



Assessing the effects of neonicotinoid insecticide on the bivalve mollusc *Mytilus galloprovincialis*

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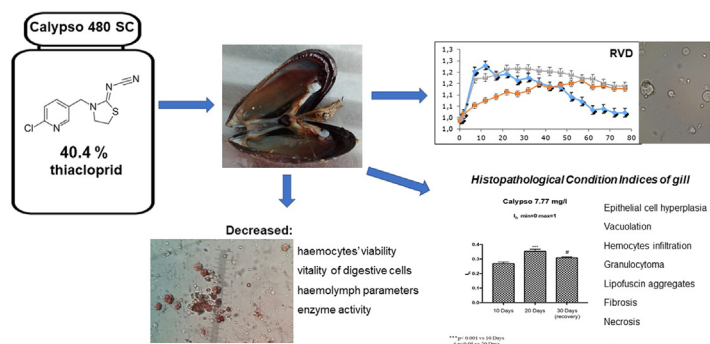
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HIGHLIGHTS

- Sub-chronic exposure significantly reduced haemolymph parameters of *Mytilus galloprovincialis*.
- To sub-chronic exposure digestive gland cells were no longer able to regulate volume.
- Neonicotinoid caused alterations in cell and tissue of *M. galloprovincialis*.

GRAPHICAL ABSTRACT



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ABSTRACT

In the present work, the marine invertebrate *Mytilus galloprovincialis* was used as model organism to evaluate the toxic effects of the neonicotinoid Calypso 480 SC (CAL) following 20 days of exposure to sub-lethal concentrations of 7.77 mg L⁻¹ (0.1% 96 h-LC50) and 77.70 mg L⁻¹ (1% 96 h-LC50), and a recovery period of 10 days in uncontaminated seawater. Results revealed that exposure to both concentrations of CAL increased significantly mortality rate in the cells of haemolymph and digestive gland, while digestive gland cells were no longer able to regulate cell volume. Exposure significantly reduced haemolymph parameters (Cl⁻, Na⁺), affected the enzymatic activities of superoxide dismutase of digestive gland and catalase of gill, and caused also histopathological alterations in digestive gland and gills. Main histological damages detected in mussels were lipofuscin accumulation, focal points of necrosis, mucous overproduction and infiltrative inflammations. Interestingly, alterations persisted after the recovery period in CAL-free water, especially for haemocyte parameters (K⁺, Na⁺, Ca²⁺, lactate dehydrogenase, glucose). A slight recovery of histological conditions was detected. These findings suggested that sub-chronic exposure to the neonicotinoid insecticide caused significant alterations in both cell and tissue parameters of *M. galloprovincialis*. Considering the ecologically and commercially important role of mussels in coastal waters, a potential risk posed by neonicotinoids to this essential aquatic resource can be highlighted.

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1. Introduction

Neonicotinoid insecticides were first introduced in the mid-1990s, and since then, they are commonly used in veterinary medicine and crop production (Wood and Goulson, 2017; Ensley, 2018). Neonicotinoids are chemically similar to nicotine, have a relatively low risk for vertebrates and are highly specific for subtypes of nicotinic receptors that occur in invertebrates. Neonicotinoids act on postsynaptic nicotinic receptors located entirely in the central nervous system of invertebrates (Ensley, 2018). Nervous system disorders are accompanied by reduced activity, tremors, miosis, incoordination, hypothermia, staggering gait, trembling, spasms, and it leads to death depending on the dose (Gupta and Milatovic, 2014). Vertebrates have relatively low affinity for nicotinic receptors and neonicotinoids do not readily pass the blood-brain barrier, further reducing the potential for toxicity in vertebrates (Gupta and Milatovic, 2014; Ensley, 2018). However, due to their widespread use, neonicotinoids are frequently detected in environment, water and food, posing a potential risk for non-target organisms (Suss et al., 2006; Klarich et al., 2017; Bradford et al., 2018; Craddock et al., 2019; Fedrizzi et al., 2019). The main source of danger is agriculture, insecticide areal spraying to the soil or plant surfaces are transported to ground-water and surface-water, through leaching, runoff, and drift and ends in the water body (Bradford et al., 2018; Fedrizzi et al., 2019). In 2013, the European Union banned three kinds of neonicotinoids (clothianidin, imidacloprid, and thiamethoxam) forbidding their use in flowering crops and in 2018 to all field crops, because of growing evidence that these pesticides affect domesticated honey bees and also wild pollinators (EFSA, 2013; PAN Europe, 2018; Valavanidis, 2018). In several cases, vertebrates and human intoxication with such insecticides has been reported in the literature (Lin et al., 2013; Vinod et al., 2015; Cimino et al., 2017; Nistor et al., 2017).

On the basis of such premises, this study was focused on the impact of widely used neonicotinoid insecticides Calypso 480 SC, containing the active ingredient thiacloprid, on the aquatic non-target species *Mytilus galloprovincialis*. Thiacloprid ([3-[(6-chloro pyridin-3-yl)methyl]-1,3-thiazolidin-2-ylidene]cyanamide) is yellowish, crystal powder, odorless, well soluble in water (at 20 °C 185 mg L⁻¹), and it is stable in anaerobic aquatic conditions (half-life is 10–63 days) (EPA, 2003). Thiacloprid acts against numerous important agricultural biting and sucking pests in cotton, pome fruits, vegetables, and potatoes (Jeschke et al., 2011). The acute toxicity of thiacloprid to agricultural target pests ranges between ≤0.6 and 18.5 mg L⁻¹, (Kramer et al., 2012). Data of thiacloprid toxicity to non-target invertebrates are summarized in Table 1. Generally, for aquatic organisms thiacloprid is classified as slightly toxic for fish and very highly toxic for invertebrates on an acute exposure basis (EPA, 2012).

Mussels are very important filtering organisms and offer the advantage of a wide geographic distribution. They are often used

world-wide as bioindicators of the contamination of the marine environment for their capability to accumulate and concentrate a large number of pollutants (Villeneuve et al., 1999; Rouane-Hacene et al., 2015; Pagano et al., 2017). *Mytilus galloprovincialis* is an ecologically important species in coastal waters. In the Mediterranean region in particular, this species of mussel represents one of the most farmed species for human consumption (Rouane-Hacene et al., 2015; Stankovic and Jovic, 2012; Carella et al., 2018; FAO, 2019).

One of the main goal of this study was to determine the acute toxicity (96 h-LC50) of the neonicotinoid pesticide Calypso 480 SC (CAL) on mussel *M. galloprovincialis*. In addition, the effects of sub-chronic exposure on haemolymph cells (haemocyte viability), digestive gland cells (viability, regulatory volume decrease assay - RVD), gills and digestive gland antioxidant enzyme activities (superoxide dismutase - SOD, catalase - CAT) and histology were evaluated. Based on the findings and knowledge about neonicotinoid, we assume that the exposure of Calypso will have a negative effect on *M. galloprovincialis*.

2. Materials and methods

2.1. Experimental design

For the tests we used insecticide product Calypso 480 SC (CAL; contains active ingredient 40.4% thiacloprid) from Bayern Crop Science Corporation.

