Ecotoxicology (2012) 21:289–296
DOI 10.1007/s10646-011-0790-2

Biomarker assessment of toxicity with miniaturised bioassays: diclofenac as a case study

Raquel Feito · Yolanda Valcárcel · Myriam Catalá

Accepted: 8 September 2011 / Published online: 22 September 2011

Abstract The development of suitable biomarker-based microbioassays with model species with ecological relevance would help increase the cost-efficiency of routine environmental monitoring and chemical toxicity testing. The anti-inflammatory drug diclofenac has been widely reported in the environment but ecotoxicological data are scarce. The aim of this work is to assess the acute and chronic sublethal toxicity of diclofenac in relevant taxa of aquatic and riparian ecosystems (the fish *Danio rerio* and the fern *Polystichum setiferum*). Reliable biomarkers of cell viability (mitochondrial activity), plant physiology (chlorophyll), growth (DNA content) or oxidative damage (lipid peroxidation) were assessed as sensitive endpoints of toxicity. DNA quantification shows that diclofenac induces acute lethal phytotoxicity at 24 and 48 h (LOECs 30 and 0.3 μg l^{-1}, respectively). Hormetic effects in mitochondrial activity in spores of *Polystichum setiferum* mask lethality, and adverse effects are only observed at 48 h (LOEC 0.3 μg l^{-1}). In chronic exposure (1 week) LOEC for DNA is 0.03 μg l^{-1}. Mitochondrial activity shows a strong hormetic stimulation of the surviving spore population (LOEC 0.3 μg l^{-1}). Little changes are observed in chlorophyll autofluorescence (LOEC 0.3 μg l^{-1}). A very short exposure (90 min) of zebrafish embryos induces a reduction of lipid peroxidation at 0.03 μg l^{-1}. Environmental concentrations of diclofenac can be deleterious for the development of significant populations of sensitive individuals in aquatic and riparian ecosystems.

Keywords Acute toxicity · Chronic toxicity · Biomarkers · Diclofenac · Early life stage · Hormesis · Bioassays

Abbreviations

Malondialdehyde	MDA
Sewage treatment plants	STPs
Thiobarbituric acid	TBA
Trichloroacetic acid	TCA

Introduction

Due to the social and legal recognition of the need for massive environmental assessment and monitoring, ecotoxicology faces at present the challenge to develop cost-effective tools, more sensitive and reliable, with elevated biological and ecological relevance, able to detect early impacts before an irreversible disturbance of the ecosystem may occur (Walker 1998; Wadhia and Thompson 2007). Pollutants, and especially micropollutants such as pharmaceuticals, do not always cause lethal effects on living organisms at environmental concentrations. However, the induction of changes in their life cycle, sex ratios, growth or varying degrees of anatomical deformities (Jjemba 2006) that can lead to a diminished biological performance of wild populations and even to their extinction. The development of suitable biomarker based microbioassays could help both lower the costs of massive toxicity tests and reduce the number of organisms used (Walker et al. 1998; Wadhia and Thompson 2007). Likewise, the use of

R. Feito (✉) · M. Catalá
Biology and Geology Department, ESCET, Rey Juan Carlos University, c/Tulipán s/n, 28933 Mostóles, Madrid, Spain
e-mail: myriam.catala@urjc.es

Y. Valcárcel
Department of Preventive Medicine and Public Health, Immunology and Microbiology, Faculty of Health Science, Rey Juan Carlos University, c/Atenas s/n, 28922 Alcorcón, Madrid, Spain

model species representative of the ecosystem studied would greatly help interpret and extrapolate laboratory results (Chung et al. 2007).

Ferns, pteridophytes, are important components of numerous plant communities. With about 9,000 living species (Smith et al. 2006), ferns are the second largest group of vascular plants. Fern spores, and spore-developed gametophytes, have long been recognized as useful models for plant research in important areas, namely: plant development, sex determination, gamete production and fertilization, response to environmental factors and evolution of plant complex traits (Banks 1999). One of the main advantages of this model is its naturally miniature size. Fern spores are single meiotic cells that develop into gametophytes which are miniature mature higher plants. In this case, the use of microtubes and microplates is imposed by the natural model. The development of rapid and reliable methods to test phytotoxicity with fern spores and gametophytes could dramatically reduce standard tests costs maintaining the biological relevance of whole plant testing. Our team has recently published a first approach to develop a bioassay of phytotoxicity based on fern spores using mitochondrial activity as biomarker (Catalá et al. 2009). Preliminary works indicate that this promising testing method could combine biological and ecological relevance together with sensitivity and simplicity, thus making it a promising cost-effective tool for high throughput toxicity screening and monitoring (Catalá et al. 2009; Rodriguez-Gil et al. 2010). The research on the cell biology and physiology of fern spores and gametophytes is very limited, and more fundamental research must be performed on them in order to develop and validate a bioassay and integrate it in testing batteries.

