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Bisphenols A and its analogues induce genotoxic damage in marine and freshwater amphipods

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ABSTRACT

Among plastic additives, bisphenol A (BPA), used mainly in manufacturing objects of everyday use, has received considerable attention for acting as a xenoestrogen and causing endocrine disruption. We now observe its rapid replacement with analogue compounds with similar structures (BPs) whose safety is not fully demonstrated. The present paper investigates the possible adverse effects of BPA, BPF, and BPS on DNA integrity of two gammarid species, *Echinogammarus veneris* and *Gammarus aequicauda,* to assess the environmental risk posed by these compounds in the aquatic ecosystem. Moreover, we analysed the cell-specific sensitivity to BPs using somatic (hemocytes) and germ (spermatozoa) cells that could have wide-ranging implications for their reproductive biology and long-term adverse effects at the population level. Results demonstrate that BPA and its analogues caused a genotoxic impact and highlighted lower genotoxic damage induced in sperm cells in both species tested. The implications of the findings of this study on the aquatic ecosystem are discussed, taking into account the critical role of amphipods within the freshwater trophic chain.

Introduction

Plastic is one of the most versatile artificial materials, and due to the social benefits produced, it has shaped our lives. Versatility, durability, and lightness are properties that make plastic an essential material for several industrial applications while at the same time constituting the main threat to the environment when dispersed. Plastics are made by polymerising monomers and other chemical compounds that are used as additives to provide required properties or incorporated to facilitate manufacturing. Among these additives, bisphenol A (BPA) is mainly used to manufacture polycarbonate and epoxy resins (Huang et al., 2012). It is an industrially important chemical because of the compounds used in plastics manufacturing, it has the highest production volumes, surpassing 8 million tons worldwide (Abraham and Chakraborty, 2019). BPA has a wide range of use, such as food packaging, bottles, straws, thermal receipt paper, toys, CDs, and medical devices (European Commission, 2018). Unfortunately, it is not stable and can leach out from these products in a process accelerated by UV light, ageing, heating, and contact with acidic or basic compounds (Frenzilli et al., 2021), causing human and environmental exposure. Because of its extensive use and production, it is estimated that over 100,000 tons of this compound are released annually into the environment (Oehlmann et al., 2009; Silveira et al., 2019). BPA has received considerable attention for being a toxic contaminant that acts as a synthetic xenoestrogen that causes endocrine disruption, cytotoxicity, genotoxicity, and reproductive toxicity (Ghosh et al., 2017). Due to the adverse biological effects exhibited, many countries, like the EU, United States, Canada, and China, have restricted or banned its use (Liu et al., 2021). These restrictions have led to its replacement with rapidly developed analogues (BPs), which are similarly structured compounds that share chemical and physical properties with BPA (Ullah et al., 2019) and have similar effects. Among them, bisphenol F (BPF) and bisphenol S (BPS) are the main substitutes for BPA used in the manufacturing of epoxy resins and polycarbonate plastics. They have the highest detection frequency in surface waters, frequently detected as the second and third-most abundant analogues in the environment, with detected concentrations even more elevated than BPA (Liu et al., 2021).

In-vitro studies have demonstrated that BPF and BPS have estrogenic and antiandrogenic effects similar to BPA, playing important roles in oxidative stress induction (Eladak et al., 2015; Nourian et al., 2017; Ullah et al., 2018) and having disturbing connections with several pathologies, including cardiovascular diseases and cancer (Yin et al.,

Abbreviations: BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S; BPs, bisphenols.

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2019).

Due to the increasing use of these alternatives in plastic products labelled BPA-free, their environmental distribution is also increasing along with the likelihood of human exposure, both dietary and non-dietary (Vrachnis et al., 2021). BPs have been detected in waters and sediments and bioaccumulate in the body of several animal species (Wang et al., 2021). The concentration of BP detected in the aquatic environment often exceeds the predicted no-effect concentration (PNEC) recommended by the European Union (1500 ng/l), reaching the highest values in industrial areas in Korea, China, Japan, and India, where concentrations up to 7200 ng/l have been detected (Morales et al., 2020). Still, even higher concentration peaks could be reached after runoff or spills.

To assess whether the move to alternatives is effective in improving health risks, we urgently need to improve our knowledge about their possible adverse effects on the physiological functions of living organisms and evaluate their toxicological properties.

Genotoxicity is considered one of the most critical toxic endpoints in ecotoxicological studies and environmental risk assessment. However, few studies have assessed the genotoxic endpoints in aquatic invertebrates exposed to BPs (Martinez-Paz et al., 2013). despite the levels of these compounds discharging into water by means of dispersed plastic particles and treatment plant effluents. The genotoxicity of BPA has been studied mainly in vertebrates, revealing its genotoxic and mutagenic potential to damage DNA through molecular breakdown (Silveira et al., 2019), chromosomal aberrations, aneuploidy, and micronuclei formation (Anet et al., 2019).

The damage to DNA caused by a xenobiotic compound may vary in different cell types in the same organism, depending on the method of exposure and defence and repair mechanisms. For this reason, in this study, we assess the cell-specific sensitivity of two gammarid species, *Echinogammarus veneris* (Heller, 1865) and *Gammarus aequicauda* (Martynov, 1931), to BPs using somatic (hemocytes) and male germ (spermatozoa) cells for an *in-vivo* Comet assay. Hemocytes are freely circulating cells that play a fundamental role in the immune system in invertebrates. Exposure to pollutants may inhibit their activity, and the effects produced can be assessed by the functional parameters of these cells (Perez and Fontanetti, 2011). On the other hand, the exposure of spermatozoa to contaminants can induce alterations of great ecological relevance because of their involvement in reproductive success.

Among invertebrates, amphipods are known for their sensitivity to toxic substances. Gammarids are mainly used as bioindicators in aquatic toxicity tests due to their abundance in nature and their sensitivity to anthropogenic toxic compounds in water (Davolos et al., 2015). According to their key role in marine and freshwater environments, the study focused on two species, *Gammarus aequicauda* and *Echinogammarus veneris*, respectively. Both species have a significant ecological role, representing a source of food for higher trophic levels and being important in the decomposition processes of macrophytes and in the natural regeneration of organic matter (Ronci et al., 2016; Kevrekidis et al., 2009; Prato et al., 2006; Prato e Biandolino, 2009).

