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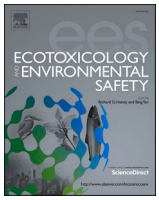

Acute effects of neonicotinoid insecticides on *Mytilus galloprovincialis*: A case study with the active compound thiacloprid and the commercial formulation calypso 480 SC

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ABSTRACT

Pesticides can enter aquatic environments potentially affecting non-target organisms. Unfortunately, the effects of such substances are still poorly understood. This study investigated the effects of the active neonicotinoid substance thiacloprid (TH) and the commercial product Calypso 480 SC (CA) (active compound 40.4% TH) on *Mytilus galloprovincialis* after short-term exposure to sublethal concentrations. Mussels were tested for seven days to 0, 1, 5 and 10 mg L^{-1} TH and 0, 10, 50 and 100 mg L^{-1} CA. For this purpose, several parameters, such as cell viability of haemocytes and digestive cells, biochemical haemolymph features, superoxide dismutase (SOD) and catalase (CAT) enzymatic activity of gills and digestive gland, as well as histology of such tissues were analysed. The sublethal concentrations of both substances lead to abatement or completely stopping the byssal fibres creation. Biochemical analysis of haemolymph showed significant changes ($P < 0.01$) in electrolytes ions (Cl^-, K^+, Na^+, Ca^{2+}, S-phosphor), lactate dehydrogenase (LDH) enzyme activity and glucose concentration following exposure to both substances. The TH-exposed mussels showed significant imbalance ($P < 0.05$) in CAT activity in digestive gland and gills. CA caused significant decrease ($P < 0.05$) in SOD activity in gills and in CAT activity in both tissues. Results of histological analyses showed severe damage in both digestive gland and gills in a time- and concentration-dependent manner. This study provides useful information about the acute toxicity of a neonicotinoid compound and a commercial insecticide on mussels. Nevertheless, considering that neonicotinoids are still widely used and that mussels are very important species for marine environment and human consumption, further researches are needed to better comprehend the potential risk posed by such compounds to aquatic non-target species.

1. Introduction

Wide range of chemicals pollutants from agriculture, industry, households etc. Get into the surface waters through various routes posing a potential risk for aquatic species (Morrissey et al., 2015; Inyinbor et al., 2018). In our study, we focused on neonicotinoid substances that are widely used insecticides in crop production and veterinary medicine since their introduction in the 1990s (Valavanidis, 2018; Craddock et al., 2019). Neonicotinoids popularity is mainly due to their relatively low risk for vertebrates and high-target specificity to invertebrates, especially insects (Fiorenza et al., 2020). Their toxicity is primarily due to considerable structural differences between vertebrate and insect nicotinic acetylcholine receptors (nAChR). The neonicotinoids have a selective effect on the invertebrates nAChR, at first stimulating postsynaptic receptors, growing Na^+ ingress and K^+ egress and subsequently paralyzing nerve conduction, leading to rapid death (Page, 2008; Crossthwaite et al., 2017). Despite their advantages, starting from 2013 the European Commission blocked the usage of 3

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neonicotinoids (imidacloprid, clothianidin, thiamethoxam) in flowering crops that appeal to honeybees and other pollinators. On April 27, 2018 the EU expanded a controversial ban on these three active substances of insecticides on all crops grown outdoors because of their unfavourable effects on pollinators (Valavanidis, 2018). Thiacloprid ([3-(6-Chloro-3-pyridinylmethyl)-2-thiazolidinylidene]cyanamide) is closely related to imidacloprid both in structure and mode of action, and it is still one of the most commonly used neonicotinoid active substance nowadays (Kammoun et al., 2019). Thiacloprid exhibits high water solubility (184 mg L^{-1}) (Thompson et al., 2020) and it is the active substance of several insecticide products, such as Biscaya, Calypso, Proteus etc., to control a variety of chewing and sucking insects (EPA, 2003). Neonicotinoids are relatively stable in the environment. Thiacloprid shows medium persistence in the anaerobic soil and aquatic environment, with a degradation half-life of 3.4–1000 days according to environment conditions (Thompson et al., 2020). The highest measured concentration of thiacloprid in the water was 4.50 μg L^{-1} in the Elbe river basin (Suβ et al., 2006). However, for other neonicotinoids, higher concentrations up to 320 μg L^{-1} for imidacloprid (Van Dijk et al., 2013) and 225 μg L^{-1} for acetamiprid (Anderson et al., 2015) have been reported. Due to their properties, high concentrations of neonicotinoids can be expected in rainfall owing to runoff from agriculture treated fields (Velisek and Stara, 2018).

Studies on toxicity of thiacloprid are mainly focused on insects, especially pollinators (Iwasa et al., 2004; Morrissey et al., 2015; Brandt et al., 2016; Raby et al., 2018). However, new studies about the harmful effects of thiacloprid on macroinvertebrates and vertebrates increased in the last few years (Sekeroglu et al., 2014; Hendawi et al., 2016; Salvaggio et al., 2018; Velisek and Stara, 2018; Stara et al., 2019). European Commission adopted a draft rule that would add thiacloprid to the list of neonicotinoids to be avoided in agriculture, mainly for its risk as endocrine disruptors in mammals. However, the approval for thiacloprid is expired on April 30, 2020 (EFSA, 2019).

Mytilus galloprovincialis is a very important shellfish species cultured in marine temperate intertidal zones and brackish waters of Europe and is a major food resource for humans (Beyer et al., 2017; FAO, 2019; Figueras et al., 2019; Freitas et al., 2019). Given that mussels continually filter a large volume of seawater, they may be the most seriously affected organisms by various pollutants, such as pesticides (Ayad et al., 2011; Karagiannis et al., 2011; Chmist et al., 2019; Peric and Buric, 2019; Stara et al., 2020), pharmaceuticals (Falfushynska et al., 2019; Freitas et al., 2020a, 2020b, 2020c; Sehonova et al., 2018), nanoparticles (Huang et al., 2018), and UV filters (Vidal-Linan et al., 2018). They are befitting and widely used for monitoring the biological effects of environmental stressors by means of molecular and cellular biomarkers (Bocchetti and Regoli, 2006; Beyer et al., 2017; Burgos-Aceves and Faggio, 2017; Nardi et al., 2017; Figueras et al., 2019; Rahman et al., 2019). In this study, we used mussel specimens from a small brackish meromictic lagoon "Faro Lake", locally called *Pantano Piccolo*, located on the Sicilian coast of Italy (Bottari et al., 2005; Capillo et al., 2018a, 2018b; Fazio et al., 2015).

Given the widespread use and the evidence of the toxic effects of thiacloprid on non-target organisms and little information about its effects to non-target macro-invertebrates, we have focused our study on this issue. The main objective of this study was to assess the acute effects of sub-lethal doses of the neonicotinoid active substance thiacloprid and the insecticide Calypso 480 SC on *M. galloprovincialis*. We hypothesised that these compounds can have a negative effect on i) cell viability of both haemolymph and digestive gland cells, ii) biochemical parameters of haemolymph, iii) activity of antioxidant biomarkers and iv) histopathology of the gills and digestive glands of mussels. Results of the present study are expected to provide new insights on the effects of mostly used neonicotinoids, focusing on the different sensitivity of the tissue analysed and on the main cellular pathways responsible of neonicotinoids detoxification and cellular homeostasis. Overall, the results obtained may allow a better understanding of *M. galloprovincialis* responsiveness towards neonicotinoids.

2. Materials and methods

2.1. Test organisms and chemicals

Mytilus galloprovincialis was supplied by a mollusc breed cooperative farm Company FARAU SRL (Frutti di Mare) Messina, Italy (Spinelli et al., 2018) from Faro Lake. Faro Lake is the deepest meromictic coastal basin in Italy reaching ~28 m depth in the central part, while it does depth from 0.50 m to 5 m around the coastal areas. Mean physical and chemical parameters of seawater are: temperature 20.3 ± 5.8 °C, pH 8.5 ± 0.3 and salinity 33.0 ± 3.5 during year (Capillo et al., 2018a c). Due to its properties, Faro Lake is a very productive environment, especially for aquaculture productions of mussels, clams or oysters, and concurrently it is part of the Naturel Oriented Reserve of Capo Peloro (Manganaro et al., 2011; Capillo et al., 2018a c). Mussels were transported to the laboratory, and acclimated for 5 days in large tanks contained continuously aerated natural brackish water with a 12 L:12D light cycle and temperature of 19.45 ± 0.98 °C.

