ASSESSING ESTROGENIC COMPOUND EXPOSURES: TIME TO INDUCTION OF THE VITELLOGENIN PROTEIN IN MALE FATHEAD MINNOWS EXPOSED TO A COMPLEX WASTEWATER EFFLUENT

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Introduction

Concerns about the potential of endocrine disruption in wildlife or human populations have been raised in the popular press, debated in the scientific literature, and have focused resources on research on understanding the problem and assessing its impact. The concerns raised by these studies led the US Congress to mandate in both the Safe Drinking Water Act of 1996 and the Food Quality Protection Act of 1996 that EPA develop a strategy for screening and testing potential EDCs for adverse effects resulting from endocrine disruption. Numerous workshops and symposia have been held by EPA and others that have reviewed the extent of the problem and recommended the approaches that should be taken to meet these mandates for evaluating chemicals for potential endocrine disruption [1-5]. Vitellogenin induction in fathead minnows is one of the endpoints in the assays being developed by EPA.

In oviparous animals, females produce vitellogenin (Vtg) in response to the production of endogenous estradiol. Vtg is a precursor protein to egg yolk production. While the gene for vtg expression is present in males, it is usually silent unless induced by exposure to exogenous estrogen or estrogenic substances. Because Vtg synthesis is directly related to the estrogen cascade pathway, it is a direct measure of biological activity resulting from exposure to estrogen or an estrogen mimic and thus is an excellent bioindicator of environmental estrogen exposure [6, 7].

Vtg induction in male fishes has become a widely used indicator of endocrine disruption [1]. Vtg induction has been reported in male fish downstream of sewage treatment plants (STP) effluents in the UK [6, 8-11]. The first report of Vtg induction in wild fish in the US was by Folmar et al. [12] who found elevated levels in males downstream but not upstream of Minneapolis-St. Paul in the Mississippi River. The US Geological Survey has found Vtg induction in fish from a number of U.S. rivers [13]. The British researchers used Toxicity Identification Evaluation (TIE) to isolate the chemicals most associated with induction, and concluded that 17ß-estradiol, estrone, and the synthetic hormone 17α-ethynylestradiol were the likely causes of induction in the STP effluent [6]. But induction from exposure to other point sources besides STPs having a different mixture of estrogenic compounds has not been well studied.

Because many ubiquitous environmental estrogens are common to STP effluent, an assay to assess effluent estrogenicity is desired. Such an exposure assay needs to be sensitive and efficient. While *in vitro* assays are faster and less expensive, *in vivo* assays are more integrative in nature, and account for the pharmacokinetics and pharmacodynamics in the organism [1]. This study explored the time required to induce Vtg in male fathead minnows exposed to a sewage treatment plant effluent in Duluth, Minnesota as the first step towards an exposure assay.

Methods and Materials

Fish exposures were carried out at the Western Lake Superior Sanitary District (WLSSD), Duluth, MN. WLSSD influent is collected from a 50-mile radius and is composed of 50 % municipal waste and

50% industrial waste. Wastewater influent is processed to the level of secondary treatment (activated sludge) and followed by an anti-fouling gravel filter and seasonal chlorination/dechlorination. WLSSD treats an average of 43-million gallons of wastewater per day discharging its effluent into the Duluth/ Superior harbor located in the western arm of Lake Superior of the Great Lakes.

Sixty-six, 5-month old, male fathead minnows (4-8 g w/w) were exposed to WLSSD effluent in August – September 2001 using a flow-through exposure apparatus. All fish were held under a constant 16:8 light:dark photoperiod at 25 °C and fed a constant ration of frozen brine shrimp twice daily for the duration of the experiment. Fatheads were exposed in three 40-L aerated aquaria (22-fish/tank) at a flow rate of 200-mL/min. Effluent exposed fatheads were then sub-sampled on exposure days 4, 7, 14, 21 and 28 to determine the level of circulating plasma Vtg. On each sub-sampling day four fish from each aquaria were removed and sacrificed preserving blood plasma for Vtg analysis. Blood was collected from anesthetized fish via the caudal vein in heparinized hematocrit tubes and spun at 10,000-rpm to separate out the plasma fraction. Separated plasma was then stored at –80 °C in aprotinin coated centrifuge vials. Plasma Vtg was measured using an enzyme-linked immunosorbent assay (ELISA) as described by Parks et al. 1999.

Plasma Vtg levels were then compared between effluent exposed fish and both a positive and negative control housed at USEPA-MED, Duluth MN. Both positive and negative controls consisted of 25 fish from the same brood as the effluent exposed fish and each was maintained in one 40-L aquarium throughout the experiment. Positive control consisted of a single IP injection of 5-mg/kg of 17ß-estradiol administered at exposure day zero. Both positive and negative control fish were maintained in Lake Superior lake water. Both positive and negative control fish were sub-sampled on the same days as the exposed fish (n=5).

