

Evaluation of the tolerance of duckweed (*Lemnaceae*) to abiotic stress factors associated with nutrient recycling systems via multispectral imaging

Lukas Audenaert^{a,b,*}, Marcella Fernandes de Souza^a, Noémie De Zutter^c,
Diederik P.L. Rousseau^b, Erik Meers^a

^a Ghent University, Department of Green Chemistry and Technology, Coupure Links 653, Ghent, Belgium

^b Ghent University, Department of Green Chemistry and Technology, Graaf Karel de Goedelaan 46, Kortrijk, Belgium

^c Ghent University, Department of Plants and Crops, Valentin Vaerwyckweg 1, Ghent, Belgium

ARTICLE INFO

Keywords:

Water Lentil
Stress Tolerance
Circular Economy
Novel Protein
Stripping/Scrubbing
Pig Manure

ABSTRACT

Lemnaceae, commonly known as the duckweed family, include the smallest flowering plants known to science. Although duckweed holds significant potential for wastewater treatment and nutrient recycling, abiotic stress factors prevailing in nutrient recycling systems can lead to die-off. Given the considerable inter-species and inter-clone morphological and physiological variability, this study aimed to assess the physiological and functional tolerance of 14 duckweed clones belonging to three different species (*Lemna minor*, *Spirodela polyrhiza*, and *Wolffia globosa*) to four abiotic stress factors: electrical conductivity (salinity stress), ammonium concentration, phosphorus concentration, and potassium concentration. In all experiments, the physiological and functional tolerance of duckweed was monitored using a multispectral imaging platform, which enabled the assessment of presymptomatic stress responses. The results demonstrated that *L. minor* clones exhibited greater physiological tolerance to the imposed stress factors, with clone *L. minor* 7022 emerging as the most tolerant. No health deterioration was observed upon exposure to high concentrations of phosphorus, ammonium, and potassium. This study uniquely demonstrates the use of multispectral imaging as a powerful and non-destructive tool for screening duckweed clones, offering a novel approach to optimize clone selection for its potential application in wastewater treatment and nutrient recovery systems.

1. Introduction

Duckweed or *Lemnaceae* is the smallest flowering plant and is widespread, growing on every continent except for Antarctica. Duckweed grows predominantly vegetatively, which allows it to duplicate its biomass in less than two days when grown in optimal conditions (Said et al., 1979). This family consists of five genera (*Lemna*, *Spirodela*, *Landoltia*, *Wolffia*, and *Wolffiella*) and 36 different species (Bog et al., 2019), with each species consisting of several clones. These different species and clones differ both morphologically and physiologically. For example, *Spirodela* is much larger than *Wolffia* (Xu et al., 2022), and unlike the other genera, *Wolffia* species are all rootless (Fig. 1).

Because of its almost exponential growth and versatility, duckweed

holds the potential to be used in a broad range of industries, e.g., food- & feed-industry, pharmaceutical industry, wastewater management industry, etc. (Coughlan et al., 2022). Due to its ability to grow on polluted wastewater and to accumulate nutrients and pollutants (phytoremediation), duckweed can be used in wastewater treatment plants, where industrial, agricultural, and municipal wastewater can be treated (Coughlan et al., 2022).

While duckweed can play a key role in valorising wastewater, the resulting biomass may offer a resource for animal feed applications. It contains a large amount of protein, ranging from 30 to 35 % of dry weight, which strengthens its applicability as a feed supplement (Verma & Suthar, 2015). In some Asian countries, duckweed is already used as food due to its high content of essential amino acids. Due to its

Abbreviations: ChlIIdx, Chlorophyll index; EC, electrical conductivity; Fv/Fm, Chlorophyll fluorescence; HS, Hoagland solution; LED, Light Emitting Diode; LM, *Lemna minor*; mARI, modified Anthocyanin reflectance index; RGB, Red-Green-Blue; RGR, Relative Growth Rate; Roi Mask, Biomass; SLF, Stripped Liquid Fraction; SP, *Spirodela polyrhiza*; WG, *Wolffia globosa*.

* Corresponding author at: Ghent University, Department of Green Chemistry and Technology, Coupure Links 653, Ghent, Belgium.

E-mail addresses: Lukas.Audenaert@UGent.be (L. Audenaert), Marcella.FernandesDeSouza@UGent.be (M. Fernandes de Souza), Noemie.DeZutter@UGent.be (N. De Zutter), Diederik.Rousseau@UGent.be (D.P.L. Rousseau), Erik.Meers@UGent.be (E. Meers).

<https://doi.org/10.1016/j.stress.2025.101198>

Received 6 August 2025; Received in revised form 9 December 2025; Accepted 22 December 2025

Available online 23 December 2025

2667-064X/© 2025 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

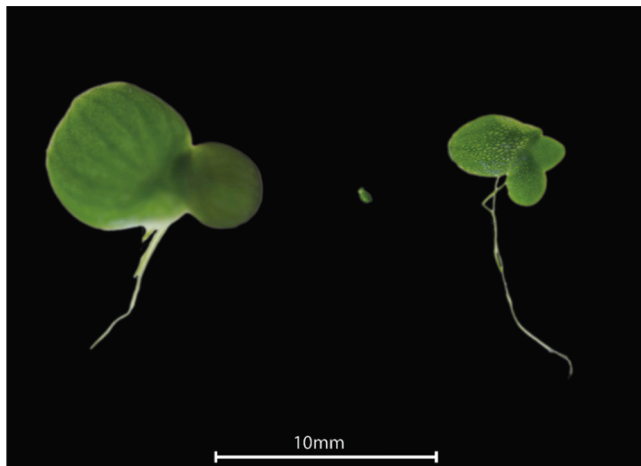


Fig. 1. Differences in morphology between the duckweed genera investigated in this study. From left to right: Spirodela, Wolffia, and Lemna.

advantageous composition, duckweed has the potential to replace other protein sources for feed, such as soy(bean) (Devlamynck et al., 2021).

This work focuses on the potential use of duckweed in wastewater treatment, specifically valorising the liquid fraction of pig manure. Currently, Europe is facing a huge manure problem. Annually, 1.4 billion tonnes of manure are produced in Europe and the United Kingdom (Euroobserver 2025). Locations with many intensive pig-rearing sites include Catalunya, Northern Italy, Germany, and Poland. Due to the excessive production of manure and the poor treatment thereof, agricultural run-off forms a real threat to the surrounding waterbodies and ecosystems. Another problematic region that has to cope with these problems and challenges is Flanders (Belgium). It has a high pig density, estimated at 5.4 million pigs in 2022 (ILVO 2025), which results in high amounts of pig manure being produced on a relatively small area (13,626 km²). A part of this manure can be land applied (max. 179 kg N/ha), but the surplus needs to be treated carefully. Recent studies have shown that duckweed might offer a solution to manage this surplus ([10], (Lambert et al., 2022)). However, even though these studies worked on quite diluted streams (a mixture of rainwater, biological effluent, and liquid fraction), duckweed die-off was observed at the end of the growing season in both studies.

Devlamynck et al. (Devlamynck et al., 2021) observed potassium accumulation in the duckweed pond at the end of the season, which caused duckweed die-off, while Lambert (Lambert, 2025) measured an increase in phosphorus concentration. Besides potassium and phosphorus, ammonium (-accumulation) also constitutes a problem for duckweed cultivation in nutrient recycling systems. In a recent study of Stadlander et al. (Stadlander et al., 2023), duckweed was cultivated on diluted chicken manure. At the end of their experiment, a decline in health and growth of the duckweed was observed, which was attributed to increased NH₄-N concentrations, ranging from 40 – 120 mg/L. It is indeed well known that ammonium can inhibit the growth of duckweed. Appenroth et al. (Appenroth et al., 1996) defined optimal growth of duckweed when using an N-medium where nitrogen is present only as nitrate. Finally, research indicates that the liquid fraction and the treated fraction of pig manure can be high in electrical conductivity (Lambert et al., 2022).

Therefore, this research investigated how electrical conductivity, ammonium, and phosphorus affect duckweed, as a step towards strengthening its potential application in such manure treatment systems. For this, a synthetic medium was spiked with the respective abiotic stressors. Different duckweed species and clones were selected to better understand how these abiotic stressors affect different duckweed and to select clones with improved physiological tolerance to the stressors for future application in a manure treatment system. The use of

multispectral imaging for these evaluations was chosen as a novel and non-destructive technique, and the selection process was validated with real manure to ensure relevance to the future intended application.

2. Material and methods

2.1. Duckweed clones and growth conditions

In this study, the effect of different abiotic stressors was evaluated on 14 duckweed clones (Table 1). The clones represented three duckweed species: *L. minor*, *S. polyrhiza*, and *W. globosa*, which significantly differ in morphology (Fig. 1).

The clones were cultivated on sterile modified Hoagland solution (HS) (Table 2; positive control). Depending on the abiotic stress imposed, as presented in overview Fig. 2, the HS was altered (Table 2 and Sections 2.2-2.3).

All experiments were carried out in 12-well plates, which were placed in a controlled growth chamber at 21 °C under sunlight LED modules (Phenovation, The Netherlands) at a light intensity of 250 μmol/m²/s with a photoperiod of 16 h.

2.2. Plant tolerance to electrical conductivity: NaCl

The physiological and functional tolerance of the 14 duckweed clones towards two NaCl concentrations (50 mM (eq. 7.4 mS/cm) and 100 mM (eq. 12.9 mS/cm)) was assessed as a proxy for increased electrical conductivity (EC). The EC values of 7.4 and 12.9 mS/cm were targeted to mimic the EC of the medium with biological effluent of pig manure (8 mS/cm) and effluent (14 mS/cm) as described by Lambert et al. (Lambert et al., 2022). As *L. minor* showed good physiological tolerance to the tested conditions, two higher NaCl concentrations were tested for this species, i.e., 150 and 200 mM. A formula correlating EC and NaCl was derived from Ali et al. (Ali et al., 2013) and is represented in Eq. (1). This formula was also verified during the experimental work, and the measured EC were 2.1, 7.4, 12.9, 17.9, and 23.9 mS/cm, respectively for the positive control, 50 mM, 100 mM, 150 mM, and 200 mM NaCl.

$$EC = 1.91 + 0.1271 \text{ (mM NaCl)} \quad (1)$$

The experiment was conducted for 14 days and the health of the duckweed clones was evaluated as described in Section 2.6.

2.3. Plant tolerance to electrical conductivity: KCl

In addition to increasing the EC with NaCl, EC-values were increased using KCl. KCl-concentrations were chosen to match the lower values tested with NaCl, i.e., 2.1, 7.5 and 12.8 mS/cm. The KCl concentration was increased to 0.05 mM, 45 mM, and 95 mM, respectively, resulting in potassium concentrations of 8.05 mM, 53 mM, and 103 mM. The control medium (positive control) was the same used during the NaCl experiment.

2.4. Tolerance test to increased ammonium (NH₄⁺) concentrations

The physiological and functional tolerance of 14 duckweed clones to elevated ammonium concentration was evaluated in a dose-response trial using two methods. In a preliminary experiment ammonium concentrations ranged between 10 mM and 42 mM, but complete duckweed death and chlorosis could be observed after 3 days of exposure. Firstly, nitrate was excluded from the medium, by which the ammonium was the only nitrogen source present (Table 2: Ammonium trial: 1 mM NH₄⁺, Ammonium trial: 3 mM NH₄⁺). Secondly, in parallel, nitrate was added to these ammonium solutions to investigate whether a certain amount of nitrate could alleviate the ammonium-toxicity. Nitrate was dosed as KNO₃ (NH₄⁺/NO₃⁻ = 3/1). In both methods potassium and calcium concentrations were maintained constant. The pH of the different

Table 1

Overview of the tested duckweed species and clones and their corresponding origin. The origin of some *Wolffia globosa* clones was not found in the database. The clones were provided by the Flemish Institute of Biotechnology (VIB).

