Variation of Tolerance to Isothiazolinones Among Daphnia pulex Clones

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Variation of Tolerance to Isothiazolinones Among *Daphnia pulex* Clones

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Abstract: Isothiazolinones are a family of broad-spectrum biocides widely used in industry and consumer products. Chloro- and methyl-isothiazolinones (CMIT and MIT) are documented as strong irritants, yet they are still used in a wide variety of applications, including cosmetics, cleansers, hygiene products and various industrial applications. The subsequent substantial release of these molecules from urban sources into freshwater environments, and their potential impacts on aquatic species, have nevertheless received little attention so far, with few studies reporting on the toxicity of either CMIT or MIT to non-target organisms. The present work addresses this current knowledge gap by evaluating CMIT/MIT (3:1) and MIT acute toxicity to *Daphnia pulex* (Cladocera), the two formulations most commonly used by manufacturers. Additionally, genetic diversity is known to be a major component of

variability in phenotypic responses, although it is largely overlooked in typical toxicity tests. Subsequently the potential range of responses inherent to genetic diversity is rarely considered. Therefore, to account for intraspecific variation in sensitivity, the design involved eight clonal lines of *D. pulex* stemming from distinct natural populations or commercial strains. Clones exhibited strong variation in their responses, with lethal concentrations (LC_{50}) ranging from 0.10 to 1.84 mg/L for the mixture CMIT/MIT, and from 0.68 to 2.84 mg/L for MIT alone. These intraspecific ranges of LC_{50} challenge the use of single clones of daphnids in standard ecotoxicological tests and the predictions based on their results. The present study brings new evidence that assessing ecological risk of chemicals while ignoring genotype diversity is neither ecologically relevant, nor a representative evaluation of the diversity of potential adverse outcomes.

Keywords: Aquatic invertebrates; Emerging pollutants; Evolutionary ecotoxicology; Freshwater toxicology; Risk extrapolation

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INTRODUCTION

Emerging pollutants are chemicals of various origins present in the environment that are not commonly monitored, and have not been studied in depth (Geissen et al., 2015). Their potential adverse effects on human health and ecosystems require action to be taken in favour of updated monitoring programmes and risk assessment tools (Geissen et al., 2015). Emerging contaminants include a wide variety

of compounds such as pharmaceuticals, surfactants, pesticides, and disinfection by-products.

Isothiazolinones are broad-spectrum biocides used in many industries due to their efficacy against myriad microorganisms (Williams, 2007). Methylisothiazolinone (MIT, CAS n°2682-20-4) and chloro-methylisothiazolinone (CMIT, CAS n°26172-55-4) are two such molecules that can be found in cosmetics, household products, paint formulations, and industrial water treatment (Williams, 2007). CMIT and MIT are two of the most commonly found isothiazolinones on the market, and MIT was found to be the second most abundant biocidal active substance after citric acid in a review of 2963 products in 131 households of Northern Germany (Wieck et al., 2016). CMIT and MIT are two of the most commonly found isothiazolinones. They are sold notably in mixture in ratios of 3:1 (CMIT:MIT) as Kathon^{TM} (DuPont), and in several formulations of Acticide® (Thor GmBH). Until the 2000s, CMIT and MIT could not be synthesized separately, but as this process has been optimised, MIT – the less toxic of the two – is increasingly used on its own (Silva et al., 2020). Conversely, CMIT is too unstable to be used in isolation from MIT.

As for many biocides, isothiazolinone leaching from building materials or urban wastewaters first affects freshwater environments (Wittmer et al., 2011; Bollmann et al., 2017; Paijens, Bressy, et al., 2020). Bester et al. (2014) reported leaching of isothiazolinones in very high concentrations in run-off waters, up to 30 mg/L of MIT from acrylate renders. Indeed, since MIT has a poor affinity with microbial cells compared to other isothiazolinone molecules, it consequently requires higher doses for antimicrobial activity (Williams, 2007). In addition, due to its reduced toxicity, MIT is allowed and present in more numerous products than CMIT

(ECHA, 2015). Therefore, the benefit of using a less toxic molecule comes at the cost of a higher level of environmental contamination. Moreover, though CMIT is more toxic, it is also more quickly degraded than MIT, which is not readily biodegradable and thus more persistent in surface waters (Baranowska & Wojciechowska, 2013). Yet, like most other emerging pollutants, the presence of CMIT and MIT in the environment is not routinely monitored at present, regardless of the potential environmental hazards these molecules represent.