Mytilus galloprovincialis, 5.60 ± 0.40 cm shell length, were obtained from “Faro Lake” from a local mollusc farm (Company FARAU SRL, Frutti di Mare) Messina, Italy. It is a small brackish lagoon (≈26 ha) consisting of a circular basin with a 500 m diameter. It is a meromictic lake which features a funnel-shape profile, with depth from 0.50 m to 5 m around the coastal part, with a steep sloping bottom that declines to a central deepest part (≈28 m). The bottom of the lake is a combination of sand, mud, interleaved with rocky areas (Spinelli et al., 2018). “Faro Lake” is part of the Oriented Natural Reserve of “Capo Peloro” that comprehends two coastal lagoons, “Faro” and “Ganzirri” one, connected each other and with both Ionian and Thyrrenian Seas (Bottari et al., 2005; Capillo et al., 2018a). “Faro Lake” is used for bivalves housing and rearing, mainly mussel (*M. galloprovincialis* Lamarck, 1819). The average annual values of parameters of Faro lagoon are: temperature ranges between the minimum in winter of 12.9 and a maximum during summer 27.4 °C, salinity ranges between 30.5–35.4 ppt, and pH in a range between 7.63–8.92 (Capillo et al., 2018b).

One-hundred-sixty-two mussels were kept in aquaria filled with continuously aerated brackish water in laboratory with day light exposure 12 h light : 12 h dark and temperature 18.17 ± 0.84 °C for 5 days acclimation before the onset of any experimental procedure. After acclimation, twenty-seven mussels

Table 1
Toxicity of thiacloprid on selected non-target invertebrates.

Order	Species	Time	LC50/LD50	References
Hymenoptera	<i>Apis mellifera L.</i>	48 h	17.32 µg bee ⁻¹	Brandt et al. (2016)
Ephemeroptera	<i>Baetis rhodani</i>	96 h	4.60 µg L ⁻¹	Beketov and Liess (2008)Van den Brink et al. (2016)
	<i>Cloeon dipterum</i>		30 µg L ⁻¹	
Lumbriculida	<i>Lumbriculus variegatus</i>	96 h	33.80 µg L ⁻¹	Raby et al. (2018)
Amphipoda	<i>Hyaella azteca</i>	96 h	55 µg L ⁻¹	Raby et al. (2018)Beketov and Liess (2008)
	<i>Gammarus pulex</i>		350 µg L ⁻¹	
Odonata	<i>Coenagrion spur</i>	96 h	5.65 mg L ⁻¹	Raby et al. (2018)
Decapoda	<i>Cherax destructor</i>	96 h	7.77 mg L ⁻¹	Stara et al. (2019)
Haptotaxida	<i>Eisenia andrei</i>	21 d	7.10 mg kg ⁻¹	De Lima e Silva et al. (2017)
Euarthropoda	<i>Folsomia candida</i>	28 d	9 mg kg ⁻¹	Van Gestel et al. (2017)

LC50 = lethal concentration killing 50% of the test organisms; LD50 = lethal dose killing 50% of the test organisms.

were randomly selected and placed into each of six aquaria (three experimental groups in duplicated) containing 20 L continuously aerated brackish water. The mussels were exposed to sub-lethal concentration of CAL: 0 mg L⁻¹ (control); 7.77 mg L⁻¹ CAL1 (0.1% 96 h-LC50); 77.70 mg L⁻¹ CAL2 (1% 96 h-LC50) for 20 days, followed by a 10-days recovery period in CAL-free water. Brackish water and exposure concentrations were renewed every 2 days to assure natural brackish food supply and avoid starvation during the exposure. Behaviour and survival of mussels and water quality (temperature 16.15 ± 1.14 °C, pH 7.72 ± 0.15, salinity 33.14 ± 1.50 ppt, 1100 m Osm kg⁻¹) were recorded daily. Samples of mussels were sampled for laboratory treatments after 10 and 20 days of exposure CAL and after 10 days of recovery.

Prior to sub-chronic exposure, a preliminary acute toxicity test was conducted for the determined 96 h-LC50 of CAL on *M. galloprovincialis*. Concentrations of CAL used for acute toxicity were 0, 1, 10, 100, 500, 800 and 1000 mg L⁻¹, the volume was 10 L of sea water continuously aerated, number of tested mussels in one tank was 10, each concentration was tested in duplicated. Brackish water was changed before the beginning of acute test and then after 48 h. Behaviour and survival of mussels and water parameters (temperature 18.33 ± 0.40 °C, pH 7.73 ± 0.15, salinity 34.40 ± 0.31 ppt, 1100 m Osm kg⁻¹) were recorded daily. Acute lethal toxicity of CAL was assessed by the number of mussels dying at individual test concentrations over a time period of 96 h median lethal concentrations (LC50). Lethal concentration was determined by linear regression and probit analysis with a confidence interval of 95% using the EKO-TOX program (v. 5.2, INGEO Liberec). The 96hLC50 for *M. galloprovincialis* correspond dose 7.77 g L⁻¹ of CAL.

2.2. Haemolymph collection

Haemolymph samples were collected from 12 mussels from each experimental group. Six pools of two mussels each were used for analyses. The haemolymph was collected from the anterior adductor muscle with a 23-gauge needle, to a 1 ml plastic syringe. Once collected, it was placed in tubes and immediately centrifuged at 1000 rpm for 10 min. The supernatant was collected and stored at -20 °C. For analytical purpose, pooling was necessary to obtain a volume of haemolymph enough.

2.3. Cell viability assays

For the experiments were used haemolymph and digestive glands cells of mussels. The viability of haemolymph and isolated digestive cells was evaluated by: 1) the Trypan blue (TB) exclusion method; and 2) the stability of lysosomal membrane by neutral red (NR) retention assay.

Through the TB exclusion method we evaluated cell viability considering unstained cells as live, while stained cells, in which the membrane permeability was altered, as damaged cells. The percentage of cell viability was calculated by the formula:

$$\text{Cell viability (\%)} = (\text{Number of viable cells} / \text{Total number of cells}) \times 100$$

To assess the stability of lysosomal membranes, the isolated cells were analysed after 15 min of incubation with NR, according to Repetto et al. (2008). The stable cells had lysosomes that were clearly visible compared to those that dispersed the dye into the cytoplasm.

2.4. Isolation of digestive cells and RVD experiments

Digestive glands, of four animals from each group, were isolated according to the method of Torre et al. (2013) with slight modifications by Pagano et al. (2016). For the RVD experiments, the isolated

cells were visualized and measured according to the method described below.

One drop of cell suspension is placed on slides treated with poly-lysine and have double-sided adhesive tape on the margins to hold the coverslip and leave a space to add the experimental solutions. An isotonic solution and then a hypotonic solution are placed on the slide using a pipette to wash the fixed cells and the excess solution is collected through strips of absorbent paper. Cells were observed with a light microscope (Leica DM6B) connected to a colour video camera that digitized the image to a PC. Individual cells were selected and the images were taken at 0 and 3 sec in isotonic solution; afterwards, the solution was rapidly changed with an hypotonic solution, and the image were taken every 1 min for the first 10 min after the change of the solution and after every 5 min for 20 min. The profiles of the cells were drawn with the aid of ImageJ (NIH, Bethesda, MD, USA). The data are reported as relative area Aexp/Ai, indeed the cell areas for each experimental condition (Aexp) were compared to the areas measured in isotonic solution (Ai) at the beginning of the experiment.

2.5. Haemolymph biochemical parameters

Determination of haemolymph parameters in this experiment, six haemolymph electrolytes (such as chloride, potassium, sodium, calcium, phosphorus and magnesium), and two different biochemical parameters lactate dehydrogenase (LDH), and glucose (GLU) were evaluated from each group for 6 samples, for each sample was by pulled 2 mussels. The total parameters were measured by a multiparametric analyser (KONELAB 60 THERMO, Milano, Italy).

