Ciência Rural, Santa Maria, v.47: 12, e20170194, 2017 http://dx.doi.org/10.1590/0103-8478cr20170194
ISSNe 1678-4596
BIOLOGY

Evaluation of eugenol toxicity in bioassays with test-organisms

Juliano Santos Gueretz[1*] Cleder Alexandre Somensi[1] Maurício Laterça Martins[2] Antonio Pereira de Souza[3]

[1]Instituto Federal Catarinense, Campus Araquari, 89245-000, Araquari, SC, Brasil. E-mail: juliano.gueretz@ifc.edu.br. *Corresponding author.
[2]Laboratório AQUOS - Sanidade de Organismos Aquáticos, Departamento de Aquicultura, Universidade Federal de Santa Catarina (UFSC), Florianópolis, SC, Brasil.
[3]Programa de Pós-graduação em Ciência Animal, Universidade do Estado de Santa Catarina (UDESC), Lages, SC, Brasil.

ABSTRACT: *Fish in both natural and farming conditions are exposed to stress of capture, handling, transport or treatment that provoke low zootechnical performance. Anesthetics like eugenol obtained from clove oil have been used strategically not only in freshwater but also in marine and estuarine fish in order to reduce the stress. Apart from the eugenol indication as anesthetic and its low toxicity for animals, its environment action is not clear. Bioassays or ecotoxicity tests with indicator organisms are used to evaluate the mode of action of the pollutants in the environment. The aim of this study was to test the acute toxicity of eugenol using the microcrustacean Daphnia magna and the bacterium Aliivibrio fischeri, and also its chronic toxicity for the algae Desmodesmus subspicatus. Eugenol in the concentrations of 50, 75 and 100mg L^{-1} were toxic to tested indicator organisms.*
Key words: *Aliivibrio fischeri, Daphnia magna, Desmodesmus subspicatus, ecotoxicology.*

Avaliação da toxicidade do eugenol em bioensaios com organismos-teste

RESUMO: *Os peixes, seja em ambiente natural ou em piscicultura, sofrem efeito do estresse na captura, manejo, transporte ou tratamentos que provocam baixo desempenho zootécnico. Anestésicos, como o eugenol, obtido a partir do óleo de cravo, tem sido usado estrategicamente não só em peixes de água doce, mas também em peixes marinhos e estuarinos para reduzir o estresse. Apesar da indicação de eugenol como anestésico em peixes e da sua baixa toxicidade para estes animais, não se tem clareza da sua ação sobre o ambiente. Bioensaios ou testes de ecotoxicidade com organismos indicadores são usados em para avaliar o modo de ação de poluentes no ambiente. O objetivo deste estudo foi testar a toxicidade aguda do eugenol utilizando o microcrustáceo Daphnia magna e a bactéria Aliivibrio fischeri e sua toxicidade crônica para alga Desmodesmus subspicatus. O eugenol, nas concentrações de 50, 75 e 100mg L^{-1}, foi tóxico para organismos indicadores testados.*
Palavras-chave: *Aliivibrio fischeri, Daphnia magna, Desmodesmus subspicatus, ecotoxicologia.*

INTRODUCTION

Fish, whether in the natural or cultivated environment, are animals that easily suffer the consequences of stress, which may be resulted from management (biometrics, treatments, collection of biological material and transport) and negatively influence growth performance parameters and even cause mortality (INOUE & MORAES, 2007; VIDAL et al., 2008; ROTILI et al., 2012; MOREIRA et al., 2015; RIBEIRO et al., 2015).

Among the strategies to minimize the negative effect of stress in fish farm is the use of anesthetics for instance eugenol. It is obtained from clove oil, natural product extracted by distillation of the *Syzygium aromaticum* and other plants. The clove oil, where the eugenol is contained, is a dark brown liquid with a peculiar odor and has important biological activities, among them anesthesia and stress reduction in fishes. (KELECOM et al., 2002; INOUE & MORAES, 2007; SIMÕES et al., 2010; SIMÕES et al., 2012; SOUZA et al., 2015). Fish anesthesia, is also used to provide greater safety to workers and technicians when handling fish (BARBOSA et al., 2007; INOUE et al., 2011).

