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Original Article

Larval Biometry as an Endpoint in Chronic Sea Urchin Assays *Echinometra lucunter* (Linnaeus, 1758)

Test based on the biometrics of *E. lucunter* larvae

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Abstract

This study was carried out with the aim of contributing to the improvement of the evaluation of sea urchin embryo-larval tests, reducing the subjectivity of the analysis and solving the problems associated with the morphology of different Echinodermata larvae used in ecotoxicological tests. In this regard, the larval size technique (biometrics) was applied as an endpoint in sub-lethal assays using the toxic reference substance zinc sulfate and potassium dichromate as standards for accuracy and reliability the EC_{50} estimate (Median Effective Concentration) and NOEC (No Observed Effect Concentration). The tests, using the sea urchin species *Echinometra lucunter*, were carried out between the years 2017 to 2019 and were analyzed by two methods, the traditional (counting normal larvae and those with developmental delay) and biometrics based on the maximum length of the larvae obtained in the treatments. It was established that the minimum number of measurements of the different stages of development (egg, embryo and larva) was 35 per replicate. For the tests carried out over 15 months, it was verified, based on the Wald Test, that there was a seasonality in the sizes of the larvae. The EC_{50} of the metals tested was similar between the traditional and biometric methods when adopting the size of 18-hour-old larvae as a reference for 100% effect, but there was disagreement between the methods when the size of the eggs was considered as the total effect. In contrast, the estimates of the NOEC were more sensitive to the biometry method, in disagreement with the traditional method.

Keywords: Echinodermata; Larval biometry; Endpoint; Reproducibility.

INTRODUCTION

The first stages of development of organisms are the most sensitive, being the weakest link in the life cycle of an organism (Rand & Petrocelli, 1985). Due to this fact, embryo-larval assays of marine invertebrates, in addition to being able to detect exposure to a wide spectrum of toxic substances, are sensitive to a low concentration of these contaminants when compared to other test organisms (Resgalla Jr. & Laitano, 2002).

The sea urchin embryo-larval test, one of the most widely used tests in the world, uses quantitative morphological alterations in the development of larvae as an effect parameter or endpoint, which requires a detailed microscopic analysis of each individual (APHA, 1998). However, there are problems associated with this technique, since it requires a deep training to be able to discriminate between the correctly and the badly developed larvae, because of the quite high possibility of commiting mistakes in toxicity evaluation. This, linked to the fact that this kind of assays are widely used in environmental risk assessment of natural matrices, makes the reproducibility of bioassay results a mandatory requirement, despite the variability in the responses of different larvae to different contaminants (Morroni *et al.*, 2023).

For a correct analysis of the adverse effects that contaminants can cause on the morphology of sea urchin larvae, the use of larval size measurements can be adopted as a criterion for classifying the degree of damage. This technique was recently presented by Saco-Álvarez *et al.*

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(2010) highlighting the advantages of eliminating the observer's subjectivity and allowing, in the near future, automated image analysis systems to take over the final phase of the tests, optimizing the analysis time (Alvarez-Mora *et al.*, 2022).

However, whatever the new proposal for analyzing or determining the responses of a test organism to chemical or polluting agents, there will always be a need of a careful evaluation of this parameter and its use in comparison with the traditionally used methods. The responses that test organisms can provide in assays are as diverse as the types of assays (USEPA, 2002). For this reason, every time a new position is presented, there is always time for adaptation and evaluation by the research group to adopt it as a routine procedure.

On the other hand, automation in toxicity tests is a reality that cannot be ignored. Today there are different examples of the use of tests with test organisms where the analyses are performed automatically by video such as those used for *Daphnia* (Reed *et al.*, 2010 and Chevalier, *et al.*, 2014) and *Danio rerio* fish (Kopman, *et al.* .2012) and real-time application.

Thus, if the first steps are presented to generate data that can enable the automatic quantification of tests with marine invertebrates, studies in environmental monitoring programs will be more applicable and faster. In this sense, the validation of effects observed in sea urchin larvae based on variations in their size may, in the future, be easily applied to equipment such as multi-size particle counters, both conduction-based and electric as laser.

