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# Toxicogenomic responses in rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to model chemicals and a synthetic mixture

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**Abstract**

As more salmon gene expression data has become available, the cDNA microarray platform has emerged as an appealing alternative in ecotoxicological screening of single chemicals and environmental samples relevant to the aquatic environment. This study was performed to validate biomarker gene responses of *in vitro* cultured rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to model chemicals, and to investigate effects of mixture toxicity in a synthetic mixture. Chemicals used for 24 h single chemical- and mixture exposures were 10 nM 17α-ethinylestradiol (EE2), 0.75 nM 2,3,7,8-tetrachloro-di-benzodioxin (TCDD), 100 μM paraquat (PQ) and 0.75 μM 4-nitroquinoline-1-oxide (NQO). RNA was isolated from exposed cells, DNAse treated and quality controlled before cDNA synthesis, fluorescent labelling and hybridisation to a 16k salmonid microarray. The salmonid 16k cDNA array identified differential gene expression predictive of exposure, which could be verified by quantitative real time PCR. More precisely, the responses of biomarker genes such as cytochrome p4501A and UDP-glucuronosyl transferase to TCDD exposure, glutathione reductase and gammaglutamyl cysteine synthetase to paraquat exposure, as well as vitellogenin and vitelline envelope protein to EE2 exposure validated the use of microarray applied to RNA extracted from *in vitro* exposed hepatocytes. The mutagenic compound NQO did not result in any change in gene expression. Results from exposure to a synthetic mixture of the same four chemicals, using identical concentrations as for single chemical exposures, revealed combined effects that were not predicted by results for individual chemicals alone. In general, the response of exposure to this mixture led to an average loss of approximately 60% of the transcriptomic signature found for single chemical exposure. The present findings show that microarray analyses may contribute to our mechanistic understanding of single contaminant mode of action as well as mixture effects, but that its use in screening of complex environmental samples will need to be further evaluated.


*Keywords:* Mixture toxicity; Gene expression; Microarray; Genomics; Dioxin; Endocrine disruption; Oxidative stress; DNA damage; Rainbow trout

## 1. Introduction

The use of gene expression studies in ecotoxicology has been limited by a lack of sequenced and identified genes in ecologically relevant species. For fish, traditionally, a range of classical biomarkers such as cytochrome P450 1A (EROD) activity or plasma levels of the egg yolk protein vitellogenin has been applied singly to investigate if environmental exposure to contaminants will result in biochemical or physiological responses. In the past few years, the generation of expressed sequence tag (EST) databases for species important in aquaculture and aquatic toxicology, including Atlantic salmon (Rise et al., 2004a) and European flounder (Williams et al., 2003), have resulted in the development of microarray platforms where expression of multiple genes can be assessed simultaneously. Due to this property, microarrays greatly facilitate the studies of signalling pathways involved in physiological and toxicological processes, which has proven useful in search for new markers of fish disease (Rise et al., 2004b; Ewart et al., 2005). Furthermore, expression profiling is an appealing alternative in ecotoxicological screening, both due to the possibility of monitoring multiple classical toxicological biomarker genes simultaneously and the possible discovery of novel biomarkers. By linking classical biomarker gene responses to other observed cellular signalling events, the establishment of a relationship between early gene expression changes and physiological damage or disease might also be strengthened (Aardema and MacGregor, 2002). Several

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microarray studies have so far focused on the effects of single compounds, pursuing the idea that each toxicant will produce a distinctive gene expression signature (Bartosiewicz et al., 2001; Amin et al., 2002; Hamadeh et al., 2002; Hook et al., 2006). However, to be a useful tool in ecotoxicological screening, the effects of simultaneous exposure to several different xenobiotics need to be evaluated. It is well known that different cellular signalling pathways influence and interact with each other, and hence, the biomarkers and effect endpoints of choice should be robust enough and the microarray sensitive enough to properly assess these complex interactions.

The *in vitro* culturing of primary cells has several advantages for use in toxicity screening, such as cost- and time efficiency, small sample requirement and possibilities for high throughput screening. Natural variation between individuals is present, but as a consequence of using cells from the same individual as both control and exposed groups, technical variation is minimized. Consequently, this approach has been used for screening single chemicals, mixtures as well as complex environmental samples, utilizing classical biomarkers and effect endpoints (Tollefsen et al., 2003, 2006a,b). In the RNA context, working with cell cultures also implies less variation of cellular origin of the isolated RNA, as the RNA is obtained from a distinct cell population (*i.e.* hepatocytes). This is in contrast to *in vivo* experiments, where liver samples from individuals will consist of several differentiated hepatic cell populations, and probably also be subject to different degrees of hepatic invasion of hematopoetic cells.