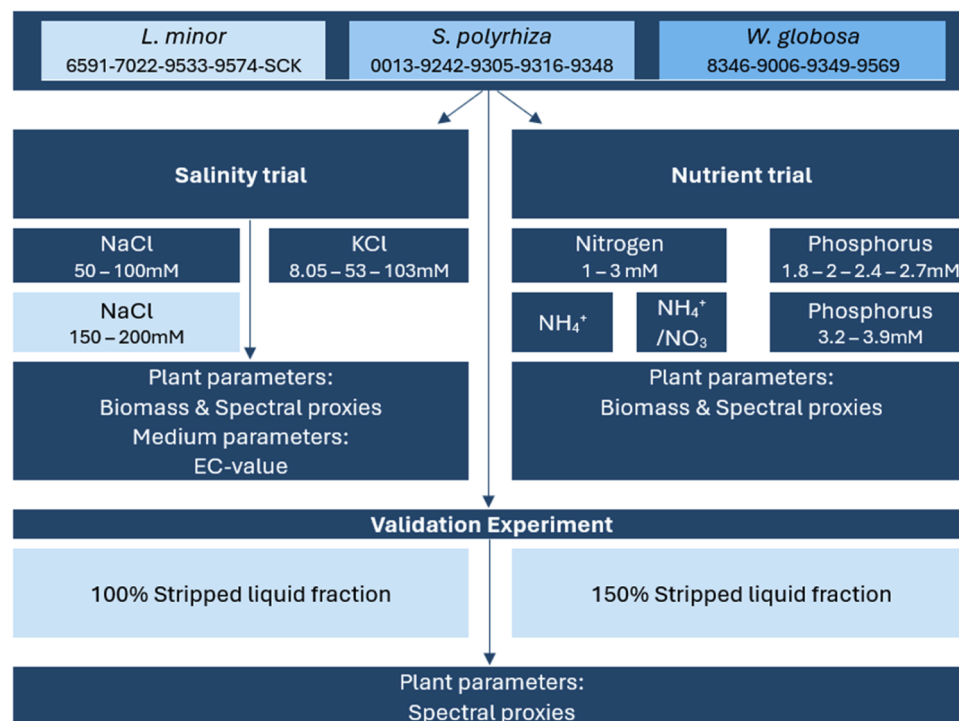
<i>Lemna minor</i>		<i>Spirodela polyrhiza</i>		<i>Wolffia globosa</i>	
Clone	Origin	Clone	Origin	Clone	Origin
6591	USA, California	0013	Vietnam, Hô Chi Minh	8346	-
7022	Spain, Cordoba	9242	Ecuador, Guayas	9006	-
9533	Macedonia, Krušje	9305	India, Andhra Pradesh	9349	Germany, Jena
9574	New Zealand, Southern Island	9316	India, Rajasthan	9569	-
SCK (5500)	County Cork, Blarney, Ireland	9346	Switzerland, Zürich		

Table 2

Composition of the different media across the different tolerance experiments. The positive control is used as a basis for the other treatments (composition derived from Ali et al. 2013). All numbers are expressed in millimolar (mM).

Component	Ammonium trial					Phosphorus trial		
	Positive control	1 mM NH ₄ ⁺	1 mM NH ₄ ⁺ +NO ₃	3 mM NH ₄ ⁺	3 mM NH ₄ ⁺ +NO ₃	Positive control	3.2 mM P	3.9 mM P
KNO ₃	6	-	0.33	-	1	6		
Ca(NO ₃) ₂ ·H ₂ O	4	-	-	-	-	4		
KH ₂ PO ₄	2					2	3.2	3.87
MgSO ₄ ·7H ₂ O	1					1		
MoO ₃	0.0005					0.0005		
H ₃ BO ₃	0.025					0.025		
FeNaEDTA	0.064					0.064		
KCl	0.05 ^a					0.05		
CuSO ₄ ·5H ₂ O	0.0005					0.0005		
ZnSO ₄ ·7H ₂ O	0.002					0.002		
MnSO ₄ ·H ₂ O	0.002					0.002		
K ₂ SO ₄	-	3	2.84	3	2.5	0.94	0.34	-
CaCl ₂	-	4	4	4	4	-		
(NH ₄) ₂ SO ₄	-	0.5	0.5	1.5	1.5	-		
NaCl	- ^a	-	-	-	-	-		

^a The concentrations of NaCl and KCl in the positive control were modified to reach the target EC values described in Sections 2.2 and 2.3.

**Fig. 2.** Flow-chart with the experimental layout of the material and methods.

treatments amounted around 5.

2.5. Phosphorus tolerance tests

Lastly, the impact of elevated phosphorus concentrations on the growth and health of duckweed was studied. In a preliminary test, the concentration range comprised of: 1.8 mM (55 mg P/L), 2 mM (P-concentration of the negative control, 62 mg P/L), 2.4 mM (75 mg P/L), and 2.7 mM (85 mg P/L). These dosages did not affect the evaluated duckweed clones. In a second experiment, the phosphorus concentration was increased up to 3.2 mM (100 mg P/L, [Table 2: Phosphorus trial: 3.2 mM P](#)) and 3.9 mM (120 mg P/L, [Table 2: Phosphorus trial: 3.9 mM P](#)).

2.6. Validation experiment

To confirm the observed outcomes, a small validation experiment, on actual stripped liquid fraction of pig manure, was conducted. Therefore, two duckweed clones were tested for their behaviour and physiological and functional tolerance towards actual pig manure effluent. In view of the results, these two clones belonged to the species of *Lemna minor*, due to their best physiological tolerance towards the investigated stressors. The chosen clones were 7022 and 9533, where 7022 was determined as most optimal, and 9533 as least tolerant within the species of *L. minor*. The tested conditions were based on the nitrogen content of the synthetic medium, and amounted 1:9 (100 % SLF, based on the nitrogen content of the synthetic medium control, [Table 2](#)) and 1:6 (150 % SLF, contains 1,5 times the nitrogen content of the synthetic medium control) ([Table 3](#)). The specific properties of the stripped fraction are shown in [Table 3](#). The pH was adapted to 6.1 ± 0.1 to mitigate the formation of ammonia (NH₃) with a 1 M H₂SO₄-solution (pKa = 9.25). Three synthetic media, the controls, were added to the experiment: Control 100 % N corresponds to the positive control ([Table 2](#)), Control 150 % N contains the increased nitrogen content corresponding to the 150 % treatment (adapted with KNO₃ and Ca(NO₃)₂·H₂O), and lastly Control NH₄-N contains only ammonium as available nitrogen source (constant nitrogen concentration, adapted with ((NH₄)₂SO₄)). The experiment lasted 13 days, whereafter a multispectral analysis was performed.

2.7. Multispectral imaging

The growth- and health-analysis of the duckweed species was carried out using a multispectral imaging platform. This platform allowed the real-time visualization of various physiological plant traits, based on specific absorption, reflection, and emission patterns at a spatial resolution of 6 μm. A monochrome camera system, including a filter wheel, allows pixel-to-pixel acquisition of RGB values, chlorophyll fluorescence, and chlorophyll content (CropReporter, Phenovation). The

Table 3

Physical properties of the different stripped liquid fractions. SLF = stripped liquid fraction.

Component	Concentration: Raw SLF (mg/L)	100 % SLF (mg/L)	150 % SLF (mg/L)
TN	3625	402	604
NH ₄ ⁺	1838	204	306
P	135	15,0	22,5
Zn	12,8	1,4	2,14
Mg	64,8	7,2	10,8
Fe	34,8	3,87	5,80
Mn	5,5	0,61	0,91
Ca	213,5	23,7	35,6
Na	1091,7	121,3	182,0
K	3777	419	629,6
Al	3,06	0,34	0,51
EC (mS/cm)	25,31	4,5	6,3
pH	8,67	6,12	6,20

(adapted)

Table 4

Overview of the screened parameters: Fv/Fm, Chlorophyll index, anthocyanin index, and biomass, with corresponding explanation and formula. Fm = maximum fluorescence, F0 = minimal fluorescence, Fv = variable fluorescence. ρ = spectral reflectance.

Parameter	Formula	Meaning	Biochemical relevance	Reference
Fv Fm	$\frac{Fv}{Fm} = \frac{Fm - F0}{Fm}$	Chlorophyll fluorescence	Dark-adapted efficiency of photosystem II	(Baker, 2008)
ChlIdx	$\frac{\rho_{770}}{\rho_{710}} - 1$	Chlorophyll index (red edge)	Proxy for foliar chlorophyll content	(Gitelson et al., 2003)
mARI	$\left(\frac{1}{\rho_{550}} - \frac{1}{\rho_{710}} \right) * \rho_{770}$	Modified anthocyanin reflectance index	Proxy for anthocyanin content in the fronds	(Gitelson et al., 2009)
Roi Mask	-	Number of detected plant pixels	Approximation for biomass*	-

* Raw biomass was not measured in the experiments, however was approximated by means of the Roi Mask (chlorophyll pixels).

parameters with their corresponding formula and biochemical relevance, are listed in [Table 4](#).

To visualize the biochemical properties of the duckweed plants, at the end of each experiment, false-color images were made. Each image shows the specific parameter (Chlorophyll fluorescence, Chlorophyll index, Anthocyanin reflectance index) of the plant at a certain treatment. For the chlorophyll fluorescence (Fv/Fm), the higher index values are displayed as yellow, while lower index values correspond to orange and red. For the chlorophyll index (ChlIdx) and anthocyanin reflectance index (ARIIdx), a higher index value is represented by a brighter look.

Besides the broad spectrum of advantages of the multispectral platform, a main disadvantage must be highlighted. The multispectral camera is not able to detect dead plant tissue, resulting in an underestimation of the obtained results (Fv/Fm-values of zero are not detected). If this phenomenon was observed during one of the tests, this was indicated in the discussion part of the corresponding tolerance test.

2.8. Statistical data analysis

Graph generation and statistical data analysis were performed in RStudio (Version 4.3.2). Graphs were created using the function ggplot (packages ggplot2 and tidyverse). Treatments and control plants were statistically compared by means of one-way ANOVA followed by a post hoc Tukey test using the functions aov and TukeyHSD respectively (package stats). Normality and homoscedasticity assumptions were verified using diagnostic plots. In the case of normally distributed data, but heteroskedasticity, a post-hoc Games-Howell test was used. In the case of violation of the two parametric assumptions a Kruskal & Wallis test and post-hoc Dunn's test were used with Bonferroni-method. All hypotheses were tested with a significance level of 5 % (α = 0.05).

3. Results and discussion

3.1. Physiological tolerance to salt stress (NaCl) in duckweed is mainly species-dependent

To assess the variability in salt tolerance of duckweed at both species and clone levels, 14 duckweed clones related to three species were grown in a base medium modified with the addition of NaCl (0, 50, 100 mM) to increase its final electrical conductivity (EC).

The chlorophyll fluorescence (Fv/Fm) of the different clones at day 14, indicative of photosynthetic activity, is shown in [Fig. 3A](#). All *S. polyrhiza* clones showed a significant reduction in photosynthetic activity when exposed to 100 mM NaCl (eq. 12.9 mS/cm), as well as

