

ASSESSMENT OF DNA DAMAGE IN BLOOD CELLS OF *Sparus aurata* L. EXPOSED TO BENZO[A]PYRENE USING THREE DISTINCT GENOTOXICITY ASSAYS

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SUMMARY

We analysed the relationships between different categories of genotoxicity biomarkers using *Sparus aurata* as model organism and benzo[a]pyrene (B[a]P) as a well-described genotoxic agent. DNA damage was assessed by means of agarose gel electrophoresis (AGE), micronuclei (MN) and flow cytometry (FC) assays. MN assay was carried out in mature erythrocytes, while the other assays were performed with all blood cell types. Both the AGE and MN assays detected significant DNA damaging effects of B[a]P, whereas almost no DNA damage was observed by FC assay. Yet, AGE and MN assays differed in the patterns of response observed. The cycles of DNA damage and repair detected in the AGE assay were not apparent in MN assay, where the genotoxic insult was consistently detected overtime.

KEYWORDS:

Genotoxicity; biomarkers; benzo[a]pyrene; *Sparus aurata*

INTRODUCTION

In recent years, one of the most serious concerns with pollution is the presence of genotoxic agents in the environment. This apprehension arises from increasing awareness that many pollutants, present in the aquatic environment, are chemical carcinogens and mutagens with the capacity to interact with DNA structure and function [1-2]. Thus, considering the risks associated with DNA damage, there is a clear need for the development and implementation of biomarkers of genotoxicity in environmental studies.

The polycyclic aromatic hydrocarbon benzo[a]pyrene (B[a]P) is widely recognized as a clastogen and potent animal carcinogen [3-4], and consequently represents one appropriate reference genotoxicant to test the sensitivity and feasibility of a set of genotoxicity biomarkers. B[a]P

has been employed as a reference toxicant to develop genotoxicity biomarkers in aquatic organisms, which address structural DNA damage and chromosomal damage [5-8]. However, there is little published work evaluating, side-by-side in the same organism and tissue, the relationship of both categories of genotoxicity responses (i.e. DNA damage and chromosomal damage biomarkers). Their integration may contribute to a better understanding of the responses of aquatic organisms to genotoxicants and improve the ability to establish more rigorously a cause-effect relationship.

Here we describe a short-term experiment, carried out under laboratory-controlled conditions, to calibrate a set of genotoxicity biomarkers and test their potential effectiveness using a well-described genotoxic agent, B[a]P, in the gilthead seabream, *Sparus aurata*. The primary aim of this study was to examine the relationships between different categories of genotoxicity biomarkers, by comparing structural DNA damage biomarkers (DNA strand breakage) with chromosomal damage biomarkers (micronuclei, nuclear abnormalities and nuclear DNA content variation) in our model organism *S. aurata*.

S. aurata is an economically valuable species that inhabits Atlantic and Mediterranean coastal waters, estuaries, and lagoons. It is intensively and extensively cultured in marine and estuarine waters, and it can be found in both pristine and contaminated sites. Furthermore, it is easy to maintain in laboratory conditions, and it is a suitable test organism. For these reasons, *S. aurata* was selected for use in this study.

MATERIALS AND METHODS

Test organisms and laboratory exposure to B[a]P

The experiment was carried out with juvenile *S. aurata*, weighing 3-4 g, from the “Ria Mãe” fish farm (Setúbal, Portugal). Two-hundred and fifty animals were acclimated

to controlled laboratory conditions, in 60-L tanks with recirculating filtered seawater, for 2 weeks before the experiment began.