Only a few studies have targeted gammarids for genotoxicity, and these were mainly conducted on freshwater species (Lacaze et al., 2010; Lacaze et al., 2011; Ronci et al., 2015; Davolos et al., 2015; Di Donato et al., 2016). *Echinogammarus veneris* has already been successfully used in previous genotoxicity studies(Marcoccia et al., 2017; Iannilli et al., 2019; Ronci et al., 2016), whereas to our knowledge, there are no genotoxicity studies that have been conducted on *G. aequicauda* even though this species has been used in several ecotoxicological investigations (Prato et al., 2013).

The present study aimed to investigate the genotoxic potential of BPF, BPS, and BPA using *G. aequicauda* and *E. veneris* as model organisms for marine and freshwater benthic invertebrates. Moreover, we analysed cell-specific sensitivity to DNA damage to improve the knowledge of genotoxic responses in amphipods. The consequences of this damage for the reproductive biology, survival, or reproduction of these species may significantly impact the food chains of their ecosystems (Prato et al., 2013; Ronci et al., 2015).

Materials and methods

Species studied, sampling and stock conditions

Gammarus aequicauda is one of the most abundant and widely distributed amphipods on the coasts, in estuaries, marine lakes, lagoons, and brackish waters of the Mediterranean and the Black Sea, living in locally abundant populations (Hupalo et al., 2019; Prato et al., 2006, 2021; Kevrekidis et al., 2009). *Echinogammarus veneris* is a common species in rivers and streams of the Peri-Mediterranean region (Pinkster, 1993), typical of both lotic and lentic environments, and well adapted to oligo-mesotrophic waters (Bazzanti et al., 2012).

Echinogammarus veneris specimens were collected from a known unpolluted spring, Fontana di Muro, in Pontinia, Latium (Italy), classified by the European Community as a Site of Community Importance (SCI), where the waters are commonly used for human consumption. *Gammarus aequicauda* specimens were collected from the saltworks of Tarquinia, a wetland of anthropogenic origin that has become an ecosystem of particular importance because it is similar to a natural retrodunal salt lagoon and classified as an SCI and a Natura 2000 Special Protection Area (SPA). Here, this species lives together with a syntopic species, *Gammarus insensibilis* Stock, 1966. For this reason, subsequent recognition in the laboratory was necessary. In both sampling sites, individuals were collected within the aquatic vegetation using a hand net, preserved in a refrigerated box, and quickly brought to the laboratory. The samples were transferred to 10 l aerated glass aquarium tanks filled with waters taken from the sampling sites and maintained under controlled conditions in a thermal cabinet: temperature15 °C, photoperiod 12/12 light/dark. The animals were fed ad libitum with dry commercial fish food (MacNeil and Platvoet, 2005).

Exposure conditions

After a laboratory acclimation period of ten days, both *E. veneris* and *G. aequicauda* specimens were exposed to three different concentrations of each bisphenol: 4,4′-methanediyldiphenol (BPA), 4,4′-dihydroxydiphenylmethane (BPF) and 4,4′-Sulfonyldiphenol (BPS). The stock solution of each bisphenol was prepared at the concentration of 100 mg/ml by dissolution in dimethyl sulfoxide (DMSO). The highest concentration of DMSO in the exposed solution is 0,027 µl/ml, the DMSO ratio is consistent in each concentration exposure group. The probability of a genotoxic effect caused by DMSO was excluded by a preliminary test conducted at the final concentration reached in the exposure conditions.

Test solutions were obtained from the stock solution through serial dilutions, using dechlorinated tap water for *E. veneris* and marine water from the sampling site for *G. aequicauda*. The amphipods were transferred into glass beakers containing 200 ml of the test solution for the exposure tests. The concentrations selected to assess the genotoxic effects were: 0.25 mg/l, 0.5 mg/l and 1 mg/l of bisphenol. Small glass beads were also added to mimic the substrate in each beaker. In the choice of concentrations, we followed the range selected by Tisler et al. (2016) for *Daphnia magna*, Morales et al. (2020) and Martinez-Paz (2013) for *Chironomus riparius*, Liu et al. (2021) for frog embryo, Ficociello et al. (2021) for *Coenorabditis elegans* and close to the non-effect concentration (NOEC) of bisphenol A on the amphipod *Hyalella azteca* (Jiang et al., 2020). Although the concentrations of BPs selected are times higher than the concentrations in the real freshwater system (Liu et al., 2021), BPs concentrations can be higher due to discharges coming from industry and hospitals or especially when landfill leachates are transported to the waters, where BPA may overcome 5 mg/l (Ferrer-Polonio et al., 2021)

Four adult specimens per species were added to each beaker and, another group of 4 specimens per species was only exposed to water as a

negative control. They were maintained into the thermal cabinet under the same conditions used for acclimatisation for a brief exposure (24 h) to highlight the direct DNA damage. All the experiments were replicated three times (independent experiments); a total of 144 specimens were analysed for each species and, for each compound, we analysed the response of 48 specimens per species.

Cell isolation and comet assay procedure

Comet assay, also known as single-cell gel electrophoresis (SCGE), is a test that allows measuring the DNA damage in individual eukaryotic cells. It is a rapid and sensitive tool that can be performed on different tissues and cell types, and it has been increasingly used in genotoxicity testing. We performed the alkaline version of the comet assay that enable the visualisation of DNA double and single-strand breaks (DNA DSBs and SSBs) but also alkali-labile sites (ALS) (Bivehed et al., 2020).

We followed the procedure described by Iannilli et al. (2019) with some modifications. After exposure, haemolymph cells were extracted from each specimen under a stereomicroscope using an insulin syringe (30G/0.30 mm needle) inserted between the cephalon and first mesosomite. To extract spermatozoa, we dissected spermatic glands (Fig. 1) located ventrally between males' third and seventh mesosomal segments. Hundreds of haemocytes and sperm cells per individual were extracted and stored in a separate tube. Each replicate consisted of a pool of four individuals. The cells were subsequently mixed with chilled PBS and kept on ice until the centrifugation at 7000 rpm for 15 min to obtain a cell pellet. Low melting agarose (0,8%) was added to the pellet at 38 °C, mixed and spotted on the precoated gelbonds (GelBond® Film, Lonza; two 40 µl spots for each exposure condition) and placed in the refrigerator to solidify for 10 min. We used entire gel bond sheets 85 × 100 mm coated with 1% normal melting agarose (NMA) to create eight spots according to the scheme in Fig. 2. After removing the coverslips, slides were placed in lysing solution at 4 °C in the dark (2.5 mol/l NaCl, 100 mmol/l Na2EDTA, 10 mmol/l Tris–HCl, 1% Triton X-100, and 10% DMSO, pH 10) for 90 min for haemocytes and overnight for sperm cells. Gelbonds were then gently placed in a horizontal electrophoresis chamber filled with freshly prepared chilled buffer (300 mmol/l NaOH, 1 mmol/L EDTA, pH 13); DNA was then allowed to unwind for 40 min. Electrophoresis was performed under 0.6 V/cm and 300 mA for 30 min. After the electrophoresis run, the gelbonds were washed three times in a neutralisation buffer (0.4 mol/l Tris–HCl, pH 7.5), then dehydrated in absolute cold ethanol and allowed to dry. Finally, the gel spots on gelbonds were cut, placed on glass slides, stained with Ethidium Bromide (20 µg/ml) and covered with a cover glass to be observed under an epifluorescence microscope (Leica DM750).

