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REVIEW

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Rethinking chemical hazard: an AOP-guided approach to non-conventional endpoints in the environmental assessment of neurotoxic, immunotoxic and metabolic toxic compounds

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Abstract

Chemical pollution is identified as a significant driver of biodiversity loss, raising concerns about the effectiveness of current environmental risk assessment (ERA) practices. Conventional ERA approaches primarily rely on the endpoints of mortality, growth, and reproduction, often failing to capture the full scope of potential effects that chemicals can have on organisms. This is potentially problematic in cases of chemicals causing neurotoxicity, immunotoxicity, and metabolic toxicity, which have recently been introduced to the discussion under REACH by the new report of the European Chemical Agency (ECHA) on *Key Areas of Regulatory Challenge*. For these modes of action (MoAs), which have to date been discussed primarily in the context of human toxicity, there is currently no established approach for addressing them in ERA. This is despite the fact that these chemicals often have sublethal effects on traits linked to potential effects on population-relevant endpoints (e.g., foraging behaviour). In this study, we evaluated the importance of non-conventional sublethal endpoints for hazard and risk practices. We categorised endpoints into conventional (CE; i.e., defined by standardised guidelines), semi-conventional (semi-CEs; i.e., defined by standardised guidelines but only for a limited number of species), and non-conventional endpoints (NCE; i.e., ecotoxicological measurements not defined by standardised guidelines and so going beyond conventional measurements). In this conceptual review, we selected case studies that evaluated both conventional and non-conventional endpoints to evaluate the importance of NCEs for the assessment of the emerging hazards in comparison to CEs, focusing on (1) sensitivity (effect levels), (2) mechanistic understanding, and (3) population-level effects. Our assessment shows that using NCEs can improve mechanistic understanding of chemical hazards and provide important information about the chemicals' MoA. Comparisons between NCEs and CEs at the individual and population levels revealed that in 13% of cases, NCEs showed effects when CEs were unaffected. NCEs were generally more sensitive, being on average 56 times more sensitive than mortality, 8 times than reproduction, and 2 times than growth—in 9 cases, the NCEs were more than 1000 times more sensitive than the CE. NCEs showed unconventional links to the population level that would have gone undetected in the current ERA system (e.g., changes in boldness behaviour affecting reproduction in fish). We propose a first approach to address environmental hazard identification and risk prediction for neurotoxic, immunotoxic, and metabolic toxic compounds by organising relevant NCEs according to an Adverse-Outcome-Pathway (AOP) structure, and a MoA-based AOP framework.

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Background

Thousands of chemicals with various modes of toxic action (MoAs, i.e., biologically plausible sequences of key cytological and biochemical events leading to an observed effect supported by robust experimental observations and mechanistic data) are designed, used, and then released into the environment, posing an enormous challenge to environmental risk assessment (ERA). Based on the exposure assessment and hazard characterization of chemicals, the current ERA aims to quantify the potential adverse effects on species in the environment by monitoring different biological levels from molecules to local population extinction [154]. Standardised tests, like the OECD and ISO guidelines, were developed to ensure consistency and reliability in ecotoxicological studies [51]. This standardisation makes it possible to compare effects among different chemicals, species, and ecosystems, which is crucial given the vast quantity of chemicals that require assessment [121]. Standardised guidelines mainly focus on the life-cycle endpoints mortality, growth, and reproduction (defined as conventional endpoints, CEs, in this study) for a narrow set of model species. Guidelines for other endpoints are limited (e.g., emergence for *Chironomus sp.*: OECD TG 218; metamorphosis for *Xenopus laevis*: OECD TG 241).

Despite our extensive chemical regulations, chemical pollution has recently been identified as one of the five main drivers of biodiversity loss in the European Union [66, 98]. We suggest that using conventional life-cycle endpoints as the main focus of hazard and risk assessment may have led to an underestimation of the harm caused by chemicals in the field. Chemicals vary in structure and have become increasingly diverse. As a result, the amount and complexity of the different chemical MoAs released into the environment have increased over time [35]. Chemical MoAs may be described as either acting non-specifically (i.e., narcotic, reactive) or specifically (e.g., targeting a specific receptor or biochemical pathway). However, due to a lack of data, many chemicals cannot be classified [104]. Compounds with specific MoAs are generally considered more potent to target species and more persistent in the target location (e.g., hormone receptor). These substances, which induce, for example, neurotoxic effects, may cause adverse effects that have an 'unconventional' connection to population dynamics, such as behavioural endpoints (e.g., feeding inhibition), which may go undetected under existing frameworks [172, 187, 196].

For plant protection products, neurotoxic active compounds have been included in regulatory assessments for several years (Commission Regulation (EU) No. 283/2013). However, to our knowledge, there is no pesticide that has been regulated due to a neurotoxic MoA based on its effects on endpoints linked to behaviour. Instead, decisions are made based on results due to effects on mortality endpoints or colony-level changes (for pollinators) without using additional neurotoxic endpoints (e.g., behavioural alterations), even in cases where such effects may have been reported in scientific studies (in cases of regulatory reassessment). Under REACH and other chemical regulations, such compounds have not yet been specifically addressed. ECHA, the European Chemical Agency, has acknowledged the importance of regulating chemicals based on specific MoAs for human and environmental health, beginning with endocrine disruption [56]. In its *Key Areas of Regulatory Challenge* report [53], ECHA now highlights compounds with MoAs that cause alterations in the neuronal, immune, or metabolic systems, which potentially can lead to adverse effects on the population level. Compounds with neurotoxic, immunotoxic, and metabolic toxic MoAs are introduced as "emerging hazards" [55], defined as chemicals that have intrinsic properties suggesting potential to cause harm (i.e., a hazard), but for which scientific evidence is still developing, standardised test methods or regulatory criteria are lacking, and current legislation does not yet adequately cover the potential risks.

It was suggested that substances exhibiting these properties might be classified as of equivalent level of concern (ELoC) to substances of very high concern (SVHCs), which include those classified as carcinogenic, mutagenic, or toxic to reproduction (CMR), persistent, bioaccumulative and toxic (PBT), and very persistent and very bioaccumulative (vPvB) under REACH [55]. To be classified as ELoC to an SVHC, "scientific evidence of probable serious effects to human health or the environment which give rise to an equivalent level of concern to those of other substances" (REACH Article 57f) is needed. For example, endocrine-disrupting chemicals (EDCs) may be considered ELoC to SVHCs. Criteria for classifying EDCs have recently been introduced into the CLP regulation [52]. These criteria require knowledge about a substance's MoA, its adversity on, e.g., the organ, individual, or population level, and the 'biologically plausible link' between the two [52]. Therefore, it can be expected that a similar level of evidence will be required for emerging hazards to be identified and regulated. As the first SVHC intention for a substance with neurotoxic properties has now been announced [55], there is a need to incorporate more MoA-specific and precise sublethal endpoints in the hazard assessment of chemicals, which are currently non-conventional.

Several studies on non-conventional endpoints (NCEs) have revealed effects at lower exposure concentrations (e.g., predator avoidance, locomotion) than for CEs [118, 145, 156, 170, 177]. However, in such cases, it is crucial to establish whether such effects (1) are substantially

different between CEs and NCEs and (2) have an adverse impact on individuals and populations linked to the ecological protection goals [34, 59, 68]. To make these linkages, it is important to understand the mechanisms by which a contaminant causes adverse effects [31, 37]. A helpful tool to unravel the mechanisms is to develop a conceptual Adverse Outcome Pathway (AOP). AOPs relate molecular-level effects to a series of key events (KE) that ultimately lead to adverse outcomes (AO) at the individual or population level [6, 181]. The conceptual AOP is built based on gathering available data on chemical effects across all biological levels. ECHA [53] has recognised AOPs as a crucial step forward in evaluating compounds associated with the emerging hazards neurotoxicity, immunotoxicity, and metabolic toxicity.

A potential reason that NCEs, such as behavioural endpoints, are not included in standardised guidelines and are rarely used for regulatory purposes may be a lack of understanding of how behavioural effects relate to adverse population effects [74]. There is also a lack of robust side-by-side comparisons between CEs and NCEs [74]. This paper aims to fill this gap by evaluating the importance of NCEs for hazard and risk practices, comparing them to CEs. For this purpose, the terms conventional, semi-conventional, and non-conventional endpoints were first defined. Next, relevant NCEs were selected, organised, and discussed according to an AOP structured framework focusing on the emerging hazards proposed by ECHA: neurotoxicity, immunotoxicity, and metabolic toxicity [53]. Case studies that evaluated both conventional and non-conventional endpoints are outlined with a focus on (1) sensitivity (effect levels), (2) mechanistic understanding, and (3) population-level effects. The findings from these studies were then used to refine a conceptual MoA-based AOP framework for the environmental hazard and risk assessment, incorporating NCEs on all biological levels for the emerging hazards.

Methodology

Definition of conventional, semi-conventional endpoints and non-conventional endpoints

To develop a consistent basis for the distinction of conventional, semi-conventional, and non-conventional endpoints, we analysed the OECD and ISO guidelines for the European ERA for animals, focusing on species, model ecosystem, animal group, test duration, and observed endpoint (Fig. 1, Table S1; last updated: July 2025). Our results show that standardised guidelines have been published for 37 animal species/groups, covering the freshwater (13), soil (8), terrestrial (7), marine (6), and sediment (3) ecosystems. Of these guidelines, 21 describe acute experiments with durations ranging from 24 h to 14 days, and a further 25 chronic experiments with durations between 48 h and 65 days. The primary ecotoxicological endpoints described in these guidelines are *mortality* (27), including its proxy, *immobilisation* (2), and the sublethal endpoints *reproduction* (19) and *growth* (14). As standardised measurements, these effects were defined as "conventional endpoints" (CEs) in the present study.

Next to mortality, growth, and reproduction, OECD and ISO guidelines also describe measurements of 17 other endpoints (see Table S1) for tests using one or two species, with a main focus on endpoints in fish test guidelines: intoxication, life cycle assessment, emergence, avoidance behaviour, locomotion, population growth inhibition, behavioural abnormalities, morphological abnormalities, (embryonic and larval) development, sexual development, sex ratio, metamorphosis, vitellogenin, secondary sexual characteristics, biomarkers for endocrine disruption (e.g., oestrogens, aromatase inhibitors), multigenerational assessment and thyroid activity screening. We defined these endpoints that are recognised in standard tests but restricted in their usage domain (i.e., species), as "semi-conventional endpoints" (semi-CEs).

Most CEs and semi-CEs are based on individual level measures that can be linked to population level effects. The direct assessment of population responses in animal species is only described in one guideline for the rotifer *Brachionus calyciflorus* as population growth inhibition (ISO 20666:2008).

