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Species interactions in three Lemnaceae species growing along a gradient of zinc pollution

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Abstract

Duckweeds (Lemnaceae) are increasingly studied for their potential for phytoremediation of heavy-metal polluted water bodies. A prerequisite for metal removal, however, is the tolerance of the organism to the pollutant, e.g., the metal zinc (Zn). Duckweeds have been shown to differ in their tolerances to Zn, however, despite them most commonly co-occurring with other species, there is a lack of research concerning the effect of species interactions on Zn tolerance. Here we tested whether the presence of a second species influenced the growth rate of the three duckweed species Lemna minor, Lemna gibba, and Lemna turionifera. We used four different Zn concentrations in a replicated microcosm experiment under sterile conditions, either growing the species in isolation or in a 2-species mixture. The response to Zn differed between species, but all three species showed a high tolerance to Zn, with low levels of Zn even increasing the growth rates. The growth rates of the individual species were influenced by the identity of the competing species, but this was independent of the Zn concentration. Our results suggest that species interactions should be considered in future research with duckweeds and that several duckweed species have high tolerance to metal pollution, making them candidates for phytoremediation efforts.

Introduction

Duckweeds are small, floating aquatic plants with a simplistic morphology. Individual duckweeds consist of single fronds with zero to multiple roots attached to the bottom surface. Duckweeds can flower; however, they rarely do so (Hicks, 1932). Their main reproduction strategy consists of asexual budding, where several daughter fronds bud and then detach from the mother frond (Laird & Barks, 2018). An individual frond can produce up to a couple dozen of daughter fronds over its life, which is only a few weeks short. Duckweeds are found around the world in slow flowing freshwater systems, when suitable anchoring possibilities are present (Landolt, 1986). Their fast reproduction cycle and their short life span make duckweeds useful model organisms for research in ecology and evolution (Laird & Barks, 2018). Duckweeds can tolerate high levels of nitrogen, phosphorus and heavy metals and different species can have different responses to temperature, light, nutrients and toxicants (Landolt, 1996). In nature, duckweed species frequently co-exist (Landolt, 1986).

Zn is an essential trace element for plant growth, but elevated concentrations inhibit growth and can lead to chlorosis. Therefore, elevated Zn levels are phytotoxic (Rout & Das, 2009). Zn is a commonly used building material and through run-off from roofs, galvanized items, and pipes it finds its way into (underground) waters, leading to Zn pollution (AWEL, 2006). In Switzerland, between 2006 and 2014, Zn concentration measurements exceeded the indicator value of 5 µg/l at 15 measuring stations, more than any other trace element measured (Bundesamt für Umwelt BAFU, 2019).

In the face of pollution of water systems, *Lemnaceae* are studied as potential organisms for phytoremediation (Liu et al., 2021). For example, one species of duckweed, *Lemna minor* L. (common duckweed), has shown to be a good accumulator of heavy metals such as Cadmium, Selenium and Copper (Zayed et al., 1998).

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Several studies have shown metal accumulation in different duckweed species (Lahive, O'Callaghan, et al., 2011), which depended both on the species (Cardwell et al., 2002) and on the metal (Gaur et al., 1994). A prerequisite for metal accumulation, however, is the tolerance of a species to elevated levels of heavy metals. Duckweed species differ in their tolerance to Zn: *L. minor* was shown to tolerate Zn concentrations above 100 mg/L but the gibbous duckweed *Lemna gibba* only tolerated concentrations up to 10 mg/L (Lahive, O' Halloran, et al., 2011). Zn tolerance of other duckweed species such as *Lemna turionifera*(red duckweed) was, to our knowledge, never investigated.

Additionally, there is a lack of research concerning the influence of species interaction on duckweed resistance to metal pollution. Previous studies suggest that species interactions in duckweeds can influence growth rates (Clatworthy & Harper, 1962; Gopal & Goel, 1993; Peeters et al., 2016). Here, we hypothesized that the presence of a second species in a mixed setting could increase heavy metal tolerance because of facilitation. The stress-gradient hypothesis predicts that interactions among plants are context dependent, shifting from competition to facilitation as environmental stress increases (Callaway, 1995). At high Zn concentrations, if the more tolerant duckweed species accumulates Zn present in the medium, this could facilitate the persistence or even growth of the co-occurring, less heavy-metal tolerant species. However, at low levels of Zn concentration, competition for nutrients could override any facilitative mechanisms, leading to a negative effect of the presence of a second species.

