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Genomic and phenotypic response of hornyhead turbot exposed to municipal wastewater effluents

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ABSTRACT

Laboratory tests with marine flatfish were conducted to investigate associations among gene expression, higher biological responses and wastewater effluent exposure. In the present study, male hornyhead turbot (*Pleuronichthys verticalis*) were exposed to environmentally realistic (0.5%) and higher (5%) concentrations of chemically enhanced advanced-primary (PL) and full-secondary treated (HTP) effluents from two southern California wastewater treatment plants (WWTP). Hepatic gene expression was examined using a custom low-density microarray. Alterations in gene expression (*vs.* controls) were observed in fish exposed to both effluent types. Fish exposed to 0.5% PL effluent showed changes in genes involved in the metabolism of xenobiotics, steroids, and lipids, among other processes. Fish exposed to 5% PL effluent showed expression changes in genes involved in carbohydrate metabolism, stress responses, xenobiotic metabolism, and steroid synthesis, among others. Exposure to 5% HTP effluent changed the expression of genes involved in lipid, glutathione and xenobiotic metabolism, as well as immune responses. Although no concentration-dependent patterns of response to effluent exposure were found, significant Spearman correlations were observed between the expression of 22 genes and molecular and/or higher biological responses. These results indicate that microarray gene expression data correspond to higher biological responses and should be incorporated in studies assessing fish health after exposure to complex environmental mixtures.

1. Introduction

The study of contaminants of emerging concern (CECs) is becoming more common and many of these compounds are increasingly being determined to cause adverse effects in exposed fish (Kidd et al., 2007). Several current-use pesticides and personal care products, as well as most CECs, are not required to be monitored or regulated under the National Pollutant Discharge Elimination System (NPDES). Effects caused by exposure to CECs capable of disrupting endocrine systems in humans and aquatic organisms are of particular concern (Baker, 2005; Brodin et al., 2013; Hutchinson et al., 2006; Swan, 2008a; Vandenberg et al., 2009). Exposure to CECs can alter plasma concentrations of steroids and thyroid hormones (Baker, 2011; Heindel and VomSaal, 2009; Oehlmann et al., 2008). Treated municipal wastewater effluents are a significant source of CECs in aquatic environments (Swan, 2008a,b; Tusher et al., 2001; Vandenberg et al., 2009). In southern

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California (USA), more than one billion gallons of these effluents are discharged into the ocean everyday (Lyon et al., 2006). To assess marine environmental condition, local sanitation districts monitor outfall discharge sites for biological impacts, including effects on hornyhead turbot (*Pleuronichthys verticalis*) (Allen et al., 1998).

A comprehensive study previously conducted in the Southern California Bight (SCB) observed biological responses that suggest endocrine disruption in hornyhead turbot living in southern California (Bay et al., 2012). This study found unexpectedly high concentrations of plasma estradiol (E2) and the presence of vitellogenin (VTG) in male hornyhead turbot. These findings could not be attributed to effluent exposure, since these responses were present among fish which were collected near effluent discharges and also at a reference area (Forsgren et al., 2012; Reyes et al., 2012). However, concentrations of thyroxine (T4) hormone were significantly lower in males and females collected near outfall discharge areas (Bay et al., 2012). Previous field studies identified several CECs in the discharged effluents, receiving seawater, as well as in sediment and fish collected at the effluent discharge areas (Maruya et al., 2012; Vidal-Dorsch et al., 2012). In addition, Maruya et al. found priority pollutants such as legacy pesticides (*e.g.*, DDTs) and PCBs in sediments and fish tissue that may have also influenced biological responses in the turbot (Maruya et al., 2012). However, the independent and additive effects of these CEC mixtures on fish remain unknown.

The present study investigated the effects of exposure to advanced primary- and secondary-treated effluents in hornyhead turbot under controlled laboratory conditions. A laboratory approach was used to resolve some of the confounding factors, such as the presence of historical DDT and PCB contamination, which complicate interpretation of data derived from field studies in the SCB. To investigate responses in different biological systems, hepatic gene expression was measured in male hornyhead turbot. Males of this species were chosen because unexpected biological responses were observed in previous field and laboratory studies (Forsgren et al., 2012; Reyes et al., 2012). A custom microarray was used to investigate genetic responses in fish exposed to two types of treated municipal wastewater effluent. The experiments included negative (natural seawater) and positive (30 nM estradiol) controls, as well as two concentrations of each effluent type: an environmentally realistic concentration (0.5%, v/v) expected to be representative of the outfall discharge sites, and a tenfold higher concentration to examine dose response relationships.

