

Bioethical principles applied to aquatic ecotoxicological studies

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Abstract

Aquatic ecotoxicology analyzes the adverse effects of chemical pollutants in the biota. The aim of this study was to analyze the adequacy of the laboratory trials with aquatic organisms, based on the principles of the 3Rs. Twenty articles on aquatic ecotoxicology published from 2010 to 2014 were reviewed. The numbers of animals utilized in ecotoxicology trials, acclimatization, research approved by the committee on ethics and utilization of anesthetics were verified. The findings indicate that the principles of refinement and replacement, of the 3Rs program, were the most encountered in the research; while most did not cite approval of experimental protocols by an ethics committee, nor the use of anesthetics on the organisms during the experiments. The research published indicates a need to reduce the number of in the bio-trials, and to apply principles of bioethics in experimentation with aquatic organisms.

Keywords: Ecotoxicology. Animal experimentation. Bioethics. Ethics-Animals.

Resumo

Princípios bioéticos aplicados aos estudos ecotoxicológicos aquáticos

A ecotoxicologia aquática analisa os efeitos adversos dos poluentes químicos na biota, utilizando-se de animais nos ensaios laboratoriais. Neste trabalho, objetivou-se analisar a adequação dos ensaios laboratoriais realizados com organismos aquáticos com base nos princípios dos 3 R. Foram realizadas revisões de 20 artigos sobre ecotoxicologia aquática, publicados no período de 2010 a 2014. A análise verificou o número de animais utilizados nos ensaios ecotoxicológicos, aclimação, aprovação da pesquisa por comitê de ética e uso de anestésicos. Os resultados indicam que os princípios de refinamento e substituição, do programa dos 3R, foram os que mais ocorreram nas pesquisas, porém a maioria delas não citou aprovação dos protocolos experimentais por um comitê de ética nem o uso de anestésicos nos organismos durante os experimentos. As pesquisas publicadas indicam a necessidade de redução do número de organismos nos bioensaios, bem como a aplicação dos princípios bioéticos na experimentação realizada com organismos aquáticos.

Palavras-chave: Ecotoxicologia. Experimentação animal. Bioética. Ética-Animais.

Resumen

Principios bioéticos aplicados a los estudios ecotoxicológicos acuáticos

La ecotoxicología acuática analiza los efectos adversos de los contaminantes químicos en la biota, utilizando animales en las pruebas de laboratorio. En este trabajo se tuvo como objetivo analizar la adecuación de las pruebas de laboratorio realizados con organismos acuáticos basadas en los principios de las 3Rs. Se realizaron revisiones en 20 artículos sobre Ecotoxicología acuática, publicados en el periodo de 2010 a 2014. Se verificó el número de animales utilizados en las pruebas ecotoxicológicas y en la aclimatación. La investigación fue aprobada por el comité de ética y para la utilización de anestésicos. Los resultados indican que los principios Refinamiento y Sustitución, del programa de las 3Rs, fueron los más encontrados en los estudios, pero que la mayoría no citó aprobación de los protocolos experimentales por un comité de ética, ni para el uso de anestésicos en los organismos durante los experimentos. Las investigaciones publicadas indican la necesidad de reducir el número de organismos en las biopruebas, así como la aplicación de los principios de la bioética en la experimentación realizada con los organismos acuáticos.

Palabras-clave: Ecotoxicología. Experimentación animal. Bioética. Ética-Animales.

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Ecotoxicology describes the relationship between chemical pollutants, the environment in which they are released and organisms that live there. Further studies of these substances are necessary in order to minimise their potential risks and to put in practice cleansing measures to attenuate their likely impacts¹. So, ecotoxicology is presented as a tool to analyse the exposure of various xenobiotics to the environment where they were inserted² and also the adverse effects of exogenous pollutants in the environment and aquatic organisms³.

The ecotoxicological assessment is extremely important in the control, regulation and classification of toxic substances regarding their potential environmental risk. The identification of the effects and the evaluation of the dose-response relationship are initial steps in the process of environmental risk analysis⁴. Many tests are also performed with the aim to evaluate both the relative sensitivity of aquatic organisms in relation to a specific toxic agent as well as the efficiency of different methods of treatment of industrial effluents⁵.