To test our hypothesis, we grew three *Lemnaceae* species in isolation and in two-species pairings along a zinc sulfate ($ZnSO_4$) concentration gradient (0, 0.45, 1.82, 11.35 mg/L Zn). We measured the Zn tolerance of three duckweeds species*L. minor, Lemna gibba,* and *L. turionifera* over 17 days in replicated microcosms under sterile and controlled conditions.

Materials and methods

Duckweed cultures

Axenic cultures of the three duckweed species were maintained at the Department of Evolutionary Biology and Environmental Studies, University of Zürich. Three strains were delivered in July from the Landolt Duckweed Collection (www.duckweed.ch). *Lemna turionifera* , strain 9478, sourced from Racibórz, Silesian, Poland, *Lemna minor* , strain 9978, sourced from Oberegg, Appenzell Innerrhoden, Switzerland, and *Lemna gibba* , strain 9965, sourced from Schloss Wartensee, Rorschacherberg, St. Gallen, Switzerland. The axenic cultures were held on Hoagland's E Medium (see Appendix for recipe used) in incubators at 18 °C with a light regime of 14/10h light/dark.

Experimental set up

The three species float occupy a similar ecological niche. They float on the water surface and are very similar in terms of their morphology. In particular, non-gibbous *L. gibba* and *L. minor*fronds cannot be distinguished by eye but the differentiation between*L. minor* and *L. turionifera* can be equally challenging (Senevirathna et al., 2021). Therefore, we separated the species using a floating ring (Figure S4). Thus, we could overcome previous limitations (Clatworthy & Harper, 1962) and investigate the competition between these closely related species.

For each treatment, 18 250ml bottles were filled with 100ml of Hoagland's E Medium. To create the Zn pollution gradient, $ZnSO_4$ * 7H2O (Alfa Aesar, Thermofisher, Germany) was added (2 mg/L, 8 mg/L, 50 mg/L). Elemental Zn accounts for 65.38 g/mol (22.7 %) of the $ZnSO_4$ * $7H_2O$ compound, the final concentration of the metal Zn was consequently 0.45 mg/L Zn, 1.82 mg/L Zn and 11.35 mg/L Zn. The medium was autoclaved before the experiment.

We grew every treatment in three replicates. In total, we had 72 bottles and 108 individual populations (36 growing in the isolated setting and 72 growing in the mixed setting). At the beginning of the experiment, we added 5-7 fronds to the 250ml bottles according to the study design (Figure 1). In the isolated setting, the 5-7 fronds were added to the inner area (inside the plastic ring). No fronds were added to the outer area. In the mixed setting, 5-7 fronds from one species were added to the inner area and 5-7 fronds from a second

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species were added to the outer area. The inside/outside position of the two respective species were kept for the replica but changed depending on the concentration, so that the position was the same for 0 mg/L Zn and 1.82 mg/L Zn, and for 0.45 mg/L Zn and 11.35 mg/L Zn. There was no significant effect of position (inside vs. outside area, Figure S1). The bottles were kept in an incubator (Figure S4) at 20 °C with a light regime of 16/8h light/dark for 17 days. During the experiment, the species competed for the same nutrients in an uncrowded culture, but there was no competition for light.

The number of fronds were counted 8 times over the course of 17 days (Figure S2). Completely white (dead) fronds were not included in the total number of fronds. During the experiment, the species got mixed in a small number of bottles. Due to their morphological similarity, the species could not be reliably distinguished any more and consequently any data from these bottles post the mixing event were excluded.

