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ALTERATION OF PHYSIOLOGICAL RESPONSES IN AQUATIC ORGANISMS EXPOSED TO ECOLOGICALLY RELEVANT INSECTICIDES

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Ad Alice

ABSTRACT

This research aims to assess the risk of two types of insecticides used in agriculture: thiacloprid, a neonicotinoid, and fenitrothion, an organophosphate, on non-target organisms such as *Mytilus galloprovincialis* and *Danio rerio* larvae.

Mussels were exposed to both the Bayer produced pesticide Calypso 480 SC (40.4% thiacloprid) and the active molecule thiacloprid (purity 99.9%, Sigma Aldrich) with acute exposure to sublethal concentrations of Calypso(10, 50and 100 mg/L) and thiacloprid (1, 5 and 10 mg/L) at two-time points (3 and 7 days) and sub-chronic exposure to Calypso (7.77 and 77.70 mg/L) and thiacloprid (4.5 and 450 μg/L) at two time points (10 and 20 days).

Acute exposure to sublethal concentration showed that in biochemical analysis of haemolymph there were significant changes in electrolytes ions, lactate dehydrogenase enzyme activity and glucose concentration following exposure to both substances. The thiacloprid-exposed mussels showed a significant imbalance in CAT activity in the digestive gland and gills. Calypso caused a significant decrease in SOD activity in gills and CAT activity in both tissues.

Sub-chronic exposure to both concentrations of Calypso significantly increased mortality rate in the cells of hemolymph and the digestive gland, while digestive gland cells were no longer able to regulate cell volume. Exposure significantly reduced hemolymph parameters, affected the enzymatic activities of SOD of the digestive gland and CAT of gills. Sub-chronic exposure to thiacloprid affected hemolymph biochemical parameters, cell viability in the digestive gland, antioxidant biomarkers in the digestive gland and gills at thiacloprid environmental relevant concentrations of 4.5 μg/L.

Twenty-four-hour exposure to environmental concentrations of fenitrothion, ranging from ng/L to low μg/L, altered the basal locomotor activity, visuomotor response and acoustic/vibrational escape response of zebrafish larvae. The computational analysis identified potential protein targets for this compound. Some of the predictions, including interactions with acetylcholinesterase, monoamine oxidases and the androgen receptor (AR), were experimentally validated. Finally, altered levels of L-DOPA, DOPAC, HVA and 5-HIAA were found, as well as a significant up-regulation of slc18a2 expression at lower fenitrothion concentrations. These data strongly suggest that the concentrations of fenitrothion commonly found in aquatic ecosystems present a significant environmental risk to fish communities.

This study attempts to improve the limited information on the effects of these insecticides on aquatic organisms, highlighting how prolonged exposure to low concentrations is harmful to non-target organisms such as mussels.

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DECLARATION OF CONTRIBUTION

I was responsible for the conception and management of the thesis project. I hereby declare that the results presented are to the best of my knowledge correct. The thesis project (2018- 20 is supported by five scientific papers and was a joint effort of several research teams from different institutes, mainly: Department of Chemical, Biological, Pharmaceutical, and Environmental Sciences and Department of Veterinary Sciences of University of Messina; Department of Biology, University of Padova; Faculty of Fisheries and Protection of Waters of University of South Bohemia in Ceske Budejovice; Institute for Environmental Assessment and Water Research (IDAEA-CSIC) of Barcelona, Spain and Zooprophylactic Institute of Sicily (IZS). I had a major role in the study design and execution of the experiments, in sampling, in the acquisition of most data, their analysis and interpretation, and in the structuring and writing of the manuscripts in which I am an author. I particularly worked on all the physiological assays. The thesis was verified with the software Noplagio, showing a percentage lower than 20%.

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ii. Stara, A., Pagano, M., Capillo, G., Fabrello, J., Sandova, M., Vazzana, I., Zuskova, E., Velisek, J., Matozzo, V., & Faggio, C. (2020). Assessing the effects of neonicotinoid insecticide on the bivalve mollusc Mytilus galloprovincialis. *Science of the total environment*, *700*, 134914.

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iv. Stara, A., Pagano, M., Albano, M., Savoca, S., Di Bella, G., Albergamo, A., Koutkova, Z., Sandova, M., Velisek, J., Fabrello, J., Matozzo, V., & Faggio, C. (2021). Effects of long-term exposure of Mytilus galloprovincialis to thiacloprid: A multibiomarker approach. *Environmental Pollution*, *289*, 117892.

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1. Introduction

1.1 Environmental impact of pesticides

The use of plant protection products is one of the most common methods of protecting plants and plant products from harmful organisms. Pesticides are special substances used primarily in agriculture, they are added to the environment to control or kill pests, including insects, rodents, fungi and weeds. Similarly, pesticides can also be used as plant regulators (to stimulate or retard plant growth), defoliants (to cause premature leaf abscission or foliage loss), desiccants (to artificially accelerate the drying of plant tissue) and as nitrogen stabilisers (to inhibit the process of nitrification, denitrification, ammonia volatilisation or urease production through action on soil bacteria) (de Souza et al., 2020; Deidda et al., 2021). They are classified by their target organ (herbicides, insecticides, fungicides) according to their chemical structure, physical state, and source of origin (synthetic or extracted). The pesticide groups with the greatest variety of chemical compounds are herbicides, fungicides and insecticides. Herbicides are synthetic chemicals whose action aims to control and eliminate undesirable pests, such as weeds. These compounds act in different ways to affect photosynthesis inhibition and pigment synthesis restriction, thus inhibiting the amino acid formation and lipid synthesis, blocking cell division and inhibiting plant growth (Herrera-Herrera et al., 2016).

Insecticides are chemicals used to kill, repel, attract or disturb insects and their functions. These substances have mechanisms of action that vary depending on the type of insecticide and can be classified into neonicotinoids, organophosphates, carbohydrates, pyrethroids and phenylpyrazoles (Bonnafé et al., 2017).

Fungicides are chemicals applied to plants or seeds to prevent fungal infections, thus protecting tubers, fruits and vegetables. Their mode of action varies depending on the type of fungicide substance; generally, they damage the cell membrane, affect specific metabolic processes, inactivate enzymes and/or alter processes related to energy production and respiration (Atmaca et al., 2018; Correia et al., 2016; Gupta, 2017).

The impact of pesticide use on public health is very high. The risks to human health are both direct (for agricultural workers) and indirect (for individuals exposed to it by chance) (Brasil et al., 2018). Global epidemiological data show that pesticides may be responsible for 350,000-440,000 deaths each year, with 99% of poisonings occurring in developing countries (Sulaj et al., 2015). Literature reviews have shown that human exposure to pesticides may be associated with chronic non-cancer effects, including dermatological, neurological, reproductive and genotoxic effects (Sanborn et al., 2007) and carcinogenic effects, such as lung, breast, pancreatic, brain, prostate, stomach, ovarian and kidney cancer, as well as non-Hodgkin's lymphoma and leukaemia (Bassil et al., 2007).

Pesticides move from the targeted application site in several ways causing dispersal mainly due to their mode of application and natural conditions. The transport pathways of pesticides in the environment and subsequent dispersal (Fig.1) are resulting from:

- wind drift - when the mixture is sprayed on the crop, a foggy mass is formed, consisting of small droplets that are carried more or less far from the treated area, mainly due to wind's speed at the moment of application or the type of spraying machine: part of the sprayed mixture falls on the soil and vegetation surrounding the crop;
- volatilization - the mixture, during the treatment or after reaching the crop or the water body (if present in the vicinity), from the soil, may evaporate in the air and be carried away by the wind;
- runoff - the mixture, once it reaches the soil after treatment, may be transported along the soil surface following a rain event or through irrigation. Similarly, the active substance of the mixture can strongly adhere to soil particles and be transported with them when erosion occurs during heavy rainfall. In this way, the active substance can reach a surface water body;
- leaching - following rainfall, the active substance that has reached the soil may leach - as a result of rainfall, the active substance that has reached the soil can penetrate through the soil, dissolved in percolating water, and thereby reach the groundwater.

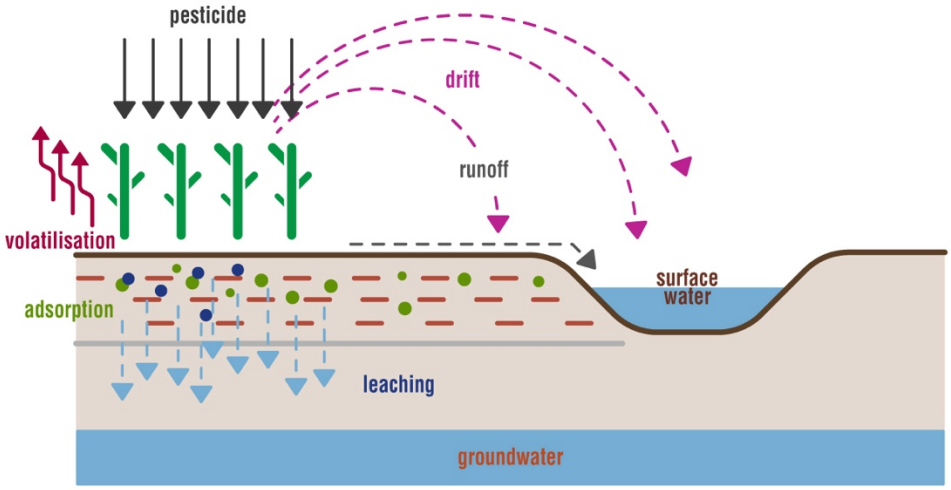


Figure 1: Pesticide dispersal mechanisms.

Exposure to chemical pesticides is extremely destructive to flora, fauna and the environment. Pesticides can pollute soil, water, grass and other plants (Prasad, 2020).

In addition to killing insects or weeds, pesticides could be poisonous to other creatures such as birds, fish, beneficial insects and non-target vegetation. Insecticides are usually more poisonous, but herbicides could also pose a danger to non-target creatures (Ali et al., 2021). The use of pesticides in agriculture may contaminate surface water through drainage, drift, runoff and leaching. Contaminated surface water has negative effects on living organisms. Aquatic life standards in the United States assess the ecological risk for pesticides, which is evaluated by the USEPA Office of Pesticide Programs (OPP) and the Environmental Protection Agency. In Europe, the predicted no-effect content depends on the European Pesticides Directive 91/414/EEC (Rani et al., 2021). Pesticides can reach groundwater not only through leaching from the polluted surface but also through accidental spills and leaks and improper disposal. Pesticide contamination of water causes the greatest risk to water bodies, mainly by lowering dissolved oxygen levels in the water, affecting aquatic species at many trophic levels, from algae to fish. The massive use of pesticides could lead to a decrease in fish numbers (Scholz et al., 2012). Aquatic animals are exposed to pesticides in three ways: dermally (direct absorption through the skin), through respiration (absorption through the gills by breathing) and orally (intake by drinking contaminated water) (Rani et al., 2021). Oxygen levels decrease rapidly due to the killing of aquatic plants by herbicides, which eventually leads to fish suffocation and a decrease in fish production. The reproductive capacity of aquatic species has also been affected due to the use of herbicides nearby fish ponds.

Although their application is functional, the very nature of pesticides is to harm unwanted organisms, thus raising important issues about their potential risks, even to non-target organisms. Indeed, many molecular targets of pesticides are shared between pests and non-target species, including humans, which can lead to a variety of adverse health effects. The overuse and/or misuse of pesticides is playing a negative role in environmental health, affecting many aquatic and terrestrial species, to the extent that they are now considered contaminants of emerging concern (CECs), i.e. 'new' pollutants in aquatic environments (Shahid et al., 2021).

Based on all these considerations, it is very difficult to strike the right balance between the benefits and harms of pesticide use.

Insecticides are toxic substances that act on various physiological systems, causing the death of insects. They are therefore used in agriculture to eliminate pests. The sites of action on which they act are (Fig.2):

- Synapses: carbamates and organophosphates can inhibit cholinesterase, which means that only acetylcholine acts on the synapses, causing the insect's muscles to contract rapidly and continuously, leading to paralysis and death. The advantage of using these insecticides is that they pollute the environment only minimally because they degrade so fast (Kaur et al., 2019). Others, such as neonicotinoids and spinosad, stimulate acetylcholine receptors by mimicking the action of the neurotransmitter acetylcholine and consequently competing with its receptor (Bass et al., n.d.; Santos & Pereira, 2019). The activity of these insecticides leads to involuntary muscle

contractions and tremors until the insect becomes paralysed and dies (Salgado, 1998). Others act on the sodium channels, leaving their binding open and causing the animal to tremble and die. Insecticides such as pyrethrin, pyrethroids and some organochlorines such as 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (DDT) modulate sodium channels (Lushchak et al., 2018).

The chlorine channel is also a target of many insecticides that interact with it through different mechanisms of action. These types of pesticides can counteract, mimic or increase the activity of the neurotransmitter γ-aminobutyric acid (GABA), a neurotransmitter found both in mammals and in insect neuromuscular junctions (L. G. Costa, 2015; Lummis, 1990). Some insecticides such as lindane, cyclodienes and fipronil bind to the picrotoxin site on the chloride channel, blocking its opening and effectively inhibiting GABA activity (Kaur et al., 2019). Other types such as avermectin amplify GABA activity, causing hyperpolarisation and muscle activity shutdown resulting in death (Batiha et al., 2020). Others, such as bifenazate, are agonists for the GABA receptor without amplifying the activity of the neurotransmitter itself (Lushchak et al., 2018).

- Growth and development: the mode of action of this type of insecticide can either target chitin synthesis or insect growth. In the former case, we find benzoyluric insecticides that can cause abortive death and defects in egg hatching, generating structural changes in the cuticle (Lushchak et al., 2018). In the latter case, we can find specific and non-specific growth regulators. The specific ones are endocrine disruptors that block metamorphosis because they are either analogue of the juvenile hormone (Jh) or its antagonists (Tunaz & Uygun, 2004) Non-specific ones do not have a clear mechanism of action, e.g. hexythiazox blocks the hatching of mite eggs, but others act at other stages of development (Alzoubi & Cobanoglu, 2008).
- Energy production: In this case, insecticides act on energy metabolism either by interfering with electron transport, as aliphatic organochlorines do (Gopalan & Chenicherry, 2018; Pany et al., 2020)or by disrupting oxidative phosphorylation, as pyrroles do, which uncouple electron transport and oxidative phosphorylation (Boukouvala et al., 2016).

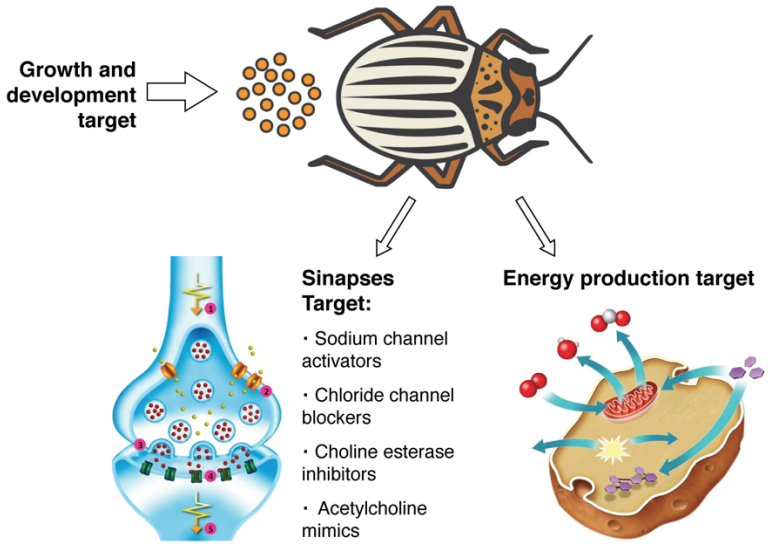


Figure 2: Most common pesticide targets.

1.2 Neonicotinoids

Neonicotinoids were developed in the late 1980s (Borsuah et al., 2020). Further development was carried out during the 1990s by Bayer (a pioneer in neonicotinoid research) and the first neonicotinoid-based product, introduced to the market in 1991, was imidacloprid. There are currently seven compounds, chemically classified as N-nitroguanidines (imidacloprid [1-((6-Cloro-3-piridinil)metil)-N-nitro-2-imidazolidinimmina], thiamethoxan [{3-[(2-Chloro-1,3-thiazol-5-yl)methyl]-5-methyl-1,3,5-oxadiazinan-4-ylidene}nitramide], clothianidin [1-(2-Chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine] and dinotefuran [2-methyl-1-nitro-3-[(tetrahydro-3-furanyl) methyl] guanidine]), nitromethylenes (nitenpyram [(*E*)-N^1-[(6-Chloropyridin-3-yl)methyl]-N^1-ethyl-N'^1-methyl-2-nitroethene-1,1-diamine]) and N-cyanoamidines (acetamiprid [*N*-[(6-chloro-3-pyridyl)methyl]-*N'*-cyano-*N*-methyl-acetamidine] and thiacloprid [{(2*Z*)-3-[(6-Chloropyridin-3-yl)methyl]-1,3-thiazolidin-2-ylidene}cyanamide]) (Bass et al.,2018) (Fig.3). The different active ingredients are marketed under different trade names and the main ones are imidacloprid produced by Bayer Crop Science, displayed as Confindor, Gaucho, Warrant, Kohinor; thiamethoxan, produced by Sygenta is found under the names Actara 25WG and Cruiser; clothianidin, produced by Sumitomo Chemical Takeda Agro Co. /Bayer Crop Science, marketed as Dantop 50WG and Poncho; dinotefuran, produced by Mitsui chemicals as Dinotefuran 20%SC; nitenpyram, produced by Sumitomo Chemical Takeda Agro Co. under the name Capstar 11,4mg; acetamiprid,

produced by Nippon Soda labelled Epik; thiacloprid, produced by Bayer Crop Science, commercialised as Sonido and Calypso.

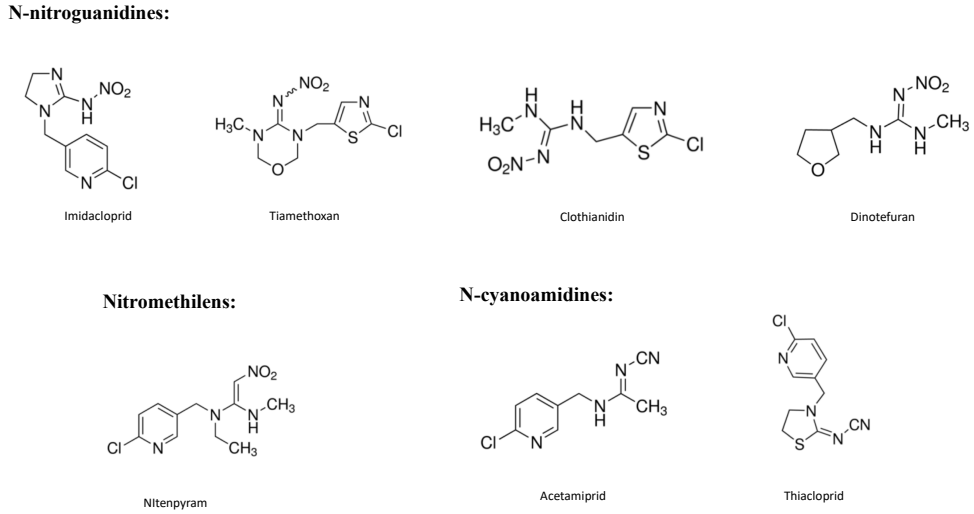


Figure 3: Structural formulae of the seven synthesised neonicotinoids.

They have been very successful in the pesticide market because, compared to classical pesticides, they are used in low doses, are absorbed and act quickly(Ahmad Kundoo et al., 2018; Buszewski et al., 2019; Hladik et al., 2018).Moreover, they have high solubility, are quite persistent in water and soil and are not volatile, which means that they can be distributed over large expanses of land (Hladik et al., 2018). Due to their versatility, they are used for various treatment techniques on foliar, seed, soil and stem applications against sap-feeding insects such as aphids, whiteflies, plant tissue-chewing insects such as beetles and the larvae of various moths (Bass et al., n.d.; Borsuah et al., 2020). As well as being employed for agricultural use in a wide variety of crops such as vegetables, pumice, stone fruits, citrus fruits, rice, cotton, maize, potatoes, sugar beet, oilseed rape and soybeans, neonicotinoids are also applied in household and garden products to control termites, ants and cockroaches and to prevent and control human and animal pests such as bedbugs, fleas and ticks (Bass et al., n.d.; Craddock et al., 2019). Neonicotinoids do not persist on the surface of the plant, but they penetrate its vascular system and reach the entire organism. This is the reason why some treatments start at the seed to protect the plant from germination to growth (Ahmad Kundoo et al., 2018).

Neonicotinoids are selective for nicotinic acetylcholine receptors (nAChRs). In mammals these are located in the central and peripheral nervous systems (CNS and PNS), whereas in insects are found only in the central nervous system. nAChRs are a large family of ligand-dependent ion channels responsible for generating rapid excitatory cholinergic neurotransmission in the CNS. In insect nervous system, acetylcholinesterase (AChE) breaks down the normal neurotransmitter acetylcholine

and this terminates nerve signals in normal synaptic transmission across nerve cells. Neonicotinoids that bind to AChR cannot be cleaved by AChE and this leads to overstimulation of the sensory system, paralysis and eventually death (Fig. 4).

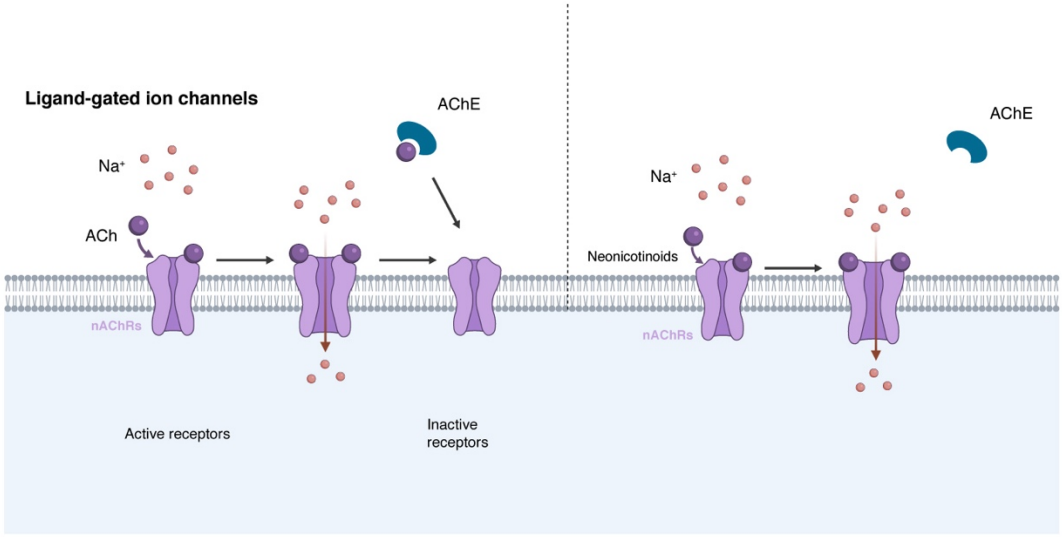


Figure 4: Schematic presentation of the acetylcholine receptor action of neonicotinoids in the presence of acetylcholine and a neonicotinoid pesticide

Neonicotinoids are thought to bind to insect nAChRs more efficiently than to vertebrates. Their selectivity for the insect CNS is linked to the presence of negatively charged nitro or cyano groups that interact with the cationic site within the receptor (Ewere et al., 2021; Rodrigues et al., 2010; Tomizawa & Casida, 2005). The nicotinic receptor is a pentameric molecule composed of 5 subunits that may be identical in the monomeric receptor or different in the heteromeric one, organised to form a central hole that is selective for Na^+, K^+ and Ca^{2+}. The full composition of insect receptor subunits is not yet fully understood. The only fully known subunit consists, as in vertebrates, of a large N-terminal domain involved in agonist binding, followed by three transmembrane regions (TM2-TM3-TM4) and an extracellular C-terminal region. A phosphorylation site is present between the TM3 and TM4 domains. Although not all subunits are known, from a pharmacological point of view, two distinct classes of nAChR receptors can be classified as more or less sensitive to **α**-Bungarotoxin: **α**-Bgt-sensitive and **α**-Bgt -insensitive (Taillebois et al., 2018; Thany et al., 2007; Tomizawa & Casida, 2001).

Neonicotinoids have been widely used in agriculture due to their great potential. This has brought an advantage in cultivation, but the non-specificity of these substances has created great ecological damage. Bees also became targets of these insecticides (Liu et al., 2020). This is precisely why the European Commission banned the use of three neonicotinoids (Clothianidin, Imidacloprid and Thiamethoxan) in 2018 because of their negative impact on bee life.

Since the main neonicotinoids in agricultural use were restricted in 2013 and then banned in 2018, thiacloprid has become the most widely used neonicotinoid. It was launched under the trade name Calypso and is active against agricultural pests on crops such as fruit, cotton, vegetables, oilseed rape, cereals, potatoes, rice (Jeschke et al., 2011). It has also proven to have a beneficial profile on bees, insomuch that it has also been applied to flowering crops (Jeschke et al., 2011). Because of its high solubility in water (184mg/L) and low octanol-water partition coefficient (log Kow: 1,26, 20°C), the potential for thiacloprid to migrate into surface water or groundwater is a major hazard. In addition, thiacloprid is stable under anaerobic aquatic conditions, with a half-life of more than one year, and degrades under aerobic aquatic conditions with a half-life of 10-63 days (Velisek & Stara, 2018a; P. Zhang et al., 2018). Due to its chemical and physical characteristics, thiacloprid is frequently found in rivers and lakes. In surface waters worldwide, it has been recorded at concentrations ranging from 0.2 to 4.50 μg/L. The highest concentration has been recorded in the surface waters of canals flowing into the Elbe River near Hamburg (Süß et al., 2006b).

