An *Artemia franciscana* bioassay for the monitoring of lipophilic phycotoxins in marine bivalve mollusc cultures: An alternative to screening testing?

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**ABSTRACT**

In Brazil, malacoculture is developed mainly in the state of Santa Catarina, followed by São Paulo and Rio de Janeiro. In the course of the development of Brazilian mariculture, legislation has addressed the sanitary requirements necessary for bivalve commercialization. However, monitoring phycotoxins is a challenge, due to often costly reference methods. In this context, this study evaluated the use of alternative ecotoxicological and bioanalytical methods using *Artemia franciscana* (brine shrimp) lethality assessments. The results confirm that, although correlations between the reference mouse assay and the brine shrimp assays were not high, the alternative brine shrimp assay may be incorporated into phycotoxin monitoring programs, as hepatopancreas methanolic extracts of mussel (*Perna perna*) containing DSP resulted in high lethality rates. Therefore, further methodological adjustment studies and the inclusion of other enzymatic and toxicological models are required to further assess these
differences, and associations between ecotoxicological methods as early-alarm methods are encouraged.

**Keywords:** Arraial do Cabo, harmful algae, *Perna perna*.

**Bioensaios com Artemia franciscana para monitoramento de ficotoxinas lipofílicas em cultivo de moluscos bivalves marinhos: Uma alternativa para teste de alarme?**

**RESUMO**

No Brasil, a malacocultura é desenvolvida principalmente no estado de Santa Catarina, seguida por São Paulo e Rio de Janeiro. No curso do desenvolvimento das atividades da maricultura brasileira, a legislação se adaptou aos requisitos sanitários necessários à comercialização de bivalves. No entanto, o monitoramento de ficotoxinas é um desafio, devido a métodos de referência frequentemente caros. Nesse contexto, o objetivo deste estudo foi avaliar o uso de métodos ecotoxicológicos e bioanalíticos alternativos usando a *Artemia franciscana* (artemia). Os resultados confirmam que, embora as correlações entre o ensaio de referência em camundongos e os ensaios com artemia não sejam altas, o ensaio alternativo com artemia pode ser incorporado aos programas de monitoramento de ficotoxinas, pois os extratos metanólicos do hepatopâncreas de mexilhões (*Perna perna*) contendo DSP resultaram em altas taxas de letalidade. Portanto, estudos adicionais de ajustes metodológicos e a inclusão de outros modelos enzimáticos e toxicológicos são necessários para avaliar essas diferenças, e são encorajadas associações entre métodos ecotoxicológicos como métodos de alarme precoce.

**Palavras-chave:** algas nocivas, Arraial do Cabo, *Perna perna*.

**1. INTRODUCTION**

Bivalve mollusc cultivation has increased in the last decades, and currently obtains high yields (FAO, 2016). However, the possibility of contamination by toxins produced by certain microalgae species (phycoxins) is an obstacle to shellfish production and consumption and may lead to product embargos when harmful algal blooms (HAB) occur in cultivation areas (Simões, 2011). HAB comprise excessive proliferation of certain phytoplankton species in short periods of time, due to favorable development conditions (Castro and Moser, 2012). As bivalve molluscs are filter feeders, many toxins can be ingested and stored in their tissues, bioaccumulating and potentially biomagnifying throughout the trophic chain (Barbosa et al., 2019), causing poisoning in several organisms, including humans, through the consumption of contaminated mollusks (Barbieri, 2009; Proença et al., 2011). In marine coastal environments, most phycotoxins are produced by dinoflagellates (Castro and Moser, 2012; Hallegraeff, 2003).

