Ecotoxicology
https://doi.org/10.1007/s10646-020-02232-1

TECHNICAL NOTE

The use of an *Allonais inaequalis* reproduction test as an ecotoxicological bioassay

M. C. Felipe[1] · A. C. Bernegossi[1] · G. B. Castro[1] · F. R. Pinheiro[1] · B. L. Nadai[1] · B. N. Cardoso-Silva[1] · J. J. Corbi[1]

Accepted: 9 May 2020

Abstract

Ecotoxicological bioassays have been widely utilized to evaluate the toxicity of substances to organisms. However, the main challenge for researchers is finding native species to assess the effects of pollutants on aquatic biota. The tropical Oligochaeta, *Allonais inaequalis*, can be used as a test organism in bioassays to understand the effects of toxicants on aquatic ecosystems and their impact on native aquatic biota. In this study, we tested four methodological designs to validate the use of our "*Allonais inaequalis* reproduction test" as an ecotoxicological bioassay. For each sample, the assay consisted of a bottle containing 10 mg of sterilized fine sand, 60 mL of dechlorinated tap water and 6 organisms, fed at the beginning of the test and again after 5 days. The assay was first established in a controlled environment and then used to evaluate a stressed environment containing one of the following three toxicants suggested by the OECD (2008) and Corbi et al. (2015): zinc chloride, copper sulfate, or potassium chloride. Our results showed that the best experimental design for reproduction analysis was a static, long-term bioassay, which lasted 10 days without aeration and allowed for the reproduction of multiple generations (10 ± 5 new organisms). The observed inhibition reproduction by toxicants (EC50 ranging between 0.2 mg L^{-1} and 1.36 g L^{-1}) validated the methods used in this paper. The use of a reproduction endpoint is a new contribution to the ecotoxicological toolbox, examining responses from a native organism to predict the effects of pollutants in an aquatic environment.

Keywords Naididae · Native species · Oligochaeta · Long-term exposure

Introduction

Ecotoxicological bioassays are important tools for assessing the toxicity of substances to living organisms (Dornfeld et al. 2001). They are carried out in laboratories under specific and controlled experimental conditions in which test organisms are exposed to different contaminant concentrations, and the effects are observed and quantified (Costa et al. 2008).

Allonais inaequalis Stephenson 1911, is a native Brazilian aquatic Oligochaeta belonging to the Naididae family (Kathman and Wetzel 2003; Gomes et al. 2017) and can be used in toxicological bioassays as a test organism. The occurrence of *A. inaequalis* has been documented in many parts of the world, including Central and South America, Oceania, Africa, and Asia (Gorni et al. 2015; Pinder 2010; Pathiratne and Weerasundara 2004; Howmiller 1974; Brinkhurst and Jamieson 1971; Naidu 1966). This species has a body length between 3 and 9 mm, lives in the water column and sediment, does not present pigmentation and usually reproduces asexually by fission (Erséus et al. 2017; Corbi et al. 2015; Bely and Wray 2001).

A. inaequalis is used as a test organism in bioassays because it is representative of benthic and water column biota in freshwater systems and is easy to rear under laboratory conditions. The species can cycle organic matter and bioaccumulates substance either by body contact or ingestion (Corbi et al. 2015; EFSA 2013; Chapman 2001; Smith 1991). Thus, it has been used by some studies for water monitoring in tropical environments (Felipe 2019; Neto et al. 2019; Rocha et al. 2018; Corbi et al. 2015). Despite its use in the area of ecotoxicology, there is a lack of protocols using this species in bioassays. Therefore, this study proposes a new protocol, named the "*Allonais inaequalis* reproduction test".

