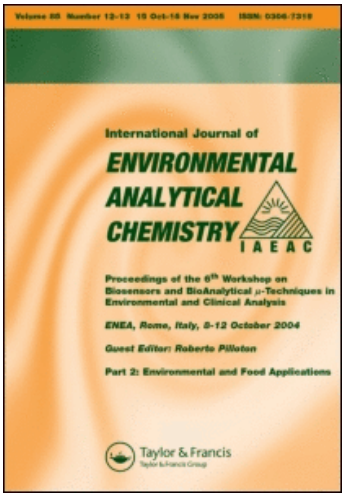

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Genotoxicity assessment of two vineyard pesticides in zebrafish

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This study deals with the use of a chronic exposure scenario of zebrafish (*Danio rerio)* in laboratory conditions to evaluate the genotoxic potential of diuron and azoxystrobin, two pesticides intensively used in vineyard agriculture. Adult male zebrafish were exposed during three weeks in the semi static mode in four 20 L aquaria. Treatment allowed to each aquarium was: negative control (untreated), positive control (methyl methane sulphonate 1 μM), diuron 4.3 nM and azoxystrobin 1.2 nM. Once per week, genotoxicity was assessed (6 fish/treatment) by the use of two complementary biomarkers: the primary DNA damages evaluated in somatic (liver) and germ (spermatozoa) cells by the alkaline version of the Comet assay and the micronucleus formation assessed in erythrocytes. Very low basal DNA damages were obtained with both biomarkers in negative control during the three consecutive weeks and a significant genotoxic response was obtained in 1 μM MMS exposed fish, both in liver and germ cells with the Comet assay and in erythrocytes with the micronucleus test, respectively starting after one and three weeks. With this chronic exposure scenario, both diuron and azoxystrobine revealed a genotoxic potential at realistic environmental concentrations and a significant response was obtained in all cell types investigated and with both biomarkers used, mainly after 7 or 14 days, thus stressing the interest of long-term exposure scenario. Further studies will be undertaken in order to evaluate whether DNA damage observed in spermatozoa of fish exposed to environmental concentration of pesticides could lead to subsequent reproductive disorders.

Keywords: genotoxicity; pesticide; vineyard; zebrafish

1. Introduction

Owing to their toxic effects on non-target organisms, the pesticides intensively used in viticulture may produce various detrimental effects on aquatic ecosystems. Complex mixtures of pesticides may contaminate rivers found below the steep slopes of vineyards through spray drift, surface water run-off and leaching from treated areas [1]. Thus, chronic contamination of watershed by pesticides dissolved or adsorbed to soil particles can endanger aquatic organisms.

Genotoxicity endpoint is often used in environmental monitoring under field conditions to characterise exposure of aquatic organisms to rather complex mixtures of pollutants. Genotoxicity assessment in aquatic organisms has been highlighted because genotoxic effects can lead to the initiation of carcinogenicity when somatic cells are

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targeted and to inheritable mutations and reproduction defects when germ cells are affected. This can impact on fitness traits such as reproductive success, genetic patterns and subsequent population dynamics [2]. The literature dealing with genotoxicity assessment of pesticides towards fish species remains poorly documented and is often restricted to the assessment in laboratory-controlled conditions after acute exposure. However, recently we have shown that a mixture of the widely used pre-emergent phenyl urea herbicide diuron and of the strobilurin broad-spectrum fungicide azoxystrobin was genotoxic towards fish erythrocytes [3].

In order to assess the genotoxic potential of each of those molecules, the present work deals with the chronic exposure of zebrafish to environmental relevant concentrations of diuron or azoxystrobin. Genotoxicity was measured both in somatic and in germinal cells by using the Comet assay: in liver cells because of their central role in xenobiotic metabolism, and in spermatozoa since recent studies have shown possible links between sperm DNA integrity and subsequent reproductive success through oxidative stress [4]. Complementary genotoxic information was gained by following micronucleus formation in erythrocytes through the micronucleus test.

