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Intraspecific diversity is critical to population-level risk assessments

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Environmental risk assessment (ERA) is critical for protecting life by predicting population responses to contaminants. However, routine toxicity testing often examines only one genotype from surrogate species, potentially leading to inaccurate risk assessments, as natural populations typically consist of genetically diverse individuals. To evaluate the importance of intraspecific variation in translating toxicity testing to natural populations, we quantified the magnitude of phenotypic variation between 20 *Daphnia magna* clones exposed to two levels of microcystins, a cosmopolitan cyanobacterial toxin. We observed significant genetic variation in survival, growth, and reproduction, which increased under microcystins exposure. Simulations of survival showed that using a single genotype for toxicity tolerance estimates on average failed to produce accurate predictions within the 95% confidence interval over half of the time. Whole genome sequencing of the 20 clones tested for correlations between toxicological responses and genomic divergence, including candidate loci from prior gene expression studies. We found no overall correlations, indicating that clonal variation, rather than variation at candidate genes, predicts population-level responses to toxins. These results highlight the importance of incorporating broad intraspecific genetic variation, without focusing specifically on variation in candidate genes, into ERAs to more reliably predict how local populations will respond to contaminants.

Keywords Risk assessment, Genetic variation, Toxicology, Daphnia, Contaminants monitoring, Environmental science

Reliable translation of toxicological responses into predictions of population-level consequences in nature is a cornerstone of environmental risk assessments (ERAs)[1–6]. These predictions, crucial for safeguarding biodiversity and preserving ecosystem services, classically rely on quantifying individual phenotypic responses under controlled laboratory conditions[7]. However, the overwhelming reliance on single-species (or surrogate species) toxicity testing and metrics such as the lethal concentration for 50% mortality (LC50) has persisted for over half a century within regulatory frameworks, despite acknowledged limitations[6,8]. It is therefore imperative to critically examine and refine traditional toxicity testing methods to better reflect ecological realism and ensure the efficacy of ERAs in protecting local populations[8].

Genetic variation within species is recognized as a critical driver of both ecological and evolutionary processes[9–11]. Within ecological contexts, this variation has profound impacts on populations, communities, and ecosystems[9–13]. Moreover, genetic variation within species is central to evolution, with natural selection acting upon this variation to drive adaptation[10,14]. Among the most visible examples of rapid adaptation are cases where populations adapt to agrochemicals[15] or contaminants[16]. More broadly, there is a growing number of demonstrations of substantial standing variation in tolerance to stressors across taxa[4,5,17–19]. Yet, despite intermittent advocacy for the relevance of intraspecific genetic variation in ERAs spanning decades, there has been little formal analysis quantifying how current practices of not accounting for intraspecific variation may ultimately limit accuracy in risk assessments for local populations encountering environmental stressors.

Investigating the magnitude of intraspecific variation within a natural population represents a crucial and understudied element in ERAs. Despite its significance, little is known about the magnitude and ecological implications of intraspecific genetic variation in toxicological responses within natural populations. The common practice of selecting genotypes based solely on availability, such as standard lab strains, may result in inferences

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that inadequately represent imperiled populations. Relying on phenotypic measurements from a single genotype for population projections overlooks the inherent, and often important, genetic diversity present[20,21]. While risk assessors apply uncertainty factors as a blanket measure to account for factors that introduce variation in responses to hazardous substances, this approach has been criticized for its arbitrary nature[22] and limited ecological relevance[23] in real populations. To address these gaps, detailed studies of specific populations are needed, integrating measures of genetic variation within focal populations alongside routine toxicity testing to better understand the potential impact of this variation on population-level responses to toxic insults[24].

While intraspecific genetic variation has not been a major focus in risk assessment, it is central to understanding the molecular mechanisms by which organisms cope with toxic insults[25,26]. The increased accessibility of transcriptomic and genomic data have led to a proliferation of functional genetic work in toxicology[5]. This includes identification of genetic variants associated with intraspecific variation in resistance through comparative genomic and transcriptomic sequencing[16,25,27]. As the scope of genomic work focused on toxicological responses has grown, calls to incorporate genomic data into risk assessment[5,28], and in the management of biodiversity more broadly[29,30], have emerged. This includes the use of identified candidate loci, typically found from genome-wide association studies or transcriptomic comparisons, to enhance accuracy of risk assessments[31]. The use of candidate loci, identified through prior mapping efforts or through changes in gene expression, provides an opportunity to test hypotheses about the relationship between genetic variation in potentially important genes and toxicological responses that could link genetic and phenotypic variation[28,32,33]. There is potential to use genotype-phenotype linkages to examine a population's susceptibility to, and potential for adaptation, in response to environmental stressors[29,30]. While undoubtedly intriguing, there is tremendous uncertainty about the overall utility of employing candidate genes to predict population-level responses to environmental stress[31,34,35].

To determine the importance of intraspecific variation in toxicity projections key to the ERA process, we sequenced the genomes of 20 *Daphnia magna* clones collected from a Belgian lake and quantified the chronic effects of the cyanobacterium *Microcystis* on their life-history traits (survival, body growth, reproductive output, number of broods). *Daphnia* (water fleas) are a robust ecological model system owing to their ease of culturing and well-documented ecological significance in freshwater ecosystems[36,37]. Widely employed in setting environmental contaminant guidelines[37], *Daphnia* studies have highlighted the considerable genetic diversity present in these organisms[2,3,7,38]. Prior work has demonstrated that 20 *D. magna* clones from one local population contain sufficient genetic diversity that could be representative of broader patterns seen across wide geographic regions, though within the same ecoregion[39]. This is consistent with the widespread distribution and adaptability of *Daphnia* to various environmental conditions, which suggests that genetic diversity observed in one lake could reflect global patterns. Furthermore, *Daphnia* are frequently studied in comparative genomics and eco-evolutionary dynamics, where the ability to resurrect resting eggs from sediments to study evolution[36,37,40–42] and a fully sequenced genome add to their utility[36,43]. Harmful algal blooms (HABs) of *Microcystis* are a prominent and toxic ecological disturbance that strongly impact a wide-range of aquatic taxa due to the production of microcystin toxins[44–49]. As a keystone zooplankton grazer, *Daphnia* shows substantial intraspecific diversity and rapid adaptation in response to HABs[40,50,51], including some evidence that particular genotypes can effectively feed on and reduce the abundance of toxic *Microcystis*[52,53]. Determining whether intraspecific variation in toxin tolerance influences vital demographic rates and population dynamics by *Daphnia* clarifies the role of intraspecific genetic variation in ERAs, with implications for regulatory agencies tasked with maintaining biodiversity under toxic conditions.