In this matter, this work presents an analysis of the endpoint protocol in tests with the sea urchin *Echinometra lucunter* using the size of the larvae as an effect criterion. For this, seasonal variations in the size of the larvae of this species were investigated and the estimates of the Median Effective Concentration (EC_{50}) and the No Observed Effect Concentration (NOEC) were compared by traditional analysis and by size of larvae in tests with the reference toxicant sulfate zinc and potassium dichromate. In addition to this measurement, it is believed that the biometrics technique can be easily applied to different species of echinoderm applied to environmental toxicology tests.

MATERIALS AND METHODS

Specimens of the sea urchin *Echinometra lucunter* were captured by scuba diving during 15 months between the years 2017 to 2019 with three to four replicates for each season of the year. The specimens were captured around Ilha Feia, north coast of Santa Catarina (Brazil) (26°44'36.96" S - 48°38'12.48" W) and, after collection, the organisms were transported in styrofoam boxes to UNIVALI's Marine Ecotoxicology Laboratory where they were kept in 360 liter tanks with sea water at 30 salinity, 23° C temperature and 12:12 photoperiod. The organisms were kept for a maximum of two weeks in the laboratory and fed with macroalgae. After spawning inductions, sea urchins were returned to the collection site. *E. lucunter* is a species of sea urchin from the Echinometridae family with thick, black spines. It exhibits burrowing behavior and it uses rocks as a substrate. They are herbivores, slow-growing, abundant and widely distributed in the western Atlantic, mainly in shallow tropical and subtropical waters (Núñez-González & Pauls, 2023). The species is registered as a test organism in chronic assays in Brazil according to the standards of ABNT/NBR (2020).

Briefly, the chronic larval development assays were performed according to Pagano et al. (1989) and APHA (1998) with some modifications. To obtain gametes, sea urchin adults were induced by injection of 2 mL of a mixture of 0.5 M KCl and 0.3 M $CaCl_2$ in a 1:1 ratio into the coelomic cavity (Resgalla Jr. *et al.*, 2020). The eggs obtained were collected in 600 mL beakers with 400 mL of the sea water where they were repeatedly washed by decantation. The spermatozoa were collected with a Pasteur pipette and deposited in beakers under ice. At the time of fertilization, a sperm solution was prepared in the proportion of 0.5 mL of concentrated spermatozoa to 24.5 mL of sea water. After homogenization, 0.5 mL of the sperm solution was added to the egg solution (400 mL) and 20 minutes later there was an examination and confirmation of a fertilization rate greater than 80%.

Embryos were exposed to six different concentrations of the toxic reference substance Zinc Sulfate (from 0.03 to 1.0 mg L^{-1} of $ZnSO_4.7H_2O$) and seven different concentrations of the Potassium Dichromate (from 0.3 to 20.0 mg L^{-1} of $K_2Cr_2O_7$) for 36 hours in 4 replicates and the control (seawater dilution) in 8 replicates. Assays were carried out in 15 mL transparent plastic flasks, containing 10 mL of test solution with approximately 300 embryos each, at a temperature of 26±2 °C, photoperiod 12:12 D:N. After 36 hours of development to the Pluteus larva, 1 mL of 4% formalin was added to each flask to finalize the test. The physical-chemical parameters monitored were pH, salinity and dissolved oxygen at the beginning and at the end of the test, in additional vials, maintaining a variation of less than 20%.

After fixation, the test flasks were analyzed in two different ways. The traditional analysis consisted of observing under a microscope using a Sedgewick-Rafter camera, recording the number of normal pluteus larvae and larvae with delayed development and/or deformities, with the quantification of 100 larvae for estimating the percentage effect. The validation of the tests was carried out with a minimum percentage of 80% of normal larvae in the control flasks. The second form of analysis was by measuring the maximum length of the larvae according to the criteria presented by Saco-Álvarez *et al.* (2010) using a Lumenera Infinity 1-3® camera, coupled to a microscope and taking measurements of the larvae by the Infinity Analyze Software from the capture of the image of the larvae. The minimum number (n) of measurements to be taken to detect significant differences in the estimates of toxicological parameters was determined through refraction analyses involving the mean and standard deviation using eight replicates of a control and taking measurements of

the maximum length of 100 larvae per replica. The test acceptability criterion, that is, the minimum size of the larvae for a test to be considered acceptable, was established from the upper percentile of 80% of the measurement values obtained in the control flasks, from 15 tests performed, totaling 4,165 larvae measurements.