Technical-grade thiacloprid (TH; purity 99.9%, Sigma Aldrich, Czech Republic) and pesticide product Calypso 480 SC (CA; contains active substance 40.4% thiacloprid; Bayern Crop Science Corporation) were dissolved in distilled water to obtain stock solutions.

2.2. Acute test conditions

Mussels, 6.85 ± 0.57 cm shell length, were randomly selected and placed into sixteen tanks each containing 20 L continuously aerated brackish water. Sixteen mussels per duplicated tank (n = 32) were exposed for seven days to concentrations of thiacloprid: 1 mg L^{-1} (TH1), 5 mg L^{-1} (TH2) and 10 mg L^{-1} (TH3); and Calypso 480 SC: 10 mg L^{-1} (CA1), 50 mg L^{-1} (CA2) and 100 mg L^{-1} (CA3). Test concentrations were selected with respect to the active substance thiacloprid content of the Calypso 480 SC and based on data from the literature (Dondero et al., 2010; Stara et al., 2020). The authors describe the effect these chemicals at these concentrations on *M. galloprovincialis* but in while monitoring another kind of indicators. Control groups without tested substances (CTH, CCA) were also prepared. Natural seawater and exposure concentrations have been restored every 2 days to provide natural brackish food and preclude starvation during the exposure. Behavioural changes and mortality of the mussels, as well as water quality (temperature 18.17 ± 0.80 °C, pH 7.65 ± 0.13, salinity 34.31 ± 0.53, 1100 m Osm kg^{-1}), were measured daily using a multiparametric probe as reported by Sanfilippo et al. (2016). Water samples were taken during the trial period (at time 0, after 24 and 48 h) for determination of actual concentrations of TH. The TH concentrations were analysed by liquid chromatography mass spectrometry (LC-MS) (Ying and Kookana, 2004) and the mean concentration of TH in the tanks was always within ±5% of the nominal concentration. Haemolymph and tissue (digestive gland, gills) samples were collected after 3 and 7 days of exposures of mussels.

2.3. Haemolymph collection

Pools of haemolymph (each pool consists of 3 animals) from each experimental condition were prepared. The pool was needed to obtain enough volumes for analysis. The haemolymph was collected from the anterior adductor muscle with a 23-gauge needle of a 1 ml plastic syringe. Once collected, it was placed in tubes and immediately centrifuged at 1000 rpm for 10 min (Pagano et al., 2017).

2.4. Isolation of digestive cells

The digestive glands of four mussels from each group were isolated to obtain digestive cells according to Pagano et al. (2016).

2.5. Cell viability assays

To evaluate cell damage, the cytotoxicity test was performed on both haemocytes and digestive gland cells. Cell viability was assessed by two assays: Trypan Blue (TB) exclusion test and lysosomal membrane stability by neutral red (NR), as described in Stara et al. (2020).

2.6. Haemolymph parameters

Eight pooled haemolymph samples (each pool containing three animals) for each group were centrifuged at 1000 rpm for 10 min at 4 °C and supernatant samples were stored at −80 °C. Haemolymph electrolytes composition (chloride, potassium, sodium, calcium, phosphorus, magnesium), lactate dehydrogenase (LDH) activity and glucose (GLU) levels were measured by using a multiparametric analyser (KONELAB 60 THERMO, Milano, Italy).

2.7. Determination of enzyme activity

Digestive gland and gill tissues of six mussels from each group were excised and frozen on −80 °C until analyses for determination of enzymatic activities (SOD and CAT). The tissues were individually homogenized on ice in 1 ml of 0.1 mM Tris-HCl buffer (pH 7, 0.15 M KCl, 0.5 M Sucrose, 1 mM EDTA, 1 mM Dithiothreitol and 40 μg L^{-1} Aprotinin) per 1 g tissue for 1 min with an Ultra-Turrax homogeniser (model T8 basic, IKA). Homogenates were then centrifuged at 12,000 rpm for 30 min at 4 °C and supernatants were collected and used for enzymatic analyses. The SOD (EC 1.15.1.1) activity was measured using the xanthine oxidase/cytochrome *c* method according by Crapo et al. (1978). The CAT (EC 1.11.1.6) activity was made by to the method described in Aebi (1984). Both enzyme activities were expressed as U per mg protein. Concentration of protein in supernatant were measured according to Bradford (1976) using bovine serum albumin as the standard.

2.8. Histology

Histopathological conditions of gills and digestive glands were assessed taking fractions of tissues from each treatment (see previous sections for detailed information). Two specimens from each exposure conditions were collected. After sampling, immersion of collected samples were performed in Immunofix (paraformaldheyde 4% in phosphate saline buffer, Bio-Optica, Milan, Italy) for 8 h at room temperature. After embedded in paraffin, tissue were sectioned by a microtome (Leica, RM2235). Five μm sections were stained using Hematoxylin and Eosin stain for a qualitative histopathological examination using a light microscope (Leitz Diaplan, Germany). Detailed procedures are reported in Pagano et al. (2016), Lauriano et al. (2019) and Zaccone et al. (2015).

2.8.1. Histopathological condition indices

Semi-quantitative weighted indices approach described by Bernet et al. (1999) for fish, with some modifications proposed by Costa et al. (2013) have been applied for the evaluation of each individual histopathological indices (I_h). Each alteration was assigned a weight (biological significance) with value ranging between 1 and 3 (maximum severity), and a score (degree of dissemination) with values between 0 (in the case that alteration is not present) and 6 (in the case alteration is diffuse). The formula for the assesment of histopathological condition indices is:

$$I_h = \frac{\sum_1^j w_j a_{jh}}{\sum_1^j M_j}$$

where I_h is the histopathological condition index for the individual *h*; w_j the weight of the *j*th histopathological alteration; a_{jh} the score attributed to the *h*th individual for the *j*th alteration and M_j is the maximum attributable value for the *j*th alteration, *i.e.*, weight × maximum score. The equation's denominator normalizes I_h to a value between 0 and 1, this is very useful for relating different situations (ie. different organs). The indices have been generally evaluated for each organ (gills and digestive gland) and subsequently related to "reaction patterns": morphological epithelial modifications (gills), tubule and intertubular tissue alterations for digestive gland. The condition weights used have been based on observations collected in this experiment and partially on literature about both invertebrate Costa et al. (2013) and vertebrate histopathology. In accordance, weight assigned are shown in Table S1.

2.9. Statistical analysis

In cell viability assays, Student's t-test was used to evaluate results obtained. The value of $P < 0.05$ was accepted as significant. The software package Prism 5.00 (Graphpad Software Ldt., La Jolla, CA 92037, USA) was used for statistical analyses. Biomarker data of haemolymph and enzymatic activities were analysed using the STATISTICA 13.4 software package (TIBCO). Significant differences between control and treatment groups for each tissue and time were analysed using the non-parametric Mann–Whitney *U* test. The data of I_h were analysed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. A P-value of less than 0.05 was considered significant.

3. Results

3.1. Conditions of the test mussels during experiments

Behaviour of animals was normal in both controls and the lowest tested concentrations of TH (1 mg L^{-1}) and CA (10 mg L^{-1}) during the experiment. Mussels produced byssal fibres that allowed a secure attachment to the bottom of tanks or with each other mussel. Concentrations of 5 and 10 mg L^{-1} of TH and 50 and 100 mg L^{-1} of CA caused a reduction in byssus fibre production in mussels, inducing detachment of mussels from the bottom and no attach to each other already after 3 days. Only one mussel died at 100 mg L^{-1} CA at the end of the experiment.

3.2. Cell viability assays

Both cell types (haemocytes and digestive gland cells) showed stability and normal parameters. No significant loss in haemocyte viability was observed at all the concentrations tested of TH and CA (Table 1). In the same way, no damage to the digestive cells was recorded (Table 1).