Nowadays, there is growing interest in the use of fish embryos as an alternative tool to examine the presence and potency of aquatic toxicants (Scholz et al. 2008; Lammer et al. 2009), and some years ago, the method to test the toxicity to the embryo, was introduced (DIR 67/548/EEC, Annex V, C15 fish short-term toxicity on embryo and sac-fry stages, or OECD TG 212). The potential of zebrafish embryo model would be greatly improved by the development of early biomarkers of damage measurable after very short exposures. Lipid peroxidation is a well known biomarker of membrane oxidative damage caused by a wide range of toxicants which could lead to disturbed cell communication and tissue organization (Valavanidis et al. 2006; Montserrat et al. 2003). These alterations in cell communication are frequently in the origin of further malformations and developmental alterations.

Diclofenac is an important inhibitor of cyclooxygenases (prostaglandin-endoperoxide synthases), that is frequently used in human therapeutics as non-steroidal anti-inflammatory. Properties of diclofenac are shown in Table 1

Table 1 Summary of properties of diclofenac

Diclofenac	
Cas NO.	15307-79-6
Molecular formula	$C_{14}H_{10}Cl_2NNaO_2$
Molecular weight	318.13
Water solubility	50 mg/ml
Log K_{ow}	4.51
Half-life in blood	1–2 h
Main metabolites	4′-hydroxydiclofenac
	3′-hydroxydiclofenac
	4′,5-hydroxydiclofenac
Metabolites half-life in blood	1–2 h
Excretion	65% in urine, 35% in bile
Excretion without metabolization	1–10%
Biodisponibility	54%
Binding to proteins	99%
Daily consumption per patient	200–300 mg
Annual world consumption	940 tons

[Zhang et al. (2008) and other basic pharmacology bibliography]. The annual world consumption of diclofenac has been estimated in 940 tons (Zhang et al. 2008). A high number of studies prove its presence in aquatic environment (Ternes 1998; Ferrari et al. 2003; Gros et al. 2007; Kasprzyk-Hordern et al. 2008; Rodriguez-Gil et al. 2010). In some studies carried out in Berlin, Heberer (2002) emphasizes diclofenac as the most important medicine present in the water cycle, reaching concentrations in the $\mu g\ l^{-1}$ level in surface water. Also diclofenac has been detected in drinking water in the $ng\ l^{-1}$ level (Heberer 2002; Jjemba 2006; Zhang et al. 2008; Van den Brandhof and Montforts 2010). These facts demonstrate that diclofenac is incompletely eliminated by current sewage/water treatment technologies (Miège et al. 2009). Although 80% elimination has been reported, most STPs eliminate from 21 to 40% (Zhang et al. 2008).

Albeit authors generally agree that environmental concentrations of pharmaceuticals are not a hazard for human health, there is a growing concern for their ecotoxicological effects. Recently, the environmental presence of diclofenac has been associated with the devastation of different species and populations of vultures (Arshad et al. 2009). Toxicological effects of diclofenac on riparian and aquatic biota must be assessed either pure or in mixture with other pharmaceuticals with which synergistic effects could result (Cleuvers 2003).

Thus, the aim of this work is the assessment of the acute and chronic sublethal toxicity of diclofenac in relevant taxa of aquatic and riparian ecosystems (the fish embryo of *Danio rerio* and the fern *Polystichum setiferum* as higher plant) using biomarker-based approaches. Reliable

biomarkers of cell energetics (mitochondrial activity), plant physiology (chlorophyll), growth (DNA content) or oxidative damage (lipid peroxidation) were assessed as sensitive endpoints of toxicity.

Materials and methods

Biological material

Polystichum setiferum

Polystichum setiferum spores, a typical fern of riparian habitats, were sampled in NW Spain, A Coruña province, in the Natural Park of As Fragas do Eume or nearby. Fragments of leaf were collected with mature but closed sporangia. Spore release was promoted by drying the fragments on smooth paper for a week in the laboratory. Spores were stored dry at 4°C in darkness until use. Spores were sieved and suspended in gametophyte culture medium, sterilised and counted as detailed in Catalá et al. (2009).

Danio rerio

Danio rerio embryos (wild type) were purchased from ZF BioLabs (Tres Cantos, Madrid) 4 h post fertilization and were carried in synthetic water: 11.80 mg l^{-1} of $CaCl_2 \cdot 2H_2O$, 4.92 mg l^{-1} of $MgSO_4 \cdot 7H_2O$, 2.52 mg l^{-1} of $NaHCO_3$ and 1.10 mg l^{-1} of KCl (Kapp et al. 2006), at a temperature of 26 ± 1°C for 1 h to Rey Juan Carlos University. Embryos, in the beginning of the gastrula period, were exposed immediately after arrival in the laboratory for 90 min (n = 8 for every concentration).

Pharmaceuticals

A logarithmic range of concentrations of diclofenac sodium salt from 3 to 3 mg l^{-1} was used (Sigma Aldrich, Germany). Dilutions were directly made in sterile spore culture medium for the plant development bioassays. Diclofenac was dissolved in synthetic water for the animal development bioassay.

