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Review

Towards improved fish tests in ecotoxicology - Efficient chronic and multi-generational testing with the killifish *Nothobranchius furzeri*

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HIGHLIGHTS

- We provide an overview of current issues with fish-based testing in ecotoxicology.
- We suggest a framework for relevant, time- and cost-efficient fish-based testing.
- We showcase killifish as a sensitive model for chronic and multigenerational tests.
- We identify future research perspectives to integrate the model in accredited tests.

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ABSTRACT

As many freshwaters are chemically polluted, one of the challenges for policy makers is to determine the potential impact of these pollutants on ecosystems and to define safe concentrations. Common practice is the use of ecotoxicological assays to assess the response of model organisms from different trophic levels such as algae, invertebrates and fish during exposure to dilutions of a specific compound. Ideally, ecotoxicological assessments of (pseudo-)persistent chemicals should be performed across the life-cycle or even multiple generations for an accurate risk assessment. Multigenerational tests with fish are, however, impractical and costly given the long lifespan and generation time of classic model species. Here, we suggest a framework for more relevant, time- and cost-efficient fish-based testing in ecotoxicology and align it with accredited test guidelines. Next, we introduce an upcoming fish model, the turquoise killifish *Nothobranchius furzeri*, and show how it facilitates such research agendas due to a short lifespan and generation time. Through a review of fish-based exposure studies with a set of reference toxicants, we position *N. furzeri* as a sensitive species, suitable for screening effects of different pollutant types. Ultimately, we perform a cost-benefit analysis and propose a plan of action for the introduction of *N. furzeri* into accredited test guidelines.

Contents

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1. Pollution and ecotoxicology

1.1. General introduction

Pollution is a key environmental problem and a major contributor to global change (Schwarzenbach et al., 2006). Although chemical pollution is rarely included in analyses of global environmental change, the rate at which a wide variety of compounds is introduced into the environment largely outpaces other drivers of global change (Bernhardt et al., 2017).

To assess and anticipate harmful effects, ecotoxicological studies investigate how pollutants may affect organisms and ecosystems as a whole (Dell'Omo, 2002; Hellou, 2011). The overall goal is to identify early signals of pollution, define environmentally safe compound concentrations and halt environmental degradation (Hellou, 2011). Practically, the approach entails a battery of standardised short-term (i.e. "acute") exposure tests on different organisms of which the results are combined to assess stressful, harmful or lethal effects of a specific compound (Klaminder et al., 2014). These tests are developed to facilitate response comparison across species and substances (Rudén et al., 2017). This way, a large body of data on organismal responses to pollutant exposure has been generated (Brady et al., 2017).

1.2. Model organisms in ecotoxicology

Ecotoxicological screening is strongly dependent on suitable model organisms. Standardised exposure tests are conducted at multiple trophic levels to test hypotheses about the impact of pollution and to allow extrapolation to other species and the ecosystem as a whole (Segner and Baumann, 2016). Typically and ideally, the model species are well-characterised, easy to breed under laboratory conditions and amenable to experimental manipulation (Fields and Johnston, 2005; Polačik et al., 2016; Russell et al., 2017). Model organisms span a large number of taxa, and include for instance *Escherichia coli* (bacteria), *Saccharomyces cerevisiae* (fungi), *Raphidocelis subcapitata* (green algae), *Lemna* sp. (duckweed, plants), *Eisenia foetida* (ringed worms), *Daphnia* spp. (water fleas, crustaceans), *Chironomus riparius* (midges, insects), *Xenopus laevis* (african clawed frog, amphibians), *Danio rerio* (zebrafish, fish) and *Coturnix japonica* (japanese quail, birds).

Fish-based tests are a fundamental component of ecotoxicological assessments (Stadnicka-michalak et al., 2015). To date, a large number of fish species are used as models. In general, they are selected for their practical convenience to study compound toxicity and the availability of technical resources (e.g., sequenced genome, established methods) (Carvan et al., 2007; Russell et al., 2017). Commonly used species include zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), fathead minnow (*Pimephales promelas*), western mosquitofish (*Gambusia affinis*), guppy (*Poecilia reticulata*), three-spined stickleback (*Gasterosteus aculeatus*) and rainbow trout (*Oncorhynchus mykiss*) (Norrgren, 2012). Since many toxicity mechanisms and responses to chemical compounds are evolutionary conserved, responses are often similar across fish species (Gunnarsson et al., 2008; Villeneuve et al., 2014).

1.3. The legal framework

Several tests are required for environmental risk assessment of chemical compounds (Klaminder et al., 2014; Rudén et al., 2017). In Europe, internationally validated guidelines for standard tests are made available by the Organisation for Economic Co-operation and Development (OECD). Similarly, aquatic toxicity tests are used by the United States Environmental Protection Agency (U.S. EPA) to develop National Recommended Water Quality Criteria for regulating the presence of pollutants and protecting aquatic life in surface waters (Brady et al., 2017). Typically, exposure tests are performed on at least three test species from three different trophic levels: primary producers (algae), primary consumers (water fleas) and secondary consumers (fish) (Tanaka et al., 2020). Several test guidelines are available for each trophic level and are developed to capture a variety of data on the toxicological responses of organisms. Standard tests for primary producers include the Growth Inhibition Test for Freshwater Alga and Cyanobacteria (OECD Test No. 201) and the Algal Toxicity test (EPA OCSPP 850.4500). For grazing zooplankton, examples of test guidelines include the *Daphnia* sp. Acute Immobilisation Test (OECD Test No. 202), the *Daphnia magna* Reproduction Test (OECD Test No. 211), and the Aquatic Invertebrate Acute Toxicity Test on Freshwater Daphnids (EPA OCSPP 850.1010). A selection of test guidelines for fish, each of which adopts specific exposure scenarios, is provided in Fig. 1. Typically, these tests are designed and optimised for the use of a specific fish model (e.g. OECD Test No. 236 on zebrafish), or several well-documented fish species (e.g. OECD Test No. 215 recommends