Bioethics requires the combination of various disciplines, aimed at the proper use of the knowledge acquired by science as well as ethical values and biological facts. The first and best-known theoretical line of thought, the principlist bioethics, is guided by the principles of beneficence, nonmaleficence, autonomy and justice⁶. The beneficence is defined as the analysis of the benefits to the individual participant of the research and, subsequently, the benefits to society; nonmaleficence is characterised by the prevention of damage during the course of the study, which could affect the subject of the research; autonomy is defined as respect for the individual's capacity for choice; and justice is related to an equitable distribution of benefits among the beings⁷. In relation to the principles proposed by the *Universal Declaration on Bioethics and Human Rights* (abbreviated as DUBDH in Brazil)⁸, benefit and harm refer to the maximisation of benefits and minimisation of harm to individuals of the study and others involved; and protection of the environment, the biosphere and the biodiversity, characterised by an understanding of the interdependence between human beings and other life forms.

In many cases, the principles of bioethics - which include, in addition to the principlism theory and the *Universal Declaration on Bioethics and Human Rights*⁸, the ideas of the 3 R, proposed by scholars Russell and - are not observed when working with most organisms used in procedures, or the information is not cited in the scientific articles

published. In addition, the bioethical discussions propose several alternative methodologies, which development and implementation in institutions which use animals in scientific studies are a complex and extensive process¹⁰.

The use of animals in research has been the cause of various bioethical discussions focused on the large number of animals and the suffering caused to them. The program of the 3 Rs - reduction, refinement and replacement - aims to decrease the number of animals, minimise their pain, discomfort, and provide alternatives for the replacement of *in vivo* studies¹⁰. And, based on the 3 Rs principles, this work aimed to analyse the adequacy of laboratory tests performed with aquatic organisms in articles published between 2010 and 2014.

Bioethics and its principles

The term "bioethics" was disseminated by the American biologist and oncologist Van Rensselaer Potter in his book "Bioethics: Bridge to the Future"¹¹. This new field of study was created with the objective of enabling biological sciences to guarantee and improve the quality of life of future generations¹². The use of animals in scientific experiments increased with the advancement of technologies, making it necessary to expand the ethical discussions about these researches. As already mentioned, the zoologist William M. S. Russell, together with the microbiologist Rex L. Burch, proposed the 3 Rs principles in 1959, known as replacement, reduction and refinement¹³. These principles redirected the scientific work involving animal research to the use of alternative models in order to reduce not only the use of animals in experiments, but also the pain and suffering that the tests caused to them¹⁴.

Historically, bioethics discussion was more closely linked to medical settings, so much that, initially, the formulation of the guiding ethical principles of scientific activity occurred more in function of studies involving human beings. This conception, later known as principlism theory, is based on four principles: nonmaleficence, beneficence, justice and respect for autonomy¹⁵. The nonmaleficence means not to harm or to prevent harm to living beings; beneficence refers to the realisation of good and maximum reduction of harm that one can cause to another, recognising their moral values; justice has equity as the main axis, that is, all must be attended in the same way, and respect for autonomy is described

as the acceptance of self-determination before the situations of the environment ¹⁶, understanding autonomy as the capacity for choice of the individual ⁷.

Another milestone in connection with the organisation and guidance of bioethical matters was the *Universal Declaration on Bioethics and Human Rights*, adopted in October 2005 by the United Nations Educational, Scientific and Cultural Organisation (UNESCO) ⁸. There are principles, in the document, such as human dignity and human rights, autonomy and individual responsibility, consent, privacy and confidentiality, social responsibility and health. In addition, it is worth noting another two norms provided by the Universal Declaration on Bioethics and Human Rights, which are of particular interest for scientific research: benefit and harm, referring to the maximisation of benefits and minimisation of harm to the research participants and others involved; protection of the environment, the biosphere and the biodiversity, which assumes the interdependence of human subjects with other forms of life.

These principles are much more often applied to studies related to human beings than to animals of different taxonomic categories, which is why there are still many ethical issues to be discussed, among them is included one that is quite relevant to this article: Can these principles of bioethics be applied to all groups of organisms?