In this study, 30 individuals per *D. magna* genotype were divided and exposed to three common gardens: *Chlorella vulgaris*-only (non-toxic diet), 2:1 chlorella: microcystis (moderately toxic diet), and 1:1 chlorella: microcystis (severely toxic diet). Common garden rearing keeps environmental conditions constant allowing for the estimation of the contribution of genetic variation to phenotypic variation. We chose 2:1 and 1:1 toxic treatments that resulted in microcystin concentrations of 3.3 ± 0.001 µg L^{-1} and 5.1 ± 0.001 µg L^{-1} (Table S1; Supporting Information, Dataset S1), respectively, which have been considered sublethal[54] to lethal[55,56] in *Daphnia* laboratory assays and are common ratios in freshwater ecosystems with HABs[46]. We sought to address the following four questions: (1) Does toxic exposure reduce the magnitude of intraspecific phenotypic variation? (2) Is there an interaction between clonal identity and the response variables (survival, body growth, reproductive output, time to first brood) under non-toxic and toxic dietary conditions? (3) Using data from these trials, how would the sampling design for intraspecific diversity influence the interpretation of the toxicity of a contaminant? (4) Is the magnitude of genomic variation overall or at candidate loci correlated with the phenotypic responses to toxic cyanobacteria? We predicted that *D. magna* clones would exhibit considerable variation in life-history traits, but this variation would be reduced when exposed to a toxic insult due to a decrease in trait values in high performing genotypes. Moreover, we predicted that certain genotypes would exhibit an interaction between clonal identity and life-history traits, with certain clones having higher reproductive output under specific levels of toxicant exposure, resulting in slower growth rates in those clones. In addition, we predicted that sampling a sufficient number of genotypes would be critical to producing an accurate estimate of reproduction in both non-toxic and toxic conditions. Finally, we predicted that the genomic architecture of fitness in response to cyanobacteria would be complex and hence would not be correlated with variation at previously identified candidate loci.

Results

Magnitude of phenotypic variation from toxic exposure

Toxic exposure reduced the overall fitness of *D. magna* genotypes following a monotonic dose response in the moderately toxic (2:1 *Chlorella* : *M. aeruginosa*) and severely toxic (1:1 *Chlorella* : *M. aeruginosa*) treatments

(Fig. 1, Table S6-S8; Supporting Information, Dataset S1). For example, mean survival across all clones for the 14-day trial declined from 94% in the non-toxic diet, to 86.5% in the moderately toxic diet, and to 53% in the severely toxic diet. Using the Brown-Forsythe test, we tested for differences in the magnitude of clonal variation with increasing exposure to *M. aeruginosa* for each of the three fitness-associated phenotypes (survival, body growth, reproductive output). Intraspecific variation in survival did not increase from the control to 2:1 ($F^* = 3.60$, $P = 0.066$) but did from 2:1 to 1:1 ($F^* = 22.59$, $P < 0.001$). Intraspecific variation in growth rate increased from the control to 2:1 ($F^* = 10.28$, $p = 0.003$) but decreased from 2:1 to 1:1 ($F^* = 11.82$, $P = 0.002$). Neonate production showed the most consistent pattern, with intraspecific variation between clones declining with increasing *M. aeruginosa* exposure (control to 2:1 ($F^* = 18.54$, $P = 0.0001$); 2:1 to 1:1 ($F^* = 7.44$, $P = 0.009$)).

### Interactions between genetic diversity and toxicity

Using GLM and LME models, we next tested for interactions associated with performance across variations in diet toxicity. On average, survival after 14 days declined with increasing toxicity by 8% in the moderately-toxic diet and 44% in the severely toxic diet relative to controls. However, we found evidence of an interaction between clonal identity and a *Microcystis* diet ($X^2 = 59.68$, $P = 0.014$). Some of these interactive effects were striking. Relative to rearing on the non-toxic diet, clones 5 and 15 showed an 80% decline in mean survival when reared on the severely toxic diet while clone 19 exhibited a 20% increase in survival. Similarly, we found a significant interaction associated with body growth across treatments ($X^2 = 244.17$, $P < 0.0001$). Finally, neonate production showed a similar pattern, with a significant interaction between clonal identity and a *Microcystis* diet ($X^2 = 264.42$, $P < 0.0001$). Of particular interest was that certain genotypes exhibited higher growth rates or produced more neonates in one of the toxic treatments compared to the non-toxic diet (e.g., genotype 6 in the 2:1 toxic treatment had greater growth and reproduction, and genotype 13 showed increased growth and reproduction in both the 2:1 and 1:1 toxic treatments). In contrast, other genotypes had higher growth and reproduction rates in the 1:1 toxic treatment compared to the 2:1 treatment (e.g., genotypes 10, 18, 19, 20). These findings indicate the relative performance of clones is dependent on the magnitude of toxicant exposure.

