Microcystin bioaccumulation in *Limnoperna fortunei* following *Microcystis aeruginosa* exposure, analysis of in vivo enzymatic phosphatase, acetylcholinesterase and carboxylesterase effects and in vitro experiments

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Abstract

Toxic cyanobacteria blooms have been reported in freshwater sources worldwide and may lead to aquatic biota toxin accumulation and trophic chain transfer, resulting in ecological and public health concerns. To assess cyanobacteria effects on microcystin uptake and accumulation and on phosphatase, acetylcholinesterase (AChE) and carboxylesterase (CarbE) enzymatic activities, an in vivo experiment was carried out employing the golden mussel *Limnoperna fortunei*. These mussels were exposed to a *Microcystis aeruginosa* NPLJ-4 strain (NPLJ-4) for 48 hours at different cell densities. Subsequently, algal cell counts were carried out and enzymatic activities were assayed. All three enzymes (Phosphatase, AChE and CarbE) were inhibited at the end of the exposure experiment. Mussels exposed to higher in vivo *M. aeruginosa* densities exhibited microcystin uptake and accumulation. In vitro assays were also carried out, exposing soluble *L. fortunei* enzyme fractions to *M. aeruginosa* extracts containing microcystin, and phosphatase inhibition was observed, whereas acetylcholinesterase and carboxylesterase were not inhibited. The results indicate that metabolites other than mycrocystin probably caused the observed in vivo esterase inhibitions, requiring further investigations.

Keywords: Cyanobacteria; golden mussel; cholinesterases; carboxylesterase; microcystin

INTRODUCTION

Toxic cyanobacteria blooms have become an important environmental and public health issue in the last decades, reported in freshwater environments in over 45 countries, and in numerous brackish, coastal, and marine environments, threatening many aquatic ecosystems (Codd et al., 2005; Paerl & Huisman, 2008). Many toxin-producing genera, such as *Microcystis*, *Anabaena*, *Anabaenopsis*, *Planktothrix*, *Aphanizomenon*, *Cylindrospermopsis*, *Raphidiopsis*, and *Nodularia*, are able to bioaccumulate in aquatic environments (Ferrão-Filho & Kozlowsky-Suzuki, 2011; Pham & Utsumi, 2018). Among these genera, *Microcystis* is the most common bloom-forming cyanobacteria worldwide and usually

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involved in wild and domestic animals poisoning cases (Stewart et al., 2008) as well as humans (Svrčev et al., 2017). Concerning microcystin, the main cyanotoxin belonging to the *Microcystis* genus, although it is able to bioaccumulate in aquatic organisms, there is no evidence of biomagnification throughout the food chain (Kozlowsky-Suzuki et al., 2012).

Some enzymes displaying important physiological and toxicological roles are affected by cyanobacterial toxins, such as phosphatase and acetylcholinesterase (AChE) (Eriksson et al., 1990; Monserrat et al., 2001). Phosphatases are intracellular enzymes that carry out the hydrolysis of phosphate groups in proteins phosphorylated by protein kinases (Cohen, 1992). Among protein phosphatases, classified as tyrosine phosphatases and serine-threonine phosphatases, two serine-threonine phosphatases are noteworthy, phosphatases 1 and 2A. Both are inhibited by cyanobacterial hepatotoxins, including microcystins (Ito et al., 2002; Mackintosh et al., 1990). Their inhibition has been associated to cytoskeleton destabilization, in combination with other effects related to cell signaling control, such as cell differentiation, growth, death (apoptosis) and tumor suppression (Mumbly, 2007; Sun et al., 2014; Toivola et al., 1994; Toivola et al., 1997).