Fish were exposed to five treatments: seawater control, solvent control (acetone) and three levels of sub-lethal B[a]P concentrations (25, 50 and 100 $\mu\text{g L}^{-1}$), during 48, 96 and 240 hours. These sub-lethal concentrations were selected according to preliminary acute toxicity tests. Since B[a]P has low solubility in seawater, it was firstly dissolved in a small amount of acetone, and then added to the seawater of the B[a]P treatments to achieve the final B[a]P nominal concentrations. In each case, the final acetone concentration was 0.008% (v/v). The natural seawater, obtained from a clean site of Tagus estuary, was filtered by a 0.45- μm sieve. The experiment was performed in 40-L glass tanks with 15 animals per aquarium. The total number of tanks was 15, one for each treatment/sampling period. Salinity and temperature were maintained at 33-34‰ and 20 °C, and aeration was provided continuously. All of the test media in each aquarium was replaced every two days and animals were fed with commercial fish food, immediately before the exposure media was changed. Survival was high in all treatments tested, only three animals died in the 50 $\mu\text{g L}^{-1}$ B[a]P concentration at the 240-h sampling period. All surviving individuals from each treatment were sampled after 48, 96 and 240 hours. Blood was collected by cutting the caudal peduncle, and stored in 1.5 ml micro-tubes with 500 μl of 25 mM EDTA for DNA strand breakage analysis, or with 800 μl of freezing buffer (250 ml $\text{H}_2\text{O}/$

21.2 g sucrose, 2.9 g citric acid and 12.5 ml dimethyl sulfoxide; pH 7.6) for flow cytometry assay. Blood samples were immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Blood smears for each individual were also prepared for the micronuclei assay.

DNA strand breakage analysis (Agarose Gel Electrophoresis assay – AGE)

DNA was isolated from all blood cells of each individual for subsequent DNA strand breakage analysis by AGE. An outline of the DNA strand breakage analysis is presented below, while detailed descriptions are provided in [9]. AGE comprised electrophoresis of the DNA extracts under alkaline (pH 12) and neutral (pH 8) conditions, thus allowing for determination of total (single and double) and double-stranded breaks in the DNA, respectively. Migration of the DNA within the gel matrix is size-dependent, and detection is easily accomplished after staining with ethidium bromide (Fig. 1). Photographs of ethidium-bromide-stained gels were analysed with the software QWin V2.3 (Leica Microsystems) in order to obtain densitometric profiles of the migration of each DNA sample. Finally the average molecular length (L_n) was computed from these data. The average molecular length is inversely proportional to the number of DNA strand breaks according to the formula:

$$(1) \text{ Number of strand breaks}/10^5 \text{ nucleotides} = 1/L_n \times 100$$

In order to normalize results among gels, it was required to determine the relative number of total (RNTSB)



FIGURE 1 - Photographs of an agarose gel run under alkaline conditions to determine total DNA strand-breakage of *Sparus aurata* exposed to benzo[a]pyrene. Lanes 1 and 32 contain molecular size standard (Hind III digests of λ DNA); lanes 2-5, 16-18 and 26 seawater control groups for 48, 96 and 240 h, respectively; lanes 6-7, 20 and 30 solvent control groups for 48, 96 and 240 h, respectively; lanes 8-10, 21 and 25 exposure to 25 $\mu\text{g L}^{-1}$ B[a]P for 48, 96 and 240 h, respectively; lanes 11-12, 21 and 31 exposure to 50 $\mu\text{g L}^{-1}$ B[a]P for 48, 96 and 240 h, respectively; lanes 13-15, 22-24 and 29 exposure to 100 $\mu\text{g L}^{-1}$ B[a]P for 48, 96 and 240 h, respectively; lane 33 and 34 molecular size standards (Coliphage T4 DNA and 100 bp ladder, respectively).

and double-strand breaks (RNDSB). This was accomplished by calculating the difference in the number of strand breaks between every treatment sample and the respective solvent control mean within each gel:

(2) Samples from total-strand break gel:

$$\text{RNTSB} = 1/L_n (s_{ij}) - 1/L_n (Cm_j)$$

(3) Samples from double-strand break gel:

$$\text{RNDSB} = 1/L_n (s_{ij}) - 1/L_n (Cm_j)$$

where s_{ij} is the sample i from gel j and Cm is the respective solvent control mean from gel j

Accordingly, the relative number of single strand breaks (RNSSB) was determined as follows:

(4) $\text{RNSSB}/10^5 \text{ nucleotides} = \text{RNTSB}_i - (2 \times \text{RNDSB}_i)$
where i is the sample number.