3.3. Haemolymph evaluation

Mussels exposed to TH showed significant differences ($P < 0.01$) in haemolymph composition during the exposure period (Table 2). Concentration of TH1 (1 mg L^{-1}) caused a significant increase ($P < 0.01$) in levels of Mg^{2+} after 3 days of exposure and glucose during the experiment, compared with control (CTH). The mussels exposed to TH2 (5 mg L^{-1}) demonstrated a significant decrease ($P < 0.01$) of haemolymph electrolytes (Cl^-, K^+, Ca^{2+}) after 3 days exposure, S-phosphor after 7 days exposure, and a significant increase in levels of Mg^{2+} and glucose after 3 days exposure, compared with control (CTH). Concentration of TH3 (10 mg L^{-1}) caused a significant decrease ($P < 0.01$) of haemolymph electrolytes Cl^-, Na^+, Ca^{2+}) after 3 days exposure and S-phosphor after 7 days of exposure, together with a significant increased LDH activity after 3 day and glucose levels during the experiment compared with controls (CTH).

Results of haemolymph parameters of mussels exposed to CA are listed in Table 2. Concentrations of CA (10, 50 and 100 mg L^{-1}) led to significant decrease ($P < 0.01$) of S-phosphor level compared to their control (CCA) during the experiment. The mussels exposed to CA1 (10 mg L^{-1}) showed a significant decrease ($P < 0.01$) of levels of electrolytes

Table 1
Percentage of viable digestive gland cells and haemocytes in *Mytilus galloprovincialis* exposed to thiacloprod (TH) and Calypso 480 SC (CA).

Digestive gland		Treatment and dose							
Cell viability test	Time (days)	CTH (0 mg L^{-1})	TH1 (1 mg L^{-1})	TH2 (5 mg L^{-1})	TH3 (10 mg L^{-1})	CCA (0 mg L^{-1})	CA1 (10 mg L^{-1})	CA2 (50 mg L^{-1})	CA3 (100 mg L^{-1})
TB (%)	3	100.00 ± 0.00	99.43 ± 0.33	97.74 ± 0.08	97.86 ± 0.55	100.00 ± 0.00	79.63 ± 0.00	97.64 ± 0.00	100.00 ± 0.00
	7	100.00 ± 0.00	99.55 ± 0.45	94.34 ± 0.23	93.17 ± 0.73	100.00 ± 0.00	100.00 ± 0.00	96.72 ± 0.00	96.99 ± 0.00
NR (%)	3	100.00 ± 0.00	99.64 ± 0.08	97.84 ± 0.27	95.80 ± 0.80	100.00 ± 0.00	76.99 ± 0.00	96.30 ± 0.14	100.00 ± 0.00
	7	99.39 ± 0.35	99.02 ± 0.56	81.50 ± 0.75	91.55 ± 0.89	100.00 ± 0.00	100.00 ± 0.00	98.94 ± 0.60	100.00 ± 0.00
Haemocytes		Treatment and dose							
Cell viability test	Time (days)	CTH (0 mg L^{-1})	TH1 (1 mg L^{-1})	TH2 (5 mg L^{-1})	TH3 (10 mg L^{-1})	CCA (0 mg L^{-1})	CA1 (10 mg L^{-1})	CA2 (50 mg L^{-1})	CA3 (100 mg L^{-1})
TB (%)	3	98.69 ± 0.86	98.79 ± 0.34	92.13 ± 1.40	98.74 ± 0.48	100.00 ± 0.00	92.43 ± 0.46	97.84 ± 0.79	96.90 ± 0.94
	7	100.00 ± 0.00	97.85 ± 0.35	72.12 ± 0.21	80.21 ± 0.71	96.26 ± 0.53	82.77 ± 0.63	94.96 ± 0.76	96.41 ± 1.24
NR (%)	3	98.45 ± 0.61	98.86 ± 0.74	98.71 ± 0.29	97.41 ± 1.23	100.00 ± 0.00	76.19 ± 0.74	100.00 ± 0.00	99.60 ± 0.40
	7	99.70 ± 0.20	98.00 ± 0.26	93.42 ± 1.18	89.62 ± 0.75	99.77 ± 0.15	100.00 ± 0.00	99.36 ± 0.42	100.00 ± 0.00

The values are mean ± SE (standard error); n = 6. Trypan blue (TB), neutral red (NR).

Table 2
Haemolymphatic parameters of *Mytilus galloprovincialis* exposed to thiacloprid (TH) and Calypso 480 SC (CA).

Thiacloprid (dose)	Time (days)	Cl^-(mmol L^{-1})	K^+ (mmol L^{-1})	Na^+ (mmol L^{-1})	Ca^{2+} (mg dl^{-1})	S-phosphor (mg dl^{-1})	Mg^{2+} (mg dl^{-1})	LDH (U L^{-1})	Glucose (mg dl^{-1})
CTH (0 mg L^{-1})	3	473.18 ± 2.04^{a}	12.78 ± 0.15^{a}	536.50 ± 0.43^{a}	56.83 ± 0.15^{a}	0.90 ± 0.06^{a}	23.90 ± 0.07^{a}	4.50 ± 0.22^{a}	1.37 ± 0.17^{a}
	7	472.03 ± 2.17^{a}	11.83 ± 0.17^{a}	545.17 ± 1.94^{a}	53.55 ± 0.11^{a}	0.75 ± 0.02^{ab}*	24.85 ± 0.06^{a}	1.50 ± 0.22^{a}*	2.60 ± 0.08^{a}*
TH1 (1 mg L^{-1})	3	465.35 ± 1.05^{a}	12.30 ± 0.04^{a}	541.33 ± 0.67^{a}	56.33 ± 0.25^{a}	0.85 ± 0.02^{a}	25.15 ± 0 04^{b}	4.50 ± 0.22^{a}	3.07 ± 0.12^{b}
	7	463.03 ± 0.32^{a}	11.87 ± 0.16^{a}	543.50 ± 4.24^{a}	53.48 ± 0.23^{a}	0.87 ± 0.02^{a}	25.05 ± 0.04^{a}	1.17 ± 0.17^{a}*	3.88 ± 0.47^{b}
TH2 (5 mg L^{-1})	3	423.10 ± 7.48^{b}	10.77 ± 0.33^{b}*	553.50 ± 2.00^{a}	51.52 ± 1.43^{b}*	0.85 ± 0.02^{a}	24.85 ± 0.12^{b}	4.50 ± 0.22^{a}	2.15 ± 0.26^{ab}
	7	458.45 ± 0.52^{a}	12.25 ± 0.06^{a}	536.33 ± 1.91^{a}	52.73 ± 0.15^{a}	0.63 ± 0.06^{b}*	24.43 ± 0.18^{a}	1.17 ± 0.17^{a}*	2.03 ± 0.04^{a}
TH3 (10 mg L^{-1})	3	449.62 ± 10.20^{b}	12.03 ± 0.38^{a}	512.17 ± 7.56^{b}*	52.92 ± 1.66^{b}	0.87 ± 0.02^{a}	24.33 ± 0.08^{a}	7.33 ± 0.67^{b}	2.77 ± 0.14^{b}
	7	459.05 ± 0.28^{a}	12.65 ± 0.04^{a}	544.50 ± 2.79^{a}	54.92 ± 0.09^{a}	0.68 ± 0.05^{b}*	25.35 ± 0.13^{a}	1.50 ± 0.22^{a}*	3.30 ± 0.46^{b}
Calypso 480 SC (dose)	**Time (days)**	**Cl^-(mmol L^{-1})**	**K^+ (mmol L^{-1})**	**Na^+ (mmol L^{-1})**	**Ca^{2+} (mg dl^{-1})**	**S-phosphor (mg dl^{-1})**	**Mg^{2+} (mg dl^{-1})**	**LDH (U L^{-1})**	**Glucose (mg dl^{-1})**
CCA (0 mg L^{-1})	3	494.30 ± 0.81^{a}	12.57 ± 0.30^{a}	498.17 ± 8.03^{a}	53.25 ± 0.53^{a}	2.22 ± 0.09^{a}	24.40 ± 0.11^{a}	2.00 ± 0.37^{a}	2.93 ± 0.28^{ab}
	7	454.62 ± 0.78^{a}*	14.52 ± 0.06^{a}	560.00 ± 1.06^{a}*	56.75 ± 0.56^{a}	2.45 ± 0.08^{a}	25.65 ± 0.06^{a}	2.83 ± 0.54^{a}	4.42 ± 0.26^{a}
CA1 (10 mg L^{-1})	3	453.35 ± 0.46^{c}	10.97 ± 0.40^{b}	450.17 ± 15.39^{b}	50.97 ± 0.70^{b}	1.20 ± 0.04^{b}	24.65 ± 0.10^{a}	4.50 ± 0.22^{b}	1.77 ± 0.32^{a}
	7	452.87 ± 0.27^{a}	13.07 ± 0.08^{b}*	541.33 ± 2.51^{a}*	62.85 ± 0.29^{c}*	1.25 ± 0.06^{b}	25.80 ± 0.40^{a}	3.50 ± 0.56^{a}	2.00 ± 0.18^{b}
CA2 (50 mg L^{-1})	3	487.08 ± 0.57^{b}	10.23 ± 0.30^{b}	492.83 ± 4.73^{a}	56.73 ± 0.17^{a}	1.40 ± 0.15^{b}	25.15 ± 0.10^{a}	3.00 ± 0.37^{a}	2.33 ± 0.18^{ab}
	7	445.52 ± 0.95^{b}*	12.60 ± 0.06^{b}*	537.33 ± 0.92^{a}	54.95 ± 0.16^{a}	1.32 ± 0.06^{b}	25.70 ± 0.17^{a}	1.33 ± 0.21^{b}*	1.37 ± 0.16^{b}
CA3 (100 mg L^{-1})	3	498.12 ± 3.51^{a}	13.02 ± 0.29^{a}	516.67 ± 4.26^{a}	55.12 ± 0.59^{a}	1.52 ± 0.20^{b}	25.30 ± 0.06^{a}	5.50 ± 0.22^{b}	3.78 ± 0.52^{b}
	7	458.47 ± 1.58^{a}*	14.05 ± 0.08^{a}	549.83 ± 6.93^{a}	59.47 ± 0.19^{b}*	1.35 ± 0.06^{b}	25.08 ± 0.06^{a}	1.50 ± 0.34^{b}*	3.37 ± 0.68^{a}

