

# Procedures for the disinfection of stock culture of duckweed

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## Introduction

The vulnerability of aquatic environments to the increasingly widespread presence of xenobiotic compounds is currently representing a major global concern. Various biological organisms are sensitive to xenobiotics and, thus, make them suitable as model species for toxicity studies. Ecotoxicological studies based on plant organisms in freshwater ecosystems are an efficient tool for evaluating and monitoring their health, as well as that of the entire food web up to the human diet. This is because plant organisms play a fundamental structural and functional role as primary producers (Gubbins et al., 2011).

Plants belonging to the Lemnoideae subfamily are recognised as bioindicators of water quality (Böcük et al., 2013; Mkandawire et al., 2014; Pietrini et al., 2022). Lemnoideae, commonly known as duckweeds, are classified by The Angiosperm Phylogeny Group (The Angiosperm Phylogeny Group, 2016) as a subfamily of Araceae. However, more recent studies, based on molecular analyses, suggest that duckweeds are phylogenetically and morphologically distinct from Araceae, and should be restored to family status as Lemnaceae (Tippary et al., 2021).

Duckweeds are aquatic plants that play an important ecological role within freshwater, estuarine and wetland habitats, forming part of the community of primary producers (Greenberg et al., 1992). Duckweed plants, particularly those belonging to the *Lemna* genus, are among the smallest representative of the vascular plants and are characterized by the fastest growth rates among flowering plants (Sree et al., 2015; Ziegler et al., 2015). They are easy to cultivate in laboratory conditions, due their fast growth rate, which is driven by mass propagation via asexual proliferation involving the budding of daughter fronds (Pietrini et al., 2019).

Due to their biological characteristics, duckweed plants are widely used to evaluate phytotoxicity of pollutants and assess their environmental risks. They have been selected as the model plant for ecotoxicity studies on the freshwater ecosystem (Baudo et al., 2015; Pietrini et al., 2015, 2016; Forni and Tommasi, 2016; Iannilli et al., 2025) and are officially used in ecotoxicological assays (OECD/OCDE 221, 2006). Moreover, duckweed plants have been indicated to be effective biological tools to remove toxic compounds from wastewater (Hegazy et al., 2009; Matamoros et al., 2012; Ceschin et al., 2019).

Over 90% of the plant-based ecotoxicological studies are conducted in a strictly laboratory setting (Ceschin et al., 2020). Obtaining stock of plant cultures free of parasites or pathogens is a major challenge in the field of ecotoxicological assays. Furthermore, axenic conditions are desirable for plants employed in lab tests in order to evaluate the respective roles of the plants and their microbiota in detoxifying chemical compounds (Khellaf and Zerdaoui, 2010; Liu et al., 2019).

To address these challenges, protocols have been developed to obtain plant cultures at different disinfection levels, ranging from “clean” to sterile, without compromising the survival or development potential of the plants (Firmin et al., 2025).

## Plant materials and culture protocol

Three duckweed species are cultivated in the growth chamber of the Research Institute on Terrestrial Ecosystems, secondary unit of Montelibretti: *Spirodela polyrhiza* (L.) Schleid. (Fig. 1), *Lemna minor* L. (Fig. 2) and *Lemna minuta* Kunth (Fig. 3).

The fronds of *L. minor* plants were purchased from a company specialising in aquatic cultivation systems and maintained in stock-culture conditions. *S. polyrhiza* and *L. minuta*, on the other hand, were collected in the wild, cleaned, and acclimatised to stock-culture conditions. The growth chamber is maintained at a constant temperature of  $25 \pm 3$  °C, with a photosynthetic photon flux density (PPFD) of  $60\text{--}80 \mu\text{mol m}^{-2}\text{s}^{-1}$  and a photoperiod of 16h light/8h dark (Fig. 4). The recipients containing duckweeds, placed in the growth chamber, are partially covered with a thin plastic sheet to minimise contamination and a light airflow from a pump is given for oxygenation.