In Brazil, Interministerial Normative Instruction 07/2012 of the Ministries of Agriculture, Livestock and Supply (MAPA) and the former Ministry of Fisheries and Aquaculture (MPA) indicate which microalgae species are capable of producing toxins and list their main symptoms. The nomenclature of each intoxication is related to the main symptom caused in humans. For example, Diarrheic Shellfish Poisoning (DSP) indicates a diarrheal syndrome which produces abdominal discomfort, nausea, vomiting and diarrhea (Grattan et al., 2016), caused by okadaic acid (AO) and derivatives such as the dinophysistoxin group (DTXs), yessotoxins (YTXs) and pectonotoxins (PTXs) (Castro and Moser, 2012; Barbieri, 2009; Hallegraeff, 2003). Okadaic acid (OA) and DTX toxins are lipophilic substances that can contaminate shellfish meat (Uchida et al., 2018) produced by planktonic dinoflagellates (Castro and Moser, 2012; Hallegraeff, 2003).
Other issues may also result from the ingestion of toxins produced by these organisms, such as tumors and mutagenic and immunotoxic effects (Kolrep et al., 2016). Specifically, cyanobacterial microcystin-LR (MC-LR), OA and DTX may also lead to inhibition of PP1 and PP2A phosphatases proteins MC-LR and OA are the most widely distributed hepatotoxins worldwide (Wu et al., 2015). Several analytical methodologies for the analysis of lipophilic phycotoxins have been applied. In this context, mass spectrometry liquid chromatography (LC-MS/MS) has been set as the reference method of the EU since 2011 (EC No. 15/2011) (Suzuki et al., 2009; Garibo et al., 2012), while mouse bioassays (Yasumoto et al., 1978) are considered an alternative method in this regard (Visciano et al., 2016). A number of other alternative or complementary techniques are, however, permitted by EC Regulation No. 15/2011, either performed individually or in combination, provided they fulfill the proper role of official methods of providing protection to public health (Garibo et al., 2012). These include immunoassays and other chromatography techniques, including high performance liquid chromatography (HPLC), thin layer chromatography (TLC), gas chromatography-mass spectrometry (GC-MS) and gas chromatography-infrared spectroscopy (GC-IV) (Fujii et al., 2004). However, these methodologies have some drawbacks, i.e., they are very expensive, require specialized labor (in the case of liquid and gas chromatographies) and use methodologies that are not ethically adequate (mouse testing). Enzymatic assays, on the other hand, are less expensive than chromatographic techniques and can be applied to detect various pollutants, such as pesticides and metals (Ochoa et al., 2013, Chouteau et al., 2005), as well as their effects. In this regard, phosphatase inhibition assays have also been proposed to detect lipophilic phycotoxin effects (Garibo et al., 2012).

Ecotoxicological assays are routinely applied in monitoring polluted environments (Anselmo et al., 2011) as well as in assessing bioactive substances and phytotoxins. Assays using species belonging to the Artemia genus are a staple in many countries (Astuya et al., 2015; Chang and Gall, 2013), and have been suggested as an early ecotoxicological alarm test for evaluating the effects of palytoxin and palytoxin-related compounds produced by benthic dinoflagellates (Faimali et al., 2012).

In this regard, this study sought to verify the associated use of alternative phosphatase inhibition tests and an artemia nauplii lethality bioassay for the detection of DSP toxins as a viable alternative to monitor phytotoxin contamination in cultivated mussels.

2. MATERIAL AND METHODS

2.1. Study area

The town of Arraial do Cabo is located in the coastal zone of the state of Rio de Janeiro, 158 km distant from the capital, between coordinates 22°57′57″ S, 42°1′40″ W. It includes approximately 152,305 km², with a population of about 27,700 (IBGE, 2020) (Figure 1).

2.2. Bivalve mollusc sampling and processing

Perna perna mussels (50 individuals per sampling) were collected between August 2013 and May 2014, always in the morning. Mean shell length was 8.5 ± 0.59 cm and mean weight of 48.61 ± 8.45 g. After transportation to the laboratory, individual hepatopancreas were removed, pooled until reaching 20 to 25 g, and stored at -20°C.