✉ M. C. Felipe
mayarafelipe@usp.br

[1] Department of Hydraulic and Sanitation (SHS), Ecology of Aquatic Environments Laboratory, School of Engineering of Sao Carlos, University of Sao Paulo – USP, CEP 13566-590 Sao Carlos, SP, Brazil

Published online: 21 May 2020

Table 1 Experimental design to determine the best conditions for the *A. inaequalis* reproduction test

Configuration test	Number of organisms	Sediment	Medium	Condition	Aeration	Type of sampling
1	6 per replicate	10 mg per replicate	60 mL of dechlorinated tap water per replicate	Static	Yes	At the end of the test
2				Static	No	At the end of the test
3				Semi-static	Yes	Daily
4				Semi-static	No	Daily

Methodology

Cultivation of the test organisms

Allonais inaequalis were collected from the Ecological Municipal Park in Sao Carlos (Sao Paulo, Brazil), and a culture was maintained at the Aquatic Ecology Laboratory in plastic trays containing sterilized fine sand (kept in a muffle furnace for 4 h at 550 °C) and dechlorinated tap water. The organisms were maintained under the following controlled conditions: a temperature of 25 ± 2 °C, light–dark cycle of 12:12 h, and constant soft aeration (one bubble per second). Feeding (2 mg of Tetramin® fish food L^{-1}) and maintenance of water level (2 L) were performed weekly. In addition, temperature, pH, and water hardness were measured as recommended by USEPA (2002).

The use of sediment in *A. inaequalis* tests was established by OECD no. 315 (2008) and is justified because this is a benthic species, and there is an interaction between substances in the water column and sediment in aquatic environments (Lundy et al. 2017; Reynoldson 1987).

Method validation

The reproduction tests were performed in 15 replicates to achieve a consistent result (although, 3 replicates of this ecotoxicological test were enough to observe reliable results). Each replicate used 6 organisms in a glass bottle containing 60 mL of dechlorinated tap water (pH ranging from 6.5 to 7.5) and 10 mg of sterilized fine sand (kept in a furnace muffle for 4 h at 550 °C). The organisms were fed at the beginning of the test and again after 5 days with 2 mg of Tetramin® fish food L^{-1} for each organism. When testing aeration, we used the cap of the glass bottle to fix the aeration hose.

Four configurations of the bioassay were tested to determine the best conditions for the *A. inaequalis* reproduction test. The configurations were divided into static or semi-static (daily sample) and aeration or unaerated conditions as described in Table 1. Temperature and light-dark cycle were the same as those used for cultivation. The exposure time was set for 10 days, in contrast to the previously published short-term test, which had a duration of 96 h (Corbi et al. 2015).

In the static condition, all organisms were counted on the 10th day, and the number of additional individuals was assumed to represent a new generation. For semi-static conditions, the number of additional individuals were counted every day and removed them from the bottle, to guarantee the measurement of daily *A. inaequalis* reproduction.

To validate the best test configuration, we applied a normality test (Shapiro-Wilk) and an ANOVA test comparing these configurations using Statistica® software (STATSOFT 2007). The best configuration was identified based on the consistency of the results, the experimental cost (including energy and equipment), and laboratory space.

Ecotoxicological application

Once the method was defined, we investigated the effects of three contaminants on the reproduction rate of *A. inaequalis* to assess the feasibility of the proposed methods. In 5 replicates over 10 days, we exposed groups of 6 organisms to 8 different concentrations of zinc chloride ($ZnCl_2$), copper sulfate ($CuSO_4$) and potassium chloride (KCl) (Table 2), which are reference substances suggested by Corbi et al. (2015) and the OECD (2008) to assess aquatic Oligochaeta sensitivity. All data were analyzed and discussed considering statistical significance.

The organism's reproduction was analyzed using EC50, NOEC (EC10) (Beasley et al. 2015), and LOEC values. EC50 and NOEC were determined in R software (Version 3.3.5) using the packages MASS and DRC (R Core Team 2014). The LOEC was determined as the next concentration tested after the NOEC value.

