Advancing environmental risk assessment: investigating the relevance of non-conventional endpoints for effect prediction

Rasmussen, A.S.B.

5 Meta-study on responses following prolonged exposure to environmental chemicals over multiple generations

Sofie B. Rasmussen, Martina G. Vijver

Abstract:

Recent advancements have demonstrated that multigenerational testing offers critical insights into ecological risk that standard one-generational assays overlook. Multigenerational approaches can more faithfully reflect real-world, prolonged exposures. Furthermore, sensitive non-conventional endpoints such as behavioural endpoints, physiological changes, molecular biomarkers, and epigenetic modifications can be addressed over these longer timespans. Therefore, the aim of the current study is to determine whether organisms exhibit altered responses under multigenerational exposure regimes compared to a single generation, and whether non-conventional endpoints demonstrate greater sensitivity across generations compared to conventional endpoints. A meta-study was conducted summarising effects trends of 176 individual multigenerational exposure scenarios using 119 uniquely testing chemicals. Only 37% of all observed effects were found to be consistent over generations. Chemical toxicity increased in 39% of all instances and 23% of all observations showed signs of acclimation over time, highlighting the inconsistencies related to predicting long-term effects. The majority of testing (60%) was done using conventional apical endpoints, where reproductive endpoints showed highest portion (49%) of observations leading to sensitisation over time. However, due to the high variety of chemicals/species combinations, generalisations of statistical significance were difficult to conclude. This review emphasises the variability in multigenerational ecotoxicity testing and advocates for continued research to enable broader, more predictive assessments beyond case-by-case evaluations.

Keywords:

Multigenerational testing, ecotoxicity, chronic, subtle effects, long-term exposure

5.1 Introduction

Environmental Risk Assessment (ERA) of chemicals has historically emphasised prospective management grounded in short-term toxicity assays following standardised protocols such as the OECD guidelines (Schuijt et al., 2021). While these methods offer reproducibility and regulatory utility, they often fall short in capturing the long-term, population-level and multigenerational consequences of chemical exposures (Heugens et al., 2003).

Recent advancements have demonstrated that multigenerational testing offers critical insights into ecological risk that the standardised bioassays do not consider. Studies on *Daphnia* spp., a common model species in aquatic toxicology testing, reveals that chronic exposure across multiple generations affects reproduction, growth, and physiological traits, and that these effects were often magnified compared to one-generational exposure (Padilla Suarez et al, 2023). Furthermore, for standard soil invertebrates, conventional laboratory assays for soil organisms based on one life stage of cycle of an individual may substantially underestimate chronic and delayed effects, especially for persistent chemicals (Guimarães et al., 2023). Both reviews highlight the ecological realism and relevance of multigenerational designs.

These findings bolster the case for expanding ERA beyond its traditional evaluation schemes. Multigenerational approaches can more faithfully reflect real-world, prolonged exposures (Nederstigt et al., 2022a; Guo et al., 2023). In addition, effects on reproduction, behaviour, and epigenetic modifications can be assessed over a longer time-period. This is important as there is evidence that these are more sensitive indicators of long-term ecological impact compared to single-generational exposures (Silva et al., 2017; Swank et al., 2021; Guo et al., 2023). Furthermore, non-conventional endpoints, which include behavioural endpoints, physiological changes, molecular biomarkers and epigenetics, are not traditionally used or described in standardised protocols (e.g. OECD guidelines). Yet, recent studies have

shown that they can be more sensitivity towards chronic stress compared to conventional endpoints (Sarma and Nandini, 2006; Raby et al., 2018; Barmentlo et al., 2019; Rasmussen et al., 2024).

Therefore, the aim of the current study is to determine to what extent organisms exhibit altered responses under multigenerational exposure regimes compared to a single generation, and whether non-conventional endpoints (e.g., behavioural or physiological markers) demonstrate greater sensitivity across generations compared to conventional endpoints. By investigating the effects in multigenerational testing in both aquatic and terrestrial model invertebrates, we aim to enhance environmental risk assessment with more ecologically relevant and predictive data. We have compiled peer-reviewed literature of the impacts of chemical multigeneration exposure, with a particular focus on sensitivity patterns between types of chemicals, organisms and tested endpoints. To evaluate the predictivity of long-term chemical effects, we decided to group population effects of multigenerational exposure to chemicals into four categories: acclimation, sensitisation, linear effects or no effects. Acclimation means becoming less sensitive or adjusted to an environmental factor after repeated exposure compared to the impacts on the first generation. In contrast, sensitisation is a process in which an organism becomes more sensitive to a certain stimulus or a series of stimuli after repeated exposure. Moreover, as we have data on a wide range of chemicals we can also determine whether the response patterns observed can be linked to group of chemicals. With this review we aimed to provide a deeper understanding of these prolonged effects that is essential for refining risk assessment methodologies and informing environmental regulations (Brander et al., 2017; Nederstigt et al., 2022b; Harayashiki et al., 2024) so that future risk predictions might be made without relying solely on case-by-case assessments.

5.2 Methods

5.2.1 Search terms and conditions

We conducted a literature search in Web of Science, using the search term "ecotox* AND multigeneration* ". The search term: "ecotox*" were selected to include toxicity testing on non-human model species relevant for environmental chemicals. Furthermore, this paper aims at long-term pollution for more than one generation, to as adequate as possible reflect actual long-term environmental exposures. Hence the search term "multigeneration*" was included. The search was performed on 25th of March 2025 and yielded a total of 256 hits. The data collection and screening process followed the principles in PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses; Moher et al. 2009).

5.2.2 Selection criteria

The papers were first screened based on title and abstract. Included papers were selected based on relevance using the inclusions criteria laid out in Table 5.1. Only experimental papers investigating organismal or sub-organismal effects on whole organisms in at least three generations exposed to chemicals were further examined. We selected three generations as several studies have found clear difference in responses of common test organisms after as little as three generations (Massarin et al., 2010; Foucault et al., 2018; Guimaraes et al., 2019a; Khosrovyan et al., 2022).

