Newsletter for the Community of Duckweed Research and Applications, edited by the ISCDRA

DUCKWEED FORUM

ISCDRA
International Steering Committee on Duckweed Research and Applications

Volume 9 (2), issue 33, pages 29 - 71 (2021)

Fronds and Turions of
Spirodela polyrhiza 9509

Cover page

Fronds and Turions of *Spirodela polyrhiza* 9509

Spirodela polyrhiza, commonly known as Greater Duckweed or Giant Duckweed is one of the duckweed species that are able to respond to cold and nutrient limiting conditions by forming a dormant tissue known as turion from its meristem pocket. In the cover picture, immature turions (dark green color) are attached to the mother fronds of *Sp. polyrhiza* 9509 under limiting phosphate. *Spirodela polyrhiza* 9509 is a clone isolated from Lotschen, Germany, for which a high-quality reference genome sequence is available and it has been used extensively in studies for turion induction mechanism in the Lam lab at Rutgers University (NJ, USA). The structure of its turions is smaller, round shaped, thicker, and dark green color with high content of anthocyanin pigments compared with those from clone 9512. See article in this issue of the *Duckweed Forum* by Pasaribu et al. for the use of this dormant state of *Sp. polyrhiza* to more efficiently manage duckweed stock collections. Photo contributed by Dr. Buntora Pasaribu (Rutgers University, USA).

In this issue

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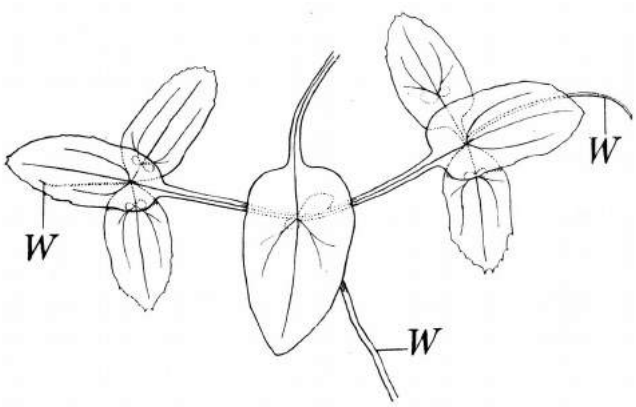
Science meets art: *Lemna trisulca* L.



The drawing of *L. trisulca* is from K. Goebel and has been published exactly 100 years ago in the journal Flora. The species can be identified as belonging to the species *Lemna trisulca* even by less experienced botanists because of its unique appearance. The stipes between mother and daughter fronds are unusually extended (up to 20 mm) and very stable. As a consequence, colonies can be formed with up to 50 fronds looking like pearls on a string. It is worth to mention the aesthetic aspect of this growth pattern. *Lemna trisulca* has a holarctic distribution and lives submerged in the water body except when flowering or fruiting. Drawing from Goebel, K. (1921) Zur Organographie der Lemnaceen. Flora **114**: 278-305. W = Wurzel (in German) = root.

Letter from the Editor:

Dear Readers of the *Duckweed Forum*,

On behalf of the International Steering Committee on Duckweed Research and Applications, I like to bid you welcome to another issue of our community's newsletter. As Spring in the Northern Hemisphere is quickly transitioning to Summer, all the plant life is awakening around us in New Jersey, USA. It brings a sense of renewal and hope, as well as a degree of optimism that we may be able to finally overcome the COVID-19 pandemic in the near future. I am sure many of you, like me, are eager to get back to work on duckweed and return to the research and application efforts that would be necessary to bring the potential of these amazing little plants to reality.

In this issue, you will find that a common theme of "methodology" runs through many of the items. On the Cover Photo and in a related article, the use of dormant turions for long term storage of *Spirodela polyrhiza* clones in a stock collection is described. A contribution from Morello et al. described a recent work applying their TBP (Tubulin-based polymorphism) approach to accurately distinguish duckweed species using PCR-based techniques. Using this method, they have uncovered the first molecular evidence that validated Landolt's suggestion of interspecific hybrid in *Lemna* species decades ago. Quite remarkable and satisfying to learn of their success, really! Another article in the category of Useful Resources is contributed by one of our committee members, Prof. Yubin Ma from China. In this, a concise description of the steps involved for carrying out genome editing in the duckweed *Lemna aequinoctialis* using CRISPR/Cas9 is presented. Last, but not least, tabulation of a survey carried out by ISCDRA members Sowjanya and Klaus for materials and methods needed to create a duckweed stock collection is presented under Useful Methods. The detailed description of all the items required should be very helpful for anyone considering to start their own duckweed collection from scratch.

We hope that sharing of these various techniques and protocols will find use by at least some of our readers and can serve as helpful reference material for others. Similarly, you will find that both our Student Spotlight (on builders of duckweed biomass-producing vertical towers) and the two Database Highlight articles are all related to novel technologies and their applications with duckweed.

In closing, my thanks go out to all our contributors for their time and effort to share knowledge and information. I do hope to hear from many of you about your opinions and ideas for contributing to the *Duckweed Forum*. After all, it is our community's newsletter and it will only be as good as we want to make it to be.

Warm regards to all, peace and happiness!

Sincerely,

Eric Lam

Chair, ISCDRA

Lemna species identification made easy

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Thirty-six species of duckweeds (Bog et al., 2020a) are quite a lot to identify in this family of tiny and very simple plants, many of which are quite similar by morphology, at least for a non-expert eye! In fact, species identification is still a challenging issue in duckweed research. Indeed, over time, the number of described species has changed often, with the synonymization of two or more species and the discovery of new ones, making duckweed taxonomy not yet fully resolved (Sree at al., 2016). So, how to unequivocally classify duckweed clones?

The traditional morphological approach (Landolt, 1980a), recently revised and updated (Bog et al, 2020b), identifies distinctive traits leading a trained eye to a successful classification in most cases, with relevant exceptions, e.g. in the particularly challenging genera *Wolffiella* and *Wolffia*. Words such as “usually, often, rarely, more or less”, are highly recurrent in the dichotomous keys for duckweed species determination, highlighting the intraspecific variability of most morphological traits. In fact, in some cases, similarity is so high and morphological markers so variable under different environmental conditions (e. g. the presence of pigmentation or the length to width ratio of the frond) that even an expert may fail in the correct identification.

More recently, the advent of molecular marker analysis has further contributed to define the genetic structure of duckweeds. Despite great improvement in phylogenetic reconstruction and a clearer delimitation of species, not all expectations were satisfied. The sequencing of at least two highly polymorphic plastid genome regions, called barcoding regions, is presently considered the gold standard for duckweed molecular taxonomy, allowing species delimitation in most but not all cases (Borisjuk et al., 2015; Bog et al., 2019). Difficulties may again arise in the genus *Wolffiella*, where the presence of hybrids was strongly suspected (Bog et al., 2018). Also in the genus *Lemna* there are a few cases of closely related species, also reported as sister species, in which intraspecific variability overlaps with interspecific one, making distinction not fully reliable even by molecular markers, e.g. *L. minuta/ L. valdiviana; L. aequinoctialis/ L. perpusilla* and *L. minor/ L. japonica*. The latest genomic technologies, like genotyping by sequencing (GBS), will further contribute to the delimitation of such “recalcitrant species”, but require extensive bioinformatics data analysis.

An alternative possibility to solve uncertainty is now offered by a very simple, PCR-based molecular fingerprinting method named TBP (Tubulin-based polymorphism), developed several years ago at the IBBA laboratory in Milan (Bardini et al., 2004), and largely applied to many different plant taxa (Braglia et al., 2020). TBP is a multilocus marker, which targets the β-tubulin gene family providing a

simple profile of DNA fragments that is typical of each species, with minor variations. This marker can easily discriminate *Spirodela, Landoltia* and *Lemna* genera at the species level (Braglia et al., 2021; Fig. 1), and we believe it can be of help to quickly distinguish similar species that might be exchanged in stock collections, to detect contamination from other species in outdoor cultivations or to distinguish similar species that can share the same habitat. For example, the common duckweed *L. minor* has a worldwide distribution, overlapping with that of many other species with which it may be confused (Bog et al, 2020a). In Europe, *L. minor* often forms mixed communities with *L. gibba*.

These two species have since long been reported as closely related and often described as the Lemna gibba-minor group (Landolt, 1975). Although *L. gibba* can be usually identified from the typical gibbosity of the ventral side of the frond, its identification becomes challenging when this distinctive trait is lost, seasonally or stably, leading to a flat form (de Lange 1984). This non-gibbous form of *L. gibba* is often interchanged with *L. minor*. Conversely, the two species are readily distinguished beyond any doubt by their different TBP fingerprinting, where a simple but distinctive band pattern can be observed by common agarose gel electrophoresis (Fig. 1). In Europe, *L. minor* can also be mistaken for the invasive American species *L. minuta*, but their TBP patterns are easily distinguishable (Fig. 1). The TBP results are therefore clear, robust and, most important, the assay can be easily performed by any basically equipped lab, in a short time and without need for DNA sequencing.

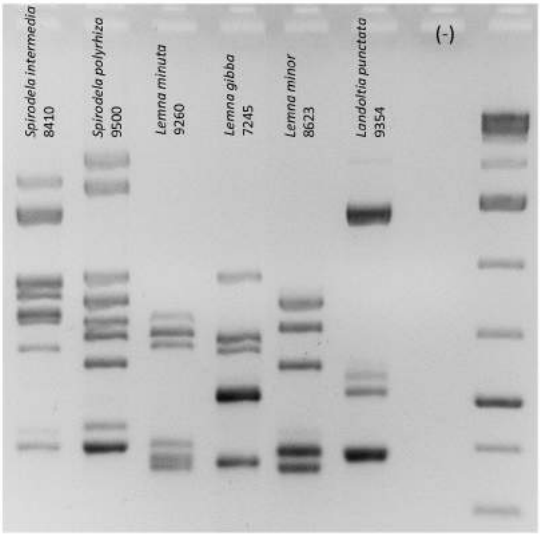

Figure 1: TBP fingerprinting of different duckweed species on agarose gel. Each band correspond to a β-tubulin locus.

However, an exception to the TBP discrimination power was found. The band pattern of L. minor was very similar to that of two strictly related East-Asian species: L. turionifera and L. japonica. However, after investigation with a higher resolution technique for fragment separation, i.e. capillary electrophoresis, we found that the TBP profile of *L. japonica* was the perfect overlap of those of the two genetically similar species *L. minor* and *L. turionifera*.

Figure 2 shows that, like in the case of DNA-based paternity testing, the mixed profile clearly indicates *L. japonica* as the "daughter" of *L. minor* and *L. turionifera* that means, translated in the plant language, it is an interspecific hybrid derived from a cross between the two parental species. This finding, confirmed by other molecular analyses, was the first direct demonstration of interspecific hybridization in duckweeds and explains why quite often *L. japonica* is mistaken for *L. minor*, from which it is morphologically distinguished by the pigmentation of the lower surface of the frond, and also why plastidic markers, maternally inherited, fail to

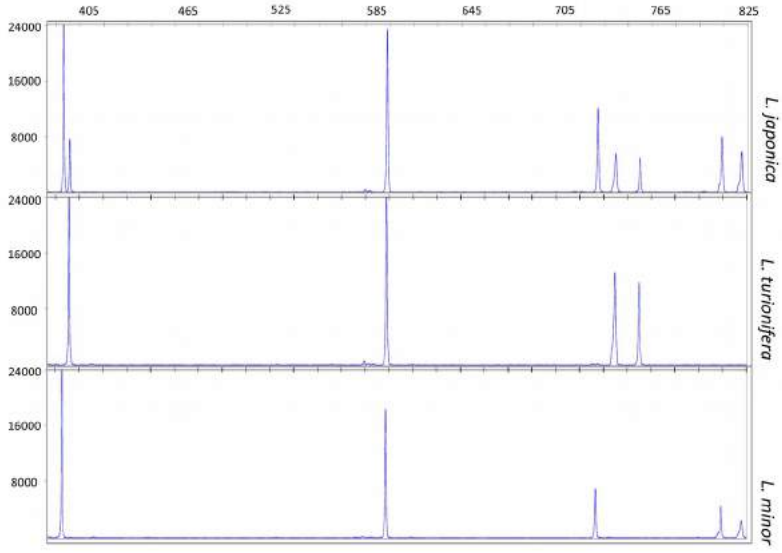

Figure 2: A section of the capillary electrophoresis TBP profile of the hybrid species *L.* × *japonica* and its parental species *L. turionifera* and *L. minor*.

distinguish the two species. So, 40 years after its description (Landolt, 1980b), molecular analysis confirmed Landolt's first intuition, based on accurate morphological analysis, of the hybrid origin of *L. japonica*.

A very simple assay, derived from TBP, can be used to distinguish these three closely related species by the PCR amplification of a single β-tubulin locus, which produce amplicons of different lengths in the two parental species (Fig. 3). This method allowed the correct determination of the three species in 42 analyzed *Lemna* clones and is presently the unique way to unambiguously distinguish *L. minor* from *L. japonica* at the molecular level (Braglia et al., 2021).

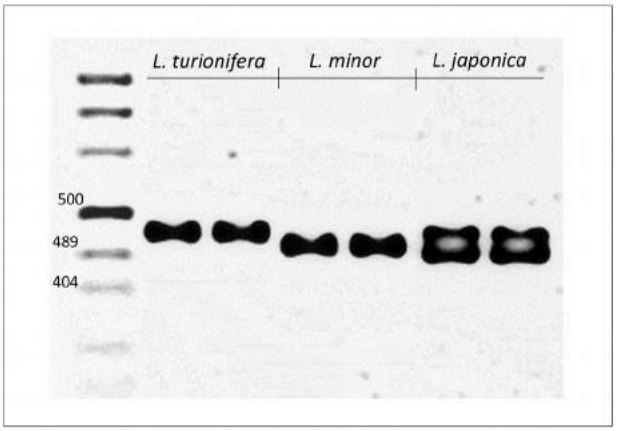

Figure 3: PCR amplification of a single β-tubulin locus in *L. turionifera, L. minor and L. × japonica.*

While offering an answer to the difficulty in *L. minor/ L. japonica* discrimination, hybrid discovery also opened new interesting questions which need further investigations:

- Did all hybrid clones *L. minor* × *turionifera* derive from the same hybridization event and can be considered as a unique taxon, namely *L. × japonica*?
- What is the chromosome set of *L. × japonica*? Is it a homoploid hybrid (two different chromosome sets with no change in number) or did it underwent chromosome rearrangements and/or allopolyploidization (duplication of the chromosome set)? Further insights on the *L. × japonica* genomic structure will soon come from genome size measurement of many clones and also from the ongoing whole genome sequencing of the *L. minor* clone 8627, which also turned out to be a hybrid.
- Is *L. × japonica* fertile? Is *L. × japonica* to be considered a true species, reproductively isolated from parents, or back-crossing may still occur giving rise to a hybrid complex? This stresses the requirement for more intensive investigations of flower induction, seed formation and cross-fertilization (Fu et al., 2017).
- What is the selective advantage of hybrids over the parent species?
- What is the true biogeographic distribution of *L. × japonica*? The species was described by Landolt as native from eastern Asia and later spread to northern Europe. Of 31 *L. minor* clones tested from our stock collection, 13 were identified as hybrids, among which some were from southern Europe (Greece, Albania and Italy) and one even from Africa. This calls for the need of an extensive re-classification of the many *L. minor* clones present in stock collections and the correct classification of newly collected clones.
- Is sexual reproduction in *L. minor* and *L. turionifera*, rarely reported to flower in nature, more common than previously thought or has it been more frequent in the past?
- Are there other cases of interspecific hybridization in duckweeds, as already suspected in many instances by both morphological and molecular approaches, and what is its role in duckweed speciation?

As usual, what we ignore is much more than what we know, but posing the right questions is a good starting point. Although some of us in Milan started working on duckweeds almost by chance less than two years ago, we have now plenty of work for the near future with these fascinating plants.

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Optimizing a Protocol for Long-term Storage of Duckweed Clones as Turions:

Organization of *Spirodela polyrhiza* accessions in the RDSC

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Introduction

Human has been collecting and maintaining plant germplasms since prehistoric time by the ways of seeds and living tissues for future use (Justice and Bass, 1978). Storage of plant germplasm is necessary to preserve and provide diverse genetic resources for crop improvement and is a primary target of conservation strategies (Migicovsky et al., 2019). Plant germplasms can be conserved through multiple methods such as seed banks, living collections, *in vitro* tissue/cell culture storage, and pollen banks (Laliberte, 1997). Unlike land plants, duckweeds are small aquatic plants growing on the surface of water bodies (Landolt, 1986). This aquatic angiosperm primarily multiplies through asexual propagation, which has limited its germplasm collection and conservation due to the labor and costs required in maintaining a living culture stock collection (Sree and Appenroth, 2020). In contrast, plant germplasm storage predominantly operates in the form of seed banks. Since viable seeds of most duckweed species are rare in either nature or the laboratory, duckweed clones are commonly maintained only as living collections. Large scale duckweed germplasm preservation was initiated by Elias Landolt at the Geobotanical Institute of the ETH Zurich, which stores well over 1000 clones at its peak (Sree and Appenroth, 2020). To date, facilities storing large duckweed collections exist in the USA, Germany, Switzerland, and China (Lam et al., 2020; Sree and Appenroth, 2020).

