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Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part I: histopathological alterations and bioaccumulation in rainbow trout☆

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Abstract

Human and veterinary pharmaceuticals have been shown to occur in considerably high amounts in sewage treatment plant (STP) effluents and surface waters. The non-steroidal inflammatory drug diclofenac represents one of the most commonly detected compounds. Information concerning possible ecotoxicological risks of the substance are rather scarce. So far there are no data available on its possible effects in fish after prolonged exposure. In order to evaluate sublethal toxic effects of diclofenac in fish, rainbow trout (*Oncorhynchus mykiss*) exposed to diclofenac concentrations ranging from 1 μg/L to 500 μg/L over a 28 day period were investigated by histopathological methods. In addition, diclofenac residues in various organs were analyzed by means of gas chromatography/mass spectrometry (GC/MS).

The histopathological examinations of diclofenac-exposed fish revealed alterations of the kidney such as an hyaline droplet degeneration of the tubular epithelial cells and the occurrence of an interstitial nephritis. In the gills, the predominant finding consisted in a necrosis of pillar cells leading to damage of the capillary wall within the secondary lamellae. The lowest observed effect concentration (LOEC) at which both renal lesions and alterations of the gills occurred was 5 μg/L. In contrast, the light microscopical examination of the liver, the gastro-intestinal tract, and the spleen did not reveal any histopathological alterations neither in diclofenac-exposed fish nor in solvent controls or control individuals.

Chemical analysis showed a concentration-related accumulation of diclofenac in all organs examined. The highest amounts could be detected in the liver, followed by the kidney, the gills and the muscle tissue. Dependent on the diclofenac concentration used, the bioconcentration factors (BCF) were 12–2732 in the liver, 5–971 in the kidney, 3–763 in the gills, and 0.3–69 in the muscle respectively. From the present findings it can be assumed, that prolonged exposure in environmentally relevant concentrations of diclofenac leads to an impairment of the general health condition of fish.

Keywords: Diclofenac; Fish toxicity; Histopathology; Bioaccumulation

☆ Dedicated to Olivia Luna

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1. Introduction

Human and veterinary pharmaceuticals have become a matter of concern in aquatic ecotoxicology due to the fact that many of these compounds have been shown to reach considerably high concentrations within surface waters (Stumpf et al., 1996; Halling-Sørensen et al., 1998; Heberer and Stan, 1998; Heberer et al., 1998; Sacher et al., 1998; Ternes, 1998; Lehmann, 2000; Ternes, 2001), STP effluents (Stumpf et al., 1996; Heberer and Stan, 1998; Ternes, 1998; Ternes, 2001) and ground waters (Heberer and Stan, 1998; Heberer et al., 1998). Whereas human drugs enter the environment via STP effluents, the use of sludges in agriculture, or leachates from waste disposal sites, veterinary pharmaceuticals may contaminate the environment through manure or directly due to aquacultural use (Henschel et al., 1997). For the notification and registration of industrial chemicals and pesticides, the evaluation of the environmental safety based on certain unified principles within the European Union is prescribed by law. In contrast to veterinary drugs, for which ecotoxicity testing is also considered as a part of the registration procedure according to Directive 92/18 EEC and the corresponding "Note for Guidance" (EMEA, 1998), for the assessment of environmental risks of human pharmaceuticals only a draft guideline has recently been released (EMEA, 2003).

Among human pharmaceuticals, the non-steroidal inflammatory drug diclofenac is one of the most commonly found substances within the aquatic environment. After therapeutic use in humans, only 15% of diclofenac is excreted unchanged (Landsdorp et al., 1990). For the most part diclofenac is eliminated following biotransformation to glucoroconjugated and sulphate metabolites which are excreted via the kidney (Davies and Anderson, 1997). As a consequence, diclofenac or its metabolites as most of the pharmaceuticals reach the aquatic environment via domestic sewage and STP effluents (Stumpf et al., 1996; Heberer and Stan, 1998; Ternes, 2001). Within the sewage, phase II human metabolism may be reversible if conjugates are exposed to microbial activities which might lead to a re-release of the biologically active parent compound within the effluents (Webb, 2001). The elimination rate of diclofenac during sewage treatment processes is in the range of 69% (Ternes, 2001). The median concentrations in the environment are reported to be 0.81 μg/L in municipal STP effluents and 0.15 μg/L in rivers and streams (Ternes, 1998). Maximum concentrations in the range of 2 μg/L in STP effluents (Stumpf et al., 1996; Ternes, 1998, 2001) and surface waters (Lehmann, 2000) have been detected.