To allow comparison of sensitivity across generations, only studies which tested the same endpoints in the parent generation, and multiple continuously exposed generations were included. The focus of this review was on continuously long-term exposure hence, only studies using continuously external exposure including pulse exposures in continually following generations were included. This means that for this review, effects of maternal transfer and transgenerational effects in unexposed generations as well as

recovery periods were excluded in the initial search. The inclusion criteria for the initial screening are listed below:

- English peer reviewed article with the full text being available
- Paper contains new empirical data
- The study is done on non-human model organisms including invertebrates and fish species
- Effects are studied over at least three continuously exposed generations
- The study is performed on a single stressor

Furthermore, the backwards snowballing method was used, in which the list of references from the included studies was checked for any missing papers. This yielded an additional 19 papers. In the end, 129 articles were included in the final assessment.

5.2.3 Extraction of key information

Based on full-text screening, key information was extracted. Key information to be extracted included: chemical, test species, number of generations, tested concentrations if mentioned, tested endpoints, and trend in effects over generation time (see *Table 5.1*). Furthermore, the mode of action of the chemical, if known, was noted down. Lastly, if the study mentioned a suggested mechanism behind the observed trend, this was included as well. If a study included individual testing of multiple chemicals or organisms, this was counted as multiple entries.

During analysis, chemicals were categorised based on their intended function and chemical structure. Studies testing on whole mixtures were counted as one entry, whereas multistress of several individual added stressors were disregarded from the analysis. For multigenerational testing, maintaining the dose at a sub-lethal effect level ensures the organism's survival until the next generation (Marinković et al., 2012); hence, the concentration range of each study was noted. Furthermore, to reconnect each effect to the potential

environmental dose and to facilitate comparisons between chemicals, the lowest concentration at which effects significantly differed from controls was noted. For studies, where no difference from controls were found, the highest tested value was used. Test organism was noted on species level. Furthermore, as the lifespan of the organism influence the number of generations during a long-term exposure duration, exposure period in this study was related to number of exposed generations.

To differentiate between effect prediction of traditional endpoints, denoted as conventional, and other less traditional endpoints, categorisation of conventional and non-conventional endpoints was done based on the nature of each endpoint. All endpoints related to mortality, reproduction and growth were noted as 'Conventional' endpoints independent of organisms. For standardised OECD testing, these are the endpoints typically used and described in the guidelines. Less standardised sublethal endpoints include behavioural, physiological, epigenetics, or molecular measures, thus these endpoints were denoted as 'Non-conventional'. Endpoints were included only if the same endpoint were tested in the parent generation and at least one of the continuously exposed generations. The last tested (or exposed) generation was then noted as the duration of the experiment. Descriptions of information of the extracted variables can be found in Table 5.1.

Table 5.1 *Descriptions of the extracted data (when reported) from papers on multigenerational ecotoxicity testing.*

Key Information	**Description**
Chemical	Name of the tested chemical
Type of chemical	Broader chemical categories such as *pesticides, metals, pharmaceuticals, nanoparticles* and *radiation*
Tested organism	Described on species level
Number of tested generations	Number of exposed generations in which endpoints are tested
Tested concentrations	The tested concentration range of each study
Effect concentration	The lowest concentration in which (if any) effects were significantly different compared to controls
Tested endpoints	Each endpoint is differentiated between being *Conventional* or *Non-conventional* depending on function of the endpoint and inclusion into standardised protocols. Conventional endpoints included: *mortality, reproductive,* and *growth*. Non-conventional *endpoints* included: *behavioural, physiological, epigenetics, or molecular.* Only endpoints tested on the first generation and on a minimum the last exposed generation, were included
Significant trend over time	Results over time were divided into four categories: *No effect, Linear effect, Acclimation or Sensitisation*

5.2.4 Categorisation of effects over time

To address how well effects can be predicted over time, we divided reported effects into the following categories: 'No effect', 'Linear effect', 'Acclimation' or 'Sensitisation'. The different categories of effects are illustrated in the Figure 5.1 showcasing the importance of being able to predict adverse outcomes in continuously exposed concentrations. Both 'Linear effect' and 'Sensitisation' are expected to adversely impact the exposed populations; however, sensitisation might lead to, for example, an early extinction of the population (see *Figure 5.1*).

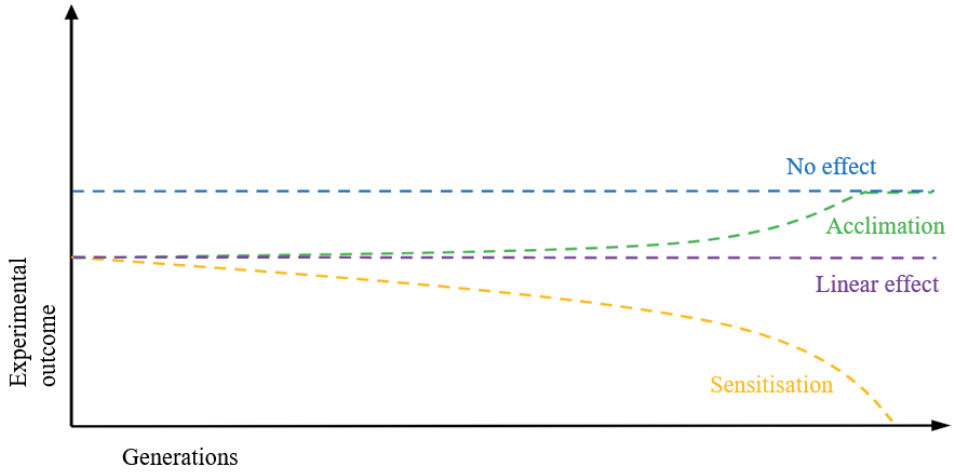


Figure 5.1 *A conceptual depiction of how effects might manifest across different generations. Effects were divided across four categories based on the nature of the effect: 'No effect', 'Linear effect', 'Acclimation' or 'Sensitisation'. 'No effect' is attributed to outcomes showing no significant difference over time, 'Linear effect' is when significant effects are found for first generation, but no significant change in effects were found over time. For 'Acclimation' effects over time became significant less compared to first generation, whereas 'Sensitisation' was found for cases, where effects significantly increased over time. Both 'Linear effect' and 'Sensitisation' adversely impact the exposed population. Sensitisation might lead to, e.g., extinction of the population.*