The values are mean ± SE (standard error); n = 6. Different superscripts indicate significant differences ($P < 0.01$) among groups at the same sample time. *Denotes significant differences among group values over time ($P < 0.01$).

ions Cl^-, Na^+, Ca^{2+} after 3 days, K^+ and glucose after 7 days and significant increase ion Ca^{2+} the seventh day of exposure compared with control (CCA). The concentration of 50 mg L^{-1} (CA2) caused a significant decrease ($P < 0.01$) in LDH activity after 7 days of exposure and electrolytes ions (Cl^-, K^+) and glucose during the experiment compared to control (CCA). Mussels kept at CA3 (100 mg L^{-1}) demonstrated significantly increased ($P < 0.01$) glucose levels, LDH activity after 3 days and Ca^{2+} after 7 days of exposure and significant decrease in LDH activity at the end of experiment, compared with control (CCA). Values of Mg^{2+} in CA-exposed mussels did not differ significantly between the groups during the exposure.

3.4. Enzyme activity

In the present study, TH was shown to affect moderately CAT activity, whereas no statistically significant ($P < 0.05$) effects on SOD activity were found (Fig. 1). CAT activity was significantly increased ($P < 0.05$) in gills after 3 days of exposure to 10 mg L^{-1} (TH3) and decreased in digestive gland after 7 days at 5 mg L^{-1} (TH2), compared to controls. Calypso caused statistically significant changes ($P < 0.05$) in CAT and SOD activities in mussels (Fig. 1). In digestive gland, CAT activity was significantly reduced ($P < 0.05$) after 3 days at the highest concentration tested (CA3, 100 mg L^{-1}). In gills, enzyme activity was significantly decreased ($P < 0.05$) in mussels exposed for 7 days to CA (10, 50 and 100 mg L^{-1}), compared to control mussels. SOD activity was significantly reduced ($P < 0.05$) in gills from mussels exposed for 3 days to three CA concentrations tested (10, 50 and 100 mg L^{-1}), compared to related controls (Fig. 1).

3.5. Histology

Results of histological observations (Figs. 2–5) showed a considerable exposure effect to toxicants in a dose and time-dependent way for both tissues, with respect to the controls. Results of histopathological condition index (I_h) are reported in Table S2 and plotted in Figs. 2–5. Statistical analyses showed significant differences among the three treated groups for both compounds; while, no statistically remarkable differences were obtained comparing the I_h of different tissues (digestive tubule modifications vs intertubular tissue alterations; gills vs digestive gland).

3.5.1. Digestive gland

For both toxicants, the most represented digestive gland tissue abnormalities comprised digestive tubule changes like degeneration of epithelial layer, altered anatomy and atrophy tendency and intertubular tissue modifications with massive haemocytes infiltration, presence of

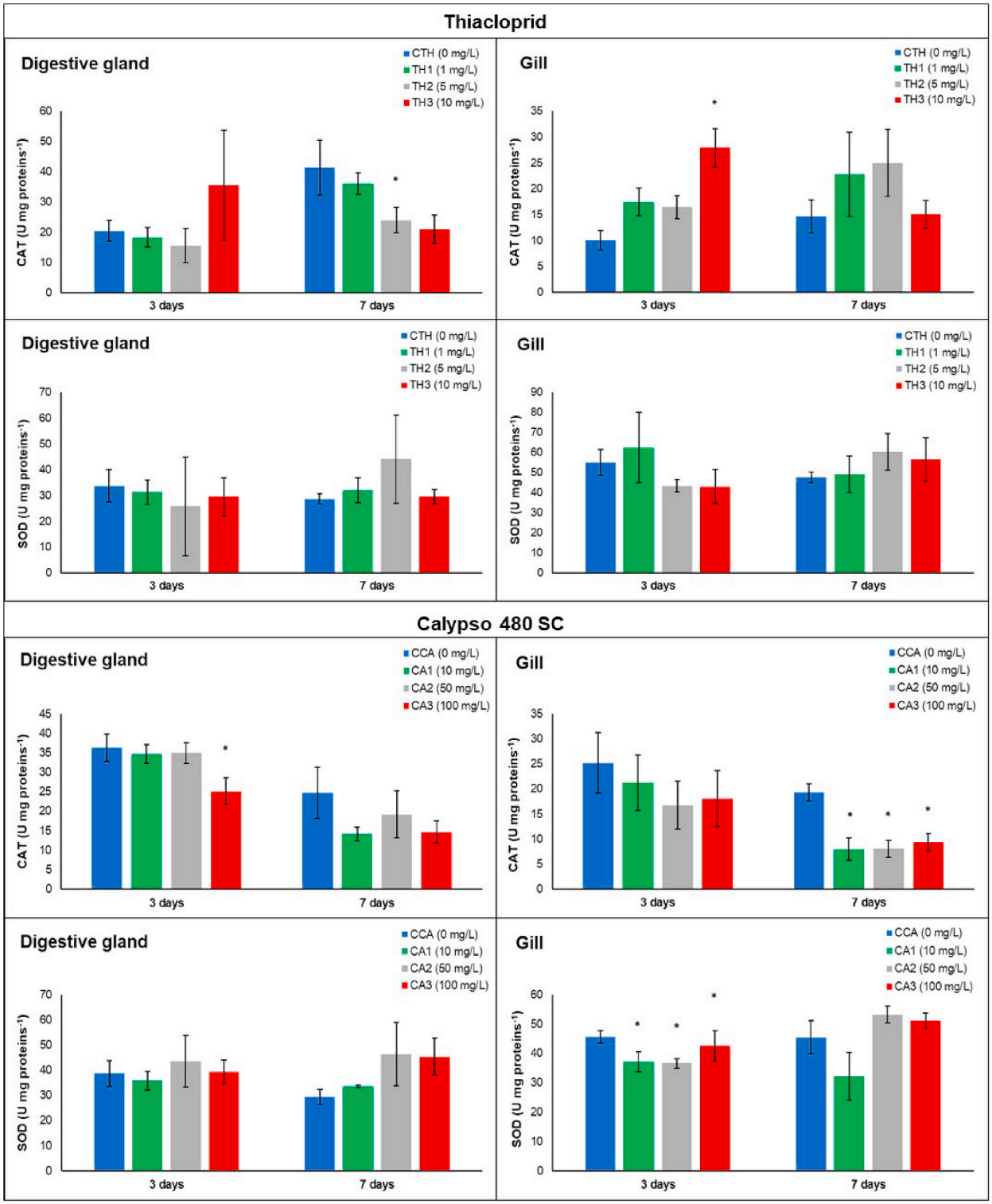

Fig. 1. Enzymatic activity of catalase (CAT) and superoxide dismutase (SOD) in digestive gland and gill in *Mytilus galloprovincialis* exposed to thiacloprid (TH) and Calypso 480 SC (CA). The values are mean ± SE (standard error); n = 6; Mann-Whitney *U* Test. *Denotes significant differences ($P < 0.05$) among groups at the same sample time.

brown cells and lipofuscin aggregates. Tubule regression, fibrosis, focal points of necrosis and nodular inflammation were the most common alterations recorded (Figs. 2–4).