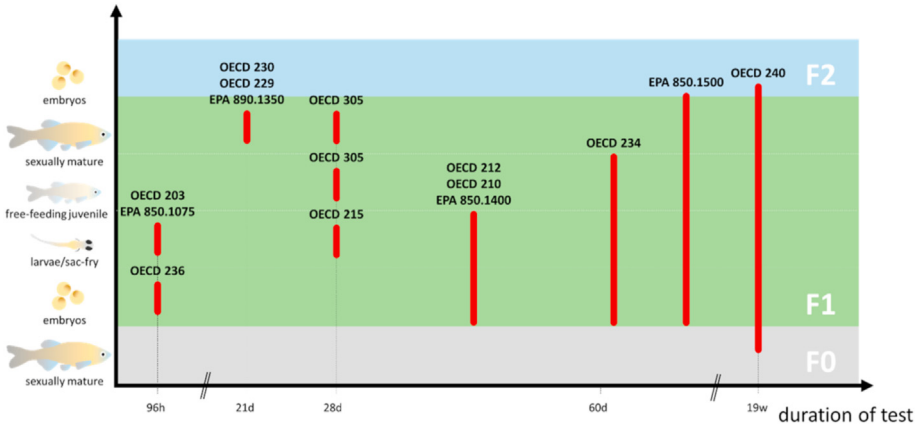

Fig. 1. Internationally validated standard test guidelines using fish as test organisms for environmental risk assessment of chemical compounds in relation to exposure window and estimated test duration. As the exact test duration typically depends on the model species, durations presented in this figure should be interpreted as indicative values of average duration only (based on canonical fish models). OECD guidelines Test No. 203: Fish Acute Toxicity Test, 210: Fish Early-Life Stage Toxicity Test, 212: Fish Short-term Toxicity Test on Embryo and Sac-Fry Stages, 215: Fish Juvenile Growth Test, 229: Fish Short Term Reproduction Assay, 230: 21-day Fish Assay, 234: Fish Sexual Development Test, 236: Fish Embryo Acute Toxicity (FET) Test, 240: Medaka Extended One Generation Reproduction Test (MEOGRT), 305: Bioaccumulation in Fish - Aqueous and Dietary Exposure; US EPA guidelines OCSPP/OPPTS 850.1075: Freshwater and Saltwater Fish Acute Toxicity Test, 890.1350: Fish Short-Term Reproduction Assay, 850.1400: Fish early life stage toxicity test, 850.1500: Fish life cycle toxicity. (OECD: Organisation for Economic Co-operation and Development; EPA: United States Environmental Protection Agency; h: hours; d: days; w: weeks; F0: parental generation; F1: first filial generation; F2: second filial generation).

rainbow trout, zebrafish or medaka).

2. Current challenges in ecotoxicology

Standard ecotoxicological tests for fish rapidly deliver information for regulatory decision making but are usually offset by a low ecological relevance (Fig. 2). Even though rigorous safety margins are adopted, pollutant effects are still often underestimated (Schäfer et al, 2012, 2019). Increasing ecological realism in laboratory exposure studies is challenging, but an improved understanding of how more realistic exposure scenarios impact organismal sensitivity to chemical pollution is imperative.

2.1. The need for full life-cycle and multi-generational fish tests

Current ecotoxicological assessment primarily focuses on acute or short-term exposure to pollutants and provides necessary toxicity benchmarks for regulatory decision making (Holmstrup et al., 2010; Peterson et al., 2017). However, information on the long-term or multi-generational impacts of chemicals is still largely lacking (Bernhardt et al., 2017; Philippe et al., 2017). Especially for persistent compounds, acute tests poorly reflect actual exposure regimes (Holmstrup et al., 2010; Santos et al., 2010). To fill this gap, standard chronic, full life-cycle and multi-generational exposure tests on vertebrates have been developed and include the Fish Sexual Development Test (OECD Test No. 234), the Fish life cycle toxicity (EPA OCSPP 850.1500) and the Medaka Extended One Generation Reproduction Test (OECD Test No. 240) (Fig. 1). However, as most fish species mature slowly and have a long lifespan (Fig. 2), such tests are resource- and time-demanding. Full life-cycle assays with medaka and fathead minnow, for instance, last typically 5–6 months (Ankley and Villeneuve, 2006).

2.2. Emerging pollutants and the need for sensitive endpoints

Endpoints of standardised tests in ecotoxicology predominantly include life-history traits such as survival, growth, development and reproduction (Morgan et al., 2018; Rudén et al., 2017), often complemented with physiological biomarkers such as vitellogenin levels in plasma, critical thermal maximum values, energy reserves and metallothionein content (Philippe et al., 2018c; Toušová et al., 2016). Still, this toolbox of exposure tests and resulting endpoints does not cover all relevant effects (Fig. 2).

Exposure to traditional pollutants such as pesticides and heavy metals usually results in increased mortality and symptoms of stress in general, including impaired growth and development rates, diminished fecundity and malformation (Klaminder et al., 2014). However, during the last decade there is increasing attention for so-called emerging contaminants that occur at very low doses in the environment, and that can impair organismal functioning in ways that are not detected by classic ecotoxicological assays (Fent et al., 2006; Klaminder et al., 2014). Neuroactive pharmaceutical compounds, for instance, affect aquatic wildlife through subtle pharmacological effects (Arnold et al., 2014; Klaminder et al., 2014). While traditional ecotoxicological tests would suggest no risks at current environmental concentrations, these compounds may still induce undetected specific biological effects such as behavioural alterations that impact individual performance and fitness (Brodin et al, 2013, 2014). For instance, OECD Test No. 210 measures hatching and survival rate, deformities and unusual behaviour such as uncoordinated swimming and body length/weight. However, changes in, for instance, spontaneous activity level, foraging and anxiety-related behaviour are not assessed.

2.3. Behavioural ecotoxicology

Ecotoxicology benefits from approaches that rapidly signal reliable information on the toxicity and environmental impact of chemical compounds. Behaviour is an integrative response to a range of internal and external stimuli (Levitis et al., 2009), and its expression is sensitive to pollutant-induced stress (Levitis et al., 2009; Melvin and Wilson, 2013). Since behavioural endpoints are generally 10–1000 times more sensitive compared to classic endpoints such as mortality and life-history effects (Hellou, 2011; Melvin and Wilson, 2013), they can serve as early warning signals of pollution and are therefore sensitive tools for ecotoxicologists (Brodin et al., 2014; Parker, 2016; Peterson et al., 2017).