Diclofenac is widely used because of its analgesic and antiphlogistic properties. These therapeutic effects are based on the fact that diclofenac as an amphiphilic acid binds to the lipid–water interphase of cell membranes thereby inhibiting the synthesis and release of prostaglandines (Kuschinsky and Lüllmann, 1981). However, apart from the therapeutical effects, several unwanted side effects of diclofenac have been described after therapeutical use in humans and in pharmacological studies using laboratory animals such as rats and dogs. These include gastropathy (Manocha and Venkataraman, 2000; Ramesh et al., 2002; Caselli et al., 1995), nonsteroidal drug colitis (Baert et al., 1995; Gut et al., 1996; Bjorkman, 1998; Puspok et al., 2000), degenerative and inflammatory liver alterations (Manocha and Venkataraman, 2000; Banks et al., 1995; Bjorkman, 1998; Hackstein et al., 1998; Dierkes-Globisch et al., 2000), and both morphological and functional renal changes (Manocha and Venkataraman, 2000; Revai and Harmos, 1999; Ramesh et al., 2002). Data on the effects of pharmaceuticals upon aquatic organisms are in most cases restricted to short-term acute responses such as lethality in algae, invertebrates and fish (Webb, 2001). In *Daphnia magna* an immobilization due to diclofenac has been described after an 24 h exposure to 74 mg/L (Kopf, pers. comm.). Investigations of Dietrich and Prietz (1999) revealed lethality and teratogenicity in diclofenac exposed zebra fish embryos after a 96 h exposure to 480 ± 50 μg/L (lethal concentration, LC50/96 h) and 90 ± 20 μg/L (effect concentration, EC 50/96 h) respectively. The only data available upon the chronic toxicity of diclofenac are restricted to a 21 d-reproduction study using *Daphnia magna* (Kopf, pers. comm.), according to which the lowest concentration (lowest observed effect concentration, LOEC) leading to reduced reproduction was 0.2 mg/L, whereas no effects could be observed after exposure to 1 mg/L (no observed effect concentration, NOEC).

Based on the facts that (1) pharmaceuticals in general display highly specific effects and are intended to be used for therapeutical purposes in vertebrates

partly over longer periods of time and (2) pharmaceuticals reach the aquatic environment continuously leading to chronic exposure of aquatic organisms, the present study was conducted in order to evaluate possible long-term effects of diclofenac on the health status of fish as a non-target vertebrate species. Histopathology was applied to detect diclofenac-induced organ lesions. In parallel, electron microscopic investigations have been conducted to characterize the cellular and sub-cellular reactions on the ultrastructural level (Triebskorn et al., 2004). In addition, residues of diclofenac in various fish organs were analyzed by chemical analytical methods. The overall intention of the study was to evaluate threshold levels at which histopathological organ changes occur in order to enable a realistic risk assessment of environmentally relevant concentrations of the compound.

2. Materials and methods

2.1. Experimental design

A 28 day experiment was carried out using rainbow trout as test organisms. Fish were obtained from the institute's breeding stock and were reared under disease-controlled conditions until they were subjected to the experiment at the age of 1.8 years (average body weight: 167.6 ± 20.28 g; average body length: 25.9 ± 1.04 cm). The exposure took place under flow-through conditions in 100 L aquaria containing 24 fish each (water flow rate: 9 L/h). Fish were exposed to nominal concentrations of 1, 5, 20, 100 and 500 μg Diclofenac/L. To control for possible side effects of the solvent used, an additional group of fish was exposed to 0.12‰ dimethylsulfoxid (DMSO). Control fish were maintained without any treatment in natural well water which was regularly checked for chemical and physical parameters (temperature: 10.3 °C; oxygen saturation: 70%; pH: 7.4; conductivity: 730 μS/cm; hardness: 21.2 dGH). During the experiment fish were fed a commercial ration (Trouvit, F4-Proaqua 18) every second day (1% of body weight). Photoperiod was maintained in a 12:12 h light:dark regime including a half-light phase of 30 min every morning and evening.

The test substance diclofenac (purity: 99.9%) was purchased from Sigma Aldrich (Deisenhofen, Germany). To obtain the final test concentrations diclofenac was diluted in DMSO and then mixed with well water to obtain a stock solution containing 5 g diclofenac/L. The stock solution was added in the appropriate amount to the aquarium water using pumping devices ((Minipuls, Gilson-Abimed, Langenfeld, Germany) via a combination of Polytetrafluorethylene (PTFE®) and Isoversinic tubes® (Abimed, Langenfeld, Germany) to obtain the final test concentrations. Diclofenac concentrations within the test waters were determined once a week throughout the exposure period. Prior to the analytic procedure a clean up of water samples was carried out by solid phase extraction (SPE) using florisil- and C_{18} columns. The chemical analyses were performed using high performance liquid chromatography with diode array detection (HPLC/DAD) and diphenylacidic acid as an internal standard. The detection limit for diclofenac within the water samples was determined to 50 ng/L. In Table 1 the measured real concentrations of diclofenac within the test waters are given.