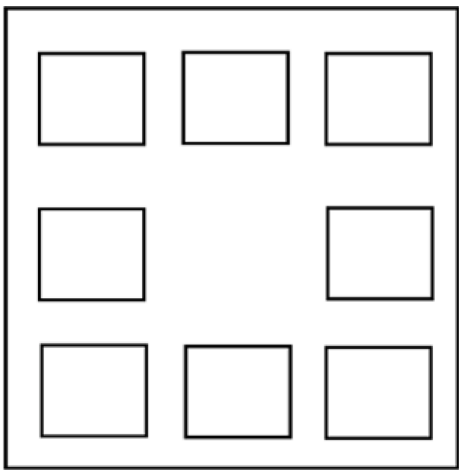

Fig. 2. Gel bond sheet 85 × 100 mm (GelBond® Film, Lonza) coated with 8 spots of 1% normal melting agarose (NMA).

Scoring and statistical analysis

The coded slides were blind scored. DNA damage was determined by quantifying the migration in 150 randomly selected nuclei each treatment, photographed at 40 × magnification by a Digital HD camera (Leica ICC50HD) and the software LAS V4.9. The images obtained were analysed by the software © 2017 TriTekCorp™ CometScore, version 2.0 measuring the Tail Moment (TM), defined as the product of the tail length and the fraction of total DNA in the tail. This widely used parameter reflects the size of migrating DNA and the number of broken DNA fragments (Roudkenar et al., 2008). Haemocytes were recognised by the rounded shape nuclei, between 15 and 20 µm, whereas spermatozoa are characterised by an unmistakable elongated shape (Fig. 3).

Results, expressed as TM values, were presented as means ± SE and analysed using the statistical analysis program PAST (version 4.06b.). Since the data were not normally distributed (Shapiro Wilk test), we used a nonparametric test (Kruskal-Wallis) to compare each treatment with the relative control group and considered it significant for $p < 0.05$. The occurrence of a dose-response relationship was tested using Pearson's linear correlation coefficient (r).

Results and discussions

Knowing the DNA damage baseline level is essential to understand

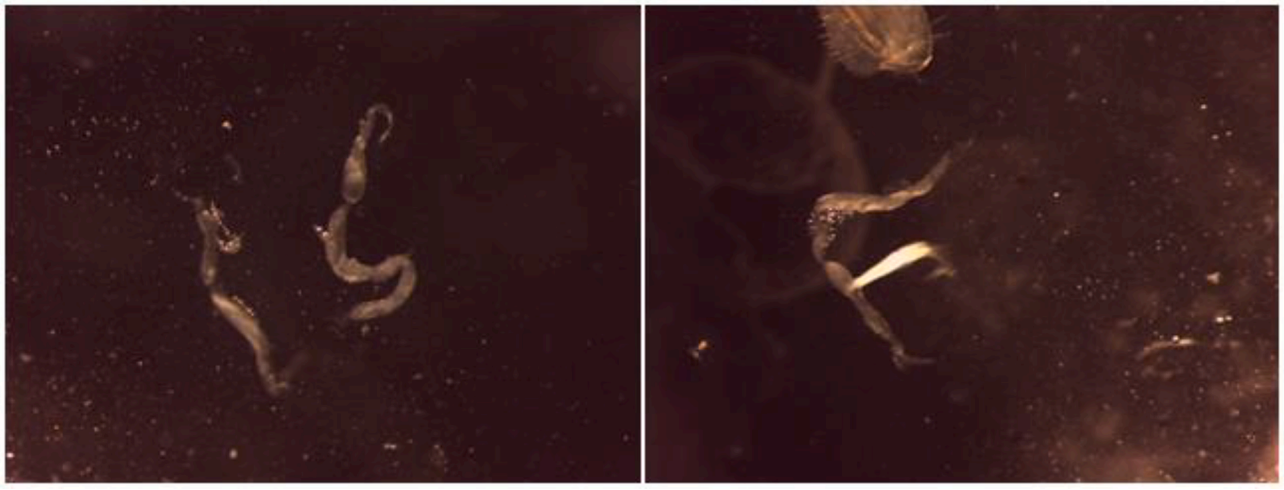

Fig. 1. Left, spermatic glands of an *Echinogammarus veneris* male specimen; right, detail of the spermatozoa as they emerge from the severed spermatic gland.

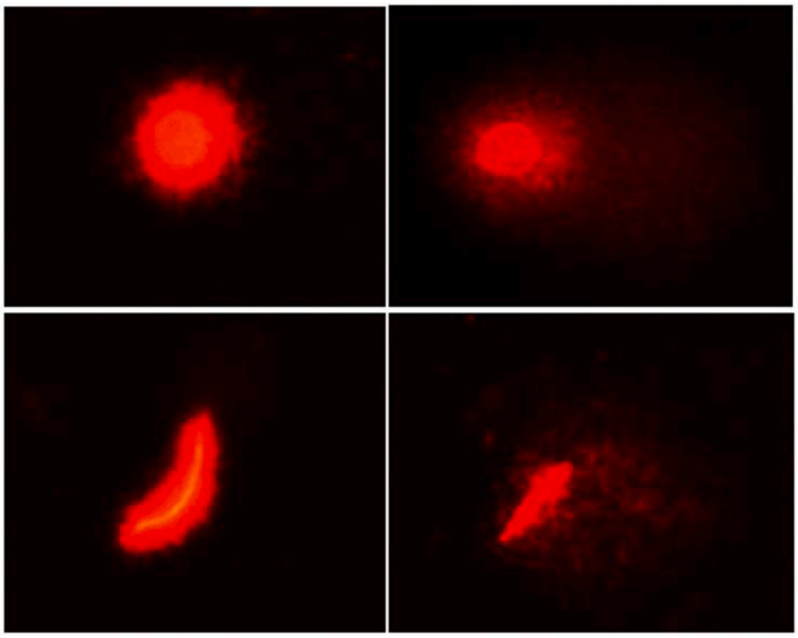

Fig. 3. Representative images of nuclei from hemolymp cells (a) and spermatozoa (b) untreated (1) and treated (2) exhibiting different DNA damage level, observed after Alkaline Comet assay procedure and EtBr staining.

the results obtained after exposure to a genotoxicant. In this regard, we analysed the data acquired in this study to evaluate the assay's discrimination power performed on both cell types. However, lab maintenance and cell collection methodology should ensure untreated samples have low biomarker variability. Both the species show a very similar basal level of DNA damage in both cell types (Fig. 4). In the haemocytes, DNA basal damage (untreated sample as negative control) varies from a TM mean value of 4.94 in *E. veneris* to 5.31 in *G. aequicauda*; in spermatozoa, it varies from 3.58 in *E. veneris* to 3.09 in *G. aequicauda*, showing no significant comparisons between species nor cell type. The DNA basal damage is low, indicating that the cell collection procedures applied were suitable and that the water used for negative controls did not affect the DNA's integrity.

