
SZENT ISTVÁN UNIVERSITY

# INVESTIGATION OF BY-PRODUCT COMPOUNDS REMAINING IN CONVENTIONAL WATER DISINFECTION TECHNOLOGIES ON FISH MODEL

**PhD thesis**

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# 1 Introduction and objectives

## 1.1 Introduction

Water is the foundation of earthly existence and the engine of human development. At the same time, however, humankind's water demand is increasing, but industrial and social development has caused severe damage to the Earth's water resources. Due to the great development and industrialization it is becoming increasingly difficult to obtain drinking water of sufficient quality and quantity.

People living in different parts of the world face different drinking water problems. People in developing countries are struggling with famine because they cannot produce enough food without water. In addition to water scarcity, pollution of available water sources is also a major problem. People suffer from many diseases (malaria, typhoid fever, salmonellosis, hepatitis, infection with Escherichia coli) in these countries due to the lack of proper hygiene conditions. In addition to biological hazards, industrial pollution in these countries is also significant, due to light or poor environmental standards.

Water sources are available in developed countries, but they are polluted by the modern way of life. In these areas, industry and agriculture are the two major sources of pollution. It is important to mention that many pollutants are also released from our households. In order to remove these substances and prevent waterborne diseases, various water treatment procedures are applied to municipal wastewater and raw water during drinking water production.

The presence and importance of disinfection by-products (DBPs) in water purification was first highlighted by Dutch chemist Johannes Rook. Over the past few decades, more than 600 substances have been identified as being produced during water treatment, but only a fraction of these have been assigned limit values under existing regulations (Government Decree 98/83, Government Decree 201/2001 (X.25.), EPA 63 FR 69390). To date, we do not have sufficient information on the toxicological aspects of many substances.

Although many publications have been published on DBP in recent years, this topic is still receiving little attention. There are many reasons for this, such as the fact that a great variety of compounds can be chemically classified in this group. They are found in very low concentrations in natural water bases and in drinking water, making them difficult to detect, even with modern analytical methods. Untreated drinking water, which can be a vector for many diseases, is generally not an acute problem for wildlife or consumers. Their

real danger lies in this, as it can take years or decades for harmful effects to develop.

So the problem is very complex. In addition to the dissolved organic matter (DOM-dissolved organic matter) in water, compounds previously formed in the water base can also form DBPs. These include the active ingredients of drugs, many of these compounds are widely used. These include hormonal contraceptives, anti-osteoporosis drugs and the most commonly used analgesic and anti-inflammatory drugs.

Many of the anti-inflammatory and analgesic formulations are available to the general public in the form of non-prescription drugs. As a result, they can easily get into the water due to improper waste management. The largest active ingredient in the world is ibuprofen (2- (4-isobutylphenyl) - propionic acid), which is present in the range of µg/l in natural waters. Concerned with this active ingredient is that it is found in the effluent of sewage treatment plants, together with its degradation products, also in the concentration range of µg/l. The mother molecule generates a number of decomposition products through heat transfer and oxidative treatment, including 4-ethylbenzaldehyde (EBA).

It is less known about the compound that it is found in the flavors of the chemical merchants' palette, because it has an almond scent, similar to the artificial almond flavor, the benzaldehyde. Unlike benzaldehyde, this SDS does not contain toxicological information. The scientific opinion of the European Food Safety Authority (EFSA) on flavorings in 2012 did not raise any concerns about the substance. All of this is surprising, because this document only mentions an unpublished publication on EBA toxicity. EFSA set the MSDI (Maximum Calculated Intake Value) for the substance at 0.37 µg/person/day. EBA is a decomposition product of the most commonly used drug, the ibuprofen's active ingradient, which can be found in effluents even after wastewater treatment. Therefore, it is vital to map its impact on living organisms. Although EFSA has established an MSDI value, as we can see, it can enter the human body from a variety of sources, so it can easily produce toxic effects.

In addition to EBA, I investigated another DBP compound, 2-4-difluoroaniline (DFA). This substance is used as an intermediate in the manufacture of pharmaceuticals, paints and pesticides. In acute cases it causes methaemoglobinaemia and is highly nephrotoxic.

Because fish spend their entire life cycle in the water, they are excellent model animals for this type of study, and zebrafish is an internationally recognized and well-known model organism. During my research, I

considered the embryo examination of both the EBA and DFA extremely important, as no information is available on the developmental effects of the substances. These experiments may be a good starting point for human health studies, especially microarray studies on fish embryos, which are designed to detect changes in gene expression during embryonic development.