Isothiazolinones are remarkably efficient as biocides against microorganisms, but this efficacy also portends their potential as human and environmental hazards (Kresmann et al., 2018; Da-Silva-Correa et al., 2022). Whilst sensitizing and allergenic effects of CMIT and MIT on human health have been well established, and have led to the restriction of their concentrations in personal care products (ECHA, 2015; Kim et al., 2019), CMIT and MIT toxicity on aquatic organisms have received less attention (ECHA, 2014, 2015). Known sublethal effects of MIT include delayed healing and impaired tissue regeneration in *Dugesia japonica* (planarian) and *Xenopus laevis* (amphibian), and in fishes, histopathological effects in *Oncorhynchus mykiss*, and developmental deficiencies in *Danio rerio* (Delos Santos et al., 2016; Capkin et al., 2017; Van Huizen et al., 2017; S. Lee et al., 2022). CMIT/MIT mixture has also been documented to induce brain damage, multiple morphological issues and decreased locomotion behaviour in *Danio rerio* (Cho & Kim, 2020; Chatterjee et al., 2021). To our knowledge, published results on CMIT/MIT acute toxicity to aquatic organisms are limited to those of Hu et al. (2014), who reported a median lethal concentration (LC_{50}) of 0.41 mg/L [0.33-0.49] (at 25°C) after 48h of exposure in the grass carp, and of Chatterjee et al. (2021) with a 96h-LC_{50} of 0.44 mg/L [0.37-0.50] in zebrafish embryos. MIT acute toxicity seems to vary widely across aquatic

invertebrate species, e.g., from 0.8 mg/L [0.55-1.19] in *Daphnia similis*, 2.06 mg/L [1.85-2.28] in *Dugesia japonica*, and up to 84.48 mg/L [70.70-100.94] *Neocaridina denticulate* (shrimp) (Li, 2019). Likewise, within species variation in sensitivity to MIT was also found to be substantial in *Daphnia magna*, with reported 48h-LC_{50} values ranging from 0.51 mg/L [0.46-0.57] (Kresmann et al., 2018) to 2.1 mg/L (Li et al., 2016). Moreover, LC_{50} values estimated in 5 planarian species after 6 hours of exposure ranged from 4.49 to 8.06 mg/L, supporting the idea that MIT lethality does not occur early upon exposure, at least in planarians (Van Huizen et al., 2017). Data on MIT toxicity to non-animal taxa are also scarce. In the microalgae *Scenedesmus* sp., MIT EC_{50} (effective concentration) was 1.0 mg/L for growth inhibition (Wang et al., 2018). Non-target prokaryotes may also be impaired. In wastewater-treatment settings, Amat et al. (2015) and Zeng et al. (2020) both reported a negative impact of MIT shocks on nitrification activity, with a modified composition of bacterial community in activated sludge. Given the extensive range of potential effects and affected organisms, and the scarcity of available data, it is crucial to expand our knowledge about environmental concentrations of isothiazolinones and their concomitant toxicity to freshwater ecosystems (Kresmann et al., 2018).

In addition to this lack of knowledge, it should be noted that ecotoxicity testing based on standard guidelines still suffers from a lack of ecological relevance, for several reasons. First, standardized bioassays tend to oversimplify environmental conditions and make results difficult to extrapolate to higher levels of biological organization (Crane et al., 2007; Forbes et al. 2008). Second, as genetic variability is deliberately disregarded in standard testing, evolutionary processes induced by toxicants cannot be addressed using current procedures of ecological risk assessment, heedless of increasing documentation and awareness of such potentials impacts (e.g.,

Medina et al., 2007; Coutellec & Barata, 2013; Weston et al., 2013; Oziolor et al., 2016; Brady et al., 2017). Genetic variation is indeed predicted to correlate positively with population adaptive potential (see Willi et al., 2006). In accordance with this, Loria et al. (2022) showed that more genetically diverse populations of daphnids persisted longer under copper stress. Also, as reported above in *D. magna* exposed to MIT (Li et al., 2016; Kresmann et al., 2018), the occurrence of variation in sensitivity between clones or genotypes points to the risk of biased assessment when based on single-clone testing of species sensitivity and derived standard parameters (e.g., species sensitivity distribution; Posthuma et al., 2001).