### Influence of genetic diversity on toxicological inferences

We next used a bootstrapping approach to determine how variation in the number of clones in the experimental sampling design could influence the robustness of the data relative to including 20 clones. Our results demonstrate that sampling individuals from a larger number of genotypes is critical to generating a robust estimate of reproduction in all three rearing conditions (Fig. 2a). Simulations of mean neonate production per surviving individual are also provided (Figure S1; Supporting Information, Dataset S1). To quantify the loss of robustness and accuracy that would result from assaying a reduced amount of genetic diversity we assessed how frequently samples at five reduced diversity levels would yield survival estimates within the 95% confidence interval (CI) obtained using all 20 clones (Fig. 2b). While including 15 clones always produced a survival estimate within the 95% CI assays, using only a single clone on average failed to produce an estimate within the 95% CI over half
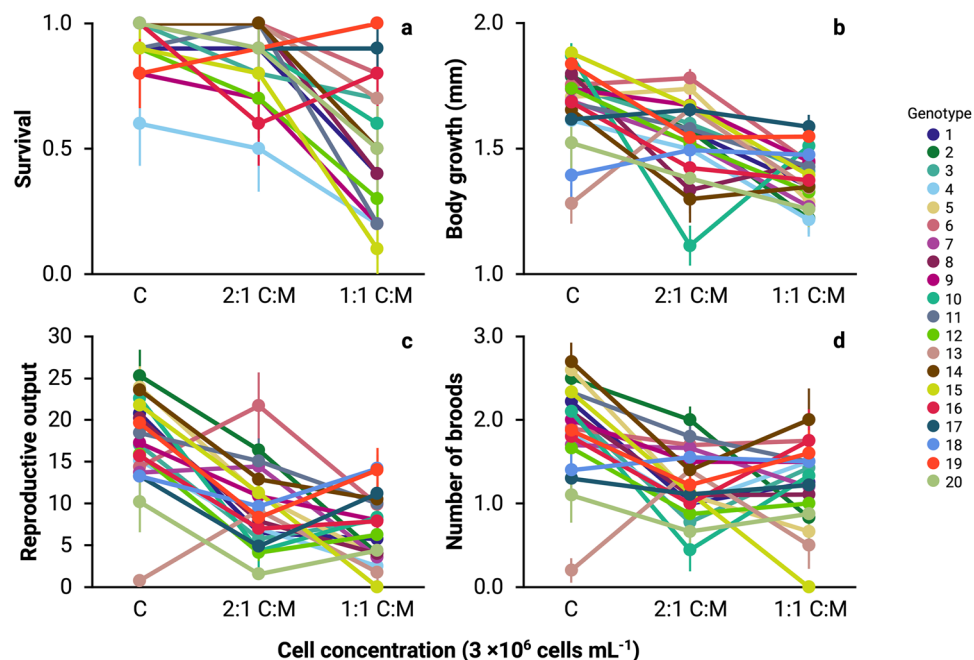
Figure 1. Phenotypic variation among the 20 *Daphnia magna* clones at the end of the 14-d chronic toxicity study. Phenotypes measured in parents were (**a**) survival, (**b**) body growth from day 0 to 14, (**c**) total number of neonates produced, and (**d**) total number of broods produced. Cell concentration was standardized across three algal food treatments: non-toxic - "C", moderately-toxic - "2:1 C: M", and severely-toxic - "1:1 C: M". Plots show means and standard errors for each clone when reared on each diet.
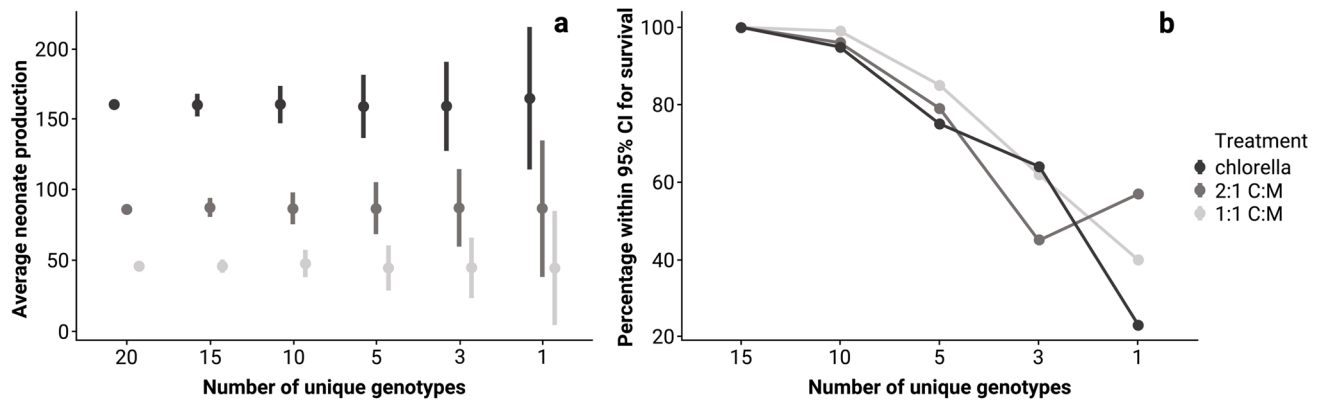
Figure 2. Panel A shows simulations of mean reproductive outputs for experimental designs with varying amounts of genetic diversity of *Daphnia magna* across three treatments of chlorella "C", 2:1 chlorella: microcystis "2:1 C: M", and 1:1 chlorella: microcystis "1:1 C: M". Symbol and error bars represent the mean and standard deviation. A 95% confidence interval was calculated for survival using all 20 clones. Panel B shows simulations of how often estimates of survival fell within this 95% confidence interval when the data were subsetted to include a reduced number of genotypes.
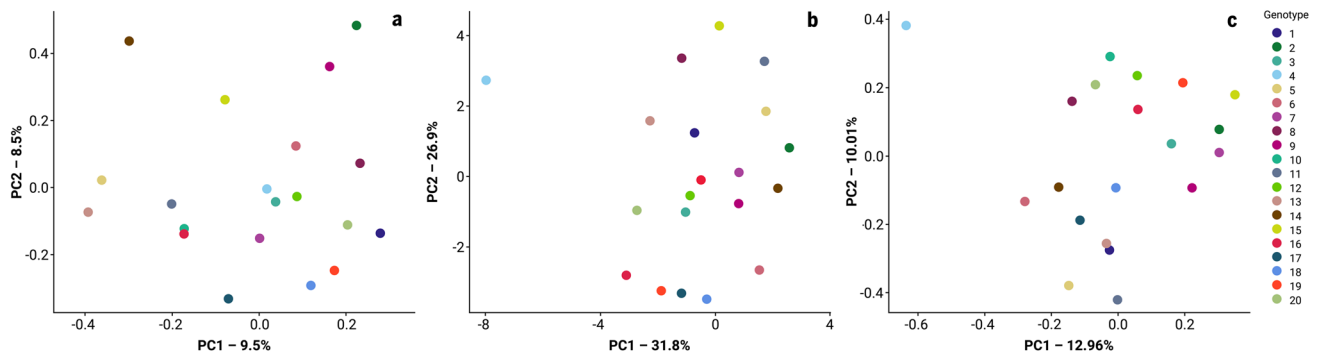
Figure 3. Multivariate (**a**) whole genome genomic and (**b**) phenotypic divergence across all treatments between 20 *Daphnia magna* clones visualized using principal component analysis (PCA). Panel (**c**) shows a PCA constructed from genetic variation at candidate loci for variation in tolerance to *Microcystis* in *Daphnia*.