### 1.2 Objectives

The aim of my work was to study the effects of two water purification by-products, EBA and DFA, on zebrafish embryos. We have very little information about EBA. It is worrying that it can be formed as a degradation product of ibuprofen, the most widely used analgesic compound. It is also available as a flavor and fragrance. My aim was to investigate the effects of EBA and DFA on embryo development and to determine the LC values of compounds in acute tests. Furthermore, my aim was to study the effect of EBA on gene expression using a microarray assay. I wanted to get an answer as to whether I could support the result of the transcriptome analysis with various classical toxicological tests, thus increasing the amount of toxicological information available on EBA.

# 2 Materials and methods

## 2.1 Used animals, housing conditions and experimental sites

Acute embryo tests, comet assay, and micronucleus test and related treatments were performed at the Department of Aquaculture, Faculty of Agriculture and Environmental Sciences, Aquaculture and Environmental Safety Institute, Szent István University. Microarray analysis was performed at the Helmholtz Environmental Research Center (*Helmholz-Zentrum für Umweltforschung* - UFZ) in Leipzig, Germany, in Dr. Stefan Scholz's research group. Primary preparation of the samples was also carried out at the Department of Aquaculture and the Regional Environmental Knowledge Center of Szent István University.

In each case I used AB line zebrafish. The fish were kept in a recirculation system (Tecniplast ZebTec, Techniplast, Buggugiate, Italy). The water temperature in the system was 25 ± 0.5 ° C, the pH was 7.5 ± 0.2 and the conductivity was 525 ± 50 μS. The fish were kept in a light program of 14 hours light and 10 hours dark. Fish were fed twice daily with SDS Small Gran (SDS - Special Diets Services Inc.) as a live feed twice a week for Artemia spp. (SERA GmbH.) nauplius.

The studies were carried out in accordance with the authorization for the animal test "Toxicological studies on fish" (XIV-I-001/2303-4/2012).

## 2.2 Experiments

### 2.2.1 Preliminary tests

As no acute toxicity data for zebrafish embryos were available for either EBA or DFA, I conducted preliminary experiments before initiating acute tests to determine appropriate treatment concentrations for the 120-hour embryo study. The range finding test for both compounds was 200; 100; 50; 25; 12.5; 6.25 mg/l concentrations were used, and the water of the recirculation system was used as a control. 20-20 embryos were placed in each group in 5 cm Petri dishes in 10 ml solution. Based on these, I was able to delimit the concentration range that proved to be appropriate for the individual substances in the 120-hour study.

### 2.2.2 120 hour embryo tests

For acute embryo tests, the eggs were collected after synchronized spawning and checked for fertility using a stereo light microscope, with normal light (Leica M205, Leica DFC 425C camera, LAS V 3.8 softver).

The tests were performed on both 2-4-difluoroaniline and 4-ethylbenzaldehyde according to the OECD 236: Fish embryo acute toxicity test with an extended study time of 120 hours. Embryos were placed in 24-well tissue culture plates (1 embryo/well) one hour after fertilization, with 12 embryos at each concentration in 4 replicates. Embryos were treated with the following concentrations: EBA: 0.2; 0.5; 1; 5; 10; 12; 15; 17; 18; 19; 21; 23; 25; 27; 29; 30; 35; 40; 50; 60 mg / l; DFA: 500; 450; 400; 350; 300; 250; 220; 200; 180; 100; 50; 1 mg/l. As mortality was not complete with DFA at 200 mg/l, treatment concentrations were significantly increased.

As a control, I used water from the ZebTEC fish housing system. Plates were incubated at 27 °C (Sanyo MIR-154). I examined all embryos daily under a microscope until 120 hours post-fertilization (120 hpf). I determined mortality, looked for morphological abnormalities, and imaged embryos 72 hours after fertilization with 30x magnification with lateral orientation. Dose-effect curves were constructed with mortality as the end point. Based on these, I was able to determine the $LC_{10}$ and $LC_{50}$ values every 24 hours up to 120 hours after fertilization (120 hpf) with the SPSS 23.0 software package. In each case, the complete experiments were repeated three times.

### 2.2.3 Determining LC values in the 96-120 hpf exposure window

By the age of 96 hours, the embryo undergoes the most intense part of its development, parts of the digestive system are developed, the mouth of the animal is opened, hormone synthesis and nuclear receptors at this time, making embryos more sensitive to hormonal effects at this stage of life.

