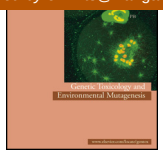
Mutation Research 794 (2015) 52–56

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox

Community address: www.elsevier.com/locate/mutres



Evaluation of genotoxicity of the acute gamma radiation on earthworm *Eisenia fetida* using single cell gel electrophoresis technique (Comet assay)

K. Sowmithra [a], N.J. Shetty [a,*], S.K. Jha [b], R.C. Chaubey [b]

[a] *Centre for Applied Genetics, Bangalore University, JB campus, Bengaluru 560056, India*
[b] *BRNS—DAE, Bhabha Atomic Research Centre, Mumbai 400 085, India*

ARTICLE INFO

Article history:
Received 15 June 2015
Received in revised form 16 October 2015
Accepted 19 October 2015
Available online 24 October 2015

Keywords:
Eisenia fetida
Gamma irradiation
Comet assay
Coelomocytes

ABSTRACT

Earthworms (*Eisenia fetida*) most suitable biological indicators of radioactive pollution. Radiation-induced lesions in DNA can be considered to be molecular markers for early effects of ionizing radiation. Gamma radiation produces a wide spectrum of DNA. Some of these lesions, i.e., DNA strand breaks and alkali labile sites can be detected by the single-cell gel electrophoresis (SCGE) or comet assay by measuring the migration of DNA from immobilized nuclear DNA. *E. fetida* were exposed to different doses of gamma radiation, i.e., 1, 5, 10, 20, 30, 40 and 50 Gy, and comet assay was performed for all the doses along with control at 1, 3 and 5 h post irradiation to evaluate the genotoxicity of gamma radiation in this organism. The DNA damage was measured as percentage of comet tail DNA. A significant increase in DNA damage was observed in samples exposed to 5 Gy and above, and the increase in DNA damage was dose dependent i.e., DNA damage was increased with increased doses of radiation. The highest DNA damage was noticed at 1 h post irradiation and gradually decreased with time, i.e., at 3 and 5 h post irradiation. The present study reveals that gamma radiation induces DNA damage in *E. fetida* and the comet assay is a sensitive and rapid method for its detection to detect genotoxicity of gamma radiation.

1. Introduction

As atomic power is gradually recognized as a potential energy source to sustain future human development, radiological protection of the environment will become an even more important environmental safety concern [1]. In the past decades, scientific and regulatory activities related to radiation protection have been concentrated on the radiation exposure of humans. The principal view has been that, if humans were adequately protected, then other living organisms would also likely to be sufficiently protected and other species would not put at risk [2]. This view has been questioned, and attention is now also put on the potential effects of exposure to ionizing radiation of non-human biota. Thus, the International Commission on Radiological Protection (ICRP) considers, that understanding the effects of ionizing radiation on non-human biota is essential for the radiological protection of the environment [1].

In most contamination situations, the majority of the radionuclide inventory in terrestrial ecosystems is found within the soil; thus, soil invertebrates can receive significant external and internal doses [3]. Soil animals have a significant part to play in the accumulation and migration of radionuclides. Earthworms are among the organisms that are most sensitive to the radionuclides, probably because intimate contact they have with soil constituents in the upper soil layers and they also lack the chitinous exoskeleton of some soil invertebrate species, which may reduce exposure from external radiation [3,4]. Thus, earthworms are useful organisms for the assessment of environmental insults due to their role in vermicomposting and nutrient cycling. Moreover, they also can act as bioindicator for the toxic effects of chemicals in soils [5–7]. Among the various earthworm species, *Eisenia fetida* is especially appropriate for the toxicity tests because it can be easily bred on a variety of organic wastes with short generation times. They have also been accepted as standard organisms for ecotoxicological testing by the European Union [8]; OECD [9,10] and included it in the list of reference animal and plants (RAP) of the International Commission on Radiological Protection (ICRP) [11,12] and as a candidate reference organism from Framework for Assessment of Environmental Impact (FASSET) [3] to study the harmful effect of ionizing radiation.

