ORIGINAL ARTICLE

Effect of four selected carrier solvents on embryonal stages of *Danio rerio*

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Abstract

OBJECTIVES: The aim of this study was to determine the effects of 96 hour exposure to selected solvents on the embryonic stages of zebrafish (*Danio rerio*). We investigated mortality and various types of changes which appeared (oedema, tail and eye defects, weak pigmentation, and deformation of the body). Based on the results, values of NOEC and LOEC for embryos of *D. rerio* were determined.

METHODS: Embryonal toxicity tests were conducted according to OECD guideline 212. Ethanol and methanol were tested at concentrations of 0.1, 0.5, 1, 1.5, 2%; acetone at concentrations of 0.1, 0.5, 1, 1.25, 1.5%; and dimethylsulfoxide at concentrations of 1, 1.5, 2, 2.5, 3%.

RESULTS: The LOEC values of ethanol and methanol were detected in the 1% concentration. Statistically significant changes (oedema) were reported in 1% ethanol, and oedema, weak pigmentation and deformation of the body were observed in 1% methanol. After exposure to acetone, the most common occurrence of oedema was in the 0.5% concentration (LOEC = 0.5%). The solvent dimethylsulfoxide caused oedema and body deformation at the 2% concentration (LOEC = 2%).

CONCLUSIONS: The NOEC concentrations of the individual solvents were as follows: ethanol and methanol, 0.5%; acetone, 0.1%; and dimethylsulfoxide, 1.5%. These concentrations of individual solvents were higher than the maximum recommended concentration for toxicity tests on fish. For this reason, it can be assumed that the concentration of solvent allowed by the norm does not affect the procedure or results of such tests.

Abbreviations:

DMSO	- Dimethylsulfoxide
NOEC	- No Observed Effect Concentration
OECD 212	- Fish, short-term toxicity test on embryo and sac-fry stages
LOEC	- Lowest Observed Effect Concentration
CAS	- Chemical Abstracts Service Registry Number
ANC4.5	- Acid Neutralizing Capacity
CODMn	- Chemical Oxygen Demand

INTRODUCTION

Chemicals with reduced solubility in water are often used in toxicity tests. Organic solvents are used in order to achieve the solubility of hydrophobic compounds. These solvents can, however, interfere with the execution of the test by having a negative effect on the tested organisms and thus affect the results of studies (El Jay 1996, Hutchinson *et al.* 2005, Rufli *et al.* 1998).

The methodology of OECD 212 (Fish, short-term toxicity test on embryo and sac-fry stages) recommends the preparation of test solutions of compounds with reduced solubility in water by means of physical methods such as stirring, sonication, or the use of a saturator column. Where absolutely necessary, the methodology of OECD 212 also allows the use of the following solvents: acetone, ethanol, methanol, dimethylsulfoxide, dimethylformamide, and triethylene glycol. The solvent must be used at a concentration which does not affect the results of tests. This study focuses on four of the most common solvents, namely ethanol, methanol, acetone and dimethylsulfoxide (DMSO). OECD defines the maximum acceptable limits for solvents used in toxicity tests on aquatic organisms. The limit for acute toxicity tests is set at 0.05%, and for chronic toxicity tests, at 0.01% (OECD 2000; Eaton *et al.* 1975; Hallare *et al.* 2006).

Zebrafish embryos and larvae are important as model organisms in developmental biology and are used for toxicity testing, ecotoxicology screening, and disease modelling, with a particular focus on drug screening experiments (Bilotta *et al.* 2001; Berghamns *et al.* 2008; Plhalova *et al.* 2011; Praskova *et al.* 2011; Redfern *et al.* 2008). The advantages of using this species include the fact that adults reach sexual maturity at 3 months of age and have the ability to produce large quantities of embryos. In addition, their eggs are transparent and their development can be examined without interference (Bilotta *et al.* 2004; Nagel 2002).

The aim of this study was to determine the effects of ethanol, methanol, acetone and DMSO at various concentrations on embryo *D. rerio*. We monitored mortality and any changes which arose (oedema; abnormalities of the body, tail and eyes; reduced pigmentation; and deformation of the body) after 96 hour exposure to these solvents, and, on the basis of the results, determined the NOEC and LOEC values.