Bioassays

Toxicity in plant development

Three different endpoints were used to evaluate sublethal phytotoxicity: mitochondrial activity, DNA quantification and chlorophyll autofluorescence. The acute phytotoxicity (24 and 48 h) was evaluated through mitochondrial activity and DNA quantification. For chronic phytotoxicity (1 week), chlorophyll autofluorescence was also measured.

Aliquots containing 8×10^4 spores were prepared and 1.5 ml of each concentration of the pharmaceutical was added (n = 6). Control treatment samples contained 1.5 ml culture medium (n = 8). Spores were incubated in controlled conditions (20°C, PAR 35 µmol m^{-2} s^{-1} 16 h photoperiod) for 24, 48 and 168 h (1 week). After exposure, the spore suspensions were sterilised with commercial bleach (1:100, 5 min) and washed twice with fresh medium.

Mitochondrial activity

The method for the measurement of the mitochondrial activity has been recently described in Catalá et al. (2009). Briefly, the tetrazolium assay was performed with 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) 0.6%, pH 8 during 2 h in darkness. The homogenization of the spores was performed in several steps: first, a mixture consisting of KOH 0.1 N and ethanol 96% (v/v) was added to the spore pellet and sonicated in a Selecta Ultrasons bath for 1 h (40 kHz, 100 W) at room temperature, then samples were incubated at 65°C during 15 min in a water bath. Finally, approximately 200 µl of sieved glass fragments (0.2–1 mm) were added to the tubes prior to 30 min of agitation (3,000 rpm, Labnet, Edison, NJ, USA) for complete homogenisation.

The coloured formazan salt, produced by the reduction of TTC in mitochondria, was extracted in 1,000 µl of n-hexane by strong agitation (3,000 rpm) during 10 min with a vortex V×100 (Labnet, Edison, NJ, USA). Phase separation was performed after 7 min of centrifugation at 1,100×*g* in a Heraeus Fresco Biofuge (Thermo Scientific, Waltham, MA, USA). The absorbance of the red TPF salt dissolved in n-hexane was read at 492 nm in a Spectronic Genesys 8 UV/Vis spectrophotometer. Mitochondrial activity of spores solely exposed to culture medium was assessed as a control treatment.

DNA quantification

After incubation with the corresponding concentration of the pharmaceutical, samples were frozen (−20°C) until DNA or chlorophyll assays were performed. After defrost, 1 ml aliquots of the treated spores were sedimented by 5 min centrifugation at 600×*g* in a Heraeus Fresco Biofuge (Thermo Scientific, Waltham, MA, USA). Supernatant was discarded and spores were resuspended in 1 ml of a 1:1 mixture of TNE buffer (Tris base 100 mM, EDTA–Na_2 10 mM, NaCl 1 M, pH 7.4) with lysis solution (0.2% Triton X-100 and 1% NaOH 1 N). Homogenation was performed by 30 min agitation (3,000 rpm, Labnet, Edison, NJ, USA) with approximately 200 µl of sieved glass fragments (0.2–1 mm).

After incubation at 37°C for 1 h in a water bath, 10 µl of the homogenates were transferred to black 96-well flat bottom Greiner plates. 190 µl of either 0.1 µg ml^{-1} bis-benzimide Hoechst 33258 (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) in TNE buffer or TNE buffer alone (for autofluorescence blanks) were added to the wells prior to measurement of fluorescence (λ_{exc}: 360 nm, λ_{em}: 465 nm) in a SPECTRAFluor Plus microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

Chlorophyll autofluorescence

After defrost, 200 µl aliquots of the exposed spores were transferred to black 96-well flat bottom Greiner plates, and chlorophyll autofluorescence was measured (λ_{exc}: 485 nm, λ_{em}: 635 nm).

Toxicity in animal development

Danio rerio embryos were individually distributed in 48 wells plates and dead embryos were discarded and replaced. Embryos were incubated in 1 ml of synthetic water with different concentrations of diclofenac for 90 min at 26°C in darkness. 16 embryos were incubated for each concentration. After incubation, embryos were washed with synthetic water and transferred to 1.5 ml microtubes and kept at −80°C until MDA measurement.

Evaluation of lipid peroxidation was performed by a variation of the thiobarbituric acid reactive substances assay described by Reilly (1999). Individually exposed embryos were taken in pairs and homogenated with glass tissue grinder in ethanol 80% supplemented with 2% butylated hydroxytoluene (BHT) as antioxidant. The homogenate was incubated at 70°C for an hour with a trichloroacetic acid (TCA)–thiobarbituric acid (TBA)–hydrogen chloride (HCl) acid (9.18×10^{-1} M of TCA, 2.57×10^{-2} M of TBA and 3.20 M of HCl) solution in a 500:900 sample:acid mixture ratio. After incubation, absorbance of the TBA–MDA–TBA complex was measured at 532 nm. Working standards were prepared by diluting a 2 mM stock solution of the malondialdehyde precursor TEP with 80% ethanol supplemented with 2% of the antioxidant BHT to suppress the decomposition of lipid peroxides during the assay. Working concentrations of 0–20 µM were prepared.