The categorisation was done by comparing observed effects per endpoint in each generation to the respective controls (see *Table 5.2*). Depending on study design, this was either done by comparing significant reported differences in LOECs or EC_x values per generation. If reported endpoints showed no effects in any generation compared to the generation controls, this was reported as 'No effect'. For endpoints showing impacts in both first generation and additional generations, but no significant change in the effects, the trend was deemed as 'Linear effect'. This category showed adverse effects of the chemical already in the first generation, and size of effects did not change with increasing generation number. For endpoints showing increasingly significant effects over time, we observe 'Sensitisation'. In this category, the adverse effects of the chemical were either not present in the first generation or were significantly smaller compared to later generations. In contrast, effects that decreased in later generations are expected to result in 'Acclimation' of the population. Effects present in the first generation but diminished significantly in later generations to a point where they are no longer significantly different from the controls, were found to acclimate. This also included results with positive effects in later generations compared to controls. All effects are based on the lowest concentration, showing significantly different results compared to controls.

Table 5.2 *Description of population trends used in the categorisation of reported effects.*

Population trend	Description
No effect	No significant adverse effects were shown in any of the exposed generations.
Linear effect	Endpoints showed significant effects of exposure over multiple generations, including the first generation. But no significant impact of generation number was found on the effect size of the effects.
Acclimation	The effect size of significant effects decreased with increasing generation number
Sensitisation	The effect size of significant effects increased with increasing generation number

5.2.5 Statistical analysis

To test what factors influence endpoint responses over generations, we performed a multinomial logistic regression with the population trend as the categorical response variable. The predictors used in the model were the number of generations, species, chemical type, and the endpoint category, all of which were applied as categorical factors. Furthermore, type 2 likelihood ratio tests were applied to indicate whether the predictors contribute significantly to the model. For post hoc testing, the analysis was redone using only the five main species (together representing over half of the collected data; or 61%), and combining additional species as others, categorised endpoints were simplified into either conventional or non-conventional for smoother running of the model. Pairwise comparisons were made using the emmeans function and p-values were adjusted for multiple testing using Tukey. All tests were performed in R software (RStudio 4.2.0, Posit, 321 Boston, USA).

5.3 Results and discussion

5.3.1 Results of the literature review

In the end, 129 papers were identified for inclusion in the final analysis after following the principles in PRISMA. A flowchart was synthesised presenting the process of selecting the papers. (see *Figure 5.2*)

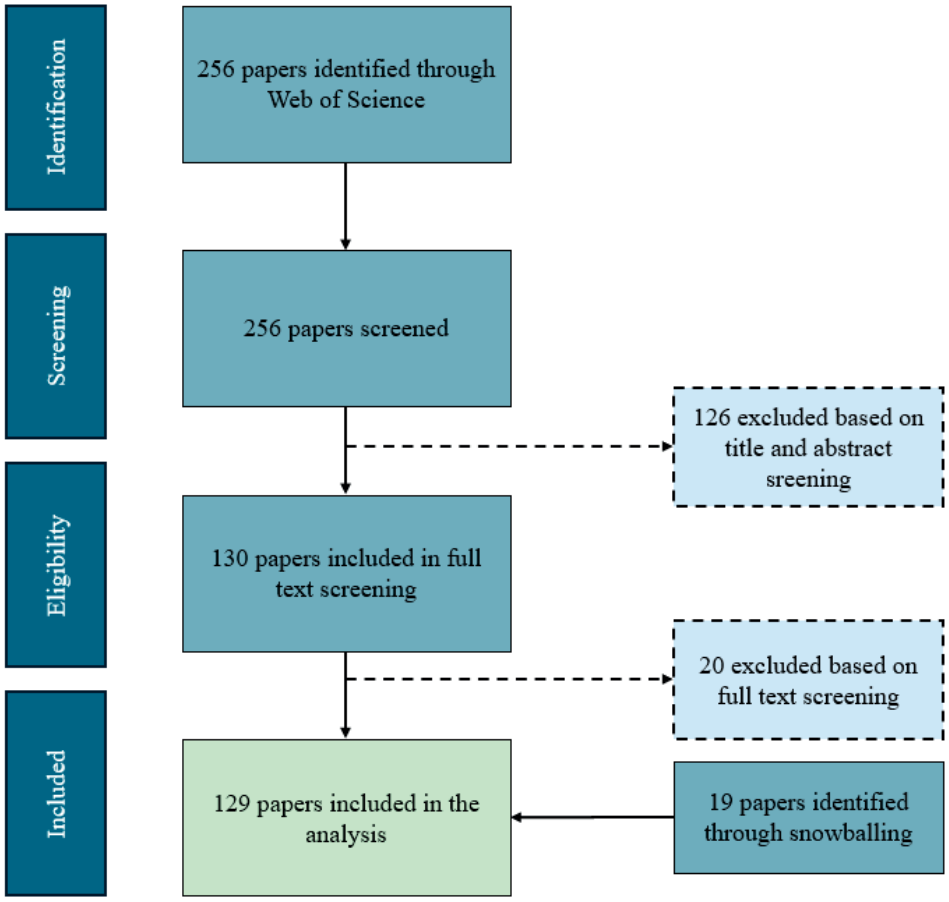


Figure 5.2 *Summary of the screening process for inclusion of publications. The number of publications included in each step is indicated. Identification of the papers followed the principles in PRISMA*

Some studies were found to test multiple chemicals or organisms separately; these were included as separate exposure scenarios for the meta-study (n = 176). Each exposure was tested on an array of endpoints, leading to a total of 730 observations included in this analysis (see *Figure 5.3*). From the included studies, a wide variety of unique chemicals were identified and tested (n = 119). Metals were the most commonly tested chemical type (n = 33), closely followed by pesticides (n = 30) and pharmaceuticals (n = 28). Among other often tested chemicals in multigenerational studies are nanoparticles and plastics, with 22 and 12 entries, respectively. Specific chemicals, cadmium (n = 9) followed by fluoxetine (n = 5), were the most tested. However, the majority of chemicals (75%) were only tested in a single individual exposure scenario. The wide variety of chemicals makes for a large dataset characterising the potential toxic effects of a multitude of different chemicals. All findings on the experimental setup of multigenerational ecotoxicity testing can be found in Figure 5.3.