A*voidance behaviour* endpoints, described for two soil species, *Eisenia fetida/andrei* and *Folsomia candida/fimetaria* (ISO 17512), are the only quantifiable behavioural endpoints at the individual level. Behavioural abnormalities are assessed in eleven species (*Eisenia fetida/andrei, fish, Enchytraeus sp., Apis Mellifera, Osmia sp., Chironomus sp., Hypoaspis (Geolaelaps) aculeifer,* and in the avian guideline; see Table S1). However, *behavioural abnormalities* are made alongside CEs and are often related to signs of *mortality* (e.g., inability to dig into the soil or lying motionless; OECD TG 220 and 222). Furthermore, details for how to assess abnormalities can be vague, lacking a standardised description, and so varying significantly between species [51]. This lack of clarity increases the challenge of applying such measurements for risk assessors. For fish and bees, *behavioural abnormality* endpoints are described in greater detail. However, these stated endpoints are still challenging to quantify and aid the interpretation of CEs such as *mortality* (fish: OECD TG 210 and 212; bees: OECD TG 245). The standard test for *Daphnia magna* (OECD TG 202) includes *immobilisation* and *locomotion* as endpoints, but they are solely used as a proxy for

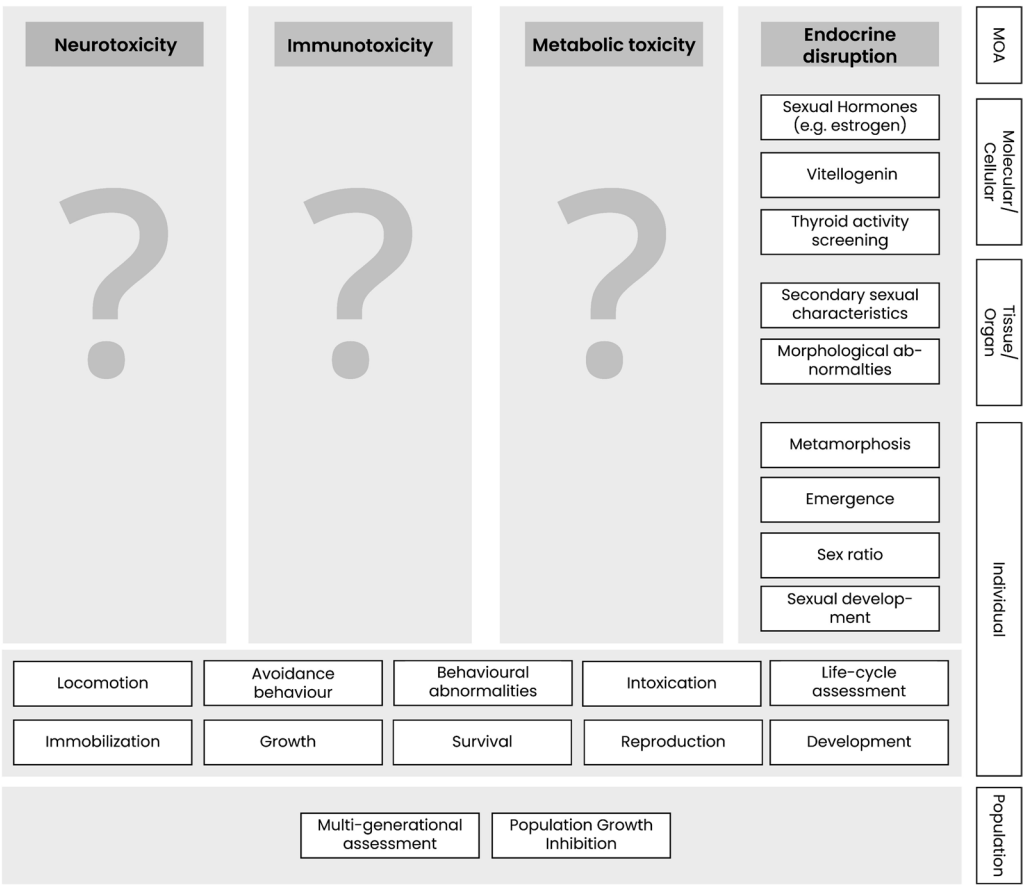

Fig. 1 Schematic overview of CEs and semi-CEs described in standardised guidelines (see summary in Table S1), highlighting their use for the assessment of the MoAs related to endocrine disruption and the emerging hazards, neurotoxicity, immunotoxicity, and metabolic toxicity, and illustrating gaps in current ERA practices by the question marks

mortality, as indicated by a lack of movement. In some cases, behavioural endpoints are included for animal welfare reasons (e.g., OECD TG 210 and 239), where behavioural changes become so severe that they cause considerable suffering, and the individual must be removed from the test system.

Endpoints on the molecular, cellular, and organ levels, as well as behavioural endpoints on the individual and population level, are still underrepresented—the exception being for chemicals with endocrine-disrupting properties (see Sect. "Implementing non-conventional endpoints in ERA guidelines: the example of endocrine disruption"). The absence of information is particularly noteworthy for the emerging hazards neurotoxicity, immunotoxicity, and metabolic toxicity [53], for which standardised guidelines and current ERA practices are absent (Fig. 1). Such emerging hazards need ecotoxicological measurements beyond CE and semi-CEs, defining them as "non-conventional endpoints" (NCEs). NCEs can provide additional information about the potential risks of environmental contaminants by (1) giving a mechanistic understanding of chemical effects and (2) detecting adverse outcomes on the population level (e.g., behavioural changes described in Sect. "Individual-level endpoints to assess adverse outcomes", Fig. 2).

Comparison of conventional, semi-conventional, and non-conventional endpoints

Bridging the gaps in current ERA practices relating to NCEs would help finding suitable approaches to identify hazards such as neurotoxicity, immunotoxicity, and metabolic toxicity [53]. To address the key steps and pathways, we conducted a conceptual review based on the relevant scientific literature. A conceptual review focuses on hypotheses, ideas, theories, and conceptual frameworks rather than presenting a comprehensive quantitative summary of empirical data or systematically summarising existing studies, as in a systematic review [95]. Therefore, this review does not aim to provide an exhaustive overview of all NCEs used in ecotoxicology but rather to illustrate their potential and limitations

within ERA. Thus, we focused on establishing the downstream consequence of the molecular changes (e.g., related to accumulation, genomics, transcriptomics, proteomics, and metabolomics) that underlie key NCEs and how these endpoints may be linked to impacts on population-relevant responses (e.g., behaviour linked to mortality through reduced predator avoidance).

Our approach was to first examine the literature discussing suitable NCEs for hazard assessment (e.g., [3, 74, 108]) for relevant endpoint groups. These endpoints were then categorised into KEs on the three main levels, following the AOP framework [129]: (1) Molecular/cellular/organ-level endpoints are, by their nature, more directly linked to the key chemical MoAs (neurotoxicity, immunotoxicity, and metabolic toxicity) and can be challenging to connect directly to adverse population outcomes; (2)–(3) Individual- and population-level endpoints, although less specific regarding mechanism, can be more directly associated with AOs and ecological protection goals. Individual-level endpoints include behavioural responses such as mobility, phototaxis, and foraging behaviour. At the population level, we focus on social behaviour and predator–prey interactions.

Upon identifying the relevant NCE groups, we selected representative and illustrative case studies that met the criterion of being supported by peer-reviewed evidence, thereby allowing us to discuss each endpoint type. Case studies were identified through searches in major scientific databases (Google Scholar, Scopus, Web of Science) using both non-conventional and conventional endpoint names as search terms, as well as by screening the reference lists of key publications. Case studies were chosen based on the following criteria: (1) inclusion of both CEs and NCEs, (2) laboratory-based (excluding field studies), (3) use of species relevant for ERA (excluding livestock and human health studies), and (4) assessment of chemicals relevant for ERA (i.e., chemicals detected as contaminants in the environment). For some endpoints, studies measuring both NCEs and CEs were challenging to find. In such cases, separate studies were selected for comparison, each using the same species, life stage, and chemical.

The search was restricted to peer-reviewed studies written in English and focused on empirical data rather than reviews. Case studies relying primarily on omics or genetic measurements were excluded. Between four and ten case studies were selected for each endpoint group. These case studies were selected to represent a conceptual review of the relevance of non-conventional endpoints for the protection goals (population-level impact; [34]), taking point of departure in three overall AOP levels (molecular/cellular/organ, individual, and population).

For each case, the NCEs are assessed in relation to CEs with respect to (1) effect concentrations (sensitivity), (2) information provided for mechanistic understanding, and (3) relevance of NCEs for adverse population outcomes and environmental protection goals. Table S2 summarises all discussed case studies, providing detailed information about endpoints, model compounds, model species, study duration, and effect concentrations. To evaluate the relative sensitivity of NCEs compared to CEs, focusing on individual- and population-level endpoints, a ratio was generated to determine the fold increase in NCE sensitivity:

$$Fold\ increase = \frac{Effect\ concentration\ CE}{Effect\ concentration\ NCE}$$

As the data spans several orders of magnitude, we calculated the geometric mean of the fold increase for mortality, reproduction, and growth, respectively, for each group of individual-level endpoints and all individual NCEs together (see Table 1 for all comparisons). Due to data limitations, it was necessary to compare effect concentrations determined by using different methodological approaches (e.g., lowest observed effect concentration (LOEC); effective concentration for 50% of the population (EC50)). The use of NOEC (No Observed Effect Concentration) and LOEC values has long been debated in ecotoxicology. However, when assessing NCEs that show high variability (e.g., behavioural alterations), data may not be suitable for fitting with the models used for EC50 estimation. Moreover, the presence of non-monotonic dose-response curves (NMDRCs) further complicates the model fitting process. Nevertheless, it is common practice in academic studies to compare effect concentrations obtained using both approaches. To highlight the methodological limitations of our study, we included the information on the different endpoints (LOEC, AC50, EC50, LC50) in Table 1.

Outcome of comparison of conventional, semi-conventional, and non-conventional endpoints

Implementing non-conventional endpoints in ERA guidelines: the example of endocrine disruption

There has been a growing concern about chemicals with endocrine-disrupting properties [56, 65]. Endocrine disruptors can have adverse effects on reproduction and development and are, therefore, relevant to population-level ecological protection goals [103, 106]. Within the new CLP guidance, the classification of endocrine disruption is considered the first hazard class that refers to a specific subgroup of endocrine MoAs, i.e., altering the

Table 1 Comparison of NCEs and CEs described in the case studies. Summary of the effect concentration of NCE and the CE mortality, reproduction, and growth, and calculation of the fold increase (CE/NCE). n.e. — No effect on this endpoint was observed in the study. Effect concentrations are presented using the following metrics: LOEC (lowest observed effect concentration), EC50 (effective concentration 50%), LC50 (lethal concentration 50%), LD50 (lethal dose 50%), and AC50 (avoidance concentration 50%)

NCE type	NCE	Mort	Repr	Grow	Unit	Mort/NCE	Repr/NCE	Grow/NCE	Chemical	Species	Study
Locomotion/mobility											
Swimming activity	0.1 (LOEC)	365 (LC50)			µg/L	3650.0			Fluoxetine	*Daphnia magna*	Nielsen & Roslev [124] (locomotion); Stremmel et al. [168] (lethality)
Locomotion	9.38 (LOEC)		37.5 (LOEC)	9.38 (LOEC)	µg/L		4.00	1.00	Benzo[a]pyrene	*Girardia tigrina*	Simão et al. [164]
Locomotion	n.e	830 (LC50)			µg/L	n.e			Phenanthrene	*Girardia tigrina*	Simão et al. [163]
Locomotion	75 (LOEC)	n.e			µg/L	n.e			Pyrene	*Girardia tigrina*	Simão et al. [163]
Locomotion	75 (LOEC)	n.e			µg/L	n.e			Benzo[a]pyrene	*Girardia tigrina*	Simão et al. [163]
Activity	0.02 (EC50)	20.9 (LC50)			mg/L	1045.0			Sertraline	*Caenorhabditis elegans*	van der Most et al. [177]
Swimming activity	30 (LOEC)	31 (LC50)			mg/L	1.0			Diuron	*Lithobates catesbeianus*	Moreira *et al.* [118]
Crawling	1000 (LOEC)	4285 (LC50)			mg/kg	4.3			Lead	*Pheretima guillelmi*	Zheng & Li [198]
Motility	52.67 (EC50)	101.07 (LC50)		15.08 (EC50)	ng/L	1.9		0.3	Bifenthrin	*Chironomus dilutus*	Hasenbein et al. [85]
Motility	22.96 (EC50)	126.16 (LC50)		26.81 (EC50)	ng/L	5.5		1.2	Permethrin	*Chironomus dilutus*	Hasenbein et al. [85]
Motility	4.81 (EC50)	17.38 (LC50)		14.48 (EC50)	ng/L	3.6		3.0	Cyfluthrin	*Chironomus dilutus*	Hasenbein et al. [85]
Motility	n.e	335.2 (LC50)		n.e	ng/L	n.e		n.e	Chlorpyrifos	*Chironomus dilutus*	Hasenbein et al. [85]
Motility	1.4 (EC50)	2.01 (LC50)		1.65 (EC50)	ng/L	1.4		1.2	Bifenthrin	*Hyalella azteca*	Hasenbein et al. [85]
Motility	38.63 (EC50)	40.9 (LC50)		4.03 (EC50)	ng/L	1.1		0.1	Permethrin	*Hyalella azteca*	Hasenbein et al. [85]
Motility	0.53 (EC50)	2.89 (LC50)		1.19 (EC50)	ng/L	5.5		2.2	Cyfluthrin	*Hyalella azteca*	Hasenbein et al. [85]
Motility	n.e	50.41 (LC50)		25.08 (EC50)	ng/L	n.e		n.e	Chlorpyrifos	*Hyalella azteca*	Hasenbein et al. [85]
Burrowing	25 (LOEC)	125 (LC50)			µg/L	5.0		0.0	Copper	*Nereis diversicolor*	Bonnard et al. [25] (burrowing); Moreira et al., [119] (mortality)
Burrowing	25 (LOEC)	500 (LC50)			µg/L	20.0		0.0	Copper	*Scrobicularia plana*	Bonnard et al. [25] (burrowing); Akberali & Black [4] (mortality)
Activity	20.1 (EC50)	116 (LC50)		105 (EC50)	µg/L	5.8		5.2	Sulfoxaflor	*Chironomus riparus*	Rasmussen et al. [145]
Mean velocity	10.6 (EC50)	116 (LC50)		105 (EC50)	µg/L	10.9		9.9	Sulfoxaflor	*Chironomus riparus*	Rasmussen et al. [145]

