Exploring natural variation in duckweed – using genomics and phenotypic approaches

By Kellie E. Smith

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**Supervisors: Professor Levi Yant,
Professor Anthony Bishopp and
Professor Erik H. Murchie**

School of Life Sciences, University of Nottingham, Nottingham, NG7 2TQ, UK

and

School of Biosciences, Sutton Bonington Campus, University of Nottingham, Loughborough, Leicestershire, LE12 5RD, UK

Abstract

The duckweeds comprise 36 species, containing both the smallest and fastest growing Angiosperms. Their aquatic growth habit and clonal production makes them ideal for phytoremediation and food applications. Duckweed contain ~45% protein by dry weight, high vitamins e.g. carotenoids and minerals including K, P and Fe. However, natural variation in these aspects have not so far been used for selection of species and accession suitability for human consumption or phytoremediation. For existing available duckweed clones domesticated in controlled environments, the original light and water environments are largely unknown. Here, a novel UK cohort with native water and light assessments are used to identify accessions for commercial applications. This work reports species and accession variation in minerals, heavy metals, aroma, metabolites and light responses in controlled environments by combined genotyping and phenotyping. The *Lemna* genus showed high elemental variation: with submerged *Lemna trisulca* hyperaccumulating five elements and *Lemna yungensis* showing variation between accessions. From the novel UK collection, higher Mg was found in *Lemna minuta* and higher K and As in *Lemna japonica,* additionally with species inhabiting higher Mg and As water environments respectively. Accessions of these species could be trialed on wastewater to maximise elemental extraction for phytoremediation, however high heavy metal contents represents a challenge for food safety. For consumption, accession and environment were manipulated to maximise growth by increasing light irradiance. Especially suitable were hybrid *Lemna japonica* accessions originating from low light, which acclimated to artificial high light by fast growth, high photoprotection and increased carotenoids. During glasshouse growth, aroma profiles varied between duckweed species but were comparable to spinach and provisionally acceptable for human consumption. Moving forward, specific functions of duckweed aroma and metabolite compounds in human health should be elucidated. Together, phenotyping, genome sequencing and environmental assessment form a tool to understand natural variation for applications.

Publications

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List of Abbreviations

Rubisco	ribulose bisphosphate carboxylase/oxygenase
VOCs	volatile organic compounds
ROS	reactive oxygen species
Mya	million years ago
AFLP	amplified fragment length polymorphisms
TBP	tubulin based polymorphisms
PPFD	photosynthetic photon flux density
PSII	photosystem II
NPQ	non-photochemical quenching
PsBS	photosystem II subunit S
C_3	three carbon compound fixation
LED	light emitting diode
UK	united kingdom
ICP-MS	inductively coupled plasma mass spectrometry
EDTA	ethylenediamine tetraacetic acid
CaOx	calcium oxalate
N medium	nutrient medium
ANOVA	analysis of variance
LOQ	limit of quantification
LOD	limit of detection
PC	principal component
PCA	principal component analysis
GBIF	global biodiversity information facility
HAS	hastings, uk
COR	cornwall, uk
BRI	bristol, uk
NEW	newport, uk
ABE	aberdeen, uk
ELG	elgin, uk

GLA	glasgow, uk
LAN	lancaster, uk
BFD	bradford, uk
YOR	york, uk
HUL	hull, uk
MID	midlands, uk
NCA	newcastle, uk
Cos2	squared cosine
L:W	length to width frond ratio
C1-2	genetic clusters 1 and 2
SNPs	single nucleotide polymorphisms
SRA	sequence read archive
GRIS	global register of introduced and invasive species
SPME PTFE	solid phase microextraction polytetrafluroethylene
HDPE	high density polyethylene
DNA	deoxyribonucleic acid
PCR	polymerase chain reaction
GATK	genome analysis toolkit
NCBI	national centre for biotechnology information
4FDS	four-fold degenerate site
dsDNA	double stranded deoxyribonucleic acid
dLL	derived from low light
dHL	derived from high light
LL	low light
HL	high light
Chl	chlorophyll
φPSII	photosynthetic efficiency of photosystem II
3D	3-dimensional
FR	far-red
R	red
G	green

B	blue
UV	ultra-violet
R:FR	red:far red ratio
QYmax (F_v/F_m)	maximum quantum yield of photosystem II
PFD	photon flux density
BIC	bayesian information criterion
RGRlog	relative growth rate (log phase)
Col	colony
FW	fresh weight biomass
FDW	freeze dried weight
rpm	revolutions per minute
HPLC	high-performance liquid chromatography
PTFE	polytetrafluoroethylene
DEPS	de-epoxidation state
XC	xanthophyll cycle pool
Car	carotenoids
L1-L11	light levels 1-11
WHO	world health organisation
GRAS	generally recognised as safe
USA	united states of america
EU	european union
OVI	other vegetation index
NDVI	normalised difference vegetation index
GI	green index
GM	green model
MeOH	methanol
SPME-GCMS	solid phase microextraction- gas chromatography mass spectrometry
GC-O	gas chromatography-olfactometry
NIFs	nasal impact frequencies
OAV	odour activity values

LC-MS/MS	liquid chromatography tandem mass spectrometry
EtOH	ethanol
HPLC-PDA	high-performance liquid chromatography-photo diode array
DW	dry weight
AU	arbitrary units
C5-C6	5- and 6-carbons
LRI	linear retention index
AAD	amino acid derivatives/precursors
FW	fresh weight biomass
BSBI	botanical society of britain & ireland

List of Terminologies

Accession	An individual collected from a specific location and defined below the level of species.
Ecotype	An individual with defining characteristics e.g. a physiological behaviour in an environmental context.
Clone	An individual unit within a population descending from an isolate. Also used to describe duckweed varieties belonging to a stock collection.
Ionome	Tissue composition of elements including macro-, micro-, trace elements and heavy metals.
Macroevolution	Differences between subfamilies or genera.
Microevolution	Differences between species or within species e.g. accessions.
Fine-scale variation	Differences between or within species, or on a small geographic scale such as within regions.
Broad-scale variation	Differences between genera or duckweeds from a wider geographical scale.
Adaptation	Features that enable survival in an environment, typically inherited and defined at species level.
Acclimation	Adjustments within a short-term (hours-weeks) to a new environment.

List of figures

Page

List of tables

Page

Table of contents

1. Chapter one. Literature review

1.1. Plant-based food systems

Sustainable and nutritious foods are vital to support a growing human population. The population is predicted to increase to between 8.9 and 12.4 billion in 2100 (United Nations, 2022). However, existing food systems are still insufficient, mainly due to limited availability of land and depletion of soil and water quality (FAO, 2021). Around 50% of the world's calories come from three cereal crops: wheat, rice and maize (Awika, 2011). However, as crop yields and availability of food are predicted to worsen due to extreme weather events including heatwaves, droughts and flooding (Mirzabaev *et al.*, 2023), food systems need to diversify to overcome these challenges. Additionally, malnutrition and micronutrient deficiencies are still problematic in mid-low income regions causing poor health and disease, especially in the women and children demographics (Lopes *et al.*, 2023). Therefore, there is a need to uncover genetic variation in resilience traits of existing food crops together with expansion into novel plant-based food sources to alleviate food insecurity.

Innovative solutions are required to reduce reliance on primary cereal crops and to support a growing human population. Comparative phenotyping to find diversity among cultivars, land races, wild relatives and elite varieties continue to be used in wheat and rice crop improvement strategies. For instance, breeding in these major crop species could maximise traits for improved tolerance to abiotic stresses, enhancement of yields, photosynthesis, nutrition and digestibility (Carmo-Silva *et al.*, 2017; Zhang *et al.*, 2017; McAusland *et al.*, 2020; Reynolds & Braun, 2022; Zafeiriou *et al.*, 2023). Similar strategies in theory could be used to maximise nutrition from wild varieties of prospective novel food crop species.

1.1.1. Duckweed as a novel food source

Duckweed development as a novel food source is of interest for sustainable global food production. This is due to many features in duckweed including: doubling in biomass every 48-72 hours, easy to harvest, and many species are readily available due to their global distributions (Classen & Bergmann, 2000). Duckweeds have global market potential as a nutritious food source, and demand is supported by increased projections in plant-based industries (Talwar *et al.*, 2024). Duckweed offers versatility in its form, particularly for human consumption as a fresh vegetable or herb, protein supplement or as an animal feed (de Beukelaar *et al.*, 2019; Liu *et al.*, 2021). The ability to grow in controlled conditions with little space requirements have seen duckweed gain interest for hydroponic vertical farming and applications in space agriculture (Coughlan *et al.*, 2022; Petersen *et al.*, 2022a; Polutchko *et al.*, 2022). For these applications, nutritional value and safety aspects of different duckweeds should be considered with careful choice of growing environment.

1.1.1.1. Duckweed as a protein supplement

One agricultural challenge is to develop a wider range of high protein sources for human consumption. The alternative protein market consists of plants, insects and microbes and plant-based protein is expected to grow at a compound annual growth rate of 7.5% to 2029 (Markets & Markets, 2023). Fundamentally, protein deficiency is still an active problem associated with malnourishment and poor health (Vissamsetti *et al.*, 2024), providing a need for alternative proteins, especially in developing countries. Moreover, plant-based sources of low cost, high protein animal feeds offering a complete range of amino acids are difficult to obtain from a single source (Ghosh *et al.*, 2012; Parisi *et al.*, 2020). While current demand for high energy proteinaceous cereal crops require both high inputs of fertilisers and a long production season, typically between three-eight months; Duckweed offer an alternative, growing quickly in both high and low Nitrogen with higher N use efficiency than cereal crops (Leng *et al.,* 1995; Liu *et al.,* 2021).

Several species of duckweed can provide a valuable protein source for human consumption (Appenroth *et al.*, 2017). Duckweeds can generate new proteinaceous biomass from low N and P sources and proteins make up to 45% of their dry biomass (Landolt and Kandeler, 1987; Oron *et al*, 1988). Comparatively, duckweed protein content on a dry weight basis is equal to cereal crops existing on the market, like wheat and soybean (Tamayo Tenorio *et al.*, 2018). Overall, duckweed forms a more sustainable protein source to soybean monoculture, which is damaging forests to largely feed animals (Dreoni *et al.*, 2022). Finally, the amino acid profiles of *Lemna* and *Spirodela* duckweed species are comparable with those of alfalfa and soybean protein, with a higher lysine content than cereals (Dewanji, 1993; Cheng & Stomp, 2009). Thus, development of duckweed as a protein supplement should not be detrimental as an alternative to existing protein sources on the market. However, this does highlight a focus towards maximising biomass and available protein production in duckweed.

Optimisation of both growing system and species of choice are key areas to develop duckweed for processing into protein powders. Recently, a combination of species *Lemna minor* and *Lemna gibba* were selected and patented in Europe for protein extraction (Gaynor, 2017; Turck *et al.*, 2023). Furthermore, Rubisco forms roughly 30% of leaf protein content, but Rubisco extraction from *Lemna gibba* has produced a relatively low yield of ~27% total Rubisco (Nieuwland *et al.*, 2021), with potential to optimise this further. In addition, duckweed Rubisco produces a desirable off-white colour and emulsification properties, leading it to be successfully used as an animal protein replacement (Tan *et al.*, 2023). For these applications, use of modulated light and automated systems were used to produce greater biomass and more consistent protein outputs in *Lemna minor* (Petersen *et al.*, 2022a,b). There is opportunity to further maximise growth, protein and Rubisco production by optimisation of growth systems and pinpointing the highest yielding *Lemna* duckweed accessions.

To pioneer duckweeds as an alternative plant protein, protein must be readily bioavailable for the human consumer. For example, essential amino acids released from *Wolffia globosa* have comparable bioavailability to cheese and pea consumption (Kaplan *et al.*, 2019), however other duckweed species were not included in this comparison. On the other hand, when combined as a poultry feed, mixed *Spirodela* and *Lemna* species provided high amino acid digestibility in chickens (Demann *et al.*, 2023). Therefore, there is some evidence emerging that duckweed provide useful protein in human food and animal feed. Further digestion studies and other dietary benefits potentially provided by duckweed supplements requires validation using other species and growing systems.

Together with high protein contents, antioxidant compounds such as phenolic compounds are prevalent in duckweed and could be incorporated into a powdered supplement. For instance, *Wolffia* commercialised as the superfood 'Mankai' showed high detectable amounts of almost 200 polyphenols including flavonoids (Yaskolka Meir *et al.*, 2021), *Lemna minor* has 12 compounds with identified antioxidant activity including phytol, ascorbic acid, tyrosols and alkylphenols (Petrova-Tacheva *et al.*, 2020; Del Buono *et al.*, 2021) and *Spirodela polyrhiza* contains 18 flavonoids including luteolin and apigenin (Qiao *et al.*, 2011). Some of these flavonoid compounds including luteolin and apigenin glucosides have proven anti-inflammatory and anti-cancer properties (Wang *et al.*, 2007; Pagliuso *et al.*, 2020; Guo *et al.*, 2023b; Kim *et al.*, 2023), giving duckweed extracts and supplements important medical applications. Furthermore, duckweed extracts have anti-microbial functions, likely attributed to antioxidants from functional secondary phenolic compounds (Gülçin *et al.*, 2010; Petrova-Tacheva *et al.*, 2020). Antioxidant functions have led to incorporation of duckweed extracts into meat and into packaging for the storage of fruit, where they successfully increased shelf-life (Luzi *et al.*, 2022; Rocchetti *et al.*, 2023). Although flavonoids were elucidated in some species individually, the functions of specific flavonoids are yet to be characterised. Future work defining phenolic compounds in different species, their functions and variability due to environment are key for development of duckweed supplements.

Prolonged shelf-life is achieved when protein supplements are produced in a powder form, predominantly reducing moisture content and thereby limiting microbial growth. However, substantial resources are required for the production of a duckweed powder, typically including costs associated with drying (Skillicorn, 1993). There are further negatives to production of a supplement powder such as the substantial loss of around 95% biomass, variable protein outputs between methods and increased bitterness in flavour (Nieuwland *et al.*, 2021). On the other hand, longevity is increased and typically an increased shelf-life is supported by various duckweed (*Lemna minor* and *Wolffia globosa*) protein extracts displaying antimicrobial functions, including reduction of important food spoilage organisms (Gülçin *et al.*, 2010; González-Renteria *et al.*, 2020; Duangjarus *et al.*, 2022). Further work is still required to characterise variable amino acid profiles, protein, flavonoid contents and shelf-life of various duckweeds when generating powdered supplements.

1.1.1.2. Duckweed as a vegetable

The *Wolffia globosa* species of duckweed was historically used as a vegetable in Asian cooking, as an additive to stir fries and curries (Bhanthumnavin & McGarry, 1971). For vegetable consumption, the total amount and freshness of duckweed biomass is preserved, however transport and storage is more costly (Iqbal *et al.*, 2019). Forming a contrast to Asia, in Western Europe, there is more resistance to duckweed vegetable consumption, due to duckweed association with wastewater. Nevertheless, the use of the naming system 'water lentils' for food contexts, its growth in sterile (axenic) controlled environments and its incorporation into commonly eaten cultural foods, are ways to further expand duckweed acceptability (de Beukelaar *et al.*, 2019). To further pioneer duckweed for nutrition with global reach, the health benefits, safety and indeed acceptability aspects can be best explored using human trials (Baek *et al.*, 2021).

Duckweeds offer promise as a sustainable source of macronutrients and trace elements including K, Fe, Zn, Ca and Mn as identified by two key studies: comparing *Wolffia* species and representatives of duckweed genera (Appenroth *et al.*, 2017, 2018). Additionally, mineral content compares well with cereal crops, in particular, *Wolffia* species can contain higher Mg, Ca, P, K, Mn and Zn than wheat (Xu *et al.*, 2023). However, there are concerns regarding mineral forms; for example, complexation of Ca as oxalate or Zn as phytate (Francheschi 1989, Steveninck, 1990). One key example is the storage of high Mg and Ca as insoluble mineral oxalates, which are known to form raphide crystals. In this form instead of being nutritious, these act as anti-nutrients for nutrient absorption and deter predation, thereby reducing acceptability of duckweed (Mazen *et al.*, 2003; Pagliuso *et al.*, 2022). Therefore, mineral oxalates currently challenge the consideration of some duckweed species (White & Wise, 1998; Fasakin, 1999; Shrivastav *et al.*, 2022). Meanwhile, both increasing e.g. macronutrients or reducing uptake of certain suites of elements e.g. heavy metals by breeding and transgenic approaches have successfully improved existing crops (Shahzad *et al.*, 2021; Tumbare & Maphosa, 2023), similar strategies are yet to be incorporated in duckweed.

Duckweed cultivation in controlled conditions offers the unique opportunity to explore how media components can influence the 'ionome' of duckweed. That is, the composition of desirable elements, including macronutrients, micronutrients, trace elements and some undesirable elements such as excessive trace elements and heavy metals. For vegetable consumption, both Zn and Se fortification in *Wolffia* species and reduction of trace elements in *Lemna gibba*/*Lemna minor* was successfully achieved using fertiliser manipulation (Appenroth *et al.*, 2018; Turck *et al.*, 2023). Higher Mn content featured in duckweed compared to other leafy vegetables was one initial safety concern for *Lemna* use as a vegetable in Europe, but this content has since been lowered by altering nutrient media (EFSA, 2024). Furthermore, increasing the salinity of a growing medium can reduce Mg, Ca, Fe, Zn and importantly the Mn composition in *Lemna minor* biomass (Ullah *et al.*, 2022). Therefore, careful consideration of duckweed species together with nutrient

inputs has proven potential to produce fortified food sources to target key nutrient deficiencies or otherwise reduce undesirable high amounts of elements.

In comparison to existing crops, duckweed starch content is comparable to maize but more productive per hectare over a shorter growing season, without acquiring new land (Cheng & Stomp, 2009). Importantly, duckweed starch content varies hugely with reports between 3-75% dry weight biomass, depending on species and growing media (Huang *et al.*, 2014). As expected, soluble sugar content also varies under different environmental conditions, with nutrient deficiency or high irradiance elevating them, possibly by starch breakdown or increasing photosynthesis (Yin *et al.*, 2015; Sree & Appenroth, 2022; Van Dyck *et al.*, 2023). Thus, duckweed can provide a significant calorific intake, with increased understanding of factors contributing to varied carbohydrate content and form. One study investigated supplementation of starch-rich *Wolffia globosa* duckweed into the diets of diabetes patients and found that it controlled blood glucose better than dairy consumption (Zelicha *et al.*, 2019), however other duckweed species were not included. Therefore, there is great potential to further explore sugar and starch optimisation by duckweed selection, controlled environment manipulation and inclusion of starch-rich biomass for digestion studies using human trials.

Health promoting compounds like carotenoids are linked with human benefits including cognitive and cardiovascular functions and maintenance during aging (Eggersdorfer & Wyss, 2018; Stewart *et al.*, 2020). Therefore, high amounts of photoprotective carotene and xanthophylls from plant sources are desirable for human dietary intake (Ekperusi *et al.*, 2019). Importantly, these compounds protect tolerant plants during exposure to damaging light irradiance and are not produced by humans (Maoka, 2020). Duckweeds represent a high source of carotenoids, whereby carotene, lutein and zeaxanthin in *Wolffia* and *Lemna* are the dominant forms and apparently high compared to other vegetable sources (Appenroth *et al.*, 2017, 2018). One study used varied light irradiance to stimulate carotenoid production, concluding that pulse modulated light is an efficient way to increase carotenoids (Stewart *et al.*, 2020, 2021). Thus, there is scope to increase understanding of the interplay of duckweed light tolerance strategies and light intensity of growing systems, to boost these important beneficial compounds for food applications.

Anthocyanins are important flavonoids typically found in vegetables, with antioxidant functions and potential benefits in human health. Anthocyanins impart red and purple coloration typically found or induced in some duckweed species including: *Spirodela polyrhiza*, *Landoltia punctata*, *Lemna turionifera* and *Lemna gibba* and other *Wolffia* and *Wolffiella* species (Landolt, 1986; Landolt & Kandeler, 1987; Lansdown, 2008; Azer, 2013) but not common in others such as *Spirodela intermedia*. Therefore, some species may be more suited for vegetable consumption than others. Of particular interest may be *Spirodela polyrhiza*, as flavonoid genes including those for anthocyanin production are reportedly more expansive compared to *Lemna* species (Landolt, 1986; Fang *et al.*, 2023b). Furthermore, a *Spirodela polyrhiza* mutant which showed 500% enhanced accumulation of anthocyanins and

flavonoids was identified from a comparative panel of duckweeds. Thus, using large duckweed panels could reveal other duckweed varieties with high levels of anthocyanins and flavonoids. In turn, the environmental landscape experienced by a plant influences anthocyanin production (Davies *et al.*, 2022). For instance, anthocyanins could be enhanced in duckweed under oxidative stress environments such as high light irradiance or copper toxicity (Böttner *et al.*, 2021). Therefore, targeted anthocyanin enrichment by exploring duckweed tolerance to these stressors could be instrumental to maximise duckweed nutrition when consumed as a vegetable.

Consumption of duckweed as a fresh vegetable requires an appealing smell, flavour and mouthfeel, all of which are involved in perception of food. One human trial has described that *Lemna minor* duckweed taste and acceptability was parallel with spinach (Mes *et al.*, 2022), but otherwise literature in this area is sparse. For instance volatile organic compounds (VOCs) are key secondary metabolites orchestrating aroma and flavour, due to high vapour pressures and unique odour thresholds (Picazo-Aragonés et al., 2020), but despite this they are not characterised in duckweed. Fundamentally, aroma combinations from VOCs together with their odour thresholds make up a food's aroma profile and are key for human decision-making about edibility and safety of foodstuffs. Aroma descriptions can be positive e.g. 'fresh' and 'fruity' or negative such as 'rotten' or 'fermented', the latter of which tending to increase with prolonged storage or inadequate processing of fresh vegetables, and are off-putting for consumption (Cantwell & Suslow, 2002; Díaz-Mula *et al.*, 2017). In nature, aroma compounds have evolved in plants to act as deterrents, attractants and defensive compounds against herbivores, insects and other plants (Aguiar et al., 2021). Therefore, VOCs can have additional health promoting antioxidant, anti-microbial and anti-inflammatory effects when consumed (Abbas *et al.*, 2023). To assess human acceptability of duckweed for consumption, aroma and flavour can be characterised by studying species' VOC profiles in context with storage and health benefits.

Duckweed show promising features for space horticulture as a live plant food due to fast growth, ability to grow in small hydroponic conditions, capability of wastewater recycling and production of medical compounds (Polutchko *et al.*, 2022; Liang *et al.*, 2023). Furthermore, *Wolffia globosa* and *Lemna aequinoctialis* can maintain or even stimulate growth in microgravity conditions (Yuan & Xu, 2017; Romano *et al.*, 2024). For this goal, characterisation of sensory properties of duckweed was identified as an area for development, to encourage astronauts to consume nutritious food (Mortimer & Gilliham, 2022). Moreover, lack of Ca, P, Na, vitamins and indeed water consumption in space has been related to deterioration of health of astronauts returning from spaceflight (Smith & Zwart, 2015). Noteworthy also is that high lutein and zeaxanthin could provide effective radiation protection during spaceflight missions (Stewart *et al.*, 2020; Polutchko *et al.*, 2022). Duckweed consumption as a microgreen with high water content, vitamins and minerals could have the potential to address these challenging health concerns caused by this specific environment. Small-scale hydroponic systems suitable for space already offer the potential to tailor light and nutrient regimes to optimise nutritional outputs (Escobar *et al.*, 2020; Nguyen *et al.*,

2023) . Duckweed panels could be compared for nutritional quality and growth responses to altered light regimes and nutrient composition in small-scale hydroponic systems, to enable identification of accessions suitable for space applications.

Conclusions. There is a need for diversification of food systems for inclusion of alternative nutritious crops and growing systems. Concordantly, there is opportunity to maximise several nutritious components in duckweed including antioxidants, micronutrients, starch and protein using varied growing environments. In duckweed, this could be achieved by using genetic diversity panels growing in varied hydroponic systems. Specifically, altered light and nutrients together with accession and species selection can maximise growth and nutritious biomass components in artificial environments. To this end, collection of a novel duckweed cohort has a powerful utility to assess natural variation of nutritional traits.

1.2. Water cleaning

Water is a vital resource to human populations across the world and is presently declining in quality. Application of fertilisers and animal organic wastes from agriculture have resulted in increased N and P leaching into water courses (Hasan & Chakrabarti, 2009; Chakrabarti *et al.*, 2018) and increased water hardness and salinity as concentrations of Ca, Mg and Na increase in run-off (Bogart *et al.*, 2019). Presently the reported levels of As, U and Pb in water are currently in excess of regulatory limits, due to mining activities in some areas (Credo, 2019). Of particular concern are the heavy metals Cd, Cu. Zn, Pb, Cr, Ni which can contaminate water courses and are toxic to animals, fish and humans, with carcinogenic and organ damaging effects (Dasharathy *et al.*, 2022; Levin *et al.*, 2023). In turn, humans and the supporting food chain are threatened due to bioaccumulation of metals (Maurya *et al.*, 2019). Therefore, the current state of water quality represents a safety issue and represents a need for water cleaning.

Cleaning polluted water to produce safe drinking water is vital to support human health. Engineering methods for remediation include bioreactors and nanoparticles but these can be both expensive and not suitable for developing countries (Yamashita & Yamamoto-Ikemoto, 2014; Chaudhary *et al.*, 2023). Plants offer a 'green' alternative, which can be grown, then harvested and consequently remove elements from water, locking them into plant biomass (Reeves *et al.*, 2018; Ekperusi *et al.*, 2019). Aquatic plants like fern (*Azolla filiculoides*) water hyacinth (*Eichhornia crassipes*), algae and duckweed act as alternative natural biological water filtration systems, otherwise known as phytoremediators (Ansari *et al.*, 2020). Therefore, combinations of aquatic plants could be used in solo or in partnership to sustainably remediate different types of wastewater (Zhao *et al.*, 2015a; Lanthemann & van Moorsel, 2022).

1.2.1. Duckweed as phytoremediators

Phytoremediation is the process by which plants uptake excess macronutrients including N and P and heavy metal contaminants from water sources. Elements are assimilated into plant biomass and should be harvested frequently to prevent release back into the water source (Szabo *et al.*, 2000). An optimum plant phytoremediator should be tolerant to high levels of nutrients and heavy metals, bioaccumulate them, maintain high rates of growth to continue this extraction process and then be easily harvested (Kafle *et al.*, 2022). Due to these features, some duckweed represent a sustainable biological option to clean up a varied range of water sources worldwide (Landesman *et al.*, 2010). Duckweed can hyperaccumulate N and P for conversion to biomass and certain species are documented for their tolerance to various heavy metals. In comparison with other aquatic plants, duckweed accumulates higher Zn than *Azolla filiculoides,* higher metals than algae and is practically easier to harvest than algae (Sharma & Gaur, 1995). Well-acclimated duckweeds can therefore be used to remediate different wastewater effluents with different elemental compositions.

1.2.1.1. Macronutrient phytoremediation

Duckweed are naturally found growing on high nutrient water bodies including agricultural ditches. They naturally absorb N and P, effectively reducing N and P levels in water, to prevent eutrophication caused by other plant species. However, excessive duckweed growth can still ultimately lead to eutrophication of water and negative effects on ecosystems. Practically, duckweed can remediate N, P, Ca, Mg and other macronutrients from farming wastes (Cheng *et al.*, 2002; Landesman *et al.*, 2005; Walsh *et al.*, 2020; Paolacci *et al*, 2022) and domestic wastewaters (Alaerts *et al.*, 1996; Al-Hashimi & Joda, 2010). Studies report variation in performance of different duckweed varieties using controlled environments, synthetic wastewater and other wastewater examples, but rarely in natural environments.

Various rooted duckweed species were described in the context of laboratory and wastewater studies exploring macronutrient phytoremediation. For instance, during phytoremediation of a sewage lagoon, an undefined *Spirodela* species removed 99% ammonium, 95% phosphorous but limited removal of nitrate from the sewage (Alaerts *et al*, 1996). Laboratory studies comparing N source using replete nutrient media also found that ammonium uptake was higher than nitrate by 2x in *Landoltia punctata*, by 11x in *Lemna minor* and in *Wolffiella hyalina* (Cedergreen & Madsen, 2002; Fang *et al.*, 2007; Petersen *et al.*, 2021). In a range of species this was accompanied by expression of nitrogen assimilation genes in response to high ammonium but not nitrate (Zhou *et al.*, 2022), suggesting this is a general preference in duckweeds. Additionally, ammonium was shown to be directly converted into protein and used for biomass (Bergmann *et al.*, 2000). Therefore, a combination of studies highlight the value of duckweed species especially for ammonium and phosphorous removal.

Macronutrient extraction and tissue contents of N and P have been compared between duckweed species. Broadly, the reported N:P tissue concentration varied five-fold between different genera and species of *Lemna*, *Wolffia* and *Spirodela* (Landolt, 1986; McCann, 2016), although growing conditions were not unified, so it is not possible to determine realistic variation in tissue accumulation for these macronutrients between species. For N and P extraction from artificial swine medium, researchers highlighted within-species variation in the best and worst accumulating accessions of *Lemna* species. Additionally, from this work, high fresh weight biomass and protein production correlated with N and P extraction, linking uptake with high nutrient efficiency (Bergmann *et al.*, 2000; Classen & Bergmann, 2000; Cheng *et al.*, 2002). However in contrast, other work indicated that high N and P remediation from dairy wastewater did not typically correlate with higher biomass or protein production (Walsh *et al.*, 2022). However, the concentration of the supplied N and P is not always reported or comparable between different laboratory or wastewater studies using different species (Pasos-Panqueva *et al.*, 2024). Therefore, a comparative assessment to study natural variation of genera, species and accessions using replete nutrient conditions could be used for discovery of candidates for N and P phytoremediation.

Hard water and dairy wastewater are particularly rich in the minerals Ca and Mg. Efficient phytoremediators should tolerate high levels by continued growth and efficiently uptake and store minerals. To this end, there are various studies documenting cell structures and mechanisms associated with storage of Ca in duckweed. This includes conjugation with oxalate and formation of raphide crystals, some of which are specific to rooted species (Landolt, 1986; Franceschi, 1989; White and Wise, 1998; Mazen *et al*, 2003). In contrast, rootless *Wolffia* species do not form Ca oxalate crystals, which may limit overall accumulation (Landolt & Kandeler, 1987; Appenroth *et al.*, 2018), emphasizing possible variation in Ca storage across the genera. Although uptake and storage potentials have not been compared specifically in the context of replete media, hardwater or dairy wastewater. Using individual species on wastewater, Mg toxicity was one limitation of Ca uptake due to antagonism between these cations. For instance, the ability of *Lemna minor* to remediate high Mg synthetic dairy wastewater and *Lemna aequinocitialis* to remediate Mg minewater were limited, due to excessively high Mg and unbalanced Ca:Mg, instead resulting in toxicity and reduced growth (Van Dam *et al.*, 2010; Walsh *et al.*, 2020). Due to differential storage mechanisms and possible different preferences for Ca:Mg, the duckweed may have different abilities to phytoremediate Ca and Mg. However, phytoremediation potential of Ca and Mg remains to be formally tested using a large panel of duckweeds in common conditions with high mineral exposure.

1.2.1.2. Heavy metal phytoremediation

Landfill leachate and acid minewater includes high levels of Fe, Mn but additionally high heavy metals and metalloids such as Cr, Cd and As, presenting a challenge for remediation. For these purposes, duckweed have been explored for remediation of heavy metal contaminated wastewaters (Teixeira *et al.*, 2014; Daud *et al.*, 2018; Sasmaz Kislioglu, 2023). One feature of tolerance is the ability to hyperaccumulate heavy metals, which was defined as bioaccumulation higher than 1000 mg/kg dry weight biomass (Zayed *et al.*, 1998). Duckweed hyperaccumulators of Zn, Mn, Cd, Co and especially Cd were described from various studies (Van Steveninck *et al.*, 1992; Xie *et al.*, 2013; Liu *et al.*, 2017b; Xu *et al.*, 2018; Hu *et al.*, 2019; Zheng *et al.*, 2023). Therefore, certain duckweed varieties have known capabilities and in some cases known mechanisms to extract heavy metals.

The tolerance of duckweeds to various heavy metals as well as their mechanisms have been elucidated in some species using laboratory experiments. Some work which compared tolerance to high Cd using different accessions of *Lemna aequinoctialis* and *Landoltia punctata* were able to identify Cd hyperaccumulators (Yin *et al.*, 2002; Xu *et al.*, 2018). Furthermore, these exceptional accumulators were defined by growth and tolerance achieved by chelation mechanisms, targeting Cd to vacuoles and antioxidant enzymes reducing reactive oxygen species (ROS). On the other hand, *Wolffia globosa* tolerated high As and accumulated up to 10x more than other duckweeds, but also effluxed the less toxic arsenite species (Zhang *et al.*, 2009). Notably the levels of Cd or As contamination in wild collection

sites for these Cd and As hyperaccumulators were not mentioned in these studies. Although, a *Lemna gibba* accession collected from a Uranium mine could reduce As pollution when optimally growing, showing that heavy metal bioaccumulators could be found from extreme environments (Mkandawire *et al.*, 2004b,a; Mkandawire & Dudel, 2005). Therefore, different mechanisms of heavy metal tolerance between species and metal types needs further resolution with inclusion of environmentally relevant contexts.

Some studies have used combined phenotypic and physiological approaches to classify toxicity of duckweed to metals. For example, reduced growth is the most common observation seen in sensitive duckweeds responding negatively to various heavy metals (Zayed *et al.*, 1998; Boonyapookana *et al.*, 2002; Alvarado *et al.*, 2008; Leblebici *et al.*, 2010; Chen *et al.*, 2020). Some studies have observed altered uptake or efflux of elements (Prasad *et al.*, 2001; Alvarado *et al.*, 2008; Leblebici *et al.*, 2010; Oláh *et al.*, 2023) or effects on photosynthesis, chlorophylls, carotenoids and antioxidants (Teisseire & Guy, 2000; Prasad *et al.*, 2001; Boonyapookana *et al.*, 2002; Jayasri & Suthindhiran, 2017; Roubeau Dumont *et al.*, 2019). Finally, chlorosis and frond separation are visual responses seen in plants experiencing heavy metal toxicity (Khellaf & Zerdaoui, 2010). Ultimately, the concentrations of metals causing 50% decline of these phenotypes can be used to compare severity of metal toxicity, as done for Cu, Ni, Cd and Zn (Khellaf & Zerdaoui, 2009) and for severity of ten heavy metals for *Lemna minor* (Naumann *et al*, 2007). One recent study resolved different distribution patterns for Ni and Cr metals within fronds and between three duckweed species (Oláh *et al.*, 2024), but did not link frond distribution to species tolerance. Although various heavy metal response phenotypes are well documented, studies typically use limited numbers of species or accessions, different heavy metal concentrations and duration of experiments. For suitability for phytoremediation, these factors could be better unified using larger panels of duckweed cohorts under standard conditions, with important linking to a real-world need for remediation.

1.2.1.3. High salinity phytoremediation

Removal of salts and minerals from seawater to produce freshwater using the process of desalination offers the prospect to increase the availability of drinking water for the human population. Existing engineering methods for desalination are high cost and energy demanding (Kalogirou, 2005), representing an opportunity to discover salt (NaCl) tolerant phytoremediators. Although, typically found on freshwater rather than seawater, both salt sensitive and salt tolerant duckweed accessions have been documented in the literature. Presence of high salinity has diverse effects on sensitive duckweeds, including reduction of growth, decreased photosynthesis and altered uptake of heavy metals. For instance, high NaCl inhibited growth of *Spirodela polyrhiza* and desensitized it to metals such as Ni and Cd (Leblebici *et al*, 2011). However, high NaCl decreased biomass and root length growth in *Lemna gibba*, resulting in reduced Ni uptake (Yilmaz, 2007). Homeostasis of macronutrients were also altered in salt sensitive individuals, whereby influx of N and P in *Lemna minor* declined as salt concentration increased and were effluxed at maximal NaCl exposure (Liu *et al*, 2017). Nevertheless, the most

complete assessment of 33 duckweeds found tolerant plants displaying high growth at exceptionally high NaCl concentrations, together with high starch accumulation (Sree *et al.*, 2015a). However, this work did not make a link with duckweed salinity tolerance and originating salinity of habitats e.g. coastal or inland water bodies. Therefore, these existing salt tolerant duckweed accessions and collection of novel accessions from saline water could be explored for potential in desalination of water.

1.2.1.4. Uncovering novel phytoremediators

Duckweed extremophiles inhabiting saline or heavy metal contaminated environments could feature unique tolerance mechanisms and therefore increased phytoremediation potentials. For instance, metal and salt tolerance mechanisms were discovered in different *Arabidopsis thaliana* (Thale cress) accessions, by using cohorts of wild varieties from varied environments, with elucidation of their genetic basis (Buescher *et al.*, 2010; Chao *et al.*, 2012; An *et al.*, 2017). Despite an extensive worldwide collection of duckweeds available for researchers (Appenroth & Sree, 2020), for the most part, knowledge of originating water environments are lacking. Some exceptions which provide a proof of principal for extremophiles, are As and U bioaccumulating *Lemna* duckweeds originating from As and U contaminated mining sites (Mkandawire *et al.*, 2004b) and the discovery of a Cu accumulating accession from an U contaminated mining site (Kanoun-Boulé *et al.*, 2009). Other works remark that duckweed accessions show remarkable plasticity when faced with Zn and Cu exposure in controlled conditions (Roubeau Dumont *et al.*, 2019; Vámos *et al.*, 2023). In these instances, local adaptation did not provide a tolerance advantage, however, Zn and Cu of originating water environments or in duckweed tissue were not quantified to affirm this conclusion. Therefore, there is scope to adopt a similar approach to *Arabidopsis* by exploring duckweed accessions in context with their natural environments.

Conclusion. There is a need for global water remediation using suitable plant species. Consideration of the 36 duckweed species and numerous accessions for nutrient uptake and metal tolerance are key for development of candidates for phytoremediation. The consideration of a duckweed ionome encapsulates a wider range of elemental variation patterns than is currently featured in existing studies comparing limited numbers of elements and species. Using large duckweed cohorts grown in common replete nutrient conditions can unify species differences in elemental bioconcentration. To identify tolerance strategies for phytoremediation, undertaking a new collection of duckweed varieties with associated water data may further address this gap by exploring tolerance in the context with natural environments.

1.3. Duckweed taxonomy

The Plant kingdom extends from the ancestral green chlorophyte clade containing algae up to flowering plants, the angiosperms (Clark *et al.*, 2023) which includes the duckweed. Terrestrial plant lineages formed about 470 mya (Harrison & Morris, 2018), followed by rapid expansion of monocotyledonous species circa 140 mya to form 77 plant families (Remizowa *et al.*, 2022). Of these, the Araceae family contains 5000 species of plants. Duckweed (Lemnaceae) are aquatic floating plants sharing a common ancestor with the Araceae (Tippery *et al.*, 2021).

1.3.1. The duckweed family

The most ancestral multi-rooted duckweeds, within the genus *Spirodela,* diverged from other terrestrial monocots 130-140 mya (An *et al.,* 2019). Duckweed propagation is in the most part asexual, enabling rapid reproduction with doubling times between 36-48 hours, and <24 hours in the fastest growing angiosperm, *Wolffia microscopica* (Sree *et al.,* 2015b; Lam & Michael, 2022). The smallest angiosperm, *Wolffia angusta* is represented by an absolute reduction in size and vasculature within the duckweeds (Romano & Aronne, 2021).

The duckweeds consist of five genera, totalling 36 species. Duckweeds are highly reduced plants deriving from the fossil genus Limnobiophyllum, which had leaves, roots and veins (Bogner, 2008). Duckweed are characterised by a simple body plan of a frond, which is proposed to be a reduced stem-leaf organ, from which daughter fronds bud, forming new clonal generations (Fig 1.1). The genera are split into two subgroups with striking morphological differences including larger, ancestral rooted duckweeds Lemnoideae (*Spirodela*, *Landoltia*, *Lemna*) and small derived rootless Wolffioideae (*Wolffia* and *Wolffiella*).

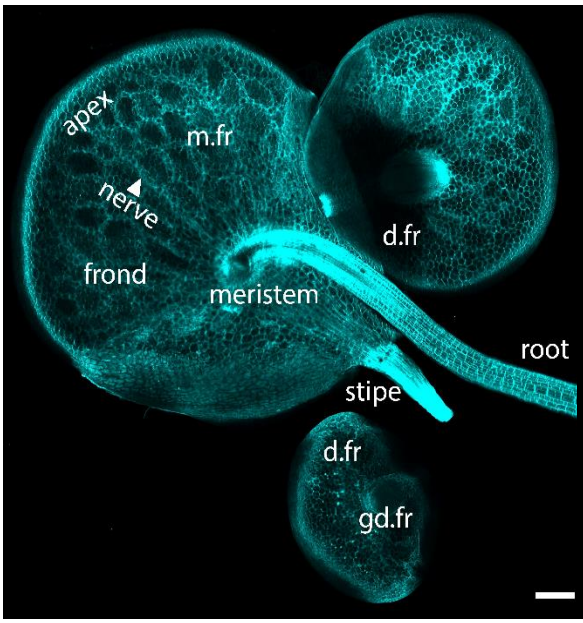

Figure 1.1. Anatomy of duckweed *Lemna gibba* ventral frond surface. A colony consisting of four fronds, a mother frond (m.fr), two daughter fronds (d.fr) one of which is detached with an emerging granddaughter frond (gd.fr). Each frond has a proximal end (apex) and a meristem from which roots, flowers or turions may emerge. Fronds nerves vary in number between species. Fronds are connected by a stalk, the stipe. Image scale bar is 200 µm.

1.3.2. Duckweed Genera

Genera differ in frond characteristics, root numbers and their genome sizes. The ancestral representative forms include species in the *Spirodela* genus which exhibit the largest fronds (3-10 mm long) and multiple roots, usually between 7-21 per frond, which perforate a scale membrane, the prophyllous sheath (Les & Crawford, 1999; Kim, 2007). The basal *Spirodela* species have the smallest genome, 158 Mb comparable to *Arabidopsis thaliana* (Wang *et al.*, 2014). *Landoltia* is the second most basal genus with large fronds (~5 mm), typically 1-7 roots and a reduced prophyllum. There is a two-fold greater genome size in *L. punctata* compared to *Spirodela* species and *Landoltia* has been considered a separate genus to *Spirodel*a since the beginning of the 21st century but contains only one species (Les & Crawford, 1999; Bog *et al.*, 2019). *Lemna* species have variable frond sizes (2-5 mm), with varying degrees of frond venation and they have a single root of differing lengths, without a prophyllum. Therefore, reduction in size and organ complexity have occurred across the duckweed family.

The most derived *Wolffia* and *Wolffiella* representative forms consist of the fastest growing, highly reduced duckweed species, which have the smallest fronds (<1 mm) and have no frond nerves or roots (Ziegler *et al.*, 2016). In fact, there are limited features in the highly reduced *Wolffia* which have been described as a reduced 'ball of cells' with limited frond architecture (Yang *et al.*, 2021; Lam & Michael, 2022). The fastest growing duckweed *Wolffia australiana* has a simplified body plan and has lost genes initiating root development and environmental responses (Michael *et al.*, 2021; Lam & Michael, 2022). A gain in genetic material has occurred in derived duckweed species, with an increase of 13-fold in the larger, variable genomes of *Wolffia* and *Wolffiella* (An *et al.*, 2018; Michael *et al.*, 2021). The organisation of duckweed family described here are given in Table 1.1.

Lemnoideae (16)			**Wolffioideae (20)**	
Spirodela (2)	*Landoltia (1)*	*Lemna (12)*	*Wolffiella (10)*	*Wolffia (11)*
Spirodela intermedia *Spirodela punctata*	*Landoltia punctata*	*Lemna obscura* *Lemna turionifera (I)* *Lemna japonica (H)* *Lemna trisulca* *Lemna minor* *Lemna gibba* *Lemna disperma* *Lemna minuta (I)* *Lemna valvidiana* (I)* *Lemna aequinoctialis (I)* *Lemna purpusilla* *Lemna tenera*	*Wolffiella lingulata* *Wolffiella oblonga* *Wolffiella gladiata* *Wolffiella neotropica* *Wolffiella welwitschii* *Wolffiella denticulata* *Wolffiella caudata* *Wolffiella repanda* *Wolffiella hyalina* *Wolffiella rotunda*	*Wolffia arrhiza* *Wolffia cylindracea* *Wolffia columbiana* *Wolffia elongata* *Wolffia neglecta* *Wolffia angusta* *Wolffia globosa* *Wolffia microscopica* *Wolffia australiana* *Wolffia borealis* *Wolffia brasiliensis*

Table 1.1. Taxonomic organisation of the duckweed family. At the highest organisational level rooted and rootless subgroups are presented with the total number of species within each subgroup. Within each subgroup, the five genera are shown with the number of species within them. From left to right genera are ordered by evolutionary forms, from ancestral to the most derived sister *Wolffia* and *Wolffiella* (Tippery *et al.*, 2015). Within the *Lemna* genus, hybrid species and invasive species of special interest in Europe are indicated with (H) and (I) respectively from the following sources (Iberite *et al.*, 2011; Kirjakov & Velichkova, 2016; Braglia *et al.*, 2021b, 2023; Fedoniuk *et al.*, 2022; Volkova *et al.*, 2023). * *Lemna valdiviana* is now a unified species with *Lemna yungensis* (Bog *et al.*, 2020).

1.3.3. The genus *Lemna*

The *Lemna* genus is organised within four sections: Lemna, Uninerves, Biformes and Alatae. *Lemna tenera* is the only species defined as Alatae due to its rare and specific ecology and possession of winged root sheaths (Crawford *et al.*, 2001). Biformes species have tapered, submerged fronds (Les *et al.*, 2001). *Lemna* duckweed show progressive reduction of frond vasculature with the section Uninerves including species with a single vascular nerve, compared to three without tracheids in Biformes and 4-7 with tracheids in section Lemna (Landolt, 1986; Tippery *et al.*, 2015).

The *Lemna* genus presently has 12 species and a handful of *Lemna* species clearly differentiate by broad morphology which is reflected in their common names (Fig 1.2). *Lemna* species *L. minor* (common duckweed), *L. gibba* (fat duckweed) recognised by its gibbosity, *L. trisulca* (ivy-leaf duckweed) and *L. minuta* (lesser duckweed). *Lemna trisulca* is vastly different and was in a unique section Hydrophylla but now forms a member of the Lemna section within *Lemna*, with its submerged growing habit and ivy-shaped fronds (Les *et al.*, 2002).

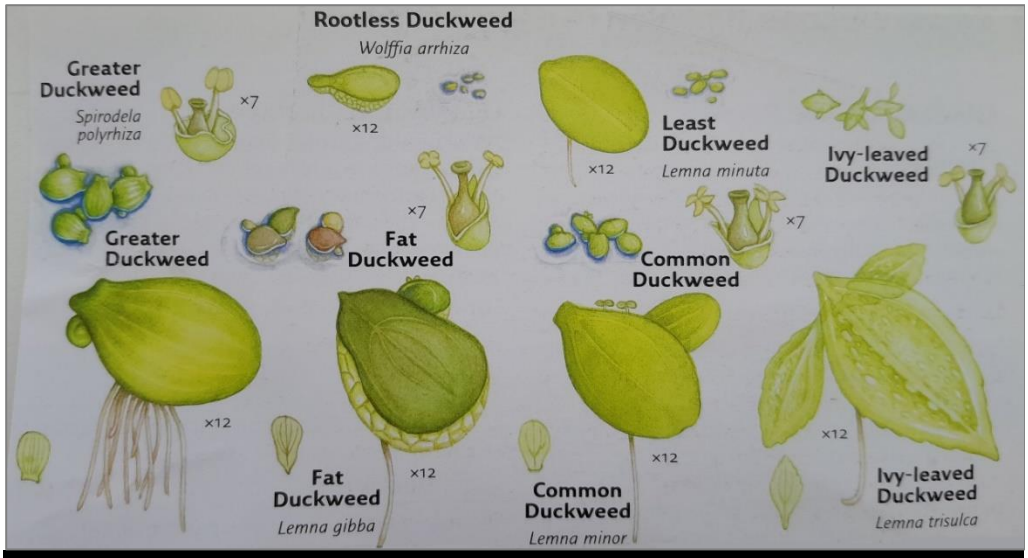

Figure 1.2. Botanical drawings of common UK duckweed species. Common and Latin names for UK duckweeds are presented with their broad and oversimplified depiction of morphology, frond, root, seed and floral structures. Various common names for *Lemna* species are shown. Additional non-*Lemna* species in the UK include: *Spirodela polyrhiza* or the 'greater duckweed' and *Wolffia arrhiza* the 'rootless duckweed'. **Source: Collins flower guide: Guide to the flowers of Britain and Ireland, Streeter, *2010*.**

1.3.4. *Lemna* species

Some closely related species are hard to discern, including others within the *Lemna* genus. Species identification is challenging due to plasticity and morphological similarity, for example, many available clones were misidentified between *L. minor* and *L. gibba* due to lack of gibbosity in the wild (Sree & Appenroth, 2020). *Lemna minor* and *L. minuta* can be distinguished by a series of frond characteristics (Mifsud, 2010; Ceschin *et al.*, 2016b). *Lemna minor* and hybrid *L. japonica* are morphologically indistinguishable and many *L. minor* clones have been reclassified from parent to hybrid species (Braglia *et al.*, 2021b; Volkova *et al.*, 2023). Species identification between *Lemna* was difficult due to similarity in genetic identities comparing allozyme loci (Crawford *et al.*, 1996, 2001). In recent times, amplified fragment length polymorphisms (AFLPs) and tubulin based polymorphisms (TBP) were used to separate clones of *Lemna gibba* from *L. minor* and find new hybrids between them, forming a new potential species (Braglia *et al.*, 2021b,a, 2023).

Within the *Lemna* genus are several native American species described as foreign invaders in Europe including the Uninerves *L. minuta* and *L. valdiviana* (indicated in Table 1.1). The length of the single frond nerve can be used to differentiate between species *L. minuta* and *L. valdiviana* (Crawford *et al.*, 1996; Iberite *et al.*, 2011). Several studies have documented species distribution within countries, highlighting the presence of several hybrid and invasive species (Xu *et al.*, 2015; Kirjakov & Velichkova, 2016; Chen *et al.*, 2022; Friedjung Yosef *et al.*, 2022; Kadono & Iida, 2022; Taghipour *et al.*, 2022; Volkova *et al.*, 2023). Indeed, *L. minuta* was shown to grow faster than native *L. minor* in high N, P and light conditions, showing its opportunism in specific environments, including Europe (Paolacci *et al.*, 2016, 2018). Excessive growth of alien species can damage the balance of the ecosystem, reducing biodiversity of native fauna and flora, and thus require expensive removal solutions (Ceschin *et al.*, 2016a, 2019). In the case of these duckweed species, reduction of frond vasculature and smaller body plans consisting of small fronds with shorter roots may be less costly to produce, equating to 'weed-like' traits involving fast growth, fast completion of life cycles, ease of spread and adaptability to new environments (Abramson *et al.*, 2022). This is exacerbated by climate change which increases invasiveness of introduced foreign species and should be monitored on a regional basis (Piria *et al.*, 2022). To assess the threat of invasive species on native species, particularly in foreign environments, a range of approaches to first robustly differentiate between species are required.

1.4. Water environments

Duckweeds are typically found in temperate and tropical waterscapes including lentic, eutrophicated ditches, ponds, lakes, lagoons and sometimes in canals and rivers (Crawford *et al.*, 1996). Distribution is widespread for most duckweed species and the habitats for colonisation can be extreme. For instance, *Lemna yungensis* species grows in an extreme physical environment, defined by growth on rocks under waterfalls with low nutrient availability in Bolivia (Landolt, 1998). Another example is *Lemna tenera* which has a specific niche environment in Australian forest swamps with low P, K, Ca and Mg availability (Landolt, 1992). Specific accessions of duckweed were also found inhabiting contaminated environments, including minewater with high heavy metals, metalloids (Cd, As) and radioactive elements (Mkandawire & Dudel, 2005). Thus, both high, low and challenging elemental compositions may be present in native duckweed environments and in turn act as selective pressures for adaptation and evolution.

1.4.1. Adaptation to high nutrient environments

Duckweeds colonise nutrient-dense environments and offer versatility in adaptation strategies to high elemental loads. For example, duckweed are commonly found growing in shallow ditches particularly high in macronutrients N and P, which receive run-off from fertiliser application (Janse and Van Puijenbroek, 1998). Duckweeds can quickly colonise and capitalise from these high nutrient loads by excessive growth to form dense mats. This strategy can make it difficult for other plants to survive, including submerged species which may suffer anoxia, giving duckweed an adaptive advantage in this environmental niche. Rapid growth strategies represents an efficient use of nutrients, as maximising growth further reduces toxicity by division of assimilated elements into daughter and granddaughter colonies (Landesman *et al.*, 2010). Thus, providing nutrient replete environments has potential for maximising growth of duckweed, in combination with controlling other environmental factors.

Duckweed have high surface areas for uptake of nutrients from water or media but show species variation. In the smallest, rootless species, only frond surfaces are in contact. In larger, floating rooted duckweed species, ventral frond surfaces and roots are in contact (Young and Sims, 1972), additionally, in submerged species the dorsal frond is in contact. Furthermore, the contribution of the frond surface relative to root surface for uptake of ammonium and nitrate increases in rooted *Lemna minor* and *Landoltia punctata*, in high N environments compared to low N environments (Cedergreen & Madsen, 2002; Fang *et al.*, 2007). Although, for *S. polyrhiza* specific frond area for uptake does not significantly change between high and low nutrient conditions (Jin *et al.*, 2021). Furthermore, low-affinity N and P transporters are plentiful in *S. polyrhiza* and unlike other plants, are unbiased between frond and roots in replete nutrient conditions (Ware *et al.*, 2023). However, details of nutrient uptake for other elements or discrepancies between rooted and rootless duckweeds are not well characterised to date.

In the context of duckweed evolution of size and root reduction, mechanisms which restrict uncontrolled uptake and transport of toxic levels of nutrients and metals are expected. Typically in plants, nutrient uptake occurs at the roots followed by long-distance root to shoot transfer of minerals. The lignified casparian strip present in the root endodermis acts by restricting apoplastic transfer of Mn, Ca, Zn, B and Na and can respond to elemental fluctuations in environments (Baxter *et al.*, 2009; Chen *et al.*, 2011; Muro *et al.*, 2023). In rooted duckweed species, an endodermal casparian strip is present, although reduced lignification may mean limited functionality in restricted transport of elements (Barnabas, 1996; Ware *et al.*, 2023). Furthermore, the cell wall components involved in apoplastic transfer of nutrients including lignin, cellulose and hemicellulose have reduced gene numbers in duckweed compared to land plants (Fang *et al.*, 2023b). The rootless *Wolffia* duckweed have expanded wax cuticle synthesis genes, presumably functioning in higher frond waterproofing and resistance against excessive uncontrolled nutrient uptake, in line with their root loss and fronds lacking vasculature (Park *et al.*, 2021; Lam & Michael, 2022). At present, there are gaps in knowledge for duckweed adaptation to high nutrient natural environments, especially between duckweed species and in comparison to other plants.

Plants which are tolerant to heavy metal contamination have several strategies including high accumulation, partitioning, conjugation or otherwise effluxion back into the environment. Examples include larger aquatic plants such as mangrove and azolla ferns, which segregate Zn and Cu into vacuoles in order to preserve photosynthesis and growth (MacFarlane & Burchett, 2000; Torasa *et al.*, 2019). In contrast, the extremely small body sizes in duckweed and limited to non-existent roots in some members give a very small distance for translocation of assimilated elements around the plant or away from photosynthetic tissues (Zhang *et al.*, 2009). Nevertheless, many duckweed hyperaccumulators have been documented (Zhou *et al.*, 2023).

Multiple laboratory studies have reported uptake of nutrients and trace elements by a single duckweed species during high exposure, however studies comparing accumulation and partitioning between species or comparing native environments are rarer. One study confirmed Zn conjugation to phytate and storage in vacuoles during exposure to high Zn in *Lemna minor* (Van Steveninck *et al*, 1992). To expand on this, Zn tolerance, uptake, accumulation and storage were reported different between three rooted duckweed species at different Zn concentrations, each varying in root and frond distributions and soluble and bound forms (Lahive *et al.*, 2011). Zn provides the only example of an element studied with this higher level of resolution of variability between species and unfortunately rootless species were not included. It is expected that the reductionist life strategy but high phenotypic variation may result in niche adaptation mechanisms in high nutrient environments.

1.4.2. Adaptation to low nutrient environments

For duckweed survival in its occupying position during extended periods of the growing season or during expansion into new locations, adaptation to low

nutrient environments may be required. It was suggested that winter may provide higher nutrient inputs due to decaying plant biomass, but summer may be nutrient-limiting due to high species richness and competition (Linton & Goulder, 1998). In turn, quiet aquatic environments can be limited in nutrient mixing, reducing floating plants' access to elements such as N and P. In such nutrient-limited environments, enhancing both surface area and sensitivity aids plants to obtain vital nutrients and survive.

Vascular plants allocate growth into belowground parts including various root morphological traits to increase available surface area for uptake in nutrient starved environments. These include root growth, root branching or root hair development. Although duckweed have relinquished development of lateral roots and root hairs (Appenroth *et al.*, 2013; Park *et al.*, 2021), *S. polyrhiza* still produces longer roots in nutrient weak solutions compared to replete nutrient solutions (Jin *et al.*, 2021). Furthermore, plants enhance expression of nutrient transporters, especially high-affinity transporters to increase influx of nutrients at the root surface (Giehl & von Wirén, 2014). Some work shows that duckweed are enriched in high affinity transporters in response to low N and P availability, as documented by high affinity P transporters in *L. punctata* (Hase *et al.*, 2004) and N and P transporters in *S. polyrhiza* (Zhao *et al.*, 2021; Kishchenko *et al.*, 2023). Therefore, rooted duckweed still respond to low nutrients by root extension and recruitment of transporters.

Adaptive mechanisms for low nutrients in duckweed species can contribute to their colonisation and success over a growing season. For example, in low N and P water environments, breakdown of stored nutrients allows expanding growth to occur as an adaptive mechanism to increase surface coverage to access more nutrients (Ziegler *et al.*, 2023). In controlled environments, *Lemna minor* species acclimated to low N and P with faster growth, but instead the apparent invasive *Lemna minuta* was less nutrient efficient and instead reduced growth (Njambuya *et al.*, 2011; Van Echelpoel *et al.*, 2016), showing differences in species adaptation to low nutrient conditions. Furthermore, when growth rates decline, duckweed respond to low N and P environments by starch accumulation (Zhao *et al.*, 2015b; Tian *et al.*, 2021) and in unfavourably low P there is a switch to overwintering as turions (Appenroth, 2002; McCann, 2016; Sree & Appenroth, 2022). Low nutrients therefore trigger seasonal duckweed growth responses in natural environments which can be studied with greater control between species using artificial environments.

1.4.3. Duckweed roots

Duckweed roots are adventitious, lacking both root hairs and lateral roots, with evolved ancestral species showing complete root loss. Generally, duckweed roots are reduced compared to land plants and other aquatic plants. Roots were lost from a number of species and function has diversified from stringent nutrient uptake to uprighting, sticking to other duckweed clones to form aggregate mats or to fauna for dispersal (An *et al.*, 2019). Contribution of roots and fronds for uptake and storage varies between replete or depleted elemental composition (Cedergreen & Madsen, 2002; Fang *et al.*, 2007; Lahive *et al.*,

2011; Ren *et al.*, 2022). Root length is also responsive to elemental concentration, growing in response to low N and P (Van Steveninck *et al.*, 1992; Cedergreen & Madsen, 2002) and reducing in high NaCl, Zn, Cd, Cr and Ag (Van Steveninck *et al.*, 1992; Yilmaz, 2007; Lee *et al.*, 2023; Zheng *et al.*, 2023) and may easily detach suggesting lack of requirement (Tae-jun & Park, 2017). The duckweed family represent a model for root vestigiality and elemental consequences of root loss (Ware *et al.*, 2023). Despite this, surprisingly nutrient uptake and storage between rooted and rootless duckweeds in replete or deplete nutrient conditions has not been actively compared.

1.4.4. Nutrient uptake and growth

Many factors affect duckweed growth rate in its native environment including light, temperature, rainfall, amounts and forms of N and P, micronutrients and toxic chemicals, water flow and depth. As proof of principal, duckweed coverage in water environments varies between regions, seasons and sites (Mkandawire & Dudel, 2007; Sullivan & Giblin, 2012; Basiglini *et al.*, 2018). Both growth and nutrient extraction potentials vary between duckweed growing in high N and P in indoor or outdoor systems. For outdoor culturing on wastewater, growth rates were relatively low giving 4.87 g m^{-2} day^{-1} for *Lemna minor* on dumpsite leachate with a slight increase on synthetic leachate to 7.03 g m^{-2} day^{-1} (Iqbal *et al.*, 2019). In outdoor ponds of waste sewerage, increases of growth to 14.3 g m^{-2} day^{-1} was achieved for *Lemna gibba* (Oron *et al.*, 1988). Overall, a higher rate of growth of 29 g m^{-2} day^{-1} and extraction of N and P by *Landoltia punctata* 7776 was achieved using synthetic swine wastewater (Cheng *et al.*, 2002). However, these varied studies do not always report the amount of starting biomass nor do they state the availability of nutrients in each wastewater. To connect the reported differences in growth and N and P uptake between duckweed species a unified study on a specific medium could be used.

There are limited descriptions of duckweed native aquatic environments and tolerance of duckweed to nutrient conditions in the environment. One study confirmed that P varied by 99% between 200 freshwater duckweed-containing habitats and is in the minority of works offering supporting evidence for duckweed potential to acclimate to varied nutrient composition (McCann, 2016). However, P uptake of collected accessions in replete laboratory conditions were not linked with originating environments. Another study linked nutrients in a medium and in pond water, finding that *Lemna minor* growth correlated with levels of N, P, Ca, Mg and K and growth was indeed faster on nutrient medium than pond water (Linton & Goulder, 1998). In general, there is a gap in knowledge documenting the range of elemental composition of water bodies experienced by duckweed within and between sites. This means any influence this might have on growth or nutrient extraction of derived accessions is not well characterised. Filling this gap could link variation in duckweed tolerance responses to water habitats, at the same time identifying a need for remediation in specific water bodies.

Controlled environments are a powerful way to control variable environmental factors and consistently provide replete nutrients. These can achieve maximal growth and influence elemental composition of duckweed tissue for phytoremediation and nutrition applications. Maximal growth rates is a common feature for both applications. Varied growth rates have been reported for duckweed accessions even when cultured on the same nutrient-rich media in controlled environments (Ziegler *et al.*, 2015), showing within-species natural variation in growth. Furthermore, various abiotic and biotic stressors can reduce growth of different accessions (Roijackers *et al.*, 2004; Khellaf & Zerdaoui, 2009; Ceschin *et al.*, 2020; Zhang *et al.*, 2020; Magahud & Dalumpines, 2021; Femeena *et al.*, 2023; Sońta *et al.*, 2023), and presumably affect accessions differently, depending on their innate ability to acclimate. Nevertheless, there is clear potential to manipulate this existing natural variation in duckweed growth and select for accessions with improved nutritional profiles for commercial applications (Coughlan *et al.*, 2022).

For this purpose, growth and elemental extraction can be manipulated by optimising independent components of the controlled environment. For instance, increasing N supply for *Lemna obscura* and *Lemna minor* improved growth (Landesman *et al.*, 2005; Ullah *et al.*, 2022) and optimising the forms of supplied N for *L. minor*, *L. punctata* and *L. gibba* further increased growth (Cedergreen & Madsen, 2002; Fang *et al.*, 2007; Shi *et al.*, 2023). Elemental uptake can also be modulated by controlling pH and nutrient composition. For instance, increased Cd uptake could be achieved using a neutral pH but increasing acidity or alkalinity could reduce Cd uptake in respective studies (Verma & Suthar, 2015; Ma *et al.*, 2023). Therefore, increasing Cd for phytoremediation or reducing Cd for food could be achieved with media manipulation. Moreover, altering pH and elemental ratios such as increasing Ca:Mg was successfully used to grow duckweed species on originally unsuitable contaminated water (Landolt & Kandeler, 1987; Van Dam *et al.*, 2010; Paolacci *et al.*, 2016; Walsh *et al.*, 2020; Jones *et al.*, 2023). Therefore, amounts, form, pH and interactions between various macronutrients, micronutrients and heavy metals are all considerations for duckweed production of biomass either for phytoremediation or food.

Conclusion. Water environments provide high and low nutrient conditions impacting growth over seasons and between regions. The use of controlled environments can increase duckweed growth and allows comparison of panels of duckweeds by standardising factors that are otherwise variable across wild environments. There is a need for inclusion of rooted and rootless genera of duckweeds with subsets of species and accessions to compare elemental compositional variation and make links with phenotypic differences. Environmental manipulation using a nutrient medium can be used to improve duckweed growth and identify candidates for food production. Collection of duckweeds from contaminated environments and using replete nutrient media to confirm hyperaccumulation could reveal tolerance traits of accessions for use in phytoremediation.

1.5. Light environments

Duckweed are versatile in light environments, as illustrated by cosmopolitan distributions and full duckweed water coverage in both sun and shade environments. In addition, shade environments give other advantages such as stable temperatures and extra nutrient input from flora above (Landolt, 1986). This is consistent with observations that duckweeds can often settle in ditches or low lying ponds. In shaded environments, terrestrial plant canopies above reduce both light intensity and light quality for aquatic plants (García-Plazaola *et al.*, 2004). Contrastingly, in exposed open water, floating plants such as duckweed can form the first penetration barrier to high irradiance in the water ecosystem (van Gerven *et al.*, 2015) implying they have coping strategies for high light exposure. In turn, the light quantity and spectra intercepted by duckweed on the surface also affects the light availability of submerged plants below including other duckweed e.g. *Lemna trisulca*. Therefore, even within a duckweed community, species would experience light differently. Despite this versatility in environments and in duckweed life strategies, their natural light habitats are not well described.

Light is not adequately described in duckweed natural environments. In the best case, measurements of light intensity at a single time point were used to describe native environments (Ceschin *et al.*, 2018a; Strzałek & Kufel, 2021; Jewell *et al.*, 2023), but daytime or seasonal effects were not considered. Therefore, the range of light duckweeds can tolerate within and between native water sites has not been measured. From an ecological comparison across Italy, invasive *L. minuta* was more prevalent in shaded sites than native *L. minor* (Ceschin *et al.*, 2018b), perhaps showing higher adaptive mechanisms to shade sites than the former species. However, only subjective visual estimations were used to describe and compare light environments.

Controlled high irradiance environments using artificial lights are more commonly used to compare growth responses of domesticated accessions but only one study considers natural environments of collected plants. A *Spirodela* accession collected from a sun site grew faster than an accession from a shade site in high artificial light, evidencing possible accession-specific or local adaptation to high light (Strzałek & Kufel, 2021). The inclusion of native and artificial light environments in duckweed research can better explore differential light acclimation strategies between duckweed accessions, species and local effects. In particular, to predict growth and spread of native and invasive species or for candidate selection for food applications.

1.5.1. High light adaptation

High light adapted or sun-loving (heliophilic) plants may seek sun by stem elongation or changing leaf incidence angle to maximise light interception. In higher plants, sun-adapted plant leaves are typically smaller to avoid excessive light absorption but thicker to increase photosynthetic efficiency per area (Lichtenthaler *et al.*, 2007). In contrast, duckweed fronds are reduced stem-leaf organs, thereby limiting phototropic responses. Instead, duckweed

canopies essentially act as two-dimensional, spreading canopies forming a relatively flat surface of fronds on the water surface. Therefore, active growth through continuous production of new colonies is a key response to light irradiance.

Duckweed can respond by high growth in naturally high light and temperatures, forming 'duckweed blooms' in open sun sites in tropical climates (Vargas-Cuentas & Roman-Gonzalez, 2019; Salcedo *et al.*, 2024). Generally for most duckweed species, growth increases with light intensity (as photosynthetic photon flux density (PPFD)) but saturates well below full sunlight (about 2000 µmol m^2 s^{-1}), beyond which light then becomes growth inhibiting. This is illuminated by various artificial light experiments (Baek *et al.*, 2021) and a summary of reported light saturation points for individual duckweed species are given in Table 1.2. Species appear to show differences in light saturation, however differences between experimental methods invalidates any conclusions made between studies. Furthermore, accession numbers are not typically included, despite the fact that this could be a contributing factor to different reported light saturation points, in particular for *L. minor*.

Species	**Light saturation (µmol m^2 s^{-1})**	**Reference**
Lemna minor	166-350	(Landolt & Kandeler, 1987)
Lemna aequinoctialis 6000	>400	(Yin *et al.*, 2015)
Lemna minor	300-450	(Thierry *et al.*, 2013)
Lemna minor	400	(Paolacci *et al.*, 2018)
Lemna minuta	400	(Paolacci *et al.*, 2018)
Lemna gibba	700	(Stewart *et al.*, 2020)
Landoltia punctata	>600	(Wedge & Burris, 1982) *
Lemna minor	<600	(Wedge & Burris, 1982) *

Table 1.2. Light saturation of growth for different duckweed species in controlled environments. Growth rate was measured under varied light intensities and temperatures. Accession numbers are included when given. *Photosynthetic saturation of light was measured by oxygen evolution.

Some studies attempted to explore light tolerance strategies and make species comparisons using common artificial high light environments. For example, an invasive *Lemna minuta* could grow faster than a native *L. minor* (Paolacci *et al.*, 2018). Although this work was limited in scope by using single accessions within each species group to draw species-wide conclusions. Other studies expanded on the meaningful physiological responses to light irradiance in duckweed. For example, the linking of positive growth responses with

higher biomass, quantum yield of photosynthesis and protein yield in *Lemna minor* and *Wolffiella hyalina,* revealing light tolerance strategies relevant for food applications (Artetxe *et al.*, 2002; Petersen *et al.*, 2022a). However, from a phytoremediation context, increasing light intensity in a synthetic wastewater system did not increase growth rate for *Lemna minor* (Walsh *et al.*, 2021). Therefore, growth response to light appears to be species and context dependent, probably determined initially by the specific duckweed's capacity for light acclimation and indeed whether it is experiencing other abiotic stresses.

Photosynthetic efficiency decreases under high light when plants are stressed and light is in excess of that required for photosynthesis. Photoprotection is generally induced to prevent photoinhibition (irreversible photosynthetic damage) which can be detrimental to growth. Before that occurs, the over-reduction of photosystem II (PSII) induces energy dissipation from excited chlorophyll molecules as heat, a photoprotective response called non-photochemical quenching (NPQ) (Ruban, 2016). NPQ is complex but largely formed via a structural reorganisation of the thylakoid membrane that is accelerated by a protein called PsBS and the operation of the xanthophyll cycle.

The xanthophyll cycle is a reversible process whereby violaxanthin is de-epoxidated via intermediate antheraxanthin and converted to form zeaxanthin in high light (Latowski *et al.*, 2011). In this respect, plant species show variation in their ability to effectively acclimate photosynthesis according to their different growth strategies (Murchie & Horton, 1997, 1998; Demmig-Adams *et al.*, 2012; Burgess *et al.*, 2023). For example, high rates of NPQ in algae compared to land plants (Ruban *et al.*, 2014) and in *L. minor* compared to *Arabidopsis* (Liebers *et al.*, 2023) support that fast growing aquatic plants have specialised light adaptation mechanisms compared to terrestrial plants. Furthermore, in conventional crops such as rice, induction of NPQ to reduce photoinhibition was linked with higher biomass (Hubbart *et al.*, 2018). Therefore, photoacclimation processes has important outcomes for growth and biomass traits which are valuable for novel crops.

In high light irradiance, plants engage a number of molecular processes such as photoprotective energy dissipation to prevent generation of ROS. This is typified by larger chlorophyll antenna consisting of higher chlorophyll a:b and more carotenoids per unit leaf area (Lichtenthaler *et al.*, 2007). Increasing carotenoid accessory pigments in chloroplasts represents a short-term light acclimation response to absorb excess light between 400-500 nm and transfer energy to chlorophyll a (Maoka, 2020). In line with other plant responses, *Lemna gibba* increased chlorophyll a:b and carotenoids, in particular lutein and beta carotene during exposure to high light irradiance (Stewart *et al.*, 2020), which are important compounds for human consumption.

Another stress response to absorb excess photons and prevent production of ROS includes accumulation of anthocyanins in vacuoles (Gould, 2004). Duckweed *Landoltia punctata* accumulated dark pigmentation in its frond

which was concluded to be an anthocyanin response to relatively low artificial light at night (Nakagawa-Lagisz and Lagisz, 2023). However, anthocyanin accumulation as a high irradiance response has not been characterised nor has species variation been compared. Furthermore, the degree to which plants acclimate their levels of photopigments and antioxidants should be linked with growth strategies to maximise compounds and biomass for nutrition.

High light irradiance can promote high photosynthetic rates, resulting in high CO_2 assimilation in source tissues. In duckweed, there are limited sink organs for assimilated carbon except for the newly developing daughter colonies (Fang *et al.*, 2023a). Therefore, this fast reductionist growth strategy continuously demands photoassimilates rather than complex body plan development and growth is uncoupled from negative feedback from starch accumulation (Stewart *et al.*, 2020). In addition, plants generally open and close stomata to regulate CO_2 uptake for assimilation but in duckweed, stomata are open and non-responsive to environmental stimuli, so presumably CO_2 is not limiting (Landolt, 1986; Shtein *et al.*, 2017). As duckweeds represent some of the fastest growing flowering plants, how growth and photosynthesis responds to varied light environments within the duckweed family remains a fundamental biological question (Ishizawa *et al.*, 2021; Stewart *et al.*, 2021).

1.5.2. Low light adaptation

Low light consists of a reduction in light quantity and concordant adjustments in light quality such as reduced red:far red ratios due to attenuation of light by plants above (Murchie & Horton, 1997). Shade avoidance strategies include stem elongation or increased specific leaf area which is the production of larger, thinner leaves to increase surface area for light interception (Puglielli *et al.*, 2017). Shade tolerance strategies include photosynthetic adjustments to grow or survive under low light (Chen *et al.*, 2016). In duckweed, a combination of shade responses may be possible depending on species and environment.

Using artificial light environments, responses to low light usually around 100 $\mu mol\ m^2\ s^{-1}$ are species dependent. For example, *S. polyrhiza* showed increases in specific frond area to maximise light interception but the smaller *L. minor* species did not show this morphological response (Strzałek & Kufel, 2021). Instead, in another study, *L. minor* total chlorophyll levels were consistently higher than *L. minuta* at a range of low light levels, indicating *L. minor* may be more shade tolerant of these two species by maintaining more chlorophyll for efficient light harvesting (Paolacci *et al.*, 2018). Taken together, these results show species variation in growth and low light responses. However, these comparisons are limited by the numbers of accessions and species included so at present it is not possible to separate species, accession and local adaptation effects.

Low light adaptation is already indicated by saturation of growth and photosynthesis at mid light levels in different duckweed species (summarised

in Table 1.2). Furthermore, duckweed are best classified as C_3 plants with high radiation use efficiency (Stewart et al., 2021; Femeena et al., 2023), indicating that they are well adapted to low light. Indeed, growth can still occur in light as low as <20 μmol m^2 s^{-1} and this ability to thrive under dim light regimes may be a key feature of some duckweed light adaptation profiles (Yin et al., 2015, Paolacci et al., 2018, Femeena et al., 2023). Additionally, using radiation efficient crops that produce high yields with low inputs of light are optimal for selection as a novel food source and were achieved by breeding in current wheat production systems (Furbank *et al.*, 2019). This means duckweed is suitable for growth under low intensity LED lighting: to minimise light inputs and maximise biomass outputs from vertical farming systems (Petersen *et al.*, 2022a). For advancement towards commercial crop production, classifying the fastest growing duckweed accessions and species with minimal light inputs remains to be tested.

Conclusion. Duckweed show high plasticity for growth in high and low light evidenced by worldwide colonisation of aquatic environments. Photoprotective mechanisms including NPQ are well developed for fast growing aquatic species but it is unknown the extent of which photosynthetic acclimation and growth rate are coupled. Studies enabling comparison of different species are limited due to disparity between light inputs and other variables used in artificial environments. Duckweed adaptation to original light environments is relatively underrepresented in the literature but could be used to identify accession or local variation in light tolerance responses. For food systems, one aim is to minimise light inputs and maximise biomass outputs, which can be best achieved by using phenotypic comparisons of duckweed panels under common light regimes. Furthermore, increased growth and compositions of protective antioxidants may be achieved in light tolerant varieties by increasing light irradiance in the growing system. These comparisons are relevant for vertical farming, hydroponics and space applications of duckweed.

1.6. Aims

The overarching aim of this research is to assess duckweed accessions and species suitability for nutrition as a vegetable crop, protein powder or for phytoremediation.

Aim 1 is to assess compositional variation of minerals and heavy metals between duckweed species. The aim is to identify suitable nutritional profiles for applications in food (high minerals, low toxic elements) or phytoremediation (high minerals and toxic elements). An additional aim is to link the reduction of roots and simplified body plan between duckweed genera to variation in elemental composition.

Aim 2 is to establish and characterise a UK panel of duckweeds with associated natural habitats including water chemistry and light environments.

Aim 3 is to discover mineral and heavy metal -excluding or accumulating accessions from the UK collection with potential for use in food or phytoremediation.

Aim 4 is to assess light tolerance strategies of UK duckweed accessions and species, to associate physiological responses with growth. This aim has a key focus towards maximising growth and carotenoids for food applications.

Aim 5 is to compare scalability of growth of UK duckweeds for powdered supplement production. An aim is to characterise aroma, antioxidant and amino acid profiles for acceptability for this application.

The aims are addressed as follows: Chapter two uses a worldwide panel of available duckweed clones and chapter three describes a large novel UK collection of accessions with associated environmental water data developed from this project. Macro, micronutrients and heavy metal composition are quantified from tissue of duckweeds from each of these panels of duckweed grown in a common environment, supplying replete nutrition conditions. Differences are discussed with a focus on biofortification as a nutritious vegetable or identification of hyperaccumulators for remediation. Chapter four focuses on responses to high light irradiance. It further highlights fast-growing species, accessions and the physiological responses linked to maximising growth in high and low artificial light environments. Small-scale production in this chapter shows how combination of light environment and accession can be applied for vegetable food or space horticulture. Chapter five focuses on scaling up UK duckweeds for growth to produce a powdered supplement. It explores variation in nutritional aspects of powders (metabolites) and aroma for human consumption, discussing these in the contexts of duckweed acceptability and health benefits.

1.7. Hypotheses

The overall hypothesis is that different duckweed species and accessions will show different suitability for phytoremediation and nutrition applications.

Different duckweeds grown in standard nutrient replete conditions will show variation in elemental contents.

Duckweed accessions and species will inhabit different regions, light and water chemistry environments in the UK.

Duckweed accessions will show differential growth and physiological responses to acclimate to high light irradiance in a controlled small-scale growing environment.

Freeze-dried supplement powders produced from different species will show marked differences in biomass, volatile and metabolite profiles using a large-scale glasshouse experiment.

1.8. Thesis layout

The thesis is written in a "thesis by publication" format, with chapters two – five in the form of published papers or submitted manuscripts. In each case the author's contribution is stated within chapters and in the supplementary methods.

Chapter one is a literature review focused firstly on the uses of duckweed in human consumption which is two-tiered: specifically focusing on duckweed as either a protein powder or eaten as a fresh vegetable. The literature supporting a role for duckweed in water remediation is also described. The literature review then follows with two key themes: duckweed and environment. The first documents the relevant background in duckweed taxonomy. The second explores environments incorporating nutrients and light variation and their impact on growth. The thesis aims and hypotheses are focused around utilizing duckweed natural variation and environmental responses for food and phytoremediation applications.

Chapter two
The evolution of the duckweed ionome mirrors loss in structural complexity.
Smith, K.E., Zhou, M., Flis, P., Jones, D.H., Bishopp, A., Yant, L. (2024). *Annals of Botany*, **133:** 7, 997–1006,. doi: https://doi.org/10.1093/aob/mcae012
This chapter takes a broad look using a range of worldwide duckweeds for their variation in the whole tissue ionome, the mineral content (macro, micro and trace elements) using standard media in a common garden experiment. It compares broad-scale variation (within genera), and fine-scale differences (within and between species) using inductively coupled plasma mass spectrometry (ICP-MS). The duckweed sampling is inclusive of phenotypic variation and finds patterns of elemental components with evolution of the duckweed body plan including root loss. It identifies new hyperaccumulating accessions which are then discussed from the perspective of applications e.g. nutrition and phytoremediation.

Chapter three
An ecological, phenotypic and genomic survey of duckweed species with their associated aquatic environments in the United Kingdom.
Smith, K.E., Cowan, L., Flis, P., Moore, C., Heatley, M., Robles-Zazueta, C., Lee, A., Yant, L. (2024). Available on bioRxiv and submitted to AoB PLANTS.
doi: https://doi.org/10.1101/2024.08.14.607898
This chapter describes a novel duckweed collection encompassing regions in the north and south UK. Species identification is achieved using combined phenotyping and genotyping strategies by inclusion of known duckweeds to aid with classification from genomic clustering. It then describes the distribution of native, invasive and new hybrid species in the UK. A description of the spatial and seasonal variation of water chemistry is provided using elemental quantification of native water sites. Then, by growing UK *Lemna* species and accessions axenically in a common garden with replete nutrients, their ionomes were quantified with ICP-MS. Hyperaccumulators are

identified from this ionomics panel and candidates discussed in the context of biofortified food or phytoremediation applications.

Chapter four
Physiological adaptation to irradiance in duckweeds is species and accession specific and depends on light habitat niche.
Smith, K.E., Cowan, L, Taylor, B, McAusland, L, Heatley, M, Yant, L, Murchie, E.H. *Journal of Experimental Botany*. 2024. **75**:7 Mar. 2046-2063. doi: https://doi.org/10.1093/jxb/erad499
This chapter first presents seasonal variation in light quality and quantity across native duckweed sites in the UK. It then demonstrates how controlled artificial low and high light can be used to compare light acclimation responses in a panel of UK duckweeds originating from these native sites. Duckweed acclimation is analysed from the contexts of species, accession, and local effects from the light levels of collection environments. The outcome of the work shows accessions with growth patterns, heightened carotenoids and anthocyanin production which could be promising for food applications including space horticulture.

Chapter five
Aroma and metabolite profiling in duckweeds: exploring species and ecotypic variation to enable wider adoption as a food crop.
Smith, K.E., Schäfer, M., Lim, M., Robles-Zazueta, C., Cowan, L., Fisk, I., Xu, S., Murchie, E.H. *Journal of Agriculture and Food Research*. 2024. 18. Dec. doi: https://doi.org/10.1016/j.jafr.2024.101263
This chapter identifies aroma compounds and metabolite profiles for UK duckweeds, including quantification of VOCs, sugars, free amino acid and flavonoid profiles. It describes UK duckweed potential for application to horticulture using a glasshouse environment to test scalability and production of freeze-dried powder supplements. The predominant aroma compounds are identified and compared with other acceptable vegetables and herbs and between duckweed species. Human participants recruited in a preliminary study describe the aromas perceived from duckweed which is discussed in the context of human acceptability. Differences in aroma and metabolite profiles are then discussed in the contexts of potential shelf-life and dietary benefits by incorporation of species as a food supplement.

Chapter six General discussion. This chapter pinpoints interesting duckweed species and accessions for elemental composition, light acclimation and those from differential natural originating environments. The use of both controlled small-scale environments and large-scale glasshouse growing systems are used to highlight accessions for either phytoremediation or food potential. Future experiments to further explore UK accessions and species from this work are postulated for food applications. Finally, hyperaccumulating duckweed accessions are discussed in the context of relevant case studies for UK regions requiring phytoremediation.