Results

Method validation

Regarding reproduction, we plotted a curve showing the cumulative number of new organisms for the semi-static tests and the final number of new organisms produced in the static tests (Fig. 1). In the semi-static test with aeration, the number of new individuals in the samples reached 48 (mean of 3 ± 2 organisms per sample), and in the semi-static test

Table 2 Concentrations used in the *Allonais inaequalis* reproduction test (exposure of 6 organisms over 10 days under static conditions)

Substance	Concentrations
$ZnCl_2$	0.20, 1.0, 1.8, 2.6, 3.4, 4.2, 5.0, 5.8 mg L^{-1}
$CuSO_4$	0.03, 0.06, 0.09, 0.12, 0.15, 0.30, 0.60, 0.90 mg L^{-1}
KCl	0.5, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5 g L^{-1}

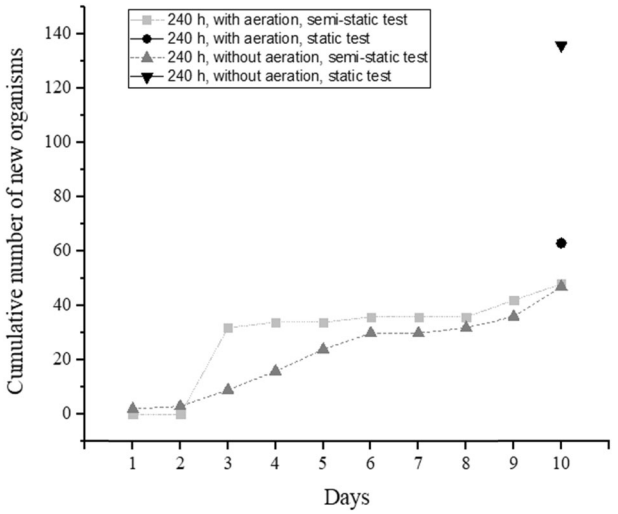

Fig. 1 Cumulative number of new organisms in the semi-static tests and final number of new organisms in the static tests

without aeration, 47 new individuals were produced (4 ± 2 organisms per sample). On the other hand, in the static test with aeration, 63 new organisms were noted after 10 days (4 ± 2 organisms per sample) and in the static test without aeration, 136 new organisms (10 ± 5 organisms per sample).

Significant differences were observed in the number of new organisms among the four experiments ($p = 0.021$) according to one-way ANOVA. The Tukey post hoc test did not indicate differences in the number of new organisms between aerated and nonaerated semi-static tests ($p = 0.93$), while in the static tests, there was evidence of significant differences in reproduction between the tests with and without aeration ($p = 0.0013$). Moreover, no significant differences in the number of new organisms were observed between the static and semi-static conditions with aeration ($p = 0.79$). However, in the absence of aeration, statistical differences were observed ($p = 0.0011$). We can conclude that aeration in semi-static tests did not influence the reproduction of the species, but the static condition without aeration is optimal, because it shows an upper limit of new organisms in the samples, prevents interferences in the experiments, and provides a lower experimental cost when taking energy, equipment and laboratory space into account.

The reproductive output of the parent animals can be expressed as the total number of new living organisms or as the mean number of new organisms which can be used to determine the effective impact on the reproduction rate. Based on appropriate statistical models, the toxic effect of the test substance/sample on reproductive output can also be expressed using the three classical ecotoxicological indices: "effective concentration" (ECx), which is the concentration responsible for causing an x% reduction in reproductive output'; "no observed effect concentration" (NOEC); and "lowest observed effect concentration" (LOEC).

Table 3 EC50, NOEC, and LOEC for $ZnCl_2$, $CuSO_4$, and KCl exposure using the *Allonais inaequalis* reproduction test

Reference substance	EC50	NOEC	LOEC
$ZnCl_2$ (mg L^{-1})	2.707	0.258	1.00
$CuSO_4$ (mg L^{-1})	0.274	0.097	0.12
KCl (g L^{-1})	1.360	0.261	0.50

Ecotoxicological application

The ecotoxicological bioassays tested here showed that the chosen methodology can be successfully applied to *A. inaequalis*. The three substances tested caused a significant reduction in the reproduction of *A. inaequalis* (Table 3). Furthermore, these results could be expressed in terms of all classical ecotoxicological indices.

