

# Anti-genotoxic ability of $\alpha$ -tocopherol and Anthocyanin to counteract fish DNA damage induced by musk xylene

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Accepted: 16 September 2015 / Published online: 25 September 2015  
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**Abstract** Many compounds released into the environment are able to interact with genetic material. The main purpose of genetic toxicology is to investigate the adverse effects of genotoxic molecules such as reduced fitness, changes in gene frequencies and their impact on genetic diversity in populations following genotoxic exposure. However, the ecological effects of many genotoxic compounds remain poorly understood. The aim of this research was to evaluate the genotoxic activity of an artificial musk (musk xylene, MX) and the potential anti-genotoxicity against this chemical compound of two antioxidant substances ( $\alpha$ -tocopherol and an anthocyanins enriched extract). The studies were performed both in vivo and in vitro, using the teleost *Danio rerio* and the DLEC (*Dicentrarchus labrax* embryonic cells) cell line. We carried out the exposure to these substances at different times. DNA and cell damage and their possible repair were detected by various experimental approaches: DNA strand breaks (Comet Assay), degree of apoptosis (Diffusion Assay) and molecular alterations at the genomic level (RAPD-PCR technique). Data were collected and analyzed for statistical significance using the Student's *t* test. The results of this study showed that MX exhibited a genotoxic activity even after short exposure times. The anti-genotoxicity experiments evidenced that both  $\alpha$ -tocopherol and

Anthocyanin were able to contrast the genotoxic effects induced by MX, both in vivo and in vitro.

**Keywords** Musk xylene · Comet Assay · RAPD-PCR technique · Antigenotoxic activity ·  $\alpha$ -Tocopherol · Bilberry anthocyanin extract

## Introduction

With the growth in urbanization and the increase in commercial activities, the risk of water pollution by Pharmaceuticals and Personal Care Products (PPCPs) has given rise to health concerns.

PPCPs are a heterogeneous group of chemical substances that includes drugs for both human and veterinary use, diagnostic agents (X-rays, contrast media), nutraceuticals (dietary supplements), and other bioactive and consumer chemical substances (cosmetics, perfumes) (Ellis 2006).

Synthetic musks also belong to the category of PPCPs; these are aromatic compounds used as substitutes for the more expensive natural musks (Daughton and Ternes 1999). PPCPs play a predominant role in eco-toxicology; this is due to their capacity of bioaccumulation and their persistence in ecosystems as well as to the consequent effects on the health of both flora and fauna, including man. The term “synthetic musk” covers four vast categories of chemical compounds, namely nitro musks, polycyclic musks, macrocyclic musks, and alicyclic musks. Among the nitro-musks, synthetic derivatives of di- and tri-nitrobenzene, commonly added to detergents and soaps, we found the musk xylene (MX) (5-*tert*-butyl-2,4,6-trinitro-*m*-xylene) (Duedahl-Olesen et al. 2005). Its levels in surface waters are in the ng- $\mu$ g L<sup>-1</sup> range (Gatermann et al. 2002;

**Electronic supplementary material** The online version of this article (doi:10.1007/s10646-015-1538-1) contains supplementary material, which is available to authorized users.

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Sumner et al. 2010). Normally the water treatment plants are not able to remove the musks completely with consequent exposure of aquatic organisms (Sumner et al. 2010).

Genotoxicity is the ability of a substance to induce modifications to the nucleotide sequence or to the double helix DNA structure in a living organism. Exposure to a genotoxic substance can cause a cascade of events able to change the integrity of the DNA leading to various types of damage, including oxidative damage. Oxidative damage can take place with the generation of free oxygen radicals, molecules that carry one or more unpaired electrons, produced either endogenously or exogenously. Agents that are catalytic intermediates, scavengers of oxide reductions, and are used as antioxidants, have been shown to inhibit these highly reactive compounds. If they are not inactivated by antioxidants, the reactive oxygen species (ROS) can potentially alter the structure and function of various cellular components, such as membrane lipids, lipoproteins, proteins, carbohydrates, RNA and DNA.

The antioxidant substances used in our study were Vitamin E ( $\alpha$ -tocopherol) and a bilberry anthocyanins extract (Anthocyanin).  $\alpha$ -Tocopherol is the most active vitamer of Vitamin E. The antioxidant activity of Vitamin E takes place thanks to the lipophilic property of the molecule, responsible for the localization and accumulation of the vitamin at the level of cellular membranes and organelles (Negis et al. 2007). Vitamin E is a good scavenger of lipoperoxide radicals and is an anti-oxidant of the "chain breaking" type due to its capacity to break the reaction chain of propagation of peroxide radicals, giving up an electron to the radical species. Natural sources are food of vegetable origin, such as seeds (and the oils derived from them), cereals, fruit and vegetables (Azzi 2007). Anthocyanins are members of flavonoids family, a large group of polyphenol compounds. Flavonoids have antioxidant properties; they are radical scavengers and they possess chelating capacity for divalent transitional ions (Wang and Stoner 2008; Ginsburg et al. 2011). Moreover, anthocyanins are able to block the production of ROS inhibiting NADPH oxidases, xanthine oxidases and myeloperoxidases and influences their release (Cotelle 2001). The richest natural source of these substances are berries, aubergines, red chard, oranges, and cherries.