2.3. Methanolic hepatopancreas extract preparation

After drying the pooled hepatopancreas samples in an oven at constant temperature of 60°C for 24 hours, methanic hepatopancreas extracts were prepared by mixing 100 mL methanol to 20 g of the dehydrated hepatopancreas samples with the aid of surgical scissors and a porcelain mortar and pestle. After homogenization and 30 min of rest, the supernatants were filtered (Milex™, 45µm, Millipore) and the same volume of methanol was added for a second
extraction step for a further 30 min. The two supernatants were then joined in order and were submitted to a rotary evaporation step at 60°C until complete evaporation.

Subsequently, the concentrated extracts were resuspended in 5 mL of deionized water and left to stand for 30 min. After this period, 1 mL of the solutions were applied to a solid phase C18 reverse phase chromatography column containing DIAION HP 20 (Sigma Aldrich, São Paulo). The mobile phase elution step was performed by successively applying different methanol concentrations. The obtained solutions were then dried by rotary evaporation and the 100% methanol fraction was stored at -20°C for subsequent toxicological and enzymatic assays.

![Map of the marine bivalve mollusc farm, Arraial do Cabo, Rio de Janeiro.](image)

**Figure 1.** Map of the marine bivalve mollusc farm, Arraial do Cabo, Rio de Janeiro.

### 2.4. *Microcystis aeruginosa* extract preparation

Freeze-dried *Microcystis aeruginosa* containing the microcystin-LR toxin was used as a positive phycotoxin control for the artemia assays, cultivated from a NPLJ-4 (Laughinghouse *et al.*, 2012) strain kindly provided from the IBCCF Ecophysiology and Cyanobacterial Toxicology Laboratory, belonging to the Federal University of Rio de Janeiro (UFRJ), and the Toxicological Biochemistry Laboratory, belonging to the State University of Rio de Janeiro (UERJ). Preparation of the *M. aeruginosa* extract followed the same methodology previously described for mussel hepatopancreas, with modifications in the methanol ratio, set at 2.5 mL methanol to 50 mg of lyophilized *M. aeruginosa* cells (NPLJ-4), followed by three methanol extractions and a centrifugation step to obtain the supernatant, instead of filtering.

### 2.5. *Artemia franciscana* ecotoxicological assays

*Artemia cysts were obtained from a commercial pet shop (Maramar Pet, Arraial do Cabo/RJ). Cysts were decapsulated using calcium hypochlorite and hatched in artificial seawater (2 g.L⁻¹ NaHCO₃ + 8 g.L⁻¹ NaCl) at room temperature (25°C) for 24 h, under constant aeration and lighting (250 μmol.m⁻².s⁻¹).

*Artemia franciscana* ecotoxicological assays were performed according to Meyer *et al.* (1982), with modifications. All assays were carried out in 6-well cell culture plates, with each well containing 10 brine shrimp nauplii. The positive control consisted of a *Microcystis aeruginosa* strain containing the microcystin-LR toxin, while the negative control consisted of 5 mL of artificial seawater. The positive control and hepatopancreas extracts were resuspended...
in 1 mL of artificial seawater solution containing 0.01% ethanol, used to improve extract. Each well received 1 mL of the extract solution to be tested and 4 mL of artificial seawater, totaling 5 mL. All assays were conducted in triplicate. The nauplii were exposed to the extract for 24 h. Lethality was considered when no nauplii movements were observed for at least 10s under observation with a binocular microscope. Results are presented as nauplii mortality percentages.

2.6. Phosphatase enzyme sample preparation and determinations

*Artemia franciscana* enzymatic extracts were prepared by weighing 1 g of brine shrimp (recently hatched nauplii at the II-III stage) and homogenizing this mass at a 1:4 ratio in a Tris/HCl 50 mmol L⁻¹ pH 7.4 containing sucrose 250 mM, EDTA 5 mmol.L⁻¹, DTT 1 mmol.L⁻¹, PMSF 0.1 mmol.L⁻¹. After thirty passes in a Potter-Elvehjem tissue homogenizer (Potter, 1955), the homogenates were centrifuged at 10,000 g for 60 min at 4°C and the obtained supernatants were used as a source of phosphatase enzymes.