Recent studies have revealed that microcystin LR is also capable of promoting neurotoxic effects by affecting AChE (Qian et al., 2018; Wu et al., 2016). This enzyme is involved in nervous impulse transmission to muscle fibers (Massoulie et al., 1993), and is a recognized biomarker of exposure and effect of several neurotoxic compounds, such as pesticides, metals and polycyclic hydrocarbons (Brown et al., 2004; Hauser-Davis et al., 2019; Oliveira et al., 2007). Anatoxin-a(s) produced by some cyanobacteria species (for example, *Anabaena spiroides*) are also important acetylcholinesterase inhibitors, and of constant concern for public health organizations (Devic et al., 2002; Molica et al., 2005). In toxicokinetic studies, biotransformation (or detoxication) processes are very useful to promote a global understanding of toxicological mechanisms, also employed to assess metabolical alterations that may indicate biomarker candidates in different ecotoxicological process. Carboxylesterase (CarbE) is a phase-1 drug-metabolizing enzyme that can hydrolyze a variety of compounds, being effective in protecting against the effects of various xenobiotics (Cashman et al., 1996). As CarbE is able to hydrolyze compounds with amide bonds to promote detoxification (Satoh & Hosokawa, 2006), its responses to microcystin exposure are important to evaluate.

Bivalves, in particular, have been reported as able to accumulate cyanotoxins at proportionally higher rates compared to other organisms, *i.e.*, fish (Vasconcelos, 1999). Concerning microcystin, due to its low hydrophobicity, the most common process observed with refgard to cyanotoxins is biodilution (BMF<1, BMF= biomagnification factor) and not biomagnification (BMF>1) in the aquatic food chain (Kozlowsky-Suzuki et al., 2012, and some bivalves species have been reported as insensitive to toxic cyanobacterial effects (Martins & Vasconcelos, 2009; Juhel et al., 2006; Vanderploeg et al., 2001), able to reproduce and survive in cyanobacterial-contaminated areas, suggesting highly successful biochemical strategies to overcome cyanobacteria toxicity (Bykova et al., 2006). Furthermore, microcystin residues have been detected larger and older mussels following toxic bloom events (Paldavicienė et al., 2015), indicating significant environmental and public health concerns, as bivalves are predated by other upper trophic level aquatic organisms and are also consumed by humans (Ferrão-Filho & Kozlowsky-Suzuki, 2011; Kozlowsky-Suzuki et al., 2012). In this regard, *Limonoperna fortunei*, an invasive bivalve mollusk recorded for the first time in South America in the early nineties (Pires et al., 2004), although not consumed by humans, exhibits high biomass and, combined with selective grazing, produces N:P ratio alterations, increasing the incidence of cyanobacterial blooms (Boltovskoy & Correa, 2015; Cataldo et al., 2012).

In this context, laboratory assessments regarding biochemical mussel mechanisms in the presence of cyanobacterial toxins may provide important insights on the risks of toxic blooms in mussel-colonized environments. Therefore, the aim of the present study was to assess *L. fortunei* microcystin bioaccumulation and determine phosphatase and esterase activities (*in vivo* and *in vitro*) following exposure to cyanobacterial toxins.

**MATERIAL AND METHODS**

*Microcystis aeruginosa NPLJ-4 strain cultivation*

The *Microcystis aeruginosa* strain (NPLJ-4) used in this study was kindly provided by Dr. Sandra Azevedo (Cyanobacteria Ecophysiology and Toxicology Laboratory, Carlos Chagas Filho Biophysics Institute, UFRJ, Rio de Janeiro, Brazil), isolated from Jacarepaguá Lagoon (Rio de Janeiro, Brazil). A 3 L culture of this strain was grown in ASM-1 until reaching 500 x 10^4 cells mL^-1, determined by counting in a Neubauer chamber.

**Mussel bioassay**

The *Limonoperna fortunei* mussel specimens used herein in a semi-static *in vivo* assay were kindly provided by the Department of Biological Oceanography at the Instituto de Estudos do Mar Almirante Paulo Moreira (IEAPM), Arraial do Cabo, Rio de Janeiro, Brazil. A preliminary assessment was performed to determine the best algal cell concentration to promote complete mussel valve-opening, as reduced or complete valve gap closure is noted in filter-feeding bivalves exposed to very low algal concentrations (Riisgård et al., 2011), which may impair toxicity assay results.

Male and female mussels (15 to 25 mm, shell length) were acclimated for 48 h at 25°C in dechlorinated freshwater, under fasting conditions in 2 L beakers, with water changes performed every 24 hours. *M. aeruginosa* cells were diluted to 514 x 10^4, 275 x 10^4, 128.5 x 10^4 and 64.25 x 10^4 cells mL^-1. Three
2 L beakers were used for each *M. aeruginosa* concentration, each containing 20 mussels. The mussels were exposed to *M. aeruginosa* cells for 48 h at 25°C in dechlorinated freshwater, under a 12:12 h photoperiod and constant aeration. Non-exposed controls were maintained in the same conditions.

