Protocol for acute and chronic ecotoxicity testing of the turquoise killifish **Nothobranchius furzeri**

TITLE:

Protocol for acute and chronic ecotoxicity testing of the turquoise killifish Nothobranchius furzeri.

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KEYWORDS: *(6 minimum, 12 maximum, separated by commas)*

- *Nothobranchius furzeri,* chronic toxicity, acute toxicity, full lifespan, protocol, fish model

SHORT ABSTRACT: *(10 word minimum, 50 word maximum)*

In this work, we describe an acute, chronic and multigenerational bioassay of the effects of single and combined stressors on the turquoise killifish *Nothobranchius furzeri*. This protocol is designed to study life history traits (mortality, growth, fecundity, weight), critical thermal maximum and physiology (energy reserves).

LONG ABSTRACT: *(150 word minimum, 300 word maximum)*

Nothobranchius furzeri is an emerging model organism in the field of ecotoxicology and its applicability in acute and chronic ecotoxicity testing has been demonstrated. Overall, the sensitivity of the species to toxic compounds is in range with, or higher than, that of other model species.

This work describes protocols for acute, chronic and multigenerational bioassays of single and combined stressor effects on the turquoise killifish *Nothobranchius furzeri*. Protocols are designed to measure life history traits (mortality, growth, fecundity, weight), critical thermal maximum and physiology (energy reserves). Due to its short maturation time and life-cycle, this vertebrate model allows the study of endpoints such as maturation time and fecundity within four months. Transgenerational full life-cycle exposure trials can be performed in as little as 8 months. Since this species produces eggs that are drought-resistant and remain viable for years, on-site culture of the species is not needed but individuals can be recruited when required.

INTRODUCTION:

Sensitivity profiles of an array of species to strategically selected toxicants have been described[1] for the European REACH legislation. Acute or short-term toxicity tests were mostly used for this purpose as they give a quick indication of a species' sensitivity. However, in their natural environment, organisms are exposed during much longer periods and full life-cycles or even several generations could be affected. Moreover, organisms in polluted environments are typically exposed to more than one stressor at a time, which may interact with each other, possibly resulting in synergistic effects. For both reasons, safe concentrations calculated based on acute, single stressor toxicity tests may underestimate the actual risks of releasing toxicants in natural environments. It is therefore advisable to also study the chronic and multigenerational effects of sublethal concentrations of toxicants in an environmentally relevant context as advocated by the European Commission [2,3] and the USEPA (United States Environmental Protection Agency) [4,5]. Especially in vertebrate research, the costs in terms of labour, money and time are high when performing chronic and multigenerational exposure studies because of the relatively long lifespan of vertebrates contrary to invertebrates. Therefore, it is advisable to choose the most appropriate fish model organism, depending on the research question. Furthermore, a wide array of vertebrate species should be available in order to test the generality of responses across species to be able to adapt regulations and protect the most sensitive species. For now, there is a need to develop new, efficient vertebrate model species with short life cycles to lower the costs of performing chronic and multigenerational exposures on vertebrates[5,6].

The turquoise killifish *Nothobranchius furzeri* is an interesting fish model to use in such long-term exposure experiments because of its short maturation time and life-cycle (generation time less than 5 weeks). As such, ecologically highly relevant endpoints such as maturation time and fecundity patterns can be studied in a time frame that is very short compared to other fish models[5]. Furthermore, these fish produce drought-resistant, dormant eggs that remain viable for several years when stored under standard conditions in petri-dishes, thereby eliminating the need of a continuous culture. In ecotoxicological studies this also means that replicate fish can all be hatched at the exact same moment, resulting in time synchrony for all animals, even among batches of eggs produced at different times.