Aquatic ecotoxicology studies and bioethics

With the high demand for new technological products and the continued destruction of natural resources by human activities, many xenobiotics have been incorporated into the environment through direct or indirect ways, be them point or diffuse sources ¹⁷. Studies on these beings can be conducted directly in the field or through tests in laboratory (experimental tests with distinct methodologies), regulated by national and international standards on the use of bioindicators organisms in such tests ¹⁸. The aquatic organisms are usually used in ecotoxicological tests for various reasons ¹⁹, among them their physiological processes, such as nutrition and breathing, as many of them breathe through gills in direct contact with water, which can carry many contaminants into the interior of these organisms, causing morphophysiological changes in them ²⁰.

To identify these morphophysiological changes (biomarkers) in aquatic species, the use of laboratory techniques able to assess the degree of contamination on an area for a certain pollutant

is necessary ²¹. In these bioassays, one can check damage to organisms (that die or keep still), estimating the extent of their impact on the environment and human beings ²². The organisms used in these studies are fish and benthic macroinvertebrates, such as, for example, crustaceans from the gender *Daphnia*, that are easily grown *in situ* and exhibit reproductive characteristics that meet the purpose of these studies ²³. In the case of fish, among the species considered an international standard of reference is the *Danio rerio* (zebrafish), mainly because its genome sequence is similar to the genome sequence of other vertebrates, including human beings ²⁴.

Currently, the scientific community has turned to the ethical aspects of research with animals of the phylum Chordata (subphylum Vertebrata), for which there is specific Brazilian legislation ²⁵. There are also specific ethics committees that evaluate studies with insects and cephalopods, but its acting is still limited, since the current legislation gives priority to sentient organisms, which means vertebrate animals, whose sentience is widely recognised in the scientific community²⁶ Now, insentient organisms, among which are included the invertebrates, do not receive the same protection intended to sentient beings ²⁷.

Most of the time, researches involving ecotoxicology tests (with invertebrates or with vertebrates) occur without approval of ethics committees. Moreover, many researchers of the aquatic ecotoxicological field still have little contact with bioethics, either in their initial training or in their laboratory work. This situation needs to be addressed and changed so we can have a positive influence on the results of researches that get published and which serve as reference to new works in the area.

Given what was shown here, the aim of this study was to analyse the adequacy of laboratory tests performed with aquatic organisms on articles published from 2010 to 2014, based on the principles of the 3 Rs. In this sense, 20 scientific articles published in 16 different journals were analysed, which address ecotoxicological studies with animals of different taxonomic categories, aimed at the identification of the most widespread bioethical principles applied to animal testing among researchers.

Methodological path

This study is a literature review which data had been gathered from scientific articles about

the procedures performed in aquatic ecotoxicological tests realised between the years 2010 and 2014. We analysed 20 articles, which were systematically searched in databases registered at the Coordination of Training of Higher Education Personnel (abbreviated as “Capes” in Brazil) and from the Scientific Electronic Library Online (SciELO), containing the following descriptors: “ecotoxicology”; “Ecotoxicological studies”; “Bioethics”; “Ethics”.

First, for the organisation of data, we read the summaries of 20 selected scientific publications in order to identify the objects of study of the works, prioritising ecotoxicological tests^{28,29}. At least one of the bioethical principles was observed in all 20 studies. The technique used by the authors for the analysis of content was based on the reading of scientific papers chosen by examining the key information such as number of animals used in ecotoxicological testing, development phase and acclimatisation, origin

of organisms (*in situ* or *ex situ*) and research approved by an ethics committee. According to the content analysis of each work, we checked the adequacy of such data to the principles of the 3Rs (Replacement, reduction and refinement)¹³. The refinement was analysed taking as base the acclimatisation, the research approved by an ethics committee, methodological protocols of the ABNT (NBR 12713/2009³⁰ e 13373/2010³¹), (ABNT- abbreviation in portuguese of Brazilian Association of Technical Standards), the use of anaesthetics. The identification technique of the bioethical principles of William M. S. Russell and Rex L. Burch⁹ was the analysis of content “thematic-categorial”, involving the following phases: pre-analysis; exploitation of material or codification; treatment of results; inference and interpretation. Statistical softwares were used in order to obtain charts and percentages for organisation of data related to scientific production. The 20 works are listed in Table 1, below.