Behavioural expression can be assessed non-invasively, is relatively inexpensive to monitor, and is amenable to high-throughput

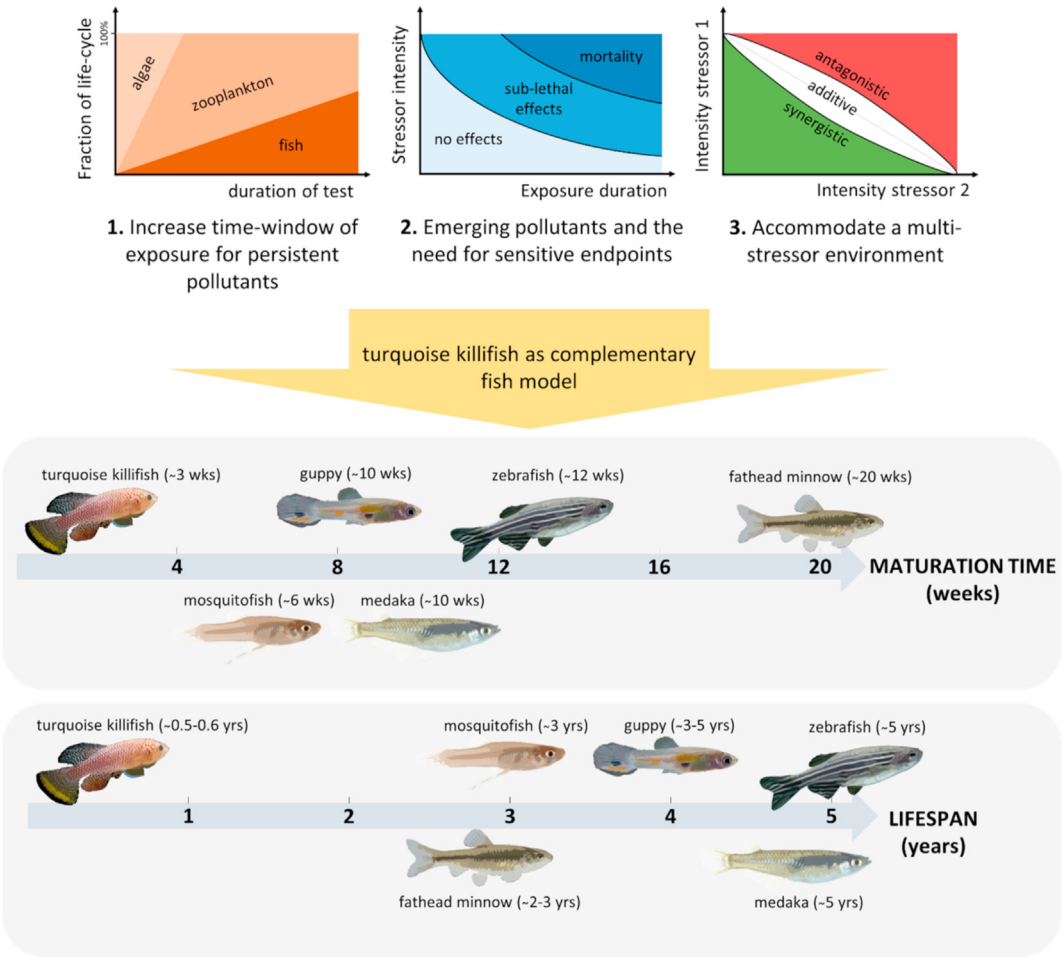

Fig. 2. Three major challenges for fish-based tests in ecotoxicology. Added ecological realism in ecotoxicological tests comes at a high cost, but an increased understanding on (1) acute vs. full life-cycle and multigenerational exposure, (2) traditional endpoints vs. specific biological effects and (3) single vs. multiple stressor effects is essential. Recent studies advocate the use of turquoise killifish as a complementary fish model to accommodate such research agendas.

and automated quantification (Gerhardt, 2007; Peterson et al., 2017). Consequently, behavioural ecotoxicology is gaining popularity, especially with regard to the screening of pharmaceutical compounds (Melvin and Wilson, 2013; Robinson, 2009). The full integration of behavioural endpoints in ecotoxicology has, however, been hampered, and standard ecotoxicological test guidelines are currently largely restricted to the occasional monitoring of abnormal behaviour such as uncoordinated swimming, atypical quiescence and atypical feeding behaviour (e.g., OECD Test No. 210, 234).

Compared to traditional tests, behavioural tests are perceived as less rigorous because they are usually less reproducible and since behavioural endpoints are often highly variable (Peterson et al., 2017). Moreover, it remains challenging to link individual-level behavioural variation to effects on higher levels of biological organisation and the link between behaviour and fitness is not always fully understood (Clotfelter et al., 2004; Gerhardt, 2007; Peterson et al., 2017). Limitations of behavioural ecotoxicology can largely be attributed to a lack of fundamental baseline data and insight in the 'behavioural norm' (i.e. typical/normal behaviour) of test organisms. Combined with the lack of standardisation of behavioural endpoints (Harris et al., 2014; Tanoue et al., 2019), this hampers reaching robust conclusions on the impact of chemicals on animal behaviour (Sumpter et al., 2014). Maximising test-retest reliability combined with more insight in fitness consequences and within- and among-individual behavioural variation is therefore pivotal to advance the field of behavioural ecotoxicology (Parker, 2016).

2.4. The relevance of test results and a multi-stressor approach

Standard test guidelines provide protocols for single-substance tests in which the effect of exposure to a single chemical is assessed (Peterson et al., 2017), while organisms are kept under optimal conditions with a control mortality of less than 10% (Klaminder et al., 2014; OECD, 1992). Although a low mortality is indicative of a healthy population, this is hardly representative for the natural environment in which organisms are simultaneously exposed to multiple stressors that can interact to have net additive, antagonistic or synergistic effects (Darling and Côté, 2008; Galic et al., 2018; Liess et al., 2016) (Fig. 2). Next to a range of chemical pollutants, also abiotic stressors can be included such as increased temperature and acidification as expected under scenarios of global change (Niinemets et al., 2017). Besides the abiotic environment, also biotic stressors including inter- and intraspecific competition for resources, predation pressure and parasite-host interactions can interact and impact organisms (Holmstrup et al., 2010). Although organisms rarely experience only one stressor in their natural habitat, interactive effects between different stressors are neglected in current standard ecotoxicity tests. This may lead to incorrect

assessments of pollutant effects and inaccurate formulation of safe concentrations (Liess et al., 2016; Philippe et al., 2019). Although it is not feasible to test all possible stressor combinations that may occur in the environment, increasing our insight in the nature of interactive effects of stressors (e.g. when to expect them, how they are expressed, how they are established) will improve our understanding of how multi-stressor environments affect organisms.