Table 1 (continued)

NCE type	NCE	Mort	Repr	Grow	Unit	Mort/NCE	Repr/NCE	Grow/NCE	Chemical	Species	Study
Fast pace	11.4 (EC50)	116 (LC50)		105 (EC50)	µg/L	10.2		9.2	Sulfoxaflor	*Chironomus riparus*	Rasmussen et al. [145]
Mobility	28.5 (EC50)	116 (LC50)		105 (EC50)	µg/L	4.07		3.68	Sulfoxaflor	*Chironomus riparus*	Rasmussen et al. [145]
Swimming velocity	0.6 (LOEC)	48.4 (LC50)			ng/L	80.1			Deltamethrin	*Palaemon serratus*	Oliveira et al. [137]
Phototaxis											
Phototaxis	1.34 (LOEC)	5 (LD50)			ng/bee	3.3			Thiamethoxam	*Apis mellifera*	Tosi & Nieh [171] (phototaxis); EFSA [57] (mortality)
Phototaxis	10.0 (LOEC)	51250 (LD50)			ng/bee	5074.3			Thymol	*Apis mellifera*	Bergougnoux et al. [19] (phototaxis); Gashout et al. [78] (mortality)
Phototaxis	0.917 (LOEC)	1.86 (LC50)			mg/L	2.0			Thallium	*Daphnia magna*	Nagel et al. [122]
Phototaxis	267.81 (LOEC)	n.e			µg/L	n.e			Benzo(b)fluoranthene	*Daphnia magna*	Martins et al. [112]
Phototaxis	1.88 (LOEC)	24.8 (LC50)			µg/L	13.2			Mercury (II) chloride	*Daphnia magna*	Martins et al. [112] (phototaxis); Tsui & Wang [174] (mortality)
Phototaxis	187.5 (LOEC)	2000 (LC50)			µg/L	10.6			Dimethoate	*Daphnia magna*	Martins et al. [112] (phototaxis); Beusen & Neven [22] (mortality)
Phototaxis	218.75 (LOEC)	6440 (LC50)			µg/L	29.4			Lindane	*Daphnia magna*	Martins et al. [112]
Phototaxis	93.75 (LOEC)	4000 (LC50)			µg/L	42.7			Linuron	*Daphnia magna*	Martins et al. [112]
Phototaxis	3000 (LOEC)	180000 (LC50)			µg/L	60.0			MCPA	*Daphnia magna*	Martins et al. [112]
Phototaxis	0.25 (LOEC)	70 (LC50)			µg/L	280.0			TBTO	*Daphnia magna*	Martins et al. [112]
Phototaxis	2343.7 (LOEC)	74450 (LC50)			µg/L	31.8			Carbon tetrachloride	*Daphnia magna*	Martins et al. [112]
Phototaxis	9.38 (LOEC)	210 (LC50)			µg/L	22.4			Thiram	*Daphnia magna*	Martins et al. [112]
Phototaxis	468.75 (LOEC)	15000 (LC50)			µg/L	32.0			2,4,6-trichlorophenol	*Daphnia magna*	Martins et al. [112]
Phototaxis	937.5 (LOEC)	15000 (LC50)			µg/L	16.0			Arsenic trioxide	*Daphnia magna*	Martins et al. [112]
Chemical avoidance											
Chemical avoidance	24 (LOEC)	138 (LC50)		n.e	µg/L	5.8		n.e	Abamectin	*Lithobates catesbeianus*	Vasconcelos et al. [178]
Chemical avoidance	102 (AC50)	606 (LC50)			µg/L	5.9			Copper	*Leptodactylus latrans*	Araújo et al. [9]
Chemical avoidance	101 (AC50)	372 (LC50)			µg/L	3.7			Copper	*Lithobates catesbeianus*	Araújo et al. [9]
Chemical avoidance	178 (AC50)	482 (LC50)			µg/L	2.7			Copper	*Pelophylax perezi*	Araújo et al. [9]

Table 1 (continued)

NCE type	NCE	Mort	Repr	Grow	Unit	Mort/NCE	Repr/NCE	Grow/NCE	Chemical	Species	Study
Chemical avoidance	10.4 (AC50)	300 (LC20)			µg/L	28.8			Sunscreen (homogenisation)	*Palaemon varians*	Araújo et al. [8]
Chemical avoidance	24.7 (AC50)	50 (LC20)			µg/L	2.0			Suncreen (direct immersion)	*Palaemon varians*	Araújo et al. [8]
Chemotaxis	0.000001 (EC50)	253 (LC50)			mg/L	253000000.0			Fluoxetine	*Caenorhabditis elegans*	van der Most et al. [177]
Chemical avoidance	0.065 (AC50)	n.e			µg/L	n.e			Atrazine	*Poecilia reticulata*	Araújo et al. [10]
Chemical avoidance	5 (LOEC)	31 (LC50)			µg/L	6.2			Diuron	*Lithobates catesbeianus*	Moreira et al. [118]
Exploratory behaviour (Boldness)											
Boldness	1.5 (LOEC)	n.e		1.5 (LOEC)	µg/L	n.e		1.0	Escitalopram	*Danio rerio*	Nielsen et al. [125]
Boldness	1 (LOEC)	9680 (LC50)			µg/L	9680.0			Cadmium	*Danio rerio*	Shelton et al. [161] (boldness); Al-sawafi et al. [5] (lethality)
Exploratory behaviour	40 (LOEC)	14270 (LC50)			µg/L	356.75			Fluoxetine	*Danio rerio*	Frese & Braunbeck [75] (exploratory behaviour); Zindler et al. [199] (mortality)
Boldness	3 (LOEC)	205 (LC50)		131.4 (EC50)	µg/L	68.3		43.8	Sertraline	*Fish Pimephales promelas*	Valenti et al. [176] (boldness) [175]; Valenti et al. [175] (lethality,growth; feeding rate)
Feeding behaviour											
Feeding rate	154 (EC50)	830 (LC50)			µg/L	5.4			Phenanthrene	*Girardia tigrina*	Simão et al. [163]
Feeding rate	85.6 (EC50)	n.e			µg/L	n.e			Pyrene	*Girardia tigrina*	Simão et al. [163]
Feeding rate	13.1 (EC50)	n.e			µg/L	n.e			Benzo[a]pyrene	*Girardia tigrina*	Simão et al. [163]
Foraging behaviour	10 (LOEC)	460 (LC50)	40.4 (EC50)	100 (LOEC)	µg/L	46.0	4.04	10	Copper	*Pimephales promelas*	Green et al. [84] (foraging behaviour); Pickering et al. [140] (mortality, growth, reproduction)
Feeding rate	30 (LOEC)	270 (LC50)			µg/L	9.0			Imidacloprid	*Gammarus pulex*	Agatz et al. [1] (feeding rate); Beketov & Liess [17] (mortality)
Feeding activity	0.39 (EC50)	20900 (LC50)			µg/L	53589.7			Sertraline	*Caenorhabditis elegans*	van der Most et al. [177]
Feeding activity	0.89 (EC50)	2.53E + 08 (LC50)			ng/L	284269662.9			Fluoxetine	*Caenorhabditis elegans*	van der Most et al. [177]
Foraging speed	10 (LOEC)	705 (LC50)	100 (LOEC)		µg/L	70.5	10.00		Fluoxetine	*Pimephales promelas*	Weinberger & Klaper [188] (feeding rate,reproduction); Brooks et al. [33] (mortality)
Feeding rate	1.83 (EC50)	100 (LOEC)	1.3 (~ EC50)	0.15 (LOEC)	mg/L	54.6	0.71	0.08	Imidacloprid	*Daphnia magna*	Agatz et al. [2]
Feeding rate	15 (LOEC)	15 (LOEC)			µg/L	1.0			Imidacloprid	*Gammarus pulex*	Nyman et al. [127]
Social behaviour											
Social behaviour	0.1 (LOEC)	n.e		0.1 (LOEC)	mg/L	n.e		1.0	Benzyl butyl phthalate	*Fundulus heteroclitus*	Kaplan et al. [99]

Table 1 (continued)

NCE type	NCE	Mort	Repr	Grow	Unit	Mort/NCE	Repr/NCE	Grow/NCE	Chemical	Species	Study
Social behaviour	1 (LOEC)	705 (LC50)	100 (LOEC)		µg/L	705.0	100		Fluoxetine	*Pimephales promelas*	Weinberger & Klaper [188] (social behaviour, reproduction); Brooks et al. [33] (mortality)
Agressiveness	100 (LOEC)	705 (LC50)	100 (LOEC)		µg/L	7.1	1.0		Fluoxetine	*Pimephales promelas*	Weinberger & Klaper [188] (aggressiveness, reproduction); Brooks et al. [33] (mortality)
Shoaling	40 (LOEC)	14270 (LC50)			µg/L	356.75			Fluoxetine	*Danio rerio*	Frese & Braunbeck [75] (shoaling); Zindler et al. [199] (mortality)
Agressiveness	0.1 (LOEC)	1260 (LC50)			µg/L	12600			Cyprodinil	*Danio rerio*	Tang et al. [170] (aggressiveness); Y. Wang et al. [184] (mortality)
Predator–prey interaction											
Antipredator behaviour	20 (LOEC)	2110 (LC50)			µg/L	105.5			Cadmium	*Danio rerio (larvae)*	Kusch et al. [105] (antipredator); Blechinger et al. [23] (mortality)
Antipredator behaviour	1 (LOEC)	9.68 (LC50)			mg/L	9.68			Cadmium	*Danio rerio (adults)*	Volz et al. [182] (antipredator); Al-sawafi et al. [5] (mortality)
Predator avoidance	0.1 (LOEC)	1260 (LC50)			µg/L	12600			Cyprodinil	*Danio rerio*	Tang et al. [170] (predator avoidance); Wang et al. [184] (mortality)
Predator avoidance	40 (LOEC)	14270 (LC50)			µg/L	356.75			Fluoxetine	*Danio rerio*	Frese & Braunbeck [75] (shoaling); Zindler et al. [199] (mortality)
Predator avoidance	1 (LOEC)	705 (LC50)	100 (LOEC)		µg/L	705	100.0		Fluoxetine	*Pimephales promelas*	Weinberger & Klaper [188] (predator avoidance); Brooks et al. [33] (mortality)

function(s) of the endocrine system with focus on different hormone systems and pathways (e.g., oestrogen, androgen, thyroid, steroidogenesis (EATS modalities)) [52]. Under this regime, it is now necessary to test the molecular event, the adverse effect (e.g., organ, individual, and population level), and the 'biologically plausible link' between both in order to classify a substance as an endocrine disruptor [52]. Already prior to these classification criteria, several guidelines were developed to assess the impact on sexual hormones, sex ratio, and secondary sexual characteristics for fish (OECD TG 229, 230, 234, 250), and reproduction and sex ratio in 12 other aquatic and terrestrial species (e.g., OECD TG 218, 219, 232; see complete list in Table S1). The Japanese rice fish, *Oryzias latipes*, is assessed using a multigenerational approach (OECD TG 240). An extensive list of guidelines and AOPs (e.g., AOP 346) can be found in OECD [130] and at https://www.oecd.org/en/topics/sub-issues/testing-of-chemicals/endocrine-disrupters.html.