Micronuclei and nuclear abnormalities (Micronuclei assay – MN)

The MN was used to determine the number of erythrocytic micronuclei (EMN) and erythrocytic nuclear abnormalities (ENA) in *S. aurata* mature erythrocytes as previous described in [10-11]. Blood smears from individuals of each treatment were fixed in absolute methanol, stained with Giemsa/phosphate buffer pH 6.8 (1:20) and mounted with Eukitt. Five slides of each treatment were randomly selected to score 1000 mature erythrocytes under 1000x magnification, and to determine the number of EMN and ENA. A single observer analyzed the randomly sorted slides. The frequencies of EMN and ENA were expressed per 1000 cells (%). The criteria for the identification of EMN and ENA followed the procedure described by [11, 12]. The ENA, classified as lobed nuclei, kidney shaped nuclei and segmented nuclei, were scored if their nuclear shape was similar to those observed in Fig. 2. ENA were considered together and separately from MN for statistical analysis.

The micronuclei assay was carried out in erythrocytes, while the other assays concerned all blood cell types. How-

ever, since blood cells are predominantly erythrocytes [13], the influence of the remaining blood cells in AGE and flow cytometry assays is most likely negligible.

Nuclear DNA content variation (Flow Cytometry assay – FC)

The FC was used to measure the nuclear DNA content variation among all blood cells of each experimental individual as previous described in [11]. The blood cell suspensions stored at -80°C were thawed and a $100\ \mu\text{l}$ aliquot was stained in the dark, for at least 15 min, with $200\ \mu\text{l}$ of fluorescent dye (500 ml $\text{H}_2\text{O}/500\ \text{mg}$ citric acid, $500\ \mu\text{l}$ Nonidet P40, 25mg propidium iodide and 25 mg RNases A, pH 7.6). The quantification of nuclear fluorescence was carried out in a flow cytometer (Coulter Epics Profile II). Fluorescence was measured from 10,000 blood cells per individual and the nuclear DNA content variation was calculated by the coefficient of variation (CV) of the mean G1 stage cells. Chicken red blood cells and fluorescent microspheres were used as standards to control the instrument alignment. Given the large sample size used in this study, it was not possible to analyze all of them at once. At each time, a set of five samples from each treatment, including controls, and from two sampling periods was measured in duplicate. Thus, to normalize the results and reduce the variability between different day runs of the flow cytometer, the CV values will be expressed as the percentage of solvent control mean of each day run: $[\text{CV}(S_{ij}) * 100] / \text{CV}(Cm_j)$ (S_{ij} is the sample i from the set j , and Cm_j is the respective solvent control mean from set j).

Statistic analyses

Statistical analyses were performed using a two-way ANOVA with sampling periods and B[a]P concentrations as factors. Post-hoc comparisons were carried out using the Least Significant Difference test (LSD) for multiple comparisons between pairs of means. The lack of normality distribution in EMN data required the use of the non-parametric tests - Kruskal-Wallis and a-posteriori Man-Whitney tests. In all tests, significant differences were established at $p < 0.05$.

