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Effects of sewage effluents on some reproductive parameters in adult Zebra fish (*Danio rerio*)

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Abstract
Sewage treatment plants are sources of anthropogenic substances in the environment. Sewage effluents elicit adverse effects on the reproductive abilities of aquatic organisms. The aim of the project was to assess the efficiency of the treatment processes at a sewage treatment plant in Sweden with zebra fish as test models for aquatic organisms. The project entailed the exposure of adult male and female zebra fish to the sewage effluents (A1- A7) for 21 days. The reproductive parameters monitored were spawning ability, fertilization, fecundity and vitellogenin concentration. Fish exposed to effluent A2 (after sedimentation treatment) had a higher number of successful spawning compared to the controls (A1 and A8). Fish in groups A3 (outlet L1), A4 (biofilter) and A5 (ozone) exhibited a decrease in spawning ability. Adult female fish exposed to effluent A4 exhibited low fecundity compared to the controls. The processes at the sewage treatment plant need to be optimized to forestall adverse effects on the reproductive abilities of aquatic organisms.

Keywords: Sewage effluents; Zebra fish; Spawning; Fecundity; Vitellogenin,

Introduction

Sewage effluents contain complex mixtures of chemicals such as natural and synthetic hormones, alkylphenols, phthalates, bisphenol A, pharmaceuticals and some pesticides (Kolpin *et al*., 2002; Vos *et al*., 2000). These chemicals disrupt the endocrine system of animals and are known as endocrine disrupting chemicals (Quinn *et al*., 2004). Endocrine disrupting chemicals (EDCs) constitute a major group of chemical pollutants in the aquatic environment and they interfere with the hormonal systems of animals (Colborn *et al*., 1993). The main sources of EDCs in the aquatic environment are effluents from paper and pulp mills, agricultural and textile industries and sewage treatment plants.

Endocrine disrupting chemicals mimic or antagonize endogenous hormones and alter the synthesis and metabolism of endogenous hormones and hormone receptors (Sonnenschein and Soto, 1998). Several adverse health effects in aquatic organisms have been attributed to EDCs, such as developmental, neurological, endocrine and reproductive alterations (Hill and Janz, 2003; Van den Belt *et al*., 2003). The aim of the study was to assess the efficiency of the treatment processes at a sewage treatment plant in Sweden with regard to the reduction of some EDCs such as pharmaceuticals and estrogens. This was conducted with the aid of the adult Zebra fish reproduction test. It is an approved test by the Organization of Economic Cooperation and Development (OECD).

Materials and Methods

Sewage Treatment Plant (STP) Effluents Studied

The sewage effluents studied were obtained from the pilot sewage treatment plant (STP) situated in Stockholm, Sweden. The pilot STP is composed of four different experimental process lines in which the first and second lines are based on aerobic processes as the main biological treatments, with activated sludge and a membrane reactor, respectively. The sewage effluents evaluated in the present study were obtained from Lines 1 and 2. Six different treatment processes were evaluated and are depicted in Table 1. The sewage effluents from the different treatment steps from Line 1 were denoted A2- A6, Line 2 was denoted A7 and A1 was used as the clean reference water for the sewage effluents. A8 was standardized laboratory water (internal control) and it consisted of deionised water, $CaCl_2$, $MgSO_4$, $NaHCO_3$ and KCl. The sewage effluents were transported to the Division of Pathology, Pharmacology and Toxicology, Swedish University of Agricultural Sciences. Grab samples were taken in 25 liters plastic containers every third day during the three-week experimental period. Subsequently, they were frozen and stored at -20ºC. The effluent

samples were thawed overnight in room temperature prior to use.

Experimental Animals
Adult zebra fish (*Danio rerio*) were procured from a local supplier in Uppsala, Sweden and were allowed to adapt to laboratory conditions for 4 weeks before the commencement of the project. The study was conducted at the Division of Pathology, Pharmacology and Toxicology, Swedish University of Agricultural Sciences. The zebra fish were handled in accordance with the ethical guidelines stipulated by the Animal Welfare Committee of Sweden. They were kept in aquaria within a temperated laboratory at 26 ± 1 ºC and a 12-hr light/dark cycle was maintained. Standardized water (ISO 7346- 1, 1996) was used for the maintenance of the adult fish. It was prepared from deionised water and the following salts were added: $CaCl_2$ x $2H_20$ (117.6 mg/l), $MgS0_4$ x 7 H_20 (49.3 mg/l), $NaHC0_3$ (25.9 mg/l) and KCl (2.3 mg /l) (Sigma Aldrich Sweden AB). The adult fish were fed commercial flake food (Sera®) and freeze-dried chironomids (Nutrafin®) 2-3 times daily.