Table 1. Articles published between 2010 and 2014 and used in the bibliographic revision of this study

| Nº | Authors | Name of the Article | Magazine | Year |
|----|--|--|---|------|
| 1 | Rechenmacher C, Siebel AM, Goldoni A, Klauck CR, Sartori T, Rodrigues MT, Rodrigues MAS, Gehlen G, Ardenghi PG, Silva LB ²⁸ | A multibiomarker approach in rats to assess the impact of pollution on Sinos River, Southern Brazil | Brazilian Journal of Biology | 2010 |
| 2 | Nepomuceno JC, Silva AC ³² | Assessment of the frequency of peripheral micronuclei in erythrocytes of the mandi-amarelo (<i>Pimelodus maculatus</i>) in the Paranaíba river | Perquirere: Revista do Núcleo Interdisciplinar de Pesquisa e Extensão do Unipam | 2010 |
| 3 | Brandolt TD, Lobo E ³³ | Ecotoxicological assessment using bioassays with <i>Daphnia magna</i> in rice paddy's water in the county of Mostardas, RS, Brazil | Tecno-Lógica | 2010 |
| 4 | Barbola IF, Moraes MFPG, Anazawa TM, Nascimento EA, Sepka ER, Polegatto CM, Milléo J, Schühli GS ³⁴ | Assessment of aquatic macroinvertebrate communities as a tool for the monitoring of a reservoir in the Pitangui river basin, Paraná, Brazil | Iheringia, Série Zoologia | 2011 |
| 5 | Morais AV, Cortez FS, Bretas ES, Cesar E, Pereira CDS, Santos AR ³⁵ | Assessment of the environmental quality of the Itagaré river, Bertioga-SP, based on toxicity tests and microbiologic indicators of current water quality | O Mundo da Saude | 2011 |
| 6 | Mendes BG, Budziak D, Stolberg J, Peixer ZI, Dalmarco JB, Simionatto EL, Pedrosa RC, Felipe KB, Ogawa J, Pegoraro C, Scheffer L, Besen MR, Oliveira LJGG, Geremias R ³⁶ | Study of the water quality of the Marombas river (SC/Brasil), using physicochemical parameters and bioassays | Revista de Ciências Ambientais | 2011 |
| 7 | Holanda JN, Maciel AP, Santos RL ³⁷ | Ecotoxicological assessment, using <i>Danio rerio</i> , of wash water from the purification of methyl soybean biodiesel | Boletim do Laboratório de Hidrobiologia | 2012 |