Embryos were placed in 24-well tissue culture plates at 1 hpf and treated at 96-120 hpf at 10, 15, 20, 22, 25, 27, 30, 32, 35, 40 mg/l concentrations, 12 replicates per concentration, the plates were incubated at 27 °C. At the end of the study, digital images of the embryos were made at 30x magnification with lateral orientation to document morphological changes. Using mortality as an endpoint, I took a dose-response curve for this stage of life to determine $LC_{10}$ and $LC_{50}$ using SPSS 23.0 software package.

### 2.2.4 Microarray assay and transcriptome analysis

I used the LC10 value in the 96-120 hpf exposure window as the highest concentration to investigate sublethal effects. At 5 concentrations (1.6; 3.2; 6.4; 12.8; 25.6 mg / l) I treated 6-6 groups and 50-50 embryos per group. The treatment was carried out in 5 cm Petri dishes and the embryos were incubated at 27 °C.

Embryos were homogenized in 1.5 ml Eppendorf tubes with 200 µl trizole (TriReagent, Izinta), and stored at -80 °C until use.

RNA isolation was performed with the 5Prime Phase Lock Gel Heavy Kit (5Prime GmbH, Germany) according to the manufacturer's protocol. The isolated RNA samples were then purified using a Quiagen RNEasy Mini Kit (Qiagen GmbH, Germany), RNA concentrations were quantified using NanoDrop 2000 (Thermo Scientific, USA), Agilent 2100 Bioanalyzer (Agilent Technologies, USA), using Agilent RNA 6000 Nano Assay Kit (Agilent Technologies, USA).

For the microarray analysis I used Agilent SurePrint G3 Custom GE 8x60k (Amadid G4102A, Agilent Technologies, USA) array. Samples were prepared, labeled, and then applied to microarray slides using the 'One-Color Microarray-Based Gene Expression Analysis, Low Input Quick Amp Labeling Protocol' specifically for Agilent Gene Expression Oligo Microarray slides (Version 6.6, September 2012, Agilent Technologies, USA). Microarray plates were read at Genovia GmbH (Zwenkau, Germany) using an Agilent DNA Microarray Scanner. The intensity of the fluorescent signal in the fields on the microarray slide was obtained using Agilent Feature Extraction software (version 10.7.3.1). I searched for mRNA transcripts whose expression differed from that of the control group at at least one concentration and then performed principal component analysis using Agilent GeneSpring 12 GX software.

After that, I examined the gene expression patterns of EBA-influenced genes and identified three gene expression clusters using the K-mean clustering algorithm, also using the Agilent GeneSpring 12 GX software.

After that, the biological functions of the genes belonging to each cluster were determined by biological process analysis by DAVID (DAVID Bioinformatics Resources 6.8, Leidos Biomedical Research Inc., Frederick, Maryland, USA).

I used the GeneMANIA prediction server to study the concentration-dependent inhibition of EBA-dependent genes in Cluster2. I used Uniprot and ZFIN databases to map the role of genes in the gene network.

### 2.2.5 Comet assay on 1 dpf embryos

The assay was based on the method of Žegura and Filipić (2004), using an organic peroxide, Luperox® (Sigma) as a positive control. 24 hpf embryos are required for testing. Embryos 1 hour after fertilization treated with 0.1; 0.5; 1; 2.5; 5; 7.5 10 and 12 mg/l EBA and 20 and 40 mg/l Luperox® solution, using ZebTEC water as a negative control. The highest

used concentration was about half of the $LC_{10}$ value determined in the acute embryo test. The embryos were treated in a 5 cm diameter Petri dish, incubated for 24 hours at 27 °C. I treated 6-6 embryos per concentration in 3 replicates. Fully frosted slides (Manzer-Gläser) were placed in methanol overnight. After drying the plates, I applied the first layer of agarose (Normal melting point agarose, 1%, Sigma), 80 µl / well (2 plates / plate), which was immediately covered with a 24x24 mm cover plate (Menzel-Gläser) and then covered and placed on ice for 2 minutes. After the agarose had solidified, the coverslips were carefully removed. Meanwhile, I prepared the embryos and removed the eggshell with 4 mg of pronase enzyme (Sigma) (4 minutes of pronase treatment and 4 rinses of system water). One embryo per field was embedded in 60 µl of 1.5% low melting point agarose (Sigma) in PBS (Phasphate buffered saline, Sigma), and the embryos were gently pressed to disperse the embryos in the gel. Slides were placed on ice for 2 minutes and then lysed in lysis solution after removal of the coverslips. An important step in reconstituting the solution is to adjust the pH to 10 and add 2.5 ml Triton X-100 (Sigma) before use and homogenize by careful shaking. Slides were placed at 4 °C for 1 hour. Because of the photosensitivity of DNA, I continued to work in looming light. After lysis, the slides were drained and transferred to an electrophoresis bath which was previously filled with electrophoresis solution (3000 ml solution: 1.11g EDTA, 90 ml 10N NaOH), incubated for 20 minutes at 4 °C, and the electrophoresis device turned on (BioRad PowerPac Basic, BioRad, USA), operated at 25 V and 400mA for 20 minutes.Then the slides were removed, drained and placed in neutralization solution (12.1144g TRIS, pH 7.5 for 250ml) and incubated for 15 minutes at 4 °C. The slides were then placed in a container lined with moist paper, sealed and stored at 4 °C until photographed. At the end of the comet assay protocol, the plates were stained with 2µg/l ethidium bromide solution and photographed with a Nikon Eclipse E600 microscope, 510-560 nm wavelength fluorescent light, QImaging MicroPublisher 3.3 RTV camera and related QImaging software. Subsequently, I determined the % of DNA in the tail of comets using TriTek CometScore ™ Freeware 1.5 software. I performed 45 measurements at 3-3 repetitions per concentration. I used the Kruskal-Wallis test ($H = 978.714$; $df = 10$, p-value $<0.00001$) to compare concentrations, and Dunn's test (SPSS 23.0 software package) was used for pairwise comparison. The experiment was repeated three times.