Eugenol has been tested over time and is suitable for the most varied species of fish, both

Received 03.21.17 Approved 08.24.17 Returned by the author 11.14.17
CR-2017-0194.R1

freshwater, saltwater and estuaries. CUNHA et al. (2010) evaluated eugenol at 50mg L^{-1} in *Rhamdia quelen* to control stress; at this concentration, the plasmatic cortisol was significantly lower related to the control group. ROTILI et al. (2012) tested eugenol as anesthetic in *Piaractus mesopotamicus* at 40, 80, 120, 160 e 200mg L^{-1} doses, and haven't observed fish toxicity. SUÁREZ-MARTÍNEZ et al. (2014) concluded that for the manipulation of the hybrid *Pseudoplatystoma metaeenseeiarius marmoratus* the concentration of eugenol is 50mg L^{-1}. PATTANASIRI et al. (2016) concluded that eugenol and clove oil were effective in combating the stress of *Betta splendens* transport.

In ecotoxicology, bioassays or ecotoxicity tests with indicator organisms, also called test-organisms or bioindicators, allow the analysis of the action of the contaminants over the environment, whether isolated or by more than one source of contamination. Chemical analysis, either isolated or complex, in spite of necessary for the identification of the pollutants, do not indicate the risk of damage or environmental impact caused by the pollutants to living organisms, unless it has been previously established, since they do not demonstrate the effects over the ecosystem. Physical-chemical analysis and bioassays with test-organisms are complementary (COSTA et al., 2008; MAGALHÃES & FERRÃO FILHO, 2008; SILVA, 2015).

Among the test-organisms indicated for bioassays, stand out the microcrustacean *Daphnia magna*, the algae *Desmodesmus subspicatus*, and the bacterium *Aliivibrio fischeri*, both for the ease in conducting tests and for the cost (COSTA et al., 2008).

In one of the most used tests (*A. fischeri*), the bioluminescence emitted by the bacteria, which acts in the environment as a decomposer, is the result of the oxidation reaction of riboflavin 5-phosphate, and a long chain aldehyde, in the presence of oxygen, mediated by the enzyme luciferase. This reaction can be inhibited by pollutants, resulting in decrease of the intensity of light (COSTA et al., 2008; POLEZA et al., 2008). This intensity of light can be measured by a luminometer.

Notwithstanding the indication, in the literature, of eugenol for fish anesthesia, the lack of clarity about the potential toxic to the environment of this substance may be a limiting factor in its use. Since water containing eugenol used in the fish anesthesia process, constitutes an effluent with possible potential polluting to the environment. This study aimed to test the acute toxicity of eugenol by means of bioassays, for the microcrustacean *D. magna*, the bacterium *A. fischeri*, and the chronic toxicity to *D. subspicatus* algae.

MATERIALS AND METHODS

Eugenol (a.p.) ($C_{10}H_{12}O_2$) at 99% Vetec® was diluted in absolute alcohol 99.6%, in the proportion of 1:20, resulting in an alcoholic stock solution of eugenol at 50g L^{-1}, in order to obtain solubility in water. From the alcoholic stock solution, three samples were prepared, diluted in distilled water, with the following concentrations: 50mg L^{-1}, 75mg L^{-1} e 100mg L^{-1}, and, three samples with the same concentrations, diluted in their own solution for *D. magna* bioassay. This solution consists of dilution water: hardness 196.02mg L^{-1} of $CaCO_3$, pH 7.72, dissolved oxygen 5.48mg L^{-1} and for the subsequent cultivation of the microcrustacean, also called culture water, with hardness of 201.96mg L^{-1} of $CaCO_3$, pH 7.78, dissolved oxygen 5.10mg L^{-1}.

