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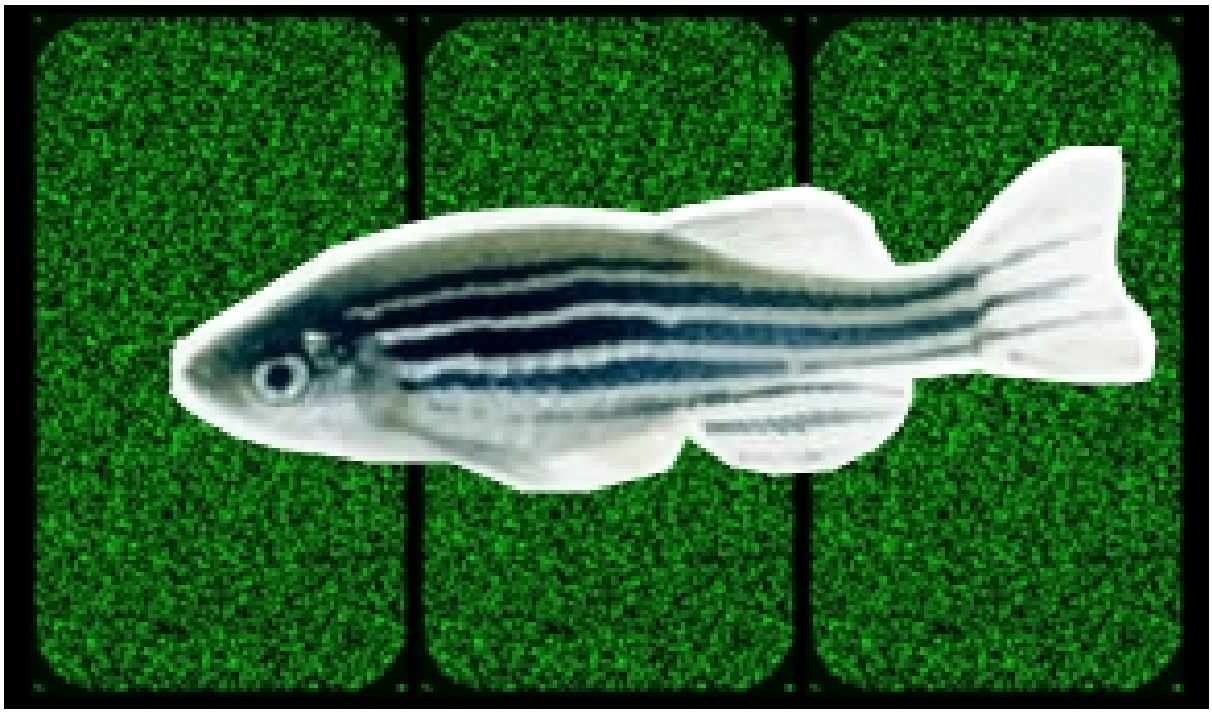
# Gene Expression Profile Assessment in Zebrafish (*Danio rerio*)

Gene expression profile in the zebrafish liver cell line ZFL exposed to environmental pollutants using DNA microarray

**Stephanie K. Bopp and Teresa Lettieri**



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# Introduction

The Zebrafish (*Danio rerio*) is a small tropical fish, which can also be commonly found in home aquaria. In the last decade Zebrafish has become a prominent model organism in biomedical, ecotoxicological and genetic fields. The exquisite features of Zebrafish render this fish unique among the vertebrate model organisms. Indeed, the development of the zebrafish is very similar to the embryogenesis in higher vertebrates, including humans, but, unlike mammals, zebrafish develop from a fertilised egg to an adult outside the female in a transparent egg. Moreover, the embryos themselves are transparent during the first few days of their lives (Wixon, 2000). So, it has been possible to generate a vast store house of mutations selected on the basis how it affects the development, providing models of human dysmorphologies (Weinstein et al 1995). Consequently, the zebrafish has become a popular model organism also for clarification of the roles of specific genes and signalling pathways during the development (Spitsbergen and Kent, 2003). An increasing interest arose also in the field of toxicology and ecotoxicology as non-mammalian vertebrate organism in risk assessment and regulation (Schirmer, 2006); e.g. the Zebrafish embryo is particularly indicated for ecotoxicological assays (Nagel, 2002; Hill et al., 2005). But what boosted the scientific community to recognize zebrafish as leading vertebrate organism in the above mentioned field, has been the complete sequencing of its genome (Sanger Institute, http://www.sanger.ac.uk/Projects/D_rerio). The availability of such information enabled the design of a DNA microarray for zebrafish (Pichler et al, 2004). As expected, gene programming, development in early life stage, receptors, and molecular processes are highly conserved among vertebrates, then, the zebrafish is also a good model for "higher" eukaryotes in gene expression studies (Hill et al, 2005). In addition, although the homologies at protein levels between zebrafish and human counterpart show less than 70 % identity, the conservation in the functional domains is approaching 100 % (Langenheinrich, 2003).

Besides the application of embryos, juvenile or adult zebrafish, the use of cell lines to completely avoid animal testing gains increasing importance. Fish cell lines in general are *in vitro* systems emerging throughout the last decade (Fent, 2001). They are often used to assess mechanisms of toxic actions, acute ecotoxicological effects (Dayeh, 2005) and are also applied looking at cellular biomarker responses (Schirmer et al, 2004). The advantages are mainly the reduction of the number of animal tests and the ease in handling instead of husbandry of living animals. Furthermore, the reproducibility is often much higher, since high variabilities occur among individual species in living animals. Nevertheless, the prediction from results gained using cell lines to a living organism is of course difficult. However, cell lines are providing valuable information and actually there are *in vitro* assays available, which are recognized as authorized alternative to animal testing (Schirmer, 2006). For zebrafish, cell lines are still relatively rarely used, but nevertheless some are commercially available: the liver cell line ZFL (Ghosh et al, 1994), the embryo cell lines ZF4 (Driever and Rangini, 1993) and ZEM2S (Ghosh and Collodi, 1994) and the caudal fin cell lines SJD.1and AB.9 (Paw and

Zon, 1999). Up to now, their use is rarely reported in the literature, probably due to the ease of working with the well-established and accepted zebrafish embryo system.

In our laboratory, we are working on the identification of gene expression signatures and molecular biomarkers, in order to develop indicators for the assessment of water quality. The aim is to investigate alterations on gene expression level due to exposure to chemical pollutants such as the polycyclic aromatic hydrocarbons (PAHs).