MATERIALS AND METHODS

Four different solvents were used in our study – MULTIPUR ethanol (CAS No. 64-17-5) CHROMSERVIS s.r.o., Czech Republic; methanol (CAS No. 67-56-1) Sigma-Aldrich, Chemie GmbH, Germany; DMSO (CAS No. 67-68-5) Sigma-Aldrich, Chemie GmbH, Germany; and acetone (CAS no. 67-64-1) CHROMSERVIS s.r.o., Czech Republic. These solvents were used in 5 different exposure concentrations, all expressed in %. Ethanol and methanol were tested at concentrations of 0.1, 0.5, 1, 1.5, 2%; acetone at concentrations of 0.1, 0.5, 1, 1.25, 1.5%; and dimethylsulfoxide at concentrations of 1, 1.5, 2, 2.5, 3%. Embryo toxicity tests were performed according to OECD 212 (Fish, short-term toxicity test on embryo and sac-fry stages). In this test, a semi-static method with solution replacement after 24 hours was used. 20 ml Petri dishes were used and kept in the incubator at a stable set temperature of 26.8 °C, the dissolved oxygen saturation being above 60%.

Testing was performed on *D. rerio* embryos. Only eggs in the blastula stage (3–4 hours after fertilization) were chosen for testing. 5 Petri dishes were prepared with different solvent concentrations and one control (C) Petri dish with dilution tap water without solvent. 10 fertilized embryos were placed in each Petri dish. Everything was done in 12 series; this means that 120 embryos were used for each concentration including control. Testing was terminated after all individuals in control were hatched, having absorbed the yolk sac.

The basic physical and chemical parameters of the dilution water used in the embryo toxicity test were: $ANC_{4,5}$ 3.6–3.7 mmol.L^{-1}, COD_{Mn} 1.4 to 1.9 mg.L^{-1}, total ammonia below the limit of determination, NO_3^- 24.5 mg.L^{-1}, NO_2^- <0.01 mg.L^{-1}, Cl^-18.9 to 19.1 mg.L^{-1}, Σ Ca + Mg 3.06 mmol.L^{-1}.

The development of embryos from blastula to early larval stages, hatching rate, and mortality were monitored at 24, 48, 72 and 96 hours. Dead embryos were removed daily from the Petri dish. Various types of changes in the surviving embryos (oedema, tail and eye defects, weak pigmentation, and deformation of the body) were recorded after 96 hours.

Statistical evaluation of results

Statistical evaluation was performed using Unistat 1.5 (Unistat Ltd., GB). Frequency differences and observed morphological changes were evaluated using contingency tables. The statistical significance of differences was evaluated at a significance level $*p<0.05$, $**p<0.01$.

RESULTS AND DISCUSSION

After 96 hours of exposure to the tested solvents, morphological changes such as oedema, tail and eye defects, weak pigmentation, and deformation of the body were recorded. The percentage of these abnormalities in the various concentrations of each tested substance was evaluated statistically.

Mortality of embryos

The mortality of embryos in the control groups was always less than 5%, which corresponds to the test requirements according to OECD methodology 212.

Ethanol

A statistically highly significant ($p<0.01$) incidence of morphological changes to embryos such as oedema (Photo 1) in the 1% ethanol concentration (Figure 1)

