Mutagenic impact on fish of runoff events in agricultural areas in south-west France

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A B S T R A C T

When heavy rainfall follows herbicide application, the intense surface runoff causes stream water contamination. Aquatic organisms are then briefly exposed to a complex mixture of contaminants. The aim of the present study is to investigate the genotoxic impact of such events on fish. A model fish, the Crucian carp (*Carassius carassius*) was exposed in controlled conditions, for 4 days, to water sampled daily in the Save River (France). The watershed of this stream is representative of agricultural areas in south-west France. Three hydrological conditions were compared: basal flow, winter flood, and spring flood. Chemical analysis of the water samples confirmed the higher contamination of the spring flood water, mainly explained by a peak of metolachlor. Genotoxicity was evaluated by micronucleus (MN) test and comet assay in peripheral erythrocytes. A significant increase in DNA breakdowns compared to controls was detected by the comet assay for all conditions. Exposure to spring flood water resulted in the highest damage induction. Moreover, induced chromosomal damage was only detected in this condition. In addition, fish were exposed, for 4 days, to an experimental mixture of 5 herbicides representative of the spring flood water contamination. Fish exhibited moderate DNA damage induction and no significant chromosomal damage. The mutagenicity induced by field-collected water is then suspected to be the result of numerous interactions between contaminants themselves and environmental factors, stressing the use of realistic exposure conditions. The results revealed a mutagenic impact of water contamination during the spring flood, emphasizing the need to consider these transient events in water quality monitoring programs.

1. Introduction

Despite increasing awareness about stream water quality (EU Water Framework Directive – 2000/60/EC), pesticides are commonly used in agriculture. In Europe, 200,000 tonnes of plant protection active substances were used in 2003 (Eurostat, 2007). Herbicides represented 38% of this amount, and France alone accounted for 26% of herbicide consumption, mainly used on cereals and maize (Eurostat, 2007). As a consequence, herbicides are found in French surface water, as reported by water quality monitoring programs (IFEN, 2007). Data from such networks provide an overall vision of global chronic contamination. However, besides chronic contamination, streams undergo pulses of contamination and it is now acknowledged that short contamination events have to be considered in the context of biological monitoring (Cold and Forbes, 2004; Whitehead et al., 2004). The highest concentrations of pesticides in agricultural streams occur during floods, when rainfall provides a major transport mechanism for pesticides through surface water runoff (Richards and Baker, 1993; Fenelon and Moore, 1998; Kuivila and Foe, 1995; Liess et al., 1999; Kreuger, 1998). This phenomenon is emphasized with rainfall occurring shortly after pesticide application. Because of the transience of these events they are not considered in the monitoring of water quality. In the Gascogne area, in south-west France, pre-emergent herbicide application in spring coincides with occasional heavy precipitation. Higher water contamination during such events has been demonstrated (Taghavi et al., 2010; Debenest et al., 2008). As a result, non-target organisms are briefly exposed to a mixture of herbicides which may induce genetic damage (Konen and Cavas, 2008; Clements et al., 1997). Moreover, interactions between contaminants may occur, affecting the overall water toxicity experienced by organisms in streams (Anderson and Lydy, 2002; Lydy and Linck, 2003). Evaluating the biological impact of brief contamination events thus requires the use of tools to integrate both temporal

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variability and multiple sources of contamination. This supports the use of biomarkers. Biomarkers are defined as biological responses related to the exposure to environmental chemicals (Peakall, 1994). Among the numerous biomarkers used in the context of stream water monitoring (Van der Oost et al., 2003; Mayon et al., 2006), genotoxicity assessment is one of the most used tool (Udroiu, 2006; Rodriguez-Cea et al., 2003; Lemos et al., 2007). The micronucleus test (MN) and comet assay have been successfully used by numerous authors. Besides providing a quick molecular response, they are a way to investigate impact at a higher, and more ecologically relevant, biological scale (Anderson and Wild, 1994; Kurelec, 1993; Depledge, 1994; Diekmann et al., 2004).

Micronuclei are whole or partial chromosomes which have not been incorporated into the daughter nucleus following mitosis due to the clastogenic (chromosome breaking) or aneugenic (mitotic spindle dysfunction) effects of a chemical. An increase in MN frequency has been demonstrated to result from exposure to various compounds found in the aquatic environment (Al-Sabti and Metcalfe, 1995; Minissi et al., 1996; Chaudhary et al., 2006; Klobucar et al., 2010; Lemos et al., 2007; Cavas and Ergene-Gozukara, 2003; Cavas and Ergene-Gozukara, 2005). MN induction indicates non-repairable mutagenicity and thus may be less sensitive than tests involving the occurrence of repairable strand breaks.

The single cell gel electrophoresis (comet assay) in alkaline conditions is a highly sensitive biomarker. It detects and quantifies DNA damage such as single- and double-strand breakage and alkali-labile sites (Tice et al., 2000). These types of damage can be induced directly by the contaminant, or indirectly via repair processes (Tice et al., 2000). The combination of comet assay and the MN test allows the detection of both the subtle repairable effects of genotoxic agents and established mutagenicity. Fish are highly suitable organisms to perform these two assays. They have been demonstrated to be sensitive to pesticides (Ali et al., 2009; Konen and Cavas, 2008; Grisolia, 2002) and their erythrocytes provide an easily accessible source of nucleated cells.

