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Research paper

What does the freshwater clam, *Corbicula largillierti*, have to tell us about chlorothalonil effects?

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ABSTRACT

Chlorothalonil (CLT) is a broad spectrum, and non-systemic fungicide applied in foliar structures to prevent and treat pathogens. This compound reaches to aquatic environments and affects the biota. In this context, the main goal of this study was to assess the effects of CLT at biochemical, tissular, and individual levels of biological organization using the invasive bivalve *Corbicula largillierti* as a bioindicator species. Clams were exposed to different sublethal concentrations (0, 10, 20 and 50 μg. L^{-1} CLT) for 96 h. At biochemical level, the enzymatic activity (Glutathione-s-Transferase, Catalase, Acetyl-, Butiryl- and Carboxyl-esterases) and lipid peroxidation were measured in gills and the visceral mass. Also, the digestive gland morphometry through quantitative histological indexes was registered at the tissular level. Finally, filtering activity and burial behavior at the individual level were measured. At the highest CLT concentration, the most significant changes were observed in enzymatic activity (except for butyrylcholinesterase), lipid peroxidation and in digestive gland morphometry. It was also registered increases of the filtering activity and the latency time to burial. Most of the biomarkers assessed showed significant responses under CLT exposure. Therefore, taking into account that *C. largillierti* was affected by CLT, it can be expected that other species could be in a potential risk if this fungicide is present in freshwater systems.

1. Introduction

Freshwater ecosystems monitoring reveals the presence of different types of pesticides, including herbicides, fungicides, and insecticides, among others (Phyu et al., 2013). Their main entering pathways are usually associated with the runoff from agricultural fields, spray drift, and direct application (Yu et al., 2013). Mainly, fungicides are among the most applied pesticides in the world. However, there is still poorly understood the risk that they represent to the non-target biota (Zhang et al., 2016). Chlorothalonil (CLT), a halogenated benzonitrile, is a non-systemic broad-spectrum fungicide (Caux et al., 1996; DeLorenzo and Fulton, 2012). It is mainly used in agriculture, in-home gardening and as a component of antifouling paints (Cima et al., 2008; Silva-Barni et al., 2018). Environmental concentrations between 0.0035 and 500 μg. L^{-1} CLT have been registered in runoff water event following application in a nearby field in Florida as well as on surface water of Mississippi river (Callicott and Hooper-Bùi, 2019; Wilson et al., 2010). In Argentina, CLT is applied in different crops such as potatoes, peanuts, tomatoes, wheat, soybean and fruit trees among others, to prevent and treat fungal infections and also to domestic pest control (SENASA, CASAFE, 2009). Studies about CLT levels in sediments or water samples in Argentina are still scarce. Recently, Silva-Barni et al. (2018), reported the occurrence of CLT in atmospheric samples from the Quequén Grande River, with values ranging between <2–160 pg.$(m^3)^{-1}$. The authors suggested that domestic application from urban areas as well as the CLT use in antifouling paints, near the Quequén harbor, are the main input pathways.

According to EPA (1999) Caux et al. (1996), CLT affects non-target organisms in the aquatic environment, being very toxic to fish and

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invertebrates. In this sense, molluscs are particularly susceptible because they can bioaccumulate this fungicide in their tissues, about a thousand times more than other invertebrates (Bringolf et al., 2007; Elskus, 2012; EPA, 1999). Effects of CLT were previously evaluated on marine and estuarine bivalves (Bellas, 2006; DeLorenzo and Fulton, 2012; Ernst et al., 1991; Guerreiro et al., 2020, 2017; Haque et al., 2019; Pariseau et al., 2009). However, there are only a few studies on freshwater bivalves species such as clams *Pisidium* sp. (Ernst et al., 1991) and mussels *Lampsilis siliquoidea*, *Dreissena polymorpha* and *Uno enlogate* (Bringolf et al., 2007; Faria et al., 2009).

The Asian clam *Corbicula* is spread into freshwater ecosystems worldwide: it is considered an invasive species in many areas which causes ecological alterations and economic losses (Reyna et al., 2018; Santos et al., 2012; Sousa et al., 2008). Since they are in direct contact with both water and sediment and filtrate high water volumes, they can accumulate several xenobiotics in their tissues (Aguirre-Martínez et al., 2018; Guo and Feng, 2018). Asian shows rapid responses to environmental changes and variable sensitivity depending on the time of exposure and the type of xenobiotic (Aguirre-Martínez et al., 2018; Guo and Feng, 2018). Moreover, *Corbicula* spp. are abundant and easy to collect as wells inhabit in some environments where native bivalves are absent or endangered (Sousa et al., 2008; Castro et al., 2018). These characteristics make them useful bioindicators at the field and under controlled laboratory conditions to assess organism responses to xenobiotics (Guo and Feng, 2018; Reyna et al., 2019; Sherman et al., 2009; Takabe et al., 2011; Uno et al., 2001).

The main goal of the present study was to assess the effect of CLT in the freshwater bivalve *Corbicula largillierti*, after being exposed to sublethal concentrations of the fungicide. Biochemical (enzymatic activity and lipid peroxidation), tissular (digestive gland morphometry) and individual (burial behavior and filtering activity) responses were selected since they have been suggested as sensitive biomarkers of effect in bivalves (Baqueiro-cárdenas et al., 2007; Bonnail et al., 2016a; Chung-Min et al., 2009; Cid et al., 2015; Esperanza et al., 2020; Reyna et al., 2019; Taylor et al., 2017; Zhou et al., 2008). The hypothesis that underlies this work is that CLT produces adverse effects at different levels of organization on *Corbicula largillierti,* and these responses can be used as biomarkers of effect under CLT exposure.

2. Material and methods

2.1. Organisms and sediment collection

Adults *of C. largillierti* (shell length: 19 – 27 mm) were manually collected in a *quasi-pristine* site along the Chorrillos brook (Wunderlin et al., 2001; Harguinteguy et al., 2014, Suquía river basin, Córdoba, Argentina, 31° 23' 59.5" S; 64° 30' 34.7" W) and transported to the laboratory in plastic jars with 5 L of aerated river water. Also, from the same sample site, superficial river sediments (from the first 5 cm depth) were collected and transported in plastic jars. Back in the laboratory, sediments were sieved to obtain homogeneous granulometry (0.1–0.2 cm) and muffled (400 °C) to eliminate the organic matter content.