Toxicity data analysis

Differences were assessed with ANOVA and a post-hoc Tukey-b test. A Levene test for homogeneity of variance was used. Kolmogorov–Smirnov test was used to verify data normality. Statistical significance was established at $p < 0.05$.

Results

Spores and embryos were exposed to diclofenac, a pharmaceutical found in surface waters. The concentrations of this compound ranged from 3 to 3 mg l^{-1}. LOEC and NOEC were calculated for diclofenac for each biomarker used.

Plant development

To assess the acute toxicity in plant development two biomarkers, mitochondrial activity and DNA quantification, have been used as measurement of cell energetics and lethality respectively. After 24 h of exposure a significant increase in mitochondrial activity was observed from 3 to 300 µg l^{-1} (Fig. 1a). NOEC and LOEC for acute exposure of 24 h are 0.3 and 3 µg l^{-1} respectively. After 48 h of exposure, the LOEC is 0.3 µg l^{-1} too (Fig. 1b). Regarding DNA quantification, the exposure to diclofenac produced a strong decrease in the quantity of DNA both after 24 h (LOEC of 30 µg l^{-1}, Fig. 1c) and 48 h (LOEC of 0.3 µg l^{-1}, Fig. 1d). DNA duplication in the germinating spores takes place after 1 week, therefore a reduction in DNA at 24 or 48 h is necessarily a biomarker of lethality (Fig. 2).

After 1 week, *P. setiferum* spores have produced chlorophyll. Therefore, to assess diclofenac toxicity in plant development, three biomarkers were used: mitochondrial activity to assess cell energetics, DNA quantification, in this case to evaluate growth, and chlorophyll auto-fluorescence that gives information about possible alterations in the photo-synthetic balance. Chlorophyll autofluorescence diminished with increasing diclofenac concentrations, but statistical significance was only achieved at 0.3 and 3,000 µg l^{-1} (Fig. 3a). A strong and significant stimulation of mitochondrial activity, around 250% above controls, was observed in Fig. 3b at concentrations of diclofenac above 300 ng l^{-1} (LOEC).

Regarding DNA quantification, Fig. 3c showed an important decrease already at the concentration of 0.03 µg l^{-1} (LOEC 30 ng l^{-1} and NOEC 3 ng l^{-1}).

Animal development

To assess the acute toxicity (90 min) of diclofenac on animal development, lipid peroxidation has been used as biomarker. In Fig. 4, a significant decrease is observed in lipid peroxidation at 0.03 µg l^{-1} (around 35% of controls). Then, a progressive increase happened in the rest of concentrations up to control level.

Discussion

Pharmaceutical products used in human medicine are of great importance in the treatment of disease and are

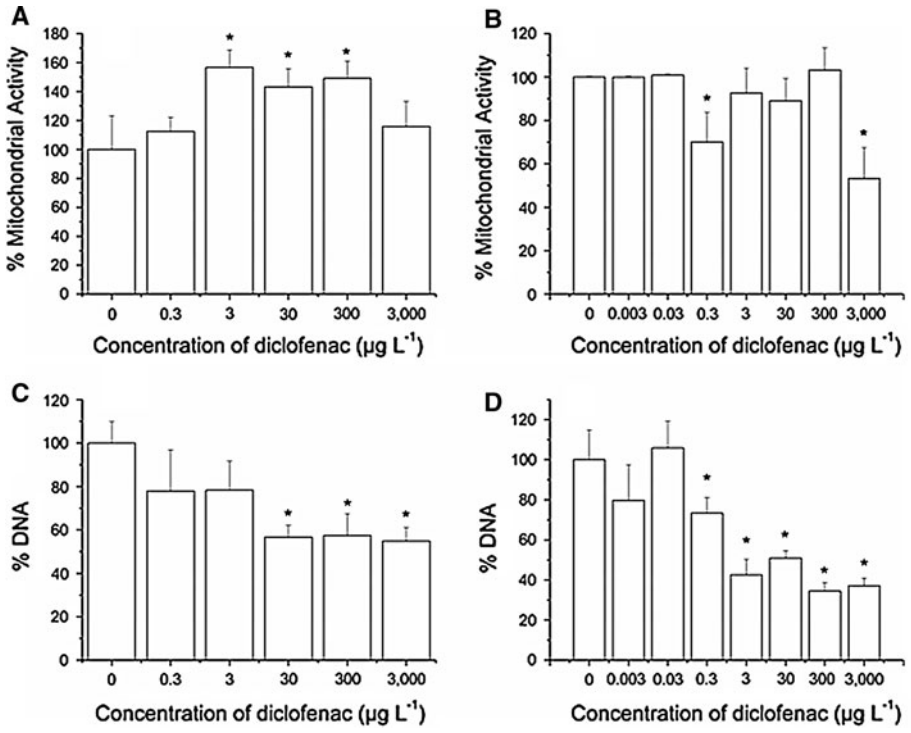

Fig. 1 Mitochondrial activity of spores exposed to diclofenac for 24 h (**a**) and 48 h (**b**). DNA quantification of spores exposed to diclofenac for 24 h (**c**) and 48 h (**d**). *Asterisks* (*) indicate significance at the $p < 0.05$ level relative to controls. The *bars* represent the mean ± standard error (n = 6)