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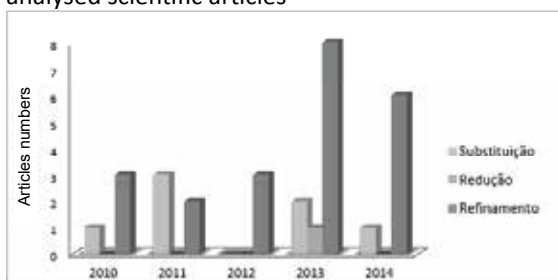
| Nº | Authors | Name of the Article | Magazine | Conclusão |
|----|--|--|---|-----------|
| | | | | Year |
| 8 | Damato M, Barbieri E ³⁸ | Study of the acute toxicity and metabolic alterations caused by the exposition of the fish <i>Hyphessobrycon callistus</i> , used as an indicator of environmental health to cadmium | O Mundo da Saúde | 2012 |
| 9 | Mannarino CF, Moreira JC, Ferreira JA, Arias ARL ³⁹ | Assessment of the impact, on the aquatic biota, of the combined effluent of the treatment of landfill leachate from municipal solid waste and sewage | Ciência & Saúde Coletiva | 2013 |
| 10 | Mohr G, Lobo EA ⁴⁰ | Assessment, using bioassays of the efficiency of a water treatment system in a small rural property | Revista Jovens Pesquisadores | 2013 |
| 11 | Gaspar N, Gago J, Repolho T, Luís O ⁴¹ | Assessment of the toxicity of lime mud on the sea urchin <i>Paracentrotus lividus</i> (Lamarck, 1816). Perspectives for new applications of this industrial waste | Revista da Unidade de Investigação do Instituto Politécnico de Santarém | 2013 |
| 12 | Hernandez OD, Castro F, Paez M ⁴² | Bioacumulación de mercurio en larvas de anuros en la zona afectada por la minería de oro en el río Dagua, Buenaventura, Valle del Cauca, Colombia | Acta Biológica Colombiana | 2013 |
| 13 | Canto T, Pich CT, Geremias R ⁴³ | Bioassays of toxicity in leachate in the landfill of the county of de Araranguá (Santa Catarina, Brasil) | Revista Biociências | 2013 |
| 14 | Sousa DBP, Almeida ZS, Carvalho-Neta RNF ⁴⁴ | histological biomarkers in two species of estuary catfish from the maranhense litoral, Brazil | Arquivo Brasileiro de Medicina Veterinária e Zootecnia | 2013 |
| 15 | Jacobowski AC, Zobiolo NN, Padilha PM, Moreno SE, Arruda EJ ⁴⁵ | Mutagenic effect of copper edetate ([Cu (EDTA)] - 2), free and nano encapsulated, in mice and fish | Ecotoxicology and Environmental Contamination – EEC | 2013 |
| 16 | Rezende KFO, Santos RM, Santos RM, Shimada JCB, Silva JRMC, Muniz CASD ⁴⁶ | Histopathology of the gills of Nile tilapia <i>Oreochromis niloticus</i> , from the Billings dam in the Area of environmental protection Bororé-Colônia | Atlas de Saúde Ambiental – ASA | 2013 |
| 17 | Pereira DP, Santos DMS, Carvalho Neta AV, Cruz CF, Carvalho Neta RNF ⁴⁷ | Morphological alterations in the gills of <i>Oreochromis niloticus</i> (Pisces, Cichlidae) as biomarkers of aquatic pollution in the Jansen Lagoon, São Luís, MA (Brazil) | Bioscience Journal | 2014 |
| 18 | Benites LM, Doncato KB, Minho TS, Perazzo GX ⁴⁸ | Assessment of the mutagenic potential of copper compounds in the water of the Uruguay river | Ciência e Natura | 2014 |
| 19 | Franco-Bernardes MF, Maschio LR, Azeredo-Oliveira MTV, Almeida EA ⁴⁹ | Biochemical and genotoxic effects of a commercial formulation of the herbicide tebuthiuron in <i>Oreochromis niloticus</i> of different sizes | Ecotoxicology and Environmental Contamination – EEC | 2014 |
| 20 | Nour EAA, Candello FP, Santos EMR, Barretto AS, Domingues LM ²⁹ | Biologic treatment of formaldehyde: residual toxicity monitored by bioassays with <i>Daphnia similis</i> | Ecotoxicology and Environmental Contamination – EEC | 2014 |

Uso dos princípios da bioética nos ensaios ecotoxicológicos

Os dados obtidos nas análises dos artigos científicos anteriormente elencados demonstram haver certa preocupação dos autores com o programa dos

3 R, já que a maioria dos trabalhos apresentava algum tipo de informação relacionada ao cuidado com os animais utilizados em estudos ecotoxicológicos. De acordo com esse programa, os conceitos encontrados foram refinamento, substituição e redução (Gráfico 1), dos quais os dois primeiros apareceram com maior frequência.

Figure 1. Distribution of the 3 Rs in relation to analysed scientific articles



Regarding the number of animals used in the ecotoxicological procedures, 4 articles did not mention the amount of animals, but other studies (16) presented that information. Most of the studies analysed (14) used between 11 and 50 individuals in the researches; however, four studies used between 300 and 18,473 specimens. Only one scientific paper used up to 10 individuals, adapting to the “reduction” principle of the 3Rs program⁵⁰. The large number of organisms used in a scientific experiment can be explained by the absence of legislation that limits the use of invertebrates in scientific studies²⁴, given that these studies were conducted with aquatic and semi-aquatic macroinvertebrates. These kinds of study using thousands of individuals do not yet include the program of the 3 R^{29,50}.