Organisms were acclimated for 30 days under laboratory conditions (temperature: 20 ± 2 °C, aerated, dechlorinated and filtered tap water, and light-dark photoperiod of 12:12 h) in plastic tanks of 50 L containing the previously mentioned sediment. During the acclimation period, the individuals were fed every 48 h *ad libitum* with lyophilized *Chlorella* sp. Also, on the same day of feeding, 50% of water was renewed to remove any debris. The water temperature, pH, conductivity and dissolved oxygen were measured using a multi-parametric probe (Multi Water Quality Checker U-50 Series, HORIBA. The levels of nitrate and ammonium were assessed with commercial tests (Tetra NH_3^+ / NH_4^+® and Tetra NO^{3-}®; Tetra Holding GmbH, Osnabrück, Germany)) (Supplementary Material, Table S1).

The clams maintenance and experimental design under laboratory conditions were performed following the guidelines of the Committee for the Care and Use of Laboratory Animals of Instituto de Ecologia y Diversidad Animal (IDEA, CONICET-Universidad Nacional de Córdoba 11/2019).

2.2. Experimental design

Clams were exposed to different sublethal concentrations of CLT during 96 h. The time of exposure was chosen to assess the biological responses of clams to the fungicide considering their persistence and biodegradation time in water (8 days, EPA, 1999; Sherrard et al., 2003). The test solutions were prepared from a commercial formulation of CLT (Odeon 72SC®), which contains 72% of chlorothalonil. It was diluted in distilled water to obtain the exposure concentrations of 10, 20, and 50 μg. L^{-1} of active ingredient at the beginning of the experiment. These sublethal concentrations were selected based on previous laboratory studies on bivalves, snails, polychaetes and shrimps (0.01–100 μg L^{-1}) (Barreto et al., 2018; Bejarano et al., 2005; Ernst et al., 1991; Haque et al., 2019; Hellou et al., 2009; Key et al., 2003).

After the acclimation period, individuals were randomly separated in glass aquaria of 1.5 L (with 1.2 L of test solutions and without sediment), corresponding to 0 (control), 10, 20 and 50 ug.L^{-1} CLT concentrations. Three clams were placed in each aquarium. This bioassay was replicated 10 times, totalizing 120 individuals and 30 for each treatment and control group (Fig. 1). Clams were not fed along the experimental period. The stability of CLT under experimental conditions was assessed using glass aquaria without clams at the same concentrations (Fig. 1). CLT bioavailability was previously evaluated under the same experimental conditions using three clams per aquarium; after 24 h, only 10% of CLT was detected. Therefore, the water of aquaria was entirely renewed every 24 h, rinsing each aquarium with tap water to remove the residues on the walls. After 96 h CLT of exposure, clams were relocated to register the behavioral responses as burial behavior, and filtering activity. The same physical and chemical parameters of the acclimation period were maintained and measured during the exposure time and behavioral biomarkers measurements. Afterwards, the individuals were dissected to biochemical and tissular biomarkers (Fig. 1).

2.3. Analytical methods

To determinate CLT concentration, aliquots of 10 mL per treatment were sampled at the beginning and the end of the experiment (96 h) (with and without clams). CLT was extracted from samples using a liquid-liquid microextraction (Benedé et al., 2014). Two calibration curves were run together with samples, one made with the technical grade CLT (ODEON 72 SC®) and the other with the pure analytical standard of CLT (1000 μg mL^{-1}, Accustandard®). Calibration curves include six CLT levels in a rank from 4.5 to 72 μg L^{-1}.

Samples were analyzed using gas chromatography coupled to mass spectrometry (Shimadzu GCMS-QP2100ULTRA-AOC20i). The ions used for quantification and qualification of CLT were m/z 266 and m/z 268/268 respectively. Limits of detection and quantitation were 3.1 μg L^{-1} and 6.8 μg L^{-1}, respectively, and the recovery was 71%.

2.4. Behavioral biomarkers

2.4.1. Burial behavior

After the exposure period, each clam was individually transferred to a small 250 mL aquarium containing 4 mL of sediment and clean water (without CLT). These small aquaria (n = 30) were located inside a bigger one (four big aquaria per concentration) where the same physiochemical parameters were maintained. Sediments were prepared according to the same protocol mentioned for sediments used in the acclimatization period. Only one individual was placed in the middle of each small aquarium and acclimated for 3 min. After this period (initial time)) the burial parameters were recorded for 30 min. This procedure was applied for all the individuals from each treatment and control

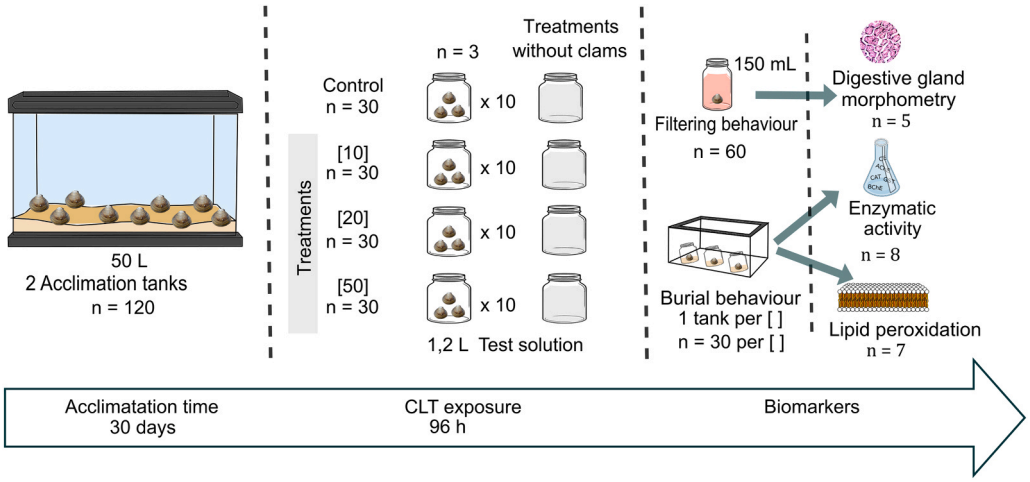

Fig. 1. The time sequence scheme is showing the acclimatization period of organisms, the experimental design of the 96-hour exposure test to CLT (control group and the three CLT treatments (10, 20 and 50 μg L^{-1}), and finally the responses measured through biomarkers at the end of the bioassays.

group. The evaluated parameters were: (1) latency time (LT), the time it takes each clam to open the valves and get the foot out; (2) activation time (AT), the time elapsed until the clam start to bury itself, and the individual is placed vertically to the sediment; (3) burial time (BT), the time it takes each clam to bury itself completely (Rodríguez et al., 2020).