We advise to use the laboratory GRZ strain to perform exposure experiments. This strain performs well under laboratory conditions, is homozygous (except for sex chromosomes) and the genome is characterised. Several methods can be used to select the appropriate range of test concentrations. Firstly, the nominal concentration range can be based on the sensitivity of a related species, such as *Nothobranchius guentheri*[7]. Second, the range can be based on

the sensitivity of species with comparable sensitivity ranges, such as *Danio rerio* [8] (Philippe et al. (subm)). In combination with both of these options, a range finding experiment should be conducted to select the nominal concentration range. For acute testing, aim for concentration treatments with 100% mortality, intermediate mortality and 0% mortality after 24h. For chronic testing, It is advisable to run such an experiment during one or two weeks to verify if larval mortality does not exceed 10%.

The protocol can serve as a baseline to perform acute and chronic exposure studies on *N. furzeri*, examining potential effects of stressors both at the individual and cellular level. It can also be used to perform multi-stressor research, mixing different toxic compounds or studying interactive effects between pollution and other natural stressors (f.e. predation) or anthropogenic stressors (f.e. warming due to climate change).

PROTOCOL:

All methods described here have been approved by the Ethical committee of KULeuven.

1) Hatching and general maintenance of *N. furzeri*

1.1 Prepare fish medium using Milli-Q water with added, standardised salts (e.g. Instant Ocean® Sea Salt) to a conductivity of 600 µS cm^{-1} (24°C). Select eggs in the DIII stage, recognisable by the presence of golden eyes[9] and gently transfer them with a soft pair of tweezers to a plastic 2L tank. Add 1 cm of fish medium at 12°C and let the water temperature gradually converge to room temperature (24°C)[9]. NOTE: In order to have a sufficient number of healthy test organisms, double the amount of eggs should be hatched.

1.2 After 24h, feed the hatchlings a concentrated dose of freshly hatched Artemia nauplii (see protocol in Polacik et al. 2016[9]) and increase the water depth to 5 cm by adding fish medium. After 36h feed the hatchlings another concentrated dose of freshly hatched Artemia nauplii and add fish medium to increase the water depth to 10 cm.

1.3 Fish containers should be housed under constant temperature conditions (e.g. in an incubator, climate room or heated water bath) under a 14h:10h light:dark regime.

1.4 Before the start of the experiment, complex fish containers with the exposure compound by filling them with the highest concentration of exposure medium and leaving it overnight.

1.5 48h after hatching, select healthy buoyant larvae to start the exposure experiment. Discard so-called belly-sliders that were unable to fill their swim bladder and continuously sink to the bottom.

2) Short-term exposure protocol

In addition to a full control treatment, a solvent control should be included if the stock solution of the compound is prepared using a solvent. The solvent control should contain the amount

of solvent equalling the solvent concentration in the highest exposure concentration. Aim for at least 20 replicates per treatment.

2.1 Prepare the experimental containers (0.5 L glass jars) by labelling them properly and filling them with the appropriate exposure medium (different toxicant concentrations). Add the compound to obtain the correct concentration.

2.2 Transfer larvae (48h post hatching) individually to the containers. Fish are exposed individually to minimise potential confounding effects of social interaction such as competition for food and aggression.

2.3 This acute exposure can last for a duration of up to two weeks. During that time, feed the fish *ad libitum* with *Artemia* nauplii (Ocean Nutrition, Essen, Belgium) twice per day, seven days per week.

2.4 Refresh the medium every other day to maintain water quality and to minimise potential effects of compound degradation. Monitor key water variables. Dissolved oxygen levels should exceed 80%, conductivity should range between 600 and 700 μScm^{-1}, pH between 7.8 and 8.2, and hardness (as $CaCO_3$) between 350 and 450 mgL^{-1}, which lies within the range of optimal rearing conditions for *N. furzeri* [9]. Take water samples before and after refreshing the medium to determine actual compound concentrations.

2.5 Endpoints

2.5.1 Check fish for mortality, stress (e.g. aberrant behaviour: swimming upside down) or sickness twice daily.

2.5.2 Calculate LC50 values based on mortality using dose-response curves (Ritz and Streibig, 2005) at different time points. This can, for instance, be done in R v3.2.3 (R Development Core Team, 2016) with the *drm* function in the drc package.