The goals of this study were to evaluate, both in vivo and in vitro, the genotoxicity of MX and the possible effect of Vitamin E and Anthocyanin on the DNA damage induced by this molecule. The two experimental animal models used were the *Dicentrarchus labrax* embryonic cell (DLEC) line and the teleost *Danio rerio*.

We used three experimental approaches: DNA strand breaks (Comet Assay), apoptotic damage (Diffusion Assay), alterations at the molecular level (RAPD-PCR technique).

The Comet assay (single-cell gel electrophoresis) is a simple method for measuring DNA strand breaks (Singh et al. 1988).

The Diffusion Assay reveals exclusively apoptotic cells (Vasquez and Tice 1997). It is a quantitative method that evidences the fragmentation of nuclear DNA at the level of the single cell. With this technique, the nuclei of the apoptotic cells, characterized by having a highly dispersed DNA, show irregular edges. The nuclei of the necrotic cells, instead, are larger and not well defined. The cells with damaged DNA have a nucleus of reduced dimensions with DNA projections, whose extension is proportional to the genetic damage (Singh 2000).

The Random Amplified Polymorphic DNA (RAPD) technique allows detection of DNA polymorphisms of the genome by Polymerase Chain Reaction (PCR) using single arbitrary primers designed independently from target DNA sequence (Williams et al. 1990). The change in the number of the bands and the variation in their intensity are associated with alterations of genetic material (Liu et al. 2005; Noel and Rath, 2006).

## Materials and methods

### Chemicals

MX tested in this study was obtained from Musk Xylene Solution 100 ng/ $\mu$ L in acetonitrile (Sigma-Aldrich<sup>®</sup>). Benzene  $\geq 99.0$  % was purchased from Sigma-Aldrich<sup>®</sup>. Vitamin E was  $\alpha$ -tocopherol  $\geq 95.5$  % (Sigma-Aldrich<sup>®</sup>) while Bilberry anthocyanin extract was acquired by Naturalin-BioResources Co.Ltd.

### Samples

The in vivo tests were carried out on 192 adult individuals of zebrafish (*Danio rerio*, Cyprinidae, Teleostei), a small-sized animal with easy feeding needs and simple to be kept in captivity, bought from a local supplier; they were allowed to stabilize for one month before beginning the experiments. Each individual was about 4.5 cm in length and 1.5 g in weight. For negative control one fish  $L^{-1}$  was transferred to small tanks each containing 5 L of water, while for the other experimental groups two fishes  $L^{-1}$  were loaded. Water quality, temperature, oxygen concentrations and pH level were routinely monitored in order to maintain fishes under optimal conditions (pH: 6.8-7.5; temperature: 28-28.5 °C; dissolved oxygen content: 6.0 ppm ( $mg L^{-1}$ ); water hardness: 50-100  $mg L^{-1}$   $CaCO_3$ ;  $NO_3^{-} < 50 mg L^{-1}$ ;  $NO_2^{-} < 0.1 mg L^{-1}$ ). Water was dechlorinated before use. Animal maintenance and experimental procedures were in accordance with the

Guide for Use and Care of Laboratory Animals (European Communities Council Directive 1986), and efforts were made to minimize animal suffering and reduce the number of specimens used. The fish were anesthetized with Tricaine methanesulfonate (Sigma-Aldrich) before being killed. The *in vitro* tests were carried out using 144 flasks containing DLEC confluent cells from the European sea bass (Buonocore et al. 2006). Five experimental groups were prepared, both *in vivo* and *in vitro*: negative control, positive control (benzene  $10.0 \mu\text{L L}^{-1}$  for *in vivo* and  $1.0 \mu\text{L mL}^{-1}$  for *in vitro* experiments), MX ( $10.0 \mu\text{g L}^{-1}$ ), MX ( $10.0 \mu\text{g L}^{-1}$ ) with Anthocyanin ( $1.0 \text{ mg L}^{-1}$ ), MX ( $10.0 \mu\text{g L}^{-1}$ ) with Vitamin E ( $1.0 \text{ mg L}^{-1}$ ). The *in vivo* exposure times were 5, 7, 14 and 21 days, while the *in vitro* exposure times were 3, 24 and 48 h. An aliquot of MX from its commercial standard solution in acetonitrile was directly added to water in order to provide the chosen final dose level. The compound, identified as a vPvB substance according to article 57(e) of Regulation (EC) No 1907/2006 (REACH), is considered to be very persistent in water (ECHA 2008). Benzene concentration was monitored throughout the entire *in vivo* experimentation by applying UV spectrophotometric analysis. To this purpose three samples (2 mL each) were daily and randomly collected from the benzene treated water tank and their absorbance was read at 253.5 nm using the Shimadzu 1700 UV-Vis double beam spectrophotometer. The amount of benzene in water tank was estimated on the basis of the benzene regression equation in pure water, which was found to be  $y = 0.0013x + 0.0166$  for a concentration range of 5–1000  $\mu\text{L L}^{-1}$ . The correlation coefficient value was 0.9998 ( $n = 6$ ). The limit of detection (LOD) was found to be  $2.54 \mu\text{L L}^{-1}$ , whereas the limit of quantification (LOQ) was  $7.69 \mu\text{L L}^{-1}$  (further information are reported in Supplementary Materials section). The final benzene concentration level was adjusted, accordingly. Analogously, when *in vitro* experiments were carried out, amounts of culture medium were collected and spectrophotometrically monitored.

