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TOXIC EFFECTS OF LAMBDA-CYHALOTHRIN AND ACETAMIPRID ON FRESHWATER MUSSELS

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Donald Romaric YEHOUENOU TESSI

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ETHICAL STATEMENT

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07/03/2023

LAMBDA-CYHALOTHRİN VE ACETAMİPRİD'İN TATLI SU MIDYESİ ÜZERİNDEKİ TOKSİK ETKİLERİ

(Doktora Tezi)

Donald Romaric YEHOUENOU TESSI

GAZİ ÜNİVERSİTESİ

FEN BİLİMLERİ ENSTİTÜSÜ

Mart 2023

ÖZET

Bu çalışma, tatlı su midyelerinin dünyanın kullanım alanı fazla olan pestisitlerden Lambda-sihalothrin (LCH) ve Asetamiprid'in (ACE) aktif maddelerine maruz kalmasının toksik etkilerinin değerlendirilmesi yoluyla su kirliliği alanındaki eko-toksikolojik çalışmalara bir katkıdır. Bu çalışmada kullanılan deneysel yaklaşım, Unio terminalis, Bourguignat (1852) tatlı su midyelerinin, farklı süre ve konsantrasyonlarda Lambda-sihalotrin ve Asetamiprid'e maruz kalması ile laboratuvarda yürütülen in vivo biyodeneylerdir. Nihai sonuç olarak, test edilen kimyasalların 96 saatlik LC_{50} değerleri asetamiprid için 33,52 mg/L (21,6846 – 50,1039) ve lambda-sihalotrin için ise 1,1 mg/L (0,7648 – 2,9747) olarak saptanmıştır. Midyelerin iki pestisitin farklı konsantrasyonlarına kronik olarak maruz kalmasından sonra, her iki pestisitin de *Unio terminalis* hemolemfinin Toplam Hemosit Sayımı (THC), Toplam Oksidatif Durum (TOS) ve Toplam Antioksidan Durum (TAS) değerleri üzerinde etkileri olduğu belirlenmiştir. LCH, oksidatif stres biyobelirteçlerinde etkiler yapmıştır. Nitekim solungaçlarda Süperoksit dismutaz (SOD) ve Katalazın (CAT) enzimatik aktivitelerinde azalma gözlemlenirken, hem solungaçlarda hem de sindirim bezlerinde Glutatyon Peroksidaz (GPx) aktivitesinde artış kaydedilmiştir. SOD ve GPx enzimlerinin, solungaçlarda ve sindirim bezlerinde aktivitelerinde artış ile ACE'ye maruz kaldıktan sonra *Unio terminalis* organizmalarının antioksidan savunmasına aktif katılımını gözlemlenmiştir. Bununla birlikte, tüm maruz kalma süreleri boyunca CAT aktivitesinde hiçbir değişiklik gözlenmemiştir. Her iki pestisitin de subletal konsantrasyonları süre ve maruziyet miktarına bağlı olarak solungaç ve sindirim bezi dokularında kontrol gruplarına kıyasla histopatolojik bulgulara neden olmuştur.

Bilim Kodu : 12704
Anahtar Kelimeler : Pestisitler, Asetamiprid, Lambda-sihalotrin, toksisite, suda yaşayan omurgasızlar, tatlı su midyeleri, Unio terminalis, kronik maruz kalma, oksidatif biyobelirteçler.
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TOXIC EFFECTS OF LAMBDA-CYHALOTHRIN AND ACETAMIPRID ON FRESHWATER MUSSELS

(Ph.D. Thesis)

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ABSTRACT

The present study is a contribution to eco-toxicological studies in the field of aquatic pollution through the evaluation of the toxic effects of the exposure of freshwater mussels (*Unio terminalis*, Bourguignat (1852)) to the active substances of the world's most frequently applied pesticides, namely Lambda-cyhalothrin (LCH) and Acetamiprid (ACE). As a final result, the 96h – LC_{50} of the chemicals tested were 33.52 mg/L (21.6846 – 50.1039) for acetamiprid and 1.1 mg/L (0.7648 – 2.9747) for lambda-cyhalothrin, respectively. After chronic exposure of the mussels to different concentrations of the two pesticides, we found out that both pesticides have an effect on the Total Hemocyte Counts (THCs) values, Total Oxidative Status (TOS) and the Total Antioxidant Status (TAS) values of *Unio terminalis* hemolymph. LCH provoked disturbances in the biomarkers of oxidative stress. Indeed, we have noted a decline in the enzymatic activities of Superoxide Dismutase (SOD) and Catalase (CAT) in the gills, while an increase in the activity of Glutathione Peroxidase (GPx) was noted in both the gills and digestive glands. We observed an active participation of SOD and GPx enzymes in the antioxidant defense of *Unio terminalis* organisms after their exposure to ACE with an increase in their activities in the gills and the digestive glands. Nevertheless, no alteration of CAT activity was observed during all exposure times in the tissues. Both pesticides affected the tissues of the species after histological analysis.

Science Code : 12704
Key Words : Pesticides, Acetamiprid, Lambda-cyhalothrin, toxicity, aquatic invertebrates, freshwater mussels, *Unio terminalis*, chronic exposure, oxidative biomarkers.
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LIST OF SYMBOLS AND ABBREVIATIONS

The symbols and abbreviations used in this thesis are presented below with explanations.

Symbols	Explanations
℃	Celsius
g	Gram
mg/L	Milligrams per liter
µg/L	Micrograms per liter
ml	Milliliter
pH	Potential hydrogen
O_2	Oxygen
H_2O	Water

Abbreviations	Explanations
ACE	Acetamiprid
ACh	Acetylcholine
ANSES	Agence Nationale de Sécurité Sanitaire de l'Alimentation
APHA	American Public Health Association
CAT	Catalase
CNS	Central Nervous System
CPMA	Canadian Pest Management Regulatory Agency
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulfoxide
EC_{25}	Concentration of a substance that has effects on 10% of the test organisms
EC_{50}	Concentration of a substance that affects 50% of the test organisms

Abbreviations	Explanations
GPx	Glutathione Peroxidase
LC_{50}	Concentration that kills 50% of the animals (Lethal Concentration 50)
LCH	Lambda-cyhalothrin
nAChRs	Nicotinic acetylcholine receptors
OECD	Organization for Economic Co-operation and Development
ROOH	Hydroperoxyl radical
ROS	Reactive oxygen species
SOD	Superoxide Dismutase
WHO	World Health Organization

1. INTRODUCTION

Pesticides represent a major evolution of chemical processes for the improvement of human living conditions. Indeed, since the end of World War II in the 1945s (Bromilow, 2005), the use of pesticides has become effective and has accelerated over time. This has led to certain advances in agriculture such as improved crop yields, reduced pest action, reduced drudgery of farm work, the reduction of crop losses especially during grain storage, the protection of animals (for example, trypanosomiasis disease in livestock), the fight against certain diseases such as malaria (use of Dichloro-diphenyl-trichloroethane (DDT)) and filariasis (Freedman, 1995; Bromilow, 2005; Wilson et al., 2001). Pesticides remain a key productivity factor in agriculture.