* Corresponding author.
E-mail address: shetty_nj@yahoo.co.in (N.J. Shetty).

http://dx.doi.org/10.1016/j.mrgentox.2015.10.001
1383-5718/

So far, most of the data obtained concerned the effects of ionizing radiation on earthworm reproduction. These studies have shown effects such as reduced reproductive ability, reduced population size, changes in the distribution of life cycle stages, and reduced number of species [13–15]. On the other hand, ionizing radiations also produce a great variety of DNA lesions that can be taken as molecular markers for early radiotoxic effects. In fact, low LET radiation such as gamma-radiation interact with DNA either directly by deposition of energy or indirectly through the generation of oxygen radicals and reactive oxygen species (ROS), creating a wide spectrum of lesions i.e., DNA double-strand breaks (DSBs), single-strand breaks (SSBs) and base damage. Among these SSBs are much frequent than DSBs, but the SSBs are generally more rapidly repaired and mostly error free [16]. Several methods have been developed to detect the damage to DNA strands. The single-cell gel electrophoresis (SCGE) or comet assay can detect DNA strand breaks and alkali labile sites by measuring the migration of DNA from immobilized nuclear DNA [17]. This technique is a quick, simple, sensitive, reliable and fairly inexpensive method for measuring DNA damage.

In earthworms, coelomocytes play an integral role in immune cell functions such as fighting microbial infections and wound healing [18]. Damage to coelomocytes can compromise these essential functions, directly affecting the health of organisms and stability of populations. Coelomocytes from a variety of terrestrial and aquatic organisms (e.g., Earthworms, bivalves, fish) have been useful bioindicators of environmental stress and are frequently used to assess genotoxicity [19–22].

The alkaline comet assay has previously been applied to detect DNA damage in coelomocytes (immune cells) from earthworms exposed in the laboratory to artificially spiked soils (e.g., Heavy metals and pesticides) or soils collected from polluted sites (e.g., Polycyclic aromatic hydrocarbons, heavy metals, uranium) [23,24]. Recently, this technique has also been used to detect the DNA damage produced by the chronic gamma radiation, X-rays [25] and radio frequency/microwave electromagnetic field [26] in *E. fetida.* Thus, by taking this as benchmark, single cell gel electrophoresis was conducted to the samples (*E. fetida*) exposed to different doses of acute gamma radiation to determine the extent of DNA damage and the time for repair of this damage.

2. Materials and method

2.1. *Culturing of* E. fetida

E. fetida were obtained from the University of Agricultural Science, Gandhi Krishi Vignana Kendra, Bengaluru. The age synchronized worms were maintained according to the procedure of Yasmin and D'souza, 2007 [27] with slight modification. The mixture contained 75% soil and 25% cow dung for the culture of worms. The dry black soil was powdered and filtered through a fine mesh sieve. The sieved soil was then moistened, and 25 adult worms were transferred to it. The air dried cow dung was supplemented as food, and the culture was covered with wet cloth. After 30 days, the adult worms were removed from the system. During the period of 30 days in the culture, the worms reproduced and laid cocoons. Once the worms were removed, the culture was left undisturbed for 4 months. Water was sprinkled to keep the soil moist. After 4 months, worms of same age were obtained from the culture. These age synchronized worms were used for the experiment.

2.2. *Irradiation*

Four months old earthworms were placed in glass containers with little amount of water and were allowed to defecate for a day. Groups of nine worms (average wet weight 400 mg) were transferred to 7 plastic Petri dishes with moist filter paper, and then each sample dish (except the control) exposed to doses of 1, 5, 10, 20, 30, 40 and 50 Gy of Cobalt-60 gamma irradiation (Source: Theratron 780-C machine) at a dose rate of 146.75 cGy/min (The exposure time ranged from 0.68 min (1 Gy) to 34 min (50 Gy) and the distance between the source and the object was 80 cm). Irradiated samples were subjected to single cell gel electrophoresis technique (Comet assay) at 1 h, 3 h and 5 h after irradiation to evaluate the genotoxicity of gamma radiation. (3 worms each for, 1 h, 3 h and 5 h). 3 worms were used for control.