3) Chronic exposure protocol

25 fish per treatment is a minimum to start a chronic exposure trial as a number of fish will die due to natural causes and to minimise chances to have a skewed sex-ratio.

3.1 Hatching (see 1.1)

3.2 Phase I (2 days post hatching – 16 days post hatching)

3.2.1 Follow protocol as described in 2.1-2.4

3.2.2 Use inert containers filled with the required amount of exposure medium for 4 refreshment trials in order to allow similar degradation of the compound of the refreshment medium as in the experimental jars throughout a week, as will be the case in phase II.

3.3 Phase II (16 days post hatching – end)

3.3.1 Prepare 2L experimental glass jars by complexing them with the compound. Fill them with the correct exposure medium and add an air tube to aerate the jar. House fish individually in these jars for the remainder of the experiment.

3.3.2 Refresh the medium once per week. Take water samples every day throughout a week to monitor the degradation of the compound in each concentration treatment. If multiple stressors are tested, a degradation curve should be calculated for each treatment. Measure abiotic parameters (pH, temperature, % dissolved oxygen, conductivity) three times per week.

3.3.2.1 From 2 days post hatching until 23 days post hatching, feed the fish twice per day, 7 days per week *ad libitum Artemia* nauplii. From 24 dph-37 dph, complement the *ad libitum Artemia* diet with chopped Chironomus larvae. From 38 dph on, feed the fish twice per day, 7 days per week *ad libitum* frozen *Chironomus* larvae.

3.4 Endpoints

3.4.1 Daily check fish for mortality, stress or sickness.

3.4.2 For growth, make weekly (9dph-16dph-21dph-…) measurements of the size of the fish by transferring them to a petri dish filled with medium from their reservoir. Take 4-5 size calibrated pictures of the fish from above and analyse them digitally using a spatial measuring program (e.g. ImageJ). It is advisable to use a tripod that is set at a fixed height in order to retain the same scale for each picture. This will facilitate the digital analysis of pictures.

3.4.3 For male maturation check fish daily for coloration from 15dph onwards. Males will show first signs of nuptial coloration on the fins. The first day at which this is visible, should be used as a proxy for male maturation time.

3.4.4 For female maturation, couple non-sexed fish with known males of the same treatment group from 30dph onwards in order to determine female maturation time (the day the first egg is deposited). For this, use the spawning protocol described in 3.4.5.

3.4.5 For fecundity, couple mature females with mature males three times per week from 30dph onwards, ideally within their treatments using a crossing scheme. Prepare a spawning tank (1L) for each couple, using exposure medium from the male aquarium supplemented with spawning substrate (fine sand or gravel). Transfer both the male and female into the spawning tank and allow them to spawn for two hours. Minimize human activity around the spawning containers during this process. Afterwards, gently transfer fish back to their original housing containers, without unnecessary mixing of the water, which will whirl up the eggs in the spawning substrate. Filter the eggs out by pouring the spawning substrate over a 500 µm mesh. Count the eggs and transfer them to damp peat moss in Petri dishes (Watters, 2009).

3.4.6 Remove dead eggs daily. After a week, seal the Petri dish with Parafilm and store it in a temperature controlled incubator at 28°C and a 14:10 light cycle.

3.4.7 Critical thermal maximum (CTmax) can be performed on adult fish. Use a water bath that is heated at a constant rate of 0.33°C min^{-1} and in which the water is continuously circulated. Add several 2L aquaria for each individual fish. NOTE: given space constraints of the water bath, it is necessary to work in several series, which should be taken into account when performing statistical analysis by including 'series' as a random factor. The trial starts when the water in the aquarium has reached the experimental rearing temperature of the fish. Monitor the temperature in the 1L aquaria every 5 minutes using a digital thermometer (0.1°C scale). The trial ends when the fish fails to maintain a dorso-ventrally upright position or starts twitching heavily [10,11]. Quickly transfer the fish back to its experimental housing for recovery and measure the temperature in the 1L aquarium. This is the critical maximal temperature.

