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Critical Review

Cryptic Species in Ecotoxicology

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Abstract: The advent of genetic methods has led to the discovery of an increasing number of species that previously could not be distinguished from each other on the basis of morphological characteristics. Even though there has been an exponential growth of publications on cryptic species, such species are rarely considered in ecotoxicology. Thus, the particular question of ecological differentiation and the sensitivity of closely related cryptic species is rarely addressed. Tackling this question, however, is of key importance for evolutionary ecology, conservation biology, and, in particular, regulatory ecotoxicology. At the same time, the use of species with (known or unknown) cryptic diversity might be a reason for the lack of reproducibility of ecotoxicological experiments and implies a false extrapolation of the findings. Our critical review includes a database and literature search through which we investigated how many of the species most frequently used in ecotoxicological assessments show evidence of cryptic diversity. We found a high proportion of reports indicating overlooked species diversity, especially in invertebrates. In terrestrial and aquatic realms, at least 67% and 54% of commonly used species, respectively, were identified as cryptic species complexes. The issue is less prominent in vertebrates, in which we found evidence for cryptic species complexes in 27% of aquatic and 6.7% of terrestrial vertebrates. We further exemplified why different evolutionary histories may significantly determine cryptic species' ecology and sensitivity to pollutants. This in turn may have a major impact on the results of ecotoxicological tests and, consequently, the outcome of environmental risk assessments. Finally, we provide a brief guideline on how to deal practically with cryptic diversity in ecotoxicological studies in general and its implementation in risk assessment procedures in particular. *Environ Toxicol Chem* 2023;42:1889–1914.

Keywords: Cryptic diversity; Evolutionary ecotoxicology; Environmental risk assessment; Sibling species; Tolerance

INTRODUCING THE PHENOMENON OF CRYPTIC SPECIES IN METAZOA

Traditional ecotoxicological testing often determines a concentration–response relationship for a given contaminant and a certain endpoint (e.g., survival or reproduction) at a specific point in time (Walker et al., 2012). Environmental risk assessment is based on comparing contaminant exposure expected or measured in the environment and the effects induced by the contaminant for a certain species by considering some uncertainty through an assessment factor. For this purpose, standardized test methods have been developed, which often make use of laboratory-reared organisms. The use of animals from the wild is an attractive alternative to extend the spectrum of study organisms within the framework of prospective risk assessment (Chapman, 2002). When one is using wild organisms, current guidelines advise collection of the species from relatively uncontaminated sites and identification of species using an appropriate taxonomic key (Amiard-Triquet et al., 2015). This approach has been challenged in recent years by the routine large-scale use of molecular methods that have

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uncovered a large number of previously unrecognized "cryptic" species, not identifiable by morphological characteristics employed in taxonomic keys. As a result, the use of field-sampled individuals may increase the uncertainty of test results because (at least so far) the taxonomic similarity of newly caught individuals with already used test specimens is not always secure.

In general, the term "cryptic species" refers to two or more species that are very similar or identical in appearance and thus difficult or impossible to recognize by morphology, but that have reproductively well-isolated and phylogenetically distinct evolutionary lineages (Fišer et al., 2018; Sáez & Lozano, 2005). In some cases, molecular studies can provide a basis for finding fine morphological diagnostic features that can separate the formerly indistinguishable species—these are often termed "pseudocryptic species" (Jabłońska et al., 2018; Rudolph et al., 2018). A recent example comes from the freshwater amphipod crustacean *Gammarus fossarum*, once considered a single species widespread in Europe, which turned out to be a species complex of at least 84 cryptic species (Wattier et al., 2020), with some known to be syntopic without any recent gene-flow between them (Bystřický et al., 2022; Lagrue et al., 2014). Another example is one of the most common terrestrial test organisms, the lumbricid earthworm *Eisenia fetida* (in the past often spelled "*foetida*"), which is now considered to contain at least two morphologically similar species (*E. fetida* and *E. andrei*; Römbke et al., 2016). These two examples are by no means exceptional phenomena; it appears that cryptic species can be found across almost all extant taxonomic groups and probably represent a significant portion of a yet largely undiscovered biodiversity (Pérez-Ponce de León & Poulin, 2016). Cryptic species seem to be most common in taxa occurring in isolated environments, presumably because allopatric isolation prevents gene flow between habitats (as in lumbricids, King et al., 2008; or amphipods, Wattier et al., 2020). Hence cryptic species could be of profound significance to our understanding of biodiversity, biogeography, and also ecotoxicology (Bickford et al., 2007; Fišer & Koselj, 2022; Struck et al., 2018). To account for this largely overlooked diversity, various molecular species delimitation methods have been widely incorporated into ecological and evolutionary studies. Based on genetic data, molecular operational taxonomic units (MOTUs) are delimited and used as approximate and handy species equivalents allowing for identification of possible cryptic species. The most commonly used markers for molecular species identification of metazoans are gene fragments within mitochondrial DNA, also referred to as DNA barcodes (Kress et al., 2015)—particularly the universally employed cytochrome oxidase subunit I (COI; Hebert et al., 2003), cytochrome *b* (cyt *b*; Hsieh et al., 2001; Parson et al., 2000), or 12S and 16S ribosomal (r)RNA (Cawthorn et al., 2012; Shu et al., 2021). These markers proved to be reliable for species delimitation, as has been demonstrated in case studies on invertebrates such as earthworms (Oligochaeta: Lumbricidae; James et al., 2010), springtails (Collembola; Hogg & Hebert, 2004), butterflies (Lepidoptera; Dincă et al., 2021), mayflies (Ephemeroptera; Ball et al., 2005), and black flies (Diptera: Simuliidae; Rivera & Currie, 2009), as well as on vertebrates such as fish (Kumar et al., 2022; Ward et al., 2009), or mammals (Clare et al., 2007). Genetic markers also help in opposite cases, for example, in phenotypic polymorphic mollusks, with different shell morphs turning out to belong to the same lineage (Osikowski et al., 2018).

However, despite their frequency, the phenomenon of cryptic species has so far rarely been addressed in ecotoxicology but has been considered to be important for interpretation of data (Feckler et al., 2013; Novo et al., 2015; Otomo et al., 2013; Römbke et al., 2016; Spurgeon et al., 2020; Weston et al., 2013). Thus, the question arises as to how many of the species regularly used in ecotoxicological testing procedures could harbor cryptic species contributing to data variability. Significant genetic differentiation including different evolutionary histories of lineages within a cryptic species complex may result in a wide range of stress responses (see Beermann et al., 2021) that can make the results of ecotoxicological assessments less accurate than expected. In the present review, we describe and discuss current ecotoxicological approaches and address two main aspects. First, we evaluate the level of attention cryptic species have received in ecotoxicology and provide an overview of regularly used organisms followed by examination of evidence for potential cryptic diversity. Second, we provide guidance on the implementation of cryptic species in routine ecotoxicological study and discuss the absorption of the knowledge into regulatory ecotoxicology.

THE ECOLOGICAL SIGNIFICANCE OF CRYPTIC SPECIES

Ecotoxicological outcomes are inherently connected to species' ecology, which is encapsulated in the concept of the ecological niche, often perceived and studied through two complementary views, the so-called Grinellian and Eltonian ecological niches (Peterson, 2011). The Grinellian niche aims to disentangle the relationship between an individual and the environment, such that it considers the limits of ecological space where species thrive and reproduce. Translated to ecotoxicology, this approach looks for the thresholds of a suite of environmental parameters beyond which the environment for a species becomes uninhabitable. Thus, it is linked to the ecotoxicological approach that looks for the concentrations above which studied parameters or chemicals become lethal or affect other population-relevant endpoints such as growth, development, and reproduction. By contrast, the Eltonian view considers individuals within the community context and looks for the individual's role within it. From an ecotoxicological and environmental safety point of view, the Eltonian niche can predict secondary changes in community structure and ecosystem functioning in the case of pollution and local extinctions.

