Influence of physiological growth parameters on duckweed (*Lemnaceae*) in a re-circulating and vertical cultivation system

Dissertation

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List of abbreviations

%	percentage
°C	degree Celsius
α	alpha
ADP	adenosine diphosphate
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATP	adenosine triphosphate
cm	centimeter
CO_2	carbon dioxide
CPC	crude protein content
Cys	cysteine
d	day
DM	dry matter
DT	doubling time
EDDHA	ethylenediamine-di-o-hydroxyphenylacetic acid
EFSA	European Food Safety Authority
EU	European Union
g	gram
Glu	glutamic acid
Gln	glutamine
Gly	glycine
GRAS	generally recognized as safe
h	hour
H^+	hydrogen
His	histidine
Ile	isoleucine
IVF	indoor vertical farm

kDA	kilodalton
kg	kilogram
l	liter
Leu	leucine
Lys	lysine
m	meter
m^2	square meter
m^3	cubic meter
µg	microgram
µmol	micromole
Met	methionine
mg	milligram
mmol	millimole
N	nitrogen
n-3 fatty acids	omega−3 fatty acids
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
nm	nanometer
NH_4^+	ammonium
NH_4^+-N	ammonium nitrogen
NH_4NO_3	ammonium nitrate
NO_2^-	nitrite
NO_3^-	nitrate
NO_3^--N	nitrate nitrogen
O_2	oxygen
P	phosphorus
pH	*Potentia hydrogenii*
Phe	phenylalanine
Pro	proline
RGR	relative growth rate
s	second

Ser	serine
t	tons
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
USA	United States of America
Val	valine
WHO	World Health Organization

1. Introduction

1.1. Overall view and purpose

A growing world population of up to 9.7 billion people by 2050 and up to 12.4 billion by 2100 (United Nations, 2022) will cause a rising future demand for plant- and meat-based food and livestock feed in general and protein in particular. Simultaneously, climate change will lead to more frequent and severe weather events, such as increasing temperatures, local heat periods resulting in droughts or heavy, long-lasting rainfalls that can cause flooding (Clarke et al., 2022). These factors make today's traditional agricultural practices vulnerable and less productive, which can result in decreased food and feed yields. Additionally, rising sea levels, caused by the melting of the global ice sheets, will result in less land area available for crop production (Despommier, 2011).

In animal nutrition, soybean meal is currently a major source of protein. To fulfill the global demand for soy, large areas of native rainforest are destroyed to obtain land for soybean production. The European Union is dependent on imports of high-protein feed, mainly from the American continent, in form of soybean meal due to its high protein content and high levels of limiting amino acids (Anderson et al., 2011; European Commission, 2018). The decoupling of land farming and livestock production caused several environmental and sustainability issues, such as deforestation in South America or nutrient surpluses in areas with intense livestock production (Demann et al., 2023). This deforestation fuels the effects of global warming. Today's agriculture is related to further ecological problems, such as eutrophication or water deficiency in certain areas due to the extensive and inefficient use of nutrients and irrigation water.

To ensure food security, meaning a sufficient global agricultural food and feed production, it requires new cultivation techniques, which should be climate-resilient, space- and resource-efficient and could potentially be operated close to the consumer and integrated into material and energy cycles. Additionally, the identification and integration of new crop plants into human and animal nutrition are required. Those new crops should be characterized, amongst others, by

- suitability for human and animal nutrition,
- fast growth rates, resulting in high biomass production,
- high nutritional value, especially regarding proteins,
- high-quality amino acid distribution and
- edibility of the whole plant or the majority of plant parts.

One plant family, which helps to fulfill all these requirements, is duckweed (*Lemnaceae*).

1.2. Duckweed

1.2.1. Taxonomy, global distribution and morphology

Duckweeds are floating fresh water plants. They are the smallest angiosperms on earth and belong to the family of *Lemnaceae* Martinov (Martinov, 1820), a subgroup of the monocot order Alismatales (Acosta et al., 2021; Tippery et al., 2021). The five genera (*Spirodela* Schleid., *Landoltia* Les & Crawford, *Lemna* L., *Wolffiella* Hegelm. and *Wolffia* Horkel ex Schleid.) consist of two, one, twelve, ten and eleven species, respectively (Table 1). This results in a total of 36 different species (Bog et al., 2019).

Table 1: Taxonomy of the five duckweed genera and their corresponding species (Bog et al., 2019)

Spirodela	***Landoltia***	***Lemna***	***Wolffiella***	***Wolffia***
S. intermedia	*L. punctata*	*L. aequinoctialis*	*W. caudata*	*W. angusta*
S. polyrhiza		*L. disperma*	*W. denticulata*	*W. arrhiza*
		L. gibba	*W. gladiata*	*W. australiana*
		L. japonica	*W. hyalina*	*W. borealis*
		L. minor	*W. lingulata*	*W. brasiliensis*
		L. minuta	*W. neotropica*	*W. columbiana*
		L. obscura	*W. oblonga*	*W. cylindracea*
		L. perpusilla	*W. repanda*	*W. elongata*
		L. tenera	*W. rotunda*	*W. globosa*
		L. trisulca	*W. welwitschii*	*W. microscopica*
		L. turionifera		*W. neglecta*
		L. valdiviana		

Duckweeds can be found worldwide, except for regions with extreme weather conditions, such as the polar areas and rarely occur in deserts (Figure 1). The genera *Lemna*, *Spirodela* and *Wolffia* are distributed globally, while *Wolffiella* is restricted to the subtropical zones of the American and African continents (Fourounjian et al., 2020; Landolt, 1986).

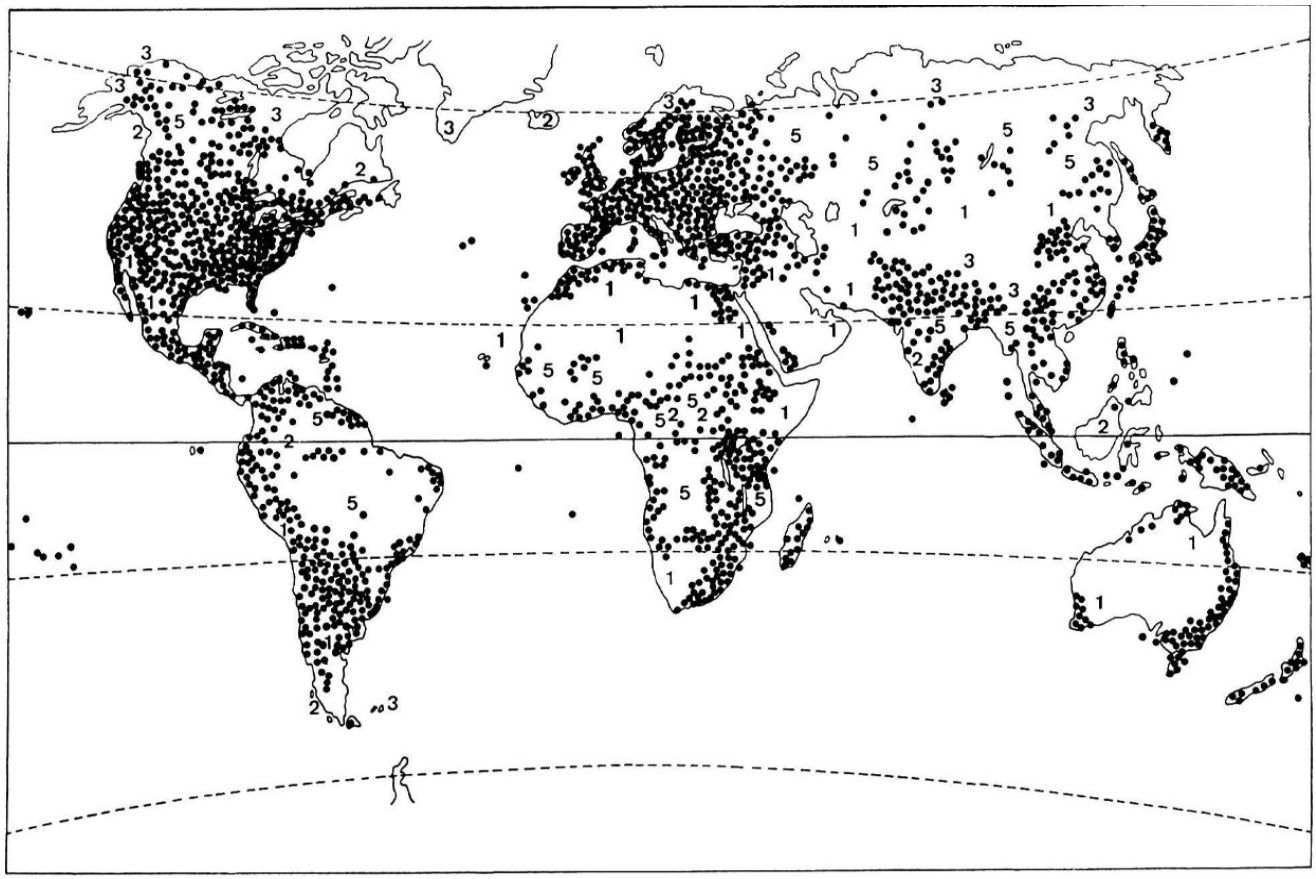

Figure 1: Map of the world with the distribution of Lemnaceae marked as dots. Numbers indicate reasons for lacking of Lemnaceae (1=too dry, 2=too wet, 3=too cold, 5= too low density of botanical exploring) (Landolt, 1986)

Duckweeds can grow in high altitudes of up to 4450 m above sea level in the southern subtropical Andes of South America. The altitude, however, is not a species-relevant growth factor, but the correlating local climatic conditions are (Landolt, 1986). Most species developed variations in their genetic and physiological properties due to geographically different conditions, named clones (Sree and Appenroth, 2020). This clonal differentiation has been proven e.g., for the nutritional quality, growth rate, turion formation capacity and starch accumulation capacity (Bog et al., 2019).

Duckweeds, as monocotyledon plants, have a simple morphological structure. Compared to other seed plants, they lack the morphological differentiation into stems, branches and leaves. Instead, their leaf-like body consists of fronds and a root, except for *Wolffia* and *Wolffiella*, which are missing the roots. The differences between the duckweed subfamilies are based on their morphology. *Lemnoideae* have roots and two lateral pouches from where new fronds emerge. *Wolffioideae* are characterized by the absence of roots and have only one such pouch (Sree and Appenroth, 2020). They reach sizes from less than 1 mm (*Wolffia angusta*) to 1.5 cm (*S. polyrhiza*) (Acosta et al., 2021). Genera can be easily distinguished by morphology (Figure 2). The identification of species and clones is more difficult due to a high similarity on a morphological basis. For clonal differentiation molecular barcoding,

fingerprinting by amplified fragment length polymorphism or genotyping-by-sequencing is required (Bog et al., 2019; Sree and Appenroth, 2020).

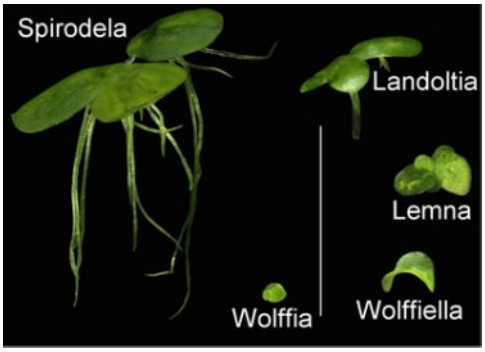

Figure 2: Morphology of the five duckweed genera. Scale: 1 cm (Yang et al., 2020)

In natural habitats, duckweeds often form a dense layer on stationary or slowly moving water bodies when growth conditions are optimal. A survival strategy for unfavorable abiotic conditions is the formation of turions, which sink to the ground of the water body and emerge when conditions become favorable for growth again. Turions are a kind of resting fronds or dormant tissue. In case of *S. polyrhiza*, the turions are morphologically different from their fronds and do not continue to grow. Those are called “true turions”. In the natural aquatic environment of the temperate zone, turions become dormant immediately after their formation in order to avoid germination before winter. They are similar to most seeds regarding their formation, dormancy and germination, however, turions are vegetative organs (Appenroth et al., 1996). Turions are rich in starch, reaching contents of 65 % in the dry matter (DM) (Xu et al., 2018). Turion formation can be induced by the limitation of nitrate, sulfate and phosphate (Appenroth et al., 1996) or low temperature (Appenroth and Nickel, 2010).

1.2.2. Growth

Duckweeds can reproduce in two different ways. By multiplication (vegetative) or by sexual (generative) reproduction. All duckweed species have the ability to flower, i.e. sexual reproduction. However, flowering occurs seldom and in small duckweed species flowering is only visible under a microscope. Factors inducing the flowering process are high duckweed densities, light intensity, photoperiod (duration of light and darkness), temperature and the application of certain chemicals, such as the chelating agent ethylenediamine-di-o-hydroxyphenylacetic acid (EDDHA), Fe-EDDHA, 8-hydroxyquinoline or salicylic acid (Khurana and Maheshwari, 1983; Khurana et al., 1986; Landolt, 1986; Seth et al., 1970)

Multiplication is the form of reproduction that occurs predominantly in most duckweed species. A mother frond built a genetically identical clone by budding within the pouch of the basal frond section. When this daughter frond matures, the connection (called stipe) to the mother frond breaks. Each daughter frond already contains two or more new generations of

daughter fronds when detaching from the mother frond. One mother frond can produce up to 24 clones in a lifespan of 5 to 10 weeks (Cao et al., 2020; Landolt, 1986; Sree et al., 2015b).

The growth rates are the highest of all higher plants and duckweeds are the fastest-growing angiosperms on earth (Sree et al., 2015c). Usually, the growth rate is specified by the relative growth rate (RGR (d^{-1})) according to equation (1), where x is the value of the measured parameter (duckweed fresh weight, dry weight or number of fronds) at two time points at the beginning (t0) and the end (t7) of a 7-day test period (Ziegler et al., 2015).

$$RGR = (lnx_{t7} - lnx_{t0})/(t7 - t0) \quad (1)$$

The RGR describes the biomass gain after one day of cultivation, starting with an initial biomass of one gram. Another parameter is the doubling time (DT (d)) according to equation (2), which describes the time needed for doubling the biomass (Ziegler et al., 2015).

$$DT = ln2/RGR \quad (2)$$

The fastest RGR in duckweeds has been achieved for the species *W. microscopica*. Table 2 shows RGRs and DTs for several selected clones.

Table 2: relative growth rates (RGR) and doubling times (DT) for several selected duckweed clones (Sree et al., 2015c; Ziegler et al., 2015)

Species	**Clone**	**RGR (d^{-1})**	**DT (d)**
W. hyalina	9525	0.519	1.34
L. gibba	7742	0.503	1.39
L. minor	9441	0.420	1.67
S. polyrhiza	7110	0.168	4.13
S. polyrhiza	9242	0.386	1.80
L. punctata	9589	0.365	1.90
W. globosa	9331	0.328	2.11
W. microscopica	2005	0.568	1.22

There are large differences in the RGR and DT between species and even between different clones of one species, as seen for *S. polyrhiza*. The reproduction rate is not only clone-specific but also depends on abiotic factors, such as nutrient composition and availability, temperature as well as light intensity and spectrum (Landolt, 1986). The above-shown RGRs and DTs were achieved under standardized, sterile in-vitro conditions with a temperature of 25 °C, replenishment of nutrient medium every seven days, a continuous white light at

100 µmol m^{-2} s^{-1} and a modified and autoclaved full medium, e.g. Schenk-Hildebrand medium (Sree et al., 2015c; Ziegler et al., 2015).

1.2.3. Cultivation and quality

Duckweeds are adapted to freshwater; they naturally do not occur in seawater. According to Sree et al. (2015a), an increasing salinity of the nutrient medium decreased the RGR of the investigated duckweed species and clones.

The nutrient composition and concentration of most freshwater sources (e.g. tap water) used for duckweed cultivation are often not optimal, therefore the supply with additional nutrients is necessary for fast growth and nutrient-rich duckweed biomass. Nitrate, ammonium, phosphate, potassium, calcium, magnesium, sulfur, iron, manganese, boron, molybdenum and zinc are among the most important nutrients. In literature, several nutrient media are described for duckweed cultivation, such as Murashige-Skoog, Hutner, Schenk-Hildebrand, Steinberg, Hoagland, Bonner-Devirian and the N-medium (Appenroth, 2015). Besides the composition and concentrations, also the nutrient ratio has an impact on duckweed biomass quantity and quality (Walsh et al., 2020). By optimizing the growth medium, the nutritional content within the biomass can be influenced and maximized (Appenroth et al., 2017; Xu et al., 2012; Xu et al., 2011).

Duckweeds can tolerate pH values in the range of 4-10, the optimum depends on the species and is usually between 5-8 (Caicedo et al., 2000; McLay, 1976).

The water temperature is another abiotic factor influencing the growth of duckweeds. The tolerable temperature range for duckweed survival is between 5 and 40 °C. The optimum depends on the species and is between 25 to 31 °C. In general, species from cooler or temperate climate regions have a lower optimum than those from tropical regions (Landolt and Kandeler, 1987; Lasfar et al., 2007).

Irradiation is essential for photosynthesis in all higher plants. It has to be distinguished between the light intensity and quality, while the duration time of radiation is another relevant factor. The light intensity is the quantity of radiation in the form of number of photons, that reaches the plant surface and is available for photosynthesis. These photosynthetic photon flux densities are given in µmol m^{-2} s^{-1}. In general, an increasing light intensity correlates with a higher growth rate in duckweeds, until reaching a species-depending photo-inhibition point (Paolacci et al., 2018; Wedge and Burris, 1982; Yin et al., 2015). The light quality, meaning the spectral distribution of photosynthetically active radiation in the range of 400 - 700 nanometers (nm), is important for photosynthesis in phototroph organisms. Most important for plant growth and health are the blue peak around 445 nm and the red peak around 660 nm. The duration of radiation is known as photoperiod and describes the rhythm between light and darkness. Theoretically, longer

periods of radiation result in longer photosynthesis phases. However, many plants are naturally adapted to phases of darkness, because of the naturally occurring day and night rhythm. This is known as the circadian clock system (Isoda et al., 2022). The optimal photoperiod for duckweeds is 12 to 13 hours of light per 24 hours when cultivated under sterile conditions (Lasfar et al., 2007).

The abiotic conditions that are optimal for duckweed growth also provide perfect habitats for other species. Therefore, if duckweed is cultivated under non-sterile conditions, the growth of ubiquitous algae and other microorganisms is inevitable. Several green algae species (*Scenedesmus conspicua. Chlorella* sp., *Chlamydomonas* sp.) reduced the growth of duckweed by the depletion of nitrogen, phosphorus, iron and manganese, as well as a pH increase above 10. Furthermore, the presence of certain algae species reduced the chlorophyll contents by up to 97 % and can be lethal for *Lemna gibba*, if the initial duckweed cover is below 50 % of the surface area. However, a high initial duckweed cover will hinder algae growth by shading and could be a solution to reduce the detrimental effects on duckweed (Roijackers et al., 2004; Szabó et al., 1998; Szabó et al., 1999; Szabó et al., 2003; Szabó et al., 2005). Also, bacteria biofilms attached to the duckweed and cultivation system, considerably contribute to nitrogen removal via uptake or nitrification/denitrification (Körner and Vermaat, 1998). Another biotic factor with a fatal impact on duckweed is the growth of certain fungus species, such as *Pythium myriotylum* (Brand et al., 2021).

Duckweeds are highly nutritious plants, which can reach protein contents of up to 45 % in the DM (Sela et al., 2020; Sońta et al., 2019; Xu et al., 2021). Appenroth et al. (2017) reported protein levels from 20 % to 35 %, fat contents from 4 % to 7 %, and starch contents from 4 % to 10 % (DM) for six different species, using a modified Schenk-Hildebrandt medium for cultivation. The amino acid composition is close to the recommendations for human nutrition of the World Health Organization (WHO, 2007). The content of the essential amino acid lysine was 4.8 %. Between 48-71 % of the fat content is present as polyunsaturated fatty acids with a high content of omega-3 fatty acids (Appenroth et al., 2017).

The nitrogen supply, especially the nitrate and ammonium concentration in the nutrient medium, influences the protein content of duckweed biomass (Iatrou et al., 2019). To maximize the starch content, several growing parameters, e.g. temperature (Cui et al., 2011; Xu et al., 2012), nutrient starvation (Zhao et al., 2015) or lighting (Yin et al., 2015), have been successfully investigated. However, high starch contents can only be achieved with simultaneously low RGRs, protein and phosphorus contents (Xiao et al., 2013). In the turions, starch contents of up to 65 % are possible (Xu et al., 2011; Xu et al., 2018).

1.2.4. Previous and current use of duckweed

Duckweed has been an important plant for research and application for centuries. They played a major role in plant biology research, for example about the photoperiodic flowering response. In the era of plant molecular biology, the physiological information obtained from duckweed research was more and more transferred to other model plants, such as *Arabidopsis thaliana* or rice (Acosta et al., 2021). Nevertheless, duckweed is still used as a model plant to investigate plant physiological and ecotoxicological processes, such as the plant circadian system, sulfur assimilation pathways, flowering response and auxin biosynthesis (Cao et al., 2020; Isoda et al., 2022). Duckweeds are well suited for biochemical *in vivo* research, for example by studying the nucleic and protein turnover in *L. minor* using radiolabeled compounds (Acosta et al., 2021). Due to their fast multiplication and their ability to accumulate high amounts of nutrients and contaminants, they are often used for biomonitoring as indicator plants for heavy metals and other pollutants in laboratory or field studies (Hegazy et al., 2009; Nasu and Kugimoto, 1981; Ziegler et al., 2019). Another popular field of application is the phytoremediation process of contaminated water bodies, including different kinds of pollutants like heavy metals, pharmaceuticals and personal care products or benzotriazoles (Gatidou et al., 2017; Hu et al., 2021).

1.3. Aims of this study

The main aim of this dissertation is to develop a standardized cultivation system and process for *Lemnaceae*. The two duckweed species *Lemna minor* and *Wolffiella hyalina* were identified as potential candidates for human and animal nutrition due to their fast growth rates (Ziegler et al., 2015) and high protein contents (Appenroth et al., 2017).

To reach the main aim, two parallel steps were pursued in the present project. On one hand, the effect of different abiotic factors (nutrients and light) on physiological and biochemical properties of *L. minor* and *W. hyalina* were investigated. On the other hand, these findings were used to develop and operate a biotechnological application, in the form of a re-circulating indoor vertical farm (IVF). In order to establish duckweed for different fields of application it requires a standardized production process, which is characterized by the following aspects:

- continuously high biomass harvest
- stable and constant product quality
- no application of pesticides
- year-round cultivation, independent of climatic conditions
- minimal input of nutrients and water
- space efficiency

2. Manuscript Overview

Manuscript I:

Finn Petersen, Johannes Demann, Dina Restemeyer, Andreas Ulbrich, Hans-Werner Olfs, Heiner Westendarp, Klaus-Jürgen Appenroth. 2021. "Influence of the Nitrate-N to Ammonium-N Ratio on Relative Growth Rate and Crude Protein Content in the Duckweeds *Lemna minor* and *Wolffiella hyalina*". Plants 10, no. 8: 1741. https://doi.org/10.3390/plants10081741

Manuscript I investigates the effect of different nitrate-N to ammonium-N ratios on the relative growth rate and crude protein content of two duckweed species. This data is important to yield a high protein content of the biomass.

Manuscript II:

Finn Petersen, Johannes Demann, Dina Restemeyer, Hans-Werner Olfs, Heiner Westendarp, Klaus-Juergen Appenroth, and Andreas Ulbrich. 2022. "Influence of Light Intensity and Spectrum on Duckweed Growth and Proteins in a Small-Scale, Re-Circulating Indoor Vertical Farm". Plants 11, no. 8: 1010. https://doi.org/10.3390/plants11081010

Manuscript II investigates the effect of different light intensities and spectral distributions on the relative growth rate and crude protein content of two duckweed species, using a small-scale and re-circulating indoor vertical farm. This data is important to achieve high growth rates at comparatively low light intensities and could provide information for a potentially energy-efficient duckweed cultivation in a new and innovative production system.

Manuscript III:

Finn Petersen, Johannes Demann, Jannis von Salzen, Hans-Werner Olfs, Heiner Westendarp, Petra Wolf, Klaus-Jürgen Appenroth, Andreas Ulbrich. 2022. “Re-circulating indoor vertical farm: Technicalities of an automated duckweed biomass production system and protein feed product quality evaluation”. Journal of Cleaner Production, Volume 380, Part 1, 134894, https://doi.org/10.1016/j.jclepro.2022.134894.

Manuscript III describes the construction, technicalities and operation as well as biomass yield and quality of a re-circulating, large-scale indoor vertical farm for duckweed production. This biomass is evaluated as a potential animal feed source.

3. Manuscripts

3.1. Manuscript I

plants

MDPI

Article

Influence of the Nitrate-N to Ammonium-N Ratio on Relative Growth Rate and Crude Protein Content in the Duckweeds *Lemna minor* and *Wolffiella hyalina*

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Abstract: In order to produce protein-rich duckweed for human and animal consumption, a stable cultivation process, including an optimal nutrient supply for each species, must be implemented. Modified nutrient media, based on the N-medium for duckweed cultivation, were tested on the relative growth rate (RGR) and crude protein content (CPC) of *Lemna minor* and *Wolffiella hyalina*, as well as the decrease of nitrate-N and ammonium-N in the media. Five different nitrate-N to ammonium-N molar ratios were diluted to 10% and 50% of the original N-medium concentration. The media mainly consisted of agricultural fertilizers. A ratio of 75% nitrate-N and 25% ammonium-N, with a dilution of 50%, yielded the best results for both species. Based on the dry weight (DW), *L. minor* achieved a RGR of 0.23 ± 0.009 d^{-1} and a CPC of 37.8 ± 0.42%, while *W. hyalina*'s maximum RGR was 0.22 ± 0.017 d^{-1}, with a CPC of 43.9 ± 0.34%. The relative protein yield per week and m^2 was highest at this ratio and dilution, as well as the ammonium-N decrease in the corresponding medium. These results could be implemented in duckweed research and applications if a high protein content or protein yield is the aim.

Keywords: amino acids; biomass production; cultivation; Lemnaceae; nutrient medium; uptake; water lentils; yield

Citation: Petersen, F.; Demann, J.; Restemeyer, D.; Ulbrich, A.; Olfs, H.-W.; Westendarp, H.; Appenroth, K.-J. Influence of the Nitrate-N to Ammonium-N Ratio on Relative Growth Rate and Crude Protein Content in the Duckweeds *Lemna minor* and *Wolffiella hyalina*. *Plants* **2021**, *10*, 1741. https://doi.org/10.3390/plants10081741

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1. Introduction

A growing world population, with an increasing demand for protein, will necessitate the efficient and increased production of food and animal feed. By 2050, the predicted global population is expected to increase to 9.5 billion, resulting in a rising global demand for protein of up to 78% under different scenarios [1]. In order to handle this challenge, cultivating plants with a high protein content is a promising option. One of the possible candidates is duckweed. However, for this purpose, duckweeds will need to be cultivated under standardized, large-scale conditions.

