High tolerance to zinc but no evidence for local adaptation in the aquatic plant *Lemna minor*

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Abstract

Duckweeds are a widely distributed and economically important aquatic plant family that have high potential for phytoremediation of polluted water bodies. We collected four ecotypes of the common duckweed (*Lemna minor*) from the four corners of Switzerland and assessed how their home vs. away environments influenced their growth. Additionally, we investigated their response to a metal pollutant (Zn) in both their home and away environments. Zn is found in freshwater systems and can become harmful at elevated concentrations. We hypothesized that growing in their home environment would help the plants buffer the negative effect of the metal pollutant. To test this, we measured *Lemna* growth in a common garden experiment in a glasshouse where the four ecotypes were grown in each of the environments, as well as in three different concentrations of Zn. To investigate whether facilitative or competitive interactions between *Lemna* and their microbial community can enhance or reduce tolerance to heavy metal pollution, we sampled chlorophyll-a as a proxy for algal biomass and measured total nitrogen and total organic carbon.

The four *Lemna* ecotypes exhibited significantly different growth rates across environments. This difference in fitness was matched with DNA sequencing revealing genetic differentiation between the four ecotypes. However, the effect of the environment on *Lemna* growth was the same for all ecotypes. We did not find evidence for local adaptation; instead, we observed strong plastic responses. *Lemna* growth rates were higher under higher Zn concentrations. This positive effect of Zn on *Lemna* growth could be in part due to reduced competition with algae. We conclude that *L. minor* ecotypes may exhibit large differences in growth rate, but that the species overall have a high Zn tolerance and strong plastic adaptive potential in novel environments.

Keywords: aquatic plant ecology, duckweed, heavy metal pollutant, home vs. away, plant-algae interactions

Introduction

Understanding how species evolve to adapt to specific environmental conditions allows us to better predict how environmental change may affect populations and communities, and thus find ways to prevent or mitigate its consequences more effectively. Within species, different populations experience different selective pressures and may thus adapt to become better suited to their own local environmental conditions (Joshi et al., 2001). When such local phenotypes demonstrate higher fitness in their local environment compared to members of populations at foreign locations and vice-versa, the population is locally adapted (Kawecki & Ebert, 2004). Populations within a species that are adapted to local environmental conditions are also referred to as ecotypes (Hufford & Mazer, 2003).

Measuring local adaptation allows for the mechanisms of natural selection to be assessed (Ruiz Daniels et al., 2019), furthers our understandings of the interactions of natural selection and gene flow and is also vital for decision-making for land managers attempting ecosystem restoration (Gibson et al., 2016). Many environmental components can select for local adaptation (Leimu & Fischer, 2008), including heavy metals (Eränen, 2006), and

interactions among species (Hoeksema & Forde, 2008) such as plant-herbivore (Hargreaves et al., 2019) and host-parasite interactions (Kaltz & Shykoff, 1998). To test for local adaptation, the fitness of a population in both its home and away environments must be measured. Two criteria are used to predict whether populations are locally adapted to an environment. Within-population comparisons require that members of a population will express higher relative fitness in their original habitat compared to members of the same population transplanted in other habitats (Blanquart et al., 2013). Between-population comparisons require that members of a native population will express higher fitness in their original habitat relative to individuals of foreign populations of the same species in that same environment (Kawecki & Ebert, 2004).

The fast-growing aquatic plant species *Lemna minor* belongs to the family Lemnaceae (duckweeds). They have a simple structure composed of a frond (its vegetative body) and a very thin thallus-like structure (Landolt, 1986; Ziegler et al., 2016). They are known as one of the smallest flowering plants in the world, but mainly reproduce asexually by budding (Landolt, 1975; O'Brien, Yu, et al., 2020). Globally distributed, *Lemna minor* grows in ponds or bodies of very slow-moving water, where they coat the surface and reach high population densities. They can take up and accumulate trace metals in their roots and fronds (Fritioff & Greger, 2006; Newman, 1991; Subramanian & Turcotte, 2020). The *Lemna* genus can tolerate a wide range of conditions, which makes it an important agent for the bioremediation of different aquatic bodies (Dirilgen, 2011; Landolt, 1996). They can remove excess macronutrients and many different substances from organic chemicals to heavy metals (Khellaf & Zerdaoui, 2009), including zinc (Lahive, O'Callaghan, et al., 2011).

Zn pollution is common in urbanized areas, including Switzerland (AWEL, 2006), and elevated Zn concentrations inhibit plant growth (Lahive, O' Halloran, et al., 2011). Heavy metal pollution is of particular relevance in freshwater ecosystems due to their long-term effects on the ecosystem integrity (Duruibe et al., 2007; Sasmaz et al., 2015). Phenotypic plasticity can arise as a fast response to lower levels of pollution, but in consistently highly toxic environments it may lead to maladaptation (Gienapp et al., 2008; Loria et al., 2019). However, organisms with short generation times, such as duckweed, have a higher probability to adapt to a fast-changing polluted environment (Vander Wal et al., 2013).

Our study aimed at testing three hypotheses: 1) *Lemna minor* ecotypes are locally adapted to their biotic and abiotic environment. According to the theory of local adaptation, we expected growth rates of *Lemna* ecotypes to be highest in their original environments and to be lower in all the other environments. 2) Their response to a heavy metal pollutant (Zn) would be influenced by the home vs. away environment. We anticipated *L. minor* to be negatively affected by an increase in Zn but that ecotypes will be more resistant to Zn when grown in their original environment due to local adaptation. 3) An interaction between *Lemna* species and local microbiome would enhance tolerance to Zn pollution (O'Brien, Laurich, et al., 2020), due to a history of co-selection with the algae (van Moorsel et al., 2020).

For 1), we collected four *Lemna* ecotypes along with water samples from four distant locations separated by several hundred kilometers across Switzerland. For 2), we grew the

Lemna ecotypes both in their home and away water and applied three Zn treatments. For 3) we measured how the addition of the metal pollutant influenced algal biomass, and whether *Lemna* fitness was related to algal biomass. To confirm species classification and study population structure, we did whole-genome sequencing and applied KmerGWAS, a novel approach that uses k-mers, i.e., short sequences derived directly from raw sequencing data (Voichek & Weigel, 2020). This approach does not require a reference genome and has been shown to have stronger statistical support.