Chapter seven Supplemental methods. A detailed supplementary methodology to each chapter is provided including associated scripts for data analysis. This chapter details the rationale for a UK duckweed collection, sampling selection, environmental measurements, and maintenance of the new collection. It gives an overall table of field equipment, laboratory equipment and reagents used for all experiments within chapters.

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2. Chapter two: The evolution of the duckweed ionome mirrors losses in structural complexity.

Preface. Existing studies of elemental accumulation in duckweed have focused on very few species, often confined to a single genus, and usually assess only a handful of elements. Therefore, to systematically assess natural variation in the duckweed ionome on a genus-wide basis, in chapter two thirty-four duckweed accessions from all genera were compiled and subjected to an ionomic analysis of whole-plant concentrations of eleven elements, using inductively coupled plasma mass spectrometry (ICP-MS). Using both within- and between-genus sampling allowed the assessment of the degree of fine-scale variation, as well as between genus variation, which may be explained by evolutionary change (*e.g.* broadly between basal *Spirodela* to more derived *Wolffia/Wolffiella*). Duckweed growing conditions were standardised and growth medium was optimised for micro- and macronutrients. Duckweed whole plant biomass was then used to measure total elemental tissue concentration by accession under identical conditions.

Aims. To characterise in detail ionome variation both at the fine- and broad-scale across all five genera of duckweed species. To identify individual accessions which may hyperaccumulate elements for nutrition or phytoremediation.

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The evolution of the duckweed ionome mirrors losses in structural complexity

Kellie E. Smith[1,#], Min Zhou[2,†,#], Paulina Flis[2], Dylan H. Jones[2], Anthony Bishopp[2] and Levi Yant[1,3,*]

[1]School of Life Sciences, University of Nottingham, Nottingham NG7 2RD, UK, [2]School of Biosciences, University of Nottingham, Sutton Bonington LE12 5RD, UK and [3]Department of Botany, Faculty of Science, Charles University, Prague, Czech Republic
[*]For correspondence. E-mail levi.yant@nottingham.ac.uk
[#]Kellie Smith and Min Zhou contributed equally.
[†]Present address: Department of Obstetrics and Gynecology, Women and Children's Hospital of Chongqing Medical University, Chongqing 401147, China and Department of Obstetrics and Gynecology, Chongqing Health Center for Women and Children, Chongqing 401147, China

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• **Background and Aims** The duckweeds (Lemnaceae) consist of 36 species exhibiting impressive phenotypic variation, including the progressive evolutionary loss of a fundamental plant organ, the root. Loss of roots and reduction of vascular tissues in recently derived taxa occur in concert with genome expansions of ≤14-fold. Given the paired loss of roots and reduction in structural complexity in derived taxa, we focus on the evolution of the ionome (whole-plant elemental contents) in the context of these fundamental changes in body plan. We expect that progressive vestigiality and eventual loss of roots might have both adaptive and maladaptive consequences that are hitherto unknown.
• **Methods** We quantified the ionomes of 34 accessions in 21 species across all duckweed genera, spanning 70 Myr in this rapidly cycling plant (doubling times are as rapid as ~24 h). We related both micro- and macroevolutionary ionome contrasts to body plan remodelling and showed nimble microevolutionary shifts in elemental accumulation and exclusion in novel accessions.
• **Key Results** We observed a robust directional trend in calcium and magnesium levels, decreasing from the ancestral representative *Spirodela* genus towards the derived rootless *Wolffia*, with the latter also accumulating cadmium. We also identified abundant within-species variation and hyperaccumulators of specific elements, with this extensive variation at the fine (as opposed to broad) scale.
• **Conclusions** These data underscore the impact of root loss and reveal the very fine scale of microevolutionary variation in hyperaccumulation and exclusion of a wide range of elements. Broadly, they might point to trade-offs not well recognized in ionomes.

Key words: Vestigiality, duckweed, ionomics, evolution, ICP-MS, *Spirodela*, *Landoltia*, *Lemna*, *Wolffiella*, *Wolffia*.

INTRODUCTION

The duckweeds (Lemnaceae) consist of 36 species exhibiting broad variation, including, in recently derived species, the progressive evolutionary loss of a fundamental plant organ, the root. This progressive loss of roots is accompanied by an overall reduction in vascular tissues in derived taxa. Given the paired loss of roots and reduction in structural complexity, we focus here on the evolution of the ionome and place it in the context of these fundamental changes in body plan.

Consisting of five genera progressively differing in the number of roots and vascular complexity, the duckweeds present broad variation in highly simplified body plans (Fig. 1). The earliest diverged lineages, *Spirodela* and *Landoltia* (Fig. 1, top), were originally both considered *Spirodela*, but are now recognized as distinct (Les and Crawford, 1999; Les *et al.*, 2002; Bog *et al.*, 2015). The three more recently diverged genera, *Lemna*, *Wolffiella* and *Wolffia*, represent novel forms, with progressively diminished roots and reduced vascular tissues (called nerves) or none at all (Fig. 1, bottom; Appenroth *et al.*, 2013; Tippery *et al.*, 2015). The divergence time between rooted *Spirodela polyrhiza* and rootless *Wolffia australiana* is estimated at 70 Myr (Park *et al.*, 2021). Since this divergence, ≥36 duckweed species have formed (Appenroth and Sree, 2020; Bog *et al.*, 2020), which vary 14-fold in genome sizes (Hoang *et al.*, 2019). The smallest is an *Arabidopsis*-scale 158 Mb genome in *Spirodela polyrhiza* (Wang *et al.*, 2011; An *et al.*, 2018), with the largest genomes in the derived *Wolffia*, which exhibit a radically simplified body plan, diminished vasculature and no roots (Fig. 1 bottom row; Park *et al.*, 2021; Yang *et al.*, 2021).

In contrast to vascular land plants, duckweeds have miniscule bodies in direct contact with water and limited to non-existent root systems. This results in small distances for ion translocation (Zhang *et al.*, 2009). However, the relative differences in translocation distance can be large: frond sizes of *Spirodela* are >1 cm, but in *Wolffia* only <1 mm. Duckweed roots are considered adventitious, lacking lateral

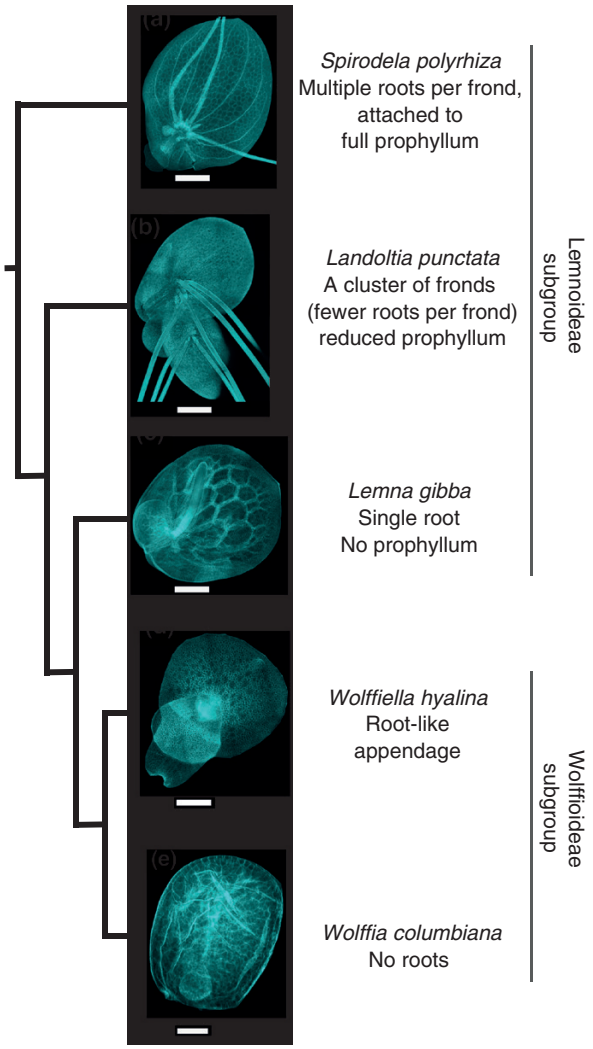

FIG. 1. Trajectory from ancestral root-harbouring duckweeds, via vestigiality, to root loss. Ancestral form (above) represented by Lemnoideae: *Spirodela*, *Landoltia* and *Lemna*. Derived from (below) shown in Wolffioideae subgroup genera *Wolffiella* and *Wolffia*. All samples were cleared, stained with Fluorescent Brightener 28 (calcofluor) following the protocol described by Kurihara *et al.* (2015) and imaged on a Leica TCS SP5 confocal microscope. Scale bars: *Spirodela* and *Landoltia*, 1000 μm; *Lemna* and *Wolffiella*, 500 μm; *Wolffia*, 100 μm. Cladogram schematic topology is based on Tippery *et al.* (2015).

roots and root hairs (An *et al.*, 2019). Root-forming species have flexibility in their root systems, which can develop or elongate in stressful situations or drop off (Landolt, 1986). Root functions in anchorage, aggregation to form duckweed mats and aiding dispersal by attachment have all been proposed (Cross, 2017; Ware *et al.*, 2023). In the highly derived Wolffioideae, the shrinking of body size and complete root loss have evolved to maximize growth rate, improve mobility and enhance adaptability to changing environments (Wang *et al.*, 2010; Michael *et al.*, 2020; Yang *et al.*, 2021). We expect that duckweeds, representing this unique example of progressive root reduction through to complete loss, will illustrate a gradient of phenotypic changes resulting in altered internal macronutrient and trace element compositions (Ware *et al.*, 2023).

At the fine scale, duckweed habitats differ in their availability of elements; thus, adaptation of accessions to their environments can occur through different elemental storage and exclusion strategies (Mkandawire and Dudel, 2007; Zhang *et al.*, 2009; Van Dam *et al.*, 2010; Lahive *et al.*, 2011). The tolerance of duckweed to elemental extremes is an important trait driving adaptive (and sometimes strongly invasive) strategies in the wild (Wang, 1991; Naumann *et al.*, 2007; Ekperusi *et al.*, 2019). To date, however, the tolerance of only a handful of duckweed accessions to external elemental concentrations has been assessed, with reports focusing on growth vigour vis-à-vis single elements in *Lemna* and *Landoltia* species. Studies quantifying elemental composition are rare, with the broadest study looking at only a single genus, *Wolffia*, with 11 species being assessed (Appenroth *et al.*, 2018). We collected existing reports of duckweed elemental variation; however, serious confounding factors plague interpretation of different studies, owing to discordant methods and quantification (Table 1).

Here, we bridge this gap, reporting whole-plant ionome compositions in 34 duckweed accessions spanning 21 species and representing the worldwide range of all five duckweed genera (Fig. 2; Supplementary Data Table S1). We place these data into an evolutionary context, focusing on 11 key macro-, micro- and trace elements, contrasting microevolutionary variation (accession-level, within-species variation) with macroevolutionary trends (between genera). These results reveal extensive ionomic variation at both the within-species and between-genus levels, with particularly clear trends for differences in Ca and Mg accumulation, in addition to possible excess Cd accumulation in the rootless *Wolffia/Wolffiella*. We discern a broad evolutionary trajectory towards very low levels of essential Ca and Mg, in addition to increased Cd accumulation, in the recently derived rootless species. This suggests a potentially deleterious consequence associated with the root loss and body-wide reduction in vasculature.

MATERIALS AND METHODS

Plant growth and care

Duckweed accessions were grown in axenic conditions from single isolates or from five to ten individuals, depending on the size of duckweeds, in 100 mL of nutrient medium (N medium) in individual sealed sterile glass conical flasks. Duckweeds were sourced from the Landolt Collection (now housed in Milan). The N medium was described by Appenroth *et al.* (1996) [KH_2PO_4, 0.15 mM; $Ca(NO_3)_2$, 1 mM; KNO_3, 8 mM; $MgSO_4$, 1 mM; H_3BO_3, 5 μM; $MnCl_2$, 13 μM; Na_2MoO_4, 0.4 μM; and FeEDTA, 25 μM]. Concentrations of elements in the supplied N medium, including the presence of other trace elements, were measured by inductively coupled plasma mass spectrometry (ICP-MS) and are presented in the Supplementary Data (Dataset S1). Weekly media changes were performed, with rinses in Milli-Q (Millipore) water to regulate nutrient composition availability. Plants were grown at 100 μmol m^{-2} s^{-1} under broad-spectrum (white) LED lights at 22 °C/18 °C with a 16 h day/night cycle. Four-week-old duckweed cultures were washed on plastic sieves using a three-step protocol for 2 min each of Milli-Q (Millipore)

Table 1. *Elemental tissue concentration of duckweeds gathered from the literature. Elements are ordered by type (macro, micro, trace elements and heavy metals) reported from the literature and included in our experiment.*

Element	Species	Fold variation (literature)	Fold variation (this study, 21 species)
P	*Wolffia* spp.	1.7[1,2]	2.4
K	*Lemna* spp., *Wolffia* spp.	2.4[1,2]	3.3
Ca	*Lemna* spp., *Wolffia* spp.	3.3[1,2]	11.4
Mg	*Lemna* spp., *Wolffia* spp.	3.1[1,2]	19.5
Na	*Lemna* spp., *Wolffia* spp.	29.5[1,2]	27.4
Fe	*Lemna* spp., *Wolffia* spp.	21.8[1,2]	111.0
Zn	*Lemna gibba*, *Lemna minor*, *Landoltia punctata*, *Wolffia* spp.	87.3[1,3,4,5]	149.6
Mn	*Spirodela polyrhiza*, *Wolffia* spp.	27.3[1,6]	4.5
Cu	*Lemna trisulca*, *Lemna gibba*, *Lemna minor*, *Wolffia* spp.	15.7[1,7,8,9]	7.6
Cd	*Landoltia punctata* 6001, *Lemna minor*, *Lemna gibba*, *Spirodela polyrhiza* sp., *Wolffia globosa*	5900[1,10,11,12,13,14]	27.3

[1]Appenroth *et al.* (2018). [2]Mkandawire and Dudel (2007). [3]Van Steveninck *et al.* (1992). [4]Khellaf and Zerdaoui (2009). [5]Lahive *et al.* (2011). [6]Liu *et al.* (2017). [7]Prasad *et al.* (2001). [8]Leblebici *et al.* (2010). [9]Landolt and Kandeler (1987).

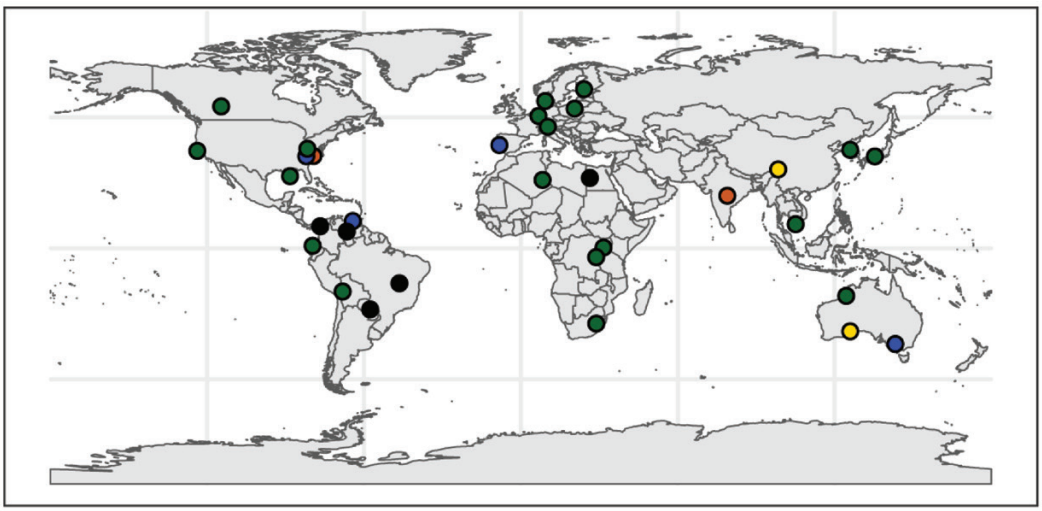

Fig. 2. Sampling of worldwide duckweeds for ionomic panel. Dots indicate sample origin locations: *Lemna* = green, *Landoltia* = yellow, *Spirodela* = black, *Wolffiella* = orange and *Wolffia* = blue. Duckweeds were derived from the Landolt collection, now housed in Milan.

water, $CaCl_2$ and Na-EDTA and harvested into individual samples from flasks of individual populations. These were harvested for ICP-MS analysis on day 1, 3 and 5 after media change, $n = 6$ per time point. Four-week-old cultures are clonally reproduced and therefore suitable replicates, given the very low generational variation and low mutation rates shown in duckweed mutation accumulation experiments (Xu *et al.*, 2019).

Imaging and microscopy

All samples were cleared, then stained with Fluorescent Brightener 28 (calcofluor) following the protocol described by Kurihara *et al.* (2015) and imaged on a Leica TCS SP5 confocal microscope. In short, plants were cleared, based on the ClearSee procedure described by Kurihara *et al.* (2015), with slight modification. Given that fluorescent markers were not being used, plants were fixed overnight in ethanol and acetic acid (3:1 v/v) rather than paraformaldehyde, because this reduced the toxicity and requirement for vacuum infiltration, which can be damaging to the air spaces. Plants were then rinsed three times with reverse osmosis water and left for 30 min, after which the reverse osmosis water was replaced with ClearSee solution (10 % xylitol, 15 % sodium deoxycholate and 25 % urea; Kurihara *et al.*, 2015) and left to clear for 2 weeks. Before imaging, plants were stained for 1 h with calcofluor in ClearSee (100 µg mL^{-1}), then washed in ClearSee for 1 h. Imaging was carried out using a confocal laser scanning microscope (Leica SP5), using a 405 nm diode laser at 12 % and hybrid detector with a range of 440–450 nm, gain of 25 % and pinhole of 0.5 Airy units.

Quantification of elemental tissue concentrations

For ICP-MS, we used a method adapted from the study by Danku *et al.* (2013). Briefly, 5–20 mg (fresh weight) was harvested per sample, placed in Pyrex test tubes and dried at 88 °C for 24 h. The dry weight was recorded, then 1 mL concentrated trace metal grade nitric acid Primar Plus (Fisher Chemicals) spiked with an internal standard was added to the samples, which were digested further in DigiPREP MS dry block heaters (SCP Science; QMX Laboratories) for 4 h at 115 °C. Before the digestion, 20 µg L^{-1} of indium (In) was added to the nitric acid as an internal standard for assessing errors in dilution, variations in sample introduction and plasma stability in the ICP-MS instrument. Then 0.5 mL of hydrogen peroxide (Primar, for trace metal analysis, Fisher Chemicals) was added to the samples and they were digested for additional 1.5 h at 115 °C. After digestion, samples and blanks were diluted to 10 mL with Milli-Q (Millipore). Direct water and elemental analysis was performed using an ICP-MS, PerkinElmer NexION 2000, with 22 elements monitored (Li, B, Na, Mg, P, S, K, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Cd and Pb) in the collision mode (He). To correct for variation between and within ICP-MS analysis runs, liquid reference material was prepared using pooled digested samples and run after every nine samples in all ICP-MS sample sets. The calibration standards were prepared from single element standard solutions (Inorganic Ventures; Essex Scientific Laboratory Supplies Ltd, Essex, UK). Sample concentrations were calculated using an external calibration method within the instrument software. Further data processing, including calculation of final elements concentrations (in milligrams per kilogram), was performed in Microsoft Excel. Log_{10}-transformations, *z*-score calculations and graphical representation were performed using R (v.3.0.2 'Frisbee Sailing'; R Development Core Team, 2023; see http://www.R-project.org), and RStudio v.1.0.136 (RStudio Team, 2020) was used for all statistical analyses. To calculate relationships between elements, the corrplot package (McKenna *et al.*, 2016) was used in R with Pearson correlations on log_{10}-transformed data.

RESULTS

Broad scale evolution of the ionome

We focus on ionomes from day 5 after media change (Fig. 3), which is representative of other time points (none of the 11 elements upon which we focus was significantly different across days by ANOVA). The full raw dataset is given in the Supplementary Data (Dataset S2); elements we considered for further analysis are shown in the Supplementary Data (Fig. S1). Concentrations were consistent for all elements for all accessions between time points except for a handful of elements in certain accessions depicted in the Supplementary Data (Fig. S2). These exceptions show a small minority of accessions decreasing in K, Ca, Fe and Cd and others still increasing (e.g. Ca, Cu and Fe). For accumulators showing the latter pattern, such as *Spirodela intermedia* 9227, the maximum concentration capacity of Ca on day 1 after media changes was still not reached, despite high nutrient provision throughout a 4-week experimental period, and the accession could still prolong uptake.

In the overall dataset of 34 accessions, the broadest contrast observed was between the Lemnoideae and Wolffioideae (rooted and rootless, respectively) for Ca, Mg and Cd accumulation (Fig. 3A). All ancestral representatives of (rooted) Lemnoideae (*Spirodela*, *Landoltia* and *Lemna*) consistently exhibited two to three times higher Ca content relative to the derived rootless Wolffioideae ($P \leq 0.01$; log_{10}, ANOVA with Tukey's post-hoc test). Likewise, on average, Mg accumulation was 1.8 times higher in the rooted species relative to the rootless *Wolffia* and *Wolffiella*. Ca and Mg showed a positive correlation (Table 2; Supplementary Data Figs S3 and S4). We observed further variation for Mg in the *Lemna* genus, where there emerged a gradient of Mg accumulation across *Lemna* sections (Figs 1 and 3A, D). The highest Mg levels were in the *Uninerves* section (Figs 3A and 4), which includes the invasive *Lemna minuta* and *Lemna yungensis* (now *Lemna valdiviana*), as described by Tippery *et al.* (2015) and Bog *et al.* (2020), both alien within Europe (Kirjakov and Velichkova, 2016; Ceschin *et al.*, 2018). This association of Mg accumulation with increased root vasculature (and with reduced frond vasculature in *Lemna*) stood in strong contrast to the uniformly very low Mg in rootless Wolffioideae. Cadmium concentrations varied significantly between rooted and non-rooted duckweeds (Fig. 3A; $P < 0.05$; log_{10}, ANOVA with Tukey's post-hoc test) in a manner inverse to Ca and Mg. The unrooted Wolffioideae species (especially *Wolffiella*) showed the highest Cd concentrations. Only the submerged *Lemna trisulca* exhibited Cd comparably high to the Wolffioideae (Fig. 3).

Rootless species exhibiting variation in at least two elements included *Wolffiella lingulata*, *Wolffiella hyalina* and *Wolffia brasiliensis* (Fig. 3E). In contrast, the species in our panel from the multi-rooted, more ancestral duckweed representatives, *Spirodela* and *Landoltia*, showed the greatest ionomic consistency across all accessions (Fig. 3B). *Spirodela* species had the highest tissue content of Ca in our panel, but other elements were not as variable between accessions.

Fine-scale ionome variation and identification of extreme accumulators in Lemna

We observed the greatest within-genus ionome variation in the *Lemna* genus ($n = 20$ accessions, six biological replicates of each; Fig. 3C, D). *Lemna* also harboured several extreme accumulators, each standing as outliers for the accumulation of three or more elements. *Lemna trisulca* 7192 has a submerged growth pattern and accumulated the greatest number of elements in amount and number from the panel, showing very high tissue concentrations of four essential elements (P, Ca, Zn and Fe), in addition to Cd, and low K levels (Fig. 3D). *Lemna yungensis* 9210 accumulated high S and Mn and also exhibited low K (Fig. 3C). The K levels trended negatively against the enhanced accumulation of other macro- and microelements in both *Le. trisulca* and *Le. yungensis* and across our panel as a whole (Table 2; Supplementary Data Fig. S4).

Fine-scale ionome variation between Lemna species

We noted variation at the level of several accession pairs, most obviously between *Le. yungensis* accessions (Fig. 3C).

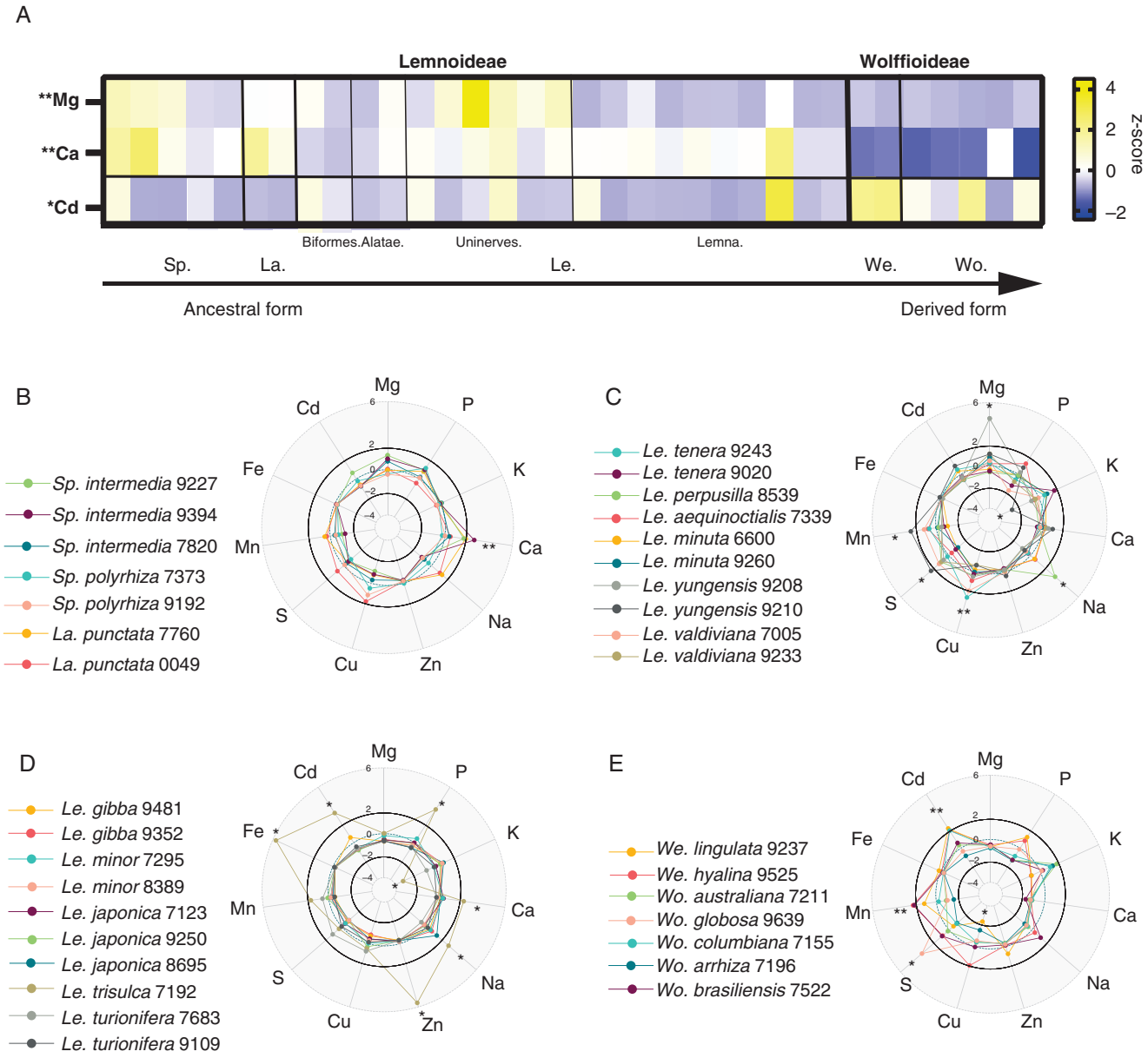

FIG. 3. The evolution of the duckweed ionome across genera, species and accessions. (A) Relative levels of elemental accumulation across rootless and rooted subgroups, respectively. The heat map is coloured by *z*-scores for the four most differentially accumulated elements. Significant differences were determind by ANOVA with Tukey's post-hoc test set at $^{**}P < 0.01$ and $^{*}P < 0.05$ between Wolffioideae and Lemnoideae. The *z*-scores (number of standard deviations away from the mean) were generated for each element using $\log_{10}$-transformation of values (in milligrams per kilogram) on day 5. The *x*-axis is arranged with basal forms on the left and derived forms on the right. Separating lines indicate genus and subgroup boundaries. We. = *Wolffiella* (2), Wo. = *Wolffia* (5), Le. = *Lemna* (20), La. = *Landoltia* (2) and Sp. = *Spirodela* (5). Within *Lemna*, sections *Biformes*, *Alatae*, *Uninerves* and *Lemna* are marked from left to right. (B–E) Radar plots showing differences in ionome profiles between individual accessions: (B) *Spirodela* and *Landoltia*; (C) *Lemna* sections *Biformes*, *Alatae* and Uninerves; (D) *Lemna* section *Lemna*; and (E) *Wolffiella* and *Wolffia* species. Species are ordered in the panels according to Tippery *et al.* (2015), from the most ancestral representative at the top left to the most derived at the bottom right. Numbers after species represent clone numbers. Asterisks represent a significant increase or decrease of ±2 relative to all normalized element concentrations for all species based on the mean and SD. The complete dataset of 17 elements and three time points is given in the Supplementary Data (Dataset S2).

Notably, *Le. yungensis* 9208 greatly accumulated Mg, and *Le. yungensis* 9210 exhibited extreme accumulation of S and Mn, but low K. When comparing *Le. yungensis* with *Lemna valdiviana* clones, none of the accessions showed large differences in ionomes between ten elements, with consistent levels of B and S (Fig. 5A). Comparing *Lemna minor* with *Lemna turionifera* and their interspecific hybrid *Lemna japonica*, *Le. japonica* accessions had lower Mo and a slight increase in Na and K in specific *Le. japonica* clones (Fig. 5B); however, neither of these ionome changes was significant in comparison to the whole duckweed panel. When contrasting native European *Le. minor* clones with invasive European *Le. minuta*, we saw

TABLE 2. *Mg and Ca were correlated strongly and positively with various elements, whereas K was negatively correlated. Element pairs were significantly correlated across 34 duckweeds at three time points. The* R *values correspond to positive or negative Pearson correlations derived from log_{10}-transformed data for eight elements. Data are given to two decimal places.*

Element	*R*
Fe/K	–0.76
Zn/K	–0.72
P/K	–0.67
Mn/K	–0.59
Mg/Ca	0.59
Fe/Mn	0.58
Zn/Mn	0.58

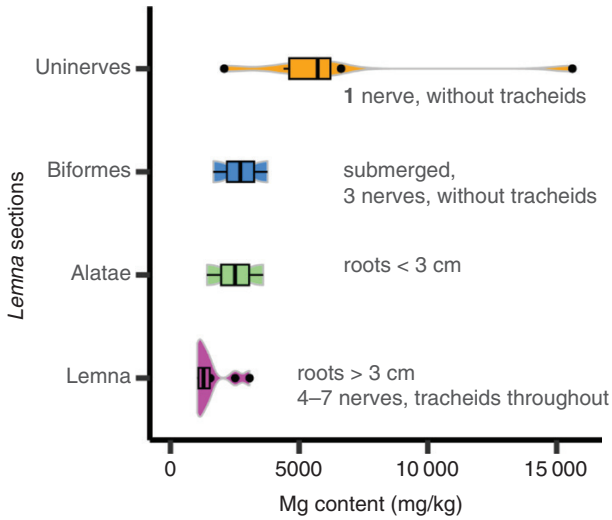

FIG. 4. Increased Mg content mirrors the reduction of frond vasculature within *Lemna*. The four sections of *Lemna* represent the highest Mg content in the species with most reduced vasculature for section *Uninerves*, with transitional sections *Biformes* and *Alatae* and the most developed frond vasculature in section *Lemna*, with reduced Mg. The Mg content is plotted from day 5 averaged values for each accession within each section: *Uninerves*, $n = 6$; *Biformes*, $n = 2$; *Alatae*, $n = 2$; and *Lemna*, $n = 10$. Sections are ordered and described according to Landolt (1986) and Tippery *et al.* (2015). Violin plots represent the spread of data for each group, with the middle line plotting the mean.

clone-level variation in some elements, but none varied significantly from the overall population by as much as one SD (Fig. 5C).

DISCUSSION

The broad variation we observed in duckweed ionomes at levels of genera, species and sister accessions is presumably attributable, in large part, to both morphological differences and adaptation to micro-environments. The most robust differences were at the genus level for Ca, Mg and Cd. The accumulation difference for Ca is perhaps explained, in part, by a storage mechanism as calcium oxalate (CaOx) within frond crystal ultrastructures in rooted genera, in the fronds of *Spirodela* and *Lemna* (Landolt and Kandeler, 1987) and in the root of *Le. minor* (Franceschi, 1989; Mazen *et al.*, 2003). In *Le. turionifera*, Ca influxes through roots and is stored in both fronds and roots, and in exceptional cases it can also be effluxed out of roots (Ren *et al.*, 2022). In contrast, Wolffioideae species have soluble Ca in cell sap and accordingly also cannot store excess Ca in the roots (Landolt and Kandeler, 1987; Appenroth *et al.*, 2017); thus Ca and Mg might be lower in Wolffioideae because they lack roots as a storage organ. Given that Ca was kept sufficiently available in our experiment through media refreshes, and rooted duckweeds use their roots as an additional storage compartment (Ren *et al.*, 2022), this might result in overall higher accumulation when compared with their rootless counterparts.

Given the broad contrasts in Ca between genera, it is interesting to consider these results alongside the importance of roots for elemental uptake and segregation of individual elements between the frond and root in duckweed species. The excision of roots makes only a modest change to the frond ionome, showing that roots are vestigial and overall not required for nutrient uptake in replete media conditions (Ware *et al.*, 2023). This supports the notion that duckweed roots might be adventitious (Landolt, 1986; An *et al.*, 2019). Although, surprisingly, removal of roots increased elemental composition in some cases (Ware *et al.*, 2023), the picture is more complicated, in that rootless species do not naturally exhibit elevated Mg or Ca in our data, indicating evolutionary adjustment of ion homeostasis upon root loss. The *Wolffia* genome harbours a derived complement of Ca export and cell wall-thickening genes, possibly minimizing potential for apoplastic transport, which, coupled with inability for storage as CaOx, results in less specialized mechanisms to manoeuvre and store Ca content overall (Michael *et al.*, 2020). In contrast, clones of *Le. aequinoctialis*, *Le. minuta* and *Le. minor* exhibit marked Ca accumulation (storage) to alleviate Mg toxicity from a contaminated mine and in high Mg:Ca ratio media or wastewater (Van Dam *et al.*, 2010; Paolacci *et al.*, 2016; Walsh *et al.*, 2020). This suggests specific adaptation of Ca storage and transport mechanisms to particular ionomic challenges.

The Mg gradient across *Lemna* species is not necessarily correlated with strict overall inferred ancestral and derived forms (Wang *et al.*, 2011; Tippery *et al.*, 2015) and root vascular complexity is not sufficiently varied between rooted duckweeds to account for this (Ware *et al.*, 2023). Instead, higher specific Mg uptake in the *Uninerves* section of *Lemna* might be associated with their reduced frond vascular complexity (Figs 3A and 4). With typical frond nerves numbering ≤16 in in *Spirodela* and between three and seven in other *Lemna* species (Les *et al.*, 2002), only one nerve is present in *Le. yungensis* and *Le. minuta*, with *Le. yungensis* (now *Le. valdiviana*) having the longer nerve of the two (Landolt, 1980; Crawford *et al.*, 1996). It is thought that this simplified vascular system might contribute to their invasive status (Kirjakov and Velichkova, 2016; Kadono and Iida, 2022). Reduced vascular complexity and ionomic differences could also offer enhanced potential for adaptation to varied environments, showing higher Mg tolerance (Paolacci *et al.*, 2016) and possibly, therefore, survival in hard water.

Although some variation in mineral content among *Wolffia* species has been reported by Appenroth *et al.* (2018), *Wolffiella*

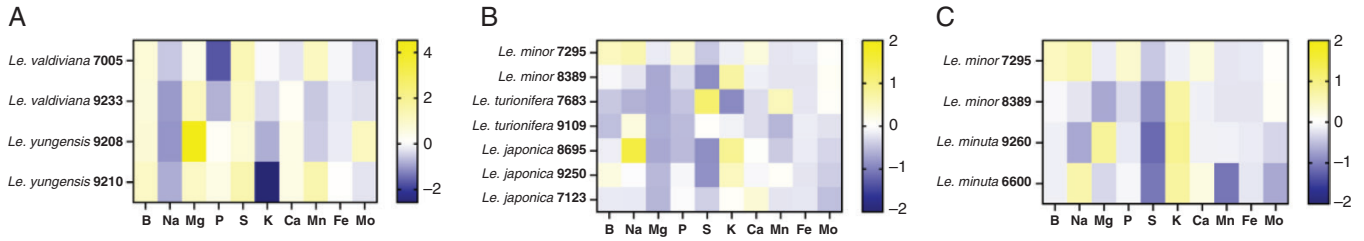

FIG. 5. Elements high in N medium show limited differences in internal ionomes between pairs of *Lemna* species. (A) *Lemna yungensis* (now merged with *Lemna valdiviana*) and *Le. valdiviana* accessions. (B) *Lemna minor*, *Lemna turionifera* and their interspecific hybrid species, *Lemna japonica*. (C) Accessions of cosmopolitan *Lemna minor* and invasive European alien *Lemna minuta*. Heat maps for *z*-scores from day 5 are presented for each accession. Ten elements were selected based on those intentionally added and present in the highest concentrations in N medium. The *z*-scores ± 2SD represent a significant increase or decrease relative to all normalized elements.

have received little attention and can be under-reported owing to clones having restricted biogeography and not being readily available (Landolt, 1986; Kimball *et al.*, 2003). Therefore, multi-elemental compositions of rooted and rootless duckweeds have not been compared directly before. In this respect, we see relative accumulation of Cd, especially in *Wolffiella* compared with the rooted species. This is somewhat surprising, because it might be expected that Cd accumulation would be detrimental to minuscule plants with no root segregation away from photosynthetically active tissue. We note, however, that *Wolffia* species also exhibit tolerance to As and have been considered as candidates for phytoremediation, accumulating more than Lemnoideae (Zhang *et al.*, 2009). Additionally, there is good evidence that *Wolffia* has moderate tolerance to Cd and increased accumulation capacity even in extreme concentrations (>200 μM). In fact, a handful of *Wolffia* species show Cd uptake in as little as 30 min from solution via apoplastic transport, which increases linearly with Cd concentration (Boonyapookana *et al.*, 2002; Xie *et al.*, 2013). We therefore speculate that loss of roots could have reduced control of heavy metal uptake whilst, at the same time, root loss removes a potential mechanism of uptake and a storage compartment available to rooted species (Verma and Suthar, 2015; Ma *et al.*, 2023; Zheng *et al.*, 2023). Wolffioideae perhaps evolved higher tolerance mechanisms to Cd toxicity, such as compartmentalization to vacuoles and complexation via conjugates (Schreinemakers, 1986). Although Cd was not supplied in a dedicated quantity in N medium preparation, we quantified the presence of Cd by ICP-MS in the media used (Supplementary Data Dataset S1) and suggest that this comes from chemical impurities, as indicated by Appenroth *et al.* (2018). We infer that *Wolffioideae* species might have a potential for heavy metal accumulation at higher dosages than those given here, perhaps also in the wild through adaptation to contaminated habitats (Zhang *et al.*, 2009).

Our results showed that the genus with the greatest diversity of specific accumulators was *Lemna*. The *Lemna* accessions with most extreme ionomes, *Le. trisulca* 7192 and *Le. yungensis* 9208, also harbour the most divergent root architecture, in comparison to other species of *Lemna*. *Lemna trisulca* is characterized by a submerged growth habit but smaller cortical cells, giving a thin, reduced root compared with other *Lemna* species, and *Le. yungensis* 9208 often displays an additional layer of cortical cells and irregularly large extracellular airspaces in the root cortex (Ware *et al.*, 2023). Thus, these differential root vasculature components, coupled with minimal frond vasculature, might play a role in producing the contrasting elemental profiles observed. Both *Le. trisulca* and *Le. yungensis* accumulated >1000 mg kg^{-1} dry weight for several elements and can therefore be considered hyperaccumulators (Zayed *et al.*, 1998; Zhang *et al.*, 2009). For this reason, these two species might have potential to be used in combination to alleviate multi-elemental toxicity in watercourses. *Lemna trisulca* accumulated greater Zn and Cd than floating species, possibly because of increased absorption through submerged fronds. Although *Le. trisulca* had the greatest variation overall and maximal micronutrient levels, the associated high Cd accumulation might be problematic for any applications in nutrition. It is also unclear whether this trait is common in other *Le. trisulca* accessions owing to limited availability of clones in stock centres; however, this species has previously been noted for its Cd accumulation potential (Kara and Kara, 2005).

A greater appreciation for duckweed variation in the micronutrients Ca, Mg, Fe and Zn is clear from our study, with particular accessions acting as hyperaccumulators for multiple nutritionally relevant elements. This is not the case for trace elements, such as Na and Cu (and especially Mn and the heavy metal Cd), for which the variation in tissue concentration was less dramatic than seen in other reports (Table 1). This is probably attributable to the combined effect of low presence of these elements in our supplied media or that comparisons across literature are confounded by variables disallowing truly quantitative comparisons between studies. This is particularly evident for Cd, which we supplied in only trace amounts (Supplementary Data Dataset S1), whereas external Cd concentrations vary 500-fold between studies.

Synthetic biology, including the tailoring of ionomic profiles in duckweeds, is an important goal of the duckweed research community (Lam and Michael, 2022). Interestingly, the *Spirodela* genome sizes are the smallest and the ionomes the least variable among all duckweeds here (Wang *et al.*, 2011; An *et al.*, 2018); additionally, the amenability of *Spirodela* to genetic transformation (Yang *et al.*, 2018*a*, *b*) makes it a strong candidate as a minimal scaffold for synthetic biology. We also suggest that because their ionomic profiles are so variable, the species harbouring larger genomes will be particularly valuable to mine natural variation to inform transgenic approaches in the smaller, highly tractable *Spirodela* genome.

For the fine-scale variation between *Lemna* species of interest, the vast ionome differences between *Le. yungensis* 9208 and 9210 can be ascribed best to local adaptation. Given that these accessions are closely related and were both originally isolated from the same region in Bolivia, one might expect more similar ionome profiles, but instead our data show that duckweeds exhibit strongly contrasting local variation in elemental uptake. Interestingly, this region of Bolivia is reported to be atypically harsh for duckweed, growing on sheer rock faces with waterfall spray with low nutrient availability (Landolt, 1998). It will be valuable to characterize *Le. yungensis* species further, in order to determine the genetic basis for their adaptation to specialized habitats. Given that *Le. yungensis* and *Le. valdiviana* showed no other significant internal differences between ten elements, this supports their unification as one species owing to lack of genetic differentiation (Bog *et al.*, 2020). *Lemna minuta* is an invasive species in introduced regions with ecological significance (Ceschin *et al.*, 2018), as an opportunist species in replete N and P with additional higher Mg tolerance (Njambuya *et al.*, 2011; Paolacci *et al.*, 2016; Ceschin *et al.*, 2020) one would expect drastic differences in the ionome in comparison to *Le. minor*. Despite this, there were no clear pattern differentiating two *Le. minuta* from two *Le. minor* clones grown in nutrient-rich medium (N medium; Appenroth *et al.*, 1996; measured here in Supplementary Data Dataset S1). Elemental differences seem to be at the clonal level, and opportunism therefore probably depends on unique situations in the wild. Recent data classified *Le. japonica* as a hybrid between *Le. minor* and *Le. turionifera* (Braglia *et al.*, 2021; Volkova *et al.*, 2023). Hybrid *Lemna japonica* clones had slightly reduced Mo compared with their parents, and one clone had significantly higher Na. It could be that hybridization might result in ionome differences important for altered adaptation to varied environments, as found in other plant species (Arnold *et al.*, 2016; Wong *et al.*, 2022). Taken together, between these groups of *Lemna* species, subtle interspecies differences for elements were clear. The physiological differences between species and their clones in light of genetic differences deserve future attention in duckweed.

Conclusions

Here, we detailed broad- and fine-scale diversity for the accumulation of physiologically and nutritionally important elements across all five duckweed genera. This variation is associated with dramatic morphological reductions in fundamental plant organs and genome expansions. Thus, disentangling the concurrent effects of dramatic genome size expansions, organ reduction and ecological adaptations will be a great challenge. However, at the more microevolutionary scale, within-species, accession-level variation points to clear promise in mapping alleles responsible for this observed variation.

One might speculate that the observed ionomic changes might be a maladaptive spandrel associated with root loss in derived taxa, but it is hard at this point to identify what the exact trade-off might be; this is for dedicated mechanistic and ecological work on the rootless taxa. Beyond highlighting these enigmatic correlates of root loss and the consequences of organ loss and vestigiality, this work serves to establish phenotypic variation across the ionome at both the fine and broad scale. This serves as a basis for future genomic characterization of causal alleles, in addition to rational development of targeted duckweed lines for both important nutritional and phytoremediation goals.

SUPPLEMENTARY DATA

Supplementary data are available at *Annals of Botany* online and consist of the following.

Figure S1: raw elemental composition of duckweed whole plants between days 1, 3 and 5 following media change by ICP-MS. Figure S2: outlier accessions with dynamic elemental concentrations over sampling days 1, 3 and 5 after media change. Figure S3: principal component analysis for 11 plant macro- and micronutrients and heavy metals. Figure S4: intensity and direction of correlations between eight elements in 34 duckweed accessions. Table S1: accessions studied in this work, with Landolt codes and locations. Dataset S1: summary elements present in N medium, as measured by ICP-MS. Dataset S2: all ionomics data (in milligrams per kilogram) for 22 elements for 34 accessions on days 1, 3 and 5 post media change quantified by ICP-MS.

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AUTHOR CONTRIBUTIONS

L.Y. conceived and oversaw the study, interpreted the data and secured funding. K.E.S. and L.Y. wrote the manuscript, with input from all authors. M.Z. performed the experiments, with assistance from A.B. and P.F. K.E.S. analysed and interpreted the data. D.H.J. performed microscopy. All authors read and contributed to the final manuscript.

DATA AVAILABILITY

The data are given as Supplemental Data to the article.

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SUPPLEMENTARY INFORMATION FOR

The evolution of the duckweed ionome mirrors losses in structural complexity

Kellie E Smith[1], Min Zhou[2], Paulina Flis[3], Dylan Jones[3], Anthony Bishopp[3], and Levi Yant[1,*]

1. School of Life Sciences, University of Nottingham, Nottingham NG7 2RD, UK

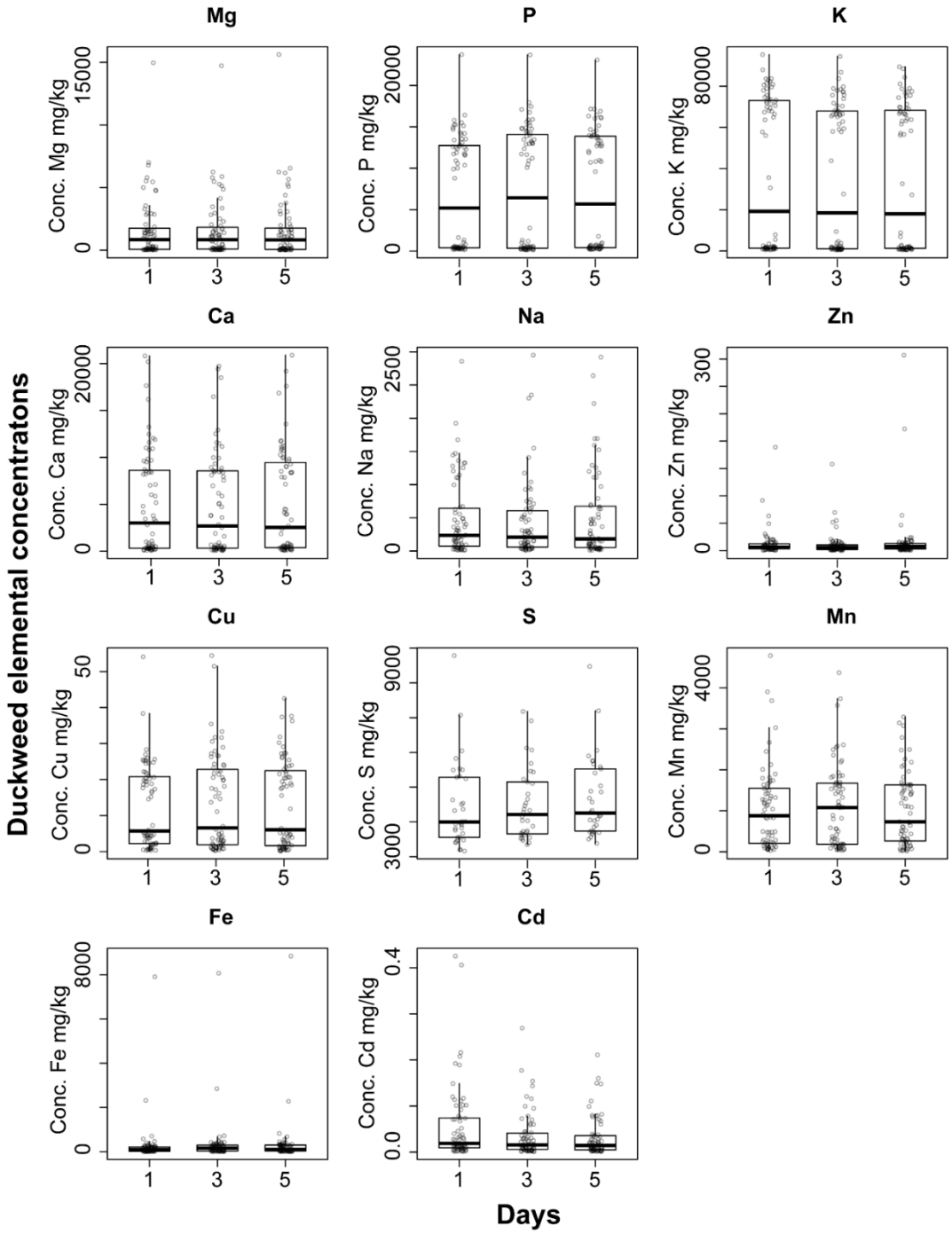
2. School of Life Sciences, Chongqing University, Chongqing 401331, China

3. School of Biosciences, University of Nottingham, Sutton Bonington LE12 5RD, UK

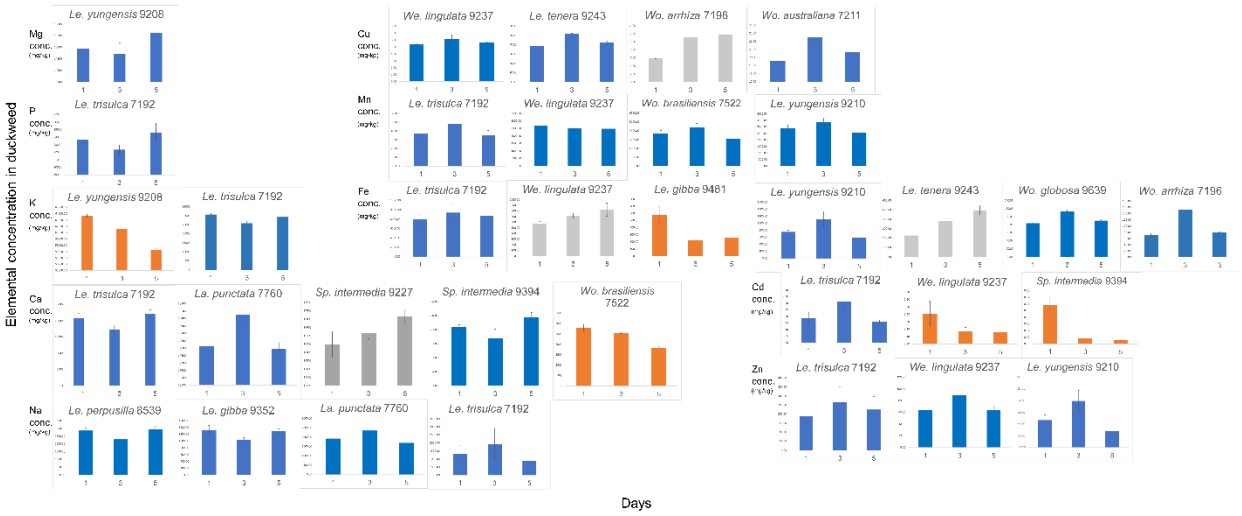
Running title: Ionomic evolution mirrors complexity reduction

*** Correspondence**: levi.yant@nottingham.ac.uk

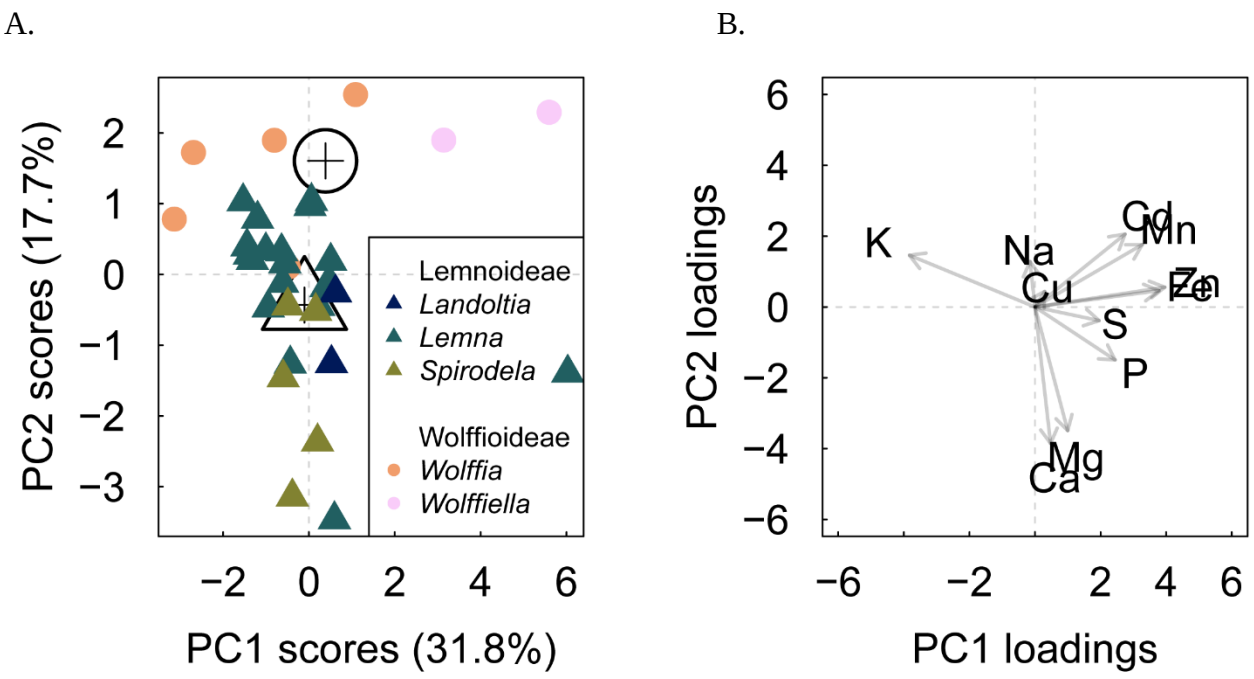
Kellie Smith and Min Zhou contributed equally.



Supplementary Figure 1. Raw elemental composition of duckweed whole plants between day 1, 3 and 5 following media change by ICP-MS. Boxplots show median value and upper (75%) and lower (25%) quartile of average concentrations (mg/kg) from 34 accessions, *n*=6 for each accession at each time point. Eleven elements are shown as independent boxplots, Mg, P, K, Ca, Na, Zn, Cu, S, Mn, Fe and Cd. The following elements were below the limit of quantification (LOQ) and limit of detection (LOD) in duckweed tissue samples using ICP-MS: Selenium LOD = 0.86 ppb/ LOQ = 2.85 ppb, Arsenic LOD = 0.034 ppb/ LOQ = 0.12 ppb. Lithium, Chromium and Lead were not above blank levels in analysed duckweed species.

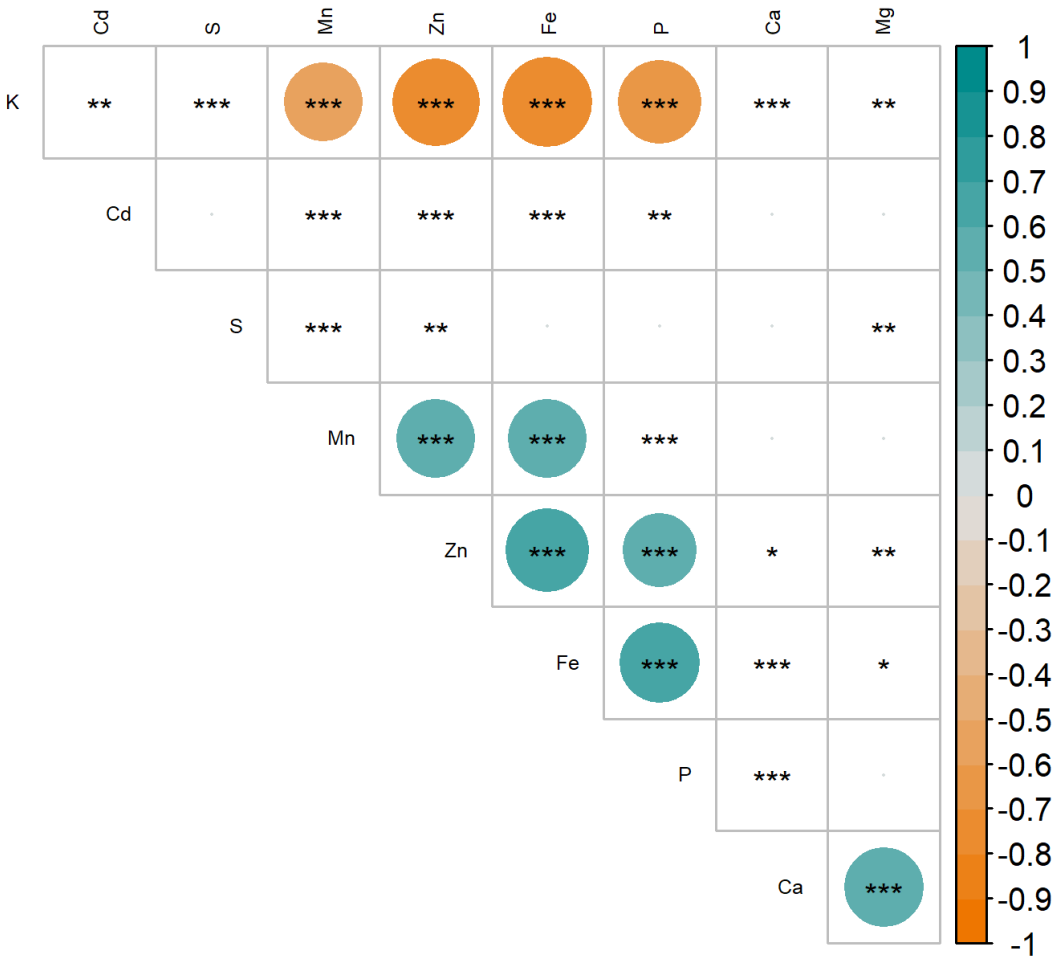


Supplementary Figure 2. Outlier accessions with dynamic elemental concentrations over sampling days 1, 3, and 5 after media change. Bar plots in blue show overall stable concentrations (mg/kg), those in grey show an increase over time and those in orange show a decrease over time. Error bars indicate standard error from 6 biological replicates from separate populations.



Supplementary Figure 3. Principal component analysis for 11 plant macro-, micronutrients and heavy metals.

A. Variable plot explaining 49.5% of the variation in PC1 and PC2 using 11 elemental concentrations averaged from three time points and colored by genus. Genera are further grouped into rooted (Lemnoideae) as triangles and rootless subgroups (Wolffioideae) as circles. Centroids are generated from means of rooted and rootless subgroups**. B.** PCA plot for 11 elemental tissue concentrations averaged from three days (1, 3, 5) for 34 accessions. *Lemna trisulca* is the major outlier across species and has been removed from this dataset to reveal underlying variation across elements and genera.



Supplementary Figure 4. Intensity and direction of correlations between eight elements in 34 duckweed accessions. Significant correlations of elements by Pearson correlation marked as *** for p values <0.001, ** for <0.01 and * for <0.05. Positive relationships indicated by large blue circles R values >0.5 and negative by orange circles with R values <-0.5. Relationships computed using log-transformed data.

Supplementary Table 1. Accessions studied in this work with Landolt codes and locations.

Species	Landolt Code	Location
Wolffiella lingulata 9237	WL9237	Africa
Wolffiella hyalina 9525	WA9525	India
Wolffia brasiliensis 7522	WB7522	North Carolina, USA
Wolffia arrhiza 7196	WA7196	Portugal
Wolffia columbiana 7155	WC7155	Florida, USA
Wolffia globosa 9639	WG9639	Venezuela
Wolffia australiana 7211	WA7211	Victoria, Australia
Lemna turionifera 9109	LT9109	Podlaskie, Poland
Lemna turionifera 7683	LT7683	Kyonggi-Do, Korea
Lemna trisulca 7192	LT7192	Kigezi, Uganda
Lemna japonica 8695	LJ8695	Kyoto, Japan
Lemna japonica 9250	LJ9250	Etela-Suomi, Finland
Lemna japonica 7123	LM7123	Saskatchewan, Canada
Lemna minor 8389	LM8389	Transvaal, South Africa
Lemna minor 7295	LM7295	Libya
Lemna gibba 9352	LG9352	Hessen, Germany
Lemna gibba 9481	LJ9481	Denmark
Lemna valdiviana 9233	LV9233	Esmeralda, Ecuador
Lemna valdiviana 7005	LV7005	Florida, USA
Lemna yungensis 9210	LY9210	La Paz, Bolivia
Lemna yungensis 9208	LY9208	La Paz, Bolivia
Lemna minuta 9260	LM9260	Trentino Alto Adige, Italy
Lemna minuta 6600	LM6600	California, USA
Lemna aequinoctialis 7339	LA7339	Burundi
Lemna perpusilla 8539	LP8539	Virginia, USA
Lemna tenera 9020	LT9020	Northern Territory, Australia
Lemna tenera 9243	LT9243	Ca Mao, Vietnam
Landoltia punctata 0049	LP0049	Sichuan, China
Landoltia punctata 7760	LP7760	South Australia
Spirodela polyrhiza 9192	SP9192	Cordoba, Columbia
Spirodela polyrhiza 7373	SP7373	Egypt
Spirodela intermedia 7820	SI7820	Formosa, Paraguay
Spirodela intermedia 9394	SI9394	Sucre, Venezuela
Spirodela intermedia 9227	SI9227	Bahia, Brazil

3. Chapter three: An ecological, phenotypic and genomic survey of duckweed species with their associated aquatic environments in the United Kingdom

Preface. Worldwide duckweed species with clone identifiers are available in stock centres, such as those used in chapter two; however they have been domesticated for years using clonal reproduction in aseptic cultivation. Additionally, most duckweed experiments are conducted in controlled environment conditions and therefore originating environments for these available clones are not considered. Accessions from the UK are limited in number in the worldwide collection and a large-scale duckweed collection has not been performed to date across the UK. The outputs from chapter two indicate that duckweed clones still show ionomic variation in a common environment, but the effect of the originating native environment (local adaptation) could not be assessed due to lack of information. To address these gaps, a novel UK collection was generated with water chemistry compared between native water sites spatially and temporarily around the UK. Species distributions and densities were characterised using combined genomics, and phenotyping to characterise UK duckweed composition.

Aims. To establish a new UK duckweed collection and complementary environmental data. To identify the range of variation (spatial and temporal) in UK water nutrient habitats. To characterise species using a combination of genotyping, phenotyping and ionomic approaches. To relate duckweed species prevalence to different UK regions. To assess the prevalence of native and invasive duckweed species including *Lemna minuta*. Finally, to identify individuals with promise as hyper- or hypo- accumulators of macro-, micronutrients and heavy metals for food-based and water cleaning applications.

This chapter is presented as an original paper available at *bioRxiv* and submitted to AoB PLANTS: https://doi.org/10.1101/2024.08.14.607898

An ecological, phenotypic and genomic survey of duckweeds with their associated aquatic environments in the United Kingdom

Kellie E. Smith[1 2 *], Laura Cowan[2], Paulina Flis[3], Chris Moore[4], Matthew Heatley[2], Carlos A. Robles-Zazueta[1], Adam Lee[5], and Levi Yant[2]

Affiliations:

[1] Division of Plant and Crop Sciences, School of Biosciences, University of Nottingham, Sutton Bonington LE12 5RD, UK

[2] School of Life Sciences, University of Nottingham, Nottingham NG7 2RD, UK

[3] Future Food Beacon, School of Biosciences, University of Nottingham, Sutton Bonington LE12 5RD, UK

[4] Deepseq DNA Sequencing Laboratory, School of Life Sciences, Queens Medical Centre, Nottingham, NG7 2UH, UK

[5] Division of Microbiology, Brewing and Biotechnology, School of Biosciences, University of Nottingham, Sutton Bonington LE12 5RD, UK

*** Author for correspondence:** kellie.smith@nottingham.ac.uk

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Running title: UK duckweed genomics and ionomics

This PDF file includes:

Main Text

Figures 1 to 5

Tables 1 to 2

Abstract

The duckweeds feature global distributions and diverse applications in phytoremediation and nutrition, as well as use in fundamental studies of development. Existing collections have minimal environmental data linked to natural habitats. Thus, there is a lack of understanding of natural variation in the context of native habitats. Here, a novel collection of 124 duckweed accessions from 115 sites across the United Kingdom were characterised by genome sequencing and ionomics. In nutrient-replete conditions all accessions hyperaccumulated P, K, Mg and Ca. Local but not large-scale associations were revealed between elemental composition of duckweed in common, replete conditions and native water profiles. *Lemna minor* was the most prevalent species in the UK, with a closely related hybrid *L. japonica* frequently found in waters with higher micronutrient concentrations. Invasive *L. minuta* was common in the southern and midland regions, but restricted in Scotland. *Lemna* accessions accumulated heavy metal contaminants typically together with macronutrients, suggesting phytoremediation potential, but some limitations as food. Furthermore, monitoring the ecological interactions between native, hybrid and invasive *Lemna* species should be ongoing in the interest of biodiversity.

Introduction

Duckweeds (Lemnaceae) represent some of the fastest growing flowering plants in the world (Sree et al., 2015; Ziegler et al., 2015) and are powerful models for studies in development (Ware *et al*.; 2023), bioremediation and ecotoxicology (Laird and Barks, 2018). There are at least thirty-six species of duckweed, consisting of simple stem-leaf structures called fronds in the rootless *Wolffia* and *Wolffiella* genera, to early diverged root-bearing genera *Spirodela*, *Landoltia* and *Lemna* (Lam and Michael, 2022; Ware et al., 2023). Duckweed have increasing roles in wastewater purification through uptake of excessive nutrients, metals and toxic elements leached from industrial and agricultural activities (Landesman et al., 2010; Ekperusi et al., 2019). Moreover, other varieties are emerging as food sources when grown hydroponically and axenically in vertical farms, providing comparable protein and nutritional contents to wheat (Appenroth et al., 2017; Xu et al., 2023). However, applications development requires novel duckweed clones with improved understanding of environmental interactions (Barton, 2024). For context, traditional crops like wheat and maize have been optimized for abiotic stress resilience and quality traits by assessing wild relatives in their natural habitats (Zhang et al., 2017; Reynolds and Braun, 2022). Adopting similar approaches with new

duckweed germplasms may unlock development of future food and phytoremediation applications.

Presently duckweed collections are generally domesticated to axenic artificial conditions (Sree and Appenroth, 2020). Collection dates, original environmental data and genome sequencing for existing clones are largely unavailable, limiting studies of accession optimisation to bioremediation or food production. Ecological characterisation at regional scales have been performed with some collections: from European (Kirjakov and Velichkova, 2016), Middle Eastern (Friedjung Yosef et al., 2022; Taghipour et al., 2022) and Asian accessions (Xu et al., 2015; Chen et al., 2022; Kadono and Iida, 2022; Tran et al., 2022). These works have uncovered various invasive and hybrid *Lemna (L.)* species. Within Europe, four invasive alien species have been identified, with *L. minuta* dominating (Lansdown, 2008; Fedoniuk et al., 2022; GBIF.org, 2022). However, characterisation is limited in the UK due to paucity of clones with only four classified as *L. minuta* species (Lam, 2018), a species that opportunistically outcompetes native *L. minor* under high nutrient and light conditions with potentially highly destructive ecological consequences (Njambuya et al., 2011; Ceschin et al., 2016; Paolacci et al., 2016; Paolacci et al., 2018a; Paolacci et al., 2018b).

Clear temporal and spatial patterns in *L. minuta* dispersal have been determined which drive species invasion fronts (Ceschin *et al.*, 2018a). For example, avian species are an important vector of dispersal (Coughlan *et al.*, 2015; Silva *et al.*, 2018) whereas, increasing ambient temperature and nutrient availability promotes invasive *L. minuta* growth (Njambuya *et al.* 2011, Peeters *et al.*, 2013). From initial invasion fronts upon European Atlantic coasts in the 1960s, *L. minuta* is now firmly established in the UK (Ceschin *et al.*, 2018a). Negative impacts of *L. minuta* dense infestation include thick mat formations which decrease light penetration, pH and oxygenation into water bodies, thereby reducing native biodiversity and causing problems for aquatic fauna (Janes et al., 1996; Ceschin et al., 2019), although *L. minor* can also form dense mats and dominate aquatic environments in optimal conditions. Wetland habitats in the UK of high conservation status are now threatened by hyper-eutrophication, ecosystem imbalance and duckweed invasion (Feller *et al.*, 2024). It is therefore timely to conduct regional surveys of both native and invasive duckweed species in wild wetlands with a view to assessing species specific adaptations in these environments.

Additionally, particularly ‘adaptable’ or ‘extremophile’ duckweed have great promise for the development of phytoremediation and food applications. Consideration of the plant

'ionome' refers to its whole-tissue or organismal levels of macro-, micro- nutrients and trace minerals (Salt et al., 2008). The applications of ionomics ranges from assessments of nutrient uptake and soil/water relations to understanding the nutritional composition of food and biofortification of crops. In duckweed, clones of *L. minor* and *Wolffia globosa* hyperaccumulate over 1 g/kg dry weight of heavy metals such as Cd, Cu and As and may have phytoremediation potential (Zayed et al., 1998; Zhang et al., 2009). From a worldwide collection, *L. yungensis* clones displayed local-scale variation in macronutrients Mg, S and Mn (Smith et al., 2024b). This suggests that nutrient uptake is linked to highly specific adaptations to micro-habitats. However, there is still a lack of understanding of the scale of variation in either water environments or the attendant accumulation potential of native duckweed accessions.

This paper presents a genomic, ecological and environmental assessment of novel UK duckweed accessions, detailing 115 environments and 124 accessions. We discover elemental variation using ionomics at local scales and document the spread of invasive *L. minuta*, as well as new reports of hybrid species. A common garden experiment with replete nutrient media was used to measure differences in duckweed whole plant tissue ionomes and native environmental water using inductively coupled mass spectrometry (ICP-MS). Overall this work provides a local-scale and UK-wide assessment of duckweed variation and water habitats, providing accessions with promising elemental accumulation profiles with potential for food and phytoremediation applications.

Results

Cohort construction and environmental assessment

To assess distributions across fine- to moderate geographic scales, duckweeds were collected from across England, Wales and Scotland. Initially, morphology was used for species determination, with later confirmation by genomic sequencing. Environmental assessment was performed concurrent with plant collections, focusing on water body analysis for elemental composition. Names and descriptions of sites are given in Table S1 and a map for sampling regions presented in Fig. S1. Site locations were chosen as described in methods. The primary latitudinal axis was between 41 sites in southern England and Wales (regions HAS, COR, BRI, NEW) and 37 sites across Scotland (regions ABE, ELG, GLA), giving a total of 103 accessions. The central UK consisted of five sampling regions LAN, BFD, YOR, HUL and MID, yielding

a total of 44 duckweed accessions. All accession names, sampling coordinates, dates and characteristics are provided in Table S2A.

Phenotype-based species identification

Morphological factors were used to determine species membership of accessions, including frond and root characteristics and turion production. Phenotypes were quantified first upon collection and then confirmed during laboratory growth for three years cultivated in controlled growth environments. Principal component analysis (PCA) was used with a subset of UK accessions suspected to be different species to discriminate species clusters based on morphological characteristics (Fig. 1B). Morphological assessment confirmed that UK duckweed consisted of species in the *Lemna* and *Spirodela* genera.

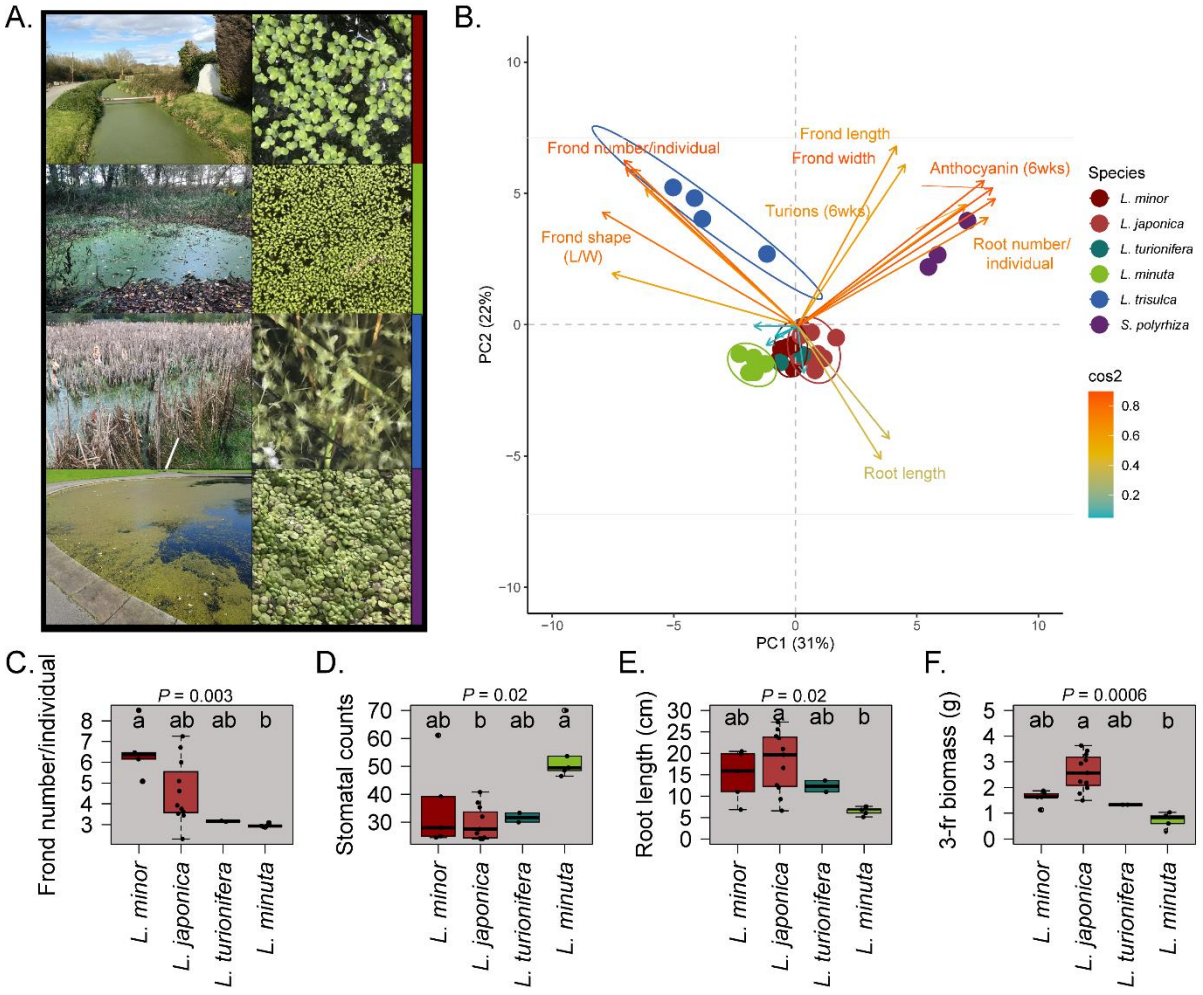

Figure 1. Twenty-two phenotypic traits used to classify five UK duckweed species. *A.* Photographs of four duckweed species growing in native sites with colour codes: genus *Lemna* (*L.*) *L. minor* (red), *L. minuta* (green), and *L. trisulca* (blue) and *Spirodela* (*S.*) *S. polyrhiza*

(purple). *B.* A subset of the UK cohort consisting of 30 accessions used for species confirmation by phenotyping. Morphological traits are represented on a PCA using principal components PC1 and PC2 to explain ~50% data variation. Ellipses display 90% confidence intervals for species groups and overlaps indicate reduced morphological criteria to differentiate between four *Lemna* species: *L. minor*, *L. minuta*, *L. turionifera* and *L. japonica*. Within species groups the number of accessions were: *L. japonica* = 11, *L. minor* = 5, *L. minuta* = 5, *L. trisulca* = 4, *L. turionifera* = 2 and *S. polyrhiza* = 3. Arrows are coloured by squared cosine (Cos2) with values > 0.5 showing phenotypic traits contributing most to dataset variation on PC1 and PC2. *C-F*. Differences in morphological traits between four *Lemna* species *C.* Frond number per individual *D.* Root length *E*. Stomatal counts and *F*. Three frond biomass per individual (3-fr biomass). Boxes display median and 25% and 75% percentiles for each species. Kruskal-Wallis $P = < 0.05$ was used to derive phenotypes significantly different between species and are indicated on the top of each plot. Different letters within the box plots indicate significant differences between species using a Dunn's post-hoc test with Bonferroni adjustment using $P = < 0.05$. For *L. minor* and *L. minuta* difference in frond number ($P = 0.001$), and for *L. japonica and L. minuta* difference in root length ($P = 0.004$), stomatal counts ($P = 0.003$) and biomass of a three-frond individual ($P = 0.0002$).

Spirodela and *Lemna* species can be differentiated by frond and root characteristics (Fig. 1). Criteria for membership in the *Spirodela* genus included larger fronds and multiple roots per individual (Landolt, 1986), anthocyanin accumulation, shorter roots and lower length-to-width frond ratios (L:W) than *Lemna* (Fig. 1A, B). Within *Lemna*, *L. trisulca* had thin, pointed fronds, giving the highest L:W ratios and higher fronds per individual connected by long stipes (Fig. 1A, B). *Lemna turionifera* were deduced from other *Lemna* species by observations of turions (overwintering bodies) produced in nutrient-depleted conditions. *Lemna minuta* produced fewer fronds (three per individual), compared to other species including *L. minor*, producing a maximum of eight (Fig. 1C). Roots were shorter in *L. minuta* but frond adaxial stomatal counts were almost two-fold higher than other *Lemna* species (Fig. 1D:E). In contrast, *Lemna japonica* and *L. minor* could not be differentiated by morphological criteria (Fig. 1B, 1C:E).

Genomics-based species identification

To extend the criteria for distinction of these two *Lemna* species and further confirm other species definitions, a genetic structure analysis was carried out. Individual whole genome

sequencing of 122 new UK accessions was performed and mapped to a common *L. minor* 7210 reference genome. These were processed with ten additional newly sequenced from the Rutgers duckweed collection and four individuals downloaded from public repositories of known clones (see Methods, Table S2A:B) for a total of 136 accessions. All individuals were classified into clusters by a variety of genomic clustering methods, including PCA and FastStructure analysis.

This PCA shows genetic groupings by species, when using the primary and secondary principal components (Fig. 2). PC1 vastly explained 72% of the variance and clearly discriminated species groups *L. minuta* and *L. minor,* with *Spirodela* emerging on PC2. Overall UK species were clustered with known species and genomic analysis aided species discrimination, compared to just using morphology alone. Native *Lemna trisulca* species formed a cluster with (*L. trisulca* 7192), invasive *L. turionifera* (*L. turionifera* 6002) and *Spirodela* (*S. intermedia* 9394) (Fig. 2A). Genome analysis was key for distinction of native *Lemna minor* and hybrid *L. japonica* which showed similar phenotypic traits. Two clusters of *L. minor (Lmo)* clones were initially observed and differentiated as C1 and C2 (Fig. 2A, C, D). These were very closely neighbouring in the PCA but were much better discriminated by FastStructure and tree-based approaches (Fig. 2A-B:D). C1 neighboured with English *Lmo*7016 and Irish *Lmo*5500, which were inferred as *L. minor*, while C2 is located between *L. minor* (C1) and *L. turionifera* clusters on the PCA (Fig. 2A). In cluster C2, very strongly admixed European *L. japonica* 9250, Canadian *L. japonica* 7123, South African *Lmo*8389 and North African *Lmo*7295 were found along with hybrid *L. japonica* (*Ljp*) species.

Structure analysis was used to estimate ancestry and to assign membership of each accession to species (Fig. 2B). This confirmed that C1 group were entirely *L. minor* species, and the C2 cluster contained accessions with substantial admixture with invasive *L. turionifera* gene pools. In the C2 cluster, the admixture between *L. minor* and *L. turionifera* species (Fig. 2B) and the intermediate cluster on the PCA is likely composed of an *L. minor* and *L. turionifera* interspecific hybrid *L. japonica (Lmo/Ltu)*. Six accessions showed admixture between *L. minuta* and *L. minor*, (*Lmu/Lmo* hybrid; Fig. 2B). Morphological criteria did not differentiate these from *L. minuta* but they likely form a presently undescribed hybrid duckweed species between native *L. minor* and invasive *L. minuta*.