### 2.2.6 Micronucleus test on adult fish

For the micronucleus test I used adult 6-8 months old individuals because of the specifics of the method. The fish were derived from the AB zebrafish line, as in the other studies. The experimental animals were kept under the housing conditions described az Chapter 2.1.. The test was done in a semi-static system and the treatment solution was changed every two days.

Based on previous literature data, the highest treatment concentration used was the $LC_{10}$ value calculated at 96 hours exposure (11 mg/l), which prevents premature death of fish, but may cause sublethal effects. The other two were $LC_{10}/2$ (5.5 mg/l) and $LC_{10}/4$ (2.75 mg/l). From each concentration I formed 3 groups with 8-8 fish per group. The treatment lasted for 21 days, sampled every 7 days, and 4-4 fish were sampled at each concentration.

For each sample, to suppress the animals were anesthetized, using MS-222 and decapitated, and their blood immediately applied in thin-layer to a microscope slide (Menzel-Gläser). After the blood samples dried, they were stained with Hoechst 33342 (5μM). Samples were examined using an epifluorescence microscope (Olympus BX-51). At 400X magnification, blue fluorescent micronuclei were counted in 2,000 randomly selected erythrocytes with intact cytoplasm on each slide). Cells considered to be micronucleated: 1. have a micronucleus smaller than 1/3 of the nucleus; 2. are in the same plane; 3. the micronuclei and nucleus fluoresce with the same color and intensity, 4. round or oval; 5. the micronucleus and nucleus are clearly separated. Descriptive statistics were generated using STATISTICA 12 software package (StatSoft, Tulsa, OK, USA). Poisson regression ($p < 0.05$) was used to compare each group.

### 2.2.7 Examination of regeneration capacity on larvae

The 96 hpf zebrafish larvae were anaesthetized in MS-222 solution prior to injury. Subsequently, the caudal end of tail of the anesthetized larvae was removed under sterile conditions with a razor blade under a light microscope. I made sure that the direction of damage was perpendicular to the longitudinal axis of the animal and resulted in a more even slaughter surface. After the injury, I photographed the animals under anesthesia, under a light microscope. Subsequently, the larvae were carefully rinsed with the water of housing system and placed individually in 24-well tissue culture plates containing the treatment solutions. After 24 hours exposure, the animals were carefully rinsed with system water following the

anesthesia described above. Photographs were taken under a light microscope and each subject was placed under control conditions. I photographed the larvae again at 48 and 72 hours after the injury. Images were analyzed with ImageJ software (NIH & LOCI, Wisconsin, USA). The level of regeneration was determined by increasing the length of the longitudinal axis is defined by the laryngeal gyrus and the length of the rectilinear section from the point of intersection of the perpendicular to it. (The starting point of the actual measured section was the well-defined end of the spine and the end was the actual end of the regenerating caudal fin.) The length of the currently regenerated section ($\Delta l$) was determined by subtracting the starting distance ($d0$) from the actual total measured distance ($dn$, $n = 24, 48, 72$ hpA).

# 3 Results

### 2.2.1 Preliminary tests

No toxicity data were available on the effects of the test compounds on embryos, so the first step was to determine the concentration range in which they are toxic.