Among freshwater organisms, daphnids are models of longstanding use in ecotoxicity testing (see e.g., OECD guidelines 202 and 211), notably due to their easy culture and short generation time, as well as their ecological status as keystone species in freshwater ecosystems. Here we assessed the toxicity of isothiazolinones to *Daphnia pulex* while emphasising within-species variation in sensitivity to highlight the importance of genetic diversity. To this end, the acute toxicity of MIT and of the mixture CMIT/MIT (ratio 3:1) to *D. pulex* was compared across eight clonal lineages stemming from various genetic and eco-evolutionary backgrounds. We hypothesized the lineages' 48h-LC_{50} for MIT to be ranging between 0.5 and 2 mg/L, as previously found in *D. magna and D. similis* (see above). We expected that CMIT/MIT would induce higher mortality than MIT alone at similar doses and that clones showing higher tolerance to MIT would also be more tolerant to the mixture.

METHODS

Study clones and pre-experimental conditions

D. pulex clonal lineages established in the laboratory originated from several populations located in Brittany, France, as well as a laboratory culture provided by

Aqualiment© (Supplementary Table S1). From each source, a single wild-caught individual was isolated and allowed to reproduce parthenogenetically to establish a clonal lineage. Lines were propagated for one year before biocide exposure. The clones were kept in dechlorinated tap water under standardized conditions (18°C, 16:8 L:D photoperiod) and fed with a mixture of two freshwater microalgae, *Chlorella vulgaris* and *Desmodesmus subspicatus*. Eight lines were selected (Supplementary Table S1) based on their potential differential response to biocide exposure (as estimated from preliminary tests), and their stability under lab conditions.

Experimental design, biocide exposure

MIT (95%; CAS 2682-20-4) and CMIT/MIT (secondary standard at 1.5% purity with a 3:1 ratio; CAS 55965-84-9) were purchased from Sigma-Aldrich. Pilot (i.e. range-finding) experiments were conducted on batches of five-to-ten neonates of each line to assess the targeted range of toxicant concentrations. This range would include at least one low dose to ensure survival, and one high dose to induce death in all lines, with a minimum of three intermediate doses, in order to interpolate reasonable LC_{50} values. Following pilot experiments, the next two series of nominal concentrations were prepared by dilution in filtered dechlorinated tap water, at the earliest two hours before the start of exposures: 0.25, 0.5, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.75, 3.0, 3.25, 3.75, and 5 mg/L for MIT and 0.06, 0.12, 0.15, 0.18, 0.24, 0.30, 0.36, 0.42, 0.5, and 0.6 mg/L for CMIT/MIT. Nominal concentrations were corrected by effective concentrations for statistical analyses.

At the start of the survival assessments, daphnia neonates (aged less than 24h) were isolated in a volume of 8 mL of control or treated water (n=50) in borosilicate glass test tubes (16 mm diameter). Renewal of the medium was performed after 24h of exposure, so as to mitigate biocide degradation and ensure a variation of less than

20% in concentration during the bioassay (see OECD (2004) and Table S3). Individuals were kept at the same temperature and photoperiod as culture conditions, but were not fed during the bioassay. Survival of each individual was examined after 24h and 48h of exposure, and tubes containing dead daphnids at 24h were emptied without renewal of the medium. Mortality was assessed using immobilization as a proxy (no observable movement for 20 seconds as recommended by OECD (2004)). The standard mortality criterion for test validity (≤ 10% of mortality in controls) was verified.

Analysis by UPLC-MS/MS

UPLC-MS/MS has been successfully used for the determination of isothiazolinones in multiple matrices (Silva et al., 2020). In the present assay, the method was developed using an Ultra-Performance Liquid Chromatograph (Acquity, Waters) coupled with a quadrupole time-of-flight mass spectrometer (XEVO G2XS QTof, Waters). For the sake of sensitivity, repeatability, and reproducibility, the final measurements were performed on a triple-quadrupole mass spectrometer (XEVO TQD, Waters). For each treatment concentration, three random samples of contaminated water were collected at 0 and 24h, then pooled per time by concentration and filtered (GF/CA, 0.22µm, Phenomenex). An isotopically labelled internal standard (2-Methyl-d3-4-isothiazolin-3-one hydrochloride (MIT-D3), analytical standard, Sigma-Aldrich; CAS 1329509-49-0) corresponding to both target compounds was added before injection. Each sample was injected ten times.