Of the included 176 exposure scenarios, the majority (61%) of testing was done on 5 commonly tested species; *Daphnia magna* (n = 41)*, Enchytraeus crypticus* (n = 9)*, Folsomia candida* (n = 15)*, Chironomus riparius* (n = 14)*,* and *Caenorhabditis elegans* (n = 29; see *Figure 5.3*). These species all have a relatively fast generative cycle, as described within the standardised ecotoxicity testing, making them ideal for multigenerational testing. Per organism, several endpoints were usually tested simultaneously for one exposure adding up to the total of 730 observations. The most commonly tested endpoints across all organisms were conventional apical endpoints, such as mortality (n = 127), growth (n = 105), and reproductive endpoints (n = 199), accounting for 59% of the total tested endpoints. Non-conventional endpoints (41%) included behavioural (n = 36), physiological (n = 68), molecular (n = 124), and epigenetics endpoints (n = 47), the molecular endpoints being the most common with 18% of the total tested entries (see *Figure 5.3*). As non-conventional endpoints are traditionally not standardised, these endpoints contained much wider variety. The wider use of many

different endpoints brings novel knowledge, however, complicates comparisons between studies.

For this review, effects were noted for the highest numbers of tested generations per study to include as much continuous information as possible. We found that most multigenerational studies, tested exposure scenarios lasting a maximum of three to five generations (73% of all observations). Numbers of experiments rapidly declines for durations lasting longer than six generations (see *Figure 5.3*). Exposure time is an important parameter in ecotoxicity testing related to how effects can manifest. Higher number of tested generations would add to the environmental realism of laboratory studies, especially considering many chemicals being either persistent (Geyer et al., 2017), or continuously released to the environment (Santos et al., 2010). Thus, majority of studies focusing on only a few generations, might become a problem if effects can manifest later. As an example, effects on *C. elegans* exposed to a wide range of chemicals tends to be more severe, the longer the test duration, highlighting the need to take generation number into account (Zhao et al., 2022).

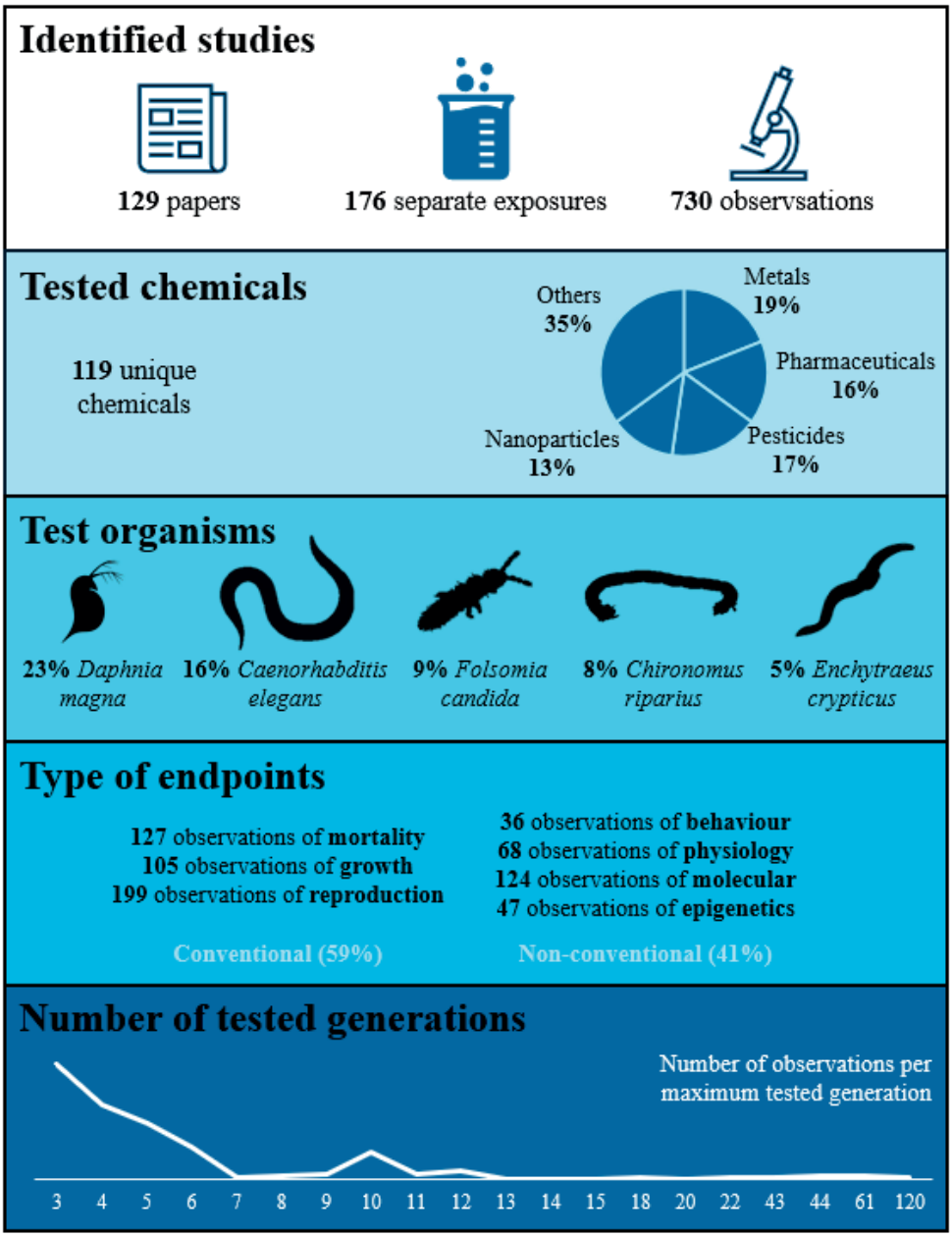


Figure 5.3 *Summary infographics on the most commonly tested chemicals, organisms, endpoints and number of generations in multigenerational ecotoxicity.*