Fig. 1 – *Spirodela polyrhiza*

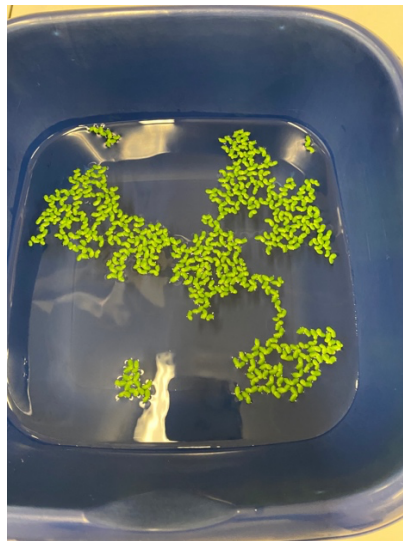


Fig. 2 – *Lemna minor*

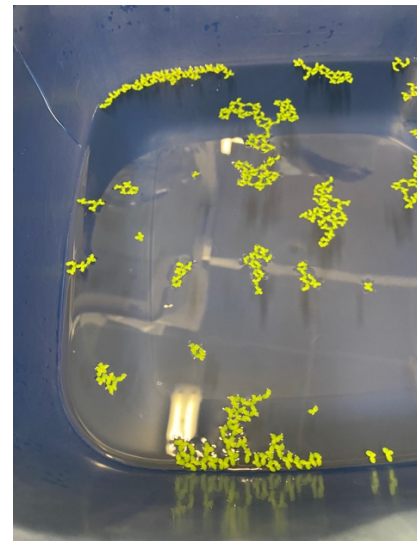


Fig. 3 – *Lemna minuta*

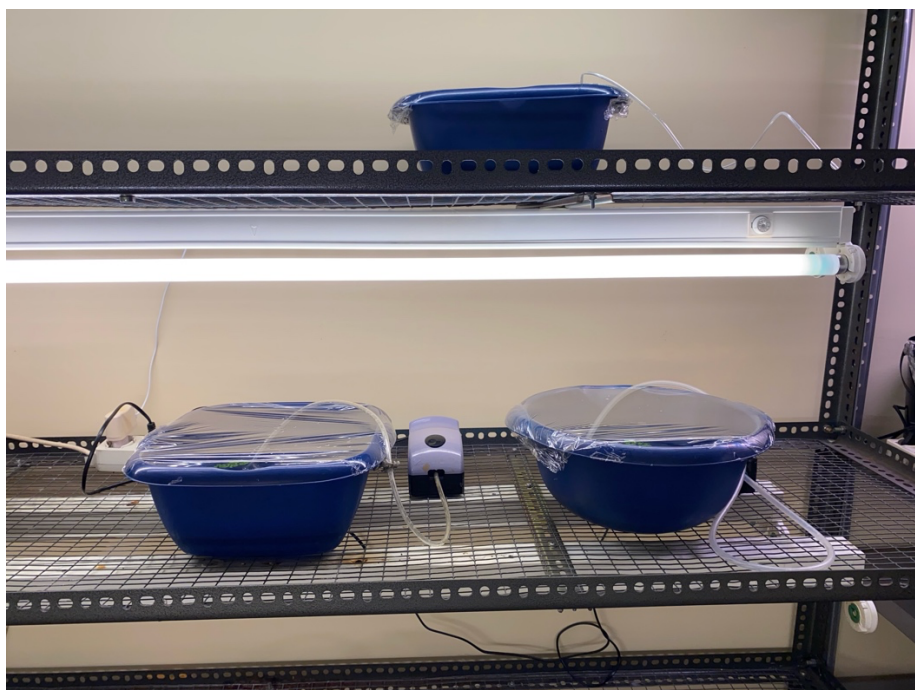


Fig. 4 – Growth chamber



The cultures are fed with Hoagland solution at half strength for *L. minor* and one-tenth strength for *S. polyrhiza* and *L. minuta* (the concentrations of the medium were determined through experimental trials). Sub-samples of each duckweed species are transferred every 10 days to fresh, clean medium, to minimise contamination by other organisms, as indicated in the OECD 221 guidelines (OECD, 2006). Only uncontaminated fronds are used for further cultivation at each transfer. The Hoagland nutrient medium comprises of macronutrients (MgSO<sub>4</sub>·7H<sub>2</sub>O, KNO<sub>3</sub>, CaCl<sub>2</sub>·2H<sub>2</sub>O), micronutrients (Fe(EDTA)Na, H<sub>3</sub>BO<sub>3</sub>, ZnSO<sub>4</sub>·7H<sub>2</sub>O, MnCl<sub>2</sub>·4H<sub>2</sub>O, (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, CoCl<sub>2</sub>·6H<sub>2</sub>O, CuSO<sub>4</sub>·5H<sub>2</sub>O) and phosphate (KH<sub>2</sub>PO<sub>4</sub>) mixed in deionised water. These nutrients are prepared and added as six solutions (Table 1). The pH of the solution is adjusted to 6.5 (McLay, 1976; Ceschin et al., 2017).