2. Experimental

2.1 *Chemicals*

Stock solutions of the pure test substances (Sigma Aldrich) were prepared in deionised water. Diuron (3-(3,4-dichlorophenyl)-1,1dimethylurea) (CAS number 330-54-1, 98% purity, water solubility 42 mg L^{-1}, log K_{ow} 2.6) and azoxystrobin (methyl (E)-2-[2-[6-(2-cyanophenoxy) pyrimidin-4-yl]oxyphenyl]-3-methoxyprop-2-enoate) (CAS number 131860-33-8, 99% purity, water solubility 6 mg L^{-1}, log K_{ow} 2.5) were respectively dissolved at concentration of 12 mg L^{-1} (51 μM) and 3 mg L^{-1} (7.5 μM) using a 48 h stirring and were both kept at 4°C in the dark until used. The model alkylating genotoxicant methyl methane sulfonate (CAS number 66-27-3, 99% purity) was dissolved at 1320 mg L^{-1} (12 mM) and aliquots were kept at −20°C until used.

2.2 *Fish and exposure protocol*

Pesticide concentrations were chosen on the basis of realistic concentrations analysed in water of vineyard rivers, especially in the spring and early summer, i.e. 1–2 μg L^{-1} for diuron and 0.5–1 μg L^{-1} for azoxystrobin, respectively [5,6]. Moreover, a mixture of diuron and azoxystrobin in the same concentration range was shown to induce genotoxicity in minnow erythrocytes in a mesocosm study [3].

A week before the start of exposure, 25 sexually mature male zebrafish (*Danio rerio*) (HB development, St Forgeux, France) were acclimatised to each of four 20 L aquaria, filled with 16 litres of oxygenated and dechlorinated tap water in an air-conditioned room (20°C) with a 12 h light/12 h dark photoperiod. Fish were fed once daily with TetraMin® flakes. Every day, one hour after feeding, 75% of the water was renewed and the aquarium bottom was cleaned by suction. On day 0, one of the four following treatments was allowed to each aquarium: negative control (dechlorinated tap water), positive control (methyl methane sulphonate/MMS 1 μM (110 μg L^{-1})), diuron 4.3 nM (1 μg L^{-1}), and azoxystrobin 1.2 nM (0.5 μg L^{-1}). These treatments were applied during 21 days, by adding daily the corresponding amount of each molecule just after water renewal. Although actual

pesticide concentrations were not analysed along the experiment and thus could be regarded with slight reservations, this daily renewal was prone to reasonably guarantee the quality of exposure. On day 0, 7, 14 and 21, six fish were removed from each aquarium for DNA damage assessment.

2.3 *Cell preparation*

Fish were anaesthetised in a 0.5% 2-phenoxy-ethanol solution and immobilised under a stereomicroscope. Five to 10 μL of blood obtained by cardiac puncture with a heparinised syringe were diluted with 10 μL of phosphate buffered saline (PBS) containing 10% bovine serum albumin and smeared onto a clean microscope slide. Liver cells were obtained by enzymatic digestion of the whole dissected liver with 1mL of PBS containing 1.25% trypsin and 0.25% collagenase (27°C for 15 min). After digestion, cells were filtered through a 100 μm mesh nylon gauze and washed twice in cold PBS buffer. Mature male gonads were excised and mildly teared up in cold PBS in order to release spermatozoa that were then washed twice in cold PBS. Both cell types were then counted and their viability was checked with the Trypan blue dye method. Cell number was adjusted to $5.10^5\,mL^{-1}$ in a cryoprotective citrate buffer (pH 7.4) containing 5% DMSO and 250 mM sucrose. This suspension was kept in liquid nitrogen until the comet assay was performed.

2.4 *Micronucleus test*

After drying, smeared blood cells were fixed in methanol for 10 minutes and stained with the Meyer's Hematoxylin solution. Slides were washed under tap water and 1500 cells per slide (one slide per fish) were observed under X 1000 magnification, using an Axioscope microscope (Zeiss, Germany). Only non-refractive particles that resembled micronucleus (MN) were scored from cells with intact nuclear and cellular membrane without discrimination of maturity stage. The statistical method of McGill *et al.* [7] was used to compare the median values of MN frequency and their 95% confidence limits between test groups and the corresponding control groups. A significant difference was defined by the absence of overlap between control and test groups of their medians within the 95% confidence limits.