Duckweeds are an aquatic plant family (*Lemnaceae*), which have been gaining increased attention as an option for human nutrition and animal feeding. Several studies showed the potential of certain duckweed species as a nutrient source [2–4]. This is due to high relative growth rates (RGR) [5,6], a protein content comparable to soybeans, and, in accordance with WHO recommendations, an amino acid composition suitable for humans. The species *Wolffiella hyalina* and *Wolffia microscopica*, in particular, have been recommended for human nutrition [2,3]. Additionally, the nutritional values and proportions within the duckweeds can be modified by changing the cultivation conditions [2,7,8].

Several duckweed species have been tested as feed for animals, such as chickens, piglets and fish [9,10]. High contents of the essential amino acids lysine and methionine make some duckweed species, such as *W. hyalina*, an interesting substitute for today's foremost feed protein source soybean. Gwaze and Mwale [11] compiled several studies, which tested duckweed in pig nutrition. The replacement of soybean meal by 40% duckweed in the feeding rations of young piglets (0 to 10 days old) led to the highest average daily gain in body weight compared to the control of 100% soybean meal [12]. Nguyen and Ogle [13] showed that replacing 75% of roasted soybeans with *Lemna minor* in 5 to 15 week old Tau Vang chickens resulted in weight gain and a feed conversion optimum.

Such promising studies have led to the challenge of yielding high quantities of protein-rich duckweed biomass from a standardized, large-scale production process to incorporate duckweed in the food and feed industry. To economically operate such a system, inexpensive and easily available resources should be used. Moreover, an optimal nutrient supply for each duckweed species must be identified.

According to Appenroth et al. [14,15] no other medium supports faster growth of duckweeds than the N-medium. However, its only nitrogen source is nitrate, but different studies have emphasised the preferential uptake of ammonium over nitrate [16–19]. Little is known about the effect of different nitrate to ammonium ratios on the growth rate and nutritional components of duckweeds.

The aim of our research was to examine how five different nitrate-N to ammonium-N ratios in modified N-media [14,15] affected the RGR, crude protein content (CPC), and relative protein yield (RPY) of *L. minor* and *W. hyalina* (Figure 1). In order to minimize the inhibiting effect of algae on duckweed growth rate and biomass production, different steps of dilution were investigated. Additionally, the decrease of NO_3^--N and NH_4^+-N concentrations in the media, due to N-uptake by the duckweeds, was measured.

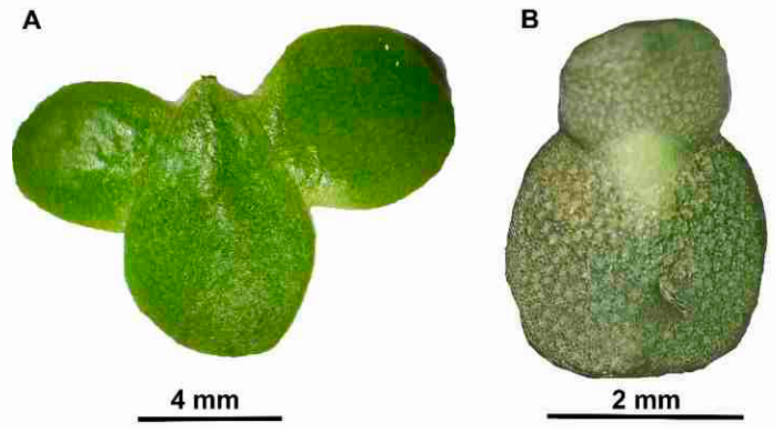

Figure 1. Investigated duckweed species. (**A**) *Lemna minor*, clone 9441. Mother frond (centre) is bearing two daughter fronds. (**B**) *Wolffiella hyalina*, clone 9525. Mother frond (bottom) is bearing a single daughter frond. Photos provided by Dr. K. Sowjanya Sree, Central University of Kerala, India.

2. Materials and Methods

2.1. Plant Material and Cultivation

Two duckweed species, *Lemna minor* L. (clone 9441; Germany) and *Wolffiella hyalina* Delile Monod (clone 9525; India), were used for the experiments due to their fast growth rates and high protein contents. The plant material was obtained from the Duckweed Stock Collection of the Department of Plant Physiology, University of Jena, Germany.

Experiments were carried out in a climate chamber (length × width × height: 4 × 3 × 3 m) at the campus of the University of Applied Sciences Osnabrück, Germany. The trials were conducted in black PE-vessels with a diameter of 24 cm, each containing 4 L

of nutrient medium. All modified media used were based on the N-medium [14,15]. The following abbreviations for five different NO_3^--N to NH_4^+-N ratios are used throughout the manuscript: [100-0] = 100% NO_3^--N - 0% NH_4^+-N; [75-25] = 75% NO_3^--N - 25% NH_4^+-N; [50-50] = 50% NO_3^--N - 50% NH_4^+-N; [25-75] = 25% NO_3^--N - 75% NH_4^+-N; and [0-100] = 0% NO_3^--N - 100% NH_4^+-N. In preliminary experiments, it was observed that the growth of duckweed was disturbed by contamination of ubiquitous algae in the cultures. In order to minimize nutrient competition and growth inhibition of the duckweed due to algae and microorganism growth, several measures were implemented. Two different dilutions (10% and 50% of the original concentration) were used for all five NO_3^--N to NH_4^+-N ratios, indicated by "/10" and "/50" following the ratios. Dilutions of 1% and 5% (/1; /5) were used in initial experiments with 100-0, but omitted in later experiments because of poor results. The temperature was kept at 20.4 ± 1.3 °C. S4W LED elements (SANlight GmbH, Bludenz, Austria), with a photosynthetically active radiation of 350 µmol m^{-2} s^{-1}, were used as the light source. The photoperiod was set to 8 h of light and 16 h of darkness.

The pH showed a minor increase throughout the experimental period, rising from pH 6.6 to a maximum value of 7.0 in the 50% dilutions and from 7.2 to a maximum value of 8.0 in the 10% dilutions.

Six stock solutions, used for all five differently modified N-media, were mainly prepared with commercially available agricultural fertilizers and deionized water. The following products were used for preparing the stock solutions: Krista MKP, Calcinit, Krista K Plus (Yara GmbH and Co. KG, Dülmen, Germany), OCI Granular 2 (OCI NV, Amsterdam, Netherlands), potassium chloride, sodium molybdate dihydrate technical grade (AppliChem GmbH, Darmstadt, Germany), ammonium chloride p.a., calcium chloride dihydrate (Merck KGaA, Darmstadt, Germany), Borax, Mangaan (Horticoop, Bleiswijk, Netherlands), Epso Combitop (K+S AG, Kassel, Germany) and Ferty 72 (Planta Düngemittel GmbH, Regenstauf, Germany). The precise formulation for each stock solution is presented in Table 1.

Table 1. Formulation of seven stock solutions (g L^{-1}) for five different nitrate-N to ammonium-N ratios ([100-0], [75-25], [50-50], [25-75], and [0-100]), based on the N-medium.

Stock Solution	Product Name	Main Components	[100-0] (g L^{-1})	[75-25] (g L^{-1})	50-50 (g L^{-1})	[25-75] (g L^{-1})	[0-100] (g L^{-1})
1	Calcinit	NO_3^--N, NH_4^+-N, Ca^+	47.2	35.4	23.6	11.8	0
1	Krista K Plus	NO_3^--N, K^+	161.8	121.3	80.9	40.4	0
2	NH_4Cl	NH_4^+-N, Cl^-	0	0	26.7	53.5	80.2
3	OCI Granular 2	NH_4^+-N, SO_4^{2-}	0	33	33	33	33
4	KCl	K^+, Cl^-	0	29.8	59.6	89.5	119.3
4	$CaCl_2 \cdot 2\,H_20$	Ca^+, Cl^-	0	7.4	14.7	22.1	29.4
5	Krista MKP	PO_4^{3-}, K^+	27.2	27.2	27.2	27.2	27.2
6	Epso Combitop	Mg^{2+}, SO_4^{2-}, Mn^{2+}, Zn^{2+}	49.3	49.3	49.3	49.3	49.3
6	Borax	BO_3^{3-}	0.06	0.06	0.06	0.06	0.06
6	Mangaan	Mn^{2+}, SO_4^{2-}	0.44	0.44	0.44	0.44	0.44
6	$MoNa_2O_4 \cdot 2\,H_20$	MoO_4^{2-}, Na^+	0.02	0.02	0.02	0.02	0.02
7	Ferty 72	Fe^{3+}	2.2	2.2	2.2	2.2	2.2

The stock solutions were diluted with local tap water (see Table S1) to obtain the initial N-medium concentrations of 100%, as shown in Table 2.

Table 2. Nutrient composition (mM) of the modified N-media with five different NO_3^--N (light grey) to NH_4^+-N (dark grey) ratios at an initial concentration of 100%. These concentrations were diluted to the final concentrations of 10% and 50%, and, in some cases, to 1% and 5%.

NO_3^--N to NH_4^+-N Ratio Substance	[100-0] (mM)	[75-25] (mM)	[50-50] (mM)	[25-75] (mM)	[0-100] (mM)
NO_3^--N	10.1	7.6	5.1	2.6	0.1
NH_4^+-N	0	2.5	5	7.5	10
PO_4^{3-}	1	1	1	1	1
K^+	9.1	9.1	9.1	9.1	9.1
Mg^{2+}	1.3	1.3	1.3	1.3	1.3
SO_4^{2-}	2.0	3.2	3.2	3.2	3.2
Ca^+	2.2	2.2	2.2	2.2	2.2
Cl^-	0.9	3.4	8.4	13.4	18.4
Fe^{3+}	0.025	0.025	0.025	0.025	0.025
BO_3^{3-}	0.005	0.005	0.005	0.005	0.005
Mn^{2+}	0.013	0.013	0.013	0.013	0.013
Zn^{2+}	0.01	0.01	0.01	0.01	0.01
MoO_4^{2-}	0.0004	0.0004	0.0004	0.0004	0.0004
Na^+	0.8	0.8	0.8	0.8	0.8

Table 3 depicts the measured concentrations for nitrate-N and ammonium-N, as well as the corresponding electrical conductivity (EC), after dilution to the final concentrations of 1%, 5%, 10%, and 50% of the undiluted medium.

Table 3. Measured concentrations of NO_3^--N and NH_4^+-N (mg L^{-1}) and EC-values (mS cm^{-1}) at the start of the experiment in the different nutrient media. For the composition of the undiluted nutrient medium, see Table 2. The diluted nutrient media contain 1, 5, 10, or 50% of the undiluted medium.

Ratio	[100-0]				[75-25]		[50-50]		[25-75]		[0-100]	
Dilution (%)	**1**	**5**	**10**	**50**	**10**	**50**	**10**	**50**	**10**	**50**	**10**	**50**
NO_3^--N (mg L^{-1})	2.7	8.8	15.3	71.2	12.1	56.7	10.1	35.3	5.2	16.6	1.1	1.4
NH_4^+-N (mg L^{-1})	0.06	0.17	0.29	1.1	3.6	17.3	7.1	32.5	10.7	51.3	14.4	64.4
EC (mS cm^{-1})	0.43	0.46	0.53	1.15	0.6	1.33	0.66	1.58	0.65	1.64	0.64	1.84

Pre-cultivation occurred for three days within each of the differently diluted and modified nutrient media in order to avoid the lag-phase effect on the RGR data. Experiments lasted for seven days and were conducted under non-axenic growth conditions. The vessels were placed in a randomized block design within the climate chamber. In order to start with a similar surface coverage of 60%, the initial fresh weight biomass of 2 g for *L. minor* and 1.5 g for *W. hyalina* was placed in the above described vessels. At the end of the experiment, the duckweeds were harvested with a metal sieve, rinsed with fresh tap water to remove the attached nutrient solution, blotted with a paper towel to remove attached water, and weighed.

2.2. Analytical Methods

The dry weight (DW) was determined from fresh weight by oven drying at 65 °C for 72 h. At time 0, four samples per species of the same fresh weight as the starting material were used to determine the DW at the beginning of the experiments.

The RGR per day was calculated according to Equation (1) [5], using the values of the DW at the start (t0) and after seven days of cultivation (t7):

$$RGR = (\ln DW_{t7} - \ln DW_{t0})/(t7 - t0) \quad (1)$$

where RGR is the relative increase of the DW per unit time of 1 day (d^{-1}). The relative weekly yield (RY; g biomass obtained after one week of cultivation starting with 1 g) was calculated from the RGR using Equations (2) and (3):

$$\ln DW_{t7} = \ln DW_{t0} + RGR \cdot (t7 - t0) \quad (2)$$

$$RY = \exp(\ln DW_{t7}) \quad (3)$$

The RY (see Figure S1) was further used to calculate the RPY (g protein $week^{-1}$ m^{-2}) by multiplying it with the CPC and extrapolating it to one square metre, according to Equation (4):

$$RPY = RY \cdot CPC/(0.0452\ m^2 \cdot 100) \quad (4)$$

where 0.0452 m^2 is the cultivation area of the vessels used in the experiment.

Dried samples were ground and homogenized using a laboratory mill and stored for further analysis. The nitrogen content of the dried samples was determined by the Dumas method [20] using a FP628 (Leco, Saint Joseph, MI, USA), and was multiplied with the factor 6.25 to determine the CPC [2,21].

Nutrient solution samples were taken at the start (day 0) and end (day 7) of the experiment from each vessel, which were filtered (MN 619 EH, Machery Nagel GmbH and Co. KG, Düren, Germany) to remove particles and instantly frozen at −18 °C. The nitrate-N and ammonium-N concentrations in these samples were measured according to German standard methods [22,23] with a Lambda 25 UV/VIS Spectrometer (Perkin Elmer, Waltham, MA, USA).

2.3. Statistics

All the data is based on four replicates, which are given as mean ± standard deviations. The data were analysed statistically by one-way ANOVA and Tukey's post hoc test at 5% significance level, using the software program SPSS 25 (IBM, Armonk, NY, USA). Datasets fulfilled the one-way ANOVA postulates (including normal distribution and homogeneity of variance).

3. Results

3.1. Growth

The RGR was determined in dependence on the different nutrient media used (Figure 2). The highest value for *L. minor* was 0.23 ± 0.009 d^{-1} at [75-25]/50. The same RGR was determined for *W. hyalina* at [100-0]/50. The two most nitrate-rich ratios ([100-0] and [75-25]) showed an increasing RGR at higher nutrient concentrations (dilutions of 50%) compared to the 10% dilutions, which was significantly higher for *L. minor* in both ratios and for *W. hyalina* only in [100-0]. In these two ratios and dilutions, *W. hyalina* had a slightly higher RGR than *L. minor*, with the exception of [75-25]/50. This was contrary to when the ammonium concentration was increased. A significant drop of the RGR was observed for the ratios [50-50], [25-75], and [0-100] for *L. minor* compared to the 50% dilutions and for *W. hyalina* compared to the 10% and 50% dilutions, but it was more severe in the 50% dilutions. This decrease resulted in a minimum RGR of 0.09 ± 0.015 d^{-1} at [25-75]/50 for *W. hyalina*, while the decrease for *L. minor* (0.12 ± 0.002 d^{-1} at [0-100]/50) was less severe. *Lemna minor* achieved higher RGRs than *W. hyalina* at an overall lower level compared to the two most nitrate-rich ratios, except for [100-0]/1.

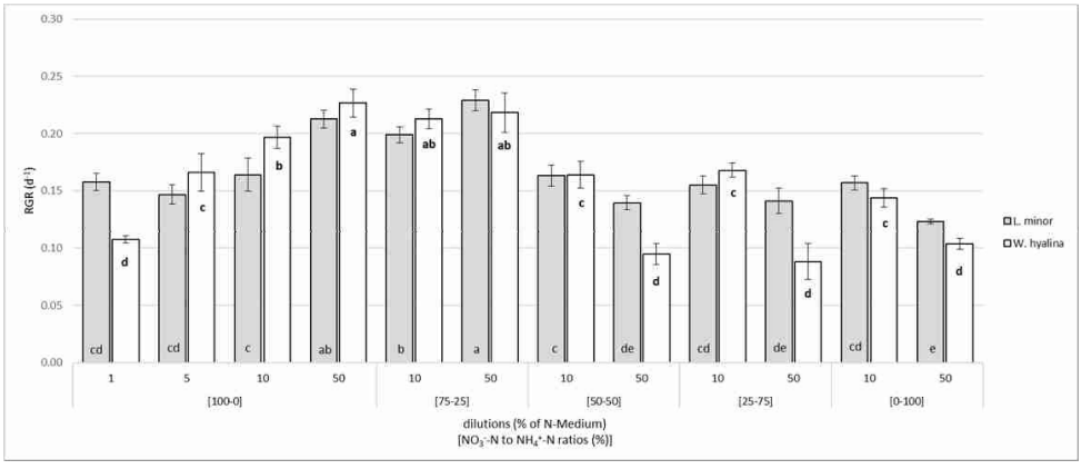

Figure 2. Relative growth rate (RGR; d^{-1}), based on dry weight, for *Lemna minor* (grey shaded columns) and *Wolffiella hyalina* (white columns). Plants were cultivated for seven days in nutrient solutions with varying nitrate-N to ammonium-N ratios (from [100-0] to [0-100]) in different dilutions (1, 5, 10, and 50% of the undiluted N-medium). For the different nutrient media see Tables 1–3. Number of parallel samples n = 4. Different letters indicate significances within a species, based on one-way ANOVA test, Tukey $p \leq 0.05$. Error bars indicate standard deviations.

3.2. Crude Protein Content and Protein Yield

The CPC increased in both duckweed species with increasing ammonium concentrations and a higher ammonium-N to nitrate-N ratio, but not significantly (Figure 3). The higher dilution of the nutrient media, i.e., lower nutrient concentrations, led to lower CPCs within each ratio. In general, *W. hyalina* had a higher CPC within each ratio and dilution compared to *L. minor*. The highest CPC in *L. minor* was reached at [0-100]/50 with 40.6 ± 0.48%, followed by 39.1 ± 0.43% at [0-100]/10. The maximum value measured for *W. hyalina* was 43.9 ± 0.34% at [75-25]/50, which is not significantly higher than the second highest CPC (43.0 ± 0.4%) at [0-100]/50. A minimum CPC of 21.1 ± 1.3% for *L. minor* and 30.3 ± 0.6% for *W. hyalina* were obtained in the ratio with the lowest concentration of nutrients available for the plants ([100-0]/1), which are significantly lower than the second lowest values for each species.

The highest RPY (g protein $week^{-1}$ m^{-2}) was obtained at [75-25]/50 for both species, with a significant difference from the second highest value (Figure 4). A total of 41.6 ± 2.2 g $week^{-1}$ m^{-2} were harvested from *L. minor* and 45.0 ± 5.7 g $week^{-1}$ m^{-2} from *W. hyalina*. A higher nutrient concentration in the ratios [100-0] and [75-25] led to a higher protein yield. *W. hyalina* yielded more protein than *L. minor* under these conditions. The protein yield significantly decreased with an ammonium concentration of 50% and more compared to [100-0]/50 and [75-25]/50 for *L. minor* and [75-25]/50 for *W. hyalina*. At dilutions of 50%, *W. hyalina* performed worse than *L. minor*, while the RPY for both species was slightly higher at 10% dilutions at an overall low level of less than 30 g $week^{-1}$ m^{-2}. The minimum RPYs of 14.1 ± 0.24 g $week^{-1}$ m^{-2} for *L. minor* and 14.2 ± 0.28 g $week^{-1}$ m^{-2} for *W. hyalina* were obtained in [100-0]/1.

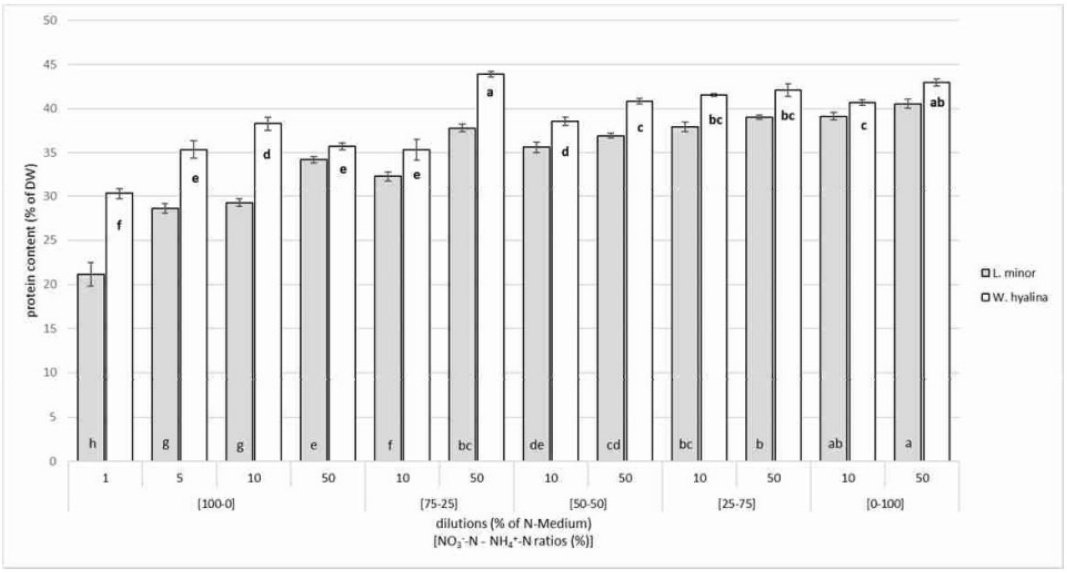

Figure 3. Crude protein content (% of DW) for *Lemna minor* (grey shaded columns) and *Wolffiella hyalina* (white columns), cultivated for seven days in nutrient solutions with different NO_3^--N to NH_4^+-N ratios in different dilutions based on N-medium. For further explanations, see Figure 2.

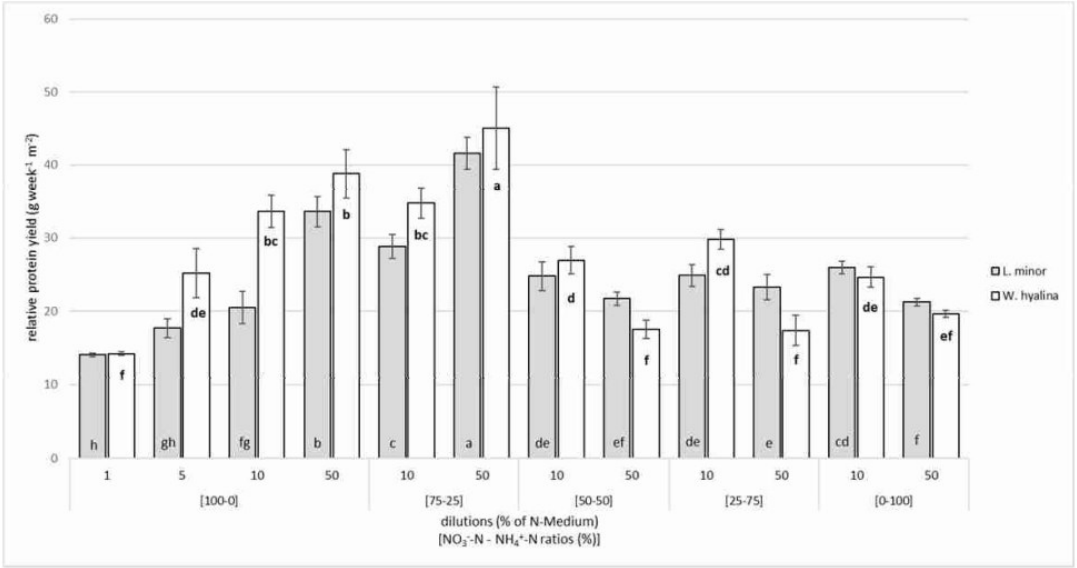

Figure 4. Relative protein yield, in grams per week and m^2 (based on DW), for *Lemna minor* (grey shaded columns) and *Wolffiella hyalina* (white columns), cultivated for seven days in nutrient solutions with different NO_3^--N to NH_4^+-N ratios in different dilutions based on N-medium. For further explanations, see Figure 2.

3.3. NO_3^--N and NH_4^+-N Reduction in the Media

Figure 5 shows the total reduction of NO_3^--N (mg L^{-1}) for each ratio and dilution at day seven. In the nitrate-only medium, [100-0], the higher NO_3^--N concentration led to a significantly higher absolute reduction in *L. minor*, but not in *W. hyalina*. The highest starting concentration of nitrate-N ([100-0]/50) led to the highest absolute decrease of nitrate-N, i.e., 6.5 mg L^{-1} and 6.9 mg L^{-1} for *L. minor* and *W. hyalina*, respectively. This corresponded to a relative reduction of 9.1% (*L. minor*) and 9.7% (*W. hyalina*). In general, higher nitrate-N concentrations resulted in a greater reduction, while an increasing NH_4^+-N concentration led to a decreasing nitrate-N reduction. These findings, however, were not significant. The maximal relative reduction of NO_3^--N for *L. minor* was found at [25-75]/10 with 29.2% and for *W. hyalina* at [75-25]/10 with 29.6%.

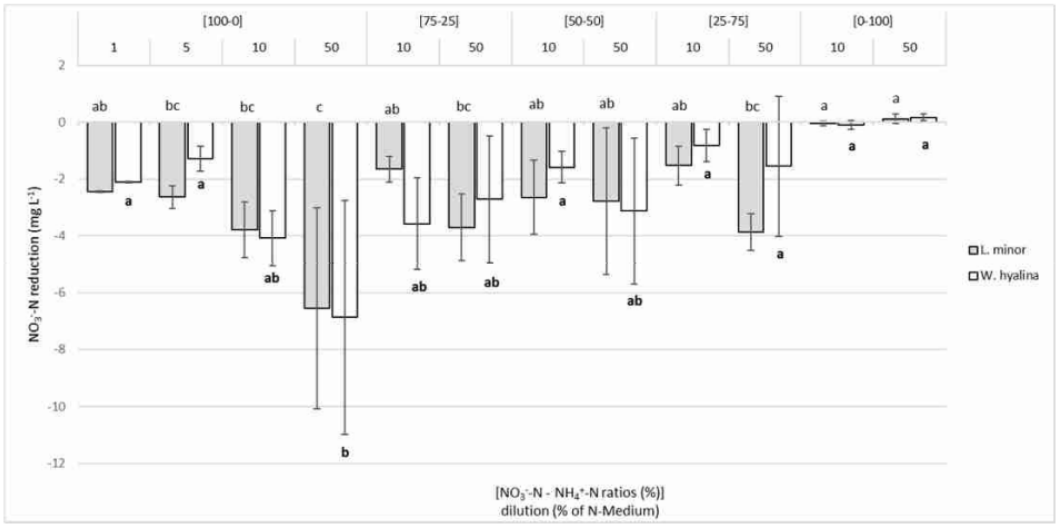

Figure 5. Decrease of the concentration of nitrate-N (mg L^{-1}) in the nutrient media for *Lemna minor* (grey shaded columns) and *Wolffiella hyalina* (white columns), cultivated for seven days in nutrient solutions with varying NO_3^--N to NH_4^+-N ratios in different dilutions. For further explanations, see Figure 2.