2.2. Histopathological examinations

After the 28 day exposure period, 10 fish per group were anaesthetized in ethylenglycol monophenylether (Merck, Darmstadt) at a concentration of 1:1000 and subsequently killed by decapitation. After complete necropsy of fish, tissue specimens of the liver, the gastro-intestinal tract (stomach wall, anterior and posterior part of the intestine), the spleen, the gills and the middle portion of the posterior kidney were re-

Table 1
Real diclofenac concentrations (μg/L; means ± S.D.) analyzed within the test water during the exposure period

	Diclofenac concentration (μg/L)				
Nominal concentration	1	5	20	100	500
Real concentration	1.06 ± 0.05	4.95 ± 0.02	20.13 ± 0.15	100.9 ± 0.80	501.2 ± 1.24

n=4; the number of water samples analyzed.

moved, fixed in buffered formalin (4%), and routinely processed for paraffin embedding. Sections were cut at 3 μm and stained with hematoxylin and eosin (H&E). Sections of kidney samples were additionally stained with periodic acid Schiff reaction (PAS) to identify glycoproteins within the renal tubular epithelial cells and the tubular lumina (Romeis, 1989). After a qualitative histopathological assessment of tissue lesions, both the degree and severity of histopathological alterations were evaluated semi-quantitatively as described by Schwaiger et al. (1997). Briefly, a ranking was used (grade 1: no pathological alterations, grade 2: focal mild to moderate changes, grade 3: extended severe histopathological alterations) to establish an overall assessment value of histopathological findings for each organ of each individual fish. From these data mean assessment values (MAV) of alterations were calculated for each exposure and control group.

2.3. Chemical analysis of diclofenac residues in fish organs

Tissue samples of liver, kidney, spleen, gills and muscle of five individuals of each test and control group were removed and stored at −20 °C until they were subjected to chemical analysis. The clean-up procedure includes solid phase extraction with Extrelut NT 20 (Merck, Darmstadt) and methylation with TMSH (Macherey-Nagel, Düren). The analysis of diclofenac sodium in these samples was carried out according to Ph.Eur. 2002, 2.2.28 using gas chromatography/mass spectrometry (GC/MS) with external standard methodology (ESTD). The detection limit was determined to <10 μg/kg with reference to the wet weight. The precision, linearity, recovery and specificity of the methodology were established prior to the beginning of the study by spiking uncontaminated tissue samples of fish with appropriate amounts of diclofenac sodium.

2.4. Statistical evaluation of data

For statistical analysis of data, the SPSS software was used. For both the histopathological findings based on a semi-quantitative evaluation and the quantitative chemical-analytical data means ± S.D. were calculated. First of all an explorative analysis of data was performed and data were proofed with regard to their normal distribution using the Shapiro–Wilk test. In case of homogeneity of variances and normal distribution of data, differences between treatment groups were determined by ANOVA. As appropriate post hoc test, the Dunnett *t*-test was applied. In case of inhomogeneity of variances, the non parametric Kruskal–Wallis test was performed followed by the Mann–Whitney *U*-test. The latter was also applied to analyze for significance of differences between two groups which did not reveal a normal distribution of data.

3. Results

3.1. Histopathological findings

The light microscopical examination of the liver, the gastro-intestinal tract, and the spleen did not reveal any histopathological alterations neither in diclofenac-exposed fish nor in solvent controls or control individuals. In contrast, in the kidney and the gills histopathological changes could be observed.

Whereas in the kidney of control animals and solvent controls the histopathological findings were restricted to a slight accumulation of protein droplets within the tubular epithelial cells, diclofenac-exposed individuals showed distinct renal changes. These alterations consisted of a severe hyaline droplet degeneration accompanied by an accumulation of proteinaceous material within the tubular lumina, and a vacuolation and single cell necrosis of tubular epithelial cells. Furthermore a distinct proliferation of the renal interstitial tissue could be observed (Fig. 1).The degree and severity of kidney lesions, expressed as MAVs, were significantly increased from a concentration of 5 μg/L up (Fig. 2).

