4420 mg L^{-1} COD (Demirel and Yenigun 2004); 55434-70150 mg L^{-1} (Malaspina et al. 1996).

Growing Lemnaceae on wastewater can lead to the reduction in the concentrations of COD and BOD (Alaerts et al. 1996; Körner et al. 1998; Li et al. 2017). However, this is highly dependent on two key conditions: the level of light provision/availability and the axenic nature of the Lemnaceae culture. In light limiting and heterotrophic conditions, organic components are a valuable source, or the only source, of carbon for Lemnaceae (Gorham 1950; Sun et al. 2020). In typical light (autotrophic) conditions, Lemnaceae do not take up large amounts of organic components, thus they do not directly contribute much to the removal of organic compounds

from wastewater (Körner et al. 1998). Although Lemnaceae might not contribute much directly to reducing COD concentrations, they do act as a vectors for biofilms of microorganisms to grow on (Körner et al. 1998). Microorganisms grow in biofilms on the roots, lower frond surface and intercellular pockets of Lemnaceae and take up and metabolise organic compounds from the surrounding medium (Li et al. 2017). In some cases the use of non-axenic Lemnaceae remediation systems can result in high reduction efficiencies between 68-99 % of COD (Alaerts et al. 1996; Li et al. 2017; Barbosa Neto et al. 2019). However, the concentration of COD in dairy processing wastewater is frequently much higher than that found in these abovementioned studies. Indeed, when Lemnaceae is cultivated on dairy processing wastewater microbial growth is strongly promoted by the availability of organic compounds, which leads to negative impacts on Lemnaceae growth and survival (Broughton 2019). Furthermore, in high COD conditions, COD removal by Lemnaceae-associated microbes may not be quick or vigorous enough to remediate the wastewater (or quick enough to curtail negative effects on Lemnaceae).

The question of how to deal with the high load of organic material in dairy processing wastewater requires an answer outside the scope of Lemnaceae-based remediation. An attractive alternative is to pre-treat wastewater, before Lemnaceae cultivation, using microbial-based digesters which can remove large amounts of organic matter in relatively short amounts of time (Malaspina et al. 1996). These digestors can also be used to generate a range of outputs, such as methane and ethanol (Charalambous et al. 2020; Sampaio et al. 2020). In this thesis, Lemnaceae cultivation has been paired with a number of aerobic and anaerobic digestion methods. One of which, aerobic dynamic feeding (ADF), promotes the accumulation of polyhydroxyalkanoates (PHAs) (Jayakrishnan et al. 2021). PHAs are polymers which can be used as precursors in bioplastic production.

In conclusion, Lemnaceae grow fast, tolerate poor water quality and have been demonstrated to be able to effectively remove N and P from a variety of wastewater types. However, the quite immense amount of literature on this subject shows how variable these traits can be. It is not always clear what the cause of the variability is as there are many factors to consider. Nonetheless, a general set of parameters for wastewater remediation can be agreed (such as excluding temperatures over 30 °C or pH over 8) but it is clear that research needs to be conducted to optimise Lemnaceae cultivation for specific wastewater types. This will include research on the wastewater itself, the environmental conditions of cultivation and the use of different species and clones. It is also clear, in the case of dairy processing wastewater, that Lemnaceae alone cannot deal with the large component of organic matter. Accordingly, a combination of Lemnaceae cultivation with microbial aerobic and anaerobic reactors should be explored.

1.6. Aim and objectives

The general aim of this study was to research the conditions under which Lemnaceae can remediate dairy processing wastewater. Specific objectives were as follows:

- Show that *L. minor* can be used to remediate dairy processing wastewater
- Assess the composition of dairy processing wastewater for suitability of Lemnaceae cultivation
- Optimise operational parameters for the remediation and biomass production
- Assess the extent to which clonal resources can be used to improve remediation and biomass production
- Assess how scaling-up a Lemnaceae remediation system affects remediation and biomass production

- Assess the ability of an integrated remediation system of Lemnaceae and microbial-based reactors to provide superior wastewater remediation

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Chapter 2

The importance of the calcium-to-magnesium ratio for phytoremediation of dairy industry wastewater using the aquatic plant *Lemna minor* L.

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Abstract

Lemnaceae are being exploited to remediate a variety of different wastewaters. Dairy processing waste is produced in large amounts, and contains a range of valuable plant nutrients, for example, nitrate, ammonium, phosphate, iron and calcium. Our aim was to remediate dairy processing wastewater with the duckweed plant *Lemna minor*. However, initial trials failed to establish growth of *L. minor* on this medium. A lack of growth can be due to a lack of essential plant micro- and macro-nutrients, or the presence of phytotoxic ingredients. In this study we show that not just nutrient concentrations, but also the ratios between them can be important in facilitating growth. Using lab-scale experiments in which *L. minor* were grown on 100 mL of synthetic wastewater, we demonstrated that the skewed Ca:Mg ratio in synthetic dairy processing wastewater is a key obstacle to good growth. Experiments showed that a ratio which favours magnesium over calcium negatively affects *L. minor* growth and photosynthetic yield, leading to RGRs as low as 0.05 d^{-1}. A change in this ratio to favour calcium, through the addition of calcium sulphate, leads to higher RGRs of 0.2–0.3 d^{-1}. Experiments lead us to conclude that a Ca:Mg ratio of 1:1.6 (by molar concentration) or greater is necessary for *Lemna minor* growth, and therefore phytoremediation of dairy industry processing wastewater.

2.1. Introduction

Dairy industry processing wastewater is generated during the production of dairy products such as milk powder, cheese and yogurt from raw milk (Ahmad et al. 2019). Typically, large volumes are produced. In Europe dairy processing is seen as one of the largest industrial food wastewater sources, with 0.5–37 m^3 of effluent per m^3 of processed milk (Slavov 2017). This processing wastewater is rich in nitrogen and phosphorous as well as other essential plant nutrients such as calcium, potassium and magnesium (Ince 1998; Demirel and Yenigun 2004; Goyal and Gandhi 2009; Carvalho et al. 2013; Ryan and Walsh 2016). As expected for a waste product from the food industry, dairy processing wastewater contains only low concentrations of contaminants such as heavy metals (Ince 1998; Demirel and Yenigun 2004). Therefore, dairy processing wastewater is well suited to remediation using a circular economy approach.

Phytoremediation refers to the process whereby plants, and associated microorganisms, are used to remove and/or degrade contaminants from soils and waters. Lemnaceae species, commonly referred to as duckweed (Landolt 1986), have been extensively studied for their phytoremediation potential. This potential relates to fast growth rates, relative tolerance to a range of pollutants, and high pollutant removal rates (Zayed et al. 1998; Cheng and Stomp 2009; Ziegler et al. 2015). Furthermore, the high protein content and good protein quality, i.e. desirable amino acid composition, make Lemnaceae biomass attractive as a potential component in animal feeds (Cheng and Stomp 2009; Anderson et al. 2011; Appenroth et al. 2017). Thus, where Lemnaceae are used to remediate uncontaminated agricultural waste streams, a circular economy approach can be considered. In this scenario, nutrients (most importantly N- and P-containing compounds such as nitrate, ammonia and phosphate) present in wastewater are recycled into animal feed. Reusing plant nutrients that are present in wastewater can generate income from waste, reduce the costs associated with storage and tertiary wastewater treatments, and prevent environmental damage (e.g. eutrophication)

associated with release of nutrient-rich waste on to surface waters (Diaz and Rosenberg 2008; Conley et al. 2009).

Duckweed species have been shown to be tolerant of a wide range of conditions and nutrient concentrations (Landolt and Kandeler 1987). Nevertheless, wastewater needs to fulfil certain criteria to facilitate growth and phytoremediation. An important criterion is the presence of adequate levels of essential plant growth nutrients. Taking nitrogen as an example, duckweed will grow on either ammonium or nitrate as a nitrogen source and can generally grow well in total nitrogen (TN) concentrations ranging between 0.2 to 25 mM, however, optimal concentrations will vary depending on the nitrogen source (Landolt and Kandeler 1987; Paolacci et al. 2016). Duckweed can tolerate a pH range from 4–8 (Landolt and Kandeler 1987), but the pH is also important in determining the tolerance of duckweed to ammonia (Körner et al. 2003). In the case of phosphate, duckweed tolerates concentrations ranging between 0.003 to 10 mM but optimally up to around 1.6 mM (Landolt and Kandeler 1987; Paolacci et al. 2016). For calcium and magnesium acceptable concentrations range between 0.2-20 mM and 0.05-10 mM, respectively (Landolt and Kandeler 1987; Van Dam et al. 2010; Paolacci et al. 2016) and the ratio between Ca and Mg ratio is also important. In general, dairy processing wastewater provides these essential nutrients in adequate amounts (Ince et al. 2000; Demirel and Yenigun 2004; Goyal and Gandhi 2009; Carvalho et al. 2013). However, there is a high degree of variability in their concentrations, and this relates to different factories and processes, as well as strong seasonal influences on milk production (Slavov 2017).

Dairy processing wastewater also contains a heavy load of organic matter, which is measured as biochemical oxygen demand (BOD_5 mg L^{-1}), chemical oxygen demand (COD mg L^{-1}) or fat (mg L^{-1}) (Janczukowicz et al. 2008; Carvalho et al. 2013). Duckweed do not require organic compounds in the medium for survival and growth (Körner et al. 1998), however, they can

contribute to the reduction in the amount of organic matter as part of a phytoremediation approach (Körner et al. 1998; Li et al. 2017).

In order to facilitate reproducible laboratory phytoremediation studies, a synthetic dairy processing wastewater has been developed (Tarpey 2016; Gil-Pulido et al. 2018). The composition of this synthetic wastewater (Table 2.1) is based on measurements of the composition of real dairy processing wastewater. Unfortunately, preliminary experiments showed that duckweed did not grow well in this synthetic dairy wastewater. The aim of the present study was to identify the reasons responsible for the poor performance of duckweed on the synthetic dairy wastewater. Synthetic dairy processing wastewater has a Ca:Mg ratio of 1:14.6. It is known that an imbalance in favour of magnesium can have a negative effect on the growth and health of duckweed (Landolt and Kandeler 1987; Paolacci et al. 2016). This antagonistic Ca:Mg relationship was first studied in terrestrial plants (Loew and May 1901). Subsequently, it was found that in soils magnesium decreases the calcium uptake in the plant, while calcium reduces magnesium uptake to a lesser extent (Halstead et al. 1958). Thus, imbalances in the soil Ca:Mg ratio can potentially aggravate calcium or magnesium deficiencies as well as magnesium toxicity (Brady et al. 2005). Therefore, based on existing literature, we hypothesised that a ratio between calcium and magnesium in favour of magnesium causes acute toxicity in *L. minor*, and that changing the ratio in favour of calcium removes this acute toxicity for *L. minor*. In this study, different levels of this imbalance between calcium and magnesium were tested in short- and long-term experiments in order to identify the concentration of calcium and magnesium most suitable for duckweed in the particular chemical environment of the synthetic dairy wastewater. This study underpins an important aspect of phytoremediation, assessing and amending wastewater composition to facilitate plant growth.

2.2. Materials and Methods

2.2.1. Cultivation of stock and experimental plants

The duckweed strain used in this study was *Lemna minor* – Blarney, strain number 5500 in Rutgers Duckweed Stock Cooperative (Lahive et al. 2011). A stock of sterile *L. minor* was kept in optimised growing conditions on half-strength Hutner's medium (Hutner 1953) in a growth room at a constant temperature of 22 °C, average light intensity of 52.66 µmol m^{-2} s^{-1} and a photoperiod of 14 hours light to 10 hours dark.

A synthetic dairy processing wastewater was used as a growing media for experimental purposes (Table 2.1). The composition of this synthetic wastewater was based on analysis of real dairy industry processing wastewater (Tarpey 2016). Control plants were grown in 100 mL of half-strength Hutner's medium and experimental plants were grown in 100 mL of synthetic wastewater. For all experiments plants were grown in their respective media in Magenta vessels (Magenta GA-7 Plant Culture Box) for 7 days in a growth room at a constant temperature of 21 °C, average light intensity of 80.82 µmol m^{-2} s^{-1} and a photoperiod of 16 hours light to 8 hours dark. This light intensity is lower than that used in standard toxicological protocols (OECD 2006), thus growth rates were moderately lower than standard, not exceeding an RGR of 0.3 d^{-1}.

2.2.2. Experimental design

This study contains two types of experiments: short-term (7 days) and long-term (42 days). In both types of studies *L. minor* plants were grown on a number of differently modified versions of synthetic dairy wastewater with half-strength Hutner's medium as a control. At the start of each experiment, three three-frond colonies were added to each Magenta. On day zero of each experiment, the starting mass (fresh weight), colony number and frond number were determined. The synthetic wastewater was not changed during the 7-day experiments. In the

case of long-term experiments (42 days), the 100mL of synthetic wastewater was replaced weekly. Furthermore, the density of plants in the long-term experiment was returned to a constant amount, three colonies, at the start of each week.

Table 2.1. Composition of synthetic dairy wastewater (Tarpey 2016).

Chemical	Concentration (mM)[1]	Concentration (mg L^{-1})[1]
Ammonium chloride	3.13	167.3
Urea	2.16	129.9
Disodium phosphate	0.35	50
Potassium bicarbonate	0.50	50
Sodium bicarbonate	1.55	130
Calcium chloride dihydrate	0.018	2
Magnesium sulphate heptahydrate	0.20	50
Manganese sulphate monohydrate	0.012	2
Iron sulphate heptahydrate	0.126	35
Zinc sulphate heptahydrate	0.007	2.15
Cobalt chloride hexahydrate	0.005	1.2
Manganese chloride tetrahydrate	0.025	4.95
Copper sulphate pentahydrate	0.005	1.25
Nickel chloride hexahydrate	0.004	0.95
Sodium molybdate dihydrate	0.005	1.1
Boric acid	0.001	0.07
Sodium selenite	0.003	0.49
EDTA	0.342	100

[1]Indicates the final concentration of each compound in the synthetic wastewater medium

2.2.2.1. Synthetic wastewater modifications

A reduction in the concentration of chloride was achieved by replacing ammonium chloride in the synthetic wastewater with ammonium sulphate, thus removing the majority of chloride. A reduction in the concentration of sodium was achieved by the removal of sodium bicarbonate. Iron and manganese concentrations were reduced through the addition of less iron sulphate heptahydrate and manganese chloride tetrahydrate, respectively. Potassium was increased

through the addition of potassium bicarbonate. Sulphate was increased through the substitution of ammonium chloride for ammonium sulphate and the addition of potassium sulphate. The calcium concentration was increased by adding calcium sulphate ($CaSO_4$) to the synthetic wastewater. The magnesium concentration was increased or decreased by altering the concentration of magnesium sulphate heptahydrate ($MgSO_4.7H_2O$). A secondary impact of altering the calcium and magnesium concentrations was that sulphate concentration was also affected. The highest concentration to which sulphate was increased, 8.3 mM, is still significantly below the maximum concentration of sulphate tolerated by *L. minor*, 60 mM (see Table 2.2 for maximum tolerated and 'optimal' concentrations). The pH of synthetic dairy wastewater is naturally around 8. However, for experiments the pH was reduced to between 4.5–5, a pH similar to that of half-strength Hutner's medium, to ensure differences observed were not due to differences in pH.

2.2.3. Measured parameters

On day seven of the short-term experiments mass (fresh biomass), colony number, frond number and chlorophyll *a* fluorescence were measured. Frond and colony numbers were counted by eye. Before weighing, plants were wrapped in tissue paper to remove excess water. The Relative Growth Rate (RGR) was calculated based on fresh weight measurements using the formula below (Connolly and Wayne 1996):

$$RGR = \frac{ln\left(\frac{W_2}{W_1}\right)}{\Delta T}$$

Where W_1 is initial fresh biomass (Day 0), W_2 is final fresh biomass (Day 7), ΔT is length of the experiment in days and ln is the natural logarithm.

Chlorophyll *a* fluorescence was measured using pulse amplitude modulated chlorophyll *a* fluorometry (WALZ Imaging fluorometer, Effeltrich, Germany). For chlorophyll *a*

fluorescence analysis, plants were dark adapted for 15 minutes immediately before measurements. Then, three random colonies from each Magenta were taken for analysis; the measured values of these three colonies were averaged together and treated as one replicate. The chlorophyll fluorescence analysis procedure is as follows; first, a low intensity modulated measuring light was turned on to measure F_0 on the dark-adapted plant, and secondly a saturating pulse of light (2700 μmol m^{-2} s^{-1}) was applied to obtain the maximum fluorescence F_m. Subsequently, actinic light (photosynthetically active light of 186 μmol m^{-2} s^{-1}) was applied to the plants and at 20 second intervals saturating pulses were applied to measure F_m', the maximum fluorescence under light-adapted conditions. F_t is the value of fluorescence immediately before the saturating pulse is applied, i.e. the steady-state value of fluorescence. F_v/F_m, the maximum quantum efficiency of photosystem II (PSII), and Y(II), the quantum efficiency of PSII under steady state light conditions were calculated according to Maxwell and Johnson (2000) using the following equations:

$$F_v/F_m = (F_m - F_0)/F_m$$

$$Y(II) = (F_m' - F_t)/F_m'$$

For the long-term 42-day experiment, mass (fresh biomass), colony number and frond number were measured every seven days before media were replaced with fresh synthetic wastewater. RGR was calculated based on growth over seven days.

2.2.4. Data analysis

Statistical analyses were conducted using R software (R 3.4.3, R Core Team (2019)). Numbers of independent replicates were 3 to 4, as stated in legends. One-way ANOVA and Welch's ANOVA were used to examine whether there were significant differences in RGR, Y(II) and F_v/F_m between treatment groups (excluding the Hutner's treatment group). Post hoc tests Tukey and Games-Howell were used in pairwise comparisons of treatment groups (also excluding the

Hutner's treatment group). Welch's ANOVA and Games-Howell tests were used when a dataset was not homoscedastic thus failing one of the assumptions of an ANOVA and Tukey test. In the 42-day experiment average RGR between treatment groups was compared each week, with the Hutner's treatment being included in this analysis.

2.3. Results and Discussion

2.3.1. Investigation of components of synthetic dairy wastewater

As part of a phytoremediation approach for dairy processing wastewater, *L. minor* was grown under laboratory conditions on synthetic dairy wastewater. However, it was found that growth rates were poor, and that colonies displayed extensive chlorosis. Chlorosis occurred relatively fast (within days) indicating toxicity rather than deficiency symptoms. Thus, a systematic desk-top study of all individual chemical components present in the synthetic medium was conducted. The concentration of each element in the synthetic wastewater was first calculated and then compared with the minimum required, the maximum tolerated and the optimal range of values (Table 2.2). Based on the data in Table 2.2, a number of elements (iron, manganese, sodium, chloride, potassium and sulphate) were selected that were present in concentrations that could potentially have a negative impact on *L. minor* growth. Iron, chloride, sodium and manganese were present at concentrations at upper end of their optimal ranges or higher than in half-strength Hutner's medium. Potassium and sulphate were both present in concentrations at the lower end of their respective optimal ranges. Concentration reductions of 100 % in chloride or sodium and 70 % in iron or manganese did not improve growth, and neither did 10-fold increases in potassium or sulphate (Table 2.3). These experiments did not reveal any candidate to explain observed growth impairment on unmodified synthetic wastewater.

Table 2.2. Concentration of compounds and elements present in half-strength Hutner's medium and synthetic wastewater, with their required and tolerated concentrations by duckweed.

Compound	Hutner's		Synthetic wastewater		Minimum Required		Maximum Tolerated		'Optimal' Range	
	mM	mg L^{-1}	mM	mg L^{-1}	mM	mg L^{-1}	mM	mg L^{-1}	mM	mg L^{-1}
Ammonium-N [a,b,c]	NP	NP	3.13	43.8	0.2	3	> 71	>1000	0.7 – 71	10 – 1000
Ammonia-N [c]					ND	ND	0.6	8	ND	ND
Nitrate-N [a]	9	126	NP	NP	0.2	3	> 71	>1000	0.2 - 21	3 – 300
Urea	NP [j]	NP	2.16	129.9	ND [k]	ND	ND	ND	ND	ND
Total Nitrogen [d]	9	126	8.17	114.4	0.005	0.07	150	2101	0.2 - 25	2.8 – 350
Phosphate-P [a,d]	3	93	0.35	10.8	0.0001	0.003	10	310	0.003 – 1.6	0.1 - 50
Total Phosphorous [a,d]	3	93	0.35	10.8	0.0001	0.003	10	310	0.01 – 1.75	0.3 – 54.2
Potassium [d]	5.96	233	0.5	19.5	0.05	1.95	40	1564	0.5 - 20	20 – 782
Sodium [d,e,f]	0.02	0.378	1.55	35.6	0	0	200	4600	0 - 10	0 – 230
Magnesium [a.d]	2.97	72.3	0.2	4.85	0.004	0.1	33	800	0.05 - 10	1.2 – 240
Calcium [a,d]	3.04	122	0.014	0.549	0.01	0.4	40	1600	0.2 - 20	8 – 800
Sulphate-S [d]	3	96.25	0.35	11.31	0.01	0.32	60	1924	0.5 - 20	16 – 641
Chloride [d,e,g]	NP	NP	3.21	113.7	0.001	0.035	100	3545	0.001 – 10	0.035 – 350
EDTA [d]	0.01	2.5	0.34	100	0	0	> 2	> 584	0 - 2	0 – 584
Iron [d,g,h]	0.004	0.229	0.13	7.035	0.001	0.06	1	56	0.001 – 0.2	0.06 - 11
Zinc [d,g,i]	0.003	0.226	0.007	0.486	0.0006	0.04	8	523	0.002 - 0.2	0.13 – 13
Cobalt [d]	NP	NP	0.005	0.3	0	0	0.05	3	ND	ND
Manganese [d,g]	0.001	0.0325	0.037	2.03	0.0001	0.005	1	55	0.001 - 0.1	0.05 – 5.5
Copper [d,g]	0.0001	0.00768	0.005	0.32	0.0001	0.006	1	64	0.0001 - 0.06	0.006 – 3.8
Nickel [d,g]	NP	NP	0.004	0.24	0	0	0.02	1	0 - 0.002	0 – 0.1
Molybdenum [d]	0.0004	0.0397	0.005	0.44	0.0002	0.02	1	96	0.0002 - 0.1	0.02 – 9.6
Boron [d,g]	0.02	0.177	0.001	0.012	0.0001	0.001	5	55	0.001 - 0.1	0.01 – 1.1
Selenium [d,g]	NP	NP	0.003	0.222	0	0	0.06	5	ND	ND

[a] Paolacci et al. (2016), [b] Caicedo et al. (2000), [c] Körner et al. (2001), [d] Landolt & Kandeler (1987), [e] Sree et al. (2015), [f] Liu et al. (2017), [g] Wang (1986), [h] Hutner (1953), [i] Lahive et al. (2012), [j] NP – Not present, [k] ND – Not determined

Table 2.3. Result of concentration change in some elements of synthetic dairy wastewater

Compound changed	Concentration change (%)	Concentration (mM)	Effect
Chloride	100 % reduction	0	No effect
Sodium	100 % reduction	0	No effect
Iron	71 % reduction	0.035	Reduction in growth
Manganese	68 % reduction	0.012	No effect
Potassium	1000 % increase	5	No effect
Sulphate	1000 % increase	3.53	No effect

2.3.2. Increasing concentrations of calcium relative to magnesium

Synthetic dairy processing water has a low concentration of calcium (0.014 mM), notably lower than the optimal range of concentrations (0.2–20 mM; Table 2.2). Furthermore, it was noted that the Ca:Mg ratio in the synthetic wastewater was 1:14.6, and this relative lack of calcium compared to magnesium might potentially also cause growth problems (Landolt and Kandeler 1987; Van Dam et al. 2010).

To explore the roles of calcium, magnesium and the Ca:Mg ratio in controlling growth, an initial experiment was conducted in which *L. minor* plants were grown on synthetic wastewater containing increasing amounts of calcium, whilst the magnesium concentration was kept constant at 0.2 mM. The calcium concentrations ranged from 0.014 to 1.21 mM, translating into Ca:Mg ratios of 1:14.6 to 6.1:1, respectively. As a control, plants were grown on half-strength Hutner's medium which has a Ca:Mg ratio of 1:1.

Under controlled growth conditions, plants on half-strength Hutner's medium displayed vigorous growth (average RGR of 0.226 d^{-1}; Figure 2.1) and had a healthy appearance. In contrast, plants grown on unmodified synthetic wastewater (Ca:Mg ratio of 1:14.6) had an average RGR of just 0.061 d^{-1} (Figure 2.1) and appeared chlorotic. When the synthetic wastewater was modified through the addition of calcium, RGR values increased significantly

from a low RGR on wastewater with a Ca:Mg ratio of 1:14.6 up until a Ca:Mg ratio of 1:1.6, where RGR-values plateaued (one-way ANOVA: $F(6, 14) = 7.606$, $p < 0.001$; Figure 2.1). The RGR of plants grown on synthetic wastewater with a Ca:Mg ratio of 1:14.6 differed significantly from that of plants grown on Ca:Mg ratios of 1:1.6, 1.2:1 and 3:1 (post hoc Tukey: $p < 0.05$). While the RGR of plants grown on synthetic wastewater with a Ca:Mg ratio of 1:8.2 differed significantly from that of plants on Ca:Mg ratios of 1:3.3, 1:1.6, 1.2:1, 3:1 and 6.1:1 (post hoc Tukey: $p < 0.05$). These results indicate that there is a positive association between the calcium concentration or the Ca:Mg ratio and the RGR of *L. minor*.

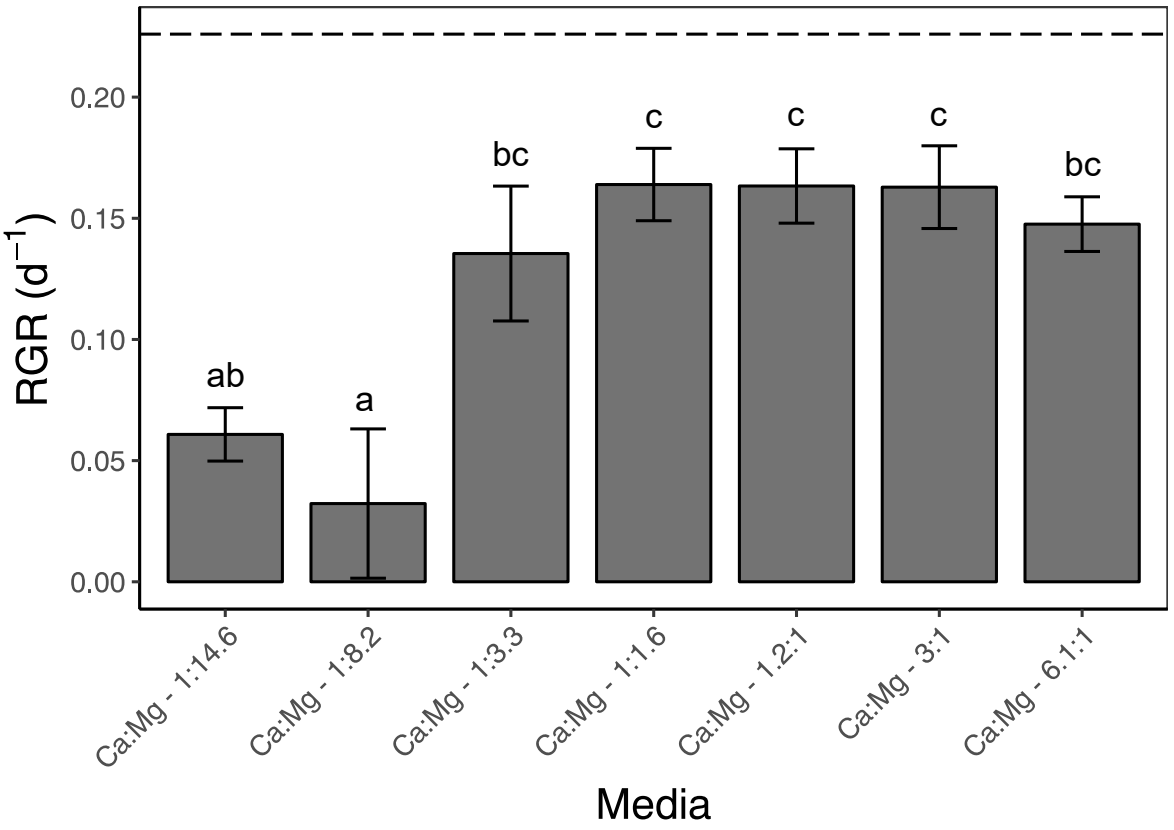

Figure 2.1. Biomass RGR (±SE, $n = 3$) of *L. minor* grown on synthetic wastewater of different Ca:Mg ratios (bars) and on half-strength Hutner's medium (dashed line)**.** The unmodified synthetic wastewater has a Ca:Mg ratio of 1:14.6. Bars that do not share a similar letter differ significantly from one another $p < 0.05$, as per post hoc Tukey.

To complement growth rate measurements, key photosynthetic parameters were measured in parallel. These parameters test if, and to what degree, a stressor is affecting photosystem II in the plant (Juneau et al. 2007). In particular F_v/F_m and Y(II) were analysed. The former refers to the maximum potential photosynthetic yield of PSII, while the latter refers to the efficiency of PSII in steady-state conditions (Maxwell and Johnson 2000). The plants on half-strength Hutner's medium displayed good photosynthetic activity, as shown by average values of 0.65 for F_v/F_m and 0.39 for Y(II) (Figure 2.2). In comparison plants grown on synthetic wastewater with Ca:Mg ratios of 1:14.6 and 1:8.2, had considerably lower F_v/F_m and Y(II) values. However, plants grown on higher Ca:Mg ratios had values that were closer or higher those grown on Hutner's (Figure 2.2). Accordingly, as the calcium concentration was increased, F_v/F_m and Y(II) values increased up until a Ca:Mg ratio of 1:1.6 where values started to plateau (one-way ANOVA F_v/F_m: $F(6, 14) = 7.843$, $p < 0.01$; one-way ANOVA Y(II): $F(6, 14) = 22.62$, $p < 0.001$; Figure 2.2). A post hoc Tukey test showed that the significant variance in F_v/F_m between the groups was between plants grown on a Ca:Mg ratio of 1:8.2 and those on ratios of 1:3.3, 1:1.6, 1.2:1, 3:1 and 6.1:1 ($p < 0.01$; Figure 2.2). For Y(II) significant variation was found between plants grown on synthetic wastewater of Ca:Mg ratios 1:14.6 and 1:8.2 and plants grown on Ca:Mg ratios of 1:3.3, 1:1.6, 1.2:1, 3:1 and 6.1:1, which had higher Y(II) values (post hoc Tukey: $p < 0.01$). These results indicate, similarly to RGR, that there is a positive association between the calcium concentration or Ca:Mg ratio and the photosynthetic efficiency of the plant.

These observations are important from a management perspective and show that the addition of calcium turns synthetic dairy processing wastewater in a suitable medium for growth of *L. minor*. However, the observations do not inform on whether the positive effects of calcium on *L. minor* growth and photosynthesis are due to a rise in calcium concentration and/or an increase in the Ca:Mg ratio.

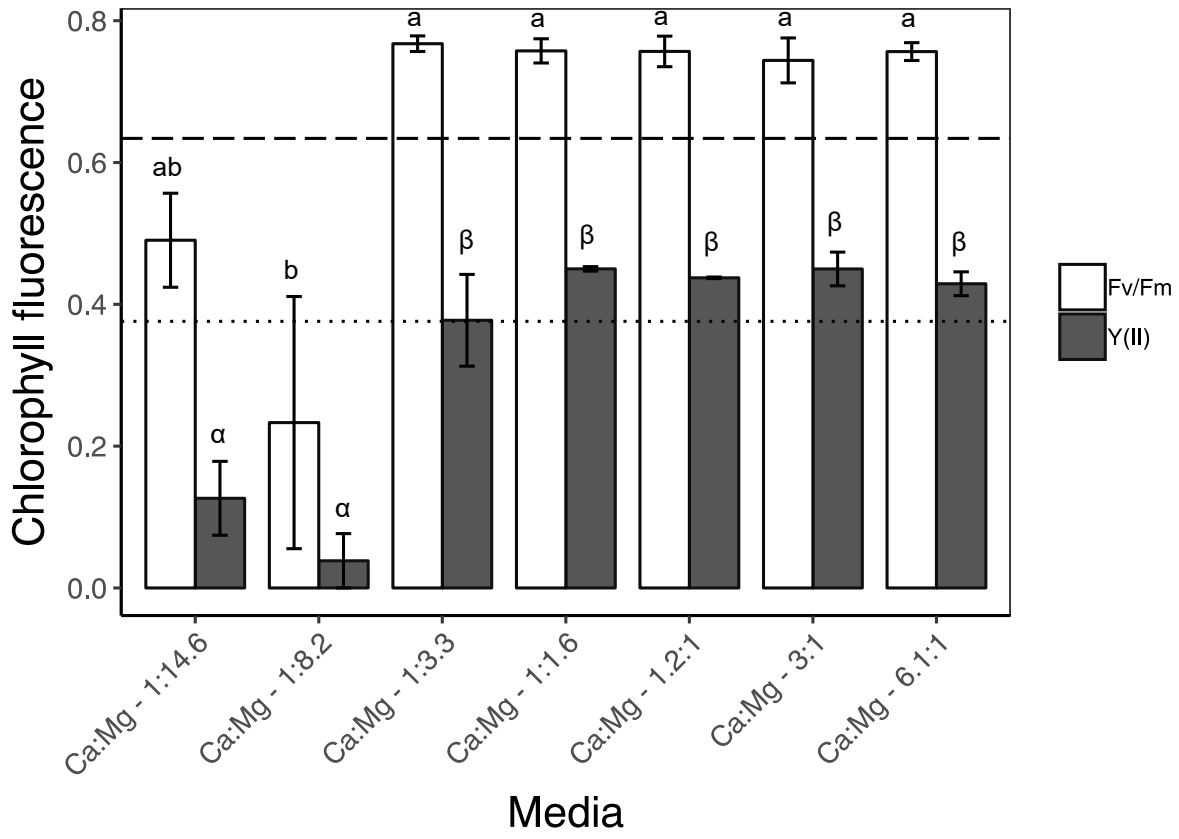

Figure 2.2. F_v/F_m and Y(II) values (±SE, $n = 3$) of *L. minor* grown on synthetic wastewater of various Ca:Mg ratios (bars) and on half-strength Hutner's medium (dashed and dotted lines, F_v/F_m and Y(II), respectively). Bars of F_v/F_m or Y(II) that do not share a similar letter differ significantly from one another $p < 0.01$, as per post hoc Tukey.

2.3.3. Increasing concentrations of magnesium relative to calcium

To explore in more detail the importance of the Ca:Mg ratio, the ratio was changed in favour of magnesium at adequate calcium concentrations. Calcium concentrations were kept constant at 0.12 mM, which was previously shown to accommodate good growth (Figure 2.1). *L. minor* was grown with magnesium concentrations ranging from 0.2 to 4.99 mM, yielding Ca:Mg ratios of 1:1.6 through to 1:41.2, respectively. It can be seen that RGR progressively decreased as the concentration of magnesium was increased in the synthetic wastewater (one-way ANOVA: $F(3, 12) = 7.996$, $p < 0.05$; Figure 2.3). The average RGR at a Ca:Mg ratio of 1:1.6 was 0.21 d^{-1} but this decreased to 0.035 d^{-1} at a Ca:Mg ratio of 1:41.2 (post-hoc Tukey: $p <$

0.01; Figure 2.3). The *L. minor* plants grown on the highest magnesium concentration (4.99 mM) were characterised by both poor growth and chlorosis.

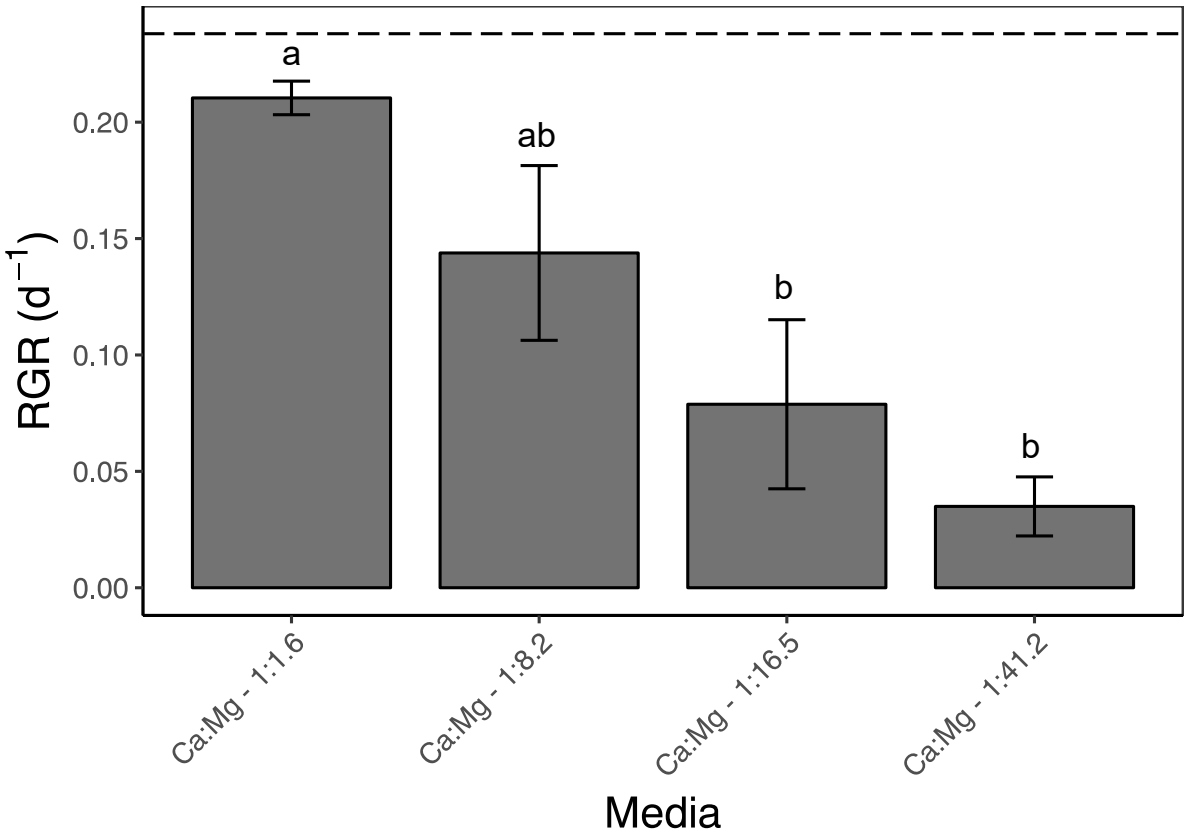

Figure 2.3. RGR (±SE, n = 4) of *L. minor* grown on synthetic wastewater of various Ca:Mg ratios (bars) and on half-strength Hutner's medium (dashed line). Bars that do not share a similar letter differ significantly from one another $p < 0.05$, as per post hoc Tukey.

F_v/F_m and Y(II) values reflect the same trend seen in the RGR data in Figure 2.3. As the magnesium concentration increased in the synthetic wastewater the F_v/F_m and Y(II) values were reduced (Welch's ANOVA F_v/F_m: $F(3) = 13.54$, $p < 0.01$; Welch's ANOVA Y(II): $F(3) = 348.34$, $p < 0.001$; Figure 2.4). At a Ca:Mg ratio of 1:1.6, the average F_v/F_m was 0.752 and Y(II) was 0.426. The lowest photosynthetic efficiencies were found at a ratio of Ca:Mg of 1:41.2, where F_v/F_m was 0.099 and Y(II) was 0.007. The F_v/F_m of plants grown on synthetic

wastewater with a Ca:Mg ratio of 1:41.2 differed significantly from that of plants grown on a Ca:Mg ratio of 1:1.6 and of 1:8.2 (Games-Howell: $p < 0.05$). Similarly, the Y(II) of plants grown on synthetic wastewater with a Ca:Mg ratio of 1:41.2 differed significantly from that of plants grown on a ratio of 1:1.6 (Games-Howell: $p < 0.001$) and 1:8.2 (Games-Howell: $p < 0.05$).