In the first preliminary experiment, 200; 100; 50; 25; 12.5 and 6.25 mg/l concentrations were used in the limit test for both compounds. DFA is shown to have low toxicity on zebrafish embryos, as there was little mortality in the treated groups at 100 mg/l after 120 hours. Further increasing the concentrations, I found that the expected $LC_{50}$ values would be between 150 and 250 mg/l.

Preliminary experiments on EBA at the highest concentrations resulted the death of embryos within a few hours. The expected $LC_{50}$ values were estimated between 15 and 35 mg/l.

## 3.1 120 hour embryo tests

In the case of DFA, I expected high LC values (low toxicity), based on the preliminary experiments, which was confirmed by the 120-hour embryo examination. $LC_{50}$ values: 24 hours after fertilization 207.2 mg/l, 48 hours after fertilization 196.5 mg/l, 72 hours after fertilization 187.5, 96 hours after fertilization 180.2 mg/l and 120 hours after fertilization 171.6 mg/l. Thus, as the exposure time increased, the toxicity of the compound also increased.

Microscopic examination of the embryos showed the presence of yolk edema and pericardial edema, which is common to zebrafish embryos, and the gray coloration of yolk. At higher concentrations, dorsal curvature of the embryo's tail was observed. Above 220 mg/l treatment concentration, complete body distortion was observed. These lesions did not occur in the control group.

Based on the preliminary experiments, I expected much lower LC values for EBA compared to DFA. This was demonstrated in the 120-hour embryo examination. The compound's $LC_{50}$ value was 24.45 mg/l at 24 hours, 27.52 mg/l at 72 hours, and 18.44 mg/l at the end of the study (120 hpf). Thus, as the exposure time increased, the toxicity of the substance increased also.

The microscopic morphological examination of this compound was performed. Zebrafish lesions such as yolk edema and pericardial edema, as well as gray coloration of the yolk, were similar to DFA. In addition, I have seen malformations in the tail, and the edge of the caudal fin was irregular. At

higher concentrations, the embryos were unable to leave the egg shell because of their severe developmental abnormalities.

Because DFA was not toxic to zebrafish embryos, I carried out further experiments with EBA.

### 2.2.3 Determining LC values in the 96-120 hpf exposure window

Pericardial edema, yolk edema, blurring of body segments, as well as gray coloration of the yolk and lack of swim bladder were observed in all treated groups. At the highest concentration (30 mg/l), head distortion was also present. The microarray study was focused on the study of sublethal effects, so in this preliminary experiment the primary determination of $LC_{10}$ was 25.59 mg/l, while $LC_{50}$ was 30, 94 mg/l.

## 3.2 Microarray assay and transcriptome analysis

I identified those mRNA transcripts, which were differentially regulated at at least one concentration ($p < 0.05$, $FC \geq 2$). 721 mRNA showed significant EBA-dependent regulation. Based on the principal component analysis (PCA), the control and treated groups were clearly differentiated. EBA treatment at the highest concentration resulted in a completely different mRNA expression profile than the control or lower concentration groups.

Then I further investigated the gene expression pattern of 721 EBA-regulated genes. Three different gene expression clusters were identified by the K-mean clustering method. Two EBA-induced gene expression clusters were identified, which showed concentration-dependent exposure. Cluster1 contains 198 EBA responsive genes, whose expression showed a concentration-dependent increase in zebrafish embryos. In contrast, the 320 genes in Cluster3 showed increased mRNA expression only at the highest (25.6 mg/l) concentration of EBA. Finally, I found 203 genes that show reduced mRNA expression after EBA exposure (Cluster2).

To characterize the functional properties of EBA regulated genes, I identified those biological functions that were significantly overrepresented in different EBA responsive gene expression clusters. By biological process analysis of DAVID (DAVID Bioinformatics Resources 6.8, Leidos Biomedical Research Inc., Frederick, Maryland, USA), I identified several biological function categories related to stress and DNA damage, including cellular stress response, response radiation, DNA repair, and response to DNA damaging stimulus whose overexpression is specific to Cluster1.

Biological process analysis has also shown that high-dose (25.6 mg/l) EBA exposure can induce the expression of genes related to the immune system and

inflammation. It can also be established that biological functional categories related to cell proliferation, such as cell division, cell cycle, processes associated with phase M (cell division, which involves segregation of chromosomes, nucleus and cell division), cell cycle phase, and the cell cycle process is significantly overrepresented among EBA-repressed genes. For further analysis of these EBA-inhibited genes, I used the GeneMANIA prediction server. I identified a 18-membered cell division gene network based on co-expression and physical interaction.