Figure 6 depicts the total reduction of NH_4^+-N (mg L^{-1}) for each ratio and dilution at day seven. In the [100-0] nutrient media, NH_4^+-N was present only in minor concentrations, which were decreased almost completely by both duckweed species. This also applied to [75-25]/10. The highest total reduction values were 8.1 $\pm$ 0.9 mg L^{-1} for *L. minor* and 7.2 $\pm$ 0.5 mg L^{-1} for *W. hyalina* in the [75-25]/50 treatments. This corresponded to a relative reduction of 46.8% (*L. minor*) and 41.6% (*W. hyalina*). The total, as well as the relative reduction, was slightly higher for *L. minor* than for *W. hyalina*. A significant drop was evident in the ammonium-only solutions, with the highest total NH_4^+-N concentration ([0-100]/50), compared to the same dilution in the ratios [75-25], [50-50], and [25-75]. The relative reduction for *L. minor* was 4.1% of the initially available NH_4^+-N, while for *W. hyalina* no reduction at all occurred. However, decreasing the total NH_4^+-N concentration, but keeping the ratio of the two N sources constant, i.e., [0-100]/10, resulted in much higher uptake rates for *L. minor* (7.8 $\pm$ 0.14 mg L^{-1}; 54% relative reduction) and *W. hyalina* (6.9 $\pm$ 0.27 mg L^{-1}; 48% relative reduction).

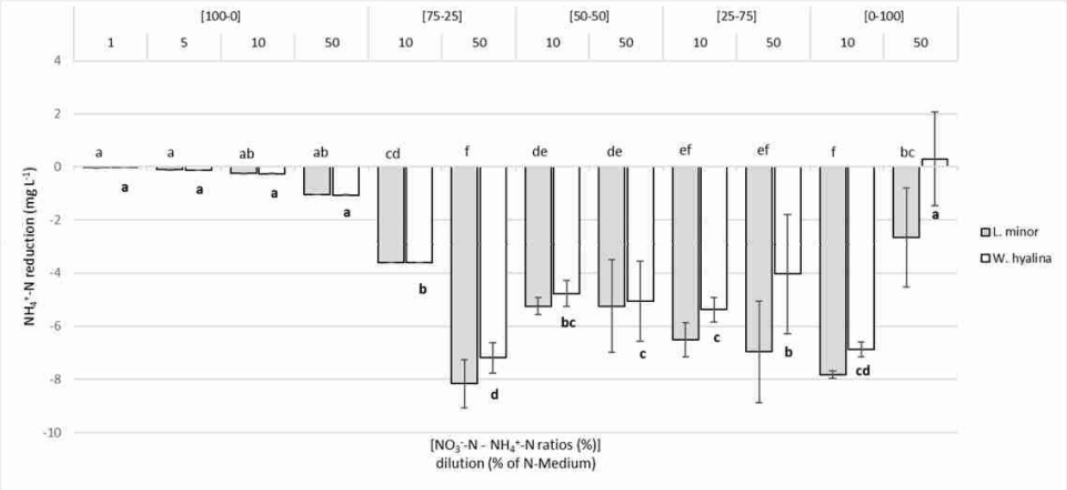

Figure 6. Decrease of the concentration of ammonium-N (mg L^{-1}) in the nutrient media for *Lemna minor* (grey shaded columns) and *Wolffiella hyalina* (white columns), cultivated for seven days in nutrient solutions with varying NO_3^--N to NH_4^+-N ratios in different dilutions. For further explanations, see Figure 2.

4. Discussion

The maximum RGR reached in this experiment was 0.23 d^{-1} for both species, which is lower compared to other studies. For *L. minor*, an RGR of 0.42 d^{-1} was reported, while *W. hyalina* had the highest RGR of all investigated species with a value of 0.519 d^{-1} [5]. The difference in the RGR was most likely caused by different growth conditions. Instead of an axenic in vitro set-up, both duckweed species in this study were cultivated under non-sterile conditions. The temperature was 5 °C lower and the photoperiod 16 h shorter, while the light intensity was 250 µmol m^{-2} s^{-1} higher compared to the experimental set-ups applied by Ziegler et al. [5]. These factors are possible explanations for the lower RGRs.

Iatrou et al. [24] achieved a maximum growth rate of 0.14 d^{-1} for *L. minor* at an ammonium-N concentration of 31.9 mg L^{-1}, using secondary treated wastewater. This was in agreement with our experimental results for the same species in the ratio of [50-50]/50 (RGR of 0.14 ± 0.009 d^{-1} at a NH_4^+-N concentration of 32.5 mg L^{-1}).

Caicedo et al. [18] observed that the highest RGR (0.3 d^{-1}) in *Spirodela polyrhiza* was obtained at the lowest total ammonium concentrations (3.5–20 mg L^{-1} N; equal to ca. 0.25–1.4 mM) and assumed an optimum NH_4^+-N concentration was below 20 mg L^{-1}. Zhang et al. [25] obtained the maximal RGR in *L. minor* at 3.5 mg L^{-1} ammonium-N. These data partly agree with our results concerning the total concentration of NH_4^+-N. High RGRs were obtained for the treatments [75-25]/10, with an initial NH_4^+-N concentration of 3.6 mg L^{-1}, and [75-25]/50, with a concentration of 17.3 mg L^{-1}. The 10% dilutions [50-50]/10, [25-75]/10, and [0-100]/10 had similar total NH_4^+-N starting values of 7.1, 10.7, and 14.4 mg L^{-1}, respectively. However, the RGR was significantly lower in these three treatments for both species. It can be assumed that other factors, such as the ratio of nitrate to ammonium, had a certain impact on the RGRs of *L. minor* and *W. hyalina*. This is in agreement with the investigations of Mehrer and Mohr [26] and Hecht and Mohr [27] on *Sinapis alba* seedlings. The explained the detrimental effects of higher ammonium concentrations by identifying that ammonium accumulation is not well regulated by plants. The stimulation of ammonium assimilation by simultaneously applied nitrate appears to explain the nitrate-mediated ammonium tolerance. A similar mechanism exists in duckweeds, as shown recently in *Landoltia punctata* [28]. A minor fraction of ammonium as

the nitrogen source seemed to stimulate duckweed growth, while proportions of 50% and higher had a growth inhibiting effect.

Approximately six NO_3^- transporters and four NH_4^+ transporters are involved in the uptake of N for *Arabidopsis thaliana*. Nitrate acts as a signalling molecule that triggers changes in the expression of genes, metabolism, and growth. Plants have evolved several NO_3^- uptake systems to survive in the changing environment. While low affinity transporters are responsible for the uptake of a large amount of nitrate in the case of available high concentrations, high affinity transporters ensure plant survival in the presence of low nitrate concentrations [29]. Acquisition of ammonium from the aquatic environment is important, as this N source for plants may be the dominating form under certain conditions. While considerable progress was made in the last two decades, many aspects of the regulation of NH_4^+ uptake and metabolism are not yet well understood [30]. *Lemna minor* grown in an NH_4NO_3 (1:1 ratio between NH_4^+-N and NO_3^--N) containing nutrient solution preferentially took up ammonium over nitrate. It was discovered that both roots and fronds take up nitrate and ammonium from the medium. At low N concentrations, the root-to-frond biomass ratio increased. This is advantageous for the plant at a morphological level, wherein there is a lower biomass investment per unit surface area for roots than for fronds [19]. Fang et al. [16] reported a preference in NH_4^+ uptake compared to NO_3^- in *Landoltia punctata*. Turions of the duckweed *Spirodela polyrhiza* absorbed $^{15}NH_4^+$ much faster than $^{15}NO_3^-$ [31]. This was confirmed by our data, which showed that the average relative uptake rate of NH_4^+-N in almost all ratios and dilutions was higher than that of NO_3^--N.

A decrease of the ammonium concentration in nutrient media can be caused by plant uptake or by volatilization depending on the pH. With a pH value of 8 at 20 °C, less than 5% of the ammonium turns into NH_3 [32]. By looking at the pH in the present experiment, it can be concluded that the majority of the NH_4^+-N was taken up by the duckweeds.

The chloride concentrations in the experiment increased with increasing ammonium supplement because ammonium chloride was partly used to increase the NH_4^+-N concentrations. Liu et al. [33] recommended an NaCl concentration below 75 mM for *L. minor* for N and P removal from water. Concentrations of 50 mM and higher caused a decrease in the fresh weight and chlorophyll content of *L. minor*. The maximum Cl^- concentration used in the presented experiment was 9.2 mM in [0-100]/50. Therefore, the significantly reduced RGRs for both species in the ratios [50-50], [25-75], and [0-100], as compared to [75-25], could not be caused by the presence of chloride.

Duckweeds (species not identified) grown in irrigation ponds in Jordan yielded an average CPC of 26% [34]. Mohedano et al. [35] investigated the CPC of duckweed species grown in anaerobically digested swine manure in two consecutive ponds. The average CPC in the primary pond was 35% (based on dry matter) and decreased to 28% in the secondary pond, which had less nutrients available. The estimated productivity of both ponds was 24 t year^{-1} ha^{-1} (ca. 46 g week^{-1} m^{-2}). This value is slightly higher than our maximum value (45 g week^{-1} m^{-2}). The lower CPC in their study was compensated for by a higher RGR (0.24 d^{-1}). Chakrabarti et al. [4] reported a yield of 703 kg month^{-1} ha^{-1} *L. minor* (ca. 17.5 g week^{-1} m^{-2}) with RGRs ranging between 0.073 d^{-1} and 0.422 d^{-1}. The duckweed was cultivated on different media containing organic manure or inorganic fertilizers. The final CPC was 36.07% and 27.12% for duckweeds grown in organic manure and in inorganic fertilizer, respectively.

The modified Schenk-Hildebrandt medium used by Appenroth et al. [2] had a nitrate-N to ammonium-N ratio of roughly [90-10]. The total ammonium-N concentration (1.3 mM) was about the same as in [75-25]/50 (1.24 mM) of the modified N-medium, while nitrate concentrations were higher in the modified Schenk-Hildebrand medium. The CPC in the presented investigation was above 25% for *L. minor* and above 35% for *W. hyalina* in almost all ratios and dilutions, which was also the result in Appenroth et al. [2] for both species. Only 100-0/1 showed a lower value of 21.1% and 30.3% for *L. minor* and *W.*

hyalina, respectively. The nitrogen availabilities in these two experiments were only slightly different, thereby confirming our own results.

If duckweed should be cultivated in an agrarian system in order to produce food and feed in the future, a standardized cultivation process needs to be implemented to yield a standardized product quality. Of high interest concerning a standardized non-axenic cultivation process is the growth of algae and microorganisms and how they influence duckweed growth and culture medium composition. The use of plant growth-promoting bacteria in particular may open up new opportunities [36]. Alongside the quality, the amount of biomass and protein yielded is of great importance. The variation of the initial biomass, hence surface coverage, could have an important impact on the productivity of a system. The higher the initial biomass, the higher the nutrient requirement over time. Therefore, highly diluted nutrient media result in low growth rates. An initial surface coverage of 20% seems optimal for a high RGR [37,38]. Such a low initial duckweed biomass, however, means less competition for other organisms competing for nutrients and light. Therefore, in these experiments, an initial surface coverage of 60% was selected. To avoid growth inhibition due to high densities ("overcrowding" [39]), a regular harvest interval should be defined. Regarding the protein yield, the RPY should be considered a good indicator for the productivity of a duckweed system.

5. Conclusions

Implementing conditions that increase the RGR and CPC, positively affect the RPY. One such condition is a suitable nutrient composition of standardized quality. The concentration of nutrients in the medium, as well as the ratio between nitrate-N and ammonium-N, influenced the RGR, CPC, and RPY in the duckweeds *L. minor* and *W. hyalina*. The modification of the promising N-medium, with a substitution of 25% nitrate-N by ammonium-N at 50% dilution, significantly increased the RPY for both species when compared to the nitrate-only ratio at the same dilution. *L. minor* yielded 41.6 ± 2.2 g week^{-1} m^{-2}, while *W. hyalina* reached 45.0 ± 5.7 g week^{-1} m^{-2}.

However, other abiotic factors, such as light intensity, light spectrum, photoperiod, temperature, water and duckweed movement, as well as biotic factors, such as the growth of algae and microorganisms and their effects on duckweed, should be closely investigated. A stable cultivation process is only possible if all the biotic and abiotic factors are complementary and optimized for the species of choice.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants10081741/s1, Figure S1: relative weekly yield (RY, week^{-1}) based on DW, for *L. minor* (grey shaded columns) and *W. hyalina* (white columns), cultivated for seven days in nutrient solutions with different NO_3^--N to NH_4^+-N ratios in different dilutions, based on N-medium. For further explanations, see Figure 1. Table S1: tap water analysis municipal utilities Osnabrueck Wittefeld.

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3.2. Manuscript II

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Article

Influence of Light Intensity and Spectrum on Duckweed Growth and Proteins in a Small-Scale, Re-Circulating Indoor Vertical Farm

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Abstract: Duckweeds can be potentially used in human and animal nutrition, biotechnology or wastewater treatment. To cultivate large quantities of a defined product quality, a standardized production process is needed. A small-scale, re-circulating indoor vertical farm (IVF) with artificial lighting and a nutrient control and dosing system was used for this purpose. The influence of different light intensities (50, 100 and 150 µmol m^{-2} s^{-1}) and spectral distributions (red/blue ratios: 70/30, 50/50 and 30/70%) on relative growth rate (RGR), crude protein content (CPC), relative protein yield (RPY) and chlorophyll a of the duckweed species *Lemna minor* and *Wolffiella hyalina* were investigated. Increasing light intensity increased RGR (by 67% and 76%) and RPY (by 50% and 89%) and decreased chlorophyll a (by 27% and 32%) for *L. minor* and *W. hyalina*, respectively. The spectral distributions had no significant impact on any investigated parameter. *Wolffiella hyalina* achieved higher values in all investigated parameters compared to *L. minor*. This investigation proved the successful cultivation of duckweed in a small-scale, re-circulating IVF with artificial lighting.

Keywords: Lemnaceae; *Lemna minor*; *Wolffiella hyalina*; red/blue ratio; standardized production; yield; light quality; light quantity; controlled environment

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1. Introduction

The term duckweed comprises 36 species [1,2] of 5 genera, belonging to the family of Lemnaceae Martinov [3,4]. They are characterized, amongst other aspects, by their fast growth rate [5,6], high nutrient uptake capacity [7,8] as well as by their edibility [9,10] and variability of nutritional values influenced by cultivation conditions [11,12]. Those are key aspects for further use in human and animal nutrition, biotechnology or wastewater treatment.

In order to continuously produce large quantities of biomass with a defined quality (e.g., for human nutrition), a standardized cultivation process is necessary. One possible solution in the future might be the cultivation of duckweed in re-circulating (also described as closed) indoor vertical farms (IVF) with artificial lighting. By stacking several layers of cultivation areas above each other, the land utilization efficiency is increased [13,14]. When operating an IVF in a controlled environment, it is possible to regulate plant-relevant abiotic factors, e.g., nutrient composition and concentration, light intensity and spectrum, photoperiod, the temperature of water and air, water flow rate or humidity according to the grower's demand. Resources, such as nutrients, water and pesticides, can be used efficiently. This can positively affect the quantity and quality of the crops. Additionally, the use of IVFs will allow year-round crop production, even in areas with short growing seasons or unfavorable climatic conditions [13–16]. One shortcoming of this cultivation

technology is the relatively high energy input, e.g., the production of one kg of curled lettuce required 7–9 kWh of electric energy [14].

However, closed hydroponic systems are already successfully used to cultivate different crops in large quantities. This includes tomatoes, cucumbers, peppers, different leafy greens, strawberries and even rice or maize [17]. The advantages of closed hydroponic systems compared to conventional farming are enormous, as up to 85% of fertilizers and 90% of water can be saved, while a productivity increase of up to 250% is possible [18]. The water and nutrient use efficiency of tomatoes cultivated in a closed hydroponic system was 23% higher compared to an open system in both cases [19]. The water use efficiency for tomatoes cultivated in a closed system in The Netherlands was 66 kg of yield per cubic meter of water applied [18]. Another study described zero discharge of nutrients and pesticides to the environment in the production of sweet peppers and autumn cucumber in a closed hydroponic system [20].

In order to also achieve an efficient system for duckweed cultivation, all necessary abiotic factors must be evaluated. Two of these abiotic factors are light intensity and the spectral light distribution. In nature, *Lemnaceae* grow in sunny as well as in shaded habitats, but the latter habitats are favorable due to lower light intensities and less extreme temperatures [21]. The plant's reaction to different light intensities is dependent on the species and abiotic factors, such as nutrients or temperature, while the light spectrum is another important parameter [22]. *Wolffia arrhiza* cultivated in steady-state conditions with blue light showed higher protein and chlorophyll contents compared to red light [22]. Increasing light intensities slightly increased the relative growth rates (RGRs) of *Lemna gibba* [23] and *Lemna aequinoctialis* [24]. Very high intensities, however, lead to light saturation. Light intensities above this point will not increase the photosynthetic activity of the plant and could lead to damages due to oxygen stress (photoinhibition). The light saturation point depends on factors such as temperature and varies for different duckweed species. A light saturation of 342 µmol m^{-2} s^{-1} for *L. minor* [25] and of 400 µmol m^{-2} s^{-1} for *L. minor* and *Lemna minuta* were observed [26], while *Landoltia punctata* (formerly *Spirodela punctata*) reached light saturation between 600 and 1200 µmol m^{-2} s^{-1} at 30 °C [27]. Considering the cost of artificial lighting, an optimum of 110 µmol m^{-2} s^{-1} was obtained for *L. aequinoctialis* [24].

The aim of our research was to evaluate the influence of different light intensities and spectral distributions on the RGR, crude protein content (CPC) and relative protein yield (RPY) in the duckweeds *L. minor* and *W. hyalina* when cultivated in a small-scale, re-circulating, aquatic IVF. Additionally, for both species, the chlorophyll a content was determined as a plant cultivation indicator. We selected clones of these two species because they showed good performance in earlier experiments concerning growth rates and protein contents [9].

2. Materials and Methods

2.1. Indoor Vertical Farm

Two duckweed species, *Lemna minor* L. (clone 9441; Germany) and *Wolffiella hyalina* Delile Monod (clone 9525; India), were chosen for the experiments due to their fast growth rates and high protein contents [28]. The plant material was obtained from the Duckweed Stock Collection of the Department of Plant Physiology, University of Jena, Germany.

Experiments were carried out in a container (length × width × height: 5 × 3 × 3 m) at the campus of the University of Applied Sciences Osnabrück, Germany. Trials were conducted in a re-circulating, aquatic IVF (Figures 1 and 2).

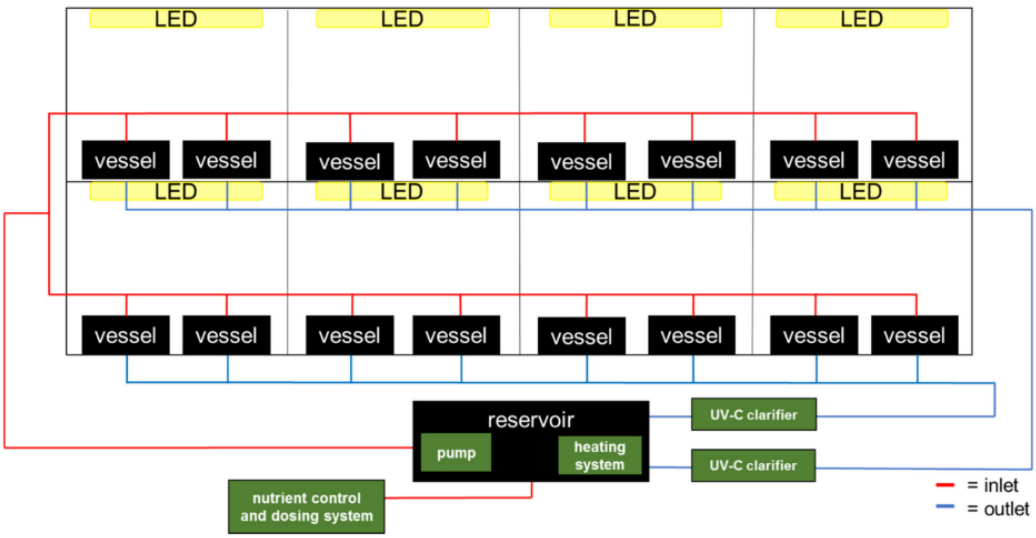

Figure 1. Scheme of the experimental set-up designed as indoor vertical farm (IVF). Black boxes depict the cultivation vessels for the duckweeds and the nutrient solution reservoir, yellow boxes depict the LEDs and green boxes depict the necessary technology to run the re-circulating system. Red lines indicate the nutrient solution inlet and blues lines the outlet.

It consisted of a 90 L reservoir for the nutrient solution connected to all duckweed cultivation vessels via flexible tubes. A submergible and adjustable pump (AquaForte DM-10000 Vario, SIBO BV, Veghel, The Netherlands) was installed at the bottom of the reservoir to create a continuous flow between reservoir and cultivation vessels. A nutrient control and dosing system (Pro Controller and PeriPods, Bluelab Corporation Ltd., Tauranga, New Zealand) added the required liquid fertilizers from stock solutions to the tap water in the reservoir. A heating system (Super Fish Smart Heater 500 W, Aquadistri BV, Klundert, The Netherlands) was installed at the bottom of the reservoir to keep a constant water temperature. The vessels (56 cm length $\times$ 37 cm width $\times$ 10 cm height) used for cultivation were positioned in a two-layer storage rack. On one side (width) of the cultivation vessel, the water inlet, a rectangular pipe leading the water inflow to the bottom of the vessel, was installed. On the opposite side, an outlet was located at 7 cm height. To guarantee no duckweed was lost from the vessel by flowing through the outlet, a wall was installed 7 cm before the outlet. The upper side of the wall was above water level, while the bottom side did not touch the ground of the vessel. This way, the nutrient solution could flow back into the reservoir, while the floating duckweed was hindered from passing the barrier. The net cultivation area per vessel decreased to 0.49 $\times$ 0.37 m = 0.1813 m^2 by applying this method. The unoccupied surface was covered with black PE in order to prevent algae growth in that area. The outlet solution from each of the two storage rack levels was led through UV-C clarifiers (OSAGA UVC36, Fischfarm Otto Schierhölter, Glandorf, Germany) in order to reduce the growth of ubiquitous algae and bacteria.

As light sources, dimmable LEDs with an adjustable spectrum (LED-LE1200-E03W-1-S, DH Licht GmbH, Wülfrath, Germany) were installed 34 cm above the water surface in the vessels. The settings were adjusted with the VisuSpectrum 3.0 software (DH Licht GmbH, Wülfrath, Germany and RAM GmbH Mess- und Regeltechnik, Herrsching, Germany).

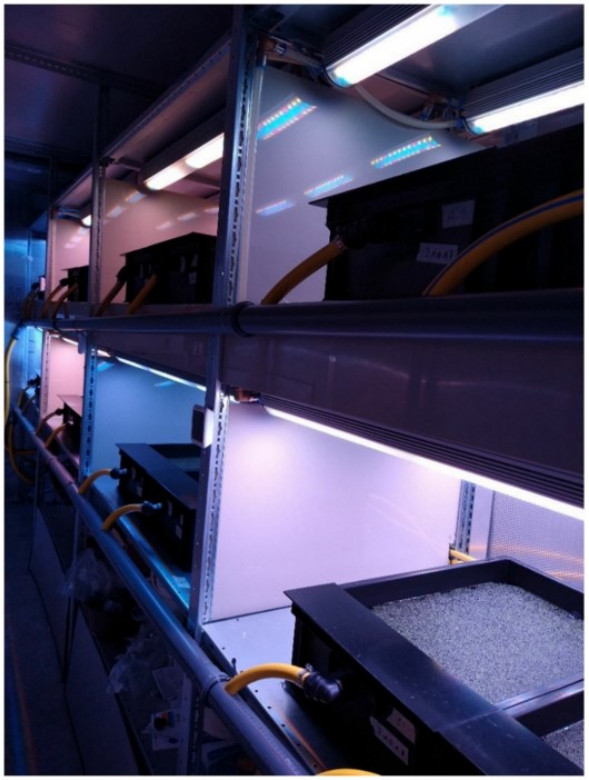

Figure 2. Experimental set-up in the container at the Osnabrück University of Applied Sciences, Germany. The light colors of the different spectral treatments are visible.

2.2. Experimental Design

Three different light intensities (50, 100 and 150 µmol m^{-2} s^{-1}) were used for the experiments. All of the three spectral treatments contained 20% light at 6500 K (white light), and the remaining 80% were split according to the following red (660 nm)/blue (450 nm) ratio: 70/30, 50/50 and 30/70 (%). This resulted in eight different treatments (Table 1). Light intensities were controlled using a Light Meter LI-250A (LI-COR Biosciences, Lincoln, NE, USA). The photoperiod was set to 12 h of light and 12 h of darkness per day.

Table 1. Applied light intensities and red/blue ratios (spectral distributions) in the experiments as well as the corresponding treatment abbreviation, as used throughout the text.

Light Intensity	Red/Blue Ratios	Treatment Abbreviation
50	70/30	50–70/30
50	50/50	50–50/50
50	30/70	50–30/70
100	70/30	100–70/30
100	30/70	100–30/70
150	70/30	150–70/30
150	50/50	150–50/50
150	30/70	150–30/70

Pre-cultivation occurred for three days under above mentioned conditions. Experiments lasted for seven days and were conducted under non-axenic growth conditions. Vessels were placed in the storage rack based on a block design. This storage rack had eight compartments, each containing two LEDs and space for two experimental vessels. Eight treatments, with four replications for each of the two species, were investigated. In total, 16 vessels could be used at a time. Two replicates per light intensity and spectral distribution per species were investigated at the same time. To start with a similar surface coverage of ca. 80% in each vessel, 20 g of *L. minor* and 15 g of *W. hyalina* fresh weight (FW) biomass was placed in each vessel.

The nutrient medium applied mainly consisted of commercially available fertilizers (see Table S1). The nutrient dosing was set to an electrical conductivity (EC) value of 0.6 mS cm^{-1}, which corresponds to a nutrient solution of 75-25/10 with the following composition and concentrations (all given in mM): NO_3^--N: 0.76, NH_4^+-N: 0.25, PO_4^{3-}: 0.1, K^+: 0.91, Mg^{2+}: 0.13, SO_4^{2-}: 0.32, Ca^+: 0.22, Cl^-: 0.34, Fe^{3+}: 0.0025, BO_3^{3-}: 0.0005, Mn^{2+}: 0.0013, Zn^{2+}: 0.001 and Na^+: 0.08 [28]. When the EC dropped below target value in the time course of cultivation, additional nutrient solution was added until the target EC was reached again.

The pH at the beginning of the experiments was 7.6. The heating system was set to a target value of 24 °C, and the pump was adjusted to a flow rate of 2 L min^{-1}.

At the end of the experiments, duckweeds were harvested with a metal sieve, rinsed with tap water, spin-dried for three minutes with a Top Spin Compact (Chal-Tec GmbH, Berlin, Germany) to remove attached water and weighed.

2.3. Analytical Methods

2.3.1. Relative Growth Rate

Dry weight (DW) was determined from FW via oven drying at 65 °C for 72 h. At time 0, four samples per species of the same FW as the starting material were used to determine the DW at the beginning of the experiments.

