



Ethoxyquin: a feed additive that poses a risk for aquatic life

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ABSTRACT: Ethoxyquin (EQ) is an antioxidant that has, to date, been commonly used in feed production. Reports on the detrimental effects of this substance on vertebrates are growing, but effects in aquatic systems have rarely been described. Therefore, the present study was conducted using serial concentrations of EQ ranging from 0.03 to 16.5 mg l⁻¹ to determine effects on 3 types of aquatic organisms. In zebrafish, 5 mg l⁻¹ EQ caused mortality (25%) and a further 62.5% of the embryos showed yolk sac edema as well as deformed bodies or missing eyes. Furthermore, all the investigated EQ concentrations decreased the heart rate of the embryos. The lowest observed effect level was 0.31 mg l⁻¹. In addition to zebrafish, the study also used water fleas *Daphnia magna* and green algae (*Scenedesmus obliquus* and *Chlorella vulgaris*). These treatments revealed that daphnids are also sensitive to EQ, exhibiting detrimental effects with a half-maximal effective concentration (EC₅₀) of 2.65 mg l⁻¹ after 48 h of exposure. The algae appeared to be at least 2 times less sensitive to EQ than fish embryos or daphnids. The results were used to calculate the risk for aquatic life resulting in a maximum tolerable level of 1 µg l⁻¹ for fish embryos and daphnids, with a safety factor of 300. According to current knowledge, this does not exceed environmental concentrations of this substance. However, this study raises further concern about the (until recently) legal maximum tolerable EQ levels in fish feeding and the rather slow pace at which authorization to use EQ as a feed additive for diverse animals in Europe is being suspended.

KEY WORDS: Feed additive · Antioxidant · Pesticide · Toxicity · Embryo · Teratogen · Zebrafish · *Danio rerio* · Water flea · *Daphnia* · Algae · *Scenedesmus* · *Chlorella*

INTRODUCTION

Initially registered as a pesticide in 1965, ethoxyquin (EQ; 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinolin; E324) is a broadly used antioxidant and has been used as a post-harvest indoor application for fruits (US EPA 2004). EQ has not been permitted as a pesticide in Europe since 2013, but can still be used as a feed additive (EFSA 2013). In addition, EQ is a major antioxidant in fish meal, and is used for long-distance overseas transport of fish meal to reduce oxidation and the subsequent danger of self-ignition (UNECE 2005). To date, as much as 150 mg EQ kg⁻¹

feed has been allowed to be added to animal feed (EEC 1990). Since EQ was suspected of being carcinogenic, the amount of EQ in feed for dogs was reduced from 150 to 75 mg kg⁻¹ in 1977. EQ is not permitted for use in human food, but considerable amounts of it enter our diet via contaminated food. The maximum allowable daily intake for humans is 5 µg kg⁻¹ body weight, a value based on studies on dogs (Dewhurst 1998, Gupta & Boobis 2005).

The major route of exposure to EQ for farmed fish is uptake through food. In carnivorous fish aquaculture, the inclusion of EQ-containing fish meal in the feed is common practice, resulting in traces of EQ

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and EQ derivatives in fish muscle. Feeding of salmonids with EQ-contaminated feed has led to detectable amounts of the parent compound and its dimeric metabolite in fish muscle (Bohne et al. 2007a,b, 2008). Similar results have also been obtained in halibut *Hippoglossus hippoglossus* and rainbow trout *Oncorhynchus mykiss* (Lundebye et al. 2010). A 2 wk recovery period was not sufficient to significantly reduce the detectable EQ values (Bohne et al. 2008). In the above-mentioned fish species, the dimeric metabolite of EQ reached approximately 10-fold higher concentrations than the parent compound. Despite the presence of this metabolite in fish feed (Thorisson et al. 1992, He & Ackman 2000), its pronounced occurrence in fish muscle was assumed to be due to the conversion of EQ to its dimer in fish (Bohne et al. 2007a,b, 2008). The reported occurrence of EQ and its metabolite in edible parts of fish at considerable concentrations led to the assumption that EQ, together with its metabolite, may reach the maximum tolerable threshold value for human consumption (Bohne et al. 2008).

The exposure of aquatic organisms to EQ via contaminated water is far less well studied. Irrespective of its extensive global use, knowledge of the occurrence of EQ in surface water is scarce. In 18 water samples from surface waters in Vietnam, EQ was detected at levels above the level of detection (LOD) of 8 ng l⁻¹, and 6 of those samples showed EQ concentrations above 0.1 µg l⁻¹ (Chau et al. 2015). The maximum level found in a water sample was 0.29 µg l⁻¹. This meant that (out of more than 1100 micropollutants that have been analysed) EQ was 1 of the 24 most frequently occurring substances in water bodies in Vietnam. The relatively low EQ concentrations in open surface waters might be due to its rapid degradation by irradiation (Bintou et al. 2015), but this might not be the case for recirculating aquaculture facilities.