As the world's population continues to grow, there is an increasing need to produce enough food and other resources to meet these demands. This has led to a rise in both the production and consumption of pesticides worldwide, with an estimated consumption of over 2.5 million metric tons as of 2019. This represents a more than 40% increase compared to 1990 levels (Sharma et al., 2019).

The UN's Food and Agricultural Organization gives the following definition of pesticides: 'Pesticides refer to insecticides, fungicides, herbicides, disinfectants and any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies. The concept comprises compounds to be used for regulating plant development, as defoliants, as desiccators, as fruit thinners or as agents for avoiding precocious fruit drops, as well as compounds which are applied to harvests prior to or immediately after picking to preserve the goods from spoilage while safekeeping and transportation (Zacharia, 2011).

This definition refers to all forms of pests against which chemical substances are deliberately used. The use of pesticides is therefore for a purpose, against a specific target organism. Its use requires information on the pests to be fought or the problem to be solved. Depending

on the forms of pests to be neutralized or repelled, there are several families of pesticides, the three largest of which are insecticides (to fight insects), herbicides (against weeds) and fungicides (against fungi). Other forms of pesticides exist, for example molluscicides, rodenticides and nematicides, etc. Pesticides consist of one or more active substances (used to destroy or prevent the pest from manifesting itself) and formulants (used as a support for the active substance to reinforce its action). A classification of pesticides is also made, according to their chemical nature, i.e., on the basis of the majority active substance. Therefore, it is common to classify them as Organochlorines, Organophosphates, Carbamates etc. These phytopharmaceutical products are constantly evolving due to the introduction or spread of new pests from other continents, decreases in the effectiveness of resistance or changes in cropping systems. The following table presents the history of the evolution of the three largest families of pesticides from the 1900s to the 2000s (Miquel, 2021).

Table 1.1. History of the evolution of the three largest families of pesticides from the 1900s to the 2000s (Miquel, 2021)

	Herbicide	Fungicide	Insecticide
Before 1990	Copper sulfate Iron sulphate	Sulphur Copper salts	Nicotine
1900 – 1920	Sulfuric acid		Arsenic salts
1920 – 1940	Nitro Dyes		
1940 – 1950	Pythotormones		Organochlorines Organophosphorus
1950 – 1960	Triazines Substituted Ureas Carbamates	Dithiocarbamates Phthalimides	Carbamates
1960 – 1970	Bipyrides, Toluidines	Benzimidazoles	
1970 – 1980	Amino-phosphonates Propionates	Triazoles Dicarboximides Amides, Phosphites Morpholines	Pyrethroids Benzoylides (Growth regulators)
1980 – 1990	Sulfonylureas		
1990 – 2000		Phenylpyrroles Strobilurins	Neonicotinoids

Despite all the advantages of pesticides, there are nevertheless disadvantages to be pointed out, highlighting the other side of the coin due to their extensive use and their dissemination in the different ecosystems with their persistence in the environment over the long term. The recurring question is the impact of these products on the health of farmers and all actors who have to handle them several times during their working life, but more importantly their impact on the food chain and non-target organisms of sensitive habitats such as aquatic ecosystems (Mahmood et al., 2016; Singh et al., 2021). The concerns raised have led researchers to investigate the poisonous impacts of these chemicals on various living organisms in order to better guide recommendations for their use.

After the Second World War, chemical substances were abundant and affordable on the market with the perception that they represented miracle solutions to pest problems. Overuse of these chemical pesticides has caused resistance in many organisms (bio-pests such as mites and various aphid species) and bacterial or viral diseases in plants (Vanoosthuyse et al., 2018). Bioaccumulation and biomagnification resulting from the abundant use of pesticides have affected several living organisms such as organisms in wildlife including birds.

The first concerns about the widespread use of pesticides appeared with the publication of Rachel Carson's novel "Silent Spring" in 1962, which showed the extreme danger of Dichloro-diphenyl-trichloroethane (DDT), whose utilization began in 1942. The objective of this book was to awaken the conscience of the citizens of the world on the problem of the poisoning of the planet by pesticides. This book emphasized the link between human activities and the ecology of all species, and the risks faced by the human species whose bodies are permeable to chemical poisons (Carson, 1962; Van Emden and Peakall, 1996). Since then, research has shown that many other pesticides accumulate in the environment (Ramade, 2022).

Despite these warnings and the environmental protection movements, pesticides are still used around the world. The global consumption of pesticides in the world reached more than 4.4 million tons in 2017 (Roser, 2019) with an increasing trend.

In the Republic of Benin, for example, agricultural production depends on pesticides. The majority of production systems use these phytosanitary products to reduce the impact of

degraded soils on production, and thus maximize yields, but because of the easy access to these chemicals. The pesticides found on the Beninese market include several hundred active substances and are mainly used in agriculture. Agriculture is practiced by more than 60% of the population and has been modernized for several years with the application of pesticides of mineral or organic nature, synthetic or natural. Benin being the first producer of cotton in Africa, this culture knows an intensification marked by the repeated application of phytosanitary products of various kinds.