Ecological differentiation of cryptic species is puzzling. Under the premise that these species are either young or they evolved under similar selective regimes (Fišer et al., 2018;

Struck et al., 2018), it seems reasonable to expect that pairs of cryptic species retained similar ecological niches and play similar roles in ecosystems. However, this hypothesis has no ground in experimental data. It was tested mainly in correlative studies, using two approaches: (1) ecological niche modeling, and (2) experimental approaches that test for sensitivity differences. Many of these studies use the first approach and calculate species' ecological niches using the environmental factors in combination with data on species' occurrence. Virtually all these studies imply that cryptic species differ in their ecological niches (Eisenring et al., 2016; Fišer et al., 2015; Macher et al., 2016). This approach, however, cannot reliably tell apart the species' ecological differentiation from the differences rooted in biogeography and depends on many assumptions, including unlimited dispersal (Warren et al., 2014). Indeed, explicit studies that tested how well ecological niche models depict species' physiological limits advise caution with interpretation because approximately one-third of such studies inadequately estimated the true niches (Lee-Yaw et al., 2016). Other studies found differences among cryptic species in feeding biology, habitat use, and even host choice (Hebert et al., 2004; Kaliszewska et al., 2005; Marchán et al., 2018; Scriven et al., 2016; Zittel et al., 2018). Experimental studies of differential sensitivity of pairs of cryptic species to ecological parameters are scarce, but consistent with correlative studies (Dallinger & Höckner, 2013; Feckler et al., 2013, 2014; Otomo et al., 2013). Therefore, most studies imply that cryptic species differ in their ecological niches, pointing toward further experimental approaches clarifying mechanisms and consequences of observed differentiation.

Fewer studies have questioned whether cryptic species play similar roles in the ecosystem, investigating their functional redundancy, with cryptic species acting as functional replicates of each other (De Meester et al., 2016; Fišer et al., 2018). This can be translated to the problem of species' coexistence. Cryptic species were commonly found co-occurring in the same habitat patch (Bystřický et al., 2022; Fišer et al., 2018; Weigand et al., 2020). The comparative analyses of coexisting cryptic species most commonly suggested that co-occurrence is an outcome of partial niche differentiation in combination with differential dispersal or predation (Montero-Pau & Serra, 2011; Scriven et al., 2016; Wellborn & Cothran, 2004). In some cases, observed patterns are seemingly consistent with the competitive exclusion scenario (Vodă et al., 2015a, 2015b). So far, only two studies experimentally addressed whether cryptic species play a similar role in a specific ecosystem, and concluded that there is no ground to treat them as functionally equivalent a priori (De Meester et al., 2011, 2016). To our knowledge, no study has tested community-level responses to the replacement of one cryptic species with another.

The scattered evidence suggests that morphological crypsis does not predict ecological similarity or equivalency. This calls for a systematic research program at the junction of ecology and ecotoxicology. Experimental approaches routinely used in ecotoxicology should more accurately detect species' sensitivity to environmental factors and refine the calculation of ecological niches. In addition, mesocosm experiments could more accurately estimate whether cryptic species really play a similar role in the ecosystem and whether the surviving cryptic species can functionally replace the extinct one. Both types of information, along with spatial distributions and population genetics, could be used in predictive models to discriminate the regions that are more sensitive to anthropogenic stressors, either because of more sensitive inhabitants or due to the irreplaceability of cryptic species in a community.

CRYPTIC SPECIES IN ECOTOXICOLOGY

Relevance of cryptic species in ecotoxicology

Millions of years of independent evolutionary pathways may have caused members of a cryptic species complex to respond very differently to stressors either because mechanisms to cope with a stressor have been lost or because such mechanisms never evolved. Consequently, being aware of the variability within a cryptic species complex and understanding the plasticity among members of such a complex allow for a more realistic reflection on the severity of ecological responses. In addition to the call to include microevolutionary processes in ecotoxicology (Coutellec & Barata, 2011), the framework of evolutionary ecotoxicology should also consider deep-rooted phylogenetic relationships to understand and interpret patterns of responses to pollutants.

In general, the physiological traits of a species determine the toxicokinetics (uptake, distribution, metabolism, and excretion) and toxicodynamics (interaction with the target sites) of chemicals and eventually, the organismal chemical sensitivity (Spurgeon et al., 2020). The extent to which ecological differences between cryptic species manifest in the outcome of ecotoxicological studies has rarely been investigated, even though their relevance has been recognized for more than two decades (see Duan et al., 1997; Fišer et al., 2018). There are a few studies that show the link between physiological (molecular) characteristics and chemical sensitivity. For example, two cryptic species of the polychaete annelid *Capitella capitata* were shown to exhibit a different ability of polycyclic aromatic hydrocarbon fluoranthene biotransformation by the overexpression of specific cytochrome P450 biotransformation enzymes in one of these species (Li et al., 2004). Similarly, in the lumbricid earthworm *Lumbricus rubellus*, different regulators of calcium physiology were determined in studied cryptic species, resulting also in different lead sensitivity (Andre et al., 2010). The most striking example comes from the amphipod species complex *Hyallela azteca*, which is widespread across North and Central America. Individuals of *H. azteca* of various origins are kept in many laboratories and used extensively in ecotoxicological bioassays. Since the 1990s, it has been recognized that *H. azteca* is a species complex (Duan et al., 1997). How differently the cryptic species within *H. azteca* respond to stressors was shown by evaluating the stress response of four different lineages toward the pyrethroid insecticide cyfluthrin. The results showed that the cryptic species studied differed by at least 550-fold in their sensitivity (Weston et al., 2013). By sequencing the primary pyrethroid target site,

the voltage-gated sodium channel, Weston et al. (2013) also identified the exact point mutations that led to increased tolerance to the pyrethroid in certain lineages. *Hyalella azteca* stocks are also regularly supplemented with wild-caught animals (Weston et al., 2013). The identification of resistance alleles in cryptic *H. azteca* species and their populations shows that mixing with other species/populations can significantly influence the outcomes of ecotoxicological studies. As a consequence, standardization of toxicity testing between laboratories—an essential component of our current environmental risk assessment—is challenged.

The example of the *H. azteca* species complex shows us that ignoring the existence of cryptic species can lead to a misinterpretation of the respective ecotoxicological data. As a result, the following three scenarios of falsification can be introduced, taking as an example two cryptic species (Figure 1). (1) Both cryptic species clearly differ in their tolerance (Figure 1A). Depending on which species is chosen, the species-specific tolerance may underestimate toxicity (Figure 1B). From a precautionary perspective, the most sensitive population/species would ideally be tested. If both species are transferred to a common stock (i.e., pooled), the tolerance of both species is taken into account, which increases the variance and might blur the overall results (Figure 1C). (2) The variance in species-specific susceptibility (i.e., population-specific differentiation; Grethlein et al., 2022) may differ significantly, resulting in a wider range of tolerance (Figure 1E). If the species with high variance is considered (Figure 1F), the species with low variance is covered. If the low-variance species is chosen, the most tolerant/sensitive populations might not be detected (Figure 1H). Increased variability (e.g., Figure 1G or when the more variable Species 1 is used) will impact hypothesis testing and result in a higher no-observed-effect concentration, due to higher variability of the control. Increased variability also translates into increased confidence intervals of *x*% effective concentration values (i.e., estimated concentration for *x*% effect relative to the control). (3) Even when cryptic species are recognized, there may also be an erroneous assumption that one has worked with Species 2, although one has actually worked with Species 1 (Figure 1J). The tolerance is then attributed to the wrong species. Such a case could occur when a species complex is insufficiently characterized because only fractions of the geographic range have been studied.

Overview of cryptic species used in ecotoxicology

To identify which species are most commonly used in ecotoxicological studies, we looked in the US Environmental Protection Agency database, searching for publication entries from the year 2000 until March 29, 2022. We distinguished between vertebrates and invertebrates, as well as between aquatic and terrestrial taxa. In each group of organisms, we selected the 15 species with the highest publication numbers and searched again for the number of publications in the Web of Science (Query: (ALL=(toxicology)) AND ALL=(Species name) Publication Date January 1, 2000 to April 7, 2022). We further checked whether each of the species was included in standard testing guidelines such as those of the Organisation for Economic Co-operation and Development (OECD), the International Organization for Standardization (ISO), and the ASTM International (ASTM; Tables 1–4).