Exposure effluent was analyzed concurrently for chemical composition using solid phase XAD-2 absorptive resin and gas chromatographic-mass spectrometry (GCMS) on exposure day 1, 21, and 28. XAD-2 resin was extracted with Soxhlet apparatus with acetone for 8-hrs followed by dichloromethane for 20-hrs, and the extracts solvent reduced and exchanged to methanol. Samples were analyzed on a DB5-MS column using a Hewlett Packard 5970 GCMS and monitoring selected ions for 17ß-estradiol (E2), 17α-ethinyl estradiol (EE2), estrone (E1), nonylphenol (NP) and octylphenol (OP).

Results and Discussion

Figure 1 shows the mean concentration of Vtg (mg/mL) in plasma of the induced fish at various times over the experiment. The concentrations in the positive control showed elevated vtg at the first time point, peaked in concentration at day 7, and then stayed at about 20 mg/mL for the remainder of the experiment. The negative controls had no measurable Vtg. The effluent exposed male fathead minnows had measurable but very low levels of Vtg until day 14 when levels were comparable to the positive controls.

The fraction of subsampled fish with significantly elevated vtg concentrations at each time point is shown in Figure 2. The top graph shows the effluent exposed fish, and the bottom graph shows the two controls. After just 4 days 50 % of the effluent exposed fish were induced, and the fraction of fish showing induction increased in a time-dependent manner to 80 % (days 7 and 10) and to 100 % by day 21. As expected, 100 % of the positive control fish were induced at the first time point, and one fish from the negative control (20 %) showed significant vtg induction at day 4 and day at day 28.

The chemical analyses of the effluent consistently indicated that the concentrations were very low (< 1 ng/L for all analytes). Thus the cause of the Vtg induction does not seem to be directly related to these chemicals alone.

These results indicate that this effluent is very estrogenic, in spite of the low levels of the target analytes. More importantly, this research demonstrated that the use of fathead minnows to monitor this

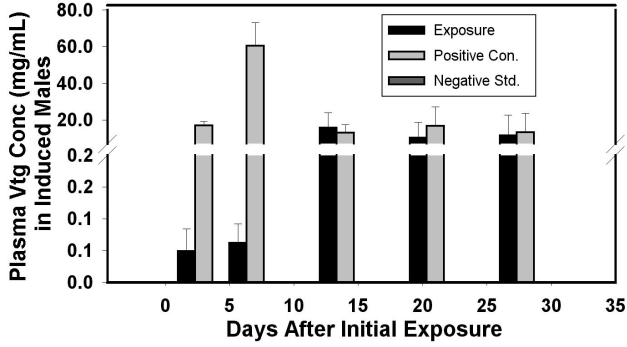

Figure 1.

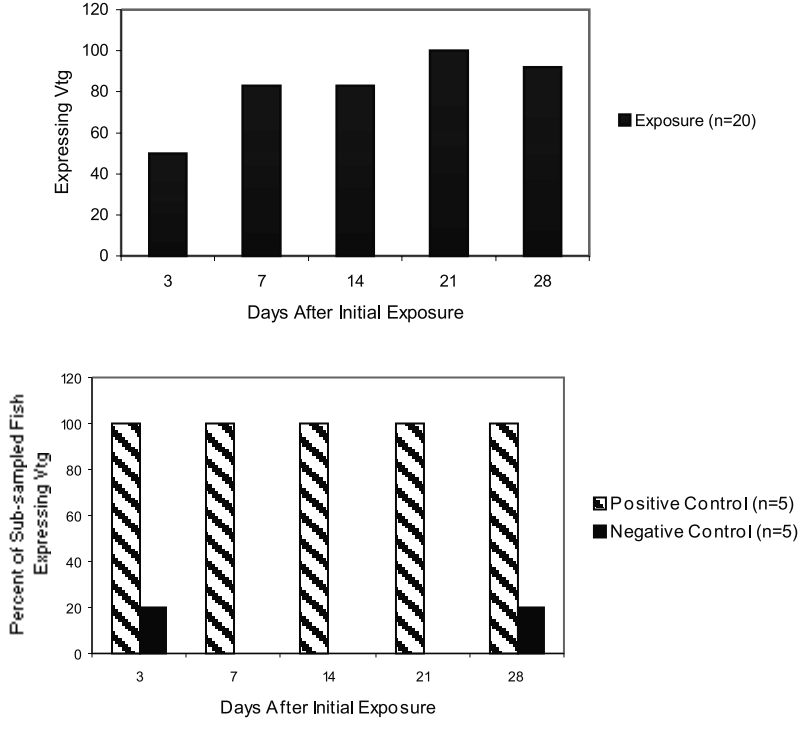

Figure 2A and B.

estrogenicity is sensitive, and that it occurs in a time frame that can be utilized by treatment facility managers. The overall impact of the presence of estrogenic compounds in the effluent on reproduction in the fathead minnow is not known.

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