Available online at www.sciencedirect.com

ScienceDirect

www.journals.elsevier.com/journal-of-environmental-sciences

JOURNAL OF ENVIRONMENTAL SCIENCES
www.jesc.ac.cn

Analysis of the genotoxic potential of low concentrations of Malathion on the *Allium cepa* cells and rat hepatoma tissue culture

Jaqueline Bianchi[1], *Mario Sérgio Mantovani*[2], *Maria Aparecida Marin-Morales*[1,*]

1. *Department of Biology, Institute of Biosciences, São Paulo State University (UNESP), Av. 24A, 1515, Bela Vista, Rio Claro, Brazil. E-mail: jaqueline_bianchi@yahoo.com.br*

2. *Department of General Biology, Biological Science Centre, Univ Estadual de Londrina, 86061990, Londrina, PR, 6001, Brazil*

ARTICLE INFO

Article history:
Received 30 December 2014
Revised 9 March 2015
Accepted 16 March 2015
Available online 11 July 2015

Keywords:
HTC
Chromosomal aberration
Micronuclei
Comet assay
Pesticide

ABSTRACT

Based on the concentration of Malathion used in the field, we evaluated the genotoxic potential of low concentrations of this insecticide on meristematic and F_1 cells of *Allium cepa* and on rat hepatoma tissue culture (HTC cells). In the *A. cepa*, chromosomal aberrations (CAs), micronuclei (MN), and mitotic index (MI) were evaluated by exposing the cells at 1.5, 0.75, 0.37, and 0.18 mg/mL of Malathion for 24 and 48 hr of exposure and 48 hr of recovery time. The results showed that all concentrations were genotoxic to *A. cepa* cells. However, the analysis of the MI has showed non-relevant effects. Chromosomal bridges were the CA more frequently induced, indicating the clastogenic action of Malathion. After the recovery period, the higher concentrations continued to induce genotoxic effects, unlike the observed for the lowest concentrations tested. In HTC cells, the genotoxicity of Malathion was evaluated by the MN test and the comet assay by exposing the cells at 0.09, 0.009, and 0.0009 mg/5 mL culture medium, for 24 hr of exposure. In the comet assay, all the concentrations induced genotoxicity in the HTC cells. In the MN test, no significant induction of MN was observed. The genotoxicity induced by the low concentrations of Malathion presented in this work highlights the importance of studying the effects of low concentrations of this pesticide and demonstrates the efficiency of these two test systems for the detection of genetic damage promoted by Malathion.

Introduction

Pesticides are used to control the proliferation of unwanted organisms and frequently end up into the environment (Galloway and Handy, 2003). As a result, many chemicals may affect species that are not their intended target.

Malathion, O,O-dimethyl-S-(1,2-dicarboethoxyethyl) phosphorodithioate ($C_{10}H_{19}O_6PS_2$), is a non-systemic organophosphate insecticide that is widely used in many countries of the world both in agriculture and in the eradication of disease-carrying insects (Flessel et al., 1993). It has a neurotoxic effect that kills insects by inhibiting the enzyme acetylcholinesterase

* *Corresponding author*. E-mail: mamm@rc.unesp.br (Maria Aparecida Marin-Morales).

http://dx.doi.org/10.1016/j.jes.2015.03.034
1001-0742/

(Kwong, 2002). Because of its widespread use, Malathion can be found even in drinking water and food (Galloway and Handy, 2003; John et al., 2001). According to Bolognesi and Morasso (2000), residues of pesticides in food and drinking water have a great potential to cause long-term changes in DNA, promoters of carcinogenesis. Some evidence suggests that the human exposure to organophosphate concentrations below those that cause inhibition of acetylcholinesterase can cause several health effects (Salvi et al., 2003; Singh and Sharma, 2000).

According to Cox (2003), Malathion is a potent contaminant of water and air. Of the 36 river basins monitored by the U.S. Geological Survey, 34 were contaminated with residues of Malathion. They also found that urban streams are more contaminated and contain higher concentrations of Malathion than agricultural streams. Furthermore, it was verified that Malathion can also contaminate the groundwater (Cox, 2003; Laine and Cheng, 2007). Studies performed by the U.S. Department of Agriculture (USDA, 2000), indicated that the application of Malathion near the water resources causes toxicity in aquatic organisms as vertebrates, amphibious and fishes. The contamination of soil by Malathion can also occur. However, the rate of its degradation will vary with the moisture/alkaline conditions (Patty, 1963).

Due to runoff and agricultural application, Malathion is frequently found in the aquatic environment in low concentrations. In Egypt, Abdel-Halim et al. (2006) evaluated the presence of several organophosphates in a drainage canal surrounding a pesticide factory and found residues of Malathion ranging from 71.9 to 466 ppb. The presence of Malathion was also detected in the studies of Sankararamakrishnan et al. (2005) in surface water samples of the River Ganges in Kanpur with a concentration of 2.6 μg/L. In the ground water located in agricultural and industrial areas, the authors detected Malathion with a maximum concentration of 29.8 μg/L.

Plant species, because they are direct recipients of biological pesticides, are important organisms for genetic testing and environmental monitoring (Ma et al., 1995). Among the plants used for this purpose, *Allium cepa* has the advantage of having few (2n = 16) and large chromosomes. In addition, an enzyme oxidase system, which is crucial to detect promutagens, is present in this species (Fiskejö, 1985). Several studies have evaluated the genotoxic action of pesticides by the chromosomal aberration (CA) and micronuclei (MN) tests in meristematic cells of *A. cepa* and have found positive results with the induction of different types of alterations, including those of clastogenic (chromosome breaks and bridges) and aneugenic origin (chromosome loss) (Ateeq et al., 2002; Chauhan et al., 1998; Fernandes et al., 2009; Rank et al., 1993; Sinha and Kumar, 2014; Srivastava and Mishra, 2009), showing that this test system is an appropriate model for these evaluations.

On the other hand, both *in vivo* and *in vitro* tests are often used to evaluate pesticide-induced DNA damage in animals (Das et al., 2007; Kumar et al., 2010; Soloneski and Larramendy, 2010). The *in vitro* tests are reproducible, rapid, and sensitive (Rogero et al., 2003) and offer a good alternative to tests performed on live animals, which require a large number of individuals (Freshney, 2005). The HTC cells are a good *in vitro* system to detect DNA damage caused by mutagens that are dependent on metabolic activation without the need for an exogenous metabolizing system (Dean et al., 1980; Marcarini et al., 2011; Tsuboy et al., 2010). Genotoxicity assays performed with these cells presented positive responses in studies that assessed the effects of environmental compounds, such as pesticides (Almeida et al., 2008; Malatesta et al., 2008), industrial effluents (Manzano et al., 2015) and mixture of environmental pollutants derived from petroleum (Mazzeo et al., 2013).

This study aimed at investigating the effectiveness of a plant test system and a cultured mammalian cell to detect genotoxic effects of low concentrations of Malathion.

1. Materials and methods

1.1. Test models

A. cepa seeds and HTC cells were used as test organisms in the genotoxicity assays of the insecticide Malathion. Seeds of *A. cepa* (TopSeed, Agristar of Brazil LTDA) (2n = 16) from the same batch and variety (Baia Periform) were commercially acquired. The HTC cells, derived from *Rattus norvegicus* hepatoma, were purchased from the Cell Bank of Rio de Janeiro/Brazil (UFRJ).