The observed negative effects of magnesium addition on both RGR and photosynthetic parameters can be attributed to the rise in magnesium concentration and/or the decrease in the Ca:Mg ratio. High concentrations of magnesium have been reported to be toxic to Lemnaceae, although this effect can be countered by increasing the Ca:Mg ratio (Van Dam et al. 2010). To further explore this point, an experiment was conducted in which the concentrations of both calcium and magnesium were increased, so as to maintain an optimised 1:1.6 Ca:Mg ratio, while using magnesium concentrations that were found to be toxic (Figures 2.3 and 2.4).

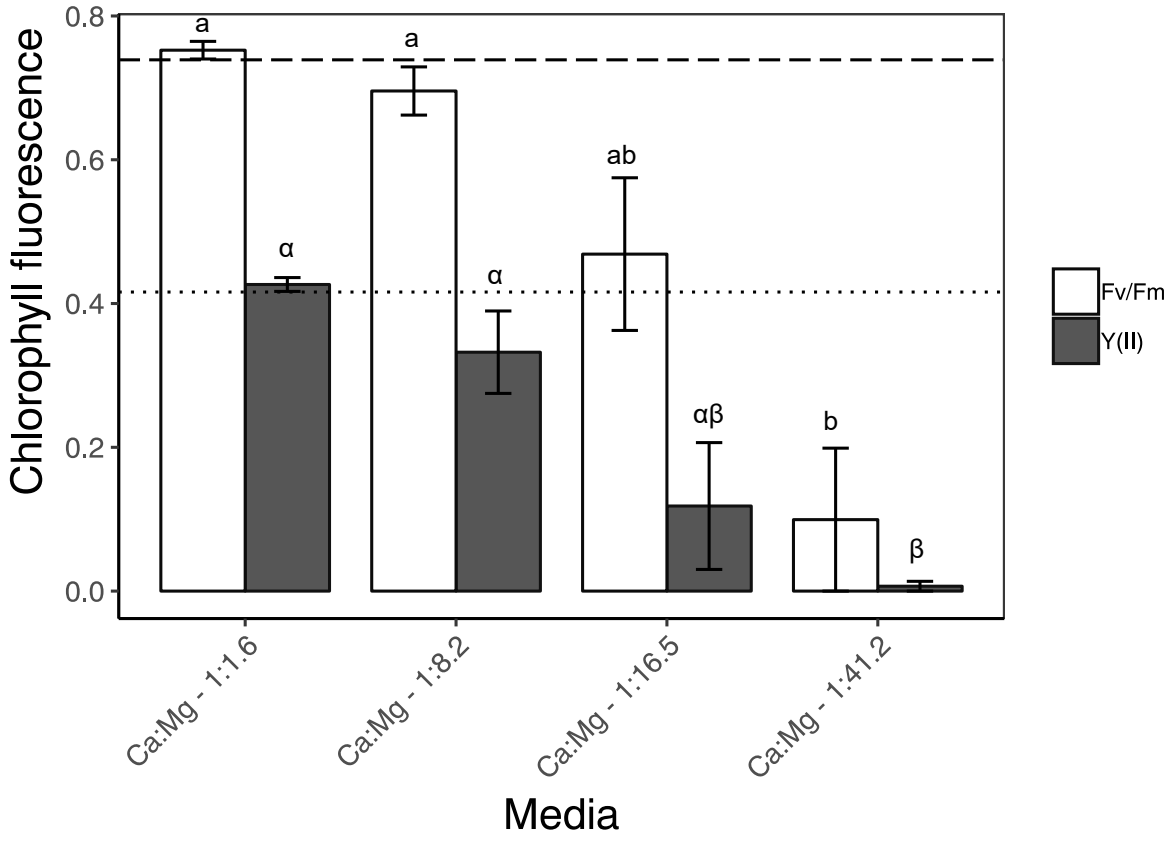

Figure 2.4. F_v/F_m and Y(II) (±SE, n = 4) of *L. minor* grown on synthetic wastewater with various Ca:Mg ratios (bars) and on half-strength Hutner's medium (dashed and dotted lines, F_v/F_m and Y(II), respectively). Bars that do not share a similar letter differ significantly from one another $p < 0.05$, as per Games-Howell post hoc test.

2.3.4. Simultaneous increase of calcium and magnesium concentrations

L. minor was grown in synthetic dairy wastewater with a Ca:Mg ratio of 1:1.6 and a range of calcium (0.12–3.12 mM) and magnesium (0.2–5.14 mM) concentrations (Figure 2.5). The RGR for plants grown on synthetic wastewater was slightly lower than that of those on half-strength Hutner's medium, across all concentrations. The highest RGR for plants grown on synthetic wastewater came from those grown at the lowest concentration of the Ca:Mg ratio of 1:1.6. As the absolute concentrations of calcium and magnesium increased plant RGR decreased moderately (one-way ANOVA: $F(4,15) = 3.89$, $p < 0.05$; Figure 2.5). Significant differences in RGR were found between plants grown on wastewater with the lowest calcium

and magnesium concentrations and that of plants grown at the two highest concentrations (post hoc Tukey; $p < 0.05$; Figure 2.5).

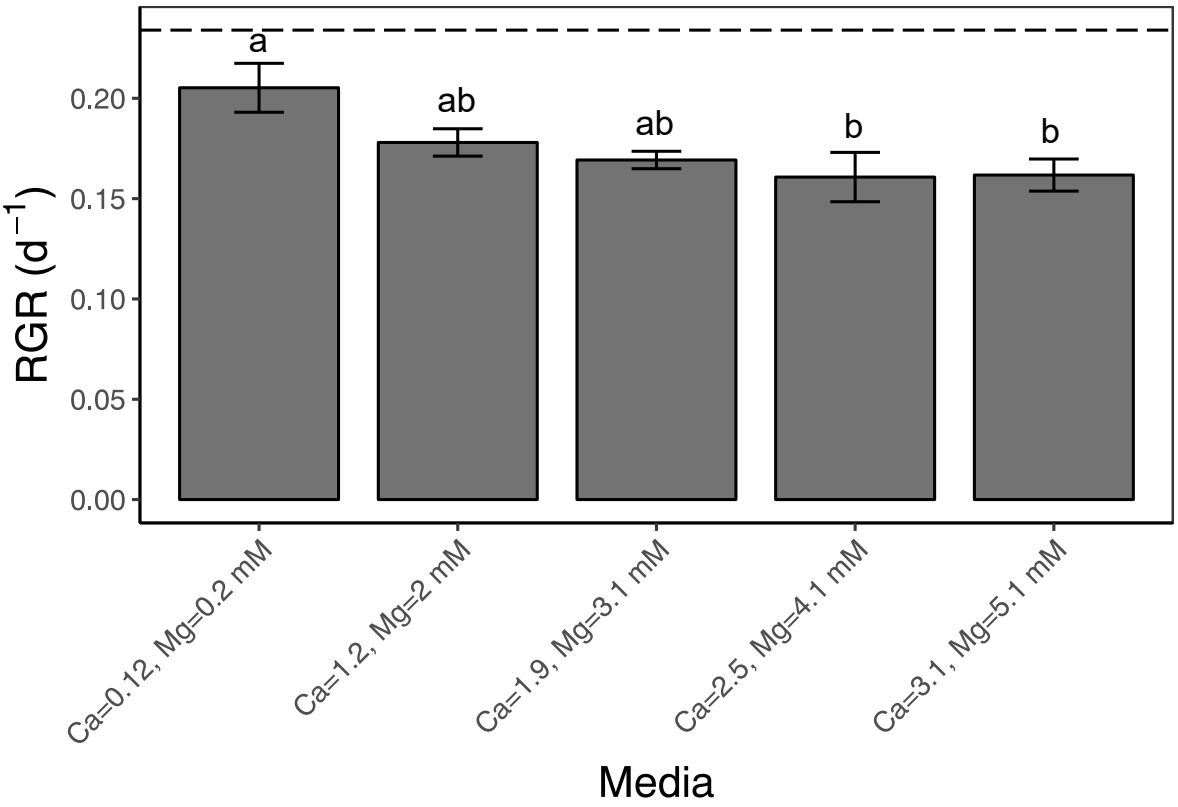

Figure 2.5. RGR (±SE, $n = 4$) of *L. minor* grown on synthetic wastewater of increasing concentrations of calcium and magnesium (bars) and on half-strength Hutner's medium (dashed line). Bars that do not share a similar letter differ significantly from one another $p < 0.05$, as per post hoc Tukey.

F_v/F_m and Y(II) measurements revealed non-significant differences between plants that were grown on synthetic wastewater containing increasing concentrations of calcium and magnesium in the same ratio (one-way ANOVA F_v/F_m,: $F(4, 15) = 0.524$, $p = 0.72$; one-way ANOVA Y(II): $F(4, 15) = 0.809$, $p = 0.538$; Figure 2.6). Furthermore, F_v/F_m and Y(II) values for plants grown on synthetic wastewater were similar to those on half-strength Hutner's medium.

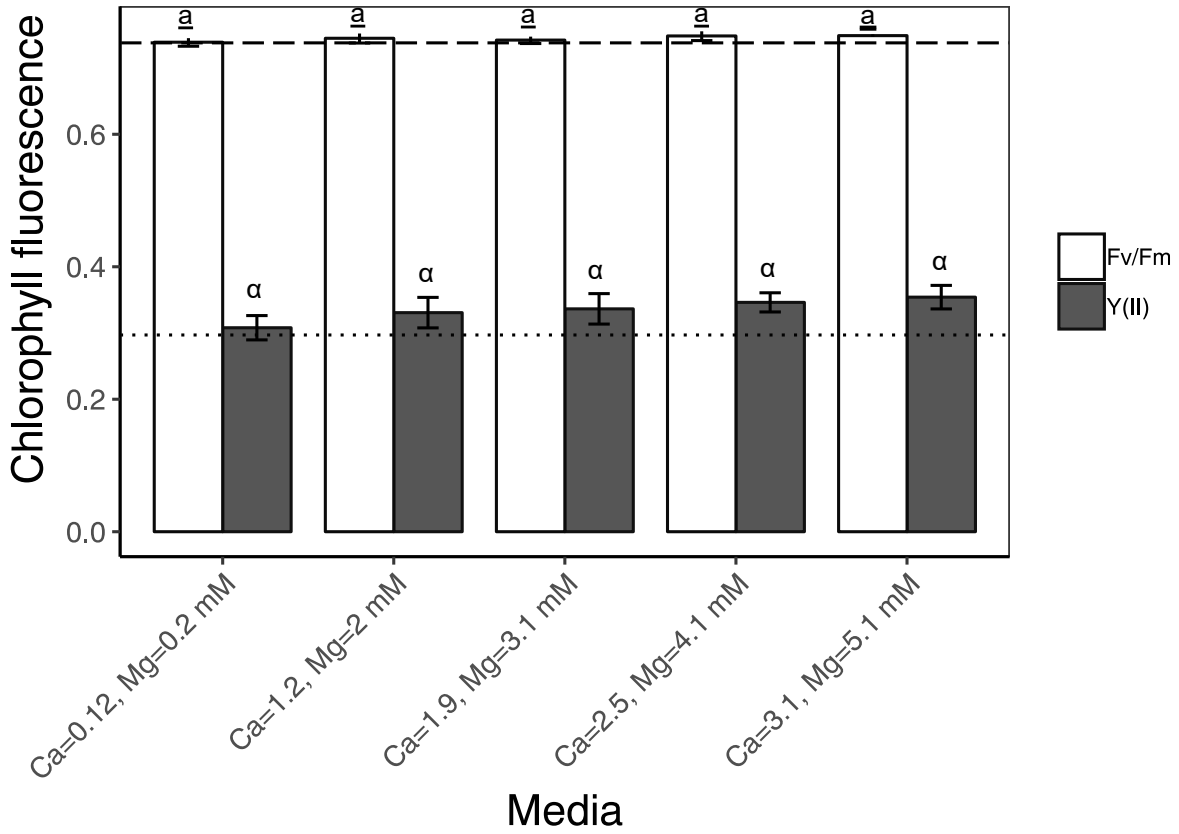

Figure 2.6. F_v/F_m and Y(II) (±SE, n = 4) for *L. minor* grown in synthetic wastewater of increasing concentrations of calcium and magnesium (bars) and on half-strength Hutner's medium (dashed and dotted lines, F_v/F_m and Y(II), respectively). Bars share the same letter as per post hoc Tukey.

A comparison of figures 2.3 and 2.4 with figures 2.5 and 2.6 shows that a magnesium concentration of 1 mM or more can have a significantly negative impact on *L. minor* growth and photosynthesis (Figures 2.3 and 2.4) when calcium concentrations are low. A magnesium concentration of 4.99 mM can even cause death of plants (Figures 2.3 and 2.4). However, the same high magnesium concentrations have only a minor effect on RGR, and no effect on photosynthesis, when the ratio of Ca:Mg is kept at 1:1.6 (Figures 2.5 and 2.6). Thus, the data emphasise the antagonism between calcium and magnesium, and the relative importance of the Ca:Mg ratio.

In Lemnaceae, antagonistic interactions between calcium and magnesium have been observed, which can impact upon the aquatic toxicity of magnesium sulphate (Van Dam et al. 2010), the degradation process of starch (Appenroth and Gabrys 2003) and the germination of turions (Appenroth et al. 1999). Van Dam et al. (2010) observed the toxic effects of magnesium on *Lemna aequinoctialis* (as well as on algae and other freshwater species), and the alleviation of these toxic effects through the addition of calcium. Similarly, Appenroth et al. (1999) observed that a high magnesium concentration, in a near calcium-free environment, inhibited turion germination in *Spirodela polyrhiza*. Furthermore, they also observed that the inhibiting effect of magnesium could be abolished by either adding calcium or by reducing the concentration of magnesium. The explanation for this observed antagonism is that magnesium is capable of competing with and inhibiting calcium uptake while also affecting calcium-dependent processes (for example turion germination).

Thus, for phytoremediation approaches the calcium concentration, the magnesium concentration and the Ca:Mg ratio in wastewater all need to be considered. This conclusion is important in the context of remediation of dairy processing wastewater which can have a highly variable Ca:Mg ratio. For example, standardised synthetic dairy wastewater has a Ca:Mg ratio of 1:14.6 but Demirel and Yenigun (2004) found a Ca:Mg ratio of 1:1.5 (1.37:2.06 mM) in milk processing waste, while Goyal and Gandhi (2009) found a Ca:Mg ratio of 5:1 (7.26:1.48 mM) from cheese whey. Thus, an unfavourable Ca:Mg ratio is a fact that needs to be considered when developing a protocol for the phytoremediation of dairy processing waste.

2.3.5. Long-term growth on modified synthetic diary wastewater

Notwithstanding the importance of the Ca:Mg ratio, we note a small decrease in RGR at higher magnesium concentrations, even where the Ca:Mg ratio is constant (Figure 2.5). It is possible that this is “pure” magnesium toxicity, which is slowly building up over time. To explore this in more detail, short term (7 day) experiments were complemented by 42-day experiments, in

which the Ca:Mg ratio was kept at 1:1.6, but at two different concentrations of calcium and magnesium. Under these conditions, healthy growth of *L. minor* was observed for the full length of the experiment, and growth rates were very similar to those obtained on half-strength Hutner's medium. ANOVA tests, confirmed this observation, showing no significant difference between the RGR of *L. minor* grown on synthetic wastewater with a Ca:Mg ratio of 1:1.6, and those grown on Hutner's throughout the 42-day experiment (Figure 2.7). Neither was there a difference between plants in low concentrations (0.12 mM Ca and 0.2 mM Mg) of calcium and magnesium, and those in high concentrations (3.12 mM Ca and 5.14 mM Mg). The general trend is that the RGR of *L. minor* increased each week up to the end of the experiment at day 42, and this is presumably due to the frequent replacement of medium.

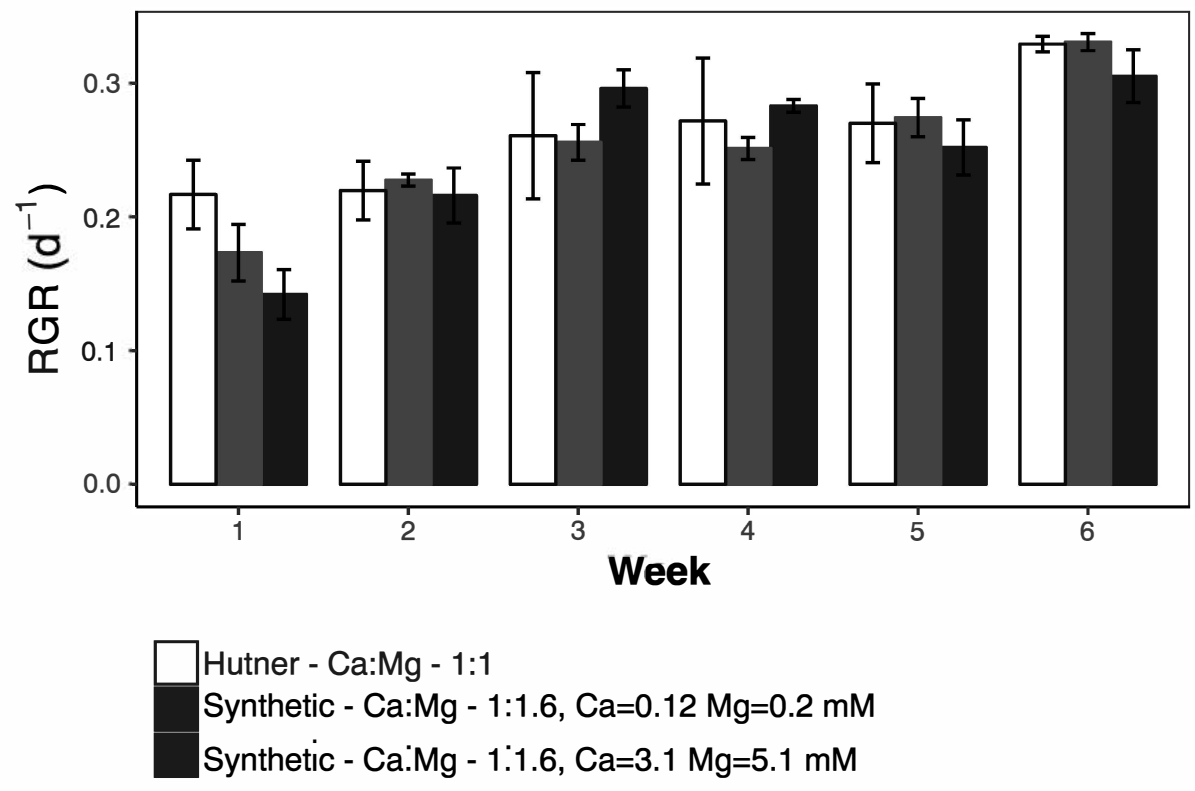

Figure 2.7. Weekly RGR of *L. minor* grown on synthetic wastewater (Ca:Mg ratio of 1:1.6 at two concentrations) and half-strength Hutner's medium throughout a 42-day experiment. ANOVA tests showed the RGR between treatments was not significantly different in any week

of the experiment (n = 4). Results for week 1-6 respectively: F(2,9) = 2.918, p = 0.105; F(2,9) = 0.112, p = 0.896; F(2,9) = 0.554, p = 0.593; F(2,9) = 0.337, p = 0.723; F(2,9) = 0.28, p = 0.762; F(2,9) = 1.319, p = 0.314.

2.4. Conclusions

Lemnaceae can be used to clean-up a variety of different wastewaters. However, some waste streams are not suitable for growth of Lemnaceae and need to be modified to facilitate growth and phytoremediation. Here we confirm the hypothesis that the Ca:Mg ratio can be a major determinant of growth and photosynthesis, both in short and long-term trials. Yet, the data also show that growth can be restored by the addition of calcium-sulphate, a procedure that is feasible in a commercial setting. An addition of calcium to the synthetic wastewater to reach a concentration of 0.12 mM, compared to 0.2 mM of magnesium, resulted in RGRs of 0.164-0.330 d^{-1}. These rates compare well with those of plants growing in half-strength Hutner's medium, which achieve RGRs between 0.226-0.330 d^{-1}. It is acknowledged that calcium can act as an antagonist for other elements, such as iron, manganese and potassium. However, initial experiments showed that a reduction in the concentrations of iron and manganese did not reverse the acute toxicity seen in plants. Thus, higher concentrations of calcium in later experiments acting as an antagonist towards these elements would not have reversed acute toxicity either. The data presented in this paper show convincingly that it is the ratio between calcium and magnesium, which is causing acute toxicity to *L. minor* in this case. A Ca:Mg ratio of 1:1.6 or greater is necessary for *Lemna minor* growth, and therefore phytoremediation of the dairy industry processing wastewater.

Author contributions

ÉW and MAKJ contributed to the study conception and design. Material preparation, data collection and analysis were performed by ÉW. All authors contributed to the interpretation of results. The first draft of the manuscript was written by ÉW and all authors contributed to writing and editing of the manuscript.

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Chapter 3

Light intensity alters the phytoremediation potential of *Lemna minor*

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Abstract

Lemnaceae, i.e. duckweed species, are attractive for phytoremediation of wastewaters, primarily due their rapid growth, high nutrient uptake rates, tolerance to a broad range of growing conditions, and ability to expeditiously assimilate a variety of pollutants. Light is essential for plant growth, and therefore, phytoremediation. Nevertheless, the effect of light intensity remains poorly understood in relation to phytoremediation, a knowledge gap that impedes the development of indoor, fully controlled, stacked remediation systems. In the present study, the effect of light intensity (10–850 μmol m^{-2} s^{-1}) on the phytoremediation potential of *Lemna minor* was assessed. Plants were grown on either an optimal growth medium (half-strength Hutner's) or synthetic dairy processing wastewater, using stationary axenic (100 mL) or re-circulating non-sterile (11.7 L) systems. The Relative Growth Rate (RGR) of *L. minor* grown on half-strength Hutner's increased proportionally with increasing light intensity. In contrast, the RGR of *L. minor* grown on synthetic dairy wastewater did not increase with light over an intensity range from 50 to 850 μmol m^{-2} s^{-1}. On synthetic dairy wastewater, total nitrogen and total phosphorous removal also remained unchanged between 50 and 850 μmol m^{-2} s^{-1}, although *L. minor* protein content (% fresh weight) increased from 1.5 % to 2 % at higher light intensities. Similar results were obtained with the larger re-circulating system. The results demonstrate interactive effects of light intensity and wastewater composition on growth and phytoremediation potential of *L. minor*. The data imply that light intensities above 50 μmol m^{-2} s^{-1} may not necessarily confer benefits in duckweed wastewater remediation, and this informs engineering of stacked, indoor remediation systems.

3.1. Introduction

Food security, including the availability of clean water, is increasingly threatened by rapid human population growth, climatic change and pollution (Porter et al. 2014; Caine et al. 2019; Hamann 2020). As such, there is an urgent requirement to develop more sustainable food production and processing methods that can deliver high nutritional value, while reducing both the consumption of finite resources and the generation of waste (Caine et al. 2019; Rufí-Salís et al. 2020). In recent years, through the efficient use of resources and waste minimisation, an economic model known as the circular economy has sought to enhance food security, environmental protection, and the socioeconomic benefits of food production systems worldwide (Morseletto 2020; Rufí-Salís et al. 2020). In principle, the circular economy is reliant on long-term value retention, reduced use of primary resources, and closed-loop production systems, whereby waste materials are recovered and transformed into new resources (Morseletto 2020).

The dairy industry is a major economic component of the global agricultural sector, and has experienced enormous growth in recent decades as the demand for milk products continues to increase worldwide (Sheng et al. 2020). However, the conversion of milk into an array of dairy products results in the generation of large volumes of dairy processing wastewater, primarily through cleaning, sanitisation, heating and cooling activities (Chokshi et al. 2016). Typically, a dairy processing facility can process between 200–550 million litres of milk a year (Baskaran et al. 2003; Gösta 2015), and it is estimated that between 0.2–10 L of wastewater are produced for every litre of milk processed (Baskaran et al. 2003; Gösta 2015; Wang and Serventi 2019). This variation in wastewater production reflects the manufacturing of a wide range of different dairy products, as well as the operational parameters of individual processing plants. Dairy processing wastewater generally contains a broad range of organic and inorganic components,

and is customarily rich in nitrogen and phosphorus compounds (Demirel and Yenigun 2004; Carvalho et al. 2013; Tikariha and Sahu 2014).

Although dairy processing plants employ a number of physicochemical and biological treatments to remediate wastewater (Wang and Serventi 2019), phytoremediation technologies have begun to emerge as alternatives for low-cost, eco-friendly and sustainable purification of tertiary, secondary and even primary dairy effluents (Lutterbeck et al. 2017; Akansha et al. 2020). Lemnaceae are a group of floating aquatic plants that are considered particularly suitable for wastewater remediation due to their rapid growth (Ziegler et al. 2015), high nutrient uptake rate (Zhao et al. 2014), relative ease of harvesting (Landolt and Kandeler 1987), and tolerance of a wide-range of growing conditions, including high ammonia levels (Landolt and Kandeler 1987; Caicedo et al. 2000). Lemnaceae biomass can be used as a biofuel, fertiliser, or feed (Ahmad et al. 1990; Cheng and Stomp 2009). In particular, there is significant interest in using duckweed as an animal feed (Anderson et al. 2011; Stadtlander et al. 2019), and even for human consumption (Appenroth et al. 2017), due to its high protein content and favourable amino acid profile (Cheng and Stomp 2009; Anderson et al. 2011). Accordingly, the integration of Lemnaceae phytoremediation technology into wastewater purification regimes could enhance the sustainability of dairy production plants, while adding value to the production chain (Adhikari et al. 2015).

Duckweed-driven phytoremediation has been employed to remove a variety of pollutants including excess macronutrients, such as nitrogen and phosphorous-containing compounds like ammonia, nitrate and phosphate (Körner et al. 2003; Cheng and Stomp 2009). The removal of these macronutrients is linked to the growth rate of the duckweed (Cheng et al. 2002), and a faster growth rate is considered to translate into greater nutrient uptake. Accordingly, to increase the phytoremediation capacity of duckweed-based systems, operational parameters should be designed to maximise duckweed growth, in relation to the nutrient composition of

wastewaters (Caicedo et al. 2000), light intensity (Paolacci et al. 2018), temperature (Wedge and Burris 1982), plant density (Driever et al. 2005; Kufel et al. 2018), and photoperiod (Yin et al. 2015). Interactive effects among some of these parameters have previously been identified (Ögren et al. 1984). The interactive effects of light and medium composition are, however, less well understood. In particular, given that nutrient deficiencies and/or surpluses can have negative effects on plant growth and health (Morales et al. 2008; Nagajyoti et al. 2010; Paolacci et al. 2016; Walsh et al. 2020), an improved understanding of potential interactive effects between wastewater composition, light and plant growth is required.

As a primary source of energy, light is a key determinant of plant growth (Paolacci et al. 2018). Duckweed growth typically increases with increasing light intensity up to a saturation point, beyond which growth is no longer accelerated by light and may even decrease due to photoinhibitory damage (Wedge and Burris 1982; Landolt and Kandeler 1987). Light intensity curves have been measured for various species of Lemnaceae (Wedge and Burris 1982; Landolt and Kandeler 1987; Paolacci et al. 2018). For example, light has been noted as a limiting factor for *L. minor* growth up to an intensity of 400 μmol m^{-2} s^{-1} (Paolacci et al. 2018). Thus, it appears that moderately high light intensities of at least 400 μmol m^{-2} s^{-1} would be required for optimisation of duckweed-based phytoremediation systems, although this will depend on possible interactive effects between medium composition and light intensity. In addition, uncertainties surrounding optimal light intensity can also impact the financial viability of large-scale indoor phytoremediation systems due to mismatch in the provision of costly lamps and their associated energy demand (including cooling systems) (Gupta and Jatothu 2013; Poulet et al. 2014). Accordingly, to advance indoor, high output duckweed remediation, there is a need to examine specific context dependencies between media types and light intensity.

Aside from possible interactive effects between wastewater composition and light intensity, an important consideration in dairy wastewater phytoremediation is increasing the scale beyond

small and highly controlled laboratory conditions. For example, a large-scale phytoremediation system may experience water currents and algal or microbial growth, unlike stationary axenic systems. In particular, excessive algal and microbial growth can compete with duckweed for the acquisition of nutrients in non-sterile systems (Körner and Vermaat 1998; Roijackers et al. 2004), and this can reduce the health, nutritional value and phytoremediation capacity of cultivated duckweed. However, the introduction of a moderate current to a phytoremediation system can decrease nutrient depletion zones and increase nutrient availability at the plant surface, as has been noted for other species (Parker 1981). The combination of these distinct influences makes upscaling an important component of the development of remediation approaches.

Overall, whilst effective remediation of dairy processing wastewater with *L. minor* has been demonstrated (e.g. Walsh et al. (2020)), possible interactive effects concerning wastewater composition and other growth parameters on duckweed health and phytoremediation capacity remain unknown. Therefore, in the present study, we assessed the interactive effects between light intensity and growing medium on key *L. minor* phytoremediation parameters, using either an optimal laboratory growing medium (i.e. half-strength Hutner's medium) or a standardised synthetic dairy wastewater, in both an axenic stationary and a non-sterile re-circulating system. We hypothesise that duckweed phytoremediation capacity will increase with greater light intensity until a plateau is reached. Results will inform the design and operational parameters of indoor duckweed-based phytoremediation systems for dairy processing wastewaters.

3.2. Materials and methods

3.2.1. Stock cultivation

The duckweed strain used in this study was *Lemna minor* L. – Blarney strain, number 5500 in the Rutgers Duckweed Stock Cooperative database (Lahive et al. 2012; Van Hoeck et al. 2015).

A sterile stock of *L. minor* was cultivated on half-strength Hutner's medium (Hutner 1953) under an average light intensity of 50 µmol m^{-2} s^{-1} PAR (photosynthetically active radiation) within a controlled growth room (22 °C, 14:10 hours light:dark photoperiod). Prior to experiments, *L. minor* plants were acclimated for seven days to experimental light conditions while grown on either synthetic dairy wastewater or half-strength Hutner's medium.

3.2.2. Experimental design

3.2.2.1. Synthetic dairy wastewater

The composition of synthetic wastewater mimics real dairy processing wastewater (Table S3.1; Tarpey 2016). A breakdown of the elemental and compound composition of this synthetic wastewater is detailed in Walsh et al. (2020). Synthetic dairy wastewater is naturally around pH 8.0 but was reduced to 4.5-5.0 using 1M H_2SO_4, to encourage optimal duckweed growth. Furthermore, additional calcium was added to maintain a favourable Ca:Mg ratio at 1:1.6 (mM) as detailed in Walsh et al. (2020).

3.2.2.2. Stationary remediation system

As an initial assessment, plants were grown for five days (days 0-5) on 100 mL of synthetic wastewater under ten different light intensities (10-850 µmol m^{-2} s^{-1}; Table S3.2) in Magenta vessels (GA-7, 7.7cm length x 7.7cm width x 9.7cm height, surface area 42.25 cm^2 with 100 mL of liquid). Following this, in a second experiment, a reduced range of three light intensities were selected (50, 200 and 850 µmol m^{-2} s^{-1}) for a comparative assessment of duckweed grown on either 100 mL of synthetic dairy wastewater or half-strength Hutner's medium in Magenta vessels for five days (days 0-5). The length of the experiment was determined by the need to achieve measurable increases in plant biomass and decreases in media nutrients, without achieving overcrowding and nutrient depletion. Eight replicates were conducted in total with the number of replicates for each measured parameter detailed in figure legends (Figures 3.1-

3.5). Both experiments were conducted under controlled laboratory conditions (18 °C, 16:8 hours light:dark photoperiod). Different light intensities were generated by placing plants at different distances from LED-based lamps (AP67 R-series, Valoya, Finland). Starting biomass was eight colonies, averaging 25-30 fronds, per replicate. Colonies were taken at random from plants acclimated for seven days to experimental light and media conditions. Throughout the experiment there was some water loss due to evaporation, but deionised water was added to maintain media volume at 100 mL.

3.2.2.3. Re-circulating remediation system

Lemna minor was grown on synthetic wastewater (Table S3.1) in a re-circulating tank system at three different light conditions (100, 300 and 900 μmol m^{-2} s^{-1} PAR, 18–21 °C, 16:8 hours light:dark photoperiod). In this re-circulating system, wastewater was pumped from a lower sump tank (at an average rate of 125 L per hour) to an upper duckweed tank and then it drained back down to the sump tank. The total re-circulating synthetic wastewater volume contained in both tanks was 11.7 L. Experiments were conducted over three days (n = 8). The length of the experiment was determined by the need to achieve measurable increases in plant biomass and decreases in media nutrients, without achieving overcrowding and nutrient depletion. Initially, tanks were seeded with duckweed to achieve a plant density of 60 % surface cover (i.e. 360 cm^2 out of 600 cm^2 surface area was plant covered). To determine how much excess material was to be removed to maintain 60 % coverage, surface coverage was monitored using the image analysis software EasyLeafArea (Easlon and Bloom 2014).

3.2.3. Measured parameters

3.2.3.1. Growth in stationary remediation system

At the start of the experiment (day 0) starting biomass was determined by measuring the biomass of ‘representative’ colonies. At the end of the experiment (day five) fresh biomass was

again measured. Fresh plant biomass was measured after removing excess water with tissue. The Relative Growth Rate (RGR) was calculated based on measurements of fresh biomass using the formula (Connolly and Wayne 1996):

$$RGR = \frac{ln\frac{W_2}{W_1}}{\Delta T} \qquad (1)$$

Where ln is the natural log, W_1 is starting biomass, W_2 is final biomass and ΔT is the length of the experiment.

3.2.3.2. Growth in re-circulating remediation system

Lemna minor cover was maintained at 60 % (or 360 cm^2) of the surface area of the tank. A proxy of RGR was calculated as per above. The biomass of excess, harvested plants was used to estimate the final fresh biomass (W_2). The initial fresh biomass (W_1) was calculated from the initial surface cover, by using the biomass per cm^2, which was determined for each replicate tank.

3.2.3.3. Chlorophyll *a* fluorometry

Chlorophyll *a* fluorescence was measured using a pulse amplitude modulated fluorometer (WALZ Imaging fluorometer, Effeltrich, Germany). Chlorophyll fluorescence measurements were taken of plants on the initial (day 0) and final days (day 5) of the stationary experiment, in which plants were grown on either half-strength Hutner's or synthetic wastewater under three different light intensities (50, 200 and 850 μmol m^{-2} s^{-1}). Day 0 measurements were not included in the final analysis but showed the baseline fluorescence characteristics in each media and under each light intensity. Chlorophyll fluorescence measurements were not taken in the re-circulating system as the purpose of these experiments was limited to the study of up-scaling. Immediately before a measurement, plants were dark-adapted for 15 minutes. For each replicate, three random colonies were selected for measurements. These three measurements were averaged together and treated as a single replicate. The chlorophyll fluorescence analysis

procedure was as follows; first, a low intensity modulated measuring light was turned on to measure F_0 on the dark-adapted plant, and secondly a saturating pulse of light (2700 μmol m^{-2} s^{-1}) was applied to obtain the maximum fluorescence F_m. Subsequently, actinic light (photosynthetically active light of 186 μmol m^{-2} s^{-1}) was applied to the plants and at 20 second intervals saturating pulses were applied to measure F_m', the maximum fluorescence under light-adapted conditions. F_t is the value of fluorescence immediately before the first saturating pulse is applied, i.e. the steady-state value of fluorescence. F_v/F_m, the maximum quantum efficiency of photosystem II (PSII), Y(II), the quantum efficiency of PSII under steady state light conditions, and NPQ, the non-photochemical quenching, were calculated according to Maxwell & Johnson 2000 using equations (2), (3) and (4).

$$F_v/F_m = (F_m - F_0)/F_m \quad (2)$$

$$Y(II) = (F_m' - F_t)/F_m' \quad (3)$$

$$NPQ = (F_m - F_m')/F_m' \quad (4)$$

Two further quenching parameters were calculated using equations (5) and (6): the yield of non-regulated energy dissipation, Y(NO), and the yield of regulated energy dissipation, Y(NPQ) (Kramer et al. 2004).

$$Y(NO) = \frac{1}{(NPQ + 1 + qL(\frac{F_m}{F_0 - 1}))} \quad (5)$$

$$Y(NPQ) = 1 - Y(II) - Y(NO) \quad (6)$$

3.2.3.4. Analysis of total nitrogen and total phosphorous in the stationary remediation system

Samples of synthetic wastewater and half-strength Hutner's medium were taken on the initial and final days of the five-day experiments in order to quantify total nitrogen (TN) and total phosphorous (TP) using a Hach machine (DR3900). For TN, Hach test LCK138 was used.

Firstly, the sample was digested with peroxo-disulphate for one hour at 100 °C causing inorganically and organically bonded nitrogen to oxidise to nitrate (Koroleff digestion). The resulting oxidised nitrate was then analysed photometrically in a reaction with 2,6-dimethylphenol. For TP, Hach test LCK348 was used. Firstly, the wastewater sample was digested using the persulphate digestion method for one hour at 100 °C. The resulting solution was then analysed photometrically through the ascorbic acid/phosphomolybdenum blue method.

3.2.3.5. Analysis of total nitrogen and total phosphorous analysis in the re-circulating remediation system

Samples of wastewater were taken for TN and TP analysis on the initial and final days of the three-day experiment. These samples were sent to an external lab for analysis (Aquatic Services Unit, Environmental Research Institute, University College Cork, Ireland). For TN, the unfiltered sample was digested with potassium persulfate and boric acid in alkaline conditions in an autoclave at 120 °C for 30 minutes. The resulting total oxidised nitrogen was analysed by automated cadmium reduction method using Lachat Quikchem 8000 by Zellweger Analytics, Inc. Milwaukee. USA (Grasshoff et al. 2009). For TP, the unfiltered sample was digested with ammonium persulfate in acidic conditions in an autoclave at 120 °C for 30 minutes. The resulting phosphate was analysed manually using the Murphy & Riley Method (Rice et al. 2005).

3.2.3.6. Protein analysis

Lemna minor biomass samples were taken on the final day of each experiment and stored in a -20 °C freezer. Protein was extracted from stored samples using 50 mM potassium phosphate buffer, pH 7, containing 0.1 mM EDTA and 0.1 mM polyvinylpyrrolidone (PVP). For protein measurements, between 50–80 mg of plant sample was weighed out, then homogenised in cold potassium phosphate buffer (1mL of buffer to 80 mg of plant sample). The homogenised

sample was centrifuged at 20,000 x *g* for 30 minutes at 4 °C (Balen et al. 2011). The supernatant was used for soluble protein analysis using the Bradford method with bovine serum albumin as a standard (Bradford 1976). Following this, 5 µL of sample was added to 1 mL of Bradford reagent in a cuvette and left for five minutes in dark conditions, then this sample was measured at 595 nm using a spectrophotometer (Shimadzu UV-160A).

3.2.4. Data analysis

Statistical analyses were conducted using R software (R Core Team (2019), R 3.4.3). Numbers of independent replicates are stated in figure legends. One-way and two-way ANOVAs were used to analyse differences in RGR, TN and TP removal rates, and protein content between treatments. A post hoc Tukey test was used for pairwise comparisons of treatment groups. For heteroscedastic datasets a Welch's ANOVA was used. A significant result indicates a *p*-value that is less than 0.05 ($p < 0.05$).