The DNA-induced damage, resulting in DNA fragmentation, was quantified by the Comet assay after a short exposure (24 h) to three concentrations of each bisphenol (0.25 mg/l, 0.50 mg/l, and 1 mg/l) selected based on previous studies (Tišler et al., 2016, Liu et al., 2021). The half-life of BPA in water is between 3 and 5 days, long enough to affect the aquatic organisms, especially those living in proximity of point source outputs, which are at the most significant risk of harmful effects (Tatar and Türkmenoğlu, 2020; Crain et al., 2007). BPA (and consequently its analogues) is not expected to be persistent in the environment because it is rapidly degraded by microbial biodegradation and photodegradation (Staples et al., 1998; Crain et al., 2007). Nevertheless, due to its continuous-release, it is regularly detected in ecosystems (Oehlmann et al., 2009; Corrales et al., 2015).

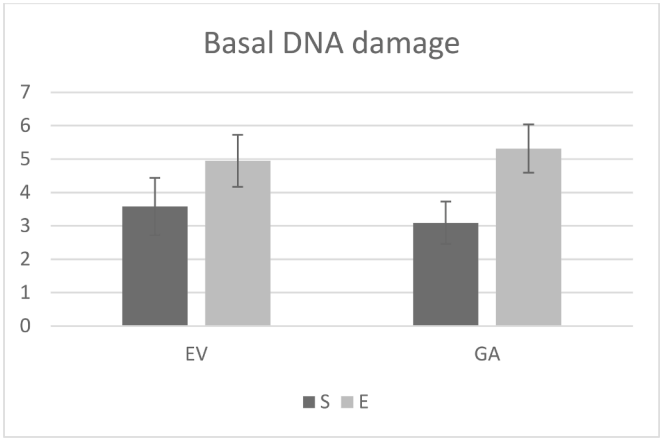

Fig. 4. Basal DNA damage in both sperm cells and hemocytes. Mean ± standard error of TM in *E. veneris* and *G. aequicauda* not exposed to toxicants. Data from 36 specimens each species.

Both species, *E. veneris* and *G. aequicauda*, showed genotoxic effects after exposure to bisphenol A, particularly for haemocytes (Fig. 5). Our data exhibited a dose-response relationship between BPA concentrations and the TM values as shown by *r* values in Tab. 1, in *E. veneris*'s haemocytes ($r_h = 0.97$). In *E. veneris*, TM trends increase with increasing concentration, and the highest causes the most significant DNA damage in both cell types (TM = 17.8 in haemocytes; TM = 10.9 in spermatozoa). On the other hand, in *G. aequicauda*, the haemocytes presented the most significant DNA damage at the intermediate concentration (TM = 31.3). The TM values were remarkably higher for all BPA concentrations tested in this species. The DNA damage induced in the haemocytes was significantly different between the two species at 0.25 and 0.5 mg/l. We also observed a dose-response relationship in sperm cells, especially in *G. aequicauda* ($r_s = 0.99$), with the most significant DNA damage at 1 mg/l in both species, resulting in statistically significant difference from the untreated group. Bisphenols commonly affect living organisms by interfering with the normal functions of the endocrine system, but they have also been reported to induce oxidative stress and cause reproductive dysfunctions (Ullah et al., 2019; Tatar and Türkmenoğlu, 2020). The results obtained are in accordance with other studies on invertebrate species. Martinez-Paz et al. (2013) observed that 0.5 and 3 mg/l BPA caused DNA fragmentation in aquatic larvae of *Chironomus riparius*, quantified by Comet assay, after both 24 h and 96 h exposure. Consistently with this evidence, a study on green mussels (*Perna viridis*) indicated that BPA might be genotoxic to adult haemocytes exposed for 7 days at the very low concentration of 98 ng/l (Juhel et al., 2017). The Comet assay has also demonstrated the genotoxic activity of BPA on *Daphnia magna* at low concentrations, 3 and 30 µg/l (Park and Choi, 2009). Studies on vertebrate species showed that bisphenols caused oxidative damage (Nourian et al., 2017; Ullah et al., 2018,), suggesting that the DNA alteration observed in the present study could be related to