1.2. Chemical substances

The chemical used was the commercially available pesticide formulation containing Malathion (Malathion 500 CE®, *w/w* 50%, Dipil Chemical Industry Ltda - Massaranduba, SC, Brazil). For the assays using *A. cepa*, 1.5, 0.75, 0.37, and 0.18 mg/mL of Malathion were employed. In tests with HTC cells, the non-cytotoxic concentrations of 0.09, 0.009, and 0.0009 mg/5 mL culture medium were used. The Malathion concentrations tested here were defined based on the concentration used in the field (1.5 mg/mL). Using it as a starting point, serial dilutions 1:1 in Milli-Q water were obtained and thus the final concentrations were defined as low concentrations. Since all concentrations tested on *A. cepa* were cytotoxic to HTC cells (data not shown), the concentrations tested on those cells were obtained from a 1:1 dilution of the lowest concentration tested on *A. cepa* (0.18 mg/mL).

Benzo[a]pyrene (20 μg/mL, CAS No. 50-32-8, Sigma, Sigma-Aldrich Brasil Ltda, SP, Brazil) was used as a positive control (PC) in tests using the HTC cells, and methyl methanesulfonate (MMS, 10 mg/L, CAS No. 66-27-3, Aldrich, Sigma-Aldrich Brasil Ltda, SP, Brazil) was used as the PC on a test employing *A. cepa*. All substances were diluted in Milli-Q water and prepared at the time of use.

1.3. *A. cepa* assays

Tests using *A. cepa* were performed according to the protocol outlined by Grant (1982). After germination of seeds in Milli-Q water, radicles measuring about 1 cm in length were exposed to different concentrations of Malathion and to MMS, and were left there for 24 hr. After exposure, 1/3 of the roots were collected (24 hr treatment), another portion remained exposed to the test substances for an additional 24 hr (48 hr treatment), and a third portion was transferred to Petri dishes

containing Milli-Q water for 48 hr to evaluate whether the damage induced by the insecticide on the cells of *A. cepa* is minimized after the removal of Malathion from the growth medium of the radicles (48 hr of recovery time). The exposure times evaluated in this study were chosen based on the duration of the cell cycle of *A. cepa* that is approximately 20 hr for the meristematic cells (Grant et al., 1981). The radicles maintained in Milli-Q water were used as negative control (NC).

After fixation in alcohol-acetic acid (Merck) for 6 hr, the radicles were subjected to the Schiff's reactive, which reacts specifically with DNA, as described by Feulgen and Rossenbeck (1924) apud Mello and Vidal (1978). The meristematic and F_1 regions of the radicles were covered with cover slips and carefully squashed with a drop of 2% acetic carmine (Vetec) to enable better visualization of individual cells by its rosy pigment that eases the delimitation of the cytoplasm and of the cell contour.

The analysis was performed by counting 500 cells per slide, totaling 5000 cells per treatment. The cytotoxic effect was ascertained by evaluating changes in the MI, and the genotoxic effects were evaluated by the presence of CA and MN in all the mitosis stages (*i.e.*, interphase, prophase, metaphase, anaphase, and telophase).

All tests were performed in duplicate, and the results were analyzed by Mann–Whitney test ($p < 0.05$).

1.4. Assays with HTC

For the MN and comet assay tests, approximately 1×10^6 HTC cells were seeded on 25 cm^2 culture flasks containing 5 mL Dulbecco's modified Eagle's medium/F12 (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) (complete culture medium). Cells were incubated at 37°C for 24 hr before being subjected to the treatments (Tsuboy et al., 2010), and then exposed for 24 hr to different concentrations of Malathion and benzo[a]pyrene. The NC was performed with complete culture medium.

1.4.1. Micronuclei test with cytokinesis block

After 24 hr of exposure to the treatments, the medium was discarded and the cells were washed twice with phosphate-buffered saline (PBS). Then, 5 mL of complete culture medium and 50 μL of a final solution of cytochalasin B 300 μg/mL (Sigma Aldrich) were added. The cells remained in this medium for 26 hr to induce binucleated cells (Marcarini et al., 2011) and then, they were harvested as described by Oliveira et al. (2006). The slides were stained with Giemsa 5% (Merck) in PBS for 10 min. Three replicates were conducted for each treatment and about 1000 binucleated cells of each replicate were analyzed, totaling approximately 3000 cells per treatment. The criteria for selection of binucleated cells and the identification of MN followed the description given by Fenech (2000). The significance of the results was ascertained using the Mann–Whitney test ($p < 0.05$).

1.4.2. Comet assay

The comet assay was performed according to the protocol described by Tice et al. (2000), with minor modifications as described in Marcarini et al. (2011). Briefly, after a 24 hr exposure to the treatment with Malathion, cells were washed with PBS, trypsinized (500 μL trypsin-EDTA 0.025%, 37°C, Gibco), centrifuged (1000 r/min, 5 min), and re-suspended in 500 μL of culture medium. The cell suspension was mixed with low melting point agarose (Invitrogen) and dropped onto pre-gelatinized microscope slides with normal melting point agarose (Invitrogen). The slides were immersed in a lysis solution for 1 hr at 4°C in the absence of light. After denaturation (20 min) and alkaline electrophoresis (0.9 V/cm, 20 min), the slides were neutralized, dried at room temperature, and fixed in ethanol (Merck) for 10 min. The slides were stained with 30 μL of a solution of Ethidium bromide 1 × (CAS No. 1239-45-8, Sigma Aldrich) obtained from a 10 × stock solution (200 μg/mL) and examined immediately using a fluorescence microscope with a 515–560 nm excitation filter and a 590 nm barrier filter at a 400× magnification.

DNA damage analysis was performed by counting 100 cells per replicate totaling 300 cells per treatment. The cells were visually classified according to the size of their tails into four classes, according to Kobayashi et al. (1995): class 0: no damage; class 1: small damage; class 2: medium damage; and class 3: big damage. An overall score (arbitrary units) was calculated for each slide by applying the formula: (percentage of cells in class 0 × 0) + (percentage of cells in class 1 × 1) + (percentage of cells in class 2 × 2) + (percentage of cells in class 3 × 3) (Azqueta et al., 2011). The results were statistically analyzed by the Mann–Whitney test ($p < 0.05$).

Concomitant with the comet assay, cell viability tests were carried out for all treatments. To this, 20 μL of cell suspension from each treatment was mixed with 20 μL 0.4% Trypan Blue (Gibco) and transferred to a Neubauer chamber. Viable (white) and non-viable (stained blue) cells were quantified. The cell viability indexes were obtained considering the percentage of viable cells in the total cells analyzed. A limit of 80% of viable cells was considered to perform the comet assay (Manzano et al., 2015).

2. Results

2.1. A. cepa tests

The results of the *A. cepa* tests are shown in Table 1. The analysis of the MI of *A. cepa* cells showed a significant reduction in dividing cells for the concentration 0.75 mg/mL of Malathion after 24 hr of exposure ($p = 0.003$). No cytotoxic effect was observed for the other tested concentrations of Malathion. In the genotoxicity assays, all concentrations of Malathion presented higher frequencies of CA and MN than those observed for the NC, although not all differences were statistically significant. In the exposures of 24 and 48 hr, a slight increase in the values of MN in meristematic cells in a dose-dependent manner was verified. During 24 hr of exposure, significant values of CA and MN were induced by the concentrations 1.5, 0.75, and 0.18 mg/mL of Malathion. During 48 hr of exposure, significant MN values were induced by the concentrations 1.5, 0.75, and 0.37 mg/mL of Malathion and significant CA values were induced by the concentrations 1.5 and 0.18 mg/mL. After the 48 hr recovery, it was observed that the cells of *A. cepa* exposed to the concentrations 1.5 and 0.75 mg/mL of Malathion continued

Table 1 – Mitotic index, chromosomal aberrations, and micronuclei in *A. cepa* cells exposed to different concentrations of the insecticide Malathion (mg/mL).