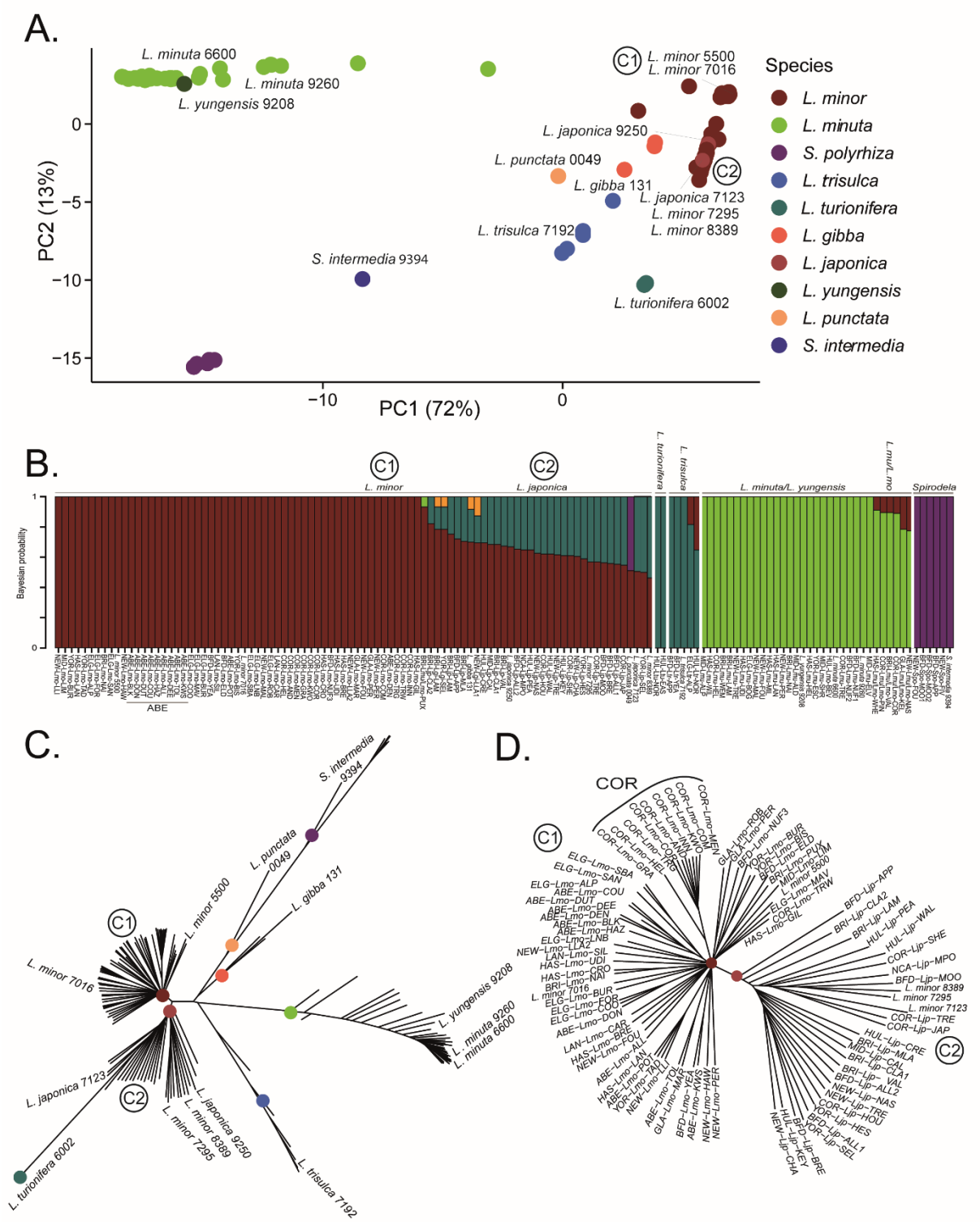

Figure 2. Genetic structure of 136 duckweed accessions, primarily from the novel UK cohort. One hundred and twenty-two novel UK accessions, four previously published (*L. minor* 7016, *L. minor* 5500, *L. gibba* 131, *L. turionifera* 6002), and ten newly sequenced duckweeds from the Rutgers duckweed collection. *A.* PCA of 11,088 quality-filtered four-fold degenerate (neutrally evolving) SNPs. Species are coloured by clusters determined by previously identified clones. The *L. minor* (red) clade shows two clusters conforming to

membership with established, previously phenotyped clones. The *L. minor* (red) clade shows two clusters labelled as cluster one (C1) and two (C2). *B.* FastStructure analysis differentiates accessions by group membership. *Lemna minor* accessions from the ABE region are labelled. Individuals with Bayesian probability assigning them to two or more species groups show admixture and are determined as hybrid species. K=9 was the model complexity with maximum marginal likelihood. The scale represents Bayesian probability of likelihood of species membership. *C.* Neighbour-joining tree showing genetic differentiation between species. *Lemna minor* (C1) clustered with *L. minor* 7016, 5500 and *L. japonica* (C2) grouped with *L. minor* 7295 and 8389 and *L. japonica* 7123. *D.* Close-up of a neighbour-joining tree distinguishing *L. minor* from *L. japonica*. *Lemna minor* accessions from COR with a common ancestor are labelled. Clone sequences from the Sequence Read Archive (SRA) or newly sequenced in this study from the duckweed stock database are labelled in italics with their corresponding identifying number.

Interestingly, some *Lemna* species are difficult to resolve by genetic structure alone. *Lemna yungensis* clone 9208 from Bolivia clustered with UK *L. minuta* accessions, showing a high degree of similarity between these species, both a part of the 'Uninerves' section of *Lemna*, consisting of one frond nerve (Fig. 2A,C, Bog *et al*; 2020). *Lemna turionifera (Ltu)* and *L. trisulca (Ltr)* are robustly separated using morphology (Fig. 1) but were undifferentiated by FastStructure (Fig. 2B), possibly due to having few representative accessions in each group (2 and 5) or low mapping efficiency to the *L. minor* reference (Table S2C).

Highly variable species distributions by region

Lemna minor (n = 81) were the most common species in number and diversity across the UK survey (Fig. 3A). This species was found both in monocultures and co-existing with other species. *Lemna minuta* were also frequent (n = 30), and exhibited a marked latitudinal contrasting distribution (Fig. 3D). *Lemna minuta* prevalence in south England and Wales was greatest (20/41 sites; 49% prevalence), with presence at 11/32 sites in central England (34% prevalence) compared to negligible presence in Scotland (3/36 sites; 8% prevalence; Fig. 3D). *Lemna minor* was the only species found across all sampling sites within the ABE region in the north of Scotland, (Figs. 2B, 3). In contrast, the southwestern BRI and NEW regions had the greatest species diversity both between sites (Fig. 3) and within sites, with up to three species co-existing in several sites (Table S2A, Fig. S2I, J and S3I, J), including the less frequent *S. polyrhiza* and *L. gibba*.

Figure 3. Different prevalence of *Lemna* species within UK sampling regions. Five *Lemna* species, coloured by species. *A. L. minor, B. L. turionifera, C. L. japonica. D. L. minuta. E. Lmu/Lmo*. The five species include common native duckweed (*L. minor*), invasive species (*L. minuta, L. turionifera*) and two hybrid species (*L. japonica (Lmo/Ltu)* and *Lmu/Lmo*). The total regions n = 12, and sample sites within regions n = <10.

Lemna turonifera was sparse throughout the UK; searches yielded only two accessions isolated in the northeast of England (Fig. 3B). The *L. japonica* (*Lmo/Ltu)* hybrids were abundant and overlapped with the two *L. turionifera* accessions, from which interspecies hybridisation may have occurred (Fig. 3B, C). *Lemna japonica* were more prevalent than the *L. turionifera* parental species but not as cosmopolitan as the *L. minor* parental species, as they were not found in Scotland (Fig. 3A, C). Conversely, *Lmu/Lmo* hybrids were found in southern regions, mirroring the pattern of parental species *L. minuta* (Fig. 3D, E). Single accessions of *Lemna minuta*, *Lemna japonica* and *Lmu/Lmo* were found in GLA. There were no hybrid or invasive species found in regions in the north of Scotland ABE and ELG during this survey (Fig. 3C, E).

Duckweed species broadly classified as native, invasive and hybrid types following morphological and genomic assessments. Native UK species included *L. minor, L. trisulca* and *S. polyrhiza*. We aimed to characterise presence *of L. minuta* invasive species, but we found that and an additional invasive species, *L. turionifera*. Furthermore, two hybrids formed between native and invasive species *L. japonica* and *Lmu/Lmo* were detected. Species types exhibited different regional distributions (Fig. 3) and further showed contrasting whole-plant ionomes in common, replete conditions, along with native water elemental differences between derived habitats (Fig. 4).

Variable ionomic profiles are species-specific

To infer relationships of genetic local adaptation, native water chemistry was compared with ionomes of plants grown in common nutrient replete conditions. In total, twenty-six elements

were measured in 116 accessions from 100 water sampling sites. After classification into species using phenotyping and genomic clustering, species differences between plant ionomes detected in a common garden and their home water chemistries were compared (Fig. 4). Overall, tissue levels of Mg, K and Mn contents varied significantly between species grown in common conditions (Fig. 4A, C, D). Interestingly, the two hybrid species *L. japonica* and *Lmu/Lmo* showed both higher and lower levels of Mg, K and Mn in both upper and lower directions, which may point to transgressive segregation (Fig. 4A-D), which can provide an evolutionary advantage for their presence in stressful environments. The highest Mg content overall was found in *L. minuta*, followed by hybrid *Lmu/Lmo* (P = < 0.0001, Fig. 4A, Table S3). Hybrid *L. japonica* had higher K levels than other *Lemna* species (P = 0.0015, Fig. 4C, Table S3) and hybrid *Lmu/Lmo* also had reduced Mn compared to other species, whilst *L. japonica* had the most (P = 0.044, Fig. 4D, Table S3). In some cases, hybrids mirrored one of their parental phenotypes, as found for internal Mg content in *L. minuta* and *Lmu/Lmo* hybrids (Fig. 4B).

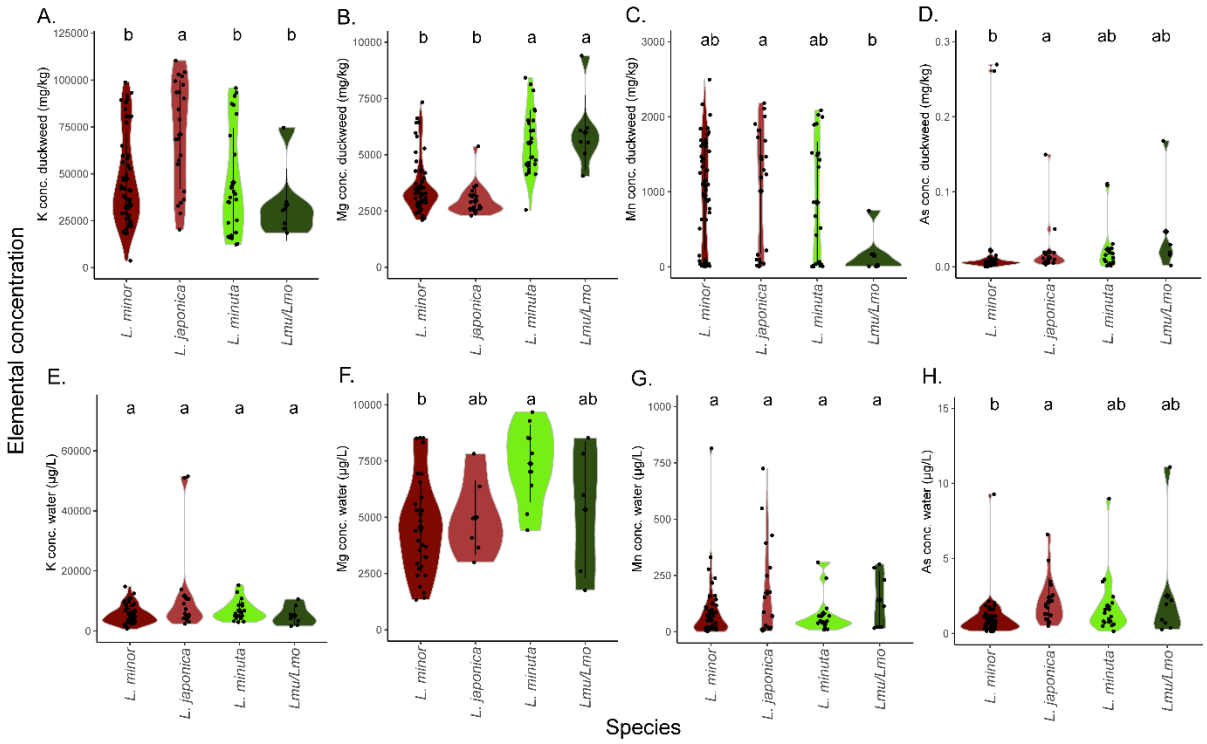

Figure 4. Macronutrients (K, Mg, Mn) and As composition varies between species, with additional variation of Mg and As between water environments. *(A-D)*. Elemental composition of K, Mg, Mn and As, for whole duckweed tissue. *(E-H)*. Environmental water concentrations of K, Mg, Mn and As, grouped by species. Whole tissue ionome

element content averaged per individual (mg/kg). Site water average elemental composition in µg/L from $n = 3$ water replicates per site. Significance was assessed by a Kruskal-Wallis test and Dunn's post-hoc test with Bonferroni adjustment using $P = < 0.05$ to indicate species significant differences using letters above plots.

Species showed contrasting accumulation profiles in replete nutrient conditions and also showed differing originating water elemental profiles. *Lemna minuta* accumulated more Mg and was found in higher Mg environments (Fig. 4B, F, Tables S3, S4). *Lmu/Lmo* accumulated comparable higher accumulation of Mg mirroring its parent, but the environmental Mg was not above average. *Lemna japonica* showed higher As accumulation and higher As contamination in water environments (Fig. 4D, H, Tables S3, S4), demonstrating one example of higher elemental compositions in both native water and in ionomes of originating species. Additionally, *L. japonica* was found on waters higher in other macro- and trace minerals Ca, B, Mo, Sr than *L. minor* ($P < 0.05$, Table S4). *Lemna japonica* accumulated higher K levels than other *Lemna* (Fig. 4A). However, originating water levels of K did not vary between species (Fig. 4E, Table S4). Similarly, there was a disconnect between higher Mn accumulation in *L. japonica* and reduced Mn in *Lmu/Lmo* respectively but no difference in species originating water levels of Mn (Fig. 4C, G, Tables S3, S4).

Widespread within-species ionomic variation in common conditions

In common conditions all accessions accumulated macronutrients P, K, Mg, Ca above the hyperaccumulation threshold of 1 g/kg. Overall, the largest variation of tissue concentrations between duckweed accessions were found for Mn and Pb, followed by S (Table S5). Almost all accessions hyperaccumulated S, with the exception of four Scottish accessions (ELG-*Lmo*-BUR, GLA-*Lmo*-PER, GLA-*Lmo*-CHA, GLA-*Lmu/Lmo*-KEL) and two from NEW (NEW-*Lmo*-LLI, NEW-*Lmo*-CHA), mostly *L. minor* species. The most variable element in the duckweed ionome was Mn, with 59/116 accessions hyperaccumulating it. In contrast, accumulation of Na was relatively rare with 17/116 accessions hyperaccumulating, but others maintaining very low levels. For other independent elements, rare, single accessions accumulated those: for example, B was hyperaccumulated by accession HAW, Si by accession BOG and Fe by accession LAN (Table 1). All other trace elements and metals were below the hyperaccumulation threshold, possibly due to limited presence of these in N-medium.

Often hyperaccumulation of one element is accompanied by changes in suites of others (Table 1). The hyperaccumulating accessions (HAW, BOG, LAN) all had higher

concentrations of multiple other elements, including heavy metals, compared to the cohort average (Table 1, Fig. S2C,J,L S3C,J,L), indicating some interdependence. BOG showed the most differential ionome, accumulating ten different elements. Overall, B was the most accumulated element in five out of seven higher accumulator accessions. Higher accumulation of elements co-occurred with reduced levels of macronutrients P, K and Fe in some instances (Table 1). High accumulators consist of several *Lemna* species and originated from a range of collection regions (Table 1, Fig. S2 and S3).

Accession	Region	Species	Duckweed ionome elemental variation
BOG	GLA	*Lmu*	▲ B, Na, Si, S, Ba, Pb, Al, Ti, Zn, Cd ▼ P
LAN	HAS	*Lmu*	▲ Fe, S, Si, Mg, Pb, Al, Ti, Zn
HAW	NEW	*Lmo*	▲ B, Ca, Fe, Cd, Ba, Pb ▼ K, Rb
ALL2	BFD	*Lmo/Ltu hybrid (Ljp)*	▲ B, Na, Sr, Ba, P ▼ Fe
MAV	ELG	*Lmo*	▲ B, Fe, Cu, Ba, Cd
APP	BFD	*Lmo/Ltu hybrid (Ljp)*	▲ Mo, Na, P
CRO	HAS	*Lmo*	▲ B, Ca, Cu

Table 1. Accessions showing accumulation of elements as measured by ICP-MS. Green triangles (▲) indicate higher accumulation and red triangles (▼) indicate reduced accumulation compared to cohort average. Accumulation differences are considered significant when z-scores exceed +/- 2 SD for n = 116 accessions. *Lmo - L. minor*, *Lmu - L. minuta*, *Ljp - L. japonica*.

Regional and local-scale site water elemental variation

Simultaneous with duckweed collection, water samples were collected for elemental composition in order to relate environmental chemistry with those ionomes of specific accessions. High nutrient water bodies included the BRI southwestern region, which exhibited higher concentrations of S, Mg, Ca and alkali metals Li and Sr and from the BFD region, higher concentrations of K, B and Mo (Table 2, Fig. S6). Different regions showed different water hardness, with ranges of over 2,000-fold in Ca, 56-fold in Mg and nearly 7,000-fold differences in Mn concentrations (Table S6). Additionally, within 19 sites in the BFD, YOR and HUL regions, the levels of Mg, Ca, Mn and Fe were highly variable due to the effect of seasonality, with the site ALL showing the largest variation overall (Fig. S7, Table S7),

Duckweed site	Region	Species	Significant elemental variation in water habitat
ALL	BFD	*Lmo/Ltu hybrid (Ljp)*	▲ K, P, Mo, B, Pb, Sn, Rb, Ni
MLA	BRI	*Lmo*	▲ Mg, Si, S, Ca, Li, Sr
NEW	BRI	*Lmu*	▲ Mg, S, Ca, Li, Sr
KEY	HUL	*Lmo*	▲ P, K, Cd, Ni, Co

Table 2. Native duckweed environmental water sites with significant levels of five or more elements as measured by ICP-MS. The table summarises water sampling sites with increased levels of macronutrients and heavy metals. Green triangles (▲) indicate higher accumulation compared to water site average and is considered significant when z-scores exceed +/- 2 SD. Species refers to the duckweed species found at water sites, *Lmo - L. minor*, *Lmu - L. minuta*, *Ljp - L. japonica*.

Duckweed ionome responses in relation to native aquatic environments

To infer whether accessions exhibited signal of specific local genetic adaptation, common garden ionomes of each accession were compared with the corresponding native water chemistry using linear models with Pearson correlation. At the grossest scale, no significant relationships were observed for twenty-two elements between water and duckweed ionomes across the sampling range as a whole. However, at a finer scale, associations by region were evident (Fig. 5) showing that region- or concentration- specific levels of elements in water habitats may therefore drive specific duckweed responses.

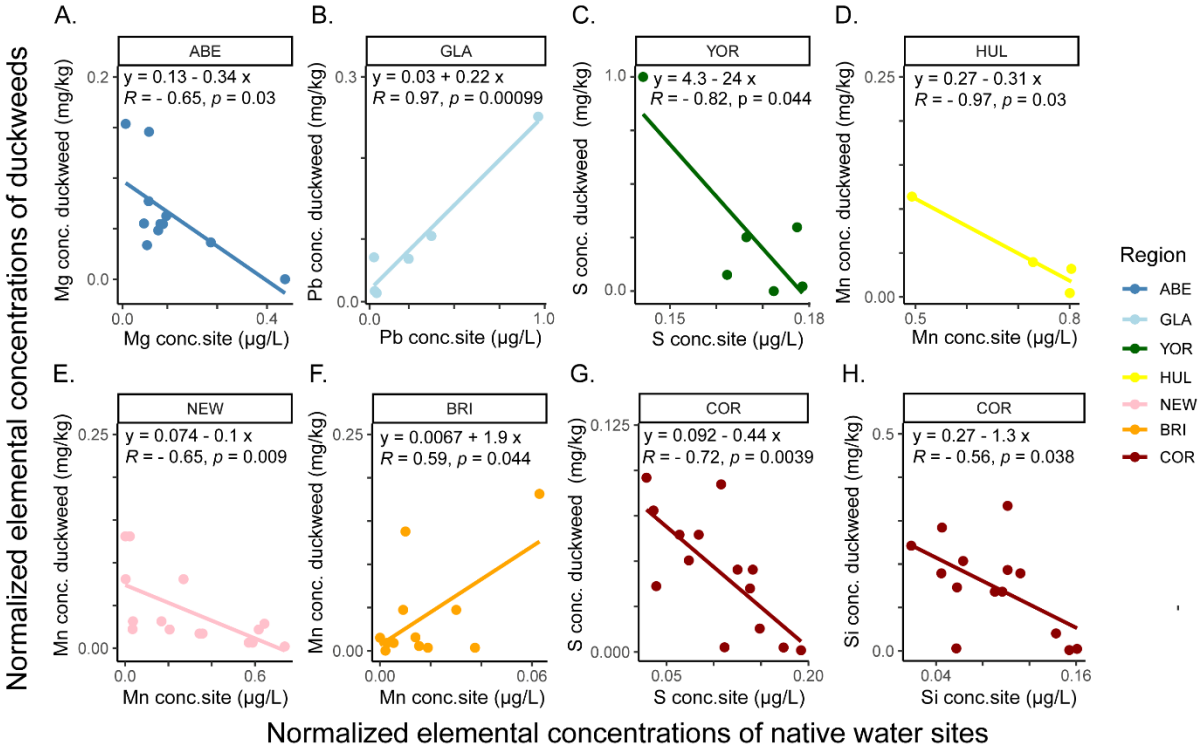

Figure 5. Specific regions show significant directional linear associations between site water elemental concentrations and *Lemna* duckweed tissue concentrations. *A-D.* Elements with significant correlations in northern UK regions. *E-H.* Elements with significant correlations in southern UK regions. Normalized water elemental concentration (µg/L) are recorded on the x axis with normalized duckweed whole tissue element concentration on the y axis (mg/kg) to scale values between 0 – 1. Duckweed accessions and sites within each region is n=>5. y and x represents line slope and intersect, R and P values depict Pearson model coefficients. R values <0.50 or <-0.50 are significant when P = <0.05. Non-*Lemna* (*Spirodela*) individuals were removed from analysis.

Together with its variability in water sites, Mn shows varied tissue-level responses in duckweeds from different regions (Fig. 5D:F). In BRI, concentrations of Mn in duckweed tissue shows an increasing trend with increasing Mn concentrations present in native sites. In contrast in NEW and HUL, duckweeds from sites associated with higher Mn concentrations show reduced Mn tissue contents in replete conditions. Higher accumulation of Pb by GLA accessions was positively associated with higher water levels of Pb in native environments (Fig. 5B). Concentrations of macro- and micro- nutrients including Mg, S and Si showed the opposite trend in specific regions, with higher concentrations in duckweed tissue associating with lower concentrations of these elements in water sites (Fig. 5A,C,G,H). Therefore, in these specific regional cases, heavy metals such as Pb tended to increase in duckweeds originating

from contaminated water environments, but for some macro- and micro- nutrients, low levels in the water environment potentially stimulated higher relative accumulation in duckweeds from these native sites when grown in replete nutrient conditions.

Water body elemental levels did not appear to drive broadly deviant plant ionomes. Whereby, none of the accessions with particularly many elements outside the normal range had higher or lower elemental concentrations compared to the UK average in their source water profiles (Tables 1, 2, Fig. S2, S5). From the seven accumulating accessions presented in Table 1, (including hyperaccumulators of B, Si and Fe), six of these came from water bodies with broadly normal levels of elements, including B, Si and Fe (Fig. S5C). An excellent example is the accession BOG which exhibited the most extreme ionome overall, hyperaccumulating ten macronutrients and heavy metals with reduced P (Figs. S2C, S3C). However, the source water chemistry from which BOG was collected harboured no elements significantly differing from the cohort average (Figs. S4C, S5C). Concurrently, accession ALL1 from the most nutrient-dense and contaminated ALL site (Figs. S4D, S5D) only highly accumulated Fe in replete conditions (Tables 1, 3, Figs. S2E, S3E). It is noteworthy that Fe, K and B were highly variable at this site, with Fe showing maximum levels in autumn 2020 but in decline until 2022 (Fig. S7).

From this survey of duckweed in the UK, native *L. minor* was diverse and commonly found. Presence of invasive species *L. minuta* and *L. turionifera* were more limited as were new reports of hybrid species *L. japonica* and *Lmu/Lmo*. Species showed different potentials to uptake macronutrients (Mg, K, Mn) and heavy metals (As) and also inhabited waters with diverse elemental profiles. Hyperaccumulators and high accumulator accessions were detected, usually coincident with higher uptake of multiple elements. Relationships between duckweed ionomes and water profiles were complex, with specificity to the element in consideration with accessions, species and in a few localised regions.

Discussion

Region-wide genomic assessments of duckweed diversity are scarce. Furthermore, existing duckweed collections lack data on source environmental parameters, and no study has assessed local-scale whole-plant ionomes in common conditions using regional sampling. Nor is there any genomic assessment of invasive duckweed impact on native accessions. In this study we fill these gaps, and further interpret this information with a view toward identification of useful accessions tailored to phytoremediation and food development applications.

405

406 **UK duckweeds are composed of native, invasive and hybrid *Lemna* species**

407 This survey confirms that native *L. minor* is cosmopolitan and thus still more prevalent than
408 invasive *L. minuta*, which had a more limited distribution, especially in the north. *Lemna minor*
409 is evidently well-adapted to the UK environments here, although hybridisation with invasives
410 is a liability. *Lemna minuta* was only prevalent in the south and midlands of England with some
411 ingress into Scotland. *Lemna minuta* has been added to the Global Register of Introduced and
412 invasive Species of Great Britain (GRIS); however observations have been in decline since
413 2019, according to the Global Biodiversity Information Facility (GBIF, 2022). Thus, presence
414 of *L. minuta* is possibly not as damaging to native species as previously reported (Paolacci et
415 al., 2018b) and this study found evidence of co-occurrence of both species in water bodies.
416 Although invasive species can be opportunistic in *in-vitro* high light and Mg conditions
417 (Paolacci et al., 2016; Paolacci et al., 2018a), dominance of invasive or non-invasive species
418 depends on competition in particular environments (Paolacci *et al.*, 2016, 2018; Ceschin *et al.*,
419 2018b; Gérard & Triest, 2018). It appears that environments in Scotland are less suited to
420 promote opportunism in *L. minuta* species.

421 Within the worldwide duckweed collection (such as the Landolt collection), many *L.*
422 *minor* species have been reclassified as hybrids including *L. japonica* and *L. mediterranea*
423 (Braglia et al., 2021; Braglia et al., 2024), indicating that the previous assignments of *L. minor*,
424 *L. japonica* and other *Lemna* species may not be fully correct. Genetic contribution from both
425 *L. minor* and *L. turionifera* in *L. japonica* accessions indicated admixture and therefore hybrid
426 presence in the UK. The presence of both *L. japonica* and *Lmu/Lmo* hybrid varieties have not
427 been previously reported in the UK. In part, because the morphology of hybrid and native
428 species can be very similar making it difficult to differentiate between them without genomic
429 or ionomic confirmation, as performed here.

430 Indeed, *L. japonica* was not easily differentiated from *L. minor* by morphology here
431 (Fig. 1) and in Eastern Europe (Volkova et al., 2023). Among these hybrids, there is
432 heterogeneity of parental introgression, putatively resulting in fitness performance attributable
433 to parental gene variants in the context of different ecological backgrounds (Gompert &
434 Buerkle, 2012). Similarly, *L. japonica* accessions exhibit variable propensities to form turions
435 under inductive conditions, probably in line with receiving varied allelic contributions from *L.*
436 *minor* (non-turionating) or *L. turionifera* (turionating) parents (Ernst *et al*, 2023).

Hybrid *L. japonica* appears more successful than its invasive parent *L. turionifera*, which showed limited geographic presence in this UK survey (Fig. 4B) and has only been reported twice previously, also localised in the east England region (Lansdown, 2008). In this instance, hybridisation has afforded wider spread than one of the parental genotypes (Volkova et al., 2023), especially in the case of *L. japonica*. Hybridisation thus may occur to access wider adaptative potential of genetic variants from native species. Supporting this, we find evidence for hybrid differences in both water elemental niches and nutrient uptake.

Species show differences in ionomes and native water chemistry

Hybridisation is a proposed mechanism to generate ionome variation in plants and aid their adapt to the water environment (Chen et al., 2017; Wang et al., 2021). Hybridisation also increases vigour in plants by increasing allelic diversity, especially in stressful environments (Washburn and Birchler, 2014). *Lemna japonica* reportedly form both diploid and triploids, and contain more transposable elements compared to parental species, hinting at possible increased capacity for environmental adaptation (Hoang *et al*., 2022, Ernst *et al*., 2023). There is also experimental support for increased adaptation to high light irradiance in *L. japonica*, relative to parental species (Smith et al., 2024a). Hybrids and parental species showed some differences in elemental composition between their water environments too, in line with possible adaptive speciation to specialised environmental niches.

Here, *L. japonica* water sites were contaminated with more As than *L. minor* sites and included higher concentrations of Ca, Mo, B and Sr. These elements were typical of BRI-region water bodies linking species distribution with adaptation to a differential water environmental niche in the southwest. Transgressive phenotypes can arise commonly in interspecific hybrid plants to give them higher abiotic tolerance during niche establishment, to enable divergence away from competition from parental species (Rieseberg et al., 1999). Transgressive phenotypes are common in hybrids, for instance to improve NaCl and Cd tolerance from that of parental species (Xue et al., 2021; Ortega-Albero et al., 2023).

In this study, invasive *L. minuta* was found in higher Mg-containing sites than native *L. minor*, accumulating more Mg than other *Lemna* species. This finding provides affirmation that this species is a high Mg-tolerator, supporting both invasive behaviour in foreign environments, possible tolerance to hardwater areas and enhanced potential for phytoremediation of high Mg-containing wastewater (Paolacci et al., 2016; Ceschin et al., 2020; Walsh et al., 2020). *Lemna minuta* and hybrid *Lmu/Lmo* had the highest internal Mg

concentrations overall, showing a mirroring phenotype between parent and hybrid species. Higher Mg in *L. minuta* is consistent with enhanced Mg accumulation occurring within species within the 'Uninerves' section of *Lemna* (Smith et al., 2024b) but for the first time this work links accumulation to higher Mg tolerance in native habitats.

From the UK panel, *L. japonica* shows higher K and Na tissue concentration compared to parent *L. minor*, which is consistent with findings from a worldwide duckweed ionome comparison (Smith et al., 2024b). As K is widely available, provided at the highest concentrations in water sites found here and all accessions accumulated it, it can be inferred that increased accumulation of K has a functional purpose in *L. japonica*, and may enhance tolerance to other elements, such as As to allow niche environment establishment. Some support comes from *Arabidopsis thaliana* and *Vicia faba* (Broad bean), whereby higher K uptake mitigated As and NaCl toxicity (Chao et al., 2013; Che et al., 2022; Shah et al., 2022).

Duckweed accumulator accessions and their potential applications

Using native water data, duckweed tolerance to macronutrients in the environment could be defined at a regional-scale, further highlighting specific UK accessions with tolerance traits for phytoremediation. Here, UK native water sites showed 81%, 74% and 44% higher maximum values for Mg, Ca and K than previously reported (Linton and Goulder, 1998). Additionally the maximum concentrations of K, Mg, Ca, Mn and Fe in native water exceeded concentrations of these elements tolerated by *L. minor* grown on dairy wastewater (O'Mahoney et al., 2022) and. Thus, inhabiting accessions may have developed useful tolerance and accumulating traits for phytoremediation and enhanced supply of macronutrients for nutrition.

That said, high accumulators did not tend to come from sites with higher elemental concentrations (accession BOG), nor did high accumulators necessarily come from contaminated UK environments (accession ALL1). Therefore, there is some degree of unlinking of elemental tolerance from accumulation potential. The accession GLA-*Lmu*-BOG showed enhanced concentrations of Ba, Pb, Al, Ti, Zn and Cd (Fig. 3D, Table 1) and can be considered for remediation of contaminated water courses, provided the tendency to accumulate can be tested on real-world water conditions. For example, Zn and Pb pollution in GLA waterbodies require remediation and the accession is already inhabiting the region (Fordyce et al., 2019; Eschenfelder et al., 2023). Unfortunately, accumulation of several macronutrients including B and Fe co-occurred with heavy metal uptake, surprisingly even in low level controlled conditions. Whilst willingness to uptake heavy metals is an optimal trait

for phytoremediation, this is problematic for direct applications in nutrition. Thus, mechanisms to retain high macronutrients but mitigate heavy metal levels such as inoculation with a synthetic microbiome or post-harvest washing or cooking steps may be future directed targets for duckweed consumption (Stout et al., 2010; Sattar et al., 2015; Amir et al., 2019).

In conclusion, this collection is the first of its nature, presenting a unique mixture of large-scale duckweed genomics, ionomics, and species assessment with environmental water data. Hybrid and invasive species were associated with novel water chemistry niches compared to parental species but were more restricted in their ranges. Therefore, high water hardness may be a predictor for future *L. minuta* colonisation in new regions as well as highlighting their potential in bioremediation. It is possible that invasive species are not currently establishing well in north Scotland but their introduction should be mitigated. Heavy metal accumulating accessions identified from this study (BOG, LAN and others) should be further explored for phytoremediation potential using outside transplantation experiments and *in-vitro* elemental spiking experiments to maximize hyperaccumulation. This UK collection serves as a useful resource to explore desired traits for human consumption, bioremediation or ecological population studies and promotes the further genetic understanding of hybrid and parental duckweed species.

Methods

Selection of site locations

An inland-coastal transect with decreasing altitude was selected locally to give seasonal time points for water collection. Original sites were chosen in May 2020, concordant with duckweed collection and further duckweed and water collections performed during autumn 2020, summer 2021, autumn 2021 and winter 2022. For spatial assessment of the UK, duckweed and water collections were conducted in spring 2021, starting at southern locations in early April and finishing mid-May 2021 in northern Scotland, to account for variation in springtime across UK. Regions were chosen to span the UK and using duckweed observations reported recently using the Global Biodiversity Information Facility (GBIF.org, 2022). Duckweed observations were particularly dense in BRI and ELG. Locations from GBIF.org were mapped onto Google maps and from that, several potential sites were searched within each region to give n=>6 local sites with duckweed presence.

Collection of duckweeds and morphological assessment

Duckweeds were collected as described in (Smith et al., 2024a) for temporal and spatial collections. In sites with more than one suspected duckweed species, these were collected and cultured separately based on size and denoted as A, B, or C. From across 19 sites along the seasonal transect, duckweeds were phenotyped at each time point, and a handful re-sequenced and denoted as 1, 2 or 3, when they showed differences from species previously characterised there. For other sites, duckweeds were collected at a single time point. Frond characteristics (length, width, length width ratio (L:W), number of fronds per individual and anthocyanin presence) were assessed initially from images taken with a Zeiss SV6 stereo microscope (Ziess, Oberkochen, Germany) (n = 10 individuals per accession). Then each of these characteristics and root lengths were measured at two later timepoints after lab cultivation. All images were analysed with Fiji (Schindelin et al., 2012). Stomatal counts were performed for n = 3 whole fronds of 24 accessions using a Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany) using preparations as described in (Kurihara et al., 2015; Smith et al., 2024b). Biomass was measured for three frond individuals (n = 3 per accession) and presence or absence of turions were assessed from cultures exhausted in nutrient media over three years.

Collection of water samples

For seasonal water collection, 100 ml samples were taken four times at each site, unless accessibility issues or water was not present at each time point. Solid Phase Microextraction Polytetrafluoroethylene (SPME PTFE) amber bottles were pre-washed with ultra-pure 10% nitric acid overnight followed by soaking in MilliQ water (Milipore, USA) and then thoroughly air dried. At each site, water bottles were washed at the top surface of the water, filled to 100 ml and 0.5% ultra-pure 1 ml nitric acid added before storage at 4 °C. Later, 18 ml water was filtered through a 1.45 µm syringe filter into 2 ml 10% Primar grade nitric acid to acidify samples and samples were stored at 4°C for elemental analysis. For UK-wide samples, water samples were collected in triplicate per site from the top water surface in Falcon™ tubes (Fisher Scientific, Loughborough, UK) and filtered through a 1.45 µm syringe filter into High density polyethylene (HDPE) Universal 25mm x 90mm 30 ml tubes (Sarstedt, Leicester, UK). HDPE tubes were pre-weighed, then 2 ml Primar grade 10% nitric acid added and then re-weighed. After addition of 18 ml water from each site into acid, tubes were stored at 4°C before ICP-MS analyses. All samples were re-weighed after water collection using a precision 5 dp balance (Mettler Toledo, Ohio, USA).

Plant care and harvesting for DNA sequencing

Duckweeds were sterilised using 0.5% sodium hypochlorite and grown in GEN2000 SH, controlled cabinets (Conviron, Winnipeg, Canada). Among all collections, four accessions from the south and 14 accessions from Scotland could not be successfully cultured in laboratory conditions. The majority of losses occurred in *L. trisulca* and provisional *L. gibba* clones, possibly due to sensitivity to sodium hypochlorite or specific adaptation to locality so were not included for sequencing or ionomics. After sterilisation and weekly media changes of successful cultures, independent sealed flasks of UK accessions were grown for four weeks to bulk tissue for DNA harvesting. Duckweeds from the Landolt collection and available at Rutger's stock database (www.ruduckweed.org) were also grown and harvested for DNA to provide known species controls. For each accession, 20-100 mg fresh duckweed tissue was harvested into liquid nitrogen and then stored at -80 °C.

DNA isolation, short-read library preparation and sequencing

Accessions were ground using a Tissuelyser II (Qiagen, Hilden, Germany) and DNA extracted using DNAeasy Plant kit (Qiagen, Hilden, Germany). DNA quantification was performed using dsDNA HS assay (Thermo Fisher Scientific, Massachusetts, USA) and Qubit 2.0. DNA was diluted to < 20 ng/µl with sterile MilliQ water. Individual Illumina DNA Prep (Illumina, San Diego, USA) sequencing libraries were prepared at the Deepseq sequencing facility, University of Nottingham, UK. on a Mosquito HV (SPT Labtech, Melbourn, UK) liquid handling robot using $1/10^{th}$ volumes at all steps. A total of 9-48 ng of DNA was used as library input and 5 cycles of Polymerase Chain Reaction (PCR) were used for the library amplification step. Final libraries were normalised and pooled on a Fluoroskan Ascent fluorometer (Thermo Fisher, Massachusetts, USA) and the resulting pools were size selected using 0.65X Ampure XP (Beckman Coulter, California, USA) to remove library fragments < 300 bp. Short read sequencing using paired end reads with Illumina HiSeq 2500 platform sequencing was performed by Novogene, Cambridge, UK using a target of 20x coverage.

Variant calling

The processing pipeline involved three parts: (1) preparing the raw sequencing data, (2) mapping and re-aligning the sequencing data and (3) variant discovery (GATK v.4 following GATK best practices). In addition to newly sequenced samples, previous sequencing data was downloaded from the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) and are summarised in Table S2B. To prepare the raw sequencing data for

mapping, the different sequencing lanes were concatenated, followed by quality trimming using Trimmomatic (Bolger et al., 2014). All genomes were then aligned to reference genome *L. minor* 7210 (SRR10958743) using BWA 0.7.17 (Li & Durbin, 2009) and processed using Samtools v1.9 (Li *et al.*, 2009) and duplicate reads flagged using 'MarkDuplicates' from picard-tools 1.13464 followed by GATK v.4 to re-align reads around indels (McKenna et al., 2010). The variant dataset was filtered for biallelic sites and mapping quality with GATK using QD < 2.0, FS > 60.0, MQ < 40.0, MQRankSum < -12.5, ReadPosRankSum < -8.0, HaplotypeScore < 13.0 and sites remaining after depth filtering DP <141 carried forward for analysis. The code for batch processing is available at https://github.com/mattheatley/ngs_pipe.

Genomic analysis

Degenotate (https://github.com/harvardinformatics/degenotate) was used to identify sites encoding fourfold degenerate sites (4FDS) as proxies for neutrally-evolving sites. These sites were further filtered >20% missingness to reduce the cohort from 143 individuals to 135. The dataset was pruned by linkage disequilibrium in order to obtain independent segregating markers out of linkage using a custom script (Hämälä et al., 2024). The final genomic analysis included only biallelic single nucleotide polymorphisms (SNPs) at allele frequencies >2.5%, with one SNP per 100 kb sliding windows with a step size of 50 kb and r2 of 0.1. Species allocation was confirmed using a mixture of PCA, tree, and structure-based approaches using R v3.6.3. The PCA was produced for variants using ggplot2 (Gómez-Rubio, 2017). Unrooted neighbour joining trees were compiled using ape v5.4 package (Paradis & Schliep, 2019) for *L. minor* and *L. japonica*. For structure plots, 4FDS variants with > 20% missingness were dropped, removing two individuals, and then converted into genotype call files using PLINK v1.9 (Chang *et al.*, 2015) based on Hardy-Weinberg equilibrium and Fisher exact tests. Allele frequencies were used for group allocation and admixture proportions by FastStructure v1 (Raj *et al.*, 2014) with K groups between 4-10. Selection of K=4 was chosen for visualisation with a Structure plot v2 using Omicsspeaks http://omicsspeaks.com/strplot2/

Duckweed growth and harvesting for ionomics experiments

After nine months of subculturing, ionomic experiments were conducted for accessions grown in controlled environment cabinets. Two individuals of each accession were grown in 500 ml Erlenmeyer flasks containing 250 ml Nutrient medium, replenished weekly for six weeks. N-medium was used as described in (Appenroth *et al.*, 1996; Appenroth & Sree, 2015) and contains KH_2PO_4 (0.15 mM), $Ca(NO_3)^2$ (1 mM), KNO_3 (8 mM), $MgSO_4$ (1 mM), H_3BO_3 (5

628 µM), $MnCl_2$ (13 µM), Na_2MoO_4 (0.4 µM) and FeEDTA (25 µM). Duckweeds were grown at
629 25 °C day and 18 °C night, with 16 h day lengths. To harvest, duckweed were rinsed with three
630 two minute MilliQ water washes. Three replicates were obtained for each accession from three
631 independent flasks using 150 mg duckweed tissue per sample. Duckweed were dried in an oven
632 at 88 °C overnight and stored in a desiccator before analysis. Weights of the dried tubes were
633 made using a 5 dp precision balance (Mettler toledo, Ohio, USA).
634

635 **Ionomics processing using inductively coupled plasma mass spectrometry (ICP-MS)**

636 Elemental analysis for water and duckweed samples were analysed on a NexION 2000 ICP-
637 MS (PerkinElmer, Massachusetts, USA) in Helium collision mode. For each set of water and
638 duckweed analyses, calibration standards were run throughout using single element standards
639 (Inorganic 226 Ventures; Essex Scientific Laboratory Supplies Ltd, Essex, UK), to subtract
640 against background samples. Concentrations of elements in water samples were measured in
641 µg/L for Na, Mg, Si, S, K, Ca, Al, P, Li, B, Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Rb, Sr, Mo,
642 Cd, Pb and Sn. Ti, Cr, Sn were removed from water analysis as they were below the limit of
643 detection (LOD). Ba was also removed as it was not measured across all sites. Duckweed
644 samples were digested with 2 ml 63% nitric acid at 115 °C for 4 hrs (spiked with the element
645 Indium as an internal standard) and then 0.5 ml hydrogen peroxide for a further 1.5 hrs at 115
646 °C, before dilution into 10 ml MilliQ water. Elements Li, B, Na, Mg, Al, Si, P, S, K, Ca, Ti,
647 Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Rb, Sr, Mo, Cd, Sn, Ba, Pb were measured in duckweed tissue
648 by dry weight (mg/kg). Elements with low levels in duckweed Li, Cr , Sn < 1 mg/kg and Ni <
649 3 mg/kg were removed from further analysis as they were below the limit of detection of ICP-
650 MS.

651 **Analysis of ionomics data**

652 For water and duckweeds analyses, elements were grouped as those present in duckweed
653 growth N-medium or trace/heavy metals (not present in N-medium) for separate analysis.
654 Water site replicates were combined to form site averages (n = 3) and replicates per accession
655 combined for accession averages (n = 3). Standardisation for each element by z-scores were
656 obtained by subtracting raw data for each element from the panel mean and dividing by
657 standard deviation (SD) to produce heat maps, radar plots and PCAs. Linear regression analysis
658 with Pearson correlation was performed between each accession's ionome and their originating
659 water elemental concentration averages, to find positive and negative relationships. For

comparison of species ionomes, those with fewer accessions were dropped including *L. gibba*, *L. turionifera* and *S. polyrhiza*. Differences between the remaining *Lemna* species ionome concentrations and originating water elemental profiles were compared separately with a Kruskal-Wallis test and a post-hoc Dunn's test with Bonferroni adjustment using $P = <0.05$.

Symbols and abbreviations

ICP-MS	Inductively coupled plasma mass spectrometry.
SPME PTFE	Solid Phase Microextraction Polytetrafluoroethylene.
HDPE	High density polyethylene.
GRIS	Global Register of Introduced and invasive Species.
LOD	Limit of detection.
NCBI	National Centre for Biotechnology Information.
SRA	Sequence read archive.
dsDNA	Double stranded deoxyribonucleic acid.
GATK	Genome analysis toolkit.
SNPs	Single nucleotide polymorphisms.
4FDS	Four-fold degenerate site.
MIS	Missingness.
MAF	Minor allele frequency.
PCA	Principal component analysis.
HAS	Hastings, UK.
COR	Cornwall, UK.
BRI	Bristol, UK.
NEW	Newport, UK.
ABE	Aberdeen, UK.
ELG	Elgin, UK.
GLA	Glasgow, UK.
LAN	Lancaster, UK.
BFD	Bradford, UK.
YOR	York, UK.
HUL	Hull, UK.
MID	Midlands, UK.

NCA	Newcastle, UK.

Author contributions

Conceptualisation, LY, KES. Methodology, LY, KES. Software, MH. Validation, KES. Formal analyses, KES, MH. Investigation, KES, LC, PF, CM. Resources, KES, MH, CARZ, AL. Writing original draft, KES, LY. Writing, review and editing, KES, LY, AL, CARZ. Funding acquisition, LY, KES. Supervision, LY. Administration, LY, KES. All authors approved the final manuscript.

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Data availability

Sequence data that support the findings of this study have been deposited in the Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra) with the primary accession code PRJNA1030266 (available at http://www.ncbi.nlm.nih.gov/bioproject/PRJNA1030266)

Supplemental information

710 Figure S7. Spatial and seasonal spikes in elemental concentrations in native water environment
711 as measured by ICP-MS.

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Supplementary figures for:

An ecological, phenotypic and genomic survey of duckweeds with their associated aquatic environments in the United Kingdom

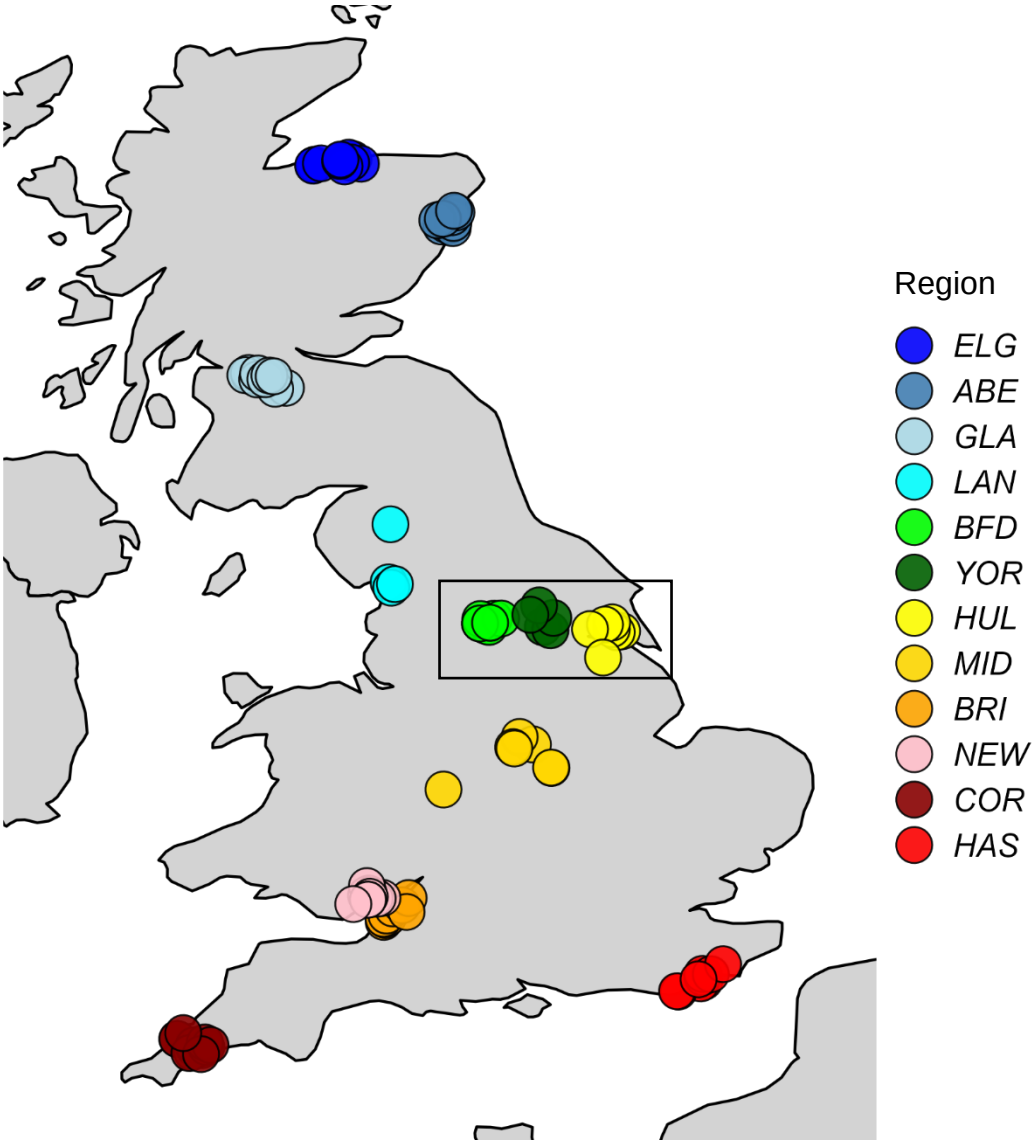
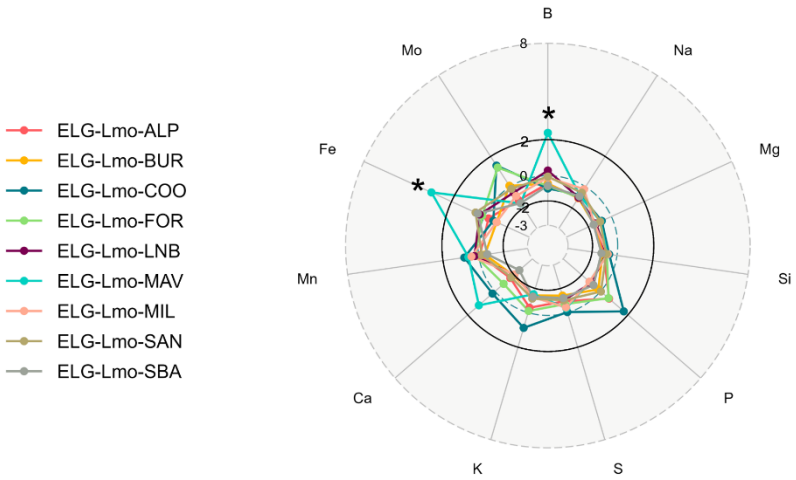


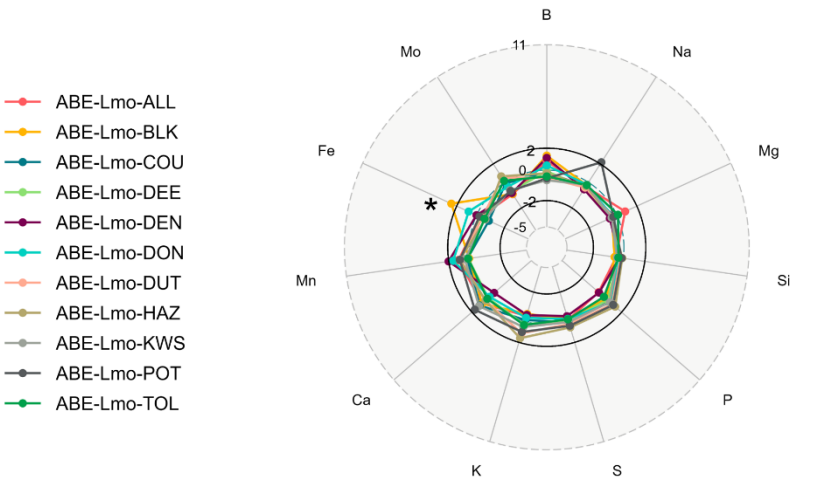
Figure S1. Duckweed sites studied, spanning twelve regions in England, Scotland and Wales on a map of the UK. Sites are coloured by regions and include from North to South: Elgin (ELG), Aberdeen (ABE), Glasgow (GLA), Lancashire (LAN), Bradford (BFD), York (YOR), Hull, (HUL) Midlands (MID), Bristol (BRI), Newport (NEW), Cornwall (COR), Hastings (HAS). The box marks a seasonal transect where duckweed plants were monitored and re-collected seasonally with longer term water assessments across 19 sites around BFD, YOR and HUL.

Scotland & north west England

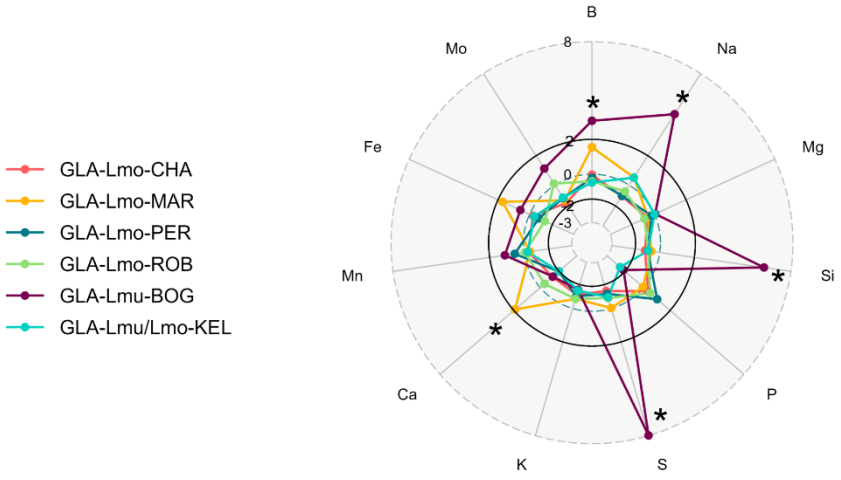
A. ELG



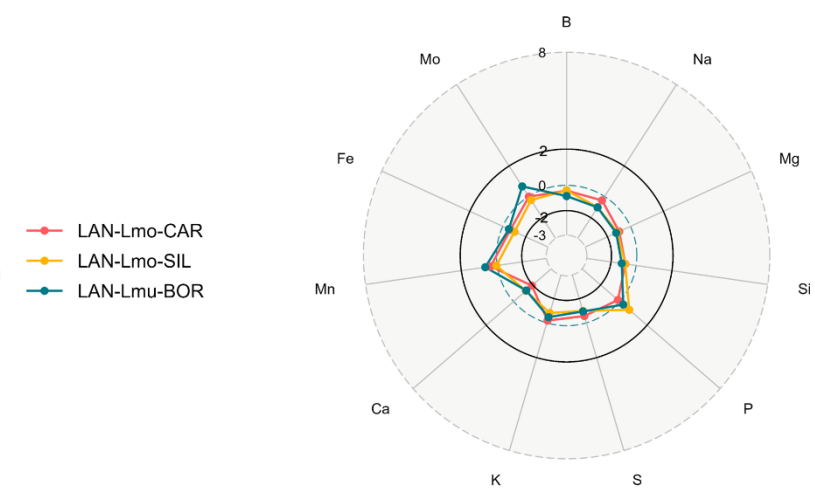
B. ABE



C. GLA



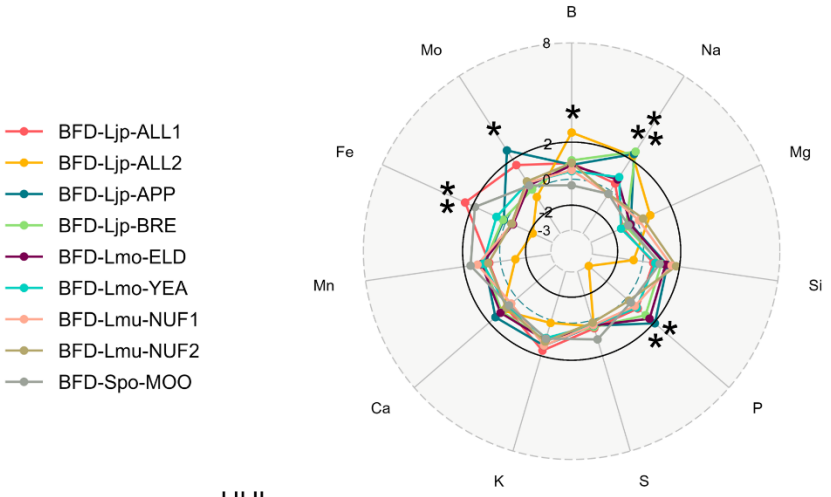
D. LAN



North east – central England

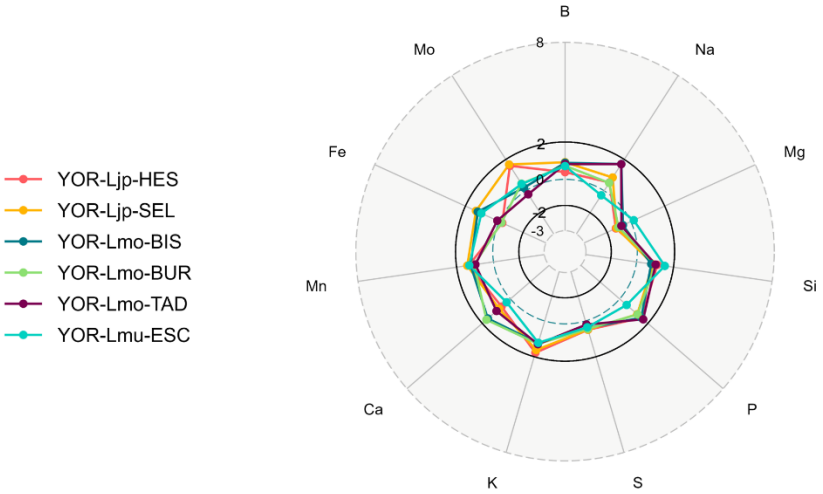
E.

BFD



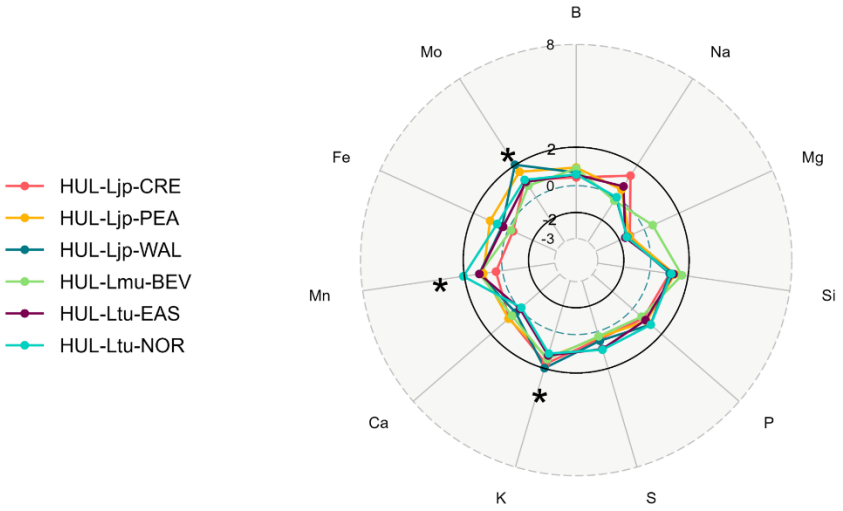
F.

YOR



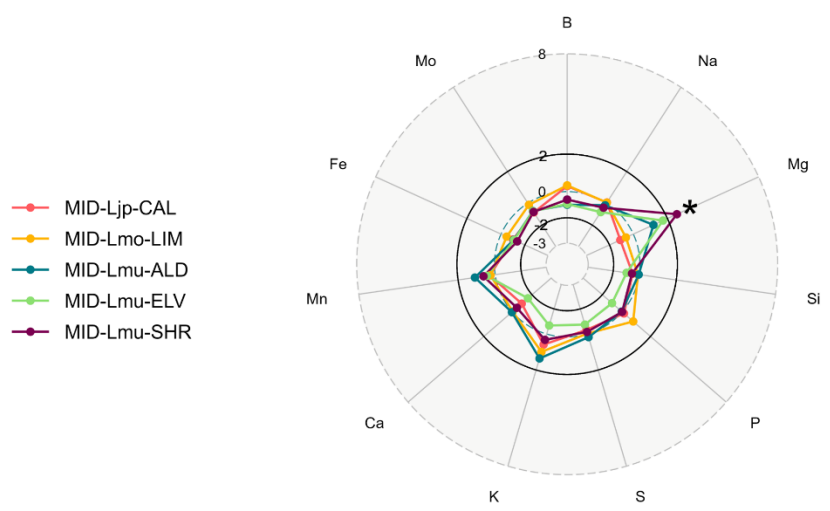
G.

HUL



H.

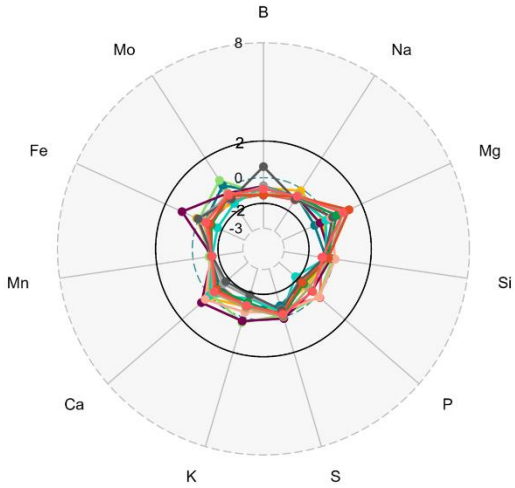
MID



South England & Wales

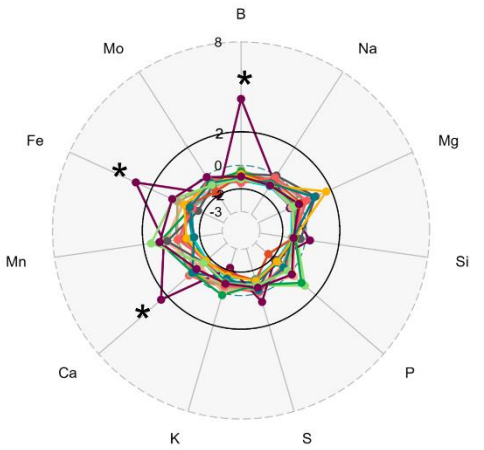
I. BRI

- BRI-Lgi-LAM
- BRI-Ljp-CLA
- BRI-Ljp-LAM
- BRI-Ljp-MLA
- BRI-Ljp-VAL
- BRI-Lmo-BRA
- BRI-Lmo-NAI
- BRI-Lmu-LAM
- BRI-Lmu-NAI
- BRI-Lmu-NEW
- BRI-Lmu-WEM
- BRI-Lmu/Lmo-PUX
- BRI-Lmu/Lmo-VAL



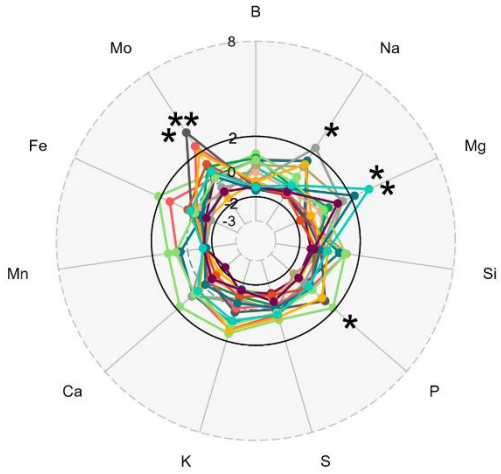
J. NEW

- NEW-Ljp-CHA
- NEW-Ljp-NAS
- NEW-Ljp-TRE
- NEW-Lmo-FOU
- NEW-Lmo-HAW
- NEW-Lmo-LLI
- NEW-Lmo-MAL
- NEW-Lmo-PER
- NEW-Lmu-CHA
- NEW-Lmu-FOU
- NEW-Lmu-LLA
- NEW-Lmu-MAL
- NEW-Lmu-PER
- NEW-Lmu-TRE
- NEW-Lmu/Lmo-NAS
- NEW-Spo-FIV
- NEW-Spo-FOU



K. COR

- COR-Ljp-HOU
- COR-Ljp-JAP
- COR-Ljp-SHE
- COR-Ljp-TRE
- COR-Lmo-AND
- COR-Lmo-COM
- COR-Lmo-COR
- COR-Lmo-GRA
- COR-Lmo-HEL
- COR-Lmo-INN
- COR-Lmo-KWO
- COR-Lmo-MEN
- COR-Lmo-TRG
- COR-Lmo-TRW
- COR-Lmu-HEL
- COR-Lmu-TRE
- COR-Lmu/Lmo-COR
- COR-Lmu/Lmo-PIN



L. HAS

- HAS-Lmo-BRE
- HAS-Lmo-CRO
- HAS-Lmo-GIL
- HAS-Lmo-LAN
- HAS-Lmo-UDI
- HAS-Lmu-HEL
- HAS-Lmu-LAN
- HAS-Lmu-MIL
- HAS-Lmu-UDI
- HAS-Lmu-WIL
- HAS-Lmu/Lmo-WHE

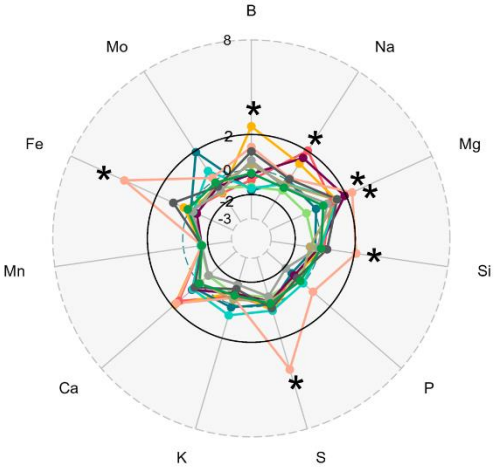
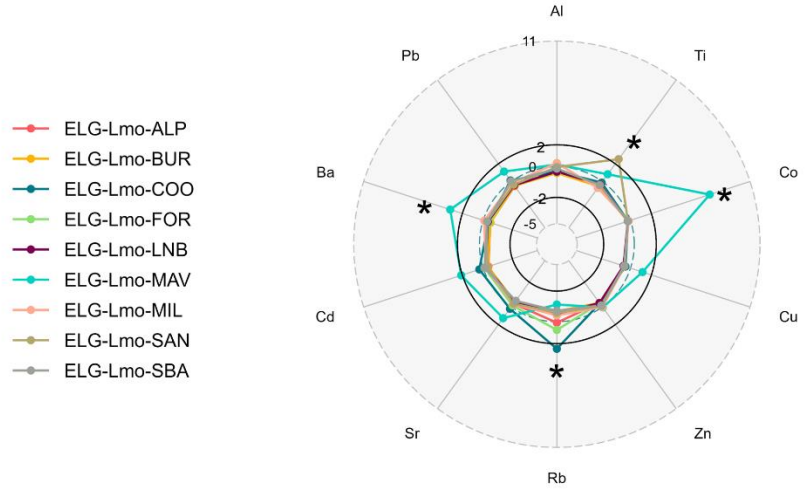


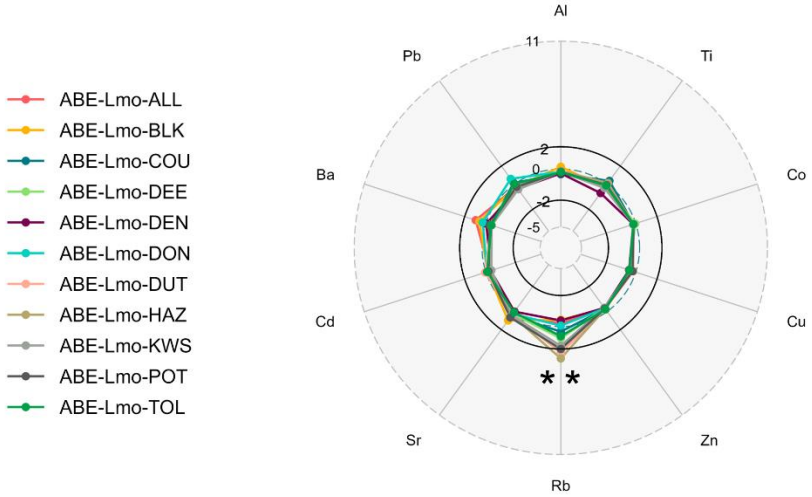
Figure S2. Radar plots for elements quantified in duckweed ionomes from 12 UK regions grown in controlled conditions as measured by ICP-MS. A-D. Scotland and north west England. E-H. North east and central England. I-L. South Wales and England. z-scores derived from normalised data for the whole duckweed panel and SD +/- 2 are considered significant. Elements are plotted at each point of the radar and include those provided in N-medium. The key corresponds to the independent site codes for each ecotype which is differentiated by its region, species and the site it was located, as indicated in Table S2A. At least *n*=3 ecotypes are included for each region.

Scotland & north west England

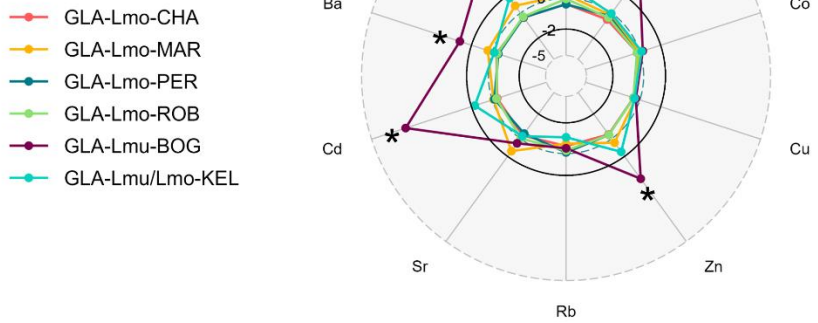
A. ELG



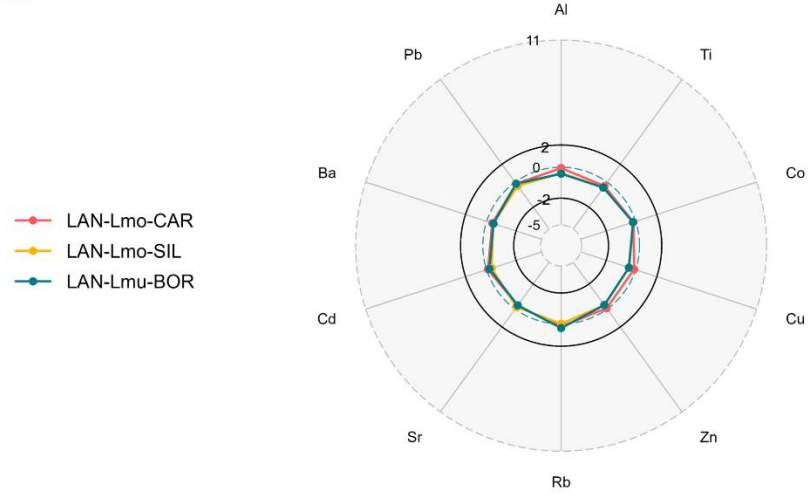
B. ABE



C. GLA

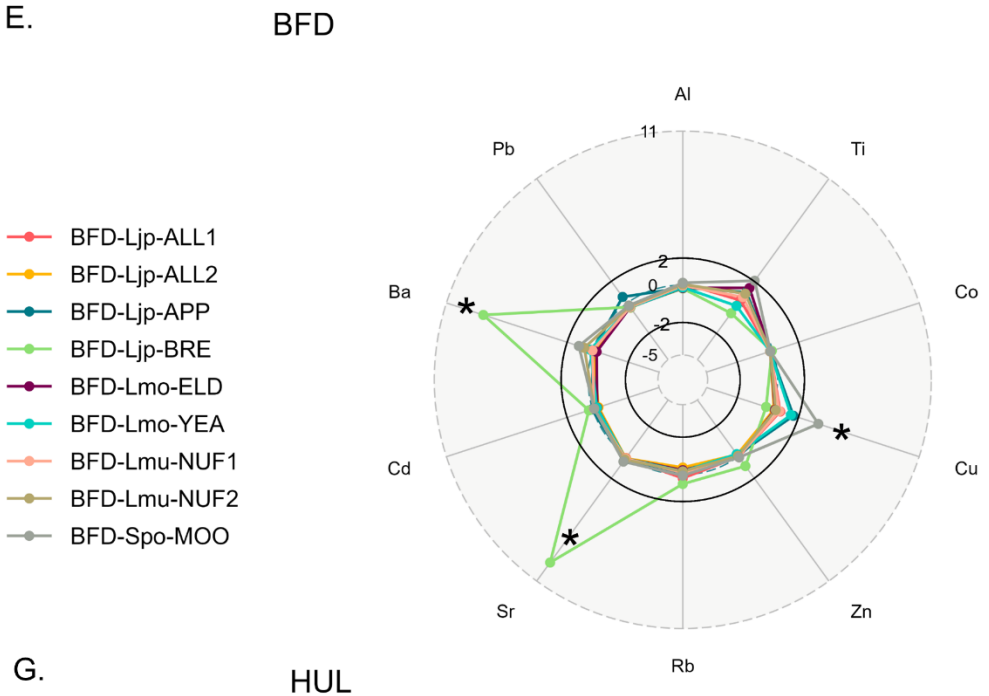


D. LAN

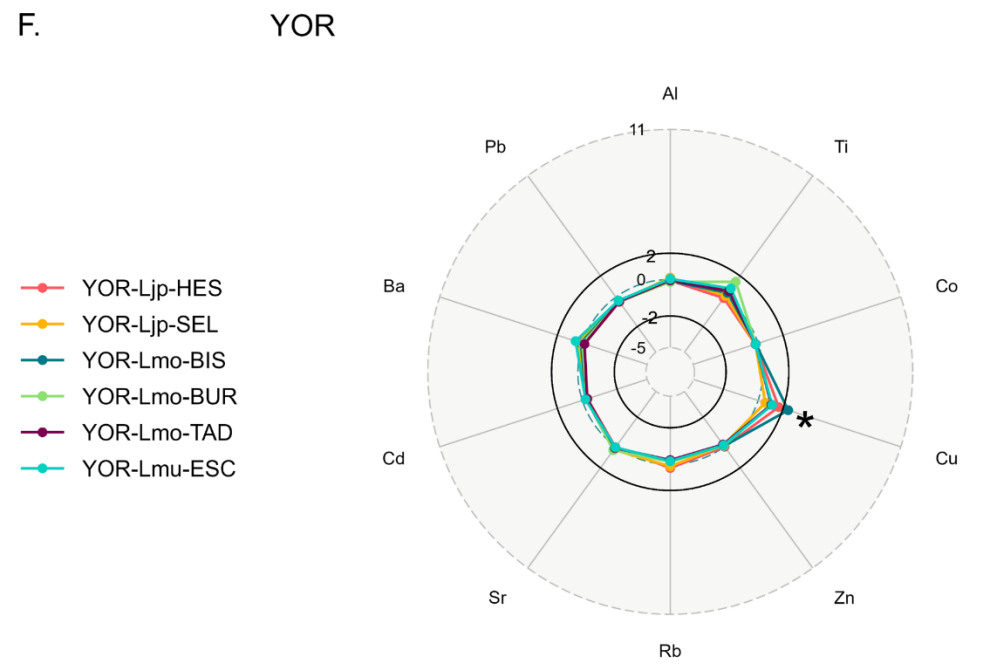


North east – central England

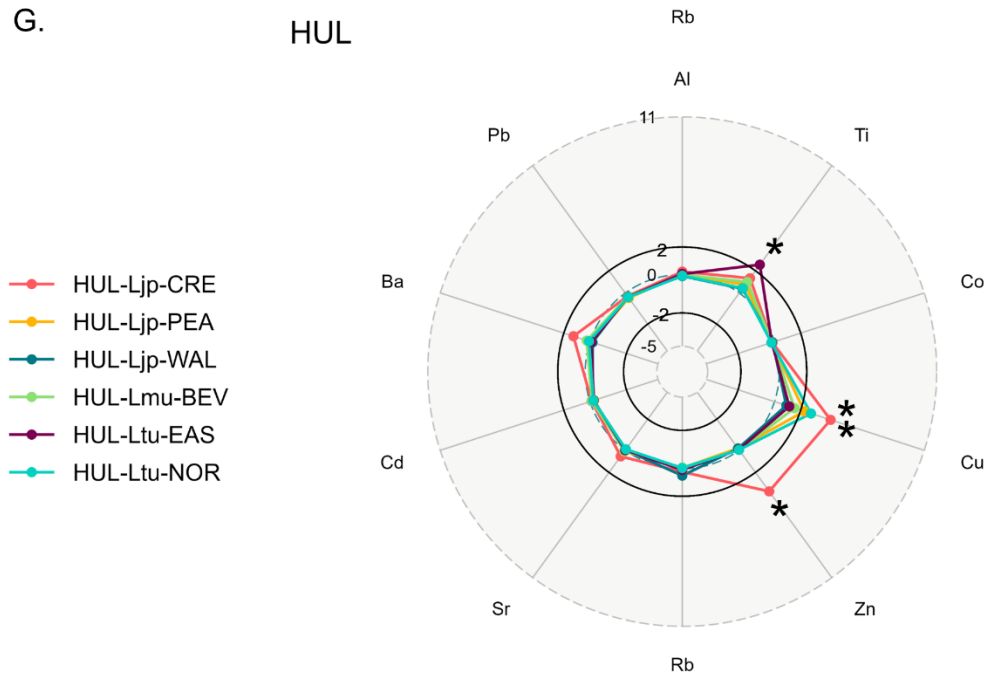
E.



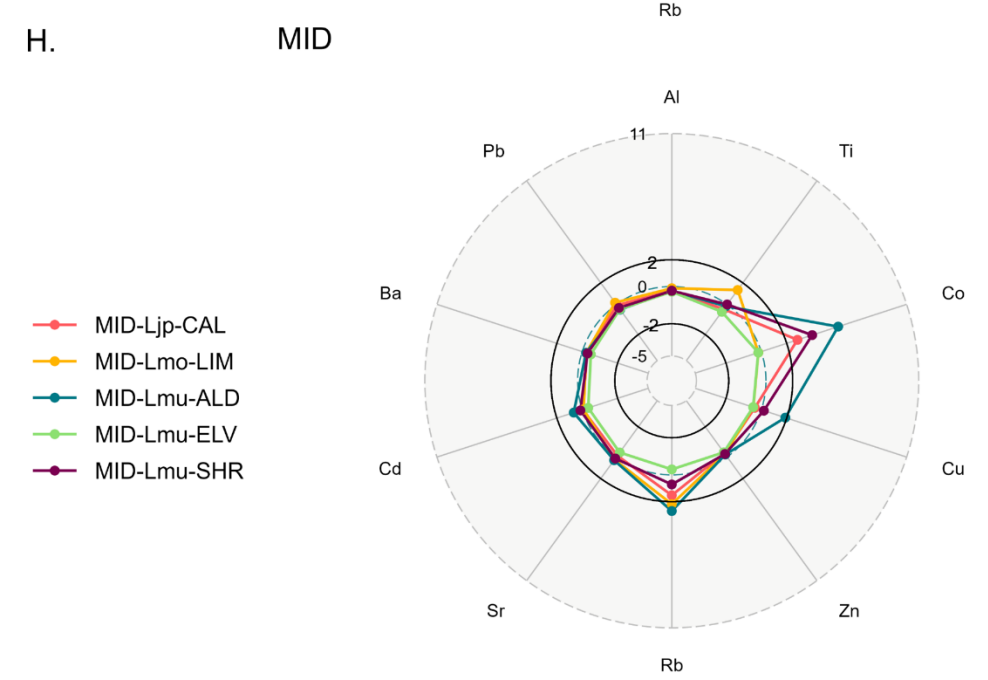
F.



G.



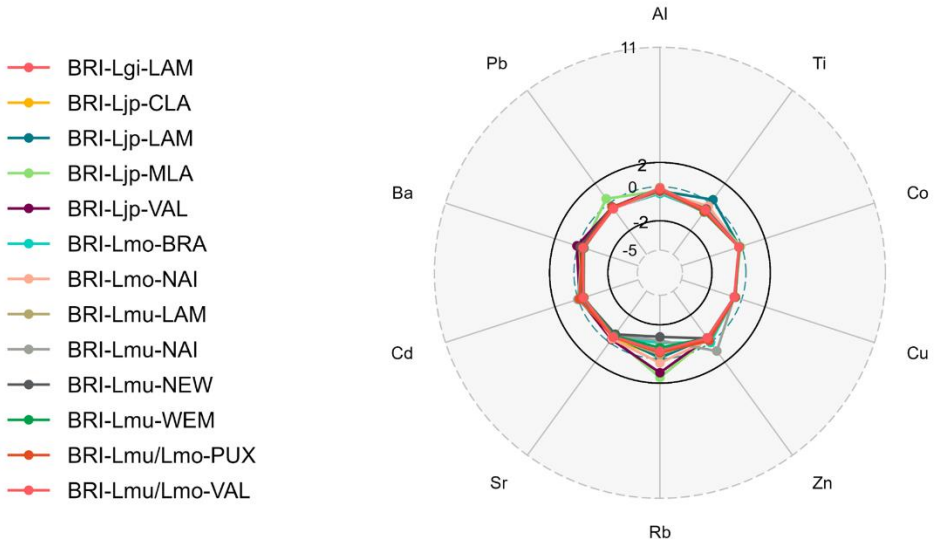
H.



South England & Wales

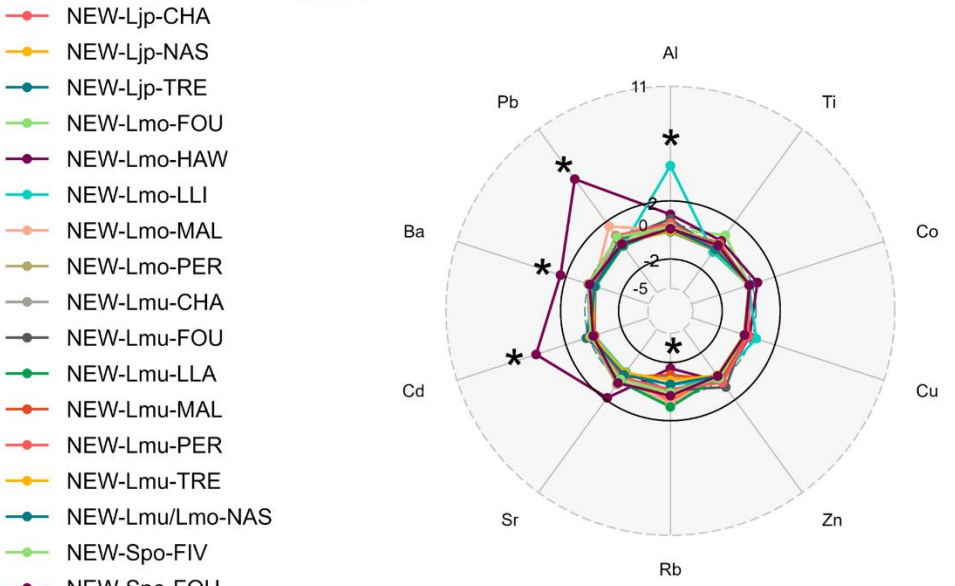
I.

BRI



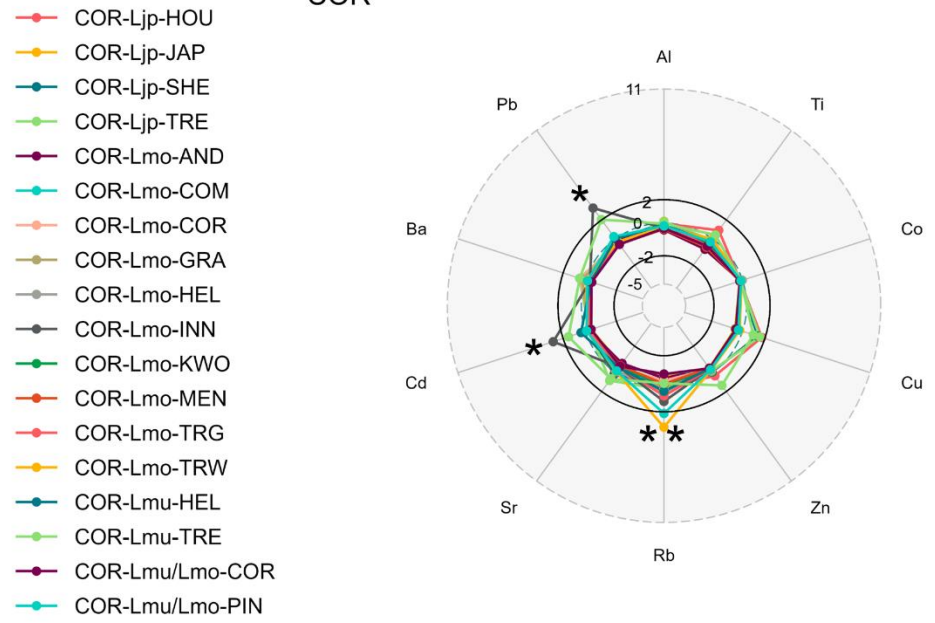
J.