#### Antioxidant substances: preparation and stability assessment

An  $\alpha$ -tocopherol stock solution was prepared in pure ethanol. It was added to water to result in a final concentration of  $\alpha$ -tocopherol ( $1.0 \text{ mg L}^{-1}$ ) and ethanol (0.005 %), respectively. An amount equal to 1.0 mg of bilberry anthocyanin extract (25 % anthocyanins, 4:1 extract) was directly added to water. As determined by the differential pH method (Giusti and Wrolstad 2001), the tested anthocyanin extract rate corresponded to a total bilberry anthocyanins of 0.183 mg cyanidin-3-*O*-galactoside equivalents per 1.0 mL of extract.

The *in vivo* stability of  $\alpha$ -tocopherol and Anthocyanin was monitored through optical investigation at different time points (24, 72, and 120 h) collecting culture media samples on the layer top of the tank. The analytical confirmation of concentrations was evaluated until five days of exposure, because the water was entirely changed after this time of treatment and the substances were added in the tank at the initial concentration. The amount of antioxidant substances was extrapolated from their relative calibration curve ( $y = 12.97x + 0.042$  for  $\alpha$ -tocopherol;  $y = 1.84x + 0.0312$  for Anthocyanin). The latter was prepared from differently standard solutions of the vitamer and bilberry anthocyanin extract (50.0, 25.0, 12.5, 6.25, 3.125, 1.56, 0.781, 0.39  $\text{mg L}^{-1}$ , final concentration levels). All the UV-Vis spectra of  $\alpha$ -tocopherol and Anthocyanin standard solutions were acquired in the range of 200–600 nm by using a Shimadzu UV-1700 double beam spectrophotometer.  $\alpha$ -Tocopherol solutions showed a maximum absorbance peak at 296 nm, whereas  $\lambda_{\text{max}}$  absorption of bilberry anthocyanin extract was peculiarly detected in the UV region at 283 nm.

#### Cell viability

Cell viability in erythrocytes of *Danio rerio* and viable DLEC were counted by Trypan Blue stain by Countess Automated Cell Counter (Invitrogen/Life Technologies). After different exposure times to Musk Xylene ( $10.0 \mu\text{g L}^{-1}$ ) alone and with Vitamin E ( $1.0 \text{ mg L}^{-1}$ ) and with Anthocyanin ( $1.0 \text{ mg L}^{-1}$ ) Trypan Blue test was performed as described by Rocco et al. (2015). In brief, 10  $\mu\text{L}$  of cell suspension for each sample, including no treated, was added to 10  $\mu\text{L}$  of 0.04 % supplied Trypan blue stain (Sigma-Aldrich Corp., St. Louis, MO, USA). Ten microliters of the solution were taken to a cell counter to determine the number of total cells, non-viable (i.e. trypan blue stained) cells and viable cells. The analysis was done in triplicate.

#### Comet assay and Diffusion assay

As regards the Comet Assay the *in vivo* protocol required the removal of a few microliters of blood from the zebrafish from below the gills with a heparinized syringe to avoid coagulation. The hematic cells were then mixed with 500  $\mu\text{L}$  of 1X PBS (Phosphate Buffered Saline) and then centrifuged at 2000 rpm for 10 min. The supernatant was removed and 300  $\mu\text{L}$  of Low Melting Point Agarose (LMPA) 0.5 % in 1X PBS was added to the pellet. Instead, the adherent DLEC to the flask were subjected to enzymatic detachment with trypsin EDTA-VERSENE ( $200 \text{ mg L}^{-1}$ ). One hundred and fifty microliters of the cell suspension obtained were added in a microtube

containing three hundred and fifty microliters of Low Melting Point Agarose (LMPA). Eighty-five  $\mu\text{l}$  of the above mentioned mixture, both in vivo and in vitro, were placed on slides previously coated with Normal Melting Point Agarose (NMPA) 1 % in 1X PBS. All the slides were maintained at 4 °C for 60 min in a lysis solution (NaCl 2.5 M, Na<sub>2</sub>EDTA 0.1 M, Tris-Base 0.4 M, TRITON-X100 1 %, DMSO 10 %) at pH 10, put on alkaline unwinding for 10 min in a basic solution (NaOH 10 N, EDTA 200 mM, pH 12.1) and then subjected to electrophoresis (25 V, 300 mA) for 20 min. The slides were then stained with 100  $\mu\text{l}$  of ethidium bromide  $\times 30$  (10  $\mu\text{g ml}^{-1}$ ), covered by a cover slide and observed by the fluorescence microscope (Nikon Eclipse E-600) equipped with an excitation filter (510-560 nm) and a suppression filter (590 nm). The images were acquired using a specific program (Komet version 6.0.0, Kinetic Imaging) (Rocco et al. 2010).