However, since 30 April 2020, the use of thiacloprid has also been banned, because it has been shown to have sub-lethal effects on bees, altering their behaviour and interacting with their immune system (Bass et al., n.d.; Ewere et al., 2021; Fent et al., 2020; Ihara et al., 2017).

1.3 Organophosphorus insecticides

Organophosphorus compounds (OP) are a group of about 250 substances produced worldwide. Of these 140 are pesticides, the rest are industrial chemicals used as flame retardants, plasticisers and industrial hydraulic fluids and solvents (Farag M et al., 2018).

Organophosphorus insecticides are anticholinesterase agents (anti-AChE). The first synthesis of this kind of compound was made in 1854 by Clermont, who synthesised the very toxic compound tetraethyl pyrophosphate (TEPP). The synthesis of these substances continued during the early years of the new century, they were first used as agricultural insecticides and then as potential chemical warfare agents (L. G. Costa, 2018; Karalliedde & Senanayake, 1988). During World War II, several highly toxic compounds were developed as nerve gases in Germany such as ethyl N-dimethylphosphomidicyanate. The use of organophosphorus pesticides increased around the 1970s, reducing the use of organochlorine pesticides (OCPs) such as DDT. POs are more toxic than OCPs; POs do not persist in the environment for more than a

few months, therefore the risk of finding them in food and then in the human food chain is reduced (Paidi et al., 2021).
These pesticides are related to phosphoric acid esters and are divided into three main classes (Fig.5):
- phosphorothioates (fenitrothion, chlorpyriphos, diazinon, methacrifos, tryazophs, parathion methyl, pirimiphos- methyl);
- phosphorodithioates (azinphos-ethyl, disulfoton, methidation, phorate);
- phosphoramidotioates (acephate, mathamidophos).

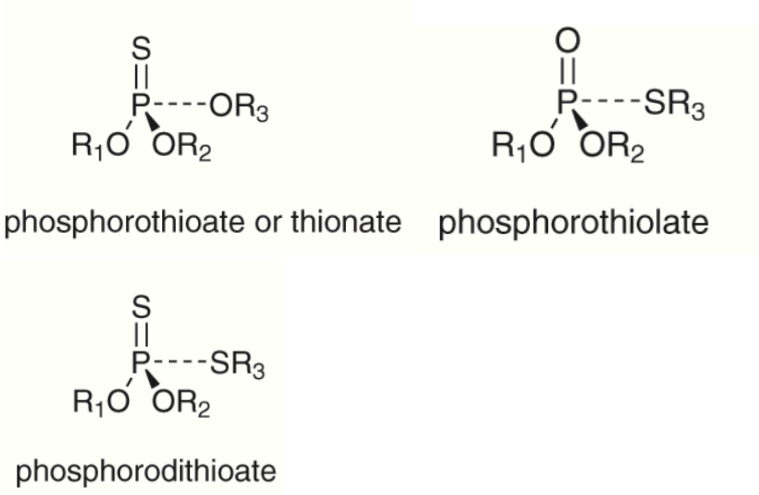


Figure 5: Structures of the main classes of organophosphorus pesticides.

The target of organophosphorus is acetylcholinesterase. The enzyme controls the hydrolysis of acetylcholine released from cholinergic nerve endings into choline and acetic acid. The active centre of acetylcholinesterase contains two reactive sites: an anionic site (negatively charged) containing a glutamic acid residue that binds the cationic part of the substrate, namely the quaternary nitrogen of choline, by both electrostatic and hydrophobic forces, and an esterase site containing the primary alcohol group of the amino acid serine that provides nucleophilic attachment to the electrophilic carbonyl carbon of the substrate (Faria et al., 2015, 2021; Pergal et al., 2020). The catalytic mechanism by which acetylcholine is hydrolysed resembles that of other serine esterases, in which the hydroxyl group of serine is rendered highly nucleophilic by a charge-shifting system involving the close apposition of an imidazole group and, presumably, a carboxyl group in the enzyme. During the enzymatic attack on the ester, a tetrahedral intermediate is formed between the enzyme and the ester, giving rise to a conjugate, the acetylated enzyme, with the concomitant release of choline. The acetylated enzyme, in turn, is rapidly hydrolysed resulting in the formation of acetate and the restoration of the active enzyme. This process is probably facilitated by a histidine residue present in the ester site of the enzyme.
Organophosphorus compounds have some structural similarities to acetylcholine. The phosphate group is attracted to the esterase site, while the rest of the molecule is

conveniently arranged in space as a result of interactions with the side groups of the amino acids that make up the entire active area of the enzyme. Organophosphorus reacts with the hydroxyl group of serine, within the enzyme's active site, phosphorylating this group and resulting in a hydroxylate generally known as a leaving group. This process results in the irreversible inactivation of the enzyme, which blocks the degradation of the neurotransmitter acetylcholine (Fig. 6). As this inhibitory activity continues over time, synaptic concentrations of acetylcholine increase, leading to the blockage of the nerve impulse in insects, resulting in their death.

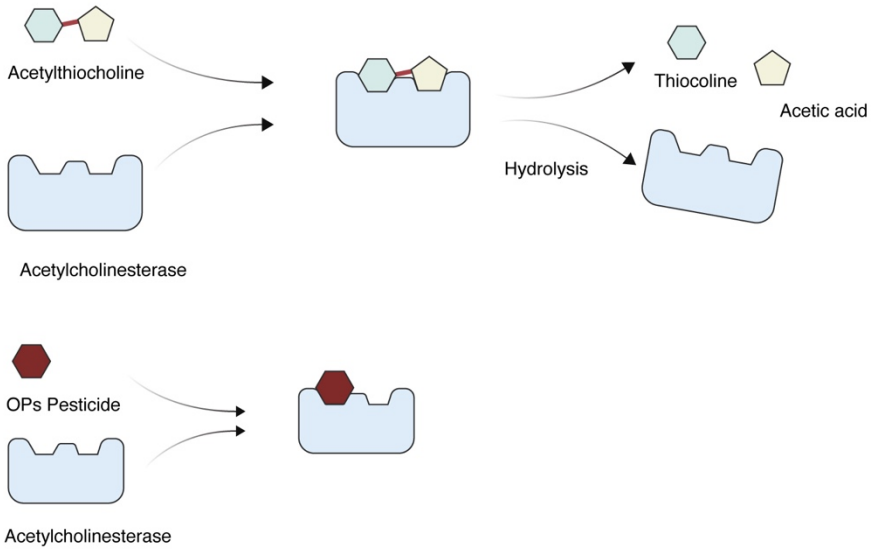


Figure 6: Mechanism of action of POs

In addition to the serious neurotoxic problems for human health, the great danger is that the chemical residues of POs that resist degradation may contaminate the food chain and thus be harmful to humans.(Paidi et al., 2021; Pergal et al., 2020).
Of these compounds, fenitrothion is the most widely used due to its cost-effectiveness and effectiveness. It is used in agriculture for cereal, rice, fruit and vegetable crops, but is also used in public health programs to control flies, mosquitoes and cockroaches (Faria et al., 2021; Ramirez-Priego et al., 2021). While in the immediate vicinity of treated areas fenitrothion concentrations are between 15 and 75 μg/L, in areas not directly exposed the concentrations are much lower between 6-600 ng/L (Faria et al., 2021).

1.4 Non-target organisms: Mytilus galloprovincialis and Danio rerio

1.4.1 Mytilus galloprovincialis

Mussels belong to the phylum Mollusca, the largest and most diverse taxa in the animal kingdom. The number of mollusc species has been estimated at 93000 and 25% of them are marine. Bivalves are morphologically characterised by the presence of a bivalve shell, filtering gills, digestive gland, and the byssus (Fig.7), which is a key feature for the filtration process (Álvarez-Ruiz et al., 2021). Marine mussels also have a significant commercial value. Their production accounts for 50% of overall EU aquaculture by weight and about 30% by value. The most important mussel species are the Mediterranean mussel (*Mytilus galloprovincialis, Lamarck 1819*) and the blue mussel (*Mytilus edulis*) (Murgarella et al., 2016). Mussels are organisms used *in vivo* and *in vitro* for ecotoxicology studies (Curpan et al., 2022; Faggio et al., 2010a, 2016a, 2016b; Pagano et al., 2017; Torre et al., 2013). Molluscs are typical bio-indicators for ecotoxicological and bioaccumulation studies of pollutants such as heavy metals, due to their abundance and presence in aquatic environments, their physiological characteristics and their ecological and economic relevance (Álvarez-Ruiz et al., 2021). The impact of dissolved substances in water is complex and can directly or indirectly affect different populations and ecosystems. The oldest ongoing biomonitoring programme is Mussel Watch in which mussels are used as 'sentinel organisms' (Azizi et al., 2018). *M. galloprovincialis* has been used for several *in vitro* ecotoxicology studies to assess the risk of exposure to pesticides(Middlemore-Risher et al., 2010; Murussi et al., 2013; Pagano et al., 2020; Patetsini et al., 2013; Stara, Pagano, et al., 2019; Stara et al., 2021; Syafrudin et al., 2021), detergents (Messina et al., 2014), heavy metals (Bouzidi et al., 2021; Faggio et al., 2010b; Pagano et al., 2017; Torre et al., 2013), industrial compounds (Li et al., 2021; Xu et al., 2020), plastics(Burgos-Aceves et al., 2021a, 2021b), and preservatives (Faggio et al., 2016a; Pagano et al., 2016).

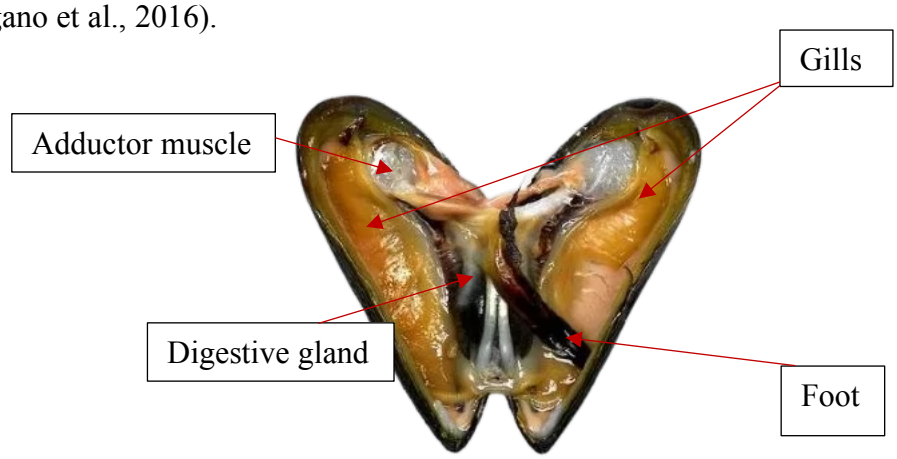


Figure 7: Anatomy of mussels

1.4.2 Danio rerio

The zebrafish (*Danio rerio)* is a small freshwater fish, a teleost cyprinid, native to the rivers of India and popular worldwide as an aquarium fish (Khan et al., 2019). It was first used as a model organism in the 1980s by George Streinsinger (Streisinger et al., 1981), who recognised its excellent experimental qualities:
- the short generation time;
- the large number of eggs produced per mating;
- fertilisation is external and therefore all stages of development are clearly visible;
- the primary organs develop within 5 days post-fertilisation;
- low maintenance cost (Basnet et al., 2019; Briggs, 2002; Streisinger et al., 1981; Trigueiro et al., 2020).

We know now from genome sequencing that most zebrafish genes are common to humans, with 84% of known genes associated with human diseases found in zebrafish. These fish also have high homology with mammalian morphology and biology, which is useful in the study of human diseases (Faria et al., 2015; Kalueff et al., 2014; Lucini et al., 2018; Trigueiro et al., 2020). Zebrafish is also a valid model organism for behavioural studies through analysis of social activities, anxiety, learning and memory usage for modelling neurological and psychiatric diseases (Basnet et al., 2019; Kalueff et al., 2014). To reduce the use of adult animals in research, following the principles of the 3Rs (Replacement, Reduction and Refinement) many studies in these fields are carried out on zebrafish larvae. In addition, zebrafish larvae are widely used to detect toxins by performing in vitro toxicity tests of substances contaminating the water (Capela, Garric, Castro, et al., 2020; Capela, Garric, Costa Castro, et al., 2020; W. Q. Wang et al., 2022; Wilson et al., 2021).

2. Aims of the study

The aim of this work is to assess the risks to the ecosystem of water contamination by insecticides widely used in both agricultural and domestic treatments. This type of study supports the concept of One Health, using integrative approaches to link the study of factors underlying responses to toxic exposures in humans and animals, to their consequences for ecosystem functioning and health.

The work was divided into two sections:

1. Acute and sub-chronic exposure of *Mytilus galloprovincialis* to the commercial product Calypso, which contains 40.4% of the active ingredient thiacloprid; and acute and chronic exposure of *Mytilus galloprovincialis* to the pure molecule thiacloprid (**PAPERS I, II, III, IV**);

2. Exposure of *Danio rerio* larvae to environmental concentrations of fenitrothion (**PAPER V)**.

3. Materials and methods

3.1 Neonicotinoid exposure to Mytilus galloprovincialis

3.1.1 Animals collections

Mytilus galloprovincialis, 6.2 ± 0.13 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. Faro is a small meromictic marine coastal lagoon. It covers an area of 26 hectares and has an almost circular shape with a diameter of about 500 m. This basin has an average depth of about 3 m, while in its central part it reaches a depth of 30 m. It communicates with the Tyrrhenian Sea through an artificial channel (commonly called the English Channel) which is open only sporadically during the summer and continuously with the Strait of Messina through the Canale del Faro (D’iglio et al., 2021; Sanfilippo et al., 2022). The Faro Lagoon is still largely exploited for bivalve rearing and cultivation.

3.1.2 Chemicals

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.

3.1.3 Experimental design for acute exposure of Mytilus galloprovincialis to Calypso 480

Sixteen mussels per duplicated tank (n= 32) were exposed for seven days to concentrations of Calypso 480SC:10 mg L^{-1} (CA1), 50 mg L^{-1} (CA2) and 100 mg L^{-1} (CA3). A control group without tested substances (CCA) was also prepared. Twelve mussels from each experimental group were randomly collected on the seventh day of exposure. Four pools of three mussels each were used for analyses of cell viability and biochemical haemolymph parameters. Immediately after haemolymph sampling, gills and digestive glands were readily dissected on ice and stored at − 80 ◦C until the analysis of antioxidant biomarkers, as well as the histopathological examination. Four mussels from each group were isolated to obtain digestive cells.

3.1.4 Experimental design for chronic exposure of Mytilus galloprovincialis to Calypso 480

One-hundred-sixty-two mussels were kept in aquaria filled with continuously aerated brackish water in the laboratory with daylight exposure 12 h light: 12 h dark and a temperature of 18.17±0.84°C for 5 days of acclimation before the onset of any experimental procedure. After acclimation, twenty-seven mussels were randomly selected and placed into each of six aquaria (three experimental groups in duplicated) containing 20 L of 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. Samples of mussels were sampled for laboratory treatments after 10 and 20 days of exposure to CAL.

3.1.5 Experimental design for acute exposure of Mytilus galloprovincialis to Thiacloprid

Sixteen mussels per duplicated tank (n= 32) were exposed for seven days to concentrations of thiacloprid: 1 mg L^{-1} (TH1), 5mg L^{-1} (TH2) and 10 mg L^{-1} (TH3) and a control group without tested substances (CTH) was also prepared. Twelve mussels from each experimental group were randomly collected on the seventh day of exposure. Four pools of three mussels each were used for analyses of cell viability and biochemical haemolymph parameters. Immediately after haemolymph sampling, gills and digestive glands were readily dissected on ice and stored at − 80°C until the analysis of antioxidant biomarkers, as well as the histopathological examination. Four mussels from each group were isolated to obtain digestive cells.

3.1.6 Experimental design for chronic exposure of Mytilus galloprovincialis to Thiacloprid

Following acclimation, mussels were randomly split into three pools of ninety individuals. Each group was transferred to 40 L experimental tanks in duplicate. One set was the Control group (n = 45/45) and was housed in thiacloprid-free seawater; two sets of experimental mussels (n = 45/45) were maintained under controlled conditions (aeration, temperature 23.03 ± 0.6 °C, pH 7.54 ± 0.16, salinity 31.79 ± 1.37, 1100 m Osm kg^{-1}, 12 h light:12 h dark) in the presence of thiacloprid for long- term exposure for 20 days. We tested two concentrations of thiacloprid: the first was an environmentally relevant concentration, namely, 4.5 µg L^{-1} (Thia env.), as referenced by (Süß et al., 2006a), whereas the second was 450 µg L^{-1} (Thia 100x), which corresponded to 100 times the environmental concentration. Twelve mussels from each experimental group were randomly collected on the 10th and 20th days of exposure. Six pools of two mussels each were used for analyses of cell viability and biochemical haemolymph parameters. Immediately after haemolymph sampling, gills and digestive glands were readily dissected on ice and stored at − 80 ◦C until the analysis of antioxidant biomarkers, as well as the histopathological examination. Four mussels from each group were isolated to obtain digestive cells.

3.1.7 Haemolymph collection

The haemolymph was collected from the anterior adductor muscle with a 23-gauge needle, in 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 purposes, pooling was necessary to obtain a volume of haemolymph enough.

3.1.8 Isolation of digestive cells

The digestive gland cells were isolated using the method described in Torre et al., 2013. The digestive glands were cut into pieces and washed with a solution Ca^{2+} and Mg^{2+} free (CMSF, Table 1). Tissue samples were cut into small pieces and transferred to a test tube containing 001% collagenase [type IV-activity ≥ 125 CDU mg^{-1}; collagenase digestion units (CDU), Sigma Aldrich, St. Louis, MO, USA] dissolved in CMSF (Table 1). The test tube was delicately stirred for 60 min at 18° C in a thermostatic bath. Afterwards, the suspension was filtered through 200 and 75mm nylon filters. The cells were suspended in physiological saline (Isotonic solution, Table 1), washed twice by centrifugation (500 rpm for 10 min at 4°C), and then put again in Isotonic solution. Before the experiments, the cells were kept in physiological saline (Isotonic solution, Table 1) at 18 ° C for 1 h to re-establish ionic concentration on either side of the cell membrane.

Table 1: Ionic composition of the solutions (concentration in millimolar)

	CMFS (1)	**ISOTONIC SOLUTION (2)**	**HYPOTONIC SOLUTION (3)**
NaCl	600	550	350
KCl	12.5	12.5	12.5
$MgSO_4$	-	8	8
$CaCl_2$	-	4	4
$MgCl_2$	-	40	40
Glucose	-	10	10
Hepes	20	20	20

CMFS: Isotonic solution without added Ca^{2+} and Mg^{2+} ,1N NaOH was added to the solutions to obtain pH = 7.3; p = 1100 ± 10 mOsm Kg1 (Sols 1 and 2), p=800±10 $mOsmKg^{-1}$ (Sol 3)

3.1.9 Cell viability assay

The viability of the isolated digestive cells was evaluated by: 1) the Trypan blue exclusion method; and 2) the stability of the lysosomal membrane by neutral red retention assay (NR). The viability of digestive cells was performed by the Trypan blue exclusion method. It is based on the principle that living cells possess intact cell membranes that exclude certain dyes, such as trypan blue (Direct blue 14, Sigma Aldrich, Steinheim, Germany), whereas dead cells do not. The percentage of unstained cells represented the percentage of viable cells in the suspension. The cytotoxicity based on alterations in plasma membrane permeability was also measured.

The viability of digestive cells was performed also by Neutral red retention assay.

This test is based on the ability of viable cells to incorporate and bind the supravital

dye neutral red (Sigma Aldrich, Steinheim, Germany) in the lysosomes (Repetto et al., 2008).

The percentage of cell viability was calculated by the formula:

Cell viability (%) = (Number of viable cells/Total number of cells) × 100

3.1.10 The Regulation of Volume Decrease experiment

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 has 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 a hypotonic solution, and the image was 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.

3.1.11 Haemolymph biochemical parameters

Haemolymph was centrifuged for 10 min at 10 000 rpm at 4 °C; subsequently, the supernatant was collected and stored in a freezer at − 20°C until the determination of haemolymph biochemical parameters. Haemolymph supernatants were thawed and used to measure urea, inorganic phosphate (PHOS), calcium (Ca^{2+}), magnesium (Mg^{2+}), albumin (ALB), alanine aminotransferase (ALT) and ammonia (NH_3) levels. Haemolymph biochemical parameters were determined using the IDEXX Catalyst One analyser (IDEXX Laboratories Inc., Maine, USA).

3.1.12 Determination of enzyme activity

The digestive gland and gill tissues of six mussels from each group were excised and frozen at - 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 to 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. The concentration of protein in the supernatant was measured according to Bradford, (1976) using bovine serum albumin as the standard.

3.1.13 Histological analysis and Histopathological condition indices

Immediately after haemolymph sampling, gills and digestive gland were quickly removed on ice and stored fixed in Immunofix (paraformaldehyde 4% in phosphate saline buffer, Bio-Optica, Milan, Italy) for 12 h at room temperature for histopathological condition evaluation. An investigation on histological conditions of digestive glands and gills was performed. Sampled fractions of both tissues from each treatment group were collected in triplicate from three specimens. Tissues were embedded in paraffin and successively sectioned to 5 μm slices by a microtome (Leica, RM2235). The obtained sections were stained using Hematoxylin and Eosin stain for a qualitative histopathological examination using a light microscope (Leitz Diaplan, Germany).

Semi-quantitative weighted indices approach described by (Bernet et al., 1999) for fish, with some modifications proposed by (P. M. Costa et al., 2013) has been applied for the evaluation of each individual histo- pathological indices (*Ih*). Each damage was assigned a weight (bio-logical significance) with a value ranging between 1 and 3 (maximum severity), and a score (degree of dissemination) with values between 0 (in the case that damage is not present) and 6 (in the case damage is diffuse). The formula for the assessment of histopathological condition indices is:

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

where I_h is the histopathological condition index for the individual h; w_j the weight of the jth histopathological damage; a_{jh} the score attributed to the hth individual for the jth damage and M_j is the maximum attributable value for the jth damage, *i.e.*, weight x 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 "different histological patterns": morphological epithelial modifications (gills), tubule and intertubular tissue damages for the digestive gland. The condition weights used have been based on observations collected in this experiment and partially on literature about both invertebrates (Costa et al., 2013) and vertebrate histopathology. In accordance, the weight assigned is shown in Table 2.

Table 2:Histological patterns. Weights assigned to each digestive gland and gill damage

Digestive Gland		Gill	
Damage	**Weight**	**Damage**	**Weight**
Tubule damages	**Cellular and morphological changes**		
Epithelial cell hyperplasia	2	Epithelial cell hyperplasia	2
Epithelial cell hypertrophy	2	Vacuolation	1
Brown cells	1	Haemocytes infiltration	1
Haemocytes infiltration	1	Granulocytoma	2
Lipofuscin aggregates	1	Lipofuscin aggregates	1
Necrosis	3	Fibrosis	2
Tubule regression	2	Necrosis	3
Intertubular damage		Loss of epithelia	3
Haemocytes infiltration	1	Lamellar fusion	1
Lipofuscin aggregates	1	Lamellar deformation	1
Brown cells	1	Epithelial detachment	1
Fibrosis	2		
Necrosis	3		
Granulocytoma	2		

3.1.14 Statistical analysis

The software package Prism 5.00 (GraphPad Software LDT., USA, 2003) was used for statistical analyses of cytotoxicity assay and RVD. One-way ANOVA and two-way ANOVA were used to test the differences between the control and treatment groups, and the Tukey test allowed pairwise comparisons among experimental conditions ($P < 0.05$). For the analysis of differences between the Control and treated groups at each sampling time point, two-way repeated-measures ANOVA was used. The results of the cytotoxicity assay were compared with Student's t-test. A value of

$P < 0.05$ was accepted as significant. The results are presented as the SD standard deviations).

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 Ih 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.

3.2 Zebrafish larvae exposed to environmental concentrations of fenitrothion

3.2.1 Fish husbandry and larvae production

Adult wild-type zebrafish were purchased from Piscicultura Superior SL (Parets del Vallès, Barcelona) and maintained in fish water [reverse-osmosis purified water containing 90 μg/mL of Instant Ocean (Aquarium Systems, Sarrebourg, France) and 0.58 mM $CaSO_4 \cdot 2H_2O$] at 28 ± 1 °C in the Research and Development Center of the Spanish Research Council (CID-CSIC) facilities under standard conditions. Embryos were obtained by in-tank group breeding with a 5:3, female: male ratio per tank. Breeding tanks are homemade and include a solid external tank and an internal plastic net (Fig. 8). Embryos deposited in the bottom of the tank were collected and maintained in 500 mL glass containers at 1 individual/mL density in fish water at 28.5 °C on a 12 light:12 dark photoperiod. Larvae were not fed before or during the experimental period (from 7 to 8 days post fertilization (dpf)). All procedures were approved by the Institutional Animal Care and Use Committees at the CID-CSIC and conducted in accordance with the institutional guidelines under a license from the local government (agreement number 9027).