Materials and methods

Study sites and sample collections

We collected *Lemna* ecotypes from four geographic region of Switzerland (Fig. 1). Ecotypes were sampled from Yverdon and Ramosch (Eastern vs. Western Switzerland), and Koblenz and Motto (Northern vs. Southern Switzerland). The distances between the ecotypes maximized the likelihood that there was no recent mixing of genotypes. The four water bodies represented also different altitudes, pond sizes, and shading conditions (Table S1). In August 2021, at each site (Koblenz: 47°36'03.3" N, 8°13'32.0" E, Yverdon: 46°47'50.8"N, 6°37'59.6"E, Motto: 46°25'43.6" N, 8°58'03.4" E, Ramosch: 46°50'01.4"N, 10°24'04.0"E, Fig. S1), we measured conductivity (WTW LF 325 conductivity meter), pH (WTW Multi 340i), water temperature, and dissolved oxygen (HQ40D Portable Multi Meter from Hach). At the same time, we collected thousands of individuals of the respective local *Lemna minor* population and 10 L of pond water. Subsequently, the water and plants were transported to the glasshouse at the University of Zurich in Zurich, Switzerland where the source water was first sieved to remove larger pieces of leaves, bark, and other aquatic organisms. A 50 mL sample of water from each location was frozen at -20° C for later analyses "pre-experiment" (natural source water) of their inorganic carbon, organic carbon, and Nitrogen concentrations (TOC/TN Analyzer, details below). A second 50 mL sample was collected and analyzed using a Fluoroprobe (see below).

Experimental design and set up of the glasshouse experiment

We conducted a common garden experiment in which each ecotype was matched to their home water and received the water from three other ecotypes (away). Additionally, we crossed this design with the application of Zn (in the form of $ZnSO_4$). We used three Zn treatment levels: no Zn (control), low Zn and high Zn. Each treatment combination was replicated four times, resulting in a total of 192 experimental units (four ecotypes x four water environments x three Zn treatments x four replicates; Fig. 1).

To create the Zn treatments, we mixed $ZnSO_4 \bullet 7H_2O$ (Alfa Aesar) with the filtered source water at a concentration of 3.4 mg [Zn]/L for the low treatment and 11.36 mg [Zn]/L for the high treatment. The high concentration level of Zn exceeded that found in a waterbody

near a mining area in Turkey (7.23 mg/L of elemental Zn, Sasmaz et al., 2015). Thus, the high concentration used would represent a heavily polluted waterbody.

Each experimental unit was contained within a 150-mL plastic cups (Semadeni, Switzerland). All cups were located within a single glasshouse compartment, which was cooled to prevent excess algal growth, but the natural daily temperature change was maintained. Artificial light was programmed to be turned on from 10 am to 4 pm if the natural light was below 30 klux. The temperature was set at a minimum of 20° C during the day, and 15°C during the night.

To make sure the collected water did not age, we started the experiment shortly after the collection of the water and the samples. Each cup received 100 mL of filtered source water and 30 *Lemna* individuals. To avoid sterilization-induced mortality, we used non-sterile populations. All individuals were rinsed in tap water to ensure that there would be no source water transferred into the cup. As some microbes may have been attached to the fronds or roots of the plant individuals, a small number of microbial cells may have transferred into the destination cups but would have met a resident microbial community with "biotic resistance" and not had the opportunity to invade or even dominate the destination community within the short time of the experiment. No nutrients were added to the cups. All 192 cups were spread onto four different tables, one table per replicate, and within each table all cups were randomly placed to account for potential variation in artificial lighting. The experiment ran for 22 days. Using a smartphone camera (iPhone 11, Apple), we took pictures of each cup on days 1, 8, 15, and 22. Using ImageJ (https://imagej.nih.gov/ij/index.html), we manually counted the number of mature green fronds. For individuals to be considered as alive, they must have contained green pigmentation. Individuals that were entirely either yellow or white were considered dead.

Laboratory analyses

At the end of the experiment (day 22), an unfiltered 50-mL water sample from each cup was analyzed for chlorophyll-a concentration (μg/L) fluorometrically through a Fluoroprobe (bbe Moldaenke, Germany). This chlorophyll-a concentration was used as a proxy for total algal biomass (see e.g., van Moorsel et al., 2021). In addition, a 30-mL water sample was analyzed for its inorganic carbon, total organic carbon (TOC) and total nitrogen (TN) concentrations (Skalar Formacs HT – I TOC/TN Analyzer). Since the samples contained more inorganic carbon than organic carbon, we were not able to analyze TOC via subtraction (i.e., TOC = TC – IC). Thus, we measured non-purgeable organic carbon (NPOC), often reported as TOC since most samples contain a negligible amount of NPOC (NPOC = TOC – POC). Part of each sample (7 mL) was acidified with 100 μL of 10% HCl and purged for two minutes with N_2 gas prior to the analyzer measurement. TN was measured simultaneously in a parallel compartment of the analyzer. We then compared the pre-experiment and post-experiment elemental concentrations.

Sample preparation and DNA extraction

From the thousands of individuals collected at each site, we collected six samples for DNA sequencing (80 individuals per sample tube). In addition, we included a *L. minor* strain and strains from closely related Lemna species from the Landolt duckweed collection. The plant tissue was immediately frozen in liquid nitrogen and stored in -80°C for downstream application. For DNA extraction 30 mg of the frozen tissue were weighted and transferred in 1.5 ml tubes (Eppendorf, Hamburg, Germany) containing two 3 mm metal beads, ground into a fine powder with a TissueLyser II (Qiagen, Germany) adjusted at 30 Hz for 2 min.

The extraction was performed by Norgen Plant and Fungi genomic DNA extraction kit (Norgen Biotek, Thorold, ON, Canada) with modification. The 750 µL of lysis buffer supplemented with 1L RNAseA (DNAse-free, 100,000 units/mL in 50% glycerol, 10 mM) and 3 µL Proteinase K (>600 u/ml ~ 20 mg/ml in 10 mM Tris-HCl pH 7.5, containing calcium acetate and 50% v/v glycerol) was added to each tube and vortexed vigorously. Afterward, 150 µL of Binding Buffer I was added to each tube, vortexed thoroughly and incubated for 5 minutes on ice. The rest of the extraction procedure was accomplished according to the manufacturer's protocol. Finally, the purified DNA was eluted in 100 ul of elution buffer and stored in -20°C. The quantification and qualification of purified DNA was performed by Qubit dsDNA HS Assay Kits (Thermo Fisher Scientific) and gel electrophoresis, respectively. The DNA samples was sequenced by Illumina NovaSeq PE150 paired-end sequencing (Novogene, Cambridge, UK). Unfortunately, only a subset of the samples yielded DNA reads of sufficient quality. Therefore, the total number of samples included in the analyses was 17 (including a technical replicate).

Data analyses

We used two metrics to evaluate population fitness. The first metric was initial population growth rate calculated as $\ln(N_2/N_1)/(t_1 - t_2)$ where N is the number of fronds, and $t_1=1$ and $t_2= 8$ represent the first and eighth day of the experiment. By focusing on population growth during the first week of the experiment (i.e., prior to reaching carrying capacity) we were able to reduce a possible effect of nutrient limitation on growth rates. The second metric was total population growth rate where $t_1=1$ and $t_2=22$, which represented the first and final days of the experiment.