Relative growth rates (RGRs) per day were calculated according to Equation (1) [6], using the values of the DW at the start (t0) and after seven days of cultivation (t7):

$$RGR = (\ln DW_{t7} - \ln DW_{t0})/(t7 - t0) \quad (1)$$

where RGR is the relative increase in the DW per day (d^{-1}).

2.3.2. Crude Protein Content and Relative Protein Yield

Dried samples were ground and homogenized using a laboratory mill and stored for further analysis. The nitrogen content of the dried samples was determined using the Dumas method [29] using an elemental analyzer (FP628, Leco, Saint Joseph, MI, USA), and CPC was calculated using the factor 6.25 [9,30].

The relative weekly yield (RY; g biomass obtained after one week of cultivation starting with 1 g) was calculated from the RGR using Equations (2) and (3):

$$\ln DW_{t7} = \ln DW_{t0} + RGR \cdot (t7 - t0) \quad (2)$$

$$RY = \exp(\ln DW_{t7}) \quad (3)$$

The RY was further used to calculate the relative protein yield (RPY; g protein $week^{-1}$ m^{-2}) by multiplying it with the crude protein content (CPC) and extrapolating it to one square meter, according to Equation (4):

$$RPY = RY \times CPC/(0.1813\ m^2 \times 100) \quad (4)$$

where 0.1813 m^2 is the cultivation area of the vessels used in the experiments.

2.3.3. Chlorophyll a

The chlorophyll a content was determined according to DIN 38409-60:2019-12 [31], using ethanol (ω(EtOH) = 90%) as a solvent. Four replicates of the starting biomass and four replicates of each treatment at the end of the experiments were analyzed. Laboratory analysis of the chlorophyll a content took place in the dark immediately after the samples were taken according to the following scheme: A net weight of 1.000 ± 0.005 g FW duckweed biomass was placed in 50 mL centrifuge tubes, filled with 10 mL of boiling solvent and homogenized for 60 s using an Ultra-Turrax. The resulting extract was cooled and treated in an ultrasonic bath for 30 min in the dark. Afterwards, the extract was filtered into a 100 mL volumetric flask, filled with ethanol to the calibration mark and homogenized again by shaking. The extract was placed into a glass cuvette. Of the remaining extract, 15 mL was put into a centrifuge tube, added with 100 µL of hydrochloric acid (2 M) and homogenized for the correction of phaeopigments. Both extracts and the pure solvent were finally put into different glass cuvettes and analyzed using a spectrophotometer (Specord 40, Analytik Jena AG, Jena, Germany) at 665 and 750 nm.

The following modified Equation (5) was applied to calculate the chlorophyll a content in the fresh duckweed biomass [31]:

$$\omega_{\text{Chlorophyll}-a} = ((A_{665v} - A_{750v}) - (A_{665n} - A_{750n})) \cdot \frac{R}{R-1} \cdot \frac{V_E}{m_P \cdot d \cdot \alpha \cdot 1000} \quad (5)$$

with

$\omega_{\text{Chlorophyll-a}}$: Chlorophyll a content (mg/g FW);
A_{665v}: Absorption of the extract before acidification, measured at 665 nm;
A_{750v}: Absorption of the extract before acidification, measured at 750 nm (for the correction of phaeopigments);
A_{665n}: Absorption of the extract after acidification, measured at 665 nm;
A_{750n}: Absorption of the extract after acidification, measured at 750 nm (for the correction of phaeopigments);
R: Ratio of A_{665v}/A_{665n} for pure Chlorophyll-a; R = 1.7;
V_E: Volume of the extract in milliliters (ml);
m_P: Net weight of the duckweed biomass sample (g);
d: Thickness of the cuvette (cm); d = 1.

Additionally, the dry matter content of each sample was determined by drying plant material at 105 °C until it reached a constant weight. The chlorophyll a FW content was then multiplied with the dry matter content to calculate the chlorophyll a DW content.

2.3.4. Nutrient Solution

A nutrient solution sample was taken at the start (day 0) and the end (day 7) of the experiments from the reservoir, filtered (MN 619 EH, Machery Nagel GmbH & Co. KG, Düren, Germany) to remove particles and instantly frozen at −18 °C. Nitrate-N and ammonium-N concentrations in these samples were measured according to German standard methods [32,33] with a Lambda 25 UV/VIS Spectrometer (Perkin Elmer, Waltham, MA, USA). Other nutrients were analyzed according to DIN EN ISO 11885:2009-09 with an ICP-OES (ICAP 7400, Thermo Fischer Scientific, Waltham, MA, USA) [34].

2.4. Statistics

All data are based on four replicates and are given as mean ± standard deviations. The data were analyzed statistically using one-way ANOVA and Tukey's post hoc test at 5% significance level, using the software program SPSS 25 (IBM, Armonk, NY, USA).

3. Results

3.1. Relative Growth Rate

Figure 3 shows the RGR based on DW. An increasing light intensity increased the RGR for both species. The highest RGR for *L. minor* was reached at 150–70/30 (0.13 ± 0.013 d^{-1}) and for *W. hyalina* at 150–50/50 (0.21 ± 0.01 d^{-1}). The minimum values were obtained at 50–30/70 for *L. minor* with an RGR of 0.078 ± 0.012 d^{-1} and at 50–50/50 for *W. hyalina* with an RGR of 0.119 ± 0.003 d^{-1}. The percentage increase from the lowest to the highest RGR was 67% for *L. minor* and 76% for *W. hyalina*. The results of all three *L. minor* treatments cultivated at 150 µmol m^{-2} s^{-1} were significantly higher compared to the 50 µmol m^{-2} s^{-1} treatments. *W. hyalina* cultivated at a light intensity of 150 µmol m^{-2} s^{-1} reached significantly higher RGRs than the 100 and 50 µmol m^{-2} s^{-1} treatments. The light spectrum showed no significant impact on the RGR in any treatment.

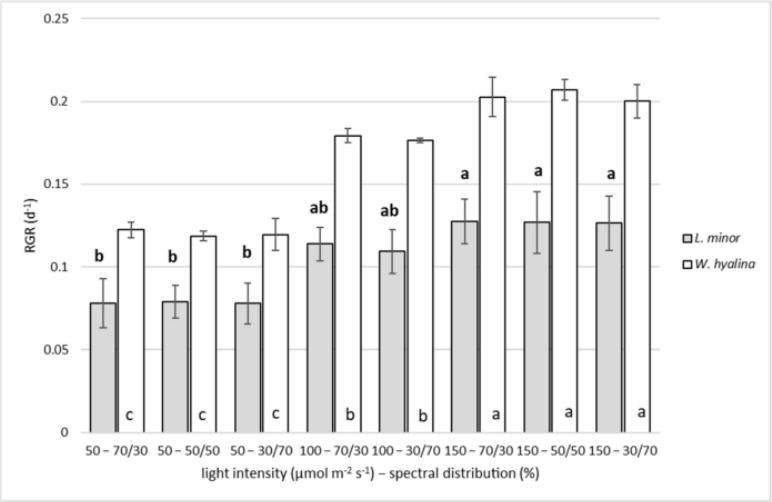

Figure 3. Relative growth rate (RGR; d^{-1}), based on dry weight, for Lemna minor (gray shaded columns) and Wolffiella hyalina (white columns). Plants were cultivated for seven days with different light intensities (50, 100 and 150 µmol m^{-2} s^{-1}) and spectral distributions (red/blue: 70/30, 50/50 and 30/70%). For the abbreviations used, see Table 1. Number of parallel samples $n = 4$. Different letters indicate significances within a species, based on one-way ANOVA test, Tukey $p \leq 0.05$. Error bars indicate standard deviations.

3.2. Crude Protein Content and Relative Protein Yield

The CPC, based on DW, varied in a narrow range between 31.8 ± 0.8% and 32.4 ± 1.2% for *L. minor* and between 39.3 ± 1.0% and 40.0 ± 0.8% for *W. hyalina* for the different treatments. No significant differences in the CPC for the different light intensities and spectral distributions within a species were detected.

The RPY in grams per week and m^2, based on DW, is presented in Figure 4. It ranged from 2.96 ± 0.30 to 4.44 ± 0.55 g $week^{-1}$ m^{-2} (50–70/30 and 150–50/50, respectively) for *L. minor*, while for *W. hyalina*, the range was from 5.01 ± 0.35 g $week^{-1}$ m^{-2} at 50–30/70 to 9.48 ± 0.39 g $week^{-1}$ m^{-2} at 150–50/50. The difference from the lowest to the highest value for *L. minor* was 50%, and for *W. hyalina*, it reached 89%. Higher light intensities resulted in higher relative protein yields. Overall, *W. hyalina* achieved higher RPYs in all treatments compared to *L. minor*. The higher the light intensity, the higher the difference between the species RPYs, meaning that at the highest light intensities (150 µmol m^{-2} s^{-1}), *W. hyalina* yielded more protein compared to *L. minor* than at the two lower light intensities. The treatments 50–70/30 and 50–30/70 were significantly lower compared to all other *L.*

minor treatments, except for 100–30/70. For *W. hyalina*, all treatments with a light intensity of 50 µmol m^{-2} s^{-1} (50–70/30, 50–50/50 and 50–30/70) were significantly lower compared to the other treatments with higher light intensities. No significant differences, in neither of the two duckweed species, were observed between the different spectral distributions.

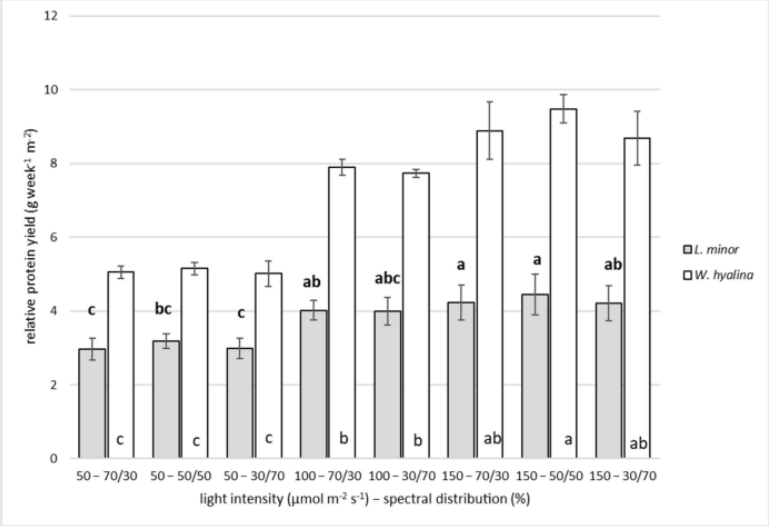

Figure 4. Relative protein yield (RPY; g $week^{-1}$ m^{-2}), based on dry weight, for *Lemna minor* (gray shaded columns) and *Wolffiella hyalina* (white columns). For further explanations, see Figure 3.

3.3. Chlorophyll a

The content of chlorophyll a for both species after seven days of experiments ranged between 5.32 ± 0.51 mg g^{-1} and 7.29 ± 0.39 mg g^{-1} for *L. minor* at 150–50/50 and 50–70/30, respectively (Figure 5). The maximum content for *W. hyalina* was 9.98 ± 1.01 mg g^{-1} chlorophyll a, achieved at 50–30/70, while the minimum content (6.83 ± 0.39 mg g^{-1}) was obtained at 150–30/70. This corresponded to a decrease of 27% for *L. minor* and 32% for *W. hyalina*.

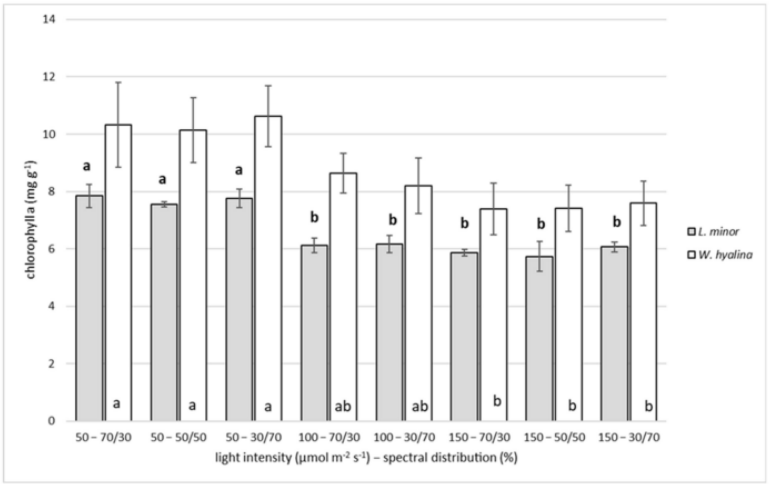

Figure 5. Chlorophyll a content, in mg g^{-1} (based on dry weight), for *Lemna minor* (gray shaded columns) and *Wolffiella hyalina* (white columns). For further explanations, see Figure 3.

A significant decline between the treatments of the lowest light intensity (50 µmol m^{-2} s^{-1}) and the two higher treatments (100 and 150 µmol m^{-2} s^{-1}) can be observed for *L. minor*. For *W. hyalina*, the 150 µmol m^{-2} s^{-1} treatments were significantly lower compared to the 50 µmol m^{-2} s^{-1} treatments. Different light spectra had no significant impact on the chlorophyll a content of both species.

3.4. Nutrients

In Table 2, the percentage reduction in different nutrient components in the nutrient medium after seven days of experiments compared to the initial concentration is presented. A percentage increase (shown as negative values) in certain substances was possible due to the EC-based nutrient dosing of the stock solutions.

Table 2. Percentage reduction in nutrient solution substances for *L. minor* and *W. hyalina*, based on one solution sample taken at the beginning and the end of experiments from the reservoir. Duckweeds were separately cultivated for seven days in a re-circulating, aquatic system. Negative values indicate an increase in the corresponding substance due to EC-based nutrient dosing.

Substance	*L. minor*	*W. hyalina*
NH_4^+-N	97.2	97.7
NO_3^--N	12.8	−6.6
PO_4^{3-}	52.8	26.6
K^+	7.9	−1.4
Mg^{2+}	−7.9	−22.6
SO_4^{2-}	−8.0	−11.7
Ca^+	−8.1	−10.1
Fe^{3+}	95.6	94.5
BO_3^{3-}	84.8	2.2
Mn^{2+}	80.3	98.6
Zn^{2+}	84.2	89.7
Na^+	29.1	23.7

A strong reduction of more than 80% can be seen for ammonium-N, iron, manganese, zinc, and in case of *L. minor*, also for boron. Nitrate-N was only slightly decreased for *L. minor* (12.8%) and showed a minor increase for *W. hyalina*. Similar results were also observed for potassium. An increase in magnesium, sulfur and calcium occurred for both species.

Compared to the start of experiments, the pH showed a minor increase with an average value of 7.8 for the *L. minor* experiments and 7.9 for the *W. hyalina* experiments.

4. Discussion

4.1. Relative Growth Rate

The RGR determined in our study differed between both investigated species and growth conditions. An increase in light intensity from 50 to 150 µmol m^{-2} s^{-1} significantly increased the RGR of *L. minor* and *W. hyalina*. Our data agree with other published investigations. Paolacci et al. [26] reported that increasing light intensities between 6 and 1000 µmol m^{-2} s^{-1} increased the RGR of *L. minor* and *L. minuta* cultivated in sterile growth rooms at 20 °C with a light:dark cycle of 16:8 h. At light intensities below 40 µmol m^{-2} s^{-1}, no significant differences were detected between the RGR of both species, while above 90 µmol m^{-2} s^{-1}, *L. minuta* had significantly higher RGRs than *L. minor*. The latter reached an RGR of 0.26 d^{-1} when grown at 150 µmol m^{-2} s^{-1}. This was higher compared to our result, but cultivation conditions varied, which might provide a possible explanation for this difference.

At comparatively low light intensities between 30 to 105 µmol m^{-2} s^{-1}, *L. aequinoctialis* reached an RGR of 0.19 d^{-1} at the highest light intensity, when cultivated in monoculture, while *L. punctata* and *Spirodela polyrhiza* reached 0.18 d^{-1} and 0.15 d^{-1} under the same growth conditions, respectively [35]. Increasing light intensity and photoperiod increased growth rate, biomass and starch production in *L. aequinoctialis*. Considering the costs for lighting, an optimum regarding those factors was reached at 110 µmol m^{-2} s^{-1} [24]. A sevenfold increase in light intensity (from 100 to 700 µmol m^{-2} s^{-1}) resulted in a 25% greater RGR of *L. gibba* [23]. This increase in RGR was lower compared to *L. minor's* RGR increase of 67% and *W. hyalina*'s increase of 76% at a 200% greater light input in our study.

The maximum obtained RGRs of 0.13 d^{-1} for *L. minor* and 0.21 d^{-1} for *W. hyalina* in the presented study are lower compared to the highest achieved values of 0.42 d^{-1} and 0.52 d^{-1} for the same clones, respectively, grown under sterile conditions in batch cultures [6]. However, under non-axenic conditions, certain cultivation adaptations due to inhibiting factors, such as algae or fungus growth, need to be considered [36,37]. A highly diluted growth medium, comparatively low light intensities and a moderate temperature were applied in our re-circulating IVF for non-axenic duckweed cultivation. Regarding the investigation of Petersen et al. [28], the same nutrient medium with a dilution of 10% resulted in an RGR of 0.21 d^{-1} for *W. hyalina*. This is in exact agreement with the results of the current study.

In contrast, other studies reported that different light intensities had no significant impact on the RGR of duckweed species. The RGR of *Lemna minor* grown on synthetic dairy wastewater did not increase with increasing light intensities between 50 and 850 µmol m^{-2} s^{-1} [38]. *Lemna gibba* reached constant high growth rates under different light intensities between 50 and 1000 µmol m^{-2} s^{-1}; however, higher intensities led to increasing zeaxanthin levels. This way, a large fraction of the absorbed light was dissipated non-photochemically [39].

The light spectra in the presented experiments had no significant impact on any investigated parameters for both species. However, it has to be kept in mind that in this study, pure red or blue light was never used. There was always a white light background of the light intensity of 20%, and the ratios between blue and red light were never higher than 70:30%.

Up to now, only a few investigations concerning this parameter have been carried out regarding duckweed RGR. *Landoltia punctata* cultivated under fluorescent white light, blue LED and white LED at 110 µmol m^{-2} s^{-1} showed no significant RGR differences [40]. There was also no significant difference in the RGR of *S. polyrhiza* when cultivated at 60 µmol m^{-2} s^{-1} using red and blue LEDs (660 and 460 nm, respectively) [41], which is in agreement with our results. Xu et al. [42] described that the application of red and blue light at the same time can be absorbed by plants more efficiently compared to other spectra and resulted in high photosynthetic efficiency. *Spirodela polyrhiza* cultured in eutrophic medium reached a significantly higher total biomass yield when a red:blue ratio of 2:1 or 4:1 at a light intensity of 110 µmol m^{-2} s^{-1} was applied compared to monochromatic (450, 630 or 660 nm) or fluorescent light sources at the same intensities.

4.2. Crude Protein Content and Relative Protein Yield

The presented crude protein contents for both species showed no significant difference between the tested light scenarios. This is in contrast to the results reported by Stewart et al. [39], who showed that the protein content of *L. gibba*, cultivated at 50 and 1000 µmol m^{-2} s^{-1}, increased from 25% to 46%, respectively. A protein content increase from 1.5% to 2% (based on FW) was observed for *L. minor* when cultivated on synthetic dairy wastewater at a light intensity of 850 µmol m^{-2} s^{-1} compared to 50 µmol m^{-2} s^{-1}. In C3 plants, such as duckweed, higher light intensities induce the increased production of Rubisco, a soluble protein [38]. A small increase in light intensity (from 200 to 400 µmol m^{-2} s^{-1}) only slightly increased the percentage of activated Rubisco in *S. polyrhiza* [43]. This could be an explanation for the relatively stable crude protein contents in our study, as the light intensity only slightly increased from 50 to

150 µmol m^{-2} s^{-1}. A more substantial increase in light intensity, as described above, will lead to rising protein contents.

The crude protein contents in the presented experiments were rather high considering the low nutrient concentration and the low light intensities, especially regarding *W. hyalina*. Appenroth et al. [9] reported a crude protein content of 35% for *W. hyalina* and 25% for *L. minor*. These duckweeds were cultivated with a modified Schenk–Hildebrandt medium at 100 µmol m^{-2} s^{-1} continuous white light. In another experiment, the highest values for crude protein of the three species *L. aequinoctialis*, *L. punctata* and *S. polyrhiza* (33.7, 32.3 and 36.8%, respectively), were reached at 105 µmol m^{-2} s^{-1} using a one-tenth strength Hoagland solution [35]. Petersen et al. [28] reached CPCs of 32.4% for *L. minor* and 35.3% for *W. hyalina* using a stationary system with the same nutrient solution as applied in these experiments. Wheeler at al. [44] assumed that a continuous supply of nitrogen caused higher protein levels in different crops (wheat, lettuce, potato and soybean) grown in a re-circulating hydroponic system compared to the same field-grown crops. Such a mechanism might also be responsible for the CPC increase in *W. hyalina*, cultivated in the re-circulating system compared to the stationary system.

A red:blue ratio of 1:2 can increase starch yield significantly, while a higher portion of the red spectrum under eutrophic conditions caused a strong inductive effect on turion formation in *S. polyrhiza* [42]. This is contrary to data reported by Zhong et al. [41], who detected an increased starch accumulation for the same species using red light, while blue light promoted protein accumulation. In *W. arrhiza*, using irradiation with wavelengths corresponding to white, red and blue light, no significant differences in amino acid concentrations of the soluble protein were detected [45]. These results fit to our findings that the spectral distribution as applied did not significantly influence CPC.

The protein productivity, given as RPY, was lower for *L. minor* compared to *W. hyalina*. The species *L. minor* reached a maximum of 4.44 ± 0.55 g $week^{-1}$ m^{-2} at 150–50/50 and *W. hyalina* of 9.48 ± 0.39 g $week^{-1}$ m^{-2} for the same treatment. This extrapolates to 2.31 and 4.93 t of pure protein per year and hectare, respectively. In the literature, a wide range of productivities are reported. For *L. minor* and *W. hyalina*, 28.8 and 34.7 g $week^{-1}$ m^{-2}, respectively, were reached using the same nutrient solution in a stationary system with smaller vessels [28]. Mohedano et al. [46] reported a protein productivity of 24 t $year^{-1}$ ha^{-1} (ca. 46 g $week^{-1}$ m^{-2}) for duckweeds. Chakrabarti et al. [47] reached a biomass yield of 703 kg $month^{-1}$ ha^{-1} (ca. 17.5 g $week^{-1}$ m^{-2}) for *L. minor*. Regarding protein content of 27.1% for duckweed grown on an inorganic fertilizer-based solution, the protein productivity resulted in 4.74 g $week^{-1}$ m^{-2}. Comparing these values to soybean with a yield of ca. 3 t $year^{-1}$ ha^{-1} and a protein content of 40% [48], the protein productivity of 1.2 t $year^{-1}$ ha^{-1} was considerably lower compared to any duckweed protein productivity projection.

4.3. Chlorophyll

The chlorophyll a content for both species was investigated as a parameter to indicate a possible color changes in the plants at different light conditions. It decreased with increasing light intensity. This negative correlation was also found for other duckweed species [23,26,38,39,49]. *L. minor* had higher total chlorophyll contents for all investigated light intensities (6 to 1000 µmol m^{-2} s^{-1}) than *L. minuta*, reaching up to ca. 1.4 mg g^{-1} of fresh biomass at the lowest light intensity [26]. *Lemna gibba* contained ca. 250 µmol m^{-2} of chlorophyll a and b at 50 µmol m^{-2} s^{-1} and ca. 300 µmol m^{-2} at 100 µmol m^{-2} s^{-1} [23,39]. The reduction in chlorophyll at high light intensities is an acclimation strategy, protecting the plant against light-induced damage due to photo oxidation [50]. Contrarily, high chlorophyll contents at low light intensities ensure maximal light absorption. Such plants are usually associated with shade tolerance [26].

The different investigated spectral distributions had no significant impact on both species' chlorophyll content. This has also been shown by Zhong et al. [41], who obtained no significant differences in *S. polyrhiza*, when cultivated under red, blue and white light. This missing effects of the light quality in our experiment might be also caused by the use of mixed light quality.

5. Conclusions

The duckweed cultivation system applied in our experiments was a small-scale, experimental prototype of a re-circulating, aquatic IVF and specifically designed and built for conducting scientific experiments. In the literature, only a theoretical approach [13], but no practical application of an IVF for duckweed cultivation has been described, neither on a small scale for experiments nor on a large scale for biomass production. This small-scale, re-circulating IVF for scientific experiments fits the criteria for a plant factory with artificial lighting regarding structure, functionality and operation goals in most aspects [16]. The results of the present study underline the idea that the cultivation of duckweeds in such a system under non-sterile conditions is feasible and might be up-scaled for mass production.

The applied system for nutrient control and dosing is based on EC values. When the actual EC values fell below the target EC, the dosing system pumped stock solution into the reservoir until the target value was reached again. This is a well-established system for nutrient dosing used in many different hydroponic applications [16,51]. However, when used in re-circulating systems, the disadvantages become obvious. An imbalance between nutrient composition of the stock solutions and actual nutrient uptake by the plants can cause increasing concentrations of certain substances in re-circulating systems, as happened in our experiments. The longer a re-circulating system runs, the greater the imbalances will become. A depletion of nutrients, such as ammonium, nitrate, sodium or magnesium, can cause reduced RGR, CPC or RPY in duckweed due to non-optimal nutrient ratios [28,52]. In the case of nitrogen, duckweeds preferentially take up ammonium over nitrate [53]. An adaptation of the stock solutions to the actual plants' demands is difficult due to plant physiological and technical reasons. Many crops have changing demands at different plant development stages. Additionally, the dosing pumps must work precisely, when dosing more than one stock solution, to keep the nutrient ratio at a given target level. The use of stationary, on-line, ion-selective sensors [54], ion-sensitive field-effect transistors [55] or mid-infrared sensors [56] might be options to solve the problem in the future, but to date, not all relevant nutrients for plant growth can be measured. Relevant aspects regarding the application in hydroponics are the frequency and complexity of sensor calibrations, lifespan and costs as well as the stability, selectivity and drift of these technologies [54,55,57]. The readiness levels of these technologies currently vary, but new components and membranes will improve the coming product generations [55].

To gain more data about the behavioral pattern of duckweed in re-circulating systems, longer-lasting experiments investigating a broad range of abiotic, and in the case of non-sterile experiments, also biotic, parameters are needed. Nonetheless, the findings and experiences of our study were already successfully implemented into the operation of a large scale, re-circulating, aquatic IVF for duckweed biomass cultivation (Figure 6).