2.4.2. Filtering activity

Once burial behavior trials were ended, the clearance rate was estimated in half of the total number of clams (n = 60 individuals) by the retention in their gills of neutral red particles from a known volume of water, as a result of the filtration processes (Martinez-Haro et al., 2016; Riisgård, 2001). Beakers of 600 mL containing 150 mL of dechlorinated tap water were used with a known concentration of a neutral red solution (5 mg L^{-1}). Individuals of each treatment (one individual per beaker) were placed and acclimated for 30 min. After this period, clams were allowed to filtrate during 1 h. The initial and final concentration of neutral red (C_O, at t = 0 and C_t, at t = 1 h, respectively) were recorded by triplicate. The concentration was estimated by triplicate through the absorbance value at λ = 530 nm using a multiplate reader (Biotek Synergy HT®). Finally, the concentrations of neutral red in the sample from each individual were calculated from a standard curve of neutral red.

The clearance rate was calculated according to Coughlan (1969):

$$\mathbf{CR} = (\mathbf{V}/\mathbf{nt})\mathbf{ln}(\mathbf{C_0}/\mathbf{C_t})$$

where *V,* the volume of water (mL); *n,* number of clams; *t,* time of test (h); C_O initial (mg L^{-1}) and C_t final neutral red concentration (mg L^{-1}).

At the end of CR and/or burial trials, clams were dissected (Committee for the Care and Use of Laboratory Animals of Instituto de Ecologia y Diversidad Animal, CONICET-Universidad Nacional de Córdoba11/2019). Afterwards, the soft tissues were individually weighted (accuracy = 0.0001 g). To avoid the interferences caused by residuals of the neutral red solution on the tissues of clams, the soft tissue (without mantle, gills and foot, called as visceral mass) of individuals from CR experiments were stored in 10% buffered formaldehyde solution for histological analysis (n = 5) of the digestive gland. The identification of the gland was made under a light microscope. On the other hand, tissues of clams from burial experiments were dissected as follows: visceral mass (0.23 ± 0.02 mg) and gills (0.13 ± 0.02 mg). They were stored at −80 °C until the biochemical analysis, enzymatic activity (n = 8) and lipid peroxidation (n = 7).

2.5. Morphometry of the digestive gland

Samples from visceral mass were prepared following routine histological techniques and stained with haematoxylin and eosin (H&E). Each individual was examined blinded, using a light microscope (Olympus X-785) and photographed with a coupled digital camera (Moticam Camera 2300, 3 Megapixels). Different measurements from tubular profiles (fifteen per sample, each called as diverticulum) of the digestive glands were taken: mean epithelial thickness (MET; μm), mean diverticulum radius (MDR; μm) and mean luminal radius (MLR; μm), according toMarigómez et al. (1990) and Garmendia et al. (2011). Both MDR and MLR were calculated from the lumen and total tubular diameter through the formula (D = 2R), where R corresponds to the radius of each tubule. As for indicators of changes in the digestive gland, the ratio of MLR / MET and MET / MDR were calculated. (Supplementary material, Fig. S1). The morphometry of the digestive gland was analyzed through the Image J software 1.51j8 (Rasband, 2012).

2.6. Enzyme activity measurement

The activity of enzymes involved in antioxidant defense and biotransformation mechanisms measured were: Catalase (CAT) and Glutathione-S-Transferase (GST). Also, the cholinesterase activities of Acetil- (AchE), Butiryl- (BchE), and Carboxyl-esterase (CE) were measured. The enzymatic extraction from each tissue was made following Bonifacio et al. (2016). CAT activity was determined using H_2O_2 as a substrate at 240 nm following Beutler (1982). GST activity was determined using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate at 340 nm, according to Habig et al. (1974); AChE, BChE, and CE were measured at 412 nm following (Ellman et al., 1961) using Acetylthiocholine Iodide, Butyrylthiocholine and S-phenyl thioacetate as substrates, respectively. Enzyme activities were measured by triplicate using a Biotek Synergy HT® microplate reader. The activity was calculated in terms of the protein content of the sample using a bovine serum as standard (Bradford, 1976) and reported in nanokatals per milligram of protein (nkat (mg prot)$^{-1}$), where 1 mol of substrate per second is equal to 1 kat.

All chemicals and reagents were purchased from Sigma−Aldrich Chemical Corporation® (USA).

2.7. Lipid peroxidation

The damage in the lipid membranes was determined by quantification of the reactive species of thiobarbituric acid (TBARs) through the protocol described following Granados-Amores et al. (2018). Each tissue

was homogenized individually. TBARs was determined by spectrophotometry at λ = 530 nm and was measured using a multiplate reader, Biotek Synergy HT®. Results were calculated through a calibration curve using 1, 1, 3, 3 tetramethoxypropane (TMP) as standard and the concentration of TBARs was expressed as nmoles of TBARs per gram of fresh weight (nmol TBARs g FW^{-1}).

2.8. Data analysis

Data were expressed as mean ± standard error (mean ± SE). At all linear models used in this study, normality and homogeneity of variance were determined by graphic methods.

Linear mixed models were used to determinate the differences in the enzymatic activity and lipid peroxidation between gills and visceral mass and among treatments and also in the measurement of the digestive gland morphology. To consider the variability of each individual, all the values of each parameter measured by triplicate in each individual were used as the random factor of the linear mixed model. Those variables that did not meet the parametric assumptions were transformed using log10 or square root depending on data distribution. When homogeneity of variance was not fulfilled, the structure of variance was also modelled using the VarIdent structure.

A general linear model was used to evaluate differences in CLT concentrations at the beginning (T0h) and the end of the experiment (T96h) and to compare concentrations between control and aquaria with clams at each treatment at 96 h. This test was also performed to evaluate significant differences in the times of burial behavior (LT, AT and BT). In particular, to evaluate burial behavior at each treatment at the end of the trial, also binomial models were carried out considering "buried individuals" (including partial and total burial) as 1 and "not buried individuals" as 0.

A *posteriori* Tukey test was applied to detect differences between pairs of means in both linear models. A p-value was set at α = 0.05.

Finally, to evaluate differences in clearance rate among treatments, a generalized least squares model (GLS) was performed because the homoscedasticity assumption was not fulfilled even when variance structure was modelled.

