



Cytotoxic effects caused by N,N-diethyl-meta-toluamide and radiation in *Perna perna* mussels

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Abstract

The aim of this study was to evaluate the biological effects of ionizing radiation in combination with DEET on marine aquatic biota. It was studied the exposure of irradiated and non-irradiated marine mussels to different concentrations of DEET. It was compared the recoverability of mussels which were exposed to DEET after suffering another stressful action by the ionizing radiation. The cytotoxicity by the neutral red retention time assay was used to verify the DEET effect on the lysosomal membrane of *Perna perna* mussels hemocytes in non-irradiated and irradiated organisms. The organisms were irradiated at 3, 11 and 107 Gy doses of ^{60}Co gamma rays and exposed to 0.1; 1.0 and 20.0 $\mu\text{g L}^{-1}$ DEET. The results were obtained 24, 48 and 72h after irradiation. It was observed statistic significance at concentrations of 1.0 and 20.0 $\mu\text{g L}^{-1}$ in non-irradiated mussels for all exposure times. In mussels irradiated with 3 Gy and 11 Gy it was observed that the retention time was not significantly different from trials in which the organisms were not irradiated. The 107 Gy dose caused some adverse effects to organisms showing a significant reduction in the number of cells compared with the other doses. The present study showed cytotoxic effect of DEET to *Perna perna* mussels at concentrations above 0.1 $\mu\text{g L}^{-1}$ a value very close to the concentrations identified in the environment suggesting attention to this concentration range.

Keywords: DEET, ionizing radiation, *Perna perna* mussel

INTRODUCTION

Each year new chemicals are synthesized and most directly or indirectly affects ecosystems, and the introduction of these compounds can cause changes in the environment, and can configure contamination events. Among the worldwide compounds, DEET (*N,N*-diethyl-meta-toluamide – $\text{C}_{12}\text{H}_{17}\text{NO}$) is used in formulation of insect repellents, and is commercially available for more than 50 years (USEPA, 1998). The presence of DEET has already been evidenced in environmental matrices, showing that this compound is persistent (Singh, 2010). DEET is one of the most commonly used active ingredients in insect repellents, and because of its effectiveness, it is produced in relatively large volumes and globally used (Weeks *et al.*, 2011). European Union (EU) conducted assessments prior to 2010, classified DEET as “harmful to aquatic organisms and may cause long-term adverse effects in the aquatic environment” (EC, 2008).

According to Weeks *et al.* (2011) there are about 225 available products with DEET as the active ingredient. Kem (2010) concludes that DEET is not a persistent, bioaccumulative, and toxic substance, and does not show a significant risk to any of the environmental compartments, but concluded that surface water assessment was warranted as a precaution. In recent years, considerable research has been conducted to define the most important physicochemical properties and fate characteristics of DEET, as well as the toxicity to aquatic and terrestrial organisms (Weeks *et al.*, 2011). The environment in regions near nuclear facilities (mostly coastal) and in regions with higher level of background radiation, many aquatic organisms may be exposed to high levels of radiation, becoming more susceptible to other contaminants. Aiming at the protection of aquatic biota, becomes desirable progress to identifying and quantifying the presence of these contaminants for an evaluation of its bioavailability and harmful effects to the aquatic ecosystem. The mussels