			Full strength	Full strength	Half strength	One-fifth strength
<b>Solution</b>	<b>Nutrients</b>	<b>Concentration</b>	<b>ml in 1 L</b>	<b>ml in 10 L</b>	<b>ml in 1 L</b>	<b>ml in 1 L</b>
Sol. 1	KH <sub>2</sub> PO <sub>4</sub>	13,6 g/L	5,00	50,00	2,5	1,00
Sol. 2	MgSO <sub>4</sub> ·7H <sub>2</sub> O	49,3 g/L	10,00	100,00	5	2,00
Sol. 3	KNO <sub>3</sub>	60,66 g/L	30,00	300,00	15	6,00
Sol. 4	CaCl <sub>2</sub> ·2H <sub>2</sub> O	73,5 g/L	16,00	160,00	8	3,20
Sol. 5	Fe(EDTA)Na	7,5 g/250 ml	0,75	7,5	0,375	0,15
Sol. 6	H <sub>3</sub> BO <sub>3</sub>	715 mg/250 ml	1,00	10,00	0,5	0,20
	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	55 mg/250 ml				
	MnCl <sub>2</sub> ·4H <sub>2</sub> O	452 mg/250 ml				
	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> ·4H <sub>2</sub> O	5 mg/250 ml				
	CoCl <sub>2</sub> ·6H <sub>2</sub> O	25 mg/250 ml				
	CuSO <sub>4</sub> ·5H <sub>2</sub> O	20 mg/250 ml				

**Table 1** - Recipe for preparing Hoagland solution.

## Disinfection procedure

Plants selected for experimental testing must be cleaned and disinfected. This condition requires a two-step protocol involving a brief rinse in a 0,5% bleach solution (sodium hypochlorite, 7% of active Cl), followed by a thorough washing in distilled, sterilised water (Pietrini et al., 2015).

### First step - Disinfection step for culture stock maintenance

This step is applied to the entire plant colony and is only necessary if algae contamination is evident. All the passages described below are intended for an inoculum of 6 g for *S. polyrhiza* e *L. minor*, 4 g for *L. minuta*. Repeat each passage as many times as necessary.

Add 200 ml of deionised water to 2 Magenta boxes. Add 199 ml of deionised water to 1 Magenta boxes.



Sterilise in an autoclave:

- Tweezers
- Spatulas
- Forks
- Tips 1 ml
- Magenta boxes previously prepared

In a laminar flow hood:

1. add 1 ml of unscented bleach to the Magenta box containing 199 ml of water;
2. using a fork, submerge the plant inoculum in the 0,5% bleach solution for 1 minute, stirring gently with a spatula or the fork;
3. using a fork, transfer the plants to a Magenta box containing deionised, sterilised water and leave to soak for 2 minutes, stirring gently with a spatula or the fork;
4. using a fork, transfer the plants to a new Magenta box containing deionised, sterilised water and leave to soak for 2 minutes, stirring gently with a spatula or the fork.

Before transferring the cleaned colony, clean the recipients containing duckweed plant colonies with bleach and rinse thoroughly.

Keep the plants in Hoagland solution at the correct concentration for one week: one-fifth for *S. polyrrhiza* (rather than one-tenth of the normal amount used for culture maintenance), one-half for *L. minor* and one-tenth for *L. minuta*.



**Fig. 5** – Transferring plants



**Fig. 6** – Stirring gently

### Second step - Disinfection step for experimental trial

This step is only applied to a part of the plant colony, resulting from the selection of the healthiest and cleanest individuals. All the passages described below are intended for an inoculum of 6 g for *S. polyrhiza* e *L. minor*, 4 g for *L. minuta*. Repeat each passage as many times as necessary.