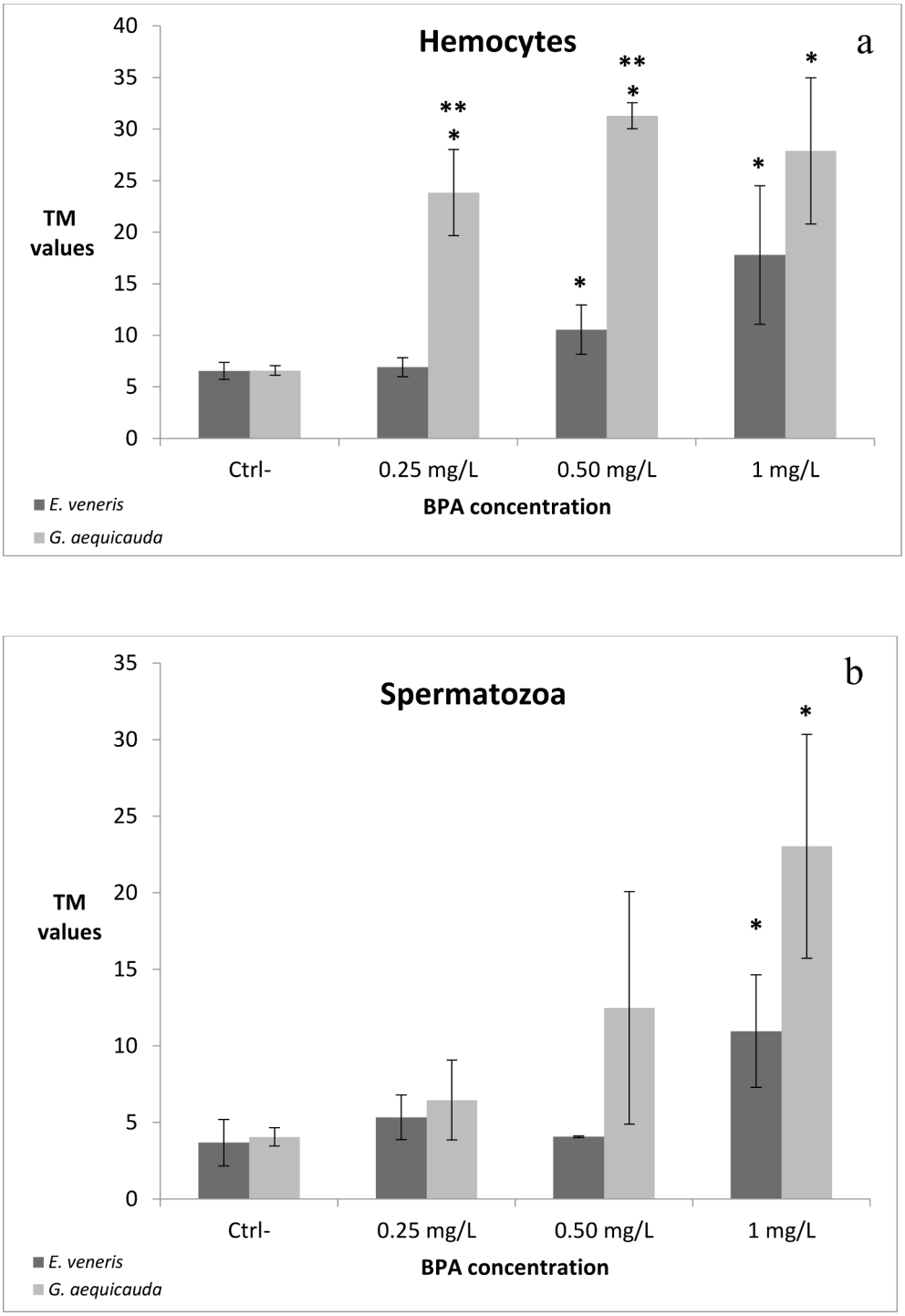

Fig. 5. DNA damage expressed as Tail Moment (mean ± standard error) of the Comet assay applied to hemocytes (a) and spermatozoa (b) of *E. veneris* and *G. aequicauda* after 24 h exposure to different concentrations of BPA. (*significantly different from negative control, $p < 0.05$; **significantly different from the other species at the same concentration, $p < 0.05$).

such damage. BPA also causes oxidative stress in amphipods, as shown in the gammarid *Gammarus pulex* at sub-lethal concentrations (Tatar and Türkmenoğlu, 2020).

In-vivo studies examining the effects of these BPA alternatives are still scarce, and the majority have been conducted on vertebrates, especially rats and fishes (Ullah et al., 2018; Ullah et al., 2019; Ji et al., 2013). BPF appears to be the primary replacement for BPA, and it is widely used to manufacture epoxy resins and different types of coatings. In both *in-vivo* and *in-vitro* toxicological studies, BPF shows endocrine-modulating capabilities, genotoxic effects, and oxidative stress (Ullah et al., 2019). In addition, a recent study revealed positive associations of urinary concentrations of BPF with obesity in children and adolescents in the United States (Liu et al., 2019). In our study, when exposed to BPF, both cell types showed genotoxic effects, both in *E. veneris* and *G. aequicauda* (Figure 6). The haemocytes showed a similar trend between the two species, decreasing at 1 mg/l only for *G. aequicauda*. A dose-response relationship can be observed in *E. veneris* haemocytes ($r_h = 0.92$). Our results show DNA damage on haemocytes of both species exposed to

Table 1
Effects of BPs exposure in haemocytes and spermatozoa DNA, Tail Moment values (mean ± standard error) after 24 h exposures of *E. veneris* and *G. aequicauda.* Pearson's linear correlation coefficient (r) is reported to test the occurrence of a dose-response relationship.

	E. veneris						*G. aequicauda*					
	TM haemocytes			TM spermatozoa			TM haemocytes			TM spermatozoa		
Conc.	BPA	BPF	BPS	BPA	BPF	BPS	BPA	BPF	BPS	BPA	BPF	BPS
0 mg/l	6,55	2,85	5,44	3,68	2,81	4,25	6,58	3,27	6,09	4,05	3	1,74
0,25 mg/l	6,9	5,32	8,26	5,33	2,21	7,63	23,8	14	10,6	6,46	4,57	2,32
0,5 mg/l	10,6	9,12	8,48	4,07	5,95	7,79	31,3	17,7	6,93	12,5	12,2	4,72
1 mg/l	17,8	9,89	10,4	11	4,11	4,7	27,9	12,6	6,41	23	11,7	5,44
Pearson's r	0,97	0,92	0,94	0,88	0,49	-0,07	0,73	0,53	-0,23	0,99	0,85	0,94