The experimental protocol of Diffusion Assay is the same as that of the Comet assay, with only one difference, namely the slides do not undergo electrophoresis. Apoptotic cells show irregular outlines with nuclei characterized by a highly diffused DNA into agarose. The Diffusion Assay slides were scored by subdividing of the degree of DNA diffusion pattern in five classes of damage such as described in Cantafora et al. (2014) and we considered only class 5 (apoptotic cell).

### RAPD-PCR technique

Zebrafish genomic DNA was extracted from about 100  $\mu\text{L}$  of whole blood according to Rocco et al. (2010). DLEC DNA was collected following the protocol reported in Rocco et al. (2014).

For DNA amplification, we used Taq DNA recombinant polymerase (ROCHE), which contains nucleotides (dNTPs 0.4 mM), magnesium chloride and DNA polymerase. It is necessary to add DNA (40 ng) and the primer 6 (5'-d[CCCGTCAGCA]-3') at the concentration of 5 pmol  $\mu\text{l}^{-1}$ . The final reaction volume was 25  $\mu\text{l}$ . The chosen primer was selected to yield amplification products with a reasonable number of bands. The amplification reaction followed this cyclic program: one initial step (2 min at 94 °C), then 1 min at 95 °C, 1 min at 36 °C and 2 min at 72 °C, all for 45 cycles. Fragments of different length were generated due to the various pairings of the primer that can be seen as bands by means of electrophoresis on 2 % agarose gel. The change in the number of the bands and the variation in their intensity are associated with alterations of genetic material (Noel and Rath, 2006). Before proceeding with the amplification reaction the DNA template was extracted using an experimental protocol with a series of passages in chloroform and

isopropanol which guarantee a sufficiently pure extraction to produce an RAPD-PCR profile of good quality. The polymorphic pattern generated by RAPD-PCR profiles allowed the calculation of Genomic Template Stability (GTS, %) as follows:

$$\text{GTS} = (1 - a/n) \times 100$$

where  $a$  is the average number of polymorphic bands detected in each treated sample and  $n$  the total number of bands in the non-treated samples. Polymorphism in RAPD profiles included disappearance of bands and appearance of new bands with respect to the control sample. The average was calculated for each experimental group at different molecules exposures. Changes in these values were considered as a percentage of their controls (set to 100 %) (Rocco et al. 2014; 2015).

### Statistical Analysis

The values for all the tests were expressed as average percentage and its standard deviation (SD). Differences in the percentage among the experimental groups were analyzed using the unpaired Student's  $t$  test. The results were considered statistically significant if  $p$ -value  $\leq 0.05$  %. The statistical analysis for GTS % was carried out using the software package SPSS 20.0 for Windows.

## Results

### Antioxidant substances stability

In order to evidence the potential beneficial effects of antioxidant substances commercially free on the musk xylene-induced DNA damage, an aliquot of 1.0 mg  $\text{L}^{-1}$  of  $\alpha$ -tocopherol or of a bilberry anthocyanin enriched extract was added to water and their stability was defined through optical investigation at different time points. The bilberry anthocyanin enriched extract, whose initial dose level was estimated to be 1.02 mg  $\text{L}^{-1}$ , was particularly stable. It seemed to undergo a weak decrease only after 120 h exposure time, when an Anthocyanin dose level equal to 0.96 mg  $\text{L}^{-1}$  was determined (a percent decrease of 5.88 %). The great stability of the water-soluble extract could be explained on the basis of copigmentation effects due to the coexistence of anthocyanins with other polyphenol compounds (e.g. flavonols and flavanols) and/or to the presence of acylated anthocyanins (Stintzing and Carle 2004). In fact it is well known that bilberry contains a variety of phenolic compounds, including quercetin, catechins, tannins, ellagitannins, and phenolic acids, but anthocyanins make by far the largest contribution to its phytochemical mix (Seeram 2008).  $\alpha$ -Tocopherol content

in water (initial extrapolated dose level =  $0.98 \text{ mg L}^{-1}$ ) reached a mean percent decrease of 5.1 % after 24 h exposure time. This value remained almost stable at 72 and 120 h exposure times.

### Cell viability

Trypan blue exclusion test showed that percentage of cell viability of zebrafish erythrocytes and DLEC was over 97 % after each different treatment and all times of exposure examined (Table 1).