Several studies have reported detrimental effects of EQ in animals, including weight loss, changes in liver and kidneys, anaemia, and increased lethargy (reviewed by Błaszczuk et al. 2013). The known effects in fish are restricted to reports of liver changes, effects on the immune system, changes to condition factors and growth performance, and increased mortality (Yamashita et al. 2009, Wang et al. 2010, 2015, Bogevik et al. 2016). The Pan Pesticide Database (www.pesticideinfo.org) listed a half-maximal lethal concentration (LC₅₀) value of 18 mg l⁻¹ after 96 h for EQ in juvenile rainbow trout. To date, the possible effects in vertebrates, including fish, of the relatively stable

metabolites of EQ remain unknown. However, feeding rats with 12.5 mg of the EQ dimer kg⁻¹ body weight d⁻¹ influenced liver enzyme activity in a similar way to the parent compound (Ørnsrud et al. 2011).

The toxicity of EQ on aquatic life has rarely been investigated, and even less is known about the toxicity of EQ metabolites. In addition, chronic studies on the effects of EQ on aquatic species are lacking. Therefore, the data obtained from the present study have been used to establish effect threshold values, which were used for further risk calculations. A toxicological profile should be established for quantitative risk assessments, and concentrations of concern should be defined (US EPA 2012). Due to the inconclusive data, a safety factor of 300 must be applied to the calculations (WHO 2012). Accordingly, the present study evaluated the toxicity of water-borne EQ for different aquatic organisms, including zebrafish *Danio rerio* embryos, daphnids *Daphnia magna*, and green algae (*Chlorella vulgaris* and *Scenedesmus obliquus*), in order to derive a risk.

MATERIALS AND METHODS

All chemicals were obtained from Sigma, including ethoxyquin (product no. E8260, lot no. 074K0026V).

Exposure of zebrafish and subsequent analyses

The zebrafish originating from the wild-type UFZ-Obi strain spawned at the Swiss Federal Institute of Aquatic Science and Technology (EAWAG) facilities (Dübendorf, Switzerland) in May and June 2016. The test was conducted according to the DIN-norm 38415-6 (DIN 2001), starting with zebrafish eggs at 4 h post-fertilization. The eggs were incubated with different EQ concentrations in sterile ISO water containing calcium chloride 2-hydrate (294 mg l⁻¹), magnesium sulfate-7-hydrate (123.3 mg l⁻¹), sodium hydrogen carbonate (63 mg l⁻¹), and potassium chloride (5.5 mg l⁻¹). The pH was 7.4. In addition, 3,4-dichloroaniline (3,4-D) was used at concentrations of between 0.25 and 4 mg l⁻¹ as a positive control, since according to the revised Organisation for Economic Cooperation and Development (OECD) guideline, a new criterion for early life stage tests with zebrafish includes the observation that an exposure to 4 mg l⁻¹ of this substance results in at least 30% mortality after 96 h of exposure (EURL ECVAM 2014).

For the range-finding experiment, 4 plates with 4 embryos for each treatment were incubated for 96 h at 28°C. Each plate contained control exposures with eggs exposed to ISO water only and to a solvent control containing the same ethanol content as the EQ treatments (0.1 % v/v). In the second experiment, the fish eggs were exposed to EQ concentrations of 5 mg l⁻¹ and lower, at an incubation temperature of 27.5°C to more accurately determine the half-maximal effective concentration (EC₅₀) value for EQ. Embryonal development was determined using a microscope (Leica Type 090-135.006) at 24, 48, 72, and 96 h post-fertilization. For the subsequent experiment, 4 plates with 4 embryos for each treatment were incubated for 96 h at 27°C. Mortality, stage of development, the presence of eyes, somites, the movement of the tail, and possible occurrence of oedema were noted after 24 h of exposure. After 48 and 72 h of exposure, pigmentation and blood flow were also recorded. At 48 and 72 h post-fertilization, the heart rate of each living embryo was assessed for 20 to 30 s and the heart rate (in beats min⁻¹) was calculated.

Exposure of daphnids and subsequent analyses

Daphnia magna specimens were obtained from the University of Basel (Switzerland) and the daphnids were grown in Aachener Daphnien Medium (ADaM) according to Klüttgen et al. (1994) for several months. Juveniles at a maximum age of 24 h were selected for exposures to EQ concentrations of 8.25 mg l⁻¹ or serial dilutions of this concentration. Test solutions in ADaM were prepared in 24-well microtiter plates (TPP, Faust Labor). The solvent control was supplemented with 0.0125 % ethanol, and the control contained only ADaM. All plates were incubated at room temperature and a 16 h light:8 h dark cycle. According to the OECD guideline for the evaluation of immobilization of daphnids, the animals were evaluated after 24 and 48 h of exposure (OECD 1984).

Exposure of algae and subsequent analyses

Serial dilutions of a 16.5 mg l⁻¹ EQ concentration in 0.1 % algae medium (Brányiková et al. 2011) were prepared in 96-well microtiter plates (TPP, Faust Labor) using 8 wells treatment⁻¹, including a negative control only containing medium supplemented with 0.0125 % ethanol. Each well contained 200 µl of medium. All treatments were prepared on triplicate

plates. The CCALA 454 strain of *Scenedesmus obliquus* and CCALA 256 strain of *Chlorella vulgaris* were obtained from the Culture Collection of Autotrophic Organisms (Trebon, Czech Republic) and were grown in the algae medium. A volume of 10 µl of algal cells was added to each well, except for the medium control wells to which the same amount of only medium was added. For *C. vulgaris*, 59 500 cells were added to the corresponding wells; 28 800 cells were used for *S. obliquus*. Cell numbers were determined by counting algal solutions in an improved Neubauer chamber (Carl Roth). The prepared microtiter plates were analysed for their absorbance at a wavelength of 750 nm and their chlorophyll fluorescence (using excitation at 420 nm and measuring light emission at 683 nm) in a plate reader (Infinite M200 Pro, Tecan). This was performed at the beginning of the experiment, then after 24 and 48 h of incubation at 25°C with 4.5 % CO₂ in a Multitron Pro incubator (Infors) under constant orbital shaking at 400 rpm on an Eppendorf Thermomixer R mixer/incubator integrated shaker (Eppendorf).