2. Materials and methods

2.1. Experimental design

This manuscript describes gene expression results in male hornyhead turbot and it is a part of a larger study that investigated hormonal responses in male and female fish of this species after exposure to wastewater effluents (Vidal-Dorsch et al., 2010). A custom low-density microarray was used to examine changes in expression of genes involved in hormone responses and xenobiotic metabolism (Baker et al., 2009). Quantitative polymerase chain reaction (qPCR) methods were used to validate the microarray results. Fish were exposed in separate experiments to advanced-primary or full-secondary treated effluent from two of the largest wastewater dischargers in the SCB.

Two separate experiments were conducted to carry out the exposures. In the first experiment, wastewater effluent from the Point Loma Wastewater Treatment Plant (PL), in San Diego, CA was used. In the second experiment, effluent from the Hyperion Treatment Plant (HTP), in Playa Del Rey, CA, near Los Angeles was used. The PL effluent received chemically enhanced advanced-primary treatment, and the HTP effluent received full-secondary treatment. Further information regarding the effluents used can be found elsewhere (Vidal-Dorsch et al., 2012).

The experimental treatments used in this study included negative (seawater only) and positive (estradiol; 30 nM nominal) controls, and two effluent concentrations (0.5% and 5%). During the PL exposure, an ammonia (NH_3) treatment was also included to investigate the effects of this common effluent constituent on endocrine responses. The NH_3 concentration represented ammonia levels found at 5% effluent concentrations (2 mg/L = 1.17 μM/L). Not enough fish were available to conduct an NH_3 exposure during the HTP experiment.

The fish were exposed to the experimental treatments for 14 days. Individual fish were placed in separate glass aquaria containing seawater. Ten replicate aquaria were used per treatment. At the conclusion of each experiment, external evaluations of gross morphology were conducted on each fish. The fish were then sacrificed and dissected. The sex of each fish was determined after dissection. Tissues of interest were harvested for subsequent storage and analysis.

Blood plasma was collected to measure hormone (T4, E2, and 11-keto testosterone (11-KT)) and VTG concentrations. The liver and the right side gonad were weighed to determine the liver somatic index (LSI) and the half gonad somatic index (1/2 GSI). To characterize the composition of the effluents and seawater a suite of 31 CECs was analyzed.

2.2. Effluents and controls

At the treatment plant the PL influent was screened and aerated, followed by chemically assisted sedimentation for particulate removal. In addition to screening and primary sedimentation, the full-secondary influent treatment at HTP included aeration in oxygen reactors and subsequent settling of activated sludge in clarifier basins (Vidal-Dorsch et al., 2010). The effluent was transported to SCCWRP facilities in Nalgene® carboys and stored at 4 °C until use. The effluent samples were peak flow grabs (collected in the late morning or early afternoon) of final effluent from each sanitation district. Three effluent batches were used for each experiment. The batches were sequentially used to prepare the experimental treatment in order to avoid long term storage; fish were exposed for 5 days to each batch. The control and dilution water was natural seawater collected from Redondo Beach (CA), which was filtered (0.45 μm) and treated with activated carbon before use.

An estradiol positive control (8 μg/L = 30 nM) was included. This concentration was selected on the basis that it would elicit a strong induction of many of the mRNA targets under study, and thereby would evaluate the performance of the microarray tool. A master-stock solution was prepared by dissolving E2 powder in HPLC grade acetone (Fisher Scientific Pittsburgh, PA) in a 10 L glass jar. Subsequently, the acetone was evaporated at room temperature for 1 h and the E2 was plated onto the jar surface. Then 10 L of seawater were added to the glass jar to create a second stock solution which was mixed for 2 h; subsequently this solution was transferred to a 20 L carboy where it was mixed with 10 L of seawater to create a final E2 stock solution. The final E2 solution was manually delivered to test aquaria where it was mixed with seawater to achieve the desired concentration. The E2 concentration measured in the test aquaria was 5.08 ± 2.28 μg/L.

2.3. Fish exposures

Hornyhead turbot were collected from Dana Point, a location distant to the largest municipal wastewater discharges in southern California, which was previously used as a reference site (Brown and Steinert, 2003; Deng et al., 2007). This area is located near a