### Comet assay and Diffusion assay

The values coming from the Comet assay on zebrafish exposed to MX, indicated a statistically significant percentage of DNA damage with respect to the negative control. Furthermore, the damage tended to increase over the days of exposure. The co-exposition to MX and to each of the two antioxidants reduced the degree of DNA integrity. The only exception was the samples simultaneously exposed to MX and Vitamin E at 5 days of exposure (Fig. 1a). The results of the Comet assay showed, also in vitro, a statistically significant DNA damage increase induced by MX at the three exposure times. The results of the simultaneous exposure to MX and Vitamin E showed a statistically significant DNA damage increase at 3 h of exposure, while there was a reduction of this damage at 24 h and 48 h. In fact, the results at 24 h and 48 h were similar to those of the unexposed cells. With the simultaneous exposure of MX and Anthocyanin, instead, the percentage of DNA in the Comet tail was notably reduced (Fig. 1b).

As regards the Diffusion Assay MX caused a high percentage of apoptotic cells (class 5) with respect to the negative control, while the simultaneous exposure of MX and one of the two antioxidants reduced this percentage, which was no longer significant both in vitro and in vivo. The only exception was the simultaneous exposure to MX and Vitamin E at 3 h and at 5 days of exposure (Fig. 1c, d).

### RAPD-PCR technique

For the molecular analyses we used primers with a high efficiency for the DNA amplification of the zebrafish and the DLEC cell line. In all the samples, the RAPD-PCR technique yielded numerous bands, some disappeared with respect to the negative control, others appeared while some varied in intensity. In the samples exposed to MX, both in vivo and in vitro, this phenomenon was present and generated a band pattern very different from that of the control. The same band pattern returned to being similar to the negative control following the simultaneous

exposure to the anti-oxidants. Particularly, in the non-treated samples, in vivo, there were bands between 200 and 850 bp (Table 2) and in vitro between 190 and 650 bp (Table 3). The polymorphic patterns generated by the RAPD profiles were used to calculate the genomic template stability (GTS, %). Changes in the RAPD profiles are expressed as decreases in the GTS. A genomic stability reduction in the samples exposed to MX at the various exposure times was found (Fig. 2). On the other hand, in the samples exposed to MX and antioxidant, especially Anthocyanin, the genomic stability increased with respect to the MX over the exposure time until, in some cases, it achieved 100 %.

### Discussion

Standard toxicity tests do not often provide a comprehensive picture of the effects MX may have upon release into the environment (Smital et al. 2004). The authors showed that MX can reduce multixenobiotic defense in aquatic organisms cells, inhibiting the activity of proteins that usually prevent the uptake of xenobiotic agents.

The adverse effects of this musk, following biotransformation and metabolism, was extensively studied in mice (Lehman-McKeeman et al. 1997) and the possible consequences on the nervous system, lungs, blood and gastrointestinal tract were evaluated in humans following inhalation, ingestion and contact with the eyes and skin (Kandyala et al. 2010).

Because the compounds released into the environment are potentially capable to interact with the genetic material, it is essential to understand the molecular and cellular events that occur between the exposure and the appearance of biological effects observed for a good interpretation of the ecosystem and health hazard associated with the presence of such contaminants. Since the alarming environmental risk associated with the presence of synthetic musks, the use of numerous and diverse biological testing is needed from which to obtain a more accurate environmental analysis (Depledge and Fossi 1994). Therefore, the simultaneous use of additional biomarkers is important to study the mechanisms by which environmental contaminants cause biological alterations (Regoli et al. 2004).

Some data present in the literature reported MX as cancerogenic (Maekawa et al. 1990; Apostolidis et al. 2002; Sarma et al. 2011). In addition, the great part of the evidences so far collected suggested that MX by itself is not genotoxic, but it may increase the genotoxicity of other chemicals (Taylor et al. 2014). In fish MX resulted embryotoxic (Carlsson and Norrgren 2004), cytotoxic and inhibited CYP1A at gene transcription level and EROD activity (Della Torre et al. 2011).

**Table 1** Percentage of DLEC viability<sup>(a)</sup> and cell viability in erythrocytes of *Danio rerio*<sup>(b)</sup> after different exposure times to Musk Xylene (10.0  $\mu\text{g L}^{-1}$ ) alone and with Vitamin E (1.0  $\text{mg L}^{-1}$ ) and with Anthocyanin (1.0  $\text{mg L}^{-1}$ )

Treatment	DLEC <sup>(a)</sup>		<i>Danio rerio</i> <sup>(b)</sup>	
	Exposure times (h)	% Viability*	Exposure times (days)	% Viability*
None			5	99.92 $\pm$ 0.09
	3	99.91 $\pm$ 0.08	7	99.54 $\pm$ 0.20
	24	99.85 $\pm$ 0.19	14	98.80 $\pm$ 0.65
	48	99.84 $\pm$ 0.18	21	99.05 $\pm$ 0.77
Musk Xylene			5	98.21 $\pm$ 1.26
	3	98.58 $\pm$ 0.75	7	97.63 $\pm$ 1.70
	24	98.30 $\pm$ 0.91	14	97.84 $\pm$ 1.47
	48	97.17 $\pm$ 2.46	21	98.51 $\pm$ 1.05
Musk Xylene + Vitamin E			5	97.98 $\pm$ 1.06
	3	98.64 $\pm$ 0.66	7	98.17 $\pm$ 1.03
	24	98.83 $\pm$ 0.79	14	98.33 $\pm$ 1.22
	48	99.11 $\pm$ 0.83	21	97.84 $\pm$ 1.79
Musk Xylene + Anthocyanin			5	98.18 $\pm$ 1.76
	3	99.51 $\pm$ 0.52	7	99.77 $\pm$ 0.15
	24	99.10 $\pm$ 0.97	14	99.04 $\pm$ 1.31
	48	99.77 $\pm$ 0.28	21	98.64 $\pm$ 1.88