3.5.2. Gills

Gill tissues indicate severe alterations following both CA and TH exposures. The main histological alterations were haemocytes infiltration, epithelial detachment, vacuolation and lipofuscin deposits (Figs. 3–5). The higher values of I_h were achieved for CA3 and TH3 groups after 7 days of exposure, as also resulted for digestive gland I_h evaluation.

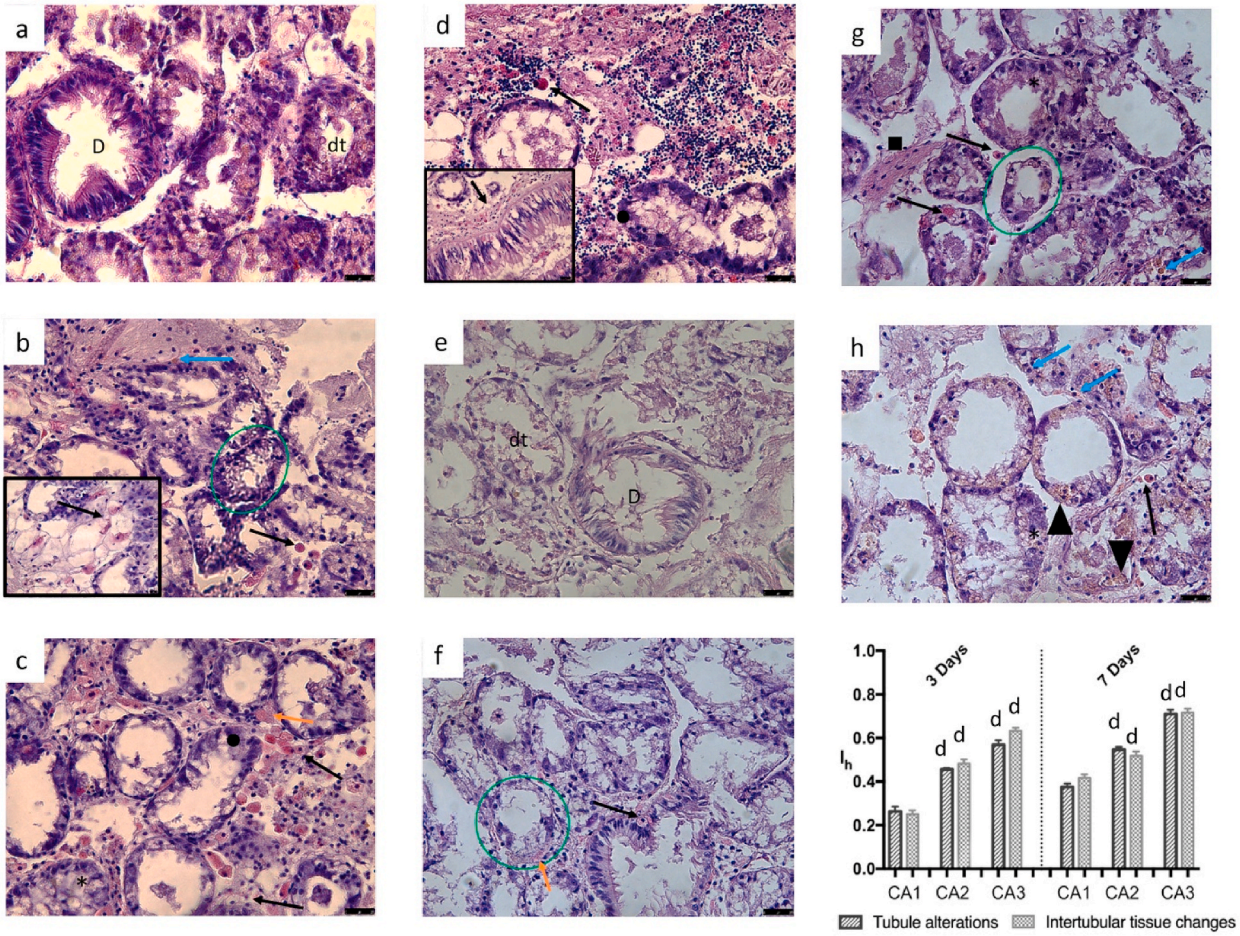

Fig. 2. *Mytilus galloprovincialis* digestive gland (DG) sections from Calypso 480 SC exposure: representative figures. a-b-c-d are respectively control, 10, 50, 100 mg L^{-1}, 3 days exposure; e-f-g-h are respectively control, 10, 50, 100 mg L^{-1}, 7 days exposure. Scale bars: 20 μm. Digestive tubule alteration is indicated by green circles, blue arrows indicate brown cells, orange arrows indicate granulocytoma, black arrows indicate haemocytes infiltrations, black filled circles indicate hyperplasia, asterisks indicate hypertrophy, arrowheads indicate lipofuscin aggregates, black square indicates fibrosis, nodular inflammation is widely diffused in d. "D" indicates duct, "dt" indicates digestive tubule. Insets in Figs b and d showed some additional details of haemocytes infiltrations from a different part of the related sample. I_h represents the graphical elaborations of histopathological condition index of DG from *Mytilus galloprovincialis* exposed to different concentration of Calypso 480 SC (CA): 10 mg L^{-1} (CA1), 50 mg L^{-1} (CA2) and 100 mg L^{-1} (CA3). The values are mean ± SD, $^{a}P < 0.0332$, $^{b}P < 0.0021$, $^{c}P < 0.0002$, $^{d}P < 0.0001$ comparing the exposed samples (One-way ANOVA test/Bonferroni's multiple comparisons test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

4.1. Conditions of mussels during experiments

With increasing of exposure time, the effects of both chemicals, at concentrations of TH 5 and 10 mg L^{-1} and CA 50 and 100 mg L^{-1}, became more evident on physiological state of mussels. *Mytilus* mussels are sessile organisms that attach themselves to the various surfaces by the producing collagenous byssus. The number or strength of the fibres may vary depending on environment conditions (Roberts, 1975; Bell and Gosline, 1996). Mussels must to be in good condition and have a large amount of energy which it is necessary to open the shells and to produce byssus. Consequently, all tissues are directly exposed to the environment and this can lead to contact with xenobiotics or molecules responsible for oxidative stress (Bell and Gosline, 1996; Burnett and Sara, 2019). Mussels protect themselves by closing the shells and reducing metabolism, helping them to cope with unfavourable conditions (Bell and Gosline, 1996; Ayad et al., 2011; Savorelli et al., 2017; Chmist et al., 2019) and simultaneously they reduce or completely stop fibres production (Bell and Gosline, 1996; Ayad et al., 2011). The apparent change fibres production under pesticide exposure has been reported in previous studies on mussels by Ayad et al. (2011), Kargiannis et al. (2011) and Stara et al. (2020). Shells opening and associated fibres formation are first good biomarkers for monitoring weak health status of mussels (Burnett and Sara, 2019; Chmist et al., 2019; Stara et al., 2020).

