Open Archive Toulouse Archive Ouverte (OATAO)

OATAO is an open access repository that collects the work of Toulouse researchers and makes it freely available over the web where possible.

Any correspondence concerning this service should be sent to the repository administrator: staff-oatao@inp-toulouse.fr

Giemsa versus acridine orange staining in the fish micronucleus assay and validation for use in water quality monitoring

T. Polard [a,b], S. Jean [a,b,*], G. Merlina [a,b], C. Laplanche [a,b], E. Pinelli [a,b], L. Gauthier [a,b]

[a] Université de Toulouse; INP-ENSAT, Avenue de l'Agrobiopôle, 31326 Castanet-Tolosan, France
[b] UMR 5245 CNRS-INP-UPS, EcoLab (Laboratoire d'écologie fonctionnelle), 31326 Castanet-Tolosan, France

ARTICLE INFO

ABSTRACT

This study concerns a comparative analysis of the acridine orange and Giemsa staining procedures for the fish erythrocyte micronucleus assay. The goal was to optimize the assay in the context of field water monitoring. Fish (*Carassius carassius*) were exposed to a reference genotoxic agent, cyclophosphamide monohydrate 5 mg l^{-1} for 2, 4, and 6 days before testing. Slides from each individual were scored using the two procedures. The results show that the assay was more sensitive when acridine orange was used. When slides were Giemsa stained, the presence of ambiguous artefacts, leading to false positives and increasing random variance, reduced the contrast between exposed and control samples. Acridine Orange staining was then applied in the context of water quality monitoring. Fish were exposed for 4 days to water sampled in two hydrological contexts: basal flow and spring flood. The results show that exposure to spring flood water in an agricultural stream can induce mutagenicity.

1. Introduction

The micronucleus assay (MN) is widely used in fish to investigate the genotoxic effects of chemicals, isolated or in mixtures, present in the aquatic environment (see Al-Sabti and Metcalfe, 1995 and Udroiu, 2006 for a review). The method is based on the detection of whole or partial chromosomes not incorporated in the daughter nucleus following mitosis due to clastogenicity (chromosomal breaking) or aneugenicity (mitotic spindle dysfunction). It has been demonstrated to respond to a large number of experimental and environmental carcinogenic pollutants, such as polycyclic aromatic hydrocarbons (PAH) (Al-Sabti and Metcalfe, 1995; Pacheco and Santos, 1997), heavy metals (Al-Sabti and Metcalfe, 1995), and pesticides (Grisolia, 2002). Moreover, many studies highlight the impact of the genotoxic burden on Darwinian fitness traits (Kurelec, 1993; Anderson and Wild, 1994; Depledge (1994); Diekmann et al., 2004). This link with a higher biological scale (population level) makes genotoxicity biomarkers highly ecologically relevant. Because fish seem to respond to xenobiotics in the same way as higher vertebrates (Al-Sabti and Metcalfe, 1995; Udroiu, 2006)

they are routinely used as sentinel organisms in biological water quality monitoring studies. However, many attempts have been made to increase the sensitivity of the MN assay in fish (Rodriguez-Cea et al., 2003). Two main directions have been explored, aiming either at increasing the MN induction potential or improving MN detection. Several strategies can be used to increase the induction potential of detectable anomalies. Carrasco et al. (1990) who first pointed out the lack of sensitivity of the MN assay in fish, proposed scoring other nuclear alterations (NA) (including blebbed, lobed, vacuolated, and notched nuclei) as complementary biomarkers beside MN scoring. This procedure is now frequently used (Ayllon and Garcia-Vasquez, 2000; Cavas and Ergene-Gozukara, 2003, 2005a,b; da Silva Souza and Fontanetti, 2006; Bolognesi et al., 2006). However, no standardization of nuclear abnormalities exists, and there is no consistent data on the origin of erythrocyte nuclear abnormalities (Strunjak-Perovic et al., 2009). Consequently, here we focus only on MN, widely recognized as an indicator of genotoxic pollution, and for which the scoring process follows well established criteria (Al-Sabti and Metcalfe, 1995). The use of tissues with a high mitotic index, and hence a high potential for MN induction, is also being explored. During the past years, several studies have successfully used embryos, cephalic kidney, liver, gills, and even regenerating fin cells (Al-Sabti and Metcalfe, 1995; Hayashi et al., 1998; Cavas and Ergene-Gozukara, 2005b; Arkhipchuk and Garanko, 2005; Deguchi et al., 2007; Talapatra and Banerjee, 2007). Such procedures

require complex cell preparation (Bolognesi et al., 2006) and often fish sacrifice. Moreover, the abundance of tissue debris slows down scoring (Frenzilli et al., 2008). The ease of collection and preparation make nucleated erythrocytes the most popular cells for fish MN assay (Udroiu, 2006).

