2011 International Nuclear Atlantic Conference - INAC 2011
Belo Horizonte,MG, Brazil, October 24-28, 2011
ASSOCIAÇÃO BRASILEIRA DE ENERGIA NUCLEAR - ABEN
ISBN: **978-85-99141-04-5**

USE OF MICRONUCLEUS TEST IN THE ASSESSMENT OF RADIATION EFFECTS IN AQUATIC ENVIRONMENTS

Edvaldo F. de A. Filho[1], Luanna R. S. Silva[2], Pedro A. de S. Lima[3], Francisco F. Amâncio[4], Edvane B. da Silva[5], Ronaldo C. da Silva[6] and Ana Maria M. de A. Melo[7]

[1] Departamento de Energia Nuclear
Universidade Federal de Pernambuco
Av. Prof. Moraes Rego, 1235 - Cidade Universitária
50670-901, Recife - PE
edvaldoaraujo18@hotmail.com

[2] Departamento de Energia Nuclear
Universidade Federal de Pernambuco
Av. Prof. Moraes Rego, 1235 - Cidade Universitária
50670-901, Recife – PE
luannaribeiro_lua@hotmail.com

[3] Departamento de Biofísica e Radiobiologia
Universidade Federal de Pernambuco
Av. Prof. Moraes Rego, 1235 - Cidade Universitária
50670-901, Recife – PE
pedroadeslima@gmail.com

[4] Departamento de Biofísica e Radiobiologia
Universidade Federal de Pernambuco
Av. Prof. Moraes Rego, 1235 - Cidade Universitária
50670-901, Recife – PE
amancioff@bol.com.br

[5] Centro Acadêmico de Vitória – UFPE
Núcleo de Ciências Biológicas
Av. Prof. Moraes Rego, 1235 - Cidade Universitária
50670-901, Recife – PE
edvborges@yahoo.com

[6] Departamento de Genética
Universidade Federal de Pernambuco
Av. Prof. Moraes Rego, 1235 - Cidade Universitária
50670-901, Recife – PE
ronaldocelerino@yahoo.com.br

[7] Departamento de Biofísica e Radiobiologia
Universidade Federal de Pernambuco
Av. Prof. Moraes Rego, 1235 - Cidade Universitária
50670-901, Recife – PE
amdemelo@hotmail.com

ABSTRACT

The study of the effects of radioactive substances on the environment is accomplished by radioecology. This science has played an important role in combating all forms of pollution. The uncontrolled use of physical and chemical agents has been a concern for environmental regulatory agencies, due to the serious damage to ecosystems. Aquatic organisms are exposed to a variety of pollutants harmful to aquatic systems. The mollusks *Biomphalaria glabrata* has been featured as a bioindicator to possess characteristics such as short reproductive cycle ease of maintenance in the laboratory and low maintenance cost. The micronucleus assay has been shown to be a great test to identify mutagenic effects caused by physical and chemical agents. In this study the frequency of micronuclei in haemocytes of *Biomphalaria glabrata* exposed to high doses of ^{60}Co gamma radiation contributing to a further standardization of this test as an indicator of the presence of radioactive contamination in aquatic environments. The young adult snails of *Biomphalaria glabrata* were divided into groups and subjected to a dose of 0 (control), 40 and 60 Gy of gamma radiation. The results showed that snails irradiated with 40 Gy showed a smaller number of haemocytes, whereas those exposed to 60 Gy had a greater quantity of these cells compared to control group. It can be concluded that the morphological analysis and the frequency of micronuclei in haemocytes of *Biomphalaria glabrata* exposed to ^{60}Co gamma radiation may be used in studies of the action of high doses of radiation in aquatic environments.

1. INTRODUCTION

As humanity increases its technological capacity to intervene in nature to satisfy their needs and desires, conflicts arise regarding the use of space, resources and increasing pollution and mutagens. It is this factor that the ecotoxicology, with the use of bioindicators, has contributed enormously to improving the life quality, minimizing the risks to human and environmental health [19].

According [9], in ecotoxicology, applied tests are intended to ascertain whether chemical substances, isolated or as complex mixtures, are harmful to biological systems, indicating the mechanisms of action and the local manifestation of their effects. Its use in risk assessment enables the detection of potentially toxic exposures well before actual adverse effects may occur.