As vegetatively propagated species, duckweed clones can be stored in the laboratory for long periods via stock cultivation with periodic sub-culturing and maintenance (Sree and Appenroth, 2020). Duckweed germplasm stock collections are cultivated in various types of nutrient media, including N-medium with phosphate concentration increased to 1 mM, Steinberg's media with Fe-EDTA concentration increased to 25 µM, and Schenk and Hildebrand's (SH) medium (Appenroth, 2015). So far, two forms of culture medium have been used to maintain duckweed germplasm for large-scale collections: liquid and solid (e.g. agar). Maintaining duckweed germplasm in liquid solution offers a more close-to-natural habitat for duckweed growth. However, the main limitations are that this method requires more space, is a bit more expensive, and is more cumbersome to maintain for a large-scale collection. Nevertheless, liquid media could be a desirable option for increasing biomass and rescuing important germplasms, although our recommended standard for

long term duckweed germplasm storage is to grow and transfer duckweed germplasm on agar plates at 15°C (RDSC website; our survey in this issue of the DF). It has been reported that all duckweed clones collected from around the world are able to survive at temperatures of around 15-17°C (Sree and Appenroth, 2020).

Current protocol for clone maintenance in the RDSC

The Rutgers Duckweed Stock Cooperative (RDSC) developed a general protocol for maintaining duckweed clones (Figure 1). Approximately 5–10 duckweed fronds are transferred to three plates. Three types of plates comprised of 0.5X SH, 0.5X SH + Cef+ Suc 0.1%, or 0.5X SH +Suc 0.5% (Cef = cefatoxime; cf. Lam and Acosta, 2019) are used. Essential nutrients and salts required by most duckweed are mimicked with 0.5X SH medium, while the antibiotic cefotaxime (Cef) is also present when indicated. This is used for backup plates, with the aim that the presence of cefotaxime could help control contaminations by bacteria (Lam and Acosta, 2019). Addition of sucrose (Suc) at 0.1 or 0.5% (wt./vol.) can stimulate more rapid growth of fronds and visualize the presence of microbiological contaminations. The main limitation of storage using solidified medium is the necessity of frequent plate examination to avoid early senescence and minimize overly rapid growth that can exhaust the nutrients in the plate. Maintaining duckweed germplasm at lower temperatures and low light fluence (15°C and 50–30 µmol m^{-2} s^{-1} white light) conditions can help to limit duckweed growth and delay senescence of duckweed clones (Sree and Appenroth, 2020) in order to extend the duration between each subculture steps and minimize the work load involved.

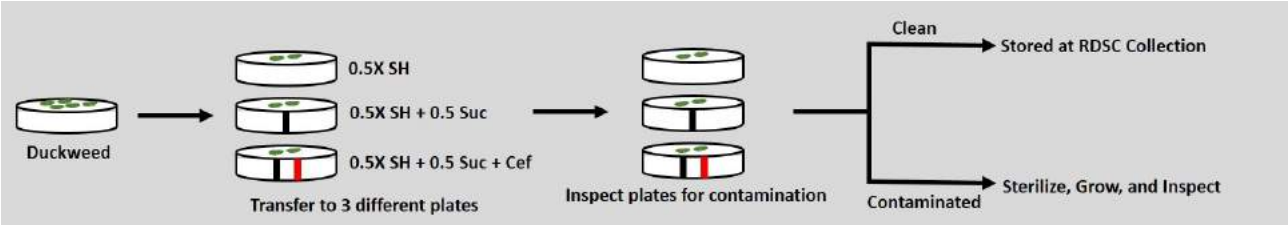

Figure 1. Scheme for duckweed stock maintenance. Healthiest duckweed fronds were selected and transferred to 3 plates with different growth medium in 0.8% agar. After 2-3 weeks, the plates were inspected for contamination. Contaminated duckweed were re-sterilized. Clean duckweed plates stored at RDSC collection.

Using the inducible dormant state of duckweeds as a storage method

As outlined in the previous paragraph, maintaining a substantial duckweed collection of over 100+ clones requires massive efforts to ensure the plants are kept in good health and minimize their loss due to contamination or accidental death. Trained staff play a key role in duckweed germplasm collection maintenance and curation; and stock cultures stored for long periods require routine observation to avoid nutrient depletion. Maintaining duckweed stocks on large scales is thus a complex and time-consuming effort. Therefore, it is necessary to develop more efficient and effective methods to maintain such collections and better serve the community.

To that end, we turn to the natural survival behavior that duckweeds and many other aquatic plants have evolved to deal with abiotic stresses. In unfavorable environments, such as low temperature and nutrient limitation, many duckweed species are known to alter the developmental program of their frond meristem to form a dormant structure that often detaches from the mother frond (Appenroth and Nickel, 2010). These dormant forms of duckweed (ex. *Spirodela polyrhiza*), called turions, are round, dark green in color, and have an average diameter of 1–3 mm in diverse clones (Smart and Trewavas, 1983b). Turions are also known to form in species from 11 genera of vascular aquatic plants (Adamec, 2018) and thus appears to be a fairly common strategy for overwintering in an aquatic environment. In the autumn, mature turions of *Sp. polyrhiza* sink to the bottom of water bodies in cold climates where they often stay unfrozen and await the return of appropriate conditions for germination and growth to resume in the spring (Smart and Trewavas,

1983a). Turion formation can also be readily induced under laboratory conditions by exposure to exogenous addition of the phytohormone abscisic acid (ABA), or through decreases in temperature and/or nutrients such as nitrogen, phosphate or sulfate (Appenroth and Nickel, 2010). While it has been noted that turions can remain dormant and viable in the media that it formed in for quite some time (Landolt, 1986), to our knowledge it has not been systematically used as a strategy for clone maintenance in a stock collection scenario for any aquatic plants. Through our project in the Lam lab to study the molecular basis of turion development, we became interested to translate our methods for turion induction and germination into a protocol for duckweed clone maintenance via turion production and storage.

Our approach to use turions for long-term storage of *Sp. polyrhiza*, the most well-studied duckweed species for turion biology, is outlined in Figure 2. In this study, *Sp. polyrhiza* 9512 (Sp9512) and 9509 (Sp9509) clones were tested as well-characterized duckweed clones forming turion with high and low Specific Turion Yield (STY), respectively (Kuehdorf et al., 2014). Two three-frond colonies of Sp9509 and Sp9512 from stock cultures were grown in liquid media containing 0.5X SH medium with 0.1% (wt./vol.) sucrose for two weeks at 25°C under illumination of 150 µmol m^{-2} s^{-1} light (16h light /8h dark). 200 mg fresh fronds of each clones were then transferred to a 177 ml baby jar containing 50 ml of N medium (60 µM KH_2PO_4, 1 mM $Ca(NO_3)_2{\cdot}4H_2O$, 8 mM KNO_3, 5 mM H_3BO_3,13 µM $MnCl_2{\cdot}4H_2O$, 0.4 µM Na_2MoO_4, 1 mM $MgSO_4{\cdot}7H_2O$ and 25 µM Fe(III) EDTA, pH 6.7) and grown for one more week under the same growth conditions. To induce turion formation, 300-500 mg fresh fronds of each clone were transferred to a 177 ml baby jar containing 50 ml of N medium with low phosphate (2 µM KH_2PO_4, 1 mM $Ca(NO_3)_2{\cdot}4H_2O$, 8 mM KNO_3, 5 mM H_3BO_3,13 µM $MnCl_2{\cdot}4H_2O$, 0.4 µM Na_2MoO_4, 1 mM $MgSO_4{\cdot}7H_2O$ and 25 µM Fe(III) EDTA, pH 6.7). This phosphate concentration is the threshold value for several clones of *Sp. polyrhiza* to induce turion formation (Appenroth and Adamec, 2015). The inoculated fronds were kept for more than 30 days under the same lighting and temperature conditions as before, until the full activation of turion production was observed, with mature turions accumulated at the bottom of the jar (Figure 2). These mature turions were harvested from the baby jars using a sterile disposable pipette and maintained in 2 µM KH_2PO_4 (pH 6.7) solution. This was followed by transfer of 5–10 turions to 2 ml Eppendorf tubes containing 1.5 ml of N medium with low phosphate and stored in the dark at 4°C.

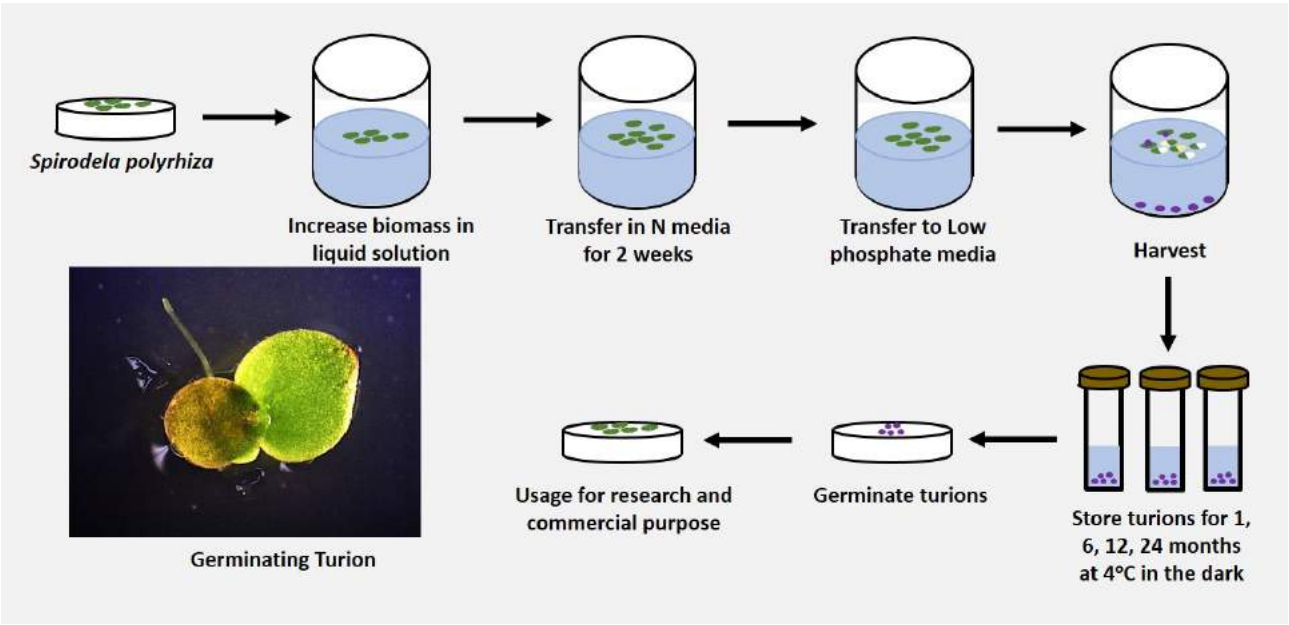

Figure 2. Turion induction and storage workflow. *Spirodela polyrhiza* biomass were increased by cultivating in 0.1% (W / V) sucrose containing liquid N-media under asceptic conditions. Biomass were then transferred to N-media only for two weeks before transferring to low phosphate N-media for 30 Days. Mature turions that have sunk to the bottom of the baby jars were harvested using a sterile disposable pipette. Turions are then stored in 2 ml Eppendorf tubes at 4°C inside a covered box and tested for viability at different length of storage times. T: turion; F: germinated frond; R: germinated root

As they mature on the mother frond of *Sp. polyrhiza*, turions developed immature root and frond under their epidermis with thickened cell wall before they detach from the mother frond (Smart and Trewavas, 1983b). After induction and formed under phosphate limitation, these turions remain dormant until after-ripening treatments with low temperature and appropriate light treatments before efficient germination can occur (Appenroth et al., 1989). For the RDSC to use turions as an effective means of general storage of clones, it is necessary to examine the turion germination rate as a parameter to ascertain the viability of turions in our storage conditions and to discern potential clonal variations in this behavior. To investigate turion germination rate post-storage, we cultivated turions on 0.5X SH agar medium. Turions were removed from the storage medium after various length of time in storage starting from 1 month post storage at 4°C and surface sterilized using sodium hypochlorite solution (10%) followed by rinsing with sterile H_2O three times. Then, 3–4 turions were placed on an agar plate with 0.5X SH medium and observed for 14 days at 25°C under illumination of 150 µmol m^{-2} s^{-1} light (16h light /8h dark). The appearance of new fronds and roots was then monitored as evidence for germination. Our study indicated that a new frond usually budded from the turion by day 2 post-germination followed by the first emerging root at day 3 to 4, and up to three developed fronds and multiple roots are observed by day 6 (Figure 3). Turion formation of different strains are known to be genetically adapted to a combination of multiple local climatic conditions including annual averages of temperature and precipitation or during the growing season (Kuehdorf et al., 2014). We designed a controlled experiment to investigate the viability of turions from two Sp9512 and Sp9509 clones that were produced in parallel under identical conditions by initiating the low phosphate medium transfer about 1 week earlier with Sp9509 fronds than those of Sp9512. Turions are then observed to initiate around the same time for both clones and can be harvested on the same day under identical conditions and with the same buffers. After harvesting the mature turions of Sp9509 and Sp9512, turions are stored for 1 month at 4°C under dark conditions (after-ripening conditions) in order to break dormancy. We then investigated the germination rates of these mature turions to compare their viability under our conditions. Germination percentage was calculated using the formula

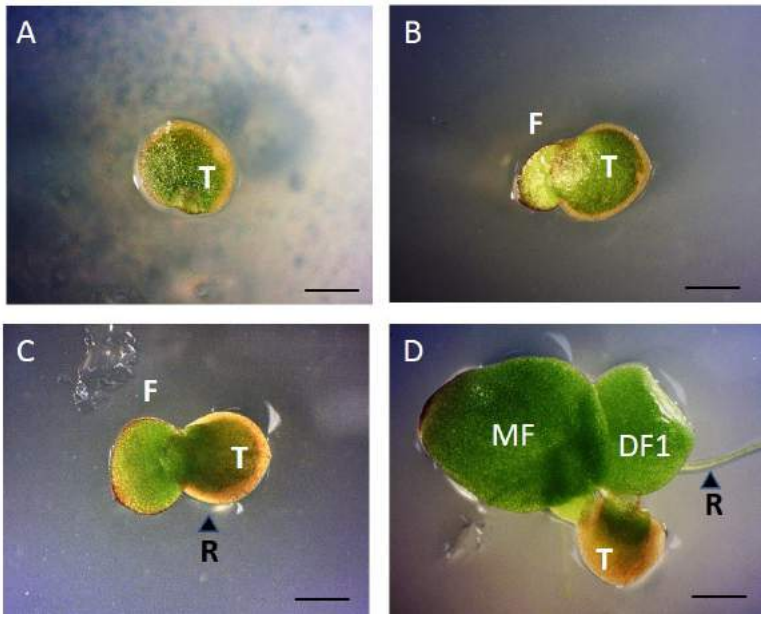

Figure 3. Stages of *Spirodela polyrhiza* 9512 turion germination. (A) Turion germinating in 0.5X SH solidified medium on Day 1. (B) After 2 days (Day 3), an expanding frond buds from the turion. (C) Day 4, the new frond grows out and an emerging root (black arrowhead) is observed. (D) Daughter fronds and root elongation observed at 6 days post-germination. Note separation of frond cluster from the remain of the turion shell. Bar: 0.5 mm; T: turion, F: frond, R: root, MF: mother frond, DF1: first daughter frond.

GP = (Total germinated turion)/(Total number of turion)*100.

Our results showed the germination rates for Sp9509 and Sp9512 turions in this trial are both 100%, thus indicating there is no significant difference in germination rates between these two *Sp* clones after 1 month of storage. We will schedule to test these stored turions later on to compare their viability over longer periods (e.g. 6 months, 1 year) to ascertain further the length of time that they could be stored. As previous experience with long term storage of turions from another *Sp. polyrhiza* strain 9500 has observed good viability after 5 years at 4°C (Appenroth, personal communication), we are optimistic that these turions should remain viable as well for at least up to a year.