Data analysis

Four populations were excluded from all analyses because they declined rapidly and went extinct despite growing in the medium without the addition of $ZnSO_4$. The excluded populations had high leverage on the results but were qualitatively very different from all other populations. The four populations were each a replicate of the pairing LgLm at 0 mg/L Zn (both species), of *L. turionifera* in the pairing LtLm at 0 mg/L Zn, and of *L. minor* in the isolated setting at 0 mg /L Zn. Subsequently, population growth rates for each individual population (n=104) were calculated as $\ln(N_2/N_1)/(t_1\text{-}t_2)$. We used the initial population abundance at the start of the experiment (t_1, 20.07.2021) and the final time point after 17 days (t_2, 6.8.2021) to calculate total growth rates. Because most other studies examining the influence of Zn on duckweed growth were conducted over less than 10 days, we additionally calculated initial growth rate comparing the first day of the experiment (t_1) with day 8 (t_2, 28.7.2021). Initial growth rates represent growth rates for when we can be confident that nutrients were not limiting. In the mixed setting, we calculated growth rates for each species separately. Total and initial growth rate were then used as response variable in the linear models. The treatment variables were species identity (*L. gibba* (Lg), *L. minor*(Lm) or *L. turionifera* (Lt)), Zn concentration (0 mg/L, 0.45 mg/L, 1.82 mg/L, and 11.35 mg/L Zn), the setting (isolated vs. mixed) and composition (isolated, pairing with species 1, pairing with species 2). lmerTest (Bates et al., 2015; Kuznetsova et al., 2017) was used for the mixed models. Composition and position were included as random factors where appropriate. All analyses were done in R (R version 4.1.0, R Development Core Team, 2021).

Results

Over all concentrations and compositions, we found that *L. turionifera* had the highest total growth rate, followed by *L. minor* , and *L. gibba* (Figure S3). However, initial growth rate was greatest for *L. minor* (Figure S3). All three species showed an overall high tolerance to Zn. *L. turionifera* was not influenced by Zn pollution. In contrast, addition of $ZnSO_4$ to the experimental cultures significantly influenced *L. gibba* and *L. minor* but only at the beginning of the experiment (Table 1, Figure 2). *L. gibba* profited from intermediate levels of $ZnSO_4$ (Figure 2). Similarly,*L. minor* grew best at the second-highest level of Zn concentration (1.82 mg /L), but in contrast to *L. gibba* , the second-lowest level (0.45 mg / L) did not increase growth (Figure 2).

The setting (isolated vs. mixed) only significantly influenced *L. gibba* growth rates (Table 1), specifically, *L. gibba* profited from growing alone and showed significantly lower growth rates (initial and total) when paired with either *L. minor* or *L. turionifera* (also mirrored in significant effects of composition, Table 1). For *L. minor* it depended on the pairing (significant effects of composition on the individual species' growth rate, Table 1, Figure 4). *L. minor* performed better when paired with *L. turionifera* and worse when paired with *L. gibba* . *L. turionifera* showed no difference in growth rate when paired with either*L. gibba* or *L. minor* (Table 1, Figure 4).

Discussion

Zn tolerance was high in all three studied species

Contrary to expectations, growth rates were not highest in the treatments without the addition of Zn.

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Low levels of Zn even increased growth rates for two of the species studied (*L. gibba* and*L. minor*). Zn is an essential plant nutrient, and Zn deficiency has been shown to reduced fresh weight production in *L. gibba*(Vaughan et al., 1982). Zn is present in the Hoagland's E Medium; nevertheless, it is possible that the *Lemna* species are Zn-limited in this medium and, therefore, show a lower growth rate as a sign of Zn deficiency. All three *Lemnaceae* species showed a very high tolerance to Zn. Only *L. minor* exhibited reduced growth rates in the highly polluted environment (11.35 mg/l). This is in line with previous results showing that Zn at lower concentrations promotes the growth of duckweeds, but inhibits duckweed growth at higher concentrations (Jayasri & Suthindhiran, 2017).

The high Zn tolerance for *L. gibba* contrasts previous work that found that concentrations of 4 mg/L of Zn inhibited growth by 50% and 10 mg/L of Zn reduced specific biomass growth by 90% (Lahive, O' Halloran, et al., 2011).

For *L. minor,* the results partially confirm previous work. Jayasri & Suthindhiran (2017) showed that *L. minor* increased biomass yield in fronds treated with a lower concentration (0.5 mg/l) of Zn by 30% compared to the control. However, they concluded that 10 mg/l had little effect on growth after 4 days. This contrasts our results that found that 11.35 mg / L reduced growth rates of *L. minor*(significantly so in comparison with 1.82 mg / L). Lahive *et al.*(2011) reported that *L. minor* tolerated Zn concentration above 100 mg/L, but specific biomass growth rate was reduced significantly at low concentrations of 3 mg/L (reduction by 20%).