2.5 *Comet assay*

Liver and spermatozoa cell suspensions were mixed with an equal volume of a 1% low melting point agarose solution immediately after rapid thawing and spread onto a microscope slide previously coated with a 0.8% normal agarose solution. Then the alkaline version of the Comet assay was performed according to the procedure of Singh *et al.* [8] that was validated for both cell types in a preliminary study. Briefly, cells were lysed in a lysing solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10, 1% (v/v) Triton X-100 and 10% (v/v) DMSO) for 1 h at 4°C. The DNA was then allowed to unwind for 40 min in an electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH > 13). The electrophoresis was run for 24 min at 20 V and 300 mA ($0.6\,V\,cm^{-1}$). Slides were then neutralised using Tris buffer (0.4 M Tris, pH 7.5) and dried in absolute ethanol. The DNA was stained with 0.05 mM ethidium bromide and scored using an Axioskop epifluorescence microscope (Zeiss, Germany) and the Comet Assay IV image analysis system (Perceptive Instruments, UK).

Randomly selected cells (100 cells per slide) were analysed and the comet parameter retained was the percentage of tail DNA. Since the distribution of DNA damage measured by the Comet assay did not follow a Gaussian distribution, both Kruskall-Wallis and Mann-Whitney non-parametric tests were used for data analysis.

3. Results

Neither fish mortality nor any behavioural changes (feeding and swimming) were observed along the experiment whatever the treatment.

The results obtained with the micronucleus test in the erythrocytes of fish are given in Table 1. Almost no micronuclei were found during the experiment in negative control. Micronucleated cells were found with a significant higher frequency in MMS exposed fish than in the unexposed control only after three weeks of exposure. An increase in micronucleus formation was obtained for both pesticides, earlier and to a larger extent than in the MMS exposed fish. Thus, the micronucleus frequency significantly increased in erythrocytes of fish exposed to azoxystrobin as soon as after 7 days and after 14 days with diuron.

Figures 1 and 2 report primary DNA damage level evaluated by the Comet assay respectively in two different target cells: liver cells and spermatozoa of zebrafish exposed during three weeks either to control conditions (negative and positive) or to diuron or to azoxystrobin at environmental concentrations. First it can be shown in the negative control fish, that the basal level of DNA damage in both isolated liver cells and spermatozoa is low, respectively $3\% \pm 2$ in liver cells and $2\% \pm 0.8$ in spermatozoa and remains stable along with the time. In the positive control, the concentration of 1 μM of the model genotoxicant MMS (chosen in a preliminary experiment to not induce any mortality), led to significant DNA damage in liver as soon as after one week. The increase factor of the damage level (about 5) was high and stable all along the experiment. In spermatozoa, DNA breaks increased one week later (day 14) but with a very high increase factor since damage level averaged 16% while being only 2% in the unexposed fish. When using our exposure scenario, both diuron and azoxystrobin revealed a genotoxic potential in various target cells of zebrafish with a time effect relationship starting after 7 and 14 days of exposure to azoxystrobin and diuron, respectively. Azoxystrobin seems more genotoxic for liver cells than for spermatozoa when the opposite phenomenon was observed with the diuron. Surprisingly, for both compounds, the absolute DNA damage level reached in some cases, values as high as with the model genotoxicant MMS after three weeks of exposure.

Table 1. Micronucleus frequency (‰) in zebrafish erythrocytes exposed to MMS (1 μM) as positive control, diuron and azoxystrobin during three weeks ($n = 6$).

Time (days)	Negative control	Positive control (MMS 1 μM)	Diuron (4.3 nM)	Azoxystrobin (1.2 nM)
0	0 (0)	0 (0)	0 (0)	0 (0)
7	0 (0)	0 (1.3)	0 (0.6)	6.5 (2.6)[a]
14	0.5 (0.64)	1.5 (1.3)	6.5 (0.6)[a]	14.5 (5.8)[a]
21	0 (0)	5.5 (1.3)[a]	7 (1.3)[a]	13.5 (2.3)[a]

Results expressed as median micronucleus frequency (confidence limit at 95%), $n = 6$.
[a]Significantly different from the corresponding negative control group.