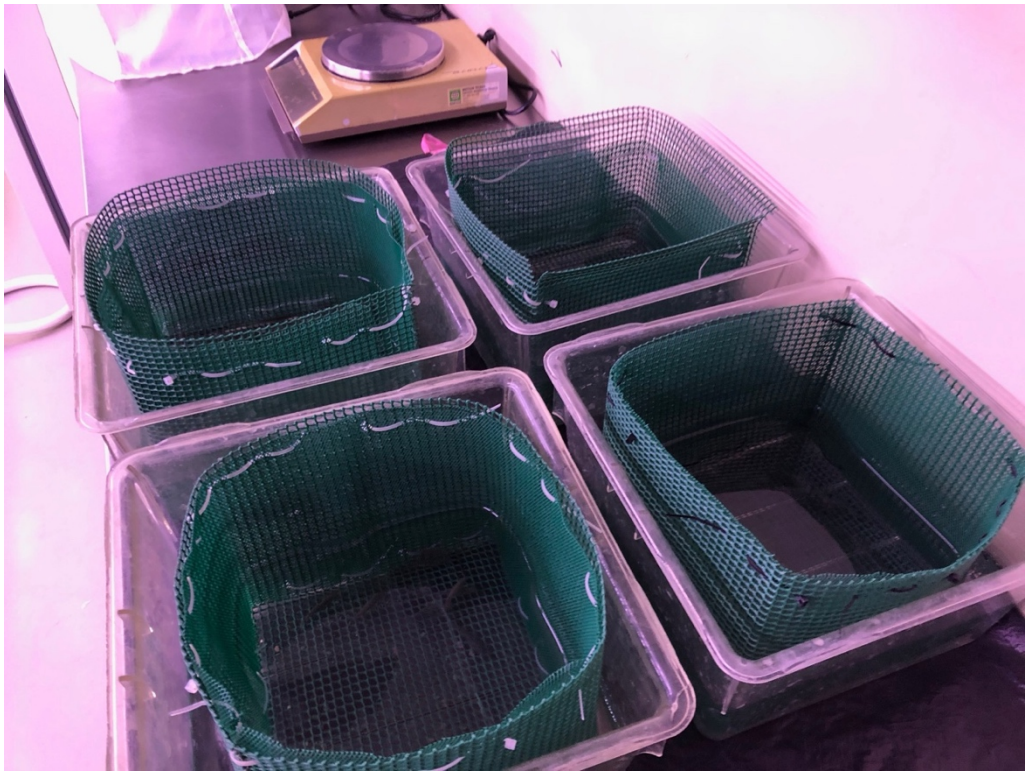


Figure 8: Breeding tanks

3.2.2 *Experimental concentration and stability of fenitrothion in fish and water*

Fenitrothion (Pestanal®, analytical standard, purity 95.4%) was obtained from Sigma-Aldrich (Steinheim, Germany). Experimental concentrations of the five nominal concentrations used in this study [17 µg /L (C5), 1.7 µg /L (C4), 170 ng/L (C3), 17 ng/L (C2), and 1.7 ng/L (C1)] have been determined using ultra-high-performance liquid chromatography with triple quadrupole detector (UPLC-MS/MS) analysis. Due to the low concentration levels analyzed, a solid-phase extraction (SPE) method was developed on a 20-port vacuum manifold (Waters, Milford, USA). OASIS HLB (200 mg, 6cc, Waters, USA) cartridges were used for water extraction since they showed good recoveries in previous studies of organophosphate pesticides (Terzopoulou et al., 2015). The cartridges were first conditioned using 5 mL dichloromethane/ ethyl acetate (50:50v/v), 5 mL hexane/dichloromethane (50:50 v/v), 1 mL methanol and 1 mL of Milli-Q water. Then, 20 mL of water sample without any pH adjustment were loaded at a 1 mL/min flow. Afterwards, cartridges were vacuum dried for 30 min ensuring that no residual water would elute with the final extract. The elution step was performed using the first 5 mL of hexane/dichloromethane (50:50 v/v) and 3 mL of dichloromethane/ethyl acetate. The final extract (8 mL) was evaporated to almost dryness under a gentle stream of N_2 in a Sample Concentrator (Techne®, Staffordshire, UK) and transferred to a 2 mL chromatographic vial with 1 mL of fish water as a washing solution. Finally, for the lowest concentrations (C2 and C1) samples were evaporated to dryness and reconstituted with 100 µL and 50 µL, respectively, of fish

water in a 150 µL insert chromatographic vial. Fenitrothion was analyzed using an ultra-high-performance liquid chromatography connected to a triple quadrupole detector (XEVO TQS, Acquity Waters, Mildford, USA) (UPLC-MS/MS). A Phenomenex Luna® Omega Polar C18 column (100 x 2.1 mm, 1.6 µm) was used. Mobile phase composition consisted of binary mixtures of 10 mM NH_4AcO in water (A) and methanol (B). Elution was performed in an isocratic mode consisting of 30% A and 70% B. The flow rate was set at 200 µL min^{-1} and 5 µL was injected. Fenitrothion was measured under positive electrospray ionization (ESI^+). Flow injection analysis (FIA) was performed to determine the optimum cone voltage in order to obtain the precursor ion ($[M+H]^+$=278), which was 40 V. Then, collision energy was optimized to obtain at least two intense fragment ions. The acquisition was performed in multiple reaction monitoring (MRM) mode using two transitions from the precursor ion to the production, in order to identify the compound: 278>245 (17 eV) and 278>109 (15 eV). The system and data management were processed using MassLynx v4.1 software package.

Three samples of each concentration were analysed. Furthermore, the stability of fenitrothion in fish water was assessed by preparing in triplicate solutions with a nominal concentration of 0.17 and 17 µg/L (representing the higher and lower levels of the concentrations studied), which were maintained under the same conditions used for zebrafish larvae (28 °C and 12 L:12D photoperiods). Aliquots of these solutions were analysed at times 0 and 24 h and analysed.

3.2.3 Experimental design

This study tested five fenitrothion concentrations 1.7 ng/L, 17 ng/L, 170 ng/L, 1.7 µg/L, and 17 µg/L. The two highest concentrations are representative of the situation in the vicinity of sprayed areas and the three lowest concentrations represent the situation in non-directly exposed areas. Fenitrothion stocks were prepared in DMSO, with a final carrier concentration of 0.1% in both vehicle control and treatment groups. The use of vehicle controls with 0.1% DMSO has been reported to be safe and is widely used to screen libraries of small chemicals in zebrafish (Maes et al., 2012; Vliet et al., 2017). The highest concentration of fenitrothion tested in this study, 17 µg/L, is already an environmentally relevant concentration that in a previous study was reported to reduce the magnitude of the vibrational-evoked startle response in zebrafish larvae after 24h exposure (Faria et al., 2020a).

Exposures were conducted in 48-well microplates with 1 larva per well and 1 mL of working solution (a total of 48 larvae per plate). After 24 h of exposure (larvae from 7 to 8 days dpf, the behaviour of the larvae was directly tested without further manipulation, or larvae were collected for the different analyses. All the exposures were performed at 28.5 °C (POL-EKO APARATURA Climatic chamber KK350, Poland) with 12 L:12D photoperiods. For each variable investigated, samples were collected from 2 to 3 trials of the same experiment setup, which were conducted on different days and with different larvae batches.

3.2.4 Behavioural analysis

The vibrational startle response assay (VSRA) was based on the automatized delivery of the vibrational stimuli using the DanioVision Tapping Device DVTD-0010, installed in a DanioVision Observation Chamber (DVOC-0040). Video tracking and the escape response were analyzed using the EthoVision XT 9 software (Noldus, Wageningen, The Netherlands). A DanioVision Temperature Control Unit (DVTCU-0011) guaranteed that all trials were performed at 28 °C. All, tapping stimulus was selected at the highest intensity (intensity level: 8), and then, sequences of the vibrational stimuli were delivered during fixed time periods referred to as inter-stimulus interval (ISI). Trails were conducted in 48 well plates, with one 8 dpf zebrafish larvae in each well containing 1 mL of exposure medium. Before delivering the first stimulus, larvae were left in the DVOC for 30 min to acclimate. Videos were recorded at 30 frames per second and the VSR was analyzed for each individual larva by measuring the distance moved (cm) over the 1 s period after the stimulus.

Video tracking acquisition was controlled and the escape response was analysed using the EthoVision XT 9 software (Noldus, Wageningen, The Netherlands). Trials were performed at 28 °C with near-infrared light. The tapping stimulus was selected at the highest intensity (intensity level: 8) and one stimulus was delivered after a 15 min acclimation period to the chamber Videos were recorded at 30 frames per second and the vibrational startle response (VSR) was analysed for each individual larva by measuring the distance travelled (cm) over the 1 s period after the stimulus. Basal locomotor activity (BLA) and visual-motor response (VMR) analyses of 8 dpf zebrafish larvae were performed using a DanioVision system running Ethovision XT 11 software (Noldus, Wageningen, the Netherlands). Whereas BLA is defined as the distance travelled by the larvae during the first period of 20 min in the dark, VMR is based on the hyperactivity period evoked by a sudden reduction in light intensity (Fernandes et al., 2012). The plate was then transferred into a behavioral testing chamber equipped with a temperature control unit (DanioVision, Noldus Information Technology, Leesburg, VA). Larvae were acclimated in the dark for 1 h before video recording. The video tracking conditions used consisted on a 50 min cycle including a 20 min dark period followed by a 10 min light period and then a second 20 min dark cycle. The position of each individual larva was recorded using an IR digital video camera Basler acA1300-60gm (Basler Inc., Exton, PA) and an EthoVision XT 9 video tracking system (Noldus Information Technology, Leesburg, VA). A dynamic subtraction method was applied, using a sampling rate of 60 images/s, dark contrast 20-250, current frame weight 1, subject-size 2-125000, and no subject contour dilatation. A minimum distance input with a filter of 10% of the total larva body, equivalent to 0.4 mm, was used to remove background noise. All measurements occurred in the afternoon between 1:00 and 6:00 pm, the optimal time interval for stability of the basal activity. Tracks were analyzed for velocity (mm s^{-1}) and total

distance traveled (cm) calculated for each dark or light period. All microplates were analyzed at 28 ± 0.5°C with the same detection and acquisition settings.

3.2.5 Kinematic analysis

The kinematics of the acoustic/vibrational-evoked escape response is a highly stereotyped complex behaviour constituted by three sequential modules: a very fast and large C-bend followed by a high amplitude counter bend and, finally, fast-swimming oriented away from the stimulus. The experiments have been carried out on the setup shown in Fig. 9, which mimics the one described in (Burgess & Granato, 2007).

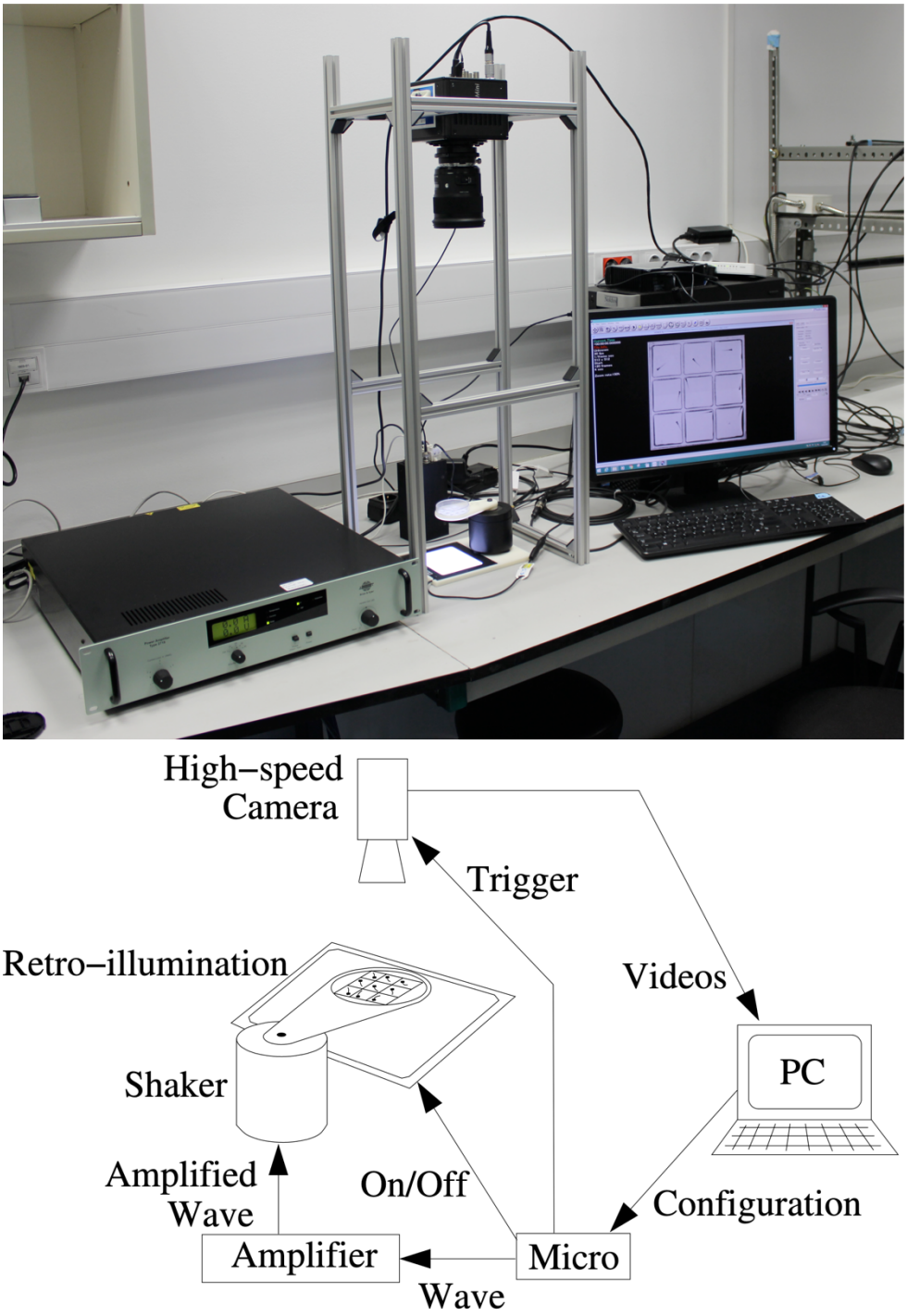


Figure 9: Setup for the kinematic analysis of the acoustic/ vibrational evoked escape response in zebrafish larvae

The setup is composed by a 50 mm Petri dish with nine arenas arranged in a 3 × 3 squared grid (Fig.10).

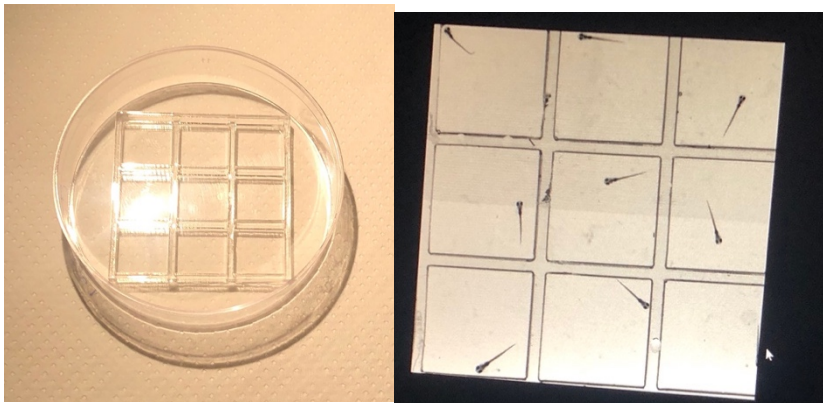


Figure 10: Petri dish with nine arenas arranged in a 3 × 3 squared grids

During the experiments, each arena holds a larva swimming in 400 µL of fish water. The dish is attached to a shaker (Mini Shaker 4810, Brüel&Kajær) with a custom, 3D-printed holder. The shaker produces the vibrational stimuli required in the experiments. Such vibrations are controlled by a microprocessor (STM32 F4 Discovery) and modulated by an amplifier (Power Amplifier Type 2718, Brüel&Kajær) set to a gain of 10 dB, a current limit of 1.8 A, and a variable gain of 50%. Larvae reactions in the nine arenas are recorded with a high-speed camera (Photron Fastcam Mini UX100) equipped with a 50 mm lens (Sigma 50 mm F1.4 DG). When active, the camera takes 512 × 512-pixel images at 1000 frames per second. The trigger of the camera is activated by the same microprocessor taking care of controlling the vibrations. In this way, a precise synchronization between the captured videos and the stimuli is obtained. To improve image quality, the Petri dish is retro-illuminated with a led light of variable intensity (Backlight Led LF-100SWZ-IU, CCS Inc.) also controlled by the mentioned microprocessor. To facilitate the use of the experimental setup, we developed LarvaCam, a software suitable both for experiments aiming at the analysis of the startle response and for experiments analysing the habituation process after multiple vibrational-evoked startle responses. LarvaCam, which runs on a Windows-based PC, allows for designing experiments composed of a variable number of phases that are executed in sequence. Each phase can include several steps where, at each step, vibration is transmitted to the larvae (Fig.11).

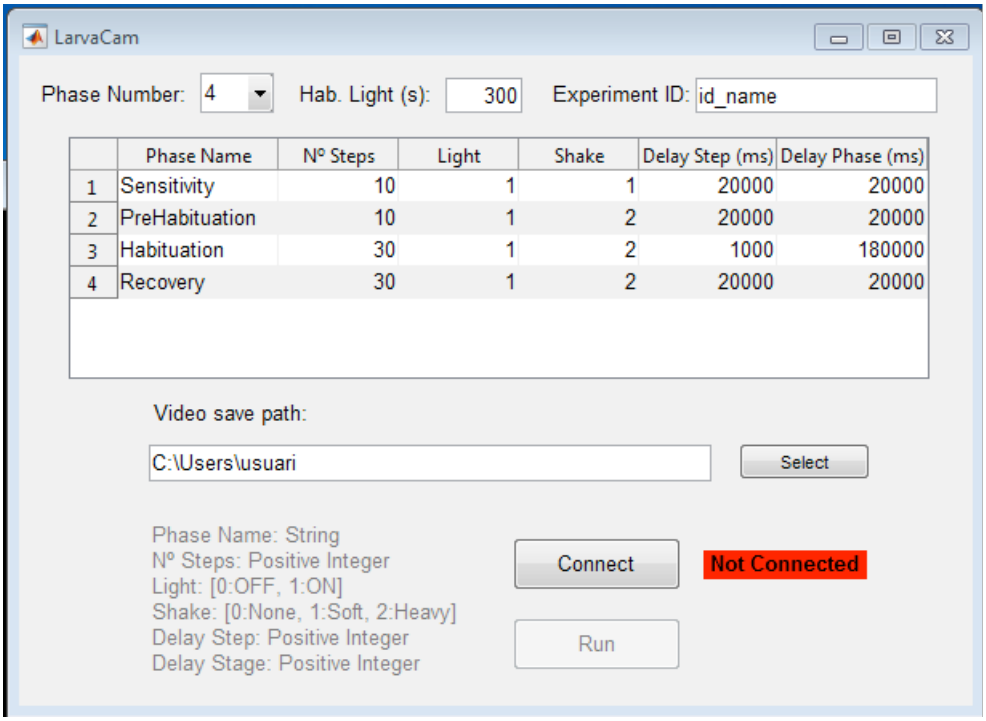


Figure 11: Main screen of the LarvaCam software. In this case, the software has designed an experiment, based in Wolman et al.(2011), to assess the habituation of the vibrational-evoked startle response. In the experiments reported in the paper only one phase with one step with sake intensity, two is used

Two different vibration intensities can be selected for all the steps of each phase: soft vibration (1 kHz, 0.6 V), and strong vibration (1 kHz, 6 V). In this study, a design with only one phase and only one step has been used. Moreover, the strong vibration was selected as the vibrational stimulus, as we found that using this intensity there were about 70% short-latency C-bends in control larvae. The camera was activated 30 ms before the vibration, and the total length of the captured video was 120 ms. LarvaCam software translates the configured experiment into low-level commands that are sent to the microcontroller via USB connection. Moreover, the software also takes care of downloading the videos captured by the high-speed camera, which is connected to the PC via a Gigabit ethernet cable. To process the videos, we developed LarvaTrack, a software that automatically identifies the patches of each frame corresponding to the nine arenas using line detection and morphological image processing techniques (Fig.12).

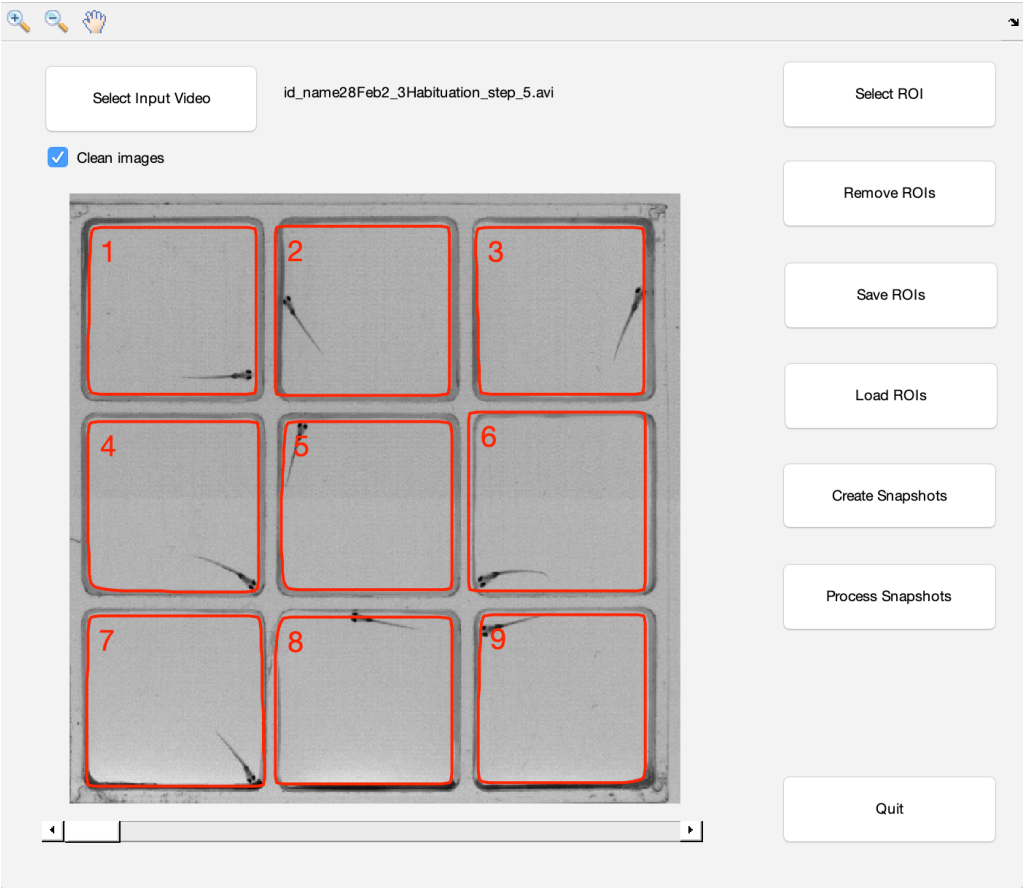


Figure 12: The main screen of the LarvaTrack software with the nine arenas automatically detected on a sample video

Then, each patch is processed, correcting the intensity and also using background subtraction and morphological image operators to detect the region corresponding to the larva. The skeleton of this region is computed next and, finally, the head of the larva is identified. The skeleton is approximated by three straight segments, and the angles shown in Fig.13 are computed. Namely, the computed angles are the one between the first and second segment (α), the one between the second and third segment (β) and the sum of both (γ), which is used to characterize the amplitude of the bend. The temporal evolution of such angles gives an accurate account of the reaction of the larvae to the stimuli.

Finally, some experiments were performed to determine the distance travelled by control and fenitrothion-exposed larvae during the short-latency C-bend (SLC). In this case, Petri dishes without a grid, containing 20 larvae, were used in the setup. The analyses of kinematic parameters of these larvae, including SLC, long-latency C-bend (LLC), and the distance travelled, were obtained by using Flote software package (Burgess & Granato, 2007).

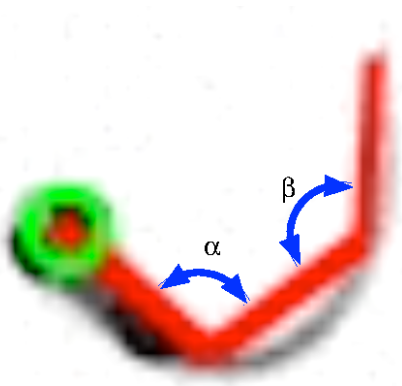


Figure 13: A larva is captured by a high-speed camera. The green circle marks the head and the three red segments approximate the position of its trunk. Bending amplitude (Burgess and Granato, 2007) is characterized as the sum $\gamma = \alpha + \beta$

3.2.6 RNA preparation and qRT-PCR analysis

Gene expression analysis was performed as previously reported by Prats et al., (2017). Total RNA was extracted from a total of 6–8 pools of 4 larvae (8 dpf) using the Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA). RNA concentration was measured by spectrophotometric absorption at 260 nm in a NanoDropTM ND-8000 spectrophotometer (Fisher Scientific) and the quality checked in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RIN (RNA Integrity Number) values ranged between 9 and 10. After DNaseI treatment (Ambion, Austin, TX), 1 µg of total RNA was used to synthesize the first strand of cDNA with First Strand cDNA synthesis Kit (Roche Diagnostics, Mannheim, Germany) using oligo(dT), following the manufacturer's instructions.

Real Time PCR was performed in a LightCycler ® 480 Real-Time PCR System using SYBR Green PCR Master Mix (Roche Diagnostics, Mannheim, Germany). Cycling parameters were 95 °C for 15 min followed by 45 cycles of 95 °C, 10 s and 60 °C, 30 s. For each experimental condition, qPCR analyses were performed from two independent experiments, with 4 biological replicates on each experiment and three technical replicates for each sample. The housekeeping gene ppia2 was used as a reference gene for normalization purposes (Prats et al., 2017b). Primers for the sixteen selected genes (gap43a, mbp, gfap, syt1a, atp2b1a, cyp19a1b, sult2st3, mao, comtb, slc6a4a, th1, th2, slc6a3, slc18a2, tph1a, tph1b) were synthesized by Sigma-Aldrich (Steinheim, Germany). The efficiency and specificity of all primers were checked before the analyses. Results were normalized to ppia2 and the relative abundance of mRNA was calculated following the ΔΔCt method (Livak & Schmittgen, 2001)deriving fold-change ratios from these values.