3.4.8 Measure the weight (0.1 mg accuracy) of the fish on the last day of the experiment by patting it dry and transferring it on a weighing boat. NOTE: All fish should be measured four hours after the last feeding to standardize food weight in the intestinal tract.

3.4.9 Euthanize the fish by sedating them in ice water and transferring them in liquid nitrogen at -196°C. Store the fish at -80°C to prevent protein degradation.

3.5 Physiology

3.5.1 Thaw the fish on ice for one hour prior to homogenisation. Eventually, fish must be diluted 15 times. To do this, multiply the weight of the fish by 15 and add that number of µL in homogenisation buffer (0.1 M TRIS–HCl, pH 8.5, 15 % polyvinyl pyrrolidone, 153 µM $MgSO_4$ and 0.2 % Triton X-100). Homogenise the fish in the tube in an ice-filled beaker to keep the temperature low. Centrifuge the content for 7 minutes at 6.0 g and 4 °C. Use the resulting supernatant to measure the energy reserves and quantify stress responses. Measure all samples in triplicate and use the means as actual measure.

3.5.2 The protocol to measure fat content is based on the protocol of Bligh and Dyer (1959). First separate polar from non-polar fats. Fill a 2.5 mL Eppendorf tube with 150 µL of supernatant and 650 µL of a 2:1 chloroform:methanol solution. Centrifuge this for 10 min (16.0 g, 4°C). For each fish sample, fill three 2-mL glass tubes with 25 µL of the polar fraction; 25 µL of the nonpolar fraction and 25 µL of the supernatant respectively and add 175 µL of H_2SO_4 (100 %) in each tube. Heat the tubes at 150°C for 20 min and allow the samples to cool down. Add 200 µL Milli-Q water and mix the sample by pipetting them up and down. Fill a transparent 384-well microtiter plate with 30 µL of each tube and measure absorbance at 340 nm. Calculate fat contents using a standard curve of glyceryl tripalmitate.

3.5.3 The protocol to measure sugar (glucose and glycogen) content is adapted from the protocol of Stoks et al. (2006b). For the measurement of the combination of glucose and glycogen, mix 37.5µL Milli-Q water and 12.5 µL of the supernatant. For the

measurement of pure glucose, mix 32.5 µL of Milli-Q water, 12.5 µL of the supernatant and 5 µL amyloglucosidase (Sigma A7420) in a 96-well microtiter plate. The difference in sugar content between the two wells will be equal to the amount of sugar stored in glycogen. Incubate the plate for 30 min at 37 °C. Add 100 µL of glucose assay reagent (Sigma G3293) to each well and incubate the plate for 20 min at 30 °C. Measure absorbance at 340 nm and calculate the glucose content based on a standard curve of known concentrations of glucose and their absorbance.

3.5.4 The protocol to measure the protein content is based on the Bradford (1976) method. Add 160 µL Milli-Q water and 1 µL of the supernatant to a 96-well microtiter plate. Then add 40 µL of Biorad protein dye and mix the sample. Incubate the plate for 5 min and measure absorbance at 595 nm. Calculate the protein content based on a standard curve of known protein concentrations.

4) Transgenerational exposure protocol

To measure transgenerational effects of pollutants on *N. furzeri*, follow the chronic exposure protocol outlined above for the first generation.

4.1 Twice weekly, check the development of the produced eggs (i.e. the second generation) by inspecting the petri dishes for embryos in the DIII phase (see Polacik et al 2016[9]). When more than 50 replicates of each parental treatment are fully developed, hatch them following the protocol in 1.1.