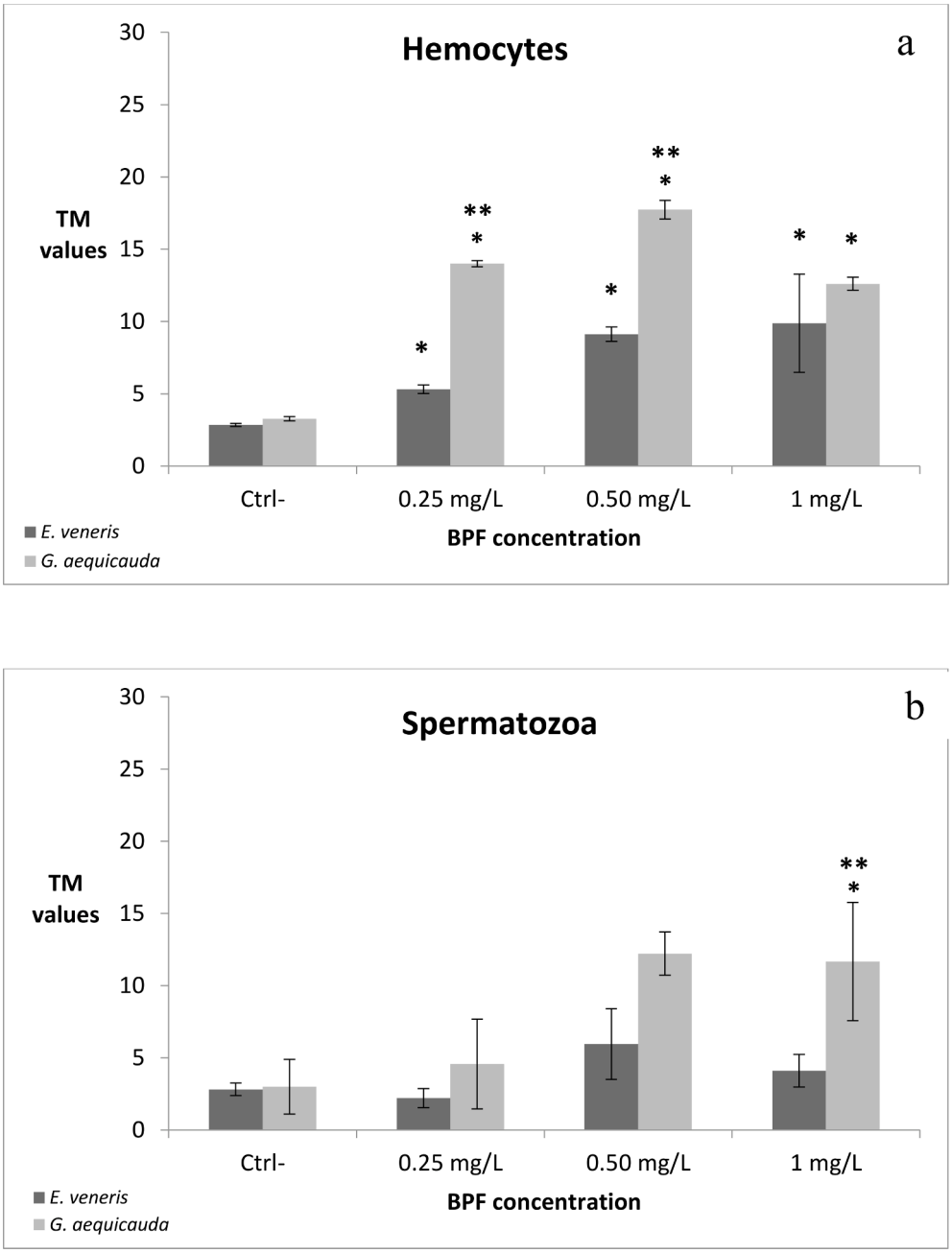

Fig. 6. DNA damage expressed as Tail Moment (mean ± standard error) of the Comet assay applied to hemocytes (a) and spermatozoa (b) of *E. veneris* and *G. aequicauda* after 24 h exposure to different concentrations of BPF. (*significantly different from negative control, $p < 0.05$; **significantly different from the other species at the same concentration, $p < 0.05$).

BPF, similar to that obtained for BPA. Remarkably, significantly higher and statistically different TM values for all the tested BPF concentrations compared to the untreated organisms were observed together, with significantly different values between the species at 0.25 and 0.5 mg/l. The test results performed on spermatozoa also exhibited a similar trend, fully comparable to that observed after BPA exposure. We obtained higher TM values in *G. aequicauda* than *E. veneris*, with a significant difference between the two species at 1 mg/l, but without any statistically significant DNA damage in relation to the untreated group. TM values increase in proportion to concentration until 0.5 mg/l, while the higher exposure concentration (1 mg/l) corresponds to a mild reduction in DNA damage in both species and cell types. A suitable explanation for this response profile could be related to the DNA repair mechanism proposed by Ching et al. (2001), described in different somatic cells. It suggested that a cell exposed to toxicants may activate a DNA repair system only after a certain level of toxicant accumulates (Lacaze et al., 2011).

BPS is one of the main alternatives to BPA as a colour developer in thermal papers, showing similar mechanisms of action to BPA, such as estrogenic activity, but a recent study revealed that it was less efficiently metabolised than BPA in humans, so its exposure might be associated with higher risks for human health than BPA (Gayrard et al., 2019). The genotoxic effects of BPS on *E. veneris* and *G. aequicauda* showed a trend not interpretable compared to that observed with the other two bisphenols (Figure 7). In the haemocytes of *E. veneris,* we observed a dose-response relationship between the concentrations of BPS and the TM values, with the TM values increasing proportionally to BPS concentration. The DNA damage observed in *E. veneris* was statistically different from the untreated group at the higher concentration (TM = 10.4 ± 1). Otherwise, *G. aequicauda* showed the greatest damage at 0.25