NEW



K.

COR



L.

HAS

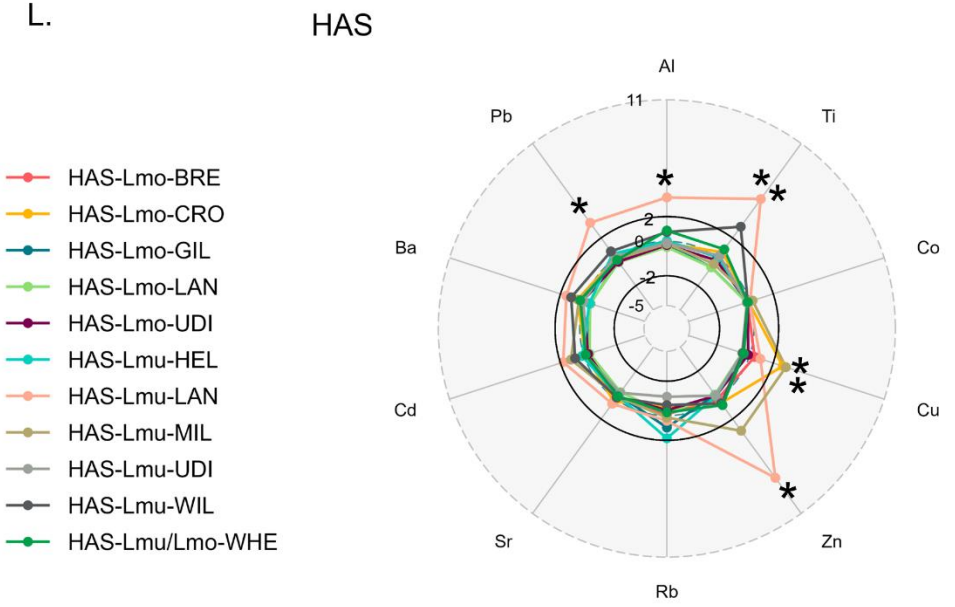
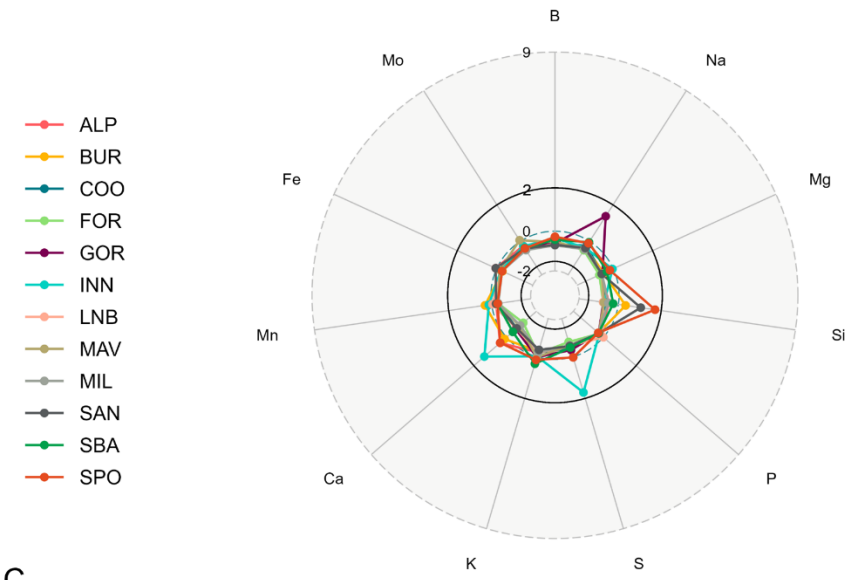


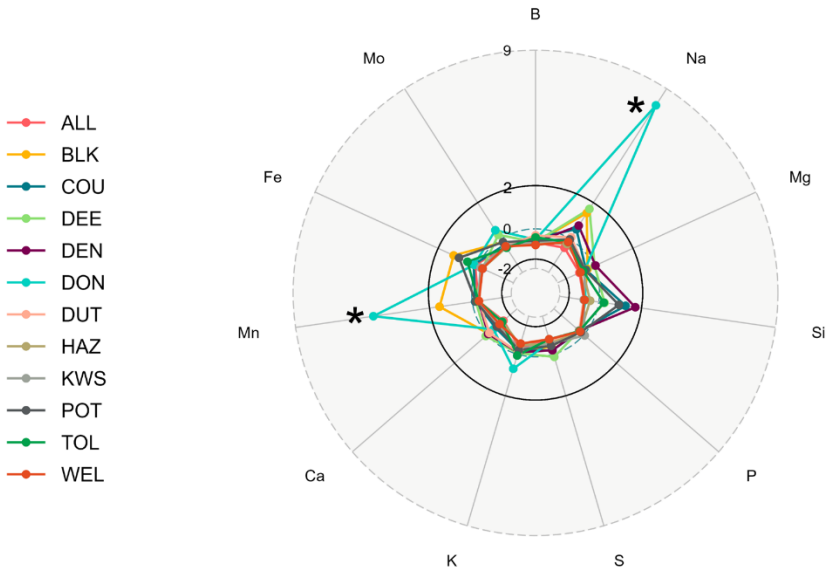
Figure S3. Radar plots for heavy metal elements quantified in duckweed ionomes from 12 UK regions grown in controlled conditions as measured by ICP-MS. A-D. Scotland and north west England. E-H. North east and central England. I-L. South Wales and England. z-scores are derived from normalised data for whole panel and SD +/- 2 are considered significant The key corresponds to the independent site codes for each ecotype which is differentiated by its region, species and the site it was located, as indicated in Table S2A.

Scotland

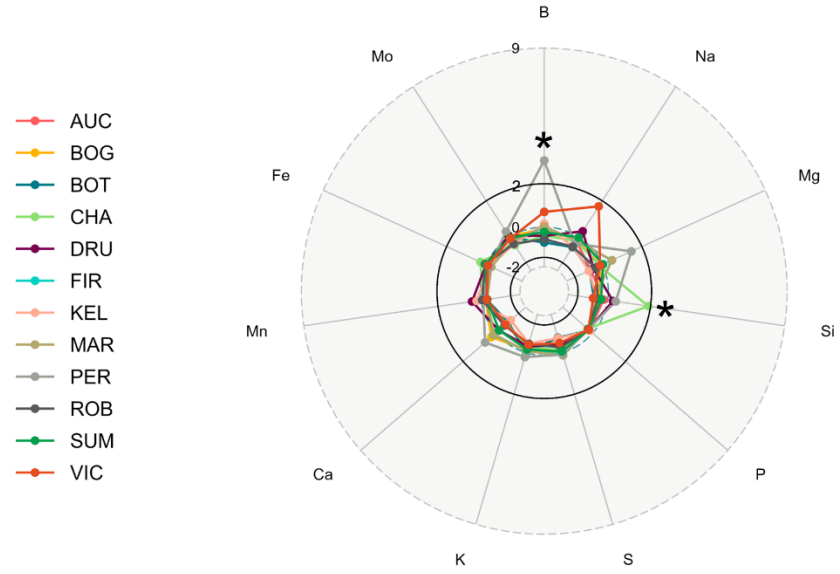
A. ELG



B. ABE

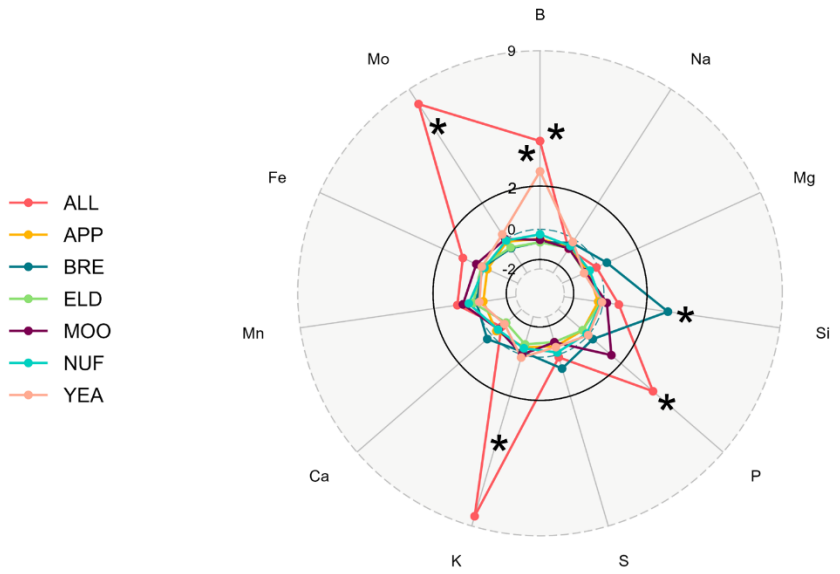


C. GLA

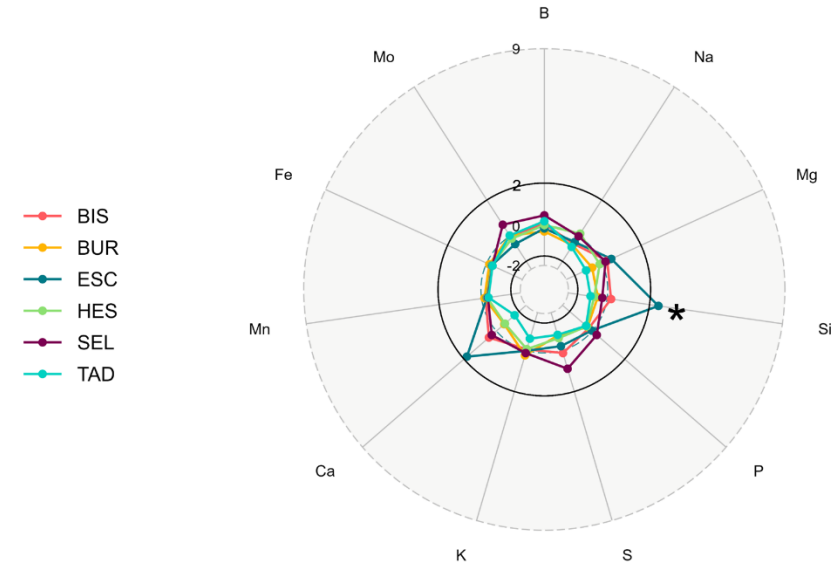


North east England

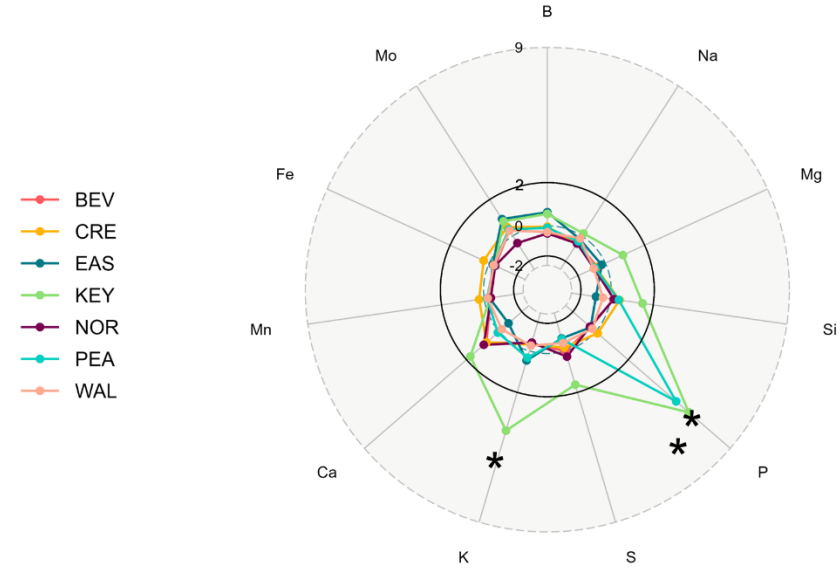
D. BFD



E. YOR

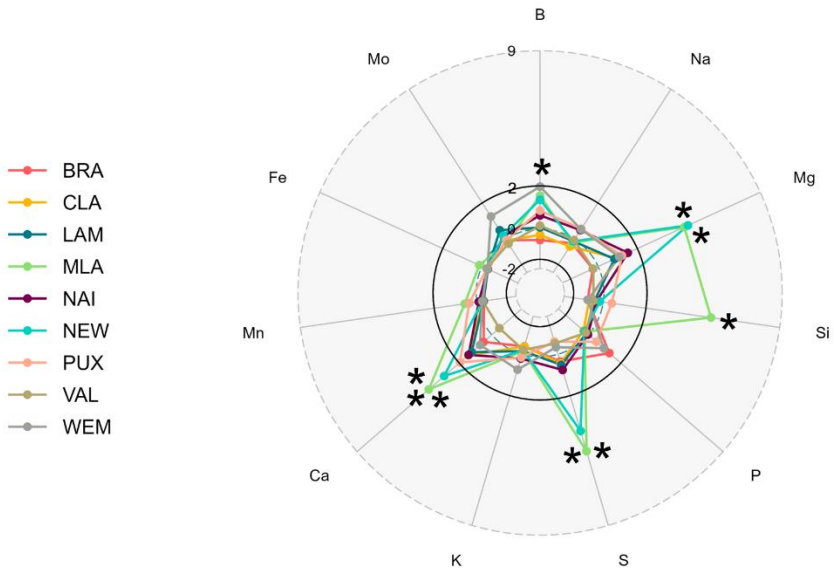


F. HUL

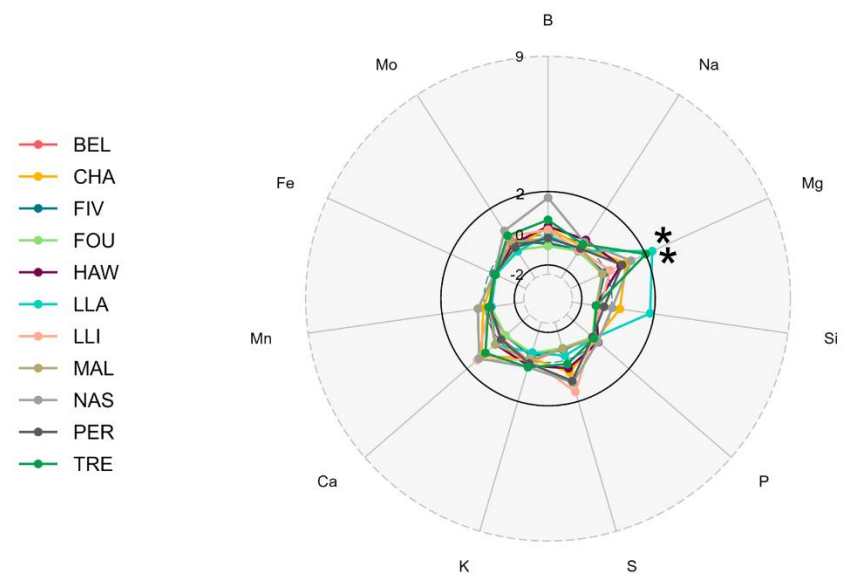


South England & Wales

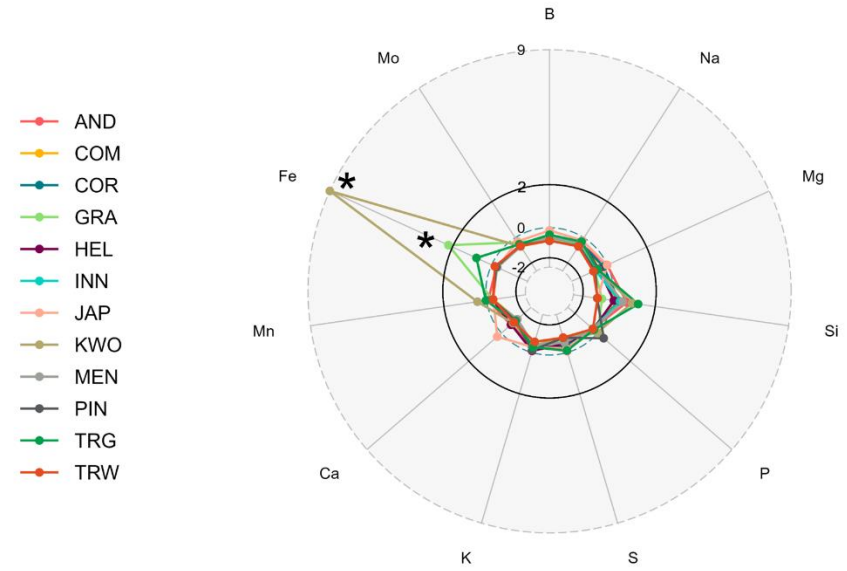
G. BRI



H. NEW



I. COR



J. HAS

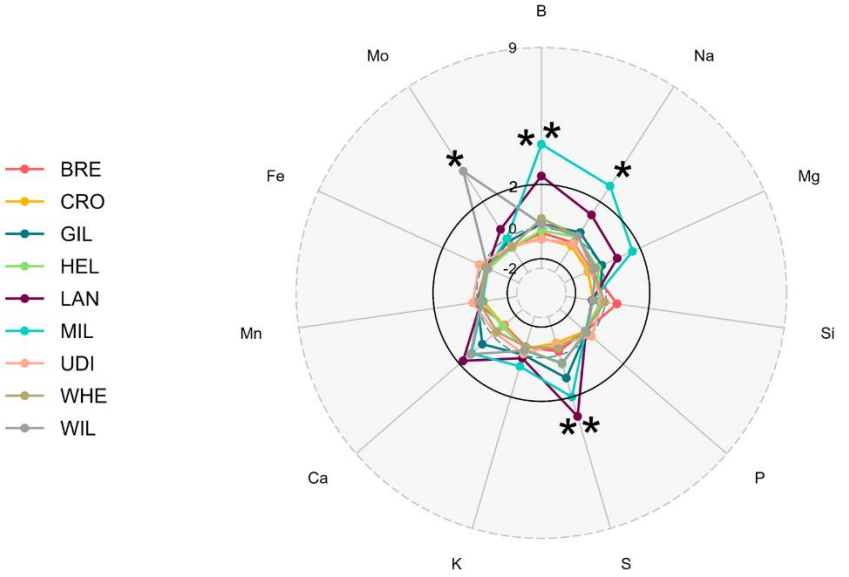
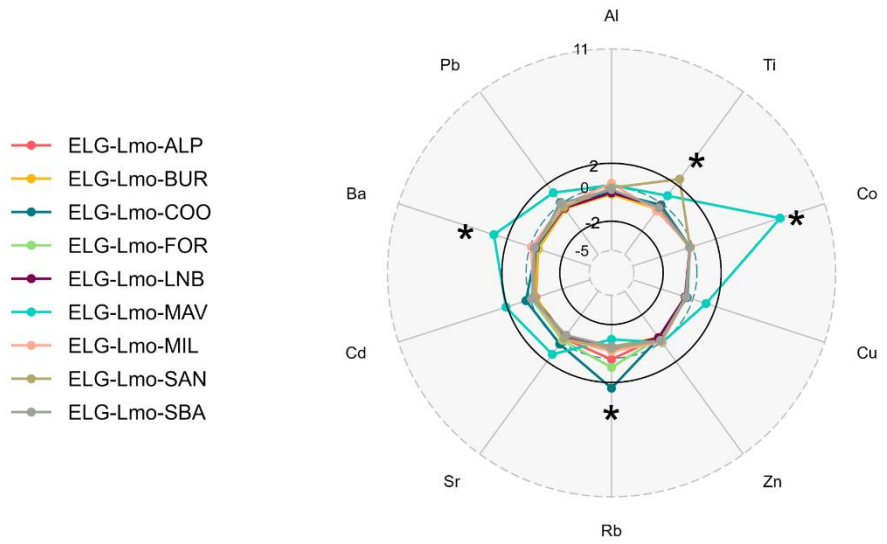


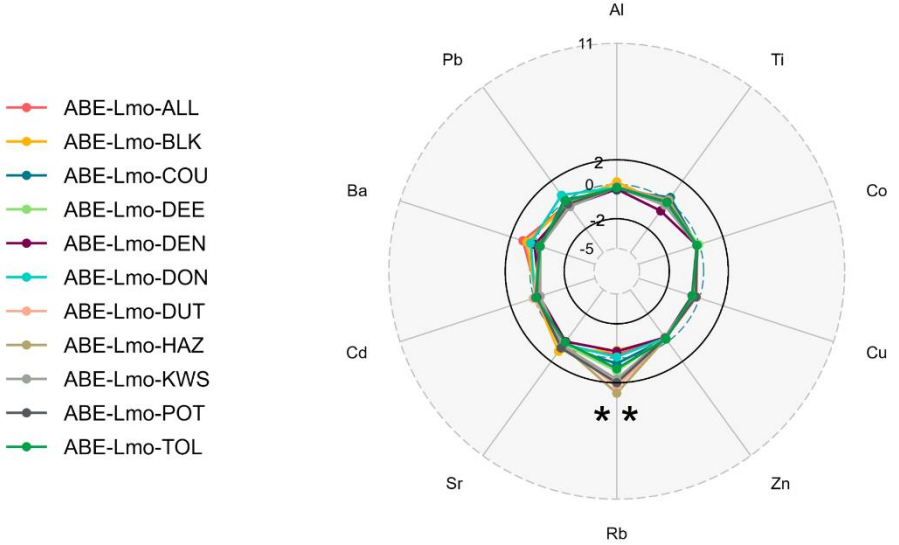
Figure S4. Spatial variation for elements measured in water environments by ICP-MS. A-C. Scotland. D-F. North east England. G-J. South Wales and England. Radar plots for macronutrients within individual water sites within ten UK regions. Macronutrients depicted at each point in the radar plot with relative concentration (z-scores) for each site coloured and named in the legend. The key corresponds to the site names in each region, as indicated in Table S1. All elements were present in duckweed lab-growth medium. Circles depict +2 or -2 of the SD for each population average and individual waters above or below this are considered extremes for that element.

Scotland & north west England

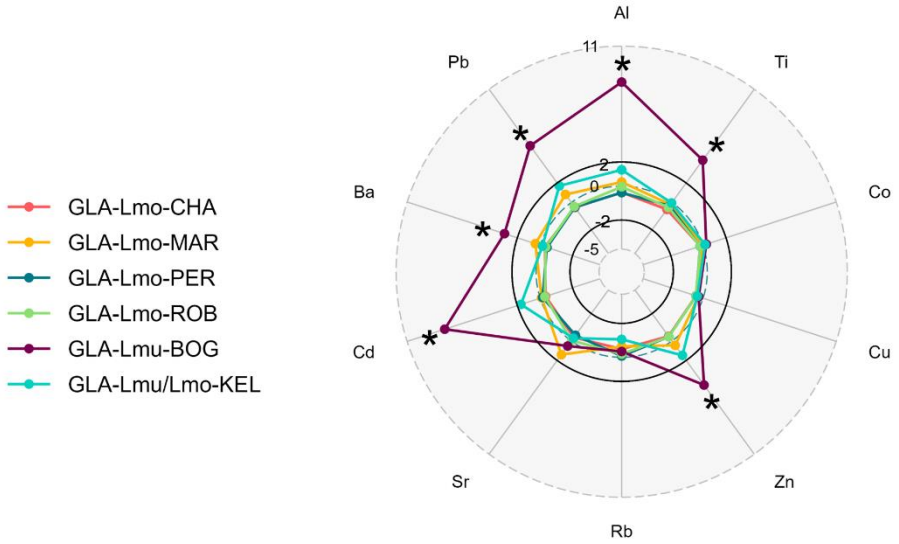
A. ELG



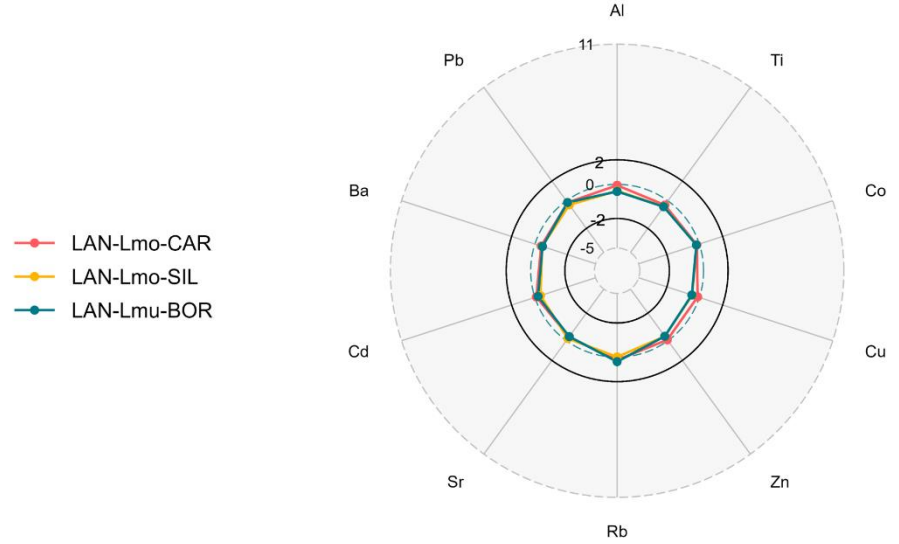
B. ABE



C. GLA

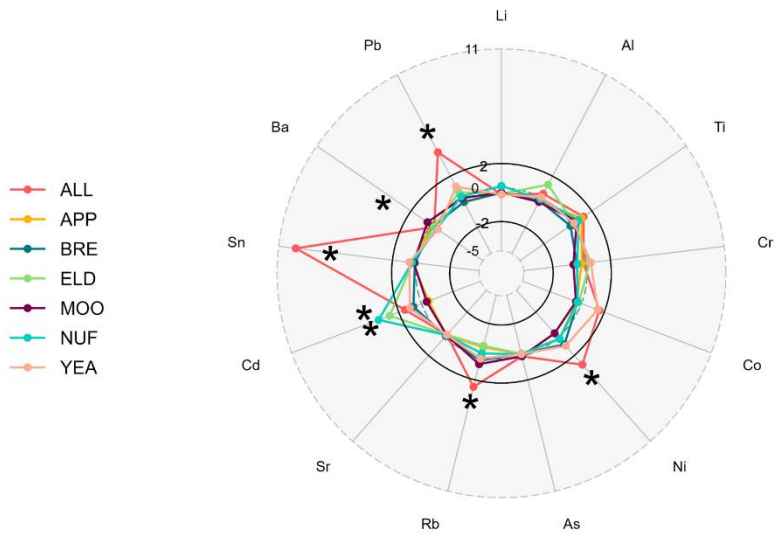


D. LAN

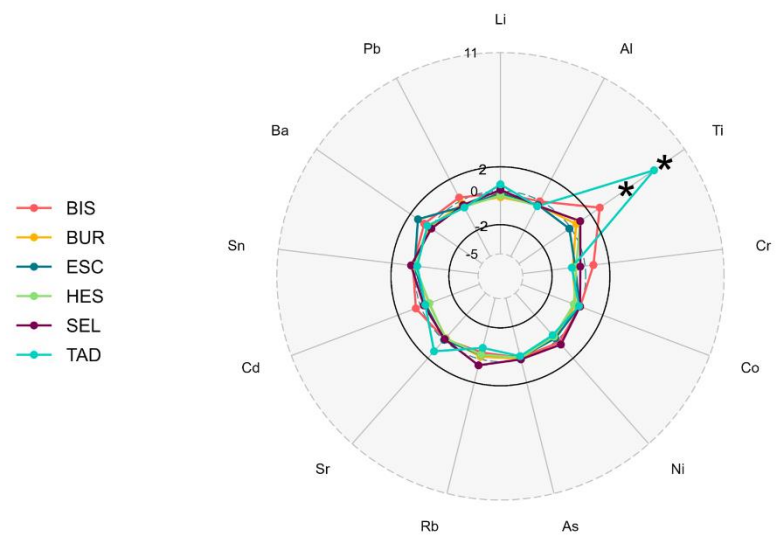


North east England

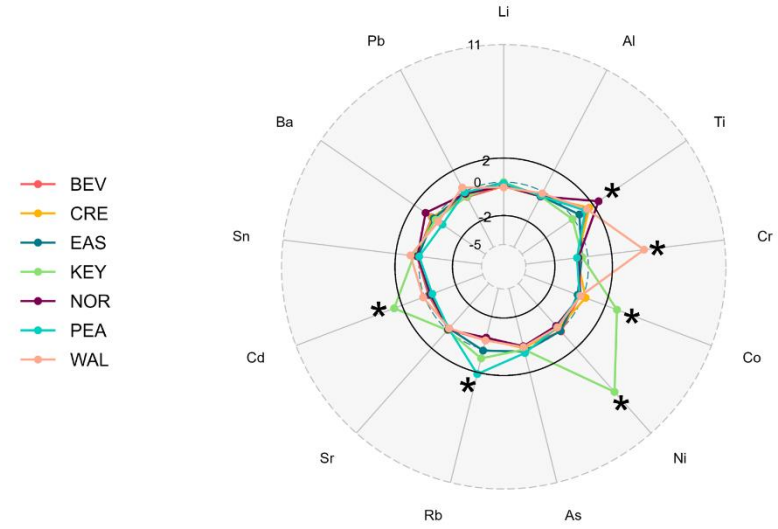
D. BFD



E. YOR

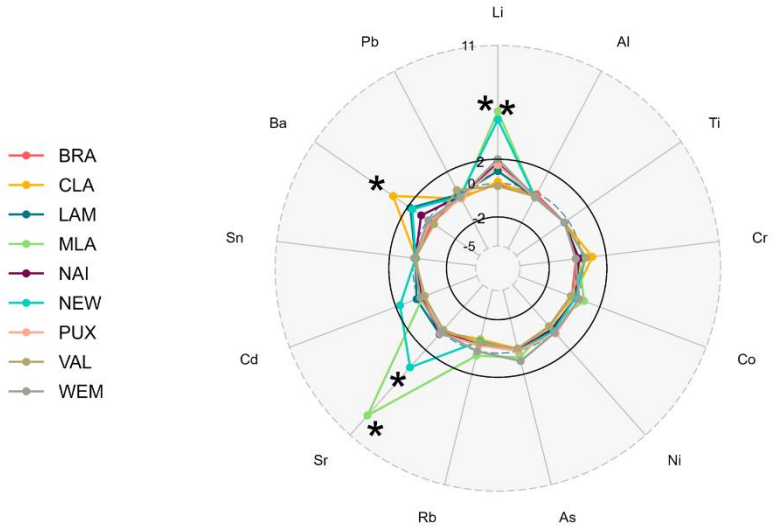


F. HUL

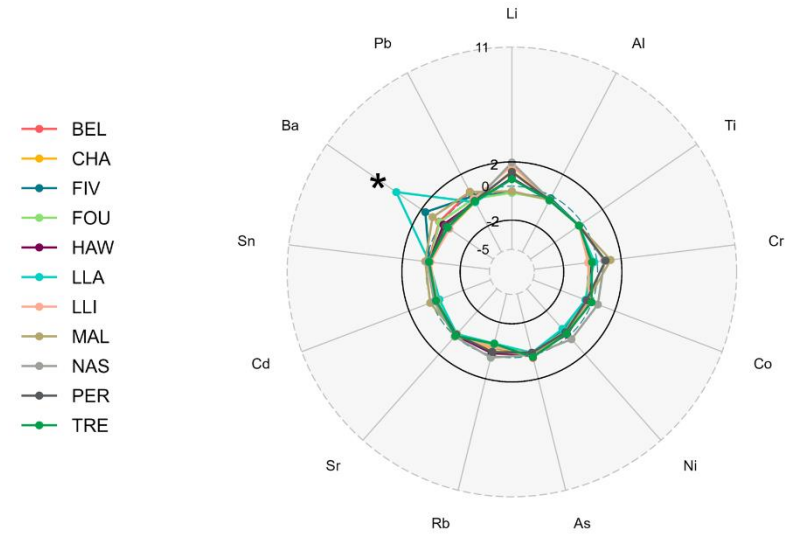


South England & Wales

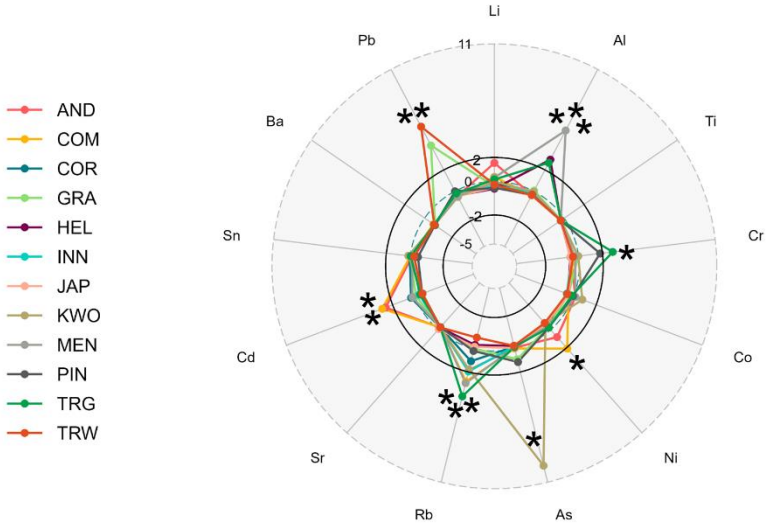
G. BRI



H. NEW



I. COR



J. HAS

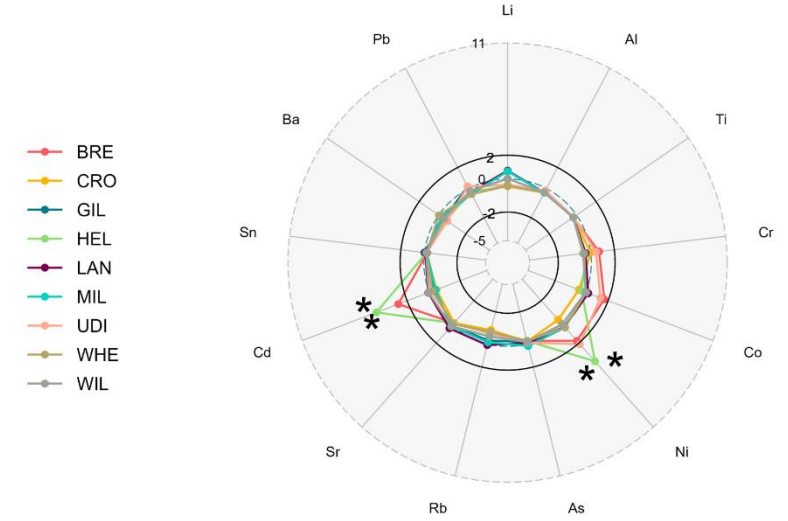


Figure S5. Spatial variation for heavy metals measured in water environments in ten UK regions by ICP-MS. A-C. Scotland. D-F. North east England. G-J. South Wales and England. The key corresponds to the site names in each region, as indicated in Table S1. Heavy metals at each axis of the radar plots and relative concentrations at each site are coloured and represented as lines from z-scores. Circles depict +2 or -2 of the SD for each population average and individual waters above or below this are considered extremes for that element.

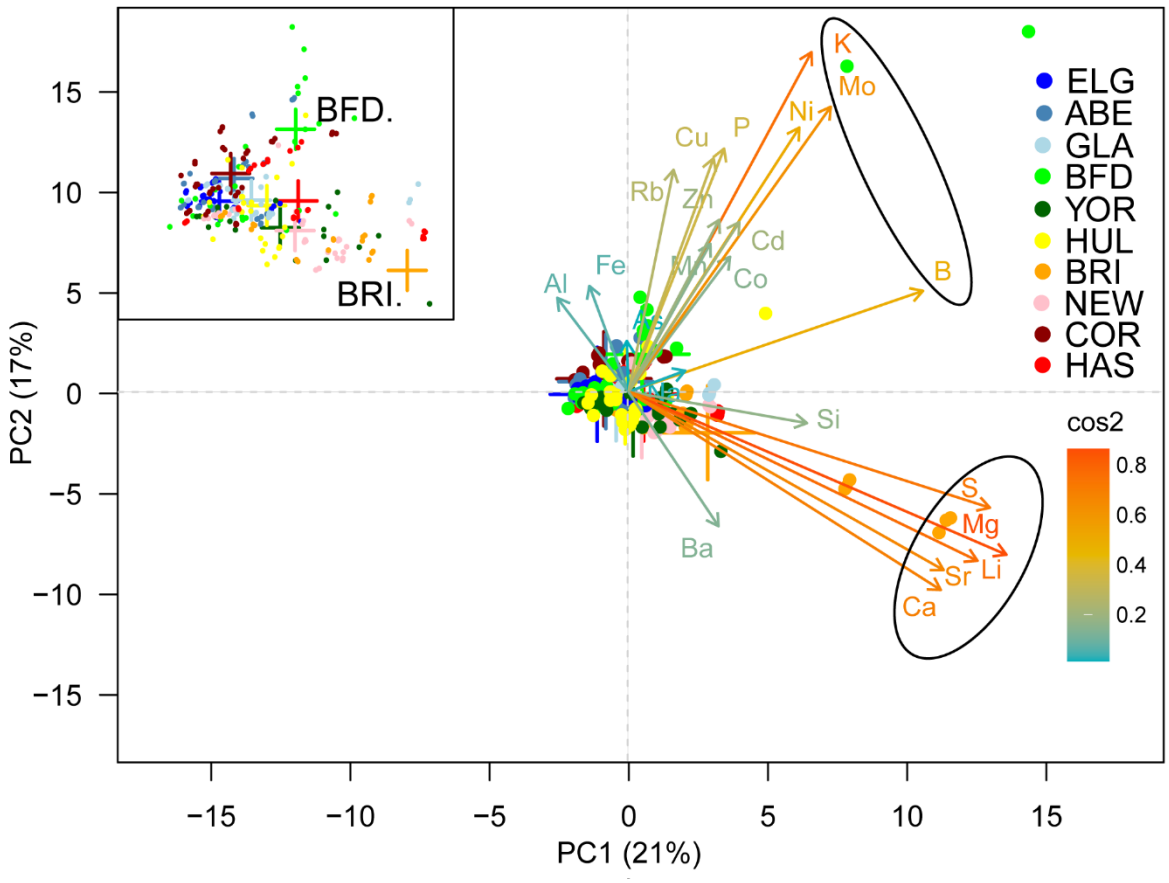
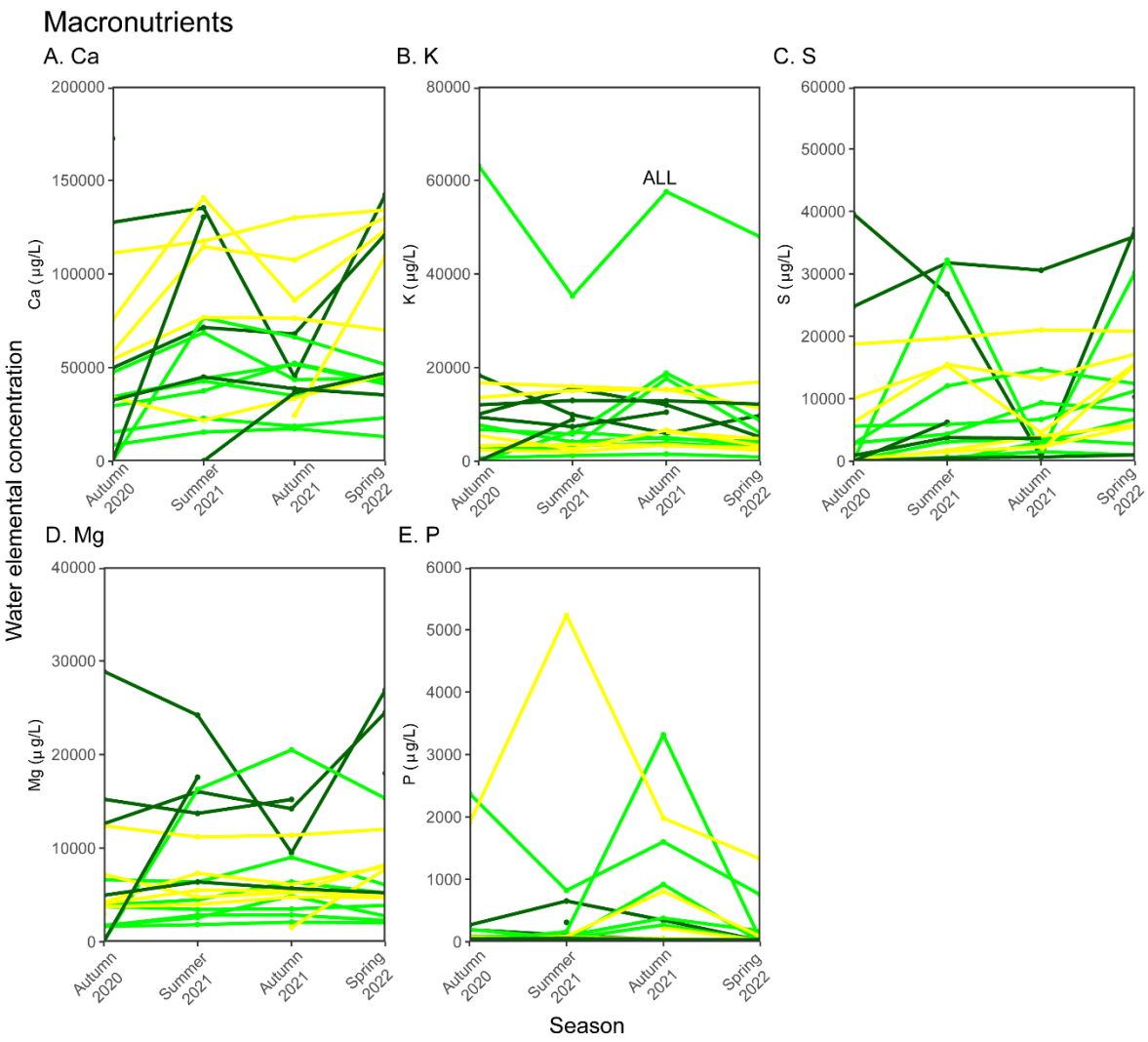
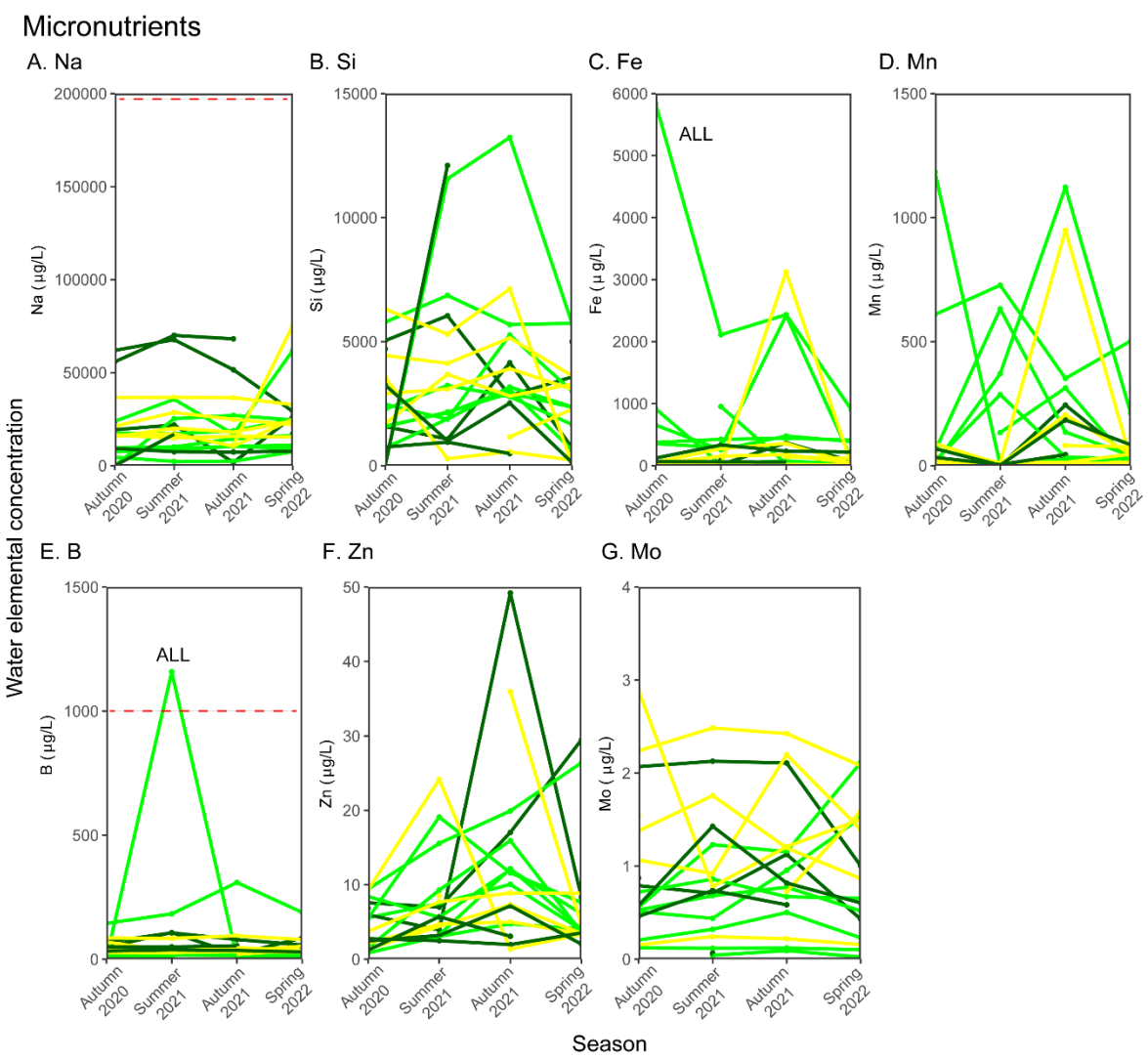


Figure S6. Footprint of regional variation from 100 water environments showing elemental compositions measured by ICP-MS. Individual sampling sites with water chemistry data including 21 elements plotted on PC1 and PC2. Ten collection regions with minimum of $n = 6$ sites sampled per region are included. The arrows on the biplot are coloured by cosine (cos2) which shows elemental relationships and degree of contribution to data set variation. Circled regions correspond to elements associated with regions BFD and BRI. *Inset:* BFD and BRI show the most variation in water chemistry. Crosses depict the averages for ten regions, outliers from BRI and BFD have been removed to produce the close up inset figure.


Macronutrients
A. Ca
B. K
C. S
D. Mg
E. P
ALL
Water elemental concentration
Ca (µg/L)
K (µg/L)
S (µg/L)
Mg (µg/L)
P (µg/L)
Season
Autumn 2020
Summer 2021
Autumn 2021
Spring 2022


Micronutrients
A. Na
B. Si
C. Fe
D. Mn
E. B
F. Zn
G. Mo
ALL
ALL
Water elemental concentration
Na (µg/L)
Si (µg/L)
Fe (µg/L)
Mn (µg/L)
B (µg/L)
Zn (µg/L)
Mo (µg/L)
Autumn 2020
Summer 2021
Autumn 2021
Spring 2022
Season

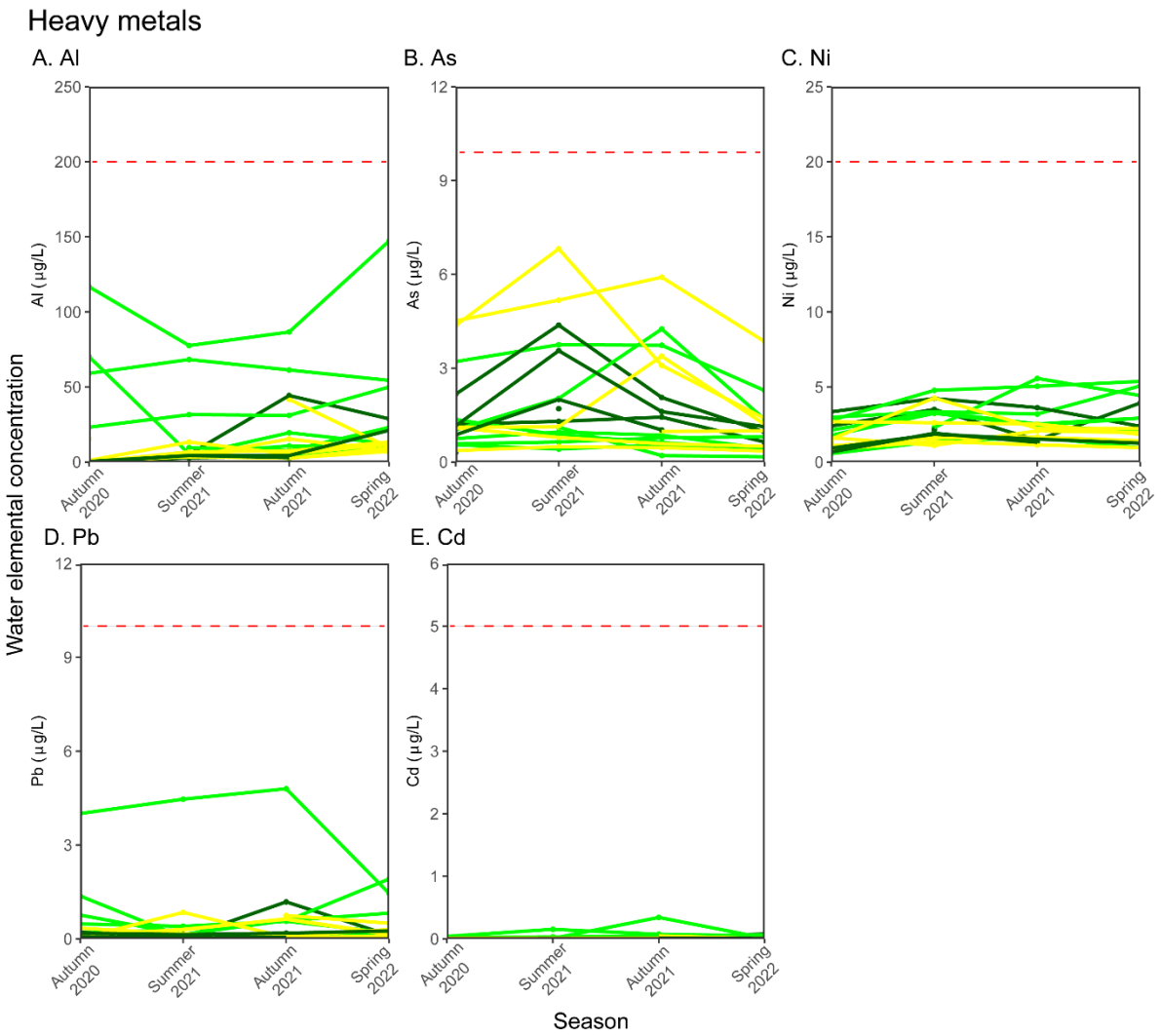


Figure S7. Spatial and seasonal spikes in elemental concentrations in native water environments as measured by ICP-MS. Panel A. Macronutrients. A. Ca, B. K, C. S, D. Mg, E. P. Panel B. Micronutrients. A. Na, B. Si, C. Fe, D. Mn, E. B, F. Zn, G. Mn. Panel C. Heavy metals. A. Al, B. As, C. Ni, D. Pb, E. Cd. Raw water elemental concentrations (in µg/L) are plotted as line plots overtime (autumn 2020, summer 2021, autumn 2021 and spring 2022). Three regions are plotted and lines are coloured by region: BFD (green), YOR (dark green), HUL (gold). Site ALL is labelled showing high and varied concentrations of K, Fe and B. The red dashed lines indicate the upper quantity limit allowed in drinking water for micronutrients Na and B and heavy metals Al, As, Ni, Pb and Cd using the Water quality regulations 2018.

Supplementary tables for:

An ecological, phenotypic and genomic survey of duckweeds with their associated aquatic environments in the United Kingdom

Table S1. UK duckweed collection with native water sites details and descriptions.

Site	Name	Region	Water source	Type	Location	Latitude	Longitude
KS02	BIS	YOR	Ditch	Ditch under bridge with high banks	Cawood common, Bishop wood, North of Scalm lane, Selby	53.79959	-1.14745
KS03	ALL	BFD	Pond	Small 2 yr old shallow pond, shaded under 1 tree	Bradford valley	53.81035	-1.755023
KS04	SEL	YOR	Canal	Selby canal with parked boats	Selby canal	53.77428	-1.066171
KS06	NUF	BFD	Pond	Isolated pond, shallow depth	Nuffield health centre, Cottingley / manor pond at end of car park	53.8307	-1.819743
KS09	YEA	BFD	Small ponds	Tarnfield family ponds. Shallow	Yeadon ponds off tarn	53.86976	-1.670763
KS12	MOO	BFD	Pond	Stagnant pond	Bradford moor park	53.80194	-1.723388
KS13	ELD	BFD	Overflow stream off dam	Shallow overflow lake, some flow from dam to stream	Eldwick hall	53.86671	-1.815469
KS14	APP	BFD	Marina	Shallow marina with moored boats	Apperley bridge marina, Bradford	53.83562	-1.711316
KS15	BRE	BFD	Bog	Smallish bog/pond in public garden	Breary marsh, Golden acre park, Leeds	53.87101	-1.594412
KS16	EAS	HUL	Pond	Large pond, stagnant area after bridge	East park, East Hull	53.76686	-0.298782
KS17	PEA	HUL	Pond	Small pond, manmade fountains	Pearson park, Hull	53.75825	-0.355821
KS18	CRE	HUL	Beck	Shallow, non-flowing beck	Creyke beck, Cottingham	53.78317	-0.408387
KS20	BEV	HUL	Canal	Wide section of canal with fishing pegs	Beverley beck, Beverley	53.83914	-0.405169
KS21	WAL	HUL	Pond	Small village pond with walkway, high banking	Walkington, Beverley	53.82131	-0.482115
KS22	NOR	HUL	Beck	Downhill flowing beck	North cave beck, Brough	53.78351	-0.642291
KS25	ESC	YOR	Bog	Shallow bog in woodlands	Escwick woods bog, near York	53.87598	-1.034457
KS27	BUR	YOR	Pond	Medium-sized village pond	Burtree avenue pond, Skelton, York	53.99645	-1.132479
KS28	HES	YOR	Swamp	Large lake/swamp in woodland	Willow's fishery, Hessay, York	53.97913	-1.187243

KS29	TAD	YOR	River offshoot	Offshoot from river, stagnant one side, flowing at other	Offshoot river wharfe, access from Wighill lane, Tadcaster	53.90209	-1.281152
KS33	SIL	LAN	Ditch	Shallow bank side between reeds and wooden walkway	Leighton moss RSPB, Silverdale	54.16884	-2.798876
KS34	CAR	LAN	Canal	Lancaster canal near Carnforth	Carnforth, before Canal turn	54.12518	-2.770829
KS35	BOR	LAN	Canal	Lancaster canal between Tewitfield and Borwick	Between Tewitfield and Borwick	54.15421	-2.731501
KS36	PEN	LAN	Small circular pond	Small circular pond with bridge	Rheged service station, off A66, Penrith	54.64717	-2.779185
KS37	UON	MID	Dried up lake, arboretum	Shaded section of dried up lake	UON, Sutton bonington campus arboretum	52.83363	-1.253883
KS38	ELV	MID	Ditch	Lake off-shoot	Elvaston castle, Derbyshire	52.89538	-1.395662
KS39	LIM	MID	Bog	Small shallow muddy bog	Calke lime pits, Derbyshire	52.81077	-1.465801
KS40	CAL	MID	Cave pond	Sunken pond bricked area above	Grotto, Calke abbey, Derbyshire	52.80043	-1.45258
AL01	ALP	MID	Pond/bog in garden	Leaky 25 yr old garden pond fed by rainwater	Leicester LE5 2HU	52.64	-1.055
AL02	ALD	MID	Small steel container by overflow from water butt	Rainwater run off	Leicester	52.64314	-1.058691
AL03	SHR	MID	Ditch	Run off from roadside	Newport Shropshire	52.46235	-2.211213
SS01	SS01	NCA	Garden pond	Garden pond	10 Wolsey Court South Shields NE34 0QU	54.97439	-1.43347
KS42	HEL	HAS	Narrow stream	Shallow wooded flowing downhill	Helen's wood, Hastings	50.88058	0.581676
KS43	GIL	HAS	Medium pond	Stagnant pond in woods with sloped landscape	Gilman's hill pond woods, St Leonards on Sea	50.86504	0.543269
KS44	LAN	HAS	Sewer	Sewer fairly steeped banks with bridge	Langney sewer, Eastbourne	50.79795	0.304928
KS45	WIL	HAS	Shallow ditch	Banked ditch with bridge, narrow beck minimal flow	Willingdon upper, shinewater park, Eastbourne	50.8006	0.289478
KS46	WHE	HAS	Medium pond	Muddy bog land	Wheel lane, Westfield, Hastings	50.91675	0.561538
KS47	BRE	HAS	Overflow dam	Reservoir overflow banked stagnant levels	Brede high woods, Battle	50.94325	0.561655
KS48	UDI	HAS	Medium pond	Medium village stagnant pond	Udimore pond, Rye	50.9405	0.651719
KS49	MIL	HAS	River	River with bridge/gate	Rother fishery, Rother river, Military road, Tenterden	51.0232	0.782752
KS50	CRO	HAS	Small pond	Shallow pond with low flow, mud sides	Crowhurst park pond, Telham lane, Battle	50.89911	0.520065

KS51	COR	COR	Shallow ditch	Shallow open ditch near river, old china clay harbour	Cornwall hotel, St Austell	50.32616	-4.795716
KS52	KWO	COR	Small sunken ditch	Shallow water and pipe opening into sunken tree area	Kingswood, St Austell	50.31427	-4.79936
KS53	GRA	COR	Shallow pond	Shallow grassy field pond, stagnant water, shaded by shrubbery	Grampound field, Truro	50.29865	-4.904628
KS54	MEN	COR	Medium pond	Spring-fed, clay-bottomed, stagnant no flow with island surrounded by plants	Menacuddle well pond, St Austell	50.34607	-4.795569
KS55	COM	COR	Medium lake	Farmyard lake on edge of farm next to streams, island in middle	Combe valley, Combe	50.33426	-4.879119
KS56	TRG	COR	Small sunken ditch	Small shallow sunken ditch walled on one side, no flow	Tregargus woods north, old china clay site, St Austell	50.35485	-4.883733
KS57	TRW	COR	Square garden pond	Square feature pond in walled garden, partial cover with net	Trewithen gardens, Truro	50.2906	-4.931766
KS58	PIN	COR	Medium pond	Medium pond, shallow with island and low flow	Pinetum gardens, Holmbush, St Austell	50.34154	-4.751426
KS59	INN	COR	Medium fishing pond	Medium fly-fishing pond, shallow at edges	Innis fly fishing, St Austell	50.37814	-4.766337
KS60	AND	COR	Large pond	Large park pond with flow	St Andrews Pond, St Andrew's Rd, Tywardreath, Par	50.35764	-4.706538
KS61	HEL	COR	Medium 'jungle' pond	~100 year old pond in jungle garden area, spring-fed, shallow 3 ft	The Lost Gardens Heligan, B3273, Pentewan, Saint Austell PL26 6EN	50.28258	-4.808394
KS62	JAP	COR	Medium pond	Medium pond with bridge, waterfall	The Japanese garden, St Mawgan, Newquay, TR8 4ET	50.45574	-4.99803
KS63	PUX	BRI	Roadside ditch	Shallow ditch banked between road/cow farm, muddy/clay sides, near river	Puxton lane, Puxton, Hewish, Weston-Super-Mare, BS246TA	51.372	-2.851253
KS64	WEM	BRI	Roadside ditch	Some flow and depth, grass banks next to horse farm	Wemberham lane, Yatton	51.38846	-2.835666
KS65	CLA	BRI	Roadside ditch	Shallow ditch with bridge, grass banks next to sheep farm	Claverham drove rhyne, Claverham drove	51.40839	-2.804857
KS66	LAM	BRI	Roadside ditch	Ditch with some depth, no flow, embankment	Lampley rhyne, Clevedon	51.39765	-2.852591
KS67	NAI	BRI	Roadside ditch	Ditch with embankment	Blind yeo, Nailsea wall	51.42396	-2.821461
KS68	VAL	BRI	Medium pond	Stagnant medium sized park pond	Vale pond, Portishead	51.48274	-2.754449
KS69	NEW	BRI	Roadside ditch	Stagnant shallow ditch. Near marsh and motorways	Newlands rhyne	51.50863	-2.656918
KS70	MLA	BRI	Roadside ditch	Muddy ditch with little water, embankment, farm nearby, connects to river via sluice	Moor lane, Bristol	51.56832	-2.58513
KS71	BRA	BRI	Park ponds	Park ponds flowing downhill	Brandon hill, Bristol	51.45379	-2.606958

KS72	TRE	NEW	Roadside ditch	Ditch on side of road, bridge, no flowing water with embankment in industrial estate	Tesco depot, Magor, Newport	51.56992	-2.865184
KS73	LLI	NEW	Large pond	Large pond, open with fishing activity, some flow on one side.	Lliswerry pond, Newport	51.58391	-2.952568
KS74	NAS	NEW	Roadside ditch	Ditch on side of road/reen, half shaded, embanked	Lakes reen, Nash road, Newport	51.56355	-2.948225
KS75	CHA	NEW	Roadside ditch with drain pipe	Reen/ditch with opening, pipe, bridge, old steel works area, near Severn river	Chapel reen, Broad St Common, Whitson, Newport	51.56428	-2.916177
KS76	MAL	NEW	Shallow brook	Minimal flow, section trapped behind permanent lock, higher level, connects to canal network either side	Malpas brook, Bettws, Newport	51.60444	-3.011118
KS77	FOU	NEW	Canal locks	Gentle flow, shallow, section between open and closed locks	Fourteen locks, Cwm lane, Rogerstone	51.59157	-3.040473
KS78	FIV	NEW	Canal locks	Gentle flow, shallow, mud edge sectioned off from main water body, water containing high sediment	Monmouth canal, five locks, Cwmbran	51.66664	-3.031435
KS79	BEL	NEW	Small park pond	Pond, different levels downstream connected by sections and bridge	Belle vue park, Newport	51.57849	-3.000884
KS80	PER	NEW	Ditch system	Reen, Ditch, muti-directions, shallow, gentle flow near edges, some stagnant	Percoed reen, Duffryn way, Duffryn, Newport	51.55498	-3.01977
KS81	HAW	NEW	Roadside ditch	Reen, ditch. Not embanked. Some depth in water, flow, lock/gated mechanism	Percoed reen, Hawse lane, Cardiff	51.55498	-3.01977
KS82	LLA	NEW	Shallow pond	Wooded shallow pond middle of circular road, stagnant, mud sediment.	Llandenis oval, Cardiff	51.51842	-3.177074
KS83	PER	GLA	Shallow pond	Shallow, shaded by reeds/long grass	Smaller perchy pond, Wishaw, Glasgow	55.77158	-3.899133
KS84	CHA	GLA	Park pond	Country park, medium pond, shallow edges, some depth	Chatelherault country park, Hamilton, Glasgow, ML3 7UE	55.76391	-4.014075
KS85	KEL	GLA	Park pond	Medium sized pond, shallow muddy water, island in middle, some flow	Kelvingrove park, Glasgow, G3 7SD	55.8692	-4.284518
KS86	BOT	GLA	Small man-made pond	Small shallow pond, some flow, mostly open	Glasgow botanical garden, Glasgow	55.87847	-4.288534
KS87	FIR	GLA	Canal overflow area	Canal link area with fishing pegs around, some flow, open, near clay pits	Firhill court, Glasgow	55.88263	-4.270283
KS88	MAR	GLA	Canal locks	Lock network, with flow, catchment pool area	Maryhill locks, Glasgow, G4 9SP	55.89368	-4.297603
KS89	VIC	GLA	Large boating lake	Park boating lake with bridge	Victoria Park, Glasgow, G14 9NW	55.87526	-4.332853
KS90	AUC	GLA	Ditch/drain system?	Sunken with bridge, in middle of housing	Auckland Wynd, Glasgow G40 4RN	55.84076	-4.20635

KS91	ROB	GLA	Small pond	Wetlands sunken shallow small pond	Robroyston Park, 220 Robroyston Rd, Glasgow G33 1JQ	55.88944	-4.19682
KS92	BOG	GLA	Marsh	Marsh near housing with embankment	Boghall road, Uddingston, Glasgow, G71	55.84264	-4.11906
KS93	DRU	GLA	Large loch	Large loch in country park	Loch lochend, Drumpellier country park, Coatbridge, ML5 2EH	55.87271	-4.075
KS94	SUM	GLA	Canal system	Canal with some flow, some depth, old steel/iron site	Summerlee industrial museum, Heritage Way, Coatbridge ML5 1QD	55.86568	-4.03001
KS95	SBA	ELG	Woodland ditch	Shaded ditch lined with trees, shallow water	Spey Bay, Fochabers IV32 7PJ	57.66333	-3.06098
KS96	SPO	ELG	Burn (stream)	Stream connecting up and flowing to sea, some flow, some depth, bridge and embankment	Dry Burn, Spey portgorden	57.66286	-3.04523
KS97	ALP	ELG	Large farm pond	Large pond with some depth, inlet/outlet from Mosstowie canal, trees with rill drain system, 20 yr old	Mossend Farm, Mosstowie, Elgin IV30 8TU	57.62975	-3.41362
KS98	FOR	ELG	Medium-sized pond	Shallow sunken bog with embankment	Forres golf course, Forres, IV36 2RD	57.60876	-3.59307
KS99	SAN	ELG	Large lake	Large lake with network of feeders/waterfall. Has island and bridge/jetty	Sanquhar Loch, Forres IV36	57.60191	-3.60725
KS100	BUR	ELG	Large pond	Large open pond, some depth in woodland arboretum garden	Burgie Arboretum Woodland Garden, Burgie Estate, Forres IV36 2QU	57.62021	-3.52381
KS101	MAV	ELG	Small pond	Small pond (apparently fed by other small pond via inlet)	Maverston golf course, Garmouth Road, Elgin IV30 8LR	57.65437	-3.17686
KS102	INN	ELG	Medium pond	Open blue pond, flowing, some depth with an island	Innes pond, Innes house, Lochhill, Elgin IV30 8NG	57.66735	-3.22723
KS103	GOR	ELG	Large pond	Large pond in estate with flow and depth	Gordon Castle Lake, Gordon Castle Estate, Fochabers IV32 7PQ	57.61726	-3.09553
KS104	LNB	ELG	Large loch	Large loch in estate with flow and depth, trees two sides, open	Loch na Bo, Elgin IV30 8QY	57.62752	-3.20042
KS105	MIL	ELG	Large loch	Large lake (with shallow stream attached), some flow, two sides tree-lined other open	Milbuies loch, Elgin	57.5954	-3.270719
KS106	EDG	ELG	Ditch in wetlands	Narrow stream some flow with embankment. Old peat bog now wetlands	Ward's wildlife site, Edgar road, Elgin, IV30	57.64044	-3.319449

KS107	COO	ELG	Medium pond	Medium sized pond, with flow, open pond in park with island	Cooper's park, Elgin, IV30 1HS	57.65186	-3.3146
KS108	TOL	ABE	Medium pond	Medium stagnant pond in woods. Some trees, treefall	Tollohill wood, Aberdeen, AB12 5XN	57.11141	-2.12911
KS109	DEE	ABE	Overflow pond	Pipe inlet/outlet with some flow	Deeside pond, Off station road, Bucksburn, Aberdeen	57.10089	-2.235427
KS110	ALL	ABE	Large pond	Large pond in park with trees around, open one side jetty with low water level, high sediment,	Allan park pond, Park Brae, Cults, Aberdeen, AB15 9HS	57.11361	-2.17897
KS111	HAZ	ABE	Medium pond	Stagnant medium pond covered in plant matter, pipe inlet/outlet	Hazeldene road pond, Hazlehead, Aberdeen, AB15	57.13494	-2.174375
KS112	COU	ABE	Medium pond	Shallow pond for fishing, with inlet, some flow	Couper's pond, 3 Macaulay Gardens, Hazlehead, Aberdeen, AB15 8FN	57.13441	-2.15543
KS113	WEL	ABE	Large pond	Large pond with flow and depth	Wellington road pond, AB12	57.0871	-2.111919
KS114	DUT	ABE	Pond network	Pond network in public park. Linked lakes, stagnant, bridges and islands	The Linked Lakes, Duthie park, Aberdeen, AB11 7BH	57.12937	-2.1056
KS115	DON	ABE	Pollution control dam	Medium-sized dam, near river. Mud/shallow water,middle - grass islands and flow, with sediment	Donside pond, Gordon Brae, Danestone, Aberdeen, AB228BN	57.17694	-2.118509
KS116	BLK	ABE	Medium pond	Medium with flow. Some depth, with sediment, near burn	Blackdog burn pond, Blackdog, Aberdeen	57.21679	-2.072611
KS117	DEN	ABE	Medium pond	Medium pond, stagnant in public park, oil in water	Denman park pond, Westhill AB32	57.15189	-2.27732
KS118	KWS	ABE	Medium pond	Medium pond, stagnant, jetty with low flow	Kingswells pond, Fairley, Aberdeen	57.16412	-2.21807
KS119	POT	ABE	Narrow stream	Narrow, shallow stream/burn, embankment, shallow, low flow.	Potterton park burn, Aberdeen AB23 8UG	57.22992	-2.09853
KS120	KEY	HUL	Ditch	Ditch under bridge. High banks	Keyingham drain, Keyingham	53.71046	-0.152052
MP01	ABB	NCA	Pond	Small garden pond	Wolsey Court South Shields NE34 0QU	54.97439	-1.43347
LY01	TRE	COR	Pond	Medium-sized pond	Trencreek holiday park	50.40796	-5.06188
LY02	SHE	COR	Stream	Slow flowing, fresh steam	Sherford stream, off Sherford road	51.00341	-3.10177
LY03	HOU	COR	Stream	Small stream no flow	Housel bay	49.96474	-5.19621
SS01	SS01	SHA	Garden pond	Garden pond	10 Wolsey Court South Shields NE34 0QU	54.97439	-1.43347

Table S2A. UK accessions and previously characterised clones newly sequenced in this study.

Accession	Site code	Species	Latitude	Longitude	Registered clone	SRA project	SRA sample	SRA clone
KS02	YOR-Lmo-BIS	*Lemna minor*	53.799	1.147	*Lemna minor* 5882	PRJNA1026139	SAMN37735463	SRR26858637
KS03	BFD-Ljp-ALL1	*Lemna japonica*	53.81	1.755	*Lemna japonica* 5883	PRJNA1026139	SAMN37735464	SRR26858636
KS04	YOR-Ljp-SEL	*Lemna japonica*	53.774	1.066	*Lemna japonica* 5884	PRJNA1026139	SAMN37735465	SRR26858625
KS06A	BFD-Lmu-NUF1	*Lemna minuta*	53.83	1.819	*Lemna minuta* 5885	PRJNA1026139	SAMN37735466	SRR26858620
KS06B	BFD-Lmu-NUF2	*Lemna minuta*	53.83	1.819	*Lemna minuta* 5886	PRJNA1026139	SAMN37735467	SRR26858619
KS09	BFD-Lmo-YEA	*Lemna minor*	53.869	1.67	*Lemna minor* 5887	PRJNA1026139	SAMN37735468	SRR26858618
KS12	BFD-Spo-MOO1	*Spirodela polyrhiza*	53.801	1.723	*Spirodela polyrhiza* 5888	PRJNA1026139	SAMN37735469	SRR26858617
KS13	BFD-Lmo-ELD	*Lemna minor*	53.866	1.815	*Lemna minor* 5889	PRJNA1026139	SAMN37735470	SRR26858616
KS14	BFD-Ljp-APP	*Lemna japonica*	53.835	1.711	*Lemna japonica* 5890	PRJNA1026139	SAMN37735471	SRR26858615
KS15	BFD-Ljp-BRE	*Lemna japonica*	53.871	1.597	*Lemna japonica* 5891	PRJNA1026139	SAMN37735472	SRR26858614
KS16	HUL-Ltu-EAS	*Lemna turionifera*	53.766	0.298	*Lemna turionifera* 5892	PRJNA1026139	SAMN37735473	SRR26858635
KS17	HUL-Ljp-PEA	*Lemna japonica*	53.758	0.355	*Lemna japonica* 5893	PRJNA1026139	SAMN37735474	SRR26858634
KS18	HUL-Ljp-CRE	*Lemna japonica*	53.783	0.408	*Lemna japonica* 5894	PRJNA1026139	SAMN37735475	SRR26858633
KS20	HUL-Lmu-BEV	*Lemna minuta*	53.839	0.405	*Lemna minuta* 5895	PRJNA1026139	SAMN37735476	SRR26858632
KS21	HUL-Ljp-WAL	*Lemna japonica*	53.821	0.482	*Lemna japonica* 5896	PRJNA1026139	SAMN37735477	SRR26858631
KS22	HUL-Ltu-NOR	*Lemna turionifera*	53.783	0.642	*Lemna turionifera* 5897	PRJNA1026139	SAMN37735478	SRR26858630
KS25	YOR-Lmu-ESC	*Lemna minuta*	53.875	1.034	*Lemna minuta* 5898	PRJNA1026139	SAMN37735479	SRR26858629
KS27	YOR-Lmo-BUR	*Lemna minor*	53.996	1.132	*Lemna minor* 5899	PRJNA1026139	SAMN37735480	SRR26858628
KS28	YOR-Ljp-HES	*Lemna japonica*	53.979	1.187	*Lemna japonica* 5900	PRJNA1026139	SAMN37735481	SRR26858627
KS29	YOR-Lmo-TAD	*Lemna minor*	53.902	1.281	*Lemna minor* 5901	PRJNA1026139	SAMN37735482	SRR26858626
LY01A	COR-Ljp-TRE	*Lemna japonica*	50.407	-5.061	*Lemna japonica* 5902	PRJNA1026139	SAMN37735483	SRR26858624
LY01B	COR-Lmu-TRE	*Lemna minuta*	50.407	-5.061	*Lemna minuta* 5903	PRJNA1026139	SAMN37735484	SRR26858623
LY02	COR-Ljp-SHE	*Lemna japonica*	51.003	-3.101	*Lemna japonica* 5904	PRJNA1026139	SAMN37735485	SRR26858622
LY03	COR-Ljp-HOU	*Lemna japonica*	49.964	-5.196	*Lemna japonica* 5905	PRJNA1026139	SAMN37735486	SRR26858621
AL01	MID-Lmu-ALP	*Lemna minuta*	52.64	-1.055	NR	PRJNA1030266	NR	SRR27840097
AL02	MID-Lmu-ALD	*Lemna minuta*	52.643	-1.059	NR	PRJNA1030266	NR	SRR27840096
AL03	MID-Lmu-SHR	*Lemna minuta*	52.462	-2.211	NR	PRJNA1030266	NR	SRR27840083
KS100	ELG-Lmo-BUR	*Lemna minor*	57.620	-3.524	NR	PRJNA1030266	NR	SRR27840072

KS101	ELG-Lmo-MAV	*Lemna minor*	57.654	-3.177	NR	PRJNA1030266	NR	SRR27840031
KS104	ELG-Lmo-LNB	*Lemna minor*	57.628	-3.200	NR	PRJNA1030266	NR	SRR27840020
KS107	ELG-Lmo-COO	*Lemna minor*	57.652	-3.315	NR	PRJNA1030266	NR	SRR27840009
KS108	ABE-Lmo-TOL	*Lemna minor*	57.111	-2.129	NR	PRJNA1030266	NR	SRR27839998
KS109	ABE-Lmo-DEE	*Lemna minor*	57.101	-2.235	NR	PRJNA1030266	NR	SRR27840053
KS110	ABE-Lmo-ALL	*Lemna minor*	57.114	-2.179	NR	PRJNA1030266	NR	SRR27840042
KS111	ABE-Lmo-HAZ	*Lemna minor*	57.135	-2.174	NR	PRJNA1030266	NR	SRR27840095
KS112	ABE-Lmo-COU	*Lemna minor*	57.134	-2.155	NR	PRJNA1030266	NR	SRR27840092
KS114	ABE-Lmo-DUT	*Lemna minor*	57.129	-2.106	NR	PRJNA1030266	NR	SRR27840091
KS115	ABE-Lmo-DON	*Lemna minor*	57.177	-2.119	NR	PRJNA1030266	NR	SRR27840090
KS116	ABE-Lmo-BLK	*Lemna minor*	57.217	-2.073	NR	PRJNA1030266	NR	SRR27840089
KS117	ABE-Lmo-DEN	*Lemna minor*	57.152	-2.277	NR	PRJNA1030266	NR	SRR27840088
KS118	ABE-Lmo-KWS	*Lemna minor*	57.164	-2.218	NR	PRJNA1030266	NR	SRR27840087
KS119	ABE-Lmo-POT	*Lemna minor*	57.230	-2.099	NR	PRJNA1030266	NR	SRR27840086
KS33	LAN-Lmo-SIL	*Lemna minor*	54.169	-2.799	NR	PRJNA1030266	NR	SRR27840085
KS34	LAN-Lmo-CAR	*Lemna minor*	54.125	-2.771	NR	PRJNA1030266	NR	SRR27840084
KS38	MID-Lmu-ELV	*Lemna minuta*	52.895	-1.396	NR	PRJNA1030266	NR	SRR27840082
KS39	MID-Lmo-LIM	*Lemna minor*	52.811	-1.466	NR	PRJNA1030266	NR	SRR27840081
KS40A	MID-Ljp-CAL	*Lemna japonica*	52.800	-1.453	*Lemna japonica* 5942	PRJNA1030266	NR	SRR27840080
KS42	HAS-Lmu-HEL	*Lemna minuta*	50.881	0.582	NR	PRJNA1030266	NR	SRR27840079
KS43	HAS-Lmo-GIL	*Lemna minor*	50.865	0.543	NR	PRJNA1030266	NR	SRR27840078
KS44A	HAS-Lmo-LAN	*Lemna minor*	50.798	0.305	NR	PRJNA1030266	NR	SRR27840077
KS44B	HAS-Lmu-LAN	*Lemna minuta*	50.798	0.305	NR	PRJNA1030266	NR	SRR27840076
KS45	HAS-Lmu-WIL	*Lemna minuta*	50.801	0.289	NR	PRJNA1030266	NR	SRR27840075
KS46B	HAS-Lmu/Lmo-WHE	*Lmu/Lmo*	50.917	0.562	*Lemna minor/minuta* hybrid 5943	PRJNA1030266	NR	SRR27840074
KS47	HAS-Lmo-BRE	*Lemna minor*	50.943	0.562	NR	PRJNA1030266	NR	SRR27840073
KS48A	HAS-Lmo-UDI	*Lemna minor*	50.941	0.652	NR	PRJNA1030266	NR	SRR27840071
KS48B	HAS-Lmu-UDI	*Lemna minuta*	50.941	0.652	NR	PRJNA1030266	NR	SRR27840070
KS49	HAS-Lmu-MIL	*Lemna minuta*	51.023	0.783	NR	PRJNA1030266	NR	SRR27840069
KS50	HAS-Lmo-CRO	*Lemna minor*	50.899	0.520	NR	PRJNA1030266	NR	SRR27840068
KS51A	COR-Lmo-COR	*Lemna minor*	50.941	0.652	NR	PRJNA1030266	NR	SRR27840067

KS51B	COR-Lmu/Lmo-COR	*Lmu/Lmo*	50.941	0.652	*Lemna minor/minuta* hybrid 5944	PRJNA1030266	NR	SRR27840066
KS52	COR-Lmo-KWO	*Lemna minor*	50.314	-4.799	NR	PRJNA1030266	NR	SRR27840065
KS53	COR-Lmo-GRA	*Lemna minor*	50.299	-4.905	NR	PRJNA1030266	NR	SRR27840064
KS54	COR-Lmo-MEN	*Lemna minor*	50.346	-4.796	NR	PRJNA1030266	NR	SRR27840063
KS55	COR-Lmo-COM	*Lemna minor*	50.334	-4.879	NR	PRJNA1030266	NR	SRR27840062
KS56	COR-Lmo-TRG	*Lemna minor*	50.355	-4.884	NR	PRJNA1030266	NR	SRR27840030
KS57	COR-Lmo-TRW	*Lemna minor*	50.291	-4.932	NR	PRJNA1030266	NR	SRR27840029
KS58B	COR-Lmu/Lmo-PIN	*Lmu/Lmo*	50.342	-4.751	*Lemna minor/minuta* hybrid 5945	PRJNA1030266	NR	SRR27840028
KS59	COR-Lmo-INN	*Lemna minor*	50.378	-4.766	NR	PRJNA1030266	NR	SRR27840027
KS60	COR-Lmo-AND	*Lemna minor*	50.358	-4.707	NR	PRJNA1030266	NR	SRR27840026
KS61A	COR-Lmo-HEL	*Lemna minor*	50.283	-4.808	NR	PRJNA1030266	NR	SRR27840025
KS61B	COR-Lmu-HEL	*Lemna minuta*	50.283	-4.808	NR	PRJNA1030266	NR	SRR27840024
KS62	COR-Ljp-JAP	*Lemna japonica*	50.456	-4.998	*Lemna japonica* 5946	PRJNA1030266	NR	SRR27840023
KS63	BRI-Lmu/Lmo-PUX	*Lmu/Lmo*	51.372	-2.851	*Lemna minor/minuta* hybrid 5947	PRJNA1030266	NR	SRR27840022
KS64B	BRI-Lmu-WEM	*Lemna minuta*	51.388	-2.836	NR	PRJNA1030266	NR	SRR27840021
KS65A	BRI-Ljp-CLA1	*Lemna japonica*	51.408	-2.805	*Lemna japonica* 5948	PRJNA1030266	NR	SRR27840019
KS65B	BRI-Ljp-CLA2	*Lemna japonica*	51.408	-2.805	*Lemna japonica* 5949	PRJNA1030266	NR	SRR27840018
KS66A	BRI-Ljp-LAM	*Lemna japonica*	51.398	-2.853	*Lemna japonica* 5906	PRJNA1074359	SAMN39856635	SRR27937329
KS66B	BRI-Lgi-LAM	*Lemna gibba*	51.398	-2.853	*Lemna gibba* 5932	PRJNA1030266	NR	SRR27840017
KS66C	BRI-Lmu-LAM	*Lemna minuta*	51.398	-2.853	NR	PRJNA1030266	NR	SRR27840016
KS67A	BRI-Lmo-NAI	*Lemna minor*	51.424	-2.821	NR	PRJNA1030266	NR	SRR27840015
KS67B	BRI-Lmu-NAI	*Lemna minuta*	51.424	-2.821	NR	PRJNA1030266	NR	SRR27840014
KS68A	BRI-Ljp-VAL	*Lemna japonica*	51.483	-2.754	*Lemna japonica* 5950	PRJNA1030266	NR	SRR27840013
KS68B	BRI-Lmu/Lmo-VAL	*Lmu/Lmo*	51.483	-2.754	*Lemna minor/minuta* hybrid 5951	PRJNA1030266	NR	SRR27840012
KS69	BRI-Lmu-NEW	*Lemna minuta*	51.509	-2.657	NR	PRJNA1030266	NR	SRR27840011
KS70	BRI-Ljp-MLA	*Lemna japonica*	51.568	-2.585	*Lemna japonica* 5952	PRJNA1030266	NR	SRR27840010
KS72A	NEW-Ljp-TRE	*Lemna japonica*	51.454	-2.607	*Lemna japonica* 5953	PRJNA1030266	NR	SRR27840008
KS72B	NEW-Lmu-TRE	*Lemna minuta*	51.454	-2.607	NR	PRJNA1030266	NR	SRR27840007
KS73	NEW-Lmo-LLI	*Lemna minor*	51.570	-2.865	NR	PRJNA1030266	NR	SRR27840006

KS74A	NEW-Ljp-NAS	*Lemna japonica*	51.584	-2.953	*Lemna japonica* 5954	PRJNA1030266	NR	SRR27840005
KS74B	NEW-Lmu/Lmo-NAS	*Lmu/Lmo*	51.584	-2.953	*Lemna minor/minuta* hybrid 5955	PRJNA1030266	NR	SRR27840004
KS75A	NEW-Ljp-CHA	*Lemna japonica*	51.564	-2.948	*Lemna japonica* 5956	PRJNA1030266	NR	SRR27840003
KS75B	NEW-Lmu-CHA	*Lemna minuta (unk)*	51.564	-2.948	NR	PRJNA1030266	NR	SRR27840002
KS76A	NEW-Lgi-MAL	*Lemna gibba*	51.564	-2.916	*Lemna gibba* 5933	PRJNA1030266	NR	SRR27840001
KS76B	NEW-Lmu-MAL	*Lemna minuta*	51.564	-2.916	NR	PRJNA1030266	NR	SRR27840000
KS77A	NEW-Spo-FOU	*Spirodela polyrhiza*	51.604	-3.011	*Spirodela polyrhiza* 5907	PRJNA1074359	SAMN39856636	SRR27937328
KS77B	NEW-Lmo-FOU	*Lemna minor*	51.604	-3.011	NR	PRJNA1030266	NR	SRR27839999
KS77C	NEW-Lmu-FOU	*Lemna minuta*	51.604	-3.011	NR	PRJNA1030266	NR	SRR27839997
KS78A	NEW-Spo-FIV	*Spirodela polyrhiza*	51.592	-3.040	*Spirodela polyrhiza* 5908	PRJNA1074359	SAMN39856637	SRR27937327
KS80A	NEW-Lmo-PER	*Lemna minor*	51.667	-3.031	NR	PRJNA1030266	NR	SRR27839996
KS80B	NEW-Lmu-PER	*Lemna minuta*	51.667	-3.031	NR	PRJNA1030266	NR	SRR27840061
KS81	NEW-Lmo-HAW	*Lemna minor*	51.578	-3.001	NR	PRJNA1030266	NR	SRR27840060
KS82A	NEW-Lgi-LLA1	*Lemna gibba*	51.555	-3.020	*Lemna gibba* 5934	PRJNA1030266	NR	SRR27840059
KS82B	NEW-Lmo-LLA2	*Lemna minor*	51.555	-3.020	NR	PRJNA1030266	NR	SRR27840058
KS83	GLA-Lmo-PER	*Lemna minor*	51.555	-3.020	NR	PRJNA1030266	NR	SRR27840057
KS85	GLA-Lmu/Lmo-KEL	*Lmu/Lmo*	51.518	-3.177	*Lemna minor/minuta* hybrid 5957	PRJNA1030266	NR	SRR27840056
KS88	GLA-Lmo-MAR	*Lemna minor*	55.772	-3.899	NR	PRJNA1030266	NR	SRR27840055
KS91	GLA-Lmo-ROB	*Lemna minor*	55.764	-4.014	NR	PRJNA1030266	NR	SRR27840054
KS92B	GLA-Lmu-BOG	*Lemna minuta*	55.869	-4.285	NR	PRJNA1030266	NR	SRR27840052
KS95	ELG-Lmo-SBA	*Lemna minor*	55.878	-4.289	NR	PRJNA1030266	NR	SRR27840051
KS97A	ELG-Ltr-ALP	*Lemna trisulca*	55.883	-4.270	*Lemna trisulca* 5935	PRJNA1030266	NR	SRR27840050
KS97B	ELG-Lmo-ALP	*Lemna minor*	55.894	-4.298	NR	PRJNA1030266	NR	SRR27840049
KS98	ELG-Lmo-FOR	*Lemna minor*	55.875	-4.333	NR	PRJNA1030266	NR	SRR27840048
KS99	ELG-Lmo-SAN	*Lemna minor*	55.841	-4.206	NR	PRJNA1030266	NR	SRR27840047
KSALL2	BFD-Ljp-ALL2	*Lemna japonica*	55.889	-4.197	*Lemna japonica* 5958	PRJNA1030266	NR	SRR27840046
KSAPP1	BFD-Ltr-APP	*Lemna trisulca*	55.843	-4.119	*Lemna trisulca* 5936	PRJNA1030266	NR	SRR27840045
KSAPP2	BFD-Spo-APP	*Spirodela polyrhiza*	55.873	-4.075	*Spirodela polyrhiza* 5937	PRJNA1030266	NR	SRR27840044
KSKEY	HUL-Ljp-KEY	*Lemna japonica*	55.866	-4.030	*Lemna japonica* 5959	PRJNA1030266	NR	SRR27840043
KSMOOR1	BFD-Spo-MOO2	*Spirodela polyrhiza*	57.663	-3.061	*Spirodela polyrhiza* 5938	PRJNA1030266	NR	SRR27840041

KSMOOR2	BFD-Ljp-MOO	*Lemna japonica*	57.663	-3.045	*Lemna japonica* 5960	PRJNA1030266	NR	SRR27840040
KSNOR1	HUL-Ltr-NOR	*Lemna trisulca*	57.630	-3.414	*Lemna trisulca* 5939	PRJNA1030266	NR	SRR27840039
KSNUF3	BFD-Lmo-NUF3	*Lemna minor*	57.609	-3.593	*Lemna minor* 5909	PRJNA1074359	SAMN39856638	SRR27937326
KSSEL1	YOR-Lgi-SEL	*Lemna gibba*	57.602	-3.607	*Lemna gibba* 5940	PRJNA1030266	NR	SRR27840038
KSYEAD1	BFD-Ltr-YEA	*Lemna trisulca*	53.869	1.670	*Lemna trisulca* 5941	PRJNA1030266	NR	SRR27840037
MP01	NCA-Ljp-MPO	*Lemna japonica*	51.015	-1.473	*Lemna japonica* 5961	PRJNA1030266	NR	SRR27840094
Clone	**Site code**	**Species**	**Latitude**	**Longitude**	**Registered clone**	**SRA project**	**SRA sample**	**SRA clone**
L. japonica 9250	WW-Lja9250	*Lemna japonica*			*Lemna japonica* 9250	PRJNA1030266	NR	SRR27840036
L. minuta 9260	WW-Lmu9260	*Lemna minuta*			*Lemna minuta* 9260	PRJNA1030266	NR	SRR27840035
L. punctata 0049	WW-Lpu0049	*Lemna punctata*			*Lemna punctata* 0049	PRJNA1030266	NR	SRR27840034
L. trisulca 7192	WW-Ltr7192	*Lemna trisulca*			*Lemna trisulca* 7192	PRJNA1030266	NR	SRR27840033
L. yungensis 9208	WW-Lyu9208	*Lemna yungensis*			*Lemna yungensis* 9208	PRJNA1030266	NR	SRR27840032
S. intermedia 9394	WW-Sin9394	*Spirodela intermedia*			*Spirodela intermedia* 9394	PRJNA1030266	NR	SRR27840093
L. japonica 7123	WW-Lja7123	*Lemna japonica*			*Lemna japonica* 7123			
L. minor 7295	WW-Lmo7295	*Lemna minor*			*Lemna minor* 7295			
L. minor 8389	WW-Lmo8389	*Lemna minor*			*Lemna minor* 8389			
L.minuta 6600	WW-Lmu6600	*Lemna minuta*			*Lemna minuta* 6600			

Registered clones refers to new accession submission on ruduckweeds.org. SRA refers to submission of genomes on Short read archive (SRA) with project, sample and individual codes for new accessions. Blue colouration refers to clones already characterised and registered. *NR - not released.