Using additive three-way ANOVAs, we tested whether the environment and the Zn treatments significantly influenced either fitness metric. Treatment variables were population, environment, Zn treatment, and their interactions. The ecotype x environment interactions were further decomposed into a 'home vs. away' contrast (Joshi et al., 2001), i.e. we matched each ecotype to its own environment and created a variable (home) for them. Zn treatment was also further decomposed into a contrast of control vs. Zn treatment followed by the comparison between the low and high Zn treatments.

We used an ANOVA to assess the effect of total chlorophyll-a concentration and its interactions with population, environment, and Zn treatment on *Lemna* population growth rate. We used the same model to test how the final TOC and TN concentrations influenced initial growth rates of *Lemna.* Since algal biomass, TOC and TN were only assessed at the end of the experiment, we limited these analyses to total growth rates.

Finally, we assessed the effect of population, environment, and Zn treatments on total chlorophyll-a concentration (log-transformed) as a proxy for algal biomass and on the final TOC and TN concentrations (proxy for nutrient levels). In the ANOVA testing for the influence of the treatment variables on total chl-a concentrations, we included "block" as a random factor because the chlorophyll concentration was measured sequentially by block. For the mixed model, we used lme() from the package nlme (Pinheiro et al., 2019). For all other linear models, we used the 'lm ()' function in R and no random factor was included (block was never significant). All analyses were conducted in R v 4.1.0 (R Development Core Team 2021).

Reads containing adaptor sequences and those with low-quality scores were removed from the raw data using Trimmomatic v. 0.39 (Bolger et al., 2014). On average, 0.33% of the reads were removed. Trimmed sequencing reads were analyzed using the kmerGWAS pipeline v.0.2 (Voichek & Weigel, 2020). The k-mer database was built using KMC v. 3 (Kokot et al., 2017) with a k-mer size of 21 bp. The kinship matrix was calculated using EMMA (Kang et al., 2008) with a minor allele frequency (MAF) of 0.05. The matrix was visualized with a heatmap and dendrogram in R, using hierarchical clustering and Euclidian distances with the package ASRgenomics v 1.1.3 (Gezan et al., 2022).

Results

Differences between the ecotypes were strong and consistent across environments but there was limited evidence for local adaptation

The effects of ecotype identity and the four environments were strong (Fig. 2, Table 1, significant main terms for environment and ecotype). But, overall, we found no evidence for local adaptation using the home vs. away approach (Table 1, non-significant main term for the contrast home vs. away). However, the significant interaction term with ecotype ($P = 0.005$ for total growth rate in Table 1 and $P = 0.016$ for initial growth rate, see Table S2) shows that for a subset of the ecotypes, there was an effect of home vs. away. Decomposing the ecotype factor into the individual ecotypes revealed that this was driven by ecotype 4 (Table 1, $P = 0.019$ for the interaction term home vs. away x ecotype 4) and by ecotype 3 (Table 1, $P = 0.008$ for the interaction term home vs. away x ecotype 3). Ecotypes 1 and 2 did not show a home vs. away effect (Table 1, $P = 0.15$ and $P = 0.084$, for ecotype 1 and 2, respectively). Thus, ecotype 3 showed evidence for local adaptation through the home vs. away approach (Fig. 2). Conversely, ecotype 4 had significantly lower growth rate in its own environment compared to the away environments.

We found limited evidence that the local ecotype would outperform all others. Ecotype 1 had significantly higher initial and total growth rates in its original environment (environment 1) compared to the other three ecotypes in that same environment for both Zn treatments (Fig. 2, $P < 0.001$ for the contrast term "ecotype 1" fitted in front of ecotype in a linear model using only data from environment 1). However, this effect was driven by the control and the low Zn treatments, and it was not present in the high Zn treatment (Fig. 2).

Ecotype 1 had on average the highest growth rates and ecotype 4 had the lowest growth rates across all treatments and environments (Fig. 2). Environment 4 generally produced the weakest growth rates across all ecotypes and treatments (Fig. 2), thus being the least suitable habitat for *Lemna* growth.

Zn increased *L. minor* growth rates in all environments

The Zn treatments only marginally affected growth rates in the early stages of the experiment (Fig. 2a, Table S2, marginally significant effect of the control vs. Zn contrast, $P = 0.081$) and low vs. high Zn treatment had no effect (Table S2, $P = 0.142$ for the contrast 'low vs. high Zn treatment'). Addition of Zn significantly increased total growth rates (Fig. 2b, Table 1, significant term for the Zn contrast 'control vs. Zn treatment', $P < 0001$). However, there was no significant difference between the low and high Zn treatments (Fig. 2b, Table 1, $P = 0.116$).

No mutualistic outcomes: Algae negatively affected *Lemna* growth

Across all levels of Zn, total growth rate generally decreased as chlorophyll-a concentration increased (Fig. 3, Table S3, $F_{1,94} = 5.7206$, $P = 0.012$). In the high Zn treatment, the different ecotypes responded very differently to increasing algal growth (Fig. S2c). Specifically, ecotype 4 had a sharp decrease in growth rate with increasing algal biomass, whereas ecotypes 1 and 2 had a flatter response. However, the interaction between mean chlorophyll-a concentration and ecotype was not significant (Table S3, $F_{3,94} = 1.3426$ and $P = 0.265$) and neither was the interaction between mean chlorophyll-a concentration and Zn treatment (Table S3, $F_{2,94} = 0.7059$ and $P = 0.496$).

In contrast, ecotype identity had significant and strong effects on algal biomass measured as chl-a concentration (Fig. 4, Table S4, $P < 0.001$ for the term 'ecotype'). Algal biomass was highest in the cultures in which they were competing with ecotype 2 and lowest in those in which they were competing with ecotype 3 (Fig. 4). In addition, the interaction with Zn treatment was significant (Table S4, $P < 0.001$ for the interaction term 'Zn treatment x ecotype'), indicating that the severity of the negative response to Zn addition further depended on the identity of the competitor in the culture.

Zn significantly reduced algae growth (Fig. 4, Table S4, $P < 0.001$) and modified community composition as resolved to the major algal group levels (Fig. S1b). In the high Zn

treatment, the subgroups diatoms and cryptophyta went extinct in nearly all environments (environment 2 had a very small concentration of diatoms remaining).

Finally, mean chlorophyll concentrations strongly differed in the field samples between the environments (Fig. S1a). This difference remained until the end of the experiment (Table S4, $P < 0.001$ for the term 'environment'). Notably, environment 2 had the highest chlorophyll-a concentration at both the beginning and end of the experiment, whereas environment 3 had very low concentrations of chlorophyll-a that exclusively algae belonged to the group "green algae". Chlorophyll-a concentrations decreased across all environments (Fig. S1a vs. b).