Given that tests for vertebrates dominate those validated test methods, there have been some criticisms of the absence of invertebrate species in the assessment of endocrine disruption [42]. Nonetheless, although gaps exist, the inclusion of endocrine endpoints in current guidelines represents a step forward in the acceptance of non-apical endpoints for their relevance for regulatory frameworks. Overall, endocrine disruption can be seen as a good example of how endpoints for the emerging hazards, neurotoxicity, immunotoxicity, and metabolic toxicity, can be integrated into environmental hazard assessment practice based on mechanistic understanding and linkage to higher-tier effects.

Molecular/cellular/organ-level endpoints for mechanistic understanding

Measuring molecular, cellular, and organ-level endpoints can provide enhanced mechanistic understanding for the emerging hazards, neurotoxicity, immunotoxicity, and metabolic toxicity [53]. Such lower organisation effects also play a crucial role in constructing AOPs [53]. As such, they offer valuable insights that less specific CEs may not be able to detect. Below, we highlight specific molecular/cellular/organ-level NCEs and their potential contribution to a greater understanding of emerging hazards compared to CEs.

Neurotoxicity

All complex organisms rely on neural activity, and any chemicals affecting nerve functionality can adversely affect an organism's biology. The morphology of the nervous system and neurotransmitter functioning can be impacted through various mechanisms, including direct damage to neurons, interference with neurotransmitter synthesis or release, disruption of ion channels, post-synaptic receptor interactions, and the induction of oxidative stress or inflammation, which alters nerve cell membrane integrity [109]. Perhaps the most direct pathways leading to neurotoxicity are through interactions with neuronal targets, including cholinesterase (ChE), glutamate decarboxylase (GABA), nicotinic acetylcholine receptor (nAChR), or dopamine and serotonin modulations [111, 123]. ChEs are crucial in the nervous system as they regulate the breakdown of their respective choline neurotransmitter [139]. Inhibition of these enzymes leads to neurotransmitter accumulation at the synapse and neurotoxicity due to nerve overstimulation. Acetylcholinesterase (AChE) activity has been a major focus as a biomarker for measuring neurotoxic effects [45], e.g., for carbamate or organophosphate insecticides [111], and so has the strongest basis from which they can be assessed as an early detection method for neurotoxic MoAs [76].

Two standardised guidelines exist for assessing neurotoxic compounds in human and mammal risk and hazard assessment (OECD TG 424 and 426). The OECD has also provided initial recommendations on in vitro testing for developmental neurotoxicity [135]. Similar guidelines remain absent for environmental risk and hazard assessment (see Table S1) [108]. With 18% of the organic chemicals found in European waterbodies being linked to a neurotoxic MoA [35], understanding how underlying mechanisms result in neurotoxicity and, ultimately, adverse effects on the individual and population level, is crucial to environmental hazard assessment.

In Table S2, we summarised five case studies measuring AChE inhibition [86, 101, 102, 165, 195], and one case study additionally measuring serotonergic, dopaminergic, and GABAergic neuron damage to assess neurotoxicity [183, 185]. The case studies show that measuring neurotoxic endpoints like ChE inhibition can be used to verify a neurotoxic MoA. The cellular measurements made could further be linked to mortality. Sismeiro-Vivas et al. [165] studied the interaction between AChE inhibition and mortality following exposure to the organophosphate chlorfenvinphos. They observed that in eastern mosquitofish (*Gambusia holbrooki*), mortality would occur only after AChE inhibition exceeded 80%. Key & Fulton [101] experimented with larval grass shrimp (*Palaemon paludosus*) to determine if AChE activity at 24 h of exposure could also be used to predict mortality at 96 h of exposure to organophosphates. These results showed strong correlations between the EC50 for both endpoints, indicating the link between AChE activity and mortality. Effects on AChE can also be much more sensitive than

mortality effects, as seen in the study by Khalil [102]. The author observed effects on AChE at 0.01 mg/kg, while the corresponding LC50 was 145.36 mg/kg.

Changes to AChE activity can be temporary, returning to baseline over time, even in the presence of the stressor. For instance, in the topmouth gudgeon (*Pseudorasbora parva*), exposure to 200 µg/L of fluoxetine increased AChE activity after 4 h, but activity was similar to controls after 42 days of exposure [38]. In mussels (*Mytilus galloprovincialis*), exposure to 75 ng/L of fluoxetine led to a spike in AChE activity after 3 days, which returned to baseline after 7 days, but then showed further inhibition after 15 days. Such results indicate a complex temporal effect dynamic [81]. While these findings suggest a potential neurotoxic MoA, their variability makes linking them to population-level outcomes difficult. However, the multi-biomarker approach in Gonzalez-Rey & Bebianno [81] indicated that fluoxetine not only acts on neurotoxic endpoints but also works as an endocrine disruptor. This highlights the importance of considering (A)ChE activity and neurotoxic endpoints in the context of other effect mechanisms to gain sufficient mechanistic understanding of interrelated effects.

Measuring neurotoxic endpoints in hazard assessment practices is highly relevant to neuronally linked MoAs. However, due to the biological variability of nervous systems, incorporating the MoAs into ERA remains challenging. Moreover, neurotoxicity can be found outside of cholinergic neurotransmission targets. For instance, Wang & Wang [186] demonstrated that Bisphenol-A exposure did not affect cholinergic neurotransmission but did affect other neurological targets in a study using multiple transgenic *C. elegans* strains. Bisphenol-A was found to block serotonin and dopamine synthesis, with the latter being affected in a dose-dependent manner, and reduced the number of GABAergic neurons, in addition to its effects on neurobehavioural and growth endpoints. This example demonstrates that markers other than (A)ChE are more complex to measure and less easily adapted to different model species, emphasizing the need for further development of neurotoxicity endpoints for ERA.

Furthermore, neurotoxicity is not always linked to direct effects on neural receptors but can also result from more generalised effects such as oxidative stress and subsequent effects on cell physiology (see Sect. "Immunotoxicity"). He & Liu [86] found that oxidative stress-related damage reduced AChE activity in *Eisenia fetida* worms exposed to phenanthrene in soil. This led to neurotoxicity by ACh accumulation at the nicotinic postsynaptic membrane. Similar pathways have been observed in response to titanium dioxide nanoparticle exposure in the worm *Pheretima hawayana* in soil [102]. Such examples highlight the relevance of measuring biochemical markers and alternative MoAs to distinguish neurotoxic-acting compounds more effectively.

Changes to neurotransmitter release, their enzymes, or upregulation of neurotransmitter pathways have also been linked to behavioural endpoints. For example, Sismeiro-Vivas et al. [165] found a significant correlation between AChE inhibition by the organophosphate chlorfenvinphos and behavioural change in the fish *Gambusia holbrooki*: effects occurring at > 40% AChE inhibition compared to the > 80% inhibition needed for a mortality response. Métais et al. [115] linked AChE inhibition in the ragworm *Hediste diversicolor* in a multi-contaminated estuary (diffuse pollution due to heavy metals, pesticides, PAHs, and PCBs) to an impairment of the burrowing activity and a lower population density and biomass. Chemicals that work through neurotoxic mechanisms other than AChE inhibition have also been linked to behavioural effects. Wang & Wang [186] found that Bisphenol-A (BPA) exposure in transgenic *C. elegans* damaged serotonergic, dopaminergic, and GABAergic neurons, without affecting cholinergic activity, and this was linked to reduced growth, shorter lifespan, and altered behaviour. Conversely, Heredia-García et al. [90] found that exposure to BPA led to AChE inhibition and altered the swimming behaviour in zebrafish. Together, these examples illustrate the limitations of relying on single biomarker approaches in complex and variable neurological systems. Further research is needed to elucidate the links between neurotoxic endpoints and NCEs linked to behavioural changes.

Immunotoxicity

The immune system is an important target to consider in ERA as: (1) it is an indicator of overall health and is critical in determining individual fitness [83], (2) unlike other systems, it is distributed throughout the body and is well connected to other systems, making it a highly vulnerable target [183, 185], and (3) it is directly related to interspecific and intraspecific interactions, as detrimental changes to the immune system can make organisms susceptible to pathogens, thereby changing individual/population disease susceptibility [24]. Immune system effects have commonly been linked to oxidative stress and reactive oxygen species. Oxidative stress is a key initiating event for various physiological processes as it modulates the transcription factors, including Nrf2, NF-kB, and FoxO, which regulate inflammatory responses and play an important role in chemical-induced immunotoxicity [49].

To date, there are only a few standardised test methods to screen for immunotoxicity of chemicals.

Immunotoxicity is tested for in pre-clinical studies during the development of pharmaceuticals and other drugs to ensure the safety for human health [62]. The immunotoxic potential of industrial chemicals can be detected following reproductive toxicity testing guidelines that investigate the T-cell-dependent antibody response (TDAR) in rodents (e.g., OECD TG 443 (EOGRT study)) or the impact on T-cells through impairments of cytokine signalling pathways in vitro (OECD TG 444A). However, immune toxicity is rarely considered in ERA [147]. This oversight may be due to the complexity of immunotoxic effects, as chemicals can cause immune impairments through a wide range of mechanisms and diverse complex targets [159]. Additionally, there is a variability in responses and a shortage of relevant studies to understand the MoA of new chemicals [183, 185]. With the acknowledgment of the emerging hazard immunotoxicity, there is a need to address endpoints to measure immune effects in ERA research and practices [26, 53].

Chemically stimulated immune modulations are present at a cellular level as well as in immune organs [47, 192]. These modulations can be divided into two main categories: immunosuppression and inappropriate immune stimulation. Immunosuppression can lead to higher susceptibility to infections, while inappropriate immune stimulation can lead to nonspecific inflammation, hypersensitivity (allergy), and/or autoimmune diseases [40, 80]. In wildlife, immune effects have been detected for various chemical groups, notably PAHs and metals such as lead, cadmium, methyl mercury, and nickel [153].

Studying immunotoxicity typically involves key steps linking exposure in the organism with co-exposure to pathogenic microorganisms or infectious agents [190]. Observations then allow study of how the immune system responds under toxic stress through measurement of immune markers or phenotypic observation of pathogen effects [179]. This approach is suitable for examining sub-lethal concentrations of chemicals during short-term or sub-chronic exposures. The case studies summarised in Table S2 identify several immunomodulatory effects, such as elevated phagocytosis, reduced spleen cellularity, increased leukocyte counts, decreased antibodies, and effects on T-cell response after exposure to nickel, copper, and PCB. These effects were, for example, observed in the two fish species *Colossoma macropomum* and *Ameiurus nebulosus* [94, 110]. The immune effects were observed at concentrations up to 40 times lower than CEs, with some studies showing effects on immunotoxicity when no effects on CEs were observed at the tested concentrations.