Table S2B. Genomes of duckweed clones downloaded from the Short Read Archive (SRA) and included in the genomic pipeline. Clones with < 5% coverage were removed from further analysis indicated in red.

Clone	SRA code	Number of reads	Percentage covered	Mean coverage
L. minor 7016	SRR10958777	8088768	86.04	55.7
L. minor 5500	SRR10958800	5970720	86.81	40.1
L. turionifera 6002	SRR23943402	1335194	46.41	7.27
L. gibba 131	SRR074103	991234	14.71	3.02
L. turionifera 9434	SRR8291590	22769	1.495	0.117
L. minuta 9484	SRR8291594	8920	0.1448	0.0371
L. minuta 6717	SRR8291596	8571	0.3825	0.034
L. minuta 7612	SRR8291595	3745	0.1921	0.0151
L. minuta 9581	SRR8291593	848	0.08327	0.00342
S. polyrhiza 9504	SRR11472010	45	0.01389	0.000146
S. intermedia 8410	ERR3957957	0	0	0

Table S2C. Genomes of new UK duckweed accessions and newly sequenced clones included in the genomic pipeline.

Accession	Site code	Species	Number of reads	Percentage covered	Mean coverage
KS02	YOR-Lmo-BIS	*Lemna minor*	792908	77.39	5.58
KS03	BFD-Ljp-ALL1	*Lemna japonica*	1642080	83.25	11.1
KS04	YOR-Ljp-SEL	*Lemna japonica*	2931134	85.41	19.6
KS06A	BFD-Lmu-NUF1	*Lemna minuta*	523797	10.35	1.53
KS06B	BFD-Lmu-NUF2	*Lemna minuta*	310907	8.41	0.98
KS09	BFD-Lmo-YEA	*Lemna minor*	1156648	79.24	8.52
KS12	BFD-Spo-MOO1	*Spirodela polyrhiza*	757884	9.833	1.98
KS13	BFD-Lmo-ELD	*Lemna minor*	1376941	81.05	10.2
KS14	BFD-Ljp-APP	*Lemna japonica*	3021601	85.79	21.2
KS15	BFD-Ljp-BRE	*Lemna japonica*	1479101	78.65	10
KS16	HUL-Ltu-EAS	*Lemna turionifera*	1081370	44.87	5.85

KS17	HUL-Ljp-PEA	*Lemna japonica*	1218293	81.99	8.21
KS18	HUL-Ljp-CRE	*Lemna japonica*	1097315	81.09	7.35
KS20	HUL-Lmu-BEV	*Lemna minuta*	347620	8.397	1.1
KS21	HUL-Ljp-WAL	*Lemna japonica*	1256535	79.44	8.49
KS22	HUL-Ltu-NOR	*Lemna turionifera*	3241725	53.42	17.1
KS25	YOR-Lmu-ESC	*Lemna minuta*	307614	8.329	0.97
KS27	YOR-Lmo-BUR	*Lemna minor*	1303766	79.93	9.68
KS28	YOR-Ljp-HES	*Lemna japonica*	1386609	82.31	9.33
KS29	YOR-Lmo-TAD	*Lemna minor*	2698291	82.75	19.9
LY01A	COR-Ljp-TRE	*Lemna japonica*	1520377	82.41	10.3
LY01B	COR-Lmu-TRE	*Lemna minuta*	431435	17.74	1.35
LY02	COR-Ljp-SHE	*Lemna japonica*	1517640	82.82	10.3
LY03	COR-Ljp-HOU	*Lemna japonica*	1259293	82.07	8.5
AL01	MID-Lmu-ALP	*Lemna minuta*	422763	14.06	1.37
AL02	MID-Lmu-ALD	*Lemna minuta*	822894	14.87	2.39
AL03	MID-Lmu-SHR	*Lemna minuta*	488595	10.95	1.31
KS100	ELG-Lmo-BUR	*Lemna minor*	4631474	85.03	32.9
KS101	ELG-Lmo-MAV	*Lemna minor*	1277761	79.99	8.92
KS104	ELG-Lmo-LNB	*Lemna minor*	4572537	85.29	32.6
KS107	ELG-Lmo-COO	*Lemna minor*	1565440	81.24	11.5
KS108	ABE-Lmo-TOL	*Lemna minor*	2318986	82.2	16.5
KS109	ABE-Lmo-DEE	*Lemna minor*	1414233	80.65	10.4
KS110	ABE-Lmo-ALL	*Lemna minor*	2433634	83.78	17.2
KS111	ABE-Lmo-HAZ	*Lemna minor*	2567984	84.07	18.4
KS112	ABE-Lmo-COU	*Lemna minor*	2991000	83.97	21.2
KS114	ABE-Lmo-DUT	*Lemna minor*	1426163	80.84	10.4
KS115	ABE-Lmo-DON	*Lemna minor*	2522849	82.2	14
KS116	ABE-Lmo-BLK	*Lemna minor*	1705583	81.43	12.4
KS117	ABE-Lmo-DEN	*Lemna minor*	476228	69.11	3.31
KS118	ABE-Lmo-KWS	*Lemna minor*	2431844	82.86	17.4
KS119	ABE-Lmo-POT	*Lemna minor*	2685678	82.97	19.1

KS33	LAN-Lmo-SIL	*Lemna minor*	4875223	84.66	34.9
KS34	LAN-Lmo-CAR	*Lemna minor*	2629542	82.95	18.8
KS38	MID-Lmu-ELV	*Lemna minuta*	468179	10.76	0.981
KS39	MID-Lmo-LIM	*Lemna minor*	3126447	83.73	22.3
KS40A	MID-Ljp-CAL	*Lemna japonica*	2437207	84.63	15.8
KS42	HAS-Lmu-HEL	*Lemna minuta*	478863	10.61	1.26
KS43	HAS-Lmo-GIL	*Lemna minor*	133422	38.44	0.948
KS44A	HAS-Lmo-LAN	*Lemna minor*	1539729	80.97	11.2
KS44B	HAS-Lmu-LAN	*Lemna minuta*	387567	9.72	0.997
KS45	HAS-Lmu-WIL	*Lemna minuta*	512705	11.29	1.25
KS46B	HAS-Lmu/Lmo-WHE	*Lmu/Lmo*	638159	27.42	2.1
KS47	HAS-Lmo-BRE	*Lemna minor*	2934396	83.88	21
KS48A	HAS-Lmo-UDI	*Lemna minor*	1415382	80.9	10.4
KS48B	HAS-Lmu-UDI	*Lemna minuta*	475833	10.7	1.26
KS49	HAS-Lmu-MIL	*Lemna minuta*	2181308	16.26	6.04
KS50	HAS-Lmo-CRO	*Lemna minor*	5379121	84.93	38.5
KS51A	COR-Lmo-COR	*Lemna minor*	1232844	80.13	8.95
KS51B	COR-Lmu/Lmo-COR	*Lmu/Lmo*	770044	31.26	2.53
KS52	COR-Lmo-KWO	*Lemna minor*	1968260	82.06	14
KS53	COR-Lmo-GRA	*Lemna minor*	1111904	79.85	8.19
KS54	COR-Lmo-MEN	*Lemna minor*	1680320	81.39	12.2
KS55	COR-Lmo-COM	*Lemna minor*	2743462	82.8	19.4
KS56	COR-Lmo-TRG	*Lemna minor*	2965827	83.23	21.2
KS57	COR-Lmo-TRW	*Lemna minor*	292621	61	2.1
KS58B	COR-Lmu/Lmo-PIN	*Lmu/Lmo*	2382832	48.82	7.17
KS59	COR-Lmo-INN	*Lemna minor*	3084228	83.49	22
KS60	COR-Lmo-AND	*Lemna minor*	2448660	82.8	17.5
KS61A	COR-Lmo-HEL	*Lemna minor*	1262225	80.47	9.28
KS61B	COR-Lmu-HEL	*Lemna minuta*	896527	12.23	2.62
KS62	COR-Ljp-JAP	*Lemna japonica*	4375540	86.27	28.3
KS63	BRI-Lmu/Lmo-PUX	*Lmu/Lmo*	517792	69.35	3.28

KS64B	BRI-Lmu-WEM	*Lemna minuta*	612677	10.88	1.76
KS65A	BRI-Ljp-CLA1	*Lemna japonica*	1530601	82.31	10.1
KS65B	BRI-Ljp-CLA2	*Lemna japonica*	1148531	80.93	7.24
KS66A	BRI-Ljp-LAM	*Lemna japonica*	1545977	82.11	10.1
KS66B	BRI-Lgi-LAM	*Lemna gibba*	1595583	78.26	8.37
KS66C	BRI-Lmu-LAM	*Lemna minuta*	450640	10.31	1.18
KS67A	BRI-Lmo-NAI	*Lemna minor*	5566145	85.18	39.5
KS67B	BRI-Lmu-NAI	*Lemna minuta*	711880	21.92	2.1
KS68A	BRI-Ljp-VAL	*Lemna japonica*	2207580	83.95	14.3
KS68B	BRI-Lmu/Lmo-VAL	*Lmu/Lmo*	874414	34.97	2.87
KS69	BRI-Lmu-NEW	*Lemna minuta*	107502	4.84	0.249
KS70	BRI-Ljp-MLA	*Lemna japonica*	1142971	81.1	7.54
KS72A	NEW-Ljp-TRE	*Lemna japonica*	1975400	83.5	12.9
KS72B	NEW-Lmu-TRE	*Lemna minuta*	694894	12.55	1.99
KS73	NEW-Lmo-LLI	*Lemna minor*	2339786	82.41	16.7
KS74A	NEW-Ljp-NAS	*Lemna japonica*	1622319	82.74	10.8
KS74B	NEW-Lmu/Lmo-NAS	*Lmu/Lmo*	154863	14.14	0.5
KS75A	NEW-Ljp-CHA	*Lemna japonica*	3081153	85.17	20
KS75B	NEW-Lmu-CHA	*Lemna minuta (unk)*	0	0	0
KS76A	NEW-Lgi-MAL	*Lemna gibba*	1907267	81.51	13.8
KS76B	NEW-Lmu-MAL	*Lemna minuta*	540172	10.62	1.39
KS77A	NEW-Spo-FOU	*Spirodela polyrhiza*	1108382	9.252	3.13
KS77B	NEW-Lmo-FOU	*Lemna minor*	1377004	80.58	10
KS77C	NEW-Lmu-FOU	*Lemna minuta*	501751	10.68	1.31
KS78A	NEW-Spo-FIV	*Spirodela polyrhiza*	433303	7.868	0.981
KS80A	NEW-Lmo-PER	*Lemna minor*	1635862	81	11.7
KS80B	NEW-Lmu-PER	*Lemna minuta*	442018	10.4	1.11
KS81	NEW-Lmo-HAW	*Lemna minor*	2124835	82.71	15
KS82A	NEW-Lgi-LLA1	*Lemna gibba*	158496	18.32	0.539
KS82B	NEW-Lmo-LLA2	*Lemna minor*	6588240	85.58	47
KS83	GLA-Lmo-PER	*Lemna minor*	1231037	80.29	9.06

KS85	GLA-Lmu/Lmo-KEL	*Lmu/Lmo*	1702567	61.17	5.92
KS88	GLA-Lmo-MAR	*Lemna minor*	2008275	81.87	14.3
KS91	GLA-Lmo-ROB	*Lemna minor*	1334701	80.4	9.76
KS92B	GLA-Lmu-BOG	*Lemna minuta*	548954	10.72	1.5
KS95	ELG-Lmo-SBA	*Lemna minor*	3365699	83.7	24.2
KS97A	ELG-Ltr-ALP	*Lemna trisulca*	1800983	54.7	9.33
KS97B	ELG-Lmo-ALP	*Lemna minor*	2545383	82.56	18.2
KS98	ELG-Lmo-FOR	*Lemna minor*	3199862	83.85	22.8
KS99	ELG-Lmo-SAN	*Lemna minor*	2914512	83.91	20.6
KSALL2	BFD-Ljp-ALL2	*Lemna japonica*	2570836	84.94	16.8
KSAPP1	BFD-Ltr-APP	*Lemna trisulca*	4168011	54.81	21.8
KSAPP2	BFD-Spo-APP	*Spirodela polyrhiza*	301646	6.988	0.663
KSKEY	HUL-Ljp-KEY	*Lemna japonica*	2756002	85.16	17.9
KSMOOR1	BFD-Spo-MOO2	*Spirodela polyrhiza*	430447	7.667	1.05
KSMOOR2	BFD-Ljp-MOO	*Lemna japonica*	1692380	82.96	11.1
KSNOR1	HUL-Ltr-NOR	*Lemna trisulca*	1809715	72.17	9.87
KSNUF3	BFD-Lmo-NUF3	*Lemna minor*	1306180	80.57	9.42
KSSEL1	YOR-Lgi-SEL	*Lemna gibba*	991069	73.64	5.13
KSYEAD1	BFD-Ltr-YEA	*Lemna trisulca*	1936634	50.48	10.2
MP01	NCA-Ljp-MPO	*Lemna japonica*	1329520	81.86	8.84

Clone	**Site code**	**Species**	**Number of reads**	**Percentage covered**	**Mean coverage**
L. japonica 9250	WW-Lja9250	*Lemna japonica*	3200303	84.32	21.3
L. minuta 9260	WW-Lmu9260	*Lemna minuta*	526680	10.8	1.38
L. punctata 0049	WW-Lpu0049	*Lemna punctata*	510974	23.93	1.75
L. trisulca 7192	WW-Ltr7192	*Lemna trisulca*	1374501	45.81	7.09
L. yungensis 9208	WW-Lyu9208	*Lemna yungensis*	1130919	14.93	2.94
S. intermedia 9394	WW-Sin9394	*Spirodela intermedia*	274124	6.735	0.562
L. japonica 7123	WW-Lja7123	*Lemna japonica*	2844367	83.47	17.9
L. minor 7295	WW-Lmo7295	*Lemna minor*	3996123	85.25	26.7
L. minor 8389	WW-Lmo8389	*Lemna minor*	5044832	86.09	33.8
L.minuta 6600	WW-Lmu6600	*Lemna minuta*	1164164	14.32	2.87

Table S3. UK duckweed species show differences between tissue concentrations of elements grown in replete N-medium. Four UK duckweed species are included in the table rows and columns, with the intersections marking significant differences between species. Red indicates the species in the row has higher accumulation, green indicates the species in the column has higher accumulation of that element. Kruskal-Wallis and Dunn's post-hoc tests were used with significance set at P=<0.05. All non-significant relationships have been removed for clarity.

A.

Mg	*L. minor*	*L. japonica*	*L. minuta*
L. japonica			
L. minuta	0.0000	0.0000	
Lmu/Lmo	0.0027	0.0000	

B.

K	*L. minor*	*L. japonica*	*L. minuta*
L. japonica	0.0065		
L. minuta		0.0066	
Lmu/Lmo		0.0038	

C.

Ca	*L. minor*	*L. japonica*	*L. minuta*
L. japonica			
L. minuta		0.0402	
Lmu/Lmo			

D.

Si	*L. minor*	*L. japonica*	*L. minuta*
L. japonica	0.0127		
L. minuta			
Lmu/Lmo			

E.

Mn	*L. minor*	*L. japonica*	*L. minuta*
L. japonica			
L. minuta			
Lmu/Lmo		0.0187	

F.

Mo	*L. minor*	*L. japonica*	*L. minuta*
L. japonica	0.0102		
L. minuta			
Lmu/Lmo			

G.

As	*L. minor*	*L. japonica*	*L. minuta*
L. japonica	0.05		
L. minuta			
Lmu/Lmo			

Table S4. Duckweed species show differences between typical elemental concentrations found in water habitats. Four UK duckweed species are included in the table rows and columns, with the intersections marking significant differences between species. Red indicates the species in the row has higher presence of that element in its water habitats, green indicates the species in the column has higher presence in its water habitats. Kruskal-Wallis and Dunn's post-hoc tests were used with significance set at P=<0.05. All non-significant relationships have been removed for clarity.

A.

Mg	*L. minor*	*L. japonica*	*L. minuta*
L. japonica			
L. minuta	0.046		
Lmu/Lmo			

B.

P	*L. minor*	*L. japonica*	*L. minuta*
L. japonica	0.0016		
L. minuta			
Lmu/Lmo			

C.

S	*L. minor*	*L. japonica*	*L. minuta*
L. japonica			
L. minuta	0.0194		
Lmu/Lmo			

D

Ca	*L. minor*	*L. japonica*	*L. minuta*
L. japonica	0.0019		
L. minuta	0.0003		
Lmu/Lmo			

E

B	*L. minor*	*L. japonica*	*L. minuta*
L. japonica	0.0081		
L. minuta	0.0039		
Lmu/Lmo			

F

Mo	*L. minor*	*L. japonica*	*L. minuta*
L. japonica	0.001		
L. minuta	0.0163		
Lmu/Lmo			

G

Sr	*L. minor*	*L. japonica*	*L. minuta*
L. japonica	0.0156		
L. minuta	0.0032		
Lmu/Lmo			

H

As	*L. minor*	*L. japonica*	*L. minuta*
L. japonica	0.0007		
L. minuta			
Lmu/Lmo			

I

Al	*L. minor*	*L. japonica*	*L. minuta*
L. japonica	0.0033		
L. minuta	0.044		
Lmu/Lmo			

Table S5. Range of internal duckweed concentrations of eight elements grown in standard replete nutrient conditions. A subset of macronutrients and toxic elemental concentrations measured in 116 duckweed accessions (in mg/kg) by ICP-MS.

(mg/kg)	**Mg**	**Ca**	**S**	**K**	**Si**	**Mn**	**Fe**	**Pb**
Min conc.	2097.29	2981.19	443.97	3660.63	0.00	3.54	70.56	0.00
Max conc.	9396.18	18703	21430	11038	1045.91	2494.01	1139.76	12.98
Fold change	3.5x	5.3x	43.7x	29.2x	11.0x	703x	15.2x	191x

Table S6. Spatial variation of eight elements found in UK water sites. A subset of macronutrients and potentially toxic elements measured in 100 UK duckweed water sampling sites. Concentrations are measured in µg/L by ICP-MS and given to 2 dp. Limits for Mn, Fe and Pb were obtained from drinking water standards (Rohlich, 1979).

(µg/L)	**Mg**	**Ca**	**S**	**K**	**Si**	**Mn**	**Fe**	**Pb**
Min conc.	1286.12	8.58	43.20	633.68	56.38	0.32	5.08	0.01
Max conc.	74149	231550	101887	305299	15731	2178	11610	6.44
Limit						**50**	**200**	**10**
Fold change	56x	2285x	2357x	2285x	278x	6807x	2285x	437x

Table S7. Seasonal variation of eight elements in UK water sites. * A subset of macronutrients and potentially toxic elements measured in 19 UK water sites at four seasonal time points. Concentrations are measured in µg/L by ICP-MS and given to 2 dp. Limits for Mn, Fe and Pb were obtained from drinking water standards (Rohlich, 1979).

(µg/L)	**Mg**	**Ca**	**S**	**K**	**Si**	**Mn**	**Fe**	**Pb**
Min conc.	1546.88	8.58	43.20	680.39	56.38	0.07	0.56	0.03
Max conc.	43199	142369	101887	63130	13229	1122.27	3123.74	4.79
Limit						**50**	**200**	**10**
Fold change	28x	16586x	2357x	93x	235x	14470x	5584x	158x

4. Chapter four: Physiological adaptation to irradiance in duckweeds is species and accession specific and depends on light habitat niche

Preface: As one of the fastest growing plants in the world, the duckweed growth and physiological responses to light are intriguing. Despite this, light variation in duckweed environments, its spatial and temporal variation and local adaptation have not been considered widely. Moreover, species differences in light responses are rarely compared using the same light conditions and environmental variables or are limited in numbers of accessions and species used to make firm conclusions. For commercial growing there is a need to maximise growth and dietary value of duckweeds using light. Using a subset of UK duckweed species and accessions together with their originating light habitats can inform variation in duckweed light tolerance. Subjecting these newly characterised accessions to a controlled high light environment was used to understand light acclimation, particularly the relationships between growth, photosynthesis and photopigments. The effects of species, ecotypes and their originating environments were determined for light acclimation responses.

Aims: To characterise the range of light variation seasonally and spatially between duckweed UK native habitats. To determine if increased artificial light irradiation can increase growth and nutritional potential of accessions in small-scale duckweed cultivation. To measure the physiological traits associated with high and low light tolerant duckweed accessions. To report optimal species, accessions and light regimes for commercialised growing of duckweeds in vertical farms or space horticulture applications.

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RESEARCH PAPER

Physiological adaptation to irradiance in duckweeds is species and accession specific and depends on light habitat niche

Kellie E. Smith[1,2,*], Laura Cowan[2], Beth Taylor[1], Lorna McAusland[1], Matthew Heatley[2], Levi Yant[2], and Erik H. Murchie[1]

[1] Division of Plant and Crop Sciences, School of Biosciences, University of Nottingham, Sutton Bonington LE12 5RD, UK
[2] School of Life Sciences, University of Nottingham, Nottingham NG7 2RD, UK

[*] Correspondence: kellie.smith@nottingham.ac.uk

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Abstract

Duckweeds span 36 species of free-floating aquatic organisms with body sizes ranging from 2 mm to 10 mm, where each plant body plan is reduced to a largely leaf-like structure. As an emerging crop, their fast growth rates offer potential for cultivation in closed systems. We describe a novel UK collection derived from low light (dLL) or high light (dHL) habitats, profiled for growth, photosynthesis, and photoprotection (non-photochemical quenching, NPQ) responses. Twenty-three accessions of three *Lemna* species and one *Spirodela polyrhiza* were grown under relatively low light (LL: 100 µmol m^{-2} s^{-1}) and high light (HL: 350 µmol m^{-2} s^{-1}) intensities. We observed broad within- and between-species level variation in photosynthesis acclimation. Duckweeds grown under HL exhibited a lower growth rate, biomass, chlorophyll, and quantum yield of photosynthesis. In HL compared with LL, carotenoid de-epoxidation state and NPQ were higher, whilst PSII efficiency (φPSII) and Chl *a*:*b* ratios were unchanged. The dLL plants showed relatively stronger acclimation to HL compared with dHL plants, especially *Lemna japonica* accessions. These achieved faster growth in HL with concurrent higher carotenoid levels and NPQ, and less degradation of chlorophyll. We conclude that these data support local adaptation to the light environment in duckweed affecting acclimation in controlled conditions.

Keywords: Carotenoids, chlorophyll, duckweed, growth, habitat, light, photosynthesis, species.

Introduction

Duckweeds are miniature, remarkably fast growing aquatic plants, with doubling times as low as 24 h and dry weight production of 106 t ha^{-1} $year^{-1}$ (Cui and Cheng, 2015; Ziegler *et al.*, 2015; Michael *et al.*, 2021). Species are widely distributed across diverse waterbodies from swamps to flowing streams and canals. Consisting of simplified units of tiny, reduced leaf–stem structures (fronds) detaching to form independent clones, vegetative reproduction allows rapid colonization and full coverage of water surfaces. The 36 identified duckweed species have great phenotypic diversity within five genera, comprising larger and rooted *Spirodela*, *Landoltia*, *Lemna*, and the reduced and rootless *Wolffia* and *Wolffiella*. Duckweeds have worldwide

market potential as human and animal feeds: growing rapidly without soil, they have global distributions and offer dietary protein including essential amino acids, with levels comparable with soybean (Cheng and Stomp, 2009). Duckweeds are also a source of high starch, fibre, and micro- and macronutrients (Appenroth *et al.*, 2017;Yahaya *et al.*, 2022). Historically used in Asian cooking, there is also growing interest in duckweeds for vertical farming and even as a live plant food for space travel (Smith *et al.*, 2009, 2015; Appenroth *et al.*, 2018; Stewart *et al.*, 2020; Polutchko *et al.*, 2022; Nguyen *et al.*, 2023).

As miniature plants, duckweeds have little control over growing position, often lodged at water edges or passively motile, carried with the water flow or by water-dwelling organisms through an array of light environments. Duckweeds are tolerant of extremely low light conditions from grates and drains where light penetrates poorly, to vast duckweed carpets in expansive open lakes such as Titicaca in Peru and Bolivia, showing tolerance of growth in full light and high temperature. Although successful worldwide colonization strategies suggest natural variability of photosynthetic apparatus to challenging light climes, this has not been explored in a dedicated fashion (Ceschin *et al.*, 2018; Paolacci *et al.*, 2018; Stewart *et al.*, 2020; Strzałek and Kufel, 2021).

Plants have many mechanisms that allow efficient acclimation to varied intensities and wavelengths of light, caused by, for example, season, cloud cover, and tree and shrub cover. Whilst light stimulates high photosynthesis under optimal growing conditions, high irradiance can cause damage to or inactivation of photosynthetic processes (photoinhibition) often in combination with other stresses such as low or high temperature. Plants can modify the area and width of leaves for shade and sun tolerance, which involves re-arrangements of the composition of light-harvesting complexes, photosystem stoichiometry, and Chl *a*:*b* ratios (Anderson *et al.*, 1995; Maxwell *et al.*, 1995; Walters, 2005; Poorter *et al.*, 2006). Light acclimation is characterized by altered photosynthetic rates per unit leaf area and increased production of carotenoids and other counteracting antioxidants (Murchie and Horton, 1997; Foyer and Harbinson, 1999; García-Plazaola *et al.*, 2004). Photoacclimation processes typically support high photosynthetic capacity in high light (HL) and more efficient light harvesting and quantum yield under low light (LL). This is commonly observed using light response curves for gas exchange and/or electron transport showing quantum yield and the saturation point of photosynthesis which can vary, for example 300–500 μmol m^{-2} s^{-1} for temperate annuals to ~1000 μmol m^{-2} s^{-1} in rice growing in the tropics (Murchie and Horton, 1997; Zhao *et al.*, 2017). Acclimation to HL is also associated with photoprotective processes such as non-photochemical chlorophyll fluorescence (NPQ) which quenches excess excitation energy as heat and helps to prevent photoinhibition. NPQ consequentially down-regulates quantum yield of photosynthesis in the short term and can restrict plant growth in prolonged dynamic light exposure in natural conditions (Kromdijk *et al.*, 2016; Ruban, 2016, 2017; Pniewski and Piasecka-Jędrzejak, 2020).

Extensive species-level variation exists in nature in terms of the ability to acclimate to light conditions, which can be linked to growth strategy (Murchie and Horton, 1997, 1998; Demmig-Adams *et al.*, 2012; Burgess *et al.*, 2023). Cultivar/accession-level variation in photosynthetic properties is seen in traditional food crops and their close relatives including wheat and rice (e.g. McAusland *et al.*, 2020; Cowling *et al.*, 2022), and indeed accession-level studies in duckweed have demonstrated broad variation in the accumulation of many elements (Smith *et al.*, 2023, Preprint). It follows from this and the varied climes where duckweed is found that light adaptation studies in duckweed selection hold promise. Indications of variation in growth and changes in thylakoid pigments and photosynthesis in response to light have been observed in single *Lemna* clones of different species (Paolacci *et al.*, 2018; Stewart *et al.*, 2020), and *Lemna* species contain an enrichment in light-harvesting proteins compared with *Spirodela* species (An *et al.*, 2018). However, there has not been a comprehensive analysis of variation in acclimation of photosynthesis to light across duckweeds, and its connection with adaptation to habitat irradiance has only begun to be explored (Strzałek and Kufel, 2021).

Duckweeds present an interesting challenge for photoacclimation due to their floating habit across diverse sites. They have unusual frond anatomy which includes large air spaces for floating, non-functional stomata, and minimal photoassimilate export to the vasculature (Shtein *et al.*, 2017; Ziegler *et al.*, 2023). Their rapid growth rate is usually assessed by clonal colony expansion rates rather than progressive (3D) canopy development and tropic responses toward light seen in other macrophytes. Growth rate in duckweed showed species and ecotypic variation in stable light conditions (Ziegler *et al.*, 2015); however, we suggest that growth rate should be measured in the context of light acclimation to better understand how photosynthetic variations can help to achieve the high growth rates in these species.

Many duckweed species lack information regarding habitat of origin and accession-specific adaptations. Here, we report a new duckweed collection composed of different species and accessions in the UK. Attention was given to the collection environments which were differentiated by light maxima and all spectral composition (FR, R, G, B, UV ,R:FR). We hypothesized that ecotypes and species would show photosynthetic and growth adaptation footprints to local light environment when grown in artificial HL and LL environments. When ecotypes derived from high (dHL) or low light environments (dLL) were cultivated under controlled light conditions, we showed the unexpected outcome that only dLL ecotypes performed well under both LL and HL. Moreover, photosynthetic processes (F_v/F_m, φPSII, and NPQ) were modulated by growth light condition and species, but originating light habitat of ecotypes did not substantially influence photosynthetic

parameters. We discuss these outcomes in terms of environmental light acclimation in duckweed and for seeking novel genetic resources for food production.

Materials and methods

Collection of duckweed accessions and measurements of environmental parameters

Twenty-four duckweed isolates were collected in May 2020 in the UK between latitudes of 49.9° and 53.9° and longitudes of –0.29° and –5.19° (Supplementary Table S1; Fig. 1A). Between 10 and 20 individuals from each site were collected into sealed tubes of local water. If mixed species were present across the site, individuals were taken of each species into separate tubes. Site KS06 was a special case where two *Lemna minuta* KS06A and KS06B varieties were sourced; however, KS06B came from a drain apparently excluding light, and was measured separately to test for potential extreme light-adaptive differences. All duckweed samples were stored at ambient temperature with local natural daylengths until return to the laboratory, where species were confirmed using genotyping by short read genome sequencing (see below).

Nineteen duckweed sites were visited to collect environmental data over a 2 d period in March 2021 and 2022 and July and October in 2020, 2021, and 2022 to monitor variation across the seasons of spring, summer, and autumn.

Duckweed coverage scores were estimated by analysing images of surface coverage. Three photos from above were taken per site with a Canon 650D camera suspended on a camera boom. A white reference and scale was provided for each photo, level with the water surface. Images were processed as follows: three representative areas of 5 × 4 cm rectangles were selected to determine duckweed coverage averages and variability per site. Images were split into red, green, and blue (RGB) stacks and the blue stack used with the threshold scale to best match the original photos. Percentage coverage was quantified using Fiji open-source software (Schindelin *et al.*, 2012). Coverage data and locational coordinate data for duckweed sites are provided in Supplementary Table S1.

Light intensity (maximum PPFD, photosynthetic photon flux density intensity) was measured above 10 cm or up to 1 m (as close as possible to) from a water source using a 400–700 nm light meter (LICOR, 0Li-250A) with an attached quantum sensor head. Using a handheld spectrometer (LICOR, LI-180), total photon flux density (PFD), PPFD, and the ratio of light wavelengths making up the PFD (380–780 nm) were split and recorded as PFD-UV (380–400 nm), PFD-B (400–500 nm), PFD-G (500–600nm), PFD-R (600–700 nm), and PFD–FR (700–780 nm) in μmol m^{-2} s^{-1}. Dominant (λd) and peak (λp) wavelengths (in nm) were recorded at each site. The dominant wavelength is defined as the colour perceived, and the peak wavelength is the highest intensity wavelength recorded per site. All measurements were taken three times over the 20 min period of each visit and the maximum was recorded. All light intensity and spectrum variables (total=50) were used together to group duckweed sites as dLL or dHL habitats using distance analysis and K-means clustering to split sites into two main groups by similarity (Supplementary Tables S2,

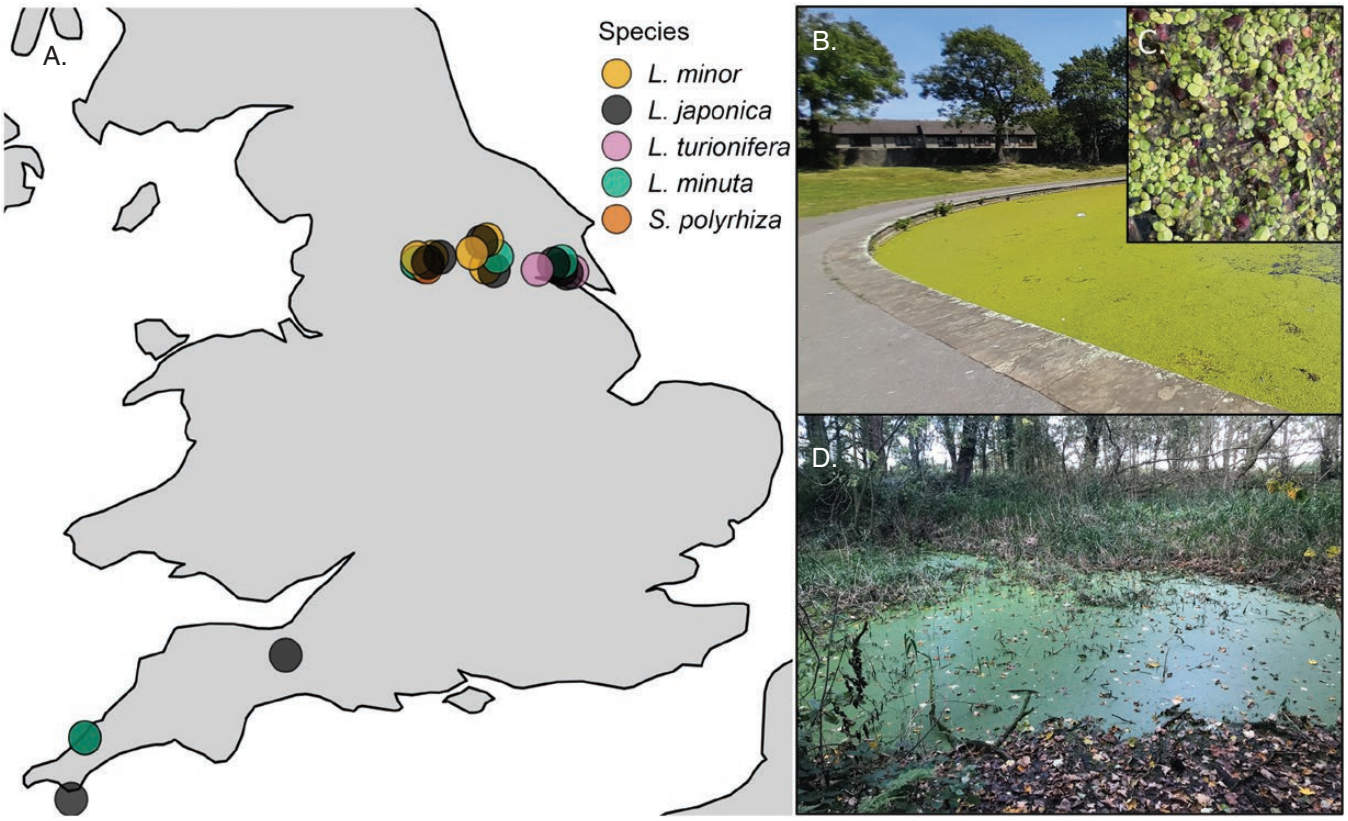

Fig. 1. Map of UK collection sites and high duckweed coverage found at high and low light sites. (A) Collection sites of duckweeds in this study, plotted by longitude and latitude coordinates (Supplementary Table S1). Coloured circles represent species groupings as shown in the key (as determined in Fig. 5). (B) High duckweed coverage in an open pond: KS12 in Bradford, UK, high light (dHL) site. (C) Close up of purple colouration in *S. polyrhiza* at KS12. (D) High duckweed coverage in a pond with steep banks and high tree cover: site KS25 in York, UK, low light (dLL) site.

S3). Distance matrices were computed using Manhattan, Euclidean, and Mikowski distances, and methods showed good consistency for two main groupings and site similarities. To measure variety in light environments, the proportion of spectral quality at each time point was calculated as the proportion of each individual spectral region/total light PFD×100 per site to give percentages, and R:FR was calculated as the ratio of R to FR from each site.

Time, weather (cloud cover), and atmospheric and water temperatures were noted across sites to account for variability across the 2 d periods and seasons. Climate data were collected for each longitude/latitude combination using bioclimatic variables extracted from worldclim.org using the R package Bioclim (Serrano-Notivoli *et al.*, 2022), and altitudes were obtained relative to sea level using Google Earth Pro. The raw and grouped light datasets are summarized in (Supplementary Tables S2 and S3, respectively) and other environmental data are shown in Supplementary Table S4.

Maintenance of duckweed accessions

Wild duckweeds were treated with 0.5% sodium hypochlorite in well plates (Greiner bio-one, Cellstar) for 1–2 min to sterilize them. The duration of treatment was dependent on size of duckweed and visible inward bleaching rates, leaving a green meristematic pocket and then dipping in Milli-Q water 18 MΩ to recover. Multiple individuals from a site were designated A or B based on size (different species) and cultured separately. Sterile colonies were grown in individual flasks containing N medium. Duckweed stocks were grown in GEN-1000 cabinets (Conviron, Winnipeg, Canada) with light provision at 50 μmol m^{-2} s^{-1} PPFD using broad-spectrum white LED lights providing 16:8 h days with a ramp of light intensity at the start and end to represent sunset and sunrise. A temperature cycle of 22/18 °C day and night was used, and relative humidity was maintained at 60%. N medium was prepared with Milli-Q water 18 MΩ and consists of KH_2PO_4 (0.15 mM), $Ca(NO_3)_2$ (1 mM), KNO_3 (8 mM), $MgSO_4$ (1 mM), H_3BO_3 (5 μM), $MnCl_2$ (13 μM), Na_2MoO_4 (0.4 μM), and FeEDTA (25 μM) as described in Appenroth *et al.* (1996). Other trace elements were confirmed in N medium as Si 23 μM, Cu 0.27 μM, and Zn 0.15 μM. N medium was autoclaved before use at 121 °C for 20 min. Each week duckweeds were re-sterilized with 0.5% sodium hypochlorite followed by dipping into sterile Milli-Q water and placed into fresh flasks containing new medium to build up sterile stock populations.

DNA isolation and genome sequencing

Each of the sterile 24 duckweed accession stocks were harvested into small populations containing 5–20 individuals, and <50 mg of whole duckweed tissue was frozen in liquid nitrogen. Duckweeds were ground using a Tissuelyser II (Qiagen) and DNA extracted using a DNAeasy Plant kit (Qiagen). DNA quantification was performed on a Qubit 2.0 using the Qubit dsDNA HS assay (ThermoFisher Scientific). Individual library preparations and short read sequencing using Illumina HiSeq 2500 platform sequencing was performed by Novogene, Cambridge, UK.

Sequencing data preparation, alignment, and genotyping

Short read sequencing data for *Lemna* and *Spirodela* accessions published in the Sequence Read Archive (SRA) were also included (*S. intermedia* 9394, *L. minuta* 9260, *L. japonica* 7123, *L. japonica* 7868, *L. minor* 7210, *L. minor* 7194, *L. minor* 9441, *L. minor* 9541, *L. minor* 7016, and *L. turionifera* 6002). Reads were quality trimmed with Trimmomatic (version 0.39) (Bolger *et al.*, 2014) and then aligned to the *L. minor* 7210 (SRR10958743) reference using BWA (version 0.7.17) (Li and Durbin, 2009) and further processed with Samtools (version 1.9) (Li *et al.*, 2009). Duplicate reads were marked using Picard (version 1.134). Indels were realigned with GATK (version 3.5) (McKenna *et al.*, 2010). The resulting variant call files (VCFs) were then filtered for biallelic sites and mapping quality (QD <2.0, FS >60.0, MQ <40.0, MQRankSum < −12.5, ReadPosRankSum < −8.0, HaplotypeScore<13.0). The VCF was then filtered by depth with a read depth cut-off of <650.

Genetic structure determination and species confirmation

For genetic structure analysis, putatively neutral 4-fold degenerate sites were extracted with DEGENOTATE (https://github.com/harvardinformatics/degenotate). These sites were then examined by a Neighbor–Joining tree using the VCFkit (Cook and Andersen, 2017) and plotted with ITOL (Letunic and Bork, 2021) using known individuals from the SRA to determine species clusters, alongside phenotypic observations (i.e. size of duckweed and presence or absence of seed formation). Species allocation was further explored using fastStructure v1 (Raj *et al.*, 2014) utilizing a genetic admixture of 4-fold degenerate sites for each accession and known individuals to map ancestry. The partition with the lowest Bayesian information criterion (BIC) was chosen for population number, K=4, which was obtained using adegenet version 2.1.3. (Jombart, 2008).

Controlled growth conditions for light acclimation experiments

GEN-2000SH cabinets (Conviron, Winnipeg, Canada) installed with broad-spectrum white LED lights were used to provide growth light treatments. Six months after collection and cabinet acclimatization at 50 μmol m^{-2} s^{-1} light, UK accessions were subcultured to ~15–20 individuals from each accession into individual flasks and continued to be grown in long days 16/8 h at 22/18 °C day/night temperatures. Accessions were either placed for 2 weeks at LL (100 μmol m^{-2} s^{-1}) (individual accessions n=24) or in 150 μmol m^{-2} s^{-1} for 1 week to acclimate to the intermediate light conditions and then transferred to HL (350 μmol m^{-2} s^{-1}) for a further week (n=24), to acclimate to conditions (Stewart *et al.*, 2020). The experiment with each light program then ran constantly for up to 6 weeks with a 1 h light and temperature linear ramp to simulate sunrise and sunset. N medium was changed weekly to maintain sterile populations and replenish nutrient dosage. Temperature and relative humidity were recorded using Datalogger (TinyTag Ultra 2, Gemini data loggers) in addition to the cabinet sensors. Light intensity and spectra were measured using a light sensor (LICOR, LI-180) (Fig. 2A).

Growth rate measurements

Single three-frond colonies from light level stock populations were added to individual conical flasks with 100 ml of N medium on day 0 (n=24) accessions for each HL and LL treatment. Growth rate was measured for each accession in each condition until ~95% surface coverage was achieved, or for up to 6 weeks in slower growing accessions. N medium was replaced each week to maintain high nutrient levels. Relative growth rate (RGR) was measured by colony gain, by counting the number of colonies in each flask, in each condition, every 7 d. Col RGRlog and RGRlog by area gain were calculated from raw data (shown in Supplementary Fig. S1). RGRlog by area gain was derived from total green area (mm^2) measured using a digital Nikon D5100 camera and an imaging pipeline for quantification (Ware *et al.*, 2023). The starting areas of the pioneering colonies were subtracted from total area gained each week to normalize differences due to size between accessions and species. RGRlog area was calculated using log(T21 area–T14 area)/7 and Col RGRlog by log(T21 colonies–T14 colonies)/7 (see Supplementary Fig. S1). The number of turions (seeds) formed between accession–light treatments was also assessed from photographs during each growth period. RGR difference by area and colony gain between treatments were obtained by mean growth in HL–mean growth in LL and proportion change calculated by RGR difference/mean growth in LL×100. Total fresh weight biomass (FW) per flask (normalized by weight of the starter

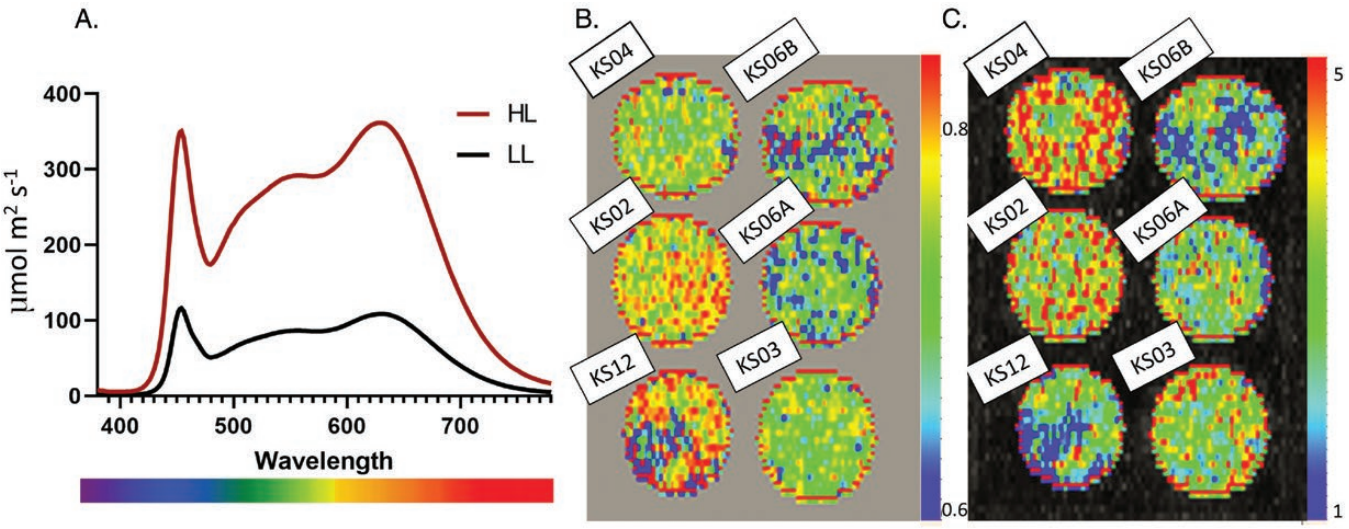

Fig. 2. Exploring duckweed photoacclimation using controlled light experiments. (A) Light spectra plotting available levels of light provided in two light treatments. The overall light input for the HL treatment (red) is PPFD 350 µmol m^{-2} s^{-1} and for LL (black) is PPFD 100 µmol µmol m^{-2} s^{-1}. (B and C) False colour images applied to pixels corresponding to populations of duckweed accessions within well plates as measured by chlorophyll fluorescence by a Fluorcam (B) F_v/F_m and (C) NPQ. Individual accessions are labelled per well and grown at HL in these image examples. Scales represent ranges of measurement values 0.6–0.83 for F_v/F_m measured in the dark and 1–5 for NPQ at maximum light intensity.

colony) was measured after 6 weeks (T42). Fresh biomass per flask was harvested and snap-frozen in liquid nitrogen before being freeze-dried and weighed to obtain freeze-dried weight (FDW).

Chlorophyll fluorescence

Chlorophyll fluorescence imaging was carried out to measure photosynthetic parameters as in McAusland *et al.* (2019), with a protocol modified for duckweeds. Populations of each accession were grown for 4 or 6 weeks in each light level, and each accession–treatment combination was used to fill the surface of clear plastic 6-well plates containing 3 ml of N medium. Duckweed plates were imaged using a closed chlorophyll fluorescence imager (800C Fluorcam, Photon System Instruments, Brno, Czech Republic); the layout in 6-well plates is shown in Fig. 2B and C. After a 1 h dark adaption, white LEDs with actinic light provided a saturating pulse set at 4500 µmol m^{-2} s^{-1} for 0.8 s to measure F_v/F_m. Then a rapid stepwise light response curve was constructed with the following light intensities (0, 20, 130, 245, 365, 480, 600, 710, 830, 950, and 1050 µmol m^{-2} s^{-1} PPFD) with a 60 s illuminating pulse applied at the end of each step. Numeric averages were exported for each accession–treatment replicate using the in-built software (Fluorcam 7, Photon System Instruments). The following parameters were extracted from the protocol: maximum PSII efficiency or quantum yield (F_v/F_m), PSII operating efficiency (F_q'/F_m'), or φPSII and NPQ (F_m–F_m')/F_m') (non-photochemical quenching, i.e. the photoprotective dissipation as heat loss) at each light level (Murchie and Lawson, 2013). These photosynthetic responses are plotted as light response curves and boxplots in Supplementary Fig. S2.

Pigment extraction and analysis

For spectrophotometry, 5 mg of freeze-dried duckweed tissue was ground in 1.5 ml of 80% acetone using a TissueLyser II (Quigen) at 24 Hz s^{-1} for 4 min and the cell debris was pelleted. Extracted supernatant was further diluted by 3.5 ml of 80% acetone to give a total volume of 5 ml. Absorbance was recorded for Chl *a* at 663 nm, Chl *b* at 646 nm, carotenoids at 470 nm, and absorbance at 750 nm as a correction turbidity factor using a UV/Visible spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences). Total chlorophyll as mg g^{-1} duckweed was calculated following Porra *et al.* (1989) and carotenoids as mg g^{-1} calculated as in Lichtenhaler and Wellburn (1983).

Pigment extraction and analysis by HPLC

For carotenoid HPLC analysis, tissue was rapidly frozen in liquid nitrogen at mid-day. A 0.8 g aliquot was ground in 2 ml of 100% acetone (HPLC grade) in LL and centrifuged at 10 000 rpm at 4 °C for 2 min. The supernatant was filtered through a 13 mm diameter 0.2 µm polytetrafluoroethylene (PTFE) syringe filter (Whatman GmbH, Dassel, Germany) into a 1.5 ml amber Eppendorf and stored at –80 °C. Pigment separation was performed by reverse-phase HPLC as described in Färber *et al.* (1997) using a Dionex BioLC HPLC system (Sunnyvale, CA, USA) with a LiChrospher® 100 RP-18 (5 µm) column (Merck, Darmstadt, Germany). The carotenoids, violaxanthin, zeaxanthin, antheraxanthin, lutein, neoxanthin, and β-carotene, and Chl a and Chl b were detected using 447 nm wavelength, shown in the chromatogram (Supplementary Fig. S3B). Each carotenoid was expressed as a percentage of the total carotenoid pool. The de-epoxidation state (DEPS) and total xanthophyll pool (XC) relate to ratios of violaxanthin, zeaxanthin, and antheraxanthin, calculated as described in Färber *et al.* (1997).

Experimental design and statistical analysis methods

Experiments were repeated on five separate occasions giving five independent sets of growth experiments for duckweed accessions growing in cabinet conditions. From each of these, three biological replicates were used for each accession–treatment combination for chlorophyll fluorescence measurements totalling 15 replicates. Pigment extraction by spectrophotometry was performed for four replicates of accession–treatment combinations from each independent experiment, maximum n=20. For HPLC analysis, 14 accessions grown in HL and LL were used, forming one data point for each accession–treatment combination. Significance of light treatment, accession, species, and light habitat (derived environmental light dHL or dLL) were determined on each growth rate parameter (RGRlog area, Col RGRlog, FW, and FDW), photosynthetic

parameters, and pigment contents using all observation data with two-way ANOVA and Welch's *t*-tests. Differences between light treatment responses amongst accession were also assessed (average HL minus LL) for growth, NPQ, φPSII, F_v/F_m, and pigment contents (total Chl *a*, Chl *b*, and carotenoids). HPLC carotenoid data were pooled for each accession to find differences between treatments. All data manipulation and analysis was performed in R (v3.6.3) using Rstudio (v1.2.5) with packages ggplot2 (Gómez-Rubio, 2017), corrplot (McKenna *et al.*, 2016), FactoMineR (Lê *et al.*, 2008), and factoextra (Kassambara and Mundt, 2020).

Results

Sites were classified as high light (dHL) or low light (dLL) intensity using distance analysis and K-means clustering utilizing all measured environmental light variables of light intensity PPFD and all spectral compositions from sites across time points (Fig. 3, and data summarized in Fig. 4 with statistics in Supplementary Table S3; see also 'Collection of duckweed accessions and measurements of environmental parameters' in the Materials and methods). Eleven sites were determined as LL and eight as HL (Fig. 3), such as ditches and locations under bridges and trees to full-scale open ponds and canals (Fig. 1B, D). Full duckweed coverage was evident in both sun and shade locations, with surface coverage variation between sites and seasons (Supplementary Table S1). Environmental light (Supplementary Table S2) was associated with surface coverage from 19 sites across the seasonal time points (Supplementary Table S1). In autumn, coverage was significantly negatively correlated with UV light (R= –0.53; P=0.02; Supplementary Fig. S4). Noteworthy also were no other correlations between light variables and coverage.

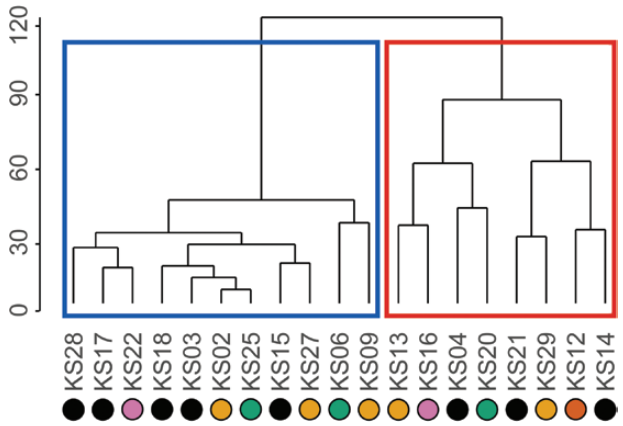

Fig. 3. Native duckweed sites can be organized into derived from high light (dHL) or low light (dLL) using measured environmental light variables. Environmental light variables (n=50) measured in either μmol m^{-2} s^{-1} (PPFD, FR, R, G, B, UV) or nm (λp, λd) for 19 sites across three seasons for 2 years were used to group sites by relatedness using a dendrogram. The distance matrix was computed using Manhattan distances and the complete method. The rectangles represent site groupings after K-means clustering set at n=2, where a blue border indicates dLL sites and red a border indicates dHL sites. Coloured circles represent species groupings as shown in the key in Fig. 1.

Seasonal light quantity and spectral quality were markedly habitat dependent

Light intensity (PPFD) and all spectral constituents (FR, R, G, B, UV, and R:FR) were higher in dHL sites than in dLL sites across all seasonal time points (Fig. 4; Supplementary Table S3). The maximum recorded light intensity overall in summer 2022 for dHL sites was 1116 μmol m^{-2} s^{-1} compared with an almost 10-fold reduction in dLL sites at 109 μmol m^{-2} s^{-1}. dLL sites received higher levels of FR light (750 nm) compared with more G light (550 nm) in dHL, especially in summer as indicated by peak wavelength measurements (Fig. 4B). Light spectral proportions of % FR and % UV were higher in dLL sites whilst % R and % G were higher in dHL sites (Fig. 4C). This spectral shift contributes to substantially lower R:FR ratios in dLL sites consistent with year-round natural canopy shading, but with the greatest differences in summer (Fig. 4D). The % PPFD differed between sites, with dLL receiving less light in the photosynthetically active region (R+G+B) in summer and autumn. For other environmental variables, no significant differences were found between dHL and dLL sites for water and atmospheric temperature measured at each time point or for bioclimatic temperature and precipitation data (Supplementary Table S4). Such contrasting differences in light intensity and spectral quality indicate a marked difference in year-round habitats which present different challenges for light acclimation.

Lemna *species were commonly identified in the UK duckweed panel*

Species were determined using clustering of genome sequences employing known sequenced individuals as controls (Fig. 5). Five species were identified in the UK cohort: the majority were *L. japonica* (n=11), followed by *L. minor* (n=5), *L. minuta* (n=5), *L. turionifera* (n=2), and *S. polyrhiza* (n=1). Indeed, putative species clustered together with their respective relatives, namely *L. turionifera* (6002), *S. intermedia* (9394), *L. minuta* (9260), *L. minor* (7016, 7194, 7210, and 9441), and *L. japonica* (7123). In addition incongruence was noted with *L. japonica* 7868 and *L. minor* 9541, which are evidently switched between species clustering patterns, indicating that these samples have been misidentified previously as these two species are notoriously difficult to distinguish (Volkova *et al.*, 2023). Individual accessions clustered into expected species groupings that corresponded with those from independent phenotypic assessments for the UK accessions. *Lemna japonica* individuals formed a cluster between *L. minor* and *L. turionifera* (Fig. 5A), and the same relationship was also apparent from fastStructure analysis, suggesting that *L. japonica* individuals arose from hybridization between *L. minor* and *L. turionifera* (see Fig. 5B).

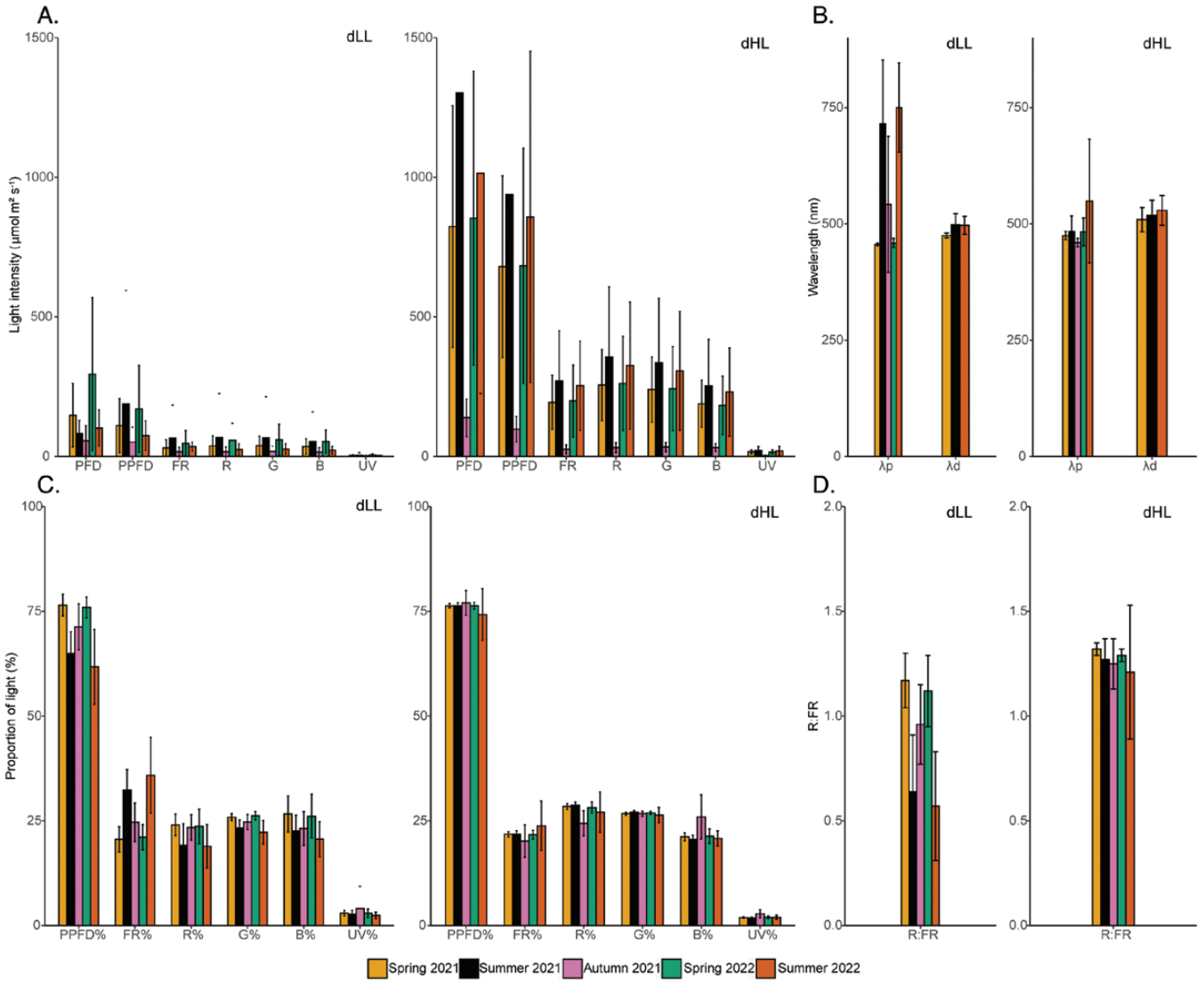

Fig. 4. dHL and dLL sites have stark contrasts in photosynthetically relevant light environments. Light measured between different seasons and years from 19 duckweed sites grouped as dHL or dLL showing averages with error bars as ±SD within site groupings. (A) Light intensity and spectral quality were measured for dLL and dHL sites in µmol m^{-2} s^{-1}, for total light PFD made up of regions of light: FR, far-red; R, red; G, green; B, blue; UV, ultraviolet; and for the photosynthetic portion PFFD (B+G+R). (B) Wavelengths of light measured in nanometres were quantified for sites as λp, peak average wavelength; and λd, dominant average wavelength for dLL and dHL. (C) Proportions of spectral quality at each time point were calculated as the proportion of spectral region/total PFD light×100 per site and grouped by dLL and dHL. (D) R:FR was calculated as the ratio of R to FR light from raw values from each site and grouped as dLL or dHL. Bars are coloured by seasonal and yearly time point in chronological order, with dLL on the left and dHL on the right of each panel. Raw and grouped summary data and *t*-test results are presented in Supplementary Tables S2 and S3.

Divergence between KS12 from *S. intermedia* 9394, as well as high anthocyanin accumulation led to the classification of this accession as *S. polyrhiza* (Fig. 5; Supplementary Fig. S5; Fang *et al.*, 2023, Preprint).

Lemna species were differentiated between both dHL and dLL environments, whilst a single *Spirodela* representative, *S. polyrhiza*, was found only at a dHL site (Figs 1B, 3, 5). There was high variation in species wild seasonal growth patterns. *Lemna minor* (e.g. KS13, KS27, and KS29) and *L. japonica* (KS17 and KS21) accessions from a mixture of dHL and dLL sites did not exceed 20% surface coverage across all time points, with low site coverage averages <5%, indicating they were often not present or only growing as sporadic colonies (Supplementary Table S1). In contrast, *L. japonica* KS03 and *L. minuta* KS06B from dLL had the highest average coverage of all sites overall, and maximum coverage in early spring and summer. The highest surface coverage in the wild in different seasons/year time points were all from dLL sites, including KS06B, KS25, KS18, and KS02, showing superior maintenance of *L. minuta*, *L. japonica*, and *L. minor* accessions in LL environments all year round. The exception was the KS12 *Spirodela* accession in summer, which had comparably high

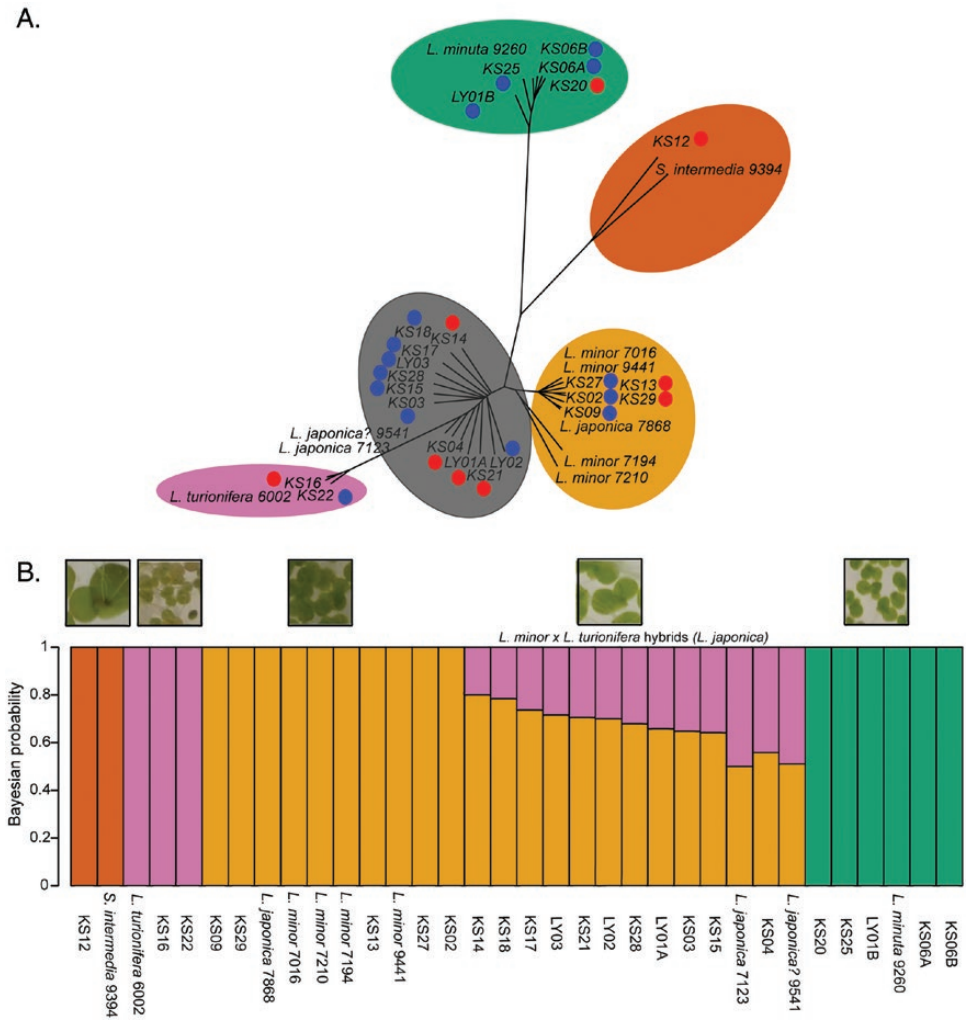

Fig. 5. Duckweed species differentiated by genome alignment and allele frequencies used to classify the UK duckweed panel. UK duckweed short read sequences and known species sequences were aligned with *L. minor* 7210 and filtered down to 4-fold degenerate coding region single nucleotide polymorphisms (SNPs), consisting of >550 000 variants presumed to be under neutral selection. (A) Phylogenetic tree of duckweed accessions with *n*=5 clusters of duckweed species. *Lemna minor* accessions group with *L. minor* 7016, 9441, 7194, and 7210, but also with *L. japonica* 7868. *Lemna japonica* individuals cluster with *L. japonica* 7123 and *L. minor* (*japonica*?) 9541. *Lemna minuta* individuals (*n*=5) distinctly cluster with *L. minuta* 9260, *L. turionifera* (*n*=2) group with *L. turionifera* 6002, and KS12 clusters with *S. intermedia* 9394, but with some genetic differentiation, and is considered to be *S. polyrhiza*. Coloured circles represent dLL or dHL ecotypes relating to habitat light environment. (B) fastStructure analysis showing the ancestral genetic makeup of each duckweed individual used to classify species (*n*=24 UK) along the *x*-axis, with Bayesian probability of population allocation on the *y*-axis. fastStructure confirms duckweed species and presence of the hybrid *L. japonica* species composed from *L. minor* and *L. turionifera* parents. K=4 was used to determine the number of populations by which to colour the structure plot. Each species group is coloured as shown in the key in Fig. 1. Representative images of each species are given above the structure plot.

surface coverage as a dHL site, highlighting different species prevalence across extremes of seasonal light and temperature in the UK.

Duckweed growth rate in controlled conditions is dependent on light intensity, species, and original habitat light environment

Differences in RGR were derived from growth curves in each light treatment measured by area or colony counts (Supplementary Fig. S1). Duckweeds generally had higher RGRs during the log phase of growth in LL compared with HL (ANOVA, $P \leq 0.001$, Supplementary Table S5A, B; Fig. 6A, B, E, F). On average, RGRlog by area was 0.77, and 0.37 by colonies in LL compared with 0.65 and 0.28 in HL ($P \leq 0.05$, Welch's *t*-test). For 18/24 accessions, HL negatively affected growth rate, three accessions had increased growth rate in HL, and three were unaltered (Supplementary Fig. S6A, B). Moreover, FW and FDW biomass positively correlated with RGRlog, especially in HL where FW/FDW and FW/RGRlog area

R=0.88, P≤0.0001, and FDW/RGRlog area R=0.84, P≤0.001 were found (Fig. 9A; Supplementary Fig. S7).

Species and light habitat were both significant for differences in RGRlog area and RGRlog colony gain by ANOVA (P≤0.0001 and P≤0.01 respectively, for RGRlog area), with an interaction for RGRlog area (Supplementary Table S5A, B). Growth responses in HL or LL were split, to consider the effects of species and derived environmental light (dHL or dLL) separately for light acclimation (shown in Fig. 6). In artificial LL, dHL and dLL accessions grew at the same rate. In HL, original light habitat had significant effects on growth rates by RGRlog area and RGRlog colony gain (Fig. 6E, F; Supplementary Fig. S6A, B), whereby the dLL accessions outperformed dHL accessions for growth rate (Supplementary Fig. S6A, B). There were no differences by colony gain, FW, and FDW between species in HL. Between treatments, *L. japonica* maintained the fastest growth by RGRlog area, and *L. minuta* growth was most severely affected by HL (Fig. 6A).

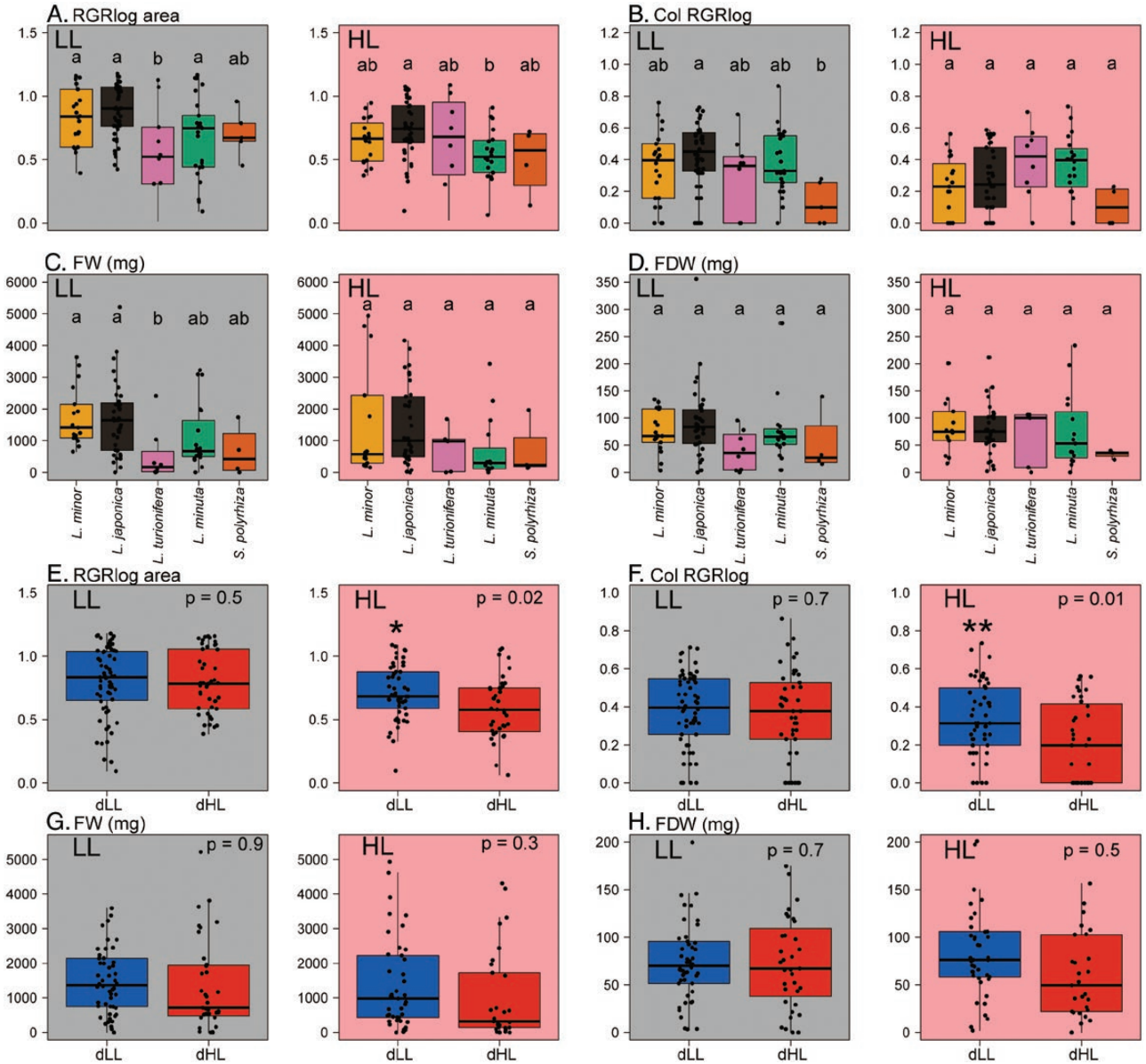

Fig. 6. Species show differences in growth rate in LL, but in HL the dLL accessions grow faster than the dHL accessions. Each pair of plots represent different growth rate parameters: RGRlog area, Col RGRlog, FW, and FDW (mg), and display LL treatment response on the left (grey) and HL on the right (red) of each panel. (A–D) Species differences coloured as shown in the key in Fig. 1. Lowercase letters indicate significant species differences within each light treatment by post-hoc Tukey test P≤0.05. (E–H) Light habitat effects by grouping accessions as dLL (blue) or dHL (red) for each boxplot. Welch's t-test is indicated above, and significant differences are shown by *P≤0.05 and **P<0.01, with insignificant differences marked with n.s. The midlines on boxplots indicate the median and 25% and 75% quartile boxes; all observations are displayed as points.

In LL, *L. japonica* and *L. minor* grew faster by area gain and produced more biomass than *L. turionifera* (Fig. 6A, C). *Lemna japonica* also produced colonies faster in LL relative to other *Lemna* species, with *S. polyrhiza* producing the least (Fig. 6B).

Photosynthetic processes are modulated by light intensity

Light response curves are presented for φPSII and NPQ for duckweed accessions grown in LL and HL treatments (Supplementary Fig. S2). At light levels equivalent to the LL growth condition (130 μmol m^{-2} s^{-1}), φPSII had already declined by 50%, with a further 20–30% decrease at 365 μmol m^{-2} s^{-1} corresponding to HL levels. Linear regression models for φPSII between 130 μmol m^{-2} s^{-1} and 365 μmol m^{-2} s^{-1} showed a higher slope and lower intercept in HL-grown plants compared with LL-grown plants (Supplementary Fig. 2A). NPQ increased with increasing light levels, with a maximum at >1000 μmol m^{-2} s^{-1} (Fig. 7B). LL plants maintained a higher photosynthetic efficiency (φPSII) while a greater photoprotective capacity (NPQ) was observed in the HL plants, with an average NPQ of 2.98 in HL and 2.33 in LL at the maximum light intensity ($P \leq 0.0001$, Welch's *t*-test).

The maximum photosynthetic efficiency measured in the dark (F_v/F_m) and maximum NPQ were strongly affected by light treatment, accession, and species (ANOVA, $P \leq 0.0001$) and for F_v/F_m (ANOVA, $P \leq 0.003$; Supplementary Fig. S2C). For F_v/F_m, the interaction between factors was also important, showing both genetic and light treatment effects (Table 1; Fig. 7C). The original light habitat did not influence photosynthetic parameters in LL or HL, whilst dHL and dLL accessions had no clear differences in HL response (Table 1; Supplementary Fig. S6C, D).

Photosynthetic processes show species-specific differences

Photosynthetic responses differed between species, with sensitivity to both growth light level and measurement light level. *Lemna minor* had naturally higher F_v/F_m and φPSII than *L. minuta* in LL and HL, in fact *L. minuta* had the lowest F_v/F_m of all species and concurrent low φPSII, showing different achievable PSII quantum yields and capacity for light acclimation between species (Fig. 7A, C). The severity and direction of changes in F_v/F_m between light levels also differed between species. *Lemna japonica* had high F_v/F_m in LL, with a reduction in HL, whilst *S. polyrhiza* had comparable F_v/F_m with other species in HL, but lower photosynthetic efficiency in LL (Fig. 7C). *Spirodela polyrhiza* also showed an additional species-specific acclimation of photosynthesis with atypical improvement of φPSII at the maximum light intensity >1000 μmol m^{-2} s^{-1}, in line with the observation that it is well adapted to high light (Fig. 7A).

Species differences for NPQ in LL-grown plants were also common, with *L. japonica* demonstrating higher inherent capacity for NPQ than *L. minuta* and *S. polyrhiza* (Fig. 7B). In HL-grown plants, when NPQ is normally higher, *L. japonica* accessions retain higher NPQ compared with all other species at specific light levels. Moving from low to high light, NPQ in *L. minor* rose whilst it declined in *S. polyrhiza*.

Higher F_v/F_m in HL was associated with faster growth in both light conditions; therefore, photoinhibition may be strongly associated with high rates of growth in HL (Fig. 9; Supplementary Fig. S7). Maintenance or increases in F_v/F_m in addition to high φPSII in HL relative to LL occurred in *S. polyrhiza* and *L. turionifera*, concordant with relatively better growth rates in HL relative to LL (Figs 6A, B, 7A, C; Supplementary Fig. S6A–C). The highest F_v/F_m values throughout were in fast growing *L. minor* accessions in LL, and the lowest F_v/F_m throughout were in *L. minuta*, which showed markedly reduced area gain in HL (Figs 6A, 7C). NPQ was not directly associated with high growth but, as *L. japonica* had high overall NPQ and high growth rates in LL and HL, it may be linked through species effects.

Changes in pigment concentrations occur in duckweed during light acclimation

Total chlorophyll and carotenoids measured spectrophotometrically were affected by light level, species, accessions, and habitat light environment (summarized in Supplementary Table 5C, D). Light treatment had significant effects (ANOVA, $P \leq 0.0001$), with total chlorophyll decline and carotenoid increase as shown in Supplementary Fig. S6E, F. *T*-tests showed that LL-grown accessions had higher Chl *a* and *b*, whilst HL-grown accessions had higher carotenoids and the carotenoid:chlorophyll (Car:Chl) ratio was also higher. Although there were clear pigment alterations in responding to the light treatments in terms of total chlorophyll and Car:Chl, duckweed accessions did not increase Chl *a*:*b* ratios to acclimate to HL (Fig. 8). The role of light habitat and species effects on pigment changes was further dissected for HL responses.

