TOXICOLOGICAL EVALUATION OF SOME SEWAGE EFFLUENTS WITH THE AID OF THE ZEBRA FISH SEXUAL DEVELOPMENT TEST

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ABSTRACT

Aquatic organisms such as fish are usually exposed to complex mixtures of environmental pollutants such as sewage effluents in the aquatic milieu. Several adverse effects such as endocrine disruption, impaired reproductive abilities of aquatic organisms, genotoxicity and immunotoxicity have been attributed to the presence of estrogenic chemicals in sewage effluents from sewage treatment plants (STPs). The major aim of the investigation was to evaluate the toxicity of the sewage effluents obtained from different treatment processes (A1- A7) at a STP situated in Stockholm, Sweden. The sewage effluents were evaluated with a fish sexual development test and zebra fish (Danio rerio) was used as a model test species. Sewage effluents A2 (After sedimentation treatment), A3 (Outlet L1) and A4 (Biofilter) evoked significant increments in vitellogenin concentration in the exposed fish compared to the controls. In addition, there were more females in the groups exposed to sewage effluents A2 (45%), A3 (46%), A4 (47%) and in the internal control A8 (46%) compared with control group A1 [clean reference water, 35%]. The results indicated that the treatment techniques at the STP were incapable of completely reducing the levels of estrogens and perhaps other aquatic pollutants in the sewage effluents. The treatment techniques should be improved upon in order to ensure successful proliferation of aquatic organisms.

Keywords: Estrogens, Fish Sexual Development Test, Sewage effluents, Toxicity, Vitellogenin, Zebra fish

INTRODUCTION

Aquatic organisms are usually exposed to complex chemical mixtures, such as effluents from industrial, agricultural and domestic sources throughout their lifespan [1]. Endocrine disrupting chemicals (EDCs), such as estrogens, phthalates, alkylphenols and bisphenol A have been detected in sewage effluents [2,3]. These compounds simulate or inhibit the transcriptional activation evoked by naturally circulating steroid hormones by binding to steroid hormone receptors [4]. Also, EDCs influence homeostasis, developmental processes and reproductive behaviour in living organisms, including humans [5]. Furthermore, EDCs increase or inhibit the metabolism of naturally occurring steroid hormones and other xenobiotics by activating or antagonizing nuclear hormone receptors. They exert genome-wide effects on DNA methylation status and alter lipid metabolism and adipose tissue formation [4].

The natural estrogens, 17β-estradiol (E2) and estrone (E1) and the synthetic contraceptive pill hormone, 17α ethinyl estradiol (EE2) have been identified as the main estrogenic substances that occur in sewage effluents [6]. These estrogens and their conjugates are eliminated in human faeces and urine and they may contribute to the estrogenic effects of sewage effluents [7]. In addition, sewage effluents have been observed to be estrogenic to fish according to several reports [7,8]. The estrogenic chemical, EE2, evoked vitellogenin induction, alterations in sex ratios, suppression of gonadal development and intersexuality in fish in some studies [9,10].
The zebra fish (\textit{Danio rerio}) is a gonochoristic, undifferentiated species and both sexes exist in an ovary-like stage \cite{11,12}. Male zebra fish initially exist as hermaphrodites prior to differentiation into the phenotypic sex and they are vulnerable to hormonal perturbations during this period. The ovaries undergo development in about 50\% of the population while in the rest, ovarian tissue regression and testicular tissue proliferation occurs \cite{13}. The transformation of ovaries into testes in zebra fish might be attributed to apoptosis \cite{11}.

The aim of the study was to evaluate the toxicity of the sewage effluents obtained from the treatment processes (A1-A7) at the sewage treatment plant (STP) situated in Stockholm, Sweden. This was conducted with the aid of a fish sexual development test (FSDT).

**MATERIALS AND METHODS**

**The Effluents Evaluated**

The effluents were obtained from the experimental sewage treatment plant (STP) situated in Stockholm, Sweden. The first and second lines had activated sludge and a membrane reactor, respectively. The main treatment processes for Lines 3 and 4 were anaerobic in nature. The investigation was based on the sewage effluents obtained from Lines 1 and 2. Sewage effluents A2 - A6 were obtained from Line 1 while effluent A7 was obtained from Line 2. A1 served as the clean reference water and control for the sewage effluents while A8 (standardized laboratory water) served as the internal control.

Effluent A2 was subjected to ‘After sedimentation’ treatment while effluent A3 was the outgoing water from Line 1. Effluent A4 had a sandfilter and a biofilter in combination with the ‘After sedimentation’ treatment while A5 had a sandfilter and an ozonation step in addition to the ‘After sedimentation’ treatment. Effluent A6 had a sand filter, ozone and a biofilter in addition to the ‘After sedimentation’ treatment. Effluent A7 was the outgoing water from Line 2 and it consisted of a filtering drum and a membrane bioreactor.