Separation was performed with an Acquity UPLC BEH C18 column (50 x 2.1 mm inner diameter, 1.7 µm particle size) from Waters, using a flow rate set at 0.7 $mL.min^{-1}$, column temperature of 30°C and injection volume of 5µL. The mobile phase through gradient elution was prepared by 0.1% formic acid in water (A) and

0.1% formic acid in acetonitrile (B), using UPLC grade solvents. The mobile phase was initially started at 5% B at 0 min and increased to 15% B within 2.9 min, and then held at 5% until 3.5 min. Electrospray ionization was performed in positive mode with a capillary voltage of 3kV and a cone voltage of 30V. The acquisition was achieved in multiple reaction monitoring (MRM) mode, with a time scan of 0.25s. Collision energy and obtained values of the precursor and product ions are presented in Supplementary Table S2.

Mass spectral data acquisition and integration were respectively conducted with MassLynx® and TargetLynx® softwares (v.4.2, Waters). Measurement of effective concentration is described further in supplementary material (Supplementary Table S3), with calibration curves built from six concentrations including blanks. For samples with both molecules, CMIT/MIT concentration was quantified by adding CMIT and MIT individual values.

Survival analysis

All analyses were conducted in R version 4.0.0 (R Core Team, 2020). Average toxicity of CMIT/MIT and MIT was evaluated by modelling survival as a function of contaminant concentration, incorporating random variation amongst clonal lines and replicate tests. We fit generalized linear mixed models (GLMM) implemented in the 'MCMCglmm' package (Hadfield, 2010), using a logit link function and a binomial error distribution, with Markov chain Monte Carlo estimation. We compared a series of nested models, beginning with a maximal model including covariance between the intercept and coefficient terms of the logit model for both random effects, and proceeding with progressively less complex structures for each of the random terms. Ultimate model selection was based on minimizing the deviance information criterion

(DIC). Models were run with a burn-in of 500 000 iterations, followed by 100 000 iterations from which each 100th point was sampled from the Markov Chain.

To test for differences between clonal lineages, additional GLMMs were run with both lineage and contaminant concentration allocated as fixed effects, and with random variation amongst replicate tests. We began with a model including both fixed effects and their interaction, followed by a model excluding the interaction term, and finally one including only contaminant concentration; note that all models shared the same random effects structure. These models were run with a burn-in of 200 000 iterations, followed by 100 000 iterations from which 1000 points were sampled from the Markov Chain. The significance of fixed effects terms was assessed via DIC comparison of nested models; significance of contrast coefficients of the parsimony model were profiled from the sampling chain.

Additionally, lethal concentrations (LC_{50}) for each clonal lineage were estimated with two methods implemented under the 'morse' R package (Baudrot & Charles, 2021). First, the mean survival rate at a given target time (48h) was described as a three-parameter log-logistic function of biocide concentration (supplementary material). Then, the same data were used to fit a toxicokinetic-toxicodynamic (TKTD) model using the GUTS framework (General Unified Threshold Model of Survival), under the assumption of differential sensitivity to chemical stress among individuals, i.e., the REDuced Individual Tolerance (RED-IT) version (Jager et al., 2011). GUTS modelling has the advantage of describing toxicant effects over time, leading to better fitting than the classical dose-response model, and can be used to predict the effect of the contaminant on survival for untested scenarios (such as time variable pulse exposure). The GUTS-RED-IT is a simple mechanistic model that describes the number of survivors in relation to time and external contaminant concentration. The

internal concentration is assumed to be driven by the external concentration, via the "dominant toxicokinetic rate constant" (k_D, Supplementary Table S4), a parameter which portrays the speed at which the internal and external concentrations equilibrate (slowest compensating process, between either toxicokinetic elimination or toxicodynamic damage repair, which governs the overall dynamics of the scaled internal concentration). In turn, survival probability is lowered by background mortality (h_b parameter) and the internal concentration dependent threshold effect. The latter threshold effect distribution is described by the two parameters m_w (median) and β (shape). In this model, once the threshold for one organism is exceeded, the organism dies immediately. Further descriptions and equations can be found in the original publication (Jager et al., 2011). For each clone, parameters were estimated using Bayesian inference, and posterior predictive checks were conducted to validate each model. To assess interclonal variation in sensitivity, we compared LC_{50} values and model parameters estimated from separated fits of the models.