Measuring immune endpoints in hazard and risk assessment provides insights into the potential MoAs and their interactions with other endpoints. For example, He et al. [88], investigated the mixture effects of two agrochemicals, imidacloprid and difenoconazole, on endpoints in yellow croakers (*Larimichthys polyactis*). Disruptions in genes related to immune function were identified, linked to alterations in oxidative stress markers and changes in endocrine and neural development pathways. This highlights the potential importance of measuring immunotoxic endpoints on the molecular and cellular level and their connectivity to other endpoints.

Immune effects can be linked to apical endpoints, as impacts can indicate a change in the host species' capacity to respond to or resist pathogens under exposure. Since chemicals and pathogens frequently coexist in the environment, studying their combined effects reveals insights into disease outbreaks. Rodgers et al. [149], for example, found disruptions in the expression of five immune genes crucial for responding to pathogenic threats. Numerous studies have also established the adverse impacts of chemicals on the immune system, resulting in disease outbreaks. For example, specific amphibian populations in contaminated environments have shown individuals with compromised immune systems, resulting in an overall rise in parasite [150] and fungal infections [151]. Similarly, PCB contamination significantly affected harbour seals due to diminished immune competence, leading to a distemper virus outbreak [16, 120]. Comparable findings were observed in fish inhabiting the estuary Puget Sound, US, with increased disease incidence following exposure to PAHs [12, 13]. Such examples indicate clearly how chemical related immune impacts can lead to adverse population effects.

Metabolic toxicity

Metabolism-disrupting chemicals (MDCs) are substances that interfere with metabolic processes [157]. In recent years, MDCs have attracted scientific and regulatory attention as emerging hazards [53] due to their association with obesity, diabetes, and fatty liver disease in humans [15, 169]. Although effects on humans are increasingly being studied, there remains uncertainty about how to evaluate MDC effects on wildlife. Consequently, environmental risks to metabolic endpoints are not included in standardised guidelines (Table S1). Metabolism can be measured in a range of ways. Direct measures can assess respiration rate as a proxy or indirectly by analysing changes in the energy reserves, or can assess cellular energy allocation (CEA) based on assessments of protein, lipids, and carbohydrate contents [44]. One example

of MDCs raising environmental concern is succinate dehydrogenase-inhibiting fungicides (SHDIs). This class has the potential to affect non-target species by targeting succinate dehydrogenase, an essential enzyme in the Krebs cycle and respiratory electron transport chain, not only in fungi but in almost all eukaryotes [196]. Energy metabolism in the fish *D. rerio* [142, 193], the earthworm *E. fetida* [87], and freshwater amphipod *Gammarus fossarum* [107] was affected following exposure to SHDIs, providing a mechanistic confirmation of the effects of these fungicides on endpoints linked to energy dynamics in non-target species [44, 82]. Any such change in available energy has the clear potential to decrease individual fitness and, ultimately, affect populations through effects on growth and reproduction [82, 166].

In Table S2, we summarised five case studies that analysed endpoints linked to energy metabolism, such as the energy reserves glycogen, lipid, and protein content, respiration rates, and aerobic energy production (measured by estimating the electron transport system—ETS) in comparison to CEs. These approaches have been used to measure the effects of a range of different chemicals, such as the insecticides endosulfan and parathion, fungicides boscalid and tebuconazole, the antidepressant fluoxetine, polyethylene microplastics, and tetradifon acaricide in species, such as the terrestrial isopod *Porcellio dilatatus,* the amphipod *G. fossarum, D. magna,* the freshwater worm *Lumbriculus variegatus* and the fish *Carassius auratus* [107, 114, 148, 162, 180]. Results showed a common effect on metabolic toxicity indicating that this a widespread potential response. Additionally, studies could establish a direct link between short-term changes in metabolic biomarkers and long-term effects on growth and reproduction [180]. The effects observed on energy dynamics were noted at concentrations orders of magnitude below those affecting CEs. For example, Ribeiro et al. [148] found effects on the energy metabolism of the isopod *P. dilatatus* after exposure to the insecticides endosulfan and parathion at 0.1 µg/g in food, while effects on growth were only detected at 500 µg/g of food for endosulfan and putatively at even higher levels for parathion.

Molecular and cellular measurements of the energy metabolism can be plausibly linked to adverse effects at higher biological levels, indicating their relevance to the protection goals for ERA. For example, effects on energy metabolism have been directly linked to the CEs of reproduction, development, and growth in *D. magna* [44, 180]. Energy metabolism has also been strongly linked to foraging behaviour [173]. For instance, insecticides that affect energy metabolism can cause changes in foraging in bees exposed to imidacloprid [194]. This link to foraging behaviour indicates the complex connections between different NCEs linked to metabolic toxic compounds and how these interact through AOPs to affect apical CEs.

Individual-level endpoints to assess adverse outcomes

Individual-level behavioural measurements are of recognised relevance as endpoints for chemical effect assessment [3, 60, 74]. As such, their use in ecotoxicology has increased significantly in recent years [20]. Drivers for a greater focus on behaviour are that they are: (1) generally more sensitive than CEs [113], (2) can be informative of MoA [2], and (3) can reveal adverse population-level effects that would go undetected with CEs [1, 2, 79]. The relevance of behavioural effects has been recognised by the European Medicines Agency (EMA) [63] for neuroactive medicinal products and by ECHA [54] for endocrine-disrupting and neurotoxic compounds. Despite this, they remain rarely used for regulatory purposes because most studies are non-standardised, often lacking relevant information or are questioned for their reliability or methodology [3]. Currently, there is a lack of understanding of how behavioural effects relate to population fitness and ecosystem-level impacts, which leads to questioning of their use in ERA [74]. Furthermore, the lack of benchmarking between behavioural testing and CEs is a major reason why behavioural studies are commonly viewed as having low regulatory relevance [74]. However, new research indicates that the majority of scientists now believe that behavioural experiments are repeatable, reliable, and relevant regulatory authorities should consider behavioural endpoints [73]. Below, we discuss behavioural endpoints and their relationships with CEs to evaluate their relevance in ERA.

Mobility/locomotion/activity

Mobility or locomotion can be assessed by measuring the activity of an organism in a given period. For species with high mobility, like most arthropods, reptiles, and fish, velocity (moving speed) can be used as a fundamental indicator, as it is a prerequisite for other behavioural actions relevant to fitness, such as mate finding, feeding, and predator avoidance [31]. Movement velocity can also be further linked to other physiological functions, such as energy metabolism [82, 191]. Quantitative methods for assessing mobility beyond simple rate include burrowing rate, head thrashes, body bends, and number of turns for nematodes [25]. Initial steps have been made toward integrating activity-linked endpoints into current ERA practices. For example, activity is included in the assessment of *D. magna* through the observation of immobilisation or locomotion (OECD TG 202 and ISO 6341:2012) and in the description of abnormal behaviour

in several guidelines (e.g., "lying motionless" in OECD TG 220). However, the endpoint primarily validates test success, is directly linked to test failure in sediment exposures, or acts as an indicator of mortality rather than an indicator of more complex population-relevant behaviour.

In Table S2, we summarised ten case studies that show how chemicals impact locomotion compared to CEs. Mobility endpoints measured in these studies include abnormal locomotory behaviour (e.g., twisting and contradiction of the body), activity, burrowing, crawling, or swimming. Chemicals assessed include PAHs, copper, lead, oxazepam, fluoxetine, sertraline, and different insecticides. Organisms tested cover both aquatic and terrestrial species, including *Girardia tigrina, Nereis diversicolor, Scrobicularia plana, C. elegans*, *D. magna*, *Aphanius dispar*, *Pheretima guillelmi*, *Chironomus dilutes, Chironomus riparius* and *Hyalella azteca* [25, 85, 137, 145, 163, 164, 177, 198]. In a set of 39 comparisons, locomotion was, on average, 10 times more sensitive than mortality and 2 times more sensitive than growth (Table 1). For example, Rasmussen et al. [145] found that mobility in *C. riparius* exposed to sulfoxaflor was up to 10 times more sensitive than mortality and twice as sensitive as the semi-CE emergence. Van der Most et al. [177] found effects on activity in *C. elegans* due to sertraline exposure at 0.02 mg/L, 1045 times lower than the LC50 (20.9 mg/L). There was only one case study looking at reproduction; this showed that mobility was four times more sensitive [164]. In 7 of the case studies, the mobility NCE showed significant changes when CEs failed to detect any effects. Such comparisons indicate that behavioural NCEs can identify hazards at lower exposure levels than for CEs.

Beyond effects on movement velocity, several different chemical MoAs can also impact more complex locomotory behaviour. Hasenbein et al. [85] compared motility to growth and mortality across various insecticides and species. They concluded that the NCEs for mobility are especially relevant when investigating neurotoxic substances like organophosphates and pyrethroids (see also [145]). Various other studies have linked changes in locomotion to neurotoxicity induced by oxidative stress or disruption of energy metabolism [82, 177]. Measurement of these more complex mobility NCEs has also indicated cases showing the response to exposure at different concentrations, resulting in NMDRCs [124, 177]. This means there is a potential for effects to occur at lower levels, which may not be apparent based on results from higher concentration exposures.

Phototaxis

Phototaxis is the responsive movement of an organism either towards or away from light (i.e., positive and/or negative phototaxis [97]). For example, marine larvae use positive phototaxis for dispersal and negative phototaxis for settling [144], and some species use phototaxis to avoid predators [18]. Neurotoxic compounds can disrupt phototactic behaviour, impacting migration, settlement, and predator–prey dynamics [171]. However, standardised testing guidelines for chemical impacts on phototaxis are lacking, although certain case studies are recommended for compounds affecting thyroid hormones or causing developmental neurotoxicity [132–134].

In Table S2, four case studies are summarised that observed phototactic behaviour and CEs for chemicals such as the herbicide linuron, metal thallium, thymol oil, and antidepressant fluoxetine in species such as *D. magna* and honeybees (*Apis mellifera*) [19, 112, 122, 171]. In total, 14 comparisons show that the phototaxis NCE is, on average, 37 times more sensitive than the CE mortality and often occurs at low and environmentally relevant concentrations (Table 1). For example, Bergougnoux et al. [19] observed reduced phototactic behaviour in honeybees exposed to thymol at 10 ng/bee, 21,000 times lower than the LC50 (51250 ng/bee), highlighting an apparent remarkable sensitivity of this endpoint.

Phototactic effects have been linked to various MoAs, such as chemicals targeting histaminergic signalling in sensory neurons, octopamine receptors, or reductions in serotonin levels [19, 112, 122, 171]. Due to its sensitivity to chemicals with several different MoAs, phototaxis is also used as an endpoint in environmental monitoring and water quality assessment. This is done, for instance, with a biomonitor constructed to observe changes in the positive phototactic behaviour of *D. magna* along a light gradient due to chemical exposure [43, 117].

The influence of chemicals on phototactic behaviour holds significant implications for individual survival and population dynamics, particularly in predator–prey relationships where negative phototaxis can correlate with effective fleeing. For example, for *D. magna*, alterations in phototactic behaviour affect their position in the water column, potentially exposing them to predators [143]. Similarly, changes in phototactic behaviour are critical for the health of populations like honeybees, where phototaxis is essential for navigating inside and outside the hive [171]. Therefore, phototaxis can be linked in a mechanistic manner to organism traits and behaviours that lead to population-relevant outcomes.

Avoidance

Avoidance or chemotaxis tests assess the organisms' ability to sense contaminant exposure (e.g., using olfactory and other chemoreceptor organs) and actively avoid exposure. Avoidance behaviour is also a proxy for habitat suitability [92, 93], and avoidance responses at a contaminated site may ultimately result in the reduction of local populations [7]. Numerous studies have shown that chemicals, like copper and antidepressants, can impair olfaction, behaviour, and chemo/mechanosensory function [84, 177]. Further, chemical avoidance has been shown to disrupt fish migration, for example, by causing salmon to alter their migration routes, e.g., in response to metal pollution [158]. The European Commission [64] has identified avoidance as an ecologically relevant endpoint for chemical hazard assessment. As such, avoidance has been included in standardised guidelines, but so far only for terrestrial species (ISO 17512–1:2008: Earthworm *E. fetida*/*andrei*; ISO 17512–2:2011: Collembola *F. candida*). However, these assays have already been demonstrated to be highly sensitive compared to CEs [77].