The aim of the present work was to investigate the biological impact of pulse herbicide contamination from agricultural runoff through genotoxicity assay in fish erythrocytes. In order to avoid the influence of physical parameters such as water flow, which can lead to stress susceptible to induce genotoxic responses beside contamination itself (Bombail et al., 2001; Winter et al., 2004; Pellacani et al., 2006), exposure took place in controlled conditions. Crucian carp, *Carassius carassius*, were used as model organisms. The fish were exposed to water sampled in the Save River, a tributary of the Garonne representative of the numerous agricultural streams in the Gascogne area. The potential genotoxicity of three different hydrological contexts were investigated. Water was first taken during a flood in winter, when contamination was expected to be low, second during a situation of basal flow and finally the mutagenicity of water from the first flood following herbicide application in spring was investigated. For each situation, water contamination was analysed and genotoxicity assayed. Finally, the effective genotoxicity of contaminants measured in higher concentrations in the spring flood was explored. Another group of fish was experimentally exposed to a mixture of the 5 most concentrated contaminants, at concentrations found to occur in the field.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals and reagents used for genotoxicity assays, heparin salt (CAS No. 9041-08-1), benzocaine (CAS No. 94-09-7), acridine orange (CAS No. 10127-02-3), and methanol were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Pesticide analyses were performed using solvents of analytical grade ("pestipure" by SDS, Solvent Documents Syntheses, Peypin, France). Anhydrous sodium sulphate from SDS was used for drying the organic phases. Pesticide Mix44, used as reference material, and pure molecules, used for preparing the pesticide mixture, were purchased from Cluzeau Information Laboratory (CIL) (Sainte-Foy-la-Grande, France).

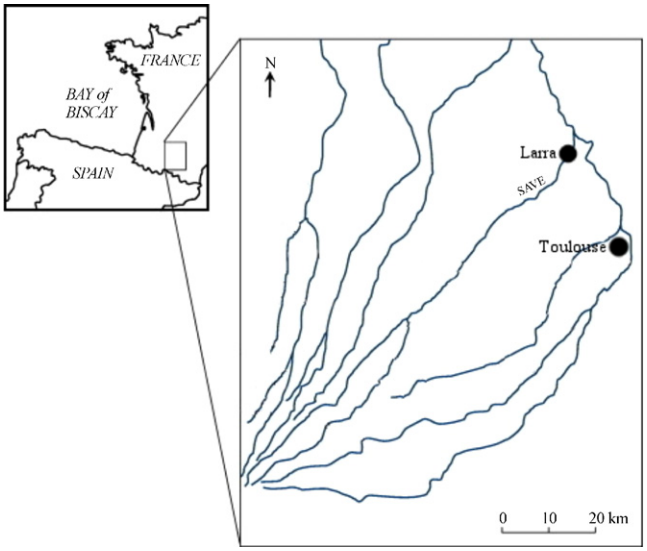

Fig. 1. France map highlighting the experimental site of Larra.

2.2. Experimental animals

The Crucian carp, *C. carassius* (Linnaeus, 1758; Cyprinidae) was chosen for its availability in a local hatchery. Specimens were aged 8–15 months, measuring 9.4 ± 0.7 cm, and weighing 12.5 ± 3 g. The fish were acclimated in filtered dechlorinated water in the rearing facilities for two weeks before the experiment. The fish were fed *ad libitum* before exposure and were not fed during exposure. The experiments were carried out in accordance with the European Ethical Guidelines, with the approval of the National Ethical Committee of the French Scientific Research National Centre (CNRS). Fish exposure and handling were conducted under the supervision of Dr. Laury Gauthier, holder of French animal handler's certificate no. 31-103, giving authorization to experiment on living vertebrates.

2.3. Experimental site

The Save River is a tributary of the Garonne located in the Gascogne area of south-west France (Fig. 1). It is 140 km long and its watershed (1150 km^2) is mainly under agriculture, with more than 75% of arable land. The main crops are maize, wheat, and sunflower, which require the use of pre-emergent herbicides. The hydrological regime is mainly pluvial with maximum discharge in May (the bi-annual flood discharge is 69 $m^3 s^{-1}$) with low flow during summer (1.3 $m^3 s^{-1}$). The mean annual discharge is 6 $m^3 s^{-1}$. There is no major city on the watershed (density: 39.3 inhabitants/km^2), suggesting no significant impact of urban pesticide contamination. Sampling was conducted at Larra (01°14′40″E–43°43′40″N), approximately 10 km upstream of the confluence of the Save river, consequently offering the maximum effective agricultural watershed.

Table 1
Herbicide concentration in the experimental mixture renewed daily for 4 days ($\mu g\,L^{-1}$). Data are shown as mean concentration and standard deviation.