4.2. Cell viability assays

Viability tests are useful tools for a preliminary evaluation of the possible damage produced by pollutants to organisms. The integrity of the cell membrane assessed by Trypan Blue showed that immunocytes (haemocytes) and the cells liable for the metabolism of the whole organism (digestive gland cells) still manage to perform their tasks. This result is confirmed by the Neutral Red test. Indeed, cells were able to absorb the dye through pinocytosis and therefore reach the lysosomes in order to evaluate their stability (Aguirre-Martínez et al., 2013; Aliko et al., 2015). Lysosomes are important targets because they are the second line of defense against oxidative stress (Burgos-Aceves et al., 2017; Domouhtsidou et al., 2004). At the cellular level, both pollutants at all the concentrations tested did not affect cell viability. Generally, after exposure to xenobiotics, both haemocytes and cells of the digestive

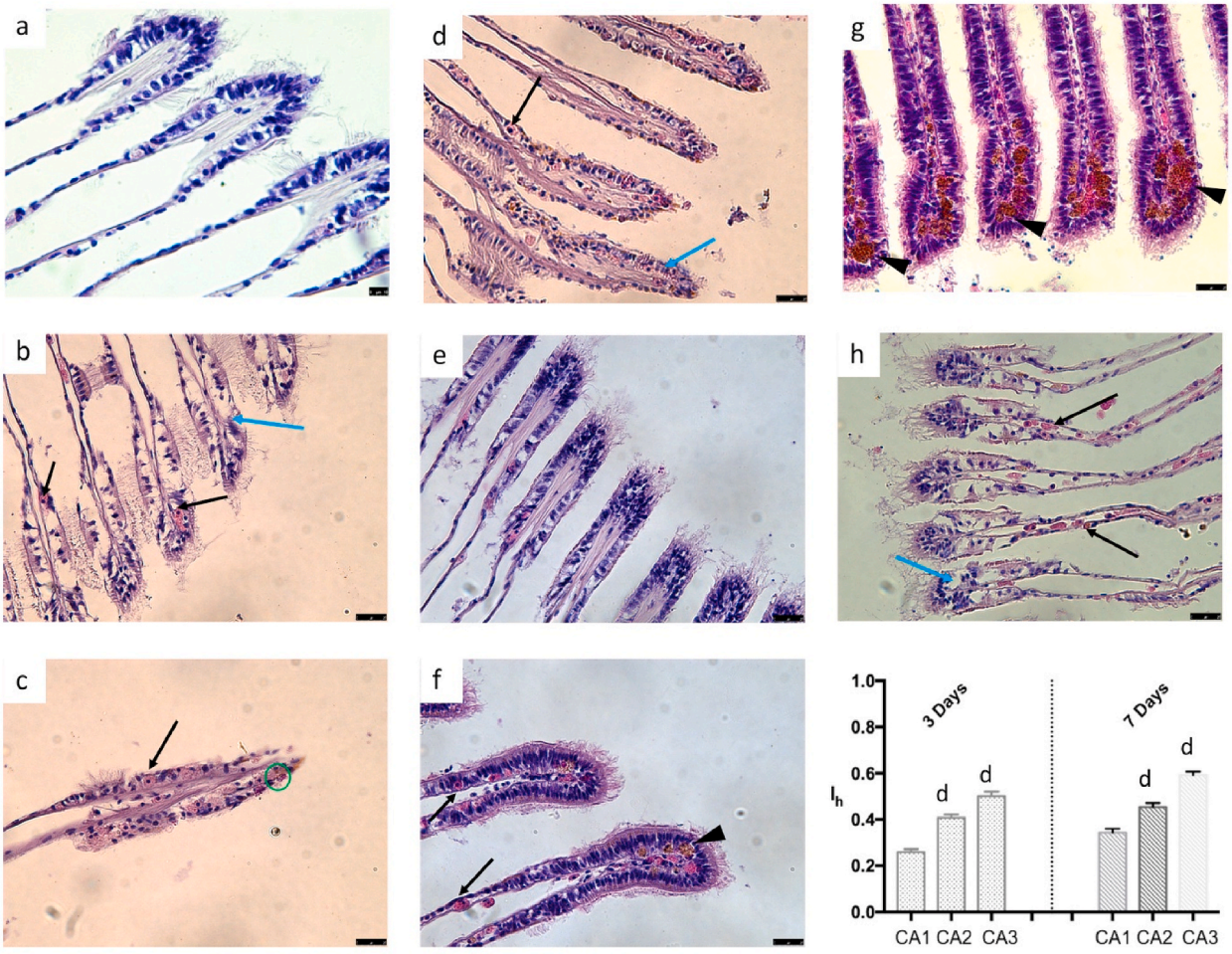

Fig. 3. *Mytilus galloprovincialis* gills sections from Calypso 480 SC exposure: representative figures. a-b-c-d are respectively control, 10, 50, 100 mg L^{-1}, 3 days exposure; e-f-g-h are respectively control, 10, 50, 100 mg L^{-1}, 7 days exposure. Scale bars: 20 μm. Green circles indicate vacuolation, blue arrows indicate epithelial alterations, black arrows indicate haemocytes infiltrations, and arrowheads indicate lipofuscin aggregates. I_h represents the graphical elaborations of histopathological condition index of gills from *Mytilus galloprovincialis* exposed to different concentration of Calypso 480 SC (CA): 10 mg L^{-1} (CA1), 50 mg L^{-1} (CA2) and 100 mg L^{-1} (CA3). The values are mean ± SD, $^{a}P < 0.0332$, $^{b}P < 0.0021$, $^{c}P < 0.0002$, $^{d}P < 0.0001$ comparing the exposed samples (One-way ANOVA test/Bonferroni's multiple comparisons test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

gland begin to be damaged and the viability percentage decreases (Faggio et al., 2016; Pagano et al., 2017; Stara et al., 2020; Torre et al., 2013). In addition, cell viability for both cell types decreased in chronic exposure to lower CA concentrations (Stara et al., 2020).

Mussels exhibit different behaviors than other aquatic organisms exposed to the same pollutants.(Stara et al., 2019; Velisek and Stara, 2018; Beketov et al., 2008). This is typically evident after exposure to high levels of heavy metals (Kraak et al., 1997), mussels closing their shells and reducing the byssus production (Roberts, 1975). This therefore prevents the entry of water and both pollutants did not reach haemolymph or the digestive gland.

4.3. Haemolymph biochemical composition

Nutrients, respiratory gases, metabolic wastes, enzymes, and toxicants are transported by haemolymph throughout the series of tissues body of bivalves and its biochemical investigations provide information relevant to health assessment (Gustafson et al., 2005; Matozzo et al., 2016), similarly to determination of serum biochemical parameters of fish (Qadir et al., 2014; Capillo et al., 2018a a). The biochemical composition of haemolymph varied identifiably between control and mussels exposed to TH (5 and 10 mg L^{-1}) and CA (10, 50 and 100 mg L^{-1}) during acute exposure. These toxicants caused most changes in haemolymph electrolytes (Cl^{-}, K^{+}, Na^{+}, Ca^{2+}, S-phosphor), glucose concentration and LDH activity. Molluscs, as osmo-comform organisms, resist changes in the environment variation of adaptation processes, such as regulation of haemolymph ions concentration (Capillo et al., 2018a a). Glucose is the main source of energy and increased concentration is indicative of stress in aquatic organisms exposed to environment contaminants. A very marked reduction of glucose indicates acute body failure due to sudden glycogen depletion (Kulkarni and Barad, 2015). Similarly, changes in activity of the LDH is considered as a good diagnostic biomarkers of cell membrane damage caused by pollutants (Kolarova and Velisek, 2012; Ateyya et al., 2015; Faggio et al., 2016). Given the importance of haemolymph evaluation, there were many studies dealing with the evaluation of bivalve haemolymph (Lopes-Lima et al., 2012; Fritts et al., 2015; Faggio et al., 2016; Capillo et al., 2018a a), little is focused on exposure to pesticides (Ayad et al., 2011), insecticides, such as neonicotinoids (Stara et al., 2020). Stara et al. (2020) observed negative effects of CA (7.77 and 77.70 mg L^{-1}) on composition of haemolymph *M. galloprovincialis* during 20 days exposure. Simultaneously, changes in these parameters were persistent even after 10 days of depuration in CA-free water. Although neonicotinoids are considered non-toxic to fish, similarly to the changes caused by CA in haemolymph of mussels, negative effects on serum biochemical parameters of fish have been also reported (Qadir et al., 2014; Vieira et al., 2018).