3.3. Results

3.3.1. Stationary remediation system

3.3.1.1. RGR for *L. minor* grown on synthetic wastewater or half-strength Hutner's medium

When grown on synthetic wastewater under a range of ten light intensities, *L. minor* kept under 50 µmol m^{-2} s^{-1} showed a higher RGR (d^{-1}) compared to plants kept under 10 µmol m^{-2} s^{-1} PAR (Figure 3.1a). However, above 50 µmol m^{-2} s^{-1} the RGR plateaued and gradually decreased as light intensity increased (Welch's ANOVA: $p = 0.1$; Figure 3.1a).

RGR for *L. minor* grown on either of two media, synthetic wastewater or half-strength Hutner's, displayed different trends in response to increasing light intensity (Figure 3.1b). Both light (two-way ANOVA: $p = 0.001$; Table 3.1) and media (two-way ANOVA: $p = 0.047$; Table

3.1) affected *L. minor* RGR. When grown on half-strength Hutner's *L. minor* RGR increased with increasing light intensity, rising from 0.24 d^{-1} at 50 µmol m^{-2} s^{-1} to 0.43 d^{-1} at 850 µmol m^{-2} s^{-1} (post hoc Tukey: $p < 0.001$; Figure 3.1b; Table S3.6). For *L. minor* grown on synthetic wastewater the mean RGR values remained close to 0.3 d^{-1} at all light intensities (post hoc Tukey: $p > 0.05$; Figure 3.1b; Table S3.6). An interactive effect was found between light intensity and media (two-way ANOVA: $p = 0.001$; Table 3.1), which indicates that light and media had a combined effect on *L. minor* RGR.

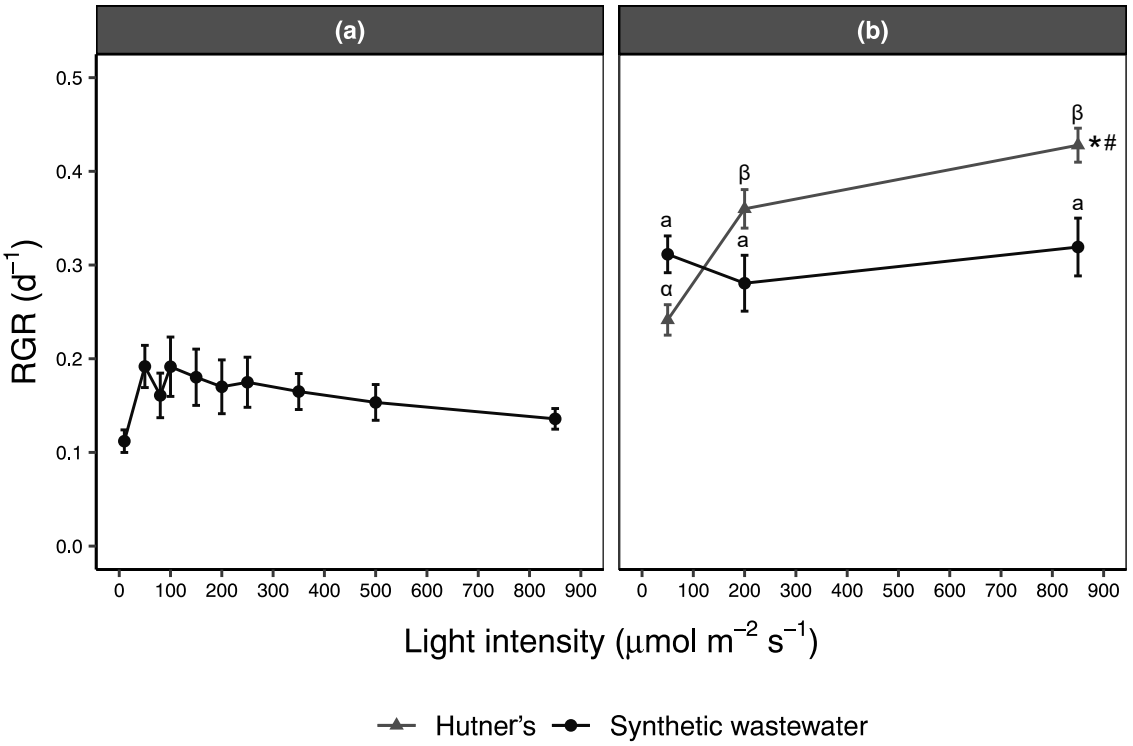

Figure 3.1. Mean (±SE) fresh biomass RGR (d^{-1}) of (a) *Lemna minor* grown on synthetic wastewater under ten light intensities ($n = 8$), and (b) *L. minor* grown under three different light intensities on synthetic wastewater or half-strength Hutner's medium ($n = 6$). An asterisk (*) denotes an effect of media for $p < 0.05$, while a hash symbol (#) denotes an effect of light intensity for $p < 0.01$, as per the two-way ANOVA (see Table 3.1). Points that do not share the same letter significantly differ from one another, as per the Tukey post hoc test, $p < 0.05$ (see Table S3.6)

Table 3.1. Summary of two-way ANOVA test for the effects of light intensity and media on *Lemna minor* RGR (d^{-1}), TN and TP removal rate (mg N/P m^{-2} d^{-1}), and protein content (%).

Measurement	Term	df	F-statistic	p-value
RGR	Light intensity	2	8.759	0.001
	Media	1	4.297	0.047
	Light intensity*Media	2	8.502	0.001
TN	Light intensity	2	1.945	0.160
	Media	1	5.447	0.027
	Light intensity*Media	2	0.405	0.671
TP	Light intensity	2	0.113	0.894
	Media	1	7.665	0.010
	Light intensity*Media	2	0.153	0.858
Protein	Light intensity	2	17.260	0.00001
	Media	1	0.000	0.997
	Light intensity*Media	2	2.066	0.144

3.3.1.2. Chlorophyll *a* fluorescence of *L. minor* grown on synthetic wastewater or half-strength Hutner's medium

Chlorophyll fluorescence measurements were taken for *L. minor* grown on either synthetic wastewater or half-strength Hutner's medium under three different light intensities on the final day of the experiment. The maximum quantum efficiency of photosystem II (F_v/F_m), decreased for *L. minor* on both media as the light intensity increased (two-way ANOVA: $p < 0.001$; Figure 3.2a; Table 3.2). F_v/F_m for *L. minor* grown on synthetic wastewater decreased from 0.78 at 50 µmol m^{-2} s^{-1} to 0.67 at 850 µmol m^{-2} s^{-1} (post hoc Tukey: $p = 0.003$; Figure 3.2a; Table S3.7). The post hoc tests did not reveal any significant decrease for plants on half-strength Hutner's.

At 50 µmol m^{-2} s^{-1}, the mean Y(II), the quantum efficiency of PSII under steady state light conditions, was almost equal irrespective of the medium. As light intensity increased, however, differences in Y(II) between *L. minor* grown on synthetic wastewater or half-strength Hutner's

were observed (two-way ANOVA: p = 0.01; Figure 3.2b; Table 3.2). As light intensity increased to 200 µmol m^{-2} s^{-1} the Y(II) of *L. minor* grown on half-strength Hutner's increased more than the Y(II) of *L. minor* grown on synthetic wastewater. At 850 µmol m^{-2} s^{-1} Y(II) decreased irrespective of medium, but this decrease was much stronger for *L. minor* grown on synthetic wastewater. In addition, an interactive effect on Y(II) between light intensity and media was observed (two-way ANOVA: p = 0.052; Table 3.2).

Y(NPQ), the yield of regulated energy dissipation, for *L. minor* grown under 50 µmol m^{-2} s^{-1} was similar irrespective of medium. At higher light intensities, Y(NPQ) values diverged depending on the medium (two-way ANOVA: p = 0.015; Figure 3.2c; Table 3.2). As light intensity increased to 200 and 850 µmol m^{-2} s^{-1} *L. minor* grown on half-strength Hutner's showed a lower Y(NPQ) value than that of *L. minor* grown on synthetic wastewater (Figure 3.2c). The Y(NO), the yield of non-regulated energy dissipation, of *L. minor* was mostly steady across all light intensities and media conditions (two-way ANOVA: p > 0.05; Figure 3.2d; Table 3.2).

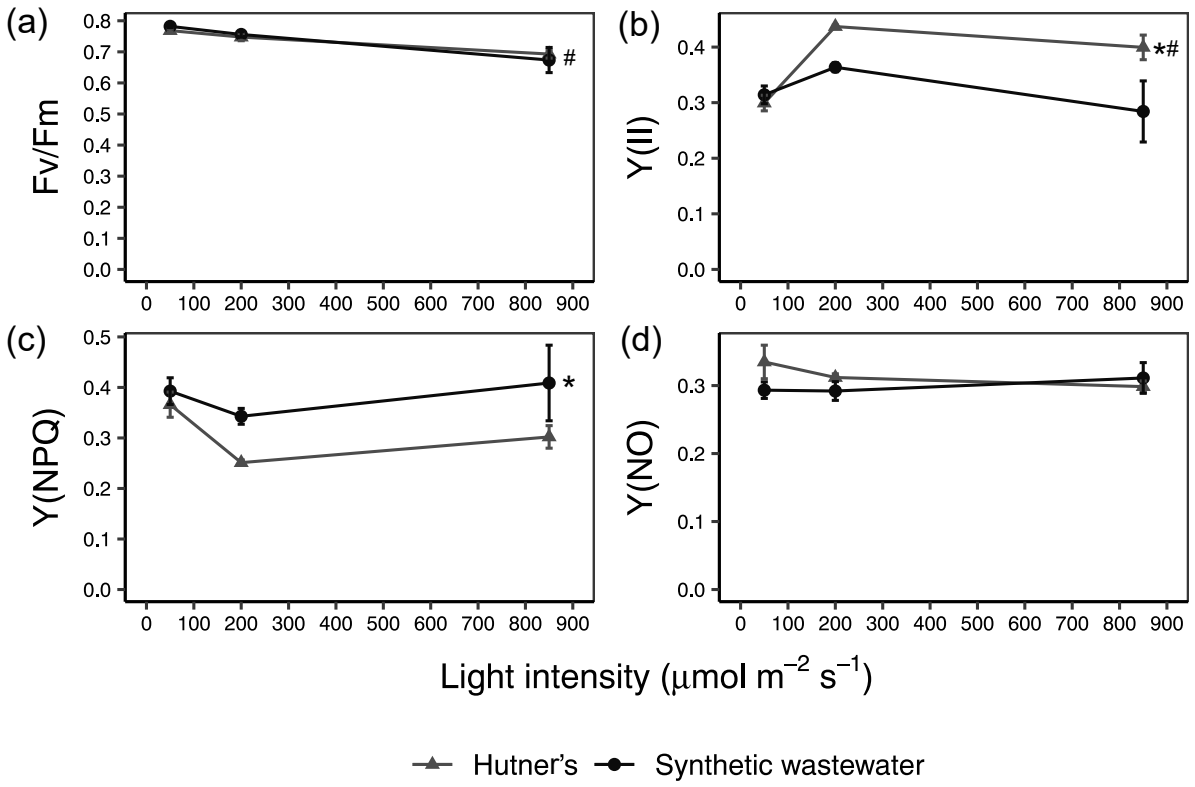

Figure 3.2. Mean (±SE) values of (a) F_v/F_m, (b) Y(II), (c) Y(NPQ), and (d) Y(NO) for *Lemna minor* grown under three different light intensities on synthetic wastewater or half-strength Hutner's medium (n = 6). An asterisk (*) denotes an effect of media for $p < 0.05$, while a hash symbol (#) denotes an effect of light intensity for $p < 0.01$, as per the two-way ANOVA (see Table 3.2)

Table 3.2. Summary of two-way ANOVA tests for the effects of light intensity and media on *Lemna minor* F_v/F_m, Y(II), Y(NPQ) and Y(NO).

Measurement	Term	df	F-statistic	p-value
F_v/F_m	Light intensity	2	13.241	0.00007
	Media	1	0.004	0.952
	Light intensity*Media	2	0.454	0.639
Y(II)	Light intensity	2	6.647	0.004
	Media	1	7.475	0.010
	Light intensity*Media	2	3.270	0.052
Y(NPQ)	Light intensity	2	2.800	0.077
	Media	1	6.596	0.015
	Light intensity*Media	2	0.704	0.503
Y(NO)	Light intensity	2	0.306	0.739
	Media	1	1.528	0.226
	Light intensity*Media	2	1.439	0.253

3.3.1.3. Total nitrogen (TN) removal for *L. minor* grown on synthetic wastewater or half-strength Hutner's medium

Removal of TN from synthetic wastewater by *L. minor* was measured under ten different light intensities (Table S3.3), and then used to calculate the mean daily TN removal rates for the duration of the experiment, i.e. mg of N removed per initial m^2 of *L. minor* per day (mg N m^{-2} d^{-1}: Day 0–5). No particular pattern or trend of TN removal was found as a function of light intensity (ANOVA: p = 0.688; Figure 3.3a). Nevertheless, mean TN removal rates were variable but standard errors were substantial.

Removal of TN from synthetic wastewater or half-strength Hutner's medium by *L. minor* was measured under three different light intensities (Table S3.4), and then used to calculate TN removal rates (mg N m^{-2} d^{-1}). Overall, *L. minor* grown on synthetic wastewater had higher TN removal rates than *L. minor* grown on half-strength Hutner's (two-way ANOVA: p = 0.027; Figure 3.3b; Table 3.1). For *L. minor* grown on half-strength Hutner's, the mean TN removal rate increased with increasing light intensity. Whereas for *L. minor* grown on synthetic

wastewater, the mean TN removal rate increased only at the highest light intensity, 850 µmol m^{-2} s^{-1}, but at all light intensities substantial standard errors were observed (Figure 3.3b). A post hoc Tukey test of TN removal rate did not show significant difference between the light intensity treatments (Table S3.6). Further analysis showed that plants grown on half-strength Hutner's displayed a strong linear correlation between growth rate (RGR) and TN removal rate (Figure 3.3c), whereas plants grown on synthetic wastewater had similar growth rates with no particular association with TN removal rate.

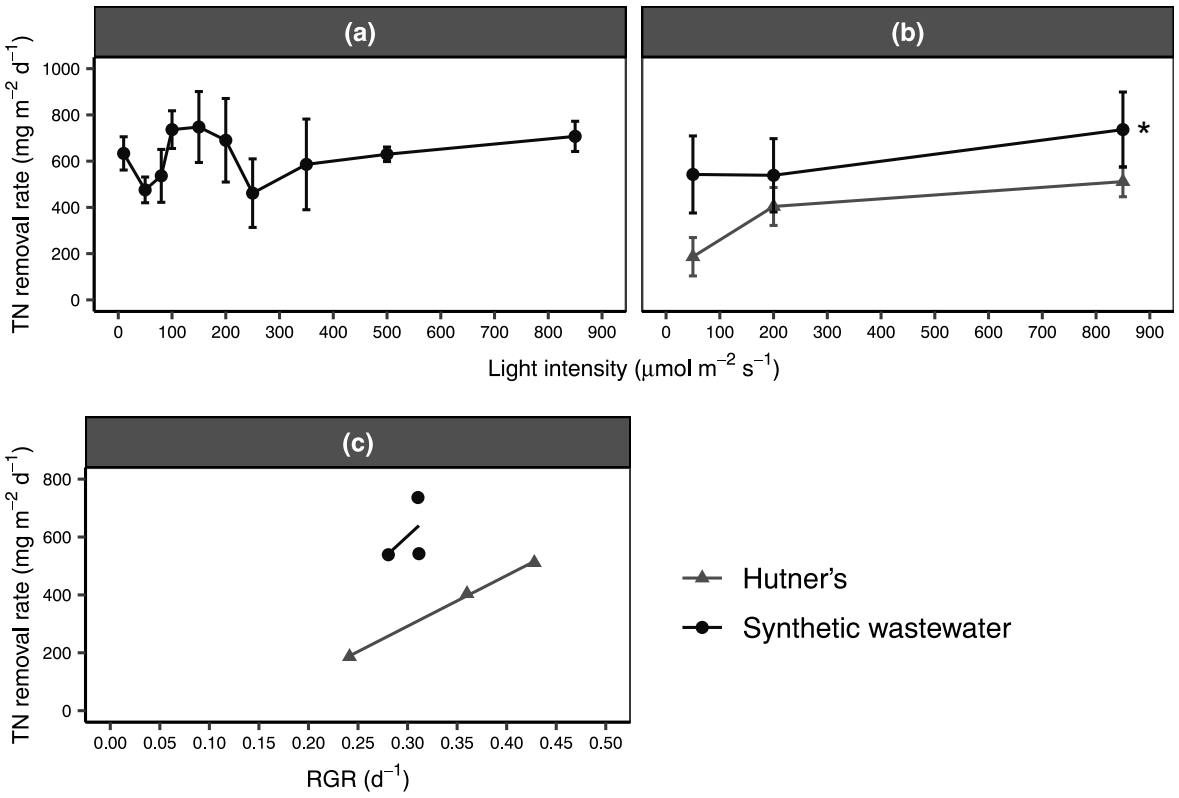

Figure 3.3. Mean (±SE) values for (a) TN removal rate (mg N m^{-2} d^{-1}) from synthetic wastewater under ten light intensities (n = 3), (b) TN removal rate (mg N m^{-2} d^{-1}) under three different light intensities from synthetic wastewater or half-strength Hutner's medium (n = 6), and (c) mean TN removal rate (mg N m^{-2} d^{-1}) against RGR for synthetic wastewater and half-strength Hutner's medium, with linear fit lines (n = 3). An asterisk (*) denotes an effect of media for $p < 0.05$, as per the two-way ANOVA (see Table 3.1)

3.3.1.4. Total phosphorous (TP) removal for *L. minor* grown on synthetic wastewater or half-strength Hutner's medium

Removal of TP from synthetic wastewater by *L. minor* was measured under ten different light intensities (Table S3.3), and then used to calculate the mean daily TP removal rates for the duration of the experiment i.e. mg P removed per initial m^2 of *L. minor* per day (mg P m^{-2} d^{-1}: Day 0-5). As observed for TN, no light-dependency of TP removal was discerned (ANOVA: $p = 0.75$; Figure 3.4a).

Removal of TP from synthetic wastewater or half-strength Hutner's medium by *L. minor* was measured under three different light intensities (Table S3.4), and then used to calculate TP removal rates (mg P m^{-2} d^{-1}). The TP removal rate differed between *L. minor* grown on synthetic wastewater and *L. minor* grown on half-strength Hutner's (two-way ANOVA: $p = 0.01$; Figure 3.4b; Table 3.1). However, light intensity did not exert a strong effect on TP removal rate (two-way ANOVA: $p = 0.894$; Figure 3.4b; Table 3.1). The mean TP removal rate for plants grown on half-strength Hutner's increased moderately from 50 to 200 µmol m^{-2} s^{-1} but then reduced at 850 µmol m^{-2} s^{-1} (Figure 3.4b). The mean TP removal for plants grown on synthetic wastewater medium increased moderately with increasing light intensity (Figure 3.4b). A post hoc Tukey test did not identify significant differences between light intensity treatments (Table S3.6).

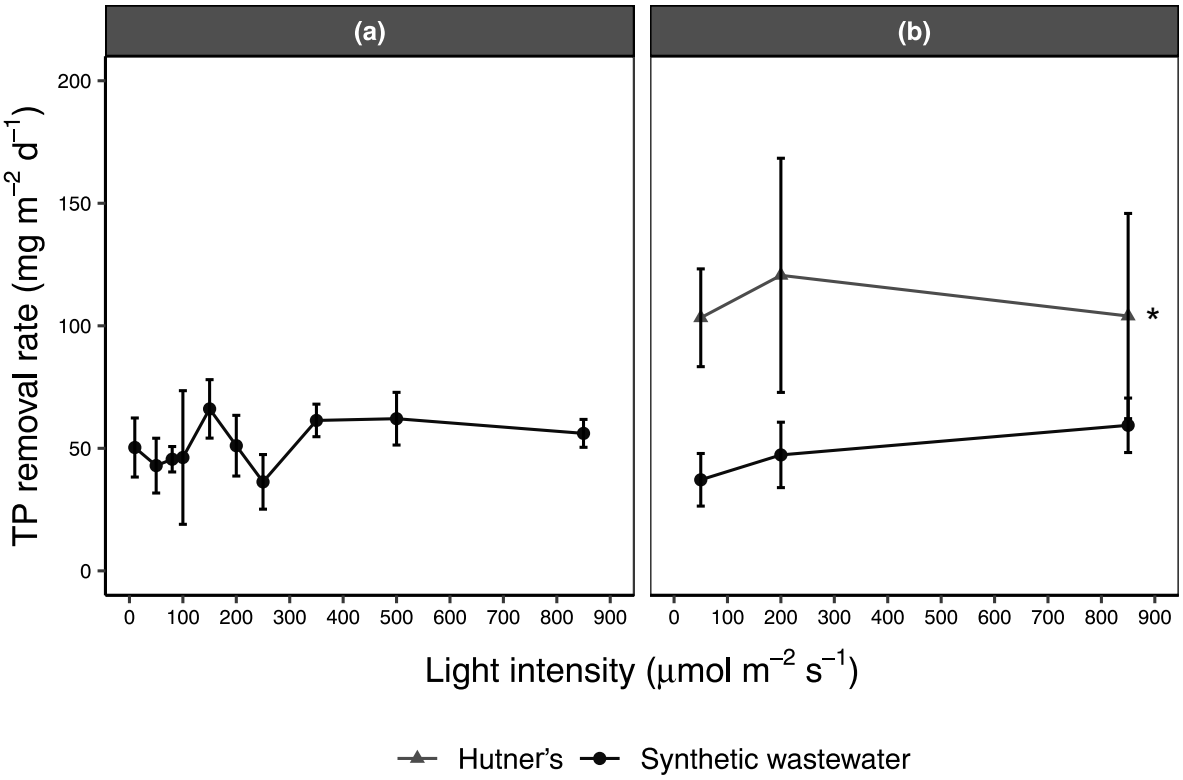

Figure 3.4. Mean (±SE) values for (a) TP removal rate (mg P m^{-2} d^{-1}) from synthetic wastewater under ten light intensities (n = 4, except at 100 and 150 µmol m^{-2} s^{-1} where n = 3), and (b) TP removal rate (mg P m^{-2} d^{-1}) under three different light intensities from synthetic wastewater or half-strength Hutner's medium (n = 6). An asterisk (*) denotes an effect of media for $p < 0.05$, as per the two-way ANOVA (see Table 3.1)

3.3.1.5. Protein content of *L. minor* grown on synthetic wastewater or half-strength Hutner's medium

When grown on synthetic wastewater under a range of ten light intensities, *L. minor* protein content (% protein of fresh biomass) increased with increasing light intensity (ANOVA: $p < 0.001$; Figure 3.5a). A post hoc Tukey test showed significant differences between plant protein content at 10 and 50 µmol m^{-2} s^{-1} and that at 350, 500 and 850 µmol m^{-2} s^{-1} ($p < 0.01$ and $p < 0.05$, respectively; Table S3.8).

Lemna minor protein content (% protein of fresh biomass) for plants grown on either half-strength Hutner's medium or synthetic wastewater increased with increasing light intensity (two-way ANOVA: $p < 0.001$; Figure 3.5b; Table 3.1). For *L. minor* grown on half-strength Hutner's, significant differences were found between protein content at 50 and 200 µmol m^{-2} s^{-1}, and that at 850 µmol m^{-2} s^{-1} (post hoc Tukey, 50-850: $p < 0.001$, 200-850: $p = 0.003$; Table S3.6). For *L. minor* grown on synthetic wastewater a borderline significant p-value of $p = 0.055$ was found (as per post hoc Tukey, Table S3.6), comparing 50 and 850 µmol m^{-2} s^{-1}.

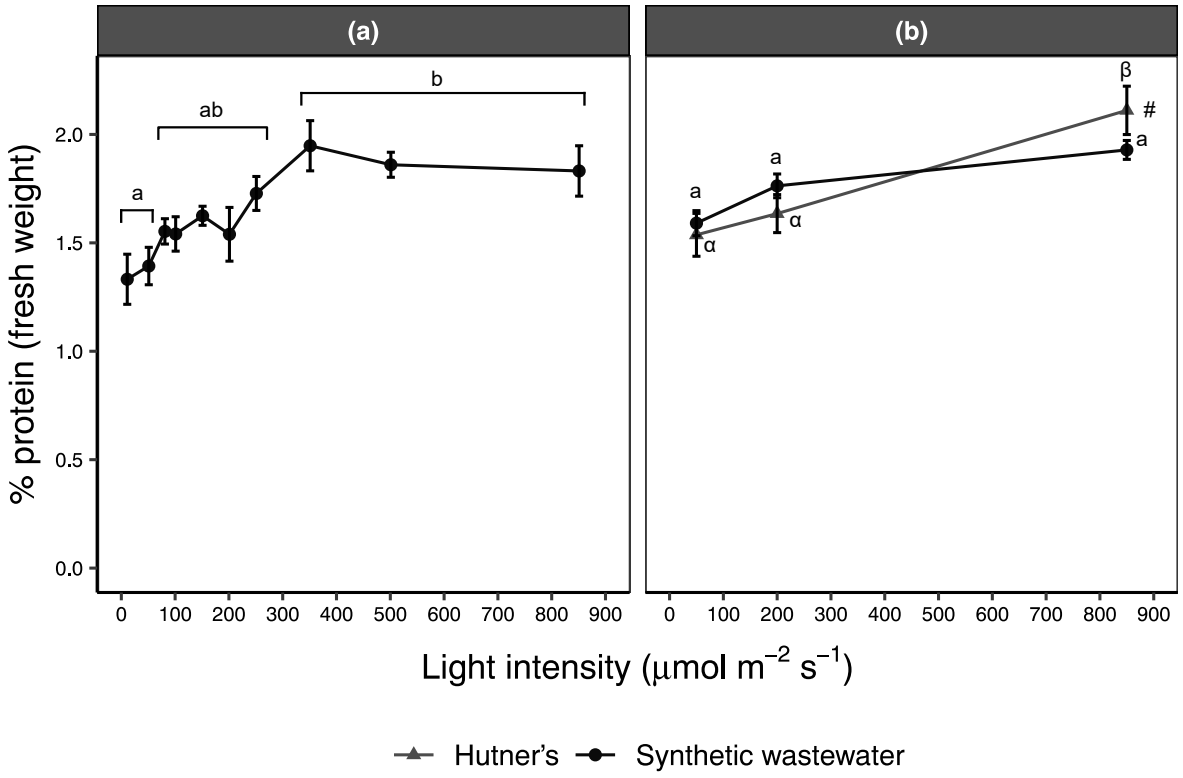

Figure 3.5. Mean (±SE) values for (a) *L. minor* protein content (% protein of fresh *Lemna minor* biomass) under ten light intensities ($n = 6$), and (b) *L. minor* protein content (% protein of fresh *L. minor* biomass) under three different light intensities on synthetic wastewater or half-strength Hutner's medium ($n = 6$). Based on a typical dry biomass content of 4%, the protein content on a dry weight basis is on average 33-50 % and 38-53 % for plants grown on half-strength Hutner's or synthetic wastewater, respectively. A hash symbol (#) denotes an effect of light intensity for $p < 0.001$, as per the two-way ANOVA (see Table 3.1). Points that do not share the same letter, significantly differ from one another for $p < 0.05$, as per the Tukey post hoc test (see Tables S3.6 and S3.8)

3.3.2. Re-circulating remediation system

The mean RGR of *L. minor* grown in re-circulating tanks under three different light intensities increased with increasing light intensity, from 0.21 to 0.27 d^{-1} (ANOVA: $p = 0.475$; Figure 3.6a). TN and TP removal from synthetic wastewater in re-circulating tanks was measured over the course of the experiment (Table S3.5). TN and TP removal rates (mg N/P m^{-2} d^{-1}) were calculated from the change in concentration between start and end date (days 0-3). The mean TN removal rate increased with light intensity but exhibited substantial standard errors at 300 and 900 µmol m^{-2} s^{-1} (ANOVA: $p = 0.479$; Figure 3.6b). The TP removal rate was similar at 100 and 300 µmol m^{-2} s^{-1} but increased at 900 µmol m^{-2} s^{-1} (ANOVA: $p = 0.008$; Figure 3.6c). Significant differences were found between the TP removal rate at 100 and 300 µmol m^{-2} s^{-1} compared to that at 900 µmol m^{-2} s^{-1} (post hoc Tukey: $p = 0.01$, $p = 0.03$, respectively; Figure 3.6c). *L. minor* protein concentration (% protein of fresh duckweed biomass) increased moderately with increasing light intensity (ANOVA: $p = 0.294$; Figure 3.6d).

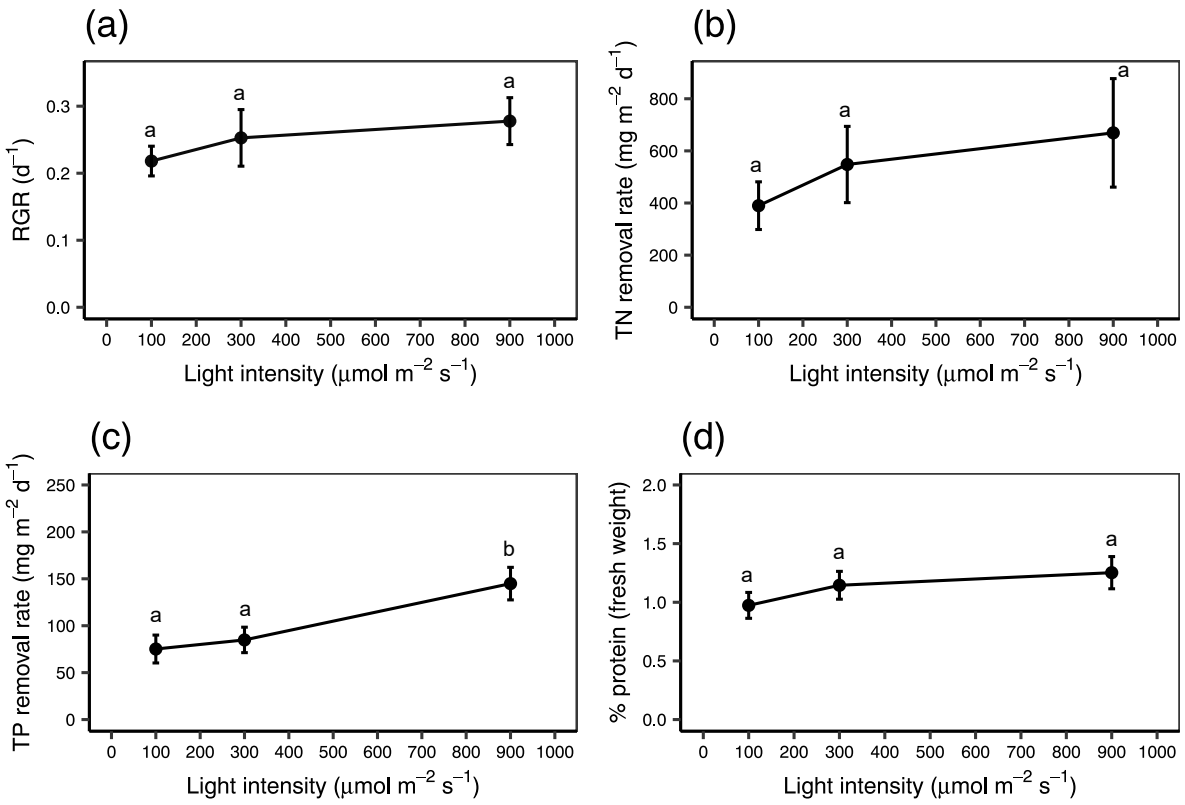

Figure 3.6. Mean (±SE) for *Lemna minor* (a) RGR, (b) TN removal rate (mg N m^{-2} d^{-1}), (c) TP removal (mg P m^{-2} d^{-1}), and (d) protein content (% protein of fresh weight) grown on synthetic wastewater under three different light intensities in re-circulating tanks (n = 8). Based on a typical dry biomass content of 4 %, the protein content on a dry weight basis is on average 24-30 %. Points that do not share the same letter, significantly differ from one another for $p < 0.05$, as per the Tukey post hoc test

3.4. Discussion

In general, growth rates documented in the present study for *Lemna minor* "Blarney", cultivated on synthetic dairy processing wastewater, are slightly lower or similar to those found in the literature for duckweed grown on optimised media (Ziegler et al. 2015; Paolacci et al. 2016, 2018), but similar or greater than those for duckweed grown on wastewater (Caicedo et al. 2000; Iatrou et al. 2015). *L. minor* grown on half-strength Hutner's medium displayed comparable growth rates to those found in the literature (Ziegler et al. 2015). However, there

was a major difference in the way *L. minor* responded to light when grown on either synthetic wastewater or half-strength Hutner's. Typically, duckweed growth rates increase until light is saturating, at which point growth rates plateau. The growth saturation point for Lemnaceae has been found to range between 250–750 µmol m^{-2} s^{-1}, depending on species and clone (Landolt and Kandeler 1987). Specifically, the saturation point for *L. minor* has been identified as between 400–600 µmol m^{-2} s^{-1} (Wedge and Burris 1982; Paolacci et al. 2018). In this context the light response curve for *L minor* grown on synthetic wastewater is unusual in that it already shows growth saturation at 50 µmol m^{-2} s^{-1}. The same pattern was found in the scaled-up 11.7 L re-circulating tank system in which *L. minor* reached growth saturation at around 100 µmol m^{-2} s^{-1}.

To explore why the growth of *L. minor* on synthetic wastewater did not increase with increasing light intensities, photosynthetic efficiency was quantified. Measurements of *L. minor* Y(II), the quantum yield of PSII (Murchie and Lawson 2013), and Y(NPQ), the proportion of energy being quenched by non-photochemical processes such as heat dissipation (Kramer et al. 2004), diverged at light intensities above 50 µmol m^{-2} s^{-1} between the two media. *L. minor* grown on synthetic wastewater was less capable of using additional light energy, as shown by lower Y(II) values (Genty et al. 1989), and dissipated increasing amounts of radiation energy through the xanthophyll cycle (Horton et al. 1996), as shown by higher values for Y(NPQ) (Klughammer and Schreiber 2008). Such regulated energy dissipation does not necessarily mean photo-inhibitory damage has occurred, and this is seen in the similar values for F_v/F_m, the maximum quantum yield of photosystem II (Murchie and Lawson 2013), and Y(NO), the proportion of light energy being dissipated in a non-regulated manner (Kramer et al. 2004), that were observed for *L. minor* grown in both media. An increase in Y(NO) would have been indicative of a plant struggling to cope with excess radiation due to photochemical damage or damage to its light-protective mechanisms (Klughammer and Schreiber 2008). Similar F_v/F_m values

indicate that PSII is not directly negatively affected in either growth medium. Rather, photosynthesis in *L. minor* growing in synthetic wastewater is likely to be disrupted at a point beyond PSII (Kanazawa and Kramer 2002; Vredenberg 2018).

A previous study, Walsh et al. (2020), which documented the concentration of elemental components in synthetic wastewater and half-strength Hutner's medium revealed some elements that, due to their concentration, may potentially impede ATP generation or the Calvin cycle. In particular, copper, which is present in higher amounts in synthetic wastewater than in half-strength Hutner's (5 and 0.12 μM, respectively), can inhibit phosphorylation by ATP synthase (Uribe and Stark 1982; Maksymiec 1998). A consequence of impeding ATP generation is the build-up of a proton gradient across the thylakoid lumen (Kramer et al. 2003). In turn, this gradient causes non-photochemical quenching, Y(NPQ), to be increased through the xanthophyll cycle (Horton et al. 1996; Li et al. 2002), as was observed in this study. Moreover, magnesium, which is present at much lower concentrations in synthetic wastewater than in half-strength Hutner's (0.2 and 3 mM, respectively), can adversely affect ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity if present in deficient amounts (Farhat et al. 2016). Whilst manganese, which can negatively affect carbon assimilation when present in excess amounts (Li et al. 2010), is present in a higher concentration in synthetic wastewater than in half-strength Hutner's (37 and 0.591 μM, respectively). Therefore, we argue that through subtle disruptions in plant physiology, the cultivation of *L. minor* on synthetic dairy wastewater resulted in increased energy dissipation, inhibition of photosynthetic yield and a lack of growth acceleration at higher light intensities. It can be concluded that synthetic dairy wastewater can support the cultivation of *L. minor* under low light intensities but is less suitable under higher light intensities due to interactive effects between light and media composition.

The detected removal rates of TN from synthetic wastewater are in-line with those documented for duckweed within the literature, which range from 500–2100 mg N m^{-2} d^{-1} (Körner and

Vermaat 1998; Cheng et al. 2002; Mohedano et al. 2012). Removal rates vary depending on the Lemnaceae species or clone used (Zhao et al. 2014), the types of nitrogen source available (Fang et al. 2007), the type of wastewater being remediated (Toyama et al. 2018) and the type of system used (e.g. outdoor or indoor) (Cheng and Stomp 2009). In addition, luxury uptake of nitrogen has been reported for some plant species (Lipson et al. 1996), and this would distort the relationship between growth and nitrogen uptake. However, to our knowledge, no records of luxury nitrogen uptake by duckweed species have been reported. In the present study, nitrogen uptake was closely linked with *L. minor* RGR for each specific medium. Although half-strength Hutner's medium contains approximately 20 % more available nitrogen than synthetic dairy wastewater (see Table S3.1; Hutner 1953), greater nitrogen removal by *L. minor* occurred for plants grown on synthetic wastewater, despite these plants having lower growth rates. The relatively high TN removal rates from synthetic wastewater are likely due to the type of nitrogen available. Synthetic wastewater contains ammonia and urea as its nitrogen sources, whereas in Hutner's medium nitrate is the sole nitrogen source (Hutner 1953). It has previously been shown that ammonia is more readily taken up than nitrate by *L. minor* (Feller and Erismann 1971; Landolt and Kandeler 1987). Furthermore, *L. minor* shows a preference for ammonia over nitrate when both nutrients are available (Feller and Erismann 1971; Porath and Pollock 1982). Thus, the relationship between RGR and TN removal is further modified by the available form of nitrogen.

Lemna minor has been shown to take up more phosphorus than it requires when this is available in high concentrations, consequently, such luxury uptake can distort the relationship between RGR and TP uptake (Chaiprapat et al. 2005). Whilst the TP removal rates observed in this study are in the lower portion of the published range (i.e. 20–590 mg P m^{-2} d^{-1}) (Körner and Vermaat 1998; Cheng et al. 2002; Mohedano et al. 2012), the TP removal rate for *L. minor* grown on half-strength Hutner's was double the rate for *L. minor* grown on synthetic

wastewater. As half-strength Hutner's medium contained substantially more phosphorus than synthetic dairy wastewater (93 mg L^{-1} and 10.9 mg L^{-1}, respectively; Table S3.1; Hutner 1953), luxury phosphorus uptake by plants grown in half-strength Hutner's may have occurred.

Typically, duckweed protein content can vary between 10 and 40 % of dry duckweed biomass (Landolt and Kandeler 1987; Bergmann et al. 2000). The protein content found in this study is presented as mg of protein per mg of fresh *L. minor* biomass, which was used to calculate protein per dry *L. minor* biomass for a comparison with literature sources (a direct measurement of dry weight would be worthwhile for future studies). Based on a *L. minor* dry biomass content of 4 % (Landolt and Kandeler 1987; Appenroth et al. 2017), the average protein content for *L. minor* grown on half-strength Hutner's was 44 % and on synthetic wastewater 42 %. The protein content of *L. minor* increased with greater light intensity, for plants grown on both synthetic wastewater and half-strength Hutner's. Previous studies have documented that higher light intensities lead to greater allocation of total nitrogen from non-protein nitrogen-containing components to soluble protein in C3 plants such as duckweed (Evans 1989; Evans and Seemann 1989). It is thought this process may be associated with the increased production of Rubisco, a soluble protein, at higher light intensities (Evans 1989). The increase in TN removal with increasing light intensity, as seen for *L. minor* grown on half-strength Hutner's, does not increase the proportion of soluble protein content in these plants any more than was observed for *L. minor* grown on synthetic wastewater. This greater TN uptake may instead have increased the overall nitrogen content of *L. minor* grown on half-strength Hutner's (Landolt and Kandeler 1987; Evans and Seemann 1989).

