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

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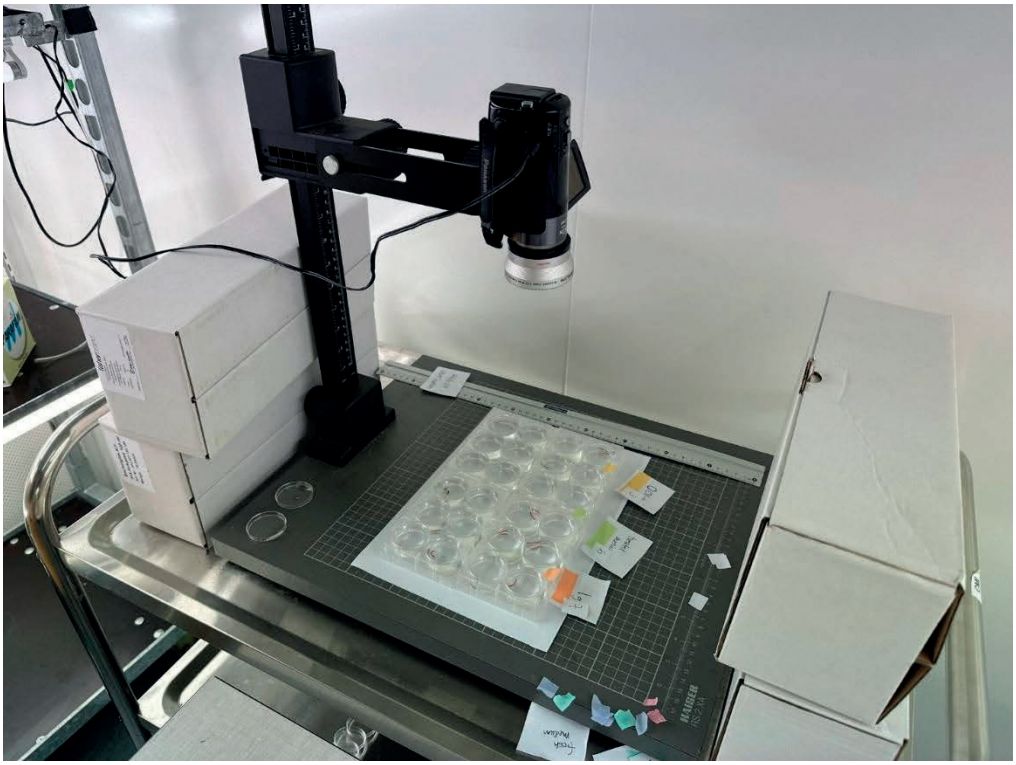

# 6 General Discussion

A growing body of scientific literature is providing stark evidence that traditional ecotoxicity testing might fall short in predicting impacts of all novel synthetic compounds. The current screening and evaluation approaches for effect assessments have their strength in the well-characterized compounds for which exposure, fate, and hazard data can be reliably generated using standardised tests methods to establish robust thresholds. However, to address the rapidly expanding body of chemicals and materials with increasingly specific either known or unknown modes of action, we need to rethink current standardised methods to achieve better environmental protection. Therefore, the overarching aim of this thesis was to:

**investigate the relevance of non-conventional[1] endpoints in predicting potential effects of chemical-induced stress**

Specifically, we set four research objectives:

1. Screen for potential sensitive non-conventional endpoints using a range of organisms exposed to a neurotoxin.
2. Assess the sensitivity of *Chironomus riparius* in a multifactorial chronic test focusing on conventional vs non-conventional endpoints for a neurotoxin under increased ecological realism.
3. Assess the sensitivity of non-conventional behavioural endpoints of *C. riparius* compared to conventional endpoints in a chronic test for mixtures of chemicals with unknown mode of action, leached from plastics and their biodegradable counterpart.

[1] Here we define non-conventional endpoints as endpoints not usually applied in regulatory frameworks for effect assessment. Furthermore, these endpoints are traditionally not standardised in commonly used guidelines such as OECD guidelines.

4. Understand how individual effects translate into multigenerational testing schemes and whether non-conventional endpoints might contribute to enhanced predictions of actual environmental effects.