the time in all three common gardens. Overall, this reinforces the importance of accounting for genetic diversity in toxicity testing, as failing to do so means estimates of reproductive output and survival are likely to be highly variable and inaccurate.

Intraspecific genomic and phenotypic variation

The magnitude of genome-wide divergence between pairwise sets of clones varied (Fig. 3a), with the most similar clones having ~ 1.2 M SNP differences and the most divergent ~ 1.6 M.

We tested for an association between the degree of genomic divergence between clones and the magnitude of their phenotypic differentiation (Fig. 3). We calculated a phenotypic distance between each pair of clones based on results of phenotypic assays for survival, growth, total reproduction, and time to reproduction when reared on non-toxic, moderately toxic, and severely toxic diets. We found no significant association between the degree of genomic divergence genome-wide and the magnitude of overall phenotypic divergence between clones (mantel $r = 0.033$, $P = 0.781$). We also examined the relationship between genetic diversity found within clones (i.e., total heterozygosity) and clonal survival and reproduction (Figure S2; Supporting Information, Dataset S1). We found no significant association between the amount of genetic diversity within clones and their ability to survive or reproduce across diet conditions, consistent with predictions from neutral theory that overall patterns of divergence may more strongly be shaped by genetic drift[57].

We used a similar analysis approach to assess the association between phenotypic differentiation among clones and genetic variation in eight gene regions previously implicated in *Daphnia* transcriptomic responses to microcystins[58,59]. A PCA of all variant sites within these eight gene regions revealed that clone 4, a phenotypically distinct clone due to its overall low survival and growth rates across conditions (Fig. 3b), is largely separated from all other clones by PC1. We found no significant association between the amount of divergence at these putatively functional gene regions and the magnitude of overall phenotypic divergence among clones (mantel $r = 0.054$, $P = 0.584$) (Fig. 3c). We also found no association between the amount of genetic diversity at these

putatively functional gene regions and their ability to survive or reproduce at high (1:1 chlorella: microcystis) toxic exposure. This suggests that genotype-phenotype linkages as ascertained from transcriptomics are not predictive of the phenotypic responses to toxicant exposure.

Discussion

Genetic variation exerts meaningful impacts on various ecological aspects such as population dynamics[60], community composition[61], ecosystem function[62], and rapid adaptation[10]. Integrating natural genetic variation into ERAs enhances the reliability of laboratory toxicity assays to accurately reflect natural populations. Our data demonstrate the importance of genetic variation, even within a population from a single locality, on toxicological responses in a widely used model system. For instance, the moderately toxic diet in our study could be interpreted as yielding an LC50 (i.e., clone 4) or an LC0 (clone 18 and 19), depending on the selected clone for the assay. Moreover, when reared on a severely toxic diet mortality ranged dramatically from 0 to 90%. Toxicity studies that overlook intraspecific genetic variation may provide inaccurate insights, leading to uncertainties in extrapolating laboratory results to field conditions and affecting risk management strategies[63]. Such oversights are likely exacerbated in local populations where natural selection may strongly shape genetic variation and toxicological responses[64,65].

The variation observed in our study, derived from a single population of *D. magna*, is doubtless an underestimate of the true magnitude of variation present across populations experiencing diverse exposures to HABs. Species from diverse ecological backgrounds, particularly those exposed to varying levels of toxicants that may have driven adaptation, are likely to exhibit greater intraspecific variation than observed within our study population. Hence, we advocate including multiple genotypes or individuals from multiple populations in toxicity studies to encompass a representative range of genetic diversity. Such robust sampling designs, though challenging, would treat 'clone' or 'population origin' as a random effect in linear mixed models, providing reasonable estimates of variation in key response variables across the populations of interest[66].

Regulatory agencies, particularly those in Europe and North America, recognize that LC50 values derived from genetically heterogeneous populations will differ across local, regional, or global scales. There is a general understanding that each geographic scale presents its own sources of variation and shapes the requirements in terms of the sampling required to achieve the stated objective of a meaningful safety limit recommendation based on an LC50. However, the approach thus far has been to apply uncertainty factors to account for these sources of variation—a strategy criticized for its arbitrariness and lack of ecological evidence[22,23]. In fact, there have been no systematic efforts to quantify the magnitude of intraspecific variation in toxicological responses that could allow for any empirically derived estimate to contribute to uncertainty factors. We acknowledge our experimental work only focused on one local population, which was reported to capture the level of genetic diversity representative across broader geographical patterns[39]. Nonetheless, testing multiple genotypes within a single population (at local/regional scales) or across multiple populations of genotypes (at international scales) and reporting CIs of LC50 values calculated from each would represent a significant advancement. This approach would provide both a more accurate and robust measure of the LC50, accounting for genetic variation and providing an indication of uncertainty arising from biological differences in toxicity responses. Reporting LC50s could then include both the estimate and the variance around this estimate stemming from genetic variation.