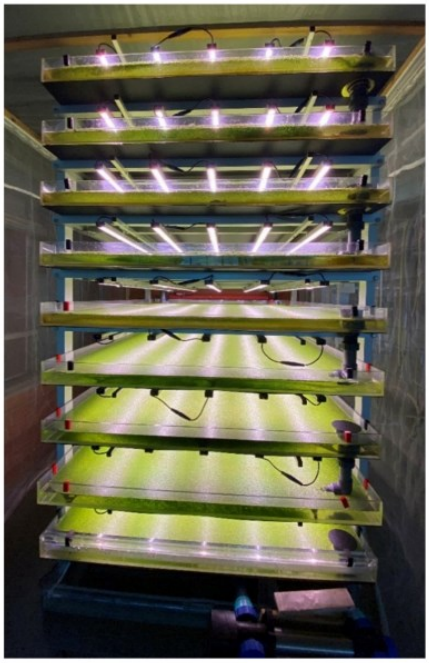

Figure 6. Large scale, re-circulating indoor vertical farm (IVF) or duckweed biomass cultivation at the University of Applied Sciences Osnabrück, Germany.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11081010/s1, Table S1. Formulation of seven stock solutions (g L^{-1}) for five different nitrate-N to ammonium-N ratios.

Author Contributions: Conceptualization, A.U., J.D., D.R. and F.P.; methodology, A.U., J.D., D.R. and F.P.; validation, H.-W.O.; investigation, J.D., D.R. and F.P.; data curation, D.R. and F.P.; writing—original draft preparation, F.P.; writing—review and editing F.P., J.D., K.-J.A. and H.-W.O.; visualization, F.P.; supervision, A.U., K.-J.A. and H.W.; project administration, H.W.; funding acquisition, J.D., A.U., H.-W.O. and H.W. All authors have read and agreed to the published version of the manuscript.

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3.3. Manuscript III

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Re-circulating indoor vertical farm: Technicalities of an automated duckweed biomass production system and protein feed product quality evaluation

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ABSTRACT

Duckweeds are fast-growing and nutritious plants, which are gaining increased attention in different fields of application. Especially for animal nutrition, alternative protein sources are needed to substitute soybean meal. The current bottleneck is the standardized production of biomass, which yields stable quantities of a defined product quality. To solve this problem, an indoor vertical farm (IVF) for duckweed biomass production was developed. It consists of nine vertically stacked basins with a total production area of 25.5 m^2. The nutrient solution, a modified N-medium, re-circulated within the IVF with a maximum flow rate of 10 L min^{-1}. Nutrients were automatically added based on electrical conductivity. In contrast, ammonium was continuously supplied. A water temperature of 23 °C and a light intensity of 105 µmol m^{-2} s^{-1} with a photoperiod of 12:12 h were applied. During a 40-day production phase, a total of 35.6 kg of fresh duckweed biomass (equals 2.1 kg of dried product) was harvested from the IVF. On average, 0.9 kg day^{-1} of fresh biomass was produced. The dried product contained 32% crude protein (CP) and high levels of proteinogenic amino acids (e.g. lysine: 5.42 g, threonine: 3.85 g and leucine: 7.59 g/100 g CP). Biomass of this quality could be used as a protein feed alternative to soybean meal. The described IVF represents a modular model system for duckweed biomass production in a controlled environment and further innovations and upscaling processes.

1. Introduction

Soybean is one of the globally most important sources of protein (Jia et al., 2020). The main production areas are located in South and North America (Tallentire et al., 2018). However, soy production is related to deforestation (Henchion et al., 2017), environmental issues (de Visser et al., 2014) and transportation issues in order to meet the global demand (He et al., 2019). The increasing demand for animal protein drives soy production and therefore the environmental issues related to soy production (Henchion et al., 2017). In the EU in particular, soybean meal accounts for 48% of the protein-rich feed with a protein content above 30% crude protein (CP) (European Commission, 2021b). This leads to a protein deficit and dependence, especially in the feeding of monogastric animals (de Visser et al., 2014). However, the growth potential for common agricultural protein crops is limited, as the European Commission (2021a) expects a further decreasing availability of land for agricultural production.

In order to reduce these issues related to soy production, novel protein sources and efficient land-use strategies for crop production have to be considered. Duckweeds are gaining increasing attention in research and application due to their high growth rates and high protein content (Acosta et al., 2021). Therefore, duckweed must be taken into account as an alternative protein source. They are small floating freshwater plants, belonging to the family of *Lemnaceae* Martinov (1820). To

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date, 36 species are known (Bog et al., 2019). Duckweeds occur worldwide, except for the polar regions and other areas with extreme climatic conditions, such as deserts (Landolt, 1986). They are the fastest-growing angiosperms in the world (Ziegler et al., 2015). The species *Wolffiella hyalina* (clone 9525) reached relative growth rates (RGR) of up to 0.512 d^{-1} and can double its biomass every 32.2 h (Ziegler et al., 2015), while *Wolffia microscopica* (clone 2005) can double its biomass every 29.3 h (RGR: 0.559 d^{-1}) under axenic in vitro conditions (Sree et al., 2015).

Duckweed biomass is highly nutritious (Acosta et al., 2021), with protein contents of up to 45%, based on dry matter (DM) (Xu et al., 2021). Duckweed protein contains a high ratio of essential amino acids, making its protein composition valuable for animal nutrition (Chakrabarti et al., 2018) and human consumption (Appenroth et al., 2017). Several experiments were conducted with livestock to investigate the effects of duckweed biomass in the total diet. Broiler chickens reacted with increased growth to a fraction of up to 6% *Lemna minor* in complete feed as a substitute for sesame oil cake (Ahammad et al., 2003). Other trials showed that rates of up to 8% duckweed in all ages of chickens (Kabir et al., 2005) and up to 10% in finisher diets (Kusina et al., 1999) are possible. Also, a partial replacement of soybean meal by duckweed for feeding piglets and growing pigs has been realized (Moss, 1999). For laying hens, *Lemna gibba* could replace up to 10% soy bean or approximately 50% fishmeal without adverse effects on egg quality or laying performance (Zakaria and Shammout, 2018). A Leghorn hens diet, containing 25% *Lemna gibba*, resulted in a higher egg protein content and significantly increased yolk pigmentation compared to the control, which contained soybean meal and fishmeal as protein sources (Haustein et al., 1990). Increased egg yolk colour has also been confirmed by Anderson et al. (2011). Moreover, the nutritional value of duckweed has been shown for feeding ducks (Khanum et al., 2005), fish (Asimi et al., 2018), cattle (Huque et al., 1996) and sheep (Zetina-Córdoba et al., 2012). Generally, trial results varied based on the quality of the used duckweed and its composition.

Consequently, product quality has to be optimized and controlled to ensure appropriate and efficient animal nutrition. In duckweeds, growth rates and the nutritional composition can be influenced by cultivation conditions, such as light and temperature (Cui et al., 2011) as well as the composition of the nutrient medium (Petersen et al., 2021).

A novel strategy for duckweed cultivation, which has not been described in detail to date, can be the use of an indoor vertical farm (IVF). Generally, this strategy consists of several horizontal cultivation levels stacked above each other, usually operated with artificial lighting (Kozai et al., 2020). This way, the land utilization efficiency, meaning the cultivation area per ground area, can be increased and thus an extensive production with less available land is feasible (Coughlan et al., 2022). Soilless cultivation methods, e.g. hydroponics, can be integrated into IVFs and are already used for the production of different crops, such as tomatoes, cucumbers, peppers, strawberries as well as lettuce and other leafy greens (Sharma et al., 2018). Hydroponic IVFs operated in a controlled environment have the advantage that the grower can set all abiotic factors according to the plant demand for most efficient growth and nutrient accumulation. This includes, amongst others, nutrient concentration and composition, light source and settings, temperature and CO_2 levels (Benke and Tomkins, 2017). It allows for year-round production, independent of weather conditions and location. Standardizing the cultivation process and all relevant abiotic parameters aims at maximizing and stabilizing yields with a defined and high nutritive value at the same time. By re-circulating the nutrient solution in the hydroponic system (also known as "closed system"), the water and nutrient input can be reduced compared to "open systems". The water and fertilizer use efficiency for the cultivation of tomatoes was 22.7% higher for "closed systems" compared to "open systems" in both cases (de la Rosa-Rodríguez et al., 2020). Compared to conventional agriculture, up to 90% of irrigation water and 85% of fertilizers can be saved in a closed hydroponic system, while a productivity increase of up to 250% is possible (AlShrouf, 2017). Nutrient leaching into the environment can be largely avoided by using this cultivation method (Keuter et al., 2021).

For duckweed, IVFs have been described theoretically (Coughlan et al., 2022; Roman and Brennan, 2021), as a greenhouse-based continuous flow plant (Fujita et al., 1999) and as a small scale version on an experimental level (Petersen et al., 2022). A vertical farming system for duckweed cultivation has been described by Everett et al. (2012), while the Israel-based company Green-Onyx has a patented vertical farming module for *Wolffia* production. In all cases detailed information about the system, operational parameters as well as yielded biomass quantity and quality are scarce.

Our team at the University of Applied Sciences Osnabrück, Germany developed a large-scale IVF, designed for duckweed biomass production. This is the first detailed report on an IVF system for duckweed cultivation. Aim of this research is the description of the construction, technicalities and operation of this automated and re-circulating IVF for the mass production of protein-rich *Lemna* biomass. The yielded qualities were evaluated for a possible use as a protein feedstuff.

2. Materials and methods

2.1. Experimental setting of the re-circulating indoor vertical farm during production

The IVF was operated for 40 consecutive days in order to produce protein-rich *L. minor* (clone 9441, Germany) biomass. The basins of the IVF were filled with local tap water (see Table S1) to a height of 5 cm and the reservoir to 25 cm, this resulted in a total volume of ca. 2000 L. A light intensity of 105 μmol m^{-2} s^{-1} and a photoperiod of 12:12 h light: dark cycle was used. The water temperature was set to 23 °C. The flow rate of the re-circulating water within the IVF was 10 L min^{-1}, which was treated with UV-C light to reduce the growth of unwanted ubiquitous organisms.

As nutrient solution, a modified N-medium with a NO_3^--N to NH_4^+-N ratio of 75%–25% was applied, which resulted in the highest RGR and protein yield of *L. minor* and *W. hyalina* (Petersen et al., 2021). The stock solutions were mainly prepared with commercially available fertilizers in order to reduce the operation cost. The EC value was set to 0.7 mS cm^{-1} to reach the target concentrations given in Table 1. No pesticides were applied throughout the whole production phase.

2.2. Sampling and analysis

Nutrient medium samples were taken at 5 time points during the production phase, while the ammonium concentration was measured at 14 time points. Ammonium and nitrate concentrations in nutrient media samples were determined with Reflectoquant® Ammonium and Reflectoquant® Nitrate Tests (0.2–7.0 mg l^{-1} NH_4^+, 5–225 mg l^{-1} NO_3^-; Merck KGaA, Darmstadt, Germany) and a RQflex®20 (Merck KGaA).

Table 1
Target nutrient concentrations (mmol l^{-1} and mg l^{-1}) in the modified N-medium.

Substance	Concentration (mmol l^{-1})	Concentration (mg l^{-1})
NO_3^--N	0.87	12.2
NH_4^+-N	0.25	3.5
PO_4^{3-}	0.1	9.5
K^+	0.98	38.4
Mg^{2+}	0.41	9.9
SO_4^{2-}	1.23	117.7
Ca^{2+}	1.34	53.5
Fe^{3+}	0.0028	0.15
B^{3+}	0.0024	0.025
Mn^{2+}	0.0013	0.07
Na^+	0.76	17.4
Zn^{2+}	0.0095	0.62
Cu^{2+}	0.0014	0.09

Other nutrients were analysed according to DIN EN ISO 11885:2009-09 (2009) with an ICP-OES (ICAP 7400, Thermo Fischer Scientific, Waltham, USA). Temperature, pH- and EC-values were logged via Pro Controller connect (Bluelab Corporation Ltd, Tauranga, New Zealand). Light intensities were measured for control with a Light Meter LI-250A (LI-COR Biosciences, Lincoln, USA).

Biomass harvesting was done six times during the 40-day production phase. The fresh biomass was subsequently oven dried at 65 °C for 72 h and stored for further use in animal feeding trials. The CP content of the dried biomass was determined using the Dumas method according to ISO 16634-1:2008-11 (2008). Acid detergent fibre and acid detergent lignin were analysed in accordance with DIN EN ISO 13906:2008-2011 (2008) and neutral detergent fibre was analysed according to ISO 16472:2006-04 (2006). The following substances were analysed by methods described by Commission Regulation (EC) No 152/2009, annex III: Tryptophan, method G; all other amino acids, method F; residual moisture and dry matter, method A; crude fibre, method I; crude fat, method H procedure B; crude ash, method M.

2.3. Calculations

In order to evaluate the protein quality of the dried biomass, the essential amino acid index (eAAI) and the amino acid ratio (AAR) were used. eAAI was calculated with an equation described by Oser and Albanese, 1959:

$$eAAI = \sqrt[n]{\frac{aa1}{AA1} * \frac{aa2}{AA2} * \ldots * \frac{aan}{AAn}} \quad (1)$$

whereas aa1, aa2, …aan are the amino acid contents in the CP of the tested sample and AA1, AA2, …AAn are the respective demands of broiler chickens (National Research Council, 1994; age of 3–5 weeks) or piglets (National Research Council, 1998; 10–20 kg body weight). The concept of ideal protein is widely used to assess the nutritive value of proteins (Santamaría-Fernández and Lübeck, 2020). For the calculations of amino acid ratios (AAR), amino acids concentrations are set in relation to the lysine content (Pastor, 2014):

$$AAR_{aa}(\%) = \frac{aa}{LYS} * 100 \quad (2)$$

whereas aa is the individual amino acid content and LYS is the lysine content in the CP of the tested sample. This ratio was calculated for every individual amino acid. This way, the amino acid ratio for feedstuffs was compared with the ideal amino acid ratio (IAAR) for broiler chickens (National Research Council, 1994) and piglets (National Research Council, 1998), matching the requirement of the respective species.

3. Results

3.1. Re-circulating indoor vertical farm

The presented IVF consists of nine rectangular production basins, positioned vertically above each other, and a reservoir at the bottom (Fig. 1A). Each basin is made of acrylic glass with a length of 195 cm, a width of 145 cm and in case of the production basins a height of 10 cm, while the reservoir has a height of 40 cm high. All production basins together amount to a total cultivation area of ca. 25.5 m^2. Acrylic glass was chosen because it is stable, break-proof, transparent and lighter than glass. All basins rest in an aluminium framework. Each basin rests on two permanently installed horizontal aluminium bars in the framework, the space between the basins is 16.7 cm. The aluminium bars are hollow to reduce the weight of the framework.

Each basin, except for the reservoir, has an outlet positioned in one

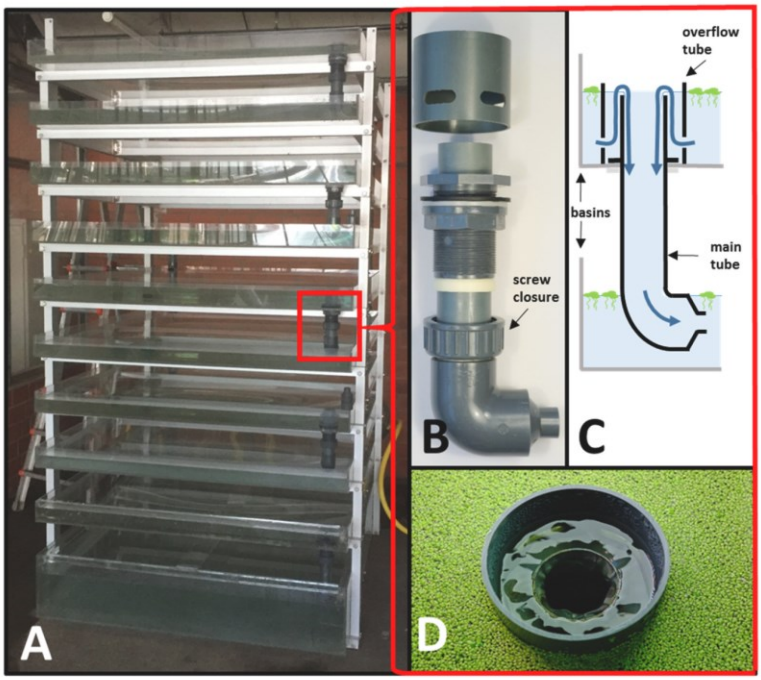

Fig. 1. Basic structure of the indoor vertical farm (IVF), consisting of the aluminium framework, nine acrylic glass production basins and one acrylic glass reservoir at the bottom (A). Close-up of the outlet, connecting the upper basin to the one below (B) and a schematic figure indicating the flow of water (C). Top view on the upper part of the outlet in an operational state of the IVF (D).

corner of the ground plate (Fig. 1B). These outlets consist of a main tube, at their bottom a 90° elbow-piece is connected. Inside rests a removable, conical reducing socket. This way, the speed of the outflow can be increased. A screw closure is implemented. In an open position, it is possible to move the main tube up and down according to the requirements. If closed, the tube rests firmly in its position. Over the top of the main tube, an overflow tube with a wider diameter is placed. This tube section has three oval shaped holes. By moving the main tube up or down, the height of the nutrient solution can be individually adjusted in each basin. If the main tube is positioned higher than the holes, nutrient solution will flow into the basin below while the floating duckweed is hindered to pass the barrier (Fig. 1C and D). This way, nutrient solution circulation throughout the whole system is achieved. The outlets are positioned on opposite sides of the basins, in order to create a circulatory nutrient solution flow within each basin.

A harvesting system is integrated in the IVF. Each basin has an outlet positioned at the shorter side wall. It consists of a T-piece. A manual gate valve is located in this structure. All T-pieces are connected to each other (Fig. 2) and this way form the complete harvesting system. All used tubes and pieces are made of polyethylene. When the gate valve is opened, the duckweed flows into the harvesting system together with the supernatant nutrient solution. At the bottom of the harvesting system, the duckweed is collected in a net. The nutrient solution flows into a container and can be pumped back into the IVF. The above-described IVF structure was completely provided by AquaLight GmbH (Bramsche, Germany).

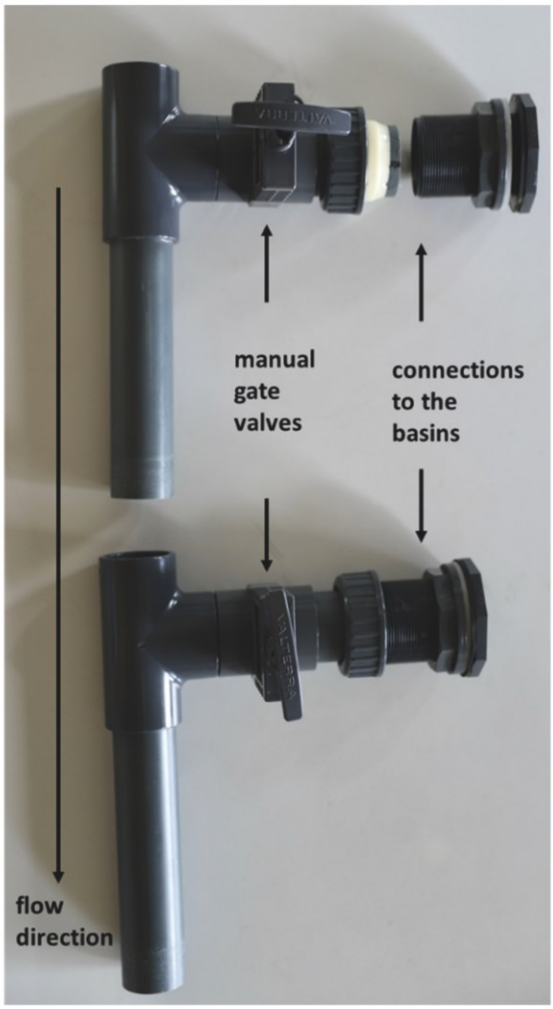

Fig. 2. Structure of two elements belonging to the harvesting system of the IVF. Such a structure is connected to each of the nine basins. By opening the manual gate valves, duckweed and supernatant nutrient solution flow out of the basins.

The described IVF is specially designed for the cultivation of duckweed. All relevant abiotic growth factors can be adjusted and partially controlled. A flow chart shows the structure and function of this IVF (Fig. 3). Local tap water and demineralized water are fed to the reservoir through flexible hoses. For both water sources, a pressure reducer valve is installed as well as an L-type ball valve to manually select between the water supply. When the IVF is firstly filled, tap water is used, while evaporation losses during the operation are replenished by demineralized water. On the inside wall of the reservoir, a mechanical float valve automatically regulates the water influx and compensates for evapotranspiration losses. By this measure, an uncontrolled water influx is prevented and the water level cannot rise above the top edge of the reservoir. The water circulation flow within the IVF is created by a submergible and adjustable feed pump (AquaForte DM-10000 Vario, SIBO BV, Veghel, The Netherlands) placed at the bottom of the reservoir. The water is pumped through a flexible hose into a UV-C clarifier (OSAGA UVC-55, Fischfarm Otto Schierhölter, Glandorf, Germany) to eliminate spores of ubiquitous algae and fungus as well as bacteria. In addition, a water smart flow meter (Gardena Deutschland GmbH, Ulm, Germany) to measure the flow rate and a gate valve to manually adjust the flow rate are installed. The water is flowing through a screen filter to avoid particles reaching the top basin. In order to reduce clogging of the filter, it is backwashed at frequent time intervals, regulated by a time switch. Magnetic valves redirect the flow direction, backwards through the screen filter for a few seconds and then into the drain. A drain valve ensures complete water outflow of the drain hose. Finally, a one-way check valve right before the top basin inlet avoids a gravity-driven backflow of water and duckweed into the UV-C clarifier when the feed pump is turned off.

A gravity-driven flow from basin to basin is realized through the outlets installed in each basin. Before the nutrient solution reaches the reservoir, it passes a second UV-C clarifier. The reservoir contains an overflow, which is connected to the drain in order to prevent flooding of the IVF. Another technical device to ensure the safe operation of the IVF devices at all times is the use of a water level sensor (WPS 3000 plus, H-TRONIC, GmbH, Hirschau, Germany). When the water level reaches the lower sensor, the pumps, heating system and UV-C clarifiers will be automatically shut down to avoid damage by overheating or running dry. When the water level reaches the upper sensor, the same technical devices are automatically turned on again.

Liquid fertilizers from stock solutions are automatically added to the tap water in the IVF by an EC- based nutrient control and dosing system (Pro Controller connect and PeriPods, Bluelab Corporation Ltd, Tauranga, New Zealand). The Bluelab Pro Controller connect regularly measured and logged EC, pH and temperature data. The corresponding probes are measuring in the reservoir. As four dosing pumps were available, the six corresponding stock solutions were combined according to the following scheme: A - stock solution 1 & 4 (Ca^{2+}, Cl^{-}, K^{+}, NH_4^{+}, NO^{3-}); B - stock solution 3 & 5 (K^{+}, NH_4^{+}, PO_4^{3-}, SO_4^{2-}); C - stock solution 6 & 7 (Fe^{3+}, Mg^{2+}, SO_4^{2-}, trace elements); D - stock solution 3 (NH_4^{+}, SO_4^{2-}). The composition of the different stock solutions is given in Table S2. Dosing pumps A, B and C were used for an EC-based nutrient dosing into the reservoir to obtain the composition of the modified N-medium. Dosing occurred for 12 s when the EC-value was below 0.7 mS cm^{-1}. In order to avoid overconcentration, a lag-phase of 15 min for dosing of stock solutions A, B and C ensured enough time for a homogenous nutrient distribution within the IVF. Dosing pump D added

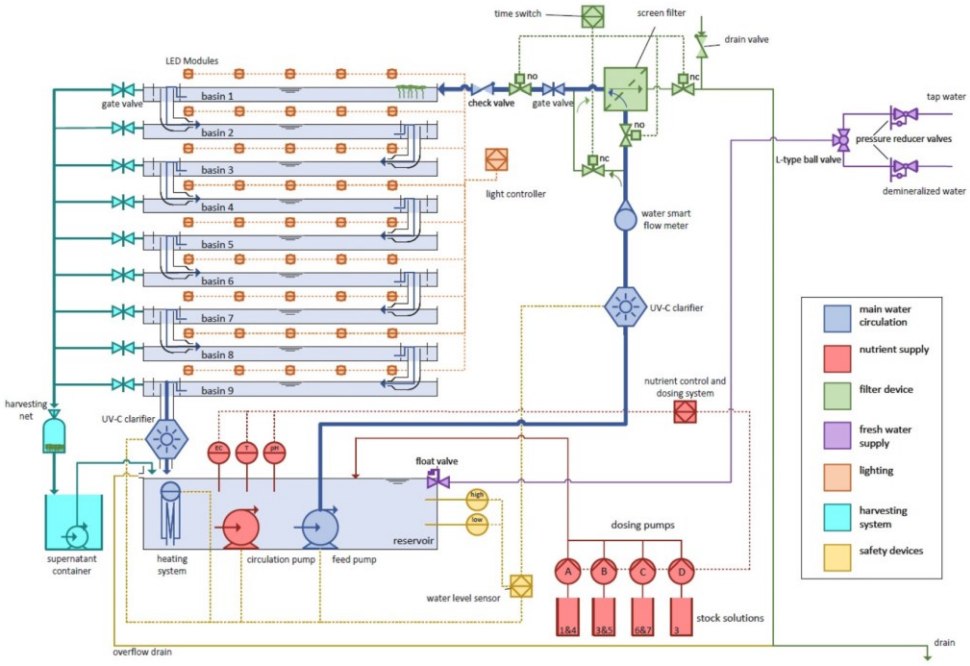

Fig. 3. Flow chart of the IVF. The continuous lines depict mass flow, while bold lines illustrate re-circulation in the IVF. Dotted lines indicate electricity or data flow.

stock solution 3 to the nutrient solution for 3 s every 40 min, independent of the actual EC-value. This continuous ammonium dosing was done to keep the NH_4^+-N concentration at a stable level.

The water temperature is adjusted and held constant by a heating system (Super Fish Smart Heater 500 W, Aquadistri BV, Klundert, The Netherlands) installed at the bottom of the reservoir. Due to the constant circulation of the water in the IVF, nutrients are equally distributed and a constant water temperature is expected throughout the whole system. A second submergible pump is placed at the bottom of the reservoir, which creates a continuous flow of the nutrient solution in the reservoir, in order to reach a fast homogenization of the added nutrients and to impede the adhesion of unwanted organisms to the reservoirs ground plate and walls. As an artificial light source, five LEDs (FLEX PRO 12 S4, SANLight GmbH, Bludenz, Austria) with a length of 1731 mm were installed 12.5 cm above the water surface in each basin. The LEDs are adjustable regarding their light intensity (0–250 μmol m^{-2} s^{-1}) and the daily operation duration (0–24 h). To adjust these parameters, a light controller with a Berryvine Farmee Client (Experior Micro Tech GmbH, Munich, Germany) is used. The LEDs are automatically turned on and off by this light controller on a daily basis. Five LED bars per layer are necessary to create an even illumination of the whole basin.