Alterations of the gills which occurred in exposed and control fish included a slight focal proliferation of interlamellar cells and chloride cells, mild inflammatory reactions and occasional epithelial lifting. The predominant findings which occurred exclusively in diclofenac-exposed fish consisted of degenerative and necrotic changes of the pillar cells as well as a dilation of the capillary walls (Fig. 3). In single cases severe teleangiectasia could be observed. Furthermore the incidence of respiratory epithelial cell necrosis increased. Expressed by MAVs (Fig. 4) the degree and

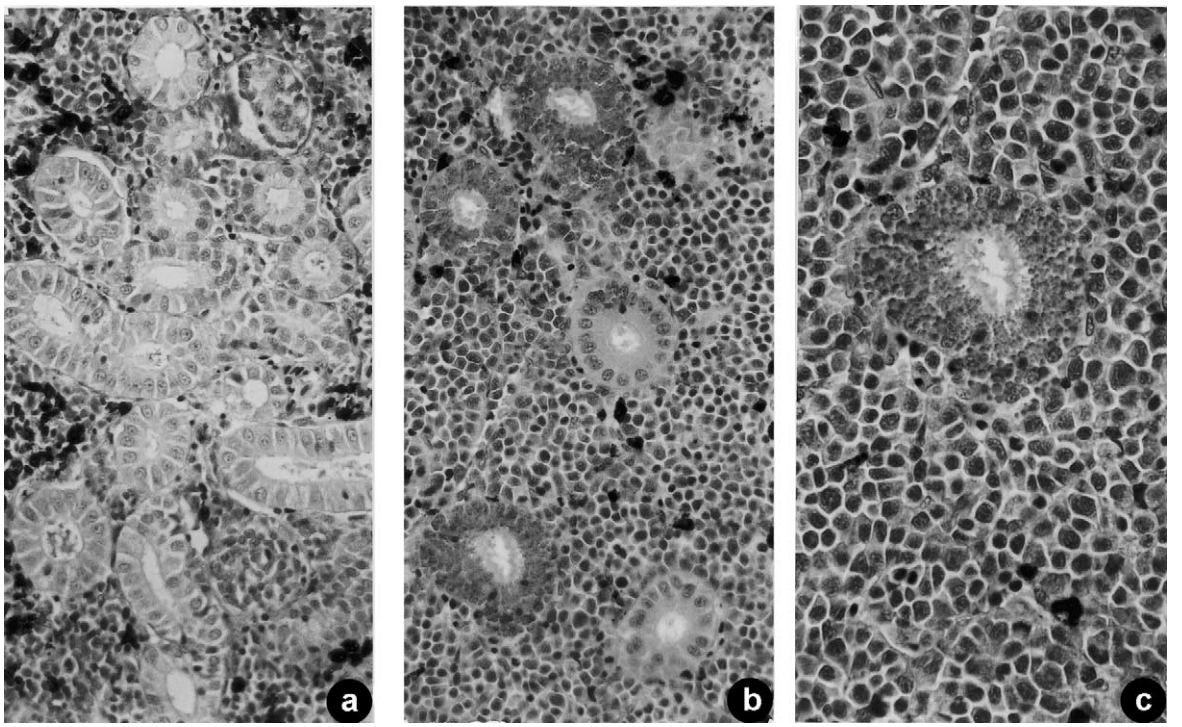

Fig. 1. Renal tissue of rainbow trout. (a) Control, (b, c) after exposure to 100 μg diclofenac/L: severe hyaline droplet degeneration of tubular epithelial cells and interstitial nephritis; original magnification (a, b) 250×; (c) 400×; H&E stain.

severity of gill damage increased significantly after exposure to 5 μg/L and higher diclofenac concentrations compared to the control level.

3.2. Diclofenac residues in fish organs

In both control fish and solvent controls the chemical analysis did not reveal any residues of diclofenac in the tissues examined such as muscle, gills, kidney, and liver.

In diclofenac-exposed individuals a concentration-related accumulation of the compound occurred in the above mentioned organs. In general, the highest concentrations were detected in the liver, followed by the kidney and the gills. In the muscle tissue only small amounts of diclofenac were analyzed. Already after exposure to 1 μg diclofenac/L residues of the compound could be detected which were with reference to the wet weight 2882.4 ± 159.1 ng/g in the liver, 1024.8 ± 67.5 ng/g in the kidney, 804.6 ± 101.9 ng/g in the gills, and 72.8 ± 23.3 ng/g in the muscle respectively. In Fig. 5 the mean concentrations of diclofenac in various tissues of fish exposed to the different test concentrations are given. The calculated bioconcentration factors (BCF) decreased with increasing test concentrations as shown in Fig. 6. Dependent on the diclofenac concentration tested, the BCFs were 12–2732 in the liver, 5–971 in the kidney, 3–763 in the gills, and 0.3–69 in the muscle, respectively.