The samples, geometrically diluted (2, 4, 8, 16, 32, 64, 128, 256 e 512 times), submitted to acute toxicity assay with *A. fischeri* bacteria and *D. magna* microcrustacean, and a chronic toxicity assay with the *D. subspicatus* algae. The assay method with *A. fischeri* bacteria followed the recommendations from ABNT NBR 15411: 2012, the *D. magna* microcrustacean from ABNT NBR 12713: 2009, and for *D. subspicatus* algae described by ABNT NBR 12648: 2011.

The influence of the sample over *D. magna* is verified by the immobility of organisms exposed to the analyte (eugenol) after 48 hours. For *D. subspicatus* the action of the analyte is verified by counting (cells mL^{-1}) of the test-organisms at the beginning and at the end of the assay (72 hours), whereas for the *A. fischeri* bacterium it is measured the initial and final bioluminescence (after 30 minutes of incubation), on a Lumistox 300 luminometer (Dr. Bruno Lange, Düsseldorf, Germany). Results for the acute tests are presented as Toxicity Factor (TF), that is, the lowest dilution of the sample in which is not observed significant effect in the mobility (in more than 10% of the organisms) and luminescence (in more than 20% of the luminescence), measured in luminometer respectively, for *D. magna* and *A. fischeri*, when compared to the control treatments. Additionally, to TF, EC_{50} results were demonstrated, that is, the effective concentration of the sample that shows a toxic effect of 50% over the indicator organism. For the chronic toxicity assay, results are presented as EC_O (lower concentration of analyte in

which significant effect is observed on algae growth), and EC_{NO} (higher concentration of analyte in which no significant effect is observed on algae growth). For this test, EC_{50} values are also shown.

RESULTS AND DISCUSION

All the concentrations, 50, 75 and 100mg L^{-1} of eugenol were toxic to the organisms tested, *D. magna*, *A. fischeri* e *D. subspicatus*. The diluent (alcohol 99.6) was not toxic to the test-organisms, under the test conditions.

Although, the ordinance 017/2002 of the Foundation of the Environment of Santa Catarina State (FATMA, 2002) does not establish maximum TF for fish farming effluents, it informs that dilution limits can be established for diverse effluents. The resolution 430 of 2011 of the Conselho Nacional do Meio Ambiente (CONAMA), also previse that, ecotoxicological assays for various effluents can be requested, using at least two distinct test-organisms. Observing the table 1, and comparing the TF's obtained in the samples tested in this study with those presented by FATMA for any type of effluent, it can be stated that, regardless of the concentration of eugenol tested, it was necessary a big dilution, so the sample could stop showing significant toxic effect. Thus, regardless the time required to occur the natural biodegradation of the compound in the environment, there is a possibility of an acute toxic effect in different trophic levels at the concentrations tested (which match the actual concentrations used). Any of the samples used in this research had to be diluted 128 times to stop showing a significant acute toxic effect (microcrustacean and bacteria).

Acute toxicity assays test severe and rapid effects that occur in a short time in the organisms tested, evaluating some effect on the organism, such as the inhibition of luminescence and immobility of these organisms, just like the tests with *A. fischeri* and *D. magna*. Chronic assays evaluate an action of the possible pollutant over part or in the entire life cycle of the tested organism (SILVA et al., 2015).

POLEZA et al. (2008) in a study about herbicide and insecticide toxicity in irrigated rice tillage, concluded that, compared to studies with other test organisms, the *A. fischeri* bacterium and the bioluminescence test showed low sensitivity to toxicity of the agrochemicals. However, for effluents released in freshwater, marine environments and estuaries, BERTOLETTI (2013) recommends tests with *A. fischeri* associated to other test organisms. In the present study, in addition to *A. fischeri*, *D. magna* and *D. subspicatus* were also used and for all, eugenol was toxic.