For the 15 most commonly used species, we then again conducted a literature search to find evidence for cryptic diversity within species. At least one of the following search terms had to be present within the publication in addition to the respective species name: cryptic, cryptic diversity, cryptic complex, phylogeography, sibling species, genetic diver*sity/gence or barcod*e/ing. We screened the first 25 results from the Google Scholar and Web of Science search. In case we found evidence for clear molecular structuring at the mitochondrial level backed up by evidence deriving from either nuclear molecular markers and/or detailed morphometric analyses within given morphospecies, the species was classified as a cryptic species complex. Species that are described as different species but cannot be distinguished from each other on the basis of morphological characteristics are marked accordingly, and are also classified as "cryptic species." When only one line of molecular evidence was supporting the presence of cryptic diversity without secondary molecular or morphological analyses included, we classified a given taxon as a "potential cryptic species complex." If no evidence for cryptic diversity was found in the first 25 results in Google Scholar or Web of Science, we classified the species as noncryptic.

Our literature search revealed that 54% of the most used aquatic invertebrates (Table 1) and 67% of terrestrial invertebrates (Table 2) contain cryptic species. For vertebrates, the proportion was lower, with 27% of aquatic vertebrates (Table 3) and 6.7% of terrestrial vertebrates (Table 4) containing cryptic species (Figure 2).

The most commonly used aquatic invertebrate test organism, *Daphnia magna*, shows no cryptic species, although *D. magna* from China and Japan are genetically different from the European ones (Bekker et al., 2018; De Gelas et al., 2005). However, because there are also morphological differences in Chinese *D. magna*, the populations in China could likely represent a separate species (Ma et al., 2020). The most commonly applied terrestrial invertebrate test organism is the honeybee (*Apis mellifera*), which is divided into at least 33 subspecies that are subdivided into five evolutionary lineages (Ilyasov et al., 2020). Different subspecies and populations are adapted to a wide range of geographic regions and hence, environmental conditions (Devillers, 2002), so it can be assumed that responses to stressors may be subspecies-specific (c.f. Weston et al., 2013). Zebrafish (*Danio rerio*) are the most commonly used aquatic vertebrates in ecotoxicology. *Danio rerio* is widely distributed over the Indian subcontinent and inhabits lowland flood plains (Spence et al., 2008). So far, there is no evidence for cryptic species within *D. rerio*. However, the large natural range of the species (Spence et al., 2008) suggests that there may be some differentiation at the population level with

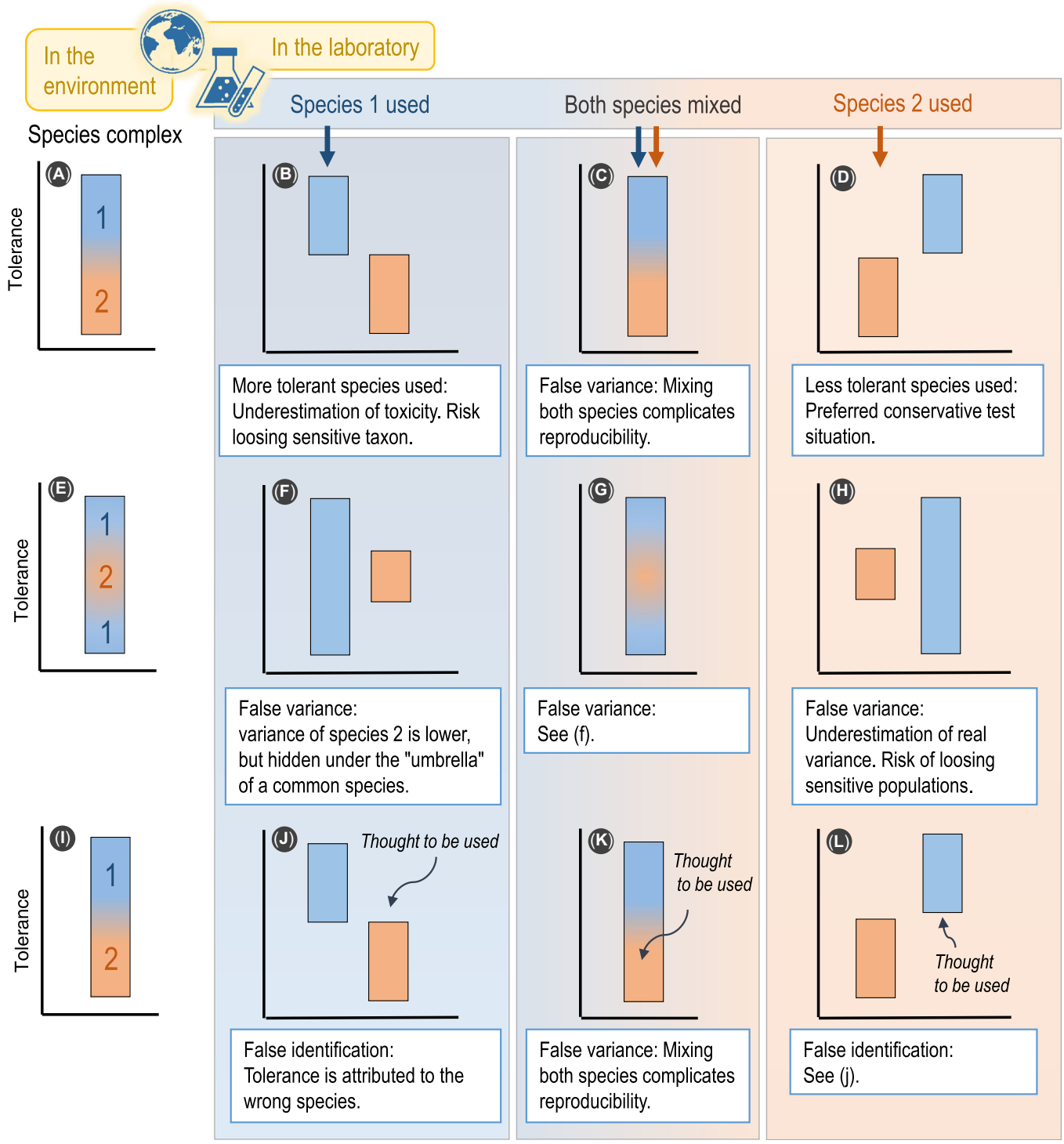

FIGURE 1: The use or mixing of a cryptic species complex in an ecotoxicological experimental setup can cause various errors. (**A**) The cryptic species may consist of a more sensitive and a more tolerant species, depending on which one is used (**B–D**); this will change the outcome of the test. (**E**) Cryptic species may also differ in variance, that is, the variance within a cryptic species is greater than the variance in another species. Depending on which species/population is used, (**F–H**) variance may be underestimated and sensitive populations may be at risk of being overlooked. Finally, (**I–L**) incorrect species identification can also cause a falsification. If researchers think they are using one species but have actually used the other, they attribute the results to the wrong species.

adaptation to different local conditions (as described for other teleost fishes; Jourdan et al., 2016; Torres-Dowdall et al., 2012). Laboratory strains of cosmopolitan Norway rats (*Rattus norvegicus*) are the most commonly used terrestrial vertebrates in ecotoxicology. A total of 13 evolutionary clusters have been described worldwide (Puckett et al., 2016), but their differentiation does not exceed a normal level of intraspecific diversity. The situation is somewhat different for the related