The most toxic substance based on reproduction rate was $CuSO_4$, which had an effective concentration of EC50 0.274 mg L^{-1}, and the least toxic was KCl, with an EC50 of 1.360 g L^{-1}. The calculated NOEC for $CuSO_4$ showed that less than 100 µg L^{-1} does not present risks for *A. inaequalis* reproduction rate and this concentration is substantially below the NOEC found for KCL (0.261 g L^{-1}). The reference substance $ZnCl_2$ presented an intermediary concentration value for all indices with an EC50 of 2.707 mg L^{-1}.

Discussion

Method validation

The proposed reproduction test method is specific to *A. inaequalis* and is an actualization of the methodology first proposed by Corbi et al. (2015). We observed during laboratory cultivation that the space required to maintain these organisms was small, so, in order to generate less contaminated water and sediment and to minimize the cost of the bioassays, we used smaller vessels and reduced the volume of test solution from 240 mL to 60 mL. The lower volume requirement was also observed by Rocha et al. (2018), who used only 10 mL of test solution in acute bioassays. The parameters of temperature and light-dark

cycle were the same as those of the acute test proposed by Corbi et al. (2015) because *A. inaequalis* is a tropical organism. In contrast to the OECD (2005), these bioassays do not aim to analyze the capacity for bioaccumulation of the substance when presented in sediment, so, despite using the reference substances to validate the methodology, the exposure time was decreased from 28 days to 10 days, and the use of aeration was discarded during methodology development.

There are few reproduction tests studies that evaluate the response of aquatic Oligochaeta to water pollutants. Most of the standardized reproduction tests found in the literature use terrestrial Oligochaeta (OECD 2004; ISO 2003), and they are normally used to test field samples of contaminated sediments or for synthetic substrate contamination (Mendonça et al. 2019; Vezzone et al. 2018; Gupta et al. 2015; Niemeyer et al. 2015; Novais et al. 2011; Spurgeon et al. 1994). Therefore, we highlight the importance of the reproduction test methodology presented in this study for a tropical aquatic Oligochaeta.

Ecotoxicological application

The application of this methodology represents a better understanding of the actions of toxicants on a tropical aquatic Oligochaeta. Acute bioassays, despite their relevance regarding substance assessments and lethality, measure extreme effects on biota. Alternately, the use of long-term exposure allowing for the determination of the Oligochaeta reproduction rate gives us detailed knowledge of the impact these toxicants have on the life cycle of this species and could help the preservation of aquatic environments to begin at the first sign of pollution.

Conclusions

This study presents the application of a reproduction test to ecotoxicological studies utilizing a native Brazilian Oligochaeta species, which can improve the evaluation of environmental impacts in tropical regions. We concluded that the best configuration for the *Allonais inaequalis* reproduction test was a static assay with a 10 day exposure time, generating 10 ± 5 new organisms in the control experiment. The results regarding the application of the reproduction test to the assessment of reference substances demonstrated the efficiency of this methodology.

Acknowledgements We are grateful to the National Council for Scientific and Technological Development (CNPq—grants# 168736/2018-7 and 131610/2018-0), the Brazilian Coordination for the Improvement of Higher Education Personnel (CAPES—grants# 88887.353028/2019-00 and 88887.339518/2019-00) and the São Paulo Research Foundation (FAPESP - grants# 2018/21901-0 and 2016/24622-9) for financing this research.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The animal used in this article is an aquatic invertebrate.