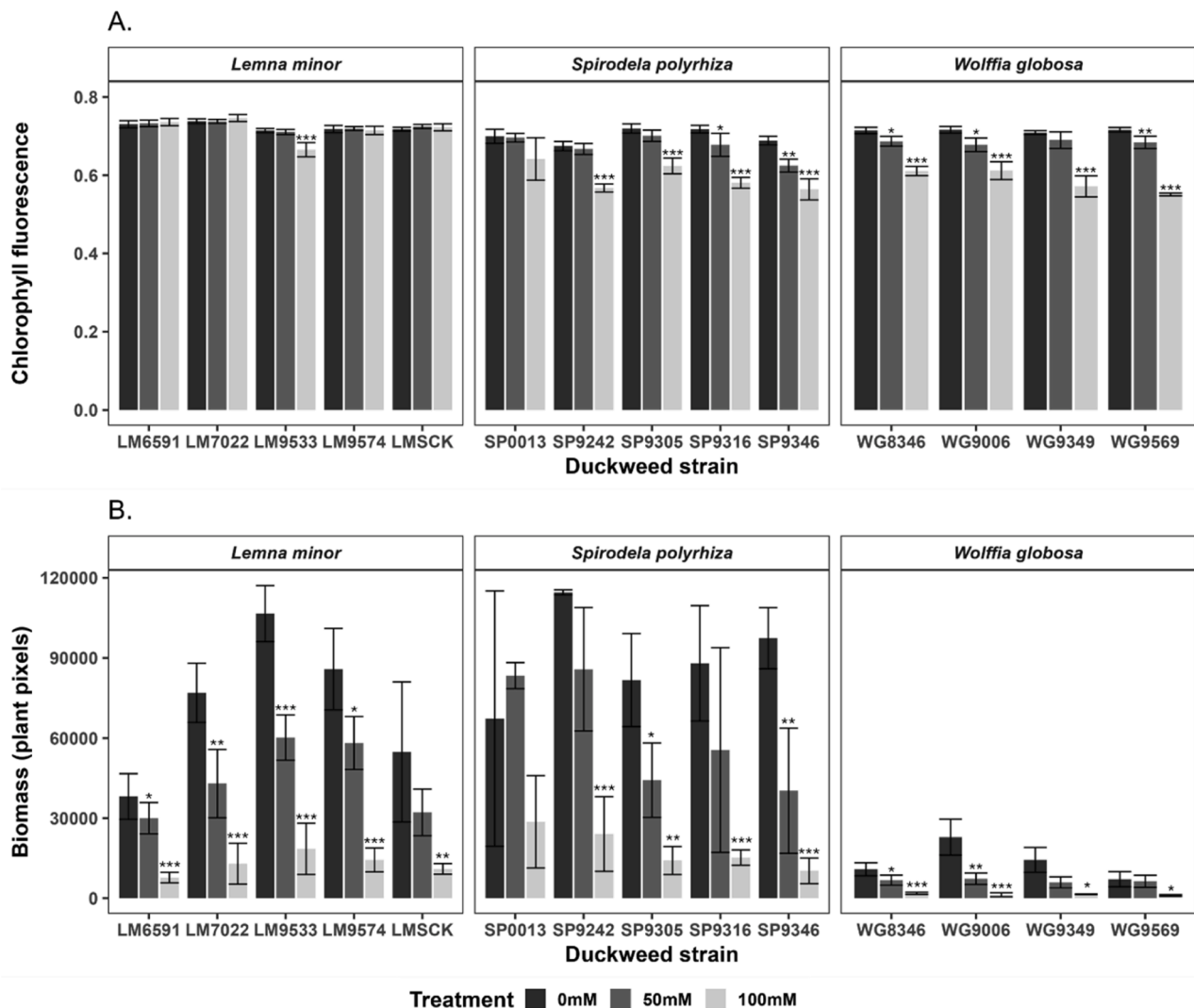


Fig. 3. A) Photosynthetic activity (Fv/Fm, chlorophyll fluorescence) and B) biomass (Roi Mask, amount of plant pixels, proxy for biomass) of different duckweed clones subjected to increasing NaCl concentrations, represented by the different grey shades in the graph. The graph is divided into three facets, where each species is represented in one facet. Values represent mean \pm sd of 4 replicates at 14 days after incubation. Significant differences in chlorophyll fluorescence values were indicated with asterisk and were determined in comparison with the control of each clone at this specific day of the experiment (*, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$). LM = *L. minor*, SP = *S. polyrhiza*, WG = *W. globosa*. 0 mM = 2.1 mS/cm, 50 mM = 7.4 mS/cm, 100 mM = 12.9 mS/cm.

macroscopic visible stress symptoms (Supplementary Material, Figure S1). The *W. globosa* clones did not show any macroscopic visible stress symptoms, but the significantly declining photosynthetic activity indicated presymptomatic stress symptoms, especially at the highest EC value tested (12.9 mS/cm).

The *L. minor* clones, except LM9533, proved to be more tolerant to 100 mM NaCl (eq. 12.9 mS/cm) and the concomitant increased EC-values, as no significant decrease in Fv/Fm values was observed. This experiment clearly showed that NaCl and EC physiological tolerance are both clone- and species-specific, with species being the dominant factor. The clone and treatment interactions were investigated with a two-way ANOVA, and interaction was observed ($p < 0.05$) for the spectral proxies.

Using the detected plant pixels as a proxy for plant biomass, the impact of increasing NaCl concentrations on plant growth was assessed (Fig. 3B). Although no clear negative effect on photosynthesis was observed in all but one *L. minor* clone, both NaCl concentrations caused a significant reduction in growth and complete growth inhibition was observed at 100 mM NaCl (eq. 12.9 mS/cm), with initial plant pixels of: LM6591, 4647; LM7022, 2911; LM9533, 7500; LM9574, 6013; LMSCK, 4361. This growth reduction pattern was also observed for the

S. polyrhiza and *W. globosa* clones. The functional tolerance could therefore be determined as insufficient for all evaluated clones.

To further assess the physiological and functional tolerance of *L. minor* clones to NaCl and the concomitant increase in EC, the NaCl concentration was increased up to 200 mM (eq. 23.9 mS/cm) for the four *L. minor* clones showing NaCl physiological tolerance in the previous test (LM6591, LM7022, LM9574, and LMSCK) (Fig. 4).

Six days after initiating the experiment, a significant health decline was observed for three *Lemna* clones (LM6591, LM9574, and LMSCK). Only *L. minor* 7022 did not show this decline and maintained acceptable health with average Fv/Fm-values of 0.712 ± 0.006 . After 10 days, a significant reduction in photosynthetic activity was observed even for clone 7022; however, chlorophyll fluorescence remained above 0.6, indicating that the plant's photosynthetic system remained healthy up to 16 days of exposure. Nevertheless, the four *L. minor* clones experienced severe growth inhibitions at both 150 mM (eq. 17.9 mS/cm) and 200 mM NaCl (eq. 23.9 mS/cm), similar to the previous experiment (Fig. 4B).

The phenotypes of the *L. minor* clones exposed to the higher NaCl concentrations are visualized in Fig. 5. For the photosynthetic activity (Fv/Fm), the red regions indicate stress; these are hardly present in

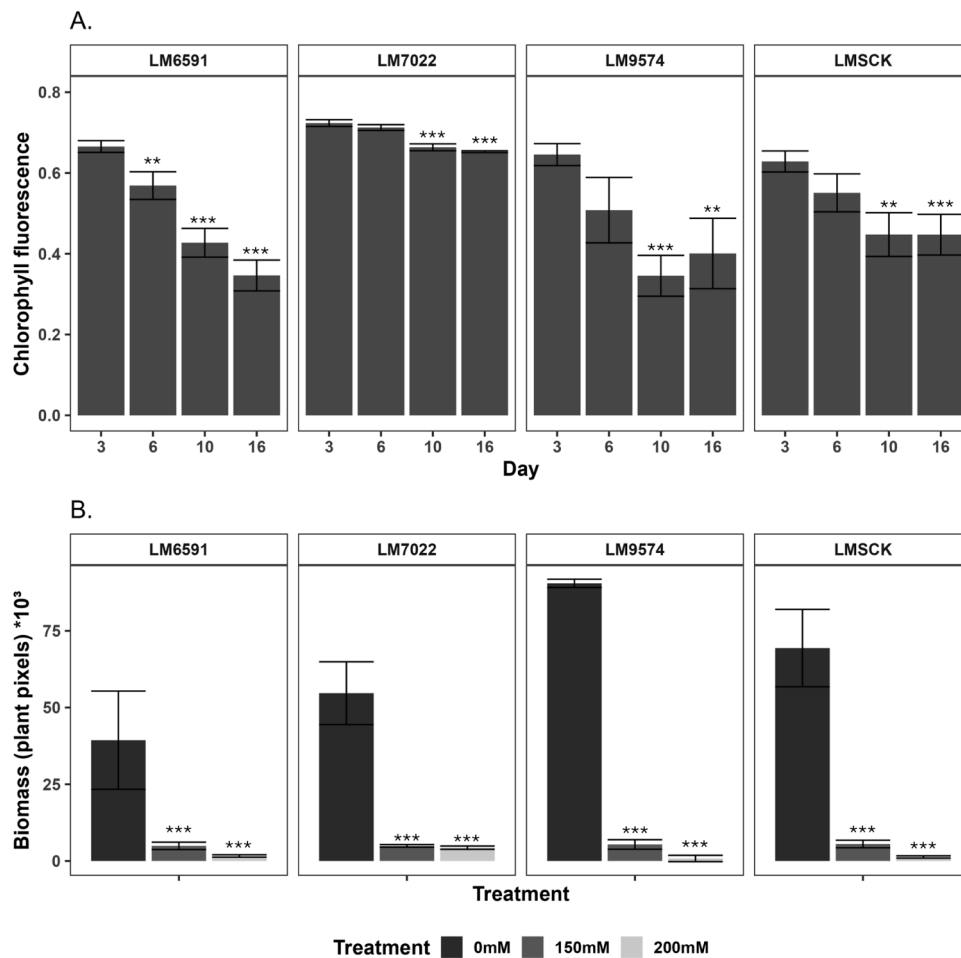


Fig. 4. A) Photosynthetic activity (Fv/Fm, chlorophyll fluorescence) of different duckweed clones subjected to 200 mM of NaCl (eq. 23.9 mS/cm) at 3, 6, 10, and 16 days after incubation. B) Biomass (Roi Mask, amount of plant pixels, proxy for biomass) of different *L. minor* clones subjected to increasing NaCl concentrations, represented by the different grey shades in the graph at day 16 of the experiment. Values represent mean ± sd of 4 replicates. The graph is divided into 4 facets, each corresponding to a different *L. minor* (LM) clone. The different grey shades correspond to the day of growth when the measurement took place. LM = *L. minor*. Significant differences in chlorophyll fluorescence values were indicated with “*”, and were determined in comparison with the first measurement of the experiment at 3 days of exposure (*, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$).

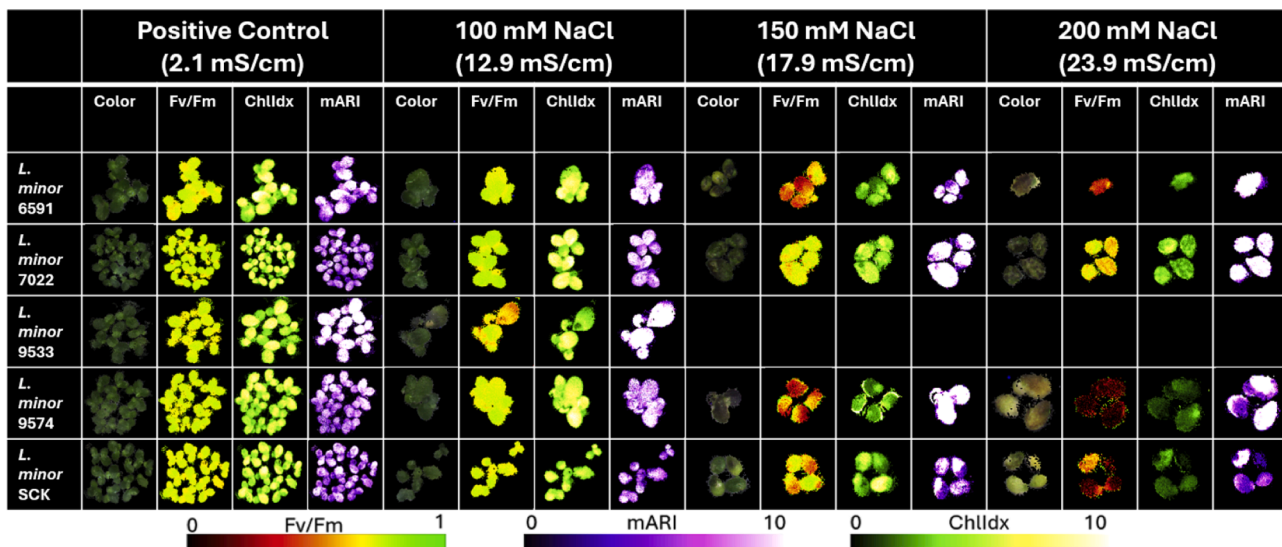


Fig. 5. Color and false-color of *L. minor* clones exposed to the different NaCl-concentrations at 14 days after exposure. Fv/Fm = chlorophyll fluorescence, proxy for photosynthetic activity; ChlI dx = Chlorophyll index, proxy for leaf greenness; mARI = modified anthocyanin reflectance index, proxy for foliar anthocyanin accumulation. The graph is based on the measurement of the corresponding parameters on the last day of the experiment (Day 14).

L. minor 7022 even when exposed to 200 mM of NaCl (eq. 24 mS/cm), confirming the higher physiological tolerance of this clone to salt stress.