All the statistical analyses were carried out using R Studio (R Development Core Team, 2011). lme() and gls() functions were used to Linear mixed and generalized least squares (GLS) models, respectively using nlme Package (Pinheiro et al., 2017). Also, lm() function was used to ANOVA and glm() function to binomial models in stats package (R Development Core Team, 2011). Furthermore, the *a posteriori* Tukey test was performed using lsmeans() function from emmeans package (Russell, 2018).

3. Results

3.1. Concentration of CLT in water

The concentration of CLT in water from containers without clams after 96 h and after three water renewals, showed that increments of 124% and 34% were observed for the treatments at 10 and 50 μg. L^{-1}, respectively, while at 20 μg. L^{-1} the concentration did not change significantly ($F = 86.16$; $p = 0.003$; $F = 46.1$, $p = 0.005$; $F = 0.02$, $p = 0.86$; respectively; Table 1). On the other hand, in water from the aquaria with clams, all values from the control group were < LOD (Table 1) at both times, t = 0 and t = 96 h. The residual CLT concentrations in treatments decrease to < LOD at 10 μg. L^{-1}, and, 72% at 20 μg. L^{-1} ($F_{20} = 120$, $p = 0.0001$) while at the highest concentration treatment, there was a 17.6% increase although it was not significant ($F_{50} = 3.79$, $p = 0.09$). Finally, when comparing treatments with and without clams, after 96 h, there were significant differences. In tanks with clams, at 10 μg L^{-1}, the CLT concentrations were < LOD. A decrease of 73% in CLT concentration was observed at 20 μg. L^{-1} ($F = 81.71$ $p = 0.003$) whereas at 50 μg L^{-1} the CLT concentration did not change after 96 h of exposure ($F = 0.52$ $p = 0.51$).

Table 1
Concentrations of Chlorothalonil (CLT) expressed as μg L^{-1} in water from treatments at the beginning (T0h) and the end of the experiment (T96h).

CLT Nominal concentration	Initial CLT concentration[a] T0h	Final CLT concentration T96h	
[0]	<LOD	Control	<LOD
		With clams	<LOD
[10]	6.19 ± 0.51	Control	13.20[*] ± 1.13
		With clams	<LOD
[20]	15.07 ± 0.56	Control	15.73[#] ± 0.81
		With clams	4.27[*,#] ± 0.86
[50]	36.19 ± 1.28	Control	51.06[*] ± 1.53
		With clams	46.09[*] ± 4.53

<LOD = limit of detection (3.1 μg L^{-1}).
[*] () Indicates significant differences between T0 and T96 within each concentration treatment.
[#] () Indicates significant differences between control and with clams within each concentration treatment.
[a] () The initial concentration was the same for treatments control (without clams) and treatments with clams.

3.2. Biochemical biomarkers

The enzymatic activities measured in gills and visceral mass at all CLT treatments are shown in Fig. 2. They presented a significant interaction between factors. Gills show the highest activity for all the enzymes (CLT treatments * tissues: $F_{GST} = 433.35$ $p < 0.05$; $F_{CAT} = 4313.54$, $p < 0.05$; $F_{AChE} = 1140.72$, $p < 0.05$; $F_{BChE} = 1145.72$, $p < 0.05$; $F_{CE} = 212.03$, $p < 0.05$).

The gill GST activity significantly decreased at 10 and 20 μg. L^1 respect to the control group. No significant differences were recorded in the GST activity of visceral mass. Besides, CAT activity had no significant changes in gills, while in the visceral mass, the activity decreased significantly at the highest concentration with respect to the other treatments.

The Carboxylesterase enzyme showed an activity increase in gills at 50 μg. L^{-1} respect to the other treatments. In visceral mass, showed a significant decrease at 20 μg. L^{-1} respect to the control group and an increase at 50 μg. L^{-1} to 20 μg.L^{-1}. Also, the AChE activity significantly decreased in both gills and visceral mass at 50 μg. L^{-1} and the BChE activity did not show significant changes at any treatment or for any organ.

The TBARs levels presented differences and interaction among factors (CLT treatments * tissues: $F = 237.72$; $p < 0.05$; Fig. 3). Both gills and visceral mass showed significant differences at 50 μg. L^{-1} CLT, although gills showed higher significantly levels of TBARs than visceral mass.

3.3. Morphological biomarkers

The morphometric analysis of the digestive gland showed an increase in MET at all the CLT treatments ($F = 4465$, $p < 0.05$) whereas MLR was significantly reduced at 50 μg. L^{-1} only ($F = 1996.8$, $p < 0.05$). On the other hand, MET/MDR ratio showed an increase at all the concentrations tested, whereas the MLR / MET ratio decreased at 50 μg. L^{-1} only ($F = 1006$, $p < 0.05$; $F = 2103.5$, $p < 0.05$, respectively; Fig. 4a).

3.4. Behavioral biomarkers

The number of individuals buried at the end of the trial (observation time 30 min) did not show differences among treatments were recorded