4. Discussion

Diclofenac represents a widely applied antiphlogistic and antirheumatic drug with an estimated prescription amount in Germany of about 75 tons per year (Landsdorp et al., 1990). Due to the facts that after therapeutical use part of the drug is excreted unchanged (Landsdorp et al., 1990), and degradation by STWs is incomplete (Ternes, 2001), maximum concentrations in the range of 2 μg/L in surface waters have been detected (Lehmann, 2000). Although such

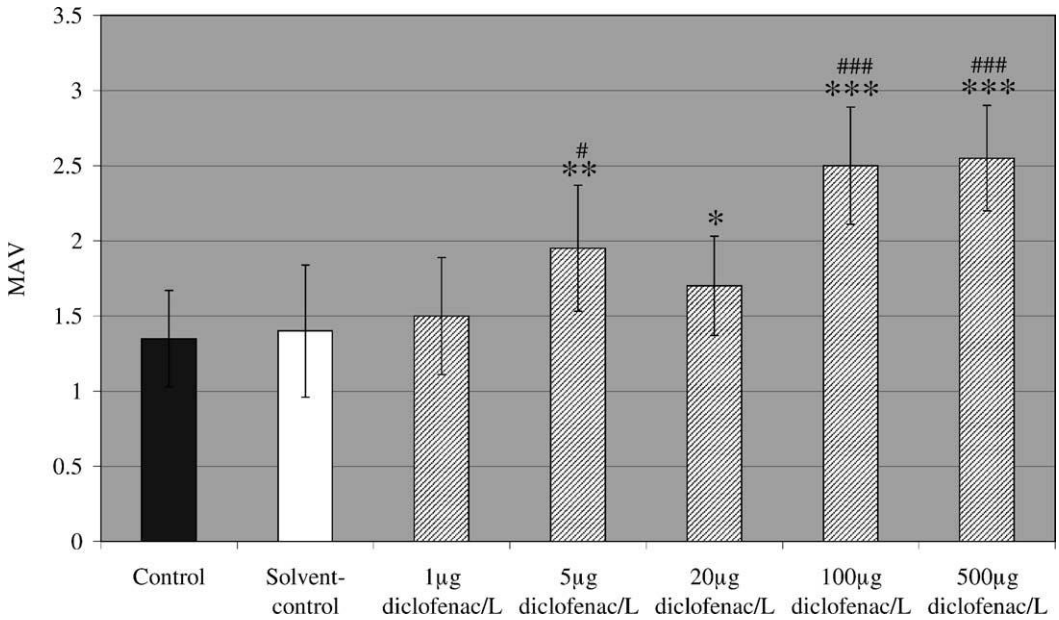

Fig. 2. Mean assessment values (MAV; means ± S.D.) of histopathological alterations in the kidney of rainbow trout after exposure to diclofenac over 28 days, calculated on the basis of semiquantitative data (grades 1–3 of histopathological alterations). *control vs. 1, 5, 20, 100, 500 μg diclofenac/L; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; # solvent-control vs. 1, 5, 20, 100, 500 μg diclofenac/L; # $P < 0.05$, ### $P < 0.001$.

a considerably high amount of diclofenac can reach the aquatic environment, so far there are no data available on the chronic fish toxicity of this compound. This could partly be explained by the fact that the acute toxicity of diclofenac on aquatic organisms such as *Daphnia magna* has been reported to be in the mg/L range (Kopf, pers. comm.), leading to the assumption that its toxicity in general seems to be very low. The present study indicates however, that a 4 weeks exposure of rainbow trout to diclofenac in an environmentally relevant concentration range leads to distinct histopathological alterations in the kidney and the gills. The threshold concentration which induced both renal lesions and alterations of the gills has been shown to be 5 μg/L. As a consequence the no observed effect concentration (NOEC) based on histopathological lesions could be established to be 1 μg/L.

The diclofenac induced renal lesions consisted in a severe hyaline droplet degeneration of tubular epithelial cells and a distinct proliferation of interstitial cells in the sense of an interstitial nephritis. At the ultrastructural level, additional glomerular damage could be observed (Triebskorn et al., 2004). Comparable alterations of the kidney as a consequence of therapeutical doses of diclofenac have also been demonstrated in mammalian laboratory animals and humans. Tubular changes such as necrosis and intraluminal secretion (Manocha and Venkataraman, 2000), or dilation (Ramesh et al., 2002) have been shown to occur after a short-term treatment in rats and dogs respectively. Commonly reported renal complications of a diclofenac treatment in humans include acute interstitial nephritis as well (Revai and Harmos, 1999). Due to the fact that acute renal failure has been recognized in diclofenac-treated humans (Radford et al., 1996; Revai and Harmos, 1999), and vulture populations (Oaks et al., 2004) an impairment of the renal function is likely to occur also in fish after chronic exposure. Apart from the kidney lesions, which at least partly seem to be comparable to those found in other species, the present study revealed severe alterations of the gills in diclofenac exposed rainbow trout. The predominant finding, which occurred already after exposure to 5 μg/L consisted in a distinct degeneration and necrosis of pillar cells and consequently in a damage of the capillary walls of the secondary gill lamellae.

