Available online at www.sciencedirect.com
ScienceDirect
Ecotoxicology and Environmental Safety 67 (2007) 149–156
Ecotoxicology and Environmental Safety
www.elsevier.com/locate/ecoenv

Evaluation of the estrogenic potential of river and treated waters in the Paris area (France) using in vivo and in vitro assays

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Received 17 December 2004; received in revised form 5 December 2005; accepted 1 March 2006
Available online 25 April 2006

Abstract

For many years, surface waters have been shown to be contaminated by endocrine-disrupting compounds (EDCs), which can cause adverse effects on human and wildlife growth, development, and reproduction. It is therefore of primary importance to determine if drinking water could be contaminated by EDCs when produced from polluted surface waters. It is also essential to determine if disinfection by-products can account for estrogenic activity in treated waters. The estrogenic potential of river and treated waters was investigated using an in vivo assay. Adult male zebrafish were placed in three drinking water treatment plants (DWTPs) in the Paris area and exposed for 1 month to the two types of waters. After exposure, vitellogenin (VTG) was measured in the plasma of fish using a competitive ELISA. In addition, an in vitro assay (MELN cells) was used to assess the estrogenic potential of 10 major chlorination by-products. No significant induction of VTG was observed in fish exposed to river or treated waters. Among the 10 chlorination by-products tested, only 2-chlorophenol was found to be weakly estrogenic at concentrations up to 1 mg/L. Therefore, the risk for the three DWTPs studied to produce drinking water with significant level of estrogenic substances appears to be low.

Keywords: Estrogenic potential; River; Treated water; Drinking water treatment plant; Vitellogenin; Zebrafish; Enzyme-linked immunosorbent assay (ELISA); Disinfection by-products; MELN cells.

1. Introduction

In recent years, concern has been raised regarding various environmental contaminants that have the potential to alter the normal functioning of the endocrine system in wildlife and humans (Colborn and Clement, 1992). These contaminants are known as endocrine-disrupting compounds (EDCs) or endocrine disruptors. EDCs encompass a variety of substances, including hormones synthesized by vertebrates (estrogens, androgens, and progesterone), phytoestrogens, and mycotoxins, as well as manmade substances such as synthetic hormones, pesticides, detergent components, and persistent environmental pollutants (PCBs, PCDDs, PCDFs). Environmental estrogens are currently the best studied EDCs. They may mimic, increase, or inhibit the action of endogenous estrogens by a number of divergent pathways (Gillesby and Zacharewski, 1998).

EDCs found in the environment, and particularly in aquatic ecosystems, originate from industrial, agricultural, and urban discharges. Natural estrogens, as well as the synthetic estrogen 17α-ethinylestradiol, the principal component of contraceptive pills, have been detected at concentrations of up to several ng/L in waste water treatment plant (WWTP) effluents and rivers (Baronti et al., 2000; Cargouët et al., 2004; Johnson et al., 2000; Kuch and Ballschmiter, 2001; Ternes et al., 1999). Alkylphenols, surfactants used in household products, have also been found in WWTP effluents and rivers. Concentrations of several hundred μg/L have been reported in Spain (Solé et al., 2000). Although the use of organochlorine pesticides has been banned in many countries for many years, they can still be detected in rivers at concentrations up to several hundred ng/L (Hung and Thiemann, 2002; Turgut, 2003). Similar concentrations of polychlorinated biphenyls have

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0147-6513/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2006.03.001

been measured in surface waters (Fernandez et al., 1999; Telli-Karakoc et al., 2002). In France, 63% of drinking water is produced from groundwater and 37% from surface waters. In the Paris area, 80% of the drinking water is produced from highly polluted rivers. Water contamination by EDCs is a major public health threat and its assessment is essential for the water industry. The possibility that EDCs could contaminate drinking water when produced from polluted surface waters has therefore to be considered.

In our previous study, conducted in the Paris area, the estrogenic potential of influents and effluents from municipal WWTPs as well as the receiving waters has been investigated (Cargouët et al., 2004). Water samples were analyzed using gas chromatography coupled with mass spectrometry to quantify natural and synthetic estrogens and an in vitro estrogenicity bioassay. We demonstrated that WWTPs contribute to the estrogenic contamination of rivers due to the incomplete removal of estrogenic compounds during wastewater treatments. The estrogenic potential of a substance or a more complex environmental sample obtained using in vitro assays does, however, not necessarily reflect the estrogenic potential in vivo. In vitro assays do not take account of absorption, distribution, metabolism and excretion. Thus, results obtained in vitro could underestimate or overestimate the risk of EDCs in an intact organism. The induction of vitellogenin (VTG) in vivo in juvenile or male fish is widely used as a sensitive and reliable biomarker of exposure to estrogenic compounds (Sumpter and Jobling, 1995). VTG is an egg yolk precursor protein normally synthesized by the liver in female fish. However, this protein can be induced in male and juvenile fish as a consequence of exposure to estrogenic substances.