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and other aquatic invertebrates, are frequently used as model organisms for toxicological tests (Depledge, 1998). Therefore, the purpose of this paper was the investigation of the biological effects of ionizing radiation in combination with the exposure of DEET on *Perna perna* mussels. To the best of our knowledge, there are no studies available in the literature reporting the sub-cellular effects caused by DEET in marine invertebrates. From this context, it was studied the exposure of marine mussels to different concentrations of DEET. In addition to the assessment of the cytotoxicity, it was compared the recoverability of mussels exposed to the compound after suffering another stressful action, in this case, the ionizing radiation. The biological effect from interaction of ionizing radiation is dose- and particle- dependent, one source is external irradiation from the surrounding contamination such as in sediment or water, mainly from gamma rays, but also beta radiation for small organisms (of sizes <1 cm), and a second source is internal irradiation due to internalization of radionuclides, whatever the physiological process involved (Fisher *et al.*, 2013). It was evidenced by Hiyama (2012) that artificial radionuclides, produced by the Fukushima nuclear power plant, caused physiological and genetic damages to a resident butterfly species and that the cumulative effects of the external and internal irradiation could have resulted in detriments at the population level. Fisher *et al.* (2013) reported that near Fukushima doses to marine biota were about two orders of magnitude below the lowest benchmark protection level proposed for ecosystems ($10 \mu \text{ Gy h}^{-1}$). According to Garnier-Laplace (2006) the Ecological Risk Assessment approaches developed for nonradioactive contaminants (chemicals) could be applied to the protection of the environment from radiation. The aquatic environment is often the ultimate recipient of a wide range of contaminants including chemical and radioactive wastes, a large proportion of which could be potentially toxic and carcinogenic (Moore *et al.*, 2004; Jha *et al.*, 2000). Therefore, in our study, in order to simulate a scenario where radiation is a potential hazard to the environment, it was determined the dose of ^{60}Co gamma radiation and the DEET concentration that causes cytotoxicity in lysosomes of *Perna perna* mussel hemocytes. The effects were evaluated (NRRT) by neutral red retention time assay on irradiated and non-irradiated organisms.

MATERIALS AND METHODS

Organism test and sea water

The organisms as well as the water used in the cytotoxicity tests were collected from Cocanha Beach – Caraguatatuba, SP. The water was packed in barrels of 50L, and maintained at a temperature of $22 \pm 2^\circ\text{C}$ at Ecotoxicology Laboratory, Nuclear and Energy Research Institute (IPEN – USP). The test organisms were *Perna perna* mussels, with 4–5 cm long, and were kept at laboratory for a week in sea water tank for the process of acclimatization. Marine bivalves have been employed as biomonitor in marine pollution assessments

all around the world because of their sessile habits, broad distribution, and economic importance, which make them suitable species to be employed in ecotoxicological studies (Farrington & Tripp, 1995).

Neutral red retention time assay (NRRT)

The mussels hemolymph was extracted from the posterior adductor muscle. An aliquot of 40 μL of sample containing hemocytes was placed carefully on top of the histological slide prepared in advance with poly-L-lysine covering. Slides were left on a rack in a light-proof humidity chamber during the assay. After 15 min, the excess solution was carefully tipped off and 40 μL of neutral red working solution were added. Slides were thereafter examined systematically under an optical microscope at 15 min intervals to determine the evidence of dye loss from the lysosomes to the cytosol of the hemocytes, and it was observed a decrease in the neutral red retention time in comparison to the control. The cytotoxicity tests with hemocytes of mussels has been an important source to knowing about quality of the environment. The bioassays using mussels cells provide a significant result on biomonitoring programs.

NRRT in non-irradiated and irradiated organisms

The NRRT method followed the protocol proposed by Lowe and Fossato (2000). In this study, the NRRT was used to verify the ionizing radiation and DEET exposure effect on the lysosomal membrane of *Perna perna* mussels hemocytes. The assay was performed by the evaluation of lysosome membrane integrity in the hemocytes. NRRT was evaluated in non-irradiated organisms exposed to different concentrations of DEET (0.1; 1.0 and 20.0 $\mu\text{g L}^{-1}$) in order to evaluate the difference that radiation causes on lysosomal membrane mussels. And for irradiated tests, the mussels ($n=15$) were placed in polypropylene bottles containing 750 mL marine water, were irradiated by ^{60}Co gamma rays with 3, 11 and 107 Gy and exposed to DEET. The results were obtained 24, 48 and 72 h after irradiation. The test endpoint was achieved when was observed the evident dye loss in at least 50% of the hemocytes.

Statistical analysis

The data are expressed as mean $\pm$ SD (mean and standard error) and statistically analyzed with variance analysis (ANOVA) followed by Bonferroni Multiple Comparisons test (different number of replicates) or Dunnett's test (same number of replicates). A p value less than 0.05 was considered statistically significant.

Data analysis was carried out with the software TOXSTAT 3.4. and graphics were plotted on Sigma Plot® 11.0 (Systat Software Inc, San Jose, CA 95131 USA). The expression of the statistical results were given by the No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC).