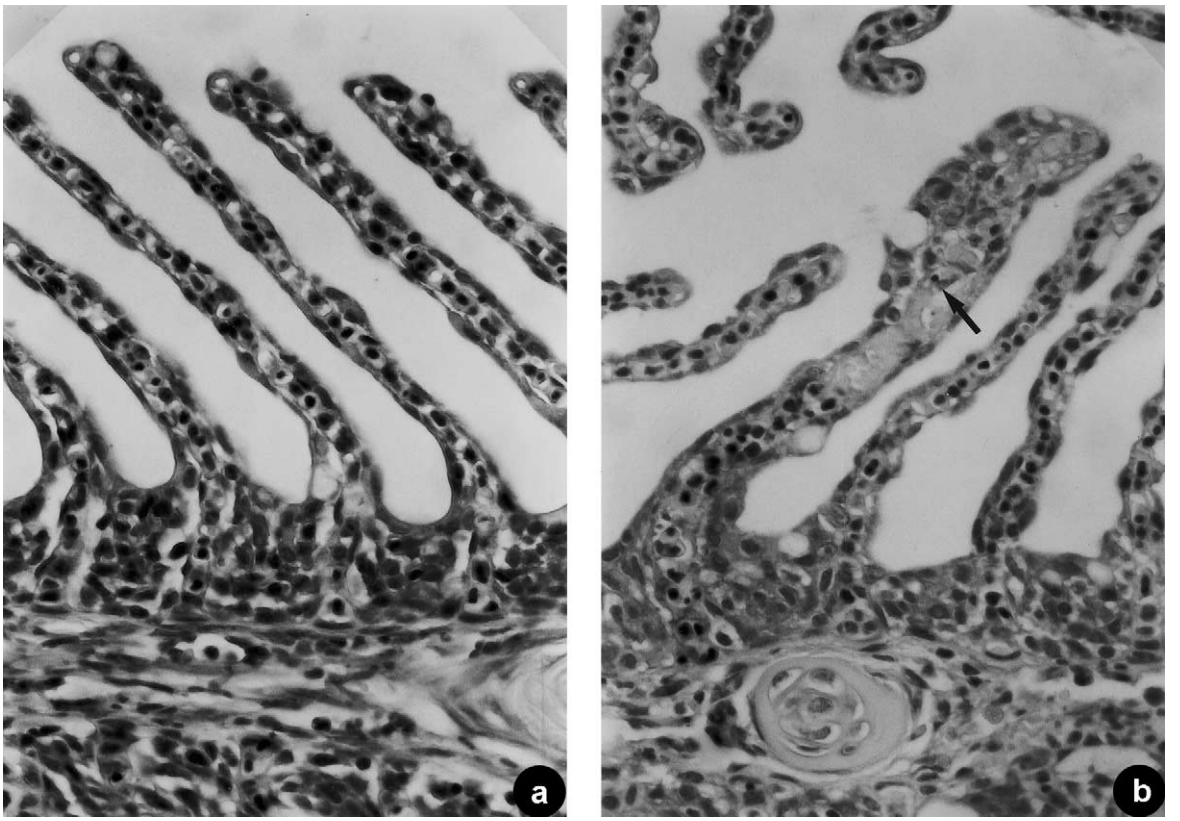

Fig. 3. Gills of rainbow trout. (a) Control, and (b) after exposure to 100 μg diclofenac/L: degeneration and necrosis of pillar cells (arrow) and dilation of capillary walls; original magnification 400×; H&E stain.

At a concentration of 20 μg/L additional degenerative changes of the respiratory epithelial cells could be observed. It is assumed that these gill alterations might interfere with normal respiratory functions and, in addition to the renal changes, might lead to an impairment of the general health condition of fish.