5.3.2 Multigenerational effects of chemicals

Prediction of long-term effects on populations are usually done through single generation testing, extrapolating the effects to account for a multigenerational timescale. This approach requires the chemicals to affect the organisms in a linear manner, however for many stressors this is not the case (Fischer et al., 2013). Based on our findings, only 37% of all observed effects were found to be consistent over generations, showing either no effect at all (21%) or similar effects in multiple generations (16%). Observed effects of the chemicals increased in 39% of all instances, making sensitisation of effects over multigenerational exposure, the most observed effect trend. This increase in the severity of the effects over time would not have been predicted, exposing only a single generation. In the worst case, this can result in the unpredicted extinction of a population over several generations (Paumen et al., 2008; Lilley et al., 2012; *Figure 5.1*).

Additionally, 24% of all observations showed signs of acclimation over time. In these instances, a potential overestimation of actual environmental impact could happen (Marinković et al., 2012). The inconsistency of the results presented here, highlights the difficulties in predicting long-term effects based on effects in a single generation (Swank et al., 2021; Zhao et al., 2022; Nederstigt et al., 2022b). Furthermore, there is a great risk in underestimating the adverse impacts compared to overestimating them, which should be taken into consideration, when considering outcomes of laboratory tests.

The changes in observed effects of multigenerational chemical exposure were in this study, not found to differ significantly depending on the type of chemical used in the experiment ($p > 0.05$, $df = 68$; see *Figure 5.4)*. Noteworthy, we did observe that exposure to pharmaceuticals and nanoparticles resulted more often in sensitisation compared to acclimation (41% vs. 15%, and 49% vs. 15% of observed effects, respectively), but this effect was not statistically significant ($p > 0.05$). In contrast, for pesticides and metals, a higher proportion of effects lead to acclimation compared to the

other types of compounds (36% and 31%; see *Figure 5.4*), this tendency were too non-significant ($p > 0.05$).

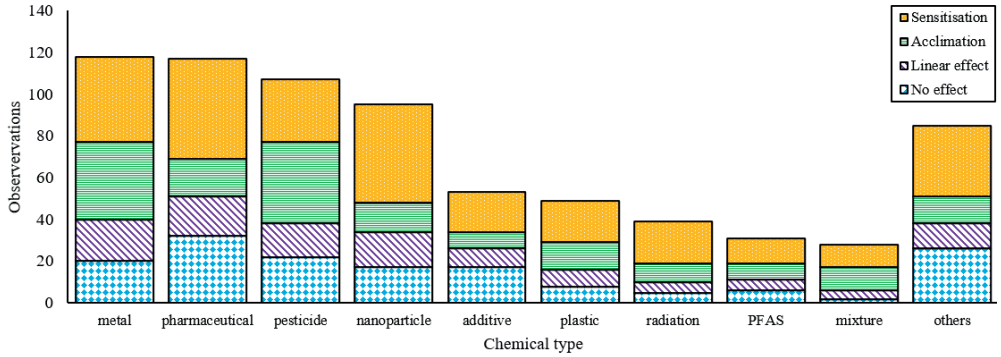


Figure 5.4 *Number of observations per tested type of chemical, where an effect over multigenerational exposure was reported (n=730). Observed effects were divided across four categories based on the nature of the effect: 'No effect', 'Linear effect', 'Acclimation' or 'Sensitisation'.*

Chemicals properties and their associated mode of action might impact how organisms are affected in multigenerational exposures. González et al. (2025) tested the effects of three compounds with different modes of action on *D. magna*: caffeine, ibuprofen, and fluoxetine. They found that the reproductive effects of caffeine and ibuprofen intensified over the course of three generations due to their likely adverse target on reproduction, whereas no effects were found for fluoxetine (González et al., 2025). Likewise, adverse reproductive effects of chlorpromazine, diclofenac, gadolinium and europium were found to increase in *Ceriodaphnia dubia* over three generations, but this pattern was not found when *C. dubia* were exposed to arsenic (Gylyte et al., 2023; Hansen et al., 2002). Lower reproductive output in several continuous generations can be related to increased fitness cost and hence a shift in energy budget, lowering the available energy for other purposes, hereunder: maintenance. Over time, this can cause accumulating of effects (Barrbosa et al., 2017; Zhao et al., 2022; Heinlaan et al., 2023), showcasing the importance

of chemicals targeting reproductive endpoints in multigenerational testing. Even though, many pharmaceuticals caused sensitisation of reproductive effects (58%); based on the limited number of studies included in this review, definitive conclusions on multigenerational effect of specific chemicals classes are not possible.

In the presented results, the most commonly tested metals in multigenerational experiments, include cadmium and zinc, both of which are essential minerals for many species. This could potentially explain, acclimation to some metal exposures, if positive selection on specific traits related to essential minerals cause phenotypic changes in populations (Lalouette et al., 2023). *Spodoptera exigua* developed resistance to cadmium over 61 generations of continuous exposure, by increasingly being adapted to breaking down the excess cadmium (Kafel et al., 2012b).

Positive selection across generations can increase the frequency of alleles conferring tolerance to specific stressors, leading to population-level acclimation through altered gene expression of the exposed population (Augustyniak et al., 2016). For *C. riparius* acclimation in emergence rates after microplastic exposure happened already in generation three, likely due to positive selection on genes related to oxidative stress pathways, lowering the adverse effects of the compounds (Khosrovyan et al., 2022). However, oxidative stress responses have also been connected to sensitisation of silver nanoparticles in *C. elegans* (Rossbach et al., 2021) and *C. riparius* exposed to a metal mixture (Im et al., 2019). Biological interactions between the organisms and specific mode of actions of compounds are relevant for predicting long-term multigenerational effects. This pleas for further research linking chemical properties to long-term toxicity outcomes.

