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The effects of continuous diazinon exposure on growth and reproduction in Japanese medaka using a modified Medaka Extended One Generation Reproduction Test (MEOGRT)

Kevin Flynn[a,*], Doug Lothenbach[a], Frank Whiteman[a], Dean Hammermeister[a], Joe Swintek[b], Matthew Etterson[a], Rodney Johnson[a]

[a] US Environmental Protection Agency, Mid-Continent Ecology Division (USEPA MED), 6201 Congdon Blvd, Duluth, MN 55804, USA
[b] Badger Technical Services, Duluth, MN, USA

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ABSTRACT

The Medaka Extended One Generation Reproduction Test (MEOGRT) is a Tier 2 test within U.S. Environmental Protection Agency's (USEPA) Endocrine Disruptor Screening Program (EDSP), designed to characterize the potential adverse effects to fish of exposure to chemical that can cause disruption of the endocrine system. The MEOGRT focuses primarily on adverse effects to reproduction while collecting information regarding effects on growth, survival, and endocrine-related endpoints. However, the risk assessment process for fish, as mandated by legislation such as the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) or the Toxic Substances Control Act (TSCA), could benefit from a more detailed assessment of effects on growth. Typically, fish growth data in support of risk assessment are obtained from full life-cycle tests or early life stage tests using the fathead minnow. As an alternative to these tests, a modified MEOGRT was conducted to assess the effects of diazinon on the various parameters measured in the MEOGRT. Diazinon is an organophosphate insecticide that is detected in the environment, and whose efficacy is a result of inhibition of the acetylcholine esterase enzyme at neuromuscular junctions and synapses of the nervous system. Diazinon (2.9, 5.2, 10.3, 19.8, and 40.2 μg/L) was tested with the MEOGRT protocol, and the lowest observable effect concentrations of 2.9 μg/L for fecundity and 5.2 μg/L for growth were determined. Additional growth measurements were added to the MEOGRT protocol to more robustly define growth rates and to determine the impact size has on reproductive performance. Fish size starting at the first measurement day (i.e. 21 days post-fertilization), and continuing through the duration of the test was reduced with exposure to 5.2 μg/L and higher, and asymptotic size predicted from growth modeling was reduced at 10.3 μg/L and higher. By simply adding non-destructive growth measurements at two additional time points, the MEOGRT provided enough data for the parameterization of growth models, which could be used to characterize the reproductive implications of growth impairment.

1. Introduction

The Endocrine Disruptor Screening Program (EDSP) of the U.S. Environmental Protection Agency (USEPA) has released a guideline for a Tier 2 fish test protocol to evaluate and define the dose-response of putative endocrine disrupting chemicals (EDCs) on fish reproduction and reproductive development (USEPA, 2015a). In addition, the Organisation of Economic Co-operation and Development's (OECD) Test Guidelines Program released a similar test guideline, as OECD TG 240 (OECD, 2015). The test, called the Medaka Extended One-Generation Reproduction Test (MEOGRT), is intended to provide information on the potential of a test chemical to adversely affect fish, putatively through endocrine disruption. Specifically, the MEOGRT has been designed to detect effects primarily on reproduction, and secondarily on growth, development, and survival, with additional measurements that could be sensitive to endocrine disrupting activities of the test chemical (Flynn et al., 2017a). Because many of these effects could be manifested later in life, especially at the time of reproduction, the MEOGRT encompasses parts of three generations covering effects from hatch to the transformation from juvenile life stages to sexual maturity.

As a test in the toolbox of the USEPA's EDSP and the OECD's Test Guideline Program, the MEOGRT has been successfully used to determine the effects of both model and environmentally relevant EDCs (Flynn et al., 2017a; Watanabe et al., 2017). As a test protocol intended

* Corresponding author.
E-mail address: flynn.kevin@epa.gov (K. Flynn).

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to be part of an EDC screening program, the MEOGRT was designed to have statistical power sufficient to detect an effect on reproduction (Flynn et al., 2017b), which was considered the primary route that an EDC would have effects at the population level. While growth measurements are recorded, less priority was given them compared to reproduction measurements. However, growth is an important parameter in toxicological risk assessments, and growth, or more accurately size, has been shown to be related to survival (Houde, 1997) and reproductive performance (Wootton, 1979) in fish. Some measures of size and/or growth are collected in other test protocols used for evaluating chemicals outside of the EDSP testing framework, including test protocols implemented as part of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Toxic Substances Control Act (TSCA). However, as the Tier 2 fish test within the EDSP, it may be advantageous for the MEOGRT to contain more robust measurements of growth. As published, the MEOGRT has only two time points at which growth measurements are recorded, once as juveniles and once as adults. To make the MEOGRT more robust for testing more diverse classes of chemicals that, at chronic levels primarily affect growth rather than directly affecting reproduction, non-destructive growth measurements were added to the protocol. The current work was initiated to determine the effectiveness of the modified MEOGRT protocol to evaluate the reproductive and growth hazard of a chemical that is not considered an EDC (i.e. no estrogen, androgen, or thyroid activity).

In support of some risk assessments, fish full life-cycle tests are performed with the fathead minnow, *Pimephales promelas*. When the test is performed according to the published guidelines (USEPA, 1996), the test is relatively long (approximately 5–6 months), and the reproduction and growth data produced is often highly variable given the low number of replicates typically used in the study, making it difficult to make reliable inferences regarding the effects of the test chemical on growth and reproduction, especially in the context of a dose-response relationship. Short-term tests that measure only mortality and growth in fish (e.g., OCSPP 850.1075 and OCSPP 850.1400) and, therefore, are much less expensive and time consuming, are more often the tests conducted as part of a risk assessment. The limitations and challenges of conducting the existing full life cycle tests provide additional incentive to ascertain the ability of a slightly modified MEOGRT to provide data that could meet the needs of chemical risk assessment.