A total of seven case studies, including ten comparisons measuring chemical avoidance responses relative to CEs, are summarised in Table S2. These case studies encompass a range of model organisms, including the amphibian *Lithobates catesbeianus*, the fish *Poecilia reticulata*, and the crustacean *Palaemon varians*, for various chemicals, including herbicides (diuron and atrazine), the pesticide abamectin, copper, and sunscreen (chemical mixture including e.g., Avobenzone, TiO2) [8–10, 118, 177, 178, 189]. Avoidance was, on average, 46 times more sensitive than mortality, and in many cases showed effects at environmentally relevant concentrations (Table 1). One case study examined found significant chemotaxis (the movement toward a chemical attractant) in *C. elegans* at 1 ng/L of fluoxetine, compared to mortality at 253 mg/L [177]. Avoidance has proved effective as an endpoint for compounds with neurotoxic, immunotoxic, and metabolic toxic effects. The NCE is especially relevant for chemicals affecting olfactory cues or downstream neurological systems. However, use of the latter should be done cautiously, as these substances can impair an organisms' ability to escape the toxic environment, leading to failure to detect avoidance effects [96].

Behavioural avoidance assays can give information about the ability of an organism to detect a chemical threat and respond. Such assays allow the determination of the avoidance concentration (AC), indicating impairment of chemosensory organs or movement. Moreira et al. [118], Vasconcelos et al. [178], and Araújo et al. [9] established threshold concentrations for avoidance, while noting that this effect could be diminished at higher concentrations if exposure compromises sensory function. In these case studies, harmful effects only occurred when the capacity to avoid chemical contamination was compromised, indicating the relevance of avoidance, both as a sublethal effect in its own right, and a compensatory mechanism that organisms use to reduce further exposure.

Avoidance assays can provide crucial ecological information. By using a multi-compartment system with several chambers that allow organisms to move between concentrations, avoidance studies have shown that chemical exposure can have important implications for habitat suitability [118]. Such effects can be linked to adverse population-level outcomes (e.g., loss of available habitat range), even if growth and reproduction are unaffected [178]. Thus, chemical avoidance could explain the local extinction or fragmentation of populations. For example, in a study by West & Ankley [189], growth and/or survival tests with contaminated sediments failed to reveal chemical effects, while avoidance tests with *Lumbriculus variegatus* showed responses that could explain the degraded state of benthic communities in the field through emigration to cleaner sediment.

Exploratory behaviour (boldness)

Exploration behaviour (boldness) is the individual's willingness to take risks, including exploring new environments [39]. This behaviour is known to be affected by the chemical exposure at environmental concentrations, e.g., pharmaceuticals [31]. As an endpoint in ecotoxicology studies, boldness can be directly linked to effects on organism survival by influencing the ability to find food and mates or by increasing predation risk [11, 31, 146]. Exploratory behaviour can be measured in assays that assess the time required for an individual to visit all zones within a new environment, often referred to as an open field test [146]. Such an approach can be adapted to behaviour for different species (e.g. great tits, zebrafish) [36, 48]. For now, measures of boldness are not implemented in current environmental risk and hazard practices (Table S1). However, when addressing emerging hazards, particularly neurotoxicity, boldness can be considered relevant for future integration.

Table S2 summarised four case studies with five comparisons of chemical effects on exploratory behaviour or boldness with CEs [75, 125, 161, 175]. These studies assess the effects of the neurotoxic drugs sertraline, escitalopram, fluoxetine, and cadmium on the fish species fathead minnow (*Pimephales promelas*), and zebrafish (*D. rerio*) [30, 124, 156, 161, 175]. Studies showed that the NCE is more sensitive, being on average 617 times more so than mortality and 6.6 times growth (Table 1),

although based on a more limited dataset than for some other NCEs. Within specific studies, Shelton et al. [161] found that cadmium impacted boldness behaviour in *D. rerio* at 1 µg/L, at a concentration 9680 times lower than for mortality, as observed by Al-sawafi et al. [5]. A study by Nielsen et al. [125], exposed *D. rerio* to the psychoactive drug escitalopram. Both behaviour and size were significantly altered at 1.5 µg/L. However, behavioural changes were gender-specific, with female boldness being significantly affected and males not, indicating the need to consider both male and female effects in studies. Changes in boldness due to pollutant effects pose risks not only for individuals, but also for populations. For example, Shelton et al. [161] found that a few individuals exposed to cadmium influenced the boldness of the unexposed majority, as even at low concentrations, cadmium exposed pairs caused changes in shoal boldness behaviour.

Foraging and feeding behaviour

Foraging behaviour, including foraging efficacy, search time, and feeding rate, has been used in several ecotoxicological studies. In the current ERA guidelines, foraging-related endpoints are currently only used as a validation criterion in studies that expose the model organisms through chemicals in their diet, such as birds (e.g., OECD TG 205), but not as a standardised and quantifiable behavioural endpoint. Several studies have shown chemical impacts on the ability to identify or reach food and feed [41, 84, 85]. Effects on foraging can be directly linked to population-relevant endpoints [41]. For example, extended periods of limited food intake can lead to starvation and ultimately to reduced growth, reproduction, and survival [1, 127] through linkage to energy budgets [2, 41]. Chemical effects on foraging can occur through multiple pathways, for example, modulating sensory organs, impacting neuronal pathways supporting food detection and by affecting foraging behaviour itself [46, 84, 177].

Table S2 summarises seven case studies with a total of 15 comparisons between NCEs for foraging behaviour, such as feeding inhibition and foraging rate/speed, and different CEs. These cases cover various contaminants such as copper, imidacloprid, fluoxetine, and PAHs, in species such as *Pimephales promelas*, *G tigrina*, or *G. pulex* [1, 2, 84, 127, 163, 177, 188]. Compared to CEs, the NCE linked to foraging were, on average, 275 times more sensitive than mortality and 4 times more sensitive than reproduction (Table 1). Van der Most et al. [177] showed that feeding was especially sensitive in *C. elegans* exposed to antidepressants, with significant changes at 0.39 µg/L for fluoxetine and 0.89 ng/L for sertraline and with effects following complex NMDRCs. These effects were compared to LC50s for fluoxetine of 253 mg/kg and sertraline of 209 mg/kg. The case studies show that foraging behaviour is relevant for a range of MoAs, revealing, for example, effects on olfactory senses. Green et al. [84] demonstrated that copper affects feeding not by inhibition but rather by loss of sensory perception. This phenomenon was also observed by van der Most et al. [177], who linked the feeding impacts of sertraline and fluoxetine to chemosensory organ changes.

For four of the comparisons, feeding was inhibited when the CE mortality was not affected at any concentration in the exposure range. Previous studies have shown that some chemicals, such as PAHs or imidacloprid, may inhibit feeding behaviour at low concentrations, leading to mortality through starvation [1, 163]. Given that mortality due to starvation occurs over a longer timeframe than for standard mortality tests [61, 91, 128], conventional acute studies are insufficient to quantify such impacts [163]. Foraging behaviour can also have important implications for survival, growth, and reproduction, indicating a need to consider the impacts of feeding inhibition in ERA practices. The development of feeding inhibition tests, such as for *D. magna*, offer the potential to conduct such screening studies [2].

Population-level endpoints to assess adverse outcomes

The primary goal of the ERA is to identify chemical effects that may lead to adverse population outcomes [34, 59, 68]. As outlined above, lower-tier NCEs can be linked to adverse population-level effects. However, some NCEs can also serve as direct measurements of population outcomes. Examples include observing dispersal or migratory behaviour, assessing group effects through social behaviour (e.g., mating, shoaling, aggressiveness), or evaluating multi-species endpoints like predator–prey interactions [27–29, 116]. While these higher organisation-level endpoints are more complex and costly to assess, they offer valuable insights that individual-level endpoints, by their reductionist nature, do not provide [71]. This makes such endpoints of significant value for future ecological hazard and risk assessment [32]. As social behaviour and predator–prey interactions are endpoints frequently observed in ecotoxicological studies and assessed under laboratory conditions, we focused on these two in the Sects. "Social behaviour" and "Predator–prey interactions".

Social behaviour

Social behaviours in animals, such as mating or aggression, are directly tied to the survival of both the individual and the group and consequently to adverse outcomes for the population [116, 155]. Given this link, including social behaviour in hazard assessment has

already been a topic of extended discussion [21, 116, 197]. NCEs for social behaviour can be measured at the individual (e.g., aggression measurements in individuals of *D. rerio*) or group level (e.g., shoaling behaviour in a group of fish). Studies using such methods have shown that social behaviours can be altered by environmentally relevant concentrations of chemicals, such as antidepressants [116], which can increase aggression in fish [170], make individual fish generally less social [188], or disrupt collective behaviours like shoaling [14, 99]. Chemical exposure can also lead to changes in the group competence of organisms through effects on mating or communication [116], for example, by affecting aggregation behaviour in the terrestrial isopod *Porcellionides pruinosus* [70]. Chemical-associated effects on such behaviours can have implications for ecological interactions, making them explicitly relevant at the population level and, therefore, for hazard and risk assessment. As such, integrating social behaviour endpoints into toxicity assessments helps to capture the complex interactions and real-world ecological impacts of chemical exposure that CEs fail to address [21].

Table S2 summarises four case studies observing chemical effects on social behaviour. Generally, these studies show that effects on these endpoints are significantly more sensitive than CEs. For example, effects have been seen for oxazepam, cyprodinil, fluoxetine, and benzyl butyl phthalate on social behaviour such as shoaling behaviour and aggressiveness of the fish species *Fundulus heteroclitus*, *Perca fluviatilis*, *Pimephales promelas,* and *D. rerio* [75, 99, 170, 188]. These NCE effects were, on average, 387 times more sensitive than mortality and 10 times more than reproduction (Table 1). For instance, Tang et al. [170] found that changes in aggressive behaviour in *D. rerio* exposed to environmentally relevant concentrations of cyprodinil (0.1 µg/L) were 12,600 times more sensitive than the LC50 [184]. Similar to exploratory behaviour, the dataset for this endpoint was too limited in size to draw robust conclusions.

Measurements of social behaviour in hazard assessment can provide further information on the ecological relevance of disruptions in social behaviour responses caused by chemicals. For example, Weinberger & Klaper [188] found that fluoxetine exposure significantly increased aggression in male fish, resulting in a reduction of female survival and reproductive success. Armstrong et al. [14] demonstrated that the shoaling behaviour of the Atlantic croaker (*Micropogonias undulatus*) was notably disrupted when just one individual was exposed to oil. Kaplan et al. [99] found that fish exposed to benzyl butyl phthalate preferred smaller shoals, potentially losing the advantages of critical ecological behaviours.

Predator–prey interactions

Predator–prey behaviours are pivotal species interactions, influencing population and community structure [29]. Chemical contamination can impact these trophic interactions [30, 31, 182]. Various methods, such as predation cues, have been developed to measure predator–prey interactions following chemical exposure in multifactorial or mesocosm studies [89, 167]. Currently, though, there are no standardised guidelines for these assays (Table S1) for application in regulatory risk assessment [3, 141].