3. Turquoise killifish: a recently emerged model organism

3.1. General biology of turquoise killifish

Nothobranchius furzeri (Jubb, 1971) is an annual fish (Cyprinodontiformes, Nothobranchiidae) occurring in temporary freshwater ponds in Zimbabwe and southern Mozambique (Reichard et al., 2009). The natural habitats are characterised by a seasonal desiccation and inundation (Cellerino et al., 2015; Polačik et al., 2016). *Nothobranchius* killifish acquired specific adaptations to cope with these harsh environmental conditions. Populations survive the dry period by producing drought-resistant eggs that remain dormant in the sediment until the next inundation (Grégoir et al., 2017; Pinceel et al., 2016). During the rainy season, eggs are stimulated to hatch (Cellerino et al., 2015), after which fish grow rapidly to reach maturity in under three weeks and produce the next generation of eggs before their habitat dries again (Cellerino et al., 2015; Polačik et al., 2016) (Fig. 3). With a lifespan of about 5–6 months, these fish are among the shortest-lived vertebrates (Blažek et al., 2016; Cellerino et al., 2015).

3.2. Turquoise killifish in the laboratory

Nothobranchius furzeri combines the perks of traditional fish models with the short generation time typical to invertebrates (Cellerino et al., 2015; Polačik et al., 2016; Reichard and Polačik, 2019). With a fast life-cycle and resulting generation time that averages about 3 months, *N. furzeri* became a popular model for biological research (Blažek et al., 2016; Cellerino et al., 2015; Terzibasi Tozzini and Cellerino, 2020). During the last decade, *N. furzeri* was introduced in many fields of biological research, including gerontology (Reichwald et al., 2015), genomics, genetics (Cellerino et al., 2015; Valenzano et al., 2015), ecology (Grégoir et al, 2017, 2018; Reichard et al., 2014) and evolutionary biology (Blažek et al., 2016). The species is naturally bold which facilitates laboratory handling and behavioural observations (Polačik et al., 2016; Thoré et al., 2020a). Moreover, *N. furzeri* is relatively easy to culture (Cellerino et al., 2015; Polačik et al., 2016) and females daily produce 20–50 eggs (Cellerino et al., 2015; Haas, 1976). As biological studies often require easy sex determination, it is an additional advantage compared to other fish models that *N. furzeri* males and females display distinct sex dimorphism in size and colouration

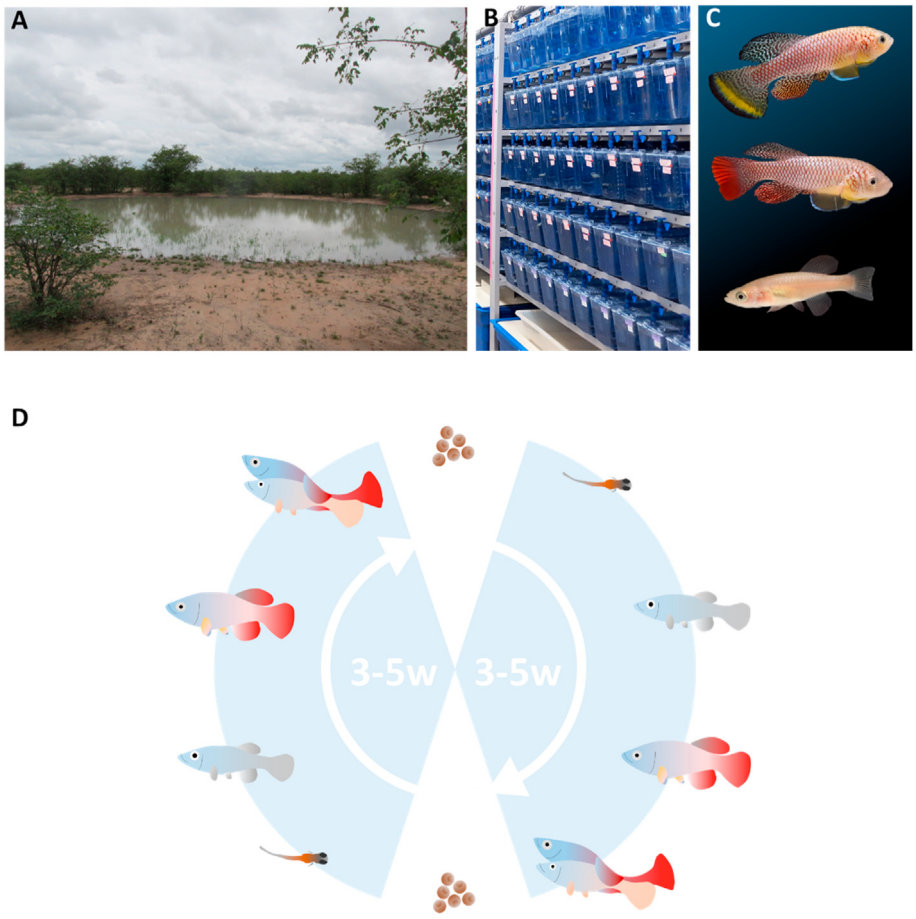

Fig. 3. General biology of turquoise killifish (*Nothobranchius furzeri*). (A) *N. furzeri* inhabits temporary freshwater ponds in southeast Africa. (B) In the laboratory, *N. furzeri* can be cultured in most available fish set-ups such as the ZebTec multi-linking system (Tecniplast group, Italy) which was initially designed for rearing zebrafish. (C) Female *N. furzeri* (bottom) are slightly smaller and not conspicuously coloured, compared to male *N. furzeri* that are larger and either have a red (middle) or yellow (top) caudal fin. (D) *N. furzeri* life-cycle: hatched juveniles can reach sexual maturity in about three weeks, after which drought-resistant eggs are produced that can be ready to hatch in 2–3 weeks or that can be stored for years until further use. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Cellerino et al., 2015) (Fig. 3). Dormant *N. furzeri* eggs can be stored dry for years and can be recruited at any moment to allow for age-synchronised culturing or experimental work (Polačik et al., 2016). This eliminates the need for a costly and labour-intensive continuous culture that is associated with most vertebrate models (Nguyen and Persoone, 2014).

Because the popularity of *N. furzeri* as a model organism for a range of biological disciplines has increased rapidly, its genetic, physiological and ecological background is well-characterised and multiple useful tools are available. These include a whole brain atlas (D'Angelo, 2013), age-related histopathological analyses and an annotated genome and transcriptome (Di Cicco et al., 2011; Reichwald et al., 2015; Valenzano et al., 2015). Moreover, CRISPR/Cas9-mediated genome editing technology was recently established for the species (Reuter et al., 2018) and various laboratory strains are developed (Hartmann and Englert, 2012; Valenzano et al., 2015). The most widely used 'Gonarezhou' strain (GRZ) has been inbred on multiple occasions and is homozygous for over 99% across the genome (Reichwald et al., 2009). Such inbred (genetically uniform) strains of laboratory animals are often used to ensure high levels of standardisation and limited between-individual variation, reducing the number of needed test animals (Brown et al., 2009; Festing et al., 2002). As genetically diverse animal strains are preferred when responses need to accurately reflect those of wild populations (Brown et al., 2009), the availability of both inbred and genetically diverse *N. furzeri* strains that can be housed under similar conditions is a valuable asset. Moreover, a range of congeneric species exists with comparably fast life-history and similar housing requirements that may facilitate multi-species experiments. These species include, but are not limited to, *N. rachovii* (van der Hoeven et al., 1982), *N. guentheri* (Shedd et al., 1999) and *N. kadleci* (Blažek et al., 2013).