TOC and TN concentrations varied between environments

TOC and TN varied significantly between environments (Table S5, $P < 0.001$ for both TN and TOC, Fig. S6). Generally, environment 2 had the highest concentrations of both TN and TOC while environment 3 had the lowest (Fig. S6). The Zn treatments significantly reduced TOC but did not have a significant effect on TN (Table S5, $F_{2,144}= 6.8561$, $P = 0.001$ for TOC and $F_{2,144}= 2.3663$, $P = 0.097$ for TN, see also Fig. S4). For TOC, there was a significant effect of ecotype ($P = 0.00015$, Table S5). TN concentrations were reduced more than TOC concentrations over the course of the experiment. This was evident because the source water samples contained much higher TN and only slightly higher TOC than post-experiment samples (Fig. S5). *Lemna* total growth rates increased with increasing TN (Fig. S3a, linear model, $F_{1, 184} = 4.172$, $P = 0.043$), whereas TOC had no effect on *Lemna* growth (Fig. S3b, $F_{1, 184} = 2.835$, $P = 0.094$).

Whole-genome sequencing confirms species identification and reveals genetic differences between ecotypes

The kinship matrix shows that samples from the same ecotypes are more closely related than samples from different ecotypes (Figure 5a). We also confirmed that all four ecotypes belong to the species *L. minor*, except for ecotype 4 (Ramosch), which may have some *Lemna japonica* mixed into the population (see also Figure S6). Yet, some variation within ecotypes remained (Figure 5b). PC1 captured the majority (76.9%) of the genetic variation, however the groups are rather overlapped, which suggests that the primary source of genetic variation was not driven by the different ecotypes but by other factors, like common genetic ancestry. PC2 explained 6.5% of the total variance, and largely separated the groups. It suggests that the second largest source of genetic variation in the data is related to population structure.

Discussion

We investigated local adaptation and the response to the metal pollutant Zn in *Lemna minor* using a common garden approach in which we grew ecotypes in their home or away waters. We asked 1) whether there is evidence for local adaptation; 2) if and how the presence of different levels of Zn affects the growth rates of the *Lemna minor* ecotypes; 3) how the level of local adaptation influences the response to the metal pollutant; and 4) how the treatments

influenced the algal biomass and the algal biomass influenced *Lemna* growth rates in turn. There were significant differences between growth rates in the different treatment groups. However, despite finding genetic differentiation between the studied ecotypes, we did not find significant evidence of local adaptation for neither of the four ecotypes. The ecotypes were positively affected by an increase in Zn in their environment, and thus had higher growth rates in the low and high Zn treatments.

No evidence for local adaptation but strong main effect of environments and ecotypes

We expected to see higher growth rates when ecotypes were raised in their home environments. Instead, we observed that an ecotype's home environment was rarely significantly better than others. Ecotype 1 (*L. minor* from Northern Switzerland) had higher growth rates in its own environment than all other ecotypes had in that same environment. However, this was only the case in the presence of Zn, thus in the modified environments. Consequently, this would constitute a false signal of adaptation potentially due to the amelioration of negative biotic interactions, such as competition with algae (Hargreaves et al., 2020).

Ecotype 4 (from Eastern Switzerland) had the lowest growth rates across all environments and treatments, and its lowest growth rates were in its own original environment. At the same time, lower growth rates of the other ecotypes in environment 4 than in other environments indicates that it was generally a less favourable environment for *Lemna*. Environment 4 had a relatively high amount of TOC and a low amount of TN, which could have contributed to the negative performance trend of the *Lemna* ecotypes. Additionally, the drastically lower growth rates in all environments could be a sign that ecotype 4 was impaired by the conditions in the glasshouse environment, e.g., light intensity or temperature. The pond where it was sampled was several degrees colder than the glasshouse conditions and the other sampled ponds (Table S1). Finally, our genomic analyses revealed that two of three analyzed samples cluster more closely with control *Lemna japonica* than with *Lemna minor* strains (Figure S6). We did those analyses with different plants than used in the experiment, so we cannot be sure which of these sister species we used in the experiment, or whether it was a mixture of the two. Not enough is known about the differences in growth rates between *L. japonica* and *L. minor* to conclude that the lower growth rate could be explained by potential mixing of these two genetically closely related (Braglia, Breviario, et al., 2021) and morphologically almost identical species (Landolt, 1986).

To our knowledge, our study is the first to test for local adaptation in multiple geographically distanced ecotypes of *L. minor*. However, a study examining the closely related and morphologically similar *L. turionifera* came to similar conclusions, i.e. they could also not demonstrate local adaptation to environmental conditions (Barks et al., 2018). Contrastingly, Muranaka *et* al. observed local adaptation to the photo period in *L. aequinoctialis* growing in rice paddies (Muranaka et al., 2022), suggesting that some traits may exhibit local adaptation, which may not be picked up when merely using growth rate as a fitness metric.

There is evidence of high genotypic diversity within duckweed species (Bog et al., 2022; Braglia, Lauria, et al., 2021). We also found evidence for genetic diversity between the studied ecotypes but also within each ecotype (Fig. 5). Thus, one of the reasons why we did not see a specific pattern here could be that genotypic diversity within the four sampled ecotypes allowed them to cope with the environmental changes they experienced. Alternatively or additionally, we explain the lack of local adaptation in these geographically very distant ecotypes of Lemna with the well-known phenotypic plasticity of the Lemna family (Hitsman & Simons, 2020; Roubeau Dumont et al., 2019; Vasseur & Aarssen, 1992a). The clonally reproducing duckweed are known to grow under many different environmental conditions (Laird & Barks, 2018) and to persist and acclimate to environmental stress from salinity (e.g., van Moorsel, 2022), and to water pollutants (e.g. copper, Roubeau Dumont et al., 2019). Thus, the lack of local adaptation further adds evidence that ecotypes and genotypes of the Lemna minor can be grown in many different environments likely due to their high levels of phenotypic plasticity (Vasseur & Aarssen, 1992b). In extension, this means that many *L. minor* ecotypes could be used for heavy metal removal of polluted waterbodies. However, at the same time, we did find strong within-species variation in growth rates, thus if high growth rates are desirable, for example for biomass production as biofuel or animal feed (Cheng & Stomp, 2009), evaluating several ecotypes may be advisable.

A more methodological explanation for the lack of strong ecotype x environment interactions could be because we only reciprocally manipulated the water conditions. Light and other environmental parameters such as air or water temperature may be equally important. Our experiment possibly underestimated the degree of local adaptation because we did not test for local adaptation to light conditions, e.g., the level of sun exposure, which may be higher in higher altitudes or the south of Switzerland, or the percent of the water body being shaded by vegetation. Future experiments should reciprocally transplant the ecotypes in the field sites to include all environmental conditions paramount for plant fitness.