Regarding the chlorophyll and anthocyanin concentrations, an increased level of both compounds is associated with a lighter appearance. Overall, increasing NaCl concentrations led to an increased anthocyanin and a reduced chlorophyll content in all evaluated clones, including 7022, further confirming that all clones were experiencing stress (Supplementary Material Figure S2). An increased anthocyanin concentration can enhance the plant's tolerance to stress (Li & Ahamed, 2023); therefore, the higher capacity for anthocyanin production in clone 7022 is possibly responsible for its higher physiological tolerance to stress. An additional explanation of the good physiological tolerance of clone 7022 to high salt concentrations is the fact that its sampling location (Cordoba) is situated within a high salinity region (Gil-Márquez et al., 2017). In contrast, *L. minor* clone 9533 originates from a non-saline region in North Macedonia, which resulted in a much lower physiological tolerance towards high salinity (Ivushkin et al., 2019). Because of the origin of clone 7022, its high-affinity carriers and transport systems may have undergone a conformational change whereby they can operate against the NaCl concentration gradient,

which would result in a more controllable ion ratio (K^+ transporters and efflux pumps) (Cushman, 2001).

Al-Dakhil et al. (Al-Dakhil et al., 2023) investigated the morphological and physiological responses of *S. polyrhiza* after being exposed to different NaCl concentrations ranging from 50 mM to 250 mM. Their results differ from the present research as *S. polyrhiza* did not show significant stress symptoms at 50 mM and 100 mM, which is in contrast with the observed health reductions in Fig. 3A. A possible explanation for this is that the sampled duckweed for their research comes from a high-salinity region (Saudi Arabia; Elhag, 2016)). Therefore, they might be more adapted to tolerate salts due to environmental pressure, indicating that acclimation or adaptive evolution of duckweed to salt stress might be possible.

Sree et al. (Sree et al., 2015) assessed 36 duckweed clones for their tolerance to NaCl ranging from 0 mM to 300 mM. In these tests, duckweed growth rates were, in general, significantly reduced when the NaCl concentration exceeded 50 mM, as observed in the present study. However, the authors also pinpointed *S. polyrhiza* clones as tolerant, which is in contrast with the results shown in Fig. 3A. The differences with this research are probably related to the different clones used, as we

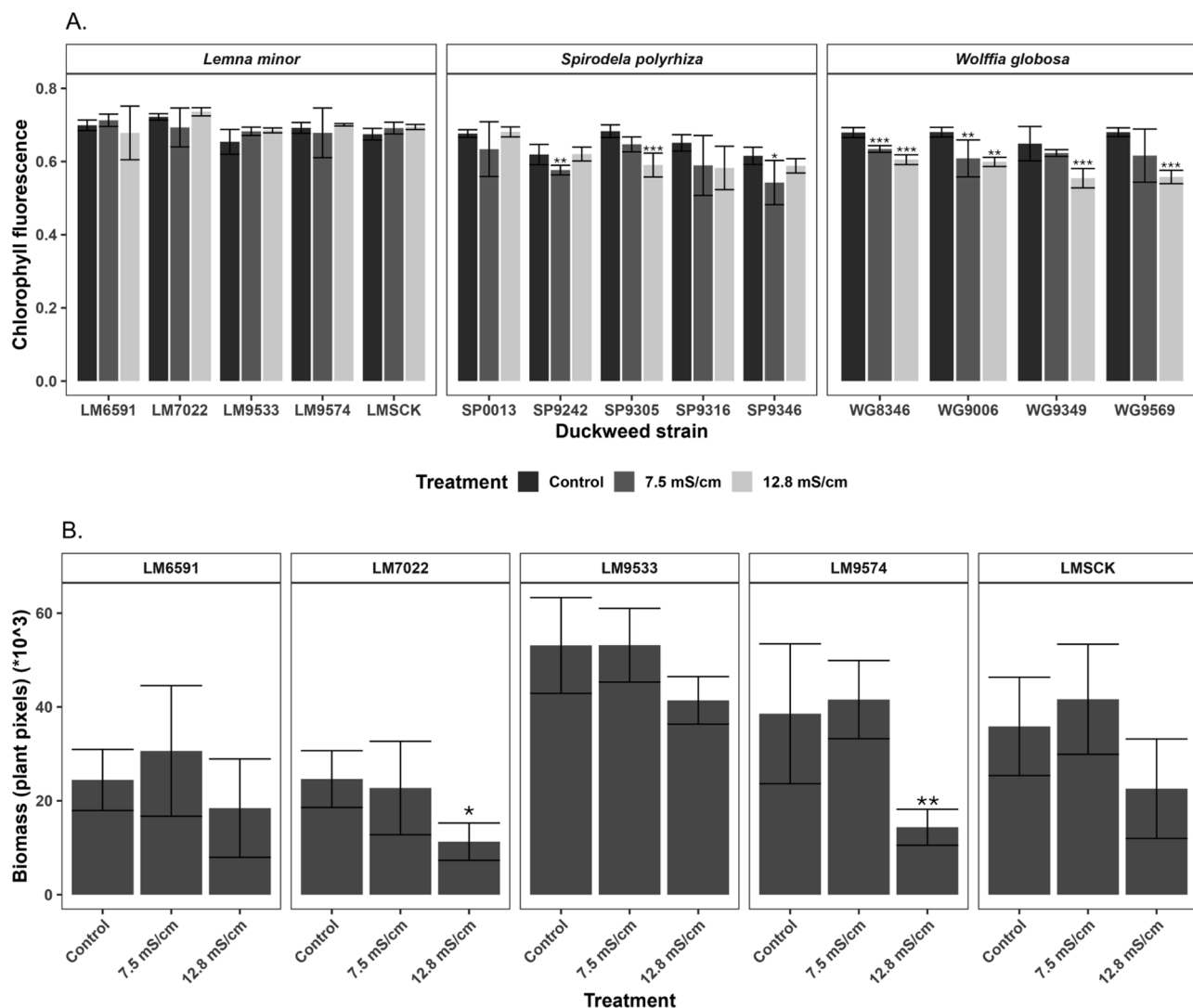


Fig. 6. A) Photosynthetic activity (Fv/Fm, chlorophyll fluorescence) and B) Biomass (Roi Mask, amount plant pixels, proxy for biomass) at 14 days after incubation of different duckweed clones subjected to increasing conductivities achieved with KCl, represented by the different grey shades. The graph is divided into different facets, where each species (A) or clone (B) is represented by one facet. Values represent mean \pm sd of 4 replicates. Significant differences were indicated with an asterisk and were calculated in comparison with the measurement of the control medium (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). LM = *Lemna minor*, SP = *Spirodela polyrhiza*, WG = *Wolffia globosa*.

could also state that salt tolerance is not only species- but also clone-specific. For the investigated *W. globosa* clones, the authors obtained the same result as the present study (Fig. 3), with a low physiological tolerance to salt stress. Regarding *L. minor* clones, they determined it a salt-tolerant species, tolerating NaCl concentrations of up to 200 mM, further confirming the present study.

In conclusion, we showed that *L. minor* had the highest physiological tolerance to NaCl compared to the other two duckweed species. On a clone level, *L. minor* 7022 was shown to be the most tolerant to increased electrical conductivities, even though its growth was affected even at the lowest tested EC values.

3.2. Duckweed tolerance to EC is cation-dependent

Devlamynck et al. (Devlamynck et al., 2021) monitored the agronomic performance of *L. minor* grown in a mixture of rainwater, liquid fraction of pig manure, and biological effluent of pig manure. In their study, they concluded that potassium accumulation had deteriorating effects on the health and growth of duckweed as it accumulated in the system above 2000 mg K/L at the end of the growing season. Therefore, a second salt tolerance test was conducted in this study in which the EC of the synthetic medium was increased with KCl. The assessed conductivities amounted to 7.5 and 12.8 mS/cm (KCl concentrations of respectively 45 mM and 95 mM), with an increased K concentration of 53 mM and 103 mM.

Regarding the chlorophyll fluorescence (Fv/Fm), similar conclusions can be drawn as in the NaCl experiment, i.e., *L. minor* clones were not affected by the increased conductivity of the medium, while some of the *S. polyrhiza* clones (9242, 9305, and 9346) and all *W. globosa* clones showed clear health reductions (Fig. 6A). However, in this experiment, the photosynthetic activity of *S. polyrhiza* 0013 and 9316 was not significantly reduced at the 12.8 mS/cm-treatment. Moreover, the impact of the increased EC on the health of the *Spirodela* and *Wolffia* clones was lower than that observed in Fig. 3A.

The chlorophyll index, which is related to the chlorophyll content, of the *L. minor* clones is shown in Fig. 6B The fronds exposed to 12.8 mS/cm

(95 mM KCl) had a significantly higher chlorophyll index compared to the positive control. This observed biological phenomenon is referred to as hormesis, in which a certain amount of stress can somehow stimulate the health or growth of the organism (Mattson, 2008).

The growth of the *L. minor*- and *W. globosa* clones was not significantly affected by the 7.5 mS/cm (eq. 53 mM K) (Fig. 6B and Supplementary Material, Figure S3). When the potassium concentration increased up to 12.8 mS/cm (eq. 103 mM), the growth of two *L. minor* (7022 & 9574) and two *W. globosa* clones (8346 & 9006) was significantly reduced. However, these observed reductions were to a much lesser extent than the ones during the NaCl test with similar electrical conductivities. The growth of almost all the *S. polyrhiza* clones was significantly reduced due to the applied potassium concentrations; only the growth of clone 9316 was not significantly reduced. The clone and treatment interactions were investigated with a two-way ANOVA, and interaction was observed ($p < 0.05$) for the spectral proxies.

To summarize the obtained results for the *L. minor* clones, a comprehensive figure was made that compares the four primary parameters analysed (Fig. 7). Despite the absence of significant health reductions, an increasing concentration of anthocyanin was observed when the conductivity was increased, indicating some level of stress.

The differences between both salinity tests (NaCl and KCl) can be explained by the fact that different ions have different effects on plants at the same value of EC, as Na^+ had a more detrimental effect than K^+ on the health and growth of the duckweed clones. This phenomenon was also observed by Lambert et al. (Lambert et al., 2022). Richter et al. (Richter et al., 2019) investigated the ion-dependent metabolic responses of fava beans to salt stress. They concluded that different ions, like Na^+ and K^+ , might induce different response mechanisms due to their different roles in cell metabolism. While sodium is not essential for most plants (Maathuis, 2014), potassium is an essential element for the neutralization of anions and macromolecules, the maintenance of the membrane potential, and pH homeostasis, among other functions (Nieves-Cordones et al., 2016). High sodium concentrations result in osmotic stress and competition with K^+ uptake (Blumwald et al., 2000). If the Na^+ concentration is high enough, it will eventually inhibit K^+

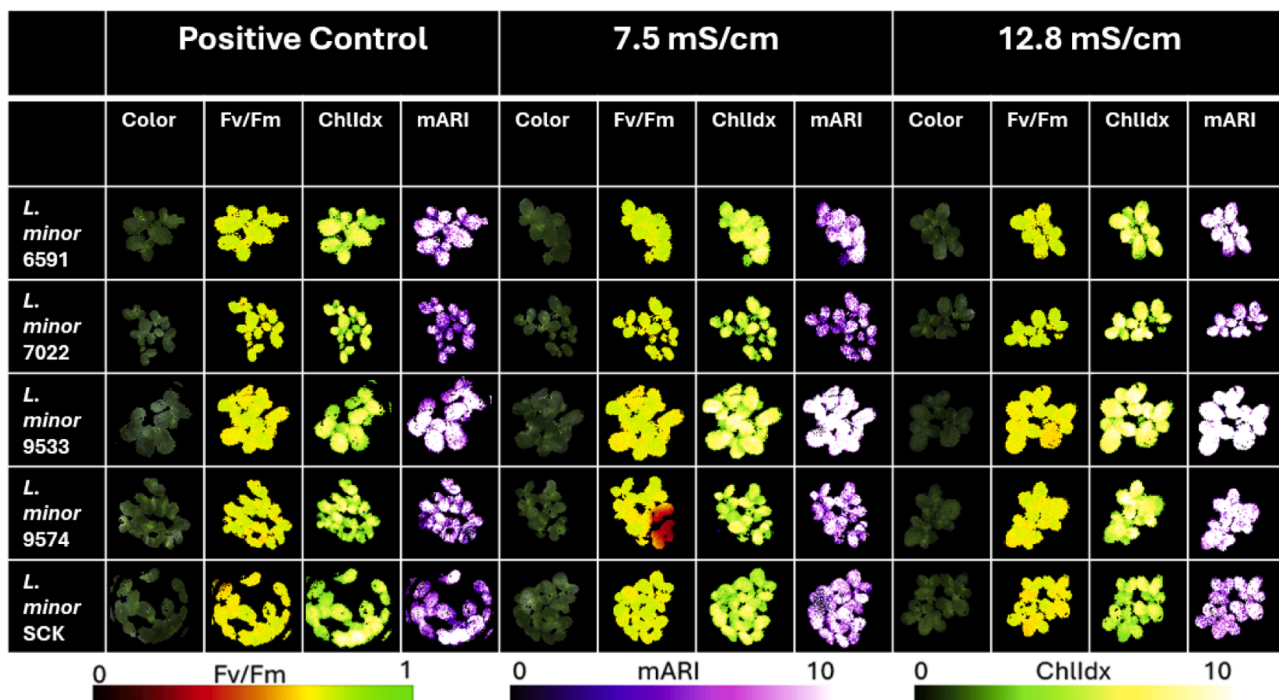


Fig. 7. Color and false-color image of *L. minor* clones exposed to the different electrical conductivities achieved with the addition of KCl, corresponding to respectively 0.05 mM, 45 mM, and 95 mM KCl at 14 days after exposure. Fv/Fm = chlorophyll fluorescence, photosynthetic activity; ChlIdx = chlorophyll index, proxy for leaf greenness; mARI = modified anthocyanin reflectance index, proxy for foliar anthocyanin accumulation.

uptake, causing metabolic disruptions.