	Metolachor	Isoproturon	Chlorotoluron	Atrazine	DEA
Nominal concentration	1.29	0.24	0.14	0.04	0.02
Mean concentration $t_{0\,h}$	1.22 ± 0.21	0.23 ± 0.00	0.74 ± 0.12	0.02 ± 0.01	0.04 ± 0.02
Mean concentration $t_{24\,h}$	1.01 ± 0.14	0.20 ± 0.00	0.45 ± 0.17	0.02 ± 0.01	0.04 ± 0.02

$t_{0\,h}$ refers to mean concentration right after each of the four renewals; $t_{24\,h}$ refers to mean concentration after 24 h of exposure.

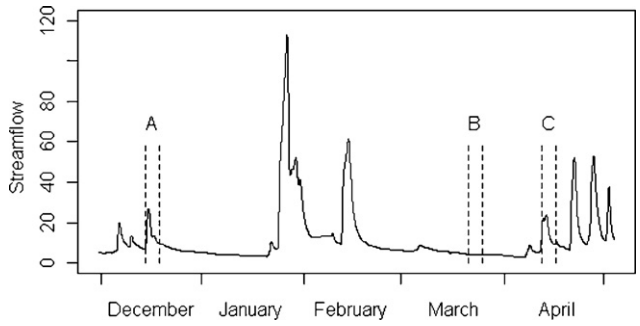

Fig. 2. Hydrogram of the Save River at Larra from December 2008 to April 2009. The line graph represents the stream flow ($m^3\,s^{-1}$). Hatched lines delimit the sampling and exposure periods. A: winter flood; B: basal flow; C: spring flood.

2.4. Experimental design

Weather forecasts were checked to predict modifications of hydrological conditions. The first sampling was timed to coincide with an average winter flood (14/12/2008 to 18/12/2008), the second with a low-flow situation (21/03/2009 to 25/03/2009) and the third with the first major rainstorm event following application of pre-emergent herbicides (12/04/2009 to 16/04/2009) (Fig. 2). Both sampled floods were 4 days long, which is consistent with the average duration of a flood in the Save River (data 1994–2008). This duration fits with the time required to induce MN in *C. carassius* (Cavas and Konen, 2007). Save river water was taken daily for fish exposure and pesticides analysis.

2.4.1. Laboratory exposure to river water

The assays were carried out in 36-L tanks each housing 10 randomly selected fish. Exposure lasted 4 days with a parallel negative control (dechlorinated tap water). Water was taken at the experimental site and renewed daily in order to follow the changes in water composition during the given period of time and to minimize changes due to metabolization, complexation, and build-up of catabolites. The exposure water samples were gently warmed (water bath, 10 min) to match the rearing water temperature thus avoiding thermal shock for the fish. Water was artificially oxygenated and suspended particles did not sediment during the 24 h periods. Light exposure was maintained on a natural cycle.

2.4.2. Laboratory exposure to the experimental mixture

In an additional experiment, 10 fish in controlled conditions were exposed to a mixture of herbicides, prepared to mimic the water contamination during the spring flood. The five herbicides detected at highest concentration during the spring flood were used, and their nominal concentrations were based on their mean concentration over the 4 days of the flood. The experimental mixture was composed of metolachlor 1.29 $\mu g\,L^{-1}$, isoproturon 0.24 $\mu g\,L^{-1}$, chlorotoluron 0.14 $\mu g\,L^{-1}$, atrazine 0.04 $\mu g\,L^{-1}$ and its metabolite deethylatrazine (DEA) 0.02 $\mu g\,L^{-1}$. The exposure lasted 4 days in 36-L exposure tanks and had parallel negative and positive controls (cyclophosphamide, 5 $mg\,L^{-1}$). Contaminated water was renewed every 24 h. Herbicide concentration in the spiked water was checked twice a day, immediately after renewal and after 24 h of exposure. Effective herbicide concentrations are shown in Table 1.

2.4.3. Water chemistry and pesticide analysis

During each exposure sequence, physicochemical parameters (temperature, pH, conductivity) and flow were checked daily in the river (Table 2). The concentration of suspended solids was estimated by filtration of a 2 L water sample through a cellulose ester filter (Millipore, 0.45 μm). Pesticide analysis was performed on 2 L raw water samples taken daily during each event. Extraction was performed following the procedure described by Devault et al. (2007). Briefly, liquid/liquid extraction was carried out with a dichloromethane/water ratio of 1:6 (V/V). Dichloromethane was then dried on anhydrous sodium sulphate and evaporated to dryness under vacuum. Finally, the dry residue was taken up in 2 mL of hexane. The contaminants were identified on a gas chromatography column from Zebra ZB-5MS 30 m, 0.25 mm i.d., 0.25 μm film from Phenomenex® (Torrance CA) with Thermo Fisher Scientific (Waltham, MA) Trace GC 2000 coupled with a DSQ II mass detector. The chromatographic conditions are described by Taghavi et al. (2010). Samples were analyzed for the 20 herbicides and the 5 fungicides most frequently used on the crops in the area (Taghavi et al., 2010; Devault et al., 2007). The detection limit, based on a signal-to-noise ratio of 3 was estimated at 0.01 $\mu g\,L^{-1}$.