Zn increased duckweed growth but reduced algal growth

We expected Zinc to be a stressful pollutant to *L. minor*, based on previous research with this species reporting that Zn impacted plant growth (O'Brien, Laurich, et al., 2020). Here, Zn treatments significantly boosted *Lemna minor* total growth rates. Therefore, we could not test one of our main hypotheses, which was that growing in the original environment would aid in the stress response. This outstanding hypothesis could be addressed in future studies using 1) a different chemical that elicits an actual stress response in *L. minor* or 2) Zn levels at higher concentrations. Here, we wanted to keep Zn levels in somewhat realistic concentrations that may be relevant for phytoremediation, which is why we did not use extremely high concentrations.

The positive effect of Zn on duckweed growth rates could be due to its negative effect on most algae, which compete with *Lemna* for resources. In the presence of abundant nutrients and similar glasshouse conditions, strong algal growth significantly reduced *L. minor* growth rates (van Moorsel, 2022). Another explanation stems from the fact that plants require Zn for their chlorophyll and protein production. Zn is an essential trace element for most organisms

and plays important roles in metabolic processes in plants (Lahive, O' Halloran, et al., 2011). This may explain the increased growth rates we observed in both low and high Zn treatments. In a different study, at the same concentrations as in this experiment, Zn increased growth rates of three *Lemna* species also under sterile conditions, i.e. in the absence of algae (Lanthemann & van Moorsel, 2022). Other studies also report positive correlations between the presence of Zn and duckweed growth (Jayasri & Suthindhiran, 2017; Khellaf & Zerdaoui, 2009), suggesting efficacy in the uptake of this metal by these macrophytes. Jayasri & Suthindhiran (2017) found high tolerance of *L. minor* to Zn^{2+} concentrations of up to 10 mg/L. A second study found *L. minor* to tolerate Zn concentrations above 100 mg [Zn]/L, whereas the closely related gibbous duckweed *Lemna gibba* only tolerated concentrations up to 10 mg [Zn]/L (Lahive, O' Halloran, et al., 2011). Taken together, these previous and our findings indicate that *Lemna* may be a candidate species for the removal of excess Zn metal and derivatives from water bodies, as long as metal concentrations in the water are not toxic to the duckweeds themselves (Ziegler et al., 2016). We did, however, not measure Zn concentrations in the water at the end of the experiment to assess the amount of it that had been taken up by the plants.

In contrast to the positive effect of Zn on the plants, algal biomass and biodiversity was significantly reduced in the presence of Zn. Zn is known to negatively affect various algal groups even at levels lower than 30 µg/L (Kayser, 1977; Wong & Chau, 1990). Zn can alter the permeability of the algal cell membrane, leading to a steep decrease in potassium and sodium cell contents, inhibition of cell multiplication, photosynthesis, and N fixation (Kostyaev, 1981). Furthermore, it has been demonstrated that algae become more sensitive to pollutants such as Zn when in competition with other plant species (Kayser, 1977).

By the end of the experiment, TN had been significantly reduced to levels below the minimum needed for continuous *Lemna* growth (about 0.2 mg/L, Roijackers et al., 2004) in all environments except for environment 2. Interestingly, environment 2 had the highest algal biomass both prior and after the experiment, compared to the other environments. This suggests that *Lemna* may have played a larger role in TN uptake than the algae present. Although TOC concentrations decreased, there was still a significant amount left by the end of the experiment. However, there is not enough research on the effects of dissolved organic carbon on macrophytes, thus we do not know how it may have affected the *Lemna* ecotypes. Initial *Lemna* growth rates were high, which together with the higher algal biomass, explains the strong TN decrease and shows that lower levels of N can have limited *Lemna* growth rates after the first week. After day 8, ecotypes could have reached carrying capacity, given that their growths afterwards were slower, reduced, or decreased.

In conclusion, despite large effects of ecotype identity and the tested environments, we did not find significant evidence for local adaptation. Instead, *Lemna* ecotypes grew well in Zn-contaminated waters, which prevented us from testing an actual stress response. Our findings suggests that for phytoremediation of heavy-metal polluted waters, many *Lemna minor* ecotypes may be suitable even though within-species differences in growth rates should be expected (Walsh et al., 2022).

Conflict of interest. None declared.

Data archiving statement. We intend to make the publicly available on Data Dryad upon final acceptance of the manuscript**.**

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Author contributions. SV and SJVM designed research. SV ran the experiment and collected data. AM did the DNA extractions. CL supported the bioinformatics. SV and SVJM analyzed data. SV wrote the initial draft of the manuscript. SJVM wrote the final draft of the paper. All authors contributed to and approved the final version of the manuscript.

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Table 1. Results for type 1 ANOVA testing the effect of ecotype identity, environment and Zn treatments and their interactions on *Lemna* total growth rates (22 days of experiment). *P* - values < 0.05 are shown in bold. For initial growth rates, see Table S2.

Source of variation	**Df**	**Sum Sq**	**Mean Sq**	***F value***	***P***
Environment	3	0.00435	0.00145	41.55	**< 0.001**
Ecotype	3	0.01306	0.00435	124.75	**< 0.001**
Home vs. away	1	0.00002	0.00002	0.49	0.485
Control vs. Zn treatment (Zn contrast)	1	0.00402	0.00402	115.11	**< 0.001**
Low vs. high Zn (Zn treatment)	1	0.00009	0.00009	2.50	0.116
Ecotype x Home vs. away	3	0.00047	0.00016	4.48	**0.005**
Environment x Ecotype	5	0.00022	0.00004	1.28	0.278
Ecotype x Zn contrast	3	0.00063	0.00021	6.00	**0.001**
Ecotype x Zn treatment	3	0.00006	0.00002	0.58	0.631
Environment x Zn contrast	3	0.00063	0.00021	6.03	**0.001**
Environment x Zn treatment	3	0.00013	0.00004	1.21	0.310
Home vs. away x Zn contrast	1	0.00009	0.00009	2.64	0.107
Environment x Ecotype x Zn contrast	8	0.00045	0.00006	1.62	0.125
Environment x Ecotype x Zn treatment	9	0.00025	0.00003	0.81	0.612
Residuals	144	0.00503	0.00003		