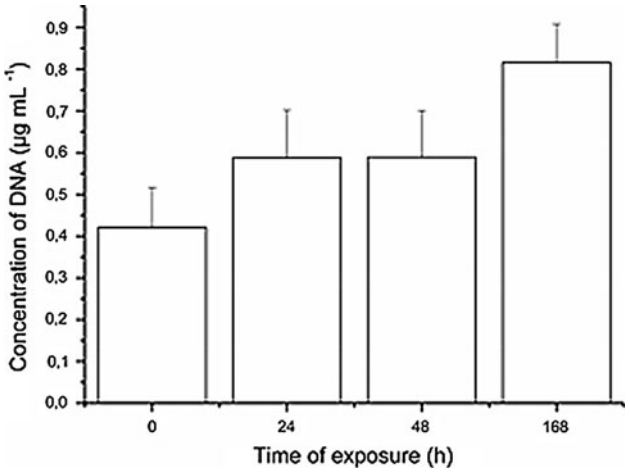

Fig. 2 DNA quantification of growing *Polystichum setiferum* gametophytes born from fern spores. The *bars* represent the mean ± standard error (n = 6)

designed to produce biological effects. The anti-inflammatory drug diclofenac is often found at concentrations in the µg l^{-1} range in surface water (Zhang et al. 2008). In order to obtain relevant information from bioassays, test organisms must be representative of the ecosystem affected and critical life stages must be considered. The development of adequate biomarkers as endpoints of toxicity will help design versatile cost-effective tools able to provide mechanistic data of toxicity. Therefore, the purpose of this study was to determine diclofenac effects on selected biomarkers using new approaches.

To assess the acute toxicity in plant development two biomarkers have been used DNA quantification and mitochondrial activity. No acute adverse effects are apparent below 3 μl^{-1} in 24 h exposure regarding mitochondrial activity, what agrees well with our previous results reported in Rodriguez-Gil et al. (2010), where natural fluvial waters containing diclofenac ranging from 0.3 to 0.9 μl^{-1} did not appear to cause alterations on this parameter. The increase observed in fern spore mitochondrial activity at 24 h of exposure is known as hormesis, in this phenomenon, moderate levels of toxic compounds induce a compensatory response in individuals, favouring the activation of defence or repair mechanisms (Calabrese 2008). This type of hormetic responses has often been considered as low or even inexistent toxicity for the cells. However, sustained compensatory responses can deplete the organism′s resources leading to a delayed biological failure. A decrease in spore mitochondrial activity, as it is observed at 48 h, implies severe toxicity. The reduction of DNA observed in the treated spores can only be due to the death and degradation of genetic material, since the duplication of the DNA of the spore has not been produced yet. Therefore, spores DNA quantification is a lethality biomarker for acute toxicity.

DNA results together with those obtained for mitochondrial activity indicate that the acute exposure to diclofenac kills an important percentage of the spore population, inducing the surviving cells to multiply several fold mitochondrial activity at the expense of spore