2.6. Determination of enzyme activity

Immediately after haemolymph sampling, 6 samples of digestive gland and gill of each group were dissected and stored at -80 °C until processing. Frozen tissue samples were thawed on ice and homogenised in four volumes of 0.1 M Tris-HCl buffer (0.15 M KCl, 0.5 M Sucrose, 1 mM EDTA, 1 mM Dithiothreitol and 40 µg L⁻¹ Aprotinin; pH 7.6) for 1 min with a Braun Labsonic U sonifier at 50% duty cycles on ice. Sample homogenates were centrifuged at 12,000g for 30 min at 4 °C and supernatant was collected for enzyme superoxide dismutase (SOD; EC 1.15.1.1) and catalase (CAT; EC 1.11.1.6) assays. Total SOD activity was measured with the xanthine oxidase/cytochrome c method spectrophotometrically at 550 nm for 1 min at room temperature according to Crapo et al. (1978). The cytochrome c reduction by superoxide anion generated by xanthine oxidase/hypoxanthine reaction was detected. The reaction mixture contained 50 mM KH₂PO₄ buffer (0.1 mM EDTA, 0.1 mM NaN₃; pH 8.6), 13 mM hypoxanthine, 1.6 mM cytochrome c, and 20 U mL⁻¹ xanthine oxidase and 30 µL supernatant. Catalase activity was measured as decreases in absorbance at 240 nm for 1 min of a 50-mM H₂O₂ solution in 50 mM phosphate buffer (pH 7.8) and 30 µL supernatant by method of Aebi (1984). The SOD and CAT activity were expressed as U mg of proteins⁻¹. Protein concentrations in homogenised tissues, according to the Biuret method were quantified (Sigma).

2.7. Histological analysis

Two specimens of *M. galloprovincialis* were collected from each experimental condition (7.77 and 77.70 mg L⁻¹) tank at three different times (10, 20 and 30 days) of exposure from the CAL and control. After sampling, gill and digestive gland were immersed in immunofix (paraformaldehyde 4% in phosphate saline buffer, Bio-Optica, Milan, Italy) for 8 h at room temperature. Tissues were then treated for paraffin embedding, sectioned using manual rotary microtome (Leica, RM2235) at 5 µm thick and stained

(H&E staining) qualitative histopathological screening under a light microscope (Leitz Diaplan, Germany). Detailed procedures are reported in [Zaccone et al. \(2015\)](#), [Pagano et al. \(2016\)](#) and [Lauriano et al. \(2018\)](#).

2.8. Statistical analyses

At each tissue sampling time, the statistical analyses of results were performed using one-way ANOVA followed by the Tukey test for pairwise comparisons among experimental conditions. The software package Prism 5.00 (2003, Graphpad Software Inc., La Jolla, CA, USA) were used for statistical analysis. Significant differences of haemolymph biochemical parameters and enzymatic activities between treatment and control groups were determined at each tissue sampling time using the non-parametric Mann-Whitney *U* test. The STATISTICA 10 software package (StatSoft, Tulsa, OK) was used for statistical analyses.

3. Results

3.1. *Calypso* acute toxicity determination

Mussels did not die in control group and at concentrations of 1, 10 and 100 mg L⁻¹ of CAL. Control mussels and those exposed to 1 and 10 mg L⁻¹ formed regularly byssus fibres, which allowed bivalves to adhere to tank walls during acute test (96 h). Conversely, mussels did not produce byssus at concentration of 100 mg L⁻¹ and higher by 48 h of acute exposure. Mussel died at concentrations from 500 mg L⁻¹ during acute toxicity test (96 h). A 96 h-LC50 value of 7.77 g L⁻¹ of CAL for *M. galloprovincialis* were determined. The acute test results allowed the determination of the concentration range to be used in the sub-chronic test.

3.2. *Calypso* sub-chronic toxicity

No deaths and changes were observed in the control mussel group during the sub-chronic toxicity test. Mussels at concentration of 7.77 mg L⁻¹ of CAL were not able to produce solid byssus fibres (they were few or absent) from 72 h throughout the exposure period and also during 10 days depuration time. Exposure to 7.77 mg L⁻¹ of CAL, which should correspond to 0.1% 96 h-LC50, resulted in a 12% mortality rate of mussels during 20 days of sub-chronic exposure. At a higher concentration of CAL, namely 77.70 mg L⁻¹ (1% 96 h-LC50), mussels were held by byssus threads weakly or not at all throughout the exposure, but they were attached strongly to the bottom of aquarium at the end of depuration phases, as well as control mussels. In addition, at this concentration, large amounts mussels (33%) died during the first 10 days of exposure to CAL and it was not possible to perform all analyses at the end of test. Therefore, mussels from the concentration of 77.70 mg L⁻¹ were used only for biochemical haemolymph parameters, haemocyte vitality and histology after 10 days recovery period.

3.3. Cell viability assays

After 10 days of exposure, the results obtained by the Trypan Blue (TB) exclusion and Neutral Red (NR) assays showed no effects of CAL on the cell viability of haemocytes, whereas digestive gland cell viability decreased of about 20% in animals exposed to the highest concentration (CAL2). However, after 20 days of exposure the viability of both group of cells exposed to the highest concentration (CAL2) changed: the haemocytes' viability decreased to about 75% but the vitality of digestive cells decreased about to 48%. After the recovery period, mussel exposed to CAL1 showed

generally unchanged viability in digestive gland cells compared to controls, whereas cell viability decreased in haemocytes ([Table 2](#)). No data on cell viability of mussels exposed to CAL2 following 10 days recovery period are available, because we have not enough animals for this evaluation at the end of experiments.

3.4. The RVD experiments

The cells exposed to the rapid change of osmolarity initially increase in size and then tended to return to their initial volume. The cells reached their maximum swelling, corresponding to a 12% increase volume, after 1 min of exposure to the hypotonic medium. Afterwards, the control cells exhibited RVD response. As shown in [Fig. 1A](#) after 10 days of exposure the cells do not reach their maximum swelling and fail to return to the initial situation. At second time, after washing with hypoosmotic solution, the cells exposed to CAL1 swell not much compared to the other and this value is significant ($P < 0.05$) ([Fig. 1B](#)), moreover, some cells exposed to CAL2 after washing with the hypoosmotic solution burst, failing to regulate the cell volume in any way. At the end of experiment, after recovery the digestive cells exposed at CAL1 improve cell volume regulation ([Fig. 1C](#)).

3.5. Biochemical haemolymph parameters

Results of haemolymph parameters are presented in [Table 3](#). The sub-chronic exposures to CAL1 showed significantly lower ($P < 0.01$) CL⁻, Na⁺ after 10 days exposure, and Na⁺, GLU after recovery period compared with controls. The CAL2 group showed significant lower ($P < 0.01$) value parameters (Cl⁻, Na⁺) after 10 days exposure, significant ($P < 0.01$) higher K⁺, and reduction Na⁺ and GLU at the conclusion of the recovery period compared with control. The indicators PHOS and Mg²⁺ were similar among groups at values comparable to control through the test.

3.6. Enzyme activity

Enzymatic activity SOD and CAT in digestive gland during the exposure period of *Calypso* (10 and 20 days) and recovery period are presented in [Fig. 2](#). In CAL1 exposed mussels there was a statistically significant decreased ($P \leq 0.01$) in SOD activity after 20 days exposure. No significant variation was detected after 10 days of recovery period in digestive gland of the CAL1-treated group compared with control ones; in the group exposed to CAL2 was not possible measure enzyme activity for lack of samples at the end of test.

The enzymatic activity of SOD and CAT in gills of mussels exposed to CAL concentrations are presented in [Fig. 3](#). Exposure to CAL1 and CAL2 induced a significant decrease ($P \leq 0.01$) of CAT activity after 20 days exposure, compared with controls.