Catecholamines and plasma cortisol at high levels, a result of stress, caused an increase at the cardiorespiratory movements in fish, interference in gas exchanges, and increased energy expenditure in nitrogen excretion (INOUE & MORAES, 2007; SIMÕES et al., 2010; SIMÕES et al., 2012). To prevent the deleterious effects of stress on fish management, the use of anesthetics such as eugenol is indicated. This one, is suitable for minimizing the stress caused by fish handling (INOUE & MORAES, 2007; ROTILI et al., 2012). Regardless of the benefits of using eugenol as a mitigator of the stress in fish, it should be taken into account that it may also be toxic to other organisms of the ecosystem, as demonstrated in this paper.

According to the results, it is possible to infer that *D. magna* was more sensitive to eugenol than *A. fischeri* and *D. subspicatus* the most tolerant. Microcrustacean are generally more susceptible to diverse contaminants than the bacteria (KUNGOLOS et al., 2004; TEODOROVIC et al., 2009). Anyhow, small concentrations of the tested solutions are sufficient to present deleterious effects to these

Table 1 - Acute toxicity, for *Daphnia magna* and *Aliivibrio fischeri* and chronic toxicity for *Desmodesmus subspicatus* for eugenol at 50mg L^{-1} * in bioassays.

	TF	EC_{50}	OEC	NOEC
Daphnia magna	128	0.70	na	na
Aliivibrio fischeri	128	2,97	na	na
Desmodesmus subspicatus	na	40,78	12,50	6,25

*Lower concentration of eugenol tested, between 50, 75 and 100mg L^{-1}. TF: Toxicity Factor is a non-dimensional number expressing the highest dilution of eugenol that does not cause acute deleterious effects on organisms at a given exposure time under the test conditions. EC_{50}: Effective concentration of the sample having a toxic effect of 50% on the indicator organism. OEC: lower concentration of eugenol in which significant effect is observed on the growth of the test organism. NOEC: higher concentration of eugenol in which there is no significant effect on the test organism. na: not applicable.

organisms, according to CE_{50} results; therefore, additional care is required for contaminated water disposal with eugenol. Eugenol may also present chronic deleterious effects to algae, in relatively low concentrations, requiring other tests of this nature to confirm this hypothesis. However, these results reinforce the need for additional care for the disposal of these wastes in the environment.

Differently from the present study, which tested eugenol in bioindicators, VIDAL et al. (2008) evaluated eugenol as an anesthetic agent in *Oreochromis niloticus* and concluded that the concentration of 50mg L^{-1} is effective for anesthesia, concentrations above 81.97mg L^{-1} were toxic, and 184.26mg L^{-1} lethal for this species. Concentrations of 50mg L^{-1}, as pointed out by the results of this study, are highly toxic to the different test-organisms used here.

SUTILI et al. (2014) evaluated the anti-helminthic activity of eugenol, at the same time tested its toxicity to *Daphnia pulex*; at 5 e 10mg L^{-1} concentrations reported no toxicity to these microcrustaceans, and found LC_{50} at 42.5mg L^{-1}. In this experiment the concentrations of 50, 75 and 100mg L^{-1} were tested and all were toxic to *D. magna*, EC_{50} for this microcrustacean was 0.70mg L^{-1}.

Test-organisms are used in ecotoxicology to evaluate the response of these organisms to the exposure of one or more pollutants, simulating a natural environment; thus constituting, an useful tool for the control and monitoring of environmental quality (LAITANO & MATIAS, 2006). Due to this, it was opted for bioassays in this study.

CONCLUSION

All the tested concentrations, 50, 75 and 100mg L^{-1} of eugenol resulted in acute toxicity for the bacterium *Aliivibrio fischeri* and for the microcrustacean *Daphnia magna*, and chronic toxicity for the alga *Desmodesmus subspicatus*. The microcrustacean was the most sensitive, followed by the bacterium and the more resistant algae. Even in the absence of results demonstrating that the concentrations tested in this study have already been detected in aquatic bodies, aquaculture effluents with these concentrations of eugenol may cause pre-dilution toxicity to the biota of these receptor bodies, suggesting additional care with waste. Testing and testing with other test organisms, including fish, are also required, using different concentrations than those tested, as well as other vehicles for the dilution of eugenol, mainly because the concentrations tested show little linearity with EC_{50} values.

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