Most of the imported pesticides are destined for the cultivation of cotton, which is the pride of Benin. Indeed, Benin ranks in Africa as the first producer of this raw material in the textile industry since the year 2018. With this in mind, the cultivation of this crop is intensifying, marked by the repeated application of various phytosanitary products and an increase in its production area. But this is not without consequences for the environment. (Sæthre et al., 2012)

Cotton cultivation in northern Benin is characterized by a non-existent tree cover on the exploited land, this is a monoculture that is repeated over several years with risks of soil degradation. In addition to the decrease in soil fertility, the use of chemicals in cotton production reduces the soil's capacity to filter water and sequester carbon. As a result, these soils are highly exposed to erosion phenomena, causing an accumulation of sediments and chemicals in the aquatic ecosystems surrounding these farming areas. This production, which aims to control pests by the application of pesticides and synthetic fertilizers on farms, threatens other non-target living organisms such as humans, wildlife and aquatic ecosystems. Table 2 presents the impacts of cotton cultivation on aquatic environments and their biodiversity. (Bärlocher et al., 1999)

Table 1.2. Cotton cultivation's pollution pathways and key consequences on freshwater ecosystems (Bärlocher et al., 1999)

Mechanism	Pollutant/Change	Impact	Cases
Run off from fields	Fertilizer	Eutrophication and pollution	China, Egypt, Uzbekistan
	Pesticides	Wildlife contamination	
	Sediments		
Drainage	Saline drainage water	Salinization of freshwater	
	Pesticide or fertilizer contaminated drainage water	Pollution of freshwater	
Application of pesticides	Insecticides, fungicides, herbicides and defoliants	Wildlife contamination Contamination of adjacent wetlands surface and ground water	
	Spray drift (e.g., aerial application)		
	Leakage of equipment	Contamination of surface and ground water	
Water withdrawal for irrigation	Use of ground water	Change of water table or depletion of ground water	New South Wales, Australia
	Use of surface water	Degradation of wetlands and lakes	Aral Sea, Yellow River Valley
Extensive irrigation	Water logging	Raising water tables and salinization of the soil surface	Australia, Indus River Valley, Uzbekistan, Pakistan
Dam construction for irrigation	Regulated water flow	Habitat destruction, change of water table and change of water flow	
Land reclamation	Change of vegetation	Habitat destruction	

Currently on the market for phytosanitary products, there are several groups of insecticides: neonicotinoids (acetamiprid, imidacloprid, clothianidin, nitenpyram, thiamethoxam, etc.) pyrethroids (lambda-cyhalothrin, cypermethrin, etc.), organophosphates (profenofos, dimethoate, chlorpyrifos- ethyl, malathion, diazinon, etc.) and carbamates (carbaryl, carbofuran, propoxur, etc.) (Karasali and Maragou, 2016; Abreu-Villaca and Levin, 2017;

Ewere et al., 2021). Among these different groups, Lambda-cyhalothrin and Acetamiprid are recognized as broadly used insecticides throughout the world (Raj et al., 2015, Singh et al., 2012; You et al., 2019; Hamer et al., 2013; Rasmussen et al., 2008; Birolli et al., 2019), in Turkey (Arıcan et al., 2019; Demirci et al., 2020, Aydogdu et al., 2017; Uğurlu et al., 2017; Berber et al., 2013), and the two most commonly used insecticides in the Republic of Benin, whether by large cotton producers or by certain producers of cereals and vegetables (Gouda et al., 2018; Guedegba et al., 2019; Cossi et al., 2020).

In accordance with a review of past studies on the toxicity of these two compounds, very little research has been carried out on aquatic invertebrates, in this case the phylum of Molluscs. This group of animals includes several genera of important species widely used to characterize the state of degradation of the aquatic environment because of their high sensitivity to environmental changes (Cossi et al., 2020). This group of animals includes several genera of important species widely used to characterize the state of degradation of the aquatic environment because it is very sensitive to environmental changes (Cossi et al., 2020). Examples include the class of bivalves, more specifically mussels, which are typically used in environmental toxicity studies to assess the degree of disturbance and adverse effects that xenobiotics and pesticides may have on behavior, survival and alteration of the functioning of the organism of aquatic species (Milam et al., 2005; Beggel et al., 2017; Günal et al., 2018).

In this generalized context of heavy use of pesticides and a beginning of awareness of their danger to living organisms; and knowing that these pesticides tend to be found very quickly in aquatic ecosystems, the primary purpose of this doctoral research is to investigate the toxicity of these two compounds on freshwater mussel species (*Unio terminalis*), which are indicator species of the state of freshwater aquatic environments. All this contributes to better orienting the use of pesticides based on these two active substances and to raise the awareness of everyone on their potential lethal and destructuring effect on aquatic invertebrate organisms, and by ricochet on man who is at the head of the food chain.

With this in mind, this study was initiated by determining the lethal concentration of these two chemicals on mussel species (*Unio terminalis*), as well as their effects on these same organisms following a period of exposure covering 48 hours, 7 and 21 days under the influence of their sublethal concentrations. Then, we determined the total number of

hemocytes and evaluated the histopathological alterations in the tissues and the effects on the enzyme levels in the gill and digestive gland tissues. In addition, extensive analyses were performed on the hemolymph collected from the organisms used in the experiments by considering some biochemical parameters.

The results of the present study will contribute to providing missing data in the national and international literature and suggest solutions for the ecosystem risk assessment of these two chemicals.

2. LITERATURE REVIEW

2.1. Pesticide Transportation in Environment and Water Ecosystems

Pesticides are dispersed into the environment by various routes and mechanisms: direct losses to the air during application of plant protection products, volatilization after application, direct losses to the soil during the application or during incidents, leaching by rain, transfers into the soil, surface transfers by runoff, wind erosion, dispersion into the atmosphere and rainfall (Aubertot et al., 2005)

While the presence of pesticides in agricultural soils is obvious and voluntary, this is not the case, for example, in aquatic ecosystems. Indeed, the presence of substances such as synthetic plant protection products, micro-organisms at thresholds beyond the normal (no risk of producing negative effects) in these ecosystems leads to their contamination or pollution with a consequent ecological imbalance. The pollution of aquatic environments concerns both surface waters (continental waters, surface) and groundwater. Nevertheless, data and evidence of pollution are more numerous in the specific case of surface waters. The contamination of the environment and aquatic environments comes essentially from losses during the application of pesticides, which is a function of the type of pesticide, the application technique, the plant cover, and the characteristics of agricultural soils (texture, structure). (Yadav, 2010)

Water contamination occurs in most cases as a result of runoff and erosion (Kreuger, 1999). Pollution can reach its maximum level during heavy rainfall occurring shortly after pesticide application or when the pesticides reach soils with poor physicochemical properties (potentially degraded) that are close to water bodies.