Table S2 summarises five case studies that have assessed the effects of a wide range of chemicals such as cadmium, cyprodinil, and fluoxetine by measuring effects on antipredator behaviour in fish species, such as *D. rerio* and *P. promelas* [75, 105, 170, 182, 188]. On average in these studies, the predator–prey interaction endpoints were 317 times more sensitive than mortality (Table 1). As examples, Tang et al. [170] found that antipredator behaviour in *D. rerio* exposed to cyprodinil was significantly altered at 0.1 µg/L compared to the corresponding LC50 of 1260 µg/L [184]. Similarly, a further study found that predator avoidance in *P. promelas* exposed to fluoxetine [188] was 100 times more sensitive than reproductive effects [33]. The case studies demonstrate that measurements of antipredator behaviour may occur at environmentally relevant concentrations, in some cases following NMDRCs [188].

It is possible to link predator–prey interactions to biochemical, physiological, and behavioural processes linked to underpinning MoAs. For example, a study by Volz et al. [182] linked antipredator responses directly to the disruption of the olfactory system in *D. rerio* after exposure to cadmium. This impairment was found to be retained even after a 14-day recovery period [105]. Tang et al. [170] showed how a neurotoxic MoA can lead to changes in predator–prey interactions. Thus, exposure of *D. rerio* to the fungicide cyprodinil led to the disruption of hypothalamic function, which caused an increase in cortisol levels that can alter antipredator behaviour at low concentrations.

Another aspect of predator–prey interactions is related to the potential for asymmetric effects between species [31, 89]. For example, Brodin et al. [31] found that exposure to oxazepam (2 µg/L) increased activity in perch (predators), but did not affect damselflies (prey), altering trophic interactions and, consequently, ecological systems. A further and potentially common effect on predator–prey relationships is through the effects of prey abundance. Conventional chemical assessments may overlook these indirect effects, underestimating their impact on populations and ecosystems. Fleeger et al. [71] emphasise the indirect effects of pollutants on aquatic

ecosystems through trophic cascades, highlighting the need for a comprehensive understanding of chemical impacts on ecological systems, especially in predator–prey interactions. Kidd et al. [103] studied the effects of EE2 (synthetic oestrogen 17a-ethynylestradiol) on a lake ecosystem, observing a 42% decrease in the top predator biomass due to population declines in prey species. Such indirect effects can even be considered early stages of community-level effects, which underscores the importance of considering trophic interactions in chemical impact assessments.

Reflection on ERA

A conceptual AOP-structured, MoA-based framework for emerging hazards

Currently, the emerging hazards, neurotoxicity, immunotoxicity, and metabolic toxicity, are primarily considered within the context of human RA. However, there is a significant knowledge gap regarding how to integrate these effects into environmental RA. We present here a first approach to addressing this challenge, outlining also possible directions for development. For any assessment, it is crucial to link the mechanistic understanding NCEs provide to population-level outcomes [52, 53]. The AOP

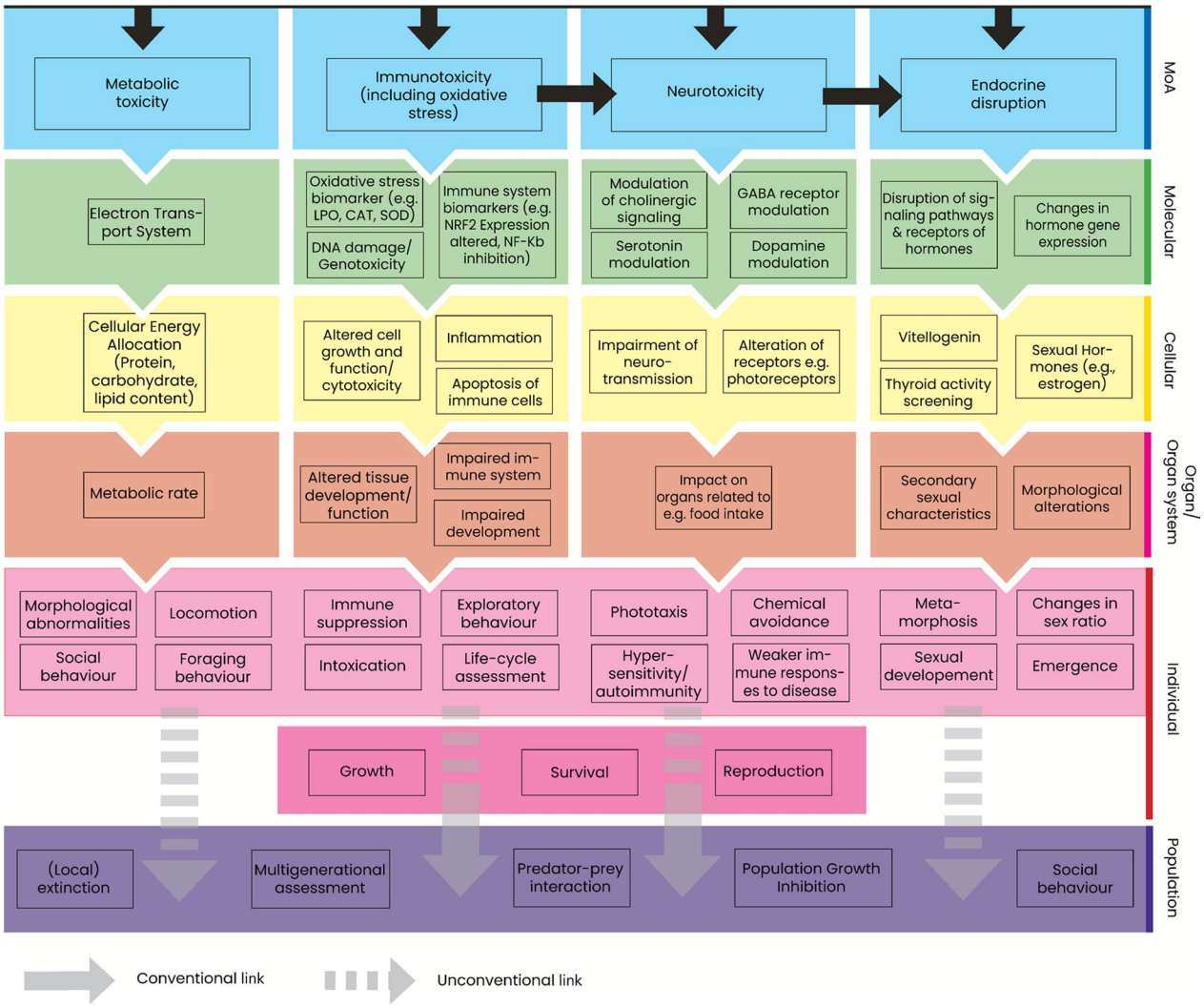

Fig. 2 This conceptual framework for a mode of action (MoA)-based environmental risk assessment (ERA) incorporates both conventional, semi-conventional, and non-conventional endpoints following the adverse outcome pathway (AOP) structure from MoA (top) to population level (bottom) for the emerging hazards neurotoxicity, immunotoxicity, and metabolic toxicity, as well as endocrine disruption. Effects through each MoA can also influence other pathways, such as oxidative stress, which may lead to immunotoxicity or neurotoxicity. Pathways converge at the individual level, where multiple causes can contribute to observed effects. These individual-level effects can then be connected to population-level outcomes through two routes: the conventional link (undashed arrow), which includes conventional endpoints such as effects on mortality, growth, and reproduction, or the unconventional link (dashed arrow) through non-conventional endpoints that links to apical effects. The figure is a conceptual example and is not exhaustive, as it does not include all possible links between MoAs, key events (KE), and adverse outcomes (AO)

framework is designed to address this question of linkage [52, 53]. The information from the described CEs and NCEs in Tables 1, S1 and S2 has been used to create an AOP-structured, MoA-based hazard assessment framework using conventional, semi-conventional, and non-conventional endpoints encompassing effects from the molecular to the population level for neurotoxicity, immunotoxicity, metabolic toxicity, and the already integrated hazard class, endocrine disruption (Fig. 2). Effects through each MoA can influence multiple pathways, such as oxidative stress, which may lead to neurotoxicity and immune effects. Pathways converge at the individual level, where multiple causes can contribute to observed effects. These individual-level effects are connected to population-level outcomes through effects on the CEs of mortality, growth, and reproduction, or via unconventional links to apical effects (e.g., chemical avoidance leading to displacement of populations).

Establishing quantitative links across molecular, individual, and population levels further provides predictive power. For example, small perturbations at the molecular level often do not translate linearly into higher levels, as the population one. Moreover, traditional AOPs typically assume a monotonic or linear relationship between key events. However, most of the time, biological systems are characterised by nonlinearities, thresholds, and context-dependent interactions, which can profoundly modulate the propagation of effects across levels. Quantitative Adverse Outcome Pathways (qAOPs) in this context can help extend the classical AOP framework by introducing mathematical and computational representations of the relationships between key events, enabling predictions of AOs from molecular perturbations [138]. Unlike qualitative AOPs, qAOPs aim to capture dose–response relationships, temporal dynamics, and nonlinear interactions at AOP levels. This quantitative approach enhances the predictive power of AOPs, potentially improving risk assessment decisions and the identification of critical thresholds for intervention [58]. Tools to establish these quantitative relationships are, for instance, Structural Equation Modeling or Bayesian Neural Networks (BNNs). Further information can be found in, for instance, Semenova et al. [160].

Mechanistic understanding through NCEs

Through development in their measurement it has now come to the point where in many cases low-level NCEs have reached a status in development where they can be considered as: (1) cost-effective and easy to measure, (2) established protocols exist in many ecotoxicological laboratories for various model species, and (3) responsive enough that effects can be detected at lower concentrations and at earlier time points than is the case for CEs. Our case studies provide further examples of endpoints that fulfill these criteria and could be considered for hazard assessment. The primary biomarker to measure neurotoxic effects is (A)ChE, which has been applied to several contaminants [45]. This NCE can provide sensitivity and informative mechanistic information, as it can be linked to behavioural and mortality effects in the observed case studies [101, 165]. Other neurotoxic endpoints, such as GABA receptor modulation [183, 185], are more complex to measure and less easily adapted to different model species, emphasizing the need for further development of neurotoxicity endpoints for ERA. For immunotoxicity, several linked biochemical endpoints can be measured, such as increased neutrophils and lymphocytes, increased phagocytes, a decrease in circulating antibodies, T-cell response, and phytohemagglutinin-P increases [94, 110]. For metabolic toxicity, the aerobic energy production (estimated from electron transport system (ETS) activity), respiration, and cellular energy allocation have been shown to be sensitive markers for chemicals known to cause metabolic toxicity, such as SDHIs [107]. Regarding immunotoxicity and neurotoxicity, biochemical measurements of oxidative stress (e.g., catalase, lipid peroxidation) can also be crucial, as oxidative stress can serve as a key event within these MoAs [49, 102].

We would like to emphasise that the nervous, immune, and metabolic systems are complex, highly interconnected networks, similar to the endocrine system. For instance, interspecific differences in physiology—particularly in neuronal, immune, and metabolic systems—can strongly influence how chemicals affect organisms. For example, the type of ChE present in a species can significantly determine its sensitivity to certain compounds [126]. To reflect this diversity, we aimed to include a range of species when selecting case studies for comparison. More information about how the reliance on a limited number of model species constrains our understanding of chemical risks and how ERA could benefit from incorporating a broader diversity of test organisms can be found in, for instance, Rosner et al. [152].

We want to point out that the biochemical endpoints discussed in this conceptual review are not the only ones required to capture all relevant MoAs, but rather serve to illustrate a conceptual approach for addressing these emerging hazards at their respective biological levels.

In this context, new approach methodologies (NAMs), including in chemico, in silico, and in vitro models, represent promising approaches to verify mechanisms of action to support the regulation of neurotoxic, immunotoxic, and metabolic toxic compounds [53]. The "Roadmap for phasing out animal testing", which is going to be published in early 2026, is expected to further underpin the regulatory acceptance of these alternative methods in ERA [67]. However, completely phasing out animal testing remains challenging at the current stage of our scientific knowledge. In line with the principles of the 3Rs (replacement, reduction, refinement), our review aims to contribute by proposing ways to refine existing standardised guidelines to better integrate NCEs.