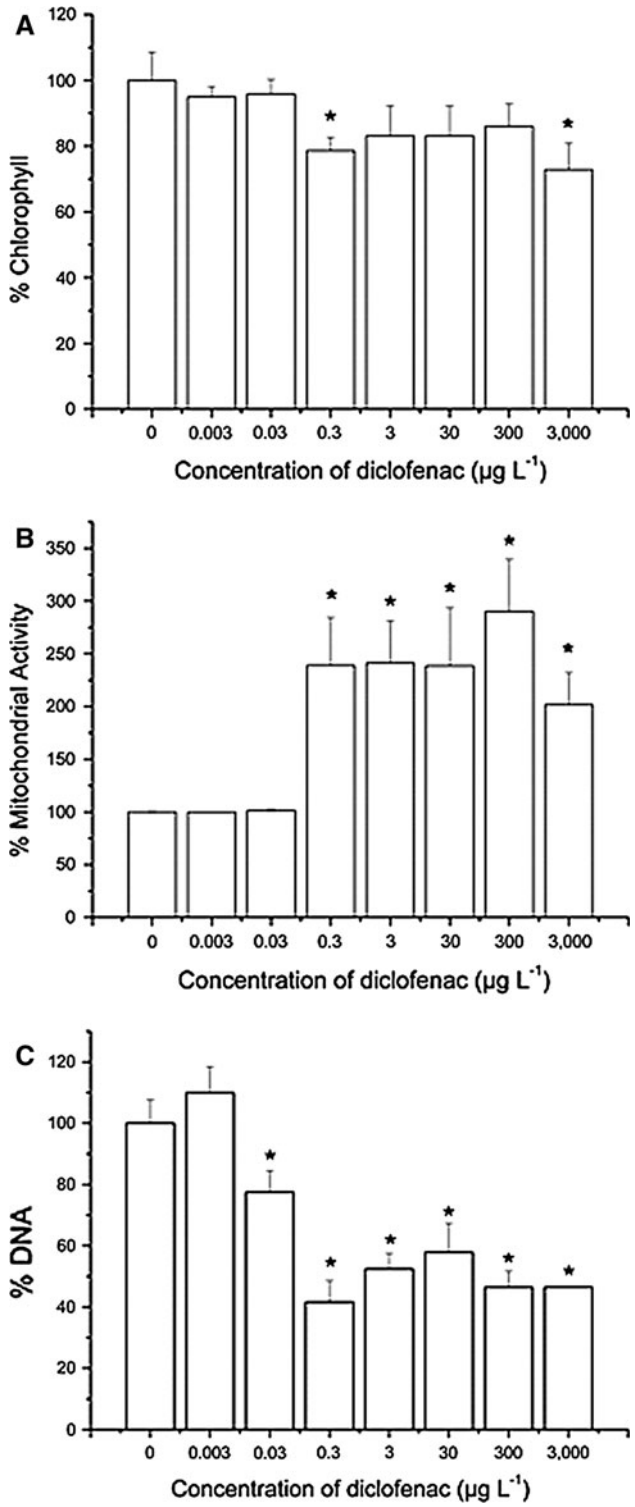

Fig. 3 Chlorophyll autofluorescence (**a**), mitochondrial activity (**b**), and DNA quantification (**c**) of spores exposed to diclofenac for 168 h. *Asterisks* (*) indicate significance at the $p < 0.05$ level relative to controls. The *bars* represent the mean ± standard error (n = 6)

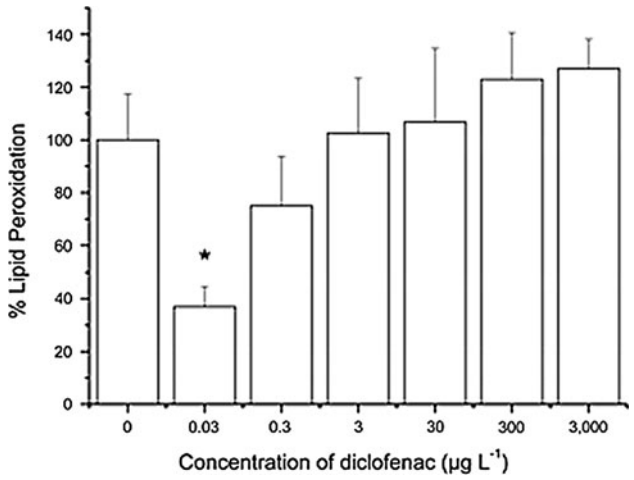

Fig. 4 Lipid peroxidation of *Danio rerio* embryos exposed to diclofenac for 90 min. *Asterisks* (*) indicate significance at the $p < 0.05$ level relative to controls. The *bars* represent the mean ± standard error (n = 8)

energetic reserves. At 48 h lethal effects of diclofenac are evident at lower concentrations (LOEC 0.3 µg l^{-1}) but still a reduced population (~40%) of spores present resistance even to high concentrations of the drug. The results indicate that diclofenac produces toxic and lethal effects in plant development in acute exposure (using spores of *P. setiferum* as model).

To assess chronic toxicity in plant development, three biomarkers have been used; chlorophyll auto-fluorescence, mitochondrial activity and DNA quantification. The results indicate that diclofenac has a dose dependent slight inhibitory effect on *P. setiferum* chlorophyll autofluorescence. Concerning mitochondrial activity, an increase is observed, that could be considered as hormesis, as we explained previously. An important decrease in DNA levels is observed, at all concentrations of diclofenac above 0.03 µg l^{-1}. These results confirm the trend of the data obtained for shorter times of exposure (24 and 48 h). The DNA reduction at 168 h, ~50% is consistent with the lethal acute effects at 48 h, also ~50%: this might indicate that the resistant population that survived acute toxicity has also been able to duplicate its DNA. However, this growth is at the expense of an extremely high mitochondrial activity. Longer exposure periods should be tested in order to find out whether the tolerant spore population develop into viable and fertile gametophytes.

It is necessary to indicate that the suspension of meiotic spores is formed by genetically different individuals, so individuals may present different levels of resistance to the toxicity of diclofenac, as it happens in natural populations of organisms with sexual reproduction. In natural conditions, physiologists describe that sexual reproduction is especially relevant when the environment is impacted, so that the genetic variability and survival opportunities of the progeny augments. This may lead to selection of tolerant populations and species adaptation to a contaminated

habitat. Therefore, the use of non-clonal populations is important to link the mechanisms and processes whereby chemicals exert their effects on ecosystems and the impact caused at the population or community level. This is one of the reasons why the fern spore approach presents an improved ecological relevance when compared to tests based on clones or parthenogenetic individuals.