Polycyclic Aromatic Hydrocarbons are environmental pollutants ubiquitously distributed. They are indirectly produced by incomplete combustion of organic materials such as wood or fossil fuels. They are reported to be carcinogenic and mutagenic (Harvey, 1991) and due to their wide distribution in the environment, the European Commission included them in the list of Priority Hazardous Substances adopted in November 2001 in the field of Water Framework Directive 2000. In our studies, we selected Benzo[a]pyrene (BaP) since its effects are well documented in many systems e.g human, animals, and mammalian cell systems, while few information is available in cellular system such as zebrafish cell lines. Particularly, using the zebrafish liver cell line, we analyzed the gene expression profiling, induced by BaP to understand the mechanistic response and correlate the specific gene expression to the selected concentrations.

Our interest was to investigate effects of chemicals at low concentrations, which are close to environmental levels and which are not directly acutely toxic, in order to detect molecular alterations as early warning signals. Therefore, we first assessed the potential cytotoxicity of the test compounds, in order to select suitable exposure concentrations for microarray gene expression analysis.

## Materials and Methods

### Culturing of ZFL cells

The zebrafish (*Danio rerio*) liver cell line ZFL (Gosh et al., 1994) was obtained from ATCC (Promochem). Cells were cultured in medium composed of 50 % Leibovitz L-15, 35 % DMEM, and 15 % Ham F-12, supplemented with 15 mM HEPES, 0.15 g/L $NaHCO_3$, 0.01 mg/mL insulin, 50 ng/mL epidermal growth factor (EGF), and 5 % fetal bovine serum (FBS) as proposed by ATCC. Cells were cultured at 28 ºC in 75 $cm^2$ cell culture flasks (Stratagene). Cells were subcultured every 5-7 days. For this purpose, they were first rinsed with 1 mL of 0.25 % trypsin EDTA (Gibco), and then detached with 1 mL of trypsin EDTA. Trypsination was stopped by addition of 5 mL of 10 % FBS containing medium. Cells were centrifuged for 5 min at 300 g. Cell pellets were resuspended in 5 % FBS

containing medium and either split to new flasks or plated in multiwell culture plates for cytotoxicity assays, or in cell culture dishes for microarray experiments.

### Cytotoxicity assessment in ZFL cells

For assessment of cytotoxic effects, cells were transferred to 48 well plates at an initial cell density of 75,000 cells/well in 400 µL of culture medium containing 5 % FBS. Cells were allowed to attach and grow to a ca. 80 % confluent monolayer within 24 h before dosing of pollutants. Benzo[a]pyrene (BaP), as a very hydrophobic substance, is sparingly soluble in water. Thus, a concentrated stock solution in DMSO (dimethyl sulfoxid, cell culture grade, Sigma, Steinheim, Germany) was prepared. Final tested concentrations were ranging from $3x10^{-14}$ to $1.96x10^{-6}$ M. BaP was dosed using a glass syringe to minimize sorptive loss. Final DMSO concentrations of 0.1 % had no significant effect on cell viability. Cells were incubated at 28º C for 24 h before cell viability was assessed.

After incubation with BaP, cell viability was assessed using the MTT cell proliferation Kit I (Roche Diagnostics, Basel, Switzerland) according to the protocol of the manufacturer. In brief, 40 µL of MTT labelling solution were added to the cells without removing the culture medium with BaP. After 4 h of incubation, 400 µL of solubilization solution was added and incubation continued over night at 28º C. The next day, absorption at 570 nm was measured (with a reference wavelength of 680 nm) using a microplate reader (Tecan Infinite F200, Segrate, MI, Italy).

### Exposure of ZFL cells for microarray experiments

For microarray analyses, the experiments were up-scaled since larger amounts of RNA were needed. BaP exposure was performed in 10 cm diameter cell culture dishes. ZFL cells were plated from confluent 7 d old cultures at an initial cell density of $1.8x10^{6}$ cells/dish in 10 mL medium. After 72 h, cells in the dishes were about 80 % confluent. After changing the medium, 10 µL of DMSO were added to the control culture and 10 µL of BaP dissolved in DMSO at 3 different concentrations were added to the fresh medium, so that final BaP concentrations were 0.03 pM, 0.3 nM, and 30 µM with a DMSO content of 0.1 % in the medium.

Cells were incubated for 24 h at 28 °C in the dark. Then, RNA was extracted from the confluent cell monolayers as described below. The experiment was preformed three times within three consecutive weeks.

### RNA isolation

RNA was extracted using Trizol LS (Invitrogen) according to the protocol of the manufacturer. Volumes of Trizol LS and solvents were up-scaled as proposed in the protocol. RNA yields were between 30 and 75 µg from 10 cm diameter petri dish.

After RNA extraction, RNA concentrations were determined using NanoDrop (NanoDropTechnologies) and RNA quality was additionally investigated using the Bioanalyzer (Agilent). Samples were then stored at -80º C.

### Preparation of labelled cRNA for microarray analysis

The labelling of the RNA samples from the three individual experiments was performed simultaneously. Preparation of labelled cRNA was performed according the Affymetrix protocol (One-Cycle cDNA Synthesis Kit). In brief, a one-cycle cDNA synthesis step was performed from 3000 ng of total RNA. An artificial RNA mixture (poly-A RNA controls) was added to the transcription reaction, in order to check the quality of the reactions during sample preparation as well as later on the scanning (see below). Double-stranded cDNA was cleaned up and transcribed to biotin-labelled cRNA. The biotin-labelled cRNA was cleaned up and quantified using NanoDrop. Then, cRNA was fragmented and its quality validated using the Bioanalyzer. The whole experimental scheme is presented in Figure 1.

### Microarray hybridization and scanning

For hybridization and scanning of the microarrays, the labelled cRNA samples were sent to the Functional Genomics Centre Zürich (University of Zürich/ETH Zürich). In brief, we applied the "Gene Chip® Zebrafish Genome Array" from Affymetrix, which contains 14,900 transcripts, spotted as 25mer oligonucleotide probes with 16 replicates per transcript. Hybridization and scanning was performed according to the protocol of the manufacturer. 15 µg of labelled cRNA were hybridized on the microarray at 45 °C under rotation for 16 h. After washing and staining steps, all arrays were scanned once using the Scanner 3000 7G (Affymetrix).

### Data evaluation

Data evaluation was performed for both experiments using the GeneSpring GX 7.3.1 software (Agilent). Data were first normalized using GCRMA upon loading into the GeneSpring software. Subsequently, the data were normalized in two steps, first respect to the 50% percentile called "per chip normalization", then "per gene". The second ("per gene") normalization was done within each experiment to the respective control sample. In a next step, gene expression data were filtered to retrieve genes with a two-fold change compared to the control. The filtering was performed for each treated condition (low, intermediate, and high BaP concentration) compared to the control. First, a 1.5 fold change looking at the average of the replicates was applied as limit, in a second step, only genes regulated more than two-fold in at least two out of three replicates were considered. The obtained gene lists were then statistically analyzed using 1-way ANOVA (as parametric test, not

assuming variances equal), followed by a Benjamini-Hochberg false discovery rate test for multiple testing correction.