**Mussel sample processing**

After 48 h in the presence of algal cells, mussels were collected and soft tissues were removed and pooled (n=10 individuals per pool, totaling five pools) for each algal concentration. Control pools were prepared in the same way. All samples were distributed into cryovials, and immediately frozen at -20°C.

Four pooled samples (n=40 individuals) were used for the enzyme assays and one (n=10) was used to determine mussel microcystin concentrations. Samples were homogenized after thawing and weighing using a Potter-Elvehjem homogenizer (Potter, 1955), in a 50 mmol L⁻¹ Tris-HCl, pH 7.4 buffer solution containing 250 mmol L⁻¹ sucrose, 5 mmol L⁻¹ EDTA, 5 mmol L⁻¹ EGTA and 1 mmol L⁻¹ DTT at a 1:6 mass/volume ratio (adapted from Toivola et al., 1994).

The homogenates were then centrifuged at 10,000 × g for 10 min in an ultracentrifuge (CP70G, Hitachi, São Paulo, Brazil) and the supernatants, hereafter termed Fraction 1, were separated.

Soluble fractions were prepared by centrifugation for 90 min at 105,000 × g at 4°C for 10 min in an ultracentrifuge (CP70G, Hitachi, São Paulo, Brazil) and the supernatants, hereafter termed Fraction 1, were separated.

**M. aeruginosa microcystin extraction**

At the end of the *M. aeruginosa* exponential growth phase, the culture was centrifuged at 500 × g at 4°C for 10 min using a Himac CR21 centrifuge (Hitachi, São Paulo, Brazil). The supernatants were discarded, and the pellets were freeze-dried using a Heto Drywinner freeze-dryer and stored at -20°C until analysis. After freeze-drying, 50 mg were treated with 2.5 mL absolute methanol containing 0.1% trifluoroacetic acid (TFA), subjected to an ultrasonic bath (Thornton T7 model) at maximum frequency for 15 min and left to stand for 30 minutes. The extracts were then centrifuged at 500 × g for 10 min, the supernatants discarded and the pellets resuspended in methanol containing 0.1% TFA. After left standing again for 30 minutes, the extracts were centrifuged again three times. The final supernatants were then collected, evaporated under a gentle N₂ stream and the residues were suspended in deionized water. The solutions were then eluted through a C18 solid phase cartridge and extracted at various methanol concentrations. The final 100% methanol extracts were evaporated, resuspended in 1 mL of deionized water and used for the *in vitro* exposure assays.

**Microcystin quantification in mussels by an Enzyme Linked Immuno Sorbent Assay (ELISA)**

Exposed mussels were freeze-dried and subjected to microcystin extractions as described above. The methanol extracts were then mixed with hexane at a 1:1 ratio. After discarding the hexane layer, the methanol was evaporated under a gentle N₂ stream and the extracts resuspended in 1 mL of deionized water for ELISA microcystin quantification using a commercial kit (Microcystin Plate Kit, EnviroLogix®), according to the manufacturer’s instructions. Data are expressed as microcystin-LR equivalents.

**Enzymatic assays - In vivo assessments - Total phosphatase (TP) activity**

TP activities were assayed according to Rivasseau et al. (1999) and Bouaïcha et al. (2002). The final reaction volume consisted in 500 μL, comprising 460 μL of the assay buffer (Tris/HCl 40 mmol L⁻¹, pH 8.4, containing 34 mmol L⁻¹ MgCl₂, 4 mmol L⁻¹ EDTA and 4 mmol L⁻¹ DTT) and a p-nitrophenyl phosphate liquid substrate at a final concentration of 9.8 mmol L⁻¹ and 40 μL of either the F1 or soluble mussel fraction. Total p-nitrophenol formation was determined by continuous kinetic readings for 90 s at 405 nm using a DU 530 spectrophotometer (Beckman, Pasadena, California). Enzyme activity was calculated using the p-nitrophenol molar absorptivity (16,890 M⁻¹ cm⁻¹). All assays were carried out in duplicate. Triplicate assays were carried out when coefficients of variation were over 10%.

