# Isolation of perfluorooctane sulfonic acid induced hepatic genes in the common carp (*Cyprinus carpio*) using microarray technology

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## Introduction

Perfluorooctane sulfonate (PFOS) is being produced over 50 years and is commonly used in lots of materials such as wetting agents, fire retardants, stain resistant treatments for leather and carpets, cosmetics and components of pharmaceuticals and insecticides[1, 2]. Due to the persistence and the bioaccumulation of this pollutant, it can be found worldwide in wildlife and humans. Marine as well as freshwater fish species from various geographic locations have been shown to have tissue PFOS concentrations ranging from <7 up to 9031 ng/g wet weight[3,4].

Biochemical effects of PFOS exposure are mainly studied in liver and serum of mammalian species an include effects on the lipid metabolism[5], intercellular communication[6], neuroendocrine effects[7], developmental effects[8,9] and effects on the alanine aminotransferase activity[10]. However, effects of PFOS on fish remain largely unknown. In juvenile carps (*Cyprinus carpio*) exposed to PFOS, inflammation-independent leakage of liver cells and disturbance of DNA metabolism homeostasis in liver tissue has been demonstrated[11]. This lack of toxicity data points out that there is an urgent need for the mechanistic molecular understanding of the mode of action of this pollutant. The use of the microarray technique is well suited to meet this challenge, because it enables us to analyse the expression of thousands of genes simultaneously. With the expression of all those genes together it is possible to create a characteristic gene expression profile and use this to unravel the mechanisms of action.

The goal of this study was to assess the impact of PFOS on the aquatic ecosystem and to characterize the biochemical effect pattern. The common carp (*Cyprinus carpio*) was chosen as test species and the liver was selected as target tissue. PFOS is known to bioconcentrate and bioaccumulate in the liver, a primary target organ in fish, as was demonstrated for rainbow trout (*Oncorhynchus mykiss*) under controlled conditions[12]. The effect of PFOS exposure on gene expression in liver tissue was assessed by constructing a liver cDNA library enriched with cDNAs that were differentially expressed between PFOS-exposed and control carps. This cDNA library has been made using Suppression Subtractive Hybridization-Polymerase Chain Reaction (SSH-PCR). SSH-PCR has been used combined with the technology of cDNA microarray hybridization. This provided the possibility of a high-throughput technique allowing screening of hundreds of individual mRNA products in a single analysis.

## Materials and Methods

Juvenile carps (*Cyprinus carpio*) were acclimased at 25°C for 3 weeks in plastic 20l aquaria filled with fully aerated water that was renewed every 48 hours. Twelve carps were housed in each aquarium and were fed with pellets on a 2% ratio once per day. After acclimatisation, carps were exposed to PFOS at concentrations of 0mg/l; 0.1mg/l, 0.5mg/l and 1mg/l during two weeks. There were 3 biological replicas for each concentration group. After 2 weeks, the fishes were sacrificed by decapitation. The liver was immediately removed, frozen in liquid nitrogen and stored at -80°C.

The concentrations of PFOS in liver tissue were determined using high pressure liquid chromatography combined with electrospray tandem mass spectrometry (HPLC-MS/MS) according to Powley and Buck[13].

Suppression Subtractive Hybridization-Polymerase Chain Reaction (SSH-PCR) has been used in combination with cDNA microarrays. Briefly, liver RNA was isolated with the Trizol method[14] and used for cDNA synthesis. SSH was performed with the PCR-Select cDNA Subtraction Kit (Clontech Laboratories, Palo Alto, CA) according to the manufacturer's instructions. cDNA clones of the enriched library were obtained and used to construct a microarray, containing 4800 cDNA spots, representing 1632 SSH clones in triplicate and Lucidea Universal ScoreCard controls (Amersham Biosciences). 9µg RNA of each exposure group and control group was labeled with respectively Cy5 and Cy3. Afterwards, all exposure groups were individually hybridized against the control groups. Microarrays were scanned at 635 nm (Cy5) and 532 nm (Cy3) with a Genepix Personal 4100A Array Scanner (Axon Instruments, Union City, CA). The images were analyzed by means of the Genpix pro Software (Axon Instruments, Union City, CA) for spot identification and for quantification of the fluorescent singal intensities. The ratio (Cy5/Cy3) was calculated for each spot, transformed into a logarithmic value ($\log_2$), and normalized using Locally Weighed Scatterplot Smoothing (Lowess). Clones with a mean dye ratio of three replicates <0,5 and >1,5 were retained for sequencing.

**Results**
The resulting hepatic PFOS concentrations of the exposed juvenile carps were 1.1µg/g for the control group, and 35.43, 232.95 and 359.28 µg/g wet weight for the exposure groups of respectively 0.1mg/l, 0.5mg/l and 1mg/l.

A total of 35 cDNAs were differentially expressed in liver tissue of juvenile carps after exposure to PFOS. After sequence analysis, it seemed that the genes were related to the endocrine system, were involved in carbohydrate metabolism and fatty acid metabolism and played a role in the cell structure.

**Discussion**
The selection of the exposure concentrations used, was based on results previously obtained in our lab. It is remarkable that there is already a very high and rapid accumulation within a rather short space of time (14 days), even at the lowest exposure doses. The observation that even fish of the control group contained detectable hepatic PFOS concentrations can be explained by the food of the fish. It consists of artificial food pellets that contains very low concentrations of PFOS. But there is still a big difference between the hepatic concentration of the control groups and these from the exposured groups.

Our toxicogenomic data reveal a broad range of biochemical pathways that may be affected by exposure to PFOS. One of the major affected genes was vitellogenin which could be an indication of endocrine disruption and plays a crucial role in the gonadal development. Another gene that was effected by exposure to PFOS is fatty acid binding protein, which plays a role in the fatty acid metabolism. A previous study has also demonstrated the differential gene expression of fatty acid metabolizing genes and genes involved in hormone regulation after PFOS exposure in the rat[15]. Also interesting in our data is the influence on genes that are involved in the carbohydrate metabolism, for example glucokinase.

This study suggests that PFOS exposure can cause up- and downregulation of some carp hepatic genes involved in a number of physiological processes.

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