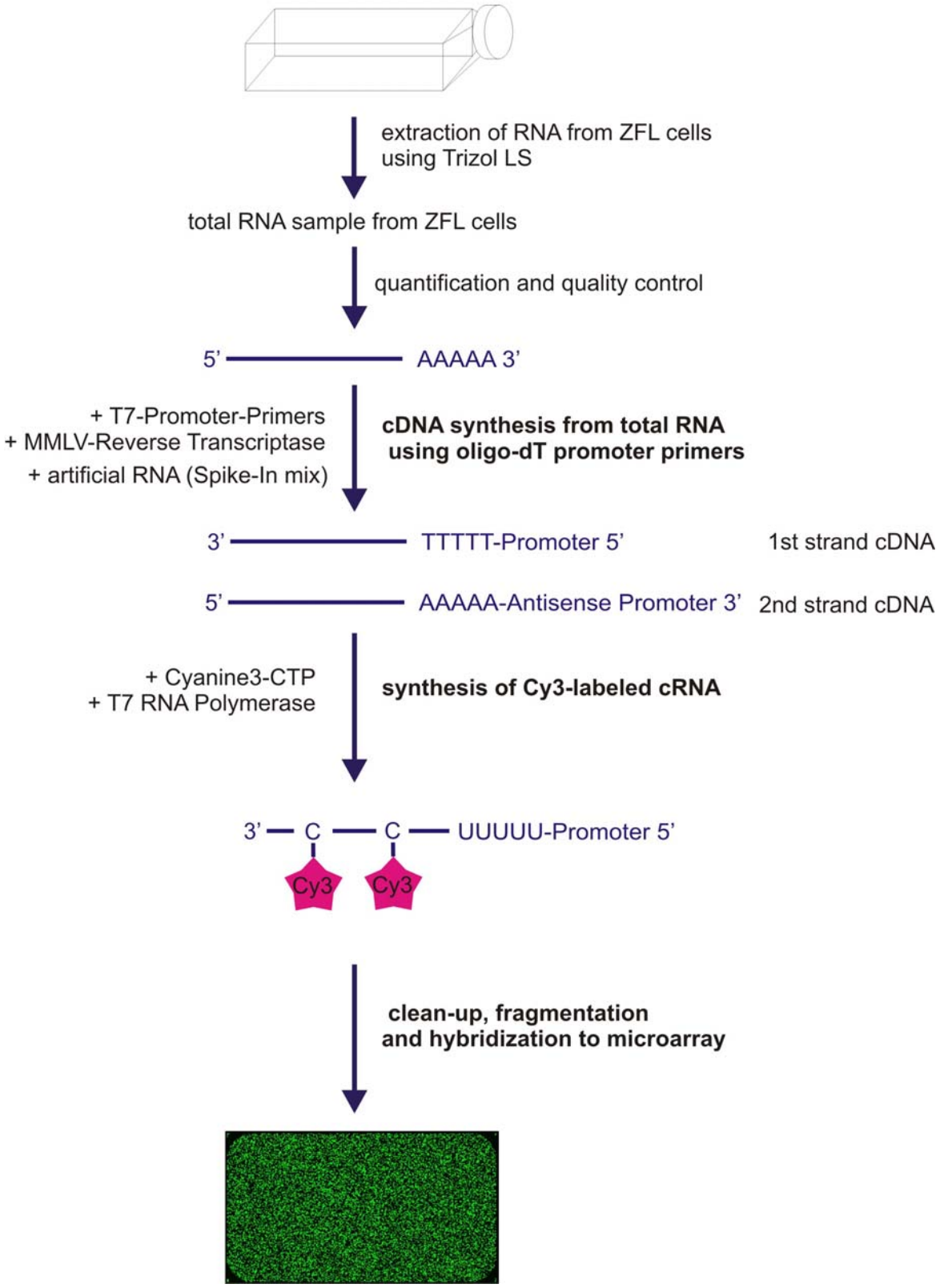



***Figure 1:*** *Scheme followed for sample preparation. Total RNA was reversely transcribed to cDNA using oligo-dT-primers, and in a next step transcribed to fluorescent cRNA by simultaneously labeling with Cyanine3 (Cy3).*

# Results

### Cytotoxicity assessment of test compounds

Figure 2 shows the results from cytotoxicity assays in ZFL cells upon exposure to the tested concentrations of BaP (see Materials & Methods). We did not observe any significant effect even at concentrations above the aqueous solubility which corresponds to 3.8 µg/L or 15 nM ( Mackay et al, 1992).

Based on the cell viability assay results and with respect to environmental concentrations of BaP, 0.03 pM, 0.3 nM, and 30 nM BaP concentrations were used for microarray experiments.

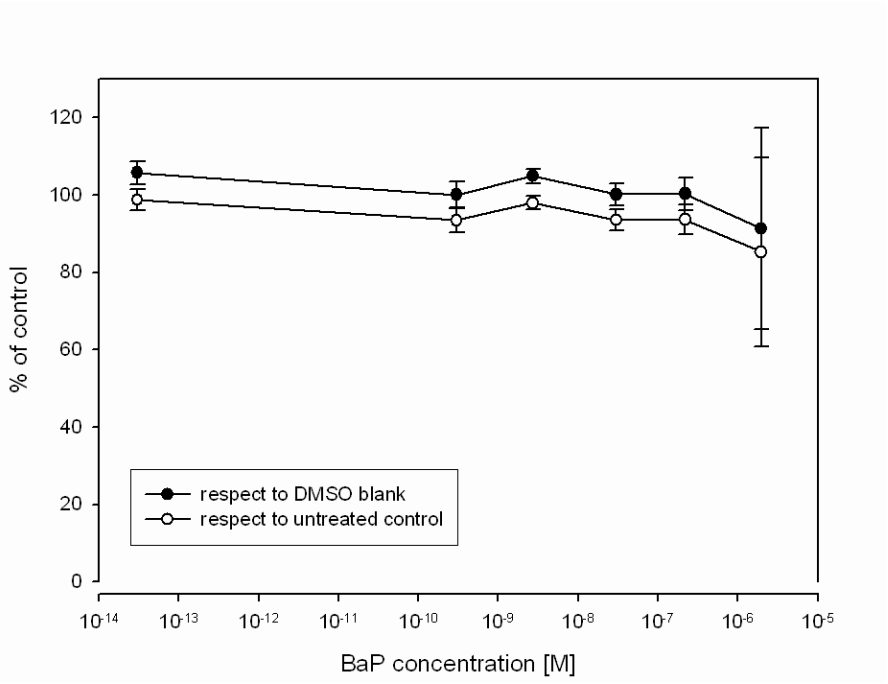



**Figure 2** *Inhibition of cell viability in zebrafish liver cells (ZFL) after 24 h exposure to benzo[a]pyrene (BaP) in 24 well plates for. MTT assay was performed as described above. Closed circles represent the results with respect to the solvent control containing DMSO, open circles represent the same data calculated as ratio with respect to the untreated control. Circles represent the average of three wells, with vertical lines indicating the standard deviation.*