In general, *L. minor* grown on synthetic wastewater in re-circulating tanks had similar growth rates, TN and TP removal rates, and protein content, as *L. minor* grown on synthetic wastewater in stationary conditions. The protein content for *L. minor* grown on synthetic wastewater in re-circulating tanks was generally lower, but exhibited the same trend under different light

intensity conditions as documented for plants grown in stationary conditions. The lower protein content detected for *L. minor* grown in re-circulating systems may reflect an unknown effect linked to upscaling and this should be considered for future research. The effects of light intensity on the measured parameters were mostly similar to the stationary system with the exception of TN and TP removal at the highest light intensities. The presence of algae and microbes in re-circulating tanks may have contributed to a modest increase in the mean TN removal at higher light intensities without an associated increase in *L. minor* RGR (Zhao et al. 2015). The presence of microbiota can lead to the loss of nitrogen from a wastewater treatment system through the bacterial nitrification-denitrification process (Thakur and Medhi 2019). Furthermore, the presence of algae and a microbial biofilm has also been reported to contribute to the removal of nitrogen and phosphorous in a duckweed-based system (Körner and Vermaat 1998). Algae in particular can compete with duckweed for nutrients (Roijackers et al. 2004), potentially decreasing duckweed-mediated uptake of nitrogen and phosphorus but increasing nutrient uptake overall. The relatively high density of duckweed in the system (60 %) may have negated a strong effect of algal competitors (Roijackers et al. 2004). The volume of wastewater to *L. minor* biomass, which is greater in re-circulating tanks, around 1.6 L to 1 g compared to 0.5 L to 1 g in stationary experiments, may have been a factor in the response of TP removal rate to different light intensities. The greater availability of phosphate may saturate luxury uptake and restore the relationship between growth and phosphorous removal (Paolacci et al. 2016). As duckweeds naturally come from still or slow-moving waters (Landolt 1986), water movement and currents, such as those introduced by the re-circulating system, can have a negative impact on *L. minor* growth (Iqbal 1999). However, the high density of duckweed seemed to have a positive effect by stabilising the duckweed and reducing the impact of moving water. Overall, no adverse effects on *L. minor* growth were noted in the re-circulating system. Despite the noted differences, it can be argued that simple stationary, sterile systems can

meaningfully inform on phytoremediation potential prior to investment in larger and more complex re-circulating systems.

3.5. Conclusion

Lemna minor has been shown to grow on and remediate a synthetic wastewater which mirrors real dairy processing wastewater. Attempts to accelerate this process by using higher light intensities showed that, remarkably, *L. minor* growth rates and TN and TP removal rates did not differ significantly across a wide range of light intensities. However, when compared to *L. minor* grown on half-strength Hutner's medium, a media-dependent effect of light intensity was detected. These findings will inform the design and operational parameters of indoor duckweed-driven phytoremediation systems, as a lower light intensity could reduce costs and energy consumption. Yet, the use of higher light intensities can also result in higher *L. minor* protein content, which may represent a supplementary source of income, improving the financial viability of the phytoremediation process. As such, an evaluation between the cost of higher light intensity and the potential financial gain of additional protein content will need to be considered.

Author Contributions

ÉW, HK and MAKJ contributed to the study conception and design. Material preparation, data collection and analysis were performed by ÉW, HK and SOB. The first draft of the manuscript was written by ÉW and all authors contributed to reviewing and editing the manuscript.

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Supplementary Material

Table S3.1. Synthetic dairy wastewater composition

Chemical	Concentration (mg L^{-1})[1]
Ammonium chloride	167.3
Urea	129.9
Disodium phosphate	50
Potassium bicarbonate	50
Sodium bicarbonate	130
Calcium chloride dihydrate	2
Calcium sulphate	14.6[2]
Magnesium sulphate heptahydrate	50
Manganese sulphate monohydrate	2
Iron sulphate heptahydrate	35
Zinc sulphate heptahydrate	2.15
Cobalt chloride hexahydrate	1.2
Manganese chloride tetrahydrate	4.95
Copper sulphate pentahydrate	1.25
Nickel chloride hexahydrate	0.95
Sodium molybdate dihydrate	1.1
Boric acid	0.07
Sodium selenite	0.49
EDTA	100

[1]Final concentration in synthetic dairy wastewater
[2]Supplemental calcium to ensure Ca:Mg ratio is favourable for *Lemna minor*

Table S3.2. Light intensity levels and measured average

Light intensity (µmol m^{-2} s^{-1})	Measured average light intensity (µmol m^{-2} s^{-1})
10	8.9
50	49.1
80	80.1
100	98.5
150	149.8
200	185.6
250	246.3
350	347.3
500	490.0
850	856.7

Table S3.3. Mean TN and TP removal (mg L^{-1} and mg) from synthetic dairy wastewater under a range of ten light intensities (n = 3 and n = 4, respectively).

Light intensity (µmol m^{-2} s^{-1})	TN removal (mg L^{-1})	TN removal (mg)	TP removal (mg L^{-1})	TP removal (mg)
10	17.8	1.78	1.44	0.14
50	13.53	1.35	1.26	0.13
80	16.73	1.67	1.43	0.14
100	23.13	2.31	0.38	0.14
150	23.33	2.33	0.87	0.20
200	21.53	2.15	1.6	0.16
250	14.47	1.45	1.13	0.11
350	18.47	1.85	1.92	0.19
500	19.8	1.98	1.95	0.19
850	22.2	2.22	1.76	0.18

Table S3.4. Mean TN and TP removal (mg L^{-1} and mg) from synthetic dairy wastewater or half-strength Hutner's medium under three different light intensities (n = 6).

Light intensity (µmol m^{-2} s^{-1})	Medium	TN removal (mg L^{-1})	TN removal (mg)	TP removal (mg L^{-1})	TP removal (mg)
50	Synthetic wastewater	14.4	1.44	1.03	0.10
50	Hutner's	6.07	0.61	3.25	0.33
200	Synthetic wastewater	15.63	1.56	1.47	0.15
200	Hutner's	12.83	1.28	3.78	0.38
850	Synthetic wastewater	20.16	2.016	1.65	0.17
850	Hutner's	15.45	1.48	3.09	0.31

Table S3.5. Mean TN and TP removal (mg $L^{-1}$and mg) from synthetic dairy wastewater in re-circulating tank experiments under three different light intensities (n = 8).

Light intensity (µmol m^{-2} s^{-1})	TN removal (mg L^{-1})	TN removal (mg)	TP removal (mg L^{-1})	TP removal (mg)
100	4.32	50.57	0.85	9.91
300	6.15	71.95	0.96	11.28
900	7.44	87.0	1.61	18.79

Table S3.6. Output of post hoc Tukey test for RGR (d^{-1}), TN and TP removal rate (mg N/P m^{-2} d^{-1}), and protein content (% fresh biomass) for *L. minor* grown on synthetic dairy wastewater or half-strength Hutner's medium under three different light intensities.

Term	Comparison	RGR p-value	TN p-value	TP p-value	Protein p-value
Light intensity:Media	200:Hutner-50:Hutner	0.013	0.811	0.998	0.951
Light intensity:Media	850:Hutner-50:Hutner	0.00005	0.445	1.0	0.0002
Light intensity:Media	50:Synthetic-50:Hutner	0.300	0.347	0.505	0.996
Light intensity:Media	200:Synthetic-50:Hutner	0.837	0.358	0.673	0.364
Light intensity:Media	850:Synthetic-50:Hutner	0.200	0.055	0.848	0.018
Light intensity:Media	850:Hutner-200:Hutner	0.331	0.989	0.998	0.0026
Light intensity:Media	50:Synthetic-200:Hutner	0.682	0.967	0.306	0.999
Light intensity:Media	200:Synthetic-200:Hutner	0.183	0.970	0.445	0.863
Light intensity:Media	850:Synthetic-200:Hutner	0.814	0.473	0.634	0.127
Light intensity:Media	50:Synthetic-850:Hutner	0.015	1.0	0.494	0.0009
Light intensity:Media	200:Synthetic-850:Hutner	0.001	1.0	0.661	0.045
Light intensity:Media	850:Synthetic-850:Hutner	0.027	0.819	0.839	0.594
Light intensity:Media	200:Synthetic-50:Synthetic	0.933	1.0	1.0	0.651
Light intensity:Media	850:Synthetic-50:Synthetic	0.999	0.893	0.991	0.055
Light intensity:Media	850:Synthetic-200:Synthetic	0.845	0.886	1.0	0.685

Table S3.7. Output of post hoc Tukey test for F_v/F_m, Y(II), Y(NPQ) and Y(NO) for *L. minor* grown on synthetic dairy wastewater or half-strength Hutner's medium under three different light intensities.

Term	Comparison	F_v/F_m p-value	Y(II) p-value	Y(NPQ) p-value	Y(NO) p-value
Light.intensity:Media	200:Hutner-50:Hutner	0.966	0.009	0.239	0.913
Light.intensity:Media	850:Hutner-50:Hutner	0.073	0.099	0.803	0.608
Light.intensity:Media	50:Synthetic-50:Hutner	0.995	0.999	0.994	0.466
Light.intensity:Media	200:Synthetic-50:Hutner	0.997	0.509	0.997	0.434
Light.intensity:Media	850:Synthetic-50:Hutner	0.013	0.998	0.956	0.902
Light.intensity:Media	850:Hutner-200:Hutner	0.324	0.907	0.912	0.991
Light.intensity:Media	50:Synthetic-200:Hutner	0.775	0.024	0.086	0.962
Light.intensity:Media	200:Synthetic-200:Hutner	0.999	0.368	0.474	0.950
Light.intensity:Media	850:Synthetic-200:Hutner	0.082	0.003	0.042	1.0
Light.intensity:Media	50:Synthetic-850:Hutner	0.022	0.215	0.486	1.0
Light.intensity:Media	200:Synthetic-850:Hutner	0.188	0.923	0.964	1.0
Light.intensity:Media	850:Synthetic-850:Hutner	0.976	0.040	0.310	0.993
Light.intensity:Media	200:Synthetic-50:Synthetic	0.916	0.754	0.919	1.0
Light.intensity:Media	850:Synthetic-50:Synthetic	0.003	0.963	0.999	0.967
Light.intensity:Media	850:Synthetic-200:Synthetic	0.040	0.282	0.781	0.957

Table S3.8. Output of post hoc Tukey test for *L. minor* protein concentration (% fresh biomass) under a range of ten light intensities

comparison	p-value
Light intensity: 50-10	1.0
Light intensity: 80-10	0.768
Light intensity: 100-10	0.819
Light intensity: 150-10	0.404
Light intensity: 200-10	0.861
Light intensity: 250-10	0.081
Light intensity: 350-10	0.0005
Light intensity: 500-10	0.005
Light intensity: 850-10	0.009
Light intensity: 80-50	0.958
Light intensity: 100-50	0.974
Light intensity: 150-50	0.716
Light intensity: 200-50	0.982
Light intensity: 250-50	0.229
Light intensity: 350-50	0.002
Light intensity: 500-50	0.019
Light intensity: 850-50	0.035
Light intensity: 100-80	1.0
Light intensity: 150-80	1.0
Light intensity: 200-80	1.0
Light intensity: 250-80	0.928
Light intensity: 350-80	0.083
Light intensity: 500-80	0.337
Light intensity: 850-80	0.474
Light intensity: 150-100	1.0
Light intensity: 200-100	1.0
Light intensity: 250-100	0.897
Light intensity: 350-100	0.066
Light intensity: 500-100	0.288
Light intensity: 850-100	0.415
Light intensity: 200-150	1.0
Light intensity: 250-150	1.0
Light intensity: 350-150	0.272
Light intensity: 500-150	0.700
Light intensity: 850-150	0.828
Light intensity: 250-200	0.917

comparison	p-value
Light intensity: 350-200	0.092
Light intensity: 500-200	0.344
Light intensity: 850-200	0.475
Light intensity: 350-250	0.772
Light intensity: 500-250	0.988
Light intensity: 850-250	0.998
Light intensity: 500-350	0.999
Light intensity: 850-350	0.995
Light intensity: 850-500	1.0

Chapter 4

Density dependence influences the efficacy of wastewater remediation by *Lemna minor*

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Abstract

As part of a circular economy (CE) approach to food production systems, Lemnaceae, i.e. duckweed species, can be used to remediate wastewater due to rapid nutrient assimilation and tolerance of non-optimal growing conditions. Further, given rapid growth rates and high protein content, duckweed species are a valuable biomass. An important consideration for duckweed-mediated remediation is the density at which the plants grow on the surface of the wastewater i.e. how much of the surface of the medium they cover. Higher duckweed density is known to have a negative effect on duckweed growth, which has implications for the development of duckweed-based remediation systems. In the present study, the effects of density (10-80 % plant surface coverage) on *Lemna minor* growth, chlorophyll fluorescence and nutrient remediation of synthetic dairy processing wastewater were assessed in stationary (100 mL) and re-circulating non-axenic (11.7 L) remediation systems. Overall, *L. minor* growth, and TN and TP removal rates decreased as density increased. However, in the stationary system, absolute TN and TP removal were greater at higher densities (50-80 % coverage). The exact cause of density related growth reduction in duckweed is unclear, especially at densities well below 100 % surface coverage. A further experiment comparing duckweed grown at 'low' and 'high' density conditions with the same biomass and media volume conditions, showed that photosynthetic yield, Y(II), is reduced at high density despite the same nutrient availability at both densities, and arguably similar shading. The results demonstrate a negative effect of high density on duckweed growth and nutrient uptake, and point towards signals from neighbouring duckweed colonies as the possible cause.

4.1. Introduction

Globally, the provision of nutritious food is a challenging endeavour (Ranganathan et al. 2016; Mehrabi et al. 2018). Climate change, a reduction in the *per capita* availability of arable land, as well as soil erosion, chemical overuse and finite resources have decreased food security (Obersteiner et al. 2013; Nearing et al. 2017; Pathak et al. 2018; Rosa et al. 2020). In recent years, the adoption of circular economy (CE) principles in food production systems has been suggested as a mechanism to improve resource-efficiency and the sustainability of food production (Del Borghi et al. 2020). In essence, CE promotes long-term retention and reuse of resources, as well as minimisation of waste generation, resulting in a reduced need for raw materials (Ghisellini et al. 2016). Thus, CE principles encourage the adoption of closed-loop production patterns, whereby waste is appropriated as a resource (Ghisellini et al. 2016), reducing emissions and energy consumption in the process (Xue et al. 2019).

Dairy products are a major and important source of nutrition, employment and trade worldwide (Gaucheron 2011; OECD-FAO 2020). However, large volumes of wastewater are created as a consequence of dairy production and processing. It is estimated that up to 10 L of wastewater is created per litre of milk processed, making dairy processing waste one of the most significant waste streams in the food industry (Wang and Serventi 2019). Dairy processing wastewaters tend to contain particularly high concentrations of organic matter, measured as chemical oxygen demand (COD): 2000-6000 mg L^{-1} COD (Ince 1998); 4420 mg L^{-1} COD (Demirel and Yenigun 2004); 55430-70150 mg L^{-1} COD (Malaspina et al. 1996). Moreover, these wastewaters generally contain high concentrations of nutrients, especially ammonium (64-270 mg L^{-1} NH_4-N), nitrate (9-30 mg L^{-1} NO_3-N) and phosphate (20-356 mg L^{-1} PO_4-P) (Ghaly and Singh 1989; Malaspina et al. 1996; Ince 1998). The disposal of such wastewater often lacks value-capture in the treatment process (Ahmad et al. 2019). For example, valuable nitrogen-containing nutrients such as nitrate and ammonium are commonly released as gaseous N_2

(Wang and Serventi 2019). Phosphate is typically precipitated using aluminium chloride, lime and similar additives, to generate a precipitate sludge (Bunce et al. 2018). The resulting non-soluble form of phosphate has arguably limited further benefit as a fertiliser (López-Mosquera et al. 2000).

Phytoremediation has been proposed as a viable alternative to traditional wastewater treatments, as phytoremediation removes plant nutrients from wastewaters and also retains these elements in a chemical form suitable for further use (Akansha et al. 2020; Walsh et al. 2020). Dairy processing wastewater is considered to be a good candidate for phytoremediation as it generally contains an abundance of essential plant nutrients, such as ammonium, nitrate and phosphate (Carvalho et al. 2013).

Duckweed, Lemnaceae, are a family of floating aquatic plants with excellent potential for phytoremediation due to a tolerance of wastewater conditions (Verma and Suthar 2014; Toyama et al. 2018; Dinh et al. 2020), fast growth rates (Ziegler et al. 2015) and high protein or starch content (Xu et al. 2011; Appenroth et al. 2017), as well as demonstrated use as feed, food and biofuel (Cheng and Stomp 2009; de Beukelaar et al. 2019; Stadtlander et al. 2019). Thus, these plants can combine efficient wastewater remediation with the creation of a valuable plant biomass. To date, few studies have attempted to assess the suitability of duckweed for remediation of dairy processing wastewater. However, in principle duckweed has been shown to remediate dairy processing wastewater that lacks organic components, such as sugars and fats (Walsh et al. 2021). As a high proportion of these organic components are generally removed by existing microbial-based treatment technologies, such as sequential batch reactors or anaerobic digesters (Kushwaha et al. 2013; Charalambous et al. 2020), the incorporation of duckweed into the remediation process is a realistic approach.

Wastewater remediation by duckweed is a surface process, whereby a layer of duckweed takes up nutrients from the underlying water column. In their natural habitats, most duckweed species grow in dense, floating mats (Landolt 1986). Once mats have filled the available space, individual colonies begin to overlap and shade each other. Such highly crowded conditions negatively impact duckweed growth rates (Driever et al. 2005; Frédéric et al. 2006), and duckweed may even start to senesce and release nutrients back into the water column (Ceschin et al. 2019). Conversely, a higher duckweed plant density can increase the potential for uptake of nitrogen and phosphorus (Xu and Shen 2011a). Given the implications for biomass production and wastewater remediation (Xu et al. 2012), an improved understanding of the relationship between plant surface density and biomass yield, as well as net nutrient uptake is required. Earlier work has shown that a low growth rate at a high plant density does not necessarily imply a low biomass yield or low N and P removal (Frédéric et al. 2006). Accordingly, to achieve effective phytoremediation, determination of optimal duckweed density for nutrient removal, plant growth and biomass yield per water surface area is required. In the present study, the effects of density on duckweed growth and remediation were quantified. This was done under axenic conditions, using stationary tanks containing either synthetic dairy processing wastewater or an optimal medium (half-strength Hutner's). Furthermore, with the aim of reproducing some of the conditions of large-scale duckweed phytoremediation systems, *L. minor* was cultivated on synthetic dairy wastewater using a larger scale non-axenic re-circulatory system. The results will inform management of duckweed-based remediation systems.

4.2. Materials and Methods

4.2.1. Stock cultivation

The duckweed strain used in this study was *Lemna minor* L. – Blarney, strain number 5500 in the Rutgers Duckweed Stock Cooperative database (Lahive et al. 2011). A sterile stock of *L. minor* was cultivated on half-strength Hutner's medium (Hutner 1953) under an average light intensity of 50 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) in a controlled growth-room (22 °C, 14h:10h light:dark photoperiod).

4.2.2. Experimental design

4.2.2.1. Synthetic dairy processing wastewater

The synthetic dairy processing wastewater used in this study is based on the composition of real dairy processing wastewater found in dairy wastewater treatment facilities (Tarpey 2016), with modifications as detailed in Walsh et al. (2020). The pH was reduced to, and maintained at, around 5.0 from a natural value of 8 with 1 M H_2SO_4 to facilitate optimal *L. minor* growth (Landolt and Kandeler 1987). H_2SO_4 was chosen to decrease pH as SO_4-S has a wide 'optimal' range (0.5-20 mM) in which it does not cause adverse or beneficial effects towards duckweed, with a high maximum tolerated concentration of 60 mM, as per Walsh et al. (2020).

4.2.2.2. Manipulation of plant density

In this paper, the term "plant density" is used to refer to the relative surface cover of the medium by *Lemna minor*, i.e. the proportional cover by duckweed as a fraction of the total available surface area. Plant density, i.e. surface cover, is linked to plant biomass per m^2. Plant biomass always refers to fresh duckweed biomass. Plant density was either measured directly, or estimated based on biomass per surface area. Direct density measurements were performed using the imaging software Easy Leaf Area (Easlon and Bloom 2014) which distinguishes duckweed frond surface cover from non-duckweed covered surface area. This non-invasive

technique could be used throughout the duration of an experiment. Alternatively, plant density, i.e. relative surface cover, was estimated based on biomass per m^2 of surface area. In this scenario, the latter values were converted to plant density using a calibration curve for *L. minor* biomass versus surface area. To generate a calibration curve, a number of colonies were taken at random from a stock culture acclimated to the relevant medium. The total surface area and mass of these 'representative' colonies were measured and the area/mass ratio was calculated.

4.2.2.3. Stationary remediation experiment 1: growth and remediation at variable plant densities

Two stationary experiments were conducted (Figure 4.1). In the first, scoping, experiment *L. minor* was grown on 100 mL of synthetic dairy wastewater for seven days (days 0-7) using a range of eight density conditions (10, 20, 30, 40, 50, 60, 70, 80 % plant coverage of total surface area, $n = 6$). The corresponding biomass per container surface area (ranging from 21 to 154 g m^{-2}) was estimated based on a mass/area ratio of *L. minor* biomass. Plants were kept in Magenta vessels (GA-7, surface area (SA) 42.24 cm^2) in a controlled growth room (average light intensity 50 µmol m^{-2} s^{-1} PAR, 22 °C, 16h:8h light:dark photoperiod). To start experiments, *L. minor* colonies were taken at random from stock cultures that had been acclimated to synthetic wastewater for seven days. The range of density conditions was created by adding varying numbers of *L. minor* colonies and determining the total frond surface cover using Easy Leaf Area imaging. Plant densities were maintained at ±2 % of target surface cover throughout the experiment by removing excess plant material every 2-3 days, and this process was guided by measurements of frond surface area, as determined by Easy Leaf Area. Excess plant biomass removed throughout the experiment was weighed and used to calculate specific growth rate (SGR) and relative growth rate (RGR) ($n = 6$, except for 40 % where $n = 4$). Total nitrogen (TN) and total phosphorous (TP) were measured from media samples taken on days 0 and 7 (*n*

= 6, except for 40 % where n = 4). Protein content was measured from plant samples taken on day 7 (n = 6, except for 40 % where n = 4).

4.2.2.4. Stationary remediation experiment 2: growth and remediation at low and high density

In the second stationary experiment, the negative effect of higher duckweed density on growth was explored in greater detail. To achieve this, two density conditions were created by using two containers with different surface areas, but containing the same medium volume (200 mL) and initial plant biomass (2 g). For both density conditions, *L. minor* was grown for seven days (days 0-7) on either synthetic wastewater or half-strength Hutner's medium. The experiment was conducted in a controlled growth room (average light intensity 50 µmol m^{-2} s^{-1} PAR, 22 °C, 16h:8h light:dark photoperiod). The plant density conditions consisted of low (25 %; 193 g m^{-2}) and high (60 %; 476 g m^{-2}) plant coverage of the total surface area (n = 4 per experimental treatment). The % density was determined using Easy Leaf Area imaging, while the density in g m^{-2} was calculated based on the ratio of the weighted inoculum (2 g) and the container surface area. The two densities were created by using two types of growing containers (Magenta vessels with 42.25 cm^2 SA for 60 % cover and larger circular glass containers with 103.87 cm^2 SA for 25 % cover). In both cases, 2 g of colonies were selected randomly from stock cultures, which had already been acclimated to their respective media for seven days within the controlled growth room. Densities were maintained at ±2% of target surface cover throughout the experiment through the removal of excess plant material every 2-3 days, a process guided by measurements of frond surface area, as determined by Easy Leaf Area. Excess plant biomass removed throughout the experiment from each replicate was weighed and used to calculate SGR and RGR (n = 4). Chlorophyll *a* fluorescence measurements were taken on randomly selected plants on days 0 and 7 (n = 4). TN and TP were measured from medium samples taken

on days 0 and 7 (n = 4). In both stationary experiments, any water loss due to evaporation was countered by adding deionised water to maintain original volumes.

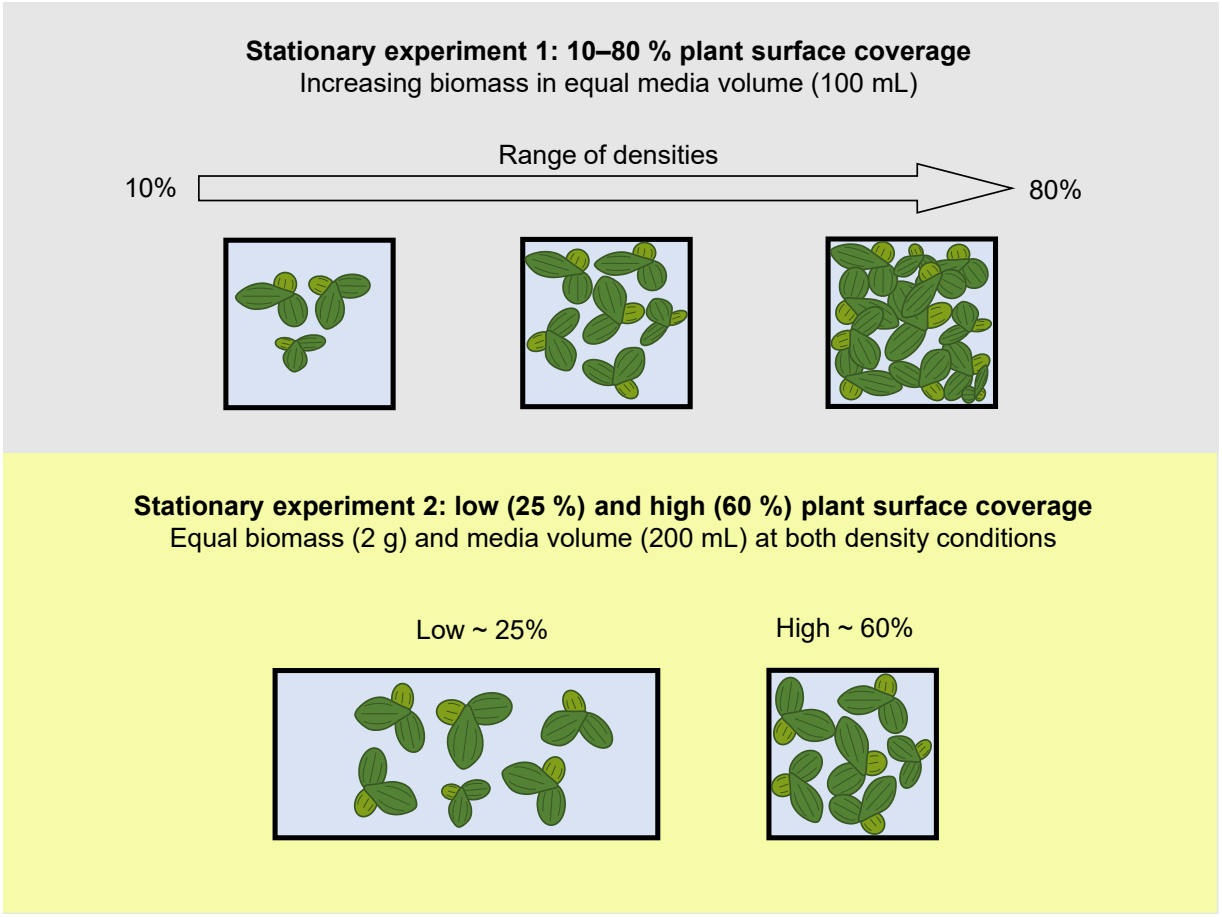

Figure 4.1. Set-up of stationary remediation experiments 1 and 2

4.2.2.5. Re-circulating remediation system: growth and remediation at variable plant densities

To determine the effect of plant density on duckweed growth and remediation capacity under more realistic operating conditions, *L. minor* was grown in a non-axenic, re-circulating system containing 11.7 L of synthetic dairy wastewater, for five days (days 0-5), and at three densities (20, 50 and 80 % plant coverage of total surface area of 600 cm^2, n = 4). Plant density was measured using Easy Leaf Area, whilst the corresponding biomass per container surface area (ranging from 50 to 187 g m^{-2}) was estimated based on a mass/area ratio of *L. minor* biomass. The experiment was conducted within a controlled environment room (300 µmol m^{-2} s^{-1} PAR, 16h:8h light:dark photoperiod). In this experimental system, synthetic wastewater was re-circulated between two tanks, an upper duckweed tank and a lower sump tank at a rate of 125 L per hour. The upper tanks of each replicate treatment were seeded at their respective plant surface densities on the initial day of the experiment, using stock plants acclimated to synthetic wastewater for seven days. Excess biomass grown over the course of the experiment was removed twice over the five-day experiment to maintain densities within ± 2% of target surface coverage, as determined using Easy Leaf Area imaging. Excess plant biomass removed throughout the experiment from each replicate was weighed and used to calculate SGR (n = 4). TN and TP were measured from medium samples taken on days 0 and 5 (n = 4). Protein content was measured from plant samples taken on day 5 (n = 4).

4.2.3. Measured parameters

4.2.3.1. Growth

All plant biomass was dried with absorbent tissue-paper to remove excess water and ensure reliable measurements before weight measurements. A specific growth rate (SGR), for growth comparisons within the present study, was calculated from estimations and measurements of fresh biomass using the formula (Chaiprapat et al. 2005):

$$SGR = \frac{W_2/W_1}{\Delta T} \qquad (1)$$

Where W_1 is starting mass, W_2 is the increase in mass over the course of the entire experiment and ΔT is the length of the experiment. Except for stationary experiment 2, starting mass (W_1) was estimated rather than measured directly and this was guided by a calibration curve of biomass versus plant surface area.

For comparison with literature sources, a relative growth rate (RGR) was calculated from estimations and measurements of fresh biomass using the formula (Connolly and Wayne 1996):

$$RGR = \frac{ln\frac{W_3}{W_1}}{\Delta T} \qquad (2)$$

Where ln is the natural log, W_1 is starting biomass, W_3 is total biomass on day 3 and ΔT is the length of time. As biomass was removed throughout each experiment to maintain a constant plant density, this formula was only used to calculate the RGR up to the first instance of removal (day 3). The total increase in mass over the course of the experiment is presented as the yield.

4.2.3.2. Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence measurements were taken on randomly selected plants on days 0 and 7 (n = 4), using a pulse amplitude modulated fluorometer (WALZ Imaging fluorometer, Effeltrich, Germany). The procedure that was followed is detailed in Walsh et al. (2021).

4.2.3.3. Total nitrogen and total phosphorous analysis

A sample of medium was taken for total nitrogen (TN) and total phosphorous (TP) analysis on the initial and final days of each experiment. For TN analysis, Hach test LCK138 was used with a Hach DR3900 spectrophotometer. Firstly, the sample was digested with peroxo-disulphate for one hour at 100°C causing inorganically and organically bonded nitrogen to oxidise to nitrate (Koroleff digestion). The resulting oxidised nitrate was then analysed

photometrically in a reaction with 2,6-dimethylphenol. For TP analysis, Hach test LCK348 was used. Firstly, the medium was digested using the persulphate digestion method for one hour at 100°C. The resulting solution was then analysed photometrically through the ascorbic acid/phosphomolybdenum blue method.

4.2.3.4. Protein analysis

Lemna minor samples, taken on the final day of experiments, were kept at -20°C until used for protein extraction and analysis. Protein was extracted using 50 mM potassium phosphate buffer (pH 7, containing 0.1 mM polyvinylpyrrolidone (PVP) and 0.1 mM EDTA). Between 50–80 mg of fresh plant material was homogenised in cold potassium phosphate buffer (1 mL of buffer to 80 mg of plant sample). The homogenised sample was then centrifuged at 20,000 x *g* for 30 minutes at 4 °C (Balen et al. 2011). The resulting supernatant was used for protein analysis using the Bradford method with bovine serum albumin as a standard (Bradford 1976). For absorbance measurements, 5 µL of sample was added to 1 mL of Bradford reagent in a cuvette and left for five minutes in dark conditions. Absorbance was measured at 595 nm using a spectrophotometer (UV-160A Shimadzu). In order to calculate the proportion of protein based on dry plant biomass, 4 % dry weight content of fresh duckweed weight was used (Appenroth et al. 2017).

4.2.4. Data analysis

Statistical analyses were conducted using R (version 3.4.3 (R Core Team 2019)). One- and two-way ANOVAs were used to analyse differences between treatments for the measured parameters. Post hoc Tukey tests were used for pairwise comparisons of treatment groups. Normality was assessed through a graphical assessment of the distribution of the residual values for data points (i.e. histogram). Homoscedasticity was assessed with ‘residuals vs. predicted values’ plots as well as Fligner-Killeen and Levene’s tests.

4.3. Results

4.3.1. Stationary remediation experiment 1: growth and remediation at variable plant densities

The absolute plant biomass yield (g) did not significantly vary over the course of the experiment although the general trend of the average yield increased with increasing density (one-way ANOVA: $F(7) = 1.57$, $p = 0.174$; Figure 4.2a). SGR (d^{-1}) exhibited the opposite trend; rates decreased as density increased (one-way ANOVA: $F(7) = 8.357$, $p < 0.001$; Figure 4.2b). The overall removal of TN (mg) from synthetic dairy wastewater increased as plant density increased (one-way ANOVA: $F(7) = 2.574$, $p < 0.05$; Figure 4.2c). However, when TN removal was expressed per frond surface area (mg N m^{-2} d^{-1}), the rate decreased as density increased (one-way ANOVA: $F(7) = 9.287$, $p < 0.001$; Figure 4.2d). A similar pattern was found for TP removal in which the overall removal of TP (mg) from synthetic dairy wastewater increased as plant density increased (one-way ANOVA: $F(7) = 5.11$, $p < 0.001$; Figure 4.2e). While the TP removal rate per frond surface area (mg P m^{-2} d^{-1}) decreased as density increased (one-way ANOVA: $F(7) = 8.158$, $p < 0.001$; Figure 4.2f). There was no difference in protein content (% dry duckweed mass) detected in relation to plant density (one-way ANOVA: $F(7) = 0.334$, $p = 0.933$; Figure 4.2g).

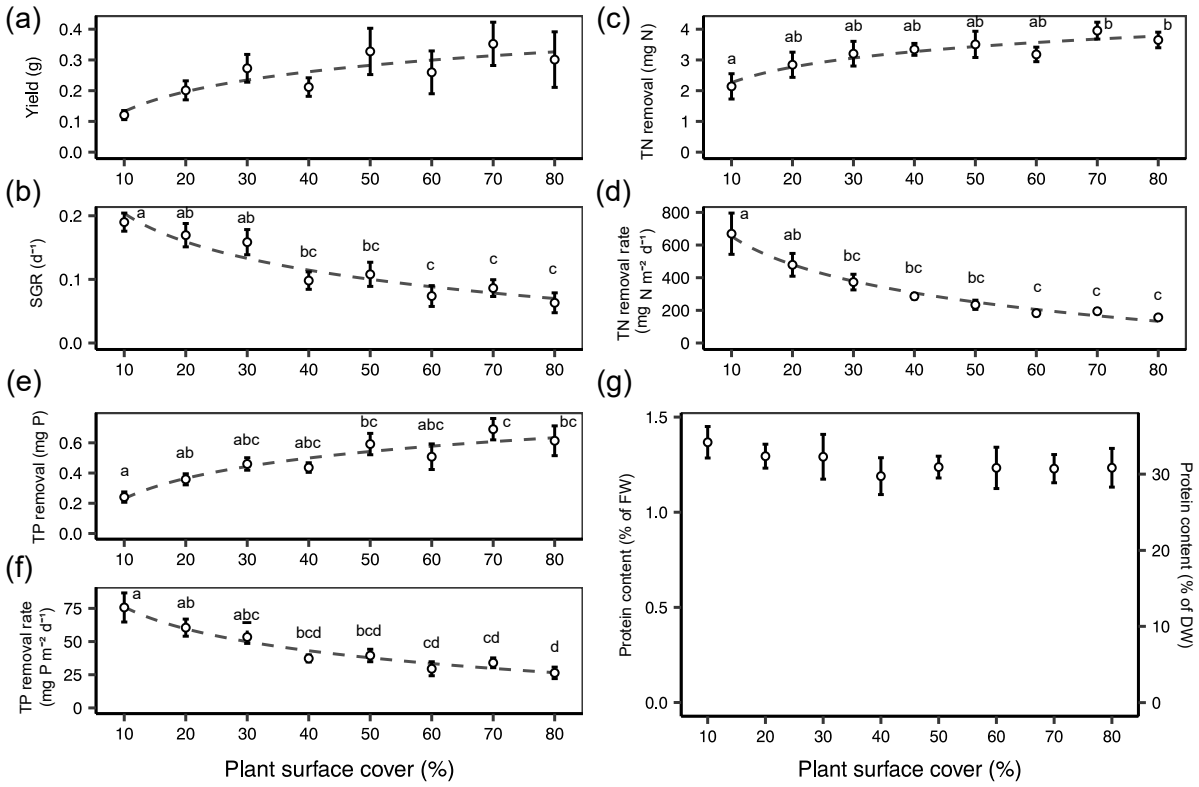

Figure 4.2. Mean (± SE) values with natural log trendline of (a) yield (g), (b) specific growth rate (SGR, d^{-1}), (c) total nitrogen (TN) removal (mg N), (d) TN removal rate per frond surface area (mg N m^{-2} d^{-1}), (e) total phosphorous (TP) removal (mg P), (f) TP removal rate per frond surface area (mg P m^{-2} d^{-1}) and (g) % protein content based on fresh duckweed biomass, FW, and dry duckweed biomass, DW, for *L. minor* grown on synthetic wastewater over 7 days under a range of plant surface covers (10-80 %). Points that do not share the same letter significantly differ from one another, as per the Tukey post hoc test, $p < 0.05$

4.3.2. Stationary remediation experiment 2: growth and remediation at low and high density

On both half-strength Hutner's and synthetic wastewater *L. minor* grown at a lower density (25 % plant surface coverage) displayed a higher absolute yield (two-way ANOVA: $F(1) = 46.607$, $p < 0.001$; Figure 4.3a) than plants grown at the higher density condition (60 % plant surface coverage). The same was found for SGR (two-way ANOVA: $F(1) = 45.994$, $p < 0.001$; Figure 4.3b). TN removal (mg) was not significantly affected by density (two-way ANOVA: F(1) =

3.642, $p = 0.0805$; Figure 4.3c), nor was TN removal rate per frond area (mg N m^{-2} d^{-1}) (two-way ANOVA: F(1) = 3.154, $p = 0.101$; Figure 4.3d), although average values were lower at the higher density. Density condition did not significantly affect TP removal (mg) (two-way ANOVA: F(1) = 2.592, $p = 0.136$; Figure 4.3e) or TP removal rate per frond area (mg P m^{-2} d^{-1}) (two-way ANOVA: F(1) = 2.293, $p = 0.158$; Figure 4.3f).