**Cholinesterase activity assays**

Cholinesterase activities were determined by Ellman’s method (Ellman et al., 1961). The final reaction medium consisted in a total of 200 μL, comprising 160 μL of a sodium phosphate 0.1 mol L⁻¹ buffer at pH 7.5 and 6.4 mmol L⁻¹ DTNB as the reaction media. The substrate (acetylthiocholine iodide) was applied at a final concentration of 1.875 mmol L⁻¹. A total of 40 μL of the mussel fractions were used for the three assays (F1 or soluble fraction). In all cases, product formation was determined by a continuous absorption kinetic test over 90 s at 412 nm on a UV 160A Shimadzu spectrophotometer. Enzyme activity was calculated using the molar absorptivity (14,150 M⁻¹ cm⁻¹) of the tioctionbenzoic (TNB) formed as product. All assays were carried out in duplicate. Triplicate assays were carried out when coefficients of variation were over 10%.

**Carboxylesterase (CarbE) activity assay**

CarbE activities were determined according to Morgan et al. (1994). The final reaction medium consisted in a total of 200 μL, comprising 160 μL of sodium phosphate 0.1 mol L⁻¹ pH 7.7 and p-nitrophenylacetate as substrate at a final concentration of 5 mmol L⁻¹ and 40 μL of the mussel fractions (F1 or soluble fraction). Total p-nitrophenol formation was determined by a continuous absorption kinetic test for 60 s at 400 nm on a Shimadzu UV-160A spectrophotometer. Enzyme activities were determined using the p-nitrophenol molar
absorptivity (13,000 M⁻¹ cm⁻¹). All assays were carried out in duplicate. Triplicate assays were carried out when coefficients of variation were over 10%.

**In vitro assessments - In vitro enzymatic M. aeruginosa methanol extract effects**

All in vitro tests were carried out as described previously for phosphatase and esterases at microcystin concentrations ranging from 0.005 to 50 mg L⁻¹, following incubation with methanolic extracts.

The methanolic M. aeruginosa extracts were incubated for 30 min with the soluble mussel fraction for IC₅₀ determinations (IC₅₀₃₀min). Another assay for the same extract was performed at 1 mg L⁻¹ of microcystin incubated for 60 min (IC₅₀₆₀min). Controls with and without methanol were incubated under the same conditions as the extract. Phosphatase, AChE and CarbE activity assays were carried as described previously. All assays were carried out in duplicate. Triplicate assays were carried out when coefficients of variation were over 10%.

**Eserine AChE and CarbE effects (IC₅₀)**

Eserine, a known AChE cholinesterase inhibitor was employed ranging from 10⁻³ to 10⁻¹² mol L⁻¹ to confirm the presence of AChE in the soluble mussel fraction. All assays were performed as previously described for AChE and CarbE. After preparation, the reaction medium was left on ice for 30 min in the presence of eserine, followed by substrate addition (acetyltiocholine iodide and p-nitrophenylacetate, for AChE and CarbE, respectively) to begin the reaction. The controls were maintained under the same conditions but without the presence of the inhibitor. All assays were carried out in duplicate. Triplicate assays were carried out when coefficients of variation were over 10%.

**Total protein contents**

Total protein contents were quantified by Peterson’s method (Peterson, 1977) using bovine serum albumin as the external standard. This protein data is used to calculate specific enzyme activities.

**Statistical analyses**

All statistical analyses were carried out using the Graph Pad Prism® software. A one-way ANOVA (p<0.05) was performed to assess differences between enzymatic activities at different M. aeruginosa algal densities and methanolic extract incubations. The IC₅₀ results were obtained by a nonlinear regression analysis (Dose-response - Inhibition) using the Graph Pad Prism® software.

**RESULTS AND DISCUSSION**

The results of this study are presented categorized as: 1) ELISA microcystin bioaccumulation detection in mussels; 2) Phosphatase and esterase enzymes (acetylcholinesterase and carboxylesterase) to verify M. aeruginosa exposure and toxic effects. Furthermore, complementary in vitro analyses were necessary to better characterize the analyzed enzymes, and are also presented.