The second way to improve the assay was thus to increase the detectability of the induced MN. The small size of the chromosomes, and thus of the MN, in many species including the widely used cyprinids is frequently pointed out to explain the difficulties encountered in detecting fish MN (Udroiu, 2006). The use of fish with bigger chromosomes, as recommended by Kligerman (1982), is not always possible in the context of ecologically relevant studies. The optimization of the staining procedure could be an interesting alternative to improving MN assay sensitivity. Surprisingly, till date, little attention has been paid to the effects of the different staining procedures currently used (mainly Giemsa and AO) on the sensitivity of the piscine MN erythrocytes assay. In mammals, fluorescent AO staining as been demonstrated to be more reliable and to allow higher sensitivity than the commonly used Giemsa staining (Hayashi et al., 1983; Tinwell and Ashby, 1989; Nersesyan et al., 2006). The AO staining procedure is now routinely used in rodent MN assay (Tiveron et al., 1996; Hamada et al., 2001; Nishikawa et al., 2002; Balachandra Dass and Ali, 2004). In fish, AO staining was first used by Ueda et al. (1992), followed by Hayashi et al. (1998), Bolognesi et al. (2006), and Costa and Costa (2007). In recent years, some authors switched from Giemsa to AO staining (Cavas and Ergene-Gozukara, 2005a). But, as yet, no comparative data are available to recommend Giemsa coloring or AO fluorescent staining in the context of the fish erythrocyte micronucleus assay. Giemsa is still by far the most used staining procedure in fish genotoxicity studies.

The aim of this work was 2-fold. First, we established a comparison between the results from the two most frequently used staining procedures (Giemsa and AO). For that purpose, we exposed a model organism (*Carassius carassius*) to a model genotoxic molecule cyclophosphamide monohydrate (CP) by immersion contact for 2, 4, or 6 days. Second, the selected procedure was validated in the context of water quality monitoring. The genotoxic potential of water from the Save River (France) was investigated during basal flow and spring flood. Because of the surface runoff due to heavy rainfall following herbicide application, the spring flood was expected to be highly contaminated with herbicides (Richards and Baker, 1993; Kuivila and Foe, 1995; Taghavi et al., 2010).

2. Materials and methods

2.1. Experimental animals

The Crucian carp, *C. carassius* (Linnaeus, 1758; Cyprinidae) was chosen for its availability. Specimens aged 8–15 months, measuring 9.6 ± 0.8 cm, and weighing 12.6 ± 3 g, were obtained from a local hatchery. The fish were acclimated in filtered dechlorinated water in the rearing facilities two weeks before the experiment. The fish were fed *ad libitum* before exposure and were not fed during exposure. The experiments have been carried out in accordance with the European Ethical Guidelines, with the approval of the National Ethical Committee of the French Scientific Research National Center (CNRS). Fish exposure and handling have been conducted under the supervision of Dr Laury Gauthier, holder of French certificate no.31–103, giving authorization to experiment on living vertebrates.

2.2. Test chemicals and reagents

All the chemicals and reagents used, heparine salt (CAS no. 9041-08-1), cyclophosphamide monohydrate (CAS no. 6055-19-2), benzocaine (CAS no. 94-09-7), acridine orange (CAS no.10127-02-3), methanol and Giemsa solution were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

2.3. Comparative study

2.3.1. Experimental design

Assays were carried out in six 36 l tanks, each containing 8 randomly picked fish. One tank was used for each exposure time (2, 4, and 6 days) (Cavas, 2008) and another for each parallel negative control. A reference genotoxic compound, cyclophosphamide monohydrate (CP), was dissolved in water at a nominal concentration of 5 mg l^{-1} (Cavas, 2008). The aim of this study was not to measure the genotoxic potential of a given concentration of CP, but to compare the test performance by scoring MN induced by CP after two different staining procedures. As each of the two staining procedures was performed on blood smears obtained from the same fish, the effective concentration of CP in water was not crucial, and no quantitative analyses were performed. Water was artificially oxygenated and renewed daily to minimize changes due to metabolization, complexation, and organisms catabolites.

Fish were anaesthetized with benzocaine 0.12 g l^{-1} (Marques de Miranda Cabral Gontijo et al., 2003). Peripheral blood samples were obtained by cardiac puncture with heparinized syringes and smeared onto slides. After fixing in pure ethanol for 15 min, the slides were allowed to air-dry for 24 h. We prepared 8 slides per fish. Four were stained using Giemsa, and 4 with fluorescent acridine orange. All AO and Giemsa stained slides were coded, randomized, and scored using a blind review by the same observer using an Olympus® BX4 microscope. A mean of 5000 cells were scored by fish. Slides of poor quality due to inefficient puncture were rejected before scoring.

2.3.2. Slide preparation

Giemsa solution was prepared immediately before staining. Solution diluted in demineralized water (12.5%) was centrifuged (10 min; 4000 g) and the supernatant was filtered (Whatman® filter paper, 1.2 μm). Slides were then immersed in solution for 25 min, rinsed thoroughly with distilled water, and air dried. Cells were scored under 1500× magnification in bright-field microscopy. As recommended by Al-Sabti and Metcalfe (1995), small, non-refractive, circular or ovoid chromatin bodies displaying the same staining and focusing patterns as the main nucleus were considered as micronuclei. An example of a positively identified Giemsa stained micronucleus is shown in Fig. 1a.

The other 4 slides for each individual were stained with a drop of AO (0.003% in Dulbecco PBS) and covered with a glass slip (Ueda et al., 1992 adapted by Cavas, 2008). Because AO stained slides were not able to be stored for a long time, observation immediately followed staining. Micronuclei, exhibiting the same yellow-green fluorescence as the nucleus were scored under epi-fluorescence with a U-MWB2 filter at 1000× magnification (Fig. 2).