Exposure times	Treatments	MI (%)	CA	MN-M	MN-F_1
24 hr	NC	15.51	1.25 ± 1.52	0.75 ± 0.79	0.55 ± 0.82
	1.5	13.92	2.70 ± 2.03* (0.01)	6.85 ± 5.53* (0.00)	7.25 ± 7.61* (0.00)
	0.75	09.64*	2.80 ± 1.76* (0.00)	3.35 ± 4.51* (0.02)	1.90 ± 2.97 (0.06)
	0.37	14.87	2.10 ± 1.44 (0.05)	1.60 ± 1.70 (0.09)	0.55 ± 0.82 (1.00)
	0.18	14.39	2.60 ± 1.73* (0.01)	0.85 ± 1.14 (0.88)	2.60 ± 2.70* (0.00)
	MMS	–	4.05 ± 2.43* (0.00)	26.25 ± 12.45* (0.00)	13.80 ± 9.72* (0.00)
48 hr	NC	13.51	0.85 ± 1.09	0.81 ± 1.32	0.95 ± 1.15
	1.5	16.22	1.95 ± 1.73* (0.03)	6.10 ± 4.51* (0.00)	6.75 ± 5.32* (0.00)
	0.75	13.77	1.85 ± 1.75 (0.06)	6.00 ± 6.50* (0.00)	4.15 ± 3.72* (0.00)
	0.37	15.00	1.60 ± 1.60 (0.15)	5.60 ± 9.27* (0.00)	7.65 ± 13.61* (0.00)
	0.18	13.10	1.75 ± 1.41* (0.03)	1.05 ± 1.00 (0.15)	1.05 ± 1.60 (0.96)
	MMS	–	4.10 ± 2.61* (0.00)	45.15 ± 31.55* (0.00)	40.45 ± 41.13* (0.00)
Recovery	NC	12.25	0.85 ± 1.04	0.90 ± 1.25	0.95 ± 1.35
	1.5	13.50	1.70 ± 1.17* (0.02)	4.55 ± 4.08* (0.00)	6.55 ± 7.66* (0.00)
	0.75	12.22	1.75 ± 1.16* (0.01)	4.20 ± 5.10* (0.01)	4.65 ± 5.49* (0.00)
	0.37	10.52	1.55 ± 1.70 (0.24)	0.95 ± 1.19 (0.87)	1.60 ± 1.87 (0.21)
	0.18	11.22	1.50 ± 1.35 (0.14)	1.60 ± 0.90 (0.64)	1.70 ± 4.50 (0.81)
	MMS	–	2.15 ± 1.63* (0.00)	13.95 ± 13.44* (0.00)	27.85 ± 27.21* (0.00)

MI: mitotic index; CA: chromosomal aberrations; MN: micronuclei; MN-M: micronuclei on the meristematic region; MN-F_1: micronuclei on the F_1 region.
Data are presented as mean ± standard deviation with *p*-value in parentheses.
* Statistically different from NC ($p < 0.05$).

to be affected by this insecticide, presenting significant rates of CA and MN.

Table 2 and Fig. 1 illustrate the various types of CA induced by Malathion on *A. cepa* root cells in all stages of the cell cycle (interphase, prophase, metaphase, anaphase, and telophase). The CAs induced by Malathion from clastogenic origin (chromosomal breaks and bridges) were more frequent than the CAs from aneugenic origin (chromosome loss, multipolar anaphases, C-metaphases, polyploid cells, chromosome stickiness, binucleated cells, and laggard chromosomes). Interphasic cells with small nuclear buds were also observed.

2.2. Comet assay and micronuclei in hepatoma tissue culture cells

The results of the MN test are summarized in Table 3. None of the three concentrations of Malathion tested induced significant values of MN in the HTC cells. The *p*-values obtained in the statistical tests were $p = 0.1904$ for the concentrations 0.09 and 0.009 mg/5 mL medium and $p = 0.3827$ for the concentration 0.0009 mg/5 mL medium. The significant induction of MN in HTC cells exposed to benzo[a]pyrene ($p = 0.0495$) indicated that these cells responded efficiently to the test.

In the comet assay (Table 4), the scores obtained showed a significant damage in the DNA of the HTC cells after exposure to the three concentrations of Malathion that were tested ($p = 0.0495$ for all treatments). Most of the damage induced by Malathion was classified as small (class 1), corresponding to approximately 78% of the damage for the concentration 0.09 mg/5 mL medium; 79% for the concentration 0.009 mg/5 mL medium; and 74% for the concentration 0.0009 mg/5 mL medium.

3. Discussion

Most of the chemical residues found in the environment end up in the water resources where they become increasingly diluted. As a consequence, they are often found at very low concentrations (Trapp, 2000, 2002). Therefore, when trying to assess the effects of environmental exposure to a given compound, analyses using high concentrations are not very relevant (Cavallo et al., 2010).

Many studies using pesticides employ animals (*in vivo* or *in vitro*) as test systems to evaluate the effects of these compounds on the DNA. However, the use of higher plants as test organisms in the genotoxicity of pesticides has grown substantially (Valencia-Quintana et al., 2013). Plants constitute an important and excellent material for genetic and monitoring tests of these contaminants, and the root meristematic cells are the most suitable for these analyses (Ma et al., 1995).

In the present study, positive results in the CA and MN tests on *A. cepa* root cells and in the comet assay on the HTC cells were induced by the insecticide Malathion.

The analyses of CA and MN on *A. cepa* cells show that the chromosomal damage observed was persistent since it was possible to ascertain that the damage was passed on to the next generation of cells, which could be visualized in the form of MN. Such inference is based on the observation that the same concentrations that induced CA in the 24 hr of exposure also induced significant levels of MN in the meristematic and/or F_1 cells of *A. cepa*. In addition, during the 48 hr of exposure, it was observed that in general there was a slight decrease in the values of CA and an increase in the values of MN in the meristematic and F_1 cells, in comparison with the values

Table 2 – Frequency (%) of the types of CA induced by the different concentrations of the Malathion (mg/mL) in meristematic cells of *A. cepa*.

		Break	Bridge	Loss	Multipolar anaphase	C-metaphase	Polyploidy	Stickiness	Binucleated	Laggard	Nuclear bud	Total number of CA[a]
24 hr	NC	0	32.00	0	0	4.00	4.00	12.00	4.00	0	44.00	25
	1.5	11.11	31.48	9.26	0	0	3.70	1.85	1.85	0	40.74	54
	0.75	5.35	26.78	8.93	1.78	0	1.78	10.71	0	0	44.64	56
	0.37	4.76	38.09	2.38	2.38	0	2.38	9.52	0	0	40.48	42
	0.18	1.92	48.08	3.85	1.92	3.85	3.85	7.69	1.92	1.92	25.00	52
	MMS	25.92	20.99	14.81	1.23	1.23	0	1.23	0	2.47	32.10	81
48 hr	NC	0	52.94	0	0	11.76	0	11.76	0	5.88	17.64	17
	1.5	2.56	74.36	5.13	0	2.56	2.56	0	2.56	0	10.26	39
	0.75	0	56.76	2.70	5.40	0	0	10.81	0	0	24.32	37
	0.37	6.25	68.75	3.12	0	6.25	0	3.12	0	0	12.50	32
	0.18	5.71	65.71	2.86	0	0	0	2.86	0	0	22.86	35
	MMS	30.49	28.05	9.76	1.22	3.66	2.44	2.44	1.22	0	20.73	82

a In a total of 10,000 cells analyzed per concentration.

observed for the 24 hr of exposure. These observations show that in 48 hr of exposure to Malathion there was no induction of more CA in *A. cepa* cells and that the high values of MN observed for this exposure time are resulted from the damage induced and unrepaired by the cells during the 24 hr of exposure. Given the results found for *A. cepa*, it can be emphasized that the analysis of CA and MN in both regions of the root (meristematic and F_1) is an important tool to assess the genotoxic potential of Malathion. The data obtained should be complementary, since the meristematic region of the *A. cepa* consists of both dividing cells as cells that have undergone the division process, whereas the F_1 region consists of daughter cells resulting from cell divisions that occurred in the meristematic region (Ma et al., 1995).