3.7. Histopathology

Histopathology results of digestive gland are presented in [Fig. 4](#). Digestive gland of control mussels exhibited normal condition for each sampling period during the experiment ([Fig. 4A, B](#)). Both CAL exposure groups exhibited several damages of digestive gland. [Fig. 4C–H](#) present marked the most pronounced abnormalities in group CAL1 as haemocyte infiltrations, lipofuscin aggregates, presence of brown cells, digestive tubule alterations, hypertrophy, hyperplasia and focal points of necrosis at 10 and 20 days, while a slight significant recovery were noted in the 10 days CAL-free water sampling are presented.

Gills of mussels in both CAL exposure groups exhibited a similar histopathological damage ([Fig. 5](#)), as it was found in tissue of digestive glands. In fact, also in this case, alterations were present in

Table 2Cell viability assays in haemocytes and digestive gland cells of *Mytilus galloprovincialis* following exposure to Calypso 480 SC.

Cell viability		Exposure time (days)	Test groups		
			Control (0 mg L ⁻¹)	CAL1 (7.77 mg L ⁻¹)	CAL2 (77.70 mg L ⁻¹)
Haemocytes	TB (%)	10	100.00 ± 0.00	99.07 ± 0.6	96.12 ± 0.97
		20	100.00 ± 0.00	95.90 ± 1.03	82.08 ± 3.18*
		R 10	100.00 ± 0.00	70.97 ± 3.20*	68.78 ± 0.25*
	NR (%)	10	100.00 ± 0.00	100.00 ± 0.00	98.94 ± 0.40
		20	100.00 ± 0.00	96.26 ± 1.66	75.06 ± 1.37*
		R 10	100.00 ± 0.00	78.16 ± 3.83*	84.10 ± 0.16*
Hepatocytes	TB (%)	10	100.00 ± 0.00	95.95 ± 0.03*	79.94 ± 1.20*
		20	100.00 ± 0.00	100.00 ± 0.00	66.01 ± 1.60*
		R 10	100.00 ± 0.00	95.34 ± 0.72	–
	NR (%)	10	100.00 ± 0.00	91.81 ± 1.6*	81.07 ± 0.01*
		20	100.00 ± 0.00	98.72 ± 0.74	48.08 ± 1.11*
		R 10	100.00 ± 0.00	90.90 ± 0.09*	–

The values are means ± SE; n = 6. One-way ANOVA has been used to test the differences between control and exposed groups and the Tukey test allowed pairwise comparisons among experimental conditions, *denotes indicate significant differences ($P < 0.05$). CAL = Calypso 480 SC, CAL1 = 7.77 mg L⁻¹, CAL2 = 77.70 mg L⁻¹, R 10 = 10 days recovery period in CAL-free water.

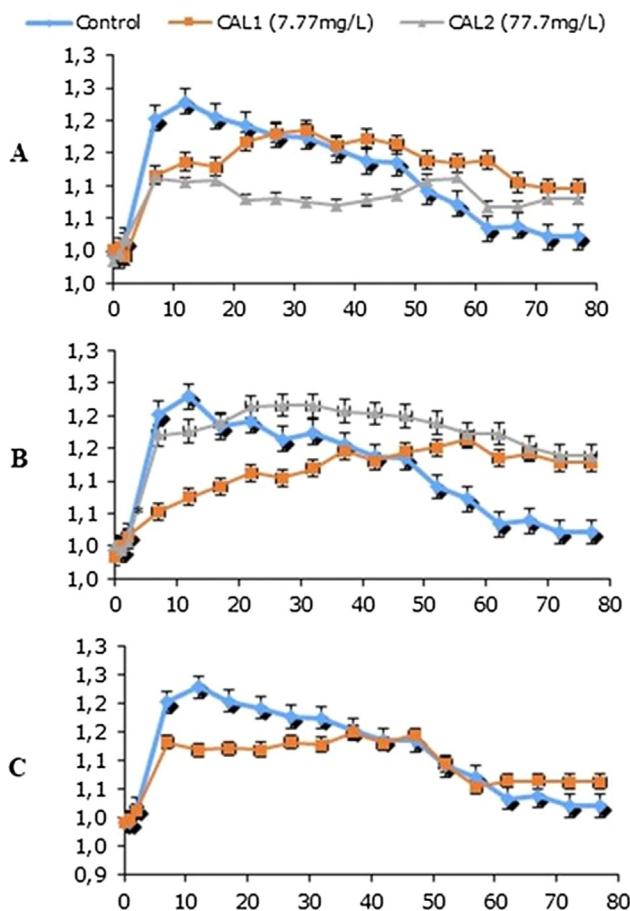


Fig. 1. Relative area changes of regulate cell volume (RVD) isolated by digestive gland of *Mytilus galloprovincialis*. (A) Cells of RVD after 10 days of exposure; (B) Cells of RVD after 20 days of exposure; (C) Cells of RVD after 10 days of recovery period. CAL = Calypso 480 SC, CAL1 = 7.77 mg L⁻¹, CAL2 = 77.70 mg L⁻¹. The values are means ± SE; * $P < 0.05$ respect to the control condition (two-way ANOVA test).

mussels from all the treatments with the higher number of elements after exposure of 10 and 20 days, and a slight recovery of gills were found after 10 days of CAL-free water. One of the most common gill tissue reaction to CAL1 exposure was lipofuscin deposit (dark brown staining) in the gill epithelium; other commonly found alterations comprehended vacuolization, mucous overproduction, haemocytes infiltration and epithelial alterations

(Fig. 5C–H). Histological condition of control mussels with normal structure are showed in Fig. 5A, B.

4. Discussion

Neonicotinoids are used relatively short period and their initial assessment have been assumed to be harmless. However, they are frequently found in aquatic ecosystems and their negative impacts to non-target organisms are a reality (Beketov and Liess, 2008; Pisa et al., 2015; Sanchez-Bayo et al., 2016). The mussels are filter feeders widely, used as bio-indicator able to accumulate a wide range of contaminants and reflect changes in the contaminant status of the aquatic environment (Faggio et al., 2016).

This study provides first data on acute and sub-chronic effects of insecticide Calypso 480 SC on *M. galloprovincialis*. The 96 h-LC50 value for *M. galloprovincialis* was 7.77 g L⁻¹ of CAL. Sublethal doses of CAL during sub-chronic test caused death in an unexpected number of mussels. Information regarding the ecotoxicological impact of neonicotinoids in marine invertebrates are very limited (Pisa et al., 2015). Studies mainly describe toxic effects of neonicotinoids on insects and freshwater invertebrates (Table 1). Dondero et al. (2010) reported half maximal effective concentration (96 h-EC50) for *M. galloprovincialis* of 1.50 mg L⁻¹ thiacloprid. The LC50 values of neonicotinoids for molluscs range from 4.00 to 86.6 mg L⁻¹, (Minakshi and Mahajan, 2012; Mukadam and Kulkarni, 2014; Prosser et al., 2016). For both tests, CAL exposures lead to diminished of byssus production. *Mytilus* attaches to solid substrate with the help of byssus threads produced by their foot. Byssal attachment requires opening the bivalve shell and extension of the muscular foot and this lead automatically expose the soft tissues of the mussels to any toxicants present in the water (Rajagopal et al., 2005). Byssus producing requires a large energy expenditure and mussels can close their shells, inherently preventing it from filter-feeding and taking in energy. Shells closure can also be followed by a decrease in the mussel's metabolism that helps the mussel to cope with the unfavourable conditions (Roberts, 1975; Ayad et al., 2011; Burnett and Sara, 2019). This suggests that the lower or stopped production of byssus might be a consequence of the amount of energy reserves in the mussel and that the inability to produce byssal attachments would affect the survival of mussels exposed to CAL. Inhibition capacity to produce byssus threads in *M. galloprovincialis* were observed by study Ayad et al. (2011) after exposure to cypermethrin at 100 µg L⁻¹, and Karagiannis et al. (2011) after exposure to atrazine at 5 mg L⁻¹. Alteration in byssal threads production is considered a good physiological biomarker of general stress conditions in mussels

Table 3
Biochemical characteristics of haemolymph of *Mytilus galloprovincialis* following exposure to Calypso 480 SC.