The whole IVF (Fig. 4) was housed in a mosquito net to reduce the risk of an infestation with insect-transmitted pathogens, as in the summer of 2020 an infection of the duckweed with the fungus *Pythium myrothulium* occurred, a soil-borne pathogen (Brand et al., 2021). It is assumed that insects acted as vectors and transferred the pathogen into the aquatic system. Another possible path of infection is through working staff. However, because the path of the infection could not be reconstructed clearly, the mosquito net was installed as a countermeasure. This way, insects are hindered to reach the IVF. Additionally, a disinfection procedure was obligatory for all people working at the IVF. During the infection, it was recognised that the fungal spots primarily occurred in the corners of the basins. In order to reduce dead zones

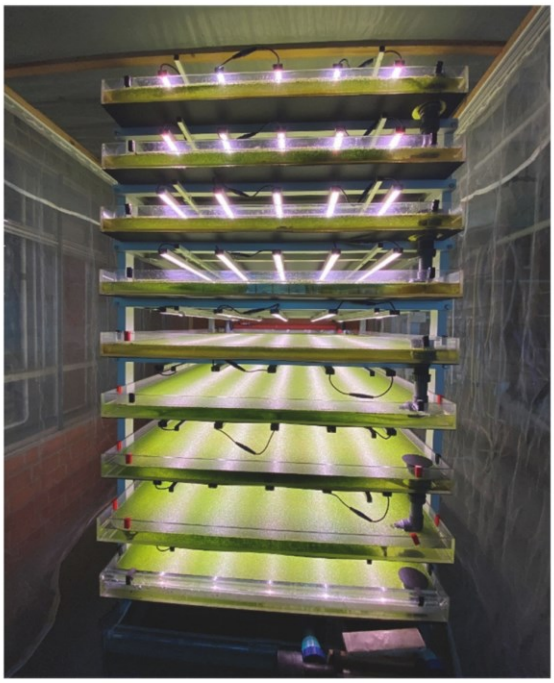

Fig. 4. Fully planted indoor vertical farm for duckweed production in operation.

regarding water and duckweed movement, the edges were rounded by installing plastic shields. This way, the nutrient solution can circulate well within each basin. After the mosquito net was installed, no infection occurred anymore.

3.2. Nutrient solution

The cumulative volume of the EC-based stock solution dosing during the 40-day production phase was 326 ml for stock solutions 1 & 4, 336 ml for stock solutions 3 & 5 and 328 ml for stock solutions 6 & 7. For the continuous ammonium dosing, a total of 722 ml of stock solution 3 was added to the IVF. The average pH during the production phase was 6.1 ± 1.1. Average measured concentrations of nutrients in the solution as well as the variation coefficient are presented in Table 2.

The concentrations of the individual nutrients were not completely constant during the production phase. The variation coefficients differed, depending on the substance. A variation coefficient of 3% for calcium and 4% for sulphate was calculated, indicating a stable nutrient concentration. In contrast, high variation coefficients of 61% for ammonium-N, 86% for zinc and 94% for manganese indicate severe fluctuations in nutrient concentrations during the production phase. On average, nitrate-N and ammonium-N were present in the nutrient solution in a ratio of 4.3:1, while the calcium to magnesium ratio was 5.5:1.

3.3. Biomass yield and quality

Over the whole 40-day production phase a total of 35.6 kg duckweed biomass (FW) was harvested from the IVF with a yield of 6 ± 1 kg FW per harvest and a harvest interval of 6.7 ± 1.4 days. On average, 0.9 ± 0.15 kg day^{-1} (FW) were yielded. One kg of fresh biomass resulted in 59 ± 2 g of dried product after oven drying. The corresponding yield for the dried product in the whole IVF was 2.1 kg in total or 53 ± 10 g day^{-1} on average. These results would extrapolate to ca. 6.3 kg of FW or 370 g dried product per week. The total dried biomass of the duckweed production in the IVF had a residual moisture of 7.8% and a protein content of 32% in DM. The biomass composition is shown in Table 3.

The CP consisted mainly of Aspartic and Glutamic acid with contents of 11.9 and 10.5 g/100 g CP, respectively, but also essential amino acids such as lysine (5.42 g/100 g CP), threonine (3.85 g/100 g CP) and leucine (7.59 g/100 g CP) are present. Proteinogenic amino acids account for 88.1% of the crude protein. The complete amino acid profile of the harvested *L. minor* biomass is shown in Fig. 5.

Carbohydrates were analysed using both the usual Weender analysis with a separation into crude fibre and N-free extracts and the more detailed detergent analysis. Crude fibre accounts for 24.9% of carbohydrates. However, considering the neutral detergent fibre (NDF), fibrous compounds are contained more extensively at a level of 66.2%. Sugar content was below the detection limit of the method (<1%) and the starch concentration was 1.08% in DM. The complete carbohydrate composition is shown in Fig. 6.

Table 2
Nutrient solution concentrations (mg l^{-1}) and variation coefficients (%) for a duckweed production phase of 40 days in the re-circulating indoor vertical farm. Number of measurements n = 5, except for ammonium (n = 14).

Substance	average ± standard deviation (mg l^{-1})	variation coefficient (%)
NO_3^--N	11.5 ± 2.3	20
NH_4^+-N	2.7 ± 1.7	61
PO_4^{3-}	6.2 ± 2.3	37
K^+	18.2 ± 5.8	32
Mg^{2+}	11 ± 1.3	12
SO_4^{2-}	160.8 ± 6.4	04
Ca^{2+}	61 ± 1.6	03
Fe^{3+}	0.008 ± 0.003	45
B^{3+}	0.01 ± 0.007	68
Mn^{2+}	0.12 ± 0.11	94
Na^+	19.1 ± 1.5	08
Zn^{2+}	0.09 ± 0.08	86
Cu^{2+}	0.013 ± 0.009	69

Table 3
Average dry matter composition of the dried *Lemna* biomass (n = 2, except where stated).

Analytical substance	Concentration in dry matter
Crude protein	32.0 ± 0.7
Crude fat (n = 1)	4.8
Crude fibre	10.7 ± 0.1
Crude ash (n = 1)	20.3

4. Discussion

4.1. Re-circulating indoor vertical farm

Cultivation in IVFs is already established for certain agricultural crops. Depending on the plant cultivated, the IVF structure has to be adapted to the crop requirements. Plant morphology and abiotic requirements have to be considered during the design and construction process of an IVF. Today's cultivation systems are adapted to plants with roots, which are traditionally grown in soil, such as tomatoes, peppers or leafy greens. Nutrient film technique (NFT), ebb and flow, drip and aeroponic are typical hydroponic production systems (Sharma et al., 2018). For duckweed, these systems are not applicable, because its morphology as an aquatic plant is not comparable to the above-mentioned crops. Duckweeds usually require a water body to float on, which can be most likely compared to a deep water culture. In order to keep the water and nutrient input to a minimum, it was decided to re-circulate the nutrient solution within the IVF instead of a batch production. To create a space efficient production a vertical structure with nine basins was built. Per square meter of ground floor 9 m^2 of production area are available. Coughlan et al. (2022) described 15 m^2 per square meter of ground floor in a theoretical approach under different constructional conditions. In general, many features and aspects of the presented IVF were also described by Coughlan et al. (2022), but our construction is the first practical IVF application for duckweed biomass production.

An IVF for duckweed production, intended for pharmaceutical use, is described by Everett et al. (2012). The former company Biolex Therapeutics Inc. (USA) chose the approach of a sterile production process using single-use seed bags, production bags and harvest bags in an IVF consisting of 8 vertical shelves. Lighting, ambient air supply, media composition and temperature were considered, but no process parameters or settings were mentioned. As the presented study aimed to produce duckweed biomass for feed purposes, a sterile production process, especially regarding the size of the IVF, seems unfeasible. Therefore, the way of duckweed production in our IVF can hardly be compared to the approach described by Everett et al. (2012).

4.2. Operation and nutrient management

A light intensity of approximately 100 μmol m^{-2} s^{-1} was chosen because it yielded good RGR results for *L. minor* (Petersen et al., 2022) and is close to the recommended optimum regarding energy input and biomass output (Yin et al., 2015). An increase in photoperiod can increase duckweed growth rate and biomass yield (Yin et al., 2015), but the growth of unwanted biotic parameters, such as ubiquitous algae and biofilm formation, has to be considered in such a non-sterile production system. The uncontrolled growth of algae can result in reduced duckweed growth (Roijackers et al., 2004). As countermeasures, the target temperature, flow rate, light intensity, photoperiod and nutrient concentration were adapted based on prior experiences (Brand et al., 2021; Petersen et al., 2021, 2022).

Nutrient management is critical to successfully cultivate duckweed

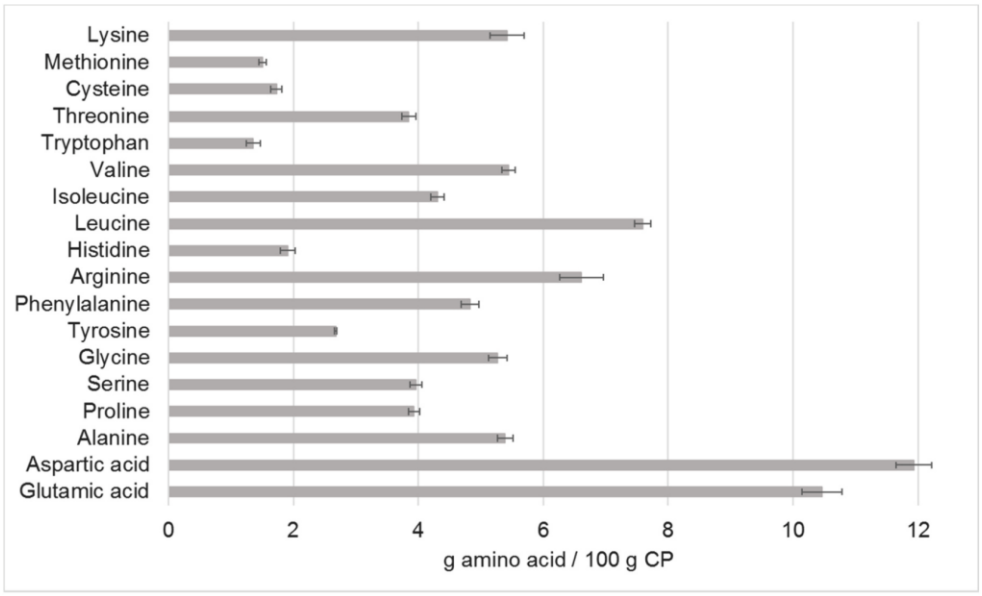

Fig. 5. Average amino acid composition of the crude protein in the total harvested *L. minor* biomass (n = 2). Error bars indicate standard deviation.

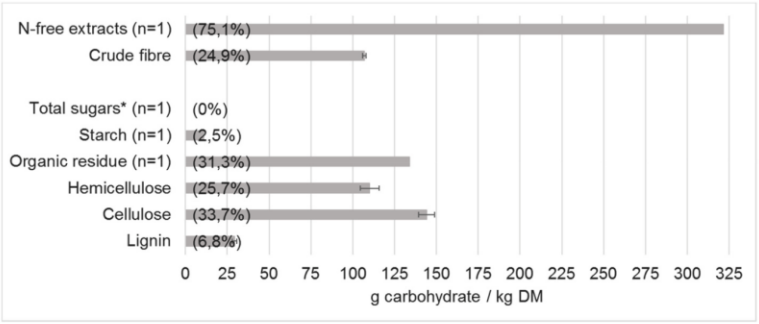

Fig. 6. Content of carbohydrates in the dry matter (n = 2, except where stated). Values in brackets indicated the share of individual compounds or fractions of total carbohydrates. Error bars indicate standard deviation.

biomass over a long time period. Different nutrient media are described and optimized for duckweed cultivation (Appenroth, 2015). However, in a continuous cultivation process, it is critical to keep the nutrient concentrations and ratios as stable as possible to maintain maximum growth rate at all times. Duckweeds quickly and preferentially take up ammonium over nitrate (Zhou et al., 2021), but keeping the concentration and ratio stable is important for high growth rates and crude protein contents (Petersen et al., 2021). Therefore, a continuous ammonium supply in intervals was installed.

The average concentration of nitrate-N in the liquid medium in the IVF was 0.7 mg l^{-1} below the target value (12.2 mg l^{-1}) and ammonium-N was 0.8 mg l^{-1} below target value (3.5 mg l^{-1}). Except for magnesium, sulphate, calcium, manganese and sodium (which were all present in high concentrations in the local tap water), all other nutrients were below the target value. In case of potassium, on average only half of the intended concentration was present in the solution, while iron was detected in a concentration more than ten times lower compared to the target value.

The presented data show that nutrient concentrations in our IVF were not stable at all times. The fluctuations differed in intensity for different nutrients. They were more intense for nutrients, such as ammonium, boron, manganese, iron and zinc, and less intense in the case of magnesium, sulphate and sodium. For calcium and sulphate, the variation coefficient is below 5%, while for manganese it is above 90%.

The ratio between nitrate-N and ammonium-N was 4.3:1 on average during the whole production phase. This is very close to the target ratio of 4:1, which achieved high RGRs, protein contents and protein yields in *L. minor* and *W. hyalina* (Petersen et al., 2021). Hecht and Mohr (1990) and Mehrer and Mohr (1989) explained that ammonium accumulation is not well regulated by plants, thus a higher ammonium concentration can have detrimental effects on plants. They called it ammonium toxicity syndrome. It has been reported that also the ratio of calcium to magnesium influences *L. minor* growth. The obtained ratio of 5.5:1 is in-between the reported ratios of 3:1 and 6.1:1, which resulted in RGRs of 0.164 d^{-1} and 0.148 d^{-1}, respectively (Walsh et al., 2020).

The continuous ammonium dosing stabilized the EC-value, meaning it decreased only slowly. As a consequence, stock solutions A, B and C were seldom dosed, which lead to a decreasing concentration of certain

nutrients over time. Furthermore, the amount of added ammonium was not adapted to the varying quantity of duckweed in the IVF at different points of time, which could explain the fluctuations in ammonium-N concentrations. These examples show that the use of EC-based dosing systems is inaccurate and can cause a diminishment or enrichment in certain substances in a re-circulating system, when there is an imbalance between the stock solution composition and the actual plant requirement. This imbalance will increase the longer a re-circulating system is in operation. A nutrient deficiency or overconcentration can cause reduced plant growth and quality and in severe cases even death of the cultivated plants. Therefore, new approaches should be tested, such as stationary ion-selective (Richa et al., 2021), ion-sensitive field-effect transistors (Bamsey et al., 2012) or mid-infrared sensors (Fan et al., 2012) coupled with a dosing system. When such a system will work reliably with mineral fertilizers in the future, the use of other promising nutrient sources, such as swine wastewater (Zhou et al., 2019) or anaerobically digested swine wastewater (Hu et al., 2019), can be tested and evaluated in an IVF.

4.3. Biomass quantity and harvesting process

The presented IVF for an automated duckweed biomass production continuously yielded fresh duckweed biomass. An average of 0.9 kg FW/53 g dried biomass was harvested per day with the above-described settings, which extrapolates to 620 kg month^{-1} ha^{-1} or 7.54 t year^{-1} ha^{-1} DM. This is comparable to the *L. minor* harvest of 702.5 kg month^{-1} ha^{-1} DM with an average protein content of 27% reported by Chakrabarti et al. (2018), when grown on inorganic fertilizers. Devlamynck et al. (2021) reported a yield of 8.1 t year^{-1} ha^{-1}, based on a 175-day growing season, when cultivating *L. minor* on a synthetic N-medium. Other studies report potential productivities of up to 68 t year^{-1} ha^{-1} (Mohedano et al., 2012), 70 t year^{-1} ha^{-1} (Calicioglu et al., 2021) or even 105 t year^{-1} ha^{-1} (Zhou and Borisjuk, 2019). These studies indicate that yields can be increased, however, growth conditions need to be considered when comparing these productivities. None of the other data sets used for these projections were obtained from a cultivation in a comparable environment. The applied conditions are optimized for a continuous production in the presented IVF.

The harvesting process in the presented IVF was executed by visual judgment at irregular time intervals. When the fronds overlap in several layers within the basins, a variable quantity of *L. minor* was removed via the installed harvesting system from the continuous production process in the IVF. After harvesting, a duckweed surface coverage of ca. 80% was always left in the basins to obtain reproducible results. However, this resulted in a slightly varying quantity of biomass left in the basins to continue growing. In order to quantify the duckweed biomass per basin at any time and thereby determine the optimal moment for harvest, in a first step the capacity limit for optimal duckweed growth in the IVF must be identified. In a second step the duckweed density in each basin must be automatically determined (e.g. by optical sensors coupled with an image processing software (Coughlan et al., 2022), in order to automatically initiate and stop the harvesting process at defined duckweed densities. In any case, a more frequent harvesting regime can be recommended. This favours nutrient recovery and biomass production (Xu and Shen, 2011). Calicioglu et al. (2021) suggested a harvest frequency of 1 day and a harvest ratio of 0.35 g g^{-1}. These suggestions, however, have to be adapted to the growth rates in the duckweed IVF.

4.4. Biomass quality

The achieved CP content of 32% (DM) has been reported previously by Kabir et al. (2005). However, widely varying compositions for *L. minor* have been investigated with CP contents ranging from 18.4 (Yilmaz et al., 2004) to 40.2% CP in DM (Khanum et al., 2005). Common protein sources for feed production are soybean meal and rapeseed meal. Those CP levels range from 38.8 to 52.1% (DM) and 30.3–37.5% (DM), respectively (Durst et al., 2021). Novel protein sources, such as insects or microalgae *Chlorella* contain CP at levels of 45.3% and 39.5%, respectively (Durst et al., 2021). The aim of our study was to produce duckweed as a protein-rich input for feed production. Therefore, strategies to increase CP content and thus nutritional value are required as part of product optimization.

Plant composition and thus CP content is influenced by cultivation conditions and, in particular, cultivation medium (Gwaze and Mwale, 2015). Comparable CP contents have been achieved using the same nutrient solution in a small re-circulating IVF for a cultivation phase of one week (Petersen et al., 2022). For the mass production of *L. minor*, cultivated on inorganic fertilizers, a CP content of 27% was reported by Chakrabarti et al. (2018), but compared to our cultivation conditions, their nitrate concentration was higher (15.3 mg l^{-1}) and no ammonium concentrations were reported. *Lemna minor* grown in a system with a constant supply of nutrients and average NO_3–N and NH_4–N concentrations of 6.3 and 0.3 mg l^{-1}, respectively, reached a CP content of 21.9%. This increased up to 39.4%, when the ammonium-N concentration increased to 39.1 mg l^{-1} (Iatrou et al., 2019). On the other hand, a decreasing ammonium-N content resulted in a decreased protein content (Hu et al., 2019). A synthetic N-medium with a concentration of 122 mg l^{-1} NO_3^--N and 0.71 mg l^{-1} NH_4^+-N yielded a CP content of 35% (Devlamynck et al., 2021). Khanum et al. (2005) reported 40.2% CP with a nutrient solution containing 26.6.mg l^{-1} NH_4^+. The reported data indicate an impact of ammonium and nitrate concentrations on CP content. In order to increase the biomass CP levels, the nutrient control and dosing system can be optimized to continuously reach stable target values for ammonium and nitrate (see 4.2). Beyond that, increasing the target NH_4^+-level in the nutrient medium is a possible strategy to improve the CP content. However, a solely increased NH_4^+-content has been associated with a decreased plant productivity (Petersen et al., 2021) and NO_3^--N contents must be adapted as well.

To obtain a high protein feedstuff, the duckweed biomass can be processed by protein extraction and protein isolation. This has been investigated with various techniques and plant protein sources e.g. alfalfa, clover, grass or macroalgae (Santamaría-Fernández and Lübeck, 2020). These processes mainly consist of three steps: First, plant material is chopped and the "green juice" is pressed out (Hojilla-Evangelista et al., 2017). In the next step, protein is precipitated with different techniques, such as coagulation or acidification. In the last step, protein is concentrated by separation and drying (Santamaría-Fernández and Lübeck, 2020). Rojas et al. (2014) observed a high digestibility in piglets and a high amino acid content for a *Lemna* protein concentrate with 68% CP indicating an improved product quality after processing. However, protein yields from leaves are mostly below 50% with an exception for alfalfa (Santamaría-Fernández and Lübeck, 2020).

In conclusion, future trials can assess, whether adaptions of cultivation conditions or further processing steps are more effective regarding protein enrichment. Therefore, plant productivity but also general efficiency of the processing steps and cultivation as well as the respective effect on nutritional value have to be weighed.

It has been stated for duckweed that the amino acid profile is stable for individual species (Appenroth et al., 2017). The findings of Amado et al. (1980) indicate that amino acid contents of different *L. minor* clones are comparable only to a limited extent. As clone 9441 (*L. minor*) was used in this study and the study of Appenroth et al. (2017), the amino acid profiles of these studies can be compared. Deviations in amino acid content are generally below 1 g amino acid per 100 g CP. However, Arginine is an exception with a difference of 1.9 g Arg/100 g CP. This can be supported by the findings of Devlamynck et al. (2021), who detected a significant influence of cultivation conditions on the Arginine content.

For all livestock species, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine are essential amino acids. Moreover, arginine is essential for poultry. Cysteine and tyrosine are semi-essential amino acids because they can

only be produced from methionine and phenylalanine, respectively (Fuller, 2004). Two methods (amino acid index and ideal amino acid ratio) were used to assess the amino acid profile of the harvested biomass. Considering the amino acid index (AAI), it appears that the nutritional value of *L. minor* protein is comparable to soybean meal and rapeseed meal, but also to other novel protein sources like algae (*A. platensis*) and insect meal of black soldierfly (BSF) larvae meal (Table 4).

The AAI neglects the fact that essential amino acids are needed in defined proportions and deficiencies of individual amino acids cannot be compensated by a surplus of other amino acids. Deficiency in one essential amino acid leads to loss of appetite and consequently to undernutrition (Santamaría-Fernández and Lübeck, 2020). In order to match the requirements of the livestock species, all essential amino acids must be supplied in a defined proportion (Kamphues et al., 2014). Therefore, the concept of ideal protein can be considered, where the ratio for individual amino acids is expressed relative to lysine (100) (Pastor, 2014). The amino acid ratios of the mentioned feedstuffs and the requirements for the ideal protein of piglets and broiler chicks can be evaluated. The respective values are shown in Table 4.

In pigs, lysine is the first amino acid to be deficient when the CP content of the diet is reduced. In poultry, this first limiting amino acid is methionine (Díaz-Gaona et al., 2021). The lysine content of the yielded biomass is comparable to rapeseed meal (5,3 g/100 g CP), but especially soybean meal (6.1 g/100 g CP (Sauvant et al., 2004)) has a higher lysine content than the *L. minor* biomass (5.4 g/100 g CP). The meal of BSF larvae has also a higher lysine content (6,6 g/100 g CP, Makkar and Ankers, 2014). With regard to the amino acid ratio, duckweed contains more sulphur-containing amino acids (SAA, Met + Cys) than soybean meal and BSF larvae meal. Moreover, *L. minor* protein is rich in tryptophan, arginine, valine, leucine and arginine, and matches the requirements of pigs and poultry with an exception for the sulphur containing amino acids. This deficiency in SAAs has previously been confirmed for *L. minor* by Devlamynck et al. (2021) and also Appenroth et al. (2017). As the described parameters only consider amino acid contents and do not regard availability, future studies might investigate the amino acid digestibility for livestock, such as broiler chickens.

5. Conclusion and further perspectives

This is the first detailed report on the construction, technicalities and operation as well as biomass yield and quality of an IVF for duckweed biomass production. An average daily FW yield of 0.9 kg with a CP content of 32% in the DM over a 40-day production phase is a first accomplishment in this field. The investigated parameters indicate that the produced duckweed biomass can be used as a soybean meal replacement in monogastric animal nutrition. However, in order to maximize the nutritional value of the duckweed biomass, CP contents should be increased.

The optimization of the presented IVF is still in progress, aiming at a maximum level of automation, biomass production and product quality with a minimum resource input. Therefore, in the future, the energy, water and nutrient input will be recorded automatically. This way, it is possible to determine the input in relation to the yield. New approaches in nutrient supply will be tested and evaluated, as well as new technologies to optimize the harvesting process.

This IVF can be used as a model system for the conduction of scientific experiments or duckweed biomass production in a controlled environment as well as for further innovations and upscaling processes.

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CRediT authorship contribution statement

Finn Petersen: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Johannes Demann:** Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Jannis von Salzen:** Investigation, Data curation, Visualization. **Hans-Werner Olfs:** Writing – review & editing, Supervision, Funding acquisition. **Heiner Westendarp:** Supervision, Project administration, Funding acquisition. **Petra Wolf:** Writing – review & editing, Supervision. **Klaus-Jürgen Appenroth:** Writing – review & editing, Supervision. **Andreas Ulbrich:** Conceptualization, Supervision, Funding acquisition.

Declaration of competing interest

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

Table 4
Amino acid ratios (%) of various protein sources compared to the ideal amino acid ratio (IAAR) of broiler chickens and piglets.

	Lemna minor clone 9441						IAAR	
	This study	Appenroth et al. (2017)	A. platensis[a]	BSF larvae[b]	Soybean meal[c]	Rapeseed meal[c]	broiler chicks[d]	piglets[e]
Lysine	100	100	100	100	100	100	100	100
Methionine	28	32	59	32	23	38	38	26
Met + Cys	60	54	72	33	47	84	72	57
Threonine	71	82	72	56	64	81	74	64
Tryptophan	25	–	43	8	21	23	15	18
Valine	100	92	87	124	78	94	82	69
Isoleucine	79	74	83	77	75	76	73	55
Leucine	140	146	181	120	120	126	109	97
Histidine	35	30	61	45	43	49	32	31
Arginine	122	94	107	85	121	113	110	40
Phenylalanine	89	90	89	79	82	73	63	59
Phe + Tyr	138	152	162	183	136	127	122	92
AAI broiler chickens	1.20	–	1.23	1.25	1.24	1.22		
AAI piglets	1.34	–	1.38	1.40	1.39	1.37		

[a] Safi et al. (2013).
[b] black soldierfly, Makkar et al. (2014).
[c] Sauvant et al. (2004).
[d] 3–5 weeks, National Research Council (1994).
[e] 10–20 kg body weight, National Research Council (1998); AAI: amino acid index.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2022.134894.

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4. General Discussion

The potential of duckweed is enormous due to its fast biomass production and high nutritional value, which can be influenced by cultivation conditions. Protein-rich duckweed biomass could become an established part of human and animal nutrition. In either case, a standardized production process aiming at large yields with stable product qualities is necessary. In the future, this process should be economically and ecologically viable, a status as Novel Food has to be granted and the acceptance of potential customers and consumers, leading to a sufficiently large demand, is required for a successful implementation of duckweed in different industries.

With regard to plant cultivation, numerous abiotic factors influence duckweed growth and biomass quality, such as water quality, nutrient supply, light conditions, pH, temperature, CO_2 content, flow rate, water depth and plant surface coverage (Coughlan et al., 2022; Landolt and Kandeler, 1987; Walsh et al., 2021) as well as biotic factors, including pathogens and competitors for nutrients and light (Brand et al., 2021; Roijackers et al., 2004). For this dissertation, a biological system was used to investigate the effect of the important abiotic factors nutrients and light on the physiological properties of two duckweed species and optimize them with regard to a biotechnological application. Especially nitrogen supply is an important factor with regard to a high protein content of the duckweed biomass.