According [15] mutagens agents can be of nature chemical and physical. Among the physical agents, there is radiation, a form of energy emitted by source and propagated in particles or electromagnetic waves. According to the energy and interaction form can be divided into ionizing radiation, where energy is enough to knock electrons around the atom, producing pairs of electrons; and non-ionizing radiation, which can cause excitation of atoms, molecules and breaking chemical bonds [13].

[10] said that livings organisms are exposed to radiation from natural or/and artificial sources, being necessary specialized sensors to detect the effects, of this sources of energies, in biological systems. Ionizing radiations are considered mutagens agents, inducing gene mutations, chromosomal aberrations and cell death [5], justifying the importance of this study.

The use of micronucleus assay in the genotoxicity detection and quantification of carcinogens and mutagens, is well established in vitro and in vivo systems [15]. Over the years, various substances were tested in numerous organisms, such as marine mollusks [6] [3] [11], freshwater mollusks (*Dreissena polymorpha* and *Planorbarius corneus*) [14], fish [1] and amphibians [7] [16].

Some studies, such as [2], seek to understand biological risks that arise after exposure of organisms to certain doses of ionizing radiation. The micronucleus test has shown a great way to identify genotoxics effects from DNA damage by gamma radiation [5]. Micronucleus (MN) are, structurally, smalls nucleus representing the genetic material that was lost by main nuclei, as consequence of genetic damage [17].

In several studies the mollusk *Biomphalaria glabrata* have been used as environmental bioindicators for chemical and physical agents [12] [10]. The mollusk show a short reproductive cycle, high resistance to radiation, easy and low maintenance cost in laboratory, have fully studied embryonic development [8], in addition to its wide geographical distribution and interference on human health.

Accordingly, the present study aims to assess the micronuclei frequency in hemolymph of *B. glabrata* exposed to high doses of ^{60}Co gamma radiation and contribute to the use of micronucleus test in *B. glabrata* as a bioindicator of DNA damage from exposure to physical agents, as gamma radiation.

2. METHODOLOGY

2.1. Origin, Creation and Mollusks Maintenance

For carry out the experiments we used young adult snails of the species *Biomphalaria glabrata* melanin (pigment), natural São Lourenço da Mata - PE and maintained in the Radiobiology laboratory, Biophysics and Radiobiology Department of Federal University of Pernambuco - Brazil in containers measuring 25 x 55 x 17 cm with dechlorinated water and filtered (filter Bella Source ®), pH around 7.0 and 25 ° C ± 2 º C. The water exchange was carried out twice per week and food was changed every day. All snails were fed with fresh organic lettuce (*Lactura sativa).*

2.2. Mollusks Selection

Wild snails *Biomphalaria glabrata* were separated, placed in individual containers with a volume of 180 ml, and observed for five consecutive days. This range, the snails were selected for shell diameter (between 10 and 14 mm), minimum age (two months) and sexual maturity.

2.3. Mollusks Irradiation

Selected snails were divided into three groups: two were subjected to doses of 40 and 60 Gy of ^{60}Co gamma radiation (Model II Excel 220 - MDS Nordion - belonging to the Nuclear Energy Department - Federal University of Pernambuco - Brazil) and one was not irradiated, which was the control group. Each group was composed of 10 snails.

The radiation dose applied in this study was established through previous studies carried out by CANTINHA (2010), in Radiobiology Laboratory of Federal University of Pernambuco - Brazil. To carry out the irradiations, snails were placed in plastic tubes of 50 ml (FALCON),

separated by thin layers of wet gases, to prevent desiccation, and then placed in the source Gammacell ® (dose rate 6.691 kGy/h - April 2010).

After irradiation, snails were separated in groups, placed in containers containing 280 ml of filtered water and fed on fresh lettuce. Only 48 hours after the snails were submitted to the micronucleus assay.

2.4. Micronucleus Assay

For this test, the hemolymph of the mollusk *B. glabrata* irradiated and not irradiated was collected by sensitizing of the dorsal part of snails. All procedures were done on the bench cleaned before. The tips were siliconized (sigmacote ® [SL2] - Sigma) according to the methodology [14].