Planned curation protocol for RDSC service

We report here a protocol that should enable us to reliably induce and harvest turions from duckweed strains. In the present study, turions were successfully induced in *Sp. polyrhiza* clones by simply lowering the phosphate concentration in the growth medium as a test case for storage. Leveraging this protocol, we could also test this method in the future for duckweed clones from the genus *Wolffia* as well as the species *Lemna turionifera* and *Le. aequinoctialis* (Table 1) that have been known for their ability to form turions (Landolt, 1986, Les et al., 1997). The objective of sharing our curation protocol is to provide guideline to help reduce operating cost and time-consuming effort when managing a substantial number of clones in these species that are able to form turions. We hope this standardized method can be applied to future collection efforts to improve the service provided by RDSC as well as by other collections. Its implementation should also simplify the effort for clone distribution pipeline to improve reliability for delivery and better viability of duckweed germplasm. As an example, 5 *Sp. polyrhiza* turions can be stored in 1.5 ml Eppendorf tubes contain 1 ml of mineral salt medium with low phosphate at 4°C in the dark. Dark and cold condition is likely to mimic the natural condition during overwintering. The stored turions will be labelled based on their clone number. As an order is received for this clone, turions will be surface sterilized using sodium hypochlorite solution (10%) followed by rinsing with sterile H_2O three times. Then the turions will be germinated on agar plates and should be ready to be sent to the requester within about 1-2 week's time. The key advantage of this method is the avoidance of having to repeatedly subculture the 100+ strains of *Sp. polyrhiza* clones that are currently in the RDSC stock collection. Furthermore, it should minimize the variability of conditions that each stock culture may be in at the time of ordering, thus giving a more reliable delivery time upon receipt of orders. Extending this method of germplasm maintenance to other turion-forming duckweed species in the future should further decrease the work-load for the RDSC while minimizing loss of culture stocks from contamination or senescence.

Table 1. List of Lemnaceae species observed to form turions (Landolt, 1986, Les et al., 1997)

Genus	Species	Distribution
Lemna	*Lemna aequinoctialis*	Africa, Asia, Europe, North America, Central America, and South America
	Lemna turionifera	Asia, Europe, and North America
Spirodela	*Spirodela polyrhiza*	Africa, Asia, Australia, Europe, North America, and South America
Wolffia	*Wolffia angusta*	Asia, and Australia
	Wolffia arrhiza	Africa, Europe, and South America
	Wolffia australiana	Australia, and Pacific
	Wolffia borealis	Asia, and North America
	Wolfia brasiliensis	North America, Central America, and South America
	Wolffia columbiana	North America, and South America
	Wolffia elongata	Asia, and South America
	Wolffia globosa	Asia, North America, and South America

Outlook

The use of turions as a storage vehicle to maintain some duckweed species could facilitate an alternative paradigm in storing duckweed germplasm. Here, we showed that turions of *Sp. polyrhiza* collected and stored using our protocol can readily germinate to produce new fronds and roots under defined medium and conditions. Using the dormant turion state may represent a more advanced method in the storage of duckweed germplasm, with the main advantages of lower labor and cost, need for fewer samples, and high viability as well as reliability of delivery. We believe this method can be used for efficient replacement of living collections of *Sp. polyrhiza* clones and we are currently taking steps to create turion stocks for the 100+ strains of this species in the RDSC. Turion storage may also be appropriate for some other duckweed species including almost all *Wolffia* species (with *W. microscopica* remaining unreported at present) and some *Lemna* species such as *Lemna turionifera* (Table 1). Thus, the standardization of turion production methods from these other species will be important next steps to follow by the RDSC. We hope to report on this effort to the community in the future.

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Useful Resources: A protocol for CRISPR/Cas9-mediated genome editing in duckweed

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Despite the potential for both basic research and industrial applications that established duckweeds as one of the most promising alternative future crops (Lam *et al*., 2014; Appenroth *et al*., 2015), current research and development in duckweeds are constrained by the lack of genetic manipulation tools that enable efficient knock-out or knock-in for a gene of interest in the duckweed genome. While using the established transformation system, one can manipulate the transcript abundance of the gene of interest through RNA interference (RNAi) or over-expression, it can suffer from low efficiency and instability due to DNA methylation of the transgene and incomplete target gene silencing (Klose and Bird, 2006; Weinhold *et al*., 2013). Therefore, to understand the function of genes and traits in duckweeds, developing tools that can stable and precisely manipulate the gene of interest is critical. To this end, the recently developed CRIPSR/Cas9 system, which was adapted from a naturally occurring genome editing system in bacteria and has been widely used in both animals and plants, is ideal (Ma *et al*., 2016; Cao *et al*., 2016). In brief, this genome editing platform utilize a silencing guide RNA (sgRNA)-based homing strategy to direct sequence-specific cleavage by the Cas9 nuclease at a chosen target site within the genome. The method is superior in its efficacy over previous DNA-based genome editing technologies while much easier to deploy through relatively simple design and introduction of one or more sgRNAs into the genome of interest. Here, we describe a protocol for stable CRISPR/Cas9-mediated genome editing in duckweed that we have successfully developed (Liu *et al*., 2019).

CRISPR/Cas9 target site selection

Potential target sequences within a duckweed gene were identified using the online tool CRISPR-P 2.0 (http://cbi.hzau.edu.cn/crispr/) described by Liu *et al.* (2017). To further selected the best candidate sequences, secondary structure analysis of potential target-sgRNA sequences was carried out with the program RNA Folding Form (http://mfold.rna.albany.edu/?q=mfold/ RNA-Folding-Form2.3) (Zuker, 2003). Target sequences were further selected to avoid those paired to the sgRNA with more than 6 consecutive bases. On the other hand, target site near the 5' end of target gene would be preferred in order to increase the chance for a resultant knock-out mutation from the

editing event after cleavage at the site. The efficiency score of candidate sgRNAs can be predicted by using a CRISPR efficiency predictor (Housden *et al.*, 2015).

Assemble the Cas9/sgRNA construct and duckweed transformation

A CRIPSR/Cas9 construct carrying three sgRNA cassettes was generated using the binary pYLCRIPSR/Cas9 multiplex genome targeting vector system provided by Yao-Guang Liu of South China Agricultural University (Ma *et al.*, 2015a). Three plasmids with sgRNA cassettes driven by the rice OsU3, OsU6a and OsU6b promoter elements, respectively, were assembled according to the golden gate cloning protocol. The CRISPR/Cas9 constructs were introduced into the *Agrobacterium tumefaciens* strain EHA105 by electroporation and used for genetic transformation.

For plant transformation, embryogenic calli of duckweed were immersed in *Agrobacterium* suspension using 350 mL tissue culture bottles. The bottles were placed in a vacuum chamber and vacuum was applied for 10 min. Then, the bottles were placed in a bath-type sonicator (Branson ultrasonic cleaner CPX2800, Branson Ultrasonics Corp., Danbury, CT, USA) and then subjected to ultrasound at a frequency of 40 kHz for 5 min at 17 °C. After sonication, the bottles were vacuum infiltrated again for 10 min. After releasing the vacuum, the callus pieces and *Agrobacteria* were incubated for 30 min with gentle shaking. Excess bacteria were removed after the incubation by transferring the infected callus pieces onto filter papers wetted with induction medium and placed in empty petri dishes in the dark at 25 °C for co-cultivation.

Three days after co-cultivation, the infected calli were transferred onto regeneration medium: Gamborg's B5 basal medium supplemented with 4.65 µM kinetin, 2.57 µM indole-3-acetic acid (IAA), 1% (w/v) sucrose, 9.48 µM hygromycin (PhytoTechnology Laboratories, Shawnee Mission, KS, USA), 600 µM timentin, and solidified with 7.9 g/L agar. After one week on the agar in darkness, the calli were cultivated under a 16 h photoperiod of approximately 50 µmol $m^{-2}s^{-1}$ of white light for 4 weeks. The regenerated fronds were transferred onto a conservation medium: SH medium supplemented with 0.6% (w/v) sucrose, 300 mM timentin, and solidified with 7.9 g/L agar. Regenerated fronds were proliferated on liquid SH medium.

Validation of stable transformations

Stable genetic transformation was validated by analyzing the integration of T-DNA into the duckweed genome. To identify the insertion sites of the T-DNA, thermal asymmetric interlaced PCR (TAIL-PCR) was performed as previously described (Liu and Chen, 2007; Wang *et al.*, 2011).

Genomic DNA extraction and detection of CRISPR/Cas9-mediated mutations

Genomic DNA was extracted from both transgenic and wild-type plants following the CTAB method (Porebski *et al*., 1997). The extracted genomic DNA was then used as a template to amplify the endogenous target gene by PCR. The PCR products were sequenced directly using specific primers with Sanger-sequencing approach. Biallelic and chimeric mutations that produced superimposed sequence chromatograms from direct sequencing were decoded using the Degenerate Sequence Decoding method (Ma *et al.*, 2015b). Biallelic mutants showed two distinct allelic mutations, but no wild-type allele, while chimeric mutations can have more than two distinct allelic mutations and an additional wild-type allele. PCR products from all biallelic mutants and some chimeric mutants were cloned into the pMD18-T Simple vector (Takara, China), and ten to twenty clones for each sample were sequenced using the same method mentioned above. DNAMAN (version 9; Lynnon Biosoft, Inc., San Ramon, CA) was used for sequence alignment analysis.

We have established genome-editing in *Lemna aequinoctialis* by this protocol and generated 15 (14.3% success rate) biallelic *LaPDS* (the gene encoding the enzyme phytoene desaturase, which is critical for chlorophyll biosynthesis) mutants that showed albino phenotype using this system (Liu *et al*., 2019). Investigations on CRISPR/Cas9-mediated mutation spectrum among mutated *L. aequinoctialis* plant lines showed that most mutations were short insertions and deletions (Liu *et al*., 2019). We anticipate that the stable gene manipulation tools will boost duckweed research and pave the way to fully utilize the remarkable duckweed plants for both industrial applications and basic research. Notably, an important foundation for our success is to establish an efficient transformation system through screening a large number of *L. aequinoctialis* clones. In addition, transient genome-editing approach via biolistic method has also successfully deployed CRISPR/Cas9 for gene knockout in duckweed tissues (Okada *et al*., 2017). Compare to the stable gene manipulation tools, the transient genome-editing approach requires less time and may be applied in some experiments where the results can be obtained more rapidly. The stable genome-editing approach described here needs the establishment of a highly efficient transformation protocol, requires more time than the transient genome-editing approach, but could generate stable genome edited mutants for long term studies in duckweed research.

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Useful methods: International survey for duckweed stock cultivation

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Fortunately duckweed research is indeed in bloom. Thus, it happens frequently that a new stock collection has to be established. This is a point to think about carefully because it will have consequences on the work for the following years. The local conditions as well as the available personnel and financial resources have to be considered. Therefore, we thought of making a survey in order to learn how the basic requirements are met with across the globe. We wrote to the managers of the stock collections listed in the "Update of duckweed stock Collections" in Duckweed Forum 9: 11-12 (2021), surveying on the questions as given below. The responses received from the nine stock collections are summarized in the sequence as mentioned for the "Name of the Institution and the manager of the stock collection", numbered 1 – 9 suffixed with the two or three letter code used by the stock collection. Further on the two or three letter code is used to refer to the stock collection. We hope that this survey will be helpful for many existing and upcoming duckweed groups in carefully selecting the modalities for successfully maintaining a stock collection of the clones in their laboratory.

Name of the Institution and the manager of the stock collection (two or three letter code)

1. Friedrich Schiller University Jena, Matthias Schleiden Institute - Plant Physiology, Jena, Germany; Klaus-J. Appenroth (KJA)
2. University Greifswald, Greifswald, Institute of Botany and Landscape Ecology, Germany; Manuela Bog (BOG)
3. School of Life Sciences, Huaiyin Normal University, China; Olena Kishchenko (NB)
4. Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei, China; Hongwei Hou (HHW)
5. Landolt Duckweed Collection, Zurich, Switzerland; Originally at the ETH Zurich, the last ca. 10 years in private rooms. Elias Landolt and Walter Laemmler (Information by K.J. Appenroth) (For this collection, there is no letter code and hence "Landolt" is being used for the same)
6. Rutgers Duckweed Stock Cooperative, Rutgers University, New Brunswick NJ, USA; Mike Chen (EL)
7. University of Debrecen, Faculty of Science and Technology, Institute of Biology and Ecology, Department of Botany, Hungary; Viktor Oláh (UD)
8. IPK Gatersleben: Ingo Schubert/ Manuela Nagel (we are using IS for Ingo Schubert)
9. Central University of Kerala, India; K. Sowjanya Sree (KSS)

Type of glassware or plasticware used for maintaining the stock cultures; Size of the glassware or plastic ware (volume/diameter)

1. KJA: Erlenmeyer flasks with cotton wool stoppers; 100 ml-flasks with 75 ml medium.
2. BOG: Wide and narrow neck Erlenmeyer flasks with cotton stoppers; 100 ml.
3. NB: Petri dishes; 6 cm diameter.
4. HHW: Erlenmeyer flasks 100 ml, 250ml and 500 ml; petri dishes 7 and 9 cm in diameter.
5. Landolt: Test tubes; 20 ml with 5 – 7 ml medium, cotton wool stoppers.
6. EL: Glass baby jars 200 ml liquid medium and with plastic lid; petri dishes 60 x 15 mm.
7. UD: Erlenmeyer flasks 100 ml.
8. IS: Erlenmeyer flasks 100 ml; plastic tubes 50 ml; plastic petri dishes 9 cm diameter.
9. KSS: Erlenmeyer flasks 150 ml, 50 ml medium; with cotton wool stoppers.

Name of the nutrient medium/ Reference

1. KJA: N medium with enhanced phosphate content (0.15 mM - 1 mM) with and without glucose (25 mM). Appenroth Duckweed Forum 3: 180-186 (2015).
2. BOG: N medium according to Appenroth et al. 1996; selected clones on sugar containing N medium (25 mM, Z medium).
3. NB: SH medium; Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 50(1):199–204. https://doi.org/10.1139/b72-026.
4. HHW: B5, SH and MS medium.
5. Landolt: For many years different media were used, depending on the duckweed species; later commercial available MS medium was used for all clones.
6. EL: ½ SH medium as base medium, is also modified by adding sucrose at 0.1% to 0.5% to promote growth or antibiotic cefotaxime at 100 µg/mL to control bacterial contamination for some duckweed strains.
7. UD: ISO 2005 modified Steinberg medium / Environment Canada EPS 1/RM/37 2nd Ed. 2007 (https://www.canada.ca/content/dam/eccc/migration/main/faunescience-wildlifescience/1ad45620-6a99-470c-8fdc-d57351d47ec8/rm37-202nded-lemnaenglish-20-20u.pdf).
8. IS: Liquid nutrient medium N (Appenroth et al. Biologia Plantarum 38: 95–106 (1996)).
9. KSS: N medium with enhanced phosphate content (1 mM) with and without glucose (25 mM). Appenroth et al. Plant Biol. 38: 95-106 (1996).

Agar/ gelrite concentration in case of solid or semisolid media

1. KJA: 0.45 % gelrite.
2. BOG: Only liquid medium.
3. NB: 0.8 % agar.
4. HHW: 1.0 % agar.
5. Landolt: 0.9 % agar.
6. EL: 0.8 % agar.
7. UD: Liquid.
8. IS: 0.45 % gelrite.
9. KSS: Liquid.

Temperature and light conditions of the stock culture room

1. KJA: 17°C; 30 µmol m^{-2} s^{-1} continuous white light by fluorescence tubes.
2. BOG: Room temperature and daylight; in winter additional light for *Wolffia* and *Wolffiella*.
3. NB: 24 ± 1°C (light period) and 20 ± 1°C (dark period) with a photon flux density of 100 µmol m^{-2} s^{-1} provided by cool white fluorescent bulbs in a 16 h light/8 h dark cycle.
4. HHW: 25 ±0.2°C, 85 µmol m^{-2} s^{-1} PAR, and a 16 h photoperiod.
5. Landolt: Room temperature (office) and day light, sometimes with an artificial illumination using a living room lamp.
6. EL: 15°C and low light (less than 50 µmol m^{-2} s^{-1}).
7. UD: 24°C, 60 µmol m^{-2} s^{-1} white light irradiation, 16/8 h photoperiod.
8. IS: 24°C; 16 h white light, 100µmol m^{-2} s^{-1}.
9. KSS: 17°C; 30 µmol m^{-2} s^{-1} continuous white light by fluorescence tubes.

How often must the cultures be renewed?

1. KJA: Each four months. *Wolffia microscopica* each month.
2. BOG: Each 3 – 4 months.
3. NB: Each 6-8 weeks.
4. HHW: Each month.
5. Landolt: If I remember correctly from my several visits in the lab of Elias Landolt, the media need to be renewed each 2 months. Klaus-J. Appenroth.
6. EL: It depends. In brief, for most *Lemna* strains, the subculture cycle is one year; for most strains of other four genera—*Spirodela, Landoltia, Wolffiella,* and *Wolffia* the cycle is better less than one year, particularly *Wolffiella,* and *Wolffia* strains should be renewed around six months. A few "weak" strains are gathered in the nursery trays to renew every 1-3 months. This strategy might be called the 12-6-3 rule for easily keeping in mind.
7. UD: Each 2 weeks.
8. IS: Liquid ~8 weeks; solid ~6 months.
9. KSS: 2-4 months depending on the species.