2.4. Experimental assessment

2.4.1. Experimental site

The Save River is a Garonne tributary located in the Gascogne area of southwestern France. It is 140 km long and its watershed (1150 km^2) is mainly dedicated to agriculture, with more than 75% of arable land. The main crops are corn, wheat, and sunflower, which require the use of pre-emergent herbicides. There is no major city on the watershed (density: 39.3 /km^2), suggesting no significant impact of urban pesticide contamination. Sampling was conducted at Larra (01°14′40″E–43°43′40″N), just before the confluence of the Save river, consequently offering the maximum effective agricultural watershed.

2.4.2. Experimental design

Assays were carried out in 36 l tanks, each containing 10 randomly picked fish. Each exposure lasted 4 days and had a parallel negative control. Exposure water was renewed every day with water collected in the Save river. Basal flow conditions sampling took place from March 21st to March 24th, 2009. Spring flood sampling was timed to coincide with the first major rainstorm event and took place in from April 12th to April 15th, 2009. Physico-chemical parameters (temperature, pH, conductivity, turbidity) and flow were measured (Table 1). Save water samples were gently heated (water bath, 10 min) to match the rearing water temperature thus avoiding thermal shock for the fish. The micronucleus assay was performed using the AO procedure as previously described.

2.5. Statistical analysis

All results are presented as mean ± standard error. For the comparative staining study, data were processed using a crossed two-way analysis of variance (ANOVA) for each staining procedure. Factors were treatment and duration of exposure. The dependent variable was micronucleus frequency, weighted by the number of cells scored. The assumption of normality and homoscedasticity of the residuals were tested through Shapiro–Wilk normality and Brown–Forsythe Levene-type test, respectively. The Tukey pairwise comparison test was performed to test differences between each sample and its respective control. The difference between residual variance of both staining procedures was tested with an *F*-test
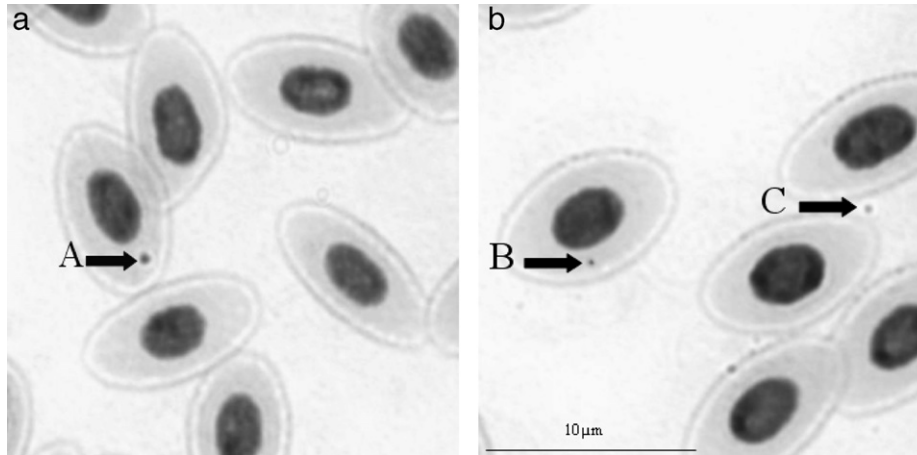
Fig. 1. Giemsa stained peripheral erythrocytes of *C. carassius* (× 1500) after 4 days exposure to CP 5 mg l^{-1}. (A) Micronucleus, (B and C) artefacts.
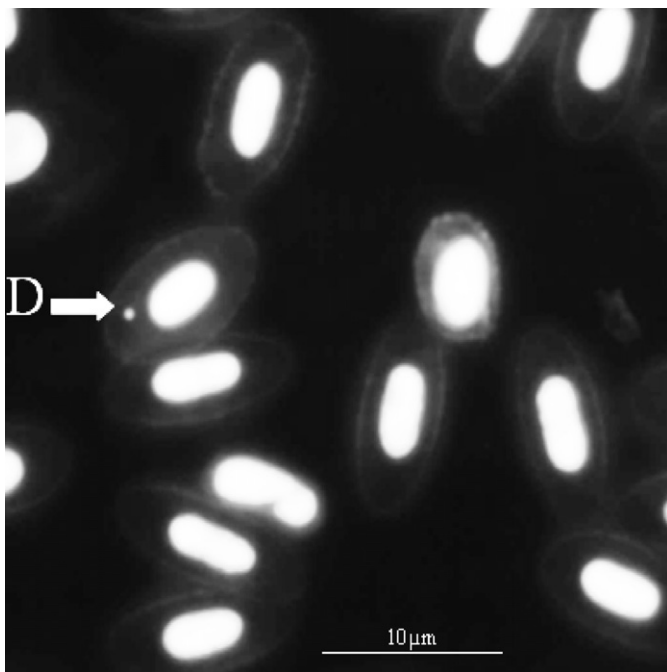
Fig. 2. Acridinde orange stained peripheral erythrocytes of *C. carassius* (× 1500) after 4 days exposure to CP 5 mg l^{-1}. (D) Micronucleus.

for two populations with correlated observations (Kanji, 2006). All statistical analyses were performed using R (Ugarte, 2008). The induction rate was estimated by the ratio of MN frequency between exposed and control samples.

3. Results

3.1. Comparative study

The frequencies of micronuclei and the induction rates observed in peripheral erythrocytes after 2, 4, and 6 days of exposure to CP at a nominal concentration of 5 mg l^{-1} are shown in Table 2. No mortality was observed in any of the 48 fish. The difference between each effective sample size and the initial number of fish exposed in each sample (8) is due to the rejection of individuals in which the blood sampling puncture did not fulfill quality requirement. Whatever the staining procedure the MN induction rates were higher after 4 days.