RESULTS AND DISCUSSION

The Fig.1 present the NRRT curves of DEET on *Perna perna* mussels. It was observed statistic significance at DEET concentrations of 1.0 and 20.0 mg L⁻¹ for all exposure times (24, 48 and 72 h) in relation to control. In this assay the NOEC was 0.1 µg L⁻¹.

The results of mussels irradiated at 3 and 11 Gy of gamma radiation from ⁶⁰Co and exposed to different DEET concentrations during 24, 48 and 72 h are presented in Fig.2 and Fig.3, respectively. It was observed that the retention time was not statistically different from the assay with non-irradiated mussels, suggesting that these doses had not been sufficient to promote damage observable within the period in which the experiment was conducted.

The number of cells decreases in irradiated mussels with 11 Gy, what demonstrates such dose level when combined with exposition of DEET in range of 0.1 – 20 µg L⁻¹ does not show irreversible effects to the *Perna perna* mussels.

The dose of 107 Gy caused some adverse effects to organisms. It was observed a significant reduction in the number of cells compared with the control (Figure 4). In addition, cell lysis occurs in most of analyzed slides (>80%). Also, mortality occurred in some organisms when exposed to 1 µg L⁻¹ and 20.0 µg L⁻¹ of DEET.

The mussels that were irradiated with 107 Gy ⁶⁰Co gamma radiation and exposed to DEET, had a shorter retention time of neutral red dye on lysosomes of hemocytes, what suggests that the toxicity increases when organisms are irradiated and exposed to compound.

It is also suggested that exposure to this radiation dose, followed by exposure to DEET have caused some disorder in physiological system of mussels, and those who failed to recover from this disorder, came to death.

The International Commission on Radiological Protection, proposed to include in 2002 molecular effects, such as DNA damage, as endpoint. Indeed, gamma irradiation is known to cause repairable and non-repairable lesions to the DNA, and lethal effect depends on competing processes of repair and mis-repair DNA (Minouflet *et al.*, 2005; Tsyusko *et al.*, 2007).

Mortality and reproduction of organisms are currently the main endpoints used to assess effects on populations exposed to radiation (Woodhead, 2003). The risk to non-human biota due to ionizing radiation exposure is a considerable current interest to International Commission on Radiological Protection (ICRP) and International Atomic Energy Agency (IAEA), and both of them recommend the radiation assessment on some natural organisms (IAEA, 1992; ICRP, 2007).

In the present study, the radiation was used as a stressor to the mussels to verify the behavior of NRRT when the organisms were exposed to DEET after irradiation. It was observed that if the mussels were exposed only by gamma radiation at 3, 11 and 107 Gy doses the NRRT did not show alterations in the assay results, in relation to control. The organisms exposition to 0.1, 1 and 20 µg L⁻¹ after irradiation, it was observed NRRT decrease with increasing DEET concentration with significance at 1 and 20 µg L⁻¹ concentrations in all used radiation doses.

Despite the DEET being the active ingredient in most insect repellents used worldwide there are still scarce data about his behavior on the environment, and the processes of degradation of the compound after treatment of waters and effluents. In recent years, the contamination of DEET has been widely disseminated, and the compound has been detected in several aquatic environmental matrices, including rivers, groundwater, ocean, wastewater, and even in water treated by conventional water treatment systems (Sandstrom, 2005; Tay, 2009; Calza, 2011). Wilson *et al.* (2013) detected concentrations of DEET in samples of surface water in Colorado River in the range of 5.5 to 8.5 ng L⁻¹. In Thompson Bay of Lake Havasu was