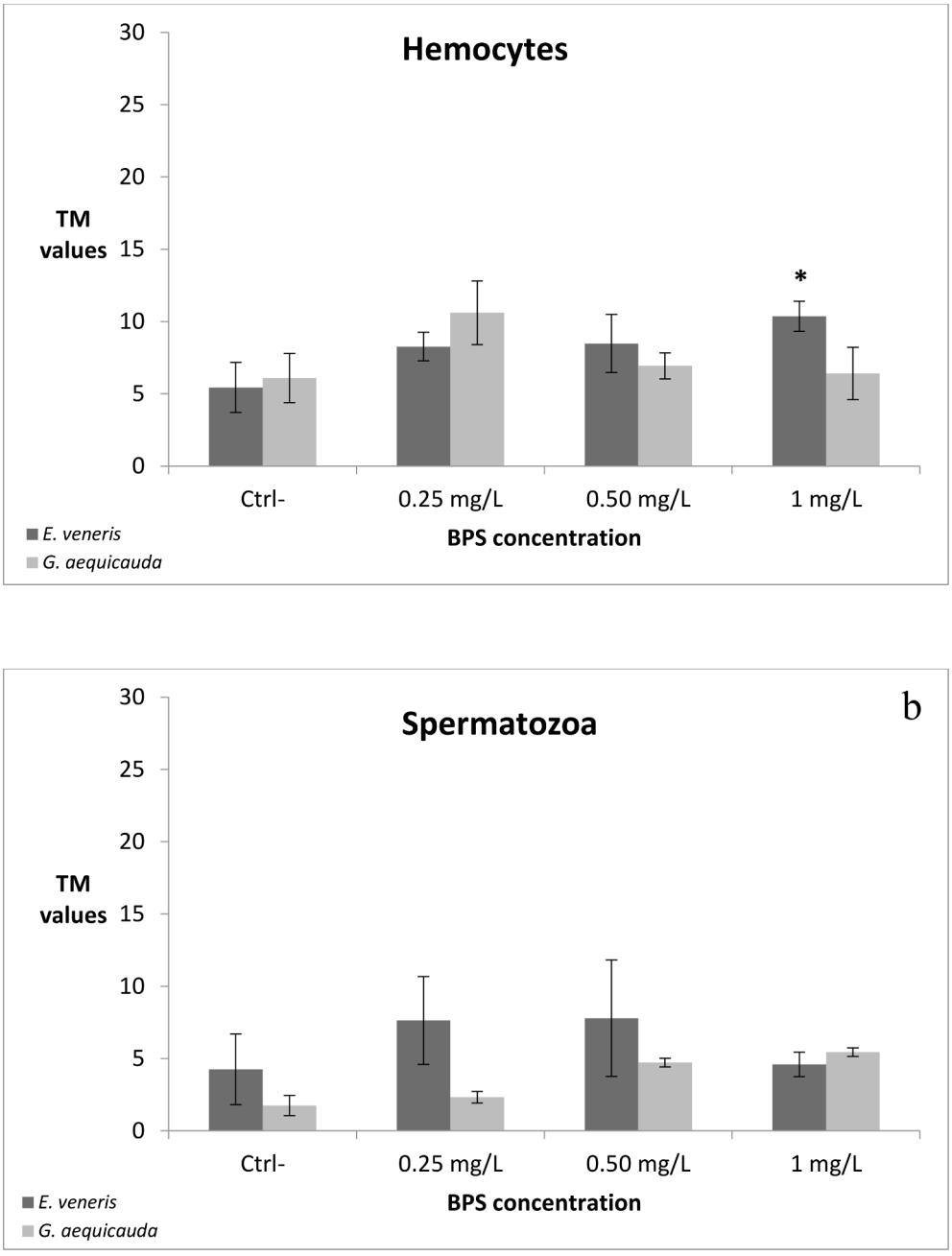

Fig. 7. DNA damage expressed as Tail Moment (mean ± standard error) of the Comet assay applied to hemocytes (a) and spermatozoa (b) of *E. veneris* and *G. aequicauda* after 24 h exposure to different concentrations of BPS. (*significantly different from negative control, $p < 0.05$).

mg/l, decreasing at higher concentrations, without statistical differences from the untreated group. The sperm cells exhibited a highest TM value at 0.5 mg/l (TM = 7.8 ± 4) in *E. veneris* and at 1 mg/l in *G. aequicauda* (TM = 5.4 ± 0.3). The TM was always maintained at low values in the spermatozoa, comparable with those of the untreated groups (not statistically different). There is only limited information available on toxicological effects of BPS on invertebrates. This is the first report about the effects on the species assayed in this study, but other studies have shown that BPS is acutely toxic in *Daphnia magna* and causes alteration of the ecdysone pathway in *Chironomus riparius* (Ji et al., 2013; Morales et al., 2020) (

Fig. 7, Table 1).

Comparing the effects of the three BPs on DNA integrity of the studied species (Fig. 8), we can observe a general higher detrimental influence of BPA compared to BPF and BPS for all concentrations tested both in hemocytes and sperm cells. BPF results were less genotoxic than BPA but caused significant DNA damage compared to the untreated group in both species and both cell types at the higher concentrations. BPS seems to cause the lowest DNA damage although the results were comparable to BPF at the higher concentration for both *E. veneris* cell types. In most cases, BPA caused the most significant genotoxic effects, confirming its adverse impact on biological systems and the necessity of its substitution. Still, the harmful physiological effects of BPA alternatives should be carefully considered because the many pieces of evidence suggest that they might not be safer than banned substances.

Of particular interest is the assessment of bisphenols' genotoxicity in spermatic cells. Spermatozoa are highly specialised cells, with a key role in reproduction and consequently in determining the continuous success of the species (Marçal et al., 2020). Although many studies assessed the genotoxic effects of BPA on germ cells, only a few of them investigated the effects produced by its analogues. (Ullah et al., 2019) studies have provided evidence of the genotoxic potential of BPA, BPF, and BPS and their oxidative stress-inducing ability in rat spermatozoa, suggesting reactive oxygen species-mediated DNA damage induced in these cell types.

In our study, spermatic cells showed generally lower genotoxic damage in both species tested compared to haemocytes. In *E. veneris,* the damage to DNA in spermatozoa exposed to BPA and BPF had a similar trend compared to haemocytes, with lower values of TM at all tested concentrations of each bisphenol. In *G. aequicauda,* we consistently observed increasing DNA damage corresponding to the growing concentration of BPA and BPF. While the highest DNA strand breaks occurred in the haemocytes at 0.5 mg/l, spermatozoa showed no decrease in DNA damage at a 1 mg/l concentration. This could mainly be due to the inability of sperm to repair DNA damage, as assumed in previous studies (Lacaze et al., 2011; Di Donato et al., 2016). As observed in the haemocytes, spermatozoa exposed to BPS show a different situation than BPA and BPF, but the trend was similar between the two gammarid species. In both species, we observed DNA damage in a linear correlation with BPS concentration, until 0.5 mg/l, followed by a decrease of TM values at the highest BPS concentration.

The present study suggests that haemocytes exhibited a higher sensitivity towards the three bisphenols than spermatozoa, unlike previous studies on gammarids (Lacaze et al., 2010; Di Donato et al., 2016) where a higher sensitivity and vulnerability to toxicants of sperm than somatic cells was observed, justified by the lack of effective protection against xenobiotics in this cell type and linked to the lack of DNA repair mechanism (Erraud et al., 2018). Nevertheless, in other studies on a similar topic, results according to our data were reported. For example, Lewis and Galloway (2008) found that sperm from the polychaete *Arenicola marina* exposed for 72 h to methyl methanesulfonate and benzo(a) pyrene showed lower levels of DNA damage than somatic cells. The