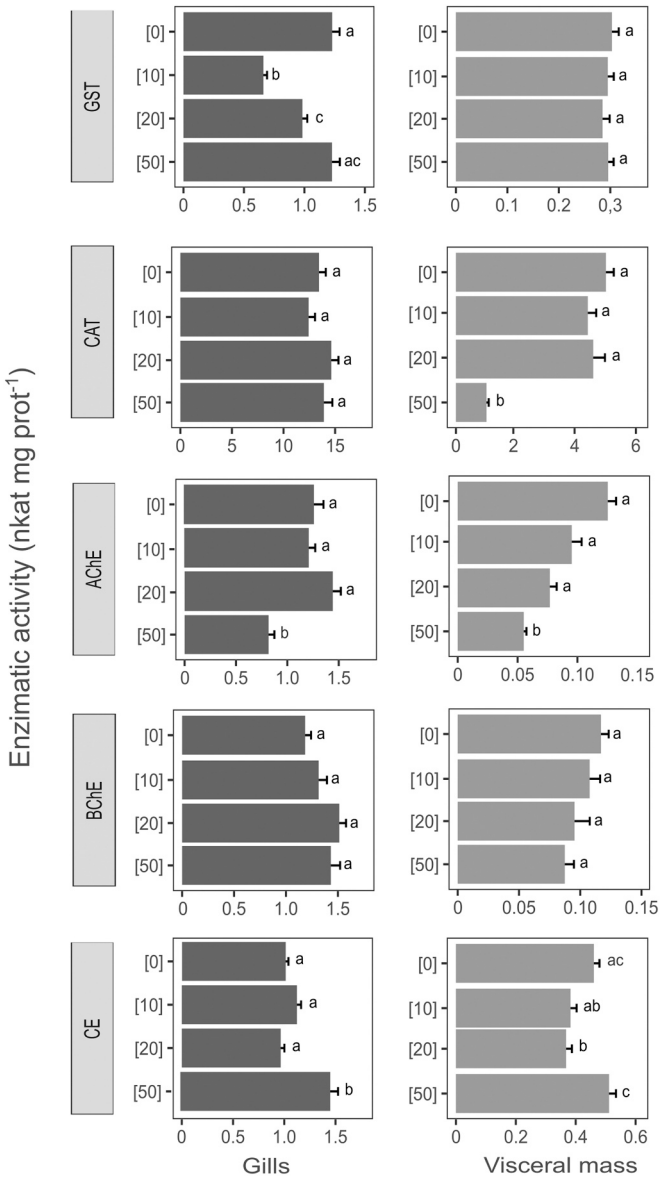

Fig. 2. Enzymatic activity (nkat mg protein^{-1}) in gills and visceral mass of *Corbicula largillierti* exposed to different sublethal concentrations of chlorothalonil (CLT; μg L^{-1}). Values expressed as mean ± standard error. Different letters indicate statistical differences among treatments for gills and visceral mass ($p < 0.05$).

($F = 107.56$, $p = 0.54$, Fig. 5a). However, a tendency to a low number of buried individuals at 50 μg. L^{-1} treatment was observed. Particularly, for the different burial times assessed, LT showed differences between the control group and individuals exposed to 50 μg. L^{-1} CLT ($F = 3.78$; $p = 0.015$). On the contrary, AT and BT did not show differences among treatments ($F = 1.48$; $p = 0.24$ and $F = 1.07$; $p = 0.38$ respectively, Fig. 5b).

Finally, the CR increased at all the CLT treatments ($F = 14.86$; $p < 0.05$. Fig. 6). The highest rates were registered at both 20 μg. L^{-1}and 50 μg. L^{-1} CLT.

4. Discussion

The present study showed that the Asian clam *Corbicula largillierti* exposed to different sublethal concentrations of CLT during 96 h were affected at biochemical, morphological and behavioral levels. Furthermore, it was demonstrated the usefulness of this species as a model of

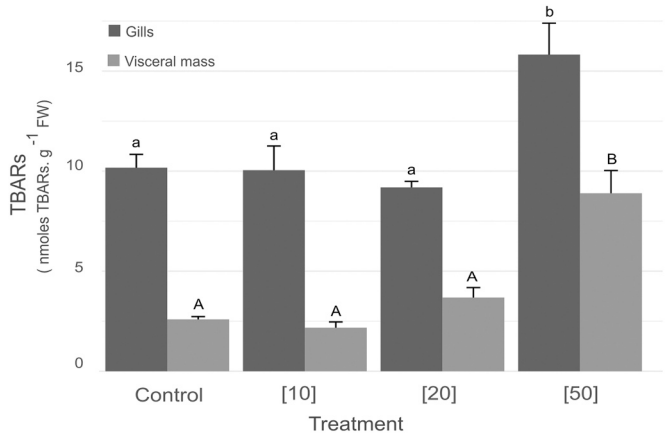

Fig. 3. Thiobarbituric acid (TBARs) levels (nmol TBARs g FW^{-1}) in gills and visceral mass of *Corbicula largillierti* exposed to different sublethal concentrations of chlorothalonil (CLT; μg L^{-1}). Values expressed as mean ± standard error. Different letters indicate statistical differences among treatments for gills and visceral mass ($p < 0.05$).

study under laboratory conditions.

4.1. Concentrations of chlorothalonil in water

The results from CLT concentrations in the aquaria with and without clams showed that after 96 h of exposure and under periodic water renewals, the concentrations in aquaria with clams decreased at 10 and 20 μg L^{-1}. This result indicates that the fungicide is incorporated, accumulated and/or metabolized by clams. However, the ability of clams for doing this was affected by CLT concentrations, since the higher the nominal CLT concentration, the lower the reduction of CLT concentration. This pattern was clearly observed at 50 μg L^{-1} treatment, where no changes in CLT concentrations were registered. It can be hypothesized that clams could have achieved the maximum concentration that are able to accumulate, reaching a balance between incorporation and elimination processes. Although it was out of the scope of the present work, further analysis of CLT bioconcentration over time by clams as well as on CLT metabolites residues in clams or water is needed to demonstrate this hypothesis. On the other hand, increases of CLT concentrations over time in aquaria without clams, suggest that some glass adsorption could occur allowing fungicide residues to remain attached to the tank walls even after a carefully water rinse at every water renewal. This situation was also evident at 10 and 50 μg. L^{-1}.

4.2. Biochemical biomarkers

Gills of *C. largillierti* showed higher enzyme activity compared with visceral mass independently from the CLT exposure concentrations. Clams are filter-feeding species; thus, gills are the first organ of contact with the surrounding environment and therefore, they interact directly with toxic compounds. Also, gills play an important role in defense and detoxification responses to coping with xenobiotic impacts (Lau and Wong, 2003; Sellami et al., 2015). A previous field study with *C. largillierti* showed the same enzymatic activity pattern between gills and visceral mass, along a gradient of river pollution (Reyna et al., 2019). It is necessary to point out that visceral mass included several organs, such as digestive gland, gonads, etc.; thus, the general responses measured could being masked the individual responses of them.