Additionally, in the case of *P. setiferum*, sporophytes have no asexual reproduction (Page 1997) and thus population recruitment is strictly dependent on spore dispersal and gametophyte fertilization (Bremer 1995). It would be reasonable to conclude that diclofenac can cause the death of an important non-tolerant population, subduing the surviving to an overload in mitochondrial activity. We must emphasize that lethality happens at environmentally relevant concentrations (Zhang et al. 2008), and that *P. setiferum* is a common riparian fern in the rivers of the temperate region. Therefore, damage on spores caused by diclofenac may be determinant for population establishment and maintenance.

Diclofenac is an inhibitor of cyclooxygenases (prostaglandin-endoperoxide synthases), as salicylic acid and other antiinflammatory compounds. Little is known about the metabolism of the derivatives of arachidonic fatty acid (such as prostaglandins) in plants and nothing in ferns. Our results provide indirect evidence that the metabolism of arachidonic acid is important for fern spore development. The results also highlight the importance of assessing several biomarkers at the same time in order to know the mechanisms of toxicity and possible effects in natural populations. Hormesis is a relevant toxicological effect that can disguise toxic effects, as we have shown with chlorophyll and mitochondrial activity results.

No higher plant toxicity data have been found in the bibliography or data banks for diclofenac. Algae growth inhibition tests have rendered LOEC and EC_{50} values in the milligram range (Ferrari et al. 2003; Crane et al. 2006). However, it is widely recognised that higher plants and algae, belonging to different kingdoms, show very different sensitivity to pollutants, and algae toxicity values should not be taken as surrogates of higher plant toxicity (Wang and Freemark 1995).

To rapidly assess the acute toxicity in animal development, a biomarker of oxidative stress damage has been used, lipid peroxidation. The decrease observed in embryo lipid peroxidation after 90 min of exposure could also be due to hormesis. It only achieves statistical significance at 0.03 $\mu g\ l^{-1}$. At higher concentrations, lipid peroxidation increases, reaching the so-called "zone of compensation" in which the hormetic stimulus does not achieve a damage reduction, and biomarker levels are indistinguishable from the control situation.

Fent et al. (2006) indicate that, in general, there is not much information about toxicity of diclofenac in fish. Nevertheless, they give information about chronic toxicity for sublethal effects in adult fish due to diclofenac, placing the LOEC at 10 $mg\ l^{-1}$, a concentration more than three fold higher than the maximum concentration we used with embryos. Dietrich and Prietz (1999) in their studies revealed lethality (LC_{50} 480 $\pm$ 50 $\mu g\ l^{-1}$) and teratogenicity (EC_{50} 90 $\pm$ 20 $\mu g\ l^{-1}$) in diclofenac-exposed *Danio rerio* after 96 h. Ferrari et al. (2003) obtained values of NOEC and LOEC of 4,000 and 8,000 $\mu g\ l^{-1}$ respectively with their bioassays of chronic toxicity in the viability of *Danio rerio* embryos exposed 10 days to diclofenac. Finally, Nassef et al. (2010) showed that the feeding behaviour of medaka fish (*Oryzias latipes*) was affected by chronic exposure to diclofenac at a concentration of 1 $mg\ l^{-1}$. Therefore, the information presented here is in conformity with that published by other authors, but our data suggest that the exposure to diclofenac has a cost for the embryo that might be negative if, as it happens in natural conditions, the organism is simultaneously exposed to combined stressors.

Conclusions

We can conclude that the exposure to diclofenac at environmental concentrations can cause acute lethal and chronic sublethal toxicity in higher plant development (fern spore bioassay), as well as hormetic effects on fish embryo′s lipid peroxidation after a very short acute exposure. Likewise, physiological function biomarkers can be subjected to strong hormetic effects masking toxic effects and therefore the use of different biomarkers is necessary in toxicity tests. The use of DNA quantifications to refer physiological measures will help detect hormesis and provide a valuable tool in order to derive ecological consequences of environmental exposure. Finally, both newly developed approaches show advantages as reliability, cost-effectiveness and biological and ecological relevance.

Acknowledgments The authors are grateful to Fondo de Investigaciones Sanitarias de Castilla-La Mancha (project FISCAM 2007/08) for funding, and to Dr Pablo J. García Cambero for revision of the work and advice.

References

Arshad M, Chaudhary MJI, Wink M (2009) High mortality and sex ratio imbalance in a critically declining White-backed Vulture population (*Gyps bengalensis*) in Pakistan. J Ornithol 150:495–503

Banks JA (1999) Gametophyte development in ferns. Annu Rev Plant Physiol Plant Mol Biol 50:163–186

Bremer P (1995) On the ecology and population dynamics of a Dutch population of *Polystichum setiferum* (Dryopteridaceae: Pteridophyta). Fern Gaz 15:11–20

Calabrese EJ (2008) Hormesis: why it is important to toxicology and toxicologists. Environ Toxicol Chem 27:1451–1474

Catalá M, Esteban M, Rodríguez Gil JL, Quintanilla LG (2009) Development of a naturally miniaturised testing method based on the mitochondrial activity of fern spores. A new higher plant bioassay. Chemosphere 77:983–988