RESULTS

The developed UPLC-MS/MS method successfully enabled the measurement of CMIT and MIT within the test range. High correlation coefficients (0.998-0.999, Figure S2 A and C) confirmed the linearity of the calibration curves in the range of tested concentrations. The quantification analysis showed concordance between effective and nominal concentrations (Figure S2 B and D), with a degradation of less than 10% over 24h for both CMIT/MIT mixture and MIT alone in all samples (Supplementary Table S3). Therefore, with the medium renewal at 24h, experimental concentrations met the 20% variation range prescribed as maximum by the OECD guideline n°202.

CMIT/MIT comparison

Parsimony dose-response models for both MIT and CMIT/MIT converged upon the same variance structure, incorporating random variation amongst lines in both the intercept ($\square_0$) and slope terms ($\square_1$), as well as random variation amongst technical replicates in the intercept (Table 1). A general view of the tolerance of daphnids to isothiazolinones (Figure 1) shows that the survival curve for CMIT/MIT is steeper than that of MIT, with an effect coefficient of CMIT/MIT concentration (-40.36) approximately 8-fold higher than that of MIT (-5.22; Table 1). This is consistent with the comparison of GUTS' *kD* parameter whose values among treatments (Figure 2) showed a globally quicker infiltration of the mixture (*mean* = 1.37 day^{-1}, *sd* = 0.281) than of MIT alone (*mean* = 0.997 day^{-1}, *sd* = 0.351). However, the difference between treatments (mixture vs MIT alone) was not significant in any lineage, as indicated by credible intervals.

D.pulex showed a very large variance in global tolerance to increasing concentrations of both contaminants, as represented by credible intervals of the curves displayed in Figure 1. Indeed random variation amongst lines in the slope parameter of the logit models represented the greatest fraction of total variance captured in each model (Table 1). Differences among lines are also supported by comparing dose-response models treating lineage as a fixed-effect: for both CMIT/MIT and MIT, model selection via DIC indicated significant line-by-concentration interaction effects (Table 2). This is further reflected in the significant differences in slope coefficients observed between many lines (Supplementary Table S6).

The estimated values of 48h-LC_{50} using GLMM (Figures 3B and 4B), GUTS-IT, or time-target analysis are presented in Table 3. The three methods reported consistent results, and the GUTS-IT model showed that CMIT/MIT mixture was almost one order of magnitude more toxic than MIT alone (LC_{50} values: 0.10 - 0.37

mg/L vs. 0.68 - 2.84 mg/L, respectively). Clones displayed different levels of tolerance (Figures 3 and 4). In terms of tolerance curve shape, AL0 and SE5 differed widely from one another and from the rest, whereas the six remaining clones formed a more homogeneous group. Clones AL0 and SE5 were the most tolerant to MIT (LC_{50} in mg/L: 2.84 and 1.84, respectively) and to CMIT/MIT (0.37 and 0.24, respectively), while at the other end, SE2 and LA0 were the most sensitive to MIT (LC_{50} in mg/L: 0.74 and 0.68, respectively) and CMIT/MIT (0.10 and 0.13, respectively).

DISCUSSION

Presence and risks to ecosystems

The present study provides data about CMIT/MIT and MIT acute toxicity to *D. pulex*, validated by the successful measurement of both compounds' concentrations. Regarding isothiazolinone detection and quantification in environmental waters, liquid chromatography methods coupled with mass spectrometry showed good performances (Speksnijder et al., 2010; Paijens, Frère, et al., 2020). In particular, to analyse CMIT and MIT in cosmetics, UPLC-MS/MS methods were developed with increased sensitivity, selectivity, and increased signal-to-noise ratio especially concerning MIT detection (Wittenberg et al., 2015; Lee et al., 2020; Ducup de Saint Paul et al., 2021). Our results also support the use of UPLC-MS/MS as a fast and sensitive method for quantifying CMIT and MIT in water samples. Accurate and easy to handle analytical techniques are required for reliable and routine environmental monitoring as well as toxicity assessment, which remains challenging in the case of MIT (ANSES, 2016). Targeted screening studies were able to detect both CMIT and MIT in various natural aqueous and soil matrices, though with mixed success, in particular regarding MIT recovery rates (Speksnijder et al., 2010; Baranowska & Wojciechowska, 2013; Nowak et al., 2020; Paijens, Frère, et al.,