### RNA quality

RNA samples were measured using NanoDrop for quantification and a first quality check. Then 300 ng of RNA were analyzed using the Bioanalyzer using the "Eukaryotic total RNA Nano Kit" according to the protocol provided by the manufacturer. Distinct peaks in the electropherograms and the clear bands on the gel images indicated that no relevant impurities or degradation processes occurred and therefore proved the high quality of the total RNA (Figure 3).

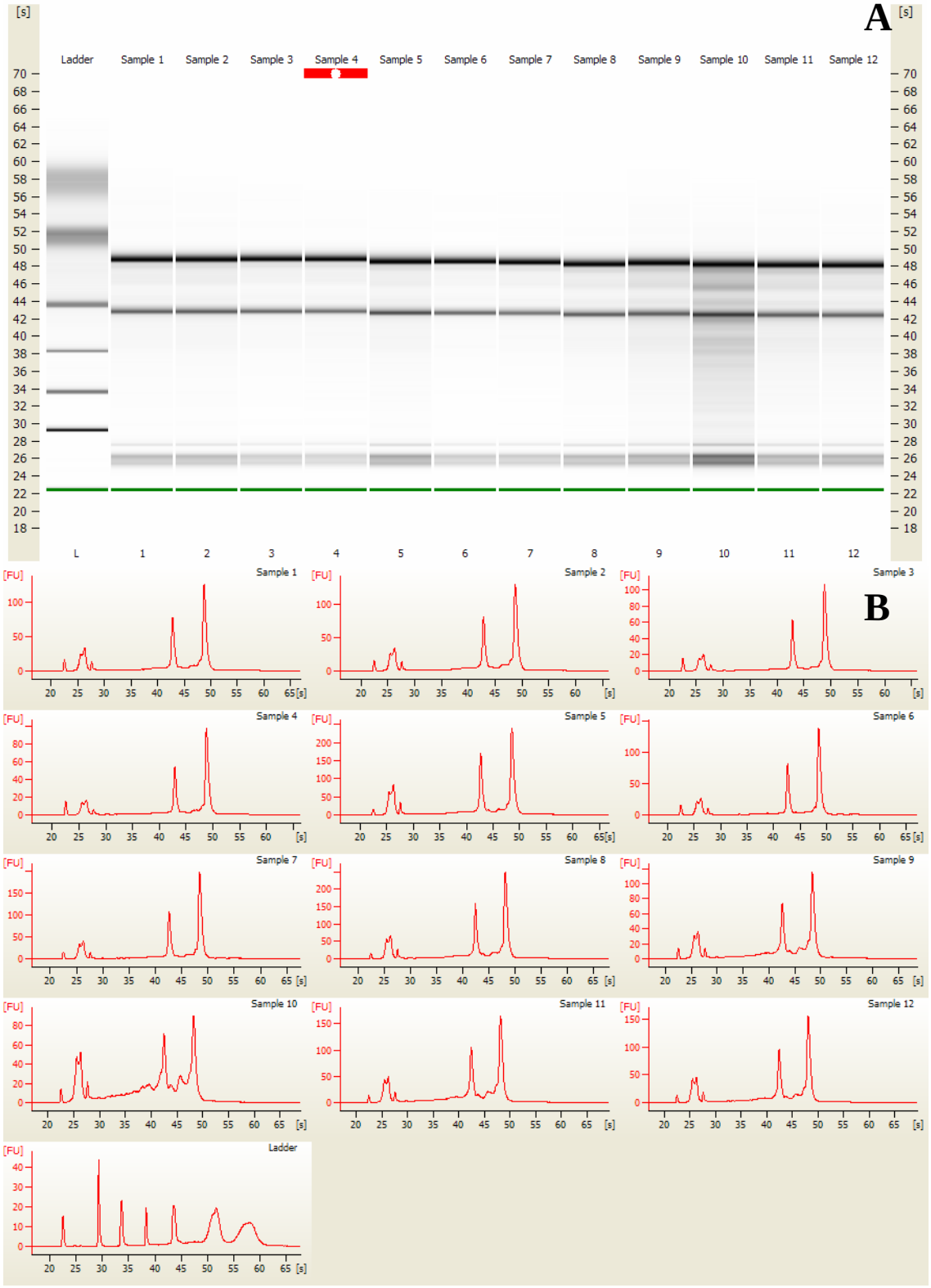



***Figure 3**: Quality control of total RNA on Bioanalyzer. In the upper panel (**A**), RNA from twelve samples from ZFL cells exposed to BaP in three biological experiments is shown. The very left lane shows the ladder containing defined RNA fragments used for estimation of RNA size. Sample 1, 5, and 9 show the control samples from the three experiments, the other samples are the BaP treated ones from the same experiments. The clear bands and the low background show the integrity of the RNA samples. This is confirmed also in panel (B), where clear peaks underline that no relevant degradation has occurred.*