To obtain the growth curve of *E. lucunter* from the measurements of the maximum length of the larvae (n of 35 per interval), a control test was carried out, in which the measurements of the larvae were taken at intervals of 8h until the end of the 36h test, observing from the first stage of development (egg) to the pluteus larval stage.

The existence of seasonality in the size of the larvae was evaluated from the generalized linear model (GLM) of an analysis of variance, with the statistical Wald Test (Siegel & Castellan, 2006) using the PAST program, since the data to evaluate the possible morphological variations in function of the time of year did not meet the precepts of normality and homogeneity of variances.

For the estimates of the percentage of effect based on the size of the larvae, two analyses were also carried out as positive controls, one considering the effect of 100% for the average size of the egg and the other considering the effect of 100% for the average size of the larvae of 18 hours old. The criterion for 18-h-old larvae was based on the prism stage, half the test time (36 hours) and because it is the limit development time for the pluteus stage larvae.

The percentage of effect was estimated according to the equation for both criteria of average size of the egg and the 18-hour-old larva:

$$PE = \frac{(M_{36h} - M_{egg\ or\ 18h}) - (M_i - M_{egg\ or\ 18h})}{(M_{36h} - M_{egg\ or\ 18h})} X100\%$$

Where:

PE = Percentage of effect (%)

M_{36h} = Mean size of larvae in 36 hours in the control

M_{egg} = Average size of eggs at the beginning of the experiment

M_{18h} = Mean size of larvae in 18 hours (control)

M_i = Average size of larvae at concentration i

The values of EC_{50} (Median Effective Concentration) were estimated by the Trimmed Spearman-Karber method, and subsequently performed ANOVA (Zar, 1996), for comparison between the traditional and biometric methods.

For the NOEC (No Observed Effect Concentration) estimates, ANOVA and the Bonferroni test were performed a posteriori (USEPA, 2002). For the NOEC estimates, the average sizes of the larvae of each control replicate were compared against the replicates of each concentration and each reference toxicant. The same was used for the traditional analysis, considering the percentages of the development classes of normal (pluteus) and delayed larvae.

RESULTS

Preliminary analysis of size measures as an endpoint criterion

Refraction analyses involving the mean maximum sizes of *E. lucunter* larvae, as well as their standard deviation based on 800 larvae from eight control replicates of an assay carried out in September 2017, indicated that a minimum n of 35 measurements would have a mean coefficient of variation of 0.41% and a mean standard error of 0.008 μm (Fig. 1). This criterion was the first to be established to enable the measurements of the larvae to be used as a comparative parameter between the tests carried out with the toxic reference substances.