5.3.3 Organism specific toxicity

When considering all tested species (n = 38), the selection of test species was found to significantly impact how effects manifest ($p < 0.001$, $df = 148$), however, this apparent difference in manifestation is likely driven by a

number of test species solely tested in one unique experiment (n = 15). Hence, chemical type, experimental setting etc. are potential covariables in these cases, not accounted for in the current statistical model. Of the five most commonly tested species, only limited differences in effects were observed (see *Figure 5.5*; $p > 0.05$, $df = 168$).

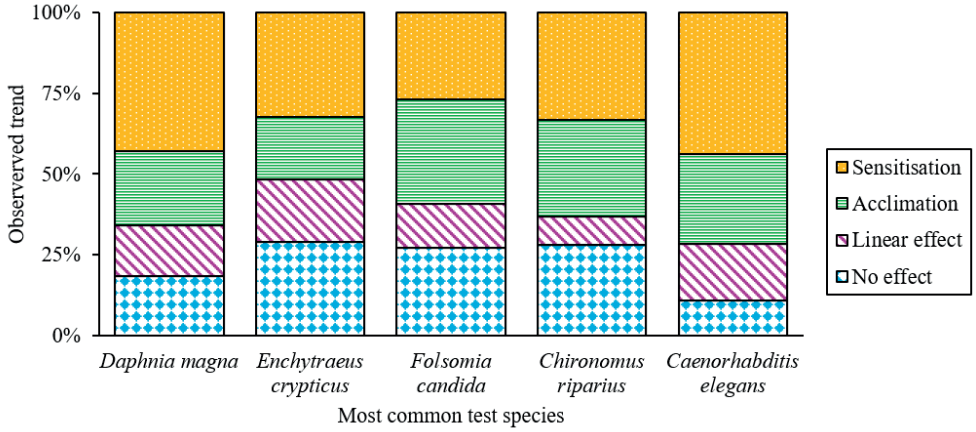


Figure 5.5 *Trends in observations of the five most commonly tested species in multigenerational ecotoxicity testing. Observed effects were divided across four categories based on the nature of the effect: 'No effect', 'Linear effect', 'Acclimation' or 'Sensitisation'.*

Noteworthy, effects tested on *D. magna* and *C. elegans* exhibited sensitisation in more than 40% of the outcomes (see *Figure 5.5*). Zhang et al. (2023) found that for high exposures to bisphenol S, sensitisation of effects lead to the extinction of the *D. magna* culture before reaching third generation, highlighting the importance of this tendency. Although not significant, toxicity testing with *F. candida*, *C. riparius*, and *S. exigua* resulted in a higher percentage of acclimatisation (32%, 30%, and 34%, respectively) compared to other commonly tested organisms. Considering these differences, acclimation to chemical exposure might be related to traits shared among these species. Traits related to development, or reproduction, which are important traits for population dynamics, could be prone to lower sensitivity

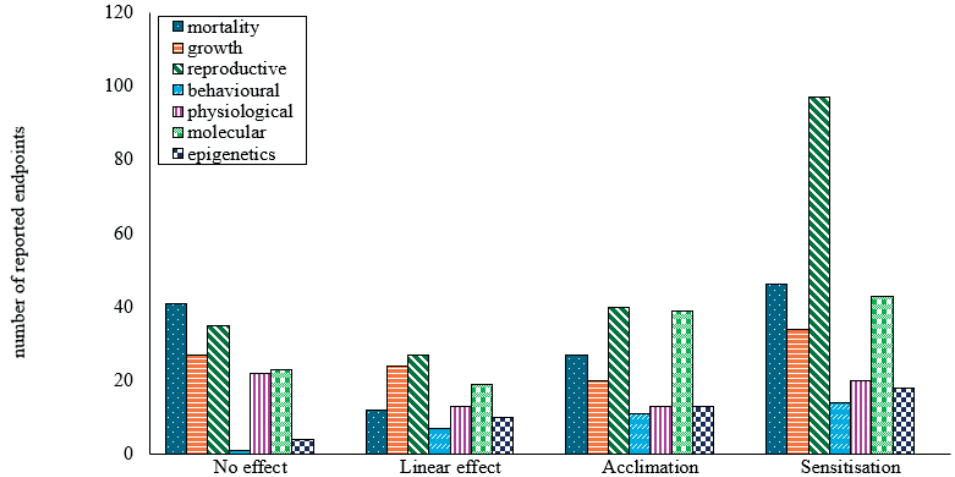
or even acclimation of *C. riparius* (Beaudouin et al., 2012; Müller et al., 2012; Khosrovyan et al. 2022) and *F. candida* (Barreto et al., 2023).

Furthermore, continuous chemical exposure can result in bioaccumulation of chemicals causing internal concentrations to increase with increasing generation number (Kadiene et al., 2022; Xie et al., 2025a). Species specific detoxification processes might aid in counteracting this increase and, upregulation of detoxification genes is prone to selective pressure (Augustyniak et al., 2016). This could, hence, potentially explain the capability of some species to better handle multigenerational exposures. Santos et al. (2024) observed increased detoxification of silver and silver nanoparticles in fifth generation of *E. crypticus* compared to earlier generations. In contrast, downregulation of genes related to detoxification processes in *C. elegans* are suggested to explain increasingly adverse effects leading to sensitisation of exposure to ketamine (Dai et al., 2022), mepanipyrim, and cyprodinil (Gai et al., 2024). Given the relative low number of studies looking at coupling specific species detoxification processes and multigenerational exposure, generalisations are still difficult.

Beside detoxification processes within the organisms, mode of actions of the chemicals can be organisms specific, which might impact manifestation of effects over time. The serotonin reuptake inhibitor fluoxetine was found to cause limited effects in *D. magna* (Stremmel et al., 2023; González et al., 2025), whereas effects of fluoxetine on survival and reproduction of *F. candida* were found to increase over generations (Oliveira et al., 2018). Potentially, fluoxetine's neurological impact related to inhibition of transporter enzymes and AChE activity (Oliveira et al., 2018), could be species specific, thus making some species more vulnerable to sensitisation of effects of this chemical, compared to species with a different biology. Species specific responses to multigenerational exposures should be carefully accounted for in the interpretation and when selecting specific representative species to minimise the risk of biased outcomes.