Chung KW, Fulton MH, Scott GI (2007) Use of juvenile clam, *Mercenaria mercenaria*, as a sensitive indicator of aqueous and sediment toxicity. Ecotoxicol Environ Saf 67:333–340

Cleuvers M (2003) Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol Lett 142:185–194

Crane M, Watts C, Boucard T (2006) Chronic aquatic environmental risks from exposure to human pharmaceuticals. Sci Total Environ 367:23–41

Dietrich DR, Prietz A (1999) Fish embryotoxicity and teratogenicity of pharmaceuticals, detergents, and pesticides regularly detected in sewage treatment plants effluents and surface waters. Toxicologist 48(1-s):151

Fent K, Weston AA, Caminada D (2006) Ecotoxicology of human pharmaceuticals. Aquat Toxicol 76:122–159

Ferrari B, Paxéus N, Lo Giudice R, Pollio A, Garric J (2003) Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. Ecotoxicol Environ Saf 55:359–370

Gros M, Petrovic M, Barceló D (2007) Wastewater treatment plants as a pathway for aquatic contamination by pharmaceuticals in the Ebro river basin (northeast Spain). Environ Toxicol Chem 26:1553–1562

Heberer T (2002) Occurrence, fate and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol Lett 131:5–17

Jjemba PK (2006) Excretion and ecotoxicity of pharmaceutical and personal care products in the environment. Ecotoxicol Environ Saf 63:113–130

Kapp T, Kammann U, Vobach M, Vetter W (2006) Synthesis of low and high chlorinated toxaphene and comparison of their toxicity by zebrafish (*Danio rerio*) embryo test. Environ Toxicol Chem 25:2884–2889

Kasprzyk-Hordern B, Dinsdale RM, Guwy AJ (2008) The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK. Water Res 42:3498–3518

Lammer E, Carr GJ, Wendler K, Rawlings JM, Belanger SE, Braunbeck T (2009) Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? Comp Biochem Physiol 149C:196–209

Miège C, Choubert JM, Ribeiro L, Eusèbe M, Coquery M (2009) Fate of pharmaceuticals and personal care products in wastewater treatment plants–Conception of a database and first results. Environ Pollut 157:1721–1726

Montserrat JM, Geracitano LA, Bianchini A (2003) Current and future perspectives using biomarkers to assess pollution in aquatic ecosystems. Comments Toxicol 9:255–269

Nassef M, Matsumoto S, Seki M, Khalil F, Kang IJ, Shimasaki Y, Oshima Y, Honjo T (2010) Acute effects of triclosan, diclofenaco and carbamazepine on feeding performance of Japanese medada fish (*Oryzias latipes*). Chemosphere 80:1095–1100

Page CN (1997) The ferns of Britain and Ireland, 2nd edn. Cambridge University Press, Cambridge

Reilly, CA, Aust SD (1999) Measurement of lipid peroxidation. In: Bus JS et al (eds). Currunt Protocols in Toxicology, 2.4.1–2.4.13. Academic Press, San Diego

Rodriguez-Gil JL, Catalá M, González Alonso S, Romo Maroto R, Valcárcel Y, Segura Y, Molina R, Melero JA, Martínez F (2010) Heterogeneous photo-Fenton treatment for the reduction of pharmaceutical contamination in Madrid rivers and ecotoxicological evaluation by a miniaturized fern spores bioassay. Chemosphere 80:381–388

Scholz S, Fischer S, Gündel U, Küster E, Luckenbach T, Voelker D (2008) The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing. Environ Sci Pollut Res 15:394–404

Smith AR, Pryer KM, Schuettpelz E, Korall P, Schneider H, Wolf PG (2006) A classification for extant ferns. Taxon 55:705–731

Ternes TA (1998) Occurrence of drugs in German sewage treatment plants and rivers. Water Res 32(11):3245–3260

Valavanidis A, Vlahogianni T, Dassenakis M, Scoullos M (2006) Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol Environ Saf 64:178–189

Van den Brandhof E-J, Montforts M (2010) Fish embryo toxicity of carbamazepine, diclofenac and metoprolol. Ecotoxicol Environ Saf 73:1862–1866

Wadhia K, Thompson KC (2007) Low-cost ecotoxicity testing of environmental samples using microbiotest for potencia implementation of the Water Framework Directive. Trend Anal Chem 26(4):300–307

Walker CH (1998) Biomarker strategies to evaluate the environmental effects of chemicals. Environ Health Perspect Suppl 106(Suppl 2):613–621

Walker CH, Kaiser L, Klein W, Lagadic L, Peakall D, Sheffield S, Soldan T, Yasuno M (1998) 13th meeting of the scientific group on methodologies for the safety evaluation of chemicals (SGOMSEC): alternative testing methodologies for ecotoxicity. Environ Health Perspect Suppl 106(Suppl 2):441–452

Wang W, Freemark K (1995) The use of plants for environmental monitoring and assessment. Ecotoxicol Environ Saf 30:289–301

Zhang Y, Geiβen S, Gal C (2008) Carbamazepine and diclofenac: removal in wastewater treatment plants and occurrence in water bodies. Chemosphere 73:1151–1161