Adult Zebra Fish Reproduction Test
Groups of adult zebra fish were placed in 30-liter aquaria containing the different sewage effluents (A1- A7) and the standardized fish water (A8). For each effluent tested, four replicates were used. The fish were placed in stainless steel net spawning cages within each aquarium and each replicate consisted of five fish (three males and two females). Effluent water was pumped from 25- liter plastic containers to each aquarium with the use of a multichannel peristaltic pump (Ismatec®, Zurich, Switzerland) through glass capillaries connected with silicon tubings. Standardized water was pumped from a stainless steel tank. The flow rate in the aquaria corresponded to a 20% daily renewal of the exposure media. The experiment was conducted at 26 ºC and in a 12-hr light/dark cycle. Each morning, the eggs from each replicate were collected by siphoning with silicon tubing from collecting containers that were placed under each spawning cage. The duration of the exposure of the fish to the effluents was 21 days. The endpoints monitored on a daily basis were spawning (yes/no), number of eggs (fecundity) and fertilization of the eggs. The eggs were examined with the aid of a stereo microscope in order to determine whether they were fertilized or not. At the end of the exposure, the male zebra fish from each replicate were anaesthetized in tricaine methane sulfonate (MS 222, 1g/L) and then sampled for vitellogenin analysis. Subsequently, the female fish were euthanized and then fixed and processed for histological evaluation of gonad morphology.

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 (Vtg) 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 frozen in liquid nitrogen and stored at -80ºC. 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 g at 4 ºC for 30 minutes and the supernatant below the fat layer was collected to determine the Vtg concentration of each fish. The supernatants were aliquoted and frozen at – 80 ºC. The measurement of Vtg was conducted by using a commercially available precoated Vtg ELISA kit (Biosense Laboratories ®, Norway). Purified Vtg from zebra fish 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 Vtg in each fish was calculated.

Histological Preparation and Evaluation
The bodies of the fish intended for histological evaluation were placed in individually labeled 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 dorso-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.

Statistical analysis

The successful spawning occasions 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. The data on number of eggs produced and fertilization were analyzed with one–way analysis of variance (ANOVA) followed by Dunnett's post hoc test for comparing exposed groups with controls. Differences in vitellogenin concentrations between exposed groups and controls were tested using the nonparametric Mann-Whitney U test. The data of the group replicates were combined if no differences were detected between the replicates. 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 0.95 ($p < 0.05$). Data was presented as mean ± standard deviation (SD).

Results

Significant differences were observed in the spawning success of zebra fish exposed to the sewage effluents. Fish exposed to effluent A2 had a higher number of successful spawnings than controls, while fish in groups A3 - A5 exhibited a decrease in spawning ability (Figure 1). Fish exposed to effluent A4 produced a fewer number of eggs per spawning compared with controls (Figure 2). There were no differences in the number of fertilized eggs between the exposed groups and controls. There were no significant differences in vitellogenin concentrations in the male fish exposed to sewage effluents compared with controls. The gonads of the male and female adult zebra fish exposed to the sewage effluents were fully developed and no differences were observed between exposed groups and controls.

Table 1: The treatment processes at the sewage treatment plant.

A2 - After Sedimentation Treatment
A3 - Sandfilter + After Sedimentation Treatment
A4 - Sandfilter + Biofilter + After Sedimentation Treatment
A5 - Sandfilter + Ozonation + After Sedimentation Treatment
A6 - Sandfilter + Ozonation + Biofilter + After Sedimentation Treatment
A7 - Filtering Drum + Membrane Reactor