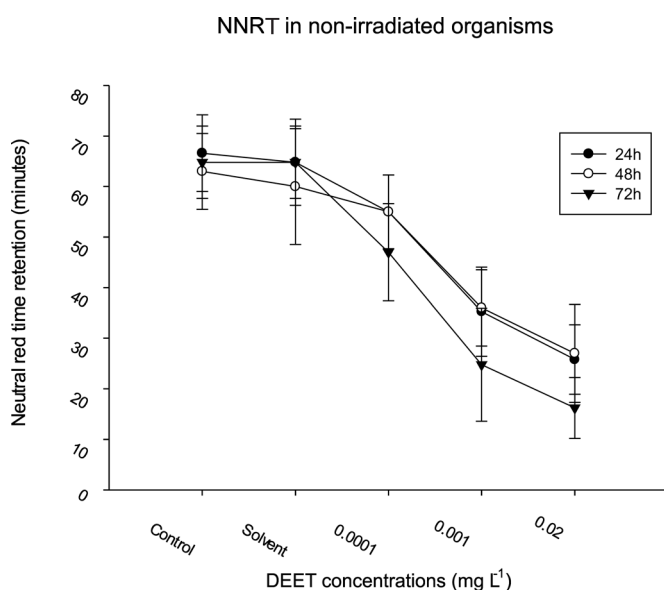


Figure 1 - Neutral red retention time results of *Perna perna* mussels exposed to different concentrations of DEET during 24, 48 and 72 h

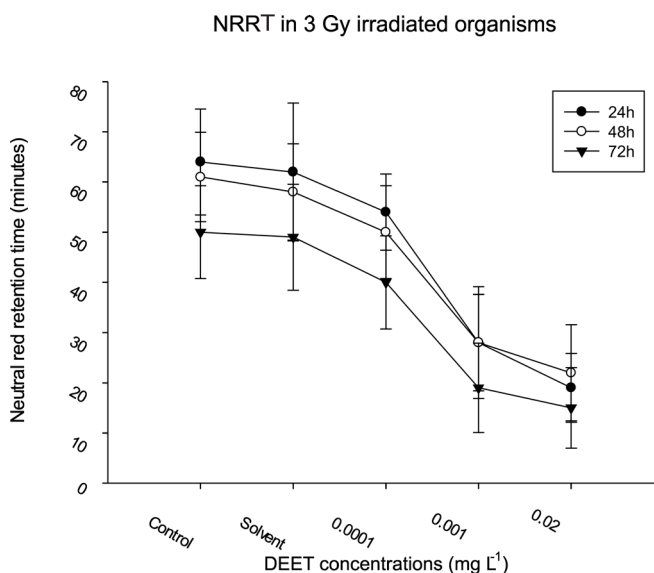


Figure 2 - Neutral red retention time results of *Perna perna* mussels irradiated at 3 Gy dose and exposed to different concentrations of DEET during 24, 48 and 72 h

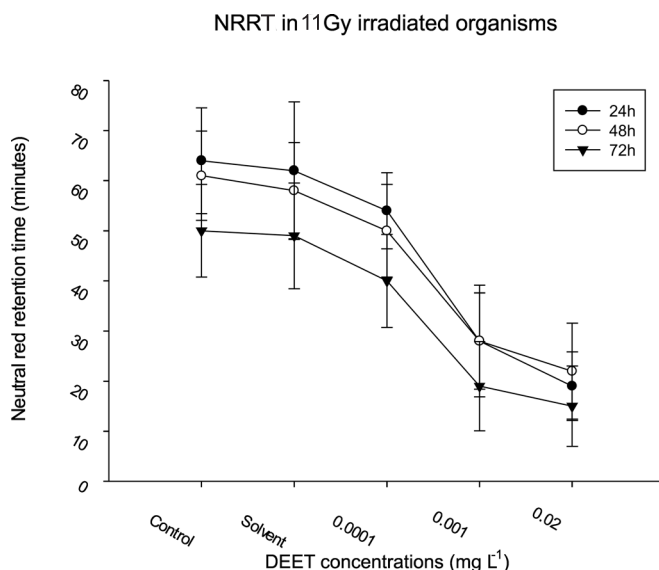


Figure 3 - Neutral red retention time results of *Perna perna* mussels irradiated at 11 Gy dose and exposed to different concentrations of DEET during 24, 48 and 72 h