Informed consent Informed consent was obtained from all individual participants included in the study.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

Beasley A, Belanger SE, Brill JL, Otter RR (2015) Evaluation and comparison of the relationship between NOEC and EC10 or EC20 values in chronic *Daphnia* toxicity testing. Environ Toxicol Chem 34:2378–2384. https://doi.org/10.1002/etc.3086

Bely AE, Wray GA (2001) Evolution of regeneration and fission in annelids: insights from engrailed and orthodenticle-class gene expression. Development 128(14):2781–2791

Brinkhurst RO, Jamieson BGM (1971) Aquatic Oligochaeta of the World. Oliver and Boyd, Edinburgh

Chapman PM (2001) Utility and relevance of aquatic oligochaetes in ecological risk assessment. In Aquatic Oligochaete Biology VIII. Springer, Dordrecht, pp 149–169

Corbi JJ, Gorni GR, Correa RC (2015) An evaluation of *Allonais inaequalis* Stephenson, 1911 (Oligochaeta: Naididae) as a toxicity test organism. Ecotoxicol Environ Contam 10(1):7–11. https://doi.org/10.5132/eec.2015.01.02

Costa C, Olivi P, Botta C, Espindola E (2008) A toxicidade em ambientes aquáticos: discussão e métodos de avaliação. Quimica Nova 31(7):1820–1830

Dornfeld CB, Masutti MB, Andrade CA, Almeida CA, Silvério PF (2001) Caracterização ecotoxicológica do sedimento da Represa do Lobo (Itirapina - Brotas, SP) e seus tributários. In: Espindola ELG (ed) Impactos ambientais em recursos hídricos: causas e consequências. Rima editora, Sao Carlos, Brazil, pp 245–259

EFSA (European Food Safety Authority) (2013) Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters EFSA J 11(7):1–268

Erséus C, Envall I, De Wit P, Gustavsson LM (2017) Molecular data reveal a tropical freshwater origin of Naidinae (Annelida, Clitellata, Naididae). Mol Phylogenet Evol 115:115–127. https://doi.org/10.1016/j.ympev.2017.07.016

Felipe MC (2019) Toxicity bioassays using aquatic invertebrates in exposure to the Linear Alkylbenzene Sulfonate. Thesis, University of Sao Paulo. Sao Carlos, Brazil

Gomes DF, Sanches NA, Sahm LH, Gorni GR (2017) Aquatic Oligochaeta (Annelida: Clitellata) in extractive reserve Lake Cunã, Western Brazilian Amazon. Biota Neotropica 17(1):e20160232. https://doi.org/10.1590/1676-0611-BN-2016-0232

Gorni GR, Peiró DF, Sanches NA (2015) Aquatic Oligochaeta (Annelida: Clitellata) from State of São Paulo, Brazil: diversity and occurrence review. Biota Neotropica 15(1):e20140063. https://doi.org/10.1590/1676-06032015006314

Gupta S, Kushwah T, Vishwakarma A (2015) Optimization of ZnO-NPs to investigate their safe application by assessing their effects on soil nematode *Caenorhabditis elegans*. Nanoscale Res Lett 10:303. https://doi.org/10.1186/s11671-015-1010-4

Howmiller RP (1974) Some Naididae and Tubificidae from Central America. Hydrobiologia 44(1):1–12. https://doi.org/10.1007/BF00036152

ISO (International Organization for Standardization) (2003) Soil quality—effects of pollutants on enchytraeidae (*Enchytraeus* sp.)—determination of effects on reproduction and survival, Guideline No. 16387. ISO, Geneva

Kathman RD, Wetzel MJ (2003) *Allonais inaequalis* (Annelida: Oligochaeta: Tubificidae) in North America. Proc Biolog Soc Wash 116(3):548–556

Lundy L, Alves L, Revitt M, Wildeboer D (2017) Metal water-sediment interactions and impacts on an urban ecosystem. Int J Environ Res Publ Health 14(7):722. https://doi.org/10.3390/ijerph14070722

Mendonça M, Rodrigues NP, de Jesus MB, Amorim M (2019) Graphene-based nanomaterials in soil: ecotoxicity assessment using *Enchytraeus crypticus* reduced full life cycle. Nanomaterials 9(6):858. https://doi.org/10.3390/nano9060858

Naidu KV (1966) Checklist of fresh-water Oligochaeta of the Indian sub-continent and Tibet. Hydrobiologia 27:208–226