2.3. *Collection of coelomocytes*

After exposure of earthworms to different doses of gamma radiation, their coelomocytes were collected at 1 h, 3 h and 5 h post irradiation using the noninvasive extrusion method described by Eyambe et al. [28]. Individual earthworms were rinsed in an extrusion medium composed of 5% ethanol, 95% saline, 2.5 mg/mL EDTA, and 10 mg/mL guaiacol glyceryl ether (pH 7.3). Coelomocytes were spontaneously secreted into the medium and washed with Lumbricus balanced solution (LBS) three times prior to the comet assay. Cells were collected by centrifugation for 10 min at 3000 × *g* and 4 °C and placed on ice prior to the comet assay.

2.4. *Cell viability assay*

Prior to the comet assay, the cell count and the cell viability were checked to ensure that there were an optimum number of living cells to perform the assay. The cell count and viability assessment were conducted with a haemocytometer and trypan blue dye exclusion test. Coelomocytes samples showing more than 90% viability and a cell count of 10^6 cells/mL were used for the comet assay.

2.5. *Comet assay*

2.5.1. *Slide Preparation*

The comet assay was performed according to Singh et al. [17], with slight modifications. The cell suspension was mixed with 100 μL of 0.7% low-melting-point agarose (LMA) in PBS at 37 °C and pipetted onto fully frosted slides precoated with a layer of 100 μL 0.8% normal-melting-point agarose (NMA). After solidification on ice, another layer of 85 μL LMA was added, and the slides were immersed in a lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA, 1% Na-sarcosinate, 10% dimethyl sulfoxide, and 1% Triton X-100), pH 10, for overnight at 4 °C.

2.5.2. *Electrophoresis*

To unwind the DNA, slides were incubated for 20 min in electrophoretic buffer containing 1 mM Na_2EDTA and 300 mM NaOH, pH 13. Then electrophoresis was run at 25 V and 300 mA for 15 min at 4 °C. After electrophoresis the slides were neutralized in cold neutralization buffer (0.4 M Tris, pH 7.5) three times at 5 min intervals, fixed in anhydrous ethanol three times at 5 min intervals, and stored in the dark at room temperature.

2.5.3. *Slide examination*

Slides were stained with ethidium bromide for analysis. Approximately, 50 cells per slide were randomly scored. All steps were conducted in dim light to prevent nonspecific additional DNA breakage. The comet images were captured, and the image analysis system (CASP) was employed to measure various comet parameters. DNA damage in coelomocytes of *E. fetida* exposed to different doses of gamma radiation measured by the comet assay was determined as percentage of comet tail DNA.