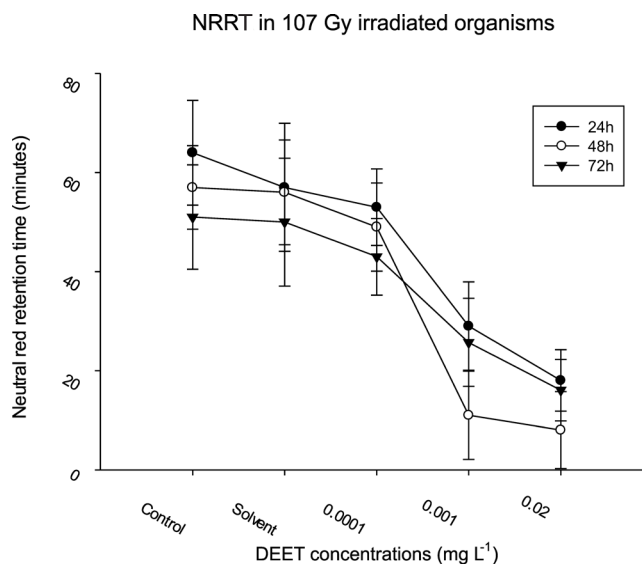


Figure 4 - Neutral red retention time results of *Perna perna* mussels irradiated at 107 Gy dose and exposed to different concentrations of DEET during 24, 48 and 72 h

also identified DEET concentration from 6.2 to 12 ng L⁻¹. Other studies also identified the presence of DEET in other environmental matrices, such as the study by Magnusson *et al.* (2013), where DEET were detected in sediments of rivers and estuaries of economic importance along the North Coast of Queensland in Australia, in Herbert River were detected 2.7 – 3.7 µg kg⁻¹ of DEET in sediment and the Daintree River 4.4 – 5.7 µg kg⁻¹. With several studies identifying concentrations of DEET in the order of µg and ng L⁻¹ in several matrices show the importance of evaluating the toxicity of this compound in non-target organisms, adverse effects caused on the biota and its possible potential for synergistic effect in combination with other substances. The present study found cytotoxic effect to DEET for *Perna perna* mussels at concentrations above 0.1 µg L⁻¹ a value very close to the concentrations identified in the environment, which highlights the need for more studies on the toxicity, persistence and degradation of this compound in the aquatic biota.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

DALLAS, L.J., KEITH-ROACH, M., LYONS, B.P., JHA, A.N.,

2012. Assessing the impact of ionizing radiation on aquatic invertebrates: a critical review. *Radiation Research* 177:693–716.
- EC: EUROPEAN COMMISSION. 2008. 31 December 2008. EU Regulation No. 1272/2008, Table 3.2. Official Journal of the European Union.
- FARRINGTON, J.W., TRIPP, B.W., 1995. International Mussel Watch Project: Initial Implementation Phase. Final Report. International Mussel Watch Committee. National Centers for Coastal Ocean Science, Silver Spring, MD, USA.
- FISHER, N. S., BEAUGELIN-SEILLERB K., HINTONB T. G., BAUMANN Z., MADIGAN D. J., GARNIER-LAPLACE J., 2013. Evaluation of radiation doses and associated risk from the Fukushima nuclear accident to marine biota and human consumers of seafood, vol. 110 no. 26, PNAS, <http://dx.doi.org/10.1073/pnas.1221834110>.
- GARNIER-LAPLACE J., 2010. A multi-criteria weight of evidence approach for deriving ecological benchmarks for radioactive substances. *J. Radiol. Prot* 30(2):215–233.
- GARNIER-LAPLACE, J., DELLA-VEDOVA, C., GILBIN, R., COPPLESTONE, D., HINGSTON, J., CIFFROY, P., 2006. First derivation of predicted-no-effect values for freshwater and terrestrial ecosystems exposed to radioactive substances. *Sci. Technol.* 40(20), 6498-6505.
- HIYAMA, A., NOHARA, C., KINJO, S., TAIRA, W., GIMA, S., TANAHARA A., OTAKI, J. M. 2012. The biological impacts of the Fukushima nuclear accident on the pale grass blue butterfly. *Nature, Sci Rep* 2, 570. <http://dx.doi.org/10.1038/srep00570>
- IAEA – INTERNATIONAL ATOMIC ENERGY AGENCY, 1992. Effects of ionizing radiation on plants and animals at levels implied by current radiation protection standards, Technical Reports Series No. 332. International Atomic Energy Agency, Vienna.
- ICRP – INTERNATIONAL COMMISSION ON RADIOLOGICAL PROTECTION. 2007 Recommendations of the International Commission on Radiological Protection. ICRP Publication 103. *Annals of ICRP* 37, 2–4.
- ICRP – INTERNATIONAL COMMISSION ON RADIATION PROTECTION. 2008. Publication 108. Environmental Protection: The Concept and Use of Reference Animals and Plants. *Ann ICRP* 37(4–6):1–242.