2.5. Genotoxicity assay

Blood samples were taken by cardiac puncture with heparinized syringes. Ten individuals were used for the MN test among which eight were also employed for the comet assay. Fish were anaesthetized with 0.12 $g\,L^{-1}$ benzocaine (Marques de Miranda Cabral Gontijo et al., 2003). Because of its higher sensitivity, the comet assay was completed before the MN assay.

The single-cell gel electrophoresis assay was performed according to Tice et al. (2000) with modifications. All the steps described were performed under red light to minimize additional UV-induced DNA damage. Freshly sampled erythrocytes were diluted in 0.5% low melting agarose (LMA) in PBS and then transferred onto degreased microscope slides dipped the day before in 1.6% normal melting agarose (NMA) for the first layer and freshly covered with 85 μL NMA. The agarose was allowed to set for 5 min on ice

Table 2
Physico-chemical parameters of the river water.

	Max flow ($m^3\,s^{-1}$)	Average flow ($m^3\,s^{-1}$)	Suspended matter ($mg\,L^{-1}$)	t (°C)	pH	Conductivity ($\mu S\,m^{-1}$)	Oxygenation rate (%)
Winter flood 12–18 December 2008	26.7	15.5 ± 5.6	451.3	11.4 ± 0.3	8.02 ± 0.12	542.6 ± 25.5	92 ± 2
Low flow 21–25 March 2009	4.48	4.3 ± 0.1	17.5	16.5 ± 0.2	8.11 ± 0.34	583.3 ± 9.6	98 ± 5
Spring flood 12–16 April 2009	23.8	16.0 ± 5.6	340.0	17.3 ± 0.3	7.97 ± 0.05	571.2 ± 8.6	86 ± 10

before removing the cover glass. After agarose solidification, the slides were placed in lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1% Triton X-100 and 10% DMSO, pH 10) for 90 min. Alkaline DNA-unwinding was carried out in an electrophoresis chamber containing a freshly prepared buffer (1 mM Na_2EDTA, 300 mM NaOH) for 20 min and electrophoresis was performed in the same buffer for 20 min at 25 V and 350 mA. After staining with 75 μL ethidium bromide (2 μg mL^{-1}), observations were made under an epi-fluorescence microscope (Olympus® BX4) equipped with a U-MWB2 filter, at 400× magnification. DNA strand breakage was quantified as the tail DNA percentage using an image-analysis system (Komet 5.5; Andor Technology®). For each individual, two slides were coded and 50 cells analyzed on each slide. Clouds of DNA fragments were not considered for the analysis.

Micronucleus test was conducted on the same individuals as the comet assay. Blood was smeared onto 5 slides for each fish. After fixing in pure methanol for 15 min, the slides were allowed to dry in air. All the slides were coded, randomized and scored using a blind review by a single observer. Slides were stained with acridine orange with a drop of AO (0.003% in Dulbecco PBS), covered right before scoring with a cover glass (Ueda et al., 1992, adapted by Cavas and Ergene-Gozukara, 2005). Micronuclei, exhibiting the same yellow–green fluorescence as the nucleus were scored in 5000 cells/individual under an epi-fluorescent microscope (Olympus® BX4) equipped with a U-MWB2 filter, at 1000× magnification.

2.6. Statistical analysis

All results are presented as mean ± standard error. For the exposure to the river water, the MN data were processed using a crossed two-way analysis of variance (ANOVA). Factors were treatment (exposed and negative control) and hydrological conditions (basal flow, winter flood, and spring flood). For the experimental exposure, the MN data were processed using an analysis of variance (ANOVA). Factor was treatment (exposed, negative control, and positive control). In each case, the dependent variable was micronucleus frequency, weighted by the number of cells scored. For the exposure to the river water, the comet assay data were processed using a 3-way ANOVA. Factors were treatment (exposed and negative control), hydrological condition (basal flow, winter flood, and spring flood) and electrophoresis. For the experimental exposure, the comet assay data were processed using a 2-way ANOVA. Factors were treatment (exposed, negative control, and positive control) and electrophoresis. The dependent variable was tail DNA percentage. For both MN and comet assays the variables were ratios and were transformed in order to respect the assumption of normality and homoscedasticity of residuals. The assumption of normality and homoscedasticity of the residuals were tested through Shapiro–Wilk normality and Brown–Forsythe test, respectively. The Tukey pairwise comparison test was performed to test differences between samples and their respective controls. The induction rate was estimated by the ratio of MN frequency between exposed and control samples. All statistical analyses were performed using R (Ugarte, 2008).

3. Results

Physical and chemical properties of the water samples are shown in Table 2. The two floods (winter and spring) were similar in terms of flow (maximum and average) and amount of suspended solid. They contrasted with the basal flow condition. The pH, conductivity, and oxygenation showed no differences between the three hydrological contexts. Temperature increased slightly from December to April, justifying the use of a control fish sample for each exposure.