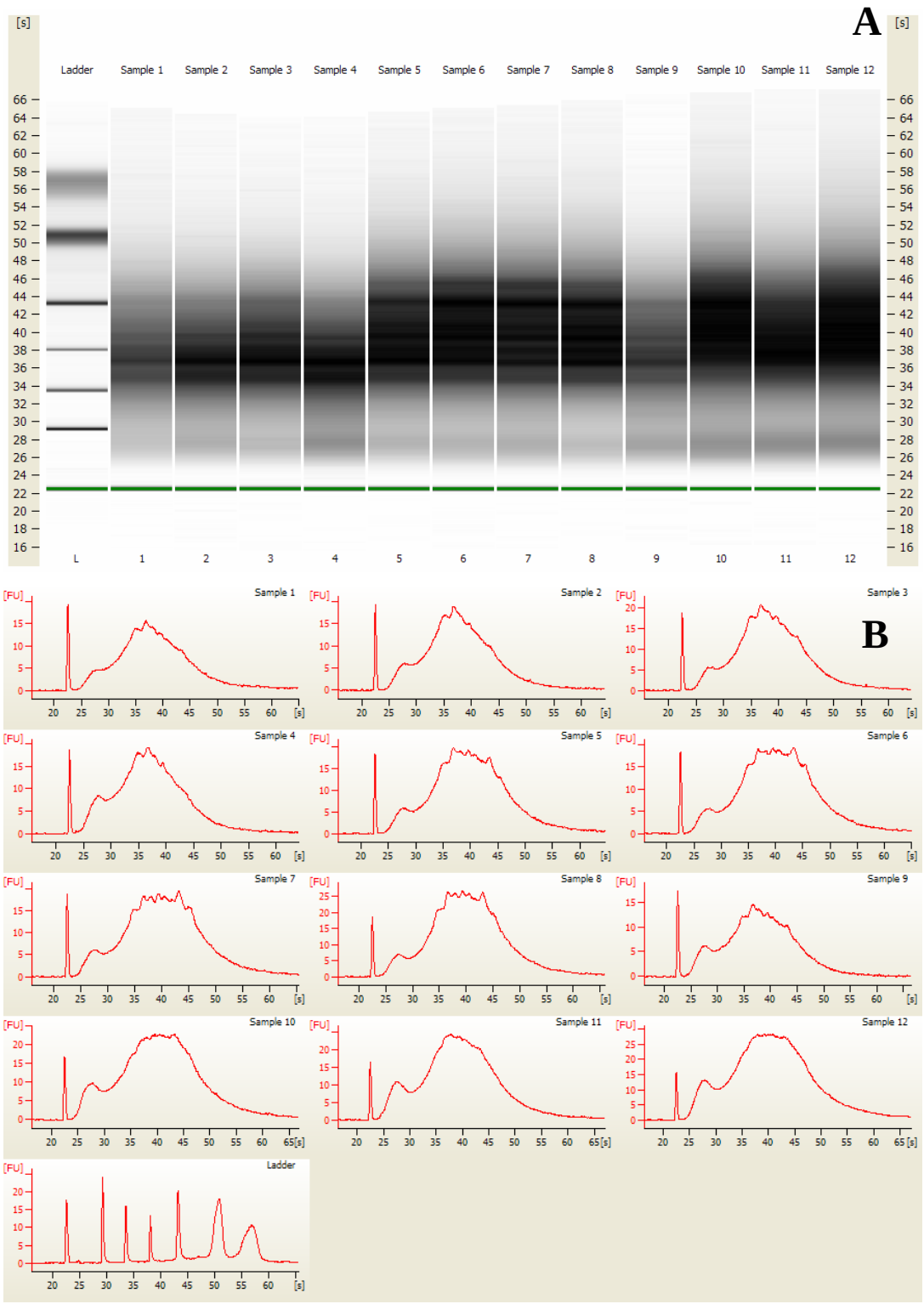



***Figure 4:*** *Quality control of the fragmented biotin-labeled cRNA on Bioanalyzer. In the upper panel (**A**), RNA from twelve samples from ZFL cells exposed to BaP in three biological experiments is shown. Sample 1, 5, and 9 show the control samples from the three experiments, the other samples are the BaP treated ones from the same experiments. The appearance of a multitude of bands of different RNA sizes occur due to the fragmentation of the cRNA samples. However, the good quality of the samples can still be confirmed looking at the electropherograms (**B**) Biotin-labeled cRNA was well-fragmented as confirmed also in this panel, showing a typical bell-like shape with a low baseline. If degradation occurred, curves would be shifted to the left due to a stronger presence of smaller fragments, which is not the case here.*

After transcription of the samples to biotin-labelled cRNA, RNA is fragmented and quality checked again on the Bioanalyzer. Due to the fragmentation, a high number of bands on the gel and peaks on the electropherogram can be observed. The good quality of the cRNA is confirmed by the bell-like shape of the curves in the electropherogram, indicating that no degradation occurred during sample processing (Figure 4). Degradation produces a high number of short fragments which leads to a shift to the left in the electropherograms, which was not observed in our samples.

**Microarray analysis**

Samples were hybridized on the microarrays and microarrays washed and scanned once. Microarray data were imported into the GeneSpring software and a GCRMA normalization was performed. Then, data were normalized per chip and per gene referring to the control within each experiment. After normalization, intensity distributions were similar for all samples (Figure 5).