2.4.1. Preparation of Slides to the Micronucleus Test

After collection, hemolymph was placed 100 mL of the same on each slide and equal amount of ethylenediaminetetraacetic acid (EDTA) at a concentration of 10 mM mixed with Ringer's solution, a solution of several salts dissolved in water for the purpose of creating an isotonic solution relative to the bodily fluids of an animal. After this procedure, the slide was placed for 30 minutes in a humid chamber. After that time, cells were fixed with 200 mL of 1% glutaraldehyde in Ringer's solution for 5 minutes, then rinsed with Ringer and stained with Giemsa. Whole process was performed according to methodology [14] with some adaptations.

2.4.3. Conventional Staining (Giemsa)

Slides were stained conventionally. We used a stock solution of Giemsa (Merck), blue-eosin-methylene blue diluted in phosphate buffer, pH 6.8, obtaining a final solution to 5%. The slides were submerged in this solution for 7 minutes and then washed with distilled water and then subjected to drying at room temperature.

2.4.4. Counting of Micronuclei

For each experimental group was made a count of 4 slides. In each slide were counted approximately 1000 cells with intact nuclear and cellular distinction. Micronuclei were identified according to the following criteria [14]:

I. Diameter smaller than 1 / 3 the size of the nucleus;
II. Color and texture similar to the nucleus;
III. Do not be in direct contact with the nuclei

All analysis was performed using an optical microscope (Medilux ®), with a 1000x magnification and a drop of immersion oil on each slide. The image capture was performed at the CCD camera and recorded in computer.

2.4.5. Statistical Analysis

Statistical analysis of results between groups was performed by the chi-square test ($\chi 2$), with 1 (one) degree of freedom and 5% (five percent) of significance through BioEstat software, version 5.0.

3. RESULTS AND DISCUSSION

3.1. Effects of ^{60}Co Gamma Radiation on Hemocytes of *B. Glabrata*

As shown in Fig.1, it can be observed that mollusks irradiated with 60 Gy had an increased number of haemocytes when compared with other experimental groups. Since snails exposed to a dose of 40 Gy showed a decrease of these cells observed in the four slides. These results suggest the existence of a defensive behavior between different groups of mollusks irradiated with high doses of ^{60}Co gamma radiation.

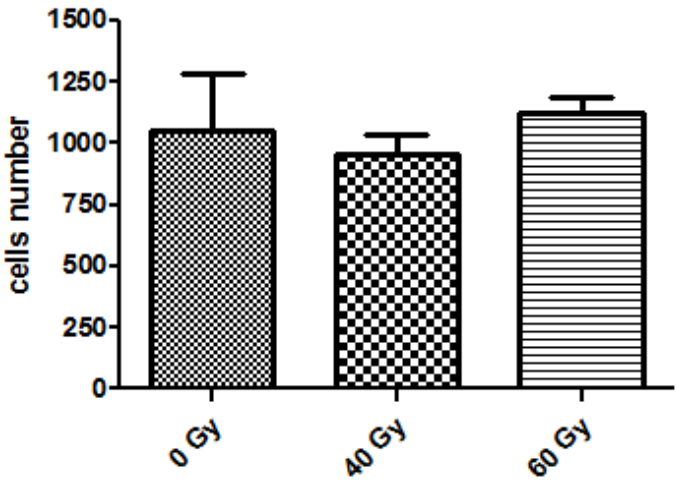

Figure 1. Number of hemocytes originate in the irradiated and non irradiated mollusks.

[2] examined effects of high doses of radiation on the fecundity *B. glabrata*, noting that the number of embryos produced by mollusks irradiated with doses of 40 and 60 Gy was higher than the control group. But these embryos had a higher amount of inviability. When compared to results obtained in our experiment, we found that the dose of 60 Gy is a critical dose for snail, because although it produces a greater number of defense cells (hemocytes) they should not able to play activities adequately due to morphological changes suffered after irradiation. These data will be shown later.

Fig. 1 also shows that quantitatively the haemocytes did not show a dose-dependent behavior and that differences are not statistically significant at doses of 40 and 60 Gy by $\chi 2$ test. In the individual comparison of each dose with the control was statistically significant.

It is known that exposure of individuals to whole-body irradiation may have different biological effects. In humans, the hematopoietic system is one of the most radiosensitive,

with the defense cells, lymphocytes. In biological dosimetry, the lymphocytes are more used to verify the biological effects suffered by individuals exposed accidentally or occupationally radiation [5]. What we see is the snails showing a similar behavior, because the defense cells, the haemocytes, exhibit morphological changes that are not observed in non-exposed haemocytes and the presence of micronuclei when exposed to ionizing radiation, suggesting its use in environmental assessment of biological dosimetry.