Stock collection at Landolt's duckweed collection, Zurich, Switzerland.

Stock collection of duckweeds at Friedrich Schiller University, Jena, Germany.

Stock collection at the Rutgers Duckweed Stock Cooperative, Rutgers University, New Brunswick, USA.

Student Spotlight: Finn Petersen

Faculty of Agricultural Sciences and Landscape Architecture, Osnabrueck University of Applied Sciences, Osnabrueck, Germany (Email: finn.petersen@hs-osnabrueck.de)

When I was young my grandparents had a dairy farm and grew a lot of different vegetables in their garden. This was the first time I came in contact with agriculture and food production. During school times, Biology and Chemistry became my favorite subjects and I developed an interest for food production and processing. This is why I decided to study "Food Technology" (B.Sc.) at Anhalt University of Applied Sciences in Germany. The main focus was on food processing and safety. Sustainability played a minor role in the studies, but environmental issues and a broad range of topics related to sustainability caught my interest at that time. Some examples would be circular production processes and economies, organic agriculture, food waste problems and strategies on how to deal with a changing climate. I graduated in "Environmental Protection and Agricultural Food Production" (M.Sc), an international program in English language, at Hohenheim University in Stuttgart, Germany. It combined agricultural, environmental and food sciences. This was also the first time I worked with duckweed. We investigated the heavy metal uptake of *Lemna minor* from polluted water.

Afterwards I was given the opportunity by Prof. Dr. Andreas Ulbrich from Osnabrueck University of Applied Sciences to do my PhD in cooperation with PD Dr. Klaus-J. Appenroth from the University of Jena. My main topic is the development of a standardized cultivation process for duckweed with a high protein content, to be used in human and animal nutrition.

Due to climate change extreme weather events, like droughts, floodings and heavy storms, become more frequent and make conventional agricultural food production more difficult and less predictable. Rising sea levels will result in less land area available for food production. Combining all of these factors with an increasing world population, the introduction of new edible plants and more productive as well as environment-friendly ways of food and feed production are necessary.

Duckweeds are a possible candidate, because of their potential for enormous biomass production and high protein contents. All parts of the plant can be used and some species have an amino acid distribution suitable for human nutrition. To reach their full potential, abiotic factors like nutrient composition and concentration, pH, light spectrum and intensity, photoperiod or temperature must be optimal for each species. I work with *Lemna minor* 9441 and *Wolffiella hyalina* 9525, both species/strains that can reach high growth rates and protein contents. In order to be independent from the weather, to ensure a year-round production, to use valuable land area more efficiently and to minimize the input of water and nutrients, a recirculating Indoor Vertical Farm (IVF) is used by our team (Figure 1).

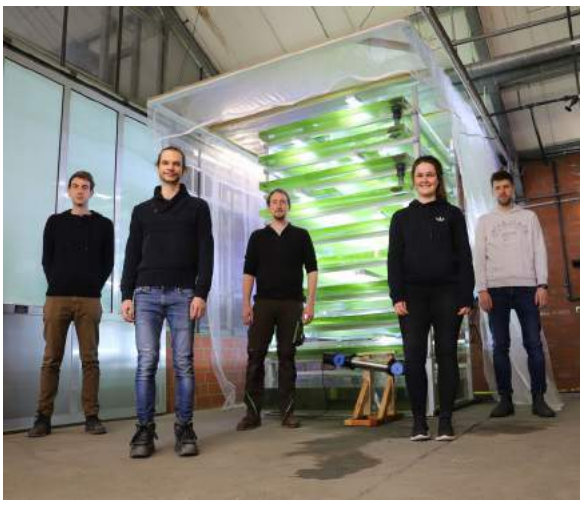

Figure 1: Our student team members. From right to left: Johannes Demann, Dina Restemeyer, Jannis von Salzen, Tim Dargatz and me. Our IVF for duckweed production is shown in the background.

The system is built out of food safe materials and is a possibility to standardize primary production. Our IVF consist of 9 basins for duckweed cultivation, with a

total area of roughly 25 m^2, and one reservoir. The water is recirculating between the reservoir and the duckweed basins, nutrients are automatically applied when necessary. Only the water lost by evapotranspiration is refilled. Lighting, temperature, pH and water flow rate can be controlled and adapted.

No pesticides are applied by us, but the growth of ubiquitously algae and other species, which are in competition for nutrients and light with the duckweed, is a challenge. Technologies, like UV-C radiation devices, can help to reduce algae growth, but can't stop it completely.

A long term goal would be the standardized biomass cultivation of duckweed for human and animal nutrition on nutrient-rich sources which are available in abundance, like wastewater or slurry. Even though many new challenges arise with this process, solutions need to be found and the recycling of valuable nutrients into a high quality product could be possible one day.

In September 2019 I attended the 5th International ICDRA Conference at the Weizmann Institute in Rehovot, Israel. I was amazed and very pleased to learn how many different people, institutes and companies around the world work on various topics related to duckweed research and application. I hope that the cooperation between actors involved in duckweed research and application will continue and intensify in the future. I'm already looking forward to the 6th ICDRA conference in 2022 in Gatersleben, Germany. In my opinion duckweeds have a huge potential to tackle the task of producing sufficient quantities of high quality food and feed for the world in the future. By trying to minimize the input of resources, using new resources and smart technologies, a more sustainable food and feed production is possible (Figure 2).

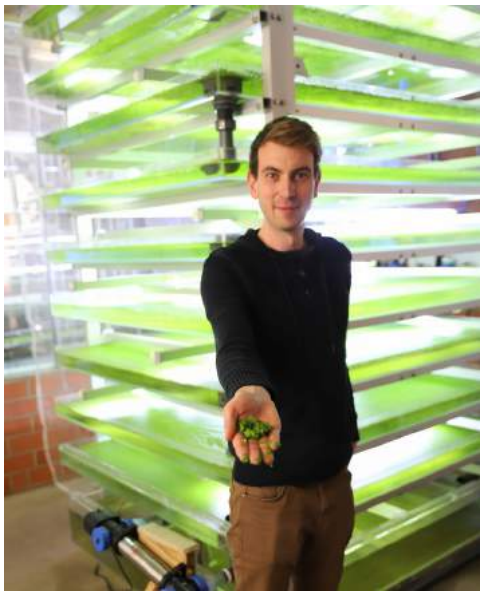

Figure 2: A small amount of *L. minor* harvested from our IVF, as presented on my palm.

Special issue in "Plants"

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Duckweed: Research Meets Applications

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Message from the Guest Editors

Dear Colleagues,

The aim of this Special Issue is to provide a comprehensive update of the current progress in duckweed research and applications. Contributions in forms of both original research papers and reviews from a broad scope of disciplines related to duckweed research and applications (e.g., morphology, taxonomy, and ecology including ecological interactions, ecotoxicology, environmental monitoring and remediation, physiology, biochemistry, genetics, omics, biotechnology, biomass production and its uses, etc.) are welcome. We hope that this overview will be of interest to all those involved in basic research or potential applications of duckweeds, and will also attract researchers from various other fields.

Deadline for manuscript submissions:
15 November 2021

mdpi.com/si/62024

Special Issue

From the Database

Highlight

Detection of uncoupled circadian rhythms in individual cells of *Lemna minor* using a dual-color bioluminescence monitoring system

Watanabe, E; Isoda, M; Muranaka, T; Ito, S; Oyama, T (2021) Plant and Cell Physiology
DOI:10.1093/pcp/pcab037

The plant circadian oscillation system is based on the circadian clock of individual cells. Circadian behavior of cells has been observed by monitoring the circadian reporter activity such as bioluminescence of AtCCA1::LUC+. To deeply analyze different circadian behaviors in individual cells, we developed the dual-color bioluminescence monitoring system that automatically measured the luminescence of two luciferase reporters simultaneously at a single-cell level. We selected a yellow-green-emitting firefly luciferase (LUC+) and a red-emitting luciferase (PtRLUC) that is a mutant form of Brazilian click beetle ELUC. We used AtCCA1::LUC+ and CaMV35S::PtRLUC. CaMV35S::LUC+ was previously reported as a circadian reporter with a low amplitude rhythm. These bioluminescent reporters were introduced into the cells of a duckweed, *Lemna minor*, by particle bombardment. Time series of the bioluminescence of individual cells in a frond were obtained using a dual-color bioluminescence monitoring system with a green-pass- and red-pass filter. Luminescence intensities from the LUC+ and PtRLUC of each cell were calculated from the filtered luminescence intensities. We succeeded in reconstructing the bioluminescence behaviors of AtCCA1::LUC+ and CaMV35S::PtRLUC in the same cells. Under prolonged constant light conditions, AtCCA1::LUC+ showed a robust circadian rhythm in individual cells in an asynchronous state in the frond, as previously reported. In contrast, CaMV35S::PtRLUC stochastically showed circadian rhythms in a synchronous state. These results strongly suggested the uncoupling of cellular behavior between these circadian reporters. This dual-color bioluminescence monitoring system is a powerful tool to analyze various stochastic phenomena accompanying large cell-to-cell variation in gene expression.

Preparation, scanning and analysis of duckweed using X-ray computed microtomography

Jones, DH; Atkinson, BS; Ware, A; Sturrock, CJ; Bishopp, A; Wells, DM (2021) Frontiers in Plant Science 11: 617830

Quantification of anatomical and compositional features underpins both fundamental and applied studies of plant structure and function. Relatively few non-invasive techniques are available for aquatic plants. Traditional methods such as sectioning are low-throughput and provide 2-dimensional information. X-ray Computed Microtomography (µCT) offers a non-destructive method of three dimensional (3D) imaging in planta, but has not been widely used for aquatic species, due to the difficulties in sample preparation and handling. We present a novel sample handling protocol for aquatic plant material developed for µCT imaging, using duckweed plants and turions as exemplars, and compare the method against existing approaches. This technique allows for previously unseen 3D volume analysis of gaseous filled spaces, cell material, and sub-cellular features. The described embedding method, utilizing petrolatum gel for sample mounting, was shown to preserve sample quality during scanning, and to display sufficiently different X-ray attenuation to the plant material to be easily differentiated by image analysis pipelines. We present this technique as an improved method for anatomical structural analysis that provides novel cellular and developmental information.

Biochemistry

Structural identification and UPLC-ESI-QTOF-MS2 analysis of flavonoids in the aquatic plant *Landoltia punctata* and their in vitro and in vivo antioxidant activities

Tsolmon, B; Fang, Y; Yang, T; Guo, L; He, KZ; Li, GY; Zhao, H (2021) Food Chemistry 343: 128392

Duckweeds have long been consumed as vegetables in several South Asian countries. In this study of the chemical constituents of duckweed *Landoltia punctata*, a new compound, apigenin 6-C-[beta;-D-apiofuranosyl-(1 -> 2)]beta;-D-glucopyranoside (1), and a previously LC-MS identified compound, quercetin 3-O-beta;-D-apiofuranoside (3), as well as three known compounds, luteolin 6-C-[beta;-D-apiofuranosyl-(1 -> 2)]-beta;-D-glucopyranoside (2), apigenin 6C-beta;-D-glucopyranoside (4), and luteolin 7-O-neohespirodise (5), were isolated and identified on the basis of MS and NMR spectroscopic analyses and chemical derivations. In total, 24 flavonoids were identified in *L. punctata* 0001 by UPLC-ESI-QTOF-MS2. In DPPH and ABTS assays, 3 exhibited significant antioxidant activity with IC50 values of 4.03 +/- 1.31 µg/mL and 14.9 ± 2.28 µg/mL, respectively. In in vivo antioxidant activity assays, 1 significantly increased the survival rate of juglone-exposed *Caenorhabditis elegans* by 2 to 3-fold, and by 75% following thermal damage. Compounds 1-5 exhibited moderate scavenging capacities of intracellular reactive oxygen species in *C. elegans* exposed to H_2O_2.

Biotechnology

Bioconversion of wastewater-derived duckweed to lactic acid through fed-batch fermentation at high-biomass loading

Lai, F; Jin, YL; Tan, L; He, KZ; Guo, L; Tian, XP; Li, JM; Du, AP; Huang, YH; Zhao, H; Fang, Y (2021) Biomass Conversion and Biorefinery DOI: 10.1007/s13399-021-01274-7

After being collected from the wastewater treatment system, duckweed biomass which is rich in starch and protein has the potential to be converted to valuable chemicals. This study aimed to establish a fermentation process for bioconversion of duckweed biomass derived from wastewater treatment to lactic acid (LA) by *Lactobacillus casei*. The *L. casei* CICC 23184 showed the best LA production ability in duckweed-based medium among five strains. Simultaneous saccharification and fermentation (SSF) and separated hydrolysis and fermentation (SHF) were conducted, and the SSF had better performance even with lower enzyme dosage. No extra nitrogen source needed supplying into duckweed-based media. In batch fermentation, biomass loading of 220 g kg^{-1} was optimal with LA concentration of 72.51 ± 2.81 g kg^{-1} and yield of 0.35 ± 0.01 g g(dry biomass)$^{-1}$. The highest LA concentration (100.4 ± 3.3 g kg^{-1}), yield (0.38 ± 0.01 g g(dry biomass)$^{-1}$), and productivity (2.09 ± 0.07 g kg^{-1} h^{-1}) were obtained through fed-batch SSF at a final biomass loading of 260 g kg^{-1}. This study demonstrated that it is feasible to utilize duckweed biomass as feedstock to produce LA through fermentation, and a fed-batch SSF at high-biomass loading was successfully established. This study provides a novel strategy for sustainable recycling of duckweed biomass derived from wastewater treatment.

Production of bioethanol from four species of duckweeds (*Landoltia punctata, Lemna aequinoctialis, Spirodela polyrhiza*, and *Wolffia arrhiza*) through optimization of saccharification process and fermentation with *Saccharomyces cerevisiae*

Faizal, A; Sembada, AA; Priharto, N (2021) Saudi Journal of Biological Sciences 28: 294-301

Duckweeds are promising potential sources for bioethanol production due to their high starch content and fast growth rate. We assessed the potential for four species, *Landoltia punctata*, *Lemna aequinoctialis*, *Spirodela polyrhiza*, and *Wolffia arrhiza*, for bioethanol production. We also optimized a possible production procedure, which must include saccharification to convert starch to soluble sugars that can serve as a substrate for fermentation. Duckweeds were cultivated on 10% Hoagland solution for 12 days, harvested, dried, homogenized, and dissolved in solutions that were tested as substrates for bioethanol production by the yeast Saccharomyces cerevisiae. First, we optimized the saccharification process, including the ideal ratio of the enzyme used to convert starch into simple sugars. The greatest starch-to-sugar conversion was obtained when the alpha-amylase and amyloglucosidase was 2:1 (v/v) and with a 24 h incubation period at 50 °C. After saccharification, the solutions were incubated with the yeast, *S. cerevisiae*. The fermentation process was carried out for 48 h with 10% (v/v) yeast inoculum. The ethanol content was maximal approximately 24 h after the start of incubation, and the sugars and protein were minimal, with little change over the next 24 h. The final ethanol concentration obtained were 0.19, 0.17, 0.19, and 0.16 g ethanol/g dry biomass for *L. punctata, L. aequinoctialis, S. polyrrhiza*, and *W. arrhiza*, respectively. We suggest that these four species of duckweed have the potential to serve sources of bioethanol and hope that the procedure we have optimized proves useful in the endeavour.

Enhancement of the biohydrogen production performance from mixed substrate by photo-fermentation: Effects of initial pH and inoculation volume ratio

Zhang, XT; Jiang, DP; Zhang, H; Wang, YJ; Zhang, ZP; Lu, CY; Zhang, QG (2021) Bioresource Technology 319: 124153

Co-digestion of substrates can improve hydrogen yield (HY) by adjusting carbon nitrogen ratio (C/N) of fermentation substrates. This study evaluated the enhancement of hydrogen production from co-digestion of duckweed and corn straw via photo-fermentation. The maximum HY of 78.0 mL/g Total solid (TS) was obtained from the mixed ratio of 5:1 (C/N of 13.2), which was 25.4% and 29.6% higher than those of single substrate of duckweed and corn straw, respectively. The effects of initial pH and inoculation volume ratio (IVR) on co digestion photo-fermentative hydrogen production (CD-PFHP) from duckweed and corn straw were further studied. A maximum HY of 85.6 mL/g TS was achieved under the optimal condition (initial pH 8, IVR 20%, mix ratio of duckweed and corn straw of 5:1). Additionally, both mix ratio and initial pH showed statistical difference ($p < 0.05$). Acetic acid and butyric acid were found to be the main metabolic by-products during CD-PFHP.