In summary, global gene expression analysis has raised the possibility that exposure to EBA can induce expression of DNA damage and inflammation-related genes and inhibit genes associated with cell proliferation. In order to verify these assumptions, I made further experiments.

### 3.3 Comet assay on 1 dpf embryos

An organic peroxide (Luperox®) was used as a positive control in the experiment, which in all cases clearly damaged the DNA spiral compared to the water used in the fish housing system. At the lowest concentration (0.5 mg/l), the amount of DNA in the tail of comets was significantly increased in the treated groups ($P < 0.05$). Based on my study, the increase of DNA fragmentation was dose-dependent.

### 3.4 Micronucleus assay on adult fish

Poisson regression showed a significant increase in micronucleus formation compared to the control group. As early as the first week, an increase in the number of micronuclei was observed, even at the lowest concentration used. However, this difference became significant only by the third week ($P < 0.05$). At the end of the experiment, in case of 2.75 mg/l concentration $2.75 \pm 2.22$, 5.5 mg/l concentration $3.5 \pm 1.29$, and 11 mg/l concentration $3.5 \pm 1.00$ micronuclei were formed. There was no statistically significant difference between the groups. It can be stated that at the concentrations used in the experiment the effect was not concentration dependent. Based on my results, the method is suitable for detecting the DNA damaging effect of EBA on adult fish at low concentrations.

### 3.5 Examination of regenerative capacity on larvae

After analyzing the results of the study, no statistically significant difrence was found between the treated and the control groups after 24 hours. After 48 hours, the rate of regeneration was significantly lower in all treated groups than in the control group, and the 6.4 mg/l group also had a significantly

shorter regenerated fin compared to the 1.6 mg/l group. Repeated measurements 72 hours after the treatment showed the same result. According to my results, EBA clearly reduces the ability of the caudal fin to regenerate, so it really has an effect on cell division and cell cycle processes.

# 4 New scientific results

1 In the case of EBA, I was the first to determine the dose-effect relationships of mortality using acute toxicity tests. Semi-lethal concentrations of 4-ethylbenzaldehyde in zebrafish embryos at 95% confidence intervals: 24 hpf: 31.45 ± 0.82 mg/l; 48hpf: 30.85 ± 0.89 mg/l; 72hpf: 27.52 ± 1.5 mg/l; 96hpf: 22.00 ± 1.39 mg/l; 120hpf: 18.44 ± 1.91 mg/l and 30.94 ± 1.85 mg/l in the 96-120 hpf exposure window.

2. For DFA, I was the first to determine the dose-effect relationships of mortality using acute toxicity tests. Semi-lethal concentrations of 2-4-difluoroaniline in zebrafish embryos with 95% confidence interval: 24 hpf: 207.2 ± 9.65 mg/l; 48 hpf: 196.5 ± 11.2 mg/l; 72 hpf: 187.5 ± 10.9 mg/l; 96 hpf: 180.2 ± 9 mg/l; 120 hpf: 171.6 ± 9.55 mg/l.

3. I was the first to investigate the effect of 4-ethylbenzaldehyde on gene expression in fish. In the analysis of biological function, I found that EBA increases the expression of genes with biological function related to DNA damage and repair mechanisms, while decreasing the expression of genes related to cell cycle and cell division, in both cases in a dose-dependent manner. Only the highest concentration of EBA (25.6 mg/l) was increased in the expression level of genes related to the immune response compared to the control.

4. I demonstrated the damaging effect of EBA on zebrafish embryos in an acute experiment by comet assay and in adult individuals in a subchronic experiment by micronucleus assay.

5. I was the first to examine the effect of EBA on regenerative capacity. According to the results of the caudal fin regeneration experiment, even at low concentrations, EBA clearly reduces the rate of tissue regeneration, thus affecting cell division.

6. Based on the combined exposure and physical interaction, I found a cell proliferation-related gene network of 18 genes, whose members show decreased expression by EBA.

# 5 Conclusions and suggestions

## 5.1 Conclusions of acute fish embryo toxicity tests

In my studies on fish embryos, the toxicity of DFA was low ($LC_{50}$=186 mg/l at 96 hours). Both the adult fish (OECD TG 203) and embryo (OECD TG 236) acute toxicity guidelines contain a limit $LC_{50}$=100 mg/l, if the toxicity test value obtained in the limit test is higher, the substance is not considered to be toxic to fish. The substance's safety data sheet (CAS 367-25-9) contains only rat specific toxicity data, the oral $LD_{50}$ value is 820 mg/kg body weight, dermal 672 mg/kg body weight, and 4 hours inhalation 6,21 $mg/m^3$. Mammalian toxicity is not high, either. Because the $LC_{50}$ value of DFA in zebrafish embryos cannot be found in the scientific literature, I considered that it is important to determine.