Prepare 500 ml of Hoagland solution, with the required concentration for the chosen duckweed plant for the trial (one-fifth for *S. polyrhiza*, one-half for *L. minor*, one-tenth for *L. minuta*), without adding iron (Fe(EDTA)Na), to prevent it from precipitating during the sterilisation process in the autoclave.

Add 200 ml of deionised water to 2 Magenta boxes. Add 199 ml of deionised water to 1 Magenta box.

Sterilise in an autoclave:

- Tweezers
- Spatulas
- Forks
- Tips 1 ml
- Tips 200 µl
- Magenta boxes previously prepared
- 2 empty Magenta boxes
- Hoagland solution without iron previously prepared

Place under a UV light in a laminar flow hood for 15-20 minutes:

- Magnetic stirrer
- Pipette (in the range for 200 µl tips)
- 1 Beaker 500 ml filled with deionised water
- Portable pH meter
- Deionised water for cleaning pH meter probe
- NaOH 1M solution
- Pasteur disposable pipette
- Some sheets of lab paper

Transfer the plant inoculum derived from the colony selection to the beaker containing deionised water that has been placed under UV light (Fig. 5).

In a laminar flow hood:

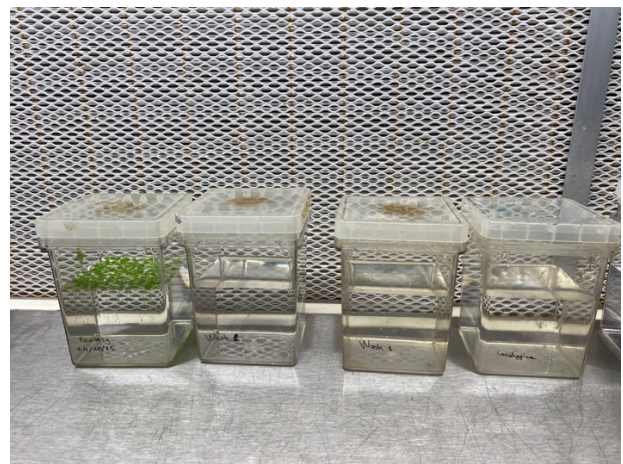
1) Prepare Hoagland solution:

- a) add the correct proportion of iron (Fe(EDTA)Na), previously filtered with a sterile, disposable syringe filter, pore size of 0.22 µm, to the sterilised Hoagland solution, taking into account the concentration and the final volume;
- b) adjust the pH to 6.5 by adding 1M NaOH using sterile tips;

- c) add 200 ml of the prepared Hoagland solution to the empty, sterilised Magenta boxes;
- 2) add 1 ml of unscented bleach to the Magenta box containing 199 ml of water;
- 3) using a fork, submerge the plant inoculum in the 0,5% bleach solution for 1 minute, stirring gently with a spatula or the fork;
- 4) using a fork, transfer the plants to a Magenta box containing deionised, sterilised water and leave to soak for 2 minutes, stirring gently with a spatula or the fork;
- 5) using a fork, transfer the plants to a new Magenta box containing deionised, sterilised water and leave to soak for 2 minutes, stirring gently with a spatula or the fork;
- 6) transfer plants to the Magenta boxes filled with the prepared, sterilised Hoagland solution;
- 7) close the Magenta boxes with parafilm.



**Fig. 7** – Materials ready for disinfection



**Fig. 8** – Magenta boxes set (from left to right: final recovery, two deionised waters, one bleach solution)

Keep the duckweed plants in Magenta boxes and ensure there is a slight, constant movement of 50 rpm under controlled conditions (constant temperature of  $25 \pm 3$  °C, photoperiod of 16h light/8h dark), for 3 days before starting the experimental trial.

### Final remarks/recommendations

During the procedure the plants experience both chemical and physical stress. The chemical stress is caused by rinsing the plants in bleach solution, while the physical stress depends on how vigorously the plants are shaken inside the Magenta boxes during this process. It is relevant that the plants are in good physiological condition, as well as clean, at the start of the experimental trial.

Some tips for reducing stress:

- pay attention when rinsing in the bleach solution: the total time should be no more than 1 minute;
- handle the plants with great care: this is especially important during rinsing;



- respect the lapse time between the two steps (1 week) and between the second step and the start of the trial (3 days): this time is important for allowing the plants to recover from stress.