4.2 Expose healthy, buoyant fish to exactly the same set-up and treatment as their parents.

REPRESENTATIVE RESULTS:

The results of the acute exposure of *N. furzeri* to different concentrations of copper show clear dose-response relationships (Fig. 1). There is an increase in mortality with increasing toxicant concentration. LC50 values decrease over time, meaning that with lowering concentrations, more time passes before 50 % of the replica die.

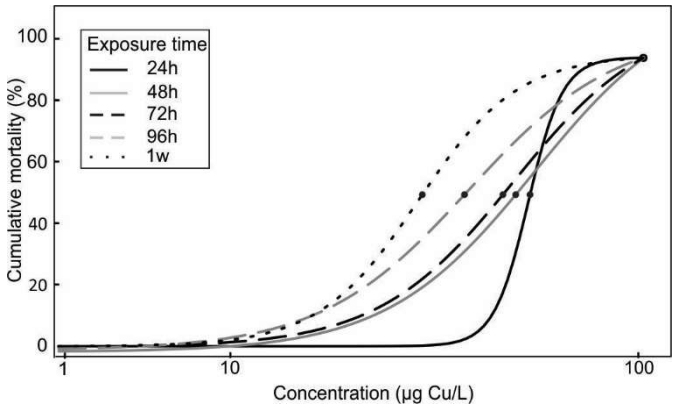

Fig. 1. Dose-response curves showing cumulative mortality of *Nothobranchius furzeri* in function of the copper concentration (in µg Cu/L) it was exposed to and in relation to exposure time. Dots indicate LC_{50} values.

In the chronic exposure trial, size and fecundity are sensitive endpoints. There can be extensive variation in the growth of the fish, depending on the temperature, feeding regime and fish strain. Adult sizes between 30 and 50 mm are considered normal in this set-up. The set-up allows to find differences between treatments (Fig. 2A), despite possible impairment of growth due to the small volume of the aquarium. For fecundity, control values should fluctuate between 10 and 50 eggs per week per female at the peak of egg production (Fig. 2B). The measurement is quite time consuming because of the handling of the fish and the plating of the eggs, but it is often the most sensitive endpoint. The measure is also sensitive to the effect of combined stressors and synergistic effects between warming and pollution on fecundity have been demonstrated (unpublished results).