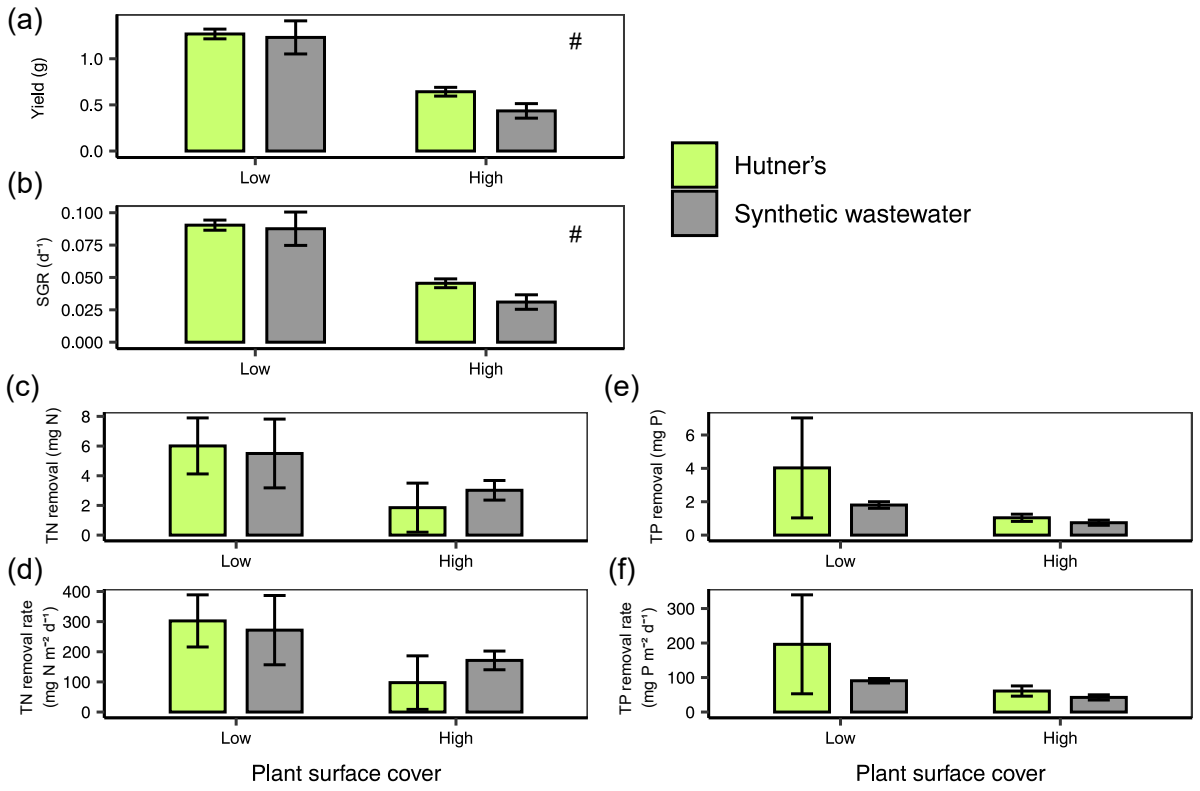

Figure 4.3. Mean (± SE) (a) yield (g), (b) SGR (d^{-1}), (c) TN removal (mg N), (d) TN removal rate per frond surface area (mg N m^{-2} d^{-1}), (e) TP removal (mg P) and (f) TP removal rate per frond surface area (mg P m^{-2} d^{-1}), for *L. minor* grown on synthetic wastewater or half-strength Hutner's medium at two plant surface covers (low, 25 % and high, 60 %) over 7 days. A hash symbol (#) denotes an effect of density for $p < 0.05$, as per the two-way ANOVA

4.3.3. Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence measurements were taken on the initial day of the stationary experiment 2 (day 0, data not shown). They showed that plants grown in both half-strength Hutner's and synthetic wastewater displayed similar values for a range of chlorophyll fluorescence parameters: F_v/F_m, Y(II), Y(NPQ) and Y(NO) (one-way ANOVAs across all treatments: F(1) = 1.006, 0, 0.049, 0.076, p = 0.354, 0.986, 0.833, 0.792, respectively). Chlorophyll fluorescence measurements taken on the final day (day 7) of the experiment revealed some differences between treatments. Similar to day 0 values, mean F_v/F_m stayed largely constant between 0.68 and 0.8, and was not significantly affected by density (two-way ANOVA: F(1) = 2.038, p = 0.179; Figure 4.4a) or medium (two-way ANOVA: F(1) = 0.911, p = 0.359; Figure 4.4a). However, measurements taken on day seven showed that higher plant density in both media resulted in a lower Y(II) (two-way ANOVA: F(1) = 34.054, $p < 0.001$; Figure 4.4b). This effect was strongest in plants grown on synthetic wastewater (post hoc Tukey test low:high density in synthetic wastewater: $p < 0.001$; Figure 4.4b). Medium alone did not significantly affect Y(II) (two-way ANOVA: F(1) = 0.907, p = 0.360; Figure 4.4b). There was, however, a significant interaction between density and medium (two-way ANOVA interaction 'density*medium': F(1) = 7.369, $p < 0.05$; Figure 4.4b). Y(NPQ) was not significantly affected by density (two-way ANOVA: F(1) = 0.594, p = 0.456; Figure 4.4c) or medium (two-way ANOVA: F(1) = 1.953, p = 0.188; Figure 4.4c). Nor was Y(NO) significantly affected by density (two-way ANOVA: F(1) = 2.016, p = 0.181; Figure 4.4d) or medium (two-way ANOVA: F(1) = 2.631, p = 0.131; Figure 4.4d).

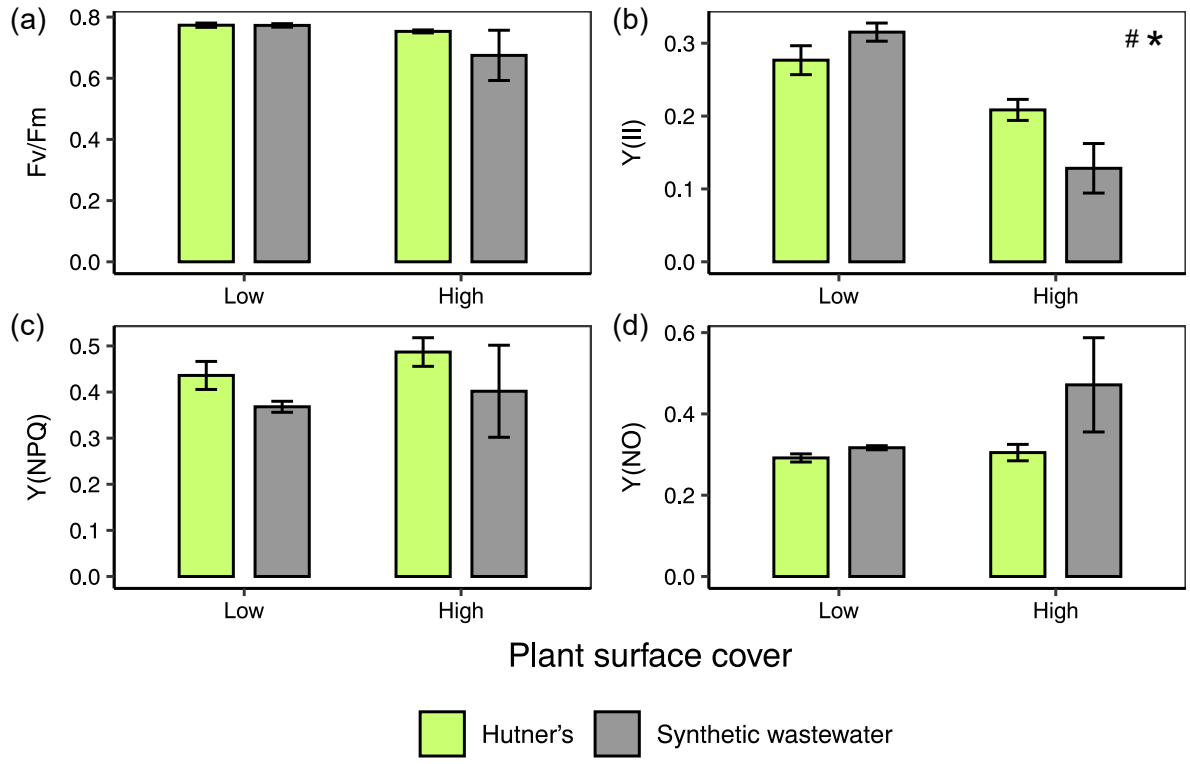

Figure 4.4. Mean (± SE) (a) F_v/F_m, (b) Y(II), (c) Y(NPQ) and (d) Y(NO), for *L. minor* grown on either synthetic wastewater or half-strength Hutner's medium at two plant surface covers (low, 25 % and high, 60 %) over 7 days. A hash symbol (#) denotes an effect of density for $p < 0.05$, and a star symbol (*) denotes an interactive effect between density and medium for $p < 0.05$, as per the two-way ANOVA

4.3.4. Re-circulating remediation system: growth and remediation at variable plant densities

The yield of *L. minor* in the re-circulating experiment was not significantly affected by the plant density (one-way ANOVA: $F(2) = 3.238$, $p = 0.087$; Figure 4.5a). However, the average biomass yield was lowest at the 20 % plant density and increased to a plateau at 50 % (post hoc Tukey: $p = 0.076$; Figure 4.5a). SGR (d^{-1}) steadily decreased with increasing density (one-way ANOVA: $F(2) = 5.143$, $p = 0.032$; post hoc Tukey 20-80: $p < 0.05$; Figure 4.5b). Overall, TN removal (mg) from synthetic wastewater was not significantly impacted by density (one-way ANOVA: $F(2) = 0.698$, $p = 0.525$; Figure 4.5c). However, TN removal rate (mg N m^{-2} d^{-1})

decreased significantly as density increased (one-way ANOVA: $F(2) = 5.701$, $p < 0.05$; Figure 4.5d). TN removal rate values dropped from around 2500 mg N m^{-2} d^{-1} at 20% plant surface cover to around 500 mg N m^{-2} d^{-1} at 80 % (post hoc Tukey: $p < 0.05$; Fig 5d). Overall, mean TP removal remained at around 17 mg across the three plant densities (one-way ANOVA: $F(2) = 0.419$, $p = 0.670$; Figure 4.5e). TP removal rate (mg P m^{-2} da^{-1}) decreased with increasing density conditions (one-way ANOVA: $F(2) = 29.240$, $p < 0.001$; Figure 4.5f). Mean TP removal rate dropped from 300 mg P m^{-2} d^{-1} at 20 % to 75 mg P m^{-2} d^{-1} at 80 % (post hoc Tukey: $p < 0.001$; Figure 4.5f). Protein content (% protein of dry duckweed mass) did not vary between plant densities, with mean values remaining between 0.7-0.8 % (one-way ANOVA: $F(2) = 0.225$, $p = 0.803$; Figure 4.5g).

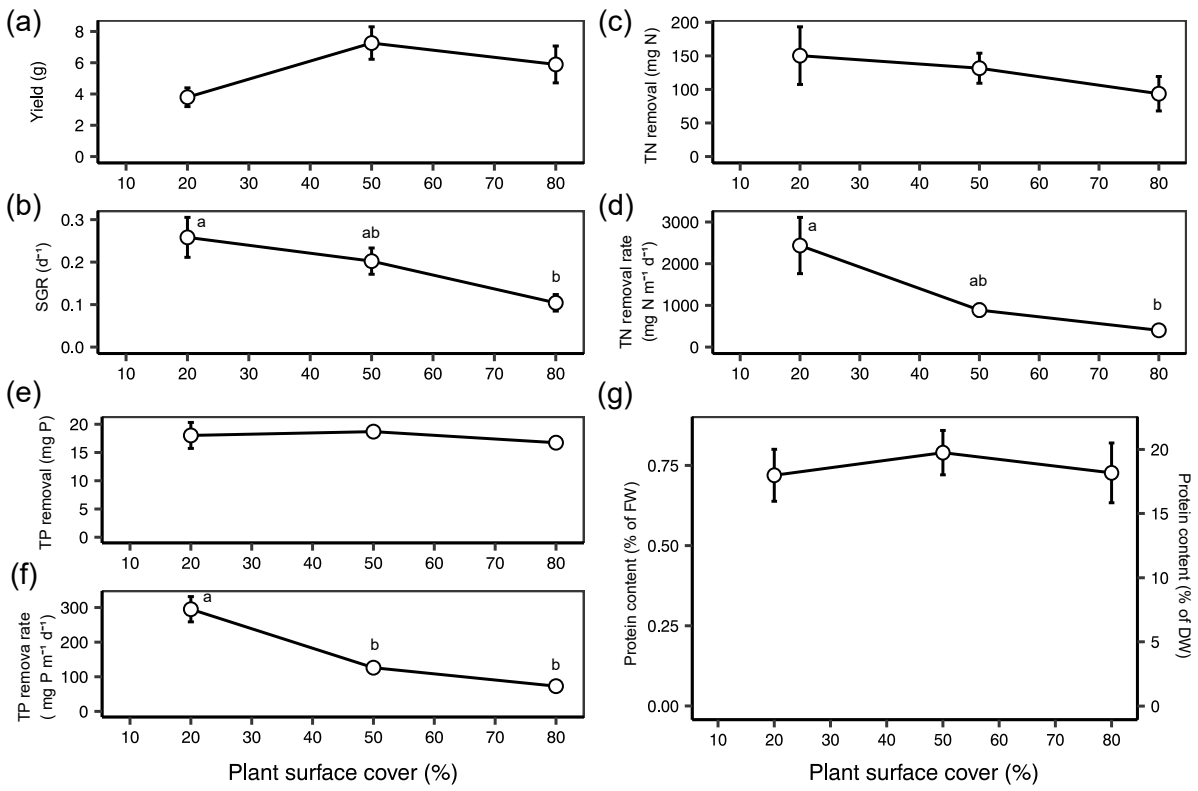

Figure 4.5. Mean (± SE) (a) yield (g), (b) SGR (d^{-1}), (c) TN removal (mg N), (d) TN removal rate per frond surface area (mg N m^{-2} d^{-1}), (e) TP removal (mg P), (f) TP removal rate per frond surface area (mg P m^{-2} d^{-1}) and (g) % protein content based on fresh duckweed biomass, FW,

and dry duckweed biomass, DW, for *L. minor* grown on synthetic wastewater in a re-circulating system at three plant surface covers (20, 50 and 80 %) over 5 days. Points that do not share the same letter significantly differ from one another, as per the Tukey post hoc test, $p < 0.05$

4.4. Discussion

4.4.1. Density effects on *L. minor* growth

RGR values recorded in the stationary experiments (based on the first 3-days of growth; 0.03-0.15 d^{-1}) are at the lower end of the range found in the literature for duckweed grown on wastewater (0.04-0.3 d^{-1}) (Al-Nozaily et al. 2000a; Iatrou et al. 2015; Dinh et al. 2020). They are also lower than those found for duckweed grown on an optimised medium (0.153-0.519 d^{-1}) (Ziegler et al. 2015). This may, in part, be due to the composition of the medium, as duckweed cultivated on wastewater often results in lower growth rates than obtained on optimised growing medium (compare growth rates in Ziegler et al. (Ziegler et al. 2015) with Al-Nozaily et al. (Al-Nozaily et al. 2000a)). As well as this, the relatively low light intensity of 50 µmol m^{-2} s^{-1} used for the stationary experiments may have also contributed to reduced growth rates (Paolacci et al. 2018). Nevertheless, it has been shown that the light saturation point is around 50 µmol m^{-2} s^{-1} for *L. minor* grown on synthetic dairy wastewater (Walsh et al. 2021), although the light saturation point for duckweed on more optimised media is 400-600 µmol m^{-2} s^{-1} (Wedge and Burris 1982; Landolt and Kandeler 1987). Commonly used experimental light intensities range from 85 to 130 µmol m^{-2} s^{-1} (Al-Nozaily et al. 2000b; Iatrou et al. 2015; Ziegler et al. 2015), which are in line with OECD guidelines for duckweed toxicity growth inhibition tests (OECD 2006). Although, a higher light intensity of 300 µmol m^{-2} s^{-1} was used in the re-circulating experiment this only led to marginally improved growth rates. In both the stationary and re-circulating experiments, SGR decreased with increasing density,

which corresponds with general trends noted within the literature (Driever et al. 2005; Frédéric et al. 2006).

4.4.1.1. Exploring the mechanism underlying density dependent changes in growth using chlorophyll fluorometry

The negative effect of high density on growth may be a result of greater competition for nutrients (Körner and Vermaat 1998), a quicker depletion of nutrients (Porath et al. 1979), or self-shading between colonies (Debusk et al. 1981). Yet, plant-plant competition has also been related to plant neighbour detection, including plant responses such as shade avoidance, root foraging, and use/induction of chemical defences (Pierik et al. 2013; Kong et al. 2018). Therefore, it is possible that *L. minor* senses the closeness of other plants and switches to a more defensive growth strategy, the trade-off of which is a reduction in growth rate. To explore the mechanism underlying the observed decrease in SGR with increasing plant surface density, plants were grown at lower and higher plant surface densities, but with identical biomass per medium volume (stationary experiment 2). This experiment confirmed the impediment of growth rate at higher plant surface densities. However, as plants at each surface density had access to the same volume of medium, the data imply that the growth impediment was due to factors other than nutrient depletion. Indeed, this point is further confirmed by the observation that similar growth impediments occurred at high plant density irrespective of whether nutrient rich Hutner's medium or more oligotrophic dairy wastewater were used.

Analysis of photosynthetic parameters in stationary experiment 2, measured after seven days of growth, showed that at high density the photosynthetic quantum yield of PSII, Y(II), was significantly depressed. A reduction in Y(II) for *L. minor* grown at high density means that the plants were using light energy less efficiently compared to those at a lower plant surface density (Murchie and Lawson 2013); an effect which was stronger for plants grown on synthetic wastewater. Y(II), together with Y(NPQ) and Y(NO) account for the partitioning of absorbed

light energy in PSII (Klughammer and Schreiber 2008), the sum of which equals 1 (Kramer et al. 2004). Accordingly, a reduction in Y(II) implies a concurrent increase in Y(NPQ) and/or Y(NO). The data reveal non-significant increases in Y(NPQ) at higher plant densities for both media, indicating minor increases in the amount of light energy dissipated in a regulated manner, i.e. through thermal dissipation (Klughammer and Schreiber 2008), at these higher densities. Thus, a key finding is a density-dependent decrease in Y(II) which is not matched by clear significant parallel increases in Y(NO) and Y(NPQ), nor a clear effect on F_v/F_m.

Overall, the data indicate that duckweed density can affect aspects of the plant's metabolism (e.g. carbon assimilation or nitrogen metabolism), rather than having a direct effect on PSII activity (i.e. F_v/F_m). As such, this would indirectly reduce photosynthetic yield (Y(II)) and therefore biomass growth (Juneau et al. 2007). Previously, Kufel et al. (2018) described complex changes in plant morphology in *L. minor* grown at different plant densities. While Zhang et al. (2020) described morphological responses of *Spirodela polyrhiza* to population density that included decreased frond and root size, as well as increased frond thickness. It is possible that shading is a driver of these changes and induces a shade avoidance response in plants at higher densities (Pierik et al. 2013; Kong et al. 2018). Nevertheless, acclimation to shade typically results in increased Y(II) and decreased Y(NPQ) at low measuring light intensities (Huang et al. 2011; Hallik et al. 2012). However, the chlorophyll fluorescence data in this study show decreased Y(II) at higher plant densities where shading might potentially have been an issue. Therefore, neither a lack of nutrient nor light supply, two well-advocated explanations, adequately explain the high-density induced impediment of growth. Rather, the data point to plant neighbour detection between *Lemna*-colonies as the most likely explanation of this (Pierik et al. 2013; Kong et al. 2018). At present, touch, volatile organic compounds, chemical exudates and possibly even acoustic signals, have all been associated with neighbour detection (Bilas et al. 2021). Although it is not known to what extent these apply to duckweed,

which tend to produce dense mats in natural habitats (Landolt 1986). Jang et al. (2007) reported that *Lemna japonica* may release interfering chemicals via its root systems, although the effects were interspecific. Similarly, Bich and Kato-Noguchi (2012) reported on interspecific allelopathic signals from *Lemna minor*. It is less clear how allelopathic signals manifest between neighbouring duckweed colonies of the same species or clone. In some duckweed species high density, or 'overcrowding', has been associated with the production of ethylene, a possible early signal for an increasing lack of space which can inhibit growth (Färber et al. 1986). Ethylene has been shown in other plant species to an inhibitor of plant growth (Dubois et al. 2018). Further studies have shown that the overcrowding-stimulated production of ethylene in duckweed is a Ca^{2+} and phytochrome-dependent process (Färber and Kandeler 1989, 1990). A transient increase in cytoplasmic Ca^{2+} is followed by an increase in ethylene production (Färber and Kandeler 1989). Nevertheless, further exploration is required to understand how plant signals, such as ethylene and other potentially unknown signals, influence *L. minor* growth and metabolism in high density conditions.

4.4.2. *L. minor* biomass yield and protein content under variable density and system conditions

Given its high protein content (Cheng and Stomp 2009; Anderson et al. 2011), and usefulness as a biofuel and a source of phytochemicals (Ge et al. 2012; Appenroth et al. 2017), *Lemna minor* biomass is an important by-product of the wastewater remediation process. As such, the absolute biomass yield is an important parameter, as it relates directly to the amount of plant mass available for further use (Xu et al. 2011). Previously, high duckweed densities of 60-80 % plant coverage (around 160-280 g m^{-2}) have been reported to result in maximum yields, in combination with different harvesting regimes (Xu and Shen 2011a, b; Verma and Suthar 2015). Data from stationary experiment 1 and the re-circulating experiment show that at higher plant densities the logarithmic relationship between plant density and yield will plateau.

Consequently, the yield increment becomes smaller. However, significant differences between density treatments were not found. Therefore, a clear benefit of higher plant density for biomass yield was not detected. The second stationary experiment shows the opposite trend between density and yield. In this experiment the use of the same plant biomass, but with different container surface areas, led to both a higher growth rate and overall yield at low density. Thus, the use of a shallower/wider container for a volume of wastewater would improve duckweed yield i.e. surface area space is an important factor in duckweed yield. However, consideration would have to be given to the impact of algae on this result, as less duckweed cover tends to increase the light availability to algae (Roijackers et al. 2004).

Duckweed density did not affect protein content in the stationary or re-circulating systems. Nevertheless, the protein content of 30-35 % on a dry weight basis found in this study compares favourably with literature sources, where duckweed protein contents up to 45 % of dry weight have been reported (Landolt and Kandeler 1987). Although, more commonly reported values are between 20-35 % (Mohedano et al. 2012; Appenroth et al. 2017). Furthermore, this compares relatively well with the commonly used high-protein feed, soybean (33-49 % (Hymowitz et al. 1972)). It should be noted that the use of the Bradford assay with BSA as a standard can underestimate plant protein content when compared to other techniques (Mæhre et al. 2018; Rekowski et al. 2021), which may affect comparisons with literature sources.

The protein content detected for plants grown in the re-circulating system ranged from 17.5-20 % of dry weight, which can be considered a low protein content relative to the published range. This demonstrates that scaling up, and the use of circulatory, non-axenic systems may have unexpected consequences. Starch content was not measured in this study, but, as has been observed in some studies, a lower protein content can result in, or be a result of, higher starch content (Xu et al. 2011). If low protein content is a consistent problem for duckweed grown on

a large-scale, there are established alternative uses for the biomass that do not depend on the protein content, such as biofuel production (Cui and Cheng 2015).

4.4.3. Remediation of TN and TP by *L. minor* from synthetic wastewater

The trend of decreasing growth with increasing density was reflected in the relationship between TN/TP removal rate and density. In both stationary and re-circulating experiments, the lower removal rates of TN and TP per plant surface area (g m^{-2} d^{-1}) at higher density conditions show that each duckweed colony is removing less nutrients at higher plant densities than those kept at lower densities. The TN and TP removal rates found in the stationary experiments were on the lower end of the wide range of values recorded in the literature: 124-4400 mg N m^{-2} d^{-1} and 14-590 mg P m^{-2} d^{-1} (Körner and Vermaat 1998; Cheng et al. 2002b; Zimmo et al. 2004; Benjawan and Koottatep 2007; Mohedano et al. 2012; Zhao et al. 2015). This can be explained by the lower growth rates observed in these experiments (Cheng et al. 2002a), which are likely to be in part due to the low light intensity used (Walsh et al. 2021), the specific medium (Toyama et al. 2018), as well as density effects on *L. minor* discussed previously. Both TN and TP removal rates from the non-axenic, re-circulating experiment compare well with values from non-axenic systems in literature sources (Körner and Vermaat 1998; Cheng et al. 2002a). Higher removal rates found in the re-circulating experiment compared to stationary may be mostly explained by the presence of algae and microorganisms, which can account for up to 50-70 % of nutrient removal in non-axenic systems (Körner and Vermaat 1998; Zhao et al. 2015), but also by improved mixing of the medium.

Another important criterium for analysing the effectiveness of a remediation system is the absolute amount of nutrients removed from the remediated wastewater. In stationary experiment 1, a higher density of plants per surface area more effectively takes up nutrients, although the growth and nutrient removal rates per frond are significantly lower (Driever et al. 2005; Frédéric et al. 2006). As the removal of nutrients started to plateau at higher densities of

50-80 %, this suggests that 50-80 % represents an optimal duckweed density for dairy wastewater phytoremediation.

There were additional issues encountered in a non-axenic re-circulating system, in which the absolute removal of TN and TP was relatively flat across the three densities. The exact cause of this is uncertain. However, microorganisms were present in substantial amounts at all plant densities in this non-axenic system. Previously, algae were shown to significantly contribute to nutrient removal (Körner and Vermaat 1998; Roijackers et al. 2004), as well as having negative effects on duckweed such as increased decomposition rates (Szabó et al. 2000). At both high and low density, a high proportion of the absolute removal of TN and TP may be attributed to microorganisms, breaking the direct link between plant growth and nutrient removal (e.g. poor plant growth, but high nutrient removal). Considering wastewater remediation alone, whether remediation is fuelled primarily by duckweed or algae, nutrient removal is the desired outcome. However, if duckweed biomass is to be generated as a valuable by-product for further use, then strong competition for nutrients with non-utilised microorganisms is undesirable. Ideally, the duckweed should be taking up the majority of the nutrients. One way to achieve this is by maximising the duckweed surface density, thereby decreasing algal growth (Szabó et al. 1999). Overall, these results show that upscaling from laboratory-based, stationary systems to larger scale, recirculatory systems is complex, and that simple extrapolations are not necessarily correct. Accordingly, management of duckweed incubators will need to be informed by the effects of plant density on biomass yield, TN and TP removal, as well as competition with algal species.

4.5. Conclusion

Lemna minor can be successfully grown on synthetic dairy processing wastewater, opening the perspective to both remediate and valorise such waste, in accordance with the principles of the

circular economy. *Lemna minor* has been shown to produce the best remediation at higher densities (50-80 %), even though growth rates and nutrient uptake rates were slowest at these densities. The decrease in growth at high density was linked to a decrease in photosynthetic yield, rather than competition for light or nutrients, which points towards signals from neighbouring colonies as the potential cause of growth restrictions. However, in non-axenic, scaled-up conditions that better reflect an industrial duckweed-based remediation system, the benefits of high density were not as clear. Compounding stresses in high density conditions led to suppressed yield and nutrient removal. Thus, despite the suitability of *L. minor* for valorisation of dairy processing waste, management of wastewater is subject to both interactions between plant density, yield and nutrient removal, as well as complex upscaling effects.

Author contributions

ÉW, HK and MAKJ contributed to the study conception and design. Material preparation, data collection and analysis were performed by ÉW, HK and SOB. All authors contributed to the interpretation of results. The first draft of the manuscript was written by ÉW and all authors contributed to writing and editing of the manuscript.

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Chapter 5

Clones collected from a small geographic region show diverse abilities to remediate dairy wastewater

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Abstract

Duckweed, Lemnaceae, are a family of aquatic plants which can be used as part of a circular economy approach to remediate dairy processing wastewater. The suitability of duckweeds relates to their tolerance of wastewater conditions, fast growth rates and valuable biomass. Duckweed species and clones display a wide range of remediation abilities and nutritional composition. The differences between clones of the same species can be as substantial as between species. In order to find duckweed species and clones with natural advantages for remediation of dairy processing wastewater, plant samples were collected from a number of locations around southern Ireland. These species and clones were kept on a synthetic dairy processing wastewater while growth, TN/TP removal and protein content were assessed. Synthetic dairy wastewater was found to be suitable for remediation by different species and clones. However, significant differences were found in relative growth rate (RGR), TN removal rate and protein content. It was found that species and clones with higher RGR tended to have a lower protein content. Furthermore, nutrient removal rate was not particularly associated with growth and protein content. The results demonstrate that important remediation parameters are not positively correlated across duckweed species and clones. Thus, clonal selection for duckweed reactors will need to consider the prime objective of remediation i.e. biomass production or wastewater remediation.

5.1. Introduction

Food production is coming under significant stress arising from multiple causes e.g. climate change, soil erosion, and decreasing availability of resources such as water, fertilisers and fuel (Wakeford and Swilling 2014; Lavrnić et al. 2017; Nearing et al. 2017; Leinweber et al. 2018; Pathak et al. 2018). Accordingly, sustainable solutions are required to promote long-term food security. The use of circular economy (CE) principles in food production has been suggested as a way to improve resource retention and avoid waste creation in an industry of vital importance (Del Borghi et al. 2020). CE fosters the use of closed-loop production patterns, of which one aspect is waste reuse (Ghisellini et al. 2016). In this scenario waste is used as a resource, with valuable materials being fed back into production, leading to reduced emissions and increased resource retention (Grimm and Wösten 2018).

Dairy processing wastewater is a waste stream created during the processing of raw milk into various dairy products (Gösta 2015), and makes up a large portion of food industry waste (OECD-FAO 2020). It is estimated that up to 10 L of wastewater are produced for every litre of milk processed, making its treatment and disposal highly relevant (Wang and Serventi 2019). Dairy wastewater generally contains high concentrations of organic material such as fats and sugars, and this can be measured as COD: 2000-70150 mg L^{-1} (Malaspina et al. 1996; Ince 1998; Demirel and Yenigun 2004). Also present in wastewater are high concentrations of essential plant micronutrients such as nitrate (0.64-2.1 mM or 9-30 mg L^{-1} NO_3-N), ammonium (4.6-19.3 mM or 64-270 mg L^{-1} NH_3-N) and phosphate (0.65-11.5 mM or 20-356 mg L^{-1} PO_4^{3-}-P) (Ghaly and Singh 1989; Malaspina et al. 1996; Ince 1998). In addition, many common elements, which are also essential for plants but in lower amounts, are present: Fe, K, Na, Cl, Ca, Mg etc. (Goyal and Gandhi 2009; Chokshi et al. 2016; Guerreiro et al. 2020). Furthermore, the salt (NaCl) concentration of dairy processing wastewater is also relatively high (Goyal and Gandhi 2009; Guerreiro et al. 2020).

The focus of remediation of this wastewater is the removal of polluting elements that are present at high concentrations such as organic compounds, ammonia, nitrate, phosphate, as well as the heavy metals (Babel and Kurniawan 2003; Carvalho et al. 2013). Phytoremediation provides an opportunity to remediate the inorganic content of this wastewater stream, according to circular economy principles (Kurniawan et al. 2021). In cases where plant material is processed for further use (animal feed or biofuel precursor), nutrients such as ammonia, nitrate and phosphate are generally the focus of removal (Kadir et al. 2020). They can be readily taken up by plants from the wastewater, and provide the plant with N and P (Cheng and Stomp 2009).

Various duckweed species, belonging to the family of Lemnaceae, show strong potential for remediation due to their fast growth and nutrient uptake rates (Cheng and Stomp 2009), coupled with high protein and starch content (Cui and Cheng 2015; Appenroth et al. 2017). The potential of a duckweed species to remediate wastewater can vary between different duckweed species (Toyama et al. 2018). Although, Ziegler et al. (2015) have shown, in a comprehensive analysis of duckweed growth, that clones from all five genera produce, on average, similar growth rates. Indeed, it was differences within species, i.e. between clones, that were most pronounced. Similar intraspecific diversity in growth rates was found for *Wolffia* by Sree, Sudakaran, and Appenroth (2015) and for *Lemna minor* and *Lemna minuta* by Paolacci, Jansen, and Harrison (2018). Given such intraspecific diversity, it is possible some clones are more suited to wastewater remediation than others. Thus, there is an opportunity to explore whether clonal resources can be utilised to optimise remediation and the nutritional content of the wastewater-cultivated duckweed. Furthermore, while differences in growth and nutritional composition between species and clones have been well established, the association between these traits and others, such as N and P removal, have been less explored.

Here, it is hypothesised that some clones will grow better on wastewater, and that this is possibly linked to the geographic site where the clone was collected. It is also hypothesised

that there is a positive correlation between remediation-relevant duckweed traits i.e. growth rates and nutrient uptake rates. Accordingly, duckweed species and clones were collected from water bodies near the sea, amongst farmland, or that were part of wastewater treatment plants, where plants would potentially be exposed to similar water quality factors as in wastewater e.g. salinity and organic or inorganic pollution. These clones were then grown in laboratory conditions on a synthetic dairy processing wastewater and growth, N and P removal as well as protein content were analysed. The results inform the selection process of duckweed for wastewater remediation, as well as the link between important growth and remediation duckweed traits.

5.2. Materials and Methods

5.2.1. Duckweed collection

Clones of *L. minor* and *L. minuta* were collected from a number of sites in southern Ireland (Figure 5.1; Table 5.1). Three clones were collected from two different dairy wastewater processing facilities, in which duckweed was growing naturally in outdoor wastewater processing tanks (MJ109-MJ111; Figure 5.1; Table 5.1). Five clones were collected from water bodies with potential saltwater influence, including estuaries in contact with the sea (MJ112-MJ116; Figure 5.1; Table 5.1). Finally, two clones were collected from rivers that are surrounded by farmland (MJ309 and MJ117; Figure 5.1; Table 5.1), a potential source of nutrient pollution (Mockler et al. 2017). The record of species from the National Biodiversity Data Centre database (https://records.biodiversityireland.ie/) was used to find previous reports of duckweed species in areas of interest. Clones that were not collected specifically for this study were taken from stock cultures kept at University College Cork, Ireland (MJ100 Blarney, *L. minor*, and MJ302 Charleville, *L. minuta*) or at Debrecen University, Hungary (UD103

Hungary, *L. gibba*). In this study the term clone is used as a collective term for all clones regardless of species, unless otherwise specified.

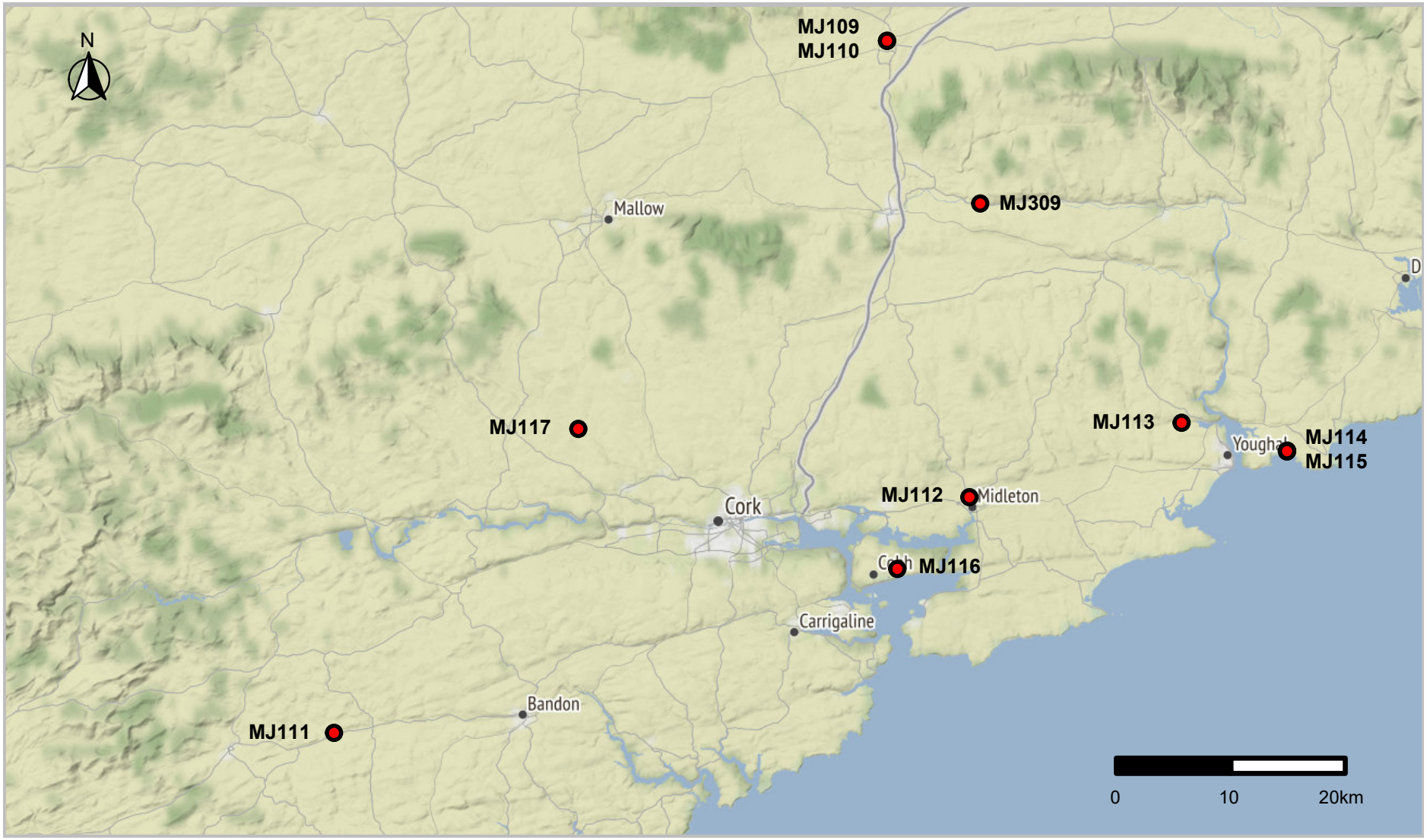

Figure 5.1. Collection points of duckweed clones in southern Ireland. Labels are ISCDRA accession ID numbers.

Table 5.1. Sampling location and details of collection points for duckweed clones collected for this study, as well as names and ISCDRA accession ID numbers for all duckweed clones used.

ISCDRA accession ID number (RDSC ID)	Common name	Origin – sampling location	Coordinates
MJ100 (5500)	Blarney	Stock (*L. minor*)	-
MJ302 (5571)	Charleville	Stock (*L. minuta*)	-
UD103	Hungary	Stock (*L. gibba*)	-
MJ109	Mitchelstown A	Outdoor clarifying tank in dairy wastewater treatment facility	52.2717123, -8.2805116
MJ110	Mitchelstown B	Outdoor thickening tank in dairy wastewater treatment facility	52.2717123, -8.2805116
MJ111	Ballineen	Outdoor tank in dairy wastewater treatment facility	51.7318492, -8.9811663
MJ309	Fermoy	Flooded land next to Blackwater river near Fermoy	52.145377, -8.162454
MJ112	Midleton	Owenacurra river at Midleton town	51.9164163, -8.1761566
MJ113	Tourig River	Tourig river where it enters Blackwater river estuary near Youghal	51.9745581, -7.9073607
MJ114	Whiting Bay A	A stream entering the sea at Whiting Bay beach	51.9524439, -7.7736677
MJ115	Whiting Bay B	A stream entering the sea at Whiting Bay beach	51.9527918, -7.7736767
MJ116	Cuskinny Marsh	Cuskinny Marsh, a pond next to Cork harbour near Cobh	51.8603034, -8.2674446
MJ117	Shournagh River	Shournagh river, northwest of Blarney	51.9697820, -8.6716304

5.2.2. Stock cultivation

In order to create axenic cultures of collected clones, individual colonies of each clone were bleached using three different concentrations (2, 2.5 and 3%) of sodium hypochlorite for 1 minute, and then washed in distilled water. After this process, individual colonies were transferred to autoclaved Magenta vessels (GA-7) with half-strength Hutner's medium (Hutner 1953) and kept in a growth room for a number of weeks, during which contamination by

microorganisms was assessed by eye. Clear contamination was the presence of flocs of microbes and/or algae in the vessel. Bleaching was repeated until cultures were free of algae and other microorganisms.