3.1. Water analysis

The chemical analysis results are shown in Table 3. The sums of the concentration of all 25 pesticides considered were 0.98 μg L^{-1}, 0.27 μg L^{-1} and 1.82 μg L^{-1} for winter flood, basal flow, and spring flood, respectively. Five of the six most abundant pesticides in water were herbicides: metolachlor, isoproturon, chlorotoluron,

Table 3
Pesticide concentrations measured daily for 4 days of the event (μg L^{-1}). Data are shown as minimum, maximum, mean concentration and standard deviation.

		Winter flood				Basal flow				Spring flood			
		Min.	Max.	Mean	S.D.	Min.	Max.	Mean	S.D.	Min.	Max.	Mean	S.D.
Metolachlor	H	nd	0.03	0.02	0.01	0.01	0.02	0.02	0.01	0.39	2.19	1.29	0.88
Isoproturon	H	0.17	0.34	0.25	0.07	0.12	0.19	0.15	0.03	0.15	0.37	0.24	0.09
Chlortoluron	H	nd	0.80	0.30	0.38	nd	nd	–	–	nd	0.32	0.14	0.16
Tebuconazole	F	nd	0.56	0.22	0.25	nd	0.03	0.01	0.01	nd	0.03	0.01	0.01
DEA	H	nd	0.07	0.03	0.03	0.03	0.03	0.03	0.00	nd	0.03	0.02	0.02
Atrazine	H	nd	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.08	0.04	0.03
Metobromuron	H	nd	0.23	0.06	0.11	nd	nd	–	–	nd	nd	–	–
Aclonifen	H	nd	0.04	0.02	0.02	nd	0.02	0.01	0.01	nd	0.02	0.01	0.01
Cyproconazole	F	nd	0.06	0.02	0.03	nd	nd	–	–	nd	0.06	0.02	0.03
Trifluraline	H	nd	0.01	0.01	0	0.01	0.01	0.01	0.00	nd	0.03	0.01	0.01
Epoxyconazole	F	nd	0.01	0.01	0	nd	0.01	0.01	0.00	nd	0.02	0.01	0.01
Fluzilazole	F	nd	0.01	0.01	0	nd	nd	–	–	nd	0.02	0.01	0.01
Linuron	H	nd	0.02	0.01	0.01	nd	nd	–	–	nd	0.04	0.01	0.02
Metazachlor	H	nd	0.01	0	0	nd	nd	–	–	nd	0.01	0.01	0.00
Hexazinone	H	nd	0.01	0	0.01	nd	nd	–	–	nd	0.03	0.01	0.01
Cyanazine	H	nd	0.02	0.01	0.01	nd	0.01	0	0	nd	nd	–	–
Simazine	H	nd	0.02	0.01	0.01	nd	nd	–	–	nd	nd	–	–
Alachlore	H	nd	0.01	0	0.01	nd	nd	–	–	nd	nd	–	–
Monolinuron	H	nd	nd	–	–	nd	nd	–	–	nd	0.02	0	0.01
Sebuthylazine	H	nd	0.02	0.01	0.01	nd	nd	–	–	nd	nd	–	–
Terbuthylazine	H	nd	0.02	0.01	0.01	nd	nd	–	–	nd	nd	–	–
Fenpropimorph	F	nd	nd	–	–	nd	nd	–	–	nd	nd	–	–
Pendimethaline	H	nd	0.01	0	0	nd	nd	–	–	nd	nd	–	–
Metoxuron	H	nd	nd	–	–	nd	nd	–	–	nd	nd	–	–
Imazamethabenz	H	nd	nd	–	–	nd	nd	–	–	nd	nd	–	–
Total				**0.98**				**0.27**				**1.82**	

H, herbicide; F, fungicide.

Table 4
Frequency of micronuclei scored in circulating erythrocytes in control fish and in fish exposed to Save River water for 96 h (mean ± standard error).

	Negative control	Exposed	Induction rate
Winter flood 12–18 December 2008	0.61 ± 0.41 (8)	0.88 ± 0.46 (10)	1.44
Low flow 21–25 March 2009	0.40 ± 0.2 (10)	0.54 ± 0.51 (9)	1.35
Spring flood 12–16 April 2009	0.44 ± 0.15 (8)	1.28 ± 0.47 (9)**	2.91

Data are shown as the mean total frequency for each treatment group, ±standard deviation (n = number of fish).
** Significant difference from the corresponding negative control group at the $p < 0.01$ level.

atrazine, and its metabolite DEA. The three main families of herbicides used in the watershed are represented by these 5 molecules: substituted ureas (isoproturon, chlorotoluron), triazines (atrazine and DEA) and amides (metolachlor). The other detected pesticides occurred at low concentrations. The main observation from this chemical analysis is the concentration peak of metolachlor during the spring flood. Its concentration was higher than any other pesticides measured in any other sample. It contributes to 70% of the total contamination of the spring flood water.

3.2. Genotoxicity assessment

3.2.1. Fish exposed to field collected water

The MN frequencies scored after exposure to water samples from Save River are shown in Table 4. All the control groups presented low MN frequencies (0.40–0.61‰). Taken together, the results demonstrated an increase of MN frequency in fish erythrocytes exposed to River Save water (ANOVA, $p < 0.01$). Post hoc test revealed significant induction of MN formation only in fish exposed to spring flood water (×2.91; $p < 0.01$). When fish were exposed to other Save water samples, no significant MN induction was detected ($p > 0.05$).