Following this two-steps protocol is possible to obtain duckweed plant cultures nearly free of parasites or pathogens in lab conditions, in order to conduct ecotoxicological assays or phytoremediation studies.

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## References

Baudo, R., Foudoulakis, M., Arapis, G., Perdaen, K., Lanneau, W., Paxinou, A.-C.M., Kouvdou, S., Persoone, G., 2015. History and sensitivity comparison of the *Spirodela polyrrhiza* microbiotest and *Lemna* toxicity tests. Knowledge and Management of Aquatic Ecosystems, 416: 23. doi: <https://doi.org/10.1051/kmae/2015019>

Böcük, H., Yakar, A., Türker, O.C., 2013. Assessment of *Lemna gibba* L. (duckweed) as a potential ecological indicator for contaminated aquatic ecosystem by boron mine effluent. Ecological Indicators, 29: 538-548, doi: <https://doi.org/10.1016/j.ecolind.2013.01.029>

Ceschin, S., Abati, S., Leacche, I., Zuccarello, V., 2017. Ecological comparison between duckweeds in central Italy: the invasive *Lemna minuta* vs the native *L. minor*. Plant Biosystems – An International Journal Dealing with all Aspects of Plant Biology, doi: <https://doi.org/10.1080/11263504.2017.1317671>

Ceschin, S., Sgambato, V., Ellwood, N.T.W., Zuccarello, V., 2019. Phytoremediation performance of *Lemna* communities in a constructed wetland system for wastewater treatment. Environmental and Experimental Botany, 162: 67–71, doi: <https://doi.org/10.1016/j.envexpbot.2019.02.007>

Ceschin, S., Bellini, A., Scalici, M., 2020. Aquatic plants and ecotoxicological assessment in freshwater ecosystems: a review. Environmental Science and Pollution Research, 28: 4975-4988, doi: <https://doi.org/10.1007/s11356-020-11496-3>

Firmin, A., Dunand, C., Elger, A., 2025. Comparative study of axenisation protocols for aquatic plants. *Plant Cell, Tissue and Organ Culture.*, 162: 23, doi: <https://doi.org/10.1007/s11240-025-03092-5>

Forni, C., Tommasi, F., 2016. Duckweed: a tool for ecotoxicology and a candidate for phytoremediation. *Current Biotechnology*, 5 (1): 2–10, doi: <https://doi.org/10.2174/2211550104666150819190629>

Greenberg, B.M., Huang, X.D., Dixon, D.G., 1992. Applications of the aquatic higher plant *Lemna gibba* for ecotoxicological assessment. *Journal of Aquatic Ecosystem Health*, 1: 147-155, doi: <https://doi.org/10.1007/BF00044046>

Gubbins, E.J., Batty, L.C., Lead, J.R., 2011. Phytotoxicity of silver nanoparticles to *Lemna minor* L. *Environmental Pollution*, 159 (6): 1551–1559, doi: <https://doi.org/10.1016/j.envpol.2011.03.002>

Hegazy, A.K., Kabil, H.F., Fawzy, M., 2009. Duckweed as heavy metal accumulator and pollution indicator in industrial wastewater ponds. *Desalination and Water Treatment*, 12 (1–3): 400–406, doi: <https://doi.org/10.5004/dwt.2009.956>

Iannilli, V., Passatore, L., Carloni, S., Massimi, I., Giusto, C., Zacchini, M., Pietrini, F., 2025. Bismuth accumulation and toxicity in freshwater biota: a study on the bioindicator species *Lemna minor* and *Echinogammarus veneris*. *Science of the Total Environment*, 975: 179263, doi: <https://doi.org/10.1016/j.scitotenv.2025.179263>

Khellaf, N., Zerdaoui, M., 2010. Growth, photosynthesis and respiratory response to copper in *Lemna minor*: a potential use of duckweed in biomonitoring. *Iran Journal of Environmental Health Science & Engineering*, 7: 299–306.