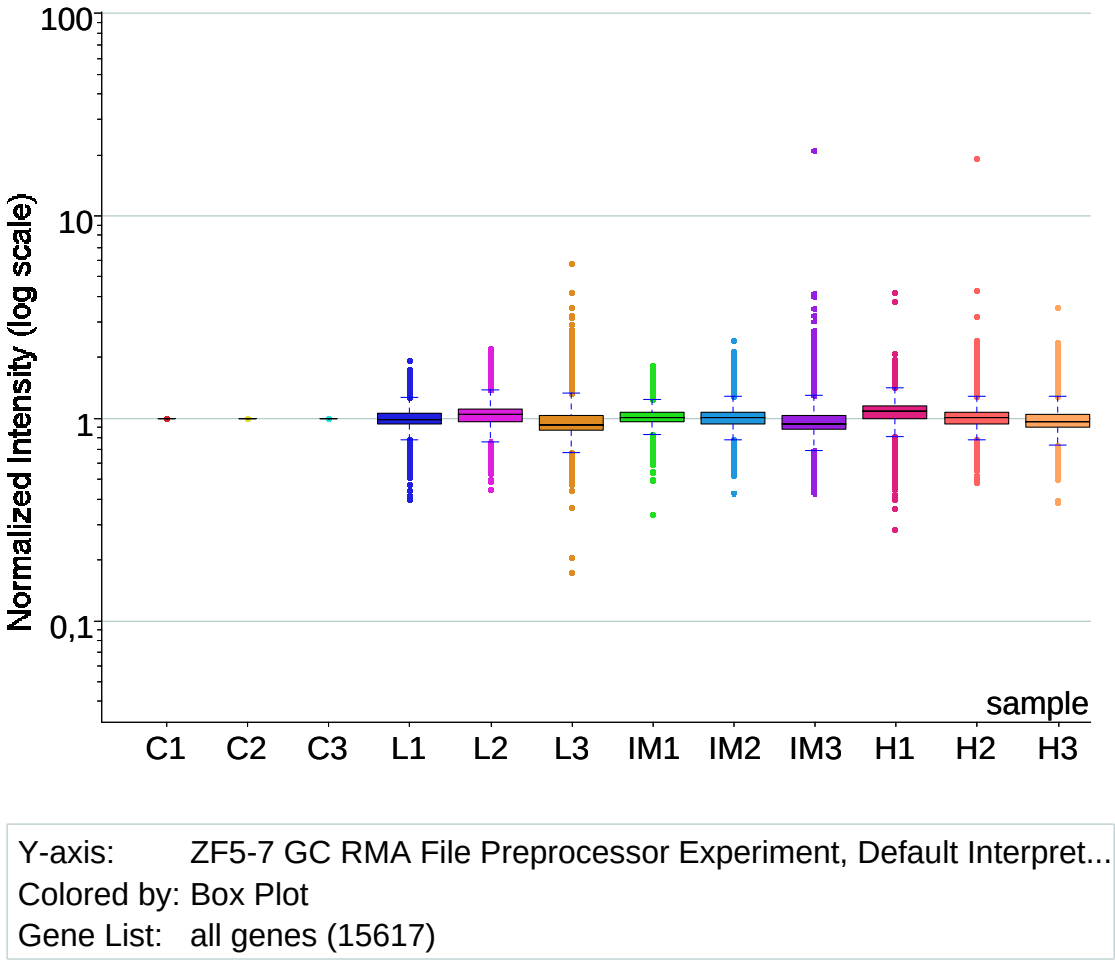
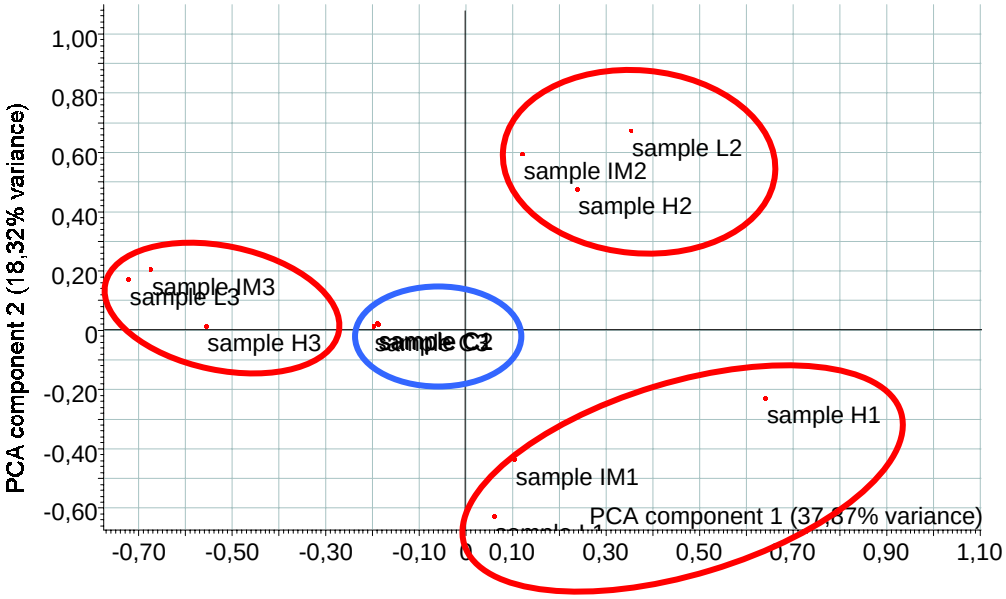



***Figure** 5: Box plot showing results from normalization of microarray data. Gene expression data were normalized first per chip on the median value and in the following per gene within each of the three biological experiments on the control sample (X-Axis: C=Control; L=low BaP concentration, 0.03 pM; IM=intermediate BaP concentration, 0.3 nM; H=high BaP concentration, 30 nM; numbers 1-3 indicate the experimental replicate.)*

After normalization, a PCA (Principal Component Analysis) on condition was performed. This analysis allows clustering the samples: the more similar the gene expression is, the closer the samples are. It can be seen that, due to the normalization method, the control samples come close together in the 2D scatter plot (Figure 6). However, instead of similarities between replicates from different treatments, samples from the same experiments were clustering together, indicating that either the exposure after 24 hours did not affect the gene expression level correlated to the concentrations or a biological variability among the replicates mask the effects so that the samples from the same experiments come closer.




X-axis: PCA component 1 (37,87% variance)
Y-axis: PCA component 2 (18,32% variance)
Conditions: ZF5-7 GC RMA File Preprocessor Experiment, Default Interpretation

***Figure 6:*** *Scatter plot showing the results of the principal component analysis on conditions. A principal component analysis clustering the samples according to there similarity was performed. It can be observed that samples from the same experiment are more similar to each other than samples from the same treatment. Red circles show the BaP treated samples, which are close together independent of BaP concentration. Only the control replicates from the three experiments (blue circle) are shown to be similar.*

Nevertheless, additional data evaluation revealed some genes which were regulated by the treatment with BaP. Normalized gene expression data from each different treatment (low, intermediate and high concentration) were filtered on "fold change" compared to the control. Applying a limit of 1.5 fold change, 14 genes were regulated due to the low concentration of BaP, 17 due to the intermediate concentration and 8 due to the high BaP concentration (Figure 7). Three of those were common to all three BaP concentrations used for treatment (Table 1).

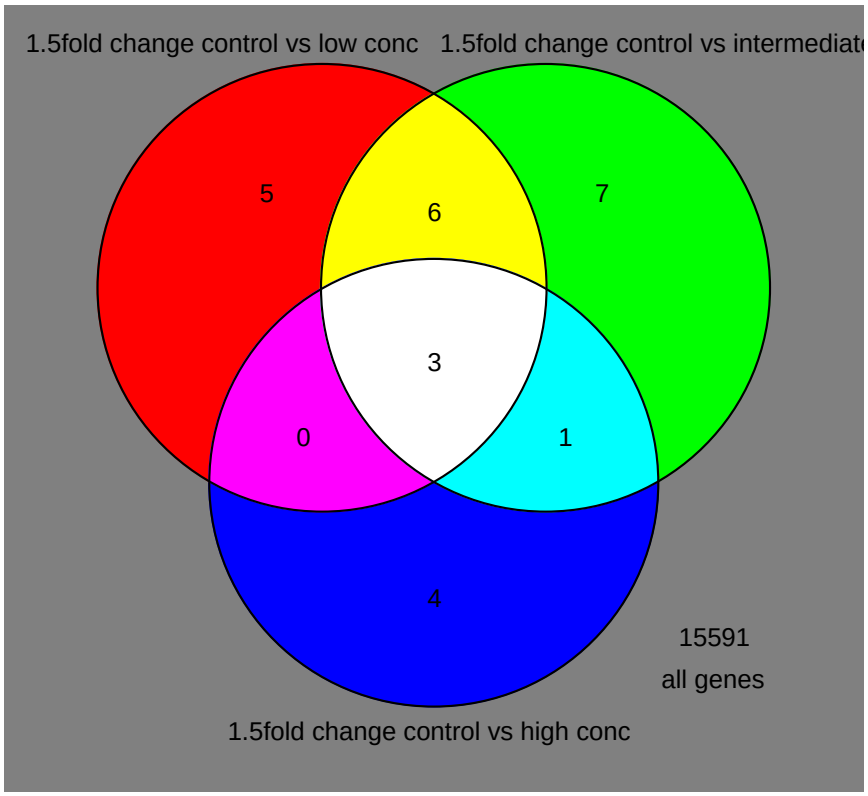



***Figure 7**: Venn Diagram showing overlap of regulated genes between the different treatments (BaP concentrations) applying a filter of 1.5 fold change in gene expression.*