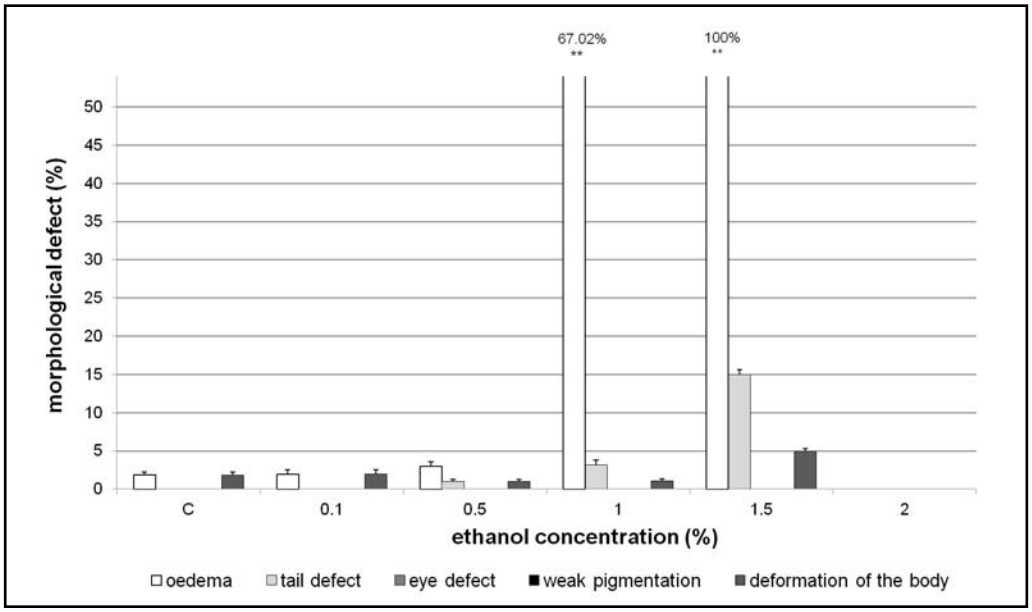

Fig. 1. The effects of solvent **ethanol** on developmental morphological defect after **96 hour** exposure. *p<0.05, **p<0.01 compared with the control

began to appear after 96 hours of exposure. Similar results were reported by Baumann and Sander (1984) and Hallare *et al.* (2006), who detected morphological defects such as oedema and very weak pigmentation starting in 1% ethanol. In addition, Chen *et al.* (2011) observed yolk-sac oedema and deformation of the body. At the 2% ethanol concentration, our highest concentration used, the end of embryonal development was already observed after 48 hours of exposure. 96 hour exposure to the 1.5% concentration caused increased mortality, and 96 hour exposure to the 2% concentration led to the death of all tested embryos (Table 1). Consequently it was not possible to record the morphological changes (Figure 1). This observation was in agreement with Hallare *et al.* (2006). The results of Bilotta *et al.* (2004) indicate that embryonic ethanol exposure affects both external and internal physical development and that the severity of these effects is a combination of both the amount of ethanol and the length of ethanol exposure.

Methanol

No morphological changes were found in the control group (Photo 2). Statistically highly significant (p<0.01) changes such as oedema were reported in the 1% methanol concentration compared with the control, and also in the 2% concentration together with deformation of the body and weak pigmentation (Figure 2). The influence of methanol with respect to morphological changes was not studied by other authors. A mortality higher than 20% was recorded in 2% methanol (Table 1).

Acetone

In the 0.5% solution, statistically significant (p<0.05) changes (oedema) appeared in comparison to the control. A statistically highly significant (p<0.01) increased incidence of tail defect (Photo 3) and deformation of the body (Figure 3) were observed in 1.25% acetone solution. Hallare *et al.* (2006) observed an insignificant reduction in pigmentation in 1% acetone, but the following statistically highly significant (p<0.01) changes

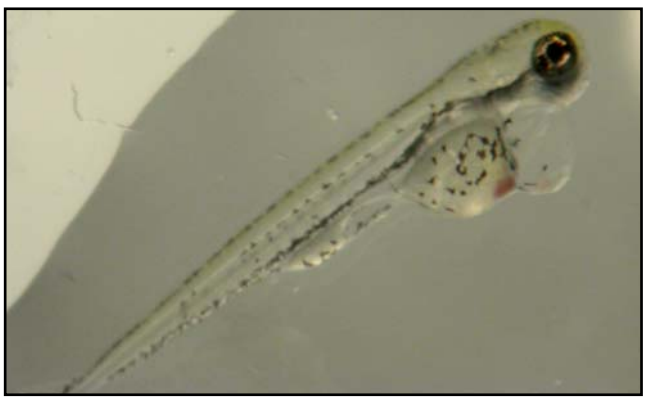

Photo 1. Oedema of embryos in the 1% ethanol concentration.