Not only LC values were determined, but also phenotypic examination was performed on embryos. In the case of DFA, I observed some general toxic effects on embryos, such as pericardial edema and grayish discoloration of the yolk. At concentrations above 100 mg / l, the spine of fish was curved in the dorsal direction, but this was not concentration dependent. This curvature is similar to the phenotype of *heart and soul* (has) mutant individuals, due to a mutation in the atypical protein kinase C (*aPKC*) gene.

Acute toxicity of EBA in zebrafish embryos was also determined and phenotypic examination was performed in EBA as well. As with DFA, generalized symptoms of zebrafish, such as pericardial edema and grayish discoloration of the yolk, have occurred. In addition to these symptoms, tail distortion and irregularity of the edges of the caudal fin were observed, but none of the lesions appeared to be concentration dependent.

The abnormality of the edges of caudal fin is not typical for ibuprofen. Distortion of the pectoral fins and its absence at higher concentrations (10-100 μg/l) can be observed. Both pericardial edema and fin developmental disorders are resulted by the inhibitory effect of ibuprofen on cyclooxygenase enzymes. As a non-selective cyclooxygenase inhibitor, it inhibits the production of prostanoids by inhibiting cyclooxygenase enzymes (COX-1, COX-2). For the development of zebrafish, prostaglandins among the prostateoids, are required for the formation of body sections. In the absence of this, the blood vessels between the gaps are shortened, and cardiac and floating developmental abnormalities develop, so I experienced that this process may also be the cause of the abnormalities.

EBA is also very similar in structure and physicochemical properties to benzaldehyde, which is known as artificial almond oil in the food industry. The symptoms observed in embryos can be traced back to the intracellular metabolism of benzaldehyde. Upon entry, it activates the cytochrome P450 system, which converts it into benzoic acid and benzyl alcohol, which is secreted from the cell during secretion. Non-transformed benzaldehyde molecules and benzoic acid damage DNA directly, and benzaldehyde reduces the activity of glutathione peroxidase (GPx) enzymes, thereby increasing oxidative stress and lipid peroxidation.

### 5.2 Conclusions of transcriptome analysis and classic toxicological studies to prove the results of transcriptome analysis

With DAVID biological function analysis, I identified several biological function categories related to stress and DNA damage in Cluster 1, including cellular stress response, radiation response, DNA repair, and response to DNA damaging stimuli. Exposure to EBA at a concentration-dependent level induces the expression of genes related to DNA damage and repair mechanisms and cellular stress response. I concluded from this that EBA has the potential to damage DNA by increasing the activity of repair processes. Microarray was performed on embryos, so I used embryo comet assay to confirm my hypothesis. EBA treatment resulted a concentration-dependent increase in DNA fragmentation, so this simple method was able to prove that EBA has an adverse effect on the genetic material in zebrafish embryos.The expression of genes related to repair mechanisms is increased, but this is not enough to compensate the adverse effects of the substance.

I have investigated the probable DNA-damaging effect of the substance in adult individuals with micronucleus assay. By day 21 of the experiment, the number of micronuclei in the treated groups increased significantly. I conclude from this that EBA has a DNA damaging effect in adult individuals, however, due to the lower treatment concentration, several weeks of exposure are required for micronuclei formation. The ibuprofen, which is the mother molecule of EBA has also DNA damaging properties.

It is most similar to EBA in its physico-chemical properties, benzaldehyde, which has already been shown to have a damaging effect on human lymphocytes DNA by comet assay and on fruitfly (*Drosophila melanogaster*) using the SMART test. The damaging effect of benzaldehyde on DNA is due to its intracellular metabolism as described in section 5.1.

Biological process analysis has also shown that high-dose (25.6 mg/l) EBA exposure can induce expression of genes related to immune function, cellular

responses to organic and inorganic substances, and intracellular metabolism of amino acid derivatives. In addition to the literature data already described, the increase in EBA-induced DNA damage and expression of related genes, as well as the biological functions and expression of Cluster 3 suggest that EBA's intracellular metabolism and effect on cell function is highly similar to benzaldehyde. This complex mechanism may be responsible for the experienced lesions.