Indices	Exposure time (days)	Test groups		
		Control (0 mg L ⁻¹)	CAL1 (7.77 mg L ⁻¹)	CAL2 (77.70 mg L ⁻¹)
Cl ⁻ (mmol L ⁻¹)	10	464.10 ± 1.55 ^a	449.53 ± 6.64 ^{ab}	443.75 ± 4.50 ^b
	20	482.57 ± 4.62 ^{a*}	500.03 ± 13.23 ^{a*}	477.85 ± 4.24 ^{a*}
	R 10	458.00 ± 1.08 ^a	450.95 ± 2.21 ^a	445.82 ± 7.34 ^a
K ⁺ (mmol L ⁻¹)	10	12.15 ± 0.02 ^a	12.53 ± 0.21 ^a	12.40 ± 0.27 ^a
	20	12.83 ± 0.08 ^a	12.70 ± 0.08 ^a	12.70 ± 0.08 ^a
	R 10	11.60 ± 0.08 ^{a*}	11.35 ± 0.02 ^{a*}	12.35 ± 0.15 ^b
Na ⁺ (mmol L ⁻¹)	10	566.33 ± 5.06 ^a	537.50 ± 6.90 ^b	532.83 ± 5.82 ^b
	20	554.50 ± 5.98 ^a	556.33 ± 7.72 ^a	536.33 ± 2.59 ^a
	R 10	549.83 ± 5.00 ^a	510.83 ± 2.93 ^{b*}	505.60 ± 5.70 ^{b*}
Ca ²⁺ (mg dL ⁻¹)	10	54.65 ± 0.37 ^a	53.48 ± 0.61 ^a	53.10 ± 0.68 ^a
	20	56.47 ± 1.52 ^a	54.20 ± 1.68 ^a	54.83 ± 0.79 ^a
	R 10	50.77 ± 0.08 ^{a*}	50.30 ± 0.15 ^{a*}	43.28 ± 5.77 ^{a*}
PHOS (mg dL ⁻¹)	10	0.80 ± 0.04 ^a	0.80 ± 0.04 ^a	0.55 ± 0.11 ^a
	20	0.80 ± 0.04 ^a	0.80 ± 0.06 ^a	0.67 ± 0.04 ^a
	R 10	0.85 ± 0.02 ^a	0.80 ± 0.00 ^a	0.83 ± 0.24 ^a
Mg ²⁺ (mg dL ⁻¹)	10	24.88 ± 0.20 ^a	25.07 ± 0.11 ^a	25.03 ± 0.07 ^a
	20	25.25 ± 0.06 ^a	24.88 ± 0.15 ^a	24.83 ± 0.10 ^a
	R 10	24.95 ± 0.02 ^a	24.65 ± 0.09 ^a	24.62 ± 0.31 ^a
LDH (U L ⁻¹)	10	2.50 ± 0.34 ^a	2.33 ± 0.21 ^a	1.67 ± 0.21 ^a
	20	2.17 ± 0.48 ^a	1.50 ± 0.22 ^a	1.67 ± 0.21 ^a
	R 10	2.50 ± 0.56 ^a	2.67 ± 0.49 ^a	3.33 ± 0.21 ^{a*}
GLU (mg dL ⁻¹)	10	0.35 ± 0.04 ^a	0.35 ± 0.02 ^a	0.25 ± 0.06 ^a
	20	0.33 ± 0.06 ^a	0.28 ± 0.07 ^a	0.28 ± 0.04 ^a
	R 10	0.47 ± 0.03 ^a	0.15 ± 0.02 ^{b*}	0.10 ± 0.03 ^{b*}

Data are mean ± SE, n = 6. Data sharing at the same superscript letter indicate no significant differences ($P < 0.01$) between groups value during the same experiment time (e.g. 10, 20 and R 10 days, respectively). *Denotes significant differences between groups ($P < 0.01$). Phosphor inorganic (PHOS), Lactate dehydrogenase (LDH), glucose (GLU), CAL = Calypso 480 SC, CAL1 = 7.77 mg L⁻¹, CAL2 = 77.70 mg L⁻¹, R 10 = 10 days recovery period in CAL-free water.

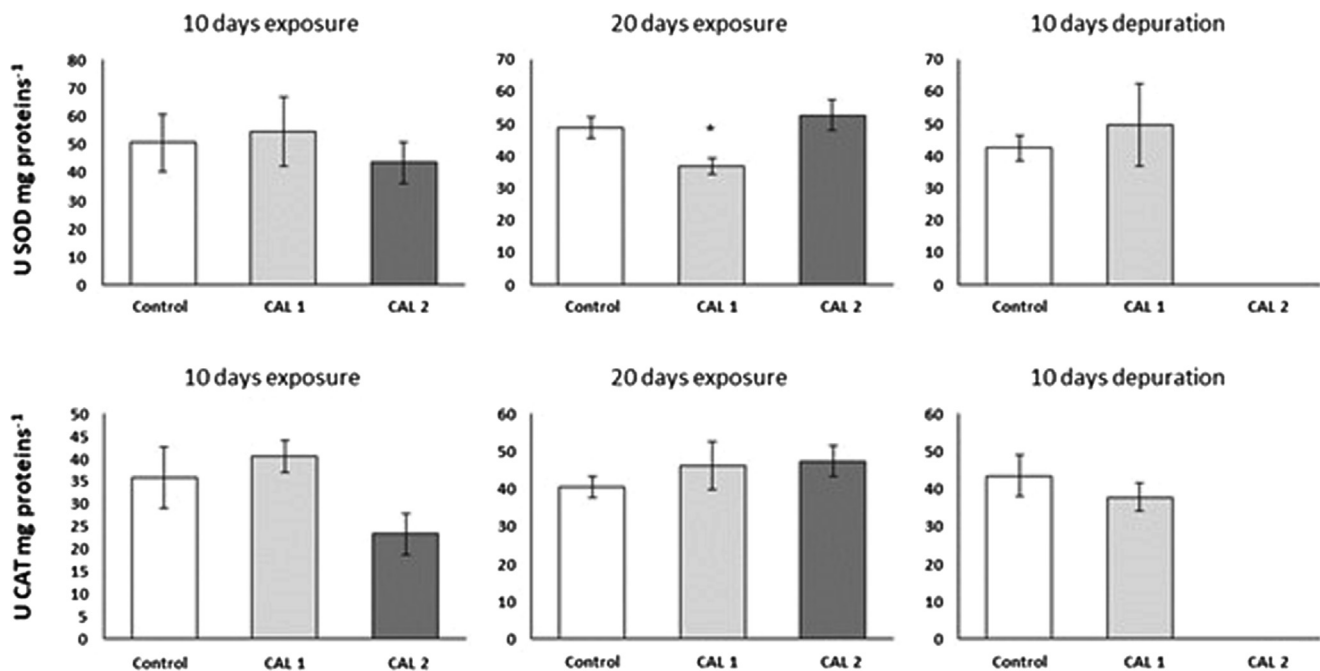


Fig. 2. Enzyme activity in digestive gland of *Mytilus galloprovincialis* following exposure to Calypso 480 SC. Data are mean ± SE, n = 6. *Denotes indicate significant differences ($P \leq 0.01$) among groups at the same sample time (Mann-Whitney *U* Test). Superoxide dismutase (SOD), catalase (CAT), CAL = Calypso 480 SC, CAL1 = 7.77 mg L⁻¹, CAL2 = 77.70 mg L⁻¹, R 10 = 10 days recovery period in CAL-free water.