To date, the concern with ethical aspects focuses on tests with animals in the phylum Chordata, subphylum Vertebrata. There are some groups and ethics committees which also evaluate studies on insects and cephalopods. This fact, however, is still the exception. As a result, organisms lower in the zoological scale, which are classified as unprotected, can also be used instead of vertebrates, characterising the “replacement” principle. One example is the use of *Daphnia pulex* (water flea), *Artemia salina* (brine shrimp) and bees in studies, mainly of ecotoxicology⁵¹. Thus, 30% of the studies analysed used species such as *Daphnia similis* (water flea), *Daphnia magna* (freshwater water flea), *Ceriodaphnia dubia* (freshwater water flea) and *Artemia* sp. (brine shrimp) and these were assessed as important in the “replacement” principle of the 3 R. program.

Just over half of the analysed studies (53%) reported that the acclimatisation of organisms was performed before the ecotoxicological procedures, indicating that the principle of refinement was observed, since the animals were kept in conditions that minimised their pain and stress⁵². This type of procedure is, moreover, provided by Brazilian law⁵³. The acclimatisation is characterised by long-lasting adaptive physiological adjustments of

organisms that result in increased tolerance to continuous or repeated exposure to multiple stressors, usually realised under field conditions⁵⁴. The acclimatisation and the reduction or alleviation of pain are considered improvements in animal care, since researchers must know how to evaluate and monitor the pain in order to provide ethical treatment to animals⁵⁵. In addition to ethical concerns, the pain and stress of the animals lead to undesirable variables in the study, as they can significantly interfere with the interpretation of the study’s results⁵⁶.

The use of anaesthesia was reported in 15% of the analysed articles, indicating that the principle of refinement was used, since the technique provides decreased suffering of organisms used in experiments⁵². During the practice of some of the laboratory procedures, the animals are subjected to various types of pain and suffering; therefore, they must be sedated and anaesthetised properly^{57,58}.

It is important to highlight that 20% of the scientific work carried out *in situ* collected peripheral blood to be smeared on microscope slides, and then the animals were returned to their habitat. This kind of research - which return all organisms to the environment, benefiting both researchers and the animals researched - are desirable because they decrease the impacts on the local wildlife and produce more reliable information on the real situation of the natural habitat of the species studied, which makes it possible to work even with mammals and endangered species⁵⁹.

Very few articles (20%) mentioned the approval of their experimental protocol by an institutional ethics committee. The vast majority of the studies (80%) did not report such approval, and did not even point out that the research involved larvae of species that are classified in the category of “least concern” by the International Union for Conservation of Nature (IUCN), and only 25% mentioned the use of protocols standardised by the ABNT (NBR 12713/2009³⁰ e 13373/2010³¹). Scientific studies involving animals should be analysed by an ethics committee, whose role is to guide and review protocols, and increase knowledge about proper moral conduct towards animals⁶⁰. It is important that the experimental protocol is analysed regarding the aspect of the methodology used, in order to identify the impacts caused to animals regarding pain, suffering, confinement, stress and death¹⁴.

Researchers have the responsibility to submit their research protocols to an Ethics Committee that guarantees the possibility to adapt best practice

guidelines which ensure the welfare of organisms subjected to a study. The use of animal species in different contexts (from ecotoxicological tests to animal production and population management within the scope of conservation programs) deserves the observation of the principles of bioethics⁶¹, so that science can evolve and respect all forms of life on the planet.

Considerações finais

The discussion on methodologies and innovative concepts introduced in scientific research with animals has been increasing in scientific pub-

lications in recent years. However, in published works containing results of ecotoxicological tests on aquatic environments, there are still several issues that need to be debated, such as the concrete need for approval of experimental protocols by ethics committees; assurance that bioethical principles are coupled up to laboratory methodologies; use of anaesthesia to minimise animal suffering; reduction of the number of organisms in laboratory tests, and adoption of alternative methods in the use of animals, without necessarily affecting the result of the research. To discuss bioethical ideas is fundamental, and the principles of the 3 Rs should be considered in the preparation of projects and in current scientific research practices in the aquatic ecotoxicology area.

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