Although the positive results of genotoxicity found in the present study corroborate the results found by other authors who assessed the effects of Malathion in plants, the predominant types of CA found in each study were different from each other. Asita and Makhalemele (2009) attributed the genotoxicity of Malathion to *A. cepa* cells, to the induction of

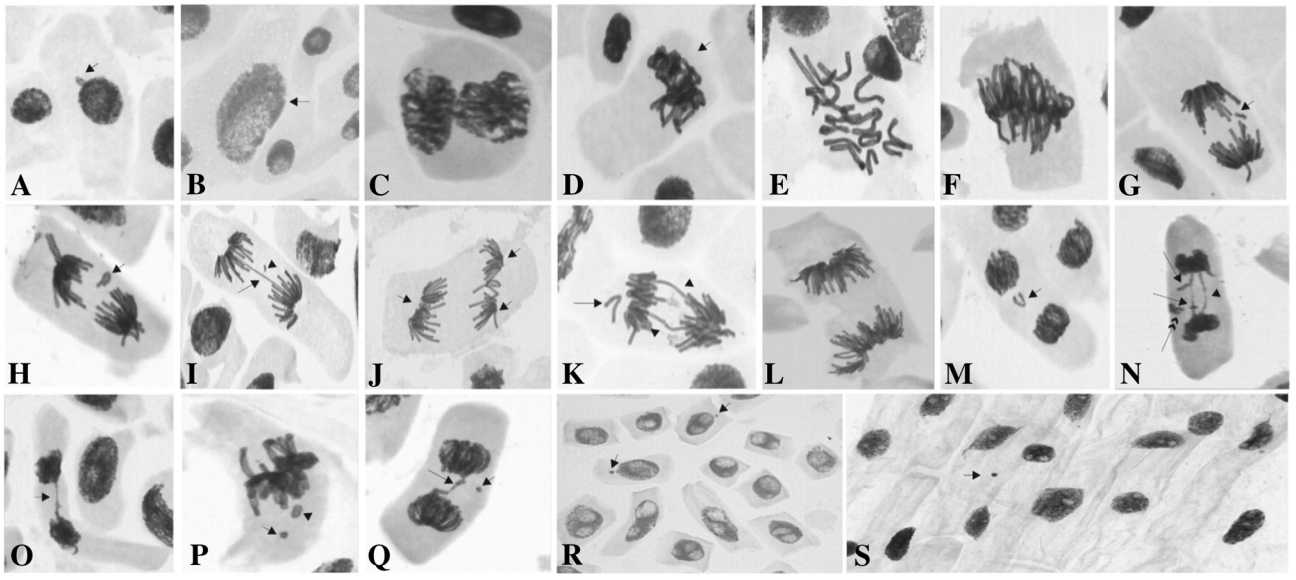

Fig. 1 – Chromosomal aberrations and micronuclei induced by different concentrations of Malathion in *Allium cepa* cells. (A) Nuclear bud (arrow); (B) cell with polyploid nucleus (arrow); (C) binucleated prophase; (D) metaphase with chromosomal stickiness; (E) C-Metaphase; (F) polyploid metaphase; (G) anaphase with chromosomal break (arrow); (H) anaphase with chromosomal loss (arrow); (I) anaphase with bridge (arrow) and chromosomal break (arrow head); (J) multipolar anaphase (arrows); (K) anaphase with bridge (arrow heads) and chromosomal loss (arrow); (L) polyploid anaphase; (M) telophase with chromosomal loss; (N) telophase with bridge (arrow head), chromosomal breaks (arrows) and chromosomal loss (arrow with double head); (O) telophase with chromosome bridge (arrow); (P) metaphase with MN (arrow) and chromosomal loss; (Q) telophase with MN (arrow) and chromosome bridge (arrow head); (R) cell on interphase with MN (arrow); (S) cells of the F_1 region with MN (arrow). Magnification: 400×.

Table 3 – Micronuclei in hepatoma tissue culture cells exposed for 24 hr to the insecticide Malathion and benzo[a]pyrene.

Treatment	Concentration	Micronuclei
NC	–	4.0 ± 1.00
Malathion	0.09 (mg/5 mL medium)	7.3 ± 2.87
	0.009 (mg/5 mL medium)	7.7 ± 3.21
	0.0009 (mg/5 mL medium)	5.7 ± 2.31
Benzo[a]pyrene	20 (μg/mL)	14.3 ± 1.53 *

Data are presented as mean ± standard deviation.
* Statistically different from the NC ($p < 0.05$).

lagging chromosomes. In *Vicia faba* root tip cells, various types of CA were induced by Malathion, such as bridges, losses, adherences, and multinucleated cells. However, most of the CA induced was the chromosomal adherence (Adam et al., 1990). In *Tradescantia* cells, Ma et al. (1983) found no genotoxicity of Malathion when the plant was exposed to the insecticide in a greenhouse. However, they observed a significant induction of MN when the plants were exposed to Malathion in gaseous form, leading to the conclusion that the use of *Tradescantia* is more appropriate for assessing the effects of the compounds present in the air.

In the present study, most of the CA induced by Malathion was derived from a clastogenic effect, principally due to the high induction of chromosomal bridges, as shown in Table 2. This result corroborates the studies of Moore et al. (2011), who found, by the CA test, that Malathion is potentially clastogenic to the bone marrow cells of Sprague–Dawley rats, and studies of Giri et al. (2002), who showed the same effects of Malathion, but by observing the induction of chromosomal breaks in bone marrow cells of mice.

Chromosomal fragments can result from DNA double-strand breaks (Hartlerode and Scully, 2009; O'Donovan and Livingston, 2010), events of base excision repair (Bull and Fenech, 2008; Dianov et al., 1991; Fenech and Crott, 2002), or breaks of nucleoplasmic/anaphasic bridges (Hoffelder et al., 2004; Shimizu et al., 2005), which can turn into MN. According to Humphrey and Brinkley (1969), the anaphasic chromosomal bridges can result from structural changes between sister chromatids or between different chromosomes due to breaks and terminal deletions or by adherence of ribosomes or persistent nucleoli.

Cells with nuclear buds were also observed in the present study. These alterations may be related to the initial process of MN formation by the elimination of extra genetic material (Fernandes et al., 2007) or aggregation of a delayed chromosome by the nuclear envelope, before being totally reincorporated to the nucleus (Serrano-García and Monteiro-Montoya, 2001), as a transient effect after which the normal morphology of the cell is restored.