\* The values are mean  $\pm$  standard-deviation

In this context, in which there are limited information about the actual genotoxicity of MX, the findings resulting from our study are placed. So far, however, there are no data in the literature both in vitro on marine fish cell lines and in vivo about genotoxicity of MX.

In the present analysis MX caused a significative genotoxic effect, revealed by all the three methods, in both experimental models and for each exposure time.

The information coming from each investigational methods used are particularly important to analyze the type of damage caused at the genomic level by MX at the concentration used. MX induced both DNA primary damage and apoptosis, as evidenced by Comet and Diffusion Assay. Besides the decrease of genomic stability due to appearance and/or disappearance of RAPD-PCR polymorphic band was due to changes in oligonucleotide priming sites related mainly to genomic rearrangements and less likely to point mutations or to DNA damage in the primer binding sites (Liu et al. 2005).

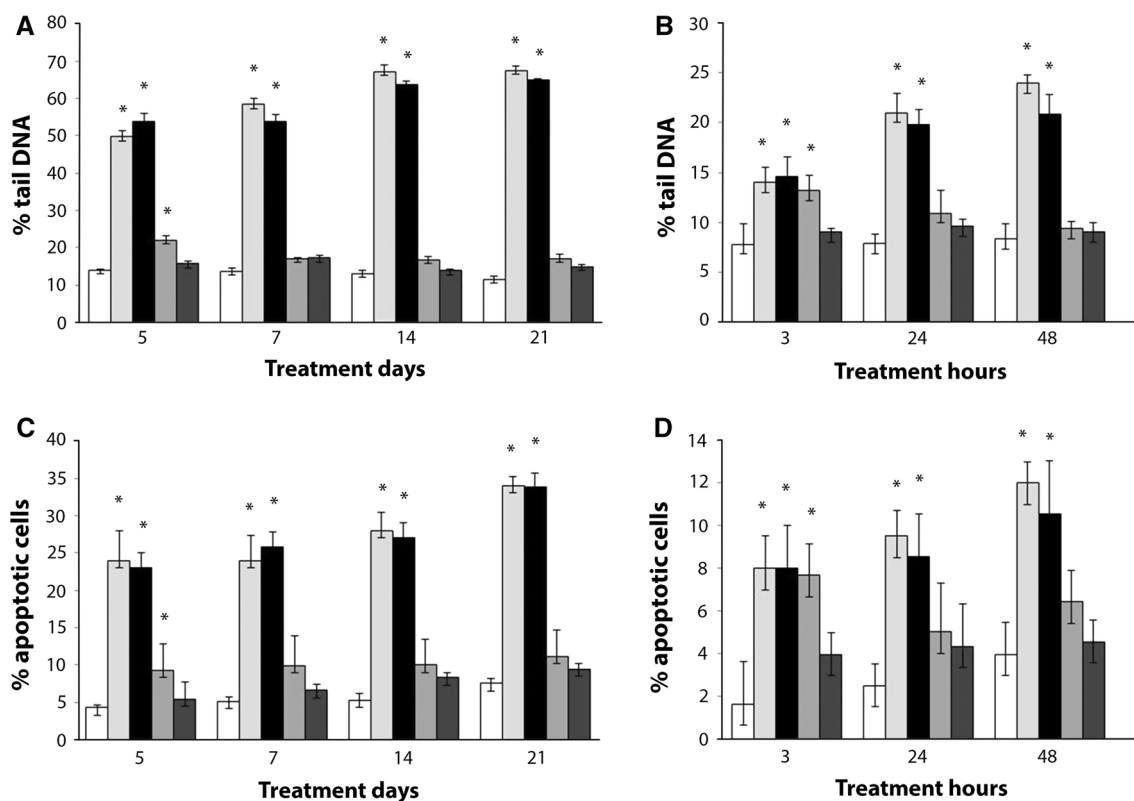
The genotoxicity of many organic compounds, as MX, is often expressed by the production of ROS generated during metabolism (Chen et al. 2000).

Antioxidants are molecules that work synergically to prevent oxidative damage to cell components such as DNA, lipids and protein. The mechanisms of action of these antioxidants at the molecular and cellular level include both roles in genetic expression and regulation, and in apoptosis and signal transduction.