Tab. 1. Mortality of embryos after 96 hour.

ethanol		methanol		acetone		DMSO	
conc. (%)	mort. (%)	conc. (%)	mort. (%)	conc. (%)	mort. (%)	conc. (%)	mort. (%)
C	1.8	C	0.8	C	1.7	C	4.1
0.1	7.3	0.1	3.3	0.1	0	1	5.8
0.5	8.2	0.5	5.8	0.5	3.3	1.5	4.1
1	14.5	1	9.2	1	18.3	2	30.3
1.5	81.8	1.5	18.3	1.25	32.5	2.5	30
2	100	2	63.3	1.5	59.2	3	79.2

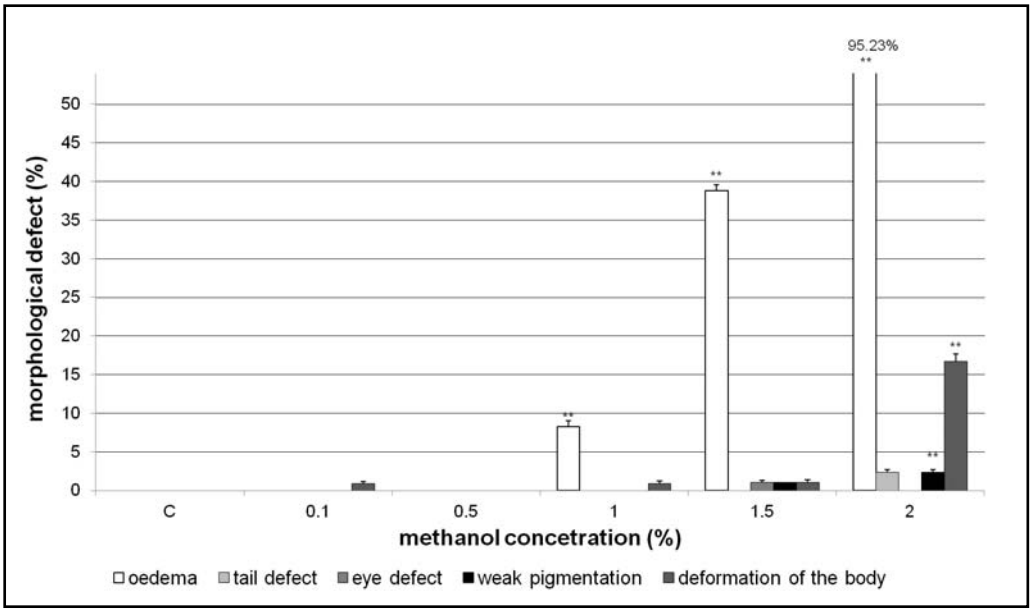

Fig. 2. The effects of **methanol** solvent on developmental morphological defect after **96 hour** exposure. *p<0.05, **p<0.01 compared with the control.