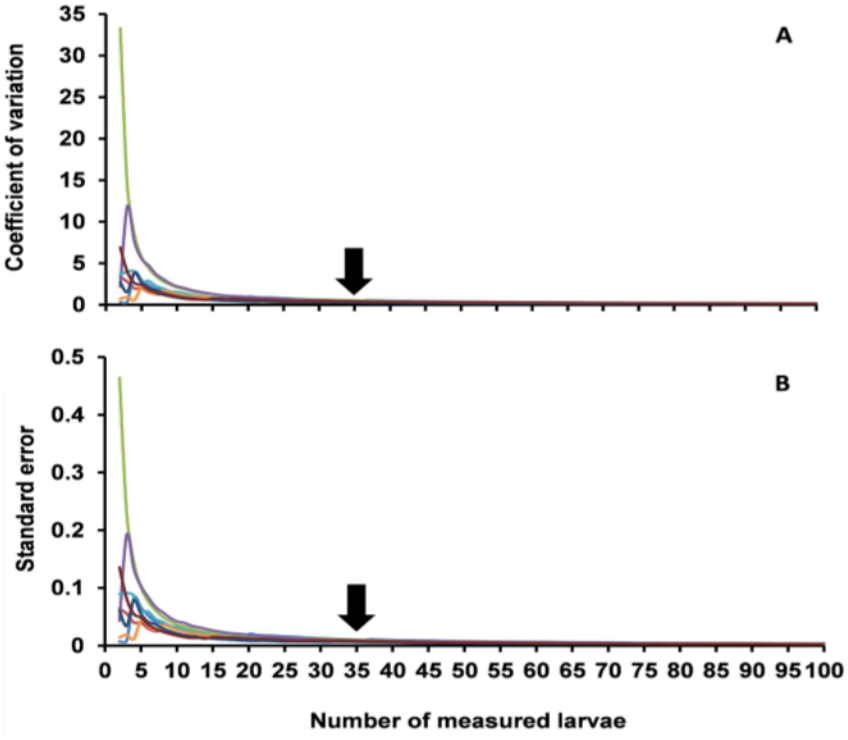

Figure 1. Coefficient of variation (A) and standard error (B) of the maximum length measurements of the larvae in the control in 8 replicates, totaling 800 measurements. The arrow indicates the n of 35 measurements with a mean coefficient of variation of 0.41% and mean standard error of 0.008 µm.

A second point to be established was the embryo larval growth curve between the maximum measurement of the organism by time (Fig. 2). Cultivation took place between June 26 and 28, 2019, with a salinity of 33 and a temperature of 26 °C, on average. The growth of the different larval stages can be divided into two phases: the first being fertilization lasting up to 18 hours (prism larva) and the second lasting 32 to 36 hours, between the undeveloped and fully developed pluteus stages. The pre-pluteus stage marks the transition between these two phases (24 hours).

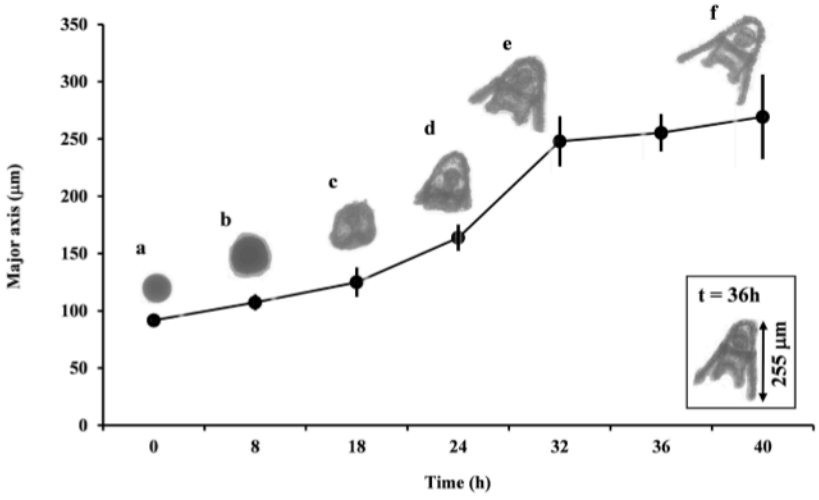

Figure 2. Embryo-larval growth curve of *Echinometra lucunter*, involving the average maximum lengths of each morphological development phase, as follows: a: Egg (91.68 µm), b: Gastrula (107.32 µm); c: Prism (124.93 µm), d: Pre-Pluteus larva (163.80 µm), e: Not fully developed Pluteus larva (247.94 µm), and f: Developed Pluteus larva with four Arms (255.44 µm). In detail, the major axis of a developed pluteus larva.

In order to assess the existence of a temporal variation in the size of eggs, 18-hour-old larvae and 36-hour larvae of *E. lucunter*, the 15 tests carried out between the years 2017 and 2019 were treated monthly (Fig. 3). On the basis of the Wald Test, a significant variation was observed in the size of all developmental stages in time ($p < 0.01$).