5.3.4 Endpoint selection

The majority of the toxicity testing was performed using conventional endpoints related to survival, growth and reproduction of the test species (59%; see *Figure 5.3*). Overall, manifestation of effects over time were found to be influenced by endpoint type ($p = 0.001$, $df = 64$), however, no clear difference between effects on conventional and non-conventional endpoints were found ($p > 0.05$, $df = 168$). Reproductive endpoints had the highest number of documented effects showing sensitisation ($n = 97$), whereas for the other conventional endpoints and non-conventional endpoints, a more even distribution between the tendencies over time was visually observed (see *Figure 5.6*).



		No effect	Linear effect	Acclimation	Sensitisation
Conventional endpoints	Mortality (%)	33	10	21	37
	Growth (%)	26	23	19	32
	Reproductive (%)	18	14	20	49
Non-conventional endpoints	Behavioural (%)	3	21	33	42
	Physiological (%)	32	19	19	29
	Molecular (%)	19	15	31	35
	Epigenetics (%)	9	22	29	40
	In total (%)	21	16	23	39

Figure 5.6 *Diagram shows number of observations per tested type of endpoint, observed effects were divided across four categories based on the nature of the effect: 'No effect', 'Linear effect', 'Acclimation' or 'Sensitisation'. (n=730). Distribution in percentages per endpoint is included for the four categories.*

Sensitisation of reproductive endpoints was found more than twice as often as any of the other tendencies measure on the same endpoint type; 49% of the reported endpoints on reproduction showed sensitisation compared to 18%, 14%, and 20% for no effect, linear effect and acclimation, respectively (see *Figure 5.6*). Compared to another commonly tested conventional endpoints; mortality, where 37% of reported observations showed sensitisation but up to 33% of observations showed no effects even after multiple generations of exposure. This broad generalisation suggest that reproductive endpoints might be more sensitive to increased effects in multigeneration tests compared to other types of endpoints. This is further highlighted by several studies, finding that the effects on mortality from chemical exposure remained unchanged over time but effects on reproductive outputs of the same species increased. This includes *Danio rerio* exposed to gamma radiation (Guirandy et al., 2022), *D. magna* exposed to polystyrene nanoplastics (Heinlaan et al., 2023) and pharmaceuticals as fluoxetine (Barbosa et al., 2017), diclofenac (Dietrich et al., 2010), and ibuprofen (González et al., 2025), and *E. crypticus* exposed to metalloid mining wastes (Barmentlo et al., 2017) and copper in nanoparticle form (Bicho et al., 2017). Increased sensitivity of reproduction can have detrimental consequences, leading to population collapse. Zhang et al. (2022) tested the toxicity of soil metals on *F. candida*. Nine different soils were tested for five generations. For all soils, reproductive sensitivity increased for the first couple of generations, rendering several of the populations extinct, due to excessively low reproductive rates (Zhang et al., 2022). Thus, highlighting the importance of reproductive endpoints as a measure of population fitness.

The involvement of pharmaceuticals as naproxen and ibuprofen in the synthesis of COX enzymes, thus interfering with prostaglandins associated reproduction functions, have been proposed as a mechanism for why these compounds might show increased effects on reproduction over time of *Daphnia* species (Soauza et al., 2025). Furthermore, estrogenic properties of chemicals might also add to the long-term sensitivity of reproductive

endpoints, as is the case for bisphenol A, when *Pimephales promelas* is exposed over three generations (Staples et al., 2011) and *Hyalella Azteca* when exposed to sublethal levels of 17a-ethinylestradiol for the same number of generations (Vandenbergh et al., 2003). However, since reproductive mechanisms may vary among species groups, more research is needed to fully understand reproductive impacts in multigenerational tests (Soauza et al., 2025).

Interestingly, non-conventional endpoints as epigenetic changes have previously been linked to changes in reproductive output. Bicho et al. (2020) found that DNA methylation over time was closely related to reproductive output of *E. crypticus* exposed to CuO nanomaterials (Bicho et al., 2020). Similarly, changes in reproduction of *D. magna* exposed to isothiazolinones was coupled with increasing DNA damage over time (Kim et al., 2023). Hence, epigenetic changes over a multigenerational period could be a sensitive and relevant non-conventional endpoint to investigate long-term effects (see *Figure 5.6*).

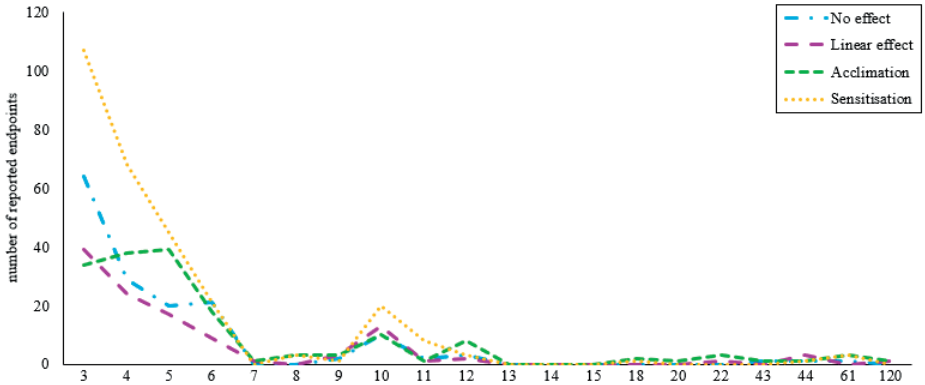
In contrast, research on *D. magna* chronically exposed to faecal coliform contaminated water showed a gradually decreasing global hypermethylation over generations albeit being significant in the first two generations (Chatterjee et al., 2019), indicating acclimation of the organisms. However, epigenetic changes, where methylation is inherited from the parental generation, could suggest a compensation strategy, selecting specific traits to accommodate increasing stress (Ho et al., 2012; Major et al., 2020; Šrutb 2022). Survival of *C. riparius* acclimated after exposure to metal-containing sediment, but epigenetic changes were found to show sensitisation over the exposure period (Im et al., 2019).