Sterile stock cultures of each axenic clone were then cultivated on half-strength Hutner's medium under an average light intensity of 50 µmol m^{-2} s^{-1} PAR (photosynthetically active radiation) in a controlled growth-room (22 °C, 14h:10h light:dark photoperiod).

5.2.3. Species characterisation of collected clones

Clones of duckweed were characterised (i.e. barcoded) through sequencing of the intergenic spacers *atpF-atpH* and *psbK-psbI*. First, DNA was extracted from around 50 mg of plant tissue using the GenElute™ Plant Genomic Miniprep DNA Kit (Sigma Aldrich). The quantity (10-50 ng) and quality of extracted DNA was analysed using a NanoDrop™. The forward and reverse intergenic spacer sequences were amplified using PCR with primers described in Borisjuk et al. (2015). DNA amplification was carried out using MiniAmp Thermal Cycler (Applied Biosystems) with the following mixture per sample (20 µL in total): 1 µL forward primer, 1 µL reverse primer, 8.6 µL ddH_2O, 10 µL PP Combi Mastermix (Top-Bio) and 1 µL DNA sample. The conditions of the PCR were: initial denaturation at 95 °C for 3 minutes followed by 35 cycles of denaturation (95 °C for 30 seconds), annealing (50 °C for 30 seconds) and extension (72 °C for 1 minute) with a final extension at 72 °C for 5 minutes at the end. PCR samples were run on a 1% agarose gel with 1x TAE buffer and SYBR™ safe DNA gel stain (Invitrogen) to determine the effectiveness of the PCR. A QIAquick gel extraction kit (QIAGEN) was used to extract the PCR product from the agarose gel. Sequencing was performed with both the forward and reverse primers of each intergenic spacer using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The mixture for each sequencing reaction (10 µL in total) was as follows: 0.32 µL primer, 1.75 µL sequencing buffer, 0.5 µL BigDye™ Direct PCR Mastermix, 6.43 µL ddH20 and 1 µL purified PCR product.

Products of the sequencing reactions were purified using the EDTA-ethanol precipitation method described in the sequencing kit handbook (Fujikura 2016) and then run on an ABI3500XL DNA analyser (Applied Biosystems).

Morphological identification of clones was carried out using a stereomicroscope (Nikon E200), using the taxonomy key by Bog, Appenroth, and Sree (2020).

5.2.4. Experimental set-up: growth and remediation on synthetic dairy wastewater

The synthetic dairy processing wastewater used in this study is based on the composition of actual dairy processing wastewater found in dairy wastewater treatment facilities (Tarpey 2016), with modifications as detailed in (Walsh et al. 2020). The pH was reduced with 1 M H_2SO_4 to pH 5, and maintained at this value to facilitate *L. minor* growth (Landolt and Kandeler 1987).

Thirteen duckweed clones (Table 5.1) were grown for 7 days (days 0-7) on 100 mL of synthetic wastewater (n = 4) in a controlled growth room (average light intensity 50 μmol m^{-2} s^{-1} PAR, 22 °C, 16h:8h light:dark photoperiod) in Magenta vessels (GA-7, surface area (SA) 42.24 cm^2). On the first day of the experiment (day 0) fifteen colonies (equating to on average 41 fronds in total) of each duckweed clone were taken at random from stock plants which had been pre-acclimated on synthetic wastewater for seven days.

5.2.5. Measured parameters

5.2.5.1. Duckweed growth

After seven days of growth plant biomass was measured (n = 4). Relative growth rate (RGR) was calculated with estimations and measurements of biomass on day 0 and day 7 using the formula:

$$RGR = \frac{\ln \frac{W_2}{W_1}}{\Delta T}$$

Where ln is the natural log, W_1 is starting biomass, W_2 is final biomass on day 7 and ΔT is the length of the experiment (7 days).

Yield was calculated as the excess biomass (g) produced over the course of the experiment (*n* = 4).

5.2.5.2. Duckweed protein

Duckweed samples (*n* = 4) taken on the final day of experiments, were kept at -20 °C until used for protein extraction. Protein was extracted using 50 mM potassium phosphate buffer (pH 7, containing 0.1 mM polyvinylpyrrolidone (PVP) and 0.1 mM EDTA). Between 50–80 mg of plant material was homogenised in cold potassium phosphate buffer (1 mL of buffer to 80 mg of plant sample). The homogenised sample was then centrifuged at 20,000 x *g* for 30 minutes at 4 °C (Balen et al. 2011). The resulting supernatant was used for protein analysis using the Bradford method with bovine serum albumin as a standard (Bradford 1976). For absorbance measurements, 5 µL of sample was added to 1 mL of Bradford reagent in a cuvette and left for five minutes in dark conditions. Absorbance was measured at 595 nm using a spectrophotometer (UV-160A Shimadzu). In order to calculate the proportion of protein based on dry plant biomass, 4 % dry weight content of fresh duckweed weight was used (Appenroth et al. 2017).

5.2.5.3. Total nitrogen and total phosphorous

Total nitrogen (TN) and total phosphorous (TP) were measured in media samples taken on days 0 and 7 of the experiment (*n* = 3). For TN analysis, Hach test LCK138 was used with a Hach DR3900 spectrophotometer. Firstly, the sample was digested with peroxo-disulphate for one hour at 100 °C causing inorganically and organically bonded nitrogen to oxidise to nitrate

(Koroleff digestion). The resulting oxidised nitrate was then analysed photometrically in a reaction with 2,6-dimethylphenol. For TP analysis, Hach test LCK348 was used. Firstly, the medium was digested using the persulphate digestion method for one hour at 100 °C. The resulting solution was then analysed photometrically through the ascorbic acid/phosphomolybdenum blue method.

5.2.6. Data analysis

Raw sequence data were examined using Chromas (2.6.6, Technelysium Pty Ltd) to resolve any ambiguous calls. Forward and reverse sequences were pairwise-aligned, to gain a full read of each sequence length, using online application EMBOSS-Needle from EMBL (https://www.ebi.ac.uk/Tools/psa/emboss_needle/, Rice, Longden, and Bleasby (2000)). Finalised sequences were compared with sequences from the GenBank database using nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi, NCBI Resource Coordinators (2018)).

Growth, TN and TP removal and protein data were analysed using R (R 3.4.3, R Core Team (2019)). One-way ANOVAs, with post hoc Tukey tests, were used to analyse differences between clonal treatments.

5.3. Results

5.3.1. Species characterisation: genetic and morphological analysis

In order to determine the species identity of collected clones, intergenic spacer sequences *psbK-psbI* and *atpF-atpH* were compared with verified sequences of *L. minor*, *L. minuta* and *L. gibba* clones present in the GenBank database. These two intergenic spacer sequences were also sequenced for stock plants of known species identity (MJ100 *L. minor*, MJ302 *L. minuta*, UD103 *L. gibba*), for comparison with the collected clones. A 100 % identity match with the reference sequences in the database, as well as with the stock species, was used as confirmation

of species identity. Identical *atpF-atpH* sequences for *L. minuta*, *L. valdiviana* and *L. yungensis* preclude final species identification on basis of that sequence alone. However, as the *psbK-psbI* sequence is unique for *L. minuta*, clones MJ302 (Charleville) and MJ309 (Fermoy) could be unambiguously identified as *L. minuta* (Table 5.2). Clone MJ302 had previously also been identified as *L. minuta*. Identical *psbK-psbI* and *atpF-atpH* sequences for *L. minor* and *L. japonica* also preclude unambiguous identification of clones MJ109-MJ117 based solely on genetic barcoding analysis (Table 5.2). The same was true for MJ100 (Blarney) but it had previously been identified as *L. minor*.

Clones that were not identified as one particular species in genetic barcoding analysis (MJ100 and MJ109-MJ117) were morphologically characterised to distinguish between *L. minor* and *L. japonica* (Figure 5.2) using a duckweed taxonomic key (Bog et al. 2020). The key morphological difference between *L. minor* and *L. japonica* is the frequent presence of a reddish lower surface (abaxial) in *L. japonica* which is more intense than the colour on the upper side (adaxial) (Bog et al. 2020). In contrast, *L. minor* rarely has a reddish colour on the abaxial surface. Microscopic images did not show a reddish colour on the abaxial side of any of the clones in question (Figure 5.2). In combination with a taxonomic key, clones MJ109-MJ117 were identified as *L. minor* rather than *L. japonica*, while MJ100 was confirmed as *L. minor* (Table 5.2).

Table 5.2. Species characterisation of duckweed clones

ISCDRA accession ID number	Common name	Identification based on *psbK-psbI*	Identification based on *atpF-atpH*	Species ID [1]
MJ100	Blarney	*L. minor/ L. japonica*	*L. minor/ L. japonica*	*L. minor*
MJ302	Charleville	*L. minuta*	*L. minuta/ L.valdiviana/ L.yungensis*	*L. minuta*
UD103	Hungary	*L. gibba (*1 *L. Japonica clone)*	*L. gibba (*1 *L. japonica clone)*	*L. gibba*
MJ109	Mitchelstown A	*L. minor/ L. japonica*	*L. minor/ L. japonica*	*L. minor*
MJ110	Mitchelstown B	*L. minor/ L. japonica*	*L. minor/ L. japonica*	*L. minor*
MJ111	Ballineen	*L. minor/ L. japonica*	*L. minor/ L. japonica*	*L. minor*
MJ309	Fermoy	*L. minuta*	*L. minuta/ L.valdiviana/ L.yungensis*	*L. minuta*
MJ112	Midleton	*L. minor/ L. japonica*	*L. minor/ L. japonica*	*L. minor*
MJ113	Tourig River	*L. minor/ L. japonica*	*L. minor/ L. japonica*	*L. minor*
MJ114	Whiting Bay A	*L. minor/ L. japonica*	*L. minor/ L. japonica*	*L. minor*
MJ115	Whiting Bay B	*L. minor/ L. japonica*	*L. minor/ L. japonica*	*L. minor*
MJ116	Cuskinny Marsh	*L. minor/ L. japonica*	*L. minor/ L. japonica*	*L. minor*
MJ117	Shournagh River	*L. minor/ L. japonica*	*L. minor/ L. japonica*	*L. minor*

[1] Species identification based on genetic and morphological information

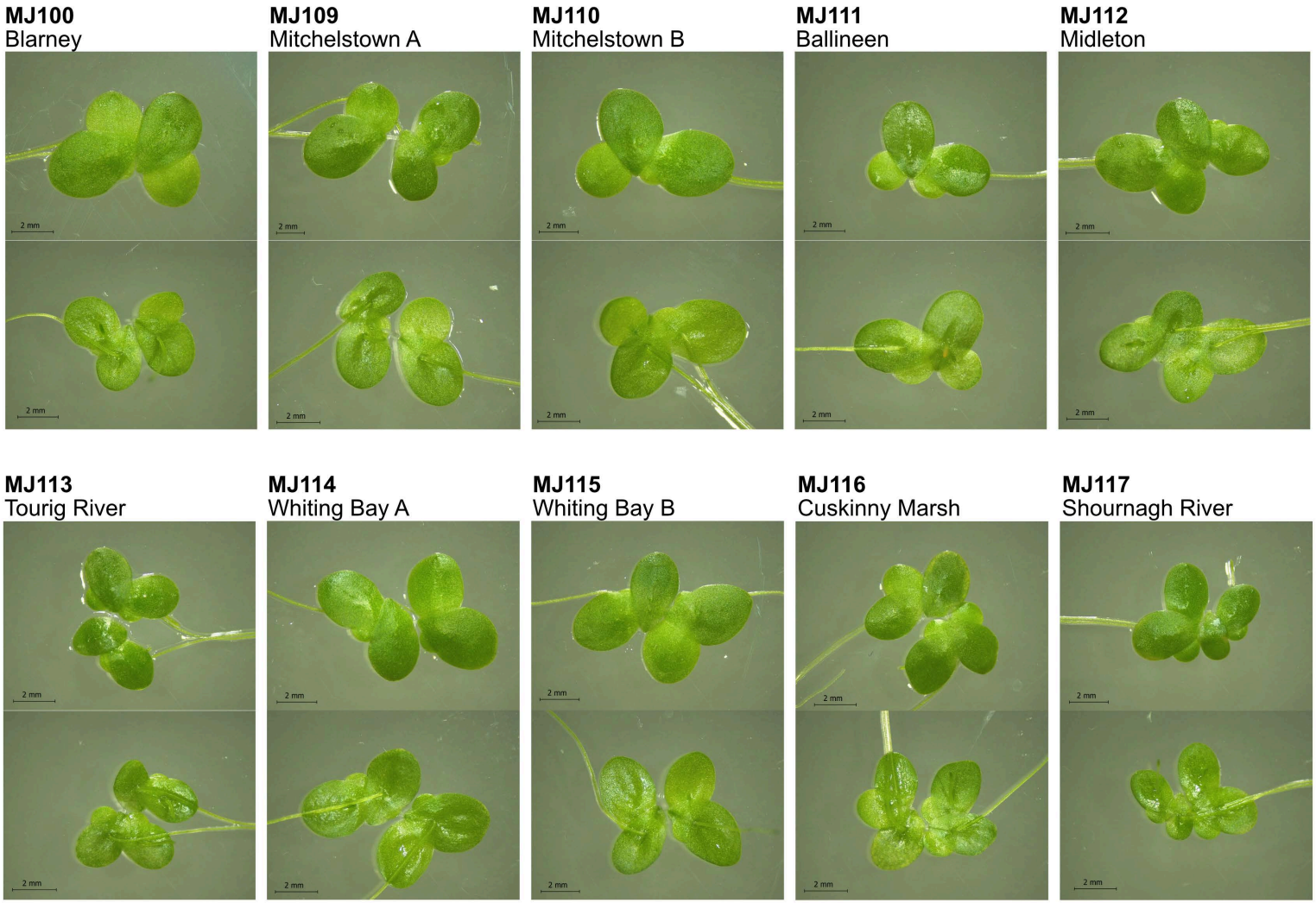

Figure 5.2. Adaxial (top row) and abaxial (bottom row) sides of *Lemna minor* clones showing the lack of a reddish lower surface colouration typical of *L. japonica*.

5.3.2. Cultivation on synthetic wastewater

5.3.2.1. Growth and protein content

The RGR (d^{-1}) on synthetic wastewater significantly differed between clones of duckweed (one-way ANOVA: $F(12) = 2.505$, $p = 0.0156$; Figure 5.3a). While most clones were not significantly different from each other, the RGR of MJ302 (Charleville) was significantly lower than that of MJ112 (Midleton) and MJ113 (Tourig River) (post hoc Tukey: $p < 0.05$; Figure 5.3a) and borderline significantly lower than that of MJ100 (Blarney) and MJ114 (Whiting Bay A) (post hoc Tukey: $p = 0.07$; Figure 5.3a). Average yield varied between clones but as the starting mass for each species and clone was not constant, yield measurements were not statistically assessed for differences.

Duckweed protein content (% protein of fresh biomass) significantly varied between species and clones (one-way ANOVA: $F(12) = 5.226$, $p < 0.001$; Figure 5.3c). UD103 (Hungary) and MJ100 (Blarney) had significantly lower protein contents than MJ111, MJ114, MJ115, MJ116 and MJ117 (Ballineen, Whiting Bay A and B, Cuskinny Marsh and Shournagh River, respectively) (post hoc Tukey: $p < 0.05$; Figure 5.3c). MJ112 (Midleton) also had a significantly lower protein content than MJ111 (Ballineen) (post hoc Tukey: $p < 0.05$; Figure 5.3c).

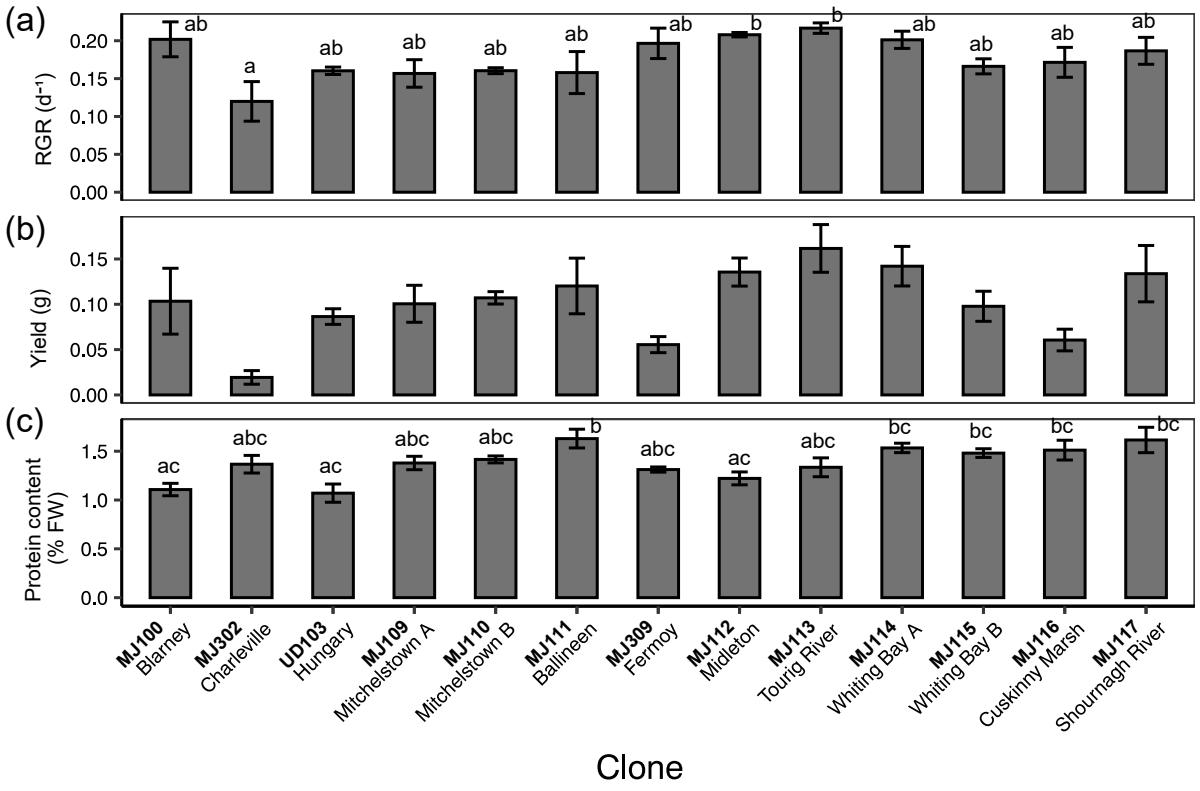

Figure 5.3. Mean (± standard error) for (a) RGR (d^{-1}), (b) duckweed biomass yield (g), and (c) protein content (% of fresh biomass) for duckweed clones grown on synthetic dairy wastewater. Bars that do not share the same letter are significantly different from each other ($p < 0.05$), as per post hoc Tukey.

5.3.2.2. TN and TP removal

TN removal expressed per g of duckweed (mg N g^{-1} d^{-1}) significantly varied between clones (one-way ANOVA: F(12) = 4.712, $p < 0.001$; Figure 5.4a). The two *L. minuta* clones, MJ302 (Charleville) and MJ309 (Fermoy), had significantly higher TN removal rates (mg N g^{-1} d^{-1}) than a number of *L. minor* clones (post hoc Tukey: $p < 0.05$; Figure 5.4a). When expressed per m^2 of duckweed (mg N m^{-2} d^{-1}) significant variation was found between clones as per a one-way ANOVA (F(12) = 2.211, $p = 0.047$; Figure 5.4b). However, a pairwise comparison using post hoc Tukey tests did not find significant differences. TP removal rates expressed per g of duckweed (mg P g^{-1} d^{-1}) or per m^2 of duckweed (mg P m^{-2} d^{-1}) did not vary significantly

between clones (one-way ANOVA: F(12) = 1.237, p = 0.31; F(12) = 0.903, p = 0.556, respectively; Figure 5.4c,d).

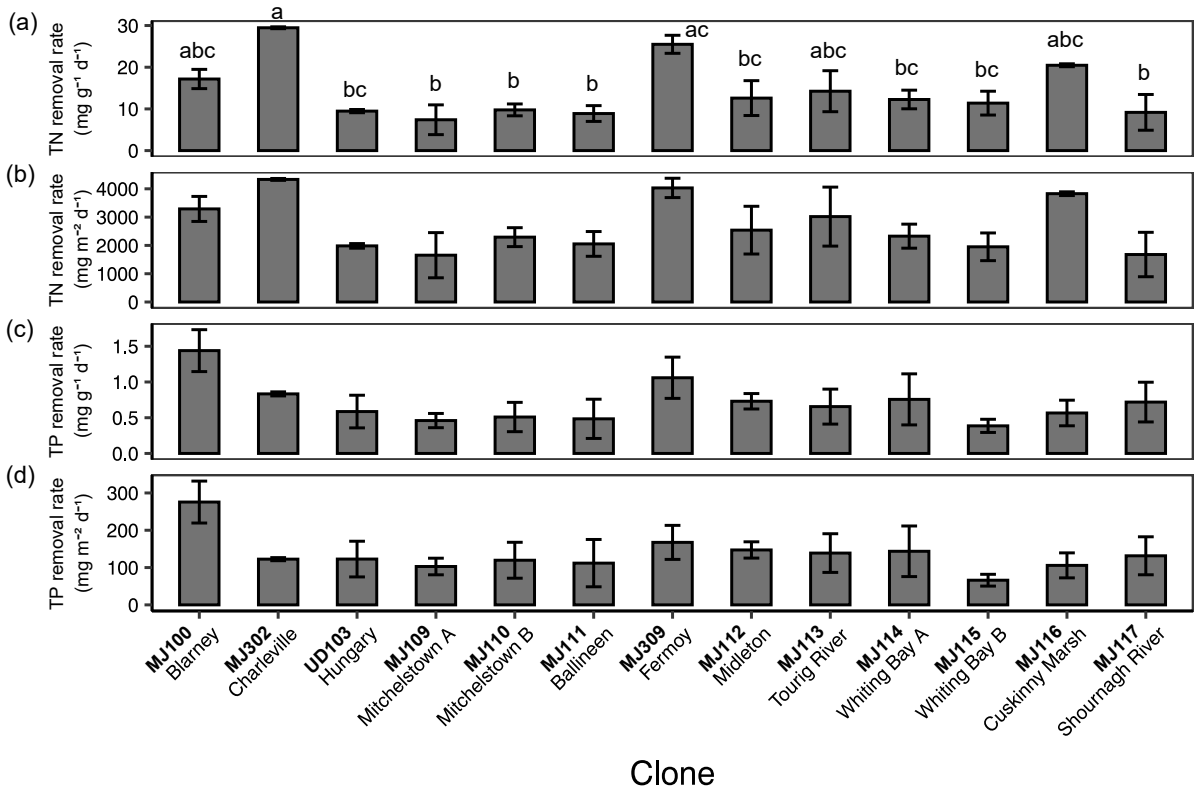

Figure 5.4. Mean (± standard error) for (a) TN removal rate (mg N g^{-1} d^{-1}), (b) TN removal rate (mg N m^{-2} d^{-1}), (c) TP removal rate (mg P g^{-1} d^{-1}) and (d) TP removal rate (mg P m^{-2} d^{-1}) for species and clones of duckweed grown on synthetic dairy wastewater. Bars that do not share the same letter are significantly different from each other ($p < 0.05$), as per post hoc Tukey test. Where no letters are included, rates were not found to be significantly different from each other.

TN removal (mg) over a 7-day period did not increase proportionally with increasing yield (Pearson's correlation: $r(11) = 0.291$, $p = 0.334$; Figure 5.5a). Clones which had higher yields only removed marginally more TN from the wastewater than clones with a lower yield. The same was not true for TP removal (mg) which increased proportionally with yield (Pearson's correlation: $r(11) = 0.695$, $p < 0.01$; Figure 5.5c). RGR had a more proportional relationship with TN removal (Pearson's correlation: $r(11) = 0.559$, $p = 0.047$; Figure 5.5b), although not

nearly as strong as observed for TP removal (Pearson's correlation: $r(11) = 0.669$, $p < 0.01$; Figure 5.5d).

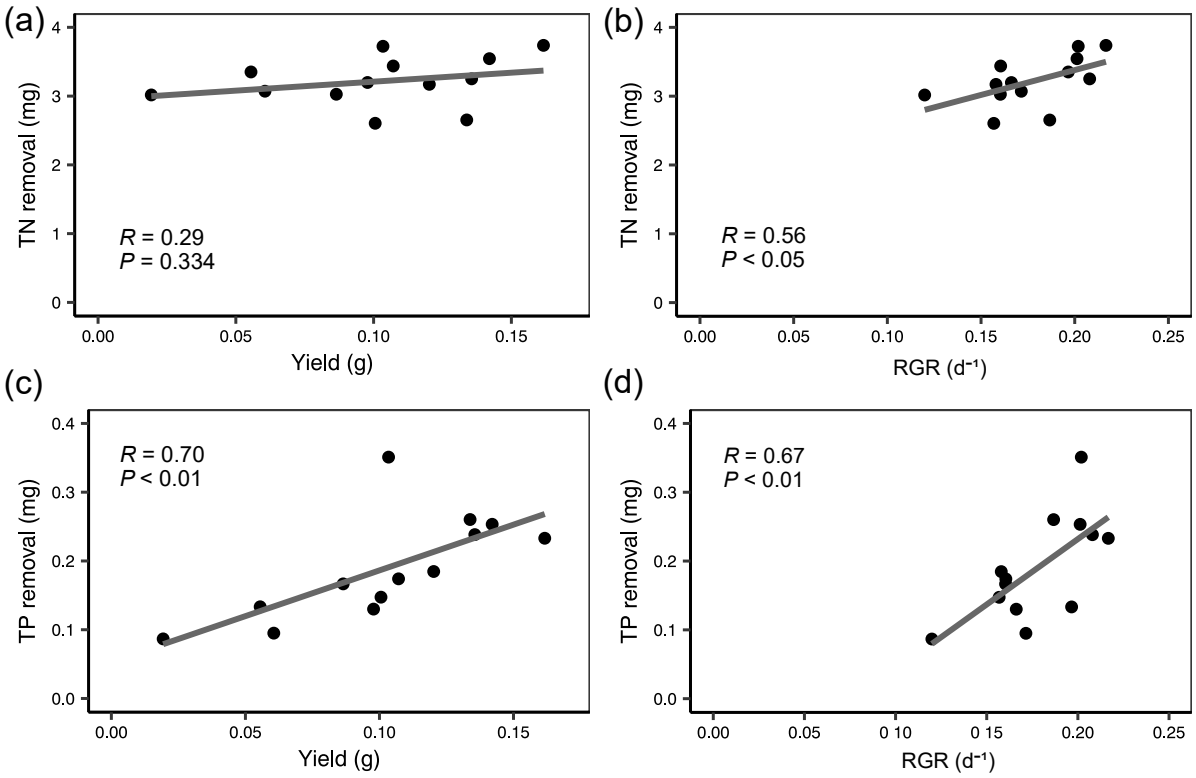

Figure 5.5. (a) TN removal (mg) vs. biomass yield (g), (b) TN removal (mg) vs. RGR (d^{-1}), (c) TP removal (mg) vs. biomass yield (g) and (d) TP removal (mg) vs. RGR (d^{-1}). Points are mean values and trendlines are linear. *R* is the correlation coefficient and *P* is the significance level of the correlation, which both come from Pearson's correlation test.

5.3.2.3. PCA analysis

A Principal Component Analysis (PCA) was conducted (Figure 5.6) for all clones with some of the measured parameters (RGR, protein content (% FW), TN removal rate (mg N g^{-1} d^{-1}) and TP removal rate (mg P g^{-1} d^{-1})). TP removal rates (mg P g^{-1} d^{-1}) were somewhat aligned with the growth parameter RGR, in accordance with the data in figure 5.5. However, the same was not true for the TN removal rate, where strong growth did not align with high TN removal

rates. Neither a strong growth rate, nor a high TN or TP removal rate co-aligned with high protein content in these species and clones. Indeed, higher removal rates (especially TP) and growth rates were associated with lower protein content.

The two *L. minuta* clones (7 and 2, bottom left of graph) were separated from *L. minor* clones due to their high TN removal rates (and to a lesser extent TP removal rates) combined with lower growth rates. Interestingly, *L. minor* clones collected from wastewater treatment plants (4, 5, 6, right of graph) converged together with slower growth rates and higher protein content. The commonly used *L. minor* clone MJ100 (Blarney) is positioned remarkably far to the left of the graph, showing good growth, and TN and TP removal, but low protein content. Most of the other *L. minor* clones did not display substantially different TN removal rates, despite differences in RGR.

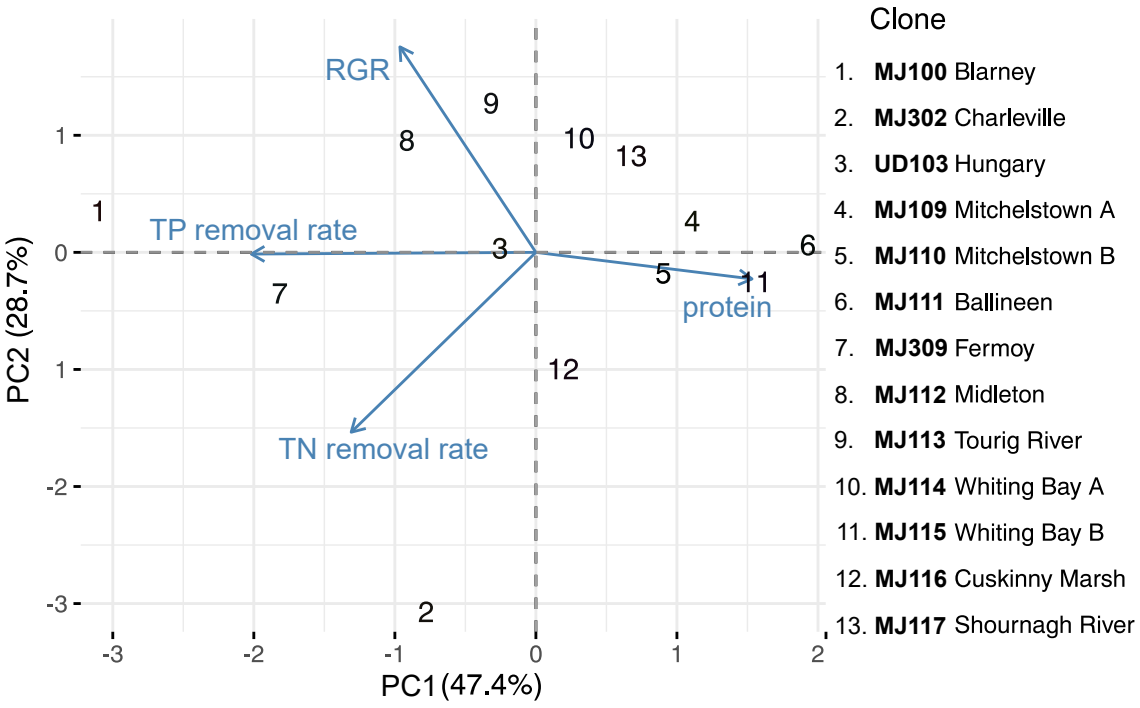

Figure 5.6. Principle component analysis (PCA) for all 13 clones of duckweed used in this study of four measured parameters: RGR, % protein content (of FW), TN removal rate (mg N

g^{-1} d^{-1}), TP removal rate (mg P g^{-1} d^{-1}). Each number on the plot (1-13) is linked to a clone, as shown in the legend. Each arrow corresponds to a particular measured parameter, as written next to each respective arrow.

5.4. Discussion

5.4.1. Species identification of clones

The ability to tell duckweed species (36 in total) apart is heavily reliant on molecular characterisation technology, such as intergenic sequencing (Wang et al. 2010). Due to a highly reduced structural complexity, closely related duckweed species appear morphologically similar (Landolt 1986). In addition, environmental conditions can complicate identification further as it influences the morphology of duckweed (Zhang et al. 2020). For seven species of duckweed, among them *L. minor*, current genetic barcoding methods cannot identify them with 100 % certainty (Borisjuk et al. 2015; Bog et al. 2019).

Rather than solely relying on genetic techniques, morphological characteristics can be used in conjunction with barcoding approaches to reliably identify certain species (Bog et al. 2020). In this study nearly all the clones collected in southern Ireland were *L. minor* (with one being *L. minuta*). Yet, the intergenic sequences *psbK-psbI* and *atpF-atpH* are identical for *L. minor* and *L. japonica* (it is hypothesised that *L. japonica* is a hybrid of *L. minor* x *L. turionifera*, Morello et al. (2021)). However, morphologically these two species can be distinguished by red pigmentation on the abaxial (lower) surface of the frond, which is often present in *L. japonica* but not in *L. minor* (Bog et al. 2020). As this study did not find any red pigmentation, it can be concluded that clones identified as *L. minor* in this study are not *L. japonica*. Indeed, the presence of *L. japonica* in Europe is also currently restricted to some Nordic countries.

5.4.2. Clonal growth and remediation

Growth and remediation parameters compare favourably with literature sources. For example, RGR varied from 0.11-0.22 d^{-1}, which compares well with RGR of 0.04-0.3 d^{-1} found in literature sources for duckweed grown on different wastewater sources (Al-Nozaily et al. 2000; Iatrou et al. 2015; Dinh et al. 2020). Protein content of dry biomass varied from 27.5 to 40 % (1.1-1.6 % of fresh biomass), which also compares favourably with commonly found literature values of 25-40 % for duckweed grown on wastewater (Oron 1994; Mohedano et al. 2012). Further, TN and TP rates varied even more than other parameters: 1678-4332 mg N m^{-2} d^{-1} and 84-332 mg P m^{-2} d^{-1}. These rates are also at the higher end of the range found in literature sources for duckweed grown on wastewater: 124-4400 mg N m^{-2} d^{-1} and 14-590 mg P m^{-2} d^{-1} (Körner and Vermaat 1998; Cheng et al. 2002b, a; Zimmo et al. 2004; Benjawan and Koottatep 2007; Mohedano et al. 2012; Zhao et al. 2015). However, it should be noted that the values from the literature include values from non-axenic studies, which usually have higher N and P removal rates (Zimmo et al. 2004; Mohedano et al. 2012). In non-axenic systems, microorganisms can account for a significant portion (50-70%) of removed nutrients (Körner and Vermaat 1998; Zhao et al. 2015). Nevertheless, some axenic systems also exhibit high N and P removal rates (Cheng et al. 2002a). Overall, it can be concluded from growth and remediation data that synthetic dairy wastewater is a suitable candidate for duckweed-based remediation for a range of species and clones.

5.4.3. Variation among clones

In this paper we show significant variation between duckweed clones in several physiological parameters. Variability between clones of the same species is a natural occurrence that derives from both random genetic drift, as well as adaptation to local conditions over prolonged periods (Leimu and Fischer 2008; Lascoux et al. 2016; Sandler et al. 2020). It has been suggested that this biological diversity may relate to the physical environment (Xue et al. 2012). Laboratory

studies have shown how genetically distinct duckweed clones differ in growth rate and nutritional composition (Sree et al. 2015b; Ziegler et al. 2015; Tang et al. 2015), although differences in nutrient uptake have been less quantified. Here it is shown that duckweed clones of the same species, collected from a relatively small geographical area can display significant variations in physiological characteristics. These findings are consistent with those by Paolacci et al. (2021), who previously showed genetic and physiological diversity of *L. minor* and *L. minuta* within Ireland, and Xu et al. (2015) who described genetic diversity within a number of duckweed species on a Chinese island.

While significant variation was found between clones, no one clone could be considered the 'best' for phytoremediation and protein-production. For example, *L. minuta* clone MJ302 (Charleville) displayed a high TN removal rate but a protein content on par with many other clones. In another example, MJ100 (Blarney *L. minor*) had a relatively high RGR on synthetic wastewater, however, its protein content, was one of the lowest among the studied clones. *L. minor* clones collected from areas with potential saltwater influence (MJ112 Midleton, MJ113 Tourig River, MJ114 Whiting Bay A, MJ115 Whiting Bay B, MJ116 Cuskinny Marsh) did not display significantly better growth or remediation ability on synthetic wastewater than most other clones. However, the Na and Cl^- concentrations (1.55 mM and 3.21 mM, respectively) in this synthetic dairy processing wastewater are not high enough for negative effects on duckweed growth and health to arise (usually around 20 mM; Sree et al. (2015)). It is possible that in conditions with higher NaCl concentrations these *L. minor* clones may have an advantage. However, this requires further characterisation of the putative salt resistance of these clones.

Three *L. minor* clones (MJ109 Mitchelstown A, MJ110 Mitchelstown B and MJ111 Ballineen) were collected directly from dairy wastewater treatment plants, where they were growing naturally on outdoor tanks containing dairy processing wastewater subject to tertiary polishing

(Ryan and Walsh 2016). The assumption that these clones may have a natural advantage over other clones when grown on a synthetic dairy wastewater was not fulfilled. A PCA analysis showed these clones group together suggesting an element of convergent evolution. However, these clones did not have higher growth or TN/TP removal than other clones, although, they generally had some of the highest protein contents.

Clones collected from water bodies in farming areas (MJ309 Fermoy, MJ117 Shournagh River) did not display overall faster growth and remediation than other clones. Although MJ309 had significantly higher TN removal rate than a number of other clones. Further analysis of water quality data of the water bodies in which they were collected did not show these water bodies being highly polluted (see https://www.epa.ie/our-services/monitoring--assessment/freshwater--marine/water-monitoring-and-assessment-/ for assessment of water quality carried out by EPA Ireland).

Overall, it is concluded that considerable clonal variation exists. However, no evidence was found that clones from specific locations (wastewater treatment plants, coastal zones, farming areas) are better adapted for growth on synthetic dairy processing wastewater.

5.4.4. Association between growth, protein and remediation traits

An interesting finding was how weakly linked TN removal rate and protein content were in the studied clones; a link which has been suggested before (Mohedano et al. 2012). A higher TN removal rate did not lead to higher protein content, although this link may have been anticipated as N makes up a significant portion of protein (Maclean et al. 2003). In fact, the opposite was generally true, plants with higher TN removal rates generally had a lower protein content. The extra N content that a clone with a higher TN uptake rate has, is not being transferred into a higher soluble protein content. It is possible then that surplus N is utilised in non-soluble

protein (e.g. thylakoid membrane proteins) or non-protein metabolites (Evans and Seemann 1989).

TN removal did not positively correlate with yield, while the correlation with RGR was not particularly strong. In contradiction to these results, studies of duckweed have shown a positive relationship between duckweed growth and N removal (Cheng et al. 2002b); the implication is that most N uptake goes directly into new growth. In this experiment the link between N uptake and new duckweed growth is not apparent. Luxury uptake of N has been recorded in duckweed at high N concentrations (8.1 mM or 114 mg L^{-1} in synthetic wastewater) (Oscarson et al. 1989). Luxury uptake of N for storage would disrupt the connection between N uptake and new biomass growth. It is speculated that duckweed are taking up significant amounts of N from the medium for storage, as described by Kufel et al. (2012). Clones appear to store N to varying degrees. This was found especially in relation to the *L. minuta* clones MJ302 (Charleville) and MJ309 (Fermoy) which take up high amounts of N, but display modest growth rates (MJ302) and yield (MJ302 and MJ309). A study from Ceschin, Crescenzi, and Iannelli (2020) suggested that unlike *L. minor*, *L. minuta* is a hyperaccumulator of nitrate. While the N-containing compounds in the synthetic wastewater used in this study are ammonium and urea, it can be speculated that hyperaccumulation of various nitrogen-compounds applies to these *L. minuta* clones.