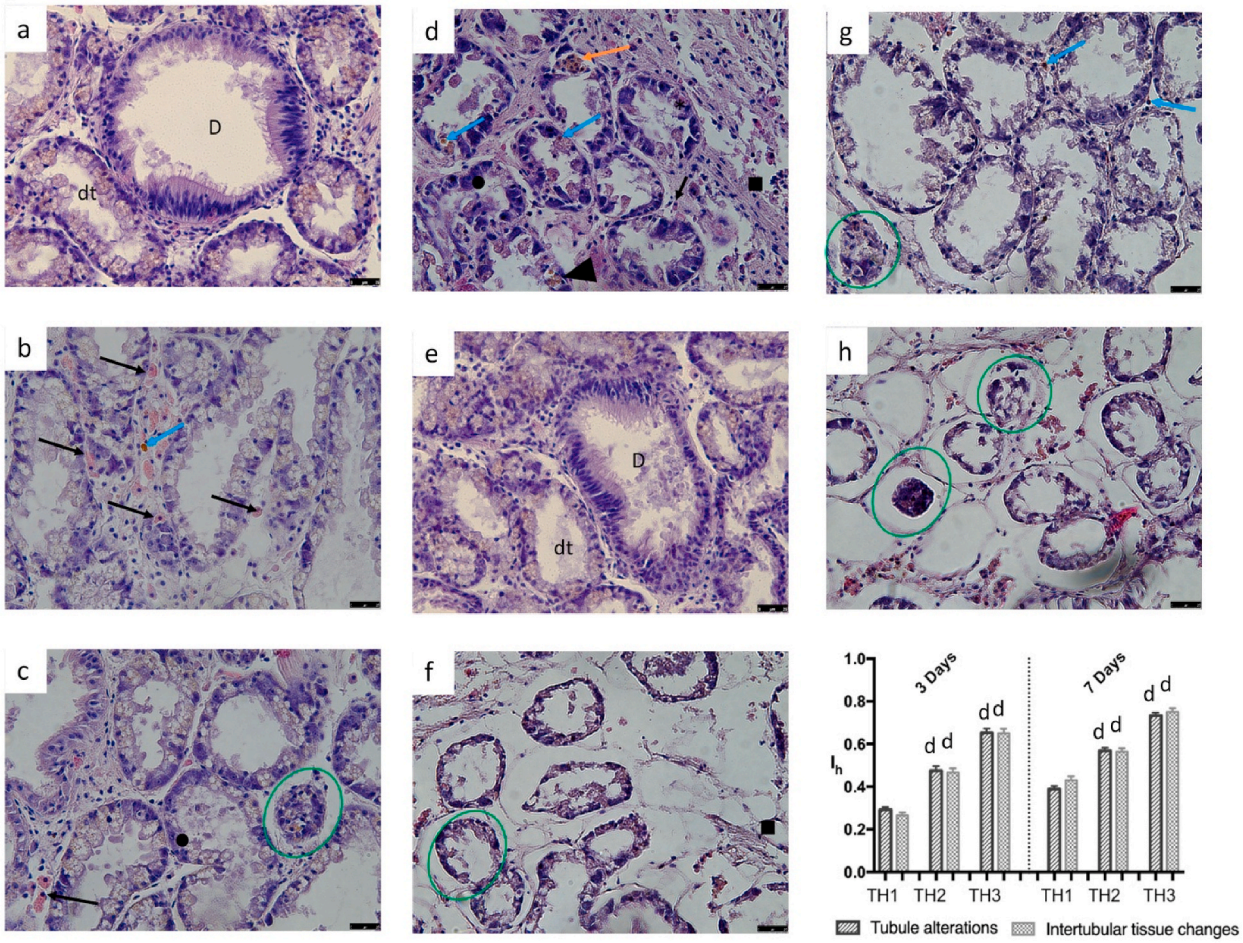

Fig. 4. *Mytilus galloprovincialis* digestive gland sections from thiacloprid exposure: representative figures. a-b-c-d are respectively control, 1, 5, 10 mg L^{-1}, 3 days exposure; e-f-g-h are respectively control, 1, 5, 10 mg L^{-1}, 7 days exposure. Scale bars: controls 25 μm, b-c-d-f-g-h 20 μm. Digestive tubule alteration is indicated by green circles, blue arrows indicate brown cells, black arrows indicate haemocytes infiltrations, orange arrows indicate granulocytoma, black filled circles indicate hyperplasia, asterisks indicate hypertrophy, arrowheads indicate lipofuscin aggregates, black square indicates fibrosis. "D" indicates duct, "dt" indicates digestive tubule. I_h represents the graphical elaborations of histopathological condition index of DG from *Mytilus galloprovincialis* exposed to different concentration of thiacloprid (TH): 1 mg L^{-1} (TH1), 5 mg L^{-1} (TH2) and 10 mg L^{-1} (TH3). The values are mean ± SD, $^{a}P < 0.0332$, $^{b}P < 0.0021$, $^{c}P < 0.0002$, $^{d}P < 0.0001$ comparing the exposed samples (One-way ANOVA test/Bonferroni's multiple comparisons test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4.4. Determination of enzyme activities

Assessment of enzyme activities has been considered as useful biomarkers of exposure of aquatic species to pollutants (Fiorino et al., 2018; Matozzo et al., 2018; Plhalova et al., 2018; et al., 2019; Vieira et al., 2018). Antioxidant enzymes play an effective antioxidant role by scavenging preferentially reactive oxygen species (ROS) and maintain a steady state in the cells (Faria et al., 2009; Vidal-Linan et al., 2016). The CAT and SOD enzymes are the first line of defense against ROS, protecting organisms from oxidative damage (Burgos-Aceves et al., 2018; Chi et al., 2019). They are found virtually in all aerobic organisms (Ewere et al., 2019). SOD catalyses the conversion of O_2^- to O_2 and H_2O_2, whereas CAT catalyses the conversion of H_2O_2 to H_2O and O_2 (Somani et al., 1997). Other biotransformation enzymes are glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), and nonenzymatic biomarkers as reduced glutathion (GSH), vitamins etc. They coordinate important cellular defence mechanisms against oxidative stress which can be impacted by xenobiotic chemicals (Banni et al., 2015; Vidal-Linan et al., 2016; Vieira et al., 2018; Ewere et al., 2019; Ulutürhan et al., 2019). Gill is a highly potent metabolic active tissue and is the first barrier which faced to xenobiotics in mussels (Vidal-Linan and Bellas, 2013) and they are often used to assess the responses of mussels to environmental stressors (Banni et al., 2015). The digestive gland of molluscs is an equally important detoxifying organ aimed at metabolic regulation, providing protection through the processes of detoxification and xenobiotic elimination (Marigomez et al., 2002; Ewere et al., 2019; Faggio et al., 2018). In the present study, TH caused an increase in CAT activity in gill at TH3 (10 mg L^{-1}) after 3 days of exposure and a decrease in digestive gland at TH2 (5 mg L^{-1}) after 7 days. Mussels exposed to CA showed a reduction of CAT activity in digestive gland at CA3 (100 mg L^{-1}) after 3 days and in gills at all the experimental concentrations tested (10, 50 and 100 mg L^{-1}) after 7 days. Similarly, all concentrations of CA decreased SOD activity in gills after 3 days. Likewise, recent studies have showed that pesticides inhibit enzymatic activity in mussels. Matozzo et al. (2018) demonstrated that glyphosate (100 and 1000 μg L^{-1}) is able to affect SOD activity in digestive gland of mussel after 7 days exposure. Similarly, Stara et al. (2020) recorded a decreased SOD activity in digestive gland of mussels exposed to CA at 7.77 mg L^{-1} after 20 days and the activity was then balanced after 10 days in CA-free water. Ewere et al. (2019) described the negative impacts of imidacloprid and Spectrum 200SC on enzymatic activities in *Saccostrea glomerata* after two weeks of exposure at environmentally relevant concentrations (0.01 and 0.05 mg L^{-1}). In this study, digestive gland CAT and GST activity, as well as gill

