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A short-term sublethal toxicity assay with zebra fish based on preying rate and its integration with mortality

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HIGHLIGHTS

- A short-term assay with zebra fish based on preying rates is proposed.
- A method to easily and precisely quantify feeding during exposure was optimized.
- The sensitivity of the 1-h feeding and 28-d growth to copper was similar.
- The impact of pH, conductivity and hardness on feeding was negligible.
- Integrating survival and feeding may improve estimates of ecosystem functioning.

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ABSTRACT

Contaminant-induced feeding inhibition has direct and immediate consequences at higher levels of biological organization, by depressing the population consumption and thus hampering ecosystem functioning (e.g. grazing, organic matter decomposition). Thus, similarly to lethality and avoidance, feeding is mechanistically linked to ecosystem processes and is therefore an unequivocal ecologically meaningful response. The objective of the present study was to develop a short-term assay with the small freshwater fish *Danio rerio*, based on feeding. For this, a methodology to easily and precisely quantify feeding was first optimized: each fish was allowed to prey on ten live *Daphnia magna* juveniles, for 1 h, just before the end of a 48-h exposure test period. Secondly, copper sensitivity of feeding relatively to survival and growth was evaluated. At the growth EC20 (40 $\mu g\ L^{-1}$), feeding was inhibited by 53%, and at the feeding EC50 (36 $\mu g\ L^{-1}$), mortality was negligible (1.3%). Integrating feeding and survival revealed a 97% depression in the population consumption at the LC50 (61 $\mu g\ L^{-1}$). Thirdly, the influence of pH, conductivity and hardness on the feeding background variability was assessed by assaying waters collected at eight reference sites and was found to be negligible, within tested ranges. Fourthly, feeding assays with natural waters contaminated with acid mine drainage confirmed the integration of lethality and feeding to be pertinent at estimating contaminant effects at higher levels of biological organization.
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1. Introduction

The decrease of the feeding rate due to contamination is expected to be fast and general, in a way that can be quantified and thus used as a toxicity test endpoint (Maltby, 1999; Forrow and Maltby, 2000; McWilliam and Baird, 2002; Moreira et al., 2005; Correia et al., 2013). As a consequence, the feeding response has been successfully adopted in aquatic toxicity testing with cladocerans (McWilliam and Baird, 2002; Lopes et al., 2007), amphipods (Forrow and Maltby, 2000; Maltby et al., 2002), decapods (Moreira et al., 2006; Satapornvanit et al., 2009), snails (Crichton et al., 2004; Krell et al., 2011; Correia et al., 2013), polychaetes (Moreira et al., 2005; Soares et al., 2005a; Rosen and Miller, 2011), midges (Soares et al., 2005a,b), and fish (Castro et al., 2004; Moreira et al., 2010).

Standard sublethal toxicity tests with animals are based on the assumption that sublethal effects measured at the organism level are extrapolated to population, community and finally to ecosystem structure and function in a time-delayed process (Maltby, 1999; Amiard-Triquet, 2009; Clements and Rohr, 2009). These responses should ideally be translatable into consequences at higher levels of biological organization; through quantitative and mechanistic linkages, though such conditions cannot always be met (Baird et al., 2007). Additionally, feeding depression at the individual level is expected to have direct and immediate consequences at the population level by inhibiting the population

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consumption, thus having direct and immediate effects on ecosystem functioning (e.g. impairment of grazing or organic matter decomposition), long before the effects at the individual level are propagated. Thus, similarly to lethality and avoidance endpoints, feeding is mechanistically linked to ecosystem functions and is therefore an unequivocal ecologically meaningful response (Maltby, 1999; Forrow and Maltby, 2000; Maltby et al., 2001; Amiard-Triquet, 2009).

The inclusion of feeding-based assays in the assessment of environmental effects may help prevent underestimation of the risks of contaminants at the ecosystem level. In effect, when contaminants depress feeding and growth/reproduction at similar concentrations, decisions based on time-delayed extrapolations from population growth rates to ecosystem functioning can underestimate risk, since an immediate inhibition on population consumption (equal to the individual feeding depression), and thus on ecosystem function, will also occur. When feeding inhibition occurs at concentrations inducing lethality, lethal assays may also result in risk underestimation at the ecosystem level, as surviving organisms will eat less than before. Under the latter scenarios, an accurate estimation of the effective concentrations inhibiting the population consumption requires integrating the effective concentrations causing lethality and inhibiting individual feeding (Maltby et al., 2001; Agostinho et al., 2012; Correia et al., 2013).

The small freshwater fish species *Danio rerio* Hamilton (Cyprinidae) is one of the most important model organism in many fields of research, including ecotoxicology (Hill et al., 2005; Bopp et al., 2006). Its scientific importance originated from several ecological, biological and practical attributes, namely its key role in the trophic chains of various water bodies, small size, rapid development and easy laboratory maintenance (Lawrence, 2007).