The results of our study showed, both in vivo and in vitro, the antigenotoxic action of Vitamin E. We evidenced that the simultaneous exposure of MX and Vitamin E reduced the DNA damage induced by MX. Instead, RAPD-PCR technique showed that only at 14 days, the profiles obtained from the simultaneous in vivo exposure to the MX and Vitamin E possessed a notable correspondence of bands with the negative control, a similarity that tended to increase with exposure time and a GTS % near to 100 at 24 and 48 h for in vitro co-exposition. The efficiency of Vitamin E could be due to its numerous intrinsic properties, among which are the greater efficiency at recycling hydroxyl radicals, the best interaction with lipoperoxidic radicals by tocotrienols (Packer et al. 2001), inhibition of prostaglandin synthesis and prevention of platelet aggregation in vitro and in vivo (Azzi and Stocker 2000; Azzi et al. 2000).

However, the antigenotoxic effects of Vitamin E could be not assigned to anti-oxidant functions (Zingg and Azzi 2004). In fact, it has been demonstrated that  $\alpha$ -tocopherol possesses functions that are independent of its anti-oxidant/radical scavenger capacity. Among these, there are the effects of Vitamin E on protein kinases C, on cell proliferation and on the genetic expression of  $\alpha$ -tropomyosin (Rimbach et al. 2002).

In addition, it was demonstrated that Vitamin E reduced both the cell mortality and the intracellular level of ROS and the expression of p53, and significantly prevented the



**Fig. 1** Percentage of DNA in the tail of the comet and of apoptotic cells (ordinate) in erythrocytes of *Danio rerio* (a, c) and DLEC (b, d) after different exposure times (abscissa) to MX (10.0 µg L<sup>-1</sup>), Vitamin E (1.0 mg L<sup>-1</sup>) and Anthocyanin (1.0 mg L<sup>-1</sup>). The white

bars are negative controls; the light gray bars are positive controls; the black bars are MX treated samples; the middle gray bars are MX plus Vitamin E treated samples; the dark gray bars are MX plus Anthocyanin treated samples. \*p ≤ 0.05

**Table 2** Molecular sizes (bp) of appeared and disappeared bands by RAPD-PCR technique in *Danio rerio* DNA exposed to MX (10 µg L<sup>-1</sup>), Vitamin E (1 mg L<sup>-1</sup>), Anthocyanin (1 mg L<sup>-1</sup>) and Benzene (10 µl L<sup>-1</sup>) for different exposure times

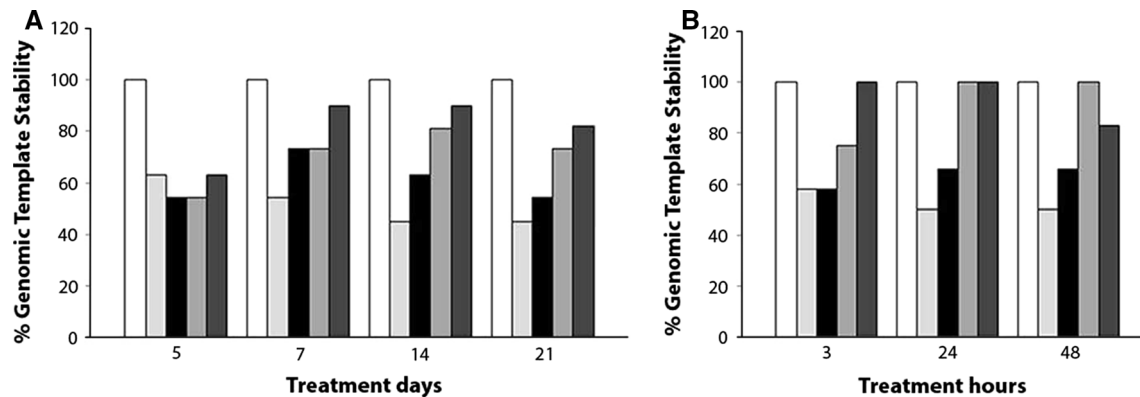
Treatment	Exposure times (days)	Appearance of new bands	Disappearance of control bands <sup>a</sup>
Musk Xylene	5	300,650	210,290,400
	7	–	210,290,550
	14	470	280,290,510
	21	300,620,820	310,420
Musk Xylene + Vitamin E	5	300	210,290,400,450
	7	–	210,290,450
	14	–	280,510
	21	300,500	450
Musk Xylene + Anthocyanin	5	300	210,290,400
	7	–	510
	14	–	280
	21	650	850
Benzene	5	250	200,450,850
	7	250,600	200,310,550
	14	350,650	280,310,450,850
	21	250,650	200,420,550,850

<sup>a</sup> Control bands are at: 200, 210, 280, 290, 310, 400, 420, 450, 510, 550, 850 bp

**Table 3** Molecular sizes (bp) of appeared and disappeared bands by RAPD-PCR technique in DLEC DNA exposed to MX ( $10 \mu\text{g L}^{-1}$ ), Vitamin E ( $1 \text{ mg L}^{-1}$ ) Anthocyanin ( $1 \text{ mg L}^{-1}$ ) and Benzene ( $1 \mu\text{l ml}^{-1}$ ) for different exposure times