In a separate analysis, carotenoids were quantified using HPLC, pooling leaf samples so that statistical analysis was possible for light treatment effects only (Supplementary Fig. S3.). Here, xanthophyll cycle (XC) carotenoids increased under HL (ANOVA, $P \leq 0.0001$), especially zeaxanthin (ANOVA, $P \leq 0.0001$) and antheraxanthin (ANOVA, $P \leq 0.05$), with a reduction in violaxanthin (ANOVA, $P \leq 0.05$). The de-epoxidation state (DES) of the XC increased in HL, showing greater conversion to zeaxanthin in HL-grown plants generally, with high levels of DES from 43% in LL to 67% in HL, indicating relatively excessive light levels in both LL and HL in comparison with other higher plants under field conditions (e.g. Murchie *et al.*, 1999). In this dataset, we note that Chl *a*:*b* was lower in LL-grown plants overall, indicating that acclimation of light-harvesting complex antenna size may have occurred.

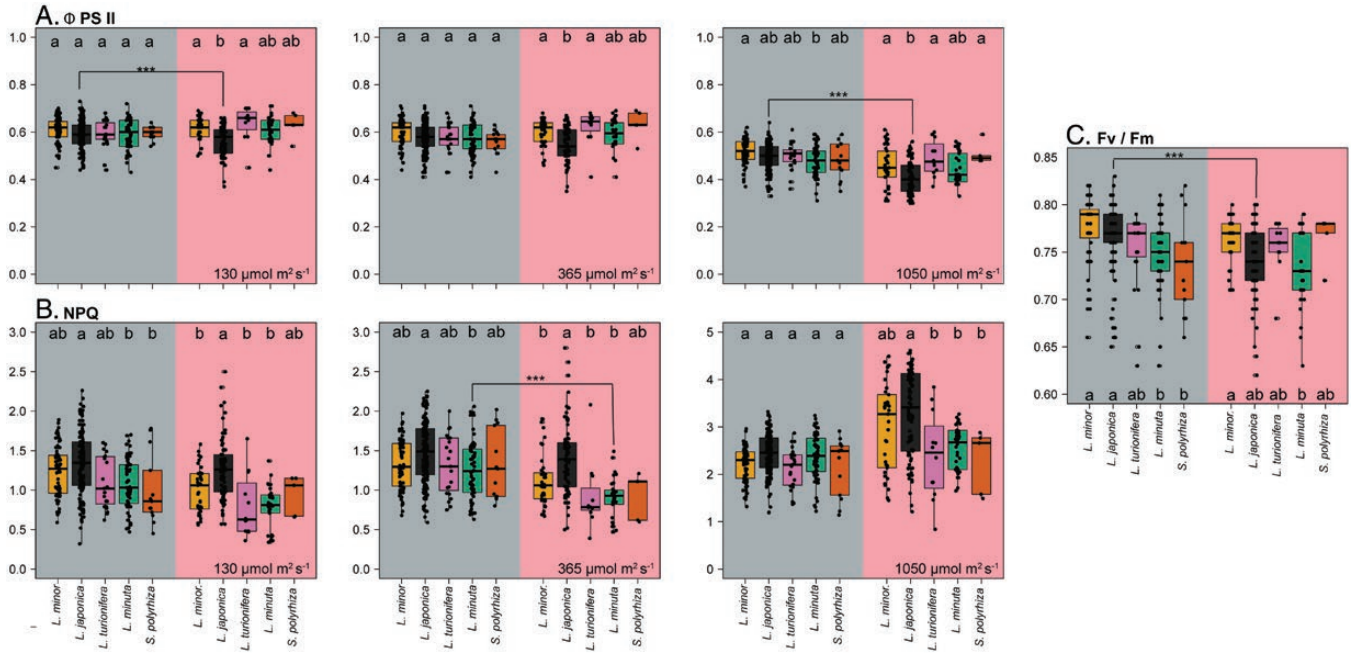

Fig. 7. Photosynthetic parameters φPSII, F_v/F_m, and NPQ display unique treatment–species effects at different light levels. (A) Boxplots showing φPSII and (B) NPQ measured in duckweed species at 130, 365, and 1050 µmol m^{-2} s^{-1}. (C) F_v/F_m measured quantum yield in the dark for duckweed species grown in two light treatments. All panels are split by treatment LL (grey) and HL (red), and all boxplots show the median and 25% and 75% quartiles, with all individual points plotted. Significant differences between species within each light treatment are indicated by lowercase letters at the top or bottom of plots by Tukey post-hoc test <0.05, and differences between the same species grown in LL and HL are indicated with bars between them and marked with asterisks.

Chlorophyll and carotenoid changes required for high light acclimation were better optimized in accessions from low light original habitats

Pigment content and the ratios normally associated with acclimation to HL differ between accessions from different light habitats. The dLL accessions acclimated to HL by increasing carotenoid and maintaining total chlorophyll, as shown by higher Chl *a*, Chl *b*, total Chl (*a*+*b*), and carotenoids in HL (Fig. 8A–C, E; Supplementary Fig. S6E, F). This response was less pronounced in dHL accessions. Interestingly, dHL accessions showed an increase in Chl *b* relative to dLL accessions only in LL. There were no significant differences in Chl *a*, total chlorophyll, and carotenoids between dHL and dLL accessions grown in the LL condition and there was no difference in overall Chl *a*:*b* or Car:Chl ratios between dHL or dLL in either treatment, which would typically be expected differences between sun- or shade-tolerant plants.

Pigment composition is naturally variable between species in controlled low light conditions

For LL-grown plants, pigment content varied among species, but this was weaker in HL (Fig. 8G–L). In LL, *L. japonica* had the highest Chl *a*:*b* and *L. minuta* the lowest, indicating sun- and shade-tolerant adaptations between species at low light. *Spirodela polyrhiza* and *L. minuta* had the highest Car:Chl in LL, with all four *Lemna* species showing significant increases due to treatment, whilst *S. polyrhiza* was unaffected. *Spirodela polyrhiza* was notably the only species with anthocyanin accumulation in response to HL treatment, contributing to its unique species adaptation to light (Supplementary Fig. S5A).

Low light-derived accessions acclimated to high light with higher pigment concentrations and strong growth performance

When accessions are plotted on the landscape of all physiological variables in light response, there is broad separation by environmental light groupings (Fig. 9B). The fastest growing accessions in HL were all *L. japonica* from dLL sites: KS03, LY02, LY03, and KS18, characterized by high chlorophyll and carotenoid contents in HL (Fig. 9). *Lemna japonica* were also fastest growing in LL, with high photoprotection via NPQ in both light intensities. Maximum quantum yield of photosynthesis in the dark (F_v/F_m) increases in *S. polyrhiza* in HL relative to LL and coincides with faster growth by colonies in HL (Fig. 6B). The centroids for dLL and dHL groupings show separation by performance in HL primarily driven by variation in growth rate and pigment composition (Figs 6, 8, 9).

Table 1. Photosynthetic efficiency, NPQ responses, and quantum yield of photosynthesis at low and high light

	Treatment	Accession	Species	Light habitat	Treatment×Accession	Treatment×Species	Light habitat×Species
A. φPSII							
φPSII 130 PPFD							
F	12.1	1.6	4.4	1.1	1.5	2.4	0.4
P	0.0005	0.04	0.001	0.3 ns	0.04	0.05	0.75 ns
φPSII 365 PPFD							
F	11.0	1.8	5.1	2.5	1.4	1.5	0.4
P	0.0009	0.01	0.0005	0.11 ns	0.10 ns	0.22 ns	0.79 ns
φPSII 1000 PPFD							
F	0.3	1.5	5.0	1.7	0.9	1.4	0.6
P	0.55 ns	0.06 ns	0.0007	0.18 ns	0.54 ns	0.24 ns	0.63 ns
B. NPQ							
NPQ 130 PPFD							
F	26.6	5.1	19.3	0.03	1.8	2.2	1.0
P	<0.0001	<0.0001	<0.0001	0.8 ns	0.01	0.06 ns	0.4 ns
NPQ 365 PPFD							
F	34.1	4.9	14.8	0.02	2.2	3.1	1.1
P	<0.0001	<0.0001	<0.0001	0.9 ns	0.001	0.02	0.3 ns
NPQ 1000 PPFD							
F	121.0	4.3	11.5	0.5	2.5	6.4	3.1
P	<0.0001	<0.0001	<0.0001	0.5 ns	0.0002	<0.0001	0.03
C. F_v/F_m							
F_v/F_m 0 PPFD							
df	1	23	4	1	23	4	3
F	31.9	2	7.1	1.5	1.8	3.3	0.8
P	<0.0001	0.003	<0.0001	0.2 ns	0.01	0.01	0.5 ns

Photosynthetic efficiency (φPSII), NPQ responses, and quantum yield of photosynthesis (F_v/F_m) vary between accessions, species, and light treatments at corresponding low (130 μmol m^{-2} s^{-1}) and high light (365 μmol m^{-2} s^{-1}). φPSII measured at maximum light (1050 μmol m^{-2} s^{-1}) was only sensitive to species differences, and light habitat does not appear to affect photosynthetic parameters at any light level. Parameters were derived from chlorophyll fluorescence.
ANOVA results for single factors and interactions of factors using significance as $P \leq 0.05$. Non-significant results are reported as ns.

Discussion

The relationship between light acclimation mechanisms and habitat has been relatively well studied in higher plants, but such information is less available for duckweeds, with their aquatic habitat complicating simple categorization. Typically, duckweed collections in stock centres are limited by unknown date of collection, specificity of collection locations, and environmental data, hampering extensive studies (Sree and Appenroth, 2020). Here we generated a novel duckweed collection from diverse sites in North and South UK alongside detailed habitat data. We then explored variation in light acclimation in controlled environments between accessions of five species from different light habitat types.

Colonization of duckweed in low light natural environments persisted all year round

The highest plant coverage was maintained all year round across dLL but not dHL sites. The differences between HL and LL habitats represented largely expected features. Diversity in light quantity by scattering and modified quality of green and FR light enhancement is caused by the presence of overhead vegetation (Lee *et al.*, 1996; De Castro, 2000; Burgess *et al.*, 2021). Previously, LL sites characterized by tree shading were proposed to also provide temperature protection for duckweeds and additionally contribute to higher nutrient injection into water from decaying biomass (Landolt, 1986; Landolt and Kandeler, 1987). Here, irradiance and spectral compositions were all significantly different between dHL and dLL sites all

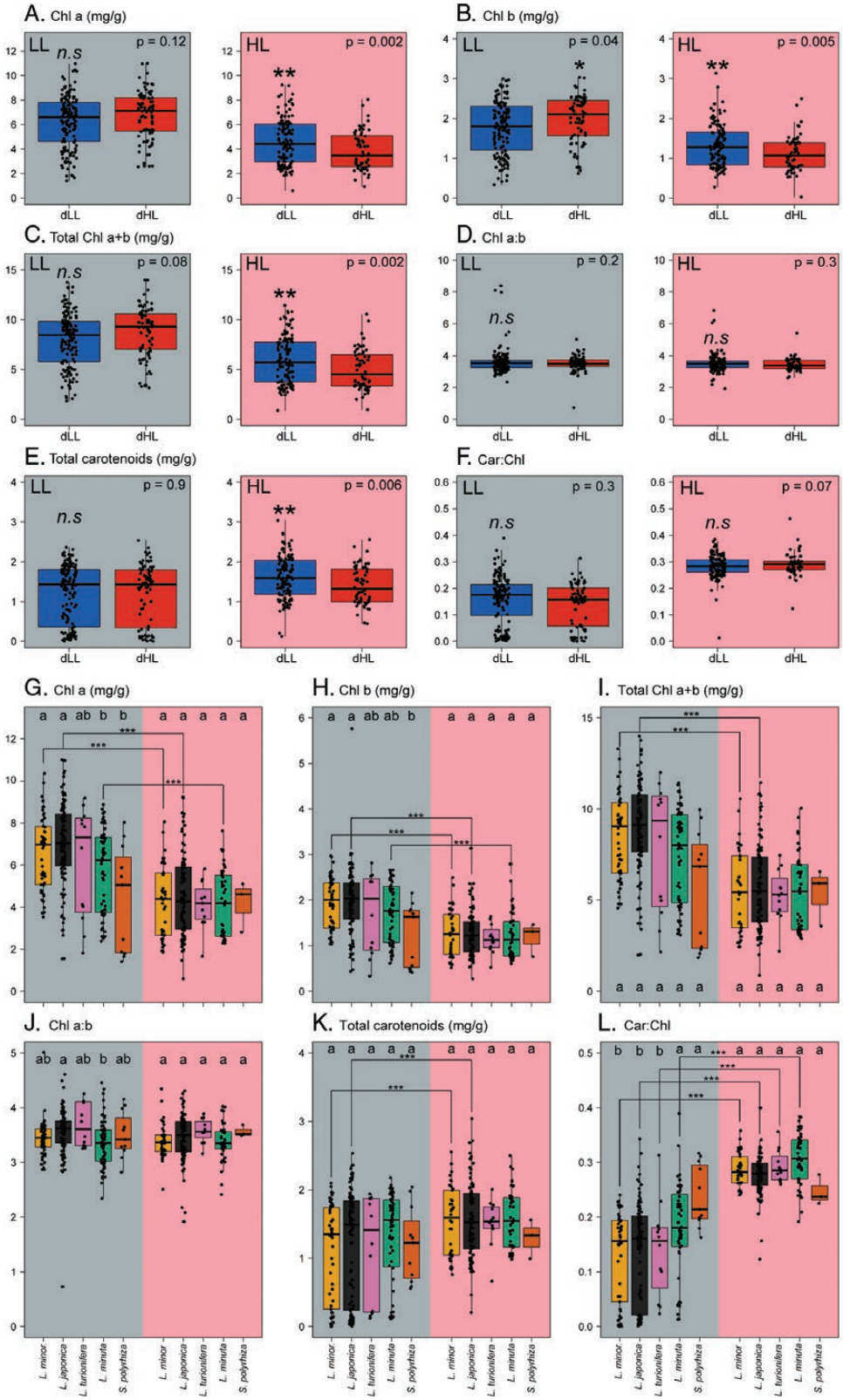

Fig. 8. Chl *a*, Chl *b*, total chlorophyll, and carotenoid contents are higher in dLL duckweed accessions than dHL accessions when grown in HL. (A–F) Pairs of boxplots for Chl *a*, Chl *b*, Chl *a*+*b*, and carotenoid contents, and Chl *a*:*b* and Car:Chl ratios of accessions grown in HL (red) and LL (grey) treatment and coloured by dLL sites (blue) or dHL sites (red) on the *x*-axis. All *P*-values by Welch's *t*-test are reported, and **P*<0.05 and ***P*<0.01

indicate significance of environmental light, with insignificant differences marked with n.s. Pigment content is different between species at LL but not different in HL. (G–L) Paired boxplots show species effects on each pigment measurement in two light treatments, LL (grey) and HL (red). Lowercase letters at the top or bottom of plots represent significance by Tukey post-hoc test <0.05 between species within the treatment. Differences between the same species grown in LL and HL are indicated with bars between them and marked with asterisks. The midline on boxplots indicates median and 25% and 75% quartile boxes, and all are observations plotted as points. *n*=20 for each accession–treatment combination.

year round. Shade-enriched components FR and green are able to drive photosynthesis in well-acclimated plants in very low light conditions (Smith *et al.*, 2017; Zhen and Bugbee, 2020). This may have contributed to dLL sites having the highest duckweed coverage of all seasons. The notable exception was *S. polyrhiza* KS12 from a dHL site which displayed high coverage in summer (Supplementary Table S1; Fig. 1B, C). UV was the only light spectral region to correlate negatively with duckweed coverage and, even in controlled conditions even at low levels, produces a stress response in *L. minor* (Farooq *et al.*, 2000). No differences in temperature were noted between dHL and dLL sites, so we conclude that HL and species-specific acclimation were important drivers of adaptation in HL sites.

Low light was more supportive for fast growth than high light in controlled conditions

Growth rates were generally higher in controlled LL. Both low (<100 μmol m^{-2} s^{-1}) and high light intensities have been cited as beneficial for biomass and growth rates in different duckweed species (Classen *et al.*, 2000; Cheng *et al.*, 2002; Paolacci *et al.*, 2018; Stewart *et al.*, 2020). At the same time, *L. gibba* grown in extremes of 50 μmol m^{-2} s^{-1} and 1000 μmol m^{-2} s^{-1} grew at the same rate, supporting better light use efficiency at LL (Stewart *et al.*, 2021). Conclusions for species as a whole have been drawn from single clones of *L. minuta*, *L. minor*, and *L. gibba* grown in HL (Paolacci *et al.*, 2018; Stewart *et al.*, 2020). However, varied RGR measurement methods by area or biomass as well as different starting densities, experimental duration, and frequency of measurements challenge comparison of growth data between species across different studies. Optimal light intensities for growth are still under debate, and here we show that they are also affected by collection origin. In the LL treatment, *L. japonica* species had the natural growth advantage and *L. turionifera* and *S. polyrhiza* showed slower growth in LL. In the HL treatment, growth was faster in dLL accessions. We hypothesize that a survival or stress tolerance strategy in dHL accessions to local adaptation of HL may be at play. Similar established trade-off strategies between growth and survival can

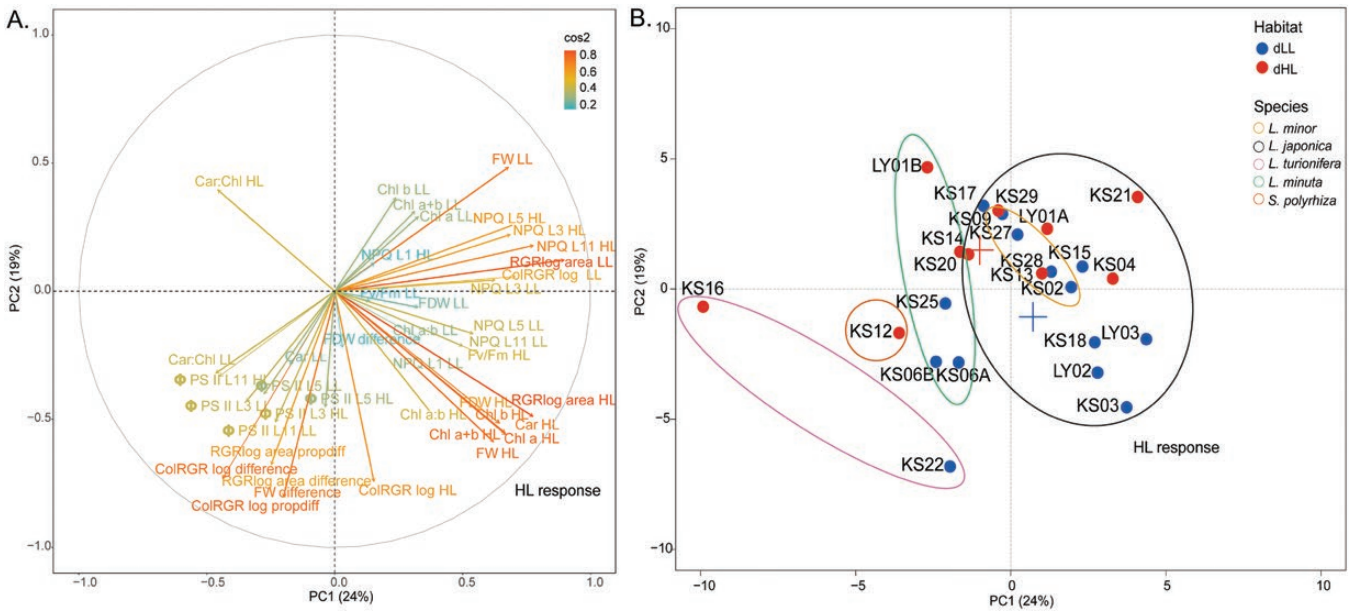

Fig. 9. (A) Fast growth in HL is associated with photosynthetic pigment contents. Principal component analysis (PCA) showing association of physiological responses from 24 accessions grown in HL and LL. The Cos2 scale shows how strongly the variables contribute to the dataset variability, and length and directions of arrows indicate the strength and relationships between variables. (B) Fast growth in HL is best achieved in dLL *L. japonica* individuals. Duckweeds broadly separate by HL response (top/bottom) and group into species (left/right) when using light physiological responses. PCA plot showing the relationship between accessions in the condensed landscape of 42 physiological variables under HL and LL treatment. Individual accession points are labelled and coloured by dHL (red) and dLL (blue). Centroids are marked with crosses for dHL and dLL accessions and represent averages for all physiological variables grouping by original habitat. Coloured ellipses represent the species types as shown in the key in Fig. 1. Ellipses overlap between *L. minor and L. japonica* species.

be seen for plants in nature (Pierce *et al.*, 2017; Zhang *et al.*, 2020). We suggest that this might be genetic in origin rather than epigenetic due to the multiple generations that took place between collection and experimentation (Huber *et al.*, 2021; Antro *et al.*, 2022).

Growth and acclimation are associated with species-specific responses

In addition to species differences in LL growth rate, we now show a dependence on habitat origin for HL growth rate. *Lemna japonica* is of note: it showed high growth in both LL and HL, higher Chl *a* and Chl *b* in LL, and highest Chl *a*:*b* and highest NPQ in LL and HL. *Lemna japonica* are hybrids between *L. minor* and *L. turionifera* reported here and recently in Braglia *et al.* (2021) and Volkova *et al.* (2023).

The turion-producing species *L. turionifera* (KS16 and KS22) and *S. polyrhiza* (KS12) were slow growing in LL but showed enhanced growth in HL. Additionally, effects on turionating capacity were evident between accessions and in response to light (Supplementary Fig. S5B). *Lemna turionifera* KS16 and *S. polyrhiza* KS12 also had higher F_v/F_m in HL than in LL. As the only *Spirodela* accession, KS12 appeared to have a distinct photosynthetic acclimation response profile. In its wild habitat, KS12 has high coverage in summer and is visibly red, and was the only accession to visibility accumulate anthocyanin in our controlled experiment (Fig. 1C; Supplementary Fig. S5A). Anthocyanin and flavonoid genes are more expansive in *S. polyrhiza* and less prevalent in *Lemna* species, suggesting alternative mechanisms for photo-oxidative stress tolerance (Landolt, 1986; Davies *et al.*, 2022; Fang *et al.*, 2023, Preprint).

Maximum quantum yield is closely linked to growth in high light

F_v/F_m is normally associated with photoinhibition and correlated positively with higher HL growth rate. The ability to limit photoinhibition is likely to contribute to the success of the dLL accessions in HL conditions. Thus it may be more important in this context to assess relative rates of damage to PSII and rate of repair, as well as the ability to cope with irreversible damage. Recovery from photoinhibition is also promoted by other mechanisms such as antioxidant production which deserve further attention in duckweeds. Related to this, accumulation of flavonoids such as anthocyanins is induced by abiotic stresses in *S. polyrhiza* (Landolt, 1986; Böttner *et al.*, 2021).

Pigment responses aid light acclimation in fast growing accessions

Chlorosis in duckweed and increases in carotenoid levels can occur in response to light up to 1000 μmol m^{-2} s^{-1} (Paolacci *et al.*, 2018; Stewart *et al.*, 2020). Overall, plants from dHL lost more chlorophyll and carotenoids in HL than plants from dLL, suggesting that chlorosis was also part of the adaption between sites.

Acclimation to HL is often accompanied by an increase in Car:Chl, as the XC pool size increases, and an increase in Chl *a*:*b* as light-harvesting antenna size decreases. Here, growth in HL did induce a higher Car:Chl across all *Lemna* species and this also negatively correlated with growth rate, perhaps consistent with the importance of limiting PSII inactivation and the need for a higher F_v/F_m for high growth rates in HL. Unchanging Chl *a*:*b* ratios have been shown for *Lemna* clones already (Paolacci *et al.*, 2018; Stewart *et al.*, 2020) and also for other species such as barley (Murchie and Horton, 1997; Zivcak *et al.*, 2014). Correlation of total Chl *a* and Chl *b* with HL growth rate indicates that total chlorophyll is an important attribute for light acclimation.

We note that Chl *a*:*b* was inconsistent in comparison with HPLC data which used pooled samples. Whilst spectrophotometry reports a Chl *a*:*b* ratio of ~3.5 and HPLC a ratio of 2.7 or 1.9 depending on the light condition, we expect that these differences in Chl *a*:*b* arose from differences in sample preparation. Whilst freeze-drying gives added accuracy when measuring pigments by mg g^{-1} (Lichtenthaler and Buschmann, 2001), low temperature and vacuum treatment can degrade chlorophyll (King *et al.*, 2001), especially Chl *b*, which could lead to an overestimation of Chl *a*:*b* from freeze-dried tissue (Lashbrooke *et al.*, 2010). Relative species differences in Chl *a*:*b* ratios indicate that *L. japonica* was more typical of sun tolerance and *L. minuta* of shade tolerance. The HPLC data also showed high de-epoxidation rates in HL consistent with this being a highly light-saturating condition and further emphasizing the role of photoinhibition in determining growth.

Reduced light intensity, extremely different peak wavelengths of light and altered proportions of spectral quality, including higher FR, and reduced R and G, would be expected to affect light acclimation and chlorophyll content of accessions from LL habitats. Indeed, shade-tolerant plants grown in controlled HL had higher chlorophyll and carotenoid contents per dry weight which correlate with HL growth. dHL accessions may be less sensitive to controlled HL, previously experiencing >1000 μmol m^{-2} s^{-1} in their habitats, and may acclimate in other ways, linked to a survival strategy rather than a dominant or competitor strategy, characterized by increased growth. The exception is species-specific acclimation as seen in *S. polyrhiza* here. Related to this, we anticipate that the dHL conditions may have been hostile enough to induce a range of stress tolerance responses which we observe as F_v/F_m reduction and pigment composition alterations.

Applications to agriculture

The species and habitat of duckweeds are important in selection for commercial purposes as environmental light contrasts were substantial. Increased growth, chlorophyll retention, and carotenoid gain were greater in *L. japonica* dLL ecotypes in increased light conditions compared with dHL ecotypes.

Further, we suggest that dHL ecotypes are less suitable for sustainable vertical farming systems, with the exception of *S. polyrhiza*, which had species-specific strategies for light adaptation, including anthocyanin accumulation.

Concluding remarks

We have focused here on accession-level and species-level variation in light adaptation, revealing widespread natural variation and broad local adaptation within species. Ecotypes from LL environments showed better acclimation of growth and pigment content to controlled HL. Pigment composition may be important in determining overall photosynthetic, growth and photoinhibitory traits because higher chlorophyll and carotenoid content in controlled conditions were related to LL habitats, suggesting biochemical or structural adaptation. How such adaptation occurs is intriguing, given the paucity of information on duckweed natural variation and adaptation to varied environmental factors. Our work provides a first step towards understanding environmental factors that are likely to select for genetic variation relevant also to subsequent breeding of duckweed accessions most useful by virtue of their distinct potentials.

Supplementary data

The following supplementary data are available at *JXB* online.

Fig. S1. Duckweed growth rates are affected by light intensity.

Fig. S2. Duckweed photosynthetic parameters are affected by light intensity.

Fig. S3. Carotenoid pigments show differential profiles in duckweeds in response to light treatment.

Fig. S4. Negative relationship between duckweed surface coverage and autumn UV light intensity.

Fig. S5. Species-specific responses to light treatment include anthocyanin production and altered turion formation.

Fig. S6. Ecotypic variation in light adaptive responses. Proportional changes in growth by area, colony gain, changes in F_v/F_m, NPQ, and pigment content between light treatments differ between accessions.

Fig. S7. Linear relationships between physiology of growth and photosynthetic responses in HL and LL treatments.

Table S1. Duckweed UK sites have five identified species, and site coverage varies across season and year time points.

Table S2. Seasonal light variables used to characterize dHL and dLL groupings.

Table S3. Grouped light variables into dHL and dLL sites show extreme light environments across seasons.

Table S4. Temperature and rainfall data across dHL and dLL sites indicate homogeneous conditions.

Table S5. Relative growth rate by area and colony gain and pigment contents between light treatments vary between accessions, original light habitats, and species.

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Author contributions

KES: performing field collection, lab experiments, and data analysis; LC and BT: aiding with pigment extractions; LM: writing the code for chlorophyll fluorescence experiments; MH: writing the wrapper for short read data processing; LY: performing field collections; EHM: supporting the project; KES and EHM: conceptualization and writing the manuscript, with all authors contributing to the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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Data availability

The sequences for duckweed genomes in this panel are deposited under project PRJNA1026139 on the NCBI Sequence read archive (SRA).

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Supplementary data

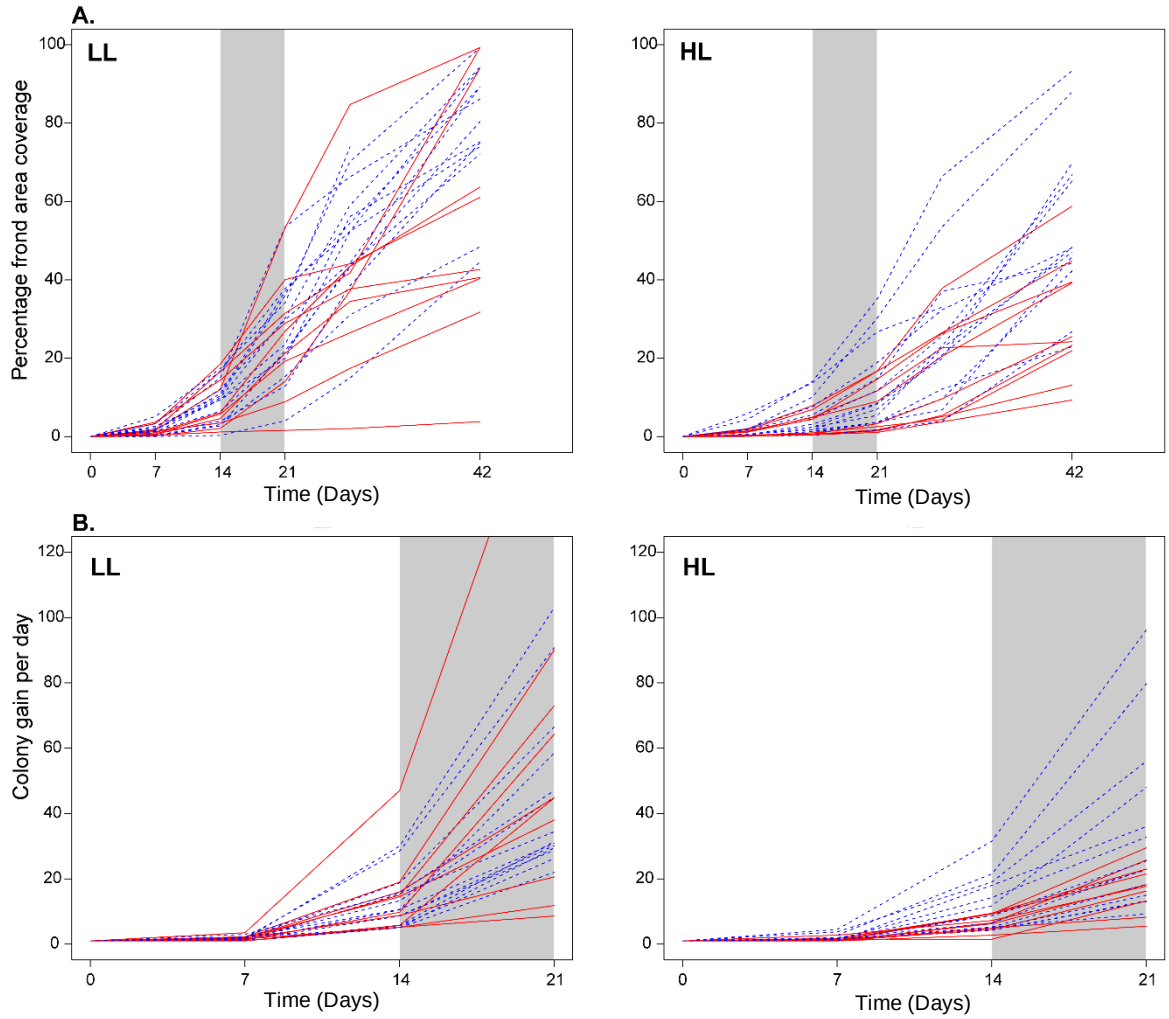

Figure S1. Duckweed growth rates are affected by light intensity. *A.* Growth rate of green tissue as percentage total area coverage plotted over time and used to derive average RGRlog per day (mm^2 gain per day in the log phase) for duckweed accessions grown in LL (left) or HL (right) light treatment. The area shaded in grey between day 14 and 21 was used to calculate RGRlog area. *B*. Growth rate by colony gain of duckweed accessions in LL (left) or HL (right) treatment. The area shaded in grey between day 14 and 21 was used to calculate Col RGRlog, the log phase of growth in colony gain per day. Duckweeds show a period of lag followed by logarithmic growth rate patterns and RGR decline in HL-grown plants relative to LL-grown plants. Each accession is plotted as average growth rate where lines were averaged from *n*=5 reps from each light treatment and each growth measurement method. Blue dashed lines represent accessions from dLL sites and red lines represent dHL accessions.

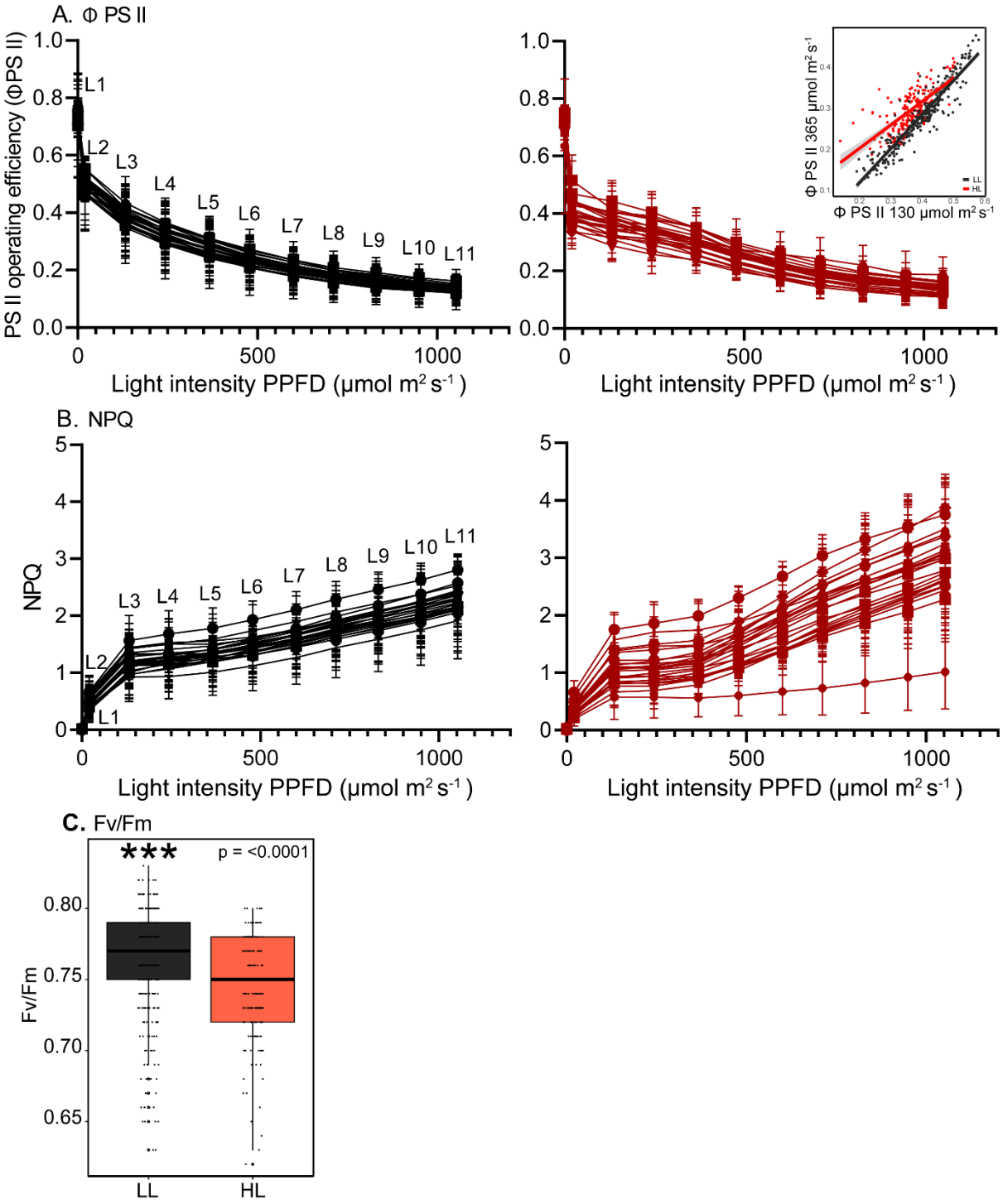

Figure S2. Duckweed photosynthetic parameters are affected by light intensity. *A.* Photosynthetic efficiency (ϕ PSII) declines faster and *B*. Higher maximum NPQ is induced in HL-grown plants in response to increasing light. ϕ PS II and NPQ measured at 11 light levels show average light response curves using a Fluorcam and parameter calculations derived from chlorophyll fluorescence (Murchie & Lawson, 2013). All accessions grown in LL (black) or HL (red) with *n* = 15 replicates for each treatment-accession combination grown from five separate growth rate experiments. Light levels allocated as L1-L11 correspond to PPFD: 0, 20, 130, 245, 365, 480, 600, 710, 830, 950, 1050 µmol m^{-2} s^{-1}. The light levels closest to growing treatment conditions for LL is L3 and HL is L5. *Inset.* ϕ PS II decline has a higher slope but lower intercept in HL-acclimated plants compared to LL- plants. Linear regression models fitted for ϕ PS II between L3 and L5 are for HL- -0.043 + 0.83 x, R2 = 0.52 and LL-

grown plants 0.092 + 0.57 x, R2 = 0.87. The linear model was a better fit for LL data. *C.* Maximum photosynthetic quantum yield F_v / F_m is higher in LL- treated plants than HL-treated plants. Boxplots indicate variation in maximum F_v / F_m across accessions measured in the dark after growth in LL (grey) or HL (red). *** P = <0.001 by Welch's T test.

A. Table of average carotenoids in each light treatment as determined from HPLC

	HL	LL
Chl a:b	2.7 **	1.9
Car:Chl	1.0	2.4 *n.s*
XC pool (V+A+Z)	31.5	29.0 *n.s*
% XC pool/Car	31.4 ***	22.3
DEPs	65.8 ***	43.1
% Neo/Car	9.0	10.0 *n.s*
% Lut/Car	46.4	57.3 ***
% Vio/Car	6.7	10.2 *
% Ant/Car	7.1 *	4.9
% Zea/Car	17.4 ***	7.1
% β-car/Car	13.0	10.2 *n.s*

B. Chromatogram at 470 nm showing carotenoids separated by HPLC by absorbance

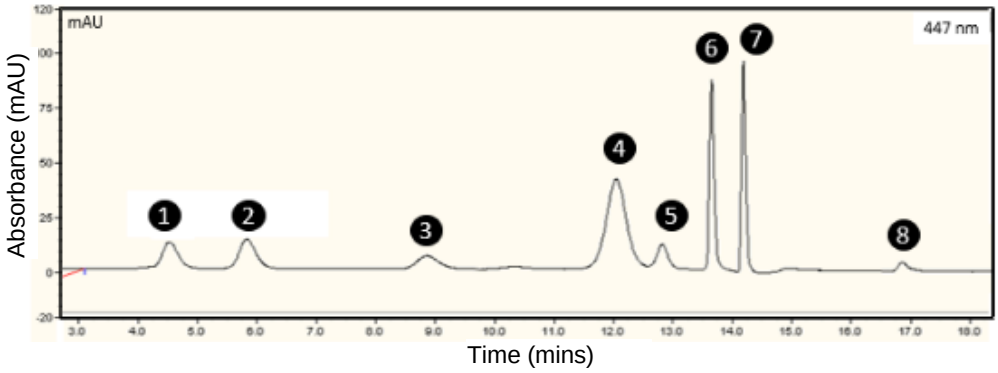

Figure S3. Carotenoid pigments show differential profiles in duckweeds in response to light treatment. *A.* Table of average carotenoids in each light treatment as determined from HPLC. *B.* Chromatogram at 470 nm showing carotenoids separated by HPLC by absorbance, peaks labelled left to right correspond to 1. Neoxanthin 2. Violaxanthin 3. Antheraxanthin 4. Lutein 5. Zeaxanthin 6. Chl b 7. Chl a 8. β-carotene. Table of light treatment variation is displaying Chl a/b and Car/Chl expressed as ratios. Total xanthophyll pool or XC pool (Vio + Ant + Zea). The %XC pool of all carotenoids, DEPs de-epoxidation state, and all independent carotenoids are expressed as percentages of total carotenoids: including Neo, Lut, Vio, Ant, Zea and β-car. Significant differences between light treatments was tested by Welch's T- test. Non-significance > 0.05 marked with *n.s,* * significant at *P* = < 0.05 *; < 0.01 **; < 0.001 ***. Higher % Xanthophyll pigments of the total carotenoids were produced by duckweed accessions in HL but there was no change in XC pools between light. De-epoxidation status related to the xanthophyll cycle was significantly higher in HL with DEPs ranging between 23 and 64% and increasing to between 26 and 88% in HL. Typically with higher DEPs in HL, Higher % Vio in LL was concordant with conversion to higher de-epoxidated forms of % Ant and % Zea in HL, indicating light stress. Neo and β -carotene remain comparable between treatments and did not form a large component of the duckweed carotenoid pool with lutein as the dominant carotenoid type overall but was significantly higher in LL treatment.

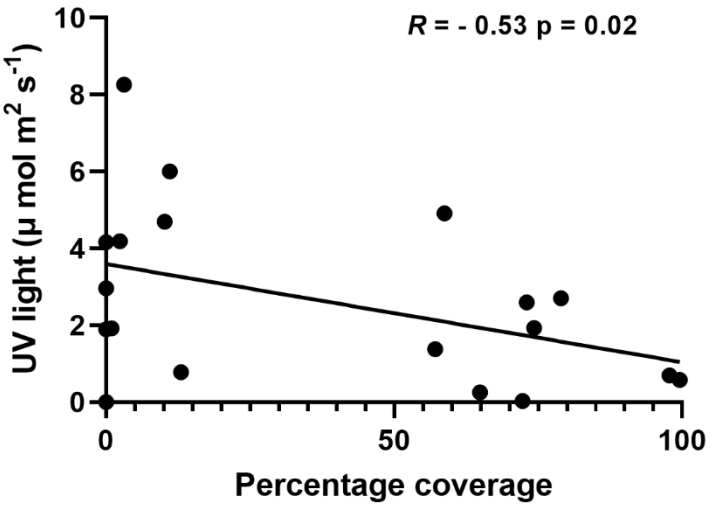

Figure S4. Negative relationship between duckweed surface coverage and Autumn UV light intensity. Linear regression model between habitat duckweed coverage and UV light intensities paired for each site as measured in Autumn 2021. Pearson model R and *P* values indicate significantly negative relationship, *n* = 19 sites.

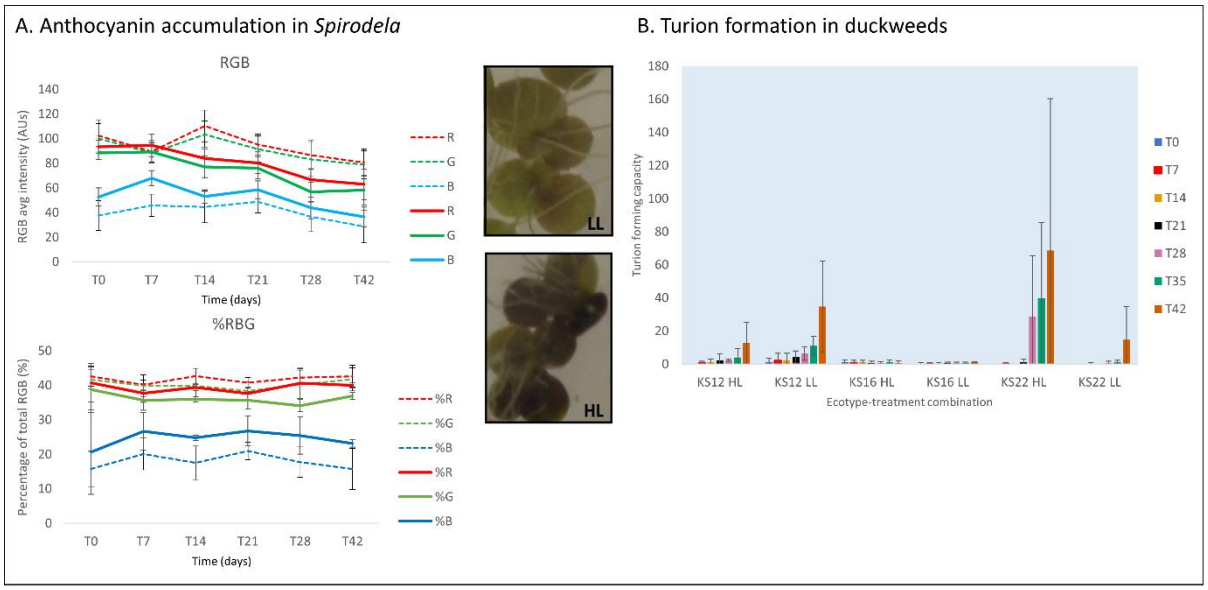

Figure S5. Species-specific responses to light treatment include anthocyanin production and altered turion formation. *A.* Line plots showing Anthocyanin accumulation in *Spirodela* shown by reflection of RGB intensity (arbitrary units) and %RGB of total intensity of duckweed *S. polyrhiza* KS12 grown in two controlled light treatments for six weeks. Dashed lines represent RGB values in LL treatment and full lines represent RGB channels in HL treatment, two representative images at LL and HL at day 42 are shown for visual comparison. Ten frond areas were selected per photograph from photographs from five growth experiments to measure independent red, green and blue channel measurements per timepoint using Fiji. Each of the ten regions were averaged and then averages from five growth rate experiments plotted, with error bars representing SD. *B. Spirodela polyrhiza* and *L. turionifera* species show differences in turion-forming capacities between accessions and light treatments overtime. Growth rate experiments *n*=5 replicates with SD as error bars. Other species showed no evidence of anthocyanin accumulation or turion formation.

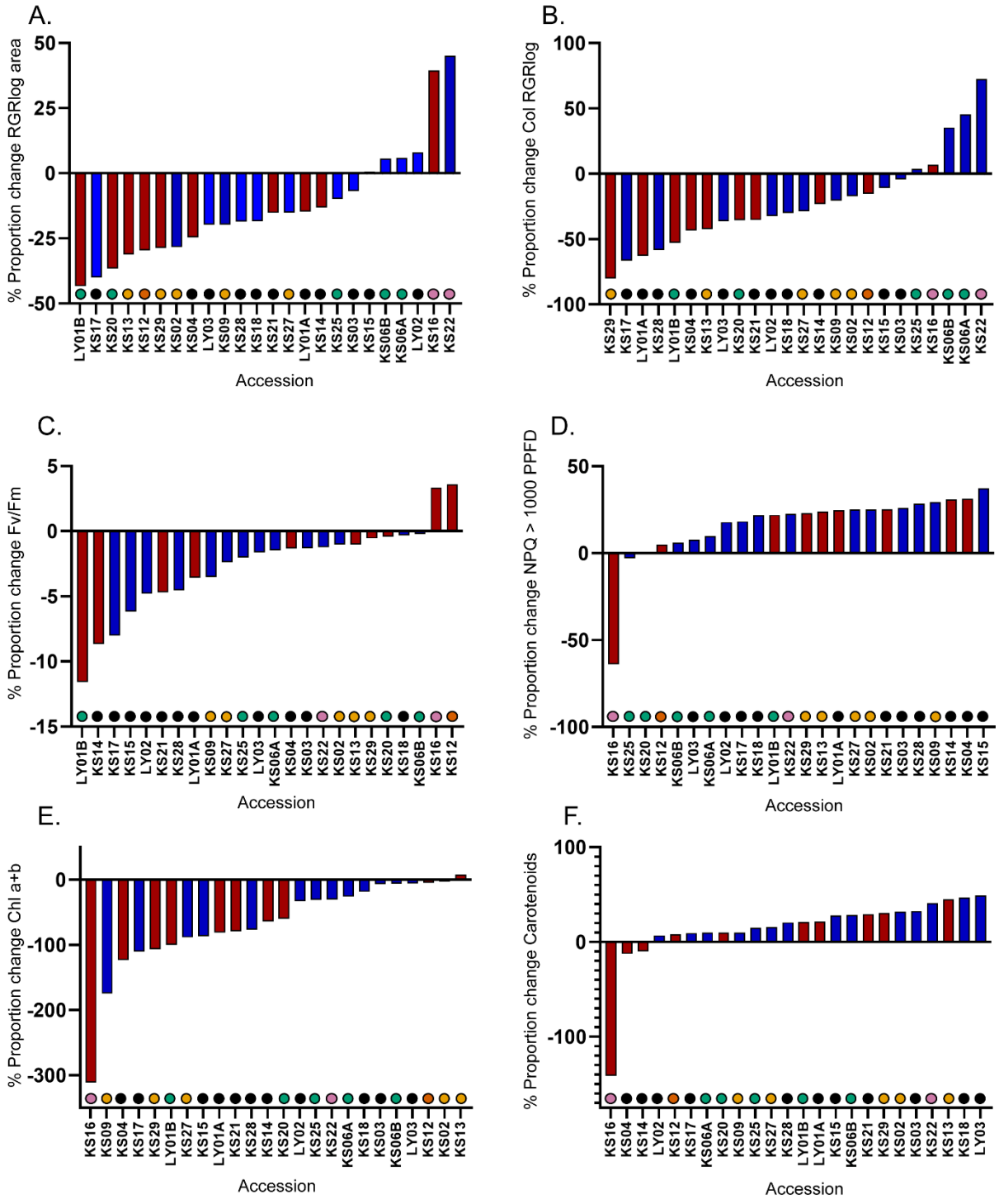

Figure S6. Accession variation in light adaptive responses. Proportional changes in growth by area, colony gain, changes in F_v/F_m, NPQ and pigment content between light treatments differ between accessions. *A.* Proportional percentage differences for difference in growth rate in HL relative to LL by RGRlog area gain difference (mm^2) for each accession. *B.* Proportional percentage difference in growth by colony gain Col RGRlog between low and high light treatments for each accession. *C.* Percentage proportional change showing difference in HL response for duckweed accessions for F_v/F_m measured in the dark. D. NPQ proportional difference between HL and LL, measured at maximum light (>1000 μmol m^2 s^{-1} PPFD). *E.* Percentage proportional change in average Chl a+b difference between light treatments for each accession. *F.* Percentage proportional change in

average total carotenoid difference between light treatments for each accession. Each bar represents average differences for each measurement for each accession *n*=5 for each growth method and *n*=15 for F_v/F_m, NPQ and *n*=20 chl and car contents. The x axis of barplots arranged in descending order of response and accessions are coloured by original site environmental data light level: dLL (blue), dHL (red). Coloured circles represent species.

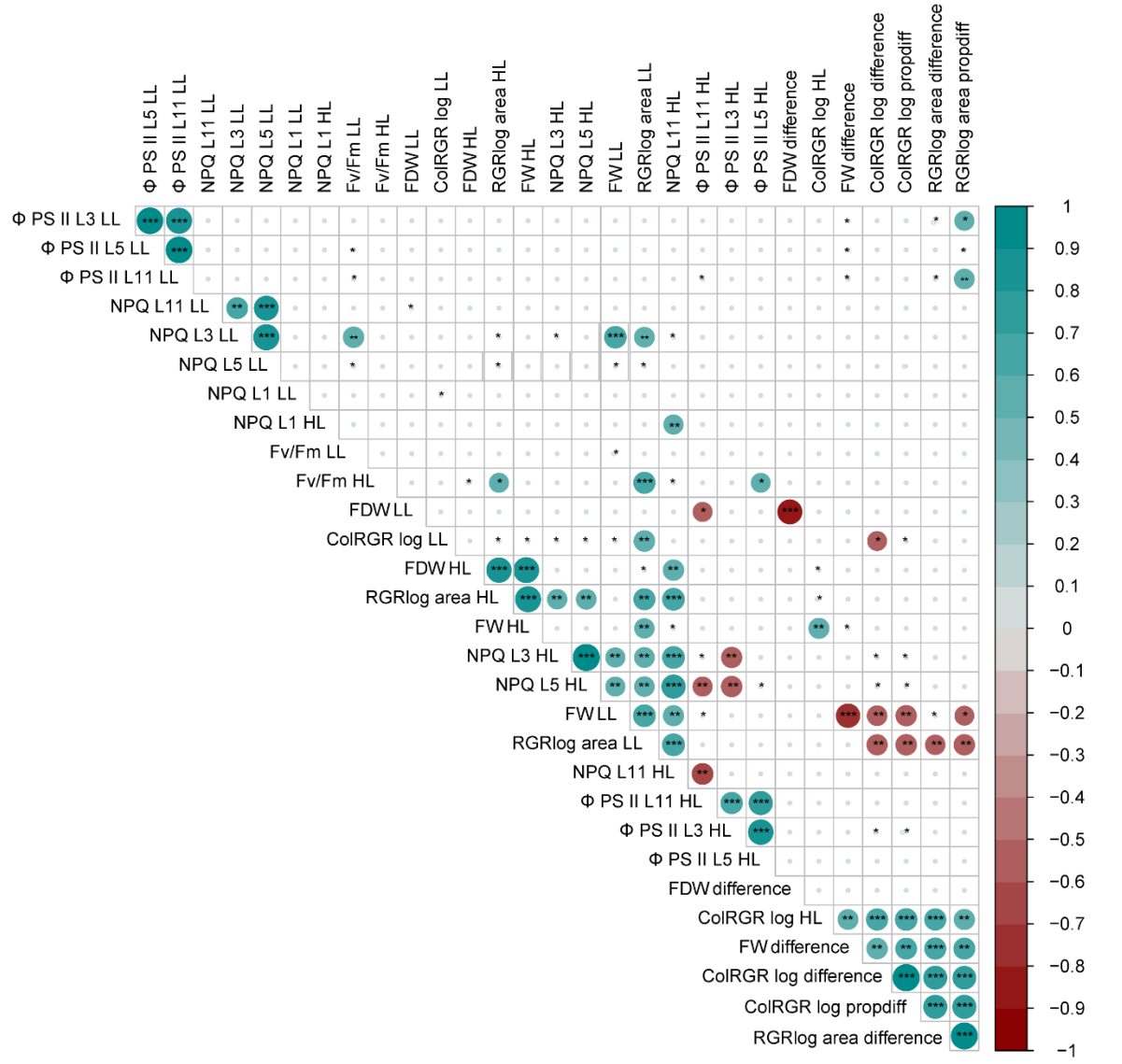

Figure S7. Linear relationships between physiology of growth and photosynthetic responses in HL and LL treatments. Corr plot for pairs of averaged physiological growth and photosynthesis variables measured from chlorophyll fluorescence during high and low light treatment, growth rate *n*=5 reps and photosynthesis 15 reps for each accession-treatment combination. Photosynthetic parameters include Fv/Fm, ϕ PSII and NPQ at light levels L1, L3, L5, L11 from light response curves for duckweed accessions grown in HL or LL treatment, corresponding to light intensities as in Supplementary Figure 2A-B and Table 1. Growth rates measured as RGRlog area, Col RGRlog and biomass as fresh weight (FW) and freeze-dried weight (FDW) and absolute and proportional differences between growth in HL and LL are included. The sizes of circles and number of stars indicate significance of relationship. Red indicates negative relationship R < -0.5 whilst blue indicates positive relationships > +0.5. Significant relationships by Pearson correlations are indicated by asterisks, *P* = <0.05 *, <0.01 **, <0.001 ***.

Table S1. Data attachment. **Duckweed UK sites have five identified species and site coverage varies across season and year timepoints.** Table displaying names of duckweed site locations, with longitude, latitude and altitude data and surface coverage of duckweed sites across seasonal timepoints, with species of duckweeds identified there and their official clone numbers as registered at ruduckweed.org. Coverage data is given to 3 dp and latitude, longitude coordinate data to 5 dp. The lowest average coverage was early spring with a minimum of 12% in March 2021 and highest in summer in July 2021 (46%) and July 2022 (44%).

Table S2. Data attachment. **Seasonal light variables used to characterise dHL and dLL groupings.** Measured light variables and derived proportional percentage of spectral composition of light for 19 duckweed locations across seasonal-year timepoints.

Table S3. Data attachment. **Grouped light variables into dHL and dLL sites show extreme light environments across seasons.** Differences in measured light variables and derived proportional percentage of spectral composition of light for 19 duckweed locations across seasonal-year timepoints. Locations are grouped into dHL and dLL sites by light data, showing averages with ± standard deviation within site groupings. Stars represent significant differences between dHL and dLL groupings for each season and year by T tests. *P* = <0.05 *. <0.01. **, <0.001 ***.

Table S4. Data attachment. **Temperature and rainfall data across dHL and dLL sites indicate homogeneous conditions.** Table summarising measured atmospheric and water temperatures at each site at each timepoint and average temperature and rainfall data from Bioclimatic databases. Locations are grouped into dHL and dLL sites as defined by light data, displaying averages with ± standard deviation within site groupings. There are no significant differences between dHL and dLL groupings for any other environmental variable by T tests.

Table S5. Data attachment. **Relative growth rate by area and colony gain and pigment contents between light treatments vary between accessions, original light habitats and species.** Table of results of ANOVA for factors and interaction between factors for *A.* growth rate RGRlog by area, *B.* RGRlog by colony gain data, *C.* Total chl a+b and *D.* Total carotenoids measured in mg/g duckweed tissue for 24 accessions grown in two treatments. Light treatment, accession and light habitat are reported significant in each case as well as species for all but carotenoid content. *n.s* represents non-significant *P* > 0.05. *n*=5 reps for each accession-treatment combination for growth rate parameters and *n*=20 reps for each accession-treatment combination for pigment contents.

5. Chapter five: Aroma and metabolite profiling in duckweeds: exploring species and ecotypic variation to enable wider adoption as a food crop

Preface: UK duckweeds were characterised in chapter three and light responses assessed in chapter four. UK *Lemna* and *Spirodela* ecotypes with variable light responses were further considered here as candidates for food application. Virtually nothing is known about aroma of duckweed e.g. VOC profiles, despite its potential applications in food. For human acceptability and towards uncovering potential health benefits from duckweed consumption, aroma compounds and metabolites present in duckweed require characterisation. A scaled up growth experiment using a glasshouse was used with UK ecotypes of four *Lemna* species and *Spirodela polyrhiza* to assess growth and nutrition for commercialisation of duckweed as a supplement powder. Multiple '-omics' experiments were used for comparisons of aroma compounds, metabolites (free amino acids, sugars, secondary metabolites) between powders produced from different species. A fieldspectrometer was used as a high-throughput method to predict growth, metabolite and health traits for quick identification of optimal accessions from a large phenotyping cohort. Defining the aroma profiles and comparing these to other herbs and vegetables fills a knowledge gap and aligns duckweed with other acceptable food sources.

Aims: To quantify key duckweed volatile compounds which may contribute to duckweed odour. To relate these aroma compounds as biomarkers for pleasant and off-putting aroma descriptors and consider these in the contexts of duckweed acceptability and storage potential. To measure aromatic profile differences in powders produced from four *Lemna* species, with *Spirodela* and with non-duckweed herbs and vegetables powders to better describe aroma. To measure accuracy of a fieldspectrometer as a prediction tool for growth and metabolite status of duckweed species and ecotypes. Finally, to suggest UK species and ecotypes which are scalable based on growth, aroma and metabolite profiles for commercialisation as a powder supplement.

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Aroma and metabolite profiling in duckweeds: Exploring species and ecotypic variation to enable wider adoption as a food crop

Kellie E. Smith [a,d,*], Martin Schäfer [b,g], Mui Lim [c], Carlos A. Robles-Zazueta [d,e], Laura Cowan [a], Ian D. Fisk [c,f], Shuqing Xu [b,g], Erik H. Murchie [d]

[a] Future Food Beacon of Excellence and School of Life Sciences, University of Nottingham, Nottingham, NG7 2RD, United Kingdom
[b] Institute for Evolution and Biodiversity, University of Münster, Münster, Germany
[c] International Flavour Research Centre, Division of Food, Nutrition and Dietetics, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, United Kingdom
[d] Division of Plant and Crop Sciences, School of Biosciences, University of Nottingham, Sutton Bonington, LE12 5RD, United Kingdom
[e] Global Wheat Program, International Maize and Wheat Improvement Center (CIMMYT), Carretera Mexico-Veracruz km. 45, El Batan, Texcoco, C.P. 56237, Mexico
[f] International Flavour Research Centre (Adelaide), School of Agriculture, Food and Wine and Waite Research Institute, The University of Adelaide, PMB 1, Glen Osmond, South Australia, 5064, Australia
[g] Institute of Organismic and Molecular Evolution (iomE), University of Mainz, 55099, Mainz, Germany

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ABSTRACT

Duckweeds (water lentils) are a nutritious human food source, with *Wolffia* species consumed traditionally in Eastern Asia. Duckweed contain up to 45 % protein by dry weight, high macronutrients, minerals and carotenoids. However, duckweed are not cultivated at scale and there are circa 35 other species to consider for food potential in other global regions. Here, we measured the suitability of four *Lemna* species and *Spirodela polyrhiza* for nutritional assessment, by scaling up growth of 25 ecotypes from the United Kingdom in a glasshouse. Here we showed intra- and inter-species variation of aromatic and metabolic profiles, together with biomass obtained from production. The dominant volatile organic compounds (VOCs) in duckweed are hexanal, 1-penten-3-one, 1-penten-3-ol, *cis*-2-pentanol and pentadecanal, with variations in amounts of 22 other compounds between species. In comparison with other leafy herbs, duckweed aroma profiles were most similar to spinach and dandelion with high 'green' and 'fresh' aroma compounds. *Spirodela polyrhiza* contained high flavonoids including apigenin and luteolin, offering potential benefits for health. Our results demonstrate that *Lemna* and *Spirodela* species have suitable flavonoid and amino acid profiles for nutrition. VOCs found here had positive aroma descriptors and can be used as biomarkers of freshness during storage of duckweed foodstuffs.

1. Introduction

Duckweeds, also known as water lentils, offer exceptionally rapid vegetative growth and high global availability, providing a sustainable alternative to both animal and plant protein such as soybean [1]. Duckweeds are additionally used in circular economy projects to produce animal and fish feeds [2,3] and are proposed for human food production due to their versatility for growth in outdoor ponds and vertical farms [4]. Small space requirements and fast growth make some species promising for space horticulture [5,6]. Duckweeds contain up to 30–45 % total dry weight protein, all nine essential amino acids, are high in potassium and iron [7–9] and contain high levels of carotenoids, especially lutein and zeaxanthin [10–12]. Furthermore, the starch content can exceed 70 %, making duckweed a potential carbohydrate source for food and biofuel [13].

Several rootless species have traditionally been used in Asian dishes known as "Khai-nam" (*Wolffia arrhiza* and *Wolffia globosa*), and are now commercially cultivated as the supergreen "Mankai" [14,15]. *Wolffia* species contain bioavailable amino acids above the world health organisation (WHO) recommended levels [9,16]. "Mankai" contains 200 polyphenol compounds from flavonoid and phenolic acid groups, with associated potential anti-cancer, anti-inflammation and anti-microbial properties [17,18]. However, with 36 species of duckweed to choose from globally, not all species have been equally considered as food crops. Additionally, variation in growth, protein, starch content, and available minerals among different species and ecotypes of duckweed

* Corresponding author. Future Food Beacon of Excellence and School of Life Sciences, University of Nottingham, Nottingham, NG7 2RD, United Kingdom.
E-mail address: kellie.smith@nottingham.ac.uk (K.E. Smith).

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have been reported [8,19,20] and furthermore will be dependent on environment and available nutrients.

More recently, other genera of duckweeds have also been evaluated for food applications, especially in regions where *Wolffia* is less prevalent (e.g. Europe). When combined in a chicken feed, dried *Lemna* and *Spirodela* species of duckweeds exhibited high amino acid digestibility [21]. *Lemna minor* protein powder now has GRAS status in the USA and is undergoing regulation in the EU as a novel food [22]. Moreover, extraction of Ribulose bisphosphate carboxylase/oxygenase (Rubisco) from mixed *Lemna minor* and *Lemna gibba* species is considered safe in Europe [23], and has been incorporated as an animal protein analogue [24].

Rooted duckweed species from the genera *Lemna* and *Spirodela* have uses in the treatment of allergies, inflammation, and tumours [25–27], likely these properties can be attributed to antioxidants, including flavonoids in *Lemna minor*, *Spirodela polyrhiza* and *Landoltia punctata* [28–31]. *Lemna minor* pills are manufactured as an herbal remedy available in the USA [32] and *Lemna minor* extracts exhibit antibacterial and antifungal activity against food spoilage microorganisms [33–35] and possess pesticidal properties against weeds [36,37]. Despite this, volatile and metabolite profiles have not been characterised largely between species and bioactivity of specific compounds have not been related to these functions.

Aroma perception is based on the unique combination of aroma compounds and their respective odour thresholds. Scents are detected by olfactory receptors both orthonasally during sniffing and retronasally during chewing, and this is key for human decision-making about edibility and safety of foods. The excess production of certain volatile organic compounds (VOCs), which are associated with undesirable smells, can limit the shelf-life of food. This is evident in leafy salads such as spinach, where the accumulation of off-notes are a limiting factor in post-harvest storage [38,39]. Enhancing compounds within duckweed aroma profiles attributed with 'pleasant' and reducing those associated with 'malodourous' could therefore be used for selecting species and ecotypes with enhanced appeal. Moreover, plant production of VOCs can act as defensive compounds against herbivores and insects, which may enhance crop resilience and foodstuff storage potential [40]. Therefore, understanding intra- and inter-species variation in VOCs, and their complex metabolic pathways could be used in synthetic engineering of future crop varieties [41].

Despite the promise of duckweeds as a new food source, resistance to duckweed acceptability in Western Europe was identified in some consumers due to association with unclean water [42]. Moreover, the drive for sustainable and resilient food systems includes using novel plant ecotypes which are well adapted to a local growing environment, stimulating regional economy and giving shorter distances for transport [43]. To address these shortcomings, we compared organoleptic value and nutritional properties among UK-derived duckweeds composed of *Lemna* species and *Spirodela.* These ecotypes previously showed variation in growth, adaptation to high light and carotenoid contents [12]. Aroma (VOC) profiles are discussed in the contexts of human acceptability for consumption and shelf-life. The aim is to recommend duckweed candidates for sustainable food development within the UK and Europe.

2. Materials and methods

2.1. Selection of duckweed ecotypes and herbs

Twenty-five duckweed ecotypes within four *Lemna* species and one *Spirodela* species were selected: *L. minor*, *L. japonica*, *L. minuta*, and *L. turionifera* and *S. polyrhiza*. Ecotypes were chosen from a UK duckweed collection consisting of >100 ecotypes which were collected between 17/05/20 and 15/07/22. Species were identified using next generation sequencing and the selected ecotypes were previously grown on a small-scale in a controlled light environment, showing varied growth rates and tolerance to light [12], The ecotypes used are detailed in Fig. S1 and summarized in Tables S1 and S2. Other leafy green vegetables were sourced as seeds including spinach (Mr Fothergills, UK), and aromatic herbs including coriander (Mr Fothergills, UK) and red sweet basil (D.T Brown, UK) from a local garden centre and used as comparative controls for aroma profiling described below.

2.2. Glasshouse system for growth of duckweed ecotypes and herbs

Large-scale duckweed production was carried out for six months during winter 2021 to spring 2022 at Sutton Bonington campus, University of Nottingham, UK. Four batches of 25 duckweed ecotypes were grown simultaneously in quadruplicate (see Fig. 1), these formed randomly positioned replicates, around a glasshouse offering 7 m^2 total growing space. Duckweed ecotypes were set up using three healthy three-frond colonies within black seed trays (32.5 × 22.5 × 5 cm) containing 1 dm^3 Nutrient (N) medium covered with Plastic propagator lids Apet (H. Smith plastics, UK). Each tray was harvested every two months, except in the slower growing ecotypes, which were harvested when trays had 95 % duckweed surface coverage. After each harvest, three colonies were used to restart growth. A maximum of three harvests over six months were completed from each tray, with an experimental end point in April 2022 (Fig. 1). Commercial seeds of spinach, coriander and red basil were grown in the same conditions in seed trays of Levington M3 soil, for subsequent aroma profiling.

2.2.1. Nutrition and growing environment

Duckweeds were grown on a large scale and non-aseptically, representing potential commercial growing conditions. N-medium is an optimum duckweed growing media described in Ref. [44], consisting of KH_2PO_4 (0.15 mM), $Ca(NO_3)_2$ (1 mM), KNO_3, (8 mM), $MgSO_4$ (1 mM), H_3BO_3 (5 μM), $MnCl_2$ (13 μM), Na_2MoO_4 (0.4 μM), and FeEDTA (25 μM) with traces of Si, Cu and Zn. N-medium was made with reverse osmosis water and sterilized at 121 °C and replaced weekly in each tray to maximize nutrient dosage. Duckweeds were washed with reverse osmosis water in sieves and returned to trays containing fresh media weekly. At timepoints in spring, media was topped up weekly with 1 dm^3 reverse osmosis water when evaporation was visible.

Duckweeds and herbs were grown in temperatures set at 23 °C and 21 °C day and night and monitored using a datalogger TGU-4500 (Gemini, UK) with this data presented in Fig. S2. Duckweeds and herbs were grown in natural day light supplemented with high pressure sodium bulbs, supplying a total maximum light intensity of 180 μmol photons m^{-2} s^{-1}. An extended photoperiod of 16 h was provided, with supplementary lighting between 7 a.m. and 23 p.m. Light intensity and light quality were measured above each replicate tray using a light meter LI-250A (LI-COR, Biosciences, NE, USA) and a handheld spectrometer LI-180 (LI-COR, Biosciences, NE, USA) (Fig. 1). All light measurements are presented in Tables S3A and 3B.

2.2.2. Measurements of duckweed health

Photographs were taken after four weeks of growth (Fig. 1) with a Canon 650D camera (Canon Inc., Tokyo, Japan) 40 cm above each tray with the whole tray in the field of view. Average greenness value and fraction cover of duckweed per tray were obtained from images and used to measure duckweed health and growth. Each parameter was derived using Fiji image processing software using five random rectangles [45] per image as described in Ref. [12]. Average greenness was obtained by extracting red-green-blue (RGB) values using ten regions within each rectangle. Growth as percentage coverage of green biomass was calculated relative to background area in each rectangle from photographs.

As a proxy for duckweed health and growth, reflectance data was collected using an ASD Fieldspectrometer (ASD Field Spec 4, Malvern Panalytical, UK) after four weeks growth (Fig. 1). Reflectance of duckweed biomass was measured with the sensor's optic fibre at 20 cm above each tray, at 1 nm increments between 350 nm and 2500 nm. Three full

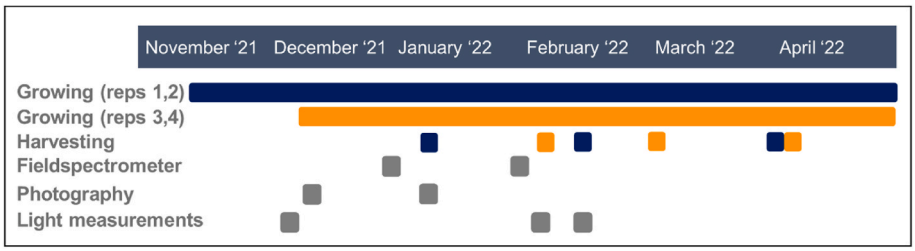

Fig. 1. Gannt chart indicating setup, harvest and end-point dates of duckweed glasshouse experiment. Growth and harvesting periods of each duckweed ecotype replicates 1 and 2 are indicated in blue and replicates 3 and 4 in orange. Photographs, fieldspectrometer and light measurement timepoints are indicated in grey and were collected for all replicates. Photograph and fieldspectrometer measurements were used for comparing duckweed health and growth during the experiment for comparison with subsequent harvesting data. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

range spectral measurements were made per tray. To estimate crop biomass and Nitrogen status, vegetation indices were calculated from reflectance data using spectral ratios of raw reflectance in the green and near infra-red regions. Nitrogen status was estimated using Other vegetation index (OVI) [46], and estimates for plant greenness and biomass used the following vegetation indices: Normalised difference vegetation index (NDVI) [47], Green index (GI) [48] and Green model (GM) [49]. All vegetation indices were calculated from reflectance where R corresponds to the wavelength of the measured reflectance in the following formulas:

$$OVI = \frac{R_{760}}{R_{730}} \quad \text{(i)}$$

$$NDVI = \frac{R_{800} - R_{680}}{R_{800} + R_{680}} \quad \text{(ii)}$$

$$GI = \frac{R_{554}}{R_{667}} \quad \text{(iii)}$$

$$GM = \frac{R_{750}}{R_{550}} - 1 \quad \text{(iv)}$$

2.3. Harvesting duckweed biomass

During each harvest, whole trays of duckweed ecotypes were washed with reverse osmosis water in sieves. Fresh biomass was then air-dried in the glasshouse for 15 min. Duckweed biomass was weighed from each tray to obtain fresh biomass per harvest. Biomass was then frozen in liquid nitrogen and stored at −80 °C until further aroma and metabolite processing.

2.4. Preparation of plant tissue

For aroma profiling, basil, coriander and spinach samples were collected from glasshouse-grown tissue and dandelion leaves were harvested from wild plants growing in Sutton Bonington, UK woodland area ($n = 4$ per herb). Duckweeds and herbs were freeze-dried for two days and re-weighed. Freeze-dried biomass were then ball-milled to a fine powder using a RETSCH PM400 ball mill (Haan, Germany). Fine freeze-dried duckweed and herb powders were then stored at −80 °C until aroma and metabolite analysis.

2.5. Aroma profiling

2.5.1. Preparation of samples for aroma profiling using SPME-GCMS

Duckweed powder (0.5 g) or dried herb samples (0.5 g) were weighed into Solid phase microextraction (SPME) amber vials. MilliQ (Merck Millipore) water (4 cm^3) and internal standard (0.001 cm^3 0.001 % 3-Heptanone in methanol (MeOH) v/v) was added to the dried samples. Samples were prepared over a two-week period with a random sampling design to process independent replicates of each ecotype from four locations within the glasshouse (25 duckweed ecotypes, $n = 4$, other herbs $n = 4$). Samples were then analysed using Solid phase microextraction Gas chromatography/mass spectrometry (SPME-GCMS).

2.5.2. SPME-GCMS to determine aroma profiles of duckweed

An untargeted volatilome approach was used to discover and semi-quantify volatile compounds. Sample volatiles were extracted from vial headspace for 30 min at 50 °C using 50/30 μm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre (Supelco, Sigma Aldrich, UK) followed by desorption for 1 min at 250 °C in spitless mode using a TriPlus robotic sample-handling (RSH) Autosampler. Analysis was conducted on a Thermo Scientific™ ISQ™ single quadruple mass spectrometer with a TRACE™ 1300 gas chromatography (GC) system. Separation was performed on a 30 m Zebron (ZB) wax column with inner diameter of 0.25 mm and 1 μm film thickness (Phenomenex Inc., Macclesfield, UK) using 18 PSI constant Helium pressure and mass separation (MS) full scan mode resolving mass to charge ratios (m/z) between 35 and 300.

2.5.3. Human aroma perception using GC-olfactory (GC-O) analysis

A small panel of five participants were selected for Gas chromatography -olfactory- mass spectrometry (GC-O-MS) for preliminary human perception of duckweed aroma. *Lemna japonica* KS18 freeze-dried powder was chosen for its high quantity. Sample volatiles were extracted using the same method in 2.5.1. and 2.5.2. but using a GC machine (TRACE 1300 GC, USA, and ISQ™ series mass spectrometer MS) customized with an olfactometry detector outlet [50]. Participants were asked to record times, descriptions and intensities of aromas. Those compounds most frequently reported at similar times during extraction contributed to aroma perception of duckweed.

2.5.4. Data processing of aroma profiles

Peak detection and integration was performed from raw data using TraceFinder 5.1 (Thermo Fisher Scientific) with deconvolution plugin 1.2. Spectral reference libraries (NIST/EPA/NIH Mass spectral library 2.0, National institute of Science and technology, Gaithersburg, MD) were used to identify compounds based on retention index and polar index. Retention time alignment was performed on all duckweed and herb samples using a threshold index of 100,000. A standard panel of Alkanes (C6–C20) (Sigma Aldrich, UK) were used to obtain linear retention index (LRI) for compound identification. Concentration of volatile compounds (μg/kg) were expressed relative to the ratio of compound peak area to the internal standard peak area.

For aroma analysis (and metabolite analysis, see 2.6. below), five species groups were formed from four replicates of 25 ecotypes: individual ecotypes within each species group were $n = 10$ *L. japonica*, $n = 5$ *L. minor*, $n = 5$ *L. minuta*, $n = 2$ *L. turionifera*, $n = 3$ *S. polyrhiza*. The total replicates per species were: *L. japonica* = 40, *L. minor* = 20, *L. minuta* =

20, *L. turionifera* = 8 and *S. polyrhiza n* = 12.

For each herb, the total replicates were $n = 4$. A Games-Howell post-hoc test was used to determine differences in amounts of VOCs between duckweed and herb pairs. Kruskal-Wallis and Wilcoxon paired post-hoc test with Benjamini-Hochberg correction were used to find inter-species differences for individual compounds. P = <0.05 was set for the significance boundary in each case.

For GC-O analysis, nasal impact frequencies (NIFs) were used to identify compounds with >50 % participant detection. These compounds contributed the most to aroma perception and are plotted as an aromagram. To determine the impact of each aroma compound relative to its concentration, odour activity values (OAVs) were calculated using odour thresholds from Ref. [51]. OAV is determined from the ratio of odour threshold and concentration in duckweed (μg/kg) whereby the lowest odour threshold and highest concentration give the greatest aroma contribution.

$$OAV = \frac{concentration}{odour\ threshold} \quad \text{(v)}$$

Odour descriptors for compounds were obtained from The Good Scents Company (thegoodscentscompany.com [52]) to associate compounds with pleasant or unpleasant aromas.

2.6. Metabolite analysis

2.6.1. Metabolite analysis via LC-MS/MS and HPLC-PDA

Metabolite analysis for soluble sugar, starch, free amino acids and secondary metabolites including flavonoid phenolic compounds was conducted with Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) as described in Ref. [53]. Secondary metabolites were additionally measured by High-performance liquid chromatography-Photo diode array (HPLC-PDA). The following adjustments were made: change of the LC-MS/MS machine and respectively the instrument settings, slight modifications in the compound list – mostly of secondary metabolites – and addition of the starch digestion step described in Ref. [54]. The steps required for phytohormone analysis were omitted.

2.6.2. Sample preparation

Each 10 mg±1 freeze-dried duckweed sample was aliquoted into 1.1 cm^3 96-well Mini tubes (Axygen) and homogenized in extraction buffer containing acidified methanol (MeOH:water:formic acid 15:4:1 v/v/v). The values represent the proximate levels of those compounds not including moieties that are bound to other compounds (therefore excluding e.g. amino acids incorporated into proteins). For soluble sugar analysis, an aliquoted sample of the extract was further diluted with 70 % MeOH containing sorbitol as internal standard. For free amino acid and secondary metabolite analysis via LC-MS/MS, another aliquot of the extract was diluted in an aqueous mix of isotope-labelled amino acids (algal amino acid mixture-13C–15N; Sigma-Aldrich). The remaining undiluted extract was used for secondary metabolite analysis via HPLC-PDA. For starch analysis, the sample pellets were re-extracted twice with 50 % ethanol (EtOH) at 80 °C, then resuspended and diluted in water, and incubated at 98 °C to gelatinize the starch, which was then digested using an enzyme mix containing amyloglucosidase and alpha-amylase overnight at 37 °C. Glucose monomers of the starch were eluted during the digestion into the aqueous phase, and subsequently diluted with 70 % MeOH containing sorbitol as internal standard.

2.6.3. LC-MS/MS measurement

Metabolite analysis was done on a Shimadzu Nexera X3 LC-System connected to a Shimadzu LCMS-8060 mass spectrometer. For soluble sugar and starch analysis the LC system was equipped with an Agilent 1290 infinity II inline filter (0.3 μm) and an apHera™ NH2 column (150 × 4.6 mm, 5 μm; Supelco). The mobile phase comprised 0.1 % acetonitrile (Fisher Chemical) in water as Solvent A and acetonitrile (Fisher Chemical) as Solvent B in gradient mode. For free amino acid and secondary metabolites, the LC system was equipped with an Agilent 1290 infinity II inline filter (0.3 μm) and a ZORBAX Rapid resolution high definition (RRHD) Eclipse XDB-C18 column (3 × 50 mm, 1.8 μm; Agilent Technologies). The mobile phase comprised 0.05 % formic acid (Fisher Chemical), 0.1 % acetonitrile (Fisher Chemical) in water as Solvent A and MeOH (Fisher Chemical) as Solvent B in gradient mode. The mass spectrometer was equipped with an Electrospray ionization (ESI) source and was operated in multi-reaction-monitoring (MRM) mode. The gradient program and column oven settings for the chromatographic separation, as well as the ESI- and MRM-settings were used as described by Ref. [54], with addition of some further secondary metabolites to method 1A.

2.6.4. HPLC-PDA measurement

Analysis of flavonoid contents was done on a Shimadzu Nexera XR liquid chromatography (LC)-System equipped with an EC 4/3 Nucleodur ® Sphinx Reversed phase (RP) pre-column (5 μm, Macherey-Nagel) and a Nucleodur ® Sphinx RP column (250 × 4.6 mm, 5 μm, Macherey-Nagel). The mobile phase comprised 0.2 % formic acid (Fisher Chemical), 0.1 % acetonitrile (Fisher Chemical) in water as Solvent A and acetonitrile (Fisher Chemical) as Solvent B in gradient mode. Measurement was performed with a PDA detector.

The gradient programs and column oven settings for the chromatographic separation, as well as the detector settings and absorption wavelength were used as described by Ref. [54] (method 1D) with addition of luteolin (absorption wavelength 348 nm, retention time 17, 880 min) and apigenin (absorption wavelength 337 nm, retention time 19,675 min).

2.6.5. Analysis of metabolite data

Metabolite analysis was performed with the LabSolutions software (Version 5.97, Shimadzu). Data were quantified based on internal and external standards, for LC-MS/MS and HPLC-PDA analysis, respectively. LC-MS/MS data of flavonoids, chlorogenic acid and shikimic acid were quantified based relative to isotopically labelled amino acid standard and are therefore reported as arbitrary units (AU). For all other data an absolute quantification is presented. The data were normalised to the dry weight (DW) of the extracted freeze-dried powdered plant material. Starch quantification was obtained in mg/g by multiplying glucose monomers with the molecular weight of anhydroglucose. Tryptamine was not detected in any duckweed and therefore excluded from analysis. Ecotypes were the same as reported in 2.5.4, with the omission of one *L. minuta* ecotype KS06A.

3. Results

3.1. Duckweeds have high prevalence of C5 and C6 volatile compounds

Duckweeds contain high amounts of five and six carbon (C5 and C6) 'green leaf volatiles' 1-penten-3-one, 1-penten-3-ol, hexanal, *cis*-2-pentanol and pentadecanal (Table 1, Fig. S3). Other C5 compounds include *trans*-2-pentenal, and the ketones 3-pentanone and 2,3-pentanedione. Other C6 compounds include 2-hexenal and the alcohols hexanol, *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol (Table S4). Carotenoid-derived beta-cyclocitral and *trans*-beta-ionone are other noteworthy compounds.