2020; Paijens et al., 2021). MIT was found in concentrations from 0.2 to 0.9 µg/L in wastewaters from the Parisian basin (France) and 0.162 µg/L in a stormwater runoff collected in Silkeborg (Denmark) (Bollmann et al., 2014; Paijens et al., 2021). CMIT concentration reached 0.16 µg/L in combined sewer overflows (Paijens et al., 2021). Based on multiple assays, including acute toxicity to *D. magna*, Kresmann et al. (2018) calculated a predicted no effect concentration (PNEC) of 0.5 µg/L for MIT, i.e., a value below some reported environmental concentrations. Although still too fragmentary, these data and predictions strongly support the need for closer attention to these emerging biocides, both in terms of environmental analysis and of ecotoxicity testing. In this respect, our analytical methodology proved to be efficient.

Considering the higher chemical reactivity of the chlorinated molecule (Collier et al., 1990), we unsurprisingly found the mixture CMIT/MIT to be about seven times more lethal than MIT alone. Whether this increase reflects additive or synergistic interaction between the two molecules cannot be established from the present study, as the toxicity of CMIT alone could not be tested. As discussed before (see introduction), the lower lethal activity of MIT among all isothiazolinones (Williams, 2007) comes with high admitted concentrations in commercial products in a very large panel of products (Silva et al., 2020), which translates into increased discharges to the environment. In the meantime, MIT sub-lethal effects remain largely unstudied, stressing once again the current underestimation of risks to ecosystems.

Intraspecific variability

With respect to intraspecific variation, we observed that tolerance to either MIT or CMIT/MIT was highly dependent on the genotype assayed, with a LC_{50} value of the most sensitive clone about 4 times smaller than that of the least sensitive clone in both cases (Table 3). As indicative of genotype-by-environment interaction, these

results suggest the possibility of selective processes induced by CMIT and MIT in exposed natural populations and to their subsequent genetic divergence, in particular from non-exposed ones. Evidences of microevolution due to chemical pollution in aquatic populations are increasingly documented (e.g., Oziolor et al., 2016; Major et al., 2018; Gouin et al., 2019). Recent studies revealed the development of insecticide resistance in populations of non-target species, such as the crustaceans *Hyallela azteca* (pyrethroids; Major et al., 2018) and *Gammarus sp.* (neonicotinoids; Shahid et al., 2018). In daphnids, Brans et al. (2021) found urban populations of *D. magna* to be more tolerant to chlorpyrifos than rural ones from the same geographical region (Flanders). In the same region, *D. magna* populations isolated from ponds located in agricultural areas presented specific pesticide resistance in line with local management practices (either chlorpyrifos or deltamethrin; Almeida et al., 2021). Romero-Blanco & Alonso (2022) also noted from database and literature review, that the sensitivity of aquatic species to contaminants depended strongly on the origin of populations, with either wild or laboratory-reared populations being the most tolerant, depending on the chemical. However, the persistence of the most tolerant populations may come not only with a reduction of genetic diversity but also with fitness or physiological costs (Coustau et al., 2000; Jansen et al., 2011). For instance, in *Gammarus pulex*, populations tolerant to the neonicotinoid clothianidin exhibited reduced fitness in pesticide-free laboratory conditions (Siddique et al., 2020).

In addition to a contribution to fill the knowledge gap on toxicity of CMIT/MIT and MIT to non-target organisms, this study provides clear evidence for a significant genetic component in *D. pulex* sensitivity to such a chemical stress. These findings point to the risk of current standard tests based on daphnids, which typically resort to single clone assays, inducing inaccuracy in environmental quality criteria

(e.g. species sensitivity distribution; Posthuma et al., 2001) and eventually leading to over- as well as under-protective measures, depending on the sensitivity of the clone used. While the latter risk is of particular concern on ecological grounds, the former may also be important for manufacturers who would need to adapt to overly stringent measures, either by deeply modifying the production line or by putting more effort into finding other less toxic molecules of interest. Also, when experimentally assessing chemical toxicity at the community-level (higher-tiered approach), the choice of a monoclonal or a genetically diverse origin population is expected to influence the outcome (Loria et al., 2022). Furthermore, one might argue that interspecific variation overtakes intraspecific variability (Roubeau Dumont et al., 2019), but the relative importance of these seems to greatly depends on species and traits studied (Vanvelk et al., 2021).