The effluents were transported to the Division of Pathology, Pharmacology and Toxicology, Swedish University of Agricultural Sciences, Uppsala, Sweden, in 25 liters plastic containers every 72 hours during the 21-day experimental period. They were frozen and stored at -20\(^{\circ}\)C and the effluents were thawed overnight at room temperature before use in the laboratory.

**Experimental Animals**

A local supplier in Uppsala, Sweden supplied the adult zebra fish (\textit{Danio rerio}) used for the investigation. The test organisms were acclimatized for 4 weeks before the investigation was conducted. The experimental set up was situated in the Division of Pathology, Pharmacology and Toxicology, Swedish University of Agricultural Sciences, Uppsala, Sweden.

The test organisms were kept in aquaria within a laboratory at 26 \pm 1\(^{\circ}\)C and a 12- h light / dark cycle was maintained. Standardised water for rearing the fish was prepared from deionised water and calcium chloride dehydrate (117.6 mg/l), magnesium sulphate heptahydrate (49.3 mg/l), sodium bicarbonate (25.9 mg/l) and potassium chloride (2.3 mg/l) (Sigma Aldrich Sweden AB) were added. The adult zebra fish were maintained on a diet of commercial flake food (Sera\textsuperscript{\textregistered}) and freeze dried chironomids (Nutrafin\textsuperscript{\textregistered}) 2-3 times daily.

**Evaluation of Toxicity**

Adult males and females were transferred to cone shaped breeding funnels for spawning. The funnels were filled with 20-25 liters of standardised water. Adult male and female fish were placed in a ratio of 2:1. Spawning substrates (glass marbles) were placed in the reproduction funnels in order to enhance breeding. The eggs in the breeding funnels were collected 24 hours later between 30 and 60 minutes after light was turned on in the laboratory. The eggs were subsequently transferred into a 10 liter aquarium containing standardized water.

The embryos were raised until 20 days post hatch after which they were transferred to 8-liter aquaria containing the different sewage effluents (A1-A7). The zebra fish juveniles were exposed to the sewage effluents and standardized water (A8) from 20-60 days post hatch. Each group (A1-A8) consisted of two replicates (A and B) with 40
juveniles each. The fish were fed with commercial flake food (Sera®, Tetra®) and freeze-dried red grubs 2-3 times daily.

Exposure was through a flow through system with a 25% daily renewal of the exposure media and this was maintained with the aid of a multichannel peristaltic pump (Ismatec®, Zurich, Switzerland). The effluents were pumped from 25 - liter plastic containers through glass capillaries connected with silicon tubings to the aquaria. Standardised water was pumped from a stainless steel tank. The experiment was conducted at 26°C and in a 12-h light /dark cycle.

At 60 days post hatch, eight males were sampled from each replicate for vitellogenin analysis (as described below). The remaining fish were euthanized in tricaine methane sulfonate (MS 222) (1g/L) and then fixed and processed for sex determination (as described below).

**Vitellogenin Analysis**

The heads and tails of the sampled fish were cut, weighed and placed in 1.5 ml eppendorf tubes. The heads and tails were removed for vitellogenin analysis while the bodies were used for the confirmation of the sex of each fish. Subsequently, the head and tail samples were cut, weighed, placed individually in 1.5 ml eppendorf tubes and then immediately frozen in liquid nitrogen and stored at - 80°C. The sampled fish were homogenized individually in buffer Tris-HCl (Tris-Ultra Pure® (ICN, Denmark) pH 7.4 + 1% Protease inhibitor cocktail, Sigma®) with the aid of a manual homogenizer.

The homogenate was centrifuged at 13000 x g at 4° C for 30 minutes and the supernatant below the fat layer was collected to determine the vitellogenin concentration of each fish. The supernatants were aliquotted and frozen at - 80°C. The measurement of vitellogenin was conducted by using a commercially available precoated vitellogenin ELISA kit (Biosense laboratories®, Norway).

Purified vitellogenin from zebrafish was used as a standard. The procedure was conducted according to the manufacturer’s instructions. The absorbance was measured using a microtiter plate reader (Lab systems Multiskan MS, Finland) and the concentration of vitellogenin in each fish was calculated.

**Histological Preparation and Evaluation for determination of Sex - Ratios**

The specimens (the bodies of the fish) intended for histological evaluation were placed in individually labelled plastic cassettes. After dehydration in 70% absolute ethanol, the specimens were treated with xylene and finally embedded in paraffin.

Each paraffin block contained 6-10 individuals. The paraffin blocks were sectioned longitudinally in a dorsal-ventral position. The paraffin blocks containing the tissues were sectioned on a microtome at about 3 - 5 microns thin sections. The sections were transferred to glass slides and placed on a heating plate for one hour in order to allow them to settle by drying. The sections were deparaffinised with xylene and rehydrated using a graded series of ethanol and finally tap water was added in order to prepare the sections for staining with haematoxylin and eosin.