Feed & Food

Lamellidens and *Wolffia* canopy improves growth, feed utilization and welfare of *Labeo rohita* (Hamilton,1822) in integrated multi-trophic freshwater aquaculture system

Nath, K; Munilkumar, S; Patel, AB; Kamilya, D; Pandey, PK; Sawant, PB (2021) Aquaculture 534: 736207

A 90 days' trial on integrated multi-trophic aquaculture (IMTA) incorporating floating weed *Wolffia globosa* as inorganic extractive and a bivalve *Lamellidens marginalis* as organic extractive was conducted to assess the growth, survival, yield, water quality and welfare of *Labeo rohita* (Rohu) in an outdoor tank culture system. Twelve cement tanks (20 m^3) were randomly allocated into four treatments (in triplicate) where *L. rohita* was used as fed species. The treatments were assigned as control (C) only rohu, T-1: Rohu and partitioned *Wolffia* canopy, T-2: Rohu, *L. marginalis* and T-3: Rohu, Wolffia and *L. marginalis*. The stocking densities for rohu and *L. marginalis* were 30,000 fingerlings ha^{-1} and 750 kg ha^{-1} while *Wolffia* was transplanted to cover 30% of the tank surface area, and fish to mussel biomass ratio maintained was 2:1. The fish were fed with a sinking pelleted

feed (30% Crude Protein) at the rate of 4% body weight. It is observed that total biomass, survival, net fish yield (NFY) was significantly highest in T-3 as compared to T-1, control and T-2. The total protein and lipid content of rohu (whole-body) differ significantly between the treatments. Among the welfare parameters, the nitroblue tetrazolium (NBT) activity was significantly higher in T-1 and T-3 while superoxide dismutase (SOD) was highest in T-1. The catalase (CAT) activity lowest in T-3 among all the treatment groups. The water quality parameters like NH_4-N, NO_3-N, PO_4-P were significantly lower in T-3 among all treatment groups. Higher water transparency was observed with no algal bloom in T-3 and T-1. The results suggest that co-culture of *L. rohita* with *W. globosa* and *L. marginalis* can improve water quality, welfare parameters, enhancing fish survival and productivity.

Duckweed protein supports the growth and organ development of mice: A feeding study comparison to conventional casein protein

Roman, B; Brennan, RA; Lambert, JD (2021) Journal of Food Science DOI: 10.1111/1750-3841.15635

As global population growth and meat consumption increases, sustainable alternatives to conventional protein-rich fodder crops for livestock are needed to reduce negative environmental impacts. Duckweed, a small floating aquatic plant, can generate 5 to 10 times higher protein yields than conventional land-grown crops. Although some in vivo feeding trials with duckweed have been conducted, those measuring animal weight are limited, and those examining organ development are nonexistent. To secure broad acceptance of new protein sources, such controlled studies are critical. This study measured the food intake, growth, and final organ and adipose tissue mass of male CF-1 mice fed a semi-purified diet containing casein or diets in which 10% or 25% of the casein was replaced with duckweed protein (DWP). Proximate analysis showed that the DWP preparation used contained 39.9% protein (w/w), and contained all of the essential amino acids with Met as the limiting amino acid. The average growth rates were not significantly different among the treatment groups: 0.21 g/day; 0.24 g/day; and 0.25 g/day for the control, 10%, and 25% DWP protein diets, respectively. The daily food intake of both DWP diets was 6.5% to 8.0% higher than the control diet, but feeding efficiency did not differ among diets. The relative weight of the liver, spleen, kidneys, heart, and epididymal fat, and colon length were not significantly different between treatment groups. The results from this study show that replacement of up to 25% dietary casein with DWP has no adverse effects on the growth rate and final organ and adipose tissue weights of laboratory mice. Duckweed can produce 5 to 10 times more protein per area than land-grown crops such as soybean. In this study, up to a 25% replacement of casein with duckweed protein had no observable effect on the growth or organ development of laboratory mice. Thus, duckweed has the potential to be used as a protein supplement for livestock, poultry, and fish, thereby decreasing environmental impacts from land-grown crops used for animal feed.

Effect of duckweed meal dietary inclusion on growth performance and survival of African catfish fingerlings

Mendez-Martinez, Y; Navarrete, YGT; Tamames, YP; Viltres, MR; Cortes-Jacinto, E (2021) Revisa de la Facultad de Agronomia de la Universidad Del Zulia 38: 84-104

Currently, the use of unconventional food sources in the inclusion of fish diets is cause of great interest. The growth performance of African catfish (*Clarias gariepinus*) was evaluated, with the inclusion of duckweed meal (*Lemna perpusilla*) in four levels (0, 6, 12 and 18 %) in the diet. African catfish with an average weight of 1.27 ± 0.03 g, were distributed in a completely randomized design, with 16 experimental cages (four replicates/treatment). The fingerlings were fed for 48 days of experimentation. An analysis of variance and a Duncan's test were performed. No significant differences were found ($p< 0.05$) for the first two levels evaluated, but with the rest there were differences, as the percentage of inclusion of the duckweed meal in the ration increased. Final weight was decreasing, as well as absolute growth rate, increase in daily weight, feed conversion ratio and food efficiency. Survival throughout the experiment was between 72 and 65 % in all treatments. The inclusion of duckweed meal in the diet did not affect the parameters of water quality. It was

concluded that the L. perpusilla meal can be included up to 12 % in diets for African catfish fingerlings, without affecting the growth performance.

Flavonoids from duckweeds: potential applications in the human diet

Pagliuso, D; Jara, CEP; Grandis, A; Lam, E; Ferreira, MJP; Buckeridge, MS (2021) RSC Advances 10: 44981-44988

Duckweeds are the smallest free-floating flowering aquatic plants. Their biotechnological applications include their use as food, bioenergy, and environmental sustainability, as they can help clean polluted water. The high growth capacity and their chemical properties make them suitable for human health applications. Here we evaluated the ethanolic extracts from five species of duckweeds by HPLC-DAD/MS-MS for chemical characterization. Sixteen compounds were identified and quantified, in which three were chlorogenic acid derivatives and eleven apigenin and luteolin derivatives. We describe for the first time the presence in duckweeds of 5-O-(E)-caffeoylquinic acid (1), 3-O-(E)-coumaroylquinic acid (2), luteolin-7-O-glucoside-C-glucoside (3), 4-O-(E)-coumaroylquinic acid (4), luteolin-6-C-glucoside-8-C-rhamnoside (5), and luteolin-8-C-glucoside-6-C-rhamnoside (6). The flavonoids diversity showed a significant content of luteolin and its derivatives, except for *Landoltia punctata* that had significant apigenin content. Flavones identified in duckweeds were mostly C-glycosides, which can benefit human diets, and its abundance seems to be related to the higher antioxidant and anticancer capacities of *Wolffiella caudata, Wolffia borealis*, and *Landoltia punctata*. Our findings reinforce the idea that duckweeds could be valuable additives to the human diet, and their potential should be further explored.

Inclusion of *Lemna* as a plant-based protein ingredient in dog and cat diets

Panasevich, M; Frantz, N; Reinhart, G (2020) Journal of Animal Science 98 (Suppl. 4): 317-317

Our objective was to evaluate the inclusion of a novel plant-based protein (*Lemna*; MC Select; Parabel®; Vero Beach, FL) in dog diets at 0, 5, and 10% and cat diets at 0, 10 and 15% for palatability, stool quality, and nutrient digestibility. We hypothesized that *Lemna* would be a viable protein source in both cat and dog diets by showing no detriments to nutrition outcomes. All feeding tests were conducted at an independent research facility (Susquehanna, PA). A standard 2 bowl palatability test over a 2-day period was done with adult dogs and cats (n = 30 each) to determine intake ratios (IR) between test diets (*Lemna* -containing diets) and control (0% *Lemna*) diet. Total tract nutrient digestibility was conducted with 18 adult dogs and 21 adult cats (n=6–7 per diet) with 5 days of diet acclimation followed by 5 days of total fecal collection. Stool quality was evaluated on a 1–5 scale where 1= non-formed/diarrhea and 5= hard, formed. Palatability data was analyzed via Wilcoxon Signed Rank, and digestibility and stool quality data were analyzed by ANOVA with a Tukey's post-hoc means separation (SAS version 9.4). Intake ratios in cats between 10% *Lemna* and control were significantly ($P < 0.05$) in favor of control, while no difference was observed between 15% *Lemna* and control. For dogs, 5% and 10% *Lemna* had significantly ($P < 0.05$) lower IR demonstrating a preference to control. Both cats and dogs fed *Lemna* diets had acceptable stool quality (3.42 avg for cat and 3.34 avg for dog). No detriments in nutrient digestibility were observed in dogs fed 5% and 10% *Lemna*; however, cats fed 10% and 15% *Lemna* had significantly ($P < 0.05$) lower dry matter, protein, and energy digestibility versus control. In conclusion, these data suggest more development is needed for *Lemna* inclusion in companion animal diets.

Effect of pH levels on duckweed's proximate composition for utilization as poultry and fish feed

Ullah, H; Gul, B; Khan, H; Hameed, I (2020) Bioscience Research 17: 2604-2613

To investigate the effect of various levels of pH on the carbohydrate, minerals, and protein contents of duckweed (*L. minor*), an experiment was conducted in Department of Weed Science; the University of

Agriculture Peshawar-Pakistan during 2016. The experiment was laid out in Completely Randomized Design (CRD) with 3 repeats. Data were taken on protein, lipid, carbohydrate and mineral contents. The laboratory trail consisted of pH levels from 4-10 as treatments. The highest protein, lipid and carbohydrate contents were recorded in control and pH 8 followed by pH 6 as compared to the lowest value in pH 4 and 10. Maximum mineral contents (Ca, Mg, Fe, Mn, and Zn combined) were recorded for pH 5, 8, 9, 10 and control, while lowest mineral contents were noted in pH 4 and 6. It is concluded from the results of the experiment that pH 7 ± 1 was the favorable pH for maximum performance of the duckweed and beyond 8 and below 4 pH level the plant efficiency was negatively affected.

Interaction with other organisms

Indigenous bacteria, an excellent reservoir of functional plant growth promoters for enhancing duckweed biomass yield on site

Khairina, Y; Jog, R; Boonmak, C; Toyama, T; Oyama, T; Morikawa, M (2021) Chemosphere 268:129247

The advantages of aquatic biomass production using wastewater as a cost-free fertilizer have recently been highlighted. Here, we report a successful study in which duckweed, *Lemna gibba*, biomass production in a food factory effluent containing low nitrogen and high salts was enhanced by employing customized plant growth-promoting bacteria (PGPB). Two common PGPB strains previously obtained from natural pond water, *Acinetobacter calcoaceticus* P23 and *Pseudomonas fulva* Ps6, hardly promoted the growth of duckweed; on the contrary, they inhibited its growth in treated factory wastewater, far different water conditions. Then, we asked if some indigenous wastewater bacteria could promote the growth of duckweed. We found that *Chryseobacterium* strains, a group of bacteria with limited nitrogen metabolism, were dominantly selected as effective PGPB. Moreover, we demonstrated that nitrogen limitation is the crucial environmental factor that induces the plant growth-inhibiting behavior of *A. calcoaceticus* P23 through competition for mineral nutrients with the host duckweed. This study uncovered points to be considered in PGPB technology to achieve efficient production of duckweed biomass in a factory effluent with unbalanced content of mineral nutrients.

Development and reproduction of *Cataclysta lemnata*, a potential natural enemy of the invasive alien duckweed *Lemna minuta* in Italy

Mariani, F; Fattorini, S; Di Giulio, A; Ceschin, S (2021) European Zoological Journal 88: 216-225

Life cycle of the aquatic moth *Cataclysta lemnata* (Lepidoptera: Crambidae) was studied in laboratory conditions to obtain a basic biological knowledge useful for predicting the possible success of the herbivorous larvae of this insect as potential control agents in limiting the spread of the invasive American duckweed *Lemna minuta* (Alismatales: Araceae) in Italy. The multivoltinism of *C. lemnata*, as well as the high overall emergence from the pupal stage (85%), the high success in mating among the formed couples (>90%), and the high number of larvae born from each egg laying (on average 310 individuals), suggest that the insect can be successfully bred in the laboratory for the purposes of an augmentative biological control. Under experimental conditions, larvae developed in 23 days (through six larval instars, distinguishable by cephalic capsule dimensions) and pupae in 10, with no difference in duration between females and males. The larval phase resulted longer than the adult one (23 vs 10 days); therefore, it can be considered the most suitable stage for releasing the insect in the field for biocontrol purposes. Indeed, the larvae having an herbivorous diet might consume a large amount of the invasive plant, contrarily to the adult phase which is focused exclusively on reproduction. Our results not only contribute to the knowledge of aquatic Lepidoptera that are scarcely known, but also support the effectiveness of a possible protocol for an augmentative biological control of the invasive alien duckweed *L. minuta*.

Molecular Biology

Improved *Spirodela polyrhiza* genome and proteomic analyses reveal a conserved chromosomal structure with high abundance of chloroplastic proteins favoring energy production

Harkess, A; McLoughlin, F; Bilkey, N; Elliott, K; Emenecker, R; Mattoon, E; Miller, K; Czymmek, K; Vierstra, RD; Meyers, BC; Michael, TP (2021) Journal of Experimental Botany 72:2491-2500

Duckweeds are a monophyletic group of rapidly reproducing aquatic monocots in the Lemnaceae family. Given their clonal, exponentially fast reproduction, a key question is whether genome structure is conserved across the species in the absence of meiotic recombination. Here, we studied the genome and proteome of *Spirodela polyrhiza*, or greater duckweed, which has the largest body plan yet the smallest genome size in the family (1C=150 Mb). Using Oxford Nanopore sequencing combined with Hi-C scaffolding, we generated a highly contiguous, chromosome-scale assembly of *S. polyrhiza* line Sp7498 (Sp7498_HiC). Both the Sp7498_HiC and Sp9509 genome assemblies reveal large chromosomal misorientations relative to a recent PacBio assembly of Sp7498, highlighting the need for orthogonal long-range scaffolding techniques such as Hi-C and BioNano optical mapping. Shotgun proteomics of Sp7498 verified the expression of ~2250 proteins and revealed a high abundance of proteins involved in photosynthesis and carbohydrate metabolism among other functions. In addition, a strong increase in chloroplast proteins was observed that correlated to chloroplast density. This Sp7498_HiC genome was generated cheaply and quickly with a single Oxford Nanopore MinION flow cell and one Hi-C library in a classroom setting. Combining these data with a mass spectrometry-generated proteome illustrates the utility of duckweed as a model for genomics- and proteomics-based education.

Limitation of current probe design for oligo-cross-FISH, exemplified by chromosome evolution studies in duckweeds

Hoang, PTN; Rouillard, JM; Macas, J; Kubalova, I; Schubert, V; Schubert, I (2021) Chromosoma 130: 15-25

Duckweeds represent a small, free-floating aquatic family (Lemnaceae) of the monocot order Alismatales with the fastest growth rate among flowering plants. They comprise five genera (*Spirodela, Landoltia, Lemna, Wolffiella*, and *Wolffia*) varying in genome size and chromosome number. *Spirodela polyrhiza* had the first sequenced duckweed genome. Cytogenetic maps are available for both species of the genus *Spirodela* (*S. polyrhiza* and *S. intermedia*). However, elucidation of chromosome homeology and evolutionary chromosome rearrangements by cross-FISH using *Spirodela* BAC probes to species of other duckweed genera has not been successful so far. We investigated the potential of chromosome-specific oligo-FISH probes to address these topics. We designed oligo-FISH probes specific for one *S. intermedia* and one *S. polyrhiza* chromosome (Fig. 1a). Our results show that these oligo-probes cross-hybridize with the homeologous regions of the other congeneric species, but are not suitable to uncover chromosomal homeology across duckweeds genera. This is most likely due to too low sequence similarity between the investigated genera and/or too low probe density on the target genomes. Finally, we suggest genus-specific design of oligo-probes to elucidate chromosome evolution across duckweed genera.

Wolffia arrhiza as a promising producer of recombinant hirudin

Khvatkov P; Firsov A; Shvedova A;Kozlov O; Chernobrovkina M; Pushin A (2021) 3 Biotech 11: 209.