Studies carried out by [10] & [2] evaluated the use of mollusks as biomarkers of exposure to ionizing radiation in aquatic environments, but the results only approached as morphological changes in fecundity, fertility and mortality of the molluscs. Some biological changes are not perceived by these tests. The results, however, indicate that it is necessary to use a tool for analyzing biological effects suffered by the DNA molecule after exposure to radiation, which makes it a more sensitive and effective test for detection of morphological changes are not noticeable.

During the analysis of slides for scoring MN, different cellular changes on irradiated mollusk hemocytes were observed at 40 and 60 Gy doses. In 60 Gy, we observed a higher number of morphological altered cells. These changes were nuclear anomaly, with many vacuoles (Fig. 2).

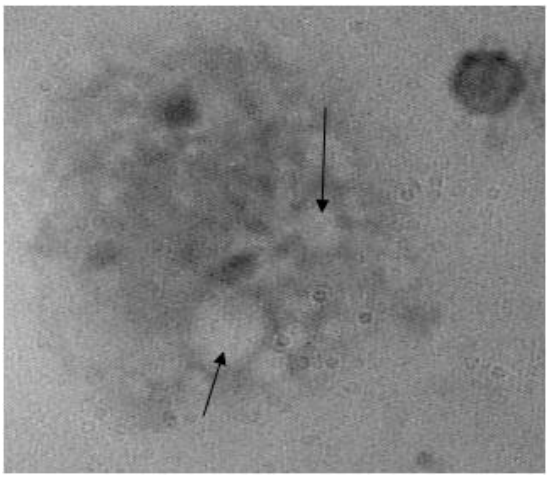

Figure 2. Hemocytes of *Biomphalaria glabrata* with deformity after receiving a dose of ^{60}Co. 1000x magnification.

Other cellular changes were exclusively observed during microscopic analysis of hemocytes, originating from mollusks exposed to a dose of 60 Gy. The haemocytes showed weak cellular beyond filamentous extensions the cell membrane (Fig. 3). However, up to now no studies to report these types of modifications.

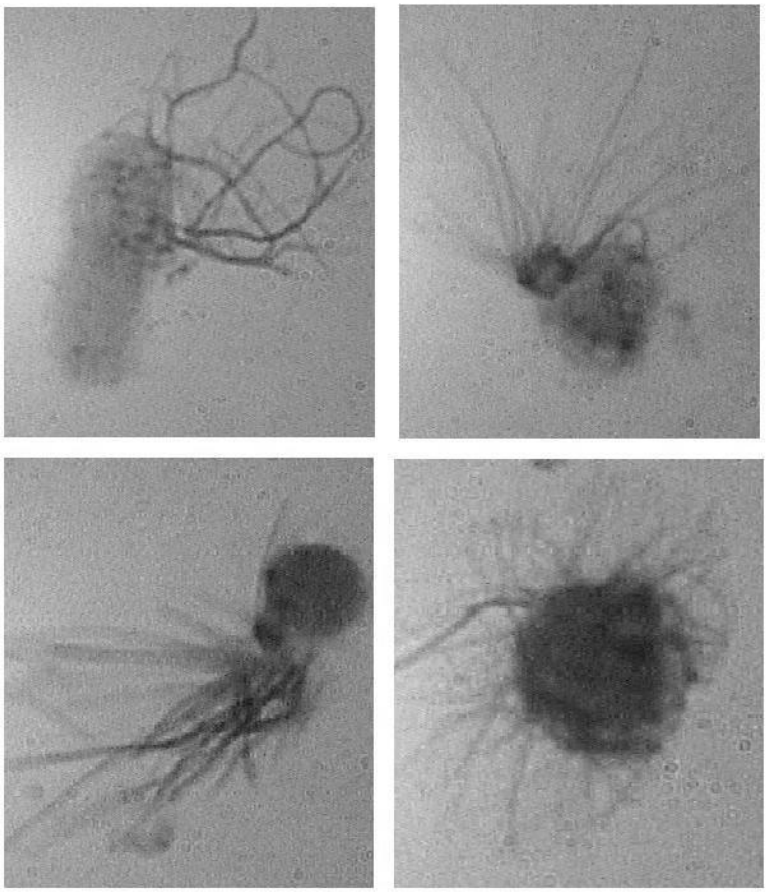

Figure 3. Hemocytes morphologically altered. Magnification1000x.