Using several lineages may help in refining the understanding of the mechanisms involved in toxicity and tolerance, particularly if a genetic basis for differential tolerance exists, as suggested by the amongst-line heterogeneity observed in the present study. The use of multiple genotypes is a cornerstone of eco-evolutionary experimental designs. For instance, in the case of daphnids, Orsini et al. (2016) recommend five to ten individuals genotyped at twenty neutral markers to obtain a good estimate of allelic richness of one population. More generally, the inclusion of evolutionary toxicology in ERA, for example through the study of the impact of reduced genetic diversity, has been discussed for decades (see Bickham et al., 2000), and leads have been opened up by focusing on more mechanistic and molecular approaches by using Adverse Outcome Pathways or high-throughput screening (Klerks et al., 2011; Côte et al., 2015; Oziolor et al., 2020). Besides documentation of evolutionary impacts, the need for a unified understanding of such

effects (see Brady et al., 2017) calls for harmonized methodologies and parameters that are meaningful for risk assessment. Yet, it is still not a common practice in ecotoxicological research, and the use of multiple genotypes is deliberately avoided in standard toxicity testing, despite regular warnings (Barata et al., 2002; Medina et al., 2007; Coutellec & Barata, 2011; Côte et al., 2015). The present study brings new evidence that ignoring genotype diversity is neither ecologically relevant nor sustainable, especially when considering that biodiversity in all its dimensions is now recognized as a protection goal in environmental risk assessment (EFSA Scientific Committee, 2016).

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Investigation; Methodology; Writing – review & editing. **David Rondeau:** Writing – review & editing. **Marie-Agnès Coutellec:** Conceptualization; Funding acquisition; Investigation; Methodology; Supervision; Writing – review & editing. **Scott McCairns:** Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Supervision; Visualization; Writing – review & editing.

Data availability—Data, associated metadata, and calculation tools are available from the corresponding author (margot.wagner@inrae.fr). This article has earned an Open Data badge for making publicly available the digitally shareable data necessary to reproduce the reported results. The data is available at https://github.com/mwagnerdeyries/Daphnia-CmitMit-ATox. Learn more about the Open Practices badges from the Center for Open Science: https://osf.io/tvyxz/wiki.

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FIGURE CAPTIONS

Figure 1. *Daphnia pulex* sensitivity represented by its mean survival proportion (mean and credible intervals) after 48h of exposure depending on contaminant concentration (mg/L, log scale), estimated from GLMM models with lineage incorporated as a random term. Daphnids' sensitivity to the mixture (solid line) is about an order of magnitude higher than sensitivity to MIT alone (dashed line).

Figure 2. Dominant rate parameter (*kD*, median and 95% credible interval) as estimated from separate fits of GUTS-IT models for each clonal lineage,

contaminated with CMIT/MIT (points, purple lines) or MIT alone (triangles, pink lines). The *kD* parameter portrays the speed at which the internal and external concentrations equilibrate (see methods).

Figure 3. Tolerance to CMIT/MIT after 48h of exposure of each *D. pulex* clonal population (n=50). In the upper panel (A), tolerance is expressed as the survival proportion, with the median survival and 95% credible interval estimated from separated fits of GUTS-RED-IT. In the lower panel (B), tolerance is expressed as 50% lethal concentration (median LC_{50}, quartile range, and 95% credible interval) estimated from GLMM models with lineage treated as a fixed effect and random variation amongst technical replicates.

Figure 4. Tolerance to MIT after 48h of exposure of each D. pulex clonal population (n=50). In the upper panel (A), tolerance is expressed as the survival proportion, with the median survival and 95% credible interval estimated from separated fits of GUTS-RED-IT. In the lower panel (B), tolerance is expressed as 50% lethal concentration (median LC50, quartile range, and 95% credible interval) estimated from GLMM models with lineage treated as a fixed effect and random variation amongst technical replicates.