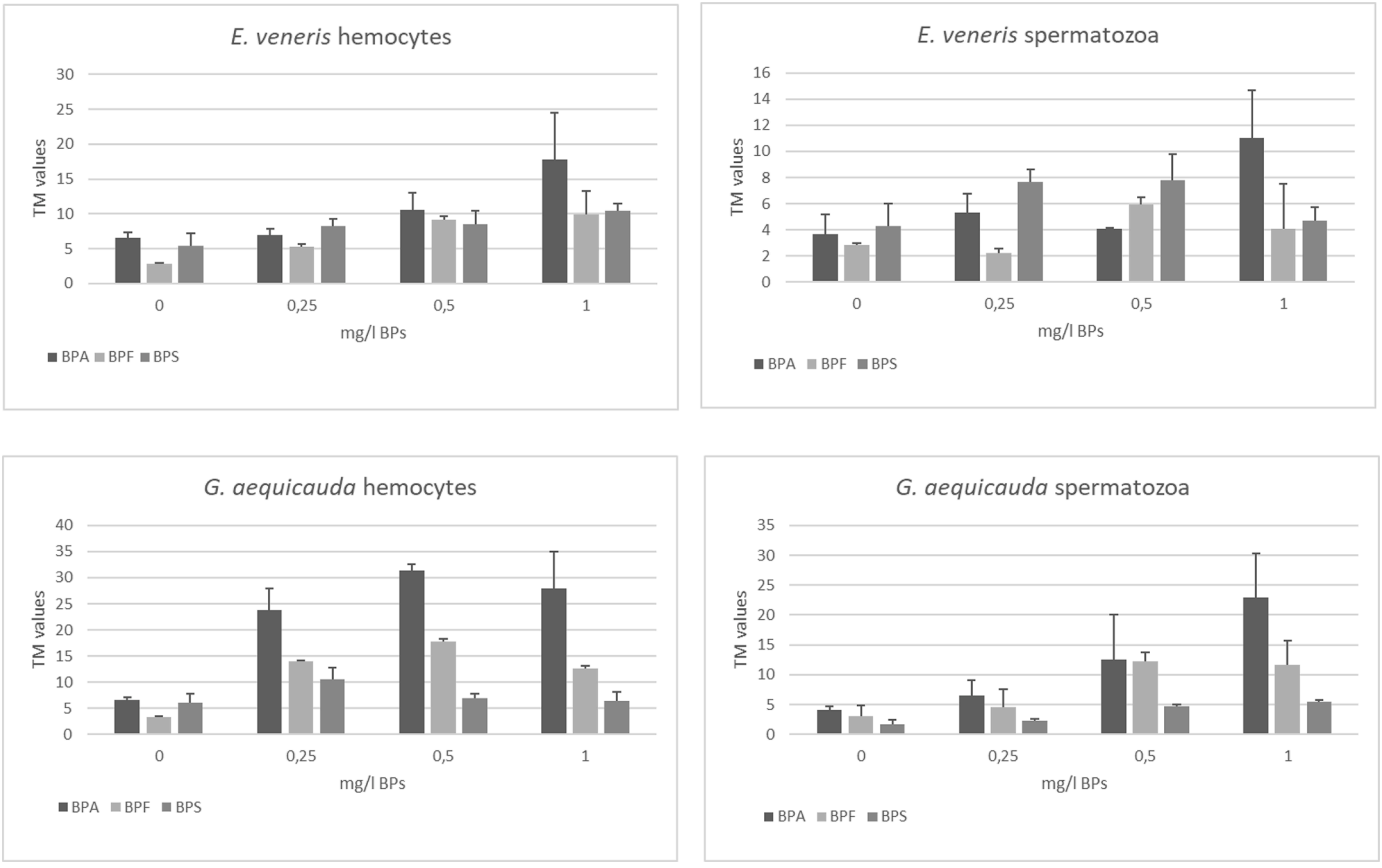

Fig. 8. DNA damage expressed as Tail Moment (mean ± standard error) of the Comet assay applied to hemocytes (a) and spermatozoa (b) of *E. veneris* and *G. aequicauda* after 24 h exposure to different concentrations of BPA, BPF and BPS.

authors suggested that sperm DNA may be only protected by their capacity to condense chromatin (Lewis and Galloway, 2008; Lacaze et al., 2011). In the present paper, we hypothesise that the lower DNA damage observed in spermatozoa compared to haemocytes can be due to the more considerable difficulty for toxicants to access the DNA because of its compact nature. Moreover, the profound differences between these two cell types, concerning metabolism and functions, could influence their different sensitivity to contaminants, including bisphenols (Silveira et al., 2019). On the other hand, we do not have enough information regarding the DNA repair capabilities of invertebrate spermatozoa. Some studies (Lewis and Galloway, 2008; Marçal, 2020) have suggested that there is an antioxidant defence system, like in the haemocytes. However, such processes appear to occur to a lower extent than somatic cells. On the contrary, other studies have speculated that sperm cells are devoid of DNA repair capabilities, and consequently, the DNA damage would represent a critical injury (Pacchierotti et al., 2018). Anyway, a direct assessment of damage to germ cells is relevant to understanding chemical-induced alterations in the genome that may potentially impact the reproductive success of populations (Lacaze et al., 2010; Erraud et al., 2018). Whereas DNA damage to somatic cells can cause negative consequences for single individuals, spermatozoa are involved in long-term changes (Lewis and Galloway, 2008; Marçal, 2020; Di Donato et al., 2016). Exposure to xenobiotics can disturb the production of high-quality sperm and maintenance of DNA integrity, preventing successful fertilisation (Erraud et al., 2018). Particularly, genotoxic pressure is suspected of causing adverse effects at the population level by altering the reproductive success of individuals and because of the potential to convey the genotoxic damage to progeny (Lacaze et al., 2010; Lewis and Galloway, 2008).

Conclusions

The description of the mode of action of these xenobiotics in aquatic organisms appears even more urgent since the aquatic environment reasonably presents the highest risk of contamination. An increase in the use of these potentially harmful alternatives to BPA is expected, given the absence of a clearly defined BPA replacement strategy. These analogues substitute BPA in products sold under the “BPA-free” label to ensure the safety of the products to reassure consumers. However, their health and mechanism of action are not yet proven.

To the best of our knowledge, no previous study has investigated the effects of BPA, BPF, and BPS in both the species involved in the present work. Only a few previous studies have dealt with the effects of BPA on other amphipods. Moreover, most of the studies in invertebrates addressed parameters other than genotoxicity, such as endocrine disruption. Our study showed that both BPA and its analogues caused a genotoxic effect on both amphipod species and cell types tested (haemocytes and sperm cells), suggesting that BPF and BPS may not be benign substitutes for BPA. Although it is not possible to predict the environmental implications of BP exposure only based on genotoxic effects, we should consider that damage at the molecular level may be reflected at the higher levels of biological organisation, from individual to ecosystems.

Results of this work highlight as particularly interesting the genotoxic impact of BPs on sperm cells. The susceptibility of these cells to environmentally induced DNA damage is relevant to understanding the chemically induced alterations in the genome that could cause adverse effects at the population level and have long term consequences. Further studies are necessary to better evaluate the impact of BPA analogues on somatic and reproductive processes in amphipods that, due to their remarkable role in the freshwater trophic chain, can affect the food web leading to humans at multiple levels.

CRediT authorship contribution statement

Serena Cosentino: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft. **Federica Aureli:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Valentina Iannilli:** Conceptualization, Validation, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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