In the recovery test, it was realized that the higher concentrations (1.5 and 0.75 mg/mL) of Malathion induced a persistent effect on the *A. cepa* cells. This was evident by the significant presence of CA and MN in the cells after the exposure to Malathion had been discontinued.

Although it has been shown that the tested concentrations of Malathion induce genotoxic damages in cells of *A. cepa*, the non-relevant results of cytotoxicity (with the MI being just affected by the concentration 0.75 mg/mL in 24 hr of exposure) may indicate that such damage does not really affect the cell division. Studies carried out by different authors about the cytotoxicity of Malathion in higher plants have found either an increase (Adam et al., 1990) or a decrease (Asita and Makhalemele, 2009) in the MI of the analyzed cells. Rank (2003), when evaluating the MI of *A. cepa* cells exposed to both tap water and synthetic fresh water and to Methyl methanesulfonate, observed a great variation in the results. Due to this fact, the author has questioned the use of the MI in the evaluation of the toxicity in *A. cepa* cells and suggested that the MI analysis should be done only to verify whether there is a sufficient amount of mitotic cells to perform the CA analysis.

MMS, an alkylating agent used in this study as PC, has induced significant levels of CA and MN in the *A. cepa* genotoxicity assays, as expected. In the cytotoxicity tests, the high MI observed in *A. cepa* cells exposed to MMS (data not presented) raised the question about using this agent as PC in these tests. As a general rule, it is expected that an alkylating agent, such as MMS, causes an inhibitory effect on the MI of the cells. However, some studies have shown that MMS did not induce any significant cytotoxic effect on the *A. cepa* cells (Bolle et al., 2004; Leme and Marin-Morales, 2008; Rank and Nielsen, 1997; Rank and Nielsen, 1998).

Table 4 – Results of the comet assay in hepatoma tissue culture cells exposed for 24 hr to the insecticide Malathion.

Treatments	Classes				Score [a]
	0	1	2	3	
NC	95 ± 4.3	4.3 ± 4.9	0	0	4.3 ± 4.9
0.09 mg/5 mL medium	85.3 ± 6.4	10.7 ± 4.6	3.0 ± 1.7 *	0	16.7 ± 8.1 *
0.009 mg/5 mL medium	79.3 ± 9.7	15.0 ± 7.0	3.0 ± 1.0 *	1.0 ± 1.7	24.0 ± 12.1 *
0.0009 mg/5 mL medium	71.7 ± 13.6 *	20.7 ± 2.5 *	4.7 ± 7.2	2.7 ± 3.8	38.0 ± 27.7 *
Benzo[a]pyrene	0.3 ± 0.6 *	11.6 ± 9.6	34.7 ± 28.5 *	50.0 ± 35.5 [a]	231.0 ± 40.3 *

Mean ± standard deviation.
Classes: classify the comets according to the length of their tails: Class 0: - no tail, no visible damage to the DNA; Class 1: tail length smaller than the diameter of the nucleoid; Class 2: tail length between 1 and 2 times the diameter of the nucleoid; Class 3: tail length greater than 2 times the diameter of the nucleoid.
* Statistically different from the NC ($p < 0.05$).
a Score = (percentage of comets in class 0 × 0) + (percentage of comets in class 1 × 1) + (percentage of comets in class 2 × 2) + (percentage of comets in class 3 × 3).

Unlike the results found in the tests with *A. cepa*, the tests performed with HTC cells showed no significant induction of MN for the three tested concentrations of the Malathion. However, a genotoxic effect was verified by the comet assay. Based on the results of this assay, it can be inferred that the tested concentrations induced mild genotoxic effects that were subsequently repaired, since most of the damage observed was small (class 1). The comet assay results also demonstrated that lower concentrations of Malathion induced more damage to the DNA than the highest concentration tested. Studies performed by Au et al. (1988, 1990) and Cavallo et al. (2010) showed that higher concentrations of a certain compound can induce less damage to the DNA than the lower concentrations. The authors explain this by the higher activity of the antioxidant enzymes in response to the high potential of inducing damage of the highest concentration. Some authors (Banerjee et al., 1999; Etemadi-Aleagha et al., 2002; Nordberg and Arnér, 2001; Rai and Sharma, 2007; Rai et al., 2009) have showed that pesticides can induce oxidative stress in mammal cells, leading to an increase in lipid peroxidation and causing a significant increase in the activities of the antioxidant enzymes. Although no tests for oxidative stress have been carried out in this study, the effect observed by the comet assay could be related with the oxidative responses suffered by the cells when they were exposed to low concentrations of the insecticide. According to Moore et al. (2010), Malathion was able to induce oxidative stress in human cells cultured *in vitro*, causing lipid peroxidation.

Another hypothesis that could explain the induction of higher damage in the DNA by the lowest concentrations of Malathion as observed in the comet assay would be a possible induction of crosslinks. According to Hartmann et al. (2001), a higher concentration of a given compound can induce a minor migration of the DNA in the comet assay if the agent studied is a potential inducer of crosslinks. The DNA repair induced by the lowest concentrations can lead to higher rates of DNA migration, whereas the high amount of crosslinks induced by the highest concentrations can lead to a decrease in the DNA migration. According to Blasiak et al. (1999); Giri et al. (2002); Shadnia et al. (2005), the oxidative stress, the alkylation, and the immunotoxicity are some of the mechanisms by which the organophosphates can induce their genotoxic effects and, according to Nackerdien et al. (1991), the induction of crosslinks in the chromatin of the cells is among the factors that can lead to oxidative stress.

In the MN tests, the non-significant results are possibly related to the repair mechanism of the HTC cells for the tested concentrations of Malathion, which prevented the fixation of the induced damage and its subsequent visualization in the form of MN. According to Valentin-Severin et al. (2003), the difference between the MN test and the comet assay is basically the type of change that these tests detect. The comet assay detects primary DNA lesions that can be repaired, whereas the MN test detects irreparable lesions. Since the comet assay is very effective in revealing the primary DNA damage, it is able to provide useful information on the effect of short-term exposures to low concentrations of certain compounds (Cavallo et al., 2010).

Different types of cells and organisms can exhibit different and specific responses to the effects of one substance. In addition, the responses may also vary according to changes in exposure time, kinds of treatments, and concentrations tested. Some researchers have investigated the potential induction of MN by the insecticide Malathion in *in vitro* systems, and the results have often been conflicting. The results for MN induction were positive for bone marrow cells of mice (Dulout et al., 1982), peripheral blood cells of fish (Kumar et al., 2010), and human lymphocytes (Titenko-Holland et al., 1997). Negative results, however, were observed for whole human blood (Titenko-Holland et al., 1997) and peripheral blood of Wistar rats subjected to acute treatment with Malathion (Réus et al., 2008). Despite such inconsistencies regarding the induction of MN by Malathion, the commercial form of this insecticide is considered genotoxic in *in vitro* assays (Blasiak et al., 1999).

For studying the genotoxic effects of the commercial form of Malathion, the best test systems are those that are able to metabolize xenobiotics, as the Malathion needs bioactivation to produce its toxic effects (ATSDR, 2003; Buratti et al., 2005; Sultatos, 1994). The HTC cells have high metabolic activity (Gad, 2000) and because they have both phase I and phase II enzymes of xenobiotic metabolizing process (Mantovani et al., 2008; Oliveira et al., 2006), they also have the ability to metabolize drugs (Marcarini et al., 2011; Tsuboy et al., 2010). Similarly, *A. cepa* cells have important enzymes that metabolize chemicals (Fiskejö, 1985), as a mixed-function oxidase system, which is essential to the activation of pro-mutagens into mutagens (Vig, 1978).