We acknowledge that uncertainty factors in ERAs account for a wide array of sources of variation, not limited to genetic diversity. These include differences in exposure scenarios, interspecies extrapolation, and limitations in data collection, among others. For instance, real-life environmental conditions often differ significantly from controlled laboratory settings, adding additional layers of complexity[67]. Likewise, uncertainty factors aim to address knowledge gaps when extrapolating toxicity data across species or geographic regions. While genetic variation is only one component, it is an underexplored source of variation in current risk assessments, and its inclusion can provide a more ecologically relevant picture of how populations will respond to toxicants in natural settings. Empirical efforts to systematically quantify the contribution of intraspecific variation to overall uncertainty remain rare. By incorporating genetic diversity estimates together with other relevant uncertainty factors, we can refine risk assessments and enhance the accuracy of safety limit recommendations, ultimately creating a more robust framework for translating laboratory findings to real-world ecological scenarios. Future work should strive to quantify the relative contributions of genetic variation compared to other sources of uncertainty, helping to position this biological factor within the broader context of ERA uncertainty.

Our study demonstrates that considerable variation is found in toxicological responses within a population. However, it is important to consider the scale and scope of biological variation. While current methods often use a single surrogate species to represent broader phylogenetic categories—such as entire family or order[67]— this approach is useful but may overlook meaningful biological variation at different levels of organization. Future work should examine variation across multiple biological scales, including within populations, across populations, between congeners, and across genera. Such investigations are critical for parsing the levels of biodiversity most relevant for risk assessment and management actions. Identifying the biological scales at which the largest fraction of functional variation occurs has proven transformative in ecology[68] and adopting a similar approach could lead to major advancements in ERA.

The substantial genomic variation observed across clones in our study highlights the extensive genetic diversity present within a single population of *Daphnia*. Genetic variation is both a primary source for phenotypic variation and is the substrate on which natural selection can act to drive rapid adaptation[10]. Prior research on *Daphnia* has suggested that rapid adaptation could be a critical mechanism of suppressing *Microcystis* blooms[52]. Field populations of *Daphnia* have shown rapid adaptation to tolerate *Microcystis* and can suppress the biomass of HABs that contain lethal concentrations of microcystins (3 to 6.5 μg L^{-1})[53]. These findings underscore the need for toxicological theory to integrate genetic diversity, and potentially rapid adaptation. It is important to recognize that dose-response relationships in routine toxicity testing may differ between chronically

exposed[40,52,53,69] and unexposed[3,70] populations due to genetic variation. It is clear that incorporating genetic diversity into toxicological assessments and ERAs are crucial for accurately understanding the responses of populations to toxicants (as evidenced by Fig. 2), but the level of genetic variation that provides the most insight for predicting population responses is less clear.

Genomic data has been transformative for toxicology, as toxicogenomics has revolutionized the identification of modes of action[5,25]. Yet, the application of genomic information to improving predictions of population-level effects[71], including of toxicant exposures, is less clear[31]. Our experiments and genomic sequencing demonstrate that genome-wide diversity is not associated with survival and reproduction, potentially fitting with the neutral theory of molecular evolution as a primary driver of overall genetic diversity[57,72]. Gene-level understanding of the genetic basis of resistance is viewed as a major goal in toxicology[73] and association genomic techniques are an increasingly common tool in toxicology[74]. However, generating the resolution needed to identify causal genomic regions, particularly specific allelic variants, demands a herculean effort, a relatively simple genetic basis, and often relies on some degree of recombinational luck—factors more likely to yield meaningful results in model systems. Moreover, there is considerable uncertainty whether genotype-phenotype relationships as measured in laboratory conditions are predictive in natural environments[34].

While there is considerable interest in understanding the genetic basis of microcystin resistance in *Daphnia*[58,59,75], our investigation into the link between variation in genes putatively involved in resistance and a clone's ability to survive and reproduce when exposed to *Microcystis* yielded no significant relationship (Table S5; Supporting Information, Dataset S1). Although Clone 4 exhibited distinct phenotypic and genotypic characteristics for putatively functional loci, it harbored a private haplotype in a region of the tyrosine-protein kinase ABL1 containing 17 singleton SNPs over a 5 kb window. However, given this clone possessed > 22,000 unique singleton SNPs, any inference regarding the function of specific genetic variants remains highly speculative.

Moreover, we saw no relationship between genetic variation at candidate loci and the effects of *Microcystis* on survival or reproduction (Fig. 3). Given the complex and environmentally variable genetic architecture for many traits, our data advocate for a focus on genetic studies at the clonal, family, or population levels in research supporting the ERA process. Explicitly incorporating information on origin into study designs and statistical models can yield powerful insights without requiring major changes in the methods most often employed in the discipline.

A key novel aspect of our study is the focus on eight gene regions previously implicated in *Daphnia* transcriptomic responses to microcystins (Fig. 3c). These regions were carefully selected based on prior research demonstrating their role in mediating responses to microcystin exposure[58,59]. Our analysis using these gene regions did not find significant genotype-phenotype linkages, suggesting that transcriptomic markers may not always predict phenotypic responses to toxicants. Given the complex and environmentally variable genetic architecture for many traits, our data suggest that genetic studies focus at the clonal, family, or population levels in research supporting the ERA process.

Studies on genetic variation have significantly advanced our understanding of ecological and evolutionary processes[74]. The case for incorporating genetic variation, and to some extent evolution, into ERAs is becoming increasingly compelling as it is recognized that meaningful levels of intraspecific genetic variation exist in key toxicological traits[24,37,63,76–78]. Neglecting genetic variation can result in inaccuracies in reporting LC50 toxicity thresholds[37], potentially undermining safety measures upheld by chemical producers and other stakeholders concerned with biodiversity preservation. In our study, we observed substantial genotypic and phenotypic differences in *D. magna* tolerance to the aquatic contaminant *M. aeruginosa* across multiple life-history traits. Moreover, significant interactions between clonal identity and body growth or reproductive output were observed among genotypes exposed to non-toxic versus toxic treatments. These findings suggest that genetic diversity can profoundly influence population dynamics both in the presence and absence of toxicant exposure. Through simulations and bootstrapping approaches, we further demonstrate that the common practice of using a single genotype to calculate toxicity tolerance in survival results in estimates falling outside of the 95% confidence interval more than 50% of the time. We propose that integrating intraspecific genetic variation into routine laboratory toxicity studies would improve the reliability and biological realism of LC50 calculations, thereby improving ERAs.