Total phosphatase (TF) activity was determined according to Rivasseau et al. (1999) and Bouaïcha et al. (2002) with modifications. Briefly, the assay buffer (40 mM Tris/HCl, pH 8.4, containing 34 mmol.L⁻¹ MgCl₂, 4 mmol.L⁻¹ EDTA and 4 mmol.L⁻¹ DDT) was mixed with p-nitrophenyl phosphate as substrate at a final concentration 9.8 mmol.L⁻¹ and 100 μL of the *Artemia franciscana* enzyme fraction, totaling a final volume of 200 μL. Product formation was measured by continuous absorption at 405 nm on a microplate reader for 6 min and enzyme activity calculations were performed using the p-nitrophenol absorption coefficient (16,890 M⁻¹.cm⁻¹). Data were expressed as one enzyme unit (U) μmol.min⁻¹ p-nitrophenol per milliliter. For the inhibition test, *Artemia franciscana* enzyme fractions and methanolic mussel hepatopancreas extracts incubated for 1 hour at 20°C were used. All assays were performed in triplicate and results are presented as enzyme inhibition percentages, to facilitate interpretation and comparison to other studies.

2.7. Statistical analyses

Data were analyzed using the Graph Pad Prism v. 6.0 and Microsoft Excel v. 2003 software packages. A simple linear regression was observed in order to verify potential correlations (Pearson’s correlation test) between the proposed brine shrimp assay and the phosphatase inhibition assay. The results are presented as means and standard deviations (means±SD) of triplicates and the t student’s test (p<0.05) was applied between control samples and the extracts.

3. RESULTS AND DISCUSSION

DSP phycotoxins are lipophilic substances that can contaminate shellfish meat (Uchida et al., 2018) and are produced by planktonic dinoflagellates belonging to the *Dinophysis* (*D. acuta*, *D. acuminata*, *D. caudata*, *D. fortii*, *D. miles*, *D. norvegica*, *D. sacculus* and *D. tripos*) and *Phalacroma* genera (*P. mitra* and *P. rotundatum*); as well as benthic species belonging to the *Prorocentrum* genus (*P. lima*, *P. arenarium*, *P. belizeanum*, *P. concavum*, *P. faustiae*, *P. hoffmannianum*, *P. mild* and *P. maculosum*) (Castro and Moser, 2012; Hallegraeff, 2003). In this regard, Souza et al. (2016) analyzed the same hepatopancreas mussel samples collected in January and May 2014 assessed herein using the mouse bioassay and reported the presence of *Dinophysis acuminata* (120). The current study assessed the same samples used by Souza et al. (2016) for the brine shrimp and phosphatase inhibition assays.

*Artemia* sensitivity to the positive control corroborates previous assessments carried out using another *Artemia* species, *Artemia salina*, exposed to cyanobacteria extracts, where an LC50 of below 50 μg mL⁻¹ was reported (Falch et al., 1995). In another study, Batista et al. (2013) evaluated *Artemia salina* sensitivity to extracts obtained from two *Microcystis* sp. strains (NPLJ-4 and TAC 95) and reported high sensitivity to these cyanotoxins (LC50 24h of 5.7
mg.L⁻¹ and 3.7 mg.L⁻¹, respectively for NPLJ-4 and TAC 95). However, when compared to other organisms (Daphnia magna, LC50 24h 2 mg.L⁻¹ and 0.8 mg.L⁻¹ and Ceriodaphnia dubia, CL5024h 1.2 mg.L⁻¹ and 0.6 mg.L⁻¹ for MPLJ-4 strains and TAC 95, respectively) Artemia salina was more resistant, and, thus, deemed the most adequate species to assess the relationship between microcystins present in cyanobacterial strain extracts.