3.2. Analysis of Micronucleus Frequency

It can be seen in Fig. 4, a significant increase in micronucleus frequency of mollusks exposed to doses of 40 and 60 Gy of gamma radiation compared to control.

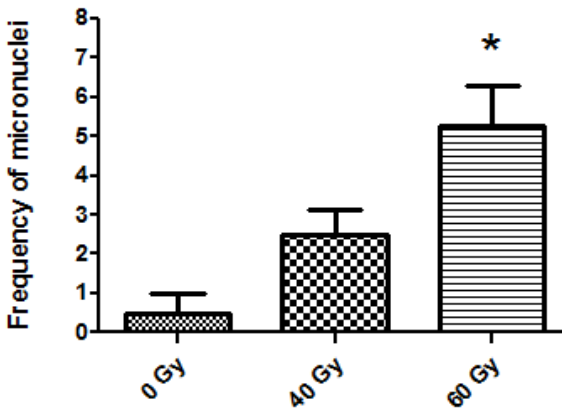

Figure 4. MN frequency of haemocytes of *B. glabrata* exposed to gamma radiation of 60Co.

Comparing the frequency of micronuclei detected in the experimental groups we can observe the existence of a dose-dependent effect. The study also found significant differences in comparison to the control.

The detection of micronuclei is a technique used to determine cytogenetic damage caused by clastogenic and aneugenic agents mainly in vertebrates [4].

Other studies have been conducted with marine mollusks (Mytilus galloprovincialis) in order to analyze the environmental contamination in these habitats. In this study it was shown that the micronucleus assay could detect changes in the DNA of mollusks exposed to different chemicals, corroborating the study of [18].

On the other hand, few studies have been published on the use of biological indicators of aquatic environments. This makes our results novel and important for ecotoxicological studies.

It was also shown that the snail *B. glabrata* is radiosensitive and the study of changes in hemocytes and the micronucleus test can serve as a new tool for studying the action of mutagens in the aquatic environment.

6. CONCLUSION

The data obtained from irradiation of the snails *Biomphalaria glabrata* adults with doses of 40 and 60 Gy of ^{60}Co gamma radiation, allowed to conclude that adult snails were sensitive to the effects of ^{60}Co gamma radiation. The number of hemocytes in a dose of 40 Gy was lower, whereas those exposed to 60 Gy a greater quantity of these cells.

The haemocytes of mollusks irradiated showed morphological type filamentous structures as well as nuclear abnormalities and apoptosis is the dose of 60 Gy the most radiotoxic. There was a significant increase in micronucleus frequency in all irradiated mollusks. This MN are generated lither in response to radiation and can be used as are indicator of the presence of radiation in aquatic enviroments.

7. REFERÊNCIAS BIBLIOGRÁFICAS

[1] BELPAEME, K.; DELBEKE, K.; ZHU, L.; KIRCHVOLDERS, M. Cytoge-netic studies of brown trout _*Salmo trutta fario*. using the micronucleus test and the alkaline comet assay. *Mutation Research,* **v. 11 _5**, pp. 485 – 492 (1996).

[2] CANTINHA, R. S.; BORRELY, S. I.; NAKANO, E.; AMARAL, A.; SILVA, L. R. S.; MELO, A. M. M. A. Effects of high dose rate gamma radiation on survival and reproduction of *Biomphalaria Glabrata*, *Int. J. Low. Radiation,* **v. 7**, No.4 pp. 245 - 252 (2010).

[3] CARRILHO. J; LEITÃO. A; VICENTE. C; MALHEIRO. I. Cytogenetics of *Anodonta cygnea* (Mollusca: Bivalvia) as possible indicator of environmental adversity; Estuarine, *Coastal and Shelf Science,* **v. 80**, pp. 303–306 (2008).

[4] CÓLUS, I. M. S., MARTINEZ, C. B. R.; Biomarcadores em peixes neotropicais para o monitoramento da poluição aquática na bacia do rio Tibagi. In: MEDRI, M. E. *et al*. Editores, Londrina - Brasil (2002).