3.2.7 Computational analyses

Based on the recently defined PHASE initiative from the US Food and Drug Administration's Center for Drug Evaluation and Research (Ellis et al., 2019, 2020a), targets for fenitrothion were predicted using two platforms, namely, CLARITY (Chemotargets CLARITY v4, 2019) and SEA (Keiser et al., 2007a). Both platforms use two-dimensional chemical structures to predict potential binding targets (Keiser et al., 2009; Vidal & Mestres, 2010). SEA uses descriptor-based similarity to compare the structure of a molecule to the chemical structures with known in vitro binding affinity in ChEMBL (Keiser et al., 2007a, 2009; Vidal & Mestres, 2010). Approximately 2300 protein targets are covered. For each predicted target, a p-value and the similarity of the closest molecule are provided. CLARITY uses six ligand-based approaches that rely on descriptor-based molecular similarity, an implementation of the similarity ensemble approach, fuzzy fragment-based mapping, quantitative structure-activity relationships, a set of machine learning methods and target cross-pharmacology indices (Gregori-Puigjané & Mestres, 2006; Vidal et al., 2011). The training set for the 4799 protein target models is generated from in vitro affinity data contained in both public and patent sources (Sharma et al., 2016). For each target prediction, the projected affinity and mode of action are provided alongside a confidence score based on the number and type of methods that independently contribute to the prediction.

3.2.8 Mammalian in vitro functional assay

For the mammalian *in vitro*, functional assays fenitrothion was tested at two concentrations, 100 nM and 10 µm.
AR Human Androgen NHR Binding Assay (Agonist Radioligand) was based on the protocol described by Zava et al., (1979), using AR(h) from human LNCaP cells. The specific ligand used was 1 nM [^{3}H] methyltrienolone, and incubation conditions were 24 h at 4°C. Binding of [^{3}H] methyltrienolone was measured by scintillation counting in the absence or presence of fenitrothion and results of the assay were expressed as a percentage of control specific binding and as a percentage of inhibition of control specific binding when incubation was performed in the presence of fenitrothion. Testosterone (1 µM) was used as a reference compound. Human Serotonin Transporter Binding Assay (Antagonist Radioligand) was based in the protocol described by Tatsumi et al., (1999), using human recombinant SERT from CHO cells. The specific ligand used was 2 nM [^{3}H] imipramine, and incubation conditions were 60 min at r.t.. Binding of [^{3}H] imipramine was measured in the absence or presence of fenitrothion by scintillation counting and results of the assay were expressed as a percentage of control specific binding and as a percentage of inhibition of control specific binding. Human Acetylcholinesterase Assay was based in the protocol described by (Ellman & Courtney, 1961), using human recombinant AChE from HEK-293 cells. The specific

substrate used was 400 µM acetylcholine, and the formation of the product 5-thio-2-nitrobenzoic acid was measured by photometry after 30 min incubation at r.t.. Assays were performed in the absence and presence of fenitrothion and results were expressed as percentage of the specific activity of the control and as the percentage of inhibition of specific activity with the control values. Human Monoamine Oxidase A (MAO-A) Assay was based on the protocol described by (Weyler & Salach, 1985), using MAO-A from the human placenta. The specific substrate used was 150 µM kynuramine, and the formation of the product 4-OH-quinoline was measured by photometry after 20 min incubation at r.t.. Assays were performed in the absence and presence of fenitrothion and results were expressed as the percentage of the specific activity of the control and as the percentage of inhibition of specific activity with the control values. Human Monoamine Oxidase B (MAO-B) Assay was based on the protocol described by (Tsugeno et al., 1995), using human recombinant MAO-B. The specific substrate used was 4 µM D-Luciferin derivative, and the formation of the product methyl ester luciferin was measured by luminescence after 60 min incubation at 37°C. Assays were performed in the absence and presence of fenitrothion and results were expressed as the percentage of the specific activity of the control and as the percentage of inhibition of specific activity with the control values.
Results showing an inhibition or stimulation higher than 50% are considered to represent significant effects of the test compounds.

3.2.9 Zebrafish acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was determined in individual larvae (n = 9 larvae per experimental group), essentially as described by Faria et al., (2015). Thus, larvae were homogenized by TissueLyser®, by adding two 3-mm stainless steel beads to each sample, and homogenizing at 50 oscillations/s during 30 s, in 200 µl of ice-cold 0.1 M phosphate buffer (pH 7.4) with 150 mM KCl and 0.1mM EDTA and further centrifuged at 10,000 ×g for 10min at 4 °C, collecting the resulting supernatant for AChE analysis. Then, 2 mM acetylthiocholine and 0.33 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were added to the S9 fraction and the formation of the product resulting from the reaction between thiocholine and DTNB ion was monitored at 405 nm for 15 min. The final results were expressed in µmol min^{-1} mg protein using the extinction coefficient of 13.6×10^3 M^{-1} cm^{-1}.

3.2.10 Extraction and analysis of neurotransmitters

Monoaminergic neurotransmitters were extracted from 8 pools of 5 larvae (8 dpf) following an extraction procedure adapted from Mayol-Cabré et al., (2020). The extraction process was based on the use of a solvent of polarity similar enough to the neurotransmitters to be able to extract them from the sample. The monoaminergic neurotransmitters profile was assessed analysing the extractions using the LC-MS/MS technique. The analysis was performed by ultra-high-performance liquid

chromatography (Acquity UPLCH-Class Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer equipped with an electrospray (ESI) source (Xevo, TQS micro, Waters, USA). The quality parameters of this method are summarized in Table 3.

Table 3: Quality parameters obtained by LC-MS/MS for the 10 monoaminergic neurochemicals. F: slope, r2: IDL: instrumental detection limit; %R: recovery; RSD: relative standard deviation; %ME: matrix effect; MDL: method detection limit.

Monoamine NTs	**Linearity ($ng\ \mu L^{-1}$)**	**Calibration type**	**F**	**r^2**	**IDL (pg)**	**MDL ($ng\ larvae^{-1}$)**	**Intra-day precision (RSD, %)**	**Inter-day precision (RSD, %)**	**%R ± RSD**	**%ME ± RSD**
5-HT	0.005–2.5	External	0.41	0.99997	1.7	1.7	4.1	4.3	77 ± 4	126 ± 7
3-MT	0.005–2.5	Internal	5.18	0.99741	0.51	14.7	8.9	13.2	123 ± 12	63 ± 8
HVA	0.005–2.5	Internal	0.10	0.99776	24.6	53.0	2.9	8.0	64 ± 17	109 ± 16
DOPAC	0.005–2.5	Internal	2.51	0.99717	0.46	11.4	2.2	4.4	115 ± 16	59 ± 8
Tryp	0.005–0.5	Internal	0.41	0.99566	2.6	4.3	0.2	2.6	126 ± 8	134 ± 17
L-DOPA	0.005–2.5	Internal	3.93	0.99973	51.4	57.8	4.3	5.4	79 ± 8	61 ± 6
DA	0.005–2.5	External	0.20	0.99860	14.2	3.8	5.8	6.9	113 ± 5	91 ± 5
NE	0.005–2.5	External	1.08	0.99831	19.8	19.6	5.2	8.2	90 ± 2	79 ± 4
HIAA	0.005–2.5	Internal	4.05	0.99593	6.4	8.8	2.2	4.9	76 ± 3	82 ± 3
Tyr	0.005–2.5	Internal	0.90	0.99974	18.2	11.3	3.7	9.2	99 ± 10	

3.2.10.1 Monoaminergic neurotransmitters extraction and analysis

Standards of dihydroxyphenylacetic acid (DOPAC), dopamine hydrochloride (DA), homovanilic acid (HVA), levodopa (L-DOPA), serotonin hydrochloride (5-HT), L-tryptophan (Trp) and L-tyrosine (Tyr) and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Besides, 5-hydroxyindoleacetic acid (5-HIAA) was provided by Toronto Research Chemicals (TRC, Toronto, Canada), 3-methoxytyramine hydrochloride (3-MT) was obtained from Merck (Darmstadt, Germany), and norepinephrine (NE) was supplied by Tocris Bioscience (Ellisville, USA).

The labeled internal standards L-tryptophan-1-^{13}C, 5-hydroxyindole-3-acetic acid-d5, L-DOPA-2,5,6-d3 and L-Tyrosine-^{13}C9,15N were all purchased from Toronto Research Chemicals (TRC, Toronto, Canada). Acetonitrile (ACN) HPLC LC-MS grade was supplied by VWR Chemicals (Leuven, Belgium) and ultra-pure water was obtained through Millipore Milli-Q purification system (Millipore, Bedford, USA). Formic acid (FA) was supplied by Fischer

Scientific (Loughborough, UK) and ammonium formate by Sigma-Aldrich (St. Louis, MO, USA).
Stock solutions of all the neurotransmitters (NTs) were prepared at 1000 ng μL^{-1} in MeOH, ultra-pure water or DMSO depending on their solubility. Calibration standards were prepared in extractant solvent. A mix solution of all labelled standards (internal standard mixture, ISM) was prepared in extractant solvent (5 ng μL^{-1} for all of them, except from 5-HT which was 2 ng μL^{-1}). These standards were kept at -20 °C in amber vials to prevent degradation. The standards used for both calibration curve and QCs were freshly prepared every day in starting the mobile phase.

Monoaminergic neurotransmitters were extracted from 8 pools of 5 larvae (8 dpf) following an extraction procedure adapted from Mayol-Cabré et al., (2020).The extraction process was based on the use of a solvent of polarity sufficiently similar to the neurotransmitters to be able to extract them from the sample. Following the described process, 300 µL of cold extractant solvent (ACN: H2O (90:10) + 1% formic acid) were added to pools of 5 zebrafish larvae. Also, each sample was spiked with 10 µL of ISM (50 ng of each labelled NTs except from 5-HT-d4, 20 ng) Then, three stainless steel beads (3 mm diameter) were placed in each pool and were homogenized using a bead mill homogenizer (TissueLyser LT, Quiagen, Hilden, Germany). The mill was programmed at 50 osc/min during 90 sec. Subsequently, samples were shaken in a vibrating plate at 4°C for 20 min and later centrifuged for 20 min at 13,000 rpm. Finally, the supernatant was filtered using 0.20 µm PTFE filters (DISMIC -13 JP, Advantec®) and kept at -80 °C until the analysis with LC-MS/MS

Temperature is a key element throughout the extraction process because NTs are very unstable compounds and high temperatures can degrade these compounds (J. Yan et al., 2017).

3.2.10.2 LC-MS/MS analysis

NTs profile was assessed analyzing the extractions using LC-MS/MS technique. The analysis was performed using ultra-high-performance liquid chromatography (Acquity UPLC® H-Class Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer equipped with an electrospray (ESI) source (Xevo, TQS micro, Waters, USA).

To retain analytes, an Acquity UPLC BEH Amide column (150 mm x 2.1 mm ID, 1.7 µm particle size) provided with an Acquity UPLC BEH Amide pre-column (5 mm x 2.1 mm ID, 1.7 µm particle size) (Waters, Milford, MA, USA) was employed. The temperature was set at 30 °C. Mobile phase composition consisted of solvent A and solvent B. Solvent A was composed of Milli-Q water and acetonitrile (H_2O:ACN) (95:5) containing 100 mM ammonium formate while solvent B was Milli-Q water and ACN (15:85) containing 30 mM ammonium formate. The pH of both solvents was adjusted to pH 3 with FA. The LC gradient started at 100 % B, decreased at 80 % B in 4 min, and held for 1 min. From 5 to 7 min, B was linearly increased to 100 %. Finally, initial conditions were re-equilibrated in 3 min. The total run time was 10 min. The flow rate was set at 250 µL min^{-1}. The samples were kept at 10 °C in the autosampler, and the injection volume was 10 µL. Regarding the MS conditions, desolvation gas flow was set to 900 L h^{-1} and the desolvation temperature was 350 °C. The cone gas flow was fixed at 150 L h^{-1}. Nitrogen was used as desolvation and cone gas. Moreover, source temperature was set at 100°C and a capillary voltage of 2.0 kV was applied. NTs were all measured under positive electrospray ionization (ESI+). The acquisition was performed in MRM mode. The optimum cone voltage (CV) and collision energies (CE) were taken from previous studies (Mayol-Cabré et al., 2020). The first transition, corresponding to the most intense fragment, was used as the quantifier ion, whereas the second as the qualifier ion. The system and data management were processed using MassLynx v4.1 software package (Waters, Manchester, UK).

3.2.10.3 Quality assurance

Calibration was performed over a concentration range from 0.005 to 2.5 ng μL^{-1}. The ISM was used as extraction and analytical quality control. Trp, L-DOPA, 3-MT, DOPAC, HVA, 5-HIAA and Tyr were quantified by internal calibration, each with the respective labeled standard, while DA, 5-HT and NE were quantified using external standard.

Instrumental detection limits (IDLs) were determined using the lowest concentrated standard 0.005 ng μL-^{1} (except for LD at 0.010 ng μL^{-1}) that yielded a S/N ratio equal to 3. Whereas method detection limits (MDLs) were calculated using samples spiked at 50 ng. Intra-day precision was determined by two consecutive injections of 1 ng μL^{-1} standard solution and inter-day precision was determined by measuring the same standard solution for four different days. Moreover, recovery studies were performed with four replicates, using samples spiked at 50 ng with the neurotransmitter standard mixture and the ISM. In addition, matrix effect (ME) was assessed by comparing the peak area of each NT from the spiked sample (A) with the peak area of the analyte from the standard solution used in calibration curve (C)according to the equation:

$$ME\ (\%)=(A-B)/C \times 100$$

where B is the peak area of each analyte from non-spiked samples (controls).

3.2.10.4 Quality parameters

All the quality parameters studied are summarized in Table 4. Great correlation coefficients (r^2) were obtained over 0.99 for all analytes in a range from 0.005 to 2.5 ng μL^{-1} in most cases, except for Trp (0.005 to 0.5 ng μL^{-1}). Furthermore, IDLs were ranged from 0.46 pg (DOPAC) to 51.4 pg (L-DOPA), while MDLs varied from 1.7 (5-HT) to 57.8 ng larvae^{-1}. Moreover, intra-day precision was ranged from 0.2% to 8.9% and inter-day precision values were from 2.6% to 13.2%. Regarding the matrix effect, this parameter is an indicator of ionization suppression or enhancement of the analytes. Compounds with values below 70% indicated signal suppression due to the matrix (3-MT, DOPAC and L-DOPA), whereas values above 130% suggested a signal enhancement (Trp).

Table 4: Quality parameters obtained by LC-MS/MS for the 10 monoaminergic neurochemicals. F: slope,r2: regression coefficient; IDL: instrumental detection limit;%R: recovery; RSD: relative standard deviation; %ME: matrix effect; MDL: method detection limit.

Monoamine NTs	Linearity (ng μL^{-1})	Calibration type	F	r^2	IDL (pg)	MDL (ng larvae^{-1})	Intra-day precision (RSD, %)	Inter-day precision (RSD, %)	%R ± RSD	%ME ± RSD
5-HT	0.005–2.5	External	0.41	0.99997	1.7	1.7	4.1	4.3	77 ± 4	126 ± 7
3-MT	0.005–2.5	Internal	5.18	0.99741	0.51	14.7	8.9	13.2	123 ± 12	63 ± 8
HVA	0.005–2.5	Internal	0.10	0.99776	24.6	53.0	2.9	8.0	64 ± 17	109 ± 16
DOPAC	0.005–2.5	Internal	2.51	0.99717	0.46	11.4	2.2	4.4	115 ± 16	59 ± 8
Tryp	0.005–0.5	Internal	0.41	0.99566	2.6	4.3	0.2	2.6	126 ± 8	134 ± 17
L-DOPA	0.005–2.5	Internal	3.93	0.99973	51.4	57.8	4.3	5.4	79 ± 8	61 ± 6
DA	0.005–2.5	External	0.20	0.99860	14.2	3.8	5.8	6.9	113 ± 5	91 ± 5
NE	0.005–2.5	External	1.08	0.99831	19.8	19.6	5.2	8.2	90 ± 2	79 ± 4
HIAA	0.005–2.5	Internal	4.05	0.99593	6.4	8.8	2.2	4.9	76 ± 3	82 ± 3
Tyr	0.005–2.5	Internal	0.90	0.99974	18.2	11.3	3.7	9.2	99 ± 10	117 ± 17

3.2.11 Risk assessment

The environmental risk of pesticides in aquatic ecosystems is commonly estimated using the hazard quotient (HQ), the ratio between the measured environmental concentration (MEC) and the predicted non-effect concentration (PNEC):

HQ 1⁄4 MEC=PNEC

MEC values used are based on the median and maximum detected concentrations, whereas PNEC values commonly use non-observed effect concentration (NOEC) for the most sensitive endpoints and species. Whereas compounds with HQ >1 are potentially hazardous for aquatic ecosystems, those with HQ < 1 values are considered slightly or not hazardous.

3.2.12 Statistical analysis

Data were analyzed with IBM SPSS v25 (Statistical Package 2010, Chicago, IL), and were plotted with GraphPad Prism 8.31 for Windows (GraphPad software Inc., La Jolla, CA) and Microsoft Excel for Mac 2011 (v14.5.7; Microsoft Corp., Redmond, WA, USA). Normality was assessed using Kolmogorov-Smirnov and Shapiro-Wilk tests. One-way ANOVA followed by Dunnett's multiple comparison test was used to test for differences between normally distributed groups, whereas the Kruskal-Wallis test followed by Dunn's multiple comparison test against the control value was used to test for differences between groups that did not meet parametric assumptions. Data are presented as the mean ± SEM or median and 25–75 percentile of 2–3 independent experiments unless otherwise stated. Significance was set at $P < 0.05$.

4. Results

4.1 Neonicotinoid exposure to Mytilus galloprovincialis

4.1.1 Acute exposure of Mytilus galloprovincialis to Calypso 480

4.1.1.1 Cell viability assay

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 CA (Table 5). In the same way, no damage to the digestive cells was recorded (Table 6).

Table 5: Percentage of viability hemocytes in *M. galloprovincialis* exposed to Calypso (CCA(0mg/L); CA1(10mg/L); CA2(50mg/L); CA3(100mg/L)) assessed by Trypan blue and Neutral red

	Exposure time (days)	TEST GROUP			
		CCA(0mg/L)	CA1(10mg/L)	CA2(50mg/L)	CA3(100mg/L)
TB (%)	3	100 ±0.00	92.43±0.46	97.84 ±0.79	96.90±0.94
	7	96.26±0.53	82.77±0.63	94.96±0.76	96.41±1.24
NR (%)	3	100 ±0.00	76.19 ±0.74	100±0.00	99.60±0.40
	7	99.77±0.15	100 ±0	99.36±0.42	100 ±0.00

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

Table 6: Percentage of viability digestive cells in *M. galloprovincialis* exposed to Calypso (CCA(0mg/L); CA1(10mg/L); CA2(50mg/L); CA3(100mg/L)) assessed by Trypan blue and Neutral red

	Exposure time (days)	TEST GROUP			
		CCA(0mg/L)	CA1(10mg/L)	CA2(50mg/L)	CA3(100mg/L)
TB (%)	3	100 ±0.00	79.63 ±0.00	97.64 ±0.00	100 ±0.00
	7	100 ±0.00	100 ±0.00	96.72 ±0.00	96.99 ±0.00
NR (%)		100 ±0.00	76.99 ±0.00	96.30 ±0.14	100 ±0.00
	7	100 ±0.00	100 ±0.00	98.94 ±0.6	100 ±0.00

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

4.1.1.2 The Regulation of Volume Decrease experiment

The cells exposed to the rapid change of osmolarity initially increased in size and then tended to return to their initial volume. The cells reached their maximum swelling, corresponding to a 12% increase in volume, after 1 min of exposure to the hypotonic medium. The exposed samples CA1, CA2 and CA3 show a response equal to that of the control CCA.

4.1.1.3 Haemolymph biochemical parameters

Results of haemolymph parameters of mussels exposed to CA are listed in Table 7. Concentrations of CA (10, 50 and 100 mg L^{-1}) led to a 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 ions Cl^-, Na^+, Ca^{2+} after 3 days, K^+ and glucose after 7 days and a 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 a significant decrease in LDH activity at the end of the experiment, compared with control (CCA). Values of Mg^{2} in CA-exposed mussels did not differ significantly between the groups during the exposure.

Table 7: Haemolymphatic parameters of Mediterranean mussel (*Mytilus galloprovincialis*) exposed to Calypso 480 SC (CA).

Treatment and 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	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}
(0 mg L^{-1})	7	$454.62 \pm 0.78^{a*}$	14.52 ± 0.06^{a}	$560.00 \pm 1.06^{a*}$	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	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}
(10 mg L^{-1})	7	452.87 ± 0.27^{a}	$13.07 \pm 0.08^{b*}$	$541.33 \pm 2.51^{a*}$	$62.85 \pm 0.29^{c*}$	1.25 ± 0.06^{b}	25.80 ± 0.40^{a}	3.50 ± 0.56^{a}	2.00 ± 0.18^{b}
CA2	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}
(50 mg L^{-1})	7	$445.52 \pm 0.95^{b*}$	$12.60 \pm 0.06^{b*}$	537.33 ± 0.92^{a}	54.95 ± 0.16^{a}	1.32 ± 0.06^{b}	25.70 ± 0.17^{a}	$1.33 \pm 0.21^{b*}$	1.37 ± 0.16^{b}
CA3	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}
(100 mg L^{-1})	7	$458.47 \pm 1.58^{a*}$	14.05 ± 0.08^{a}	549.83 ± 6.93^{a}	$59.47 \pm 0.19^{b*}$	1.35 ± 0.06^{b}	25.08 ± 0.06^{a}	$1.50 \pm 0.34^{b*}$	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 ($P < 0.01$)

4.1.1.4 Determination of enzyme activity

Calypso caused statistically significant changes ($P < 0.05$) in CAT and SOD activities in mussels (Fig. 14). In the 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. 14).

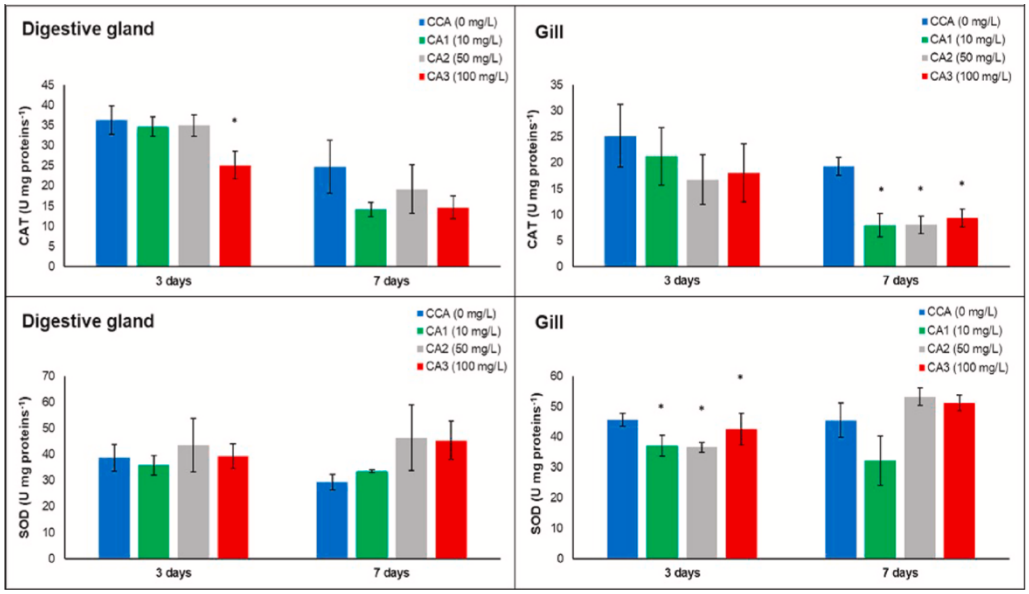


Figure 14: Enzymatic activity of catalase (CAT) and superoxide dismutase (SOD) in digestive gland and gill in Mediterranean mussel (*Mytilus galloprovincialis*) exposed to thiacloprid (TH). The values are mean ± SE (standard error); n = 6; Mann-Whitney U Test

4.1.1.5 Histological analysis and Histopathological condition indices

Histological findings of both the digestive gland and gills show no marked lesions associated with pesticide exposure.

4.1.2 Sub-chronic exposure of Mytilus galloprovincialis to Calypso 480

4.1.2.1 Cell viability assay

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 by about 20% in animals exposed to the highest concentration (CAL2). However, after 20 days of exposure, the viability of both groups 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% (Table 8).

Table 8: Cell viability assays in haemocytes and digestive gland cells of *Mytilus galloprovincialis* following exposure to Calypso 480 SC.

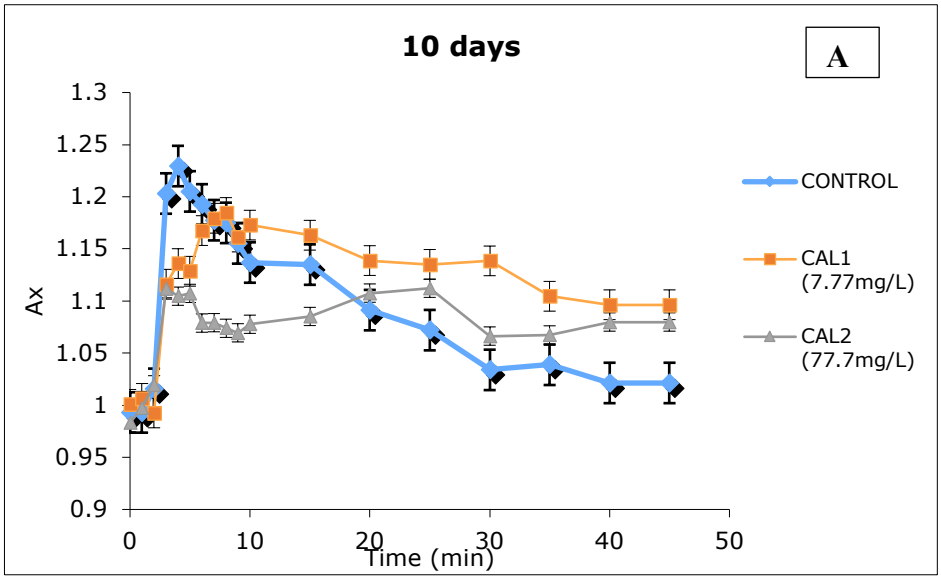
Cell viability		Exposure time (days)	Test groups		
			Control (0 mg L^{-1})	CAL1 (7.77 mg L^{-1})	CAL2 (77.7 mg L^{-1})
Haemocytes	TB (%)	10	100.00 ± 0.00	99.07 ± 0.6	96.12 ± 0.97
		20	100.00 ± 0.00	95.90 ± 1.03	82.08 ± 3.18*
	NR	10	100.00 ± 0.00	100.00 ± 0.00	98.94 ± 0.40

	(%)	20	100.00 ± 0.00	96.26 ± 1.66	75.06 ± 1.37*
Digestive gland cells	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*
	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*

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 ($P < 0.05$). CAL = Calypso 480 SC, CAL1 = 7.77 mg L^{-1}, CAL2 = 77.7 mg L^{-1}.