The DNA breakdown data, measured as the percentage tail DNA by comet assay, are presented in Table 5. DNA damage was significantly higher in all fish samples exposed to Save water than in control fish. Moreover, fish exposed to river water sampled during the spring flood expressed the highest DNA damage induction (×2.82).

3.2.2. Fish exposed to the experimental mixture

Fish exposed, in controlled conditions, to the experimental mixture (metolachlor 1.29 $\mu g\,L^{-1}$, isoproturon 0.24 $\mu g\,L^{-1}$, chlorotoluron 0.14 $\mu g\,L^{-1}$, atrazine 0.04 $\mu g\,L^{-1}$, DEA 0.02 $\mu g\,L^{-1}$) did not exhibit the genotoxic damage seen in the comet assay of fish exposed to spring flood water (Table 6). The induction of DNA damage measured by comet assay was significant (×2.08). The value is comparable to that measured in fish exposed to winter flood water. However, no significant MN induction was detected.

4. Discussion

4.1. Contamination pattern

The temporal pattern of agricultural stream water contamination is driven both by hydrological events and agricultural practices (Kadoum and Mock, 1978; Thurman et al., 1992; Pratt et al., 1997). In this study, the lowest total pesticide concentration occurred during basal flow (0.27 $\mu g\,L^{-1}$). The low contamination of water collected during the basal flow event can result from several weeks without rain, precluding surface runoff as a transport mechanism. In contrast, the winter flood occurred after successive rainfall events. The resulting washing off of applied pesticides from the field surface led to moderate contamination (0.98 $\mu g\,L^{-1}$). The spring flood presented the highest level of contamination (1.82 $\mu g\,L^{-1}$). This event happened following several weeks without rainfall (Fig. 2). Moreover, it coincided with increased herbicide application in this agricultural catchment. The higher water contamination (twice the total concentration measured in winter) results from the simultaneous transport of the pesticides applied during the preceding weeks. The molecules detected were mainly pre-emergent herbicides such as metolachlor, isoproturon, chlorotoluron, atrazine and its metabolite DEA. Such herbicides are used for weed control in crops including wheat, maize, and sunflower, accounting for most of the crops in the Save watershed. This type of contamination is in accordance with previous studies concerning water contamination in the Gascogne area (Devault et al., 2007)

Table 5
Level of the DNA damage measured by tail DNA percentage in circulating erythrocytes in control fish and in fish exposed to Save River water for 96 h (mean ± standard error).

	Negative control	Exposed	Induction rate
Winter flood 12–18 December 2008	10.08 ± 2.15 (4)	16.40 ± 2.45** (4)	1.63
Basal flow 21–25 March 2009	15.42 ± 1.95 (8)	17.39 ± 2.06** (8)	1.13
Spring flood 12–16 April 2009	10.19 ± 3.13 (8)	28.68 ± 7.25*** (8)	2.82

Data are shown as the tail DNA percentage for each treatment group, ±standard deviation (n = number of fish).
** Significant difference from the corresponding control group at the $p < 0.01$ level.
*** Significant difference from the corresponding control group at the $p < 0.001$ level (Tukey).

Table 6
Level of DNA damage measured by tail DNA percentage and frequency of micronuclei in circulating erythrocytes in control fish and in fish exposed to the experimental mixture for 96 h (mean ± standard error).

	MN test		Comet assay	
	MN frequency (‰)	Induction rate	Tail DNA (%)	Induction rate
Negative control	0.64 ± 0.45 (8)		8.04 ± 1.36 (8)	
Positive control	1.43 ± 0.80* (6)	2.23	18.43 ± 4.12** (7)	2.29
Experimental mixture	0.50 ± 0.26 (8)	0.78	16.76 ± 1.33**(8)	2.08

MN test data are shown as the mean total frequency for each treatment group, ±standard deviation; comet assay data are shown as the tail DNA percentage for each treatment group, ±standard deviation (n = number of fish).
* Significant difference from the corresponding negative control group at the $p < 0.05$ level.
** Significant difference from the corresponding negative control group at the $p < 0.01$ level (Tukey).

and more precisely in the Montoussé river, a tributary of the Save (Taghavi et al., 2010). Metolachlor contributed most to the high contamination measured in the spring flood reaching concentrations of up to 2.19 $\mu g\,L^{-1}$. The use of atrazine has been forbidden in France since 2003, but it remains among the frequently detected molecules in streams (28.1% of detection when tested for; IFEN, 2007). The detection of both atrazine and DEA, indicates that the contamination can be attributed both to the persistence and slow degradation of this molecule in the soil (Wiegand et al., 2001) and to marginal residual use. Tebuconazole was the only fungicide detected in significant amounts among the molecules we looked for. It is generally used on wheat and maize.