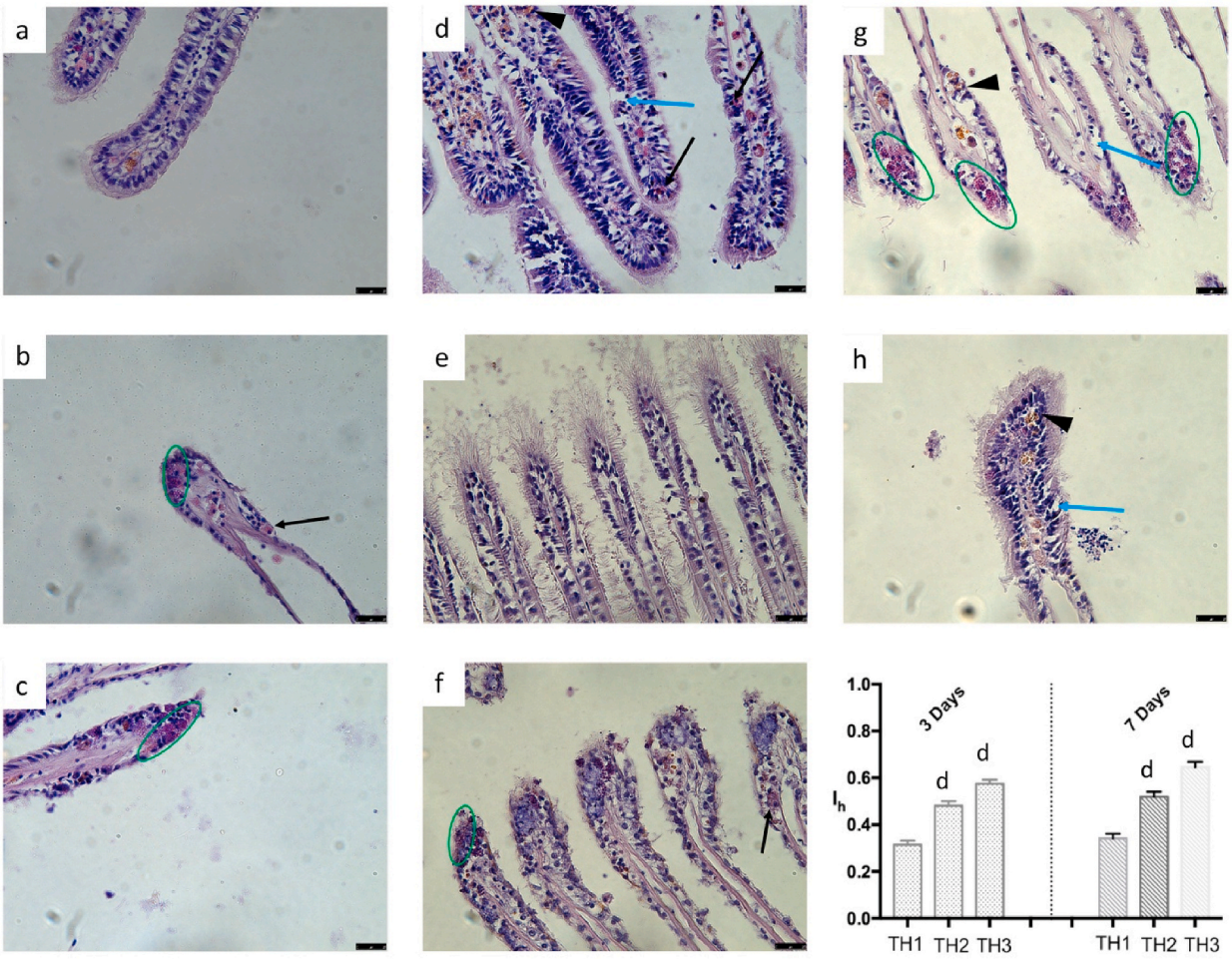

Fig. 5. *Mytilus galloprovincialis* gills sections from thiacloprid exposure: representative figures. a-b-c-d are respectively control, 1, 5, 10 mg L^{-1}, 3 days exposure; e-f-g-h are respectively control, 1, 5, 10 mg L^{-1}, 7 days exposure. Scale bars: 20 μm. Green circles indicate vacuolation, blue arrows indicate epithelial alterations, black arrows indicate haemocytes infiltrations, and arrowheads indicate lipofuscin aggregates. I_h represents the graphical elaborations of histopathological condition index of gills from *Mytilus galloprovincialis* exposed to different concentration of thiacloprid (TH): 1 mg L^{-1} (TH1), 5 mg L^{-1} (TH2) and 10 mg L^{-1} (TH3). The values are mean ± SD, $^{a}P < 0.0332$, $^{b}P < 0.0021$, $^{c}P < 0.0002$, $^{d}P < 0.0001$ comparing the exposed samples (One-way ANOVA test/Bonferroni's multiple comparisons test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

GST activity were markedly affected by the exposure. In addition, neonicotinoids can induce antioxidant imbalance and provoke oxidative stress in fish and crayfish. Velisek and Stara (2018) described effect of TH at concentration of 4.50 μg L^{-1} on antioxidant activity of SOD and GR during embryo-larval development of *Cyprinus carpio*. Vieira et al. (2018) observed differences in antioxidant biomarkers SOD, CAT, GSH, GPx and GST in tissues of *Prochilodus lineatus* exposed for 5 days to imidacloprid at concentrations of 1.25–1250 μg L^{-1}. These alterations in the antioxidants can lead to high levels of lipid peroxidation (LPO) and protein carbonyl content production, which indicates overproduction of ROS and a state of oxidative stress (Stara et al., 2019).

4.5. Histology

Digestive gland is the principal site for xenobiotics detoxification in bivalve (Minier et al., 2000; Gomes et al., 2012) and it is also involved in bioaccumulation of xenobiotics and in challenging environmental and/or induced stressors (Costa et al., 2013). Gills tissue is the first contact surfaces between the organisms and the aquatic environment and are the organs responsible for respiratory function and nutrient assimilation, as for the uptake of contaminants (Azevedo et al., 2015). For these reasons, these tissues have been selected for determining of histological alteration using histopathological condition index as suggested by Costa et al. (2013). Higher values of I_h were obtained for both tissues, as expected, in the higher concentration experimental condition at 7 days of exposure. The I_h took in account several histological abnormalities and alteration as reported in Table S1. Alterations in digestive tubule and intertubular tissues were recorded with no important differences between them.

Among the various alterations, melanosis widely occurred. Melanosis is a reported alteration in mussels as in other organisms. This condition reflects an inflammatory process related to the exposure to toxicants. Melanosis derives, in fact, from a series of events known as "pro-phenoloxidase (PO) activating systems". The PO activating system is responsible of recognition, cytotoxicity and encapsulation of toxicants (De Vico and Carella, 2012; Luna-Acosta et al., 2010; Pagano et al., 2016). The same histological alteration was observed in *M. galloprovincialis* in a recent study that evaluated the effect of chronic exposure to CA at sub-lethal concentrations (Stara et al., 2020). The other histological abnormalities detected, confirmed a severe inflammatory response of these organisms to the pollutant. For example, it is well known that brown cells, highly present in digestive gland of treated organisms, are involved in recognition, accumulation and detoxification of pollutants (De Vico and Carella, 2012; Usheva and Frolova, 2006). Moreover, nodular inflammation has been recognized in many digestive glands of exposed mussels. Nodular inflammations occur when

haemocytes phagocyte little and abundant particles, forming different sized aggregates in haemolymph and interstices (Galloway and Depledge, 2001). Despite normally nodular formations are a consequence of microbial invasions (Rahmet-Alla and Rowley, 1989), which have shown that the aggregative properties of haemocytes are stimulated by the exposure to xenobiotics and other contaminants (Auffret and Oubella, 1997; Carella et al., 2015).

The pathological inflammatory condition has also been observed in gill tissues, with abnormalities comprehending abundant deposits of melanin, intense vacuolation, haemocytes infiltrations and presence of brown cells. Focal and/or diffuse haemocytes infiltrations in tissues, without the tendency to form aggregates, characterize the infiltrative inflammations (Kim et al., 2006; Kim and Powell, 2004, 2007). Infiltrative haemocytes phagocyte pathogens and foreign bodies in order to activate a reaction to the toxicant, activating the Multixenobiotic defence mechanism (MXDM) (Pain and Parant, 2003). The MXDM can act as protection for cells from negative actions of toxic compounds by reducing access and in promoting efflux of toxicants (Pagano et al., 2016). Haemocytes infiltrations are widely reported in gill tissues of molluscs exposed to environmental pollutants.

5. Conclusions

This study investigated for the first time the acute effects of the active neonicotinoid substance thiacloprid and the commercial insecticide Calypso 480 SC on *M. galloprovincialis*. Our results revealed a series of biological alterations following exposure to the compounds. Thiacloprid and also Calypso mainly affected the production of byssus fibres, biomarkers of biochemical profile of haemolymph (glucose, LDH, S-phosphor, Ca^{2+}, Cl^{-}, K^{+}, Na^{+}), activity of antioxidant biomarkers (SOD, CAT) and caused histopathological alterations in the digestive glands and gills. The results also show that thiacloprid in low concentrations has a stronger effect on mussels than an insecticide which containing another concomitant substances. The study of biological responses of exposed organisms is essential for understanding of the mechanisms of toxicity. To better comprehend the mechanisms of action of emerging contaminants in aquatic organisms, it is desirable to find particularly sensitive early warning signals to predict the risks posed by such substances in environment.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.110980.

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