The run-off water flowing from the agricultural fields always carries a part of the sprayed fertilizers and pesticides and consequently contaminates the water habitats. In fact, pesticides from farming activities are regularly accumulated in bottom sediments and organisms of water ecosystems. (Kreuger et al., 1999).

The world's water resources are increasingly threatened by a wide range of contaminants, leading to degraded water quality in streams, reservoirs, and groundwater aquifers (Stehle and Schulz, 2015). Degradation of water quality limits its availability for a variety of human uses and ecosystem functioning. In addition, water contamination has been aggravating in several parts of the world and major contamination menace have been identified in European countries, Asia (India, Bangladesh, etc.), South and North America, and some regions of Africa. (Kraemer et al., 2001; Fernandez-Luqueno et al., 2013)

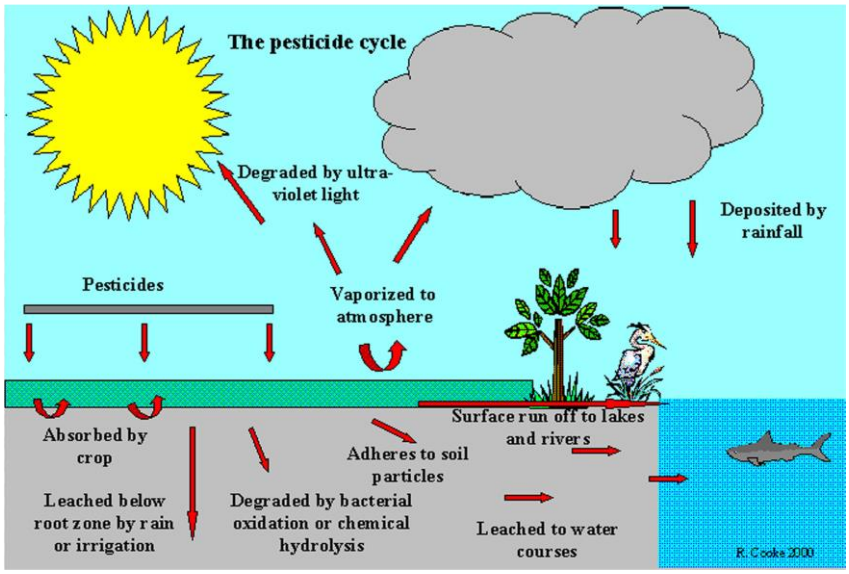


Image 2.1. Processes explaining fate of the pesticides in the environment (Shakeel et al., 2017)

2.2. The Pesticides Studied in this Thesis

To meet the challenges of crop protection and increased agricultural production, the synthesis of many active substances with varying mechanisms of action has led to the development of several chemical substances whose families exist for each type of substance. There are several different modes of action on the intended target for insecticides, and for each different family of pesticides, Examples of neurotoxic (organophosphates, pyrethroids, avermectins, neonicotinoids, etc.); Insect growth regulators (etoxazole, diacylhydrazines, enoxicarb, etc.); Cellular respiration inhibitors (stanic derivatives, METI); Muscle contraction inhibitors (anthranilamides), etc.

Neurotoxic insecticides are in the majority and include eight different chemical families whose modes of action are to disrupt the transmission of nerve impulses according to the intended cellular target. For the studies concerning this thesis, two families of neurotoxic insecticides are of particular interest to us:

- The pyrethroids, pyrethrins (Mode of action/Target: Modulators of the sodium channel);
- Neonicotinoids (Mode of action/Target: Agonist of the nicotinic acetylcholine receptor nAChR).

2.2.1. Lambda-cyhalothrin

General information on lambda-cyhalothrin (LCH) and pyrethroids

Pyrethroids are ranked as the third most widely used insecticides especially in agriculture and various other areas (residential, forestry, etc.) (Starr et al., 2014). Composed of multiple active substances, with different properties, pyrethroids include several groups of which the first is pyrethrin (isolated from chrysanthemum flowers). However, this group (pyrethrin) has high and rapid degradation characteristics (photodegradation by ambient light of the sun and hydrolysis in case of humidity). Thus, to improve the properties of pyrethrin, synthetic pyrethroids have been created and they are more stable. In this family of insecticides, two groups are distinguished, differentiated by the presence (type I) or absence (type II) of a cyano group (made from nitrogen and carbon) on the alpha carbon (first position attached to a functional group) of the molecule of each active substance (Figure 2.1).

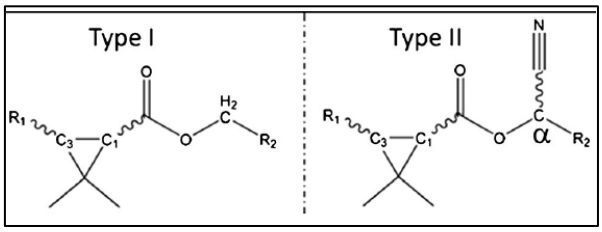


Figure 2.1. Differential structure of type I and type II pyrethroids

Due to their decreased volatility and quick metabolic inactivation, their photostability, great effectivity at paltry doses, facile disaggregation and marginal toxicity to species like

mammals and birds (Maud et al. 1998), pyrethroids, which are lipophilic insecticides, are used as alternatives to organophosphates (Barr et al., 2010).

Lambda-cyhalothrin (LCH or λ-cyhalothrin) is a chemical compound, which belongs to the family of synthetic pyrethroids (type II), used as an active ingredient in several insecticides. It is commonly used against crop pests (wheat, grapevine, cotton, etc.) across several agricultural areas but also against some external pests, e.g., domestic insects (WHO, 1999; Mazmanci et al., 2008). It is used for the control of various vineyard pests, including moths, leafrollers, leafhoppers and thrips.

Table 2.1. Description of lambda-cyhalothrin

Parameter	Name or number
Common name	Lambda-cyhalothrin
Chemical name	1:1 mixture of (*S*)-α-cyano-3-phenoxybenzyl (*Z*)-(1*R*,3*R*)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate and (*R*)-α-cyano-3-phenoxybenzyl (*Z*)-(1*S*,3*S*)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate
CAS number	91465-08-6
Mode of action	The pyrethroid: interaction with pre-synaptic sodium channels.