The production of recombinant proteins in transgenic plants is becoming an increasingly serious alternative to classical biopharming methods as knowledge about this process grows. *Wolffia arrhiza*, an aquatic plant unique in its anatomy, is a promising expression system that can grow in submerged culture in bioreactors. In our study 8550 explants were subjected to *Agrobacterium*-mediated transformation, and 41 independent hygromycin-resistant *Wolffia* lines were obtained, with the transformation efficiency of 0.48%. 40 of them

contained the *hirudin-1* gene (codon-optimized for expression in plants) and were independent lines of nuclear-transformed *Wolffia*, the transgenic insertion has been confirmed by PCR and Southern blot analysis. We have analyzed the accumulation of the target protein and its expression has been proven in three transgenic lines. The maximum accumulation of recombinant hirudin was 0.02% of the total soluble protein, which corresponds to 775.5 ± 111.9 ng g^{-1} of fresh weight of the plant. The results will be used in research on the development of an expression system based on *Wolffia* plants for the production of hirudin and other recombinant pharmaceutical proteins.

Physiology & Stress

Ammonium detoxification mechanism of ammonium-tolerant duckweed (*Landoltia punctata*) revealed by carbon and nitrogen metabolism under ammonium stress

Tian, X; Fang, Y; Jin, Y; Yi, Z; Li, J; Du, A; He, K; Huang, Y; Zhao, H (2021) Environmental Pollution 277:116834

In this work, the ammonium-tolerant duckweed *Landoltia punctata* 0202 was used to study the effect of ammonium stress on carbon and nitrogen metabolism and elucidate the detoxification mechanism. The growth status, protein and starch content, and activity of nitrogen assimilation enzymes were determined, and the transcriptional levels of genes involved in ion transport and carbon and nitrogen metabolism were investigated. Under high ammonium stress, the duckweed growth was inhibited, especially when ammonium was the sole nitrogen source. Ammonium might mainly enter cells via low-affinity transporters. The stimulation of potassium transport genes suggested sufficient potassium acquisition, precluding cation deficiency. In addition, the up-regulation of ammonium assimilation and transamination indicated that excess ammonium could be incorporated into organic nitrogen. Furthermore, the starch content increased from 3.97% to 16.43% and 26.02% in the mixed-nitrogen and ammonium-nitrogen groups, respectively. And the up-regulated starch synthesis, degradation, and glycolysis processes indicated that the accumulated starch could provide sufficient carbon skeletons for excess ammonium assimilation. The findings of this study illustrated that the coordination of carbon and nitrogen metabolism played a vital role in the ammonium detoxification mechanism of duckweeds.

Combined action of gamma radiation and exposure to copper ions on *Lemna minor* L.

Bodnar, IS; Cheban, EV (2021) International Journal of Radiation Biology DOI:10.1080/09553002.2021.1894655

Under natural conditions, the reaction of living organisms to the action of acute γ-radiation depends on other stressors, including heavy metals. The aim of this work was to study changes in morphometric parameters, the content of photo assimilation pigments and the level of oxidative stress in irradiated duckweed at various copper concentrations in the culture medium. As a model organism, we used *Lemna minor* L. Duckweed was exposed to acute γ-radiation at doses of 18, 42, 63 Gy. After irradiation, the plants were transferred into a medium containing 3, 5, 6.3 μM Cu. On the 4th day of exposure, the levels of chlorophyll, carotenoids, malondialdehyde (MDA) were measured; after 7days, the specific growth rate, the level of damage, the change in the frond area, copper concentration in plant tissues were determined. The action of γ-radiation (18, 42, 63 Gy) and copper ions (3, 5, 6.3 μM) reduced the growth rate, increased the membrane lipid peroxidation, reduced the area of the fronds more significantly than under the separate action of the factors. The factors acted antagonistically on the specific growth rate. The content of copper in the tissues of irradiated plants (42, 63 Gy) increased. Irradiation of duckweed with acute doses of γ-radiation reduced the resistance of plants to excess copper in the environment.

Flowering and seed production across the Lemnaceae

Fourounjian, P; Slovin, J; Messing, J (2021) International Journal of Molecular Sciences 22: 2733

Plants in the family Lemnaceae are aquatic monocots and the smallest, simplest, and fastest growing angiosperms. Their small size, the smallest family member is 0.5 mm and the largest is 2.0 cm, as well as their diverse morphologies make these plants ideal for laboratory studies. Their rapid growth rate is partially due to the family's neotenous lifestyle, where instead of maturing and producing flowers, the plants remain in a juvenile state and continuously bud asexually. Maturation and flowering in the wild are rare in most family members. To promote further research on these unique plants, we have optimized laboratory flowering protocols for 3 of the 5 genera: *Spirodela; Lemna*; and *Wolffia* in the Lemnaceae. Duckweeds were widely used in the past for research on flowering, hormone and amino acid biosynthesis, the photosynthetic apparatus, and phytoremediation due to their aqueous lifestyle and ease of aseptic culture. There is a recent renaissance in interest in growing these plants as non-lignified biomass sources for fuel production, and as a resource-efficient complete protein source. The genome sequences of several Lemnaceae family members have become available, providing a foundation for genetic improvement of these plants as crops. The protocols for maximizing flowering described herein are based on screens testing daylength, a variety of media, supplementation with salicylic acid or ethylenediamine-N,N '-bis(2-hydroxyphenylacetic acid) (EDDHA), as well as various culture vessels for effects on flowering of verified Lemnaceae strains available from the Rutgers Duckweed Stock Cooperative.

Differential localization of flavonoid glucosides in an aquatic plant implicates different functions under abiotic stress

Bottner, L; Grabe, V; Gablenz, S; Bohme, N; Appenroth, KJ; Gershenzon, J; Huber, M (2021) Plant Cell and Environment 44: 900-914

Flavonoids may mediate UV protection in plants either by screening of harmful radiation or by minimizing the resulting oxidative stress. To help distinguish between these alternatives, more precise knowledge of flavonoid distribution is needed. We used confocal laser scanning microscopy (cLSM) with the "emission fingerprinting" feature to study the cellular and subcellular distribution of flavonoid glucosides in the giant duckweed (*Spirodela polyrhiza*), and investigated the fitness effects of these compounds under natural UV radiation and copper sulphate addition (oxidative stress) using common garden experiments indoors and outdoors. cLSM "emission fingerprinting" allowed us to individually visualize the major dihydroxylated B-ring-substituted flavonoids, luteolin 7-O-glucoside and luteolin 8-C-glucoside, in cross-sections of the photosynthetic organs. While luteolin 8-C-glucoside accumulated mostly in the vacuoles and chloroplasts of mesophyll cells, luteolin 7-O-glucoside was predominantly found in the vacuoles of epidermal cells. In congruence with its cellular distribution, the mesophyll-associated luteolin 8-C-glucoside increased plant fitness under copper sulphate addition but not under natural UV light treatment, whereas the epidermis-associated luteolin 7-O-glucoside tended to increase fitness under both stresses across chemically diverse genotypes. Taken together, we demonstrate that individual flavonoid glucosides have distinct cellular and subcellular locations and promote duckweed fitness under different abiotic stresses.

Birth order as a source of within-genotype diversification in the clonal duckweed, *Spirodela polyrhiza*

Morris, RS; Compton, ME; Simons, AM (2021) Biological Journal of the Linnean Society 31: 1002-1010

Organismal persistence attests to adaptive responses to environmental variation. Diversification bet hedging, in which risk is reduced at the cost of expected fitness, is increasingly recognized as an adaptive response, yet mechanisms by which a single genotype generates diversification remain obscure. The clonal greater duckweed, *Spirodela polyrhiza* (L.), facultatively expresses a seed-like but vegetative form, the 'turion', that

allows survival through otherwise lethal conditions. Turion reactivation phenology is a key fitness component, yet little is known about turion reactivation phenology in the field, or sources of variation. Here, using floating traps deployed in the field, we found a remarkable extent of variation in natural reactivation phenology that could not be explained solely by spring cues, occurring over a period of ≥ 200 days. In controlled laboratory conditions, we found support for the hypothesis that turion phenology is influenced jointly by phenotypic plasticity to temperature and diversification within clones. Turion 'birth order' consistently accounted for a difference in reactivation time of 46 days at temperatures between 10 and 18 degrees C, with turions early in birth order reactivating more rapidly than turions late in birth order. These results should motivate future work to evaluate the variance in turion phenology formally as a bet-hedging trait.

Effects of homeopathic preparations of Mercurius corrosivus on the growth rate of moderately mercury-stressed duckweed *Lemna gibba* L.

Jager, T; Wurtenberger, S; Baumgartner, S (2021) Homeopathy : The Journal of the Faculty of Homeopathy DOI:10.1055/s-0040-1718743

A bioassay with severely mercury-stressed duckweed (*Lemna gibba* L.) had revealed growth-inhibiting effects of homeopathically potentised mercury(II) chloride (Mercurius corrosivus, Merc-c.). We hypothesised that effects of potentised preparations are dependent on the stress level of the organisms used in the bioassay. The aim of the present investigation was to examine the response of duckweed to potentised Merc-c. at a lower stress level. Duckweed was moderately stressed with 2.5 mg/L mercury(II) chloride for 48 hours. Afterwards plants grew in either Merc-c. (seven different potency levels, 24x – 30x) or water controls (unsuccussed or succussed water) for 7 days. Growth rates of the frond (leaf) area were determined using a computerised image-analysis system for day 0-3 and 3-7. Three independent experiments with potentised Merc-c. and three systematic negative control experiments were performed. All experiments were randomised and blinded. Unsuccussed and succussed water did not significantly differ in their effects on duckweed growth rate. The systematic negative control experiments did not yield any significant effects, thus providing evidence for the stability of the experimental system. Data from the two control groups and the seven treatment groups (Merc-c. 24x-30x) were each pooled to increase statistical power. Duckweed growth rates for day 3-7 were enhanced ($p<0.05$) after application of Merc-c. compared with the controls. Growth rates for day 0-3 were not influenced by the homeopathic preparations. Moderately mercury-stressed *Lemna gibba* L. yielded evidence of growth-enhancing specific effects of Merc-c. 24x - 30x in the second observation period (day 3-7). This observation is complementary to previous experiments with severely mercury-stressed duckweed, in which a decrease in growth was observed in the first observation period (day 0-3). We hypothesise that the differing results are associated with the level of stress intensity (moderate vs. severe).

Phytomedicine

A combination of *Olea europaea* leaf extract and *Spirodela polyrhiza* extract alleviates atopic dermatitis by modulating immune balance and skin barrier function in a 1-chloro-2,4-dinitrobenzene-induced murine model

Lee, YS; Ryu, HW; Yang, WK; Park, MH; Park, YC; Kim, DY; Kwon, HJ; Kim, SY; Oh, SR; Kim, SH (2021) Phytomedicine 82: 153407

Atopic dermatitis is a chronic inflammatory skin disease in humans. Although *Olea europaea* leaf extract (OLE) and *Spirodela polyrhiza* extract (SPE) have been used to protect against skin damage, the effects of their combined administration on atopic dermatitis have yet to studied. In this study, we evaluated the potential therapeutic effects of an OLE and SPE combination on the progression of atopic dermatitis and the possible mechanisms underlying these effects in 1-chloro-2,4-dinitrobenzene (DNCB)-treated NC/Nga mice. Atopic

dermatitis was induced by topical application of 0.2% w/v DNCB prepared in an olive oil: acetone solution (1:3), and thereafter OLE, SPE and OLE + SPE were administered orally for 5 weeks. We determined atopic dermatitis symptoms, serum IgE levels, and levels of cytokine- and gene expression in the dorsal skin and splenocytes, and performed histological and immune cell subtype analyses. The expression of skin barrier-related proteins (filaggrin, sirtuin 1, and claudin 1) was also evaluated. The OLE + SPE combination significantly ameliorated atopic dermatitis symptoms, including dermatitis scores, and reduced epidermal thickness and infiltration of different inflammatory cells in mice with DNCB induced atopic dermatitis. It also significantly reduced the number of CD4(+), CD8(+), and CD4(+)/CD69(+) T cells; immunoglobulin E-producing B cells (CD23(+)/B220(+)) in the axillary lymph nodes; CD3(+) T-cell eosinophils (chemokine-chemokine receptor 3(+)/CD11b(+)) in the skin; and CD3(+) T cells, immunoglobulin E-producing B cells (CD23(+)/B220(+)), and eosinophils in peripheral blood mononuclear cells. Additionally, the experimental combination lowered levels of serum immunoglobulin E and histamine, as well as Th2-mediated cytokines, and interleukin-4, -5, and -13, whereas it increased the levels of Th1-mediated cytokine interferon-gamma in splenocytes. Furthermore, the preparation significantly restored expression of the skin barrier-related proteins filaggrin, sirtuin 1, and claudin 1, and also reduced the expression of the inflammatory cytokine interleukin-6 and chemokine-chemokine receptor 3, as well as the pruritus-related cytokine interleukin-31 and interleukin-31 receptor, in atopic dermatitis skin lesions. Taken together, our findings indicate that administration of a combination of OLE and SPE can alleviate atopic dermatitis symptoms by regulating immune balance and skin barrier function and may be an effective therapeutic option for the treatment of atopic dermatitis.

Effect of green-Mediterranean diet on intrahepatic fat: the DIRECT PLUS randomised controlled trial

Yaskolka Meir, A; Rinott, E; Tsaban, G; Zelicha, H; Kaplan, A; Rosen, P; Shelef, I; Youngster, I; Shalev, A; Bluher, M; Ceglarek, U; Stumvoll, M; Tuohy, K; Diotallevi, C; Vrhovsek, U; Hu, F; Stampfer, M; Shai, I (2021) Gut DOI:10.1136/gutjnl-2020-323106

To examine the effectiveness of green-Mediterranean (MED) diet, further restricted in red/processed meat, and enriched with green plants and polyphenols on non-alcoholic fatty liver disease (NAFLD), reflected by intrahepatic fat (IHF) loss. For the DIRECT-PLUS 18-month randomized clinical trial, we assigned 294 participants with abdominal obesity/dyslipidaemia into healthy dietary guidelines (HDG), MED and green-MED weight-loss diet groups, all accompanied by physical activity. Both isocaloric MED groups consumed 28 g/day walnuts (+440 mg/day polyphenols provided). The green-MED group further consumed green tea (3-4 cups/day) and Mankai (a *Wolffia globosa* aquatic plant strain; 100 g/day frozen cubes) green shake (+1240mg/day total polyphenols provided). IHF% 18-month changes were quantified continuously by proton magnetic resonance spectroscopy (MRS). Participants (age=51years; 88% men; body mass index=31.3 kg/m^2; median IHF%=6.6%; mean=10.2%; 62% with NAFLD) had 89.8% 18-month retention-rate, and 78% had eligible follow-up MRS. Overall, NAFLD prevalence declined to: 54.8% (HDG), 47.9% (MED) and 31.5% (green-MED), p=0.012 between groups. Despite similar moderate weight-loss in both MED groups, green-MED group achieved almost double IHF% loss (-38.9% proportionally), as compared with MED (-19.6% proportionally; p=0.035 weight loss adjusted) and HDG (-12.2% proportionally; p<0.001). After 18 months, both MED groups had significantly higher total plasma polyphenol levels versus HDG, with higher detection of Naringenin and 2-5-dihydroxybenzoic-acid in green-MED. Greater IHF% loss was independently associated with increased Mankai and walnuts intake, decreased red/processed meat consumption, improved serum folate and adipokines/lipids biomarkers, changes in microbiome composition (beta-diversity) and specific bacteria (p<0.05 for all). The new suggested strategy of green-Mediterranean diet, amplified with green plant-based proteins/polyphenols as Mankai, green tea, and walnuts, and restricted in red/processed meat can double IHF loss than other healthy nutritional strategies and reduce NAFLD in half.

Phytoremediation

Long-term effect of sediment on the performance of a pilot-scale duckweed-based waste stabilization pond

Tu, Q; Lu, Y; Zhao, Y; Duan, C; Huang, J; Fang, Y; Li, B; Zhao, H (2021) The Science of the Total Environment 770:145216

Duckweed-based waste stabilization ponds (DWPs) have been widely used in wastewater treatment. However, the effects of sediment, an essential component of DWPs, on their performance have rarely been studied. In this study, two pilot-scale DWPs (12 m^2) with sediment (DPS) and without sediment (DP) were evaluated over more than 1year to determine the effects of sediment on duckweed growth, wastewater treatment, and greenhouse gas (GHG) production and emission in DWPs. The results indicated that the annual average duckweed growth rate were comparable, but protein content, carbon (C) and nitrogen (N) recovery rates of duckweed were slightly higher in the DPS than in the DP. Meanwhile, the dissolved oxygen (DO) and oxidation reduction potential (ORP), removal efficiencies of COD, TP, TN, NH_4^+-N, and turbidity of pond water from the DPS were significantly lower than for DP. More importantly, the DPS had considerably higher CH_4 production/emission and global warming potential (GWP) than the DP, even though more than 90% of CH4 released from the sediment was consumed during its passage through the water column and duckweed layer. Sediment increased the recoveries of C and N by 7.94% and 8.82%, respectively. Influencing degree for COD, TP, TN, NH_4^+-N and turbidity were -27.92%, -20.98%, -22.61%, -24.13% and -14.91%, respectively; for pond water DO and ORP, the values were -35.68% and -44.59%, respectively; and for CO_2, CH_4 and N_2O emission and "combined GWP", they were 21.66%, 271.67%, -8.47% and 178.02%, respectively. Thus, this study indicates that sediment formed in the DWPs has a multi-faced effect on the performance of a DWP. In particular, sediment has an unfavourable effect on the wastewater treatment and the GHGs mitigation, but a favourable effect on the protein content and the C and N recoveries in duckweed.