By looking further into the research of Devlamynck et al. (Devlamynck et al., 2021) in light of our results, it may be concluded that potassium alone would not have been the reason why the duckweed cultivation eventually collapsed.

3.3. Ammonium tolerance is clone-dependent

Beyond salt stress, duckweed is also exposed to potentially high ammonium concentrations when grown on recycled nutrients. Therefore, a base medium was adapted by excluding nitrate and adding $(\text{NH}_4)_2\text{SO}_4$ to assess the physiological and functional tolerance of duckweed to elevated ammonium concentrations. Due to the complete health deterioration of all clones at 10 mM NH_4^+ (eq. 180 mg/L) in a preliminary test, the ammonium dosages were lowered to 1 mM (eq. 18 mg/L) and 3 mM (eq. 54 mg/L). The photosynthetic activity of duckweed clones at day 14 is shown in Fig. 8A.

Additionally, a nitrate treatment ($[\text{NH}_4^+]/[\text{NO}_3^-] = 3/1$) was added to assess the possible mitigation of the ammonium toxicity by the availability of a non-toxic nitrogen source. However, no mitigation activity of nitrate was observed (Supplementary Material, Figure S4).

All *S. polyrrhiza* and *W. globosa* clones showed significant health reductions when exposed to the applied ammonium treatments (Table 5). Focusing on the *L. minor* clones, inter-clone variability was observed. *L. minor* 9574 and SCK experienced the same response as the *Spirodela* and *Wolffia* clones, characterized by declining health after 6 days of ammonium exposure, which was also observed for the 6591 clone under the highest ammonium concentration, except for *L. minor* 7022 and 9533 (Table 5). Specifically for *L. minor* 7022, a slight decrease in photosynthetic activity, in comparison with the measurement on day 1, was observed with a stabilization after 10 days and without further reduction after 14 days (Table 5; Fig. 8).

The anthocyanin accumulation in evaluated duckweed clones is shown in Fig. 8B. Upon comparison between the two most resistant *L. minor* clones (7022 and 9533), the production of anthocyanins by clone 7022 is significantly lower than in clone 9533, suggesting that clone 7022 allocates less energy toward maintaining its physiological health.

All *S. polyrrhiza* clones were affected by the elevated ammonium concentrations, and a correlation between the production of anthocyanins and photosynthetic activity can be seen. For *S. polyrrhiza* 9242, a strong inhibition of the photosynthetic activity was seen at both ammonium treatments, while this was the clone with the lowest anthocyanin production. A distinction at the frond level can be made for the other *S. polyrrhiza* clones; the plant tissues with a lower Fv/Fm (red spots) have lower production of anthocyanins, as these clones are dying.

In general, plants exposed to a certain stressor experience a faster diffusion of Reactive Oxygen Species (ROS) across the membranes (Mittler, 2002). Anthocyanins have an antioxidant action as they scavenge the stress-induced ROS whereby they eventually reduce the oxidative stress (Xu & Rothstein, 2018).

No macroscopic stress symptoms were observed for the *W. globosa* clones during the experimental period. However, significant health reductions ($\sim\text{Fv}/\text{Fm}$) were observed for all clones at both ammonium treatments, which indicates the presence of presymptomatic stress.

Besides the chlorophyll fluorescence and anthocyanin production, the biomass was monitored as well (Fig. 8 and Supplementary Material Figure S5). Significant growth reductions were observed for almost all *S. polyrrhiza* and *W. globosa* clones, but only for one *L. minor* clone (SCK) in the highest ammonium concentration.

Huang et al. (Huang et al., 2013) investigated the effect of ammonium-induced oxidative stress on the health and growth of *L. minor*. They concluded that *L. minor* grew well in the presence of an ammonium concentration ranging between 0.5 mM and 3 mM. When the ammonium concentration was increased to 4 mM, mild chlorosis was observed. In the present study, chlorosis was already observed at 3 mM

for *L. minor* 9574 and SCK, but not for the other *L. minor* clones, further confirming intra-species differences in response to stress factors. The origin of the clones is of great importance, as clones sampled from an ammonium-containing waterbody, developed a certain physiological tolerance to ammonium. However, the ammonium concentrations in the waterbodies from which the clones used in this study were sampled are not known to the authors.

Chong et al. (CHONG et al., 2005) investigated the impact of elevated ammonium concentrations on *S. polyrrhiza*. They concluded that this species tolerates NH_4^+ concentrations up to 28.6 mM, which is much higher than the determined 1 mM NH_4^+ in the present study. The *S. polyrrhiza* clone used by Chong et al. (CHONG et al., 2005) was sampled from a waterbody already containing 2.86 mM NH_4^+ . Due to its origin, the duckweed might already be acclimated to the presence of ammonium, which explains the different outcomes when compared to the present study.

Zhou et al. (Zhou et al., 2021) concluded that *W. globosa* preferred ammonium over nitrate as a primary nitrogen source. The health and growth of *W. globosa* were not negatively affected when the concentration was increased up to 5 mM NH_4^+ . These observations were not confirmed by the present study, in which *W. globosa* clones showed presymptomatic stress, along with small reductions in growth at 3 mM NH_4^+ .

3.4. High tolerance to elevated phosphorus concentrations

In Flanders, a duckweed system for the treatment of pig manure was investigated in a previous study (Lambert, 2025). After one growing season, phosphorus accumulation was observed in the nutrient recycling system, accompanied by duckweed die-off, suggesting a correlation between the two occurrences. Therefore, in the current study two experiments were set up to assess the individual impact of increased phosphorus concentrations on the 14 studied duckweed clones.

At first the impact of 1.8 mM (eq. 55 mg P/L), 2.4 mM (eq. 75 mg P/L), and 2.7 mM (eq. 85 mg P/L) on the health and growth of the clones was investigated. At the end of the experimental period neither growth nor health reductions were observed. Hence, the phosphorus concentration was increased to 3.2 mM (eq. 100 mg P/L) and 3.9 mM (eq. 120 mg P/L) (Fig. 9).

S. polyrrhiza clones were proven to be the most susceptible to a phosphorus concentration of up to 3.9 mM (eq. 120 mg/L) ($p < 0.002$). Nevertheless significant health reductions were only observed at the highest phosphorus concentration. Within the species of *S. polyrrhiza*, only clone 9346 was able to maintain its health during the experimental period at both treatments. However, the biggest part of the fronds did not contain enough chlorophyll to be detected by the camera system, by which only the healthy tissues were monitored, resulting in an incorrect analysis and outcome. There were health reductions visible. Despite significant health reductions for *S. polyrrhiza* clones, no significant growth reductions were observed across the different phosphorus treatments, except for clone 0013 ($p < 0.05$) (Supplementary Material, Figure S6).

For the *L. minor* and *W. globosa* clones, no significant differences in anthocyanin accumulation were observed upon increased the phosphorus concentrations, indicating that the clones are easily maintaining their health (Supplementary Material, Figure S7A). Additionally, the chlorophyll content was high across all treatments, and was not further reduced with increased phosphorus concentrations (Supplementary Material, Figure S7B). Generally, no significant growth reductions were observed for *W. globosa* and *L. minor* clones ($p > 0.05$) (Supplementary Material, Figure S6). The color and false-color images of the *L. minor* and *W. globosa* clones are shown in the Supplementary Material, Figure S8. The clone and treatment interactions were investigated with a two-way ANOVA, and interaction was observed ($p < 0.05$) for the spectral proxies.

Landolt & Kandler (Landolt & Kandler, 1987) stated that the

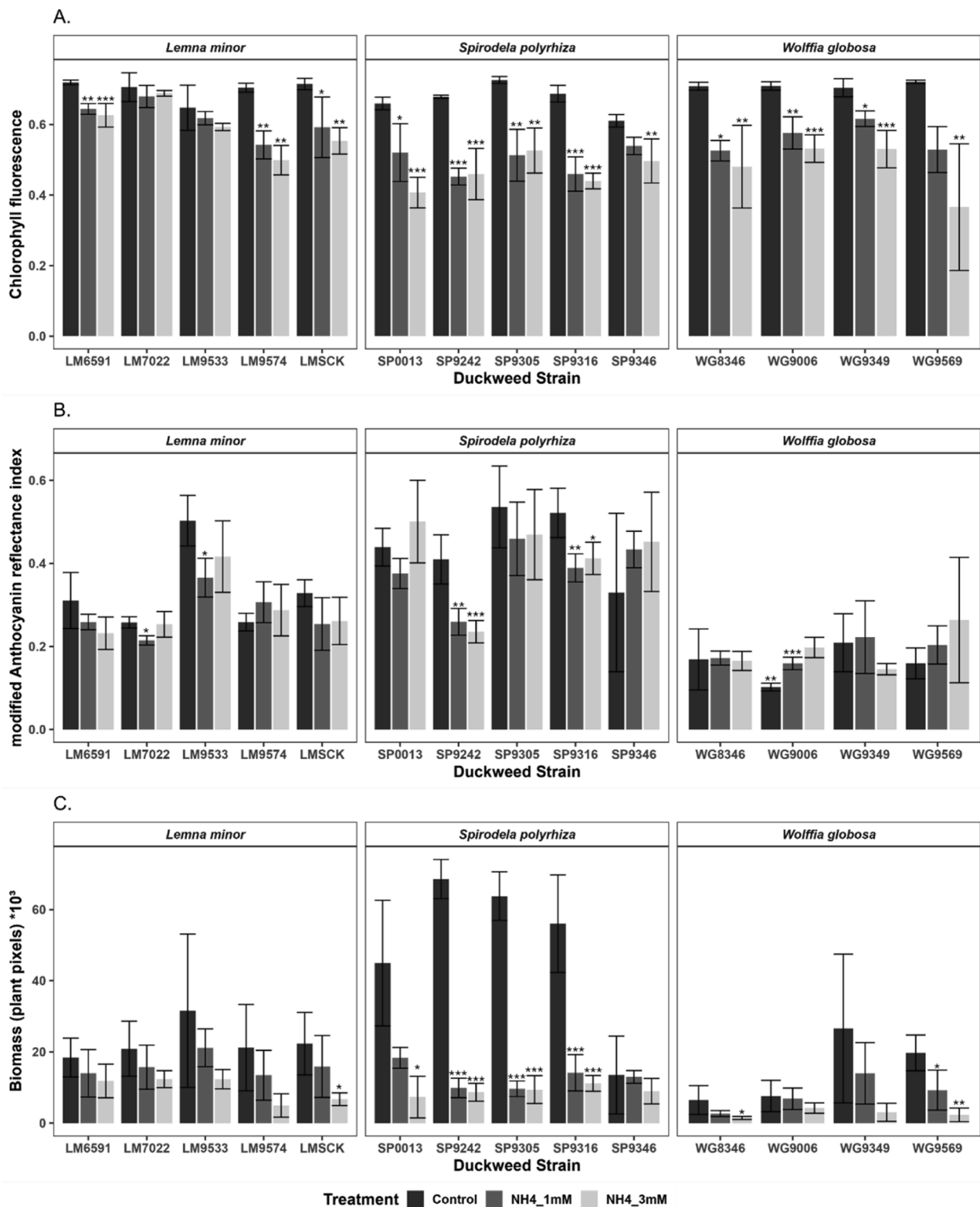


Fig. 8. A) Chlorophyll fluorescence (Fv/Fm, proxy for photosynthetic activity), B) Modified anthocyanin reflectance index (mARI, proxy for foliar anthocyanin accumulation) and C) Biomass (Roi Mask, amount of plant pixels, proxy for biomass) of the evaluated duckweed clones subjected to the ammonium concentrations at 14 days of incubation. Both graphs are divided into 3 facets, each corresponding to a different species: *L. minor* (LM), *S. polyrhiza* (SP), *W. globosa* (WG). The different grey shades correspond to the applied treatment. Values represent mean \pm sd. Significant differences were indicated with an asterisk and were calculated in comparison with the control (*, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$). 1 mM NH₄⁺ = 18 mg/L, 3 mM NH₄⁺ = 54 mg/L.