3.3. The introduction of turquoise killifish in ecotoxicology

The unique combination of characteristics of *N. furzeri* fuelled its introduction as a complementary model organism for ecotoxicological research. The addition of a new model organism increases the resolution of ecological risk assessments, as fish with different life-histories may respond differently to chemical compounds (Forbes and Calow, 2002), even though several toxicity mechanisms are evolutionary conserved across species (Villeneuve et al., 2014). As a first step to explore the potential of *N. furzeri* for ecotoxicological testing, its sensitivity to several pesticides and heavy metals as conventional reference contaminants was assessed (Fig. 4). Philippe et al. (2017; 2018b) acutely exposed larval *N. furzeri* (2 days post hatching) to dilution series of a set of reference toxicants, allowing to compare the resulting 24 h-LC_{50} and 96 h-LC_{50} values with results on canonical fish models. Overall, the results indicate that the sensitivity of *N. furzeri* to short-term pollutant exposure is comparable to - or higher than - that of traditional models (Fig. 4). These findings underscore the potential of *N. furzeri* as a model for ecotoxicological assessments, since the determination of safe concentrations for regulatory decision making hinges largely on assays making use of the most sensitive test species (Forbes and Calow, 2002).

4. How turquoise killifish may fuel advancements in ecotoxicology

Acute, chronic and multi-generational exposure tests with a variety of different toxicants and other stressors were performed to assess the effects on a diverse set of endpoints with relation to exposure duration in a single- vs. multi-stressor environment.

4.1. Compiling test protocols and assembling baseline information on sensitivity

Not only is *N. furzeri* responsive to sub-lethal doses of different stressors, the impact of pollution can also be assessed on a set of classic life-history and physiological endpoints over timescales that span the whole life-cycle or even multiple generations (Table 1). Combined, this greatly adds to the value of *N. furzeri* as a model organism in stress ecology and ecotoxicology. Moreover, exposure studies indicate that its sensitivity is stressor-dependent and show that exposure duration affects the induced stress response (Table 1). For instance, Philippe et al. (2017) showed that acute exposure (96 h) to 10.27 μg/L copper did not reveal any adverse effects, while prolonged exposure to the same concentration induced mortality. In addition, a study across two successive generations revealed that stressor impact may differ across generations (Table 1; Thoré et al., 2021), underlining the need of assessing the impact of pollution across such timescales. As full life-cycle and multigenerational studies were performed (using MZCS-222 and -414 strains) within a period of 3–4 and 6 months, respectively, *N. furzeri* can be a time- and cost-efficient alternative model for fish-based tests to assess environmental risks of chemical pollution (Philippe et al., 2018c; Thoré et al, 2018b, 2021).

4.2. N. furzeri behaviour is sensitive to chemical exposure

Behavioural expression of *N. furzeri* is sensitive to pharmaceutical and pesticide exposure (Table 2), depending both on exposure duration and pollutant concentration. For instance, for most traits a monotonous relationship between behavioural expression and fluoxetine concentration (a neurochemical pharmaceutical) was apparent, with more pronounced effects at increasing concentrations. Yet, also inverse or non-linear relationships sometimes occur, and were shown for both pharmaceuticals and pesticides (Table 2; Thoré et al., 2018b). For instance, 50 μg/L but not 100 μg/L 3,4-DCA induced an increase in risk-prone- and feeding behaviour (Table 2; Thoré et al., 2021). Further, pollutant-induced responses are sometimes sex specific, such as a 3,4-DCA-induced increase in female but not male feeding behaviour (Thoré et al., 2021) and a fluoxetine-induced increase in male but not female sociability (Table 2; Thoré et al., 2020b). Combined, these findings illustrate not only the relevance of including behavioural endpoints in ecotoxicological assessments, but also that *N. furzeri* is a potential model to study the behavioural impact of pollutant-exposure across ecologically relevant timescales (Fent et al., 2006; Santos et al., 2010).

To date, baseline data for test organisms are often lacking in behavioural ecotoxicology. Still, characterisation of the 'behavioural norm' is needed to better understand the significance of observed behavioural changes (Harris et al., 2014; Tanoue et al., 2019). Several recent studies identified the components of normal behaviour in *N. furzeri* to calculate behavioural repeatability (e.g. Thoré et al., 2019; 2018a) (Table 3). In addition, to further facilitate the use of *N. furzeri* in behavioural ecotoxicology, Thoré et al. (2020a) studied how laboratory conditions, including aquarium infrastructure to accommodate fish wellbeing, may affect its behaviour. Specifically, the results of this study suggest that structural enrichment of the rearing environment affects anxiety and exploration behaviour while fish rearing density also has an impact on locomotor activity, feeding behaviour and total body length. Therefore it is important to correct for or standardise such environmental differences in behavioural studies.