The duration of our experiment exceeded that of previous experiments, which were conducted over four to seven days (e.g., Jayasri & Suthindhiran, 2017; Lahive, O' Halloran, et al., 2011; Megateli et al., 2009). We speculated that these previous studies may have underestimated the toxicity of Zn (and overestimated the duckweed Zn tolerance) because the plants may become more sensitive over time, as their tissue accumulates more Zn. However, the overall high tolerance to Zn we found after 17 days suggests that toxicity does not increase over time. The high tolerance of duckweed to polluted environments might be an explanation as to why duckweeds are common all around the world (Landolt, 1986).

Mixing two species can have contrasting effects on the focal species

Based on the stress-gradient hypothesis (SGH, e.g. Callaway et al., 2002), we hypothesized that in a more stressful environment we would observe more facilitative interactions while in the medium without Zn pollution we would observe more competitive interactions. We did not find evidence for this hypothesis, since the levels of Zn pollution we used in our experiment were not high enough to impact duckweed growth rates. Instead, we found that species interactions were highly dependent on focal species identity and competitor identity. For example, the effect of the setting on growth rate was only consistent for one of the three species, *L. gibba* . However, contrary to expectations, it did not profit from growing in the presence of a second species that could have helped it to accumulate Zn. Instead, *L. gibba* grew the best alone. Previous research with *L. gibba* competing against a different duckweed species, the greater duckweed *Spirodela polyrhiza* , showed that *L. gibba* was highly competitive due to its gibbous fronds which could overgrow *S. polyrhiza* (Clatworthy & Harper, 1962). However, in our experiment there was no physical contact between the species and *L. gibba* only produced non-gibbous fronds. The outcome of species pairings could, therefore, be dependent on frond morphology, which in turn is dependent on the environmental conditions. Gibbosity in *L. gibba* depends on the environmental conditions, and may be restricted to optimal growth conditions, including very high nutrient availability (Vaughan & Baker, 1994). Under our conditions in the experiment, *L. gibba* may have had a disadvantage due to the lack of gibbous fronds and the inability to overgrow the competitor.

For the other two species, growth performance depended on the pairing, but there was no evidence of facilitation. Instead, we observed strong competition between *L. minor* and *L. turionifera* , with *L. minor* outcompeting *L. turionifera* . To the best of our knowledge, this is the first study investigating *L. turionifera* growth rates in the presence or absence of a competitor. Recently, it has been found that some populations previously thought to be *L. minor* were in fact *L. turionifera* (Senevirathna et al., 2021). It is thus possible that *L. turionifera* has a wider geographic distribution than previously thought and is, therefore, a good

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candidate for phytoremediation in many regions of the world.

For *L. minor,* we found that, even though it profited from growing with *L. turionifera* , the presence of *L. gibba* had a negative impact on its growth. Simultaneously, *L. gibba* was negatively influenced by the presence of *L. minor* . Thus, this pairing had a negative effect on both interacting species, which is surprising given that they frequently co-exist in nature. Due to their morphological similarity, *L. gibba* and *L. minor* have rarely been used in competition experiments. In instances where they did compete in experiments the analysis was not completed (Clatworthy & Harper, 1962; Wołek, 1972), or the two species were even grouped together (Peeters et al., 2016). As an exception, Rejmánková (1975) found that in gibbous form, *L. gibba* was always the stronger competitor, overgrowing *L. minor* . Thus, *L. gibba* may have had the disadvantage of non-gibbous fronds in our experiment.

Duckweeds have great potential for heavy metal removal in wastewaters (Abdel-Gawad et al., 2020) but many questions remain, in particular in terms of how polycultures of multiple species may improve efficiency. Our study is a first assessment of the interaction between competition and tolerance to Zn pollution for a subset of *Lemnaceae* species. A next step could be to test the accumulation of Zn not only for species in an isolated setting across different metal concentrations (Lahive, O'Callaghan, et al., 2011) but also in a mixed setting and, importantly, in a natural environment. In addition, due to their fast growth, it is possible that duckweed species will evolve in response to their competitor (Hart et al., 2019). For future phytoremediation efforts, species mixing could be interesting, but the effects of a second species need to be evaluated first as they cannot be readily predicted from the performance in individual cultures.