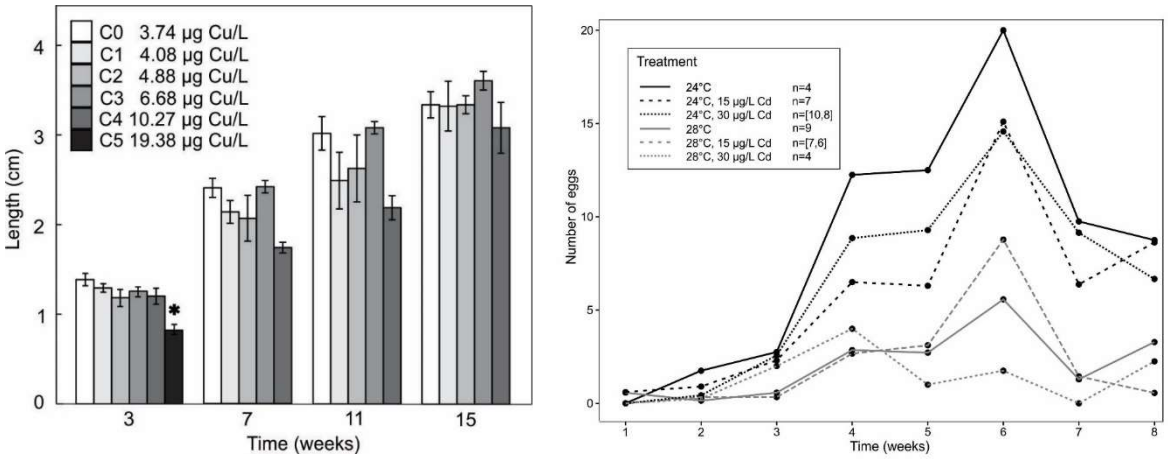

Fig. 2A. Size (in cm) of Nothobranchius furzeri exposed to different concentrations of copper at week 3, 7, 11 and 15. Asterisk indicates that C5 fish are smaller after three weeks at the significance level of $P<0.05$. Values are presented as mean±SEM. Sample sizes are n = 6; 6; 7; 7; 7; 7 in week 3, n=6; 6; 7; 7; 4 in week 7, n = 5; 4; 5; 5; 3 in week 11 and n = 5; 3; 3; 5; 2 in week 15. Figure 2B: Fecundity through time of fish exposed to different concentrations of cadmium, crossed with two temperature treatments, measured as number of eggs per week. To improve the readability and interpretability of the figure, error bars are not shown on the graphs. The number of females in each treatment at the begin and end of the egg laying period is indicated using the letter n.

Maturation time is most often affected by pollutants in males (Fig. 3A). This response should, however, be interpreted with caution since maturation time is scored indirectly by determining the onset of nuptial colouration as a proxy. Although males are considered to mature a few days after the appearance of colouration[9], there may be some error on the exact timing of maturation using this measure.

Near the thermal optimum, fish exhibit erratic swimming, increased opercular movement and loss of ability to remain in a dorso-ventrally upright position [10,11]. CTmax values differ between *N. furzeri* strains. Natural populations have CTmax values between 39°C and 42°C when reared in temperatures between 24 and 28°C (Fig. 3B). The inbred GRZ strain, however, already reaches its thermal maximum at around 37-38°C, even when reared at 28°C. Exceptionally, fish do not survive the CTmax measurement. Such fish are best excluded from the CTmax analysis.

Previous results mostly showed that CTmax was affected by the rearing temperature ($\chi^2_{1,71}$=322.0, P<0.001) and that fish that were reared at 28°C had a 1.3°C higher CTmax compared to fish that were reared at 24°C. Also, CTmax can be affected by the pollutant, in this case 3,4-DCA ($\chi^2_{2,71}$=17.65, P<0.001) with fish exposed to 0.1 mg/L 3,4-DCA having a 0.32°C lower thermal maximum compared to control fish (P<0.001).