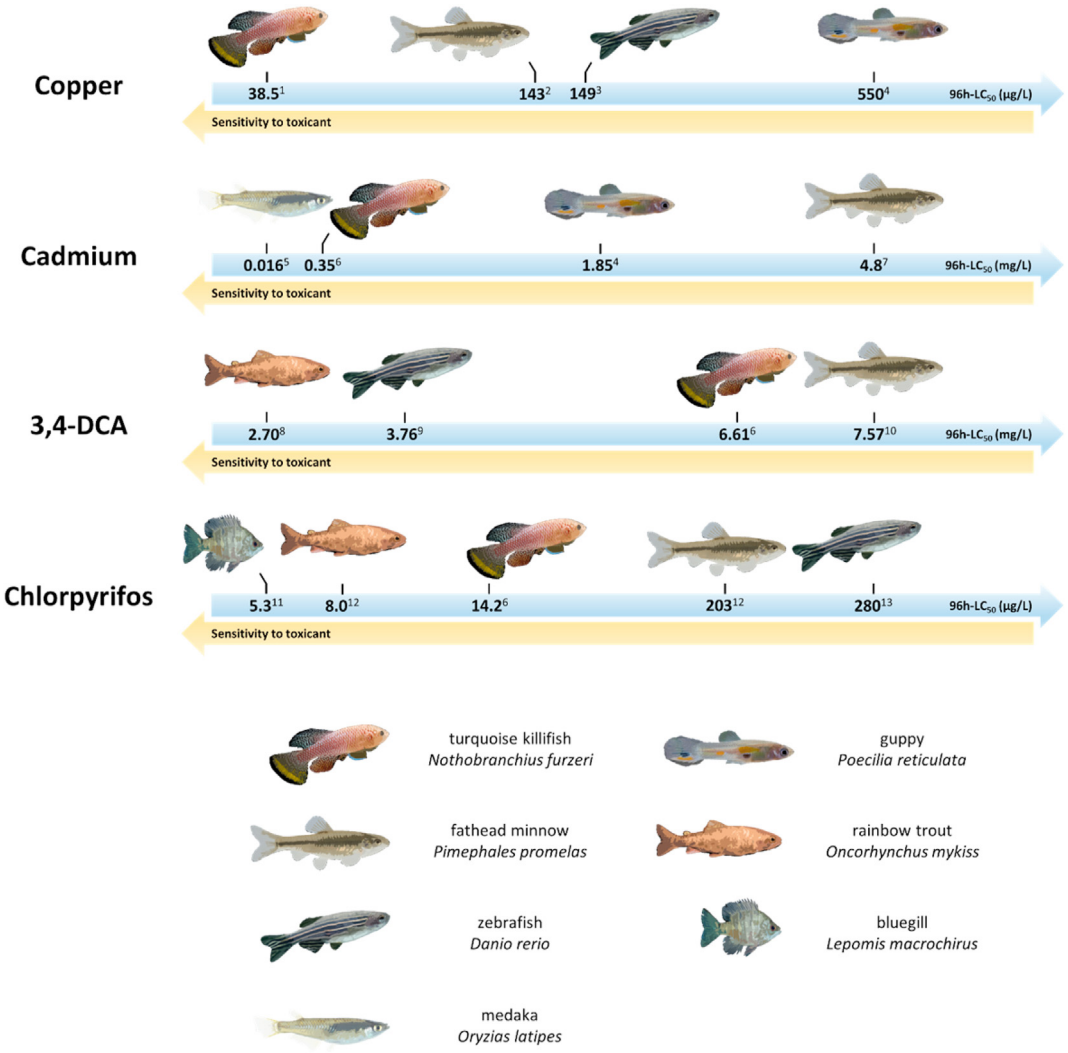

Fig. 4. Comparison of the acute sensitivity to metal (copper, cadmium) and pesticide (3,4-DCA, chlorpyrifos) between *N. furzeri* and traditional fish models. The relative sensitivity of different fish models to acute toxicant exposure is illustrated by their 96 h-LC_{50} value (concentration of the toxicant that causes 50% mortality in the tested organisms after 96 h of exposure) when exposed to the respective compounds in early life stages. (3,4-DCA: 3,4-dichloroaniline) [1]Philippe et al. (2017), [2]Erickson et al. (1996), [3]Alsop and Wood (2011), [4]Slabbert and Venter (1999), [5]Foran et al. (2002), [6]Philippe et al. (2018b), [7]Suedel et al. (1997), [8]Crossland (1990), [9]Bichara et al. (2014), [10]Russom et al. (1997), [11]Tyler Mehler et al. (2008), [12]Holcombe et al. (1982), [13]Wang et al. (2017).

4.3. Single vs. multi-stressor assays

A number of studies have explored potential interactive effects between different stressors in *N. furzeri*. Philippe et al. (2018b), for example, showed that body length was only reduced when fish were simultaneously exposed to both heavy metals and temperature stress while exposure to one of both stressors had no perceivable effect. Likewise, simultaneous exposure to heavy metal and temperature stress decreased fecundity and embryonic survival (Philippe et al., 2018b). Responses to pollutants were also shown to depend on the presence of other chemical stressors. Thoré et al. (2021), for instance, uncovered that combinations of pesticides and neuroactive chemicals induce different effects in single exposure trials as compared to simultaneous exposure (Table 2).

5. Remaining challenges and future perspectives

Although *N. furzeri* is especially promising as a model organism because of its very short life-cycle, this characteristic also brings limitations as it renders the species less suitable for studies on long-term accumulation. Rapid integration of the model in official ecotoxicological test frameworks is further challenged by aspects inherent to any new study system: a need for further standardisation in husbandry and testing, extrapolation of laboratory studies to the natural environment, and ethical issues on the use of the species in accredited test frameworks.

5.1. The need for standardised husbandry

To date, the development of standardised husbandry protocols for *N. furzeri* failed to keep pace with the increasing use of the species across laboratories and companies (*Nothobranchius* Symposium, Jena 2016). For instance, an optimised formulated dry feed is lacking but preferable to ensure optimal maintenance and high levels of standardisation across time and institutions. Furthermore, a wide variety of husbandry methods are currently adopted across institutions, ranging from static units to multi-linking flow-through systems which contributes to extensive variation in fish density, water chemistry, temperature and light-conditions. Future research

Table 1
Chronic exposure studies with *N. furzeri* to assess the expression of life-history and physiological endpoints in *Nothobranchius furzeri* in response to stressors with different modes of action. Affected endpoints are indicated in bold and the direction of the response is given between parentheses. (MT: metallothionein, GST: Glutathione S-Transferase, LDH: Lactate Dehydrogenase).

Stressor	Concentration (μg/L) or degree of stressor	Exposure duration	Measured endpoints	Reference
copper	10.27	96 h (larvae)	survival	Philippe et al. (2017)
copper	10.27	whole life	**growth rate** (↘), **female maturation time** (↗), **MT induction** (↗), **survival** (↘)	Philippe et al. (2017)
cadmium	15–30	whole life	adult body length and mass, energy reserves and physiological stress response, fecundity, growth rate, maturation time, parental and offspring survival, thermal tolerance	
fluoxetine	0.5	3 weeks (adult)	body condition	Thoré et al. (2018b)
fluoxetine	0.5	whole life	body length, egg fertilisation rate, **fecundity** (↗)	Thoré et al. (2020b)
fluoxetine	0.5	whole life for 2 successive generations	**body length** (↘, 2nd gen. only), egg fertilisation rate, **fecundity** (↘, 1st gen. only)	Thoré et al. (2021)
fluoxetine	5	3 weeks (adult)	body condition	Thoré et al. (2018b)
fluoxetine	5	whole life	**body length** (↘), egg fertilisation rate, **fecundity** (↗)	Thoré et al. (2020b)
3,4-DCA	50	whole life	body condition, energy reserves and physiological stress response, fecundity, growth rate, maturation time, survival, thermal tolerance	Philippe et al. (2019)
3,4-DCA	50	5–6 weeks (adult) for 2 successive generations	body length, egg fertilisation rate, **fecundity** (↘, 1st gen. only)	Thoré et al. (2021)
3,4-DCA	100	whole life	body condition, energy reserves and physiological stress response, fecundity, growth rate, maturation time, survival, **thermal tolerance** (↘)	Philippe et al. (2019)
temperature rise	+4 °C	whole life	**body length** (↘), body condition, **fecundity** (↘), **female glucose levels** (↗), glycogen content, **GST activity** (↘), **LDH activity** (↘), **male body mass** (↘), maturation time, protein content, survival, **thermal tolerance** (↗), total fat content	Philippe et al. (2019)

Table 2
Chronic exposure studies with *N. furzeri* to assess the expression of behavioural traits in response to selected pollutants. Affected traits are indicated in bold and the direction of the response is given between parentheses.