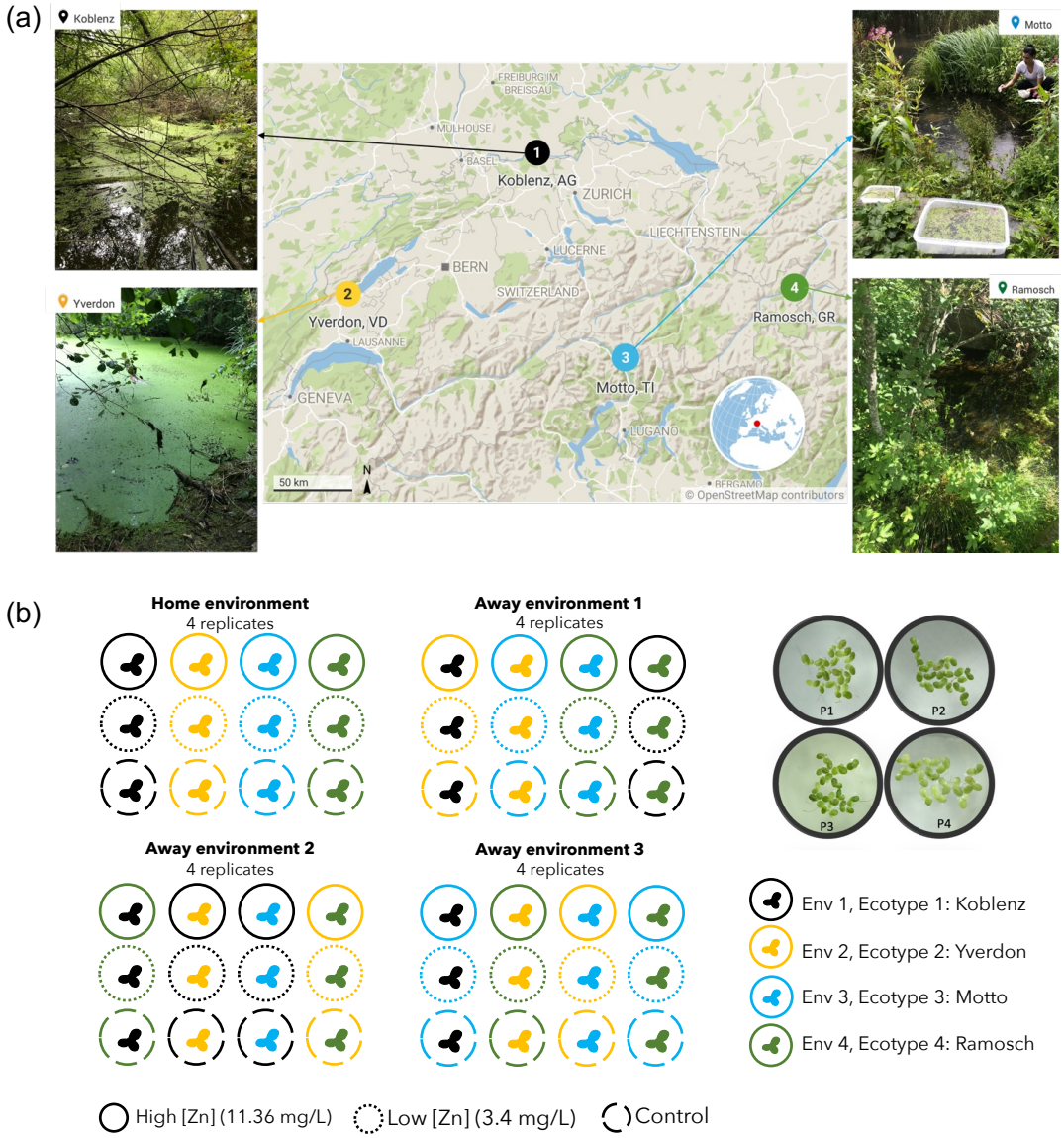
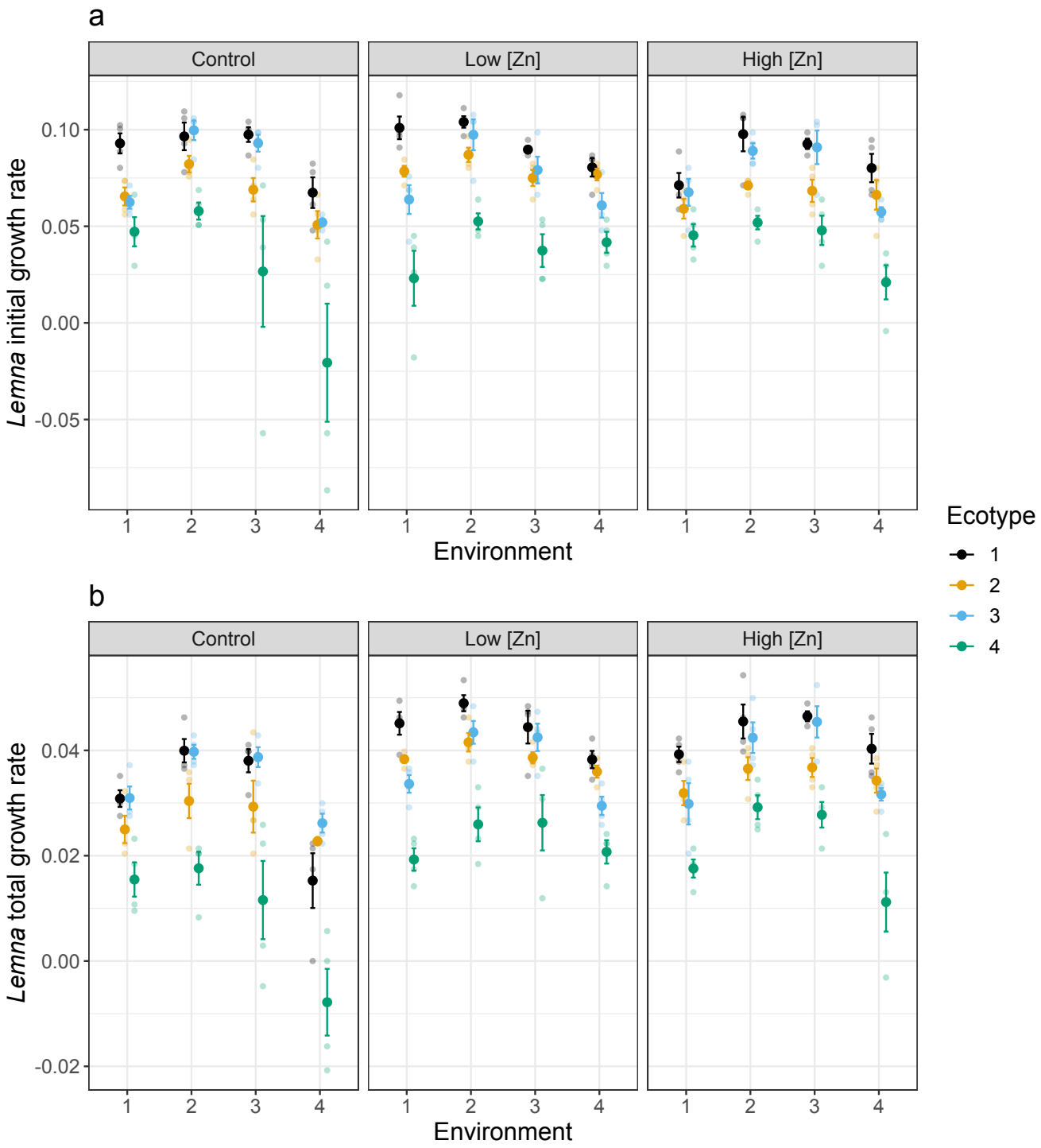


Fig. 1. Schematic of the study design. (**a**) Field collection locations across Switzerland. Map created with Datawrapper. (**b**) *Lemna* fronds in different colors to represent each ecotype; Circles represent the cups, and the different outlines represent the Zn treatment. E1-E4 represent each environment. Inset in (b): Photo taken of each ecotype from above.