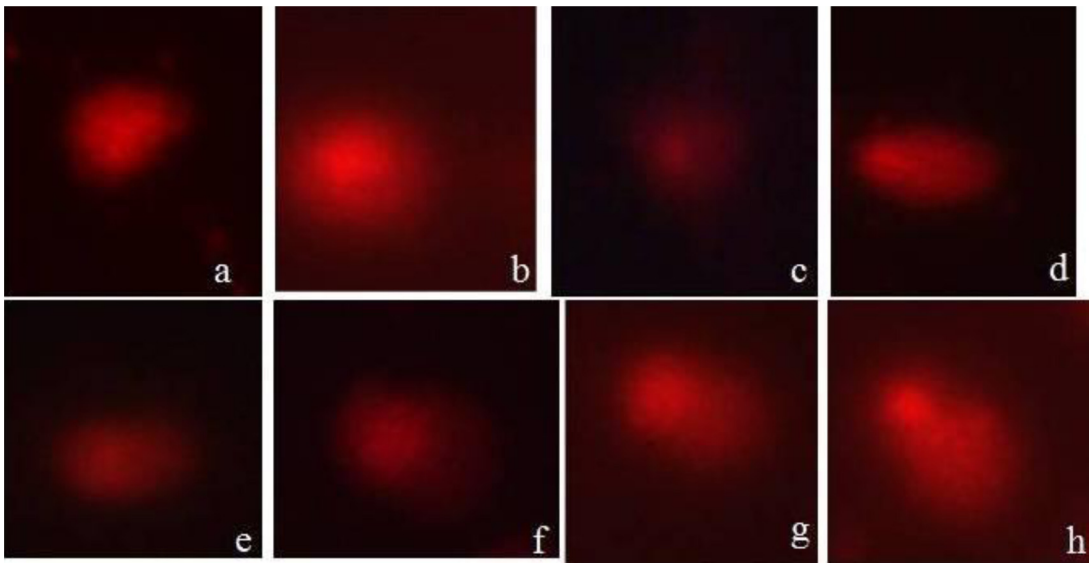


Fig. 1. Images of the comet obtained from the coelomocytes of the earthworms exposed to different doses of gamma radiation. a) Control, b)1 Gy, c) 5 Gy, d) 10 Gy, e) 20 Gy, f) 30 Gy, g) 40 Gy and h) 50 Gy.

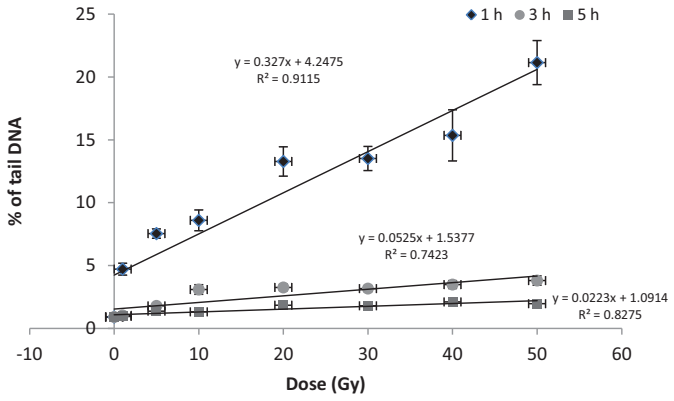


Fig. 2. Dose–response relationship for DNA damage at1 h, 3 h and 5 h after acute gamma irradiation of *E. fetida*.

2.6. Statistical analysis

The effects of acute gamma radiation on DNA integrity were evaluated by comparing all doses using the ANOVA GLM followed by Tukey's post hoc test. In addition, trend analysis in the form of linear regression was performed using SPSS, and a significant dose–rate response relationship was indicated by a slope significantly different ($P \leq 0.05$) from zero [12].

3. Result and discussion

Results obtained from the comet assay showing DNA damage on exposure to acute gamma radiation is given in Fig. 1, and the DNA damage was determined as percentage of DNA in tail (% tail DNA or also expressed as relative tail intensity). Although the tail length and tail moment could also be used as a parameter of DNA damage, % tail DNA is the most useful parameter, as it bears a linear increase in percentage of DNA in tail that corresponds to DNA damage up to about 2.5 breaks per 109 Dalton. It is relatively unaffected by threshold settings, and allows discrimination of damage over the widest possible range [29].

3.1. DNA damage in E. fetida

The dose and the time dependent increase of DNA damage induced by gamma radiation and their statistical significance are represented in Fig. 2, respectively.

3.2. Dose response study

Significant DNA damage was seen in all individuals of *E. fetida* irradiated by various doses of gamma radiation except 1 Gy of 1 h post irradiation, 1 and 5 Gy of 3 h post irradiation and 1, 5, and 10 Gy of 5 h post irradiation ($p \geq 0.05$), which did not show a significant increase in percentage tail DNA in comparison to their respective controls as per the ANOVA. Dose response study showed that there is a dose dependent increase in the intensity of radiation and DNA damage with a minimum (4.72 ± 0.47) at the lowest dose of 1 Gy and the maximum (21.13 ± 1.75) at the highest dose of 50 Gy at 1 h of post irradiation. It was also observed that the dose response effect was linear. When significant dose-rate response trends were found using linear regression, the goodness of the fit (R^2 adj.) was high for all the samples exposed to different doses of gamma radiation and at all the time intervals (Fig. 2) i.e., 1 h, 3 h and 5 h after

radiation. (1 h; slope 0.327, R^2 = 89%, $p \leq 0.05$, 3 h; slope = 0.053, R^2 = 69%, $p \leq 0.05$, 5 h; slope = 0.022, R^2 = 79%, $p \leq 0.05$). There were few comets of the apoptotic types found in each dose (only at 1 h after exposure) except for 5 Gy. Since these comets showed a very high percentage of tail DNA (ranging from 50 to 80%), they were not considered for the count as it gives high variation in the mean percentage of tail DNA.