Treatment	Exposure times (h)	Appearance of new bands	Disappearance of control bands*
Musk Xylene	3	150,180,550	380,400
	24	510,800	300,410
	48	550	210,500,650
Musk Xylene + Vitamin E	3	150,550	380
	24	–	–
	48	–	–
Musk Xylene + Anthocyanin	3	–	–
	24	–	–
	48	–	190,320
Benzene	3	450	210,350,400,650
	24	200,450,600	210,290,400
	48	450,550	190,210,290,380

\* Control bands are at: 190, 210, 290, 300, 320, 350, 380, 400, 410, 500, 520, 650 bp



**Fig. 2** Changes in percentage of Genome Template Stability (ordinate) in *Danio rerio* erythrocytes (a) and DLEC (b) after different exposure times (abscissa) to MX ( $10.0 \mu\text{g L}^{-1}$ ), Vitamin E ( $1.0 \text{ mg L}^{-1}$ ) and Anthocyanin ( $1.0 \text{ mg L}^{-1}$ ) as evidenced by RAPD-PCR

technique. The *white bars* are negative controls; the *light gray bars* are positive controls; the *black bars* are MX treated samples; the *middle gray bars* are MX plus Vitamin E treated samples; the *dark gray bars* are MX plus Anthocyanin treated samples

percentage of chromosomal aberrations induced by patulin (Ayed-Boussema et al. 2013). Thus, it could be deduced that prolonging exposure time and increasing the concentration, the anti-genotoxic and anti-oxidant action would be greater.

As regards the second antioxidant tested simultaneously with MX, on both experimental models, the bilberry anthocyanin enriched extract (Anthocyanin), the results from both the Comet assay and the Diffusion assay have shown that Anthocyanin reduced the DNA damage already at 3 h of exposure in DLEC and at 5 days in zebrafish. Moreover, its action tends to increase over time, reaching a peak, at 48 h and 21 days of exposure, respectively. Also the RAPD molecular analysis confirmed the results obtained from the other two tests. In fact, there is a possible

overlap between the electrophoretic profiles of the cells exposed simultaneously to the MX and Anthocyanin, and the negative control. Further confirmation came from the analysis of GTS (ranging between 90 and 100 %).

The antioxidant properties of bilberry anthocyanin enriched extract probably derive from its high content of polyphenols compounds, beyond the anthocyanins, all characterized for their strong reactivity as hydrogen or electron donor, and for the capacity of the radicales derived therefrom to stabilize and delocalize the unpaired electron, as well as to chelate transition metallic ions (termination of the Fenton reaction) (Shin et al. 2006). From the results of the present study, it has also been shown that Anthocyanin played an antigenotoxic role. In fact, intrinsic properties of the anthocyanin molecules, which could justify the action



of our investigated extract against genotoxic damage, are the capacity to modulate the activity of arachidonic acid, inhibiting the activity of phospholipases A2 (PLA2) and enzymatic activity or protein expression of cyclooxygenases (COX) and the lipooxygenases (LOX), the capacity to modulate the production of factor NF- $\kappa$ B, an inducible transcription factor expressed in various cell types and that plays a central role in the inflammatory response and in the innate immune response (Marzocchella et al. 2011).

The present study has brought to light two important aspects: the genotoxic effect of MX, and the more evident antigenotoxic action that Anthocyanin exhibited with respect to Vitamin E. In fact, in particular the results of RAPD-PCR technique showed that Vitamin E began to exert its antigenotoxic action in vivo only at 14 days of co-exposure and did not reset the genomic instability, a phenomenon that was evident when MX has been given with Anthocyanin. This could be explained by the fact that Vitamin E needs, due to its hydrophobicity, special transport mechanisms in plasma, body fluids and cells. In humans, for example, Vitamin E is reabsorbed in the proximal part of the intestine; it is emulsified with liposoluble elements of food and absorbed at the membrane level of the mucosa by means of passive diffusion (Brigelius-Flohé and Traber 1999). Another possible explanation might be simply related with the greater beneficial activity of polyphenol constituents of bilberry extract. It is known, for instance, that anthocyanins not only play a role as radical scavenger, but they also play a fundamental role in the protection against cardiovascular diseases by their anti-hypertensive, endothelium-protective and anti-atherogenic effects, in the control of growth/differentiation and suppression of tumors, and of cell cycle in the apoptosis induction (Domitrovic 2011).

Given the genotoxicity and mechanism of action of MX, we have demonstrated in this paper that both Vitamin E and Anthocyanin, in addition to a well-known anti-oxidant action, stabilizing radicals in such a way as to allow the natural defense systems of the cell to become activated, possess an antigenotoxic action, reducing DNA damage; thus providing stimulus for new studies in the field of genotoxicity. It can thus be hypothesized that the use of Vitamin E and Anthocyanin could reduce the toxic effects on human health due to the potential transfer of this environmental contaminant through food chain.

**Acknowledgments** We thank Dr. Ippolita Veronica Valentino for her technical help in the tests. We would like to express our gratitude to Dr. Antony Bridgewood for his assistance in revising the English of this manuscript.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflicts of interest.

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