Analysis of EQ

Cooled samples were sent to Eurofins Scientific (Schönenwerd, Switzerland) for EQ analysis by means of an accredited GC-MS method. In the medium used for zebrafish embryo exposures, only EQ was analysed. In the daphnia and algae medium, EQ and the concentration of the EQ dimer were assessed.

Statistics

Chi-squared statistics were employed to compare the incidence of lethal and non-lethal damage to the embryos by using the Monte Carlo approximation (with a confidence interval of 99%) to the Pearson chi-squared test. The heart rates were compared by using Mann-Whitney *U*-tests and Kruskal-Wallis tests in SPSS v.21 (SPSS). Differences between treatment groups were considered statistically significant when $p < 0.05$.

Risk assessment

Based on recommendations (US EPA 2012), toxicological profiles were established and concentrations of concern were derived. The first part of such

a risk assessment is the assessment of toxicity of a chemical.

To assess risk, it was necessary to perform an exposure assessment. For substances in the environment, it is generally assumed that the more the substances are added to ecosystems, the higher the exposure potential. EQ is a widely used industrial chemical that is produced every year in large amounts. Exposure of fish in aquaculture certainly includes exposure from food. Since EQ is a lipophilic compound, this exposure route is the most important one. However, recent reports on EQ detection in surface water samples have raised concerns about how common a micropollutant EQ might be (Chau et al. 2015). No sufficient data on the occurrence in water of the more hydrophilic metabolites of EQ are currently available, and they have rarely been investigated, even in aquatic organisms (Bohne et al. 2007a,b, 2008). For this reason, metabolites of EQ cannot be included in this study.

In order to characterise the risk, toxic levels need to be compared to the actual exposure situation and specific safety factors applied. According to Annex VI of Directive 91/414/EEC of EU Legislation (EEC 1991), a factor of 10 should be chosen for risk assessment of chronic effects. However, up-to-date chronic studies are not available for EQ. In conventional risk assessments, a safety factor of 50 is used to derive predicted no-effect concentrations (PNECs) to compensate for uncertainty in measurements due to accumulation rates, metabolism, intra-species and inter-species variation, differences related to age, sex, nutritional status and time of exposure, and the uncertainty of extrapolation of laboratory studies to the field. Further recommendations include basing calculations on the most sensitive species, which is also unknown for EQ. Thus, this approach was not applicable to the current data set. Given these serious limitations, a safety factor of 50 was recommended to derive PNECs, since this is the value recommended for chronic toxicity data not necessarily derived from the most sensitive species (OPP 2002). The reasons for this decision are as follows: for acute studies, a more conservative factor of 100 is recommended, especially when the above-mentioned sources make a strong contribution to overall uncertainty (OPP 2002). Consequently, the US EPA recommended a safety factor of 100 for acute, dietary exposure of higher vertebrates (10 for each, inter- and intra-specific variation, respectively).

The US EPA safety factors also incorporate a factor 3 for uncertainties related to inter- and intraspecies-

specific differences, extrapolation of sub-chronic to chronic values and the use of lowest observable effect levels (LOELs) instead of no observed effect levels (NOELs). Finally, the Food Safety Commission of Japan (FSCJ 2014) conducted a risk assessment for EQ and applied a safety factor of 300 (10 for species difference, 10 for individual difference, and 3 because it was assumed to be more appropriate to adopt the lowest observed adverse effect level [LOAEL]). So far, no safe values could be derived from summarising studies on Atlantic salmon, Atlantic croaker, and Nile tilapia (Bohne et al. 2008, Yamashita et al. 2009, Wang et al. 2010, EFSA FEEDAP 2015). Therefore, dose-response studies were conducted in different aquatic organisms for the present study.

RESULTS

The toxicity data in the different assays were used to derive LOELs after 24 and 48 h of exposure to EQ (Table 1). The results of the individual experiments are presented in the following sections.

Expt 1 with zebrafish

After 24 h of exposure, all fish eggs exposed to EQ concentrations of 10 mg l⁻¹ or higher died (Fig. 1). Mortality in the control and in the solvent control was 12.5 %. Fish eggs exposed to 5 mg l⁻¹ EQ showed a significantly higher mortality of 25 % and a further 62.5 % of the embryos showed damage, such as severe yolk sac deformation and reduced yolk sac transparency. At this time point the larvae were not yet pigmented.