## 6.1 Summary of findings

This section provides a brief overview of the findings of this thesis (Chapter 2-5) in preparation for a collective discussion on the main findings. In **Chapter 2** we conducted a large-scale hackathon and found that behavioural endpoints such as locomotion of *C. riparius* larvae is a sensitive, relevant and reliable endpoint for at least one neurotoxin: sulfoxaflor. This was further investigated in **Chapter 3**, where we found that non-conventional endpoints such as behavioural changes in *C. riparius* larvae showed interactions between toxicity of the neurotoxin sulfoxaflor and environmental stressors. These interactions were missed if only conventional endpoints were considered. We continued in **Chapter 4**, where exposing *C. riparius* larvae to a complex set of chemical leachates [derived from both conventional plastic (LDPE) and biodegradable plastics (PBAT)] caused developmental effects in high concentrations of PBAT leachate. However, in contrast to the known mode of action of the tested neurotoxin, no effects were found on the behaviour of the larvae, highlighting the importance of considering the mode of action of the pollutants when determining the most suitable endpoints. Lastly, in **Chapter 5**, we conducted a systematic review and found that standardised one-generation experiments miss potential long-term adverse outcomes in later generations. However, multigenerational effects were in general not better predicted by non-conventional endpoints compared to conventional endpoints. High variability in the prediction of multigenerational effects was observed across endpoints, species and pollution type highlighting the complexity of environmental long-term impacts. We warrant the execution of more studies to better understand the chemical-species interaction across time. Table 6.1 highlights the outcomes of each research chapter included in this thesis.

***Table 6.1*** *Highlights of the outcome of each research chapter.*

| Chapter | Research gap | Approach taken | Scientific highlights |
|---|---|---|---|
| **2** | Ecotoxicity data beyond standard endpoints, which can still fast and efficiently indicate effects on aquatic populations from a chemical with a known mode of action | Hackathon identifying sensitive organisms and endpoints for the neurooxin: sulfoxaflor | Participatory research conducted during a Hackathon provided creative and realistic insights into how to measure non-conventional endpoints. This approach is especially valuable for those cases where standard toxicity testing might not be sufficient to address potential environmental effects.<br><br>We demonstrated that changes in locomotion of *C. riparius* larvae measured as their mean velocity in water showed impacts at concentrations levels 5-10 times lower compared to mortality. |
| **3** | How a combination of environmental factors affecting similar endpoints jointly impact neurotoxic-induced effects on ecologically relevant sub-lethal | Chronic experiments with *C. riparius* exposed to a neurotoxin in combination with environmental stressors: increased temperature and predation stress, using non- | Conventional endpoints do not pick up on possible stronger effects of sulfoxaflor in combination with environmental stressors. We observed increased effects of sulfoxaflor and a combination of stressors (increased temperature and predation cues) on the size of emerged adults, exploration behaviour of the larvae, and swimming |

| | | | |
|---|---|---|---|
| | biomechanical endpoints | conventional endpoints including behavoiral endpoints. | behaviour that we could not explain solely by the Independent Action model. |
| **4** | Assessment of the effects associated with biodegradable plastics compared to conventional plastics using non-conventional endpoints such as behaviour | Life-cycle experiment testing conventional and non-conventional endpoints of *C. riparius* exposed to mixtures of chemicals of unknown mode of action. | Both conventional and non-conventional endpoints showed none to negligible responses of the non-target organism *C. riparius* larvae exposed to different types of plastics. |
| **5** | Lack of prediction of effects over time, as well as on the organismal responses that may facilitate sensitisation or acclimation over generations | Meta-study on multigenerational ecotoxicity testing of exposures to chemicals with different mode of action including both conventional and non-conventional endpoints. | Varying trends of multigenerational exposures across similar pollutants, test species and endpoints were found. Addressing long-term effects of chemicals with different mode of actions is still troublesome and the sensitivity across endpoints are not consistent. |