Table 1
Physico-chemical properties of the test water.

	Basal flow		Spring flood	
	Exposed	Control	Exposed	Control
pH	8.11 ± 0.34	8.01 ± 0.05	7.97 ± 0.05	8.1 ± 0.14
Temperature (°C)	16.5 ± 0.2	16.7 ± 0.17	17.3 ± 0.3	17.5 ± 0.45
Conductivity (μS/cm)	583.3 ± 9.6	558 ± 2.9	571.2 ± 8.6	564 ± 10.4
Oxygenation rate	$98 \pm 5\%$	$94 \pm 2\%$	$85.6 \pm 10\%$	$89.5 \pm 3\%$

Values are the average value of daily measurements for the 4 days of experiment $\pm$ standard error.

Results of the crossed two-way analysis of variance (ANOVA) for each staining procedure are shown in Table 3. Considering all the durations of exposure together, the exposure to CP at a nominal concentration of 5 mg l^{-1} led to micronucleus induction in a significant way, for both AO and Giemsa staining ($p < 0.001$ and $p = 0.01$, respectively). There was no time-dependent change in micronucleus induction between 2, 4, or 6 days of exposure for AO ($p > 0.05$). Time had a significant impact on MN frequencies when Giemsa was used ($p < 0.05$). Tukey tests revealed no difference between 2 and 4 days ($p > 0.05$), but a higher MN frequency after 6 days ($p < 0.05$) in Giemsa stained slides. Although not significantly different from other control MN frequencies ($p > 0.05$), the higher MN frequency for the negative control at 6 days could explain this result. Moreover, the intra-sample variance was lower with AO than with Giemsa, 0.27 and 1.195, respectively. The *F*-test with correlated observations showed that residual variance with Giemsa was greater than with AO (Table 3).

When each experimental sample was compared with its respective control sample for each duration of exposure, MN induction was not significant at any time in Giemsa stained slides (Tukey, $p > 0.05$). In contrast, micronucleus frequency was always significantly higher in exposed fish on AO staining (Tukey, $p < 0.05$) (Table 2).

3.2. Experimental assessment

The frequencies of MN after 4 days of exposure to water sampled in the Save River are shown in Table 4. There was no significant difference in MN frequency between fish exposed to water taken during basal flow and to control water. MN frequency in fish exposed to water taken during the flood was significantly higher than in the controls.

Table 2
Frequency (‰) of micronuclei scored in control fish and in fish exposed to 5 mg l^{-1} CP and MN induction rate.

Exposure (days)		Giemsa (n)	Induction rate	Acridine orange (n)	Induction rate
2	Control	2.41 ± 0.76 (8)	1.24	0.66 ± 0.36 (8)	2.40
	Exposed	2.97 ± 1.28 (8)		1.58 ± 0.79 (8)*	
4	Control	2.04 ± 1.05 (7)	1.70	0.59 ± 0.32 (7)	2.67
	Exposed	3.46 ± 1.35 (7)		1.59 ± 0.57 (7)*	
6	Control	3.41 ± 1.03 (7)	1.16	0.68 ± 0.43 (7)	2.32
	Exposed	3.96 ± 0.91 (7)		1.57 ± 0.47 (6)*	

Data are shown as the mean frequency of MN for each treatment group (‰), ± standard deviation, (n=number of fish).

   * Denotes a significant difference from the corresponding control group at the $p < 0.05$ level (Tukey).

Table 3
Analysis of variance of MN frequency as a function of treatment and time.

	Acridine orange		Giemsa	
	Mean square	p	Mean square	p
Treatment	9.41	10^{-6}*	8.56	0.01*
Time	0.0043	0.98	3.86	0.013*
Treatment × time	0.0091	0.97	0.83	0.73
Residuals	0.27		1.195	

Mean square refers to the estimate of the variance based on the variability among the set of measures.

   * Denotes a significant effect of the factor at the $p < 0.05$ level.

Table 4
Frequency of micronuclei scored in control fish and fish exposed to basal flow water or spring flood water for 96 h.

	Basal flow (n)	Spring flood (n)
Control	0.40 ± 0.2 (10)	0.44 ± 0.15 (8)
Exposed	0.54 ± 0.5 (9)	1.275 ± 0.46 (9)*

Data are shown as the mean total frequency for each treatment group, ± standard deviation (n=number of fish).

   * Denotes a significant difference from the corresponding control group at the $p < 0.05$ level (Tukey).

4. Discussion

4.1. Comparative study

The MN frequencies observed in the present study are in agreement with the literature. The baseline MN frequencies measured are consistent with the results observed in *C. auratus* by Cavas (2008) and Cavas and Konen (2007) and in the range of variability of 1–2 orders of magnitude reported by Bolognesi et al. (2006). The rate of induction by the CP solution at nominal concentration of 5 mg l^{-1} is low compared to previous studies (Cavas and Konen, 2007; Cavas, 2008). As MN induction requires cell division, this difference can be explained by a lower mitotic rate caused by a lower average temperature and by the use of older fish.