TABLE 1: Aquatic invertebrates

	Species	No. of publications (USEPA)	No. of results in the WOS	Test guidelines	Cryptic	Potentially cryptic	Noncryptic	Reference	Notes
1	*Daphnia magna*	681	1807	OECD 202 (2004), 211 (2012a); ISO 6341 (2012a), 10706 (2020a); ASTM E1193-20 (2021a)			x	Bekker et al. (2018); De Gelas et al. (2005)	No cryptic species in Europe, but clear COI divergence from North American and Japanese populations. However, Bekker et al. (2018) concluded that they likely do not represent cryptic species.
2	*Ceriodaphnia dubia*	175	362	ISO 20665 (2008b); ASTM E1295-22 (2022b)		x		Elias-Gutierrez et al. (2008)	A taxon described as *C.* cf. *dubia* was found from Canada to Guatemala. Also, morphological variation is described, and thus the possibility exists that there are previously unrecognized (cryptic) species within *C. dubia* (Elias-Gutierrez et al., 2008).
3	*Hyalella azteca*	130	390	ISO 16303 (2013a); ASTM E1706-20 (2020b)	x			Weston et al. (2013); Witt et al. (2006)	Known cryptic species complex. So far, no study is available that covers the entire distribution range.
4	*Aedes aegypti*	115	99	—	x			Gloria-Soria et al. (2016); Mousson et al. (2005)	Two genetically distinct subspecies.
5	*Chironomus riparius*	105	276	OECD 218 (2004b), 219 (2004c), 233 (2010a), 235 (2011c); ISO 14371 (2012c); ASTM E1706-20 (2020b), E1688-19 (2020c)	(x)			Pedrosa et al. (2017); Pfenninger and Nowak (2008); Schmidt et al. (2013)	High morphological similarity to cryptic sister species *Chironomus piger*. Foucault et al. (2019) recommend using DNA barcoding for differentiation.
6	*Mytilus galloprovincialis*	85	667	ISO 17244 (2015), 21716-3 (2020c); ASTM E724-21 (2021b)	x			Lourenco et al. (2015); Westfall et al. (2010); Zbawicka et al. (2019)	Evidence for intraspecific cryptic diversity in *M. galloprovincialis* with clear Northern- and Southern-hemisphere entities. Furthermore, two cryptic sister species exist, *Mytilus edulis* and *Mytilus trossulus*.
7	*Gammarus pulex*	61	164	—	x			Hupało et al. (2020); Lagrue et al. (2014)	Known cryptic species complex. No large-scale study available so far. Often also confused with the very similar appearing *Gammarus fossarum* species complex.
8	*Daphnia pulex*	59	144	ASTM E1193-20 (2021a)	x			Chin and Cristescu (2021); Colbourne et al. (1998); Crease et al. (1990); Lynch and Spitze (1994)	Known cryptic species complex with high diversity. European and North American *D. pulex* diverging over 5 million years ago (Ma) and having distinct evolutionary histories and endemism within continents.
9	*Brachionus calyciflorus*	58	75	ISO 15799 (2019a), 19827 (2016b), 20666 (2008c)			(x)	Gilbert and Walsh (2005); Michaloudi et al. (2018)	Has been partially classified as a species complex with four cryptic species, but described as separate species by Michaloudi et al. (2018),

(*Continued*)

TABLE 1: (*Continued*)

	Species	No. of publications (USEPA)	No. of results in the WOS	Test guidelines	Cryptic	Potentially cryptic	Noncryptic	Reference	Notes
									who also found morphological differences. Morphological similarity between sister species still calls for caution.
10	*Chironomus tentans*	51	128	OECD 218 (2004b), 219 (2004c)		x		Gunderina et al. (2007); Kiknadze et al. (1996); Polukonova et al. (2015)	Cryptic species complex until 1999. Now divided into two species, Palaearctic *C. tentans* and Nearctic *Chironomus dilutus* (Butler et al., 1999). However, there is still high genetic variability within European *Chironomus tentatus*.
11	*Lymnaea stagnalis*	50	106	OECD 243 (2016e)	(x)			Remigio (2002); Vinarski (2015)	Clear genetic differentiation between countries/continents. At least subspecies should be considered.
12	*Culex quinquefasciatus*	50	31	—			(x)	Dumas et al. (2016); Weitzel et al. (2009)	Morphological (and to a degree molecular) similarity with *Culex pipiens*, yet no evidence of cryptic diversity within.
13	*Lumbriculus variegatus*	48	163	OECD 225 (2007a), 315 (2008a); ASTM E1688-19 (2020c)	x			Gustafsson et al. (2009)	At least two distinct clades, both occurring in Europe and North America.
14	*Palaemonetes pugio*	47	57	—			x		No evidence for cryptic species.
15	*Mytilus edulis*	46	675	ISO 17244 (2015), 21716-3 (2020c); ASTM E724-21 (2021b)			(x)	Khaitov et al. (2021); Lourenco et al. (2015); Zbawicka et al. (2019)	High morphological similarity to *M. galloprovincialis*. Hybrid zones of both species.

Evaluation of cryptic status through literature research for the 15 most used species in ecotoxicological studies. Cryptic status was assumed when a distinct genetic structuring was found within the morphospecies (indicated by x in the category "cryptic"). "Potentially cryptic" refers to species for which there is indicative evidence of cryptic species but this has not yet been confirmed by more than one molecular analysis or accompanying morphological analyses. In the case that there are cryptic sister species, that is, species that have been described as separate species but cannot be distinguished on the basis of morphological characteristics, we have classified them as (x) in the category "cryptic." If there are sister species that are difficult to identify but still morphologically identifiable, we have categorized them as (x) in the "noncryptic" category.

ASTM = ASTM International; COI = cytochrome oxidase subunit I; ISO = International Organization for Standardization; OECD = Organisation for Economic Co-operation and Development; USEPA database = US Environmental Protection Agency; WOS = Web of Science database.

TABLE 2: Terrestrial invertebrates

	Species	No. of publications (USEPA)	No. of results in the WOS	Test guidelines	Cryptic	Potentially cryptic	Noncryptic	Reference	Habitat	Notes
1	Western honey bee (*Apis mellifera*)	305	184	OECD 213 (1998b), 214 (1998c), 237 (2013c), 245 (2017b)	x			Ilyasov et al. (2020)	Epigean	At least 33 subspecies with additional ecotypes and reproducing lines. Many of them cannot be discriminated by morphometry.
2	Red wiggler (*Eisenia fetida*)	223	202	OECD 207 (1984c), 222 (2016a), 317 (2010b); ISO 11268-3 (2014b), 11269-1 (2012b), 15799 (2019a), 17512-1 (2008a); ASTM E1676-12 (2021c)	x			Latif et al. (2017); Römbke et al. (2016)	Subterranean	Hardly distinguishable morphologically from *Eisenia andrei*. Evidence for different genetic lineages.
3	*Caenorhabditis elegans*	130	590	ISO 10872 (2020b); ASTM E2172-01 (2008)	(x)			Barrière and Félix (2005); Kiontke et al. (2011)	Subterranean	Mostly hermaphroditic organisms. Numerous morphologically indistinguishable species in the *C. elegans* supergroup. Most studies use a single genetic background of the N2 strain.
4	Pomace fly (*Drosophila melanogaster*)	86	610	—	(x)			Begun and Aquadro (1993); David et al. (2007); Kapun et al. (2020)	Epigean	Morphologically hardly distinguishable from sister species. David et al. (2007) recommend DNA barcoding for easy recognition of morphologically similar species within the *D. melanogaster* group.
5	*Folsomia candida*	82	123	OECD 232 (2016c); ISO 11267 (2014a), 17512-2 (2011)	x			Tully and Potapov (2015); Tully et al. (2006)	Subterranean	At least two distinct lineages.
6	*Eisenia andrei*	77	103	OECD 207 (1984c), 222 (2016a), 317 (2010b)	(x)			Dhakane and Shinde (2020); Latif et al. (2017); Römbke et al. (2016)	Subterranean	Hardly distinguishable morphologically from *E. fetida*. Less evidence of cryptic lines so far (compared to *E. fetida*).
7	Diamondback moth (*Plutella xylostella*)	63	13	—			(x)	Perry et al. (2018); Pichon et al. (2006)	Epigean	Morphologically similar to *Plutella australiana*.
8	Red spider mite (*Tetranychus urticae*)	54	14	—	(x)			Ros and Breeuwer (2007)	Epigean	Morphologically hardly distinguishable from sister species. Many misidentifications in the past, resulting in erroneous phylogenetic patterns (Ros & Breeuwer, 2007).