Compound	Concentration (μg/L)	Exposure duration	Measured traits	Reference
fluoxetine	0.5	3 weeks (adult)	anti-predator response, exploration tendency, feeding behaviour, locomotor activity, **risk-prone behaviour in novel environment** (↘)	Thoré et al. (2018b)
fluoxetine	0.5	whole life	aggressiveness, frequency of mating, latency to initiate mating, sociability	Thoré et al. (2020b)
fluoxetine	0.5	whole life for 2 successive generations	**feeding behaviour** (↘, except in combination with 50 μg/L 3,4-DCA), **locomotor activity** (↗, 2nd gen. only and in combination with 50 μg/L 3,4-DCA), **risk-prone behaviour** (↘, 1st gen., in 2nd gen. only in combination with 50 μg/L 3,4-DCA), swimming acceleration, swimming velocity	Thoré et al. (2021)
fluoxetine	5	3 weeks (adult)	**anti-predator response** (↗), exploration tendency, **feeding behaviour** (↘), locomotor activity, risk-prone behaviour in novel environment	Thoré et al. (2018b)
fluoxetine	5	whole life	aggressiveness, **frequency of mating** (↗), latency to initiate mating, **sociability** (↗, males only)	Thoré et al. (2020b)
3,4-DCA	50	5–6 weeks (adult) for 2 successive generations	**feeding behaviour** (↗, in 2nd gen. females only), locomotor activity, **risk-prone behaviour** (↗), swimming acceleration, swimming velocity	Thoré et al. (2021)
3,4-DCA	100	5–6 weeks (adult) for 2 successive generations	feeding behaviour, locomotor activity, risk-prone behaviour, swimming acceleration, swimming velocity	Thoré et al. (2021)

should focus on developing uniform and optimised husbandry protocols that maximise cost-effectiveness and animal welfare.

Ready-to-hatch *N. furzeri* eggs hatch simultaneously upon inundation and allow for age-synchronised experiments. However, storage conditions such as temperature, moisture level and light regime may influence embryonic development (Polačik et al., 2016) and future research should therefore examine potential shifts in other phenotypic traits, including stressor sensitivity. As the popularity of *N. furzeri* increases, controlled and centralised production and distribution of dormant eggs from specific strains would ensure quality control. To this end, controlled production of *N. furzeri* eggs was recently started by private companies, although research is needed to assess how potential differences in transport conditions may affect egg condition and performance of hatched fish.

5.2. From *N. furzeri* behaviour to population-level responses to pollution

A full understanding of animal behaviour requires the integration of both proximate (how-questions) and ultimate (why-questions) analyses across different levels of biological organisation. More specifically, the underlying physiological mechanisms and ontogenetic development of behaviour should be scrutinised along with its adaptive value and phylogenetic history. Yet, integrative work in behavioural ecotoxicology is still scarce (Peterson et al., 2017). For instance, a major challenge is to link pollutant-induced behavioural effects in the laboratory to population- and ecosystem-level responses in a natural environment (Mcdonald, 2017; Peterson et al., 2017). First steps have been taken to demonstrate actual fitness consequences of specific behavioural

Table 3
Repeatability values for a set of behavioural traits in *Nothobranchius furzeri*. In Thoré et al. (2020b; 2021), repeatability values were calculated across experimental conditions. Values between brackets were obtained with control fish only. Different behavioural tests were used to assess the listed traits, and are explained in detail in the respective publications.

	Behavioural trait	Publication	Repeatability (R)	Confidence interval (95%)
Locomotor activity				
	Activity level	Thoré et al. (2019)	0.550	0.426–0.658
	Diurnal change in activity level	Thoré et al. (2019)	0.731	0.393–0.999
	Total travelled distance	Thoré et al. (2018a)	0.457	0.159–0.654
	Total travelled distance	Thoré et al. (2021)	0.296 (0.389)	0.209–0.391 (0.232–0.530)
	Total time moving	Thoré et al. (2021)	0.321 (0.386)	0.219–0.409 (0.229–0.525)
	Mean velocity	Thoré et al. (2021)	0.262 (0.276)	0.189–0.351 (0.194–0.364)
	Maximum acceleration	Thoré et al. (2021)	0.088 (0.017)	0.039–0.157 (0–0.128)
Boldness/exploratory behaviour				
	Number of entries in centrum	Thoré et al. (2018a)	0.266	0.058–0.526
	Total time spent in centrum	Thoré et al. (2018a)	0.259	0.005–0.485
	Total time spent in centrum	Thoré et al. (2021)	0.158 (0.196)	0.109–0.234 (0.053–0.347)
	Mean distance to centrum	Thoré et al. (2021)	0.169 (0.150)	0.118–0.238 (0.008–0.302)
	Habitat choice	Thoré et al. (2018a)	0.178	0.001–0.394
	Latency time to enter novel environment	Thoré et al. (2018a)	0.334	0.072–0.541
Life skills				
	Latency time to initiate feeding	Thoré et al. (2018a)	0.174	0–0.405
	Latency time to initiate feeding	Thoré et al. (2021)	0.316 (0.336)	0.248–0.400 (0.187–0.503)
	Latency time to resume feeding	Thoré et al. (2018a)	0.108	0–0.384
	Time till movement after predator attack	Thoré et al. (2018a)	0.320	0.043–0.593
Social behaviour				
	Aggressiveness	Thoré et al. (2020b)	0.360 (0.477)	0.214–0.492 (0.194–0.681)
	Sociability	Thoré et al. (2020b)	0.195 (0.167)	0.104–0.287 (0.011–0.317)