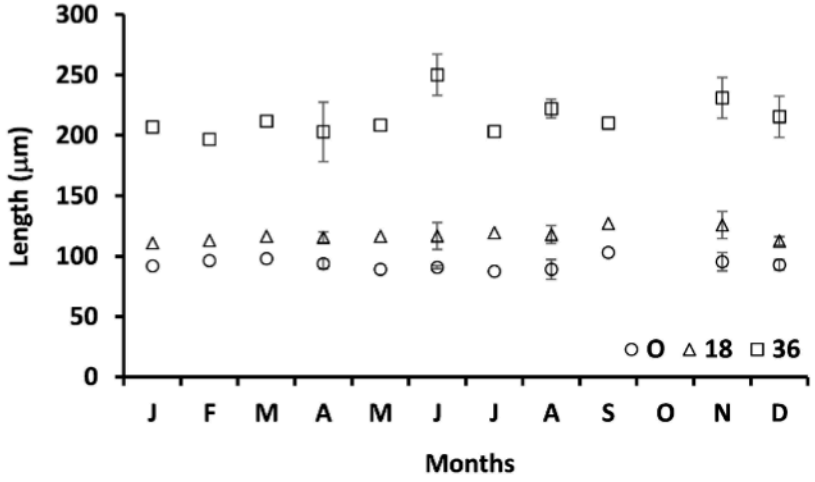

Figure 3. Mean values (and standard deviation) of the maximum lengths in the embryo-larval development stages of *Echinometra lucunter*, being Egg (○), 18-h larva (△) and 36-h larva (□), for each month (except October) of analysis involving the years 2017 to 2019.

Finally, the minimum size of the larvae for a test to be considered valid, given the quality of the batch of biological material used in the tests, was determined from the upper percentile of 80% of the measurement values obtained in the control flasks of all tests carried out and based on 4,165 measurement data. This validation corresponds to the average larval size of 243.45 µm or close to 32 hours of age (Fig. 4).

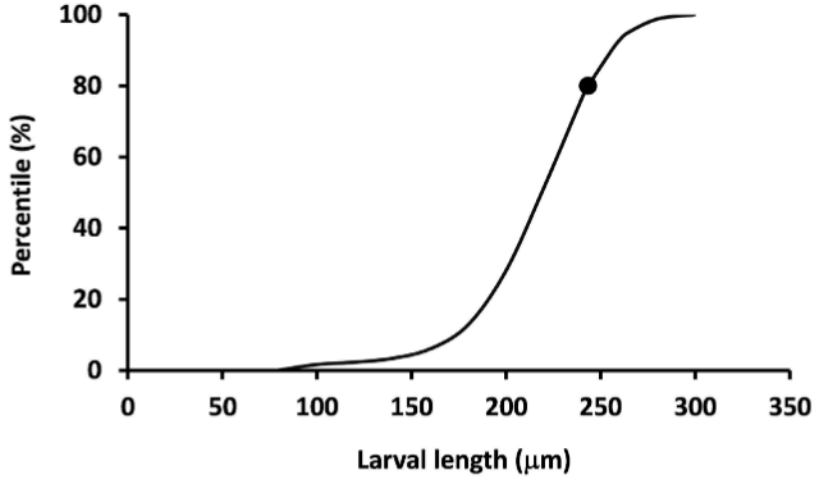

Figure 4. Accumulated percentage of larval lengths of controls obtained in 15 tests performed between 2017 and 2019, totaling 4,165 length data. The dot on the curve indicates the 80% percentile (243.45 µm or 32 hours of age).

Median Effective Concentration (EC_{50}) and No Observed Effect Concentration (NOEC)

According to Table 1 and figure 5, there is an agreement of the results based on EC_{50} for the traditional endpoint and effect-based biometry tests considering the size of the larvae at 18 hours.

Table 1. Probability values for the Analysis of Variance (ANOVA) between the EC_{50} estimation methods for the toxic reference substances $ZnSO_4$ and $K_2Cr_2O_7$.

Methods	$ZnSO_4$	$K_2Cr_2O_7$
Traditional x Larva 18h (positive control)	0.086	0.358
Traditional x Egg (positive control)	0.048*	0.00003*

*p<0.05 indicate that there is a significant difference.