Table 1. Lowest observable effect levels (LOELs) of ethoxyquin (EQ) for the corresponding toxicity tests and calculation of predicted no effect concentrations (PNECs) when applying various safety factors

Organism	LOEL (mg l ⁻¹)	
	24 h	48 h
<i>Danio rerio</i>	1.25	0.31
<i>Daphnia magna</i>	0.26	0.52
<i>Chlorella vulgaris</i>	2.06	4.31
<i>Scenedesmus obliquus</i>	4.31	4.31
PNEC plus safety 50	0.039	0.047
PNEC plus safety 100	0.020	0.024
PNEC plus safety 300	0.007	0.008
PNEC plus safety 300 (animals only)	0.003	0.001

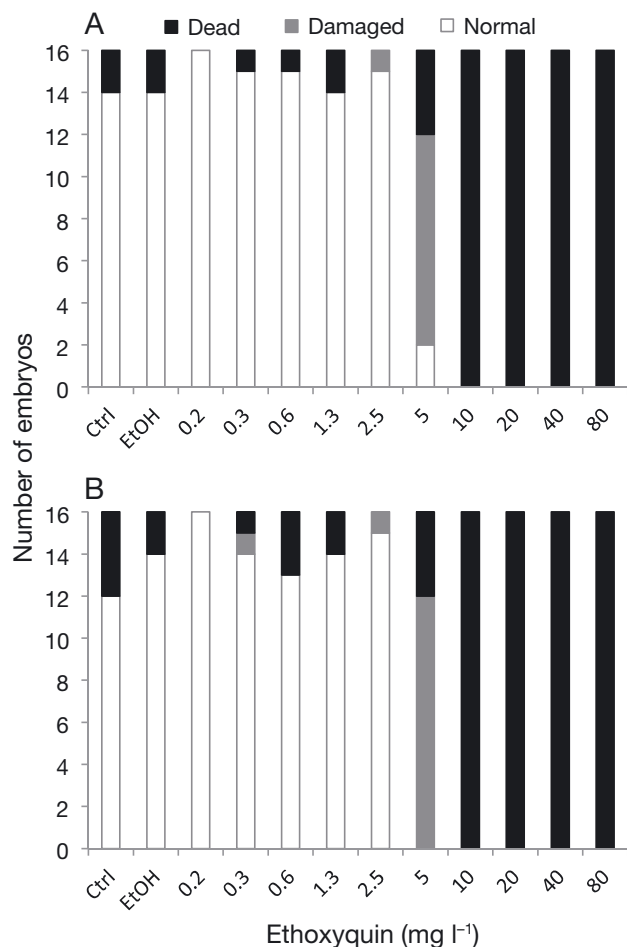


Fig. 1. Differences in the development of zebrafish embryos (*Danio rerio*) after (A) 24 h and (B) 48 h of exposure to ethoxyquin in the first experiment ($n = 16$ treatment⁻¹). Negative control with ISO water

After 48 h of exposure, the pigmentation of the embryos had started, the eye of normally developed embryos was dark-pigmented, and yolk sac depletion was in progress. Mortality increased further in the groups exposed to 5 mg l⁻¹ EQ (Fig. 1). Some EQ-exposed embryos not only showed yolk sac deformations, but also reduced eye development, and pigmentation was missing in some embryos (Fig. 2). One embryo also showed deformation of the spine (Fig. 2B). From this first range-finding experiment, it was observed that the LC₅₀ for EQ in zebrafish embryo ranged between 10 and 20 mg l⁻¹. Calculated LC₅₀ values were found to be 14.7 to 14.8 mg l⁻¹ for 24 and 48 h of exposure, respectively. The EC₅₀ values for any damage (including dead embryos) were found to be 8.1 and 7.8 mg l⁻¹ for 24 and 48 h of exposure, respectively. A second experiment was conducted to further define the EC₅₀ values.

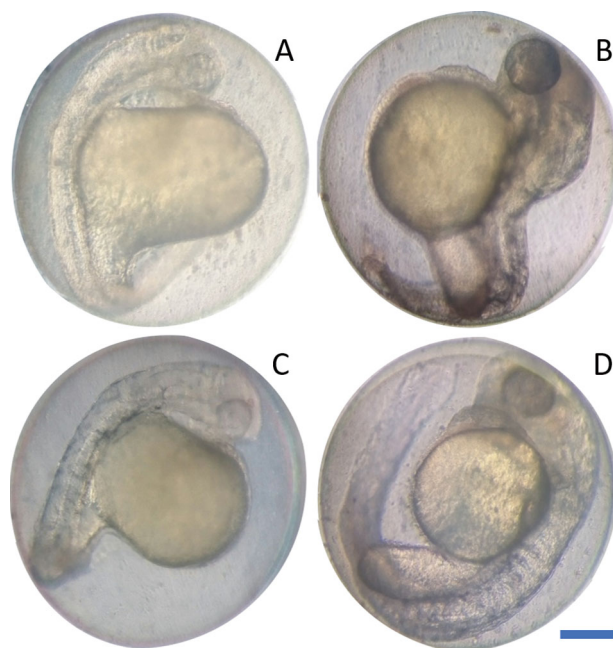


Fig. 2. Developmental problems of zebrafish *Danio rerio* in the first experiment, showing (A,C) increased yolk turbidity, yolk sac deformation, and a lack of eye pigmentation, and (B) spinal deformation in embryos after 48 h of exposure to 5 mg l⁻¹ ethoxyquin (A–C) compared with a control embryo (D). Magnification 100× (Leica microscope). Scale bar = 200 μm

Expt 2 with zebrafish

In this experiment, the detrimental effects on zebrafish embryos were confirmed and the resulting calculations exhibited a polynomial relationship ($y = -2.3817x^2 + 22.78x + 24.355$; $r^2 = 0.67$). Consequently, an EC₅₀ value of 1.30 mg l⁻¹ was calculated after 24 h of exposure. However, the detrimental effects on the embryos showed an EC₅₀ value of 3.70 mg l⁻¹ ($y = 1.2957x^2 + 1.4452x + 26.934$; $r^2 = 0.64$) after 48 h of exposure.