(*Continued*)

TABLE 2: (*Continued*)

	Species	No. of publications (USEPA)	No. of results in the WOS	Test guidelines	Cryptic	Potentially cryptic	Noncryptic	Reference	Habitat	Notes
9	Cotton bollworm (*Helicoverpa armigera*)	51	32	—			x	Behere et al. (2007)	Epigean	Minimal differentiation among global samples.
10	Buff-tailed bumblebee (*Bombus terrestris*)	40	7	OECD 246 (2017c), 247 (2017d)			(x)	Murray et al. (2008); Rasmont et al. (2008); Williams et al. (2012)	Epigean	Lack of consistent morphological characters to discriminate between workers of *Bombus cryptarum*, *Bombus magnus*, *Bombus lucorum*. Several subspecies described based on morphological characteristics (Rasmont et al., 2008).
11	Common green lacewing (*Chrysoperla carnea*)	40	8	–	x			Henry et al. (2013); Taylor (2020)	Epigean	Described as a cryptic species complex with ecotypes that can only be distinguished by courtship songs (Henry et al., 2013).
12	Beet armyworm (*Spodoptera exigua*)	38	10	—		x		Satiman et al. (2022); Shashank et al. (2015)	Epigean	No large-scale studies available. Australian populations of *S. exigua* differ genetically from Asian, European, and North American populations (Shashank et al., 2015).
13	Tobacco cutworm (*Spodoptera litura*)	36	10	—			x	Shashank et al. (2015)	Epigean	No evidence for cryptic species.
14	Common earthworm (*Lumbricus terrestris*)	36	60	ISO 11268-3 (2014b), 23611-1 (2018), 23611-6 (2012d)	(x)			James et al. (2010); Martinsson and Erséus (2017)	Subterranean	Recently split into two species, *L. terrestris* and *Lumbricus herculeus* based on large genetic distances (James et al., 2010; Martinsson & Erséus, 2017).
15	*Enchytraeus crypticus*	?	?	ISO 15799 (2019a), 16387 (2023), 22190 (2020d), 23611-3 (2019c)	(x)			Erséus and Gustafsson (2009); Schmelz et al. (2017)	Subterranean	Morphologically indistinguishable from *Enchytraeus variatus*, but described as a separate species in 1992 due to genetic differentiation.

Evaluation of cryptic status through literature research for the 15 most used species in ecotoxicological studies. Cryptic status was assumed when a distinct genetic structuring was found within the morphospecies (indicated by x in the category "cryptic"). "Potentially cryptic" refers to species for which there is indicative evidence of cryptic species but this has not yet been confirmed by more than one molecular analysis or accompanying morphological analyses. If there are cryptic sister species, that is, species that have been described as separate species but cannot be distinguished on the basis of morphological characteristics, we have classified them as (x) in the category "cryptic." If there are sister species that are difficult to identify but still morphologically identifiable, we have categorized them as (x) in the "noncryptic" category.
ASTM = American Society for Testing and Materials; COI = cytochrome oxidase subunit I; ISO = International Organization for Standardization; OECD = Organisation for Economic Co-operation and Development; USEPA database = US Environmental Protection Agency; WOS = Web of Science database.

TABLE 3: Aquatic vertebrates

	Species	No. of publications (USEPA)	No. of results in the WOS	Test guidelines	Cryptic	Potentially cryptic	Non cryptic	Reference	Notes
1	Zebrafish (*Danio rerio*)	995	2207	OECD 203 (2019a), 210 (2013a), 212 (1998a), 215 (2020a), 229 (2012b), 230 (2009a), 234 (2011b), 236 (2013b), 250 (2021b), 305 (1996); ISO 15088 (2007), 12890 (1999)			x		No evidence for cryptic species.
2	Rainbow trout (*Oncorhynchus mykiss*)	496	1996	OECD 203 (2019), 210 (2013a), 212 (1998), 215 (2020a), 249 (2021a), 305 (1996), 319A (2018a), 319B (2018b); ISO 23893-3 (2013b); ASTM E1241-22 (2022a)			x	McCusker et al. (2000)	Two clades overlapping geographically exist. Both clades survived last glaciation in both coastal and inland refugia followed by postglacial gene flow and secondary contact. Differentiation at a level of regular intraspecific variation.
3	Eurasian carp (*Cyprinus carpio*)	306	944	OECD 203 (2019a), 212 (1998a), 305 (1996)			x	Mabuchi et al. (2008)	No evidence for cryptic species.
4	Fathead minnow (*Pimephales promelas*)	295	937	OECD 203 (2019a), 210 (2013a), 212 (1998a), 229 (2012b), 230 (2009a), 234 (2011b), 305 (1996); ASTM E1241-22 (2022a)	x			Ballesteros-Nova et al. (2019); Vandermeer (1966)	Cryptic species described for US (Vandermeer, 1966) and Mexican populations (Ballesteros-Nova et al., 2019).
5	Japanese rice fish (*Oryzias latipes*)	244	735	OECD 203 (2019a), 210 (2013a), 212 (1998a), 215 (2020a), 229 (2012b), 230 (2009a), 234 (2011b), 240 (2015a), 305 (1996)	(x)			In et al. (2013); Parenti (2008)	High morphological similarity to cryptic sister species *Oryzias sinensis* (In et al., 2013) and other *Oryzias* species (Parenti, 2008).
6	African clawed frog (*Xenopus laevis*)	213	565	OECD 231 (2009b), 241 (2015b), 248 (2019b); ISO 21427-1 (2006); ASTM E1439-12 (2019b), E2591-22 (2022c)		x		Furman et al. (2015)	Widely distributed over a large part of sub-Saharan Africa. Several subspecies described (Kobel, 1996) which should be reassigned/ revalidated as distinct species (e.g., *Xenopus petersii*, *Xenopus poweri*, *Xenopus victorianus*) based on recent molecular analyses (Furman et al., 2015).
7	Nile tilapia (*Oreochromis niloticus*)	203	544	—	x			Agnèse et al. (1997); Lind et al. (2019); Mojekwu et al. (2021)	Several subspecies and significant spatial genetic structuring described.
8	Goldfish (*Carassius auratus*)	129	353	OECD 212 (1998a)			x	Gu et al. (2022); Wang et al. (2013)	Old debate whether goldfish are subspecies of *Carassius carassius*, or *Carassius gibelio*. Genetic data, however, recommend that goldfish are domesticated forms of *C. auratus* that are native to Southern

(*Continued*)

TABLE 3: (*Continued*)

	Species	No. of publications (USEPA)	No. of results in the WOS	Test guidelines	Cryptic	Potentially cryptic	Non cryptic	Reference	Notes
									China. Hybridization of the *C. auratus* complex with other *Carassius* is possible.
9	Atlantic salmon (*Salmo salar*)	85	318	ASTM E1241-22 (2022a)			x	Finnegan et al. (2013); Säisä et al. (2005)	Common degree of intraspecific differentiation among populations. No evidence for cryptic species.
10	Northern leopard frog (*Lithobates pipiens*)	78	23	ASTM E2591-22 (2022c)			x	Hoffman and Blouin (2004); Schlesinger et al. (2018)	Morphologically similar species (e.g., *Rana sphenocephala* and *Rana kauffeldi*) have often led to confusion. However, species differentiation is possible (Schlesinger et al., 2018).
11	Spotted snakehead (*Channa punctata*)	75	27	—			x	Baisvar et al. (2019); Kundu et al. (2019)	No evidence for cryptic species.
12	African sharptooth catfish (*Clarias gariepinus*)	70	136	—		x		Alal et al. (2021); Dahruddin et al. (2017)	Evidence for high haplotype diversity and genetically distinct populations.
13	Rohu (*Labeo rohita*)	64	76	—			x	Ayesha et al. (2019); Luhariya et al. (2014)	Common degree of intraspecific differentiation among populations. No evidence for cryptic species.
14	Chinese rare minnow (*Gobiocypris rarus*)	62	109	—			x		Frequently used laboratory animal in China (He et al., 2013). So far, no evidence for cryptic species.
15	Guppy (*Poecilia reticulata*)	61	89	OECD 203 (2019a)	(x)			Alexander et al. (2006); Schories et al. (2009)	High morphological similarity to cryptic sister species *Poecilia wingei* and *Poecilia obscura*. Five major lineages within *P. reticulata* (Alexander et al., 2006).

Evaluation of cryptic status through literature research for the 15 most used species in ecotoxicological studies. Cryptic status was assumed when a distinct genetic structuring was found within the morphospecies (indicated by x in the category "cryptic"). "Potentially cryptic" refers to species for which there is indicative evidence of cryptic species but this has not yet been confirmed by more than one molecular analysis or accompanying morphological analyses. If there are cryptic sister species, that is, species that have been described as separate species but cannot be distinguished on the basis of morphological characteristics, we have classified them as (x) in the category "cryptic."