For TP removal there was a strong, proportional relationship with biomass yield and RGR. The concentration of P in the synthetic wastewater is relatively low, 0.35 mM or 10.9 mg L^{-1} which, although not a limiting factor, may not trigger luxury uptake (Walsh et al. 2020).

In general, differences in RGR between clones translated into faster growing clones being partially better wastewater remediators i.e. removing more P but not N. P is a significant contributor to pollution e.g. eutrophication (Bol et al. 2018), and therefore its emission limit is

set low (European Commission 2019). Yet, from a remediation standpoint, rapid removal of both N and P is important. However, the data presented here also show an antagonism between N and P uptake, with some of the best clones for N uptake less efficient in P uptake, and forcing operators of remediation systems to make a choice about their primary objective. Furthermore, in a circular economy-based system the remediation aspect is balanced with the extracted value (in this case the duckweed biomass and protein content) (Ahmad et al. 2019). PCA analysis shows that protein content is not associated with N and P-uptake, thus further complicating management decisions. Indeed, no measured parameter was a good predictor for another parameter.

5.5. Conclusion

Dairy processing wastewater is a suitable candidate for duckweed-based remediation. Duckweed clones assessed for this study showed a significant diversity in remediation-relevant parameters. It is shown that duckweed clones of the same species, collected from a relatively small geographical area can display significant variations in physiological characteristics. Clones with higher protein content tended to have slower growth rates and were not associated with better TN and TP removal rates. Thus, it is unlikely that remediation approaches can be optimised for high nutrient removal and high protein yield. Overall, this study did not identify an "optimal" duckweed clone and/or species for remediation of dairy processing wastewater, but rather emphasises that some clones are better than others for specific aspects of the process of remediation and valorisation of wastewater.

Author contributions

ÉW and MAKJ contributed to the study conception and design. Plant samples were collected by ÉW and EC. Material preparation, data collection and analysis were performed by ÉW and

ED. All authors contributed to the interpretation of results. The first draft of the manuscript was written by ÉW. ÉW and MAKJ contributed to writing and editing of the manuscript.

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Chapter 6

Remediation of dairy processing wastewater through the integration of microbial digestion with duckweed

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Abstract

The dairy industry generates large volumes of wastewater in the processing of milk into dairy products. This dairy processing wastewater predominantly consists of organic material but also contains significant amounts of N, P, salt (NaCl), and lower concentrations of metals (Ca, Mg, Fe, K). Due to the wealth of compounds contained in this wastewater, it can be used as a valuable resource i.e. valorisation. One novel method, as examined in this study, of remediating and extracting value from this wastewater is to use an integrated system of microbial digestion with phytoremediation. Accordingly, wastewater was taken from a dairy processing facility and a number of microbiological treatment methods (A/O, IASBR, ADF) were combined with cultivation of the aquatic plant *Lemna minor* (duckweed). Highly successful was the combination of an ADF reactor (aerobic dynamic feeding) with subsequent duckweed cultivation, which removed nearly all organic compounds (97 % of COD) and total nitrogen (97 %) from the wastewater. However, significant amounts of P remained in the wastewater (it was reduced by 73 % overall), which would necessitate further processing to bring within discharge limits. Furthermore, in combination with the ADF reactor the duckweed biomass had a high protein content and chlorophyll fluorescence measurements showed that the plant was healthy. Thus, dairy processing wastewater can be remediated to a high level with a combination microbial reactors and duckweed cultivation. This will inform on the integration of duckweed cultivation into existing industrial wastewater treatment systems.

6.1. Introduction

The dairy processing industry consumes a significant portion, 12 %, of total water used by the food processing sector worldwide (Bustillo-Lecompte and Mehrvar 2015). Such water use results in a substantial production of wastewater. It is estimated that up to 10 L of wastewater is produced per litre of milk processed into products such as cheese, milk powder and yoghurt (Wang and Serventi 2019). In general, this dairy processing wastewater contains high amounts of organic components such as sugars and fats, measured as chemical oxygen demand (COD): 2000-6000 mg L^{-1} (Ince 1998; Demirel and Yenigun 2004). Furthermore, cheese whey in particular exhibits a notably high COD of up to 77000 mg L^{-1} (Carvalho et al. 2013). Additionally, dairy processing wastewater contains a considerable amount of non-organic nutrients: 4.6-19.3 mM NH_3-N (64-270 mg L^{-1}), 0.64-2.1 mM NO_3-N (9-30 mg L^{-1}), 0.65-11.5 mM PO_4-P (20-356 mg L^{-1}), as well as metals (e.g. Ca, Mg, K, Fe) (Ghaly and Singh 1989; Malaspina et al. 1996; Ince 1998; Menchik et al. 2019).

The disposal of dairy processing wastewater is restricted by regional and/or country-level regulations, which dictate allowable emission limits (European Commission 2019). Accordingly, the wastewater must be treated chemically and biologically to bring it within disposal limits (Slavov 2017). The operation of treatment systems traditionally comprised a cost for the dairy industry. Although, modern treatments systems of dairy processing wastewater have been developed to capture the energy content of the wastewater, for example through anaerobic digestion (AD) which produces the biofuel methane (Charalambous et al. 2020). However, in general there is a lack of value-capture of non-organic constituents of wastewater, such as N and P (Ahmad et al. 2019). N-containing nutrients such as ammonia and nitrate are frequently removed from the wastewater through the nitrification-denitrification cycle which results in the production and release of N_2 gas (Gil-Pulido et al. 2018). Phosphorous is typically precipitated through the addition of aluminium salts or lime (Bunce

et al. 2018) with the resulting sludge being used, to arguably limited effect, in land spreading (López-Mosquera et al. 2000). Such dissipation of beneficial plant nutrients is regrettable, given that the resource of mineable rock-phosphate is rapidly dwindling (Cordell et al. 2009) and given that production of N-fertiliser from atmospheric N_2 through the Haber-Bosch process requires substantial amounts of energy, typically in the form of fossil fuels (Smith et al. 2020).

Applying the principles of the circular economy (CE) to the wastewater treatment process can stem the loss of organic and inorganic resources through value-capture methods. CE is an economic model which promotes closed-loop production patterns, reduced raw material use and waste minimisation (Morseletto 2020). Key to this is the appropriation of waste as a resource (Gherghel et al. 2019). Capturing the value from dairy processing waste requires a cascading approach whereby microorganisms create value from sugars and fat, while the resulting effluent that is mostly devoid of organic constituents, is further polished by plants that take up inorganic elements to sustain their growth (Ahmad et al. 2019; Akansha et al. 2020). Such an approach will generate value, as well as clean dairy processing wastewater such that it can be released on surface waters without any further treatment.

Here, a novel integrated approach to valorising dairy processing wastewater is presented. The approach comprises of microbial-driven fermentation, followed by duckweed-based remediation. Microbial-based techniques can remove organic components from wastewater and convert them into valuable products such as methane gas (Li et al. 2019; Mannina et al. 2020), bioethanol (Sampaio et al. 2020), and/or polyhydroxyalkanoate (PHA) i.e. a bioplastic precursor molecule (Serafim et al. 2004).

Four different biological nutrient removal (BNR, Barker and Dold (1997)) methods were used in this study to valorise and/or remove the organic and inorganic components of dairy processing wastewater. First, a commercial biogas-producing anaerobic digester (AD) was

used, which removes organic matter from wastewater and converts it into biogas in an oxygen free environment (Charalambous et al. 2020). Second, a laboratory-scale anoxic-oxic reactor (A/O) was used, which employs separate anoxic and oxic zones to remove N, P and COD from wastewater (Carrera et al. 2004). Third, a laboratory-scale intermittently aerated sequencing batch reactor (IASBR) was employed, which reduces N, P and COD in wastewater but uses only a single reactor with intermittent aeration throughout the treatment to create aerobic periods between anoxic periods (Gil-Pulido et al. 2018). Lastly, a laboratory-scale aerobic dynamic feeding (ADF) reactor was used. This reactor generates a so-called 'feast and famine' treatment, in which a short period of excess carbon, feast, is followed by a longer period of starvation, famine, which promotes the accumulation of the storage molecule PHA (Serafim et al. 2004). The ADF and IASBR systems both offer single, sequencing batch reactor operation strategies, however the former is designed to enrich for bacteria with storage mechanisms for organic carbon rather than metabolising it to CO_2.

All these methods generally achieve high removal efficiencies (> 90 %) of COD (Ince 1998; Pan et al. 2013), which makes the wastewater potentially suitable for subsequent phytoremediation. Plants can take up nutrients from a wastewater source, creating valuable plant biomass in the process (Ahmad et al. 2019; Hu et al. 2020). Duckweed, Lemnaceae, is a family of aquatic plant species which are of particular interest for use in phytoremediation due to their tolerance of wastewater conditions (Toyama et al. 2018), fast growth rates (Ziegler et al. 2015) and nutritional composition (Cui and Cheng 2015; Appenroth et al. 2017). Duckweed species have been successfully cultivated on, and used to remediate, a wide range of wastewater types ranging from farm wastewater (Mohedano et al. 2012) and municipal wastewater (Verma and Suthar 2014) to industrial wastewaters (Teixeira et al. 2014) and even a synthetic dairy processing wastewater (Walsh et al. 2020, 2021).

The overarching aim of this study was to develop an integrated system for the treatment of dairy processing wastewater. Specifically, it was explored which combinations of microbial wastewater treatment are compatible with duckweed-based polishing, and result in effective remediation. Furthermore, the relative contribution of remediation by microbial and duckweed constituents to water remediation was ascertained. Once suitable microbial wastewater pre-conditioning methods were established, impacts on important plant parameters, such as biomass production and protein content were assessed to determine potential waste valorisation.

6.2. Materials and Methods

6.2.1. Wastewater origin

Dairy processing wastewater was sourced from a large scale, commercial dairy processing facility in the Munster region in the south of Ireland. This facility produces a range of dairy products including cheese, butter and milk powder. The aqueous waste streams from all production-lines, along with water and chemicals used for cleaning, are screened for the removal of large lumps of solids and then collected together in a balancing tank. Then the wastewater is fed into an on-site anaerobic digester (AD). The 45 000 m^3 AD digests (anoxically) a high percentage (average 90 %) of the raw wastewaters biochemical oxygen demand (BOD) and total suspended solid (TSS) loadings while converting the degradable organics into energy-rich biogas and a small amount of waste biomass (sludge).

Wastewater from the balancing tank was used as a feed-stock for experimental, microbial-based wastewater treatments. Furthermore, both untreated dairy wastewater from the balancing tank, as well as wastewater that had passed through a commercial AD, i.e. AD effluent, were used for duckweed growth experiments.

6.2.2. Experimental microbiological wastewater treatment

6.2.2.1. A/O system

The A/O reactor consisted of two reactor vessels, anoxic and oxic, followed by a clarifier, with a total system volume of 7 L. Aeration was provided to the oxic vessel at ≥ 5 L min^{-1} (Welch® 2511 dry vacuum pump/compressor, Denver, USA). Influent flow rates were set at 200 mL h^{-1} while spatial configuration of the system allowed for gravity feeding between reactors and clarifier (MasterFlex Peristaltic Pump 6-300 RPM). After the reaction vessels, the clarifier separated solids from the effluent liquid phase. The solid sludge was returned to the treatment system periodically to maintain mixed liquor suspended solids at approximately 4 g L^{-1} and excess sludge autoclaved before disposal. The clarified effluent phase provided the feedstock for downstream polishing treatment via the duckweed cultivation system. The hydraulic retention time (HRT) for the whole system was 1.46 days. The system was run for a total of 210 days during which influent and effluent samples were subjected to pH measurements and Hach spectrophotometric analyses to monitor COD, NO_3, NH_4 and PO_4 levels 2-3 times per week.

6.2.2.2. IASBR system

The IASBR system consisted of a single vessel with a 2 L system volume running on a 12 h cycle. At these start of a cycle 500 mL of wastewater was pumped into the reactor (MasterFlex Peristaltic Pump 6-300 RPM). Over 12 hours, two-hour aeration periods (Welch® 2511 dry vacuum pump/compressor) were followed by 20-minute non-aeration periods, with continuous mixing the whole time. After 12 hours there was a settling phase of 40 minutes, after which 300 mL of the liquid phase was removed for further treatment via duckweed cultivation. A further 200 mL of the settled wastewater was removed for disposal. The IASBR system was run for 265 days during which influent and effluent samples were subjected to pH

measurements and Hach spectrophotometric analyses to monitor COD, NO_3, NH_4 and PO_4 levels 2-3 times per week.

6.2.2.3. ADF system

The ADF reactors had an operational volume of 2 L and was heterogeneously mixed via overhead, motor driven, stirring shafts with a single impeller. Reactors were operated in 8 h automated cycles wherein 450 ml of AD effluent was added to each reactor at the start of a cycle together with mixing and oxygen, supplied at a rate of 0.5 L min^{-1} (Fisher Scientific vacuum/aerator pump). Mixing and aeration ceased after 7 hours and the biomass was allowed to settle under gravity for 40 minutes before withdrawing 450 ml of clarified supernatant over a 15-minute period (Watson Marlow 454S peristaltic pump). Manual biosolids removal was performed periodically at the end of the supernatant withdrawal phase to maintain mixed liquid suspended solids between 3-4 g L^{-1}. Samples of influent and effluent were taken over the course of its run for full physico-chemical analysis.

6.2.3. Duckweed cultivation on dairy processing wastewater

Duckweed was grown on the following types of wastewater (Figure 6.1):

(1) Untreated commercial dairy processing wastewater

(2) Effluent from a commercial AD

(3) A/O effluent

(4) IASBR effluent

(5) ADF effluent

The basic duckweed culture system for remediation of wastewaters (1), (2), (3) and (4) comprised of *L. minor* Blarney (strain 5500 RDSC, Lahive, O'Halloran, and Jansen (2012)) cultivated on 100 mL of wastewater for 7 days (days 0-7) in Magenta vessels (GA-7) in a controlled growth room (average light intensity 50 µmol m^{-2} s^{-1} PAR, 22 °C, 16h:8h light:dark

photoperiod). On day 0 each replicate was started with three colonies that had been growing on an optimised medium. Total fresh biomass was measured on day 7.

6.2.3.1. Untreated wastewater, AD, A/O and IASBR effluent

Untreated wastewater and AD effluent taken from a commercial dairy production facility were used immediately for duckweed cultivation or stored at 4 °C until used. Untreated wastewater was taken from the wastewater treatment facility at two separate time points for duckweed cultivation ($n = 8$). Samples of AD effluent were also taken at two time points. The total volume of AD effluent was separated in half, with the pH in one half being reduced from 8 to on average 4.8. The pH of the other half of the effluent was left unchanged. Duckweed was cultivated on both naturally alkaline AD effluent ($n = 10$) and pH-reduced effluent ($n = 6$).

Wastewater effluent was taken from the A/O system at two time points for duckweed cultivation ($n = 10$) and the IASBR system at one time point for duckweed cultivation ($n = 3$) over the course of their operation.

6.2.3.2. ADF effluent

ADF effluent had a natural pH ranging from 8.6-8.8 and an NH_3-N concentration range of 0.21-0.5 mM (1.75 to 7 mg L^{-1}). To explore the potential negative effect of ammonia on duckweed growth, the effect of combinations of different pH and ammonia levels on duckweed growth was assessed using 3 samples of ADF effluent taken over a month's period. A factorial design with two pH levels (low, pH 5, and high, pH 9) and two ammonia levels (low, 0.21-0.5 mM, and high, 2.1 mM) was used. The pH of ADF effluent was reduced to around 5 with 1 M H_2SO_4. The NH_3-N concentration was increased to 2.1 mM (30 mg L^{-1}) through the addition of ammonium sulphate $(NH_4)_2SO_4$.

ADF wastewater was further used to analyse wastewater remediation and duckweed biomass growth. 500 mL samples of ADF reactor effluent were taken at four points over the course of

3 months. The pH of the effluent sample was reduced to around pH 5 with 1 M H_2SO_4, and then split between 5 Magenta vessels. The environmental conditions were as described above. Colonies of *L. minor*, taken from stock cultures acclimated to real wastewater conditions for 7 days, were used to generate 50 % plant surface coverage (as determined by EasyLeafArea) for each Magenta. Densities were maintained at ±2 % of target surface cover throughout the experiment through the removal of excess plant material every 2-3 days. This process was guided by measurements of duckweed surface area, as determined by Easy Leaf Area. Removed excess plant biomass was weighed and used to calculate the biomass yield (n = 5). A 3-day RGR (n = 5) was calculated based on the increase in biomass at the first harvest time point. Plant samples were taken on day 7 for protein content analysis (n = 5) and amino acid analysis (n = 3). Chlorophyll *a* fluorescence measurements were taken on randomly selected plants on day 0 and day 7 (n = 5) to assess plant health.

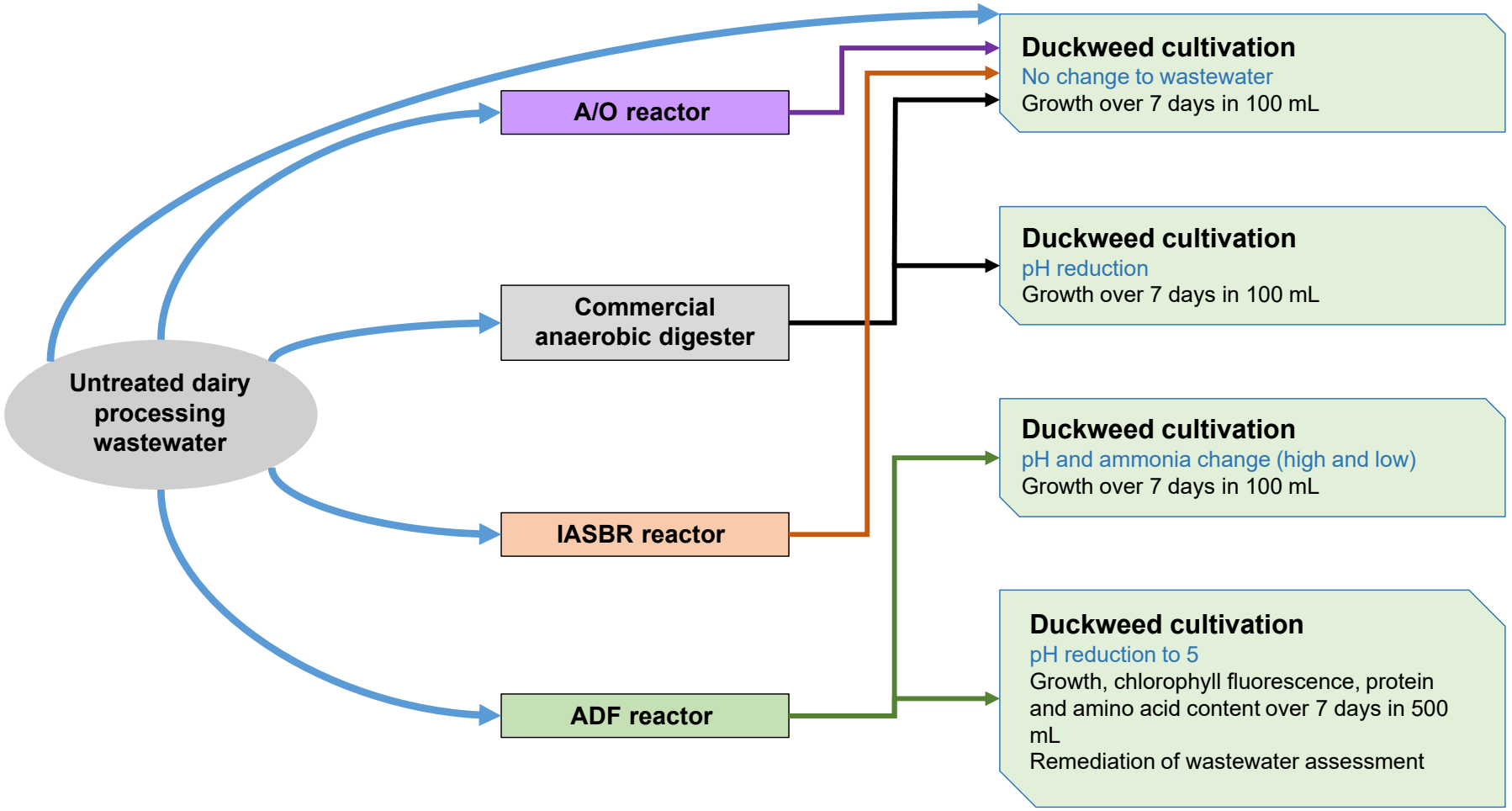

Figure 6.1. Overview of experiments assessing the integration of microbial and duckweed systems for the remediation of dairy processing wastewater.

6.2.4. Duckweed biomass measurements

6.2.4.1. Growth

Relative growth rate (RGR) was calculated with estimations and measurements of starting and final fresh biomass using the formula:

$$\mathrm{RGR} = \frac{\ln \frac{W_2}{W_1}}{\Delta T}$$

Where ln is the natural log, W_1 is starting fresh biomass, W_2 is fresh biomass on the final day of the experiment and ΔT is the length of the experiment.

For duckweed cultivated on ADF effluent, biomass was removed throughout the experiment to maintain a constant plant density. In this case, this formula was used to calculate the RGR up to the first instance of removal (day 3) for comparison with literature sources. Yield (g) is the total amount of excess biomass removed over the course of the experiment.

6.2.4.2. Protein content

Lemna minor samples, taken on the final day of the ADF experiment (day 7), were kept at -20 °C until used for protein extraction and analysis. Protein was extracted using 50 mM potassium phosphate buffer (pH 7, containing 0.1 mM polyvinylpyrrolidone (PVP) and 0.1 mM EDTA). Between 50–80 mg of fresh plant material was homogenised in cold potassium phosphate buffer (1 mL of buffer to 80 mg of plant sample). The homogenised sample was then centrifuged at 20,000 x *g* for 30 minutes at 4 °C (Balen et al. 2011). The resulting supernatant was used for protein analysis using the Bradford method with bovine serum albumin as a standard (Bradford 1976). For absorbance measurements, 5 μL of sample was added to 1 mL of Bradford reagent in a cuvette and left for five minutes in dark conditions. Absorbance was measured at 595 nm using a spectrophotometer (UV-160A Shimadzu). In order to calculate the

proportion of protein based on dry plant biomass, 4 % dry weight content of fresh duckweed weight was used (Appenroth et al. 2017).

6.2.4.3. Amino acid content

This method involved hydrolysing amino acids from standards and samples (Galdón et al. 2010; Mišurcová et al. 2014), prior to derivatising the hydrolysates with diethyl ethoxymethylenemalonate (DEMM) as described by Ortiz et al. (2006) and Alaiz et al. (1992) before detection with HPLC-UV (1290 Infinity II system with 1260 Infinity II Diode Array Detector; Agilent Technologies). First, 25 mg of each freeze-dried duckweed sample was digested in 6 M HCl at 110 °C for 24 hours. Each hydrolysate sample was then evaporated to dryness using a vacuum concentrator (MiniVac evaporator, ScanVac). Amino acid standards (Sigma Aldrich) were made up to concentrations 12.5, 25, 44, 63, 125 and 186 µM.

1 µL of DEMM was added to the concentrated hydrolysate or the amino acid standard or Milli-Q water (for a blank), which were then reconstituted with 1 mL of 1 M borate buffer (boric acid in MilliQ water adjusted to pH 9 with NaOH). Samples were then placed on a heating block at 50 °C for 50 minutes for the derivatisation reaction. They were then left to cool to room temperature.

Standards and samples were run through a Supelco® C18 HPLC column (3-µm particle size) with two mobile phases: 25 mM sodium-acetate with 0.05 % sodium azide (pH 6) and 100 % acetonitrile. 10 µL of each sample and standard was run through the column at 0.9 mL min^{-1} (50 minutes for each sample) and detection was set at 280 nm. Aspartame and aspartic acid were detected as a single peak (aspartic acid) as were glutamine and glutamic acid (which were identified as glutamic acid).

6.2.4.4. Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence measurements were taken on randomly selected plants on days 0 and 7 (n = 4), using a pulse amplitude modulated fluorometer (WALZ Imaging fluorometer, Effeltrich, Germany). The procedure that was followed is detailed in Walsh et al. (2020).

6.2.5. Water quality measurements

6.2.5.1. A/O and IASBR remediation

For the assessment of remediation in A/O and IASBR systems, measurements of COD, NH_4^+, NO_3^- and PO_4^{3-} were made using a Hach DR2800 spectrophotometer. Hach test method 8000 (20-1500 mg L^{-1}) was used for COD measurements. Hach method 8036 (Nessler method, 0.02-2.5 mg L^{-1}) was used for NH_4^+ measurements. Hach method 8171 (0.1-10 mg L^{-1}) was used for NO_3^- measurements. Hach method 8114 (molybdovanadate method, 0.3-45 mg L^{-1}) was used for PO_4^{3-} measurements.

6.2.5.2. Physico-chemical analysis of wastewater

For full physico-chemical assessment of untreated wastewater, AD effluent, ADF effluent and duckweed reactor effluent, samples of wastewater were sent to the Aquatic Services Unit (Environmental Research Institute, UCC, Lee Road, Cork , Ireland). BOD, COD, total solids, total nitrogen (TN) and total phosphorous (TP) were measured on whole wastewater samples, i.e. unfiltered sample, as per standard methods for wastewater analysis (Rice et al. 2005; Grasshoff et al. 2009). For the following compounds the wastewater was filtered at 0.45 µm to determine the dissolved concentration. Ammonia, nitrate, nitrite and orthophosphate were measured on filtered wastewater using the Lachat QuikChem 8000 by Zellweger Analytics, Inc. Milwaukee. USA (QuikChem Methods 10-107-06-3-D, 10-107-04-1-C, 10-107-04-1-C and 10-115-01-1-B, respectively). Sodium, potassium, calcium, magnesium, zinc and iron were measured in filtered wastewater using flame AAS (Varian Australia Ply Ltd. 1989). Copper

and manganese were measured using graphite furnace AAS (Varian Australia Ply Ltd. 1989). Chloride was measured using ferricyanide method on filtered wastewater (Rice et al. 2005).

6.2.6. Data analysis

Statistical analyses were conducted using R (version 3.4.3, R Core Team (2019)). One-way ANOVAs were used to analyse differences between treatments. Two-sample t-tests were used to compare chlorophyll fluorescence measurements taken on day 0 and day 7. Normality was assessed through a graphical assessment of the distribution of the residual values for data points (i.e. histogram). Homoscedasticity was assessed with 'residuals vs. predicted values' plots as well as Fligner-Killeen tests. Multiple linear regression was used to analyse the effect of pH and ammonia on duckweed RGR. The presence of multicollinearity was assessed using Variance Inflation Factor (VIF) values.

6.3. Results

6.3.1. Commercial dairy wastewater: untreated wastewater and AD integrated with duckweed cultivation

The use of commercially generated wastewaters, untreated wastewater and AD effluent, was explored first, and this included both the physicochemical analysis of two types of wastewater, as well as an assessment of their suitability for duckweed cultivation. Compositional differences between untreated wastewater and AD effluent were mainly in the concentrations of COD and BOD, with a dramatic decrease in both BOD and COD following anaerobic digestion (Table 6.1). For example, BOD was reduced from 1197 to 33 mg L^{-1} on average, as a result of the passage through the AD reactor. Most other elements remained at similar concentrations (Table 6.1). Additionally, the pH was generally lower in the untreated wastewater, 6.5, compared to anaerobic digester effluent, 8-9.

L. minor grew on untreated wastewater to different degrees of success over 7 days (Figure 6.2a). However, at the end of 7 days a layer of microbial gunk had usually formed on the surface of the medium, and plants were discoloured and dying.

L. minor RGR on AD effluent was on average a low 0.02 d^{-1} (Figure 6.2a) and by day 7 of growth the plants were discoloured and dying. However, duckweed cultivated on AD effluent with pH manually reduced to 5, had a significantly higher RGR (one-way ANOVA: $F(2) = 117$, $p < 0.001$; Figure 6.2a).

Table 6.1. Composition of untreated dairy wastewater, AD effluent, ADF effluent and duckweed reactor effluent.

Parameter	Untreated wastewater (mean±SD, *n* = 5)	AD effluent (mean±SD, *n* = 3)	ADF effluent (mean±SD, *n* = 4)	Duckweed reactor effluent (mean±SD, *n* = 4)
pH	6.5 (±0.5)	8.5	8.9 (±0.1)	7.9 (±0.3)
BOD (mg L^{-1})	1496 (±594)	33.2 (±3)	22.3 (±18)	9 (±3.9)
COD (mg L^{-1})	2663 (±459)	292.3 (±181)	60 (±32)	77 (±43)
Total Solids (mg L^{-1})	4198 (±659)	3240 (±122)	2825 (±555)	2870 (±0.5)
Total nitrogen (mM)	7.8 (±1.6)	6.7 (±0.8)	1.4 (±0.6)	0.3 (±0.1)
Ammonia-N (mM)	2.7 (±0.8)	5.1 (±0.6)	0.6 (±0.3)	0.001 (±0.0001)
Nitrate-N (mM)	BD (<0.0007)	BD (<0.0007)	0.8 (±0.4)	0.009 (±0.01)
Nitrite-N (mM)	BD (<0.0001)	BD (<0.0001)	NM	NM
Total phosphorus (mM)	1.0 (±0.2)	0.91 (±0.003)	0.5 (±0.1)	0.3 (±0.1)
Orthophosphate-P (mM)	0.8 (±0.2)	0.86 (±0.02)	0.4 (±0.1)	0.3 (±0.1)
Sodium (mM)	36.5 (±7.7)	45.2 (±0.5)	38.7 (±8.7)	36 (±8.5)
Chloride (mM)	26 (±8.6)	26.9 (±1.3)	24.7 (±8.9)	23.5 (±10)
Potassium (mM)	2.7 (±0.6)	2.3 (±0.08)	2.7 (±0.5)	2.5 (±0.7)
Calcium (mM)	2.3 (±1.3)	2.2 (±0.06)	1.2 (±0.3)	1.1 (±0.2)
Magnesium (mM)	1.9 (±3.3)	0.42 (±0.03)	0.4 (±0.1)	0.3 (±0.1)
Iron (mM)	0.006 (±0.004)	0.004 (±0.0003)	0.003 (±0.0008)	0.003 (±0.001)
Zinc (mM)	0.05 (±0.06)	0.0003 (±0.0001)	0.06 (±0.1)	0.009 (±0.006)
Copper (mM)	0.0003 (±0.0002)	0.0001 (±0.0002)	0.0002	0.0007 (±0.0005)
Manganese (mM)	0.007 (±0.01)	0.0003 (±0.00005)	0.0001 (±0.00006)	0.0002 (±0.0001)

BD – Below detection
NM – Not measured

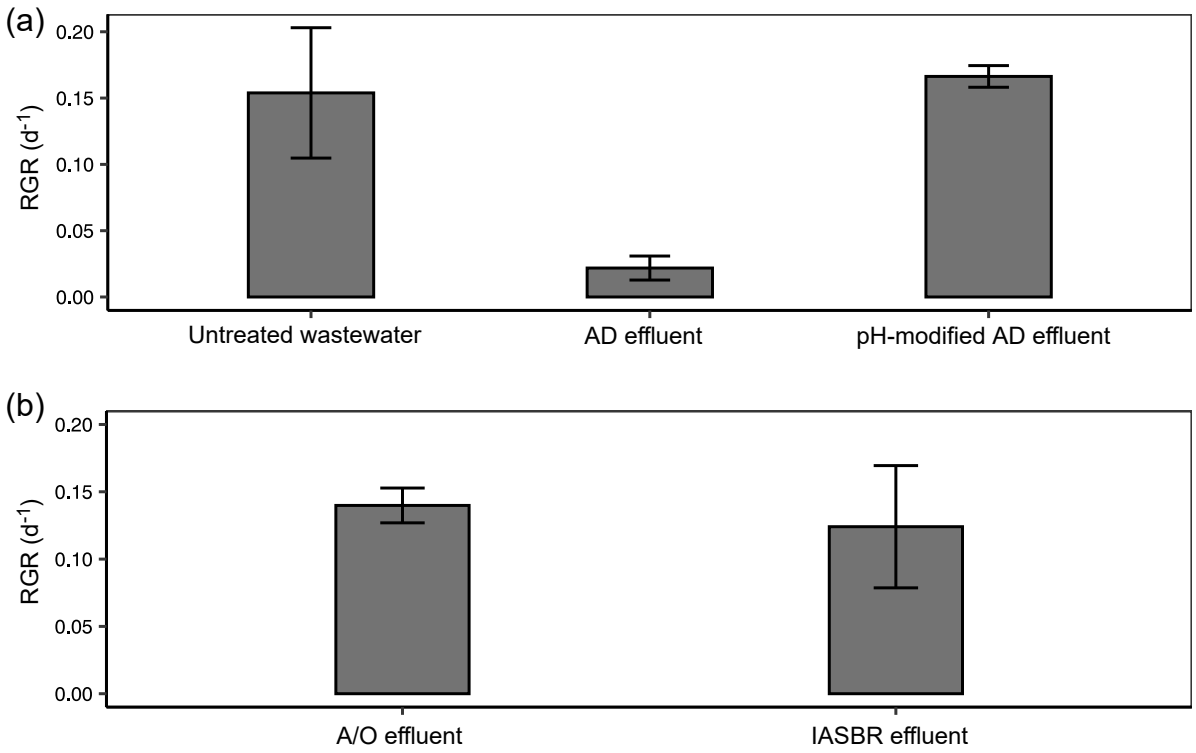

Figure 6.2. Mean RGR (d^{-1}) ± SE for *L. minor* grown on fives types of wastewater: (a) untreated wastewater, anaerobic digester (AD) effluent, and AD effluent in which the pH was reduced to 5 and (b) A/O reactor effluent and IASBR reactor effluent.

6.3.2. A/O and IASBR systems integrated with duckweed cultivation

The A/O and IASBR systems removed up to 98 % of COD from untreated dairy processing wastewater, although sometimes efficiency dipped below these levels for short periods (Table 6.2). Removal of NH_3-N was up to 99 % in the A/O system and up to 94 % in the IASBR system (Table 6.2). Although sometimes the removal dropped significantly for short periods. NO_3-N removal was 100 % for both A/O and IASBR systems (Table 6.2). Removal of PO_4^3-P was generally lower but reached up to 95 % in the A/O system and 80 % in the IASBR (Table 6.2).

L. minor grown on wastewater on samples from the A/O system had an average RGR of 0.14 d^{-1} (Figure 6.2b). Duckweed maintained a similar RGR, 0.12 d^{-1}, on effluent from the IASBR system (Figure 6.2b).

Table 6.2. Removal efficiencies in A/O and IASBR systems

Parameter	A/O system (% removal)	IASBR system (% removal)
COD	80-98	60-97
Ammonia-N	20-99	4-94
Nitrate-N	100	100
Orthophosphate-P	50-95	30-80

6.3.3. Duckweed cultivation on ADF reactor wastewater

Initial growth trials on ADF effluent wastewater showed poor *L. minor* growth on some samples, but successful growth on others. The wastewater contained significant amounts of solids that were being carried over from the ADF reactor (Table 6.1). However, filtration of the samples to 1.2 µm had no effect on growth.

An analysis of the composition of the ADF effluent showed that the wastewater had consistently high pH (8-9) with a fluctuating ammonia concentration (Table 6.1). Therefore, the phytotoxicity of ammonia was assessed through ammonia addition and pH reduction (Figure 6.3). It was shown that a combination of high pH, 8.6-8.8, with an NH_3 concentration of 0.5 mM (7 mg L^{-1}), or above, negatively affected duckweed RGR, leading to no growth when NH_3 concentration was increased to 2.14 mM (30 mg L^{-1}). However, when the pH was neutralised, by adding H_2SO_4, to values ranging between pH 4.9 and 5.1, no negative effects on RGR were observed, irrespective of the ammonia concentration. A multiple linear regression ($F(3, 32) = 46.29$, $p < 0.001$, $R^2 = 0.81$; Figure 6.3) showed that pH and ammonia explained a high amount of the variation in RGR.

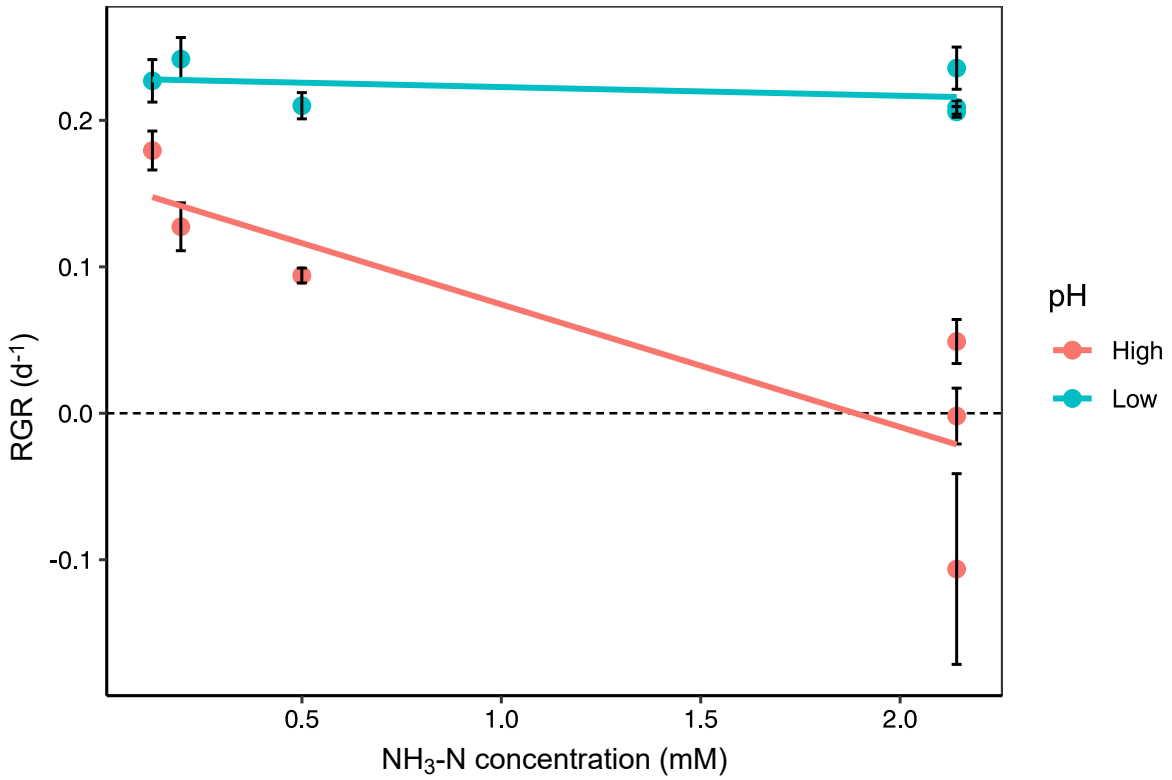

Figure 6.3. Mean RGR (d^{-1}) ± SE of *L. minor* grown on ADF wastewater effluent vs. concentration of NH_3-N (mM) in ADF wastewater effluent. 'High' pH denotes values from 8.6-8.8 and 'low' pH denotes values from 4.9-5.1. Trendlines are fitted as per multiple linear regression analysis.

6.3.4. Assessment of plant parameters for duckweed grown on ADF effluent

Chlorophyll *a* fluorescence measurements, as well as a physical assessment, of *L. minor* grown on ADF effluent showed that these plants were healthy when grown on pH-amended wastewater effluent. Significant differences between chlorophyll *a* fluorescence measurements taken on day 0 and day 7 for *L. minor* grown on ADF effluent were not found (t-test F_v/F_m: $t(6) = 0.37$, $p = 0.72$; t-test Y(II): $t(6) = 1.56$, $p = 0.17$; t-test Y(NPQ): $t(6) = 0.74$, $p = 0.49$; t-test Y(NO): $t(6) = 0.005$, $p = 1.0$ Figure 6.4). As the health of *L. minor* cultivated on ADF effluent was shown to be regular, plant growth and biomass quality were analysed in further detail.