At 48 h post-fertilization, the heart rates of the embryos were assessed and combined with the results from the first experiment (Fig. 3), which showed a decrease in heart rate as EQ concentration increased ($y = -1.0263x^2 + 0.15x + 96.533$; $r^2 = 0.94$), and an EC₅₀ of 6.81 mg l⁻¹ was estimated. Fig. 4 shows that the heart rate was significantly reduced in embryos exposed to 0.31 mg l⁻¹ EQ. After 72 h of exposure, only the differences in heart rates between the control animals and embryos exposed to the 2 highest concentrations were assessed. These measurements showed that the heart rate of embryos exposed to these EQ concentrations were significantly lower than in the control animals. At 72 and 96 h of exposure, there was no pronounced increase in mortality. All embryos still living at 48 h of exposure also hatched by 96 h, but the embryos ex-

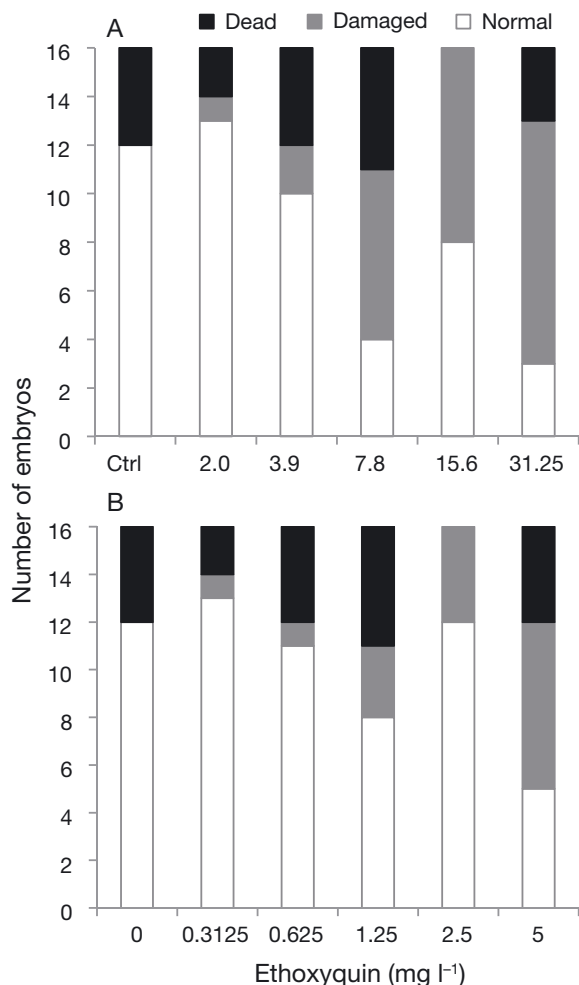


Fig. 3. Differences in the development of zebrafish embryos (*Danio rerio*) after (A) 24 h and (B) 48 h of exposure to ethoxyquin in the second experiment ($n = 16$ treatment⁻¹)

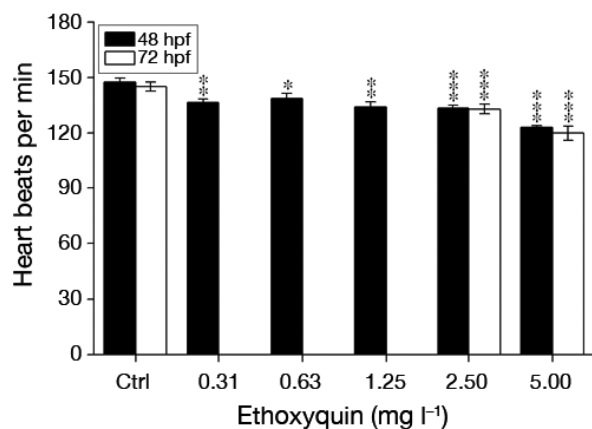


Fig. 4. Mean ($\pm$ SE) heart rate in zebrafish embryos (*Danio rerio*) after 48 and 72 h of exposure to different concentrations of ethoxyquin in the second experiment ($n = 8$ to 12 treatment⁻¹). Asterisks indicate significant differences to control embryos tested with the Mann-Whitney *U*-test and the Kruskal-Wallis test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

posed to the highest EQ concentration in this experiment (5 mg l^{-1}) showed the slowest development.

As expected, exposure to the reference compound 3,4-D resulted in mortality of the fish embryos (Fig. 5). After 24 h of exposure, significant damage occurred in embryos that had been treated with 4 mg l^{-1} 3,4-D, but no significant increase in lethal damage was noted. After 48 h of exposure, damaged embryos were observed, delivering an EC_{50} value of 0.66 mg l^{-1} , whereas the LC_{50} value was 3.03 mg l^{-1} (Table 2).