**Cells counts and microcystin accumulation**

In general, studies carried out on bivalve mollusks and cyanobacteria are applied to determine toxin accumulation and clearance, as this information is very useful for environmental and public health risk assessments regarding potentially toxic cyanobacteria blooms (Cataldo et al., 2012; Dionisio Pires & Van Donk, 2002; Sipiä et al., 2001; Vasconcelos et al., 2007; Williams et al., 1997).

In this experiment, the golden mussels removed considerable amounts of M. aeruginosa cells from the water (Table 1). However, we observed that the reduction of algal cells in the water did not correlate with toxin accumulation by toxin ingestion, since higher cell densities lead to lower ingestion rates. It is likely that higher cell densities lead to inhibition of filtering rates (Juhel et al., 2006). Moreover, Microcystis sp. can be rejected in the form of pseudofeces, decreasing ingestion (Juhel et al., 2006; Gazulha et al., 2012a; Gazulha et al., 2012b).

The ELISA method detected the presence of microcystin in mussels in contact with only the higher M. aeruginosa cell density; Right Axis - L. fortunei LR microcystin content.

### Table 1. Microcystis aeruginosa cell counts at the initial exposure time (T₀) and after 48 h (T₄₈) of exposure in Limnoperna fortunei.

| Cell density (Cells mL⁻¹) | Cells mL⁻¹ (x10⁴) | % Reduction |
|---------------------------|-------------------|-------------|
|                            | Beginning (T₀)    | End (T₄₈)   |
| 64 x10⁴                   | 64                | 14          | 78.2 |
| 129 x10⁴                  | 129               | 38          | 70.4 |
| 257 x10⁴                  | 257               | 132         | 48.5 |
| 514 x10⁴                  | 514               | 263         | 48.8 |

To – Initial exposure time; T₄₈ – After 48 h of exposure.

**Figure 1.** Cell densities and microcystin contents in L. fortunei in the presence of the M. aeruginosa NPLJ-4 strain cells. Left axis - M. aeruginosa cell density; Right Axis - L. fortunei LR microcystin content.
densities (257 x 10^4 cells mL^-1, 75.4 ng g^-1 wet weight and 514 x 10^4 cell mL^-1, or 76.5 ng g^-1 wet weight) (Figure 1), not detected at lower cell densities exposures (64x10^4 and 129 x 10^4 cells mL^-1). As this study did not aim to evaluate the grazing efficiency of L. fortunei, we cannot adequately estimate the filtration rates of different M. aeruginosa cell densities. However, there are no doubts concerning M. aeruginosa accumulation, as expected. Corroborating these data, Kim et al. (2017) reported that three bivalve mollusk species exposed to M. aeruginosa bloom accumulated microcystins in their digestive glands and muscle tissue, presenting different clearance and detoxification systems.

Another relevant aspect concerning L. fortunei refers to its inability to distinguish between microcystin-producing and non-producing M. aeruginosa cells, which reinforces the fact that the species is an important vehicle for microcystin transfer throughout the trophic chain (Gazulha et al., 2012a; Von Ruckert et al., 2004). Another mussel species, Dreissena polymorpha, has been reported as able to distinguish between toxic and non-toxic M. aeruginosa cells in laboratory experiments (Dionizio Pires & Van Donk, 2002; Vanderploeg et al., 2001). According to Martins and Vasconcelos (2009), although both freshwater and marine mussels can accumulate cyanotoxins during toxic cyanobacteria blooms, this accumulation pattern depends on the species. Interspecific differences can be caused by food intake selection, reproductive period, microcystin metabolism and clearance rate (Martins & Vasconcelos, 2009).

**Enzymatic assays following in vivo L. fortunei exposure to M. aeruginosa**

These enzymatic assays were performed after 48 hours of exposure of L. fortunei to M. aeruginosa and reflect the toxicological biochemical condition of the specimens concerning three enzymatic parameters: phosphatase (microcystin inhibition), CarbE (phase 1 biotransformation) and AChE (neurotoxic exposure). The enzymatic assay results after M. aeruginosa mussel exposure are displayed in Figure 2. All M. aeruginosa cell density mussel exposures led to phosphatase activity inhibition in the F1 mussel fraction, reaching about a 90% decrease, while about 50% activity inhibitions were observed at all cell densities for both CarbE and AChE (Figure 2).