However, since these were not all statistically significant (1-way ANOVA, $p<0.1$) we applied a threshold of 2 fold regulation. Utilizing a filter of 2fold change, we identified the following genes as regulated (Table 2):

*cyp1a1*, which is involved in the metabolism of xenobiotics, was upregulated up to 4.0 fold in the highest BaP concentration and slightly (2.8 fold) in the intermediate. However, it was not included in the regulated genes in the lowest BaP concentration.

*zgc:86759* was down-regulated 2.1 fold only in the lowest BaP concentration treatment. Its function is not yet known, it encodes for a hypothetical protein (LOC415222).

cb508 was the only gene identified which was down-regulated in all three BaP concentrations in comparison to the control. It encodes for a protein belonging to the aldehyde dehydrogenase 3 family (member D1). It was down-regulated by a factor of only slightly above 2, but still significantly regulated (1-way ANOVA, $p<0.1$).

*Table 1: Genes which were found to be more than 1.5-fold regulated in the different BaP treatments compared to the control in all 3 replicates.*

| Gene Name | Description | regulation due to treatment[1] |
|---|---|---|
| Dr.24938.1.S1_a_at | aldehyde dehydrogenase 3 family, member D1 | L, IM, H |
| Dr.24938.1.S1_x_at | aldehyde dehydrogenase 3 family, member D1 | L, IM, H |
| Dr.13774.1.S1_at | Transcribed locus | H |
| Dr.25322.1.S1_at | Wu:fb75b09 | H |
| Dr.5040.1.S1_at | cytochrome b5 type A (microsomal) | H |
| Dr.6174.1.A1_at | wu:fj39b01 | H |
| Dr.9478.1.S1_at | cytochrome P450, family 1, subfamily A | IM, H |
| DrAffx.3.1.A1_at | zgc:86759 | IM, H |
| AFFX-Dr-acta1-5_at | similar to skeletal muscle actin | IM |
| Dr.13563.1.S1_at | methionyl aminopeptidase 2 | IM |
| Dr.20715.1.S1_at | signal sequence receptor, alpha | IM |
| Dr.26388.1.S1_at | hypothetical LOC566409 | IM |
| Dr.3208.1.S1_at | zgc:101580 | IM |
| Dr.8301.4.S1_a_at | heat shock transcription factor 1 | IM |
| Dr.14555.1.S1_at | influenza virus NS1A binding protein a | L, IM |
| Dr.19537.1.S1_a_at | si:dkey-31f5.7 | L, IM |
| Dr.20106.1.S1_at | apoptosis inhibitor 5 | L, IM |
| Dr.350.1.S1_at | retinoid x receptor, beta b | L, IM |
| Dr.5418.2.S1_at | topoisomerase (DNA) II alpha | L, IM |
| Dr.7539.2.S1_at | similar to zinc finger protein 289, ID1 regulated | L, IM |
| DrAffx.1.55.S1_at | ELOVL family member 5, elongation of long chain fatty acids (yeast) | L, IM |
| Dr.18643.1.A1_at | Transcribed locus | L |
| Dr.20948.1.S1_at | MCM3 minichromosome maintenance deficient 3 (S. cerevisiae) | L |
| Dr.25180.1.S1_at | WD repeat domain 3 | L |
| Dr.25758.1.S1_at | knypek | L |
| Dr.5760.1.S1_at | activin A receptor, type IB | L |

[1] Regulation of more than 1.5-fold change in all three replicates upon exposure to BaP at high (H), intermediate (IM) or low (L) concentrations.

*Table 2: Genes which were found to be regulated in the different BaP treatments compared to the control in at least 2 out of 3 replicates for more than 2fold.*

| Gene Name | Description | regulation due to treatment[1] |
|---|---|---|
| Dr.9478.1.S1_at | cytochrome P450, family 1, subfamily A | H (4.0) |
| Dr.5040.1.S1_at | cytochrome b5 type A (microsomal) | H (2.5) |
| Dr.20106.1.S1_at | apoptosis inhibitor 5 | H (1.0) |
| Dr.13774.1.S1_at | Transcribed locus | H (2.0) |
| AFFX-Dr-acta1-5_at | similar to skeletal muscle actin | H (1.2) |
| DrAffx.3.1.A1_at | zgc:86759 | IM (0.6) |
| Dr.24938.1.S1_a_at | aldehyde dehydrogenase 3 family, member D1 | L (0.4), IM (0.4) |
| DrAffx.1.55.S1_at | ELOVL family member 5, elongation of long chain fatty acids (yeast) | L (1.3) |
| Dr.25322.1.S1_at | Wu:fb75b09 | L (1.2) |

[1] Regulation of more than two-fold change in at least 2 out of 3 replicates upon exposure to BaP at high (H), intermediate (IM) or low (L) concentrations. The average value of fold change is shown in brackets.

## Discussion

Biological effects elicited by environmental pollutants such as BaP can be detected at various levels. Early effects will be found at molecular level, as this is one of the first targets in the cell (Moore, 2002). Therefore, the detection of effects at gene expression level can serve as early-warning tool.
The measurement of gene expression levels upon exposure to a chemical can both provide information about the mechanism of action of toxicants and form a sort of "genetic signature" from the pattern of gene expression (Lettieri, 2006). For screening a large number of genes and identification of gene expression patterns, DNA microarrays are the method of choice since they allow a simultaneous investigation of complete genomes.

Before starting with the microarray experiments, BaP acute toxicity was assessed in the MTT cytotoxicity assay. No significant acute toxicity was found at none of the concentrations, which might be due to the really low acute effects, or due to the exposure time of 24 h. The doubling time assumed for ZFL cells is 72 h (Gosh et al., 1994). Thus, acute effects of BaP on cell proliferation might not be detected after 24 h. However, also in other studies using e.g. rainbow trout cells (Bopp et al, 2006) no direct cytotoxic effects were found.