Additionally, contents of 1-penten-3-one are higher in northern UK ecotypes, benzaldehyde is higher in southern ecotypes and 1,3-di-*tert*-butylbenzene is higher in ecotypes from high light intensity environments (Table S5). Furthermore, twenty-two other compounds varied significantly between duckweed species (Fig. 2, Table S4).

3.2. Lemna minuta displays the most decreased aromatic profile

The duckweed species and ecotypes used in this study, the coordinates of origin and environmental data for origins are given in

Table 1
Top five duckweed volatile compounds by amounts as detected by semi-quantitative SPME-GCMS.

Compound	Retention time	LRI	CAS number	Descriptor	Function
1-penten-3-one	7.098	1041	1629-58-9	Spicy, pungent, peppery	Wound response, fungal resistance, ripening [55–57]
Hexanal	8.639	1101	66-25-1	Green, fresh, grassy.	Antimicrobial, enhance shelf-life [58–60]
1-penten-3-ol	10.632	1175	616-25-1	Ethereal, horseradish, green	Wound response, fungal resistance, ripening [55–57]
cis-2-pentenol	14.839	1337	1576-95-0	Green, phenolic, nasturtium	Reduce insect attraction, released from intact and mechanically-damaged leaves [61]
Pentadecanal	30.246	NA	2765-11-09	Fresh, waxy	Antimicrobial [62,63]

Functions are derived from the following sources [55]: Fisher et al., 2003 [56] Gorman et al., 2021 [57] Moummou et al., 2012 [58] (Song et al., 1996) [59] El Kayal et al., 2017 [60] Dhakshinamoorthy et al., 2020) [61] Tang et al., 2012 [62] Venuti et al., 2022 [63] Togashi et al., 2007. Descriptors are derived from The Good Scents Company (thegoodscentscompany.com). LRI = Linear retention index. CAS = Chemical abstracts service.

Fig. S1 (and Tables S1 and S2). Duckweed ecotypes were grouped by species for comparison. *Lemna minuta* has the lowest quantities for a range of aromatic compounds compared to other species. *Lemna minuta* has less 'green' descriptor compounds including tridecanal, 1-octen-3-ol, *trans*-geranylacetone but also fewer negative 'pungent/fatty' compounds like 2-tetradecanal and tetradecanal than *L. minor* (Fig. 2, Fig. S4). *Lemna minuta* has 'other' descriptor compounds higher than *L. minor* including butanol,3-methyl and 2-ethylfuran but lower levels of compounds without aroma descriptors. *Lemna minor* has the highest quantities of 'green' compounds including heptanal, *cis*-3-hexen-1-ol and 1-octen-3-ol. Pyrolle has a nutty aroma and was the only compound higher in *L. turionifera*. *Trans*-2-hexen-1-ol and 2-hexenal 'fresh and 'green' aromas are higher in *S. polyrhiza* than in *Lemna* (Fig. 2. Fig. S4A). Levels of VOCs also vary between individual ecotypes within a species but *L. minuta* ecotypes show the greatest consistency in profiles by clustering (Fig. S4B).

3.3. Duckweed volatile composition is similar to spinach and dandelion

Duckweed ecotypes are grouped by species and volatile profiles compared with other fast-growing herbs and leafy green vegetables. Comparisons of compounds common between duckweed and other herbs are given in Table S6. Duckweeds, dandelion, spinach and coriander have a comparable number of aromatic compounds; however basil contains more than double the number of total compounds at the same detection threshold, supported by its strong aroma. Duckweeds contain more hexanal and 'green', 'ethereal' C5 compounds: 3-pentanone, *trans*-2-pentenal, *cis*-2-pentenol, penten-3-one and 1-penten-3-ol than coriander or basil. Instead, basil and coriander have more 'woody' compounds including naphthalene- and sesquiterpenes with additional terpenes in basil (Table S6A). To detect differences between duckweed species, 32 compounds with concentrations <1 μg/kg in duckweeds were removed from analysis.

Duckweeds have the most similar aromatic profiles to spinach and dandelion, and are dissimilar to basil and coriander (Fig. 3A and B, Table S6). Duckweeds are higher than dandelion for 'ethereal' and 'pungent' aromas including 3-pentanone, naphthalene and 2-methyl naphthalene compared to higher 'minty', 'fresh', 'cheesy' and 'woody' aromas associated with dandelion. Nine compounds were found in duckweeds but not in spinach, with duckweed having higher 'green', 'fresh' and 'ethereal' positive descriptor compounds.

3.4. Penta-volatile compounds were frequently detected by participants

GC-O was conducted to identify key aroma compounds associated with duckweed (*L. japonica* KS18). Participants frequently identified fifteen compounds during headspace extraction, these are shown in Fig. 4. These include C5 alcohols and aldehydes, notably 1-penten-3-ol and beta-cyclocitral which are reported as odour active by all participants. Some compounds extracted from the headspace at similar run times and were hard to resolve, e.g. 1-pentanol with *cis*-4-heptanal (Fig. 4). Common aromatic compounds identified by the panel and their aroma descriptors are summarized in Table 2 to indicate 'pleasant' and 'malodourous' smells.

Of the 15 volatile compounds found in the duckweed species studied, only 7 have an odour activity value (OAV) high enough to significantly contribute to aroma profile of duckweed (Table 2). Butanal-3-methyl and tridecanal have the highest OAVs and 1-penten-3-ol and beta-cyclocitral have relatively low OAVs. The main positive aroma descriptors for *L. japonica* KS18 is a mixture of 'green', 'fruity', 'fresh' and on the negative end of the scale 'waxy', 'fatty' and 'oily' descriptors are common. Similar descriptors were identified between SPME-GCMS and GC-O for common duckweed VOCs (Table 1, Table 2, Fig. 4). Those odour active VOCs identified by participants in *L. japonica* including *trans*-geranylacetone, tridecanal, pentadecanal and butanal,3-methyl, are compounds which were significantly different in *L. minuta* compared to other species, therefore human perception of different species is expected to differ.

3.5. Spirodela and Lemna duckweed species have different free amino acid profiles but limited differences in sugar content

Duckweeds show a complete profile of free amino acids for human consumption, but show inter-species differences when grown in a common glasshouse environment. *Lemna minuta* and *S. polyrhiza* show decreased levels of the essential amino acids histidine and tryptophan compared to *Lemna minor*. In *Spirodela polyrhiza,* the aromatic amino acid precursor, shikimic acid is higher than *Lemna* species but otherwise *S. polyrhiza* displays lower levels of aromatic amino acid levels (Fig. 5). In *Lemna* and *Spirodela* duckweeds, the predominant sugar storage is starch, with lower levels of soluble sugars glucose and fructose. Sucrose levels were the lowest sugar detected but show a comparable average between species (Table 3). Sugar content is not significantly different between species but shows high variation between ecotypes and replicates.

3.6. Flavonoids are dominant in Spirodela polyrhiza compared to Lemna species

Spirodela polyrhiza in contrast to the four *Lemna* species is highly abundant in cyanidine- and chlorogenic-compounds, apigenin, luteolin and apigenin/luteolin 7-O-glucoside forms (Fig. 6, Fig. S5). *Lemna turionifera* has comparable luteolin 8-C-glucoside and more apigenin 8-C-glucoside than *S. polyrhiza*. Interestingly, these flavonoid compounds are low and not detected in other *Lemna* species. These findings were consistent between HPLC-PDA (Fig. S5) with LC-MS/MS (Fig. 6) using multiple ecotypes within species. *Lemna* species do however show higher levels of free amino acids compared to *Spirodela polyrhiza* (Figs. 5 and 7).

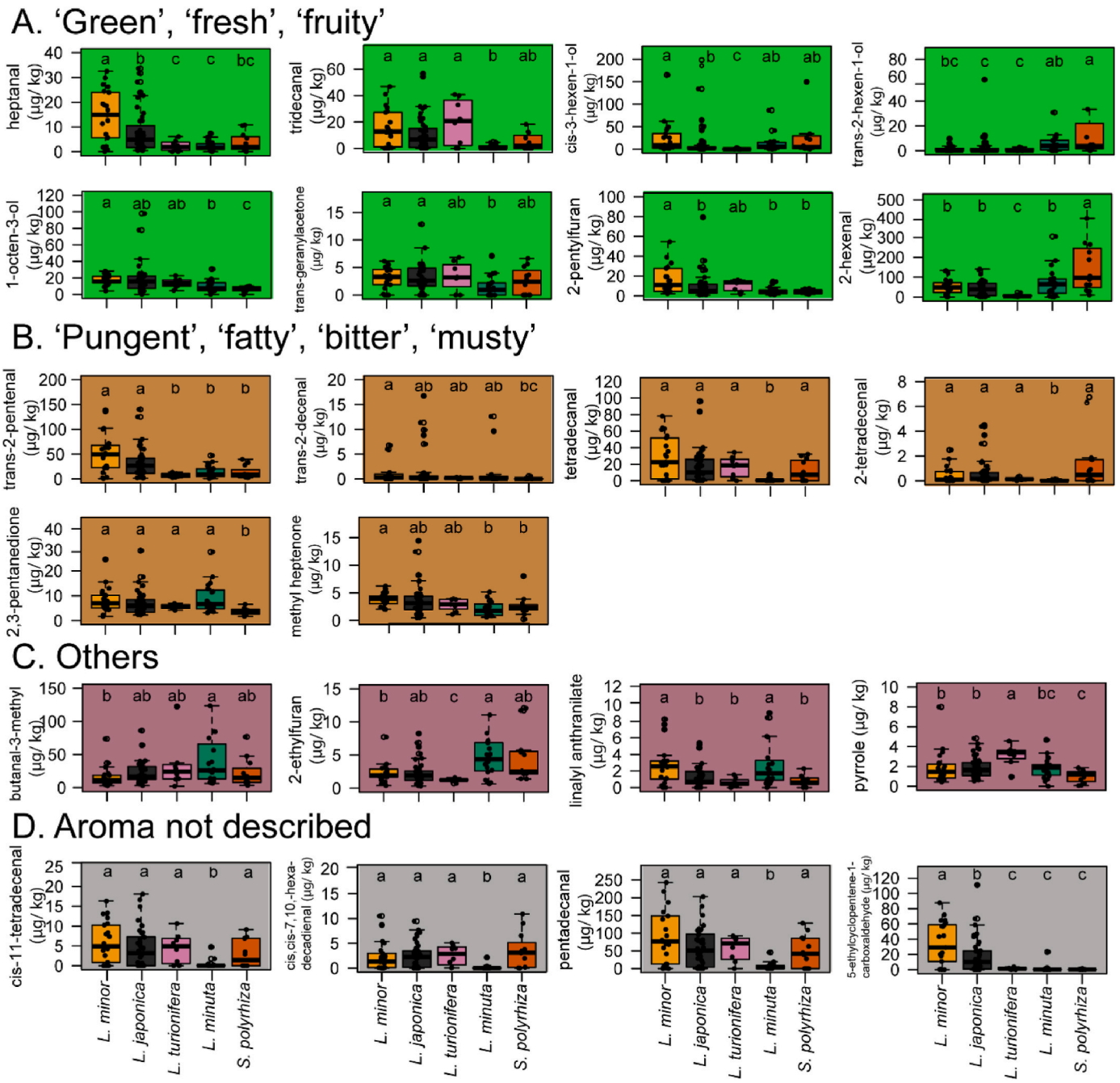

Fig. 2. Duckweed species had significantly different amounts of twenty-two aromatic compounds. Different aroma compounds found in duckweed freeze-dried powder grouped by species. *A*. Compounds with 'green', 'fresh' and 'fruity' positive descriptors. *B*. Compounds with 'pungent' 'fatty' 'bitter' and 'musty' negative descriptors. *C*. Others unique aroma compounds 'ethereal', 'chocolate', 'nutty'. *D*. Compounds lacking aroma data. Plots show median and 25 % and 75 % percentiles of concentrations of VOCs in µg/kg. Letters indicate significant differences for species by Paired Wilcoxon test using P = <0.05.

3.7. Analysis of duckweed growth using an ASD fieldspectrometer

Fresh and freeze-dried biomass for each ecotype is given in Table S7 and in Fig. S6. However the development of high-throughput detection methods will be useful to assess growth, physiological status and composition of duckweed to select new crop varieties. Green area was quantified by splitting photos into RGB channels as a measure of growth, and results are shown in Fig. S6. Hyperspectral vegetation indices were derived from an ASD fieldspectrometer in order to detect plant status (Fig. 1). Other vegetation index (OVI) has been used to estimate Nitrogen status in plants, and green model (GM), green index (GI) and normalised difference vegetation index (NDVI) used to predict greenness and plant health. All three greenness estimation parameters NDVI, GM and GI show an R^2 between 0.7 and 0.8 showing strong positive correlations with each other and with green area from RGB values of images.

Green model (GM) has moderate positive correlation with a range of amino acids (Fig. 8). OVI correlates positively and strongly with phenylalanine, histidine and ethanol content and total fresh weight biomass (FW) after six months growth (Fig. 8B and C). Vegetation indices cluster adjacent to health, growth and amino acid contents and opposite to sugar contents on a PCA biplot (Fig. 8D). Therefore GM and OVI could be useful detection methods for growth, biomass and nutritional quality.

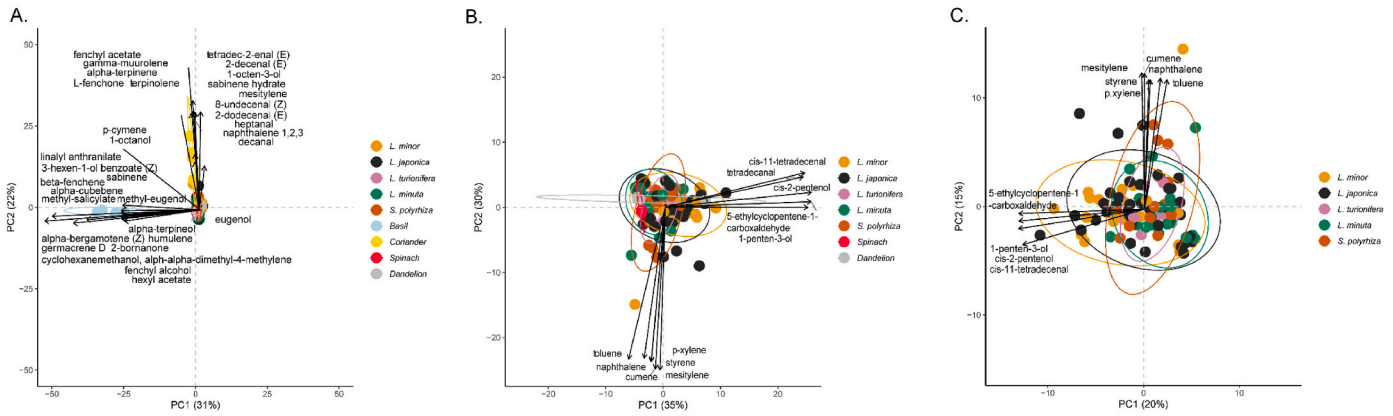

Fig. 3. Duckweeds have similar volatile profiles to spinach and dandelion. *A.* Basil and coriander form diverse clusters dissimilar to duckweeds using 92 compounds. Principal component analyses (PCA) for 92 compounds condensed onto two axis and coloured by five duckweed species and four herbs: basil, coriander, spinach and dandelion. *B.* Duckweed species do not differentiate from spinach and dandelion using 59 VOCs, after removal of low quantity compounds (>1 μg/kg) in duckweed. *C. Lemna japonica* and *Lemna minor* cluster away from *S. polyrhiza*. PC1 and PC2 account for approx. 50 % of the VOCs profile data variation. The VOCs contributing most to data variation are plotted with a cos2 value set at >0.7 using arrows to show direction of contribution.

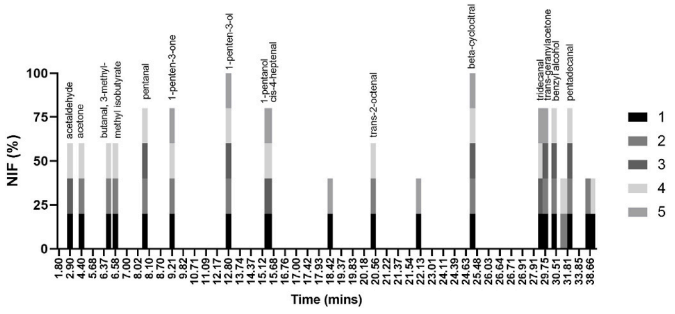

Fig. 4. Fifteen volatile compounds in duckweed were detected frequently by participants. An aromagram depicting aromatic compounds detected by GC-O as a time series and coloured in greyscale for each of the five participants. Nasal intensity frequency (NIF) is given between 0 % (not smelt) and 100 % is smelt by five participants. Aromatic compounds >50 % are indicated as frequently detected.

4. Discussion

4.1. Volatile profiles

Duckweed species not traditionally considered for human consumption were compared in a large-scale growth experiment for aroma perception to identify UK species with potential acceptability and usability in local food systems. Here we defined common aroma compounds in duckweeds, identified variation in amounts of VOCs within the *Lemna* and *Spirodela* species and found consistency between positive descriptor compounds identified with SPME-GCMS and those perceived by humans using GC-O. Aroma profiles of duckweeds were compared with those of commonly consumed leaf crops.

4.1.1. Volatile profiles of duckweed species had promising descriptors for food applications

Flavourings which are responsive to human taste includes sweet, sour, salty, bitter and savoury, which can be perceived as aromas by retronasal and orthonasal olfaction and are ultimately involved in decision-making regarding consumption and food likeliness [64]. Moreover, there is an innate preference for more 'sweet' smelling and tasting foods in human infants [65]. Human participants used

Table 2
L. japonica volatile compounds detected by GC-O with corresponding compound odour frequencies, odour activity values and descriptions.

Compound	Retention time	Concentration (μg/kg)	Odour threshold	Odour activity value	Descriptors
Acetaldehyde	2.90	5.86	120	0.05	**Pungent** ethereal aldehydic *fruity*
Acetone	4.40	5.62	500,000	0.00	Solvent ethereal *apple pear*
butanal,3-methyl-	6.48	13.98	2	69.91	Ethereal aldehydic chocolate *peach* **fatty**
methyl isobutyrate	6.58	12.15	7	1.74	*Fruity* floral *apple pineapple*
pentanal	8.06	16.58	42	0.39	**Fermented** bready *fruity* nutty *berry*
1-penten-3-one	9.21	9.02	1.3	6.49	Spicy **pungent** peppery mustard garlic onion
1-penten-3-ol	12.80	37.42	400	0.09	Ethereal horseradish *green* radish chrysanthemum vegetable *tropical fruity*
1-pentanol	15.28	8.88	4000	0.00	Fusel **fermented oily** sweet balsamic
cis-4-heptanal	15.38	0.89	0.8	1.12	**Oily fatty** *green* dairy milky creamy
trans-2-octenal	20.46	3.29	3	1.10	**Fatty** fresh cucumber *green* herbal *banana* **waxy** *green* leafy
beta-cyclocitral	25.29	0.42	5	0.08	*Tropical* saffron herbal clean rose sweet tobacco *green fruity*
tridecanal	28.95	0.55	0.01	55.33	Fresh clean aldehydic **soapy** *citrus* petal **waxy** *grapefruit peel*
trans-geranylacetone	29.75	0.69	60	0.01	Fresh *green* fruity **waxy** rose woody magnolia *tropical*
benzyl alcohol	30.33	0.11	10,000	0.00	Floral rose phenolic balsamic
pentadecanal	33.05	8.05	1	8.05	Fresh **waxy**

[a] Odour frequencies for compounds were retrieved from Ref. [51] and descriptors from The Good Scents Company [52]. Compounds with positive 'green' and 'fruity' descriptors are highlighted in italics. Descriptors in bold are considered to have negative associations. Compounds with odour activity values (OAV) > 1 are marked in grey and are expected to contribute most to human aromatic perception.

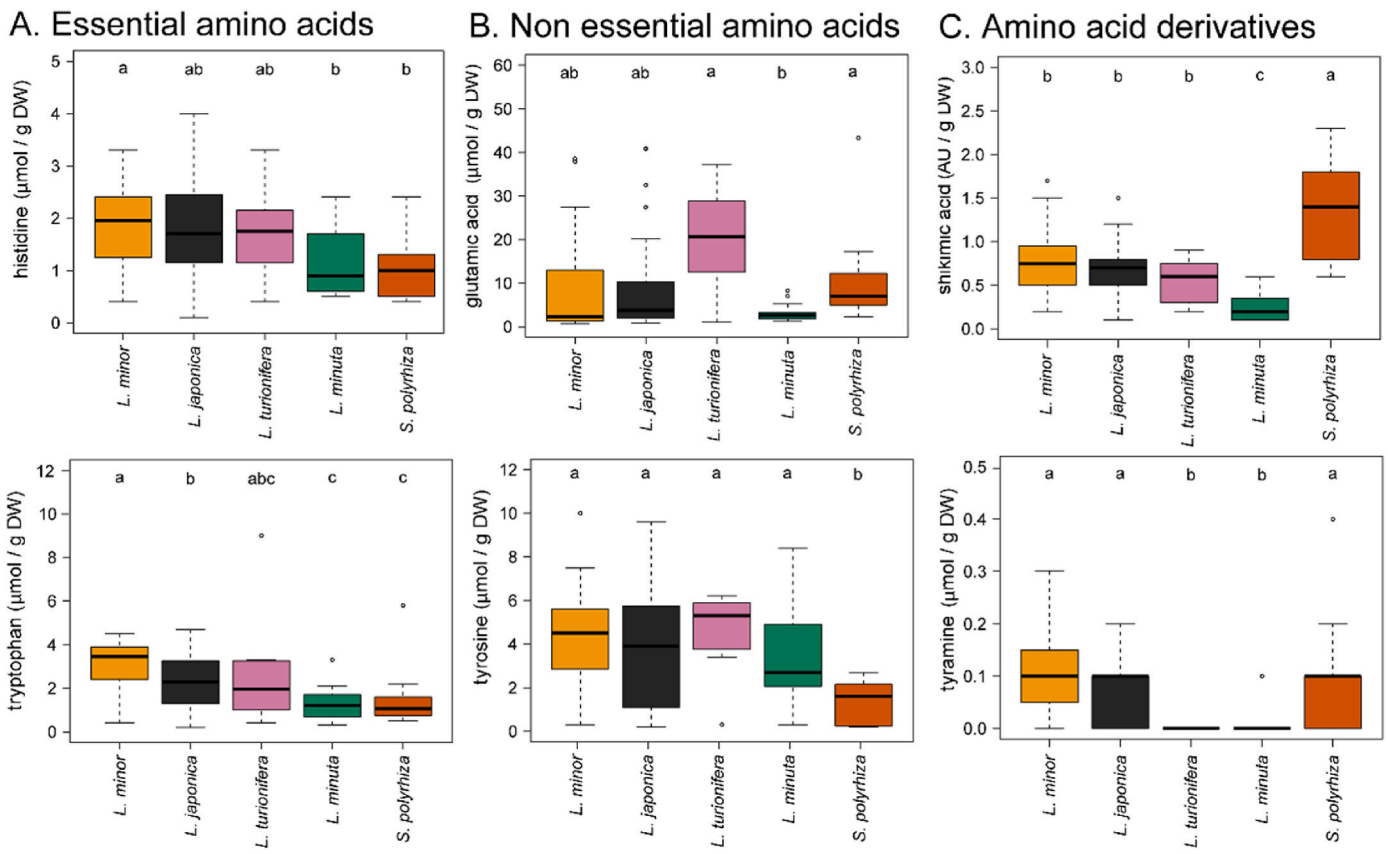

Fig. 5. ***Spirodela polyrhiza*** **has decreased essential amino acid profiles compared with** ***Lemna****. A:D.* Metabolite contents measured in duckweed species normalised to duckweed dried weight and presented as µmol/g DW or AU/g DW. *A*. essential amino acids, *B*. non-essential amino acids and *C*. amino acid precursors/derivatives. Plots show median values with 75 % and 25 % percentiles. Letters indicate significant differences between species from Kruskal-Wallis and post-hoc paired Wilcoxon test P = <0.05. *S. polyrhiza* and *Lemna minuta* have 1–2 fold lower histidine and tryptophan compared to *L. minor*. *Spirodela polyrhiza* species show 3-fold reduction in tyrosine but shikimic acid is 5-fold higher than *Lemna* species. *Lemna minuta* is 2–6 fold lower in glutamic acid compared to other species, with the highest levels in *L. turionifera*. Both *L. turionifera* and *L. minuta* have 18-fold reduction in tyramine compared to other duckweed species.

Table 3
Sugar content shows high variation within duckweed species.

Sugar	Average concentrations (µmol/g DW or mg/g DW for starch)				
	L. japonica	*L. minor*	*L. minuta*	*L. turionifera*	*S. polyrhiza*
glucose	57.02	42.76	45.26	72.84	50.53
fructose	70.93	47.16	62.68	94.38	94.94
sucrose	4.63	0.19	2.25	1.59	7.73
starch	99.65	126.04	154.84	150.43	101.12
Sugar	Range of concentrations (µmol/g DW or mg/g DW for starch)				
	L. japonica	*L. minor*	*L. minuta*	*L. turionifera*	*S. polyrhiza*
glucose	3.2–539	17.3–209.3	16.8–167.4	18.6–298.7	22.7–126.9
fructose	6.2–580.7	17.1–262	16.5–366.3	21–429	11.6–433.5
sucrose	0–36.4	0–0.7	0.1–27.5	0.1–11.6	0.1–28.7
starch	17.2–346.1	16.7–477.6	10.8–369.5	25.2–323.9	4.8–310.5

[a] Concentrations of sugars measured in multiple ecotypes within duckweed species by LCMS/MS. For each species, all raw values are presented as averages and ranges. Soluble sugars were measured in µmol/g DW and starch is presented in mg/g DW. Individual ecotypes within each species group were $n = 10$ *L. japonica*, $n = 5$ *L. minor*, $n = 4$ *L. minuta*, $n = 2$ *L. turionifera*, $n = 3$ *S. polyrhiza* and each ecotype was replicated four times positioned around a glasshouse. A Kruskal-Wallis test was used to derive significant differences between species averages with P = <0.05.

descriptors including 'green', 'fruity' and 'floral' for the scents of *L. japonica* during orthonasal sensory analysis (GC-O). Coincidently these descriptors are most correlated with the appealing perception of 'sweet' flavour [66] and 'green', 'grassy' and 'floral' descriptors are used generally to describe the aromas of other dietary vegetables available for consumption [67].

The negative descriptors associated with duckweeds included 'bitter' and 'pungent' which have also been descriptors associated with sulfur-containing nitriles, aldehydes and alcoholic compounds in green vegetables, for example broccoli [68]. No severely negative descriptors such as 'cheesy', 'eggy', 'fishy' or 'rotten' were found in freeze-dried duckweed. Therefore, UK-sourced species could be an acceptable novel vegetable depending on amounts, ratios and interactions between aroma compounds.

It is noteworthy that processing of foodstuffs can affect volatile profiles and aroma perception. For example, freezing duckweed

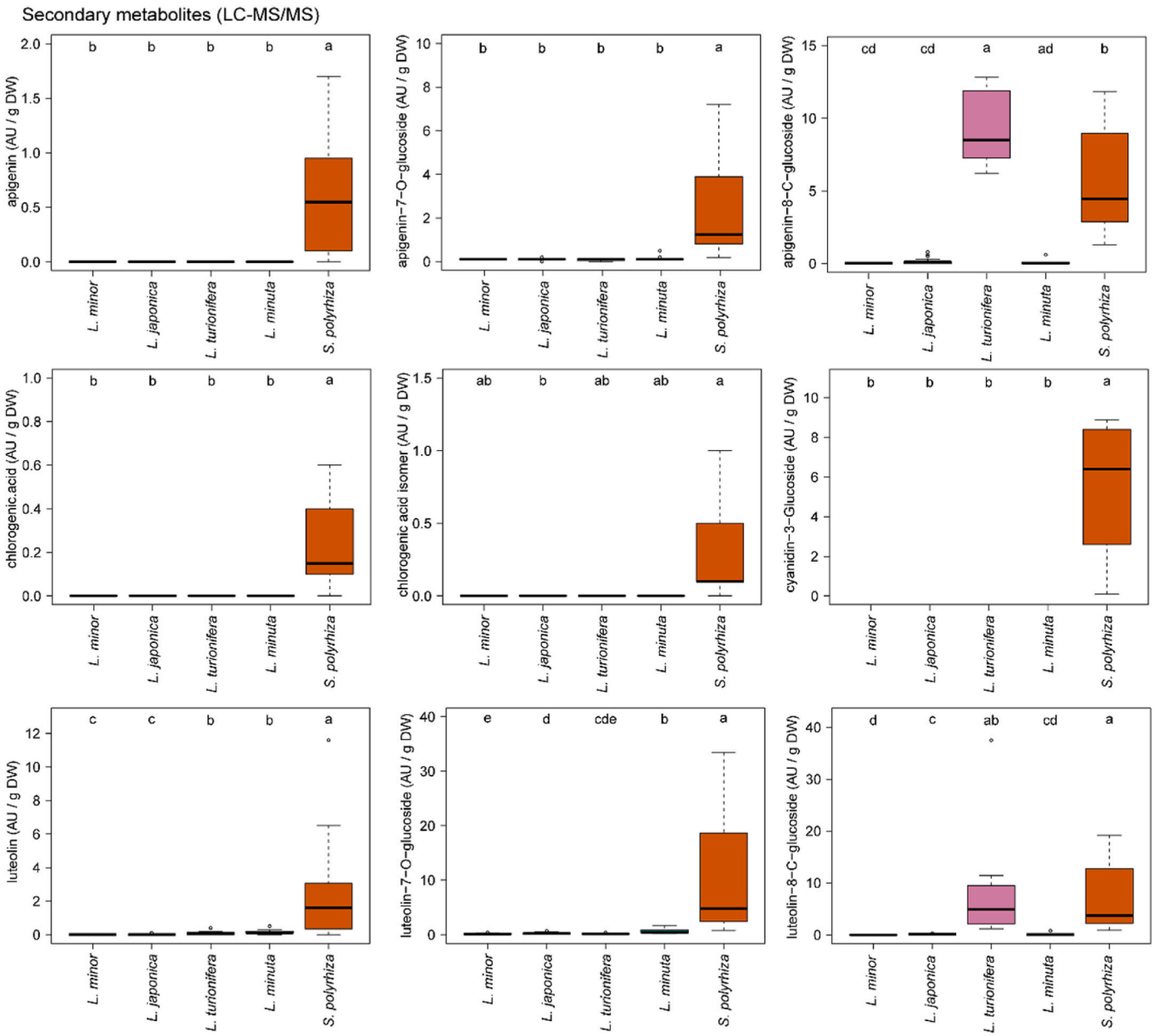

Fig. 6. Secondary metabolites including flavonoid compounds are dominant in *S. polyrhiza* compared to *Lemna* species. Boxplots indicating relative amounts of polyphenols including flavonoids in duckweed species (in AU/g DW). Boxplots are arranged by compounds alphabetically with conjugated forms next to their corresponding base compound in each row. Plots show median values and 25 % and 75 % percentiles and are coloured by species groupings. Letters indicate significant differences by Wilcoxon paired statistic <0.05.

concentrate increased bitterness and decreased protein content [69], and drying under direct sunlight decreased beta-carotene [70], which are volatile precursors for apocarotenoids such as beta-cyclocitral. Fresh duckweed may show differences in odour active compounds from that found in freeze-dried duckweed here. However, freeze-drying maintains high quality of many herbs and spices and preserves phenolic contents compared to other preparation methods [71]. It is also in line with the development of duckweed freeze-dried powders for the health and protein supplement markets.

Short chain volatile compounds (penta- hexa- and hepta-) in duckweeds are similar to other leafy vegetables, like broccoli and in tomato and olive fruits [72–74]. Duckweeds have higher amounts of 'green' and 'fresh' descriptive compounds than basil, coriander and spinach overall. *Lemna minor* and spinach had equal acceptability as inputs in foodstuffs in human feeding trials [75], perhaps because of their similar aroma profiles.

From preliminary olfactory analysis of a *L. japonica* duckweed sample, the most frequently detected compounds were beta-cyclocitral and 1-penten-3-ol (Fig. 4), the latter was also one of the compounds with the highest abundance from GCMS (Table 1, Fig. S3). Despite this, these were indicated to not have significantly high OAVs to be detected by humans. Additionally, beta-cyclocitral is reported as a substance with high anosnia, where ~34 % participants are not expected to smell it [76]. In contrast, hexanal was highly abundant but surprisingly not detected amongst the duckweed sensory panel, despite previous detection in other vegetables and salad crops [77]. Additional compounds without peaks in GCMS were found to contribute to human perception of aroma from GC-O in duckweed, due to low odour detection thresholds [78,79]. As aroma compounds vary between species they are likely to vary too in both human acceptability and storage potential.

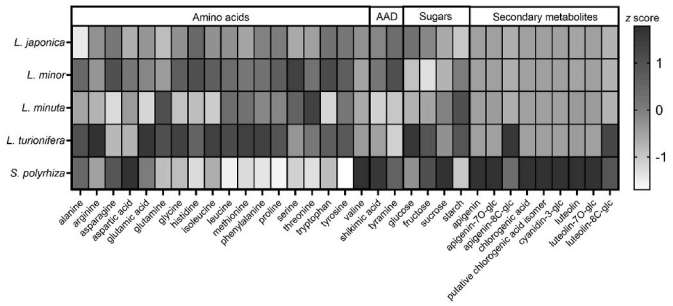

Fig. 7. *Spirodela* is higher in flavonoid secondary metabolites than *Lemna* species but has decreased free amino acids. Heat map with false colour greyscale for average metabolite abundance per dry weight of freeze-dried duckweed powder. Metabolites include free amino acids, amino acid precursors/derivatives (ADD), sugars, starch and secondary metabolites measured in ecotypes of five duckweed species. *z* scores for each compound were calculated using standardisation to the mean and SDs for the whole sample size. Compounds were measured by LC-MS/MS.

Lemna minor have the highest range and more 'green' and 'fruity' descriptors of the included *Lemna* species. Decreased levels of 'green' aroma compounds in *L. minuta and L. turionifera* and more compounds with 'other' descriptors has likely outcomes for uniqueness of their aroma profiles. *Lemna minuta* had a distinct aroma profile with decreased numbers of several aromatic compounds, including those smelt by participants of the *L. japonica* sample (*trans*-geranylacetone, tridecanal, pentadecanal) but higher presence of butanal, 3-methyl, a compound with the highest OAV attributing an 'ethereal' aroma.

4.1.2. *The role of VOCs in the storage potential of duckweeds*

Fresh-stored duckweed is reportedly unspoiled for 28 days [80,81] supporting general opinion of good longevity in post-harvest storage, possibly due to high phenolic contents. Furthermore, duckweed extracts show antibacterial activity against food spoilage microorganisms including *Bacillus*, *Staphylococcus* and *Pseudomonas* species [33,34]. Supporting this, incorporation of *Lemna minor* extract into beef burgers decreased protein oxidative products from meat [35] and application into polyvinylalcohol packaging limited fungal spoilage during storage of avocados [82]. The five highest VOC contents in duckweed here are recognized compounds with antimicrobial functions, possibly contributing to extending shelf-life (Table 1). Moreover, hexanal and heptanal provide fungal resistance in other plants [60,83], and are produced in varying amounts by duckweeds. Further exploration of the roles of specific VOCs and phenolics in post-harvest storage are required in the future.

4.2. *Metabolites in duckweed for food applications*

Here we show UK duckweed species grown under common

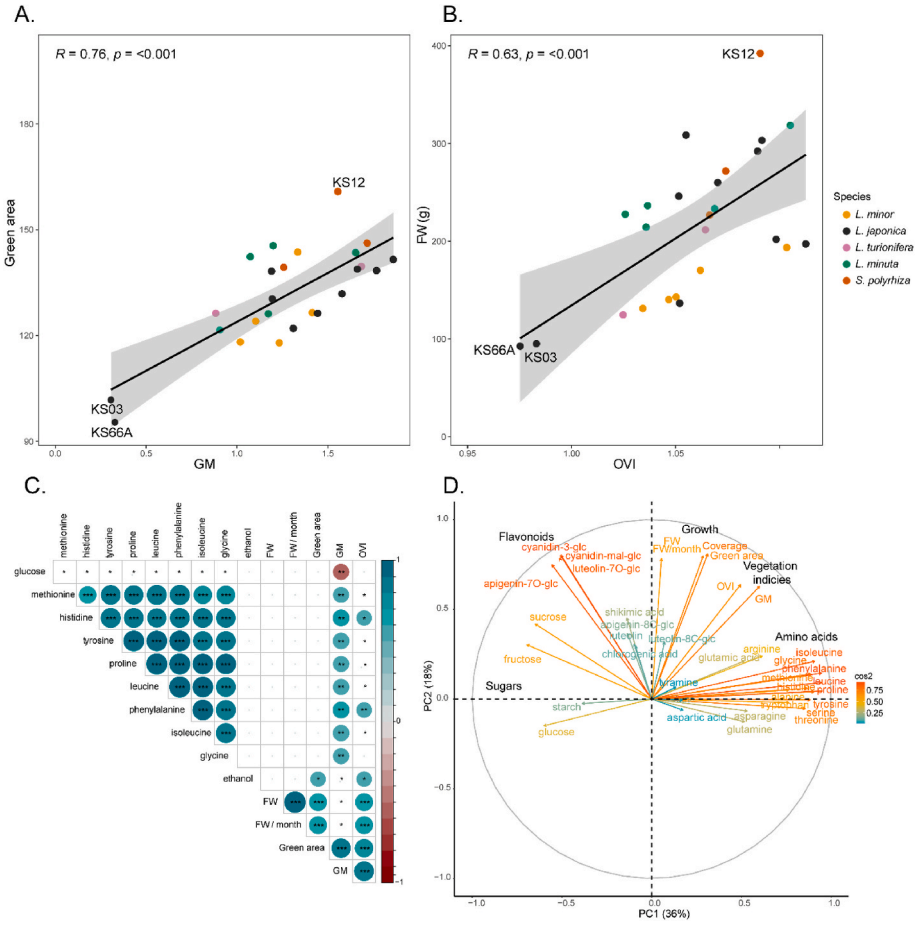

Fig. 8. Vegetation indices can be used as a proxy for health, growth and metabolite profiles of duckweeds. *A.* Linear model showing positive correlation between ASD fieldspectrometer derived GM values with green area as obtained by RGB from photographs at four weeks growth averaged by ecotypes. *B.* Linear model showing correlation between OVI measured by ASD fieldspectrometer at four weeks growth with total fresh weight at six months averaged by ecotypes. Points are coloured by species and outlying individuals are labelled. *C.* Correlation plot matrix of significant relationships by Pearson's correlation co-efficient of amino acids, sugars, growth, health and vegetation indices. *D.* PCA biplot for variable relationships showing trade-offs for growth/amino acid content and sugars/flavonoids.

glasshouse conditions have different nutritional potentials relevant in food applications.

4.2.1. *Spirodela is dominant in flavonoids compared to Lemna species*

Our work shows species-dependent variation in beneficial secondary metabolite contents of duckweed. *Lemna minor* was identified with potential as a future food due to a range nutritional qualities including higher composition of the polyphenol naringenin compared to a *Wolffia* species [84]. Here, *Spirodela polyrhiza* has a higher polyphenol content and greater number of different flavonoid polyphenolic compounds compared to four *Lemna* species (Figs. 6 and 7). *Spirodela* is high in cyanidine-3-glucoside, chlorogenic acid and shikimic acid which are supposedly anti-inflammatory and anti-cancer compounds. Luteolin and apigenin and their 7-O- and 8-C-gluc. conjugates are abundant in *Spirodela* [85] acting as antioxidants and may even contribute to anti-tumour and anti-inflammatory medical properties of *Spirodela* [25,26,30]. Chlorogenic acid was previously found in *Spirodela* but not in *Landoltia* or *Wolffia* species [31], the lack of detection in *Lemna* provides supporting evidence that it may be exclusive to the *Spirodela* genus of duckweeds.

4.2.2. *Spirodela contains less free amino acid compositions compared to Lemna*

In contrast to its higher flavonoid content, *S. polyrhiza* has decreased contents of three free amino acids compared to *Lemna* species. Amino acids are precursors for certain secondary metabolites, such as flavonoids. Therefore, the flavonoid biosynthesis in *S. polyrhiza* might lead to a reduction of free phenylalanine and related amino acids. *Lemna minor* has the highest levels of the essential amino acids tryptophan and histidine, and displayed previously higher amounts by dry weight than soya, rice and wheat [86]. However, both tryptophan and histidine impart bitter flavours in mushoom species and may contribute to increased bitterness of *L. minor* in comparison with *L. minuta* and *S. polyrhiza* [87]. *Lemna turionifera* had more free glutamic acid and arginine than other species (Figs. 5 and 7), with glutamic acid one of the highest contributing free amino acids to flavour, imparting savoury or satisfactory tastes, whilst arginine contributes heavily to bitterness [88]. Further analysis is required to assess which species show the most promise for providing 'complete' amino acid composition for human nutrition [89,90] with the lowest trade-off in bitterness possible.

4.3. *Relationship between metabolites and growth for optimisation for food production*

Growth rate potential is important when selecting duckweed ecotypes for commercial purposes. In this context the cost to the plant of synthesizing secondary compounds and amino acids may need to be considered too, creating a complex trade-off. Starch content is not a good indicator of growth rate in duckweed, as it is in staple cereal grains [91]: the negative relationship between growth and starch content here and in Sree & Appenroth (2014) [92] indicates a stress response or a lack of ability to utilise storage sugars in growth. A closer relationship between flavonoids and sugar content was seen here (Fig. 8D), possibly because of conjugation of flavonoids through glycosylation.

4.4. *Optimisation of duckweed ecotypes for food production*

For commercial applications as either a fresh vegetable herb or dried protein supplement, high and consistent yields (dry and fresh weight) are required. Biomass showed variability between a trio of harvests conducted over a six month period here (Fig. S6C:F), and previously [93], so the conditions required for predictable harvests represents an ongoing challenge for duckweed development as sustainable food. However, we conclude that *Spirodela polyrhiza* ecotype KS12 has the highest greenness values, surface coverage and biomass with the benefits of high flavonoid content (Fig. 8A,B and Figs. S5 and S6). Additionally, this work recognizes indoor duckweed production as being intensive i.e. requiring high resources [70] and supports a drive towards automation to monitor growth, water and nutrient supply and harvest.

5. Conclusion

Wolffia species are commonly utilised for human consumption worldwide, with additional recent inclusion in spaceflight missions [94,95]. Here we recognize *Lemna* and *Spirodela* species, which also show positive sensory and nutritional properties. Specific *Spirodela* and *Lemna* species may be suitable for food applications based on aroma, flavonoid content and free amino acid composition. In future, wider human feeding studies and digestion assays with *Lemna* and *Spirodela* species should be conducted to assess taste and mouthfeel, any acceptability issues, and negative effects from duckweed consumption.

Future studies measuring stability of VOC profiles during processing methods, together with antimicrobial activity and storage potential of specific species are required. Future aims include isolation of genetic components to increase compounds associated with positive aroma. Concordantly, to increase acceptability, mitigating the few identified negative aroma traits should be a goal akin to that performed in tomato and wheat [96,97]. Future selection of other underutilized duckweed species and ecotypes should use high-throughput techniques to detect high growth in parallel with '-omics' technologies for nutritional assessment. This work supports the use of digital media to educate the population about food research [98], in particular here the aroma profiles and potential benefits of different duckweed species as a novel food.

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CRediT authorship contribution statement

Kellie E. Smith: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Martin Schäfer:** Writing – review & editing, Resources, Investigation, Formal analysis, Data curation. **Mui Lim:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Data curation. **Carlos A. Robles-Zazueta:** Writing – review & editing, Resources, Data curation. **Laura Cowan:** Writing – review & editing, Data curation. **Ian D. Fisk:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization. **Shuqing Xu:** Writing – review & editing, Writing – original draft, Supervision, Resources, Conceptualization. **Erik H. Murchie:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

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.

Data availability

The sequences for duckweed genomes in this panel are deposited under project codes PRJNA1026139 and PRJNA1074359 on the NCBI Sequence read archive (SRA). Scripts used for analysis are available at: https://github.com/Duckweed-KS/Flavour_metabolites

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Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2024.101263.

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Supplementary figures for Smith *et al*; Aroma and metabolite profiling in duckweeds: exploring species and ecotypic variation to enable wider adoption as a food crop

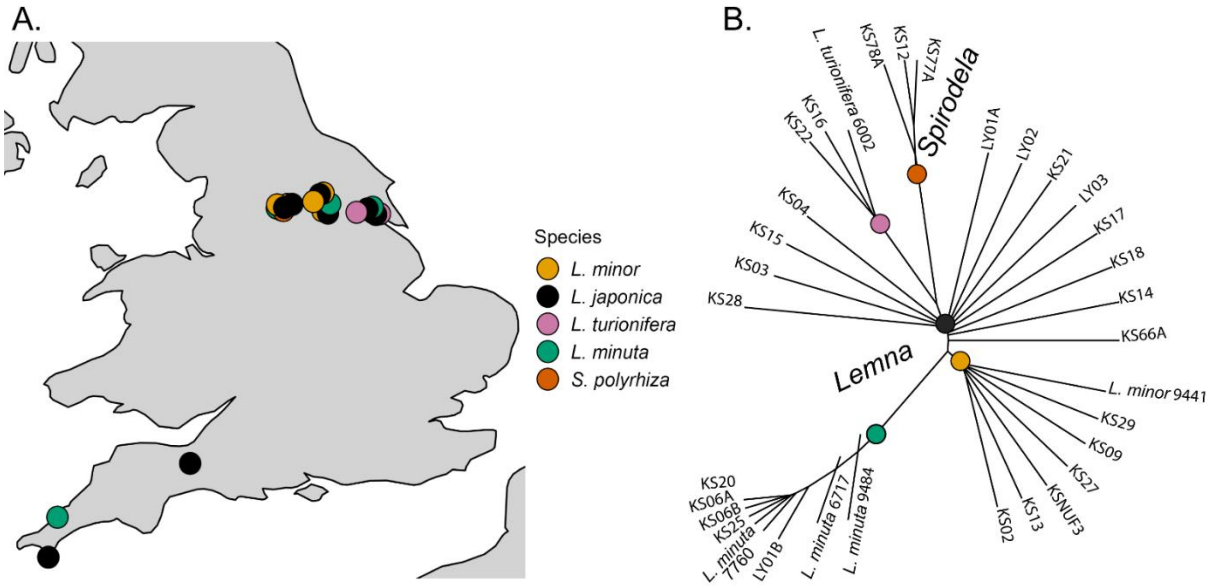


Fig. S1. **UK *Lemna* and *Spirodela* duckweed ecotypes.** *A.* Map of United Kingdom showing geographic distributions of collected duckweed ecotypes. *B.* Phylogenetic tree showing clusters of ecotypes within five duckweed species. Geographical coordinates in A and nodes of the tree in B are coloured by species classifications.

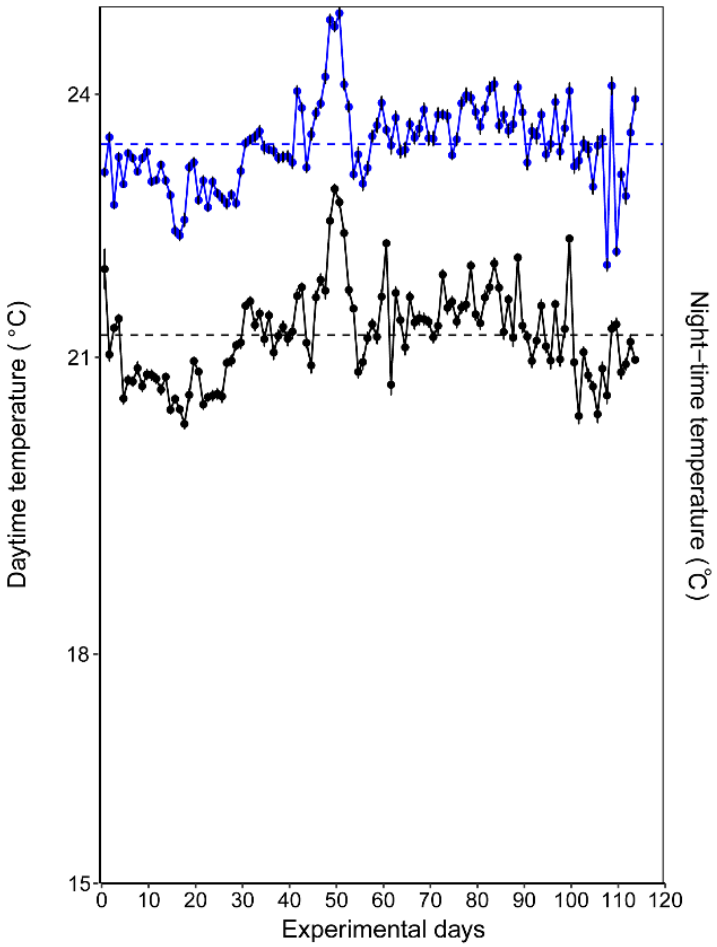


Fig. S2. **Temperature range within the glasshouse environment used for scaling up duckweed growth.** Glasshouse temperature range during four months of duckweed growth period. Daytime temperature ranged between 22-25°C (blue) and night-time temperature between 20-23°C (black). Measurements were made every five minutes and averaged for each day and night period.

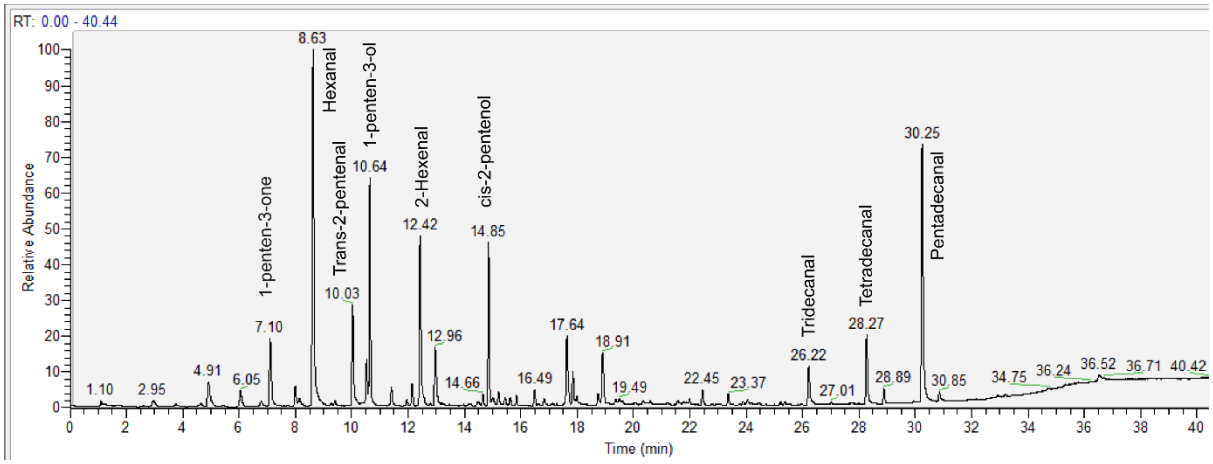
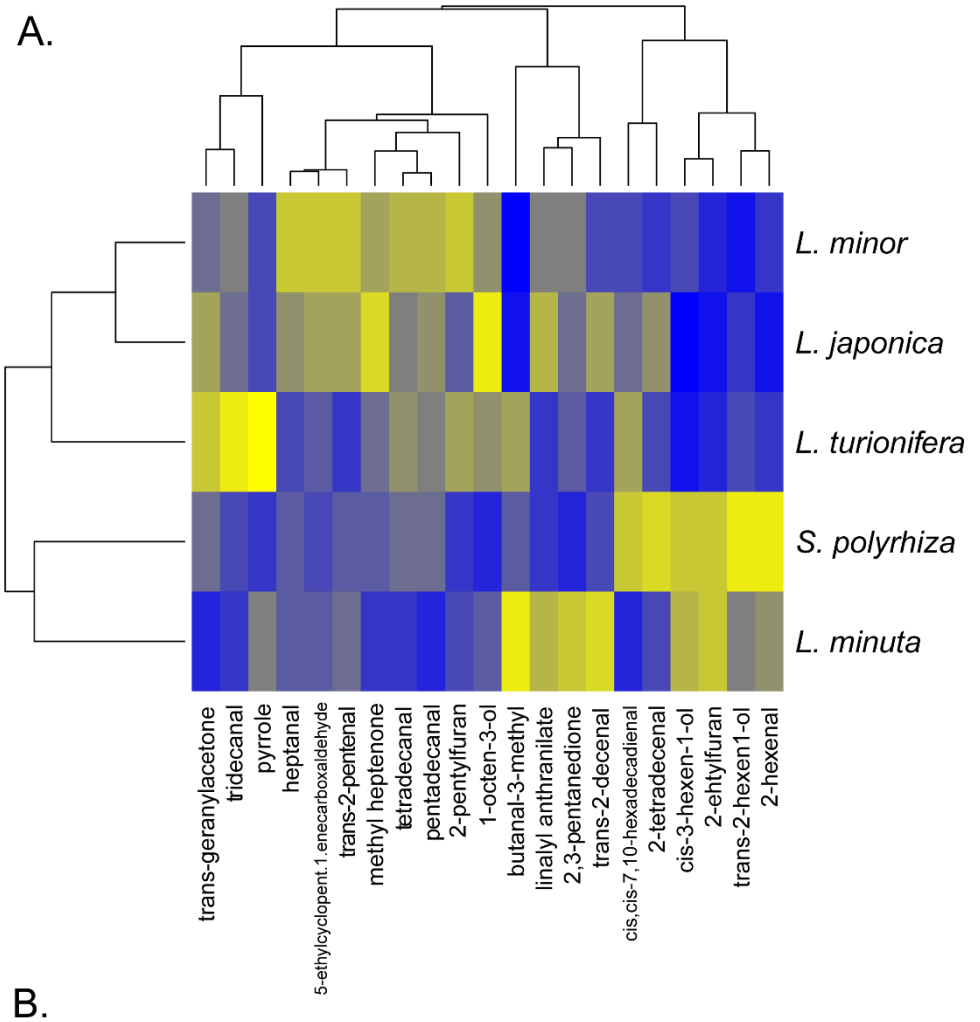


Fig. S3. **The duckweed volatile profile is characterized by high amounts and numbers of different penta- and hexa- aldehydes and alcohols.** SPME-GCMS Chromatogram showing peaks associated with VOCs.


A.
L. minor
L. japonica
L. turionifera
S. polyrhiza
L. minuta
trans-geranylacetone
tridecanal
pyrrole
heptanal
5-ethylcyclopent-1-enecarboxaldehyde
trans-2-pentenal
methyl heptenone
tetradecanal
pentadecanal
2-pentylfuran
1-octen-3-ol
butanal-3-methyl
linalyl anthranilate
2,3-pentanedione
trans-2-decenal
cis,cis-7,10-hexadecadienal
2-tetradecenal
cis-3-hexen-1-ol
2-ehtylfuran
trans-2-hexen1-ol
2-hexenal

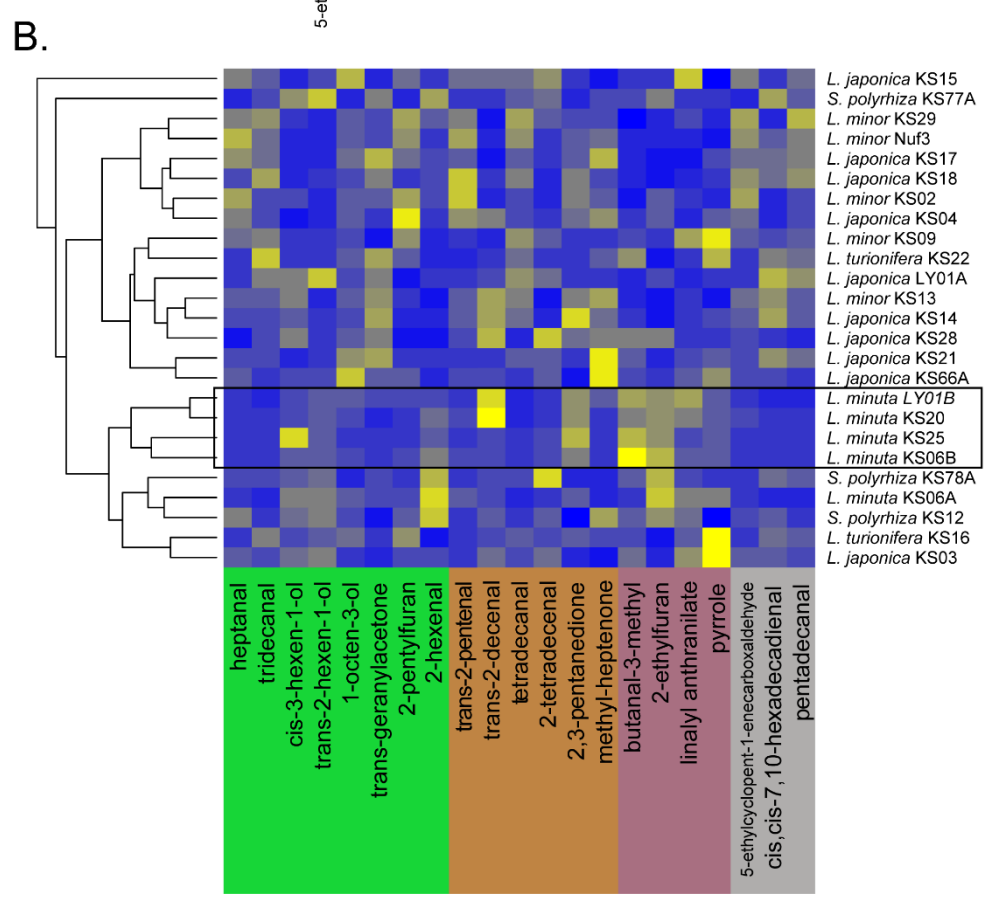

B.
L. japonica KS15
S. polyrhiza KS77A
L. minor KS29
L. minor Nuf3
L. japonica KS17
L. japonica KS18
L. minor KS02
L. japonica KS04
L. minor KS09
L. turionifera KS22
L. japonica LY01A
L. minor KS13
L. japonica KS14
L. japonica KS28
L. japonica KS21
L. japonica KS66A
L. minuta LY01B
L. minuta KS20
L. minuta KS25
L. minuta KS06B
S. polyrhiza KS78A
L. minuta KS06A
S. polyrhiza KS12
L. turionifera KS16
L. japonica KS03
heptanal
tridecanal
cis-3-hexen-1-ol
trans-2-hexen-1-ol
1-octen-3-ol
trans-geranylacetone
2-pentylfuran
2-hexenal
trans-2-pentenal
trans-2-decenal
tetradecanal
2-tetradecenal
2,3-pentanedione
methyl-heptenone
butanal-3-methyl
2-ethylfuran
linalyl anthranilate
pyrrole
5-ethylcyclopent-1-enecarboxaldehyde
cis,cis-7,10-hexadecadienal
pentadecanal

Fig. S4. **Duckweeds show species and ecotypic variation in aromas associated with positive and negative aroma perception.** *A.* Heatmap showing species clustered by similarity of volatile profiles. *B.* Heatmap showing duckweed ecotypes and their relative levels of individual VOCs grouped and coloured by descriptor type, in green ‘green’, ‘fresh’ and ‘fruity’, in brown ‘pungent’, ‘fatty’, ‘bitter’ and ‘musty’ negative associated compounds, in pink other compounds giving differential aromas e.g. ‘chocolate’, ‘nutty’ and in grey non-descript aromas. Twenty-one compounds are included for which there were species differences in amounts by Kruskal-Wallis statistical analysis.

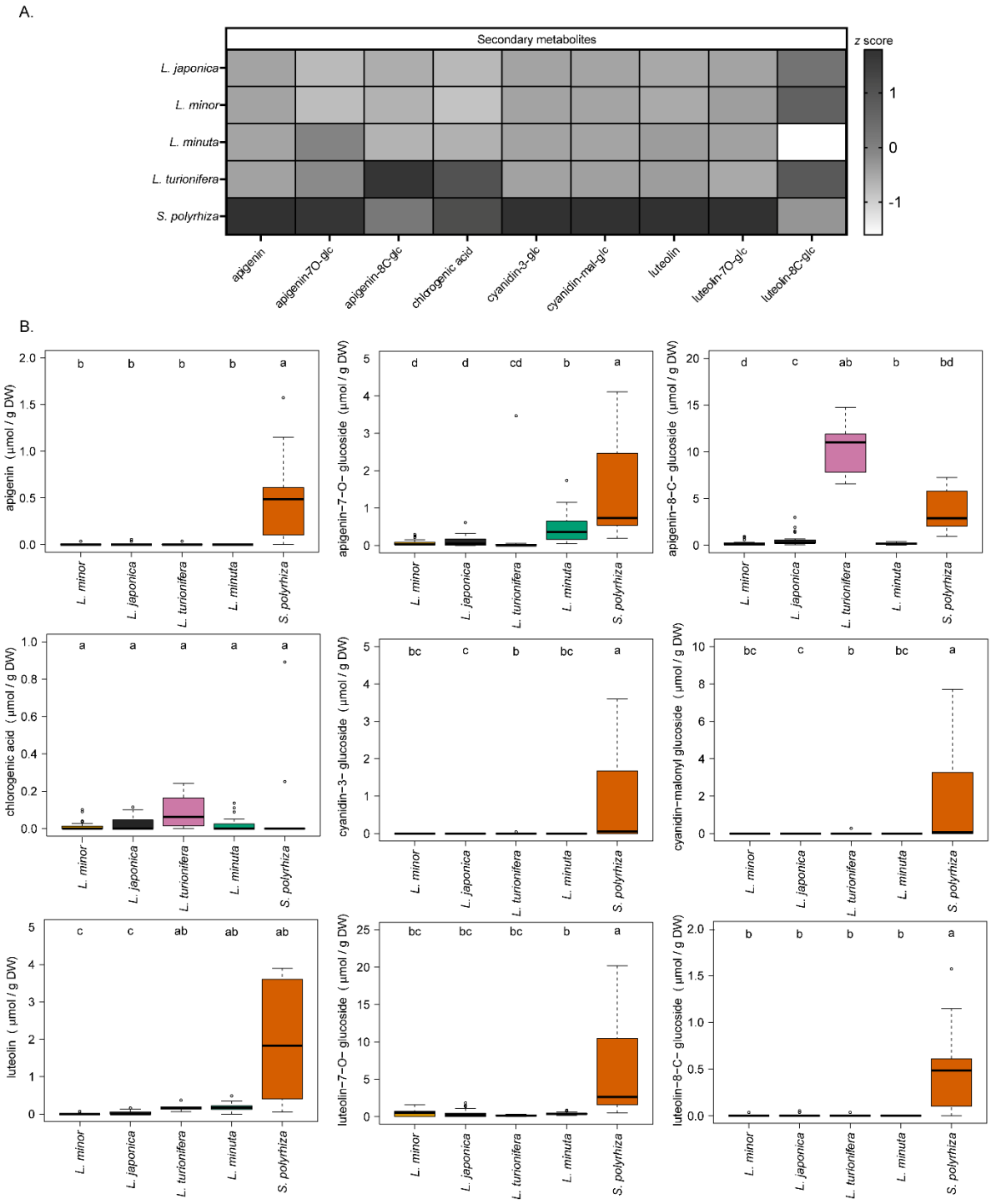


Fig. S5. ***Spirodela polyrhiza*** **is dominant in flavonoid secondary metabolites compared to** ***Lemna*** **species.** *A.* Heat map with false colour greyscale for average flavonoid abundance per dry weight measured in ecotypes of five duckweed species by HPLC-PDA. z scores for each compound were calculated using standardization to the mean and SDs for the whole sample size. *B.* Boxplots showing individual amounts of different flavonoid compounds in (µmol g / DW) in different duckweed species as measured by HPLC-PDA. Boxplots indicate medians and 75% and 25%

percentiles. Letters indicate significant differences between species by Kruskal-Wallis statistical analysis. Heatmap and boxplots are arranged by compounds alphabetically with conjugated forms next to their corresponding base compound in each row.

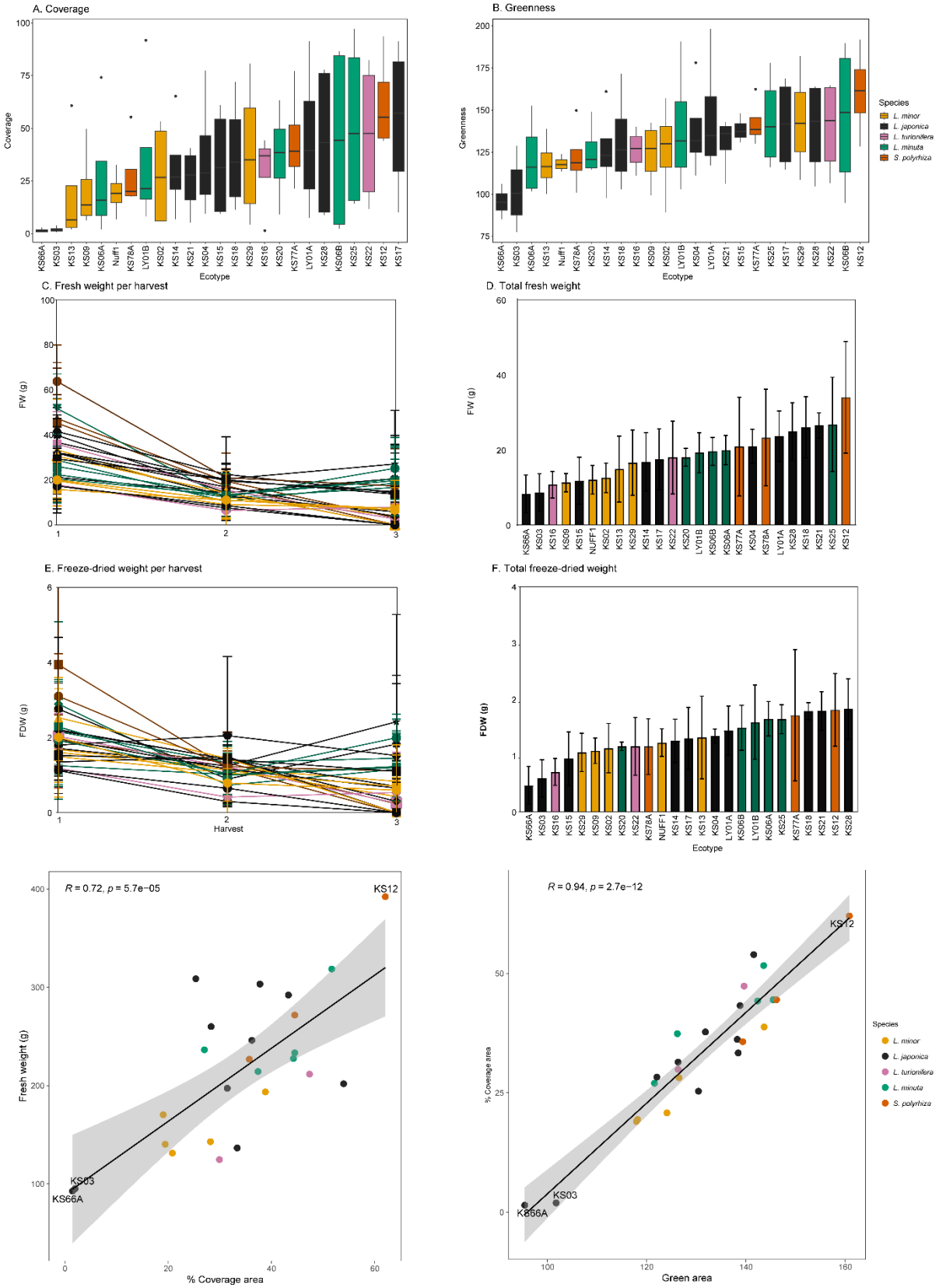


Fig. S6. ***S. polyrhiza* ecotype KS12 had exceptional growth, health and biomass in glasshouse experiment.** *A.* Percentage surface coverage of duckweeds after four weeks growth, error bars *n*=4 replicate trays per ecotype. *B.* Greenness values from photos at four weeks growth, from five average green channel values from each photo and four photos taken of each tray replicate of each ecotype. Error bars indicate SD between trays for each ecotype. *C.* Biomass split by harvest period: Line plots showing average fresh and freeze dried weights (g) with error bars indicating SEM between four trays for each ecotype (*n*=25) per harvest period over a six month period. *D.* Biomass split by ecotypes: Bar plots showing fresh weight and freeze dried weight averages for each ecotype with four replicates represented over three harvest periods over six months *n*=12 with error bars shown as SEM. *E.* Strong positive correlation between percentage surface coverage area from photos at four weeks and total fresh weight biomass (g) after six months. *F.* Average green area and percentage surface coverage area show strong positive correlation. R^2 and P values are reported from linear model fitting using Pearson's model. Points are coloured by species groupings of ecotypes: ● *L. minor*, ● *L. japonica*, ● *L. turionifera*, ● *L. minuta*, ● *S. polyrhiza*. The outlying ecotypes are labelled.

Supplementary tables for Smith *et al*; Aroma and metabolite profiling in duckweeds: exploring species and ecotypic variation to enable wider adoption as a food crop

Table S1. Duckweed ecotypes of *Spirodela* and *Lemna* species collected from around the UK from different environmental conditions.

Ecotype	Registered clone	Latitude	Longitude	Light (PPFD)	Water pH	Water temp (ºC)	Air temp (ºC)	Light habitat	Location
KS02	*Lemna minor* 5882	53.799	1.147	11.33	7.11	6.4	8.6	dLL	North
KS03	*Lemna japonica* 5883	53.81	1.755	67.34	6.41	6.6	8.9	dLL	North
KS04	*Lemna japonica* 5884	53.774	1.066	NA	8.44	6.8	8.5	dHL	North
KS06A	*Lemna minuta* 5885	53.83	1.819	NA	NA	NA	NA	dLL	North
KS06B	*Lemna minuta* 5886	53.83	1.819	NA	NA	NA	NA	dLL	North
KS09	*Lemna minor* 5887	53.869	1.67	181.3	6.45	8.4	11.1	dLL	North
KS12	*Spirodela polyrhiza* 5888	53.801	1.723	835.4	8.25	5.6	8.2	dHL	North
KS13	*Lemna minor* 5889	53.866	1.815	297.4	7.56	8.9	12.5	dHL	North
KS14	*Lemna japonica* 5890	53.835	1.711	784.9	8.59	8.5	11.5	dHL	North
KS15	*Lemna japonica* 5891	53.871	1.597	155	5.91	7.7	9.9	dLL	North
KS16	*Lemna turionifera* 5892	53.766	0.298	246.7	8.99	8.3	11.5	dHL	North
KS17	*Lemna japonica* 5893	53.758	0.355	46.29	7.98	7.7	11.4	dLL	North
KS18	*Lemna japonica* 5894	53.783	0.408	52.86	7.91	8.9	10.5	dLL	North
KS20	*Lemna minuta* 5895	53.839	0.405	832.1	7.46	8.8	12.8	dHL	North
KS21	*Lemna japonica* 5896	53.821	0.482	1006	7.37	8.8	12.1	dHL	North
KS22	*Lemna turionifera* 5897	53.783	0.642	126.5	8.12	8.9	12.2	dLL	North
KS25	*Lemna minuta* 5898	53.875	1.034	39.59	7.18	8.4	12.1	dLL	North
KS28	*Lemna japonica* 5900	53.979	1.187	87.06	7.24	8.8	12.2	dLL	North
KS29	*Lemna minor* 5901	53.902	1.281	875.1	7.19	8.2	10.8	dHL	North
LY01A	*Lemna japonica* 5902	50.407	-5.061	NA	NA	NA	NA	dHL	South
LY01B	*Lemna minuta* 5903	50.407	-5.061	NA	NA	NA	NA	dHL	South

KSNuf3	*Lemna minor* 5909	53.83	1.819	NA	NA	NA	NA	dLL	North
KS66A	*Lemna japonica* 5906	51.397	-2.852	1185.2	8.83	10.3	16.6	dHL	South
KS77A	*Spirodela polyrhiza* 5907	51.591	-3.04	1457.9	7.45	16.1	22.5	dHL	South
KS78A	*Spirodela polyrhiza* 5908	51.666	-3.031	1440.4	7.31	19.1	23.2	dHL	South

[a] Ecotypes included in study with their 4 digit registration codes, species allocations, latitude, longitude coordinates and environmental data.
[b] Registration codes correspond to the Rutger's duckweed collection (ruduckweed.org) and environmental data was collected in spring 2021.
[c] PPFD = photosynthetic photon flux density (blue + green + red light).
[d] Light habitat corresponds to dLL derived from low light and dHL derived from high light from [12].

Table S2. Southern UK ecotypes experienced higher light and temperatures in their environments compared to northern ecotypes.

	T	Df	P value
Light (PPFD)	-7.91	7.5	0.0000652 ***
Air temperature (°C)	-4.65	2.1	0.04 *
pH	-0.62	2.7	0.584 n.s
Water temperature (°C)	-2.77	2	0.107 n.s

[a] Welch t-tests were performed for north and south for each geographical parameter. P = <0.001 and <0.05 used for significance. The full dataset was collected in spring 2021 and is provided in Supplementary Table 1.

Table S3A. Light intensity and spectra in a glasshouse environment in December did not significantly vary between ecotypes.

Ecotype	LI	PFD	PPFD	FR	R	G	B	UV
KS02	60 ± 10	90 ± 20	70 ± 20	20 ± 3	30 ± 9	30 ± 9	20 ± 2	1.5 ± 0.1
KS03	60 ± 10	80 ± 20	70 ± 10	10 ± 2	20 ± 7	30 ± 7	20 ± 1	1.5 ± 0.1
KS04	60 ± 20	90 ± 20	80 ± 20	20 ± 4	30 ± 9	30 ± 10	20 ± 2	1.6 ± 0.1
KS06A	50 ± 20	80 ± 20	60 ± 20	10 ± 3	20 ± 8	20 ± 9	20 ± 2	1.5 ± 0.2
KS06B	50 ± 20	80 ± 20	60 ± 10	10 ± 2	20 ± 7	20 ± 7	20 ± 1	1.4 ± 0.1
KS09	50 ± 10	80 ± 20	60 ± 20	10 ± 3	20 ± 7	20 ± 8	20 ± 2	1.5 ± 0.1
KS12	60 ± 20	80 ± 20	70 ± 20	10 ± 3	20 ± 10	30 ± 11	20 ± 2	1.5 ± 0.1
KS13	50 ± 20	80 ± 30	60 ± 30	10 ± 4	20 ± 11	20 ± 11	20 ± 3	1.6 ± 0.2
KS14	50 ± 10	70 ± 10	60 ± 10	10 ± 2	20 ± 3	20 ± 4	20 ± 1	1.5 ± 0.1
KS15	60 ± 20	100 ± 10	80 ± 10	20 ± 3	30 ± 5	30 ± 4	20 ± 1	1.5 ± 0.1
KS16	40 ± 10	80 ± 20	60 ± 20	10 ± 3	20 ± 9	20 ± 9	20 ± 3	1.5 ± 0.2
KS17	50 ± 20	90 ± 20	70 ± 20	20 ± 4	30 ± 9	30 ± 9	20 ± 2	1.5 ± 0.2
KS18	50 ± 20	80 ± 10	70 ± 10	10 ± 2	30 ± 5	30 ± 6	20 ± 1	1.5 ± 0.1
KS20	50 ± 20	90 ± 20	70 ± 20	20 ± 4	30 ± 9	30 ± 9	20 ± 2	1.6 ± 0.1
KS21	60 ± 20	100 ± 30	80 ± 20	20 ± 4	30 ± 10	30 ± 11	20 ± 3	1.6 ± 0.2
KS22	50 ± 10	90 ± 30	80 ± 30	20 ± 5	30 ± 11	30 ± 12	20 ± 3	1.6 ± 0.2
KS25	50 ± 10	80 ± 30	60 ± 30	10 ± 5	20 ± 12	20 ± 12	20 ± 4	1.6 ± 0.3
KS28	50 ± 20	80 ± 20	70 ± 20	10 ± 3	30 ± 9	30 ± 9	20 ± 2	1.4 ± 0.2
KS29	60 ± 10	80 ± 20	70 ± 10	10 ± 2	20 ± 6	30 ± 7	20 ± 1	1.5 ± 0.1
LY01A	60 ± 20	100 ± 30	80 ± 20	20 ± 6	30 ± 10	30 ± 9	20 ± 2	1.6 ± 0.1
LY01B	50 ± 10	80 ± 20	60 ± 20	10 ± 3	20 ± 8	30 ± 9	20 ± 1	1.5 ± 0.1
KSNuf3	60 ± 20	100 ± 30	80 ± 20	20 ± 4	30 ± 11	30 ± 12	20 ± 3	1.6 ± 0.2
KS66A	50 ± 10	70 ± 20	60 ± 20	10 ± 2	20 ± 8	20 ± 7	20 ± 2	1.4 ± 0.2
KS77A	50 ± 10	80 ± 30	70 ± 20	10 ± 4	20 ± 11	30 ± 11	20 ± 3	1.6 ± 0.2
KS78A	60 ± 10	90 ± 20	70 ± 20	10 ± 3	30 ± 9	30 ± 10	20 ± 2	1.6 ± 0.1

[a] Measured light in December at each replicate tray position displaying average and SD for each ecotype *n*=4.

[b] Light intensity (LI), Photon flux density (PFD), photosynthetic photon flux density (PPFD: blue + green + red), blue, green,

red, far-red and ultra-violet light measured in µmol photons m^{-2} s^{-1}.

Table S3B. Light intensity and spectra in a glasshouse environment in February did not significantly vary between ecotypes.

Ecotype	LI1	LI2	PFD	PPFD	FR	R	G	B	UV
KS02	140 ± 20	110 ± 20	140 ± 20	120 ± 20	20 ± 4	50 ± 8	50 ± 7	20 ± 4	1.9 ± 0.4
KS03	160 ± 10	100 ± 20	140 ± 30	110 ± 20	20 ± 4	50 ± 10	50 ± 10	20 ± 3	1.9 ± 0.3
KS04	160 ± 30	120 ± 20	150 ± 30	120 ± 20	30 ± 6	50 ± 9	50 ± 9	20 ± 6	2.0 ± 0.5
KS06A	170 ± 40	120 ± 20	140 ± 30	110 ± 30	20 ± 6	50 ± 12	50 ± 12	20 ± 5	1.9 ± 0.4
KS06B	150 ± 20	120 ± 20	140 ± 20	120 ± 20	30 ± 5	50 ± 8	50 ± 9	20 ± 4	2.0 ± 0.2
KS09	150 ± 30	100 ± 10	140 ± 30	110 ± 30	20 ± 4	50 ± 11	50 ± 11	20 ± 4	1.9 ± 0.4
KS12	160 ± 20	140 ± 20	160 ± 20	130 ± 20	30 ± 6	50 ± 6	50 ± 6	30 ± 5	2.2 ± 0.4
KS13	180 ± 40	130 ± 10	160 ± 20	130 ± 10	30 ± 4	50 ± 7	50 ± 7	20 ± 3	2.1 ± 0.2
KS14	170 ± 30	130 ± 20	160 ± 20	130 ± 10	30 ± 2	50 ± 5	50 ± 6	30 ± 3	2.2 ± 0.3
KS15	100 ± 20	110 ± 10	130 ± 20	100 ± 20	20 ± 4	40 ± 8	40 ± 8	20 ± 3	1.9 ± 0.2
KS16	160 ± 20	100 ± 10	140 ± 20	110 ± 20	20 ± 4	50 ± 9	50 ± 9	20 ± 3	1.9 ± 0.2
KS17	140 ± 30	120 ± 10	130 ± 30	110 ± 20	20 ± 5	40 ± 10	40 ± 10	20 ± 4	1.9 ± 0.3
KS18	150 ± 10	130 ± 20	150 ± 20	120 ± 20	30 ± 5	50 ± 7	50 ± 6	20 ± 5	2.0 ± 0.4
KS20	130 ± 30	110 ± 10	140 ± 10	110 ± 10	20 ± 2	40 ± 4	40 ± 5	20 ± 1	1.9 ± 0.2
KS21	160 ± 10	130 ± 10	140 ± 20	110 ± 20	20 ± 3	50 ± 8	50 ± 8	20 ± 3	1.8 ± 0.2
KS22	150 ± 30	100 ± 20	140 ± 30	110 ± 20	20 ± 5	50 ± 10	50 ± 10	20 ± 4	1.8 ± 0.3
KS25	160 ± 30	120 ± 10	160 ± 20	130 ± 10	30 ± 4	50 ± 5	50 ± 4	30 ± 4	2.2 ± 0.3
KS28	120 ± 20	120 ± 10	150 ± 10	120 ± 10	30 ± 5	50 ± 4	50 ± 5	20 ± 4	2.1 ± 0.2
KS29	150 ± 1	110 ± 10	140 ± 10	110 ± 10	20 ± 3	40 ± 6	50 ± 6	20 ± 2	1.9 ± 0.2

LY01A	130 ± 30	130 ± 2	140 ± 10	110 ± 10	30 ± 3	50 ± 4	50 ± 4	20 ± 3	2.0 ± 0.3
LY01B	160 ± 20	110 ± 10	130 ± 10	110 ± 5	20 ± 2	40 ± 3	40 ± 1	20 ± 3	1.8 ± 0.3
KSNuf3	170 ± 1	120 ± 10	160 ± 20	130± 10	30 ± 4	50 ± 5	50 ± 5	20 ± 3	2.0 ± 0.2
KS66A	130 ± 20	110 ± 30	140 ± 30	110 ± 30	20 ± 3	40 ± 10	50 ± 13	20 ± 3	1.9 ± 0.3
KS77A	160 ± 50	110 ± 3	140 ± 30	110 ± 20	20 ± 5	50 ± 10	50 ± 10	20 ± 4	1.8 ± 0.3
KS78A	160 ± 8	110 ± 10	150 ± 30	120 ± 30	20 ± 6	50 ± 11	50 ± 11	20 ± 5	1.9 ± 0.4

[a] Measured light in February at each tray position displaying average and SD for each ecotype *n*=4.

[b] Photon flux density (PFD), photosynthetic photon flux density (PPFD: blue + green + red), blue, green, red, far-red and ultra-violet light measured in µmol photons m^{-2} s^{-1}.

[c] LI- light intensity in Feb LI1 and LI2 indicates two measurements on different days.

Table S4. Duckweeds have over 60 volatile compounds and 22 of these vary between duckweed species type.