Methods
Daphnia magna field collection and culturing
Twenty distinct genotypes of *D. magna* were collected from 'Langerodevijver' (LRV; 50° 49' 42.08", 04° 38' 20.60"), a small lake (surface area = 140,000 m^2, max depth = 1 m) within the nature reserve of Doode Bemde, Vlaams-Brabant, Belgium[79], in late spring and early summer. LRV has a single basin and has seasonal *Microcystis* HABs typical to many lakes globally and has a large resident population of *D. magna*. This lake was chosen based on prior work across multiple *D. magna* populations (spanning less than 100 m to more than 500 km) demonstrating 20 clones from a single population can contain sufficient genetic diversity to test our hypotheses[39]. Each of these 20 genotypes was maintained as a separate parthenogenetic line for over five years (approximately 300 generations) in continuous cultures at 20 ºC in UV-filtered dechlorinated municipal tap water containing 2 mg C L^{-1} of the green alga *C. vulgaris* (strain CPCC 90; Canadian Phycological Culture Centre, Waterloo, ON, Canada). *C. vulgaris* was grown in COMBO medium [80].

To prepare for this study, we isolated one adult female *D. magna* per genotype in separate 50-mL glass tubes inoculated with COMBO medium and *C. vulgaris* at 2 mg C L^{-1}, and monitored them daily for reproduction. *D. magna* neonates born within 24 h were collected from their respective genotypes and individually separated into 50-mL glass tubes as previously described, totalling 10 replicates per genotype and 200 tubes total. These 200 *D. magna* representing 20 genotypes were the founding mothers of this study. All *D. magna* were incubated under

constant conditions (temperature of 21 ± 1 ºC, cool-white fluorescent light of 600 ± 15 lx, with a photoperiod of 16:8 h light: dark).

Microcystis aeruginosa culturing

Following our previously described method[81], *M. aeruginosa* (strain CPCC 300; Canadian Phycological Culture Centre, Waterloo, ON, Canada) was cultured in BG-11 media and kept in a growth chamber under axenic conditions with a fixed temperature of 21 ± 1 ºC, cool-white fluorescent light of 600 ± 15 lx, with a photoperiod of 16:8 h light: dark. The culture was grown for a minimum of one month before preparation for the chronic study. *M. aeruginosa* CPCC 300 produces microcystins-LR (CAS: 101043-37-2, $C_{49}H_{74}N_{10}O_{12}$) and its desmethylated form [D-Asp³]-microcystin-LR (CAS: 120011-66-7, $C_{48}H_{72}N_{10}O_{12}$), which occur widely in freshwater ecosystems[44,46] and are toxic to zooplankton.

To prepare *M. aeruginosa* for testing on *D. magna*, an aliquot of the stock was inoculated in 100% COMBO medium for two weeks prior to test initiation and cultured to a cell concentration of $1.25 \pm 0.02 \times 10^7$ cells mL⁻¹. This medium was chosen because it supports the growth of algae and cyanobacteria and is non-toxic to zooplankton [80].

Chronic toxicity study

A 14-d chronic toxicity study was conducted to assess the effects of toxicants on survival, body growth, reproductive output, and the time to first brood of *D. magna*. Three treatments were included in this study: 100% chlorella (non-toxic diet), 2:1 chlorella: microcystis (moderately toxic diet), and 1:1 chlorella: microcystis (severely toxic diet). Following these ratios all *D. magna* were fed 2 mg C L⁻¹, corresponding to 3×10^6 cells total, which corroborates with previous literature exposing daphnids to dietary combinations of green algae and cyanobacteria[51,54,82].

D. magna neonates born within 24 h were collected from their respective genotype, and 10 neonates were individually placed in 50-mL glass tubes containing 50 mL of UV-filtered water and its respective treatment. To ensure no systematic differences in ages between *D. magna* neonates that could drive variation in responses within and across clones, a stringent protocol was implemented to remove neonates twice daily, in the morning and at night, in preparation for the toxicity test. The UV-filtered water met the validity criteria for the test following an accredited method for *D. magna* culturing and toxicity testing[83]. A total of 600 animals were used for this study (i.e., 10 replicates per genotype, 200 animals per treatment). Since this was a semi-static test, solutions were renewed 3 × wk on Mondays, Wednesdays, and Fridays by transferring *D. magna* from old to new glass tubes, followed by supplying each *D. magna* with 3×10^6 cells of food (e.g., the 2:1 treatment received 2×10^6 *C. vulgaris* cells and 1×10^6 *M. aeruginosa* cells, corresponding with 2 mg C L⁻¹). Survival, body growth, reproductive output, and number of broods were recorded to assess interactions between genotype and treatment effects. Body growth (mm) for each replicate was measured on days 0, 7, and 14. The study was incubated under 400–800 lx cool-white fluorescent light at 20 ± 1 °C with a 16:8 light: dark cycle.