(Roberts, 1975; Ayad et al., 2011; Rajagopal et al., 2005; Burnett and Sara, 2019).

The changes in the health status of the mussels was highlighted in haemocytes and digestive cells through the TB exclusion and NR assays, that allow an effective measurement of the integrated

physiological function (Patetsini et al., 2013; Faggio et al., 2016; Matozzo et al., 2016; Pagano et al., 2017; Rizzo et al., 2017; Moore et al., 2018). In mussels the haemocytes have a primary role in the innate immune defence. These cells are also activated by many pollutants and participate directly by eliminating the patho-

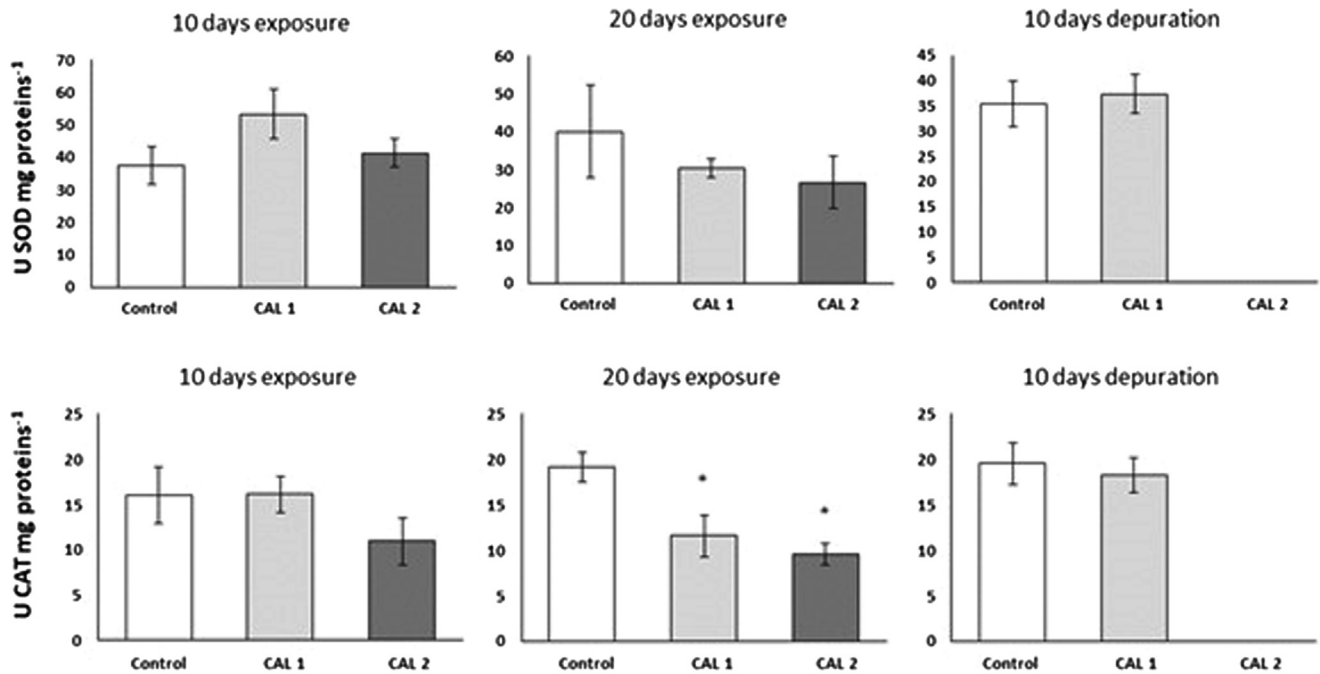


Fig. 3. Enzyme activity in gill of *Mytilus galloprovincialis* following exposure to Calypso 480 SC. Data are mean \pm SE, $n = 6$. *Denotes indicate significant differences ($P \leq 0.01$) among groups at the same sample time (Mann-Whitney U Test). Superoxide dismutase (SOD), catalase (CAT), Control = 0 mg L^{-1} , CAL = Calypso 480 SC, CAL1 = 7.77 mg L^{-1} , CAL2 = 77.70 mg L^{-1} .

gen through phagocytosis and producing lysosomal enzymes and antimicrobial molecules that help to destroy and eliminate the pathogen (Renault et al., 2011; Sureda et al., 2013). Our experiments show that CAL is harmful to the haemocytes after longer exposure (20 days). Prolonged exposure to both concentrations caused an overload in lysosomal storage, which has led to low membrane stability, which is an obvious reaction to toxic damage. The result of evident toxicity was confirmed by the TB exclusion test of cell viability. The digestive gland is responsible for digestion and metabolism in *M. galloprovincialis*. Because of this function, substances accumulate in this organ. Damage to this organ has allowed us to evaluate the toxicity of many substances (Regoli et al., 2004; Torre et al., 2013; Messina et al., 2014; Pagano et al., 2016; Faggio et al., 2018; Mezzelani et al., 2018). In our experiments, the cells of the digestive gland already have a high mortality rate in both concentrations of CAL after a short exposure. The interaction of the cells with the pesticide is demonstrated not only by the lysosomal accumulation and by the consequent lysosomal instability, but as the figures show, also by the impossibility of the cells to recover their volume. This suggests that cellular damage occurred at the membrane level, as evidenced by the TB exclusion test and at the cytoskeletal and channel protein level, as demonstrated by the RVD assay. The regulation of cell volume for digestive cells is necessary to maintain organ functions. In fact, under controlled conditions, the cells are able to respond to osmolar variations. The presence of pesticides probably inhibits the cellular structures that regulate the osmotic variations to which animals is exposed. In fact, the cells after 10 days of exposure immediately after washing with the hypoosmotic solution swell for a passive movement of the water, but due to the damage present in the channels they are unable to return to the initial volume. After 20 days of exposure, the cells exposed to the maximum concentration of CAL, after washing with hypoosmotic solution, swell little and slowly and do not adjust their volume at the end of the experiment. This suggests that damage is related to the transports of K^+ and Cl^- ions and the cytoskeletal components that normally

is involving in the processes of regulation of cell volume (Torre et al., 2013; Lungu-Mitea et al., 2018).

Mussels as osmoconformer organisms react to pollutants by variations in adaptation processes, one of them being regulation of the haemolymph minerals ions concentration (Cl^- , Na^+ , K^+ , Ca^{2+} , Mg^{2+}) (Capillo et al., 2018) and biochemical parameters (LDH, GLU) (Gustafson et al., 2005; Faggio et al., 2016). Exposure to both CAL concentrations induced a significant decreased in Cl^- and Na^+ ions, moreover further reduction was also observed in K^+ , Na^+ , Ca^{2+} and GLU after 10 days depuration period, in CAL-free water. Furthermore, at the high concentration of CAL, LDH show an increase values after depuration period. Glucose is the main source of energy and it's indicates failure of digestive gland function and sudden glycogen depletion (Sanchez-Paz et al., 2007). Lactate dehydrogenase is a ubiquitous cytoplasmic enzyme important in the differential diagnosis of digestive gland damage and it is released from the cells with damage to the cell membranes. An increase in LDH activity indicates acute failure, dystrophy, and toxic damage of digestive gland (Matozzo et al., 2012; Faggio et al., 2016; Pagano et al., 2016). Haemolymph minerals ions are essential components of many systems, including skeleton, enzymatic activity, muscle metabolism, osmoregulation etc. (Gustafson et al., 2005). Changes in K^+ and Cl^- levels could support the results obtained in RVD test. These findings correspond to our results identified in the other parameters and confirms that CAL at sublethal doses has a negative impact on mussels. Changes in haematology indices of mussels caused by exposure to xenobiotics have been described in several studies (Matozzo et al., 2012; Faggio et al., 2016; Pagano et al., 2017; Milan et al., 2018). These studies establish that haemolymph chemistry parameters are affected by conditions that can compromise the health of aquatic invertebrates. Determination of haemolymph biochemical profile is a good bioindicator for assess levels of damage to *M. galloprovincialis* by pollution as well as blood samples routinely used in disease surveillance and diagnosis in mammals, at times providing the first indication of abnormalities (Gustafson et al., 2005).