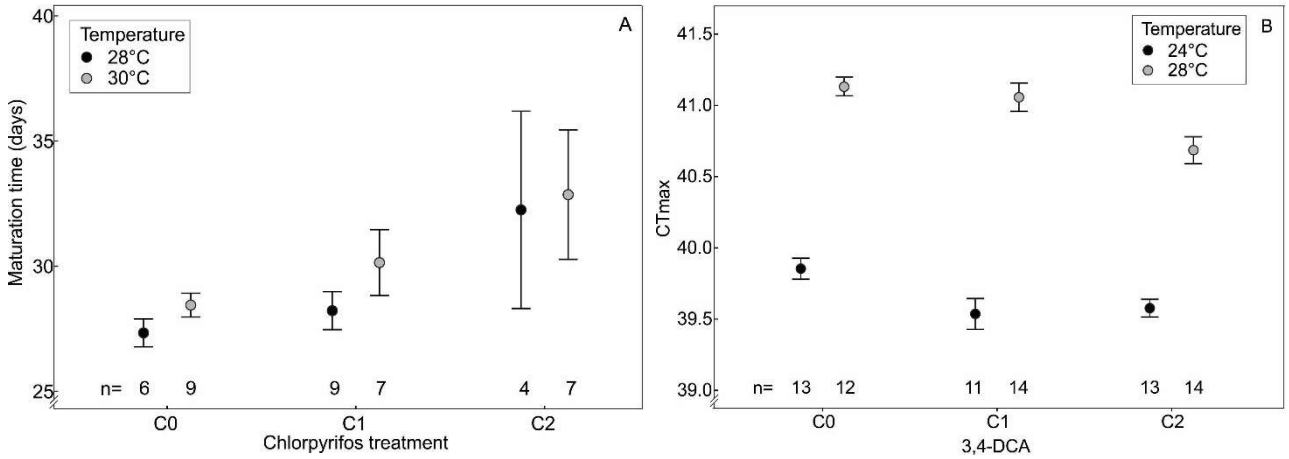

Figure 3A: Mean age (in days) at which the first signs of colouration appeared in males of *Nothobranchius furzeri* exposed to different chlorpyrifos concentrations (0 µg/L (C0), 2 µg/L (C1) and 4 µg/L CPF (C2)) and two temperatures (28°C and 30°C). Figure 3B: Mean critical thermal maximum (CTmax) of fish exposed to different concentrations of 3,4-DCA (0 mg/L 3,4-DCA (C0), 0.05 mg/L 3,4-DCA (C1) and 0.1 mg/L 3,4-DCA (C2)) and two temperatures (24°C and 28°C). Nominal concentrations are Values are presented as mean ± SE.

FIGURE AND TABLE LEGENDS:

Figure 1. Dose-response curves showing cumulative mortality of *Nothobranchius furzeri* in function of the copper concentration (in µg Cu/L) it was exposed to and in relation to exposure time. Dots indicate LC_{50} values.

Figure 2A) Size (in cm) of Nothobranchius furzeri exposed to different concentrations of copper at week 3, 7, 11 and 15. Asterisk indicates that C5 fish are smaller after three weeks at the significance level of P<0.05. Values are presented as mean±SEM. Sample sizes are n = 6; 6; 7; 7; 7; 7 in week 3, n=6; 6; 7; 7; 4 in week 7, n = 5; 4; 5; 5; 3 in week 11 and n = 5; 3; 3; 5; 2 in week 15. **Figure 2B**): Fecundity through time of fish exposed to different concentrations of cadmium, crossed with two temperature treatments, measured as number of eggs per week. To improve the readability and interpretability of the figure, error bars are not shown on the graphs. The number of females in each treatment at the begin and end of the egg laying period is indicated using the letter n.

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DISCUSSION:

This work describes a new bioassay using *Nothobranchius furzeri*, a novel model organism to study the individual and combined long-term effects of toxicants and other stressors, that was successfully applied to measure the sensitivity of the species to an array on toxicants (Cu, Cd, 3,4-DCA and chlorpyrifos). Due to its fast life cycle, this vertebrate model allows for assessment of sublethal and transgenerational effects within four months. Another major advantage of using this fish species as a model for toxicity screening is the fact that it produces drought-resistant eggs. This enables researchers to store eggs or obtain them from a supplier and eliminates the need for a costly and time consuming on-site culture. Moreover, the embryos can be stored for several months up to a year until hatchlings are needed [7].

After studying its sensitivity to a number of reference toxicants, we can add that the sensitivity of the species is in range with, or higher than, that of other model species, depending on the tested compound. The extent to which multiple stressors exert adverse effects individually or combined is dependent on the endpoint evaluated as well as the intensity of stress. It is possible to measure a battery of endpoints on this fish species in a short time span.

There are still some limitations when working with the model organism. One of the most important ones is the standardisation of food. Batches of Artemia cysts or bloodworms can differ in quality and can, as such, impact the results of the study. It is therefore advisable to order a large batch of food to use during the whole length of the experiment. Furthermore, we want to add that the larval phase is a critical step in the protocol and cleaning aquaria, as well as working in the absence of infectious water is recommended. Finally, left-over Artemia could induce infections, which could be of a bigger extent in control treatments compared to pollutant treatments. We, therefore, advise to remove left-over food every other day during the larval stage.

We believe that this protocol is widely applicable for ecotoxicological screening. *N. furzeri* is rapidly developing into a standard test species in ecotoxicology. The availability of this standard protocol may fuel its introduction.

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DISCLOSURES:
The authors have nothing to disclose.

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