4.1. Nitrogen assimilation

Nitrogen, after carbon, is the element required the second most by plants in terms of quantity, amounting to 1 - 5 % of the total plant DM (Hawkesford et al., 2012). In soils, nitrogen is present in inorganic forms (mainly nitrate and ammonium) and organic forms (mainly urea, free amino acids and short peptides) (Muratore et al., 2021). Nitrate is the major nitrogen source for plant growth in agriculture (Miller and Cramer, 2005). In general, nitrate assimilation occurs in the leaves and the roots of plants. In herbaceous plants, nitrate assimilation predominantly occurs in the leaves, while in woody plants or legumes it mainly takes place in the roots. Nitrate is taken up by the roots via a symporter with two protons (Figure 3). It can be temporary stored in the vacuole. Nitrate can be assimilated in the roots, until the capacity for nitrate assimilation in the roots is exhausted. The remaining nitrate can be released into the xylem and is carried by the transpiration stream into the mesophyll of the leaves. Nitrogen assimilation occurs in a multi-step process. In a first step, nitrate is reduced to nitrite by the enzyme nitrate reductase, which is present in the cytosol of the mesophyll. NADH acts as a reducing agent for this process. In a second step, nitrite is reduced to ammonium. The enzyme nitrite reductase, located in the plastids, causes this reaction by the uptake of six electrons from reduced ferredoxin (Heldt and Piechulla, 2021).

Triggered by glutamine synthase, which is present in the chloroplasts, ammonium is incorporated by glutamic acid in ammonium assimilation to form glutamine, using ATP. Glutamine is then reduced by the enzyme glutamate synthase through transamination into glutamate (Heldt and Piechulla, 2021).

Ammonium is assimilated in the roots of plants. Theoretically, ammonium uptake for plants is easier than nitrate uptake, as the electrochemical gradient is lower. The assimilatory nitrate reduction is omitted, as ammonium can be directly used by the plants to metabolize amino acids. Nevertheless, many plant species preferentially take up nitrate over ammonium, even though it is correlated with an increased energy consumption for ammonium reduction. One reason might be an ammonium-triggered toxicity, which, however, differs between species (Zhou et al., 2021).

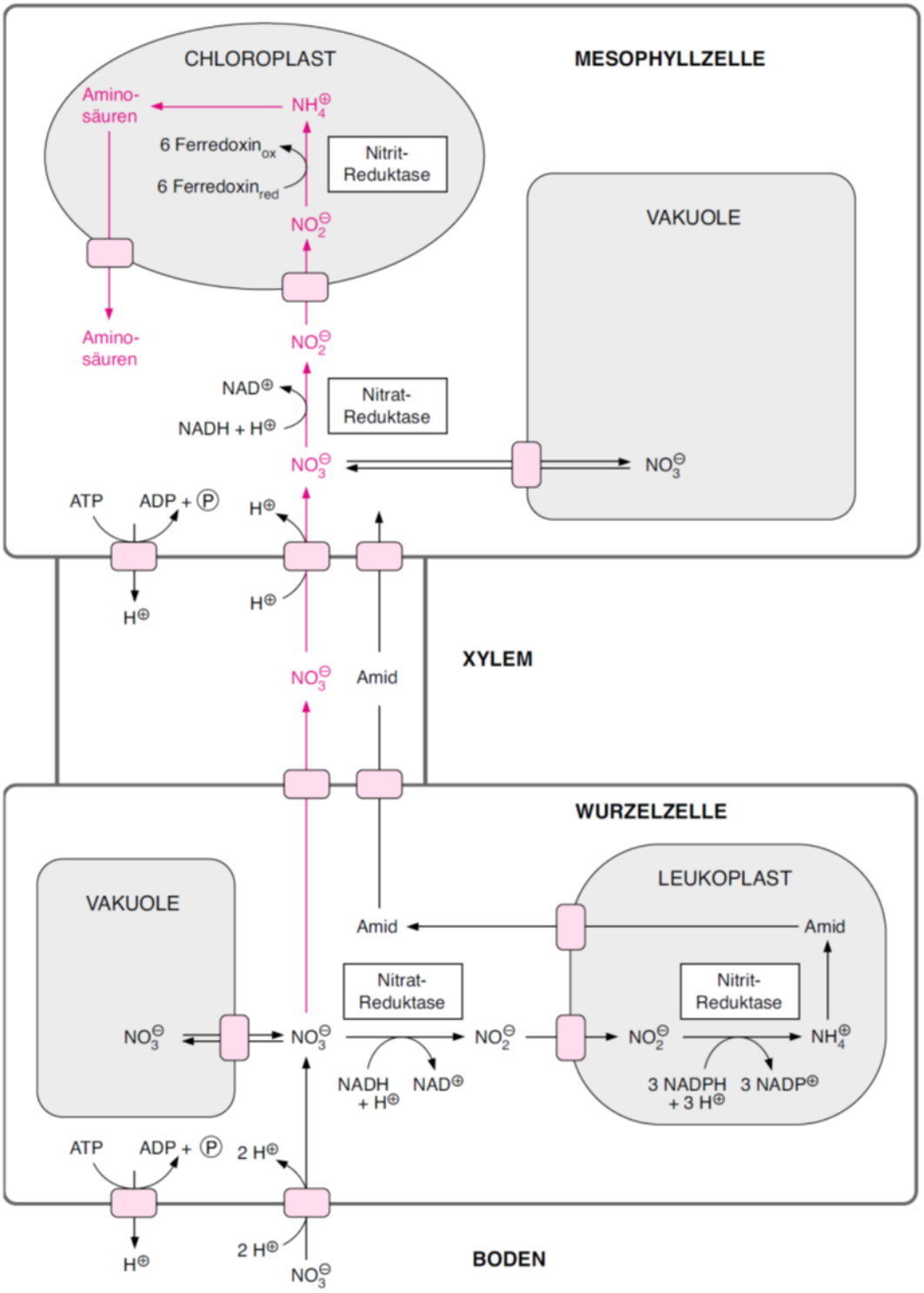

Figure 3: Nitrate assimilation process in the root and leaves of plants (Heldt and Piechulla, 2014)

The content and form of nitrogen in the soil or nutrient solution is therefore an important growth factor. Plant physiologically, this has several reasons, as nitrogen is needed in many different compounds in plants. These include amino acids, proteins, amides, ureides, nucleic acids, chlorophyll, ATP, NAD(P)H, phytohormones, such as auxins and cytokinins, vitamins, such as thiamine and riboflavin, and various secondary plant compounds (Hawkesford et al., 2012). As nitrogen is essential for the synthesis of a large number of different substances, a change in the nitrogen content of the soil or nutrient solution also affects the composition of the plant (Lieberei and Reisdorff, 2012).

In soils, crop performance is often limited by nitrogen availability (Muratore et al., 2021). As a consequence, large amounts of nitrogen fertilizer are applied to the fields by conventional agriculture. As only about 50 % of the applied fertilizers is taken up by the plants, the remaining part is leached into the environment, leading to severe consequences, such as water eutrophication, air pollution, soil acidification (Zhou et al., 2021) and groundwater contamination (Mahvi et al., 2005). Hence, a target-oriented application of nitrogen fertilizers is needed. This includes minimal losses into the environment and an efficient uptake and conversion into usable biomass. Duckweeds cultivated in a re-circulating IVF could be a possible solution.

Duckweeds take up nitrogen with their roots and fronds. In case of *Wolffioideae* uptake occurs only via the fronds, as they possess no roots. Increased root length and root to frond dry weight ratio at nutrient deficiency indicate the role of roots in nutrient uptake (Cedergreen and Vindbæk Madsen, 2002). Cedergreen and Vindbæk Madsen (2003) demonstrated that nitrate reductase activity in *L. gibba* is eight times higher in the root than in the fronds. As the uptake of ammonium and nitrate in duckweed occurs through the leaf-like parts in addition to the roots, this indicates that nitrate, after uptake via the leaves, is transported through the phloem into the root where it is reduced to ammonium. This hypothesis can be supported by Cedergreen (2001). However, the uptake rate for nitrate and ammonium differed between roots and fronds of *L. minor*, while the area-specific uptake capacity only differed for ammonium. The uptake rate (µmol g^{-1} plant dry weight h^{-1}) of nitrate and ammonium at low and intermediate NH_4NO_3 concentrations (5 and 50 mmol m^{-3}, respectively) was higher for roots than fronds. Contrarily, at high NH_4NO_3 concentrations (250 mmol m^{-3}) the uptake rate for both N-forms was higher in the fronds. The area-specific ammonium uptake capacity (µmol m^{-2} root or frond h^{-1}) of fronds was significantly increased compared to roots. With increasing nitrogen availability, the uptake capacity of roots and fronds declined. No differences for roots and fronds regarding the area-specific nitrate uptake capacity were determined. The ammonium uptake capacity was three to eleven times higher than the nitrate uptake capacity for both, roots and fronds. This indicates a preferential uptake of ammonium over nitrate and was confirmed by a two to 30 times higher affinity for ammonium than nitrate (Cedergreen and Vindbæk Madsen, 2002). Nitrate was only taken up by six different investigated duckweed species when ammonium was depleted (Zhou et al., 2021).

However, previous studies did not investigate the effect of different nitrate to ammonium ratios on duckweed growth, protein content and nutrient uptake. In our study (Petersen et al., 2021) we identified that the nitrate-N to ammonium-N ratio significantly influences RGR, crude protein content (CPC) and relative protein yield of the two duckweed species *L. minor* and *W. hyalina* as well as the nitrate-N and ammonium-N content in the nutrient solution. A ratio of 75 % nitrate-N and 25 % ammonium-N [75-25] resulted in the highest RGR, which was significantly reduced with an increasing ratio of ammonium in the nutrient solution. The CPC increased with increasing ammonium concentration, except for a solution at the [75-25] ratio. The resulting relative protein yield, however, was highest at the ratio [75-25]. The total reduction of ammonium-N (mg l^{-1}) in the nutrient solution was highest at the ratio [75-25], even though it only contributed to 25 % of the total nitrogen available. A possible explanation is the highest RGR at this ratio, which led to an increased nitrogen demand of the duckweeds. The highest nitrate reduction was achieved at the ratio [100-0], as nitrate was the sole nitrogen form available. In the other ratios containing ammonium, the nitrate reduction was significantly lower, as ammonium was presumably taken up preferentially. This data shows that for a larger-scale application aiming at protein-rich biomass, the nutrient solution has to be optimized firstly concerning the N-sources to reach fast growth rates and high protein contents.

4.2. Protein biosynthesis

The synthesis of amino acids, products of nitrogen assimilation, occurs mainly in the chloroplasts of plants. Different amino acids are synthesized in different ratios, depending on the species and the metabolic conditions. Usually, glutamate and glutamine are synthesized predominantly, while large quantities of alanine can often be detected in C_4-plants (Heldt and Piechulla, 2021).

For the synthesis of the different amino acids carbon structures are needed, which are synthesized in the Calvin cycle during CO_2 assimilation. 3-Phosphoglycerate is the most important carbon source for amino acid synthesis (Heldt and Piechulla, 2021). A variety of different metabolic pathways are involved to synthesize the different amino acids (Figure 4).

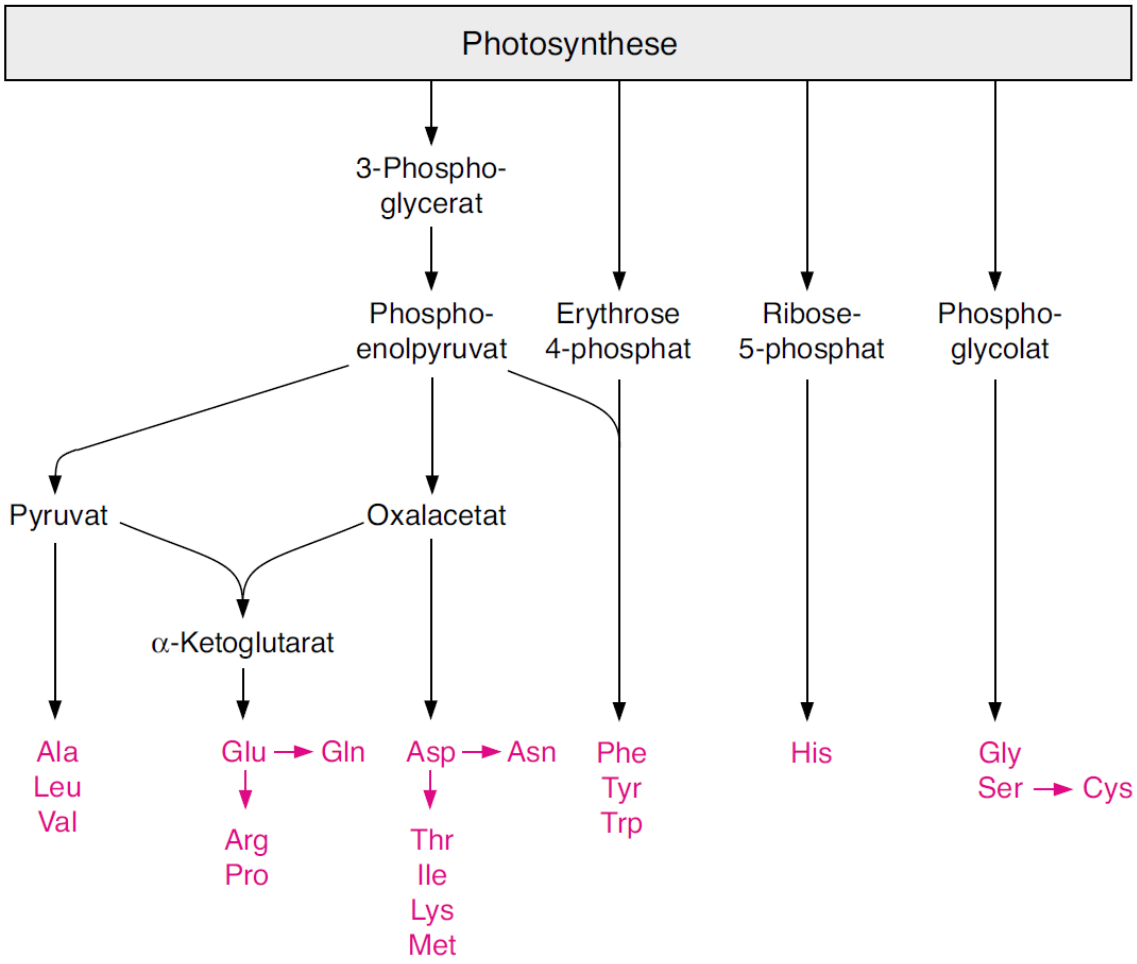

Figure 4: Involvement of different carbon sources for the synthesis of the different amino acids (Heldt and Piechulla, 2014)

Proteinogenic amino acids are the structures needed for protein biosynthesis, which takes place at the ribosomes. These synthesized proteins are reserves for amino acids, mainly in the form of storage proteins, which do not possess any enzymatic functions (Heldt and Piechulla, 2021). Storage proteins accumulate primarily in the protein storage vacuoles of terminally differentiated cells of the embryo and endosperm and as protein bodies directly assembled within the endoplasmic reticulum (Herman and Larkins, 1999). Those storage proteins can be fractioned based on their solubility into albumin (soluble in water), globulin (soluble in diluted saline solution) and prolamin (soluble in ethanol). The predominant form in plants is globulins, however, in different quantities. In duckweed *S. polyrhiza* high quantities of storage proteins are suspected, due to a high amount of larger molecular proteins with low solubility. The molecular weights of duckweed protein fractions ranged from 14 kDa to more than 160 kDa. Duckweed protein is more hydrophobic than soy protein at the same pH value and thermally stable up to 250°C (Yu et al., 2011).

4.3. Light and photosynthesis

Plants use the energy of light to transform water and CO_2 into glucose and O_2. This process is called photosynthesis, which made life on earth possible in the first place, as all living biomass and O_2 in the atmosphere are derived from this process.

For this process, plants capture the light of the sun or an artificial light source and use its energy. The electromagnetic radiation comprises a large spectrum. With increasing wavelength, the energy content is decreasing (Figure 5).

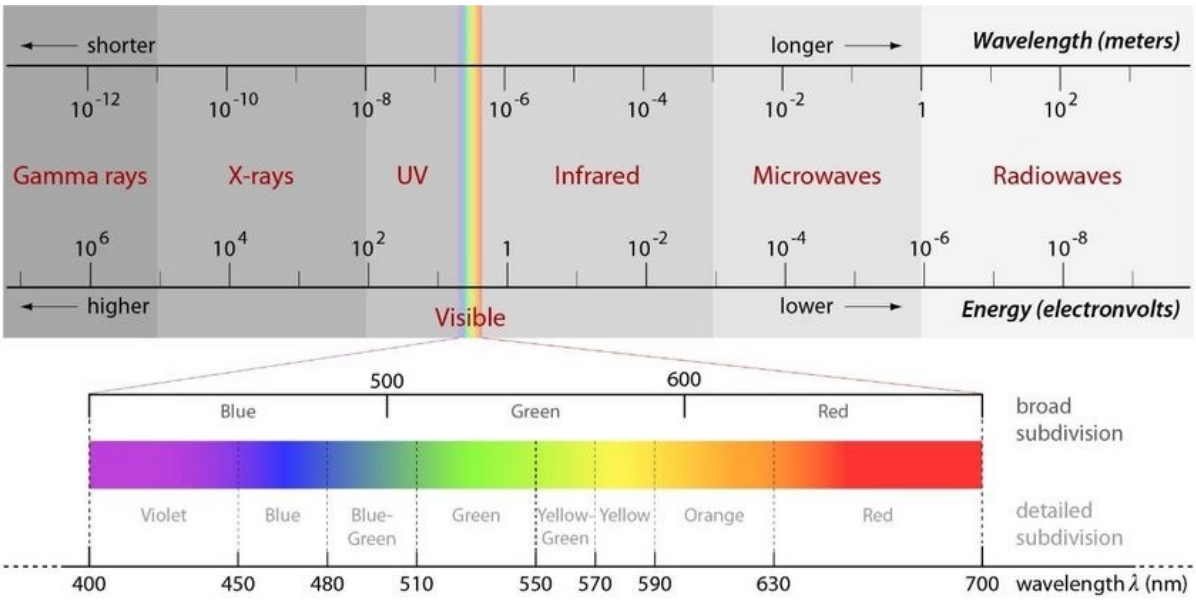

Figure 5: The electromagnetic spectrum (Verhoeven, 2017)

In nature, plants need to react to large temporal and spatial variations in light conditions, such as intensity, spectral distribution and photoperiod (Ruban, 2009). Plants absorb photons as photosynthetic active radiation in the wavelength spectrum between 400 and 700 by specific protein complexes, called reaction centers.

These reaction centers, mainly located in the leaves of plants, contain chlorophyll. It is the predominant absorber of the photons of the red and blue wavelengths. As it doesn't absorb much green light (480 – 550 nm), but partially reflects it, leaves appear in green color. Plants contain two types of chlorophyll (a and b), usually in a ratio of 3:1 (Heldt and Piechulla, 2021). Other pigments for light absorption are carotenoids. For efficient photosynthesis, the energy of the photons is captured by antennas. They consist of protein-bound chlorophyll molecules, that absorb photons and transfer their energy to the reaction centers. Also, carotenoid pigments are present in the antennas. Two subsequent reaction centers, in photosystem II with an absorption maximum at 680 nm and photosystem I with an absorption maximum at 700 nm, are connected in plants (Heldt and Piechulla, 2021).

Light can affect plants in two different ways. On the one hand, light affects the growth rate and the formation of metabolites of plants via photosynthesis. On the other hand, light can influence the development of plants due to light receptors (Table 3) (Nover et al., 2008). The influence of light on the plant's phenotype is called photo morphogenesis (Kadereit et al., 2021; Schäfer and Nagy, 2006).

Excessive light irradiation can lead to a reduction in photosynthetic intensity. In light-sensitive species, this can even lead to bleaching or death of the leaves. In this process, known as photo-inhibition, the photosynthetic apparatus of the plants is supersaturated with light energy, in particular Photosystem II is affected. Plants have protective mechanisms for excessive light irradiation, such as carotenoids or certain radical scavenging reactions, but these are often insufficient to completely prevent photo-oxidative destruction. Photo-inhibition often occurs when plants are exposed to high light intensities after a dark period (Schopfer and Brennicke, 2016).

With the help of various light receptors, plants are able to perceive seasonal and daily changes in day length in the form of circadian rhythms and to adapt their metabolic and developmental processes (Nover et al., 2008).

The day/night ratio can influence the plant morphoses, such as flower induction, beginning and end of dormant periods, cambium activity, growth rate, formation of storage organs, formation of frost resistance, leaf fall, branching, adventitious rooting, leaf shape and succulence, and pigment formation (Kadereit et al., 2014).

Table 3: Photosynthetic pigments and light receptors of plants with the respective absorption ranges and their effects (according to Schopfer and Brennicke (2016) and Nover et al. (2008))

photosynthesis pigments	absorption spectrum	effect
chlorophyll	400-480 nm/550-700 nm	absorbing light for photosynthesis
carotenoids	460-520 nm	absorbing light for photosynthesis
photoreceptors		
phytochromes	660/730 nm	control of photo-morphogenesis, circadian rhythm, photoperiod and shade avoidance; photo tropism
cryptochromes	340 – 520 nm	Inhibition of hypocotyl elongation; promotes leaf development, synthesis of anthocyanin, stomata opening; circadian control of flowering time
phototropins	340 – 520 nm	controls phototropism, movement of the chloroplasts and stomata opening; optimizing photosynthesis
UV-B-photoreceptor	280 – 350 nm	Inhibition of hypocotyl growth; regulates the transcription of several genes; sunscreen; regulation of stomata opening
Zeitlupe-family	340-520 nm	control of circadian rhythm and phototropism

The effect of light intensity on duckweed growth is well described in the literature. Increasing light intensities increase the RGR of duckweed until the point of photo-inhibition is reached (Paolacci et al., 2018; Stewart et al., 2020). However, little knowledge about the spectral effect on duckweed growth and protein content is available. According to Zhong et al. (2021), red light promoted starch accumulation in *S. polyrhiza* (clone 5510), while blue light increased biomass and protein accumulation. Contrarily, wavelengths corresponding to red, blue and white light did not significantly influence the amino acid concentrations of the soluble protein in *W. arrhiza* (Appenroth et al., 1982). Therefore, we investigated the effect of different light intensities and spectral distributions as well as their interaction on *L. minor* and *W. hyalina* growth and quality. The RGR increased with increasing light intensity for both species, while the spectrum did not significantly impact RGR. The CPC ranged between 31.8 ± 0.8 % and 32.4 ± 1.2 % for *L. minor* and between 39.3 ± 1.0 % and 40.0 ± 0.8 % for *W. hyalina* for the different treatments. No significant differences in the CPC for the different light intensities and spectral distributions within a species were detected (Petersen et al., 2022a).

In the course of this dissertation, an issue occurred regarding the growth of unwanted ubiquitous algae, which compete for nutrients and light and could overgrow a duckweed population, if no adequate measures were taken. Therefore, comparatively low light intensities were applied to avoid this effect.

For large-scale applications, aiming at maximum biomass production, abiotic conditions closer to the respective duckweed species optimum have to be applied. However, measures to prevent uncontrolled algae growth or infections with pathogens have to be implemented as well.

4.4. Cultivation system

Different research institutes and companies around the world work on different approaches toward a standardized duckweed cultivation process. A re-circulating IVF operated in a controlled environment offers the possibility to standardize most of the relevant growth factors (Petersen et al., 2022b). The pH, nutrient composition and concentrations of the medium can be monitored and controlled, lighting systems can be automated and water temperature and movement can be adjusted. This way the quality and quantity of the yield can be regulated. Due to an indoor cultivation, a year-round production is possible, as the system is independent of the climatic conditions. Furthermore, some unwanted biotic factors, such as animals feeding on the duckweed and thereby damaging the harvest, can be excluded. The application of pesticides is not required. By re-circulating the nutrient solution within the system and constantly adding missing nutrients, the water and nutrient efficiency of such a system can be increased. Challenges, however, are the growth of ubiquitous algae and microorganisms. Different kind of filter systems and the adaption of growth factors can reduce their impact.

High costs for building cultivation systems, energy supply for technical devices (e.g. light sources, pumps, heaters or coolers, nutrient dosing systems) and providing the infrastructure (e.g. water supply, nutrient sources, electrical energy) must be named on the

downside. To economically operate such a system, the input of resources and energy must be minimized, by simultaneously maximizing the output (quantity and quality of duckweed biomass). The use of cheap and easily available resources must be aimed at, while still trying to keep the process as standardized as possible. A medium mainly prepared with agricultural fertilizers and tap water fulfills these requirements and allows a quite standardized cultivation (Petersen, 2022; Petersen et al., 2021).

4.5. Potential use of duckweed biomass

Due to their beneficial properties, duckweeds are recently getting into the spotlight of interest for different applications (Acosta et al., 2021; Rozman and Kalčíková, 2022). Depending on the cultivation conditions, duckweed can either accumulate proteins or carbohydrates. The biomass can be used as fresh material or in a processed form, e.g. dried, milled or as an isolated fraction of the biomass. To demonstrate the variability of duckweed biomass applications, a potential use as food, feed and biofuel is discussed in this Chapter.

4.5.1. Human nutrition

Duckweeds are suitable for human nutrition (Appenroth et al., 2018; Appenroth et al., 2017; Beukelaar et al., 2019; Sree et al., 2019; Xu et al., 2021). The species *W. globosa* has been consumed as an inexpensive protein source by the less wealthy people in parts of southeastern Asia (Myanmar, Laos and northern Thailand) for many decades. They call it “khai-nam”, which translates to “eggs of the water” (Bhanthumnavin and McGarry, 1971). Recently, the nutritional composition and value of several duckweed species have been investigated more in detail. Duckweed possesses a high-quality protein, containing 4.8 % lysine, 2.7 % methionine + cysteine and 7.7 % phenylalanine + tyrosine (Appenroth et al., 2017). The content of all essential amino acids is above the recommendations of the WHO for human nutrition (WHO, 2007). The starch content differed between 4 and 10 %, while the fat content (both based on DM) reached more than 6 %, of which up to 71 % were polyunsaturated fatty acids. A high share of n-3 fatty acids (mainly α-linolenic acid) resulted in a favorable n6/n3 ratio of less than 0.5 for most of the investigated species (Appenroth et al., 2017). According to Appenroth et al. (2018) the species *W. microscopica* had an ash and fiber content of 16.5 % and 28.6 %, respectively, based on DM. Its biomass is rich in potassium, copper, iron, manganese and zinc and is characterized by a low content of sodium. All-E-lutein (70 mg per 100 g DM), (all-E)- violaxanthin (46 mg/100 g DM) and (all-E)-b-carotene (28 mg/ 100 g DM) were the main components of carotenoids present in *W. microscopicas* biomass. The content of sterol, an important substance for serum cholesterol-lowering processes, was about 50 mg g^{-1} lipid. However, the content of certain components, such as proteins, starch or micro elements, mainly depends on the nutrient medium applied (Appenroth et al., 2017). A strain of *W. globosa*, called Mankai®, is not only rich in proteins (about 45 % DM) but also has a high content of Vitamin B12 (2.8 µg per 100 g DM) (Sela et al., 2020). This vitamin is usually present in animal products, such as meat, eggs or dairy

products, making this strain especially interesting for the increasing consumer group of vegans. It is, however, not yet clear whether vitamin B12 is indeed produced by *W. globosa* or by associated bacteria. Mankai® has a mitigating postprandial effect on the Glycemic Index of abdominally obese participants compared to yogurt (Zelicha et al., 2019). Sree et al. (2019) discovered, that seven different duckweed species covering all five genera do not contain anti-proliferative or cytotoxic effects, meaning that the high nutritional value of duckweed is not diminished by these harmful factors. Biomarkers in the blood and urine of adults consuming *L. minor* did not show significant differences in kidney function, liver function, cardiovascular health, inflammation and iron status compared to a spinach-based diet (Mes et al., 2022). The consumption of *L. minor* compared to green peas resulted in lower plasma glucose and insulin levels as well as significantly reduced blood concentrations of amino acids, indicating a lower digestibility of the duckweed (Zeinstra et al., 2019). Beukelaar et al. (2019) showed, that the knowledge about nutritional and sustainability benefits as well as an appealing meal preparation (e.g. in form of a salad) of duckweed increased the acceptability of Dutch consumers. Romano and Aronne (2021) suggest that the genus *Wolffia* can be used for food production and wastewater remediation in bioregenerative life support systems of future space missions.

