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RESEARCH

# Subacute Toxicity Assessment of Water Disinfection Byproducts on Zebrafish

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**Abstract** Disinfection of raw water is essential to the production of drinking water. However, by-products of disinfection may exert toxic effects. The potential toxic effects of two of these compounds, 4-ethylbenzaldehyde (EBA) and 2,4-difluoroaniline (DFA) were investigated using the zebrafish (Danio rerio) model. The two compounds, dissolved, were introduced in duplicate aquariums containing zebrafish in two different concentrations based on LC50 values. The aquarium water containing EBA or DFA was changed every 96 h throughout the 3 months of treatment. Behavior of the fish in each replicate was inspected twice daily. In course of treatment with both concentrations, fish exposed to DFA displayed behavior associated with visible anxiety, while EBA treated were lethargic and did not evade capture. Application of both concentrations of each component into the aquarium water resulted in dystrophic lesions in the liver, kidney and skin of the fish while preneoplastic lesions and tumors were not observed.

**Keywords** 4-ethylbenzaldehyde · 2,4-difluoroaniline · Zebrafish · Water disinfection byproducts

**Abbreviations**

| | |
|---|---|
| EBA | 4-ethylbenzaldehyde |
| DFA | 2,4-difluoroaniline |
| DBP | Disinfection byproduct |
| ASV | Air saturation volume |

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## Introduction

The disinfection of water for human use and consumption is among the major public health advancements of the 20th century. The production of potable water, principally by chlorination, and widespread implementation has served as an effective means of reducing—and in some cases eradicating—water-borne illnesses including typhoid fever, cholera, and dysentery, among others [1]. However, chemicals added during disinfection have been shown to react with dissolved organic matter (humic and fulvic acids) to produce

a wide variety of chlorine-substituted carbon and nitrogen containing byproducts, which may induce mutagenic and also carcinogenic effects [2, 3]. Several epidemiologic studies have suggested that the consumption of treated drinking water may be associated with the development of certain malignancies in humans [4–6]. Furthermore, during previous analyses utilizing in vitro methods (AMES test, human lymphocyte cultures), mutagenic and apoptosis-inducing effects were demonstrated in studies using a mixture of substances eluted from resin columns through which drinking water produced by the Budapest water works had been passed [7, 8]. The chromatographic analysis of this mixture revealed more than 200 well-defined chemical compounds, dozens of which are likely among the now more than 700 unique disinfection byproducts (DBPs) identified in the literature [7, 9].

Computer-assisted analysis relating the structure of these compounds to data obtained from the literature revealed that 12 such compounds may be mutagenic, carcinogenic, or have other toxic capacity, in vitro. Out of these, two commercially available compounds, 2,4-difluorianiline (DFA) and 4-ethylbenzaldehyde (EBA) were chosen to start a series of in vivo studies, using zebra fish (*Danio rerio*) as the test object. Laboratory fish are widely used in various fields of biological and medical research and in exposure analysis [10]. Most compounds which are carcinogenic to rodents and humans, are shown to cause tumors in fish, and compounds causing malignancies in fish often do the same to other species [11]. Due to favorable biological characteristics and for technical reasons, *Danio rerio* is the most frequently used fish in experimental settings [12].

The present study observes the effects of the two mentioned chemical agents selected as suspicious regarding mutagenic, carcinogenic or other toxic effect based on chemical structure upon the survival, behavior and morphological attributes of *Danio rerio*.

## Materials and Methods

### Chemicals

4-Ethylbenzaldehyde (EBA) 98% (Sigma 23,363-3) 2,4-Difluorianiline (DFA) 99% (Sigma D10,140-0) were purchased from Sigma Aldrich Hungary (Budapest, Hungary). Stock solutions were made in distilled water by means of ultrasonic dispersion. All subsequent solutions were also created with distilled water.

### Animal Care and Handling

The zebrafish AB strain was used in our study. Adult fish were maintained at 25°C, pH 7.0±0.2, conductivity 525± 50 μS with a 14-h light/10-h dark cycle in a recirculation system (Zebtec, Tecniplast S.p.a., Italy). Fish were fed twice a day with SDS Small Gran granulated feed (Dietex International Limited Special Diets Services G.B.). Additionally, all fish were fed twice a week with artemia. The Animal Protocol was approved by the Hungarian Animal Welfare Law (22.1/518/003/2008).

### Acute Toxicity Test

The $LC_{50}$ were determined using the OECD guideline [13] that describes the Fish Acute Toxicity Test. The stock-solution was 1,000 mg/l. The $R^2$ values were: DFA: $y$= 94.538 Ln(x)-277.77 $R^2$=0.95; EBA: $y$=72.135 Ln(x)-180.38 $R^2$=0.80. A semi-static test was applied, by changing the solution every 48 h.