Genomic sequencing and analysis

Pools of individuals were collected from each clone and DNA was extracted using the QIAGEN MagAttract HMW DNA kit. Extractions were checked for DNA concentration using a Qubit 2.0 fluorometer and libraries for each clone were prepared using the KAPA Hyper Prep kit. The mean DNA concentration and mean total quantity of DNA used for library construction was 6.0 ± 0.4 ng μL⁻¹ and 304.3 ± 15.6 ng, respectively. Sequencing of these libraries was generated on a NovaSeq 6000 generating 150 bp paired-end reads. Sequences were then trimmed using *trimmomatic/0.39* using a quality threshold of 20 and a min length of 50 and subsequently mapped to the *D. magna* reference genome (GCA_020631705.2) using *bwa/0.7.17* (default parameters). Duplicates were marked using *Picard tools/2.21.2* and then the bam files were input into *freebayes/1.2.0* to generate a VCF of all variant positions among all individuals on the 10 *D. magna* chromosomes (not scaffolds). *Vcftools* was then used to trim to a minimum quality score of 30. This produced a whole genome dataset with an average genome sequencing coverage of 29.5 (min = 26.6, max = 35.5, SE = 0.34) for each of the 20 clones. The total number of variant sites and the number of heterozygous sites for each clone was extracted using *VCFtools*. Genetic distance was calculated using *VCF2dis* (https://github.com/BGI-shenzhen/VCF2Dis). Genotype likelihood (beagle) files were generated using *angsd* (-GL 1 -out D23Genolike -doMaf 2 -SNP_pval 1e-6 -doMajorMinor 1 -doGlf 2), and *pcangsd* was used to generate principal component analysis. A heatmap of pairwise genetic distances between clones was additionally constructed and provides a visual representation of this variation (Figure S3; Supporting Information, Dataset S1). The genetic distance was calculated as the proportion of SNPs that differed between two clones, with darker red shades in the heatmap indicating fewer shared SNPs, and lighter shades signifying more similarity.

Water sampling for analysis

Water samples were collected from each treatment at test initiation, solution changes, and termination to quantify the cell concentration, fluorescence, and standard water parameters and were quick-frozen at -80 °C before analysis for microcystins and oligopeptides (Table S1; Supporting Information, Dataset S1).

Chemicals and reagents for water analysis

Water, acetonitrile, methanol, acetic acid, and formic acid were all Optima LC/MS grade solvents and purchased from Fisher Scientific (Tewksbury, MA, USA). Microcystin standards D-Asp³-RR, MC-RR, YR, HtyR, LR, D-Asp³-LR, HilR, WR, LA, LY, LW, and LF, as well as nodularin and cylindrospermopsin were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Microcystin Dha⁷-LR was purchased from National Research Council Canada (Ottawa, ON, Canada). Microcystin Leu¹-LR was purchased from GreenWater Laboratories

(Palatka, FL, USA). D-Asp^3-E-Dhb^7-RR was purchased from Sigma Aldrich. Anabaenopeptin A and B, oscillamide Y, oscillaginin A, microginin 690 methyl ester, aeruginosamide B and C, cyanopeptolin 1040 MB and 1007, and micropeptin 1106 were purchased from MARBIONC (Wilmington, NC, USA). Anabaenopeptin F was synthetically created by the Stockdill laboratory at Wayne State University (Detroit, MI, USA)[15]. N MC-LR was purchased from Cambridge Isotope Laboratories, INC. (Tewksbury, MA, USA).

Microcystin and cyanopeptide water analysis

Microcystins were measured using a method based on the US EPA method 544 [84]. Quality assurance and quality control met the criteria set by the US EPA method 544. The standard curve range, retention time, and linearity, as well as the quantitative and qualitative ions that were used for further identification of the microcystins were also reported (Table S2; Supporting Information, Dataset S1). Surrogate recovery for the samples were within the US EPA method 544 limit of 60–130%, and the control standards were within the limits of 70–130% set by this same method. All duplicates relative % differences were calculated to be < 30%.

Microcystin and cyanopeptide standards and samples were analyzed using a Thermo Scientific TSQ Altis™ triple quadrupole mass spectrometer (Waltham, MA, USA) with a TriPlus™ RSH EQuan 850 system. Both online concentration analysis and direct inject analysis were completed[84]. Quantitative results shown use the direct inject analysis method. Detection limits for the online concentration method were between 0.5 and 5 ng L^{-1}, and the standard curve range was between 0.5 and 500 ng L^{-1}.

For direct injection analysis, 10 μL injections of standards and samples were loaded onto a Thermo Hypersil GOLD™ C18 2.1 × 50 mm, 1.9 μm column using a TriPlus RSH autosampler equipped with a 100 μL sampling syringe. The analytical separation gradient consisted of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). At a flow rate of 0.5 mL/min, the gradient started at 24% B and was held for 0.7 min. It was then ramped to 26% B from 0.7 to 1.7 min, from 26 to 50% B from 1.7 to 5.5 min. The column was washed at 98% B from 5.51 to 6.5 min at a flow rate 1.0 mL/min, then brought back down to original conditions to equilibrate. The total run time was 8.5 min. The column oven temperature was held at 35 °C. The standard curve was prepped in LC-MS water and the range measured between 0.05 and 50 μg L^{-1}.

Mass spectrometry analysis was performed using electrospray ionization source (ESI). The MS source settings were as follows: spray voltage 3500 V, ion transfer tube temperature at 325 °C, vaporizer temperature at 350 °C, sheath gas 35 (arbitrary units), auxiliary gas 10 (arbitrary units), sweep gas 0 (arbitrary units), collision gas at 1.5 mTorr, and Q1/Q3 resolution set to 0.7 Da.

Control samples and standards were performed every 20 samples during each run. Control standards included negative control (blank) and positive control (10 ppb standard) as well as duplicate and fortified samples. For each standard, a quantitative and qualitative ion transition were chosen for use with selected reaction monitoring (SRM).

Selective ion mode analysis

For selective ion mode (SIM) analysis, the following masses (Table S3; Supporting Information, Dataset S1) were investigated because they are known to be produced by *M. aeruginosa* CPCC 300 (Racine et al. 2019). The gradient for the method consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). At a flow rate of 0.35 mL min^{-1}, the gradient started at 0% B and was held for 1.0 min. It was then ramped to 60% B from 1.0 to 8.5 min. The column was washed at 98% B from 8.51 to 10.5 min at a flow rate of 0.6 mL min^{-1}, then brought back down to original conditions to equilibrate. The total run time was 14 min. The injection volume was 10 μL and the column oven temperature was held at 35 °C. Mass spectrometer source settings were the same as the quantitative method above. The SIM method was accomplished in Q3. The area under the m/z peak was calculated and reported.