The objective of the present study was to contribute to develop a sensitive and ecologically relevant short-term laboratory/in situ assay with *D. rerio* based on feeding to evaluate secondary production impairment due to chemical contamination, for individual substances as well as complex mixtures occurring in natural waters. Firstly, a methodology to easily and precisely quantify feeding in *D. rerio* during exposure was optimized. Secondly, the sensitivity of feeding relative to survival and growth, after exposure to the reference chemical copper, was evaluated. Thirdly, the influence of environmental parameters on the background variability of feeding rates was determined. And fourthly, the integration of feeding depression and mortality was evaluated with natural contaminated waters.

2. Materials and methods

2.1. Assay organism

Zebra fish *D. rerio* juveniles were obtained from a commercial supplier, the stock quality being verified as recommended in standard guidelines for fish toxicity testing (OECD, 1992, 2000). Cultures were acclimated for at least two weeks and maintained in 50-L glass aquaria filled with dechlorinated tap water, at a temperature of 21–23 °C under a 14:10 h L:D photoperiod (OECD, 1992, 2000). Fish were fed once daily either on a commercial flake food diet (Tetramin, TetraWerk, Melle, Germany) or on live juveniles (less than 72-h old) of the freshwater cladoceran *Daphnia magna* Straus. Fish were not fed for 24 h prior to feeding quantification experiments and toxicity assays.

2.2. Feeding quantification

Although the use of nonliving food greatly simplifies estimations of feeding rates, only live food (e.g. small cladocerans) integrates all behavioral aspects of feeding (Atchison et al., 1996; Kasumyan, 2001). Under optimal conditions, fish eat voraciously all the food available (also from previous preliminary experiments with sequential small doses given to each fish; results not shown). Thus, a method to quantify feeding should allow the control fish to eat most, if not all of the prey offered to them in the shortest time possible, to maximize the discriminative power among treatments. The food source selected was live *D. magna* juveniles (less than 48-h old) obtained from laboratory cultures (for details on *D. magna* culturing see Rosa et al. (2010)).

Fish were individually transferred to 175-mL glass vials filled with 100 mL of the fish culture medium (dechlorinated tap water) and wrapped with white paper to isolate fish from external stress factors. After 1 h of acclimation, each fish was provided once a dose of ten daphnids for different feeding periods. At the end of each feeding period – 1, 3, 15, 45, and 60 min, with at least 12 replicates/feeding period – the number of remaining daphnids was immediately counted. Feeding rates were estimated as the mean number of eaten daphnids/fish/h.

2.3. Endpoint sensitivity

The sensitivity of the feeding response was investigated in the laboratory by comparing feeding rates with sublethal (growth) (OECD, 2000) and lethal endpoints (OECD, 1992), after exposure to the reference toxicant copper. Test solutions were prepared by adding the appropriate amount of dechlorinated tap water, also used as the control medium, to a stock solution of copper (100 mg L^{-1}) prepared in nanopure water (conductivity <5 μS cm^{-1}; Seralpur PRO 90 CN, Seral, Ransbach-Baumbach, Germany), using copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) (Merck, Darmstadt, Germany). Eleven nominal copper concentrations were tested for the 48-h lethality test, using a dilution factor of 1.35 (0, 13.5, 18.0, 24.5, 33.0, 45.0, 60.0, 81.5, 110, 148, and 200 μg L^{-1}). To estimate the copper concentrations inhibiting feeding, fish were exposed for 48 h to seven nominal concentrations using a dilution factor of 1.5 (0, 5.93, 8.89, 13.3, 20.0, 30.0, and 45 μg L^{-1}), followed by a 1-h acclimation period plus a 1-h feeding period, both periods in the respective test solutions, as described in the previous section. For the 28-d growth test, nine nominal concentrations of copper using a dilution factor of 1.5 (0, 2.34, 3.51, 5.27, 7.90, 11.9, 17.8, 26.7, and 40 μg L^{-1}) were used. Specific growth rates per d were estimated as the difference between the final and initial logarithmized wet weights divided by the exposure period (28 d).

The tests were carried out in transparent polyethylene terephthalate vials (8 cm inner diameter), incubated at 19–21 °C under a 14:10 h L:D light regime and isolated from the surrounding environment with a cardboard. During all tests, a source of aeration was connected to each replicate; it was started 1 h before fish introduction and stopped for 5 and 20 min to introduce the fish and allow fish to eat during the growth test, respectively. Mortality was checked every 24 h and dead fish were removed without replacing them. Water levels were adjusted daily with distilled water.

For the 48-h lethality test, four replicates per concentration with 300 mL of test solution and one fish each were used, whereas for the 48-h feeding test, three replicates per concentration with 750 mL of test solution and three fish each were set up. The mean (±standard deviation [SD], *n*) wet weight of the fish used was 0.386 (±0.064, 10) and 0.241 (±0.029, 12) g for the lethality and feeding tests, respectively. During both the lethality and feeding tests, no food was provided and no medium renewal was performed. After the 48-h exposure, mortality was recorded and, for the feeding test, individual feeding rates were determined as described above, using for each fish the respective test solution. For the 28-d growth test, fish with a mean wet weight (±SD, *n*) of 0.221 (±0.039, 12) g (between 2 and 3 cm total length) were exposed in four replicates

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