Liu Y, Wang Y, Xu S et al. (2019) Efficient genetic transformation and CRISPR/Cas9-mediated genome editing in *Lemna aequinoctialis*. *Plant Biotechnology Journal*, 17: 2143–2152, doi: <https://doi.org/10.1111/pbi.13128>

Matamoros, V., Nguyen, L.X., Arias, C.A., Salvadó, V., Brix, H., 2012. Evaluation of aquatic plants for removing polar microcontaminants: a microcosm experiment. *Chemosphere*, 88 (10): 1257–1264, doi: <https://doi.org/10.1016/j.chemosphere.2012.04.004>

McLay, C.L., 1976. The effect of pH on the population growth of three species of duckweed: *Spirodela oligorrhiza*, *Lemna minor*, *Wolffia arhizza*. *Freshwater Biology*, 6: 125-136, doi: <https://doi.org/10.1111/j.1365-2427.1976.tb01596.x>

Mkandawire, M., Teixeira Da Silva, J.A., Dudel, E.G., 2014. The *Lemna* bioassay: contemporary issues as the most standardized plant bioassay for aquatic ecotoxicology. Critical Reviews in Environmental Science Technology. 44: 154–197, doi: <https://doi.org/10.1080/10643389.2012.710451>

OECD, 2006. OECD Guidelines for the Testing of Chemicals, Section 2. Test No. 221: *Lemna* sp. Growth Inhibition Test, Paris, France.

Pietrini, F., Di Baccio, D., Aceña, J., Pérez, S., Barceló, D., Zacchini, M., 2015. Ibuprofen exposure in *Lemna gibba* L.: evaluation of growth and phytotoxic indicators, detection of ibuprofen and identification of its metabolites in plant and in the medium. Journal of Hazardous Materials, 300: 189–193, doi: <https://doi.org/10.1016/j.jhazmat.2015.06.068>

Pietrini, F., Bianconi, D., Massacci, A., Iannelli, M.A., 2016. Combined effects of elevated CO<sub>2</sub> and Cd-contaminated water on growth, photosynthetic response, Cd accumulation and thiolic components status in *Lemna minor* L. Journal of Hazardous Materials, 309: 77–86, doi: <https://doi.org/10.1016/j.jhazmat.2016.01.079>

Pietrini, F., Passatore, L., Fischetti, E., Carloni, S., Ferrario, C., Polesello, S., Zacchini, M., 2019. Evaluation of morpho-physiological traits and contaminant accumulation ability in *Lemna minor* L. treated with increasing perfluorooctanoic acid (PFOA) concentrations under laboratory conditions. Science of the Total Environment, 695: 133828, doi: <https://doi.org/10.1016/j.scitotenv.2019.133828>

Pietrini, F., Iannilli, V., Passatore, L., Carloni, S., Sciacca, G., Cerasa, M., Zacchini, M., 2022. Ecotoxicological and genotoxic effects of dimethyl phthalate (DMP) on *Lemna minor* L. and *Spirodela polyrhiza* (L.) Schleid. plants under a short-term laboratory assay. Science of the Total Environment, 806 (4): 150972, doi: <https://doi.org/10.1016/j.scitotenv.2021.150972>

Sree, K. S., Sudakaran, S., and Appenroth, K. J., 2015. How fast can angiosperms grow? Species and clonal diversity of growth rates in the genus *Wolffia* (Lemnaceae). Acta Physiologiae Plantarum, 37:204. doi: <https://doi.org/10.1007/s11738-015-1951-3>

The Angiosperm Phylogeny Group, M. W. Chase, M. J. M. Christenhusz, M. F. Fay, J. W. Byng, W. S. Judd, D. E. Soltis, D. J. Mabberley, A. N. Sennikov, P. S. Soltis, P. F. Stevens, 2016. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV. Botanical Journal of the Linnean Society, 181 (1): 1–20, doi: <https://doi.org/10.1111/boj.12385>

Tippery, N.P., Les, D.H., Appenroth, K.J., Sree, K.S., Crawford, D.J., Bog, M., 2021. Lemnaceae and Orontiaceae are phylogenetically and morphologically distinct from Araceae. Plants, 10, 2639, doi: <https://doi.org/10.3390/plants10122639>



Ziegler, P., Adelman, K., Zimmer, S., Schmidt, C., Appenroth, K.J., 2015. Relative in vitro growth rates of duckweeds (Lemnaceae) - the most rapidly growing higher plants. *Plant Biology (Stuttg)*, 17 (1): 33-41, doi: <https://doi.org/10.1111/plb.12184>



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