The difference between the MN results in hepatocytes cultured *in vitro* and *A. cepa* cells also was observed by Feretti et al. (2008). The authors evaluated the genotoxicity of chlorite and chlorate ions in *A. cepa* cells and human hepatocytes cultured *in vitro* (HepG2 cells) and found MN induction just in cells of *A. cepa*. According to the authors, the detoxification of xenobiotics and the activity of the DNA repair enzymes are more relevant in animal cells (especially in liver cells) than in plant cells. Thus, as observed in the present study, it can be added that the enzymatic particularities of each organism may contribute to the difference in results obtained when analyzing the genotoxic effects of the same compound in different organisms.

4. Conclusions

Based on the results found in this study and on the observations mentioned earlier, it can be concluded that the commercial Malathion, in low concentrations, has a genotoxic potential in *in vitro* assays with the HTC cells and in tests with *A. cepa*. In addition, both test systems were effective in the detection of the genotoxicity induced by the insecticide and can be indicated as good systems for assessing genotoxic effects of low concentrations of pesticide with indirect action on the cells, such as Malathion.

Acknowledgments

The authors would like to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (Foundation for Research

Support of the State of Sao Paulo) — FAPESP for funding the research (Process no. 2005/57857-4).

REFERENCES

Abdel-Halim, K.Y., Salama, A.K., El-Khateeb, E.N., Bakry, N.M., 2006. Organophosphorus pollutants (OPP) in aquatic environment at Damietta Governorate, Egypt: implications for monitoring and biomarker responses. Chemosphere 63 (9), 1491–1498.

Adam, Z.M., Ebad, F.A., Abo-El-Kheir, Z.A., El-Sheikh, A., 1990. Alterations in nucleic acids, protein content and mitotic division of *Vicia faba* root tip cells as affected by malathion and tamaron insecticides. Cytologia 55 (3), 349–355.

Agencia para Sustancias Tóxicas yel Registro de Enfermedades, 2003. Reseña Toxicológica del Malation. Atlanta, A.T.S.D.R., GA: Departamento de Salud yServicios Humanos de los EE.UU, Servicio de la Salud Pública.

Almeida, D.V., Trindade, G.S., Geracitano, L.A., Barros, D.M., Monserrat, J.M., Marins, L.F., 2008. Initial results in the development of a reporter cell line for toxicology studies at gene expression level: activation of the electrophile-responsive element by copper and methyl parathion. Mar. Environ. Res. 66 (1), 158–160.

Asita, O.A., Makhalemele, R., 2009. Genotoxic effects of dithane, malathion and garden ripcord on onion root tip cells. Afr. J. Food Agric. Nutr. Dev. 9 (5), 1191–1209.

Ateeq, B., Farah, M.A., Ali, M.N., Ahmad, W., 2002. Clastogenicity of pentachlorophenol, 2,4-D and butachlor evaluated by *Allium* root tip test. Mutat. Res. 514 (1-2), 105–113.

Au, W.W., Ward, J.B., Ramanujam, V.M.S., Harper, B.L., Moslen, M.T., Legator, M.S., 1988. Genotoxic effects of a sub-acute low-level inhalation exposure to a mixture of carcinogenic chemicals. Mutat. Res. 203 (2), 103–115.

Au, W.W., Cantelli-Forti, G., Hrelia, P., Legator, M.S., 1990. Cytogenetic assays in genotoxic studies; somatic cell effects of benzene and germinal cell effects of dibromochloropropane. Teratog. Carcinog. Mutagen. 10 (2), 125–134.

Azqueta, A., Meier, S., Priestley, C., Gutzkow, K.B., Brunborg, G., Sallette, J., et al., 2011. The influence of scoring method on variability in results obtained with the comet assay. Mutagenesis 26 (3), 393–399.

Banerjee, B.D., Seth, V., Bhattacharya, A., Pasha, S.T., Chakraborty, A.K., 1999. Biochemical effects of some pesticides on lipid peroxidation and free radical scavengers. Toxicol. Lett. 107 (1-3), 33–47.

Blasiak, J., Jaloszynski, P., Trzeciak, A., Szyfter, K., 1999. *In vitro* studies on the genotoxicity of the organophosphorus insecticide Malathion and its two analogues. Mutat. Res. 445 (2), 275–283.

Bolle, P., Mastrangelo, S., Tucci, P., Evandri, M.G., 2004. Clastogenicity of atrazine assessed with the *Allium cepa* test. Environ. Mol. Mutagen. 43 (2), 137–141.

Bolognesi, C., Morasso, G., 2000. Genotoxicity of pesticides: potential risk for consumers. Trends Food Sci. Technol. 11 (4-5), 182–187.

Bull, C., Fenech, M., 2008. Genome-health nutrigenomics and nutrigenetics: nutritional requirements or 'nutriomes' for chromosomal stability and telomere maintenance at the individual level. Proc. Nutr. Soc. 67 (2), 146–156.

Buratti, F.M., D'Aniello, A., Volpe, M.T., Meneguz, A., Testai, E., 2005. Malathion bioactivation in the human liver: the contribution of different cytochrome P450 isoforms. Drug Metab. Dispos. 33 (3), 295–302.

Cavallo, D., Ursini, C.L., Fresegna, A.M., Ciervo, A., Maiello, R., Rondinone, B., et al., 2010. Direct-oxidative DNA damage and apoptosis induction in different human respiratory cell exposed to low concentrations of sodium chromate. J. Appl. Toxicol. 30 (3), 218–225.

Chauhan, L.K.S., Saxena, P.N., Sundararaman, V., Gupta, S.K., 1998. Diuron-induced cytological and ultrastructural alterations in the root meristem cells of *Allium cepa*. Pestic. Biochem. Physiol. 62 (3), 152–163.

Cox, C., 2003. Insecticide factsheet–malathion. J. Pest. Reform. 23 (4), 10–15.

Das, P.P., Shaik, A.P., Jamil, K., 2007. Genotoxicity induced by pesticide mixtures: *in-vitro* studies on human peripheral blood lymphocytes. Toxicol. Ind. Health 23 (8), 449–458.

Dean, R., Bynum, G., Kram, D., Schneider, E.L., 1980. Sister-chromatid exchange induction by carcinogens in HTC cells. An in *vitro* system which does not requires addition of activating factors. Mutat. Res. 74 (6), 477–483.

Dianov, G.L., Timchenko, T.V., Sinitsina, O.I., Kuzminov, A.V., Medvedev, O.A., Salganik, R.I., 1991. Repair of uracil residues closely spaced on the opposite strands of plasmid DNA results in double strand break and deletion formation. Mol. Gen. Genet. 225 (3), 448–452.

Dulout, F.N., Olivero, O.A., Von Guradze, H., Pastori, M.C., 1982. Cytogenetic effect of Malathion assessed by the micronucleus test. Mutat. Res. 105 (6), 413–416.

Etemadi-Aleagha, A., Akhgar, I.M., Abdollahi, M., 2002. A brief review on oxidative stress and cardiac diseases. Middle East Pharm. 10, 8–9.

Fenech, M., 2000. The *in vitro* micronucleus technique. Mutat. Res. 455 (1-2), 81–95.

Fenech, M., Crott, J.W., 2002. Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes: evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay. Mutat. Res. 504 (1-2), 131–136.