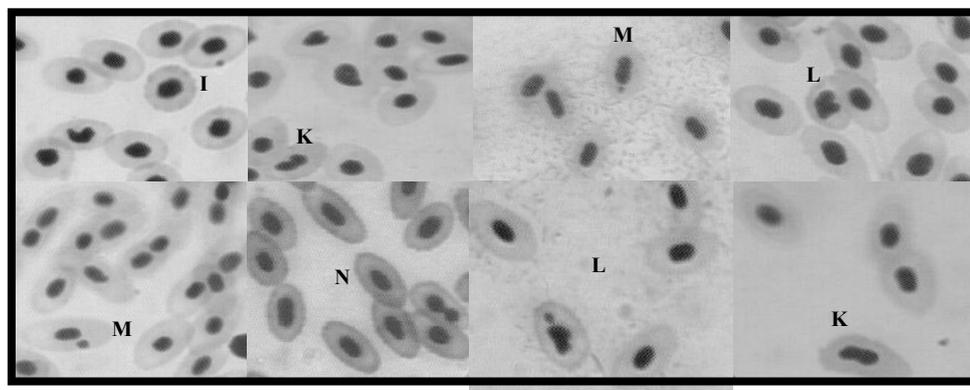


FIGURE 2 - Erythrocytes of *Sparus aurata* with normal nuclear shape and nuclear abnormalities: N = normal shaped nucleus, M = micronucleus, L = lobed nucleus, K = kidney-shaped nucleus and S = segmented nucleus. The immature erythrocytes (I) were not scored. Giemsa stain, 1000 x.

RESULTS AND DISCUSSION

Relative number of total (RNTSB), double (RNDSB) and single (RNSSB) strand breaks, as a function of B[a]P doses and exposure periods, are shown in Fig. 3. No significant differences in DNA strand breakage parameters were observed over the 240-h exposure between control (seawater control) and solvent control groups. B[a]P was able to induce significant effects on RNTSB ($p < 0.05$, Fig. 3a) and on RNSSB ($p < 0.05$, Fig. 3c), but no effects were observed on RNDSB ($p > 0.05$, Fig. 3b) over all exposure time. The highest B[a]P concentration ($100 \mu\text{g L}^{-1}$) showed values significantly different from solvent control after 48, and 240 h of exposure, both for RNTSB and RNSSB ($p < 0.05$).