3.3. Time response study

Time-response study indicated significant DNA damage at all the time intervals for all the doses of gamma radiation studied (except 1 Gy of 1 hour post irradiation, 1 and 5 Gy of 3 h post irradiation and 1, 5, and 10 Gy of 5 h post irradiation). The highest DNA damage (21.13 ± 1.75) was observed in 1 h post treatment of 50 Gy exposed samples, and it decreased at the later time points reaching a minimum (1.96 ± 0.24) at 5 h. A similar trend was also observed for the other doses (5, 10, 20, 30 and 40 Gy) (1 h; df = 7, F = 31.58, $p \leq 0.05$; 3 h; df = 7, F = 18.34, $p \leq 0.05$; 5 h; df = 7, F = 7.34, $p \leq 0.05$).

A Significant increase of DNA damage (mean percentage of tail DNA) observed by comet assay in the present study at all the exposure levels indicates the induction of genotoxic effects by gamma radiation in the earthworm *E. fetida*. Earthworms, as members of the phylum Annelida, which is the largest group of soil invertebrates, have been used for vermicomposting and also used for biomonitoring of different pollutants. As of genotoxicity of radiation concerned there are very few papers available [25,26]. This is the first attempt, where we used this technique to study the genotoxicity of acute gamma radiation.

The dose-dependent increase of DNA single-strand breaks, in the form of comet induction (% tail DNA) induced by acute gamma irradiation in *E. fetida* in the present study is in line with the observations of Hertle-Aas et al. [25] where *E. fetida* was exposed to a series of chronic gamma radiation and acute X- radiation accordingly observed a dose-dependent increase of DNA damage in coelomocytes. The highest DNA damage was observed at 1 h post irradiation, which gradually decreased over time, with a significant reduction at 3 h and reaching the minimum at 5 h, which suggests that the genotoxic effect of gamma radiation does not last for a long period for the above mentioned doses. This is in agreement with the observation of Hertle-Aas et al. [25] who observed that the half-life (t 1/2) of SSBs using monophasic repair kinetics was 36 min. Decrease in genetic damage during post irradiation time time may indicate either repair of damaged DNA or loss of heavily damaged cells (by apoptosis, cell turnover and dilution by cell replication) or both [25,30,31].

The comet assay used in the present study seems to be less sensitive compared to reproduction end point. When the present data compared with our earlier studies where the effect of gamma radiation studied on reproduction [15], it is observed that the dose which produced 90% sterility i.e., 50 Gy could only show 21% DNA damage in coelomocytes (percentage tail DNA) at 1 h post irradiation. This is because the germ cells are more sensitive to radiation than the somatic cells. Cells in the process of spermatogenesis are highly radiosensitive and apparently are easily killed [32]. Hertle-Aas et al. observed that the DNA repair ability for SSB in coelomocytes are faster than that of the spermatocytes, this is because the ability to repair certain DNA lesions decreases as the spermatogenesis proceeds with DNA chromatin becoming highly compacted together with reduced cytoplasm in the sperms [25].

4. Conclusion

The result of the single cell gel electrophoresis on gamma irradiated earthworm has confirmed the genotoxic effect of acute gamma radiation on earthworm *E. fetida*. A dose-related increase and a time-dependent decrease of genotoxicity of acute gamma radiation were also observed in the coelomocytes of earthworm. Thus the study confirms that earthworms are able to serve as indicators of environmental toxicants like ionising radiation, and coelomocytes are suitable cell types for such genotoxic studies. Further, the alkaline comet assay appears to be a promising technique to assess the genotoxic potential of acute gamma radiation in the earthworm at doses above 1 Gy.