Feretti, D., Zerbini, I., Ceretti, E., Villarini, M., Zani, C., Moretti, M., et al., 2008. Evaluation of chlorite and chlorate genotoxicity using plant bioassays and *in vitro* DNA damage tests. Water Res. 42 (15), 4075–4082.

Fernandes, T.C.C., Mazzeo, D.E.C., Marin-Morales, M.A., 2007. Mechanism of micronuclei formation in polyploidizated cells of *Allium cepa* exposed to trifluralin herbicide. Pestic Biochem. Physiol. 88 (3), 252–259.

Fernandes, T.C.C., Mazzeo, D.E.C., Marin-Morales, M.A., 2009. Origin of nuclear and chromosomal alterations derived from the action of an aneugenic agent Trifluralin herbicide. Ecotoxicol. Environ. Saf. 72 (6), 1680–1686.

Feulgen, R., Rossenbeck, H., 1924. Mikroskopisch-chemischer nachweis einer nukleinsäure vom typus der thymonukleinsäure und die darauf beruhende elektive färbung von zellkernen in mikroskopischen präparaten. Hoppe Seylers Z. Physiol. Chem. 135, 203–248.

Fiskejö, G., 1985. The *Allium* test as a standard in environmental monitoring. Heredita 102 (1), 99–112.

Flessel, P., Quintana, P.J.E., Hooper, K., 1993. Genetic toxicity of Malathion: a review. Environ. Mol. Mutagen. 22 (1), 7–17.

Freshney, R.I., 2005. Culture of Animal Cells—A Manual of Basic Technique. John Wiley & Sons, Inc, New York.

Gad, S.C., 2000. Primary hepatocyte culture as an *in vitro* toxicologic system for the liver. In: Gad, S.C. (Ed.), *In Vitro* Toxicology. Taylor & Francis, New York, p. 410.

Galloway, T., Handy, R., 2003. Immunotoxicity of organophosphorous pesticides. Ecotoxicology 12 (1-4), 345–363.

Giri, S., Prasad, S.B., Giri, A., Sharma, G.D., 2002. Genotoxic effects of Malathion: an organophosphorus insecticide, using three mammalian bioassays *in vivo*. Mutat. Res. 514 (1-2), 223–231.

Grant, W.F., 1982. Chromosome aberration assays in *Allium*, a report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat. Res. 99 (3), 273–291.

Grant, W.F., Zinov'eva-Stahevitch,, A.E., Zura, K.D., 1981. Plant genetic test systems for detection of chemical mutagens. In: Stich, H.F., San, R.H. (Eds.), Short-term Tests for Chemical CarcinogensTopics in Environmental Physiology and Medicine. Springer-Verlag, New York, pp. 200–216.
Hartlerode, A.J., Scully, R., 2009. Mechanisms of double-strand break repair in somatic mammalian cells. Biochem. J. 423 (2), 157–168.
Hartmann, A., Elhajouji, A., Kiskinis, E., Poetter, F., Martus, H.-J., Fjällman, A., et al., 2001. Use of the alkaline comet assay for industrial genotoxicity screening: comparative investigation with the micronucleus test. Food Chem. Toxicol. 39 (8), 843–858.
Hoffelder, D.R., Luo, L., Burke, N.A., Watkins, S.C., Gollin, S.M., Saunders, W.S., 2004. Resolution of anaphase bridges in cancer cells. Chromosoma 112 (8), 389–397.
Humphrey, R.M., Brinkley, B.R., 1969. Ultrastructural studies of radiation-induced chromosome damage. J. Cell Biol. 42 (3), 745–753.
John, S., Kale, M., Rathore, N., Bhatnagar, D., 2001. Protective effect of vitamin E in dimethoate and malathion induced oxidative stress in rat erythrocytes. J. Nutr. Biochem. 12 (9), 500–504.
Kobayashi, H., Sugiyama, C., Morikawa, Y., Hayashi, M., Sofuni, T., 1995. A comparison between manual microscopic analysis and computerized image analysis in the single cell gel electrophoresis assay. MMS Commun. 3, 103–115.
Kumar, R., Nagpure, N.S., Kushwaha, B., Srivastava, K.S., Lakra, W.S., 2010. Investigation of the genotoxicity of Malathion to freshwater teleost fish *Channa punctatus* (Bloch) using the micronucleus test and comet assay. Arch. Environ. Contam. Toxicol. 58 (1), 123–130.
Kwong, T.C., 2002. Organophosphate pesticides: biochemistry and clinical toxicology. Ther. Drug Monit. 24 (1), 144–149.
Laine, D.F., Cheng, I.F., 2007. The destruction of organic pollutants under mild reaction conditions: a review. Microchem. J. 85 (2), 183–193.
Leme, D.M., Marin-Morales, M.A., 2008. Chromosome aberration and micronucleus frequencies in *Allium cepa* cells exposed to petroleum polluted water–a case study. Mutat. Res. 650 (1), 80–86.
Ma, T.H., Anderson, V.A., Harris, M.M., Bare, J.L., 1983. Tradescantia-micronucleus (Trad-MCN) test on the genotoxicity of Malathion. Environ. Mutagen. 5 (2), 127–137.
Ma, T.H., Xu, Z.D., Xu, C.G., McConell, H., Rabago, E.V., Arreola, H., et al., 1995. An improved *Allium/Vicia* root tip micronucleus assay for clastogenicity of environmental pollutants. Mutat. Res. 334 (2), 185–195.
Malatesta, M., Perdoni, F., Santin, G., Battistelli, S., Muller, S., Biggiogera, M., 2008. Hepatoma tissue culture (HTC) cells as a model for investigating the effects of low concentrations of herbicide on cell structure and function. Toxicol. *in Vitro* 22 (8), 1853–1860.
Mantovani, M.S., Bellini, M.F., Angeli, J.P.F., Oliveira, R.J., da Silva, A.F., Ribeiro, L.R., 2008. β-Glucans in promoting health: prevention against mutation and cancer. Mutat. Res. 658 (3), 154–161.
Manzano, B.C., Roberto, M.M., Hoshina, M.M., Menegário, A.A., Marin-Morales, M.A., 2015. Evaluation of the genotoxicity of waters impacted by domestic and industrial effluents of a highly industrialized region of São Paulo State, Brazil, by the comet assay in HTC cells. Environ. Sci. Pollut. Res. Int. 22 (2), 1399–1407.
Marcarini, J.C., Tsuboy, M.S.F., Luiz, R.C., Ribeiro, L.R., Hoffmann-Campo, C.B., Mantovani, M.S., 2011. Investigation of cytotoxic, apoptosis-inducing, genotoxic and protective effects of the flavonoid rutin in HTC hepatic cells. Exp. Toxicol. Pathol. 63 (5), 459–465.
Mazzeo, D.E.C., Matsumoto, S.T., Levy, C.E., de Angelis, D., de, F., Marin-Morales, M.A., 2013. Application of micronucleus test and comet assay to evaluate BTEX biodegradation. Chemosphere 90 (3), 1030–1036.
Mello, M.L.S., Vidal, B.C., 1978. A reação de Feulgen. Ciência e Cultura, São Paulo.
Moore, P.D., Yedjou, C.G., Tchounwou, P.B., 2010. Malathion-induced oxidative stress, cytotoxicity and genotoxicity in human liver carcinoma ($HepG_2$) cells. Environ. Toxicol. 25 (3), 221–226.
Moore, P.D., Patlolla, A.K., Tchounwou, P.B., 2011. Cytogenetic evaluation of Malathion-induced toxicity in Sprague–Dawley rats. Mutat. Res. 725 (1-2), 78–82.
Nackerdien, Z., Kasprzak, K.S., Rao, G., Halliwell, B., Dizdaroglu, M., 1991. Nickel(II)- and cobalt(II)-dependent damage by hydrogen peroxide to the DNA bases in isolated human chromatin. Cancer Res. 51 (21), 5837–5842.
Nordberg, J., Arnér, E.S.J., 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic. Biol. Med. 31 (11), 1287–1312.
O'Donovan, P.J., Livingston, D.M., 2010. BRCA1 and BRCA2: breast/ovarian cancer susceptibility gene products and participants in DNA double strand break repair. Carcinogenesis 31 (6), 961–967.
Oliveira, R.J., Ribeiro, L.R., da Silva, A.F., Matuo, R., Mantovani, M.S., 2006. Evaluation of antimutagenic activity and mechanisms of action of -glucan from barley, in CHO-K1 and HTC cell lines using the micronucleus test. Toxicol. *in Vitro* 20 (7), 1225–1233.
Patty, F.A., 1963. Industrial Hygiene and Toxicology. Interscience Publishers, New York.
Rai, D.K., Sharma, B., 2007. Carbofuran-induced oxidative stress in mammalian brain. Mol. Biotechnol. 37 (1), 66–71.
Rai, D.K., Rai, P.K., Rizvi, S.I., Watal, G., Sharma, B., 2009. Carbofuran induced toxicity in rats: protective role of vitamin C. Exp. Toxicol. Pathol. 61 (6), 531–535.
Rank, J., 2003. The method of *Allium* anaphase–telophase chromosome aberration assay. Ekologija 1, 38–42.
Rank, J., Nielsen, M.H., 1997. *Allium cepa* anaphase–telophase root tip chromosome aberration assay on *N*-methyl-*N*-nitrosourea, maleic hydrazide, sodium azide, and ethyl methanesulfonate. Mutat. Res. 390 (1-2), 121–127.
Rank, J., Nielsen, M.H., 1998. Genotoxicity testing of wastewater sludge using the *Allium cepa* anaphase-telophase chromosome aberration assay. Mutat. Res. 418 (2-3), 113–119.
Rank, J., Jensen, A.-G., Skov, B., Pedersen, L.H., Jensen, K., 1993. Genotoxicity testing of the herbicide Roundup and its active ingredient glyphosate isopropylamine using the mouse bone marrow micronucleus test, *Salmonella* mutagenicity test, and *Allium* anaphase–telophase test. Mutat. Res. 300 (1), 29–36.
Réus, G.Z., Valvassori, S.S., Nuernberg, H., Comim, C.M., Stringari, R.B., Padilha, P.T., et al., 2008. DNA damage after acute and chronic treatment with Malathion in rats. J. Agric. Food Chem. 56, 7560–7565.
Rogero, S.O., Lugão, A.B., Ikeda, T.I., Cruz, A.S., 2003. Teste *in vitro* de citotoxicidade: estudo comparativo entre duas metodologias. Mater. Res. 6 (3), 317–320.
Salvi, R.M., Lara, D.R., Ghisoldfi, E.S., Portela, L.V., Dias, R.D., Souza, D.O., 2003. Neuropsychiatric evaluation in subjects chronically exposed to organophosphate pesticides. Toxicol. Sci. 72 (2), 267–271.
Sankararamakrishnan, N., Sharma, A.K., Sanghi, R., 2005. Organochlorine and organophosphorous pesticide residues in ground water and surface waters of Kanpur, Uttar Pradesh. India Environ. Int. 31 (1), 113–120.
Serrano-García, L., Monteiro-Montoya, R., 2001. Micronuclei and chromatid buds are the results of related genotoxic events. Environ. Mol. Mutagen. 38 (1), 38–45.
Shadnia, S., Azizi, E., Hosseini, R., Khoei, S., Fouladdel, S., Pajouman, A., et al., 2005. Evaluation of oxidative stress and