Total phosphatase inhibition was observed in L. fortunei exposed to M. aeruginosa, as reported in other studies. For example, a significant reduction in PP2A activity with a concomitant enhancement of its gene expression in the freshwater clam Corbicula fluminea has been reported after exposure for 96 h to 5 µg.L^-1 of dissolved microcystin-LR (Martins et al., 2011). On the other hand, another freshwater mussel Dreissena polymorpha exposed to 100 µg L^-1 microcystin-LR for up to 72 h exhibited no alteration in protein phosphatase 2A (PP2A) gene expression (Contardo-Jara et al., 2008).

The CarbE inhibition observed herein is indicative of a carboxylesterase inhibitor present in the employed M. aeruginosa NPLJ-4 strain. In this regard, a cell culture (HEK293-OATP1B3 cells) exposed to microcystin-LR displayed increased CarbE gene expression, attenuating the cytotoxicity of the microcystin strain (Takumi et al., 2017). As an enzyme related to the detoxification of various xenobiotics, such as organophosphates, pyrethroids and drugs (Whellock et al., 2008), CarbE inhibition may indicate a toxicokinetic flow of toxic metabolites from M. aeruginosa. To the best of our knowledge, this is the first record of cyanobacterial metabolites effects on shellfish CarbE. AChE activities were lower at all algal densities, indicating the presence of a cholinergic inhibitor in the M. aeruginosa NPLJ-4 strain. This corroborates other reports, such as the study carried out by Kankaanpää (2007) concerning Mytilus edulis exposed to Nodularia spumigena cyanobacteria. AChE inhibition was also reported in Macoma balthica specimens exposed to the cyanobacterium Nodularia spumigena, which produces the hepatotoxin nodularin, at 20 mg dry weight L^-1 after a 96 h assay (Lehtonen et al., 2003). In addition, some cyanobacteria species belonging to the Anabaena genus produce anatoxin-a(s), a known AChE inhibitor (Molica et al., 2005; Matsunaga et al., 1989). However, anatoxin-a (s) was not assessed in

![Figure 2](image-url)
the present study, as the NPLJ-4 strain is a microcystin-LR producer (Silva-Stenico et al., 2009).

**Enzymatic assays concerning in vitro L. fortunei exposure to M. aeruginosa extracts and eserine**

The use of inhibitors is a valuable tool to identify an enzyme or group of enzymes, especially when obtained from a crude extract, non-purified enzyme (Copeland 2000). Therefore, the following tests were used to briefly characterize the afore mentioned enzymes.

As a way of verifying whether the methanolic extract rich in microcystin-LR would be able to inhibit the *L. fortunei* enzymes evaluated herein in vitro, samples were incubated as described in the material and methods section. After 1 h of incubation at 1 mg L\(^{-1}\) microcystin, 48% of the soluble fraction phosphatase activity was inhibited. No AChE or CarbE inhibition was observed, even after 1 h of incubation with the methanolic extract (Table 2). An inhibition curve was also constructed for *L. fortunei* phosphatase, in order to obtain the phosphatase IC\(_{50}\) determined as 3.5 nmol L\(^{-1}\) microcystin.

Phosphatases were significantly inhibited by the *M. aeruginosa* extracts, justifying the high percentage of phosphatase activity inhibition obtained in the in vivo experiment. Rivas et al. (2000) also reported *Mytilus chilensis* phosphatase inhibition by microcystin-LR in an in vitro study conducted with purified enzymes and cyanotoxin, where the inhibition was more toxic than that of other phyctoxins (ocadia acid and dinophytoxistoxin). As expected, the in vitro results with the *M. aeruginosa* extracts gave no indication that microcystins are involved in the observed in vivo esterase inhibition. Thus, bioactive cyanobacteria compounds other than microcystins may explain the inhibition results for the other evaluated enzymes, requiring further studies.