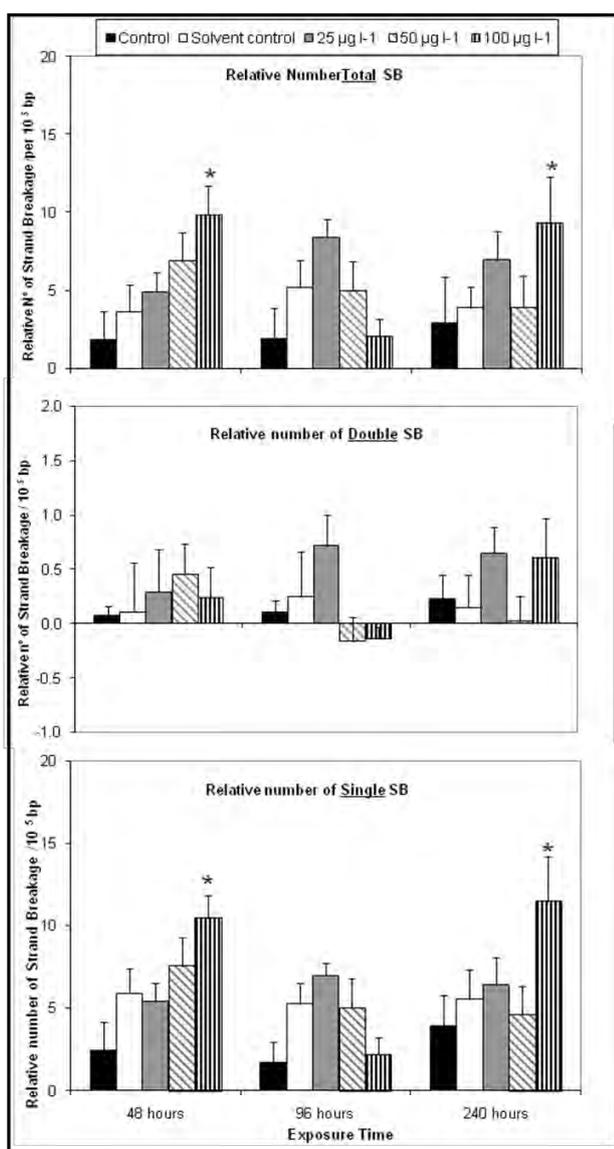


FIGURE 3 - DNA strand breakage in blood cells of *Sparus aurata* exposed to benzo[a]pyrene: relative number of (a) total, (b) double and (c) single strand breaks as a function of B[a]P dose and exposure period. Bars and error bars represent means and standard errors. Asterisks indicate significant effects relative to solvent control: * = $p < 0.05$. Control represents seawater control and solvent control is acetone control

It is well-established that DNA strand breaks is an early biological result of exposure to genotoxicants that may or may not persist in time [14]. The persistence of DNA strand breakage depends, among other factors, on the organism ability to repair its damaged DNA, and on the extent and dose of exposure to the genotoxicant [14, 15]. In addition, it has been observed that genotoxicant concentrations in tissues have to reach a threshold value before the repair systems become activated [16]. Ultimately, continuous exposure to genotoxicants can overwhelm the repair mechanisms of the cells, resulting in a marked increase of DNA damage [6]. On this basis, the results obtained in AGE assay (RNTSB and RNSSB) for the highest BaP concentration ($100 \mu\text{g l}^{-1}$) suggest the occurrence of a 3-stage biological response, which could be explained by the alternation of the prevalence of DNA damage versus DNA repair mechanisms: 1) Acute phase - significant genotoxic insult detected after 48 h of exposure, 2) Compensatory phase - activation of DNA repair mechanisms and effective compensation reducing the DNA damage to control levels after 96 h of exposure, and 3) Chronic phase - continuation of exposure ultimately led to saturating or impairing DNA repair mechanisms, resulting in the prevalence of DNA damage again at 240 h.

Similar patterns of alternation of DNA damage and repair over the exposure period have been observed in various animals, such as fish and crustaceans exposed to various genotoxicants, including B[a]P [5-7, 9, 17]. For example, in one of these studies, it was reported a fluctuating response of DNA integrity in the digestive gland of *Perna viridis* measured by the alkaline unwinding method [6]. In that study, it was observed that the levels of DNA strand breaks increased significantly after one day of exposure at 0.3 and $3 \mu\text{g L}^{-1}$ B[a]P, then the DNA damage gradually decreased back to the control levels at day 12, and, finally, a second increase of DNA strand breaks was detected from day 12 to 24. These authors suggested an activation of DNA repair mechanisms at day 12, and an overwhelmed one of DNA repair systems after day 12. In another study, that measured the DNA integrity by comet assay, an identical phenomenon of DNA damage/repair was reported in blood cells of black porgy exposed to $2 \mu\text{g L}^{-1}$ B[a]P [17]. The levels of DNA strand breaks increased significantly after 2-h exposure, decreased at 5 h and increased again at 24 h. Rixian et al. [17] suggested that the recovery of DNA damage observed at 5-h exposure may be explained by the inbuilt processes of DNA repair of living cells. It is important to point out that the DNA damage effects reported in such studies were detected at lower B[a]P concentrations than those used in the present investigation. These differences suggest that DNA strand breakage is a tissue/species dependent response as was already reported by Monteith and Vanstone [18].

No significant induction of DNA double-strand breaks (DSB) was detected. DSB are more difficult to generate than SSB, since they occur in two adjacent DNA strands in close proximity [1, 14]. A possible reason for the absence of effects on DSB is the insufficient dose of B[a]P.