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TABLE 4: Terrestrial vertebrates

	Species	No. of publications (USEPA)	No. of results in the WOS	Test guidelines	Cryptic	Potentially cryptic	Noncryptic	Reference	Notes
1	Brown rat (*Rattus norvegicus*)	1191	79	OECD 402 (2017e), 403 (2009c), 407 (2008b), 408 (2018c), 411 (1981b), 412 (2018d), 413 (2018e), 416 (2001), 417 (2010c), 420 (2002a), 421 (1995b), 422 (2016f), 423 (2002b), 424 (1997a), 425 (2022a), 426 (2007b), 427 (2004d), 430 (2015d), 433 (2018g), 436 (2009d), 440 (2007c), 441 (2004e), 443 (2018j), 451 (2018k), 452 (2018l), 453 (2018m), 474 (2016g), 475 (2016h), 486 (1997b), 488 (2022b), 489 (2016l)			x	Puckett et al. (2016)	Common degree of intraspecific differentiation among populations. No evidence for cryptic species.
2	House mouse (*Mus musculus*)	458	82	OECD 429 (2010d), 432 (2019c), 442A (2010e), 442B (2018i), 474 (2016g), 476 (2016i), 478 (2016j), 483 (2016k), 485 (1986), 488 (2022b), 490 (2016m)	(x)			Didion and de Villena (2013); Fujiwara et al. (2021); Hardouin et al. (2015)	Cosmopolitan species, with at least three distinct genetic lineages described. Lineages (regularly referred to as subspecies) cannot be clearly distinguished phenotypically (Boursot et al., 1993).
3	Domestic chicken (*Gallus gallus*)	196	138	OECD 148 (2017a), 419 (1995a), 438 (2018h), 503 (2007d), 505 (2007e)			x		No evidence for cryptic species.
4	Japanese quail (*Coturnix japonica*)	65	83	OECD 205 (1984a), 206 (1984b), 223 (2016b); ASTM E857-05 (2019a)			x		No evidence for cryptic species.
5	European rabbit (*Oryctolagus cuniculus*)	45	17	OECD 404 (2015c), 405 (2021c), 410 (1981a), 411 (1981b), 414 (2018f), 491 (2020c); ISO 10993-23 (2021)			x	Branco et al. (2002); Queney et al. (2001)	Origin in southwestern Europe. Can be found throughout the world today. Two distinct parapatric mitochondrial DNA clades A and B in the native range. However, no evidence for a deep divergence split.
6	Cattle (*Bos taurus*)	35	8	OECD 437 (2020b), 503 (2007d), 505 (2007e)			x	Li et al. (2007)	Ongoing debate about taxonomic classification. Numerous interbreeding of related species into modern domesticated *Bos taurus* complicates taxonomy. No evidence for cryptic species.
7	Sheep (*Ovis aries*)	31	7	–			x	Guo et al. (2005); Hiendleder et al. (2002)	The origin of domestic sheep (*O. aries*) remains uncertain and controversial. At least three major mtDNA lineages in domestic sheep exist. No evidence for cryptic species.

(*Continued*)

TABLE 4: (*Continued*)

	Species	No. of publications (USEPA)	No. of results in the WOS	Test guidelines	Cryptic	Potentially cryptic	Noncryptic	Reference	Notes
8	Wild boar (*Sus scrofa*)	30	53	—			x	Vernesi et al. (2003)	Subspecies described in different regions of Europe. Minor genetic differences and evidence for hybridization. No evidence for cryptic species.
9	Northern bobwhite (*Colinus virginianus*)	28	57	OECD 205 (1984a), 206 (1984b), 223 (2016b); ASTM E857-05 (2019a)			x	Eo et al. (2010)	Numerous subspecies described based on phenotypic differences. No clear support from genetic data.
10	American kestrel (*Falco sparverius*)	28	70	–		x		Ruegg et al. (2021)	Widespread from South to North America. 17 subspecies described, some of them supported by genomic data.
11	Goat (*Capra hircus*)	26	7	—			x	Tabata et al. (2019)	Domesticated from the wild goat (*Capra aegagrus*). Up to 600 goat breeds have been reported world-wide
12	Mallard (*Anas platyrhynchos*)	21	70	OECD 205 (1984a), 206 (1984b); ASTM E857-05 (2019a)			x	Kulikova et al. (2012); Lavretsky et al. (2019)	Some genetic structuring between Eurasia and the Americas. Little genetic structuring within the continents. No evidence for cryptic species.
13	Zebra finch (*Taeniopygia guttata*)	19	35	—			x	Forstmeier et al. (2007)	No evidence for cryptic species.
14	Mongolia racerunner (*Eremias argus*)	17	2	—			x	Zhao et al. (2011)	Two syntopically occurring haplotype lineages. No evidence of cryptic species.
15	Common pigeon (*Columba livia*)	16	25	OECD 205 (1984a)			x	Shivambu et al. (2020)	12 subspecies described based on different geographic origins and phenotypic differences. No evidence for cryptic species.

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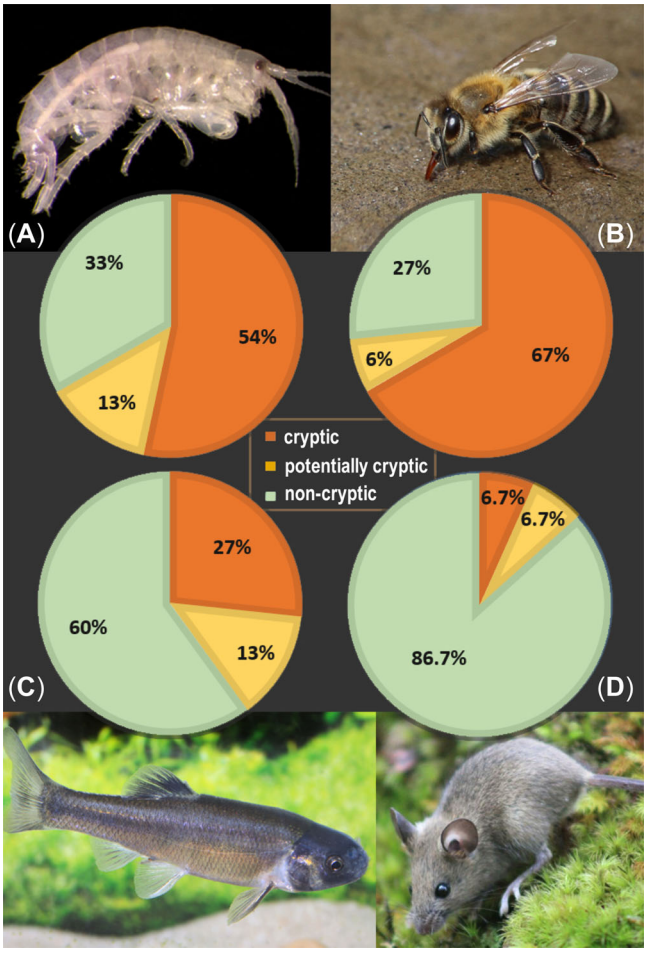

FIGURE 2: Results of the literature review investigating whether there is evidence for the presence of cryptic species in the 15 most commonly used species in ecotoxicology. The evaluation was performed within the following organisms' groups: (**A**) aquatic invertebrates, (**B**) terrestrial invertebrates, (**C**) aquatic vertebrates, and (**D**) terrestrial vertebrates. The pie charts represent the share of cryptic, potentially cryptic, and noncryptic species within a certain organism group. Photos show a cryptic representative of each organism group: the cryptic species complexes *Hyalella azteca* (aquatic invertebrate), *Apis mellifera* (terrestrial invertebrate), *Pimephales promelas* (aquatic vertebrate; picture by Frank Schäfer), and *Mus musculus* (terrestrial vertebrate; picture by Paul Norwood).

house mouse (*Mus musculus*). The house mouse diverged into three major lineages approximately 350 000–500 000 years ago (Fujiwara et al., 2021). Morphological differentiation of the three distinct lineages is not possible due to a high phenotypic variability within lineages, which is further complicated by secondary contact and hybridization of lineages (Boursot et al., 1993; Fujiwara et al., 2021). Therefore we classified *M. musculus* as a cryptic species complex. Although some authors consider these distinct lineages to be separate species (see Geraldes et al., 2008), they are mostly referred to as three subspecies, the North Eurasian *Mus musculus musculus*, South Asian *Mus musculus castaneus*, and western European *Mus musculus domesticus*. Inbred laboratory strains originate from all three wild subspecies (Didion & de Villena, 2013; Fujiwara et al., 2021).