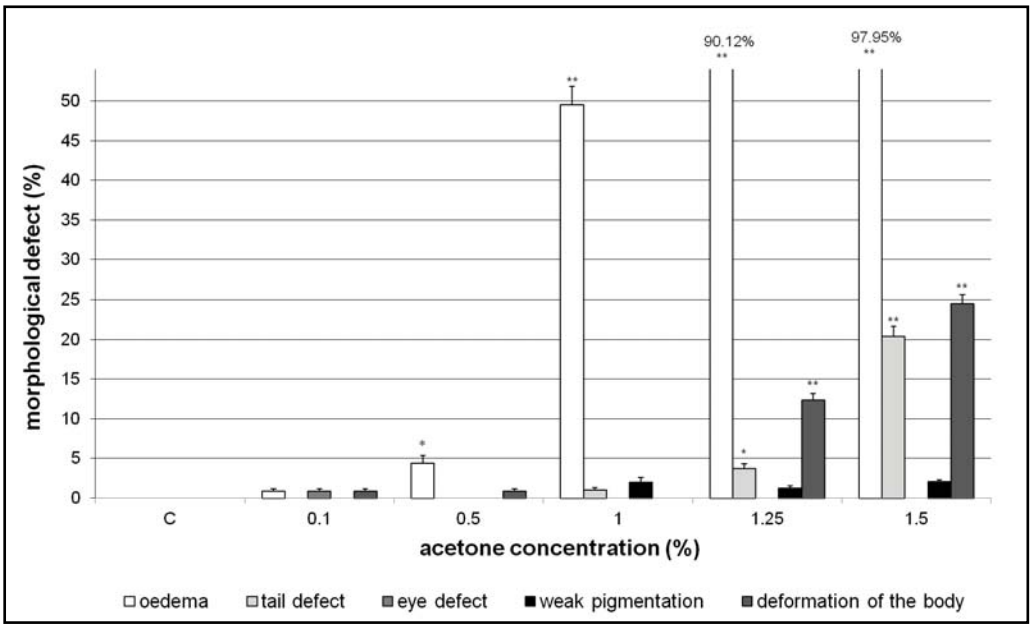

Fig. 3. The effects of solvent **acetone** on developmental morphological defect after **96 hour** exposure. *p<0.05, **p<0.01 compared with the control.

Photo 2. Hatching embryo without defect in the control group.

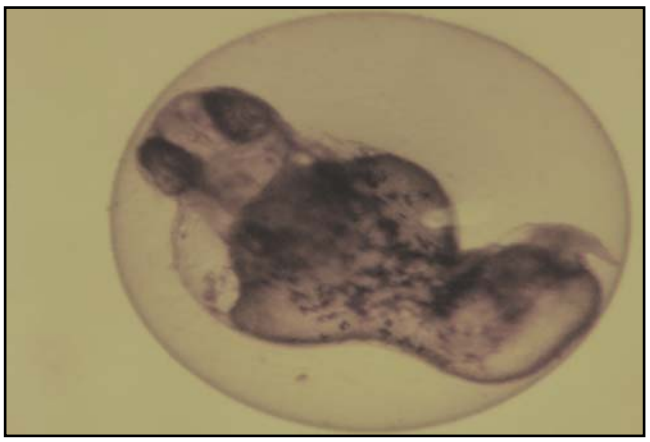

Photo 3. Tail defect in the 1.5% acetone concentration.

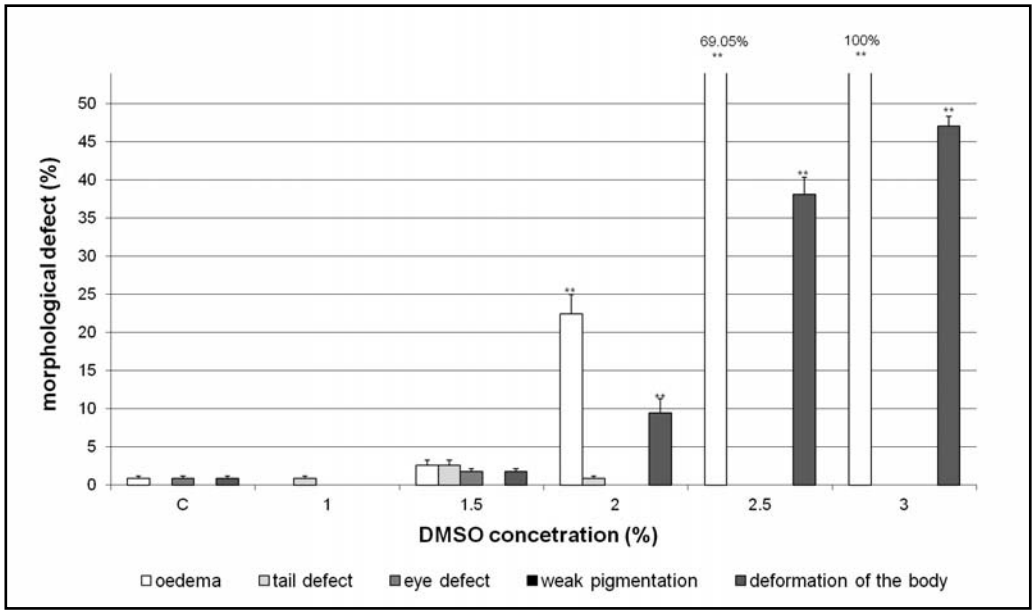

Fig. 4. The effects of solvent **DMSO** on developmental morphological defect after **96 hour** exposure. *$p<0.05$, **$p<0.01$ compared with the control.

at higher concentrations: weak pigmentation in 1.5% acetone, and oedema in 2% acetone. Mortalities of 32.5% were found in 1.25% acetone, and 59.2% in 1.5% acetone after 96 hours (Table 1).