Coupling ecological wastewater treatment with the production of livestock feed and irrigation water provides net benefits to human health and the environment: A life cycle assessment

Roman, B; Brennan, RA (2021) Journal of Environmental Management 288:112361

Ecologically designed wastewater treatment systems (ex., Eco-Machines) utilize a diverse ecosystem to treat wastewater to the same extent as conventional treatment, but require less energy and chemical inputs. The environmental benefits of Eco-Machines can be theoretically maximized by incorporating hyperaccumulating aquatic plants (ex., duckweed) to facilitate nutrient recovery and conversion into protein-rich biomass, which can then be harvested for a range of agricultural and bioenergy applications. Although it has been established that ecological wastewater treatment systems are more cost- and energy-efficient than conventional wastewater treatment systems, a systematic life cycle assessment (LCA) of an Eco-Machine coupled with its beneficial by-products has not been conducted. In this study, a series of LCAs were performed on different operational scenarios for a 1000 gallon per day, pilot-scale Eco-Machine that, in addition to producing irrigation-quality water, also produces duckweed biomass for aquaculture. The analysis revealed that Eco-Machines located in warm climates, which do not require a greenhouse or supplemental heating, use approximately a third of the energy and produce half of the greenhouse gas emissions compared to conventional wastewater treatment systems in similar locations, while also providing benefits to human health, ecosystem quality, climate change, and resources. In addition, increasing the growth area for duckweed using vertical farming techniques improves the overall impact of the system. This study suggests that with proper management, ecological wastewater treatment systems that upcycle nutrients and water into beneficial products can provide a net benefit to human health and the environment.

Lemna minor, a hyperaccumulator shows elevated levels of Cd accumulation and genomic template stability in binary application of Cd and Ni: a physiological and genetic approach

Ozyigit, II; Arda, L; Yalcin, B; Yalcin, IE; Ucar, B; Hocaoglu-Ozyigit, A (2021) International Journal of Phytoremediation DOI:10.1080/15226514.2021.1892586

In this study, to determine whether having potential to be used as hyperaccumulator for Cd and Ni, numerous experiments were designed for conducting assessments for physiological and genotoxic changes along with defining possible alterations on mineral nutrient status of *Lemna minor* L. by applying Cd-Ni binary treatments (0, 100, 200 and 400 µM). Our study revealed that there were increases in the concentrations of B, Cr, Fe, K, Mg, and Mn whereas decreases were noticed in the concentrations of Na and Zn and the levels of Ca were inversely proportional to Cd-Ni applications showing tendency to increase at the low concentration and to decrease at the high concentration. Randomly Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) analyses revealed that rather than band losses and new band formations, mostly intensity changes in the band profiles, and low polymorphism and high genomic template stability (GTS) were observed. Although, to date, *L. minor* was defined as an efficient hyperaccumulator/potential accumulator or competent phytoremedial agent by researchers. Our research revealed that *L. minor* showing high accumulation capability for Cd and having low polymorphism rate and high genomic template stability is a versatile hyperaccumulator, especially for Cd; therefore, highly recommended by us for decontamination of water polluted with Cd. Many studies have been focused on the effects of individual metal ions. However, heavy metal contaminants usually exist as their mixtures in natural aquatic environments. Especially, Cd and Ni coexist in industrial wastes. In this study, the accumulation properties of *Lemna minor* for both Cd and Ni were investigated and the effects of Cd and Ni on the bioaccumulation of B, Ca, Cu, Fe, Mg, K, Mn, Na, Pb and Zn in *L. minor* were also determined. This study furthermore aimed to assess the genotoxic effects of Cd and Ni found in being extended concentrations on DNA using the Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) method.

Duckweed - *Lemna minor* as green route for removal of chromium(VI) from aqueous solution

Nassar, HF; Ibrahim, M (2021) International Journal of Environmental Research 15: 275-284

Dried duckweed- Lemna minor aquatic plant was subjected to molecular modifications using quantum mechanical calculations. The molecular modelling simulates aquatic weed as a composite of cellulose/lignin/glycine. This model suggests the ability to remove inorganic pollutants from wastewater according to unique hydrogen bonding and high total dipole moment. For verifications, experimental efforts are performed for the phytoremediation of Cr(VI) from aqueous solution using a species of Duckweed *Lemna minor* (Lm) through adsorption process. Duckweed-Lm biomass was prepared and characterized by X-ray diffraction (XRD), scan electron microscope (SEM), Fourier-transform infrared (FTIR) and Brunauer- Emmett-Teller (BET). The effect of several important parameters, such as pH, sorbent dose, contact time, temperature, sorption and desorption processes, was studied. The phytoremediation of Cr(VI) ion was found to be pH-dependent with a maximum removal of 64% achieved at pH of 3.8 and a lowest removal of 25% at pH of 9.5. The obtained adsorption results indicated that duckweed-Lm can be effective for chromium ion removal from aqueous solutions.

The enhancement of treatment capacity and the performance of phytoremediation system by fed batch and periodic harvesting

Ng, YS; Chan, DJC (2021) RSC Advances 11: 6049-6059

Floating macrophyte phytoremediation could be the most relevant solution to the ever-increasing finfish farm pond effluent worldwide. However, the information of *Spirodela polyrhiza* monoculture system in fed batch

mode, with periodic harvesting and increased macrophyte density is limited. In this study, the effect of fed batch and periodic harvesting on the treatment capacity and performance of the *S. polyrhiza* monoculture system (with increased the macrophyte density) in fish farm wastewater were evaluated. Results showed that the system with fed batch and harvesting could treat a greater volume of wastewater, remove a higher amount of pollutants in terms of ammonia (NH_3-N), phosphate (PO_4^{3-}), total suspended solids (TSS) and chemical oxygen demand (COD), while meeting the effluent limits. The system with *S. polyrhiza* macrophyte density of 11.67 g fresh weight (FW) per L wastewater was able to decrease nitrate (NO_3^--N) and nitrite (NO_2^--N) to an undetected level. This study suggested that the *S. polyrhiza* monoculture system with fed batch, optimal harvesting and frequent sediment removal is feasible and effective in treating the fish farm wastewater, and produces biomass with superior protein content for fish feed supplement and poultry diet. The obtained data provided insights into the system reliability in wastewater treatment and ways of improvement for the system. The treated wastewater could achieve exceptional quality with minimal toxicity before discharge to receiving waters, and potentially be reused for water flow recharge, aquaculture and irrigation purposes, minimizing the pollution and ecological impacts.

Agronomic and environmental performance of *Lemna minor* cultivated on agricultural wastewater streams - A practical approach

Devlamynck, R; de Souza, MF; Michels, E; Sigurnjak, I; Donoso, N; Coudron, C; Leenknegt, J; Vermeir, P; Eeckhout, M; Meers, E (2021) Sustainability 13: 1570

This study investigated the potential of *Lemna minor* to valorise agricultural wastewater in protein-rich feed material in order to meet the growing demand for animal feed protein and reduce the excess of nutrients in certain European regions. For this purpose, three pilot-scale systems were monitored for 175 days under outdoor conditions in Flanders. The systems were fed with the effluent of aquaculture (pikeperch production-PP), a mixture of diluted pig manure wastewater (PM), and a synthetic medium (SM). PM showed the highest productivity (6.1 ± 2.5 g DW m^{-2} d^{-1}) and N uptake (327 ± 107 mg N m^{-2} d^{-1}). PP yielded a similar productivity and both wastewaters resulted in higher productivities than SM. Furthermore, all media showed similar P uptake rates (65-70 P m^{-2} d^{-1}). Finally, duckweed had a beneficial amino acid composition for humans (essential amino acid index = 1.1), broilers and pigs. This study also showed that the growing medium had more influence on the productivity of duckweed than on its amino acid composition or protein content, with the latter being only slightly affected by the different media studied. Overall, these results demonstrate that duckweed can effectively remove nutrients from agriculture wastewaters while producing quality protein.

Light intensity alters the phytoremediation potential of *Lemna minor*

Walsh, E; Kuehnhold, H; O'Brien, S; Coughlan, NE; Jansen, MAK (2021) Environmental Science and Pollution Research 28: 16394-16407

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

light intensities. Similar results were obtained with the larger re-circulating system. The results demonstrate interactive effects of light intensity and wastewater composition on growth and phytoremediation potential of *L. minor*. The data imply that light intensities above 50 µmol m^{-2} s^{-1} may not necessarily confer benefits in duckweed wastewater remediation, and this informs engineering of stacked, indoor remediation systems.

Biosorption of Co^{2+} ions from aqueous solution by K_2HPO_4-pretreated duckweed *Lemna gibba*

Reyes-Ledezma, JL; Cristiani-Urbina, E; Morales-Barrera, L (2020) 8: 1532

The wastewater of the many industries that use divalent cobalt (Co^{2+})-containing compounds has elevated levels of this metal. Thus, novel technology is needed to efficiently remove Co^{2+} ions from aqueous solutions. Biosorption is a low-cost technique capable of removing heavy metals from contaminated water. This study aims to evaluate the performance of KH_2PO_4-pretreated *Lemna gibba* (PLEM) as a biosorbent of Co^{2+} in aqueous solutions tested under different conditions of pH, particle size, and initial Co^{2+} concentration. Kinetic, equilibrium, and thermodynamic studies were conducted. The capacity of biosorption increased with a greater initial Co^{2+} concentration and was optimal at pH 7.0 and with small-sized biosorbent particles (0.3-0.8 mm). The pseudo-second-order sorption model best describes the experimental data on Co^{2+} biosorption kinetics. The Sips and Redlich-Peterson isotherm models best predict the biosorption capacity at equilibrium. According to the thermodynamic study, biosorption of Co^{2+} was endothermic and spontaneous. The effect of pH on the biosorption/desorption of Co^{2+} suggests that electrostatic attraction is the main biosorption mechanism. SEM-EDX verified the presence of Co^{2+} on the surface of the pretreated-saturated biosorbent and the absence of the metal after desorption.

Application of *Lemna gibba* L. and a bio-based aerogel for the removal of metal(loid)s from stream waters near three gold deposits in northwestern Iran

Torbati, S; Keshipour, S (2020) Environmental Technology and Innovation 20: 101068

Mining activities can be regarded as one of the main reasons for heavy metals pollution in the environment. In the present study, the capability of duckweed (*Lemna gibba* L.) and a modified cellulose aerogel, in removing of some metal(loid)s from stream waters near three main gold deposits in the Northwestern Iran was evaluated. The amount of metal(loid)s in water samples and the plant was determined by inductively coupled plasma-mass spectroscopy (ICP-MS). It was found that using plant and aerogel led to the significant reduction of the metal(loid)s concentration in water samples and the performance of the bio-based aerogel in metal remediation was more efficient. Simultaneous use of plant and the aerogel (P+A) for the removal of As, Pb and Cu from the most polluted water sample (S1) was led to the statistically significant increase of removal efficiency (near to 18%, 42% and 63%, respectively), as compared to the use of the aerogel alone. Duckweed could accumulate the metal(loid)s in its body a few hundred times in comparison to the water samples. Photosynthetic pigments and malondialdehyde contents, as well as antioxidant activities in the plant were increased by the enhancement of the amount of the metal(loid)s concentration in treated waters; so, the plant might create tolerable conditions by the induction of its defense system after treatment by the stream waters of the mining areas.

Assessing and modelling the efficacy of *Lemna paucicostata* for the phytoremediation of petroleum hydrocarbons in crude oil-contaminated wetlands

Ekperusi, AO; Nwachukwu, EO; Sikoki, FD (2020) Scientific Report 10: 8489

The potentials of the invasive duckweed species, *Lemna paucicostata* (DF: the correct name is *Lemna aequinoctialis*) to remove pollutants from aquatic environment was tested in a constructed wetland as an ecological based system for the phytoremediation of petroleum hydrocarbons in crude oil-contaminated waters within 120 days. Total petroleum hydrocarbons in wetlands and tissues of duckweed were analyzed using gas chromatography with flame ionization detector following established methods while the experimental data were subjected to the first-order kinetic rate model to understand the remediation rate of duckweed in wetlands. *L. paucicostata* effected a significant ($F=253.405$, $P<0.05$) removal of hydrocarbons from wetlands reaching 97.91% after 120 days. Assessment on the transport and fate of hydrocarbons in duckweed indicated that *L. paucicostata* bioaccumulated less than 1% and significantly biodegraded 97.74% of hydrocarbons in wetlands at the end of the study. The experimental data reasonably fitted ($r^2=0.938$) into the first-order kinetic rate model. From the result of the study, it is reasonable to infer that *L. paucicostata* is an effective aquatic macrophyte for the removal of petroleum hydrocarbons in moderately polluted waters.

Phytotoxicity

Interactions between *Lemna minor* (common duckweed) and PFAS intermediates: Perfluorooctanesulfonamide (PFOSA) and 6:2 fluorotelomer sulfonate (6:2 FTSA)

Zhang, W; Liang, Y (2021) Chemosphere 276:130165

Perfluorooctanesulfonamide (PFOSA) and 6:2 fluorotelomer sulfonate (FTSA) are widely present intermediates of per- and polyfluorinated substances (PFAS). Although detected at high concentrations in landfill leachate and groundwater, the interactions of these two compounds with plants have not been investigated much. In this work, uptake of these two PFAS intermediates at 10 and 200 μg/L by *Lemna minor* (common duckweed) were studied in detail. It was found that the biomass production of *L. minor* was not impacted negatively by PFOSA and FTSA at concentrations equal to or lower than 200 μg/L. Between these two target compounds, FTSA had much higher concentrations in *L. minor* when the concentrations and exposure times were the same as those for PFOSA. In addition, this compound at 200 μg/L inhibited the activities of catalase in *L. minor* significantly compared to the controls. This study indicates that PFOSA with low water solubility has low toxicity to *L. minor*, while FTSA at high concentration may accumulate in the floating plants and cause adverse effects on plant's antioxidative defence system. Longer-term studies of *L. minor* with these two and other PFAS are warranted given the important role of this floating plant in the ecosystem.

Toxic responses of Palladium accumulation in duckweed *Lemna minor*: Determination of biomarkers

Jmii, S; Dewez, D (2021) Environmental Toxicology and Chemistry DOI:10.1002/etc.5011

Palladium (Pd) is a trace metal of the platinum group elements representing an emerging contaminant for the environment. It is of great interest to characterize the bioaccumulation and toxicity of Pd to improve our toxicological knowledge for this contaminant. Under standardized toxicity testing conditions, we analyzed Pd accumulation and toxicity effects on the duckweed *Lemna minor* exposed to nominal concentrations from 2 to 50 μM. The inhibitory effect was significant ($p < 0.05$) from 8 μM of Pd, starting with 9.5 % of growth inhibition and a decrease of 1 cm for the root size. Under 12.5 μM of Pd, the bioaccumulated Pd of 63.93 g/g fresh weight inhibited the plant growth by 37.4 %, which was caused by a strong oxidative stress in the cytosol and organelles containing DNA. Under 25 and 50 μM of Pd, bioaccumulated Pd was able to deteriorate the entire plant physiology including the chlorophylls synthesis, the photosystem II antenna complex, and the photochemical reactions of photosynthesis. In fact, Pd-treated plants to 50 μM accumulated Pd up to 256 g/g fresh weight, causing a strong decrease in total biomass and root elongation process. Therefore, we showed several growths, physiological, and biochemical alterations which were correlated with the bioaccumulation of

Pd. These alterations constituted toxicity biomarkers of Pd with different Lowest Observed Effect Dose (LOED), following this order: Root size = Growth inhibition < Catalase activity = Carotenoids content = ROS production = Total thiols < Chl a/b = FV /FM = ABS/RC = PIABS < VJ . Therefore, this work presented new knowledge on the toxicity mechanism of Pd in *L. minor* plants under standardized testing condition.