[5] FERNANDES, T. S.; LLOYD, D.; AMARAL. A.. A comparison of different cytological stains for biological dosimetry; *International Journal of Radiation Biology*; v. 84, n. 8, pp. 703-711 (2008)**.**

[6] GODOY, J..M.; OLIVEIRA, M. S.; ALMEIDA, C. E. B.; CARVALHO, Z. L.; SILVA, E. R.; FERNANDES, F. C.; PITANGA, F. L.; DANELON, O. M.. 210Po concentration in *Perna perna* mussels: looking for radiation effects; *Journal of Environmental Radioactivity*, **v. 99**, pp. 631-640 (2008).

[7] JAYLET, A.; DEPARIS, P.; FERRIER, V.; GRINFELD, S.; SIBOULET, R.; A new micronucleus test using peripheral blood erythrocytes of the newt *Pleurodeles waltl* to detect mutagen in freshwater pollution. *Mutation Research,* **v.** 164, pp. 245–257 (1986).

[8] KAWANO, T.; OKAZAKI, K.; RÉ, L.; Embryonic development of *Biomphalaria glabrata* (SAY, 1818) (Mollusca, Gatropoda, Planorbidae): a practical guide to the main stages. *Malacologia*, v. 34, pp. 25-32 (1992).

[9] KNIE, J. L. W.; LOPES, E. W. B**.**. *Testes Toxicológicos: Métodos, técnicas e aplicações.* FATMA/GTZ, Florianópolis – Brasil (2004).

[10] MELO, A. M. M. A.; CARVALHO, E. B.; MOTTA, M. A.. DL50 of Co-60 gamma rays applied on *Biomphalaria glabrata* (SAY, 1818). Bulletin Of The Institute Of Malacology, Japão, v. 3, n. 8, pp. 125-130 (2001).

[11] MERSCH, J.; BEAUVAIS, M. N.; NAGEL, P.. Induction of micronuclei in haemocytes and gill cells of zebra mussels, *Dreissena polymorpha*, exposed to clastogens. *Mutation Research,* **v. 371**, pp. 47–55 (1996).

[12] NAKANO, E.; WATANABE, L. C.; OHLWEILER, F. P.; PEREIRA, C. A. B.; KAWANO, T. Establishment of the dominant lethal test in the freshwater mollusk *Biomphalaria glabrata* (SAY, 1818). *Mutation Research*, London, **v. 536**, n° 1-2, pp. 145 154 (2003).

[13] OKUNO, E.. *Radiação: efeitos, riscos e benefícios*. HARBRA, São Paulo – Brasil (1988).

[14] PAVLICA, M.; KLOBUCAR, G. I. V. M.; VETMA, N.; ERBEN, R.; PAPES, D.. Detection of micronuclei in haemocytes of zebra mussel and great ramshorn snail exposed to pentachlorophenol. *Mutation Research*, v. 465, pp. 145 – 150 (2000).

[15] RABELLO-GAY, M. N.; RODRIGUES, M. A. L. R.; MONTELEONE-NETO, R.. Mutagênese, Carcinogênese e Teratogênese: Métodos e critérios de avaliação. *Sociedade Brasileira de Genética/ Revista Brasileira de Genética*. Ribeirão Preto - Brasil, pp. 246 (1991).

[16] VAN HUMMELEN, P.; ZOLL, C.; PAULUSSEN, J.; KIRSCH-VOLDERS, M.; JAYLET, A.. The micronucleus test in *Xenopus*: a new and simple in vivo technique for detection of mutagens in fresh water, *Mutation Research*, v. 4, pp. 12– 16 (1989).

[17] VILLELA, I.V.; OLIVEIRA, I. M.; SILVA, J.; HENRIQUES, J. A. P.; DNA damage and repair in haemolymph cells of golden mussel (*Limnoperna fortunei*) exposed to environmental contaminants; *Mutation Research,* **v. 605**, pp. 78–86 (2006).

[18] VUKMIROVIC, M.; BIHARI, N.; ZAHN, R. K.; MULLER, W. E. G.; BATEL, R. DNA damage in marine mussel *Mytilus galloprovincialis* as a biomarker of environmental contamination. *Marine Ecology Progress Series,* **v. 109**, pp. 165 -171 (1994).

[19] ZAGATTO, P. A.; BERTOLETTI, E., *Ecotoxicologia Aquática: Princípios e Aplicações*. Editora Rima, São Paulo – Brasil (2008).