The fish were exposed to the test substances for a period of 96 h. Mortalities were recorded after 96 h and the concentrations which kill 50% of the fish (LC50) are determined where possible. Records were kept of visible abnormalities (e.g. loss of equilibrium, swimming behavior, respiratory function, pigmentation, etc.). Measurement of pH, dissolved oxygen and temperature were carried out at least daily.

### Exposure of Zebrafish

In duplicate, fish cohorts were independently treated with two concentrations of each compound. The working solution contained 2.5 mg/l and 5 mg/l of EBA and 5 mg/l and 10 mg/l of DFA. Applied concentrations were administered in situ at levels determined to be at sub-acute levels, below $LC_{10}$, based on the previously determined $LC_{50}$ values. Control groups, free of exposure to either compound were also generated in duplicate. Twenty-five adult fish, not differentiated by sex, were used in each replicate. The total density of fish was 0.4–0.5 g/l in each treatment.

The solutions were changed every 96 h. The treated fish were fed with artemia daily and with SDS feed prior to replacing (host) solution. No aeration was applied to the water; Air Saturation Volume (ASV) was over 80%.

Behaviour of the fish was observed twice daily and two fish were sampled each week from every replicate in the manner described as follows: prior to euthanasia, fish were anesthetized with MESAB (0.4% Tricaine Methanesulfonate, 1% $Na_2HPO_4$ in 10% Hanks' Balanced Salt solution).

### Histology of Zebrafish

Zebrafish were fixed in 4% buffered formaldehyde at 4°C for 24–48 h, washed with Phosphate buffered saline PBS, and tissues were dehydrated in a series of graded ethanol solutions and xylene before embedment in paraffin. The fish were cut in half sagittally just left of the midline and both

halves of the fish were placed into the cassette for sectioning. Sections were 4–6 μm thick and were stained with hematoxylin and eosin (HE), Periodic Acid-Schiff reaction (PAS), and Congo-Red.

### Quantitative Analysis of the Effect of DBP Exposure on Fatty Change of the Liver Using Digital Microscopy

Here, the effect of EBA and DFA were studied on the liver, using digital microscopy based on automated image analysis (also see ref.[14]) to detect and quantify changes in the amount on fatty degradation within hepatocytes. For each DBP, two different concentrations were used (2.5 and 5 mg/l for EBA and 10 and 5 mg/l for DFA). We exposed two groups of fish for each condition for the indicated time. Control groups were kept in EBA and DFA free medium. Random fields from the liver tissue were recorded and analyzed at 450x magnification to detect and quantify the area occupied by the non-stained lipid droplets within hepatocytes.

We generated digital slides from HE stained liver tissue of the studied fish. These digital slides are ideal to extract microscopic information at any magnification with easy navigation, annotation and measurement. Digital signals permit image segmentation along color, intensity, and size for automated object quantification while digital slides offer superior imaging features and batch processing. In this study we used the PANNORAMIC system developed by 3DHISTECH[15].

## Results

### Acute Toxicity Test

$LC_{50}$ of EBA for adult zebrafish was 23.49 mg/l.
$LC_{50}$ of DFA for adult zebrafish was 29.36 mg/l.

### Behavioral Observations

In both EBA exposure groups, throughout the course of the 3 months exposure all fish were lethargic and did not evade capture. No visible change was observed in the balance or upright orientation among these replicates, however.

All fish exposed to DFA also demonstrated different behavior compared to the control. Among fish in these replicates, behaviour change was evident in the dominant observed swimming pattern and the display of behaviour associated with anxiety[16]. Most notably, DFA exposed-fish behaviour may be characterized by frequent and rapid changes in the direction of travel and was not observed among the control cohort.

### Histopathology

Alterations were found in the liver, kidney, and skin of the treated animals beginning in week 3 after the onset of the experiment. Among each high-dose exposure scenario (10 and 5 mg/l for EBA and DFA, respectively), the severity of augmentation increased gradually and reached its peak by the end of the second month. For the low-dose exposure scenario, however, effects were less consistent and seemingly time-independent among replicates exposed to 2.5 mg/l DFA and 5 mg/l EBA.

*Liver alterations due to EBA exposure:* within the liver parenchyma cells, changes were observed in the relative content and distribution of fat. The fat droplets varied in size, but at the experiments duration, nearly filled the whole cytoplasm. (Figure 1) Furthermore, relative to the control, the glycogen content of the parenchyma decreased. These lesions were observed in both males and females. No hepatocyte megalocytosis, foci of hepatocellular alterations, or adenofibrosis were found.