The responses of biotransformation and antioxidant enzymes (an activity can increase or decrease) depending on the intensity and duration of the xenobiotic exposure (Livingstone, 2001). When clams were exposed to CLT during 96 h, a reduction in GST activity was observed in gills (at 10 μg. L^{-1} and 20 μg. L^{-1}) respect to the control

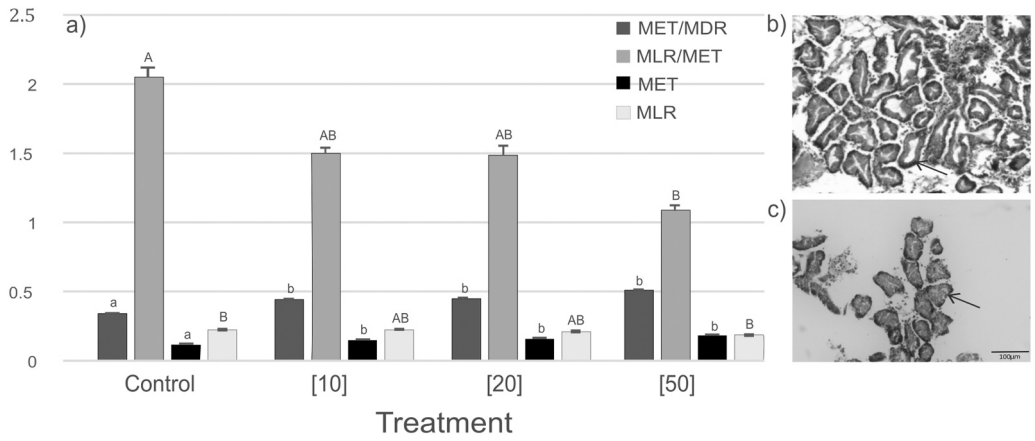

Fig. 4. Morphometric analysis of the digestive gland. (a) The MET/MDR and MLR/MET ratios, mean epithelial thickness (MET; μm) and mean luminal radius (MLR; μm). (b) Examples of digestive gland from a control clam. (c) Altered digestive gland of clam from 50 μg. L^{-1} CLT group. Values are expressed as mean ± standard error. Different letters indicate statistical differences among treatments ($p < 0.05$).

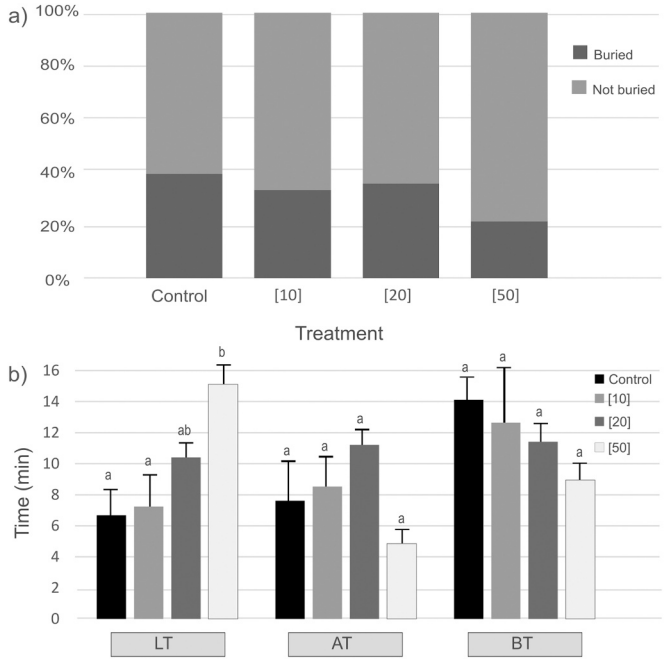

Fig. 5. Burial behavior recorded in *Corbicula largillierti* after different sublethal chlorothalonil concentration exposure (CLT; μg. L^{-1}). a) Percentage of individuals buried in each treatment at the end of the trial (30 min), b) Burial parameters: Latency time (LT), activation time (AT), burial time (BT). Values are expressed as mean ± standard error. Different letters indicate statistical differences among treatments inside each measured burial parameters ($p < 0.05$).

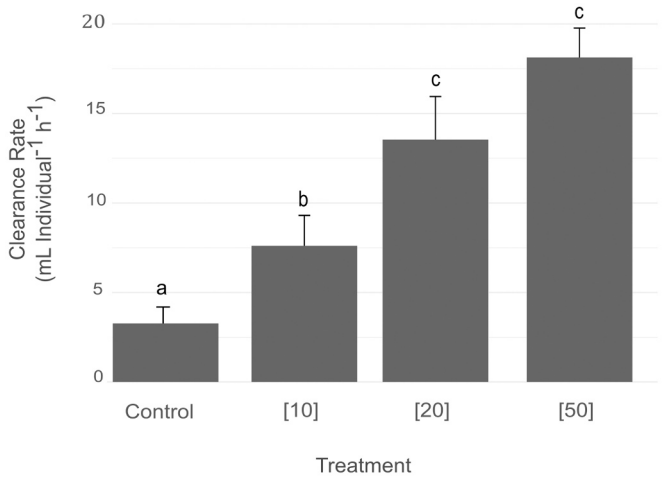

Fig. 6. Clearance rate (mL Individual^{-1} h^{-1}) of *Corbicula largillierti* performed after different sublethal chlorothalonil concentration exposure (CLT; μg. L^{-1}). Values expressed as mean ± error. Different letters indicate statistical differences among treatments ($p < 0.05$).

group, whereas no changes were registered in visceral mass. In gills, it can be seen that the GST activity has a hormetic response. This kind of biological dose-response has a U-Shape where the toxic compound shows an inhibitory effect at low concentrations and a stimulatory one at high concentrations (Calabrese and Blain, 2011). The GST is an enzyme involved in the biotransformation process (phase II) which conjugates a molecule of glutathione with the exogenous compound making it more water-soluble and easily to be excreted (Tan et al., 1987; Vrankovic and Slavic, 2015). In fish and molluscs, it was demonstrated that GST activity is involved in the metabolism of CLT since the detection of chlorothalonil-glutathione conjugates suggests the role of this enzyme in the CLT detoxification processes (Gallagher et al., 1991; Guerreiro et al., 2020; Rosner, 1996). Contrary to our results, Guerreiro et al. (2020) did not register changes in gills GST activity in *Perna perna* exposed to similar concentrations of CLT (1–10 μg. L^{-1}). However, these authors observed a decrease in the GST activity in the digestive gland at 24 h, suggesting that the animals were unable to synthesize enough GST to cope with the fungicide exposure. This decrease in GST activity could lead to an imbalance between phases I and II of the biotransformation process. As a consequence, an accumulation of metabolites from phase I, which could be more toxic than the parental compound, could not be finally excreted and at the end produce oxidative damage (Fernández et al., 2012).