genotoxicity in organophosphorus insecticide formulators. Hum. Exp. Toxicol. 24 (9), 439–445.
Shimizu, N., Shingaki, K., Kaneko-Sasaguri, Y., Hashizume, T., Kanda, T., 2005. When, where and how the bridge breaks: anaphase bridge breakage plays a crucial role in gene amplification and HSR generation. Exp. Cell Res. 302 (2), 233–243.
Singh, S., Sharma, N., 2000. Neurological syndromes following organophosphate poisoning. Neurology 48 (4), 308–313.
Sinha, V.S., Kumar, N., 2014. Assessment of mito-inhibitory and genotoxic effects of two organophosphate pesticides in the root tip cells of *Allium cepa* L. Ann. Plant. Sci. 3 (5), 699–703.
Soloneski, S., Larramendy, M.L., 2010. Sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary (CHO-K1) cells treated with the insecticide pirimicarb. J. Hazard. Mater. 174 (1-3), 410–415.
Srivastava, K., Mishra, K.K., 2009. Cytogenetic effects of commercially formulated atrazine on the somatic cells of *Allium cepa* and *Vicia faba*. Pestic. Biochem. Physiol. 93 (1), 8–12.
Sultatos, L.G., 1994. Mammalian toxicology of organophosphorus pesticides. J. Toxicol. Environ. Health 43 (3), 271–289.
Tice, R.R., Agurell, E., Erson, D., Burlinson, B., Hartmann, A., Kobayashi, H., et al., 2000. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ. Mol. Mutagen. 35 (3), 206–221.
Titenko-Holland, N., Windham, G., Kolachana, P., Reinisch, F., Parvatham, S., Osorio, A.M., et al., 1997. Genotoxicity of Malathion in human lymphocytes assessed using the micronucleus assay *in vitro* and *in vivo*: a study of Malathion-exposed workers. Mutat. Res. 388 (1), 85–95.
Trapp, S., 2000. Modelling uptake into roots and subsequent translocation of neutral and ionisable organic compounds. Pest Manag. Sci. 56 (9), 767–778.
Trapp, S., 2002. Dynamic root uptake model for neutral lipophilic organics. Environ. Toxicol. Chem. 21 (1), 203–206.
Tsuboy, M.S., Marcarini, J.C., Luiz, R.C., Barros, I.B., Ferreira, D.T., Ribeiro, L.R., et al., 2010. *In vitro* evaluation of the genotoxic activity and apoptosis induction of the extracts of roots and leaves from the medicinal plant *Coccoloba mollis* (polygonaceae). J. Med. Food 13 (3), 503–508.
U.S. Department of Agriculture, U.S.D.A., 2000. Programa Cooperativo de Erradicación de la Mosca de la Fruta de las Antillas Valle Más Bajo del Río Grande. Texas, Evaluación Ambiental.
Valencia-Quintana, R., Alarcón, J.S., Gómez-Arroyo, S., Eslava, J.C., Waliszewski, S.M., Fernández, S., et al., 2013. Genotoxicidad de plaguicidas en sistemas vegetales. Rev. Int. Contam. Ambie. 29, 133–157.
Valentin-Severin, I., Hegarat, L.L., Lhuguenot, J.-C., Le Bon, A.-M., Chagnon, M.-C., 2003. Use of HepG2 cell line for direct or indirect mutagens screening: comparative investigation between comet and micronucleus assays. Mutat. Res. 536 (1-2), 79–90.
Vig, B.K., 1978. Somatic mosaicism in plants with special reference to somatic crossing over. Environ. Health Perspect. 27, 27–36.