*Kidney alterations due to EBA exposure:* HE and PAS stained sections showed small, clear, PAS positive vacuoles within the cytoplasm of the epithelial cells of the distal tubuli. Pycnotic chromatic condensations were found in 5–10% of these cells. Epithelial cells of the proximal tubuli showed larger, PAS positive, supranuclear droplets. However, the nuclei were without any observed alteration.

*Skin alterations due to EBA exposure:* the mucin producing cells of the epidermis increased in a time-dependent manner.

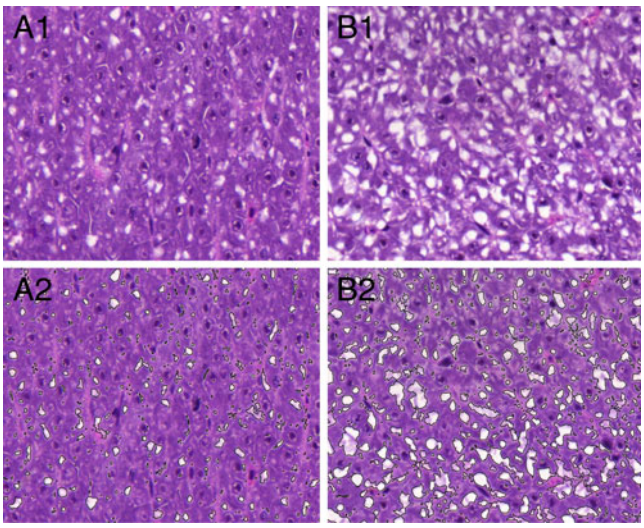


**Fig. 1** Images showing liver of a control fish with moderate fatty change (**A1**) and severe diffuse fatty change in the liver of a fish treated for 3 months with EBA (**B1**). **A2** and **B2** panels demonstrate digital image processing of the liver tissue shown on A1 and B1. (H-E, × 360)

*Liver alterations due to DFA exposure:* throughout the study, diffuse fatty change was observed and most notable was the appearance of small fat droplets. The glycogen content of the liver parenchyma cells increased compared to the control. No differences were observed in liver alterations between males and females. Preneoplastic alterations were not observed.

*Kidney alterations due to DFA exposure:* observed histological changes were similar to those encountered with EBA.

*Skin alterations due to DFA treatment:* an increase in the number of mucus-producing cells was observed throughout the treatment.

Congo red staining for amyloid detection was negative in all organs of the fish treated with both EBA and DFA. No preneoplastic lesions or tumors of any kind were observed among fish exposed to either EBA or DFA.

### Quantitative Analysis of the Effect of DBP Exposure on Fatty Change of the Liver

First differences in liver histology were investigated between two fish cohorts of 25 that were exposed to the same conditions (i.e. to the same concentrations of EBA and DFA). No significant difference in the amount of cells showing fatty degradation was found between groups exposed to same conditions (Table 1). Since similar exposure concentrations did not reveal any significant differences within the given two groups of fish, we pooled the data acquired from each similarly exposed group and further studied the effect of different dosage of the two DBPs.

Low dose of EBA and DFA exposure did not change the fatty degradation of the liver parenchyma significantly ($P>0.7$ and $P>0.4$, respectively). However, high dose exposure to these DBPs caused significant elevation of the fat content of the liver cells ($P<0.002$ for both groups, $n=50$ in each group; Student-$t$ test, unpaired data with unpaired variance). Thus, higher exposure concentration significantly increased the degree of fatty change of the liver cells. This difference between liver-alterations at low- and high-dose DBP exposure can be explained by the detoxifying capability of liver-enzymes: only exposure to the high concentration level saturated their enzymatic activity, resulting in the degradation that was noted.

## Discussion

Beginning with the discovery of chloroform formation during water treatment, the presence of DBPs in drinking water has been known for nearly three decades [17, 18]. The mutagenic and possible carcinogenic potential of these byproducts has been previously demonstrated [19, 20]. In our in vitro studies concentration-dependent mutagenic effects of several DBPs were identified by Ames-test[7], and a similarly concentration-dependent, significant apoptosis-inducing effect of these DBPs appeared when incubated with cultures of human peripheral blood lymphocytes[8].