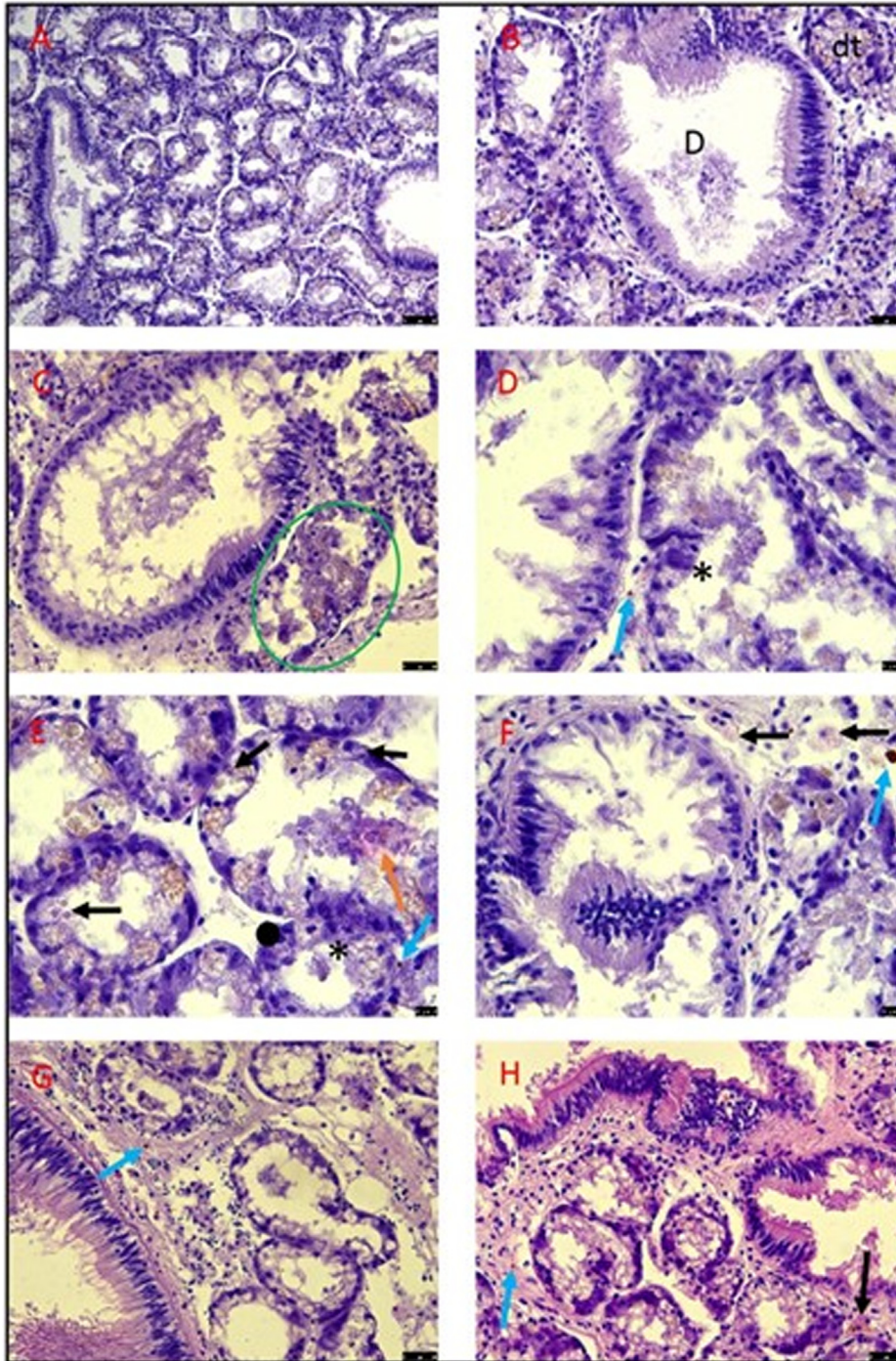


Fig. 4. Representative figures of mussels' digestive gland from *Mytilus galloprovincialis* following exposure to Calypso 480 SC. (A, B) Control with typical structure (Scale bars: 50 μm , 25 μm respectively); (C, D) CAL1 for 10 days (Scale bars: 25 μm , 10 μm respectively); (E, F) CAL1 for 20 days (Scale bars: 10 μm), (G, H) CAL1 after 10 days recovery period in CAL-free water (Scale bars: 25 μm). Green circles indicate digestive tubule alterations, blue arrows indicate brown cells, orange arrows indicate granulocytoma, black arrows indicate haemocytosis, black filled circles indicate hyperplasia, asterisks indicate hypertrophy, and arrowheads indicate lipofuscin aggregates. CAL1 = 7.77 mg L^{-1} of Calypso 480 SC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

How extensively reported, exposure to pesticides can lead to imbalance between the generation of reactive oxygen species (ROS) and antioxidant defences (Lushchak, 2011). Enzymes CAT and SOD play the first important defensive line against overproduction of ROS during the biotransformation of pollutants (Freitas et al., 2019; Lushchak, 2016; Sillero-Rios et al., 2018; Vieira et al., 2018). The SOD enzyme plays an important role in oxidative defence by catalyzing the dismutation of superoxide radicals to

oxygen and hydrogen peroxide. Catalase protecting the cell from hydrogen peroxide by converting them into oxygen and water (Lushchak, 2016; Bhagat et al., 2016). In *M. galloprovincialis* exposed to CAL for 20 days, a decreased SOD activity in digestive gland at CAL1 and the inhibition of CAT activity in gill at both CAL concentrations were detected. Thus, the significant decrease of enzymes activity might have resulted from their inactivation by the superoxide radical triggered by CAL exposures. Moreover,