## 6.2 Relevance of non-conventional endpoints

As part of an Environmental Risk Assessment (ERA), a hazard assessment is included to identify intrinsic properties of an active substance that could render it harmful to the environment (EMA, 2024). Traditionally, this assessment is based on single-species laboratory setups using standardised conditions and focusing on whole-organism responses: such as mortality, growth and reproduction (Rohr et al., 2016; Schuijt et al., 2021). These conventional testing methods have been observed in many cases to adequately predict effects of the compound in question (Van den Berg et al., 2019; Gomes et al., 2021; Chapter 4 and 5 in this thesis). However, a consensus among research is growing towards predicted risk of certain stressors is lacking by traditional risk assessment, leaving unintended consequences to our ecosystems (Oldenkamp et al., 2022; Harrison et al., 2025; Rivetti et al., 2025; Ford et al., 2025).

Especially biologically active molecules with novel and/or specific Mode of Actions (MoAs) can lead to implications for wildlife populations. Failure to identify potential adverse effects of these chemicals are likely due to a disconnect between apical conventional endpoints and the mechanisms of action of these chemicals (Rasmussen et al., 2013; Legradi et al., 2018; Oldenkamp et al., 2022). Non-conventional endpoints, such as behavioural endpoints, physiological and molecular biomarkers, can add information of the toxicity at lower levels of biological organisation (Gomes et al., 2021; Bertram et al., 2025). Hence, non-conventional endpoints seem promising for enhanced prediction of effects, by closer linking the MoA of the chemical to adverse outcomes of specific species (Van den Berg et al., 2019).

Chemicals with a neurotoxic mode of action (e.g., neurotoxic insecticides) are of growing environmental concerns (Casillas et al., 2022). However, direct assessment of neurotoxicity in ERA is still lacking (Legradi et al., 2018; EMA, 2024). By directly focusing on the expected target of compounds with known neurotoxic MoA, behavioural endpoints can greatly add to our

understanding of impacts (Bownik and Wlodkowic, 2021). Behavioural endpoints show higher sensitivity towards effects of neurotoxic compounds compared to conventional endpoints, highlighting the relevance of these compounds (Rasmussen et al., 2013; Barmentlo et al., 2018; Raby et al., 2018; Chapter 2 and 3 in this thesis). One concern about behavioural endpoints is the lack of reliability, standardisation and robustness connected to estimating potential environmental effects using measures with low level of standardisation (Ågerstrand et al., 2020). Development of video-tracking in animal behavioural research have greatly advanced the production of high-quality and robust data and lowered the observer bias compared to manual tracking (Bownik and Wlodkowic, 2021; Bertram et al., 2025). Coupled with high-throughput, high quality data, with increased sensitivity towards neurotoxins, behavioural endpoints have proved to be a relevant and reliable way to estimate potential adverse effects of these compounds (Augusiak and Van den Brink, 2016; Ågerstrand et al., 2020; Bownik and Wlodkowic, 2021; Chapter 2 in this thesis)

## 6.3 Increased testing and endpoint selection

Knowing that current risk assessments schemes are not up to date with increasing demands of novel compounds, it is crucial that we reconsider how we assess environmental effects (Rivetti et al., 2025). With rapidly increasing demand on chemical production (Alpizar et al., 2019), risk assessments plea for fast and reliable data, mainly produced by standardised setups to minimise assessment time (Moermond et al., 2016a; ECHA, 2023; EMA, 2024). Non-standard testing that highlights effects are missed in the standardised setups is commonly performed in academic publications, yet currently these types of setups are often excluded from being key studies and only limited included as supportive evidence (Ågerstrand et al., 2020). Standardisation of these setups which include non-conventional endpoints would increase their usability for ERAs (Rohr et al., 2016, Ågerstrand et al., 2020). Through standardisation and optimisation, these endpoints can be more easily

integrated into already existing regulatory testing guidelines (e.g., from the US EPA or OECD), thereby increasing the chance of these tests to detect potential adverse effects (Ford et al., 2021).