Acknowledgements

This work was supported by the grants of Board of Research in Nuclear Science (BRNS)–Department of Atomic Energy (DAE), Bhabha Atomic Research Centre (BARC), Mumbai (No.2009/36/80-BRNS/2394 Dated 9/12/2009). We are thankful to the radiation physics department of the Kidwai Memorial Institute of Oncology, Bangalore for providing radiation facility and the details for dosimetry and dose rate.

References

[1] ICRP, A framework for assessing the impact of ionizing radiation on non-human species. Publication 91, Pergamon Press, Oxford, Ann. ICRP 33, 2003, pp. 3.
[2] ICRP, Recommendations of the International Commission on Radiological Protection, Publication 60, Pergamon Press, Oxford, Ann. ICRP 21,1991 pp. 1–3.
[3] A. Agüero, C.L. Barnett, J. Brown, M. Gilek, B.J. Howard, E. Ilus, U. Kautsky, L. Kumblad, B. Naeslund, S.M. Wright, Identification of candidate reference organisms from a radiation exposure pathways perspective, deliverable 1, in: P. Strand, N. Beresford, R. Avila, S.R. Jones, C.M. Larsson (Eds.), FASSET, Framework for Assessment of Environmental Impact, European Community, Luxembourg, 2001, pp. 1–48.
[4] D.A. Krivolutsky, Radiation ecology of soil animals, in: B.R. Striganova (Ed.), Biology and Fertility of Soils, Nauka, Moscow, 1987, pp. 51–55.
[5] C.A. Callahan, Earthworms as ecotoxicological assessment tools, in: C.A. Edwards, E.F. Neuhauser (Eds.), Earthworms in Waste and Environmental Assessment, SPB Academic Publishing, The Hague, Netherlands, 1988, pp. 295–301.
[6] G.C. Goats, C.A. Edwards, The prediction of field toxicity of chemicals to earthworms by laboratory methods, in: C.A. Edwards, E.F. Neuhauser (Eds.), Earthworms in Waste and Environmental Assessment, SPB Academic Publishing, The Hague, Netherlands, 1988, pp. 283–294.
[7] M.B. Bouche, Earthworm species and ecotoxicological studies, in: P.W. Greig-Smith, H. Becker, P.J. Edwards, F. Heimbach (Eds.), Ecotoxicology of Earthworms, Intercept, Andover, 1992, pp. 20–35.
[8] EEC (European Economic Community). Directive 79/831/ EEC, Annex V, Part C. Method for the determination of ecotoxicity. Level 1. Earthworms: artificial soil test. Commission of the European Communities, DGXI/128/82. Rev. 5, Brussels, 1984.
[9] OECD, Earthworm, acute toxicity tests, in: Organization for Economic Co-operation and Development (ed.) OECD guidelines for testing of chemicals, Paris 1984.
[10] OECD, Earthworm reproduction test (Eisenia fetida/andrei), in: Guidelines for testing of chemicals OECD (ed.) Paris 2000.
[11] ICRP, Recommendations of the International Commission on Radiological Protection. Publication 103, Pergamon Press., Oxford and New York, Ann. ICRP 37, 2007, pp. 2–4.
[12] ICRP, Environmental protection–the concept and use of reference animals and plants. Annals of the International Commission on Radiation Protection, Publication 108, Pergamon press, Oxford, Ann. ICRP 38, 2008, 4–6.
[13] T. Hertel-Aas, D.H. Oughton, A. Jaworska, H. Bjerke, B. Salbu, G. Brunborg, Effects of chronic gamma irradiation on reproduction in the earthworm *Eisenia fetida* (Oligochaeta), Radiat. Res. 168 (2007) 515–526.
[14] T. Nakamori, Y. Kubota, T. Ban-nai, Y. Fujii, S. Yoshida, Effects of acute gamma irradiation on soil invertebrates in laboratory tests, Radioprotection 44 (2009) 421–424.
[15] K. Sowmithra, N.J. Shetty, B.P. Harini, S.K. Jha, R.C. Chaubey, Effects of acute gamma radiation on the reproductive ability of the earthworm *Eisenia fetida*, J. Environ. Radioact. 140 (2015) 11–15.
[16] P.A. Jeggo, A break is not the End; insight into the damage response to DNA double strand breaks, DNA Repair 9 (2010) 1217–1218.
[17] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, Exp. Cell Res. 175 (1988) 184–191.
[18] L.C. Smith, J. Ghosh, K.M. Buckley, K.A. Clow, N.M. Dheilly, et al., Echinoderm immunity, in: K. Soderhall (Ed.), Invertebrate Immunology, Springer Science+Business Media, LLC, Landes Bioscience, 2010, pp. 260–301.