639 **Fig. 2**. ***Lemna*** **growth rates per ecotype in response to environment and Zn treatments.**
640 Mean initial (a) and total (b) growth rates (n = 4) of all four ecotypes across all four
641 environments and the three Zn treatments with the associated standard errors. For test statistics
642 see Table 1 and Table S2.

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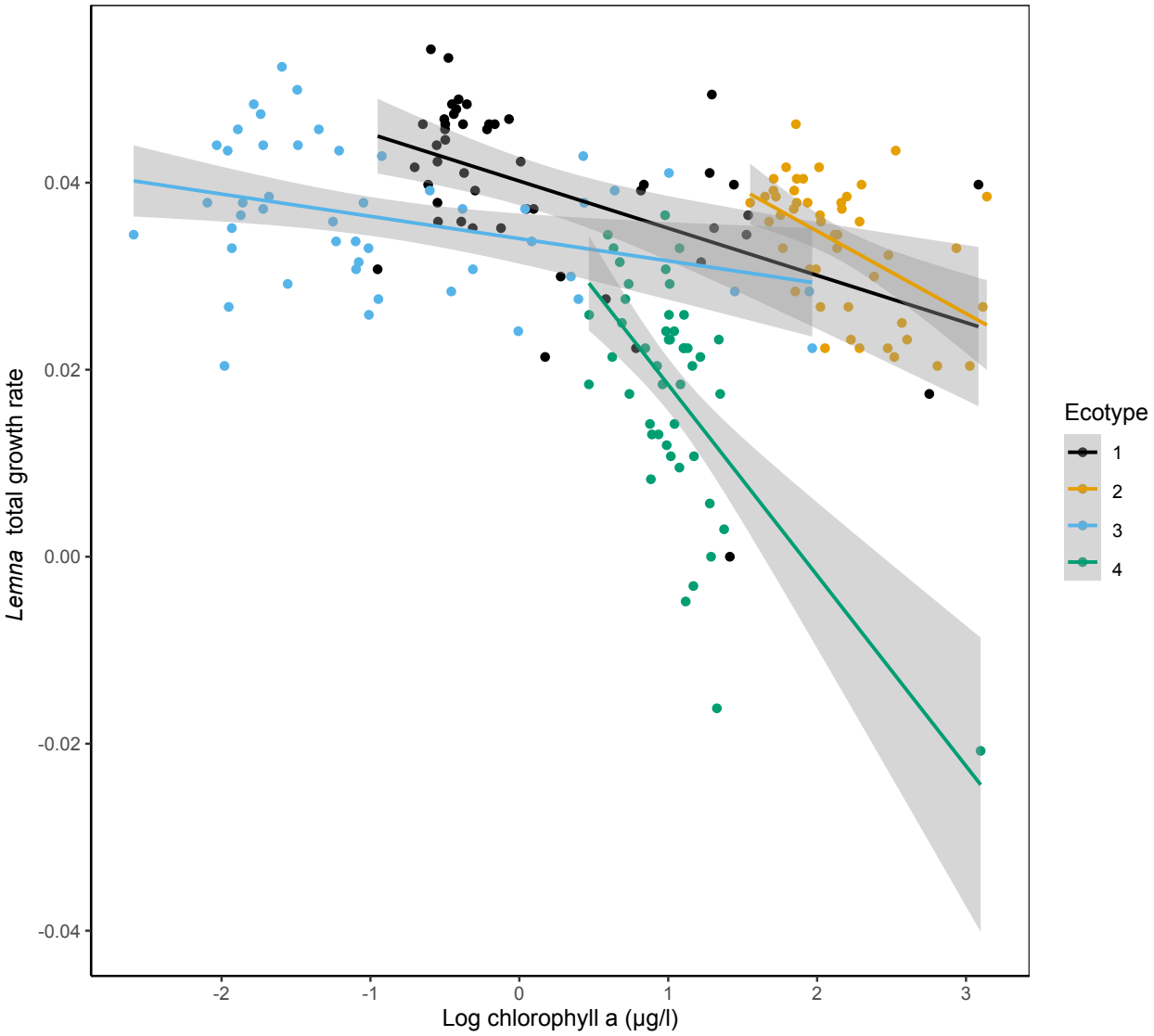

644 **Fig. 3.** *Lemna* growth rates per ecotype in response to chlorophyll-a. Total growth rates of all
645 ecotypes vs. total algal biomass (log-transformed mean chl a-concentration) across all Zn
646 treatments. See Figure S2 for the regressions for each Zn separately. Shaded areas correspond
647 to 95% confidence intervals.