Undetected adverse outcomes on individual and population level

To be relevant in ecological risk assessment, it is essential that neurotoxicity, immunotoxicity, and metabolic NCEs are qualitatively and quantitatively linkable to population-level effects. As detailed, chemical impacts on phototaxis, locomotion, avoidance, exploratory behaviour, foraging behaviour, social behaviour, and predator–prey interactions can all be linked to adverse outcomes at the population level through impacts on ecological and trophic interactions [1, 2, 10, 118, 163]. These NCEs are also now more broadly operational, as they: (1) can be effectively measured in several model species (e.g., locomotion, phototaxis, foraging), (2) can be quick and cost-efficient to assess (e.g., locomotion, phototaxis, foraging), (3) can be robustly quantified (locomotion, phototaxis, foraging), (4) can be influenced by multiple chemical MoAs (locomotion, phototaxis, chemical avoidance, predator–prey interaction), and (5) can be measured in a non-invasive assessment (aligning with the principles of refinement within the 3Rs). These NCEs can also provide more ecologically relevant information by identifying gender-specific responses relevant to mortality and reproductive behaviours, such as territoriality, courtship, and boldness [125]. Group effects can be studied through changes in behaviour, like social and exploratory behaviour endpoints affecting group dynamics and social hierarchy [161]. Further by incorporating social behaviour or predator–prey interactions, it is possible to provide a better understanding of species dynamics and asymmetric effects that can affect the stability and energy flows of trophic networks [31]. Finally, our comparisons showed that NCE effects were often observed when there was no significant change in the CE (13 cases, Table 1), showing that the NCEs revealed population-level effects that would have gone undetected under current standardised testing methods [98].

However, the regulatory value of endpoints depends on demonstrating a consistent and predictive linkage to adverse outcomes of ecological significance. For now, the individual and population-level NCEs should be considered complementary diagnostic tools within a tiered or AOP-based framework. In the long run, we aim for these endpoints to be considered as stand-alone adverse outcomes on the population level, having similar weight as measurements based on conventional endpoints.

Sensitivity of NCEs

A key aim of the conceptual review was to evaluate the sensitivity of NCEs compared to CEs, focusing on individual- and population-level endpoints. To summarise the results: Table 1 gives an overview of 43 case studies that measured individual- and population-level NCEs (locomotion, phototaxis, avoidance, exploratory behaviour, foraging, social behaviour, predator–prey interaction) alongside CEs (mortality, growth, and reproduction). As some studies included several species, chemicals, and NCEs/CEs, we were able to make a total of 96 comparisons of CE and NCEs in terms of their response sensitivity. In 13 cases (i.e., 13.5%), significant effects were observed for the NCEs, while no effects on the CE were seen in any test treatment. Many of the changes in NCEs were observed at environmentally relevant concentrations, indicating their real-world relevance [10, 85, 171]. One reason why these effects go undetected in some assessments and observations is that the NCE shows effects at low concentrations not evident at higher concentrations due to the complex NMDRC response [124, 188]. This points to a need to conserve a range of exposure levels, including low concentrations in studies of such NCEs.

For those remaining comparisons where studies found effects both for the CE and NCE, we determined the fold increase in NCE sensitivity. In nine cases (i.e., 9.4%), the NCE was more than 1,000 times more sensitive than the CE. As the data spans several orders of magnitude, we calculated the geometric mean of the fold increase for mortality, reproduction, and growth, respectively. The comparative analysis showed that NCEs were, on average, 56 times more sensitive than mortality (GSD = 55.9, n = 59), 8 times more sensitive than reproduction (GSD = 7.6, n = 8), and 2 times more sensitive than growth (GSD = 1.7, n = 15) (Table 1; Fig. 3). Although few review papers directly compare NCEs and CEs, the range of relative sensitivity is consistent with that of Melvin and Wilson [113], who found through meta-analysis that behavioural endpoints (mainly locomotive endpoints) were 4 times more sensitive than the CE development (which can be related to growth), and 2 times more

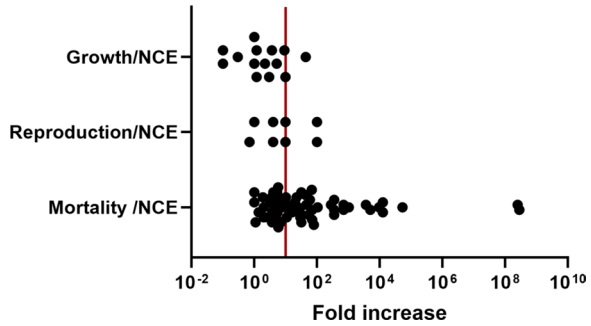

Fig. 3 Comparison of effect levels of the conventional- (CE; mortality, reproduction, growth) and non-conventional endpoints (NCEs) on the individual- and population-level (locomotion, phototaxis, avoidance, exploratory behaviour, foraging, social behaviour, predator–prey interaction) in fold increase. Excluded from this graph were studies where no effects were observed for CEs (i.e., 24.2% of the cases). In 9.4% of all 96 comparisons, the NCE was 1000 times more sensitive than the CE (range 1045–253000000 times higher). The red line represents the assessment factor of 10 to account for differences in acute-chronic effects

sensitive than reproductive endpoints. Faimali et al. [69] also found in their reviewed literature that swimming behaviour was, on average, 16 times more sensitive than mortality/immobility.

The greater sensitivity of NCEs raises the question of how behavioural NCEs should be considered in ERA practices, especially given that many such endpoints can be readily related to population-relevant outcomes (mating, trophic interactions, predation, etc.). Differences in acute-chronic effects are usually covered by an assessment factor of 10 [50]. However, if the behaviour is, on average, 56 times more sensitive than mortality or 8 times more sensitive than reproduction, this identifies that placing an assessment factor of 10 on reproduction data would often, but not always, be sufficient to account for links through NCE, but for mortality would not be sufficient in the majority of cases. It should be noted that the comparative analysis is based on averages and will, as such, not cover the species where NCEs are impacted at concentrations more than 10 times (i.e., up to > 1000 times) lower than the CE, or cases where no effects on CEs were observed at all. Similar to Forbes et al. [72], we conclude that the assessment factor seems to be protective, but we also identified scenarios in our system where it is highly underprotective, and we raise the question whether these outliers (> 1000 times higher effect concentration) should be given more attention.

Food for thought for scientists and regulators

Including NCEs, building on standardised guidelines, into risk assessment research to refine ERA practices has the potential to improve understanding of chemical impacts on species in a manner in line with the 3Rs. A transition to their greater integration into ERA would support more ecologically relevant hazard assessments, ultimately leading to more robust environmental risk assessment. NCEs should only be included in ERA practices if they provide a higher sensitivity than CEs, specificity and accuracy. While sensitivity and specificity were discussed in the prior sections, we still need to address accuracy, which is provided by the validation of the chosen endpoint and standardisation of methods. Some endpoints are already ahead of the curve, showing greater readiness for standardisation of methods. Quantified effects on locomotion, for instance, are already partially integrated into existing guidelines (see Sect. "Definition of conventional, semi-conventional endpoints and non-conventional endpoints"). This is, therefore, an endpoint that could be more immediately integrated. Similarly, for phototaxis, which is reproducible and readily analysed, established protocols are already available for several model species (e.g., *D. magna*, [112]). Avoidance is already included in standardised guidelines for soil organisms [92, 93], which could be extended to further species and ecosystem compartments (e.g., sediment organisms). Social behaviour could be included by integrating shoaling endpoints into testing protocols, such as OECD TG 210, as proposed by Frese & Braunbeck [75]. However, to include these endpoints, further development and ring-testing would be necessary. Integration of NCEs in hazard assessment within IATAs (Integrated Approaches to Testing and Assessment) is also a way in which these approaches can be integrated with more recognised ERA practice [131].

The greater research use of NCE studies has the potential to support regulatory decisions about chemical risks. However, such endpoints are often excluded due to concerns about reliability and ecological relevance [3]. Especially the evaluation framework Klimisch, used for the weight of evidence approach under REACH, does not consider non-standard studies as reliable without restrictions (Klimisch Category 1). Therefore, they always get less weight in regulatory decision-making than standard studies, for instance, when they are handed in as supporting studies as part of the registration dossier. To address this, researchers can follow the Criteria for Reporting and Evaluating Ecotoxicity Data (CRED) or EthoCRED for behavioural studies, ensuring a robust evaluation of ecological relevance and reliability [20, 100]. A detailed guidance for integrating non-standard studies in regulatory risk assessment has now also been presented in the new *OECD Guidance document on the Generation, Reporting and Use of Research Data for Regulatory Assessment* [136]. Developed non-conventional methods can also be submitted in the OECD's Standard Project Submission

Form (SPSF) to make these methods both more transparent and accessible for their use. Progress through the combined action can support the greater uptake and use of NCEs to understand the wider hazards of chemicals not fully understood through current testing. Something that will benefit knowledge and capacity to manage chemical hazards in the environment, especially for the emerging hazards, neurotoxicity, immunotoxicity, and metabolic toxicity.

Abbreviations

AChE	Acetylcholinesterase
AC50	Avoidance concentration 50%
AO	Adverse outcome
AOP	Adverse Outcome Pathway
AC	Avoidance Concentration
BNN	Bayesian Neural Network
CE	Conventional Endpoint
CEA	Cellular Energy Allocation
ChE	Cholinesterase
CLP	Classification, Labelling and Packaging
CMR	Carcinogenic, Mutagenic or toxic to Reproduction
CRED	Criteria for Reporting and Evaluating Ecotoxicity Data
ECHA	European Chemical Agency
EDC	Endocrine Disrupting Compound
EC50	Effective Concentration 50%
EE2	17α-Ethinylestradiol
EFSA	European Food Safety Authorisation
ELoC	Equivalent Level of Concern
EMA	European Medicines Agency
ERA	Environmental Risk Assessment
ETS	Electron Transport System
GABA	Glutamate Decarboxylase
GSD	Geometric Standard Deviation
KE	Key Event
LC50	Lethal Concentration 50%
IATA	Integrated Approaches to Testing and Assessment
ISO	International Organization for Standardization
MoA	Mode of Action
MDC	Metabolism-Disrupting Chemicals
MCPA	2-Methyl-4-chlorophenoxyacetic acid
nAChR	Nicotinic Acetylcholine Receptor
NCE	Non-Conventional Endpoint
NMDRC	Non-Monotonic Dose–Response Curve
OECD	Organisation for Economic Co-operation and Development
PAH	Polycyclic Aromatic Hydrocarbons
PCB	Polychlorinated Biphenyls
qAOP	Quantitative Adverse Outcome Pathways
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
semi-CE	Semi-conventional endpoint
SHDI	Succinate dehydrogenase inhibiting fungicide
SPSF	Standard Project Submission Form
SVHC	Substance of Very High Concern
TG	Test guideline
TiO2	Titanium Dioxide
TBTO	Tributyltin Oxide
vPvB	Very Persistent and Very Bioaccumulative

Supplementary Information

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Additional file1 (XLSX 24 KB)

Additional file2 (XLSX 100 KB)

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Author contributions
All authors contributed to the conceptualisation and design of the study. Data collection was carried out by JH, HL-Q, KN, SR, and WC. JH performed the data analysis. The main manuscript text was written by JH, with HL-Q, SR, and KN authoring specific sections. JH prepared the Tables 1, S1 and S2, and Figs. 1 and 2. J.H., H.S. and D.S. contributed to the discussion. All authors reviewed and approved the final version of the manuscript.

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Availability of data and materials
Data is provided within the manuscript or in the supplementary information files, Tables S1 (additional file 1) and S2 (additional file 2).

Declarations

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Competing interests
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