4.5.2. Animal nutrition

Duckweeds are a feed source for different animals in the wild, such as ducks and fish. Traditionally, humans have used duckweed for feeding their domestic animals, especially in south east Asia (Fourounjian et al., 2020). For example, they have been produced for native chickens in Cambodia in an integrated system with duckweed ponds and a bio digester (Samnang, 1999). Moreover, it has been discovered that they pose a possibility for local farmers to minimize feed costs and increase income in Cambodia, Bangladesh or Nigeria (Ahammad et al., 2003; Olorunfemi, 2006; Samnang, 1999).

The main characteristic of duckweed, as mentioned above, is its high protein content. The high share of essential amino acids in the protein is not only favorable for humans but also for farm animals. The fatty acid profile is also worth mentioning, as more than 50 % of the duckweed fatty acids profile consists of linolenic and linoleic acid (Appenroth et al., 2018; Appenroth et al., 2017; Yan et al., 2013). These are indispensable not only for humans but also for animals.

In order to evaluate the nutritional quality of duckweed for farm animals, digestibility has to be considered. Especially protein as a main factor for growth or reproduction but also the phosphorus content has been investigated. A *Lemna* protein concentrate tested in growing pigs had a high digestibility of amino acids (Met 80.1 and Lys 81.2 %) and phosphorus (72.8 %) (Rojas et al., 2014). Several trials were conducted with livestock to investigate the effect on performance associated with the proportion of duckweed in the complete diet. Broiler chickens reacted with increased growth to *L. minor* up to a share of 6 % in complete feed as

a substitute for sesame oil cake (Ahammad et al., 2003). Other trials showed, that rates of up to 8 % duckweed in all ages (Kabir et al., 2005) or up to 10 % in finisher diets (Kusina et al., 1999) could be realized. A partial replacement for soybean meal for piglets and growing pigs has been realized (Moss, 1999). For laying hens, *L. gibba* could replace up to 10 % soy bean or approximately 50 % fishmeal without adverse effects on the egg quality or laying performance of hens (Akter et al., 2011; Zakaria and Shammout, 2018). Increased egg yolk color has been confirmed by various authors (Anderson et al., 2011; Chantiratikul et al., 2010; Zakaria and Shammout, 2018), as well as increased n-3 fatty acid content in eggs (Anderson et al., 2011). Moreover, the nutritional value of duckweed has been shown for ducks (Khandaker et al., 2007; Khanum et al., 2005), fish (Asimi et al., 2018; Da et al., 2013; Fasakin et al., 1999), cattle (Huque et al., 1996) and sheep (Damry et al., 2001; Zetina-Córdoba et al., 2012). Generally, trial results varied based on the quality of the used duckweed and its composition. It has been assumed, that oxalates or other anti-nutritives may limit the potential use of duckweed in feeding animals (Fourounjian et al., 2020). Consequently, product quality has to be optimized and controlled to ensure appropriate and efficient animal nutrition (Demann et al., 2023).

4.5.3. Biofuel

Biofuel is considered a more sustainable alternative to fulfill energy demands. Besides conventional plants, duckweed can accumulate starch as the main feedstock for biofuel production in actively growing plants as well as in turions. Other plant compartments can also be used energetically, such as pectin or cellulose. Main advantage of the use of duckweed is, that as an aquatic plant, it doesn't compete with other crop plants for arable land (Appenroth et al., 2021). Moreover, high amounts of starch can be yielded, e.g. 28 t per hectare and year (Cheng and Stomp, 2009).

Biofuel production can be divided into biomethane or biogas production and bioalcohol production. In these fields, several approaches to produce biofuel with duckweed have been described. According to Appenroth et al. (2021), the starch content in duckweeds can be increased by manipulation of the cultivation conditions, e.g. nutrient deficiency, salt stress or the phytohormone abscisic acid. Chen et al. (2012) found out that bioethanol yield could be maximized with pectinase pre-treatment of *L. punctata* as feedstuff for *Saccharomyces cerevisiae*. The maximum glucose yield of enzymatic liquefaction was 218.64 ± 3.10 mg g^{-1} DM and was increased by 142 % compared to the not pre-treated plant material. Hydrolysates of duckweed have also been fermented with a modified *Corynebacterium crenatum* to higher alcohols (Su et al., 2015). Cheng and Stomp (2009) optimized duckweed production and yielded up to 45.8 % starch with a bioethanol yield of 25 % of the original biomass. Moreover, an N-supplementation of the yeast fermentation mesh may not be necessary because of the relevant N content in duckweed (Cheng and Stomp, 2009). Ge et al. (2012) reached a level of 36 % starch and the bioethanol yield was 8.5 % of the original

biomass. As mentioned above starch can also be accumulated in turions. Not only for bioalcohol but also for biogas production, the usability of duckweed biomass has been investigated. The duckweed species *S. polyrhiza* is a possible substrate for anaerobic digestion. A co-digestion with 20 % duckweed with activated sludge improved methane yield significantly (Ghosh et al., 2019). Combined fermentation with pig manure also resulted in higher methane yields (Cui and Cheng, 2015).

As mentioned in Chapter 1.2.3., a high starch content is only possible with a simultaneously low RGR, protein and phosphorus content. This is useful for energy purposes but detrimental when a nutritional use is intended.

4.6. Legal status

Duckweed biomass intended for human nutrition purposes is considered a “Novel Food” in most parts of the world. As such, it is subject to thorough procedures for approval. This regulatory status includes the assessment of potential adverse effects on animals, plants, the environment and human health, specifically the nutritional composition, and the presence of toxins, anti-nutrients or allergens. This approval process is avoided if duckweed is assessed as a traditional food by the responsible authorities. In general, it depends on three criteria:

- Duckweed species and strain:
  W. globosa and *W. arrhiza* are considered traditional eligible vegetables, while other duckweed species and strains (e.g. *L. minor*) are evaluated as Novel Food.
- Natural and proprietary strains:
  Naturally occurring strains can be considered traditional food, while strains altered by human effort (e.g. breeding) will be considered Novel Food.
- Intended use:
  only fresh produce will be evaluated as traditional food, while any processed or modified products will be considered Novel Food

If not considered a traditional food, duckweed plants and their products are subject to the application procedures of the responsible authorities (Shoham, 2022). Table 4 shows the current application status of different duckweed species and their intended use.

Table 4: Current application status for different duckweeds and their products intended as human food in the EU, USA and Israel (Shoham, 2022)

REGULATORY APPROVAL STATUS	APPLICANT	REGION / MARKET	TYPE	TYPE	COMPANY / APPLICANT
APPROVALS	Mix Lemna & Wolffia	USA	GRAS affirmation	Powder ingredient	Parabel
	Wolffia globosa *Wolffia arrhiza*	USA	GRAS affirmation Traditional food	Fresh produce Processed ingredient	GreenOnyx
	Wolffia globosa	USA	GRAS affirmation	Powder ingredient Dried frozen	Hinoman
	Wolffia globosa *Wolffia arrhiza*	EU	EFSA, Traditional food	Fresh produce	GreenOnyx
	Wolffia globosa	Israel	Novel food Ministry of Health	Powder ingredient Dried frozen	Hinoman
	Wolffia globosa *Wolffia arrhiza*	Israel	Novel food Ministry of Health	Fresh produce	GreenOnyx
REJECTIONS	Mix Lemna & Wolffia	EU	EFSA	Powder ingredient	Parabel
	Wolffia globosa	EU	EFSA	Powder ingredient	Hinoman
PENDING APPLICATIONS	*Lemna minor* *Lemna gibba*	EU	EFSA	Fresh produce	Wageningen University
	Lemna minor *Lemna gibba*	EU	EFSA	Concentrates Protein ingredient	Rubisco Foods

The European Food Safety Authority did not raise safety objections regarding the duckweed product of Israel-based company GreenOnyx for the species *W. globosa* and *W. arrhiza* (EFSA, 2021). This means that these two species can be used for human nutrition in the EU. The permission for *L. minor* as a novel food in the EU is still pending, but it's already been approved as animal feed in Germany (Normenkommission für Einzelfuttermittel, 2017).

5. Outlook

Besides the application of mineral fertilizers as a nutrient source, alternative nutrient-rich resources could be used for duckweed cultivation. Manure and wastewater are available in abundance in most parts of the world, easily available and cheap, however, they often contain a mixture of contaminants, such as pharmaceuticals, pesticides, biocides or heavy metals (Helmecke et al., 2020; Hölzel et al., 2012). The occurrence and concentration of contaminants depends on the origin of these nutrient sources. A lot of money and a complex infrastructure are provided to process these resources, for example the intensive purification of wastewater, which is afterwards pumped into rivers or streams. However, they still impact ecosystems, when they are released into nature. Another example would be the excessive spread of manure on fields, which leads to increasing nitrate concentrations in groundwater. Valuable nutrients are this way removed or lost during these processes. By implementing treated manure (Devlamynck et al., 2021a; Devlamynck et al., 2021b; Lambert et al., 2022), wastewater (Keuter et al., 2021) or dairy effluent (O'Mahoney et al., 2022) as a nutrient source to produce protein-rich duckweed biomass for the food and feed industry, two of the most urgent problems in today's agriculture could be tackled.

As those resources are not standardized and contain possible pollutants, several processing steps are necessary before use. The manure or wastewater has to be processed in a way, that the majority of pollutants are removed, but only a minor fraction of the nutrients. Possible treatments are a process using membrane bioreactors and electrodialysis (Gurreri et al., 2020) or the separation of the solid and liquid fractions (Porterfield et al., 2020). The liquid fraction can be added into the hydroponic production system but doesn't contain the optimal nutrient composition needed for duckweed cultivation. Therefore, missing nutrients must be supplemented by adding mineral fertilizers. It has to be assured, however, that the concentrations of contaminants are below the respective threshold values and the growth rate and nutritional composition are similar compared to duckweeds from a cultivation process based solely on mineral fertilizers.

The production management of a re-circulating IVF is an essential factor regarding the success or failure of the operation. To ensure constant yields with a defined product quality from a re-circulating IVF, close monitoring of the nutrient solution is inevitable. With regard to a practical application, these datasets should be available at regular time intervals online and on demand for the grower. Ion-selective sensors (Bamsey et al., 2012) or ion-sensitive field-effect transistors (Fan et al., 2012) for all relevant macro- and micronutrients are required, however, these technologies need to be improved firstly.

To continuously obtain and assess data about the growth patterns of duckweed in a continuous production process, the implementation of image analysis software coupled with artificial intelligence is a possible solution (Romano et al., 2022). This way the harvest process could be automated.

The use of plant growth-promoting bacteria (PGPB) can increase the growth rate of duckweed by up to 32.1 % compared to the control group without PGPB. This effect, however, only lasted for a few days after inoculation, after seven days the natural duckweed

microbiome significantly reduced the PGPB (Ishizawa et al., 2020). The implementation of PGPB in a non-sterile and re-circulating IVF has not been investigated yet.

Contrarily, the growth of unwanted organisms in the re-circulating IVF can negatively influence duckweed cultivation and has to be suppressed by adequate measures, such as water filter devises or automated cleaning tools for the production basins. The current adaptations of the abiotic factors (e.g. low nutrient concentration and light intensity), which unfortunately also reduce the full potential of duckweed, have to be superseded by technological advances.

To operate such a re-circulating IVF resource-efficiently and economically, the input of electrical energy, water and fertilizers have to be monitored closely. This way the input of resources can be correlated with the product (biomass) output. This enables to calculate the costs for a large-scale production system. Additionally, the process of drying fresh duckweed biomass to preserve it has to be investigated, as the energy input for oven drying is a major cost factor.

To economically run a standardized cultivation system is only possible if all biotic and abiotic factors complement each other, are optimized for the species of choice, the output exceeds the input and if it's able to compete with today's protein sources regarding quality and quantity.

In order to transfer the presented results to a larger and even more practical-oriented surrounding, longer-lasting trials in larger IVFs have to be conducted. Only this way the overall aim of providing large quantities of a high-quality, protein-rich product at all times can be realized.

6. Summary

Duckweeds (*Lemnaceae*) are the fastest-growing angiosperms on earth. Five different genera, compromising 36 species of these floating freshwater plants, are distributed around the world. They differ in size, between less than 1 mm and up to 1.5 cm. They are highly interesting for different fields of application due to their fast growth rate and biomass production, their high nutritional value and their variability of nutritional composition, which can be influenced by cultivation conditions. Duckweeds are already used in wastewater remediation or as indicator plants in laboratories and could be implemented in human and animal nutrition, bioethanol production and for pharmaceutical purposes.

For industrial purposes, large amounts of duckweed with a certain quality are required at any time of the year. Therefore, a standardized production process with a focus on plant physiological growth parameters is needed. The effect of the nutrient medium, especially the nitrate-N to ammonium-N ratio and nutrient concentration, on the relative growth rates (RGRs) and crude protein contents (CPCs) of two duckweed species (*Lemna minor* and *Wolffiella hyalina*) was investigated under non-axenic cultivation conditions. A nitrate-N to ammonium ratio of 75 to 25 % and a half-strength nutrient concentration compared to the original N-medium resulted in high RGRs of 0.23 d^{-1} for *L. minor* and 0.22 d^{-1} for *W. hyalina* and high CPCs (37.8 % for *L. minor* and 43.9 % for *W. hyalina*).

The effect of different light intensities and spectral distributions on RGR, CPC and chlorophyll a content was investigated using a small-scale indoor vertical farm under non-sterile cultivation conditions. As ubiquitous algae can reduce duckweed growth in longer-lasting cultivation, for this 16-day experiment a nutrient concentration of 10 % compared to the original N-medium was applied. It resulted in the highest RGR for both species at the highest light intensity (150 µmol m^{-2} s^{-1}), while the spectral distribution had no significant influence on the RGR. The CPC was not significantly influenced by any of the investigated cultivation parameters and reached between 31.8 % and 32.4 % for *L. minor* and between 39.3 % and 40.0 % for *W. hyalina* for the different treatments. The chlorophyll a content was highest at the lowest light intensity (50 µmol m^{-2} s^{-1}) and decreased with an increasing light intensity in both species.

Based on these results and experiences with the previously used cultivation systems, a large-scale Indoor Vertical Farm was developed. It consists of nine vertically stacked production basins (1.95 x 1.45 x 0.1 m), resulting in a total production area of ca. 25.5 m^2. The nutrient solution is re-circulating within the system and the nutrients are dosed automatically based on the electrical conductivity. Most abiotic growth parameters are controllable and adjustable. During a 40-day production phase a total of 35.6 kg of fresh *L. minor* biomass was harvested, which corresponds to ca. 900 g per day.

To further optimize the re-circulating cultivation system, an advanced method to identify the concentration of each nutrient in the medium at any time is required to avoid overconcentration or lack of single nutrients. Additional filter systems and cleaning devices should be implemented to reduce unwanted algae growth. A stable and standardized production process, resulting in stable yields with a defined product quality, is required to promote the implementation of duckweed for industrial applications.

7. Zusammenfassung

Wasserlinsen (*Lemnaceae*) sind die am schnellsten wachsenden Blütenpflanzen der Erde. Es gibt fünf verschiedene Gattungen mit insgesamt 36 Arten dieser Süßwasserpflanzen, die eine Größe von weniger als einem Millimeter bis zu 1,5 cm erreichen können. Sie sind aufgrund ihres schnellen Wachstums und Biomasseproduktion, ihres hohen Nährwerts und ihrer Variabilität der Nährstoffzusammensetzung, die durch die Anbaubedingungen beeinflusst werden kann, für verschiedene Anwendungsbereiche von großem Interesse. Wasserlinsen werden bereits in der Abwassersaufbereitung und als Indikatorpflanze in Laboren eingesetzt und könnten in der Human- und Tierernährung, der Produktion von Bioethanol und für pharmazeutische Zwecke verwendet werden.

Für industrielle Zwecke werden ganzjährig große Mengen an Wasserlinsen von definierter und stabiler Qualität benötigt. Daher ist ein standardisiertes Produktionsverfahren mit Fokus auf pflanzenphysiologische Wachstumsparameter erforderlich. Der Einfluss des Nährmediums, insbesondere des Nitrat-N- zu Ammonium-N-Verhältnisses und der Nährstoffkonzentration, auf die relativen Wachstumsraten und Rohproteingehalte von zwei Wasserlinsenarten (*Lemna minor* und *Wolffiella hyalina*) unter nicht-sterilen Kultivierungsbedingungen wurde untersucht. Ein Nitrat-N zu Ammonium-N Verhältnis von 75 zu 25 % sowie eine Nährstoffkonzentration von 50 % im Vergleich zum ursprünglichen N-Medium führten zu hohen relativen Wachstumsraten von 0,23 d^{-1} für *L. minor* und 0,22 d^{-1} für *W. hyalina* und hohen Rohproteingehalten (37,8 % für *L. minor* und 43,9 % für *W. hyalina*).

Die Auswirkung verschiedener Lichtintensitäten und -spektren auf die relative Wachstumsrate, den Rohprotein- und Chlorophyll a-Gehalt wurde in einer kleinen, vertikalen Indoor-Farm unter nicht sterilen Anbaubedingungen untersucht. Da ubiquitär vorkommende Algen das Wachstum von Wasserlinsen bei längerer Kultivierdauer beeinträchtigen können, wurde in diesem 16-tägigen Experiment eine Nährstoffkonzentration von 10 % im Vergleich zum ursprünglichen N-Medium verwendet. Bei der höchsten Lichtintensität (150 µmol m^{-2} s^{-1}) wurden die höchsten relativen Wachstumsraten für beide Arten erzielt, während die spektrale Verteilung keinen signifikanten Einfluss auf die relativen Wachstumsraten hatte. Der Rohproteingehalt wurde durch keinen der untersuchten Kultivierungsparameter signifikant beeinflusst und erreichte bei den verschiedenen Behandlungen zwischen 31,8 % und 32,4 % für *L. minor* und zwischen 39,3 % und 40,0 % für *W. hyalina*. Der Chlorophyll a-Gehalt war bei der niedrigsten Lichtintensität (50 µmol m^{-2} s^{-1}) bei beiden Arten am höchsten und nahm mit steigender Lichtintensität ab.

Auf der Grundlage dieser Ergebnisse und der Erfahrungen mit den zuvor verwendeten Anbausystemen wurde eine große Indoor Vertical Farm entwickelt. Sie besteht aus neun vertikal angeordneten Produktionsbecken (1,95 x 1,45 x 0,1 m) mit einer Gesamtproduktionsfläche von ca. 25,5 m^2. Die Nährlösung zirkuliert innerhalb des Systems und die Nährstoffe werden automatisch auf Basis der elektrischen Leitfähigkeit dosiert. Ein Großteil der abiotischen Wachstumsparameter kann kontrolliert und angepasst werden. Während einer 40-tägigen Produktionsphase wurden insgesamt 35,6 kg frische *L. minor*-Biomasse geerntet, was ca. 900 g pro Tag entspricht.

Zur weiteren Optimierung des Kreislaufkultivierungssystems sind fortschrittlichere Konzentrationsbestimmungen eines jeden Nährstoffs im Medium zu jedem Zeitpunkt nötig, um eine Überkonzentration oder einen Mangel an Einzelnährstoffen zu vermeiden. Zusätzliche Filtersysteme und Reinigungsvorrichtungen sollten implementiert werden, um unerwünschtes Algenwachstum zu reduzieren. Ein stabiles und standardisiertes Anbauverfahren, das zu stabilen Erträgen mit einer definierten Produktqualität führt, ist erforderlich, um den Einsatz von Wasserlinsen für industrielle Anwendungen zu fördern.

8. References

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9. Ehrenwörtliche Erklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Fakultät für Biowissenschaften der Friedrich-Schiller-Universität Jena erkläre ich, dass ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die bei der Durchführung der Experimente, Auswertung sowie der Fertigstellung der Manuskripte mitgewirkt haben sind vor den jeweiligen Publikationen sowie im Anhang aufgeführt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht. Weiterhin wurde keine ähnliche oder andere Abhandlung als Dissertation anderswo eingereicht.

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Finn Petersen

10. Appendix

10.1. Supplementary material

Manuscript I

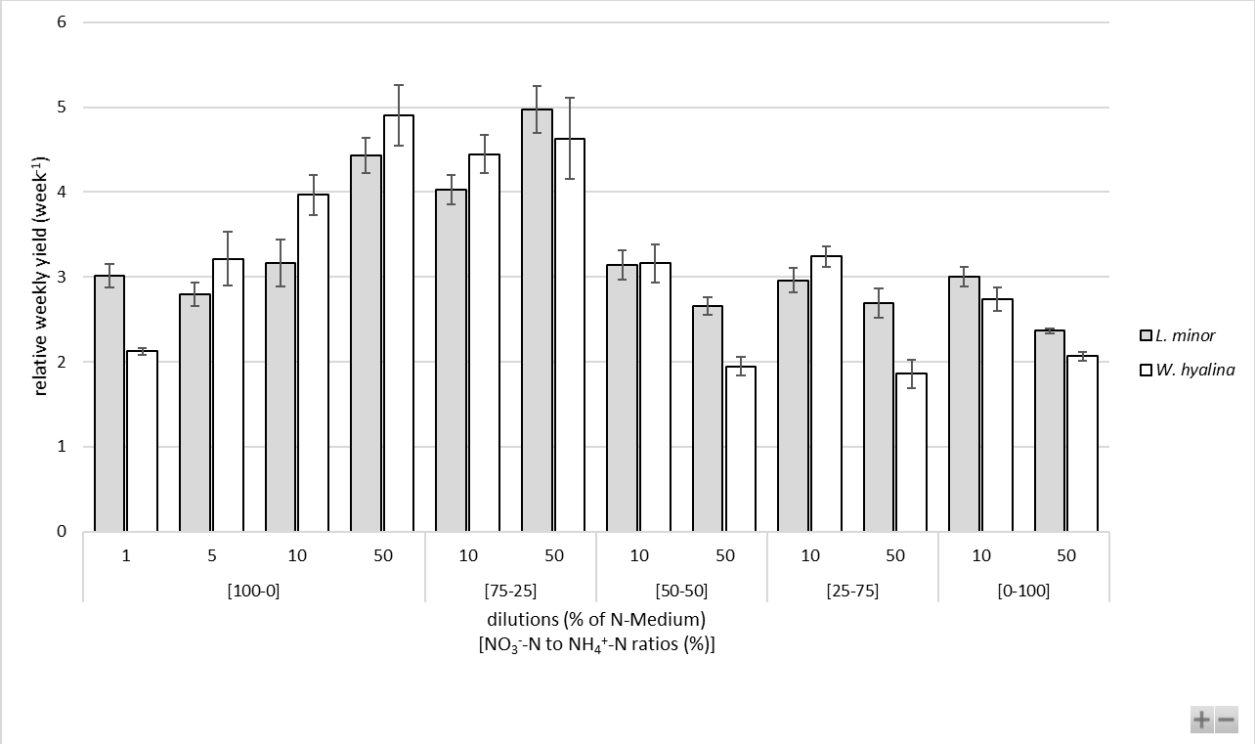

Figure S1: relative weekly yield (RY, week^{-1}) based on DW, for *L. minor* (grey shaded columns) and *W. hyalina* (white columns), cultivated for seven days in nutrient solutions with different NO_3^--N to NH_4^+-N ratios in different dilutions, based on N-medium. For further explanations, see Fig. 1.

Table S5: tap water analysis municipal utilities Osnabrueck Wittefeld

substance	unit	german drinking water ordinance limits	measured concentration
boron	mg L^{-1}	1	0.02
nitrate	mg L^{-1}	50	7.7
ammonium	mg L^{-1}	0.5	<0.02
chloride	mg L^{-1}	250	32
iron	mg L^{-1}	0.2	0.014
manganese	mg L^{-1}	0.05	-
sodium	mg L^{-1}	200	17.4
sulfate	mg L^{-1}	250	94
potassium	mg L^{-1}	-	3.26
calcium	mg L^{-1}	-	49.5
magnesium	mg L^{-1}	-	7.5

Manuscript II

Table S1. Formulation of seven stock solutions (g L^{-1}) for five different nitrate-N to ammonium-N ratios ([100-0], [75-25], [50-50], [25-75], [0-100]), based on the N-medium.

Stock solution	Product name	Main components	[100-0] (g L^{-1})	[75-25] (g L^{-1})	[50-50] (g L^{-1})	[25-75] (g L^{-1})	[0-100] (g L^{-1})
1	Calcinit	NO_3^--N, NH_4^+-N, Ca^+	47.2	35.4	23.6	11.8	0
1	Krista K Plus	NO_3^--N, K^+	161.8	121.3	80.9	40.4	0
2	NH_4Cl	NH_4^+-N, Cl^-	0	0	26.7	53.5	80.2
3	OCI Granular 2	NH_4^+-N, SO_4^{2-}	0	33	33	33	33
4	KCl	K^+, Cl^-	0	29.8	59.6	89.5	119.3
4	$CaCl_2 * 2\ H_20$	Ca^+, Cl^-	0	7.4	14.7	22.1	29.4
5	Krista MKP	PO_4^{3-}, K^+	27.2	27.2	27.2	27.2	27.2
6	Epso Combitop	Mg^{2+}, SO_4^{2-}, Mn^{2+}, Zn^{2+}	49.3	49.3	49.3	49.3	49.3
6	Borax	BO_3^{3-}	0.06	0.06	0.06	0.06	0.06
6	Mangaan	Mn^{2+}, SO_4^{2-}	0.44	0.44	0.44	0.44	0.44
6	$MoNa_2O_4 * 2\ H_20$	MoO_4^{2-}, Na^+	0.02	0.02	0.02	0.02	0.02
7	Ferty 72	Fe^{3+}	2.2	2.2	2.2	2.2	2.2

Manuscript III

Table S1: same as Table S1 in “Supplementary Material Manuscript I”

Table S2: same as Table S1 in “Supplementary Material Manuscript II”

11. Danksagung

Diese Dissertation wäre ohne die tatkräftige Unterstützung vieler fantastischer Leute gar nicht möglich gewesen.

Zuallererst möchte ich mich bei meinen Betreuern bedanken.

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