Test species and strains in standardized ecotoxicity tests

Standardized test procedures (e.g., OECD and ISO) play a central role in ecotoxicology. These regulations are in place to ensure that results of environmental risk assessment are legally binding and recognized by different national and supranational authorities. The regulations also prevent unnecessary multiple testing of the same toxicant. This is not only for economic and ethical reasons but also follows the assumption that standardization provides more robust, ecosystem-relevant results (Chapman, 2002; Walker et al., 2012).

The standardization procedures also include specific requirements for the test organisms. In most tests, individuals from standardized laboratory strains are used. Wild-caught animals are more commonly used in so-called higher tier tests such as soil ecotoxicology field tests, which are presently required, for example, as part of the registration process of pesticides (Römbke et al., 2017). In contrast, the guidelines for laboratory tests stipulate that the test organisms should come from laboratory cultures and not from the field, although exceptions are possible, such as in the case of OECD test guidelines 221 (2006a) for *Lemna* sp., 239 (2014) for *Myriophyllum spicatum*, and 243 (2016e) for *Lymnaea stagnalis*. Some test guidelines allow the use of several alternative types. For example, OECD test guideline 202 (2004a) allows use of other "suitable *Daphnia* species" in addition to *D. magna*, such as *Daphnia pulex*. The OECD test guidelines 201 (2011a), 203 (2019a), and 208 (2006b) list numerous species, specifically 5 algae, 11 fish, and 52 plant species, respectively, that can be used as alternatives for the tests. However, even then, as for example in the case of OECD test guidelines 201 (2011a), 211 (2012a), and 242 (2016d), the use of specific and clearly defined strains or even clones or haplotypes is prescribed or at least recommended.

Although numerous organisms from various taxonomic groups with well-known cryptic species are frequently used (Tables 1–4), the relevant guidelines have so far not required any genetic characterization of the test organisms to verify the identity of cryptic species. Earthworms of the genus *Eisenia*, which are among the most commonly used test organisms in terrestrial ecotoxicology, serve as a prime example (Table 2). In 1984, OECD test guideline 207 (1984c) listed *Eisenia fetida* and *E. andrei* as subspecies, whereas in test guideline 222 (2016a) from 2016 they are already delineated as separate species. However, both taxa cannot be reliably differentiated on the basis of morphological differences according to Römbke et al. (2016). The same authors also report results of a DNA barcoding survey of *Eisenia* samples from 28 ecotoxicological laboratories in 15 countries: two cryptic species within the *E. fetida* species complex were identified. Based on morphology, those samples were identified as *E. fetida*, even including distinct *E. andrei* molecular clusters (Römbke et al., 2016). Accordingly, the authors recommend inclusion of a regular DNA barcoding step in all ecotoxicological tests with *Eisenia* earthworms. Although it is still unclear whether the cryptic species of the *E. fetida/andrei* complex differ in terms of their sensitivity to certain toxicants,

this finding shows the high relevance of considering intraspecific genetic variability, particularly when cryptic diversity has already been reported.

Learning from cryptic species: Similar challenges below the species level

Although the variability within cryptic species is indeed a challenge for risk assessment of chemicals, it is also a reflection of a general problem at the subspecies level: evolution operates on a population level, and a well-supported species may not be a homogenous entity. The processes of divergence may take place well below the emergence of cryptic species, and the challenges are identical to those related to cryptic species. Populations can differ significantly, sometimes over small geographic distances, and the effect can be reinforced by laboratory inbreeding. Adaptive processes have repeatedly been identified in chronically exposed populations, in which concentration–response patterns have differed across populations, which can be explained by microevolutionary processes (see Barata et al., 2002; Grethlein et al., 2022; Jourdan et al., 2019; Morgan et al., 2007; Shahid et al., 2018; Weston et al., 2013). In contrast, cryptic species usually represent much older evolutionary splits and thus reflect macro-evolutionary processes. Resolving the issue of cryptic species may eventually also improve ecotoxicological testing of the standard, noncryptic species that are genetically divergent and possibly locally adapted.

ACCOUNTING FOR CRYPTIC SPECIES IN ROUTINE ECOTOXICOLOGICAL STUDY

Laboratory approaches: Genetic characterization of your test organism

Taxonomists have traditionally defined species based on morphological criteria. Thus the limited morphological differentiation or lack thereof among cryptic species poses a significant challenge for their identification. This is further exacerbated by the declining number of taxonomists worldwide (Engel et al., 2021). Nevertheless, speciation is a heterogenous process that often involves divergence along multiple axes of differentiation (genetic, physiological, ecological, behavioral, etc.; De Queiroz, 2007), providing researchers with plenty of other potential means of species identification besides morphology. Consequently, cryptic species have been differentiated by various approaches ranging from molecular genetics (Fišer et al., 2018), to proteomics (Wilke et al., 2020), to pheromones (Lassance et al., 2019), and acoustic signals (Stiffler et al., 2018), to name a few. Naturally, some methods are taxon specific whereas others have broad applicability across the tree of life, making them ideal for standardization efforts.

To date, genetic methods have been the most frequently employed tools to detect cryptic species, most likely as a consequence of the exponentially decreasing costs of DNA sequencing and advances in polymerase chain reaction techniques (Bickford et al., 2007). The formal introduction of DNA barcoding two decades ago has greatly facilitated species identification and cryptic species discovery (Hebert et al., 2003). The method is based on sequencing of a short DNA fragment of a specific gene (i.e., barcode) to identify specimens by comparison with a reference database. Therefore, the barcode needs to be species specific, with the interspecific divergence exceeding the intraspecific one. The emergence of universal primers that can amplify the same barcode region across phylogenetically distant groups (see Folmer et al., 1994), and the rise of Next Generation Sequencing (Shokralla et al., 2014) has further increased the feasibility and popularity of the method, leading to an exponential increase in the number of DNA barcodes. To date, there are more than 11 million barcodes for almost 340 000 species in online databases, such as the Barcode of Life Data System (BOLD; BOLD Systems, 2019) and GenBank (National Library of Medicine, 2013), which serve as important reference libraries.

Due to the morphological bias in taxonomy, most cryptic species are not formally described, and are thus not legally recognized, further hampering standardization efforts in ecotoxicology. Here, DNA barcoding again proves useful because individuals belonging to undescribed or morphologically cryptic lineages can automatically be assigned to provisional species called MOTUs. One prominent example is the Barcode Index Number (BIN) system (implemented in BOLD) whereby each BIN (i.e., MOTU equivalent) has a unique identifier. Each BIN can be further validated and annotated with additional data and also provided with a digital object identifier (DOI), allowing one to treat it analogically to published species names (Ratnasingham & Hebert, 2013). Being associated with unique DOIs, BINs are traceable with the addition of new data, even if they are merged or further split. Furthermore, the BIN MOTUs match true species in almost 90% of cases (particularly in insects and vertebrates) and have an overall higher accuracy than other MOTU-delimiting approaches (Ratnasingham & Hebert, 2013). Nevertheless, it must be stressed that species-level identifications via BOLD are as reliable as the data already present in the database and used as a reference library. Thus the uploaded barcode sequences should be supplemented with all possible metadata, including the collection locality, date, photo, and name of the person who identified the specimen. Only a strategy such as this will allow proper taxonomic curation of the reference library, including flagging and eliminating misidentified entries or detecting possible hybridization/introgression events that blur the taxonomic assignments.

In sum, molecular species delimitation is still in process, with technical achievements providing new opportunities (Fontaneto et al., 2015). Single-locus Sanger sequencing has already been largely replaced by second- and third-generation high-throughput sequencing (HTS), which makes it possible to sequence multiple DNA molecules in parallel with long reads and high accuracy. For example, the PacBio Sequel HTS can be used for DNA barcode-based taxonomic identification of hundreds or, given the capacity of a single SMRT Cell, even thousands of individuals at once with a higher success rate than Sanger sequencing but with a comparable cost (Runnel et al., 2022). Alternatively, the new types of MinION flow cells with high-accuracy base-calling, developed most recently by Oxford

Nanopore Technologies, offer handy and cost-effective solutions for both small- and large-scale DNA barcoding projects (Srivathsan et al., 2021). Oxford Nanopore Technologies' MinION barcoding seems a particularly promising tool for ecotoxicology practice because it requires minimal laboratory equipment and the procedure can be learned within a few days, reducing the barcode sequencing cost to <10 cents. In addition, the MinION sequencer is portable, and it can be operated (and the sequence data analyzed) with a standard laptop containing the most popular operating systems. As a result, the turnaround from specimen to sequence is fast. Last but not least, the whole procedure can be done away from a molecular laboratory, even during the fieldwork and by nonprofessionals (Maestri et al., 2019; Schilthuizen et al., 2022).