Catalase is an antioxidant enzyme which breaks down H_2O_2 into O_2 and H_2O. According to our results gill CAT activity did not have any significant change at any CLT treatment. This result agrees with those obtained for *Perna perna* expose to 1 μg. L^{-1} and 10 μg. L^{-1} CLT during 24 h and 96 h Guerreiro et al. (2020). The absence of significant changes in CAT activity in gills of *C. largillierti* could be due to the antioxidant defense system acting through another enzyme pathway such as glutathione peroxidase (GPx), which is also able to metabolize organic peroxides, or through the superoxide dismutase (SOD) activity, involved in O_2^- excretion. Changes in SOD and GPx activity in gills of estuarine bivalves associated with CLT exposure have been registered by Guerreiro et al. (2020) Haque et al. (2019). Another work carried out by Almeida

et al. (2005) suggested that a low gills CAT activity in the mussel *Perna perna* could be explained by the ability of this organ to excrete hydrogen peroxide directly to the surrounding water environment. According to our results, we suggest that future studies, including the measurement of other enzymes such as SOD, GPx and GSH molecule are necessary to support these statements. On the other hand, CAT activity showed a decrease in visceral mass at the highest CLT concentration tested (50 μg. L^{-1}) respect to the control group, demonstrating a negative effect on the antioxidant defense system in visceral mass. Thus, the detoxifying and antioxidant capacity of *C. largillierti* were affected via GST and CAT enzymes in gills and visceral mass by CLT, respectively.

The AchE is an enzyme involved in cholinergic neurons of the central nervous system mainly at neuromuscular junctions. It breaks down the acetylcholine neurotransmitter into choline and acetic acid (van der Oost et al., 2003). This enzyme has been usually considered as a biomarker of the neurotoxic effect of xenobiotics. It has been widely demonstrated that some pesticides cause AchE inhibition, such as organophosphorus and carbamates, but also organochlorine pesticides, heavy metals and polycyclic aromatic hydrocarbons (PAHs) (Ballesteros et al., 2009; Domingues et al., 2010; Ghedira et al., 2009; Mora et al., 1999; Ramos et al., 2012). The AchE activity in both gills and visceral mass was inhibited at the highest CLT concentration (50 μg. L^{-1}). In a previous study, Haque et al. (2019) demonstrated an AChE inhibition in gills of *Crassostrea gigas* exposed to 10 μg. L^{-1} CLT at 96 h whereas in *Mytilus edulis* the enzyme was inhibited at a lower concentration and shorter exposure period (1 μg. L^{-1} at 48 h). It is important to point out that the CLT concentration where AChE of *C. largillierti* was inhibited is five times higher than those used by Haque et al. (2019). However, they used the analytical standard while in the present study, the commercial formulation of the fungicide was used, since this is the form which the pesticide enters the aquatic environment. Thus, our results provide more realistic results.

The BChE is an unspecific esterase enzyme with a protective role against the effects of toxic compounds containing carboxylic or phosphoric acid esters. Therefore this enzyme acts as an endogenous scavenger of anticholinergic compounds and breaking them down before they could cause damage to other enzymes such as AChE (Çokuğraş, 2003; Valbonesi et al., 2003). Nevertheless, this enzyme did not show changes in the studied organs indicating a lower sensitivity than AChE activity under the CLT concentrations tested and thus, it does not fulfil its protective role. According to the no effects registered in BChE activity by CLT, we suggest that the fungicide could affect a different pathway than the BChE activity to cause the observed inhibition in AChE.

Carboxylesterases are also unspecific esterase enzymes involved in detoxification of carboxylic-ester compounds. It is especially sensitive to organophosphate, carbamates and pyrethroids pesticides (Escartin and Porte, 1997; Galloway et al., 2002; Solé et al., 2018; Wheelock et al., 2008). In the present study, CE activity in gills of *C. largillierti* showed an activity increase after the exposure to 50 μg.L^{-1} suggesting an activation of the detoxification process through this enzyme. In contrast, in visceral mass, the activity was inhibited at 20 μg.L^{-1} only, suggesting a hormetic response as was also observed in gills GST activity (Calabrese and Blain, 2011). The present study demonstrates that a halogenated benzonitrile compound can cause damage in this enzyme; therefore, it could not be ruled out that CE can be sensitive to other compounds apart from insecticides.

The determination of damage in lipid membranes allows estimating the effect that comes from the excess of ROS on an organism when is subject of a stressor, such as a xenobiotic compound (Almeida et al., 2005; Chen et al., 2015; Taylor et al., 2017). The gills of *C. largillierti* presented the twice-fold higher levels of LPO compared with visceral mass. These results suggest that gills are more prone to suffer oxidative damage due to exposure to CLT. The differences in TBARs levels between gills and visceral mass can be explained by the different function of them. As we stated before, gills have a large surface in direct contact with the aquatic environment, and therefore, they are also in direct contact with toxic compounds. Also, in the present study, gills and visceral mass showed a significant increase in TBARs at 50 μg.L^{-1} of CLT. These results are in agreement with previous studies in gills of bivalves (*C. gigas* and *M. edulis*) and polychaete body (*Laenoreis acuta*), that registered LPO increases at 10 μg. and 100 μg.L^{-1} CLT, respectively after 96 h of exposure (Barreto et al., 2018; Haque et al., 2019). Our results suggest once again that *C. largillierti* is more resistant than the mentioned species to CLT exposure. Although the CE activity increased, it was not enough to counteract the CAT inhibition and the absence of GST responses. Thus, the biotransformation and detoxification processes were not enough to neutralize the possible ROS increase and then to protect the cell against oxidative damage. This inverse relationship between LPO levels and CAT in visceral mass was also supported by Saidani et al. (2019) who showed that after exposure of clam *Ruditapes decussatus* to a 100 μg.L^{-1} of titanium dioxide nanoparticles, LPO levels increases when CAT activity decreases.

4.3. Morphometric biomarkers

The digestive gland in molluscs is involved in intracellular digestion processes, contaminant accumulation and detoxification metabolism, (Vale et al., 2014). At the tissue level, the digestive gland morphometry of *C. largillierti* was altered by CLT. The estimated values of MLR/MET and MET/MDR indicate significant effects on this organ. Both the increase of MET/MDR and the decrease of MLR/MET are due to the increase of the epithelium thickness. The increase of the epithelium thickening in clams exposed to CLT together with a significant decrease in lumen radio at 50 μg.L^{-1} respect to the control group indicates hyperplasia or hypertrophy events of the epithelium tissue. These kinds of responses occur as a primary adaptive response due to toxic exposure to xenobiotic compound (Benjamin et al., 2019; Marigómez et al., 2006; Rocha et al., 2016). Therefore, damage on the digestive gland caused by CLT exposure could compromise the general health of the individuals by reducing the energy supply and detoxification capability (Cuevas et al., 2015; Rocha et al., 2016).