Experiments with daphnids

In the first experiment with daphnids, all animals exposed to concentrations higher than 10 mg l^{-1} died. The LC_{50} after 24 h of exposure to EQ was calculated to be 5.72 mg l^{-1} . However, in the second experiment, the EC_{50} for detrimental effects (including damage or death) to daphnids was calculated to be 13.54 mg l^{-1}

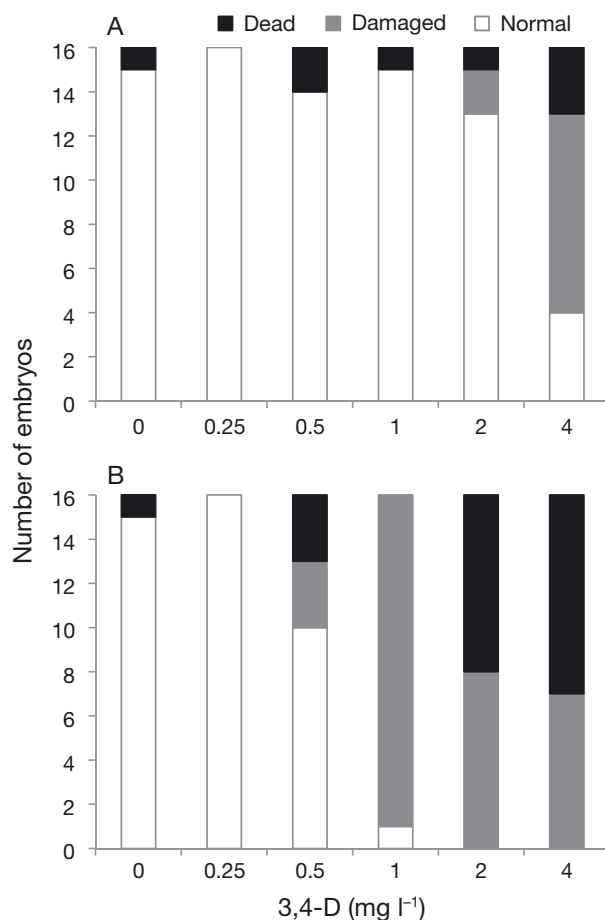


Fig. 5. Differences in the development of zebrafish embryos (*Danio rerio*) after (A) 24 h and (B) 48 h of exposure to the reference compound 3,4-dichloroaniline (3,4-D) ($n = 16$ treatment⁻¹)

Table 2. Median effective concentration (EC_{50}) and mean lethal concentration (LC_{50}) values obtained for the exposure of *Danio rerio* embryos ($n = 18$) to 3,4-dichloroaniline for different test durations

Exposure duration (h)	EC_{50} (mg l ⁻¹)	LC_{50} (mg l ⁻¹)
24	3.28	7.03
48	0.66	3.03

after 24 h of exposure to EQ and 2.65 mg l⁻¹ after 48 h (Fig. 6).

Experiments with green algae

The cell numbers of *Chlorella vulgaris* increased over time and evaluation of the cell numbers after 24 h of exposure to EQ did not reveal a linear dose-response relationship (Fig. 7, Table 3). However, exposure for 48 h to the highest EQ concentrations

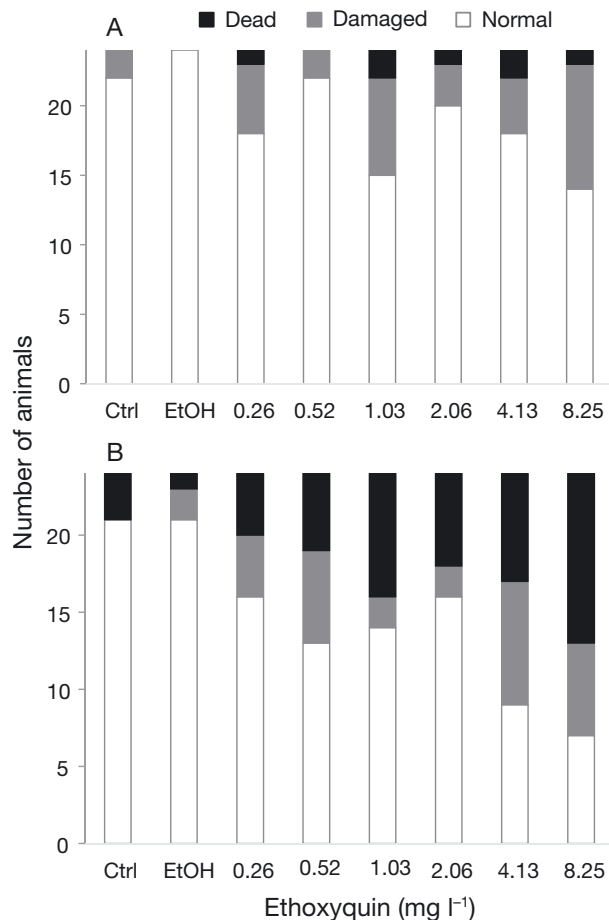


Fig. 6. Effects of ethoxyquin on water fleas *Daphnia magna* after (A) 24 h and (B) 48 h of exposure to different concentrations of ethoxyquin in the second experiment ($n = 24$ treatment⁻¹)

reduced the cell numbers estimated by absorption at 750 nm by 12 %. Consequently, LC_{50} values for this species could not be observed during the experiments, but a relationship between the EQ concentrations and the reduction in cell numbers could be described ($y = 0.067x^2 - 1.7874x + 99.702$; $r^2 = 0.92$). Since only viable cells respond with chlorophyll fluorescence emissions, the measured emitted light units were calculated based on a common light absorption value of 0.1 at 750 nm in order to allow comparison of the values (Fig. 8). For *C. vulgaris*, the relative fluorescence values after 48 h of exposure were 20 % higher in the incubations with the highest EQ concentration than in the control incubations. Therefore, a decreasing fluorescence trend with increasing concentrations could be assumed from the data, which allowed an EC_{50} value of 27.03 mg l⁻¹ ($-0.2907x^2 + 5.9696x + 101.07$; $r^2 = 0.92$) to be calculated.