Neto AJG, Moreira RA, dos Santos Lima JC, Daam MA, Rocha O (2019) Freshwater neotropical oligochaetes as native test species for the toxicity evaluation of cadmium, mercury and their mixtures. Ecotoxicology 28(2):133–142. https://doi.org/10.1007/s10646-018-2006-5

Niemeyer JC, Moreira-Santos M, Ribeiro R, Rutgers M, Nogueira MA, da Silva, E. M., & Sousa, J. P. (2015) Ecological risk assessment of a metal-contaminated area in the Tropics. Tier II: detailed assessment. PLoS ONE 10(11). https://doi.org/10.1371/journal.pone.0141772

Novais SC, Gomes SI, Gravato C, Guilhermino L, De Coen W, Soares AM, Amorim MJ (2011) Reproduction and biochemical responses in *Enchytraeus albidus* (Oligochaeta) to zinc or cadmium exposures. Environ Pollut 159(7):1836–1843. https://doi.org/10.1016/j.envpol.2011.03.031

OECD (Organization for Economy Co-Operation and Development) (2008) Test No 315: bioaccumulation in sediment-dwelling benthic oligochaetes, OECD guidelines for the testing of chemicals, section 3. OECD Publishing, Paris, 10.1787/9789264067516-en

OECD (Organization for Economy Co-Operation and Development) (2004) Test No 220: enchytraeid reproduction test. OECD Publishing, Paris, 10.1787/9789264070301-en

OECD (Organization for Economy Co-Operation and Development) (2005) Attachment 3, Technical Report: Validation of a sediment toxicity test with the endobenthic aquatic oligochaete Lumbriculus variegatus by an International Ring Test, OECD Publishing, Paris

Pathiratne A, Weerasundara A (2004) Bioassessment of selected inland water bodies in Sri Lanka using benthic oligochaetes with consideration of temporal variations. Int Rev Hydrobiol 89 (3):305–316. https://doi.org/10.1002/iroh.200310676

Pinder A (2010) Tools for identifying selected Australian aquatic oligochaetes (Clitellata: Annelida). Museum Victoria Science Reports 13:1–26. https://doi.org/10.24199/j.mvsr.2010.13

R Core Team (2014) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/

Reynoldson TB (1987) Interactions between sediment contaminants and benthic organisms. Hydrobiologia 149:53–66

Rocha O, Neto AJG, Lima JCS, Freitas EC, Miguel M, Mansano AS, Moreira RA, Daam MA (2018) Sensitivities of three tropical indigenous freshwater invertebrates to single and mixture exposures of diuron and carbofuran and their commercial formulations. Ecotoxicology 27:834–844. https://doi.org/10.1007/s10646-018-1921-9

Smith DA, Kennedy JH, Dickson KL (1991) An evaluation of a naidid oligochaete as a toxicity test organism. Environ Toxicol Chem 10:1459–1465

Spurgeon DJ, Hopkin SP, Jones DT (1994) Effects of cadmium, copper, lead and zinc on growth, reproduction and survival of the earthworm *Eisenia fetida* (Savigny): Assessing the environmental impact of point-source metal contamination in terrestrial ecosystems. Environ Pollut 84(2):123–130. https://doi.org/10.1016/0269-7491(94)90094-9

Statsoft (2007) Statistica (data analysis software system) ver. 8.0. Tulsa, StatSoft, Inc. CD-ROM

USEPA (United States Environmental Protection Agency) (2002) Methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. USEPA. Washington, EUA, pp 1–350

Vezzone M, Cesar R, Polinavov H, Serrano A, Siqueira D, Abreu L, Bianchi M, Correia ME, Castilhos Z, De Campos T (2018) Ecotoxicological evaluation of dredged sediments from Rodrigo de Freitas Lagoon (Rio de Janeiro State, Brazil) using bioassays with earthworms and collembolans. Environ Earth Sci 77:743. https://doi.org/10.1007/s12665-018-7930-4