Title of the manuscript: Variation of tolerance to isothiazolinones among Daphnia pulex clones

TABLES

Table 1. Fixed-effects coefficients and variance estimates of random-effects for parsimony models of mean dose-survival curves. Interval estimates are profiled from the posterior density estimates sampled from 1000 points of the Markov chain.

CMIT/MIT				
Model Coeff.	**Est.**	**$PDI_{0.025}$**	**$PDI_{0.975}$**	**pMCMC**
β_0	5.827	5.328	6.403	< 0.001
β_1	-40.365	-50.613	-27.480	< 0.001
Var. Comp.	**Var**	**$Var_{0.025}$**	**$Var_{0.975}$**	
Var{β_0 Line}	0.2169	0.0003	0.7636	
Var{β_1 Line}	278.4327	68.6200	667.4698	
Var{β_0 Rep|Line}	1.3130	0.8085	1.7420	
Var{residual}	0.0488	0.0007	0.1581	

MIT				
Model Coeff.	**Est.**	**$PDI_{0.025}$**	**$PDI_{0.975}$**	**pMCMC**
β_0	5.152	4.596	5.746	< 0.001
β_1	-5.216	-7.078	-3.333	< 0.001
Var. Comp.	**Var**	**$Var_{0.025}$**	**$Var_{0.975}$**	
Var{β_0 Line}	0.1308	0.0003	0.5308	
Var{β_1 Line}	6.7456	1.2543	16.2181	
Var{β_0 Rep|Line}	2.2950	1.6260	3.1530	
Var{residual}	0.4548	0.0440	1.2830	

Table 2. Comparison of nested hierarchical dose-response models. Random-effects structures are constant between models, and include only random variation amongst technical replicates in the intercept term. Models exhibiting the lowest values of the Deviance Information Criterion (DIC), are determined to represent a parsimony model, explaining the greatest amount of variance relative to model complexity.

CMIT/MIT	
Model Formulation	**DIC**
Conc + Line + Conc × Line	1,540.73
Conc + Line	1,639.52
Conc	1,602.75
MIT	
Model Formulation	**DIC**
Conc + Line + Conc × Line	2,260.16
Conc + Line	2,314.13
Conc	2,288.54

Table 3. Lethal concentrations for 50% (LC_{50}) at 48h values and 95% credible interval, in mg/L, computed with either GLMM, GUTS-RED-IT or an exposure-response log-logistic model for 8 clonal lines of *Daphnia pulex*.

Clone	CMIT + MIT			MIT		
	GLMM	GUTS-IT	Log-logit	GLMM	GUTS-IT	Log-logit
AL0	0.40	0.37	0.37	2.86	2.84	2.73
	[0.34-0.47]	[0.35-0.39]	[0.36-0.39]	[2.50-3.19]	[2.66-3.03]	[2.59-2.88]
GO6	0.15	0.15	0.157	0.78	0.78	0.79
	[0.13-0.17]	[0.14-0.16]	[0.149-0.164]	[0.64-0.91]	[0.70-0.87]	[0.70-0.92]
LA0	0.12	0.13	0.13	0.63	0.68	0.66
	[0.10-0.14]	[0.12-0.14]	[0.12-0.14]	[0.47-0.80]	[0.60-0.75]	[0.59-0.89]
RE0	0.12	0.14	0.146	1.02	0.96	1.14
	[0.11-0.14]	[0.13-0.15]	[0.137-0.155]	[0.88-1.15]	[0.83-1.08]	[0.96-1.24]
SE2	0.10	0.10	0.10	0.71	0.74	0.74
	[0.08-0.12]	[0.09-0.11]	[0.09-0.11]	[0.56-0.87]	[0.66-0.82]	[0.65-0.86]
SE5	0.23	0.24	0.24	1.76	1.84	1.86
	[0.20-0.26]	[0.22-0.25]	[0.22-0.25]	[1.53-2.00]	[1.74-1.95]	[1.74-1.98]
P16	0.13	0.13	0.13	0.96	1.08	1.12
	[0.11-0.15]	[0.12-0.14]	[0.12-0.14]	[0.73-1.17]	[0.99-1.17]	[1.04-1.20]
PE7	0.13	0.13	0.14	1.00	0.92	0.96

	[0.10-0.15]	[0.12-0.14]	[0.13-0.15]	[0.80-1.20]	[0.83-1.02]	[0.85-1.07]