Source: L.C. Van Leeuwen et al. (2008)

LCH is a substance capable of either killing or repelling pests. It is one of the most widely utilized pyrethroids in the world (He et al., 2008) due to its advantages in terms of effectiveness and less toxicity (Anadon et al., 2006). This active substance consists of two of the four enantiomeric forms of cyhalothrin (figure 2.2).

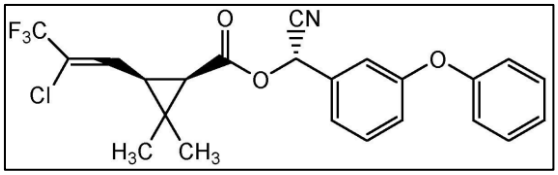


Figure 2.2. Structure of lambda-cyhalothrin

Lambda-cyhalothrin is mainly used to control defoliator caterpillars (caterpillars that eat leaves). It is also used to control tomato moths, aphids, leafhoppers, and cucurbit flies (melon, squash). Lambda-cyhalothrin is more rarely offered for control of thrips and whitefly.

Table 2.2. Lambda-cyhalothrin physico-chemical proprieties

Parameter	Unit	Value	Remark
Molecular weight	[g/mol]	449.9	
Water solubility	[mg/L]	0.004	20°C, pH 5
		0.005	20°C, pH 6.5
		0.004	20°C, pH 9.2
pK_a	[-]	>9	No dissociation at environmentally relevant pH values
Log K_{ow}	[-]	7	20°C
Log K_{oc}	[-]	5.20	Mean of 4.58, 4.68, 5.30 and 5.54
Vapour pressure	[Pa]	$2x10^{-7}$	20°C, extrapolated
Melting point	[°C]	49.2	
Boiling point	[°C]	-	No measurable boiling point (decomposes)
Henry's law constant	[Pa.m3/mol]	0.02	20°C

Source: L.C. van Leeuwen et al. (2008)

Mode of action

The pyrethroids impact the neurological system of organisms by interfering with the triggering mechanisms of sodium channels, which are fundamental for the production and transfer of nerve impulses (WHO, 1990). They interfere with the activation of sodium channels by keeping the gate open. The delay in gate closure results in nerve fiber excitation for an extended period (WHO, 1990).

LCH acts by contact and ingestion on a large number of insects at very low doses, then continues to protect the crops over a period of 2 weeks even in hot and windy conditions. It is a compound that intervenes with the sodium channel inside the nervous system, leading to both paralysis and death of insects (Tomlin et al., 1997). It has a braking action on phytophagous mites as well as an ovicidal action on lepidopteran eggs (butterflies). Lambda-cyhalothrin has properties that can repel insects (Macbean, 2002).

As LCH is one of the most widely used active substances in the world, several studies have evaluated its environmental fate. Below is a table that summarizes some of these environmental characteristics (Table 2.3).

Table 2.3. Lambda-cyhalothrin's environmental characteristics

Parameter	Unit	Value	Remark
Hydrolysis half-life	DT50 [d]	Stable at environmentally relevant pH values	No significant hydrolysis at pHs 5.2 and 6.9. At pH 9.0, 43-45% of substance stays intact for 7 days.
Photolysis half-life	DT50 [d]	13 days 4.1 hours	Water Air
Relevant Metabolites		(Z)-3-(2-chloro-3,3,3-trifluoro-propenyl) 2, 2-dimethylcyclopropanecarboxylic acid; 3-phenoxybenzoic acid	> 10%

Source: L.C. Van Leeuwen et al. (2008)

On plant surfaces, lambda-cyhalothrin has a five-day half-life (Knisel, 1993). In the experimental laboratory investigations, LCH hydrolyzed in water at pH=9 with a half-life of about 7 days. There was no hydrolysis in water at lower pH levels (5; 7) (WHO, 1990). In the soil and water studies, it was revealed that LCH, when exposed to sunlight, photodegrades over periods of time with half-lives of the order of 30 days and <30 days respectively (WHO, 1990). The limited possibility for ground water contamination is indicated by lambda-cyhalothrin's strong binding affinity and poor water solubility (Vogue et al., 1994).

Toxicity of lambda-cyhalothrin

As a result of their intensive use, the pyrethroid residues are becoming present in the environment with evidence of detection (Tang et al., 2018). Par example in Pakistan, concentrations of these insecticides hit 1.2 μg/g in the soil (Sabzevari and Hofman, 2022). In comparison to other species (mammals and birds), fish metabolize and eliminate pyrethroids more slowly, which accounts for the greater toxicity results for these compounds in fish (Atamanalp, 2002).

Like most insecticides used in agriculture, the use of Pyrethroids in agriculture could have effects on human health. For example, Thiphom et al. (2012) noted the presence of 3-phenoxybenzoic acid (3 PBA) in the human plasma of producers and consumers. But 3-phenoxybenzoic acid is present in almost all Pyrethroids (lambda-cyhalothrin, cypermethrin, deltamethrin, etc.). According to these authors, the presence of 3-PBA in consumers is due to the occurrence of traces in fruits and vegetables. Other studies have also shown that adverse health effects such as anorexia, abdominal cramps, anxiety were the result of acute exposure to high doses of pyrethroids and not to metabolites (Leng et al., 1999). In addition, a survey reported by Temple et al. (1996) on a study conducted in China from 1983 to 1988 reported 573 cases of poisoning due to Pyrethroids. Adverse health effects (such as lymph nodes and various types of cancers) have also been observed following long-term exposure to Pyrethroids (Hallenbeck et al., 1985). According to Zhang et al. (1991), these Pyrethroids have adverse effects (abnormal sensations on the face, disturbances on the central nervous system and systemic effects) on cotton growers who are contaminated through the skin. Thus, we can say that the use of pyrethroids is not without consequences on human health.

The pyrethroid cyhalothrin, which is a combination of 4 isomers, is comparable to LCH, which is made of 2 of these isomers. LCH has the same range of insecticidal properties as cyhalothrin. Because of their similarity, researchers occasionally employ toxicity studies done with cyhalothrin to assess the LCH toxicity (Armour, 1998).