alterations in *N. furzeri* (Thoré et al., 2020b). Still, considerable life-history and behavioural variation exists between natural populations of the species (Blažek et al., 2016; Thoré et al., 2019) that could be associated with sensitivity-differences to chemicals. Therefore, the degree to which commonly-studied strains are representative of wild *N. furzeri* populations – and perhaps congeneric species – should be validated (Brown et al., 2009). Moreover, animal behaviour is intrinsically variable and may reflect a diversity of underlying motivational and cognitive mechanisms (Budaev and Brown, 2011). This often hampers clear-cut interpretations of observed behavioural changes (Thoré et al., 2020a) and suggests that behavioural tests with *N. furzeri* should be validated for further use in ecotoxicology. This includes a better understanding of normal behaviour and its (natural) variability (Tanoue et al., 2019; Thoré et al., 2018b), but also assessment of how experience and housing conditions impact baseline behavioural expression in *N. furzeri* across life-stages (Thoré et al., 2020a). Moreover, follow-up research on the individual-level is imperative as it has been shown that pollutant exposure does not only impact the average level of behaviour but also individual behavioural variability in certain fish species (Dzieweczynski and Hebert, 2012). Whether or not this is also the case for *N. furzeri* has not been examined.

Other remaining questions relate to the physiological underpinnings of most pharmacological effects, which should best be assessed by an integrative approach combining life-history, behaviour and physiological endpoints across developmental stages. Through population-level modelling, modes of action and individual-level responses can be related to populations via adverse outcome pathways to bridge the gap between laboratory observations and the ecological consequences of pollution (Kramer et al., 2011; Mcdonald, 2017). This requires reconstruction of dose-response relationships for effects that may compromise the organism's ability to carry out its ecological role. Subsequently, such effects should be related quantitatively to survival or fecundity as key determinants of population demographics. To provide context for such models, increasing knowledge of baseline biological data is essential (Kramer et al., 2011) and an improved understanding of *N. furzeri* behaviour across different levels of organisation is needed.

5.3. Towards accredited test frameworks for N. furzeri

Several studies show that *N. furzeri* can be used for whole life-cycle and multigenerational testing (Table 1, Table 2). Based on available laboratory husbandry protocols (Polačik et al., 2016), Philippe et al. (2018a) refined hatching and general maintenance protocols for application in ecotoxicological tests. In addition, a procedure was published for short-term exposure studies, optimised to monitor survival and general stress symptoms (Philippe et al., 2018a). Still, a formal framework is needed before *N. furzeri* can be used as a model organism in official environmental risk assessment procedures (Hund-Rinke et al., 2016; Tanaka et al., 2020). To this end, suitable endpoints should be selected and existing standard test guidelines (e.g., OECD guidelines for testing of chemicals) adapted for a wide variety of different endpoints (life-history, physiology, behaviour). In addition, guidelines should be extended by incorporating behavioural tests to assess effects of pharmaceuticals and other emerging contaminants. To this end, not only further fine-tuning of the current toolbox (Table 2, Table 3) with behavioural tests is essential, but tests and endpoints also need to be validated before they can be included in formal guidelines.

Priority candidate test guidelines in which *N. furzeri* can be included are, for instance, the Fish Sexual Development Test (OECD Test No. 234) and the Extended One-Generation Reproduction Test (OECD Test No. 240). These tests can become 25–50% more time-efficient when conducted with *N. furzeri* as compared to traditional models such as zebrafish and medaka. For proper validation of *N. furzeri* as a complementary model, comparative tests with established fish models should still be performed. These tests should be repeated in time and across research institutes, not only to further optimise and standardise test protocols but also to evaluate test repeatability. Once such data are available, full cost-benefit analyses should be performed, taking into account several criteria including the ecological relevance of the test, relative

sensitivity of *N. furzeri* to different classes of contaminants, test-retest reliability, time- and monetary costs, high-throughput implementation, and compatibility with measures to improve animal welfare.

5.4. Ethical considerations on the use of N. furzeri in ecotoxicology

Animal welfare is fundamental to laboratory animal use, which should adhere to the highest ethical standards (Broom, 2011). This is implemented by current guidelines which are rooted in the "three R" guiding principles of Russel and Burch to prevent animal cruelty: Replacement, Reduction and Refinement (Fenwick et al., 2009). When alternative methods (e.g. modelling, cell lines) are not available or possible, efforts should be directed at reducing the number of used animals and at optimising husbandry and experimental procedures to lower animal suffering and distress.

Behavioural endpoints can serve as early-warning signals that can be assessed non-invasively at sub-lethal concentrations, thereby limiting animal stress and suffering (Peterson et al., 2017). Moreover, because behavioural effects may be reversible (Gerhardt, 2007), multiple research objectives can be established and animals may be reused for other purposes in the absence of confounding experiential effects (Fenwick et al., 2009). However, it should also be considered that behaviour is naturally variable and higher sample sizes may be required to avoid spurious effects. In part, this can be counteracted through repeated-measures designs in which individuals are repeatedly scored for the same trait, which increases statistical power and reduces the number of necessary test animals.

To further accommodate animal welfare in *N. furzeri* ecotoxicology, husbandry protocols and experimental procedures are being developed with consideration for environmental enrichment to enhance fish wellbeing (Thoré et al., 2020a). Moreover, standardisation of these procedures will promote experimental reproducibility (Parker, 2016) and thereby contribute to an overall reduction in number of laboratory animals needed for testing.

Lastly, ethical use of animals for experimentation should not only consider animal welfare but also the integrity of natural populations. The availability of natural populations in addition to laboratory strains is a valuable asset for research with *N. furzeri*. However, as growing research interest (including collection of wild individuals) may also increase the pressure on natural populations, ecological risks should be carefully considered and justified.

6. General conclusion

Current standard fish-based tests in ecotoxicology suffer from limited ecological relevance as they rely on species with a relatively slow life-cycle that do not readily facilitate chronic or multigenerational exposure tests. There is also a need for sensitive sub-lethal endpoints that signal effects of environmental pollutants even at low concentrations, including emerging contaminants.

Here, we provide a comprehensive overview of current issues with fish-based testing in ecotoxicology. Next, we showcase the recently-introduced turquoise killifish (*N. furzeri*) as a sensitive model for time-efficient full life-cycle and multigenerational testing of various pollutant types in a multi-stressor environment. We compile a battery of relevant life-history, physiological and behavioural endpoints that are easy to measure in *N. furzeri*. Finally, we also pinpoint key issues pertaining to standardisation, extrapolation – including a better understanding of the behavioural norm – and ethical use of *N. furzeri* that should be addressed before the model can be integrated in accredited test frameworks. Together, this information offers a route for tailored ecotoxicological testing to further map the environmental impact of pollution.

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Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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