[19] C. Bolognesi, M. Hayashi, Micronucleus assay in aquatic animals, Mutagenesis 26 (2011) 205–213.
[20] M.N. Canty, T.H. Hutchinson, R.J. Brown, M.B. Jones, A.N. Jha, Linking genotoxic responses with cytotoxic and behavioural or physiological consequences: differential sensitivity of echinoderms (*Asterias rubens*) and marine molluscs (*Mytilus edulis*), Aquat. Toxicol. 94 (2011) 68–76.
[21] L.J. Dallas, T.P. Bean, A. Turner, B.P. Lyons, A.N. Jha, Oxidative DNA damage may not mediate Ni-induced genotoxicity in marine mussels: assessment of genotoxic biomarkers and transcriptional responses of key stress genes, Mutat. Res. Genet. Toxicol. Environ. Mutagen. 754 (2013) 22–31.
[22] S. Kolarevic, J. Knezevic-Vukcevic, M. Paunovic, M. Kracun, B. Vasiljevic, et al., Monitoring DNA damage in haemocytes of freshwater mussel Sinanodonta woodiana sampled from the Velika Morava River in Serbia with the comet assay, Chemosphere 93 (2013) 243–251.
[23] P. Muangphra, R. Gooneratne, Comparative genotoxicity of cadmium and lead in earthworm coelomocytes, Appl. Environ. Soil Sci. (2011) 1–7.
[24] S.A. Reinecke, A.J. Reinecke, The comet assay as biomarker of heavy metal genotoxicity in earthworms, Arch. Environ Contam. Toxicol. 46 (2004) 208–215.
[25] T. Hertel-Aas, D.H. Oughton, A. Jaworska, G. Brunborg, Induction and repair of DNA strand breaks and oxidised bases in somatic and spermatogonic cells from the earthworm *Eisenia fetida* after exposure to ionizing radiation, Mutagenesis 26 (2011) 783–793.
[26] K. Malaric1, A. Stambukz, M. Srutz, M. Tkalecz, Evaluation of genotoxic potential of radiofrequency/microwave electromagnetic field (RF/MW EMF) using comet assay in earthworms (*Eisenia fetida*), in: 16th IMEKO TC4 Symposium, Exploring New Frontiers of Instrumentation and Methods for Electrical and Electronic Measurements, Florence Italy, 2008.
[27] S. Yasmin, D. D'souza, Effect of pesticides on the reproductive output of *Eisenia fetida*, Bull. Environ. Contam. Toxicol. 79 (2007) 529–532.
[28] G.S. Eyambe, A.J. Goven, L.C. Fitzpatrick, B.J. Venables, E.L. Cooper, et al., A non-invasive technique for sequential collection of earthworm (*Lumbricus terrestris*) leukocytes during subchronic immunotoxicity studies, Lab. Anim. 25 (1991) 61–67.
[29] A.R. Collins, The comet assay for DNA damage and repair, Mol. Biotech. 26 (2004) 249–261.
[30] S.B. Banu, K. Danadevi, M.F. Rahman, Y.R. Ahuja, K. Jamil, Genotoxic effect of monocrotophos to sentinel species using comet assay, Food Chem. Toxicol. 39 (2001) 361–366.
[31] R.R. Preeti, S.K. Shyama, Genotoxic effects of monocrotophos, an organophosphorous pesticide, on an estuarine bivalve, Meretrix ovum, Food Chem. Toxicol. 47 (2009) 1618–1623.
[32] M. Hasan, M. Khalequzzaman, A.R. Khan, Development of *Tribolium* anaphe irradiated as larvae of various ages with gamma rays, Entomol. Exp. Appl. 53 (1989) 92–94.