Testing criteria and toxin analysis

Measured microcystin concentrations were 3.3 ± 0.001 μg L^{-1} and 5.1 ± 0.001 μg L^{-1} in the 2:1 and 1:1 toxic treatments, respectively (Table S1; Supporting Information, Dataset S1). Water parameters before and after solution renewals were also within the test criteria (Table S4; Supporting Information, Dataset S1). There were no signs of hypoxia stress on *D. magna* behavior. Following the criteria for survival and reproduction of controls, all test acceptability criteria were met. Mortality and immobilization were first observed within 48 h after microcystin exposure in either treatment, and continued throughout the test. This was expected as the microcystin concentrations chosen can be sublethal[54] to lethal[55,56] in *Daphnia* laboratory assays and are commonly measured in freshwater ecosystems with HABs[46].

Statistical analysis

For survival, growth, and neonate production
To test the magnitude of clonal variation, we used a Linear Mixed Effects (LME) model (or a Generalized Linear Model (GLM) for binomial survival data) for each response. 'Clone' was treated as a random effect, and we tested for significant differences in model fit with and without 'clone' being included as a random effect[85]. Second, to determine whether the concentration of Microcystis influenced the magnitude of clonal variation, we conducted Brown-Forsythe tests to assess the homogeneity of variances for each response variable across different clones. These Brown-Forsythe tests were done sequentially—meaning comparisons were between *Chlorella* and 2:1, then 2:1 and 1:1. Finally, to test for interactions between response variables across *Microcystis* concentrations we used linear models with concentration and clone as fixed effects (*glm* with binomial for survival, *lm* for growth, and *glm* with a gamma distribution for neonate production).

Association between clonal genomic diversity, genomic divergence, and phenotypic distance
We tested for an association between the amount of genetic diversity found across clones and clonal survival and reproduction across each of the three diet conditions. To do so we regressed the total number of heterozygous sites and each response variable for each rearing condition. To test for associations between genomic diversity and phenotypic distance, we first generated distance matrices from mean values for all 20 clones. The genomic distance matrix was constructed from all polymorphic sites. The phenotypic distance matrix was constructed using neonate production, time to first brood, total broods, week one growth (day 0–7), week two growth (day 7–14), week one survival, and week two survival for each clone for each diet condition. A two-tailed Mantel test was then conducted to determine whether there was a significant correlation between the matrices that differed from 0. The Mantel test is useful because it assesses the relationship between two distance matrices, allowing us to determine if genetic diversity (measured by genomic distance) is related to phenotypic variation (measured by phenotypic distance) across different diet conditions. A correlation different from 0 would indicate that genetic diversity within clones is associated with differences in their phenotypic traits, suggesting that genetic variation may contribute to the observed phenotypic responses.

We conducted a similar set of analyses exclusively using genes previously identified as involved in microcystin tolerance. These two unique *Daphnia* studies identified potential candidate genes for microcystin tolerance in *Daphnia* through a combination of differential expression analysis (RNAseq) and employing functional annotations[58,59]. We searched the gene names provided by the authors in the NCBI database. If only a single gene region matched the name we included it in our downstream analysis. For gene names that had multiple matches we selected from the most exact. Finally, when authors provided primer regions we used ePCR to identify the gene region that would be amplified in the reference. Using these methods we were able to locate 8 potential candidate loci for genetic variation in microcystin tolerance. We then identified all variant sites across the 20 *Daphnia* clones within these eight candidate gene regions, including 1,000 bp up and downstream of the demarcated gene, for each of 20 clones. We constructed a PCA using this data, conducted a two-tailed Mantel test to determine whether there was significant correlation between differences at these loci and phenotypic variation, and tested for associations between the number of variants in these eight genes and survival and reproduction in high (1:1) *Microcystis* conditions (Table S5; Supporting Information, Dataset S1).

Examining the effects of assays with reduced diversity
We sought to determine how the choice to conduct lower diversity assays might influence the estimate of the effects of the toxicant on reproduction and survival. For reproductive output we calculated means and standard errors for all 20 clones and for 15, 10, 5, 3, and 1 clonal diversity groups created by subsetting the full dataset 100 times at each diversity level. For survival, we calculated the 95% CI of survival probability using the means of all 20 clones. We then created subsamples containing 15, 10, 5, 3, and 1 clones and determined how frequently they would produce survival estimates within the 95% CI obtained from the full dataset.

All analyses were completed in R version 4.2.2[86]. The data supporting the results are archived in the public repository Dryad under DOI https://doi.org/10.5061/dryad.5tb2rbpck (https://datadryad.org/stash/share/TCmFLjtyfP6G0hhmx5iCvMYu4eaAx9I7m_Vol4kl3ew).

Data availability
The data supporting the results are archived in the public repository Dryad under DOI 10.5061/dryad.5tb2rb-pck.For peer review, click here: https://datadryad.org/stash/share/TCmFLjtyfP6G0hhmx5iCvMYu4eaAx9I7m_Vol4kl3ew.

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Data accessibility
The data supporting the results are archived in the public repository Dryad under DOI https://doi.org/10.5061/dryad.5tb2rbpck.

For peer review, click here: https://datadryad.org/stash/share/TCmFLjtyfP6G0hhmx5iCvMYu4eaAx9I7m_Vol4kl3ew.

Statement of authorship
R.S.S., S.M.R., L.D.M., and J.M.F. designed the research. R.S.S. and S.M.R. conducted the experiments. J.A.W. and X.W. conducted supporting data collections. S.M.R., R.S.S., and C.I.C. analyzed the data. R.S.S. and S.M.R. wrote the paper with insights from L.D.M., C.I.C., and J.M.F. All authors read, amended, and approved the final manuscript.

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R.S.S., S.M.R., L.D.M., and J.M.F. designed the research. R.S.S. and S.M.R. conducted the experiments. J.A.W. and X.W. conducted supporting data collections. S.M.R., R.S.S., and C.I.C. analyzed the data. R.S.S. and S.M.R. wrote the paper with insights from L.D.M., C.I.C., and J.M.F. All authors read, amended, and approved the final manuscript.

Declarations

Competing interests
The authors declare no competing interests.

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