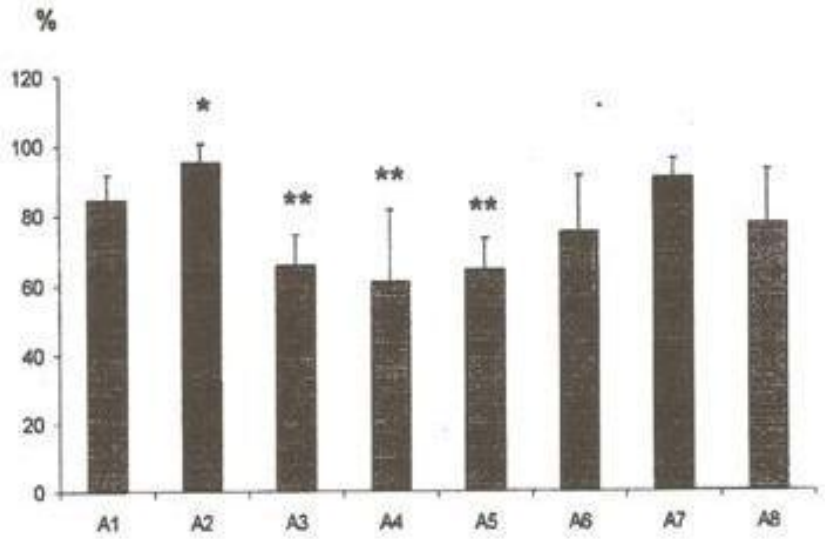

Figure 1. *Percentages of successful spawning occasions in zebrafish exposed to different sewage effluents for 21 days. ** indicate significant differences at p< 0.01 level.*

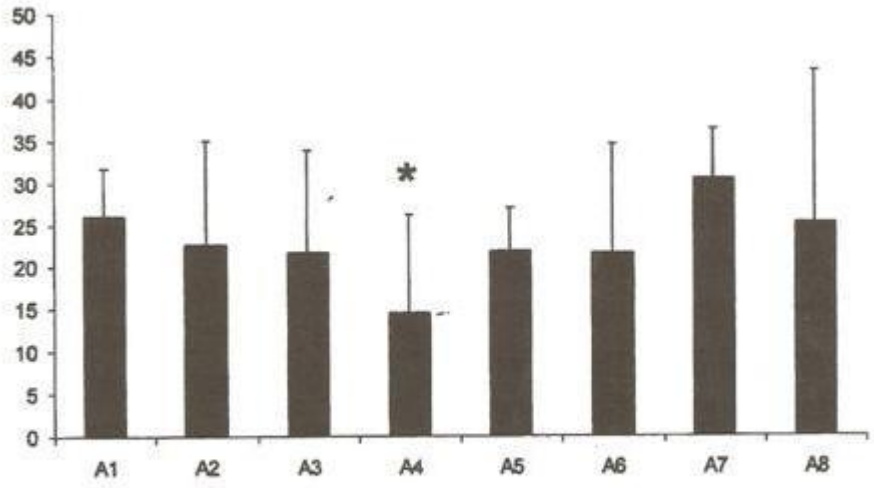

Figure 2. *The mean number of eggs/spawning in zebrafish exposed to different sewage effluents for 21 days.*

Discussion

In this study, it was observed that the zebra fish exposed to effluents A2 and A7 had a higher number of successful spawnings compared to the controls (A1 and A8), while the zebra fish exposed to effluents A3 - A6 exhibited a decrease in spawning ability compared to the controls. However, the concentrations of endocrine disrupting chemicals (EDCs) in sewage effluents A3 - A6 were slightly higher compared to that of sewage effluent A2. This is based on a chemical analysis that was conducted on the sewage effluents in a previous investigation (unpublished observations). This might have contributed to the adverse effects observed in the zebra fish exposed to these effluents. Endocrine disrupting chemicals such as estrogens elicit adverse effects on the reproductive ability of zebra fish. These include delayed or inhibited onset of spawning, reduced number of spawning females, reduced fecundity and reductions in the fertilization and hatchability of the eggs (Hill and Janz, 2003; Van den Belt *et al*., 2003). The effects observed on reproduction in the current study might be partially due to the presence of EDCs, especially estrogens in the effluents since they are capable of eliciting adverse effects on the reproductive capabilities of aquatic organisms. This could have deleterious effects on the population and survivability of aquatic organisms, thereby causing a disruption in the aquatic ecosystem.

Conclusion

From the results of this investigation, it is apparent that sewage effluents A3 – A6 elicited adverse effects on the reproductive parameters of the adult zebra fish. The effects might be attributed to the presence of EDCs in the sewage effluents. The sewage treatment processes particularly A3 (outlet L1), A4 (biofilter), A5 (ozone) and A6 (ozone and biofilter) should be optimized. This would ensure the complete reduction of EDCs in the sewage effluents thereby forestalling adverse effects on the reproductive capabilities of aquatic organisms.

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