In the study at hand, an interesting in vivo vertebrate model was chosen to investigate possible toxic, mutagenic, and carcinogenic effects of two selected DBPs. The zebrafish has proved to be a good model system in which to study toxicology[21, 22], carcinogenesis [23], and infectious disease and immune function [24, 25]. Moreover, zebrafish are easy to grow and care for and can be maintained inexpensively in large quantities. For histopathological analysis, the fish's small size allows examination of all organs with relatively few histologic sections placed on relatively few microscope slides. Moreover, the fish offer exceptional transparency which is an advantage for gross and stereomicroscopic examination.

Our previous studies revealed a series of chemical compounds which may be responsible for mutagenic effects [7]. Two such compounds, ethyl-benzaldehyde (EBA) and 2,4-difluoroaniline (DFA), were investigated by our group to address such concerns regarding toxicity.

When treated for 3 months with two doses chosen based upon acute toxicity, DFA and EBA did not induce hepatocyte

**Table 1** Quantitative analysis of fatty change in the liver of fish-groups exposed to the same condition. There was no significant difference between groups exposed to similar conditions. Data are given as mean ± SE, number of animals in each group $n=50$

| | Exposure condition | Per cent of fatty change in the liver | *P*-value |
|---|---|---|---|
| Low dose | 5 mg/l EBA group A | 18.47±2.99 | $P>0.65$ |
| | 5 mg/l EBA group B | 16.94±2.47 | |
| | 2.5 mg/l DFA group A | 11.90±1.87 | $P>0.15$ |
| | 2.5 mg/l DFA group B | 16.36±2.4 | |
| High dose | 10 mg/l EBA group A | 22.88±2.18 | $P>0.35$ |
| | 10 mg/l EBA group B | 18.91±2.29 | |
| | 5 mg/l DFA group A | 24.77±3.09 | $P>0.4$ |
| | 5 mg/l DFA group B | 21.52±2.52 | |
| Control | group A | 17.60±2.1 | $P>0.5$ |
| | group B | 15.26±3.0 | |

megalocytosis, foci of hepatocellular alteration, adenofibrosis or other lesions typical of carcinogen exposure in the liver of zebra fish. According to our previous findings and data encountered in the literature, pre–neoplastic lesions and neoplasms are capable of developing in laboratory fish after 3 months of exposure to a carcinogenic substance [26]. Based upon our findings, DFA and EBA do not seem to cause preneoplastic lesions. Extended exposure to these compounds, however, could lead to evidence of carcinogenic activity. These experiments are in progress. Dystrophic lesions affecting the liver, kidney, and skin were caused by both compounds, in both time- and dose-dependent manners.

These lesions are not specific for either of the compounds, but drew attention to the possible toxicity. The principal finding is suggestive of alterations in carbohydrate and fat metabolism exerted by DFA and EBA.

The observed effects upon behavior and in response to external stimuli among zebra fish exposed to EBA and DFA also seems to be of considerable importance.

Since the behavioral effects exhibited by exposure to the two compounds are characteristically different and appear to impact the function of the fish nervous system in different manners, it is likely that specific modifying mechanisms are in the background. Such observations may be of interest also in context of human toxicology or even offer therapeutic considerations. Despite observed behavioral changes, no alterations were observed in the brain of the fish with analysis by light microscopy. Further, more detailed studies are needed to explain the nature and significance of the action of EBA and DFA upon the central nervous system as this was not a principal objective of this study.

As is often typical of animal studies, the component doses applied to the fish were several orders of magnitude higher compared to levels anticipated in treated water resulting in human exposure. Consider, however, the chronic exposure associated with the prolonged daily consumption and contact with these (and similar) compounds in the specific context of human toxicity. Such exposure includes the daily consumption of 1.5 l of drinking water and more water consumed and utilized in food preparation, as well as the multiple routes of dermal exposure throughout one's day, and even the potential for inhalation during a shower. Since this water sample contains approximately 200 compounds including EBA and DFA the toxicity exerted by these compounds may be expected only upon continuous human exposure. Moreover, there is a possibility for the compounds to accumulate within certain tissues of the body as well as the potential for synergistic or antagonistic effects of these compounds with the myriad of chemical compounds documented, assumed, or suggested to be present in the environment. On the basis of such considerations of chronic exposure, data presented here should be considered among risk factors of human health.

Since disinfection of drinking water is of the utmost importance in the prevention of sudden, acute, and potentially fatal health endpoints, further studies into to the prevention of DBP formation or removal of compounds after formation are needed. Following thorough analysis, the costs associated with the mitigation of DBP exposure can be coupled with the benefits associated with the prevention of undesirable health endpoints associated with deleterious components found in treated water as they are brought to attention in toxicology studies such as these. In the latter interest, further research on the in vivo effects of compounds in water disinfection byproducts using the zebrafish model is underway.

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