Frequencies of EMN and ENA were influenced by exposure to B[a]P as shown in Fig. 4. No significant differences were detected over all exposure times between control and solvent control groups. Temporal variation in both EMN and ENA were similar. EMN and ENA maximum induction in each exposure period was always observed for the highest B[a]P concentration (100 $\mu\text{g L}^{-1}$). A significant increase of EMN and ENA was observed at 96 hours ($p < 0.05$ and $p < 0.01$, respectively), or at 240 hours of exposure ($p < 0.05$), when compared with solvent control. Furthermore, a significant EMN increase was detected after the 48-h exposure ($p < 0.05$), which was followed by an ENA frequency increase, but this increase was not statistically significant. These results confirmed the genotoxic potential of B[a]P already observed by the total and single strand breaks formation. Other studies also detected significant higher frequencies of EMN and ENA in fish erythrocytes exposed to B[a]P [7-8, 10, 19].

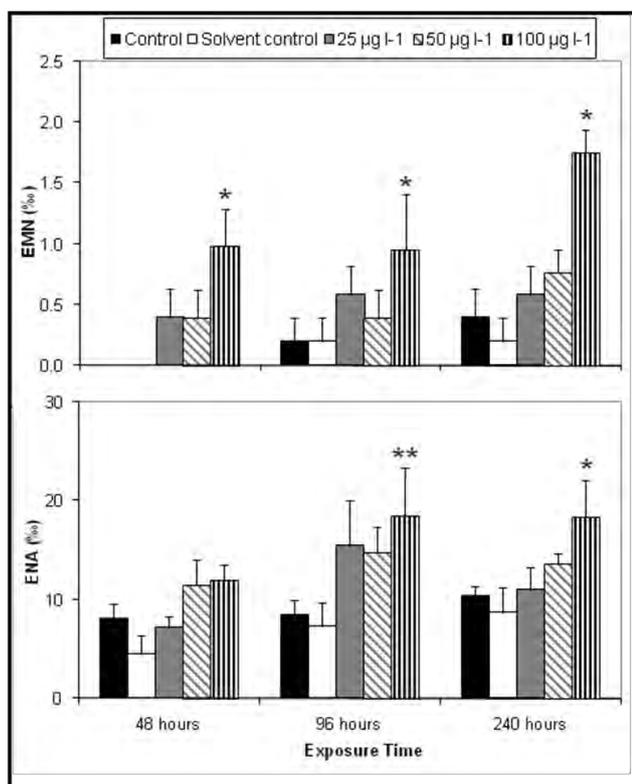


FIGURE 4 (a) - Erythrocytic micronuclei (EMN) and (b) Erythrocytic nuclear abnormality (ENA) frequencies in *Sparus aurata* as a function of B[a]P dose and exposure period. Bars and error bars represent means and standard errors. Asterisks indicate significant effects relative to solvent control: * = $p < 0.05$ and ** = $p < 0.01$. Control represents seawater control and solvent control is acetone control.

The persistence of DNA strand breaks, as a result of faulty DNA damage repair, can give rise to lesions in somatic cells. During cell division, DNA damage may become permanent and passed on to daughter cells, biomagnifying the initial structural DNA damage and given way to more insidious chromosomal damage. This type of damage can

then be detected as MN, or by way of higher variation of nuclear DNA content in a target tissue (FC assay). This may explain why, compared to AGE assay, the biological response detected by the MN assay shows a more steady and cumulative type of overtime variation in DNA damage. Comparison of the results, obtained at 96 hours, clearly shows the difference in the type of response detected by each of the assays. With the MN assay, as opposed to AGE assay, no cycles of DNA damage and repair were apparent, but the presence of genotoxic insult was consistently detected overtime.