Although the additional complexity in terms of non-conventional endpoints might help to predict the risk in specific cases, it does not necessarily mean increased environmental protection for all compounds (Harrison et al., 2025). In Chapter 4, where we focussed on plastics and their leachates, we used the methods we developed for a neurotoxin (in Chapter 2 and 3). However, the additional testing of *C. riparius* larvae's locomotion after exposure to microplastics did not add further information of predicted toxicity compared to conventional testing approach focussed on growth and emergence. We did observe some effects on development from high concentrations of biodegradable plastic leachate, but no effects of the exposure, when we tracked their locomotion (Chapter 4 of this thesis). Hence, likely the MoA of the microplastic and their associated leachates were developmental and not related to the behaviour of *C. riparius* larvae.

Selection of appropriate study design including endpoint selection should depend on the (environmental) relevance (Moermond et al., 2016a). Whether the settings are relevant depends on selected species, the MoA of the compound in question, exposure scenario or even relevant environmental stressors (Casado-Martinez et al., 2024; Bertram et al., 2025). Preferably, this would be considered on a case-by-case basis, considering the environmental realism when performing ecotoxicity studies. This is often the case for non-standard studies produced in academia; where extensive considerations of testing schemes can be included for maximising testing outcome (Rohr et al., 2016).

## 6.4 A tailored approach

The above examples highlight that the approach in effect assessment ideally should happen on a case-by-case basis. However, testing all combinations of

how a chemical might induce adverse environmental effects are impossible (Rohr et al., 2016; Van den Berg et al., 2019). Therefore, a prioritisation of type of testing through a tailored approach depending on specific exposure setting, could be beneficial. This requires inclusion of all available data and better collaboration across assessment frameworks (Roth & Ciffroy, 2016). Rivetti et al. (2025) propose a tailored approach, integrating new approach methodologies (NAMs) to identify priority research gaps based on existing knowledge of MoAs into decision making. Ideally selecting relevant endpoints and allowing flexible testing depending on the predicted known MoA, can streamline regulatory processes (Rivetti et al., 2025). Recently, new guidelines on ERA of pharmaceuticals have opened the door for inclusion of specific testing for known endocrine disruptors and neurotoxins (EMA, 2024).

Furthermore, grouping compounds with similar MoAs can aid in the determination of species sensitivity (Van den Berg et al., 2019). MoAs of most chemicals are not constant across species depending on their specific traits; connecting organisms to certain mechanisms of actions of compounds, hence is valuable for prioritisation of sensitive species (Van den Berg et al., 2019; Rivetti et al., 2025). To further elaborate on a sub-organismal level, biomarkers can add higher mechanisms information for relevant MoAs (Forbes et al., 2006; Casado-Martinez et al., 2024). Better linking between known MoAs of compounds by inclusion of all available data on species sensitivity and integration of NAMs, can help save valuable testing resources and likely decrease the level of live animals use for testing (Van den Berg et al., 2019; Brooks et al., 2024; Rivetti et al., 2025).

However, sometimes very little information on the potential MoAs in non-target organisms can be gathered or information do not depict a clear image of the mechanism of toxicity, resulting in a mixed unknown outcome (Rivetti et al., 2025). Furthermore, evolutionary considerations on species sensitivities and environmental influences can impact potential chemical effects. It remains a significant challenge what determines chemical

sensitivity (Brooks et al., 2024). To efficiently determine potential sensitive focus points for effect assessment, a scattergun approach could be applied, keeping cost of long resource-intensive testing low, while accurately screen for sensitivity. Ideally, this would include different levels of biological organisation to maximise output (Rohr et al., 2016; Gomes et al., 2021). We used the approach on a known neurotoxin, sulfoxaflor, in Chapter 2, acquiring high output of effect data used to prioritise further in-depth assessments (Chapter 2). Likewise, we can assume this method should work similarly with chemicals with unknown MoAs or mixtures, where the main driver of toxicity is undetermined (Oldenkamp et al., 2022).

## 6.5 Rethinking our ERA – Implications for stakeholders

Rethinking our current ERA frameworks, however, will come with associated conflicts between the stakeholders. Standardised laboratory setups under controlled conditions, only assessing effects on very limited apical endpoints ensures a streamlined process, needed in regulation to keep assessment time down when evaluating risk of many chemicals (Moermond et al., 2016b; Gomes et al., 2021). While, currently, this process is widely used across frameworks, we know it falls short in considering all harmful effects of all anthropogenic pollutants, hence a change is necessary (Harrison et al., 2025; Rivetti et al., 2025).