Diazinon is an organophosphate (OP) insecticide used on orchards (almonds, stone fruit, and pome fruit), ground fruit, some vegetable crops, and outdoor nurseries that is registered under FIFRA and can affect fish growth (Jarvinen and Tanner, 1982), but does not appear to affect reproduction directly as an EDC. Therefore, a test with diazinon can provide a good demonstration of the potential of the MEOGRT protocol to define the dose-response of a chemical that does not interact with the estrogen, androgen, or thyroid pathways (USEPA, 2015b), but would still be expected to impact growth or survival, and perhaps reproduction through a non-endocrine mechanism. Diazinon has been detected in the aquatic environment (Bailey et al., 2000; Dubus et al., 2000). While use of diazinon has steadily declined in the U.S. since being phased out of residential use, indoors in 2002 and outdoors in 2004, the total annual agricultural use in fruit, vegetable, nut, and ornamental production is still approximately 100 metric tons (Ryberg and Gilliom, 2015). Therefore, diazinon remains a potential contaminant of some U.S. surface waters.

Diazinon, or more accurately its oxon metabolite, diazoxon, inhibits acetylcholinesterase (AChE) at the synaptic cleft of neuromuscular junctions (Fukuto, 1990) and cholinergic synapses in the nervous system (Anasari et al., 1987). Fish are susceptible to the toxic effects of diazinon as the molecular target (i.e. AChE) is present in many fish tissues, and fish, via P450 enzymes, metabolize diazinon to the active diazoxon (Hamm et al., 2001; Keizer et al., 1991).

While there are several published studies detailing acute effects of diazinon exposure to fish (Allison and Hermanutz, 1977; Jarvinen and Tanner, 1982; Palacio et al., 2002) and there are tests with durations longer than typical acute tests (Dutta and Meijer, 2003; Norberg-King, 1989; Oruc et al., 2006), there are relatively few studies detailing effects of lifecycle exposures. Therefore, there is interest in determining the extent of effects on growth and reproduction in fish chronically exposed to low-doses of diazinon. The current study was undertaken for two primary reasons: 1) to provide information on the ability of a modified MEOGRT to define the effects of a non-EDC (in this case, diazinon) on growth, and 2) to specifically provide effects information from a chronic diazinon exposure of fish.

2. Material and methods

2.1. Chemical information, measurement, and dilution

Diazinon (CAS number: 333-41-5) was purchased from RIA International (East Hanover, NJ, USA) at greater than 97% purity. A continuous flow diluter system was used to deliver the final test concentration to each aquaria. Diazinon stock solutions at a concentration of 37 mg/L were diluted with Lake Superior water (LSW) first to 190 µg/L. This solution was further diluted with LSW to each of the test concentrations (nominally, 40, 20, 10, 5, and 2.5 µg/L, plus a 0 µg/L control), which were then delivered directly to the appropriate test aquaria by peristaltic pumps. All dilutions were accomplished using proportional metering pumps (Q2V Ratio:Matic; Fluid Metering, Inc., Syosset, NY, USA).

Diazinon concentrations in stock solutions and test aquaria were determined on a weekly basis by HPLC (1200 Series; Agilent Technologies, Santa Clara, CA, USA) using a reverse phase LC column (Synergi 2.5 µm Max-RP; Phenomenex, Torrance, CA, USA) with acetonitrile and water as the mobile phase and UV detection at 206 nm. In addition, two diazinon fortified matrix samples, both procedural and matrix blanks, and technical duplicates were analyzed. Diazinon recoveries from the fortified samples were always between 80% and 120%, blanks were always below the method detection limit (0.4 µg/L), samples for test aquaria were above level of quantification (1 µg/L), and technical duplicates had a relative percent difference less than 20%.

2.2. Test fish, test timeline and protocol

Japanese medaka (*Oryzias latipes*) were obtained from an on-site culture unit at the USEPA Mid-Continent Ecology Division (MED; Duluth, MN, USA). Pairs of fish that were 12 weeks post fertilization (wpf), met the size requirements (females ≥ 300 mg, males ≥ 250 mg, and individual weights, by sex, were within ± 20% of the mean weight), and fecundity requirements (mean daily fecundity of greater than 20 eggs per day) of the guideline for inclusion in a MEOGRT (USEPA, 2015a) were used to initiate the test. Fish were reared on a 16:8 h light:dark photoperiod and were fed less than 24-h post-hatch *Artemia nauplii,* twice a day. The amount fed was increased through time, based upon historical control growth rates, to provide ad libitum food. All laboratory procedures involving medaka were reviewed and approved by the Animal Care and Use Committee of the USEPA MED in accordance with the Animal Welfare Act and Interagency Research Animal Committee guidelines.

Essentially, the published MEOGRT guideline (OSCPP 890.2200) was followed (USEPA, 2015a). Briefly, the MEOGRT is a multi-generation test used to characterize the likelihood, nature, and concentration-response relationship of endocrine disrupting chemicals on fish. The test begins by exposing breeding pairs of adult medaka (F0 generation), one pair per aquarium. The exposure continues through development and successful reproduction by the F1 generation, and is terminated upon the hatch of embryos constituting the F2 generation. At various times during the MEOGRT, endpoint measurements are taken, including fecundity, fertility, hatching, survival, weight, length, anal fin papillae, vitellogenin gene expression, and histopathology (Supplemental Fig. 1). The reproductive data (fecundity and fertility) is