Over the last decade, the occurrence of nuclear abnormalities has been reported in addition to micronuclei in several aquatic organisms, such as bivalves and fish, after exposure to various genotoxicants, including B[a]P. A number of authors [8, 20-21] have described and-photographed various alterations of the nuclear morphology and interpreted them as nuclear lesions analogous to micronuclei. These authors suggested that these nuclear alterations should be enumerated as an indicator of clastogenic activity. However, the responsible mechanism for these nuclear abnormalities is not yet fully understood [21-22]. In the current study, we observed three different types of nuclear abnormalities, according to those described by the above authors, lobed nuclei, segmented nuclei and kidney-shaped nuclei. The frequency of these nuclear abnormalities followed a similar pattern of micronuclei; therefore, our results also indicate that nuclear abnormalities are analogous to micronuclei. However, more research is needed to understand the origin of these abnormalities and for standardizing the use of this biomarker, before including it in routine genotoxicity tests.

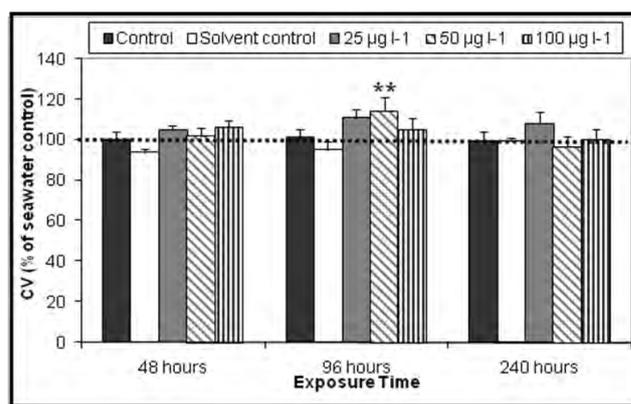


FIGURE 5 - Nuclear DNA content variation (CV) in blood cells of *Sparus aurata* as a function of benzo[a]pyrene dose and exposure period. All results are expressed as a percentage of control CVs. Bars and error bars represent means and standard errors. Asterisk indicates significant effects relative to solvent control: ** = $p < 0.01$. Control represents seawater control and solvent control is acetone control.

The results of the flow cytometry analysis presented in Fig. 5 point to a similar response of control and exposed individuals over all exposure times, except 96 h, where CV values from 50 $\mu\text{g L}^{-1}$ B[a]P concentration were 19% higher

than the solvent control ($p < 0.01$). No significant differences were detected over all exposure times between control and solvent control groups. In contrast to the remaining biomarkers, there was a lack of response of the FC assay to detect B[a]P-induced genotoxicity. Although it would be anticipated that the response of the FC assay might differ from AGE assay, a general conformity with the MN results would be expected. In fact, in a companion study [11], both MN and FC assays showed a fine conformity in detection of DNA damage in blood cells of juvenile *S. aurata* exposed to endosulfan for 96 hours. Flow cytometry is recognized as very efficient and fast method [23-24], and it is an established assay for genotoxicity detection [23, 25]. Nevertheless, the results are also sensitive to overtime variation in the operation of the flow cytometer (see [26], where this issue is discussed). Ideally, all samples should be read in a single run of the equipment, or alternatively the number of runs should be kept to a minimum. Given the high number of samples analyzed in the current study, several runs were required. This may have resulted in high enough inter-run variation that masked the effects of exposure to B[a]P.

On the whole, our results of genotoxicity biomarkers indicate that the information produced by each assay does not overlap. The AGE assay is especially appropriate to address the ethiology of DNA damage and repair mechanisms, or to obtain qualitative information on the type of genotoxic insult (single versus double strand breaks). The MN and, eventually, FC assay provide a consistent means of detection of DNA damage, integrating the genotoxic insult overtime.

CONCLUSIONS

The comprehensive approach developed in this study illustrated the usefulness of comparing side-by-side, in the same organism and tissue, the information given by different genotoxicity biomarkers. This approach also highlights the utility of integrating different genotoxicity biomarkers to gain insights into the dynamics of genotoxins in fish. Application of these biomarkers in biomonitoring studies is advisable, namely as components of weight of evidence approaches. This should be preceded by additional investigations, either to optimize the FC assay, or for better understanding the relationships of all the tested biomarkers.

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