4.4. Behavioral biomarkers

There was not an apparent CLT effect on burial behavior of clams, because no differences among treatments were observed (in terms of buried/not buried clams). Considering all the CLT treatments together, a low proportion of clams were buried at the end of the trials. Nevertheless, at the highest concentration (50 μg.L^{-1}) there was a tendency of this proportion to decrease (less than 30%), and also it was observed that the clams took longer time to start the burial behavior (as it is showed through the significant LT increase). Similar results were registered by Hellou et al. (2009), who demonstrated the adverse CLT effects on the behavioral movement of mud snail *Ilyanassa obsolete,* after the exposure to 100 μg.L^{-1} during 24 and 48 h. Moreover, Bonnail et al. (2016b) and Further et al. (2006) observed that individuals of *C. fluminea* took more time to rebury after the exposure to different concentrations of contaminants. Bonnail et al. (2016b), demonstrated that the low number of clam reburials after 48 h of exposure to contaminated sediment with increasing levels of metals, whereas Further et al., (2006) found that clams reduced the ability to reburial after 96 h of exposure to chlorpyrifos (500–1000 μg.L^{-1}). On the other hand, the LT increase and the decreasing tendency observed in AT and BT in the present study could be explained by the inhibition of AchE at the highest concentration. Other studies have demonstrated that some xenobiotics such as chlorpyrifos produced a reduction of burial ability in *C. fluminea* and the rotation behavior in the snail *Gibbula umbilicalis* as a consequence of the inhibition of AChE activity (Cooper and Bidwell, 2006; Silva et al., 2019).

In bivalves, the filtration behavior is sensitive to external stimulus and regulated by the movement of valves (Cranford et al., 2011; Riisgård and Petersen, 2004). This response of valves (open-close movement) could reflect the environmental conditions and a way to cope with a

stressor. In this sense, when bivalves are in a contaminated environment, they respond by closing their valves leading to a decrease in the filtration rate (Castro et al., 2018; Chen et al., 2010). Contrary to our expectations, *C. largillierti* showed an increase in clearance rate (CR) in a CLT concentration-dependent manner. This result suggests that the mechanisms of opening–closing the valves are affected by the fungicide. Riisgård and Petersen (2004) demonstrated a direct correlation between the gape of valves and the clearance rate in bivalves. It was expected that because of the inability of *C. largillierti* to close the valves to avoid the CLT, which leads the clams to remain to filter the surrounding water and to produce a continuous input of the toxic. This inability could be the consequence of the inhibition of AChE activity at the highest CLT concentration. Our hypothesis could be related to the statement proposed by Geraudie et al. (2016). They suggested that inhibition of AchE in the adductor muscle in scallops (*Chlamys islandica*) affects the valve closure capacity and this could be altering other physiological processes associated with this opening/closing valves behavior such as the filtration rates, spawning, and others.

Furthermore, a constant filtering activity could lead to clams to a higher energy reserves consumption which would be necessary to the antioxidant defence system and reproduction. On the other hand, Castro et al. (2018) did not find inhibition of filtration rate in *C. fluminea* individuals exposed to high concentrations of the organophosphorus insecticide dichlorvos (5810 $\mu g\ L^{-1}$). These authors suggest that the chemoreceptors involved in the behavior of the open-close valve did not detect the insecticide or this compound could interfere with other mechanisms involved in the neurotransmission activity in the abductor muscle contraction related to the valves opening behavior. Therefore, it is an interesting hypothesis that could be applied in future studies in *C. largillierti,* where the effect of CLT on muscle function and this behavior could be evaluated.

The increase in the CR registered in *C. largillierti* was also in contraposition with the results obtained in *C. fluminea.* The filtration rate of this species was negatively affected as was demonstrated by Cooper and Bidwell (2006). They showed a decrease in the CR after the exposure to chlorpyrifos during 96 h (3130 $\mu g.L^{-1}$). On the other hand, Castro et al. (2018) analyzed the inhibition of CR in *C. fluminea* after the exposure to different kinds of toxic compounds (including metals, such as copper sulfate, salts, sodium chloride and nitrogen fertilizers) and related it to the closure of the valves. Also, Al-Subiai et al. (2011) demonstrated inhibition of CR in *M. edulis* due to the loss of the ciliary function of the gills after the exposure to different cooper concentration (8–56 $\mu g.L^{-1}$ at 96 h).

The behavior of an organism depends on a wide variety of biochemical and physiological process (Amiard-triquet, 2009). In this sense, the behavioral changes showed by *C. largillierti* reflect the conditions imposed by the toxic and the responses registered through the different biomarkers measured. Since the toxics compounds do not occur individually but accompanied by others in the natural environment, the survival of native bivalve populations could be significantly affected. For instance, the effect on burial behavior would increase the drift and therefore, depredation risk (Bowers et al., 2005). On the other hand, as we stated before *Corbicula* sp. is an invasive species, characterized by high filtration rates hence produce a negative impact on nutrient cycling, primary production and excessive consumption of plankton in the invaded environments (Argente, 2016; Ilarri and Sousa, 2012; Mackie and Claudi, 2001; Sousa et al., 2008). Therefore, it is expected that increases in the filtration behavior caused by the exposure to CLT have an increased ecological impact in high biological organization levels.

5. Conclusions

The exposure to CLT evidences the adverse effects on clams at all the studied biological organization levels. The gills were the most compromised organ followed by the visceral mass. The CLT produced responses in antioxidant, biotransformation and cholinesterase enzymes. These alterations, together with the lipid peroxidation, digestive gland damages and changes in clam burial and filtration behaviors registered in the present work, indicated the negative impact of this fungicide. Our results allow inferring the potential risk for the freshwater molluscs and other invertebrates that inhabit ecosystems where this fungicide is present.

CRediT authorship contribution statement

P.B. Reyna: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **M.L. Albá:** Investigation, Resources, Formal analysis. **F.A. Rodríguez:** Investigation, Resources, Formal analysis. **M. Gonzalez:** Formal analysis, Writing - review & editing. **C. Pegoraro:** Formal analysis, Writing - review & editing. **A.C. Hued:** Conceptualization, Writing - review & editing, Supervision. **M. Tatián:** Conceptualization, Writing - review & editing, Supervision. **M.L. Ballesteros:** Conceptualization, Investigation, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111603.

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