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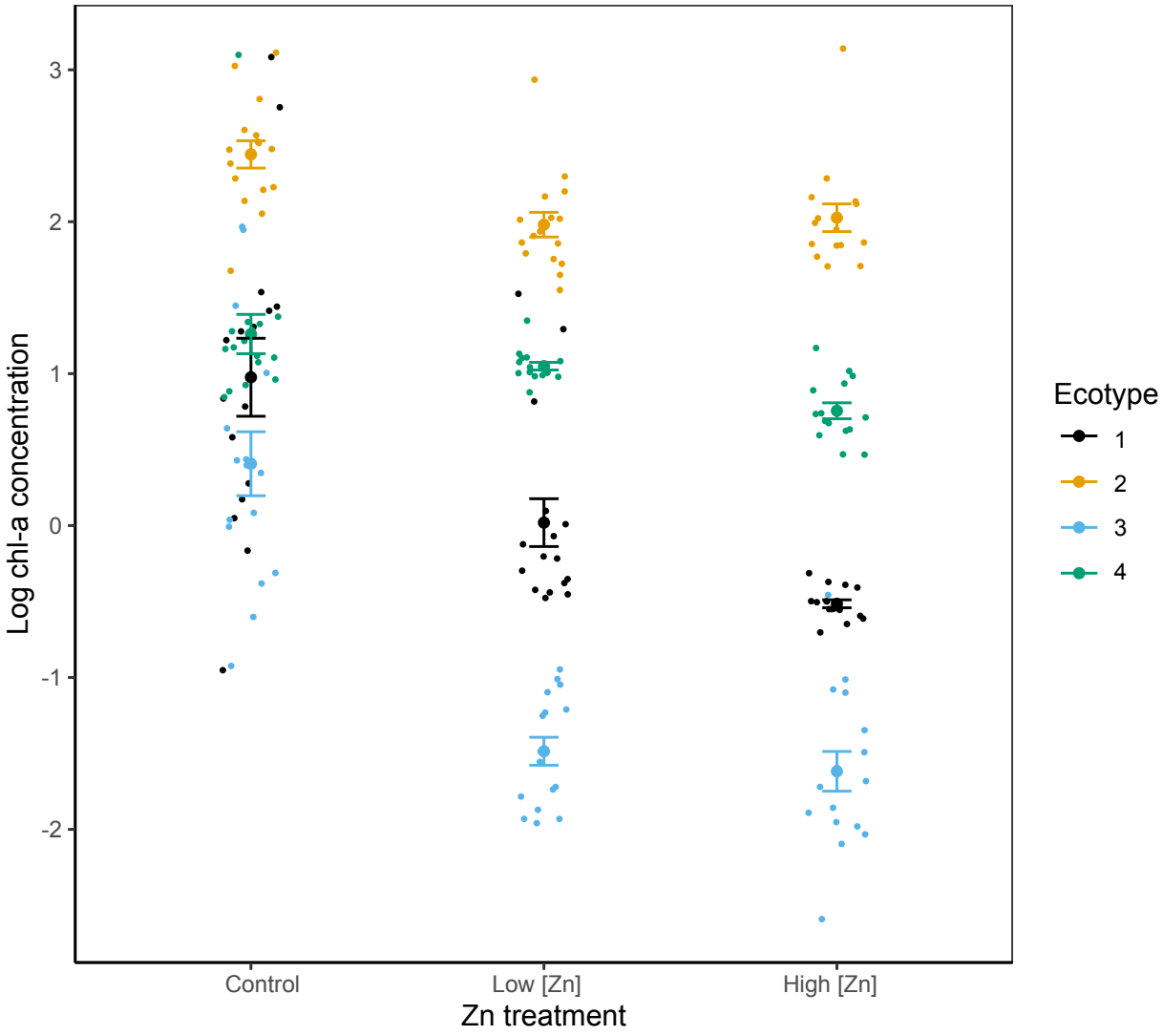

650 **Fig. 4**. Response of algal growth (log-transformed mean chl-a concentration) to Zn treatments
651 and ecotype identity (i.e., competitor identity). Shown are means (n = 16) and associated
652 standard errors. For test statistics see Table S4.

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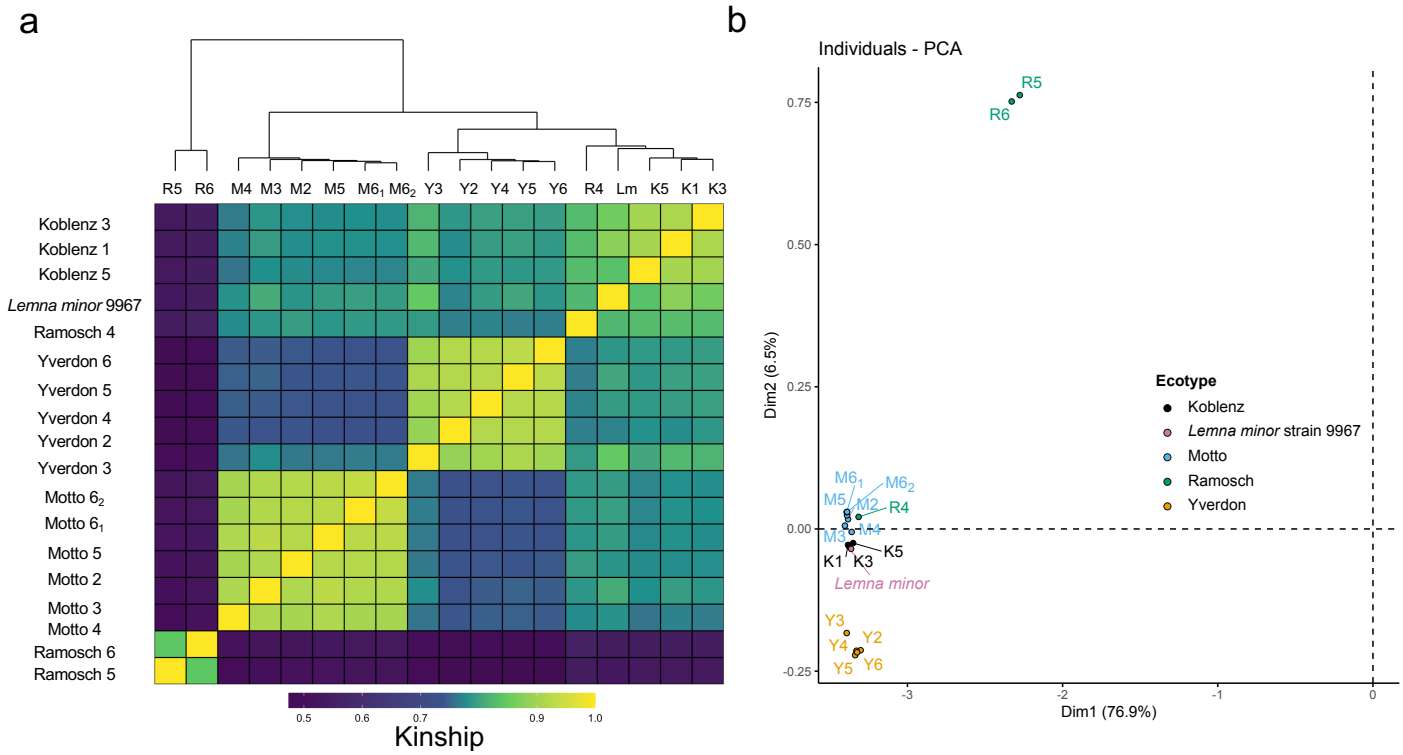

Fig. 5. (**a**) Kinship matrix. Yellow color indicates high kinship, blue indicates low kinship. Each sample contained multiple individuals per site, thus does not represent a single clone. $M6_1$ and $M6_2$ are technical replicates (same DNA after the extraction protocol). Top row: ecotype abbreviated with a single letter (K: Koblenz, M: Motto, Y: Yverdon, R: Ramosch, Lm: *Lemna minor* strain 9967). (**b**) PCA based on the kinship matrix.