Table 5

Chlorophyll fluorescence (Fv/Fm, proxy for photosynthetic activity) at 3, 6 and 10 days after exposure at 3 mM NH₄⁺. Significant differences were indicated with an asterisk and were calculated per clone in comparison with the first measurement of the experiment at 3 days of exposure (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). LM = *Lemna minor*, SP = *Spirodela polyrhiza*, WG = *Wolffia globosa*.

Duckweed Clone	Day 3	Day 6	Day 10
<i>L. minor</i> 6591	0.697 ± 0.004	0.683 ± 0.019	0.642 ± 0.004***
<i>L. minor</i> 7022	0.718 ± 0.005	0.702 ± 0.013	0.698 ± 0.006
<i>L. minor</i> 9533	0.689 ± 0.010	0.677 ± 0.011	0.662 ± 0.014*
<i>L. minor</i> 9574	0.672 ± 0.011	0.624 ± 0.01	0.579 ± 0.028*
<i>L. minor</i> SCK	0.703 ± 0.01	0.692 ± 0.012	0.583 ± 0.039***
<i>S. polyrhiza</i> 0013	0.653 ± 0.04	0.628 ± 0.009	0.553 ± 0.074
<i>S. polyrhiza</i> 9242	0.630 ± 0.007	0.616 ± 0.016	0.523 ± 0.062
<i>S. polyrhiza</i> 9305	0.661 ± 0.035	0.628 ± 0.023	0.632 ± 0.025
<i>S. polyrhiza</i> 9316	0.693 ± 0.004	0.658 ± 0.022	0.534 ± 0.025***
<i>S. polyrhiza</i> 9346	0.654 ± 0.005	0.603 ± 0.028	0.621 ± 0.008
<i>W. globosa</i> 8346	0.633 ± 0.013	0.618 ± 0.005	0.570 ± 0.010**
<i>W. globosa</i> 9006	0.697 ± 0.017	0.704 ± 0.013	0.629 ± 0.015***
<i>W. globosa</i> 9349	0.630 ± 0.009	0.630 ± 0.003	0.583 ± 0.0255
<i>W. globosa</i> 9569	0.683 ± 0.017	0.670 ± 0.011	0.569 ± 0.029***

maximum tolerable phosphorus concentration in the medium, reducing the maximum growth rate by half, amounts to 1.75 mM (eq. 54 mg/L) for both *L. minor* and *S. polyrhiza*. In a more recent study Lasfar et al. (Lasfar et al., 2007) observed that, for a clone of *L. minor*, the intrinsic growth rate remained maximal up to 10 mg P/L with a slight reduction with an increasing phosphorus concentration up to 1.75 mM (eq. 54 mg P/L); however, at this higher concentration, the growth rate was not reduced by half, as reported by Landolt & Kandeler (Landolt & Kandeler, 1987). Additionally, in the present study, no significant growth reductions were observed when the phosphorus concentration was further increased from 1.75 mM (eq. 54 mg/L) to 3.9 mM P (eq. 120 mg/L) for a number of clones. These observations give further insight into duckweed

growth when the phosphorus concentration exceeds 1.75 mM (eq. 54 mg/L), and indicate that such higher concentrations do not negatively affect the photosynthesis, chlorophyll content, and growth of some duckweed clones, once more confirming the clone-specificity of the physiological tolerance of stress.

In contrast with the observed duckweed death in the mentioned nutrient recycling system (Lambert, 2025), which was pointed out to be caused by phosphorus, no health reductions were seen when only the phosphorus concentration was raised. Therefore, phosphorus accumulation was probably not the only cause for the observed duckweed die-off.

3.5. Validation: stripped liquid fraction of pig manure

In the validation experiment, two *Lemna minor* clones, 7022 and 9533, were cultivated on two different dilutions of stripped liquid fraction of pig manure. These were chosen due to their high and low physiological tolerance as observed in the previous tests. The results of the experiment are shown in Figs. 10 and 11, and the significant differences were calculated in comparison with the corresponding control.

When exposed to the treatment of 100 %-SLF for 13 days, no significant health reductions could be observed for *L. minor* clone 7022, while clone 9533 experienced a significant health reduction when compared to the Control 100 % N. Additionally, the *L. minor* 7022 clone had a significantly higher ($p = 0.00374$, TukeyHSD) chlorophyll fluorescence (photosynthetic activity, Fv/Fm) than the *L. minor* clone 9533 at the treatment of 100 % SLF.

Despite its lower photosynthetic activity, the chlorophyll content and anthocyanin production of *L. minor* clone 9533 were not significantly different from *L. minor* clone 7022. Nevertheless, in comparison with the controls, the chlorophyll content of clone 9533 was significantly reduced ($p < 0.001$, TukeyHSD) after 13 days of exposure at the

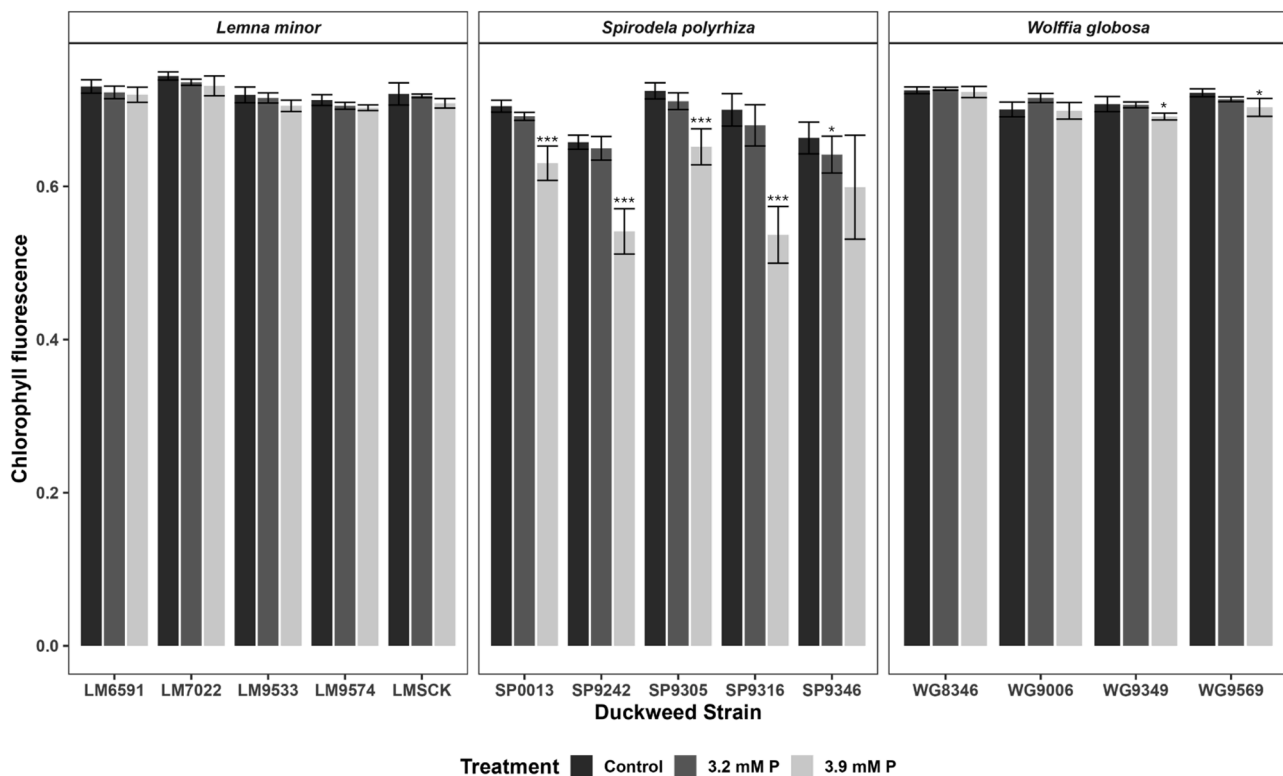


Fig. 9. Chlorophyll fluorescence (Fv/Fm, proxy for photosynthetic activity), at 14 days after incubation of different duckweed clones subjected to increasing phosphorus concentrations, represented by the different grey shades in the graph. The graph is divided into three facets, each corresponding to the evaluated duckweed species. Values represent mean ± sd. Significant differences were determined in comparison with the control of each clone at this specific day of measurement (*, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$). LM = *L. minor*, SP = *S. polyrhiza*, WG = *W. globosa*. 3.2 mM (eq. 100 mg P/L) and 3.9 mM (eq. 120 mg P/L).