DMSO

Our testing indicated a statistically highly significant incidence of morphological changes, such as oedema (Photo 4) and body deformation, which started to appear in 2% DMSO (Figure 4). Hallare *et al.* (2006) found weak pigmentation in 1.5% DMSO and oedema in 2% DMSO, which is in accordance with our observations. Chen *et al.* (2011) and Hallare *et al.* (2006) observed no detectable developmental differences at concentrations of 0.01–0.1%. In another study Hallare *et al.* (2004) used DMSO for the better dissolution of diclofenac in concentrations ranging from 0.00002% to 0.04% and showed that there was no change compared to the control group, in which DMSO was not used. Despite the fact that zebrafish larvae have been shown to be extremely tolerant to DMSO up to 4%, and that 1% is routinely used in short term drug toxicity experiments, Winter *et al.* (2008) and David *et al.* (2012) suggest that concentrations of solvents be minimised to the lowest level possible, especially in longer term tests of CYP-mediated xenobiotic metabolism. Machova *et al.* (2009) studied the effect of DMSO on the growth, development and mortality of the embryo and embryo-larval stages of common carp at test concentrations of 0.2, 0.5, 1.0 and 5 $ml.L^{-1}$ (0.02, 0.05, 0.1 and 0.5%). Organisms exposed to concentrations from 0.02 to 0.1% DMSO showed no change compared to controls. Significant changes in growth, however, occurred in the 0.5% concentration. Comparing these results, *D. rerio* embryos may appear to be less sensitive to the effects of

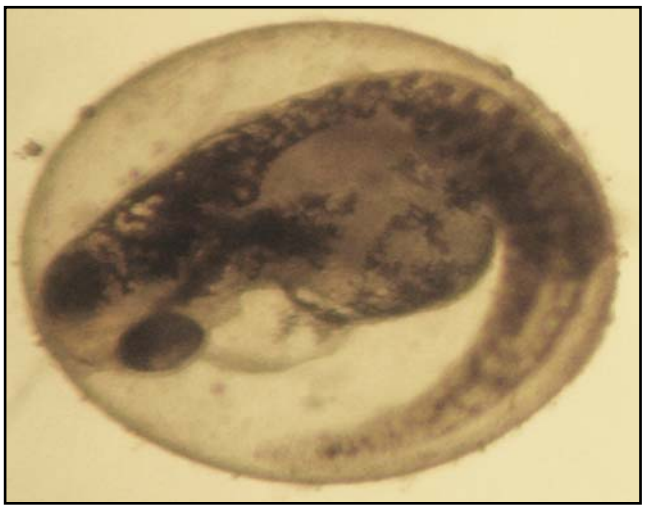

Photo 4. Oedema of no hatching embryo in the 2% DMSO concentration.

DMSO than larval stages of carp. However, it must be remembered that the exposure times were significantly different in each study: 96 hours for *D. rerio*, and 29 days for the embryo-larval stages of a common carp.

Our results do not contradict the recommended maximum acceptable solvent concentration of 0.05% for acute toxicity tests (Eaton *et al.* 1975; Hallare *et al.* 2006; OECD 2000). The NOEC values of individual solvents for *D. rerio* embryos determined in our study were 0.5% for ethanol and methanol, 0.1% for acetone, and 1.5% for DMSO. As these were higher than the maximum recommended concentrations for toxicity tests on fish, it can be assumed that the maximum recommended concentrations would not affect the results of embryo toxicity tests on *D. rerio*.

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Potential Conflicts of Interest: *None disclosed.*

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