Scenedesmus obliquus showed less pronounced growth over time compared to *C. vulgaris* (Table 3) and also did not exhibit a reduction in cell numbers after exposure to EQ for 24 h (Fig. 7). In contrast to the other algal species, *S. obliquus* was found to be more sensitive to EQ. A LC_{50} value of 31.48 mg l⁻¹

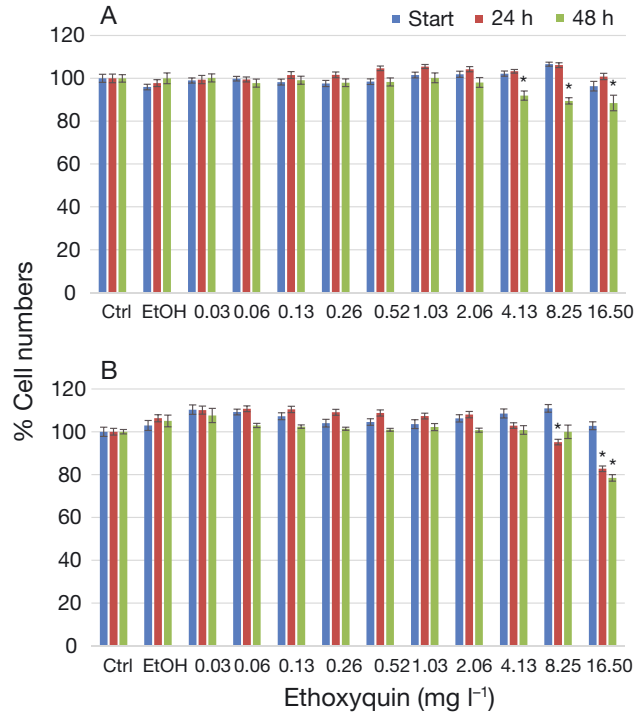


Fig. 7. Mean ($\pm$ SE) cell numbers estimated by absorption at 750 nm for green algae (A) *Chlorella vulgaris* and (B) *Scenedesmus obliquus* at the start of the experiment and after 24 and 48 h of exposure to different concentrations of ethoxyquin ($n = 21$ wells treatment⁻¹). *Significant difference to control ($p < 0.05$)

Table 3. Increase in biomass (assessed according to the difference in light absorption [OD] at 750 nm to evaluate cell numbers) obtained on exposure of *Chlorella vulgaris* and *Scenedesmus obliquus* algae (n = 21 wells treatment⁻¹) to ethoxyquin (EQ) over different test durations. Data are mean $\pm$ SE

EQ concentration (mg l ⁻¹)	Biomass increase (% of initial OD)			
	<i>Chlorella</i>		<i>Scenedesmus</i>	
	24 h	48 h	24 h	48 h
Control	109.6 $\pm$ 5.8	340.0 $\pm$ 17.9	73.7 $\pm$ 4.0	225.5 $\pm$ 12.0
Ethanol	113.7 $\pm$ 6.5	358.1 $\pm$ 21.0	79.5 $\pm$ 4.5	232.4 $\pm$ 14.8
0.03	110.2 $\pm$ 6.0	344.5 $\pm$ 18.6	73.2 $\pm$ 3.8	218.2 $\pm$ 16.1
0.06	109.1 $\pm$ 6.0	330.2 $\pm$ 17.9	75.5 $\pm$ 3.8	205.6 $\pm$ 10.3
0.13	116.7 $\pm$ 6.7	343.7 $\pm$ 19.0	78.4 $\pm$ 4.1	209.9 $\pm$ 10.6
0.26	118.2 $\pm$ 6.3	342.0 $\pm$ 19.5	81.9 $\pm$ 4.4	217.2 $\pm$ 11.3
0.52	122.9 $\pm$ 6.7	339.4 $\pm$ 19.4	80.3 $\pm$ 4.3	213.6 $\pm$ 11.1
1.03	117.9 $\pm$ 6.4	333.5 $\pm$ 18.7	80.0 $\pm$ 4.4	221.7 $\pm$ 13.2
2.06	114.6 $\pm$ 6.2	323.3 $\pm$ 18.7	76.3 $\pm$ 4.0	208.4 $\pm$ 11.2
4.13	112.2 $\pm$ 6.2	295.7 $\pm$ 17.2	65.0 $\pm$ 3.6	202.3 $\pm$ 11.8
8.25	108.7 $\pm$ 5.7	269.3 $\pm$ 14.6	49.4 $\pm$ 2.5	192.8 $\pm$ 13.3
16.50	120.3 $\pm$ 6.9	305.4 $\pm$ 22.6	40.4 $\pm$ 2.1	148.5 $\pm$ 8.2