Because of their lipophilic properties, it is almost impossible for them to be eliminated once they are in the organism. The toxic effects of pyrethroids to organisms subjected to low doses for long periods of time can manifest themselves in the genetic and nervous systems inducing carcinogenicity, etc. (Ma, 2009); or undesirable effects on the male reproductive system (Koureas et al., 2012).

According to the European Parliament Regulation (EC) number 1272/2008, LCH is categorized as a substance with variable acute toxicity depending on the mode of intoxication (fatal by inhalation; toxic by ingestion; harmful by skin contact); and is recognized as extremely toxic to aquatic ecosystem species, which may result in long-term effects (ANSES, 2014). Extensive studies by ANSES (2013) have revealed that some of the organic compounds intermediate to LCH or resulting from its metabolism (metabolites) are less toxic than it.

It should be noted that type II pyrethroids are more toxic than those of type I, since they are synthetic, more stable, and degrade less quickly in sunlight and in the presence of humidity. Although synthetic pyrethroids have a greater degree of toxicity and selectivity for the insect nervous system, local effects on human skin leading to paresthesia are likely to be produced with overexposure. Humans seldom experience systemic poisoning since there doesn't seem to be much of a chemical absorption via the skin. (Amour, 1998; Tomlin, 1997). The majority of recorded cases of systemic poisoning and Central Nervous System (CNS) consequences from synthetic pyrethroids have been linked to purposeful consumption and occupational overexposure (Proudfoot, 2005).

According to studies conducted in the United States, lambda-cyhalothrin has been found in food, dust, and residential settings. However, it is presently believed that consuming pesticide residues through food and beverages is the main means of exposure to this active ingredient (Julien et al. 2008; Melnyk et al., 2012; Barr et al., 2010). The exposure of some individuals to Lambda-cyhalothrin creates excitation in their behavior (Martinez-Larrantildeaga et al., 2003). On the other hand, for living organisms (animals/humans) that experience acute poisoning, other symptoms (vomiting, nausea, etc.) related to more serious diseases can be observed (Hossain et al., 2005). In relation to the dose, Lambda-cyhalothrin induces liver and kidney tissue damage in mammalian (Mazmanci et al., 2008).

Because of its recognized toxicity, the European Food Safety Authority (EFSA), which produces autonomous scientific recommendations on the hazards related to the food, in 2014 set the Acceptable Daily Intake (ADI) for LCH at 0.0025 mg/kg body weight per day.

Numerous investigations have demonstrated that this substance's exposure by ingestion in rats resulted in a considerable reduction in food and water consumption as well as a subsequent deterioration of motor function, coordination, and tremor. In small mammals (e.g., rabbit, rat and mouse), laboratory tests have revealed neurotoxic effects of this chemical (El-Demerdash, 2007); with consequences such as reduced body weight in some of the exposed animals (Ali et al., 2014).

These are neurotoxic substances that influence neuronal signals by triggering a series of electrical impulses in the neurons causing nervous disorders and even paralysis in the organism having undergone the exposure (Stenersen, 2004; Hénault-Ethier, 2015). Through

laboratory bioassays on the toxic effects of this pesticide, it was concluded that lambda-cyhalothrin causes cytotoxic and genotoxic effect on non-target organisms (Aydogdu et al., 2017) and high toxicity for fish, other aquatic organisms and mammals (Aydogdu et al., 2017; Amweg and Wetson, 2005; Oros and Werner, 2005). During high exposure duration of fish, Lambda-cyhalothrin can cause severe cytopathological alterations on the gills (Uğurlu et al., 2017).

LCH is extremely hazardous to bees whether it is consumed (LD_{50} = median lethal dose= 0.97 μg/bee) or applied topically (LD_{50} = median lethal dose= 0.051 μg/bee), and then present a problem not only for the targeted insects, but also for other insects (WHO, 1990). In relation to fish, the high rate of absorption of pyrethroids through the gills of the latter is essentially related to the lipophilic nature of these pesticides, which directly increases the sensitivity of these groups of animals. This has the direct consequence of greatly increasing the sensitivity of the fish (Polat et al., 2002). Concrete examples of studies on the *Clarias gariepinus* species by Oluah et al. (2014) resulted in lethal concentrations (LC_{50}) on the order of 0.103 mg/L for LCH. Due to these results, pesticides based on this active substance have been prohibited from application to rice cultivation in China (Köprücü and Aydin, 2004).

LCH is extremely poisonous to fish and aquatic invertebrates with lethal concentration values hovering around 0.078-2.3 μg/L (LC_{50}) and 0.0023-3.3 μg/L (EC_{50}), respectively (Maud et al., 1998). It is possible for LCH to bond to species (fish, mammals, etc.) tissues due to its high affinity for organic solvents and its low affinity (attraction) for water. Cyhalothrin may bioconcentrate in fish, according to laboratory research. The study by Clasen et al. (2018) showed that LCH can bioaccumulate in fish species (an example of its bioaccumulation in carp muscles through 100 days of exposition) and therefore poses a great risk to human health due to this process. This implies that they can easily pass through the food chain to enter organisms. In fact, through food chains, pesticides that build-up (bioaccumulate) within aquatic species organisms are biomagnified. Those organisms not initially targeted by pesticides, but which become contaminated, such as fish that are a real source of protein for humans, can constitute an evident threat to consumers and public health problems.

2.2.2. Acetamiprid

General information on neonicotinoids and acetamiprid

Insecticides of the neonicotinoid type were introduced in the 1990s to address concerns about the use of other classes of insecticides to which pests showed resistance (organophosphates and carbamates, etc.). In addition, the consequences of the use of the same pesticides on the neuronal growth of children have been documented (Eskenazi et al., 1999). Homologated in over 120 countries since their entry into the global market in 1990, neonicotinoids alone account for more than 25% of insecticide sales, resulting in the total elimination of current uses of older pesticide families (organochlorines, organophosphates) (Hladik et al., 2018).