The MN frequency was always higher measured with Giemsa than with OA in both exposed and control samples. Because of the high contrast between green AO stained DNA and the dark background, we assume that no MN were missed during the scoring (Tinwell and Ashby, 1989). In accordance with the literature, even with the highest care in solution preparation, Giemsa does not stain solely actual MN, but also stains ambiguous artefacts such as cell debris or protein granules (Nersesyan et al., 2006; Winter et al., 2007; Costa and Costa, 2007). The identification of authentic MN among all the artefacts is then time consuming because the focusing pattern is often the only reliable distinctive criterion. Fig. 1b exhibits examples of staining artefacts. 'C' indicates a confirmed artefact, because of its location outside the cells. On the other hand, 'B' is susceptible to be mistaken for a micronucleus, leading to a false-positive detection. Only a detailed analysis of its slightly different focusing pattern, compared with the main nucleus, allows a conclusion to be reached concerning the real status of the material observed. Therefore, the higher MN score in Giemsa slides should be attributed to artefacts mistaken for MN even with time-consuming checking and rejection of doubtful items (Heddle and Salamone, 1981). Such an overestimation of MN frequency has already been pointed out for Giemsa stained slides, in the case of human exfoliated oral mucosa cells (Nersesyan et al., 2006). Nersesyan et al. demonstrated that the keratin bodies that form in mucosal cells resemble micronuclei with non-specific staining, leading to false positives. In fish erythrocytes, such keratinization cannot be considered, and artefacts are most likely to be coloration grains or protein granules (Costa and Costa, 2007), which are not expected to be more represented in either exposed or control samples. AO is a nucleic-acid-selective fluorescent cationic dye, which emits green light (525 nm) when exited (502 nm) only if bound to DNA. Thus, no ambiguous artefacts may confuse the observer in AO stained slides (Fig. 2). As a result, the scoring process is faster and more reliable (Hayashi et al., 1983).

False-positive artefact scoring in Giemsa-stained slides is responsible for background noise. This is illustrated by the random variance in ANOVA, higher with Giemsa than with AO. Given the low MN induction rate, this background noise significantly reduces the contrast between control and exposed samples. The resulting loss of detection power is stressed in the present study. When considering all three exposure times together (44 individuals), the Giemsa staining detected a statistical difference between exposed and control individuals, whereas when samples of different exposure times are considered separately in the post-hoc Tukey test (16 individuals), the difference appears to be non-significative. Considering the time required for scoring with Giemsa staining, improving the statistical power by increasing the number of individuals is not desirable.

The usual drawbacks of florescent staining cannot be considered in the context of MN scoring. No fading was observed while scoring during this study and the one-by-one staining strategy allow quick scoring of stained slides. Using AO, MN can be scored selectively in immature erythrocytes (Ueda et al., 1992; Cavas and Ergene-Gozukara, 2005a; Cavas, 2008). AO maximum excitation shifts to 460 nm when bound with RNA and the maximum emission shifts to the red (650 nm). A red-stained cytoplasm is then characteristic of an immature RNA-containing

cell. However, immature erythrocyte frequency depends on the erythropoiesis intensity and is low if the organism exhibits little mitosis. In our experimental conditions, immature erythrocyte frequency was too low to restrict MN scoring to this type of cell. Nevertheless the assay was sensitive enough to allow discrimination between exposed and control samples when AO was used.

The time course study supports previous studies dealing with MN assay in *Carassius* sp. (Cavas, 2008). More widely, MN are generally induced in fish after 1–6 days of exposure (Al-Sabti and Metcalfe, 1995; Grisolia and Cordeiro, 2000; Udroiu, 2006). In this study, the highest induction rates were measured after 4 days of exposure whatever the staining procedure. Statistical analyses revealed a higher MN frequency after 6 days of contact when slides were stained with Giemsa. In this case, MN frequencies were high in both control and exposed samples, resulting in a low induction rate. No difference in MN frequencies was detected between each duration of exposure for AO stained slides. Stability in control MN frequencies from one exposure time to another confirms the high reproducibility of the results in our negative control conditions. A longer exposure time could have been performed in order to determine the MN induction kinetics in our experimental conditions. Given that no significant time variation occurred in AO stained slides, the three times of exposure can be used in experimental assessments without distinction. Analysis of the flow data from the Save River (1994–2008) revealed that the average duration of a flood was 4 days. Given that this duration coincides well with high induction rates, the exposure time of 4 days was retained for stream water monitoring.

4.2. Experimental assessment

MN assay was used for field water quality monitoring (Lemos et al., 2007). In order to validate the AO staining procedure in this context, we investigated the mutagenicity of water collected in two hydrological conditions typical of an agricultural stream contamination pattern. Short contamination events are rarely considered in water quality monitoring programs. However, this type of contamination occurs during floods in agricultural streams. It has been demonstrated that the highest contaminant loads in agricultural streams are measured when rainfall causes surface water runoff, providing a major transport mechanism for pesticides. This phenomenon is emphasized in spring when the rainfall happens shortly after pesticide application (Richards and Baker, 1993; Kuivila and Foe, 1995; Schulz, 2001; Ferenczi et al., 2002). Fenelon and Moore (1998) describe an increase in atrazine level from trace level to $14 \, \mu g \, l^{-1}$ during spring floods in a small Indiana watershed where corn and soybean are the main crops. In the Gascogne area, where the Save River is located, the major contaminants are pre-emergent herbicides used for crops of corn, wheat, and sunflower (Devault et al., 2009). These herbicides, belonging to the families of triazines (atrazine, DEA, cyanazine), ureas (isoproturon, linuron, chlorotoluron) and anilides (metolachlor, metazachlor), are mostly applied in spring. High contamination of water during spring flood has been reported (Debenest et al., 2008). Taghavi et al. (2010) reported a 10-fold increase in chlorotoluron and linuron concentration in the Montoussé creek, a tributary of the Save river, during a flood in May 2008. Aquatic organisms are then exposed to a pulse of a mixture of herbicides. Genotoxicity of metolachlor and atrazine in aquatic organisms has already been discussed (Clements et al., 1997; de Campos Ventura et al., 2008), but little is known about the impact of pulse contaminations during floods. Few authors have pointed out DNA damage induction (single, double strand breakage or alkali-labile sites) by agricultural runoff (Whitehead et al., 2004; Bony et al., 2008). Mutagenicity has been demonstrated by Ames bacterial