4.1.2.2 The Regulation of Volume Decrease experiment

The cells exposed to the rapid change of osmolarity initially increased in size and then tended to return to their initial volume. The cells reached their maximum swelling, corresponding to a 12% increase in volume, after 1 min of exposure to the hypotonic medium. Afterwards, the control cells exhibited RVD response. As shown in Fig. 15A after 10 days of exposure the cells did not reach their maximum swelling and failed to return to the initial condition. Subsequently washing with hypoosmotic solution was done; the cells exposed to CAL1 did not swell substantially compared to the others and this value is significant (P < 0.05) (Fig. 15B), moreover, some cells exposed to CAL2 burst after washing with the hypoosmotic solution was done , failing to regulate the cell volume in any way.



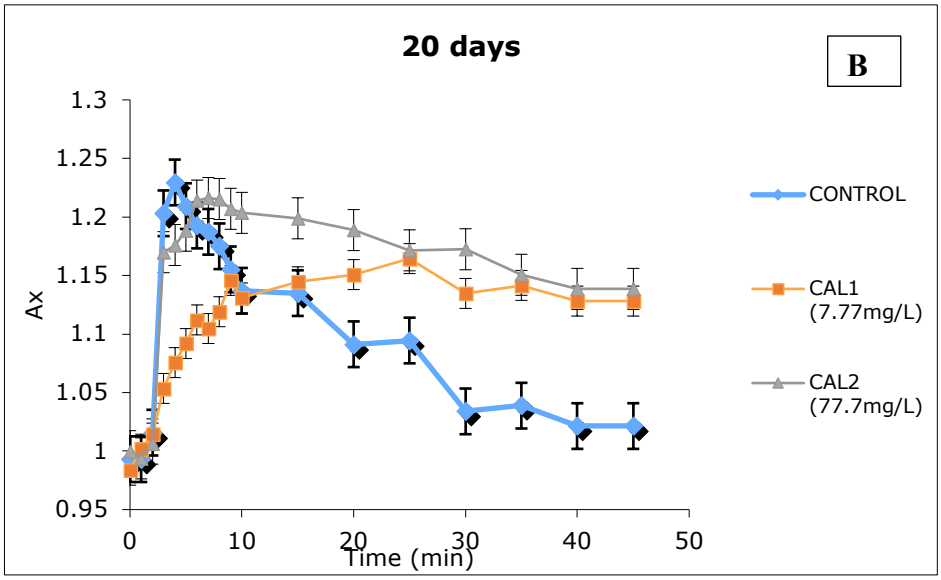


Figure 15: Relative area changes of regulating cell volume (RVD) isolated by digestive gland of *Mytilus galloprovincialis*. (A) Cells of RVD after 10 days of exposure; (B) Cells of RVD after 20 days of exposure. CAL=Calypso 480 SC, CAL1=7.77mgL1, CAL2=77.70mgL1. The values are means ± SE; *P < 0.05 respect to the control condition (two-way ANOVA test).

4.1.2.3 Haemolymph biochemical parameters

Results of haemolymph parameters are presented in Table 9. The sub-chronic exposures to CAL1 showed significantly lower ($P < 0.01$) CL, Na^+ after 10 days of exposure compared with controls. The CAL2 group showed significant lower ($P < 0.01$) value parameters (Cl^-, Na^+) after 10 days of exposure, significant ($P < 0.01$) higher K^+, and a reduction Na^+ and GLU at the conclusion of the recovery period compared with control. The indicators PHOS and Mg^{2+} were similar among groups at values comparable to control through the test.

Table 9: 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 (77.7 mg L^{-1})
(Cl^-	10	464.10 ± 1.55^a	449.53 ± 6.64^{ab}	443.75 ± 4.50^b
(mmol/L)	20	482.57 ± 4.62^a*	500.03 ± 13.23^a*	477.85 ± 4.24^a*
K^+	10	12.15 ± 0.02^a	12.53 ± 0.21^a	12.40 ± 0.27^a
(mmol/L)	20	12.83 ± 0.08^a	12.70 ± 0.08^a	12.70 ± 0.08^a
Na^+	10	566.33 ± 5.06^a	537.50 ± 6.90^b	532.83 ± 5.82^b
(mmol/L)	20	554.50 ± 5.98^a	556.33 ± 7.72^a	536.33 ± 2.59^a
Ca^{2+}	10	54.65 ± 0.37^a	53.48 ± 0.61^a	53.10 ± 0.68^a
(mg/dl)	20	56.47 ± 1.52^a	54.20 ± 1.68^a	54.83 ± 0.79^a
PHOS	10	0.80 ± 0.04^a	0.80 ± 0.04^a	0.55 ± 0.11^a
(mg/dl)	20	0.80 ± 0.04^a	0.80 ± 0.06^a	0.67 ± 0.04^a
Mg^{2+}	10	24.88 ± 0.20^a	25.07 ± 0.11^a	25.03 ± 0.07^a
(mg/dl)	20	25.25 ± 0.06^a	24.88 ± 0.15^a	24.83 ± 0.10^a
LDH	10	2.50 ± 0.34^a	2.33 ± 0.21^a	1.67 ± 0.21^a
(U/l)	20	2.17 ± 0.48^a	1.50 ± 0.22^a	1.67 ± 0.21^a
GLU	10	0.35 ± 0.04^a	0.35 ± 0.02^a	0.25 ± 0.06^a
(mg/dl)	20	0.33 ± 0.06^a	0.28 ± 0.07^a	0.28 ± 0.04^a

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 days). *Denotes significant differences between groups value for a test group along time ($P < 0.01$). Lactate dehydrogenase (LDH), glucose (GLU), CAL = Calypso 480 SC, CAL1 = 7.77 mg L^{-1}, CAL2 = 77.7 mg L^{-1}.

4.1.2.4 Determination of enzyme activity

Enzymatic activity SOD and CAT in the digestive gland during the exposure period of Calypso (10 and 20 days) are presented in Fig. 16. In CAL1 exposed mussels there was a statistically significant decrease (P 0.01) in SOD activity after 20 days of exposure.

The enzymatic activity of SOD and CAT in gills of mussels exposed to CAL concentrations are presented in Fig. 17. Exposure to CAL1 and CAL2 induced a significant decrease (P 0.01) of CAT activity after 20 days of exposure, compared with controls.

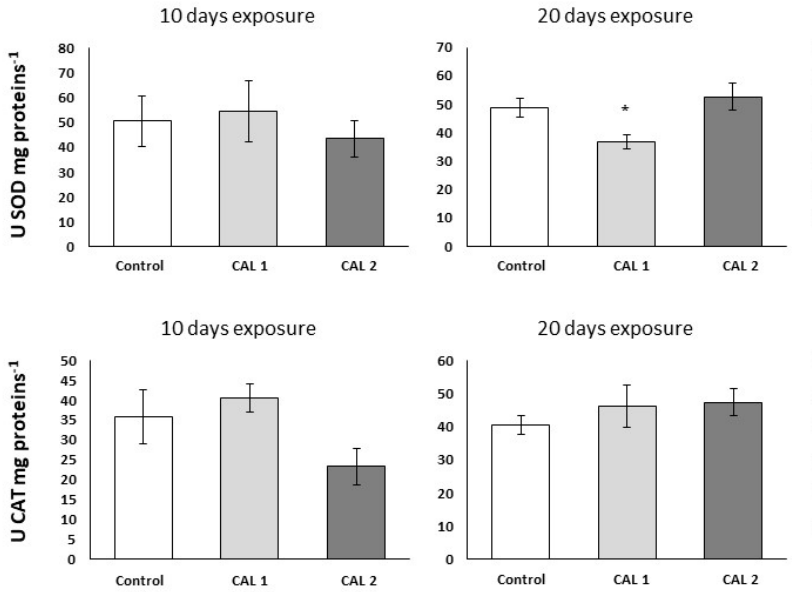


Figure 16: Enzyme activity in the digestive gland of *Mytilus galloprovincialis* following exposure to Calypso 480 SC. Data are mean ± SE, n = 6. *Denotes indicate significant differences ($P \leq 0.01$) among groups at the same sample time (Mann-Whitney U Test).). Superoxide dismutase (SOD), catalase (CAT), CAL = Calypso 480 SC, CAL1 = 7.77 mg L^{-1}, CAL2 = 77.7 mg L^{-}.

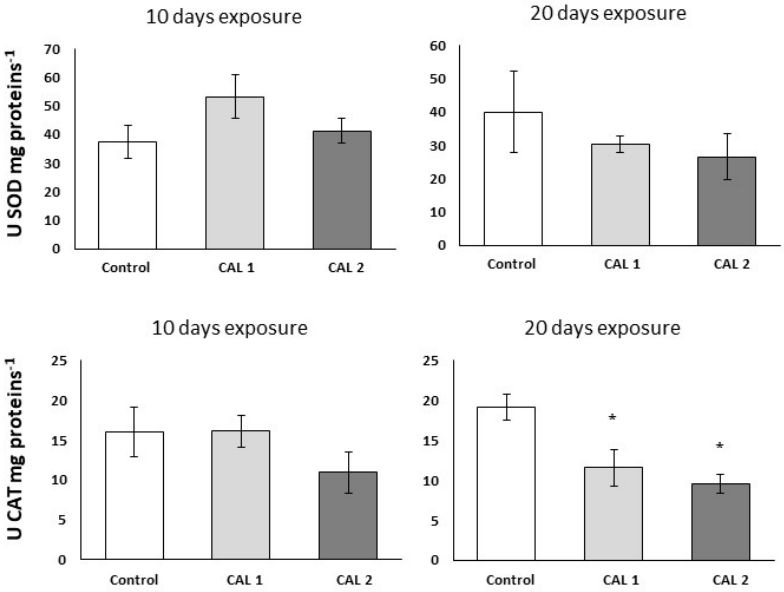


Figure 17: Enzyme activity in gill of *Mytilus galloprovincialis* following exposure to Calypso 480 SC. Data are mean ± SE, n = 6. *Denotes indicate significant differences ($P \leq 0.01$) among groups at the same sample time (Mann-Whitney U Test). Superoxide dismutase (SOD), catalase (CAT), Control = 0 mg L^{-1}, CAL = Calypso 480 SC, CAL1 = 7.77 mg L^{-1}, CAL2 = 77.70 mg L^{-1}

4.1.2.5 Histological analysis and histopathological condition indices

Histological findings of both the digestive gland and gills show no marked lesions associated with pesticide exposure.

4.1.3 Acute exposure of Mytilus galloprovincialis to Thiacloprid

4.1.3.1 Cell viability assay

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 (Table 10). In the same way, no damage to the digestive cells was recorded (Table 11).

Table 10: Percentage of viability hemocytes in *M. galloprovincialis* exposed to Thiacloprid (CTH(0mg/L); TH1(1mg/L); TH2(5mg/L)).

	Exposure time (days)	CTH(0mg/L)	TH1(1mg/L)	TH2(5mg/L)	TH3(10mg/L)
TB (%)	3	98.69±0.86	98.79±0.34	92.13±1.40	98.74± 0.48
	7	100 ± 0	97.85±0.35	72.12±0.21	80.21 ±0.71
NR (%)	3	98.45 ±0.61	98.86±0.74	98.71±0.29	97.41± 1.23
	7	99.70± 0.20	98.00±0.26	93.42± 1.18	89.62±0.75

Table 11: Percentage of viability digestive cells in *M. galloprovincialis* exposed to Thiacloprid (CTH(0mg/L); TH1(1mg/L); TH2(5mg/L)).

	Exposure time (days)	CTH(0mg/L)	TH1(1mg/L)	TH2(5mg/L)	TH3(10mg/L)
TB (%)	3	100 ±0	99.43 ±0.33	97.74 ±0.08	97.86 ±0.55
	7	100 ± 0	99.55 ±0.45	94.34 ±0.23	93.17 ±0.73
NR (%)		100 ±0	99.64 ± 0.08	97.84 ±0.27	95.80 ±0.80
	7	99.39 ±0.35	99.02 ±0.56	81.50 ±0.75	91.55 ±0.89

4.1.3.2 The Regulation of Volume Decrease experiment

The cells exposed to the rapid change of osmolarity initially increased in size and then returned to their initial volume. The cells reached their maximum swelling, corresponding to a 12% increase in volume, after 1 min of exposure to the hypotonic medium. The exposed samples TH1, TH2 and TH3 show a response equal to that of the control CTH.

4.1.3.3 Haemolymph biochemical parameters

Mussels exposed to TH showed significant differences ($P < 0.01$) in haemolymph composition during the exposure period (Table 12). Con- centration 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). The 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 significantly increased LDH activity after 3 day and glucose levels during the experiment compared with controls (CTH).

Table 12: Haemolymphatic parameters of Mediterranean mussel (*Mytilus galloprovincialis*) exposed to thiacloprid (TH).

Treatment and 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	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}
(0 mg L^{-1})	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	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}
(1 mg L^{-1})	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	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}
(5 mg L^{-1})	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	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}
(10 mg L^{-1})	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}

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 ($P < 0.01$).

4.1.3.4 Determination of 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. 18). CAT activity was significantly increased ($P < 0.05$) in gills after 3 days of exposure to 10 mg L^{-1}(TH3) and decreased in the digestive gland after 7 days at 5 mg L^{-1} (TH2), compared to controls

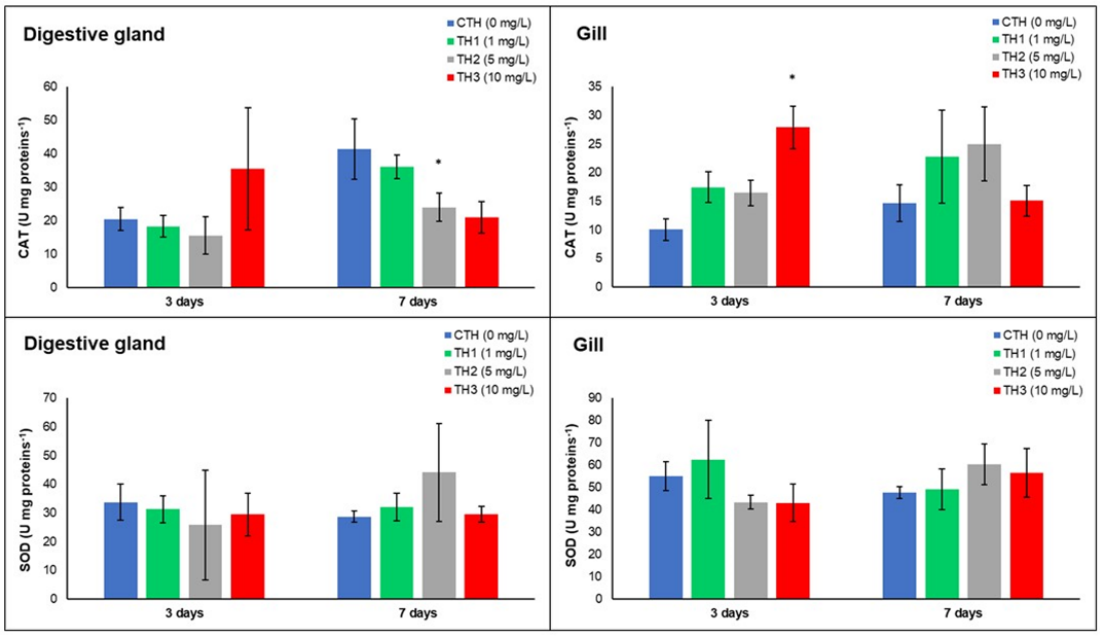


Figure 18: Enzymatic activity of catalase (CAT) and superoxide dismutase (SOD) in the digestive gland and gill in Mediterranean mussel (*Mytilus galloprovincialis*) exposed to thiacloprid (TH). The values are mean ± SE (standard error); n = 6; Mann-Whitney U Test

4.1.3.5 Histological analysis and histopathological condition indices

Histological findings of the digestive gland and gills show no marked lesions associated with pesticide exposure.

4.1.4 Sub- chronic exposure of Mytilus galloprovincialis to Thiacloprid

4.1.4.1 Cell viability assay

After 10 and 20 days of exposure, cells from animals exposed to all the concentrations tested, namely, Control (0 µg L^{-1}, Thia env (4.5 µg L^{-1}), and Thia 100x (450 µg L^{-1}), showed a high level of vitality and high stability of the lysosomal membranes and high viability (>80 %) (Table 13).

Table 13: Lysosomal stability assay and Trypan blue exclusion test in haemocytes and digestive gland cells of Mytilus galloprovincialis following exposure to thiacloprid.

Cell viability		Exposure time (days)	Test groups		
			Control (0 µg L^{-1})	Thia env. (4.5 µg L^{-1})	Thia 100x (450 µg 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*
	NR (%)	10	100.00 ± 0.00	97.87 ± 0.80*	81.03 ± 0.39*
		20	98.81 ± 0.45	89.63 ± 1.01*	89.85 ± 1.67*

Digestive gland cells	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*
	NR (%)	10	100.00 ± 0.00	98.68 ± 0.86	93.03 ± 2.66*
		20	100.00 ± 0.00	99.31 ± 0.45	98.32 ± 0.69

The values are shown as mean ± SE; One-way ANOVA followed by the posthoc Tukey test were used for statistical comparisons among experimental conditions.*$P < 0.05$ versus control condition. Thia env. (thiacloprid 4.5 µg L^{-1}), Thia 100x (thiacloprid 450 µg L^{-1}).

4.1.4.2 The Regulation of Volume Decrease experiment

After exposure to hypotonic solution, the digestive cells increased their volume up to a maximum of 18%. At all concentrations and sampling times, the cells were able to regulate this change in volume and return to the starting condition, as shown in the graphs in Fig. 19.

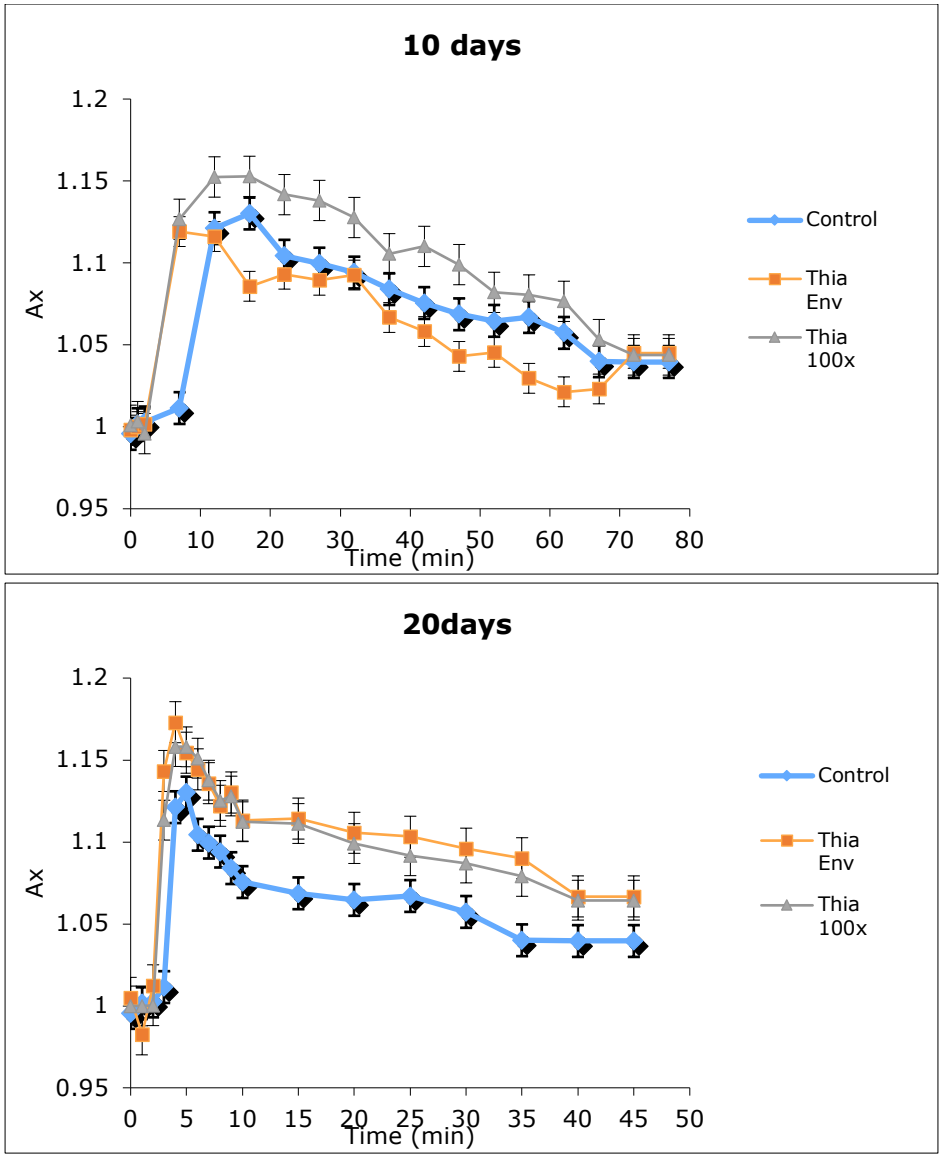


Figure 19: Relative area changes of digestive cells of *Mytilus galloprovincialis* exposed to hypotonic solution. The values are shown as mean ± SE; significant differences compared with control value ($P < 0.05$) (Two-way ANOVA test). Control (0 mg L-1) (◆), Thia env (4.5 µg L^{-1}) (■), Thia 100x (450 µg L^{-1}) (▲)

4.1.4.3 Haemolymph biochemical parameters

Effects of exposure to thiacloprid on haemolymph biochemical parameters of mussels are shown in Table 14. Both thiacloprid concentrations (4.5 and 450 µg L^{-1}) caused a significant increase in Ca^{2+} ($P < 0.05$) and NH_3 ($P < 0.01$) and a decrease in PHOS levels ($P < 0.05$) in the haemolymph of mussels after 10 days of exposure, compared to the control. It was also found that the level of urea decreased significant ($P < 0.05$) and Mg^{2+} increased significant ($P < 0.01$) in group thiacloprid 100x environmental after 10 days exposure compared with control group. After 20 days of exposure, thiacloprid did not affect other haemolymph parameters of mussels.

Table 14: Haemolymph biochemical indices of *Mytilus galloprovincialis* exposed to thiacloprid.

Haemolymph Indices	Exposure time (days)	Test groups			Value *P*
		Control (0 μg L^{-1})	Thia env. (4.5 μg L^{-1})	Thia 100x (450 μg L^{-1})	
Urea (mmol L^{-1})	10	0.760 ± 0.049[a]	0.767 ± 0.052[a]	0.700 ± 0.002[b]	< 0.05
	20	0.700 ± 0.001[a]	0.700 ± 0.001[a]	0.700 ± 0.002[a]	> 0.05
NH_3 (μmol L^{-1})	10	131.833 ± 16.678[a]	489.000 ± 48.237[b]	560.500 ± 37.179[b]	< 0.01
	20	142.500 ± 18.908[a]	105.500 ± 29.440[a]	141.833 ± 28.701[a]	> 0.05
PHOS (mmol L^{-1})	10	0.374 ± 0.112[a]	0.317 ± 0.027[ab]	0.253 ± 0.043[b]	< 0.05
	20	0.320 ± 0.056[a]	0.308 ± 0.040[a]	0.348 ± 0.074[a]	> 0.05
Ca^{2+} (mmol L^{-1})	10	8.145 ± 2.258[a]	17.575 ± 7.335[b]	14.417 ± 2.539[b]	< 0.05
	20	7.383 ± 4.395[a]	5.233 ± 2.270[a]	4.250 ± 0.476[a]	> 0.05
Mg^{2+} (mmol L^{-1})	10	46.967 ± 5.918[a]	59.867 ± 12.307[ab]	69.100 ± 7.583[b]	< 0.01
	20	62.867 ± 12.312[a]	70.533 ± 11.932[a]	66.733 ± 5.737[a]	> 0.05
ALB (g L^{-1})	10	4.000 ± 0.002[a]	4.500 ± 0.548[a]	4.667 ± 0.516[a]	> 0.05
	20	4.000 ± 0.001[a]	4.167 ± 0.408[a]	4.500 ± 0.548[a]	> 0.05
ALT (U L^{-1})	10	17.600 ± 2.154[a]	16.667 ± 2.066[a]	17.833 ± 2.041[a]	> 0.05
	20	18.833 ± 1.169[a]	17.833 ± 1.941[a]	21.000 ± 2.757[a]	> 0.05

The values are mean ± SD; n = 6; significant differences compared with control value (*P*). Thia env. (thiacloprid 4.5 μg L^{-1}), Thia 100x (thiacloprid 450 μg L^{-1}). PHOS (inorganic phosphate), ALB (albumin), ALT (alanine aminotransferase).