- JHA, A.N., CHEUNG, V.V., FOULKES, M.E., HILL, S.J., DEPLEDGE, M.H., 2000. Detection of genotoxins in the marine environment: adoption and evaluation of an integrated approach using the embryo larval stages of the marine mussel, *Mytilus edulis*. *Mutation Research* 464:213–228. [http://dx.doi.org/10.1016/S1383-5718\(99\)00188-6](http://dx.doi.org/10.1016/S1383-5718(99)00188-6)
- KEM, I. 2010. Assessment Report, N,N-diethyl-meta-toluamide (DEET), Product-type 19 (Repellents and Attractants). Rapporteur Member State: Sweden. KemI, Swedish Chemicals Agency. Sundbyberg. 11 March 2010.
- LOWE, D.M.; V.U. FOSSATO, 2000. The influence of environmental contaminants on lysosomal activity in the digestive cells of mussels (*Mytilus galloprovincialis*) from the Venice Lagoon. *Aquatic Toxicology*, 48(2–3): 75–85. [http://dx.doi.org/10.1016/S0166-445X\(99\)00054-5](http://dx.doi.org/10.1016/S0166-445X(99)00054-5)
- MINOUFLET, M., AYRAULT, S., BADOT, P.M., COTELLE, S., FERARD, J.F., 2005. Assessment of the genotoxicity of 137 Cs radiation using Vicia-micronucleus, Tradescantia- micronucleus and Tradescantia-stamen-hair mutation bioassays. *J. Environ. Radioact.* 81(2-3):143–153. <http://dx.doi.org/10.1016/j.jenvrad.2005.01.003>
- MOORE, M.N., DEPLEDGE, M.H., READMAN, J.W., PAUL LEONARD, D.R., 2004. An integrated biomarker based strategy for ecotoxicological evaluation of risk in environmental management. *Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis*, 552(1–2):247–268. <http://dx.doi.org/10.1016/j.mrfmmm.2004.06.028>
- SINGH, S. P. A., AZUA, A.; CHAUDHARY, A.; KHAN, S.; WILLETT, K.L.; GARDINALI, P.R. 2010. Occurrence and distribution of steroids, hormones and selected pharmaceuticals in South Florida coastal environments. *Ecotoxicology*. <http://dx.doi.org/10.1007/s10646-009-0416-0>
- TSYUSKO, O., YI, Y., COUGHLIN, D., MAIN, D., PODOLSKY, R., HINTON, T.G., GLENN, T.C., 2007. Radiation-induced untargeted germ line mutation in Japanese medaka. *Comp. Biochem. Phys. C.*, 145:103–110. <http://dx.doi.org/10.1016/j.cbpc.2006.08.010>
- USEPA - UNITED STATES ENVIRONMENTAL PROTECTION AGENCY. 1998. Reregistration Eligibility Decision (RED) DEET. Prevention, pesticides and toxic substances. p.134.
- WEEKS, J.A., GUINEY, P. D., NIKIFOROV, A. I.; 2012. Assessment of the Environmental Fate and Ecotoxicity of N,N-Diethyl-m-toluamide (DEET). *Integrated Environmental Assessment and Management*, 8 (1):120–134. <http://dx.doi.org/10.1002/ieam.1246>
- WOODHEAD, D.S., 2003. A possible approach for the assessment of radiation effects on populations of wild organisms in radionuclide-contaminated environments. *J. Environ. Radioact.* 66 (1-2):181–213. [http://dx.doi.org/10.1016/S0265-931X\(02\)00123-6](http://dx.doi.org/10.1016/S0265-931X(02)00123-6)