With the recognized success of the first neonicotinoid, Imidacloprid, of the neonicotinoid class, the development of other types of this insecticide followed with record sales in the 2000s with active substances such as Acetamiprid and others marketed under several brand names. In the insecticide market, neonicotinoids are considered in recent years as the most sold class with 85% of the market share worldwide (Sultana et al., 2018). Neonicotinoids are exceptionally well liked for pest management on a variety of crops because of their systemic action and simplicity of usage as seed treatments (Elbert et al., 2008; Main et al., 2014). Neonicotinoids are systemic insecticides which, due to their physicochemical characteristics, are absorbed by the plant and can be transported, via the phloem or xylem, in all tissues: foliage, stem, roots, flowers, pollen and fruits. Because the parent molecules of neonicotinoids and their metabolites circulate through plant tissues, these insecticides provide protection against a large number of phytophagous arthropods that will be affected following contact with the insecticide or ingestion of a piece of the plant (Bonmatin et al., 2015). Thus, they have a broad spectrum of action.

The discovery of Acetamiprid (ACE) was made in 1989. It is an organochlorine compound (α-chloro-N-heteroaromatic) of the first generation of neonicotinoids that was introduced in 1995. Acetamiprid was first launched in 1995 by Nippon Soda in Japan. It is now sold under a number of brand names with various formulations, including Mosipilan®, Rescate®, Epik®, Assail Intruder®, Tristar®, Saurus®, and Chipco TM. It comes as an odorless white powder. The commercial preparations are available as soluble concentrates (5, 10, 20, 50, 75 or 95% active substance), wettable powders or granules dispersible in water. In some

liquid formulations the insecticide is in solution in an organic solvent (Couteux and Lejeune, 2012; Testud, 2014).

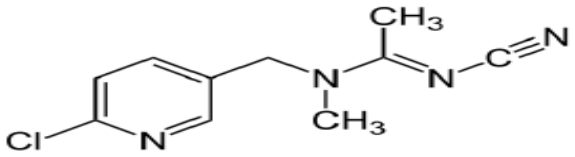
It is one of the most used neonicotinoids in the world with a direct action on the nervous systems of target organisms and others, thus causing paralysis or death (Jeschke et al., 2011, Terayama et al., 2016). Developed for pest control, it is a systemic insecticide that is primarily used against sucking insects (e.g. aphids and whiteflies) on leafy vegetables, cabbage, citrus, cotton, ornamentals and fruiting vegetables. It also has domestic use in the protection of pets such as cats and dogs against fleas (Couteux and Lejeune, 2012).

Physico-chemical properties and functioning

The IUPAC name for Acetamiprid is (E)-N1-[(6-chloro-3-pyridyl)methyl]-N2-cyano-N1-methylacetamidine. Physicochemically, Acetamiprid (ACE) is composed of a chloropyridine ring (Het) and an imidazolidine ring that carries a cyano- (N-cyanoimine, =NCN) group (Kabagu et al., 2007).

According to their chemical structure, neonicotinoids can be classified into 2 and 3 groups depending on the type of pharmacophore ([-N-C(E)=X-Y]) they possess (5- and 6-membered ring compounds; open chain compounds). Acetamiprid, according to its pharmacophores belongs to the group of open chain compounds. ACE is a member of the N-cyanoamidine class of neonicotinoid compounds like the insecticide thiacloprid (THC). The nitro- and cyano- groups are essential for the insecticidal activity of neonicotinoids.

Table 2.4. Molecular structure and physicochemical properties of acetamiprid (ACE)

Molecular structure	
Common name	Acetamiprid (ACE)
CAS	135410-20-7
Chemical name	(*E*)-*N*1-[(6-chloro-3-pyridyl)methyl]-*N*2-cyano-*N*1-methylacetamidine
Chemical formula	C10H11ClN4
Molecular structure	
Physicochemical properties	
	White powder with low water solubility (3 to 4 g/L depending on pH) and nonvolatile (vapor pressure < 1 µPa at 25◦C)
Maximum residue levels in foodstuffs	0.01 to 5 mg/kg depending on the type of crop
Molecular weight (g/mol)	222.67
Pressure of vapour (mm Hg)	4.5×10^{-05}
Relative density	1.33 g cm^{-3}
Solubility (pH7)	2950 mg L-1 (at 25 °C)
Octanol-water coefficient (Kow)	6.27
Henry's constant (atm m3/mole)	7.92×10^{-08}
Melting point (degree C)	98.9
Anaerobic aquatic half-life (days)	45
Aqueous photolysis half-life (hours)	>34
Water solubility (mg/L at 20 degree C)	4200
Soil photolysis half-life (days)	25.1
Field dissipation half-life (days)	<18
Soil adsorption coefficient (Kd)	<4.1

Source: Testud, 2014; Ewere et al., 2021

The description of ACE molecular structure (figure 4) shows that it is also composed of a chloromethylpyridine group coupled to a derivative of a cyanoamidine, methylated N-cyanoacetamidine, hence its name (Takahashi et al., 1992; Tomizawa and Casida, 2005).

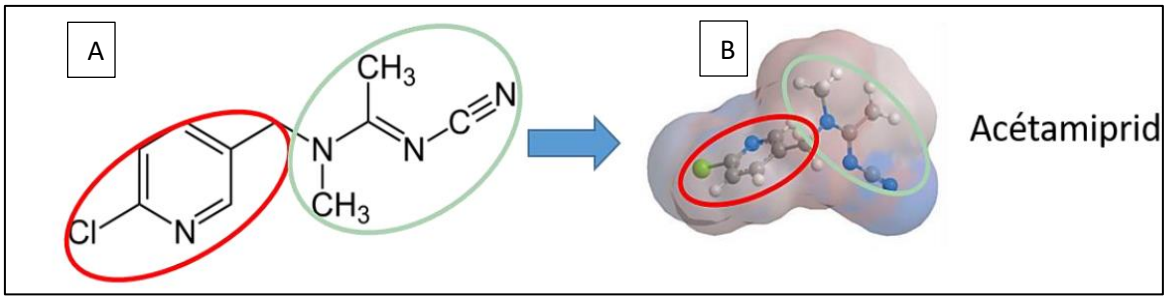


Figure 2.3. Acetamiprid molecule
*In A, the Lewis representation; in B: Acetamiprid molecule represented with positive electrostatic potential in red and negative in blue and chemical groups in a 3D structure. The circled chemical groups are: **chloromethylpyridine,** **N-cyanoacetamidine methyl group**

The electrostatic potential makes it possible to visualize a characteristic of neonicotinoids which is to mimic the natural neurotransmitter: acetylcholine. Acetylcholine (ACh) is the most important excitatory neurotransmitter in the central nervous system of insects. It plays a primary role in insect neuronal synaptic transmission (Thany et al., 2007). Neonicotinoids act within the central nervous system by interfering with neuronal transmission.