4.1.4.4 Determination of enzyme activity

In digestive gland of mussel changes in antioxidant biomarkers were observed (Fig. 20). CAT activity was significantly decreased ($P < 0.01$) after 10 days in mussels exposed to thiacloprid 100x (450 μg L^{-1}) and after 20 days in those exposed to both concentrations of thiacloprid compared to the control. SOD activity did not change between groups during the experiment.

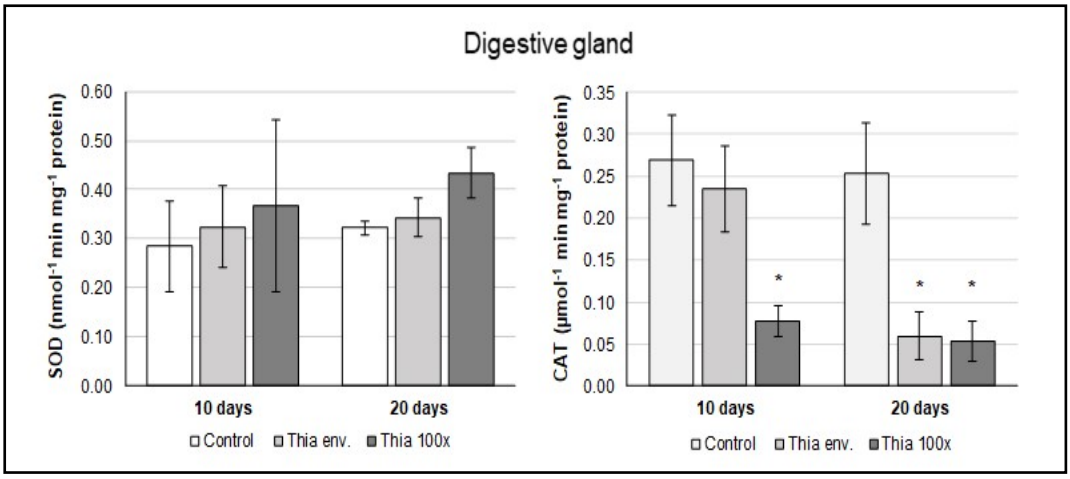


Figure 20: Antioxidant biomarkers and lipid peroxidation in digestive gland of *Mytilus galloprovincialis* exposed to thiacloprid. The values are mean ± SD; n = 6; significant differences compared with control value (P < 0.01). Thia env. (thiacloprid 4.5 mg L-1), Thia 100x (thiacloprid 450 μg L^{-1}), SOD (superoxide dismutase), CAT (catalase)

In gills, significant increase ($P < 0.01$) of SOD activity were observed after 20 days of exposure in both the exposed groups to thiacloprid (4.5 and 450 μg L^{-1}), compared with control group (Fig. 21). No significant alteration in CAT activity was observed during the experiment.

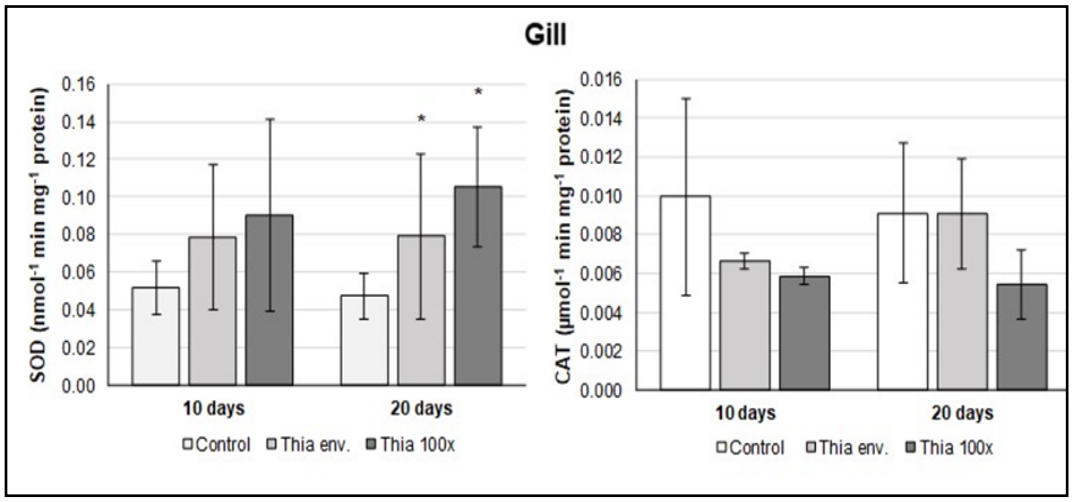


Figure 21: Antioxidant biomarkers and lipid peroxidation in gills of *Mytilus galloprovincialis* exposed to thiacloprid. The values are mean ± SD; n = 6; significant differences compared with control value ($P < 0.01$). Thia env. (thiacloprid 4.50 mg L-1), Thia 100x (thiacloprid 450 µg L^{-1}), SOD (superoxide dismutase), CAT (catalase)

4.1.4.5 Histological analysis and histopathological condition indices

Histological findings of both the digestive gland and gills show no marked lesions associated with pesticide exposure.

4.2 Zebrafish larvae exposed to environmental concentrations of fenitrothion

4.2.1 Analytical Chemistry

Table 15 shows the experimental concentration of fenitrothion for the five nominal concentrations used in this study. The experimental concentrations were calculated as the mean values of three replicates, showing values between 94-104% of the nominal concentrations. The recoveries obtained in the SPE procedure were in the range 94-102% (Table 15). Regarding the degradation assay, 0.17 and 17 µg/L were analyzed at times 0 and 24 hours after sample preparation. When the experimental concentrations of the 0.17 and 17 µg/L fenitrothion solutions were determined 24 hours after their preparation, no degradation was found for the 0.17 µg/L solution whereas the fenitrothion content in the 17 µg/L solutions decreased by only 7% (Table 15).

Table 15: Experimental concentrations of fenitrothion, recoveries obtained in the SPE procedure and stability of two of the concentrations 24h after preparation.

Nominal concentration (µg/L)	Experimental concentration (µg/L)	% Recovery	Experimental concentration (µg/L) after 24h
0.00170	$0.00174 \pm 1.95 \times 10^{-5}$	102.1 ± 1.15	-

0.0170	$0.0166 \pm 1.97x10^{-5}$	97.5 ± 0.12	-
0.170	$0.169 \pm 4.46x10^{-4}$	99.4 ± 0.37	$0.171 \pm 0.624x10^{-4}$
1.70	1.643 ± 0.0135	96.7 ± 0.79	-
17.0	16.063 ± 0.02	94.5 ± 0.1	14.947 ± 0.377

4.2.2 Experimental concentration of fenitrothion impair locomotor behaviors

None of the five selected concentrations resulted in signs of systemic toxicity (impaired gross morphology or lethality). Locomotor behaviour of the control and fenitrothion-treated larvae was analyzed using a battery of tests including basal locomotor activity (BLA), visual-motor response (VMR) and acoustic/vibrational escape response.

A significant effect on BLA was found in zebrafish larvae across the selected fenitrothion concentrations ($F_{5,587} = 13.831$, $P = 8.6 \times 10^{-13}$). This behavior decreased in larvae exposed to 1.7–170 ng/L and 17 µg/L fenitrothion compared to controls (Fig. 22A).

The analysis of VMR reported also significant effects on this behavior across fenitrothion concentrations ($F_{5,538} = 18.440$, $P = 6.4 \times 10^{-17}$). VMR increased in larvae exposed to 170 ng/L to 17 µg/L (Fig. 22B).

We then analyzed the effect of the five environmental concentrations of fenitrothion on the acoustic/vibrational escape response in zebrafish larvae, Fig. 22C shows that fenitrothion exhibited a non-monotonic dose-response effect on this behavior ($F_{5,528} = 18.778$, $P = 3.4 \times 10^{-17}$), with an increase in the startle response at 17 ng/L and then the startle magnitude decreases from 170 ng/L to 17 µg/L. Finally, we analyzed the effect of fenitrothion on the kinematic of the acoustic/vibrational-evoked escape response, a highly stereotyped complex behavior constituted by three sequential modules: a very fast and large C-bend followed by a high amplitude counter bend and, finally, the fast swimming oriented away from the stimulus. Two different types of escape responses can be evoked by an acoustic/vibrational stimulus, a short latency C-bend (SLC) and a long latency C-bend (LLC), although only SLC can be considered a startle response (Burgess & Granato, 2007). In order to determine if the decrease in the magnitude of the vibrational-evoked escape response found by tracking the larvae on the DanioVision was in fact the result of a change in the SLC/LLC ratio, an automatized setup was built including a vibration exciter to provide the stimuli and a high-speed camera to record the larvae movement at 1000 fps. The intensity of the vibrational stimulus was selected to evoke SLC in about 70% of the control larvae (Wolman et al., 2011b). Fig. 22D shows that after 24 h of exposure to 17 µg/L fenitrothion there was a significant increase in the SLC/LLC

ratio. As the distance travelled during SLC has been reported to be higher than during LLC (Marquart et al., 2019), the increased percentage of SLC found in fenitrothion-exposed larvae should lead to an increase in the total distance travelled after the stimulus rather than a decrease in the distance travelled by the larvae consistently found in the DanioVision response to the vibrational stimulus. One potential explanation would be that fenitrothion specifically targeted the last step of SLC in zebrafish larvae, the fast swimming bouts, reducing the total distance travelled. In order to assess this hypothesis, a different design was used, recording the movement of the larvae for 300 ms (30 ms before the stimulus delivery and 270 ms after) in a Petri dish without any grid. In this case, kinematic analysis was performed using Flote software. Surprisingly, kinematic analysis showed that the main parameters characterizing SLC, including latency (9.06 ± 0.32 ms vs 8.28 ± 0.34 ms for control and fenitrothion- treated larvae, respectively; n = 50–53; P = 0.100, Student's t-test), amplitude of the C-bend (118.34 ± 3.53 degrees vs 119.64 ± 2.05 degrees for control and fenitrothion-treated larvae, respectively; n = 50–53; P = 0.749, Student's t-test), duration of the C-bend (10.16 ± 0.11 ms vs 9.85 ± 0.13 ms for control and fenitrothion-treated larvae, respectively; n = 50–53; P = 0.075, Student's t-test) and distance traveled in 270 ms after the stimulus (11.82 ± 0.34 mm vs 12.42 ± 0.45 mm for control and fenitrothion-treated larvae, respectively; n = 50–53; P = 0.298, Student's t-test) remained unchanged in fenitrothion- treated larvae.

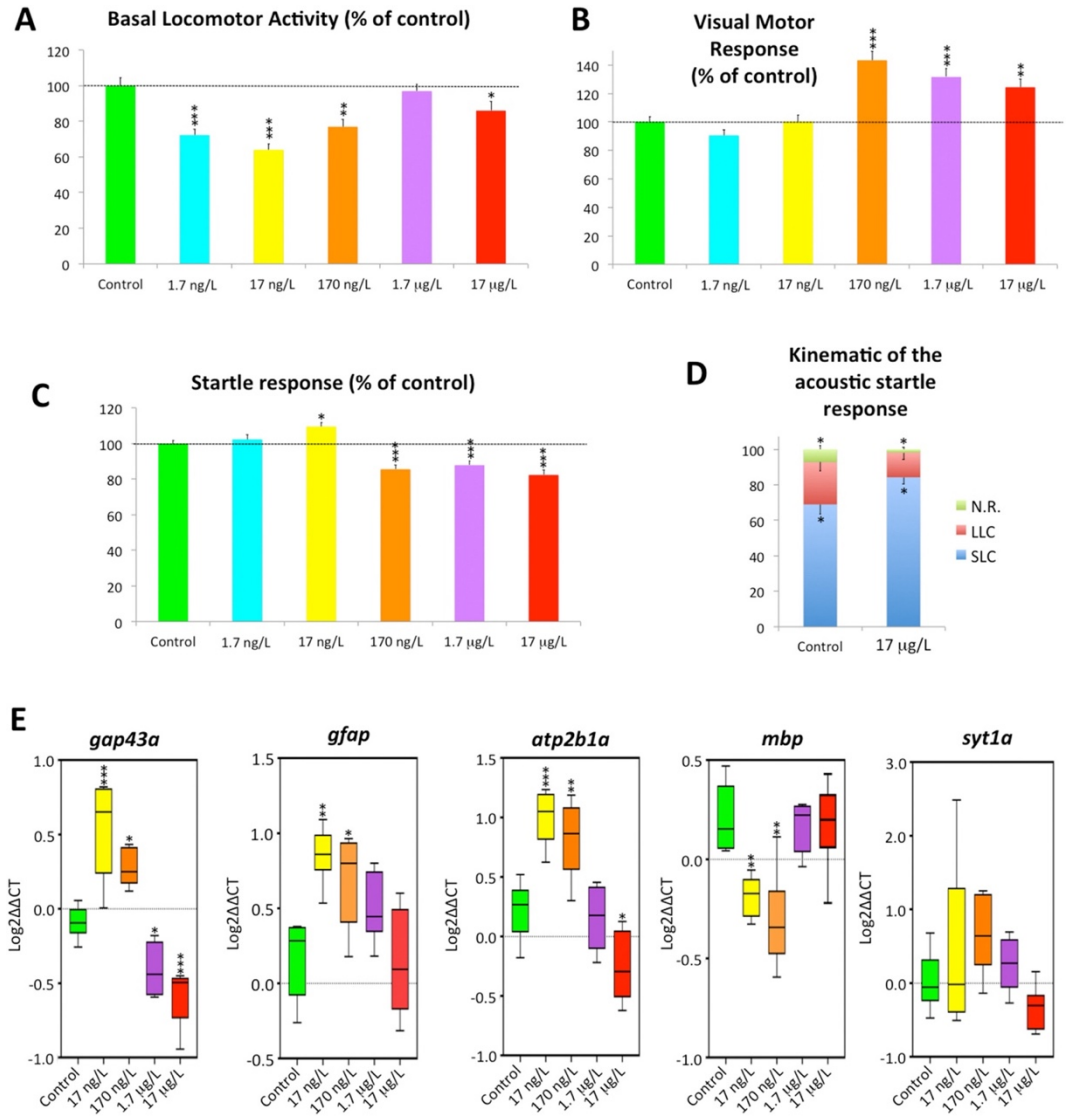


Figure 22: Short-term (24 h) exposure to environmental concentrations of fenitrothion induces neurotoxicity in 8 days-post fertilization zebrafish larvae. (A) Analysis of the basal locomotor activity (BLA), shows the hypoactivity of larvae exposed to 1.7–170 ng/L and 17 µg/L. (B) Analysis of the visual-motor response (VMR) showed a significant increase in larvae mobility exposed to 0.17–17 µg/L during the first 2 min of the light/dark challenge. (C) Analysis of the acoustic/vibrational escape response shows a significant decrease of the escape response evoked by one tapping stimulus in larvae exposed to 0.17–17 µg/L fenitrothion, and an increase in the response in larvae exposed to 17 ng/L. (D) Kinematics of the vibrational escape response shows an increase in the SLC/LLC ratio in larvae exposed to 17 µg/L (N.R.: non-responder; SLC: short-latency C-bend; LLC: long-latency C-bend). Data reported as mean ± SEM (n = 81–167 for BLA, n = 76–154 for VMR, n = 73–148 for acoustic/vibrational escape response and n = 12–15 for kinematic analysis SLC/LLC ratio) (E) Gene expression of five selected transcriptional markers of neurotoxicity in zebrafish larvae control or exposed to four Environmental concentrations of fenitrothion. Boxplot representation of ΔΔCt values, with the box indicating the 25th and 75th percentiles and the whiskers the maximum and minimum values. The thin line within the box marks the median (n = 6–8 pools). *P < 0.05, **P < 0.01, ***P < 0.001; one-way ANOVA with Dunnett's multiple comparison test. Data from 2 to 4 independent experiments.

4.2.3 *Non-monotonic concentration-response relationship between fenitrothion and some transcriptional neurotoxicity markers*

In order to better characterize the neurotoxic effects of fenitrothion at the molecular level, the expression of five genes involved in different functions in the central and peripheral nervous system was analyzed (Fig. 22E). A non-monotonic concentration-response curve (NMCRC) with an inverted U-shape was found to describe the relationship between fenitrothion and gap43a expression (Fig. 22B), with a significant up-regulation in the expression of this gene at the two lowest concentrations and a down-regulation at the two highest concentrations ($F_{4,25}$ = 32.983, P = 1.2×10^{-9}). An inverted U-shaped NMCRC also described the relationship between fenitrothion concentrations and gfap expression, with a significant increase in the expression of this gene at the two lowest concentrations and no significant effects at the higher concentrations ($F_{4,25}$ = 8.003, P = 0.00026). Glial fibrillary acid protein (GFAP), the protein encoded by gfap, is a classical marker of astrogliosis.

Expression of atp2b1a, a marker of the integrity of the hair cells in inner ear and neuromasts (Go et al., 2010), exhibited also a U-shaped non-monotonic relationship with fenitrothion concentrations ($F_{4,25}$ = 21.548, P = 8.5×10^{-8}). Whereas larvae exposed to 17–170 ng/L fenitrothion exhibited a significant up-regulation of atp2b1a, the expression of this gene in larvae exposed to 17 µg/L fenitrothion was down- regulated.

In contrast to gap43, gfap and atp2b1a, the relationship between myelin basic protein (mbp) expression and fenitrothion was better described with an inverted U-shaped NMCRC, since the expression of this gene was significantly reduced at 17–170 ng/L fenitrothion, but not at higher concentrations. Finally, expression of syt1a, a transcriptional marker of synaptic vesicle cycling, was not significantly altered by any of the tested concentrations of fenitrothion.

4.2.4 *Predicted target profile for fenitrothion*

In order to explore the toxicological targets of fenitrothion potentially behind the observed neurotoxic effect, the CLARITY (Chemotargets CLARITY v4, 2019) and SEA (Keiser et al., 2007b) platforms recommended in the PHASE initiative (Ellis et al., 2019, 2020b) were used to predict its off-target pharmacology.

The list of predicted human targets for fenitrothion is collected in Table 16. Taking the confidence/significance thresholds recommended by each platform, a total of six and four targets were identified by CLARITY and SEA, respectively. Among them, the only common target of fenitrothion predicted by both platforms is carboxylic ester hydro- lase, a protein with acetylcholinesterase activity.

Focus was then put on selecting, for further consideration, any predicted targets known to be linked to neurotoxic effects. Within the list of predictions from CLARITY above the recommended confidence score, two neurotoxic-associated targets were detected, namely, the androgen receptor (AR) and the sodium-dependent serotonin transporter (SLC6A4, also referred as SERT). In addition, five additional neurotoxic-associated targets could be recovered from predictions below the recommended confidence scores. These are the amine oxidase [flavin-containing] A and B (MAOA and MAOB), the tyrosine 3-monooxygenase (TH), the dopamine beta-hydroxylase (DBH) and the catechol O-methyltransferase (COMT).

Further experimental studies were then designed to explore the possibility that any of these predicted targets could be responsible for the observed neurotoxic effect of fenitrothion in zebrafish larvae.

Table 16: Predicted targets of fenitrothion by CLARITY and SEA. Confidence scores from CLARITY and p-values from SEA are provided.

Protein(s)	Gene name(s)	CLARITY	SEA
Carboxylic ester hydrolase	ACE2	1.00	3.4E-43
Hepatocyte nuclear factor 4-alpha	HNF4A	0.38	4.2E-07
G protein-coupled receptor 35	GPR35	0.47	-
Hydroxycarboxylic acid receptor 2/3	HCAR2/3	0.47	-
Kynurenine 3-monooxygenase	KMO	0.42	-
Palmitoleoyl-protein carboxylesterase	NOTUM	0.34	-
Transthyretin	TTR	0.32	-
Krueppel-like factor 10	KLF10	-	8.2E-33
Free fatty acid receptor 4	FFAR4	-	9.9E-20

4.2.5 Validating in silico prediction with human in vitro functional assay

In vitro binding and enzymatic assays were performed to validate some of the fenitrothion interactions suggested by the in silico predicted target profile (Table 17). Whereas no inhibition of human AChE, MAO-A and MAO-B activities was found in these assays, the binding of fenitrothion to the human AR was confirmed, resulting in inhibitions of 44.3% and 96.4% of the binding of the synthetic radiolabeled androgen [3H] methyltrienolone to the human AR when cells were

obtained after exposure to 100 nM and 10 µM fenitrothion, respectively. These results demonstrate that the primary toxicological target of fenitrothion in humans is AR and not AChE. Both human and rat serotonin transporter (SERT) has been reported to be inhibited by fenitrothion (https://comptox.epa.gov/ dashboard). Therefore, in addition to validating the interactions predicted in our in-silico target profile, an *in vitro* binding assay was performed to determine potential interactions of fenitrothion with human SERT. While exposure to 10 µM fenitrothion resulted in a 66.5% inhibition of the binding of [^{3}H] imipramine to human SERT, no effect was found when using 100 nM fenitrothion.

Table 17: Binding and Enzymatic assays (Eurofins cerep). Numbers in red are considered significant (>50% inhibition)

ASSAY	ASSAY ITEM #	% Inhibition (100 nM)	% Inhibition (10 uM)
AR(h) (agonist radioligand)	933	44.3%	96.4%
5-HT transporter (h) (antagonist radioligand)	439	7.6%	66.5%
Acetylcholinesterase (h)	363	0.7%	-2.5%
MAO-A (h)	191	1.5%	3.2%
MAO-B (h)	3477	-9.5%	-6.0%

4.2.6 *Exploring fenitrothion interactions with zebrafish proteins*

In vitro functional assays to validate some of the predicted interactions of fenitrothion with proteins were performed using human proteins, since these assays are not currently commercially available for zebrafish proteins. Therefore, we decided to directly explore the potential interactions of fenitrothion with zebrafish proteins *in vivo* (Fig. 23).

It is generally assumed that the molecular initiating event of fenitrothion neurotoxicity is AChE inhibition. Thus, in order to understand the molecular mechanisms involved in the behavioral changes observed in zebrafish larvae exposed to environmental concentrations of fenitrothion, the AChE activity of control and fenitrothion-exposed larvae was determined. As Fig. 23A shows, concentrations of fenitrothion in the range 17 ng/L to 17 µg/L were not able to significantly inhibit AChE activity ($F_{4,39} = 0.531$, $P = 0.713$). In fact, in the exposed larvae fenitrothion was unable to inhibit AChE activity even at concentrations as high as 1.7 mg/L (Table 18). These results indicate that the effect of fenitrothion on larvae behavior was mediated through AChE-independent mechanisms.

A potential androgen receptor agonistic role of fenitrothion at environmentally concentrations was then explored by analyzing the expression of two androgen-

responsive genes, cyp19a1b and sult2st3 (Fig. 23B). An inverted U-shaped NMCRC described the relationship between the expression of cyp19a1b ($F_{4,26}$ = 4.490, P = 0.007) and sult2st3 ($F_{4,26}$ = 5.185, P = 0.003) and the fenitrothion levels, with a significant up-regulation of both genes after exposure to the lowest concentrations and no effect at higher concentrations.

In order to validate the interactions of fenitrothion with zebrafish SERT, the expression of slc6a4a, the gene encoding this transporter, was determined (Fig. 23C). However, as Fig. 23C shows, fenitrothion had no a significant effect on the expression of this gene (H(4) = 7.368, P = 0.118).

Finally, in order to validate potential interactions of fenitrothion with zebrafish MAO, both gene expression and enzymatic activity were determined in control and treated larvae (Fig. 23D). Fenitrothion was able to alter MAO expression (F4,26 = 3.122, P = 0.032), and a mild but significant up-regulation in the expression of this gene was found in larvae exposed to 17 ng/L and 1.7 µg/L fenitrothion. Larvae exposed to 170 ng/L fenitrothion also exhibited a similar trend to up-regulate MAO expression (P = 0.069), while no effect was found for larvae exposed to 17 µg/L (P = 0.114).). Then, in order to better understand the potential role of MAO in the observed behavioral changes, zebrafish MAO activity was determined in larvae exposed to 17 ng/L - 17 µg/L fenitrothion for 24 h, using 1.1 mg/L deprenyl, a specific MAO inhibitor, as positive control (Fig. 23D). Despite the significant up-regulation of MAO expression found in larvae exposed to 17 ng/L and 1.7 µg/L fenitrothion, this insecticide was no able to inhibit MAO activity at any tested concentration (H(4) = 6.771, P = 0.149).