4.2. Genotoxicity

All fish exposed to Save water exhibited significant DNA breakdown compared to the controls. This reveals the genotoxic potential of the Save stream in a context of possible chronic contamination. Moreover, genotoxicity measurements are in accordance with the temporal contamination pattern brought to light by the chemical analysis of the river water. DNA breakdown, detected by the comet assay, was the lowest during basal flow and the highest during spring flood. The MN assay results confirmed the higher genotoxic impact of the water during spring flood: it was the only exposure condition which induced significant MN formation. The level of oxygen, conductivity, and pH of the water were almost identical at the three sampling times. It is unlikely that they affected the assay results significantly. Previous studies demonstrated the impact of temperature on genotoxicity assay responses (Moraes de Andrade et al., 2004). However, the temperature variation in this study was low compared to the high range of temperatures *C. carassius* can withstand (from 0 °C to 36 °C; Horoszewicz, 1973). In addition, it is important to point out that water sampled *in situ* was gently warmed to reach the temperature of water in which the fish were kept in the rearing facilities. Heat-shock, inducing genotoxic damage (Anitha et al., 2000), was then avoided. Due to field runoff from heavy rainfall, flood water presents an increase of suspended matter resulting from alluvial terraces and tertiary clay-rock slope erosion (Bornand et al., 1989). The winter flood exhibited slightly higher suspended matter concentrations than spring flood, but did not induce MN formation. Direct impact of clay on mutagenicity can thus be excluded. These results suggest an increased genotoxic potential of stream water associated with the pulses of contamination occurring during runoff events.

Genotoxicity in fish associated with agricultural runoff has already been demonstrated using the comet assay in previous studies (Whitehead et al., 2004; Bony et al., 2008). Based on data from the literature, the genotoxicity of the molecules detected in higher concentrations during spring flood remains unclear. Clements et al. (1997) showed induction of DNA damage in *Rana catesiana* tadpoles exposed by immersion to a commercial formulation of metolachlor (Dual-960E) at 0.272 $mg\,L^{-1}$, but Grisolia and Ferrari (1997) concluded to non-mutagenicity in mice after injection of metolachlor at concentrations up to 40 $mg\,kg^{-1}$. No genotoxicity associated with exposure to isoproturon was detected, either in the mouse bone-marrow micronucleus test (Gebel et al., 1997) or by the comet assay and the chromosomal aberration test in Chinese Hamster ovary cells (Vigreux et al., 1998). In contrast, Behera and Bhunya (1990) and Chauhan et al. (2001) revealed the genotoxic impact of isoproturon, which was the second most concentrated herbicide during spring flood. Chlorotoluron was the third most concentrated herbicide in spring flood water. Based on the literature, chlorotoluron and its metabolites have shown no evidence of genotoxicity (European Commission, 2005). In addition, in our study, the chlorotoluron concentration, which was highest in the winter flood water, did not induce MN formation. Atrazine has been under focus in many genotoxicity studies, and the results are often conflicting. Atrazine genotoxicity has been pointed out using many tools, such as flow cytometry (Biradar and Rayburn, 1995a,b), drosophila wing-spot assay (Torres et al., 1992), and electronic DNA-biosensor (Oliveira-Brett and da Silva, 2002). Micronucleus test and comet assay have also shown DNA damage induction by atrazine (de Campos Ventura et al., 2008; Chang et al., 2005; Clements et al., 1997; Conners and Black, 2004; Ribas et al., 1995). In contrast, other studies concluded there was no atrazine genotoxicity (Kligerman et al., 2000a,b; Freeman and Rayburn, 2004) or marginal genotoxicity (Tennant et al., 2001; Gebel et al., 1997; Rayburn et al., 2001). Data concerning DEA are scarce. Using the Microtox method, it has been demonstrated to be less toxic than atrazine (Kross et al., 1992), but no genotoxicity data are available. The other herbicides were measured at low concentrations and are unlikely to be responsible for MN induction in fish.

To determine the implications of these molecules in the genotoxic effects of spring floods, a complementary experiment was performed in controlled conditions. Fish were exposed to a mixture of the five herbicides detected at the highest concentrations during the mutagenic spring flood. The genotoxicity measured (Table 6) did not match the results of the exposure to the spring flood water. The DNA breakdown measured with the comet assay was significant but lower than compared to the spring flood, and no MN induction was detected. The comet and MN assays bring to light different genetic damage. The comet assay can detect less severe and repairable DNA damage, such as single and double strand breakage (Tice et al., 2000). Alkali-labile sites are also detected as they are expressed as single-strand breaks in alkaline conditions (Tice et al., 2000). This assay does not require cell division to reveal damage. DNA breakdown occurring at all phases of the cell cycle, including in circulating erythrocytes, can then be quantified. In contrast, MN formation occurs during cell division (Al-Sabti and Metcalfe, 1995). It can result from two events. Chromosomal breakage and/or dysfunction of the mitotic spindle, both can lead to an incomplete distribution of chromosomes in daughter cells during mitosis. MN frequency in peripheral erythrocytes is then the result of the dynamic balance between the formation of micronucleated cells and their elimination. The detection of a modification of basal MN frequency reveals the alteration of one or both of these processes. The input of erythrocytes in circulating blood can be caused either by the production of new cells by the cephalic kidney or, under stressful conditions, by the release of cells stored in the spleen. An increased production of micronucleated cells could be explained by an aneugenic and/or a clastogenic impact in the erythropoietic organ during the duration of cell differentiation. In the present study, the clastogenic potential of the mixture has been demonstrated by the comet assay. The cells which are involved in the differentiation process during the 4-day exposure are then susceptible to be micronucleated. Splenic contraction has been demonstrated in fish in response to exercise and hypoxia (Yamamoto, 1987; Lai et al., 2006) and more recently to metal contamination (Witeska, 2005). In the frame of our study, no argument supports the hypothesis of a higher MN frequency in the cells stored in spleen compared to circulating erythrocytes. Then, such a release is unlikely to induce an increase in total MN frequency. Beside micronucleated cells formation, the rise in MN frequency could be caused by a decrease in micronucleated cells elimination, either through apoptosis or MN removal. Decordier et al. (2002) demonstrated that micronucleated cells can undergo apoptosis. An inhibition of this process would then lead to an increase in MN frequency. To our knowledge, no experimental data support such inhibition of apoptosis by contaminant exposure. Splenic removal of micronucleated cells has been demonstrated in mammals (Ramirez-Munoz et al., 1999; Cristaldi et al., 2004). In the review published in 2006, Udroiu highlighted the