Following staining, the sections were dehydrated again in ethanol and xylene and then mounted with cover slips. Subsequently, the determination of the sex-ratios of the zebra fish sections were conducted based on the presence of oocytes (female) and spermatozoa (males).

**STATISTICS**

The sex ratios were tested for differences between exposed groups (A2-A7) and controls A1 and A8 using the Chi square test, with subsequent Bonferroni - Holm adjustment of p-values. Differences in vitellogenin concentrations between exposed groups and controls were tested using the non-parametric Mann - Whitney U test. The software used for analyzing the data were Statview 5.0.1 (SAS Institute Inc.) and MINITAB release 14 (Minitab Inc.). The level of significance was set at 0.95 (p < 0.05). Data was presented as mean ± standard deviation (SD).
RESULTS

Significant increases in vitellogenin concentrations were observed in zebra fish exposed to sewage effluents A2, A3 and A4 (Figure 1). There were no differences in sex ratios between the exposed groups and the control groups (Figure 2). However, there were more females in the groups exposed to sewage effluents A2, A3 and A4 (45-47%) and in the internal control A8 (46%) compared with control A1 (35%). There were no intersex fish in all the groups.

![Vitellogenin concentrations graph](image1)

**Figure 1.** Mean (± S.D.) vitellogenin concentrations at 60 days post hatch (dph) in zebrafish exposed to sewage effluents (A1- A7). A8 was used as an internal control. ** indicate significant differences at p < 0.01 level.

![Sex ratio graph](image2)

**Figure 2.** Mean percentages of females in groups of zebrafish exposed from 20 to 60 days post-hatch to different sewage effluents (A1 - A7). A8 was used as an internal control.

DISCUSSION

The period of 20 to 60 days post hatch in the zebra fish (*Danio rerio*) is regarded as a very sensitive period during which exposure to endocrine disrupting chemicals (EDCs) is likely to elicit effects on sensitive endpoints such as vitellogenin induction and sex ratios [14]. Vitellogenin (Vtg) is a large serum phosphoglycolipoprotein that is
synthesized by the liver of many oviparous organisms such as fish. The gene for Vtg exists in the liver of both females and males and it undergoes activation after exposure to estrogen [15]. Following exposure to estrogens, both estrogen receptors and Vtg genes undergo instantaneous stimulation in the liver [16].

In addition, Vtg is transported via the blood to the gonads, where it is processed into lipovitellin and phosvitin, the nutrient sources of the developing embryo [17]. The synthesis of Vtg is regulated by the endocrine system through the hypothalamus-pituitary-gonadal – liver axis, 17-β estradiol and testosterone. Levels of Vtg in the range of 10-20 mg/ml have been detected at peak activity in the plasma of females [18, 19]. It is noteworthy that Vtg levels in male or juvenile fish are minimal.

However, in our investigation, Vtg was significantly induced in males exposed to the sewage effluents A2 (after sedimentation treatment), A3 (outlet 1) and A4 (biofilter). The induction of Vtg in male or juvenile fish has been identified as an effective biomarker for the detection of estrogenic aquatic contaminants [20, 21, 22] and the Vtg induction detected in our investigation might be attributed to the presence of estrogens and other EDCs in the sewage effluents [23, 24].

Various enzyme-linked immunosorbent assays (ELISAs) have been developed to detect Vtg in fish [1]. They are based on the application of polyclonal antibodies against Vtg [25, 26], lipovitellin [27] or a mixture of monoclonal and polyclonal antibodies against Vtg [28]. In addition, Vtg has been measured in liver homogenates and whole-body fish [16, 27].

In some studies, elevated levels of Vtg were detected in fish placed in cages downstream of sewage treatment works (STW) [28, 29]. Also, increased Vtg production was detected in wild or caged fish following exposure to STW effluents [7,8]. The observations in these studies substantiate our findings and suggest that sewage effluents are sources of EDCs that are capable of inducing Vtg.

Furthermore, sophisticated techniques such as ozonation, membrane filtration and activated carbon have been identified as efficient treatment processes for the elimination of environmental pollutants such as EDCs in sewage treatment plants [29]. Ozonation is regarded as a useful method for the attenuation of the toxic effects of sewage effluents [30]. In the current study, the results indicate that the ozone sewage treatment processes (A5 and A6) might be efficient solutions since the sewage effluents that emanated from them did not elicit toxicity in the test organisms.

CONCLUSION AND RECOMMENDATION

Sewage effluents A2 (After sedimentation), A3 (Outlet 1) and A4 (Bio filter) elicited toxic effects in the zebra fish in the exposed groups. It is recommended that the treatment processes denoted by A2, A3 and A4 should be enhanced in order to prevent the disruption of the reproductive functions of aquatic organisms.

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