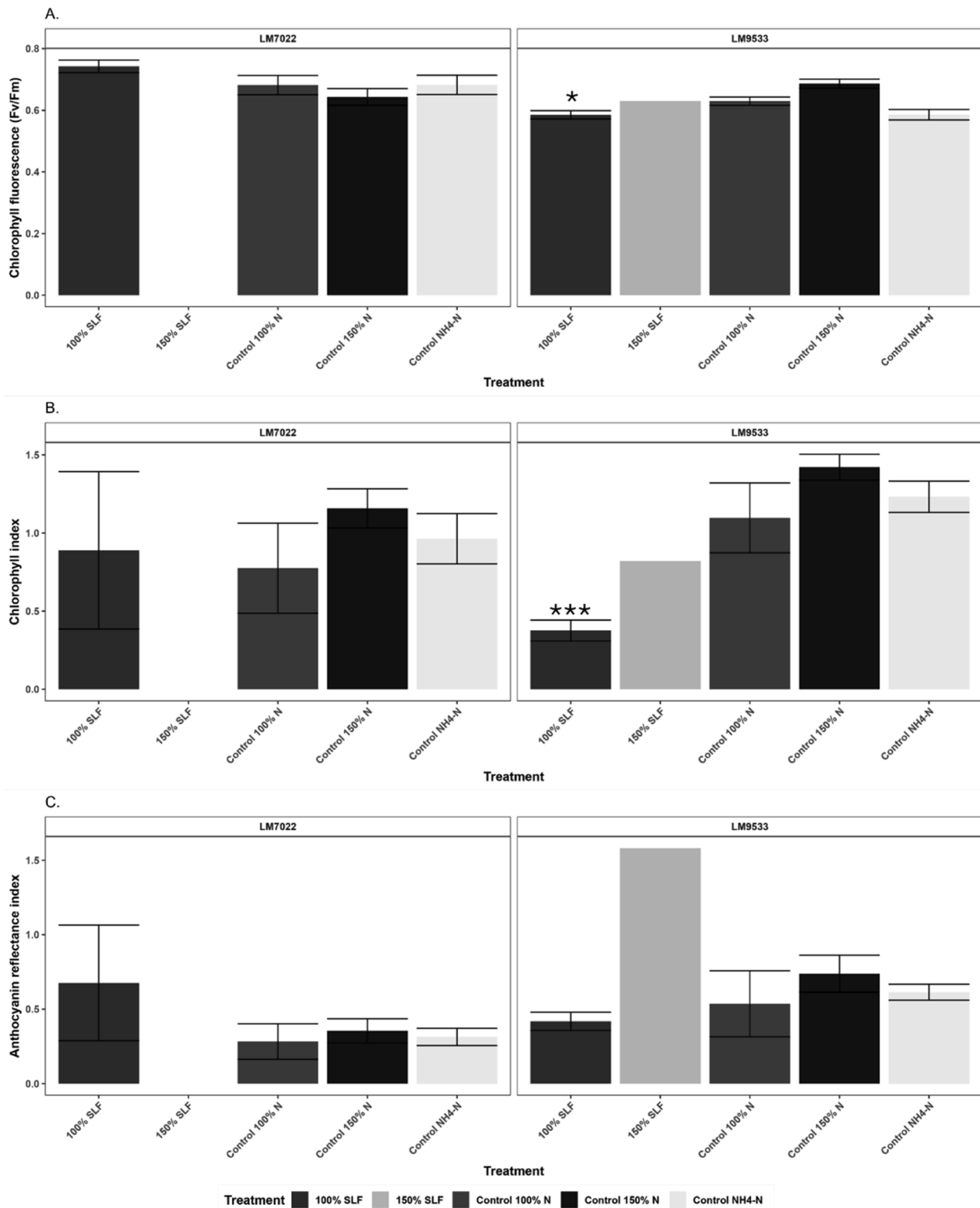


Fig. 10. Chlorophyll fluorescence (Fv/Fm, proxy for photosynthetic activity), B) Chlorophyll index (ChlIdx, proxy for chlorophyll content), C) Modified anthocyanin reflectance index (mARI, proxy for foliar anthocyanin accumulation) after 13 days of exposure to the stripped liquid fraction. The 100 % SLF (NH4-N), Control 100 % N and Control NH4-N have the same nitrogen concentration of ± 200 mg/L, while the 150 % SLF (NH4-N) and Control 150 % N have the same nitrogen concentration of ± 300 mg/L. LM = Lemna minor, SLF = Stripped liquid fraction. Values represent mean ± sd of 3 replicates. Due to the complete death and chlorosis of the *L. minor* 7022 fronds at 150 % stripped liquid fraction, no bar is plotted. Significant differences were determined in comparison with the corresponding control of each clone at this specific day of measurement (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$).

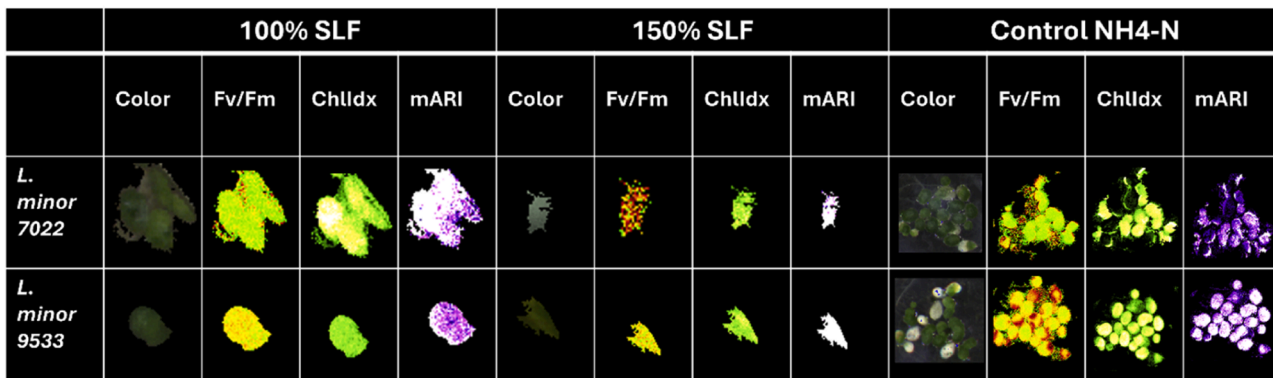


Fig. 11. Color and false-color image of the two *L. minor* clones exposed to the different dilutions of stripped liquid fraction of pig manure at 13 days after exposure. The 100 % SLF (NH4-N), Control 100 % N and Control NH4-N have the same nitrogen concentration of ± 200 mg/L, while the 150 % SLF (NH4-N) and Control 150 % N have the same nitrogen concentration of ± 300 mg/L. Fv/Fm = chlorophyll fluorescence, photosynthetic activity; ChlIIdx = chlorophyll index, proxy for leaf greenness; mARI = modified anthocyanin reflectance index, proxy for foliar anthocyanin accumulation. LM = Lemna minor, SLF = Stripped Liquid Fraction.

100 % SLF-treatment. These observed phenomena confirm the outcomes of the tolerance tests, validating that *L. minor* clone 7022 is more capable of maintaining its health when grown on these specific agricultural wastewaters.

For both clones, complete death and chlorosis were observed after 14 days when exposed to the treatment of 150 % stripped liquid fraction (SLF); for *L. minor* 7022, insufficient (active) biomass was detected for a multispectral analysis (Figs. 10 and 11). For *L. minor* clone 9533, only a limited amount of biomass was detectable for a multispectral analysis, whereby the measured spectral proxies are not representative of the actual physicochemical properties of the fronds. Therefore, the

statistical analysis was not performed on this specific treatment.

Focusing on the biomass, growth was observed for both clones in the 100 % SLF treatment. However, a growth reduction was observed when compared to the Control 100 % N of respectively 54 % and 41 % for *L. minor* 7022 and *L. minor* 9533 (data not shown). For the 100 % SLF treatment, the relative growth rates (RGR) estimated respectively 0.0527 and 0.0684. Nonetheless, the RGR of *L. minor* 7022 at 100 % SLF was 18 times higher than the RGR at the Control NH4-N.

As expected and described in Section 3.3, both clones were clearly stressed when exposed to the medium with ammonium as the only nitrogen source (Control NH4-N, Fig. 11). These health reductions were

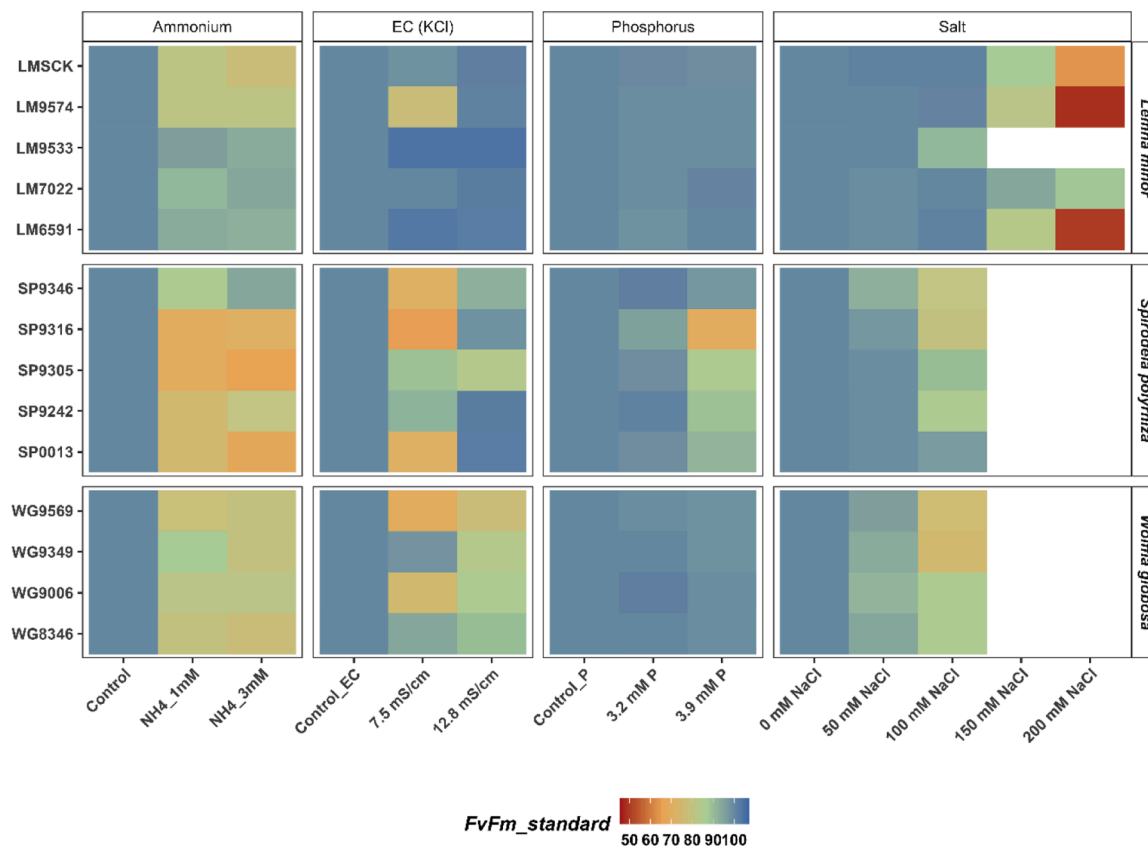


Fig. 12. Heatmap of the screened parameters for the impact on the chlorophyll fluorescence (Fv/Fm, proxy for the photosynthetic activity) of the 14 duckweed clones. The graph is divided based on duckweed species and the investigated parameter. How darker the square, the more resistant the clones to the specific abiotic stress factor. LM = *L. minor*, SP = *S. polyrrhiza*, WG = *W. globosa*.

not seen when the clones were grown on the 100 % SLF (containing the same concentration of ammonium). Moreover, in the ammonium tolerance tests, 3 mM was the threshold value for both clones, while in the validation experiment, the clones maintained their health at ammonium concentrations of 11 mM. This can be explained by the fact that (treated) pig manure is a complex organic matrix, in which interactions between the different components can have an impact in the toxicity of a certain stressor.

Based on this validation experiment, the results of the tolerance tests were preliminarily confirmed and point to the importance of the use of a specific clone in nutrient recycling systems. Nevertheless, further validation is necessary in a more realistic system, in which duckweed is harvested constantly to favor its continuous growth and nutrient uptake.

3.6. Further guidelines for a nutrient recycling system

Heatmaps were made summarizing the obtained results, based on the chlorophyll fluorescence (Fv/Fm, Fig. 12) and the biomass (Roi Mask, Supplementary Material, Figure S9) at the last day of each experiment. Both heatmaps are standardized, meaning that the control was considered as 100 %. In none of the experiments, a complete growth inhibition was seen, except in the NaCl experiment (Supplementary Material, Figure S9), although the *L. minor* plants were still considered healthy (Fv/Fm > 0.7). The extreme conductivities tested represent raw pig manure and raw stripped pig manure, while in wastewater treatment systems, duckweed will never be grown on pure liquid fraction, but always on more diluted one, thereby mitigating complete growth inhibition.

Based on the research outcomes (Fig. 12), the ionic stress was mainly determined at the species level while the ammonium-induced stress was mainly clone-dependent. A possible hypothesis for this observation is that physiological salt tolerance mainly lies in mechanisms like ion transporters and ion homeostasis, which control the abiotic stress (Duque et al., 2013). On the other hand, ammonium stress is more complex and relies on enzyme regulation and reactive oxygen species, which can differ between different clones (Shilpha et al., 2023).

Duckweed can be introduced as a sustainable alternative in wastewater treatment due to its bioremediating activity. However, as pointed out in the present study, inter-species variability is a dominant factor when it comes to the exposure to certain stressors. Wastewaters from different origins often contain elevated concentrations of specific nutrients, depending on their source. To assure good health and growth of the duckweed, the provided heatmaps may serve as a framework to know which species to use.

Based on the present study, *S. polyrhiza* clones were determined as the least optimal for wastewater treatment due to the declining health in each experiment, followed by growth limitations. The *Wolffia* clones mainly showed presymptomatic stress, which was confirmed by the absence of macroscopic health issues but accompanied by a declining photosynthetic activity. The good health and growth of the *L. minor* clones in the present study confirm that this duckweed species is well-suited for use in nutrient recycling systems with elevated concentrations of phosphorus and potassium (and corresponding elevated conductivities).

In general, both ammonium and high NaCl concentrations were shown to be the most detrimental factors for the growth, regardless of the evaluated clone or species. These parameters need to be properly managed in nutrient recycling systems to ensure beneficial health and growth of the used duckweed clone.