Growing acceptance of non-conventional endpoints such as behavioural endpoints to identify adverse effects are found for academia and government-based researchers (Ford et al., 2025). However, at the moment, inclusion of these types of effects to conclude potential harm to the environment relies heavily on expert judgement prone to biased opinions, slowing down inclusion of them for standard risk assessment (Casado-Martinez et al., 2024). Increasing inclusion of specific non-conventional endpoints as behavioural endpoints through standardised frameworks can help predict novel effects and lower the assessment time associated with these types of effects (Ågerstrand et al., 2020; EMA, 2024; Bertram et al., 2025). Furthermore, it broadens the

currently accepted battery of test approaches, and stimulate further research using these approaches, which is needed for keeping up with the development of novel bioactive compounds.

While standardisation ensures easier compliance for regulatory frameworks, it comes with a cost of lowering the complexity. A challenge in ERA is still the extrapolation of measured responses on individuals to relevant effects at the population, community and ecosystem level (Schuijt et al., 2021). Individual species sensitivities and associated species interactions across a complete ecosystem after exposure to toxicants remains a challenge (Van den Berg et al., 2019; Brooks et al., 2024). Furthermore, increasing the environmental realism including species interactions and environmentally relevant conditions increases the complexity, otherwise not included under a standardised framework (Holmstrup et al., 2010; Jackson et al., 2016; Rohr et a., 2016). Research performed in academia, allows for less rigid limitations, and more room for inclusion of increased relevance in each unique situation (Moermond et al., 2016b). However, reality is that we will never be able to test all possible environmental combinations (Rohr et al., 2016), and even if we could, the risk of unexpected exposure or effects will always be there (Harrison et al., 2025).

Industry tends to lean towards a less conservative way of handling effect assessment arguing that intensified and expensive testing would hamper product innovation (Harrison et al., 2025; Ford et al., 2025; personal experiences at Syngenta). In a survey, conducted on scientist working in the field of ecotoxicity, 91% of academia agreed that chemicals are impacting wildlife, while only 52% of people working in industry agreed on the same statement (Ford et al., 2025). This opens the conflict of which level of protection we should strive towards. Regulatory goals and the publics perceptions of protection are on whole ecosystem level (Rohr et al., 2016). However, testing all (novel) chemicals at the ecosystem level is just not feasible if we still want development of newer and safer product in the future (Rohr et al., 2016; Harrison et al., 2025).

Combining the above discussions, communication and collaboration are key in moving towards increased environmental protection (Roth & Ciffroy, 2016). This will only be more relevant in the future; novel technologies require experts from all actors mentioned above (Gomes et al., 2021; Harrison et al., 2025) and flexibility to combine this knowledge of synthesising to the safety testing. Increasing the use of tailored approaches, where combining the efficiency of standardised protocols in regulatory frameworks with inclusion of relevant non-standard studies can keep cost and resource use at a minimum while enhancing the environmental protection (Rivetti et al., 2025).

## 6.6 Highlights in brief

Overall, this thesis aimed at addressing the use of non-conventional endpoints for effect assessments. Throughout the thesis, we found that non-conventional endpoints such as behaviour of *C. riparius* are effective in predicting potential adverse effects of a known neurotoxin sulfoxaflor in both standardised settings (Chapter 2) and under increased environmental realism (Chapter 3). This highlights the relevance of including of non-standard toxicity studies, when assessing the actual environmental risk. However, while these specific non-conventional endpoints seemed promising for a neurotoxin, non-conventional endpoints in general did not provide additional information on toxicity of mixtures with unknown mode of action (Chapter 4) and when predicting effects over multiple generations (Chapter 5). The importance of considering environmental relevance in terms of mode of action of the chemical, sensitivity of species and environmental realism is evident for enhanced effect prediction. Uncertainties regarding anthropogenic stressors full capabilities to induce adverse effects on the environment persist. Therefore, this thesis encourages a more tailored and collaborative approach for future risk assessment, allowing for flexible testing taking environmental relevance into account, while streamlining regulatory processes and keeping cost at a minimum.