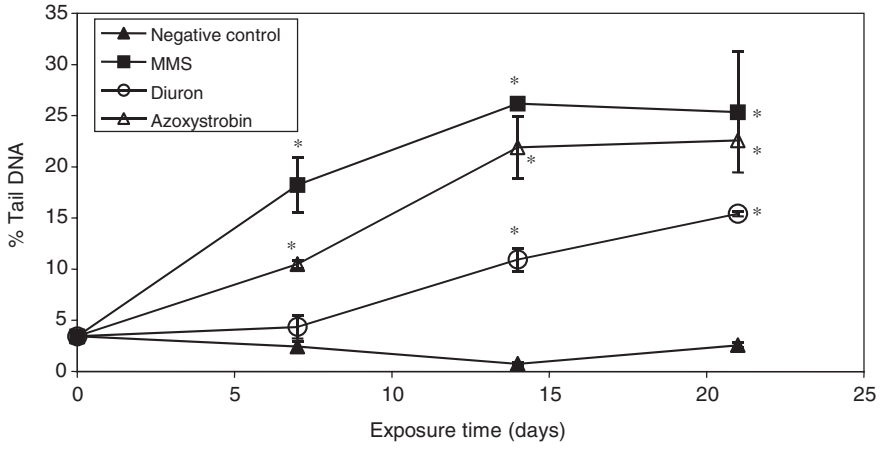

Figure 1. DNA damage evaluated by the comet assay in liver cells of zebrafish exposed to MMS (1 μM) as positive control, diuron (4.3 nM) and azoxystrobin (1.2 nM) during three weeks. Results are expressed as mean ±SD ($n = 6$) of % tail DNA median values (% of damaged DNA measured in 100 cells per fish).

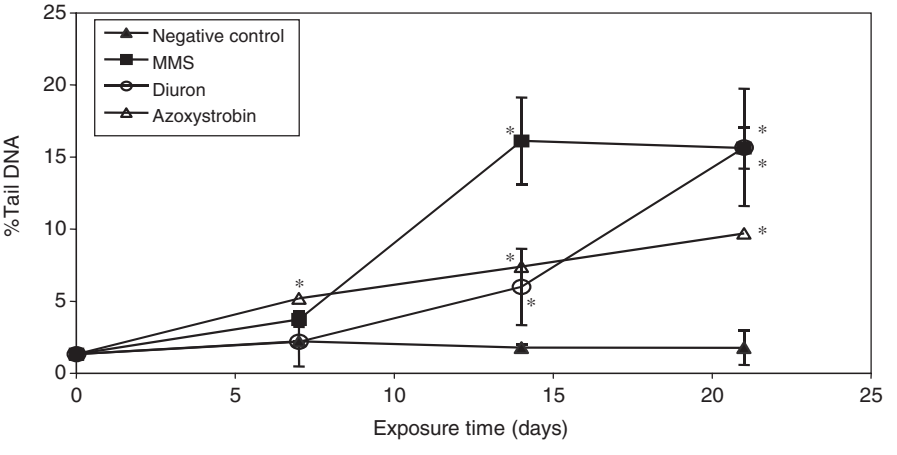

Figure 2. DNA damage evaluated by the Comet assay in spermatozoa of zebrafish exposed to MMS (1 μM) as positive control, diuron (4.3 nM) and azoxystrobin (1.2 nM) during three weeks. Results are expressed as mean ±SD ($n = 6$) of % tail DNA median values (% of damaged DNA measured in 100 cells per fish).

4. Discussion

Recently, genotoxic events have been observed in trout early life stages exposed in a vineyard watershed river chronically contaminated by pesticides including diuron and azoxystrobin [3]. The present study aimed to evaluate at realistic environmental concentrations, but in laboratory well-controlled conditions, the genotoxic potential of these two main pesticides. Thus, each chemical was studied here at its average low river flow concentration as described by Rabiet *et al.* [6]: 4.3 nM for diuron and 1.2 nM for azoxystrobin.

The choice of the two genotoxicity endpoints was made since the Comet assay is known as a very sensitive and versatile tool to evaluate primary DNA damage and the micronucleus

test, often described to be less sensitive [9], but revealing unrepaired genotoxic lesions such as chromosome breakage and chromosome loss prone to long-term consequences [10].