fact that this removal mechanism involves the interendothelial slits of sinusal spleen, and conclude that the application of the test is not compromised in fish as their spleen is non-sinusal. Furthermore, the balance between the processes which are driving the MN frequency may vary in time. Previous studies suggested that the exposure to contaminants inhibits erythropoiesis (Das and Nanda, 1986; Dinnen et al., 1988). Consequently, less erythroid stem cells are undergoing differentiation into potentially micronucleated erythrocytes. The MN formation is then lowered, leading to a shift in the equilibrium. As a result, the peak of micronucleated erythrocytes is observed between 1 and 5 days after exposure to contaminants (Al-Sabti and Metcalfe, 1995; Udroiu, 2006). This duration is short compared to the average life span of circulating erythrocytes which has been estimated at 51 days for *C. auratus langsdorfii* (Fischer et al., 1998). In fact, the results from the experimental exposure indicated that the increased MN frequency measured in fish exposed to the spring flood water cannot be exclusively attributed to the five herbicides tested alone, even if they represent the major part of the contamination detected. Other parameters must be involved in the higher mutagenicity of the spring flood water.

Sorensen et al. (2005) highlighted the interaction between clay and pesticide genotoxicity. The genotoxicity of pesticides can be either enhanced (dicamba) or lowered (oxamyl) by the molecule–clay interaction. As no suspended particles were present in the water of the experimental exposure, such interactions were not possible. This dissimilarity between field water and the experimental mixture may explain part of the divergence of the results. A direct mutagenic impact of clay is unlikely, as no MN induction was detected during the winter flood, but an interaction with the contaminant mixture present in the spring water can be suspected.

Numerous environmental and biological processes can modify the original molecule and, consequently, its toxicity. Osano et al. (2002) demonstrated that the genotoxic potential of metolachlor increased with its degradation. A stable aniline degradation product of metolachlor (2-ethyl-6-methylaniline) exhibits higher toxic effects than the parent molecule. Metabolites resulting from the degradation of every detected molecule could be present in the Save water, but were not included in the experimental mixture. Moreover, we cannot exclude that molecules which were unsuspected *a priori* were actually present in the Save. The glyphosate and diuron require specific analytical processes. Because they were not expected in such an agricultural stream, they were not looked for in our study. However, diuron and glyphosate are present in French streams. The detection rates were respectively 31.3% and 25.9% in 2006 (IFEN, 2007). Based on the literature, diuron appears genotoxic at high concentrations (Canna-Michaelidou and Nicolaou, 1996; Agrawal et al., 1996). It has been suspected to induce DNA breakdown in fish (Bony et al., 2008). In controlled conditions, glyphosate has been demonstrated to be genotoxic in fish. DNA breakdown was detected in *Prochilodus lineatus* (Cavalcante et al., 2008), and MN induction and DNA breakdown has been demonstrated in *Carassius auratus* (Cavas and Konen, 2007). Nevertheless, the concentrations tested in these studies (5–15 mg L^{-1} and 10 mg L^{-1} respectively) were significantly higher than those encountered in French rivers (maximum 17 μg L^{-1} in 2005; IFEN, 2007). Finally, due to the complexity of realistic environmental mixtures tested, chosen in order to favour the ecological relevancy of the study, the causes of the higher mutagenicity observed in spring flood can not be fully identified.

In conclusion, detecting the contaminants in an exhaustive way and mimicking the full complexity of field contamination in controlled conditions seems unrealistic. The present results point out the challenge of predicting and evaluating the biological impact of contaminants in field conditions. However, the experimental design used in the present study made it possible to demonstrate the mutagenicity of a brief exposure to an environmental mixture. Moreover, the increased genotoxicity associated with a pulse of contamination in an agricultural stream is highlighted. By altering the genetic integrity of the aquatic organisms, these events may represent a threat for aquatic ecosystems.

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