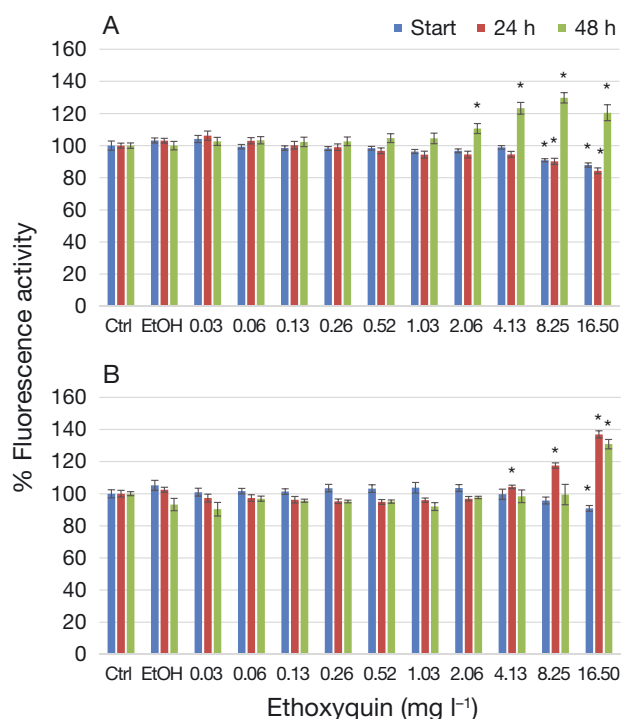


Fig. 8. Mean ($\pm$ SE) chlorophyll fluorescence activity of (A) *Chlorella vulgaris* and (B) *Scenedesmus obliquus* at the start of the experiment, and after 24 and 48 h of exposure to different concentrations of ethoxyquin (EQ) expressed as fluorescence units emitted at 683 nm per 0.1 nm of light absorption at 750 nm, calculated as percentage of control incubations (n = 21 wells treatment⁻¹). *Significant difference to control (p < 0.05)

was observed for this species ($y = -0.0188x^2 - 1.2611x + 108.34$; $r^2 = 0.87$) after 24 h of exposure and a LC₅₀ value of 23.84 mg l⁻¹ after 48 h ($y = -0.1047x^2 +$

$0.2908x + 102.59$; $r^2 = 0.91$). However, calculation of relative fluorescence values for this algal species showed that in the highest EQ incubation, 30% higher fluorescence values were obtained compared to the control incubations (Fig. 8).

DISCUSSION

This study revealed that EQ is toxic to aquatic organisms, with aquatic animals being more sensitive than algae to water-borne EQ exposure. During the experiments the algae displayed expected growth, and compared to the solvent controls, there were only minor reductions in cell

numbers due to exposure to EQ after 24 and 48 h of exposure. This implies that algal EQ toxicity is slower than the toxicity in animal models. Increased fluorescence activity in the algae exposed to the higher EQ concentrations indicates a disturbance to photosystem function (Maxwell & Johnson 2000). In contrast to the algae tests, the experiments with zebrafish embryos and daphnids allowed the calculation of EC₅₀ and LC₅₀ values.

Exposure to 3.03 mg l⁻¹ 3,4-D resulted in 50% mortality in fish embryos after only 48 h of exposure, and therefore fulfilled the criteria for the early life-stage test based on the revised OECD guidelines (EURL ECVAM 2014). Using a safety factor of 300, a threshold EQ value for potential damage to aquatic fauna of 1 μ g l⁻¹ could be established. Since the maximum level that has been found in a water sample is 0.29 μ g l⁻¹ (Chau et al. 2015), the environmental concentration of EQ can be assumed to be close to the PNEC in this study.

It can be assumed that the toxicity observed due to EQ exposure was due to the phenomenon that antioxidants can act as prooxidants when applied at high concentrations. In addition, phenolic antioxidants such as EQ are able to form phenoxyl radicals which show typical prooxidant activities (Decker 1997, Sakihama et al. 2002). In solution, at least a part of EQ has been reported to exist in the free radical form (Skaare & Henriksen 1975, Sakihama et al. 2002). As a consequence, oxygen species can occur which cause oxidative damage. In fish, liver changes and adverse effects on the immune system, growth, and increased mortality have been observed (Yamashita et al. 2009, Wang et al. 2010, 2015, Bøgevik et

al. 2016), which may be related to the prooxidant characteristics of EQ. The reason why the green algae were less affected by EQ than the animal species may be the high antioxidative capacity of algae of the genera *Chlorella* and *Scenedesmus*, probably bestowed by their considerable polyphenolic and flavonoid contents (Stoica et al. 2013, Ahmed 2016, Strejckova et al. 2017).

Compared to exposure to EQ in diets, the sensitivity of zebrafish embryos to EQ via the surrounding medium was high. Juvenile Japanese seabass *Lateolabrax japonicus* should not be exposed to food-borne EQ concentrations of more than 13.78 mg EQ kg⁻¹ diet for 12 wk in order to avoid growth depression as a result of EQ exposure (Wang et al. 2015). Nevertheless, environmental concentrations are considerably lower in water than in feed, although exact data for recirculating aquaculture systems are currently not available.

CONCLUSION

The present study raises concern about the (until recently) legal maximum tolerable EQ levels in fish feed in the European Union, and the relatively slow pace at which EQ authorization as a feed additive for diverse animals in Europe is being suspended. However, further studies are needed to establish no-effect concentration levels and to increase our knowledge of environmental EQ concentrations in different countries.

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