Based on the chosen stress conditions, number of clones, monitored parameters, and outcomes, the present work innovates in methodology and the evaluation of candidate duckweed clones for wastewater treatment. Nevertheless, further research is necessary to couple these outcomes to the mechanistic understanding of the duckweed stress physiology to potentially guide the improved use of duckweed in nutrient recovery systems.

4. Conclusion

The present study aimed to investigate and visualize the inter-species and inter-clone variability in the family of *Lemnaceae* for their physiological and functional tolerance towards stressors related to nutrient recovery systems. The physiological tolerance of duckweed was shown to be mainly dependent on species rather than on clone level. However, inter-clone variability was observed when the clones were exposed to higher ammonium concentrations. On the species level, *L. minor* clones were proven to be the most tolerant, while the clone *L. minor* 7022 showed the highest physiological tolerance. This clone showed only minor growth reductions, with no health deterioration, at high concentrations of potassium and phosphorus. Its growth was only drastically reduced when exposed to high NaCl concentrations, where only 15 % of its growth was maintained in comparison to the control. Its high photosynthetic activity was combined with a relatively low production of anthocyanins, which states that this plant easily maintains its health in extreme conditions. The inter-clone variability and the physiological tolerance of *L. minor* 7022 were further confirmed when clones were grown on stripped pig manure, indicating that multispectral imaging is a useful measurement technique for duckweed clone screening under induced stress conditions. Furthermore, this research strengthens the potential application of duckweed in manure treatment systems.

CRedit authorship contribution statement

Lukas Audenaert: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marcella Fernandes de Souza:** Writing – review & editing, Supervision, Software, Methodology, Conceptualization. **Noémie De Zutter:** Writing – review & editing, Visualization, Supervision, Software, Methodology. **Diederik P.L. Rousseau:** Writing – review & editing, Supervision, Software, Resources, Project administration. **Erik Meers:** Writing – review & editing, Supervision, Software, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Erik Meers reports financial support was provided by Horizon Europe. Noemie De Zutter reports financial support was provided by Hercules Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study has been developed within the framework of the European Union HORIZON—CL6-2021-ZEROPOLLUTION-01 Grant Agreement No.101060455 (NutriBudget project). The authors acknowledge the financial support from the Hercules Foundation of the Flemish Government for the multispectral imaging platform (AUGE/15/17). We thank Quinten Bafort and the Flemish Institute of Biotechnology (VIB) for the delivery of the different duckweed species and clones. We thank Trang Tran Minh for the creation of Figure 1. We thank Detricon for the delivery of the stripped liquid fraction of pig manure.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.stress.2025.101198](https://doi.org/10.1016/j.stress.2025.101198).

Data availability

Data will be made available on request.

References

- Al-Dakhil, M., Ben Romdhane, W., Alghamdi, S., Ali, A.A.M., 2023. Differential morpho-physiological and biochemical responses of duckweed clones from Saudi Arabia to salinity. *Plants* 12 (18), 3206. <https://doi.org/10.3390/plants12183206>.
- Ali, Z., Khan, D., Ahmed, N., 2013. Salt tolerance of three Sorghum cultivars during germination and early seedling growth. *Int. J. Biol. Biotechnol.* 10 (2), 193–202.
- Appenroth, K.-J., Teller, S., Horn, M., 1996. Photophysiology of turion formation and germination in *Spirodela polyrrhiza*. *Biol. Plant* 38, 95–106. <https://doi.org/10.1007/BF02879642>.
- Baker, N.R., 2008. Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annu. Rev. Plant Biol.* 59, 89–113. <https://doi.org/10.1146/annurev-arplant.59.032607.092759>.
- Blumwald, E., Aharon, G.S., Apse, M.P., 2000. Sodium transport in plant cells. *Biochim. Biophys. Acta (BBA)-Biomembr.* 1465 (1–2), 140–151. [https://doi.org/10.1016/S0005-2736\(00\)00135-8](https://doi.org/10.1016/S0005-2736(00)00135-8).
- Bog, M., Appenroth, K.-J., Sree, K.S., 2019. Duckweed (Lemnaceae): its molecular taxonomy. *Front. Sustain. Food Syst.* 3, 117. <https://doi.org/10.3389/fsufs.2019.00117>.
- CHONG, Y.-x., HU, H.-y., QIAN, Y., 2005. Improvement on tolerance of *Spirodela polyrrhiza* to ammonia at presence of nitrate. *华南农业大学学报* 26 (3).
- Coughlan, N.E., Walsh, É., Bolger, P., Burnell, G., O'Leary, N., O'Mahoney, M., Jansen, M.A., 2022. Duckweed bioreactors: challenges and opportunities for large-scale indoor cultivation of Lemnaceae. *J. Clean Prod.*, 130285 <https://doi.org/10.1016/j.jclepro.2021.130285>.
- Cushman, J.C., 2001. Osmoregulation in plants: implications for agriculture. *Am. Zool.* 41 (4), 758–769. <https://doi.org/10.1093/icb/41.4.758>.
- Devlamynck, R., de Souza, M.F., Leenknecht, J., Jaxsens, L., Eeckhout, M., Meers, E., 2021. Lemna minor cultivation for treating swine manure and providing micronutrients for animal feed. *Plants* 10 (6), 1124. <https://doi.org/10.3390/plants10061124>.
- Devlamynck, R., de Souza, M.F., Michels, E., Sigurnjak, I., Donoso, N., Coudron, C., Meers, E., 2021. Agronomic and environmental performance of Lemna minor cultivated on agricultural wastewater streams—a practical approach. *Sustainability* 13 (3), 1570. <https://doi.org/10.3390/su13031570>.
- Duque, A.S., et al., 2013. Abiotic stress responses in plants: unraveling the complexity of genes and networks to survive. *Abiotic Stress-Plant Responses and Applications in Agriculture*. IntechOpen. <https://doi.org/10.5772/52779>.
- Elhag, M., 2016. Evaluation of different soil salinity mapping using remote sensing techniques in arid ecosystems, Saudi Arabia. *J. Sens.* 2016 (1), 7596175. <https://doi.org/10.1155/2016/7596175>.
- Euroserver, 2025. Adapted from: In deep shit: EU's Massive Subsidised Manure Problem.
- Gil-Márquez, J.M., et al., 2017. Hydrological and geochemical processes controlling groundwater salinity in wetland areas related to evaporitic (karst) systems. A case study from Southern Spain. *J. Hydrol.* 544, 538–554. <https://doi.org/10.1016/j.jhydrol.2016.11.062>.
- Gitelson, A.A., Chivkunova, O.B., Merzlyak, M.N., 2009. Nondestructive estimation of anthocyanins and chlorophylls in anthocyanic leaves. *Am. J. Bot.* 96 (10), 1861–1868. <https://doi.org/10.3732/ajb.0800395>.
- Gitelson, A.A., Gritz, Y., Merzlyak, M.N., 2003. Relationships between leaf chlorophyll content and spectral reflectance and algorithms for non-destructive chlorophyll assessment in higher plant leaves. *J. Plant Physiol.* 160 (3), 271–282. <https://doi.org/10.1078/0176-1617-00887>.
- Huang, L., Lu, Y., Gao, X., Du, G., Ma, X., Liu, M., Chen, Y., 2013. Ammonium-induced oxidative stress on plant growth and antioxidative response of duckweed (*Lemna minor* L.). *Ecol. Eng.* 58, 355–362. <https://doi.org/10.1016/j.ecoleng.2013.06.031>.
- ILVO, 2025. Varkensloket, Bedrijfsmanagement - Sector- en marktinformatie. Available from. www.varkensloket.be.
- Ivushkin, K., et al., 2019. Global mapping of soil salinity change. *Remote Sens Environ.* 231, 111260. <https://doi.org/10.1016/j.rse.2019.111260>.
- Lambert, M. (2025). [Unpublished manuscript]; Ghent University.
- Lambert, M., Devlamynck, R., Fernandes de Souza, M., Leenknecht, J., Raes, K., Eeckhout, M., Meers, E., 2022. The impact of salt accumulation on the growth of duckweed in a continuous system for pig manure treatment. *Plants* 11 (23), 3189. <https://doi.org/10.3390/plants11233189>.
- Landolt, E., Kandel, R., 1987. *Biosystematic Investigations in the Family of Duckweeds (Lemnaceae)*, Vol. 4: the Family of Lemnaceae—a monographic study, Vol. 2 (phytochemistry, physiology, application, Bibliography). *Veröffentlichungen des Geobotanischen Instituts der ETH, Stiftung Ruebel (Switzerland)*.
- Lasfar, S., Monette, F., Millette, L., Azzouz, A., 2007. Intrinsic growth rate: a new approach to evaluate the effects of temperature, photoperiod and phosphorus-nitrogen concentrations on duckweed growth under controlled eutrophication. *Water Res.* 41 (11), 2333–2340. <https://doi.org/10.1016/j.watres.2007.01.059>.
- Li, Z., Ahammed, G.J., 2023. Plant stress response and adaptation via anthocyanins: a review. *Plant Stress*, 100230. <https://doi.org/10.1016/j.stress.2023.100230>.
- Maathuis, F.J., 2014. Sodium in plants: perception, signalling, and regulation of sodium fluxes. *J. Exp. Bot.* 65 (3), 849–858. <https://doi.org/10.1093/jxb/ert326>.
- Mattson, M.P., 2008. Hormesis defined. *Ageing Res. Rev.* 7 (1), 1–7. <https://doi.org/10.1016/j.arr.2007.08.007>.
- Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 7 (9), 405–410. [https://doi.org/10.1016/S1360-1385\(02\)02312-9](https://doi.org/10.1016/S1360-1385(02)02312-9).
- Nieves-Cordones, M., Al Shibli, F.R., Sentenac, H., 2016. Roles and transport of sodium and potassium in plants. *Alkali Met. Ions: Their Role Life* 291–324.
- Richter, J.A., Behr, J.H., Erban, A., Kopka, J., Zörb, C., 2019. Ion-dependent metabolic responses of *Vicia faba* L. to salt stress. *Plant Cell Environ.* 42 (1), 295–309. <https://doi.org/10.1111/pce.13386>.
- Said, M.Z.M., Culley, D.D., Standifer, L.C., Epps, E.A., Myers, R.W., Boney, S.A., 1979. Effect of harvest rate, waste loading, and stocking density on the yield of duckweeds. In: Paper presented at the Proceedings of the World Mariculture Society. <https://doi.org/10.1111/j.1749-7345.1979.tb00076.x>.
- Shilpha, J., et al., 2023. Ammonium phytotoxicity and tolerance: an insight into ammonium nutrition to improve crop productivity. *Agronomy* 13 (6), 1487. <https://doi.org/10.3390/agronomy13061487>.
- Sree, K.S., Adelman, K., Garcia, C., Lam, E., Appenroth, K.-J., 2015. Natural variance in salt tolerance and induction of starch accumulation in duckweeds. *Planta* 241 (6), 1395–1404. <https://doi.org/10.1007/s00425-015-2264-x>.
- Stadlander, T., Schmidtke, A., Baki, C., Leiber, F., 2023. Duckweed production on diluted chicken manure. *J. Anim. Feed Sci.* 33 (1). <https://doi.org/10.22358/jafs/169431/2023>.
- Verma, R., Suthar, S., 2015. Utility of duckweeds as source of biomass energy: a review. *Bioenergy Res.* 8, 1589–1597. <https://doi.org/10.1007/s12155-015-9639-5>.
- Xu, J., Shen, Y., Zheng, Y., Smith, G., Sun, X.S., Wang, D., Li, Y., 2022. Duckweed (Lemnaceae) for potentially nutritious human food: a review. *Food Rev. Int.* 1–15. <https://doi.org/10.1080/87559129.2021.2012800>.
- Xu, Z., Rothstein, S.J., 2018. ROS-Induced anthocyanin production provides feedback protection by scavenging ROS and maintaining photosynthetic capacity in Arabidopsis. *Plant Signal Behav.* 13 (3), 1364–1377. <https://doi.org/10.1080/15592324.2018.1451708>.
- Zhou, Y., Kishchenko, O., Stepanenko, A., Chen, G., Wang, W., Zhou, J., Borisjuk, N., 2021. The dynamics of NO₃⁻ and NH₄⁺ uptake in duckweed are coordinated with the expression of major nitrogen assimilation genes. *Plants* 11 (1), 11. <https://doi.org/10.3390/plants11010011>.