Exposure to chemicals, might induce a trade-off between other valuable traits as well. Behavioural endpoints on locomotion of *D. magna,* have been found to be more sensitive to long-term effects compared to mortality (Silva et al., 2017). Likewise, foraging, courtship behavioural and boldness of *P. promelas* have been shown sensitisation over generational exposure to a mixture of

pharmaceuticals, where effects on growth found acclimation (Swank et al., 2021). This highlights the importance of a better understanding of non-conventional endpoints as suggestive of a compensation strategy in multigenerational studies to enhance the knowledge on how populations might acclimate over time to certain stressors (Brander et al., 2017).

5.3.5 The role of time in multigenerational sensitivity

We found that most multigenerational studies (73%) tested exposure scenarios lasting a maximum of three to five generations. Numbers of studies rapidly declines after durations of six generations (see *Figure 5.7*).



	3	4	5	6	7	8	9	10	11	12	13	14	15	18	20	22	43	44	61	120
No effect (%)	26	18	17	30	0	0	22	19	17	19	0	0	0	0	0	0	50	17	14	0
Linear effect (%)	16	15	14	13	50	0	33	25	8	13	0	0	0	0	0	25	0	50	0	50
Acclimation (%)	14	24	32	26	50	50	33	19	8	50	0	0	0	67	100	75	50	17	43	50
Sensitisation (%)	44	43	37	30	0	50	11	38	67	19	0	0	0	33	0	0	0	17	43	0

Number of tested generations

Figure 5.7 *Number of observations reported per tested generations in multigenerational experiments lasting at least three generations (n=730). Effects were noted for the final tested generation. Observed effects were divided across four categories based on the nature of the effect: 'No effect', 'Linear effect', 'Acclimation' or 'Sensitisation'.*

For this review, three generations were chosen as the lowest number of tested generations in a multigenerational study. After three generations of exposure,

58% of the reported effects differed significantly from those observed in the respective first generations (indicating either acclimation or sensitisation; see *Figure 5.7*). This proportion increased to 67% and 69% for the fourth and fifth generations, respectively. Hence, it would be premature to conclude that prolonged exposure beyond three generations has no additional impact on the observed effects. This is particularly important given the limited number of studies extending beyond six generations. Therefore, a more cautious, generic conclusion is that while notable changes tend to manifest within the first few generations, potential long-term or cumulative effects cannot yet be ruled out due to insufficient multigenerational data.

5.3.6 Future perspectives

Natural populations are by definition not exposed to one stressor at a time. Multi-stress such as simultaneous exposure to chemicals and elevated temperature or mixtures of chemicals, will only gain importance in the future (Holmstrup et al., 2010). In this review, we only looked at single stressors, however, it is important to notice that effects over generations can be influenced by joint exposure, where the organism's ability to cope with each individual one is interfered by other stressors (Guttman 1994; Backhaus, & Faust, 2012; Barmentlo et al., 2017). While acclimation to one stressor can enhance performance under that specific condition, it may simultaneously reduce tolerance and increase sensitivity when the population is later exposed to a different stressor. For example, Salice et al. (2010) found that acclimation to cadmium for snails came at the expense of resistance to a second stressor whether that was increased temperature or parasites (Salice et al., 2010). On the contrary, Augustyniak et al. (2017) found that acclimation to cadmium of beet armyworms over 130 generations induced increased tolerance to additional changes in temperature and joint exposure to a pesticide. How effects can manifest under more ecologically relevant settings, is important for understanding actual effects, but this is outside the scope of the current review.

Emerging and innovative chemicals and materials, with a specific mode of action and feature slow-release properties is designed to reduce usage volumes. Many of these substances exhibit no immediate or acute toxicity in standard toxicological assessments, however growing concern surrounds their potential delayed or cumulative effect causing long-term impacts on human health and the environment (Nel et al., 2006; Vijver et al., 2018). Data to predict long-term risks of these chemicals are often limited (Keller et al., 2020), despite evidence that chronic exposure may lead to subtle yet significant biological changes (Scheringer et al., 2014; Wang et al., 2017). Consequently, there is an urgent need for novel methodologies and long-term studies to elucidate the chronic toxicity, and we foresee this mismatch between acute- and chronic effects will only occur more often with innovative chemicals and materials. Titanium and silver nanoparticles have shown to induce increased sensitivity on survival, growth and reproduction of *D. magna* after four generations of continuous exposure compared to one generational exposure (Ellis et al., 2020; 2021). Furthermore, novel use of non-conventional endpoints might add to better screening of these compounds. Hu et al., (2020) found that molecular measurements on total gsh of *C. elegans* caused sensitisation after four generations of exposure to TiO2 nanoparticles, compared to conventional endpoints on survival and growth showing linear effects or even acclimation over time (Hu et al., 2020). Similarly, exposure to silver nanoparticles for six generations caused acclimation of the same organisms when looking at survival, growth and reproduction whereas a behavioural endpoint on locomotion caused sensitisation (Rossbach et al., 2019). This highlights the challenges of predicting long-term effects of these kinds of materials, especially considering the use of non-conventional endpoints are still limited in risk assessment.

5.4 Conclusion

Here we present a meta-study on how the effects of chemicals can manifest over several generations of exposure. We included data from 176 exposure scenarios on multigenerational testing on an array of different chemicals, endpoints and test organisms, highlighting the variety of exposure scenarios used to address multigenerational effects. Overall, the variety of different exposure scenarios gave only limited statistical power to address actual trends across similar chemicals, test species and endpoints. Extrapolation from single generation testing to multiple generations is still difficult, with varying results even within comparable studies on the same chemicals and organisms. This highlights the complexity of effect prediction and warrants the incorporation and execution of more multi-generational studies in assessing the actual long-term effects of a chemical in the environment.

5.5 Acknowledgements

The authors would like to thank Martina Santobuono, Shivani Ronanki, Luca Boldrini, Matteo Schiavinato, Colette Gaillard, and Nico van den Brink for the collaborative work on idea creation and data curation and the authors are looking forward to the continuous collaboration with the goal of publishing the chapter.