In addition to being a non-selective inhibitor of cyclooxygenase, ibuprofen has been shown to stimulate glutathione peroxidase (GPx) and glutathione-S-transferase (GST) among antioxidant enzymes in zebrafish. This can be explained by the fact that ibuprofen causes oxidative stress in the cells, thus increasing the activity of these enzymes in response. However, glutathione reductase (GR) and catalase (CAT) enzymes were unaffected and malondialdehyde levels in lipid peroxidation processes were also lower in the treated groups than in the control groups. In contrast to benzaldehyde, ibuprofen does not induce oxidative stress in zebrafish and even has a role in the prevention of lipid peroxidation. Thus, the mechanism of action of EBA , which I investigated, is closer to benzaldehyde, which is more similar in its chemical structure and physico-chemical properties, than to the ibuprofen, the mother molecule.

It can also be established that biological functional categories which related to cell proliferation, such as cell division, cell cycle, M phase of cell division, cell cycle phase and cell cycle process, are significantly over-represented among EBA-suppressed genes (Cluster 2). To further investigate the EBA-inhibited genes in the cluster, I identified the 18-cell cell division-related gene network by GeneMANIA prediction server based on co-expression and physical interaction Information from UniProt and ZFIN databases indicates that all 18 genes play a key role in cell division. Most of the genes are involved in the formation of the elements required for division (*ercc6l, mis12, esco2, nusap1, ndc80, nuf2, sgol1, plk1, kif23*), while some genes mainly regulate the division processes (*ttk, cks1b, mad2l1, cdca8, ccnb1, ccnb2, cdc20, cdca7a, fbxo5*). Mutation of the *sgol1* gene in humans is the cause of CAID syndrome (chronic atrial and intestinal dysrhythmia). During previous studies in zebrafish with the morpholino oligonucleotide, the *sgol1* gene was knocked out and the experimental animals showed symptoms similar as humans. Thus, if the expression of this gene is reduced by external influences, it may be detrimental to the proper functioning of the heart, but further studies are needed to confirm this. The two members of the gene network described in my thesis, *esco2* and *ttk* are involved in the regeneration of the caudal fin, and

the *mad2l1, fbox5, kif23* genes are also expressed in their proliferative regions. To confirm my hypothesis, I performed a caudal fin regeneration study on 96 hpf embryos, and I concluded that my conclusion was correct, because the rate of regeneration was significantly reduced in the treated groups.

Based on my results, more attention should be paid to the detailed toxicological examination of both water by-product compounds and substances in food, as they may pose serious risks to both consumers and the environment.

## 5.3 Suggestions

- It is clear from the example of the EBA that despite the limited available data, certain compounds may be marketable. These can be harmful to human health and the environment. Therefore, I suggest further testing of EBA in other test organizations as well as complex toxicological testing of other water disinfection by-products and food additives in acute, subchronic and chronic studies.
- As the 72 hpf embryos have a dorsal curvature of the tail at each treatment concentration above 100 mg/l, it is possible that DFA has an estrogen-like effect, as this disorder has already been described by other authors for estrogenic substances. The curvature of the tail shows similarity to the phenotype of *heart and soul* (has) mutant individuals, which is the aftermath of a mutation in the atypical protein kinase C (*aPKC*) gene. I recommend the use of a specific morpholino oligonucleotide to study the mutation of the *aPKC* gene. In addition, the estrogen-sensitive hepatic transgenic zebrafish line *Tg(vtg1: mCherry)* may be useful in investigating potential estrogen-like effects of the substance.
- Based on my microarray study, EBA has been shown to have potential DNA damaging. It enhances the expression of several genes related to DNA repair mechanisms. In addition, it reduces the expression of a number of genes involved in cell division, suggesting that it has a negative effect on cell division and thus on regenerative capacity. These assumptions were supported by classical methods (comet assay, micronucleus assay and caudal fin regeneration assay). I recommend the FISH-comet assay, which is a combination of comet assay and fluorescent in situ hybridization, to give a more accurate picture of the effect of the material and the expression of certain genes.

- Based on literature data, the cellular mechanism of action of EBA is may be similar to benzaldehyde. This may affect GPx and GST antioxidant enzymes. I suggest examining the effect of EBA on oxidative stress.

# Publications related to the topic of the thesis

Publications in scientific journals:

**Bencsik D.**, Gazsi Gy., Urbányi B., Szende B., Rácz G., Véha A., Csenki Zs. (2018): Preliminary study: Assesment of subacute genotoxic and histopathological effects of a food flavour, 4- ethylbenzaldehyde (EBA) on zebrafish (Danio rerio) model, ACTA ALIMENTARIA: AN INTERNATIONAL JOURNAL OF FOOD SCIENCE 47: 2 pp. 245-251. (IF: 0,384)

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## Publications not related directly to the topic of the thesis

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