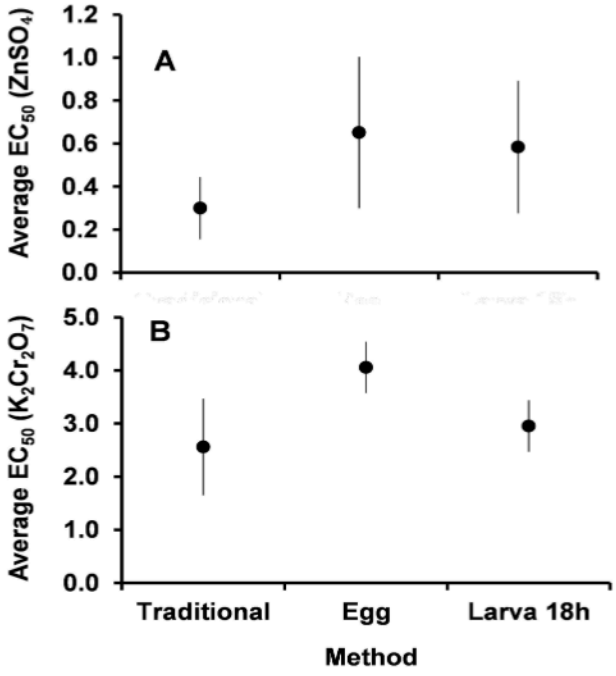

Figure 5. Mean values (point) and standard deviation (line) of EC_{50} for $ZnSO_4.7H_2O$ (A) and $K_2Cr_2O_7$ (B) estimated in the traditional way (counting method) and by measuring the larvae using the effect percentages obtained in based on egg and larval size at 18h (positive controls). Based on 15 tests carried out between the years 2017 to 2019.

For the NOEC estimates, the comparison between the average size of the larvae in the control in relation to the treatments (different concentrations) of the two toxic reference substances, indicated a greater sensitivity of the method by biometrics. The traditional classification indicated that changes in morphological classes occurred only at intermediate concentrations of Zn and Cr salts, while the measurement method indicated significant differences even at the lowest concentration tested (Fig. 6).

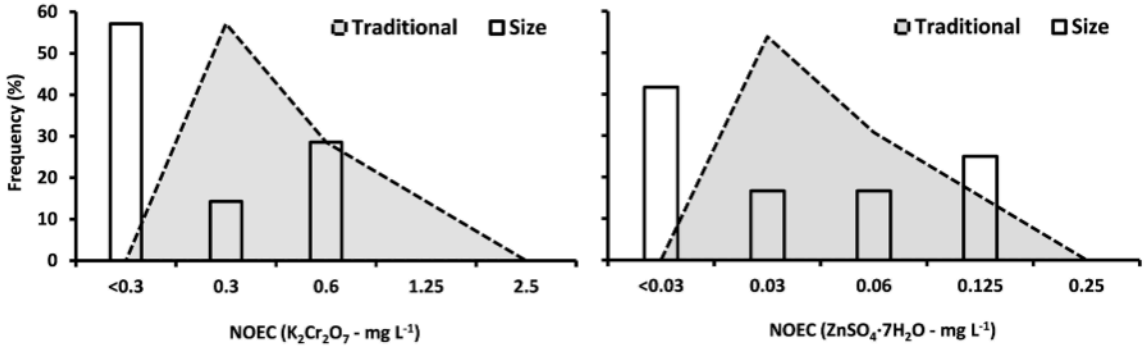

Figure 6. Frequency of NOEC values of $K_2Cr_2O_7$ and $ZnSO_4.7H_2O$ for *Echinometra lucunter* larval embryo assays analyzed by the traditional method (identification of the morphological state of the larvae – hatched area) and by the method of maximum larval size (biometrics - columns). Based on 15 tests carried out between the years 2017 to 2019.

DISCUSSION

This study presents a contribution to optimize the analysis of ecotoxicological tests using sea urchin larvae in an automated way. It is known that this type of test has a subjectivity in the form of traditional analysis, which can present a variability dependening on the technician, in addition to the fact of the length of training and the issue of results. Therefore, this new method of analysis, proposed by Saco-Álvarez *et al.* (2010), must be validated for different species in addition to determining the existence of application limits normally associated with different protocols existing worldwide.

The first advantage observed in the use of biometrics as an endpoint was the determination of the minimum number of organisms to be measured per test flask. According to the traditional method of analysis, a visual observation of at least 100 individuals per replicate in the assays is required (APHA, 1998). Concerning the biometry method, due to its low variability, the number of measurements of 35 specimens per test flask is in agreement with Saco-Álvarez *et al.* (2010) who confirmed the need for a minimum n of 30 organisms measured per replicate.

The criteria for validating an assay are important for the reliability of the results. In the traditional method, the count of normal and abnormal larvae in the control is adopted, and it is necessary to obtain a percentage of normality greater than 80% for it to be validated (APHA, 1998). The criterion used for the biometry method can also, based on the same assumption, adopt the same percentage of the cumulative distribution of the measurements of the organisms obtained in the control flasks as a validation criterion. The minimum average size of 243.45 μm corresponds to the 80% percentile of the observed larvae, as it considers the seasonal variability of the species' larvae throughout the year. This size corresponds to a development time of 32 hours, the same time in which the larvae complete their exponential growth (Fig. 2), which may also suggest a test time of less than proposed by the ABNT/NBR (2020) protocol, test time can vary from 36 to 42 hours.