Besides histopathology, chemical analysis of diclofenac residues in various organs have been carried out. Despite an octanol–water partition coefficient $\log P_{ow}$ of 0.7 (Yoon et al., 2002) indicating a high water solubility, diclofenac accumulated to a various degree in all organs examined. The fact that the calculated bioconcentration factors have been shown to be inversely proportional to the exposure concentrations is assumed to be a consequence of an almost complete saturation of tissues by diclofenac in the highest concentration group. Corresponding to the histopathological findings remarkable concentrations of diclofenac have been detected in the gills and the kidney, the latter representing the organ responsible for the excretion of the compound (Davies and Anderson, 1997). The highest concentrations however could be found in the liver, where according to Bort et al. (1999) the cytochrome P450-dependent metabolization of diclofenac takes place. In contrast to diclofenac treated humans (Banks et al., 1995; Bjorkman, 1998; Hackstein et al., 1998; Dierkes-Globisch et al., 2000) and rats (Manocha and Venkataraman, 2000) in which liver alterations such as parenchymal necrosis and inflammation have been demonstrated, in the liver of rainbow trout, no severe histopathological alterations were observed. However, at the ultrastructural level, diclofenac induced cellular reactions indicating an activated hepatic metabolism became visible in the trout liver already after exposure to 1 μg/L (Triebskorn et al., 2004).

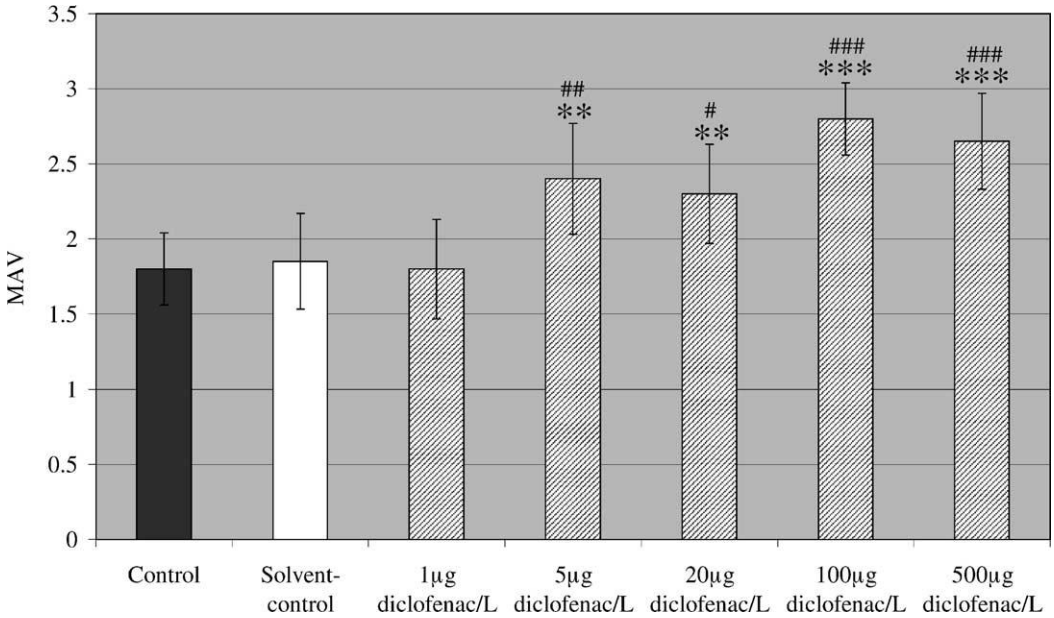

Fig. 4. Mean assessment values (MAV; means ± S.D.) of histopathological alterations in the gills of rainbow trout after exposure to diclofenac over 28 days, calculated on the basis of semiquantitative data (grades 1–3 of histopathological alterations). *control vs. 1, 5, 20, 100, 500 µg diclofenac/L; $^{**}P < 0.01$, $^{***}P < 0.001$; $^{\#}$ solvent-control vs. 1, 5, 20, 100, 500 µg Diclofenac/L; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