Studies with zebrafish *in vivo* mostly use concentrations at or above the aqueous solubility limit of the test compound. Since no significant acute toxicity was found, the following three concentrations were chosen for microarray experiments: the lowest concentration was chosen at environmental background level with 0.03 pM, the intermediate concentration at 0.3 nM which should not have an acute effect as well, and to compare to other studies at the highest concentration which lies around the aqueous solubility with 30 nM.

We analyzed the gene expression profiling of the liver zebrafish cell lines (ZFL) exposed to Benzo[a]pyrene (BaP). The labeled RNA of three experimental replicates were hybridized on a 14K array (Affymetrix) and analyzed using the software Gene Spring (Agilent). Nine genes were regulated in two out of three replicates with a fold change of more than 2.0. The highest up regulated gene was the gene encoding for the cytochrome P450 CYP1A with a 4.0 fold and 2.8 fold change with respect to the control in the high and intermediate condition, respectively. An up-regulation of CYP1A upon exposure to dibenzodioxins and dibenzofurans, which act similar to BaP, but which are mostly more potent inducers, was shown already for the ZFL cell line, confirming the regulation of that pathway due to dioxin-like acting pollutants (Miranda et al, 1993; Henry et al, 2001). Moreover, our results confirm the role of the cytochrome P450 in response to the environmental pollutants as shown in other organisms (Ma, 2001).

Zebrafish CYP1A gene clusters in the group of fish CYP1A1s, but, in respect to other fishes which often have multiple CYP1A genes like in the rainbow trout (Råbergh et al, 2000), only one gene has been found in zebrafish (Yamazaki et al., 2002). Further in comparison with other CYP1As of various species the zebrafish CYP1A shows high homology with human CYP1A1, A2, rat CYP1A1 and A2 and rabbit CYP1A1, implying a relatively greater similarity with mammalians CYP1As. Studies performed *in vitro*, showed that the recombinant CYP1A expressed in yeast (Chung et al, 2004) was active and capable also to metabolize two common substrates such as BaP, and 7-ethoxyresorufin (7-ER). The same authors recently analysed the formation of metabolites by the recombinant zebrafish CYP1A in yeast and they could identify at least six metabolites (Miranda et al, 2006) producing DNA adducts believed to play a major role in chemical carcinogenesis (Peltonen and Dipple, 1995). The increased transcription of CYP1A would explain, then, why we did not observe any effect on cell viability after 24 hours since BaP is metabolized and its products would bind to DNA. The DNA-adducts could probably affect the cell cycle which occurs in a 72 hours cycle, longer than the experimental exposure time.

Another regulated gene is the cytochrome b5 type A with a fold change of 2.5 fold in the highest condition (see Table 2). Cytochrome b5 is a membrane bound heme-protein which acts as an electron carrier for several membrane bound oxygenases. It belongs to the family of cytochrome b5, and is homologous to the human and other species. The role of the protein, which serves as an electron donor in a number of biochemical reactions, includes microsomal fatty acid desaturation and

hydroxylation. The possible explanation of its up regulation could be its regulatory role in CYP-mediated reaction as shown by Duarte et al. (2007), who found that human cytochrome b5 is stimulating the activity of CYP1A2, 2A6 and 2E1.

In the other two conditions (intermediate and low concentrations), two genes could be annotated, with one encoding for the aldehyde dehydrogenase 3 family, member D1 (ALDH) which is down-regulated in all three tested concentrations with a 2.0 fold change, while the other encodes for ELOVL slightly up regulated only at low concentration.

Aldehyde dehydrogenase 3 family, member D1 (aldh3d1) is an enzyme NAD Oxidoreductase that catalyzes the irreversible oxidation of a wide range of aldehydes to their corresponding carboxylic acids (Xie et al, 1996). In mammals, the ALDH class 3 gene is activated in liver by a variety of xenobiotics including polycyclic aromatic hydrocarbons (PAH) such as 3-methylcholanthrene (3MC), and dioxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Torronen et al, 1981; Dunn et al, 1989). The activation of the gene requires the functional Ah receptor and Ah receptor (Sogawa et al, 1995). Interestingly we observed a down regulation in all conditions. This occurs probably due to impairment of the functional Ah receptor and this would enhance the toxicity in long term effects at molecular level. Additional studied will be required, such as using quantitative PCR to confirm the down regulation.

## Conclusion

Our microarray experiments had two major aims (i) to characterize gene expression profiles and (ii) to identify sensitive molecular biomarkers upon exposure to these BaP concentrations explained above.

(i)The identification of gene expression profiles allows the comparison of pathways involved in the mode of action of different pollutants. In the case of BaP exposure at the concentrations used in this study, only few genes were regulated more than two-fold compared to the control. So the possibilities for interpretation of the gene expression profile are limited. However, some single genes could be identified as regulated.

(ii)Among the single genes, some were regulated strongly e.g. CYP1A and cyb5 confirming their role in the xenobiotic response. The other gene is aldh3D1, which is down-regulated in all three conditions and potentially could be used as molecular biomarker. More investigation will be required to confirm the results. If it can be proved, this gene will constitute a very useful and easy to measure biomarker of exposure to BaP.

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**Abstract**
The Zebrafish *Danio rerio*, in the last decade, has been recognized as the best model among the vertebrate model organisms. The exquisite features and the high homology with the mammalian system have facilitated its application in many field e.g. eco-toxicology, biomedicine, genetics and physiology. The complete sequencing of the genome provided enormous value giving the possibility to develop a DNA microarray for gene expression analysis. Gene expression represents a unique way of characterizing how cells and organisms adapt to changes in the external environment (Lettieri, EHP, 2006). The measurements of gene expression levels, upon exposure to a chemical, can be used both to provide information about the mechanism of action of the toxicant, and also to form a sort of "genetic signature" for the identification of toxic products. Benzo[a]pyrene (BaP) is a well-known chemical pollutant, which belongs to the family of polycyclic aromatic hydrocarbons (PAHs) which are formed during combustion processes. Extensive studies have been published on the metabolism and the toxicity of PAHs, as well as epidemiologic studies which suggest a role of the PAHs in carcinogenetic and mutagenic effects in many species.
BaP as well as other PAHs is included in the Priority Hazardous Substances adopted in November 2001 by the new Water Framework Directive (2000).
Our studies focused on the analysis of gene expression changes in a liver cell line of Zebrafish upon exposure to BaP concentrations close to environmental level using DNA Microarrays.
In our gene expression analysis, we identified nine genes, among them the cyctochrome P450 CYP1A involved in the xenobiotic response and the cyb5 whose role in the activation of human CYP1A2 was recently reported in the literature.

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