Other HTS-based techniques, such as Restriction site-Associated DNA sequencing (RAD-seq), genome skimming, or even whole-genome sequencing, are currently being developed and used for detecting cryptic diversity in various taxa. Nevertheless, as a handy, easy to establish, and widespread identification tool, and due to the presence of huge reference library, DNA barcoding of metazoans will continue to develop in the future, most likely in the direction of multimarker DNA barcoding, as it is currently practiced in fungi and plants (Coissac et al., 2016; DeSalle & Goldstein, 2019; Grant et al., 2021). We therefore highly recommend characterization of test animals via DNA barcoding. Depending on the test organism involved, this can be done before or after the experimental procedures. Ideally, the barcodes obtained (along with all metadata and photos of barcoded individuals) should be submitted to BOLD, which offers both taxonomic identification and sequence analytical tools; the specimen vouchers and corresponding DNA isolates should be kept for further reference (BOLD Systems, 2019).

Although DNA barcoding has become the most reliable method for cryptic species identification, certain emerging methods are potentially even more cost effective. One of the most promising examples is matrix-assisted laser desorption ionization–time of flight mass spectrometry, which characterizes species or strains based on their unique proteomic fingerprint (Singhal et al., 2015). The method is significantly faster and cheaper than DNA barcoding, does not require trained laboratory personnel, and at the same time offers a similar accuracy as DNA barcoding in characterizing cryptic species (Rossel et al., 2019). Procedural standardization has allowed the constant accumulation of species-specific protein mass spectra in reference libraries, thereby facilitating organism identification (Singhal et al., 2015). However, the lack of a universal automatic MOTU assignment algorithm (similar to the one in BOLD) limits its use as a standardized tool for cryptic species identification for now.

OUTLOOK ON PERSPECTIVES AND SUGGESTIONS FOR IMPLEMENTATION

Perspective for implementation of the knowledge into regulatory ecotoxicology

In light of the regular appearance of cryptic species complexes among the most frequently tested morphospecies in ecotoxicology (Tables 1–4), addressing the challenge of considering cryptic diversity in ecotoxicology seems overdue. This is actually an ongoing discussion, for example, at ISO, both for the identification of individual (test) species (ISO, 2019b) and for the characterization of species communities in higher tier (semifield or field) investigations. This holds true in particular for microbes (ISO, 2016a), but similar documents will be prepared by the OECD as well. Confirmation, potentially at regular time intervals, of the existence of one cryptic lineage within the laboratory culture is certainly helpful to clarify the status of the test population. The recommendation is even more important for wild-caught test organisms: confirming that the sample population is not harboring several cryptic species will probably have positive implications for the reliability of the data set generated. If several cryptic species co-occur, the species identity (e.g., the BIN) of each replicate should be assessed and considered in the statistical evaluation as a covariate. Recently, ISO decided to set up a new Technical Committee for standardization in the field of biodiversity, to develop requirements, principles, frameworks, guidance, and supporting tools in a holistic and global approach for all relevant organizations, to enhance their contribution to sustainable development. In parallel, the OECD is preparing a guidance document for an improved field study design (mainly for earthworms), which will also be helpful to assess the status of the respective communities (Römbke et al., 2020).

Being transparent about the cryptic status of the test population will not only support the interpretation of sensitivity differences within a morphospecies but enlighten the scientific and regulatory communities about systematic differences in sensitivity among cryptic lineages and strengthen the reliability of conclusions that can be drawn from the very few published case studies (Feckler et al., 2012, 2014; Weston et al., 2013). Ideally, the data generated in the course of ecotoxicological study are supplemented by data from studies targeting the traits of cryptic species, including toxicokinetic as well as physiological, behavioral, and ecological parameters. This strategy will ultimately contribute to better reproducibility of already published results and consequently, the transfer of scientific insights to regulatory practice, a strategic goal of the Society of Environmental Toxicology and Chemistry.

Furthermore, identifying cryptic species and knowing their sensitivity to chemicals is very important in species sensitivity distribution (SSD) and adverse outcome pathway (AOP) studies, which are widely used in regulatory risk assessment schemes (Posthuma et al., 2019). Species sensitivity distribution is a statistical approach that is used to estimate either the concentration of a chemical that is hazardous to a fraction of all species (the hazardous concentration, usually 5%) or the proportion of species potentially affected by a given concentration of a chemical (Fox et al., 2021). The AOP is a conceptual framework that links direct molecular initiating events (e.g., a molecular interaction between a xenobiotic and a specific biomolecule) and an adverse outcome at a higher biological level of organization relevant to risk assessment (Ankley et al., 2010). Designing relevant SSD plots and AOP frameworks relies on the inclusion of exact data on species

sensitivity, which depends on the exact identity of the species under study. Both SSD and AOP are relevant tools for prospective chemical risk assessments to extrapolate data from the small number of ecotoxicological test species that we currently use to the variety of species present in the ecosystem as well as to predict effects for the vast majority of emerging chemicals, which in reality we will never be able to test.

Implications for species conservation

It is crucial for future management strategies to recognize the existence of cryptic species that are vulnerable to different threats and require different protection measures. Small-range species may be much more vulnerable to external disturbances than widespread morphospecies (i.e., a complex of undescribed cryptic species; Yan et al., 2022). In such a case, even small-scale disturbances can lead to the complete loss of a species (Delić et al., 2017; Niemiller et al., 2013). Our examples show that some cryptic species may have evolved unique adaptations that make them more resilient to pollutants, despite their phylogenetic relationships with more vulnerable species of the cryptic species complex (see Monteiro et al., 2018; Rocha-Olivares et al., 2004; Weston et al., 2013). Therefore, although phylogenetic similarity may provide some clues about vulnerability to pollutants, it is not always a reliable predictor and must be considered in the light of individual evolutionary history and possible points of contact with stressors (Best & Stachowicz, 2013; Grethlein et al., 2022). Therefore conservationists and policymakers should consider this complexity when making informed decisions on species diversity and distribution. That means we need to change our thinking away from protecting morphospecies to the protection of significant evolutionary units (Coates et al., 2018; Hoban et al., 2023; Moritz, 1994). One way forward could be the path proposed by Fišer et al. (2018): to develop continental maps of species richness and geographic distribution size using different methods of species delineation and highlighting regions of incongruence that reveal geographic variation in the speciation process.

Suggestions for addressing cryptic species in the future

In the present review, we were able to show the limitations of current standardization approaches, which can be attributed to an incomplete taxonomy. We have the following suggestions to reduce misleading data in the future: (1) All widely used test species should be checked for their potential cryptic species status. Furthermore, international standard test guidelines should be amended to account for cryptic species: The MOTUs or BINs used in the tests should be reported together with the test results. To test for cryptic diversity, wild-caught animals should be barcoded. Barcode information should be included in the raw data file and the documentation of the test results. This approach warrants revision of taxon status when the study is complete. (2) A system of MOTUs should be adopted in parallel, to secure implementation of cryptic species. We recommend using the BOLD platform because it automatically assigns the submitted sequences to new or existing MOTUs (i.e., BINs) that have traceable unique identifiers, thus helping with standardization efforts. Voucher specimens along with DNA extracts should be BioBanked, allowing for corrections of taxonomic status, if necessary, in the future. (3) An evo–ecotox database should be established, or existing databases should be supplemented with an evolutionary aspect of the test organism. The database could then link information on cryptic species to the outcome of ecotoxicological studies, keeping an overview of how many cryptic species/test system exist and which tests were performed on which MOTU. Such a database would allow cross-validation of past research and the possibility of running post-hoc species delimitation of one's own and past species identification data. Thus, it can be considered retrospectively whether one was dealing with the same taxonomic unit, which would also address challenges at the subspecies level.

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