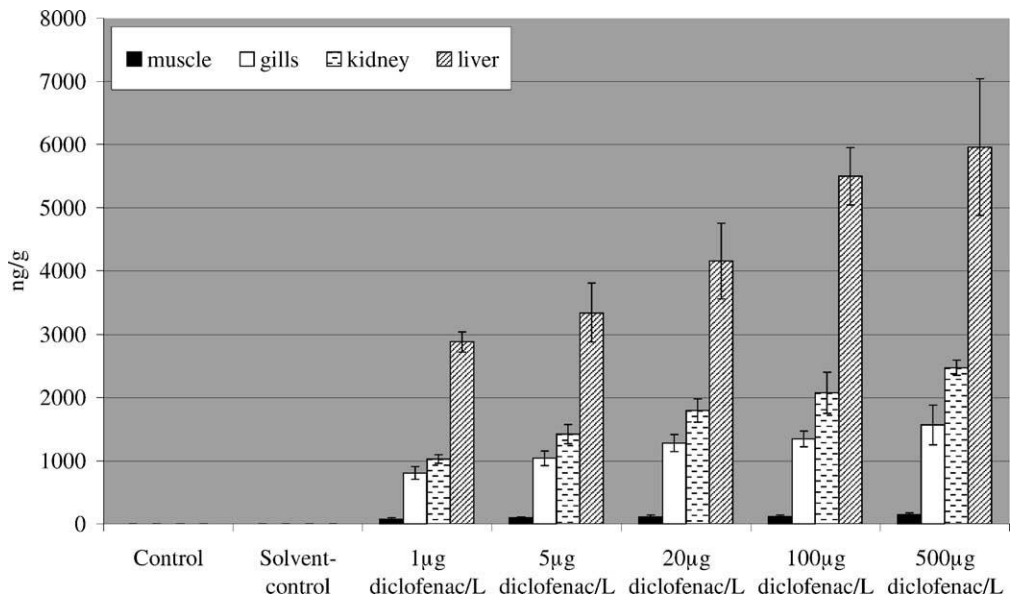

Fig. 5. Concentrations (ng/g (w/w)) of diclofenac in various tissues of rainbow trout after 28 days of exposure to diclofenac (means ± S.D.).

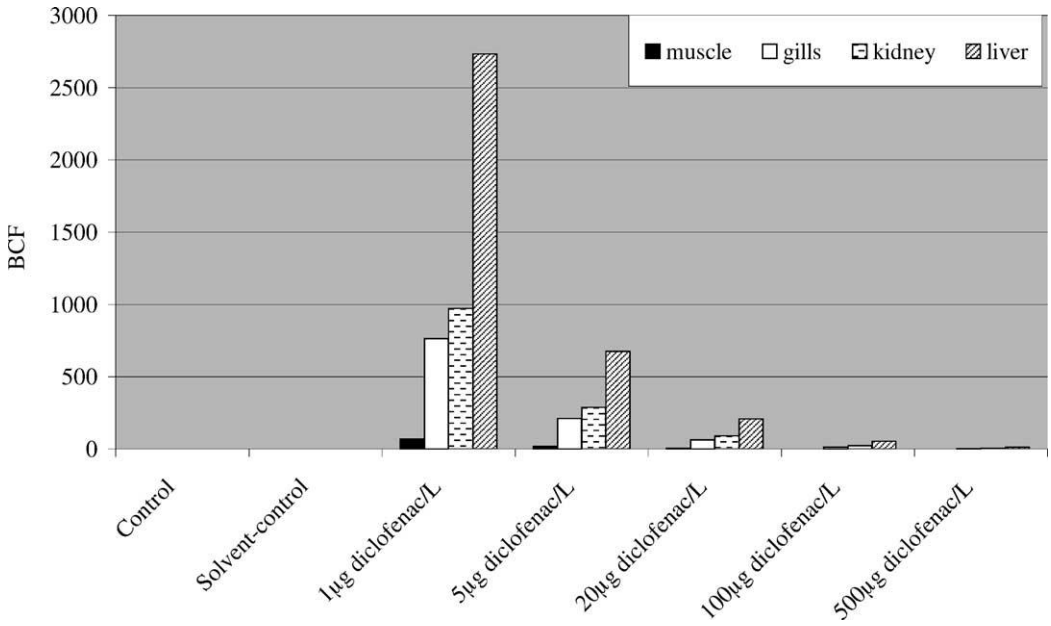

Fig. 6. Bioconcentration factors (BCF) in various tissues of rainbow trout after 28 days of exposure to diclofenac.

The present study indicates a toxic potential of diclofenac towards fish. The threshold level of 5 μg/L leading to histopathological organ lesions after 28 days of exposure is about 10^2-fold lower than that reported to induce sublethal effects in invertebrates (*Daphnia magna*, 21 d reproduction test; Kopf, pers. comm.). An extrapolation of toxicity data deriving from tests carried out in invertebrates on the situation in vertebrates such as fish therefore seems to be rather unsuitable. This is especially the case for pharmaceuticals which are designed to induce specific biological effects in man, and thus, can be expected to be effective also in other vertebrates. Compared to acute toxicity data based on the endpoint lethality in fish (Dietrich and Prietz, 1999) or *Daphnia magna* (Kopf, pers. comm.), the effect concentrations leading to sublethal histopathological alterations in rainbow trout after prolonged exposure have been proved to be at least 10^2 (fish) and 10^4 (*Daphnia magna*) -fold lower. From these data it can be concluded that for a realistic risk assessment of pharmaceuticals in the aquatic environment (1) prolonged exposure, (2) the application of sensitive endpoints, and (3) the use of susceptible test species is required.

Acknowledgements

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