However, eggs and larvae show a seasonal variation in their size, which could be explained by the gametogenesis cycle of the species, possibly related to the physicochemical parameters of the environment water and the availability of food. These abiotic and biotic factors can induce different reproductive and resting periods, determining the reproductive seasonality of organisms, as observed by Lima *et al.* (2009), Mariante *et al.* (2009) and Siikavuopio (2009). This variability cannot be avoided, but was considered in determining the minimum size of the 32-hour larvae for assay validation. In any case, the use of data (biometrics) from the control in trials throughout the year is of fundamental importance both for estimating the percentage of effect for the EC_{50} calculation and for comparisons with the treatments for the NOEC estimates.

According to Saco-Álvarez *et al.* (2010), conducting tests with toxic reference substances may be more useful to verify the biological quality of the organisms used and may contribute to the creation of a database to be used by different laboratories as criteria for validating the tests. The use of biometrics as an endpoint and its application in the estimates of percentage of effect for the calculation of the EC_{50} indicated that only using 18-hour-old larvae as a criterion of 100% effect, or positive control, showed significant similarity with the traditional analyses for the two toxic reference substances. In this way, it is mathematically unfeasible to reach 100% of effect, in chronic assays, adopting the size (diameter) of the eggs as an effect criterion. The effect percentage of 100% could only be obtained if embryo mortality occurred or before the formation of the Gastrula (Fig. 2). Although the proposal by Saco-Álvarez *et al.* (2010) does not adopt this criterion, since the study did not i predict a comparison with traditional tests, the work of Resgalla Jr. (2016) confirms the results obtained here using the same techniques in embryo-larval tests of Mediterranean mussel (*Mytilus galloprovincialis*). The use of larvae aged less than 18 hours could also be applied to estimate the effect percentages, but, for reasons of time practicality, other ages of the larvae may be unfeasible.

For NOEC estimates, the use of biometry in control flasks for comparison with treatments (different concentrations for each reference toxicant) did not show agreement with the traditional method. In general, NOEC values below the lowest concentration tested for both toxicants were the most frequent. Thus, the control larvae were significantly larger in relation to

the larvae of the lowest tested concentration. This result was maintained even when using the 80th and 90th percentiles of the larval size of all tests carried out in the control for comparison with the treatments in the individualized tests. With this, it is confirmed that the use of the size of the larvae as an endpoint presents a greater sensitivity, becoming imperceptible by the analysis of the human eye and highlighting greater toxicity than expected and/or known. On the other hand, this greater sensitivity can also be questionable, since the metals tested as toxic reference substances can be considered essential, being able to accelerate the growth rates of organisms and thus interfering with their final size, and not necessarily being toxic, but acting as a limiting substance in physiology. Taslima *et al.* (2022) highlights the variability of the effects of metals on fish, involving both Zn and Cr, which can inhibit (toxic) or stimulate (limiting element that does not exceed its tolerance) the physiology of these organisms. Likewise, El Idrissi *et al.* (2022) working with a mixture of essential elements, including metals, observed differences in the growth rates of sea urchin larvae *Paracentrotus lividus* (Lamarck, 1816). In any case, the interpretation of the NOEC can be redefined, in the future, based on a positive control, which could adapt the technique using biometrics, as was observed for the EC_{50} parameter.

CONCLUSIONS

The use of the maximum larvae length methodology requires a lower sample n than the traditional method, allowing faster analyses and, since it is based on measurements, the subjectivity of the analyst is eliminated, and may even prove to be more sensitive than the traditional method, leading to lower-than-expected NOEC values. With the elimination of subjectivity through biometrics, it is possible to meet and contribute to the standardization of interlaboratory tests. However, there is a need to include a positive control for EC_{50} estimates.

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AUTHOR CONTRIBUTIONS

KND: Methodology, Data curation, Writing-Original draft preparation. DCV: Methodology. CRJ: Conceptualization, Supervision, Writing-Reviewing and Editing.

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