3.1. Artemia franciscana lethality assay

High A. franciscana mortality rates of over 50% for all assessed months were observed after 24 hours of exposure to the methanolic extracts. In August 2013 and May 2014, the mortality rates of the metanolic extract was comparable to the effects of the pure microcystin-LR positive control, of 90% or more (Figure 2).

The dinoflagellate Dinophysis acuminata was detected in seawater (120 and 7040 Cell.L⁻¹) and in Perna perna hepatopancreas samples collected in January and May 2014 by Souza et al. (2016), indicating the presence of a lipophilic toxin causing DSP in the reference mouse bioassay, which may have been responsible for the high toxicity of around 90% observed for these mussel extracts. No correlations were detected between high nauplii mortality and mouse bioassay toxicity for the other assessed months.

Several brine shrimp assays have been applied to assess the toxicity of several dinoflagellates and diatoms in the literature. For example, these shrimp assays have been reported as adequate for the assessment of the toxicity of the dinoflagellate species Amphidinium carterae, Coolia monotis and Ostreopsis ovata in a study conducted in the Northern Ionian Sea (Mediterranean Sea), applied comparatively and alongside phytotoxin evaluations concerning the larval development of sea urchin (Paracentrotus lividus) and hemolysis tests on human erythrocytes (Pagliara and Caroppo, 2012). Comparative toxicity studies of the effects of different Ostreopsis ovata concentrations on artemia nauplii and fish larvae have also been reported, and the results indicate that artemia nauplii are an adequate test...
organism due to their high sensitivity (Faimali et al., 2012).

Artemia nauplii assessments expressed as cyst hatching success have also been deemed efficient in toxicity evaluations regarding Skeletonema costatum and Nitzschia commutata diatoms and short-chain aldehydes (Caldwell et al., 2003). However, low acute toxicity was observed when artemia nauplii were exposed to Pseudo Chattonella and Chattonella marina strains, which are ichthyotoxic (Skjelbred et al., 2011).

### 3.2. Phosphatase Enzyme Assay

The phosphatase enzyme assay has been routinely used for the detection of cyanobacterial microcystins and okadaic acid produced by certain dinoflagellate species (Triantis et al., 2010; Eberhart et al., 2013), and no differences have been reported when comparing this method with reference methods, such as mouse bioassays and fluorometric detection high performance liquid chromatography (HPLC-FLD) using 1-bromoacetylpyrene (BAP) as the pre-column derivative reagent (Prassopoulou et al., 2009).

Only exposure to extracts obtained from samples collected in January resulted in phosphatase enzyme inhibition of about or over 50%, while the other samples displayed lower than 40% inhibition rates (Figure 3).

![Figure 3. Enzymatic phosphatase activity (mean±SD, n=3)) in Artemia franciscana submitted to methanolic mussel hepatopancreas extracts. (*) Indicates significant difference between the means extract vs control (Student’s t test, p<0.05).](image)

The inhibition pattern of around 50% is similar to that reported in another study for genetically engineered phosphatases 2A and purified red blood cell enzymes in samples that had accumulated lipophilic toxins, carried out by Garibo et al. (2012) when analyzing mussels and oysters collected in Catalonia and Galicia.

### 3.3. Correlations between the brine shrimp lethality assay and phosphatase inhibition assay

No correlation between phosphatase inhibition and artemia mortality was observed (Figure 4). It is probable that acute DSP toxin toxicity (okadaic acid and derivatives) may not only related to phosphatase inhibition (Munday, 2013), and that other mechanisms of action may play a role in toxicity and artemia nauplii mortality (Rossini and Hess, 2010). This has
been reported previously in other assessments, where derivatives such as DTX-4 and 7-O-docosahexaenoyl-OA, for example, display high acute mouse toxicity and low protein phosphatase 1 and 2A inhibition (Munday, 2013).

**Figure 4.** Correlations between artemia nauplii phosphatase activity inhibition and lethality when exposed to methanolic extracts. No correlation (Pearson’s correlation coefficient p< 0.05) was observed between the assessed variables.