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Data accessibility

Data and code will be made publicly available upon acceptance of the manuscript.

Conflict of interest

None declared.

Author contribution

L.L. designed and carried out the experiment, supported data analysis and wrote the first draft. S.J.V.M supported experimental design, analyzed the data and wrote the manuscript. Both authors contributed to the final version of the paper.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Recipe for Hoagland's E Medium.

Table S2. ANOVA results with interactions.

Figure S1. Influence of position over time.

Figure S2. Growth of all duckweed populations.

Figure S3. Total and initial growth rate per species across all compositions and concentrations.

Figure S4. Photos of the experimental microcosms.

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Table 1. Summary of the Type 3 ANOVA's showing the influence of the setting (isolated vs. mixed), the Zn concentration (factorial) and the composition (isolated, pairing with species 1, pairing with species 2) on the three study species. Significant (<0.05) and near-significant (<0.06) p-values are in bold. The linear mixed models for setting and composition included position (outer vs. inner) as random factor, the linear mixed model for concentration included composition as random factor. Within species, interaction terms were never significant and thus not shown here (but see Table S2).

Species	Total growth rate	Total growth rate	Initial growth rate	Initial growth rate
Zn concentration	*F*	*P*	*F*	*P*
Lemna gibba	1.700	0.190	3.227	**0.037**
Lemna minor	2.742	0.063	3.660	**0.024**
Lemna turionifera	0.073	0.974	1.454	0.246
Setting (isolated vs. mixed)	*F*	*P*	*F*	*P*
Lemna gibba	5.096	**0.040**	11.394	**0.002**

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Species	Total growth rate	Total growth rate	Initial growth rate	Initial growth rate
Lemna minor	0.017	0.898	0.377	0.544
Lemna turionifera	0.065	0.800	0.017	0.897
Composition	*F*	*P*	*F*	*P*
Lemna gibba	3.291	**0.057**	6.842	**0.003**
Lemna minor	34.052	**< 0.001**	35.594	**< 0.001**
Lemna turionifera	0.371	0.693	0.334	0.719

Table 2: Overview facilitative (+) and inhibiting (–) effects between the species in the experiment (effect of species on the left on the species on the right). Significant effects are labelled with a**.** (P < 0.1), * (P < 0.05), ** (P< 0.01), *** (P < 0.001). See also Figure 3.

Total growth rates	on *L. gibba*	on *L. minor*	on *L. turionifera*
Effect of *L. gibba*		– *	no effect
Effect of *L. minor*	– *		– ns
Effect of *L. turionifera*	– ns	+ **	
Initial growth rates			
Effect of *L. gibba*		– **.**	no effect
Effect of *L. minor*	– ***		– ns
Effect of *L. turionifera*	– *	+ ***	

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Figure 1. Experimental design with the three different species used. Left: schematic of species growing in the isolated setting (12 treatments replicated three times for a total of 36 bottles). Right: Schematic of species growing in the presence of a competitor (mixed setting). Every treatment x species combination was replicated three times for a total of 36 experimental units. The whole experiment had consequently 72 bottles.

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Figure 2. Growth of the three duckweed species in the four zinc (Zn) treatments over 17 days. Shown are means and standard errors for each sampling date, across settings and compositions. Orange lines, 0 mg/ L Zn, blue lines, 0.45 mg/L Zn, green lines, 1.82 mg / L Zn, yellow lines, 11.35 mg/L Zn.

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Figure 3. Growth rates across the entire time series (total growth rate, **A**) and growth rates during the first 8 days of the experiment (initial growth rates, **B**) of three species separately and in the two settings across the Zn gradient. Orange: isolated setting, blue: mixed setting. Shown are means and associated standard errors across replicates and, in the mixed setting, across the two pairings.

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Figure 4. (**A**) Growth rates across the entire time series and (**B**) for the first 8 days of the experiment (initial growth rates) of the three species for each composition separately. Means and associated standard errors across all Zn treatments (including no Zn) are presented. For initial growth rate, the interaction between composition and concentration was not significant, but for total growth rate it was (Table S2). Dashed lines are drawn to visualize the difference between the isolated setting and the two different pairings.