Concerning the positive control, all the cell types studied here significantly responded to the model genotoxicant MMS. Although genotoxic potency of this compound (acting as a direct alkylating agent) has been mainly described *in vitro*, our results are in accordance with some *in vivo* studies demonstrating its genotoxicity (or of the parent compound EMS) in fish using the micronucleus or comet assay in erythrocytes and liver cells through water exposure. Thus, a significant increase in micronuclei frequency was demonstrated in zebrafish erythrocytes exposed for 6 to 12 days to micromolar concentrations of MMS and a significant increase in strand breaks formation in liver cells after 4 days [11]. Moreover, water exposure for 14 days of the fish *Clarias lazera* and for one day of *Oreochromis niloticus* to micromolar concentrations of EMS and MMS respectively, led to a significant increase in DNA damage measured in erythrocytes using either the micronucleus or the comet assay [12,13].

Diuron, one of the 33 priority substances of the EU Water Framework Directive has been described as a potential genotoxicant with the mice micronucleus bone marrow test [14] and in few *in vitro* studies [15]. Very few studies have demonstrated diuron genotoxicity in aquatic animals. A significant increase in tail DNA was measured in the erythrocytes of European minnow chronically exposed for one month to a mixture of diuron and azoxystrobin in the same range of concentrations as in the present study [3]. Besides, aneuploidy was showed in oyster exposed for 11 weeks to similar diuron concentrations [16]. Such studies underline the interest of long-term experiments as already shown in zebrafish exposed to other genotoxicants [17]. However, because exposure to pesticides in the field is most often discontinuous and linked with the treatment period, the cell turnover and the possibility that DNA repair occur after exposure, must be considered.

It has to be highlighted that the increase in tail DNA observed here in spermatozoa of zebrafish exposed during 21 days to environmental concentrations of pesticides reached an 8-fold value, which is in the high range when compared with the genotoxicity level observed with other pesticides. For example, exposure of the fish *Mystus vittatus* to environmental endosulfan concentrations led to a maximum 5-fold increase in erythrocyte tail DNA [18]. A maximum 5-fold increase in tail DNA of the fish *Channa punctatus* lymphocytes was also found after a 4 days' exposure to high concentrations of the organophosphates monocrotophos or chlopyriphos [19,20]. Furthermore, Sacramento sucker (*Catostomus occidentalis*) caged up to 13 days in a river receiving heavy agricultural chemical run-off (mainly the insecticide diazinon and herbicide simazine), exhibited a maximum 4-fold increase in erythrocyte tail DNA [21].

The mechanisms of diuron genotoxicity remains to be elucidated but it can trigger oxidative stress in fish and oysters, thus producing reactive oxygen species (ROS) possibly responsible for further primary DNA damages [22,16]. Additionally, diuron was suggested to disturb the metaphase spindle microtubule and to induce cytogenetic effects such as aneuploidy [16]. The micronucleus formation observed here in erythrocytes could be related to such a dysfunction of the chromosome segregation step during mitosis since micronucleus formation can be due to an aneugenic event related to the spindle apparatus.

Regarding azoxystrobin, as far as we know, its genotoxicity had never been demonstrated before. However, as well as diuron, this chemical has been described as a ROS inducer leading to toxicity both in animals and plants [23]. Strobilurin family molecules act as inhibitors of the mitochondrial respiratory chain targeting the

ubiquinone–cytochrome bc1 of complex III [24]. In liver, complex III has been demonstrated as one of the most critical ROS production site [25] and thus, the genotoxic effect observed in liver cells of fish exposed to azoxystrobin could be related to oxidative DNA damage resulting from an unbalanced redox status. Such a phenomenon could have occurred in spermatozoa in which complex III is also represented [26]. However, the precise mechanisms involved in genotoxicity of the studied pesticides remain rather speculative and will require further investigations.

In this study, primary DNA damage was quantified in two target cells, chosen for their respective physiological relevance. These were: liver cells, involved in xenobiotic biotransformation and rather well equipped with antioxydant defences, and spermatozoa, characterised by a low DNA repairing ability and also by weak antioxidant capacities. Both cell types responded to the studied pesticides and could lead to a great variety of functional consequences for exposed animals. However, results obtained in this work, showing the high response level of mature spermatozoa, stress the relevance of the increasing concern about genotoxicity assessment of chemicals in germ cells. There is here a real future challenge to clearly demonstrate the possible links between sperm DNA damage and reproduction impairment in fish exposed to pesticides. Until now, only some rare studies have shown such a correlation between sperm DNA integrity and sperm motility, or fertilisation and hatching rate in fish exposed to model genotoxicants such as duroquinone or hydrogen [4–27]. To this purpose, experiments are currently in progress.

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