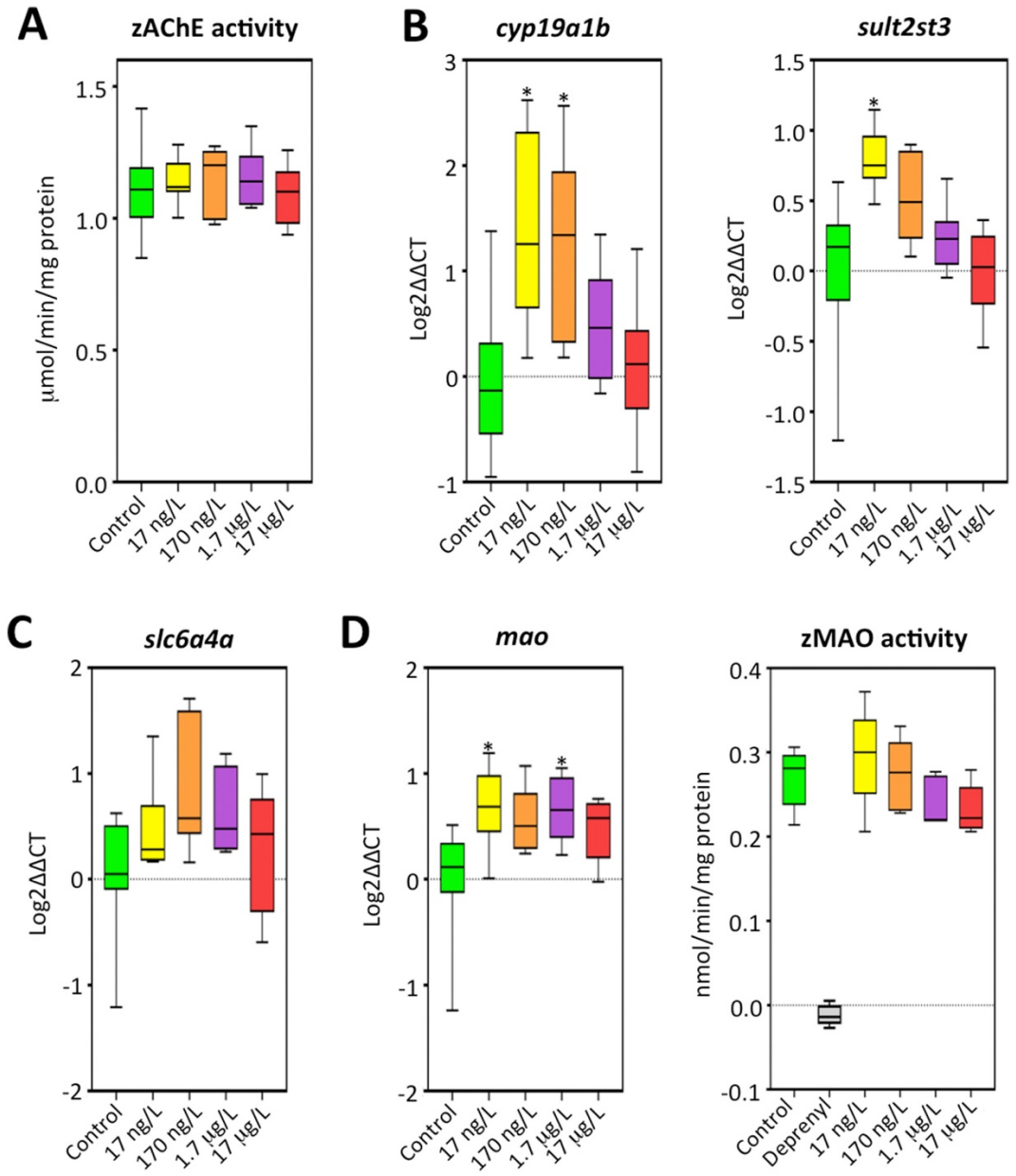


Figure 23: Validation in zebrafish larvae of in silico predicted molecular targets of fenitrothion(A) Zebrafish acetylcholinesterase (zAChE) activity in control and fenitrothion exposed 8 days-post fertilization zebrafish larvae, showing that the selected concentrations of fenitrothion are not able to induce a significant inhibition of AChE activity ($P > 0.05$; one-way ANOVA with Dunnett's multiple comparison test). Data from 2 independent experiments (n = 8–9). (B) The potential androgenic or anti-androgenic effect of fenitrothion on zebrafish larvae was assessed by analyzing the expression of *cyp19a1b* and *sult2st3*, two well established markers of androgenicity in zebrafish. (C) Expression of *slc6a4a* (zebrafish SERT) in control and fenitrothion exposed zebrafish larvae. (D) Expression of *mao* and MAO activity in control and fenitrothion exposed zebrafish larvae. Boxplot representation with the box indicating the 25th and 75th percentiles and the maximum and minimum values whiskers. The thin line within the box marks the median. *$P < 0.05$; One way ANOVA followed by Dunnett's multiple comparison test was used for zAChE activity and *cyp19a1b*, *sult2st3* and *mao* expression, whereas Kruskal Wallis test was followed by Dunn's multiple comparison test against the control values was used for *slc6a4* expression and MAO activity.

Table 18: AChE activity, expressed as a percentage of the control values, in zebrafish 8 days post-fertilization larvae waterborne exposed for 24 h to fenitrothion concentrations in the range 17 ng/L-1.7 mg/L

	AChE activity (% control)		
Fenitrothion concentration	**Mean**	**SE**	***P***
17 ng/L	103.03	2.63	0.59
170 ng/L	103.12	4.06	0.64
1.7 µg/L	104.29	3.15	0.47
17 µg/L	97.84	3.35	0.71
170 µg/L	88.53	6.74	0.17
1.7 mg/L	94.97	11.30	0.69

4.2.7 Effect of fenitrothion on monoaminergic system

The in silico predictions suggested that fenitrothion may also directly interact with key proteins involved in the synthesis (TH) or metabolism (DBH, COMT) of monoaminergic neurotransmitters. Therefore, the monoaminergic profile of the control and exposed larvae, as well as the expression of main monoaminergic-related genes have been determined (Fig. 24).

As Fig. 24A shows, whereas dopamine (DA) levels remained unchanged, fenitrothion significantly altered the levels of its precursors tyrosine ($F_{4,35} = 21.392$, $P = 5.3 \times 10^{-9}$) and L-DOPA ($F_{4,35} = 43.261$, $P = 4.4 \times 10^{-13}$), with the former increasing at concentrations in the range 17–170 ng/L and the latter at all tested concentrations. Fenitrothion also exhibited a significant effect on the levels of the DA metabolites DOPAC (H(4) = 23.354, P = 0.0001) and HVA (F4,35 = 14.306, $P = 5.1 \times 10^{-7}$), with a significant decrease of the former in larvae exposed to all tested concentrations and a significant increase of the latter only in those larvae exposed to 17–170 ng/L. Norepinephrine levels were also significantly altered by fenitrothion ($F_{4,35} = 2.799$, $P = 0.041$), with decrease in the levels of this catecholaminergic neurotransmitter after exposure to 1.7 µg/L fenitrothion. Similarly to DA, serotonin levels remained unchanged after fenitrothion exposures (Fig. 25). Finally, although fenitrothion did not show a significant effect ($F_{4,35}$ =

2.031 P = 0.111) on 5-HIAA, the main serotonin metabolite, its levels were found significantly reduced after exposure to 17 ng/L fenitrothion (Fig. 24).

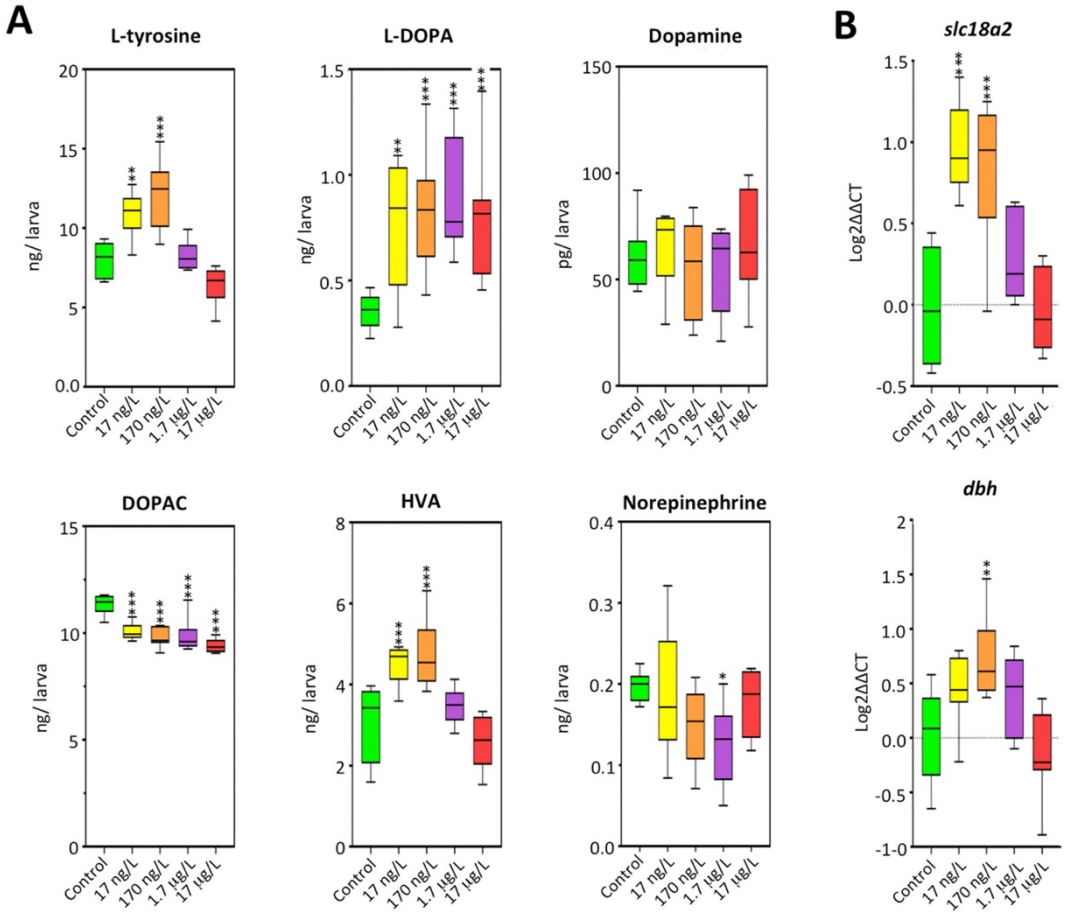


Figure 24: Effect of environmental concentrations of fenitrothion on the monoaminergic system in zebrafish larvae. (A) Effects of fenitrothion on the levels of some neurochemicals from the monoaminergic system. (B) Effects of fenitrothion on the expression levels of *slc18a2* (gene encoding zebrafish VMAT2) and *dbh*. Boxplot representation of ng or pg of the neurochemical per larva (A) or ΔΔCt values (B). The box indicates the 25th and 75th percentiles and the whiskers the maximum and minimum values. The thin line within the box marks the median [n = 8 pools (A) or 6–8 pools (B)].*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; one-way ANOVA with Dunnett's multiple comparison test. Data from 2 to 4 independent experiments.

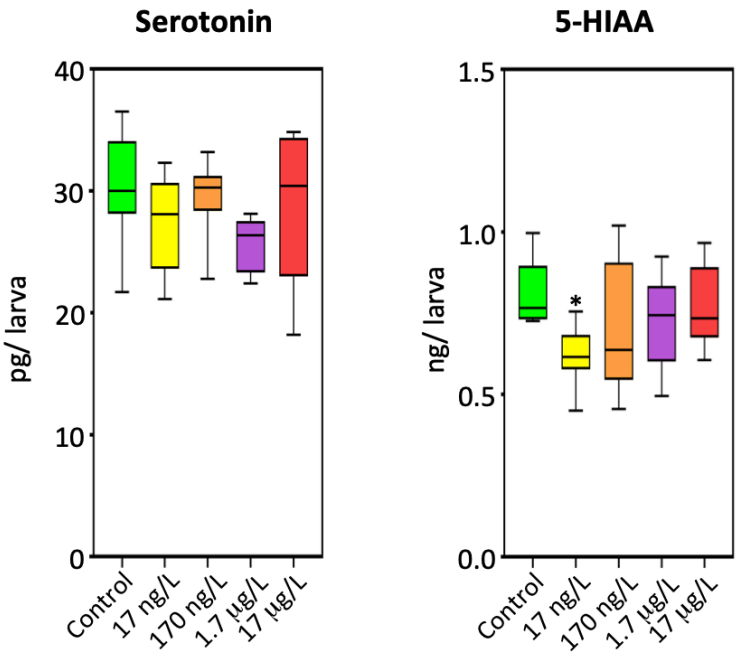


Figure 25: Neurotransmitter profile of serotonergic system in control and fenitrothion-exposed larvae. Whereas no effect of fenitrothion on serotonin levels was found, larvae exposed to 17 ng/L fenitrothion exhibited a significant decrease in the 5-HIAA levels. (*P< 0.05; one-way ANOVA with Dunnett's multiple comparison test). Data from 2 independent experiments (n = 8)

Whereas the expression of the monaminergic-related genes slc6a4a and mao was already analyzed (Fig. 24C, D) as a part of the validation process of the in silico predictions, the expression of genes involved in the synthesis of dopamine (th1 and th2), serotonin (tph1a and tph1b) and norepinephrine (dbh), the vesicular monoamine transporter 2 (slc18a2, encoding VMAT2), dopamine active transporter (slc6a3, encoding DAT), as well as the gene involved in dopamine metabolism catechol-O-methyltransferase (comtb) was determined to evaluate the effect of fenitrothion exposure on this essential modulatory neurotransmitter system (Fig. 24 and Fig. 25). Whereas no effect of fenitrothion on th1, th2, tph1a, tph1b, slc6a3 and comtb expression levels was found when compared with the control values ($P > 0.05$; One way ANOVA followed by Dunnett's multiple comparison test; Fig. 26), an inverted U-shaped NMCRC described the relationship between the slc18a2 expression and the fenitrothion levels ($F_{4,33} = 15.189$, $P = 3.8 \times 10^{-7}$), with a significant up-regulation of this gene after exposure to the lowest concentrations and no effect at higher concentrations (Fig. 24B). Finally, fenitrothion exhibited also a significant effect on dbh expression ($F_{4,33} = 6.397$, $P = 0.0006$), although only larvae exposed to 170 ng/L of these chemicals presented dbh levels significantly higher than the corresponding controls (Fig. 24B).

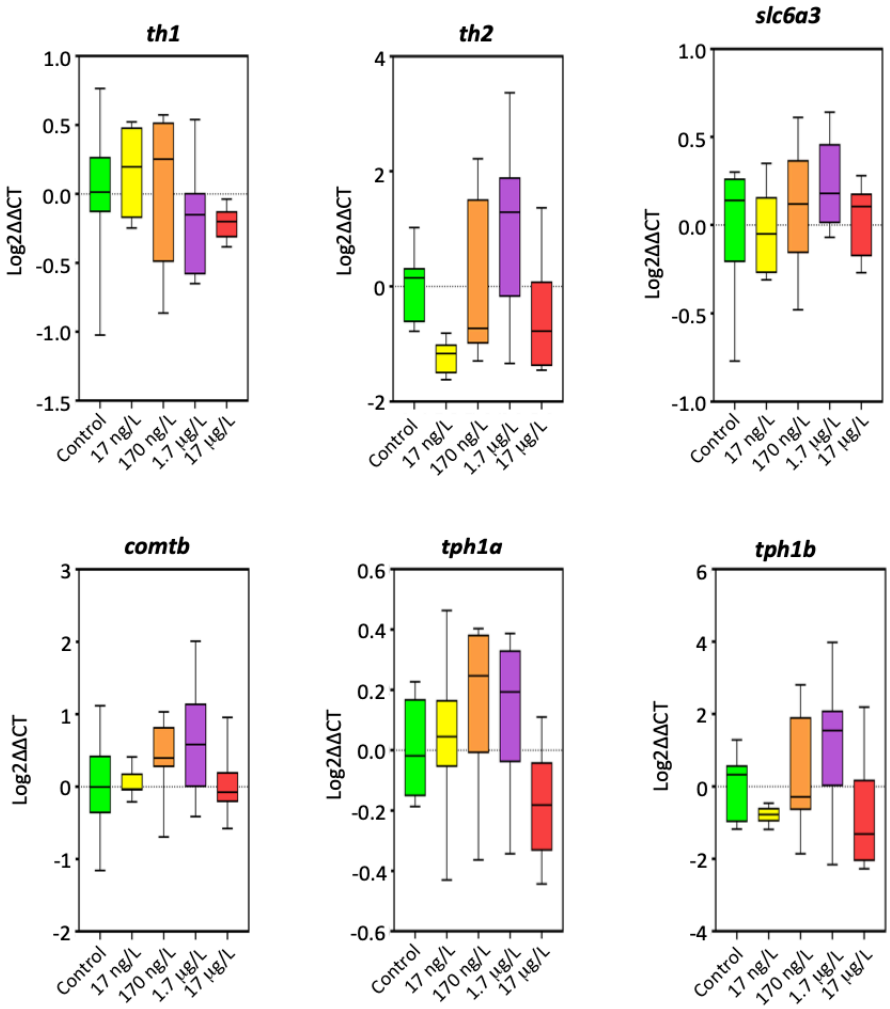


Figure 26: Fenitrothion has no significant effect on the expression of some genes directly involved in the monoaminergic system of zebrafish larvae. Boxplot representation of ΔΔCt values, with the box indicating the 25th and 75th percentiles and the whiskers the maximum and minimum values. The thin line within the box marks the median (n= 6-8 pools; one-way ANOVA with Dunnett's multiple comparison test. Data from 2 independent experiments)

4.2.8 Revisiting the risk assessment for fenitrothion

The finding that environmental concentrations of fenitrothion are able to alter zebrafish larvae behaviour strongly suggests that the environmental risk of this pesticide should be revisited. The predicted non-effect concentration (PNEC) of fenitrothion in zebrafish larvae can be determined by using the non-observed effect concentration (NOEC) for the different behaviours analyzed. Whereas the basal locomotor activity of the larvae was altered even at the lower concentration tested, NOEC for the vibrational startle response was 1.7 ng/L fenitrothion.

5. Discussion

5.1 *Neonicotinoid exposure to Mytilus galloprovincialis*

5.1.1 Cells viability

Neonicotinoids are used relatively little and their initial assessment was assumed to be harmless. However, they are frequently found in aquatic ecosystems and their negative impacts on non-target organisms are a reality (Beketov & Liess, 2008a; Pisa et al., 2015; Sánchez-Bayo et al., 2016). Mussels are widely used filter feeders, as bioindicators that can accumulate a wide range of contaminants and reflect changes in the contaminant status of the aquatic environment (Faggio et al., 2016a). To assess the interaction with the pollutant, we evaluated cell membrane integrity by Trypan Blue and lysosome integrity by Neutral Red in haemocytes and digestive gland cells. Lysosomes are important targets because they are the second line of defence against oxidative stress (Burgos-Aceves et al., 2016; Domouhtsidou et al., 2004). Assessing the health status of haemocytes can also evaluate the action of pollutants as these cells play a primary role in innate immune defence. They are also activated by many pollutants and participate directly by eliminating the pathogen through phagocytosis and producing lysosomal enzymes and antimicrobial molecules that help destroy and eliminate the pathogen (Renault et al., 2011; Sureda et al., 2013). 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 the assessment of the toxicity of many substances(Faggio et al., 2018; Messina et al., 2014; Mezzelani et al., 2018; Pagano et al., 2016; Regoli et al., 2004; Torre et al., 2013).

During acute exposure of mussels to both Calypso 480 and thiacloprid, we found no damage either at the level of haemocytes or at the level of digestive gland cells. In fact, both cell lines showed high viability. This was maintained even after sub-chronic exposure to thiacloprid. Previous studies (Faggio et al., 2016a, 2016b; Pagano et al., 2017), confirm that even exposure to similar pollutants or heavy metals in *M. galloprovincialis*, does not affect cell viability and normal animal functions. Long-term exposure to thiacloprid did not alter cell viability in either haemocytes or digestive gland cells.

These results show that mussels exhibit different behaviour than other aquatic organisms exposed to the same pollutants (Beketov & Liess, 2008b; Stara, Bellinvia, et al., 2019; Velisek & Stara, 2018b).

An inverse behaviour was shown during sub-chronic exposure to Calypso 480. Our experiments show that CAL is harmful to haemocytes after longer exposure (20 days). Prolonged exposure to both concentrations caused an overload in lysosomal storage, leading to low membrane stability, which is an obvious reaction to toxic damage. The result of the overt toxicity was confirmed by the TB exclusion test of cell viability.

5.1.2 *Regulation volume decrease*

After acute exposure to both Calypso 480 and thiacloprid, digestive gland cells showed no changes in volume regulation. The absence of interaction between the pollutants and the membrane revealed by the trypan blue test is confirmed by normal physiological responses of the cells after exposure to a hypotonic solution in cell volume regulation tests. In contrast, following sub-chronic exposure to thiacloprid, the cells respond physiologically after washing with hypotonic solution, but we noticed that the cells of animals treated with thiacloprid swelled more than the control, so we can assume that there is an interaction between the cells and the pollutant, but it does not alter their function. In contrast, sub-chronic exposure to Calypso 480 showed that the presence of pesticides probably inhibits the cellular structures that regulate the osmotic variations to which animals are 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 are normally involving in the processes of regulation of cell volume (Lungu-Mitea et al., 2018; Torre et al., 2013).

5.1.3 *Haemolymph biochemical parameters*

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 the determination of serum biochemical parameters of fish (Capillo et al., 2018). Changes in haematology indices of mussels caused by exposure to xenobiotics have been described in several studies (Faggio et al., 2010a, 2018; Matozzo et al., 2012; Milan et al., 2018).

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-conform organisms, are resistant to environmental variables and trigger adaptive processes, such as regulation of haemolymph ions concentration (Capillo et al., 2018). Glucose is the main source of energy and increased concentration is indicative of stress in aquatic organisms exposed to environmental contaminants. A very marked reduction of glucose indicates acute body failure due to sudden glycogen depletion (Kulkarni & Barad, 2015). Similarly,

changes in the activity of the LDH are considered a good diagnostic biomarker of cell membrane damage caused by pollutants (Ateyya et al., 2015; Faggio et al., 2016b; Kolarova & Velisek, 2012).

Sub-chronic exposure to Calypso induced a significant decrease in Cl^- and Na^+ ions. Glucose is the main source of energy and it indicates failure of the digestive gland function and sudden glycogen depletion (Sánchez-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 to the digestive gland (Matozzo et al., 2012; Pagano et al., 2016). Haemolymph minerals ions are essential components of many systems, including the skeleton, enzymatic activity, muscle metabolism, osmoregulation etc. (Gustafson et al., 2005). Changes in K^+ and Cl^- levels could support the results obtained in the RVD test, in fact, the increase is visible only after sub-chronic exposure to Calypso. These findings correspond to our results identified in the other parameters and confirm that CAL at sublethal doses has a negative impact on mussels.

An environmental concentration of thiacloprid (4.5 μg L^{-1}) significantly increased the levels of Ca^{2+}, Mg^{2+} and NH_3 and decreased PHOS levels after exposure for 10 days. In addition, at 100 times higher concentrations of thiacloprid, urea levels were also reduced. However, after 20 days of exposure, all parameters of haemolymph in mussels haemolymph exposed to both concentrations of thiacloprid were at values comparable to the Control. Haemolymph composition depends on the water parameters (pH, temperature, ionic concentration, salinity, etc) and quality of the aquatic environment (Capillo et al., 2018). The ions Ca^{2+}, Mg^{2+}, PHOS, K^+, Na^+, Cl^-, are essential components of haemolymph. They are involved in metabolism, enzyme function and shell formation (Gustafson et al., 2005; Stara et al., 2020). Changes in PHOS and Ca^{2+} levels indicate kidney damage; in addition, Ca^{2+} indicates defects in calcium metabolism (Kolarova & Velisek, 2012; Wimalawansa, 1960). The precise function of Mg^{2+} is not well understood in bivalves (Fritts et al., 2015). In general, Mg^{2+} is important in osmoregulation, ion homeostasis, and enzyme activation and is involved in a number of anabolic and catabolic processes (Fritts et al., 2015; Kolarova & Velisek, 2012). Ammonia is produced by nearly all living cells, usually as a catabolic product of the digestion and metabolism of proteins (Weihrauch & Allen, 2018). As NH_3 is highly toxic, it must either be excreted or converted to less toxic end products, such as urea. Marine and freshwater fishes, crustaceans and bivalves excrete 80–90% of their nitrogenous wastes as NH_3 and the remainder as urea (Weihrauch & Allen, 2018; Wilkie, 2002; Zhen et al., 2010). Concentrations of pollutants in the aquatic environment are clearly reflected in the changes and composition of the haemolymph in the first days of exposure, and the organism is likely to cope with exposure to the toxic effect of thiacloprid. Balancing biochemical parameters of haemolymph to physiological values comparable to the Control after prolonged exposure time can be considered

the acclimatization ability of mussels to adapt to the changing environment, based on our results, as well as those of other studies (Capillo et al., 2018; Matozzo et al., 2020; Zhen et al., 2010).

Given the importance of haemolymph evaluation, there were many studies dealing with the evaluation of bivalve haemolymph (Capillo et al., 2018; Faggio et al., 2016a; Fritts et al., 2015; Lopes-Lima et al., 2012), few are focused on exposure to pesticides (Ait Ayad et al., 2010). 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 assessing 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).

5.1.4 Determination of enzyme activity

The assessment of enzyme activities has been considered a useful biomarker of aquatic species exposure to pollutants (Fiorino et al., 2018; Matozzo et al., 2018; Plhalova et al., 2018; Sehonova et al., 2019; Vieira et al., 2018a). Antioxidant enzymes play an effective antioxidant role by preferentially scavenging reactive oxygen species (ROS) and maintaining a steady state in cells (Faria et al., 2009; Vidal-Liñán & Bellas, 2013). CAT and SOD enzymes are the first lines of defence against ROS, protecting organisms from oxidative damage (Burgos-Aceves & Faggio, 2017; Chi et al., 2019). They are found in virtually all aerobic organisms (Ewere et al., 2019). SOD catalyses the conversion of O_2^- to O_2 and H_2O_2, while CAT catalyses the conversion of H_2O_2 to H_2O and O_2 (Somani et al., 1997). The gill is a very potent metabolically active tissue and is the first barrier faced by xenobiotics in mussels (Vidal-Liñán & Bellas, 2013) and the gills are often used to assess mussel responses 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 elimination of xenobiotics (Ewere et al., 2019; Faggio et al., 2018; Marigómez et al., 2002). During acute exposure, Thiacloprid caused an increase in CAT activity in the gills at TH3 (10 mg L^{-1}) after 3 days of exposure and a decrease in the digestive gland at TH2 (5 mg L^{-1}) after 7 days. Mussels exposed to CA showed a decrease in CAT activity in the digestive gland at CA3 (100 mg L^{-1}) after 3 days and in the gills at all experimental concentrations tested (10, 50 and 100 mg L^{-1}) after 7 days. Similarly, all CA concentrations decreased SOD activity in the gills after 3 days. Similarly, recent studies have shown that pesticides inhibit enzyme activity in mussels. Matozzo et al., (2018) demonstrated that glyphosate (100 and 1000 μg L^{-1}) is able to affect SOD activity in the digestive gland of mussels after 7 days of exposure. Similarly, sub-chronic exposure to Calypso recorded a decrease in SOD activity in the digestive gland of mussels exposed to CA at 7.77 mg L^{-1} after 20 days. Ewere et al., (2019) described the negative impacts of imidacloprid

and Spectrum 200SC on enzyme activities in *Saccostrea glomerata* after two weeks of exposure to environmentally relevant concentrations (0.01 and 0.05 mg L^{-1}). In this study, the digestive gland CAT was markedly affected by exposure. In addition, neonicotinoids can induce antioxidant imbalance and cause oxidative stress in fish and shrimp. Velisek & Stara, (2018b) described the effect of TH at a concentration of 4.50 µg L^{-1} on the antioxidant activity of SOD and GR during embryo-larval development of *Cyprinus carpio*. Vieira et al., (2018b) observed differences in antioxidant biomarkers SOD, CAT, in Prochilodus lineatus tissues exposed for 5 days to imidacloprid at concentrations of 1.25-1250 µg L^{-1}. These alterations in antioxidants may lead to high levels of lipid peroxidation (LPO) and production of protein carbonyl content, indicating overproduction of ROS and a state of oxidative stress (Stara et al., 2019).