The purpose of the present study was, by means of the GRASP 16k salmonid microarray (von Schalburg et al., 2005) to validate toxicogenomic responses of *in vitro* cultured rainbow trout primary hepatocytes exposed to model chemicals, and to investigate mixture toxicity effects of these chemicals. The salmonid array contains spotted cDNA from Atlantic salmon and rainbow trout, and it has been demonstrated that rainbow trout cDNA has a good binding affinity to spots on the salmonid array (von Schalburg et al., 2005). The chemicals chosen for this study have previously been reported to cause the activation of arylhydrocarbon receptor (AhR) pathways (2,3,7,8-tetrachloro-di-benzodioxin), to cause endocrine disruption (17α-ethinylestradiol), oxidative stress (paraquat) or direct mutagenicity (4-nitroquinoline-1-oxide) in rainbow trout hepatocytes (Tollefsen et al., 2003, 2006a,b). A mixture of the four compounds, with identical concentrations as for single chemical exposures, was included in the study to clarify the extent to which transcriptomic responses of individual chemicals will be retained in the presence of compounds acting through dissimilar modes of action, as will be the case in the environment.

## 2. Materials and methods

### 2.1. Chemicals

Paraquat (PQ), 17α-ethinylestradiol (EE2), 4-nitroquinoline-1-oxide (NQO), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and dimethyl sulphoxide (DMSO) were obtained from Sigma–Aldrich (St. Louis, OR, USA). All the test chemicals had a minimum purity of 98%. Prior to use in the *in vitro* bioassays all chemicals were diluted in utrapure DMSO (99.8%).

### 2.2. Fish

Rainbow trout (*Oncorhynchus mykiss*) (200–500 g) were obtained from Killi Oppdrettsanlegg (Dombås, Norway) and were kept in tanks at the Department of Biology, University of Oslo (Norway), at a water temperature of 12 °C, oxygen saturation of approximately 100% and pH 6.6. The fish were fed daily with commercial fish pellets (EWOS, Bergen, Norway) in amounts corresponding to 0.5% of total body mass. The tanks received artificial illumination (100 lux) with a photoperiod of 12 h. Four individual male fish were used in this experiment.

### 2.3. Isolation, culturing and exposure of hepatocytes

A two-step perfusion of the liver was performed as described by Tollefsen et al. (2003). Post-perfusion, the cells were plated as a mono-layer culture at a density of $5 \times 10^5$ cells/ml (3 ml/well) in 6-well Falcon Primaria plates (Becton Dickinson, Franklin Lakes, NJ, USA), using serum free L-15 medium containing 1000 U Penicillin/ml, 1000 μg Streptomycin/ml and 1 mM L-glutamine (all from BioWhittaker, Walkersville, MD, USA). After a 24 h pre-culture period, the cells were exposed to either 10 nM EE2, 0.75 mM NQO, 0.1 mM PQ, 0.75 nM TCDD, or a mixture of the same compounds (MIX), where concentrations of each compound were equivalent to the single chemical exposure. The concentrations of test compounds were chosen on basis of reported (Tollefsen et al., 2003, 2006a,b) or unpublished $EC_{50}$ for typical biomarker responses in separate dose–response studies for the chosen chemicals. All chemicals were dissolved in DMSO, resulting in a final concentration of 0.1% DMSO when exposing the cells. At 24 h of exposure, cytotoxicity was measured directly in the cell culture by the fluorescent dyes 5-Carboxyfluorescein Diacetate Acetoxymethyl Ester (CFDA-AM) (Molecular Probes, Leiden, The Netherlands) and Alamar Blue (BioSource, Nivelles, Belgium) according to the method described by Tollefsen et al. (2006b). Briefly, the exposure media was removed from the wells and exchanged with 100 μl of DMEM (Sigma–Aldrich) containing 5% AB (BioSource International, Camarillo, CA, USA) and 4 μM CFDA-AM (Molecular Probes, Eugene, OR, USA). The cells were incubated for 30 min in the dark (20 °C) and the concentrations of the metabolites of the fluorescent probes AB and CFDA-AM were measured simultaneously using the wavelength pairs of 530–590 and 485–530 nm (excitation–emission), respectively. The viability of the cells was determined on basis of the fluorescence of cells exposed to the solvent control DMSO (no effect) and the maximum toxicity obtained for $CuSO_4$ (10 mM).

### 2.4. RNA isolation and microarray hybridisation

After 24 h exposure, cells were lysed and total RNA isolated and DNAse treated using the RNeasy mini kit and RNase free DNAse kit, both from Qiagen (Hilden, Germany). The RNA