Aquatic Toxicology 95 (2009) 173–181

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Developmental effects of a municipal wastewater effluent on two generations of the fathead minnow, *Pimephales promelas*

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ARTICLE INFO

Article history:
Received 11 May 2009
Received in revised form 19 August 2009
Accepted 23 August 2009

Keywords:
Wastewater effluent
Endocrine disruption
Fathead minnow

ABSTRACT

Municipal wastewater effluents have been shown to contain a variety of anthropogenic compounds, many of which are known to display estrogenic properties. While multiple laboratory studies have shown the effects of such compounds on an individual basis at elevated concentrations, little research has attempted to characterize the effects of exposure to environmentally relevant mixtures of estrogenic compounds. The current study examined the effects of long-term exposure to graded concentrations (0, 50, 100%) of wastewater effluent on the fathead minnow, *Pimephales promelas*. The F1 generation was cultured in control water to test for transgenerational effects from parental exposure to wastewater effluent. Total estrogenic activity in the wastewater was determined to be approximately 1.7 ng/L 17β-estradiol equivalents. Survival, growth, and reproduction in the parent generation were not affected by exposure to the wastewater treatments. An increase in the gonadosomatic index and a reduction in the expression of secondary sex characteristics in male fathead minnows exposed to 100% wastewater in the parent generation were observed. Conversely, the expression of secondary sex characteristics was greater in males from the F1 generation of wastewater-exposed parents. Additionally, a positive relationship between parental exposure to wastewater and the onset of reproductive activity in the F1 generation was observed. Results of this study suggest that exposure to wastewater effluent did not pose a significant threat to the successful growth, development, and reproduction of the fathead minnow. Early onset of reproductive activity observed in the F1 generation of wastewater-exposed parents in subsequent generations should be studied further.

1. Introduction

Endocrine disrupting compounds (EDCs) can be defined as exogenous substances that disrupt endocrine system-mediated physiological functions. One of the major pathways for EDCs to enter the aquatic environment is through municipal wastewater effluents. Effluents around the world have been shown to contain measurable concentrations of pharmaceuticals, personal care products, and natural and synthetic hormones that are capable of acting as EDCs (Nakada et al., 2004, 2006; Snyder, 2008). A survey conducted in the United States between 1999 and 2000 by the United States Geological Survey that targeted areas downstream of urbanization and/or livestock production found the presence of organic wastewater-derived contaminants, many of which are known EDCs, in 80% of 139 surface waters (Kolpin et al., 2002).

The presence of estrogenic compounds in wastewater effluents is a potential risk to aquatic ecosystems and has been studied extensively (Byrns, 2001; Murk et al., 2002; Nakada et al., 2004; Servos et al., 2005). Estrogenic compounds of interest include the natural estrogen, 17-β estradiol (E2); the synthetic oral contraceptive-derived estrogen, 17-α ethynylestradiol (EE2); bisphenol-A (BPA), which is a component of polycarbonate plastics; and a class of industrial compounds known as the alkylphenols, which include nonylphenol (NP) and octylphenol (OP). The estrogen receptor (ER) affinities of E2 and EE2 are orders of magnitude higher than those of BPA, NP, and OP (Rutishauser et al., 2004), although in some wastewater effluents such industrial compounds may reach concentrations in the parts per billion range making them relevant to study as endocrine disruptors despite their low ER affinities.

In addition to studying the occurrence of estrogenic EDCs, a great deal of research has focused on their effects on aquatic organisms. The developmental and reproductive status of EDC-exposed fish species has been studied in detail. Altered secondary sexual characteristics as well as a decrease in reproductive success have been observed in fathead minnows exposed to EE2 (Parrott and Blunt,

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doi:10.1016/j.aquatox.2009.08.012

2005) and alkylphenolethoxylates (Bistodeau et al., 2006). Intersex fish, defined as individuals containing both male and female gonadal tissue, have been sampled downstream from wastewater effluent discharge sites (Jobling et al., 1998; Woodling et al., 2006; Vajda et al., 2008). Laboratory dosing studies have also revealed relationships between exposure to estrogenic EDCs and an increased intersex incidence (Blazquez et al., 1998; Seki et al., 2002; Orn et al., 2003). Kidd et al. (2007) observed a near extinction of a fathead minnow population over a seven-year period in an experimental lake after exposure to an environmentally relevant concentration of EE2. This near extinction coincided with an increase in male vitellogenin production, an increase in male intersex gonads, and an altered female oogenesis.

While the effects of EDC exposure to fish have been studied thoroughly, the existing knowledge base has a few limitations. Much of the work characterizing the effects of EDC exposure on wild caught fish lacks data regarding the exposure history of the sampled organisms. In addition, much of the laboratory EDC work has either been conducted at concentrations greater than those found in the environment or has not adequately addressed the issue of complex mixture exposures that are likely to be found in the environment. Also, not much is known about the responses of F1 generation fish after exposure to the parent generation. Recent research using mammalian models has shown the potential for EDCs to elicit altered sexual development in subsequent generations after parental exposure (Anway et al., 2005, 2006; Anway and Skinner, 2006, 2008; Chang et al., 2006). The potential for EDCs to cause such transgenerational developmental effects in teleost fish models has not been adequately studied; therefore, the evolutionary significance of endocrine disruption in teleost fish is not well understood and warrants further study. The goal of this study was to characterize the responses of laboratory-cultured fathead minnows, *Pimephales promelas*, to wastewater effluent exposure and subsequently examine the development of male fish in the F1 generation of an exposed parental generation.