As widely reported, pesticide exposure can lead to an in-balance between reactive oxygen species (ROS) generation and antioxidant defences (Lushchak, 2011). CAT and SOD enzymes play the first important line of defence against ROS overproduction during pollutant biotransformation (Freitas, Coppola, et al., 2019; Lushchak, 2015; Sillero-Ríos et al., 2018; Vieira et al., 2018b). In *M. galloprovincialis* exposed to CAL for 20 days, inhibition of CAT activity in the gills was also detected at both CAL concentrations. Thus, the significant decrease in enzyme activity might have resulted from their inactivation by the superoxide radical triggered by CAL exposures. In addition, results obtained after a 10-day purification phase in CAL-free water demonstrated a reversible balance of digestive gland and gill enzyme activities. In general, toxic radicals inactivate the binding of antioxidant enzymes.

As shown in previous studies (Shukla et al., 2017; Stara, Bellinvia, et al., 2019; Vieira et al., 2018b; N. Yan & Chen, 2015), neonicotinoids lead to damage to the antioxidant balance in aquatic organisms, suggesting elevated ROS production, which triggers a state of oxidative stress. Similarly, changes in the activity of antioxidant enzymes have been found in *M. galloprovincialis* exposed to different pollutants (Faggio et al., 2016a; Freitas, Silvestro, et al., 2019; Milan et al., 2018; Munari et al., 2018) but not after exposure to neonicotinoids.

The toxic effect of thiacloprid appears to be time-dependent, with mussels more susceptible to lipid peroxidation and antioxidant changes after twenty days of exposure rather than after 10 days of exposure. At the same time, the toxic effect of thiacloprid was most evident in mussels exposed to the highest concentration tested.

The first line of defence against ROS overproduction includes CAT and SOD in particular (Lushchak, 2011; Niamul Haque et al., 2019). Several studies have focused on the antioxidant capacity of bivalves after exposure to neonicotinoids, especially imidacloprid.(Ewere et al., 2019, 2021; Shan et al., 2020) Imidacloprid caused significant differences in SOD, CAT activities in *Saccostrea glomerata* and *Corbicula fluminea* (Ewere, et al., 2019; Shan et al., 2020).

These results suggest a strong involvement of antioxidants in the regulation of cellular metabolism in case of perturbation of their balance following neonicotinoid

damage. ROS production and the possibility of cellular damage after neonicotinoid exposure were only addressed by Shan et al., (2020). They observed an increased level of malondialdehyde in Corbicula fluminea after exposure to imidacloprid.

5.1.5 Histology

The digestive gland is the main site for xenobiotic detoxification in bivalves (Gomes et al., 2012; Minier et al., 2000) and is also involved in xenobiotic bioaccumulation and challenge to environmental and/or induced stressors (Costa et al., 2013). This tissue has been attributed a key role in metabolism that protects against stressors and essential metals (Costa et al., 2013; Viarengo et al., 1981). The gill tissue is the first contact surface between organisms and the aquatic environment and gills are the organs responsible for respiratory function and nutrient assimilation, as well as contaminant uptake (Azevedo et al., 2015). For these reasons, these tissues were selected to determine histological alteration using the histopathological condition index as suggested by (P. M. Costa et al., 2013).

The pathological inflammatory state has not been observed in tissues after exposure to pesticides. Nor was the presence of infiltrated haemocytes, especially in the gills, indicative of an inflammatory process. Indeed, infiltrated haemocytes phagocytose pathogens and foreign bodies to trigger a toxic response, activating the multi-xenobiotic defence mechanism (MXDM) (Pain & Parant, 2003). The MXDM may act as protection of cells from the negative actions of toxic compounds, reducing access and promoting toxin efflux (Pagano et al., 2016).

Acute and sub-chronic exposure probably did not allow any appreciable damage to be observed.

5.2 Zebrafish larvae exposed to environmental concentrations of fenitrothion

Fenitrothion is an organophosphorus insecticide usually found in aquatic ecosystems at concentrations in the range of ng/L (Carrasco et al., 1987; Derbalah et al., 2019), whereas the acute systemic toxicity of this compound in fish is in the range of mg/L ((Organization, 1991)). As a result, it is generally assumed that the environmental risk of fenitrothion for the fish communities is very low. Therefore, the first objective of this study was to determine if short-term exposures to environmental concentrations of fenitrothion were able to induce neurotoxicity in fish. In order to answer this biological question, an apical and ecologically relevant endpoint, behaviour, and a molecular endpoint, changes in the expression of different transcriptional markers of neurotoxicity, have been analyzed. After only 24 h exposure, fenitrothion concentrations ranging from low ng/L to low µg/L altered three essential behaviours, BLA, VMR, and acoustic/vibrational escape response, in zebrafish larvae. Since VMR and the acoustic/vibrational escape response integrate sensory and motor responses, the effect of fenitrothion could be mediated by the impairment of the mechanisms involved in the detection of the

stimulus or by an effect on the specific motor circuits involved in the evoked motor response (Emran et al., 2007; Fero et al., 2011). The fact that fenitrothion increases the motor activity in response to a sudden decrease in light intensity but decreases this activity in response to vibrational stimuli strongly suggests that sensory and/or integrative circuits at the CNS, and not the neuromuscular system, are the main targets of fenitrothion. Interestingly, different effects on the visual system have also been reported after exposure to fenitrothion in humans and experimental animal models (Mecklenburg & Schraermeyer, 2007). Although the effect of 17 μg/L fenitrothion on the vibrational escape response was previously reported (Faria et al., 2020b), our data show that fenitrothion is able to impair this response even at low ng/L concentrations. One interesting finding during the behavioural analysis is the apparently contradictory results obtained with the vibrational startle analysis performed with the DanioVision and the kinematic analysis. Whereas larvae exposed to 17 μg/L fenitrothion exhibited a reduced response to the vibrational stimulus in the DanioVision setup, the percentage of larvae responding with an SLC to a similar stimulus increased when the kinematic of the vibrational evoked escape response was analyzed. The important methodological differences between the DanioVision and the setup used for the kinematic analysis, including the type of vibration (solenoid vs mini shaker) and the time of the analysis (1000 ms vs 270 ms), could explain the apparent differences observed between the two approaches analyzing the vibrational-evoked escape response. After determining the effects on behaviour, the neurotoxic potential of fenitrothion was also determined through the analysis of the expression of gap43a, gfap, atp2b1a, mbp, and syt1a. Growth-associated protein 43, the protein encoded by gap43a, is expressed in both neurons and astrocytes, and neurotoxic damage induces up-regulation of gap43a in both neurons, where this protein promotes axonal regeneration, and in astrocytes, which attenuates the astrogliosis-induced neurotoxicity (Hung et al., 2016). Increased levels of GFAP have been also found in mammalian and zebrafish brains after exposure to different neurotoxicants and measurement of this protein has been included in the neurotoxicity screening panel recommended by the US Environmental Protection Agency (McGrath & Li, 2008). Interestingly, the expression of gap43 and gfap is regulated by STAT3, a transcription factor involved in astrogliosis activation (Tsai et al., 2007; Yeo et al., 2013). Down-regulation of atp2b1a has been reported in zebrafish larvae exposed to ototoxic compounds, probably reflecting the death of the hair cells of neuromasts (Han et al., 2019; Monroe, 2015; Sonnack et al., 2018). Interestingly, a non-monotonic concentration-response curve (NMCRC) with an inverted U-shape was found to describe the relationship between fenitrothion concentrations and the levels of transcripts of gap43a, gfap, and atp2b1a, with a significant up-regulation in the expression of these genes at the lowest fenitrothion concentrations and a down-regulation at the highest concentrations. The concept of a non-monotonic dose-response relationship is well-established in ecological risk assessment, and many endocrine-distrupting compounds have been reported to produce non-monotonic effects at environmental

concentrations (Agathokleous et al., 2019; Vandenberg et al., 2012). Moreover, the up-regulation of gap43a and gfap in zebrafish larvae exposed to a similar range of fenitrothion concentrations strongly suggests that this chemical is able to trigger astrogliosis even at ng/L concentrations. On the other hand, the atp2b1a up-regulation found in the exposed larvae strongly suggests that the observed increase in the vibrational escape response is not the result of a specific toxic effect on the neuromasts and/or inner ear. Once demonstrated that short-term exposures to environmental concentrations of fenitrothion resulted in relevant neurotoxic effects in zebrafish larvae, the molecular modes of action behind these effects were further explored. Different toxicological modes of action (MoA) have been proposed for fenitrothion, including the direct inhibition of the activity of the enzyme acetylcholinesterase (AChE) (Sancho et al., 1997; Zinkl et al., 1991) and binding to the androgen receptor (AR) (Sebire et al., 2008; Sohoni et al., 2001; Tamura et al., 2001, 2003). Our computational analysis predicted several potential safety-associated human targets, including AChE, AR, MAO-A, MAO-B, tyrosine hydroxylase (TH), dopamine beta hydroxylase (DBH), and catechol O-methyltransferase (COMT). During the validation process, however, AChE was discarded as a toxicological target, since no inhibition of this activity was observed in either the *in vitro* (human AChE) or the in vivo (zebrafish AChE) assays. As fenitrothion needs to be oxidized into fenitrooxon by the cytochrome P450 monooxygenase system to be able to irreversible inhibit AChE (Escartín & Porte, 1996), results from the *in vitro* test, without any metabolic activation, were somehow expected. Moreover, considering that 24 h exposure to 20 μg/L fenitrothion has been reported to significantly inhibit AChE activity in eel brain (Sancho et al., 1997), the absence of inhibition in 8 dpf zebrafish larvae exposed 24 h to 17 ng/L-1.7 mg/L fenitrothion strongly suggests that at this developmental stage larvae lack fully functional biotransformation enzymes specifically involved in oxidizing fenitrothion into its oxon form (D. Yang et al., 2011). MAOA from rat brain has been found to be inhibited by fenitrothion *in vitro* assays (Sallinen et al., 2009; Williams et al., 2017), and both MAOA and MAOB were identified as potential targets of fenitrothion in our in silico predictions. Furthermore, inhibition of MAO with deprenyl in zebrafish larvae has been reported to induce a decrease in the BLA (Sallinen et al., 2009), a similar effect to that observed in fenitrothion-treated larvae. Despite of the mild but significant up-regulation of mao expression found in larvae exposed to 17 ng/L and 1.7 μg/L fenitrothion, the absence of MAO inhibition in larvae exposed to all fenitrothion concentrations indicates that this enzymatic activity is not directly involved in the observed neurotoxic effects. Our *in vitro* functional assays show that human SERT was significantly inhibited by fenitrothion, a result consistent with a previous report with human and rat SERT (Williams et al., 2017). Moreover, in a recent study (Faria et al., 2019) using also zebrafish larvae, inhibition of the serotonin transporter (SERT; slc6a4a) with fluoxetine resulted in a decreased startle response, a behavioural phenotype similar to that observed in larvae exposed to 170 ng/L-17 μg/L fenitrothion in our study.

However, the fact that slc6a4 expression remained unchanged in larvae exposed to all fenitrothion concentrations suggests that zebrafish SERT is not a highly relevant target of fenitrothion at the selected concentrations. TH, COMT and DBH are three essential proteins of the monoaminergic system that were also predicted to interact with fenitrothion in our in silico analysis. However, the expression of th1, th2 and comtb in fenitrothion exposed larvae were similar to the control values. Moreover, only larvae exposed to 170 ng/L fenitrothion exhibited altered dbh expression, whereas the only effect on NE levels was found at 1.7 µg/L. These results suggest that fenitrothion interaction with TH, COMT or DBH is not playing an essential role in the observed neurotoxic effects of this insecticide. Androgen receptor (AR) binding properties of fenitrothion were first reported about 20 years ago (Freyberger & Ahr, 2004), and these properties have been confirmed in the present study by an *in vitro* binding assay with human AR. Although fenitrothion is generally considered as a weak anti-androgenic compound (Freyberger & Ahr, 2004; Sebire et al., 2008; Tamura et al., 2003), other reports indicate that fenitrothion could also behave as an androgen agonist in biological context with very low concentrations of androgens (Tamura et al., 2003), as probably happens in 7 dpf zebrafish larvae (Gorelick et al., 2008). In order to better understand if environmental concentrations of fenitrothion were able to bind zebrafish AR inducing an androgenic/anti-androgenic effect on zebrafish larvae, the expression cyp19a1b and sult2st3, transcriptional markers commonly used to identify androgenic compounds in zebrafish(Agathokleous et al., 2019; Campos et al., 2013; Fent et al., 2018; Fetter et al., 2015; Vandenberg et al., 2012) was analyzed, and a significant up-regulation of both genes was found. Interestingly, the relationship between fenitrothion concentration and cyp19a1b and sult2st3 expression was also described by an inverted U- shaped NMCRC, the typical relationship found between endocrine disruptors concentrations and biological effects(Agathokleous et al., 2019; Campos et al., 2013; Vandenberg et al., 2012) . Moreover, the relationship found in this study between fenitrothion and mbp expression, a U-shaped NMCRC, is similar to the relationship previously reported for mbp expression and 17β-trenbolone, a potent androgen receptor agonist, in the medial prefrontal cortex of mice (Zhang et al., 2020). Interestingly, in addition to the altered mbp expression, mice exposed to 17β-trenbolone also exhibited behavioral changes. All these results, together with the higher potency exhibited by the binding fenitrothion-AR respect other predicted interactions and the absence of evidences supporting the involvement of other predicted targets strongly support the hypothesis that fenitrothion binding to AR is the molecular initiating event (MIE) of the neurotoxic effect induced by this chemical in zebrafish larvae. AR are widely expressed in the brain and endogenous and exogenous androgens may regulate neuronal activity, synaptic plasticity, cognition and different behaviors (Sarkey et al., 2008). For instance, administration of exogenous testosterone to animals and humans has an anxiolytic effect, reducing vigilance, startle response and threat detection, increasing risk-taking (Domonkos et al., 2018; Hodosy et al., 2012; Oliveira & Oliveira, 2014). Treatment with the

anti-androgen flutamide has been associated with depression in humans (Dinh et al., 2016). Although the monoaminergic system, and in particular the dopaminergic system, are controlled by androgens (Barclay & Harding, 1990; Fabre-Nys, 1998; Jardí et al., 2018; Sotomayor-Zarate et al., 2015), the precise mechanisms of this control remain to be established. Moreover, our in-silico predictions suggested that fenitrothion may not only impair the dopaminergic system through its androgenic effect, but might also directly interact with some key components of the dopaminergic system involved in the synthesis (TH) and metabolism (COMT, DBH) of dopamine. The significant decrease in DOPAC levels with a concomitant increase in HVA observed in zebrafish larvae exposed to low fenitrothion concentrations (17–170 ng/L) was previously reported in the brain of rats treated with amphetamines (Westerink & Korf, 1976) and methamphetamines (Yang et al., 1997), drugs targeting the vesicular monoamine transporter. Even knowing that results from neurochemical analyses performed on whole larvae should be always taken with caution, as many of the analyzed chemicals are also expressed in non-neural tissue, the fact that expression of slc18a2, the gene encoding zebrafish VMAT2, was also significantly altered at the same two fenitrothion concentrations suggests that VMAT2 could play an important role in the observed changes in behaviour. Furthermore, slc18a2 expression in fenitrothion-exposed larvae follows an inverted U- shaped NMCRC typical of endocrine disrupters and it has been recently demonstrated in rats an increase in slc18a expression after treatment with an androgenic drug, propionate of testosterone has been recently demonstrated in rats (Wang et al., 2016). The results presented are not only relevant for environmental risk assessment, but also for the human risk assessment methodology. The reference dose (RfD) for fenitrothion, 0.0013 mg/kg/day, was established by US EPA based upon a NOEL for systemic effects (histopathological changes in lymph nodes) and plasma cholinesterase inhibition observed in a long-term feeding study in dogs (EPA, 1995.). Our results show that relevant adverse effects on behaviour are observed at fenitrothion concentrations several orders of magnitude lower than those inhibiting AChE activities or producing changes in morphology. Therefore, the potential behavioural effects of low levels of fenitrothion should be tested in neonatal rodents and, if necessary, the current RfD for fenitrothion should be re-adjusted.

6. Conclusion

The aim of this work was to evaluate the effects of Thiacloprid and Fenitrothion, two pesticides widely used in agriculture, on two non-target model organisms such as *Mytilus galloprovincialis* and *Danio rerio*.

Through the investigations carried out on *M. galloprovincialis*, we were able to assess that the most damaging effects, leading to an alteration in the physiology of the organism, was sub- chronic exposure to the commercial product Calypso, which contains not only the active ingredient but a series of other compounds and which, in fact, is normally used in agriculture.

European legislation has banned thiacloprid, but thiacloprid and other neonicotinoids continue to be used in Europe and sold in other non-EU countries.

Studies on zebrafish larvae exposed to fenitrothion have shown that even low concentrations of the pesticide, which do not actually inhibit AchE, cause various behavioural changes in zebrafish. Therefore, the potential behavioral effects of low levels of fenitrothion should be tested in neonatal rodents and, if necessary, the current RfD for fenitrothion should be re-adjusted.

The study of the biological responses of organisms exposed to pesticides is essential for understanding the mechanisms of toxicity, and the aim of this PhD project was to better understand the mechanism of action of emerging contaminants in aquatic organisms, to try to find particularly sensitive early warning signals so as to predict the risks posed by these substances in the environment.

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

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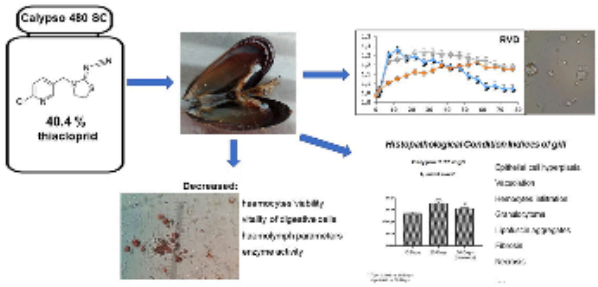
HIGHLIGHTS

- 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^+, Ca^{2+}, 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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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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Biochemical parameters

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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Review

Impact of Neonicotinoids to Aquatic Invertebrates—In Vitro Studies on *Mytilus galloprovincialis*: A Review

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Abstract: The use of pesticides in agriculture has always had a strong impact on environmental contamination. Since the 1990s, neonicotinoids have grown increasingly more popular, targeting specific receptors for insects, especially bees, which is why the use of some neonicotinoids has been banned. Much is known about the effects they have on insects, but very little about the effect they can have on non-target organisms. Several studies have shown how these neonicotinoids interact negatively with the normal physiology of aquatic organisms. For the genus *Mytilus*, even though the neonicotinoids did not show an interaction with specific receptors, a chronic and acute exposure to them causes damage. In these animals, a reduced production of byssus, alteration of the normal antioxidant systems and tissue damage have been found. Therefore, an analysis of the entire ecosystem in which the pollutant enters is of great importance in evaluating any possible alterations.

Keywords: neonicotinoids; mussels; pesticide

1. Introduction

The worldwide use of pesticides in agriculture, a practice that has been going on for decades, means that residues of pesticides, most often substances of the group of insecticides and herbicides, are commonly found in many environments, from cultivated fields to rivers and oceans, and even in urban environments [1,2]. While insecticides are useful for controlling and avoiding pest infestations, it is also true that the pollution they cause is one of the many problems that our society must face. This is because the extreme toxicity of most of these chemical substances, whether natural or artificial, affects not only the target organisms, but also many other species of animals, albeit obviously to different degrees [2,3]. In fact, insecticides are able to alter the ecological structure of the soil in which earthworms and arthropod communities live, and in turn affect birds and other vertebrates that feed on these organisms. Additionally, residues that float in water for short or long periods of time can decimate zooplankton, aquatic crustacean larvae and insects, or impair the growth and development of tadpoles and fish. On the other hand, just because insecticides are designed to kill insects does not mean that they are excluded from killing other animals, including humans. It is therefore necessary for

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Effects of long-term exposure of *Mytilus galloprovincialis* to thiacloprid: A multibiomarker approach☆

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ABSTRACT

Thiacloprid is a neonicotinoid insecticide widely exploited in agriculture and easily mobilized towards aquatic environments by atmospheric agents. However, little information about its toxicological effects on aquatic invertebrate bioindicators is available. In this study, specimens of the mussel *Mytilus galloprovincialis* were exposed to thiacloprid at environmental (4.5 μg L^{-1}) and 100 times higher than environmental (450 μg L^{-1}) concentrations for 20 days. Thiacloprid affected haemolymph biochemical parameters, cell viability in the digestive gland, antioxidant biomarkers and lipid peroxidation in the digestive gland and gills at environmentally relevant concentrations (4.5 μg L^{-1}). In addition, thiacloprid exposure caused histological damage to the digestive gland and gills. Interestingly, the pesticide was detected at levels equal to 0.14 ng g^{-1} in the soft tissues of sentinels exposed for 20 days to 450 μg L^{-1} thiacloprid in seawaterμ. Due to its harmful potential and cumulative effects after long-term exposure of *M. galloprovincialis*, thiacloprid may pose a potential risk to nontarget aquatic organisms, as well as to human health. This aspect requires further in-depth investigation.

1. Introduction

Water pollution prevention is an important part of the preservation of a healthy environment. An understanding of the basic principles and effects of potentially toxic substances on nontarget organisms is essential (Gorinsterin et al., 2008; Albano et al., 2021a, 2021b).

Bivalve molluscs are abundant in aquatic ecosystems and have an ecological and economic importance. Bivalves are filter feeders that couple the water column and benthos; they alter nutrient cycling, create and modify habitats, and affect food webs and the movement of nutrients and energy (Vaughn and Hoellein, 2018). Marine bivalves are an important part of the livelihood of people in coastal areas. Their global production for human consumption is on average more than 15 million tonnes per year, which is approximately 14 % of the total marine production in the world (Wijsman et al., 2018). The area of the world with the highest production of *Mytilus galloprovincialis* is China, and in Europe, Spain, Italy, Portugal, and France are by far the largest producers (Orban et al., 2002; FAO, 2012; Seafood Source, 2019). Moreover, *M. galloprovincialis* has been notoriously exploited as a bioindicator species in ecotoxicological studies and is able to bioaccumulate a wide range of inorganic and organic contaminants from the surrounding aquatic environment due to its filter feeding traits (Albergamo et al., 2016; Freitas et al., 2020a, 2020b; 2020c; O'Connor, 2002; Pagano et al., 2020). Mussels reach their commercial size within 12 months, which is a long period during which many factors potentially affect their health and safety for consumers (Sara et al., 1998).

Thiacloprid ([3-(6-chloro-3-pyridinylmethyl)-2-thiazolidinylidene] cyanamide) (Schäfer et al., 2008), similar to imidacloprid, is classified as

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Androgenic activation, impairment of the monoaminergic system and altered behavior in zebrafish larvae exposed to environmental concentrations of fenitrothion

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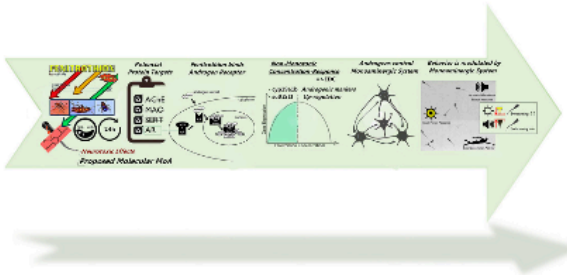
HIGHLIGHTS

- Environmental concentrations of fenitrothion induce behavioral changes in zebrafish.
- Fenitrothion alter the expression of neurotoxicity markers in a non-monotonic fashion.
- At low environmental concentrations, fenitrothion effects are AChE-independents.
- Binding to androgen receptor is the most suitable candidate to MIE.

GRAPHICAL ABSTRACT



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ABSTRACT

Fenitrothion is an organophosphorus insecticide usually found in aquatic ecosystems at concentrations in the range of low ng/L. In this manuscript we show that 24 h exposure to environmental concentrations of fenitrothion, from ng/L to low μg/L, altered basal locomotor activity, visual-motor response and acoustic/vibrational escape response of zebrafish larvae. Furthermore, fenitrothion and expression of *gap43a*, *gfap*, *atp2b1a*, and *mbp* exhibited a significant non-monotonic concentration-response relationship. Once determined that environmental concentrations of fenitrothion were neurotoxic for zebrafish larvae, a computational analysis identified potential protein targets of this compound. Some of the predictions, including interactions with acetylcholinesterase, monoamine-oxidases and androgen receptor (AR), were experimentally validated. Binding to AR was the most suitable candidate for molecular initiating event, as indicated by both the up-regulation of *cyp19a1b* and *sult2st3* and the non-monotonic relationship found between fenitrothion and the observed responses. Finally, when the integrity of the monoaminergic system was evaluated, altered levels of L-DOPA, DOPAC, HVA and 5-HIAA were

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