Eserine (physostigmine) is an alkaloid extracted from calabar beans, considered a strong reversible cholinesterase inhibitor (Battha et al., 2020). The concentration able to inhibit 50% of enzyme activity in 30 min (IC\(_{50}\)) was of 5.53 x 10\(^{-4}\)mmol L\(^{-1}\) for AChE and 5.17 x 10\(^{-3}\) mmol L\(^{-1}\) for CarbE. The IC-50 determination for esterases using eserine aimed to identify golden mussel esterase similarities to other aquatic organisms, and the values obtained herein indicate that the analyzed *L. fortunei* cholinesterase, probably AChE, is similar to those observed in other mollusks. Eserine data from the present study are compared to other reports in the literature for bivalves, displayed in Table 3.

Table 2 - Inhibitory effect of *M. aeruginosa* extracts on acetylcholinesterase (AChE), Total phosphatase (TP) and Carboxylesterase (CarbE) activities in soluble *L. fortunei* fractions.

| Enzyme | Enzyme activity inhibition (%) |
|--------|--------------------------------|
| TP     | 48                             |
| AChE   | 0                              |
| CarbE  | 0                              |

The phosphatase results were similar in both the in vivo and in vitro experiments. However, esterase (AChE and CarbE) in vivo inhibition was not confirmed by the in vitro experiment. This has an interesting field of study in microcystin ecotoxicology, as the recognition that microcystin can accumulate in the brain has led to several assessments aiming to better understand its neurotoxicity (Hinojosa et al., 2019; Yu et al., 2021; Wang et al., 2010). In fact, in vivo studies carried out with another mollusc, *Macoma balhica*, indicated significant AChE activity inhibition following exposure to 50 μg L\(^{-1}\) nodularin, also a hepatoxin, and 20 mg L\(^{-1}\) of *Nodularia spumigena*. A slight increase in activity, albeit non-significant, was observed at lower doses of both nodularin and *Nodularia spumigena* (Lehtonen et al., 2003). In fish, *Danio rerio* exposed to an acute microcystin concentration in water (100 μg L\(^{-1}\)) demonstrated an increase in the activity and transcription of AChE mRNA (Kist et al., 2012). Increased AChE activity was also verified in another experiment following *Geophagus brasiliensis* exposure to sublethal microcystin concentration of 1 μg L\(^{-1}\) during a clearance period (Calado et al., 2019).

No conclusive responses concerning the integrated relationship between *L. fortunei* bioaccumulation results and the observed biochemical responses were observed herein. However, our study demonstrated the ability of this species to consume *M. aeruginosa* cells followed by efficient clearance, also indicating accumulation without leading to death. The investigated enzymes were rapidly inhibited, although this does not appear to be dose dependent. Subsequent studies include investigations on Glutathione (GSH) concentrations and Glutathione S-transferase (GST) activities, as a way to verify the main microcystin biotransformation or detoxification main process in this bivalve (Amado et al., 2011).

**CONCLUSIONS**

The golden mussel *L. fortunei* is an invasive species in Brazilian aquatic environments and displays a high capacity to accumulate microcystins during cyanobacterial blooms, enabling the transfer of this toxin to other trophic levels, although scarce knowledge on the biochemical behavior of this mussel following microcystins exposure is available. Herein, phosphatases, a common toxicodynamic microcystin target, were significantly affected, although not enough to cause *L. fortunei* death. Acetylcholinesterase and carboxylesterase

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Table 3. In vitro enzymatic activity inhibition (IC\(_{50}\) eserine) for AChE

| Organism                        | AChE (mmol L\(^{-1}\)) | Reference                     |
|---------------------------------|------------------------|--------------------------------|
| *Linnoperna fortunei*           | 5.53 x 10\(^{-3}\)     | Present study                  |
| *Potamopyrgus antipodarum*      | 0.034 x 10\(^{-3}\)    | Gagnaire et al. (2008)         |
| *Valvata piscinalis*            | 1.39 x 10\(^{-3}\)     | Gagnaire et al. (2008)         |
| *Ostrea edulis*                 | 1.04 x 10\(^{-4}\)     | Valbonesi et al. (2003)        |
| *Mytilus galloprovincialis*     | 2.06 x 10\(^{-4}\)     | Valbonesi et al. (2003)        |
| *Mytilus edulis*                | <0.01                  | Galloway et al. (2002)         |
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were also inhibited, although probably due to compounds other than the assessed microcystin, may according to the in vitro findings reported herein. Therefore, further studies should be conducted to investigate which metabolite is responsible for these inhibitions.

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