2. Materials and methods

2.1. Solution preparation

Reconstituted moderately hard water (nominal hardness and alkalinity of 80 and 60 mg/L as $CaCO_3$, respectively) was made from 18 MΩ water and reagent grade salts. Final wastewater effluent was collected after chlorination and before reaching the outfall at the Mauldin Road wastewater treatment plant in Greenville, SC, USA and transported to the Clemson University Institute of Environmental Toxicology (CU-ENTOX) in Pendleton, SC, USA. The effluent was diluted with moderately hard water to produce treatments of 0, 50, and 100% wastewater.

2.2. Mauldin Road wastewater treatment plant

The Mauldin Road wastewater treatment plant in Greenville, SC, USA, has a drainage area of 66 square miles with a maximum daily flow of 45 million gallons per day (MGD). Approximately 37,000 residences comprise the service area. The average daily plant flow is 16.5 MGD, of which 2.5 MGD comes from industrial sources (Dr. Stephen Graef, personal communication, 4/1/09).

2.3. Experimental setup

Nine-hundred 24–36 h old larval fathead minnows were taken from an in-house culture at CU-ENTOX, divided into groups of 100, and stocked into glass dishes containing 2 L of test solution. Each treatment was replicated three times and each dish contained 100 organisms. Larval fish were fed brine shrimp twice daily. Test solutions were renewed 100% twice a week with fresh wastewater effluent collected on the day of the renewal. Partial renewals (33% of total volume) were conducted daily using excess effluent from the most recent collection event and fresh control water. Test organisms were maintained in a climate controlled test room at CU-ENTOX on a 16:8 light:dark cycle and were continuously aerated. Treatment mean water temperature, pH, and dissolved oxygen ranged from 22.32 to 22.42 °C, 7.80 to 8.07, and 7.21 to 7.32 mg/L, respectively.

On day 7 of the experiment, 10 organisms were sampled from each replicate and frozen at −80 °C for later analysis. On day 24 of the experiment, test organisms were moved to 30 L glass aquaria containing 10 L of test solution and the food source was changed to Tetramin™ commercial fish food. As the test organisms grew, test solution volumes were gradually increased to a maximum of 20 L. On day 68, replicates were thinned to 25 fish apiece and two breeding tiles were placed in each aquarium. Mortality up to day 68 was recorded. Egg production was quantified until day 145. On day 145, male secondary sexual characteristics (SSCs) were assessed following the methods of Parrott et al. (2003). Banding strength (0–3 points) and the presence of nuptial tubercles, dorsal fat pad, and dorsal fin dot (1 point each) were assessed for the two males in each tank who had established dominance and successfully guarded a nesting site (breeding tile) to create a subjective index score ranging from 0 to 6. These dominant males also possessed the most prominent SSCs in each tank. At this time, four males and eight females were removed from each tank and placed in separate 30 L aquaria for an isolated breeding experiment. Males displaying SSCs and females displaying distinct ovipositors were selected. The four males and eight females that were selected from each tank were divided into two groups, each containing two males and four females. One group was stocked in a breeding tank containing the same test solution in which they had previously been exposed. The other group was placed in a test solution that differed from their previous exposure and went as follows: fish from the control treatment were placed in 100% effluent, while fish from the 100 and 50% effluent treatments were placed in control solutions (Fig. 1). Organisms were allowed to acclimate to the breeding tanks for 48 h after which two breeding tiles were placed in each tank. Over the next 14 days, tiles were checked for eggs three times daily. Egg production was recorded as the total number of eggs laid per female over the 14 days. At the conclusion of the breeding experiment, all adult organisms were euthanized. Survival at the end of the experiment was recorded. Total lengths and weights were measured and used to calculate a condition factor [(weight/length^3) × 10^5]. Ovipositor size in females was measured. Organisms were sexed internally and gonads were weighed to calculate a gonadosomatic index (GSI) [(gonad weight/total weight) × 100] before being fixed in 10% neutral-buffered formalin for histological analysis. Livers were weighed to determine a hepatosomatic index (HSI) [(liver weight/total weight) × 100]. A small number of individuals (five in the control treatment, three each in 50 and 100% treatments) were determined to be immature individuals. These individuals were not used in the analysis of growth and development endpoints or during the isolated breeding experiment. Fish that spawned in the test solution that differed from their original treatment were not used for HSI or GSI analysis.

During the final 48 h of the isolated breeding experiment, eggs from all tanks were collected, pooled by treatment, and allowed to hatch in moderately hard water. At 10–11 days post-hatch, F1 generation larval organisms from each treatment were split into three replicates, each containing 50 organisms, and placed in 30 L glass aquaria containing moderately hard water. F1 treatment groups were labeled to correspond with the parent breeding group that produced them (i.e. 0 M, 0 W, etc.) (Fig. 1). On day 93 of the F1 generation experiment, all tanks were thinned to 30 organisms and