In the present work, we evaluated the estrogenic potential of rivers and treated waters using an in vivo assay. Adult male zebrafish (*Danio rerio*) were exposed to river waters at the inlets of drinking water treatment plants (DWTPs) in the Paris area for 1 month. In addition to EDCs that could be found in rivers, it is important to determine if by-products produced during the disinfection treatments (ozonation, chlorination) can account for estrogenic activity in treated waters. Adult male zebrafish were therefore exposed to treated waters (after ozonation, prior to chlorination). Only the estrogenic potential of ozonation by-products was investigated in vivo, since the chlorinated water would be too toxic for the fish. The estrogenic potential of 10 major chlorination by-products was then evaluated using an in vitro assay. VTG was used as the endpoint to measure the estrogenic effect in exposed fish.

2. Material and methods

2.1. Chlorination by-products and in vitro estrogenicity bioassay (MELN cell line)

Bromodichloromethane (98% pure), bromoform (>99% pure), trichloroacetic acid (98% pure), dichloroacetonitrile (98% pure), and trichloroacetonitrile (98% pure) were purchased from Aldrich. Dibromochloromethane (98.5% pure), dichloroacetic acid (99.7% pure), chloral hydrate (trichloroacetaldehyde monohydrate) (99.5–101% pure), dichloromethane (>99% pure), and 2-chlorophenol (99.2% pure) were supplied by Riedel–De Haën (Seelze, Germany). Stock solution of chloral hydrate was prepared in MilliQ water (Millipore, France). The other compounds were dissolved in ethanol.

The MELN cells were kindly provided by Dr. P. Balaguer (INSERM U439, Montpellier, France). This cell line derived from the MCF-7 human breast carcinoma cell line in which an estrogen-regulated luciferase gene was stably transfected (Balaguer et al., 1999; Pons et al., 1990). A detailed description of the routine cell culture and estrogenicity bioassay conditions was previously described (Cargouët et al., 2004). Briefly, cells were seeded in 35-mm tissue culture dishes with approximately 4×10^5 cells per dish. They were allowed to attach for 24 h and then incubated with a range of chlorination by-products (CBPs) concentrations for 24 h. The compound solvents, ethanol or MilliQ water, never exceeded 0.1% and 1% of the culture medium, respectively. For each experiment, a range of 17β-estradiol (E2) concentrations (from 10^{-12} to 10^{-9} nM) was used as positive controls whereas cells treated with ethanol or MilliQ water only were used as negative controls. After treatment, the luciferase activity was measured using a Lumat LB 9507 luminometer (Berthold, France). All CBPs were tested in duplicate for at least two independent assays.

Prior to the assessment of the CBP estrogenic activity, the cytotoxicity of the compounds for the MELN cells was investigated using the MTT assay (Mosmann, 1983). The cells were exposed to the CBPs under the same conditions as described above.

2.2. Measurement of vitellogenin

2.2.1. Isolation of vitellogenin

VTG synthesis was induced by aqueous exposure of female zebrafish to E2 (100 µg/L) for 5 days. Following exposure, plasma was purified using a combination of gel permeation chromatography and anion exchange chromatography according to a previously described method with some modifications (Brion et al., 2000). Briefly, a Superdex 200 HR (Amersham Biosciences) was equilibrated with 2 column volumes of 0.1 M Tris–HCl, 1 mM PMSF (pH 8.5) at a flow rate of 1 mL/min. Plasma was diluted in the Tris–HCl/PMSF buffer and applied onto the column using a sample loop of 500 µL. The proteins were eluted isocratically at a flow rate of 0.2 mL/min. Fractions were collected and those containing VTG were identified using a noncompetitive ELISA and pooled. The primary antibody used was polyclonal rabbit anti-zebrafish VTG (Biosense Laboratories).

One millitre of "prepurified" VTG sample was applied on a Resource Q column (Amersham Biosciences) equilibrated with 10 volumes of the Tris–HCl/PMSF buffer. The unbound proteins were eluted with 2 volumes of buffer. The bound proteins were separated by a 10 column volumes linear gradient of 0–0.5 M NaCl at a flow rate of 2.5 mL/min. Fractions of 1 mL were collected. The column was washed with 8 volumes of 0.5 M NaCl at the same flow rate. Fractions were analyzed by noncompetitive ELISA. Positive fractions were pooled and concentrated by ultrafiltration (5000 g, 4 °C) using Nanosep (Pall Filtron, USA) with 100,000 molecular mass cut off. Total protein concentration was determined using Bradford assay (Bradford, 1976). Purified VTG was stored in 50% glycerol and aprotinin (2 TIU/mL) at −20 °C.

2.2.2. Competitive ELISA

Using a procedure modified from Tyler et al. (1999), a competitive ELISA was used to measure VTG in zebrafish plasma samples. Microtiter plates with 96 wells (Nunc Maxisorp) were coated with 100 µL/well of purified VTG diluted to 600 ng/mL in coating buffer. Two wells were coated with only coating buffer and used as blank. To determine the nonspecific bindings, two wells were coated with control male zebrafish plasma diluted in coating buffer to obtain a total protein concentration of 600 ng/mL. The plates were incubated overnight at 4 °C. In parallel, 60 µL/well of zebrafish plasma sample or purified zebrafish VTG