### 3.4. Correspondence with bioassays from another study

The dinoflagellate *Dinophysis acuminata* was detected in the January and May 2014 hepatopancreas samples by Souza *et al.* (2016), which may have been the cause for the high mortality *Artemia franciscana* rates observed herein for these months. The phosphatase inhibition was significant for these sampling points.

Souza *et al.* (2016), using the same samples as assessed herein, carried out the DSP bioassay which assessed mouse deaths. The mouse deaths observed for the January and May samples also coincide with high mortality rates and phosphatase inhibition in the *Artemia franciscana* trial carried out herein, corroborating the dinoflagellate *Dinophysis acuminata* as the toxic agent responsible for these deaths.

A simple comparison of the brine shrimp bioassay and phosphatase assays carried out herein with the results reported by Souza *et al.* (2016) for the same samples concerning *Perna perna* microalgae content and mouse bioassay results is displayed in Table 1. In the present study, both *Artemia franciscana* nauplii and phosphatase enzyme displayed sensitivity to the *Microcystis aeruginosa* standard used as the positive control in the assays (Table 1).

#### Table 1. Comparison of brine shrimp bioassay and phosphatase assays results with *Perna perna* hepatopancreas microalgae content and mouse bioassay assessments.

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>Percentage brine shrimp lethality (CV%)</th>
<th>Percentage phosphatase inhibition (CV%)</th>
<th>Detected microorganism (cells.L⁻¹) (Souza <em>et al.</em>, 2016)</th>
<th>Mouse DSP assay (Souza <em>et al.</em>, 2016)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>93(6)</td>
<td>72(8)</td>
<td><em>M. aeruginosa</em> (NPLJ-4)</td>
<td>-</td>
</tr>
<tr>
<td>Jan-14</td>
<td>74(6)</td>
<td>48(11)</td>
<td><em>Dinophysis acuminata</em> (120)</td>
<td>Positive</td>
</tr>
<tr>
<td>May-14</td>
<td>96(6)</td>
<td>42(17)</td>
<td><em>Dinophysis acuminata</em> (7040)</td>
<td>Positive</td>
</tr>
</tbody>
</table>

(CV%) Coefficient of variation.
A 100% agreement between fluorometric detection high performance liquid chromatography (HPLC-FLD), using 9-athryldiazomethane (ADAM) as the precolumn derivative reagent, with mass spectrometry-coupled liquid chromatography method in tandem (LC-MS/MS), applied for detection of okadaic acid has been previously reported, as well as a 97.1% agreement observed between each of these methods with the reference mouse bioassay (Louppis et al., 2010). Although a high agreement was not noted herein between brine shrimp lethality assay, the phosphatase inhibition assay and the reference mouse assay, the first two display the potential to be applied as alarm methods to aid decision-making in malaculture activities prior to further, more costly, methods using mice, as the same the January and May samples containing Dinophysis acuminata led to both high Artemia franciscana mortality rates and mice deaths in the DSP assay carried out by Souza et al. (2016).

In this regard, other enzymatic and toxicological models are required to further assess these differences, identifying the minimum inhibitory DSP concentrations and validation parameters, such as selectivity, limits of detection and quantification, accuracy, precision, linearity, sensitivity, robustness and reproducibility for each of the evaluated factors should be taken into account.

4. CONCLUSIONS

The present study indicates that the early-alarm method of brine shrimp assay may be incorporated into phytotoxin monitoring programs in marine-farmed bivalve molluscs. Artemia nauplii efficiently demonstrated lethality and phosphatase inhibition due to exposure to hepatopancreas methanolic extracts containing DSP, corroborated by mouse deaths caused by exposure to the same samples.

Contrasting results for the brine shrimp phosphatase inhibition assay and lethality assays may be due to different toxicodynamic pathways in place between brine shrimp and mice, probably occurring not only through phosphatase inhibition. Because of this, further methodological adjustments studies and the inclusion of other enzymatic and toxicological models are required to further assess these differences, and associations between methods for application as early-alarm methods are encouraged.

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