mutagenicity assay (Whitehead et al., 2004), but until now, MN induction had not been investigated in this context. In this study, MN induction was detected in fish exposed to Save River water sampled during the spring flood, but not in fish exposed to water sampled during basal flow. The pulse of agricultural contamination associated with the flood, as described in the literature (Richards and Baker, 1993; Kuivila and Foe, 1995; Fenelon and Moore, 1998; Schulz, 2001; Ferenczi et al., 2002; Debenest et al., 2008; Taghavi et al., 2010), appears to be mutagenic in the context of our study. This is the first time that an agricultural flood is demonstrated to induce MN in fish, revealing the mutagenic potential of short contamination events. At this point, the detected genotoxicity cannot be attributed to a contaminant in particular. Further studies are required in order to explore the chemicals, or mixture of chemicals, which exhibits the higher genotoxic potential in the Save river. This result constitutes evidence that short contamination events should be taken into consideration when monitoring water quality.

5. Conclusion

The results of the present study add further evidence about the use of the MN assay as a relevant biomarker for water quality monitoring when used with AO staining. Statistical analyses revealed that fluorescent AO staining is better adapted to piscine erythrocyte MN assay than classical Giemsa staining. DNA-specific staining reduces false-positive MN scoring due to artefacts. This provides higher reliability and sensitivity for the assay. In addition, the scoring process is faster. The sensitivity of the MN assay using AO is validated by the field experiment. The mutagenic potential of a spring flood in the Save river was investigated. In such an agricultural watershed, where the main crops are corn, wheat, and sunflower, spring floods are heavily contaminated by pre-emergent herbicides transported from field to stream by surface runoff. Significant MN induction was detected in fish exposed to flood water under our experimental conditions. This mutagenicity is most likely associated with the short pulse of herbicides as no such results were observed with water sampled during basal flow. This contribution to the optimization of the MN assay highlights (i) the advantages of using AO rather than Giemsa staining and (ii) the applicability of the assay for monitoring biological impacts of short contamination events in the field.

References

Al-Sabti, K., Metcalfe, C.D., 1995. Fish micronuclei for assessing genotoxicity in water. Mutat. Res 343, 121–135.
Anderson, S.L., Wild, G.C., 1994. Linking genotoxic responses and reproductive success in ecotoxicology. Environ. Health Perspect. 102, 9–12.
Arkhipchuk, V.V., Garanko, N.N., 2005. Using the nucleolar biomarker and the micronucleus test on in vivo fish fin cells. Ecotoxicol. Environ. Saf. 62, 42–52.
Ayllon, F., Garcia-Vasquez, E., 2000. Induction of micronuclei and other nuclear abnormalities in European minnow *Phoxinus phoxinus* and mollie *Poecilia latipinna*: an assessment of the fish micronucleus test. Mutat. Res. 467, 177–186.
Balachandra Dass, S., Ali, S.F., 2004. Evaluation of γ-hydroxybutyric acid for genotoxicity in the mouse micronucleus assay. N. Y. Acad. Sci. 1025, 538–542.

Bolognesi, C., Perrone, E., Roggieri, P., Pampanin, D.M., Sciutto, A., 2006. Assessment of micronuclei induction in peripheral erythrocytes of fish exposed to xenobiotics under controlled conditions. Aquat. Toxicol. 78, 93–98.

Bony, S., Gillet, C., Bouchez, A., Margoum, C., Devaux, A., 2008. Genotoxic pressure of vineyard pesticides in fish: field and mesocosm surveys. Aquat. Toxicol. 89, 197–203.

Carrasco, K., Tilbury, K.L., Mayers, M.S., 1990. Assessment of the piscine micronuclei test as in-situ biological indicator of chemical contaminant effects. Can. J. Fish Aquat. Sci. 47, 2133–2136.

Cavas, T., Ergene-Gozukara, S., 2003. Micronuclei, nuclear lesions and interphase silver-stained nucleolar organizer regions (AgNORs) as cyto-genotoxicity indicators in Oreochromis niloticus exposed to textile mill effluent. Mutat. Res. 538, 81–91.

Cavas, T., Ergene-Gozukara, S., 2005a. Genotoxicity evaluation of metronidazole using the piscine micronucleus test by acridine orange fluorescent staining. Environ. Toxicol. Pharmacol. 19, 107–111.

Cavas, T., Ergene-Gozukara, S., 2005b. Induction of micronuclei and nuclear abnormalities in Oreochromis niloticus following exposure to petroleum refinery and chromium processing plant effluents. Aquat. Toxicol. 74, 264–271.