The 3-day RGR was 0.09 d^{-1} with some variation between runs (Table 6.3). *L. minor* protein content was on average 1.54 % of fresh weight and 38.5 % of dry weight (Table 6.3). Low protein content in some samples was associated with high NaCl concentrations in the wastewater medium (Na: R = -0.90; Cl: R = -0.85; Figure 6.5). The association of other elements with protein content was assessed as well, such as with K, Fe, Zn and Mn. K and Fe had some association with protein content, but were both present in optimal amounts.

The total essential amino acid (EAA) as a percentage of total protein content was 20 % (excluding methionine and tryptophan which were not measured; Table 6.3). Amino acids such as aspartate, glutamate, alanine, leucine and serine were present on average in higher relative amounts than other measured amino acids for duckweed grown on ADF effluent (Table 6.3). Amino acid content for duckweed grown on ADF effluent was in general similar to duckweed grown on optimised half-strength Hutner's medium (Table S6.1), although EEA content was lower for duckweed grown on Hutner's at 16.4 % of total protein content.

Table 6.3. Biomass parameters for *Lemna minor* Blarney grown on ADF effluent

Parameter	Mean (±SE)
3-day RGR (d^{-1})	0.09 (±0.014)
Yield (g)	0.36 (±0.027)
Protein content (% FW)	1.54 (±0.22)
Protein content (% DW)	38.5 (±5.5)
Essential amino acid (% of total protein)	20 (±0.7)
Alanine (g/100 g protein)	4.6 (±0.05)
Arginine (g/100 g protein)	4.1 (±0.3)
Aspartate (g/100 g protein)	9.4 (±0.3)
Glutamate (g/100 g protein)	8.9 (±0.1)
Glycine (g/100 g protein)	4 (±0.08)
Histidine (g/100 g protein) [1]	1.6 (±0.06)
Illeucine (g/100 g protein) [1]	1.5 (±0.08)
Leucine (g/100 g protein) [1]	5 (±0.2)
Lysine (g/100 g protein) [1]	3.7 (±0.07)
Phenyalanine (g/100 g protein) [1]	2.8 (±0.1)
Serine (g/100 g protein)	4.5 (±0.05)
Threonine (g/100 g protein) [1]	2.7 (±0.2)
Tyrosine (g/100 g protein)	2.1 (±0.05)
Valine (g/100 g protein) [1]	2.7 (±0.03)

[1] essential amino acid (EAA) (Kaplan et al. 2019)

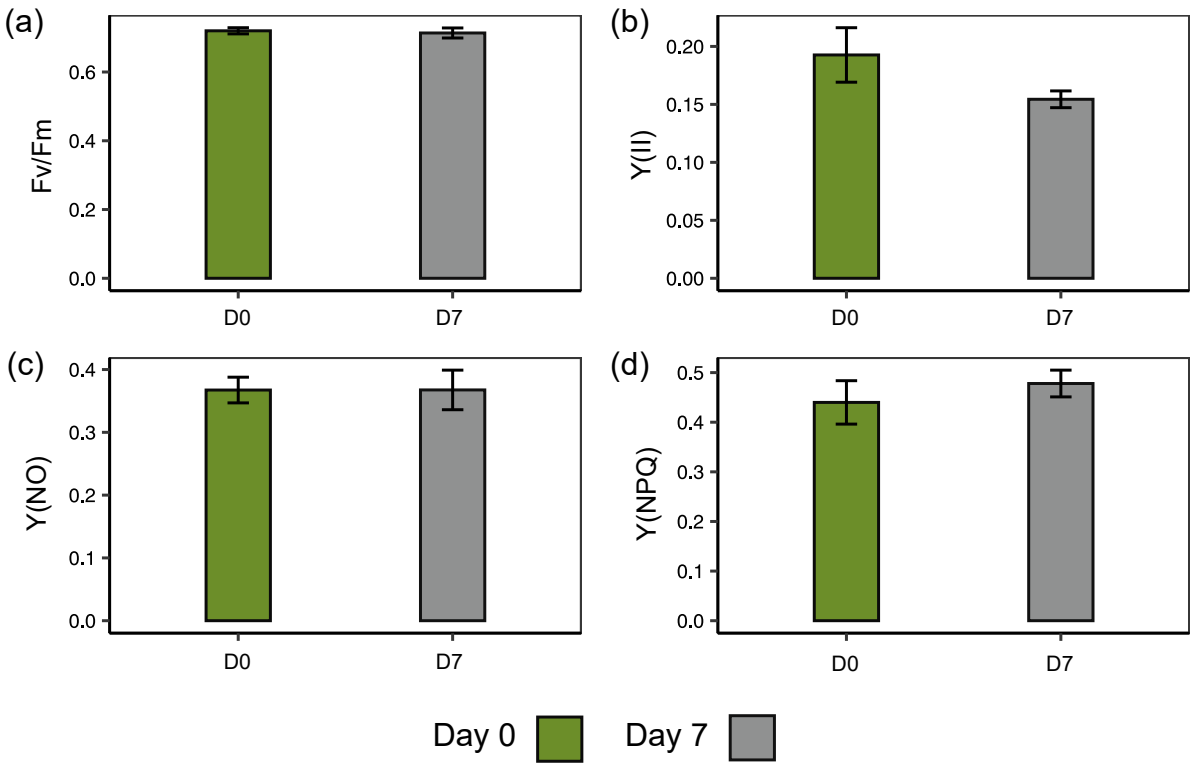

Figure 6.4. Mean (±SE) (a) F_v/F_m, (b) Y(II), (c) Y(NPQ), (d) Y(NO) on day 0 and day 7 for *L. minor* grown for 7 days on ADF effluent.

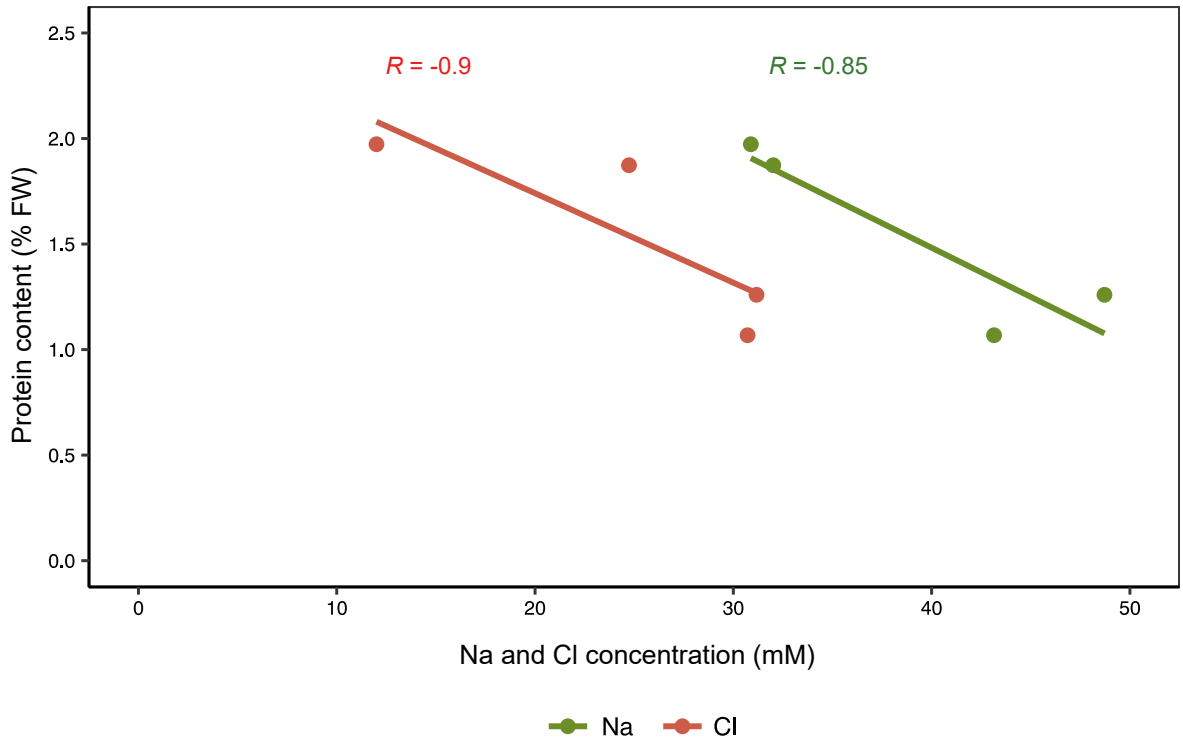

Figure 6.5. *L. minor* protein content (% of fresh weight) vs. Na and Cl concentrations in ADF effluent (mM). Points are single values and trendlines are linear. *R* is the correlation coefficient, as per Pearson's correlation test.

6.3.5. Assessment of combined ADF and duckweed-based remediation of wastewater

The untreated wastewater from a commercial dairy facility was rather variable and had particularly high concentrations of BOD, COD and total solids (Table 6.1). While the effluent from the ADF reactor had substantially reduced levels of BOD, COD and total solids. The ADF reactor removed on average 98 % of COD and 99 % of BOD (Table 6.4). Thus, the medium used for duckweed cultivation contained low levels of organic compounds (Table 6.1). Duckweed cultivation did not have a large impact on the levels of organic compounds, although there was a noticeable increase in COD, by 37 %, from a low base (Table 6.4).

Untreated wastewater had a relatively high concentration of TN, 7.8 mM, of which a significant portion was NH_3, 2.7 mM, while NO_3 could not be detected in the wastewater and was essentially not present (Table 6.1). While, the concentration of TN was reduced quite a bit in the ADF reactor (down 83 % to 1.4 mM; Table 6.1; Table 6.4), the concentration of NH_3 and NO_3 taken together was still around half, 1.4 mM, of the concentration of NH_3 in the untreated wastewater. Up to 89 % of NH_3-N was removed in the ADF reactor but NO_3-N actually increased from an undetectable level to 0.8 mM (Table 6.4; Table 6.1). N nutrients, NH_3 and NO_3, were effectively removed by the duckweed system (up to 99 %) and overall, TN was further removed by on average 81 % (Table 6.4).

Untreated wastewater had a relatively low concentration of TP, 1 mM, most of which was PO_4^{3-} (Table 6.1). On average the ADF reactor removed around half of P: 58 % of TP and 53 % of PO_4^{3-}-P (Table 6.4). Thus, the plant nutrient PO_4^{3-} was available on average at a concentration

of 0.4 mM for duckweed cultivation (Table 6.1). The duckweed reactor reduced TP and PO_4^{3-} by a further third (Table 6.4).

The concentrations of Na and Cl, i.e. NaCl salt, were high but stable in the untreated wastewater (Table 6.1) and the ADF and duckweed reactors had no effect on the concentrations of the elements (Table 6.4).

Most measured metals were present in low concentrations in the untreated wastewater (Table 6.1). However, Zn in particular was present in some samples at a higher concentration. The ADF reactor caused the reduction of all measured metals: K, Ca, Mg, Fe, Zn, Cu, Mn (Table 6.4). However, this was almost negligible for K. In contrast, the duckweed reactor caused little impact on the absolute concentration of most metals (Table 6.1), except for Cu and Mn where there was a notable increase. The ratio of Ca:Mg was 1:0.8 in untreated wastewater. However, this changed to Ca concentrations being on average 3 times higher than Mg concentrations in the ADF effluent, 1:0.33.

The pH of untreated wastewater, 6.5, increased to near 9 in ADF effluent. The duckweed reactor also increased the pH of the wastewater over 7 days (Table 6.1). Before duckweed cultivation pH was reduced manually to 5 but had increased to 8 post-duckweed cultivation.

When the two reactors are taken together, nearly all compounds in the wastewater are reduced compared with the untreated wastewater (Table 6.4). One exception is nitrate as discussed above. Another exception is copper which had on average a concentration that was higher at the end of the remediation process.

Table 6.4. Wastewater composition change as a result of the ADF reactor, duckweed reactor and both reactors taken together.

Parameter	% change ADF reactor [1]	% change duckweed reactor [2]	Total % change [3]
BOD	-98.6	-28.5	-99.3
COD	-97.7	36.8	-97.2
Total Solids	-32.8	1.9	-31.5
Total Nitrogen	-83.1	-80.8	-96.8
Ammonia-N	-88.7	-98.9	-100
Nitrate-N	111883	-99.3	1107.5
Total Phosphorus	-58.3	-34.5	-72.6
Orthophosphate-P	-53.6	-34.3	-68.9
Sodium	2.4	-7.0	-4.6
Chloride	1.1	-5.9	-4.2
Potassium	-0.6	-8.2	-8.6
Calcium	-54.3	-7.1	-57.4
Magnesium	-18.8	-12.4	-28.5
Iron	-45.3	33.5	-28.1
Zinc	-26.9	91.4	-40
Copper	-38.8	285.8	160.7
Manganese	-84.4	102.6	-87.2

[1] Change from untreated wastewater to ADF effluent
[2] Change from ADF effluent to duckweed reactor effluent
[3] Change from untreated wastewater to duckweed reactor effluent

6.4. Discussion

6.4.1. Commercial dairy processing wastewater as a resource

Dairy processing wastewater contains high concentrations of organic and inorganic nutrients which can facilitate both microbial and plant growth (Gil-Pulido et al. 2018; Walsh et al. 2020). Disposal of such waste is restricted by regional and/or country-level regulations, thus necessitating the need for wastewater treatment (European Commission 2019; Lu et al. 2019). The complex character of the waste, and in particular the combination of organic and inorganic nutrients, necessitates a multifaceted waste management system. Here, we have explored how an integrated system comprised of microbial and duckweed-based reactors can be used to remediate such wastewater. While microbial systems tend to be particularly effective in

remediating large amounts of the organic components of waste (Charalambous et al. 2020), duckweed species tend to be especially effective in the removal of inorganic elements (Cheng and Stomp 2009).

Despite the mainly adequate wastewater composition, initial growth trials on untreated dairy processing wastewater directly from the treatment plant led to poor-quality duckweed, although there was passable growth observed on some samples. Poor growth may be explained by very high organic loads. It was noticeable that after 7 days the plants were in the process of dying and that a surface slime had completely enclosed the plants on the wastewater surface. Although an upper limit of COD/BOD that duckweed tolerates cannot be found in the literature, this surface slime, a product of the high load of organic material, microbial growth and the stationary nature of the system, has been shown to physically restrict duckweed growth and lead to death (Broughton 2019).

Another problem with using untreated wastewater for cultivation, is that duckweed itself removes relatively small amounts of organic matter in autotrophic (presence of light) conditions (Landolt and Kandeler 1987). Although, duckweed-associated microbes can remove significant amounts of COD (Körner et al. 1998) and in heterotrophic and mixotrophic conditions duckweed does use organic compounds as a carbon source (Sun et al. 2020). Nevertheless, the removal of the organic material before duckweed cultivation is preferred as this reduces any complications arising from the addition of significant amounts of COD to the duckweed cultivation system. Furthermore, there are numerous effective microbial-based methods that can achieve near 100 % removal of COD and BOD within short spaces of time (Latif et al. 2011; Kushwaha et al. 2013). These results underline the necessity of integrating microbial and duckweed-based systems.

6.4.2. Treatment of dairy processing wastewater

While the commercial AD effectively removed the majority of organic matter from the wastewater, cultivation of duckweed on this wastewater was still poor. However, it was found that a reduction in pH in the AD effluent significantly improved duckweed growth. Indeed, the pH was at a level (pH 8.5) that can restrict duckweed growth (McLay 1976). The high pH is partly the result of a low presence of volatile fatty acids (VFAs) which are being consumed by bacteria (Lyberatos and Skiadas 1999); pH usually declines if the AD starts to fail (Graef and Andrews 1974). Nevertheless, further experimental microbial treatment methods were used to determine whether more suitable wastewater could be provided for duckweed cultivation.

The three experimental microbial treatment methods used in this study (A/O, IASBR and ADF) all removed the majority of COD from the wastewater (up to 99 %). However, for the A/O and IASBR systems in particular, high N and P removal was also achieved. Manipulation of A/O and IASBR systems to reduce N and P removal led to, in the long term, biomass instability, poor settling and floc washout in the effluent (Broughton 2019). The successful growth of duckweed on any medium depends heavily on the presence of N and P (Körner et al. 2003). So, while the plants can grow on this N and P-depleted A/O and IASBR effluent by relying on internal nutrient stocks (Kufel et al. 2012), growth is restricted and will slow over time if nutrients are not replenished. The two microbial systems work effectively but the effluent they are providing is not suitable for sustained duckweed growth.

The ADF effluent retained substantial amounts of both N and P, but also had high concentrations of ammonia with a pH that was fairly alkaline (pH 8.9). Nonetheless, the combination of a high pH and ammonia is a major hindrance for successful duckweed growth (Caicedo et al. 2000). The drastic difference between plants grown at low pH compared to those at high pH in higher ammonia concentrations shows how important it is to manage these

parameters. Commercial pH adjustment systems should therefore be incorporated in future ADF-duckweed, integrated remediation systems.

6.4.3. Growth and health of duckweed on ADF effluent

The ADF system was chosen for further integration with duckweed cultivation as it provided nutrients for the duckweed while also converting organic compounds into the bioplastic precursor PHA.

The effects of cultivation on ADF effluent on duckweed photosynthesis were quantified by measuring chlorophyll *a* fluorescence parameters. Overall, there was no negative impact on photosynthesis, as seen in chlorophyll fluorescence parameters which did not significantly differ between day 0 and 7. Overall, the efficiency of the photosynthetic machinery was not negatively affected, as shown by good F_v/F_m and Y(II) values. Also, plants were able to dissipate excess light energy in a regulated manner, Y(NPQ) (Maxwell and Johnson 2000; Klughammer and Schreiber 2008). In the literature, there are reported inhibitory effects of high NaCl concentrations on photosynthesis, although this is observed only from 100 mM (Oukarroum et al. 2015).

Once duckweed could be shown to grow consistently and healthily on ADF effluent (with reduced pH), important duckweed parameters such as growth, protein content and amino acid content could be quantified. Using an estimate for the starting biomass, RGR (3-day) for *L. minor* grown on ADF effluent was relatively low compared to the literature sources for duckweed grown on wastewater: 0.05-0.3 d^{-1} (Al-Nozaily et al. 2000; Caicedo et al. 2000; Iatrou et al. 2015; Dinh et al. 2020).

In the context of use as a feed, the ability of duckweed to meet daily nutrition requirements can be discussed in terms of amino acid quantity and quality (Roman et al. 2021). Average protein content measured in this study of 38.5 % of dry biomass (1.54 % of fresh biomass) compares

well with common values from literature sources of 25-40 %. However, protein content was nearly double in some replicates compared to others (full range was 25-50 %). As the duckweed were kept in standardised conditions before being used in the experiment it is most likely that the composition of the wastewater was the cause of this difference in protein content. Indeed, lower protein content was associated with higher Na and Cl concentrations. These higher NaCl concentrations were close to or within the range of concentrations at which negative effects on duckweed growth begin to arise (around 30-50 mM, (Sree et al. 2015)). Indeed, Ullah et al. (2021) showed that an increasing NaCl concentration causes a reduction in protein content in duckweed. It has also been shown that salt stress can induce starch production (de Morais et al. 2019), which can have the knock-on effect of reducing protein content (Xu et al. 2011). The association between protein content and other wastewater elements was also tested with K, Fe, Zn and Mn. Only K and Fe had some association with protein content but they were both present at low concentrations in all runs, which are firmly within optimal amounts (Walsh et al. 2020).

A key aspect of protein quality is the content of essential amino acids (EAA) i.e. histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Kaplan et al. 2019). In order to assess amino acid quality a comparison is commonly made with soybean and other widely-used feed supplements consumed in animal husbandry, as well as with human and animal dietary requirements (Cheng and Stomp 2009; Gorissen et al. 2018). In general, the amino acid values found in this study were on par or lower than values found in the literature. EAAs isoleucine, lysine, phenylalanine and valine were distinctly lower than some literature values (Cheng and Stomp 2009; Appenroth et al. 2017). Although in a comparison with values from one study by Chakrabarti et al. (2018), many EAAs were markedly higher. Nevertheless, a number of EAAs do not reach recommended nutritional requirements for adults as set out by a WHO/FAO/UN expert panel (Joint FAO/WHO/UNU

Expert Consultation 2007). Indeed, the overall proportion of protein that was EAAs was on average 20%, which is below the 29 % recommended for sufficient nutrition (Gorissen et al. 2018), although methionine and tryptophan were not included in the value calculated for this study (they usually add another 3 %). There was a similar, albeit lower, amino acid quality found for *L. minor* Blarney grown on half-strength Hutner's medium (Table S6.1), so it may be that this is a clone with a lower EAA content rather than an effect of the wastewater. Indeed, it has been shown that amino acid content varies a lot between species and clones (Cheng and Stomp 2009; Appenroth et al. 2018). Clonal resources could be explored to reach higher overall proportions of EAAs of protein content.

6.4.4. ADF and duckweed-based remediation of dairy wastewater

Licenced discharge limits in the EU depend on the local environmental conditions e.g. the volume of a river or the underlying pollution (Preisner et al. 2020). Licenses are given by regulatory bodies, for example the Environmental Protection Agency (EPA) in Ireland, to specific processing plants which dictate their limit. Nevertheless, there are indicative ranges for allowable daily concentrations for direct emissions to water bodies based on the use of 'Best Available Techniques': 25-100 mg L^{-1} COD, 4-50 mg L^{-1} TN (0.2-3.6 mM) and 0.2-2 mg L^{-1} TP (0.006–0.06 mM) (European Commission 2019).

The ADF reactor alone reduced COD below those legally binding limits. As expected, the duckweed reactor did not contribute much to the removal of COD and BOD (Sun et al. 2020). Indeed, the ADF reactor was responsible for almost all of the removal of organic components from the wastewater; close to 100 % of COD/BOD was removed overall. Although, significant amounts of suspended solids were left in the ADF effluent. Better separation of suspended solids from the effluent would reduce the retention of microbial flocs in the wastewater effluent that is used for duckweed cultivation (Caixeta et al. 2002).

The removal of N from the wastewater was assessed through three parameters, TN, NH_3 and NO_3, with the majority of N in the wastewater in the form of NH_3 and NO_3. The ADF reactor, on the one hand, reduced NH_3 concentrations but at the same time increased NO_3 concentrations. In the ADF reactor, NH_3 is oxidised to NO_3 in the nitrification reaction but a denitrification reaction does not occur due to a lack of energy-rich carbon compounds, which have already been consumed (this is in contrast to the A/O and IASBR systems where residual carbon allows denitrification to occur). A rapid removal of NH_3 by ADF has also been shown in literature sources (Serafim et al. 2004; Lemos et al. 2006). TN was reduced to below EU-designated emission limits already in the ADF effluent. Although, there was still enough N left to support duckweed growth. Therefore, the duckweed reactor can be used to reduce TN even further, with the main N species NH_3 and NO_3 being reduced to near undetectable levels.

The duckweed TN removal rates from ADF effluent (402-931 mg N m^{-2} d^{-1}) compare satisfactorily, considering the limited availability of N in the medium, with values from the literature: 124-4400 mg N m^{-2} d^{-1} (Körner and Vermaat 1998; Cheng et al. 2002; Benjawan and Koottatep 2007; Mohedano et al. 2012). The removal rates from this study would be mostly likely be increased in media with higher N concentration (Cheng et al. 2002). For example, Mohedano et al. (2012) achieved a TKN removal rate 4400 mg N m^{-2} d^{-1} for duckweed cultivated on wastewater with an average NH_3-N concentration of 48.85 mM (832 mg L^{-1}). While Körner and Vermaat (1998) achieved 124 mg N m^{-2} d^{-1} on 0.68 mM TKN (11.6 mg L^{-1}).

In contrast to COD and TN, TP is not reduced to below EU emission limits. Indeed, the concentration remained around 4 times above the upper limit after both the ADF reactor and duckweed cultivation. The overall removal of P, which is mostly PO_4^{3-}, was lower than N, with the ADF reactor removing more in absolute and percentage terms than the duckweed reactor. P is generally removed in microbial treatment systems through the metabolic activity of

microorganisms (Yeoman et al. 1988). However, in the literature on ADF systems P is not generally measured or presented, so knowledge of the typical removal is lacking. Duckweed TP removal rates of 123-230 mg P m^{-2} d^{-1} were achieved in this study, compared to 14-590 mg P m^{-2} d^{-1} found in literature sources (Körner and Vermaat 1998; Al-Nozaily et al. 2000; Cheng et al. 2002; Mohedano et al. 2012).

The duckweed TP uptake rate is normally slower than the TN uptake rate (Cheng et al. 2002), which does make the removal of TP from wastewater slower. Indeed, the ratio of N:P concentrations in duckweed is usually 5:1 (Alaerts et al. 1996), which reflects the ratio in which plants need these nutrients (Güsewell 2004). However, in this case there was just under half as much TP as TN in the ADF reactor effluent. The N-species that are most relevant to plants, ammonium and nitrate, were rapidly removed from the wastewater, leaving significant amounts of P (mostly PO_4^{3-}) in the wastewater. The significant amount of remaining P requires further intervention to remove. Plants could be kept for longer on the wastewater to bring down the P concentration further. Even without growth, duckweed can still remove significant amounts of P (Paterson et al. 2020). Alternatively, a nitrogen source, for example urea, could be added to the wastewater to help bring down P quicker (Ramanna et al. 2014), but this would be an added cost and not within circular economy framework. Otherwise, a species or clone of duckweed with a faster TP removal rate could be used (although *L. minor* Blarney has one of the highest measured for this thesis – Chapter 5).

6.5. Conclusion

Dairy processing wastewater has been shown to be remediated by a combination of a microorganism-based reactors and duckweed cultivation. The combination of an ADF reactor with subsequent duckweed cultivation removed nearly all organic compounds and TN from the wastewater. However, significant amounts of TP remained in the wastewater and would require

further processing to remove to within discharge limits. For successful duckweed cultivation of duckweed on either AD or ADF effluent, only a small modification was required, namely a lowering of wastewater pH. Under these modified conditions *L. minor* has good growth rates and protein content and generally displays signs of a healthy plant.

Author Contributions

ÉW and MAKJ contributed to the duckweed-based remediation conception and design. AF, RB, DW and NDO'L contributed to the microbial remediation conception and design. Material preparation and data collection were performed by ÉW, AF, UD and RB. The analysis and interpretation of results was performed by ÉW, UB, NDO'L and MAKJ. The first draft of the manuscript was written by ÉW. ÉW, NDO'L and MAKJ contributed to writing and editing of the manuscript.

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Supplementary Material

Table S6.1. Amino acid content of *L. minor* grown on half-strength Hutner's medium.

Parameter	Mean (±SE)
Essential amio acid (% of total protein)	16.4 (±0.3)
Alanine (g/100 g protein)	4.4 (±0.03)
Arginine (g/100 g protein)	2.8 (±0.2)
Aspartate (g/100 g protein)	9.2 (±0.3)
Glutamate (g/100 g protein)	7.2 (±0.1)
Glycine (g/100 g protein)	3.8 (±0.03)
Histidine (g/100 g protein)	1.3 (±0.04)
Illeucine (g/100 g protein)	1.2 (±0.02)
Leucine (g/100 g protein)	4.1 (±0.02)
Lysine (g/100 g protein)	3.3 (±0.03)
Phenyalanine (g/100 g protein)	2.2 (±0.02)
Serine (g/100 g protein)	4 (±0.07)
Threonine (g/100 g protein)	2.3 (±0.1)
Tyrosine (g/100 g protein)	1.9 (±0.02)
Valine (g/100 g protein)	2 (±0.03)

Chapter 7

Thesis conclusion and outlook

7.1. Key conclusions

The following key conclusions can be drawn from the experiments presented in this thesis:

- Synthetic and real dairy processing wastewater are suitable for duckweed remediation.
- Some changes to the composition of the wastewater are required to make it suitable for duckweed cultivation, for example, in the Ca:Mg ratio or pH value.
- Conditions, such as light and density, can be manipulated to increase growth, remediation or protein content.
- Some duckweed clones show higher abilities in certain parameters, such as protein content and N removal, and these clones can be exploited to improve remediation or the value of duckweed biomass
- Moderately scaling-up the duckweed remediation system mainly results in similar outcomes, with the exception of a slightly lower protein content
- Integration of duckweed cultivation with microbial-based bioreactors provides good remediation of dairy processing wastewater

These conclusions show that dairy processing wastewater is suitable for remediation by duckweed and results can inform development of large-scale duckweed-based remediation of dairy processing wastewater.

7.2. Making wastewater suitable for duckweed

In line with one of the main objectives of this thesis, these experiments clearly show that both synthetic and real versions of dairy processing wastewater are suitable for duckweed remediation, even though the unmodified synthetic wastewater and some variations on real dairy processing wastewater were not initially conducive to duckweed cultivation. Therefore, adjustments had to be made to accommodate duckweed cultivation.

In general, it was found that adjustments made to facilitate duckweed cultivation can be broken into two groups: adjustments made to the medium itself that are required for duckweed survival and adjustments made (often to external conditions) for optimisation. In relation to the first group, the ratio of Ca:Mg favouring Mg in synthetic wastewater and the combination of high pH with high ammonia concentration in the ADF and AD effluents resulted in severe limits on duckweed growth. However, these barriers to growth and survival can be addressed relatively easily to make a suitable medium for duckweed cultivation. Importantly, they involve the addition of easily accessible compounds to the medium (in the case of this research, calcium sulphate or sulphuric acid) rather than removal, which is in general harder to achieve.

7.3. Optimising wastewater for duckweed cultivation

Once wastewater is made suitable for duckweed cultivation, the question thereafter is really one of optimisation; the second category of adjustments. Changes can be made to the light provision, plant density, temperature, length of cultivation, even the clone of duckweed used, among many other parameters (Sree et al. 2015; Yin et al. 2015; Verma and Suthar 2015). Some of these parameters (light and density) were taken and studied in detail to get a better understanding of how they affect duckweed remediation ability and biomass production but also to understand on a deeper level why they produce such effects on duckweed. The changes in light provision and plant density had clear effects on growth, yield and protein content. Some of these effects have been shown before (Driever et al. 2005; Paolacci et al. 2018), but there were specificities for duckweed grown on dairy processing wastewater. A particularly interesting observation was the interactive effects between light intensity and medium which produced a much lower light saturation point than normal.

7.4. Further research on duckweed biology

With the principle of duckweed-based remediation of dairy processing wastewater shown to be valid, further research in this area can be divided into two broad categories: (1) the particulars of the biological basis of changes in duckweed in response to changing parameters and (2) scaling-up for industrial-level remediation and biomass production.

For the first category, there are some interesting research avenues to explore, particularly in relation to the mechanism of action of some observed effects. This study showed that in certain conditions it is most likely signalling between individual colonies of duckweed (and not a lack of light or nutrients) that is responsible for the effects of high density on growth and nutrient removal rates. The effect of plant density on duckweed growth is relatively well understood (Frédéric et al. 2006) but there remains much more to be learned about the signalling between individual duckweed colonies that lies behind these effects. Ethylene is clearly a signal (Färber et al. 1986), but it is not clear whether there are other signals and how this type of signalling functions in plants which frequently grow in dense mats. Another area of consequence which this research shone a light on is species and clonal diversity. In this analysis, there was an interesting lack of association between some of the measured parameters. The quality of the duckweed biomass, i.e. the protein content, did not associate with higher growth or N and P removal rates. Certainly, it is worth exploring these associations with a larger and broader group of duckweed clones, as this study is limited to 13 clones only. Clone selection is quite a significant choice for remediation and biomass quality (Ziegler et al. 2015; Appenroth et al. 2018), so it would be important to understand how choice of a clone for a certain trait may affect outputs and remediation.

Lastly, one big topic that has not been directly explored in this thesis, but is gaining more and more interest in the duckweed researching community, is duckweed-associated microbes.

Indeed, it is now clear these microbes are involved many aspects of duckweeds activity, for example, in remediation and growth (Ishizawa et al. 2019; Muerdter and LeFevre 2019). However, there is much more to be understood about how they can be utilised for the benefit of duckweed production. Most of the experiments conducted on a lab-scale in this study were axenic cultures and it would be worth exploring how duckweed remediation and growth would be affected by non-axenic cultures. Furthermore, the effects, if any, of duckweed-associated microbes on the nutritional composition of duckweed is an area of further exploration.

All research into the duckweed biology can inform on the use of duckweed in remediation but, furthermore, can also help with other uses for duckweed, for example as a model organism or a chassis plant in synthetic biology (Liu et al. 2021).

7.5. Scaling-up the research

The second category of future research regards an increase in scale and outlook. The overarching purpose of researching duckweed-based remediation is to be able to remediate large volumes of wastewater, in conjunction with other remediation methods, while also producing high-quality plant biomass. This research showed how moderately scaling-up (11.7 L) mostly resulted in similar outcomes as found at a small laboratory-scale (100 mL). However, upscaling also led to negative effects on duckweed i.e. lower protein content. It is unclear what exactly caused the lower protein content in these systems (possibly stress from water currents or competition with algae) but it has consequences for the value of the biomass as a feed.

Nevertheless, this research must now take a step forward into dealing with the large wastewater volumes that are produced by the dairy industry (single facilities can process up to 550 million litres of milk per year with the resultant wastewater that comes with that (Baskaran et al. 2003)), as well as delving further into the potential uses of the duckweed biomass. Duckweed has previously been shown to be suitable for wastewater remediation in large-scale and pilot-scale

facilities (Zhao et al. 2015; Ceschin et al. 2019). Furthermore, a number of companies are implementing duckweed cultivation at this scale for use as a food, such as Hinoman (https://www.hinoman.com/) and Rubisco Foods (https://rubiscofoods.com/). Although, for these companies the intention is not wastewater remediation and as such they do not use wastewater as a cultivation medium. Other enterprises (http://mamagrande.org/) are remediating wastewater with duckweed at a large scale and producing duckweed biomass for multiple uses, such as biofuel. However, dairy processing wastewater, of which there is vast quantities worldwide, is not currently being utilised for duckweed-based remediation at a large scale.

There are a number of areas that still need to be addressed to scale-up duckweed-based wastewater remediation and to establish industrial take-up of the technology. The design of the cultivation system, the integration with other remediation technologies, as well as defining pathways and local facilities for processing duckweed biomass would all be key.

7.6. Type of remediation system

Up to the present moment, scaled-up research has mostly focused on outdoor, pond settings, which are open to the elements (Guo et al. 2020) or have some form of covered glasshouse (see Rubisco Foods). However, the outlook for scaled-up technology may be in the direction of indoor, multi-level, stacked systems kept in controlled settings (Park et al. 2019). First of all, this type of setting means that these systems can be set up anywhere in the world, regardless of the climate conditions. This is one major barrier to the use of outdoor remediation ponds. In northern Europe, for example, the winter months are not conducive to duckweed growth (Landolt 1986). Plants can certainly survive these periods but do not necessarily thrive.

Another driver of the move to indoor units would be the efficiency of space use. Already, there has been a push towards these types of systems for food production in urban areas in which

space is premium (Benke and Tomkins 2017). However, in non-urban areas these systems are just as applicable, as usable arable land is in decline (Benke and Tomkins 2017). For duckweed the technology or physical infrastructure for these stacked systems can be developed or built upon existing technologies for hydroponic food production (Touliatos et al. 2016). There would be more inputs required than in outdoor systems, such as light provision and temperature control, but the benefits of being able to produce duckweed biomass 365 days a year may balance out these costs (Gentry 2019).

Duckweed is suitable to cultivation in this controlled system design, because, as shown in this research, great benefit can be reaped in growth, nutrient uptake rates and nutritional content, when conditions are manipulated.

7.7. Integrated of duckweed with microbial-based reactors

This research shows that a combination of duckweed remediation with other methods is required for superior wastewater remediation. There are some instances when duckweed will remove significant amounts of COD from the surrounding medium (Li et al. 2017). However, the best use of this organic resource is through microbial uptake and metabolism, as microbial-based systems have an array of useful outputs e.g. biomethane, bioplastic (PHA), bioethanol (Li et al. 2019; Mannina et al. 2020; Sampaio et al. 2020).

Fortunately, in the dairy industry the use of microbial based treatment methods is widespread and available to tap into, as such, for integration with duckweed cultivation. The use of dairy processing wastewater as a resource is being explored within the industry already, particularly in relation to the creation of biogas, e.g. methane, where the digestion system, anaerobic digestion, is well understood and widely employed (Ahmad et al. 2019).

The ideal future situation would involve dairy industry facilities adding duckweed to their existing wastewater remediation treatments. However, the future for duckweed is not restricted

to the dairy industry. Repeatedly duckweed has been shown to remediate a wide range of wastewater types.

7.8. Duckweed as food/feed

In order to generate interest in using duckweed-based remediation and production, defined routes for duckweed biomass use would need to be established. First of all, this requires research into the composition of duckweed and how that composition is influenced by external conditions. We have shown here how certain conditions such as high light intensity or high salinity can reduce the protein content of duckweed. Other studies have shown how conditions can be changed to shape the nutritional content of duckweed, for example, the increase starch content due to salt stress or nutrient limiting conditions (Xu et al. 2011; de Morais et al. 2019). Accordingly, outputs could be more strongly linked with conditions for duckweed production i.e. manipulate the system to produce high-starch plants for bioethanol production, or conversely high-protein plants for feed.

In this thesis the focus of use for duckweed biomass was as a feed, and thus protein and amino acid content was assessed. However, there are some limits on its use in this form. Duckweed grown on wastewater is perhaps unlikely to be used as a food or food supplement for human consumption due to the manner of its production. Although, if the wastewater is treated to a high degree before duckweed cultivation (for example, BOD and TSS would have to be ≤ 10 mg L^{-1}), it may become acceptable for food production (Magwaza et al. 2020). A cautionary regulatory framework can also inhibit the use of wastewater in food production (Molle et al. 2012; Rock et al. 2019). Furthermore, it is not clear how high public acceptance is for wastewater-cultivated food. In this context, the use of the word waste is, in some way, the antithesis to food and conjures up distasteful imagery in the imagination (Ricart and Rico 2019). Some of the population have expressed interest in duckweed as a food (de Beukelaar et

al. 2019) and there is some hope that waste-derived food products could be acceptable to consumers when explained (Aschemann-Witzel and Peschel 2019).

When wastewater is used as a duckweed production medium for animal feed, the standard is slightly lower than that for human food production (Magwaza et al. 2020). Nevertheless, even if the main aim is to produce duckweed for agricultural animal consumption, as in this project, feed purchasers and farmers must be interested. However, while it's nutritional content mostly fulfils requirements (Cheng and Stomp 2009), more feeding studies, particularly for ruminants, are needed, to show its safety and efficacy in these animals. This is pertinent in a country such as Ireland where there is a large national herd of cows and cattle.

Duckweed is not an animal feed that is currently used extensively in Europe (although there is sustained interest in its use as a feed (Tallentire et al. 2018)), so it is starting from zero in terms of recognisability. However, a key point that wastewater-grown duckweed has in its favour is its sustainable credentials (Calicioglu et al. 2021). More and more this is an aspect of food production that is sought after and even expected (Bosona and Gebresenbet 2018). Indeed, it has been shown that food production accounts directly for around 10 % of greenhouse gas emissions worldwide (higher in countries such as Ireland where it is 33 % (Sustainable Energy Authority of Ireland 2020)) and the agro-industry will come under increasing pressure to change its practices (Frank et al. 2017).

I think the most pertinent questions now are on how to create wider systems in which duckweed can be produced and processed on a large-scale, which requires companies and processers to be interested in the idea. This research will be disseminated to a broader audience in a subsequent EPA report. This is important for connecting the outputs of this research with non-academic dairy industry sources.

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