Joint effects of naphthalene and microcystin-LR on physiological responses and toxin bioaccumulation of *Landoltia punctata*

Yang, GL; Huang, MJ; Tan, AJ; Lv, SM (2021) Aquatic Toxicology 231: 105710

The co-contamination of naphthalene (NAP) and microcystin-LR (MC-LR) commonly occurs in eutrophic waters. However, the joint effects of NAP and MC-LR on plants in aquatic environments remain unknown. *Landoltia punctata* is characterized by high starch yields and high biomass in polluted waters and has been proven to be a bioenergy crop and phytoremediation plant. In this study, *L. punctata* was cultured in a nutrient medium with environmentally relevant NAP (0.1, 1, 3, 5, and 10 μg/L) and MC-LR (5, 10, 25, 50, and 100 μg/L) to determine individual and joint toxic effects. The effects of NAP and MC-LR on physiological responses of *L. punctata*, including growth, starch accumulation, and antioxidant responses, were studied. Bioaccumulation of MC-LR in *L. punctata*, with or without NAP, was also examined. The results showed that growth and chlorophyll-a contents of *L. punctata* were reduced at high concentrations of MC-LR (≥ 25 μg/L), NAP (≥ 10 μg/L) and their mixture (≥ 10 ± 1 μg/L) after exposure for 7 d. Starch accumulation in *L. punctata* did not decrease when exposed to NAP and MC-LR, and higher starch content of 29.8 % ± 2.7 % DW could be due to the destruction of starch-degrading enzymes. The antioxidant responses of *L. punctata* were stronger after exposure to MC-LR + NAP than when exposed to a single pollutant, although not enough to avoid oxidative damage. NAP enhanced the bio- accumulation of MC-LR in *L. punctata* when NAP concentration was higher than 5 μg/L suggesting that higher potentials of MC-LR phytoremediation with *L. punctata* may be observed in NAP and MC-LR co-concomitant waters. This study provides theoretical support for the application of duckweed in eutrophic waters containing organic chemical pollutants.

The effects of solubility of silver nanoparticles, accumulation, and toxicity to the aquatic plant *Lemna minor*

Souza, LRR; Correa, TZ; Bruni, AT; da Veiga, MAMS (2021) Environmental Sceince and Pollution Research 28: 16720-16733

The use of silver nanoparticles (AgNPs) in commercial products has increased due to their antibacterial properties and their impacts on the environment must be investigated. This scenario has motivated the conduction of this study, which relates different factors that affect the toxicity of AgNPs to the aquatic plant *Lemna minor* such as size, accumulation, concentration, and dissolution of AgNPs. To this end, synthesized AgNPs measuring 30, 85, and 110 nm were added into the culture medium to observe toxicity for 30 days. The mapping by SEM showed that the smallest AgNPs can translocate from roots to leaves due to its mobility and internalization. As predicted by the Ostwald equation, the solubility for 30-nm AgNPs increased almost 3 times at the end of 30 days, while for 85 and 110 nm size nanoparticles, after 7 days, the solubility decreased due to "Ostwald ripening" process. Plant mortality was assessed and, after 1 month, the size of 30 nm was the most toxic with negative growth in all studied concentrations, with 60% mortality in the worst case. The concentration of 50 μg $mL^{-1}$was toxic in all sizes with negative growth in the period. Therefore, the investigation of AgNPs' toxicity needs to consider a different factor to better understand their effects on aquatic plants and the environment.

Application of stress induces ascorbate peroxidases of *Spirodela polyrhiza* for green-synthesis Cu nanoparticles

Patel, VR; Bhatt, N (2020) Arabian Journal of Chemistry 13: 8783-8792

The objective of this study was to assess the effects of stress on physiology/biochemical component of *S. polyrhiza* and its impact on CuNPs synthesis and bioethanol production. NaCl with RV5 provokes oxidative stress in *S. polyrhiza* and significantly increase MAD, Proline, H_2O_2, ROS, SOD and APX activity compare to control condition. Starch accumulation in S. *polyrhiza* was found 354% higher and correspond 4.4 times higher ethanol yield under stress condition compare to control. CuNPs were synthesized with an average size of 23-26 nm by purified fraction of APX having 37 KDa MW, 1.44 IU specific activity. Synthesized CuNPs were stable up to 15 consecutive cycles and potency against wide range of reactive dyes. The maximum remedial efficiency of synthesized CuNPs for COD and BOD was 55263 ± 3298 mg/m^3 min and 30560 ± 1987 mg/m3 min, respectively for RV5 wastewater. 0.072 mg/g of bioethanol was produced from the wet pulp remaining after nanoparticles synthesis. High efficiency of CuNPs and significant production of ethanol, indicate that the feasibility for circular model for continuous industrial wastewater treatment.

Effect of cadmium on the level of isoprenoid-derived phytohormones in duckweed *Wolffia arrhiza*

Chmur, M; Bajguz, A; Piotrowska-Niczyporuk, A (2020) Journal of Plant Growth Regulation 39: 1518-1530

Wolffia arrhiza (L.) Horkel ex Wimm. is an aquatic plant belonging to the Lemnaceae family. It does not have leaves, stems, and roots, flowers rarely occur, while body size can reach 1 mm of width and 1.3 mm of length. The present study demonstrates the endogenous level of isoprenoid-derived phytohormones and their changes under the influence of different cadmium (Cd) concentrations (0.1, 1, 10, and 100 μM). A liquid chromatography quadrupole-time-of-flight mass spectrometry analysis indicated the presence of abscisic acid, eight brassinosteroids (6-deoxocastasterone, 6-deoxotyphasterol, cathasterone, typhasterol, castasterone, 24-epicastasterone, brassinolide, and 28-homobrassinolide), seven free bases of cytokinins [trans-zeatin (tZ),cis-zeatin (cZ), dihydrozeatin (DHZ),N-6-isopentenyladenine,N-6-isopentenyladenosine,ortho-topolin, and meta-topolin], eight conjugates of cytokinins (tZ riboside,tZ-9-glucoside,tZ-7-glucoside,tZ-O-glucoside riboside,cZ-9-glucoside, DHZ riboside, DHZ-O-glucoside, and N(6)-isopentenyladenosine-7-glucoside) and gibberellic acid (GA(3)) in this duckweed. The level of phytohormones in plants treated with Cd has changed, e.g., the ABA level increased while GA_3 decreased. Whereas the amount of BRs and CKs was different in Cd dose-dependent manner. Besides, it is worth noting that the distribution of 25 various phytohormones in *Wolffia arrhiza* is reported for the first time.

Effects of silver(I) toxicity on microstructure, biochemical activities, and genic material of *Lemna minor* L. with special reference to application of bioindicator

Li, HB; Mo, F; Li, YH; Wang, MS; Li, Z; Hu, HY; Deng, WH; Zhang, R (2020) Environmental Science and Pollution Research 27: 22735-22748

In this research, several biochemical variations in plant of *Lemna minor* L. were investigated to reflect Ag^+ toxicity. *Lemna minor* L. changed colorless $AgNO_3$ to colloidal brown at doses equal to and greater than 1 mg L^{-1}. Optical and fluorescence microscopy revealed the presence of bright spots in roots of tested plant related to Ag/Ag_2O-NPs. Photosynthetic pigment contents of *Lemna minor* L. declined upon exposure to Ag^+ with an evidently higher decrease in chlorophyll a than in chlorophyll b. Similarly, Ag^+ treatment caused an evident reduction in the activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). The reduction in antioxidase activity was significantly higher in POD than in SOD and CAT. Ag^+ treatment resulted in a significant increment in the level of malondialdehyde (MDA) content as the judging criteria of cellular injury which showed sign of dose-related. The alterations occurred in RAPD profiles of treated samples following Ag^+ toxicity containing loss of normal bands, appearance of new bands, and variation in band intensities compared with the normal plants. In addition, morphological character and biomass of *Lemna minor* L. subjected to increasing Ag^+ concentrations were evaluated to reveal Ag^+ toxicity. Our study demonstrated that *Lemna minor*

L. have a high sensitivity to indicate fluctuation of water quality. It would be beneficial that modulating the genotype of *Lemna minor* L. to bear high proportion of contaminates.

Reviews

Progress in thermochemical conversion of duckweed and upgrading of the bio-oil: A critical review

Djandja, OS; Yin, L; Wang, Z; Guo, Y; Zhang, X; Duan, P (2021) The Science of the Total Environment 769:144660

The processing of duckweed has been included in the list of promising pathways for biofuels production. This property is attributed to its simple manual harvesting method and its ability for high protein or starch content, depending on its species and growing environment. The biofuels production from duckweed is not only a solution to energy and environmental problems, but also a reliable way to realize the utilization of duckweed. This critical review focuses on the bio-oil production from duckweed via pyrolysis and hydrothermal liquefaction processes. First, characteristics and eco-environmental benefits of duckweed are reviewed. Next, the impacts of different parameters on the properties and distribution of bio-oil from pyrolysis and hydrothermal liquefaction are discussed in detail. Subsequently, the effect of hydrogen donor solvents (as reaction media for upgrading) and catalysts on the upgrading of duckweed bio-oil are extensively discussed. This paper ends with the prospects for further development in thermochemical valorization of duckweed.

Research Progress of a Potential Bioreactor: Duckweed

Yang, GL; Feng, D; Liu, YT; Lv, SM; Zheng, MM; Tan, AJ (2021) Biomolecules 11: 93

Recently, plant bioreactors have flourished into an exciting area of synthetic biology because of their product safety, inexpensive production cost, and easy scale-up. Duckweed is the smallest and fastest-growing aquatic plant, and has advantages including simple processing and the ability to grow high biomass in smaller areas. Therefore, duckweed could be used as a new potential bioreactor for biological products such as vaccines, antibodies, pharmaceutical proteins, and industrial enzymes. Duckweed has made a breakthrough in biosynthesis as a chassis plant and is being utilized for the production of plenty of biological products or bio-derivatives with multiple uses and high values. This review summarizes the latest progress on genetic background, genetic transformation system, and bioreactor development of duckweed, and provides insights for further exploration and application of duckweed.

Taxonomy

Duckweed species genotyping and interspecific hybrid discovery by tubulin-based polymorphism fingerprinting

Braglia, L; Lauria, M; Appenroth, KJ; Bog, M; Breviario, D; Grasso, A; Gavazzi, F; Morello, L (2021) Frontiers in Plant Science 12: 625670

Duckweeds (Lemnaceae) are the smallest and fastest-growing angiosperms. This feature, together with high starch production and good nutritional properties, makes them suitable for several applications, including wastewater treatment, bioenergy production, or feed and food supplement. Due to their reduced morphology and great similarity between diverse species, taxonomic identification of duckweeds is a challenging issue even for experts. Among molecular genotyping methods, DNA barcoding is the most useful tool for species identification without a need for cluster analysis. The combination of two plastid barcoding loci is now considered the gold standard for duckweed classification. However, not all species can be defined with confidence by these markers, and a fast identification method able to solve doubtful cases is missing. Here we show the potential of tubulin-based polymorphism (TBP), a molecular marker based on the intron length

polymorphisms of beta-tubulin loci, in the genomic profiling of the genera *Spirodela, Landoltia*, and *Lemna*. Ninety-four clones were analyzed, including at least two representatives of each species of the three genera, with a special focus on the very heterogeneous species *Lemna minor*. We showed that a single PCR amplification with universal primers, followed by agarose gel analysis, was able to provide distinctive fingerprinting profiles for 10 out of 15 species. Cluster analysis of capillary electrophoresis-TBP data provided good separation for the remaining species, although the relationship between *L. minor* and *Lemna japonica* was not fully resolved. However, an accurate comparison of TBP profiles provided evidence for the unexpected existence of interspecific hybrids between *Lemna turionifera* and *L. minor*, as further confirmed by amplified fragment length polymorphism and sequence analysis of a specific beta-tubulin locus. Such hybrids could possibly correspond to *L. japonica*, as originally suggested by E. Landolt. The discovery of interspecific hybrids opens a new perspective to understand the speciation mechanisms in the family of duckweeds.

In Books

Duckweeds for the production of therapeutic proteins

Khvatkov P., Firsov A., Mitiouchkina T., Chernobrovkina M., Dolgov S. (2021)

In: Malik S. (ed) Exploring Plant Cells for the Production of Compounds of Interest. Springer, Cham. https://doi.org/10.1007/978-3-030-58271-5_5

Instructions to Contributors for the Duckweed Forum

The Duckweed Forum (DF) is an electronic publication that is dedicated to serve the Duckweed Research and Applications community by disseminating pertinent information related to community standards, current and future events, as well as other commentaries that could benefit this field. As such, involvement of the community is essential and the DF can provide a convenient platform for members in the field to exchange ideas and observations. While we would invite everyone to contribute, we do have to establish clear guidelines for interested contributors to follow in order to standardize the workflow for their review and publication by the Duckweed Steering Committee members.

Contributions to DF must be written in English, although they may be submitted by authors from any country. Authors who are not native English speakers may appreciate assistance with grammar, vocabulary, and style when submitting papers to the DF.
DF is currently arranged in sections, which may be chosen by a prospective author(s) to contribute to: Main text, Opinion paper, Discussion corner, Useful methods, Student experiments, Student spotlight, Science meets art, and Cover photo(s). 1,000 words are suggested as the upper limit for each contribution, but can be extended on request to the Steering Committee if the reason for the waiver request is warranted.

Presubmissions

In addition to invitees by a Duckweed Steering Committee member, if you are considering submitting a contribution to DF but are unsure about the fit of your idea, please feel free to contact one of the members in the Duckweed Steering Committee in order to obtain feedback as to the appropriateness of the subject for DF. Please include a few sentences describing the overall topic that you are interested to present on, and why you think it is of interest to the general duckweed community. If you have the abstract or draft text prepared, please include it. The Duckweed Steering Committee will discuss the material in one of its meetings and the decision to formally invite submission will be given shortly afterwards.

Copyright and co-author consent

All listed authors must concur in the submission and the final version must be seen and approved by all authors of the contribution. As a public forum, we do not carry out any Copyright application. If you need to copyright your material, please do so beforehand.

Formatting requirements:

- A commonly used word processing program, such as Word, is highly recommended.

- Formatting requirements: 8.5-by-11-inch (or 22 cm-by-28 cm) paper size (standard US letter).
- Single-spaced text throughout.
- One-inch (or 2.5 cm) left and right, as well as top and bottom margins.
- 11-point Times New Roman font.
- Number all pages, including those with figures on the bottom and center of each page.

Title:

- Should be intelligible to DF readers who are not specialists in the field and should convey your essential points clearly.
- Should be short (no more than 150 characters including spaces) and informative.
- Should avoid acronyms or abbreviations aside from the most common biochemical abbreviations (e.g., ATP). Other acronyms or abbreviations should either:
    - be introduced in their full form (e.g., Visualization of Polarized Membrane Type 1 Matrix Metalloproteinase (MT1-MMP) Activity in Live Cells by Fluorescence Resonance Energy Transfer (FRET) Imaging); or
    - be clarified by use as a modifier of the appropriate noun (e.g., FOX1 transcription factor, ACC dopamine receptor).

Authors:

- All authors are responsible for the content of the manuscript.
- Provide the complete names of all authors.
- Identify which author will receive correspondence regarding the contribution.
- Provide the corresponding author's name, telephone number, and current e-mail address.

Image resolution and submission:

It is extremely important that figures be prepared with the proper resolution for publication in order to avoid inaccurate presentation of the data. The minimum acceptable resolution for all figures is 300 dpi. Excessive file compression can distort images, so files should be carefully checked after compression. Note that figures that contain both line art (such as graphs) and RGB/grayscale areas (such as photographs) are best prepared as EPS (vector) files with embedded TIFF images for the RGB/grayscale portions. The resolution of those embedded TIFF images should be at least 300 dpi. Original images should be submitted as a separate file to the text file. It would be helpful to insert the intended into the Word file as well, if desired, to indicate the location for it. The legend to the image/figure should be added at the end of the text file and labeled as "Legend to Figures".

Links for Further Reading

http://www.ruduckweed.org/ Rutgers Duckweed Stock Cooperative, New Brunswick, New Jersey State University. Prof. Dr. Eric Lam

http://www.InternationalLemnaAssociation.org/ Working to develop commercial applications for duckweed globally, Exec. Director, Tamra Fakhoorian

http://thecharmsofduckweed.org Comprehensive site on all things duckweed-related, By Dr. John Cross, maintained by Paul Fourounjian.

http://plants.ifas.ufl.edu/ University of Florida's Center for Aquatic & Invasive Plants.

Community Resources - Updated Table for Duckweed Collections in the Community

For information related to the location, collection size and contact email for duckweed collections in our community, please access the website of the RDSC (Rutgers Duckweed Stock Cooperative) under the heading "List of Worldwide Duckweed Collections". This Table will be updated as new entries for duckweed collections are being supplied to members of the International Steering Committee for Duckweed Research and Applications (ISCDRA). We also plan to publish the updated table in the first issue of each Duckweed Forum newsletter volume starting in 2021.

Note to the Reader

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Please let us know via email to the Chair of ISCDRA, Prof. Eric Lam: ericL89@hotmail.com