Cavas, T., Konen, S., 2007. Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (Carassius auratus) exposed to a glyphosate formulation using the micronucleus test and the comet assay. Mutagenesis 22, 263–268.

Cavas, T., 2008. In vivo genotoxicity of mercury chloride and lead acetate: Micronucleus test on acridine orange stained fish cells. Food Chem. Toxicol. 46, 352–358.

Clements, C., Ralph, S., Petras, M., 1997. Genotoxicity of select herbicides in Rana catesbeiana tadpoles using the alkaline single-cell gel DNA electrophoresis (comet) assay. Environ. Mol. Mutagen 29, 277–288.

Costa, P.M., Costa, M.H., 2007. Genotoxicty assessment in fish peripheral blood: a method for a more efficient analysis of micronuclei. J. Fish Biol. 71, 148–151.

da Silva Souza, T., Fontanetti, C.S., 2006. Micronucleus test and observation of nuclear alterations in erythrocytes of Nile tilapia exposed to waters affected by refinery effluent. Mutat. Res. 605, 87–93.

Debenest, T., Silvestre, J., Coste, M., Delmas, F., Pinelli, E., 2008. Herbicide effects on freshwater benthic diatoms: Induction of nucleus alterations and silica cell wall abnormalities. Aquat. Toxicol. 88, 88–94.

de Campos Ventura, B., de Fransceschi de Angelis, D., Aparecida Marin-Morales, M., 2008. Mutagenic and genotoxic effects of the atrazine herbicide in Oreochromis niloticus (Perciformes, Cichlidae) detected by the micronuclei test and the comet assay. Pestic. Biochem. Phys. 90, 42–51.

Deguchi, Y., Toyoizumi, T., Masuda, S., Yasuhara, A., Mohri, S., Yamada, M., Inoue, Y., Kinae, N., 2007. Evaluation of mutagenic activities of leachates in landfill sites by micronucleus test and comet assay using goldfish. Mutat. Res. 627, 178–185.

Depledge, M.H., 1994. Genotypic toxicity: implications for individuals and populations. Environ. Health Perspect. 102, 101–104.

Devault, D.A., Gérino, M., Laplanche, C., Julien, F., Winterton, P., Merlina, G., Delmas, F., Lim, P., Sánchez-Pérez, J.M., Pinelli, E., 2009. Herbicide accumulation and evolution in reservoir sediments. Sci. Total Environ. 407, 2659–2665.

Diekmann, B., Waldmann, P., Schnurstein, A., Grummt, T., Braunbeck, T., Nagel, R., 2004. On the relevance of genotoxicity for fish populations II: genotoxic effects in zebrafish (Danio rerio) exposed to 4-nitroquinoline-1-oxide in a complete life-cycle test. Aquat. Toxicol. 68, 27–37.

Fenelon, J.M., Moore, R.C., 1998. Transport of agrichemicals to ground and surface water in a small central Indiana watershed. J. Environ. Qual. 27, 884–894.

Ferenczi, J., Ambrus, A., Wauchope, R.D., Sumner, H.R., 2002. Persistence and runoff losses of 3 herbicides and chlorpyrifos from a corn field in the Lake Balaton watershed of Hungary. J. Environ. Sci. Health B. 37, 211–224.

Frenzilli, G., Falleni, A., Scarcelli, V., Del Barga, I., Pellegrini, S., Savarino, G., Mariotti, V., Benedetti, M., Fattorini, D., Regoli., F., Nigro., M., 2008. Cellular responses in the cyprinid Leuciscus cephalus from a contaminated freshwater ecosystem. Aquat. Toxicol. 89, 188–196.

Grisolia, C.K., Cordeiro, C.M.T., 2000. Variability in micronucleus induction with different mutagens applied to several species of fish. Genet. Mol. Biol. 23, 235–239.

Grisolia, C.K., 2002. A comparison between mouse and fish micronucleus test using cyclophosphamide, mitomycin C and various pesticides. Mutat. Res. 518, 145–150.

Hamada, S., Sutou, S., Morita, T., Wakata, A., Asanami, S., Hosoya, S., Ozawa, S., Kondo, K., Nakajima, M., Shimada, H., Osawa, K., Kondo, Y., Asano, N., Sato, S., Tamura, H., Yajima, N., Marshall, R., Moore, C., Blakey, D.H., Schechtman, L.M., Weaver, J.L., Torous, D.K., Proudlock, R., Ito., S., Namiki, C., Hayashi, M., 2001. Evaluation of the rodent micronucleus assay by a 28-day treatment protocol: summary of the 3th Collaborative Study by The Collaborative Study Group for the Micronucleus Test (CSGMT)/Environmental Mutagen Society of Japan (JEMS)–Mammalian Mutagenicity Study Group (MMS). Environ. Mol. Mutagen. 37, 93–110.

Hayashi, M., Sofuni, T., Ishidate Jr, M., 1983. An application of acridine orange fluorescent staining to the micronucleus test. Mutat. Res. 120, 241–247.

Hayashi, M., Ueda, T., Uyeno, K., Wada, K., Kinae, N., Saotome, K., Tanaka, N., Takai, A., Sasaki, Y.F., Asano, N., Sofuni, T., Ojima, Y., 1998. Development of genotoxicity assay systems that use aquatic organisms. Mutat. Res. 399, 125–133.