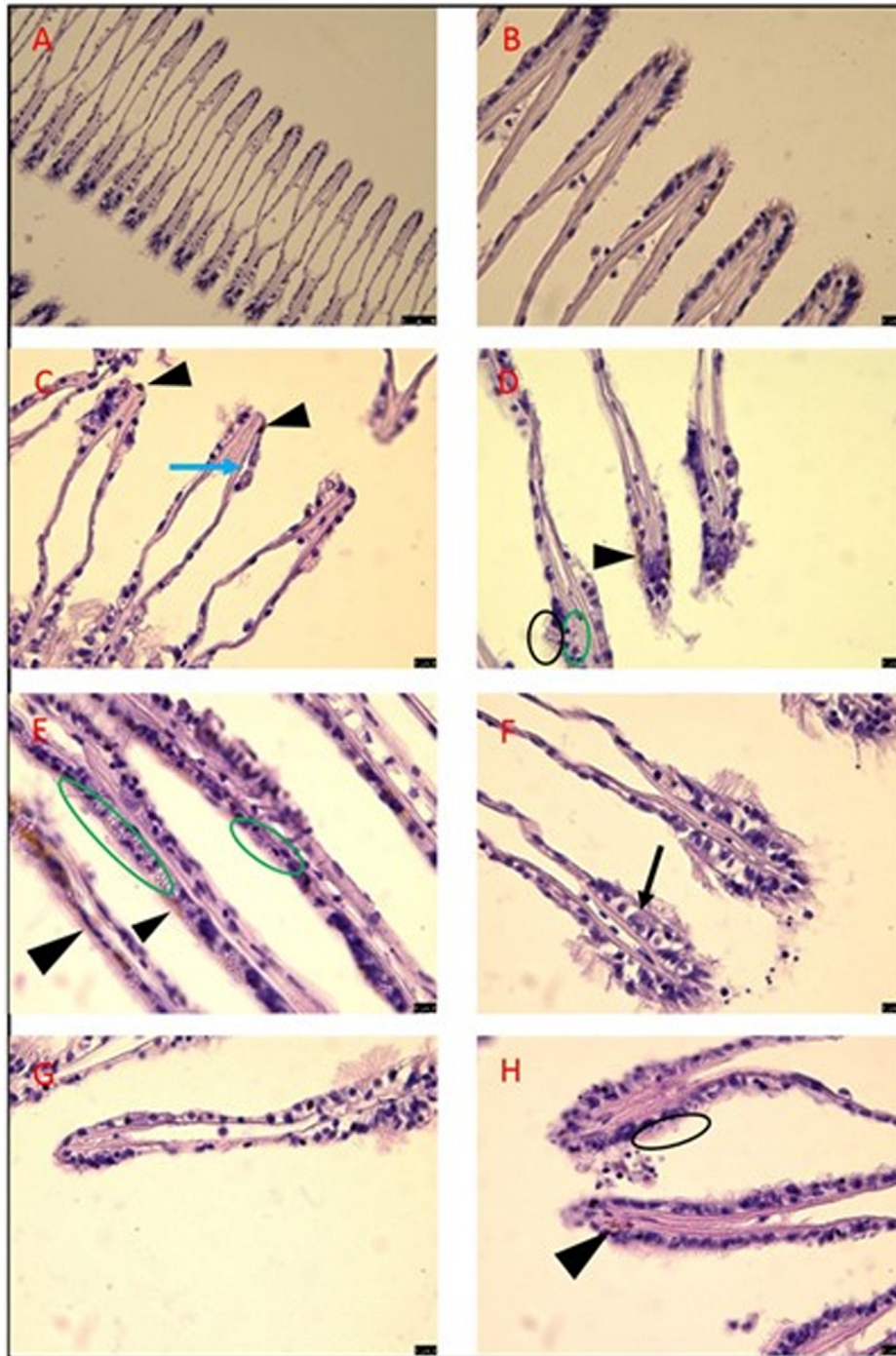


Fig. 5. Representative figures of mussels' gill of *Mytilus galloprovincialis* following exposure to Calypso 480 SC. (A, B) Control with typical structure (Scale bars: 50 μm , 10 μm respectively); (C, D) CAL1 for 10 days (Scale bars: 10 μm); (E, F) CAL1 for 20 days (Scale bars: 10 μm), (G, H) CAL1 after 10 days recovery period in CAL-free water (Scale bars: 10 μm). Green circles indicate vacuolation, black circles indicate mucous overproduction, blue arrows indicate epithelial alterations, black arrows indicate haemocytes infiltrations, asterisks indicate hypertrophy, and arrowheads indicate lipofuscin aggregates. CAL1 = 7.77 mg L^{-1} of Calypso 480 SC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

results obtained after 10 days depuration phase in CAL-free water demonstrated reversible balance of enzyme activities of digestive gland and gill. General, that toxic radicals inactivate the binding of antioxidant enzymes. How it has been shown in previous studies (Shukla et al., 2007; Yan et al., 2015; Velisek and Stara, 2018; Vieira et al., 2018; Stara et al., 2019), neonicotinoids lead to damage to the antioxidant balance in aquatic organisms, suggesting a high production of ROS, which triggers a state of oxidative stress. Similarly, the changes in activity of antioxidant enzymes were found in

M. galloprovincialis exposed to different pollutions (Faggio et al., 2016; Milan et al., 2018; Munari et al., 2018; Freitas et al., 2019) but not after exposure to neonicotinoids.

The digestive gland of bivalve molluscs, is involved in metabolic detoxification and/or bioaccumulation of xenobiotics (Stegeman, 1985; Cajaraville et al., 1990; Metian et al., 2009), and reflects exposure to environmental stressors (Costa et al., 2013). Concerning the histopathological evaluations of the exposed mussels to the toxicant the first evidence of tissue alteration was given by the

massive deposit of melanin. This condition, known as melanosis, highlight the inflammation processes resulting from the exposure to CAL. Melanosis, in molluscs, is the results of a cascade of events if the “pro-phenoloxidase (PO) activating systems”; this system is involved in mechanisms of recognition, cytotoxicity and encapsulation of foreign bodies (De Vico and Carella, 2012; Pagano et al., 2016). Other histopathological lesions as digestive tubule alterations and intertubular tissue alteration revealed the triggering of pathological events of mussels to the toxicant. It has been demonstrated, in fact, that regression of digestive tubule could be an indicator of a “reconstituting phase” as an attempt to restore the detoxification potential of digestive gland (Robinson and Langton, 1980). In some cases, focal points of tubular necrosis were present demonstrating the heavy toxic effect of CAL (Auffret, 1988). Moreover, presence of brown cells jointly with granulocytoma, haemocytosis infiltrations, hyperplasia and hypertrophy, confirmed the inflammatory condition of treated molluscs (De Vico and Carella, 2012). In particular, brown cells presence can confirm the aetiology of pathological lesions; these cells are, in fact, known to participate in the accumulation and detoxification of pollutants (Usheva and Frolova, 2006; De Vico and Carella, 2012).

For what in concerning the gills, these represent the first interface with the surrounding water of bivalve, and then are structure that primarily suffers the environmental stressors (Costa et al., 2013). As for digestive gland, deposits of lipofuscin/melanin and presence of brown cells leaved no doubts about the inflammatory reactions also in gill tissues (Carella et al., 2018). Intense vacuolation and relative mucous overproduction indicate an alteration of goblet cells functionality confirming the reaction of the tissue to inflammation as already demonstrated by El-Shenawy et al. (2007). These features could lead to the hypothesis that mussels exposed to CAL react with a particular defence mechanism known as Multixenobiotic defence mechanism (MXDM) (Pain-Devin and Parant, 2003). The MXDM can protect cells from toxic compounds by limiting entry and in facilitating efflux of these compounds (Pagano et al., 2016).

All the inflammatory processes occurred in the gills and digestive glands led to a resistance performance by the exposed bivalves that allowed a slight recover to lesser pathological condition after the non-exposure period (last 10 days of experiment).

5. Conclusions

Here, we described the acute and sub-chronic toxic effects of the neonicotinoid insecticide Calypso 480 SC on *M. galloprovincialis*. Although CAL is seemed that acute dose is too high (96 h-LC50 = 7.77 g L⁻¹) on *M. galloprovincialis*, the sub-lethal doses (0.1 and 1% 96 h-LC50) of CAL during sub-chronic test caused negative effects (from cell damage to histopathological changes etc.) on mussels. This study provides new information to support the growing evidence that this product is not safe on the aquatic ecosystem. Thiocloprid is still widespread use at present. Therefore, it is necessary to carry out other similar studies to evaluate better the risk posed to non-target organisms. However, it is necessary to highlight that mussels affected by insecticide recovered their physiological state after few days in insecticide-free water.

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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