Compound	Retention time	LRI	CAS number	*L. minor*	*L. japonica*	*L. turionifera*	*L. minuta*	*S. polyrhiza*
Aldehydes								
acetaldehyde	1.823	713	75-07-0	9.38 a	21.23 a	6.54 a	22.09 a	7.27 a
2-methyl-1-propanal	2.84	824	78-84-2	1.71 a	3.09 a	3.56 a	4.46 a	3.73 a
butanal, 3-methyl-	4.629	NA	590-86-3	16.01 b	22.16 ab	33.46 ab	49.17 a	22.13 ab
2-butenal	7.69	NA	4170-30-3	2.51 a	2.39 a	1.34 a	3.39 a	2.31 a
hexanal	8.639	1101	66-25-1	243.12 a	187.47 a	53.49 a	293.43 a	113.14 a
trans-2-pentenal	10.023	1153	1576-87-0	52.75 a	32.87 a	7.47 b	14.66 b	13.24 b
heptanal	11.397	NA	111-71-7	14.68 a	8.15 b	2.36 c	2.17 c	3.72 bc
2-hexenal	12.421	1244	505-57-7	52.38 b	40.05 b	6.97 c	73.53 b	148.21 a
trans-2-heptenal	15.193	NA	18829-55-5	4.17 a	3.40 a	1.84 a	2.98 a	1.97 a
ethylcyclopentene-1-carboxaldehyde	17.634	1448	36431-60-4	34.82 a	17.88 b	1.60 c	1.58 c	0.28 c
decanal	19.413	NA	112-31-2	1.51 a	2.12 a	0.60 a	4.17 a	0.76 a
benzaldehyde	20.298	1563	100-52-7	31.86 a	21.49 a	13.49 a	12.83 a	16.11 a

beta-cyclocitral	22.437	1657	432-25-7	5.27 a	4.51 a	3.74 a	6.25 a	4.69 a
trans-2-decenal	22.824	NA	3913-81-3	0.98 a	1.48 ab	0.16 ab	2.45 ab	0.07 bc
tridecanal	26.208	NA	10486-19-8	15.30 a	11.28 a	19.92 a	1.21 b	4.91 ab
tetradecanal	28.273	NA	124-25-4	28.62 a	17.33 a	16.68 a	0.95 b	11.59 a
pentadecanal	30.246	NA	09/11/2765	100.40a	70.86 a	57.25 a	6.70 b	48.59 a
cis-11-tetradecenal	30.844	NA	35237-64-0	6.02 a	4.44 a	4.42 a	0.38 b	3.06 ab
2-tetradecenal	31.242	NA	51534-36-2	0.42 a	0.64 a	0.14 a	0.04 b	1.19 a
cis,cis-7,10,-hexadecadienal	35.306	NA	56829-23-3	2.31 a	2.39 a	2.57 a	0.14 b	3.50 a
Alcohols								
ethanol	5.027	951	64-17-5	23.19 a	14.52 a	10.29 a	16.93 a	23.80 a
1-undecanol	9.826	1145	112-42-5	15.27 a	16.57 a	2.42 a	4.80 a	0.41 a
1-penten-3-ol	10.632	1175	616-25-1	538.00 a	428.01 a	392.42 a	282.94 a	256.70 a
cis-2-pentenol	14.839	1337	1576-95-0	200.36 a	142.42 a	85.01 a	76.17 a	80.72 a
hexanol	15.621	1367	111-27-3	29.99 a	43.70 a	14.31 a	107.89a	71.70 a
cis-3-hexen-1-ol	16.488	1401	928-96-1	25.45 a	15.09 b	0.45 c	39.84 ab	49.09 ab
trans-2-hexen-1-ol	16.995	NA	928-95-0	1.53 bc	5.94 c	0.78 c	5.49 ab	27.35 a
1-octen-3-ol	17.968	1463	3391-86-4	17.28 a	17.98 ab	13.76 ab	9.94 b	6.60 c
Ketones								
3-pentanone	6.017	997	96-22-0	85.31 a	73.12 a	39.73 a	41.45 a	30.90 a
1-penten-3-one	7.098	1041	1629-58-9	189.53 a	90.10 a	58.88 a	38.09 a	22.48 a
2,3-pentanedione	8.064	1078	600-14-6	8.27 a	7.03 a	5.69 a	9.15 a	3.73 b
1-cyclopropylpropan-1-one	8.231	1086	6704-19-4	1.46 a	2.66 a	1.68 a	1.74 a	1.49 a
3-octanone	13.237	NA	106-68-3	1.22 a	1.31 a	1.02 a	1.10 a	0.65 a
cistus cyclohexanone	15.026	1345	2408-37-9	7.81 a	5.33 a	4.04 a	4.04 a	4.66 a
methyl heptenone	15.407	1360	110-93-0	3.82 a	3.56 ab	2.81 ab	2.09 b	2.65 b
L-fenchone	17.131	NA	7787-20-4	0.73 a	0.62 a	0.14 a	0.75 a	1.02 a
trans-geranylacetone	26.998	1878	3796-70-1	3.09 a	3.14 a	3.42 ab	1.48 b	2.45 ab

trans-beta-ionone	28.878	NA	79-77-6	11.91 a	9.78 a	9.84 a	15.05 a	11.40 a
Alkenes								
toluene	7.568	1059	108-88-3	4.16 a	3.32 a	2.84 a	3.40 a	7.13 a
p-xylene	11.445	1208	106-42-3	1.91 a	1.47 a	1.46 a	1.29 a	2.32 a
styrene	13.438	1283	100-42-5	1.47 a	1.23 a	1.31 a	1.02 a	2.10 a
cumene	13.502	NA	98-82-8	1.06 a	0.82 a	0.99 a	0.66 a	1.22 a
mesitylene	15.553	NA	108-67-8	1.09 a	0.93 a	1.05 a	0.72 a	1.09 a
benzene, 2-propenyl-	16.084	NA	300-57-2	1.44 a	0.74 a	1.06 a	0.95 a	1.79 a
1,3-di-tert-butylbenzene	17.536	NA	1014-60-4	6.42 a	9.17 a	8.11 a	10.86 a	11.01 a
naphthalene	25.232	1788	91-20-3	5.47 a	5.69 a	6.22 a	4.89 a	7.74 a
2-methyl naphthalene	27.531	NA	91-57-6	0.86 a	0.89 a	0.93 a	0.73 a	1.08 a
Others								
2-ethylfuran	5.428	970	3208-16-0	2.31 b	2.28 ab	1.21 c	4.57 a	4.54 ab
2-pentylfuran	12.55	1249	3777-69-3	16.55 a	9.53 b	10.70 ab	4.95 b	4.32 b
vinyl hexanoate	14.972	1343	3050-69-9	4.99 a	5.58 a	3.99 a	3.07 a	4.41 a
pyrrole	19.873	1543	109-97-7	1.89 b	1.89 b	3.14 a	1.81 bc	1.01 c
D-camphor	20.148	NA	464-49-3	2.26 a	1.70 a	0.73 a	2.80 a	1.37 a
linalyl anthranilate	20.366	NA	7149-26-0	2.66 a	1.76 b	0.61 b	2.79 a	0.79 b
2-ethyl-3-methyl maleimide	34.664	NA	20189-42-8	2.21 a	1.45 a	0.96 a	2.07 a	0.96 a

[a] Compounds found in freeze-dried duckweed in concentrations > 1 µg/kg from SPME-GCMS.

[b] Retention time of peaks corresponding to chromatograms and LRI linear retention index as calculated from alkanes C6-C19.

[c] Unique identifying compound CAS numbers are retrieved from the National Institute of Standards and Technology (NIST) database.

[d] Corresponding letters for groupings from paired Wilcoxon test, P = <0.05 used for significantly different groups for letter allocation.

[e] The compounds highlighted in dark grey (*n*=5) found in highest prevalence associated with chromatogram peaks (Table 1, Supplementary Fig. 3).

[f] The compounds in light grey (*n*=22) have significant differences in amounts between species.

[g] pentadecanal was in both groups: high prevalence with significant differences between species.

Table S5. Three volatile compounds are significantly different between accessions due to geographical differences.

1,3-di-tert-butylbenzene	**df**	**Chi sq**	**P value**
Light environment	1	4.05	0.04
Location	1	0.02	0.89
benzaldehyde	**df**	**Chi sq**	**P value**
Light environment	1	2.66	0.10
Location	1	3.88	0.05
1-penten-3-one	**df**	**Chi sq**	**P value**
Light environment	1	1.70	0.19
Location	1	4.44	0.04

[a] Compounds quantified in µg/kg in duckweed

[b] Ecotypes grouped by location (north/south UK) or by light (HL or LL).

[c] Kruskal-Wallis test used to derive significance using P value = <0.05.

Table S6. Duckweed aroma profiles compared to other herbs: basil, dandelion, spinach and coriander show most similarity with spinach and dandelion.

S6A. Compound	Duckweed (µg/kg)	Basil (µg/kg)	CAS number	Aroma
(+)-2-bornanone	1.77	1269.15	464-49-3	camphoreous, minty
gamma-muurolene	0.02	34.85	30021-74-0	woody
beta-cyclocitral	5.18	4.66	432-25-7	tropical
1-octanol	0.54	88.36	111-87-5	waxy
1-octen-3-ol	13.11	613.33	3391-86-4	earthy
2-butenal	2.4	0.33	4170-30-3	NA

1-propanone, 1-cyclopropyl-	1.8	0	6704-19-4	NA
2-thiapropane	9.13	33.48	75-18-3	sulfurous
3-hexen-1-ol, (Z)-	25.99	47.95	928-96-1	fresh, green
3-pentanone	54.11	2.68	96-22-0	ethereal
sabinene hydrate	0.06	465.27	546-79-2	minty, eucalyptus
acetaldehyde	13.3	5.21	75-07-0	ethereal
bergamotene <alpha-, cis->	0.03	1185.77	18252-46-5	NA
butanal, 3-methyl-	28.59	3.22	590-86-3	ethereal, aldehydic
cubebene <alpha->	0	59.09	17699-14-8	herbal
cymenene <para->	0.3	28.17	1195-32-0	phenolic
eugenol	0.05	2278.68	97-53-0	spicy
eugenol <methyl->	0.14	1966.92	93-15-2	spicy
fenchyl acetate	0.08	885.6	13851-11-1	balsamic, fresh
furan <alpha-, ethyl->	2.98	1.45	3208-16-0	chemical, beany
furan, 3-methyl-	1.53	0	930-27-8	NA
germacrene D	0.14	237.63	23986-74-5	woody
hept-5-en-2-one <6-methyl->	2.99	1.59	110-93-0	citrus, green
heptanal <n->	6.21	2.35	111-71-7	fresh, green
hexanal	178.13	13.82	66-25-1	fresh, green
linalyl anthranilate	0.96	1737.24	7149-26-0	floral
naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.beta.,8a.alpha.)-	0.04	242.91	39029-41-9	herbal, woody
octan-3-one	1.06	8.19	106-68-3	fresh, herbal

p-cymene	0.37	90.75	99-87-6	fresh, citrus
pent-(2E)-enal	24.2	1.91	1576-87-0	pungent, green
pent-(2Z)-enol	116.93	2.82	1576-95-0	green
penten-3-one	79.81	1.41	1629-58-9	spicy
1-penten-3-ol	379.62	12.52	616-25-1	green, ethereal
propanal, 2-methyl-	3.31	0.99	78-84-2	aldehydic, fresh
terpinene <alpha->	0	36.68	99-86-5	woody
terpineol <alpha->	0.08	460.55	98-55-5	terpenic, pine
terpinolene	0.05	303.52	586-62-9	herbal, fresh
tetradecanal	15.04	0	124-25-4	waxy

[a] Compounds significantly different between duckweed and basil by Games-Howell test P value = < 0.05

[b] 3 not in basil (red), 2 not in duckweed (red), 17 higher in duckweed (green), 21 higher in basil (green)

[c] Individual CAS numbers and aroma descriptors are provided.

[d] Duckweed species were averaged to give an average value for duckweed in each comparison

S5B. Compound	Duckweed (µg/kg)	Dandelion (µg/kg)	CAS number	Aroma
3-pentanone	54.11	18.01	96-22-0	ethereal
sabinene hydrate	0.06	0.29	546-79-2	minty, eucalyptus
p-cymene	0.37	0.65	99-87-6	fresh, citrus
dec-(2E)-enal	1.03	11.16	3913-81-3	fatty, waxy
naphthalene	6	1.31	91-20-3	pungent
2-methyl naphthalene	0.9	0.13	91-57-6	sweet, floral
5-hydroxypentanoic acid	0.69	1.25	166273-37-6	cheesy
terpinene <alpha->	0	0.1	99-86-5	woody

[a] Compounds significantly different between duckweed and dandelion by Games-Howell test P value = < 0.05

[b] 0 not in dandelion, 1 not in duckweed (red), 3 higher in duckweed (green), 5 higher in dandelion (green)

[c] Individual CAS numbers and aroma descriptors are provided.

[d] Duckweed species were averaged to give an average value for duckweed in each comparison

S5C. Compound	Duckweed (µg/kg)	Spinach µg/kg)	CAS number	Aroma
(+)-2-bornanone	1.77	3.36	464-49-3	camphoreous, minty
1-hexanol	53.52	57.88	111-27-3	ethereal
1-propanone, 1-cyclopropyl-	1.81	0	6704-19-4	NA
2-butenal	2.39	0	4170-30-3	NA
2-hexenal	64.23	7.02	505-57-7	green
3-hexen-1-ol, benzoate, (Z)-	0.01	0	25152-85-6	green
3-pentanone	54.11	20	96-22-0	ethereal
sabinene hydrate	0.06	0.13	546-79-2	minty, eucalyptus
acetaldehyde	13.3	0	75-07-0	ethereal
acetic acid, methyl ester	3.05	19.67	79-20-9	ethereal
bergamotene <alpha-, cis->	0.03	0.42	18252-46-5	NA
butanal, 3-methyl-	28.59	9.77	590-86-3	ethereal, aldehydic
cis,-cis-7,10,-hexadecadienal	2.19	0	56829-23-3	NA
ethanol	17.75	50.87	64-17-5	alcoholic
eugenol <methyl->	0.14	2.32	93-15-2	spicy
furan <alpha-, ethyl->	2.98	0.98	3208-16-0	chemical, beany

furan, 2-pentyl-	9.21	3.42	3777-69-3	fruity, green
hept-(2E)-enal	2.87	0.05	18829-55-5	green
hept-5-en-2-one <6-methyl->	2.99	0.45	110-93-0	citrus, green
heptanal <n->	6.22	0.12	111-71-7	fresh, green
hexanal	178.13	1.97	66-25-1	fresh, green
L-Fenchone	0.66	5.14	7787-20-4	camphoreous, herbal
linalyl anthranilate	0.96	13.51	7149-26-0	floral
naphthalene	6	2.96	91-20-3	pungent
naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.beta.,8a.alpha.)-	0.04	0.08	39029-41-9	herbal, woody
2-methyl naphthalene	0.9	0.4	91-57-6	sweet, floral
octan-3-one	1.06	1.19	106-68-3	fresh, herbal
pentadecanal	56.76	0	2765-11.-9	NA
pent-(2E)-enal	24.2	0.71	1576-87-0	pungent, green
pent-(2Z)-enol	116.94	9.11	1576-95-0	green
penten-3-one	79.82	1.88	1629-58-9	spicy
1-penten-3-ol	379.62	43.65	616-25-1	green, ethereal
terpineol <alpha->	0.08	0.64	98-55-5	terpenic, pine
tetradecanal	15.04	0	124-25-4	waxy
tetradec-(11Z)-enal	3.67	0	35237-64-0	NA
tetradec-2-enal <-trans>	0.49	0	51534-36-2	citrus
tridecanal <n->	10.52	4.85	10486-19-8	aldehydic, fresh

[a] Compounds significantly different between duckweed and spinach by Games-Howell test P value = < 0.05

[b] 9 not in spinach, 26 higher in duckweed (green), 11 higher in spinach (green)

[c] Individual CAS numbers and aroma descriptors are provided.

[d] Duckweed species were averaged to give an average value for duckweed in each comparison

S5D. Compound	Duckweed (µg/kg)	Coriander (µg/kg)	CAS number	Aroma
gamma-muurolene	0.02	0.05	30021-74-0	woody
1-octanol	0.54	76.65	111-87-5	waxy
2-hexen-1-ol, (E)-	8.22	0	928-95-0	fruity
3-pentanone	54.11	7.17	96-22-0	ethereal
dec-(2E)-enal	1.03	1360.24	3913-81-3	fatty, waxy
germacrene D	0.14	0.19	23986-74-5	woody
hexanal	178.13	71.11	66-25-1	fresh, green
furan, 3-methyl-	1.54	0	930-27-8	NA
linalyl anthranilate	0.96	20.14	7149-26-0	floral
naphthalene	6	10.66	91-20-3	pungent
naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.beta.,8a.alpha.)-	0.04	0.21	39029-41-9	herbal, woody
2-methyl naphthalene	0.9	1.97	91-57-6	sweet, floral
pent-(2E)-enal	24.2	2.7	1576-87-0	pungent, green
pent-(2Z)-enol	116.94	10.34	1576-95-0	green
penten-3-one	79.82	5.75	1629-58-9	spicy
1-penten-3-ol	379.62	38.33	616-25-1	green, ethereal

tridecanal <n->	10.52	10.7	10486-19-8	aldehydic, fresh

[a] Compounds significantly different between duckweed and coriander by Games-Howell test P value = < 0.05

[b] 2 not in coriander (red), 8 higher in duckweed (green), 9 higher in coriander (green)

[c] Individual CAS numbers and aroma descriptors are provided.

[d] Duckweed species were averaged to give an average value for duckweed in each comparison

Table S7. Overall fresh weight (FW) biomass and fresh weight per month varied between ecotypes.

Ecotype	Registered clone	FW(g)	FW (g/ month)
KS02	*Lemna minor* 5882	142.93	23.82
KS03	*Lemna japonica* 5883	95.07	15.84
KS04	*Lemna japonica* 5884	246.1	41.02
KS06A	*Lemna minuta* 5885	236.43	39.41
KS06B	*Lemna minuta* 5886	233.35	38.89
KS09	*Lemna minor* 5887	131.33	21.89
KS12	*Spirodela polyrhiza* 5888	392.22	65.37
KS13	*Lemna minor* 5889	170.3	28.39
KS14	*Lemna japonica* 5890	197.28	32.88
KS15	*Lemna japonica* 5891	136.59	22.77
KS16	*Lemna turionifera* 5892	124.73	20.79
KS17	*Lemna japonica* 5893	201.9	33.65
KS18	*Lemna japonica* 5894	303.3	50.55
KS20	*Lemna minuta* 5895	214.36	35.73
KS21	*Lemna japonica* 5896	308.69	51.45
KS22	*Lemna turionifera* 5897	211.66	35.28
KS25	*Lemna minuta* 5898	318.68	53.11
KS28	*Lemna japonica* 5900	292.17	48.7
KS29	*Lemna minor* 5901	193.59	32.27

LY01A	*Lemna japonica* 5902	271.79	45.3
LY01B	*Lemna minuta* 5903	226.85	37.81
KSNuf3	*Lemna minor* 5909	140.34	23.39
KS66A	*Lemna japonica* 5906	92.69	15.44
KS77A	*Spirodela polyrhiza* 5907	227.58	37.93
KS78A	*Spirodela polyrhiza* 5908	260.08	43.35

[a] Total biomass over the six month period divided by 6 to estimate FW/month.

[b] FW and FW/month per ecotype is given to 2 dp.

[c] Registration codes correspond to the Rutger's duckweed collection (ruduckweed.org)

6. Chapter six. General discussion

The overall overarching aim was to study the different potentials of duckweed species and accessions for phytoremediation and nutrition. Hyperaccumulators of heavy metals for phytoremediation and macronutrients for food were found from chapters two and three. Duckweeds with high biomass, antioxidant contents, pleasant aroma profiles and efficient light responses are marked for development as a biofortified vegetable or protein supplement from chapters four and five.

6.1. Duckweed for nutrition

Nutrient deficiencies in Fe, Ca, Mg and Zn are common worldwide and linked with diseases and shortened life expectancy (Kiani *et al.*, 2022). Thus, research-guided duckweed selection using mineral composition holds promise for application as a vegetable or supplement. To this end, whole biomass from *Lemna minuta* and *Lemna yungensis* 9208 displayed high Mg, *Spirodela polyrhiza* and *Landoltia punctata* species were high in Ca and *Lemna minuta* and *Lemna japonica* had higher macronutrients Mg and K (Chapter two, three). More studies on elemental distributions between fronds, roots and organelles alongside genetic components would further expand on species ionome variation (Lahive *et al.*, 2011; Oláh *et al.*, 2024).

Elemental form is also important for digestion, for instance Mg and Ca oxalates act as anti-nutrients in vegetables such as spinach and kale (Hemmige Natesh *et al.*, 2017). As some duckweed species contain mineral oxalates (Mazen *et al.*, 2003), high overall whole tissue concentration is not the only factor to consider. Application of novel species and accessions in human trials can be used to assess this degree of digestion and bioavailability of minerals, as documented for limited other duckweed species (Kaplan *et al.*, 2019; Zeinstra *et al.*, 2019; Yaskolka Meir *et al.*, 2021). Further consideration for processing duckweed biomass (e.g. cooking, blanching) to reduce oxalates and increase mineral availability warrant further exploration too (Chai & Liebman, 2005).

Towards food safety, the whole ionome of a vegetable cannot be ignored, including relationships between different elements. This work found biofortification of B and Fe in UK accessions (e.g. LAN, MAV) and enhanced macro- and micronutrient contents e.g. in *Lemna trisulca 7192*, however undesirable increased heavy metals also featured (Chapter two, three). Despite negligible heavy metals provided in the nutrient growth medium (Appenroth *et al.*, 2018), this reinforces a need to manipulate growth medium to produce safe duckweed biomass for consumption. Furthermore, methods that retain high macronutrients but reduce damaging heavy metal composition should be in focus. For instance, applying a synthetic microbiome community during growth could impact the ionome (Egamberdiyeva, 2007; Stout *et al.*, 2010; Jewell *et al.*, 2023). Otherwise, applying post-harvesting washing and extraction techniques may further reduce metal contamination of duckweed foodstuffs (Sattar *et al.*, 2015; Amir *et al.*, 2019).

Specifically, Mn reduction is one goal for development of *Lemna minor/gibba* powder for consumption (Turck *et al.*, 2023). This work found a negative relationship between Mn and K composition in duckweed (Chapter two). Therefore, accession selection and environmental manipulation can inform production of biomass for consumption. For instance, selection of a high K accession or otherwise provision of additional K in the growth media could result in a targeted desirable Mn reduction in duckweed biomass.

Both high light responses of *L. japonica* ALL1 (high carotenoids) and *S. polyrhiza* MOO (high anthocyanin) offer potential for food or space horticulture. Increased light irradiance in vertical farms is a considerable factor to maximise duckweed growth, while increasing carotenoids and anthocyanins for plant defence. To this end, consumers of accessions MOO and ALL1 produced using the growth methods here could provide antioxidants with health benefits including radiation protection (Smith & Zwart, 2008, Smith *et al.*, 2015; Stewart *et al.*, 2020). Furthermore, high natural photoprotection or NPQ can be coupled with crop yield benefits, for example in rice (Hubbart *et al.*, 2018) and documented in *Lemna japonica* from this work (Chapter five).

Additionally, provision of minimal light inputs to maximize biomass outputs is a goal for year-round crop production in sustainable farming systems (Jayalath & van Iersel, 2021). Overall, *L. japonica* ALL1 grew fastest in low light artificial light regimes and may be suited for admission as a radiation efficient accession for small-scale hydroponics. For duckweed biomass scalability, accessions showed different responses in a glasshouse setting suitable for commercialisation as an animal feed or protein supplement. To this end, *S. polyrhiza* MOO produced high biomass with a multitude of flavonoid compounds for powder supplement production, however *Lemna japonica* ALL1 only performed well under small-scale LED artificial lighting relevant for space horticulture, and limited growth in a glasshouse. Thus, ongoing work should apply accessions in a setting close to the intended commercial application.

6.2. Duckweed for phytoremediation

Specifically for phytoremediation in the UK, abandoned metal mines in England and Wales have leached concerning levels of Cd, Cu, Zn, Pb into lakes and rivers, effecting coastal and marine life (Environment Agency, 2008, 2023). This UK survey additionally uncovered high concentration of Mn and Fe in freshwater duckweed habitat sites. The southwest Cornwall region is of particular concern for metallic and acidic minewater including As in both soil and drinking water (Mitchell & Barre, 1995, Braungardt *et al.*, 2020). and therefore requires remediation strategies (Environmental Agency, 2008; Wyatt *et al.*, 2013). From south England, *L. minor* HAW highly accumulates Zn, Pb and Fe and *L. minor* LAN Pb and Fe. These accessions could be recommended for minewater remediation in nearby contaminated watercourses, provided they continue to hyperaccumulate in the context of real-world conditions.

In the North, there are high levels of Zn and Pb contamination which are in need of remediation in Glasgow (Fordyce *et al.*, 2019; Rodgers *et al.*, 2020). In this instance, *Lemna minuta* BOG could be trialled on wastewater for phytoremediation here as it showed potential to accumulate Zn, Cd and Pb plus other metals and indeed originates from Glasgow.

Duckweed show fine-scale ionomic differences in common environments even when originating from nearby locations (e.g. *L. yungensis* 9210 & 9208 and UK accessions). In general, *Lemna minuta* species are high Mg accumulators and *L. japonica* As accumulators both found to grow in high Mg and As native UK waters. Therefore, *Lemna minuta* accessions could be tested for their potential in remediation of dairy wastewater exploring the limits of Mg:Ca ratio tolerance (Walsh *et al.*, 2020) and *Lemna japonica* could be tested on contaminated minewater. While variation of duckweed ionomes on native water and controlled nutrient media are still not unified, water chemistry data discovered here can be used to tailor synthetic media reflecting wastewater conditions. This could be a powerful technique to further explore limits of tolerance and accumulation in potential accessions for phytoremediation going forward (Bergmann *et al.*, 2000; Walsh *et al.*, 2020; Sasmaz Kislioglu, 2023).

Both UK hybrid and invasive species may be recommended for remediation in the southern and midland parts of the UK where they are already established. However, using invasive and hybrid species for phytoremediation particularly in north Scotland should be avoided. This is in the interest of biodiversity, as they are currently not naturally prevalent from this UK survey and introduced invasive species already show capability to hybridise with native species elsewhere in the UK.

6.3. Conclusion

The outcomes of this work are the establishment of a novel diversity panel of duckweeds, characterised at the levels of accessions, species and environments. It provides local and research-driven duckweed candidates with physiological traits for bespoke applications in phytoremediation and nutrition. The UK collection of duckweeds provide a tool to further explore the genetic basis for natural variation - specifically in the areas of light responses, ionomics, metabolomics, and traits involved in invasive strategies. Each of these traits were highlighted from phenotyping and environmental studies undertaken here. Accessions from this thesis have been registered to the Rutger's database with assignment of clone numbers available at www.ruduckweed.org and all sequencing data have been deposited at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1026139, /PRJNA1074359 and /PRJNA1030266

6.4. References

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7. Chapter seven. Supplementary methods

Consider this Methods section as a supplementary to the methods detailed in chapters two, three, four and five.

7.1. Supplementary methods for chapter two

For this chapter, duckweed clones/species were sourced from a worldwide duckweed collection - the Landolt collection, originally from Eidgenössische Technische Hochschule Zürich, and now housed in Milan. The varied clones and species across genera used for chapter two are documented in Supplementary table s1 from chapter two. Duckweed sampling and inductively coupled mass spectrometry (ICP-MS) are described in the methods of chapter two.

The duckweed growth, harvest and ICP-MS experiments were performed by collaborators prior to starting the PhD project. I analysed and interpreted the data and had a major role in writing the manuscript.

7.1.1. Statistical analysis methods for ICP-MS analysis

Raw data for whole duckweed elemental composition from day one, three and five from inductively coupled plasma mass spectrometry (ICP-MS) for each duckweed replicate (n=6) were grouped into averages for each clone. Comparisons at the subgroup and genus levels were made due to vast phenotypical and genotypical differences and was enabled by the number of species within each group. Shapiro Wilk tests showed that data was not normally distributed for any element and visual quantile-quantile QQ plots confirmed raw data was skewed. Additionally, Bartlett's tests showed that data variance was different between groups. Therefore elemental data was log transformed for further analysis using log10 which gave QQ plots with reduced skew. Log data was then used for significance tests to find elemental differences between rooted/rootless subgroups and for Pearson's correlations to assess relationships between variables.

To plot a Principal component analysis (PCA), day one, three and five data were averaged and then scaled and centered (Chapter two, Supplementary figure 3). The entire dataset for each elemental concentration amongst duckweeds was plotted as a boxplot as a function of day, which determined no difference between days as boxplots greatly overlapped. Outliers plotted outside of the boxplots (outside of 10% and 90% data variance) were exceptions to this (Chapter two, Supplementary figure 1). Outlying duckweed clones had significant differences between days for certain elements and each were plotted as bar plots using Excel to show transient differences in that element over time (Chapter two, Supplementary figure 2). For the rest of the analysis, day five was chosen to represent the dataset. To quantify elements denoted as high or low accumulating within clones, data was normalised to give z-scores to scale each elemental concentration against the full dataset. Normalisation was conducted using the Microsoft Excel function

‘Standardise’ whereby the average mean and standard deviation for all clones were used to scale all values against the rest of the panel. Heat maps were generated using Graphpad prism 9.4.1. for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com”. All other plots and statistical tests were performed using R (version 3.0.2 “Frisbee Sailing”; R Development Core Team, 2013; see http://www.R-project.org) and R Studio v 1.0.136 (RStudio Team, 2020) (v 1.0.136).

All R scripts are included at: https://github.com/Duckweed-KS/Worldwide-duckweed-ionomics

1. Normalisation tests for each element including Shapiro Wilk, QQ plots, Bartlett’s tests.
2. Create boxplots between days for each element.
3. PCA to plot subgroup and genera differences for all elements.
4. Radar plots for elements.
5. Linear models and Corr plot between variables.
6. World map of sampling sites.
7. *Lemna* section violin plots for Mg content.

7.2. Establishing a UK novel duckweed collection

A novel UK duckweed collection was established and maintained from this project. During the course of this study >125 duckweed accessions were collected from >100 sites with environmental data collection across the UK. These accessions and environmental data feature in chapters three, four and five. The equipment used for collection are included in Table 7.1.

Field (light)	**Supplier**
Light meter (LI-250)	LI-COR Biosciences, Lincoln, Nebraska, USA.
Light spectrometer (LI-180)	LI-COR Biosciences, Lincoln, Nebraska, USA.
Field (water)	
Handheld pH field probe	Hanna instruments, Woonsocket, Rhode Island, USA.
Absorbent paper towel, rolled blue Paper Towel, 198 x 200 mm, 7200 Sheets	Kimberley-Clark, Reigate, UK.
Ziplock bags 279 – 406 mm	Fisher Scientific Ltd, Loughborough, UK.

Plastic jerrican 12.5 L	Scientific Laboratory Supplies Ltd, Nottingham, UK.
HDPE Universal 25mm x 90mm tubes with screw caps (30 ml)	Sarstedt, Leicester, UK.
HDPE Wide-mouth amber reagent bottles (125 ml)	United Scientific Supplies, Libertyville, Illinois, USA.
Schott Duran® glass laboratory bottles, with caps (100 ml)	Merck, Massachusetts, USA
Schott Duran® laboratory bottles, with caps (500 ml)	Merck, Massachusetts, USA
Sterile terumo syringe (30 ml)	Terumo, Leuven, Belgium
Sterile Henke-ject syringe (12 ml)	Henke Sass Wolf, Tuttingen, Germany
Sterile PES Syringe Filter (0.45 um)	Fisher Scientific Ltd, Loughborough, UK.
SBB Aqua Plus boiling water bath	Fisher Scientific Ltd, Loughborough, UK.
Field (other)	
Anemometer	RUZIZAO®, Shenzhen, China
Field (duckweed)	
Falcon™ 50 mL sterile Conical Centrifuge Tubes	Fisher Scientific Ltd, Loughborough, UK.
Canon 650D camera	Canon, Tokyo, Japan.
Modified equipment	
Fishing rod with adapted ping pong (7.5 cm) reference	NA
Reach-in device with adapted ends for fitting falcon, or two sizes of HDPE bottles	NA

Table 7.1. Field equipment used for duckweed collection in 7.2.

7.2.1. Local duckweed collection sampling choice

A core collection of duckweeds commenced in the spring and summer of 2020. A local collection of sites was chosen for several reasons: 1. Sites can

be revisited seasonally to assess species density over time 2. They allow frequent re-collection of environmental data to observe both spatial and temporal fluctuations. 3. COVID-19 restrictions allowed limited travel at this time. The regional transect for the local collection is within north England extending from west to east Yorkshire. The collection regions include Bradford (BFD), York (YOR) and HULL (HUL). These were selected as a transect extending from inland to coast, with variation in both bedrock and altitude, thus expecting to yield local variation. Duckweed sites were chosen from local knowledge and using Google Maps to virtually locate sites with public access within each region. Up to ten sites were selected for each region and searched until at least six were found to contain duckweeds. Each duckweed site was at least 1 m away from other sites to be considered as independent sites. On selection of 19 duckweed-containing sites these were then revisited every season up to summer 2022. The site names, details and locations are presented in Chapter 3, supplementary table 1. The local cohort of duckweeds arising from the initial 2020 collection sites were characterised in chapters three, four and five.

7.2.2. UK-wide duckweed collection sampling choice

A UK-wide duckweed collection was conducted for the following reasons: 1. To characterise species density across UK regions. 2. Assess north-south spread of native vs invasive species. 3. Collect over 100 ecotypes to find potential elemental hyperaccumulators. 4. Collect water data from over 100 sites for assessment of elemental tolerance ranges supporting duckweed presence.

The UK-wide duckweed collection was built on the local collection with additional regional collections in autumn 2020: Midlands (MID) and incorporation of north west: Lancaster region (LAN). In spring 2021, the north and south latitudinal span of the UK was captured, starting with the south in early April and ending in north Scotland in mid-May to incorporate variation in UK spring-time. All duckweed sampling regions are provided in Fig 7.1 which is taken from Google My Maps. Four regions were sampled in the south: Hastings (HAS), Cornwall (COR), Bristol (BRI) in England and Newport (NEW) in Wales. Three regions were sampled in Scotland: in Glasgow (GLA), Elgin (ELG) and Aberdeen (ABE).

Figure 7.1. Duckweed collection sites across the latitudinal and longitudinal axes of the UK forming a novel UK cohort. Map of locations included in the UK duckweed collection panel. Purple points were collected in 2020 and blue points in 2021. Map available to access on Google My maps with higher resolution, with request permissions.

In determining choice of sampling regions the Global Biodiversity Information Facility (GBIF.org, 2022) and Botanical Society of Britain and Ireland (BSBI.org, 2022) botanical databases were consulted. GBIF was used to find reports of species detected and prevalence over seasons and years. The total number of observations for key UK duckweed species is shown in Fig 7.2. *Lemna minuta* is considered as an invasive species and is on the Global Register of Introduced and Invasive Species for Great Britain (GRAS). Following GBIF observations, *L. minuta* is increasing in observations, supporting that reported in Italy in (Ceschin *et al.*, 2018). *Lemna* and *Spirodela* species had increasing observations in spring, so spring was chosen for collection in order to unbiasedly capture all species and at the same time find ecotypes thriving early in the season. Maps from BSBI.org were used to determine regions of interest by using the search terms '*Lemna*' and 'post 2020'. Maps were accessed in January 2021 and were filtered by where *Lemna* had been recently thriving. BRI and ELG were particular hotspots so were included in the collection. By using the search terms '*Lemna minuta*' and '*Lemna turionifera*' the first reported observation on GBIF.org for both species was also in BRI supporting its inclusion. As invasive *L. minuta* was reported as entering the UK from Europe via ports, including '*Lemna minuta*' as a search term found observations in England and nouth Scotland. ABE has a port and was therefore included in the north sampling. HAS and COR offer extreme variation of the south coast in closest proximity to Europe and additional differences in bedrock. NEW was used as the solo sampling region in Wales, because of its proximity to BRI but separation geographically by a river.

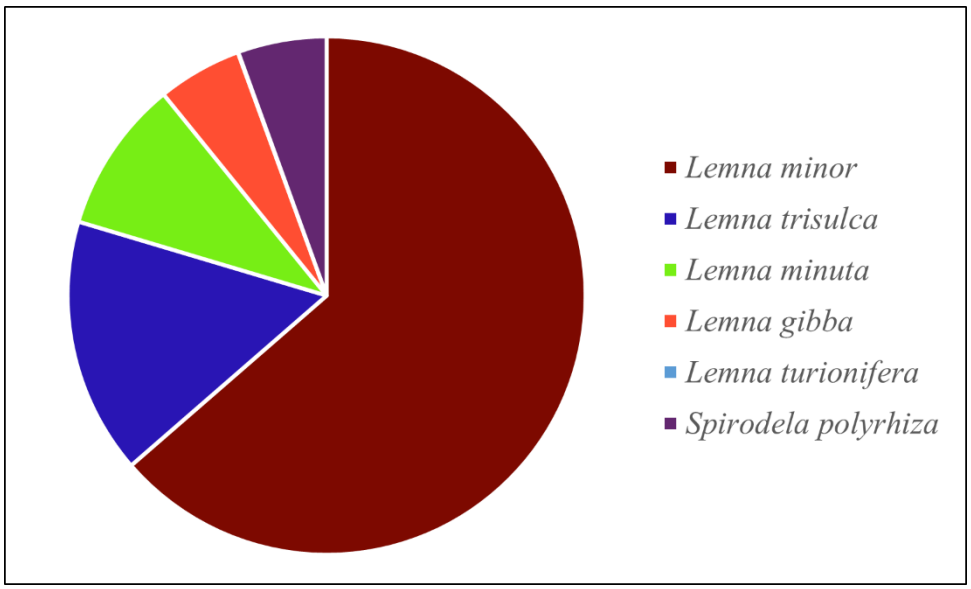

Figure 7.2. Native *Lemna minor* holds the major share of duckweed species observations in the UK. Pie chart showing prevalence of five *Lemna* species and *Spirodela polyrhiza* within the UK. Species density is presented as records per kilometres using all observations as reported on GBIF.org. The region of United Kingdom includes Great Britain and northern Ireland. Observations were accessed on 14th December 2020.

Potential sites within each region were decided using bsbi.org using search terms '*Lemna*' and 'post 2010'. Observations were plotted on maps and regions of high density were overplotted onto Google Maps to find locations. In some cases Google Street View was used to assess accessibility and duckweed presence beforehand. Up to 30 potential sites were chosen virtually and then over the three-five days in each region, each potential site was searched until n=10 sites per region containing duckweeds were successfully sampled. Sites and regions sampled in the UK duckweed collection are included in Chapter three, supplementary table 1 and plotted on My Google maps.

7.2.3. Collection of duckweed accessions

Sites were used if they contained one or more duckweeds. Where water was easily and safely accessible a branch from the area was used to collect a cluster of duckweeds. Where sites were deep or unsafe to reach, a modified water-reach in device was used to provide a reach of 2 m. After collection, the water reach in device was washed thoroughly in local water, or with deionized water, where sites were dense with duckweed mats, in order to remove the possibility of contamination between sites. Where multiple accessions were found in the same location, individuals of each different type were collected into seperate sealed Falcon™ containers and labelled A, B, C depending on size of accessions. At each site, duckweed species were estimated using size, number of fronds per colony, number of roots per colony and length of roots. Duckweeds were stored in local water in natural daylight cycles and temperature until return to laboratory, where species type was confirmed by further phenotyping and genome sequencing. All duckweed accessions from the UK used in this project are provided in Chapter 3, Supplementary table 2A.

7.2.4. Collection of environmental data

Collection of water samples to quantify dissolved elements are described in depth in chapter three, and differ slightly depending on local (repeated seasonal collections) and single site UK wide sampling. Diluted nitric acid concentrations of 0.5% and 10% were used for each water sampling method respectively and prepared from concentrated 68% nitric acid (Primar grade), prior to collections. The 10% stock was made with 882.35 ml conc. nitric acid into 6 L Milli-Q water and stored in a 10 L HDPE bottle. For the UK wide collection, one ml of 10% nitric stock was aliquoted into HDPE Universal sampling bottles, covered with foil, placed into ziplock bags and taken to the field. Each bottle was labelled and pre-weighed empty and again with the addition of nitric acid using a 5 dp precision balance. Three randomly labelled bottles were used to collect water per site and water was filtered on site.

For seasonal water collection amber HDPE bottles were washed using 10% nitric stock and Milli-Q water over night. For the field, 0.5% nitric acid was made by half dilution of the 10% stock and taking 5 ml of this into 50 ml Milli-

Q water. This was taken into the field in a 100 ml Duran bottle covered in aluminum foil and made fresh for each seasonal collection.

Collection of seasonal light intensity and light spectral measurements are well-described in chapter four. Light measurements were made for the local collection around the same relative time differences for each site during seasonal visits over a two day period. BFD light measurements were taken on day one, followed by YOR and then HUL on day two. All light measurements were taken between nine am and five pm in summer and ten am and three pm in seasons with shorter day lengths.

At each site, pH, water temperature, atmospheric temperature and wind speed was also recorded. Before use in the field, a pH probe (Hanna instruments) was calibrated using standard solutions pH 4 and pH 7 using manufacturer solutions and instructions. In the field, fresh sealed Falcon™ tubes were washed in local water three times and water from the top surface was collected. The pH and water temperature were directly measured by stirring until readings stabilized. The pH probe was washed with deionized water between sites and dried with absorbent paper towels. On returning to the lab, pH was measured again using a bench pH probe. Water temperature was measured using the Hanna pH probe at each site. An anemometer (Ruzizao) was used as close to the water as possible and held vertically for one minute to record the maximum wind speed in m/s, at the same time the air temperature was taken with this device in °C.

7.2.5. Measuring duckweed site coverage

At each site, duckweed coverage was estimated visually by scoring between zero-five. Using the following classifications: 0 – zero duckweeds or null site, 1 – 1 duckweed, 2 – 2 -10 duckweeds, 3 – reasonable duckweed coverage, 4 – >10% water visibility, 5- full duckweed coverage, matting. Where coverage showed variability at a site, the highest recorded coverage score was noted.
Photographs were used for quantification of surface coverage and are described in chapter four. Sites were variable and fraught with confounding variables e.g. quality of light, distance from water, nature of site for reachability and other objects in the water including debris, and other green plant biomass. To combat this, a canon camera was suspended on a camera boom with an umbrella to improve photo quality and reduce light scattering and water reflection. A white reference was also used for size and white referencing on photos, consisting of a white ping pong ball 7.5 cm suspended on a 2 m fishing rod. The white ping pong ball could be changed between sites and each were used to photograph level with the water surface.
For analysis, a FIJI image processing program was used with a pipeline for each image, using three representative squares per image to assess average and degree of variability per site. The following was used to select representative areas: If duckweed had full coverage or were not present then all areas containing water were representative due to homogeneity. Where coverage was patchy, areas of both sparse and fuller coverage regions were used to account for this in averages. Where 1-5 colonies were found, one area containing duckweed were selected and naturally other areas did not contain

any, therefore giving low coverage > 5%. This method gave reflective areas of the water course at each site, and enabled comparisons of sites with each other. This quantitative coverage data was compared against original scores determined at the field sites.

During establishment of the novel UK duckweed collection all duckweed accessions were sterilised and cultivated in controlled environments. The biomass was harvested for DNA extraction and sequencing, growth and root assessments, light acclimation experiments including chlorophyll fluorescence, pigment extraction for analysis using spectrophotometry and HPLC and micronutrient analysis using ICP-MS in 7.3 and 7.4. Then ecotypes were grown at scale in a glasshouse environment in 7.5, their growth and health were measured using a Fieldspectrometer. Ecotypes were harvested for freeze-dried powder for '-omics' analysis with SPME-GCMS, GCO-MS, HPLC-PDA and LC-MS/MS for aroma and metabolite profiling. The lab equipment and reagents used for these experiments are presented in table 7.2.

Laboratory (apparatus)	**Supplier**
CELLSTAR® six-well cell culture plate, sterile with lid	Greiner bio-one, Frickenhausen, Germany.
Growth cabinet A-1000	Conviron, Winnipeg, Canada
Growth cabinet GS-2000-SH	Conviron, Winnipeg, Canada
Academy Conical Flask Narrow Neck 500ml	Better Equipped, UK
Academy Conical Flask Narrow Neck 250ml	Better Equipped, UK
Cotton Wool White Non Absorbent 500g	Zoro, UK
Sterilising oven GS-150	LTE Scientific LTD, Oldham, UK
SafeFAST Classic microbiological safety cabinet class II	Faster-air, Cornaredo, Italy
TissueLyser II	QUIGEN, Hilden, Germany.
Pico™ 21 Microcentrifuge	ThermoFisher Scientific. USA.
Vortexer, TopMix	Fisher Scientific, Loughborough, UK.
Nanodrop 2000 spectrophotometer (ND-2000)	ThermoFisher Scientific, USA.
Analytical balance (Model: PAS214)	Fisher Scientific, Loughborough, UK.

Analytical balance (Model: PM 600)	Mettler Toledo, Ohio, USA.
Bench pH meter (Model: HI-110)	Hanna instruments, Woonsocket, Rhode Island, USA.
Precision Balance (5 dp)	Mettler Toledo, Ohio, USA.
APEX tough microcentrifuge tubes (2 ml)	Alpha laboratories, Hampshire, UK.
ULT freezer C66085	New Brunswick Scientific, Connecticut, USA.
Simax glass measuring cylinder 1 L	Better Equipped, UK
Teflon rods (20 cm)	Fisher Scientific, Loughborough, UK.
Plastic sieves (9 cm)	Fackelmann, Hersbruck, Germany.
Ansell™ Microflex™ 93-260 Chemical-Resistant Disposable Glove	Fisher Scientific, Loughborough, UK.
Fisherbrand™ Stainless steel spatula	Fisher Scientific, Loughborough, UK.
Fisherbrand™ Stainless steel Micro spatula	Fisher Scientific, Loughborough, UK.
Fluorometer 4	Thermo Fisher Scientific, Massachusetts, USA
96 well plate with strip covering	Thermo Fisher Scientific, Massachusetts, USA
Pyrex® Borosilicate rimless glass digestion tubes (100 x 116 mm)	Corning, New York, USA.
Aluminum foil, Kirkland Signature	Costco Wholesale UK Ltd., Watford, Hertfordshire, UK.
Disposable innoculation sterile loop (10 µl)	Sarstedt, Leicester, UK.
Falcon™ 15 mL sterile Conical Centrifuge Tubes	Fisher Scientific, Loughborough, UK.

Schott Duran® laboratory bottles, with caps (1 L)	Merck, Massachusetts, USA.
Bench-top refrigerated microfuge	Thermo Fisher Scientific, Massachusetts, USA.
Pestle and mortar	Fisher Scientific, Loughborough, UK.
Acrodisc filters 13 mm (0.2 µm)	Pall corporation, New York, USA.
Microcentrifuge tubes amber (1.5 ml)	Fisher Scientific, Loughborough, UK.
NexION 2000 ICP-MS	PerkinElmer, Massachusetts, USA.
Zeiss Stemi SV6 Stereo Microscope On Transmitted Light Mirror Base	Zeiss, Oberkochen, Germany.
Fluorcam FC 800-C	Photon Systems Instruments, Brno, Czech Republic.
TinyTag Ultra 2 data loggers	Gemini, Chichester, UK.
Ultrospec 2100 pro UV/Visible Spectrophotometer	Amersham biosciences, Buckinghamshire, UK.
Bench top freeze drier L-series	Lablyo, York, UK
Glass cuvettes	Fisher Scientific, Loughborough, UK.
Whatman® qualitative filter paper, Grade 1 110 mm	Merck, Massachusetts, USA.
Planetary Ball Mill PM 400/2	Retsch™, Haan, Germany.
Grinding Jars, Agate PM 400 (80 ml)	Retsch™, Haan, Germany.
Agate Grinding balls	Retsch™, Haan, Germany.
Heavy weight black seed trays without holes (32.5 x 22.5 x 5 cm)	H. Smith plastics, Wickford, Essex.
Apet plastic propagator lids	H. Smith plastics, Wickford, Essex.
ASD Field Spec® 3	Malvern Panalytical, Boulder, CO, USA.

Seeds (spinach, coriander)	Mr Fothergills, Suffolk, UK.
Seeds (Red sweet basil)	D.T. Brown, Suffolk, UK.
Laboratory (modified)	
Square petri dish, 120mm	Greiner bio-one, Frickenhausen, Germany.
Duckweed phenotyping dock	NA
Laboratory (reagents)	
Acetone 99.8% HPLC grade	Fisher Scientific, Loughborough, UK.
Hydrogen peroxide	Fisher Scientific, Loughborough, UK.
ICP-MS Element standards	Inorganic 226 Ventures, Essex Scientific Laboratory Supplies Ltd, Essex, UK.
Nuclease free water	Thermo Fisher Scientific, Massachusetts, USA.
Milli-Q water (18 MΩcm[1)]	Merck Millipore, Massachusetts, USA.
Ethanol, absolute 99.8% Analytical reagent grade	Fisher Scientific, Loughborough, UK.
Sodium hypochlorite solution	Fisher Scientific, Loughborough, UK.
68% Nitric acid (Primar grade)	Fisher Scientific, Loughborough, UK.
Qubit™ 1X dsDNA High sensitivity Quantification Assay Kit	Thermo Fisher Scientific, Massachusetts, USA.
DNeasy® Plant Pro Kit	QUIGEN, Hilden, Germany.

Table 7.2. Lab apparatus and reagents for 7.3, 7.4, and 7.5.

7.3. Supplementary methods for chapter three

The duckweed collection and water collection detailed in 7.2. are relevant for chapter three. Supplementary methods include details for maintenance of the duckweed collection, methods and modifications for ICP-MS of duckweed from that conducted in 7.1. for the worldwide duckweed panel. It also includes methods of ICP-MS for water samples, selection of known species for

genotyping and details of the genome processing pipeline and demographic analysis methods.

The planning for the duckweed collection was coordinated by me and the collection was completed by me and several other contributors. I collected water samples and prepared them for ICP-MS. Sterilisation, growth and duckweed harvesting for DNA purification and ICP-MS were performed in collaboration with a technician. I performed phenotyping on duckweed accessions for frond and root morphology. ICP-MS analysis and DNA library preparations were performed by collaborators. DNA sequencing was performed by an external company. I processed genomes using a pipeline developed by a collaborator. I analysed and interpreted the data and had a major role in writing the manuscript.

7.3.1. Sterilisation and maintenance of UK duckweed stock cultures

Sterilisation of wild isolates were performed in six-well plates containing 0.5% sodium hypochlorite. A group of duckweeds of each ecotype were sterilised with bleach until visible bleaching rates towards meristem. This varied between 1-2 mins depending on size of duckweed and *Lemna trisulca* were particularly sensitive and did not survive bleach treatment > 15 secs. Duckweeds were dipped into six-well plates containing Milli-Q (MQ) water to recover and added to conical flasks containing Nutrient (N) medium for growth. N medium was made with MQ water using four stocks made up as below in table 7.3 and then adding 1 ml of each stock to 980 ml MQ water to make 1000 L stocks. Stocks were autoclaved at 121°C for 30 mins in 1 L Duran bottles. For each duckweed stock culture 100 ml media was added to 200 ml conical flasks and plugged with cotton wool. Conical flasks were sterilised beforehand using a heat sterilisation oven for 5 hrs before use. Each week conical flasks were changed with fresh medium provided. When stocks had traces of algae or cloudiness of the medium, the sterilisation process was repeated until stocks were acceptably axenic. Stocks were kept in a A-1000 growth cabinet under broad-spectrum LED lights providing low light at 50 µmol m^{-2} s^{-1}.

Stock number	Stock compound	Stock concentration (mM or M)	Stock concentration (mg/L or g/L)	Final concentration (µM or mM)
1	KH_2PO_4 (136.1)	30 mM	4.083 g/L	0.15 mM
2	$Ca(NO_3)_2$ $4H_2O$ (236.2)	0.2 M	47.23 g/L	1 mM
3	KNO_3 (101.1)	1.6 M	161.8 g/L	8 mM
	H_3BO_3 (61.83)	1 mM	61.8 mg/L	5 µM
	$MnCl_2$ $4H_2O$ (197.9)	2.6 mM	514.5 mg/L	13 µM
	Na_2MoO_4 $2H_2O$ (241.95)	80 µM	9.4 mg/L	0.4 µM
	$MgSO_4$ $7H_2O$ (246.48)	0.2 M	49.30 g/L	1 mM
4	Fe(III)EDTA (345.07)	5 mM	1.725 g/L	25 µM

Table 7.3. Recipe for four stocks used to make Nutrient medium. The original recipe was described in (Appenroth *et al.*, 1996). The table was copied from (Appenroth., *et al.*, 2015). Stock four was stored covered in aluminum foil due to possibility for light degradation. All stocks were well mixed by vigorous shaking before making N medium. N medium was used for all stock cultures and ICP-MS experiments as it is described as the medium producing fastest growth in (Appenroth, 2023).

7.3.2. Identifying duckweed accessions by morphology

Duckweeds were assessed morphologically in the field and on return to the laboratory to aid species identification. On return to the laboratory, a Zeiss SV6 stereo microscope was used to photograph ten random individuals of each ecotype in 3 ml N medium within six-well plates to make presumptions based on size and number of fronds per colony. Size and morphology (e.g. root length/number) were used to easily differentiate *Spirodela polyrhiza*, *Lemna trisulca*, *L. minor* and *L. minuta*. Maximum frond numbers was also used, whereby *L. minuta* had 3 fronds, in *L. turionifera* maximum 4 fronds are common, *L. minor/L. japonica* were less distinguishable but *L. minor* had the most fronds, 8 per colony. As duckweeds show plasticity in different environments, during maintenance of sterile stock cultures, duckweeds were grown in the same light, temperature and nutrient conditions and were compared on a duckweed phenotyping platform by photography using the same morphological traits to reconfirm species allocation. No species were seen flowering visually in growing conditions during the three year study. *Spirodela polyrhiza* and *L. turionifera* produced turions (overwintering bodies) when media nutrition became depleted, therefore any *Lemna* accessions producing turions in three years were determined as *L. turionifera*.

Naming of accessions by species type were provisionally chosen from these methods until confirmation with genome sequencing.

A subset of ecotypes were grown for four weeks, 15 randomly selected colonies from each flask were placed in a modified plastic cassette square petri dish (Greiner bio-one) stood vertically with the top section cut out to allow placement of duckweeds on the surface of water. 175 ml MQ water was added to create a flat floating surface of which roots could be accurately measured. The total number and maximum length of roots per ecotype were measured by photography by inclusion of a ruler next to the petri dish. FIJI image processing was used to scale the image to the ruler and then the segmentation tool drawing a line to measure each root in mm. *Lemna minor* and *L. japonica* were indistinguishable by root length but the short-rooted *L. minuta* (shortest roots) and *L. turionifera* and *S. polyrhiza* could be differentiated with this method.

7.3.3. DNA extraction

Between 20-100 mg sterile whole duckweed biomass for each accession was harvested from stock cultures into 2 ml microcentrifuge tubes and snap frozen in liquid nitrogen and stored at -80°C in an ultra-low temperature freezer. The DNeasy plant pro kit reagents (QUIGEN®) were prepared and stored per manufacturer instructions. The protocol was carried out as per the manufacturer's handbook with one addition. The final steps for elution involved two spins at 16,000 xg for 1 min eluted into a 75 µl total volume of EB buffer in a two-step process first using 50 µl and then 25 µl EB buffer. DNA quantity was confirmed using a Nanodrop and 260 nm/ 230 nm ratios ~2 to indicate purity. At least 5 ng/µl pure DNA was collected for each accession and stored at -80°C. If DNA quantity was below this value or considered contaminated for quality, duckweeds were re-harvested and extracted until these standards were met.

7.3.4. Library preparation for sequencing

To quantify DNA, Qubit was performed as per the assay kit manufacturer's handbook using 10 µl of two standard solutions into 190 µl working solution and 198 µl working solution in 2 µl genomic duckweed DNA samples. Samples were quantified using a Qubit 4 Fluorometer using dilution where readings were outside of measurement scope. Where genomic samples were higher than 16 ng/µl overall they were diluted in nuclease free water and each genomic sample was arranged in a 96 well plate. Samples below 2 ng/µl were re-harvested and DNA re-extracted. Collaborators performed library preparation steps using a Mosquito handling robot at Deepseq, Nottingham and Illumina short-read sequencing was performed at Novogene, Cambridge.

7.3.5. Selection of duckweeds from worldwide collection for sequencing

Clones from the Landolt Collection already available at the University of Nottingham were re-sequenced. This includes interesting clones from chapter two for elemental accumulation including: *S. intermedia* 9394, *L. punctata*

0049, *L. minuta* 9260, *L. minuta* 6600, *L. yungensis* 9208, *L. trisulca* 7192. Additionally, multiple species of *Lemna minor* and *Lemna japonica* were sequenced as they are notoriously difficult to identify by morphology: *L. japonica* 7123, *L. japonica* 9250, *L. minor* 7295, *L. minor* 8389. Genome sequence data from the Short Read Archive (SRA) for the following accessions and sample codes were also used: ERR3957957 - *S. intermedia* 8410, SRR11472010 - *S. polyrhiza* 9504, SRR074103 - *L. gibba* 131, SRR10958777, *L. minor* 7016, SRR10958800 - *L. minor* 5500, SRR8291593 - *L. minuta* 9581, SRR8291596 - *L. minuta* 6717, SRR8291594 - *L. minuta* 9484, SRR8291595 - *L. minuta* 7612, SRR8291590 - *L. turionifera* 9434, SRR23943402 - *L. turionifera* 6002. Clones were chosen as representatives of relevant UK species. A Python 3 pipeline for downloading SRA sequences and conversion to fastq file format used in this study is available at: https://github.com/mattheatley/sra_download.

7.3.6. Processing genomic data

Genomic sequences were sorted and merged for each genotype. The UK cohort were sequenced as paired end reads however some of the genotypes derived from SRA were processed as single reads. Where genotypes had been sequenced and had low coverage < 5% across the genome, DNA was re-extracted, so multiple pairs of paired end reads had to be sorted for individuals in this case. Genomes were processed using a Python 3 pipeline. Reads were concatenated and then quality trimmed with Trimmomatic (version 0.39) using -phred 33 function with settings: LEADING:10 TRAILING:10 SLIDINGWINDOW:4:15 MINLEN:50 (Bolger *et al.*, 2014). and then aligned to the *L. minor* 7210 (SRR10958743) reference using BWA (version 0.7.17) (Li & Durbin, 2009) and further processed with Samtools (version 1.9) (Li *et al.*, 2009). Duplicate reads were marked using Picard (version 1.134) using MarkDuplicates and AddOrReplaceReadGroups functions. GATK (version 3.5) was used for haplotyping using HaplotypeCaller with the following settings --ploidy 2, --min-base-quality-score 25, --minimum-mapping-quality 25, followed by merging and further processing of genotypes using CombineGVCFs, GenotypeGVCFs and GatherVcfs (McKenna *et al.*, 2010). The resulting variant call files (VCF) files were then filtered for biallelic sites and mapping quality (QD < 2.0, FS > 60.0, MQ < 40.0, MQRankSum < -12.5, ReadPosRankSum < -8.0, HaplotypeScore < 13.0). The VCF was then filtered by depth with a read depth average x 1.6 following GATK best practice as in (Marburger *et al.*, 2019; Monnahan *et al.*, 2019) The full pipeline is accessible at: https://github.com/mattheatley/ngs_pipe.

7.3.7. Further genomic processing and quality controls

For genetic analysis, putatively neutral fourfold degenerate sites were extracted with DEGENOTATE Python 3 script available at: https://github.com/harvardinformatics/degenotate reducing variant number from 23768794 to 572443. Depth for each genotype was determined by samtools with the coverage function. Those with low coverage <5% were filtered out by vcftools --remove-indv function thereby removing several SRA individuals: ERR3957957, SRR11472010, SRR8291593, SRR8291594,

SRR8291595, SRR8291596, SRR8291590 and KS75B, namely removing *S. intermedia* 8410, *S. polyrhiza* 9504, *L. minuta* 9581, *L. minuta* 9484, *L. minuta* 7612, *L. minuta* 6717, *L. turionifera* 9434 and ecotype NEW-Lmu-Cha respectively from further analysis. To prune for linkage disequilibrium (<0.1) allele frequencies greater than 2.5%, with one SNP per 100 kb sliding windows with a step size of 50 kb with <20% missing data were kept using a filtering script written in C described in: (Hämälä *et al.*, 2024). This left 11,000 variants for further analysis.

7.3.8. Genome demographic analysis to determine species

A PCA of genetic data for all duckweed ecotypes was composed using an R script available at: https://github.com/thamala/polySV/blob/main/est_cov_pca.r.Principal components 1 and 2 were also plotted using adegenet version 2.1.3. (Jombart, 2008) for comparison. Species were determined by clustering on a PCA when groupings of UK accessions clustered with known species either re-sequenced or downloaded from the SRA. Neighbour-joining trees (NJ trees) and Structure plots were generated as described in chapter three and used for species determination. ChooseK Python 3 script was used to obtain K number of populations for structure analysis, generating values of 9 for model complexity that maximizes marginal likelihood and 7 for model components used to explain structure in data.

7.3.9. Modifications to ICP-MS protocol for UK duckweed

Duckweed ionomics experiments were performed in triplicate for each UK ecotype and over three batches staggered between collection time. The local north west ecotypes were processed first in February 2021. Southern ecotypes were grown from August and sampled for ICP-MS in September 2021 and Scottish ecotypes were grown from late September and harvested in November 2021, both four months apart from initial collection. Duckweed populations were seeded from two three-frond colonies and then grown for six weeks in 500 ml conical flasks with 250 ml N medium, as shown in Fig 7.3. Duckweeds were grown in a GS-2000-SH cabinet in low light set at 100 µmol m^2 s^{-1}. During harvesting of flasks, wash steps consisting of 3 x MQ water replaced the original MQ water single step followed by $CaCl_2$ as wash two and NA_2EDTA as wash three, as used in chapter two. These wash steps were found to affect the ionome as they increased Ca and Na relative to MQ washes for both *L. minor* and *L. minuta*. Each MQ wash lasted for two mins using duckweeds on a plastic sieve which were passed across beakers along a series containing fresh MQ water and then harvesting 150 mg whole duckweed tissue into individual glass digestion tubes. Beakers were emptied and fresh MQ water added for every ecotype and all samples harvested within a five hour period on the same day. Additional trace/heavy metals included in the ICP-MS analysis include Si, Sn, Al and Ba. These were included as contaminants expected to be in water samples so were quantified in both water and duckweed for direct comparison.

Figure 7.3. Duckweed UK ecotypes growing in conical flasks for ICP-MS displaying differential growing rates under broad spectrum LED lights. Illustration of the controlled growth cabinet setup for duckweed ecotypes for ICP-MS experiment in a GS-2000-SH cabinet (Conviron, Winnipeg, Canada).

7.3.10. ICP-MS protocol for dissolved elements in water

During collection and before analysis, site water samples were stored in the dark at 4°C. For each water sample, 8 ml was filtered and then acidified with 2 ml of 10% HNO3. Calibration standards for water analysis were different to duckweed samples to account for higher levels and variation in water. Nine standards and ten blanks were run throughout each batch of water samples. Averages of blank values and their standard deviations were used to calculate limits of quantification, the lowest analyte concentration that can be reliably quantified is calculated as LOQ = 10 x SD of 10 measured blanks. The limit of detection is the lowest concentration of the analyte that can be reliably detected LOD = 3 x SD of 10 measured blanks. Elements below LOD or LOQ were not included in further analysis. The average blank values were subtracted from water elemental concentrations to give the final concentrations in mg/l or µg/l.

7.3.11. Other data analysis/scripts.

Each ecotype and site ICP-MS data was averaged and z-scores determined as in 7.2.1. All data processing of duckweed and water ICP-MS samples were done in R. The inbuilt 'prcomp' function was used to produce PCAs. PCAs and line plots were produced with R inbuilt 'plot' function. The inbuilt heatmap function was used to plot z-scores for each element for each ecotype or water site using 11 scale variation using two false colours. Packages 'ggradar' (https://github.com/ricardo-bion/ggradar) and ''ggforce' (https://ggforce.data-imaginist.com/index.html) were used to make radar plots. Violin plots, scatter plots and UK map were made using 'ggplot2' and 'maps'.

The following scripts are available at: https://github.com/Duckweed-KS/UK_genomics_ionomics

A. Genomics scripts - Python and bash scripts -
1. Pruning data.
2. ChooseK for k number determination for structure analysis.
3. Plink script for generating files for structure analysis.
4. Running structure analysis.
5. Splitting species by vcf.
6. Generate metrics for population comparisons – FST, Pi, Tajima D.

B. Genomics scripts - R scripts -
1. Generating a PCA.
2. Adegenet script for PCA, NJ tree generation and computing Nei's distances.
3. Demographic metric viewer.

C. ICP-MS ionomics - R scripts -
1. Water ICP-MS analysis (PCA, heat maps).
2. Water ICP-MS radar plots for elements.
3. Water analysis between species.
4. Duckweed ICP-MS analysis (PCA, heat maps).
5. Duckweed ICP-MS radar plots for elements.
6. Duckweed ICP-MS differences between species as violin plots.
7. Seasonal line plots for water elemental composition.
8. Correlation/ scatter plots between water and duckweed ionomes.
9. Map of the UK with site coordinates plotted.

7.4. Supplementary methods for chapter four

Duckweed and environmental collections for light were performed by me. Collaborators wrote the scripts for chlorophyll fluorescence analysis and genome alignment and were ran by me. I performed all growth experiments and chlorophyll fluorescence. Myself and technicians performed chlorophyll extractions for spectrophotometry. External collaborators performed the HPLC analysis for carotenoid profiling. All analysis were performed by me. The conceptualization, interpretation and writing of the manuscript were performed by myself and my secondary supervisor.

7.4.1. Genome analysis of the light acclimation cohort

UK plants used in light acclimation work are included in (Chapter four, supplementary table 1. Genome analysis as in 7.3. was conducted for this smaller local cohort with the following additional *L. minor* and *L. japonica* accessions from the SRA: SRR10958743 - *L. minor* 7210, SRR10958765 – *L. minor* 9441, SRR10958787 – *L. japonica* 7868, SRR10958744 – *L. minor* 7194, SRR10958760 – *L. japonica* 9541. These clone sequences were used in addition to other previously mentioned to confirm species identify of the UK cohort. All steps for DNA extraction were the same as 7.3.3. however both library preparation and sequencing of these UK genotypes were performed at Novogene, Cambridge. After filtering for depth the variant number was 17708888 which was then reduced to 566849 to include only four-fold

degenerate sites. Four-fold degenerate sites were plotted on a PCA using adegenet version 2.1.3. (Jombart, 2008). K means clustering with the find.clusters function was used for goodness of fit and determine Bayesian information criterion (BIC) (Thibaut *et al.*, 2010), BIC was used to determine K=4 for the population number for structure analysis.

7.4.2. Light acclimation and artificial light treatments

For light acclimation experiments plants were either placed for two weeks at low light (100 µmol m^2 s^{-1}) or in intermediate light 150 µmol m^2 s^{-1} for one week and then transferred to high light 350 µmol m^2 s^{-1} for another week (n=24), to acclimatise to the two treatments. This acclimation strategy was derived from (Stewart *et al.*, 2020). Three week old populations were then sub-cultured using a single three-frond colony for each accession for the start of light treatment experiments.

Plants were grown under two light regimes for six weeks. Replicates were individual flasks of the same ecotype grown on five separate experiments in each light condition. Light intensity and spectral measurements were made for each position of flasks to account for light variation for data analysis. Temperature and relative humidity were measured throughout the duration of the experiments using data loggers (TinyTag Ultra 2, Gemini data loggers).

7.4.3. Growth and morphological phenotyping

Growth rate was measured using an imaging pipeline to determine total area of duckweed and percentage surface area coverage of the available surface of conical flasks. The phenotyping protocol is described in (Ware *et al.*, 2023). Green area was used to work out relative growth rate (RGR) $RGR = (\ln x_{t14} - \ln x_{t21})/(t14 - t21)$ as described in (Ziegler *et al.*, 2015). Colonies were also counted weekly following changes of N medium. Turions formed by turion-forming species were counted each week once the transformative fronds had sunk to the bottom of flasks and counts later confirmed using photography. Anthocyanin content of *Spirodela polyrhiza* species was visualised and quantified with photography. RGB values of duckweed fronds were averaged from ten areas and quantified each week from photos to determine the proportion of reflectance from the red channel relative to masking by green and blue channels.

7.4.4. Chlorophyll fluorescence using a Fluorcam FC 800-C

At six weeks, whole flasks were split into three samples and spread out across six-well plates filling the surface area containing 3 ml N medium. Any remaining colonies from the flask were used for 7.4.5. biomass harvesting. Replicates were randomized between plates and between positions in plates and arranged in a closed Fluorcam, a chlorophyll fluorescence imager (see Fig 7.4 and (McAusland *et al.*, 2019). Plants were always harvested before 9 am and then dark adapted for 1 hr on the floor of the Fluorcam imager and arranged around the centre, where light variation is most reduced. Actinic white light providing both blue and red spectra provides a maximum intensity

>1000 µmol m^2 s^{-1} and is set at percentages of the maximum to provide sequential steps. An adequate sensitivity value for detection was first derived using a saturating pulse set at 80% to check all pixels were within measurable range and data not lost and consequently this was set at 5 for each run.
A custom light induction curve script for chlorophyll a fluorescence measurements was used. The F0 is measured at the start for 5 secs followed by a saturating pulse 800 ms to determine the dark adapted Fm measurement. F0', Fm' and Ft' are then measured after each light pulse for 60 secs with 20 ms gap between each light level. The protocol was composed of eleven steps of increasing light – 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% total light. A Li-250A light meter was used to measure photosynthetic photon flux density (PPFD) at each step and equated to: 0, 20, 130, 245, 365, 480, 600, 710, 830, 950, 1050 µmol m^2 s^{-1} PPFD. The minimum fluorescence in dark-adapted state (F0) and the maximum fluorescence in dark-adapted state (Fm) and steady-state fluorescence (Ft) are all measured in the dark and at each light level. Other parameters are derived from this data to measure acclimation of photosynthesis to each light level. Fv is determined as the difference between F0 and Fm (Fm - F0) in the dark. Fv/Fm is the maximum photosystem II (PS II) quantum yield, which is calculated as the proportion of the difference between fluorescence min and max over max (Fm-F0/Fm). ϕPS II or Fq'/Fm' is the operating efficiency of PS II and non-photochemical quenching (NPQ) NPQ = (Fm-Fm')/ Fm') measured in the light indicating the difference between maximum fluorescence in light and dark estimating heat loss. Parameters using chlorophyll fluorescence are described in detail in (Murchie & Lawson, 2013). Pixel areas are defined and their averages are exported to obtain numerical values with reduced noise for each parameter for each ecotype.

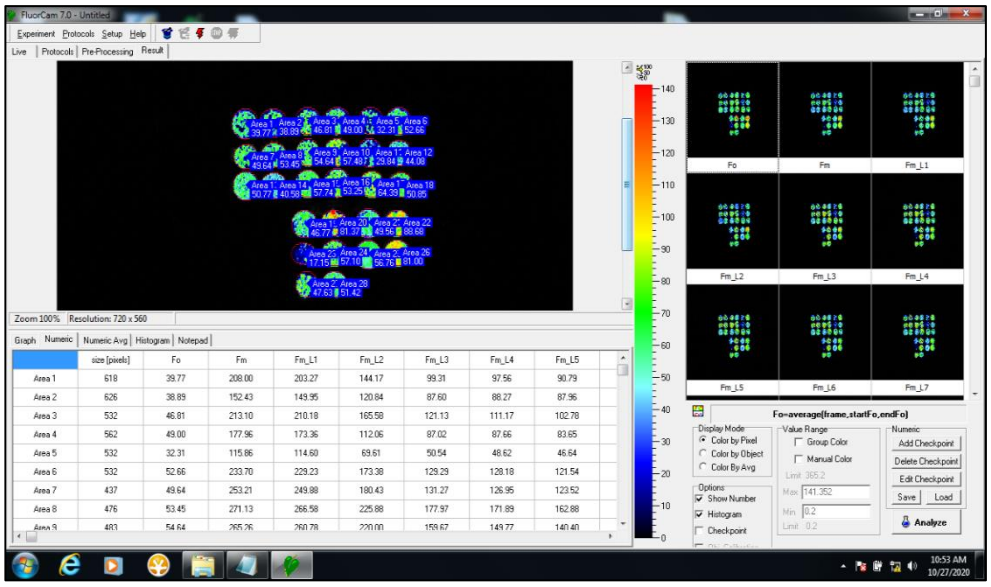

Figure 7.4. Visualisation of duckweed ecotypes grown in high or low light with a closed Fluorcam FC 800-C measuring chlorophyll fluorescence. Six-well plates containing duckweed ecotypes were arranged on the floor of the Fluorcam in the centre. False-colour scales were used to colour each pixel area and can be coloured according to photosynthetic parameters of interest.

7.4.5. Fresh and freeze-dried biomass

After measuring chlorophyll fluorescence the three replicates (plus any residual from the flask in excess of three replicates) from each ecotype-treatment were harvested and air dried for 10 mins and then weighed to give fresh biomass. The total fresh weight for each ecotype was composed of the combination of weights between three (or four) replicates. Fresh biomass samples were stored in foil packages then briefly in liquid nitrogen followed by storage in a -80°C Ultra-low freezer. Samples were placed in a Freeze-drier for two days to remove water content. Packages were re-weighed and the weight of the foil subtracted from the total weight to give freeze-dried weights per sample and per flask by combining the three (or four) replicates. Freeze-dried samples each formed independent replicates for pigment extraction to obtain concentrations in mg/g.

7.4.6. Pigment extraction and analysis by spectrophotometry

Five mg freeze-dried duckweed samples from each foil package were weighed into 2 ml microcentrifuge tubes with a 2 dp balance. They were stored at -80°C in the dark until analysis. All pigment extractions were performed on a morning at low light between 9 and 11 am. Samples were analysed in batches of 24. Acetone was diluted to 80% with MQ water fresh each time before use. Two metal beads were added into each tube with 1.5 ml 80% acetone. Samples were arranged in an adapter and ground in a Tissuelyser II at 24 Hz at 400/sec for 2 mins and then samples re-arranged on the opposite side and the program run again. Samples were pelleted using a centrifuge on 16000 xg for 2 mins. Then the supernatant from each sample was taken into fresh 15 ml labelled

falcon tubes. All metal beads were emptied into a waste beaker and washed with 70% ethanol and then air dried and autoclaved between uses. In falcon tubes, all samples were topped up to a total of volume of 5 ml using around 3.5 ml 80% acetone. Samples were read on a UV-VIS spectrophotometer by adding 3 ml of each well mixed sample into glass cuvettes using an 80% acetone sample to blank before reading at each wavelength.

7.4.7. Calculating photosynthetic pigment content

The following formulas were used to calculate chl a, b and carotenoid contents in mg/g freeze dried duckweed.

Chl a (mg/g) = {[12.25*(A663.6-A750)] – [2.55*(A646.6-A750)] *V}/(1000*W)
Chl b (mg/g) = {[20.31*(A646.6-A750)] – [4.91*(A663.6-A750)] *V}/(1000*W)
where A= absorbance, V= volume of extract (ml), w = weight of material.
Total carotenoids (mg/g) =
a) [(1000*A470) – (3.27*Chl a value) – (104*Chl b value)]/229
b) (answer a * V)/(1000*W)

Calculations were done using an Excel template, including subtraction of absorbance at 750 nm to correct for turbidity. Coefficients for elution in 80% acetone for chl a and b are provided in (Porra *et al.*, 1989) and for carotenoids in (Lichtenhaler & Wellburn, 1983).

7.4.8. Carotenoid profiling by HPLC

Conical flasks of duckweed were harvested at mid-day after six weeks growing under high or low light in their respective growing position. Accessions which had not grown enough in six weeks were not included. Each flask was harvested into foil packages and immediately snap frozen in liquid nitrogen and stored at -80°C until further preparation. All sample preparation was performed in a dimly lit room away from acids. One gram of duckweed sample were ground with a pestle of mortar in one ml of 100% acetone. Samples were aliquoted into 1.5 ml amber vials and centrifuged at 10,000 rpm for 2 mins at 4°C. The supernatants were then filtered through 0.2 µm HPLC filters and stored in amber microfuges at -80°C. For HPLC samples were diluted by half, added to amber vials with inserts and then 20 µl of each sample was injected for a run time of 20 mins each with blank measurements every three samples. HPLC is detailed in chapter four and was run by collaborators at Queen Mary University of London. Calculations of carotenoids were performed using Microsoft Excel.

7.4.9. Statistical analysis of light data

Statistical analysis is described in Methods of chapter four. Genome processing scripts are the same as in 7.3. R scripts and the custom script for chlorophyll fluorescence are provided at: https://github.com/Duckweed-KS/Light_adaption

R scripts -

7. Seasonal light data bar charts and statistics.
8. Coverage analysis.
9. Correlations between seasonal light data/coverage and grouping light data.
10. Extracting climate variables (Bioclim).
11. Weather/temperature/climate analysis.
12. Adegenet/PCA for species determination.
13. Growth analysis – RGR calculations, biomass, line plots.
14. Photosynthesis analysis – parameter calculations, boxplots, light curve plots, difference between light treatment calculations.
15. Chl car analysis, boxplots and difference between light treatment calculations.
16. Carotenoid profiling script, boxplots and statistics.
17. Correlations and PCA for combined physiology.

Chl fluorescence script -

18. Fluorcam (PSI) chl fluorescence script for light response curve.

7.5. Supplementary methods for chapter five.

Duckweed growth in a glasshouse was setup and maintained by myself in collaboration with a technician. Light measurements, fieldspectrometer and duckweed harvests were completed by myself. A technician freeze-dried all samples. Myself and collaborators prepared samples for '-omics' techniques. Collaborators setup and ran the SPME-GCMS and GCO-MS machines. External collaborators setup and ran the LC-MS/LS and HPLC-PDA machines. Analysis was performed by myself and interpretation by myself and collaborators. Funding, conceptualization and major writing of the manuscript were completed by myself and secondary supervisors.

7.5.1. Duckweed panel for scaling up growth

The UK panel described in 7.4. show variation in growth rate, light acclimation and nutritive pigments like carotenoids. The panel included in this chapter are similar with the following changes: *L. japonica* LY02 was no longer available, *L. minor* KS27 and KS29 were replaced by *L. minor* KSNuf3 and *L. japonica* KS66A. Two extra *S. polyrhiza* ecotypes were added for additional power to enable species inferences, namely accessions KS77A and KS78A. All included accessions in this cohort are in Chapter five, supplementary table 1.

7.5.2. Growing duckweed cultures in a glasshouse environment

Twenty-five ecotypes were replicated in fours and arranged randomly in numbered seed trays around a glasshouse at Sutton Bonington, Leicestershire, UK. Ecotypes were started as three three-frond colonies and positioning in numbered trays was decided using a random number generator. Lids were placed on trays to maintain high humidity, stop cross contamination and evaporation of medium. N medium was made up to a large volume of >100 L

using RO water each week. Between weeks, trays were scrubbed with bristle brushes with washing up liquid to remove algae growth, dried with paper towels and replaced with fresh N medium weekly. Duckweeds from each tray were washed in a sieve with RO water between weeks and replaced into trays. Sieves were sterilised in 5% bleach between uses and flushed with reverse osmosis water before use. In the last two months of experimentation (spring) when light was naturally increasing, N medium was replaced with 1 L reverse osmosis water instead to reduce proliferative algae growth.

A datalogger (Gemini) recorded the temperature every five minutes for four months of the experiment. Light levels and spectral composition measurements were taken with LI-250A and LI-180 handheld devices to compare light variation at each central position above each tray once in the morning in December and in a morning and afternoon in February.

7.5.3. Measuring duckweed health/greenness and coverage

Photos were taken with a Canon camera at four weeks growth 40 cm above each tray replicate with a ruler next to it. Photos were used to quantify both duckweed coverage and greenness using FIJI image processing software. Five 10 cm x 10 cm areas were selected per photo to account for any heterogeneity in surface coverage. The total area (in pixels) was cropped down and then channels split into RGB, the green channel was selected and threshold used to trace the biomass of duckweed and then percentage total area was calculated. To quantify greenness, average values from the green channel were taken for five sections within the photographs.

7.5.4. Harvesting for biomass

Harvesting was completed as detailed in 7.4.5. with the following modifications: Each tray was harvested every two months or when 95% surface coverage was achieved, whichever occurred first. Each tray was harvested up to three times in the six month growing season. There was no use of a sterile environment as all growth and harvest steps were completed in the glasshouse. A greater biomass was obtained per tray compared to flasks, so harvests were dried on paper towels under glasshouse lights for 20-30 mins. Fresh weight was measured into large foil packages and stored at -80°C. Freeze drying run-times increased to four days to allow removal of higher water content.

7.5.5. Spectroradiometer measurements in the glasshouse

Biomass reflectance was measured using a field spectroradiometer (ASD Field Spec® 3) as a potential high throughput method to predict growth/health and metabolite status. The spectroradiometer has a spectral range from 350 nm to 2500 nm measuring in 1 nm increments and reflectance was measured at 20 cm above each tray with an optic fibre with a field of view of 25°. At four weeks growth, three measurements were made per tray including central and outer growth regions. Vegetation indices were calculated from raw reflectance values at different wavelengths for each tray. All calculations were done in R and include the following:

$OVI = \frac{R_{760}}{R_{730}}$ (Erdle *et al.*, 2011)

$NDVI = \frac{R_{800}-R_{680}}{R_{800}+R_{680}}$ (Rouse *et al.*, 1974)

$GI = \frac{R_{554}}{R_{677}}$ (Smith *et al.*, 1995)

$GM = \frac{R_{750}}{R_{550}} - 1$ (Gitelson *et al.*, 2005)

R = reflectance at the given wavelength.

7.5.6. Sample preparation for '-omics'

Preparation of samples and analytical methods for '-omics' experiments are well described in chapter five. SPME-GCMS was used to generate volatilome (aroma) data, LCMS-MS was used to analyse amino acids, sugar, starch and flavonoid composition and additionally HPLC-PDA used for confirmation of flavonoids. SPME-GCMS and GCO-MS were run by collaborators in Food science, University of Nottingham and LC-MS/MS and HPLC-PDA at the University of Münster.

7.5.7. Candidate selection for GCO-MS

For an aroma smelling panel we recruited participants via internal university email. Interested candidates had a 30 min training session using 0.5 g custom test mix composed of various aromas. If participants were able to distinctly pick out individual smells and accurately describe them during the extraction they were selected for duckweed smell tests via GCO-MS. This reduced participants by half and five were used for smelling trials. Participants were instructed to not eat, drink, smoke or apply perfume which could interfere with the tests. Duckweed sample, extraction and compound identification was performed using the same methods as volatile testing by SPME-GCMS. Each participant's sheet were aligned with a chromatogram to derive an overall list of odour active compounds.

7.5.8. Statistical analysis of glasshouse environment, '-omics' and aroma perception datasets

Each compound from GCO-MS was given a nasal impact factor (NIF) to indicate frequency of detection where 0 is not detected and 100 is detected by five participants as in (McAusland *et al.*, 2020). An aromagram (stacked bar chart) was generated to display NIFs for each compound respective to its retention time during the extraction (mins) along the x axis. Compounds with NIFs >50 were considered to be frequently detected. For each compound amounts in ppb were quantified using:

peak area of compound / peak area of 3-heptanone standard x concentration of 3-heptanone in sample

For SPME-GCMS data, aroma compounds quantified in herbs and duckweed species were plotted on PCAs with a cos2 > 70 to plot only compounds driving the biggest dataset variation on PC1 and PC2. For each PCA, subsequently dropping herbs in turn based on dissimilarity to duckweed showed compounds driving dataset differences between duckweed/herb pairs. To obtain lists of significant compounds different between duckweed and herb pairs, a Games-Howell post-hoc analysis was used to find mean differences and P= < 0.05 used to determine significance. Compounds which were not detected in duckweed or herb could not be included in the post-hoc analysis but were still concluded as different between duckweed/herb pairs due to presence or absence. Compounds <1 ppb in duckweed were removed from further analysis for between duckweed species differences.

Volatiles and metabolites were compared between duckweed species with Kruskal-Wallis and Wilcoxon paired non-parametric statistical tests for each compound. Metabolites were averaged and scaled for each species and duckweed accession in R to find relative differences between them and plotted as heat maps. Aromagram, biomass plots and heat maps were generated on Graphpad prism 9.4.1. The Games-Howell analysis was performed using Microsoft Excel and IMB SPSS v 29.01. All other statistics and graphical analysis was conducted in R and scripts are provided at: https://github.com/Duckweed-KS/Flavour_metabolites

R scripts -

7. Glasshouse environment – temperature line plot, statistics of light variables.
8. Greenness and coverage measurements analysis for each accession.
9. Volatilome – Calculating amounts in ppb, plotting differences as PCAs and species boxplots.
10. Metabolites – Plotting differences as species boxplots.
11. Calculating vegetation indices from fieldspectrometer data.
12. PCA, corr plot and linear models for observed vs predicted traits.

7.6. References

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