Heddle, J.A., Salamone, M.F., 1981. The micronucleus assay, 1, in vivo. In: Stich, H.F., San, R.H.C. (Eds.), Short Term Tests for Chemical Carcinogens. Springer Verlag, Berlin, pp. 243–249.

Kanji, G.K., 2006. 100 Statistical Tests third ed. SAGE Publications.

Kligerman, A.D., 1982. Fishes as a biological detector of the effects of genotoxic agents. In: Heddle, J.A. (Ed.), Mutagenicity: New Horizons in Genetic Toxicology. Academic Press, New York, pp. 435–453.

Kuivila, K.M., Foe, C.G., 1995. Concentrations, transport and biological effects of dormant spray pesticides in the San Francisco estuary, California. Environ. Toxicol. Chem. 14, 1141–1150.

Kurelec, B., 1993. The genotoxic disease syndrome. Mar. Environ. Res. 35, 341–348.

Lemos, C.T., Milan Rodel, P., Terra, N.R., D'Avila de Oliveira, N.C., Erdtmann, B., 2007. River water genotoxicity evaluation using micronucleus assay in fish erythrocytes. Ecotoxicol. Environ. Saf. 66, 391–401.

Marques de Miranda Cabral Gontijo, Á., Barreto, R.E., Speit, G., Valenzuela Reyes, V.A., Luiz Volpato, G., Favero Salvadori, D.M., 2003. Anesthesia of fish with benzocaine does not interfere with comet assay results. Mutat. Res. 534, 165–172.

Nersesyan, A., Kundi, M., Kambis, A., Schulte-Hermann, R., Knasmuller, S., 2006. Effect of staining procedure on the results of micronucleus assay with exfoliated oral mucosa cells. Cancer Epidemiol. Biomarkers Prev. 15, 1835–1840.

Nishikawa, T., Haresaku, M., Fukushima, A., Nakamura, T., Adachi, K., Masuda, M., Hayashi, M., 2002. Further evaluation of an in vivo micronucleus test on rat and mouse skin: results with five skin carcinogens. Mutat. Res. 513, 93–102.

Pacheco, M., Santos, M.A., 1997. Induction of EROD activity and genotoxic effects by polycyclic aromatic hydrocarbons and resin acids on the juvenile eel (Anguilla anguilla L.). Ecotoxicol. Environ. Saf. 38, 252–259.

Richards, R.P., Baker, D.B., 1993. Pesticide concentration patterns in agricultural drainage networks in the Lake Erie Basin. Environ. Toxicol. Chem. 12, 13–16.

Rodriguez-Cea, A., Ayllon, F., Garcia-Vazquez, E., 2003. Micronucleus test in freshwater fish species: an evaluation of its sensitivity for application in field surveys. Ecotoxicol. Environ. Saf. 56, 442–448.

Schulz, R., 2001. Rainfall-induced sediment and pesticide input from orchards into the lourens river, western cape, south Africa: importance of a single event. Water Res. 35, 1869–1876.

Strunjak-Perovic, I., Coz-Rakovac, R., Topic Popovic, N., Jadan, M., 2009. Seasonality of nuclear abnormalities in gilthead sea bream Sparus aurata (L.) erythrocytes. Fish Physiol. Biochem. 35, 287–291.

Taghavi, L., Probst, J.-L., Merlina, G., Marchand, A.-L., Durbe, G., Probst, A., 2010. Flood event impact on pesticide transfer in a small agricultural catchment (Montousse at Aurade, south west France). Int. J. Environ. Anal. Chem. 90, 390–405.

Talapatra, S.N., Banerjee, S.K., 2007. Detection of micronucleus and abnormal nucleus in erythrocytes from the gill and kidney of Labeo bata cultivated in sewage-fed fish farms. Food Chem. Toxicol. 45, 210–215.

Tinwell, H., Ashby, J., 1989. Comparison of acridine orange and Giemsa stains in several mouse bone narrow micronucleus assays-including a triple dose study. Mutagenesis 4, 476–481.

Tiveron, C., Russo, A., Bassani, B., Pacchierotti, F., 1996. Genotoxicity of trophosphamide in mouse germ cells: assessment of micronuclei in spermatids and chromosome aberrations in one-cell zygotes. Mutagenesis 11, 125–130.

Udroiu, I., 2006. The micronucleus test in piscine erythrocytes. Aquat. Toxicol. 79, 201–204.

Ueda, T., Hayashi, M., Ohtsuka, Y., Nakamura, T., Kobayashi, J., Sofuni, T., 1992. A preliminary study of the micronucleus test by acridine orange fluorescent staining compared with chromosomal aberration test using fish erythropoietic and embryonic cells. Water Sci. Technol. 25, 235–240.

Ugarte, M.D., 2008. Probability Statistics with R. Chapman & Hall.

Whitehead, A., Kuivila, K.M., Orlando, J.L., Kotelevtsev, S., Anderson, S.L., 2004. Genotoxicity in native fish associated with agricultural runoff events. Environ. Toxicol. Chem. 13, 2868–2877.

Winter, M.J., Ellis, L.C.J., Hutchinson, T.H., 2007. Formation of micronuclei in erythrocytes of the fathead minnow (Pimephales promelas) after acute treatment with mitomycin C or cyclophosphamide. Mutat. Res. 629, 89–99.