Like most neonicotinoid insecticides, Acetamiprid is an agonist of acetylcholine that has a high affinity for nicotinic receptors (nAChRs) located in post-synaptic neurons. At low concentrations, neonicotinoids induce central nervous system stimulation in insects (van Lexmond et al., 2015). However, at high concentrations, they induce neuronal hyperactivity. This malfunction causes the nAChRs receptors to become blocked, interrupting the transmission of nerve impulses and causing acetylcholine to build up in the intrasynaptic region. This neuronal modification leads incessantly to paralysis and death of the insect. Thus, the effect on the central nervous system is similar to that of nicotine. Insects are particularly sensitive to this since their proportion of nAChRs receptors is much higher than in other organisms (Jeschke et al. 2013; Anderson et al., 2015).

The lipophilic nature of neonicotinoids such as acetamiprid facilitates their passage through all biological barriers. The digestive distribution is rapid and complete; the peak plasma peak is obtained at the second hour. The mammalian blood-brain barrier is very low permeability to acetamiprid (David et al., 2007; EFSA, 2014; Terayama et al., 2016).

Toxicity of neonicotonoids and acetamiprid

Neonicotinoids are very popular because they are often associated with systemic insecticides, which work with low application rates, effective action on crop pests, and low toxicity to mammals (Jeschke et al., 2011; Simon-Delso et al., 2015). Nevertheless, neonicotinoid residues are increasingly detected in surface waters of aquatic ecosystems near their areas of applications due to their high-water solubility and persistence in the environment (Morrissey et al., 2015). In fact, by being more water soluble than other insecticides (e.g., organochlorines and pyrethroids, neonicotinoids are likely to enter aquatic environments through runoff and erosion from farmlands (Stehle et al., 2018).

By assessing the number of neonicotinoid residues in the space between treated agricultural areas, adjacent land parcels and then aquatic environments, Bonmatin et al. (2019) demonstrated the existence of residues of this insecticide in the different target areas, at variable concentrations but with higher values at the application areas. Neonicotinoids and as well as their metabolites are persistent in soil and accumulate there (Goulson, 2013).

They are not without risk to living organisms and the balance of ecosystems in the vicinity of their application area. They have chemical properties that increase their persistence in the environment and their predisposition to being transported into aquatic environments by surface runoff and drainage from agricultural sites (Armbrust and Peeler, 2002). Research data show that in environments where neonicotinoids are present, a decrease in macroinvertebrate abundance is observed (Van Dijk et al., 2013), as well as a change in the specific composition, especially of aquatic communities (Vehovszky et al., 2015).

Based on the potential threats of neonicotinoids to living organisms, several research results have demonstrated risks of acute or chronic toxicity following long-term exposure, and ecological consequences for aquatic environments and organisms (Chen et al., 2019).

Although being selective for insect nAChRs, neonicotinoids may have deadly and sublethal consequences on non-target species. Due of the significant harm that neonicotinoids do to bee populations, bees are the primary subject of most investigations on their impacts (Henry et al., 2012). Nevertheless, following neonicotinoid exposures, some scientists have noted

impacts on a number of biomarkers in invertebrates' non-insects, such as molluscs (bivalves, annelids, etc.).

Neonicotinoids, having a high capacity of accumulation in plants, are at the origin of an increased risk of environmental pollution and toxicity to non-target organs because of this characteristic (Ihara et al., 2018).

Given that the placental barrier enables the transfer of several xenobiotics to the fetus that ACE is detected in the brain after ingestion, and that its known metabolites are detected in the urine of human neonates on post-natal day 2, it is hypothesized that neonicotinoids may be able to traverse the placental barrier (Gomez et al., 2020).

Available studies on acetamiprid toxicity are limited in some regions of the world where the sale of this neonicotinoid is free and cheap such as in Southeast Asia and most African countries (Kimura et al., 2012; Testud, 2014; Terayama et al., 2016). In Europe, ACE has recently been added to the list of chemicals to be monitored as a possible water contaminant (2018/840/EC).

Acetamiprid is not permanent in soil environments, but it could be under some circumstances quite durable in freshwater ecosystems (Lewis et al., 2016).

According to Kimura-Kuroda et al. (2012) acetamiprid leads to morphological aberrations in brain development and behavioral disturbance in mammals. The stimulation of the central nervous system is produced, clinically translated by violent tonic-clonic convulsions. Nausea, vomiting, diarrhea, paresthesia of the tongue and face also occur.

At the organism level, ACE is fully incorporated and diffused into the tissues and is often found in high concentrations in the liver, kidney, adrenal gland, etc.; its elimination is via urine and bile (Mondal et al., 2014). ACE poisoning leads to several symptoms, ranging from those observed in the neural centers, lungs, muscles, and mouth (dry mouth) (Terayama et al., 2016). As a result of inhalation of ACE, symptoms such as vomiting, dizziness, etc. are common.

Terayama et al. (2016) showed accumulation of acetamiprid in the brain of mammals such as rats and murines. In addition, it has been reported that continuous exposure of rats to ACE caused dysfunction of matrix oxidative state and loss of mitochondrial membrane integrity in the brain (Salim, 2018). ACE has also known damage to mammalian reproductive systems as shown by Kong et al. (2016). Indeed, this active substance disrupts the testosterone biosynthesis mechanism by influencing the cholesterol conversion rate and blocking its entry into the mitochondria existing in the intestinal cells (Leydig cells).

The study by Jeschke et al. (2011) identified a potential harmful effect on the development of neurons and brain structures in a fetus or young child. Cardiac and respiratory failure results in death within hours. Inhalation of acetamiprid dust results in mouth and lung irritation with bronchial hypersecretion (Woodcock et al., 2016). The Canadian Pest Management Regulatory Agency (CPMA) considers acetamiprid as a potential endocrine disruptor and suspects reproductive effects in animals (Jeschke et al., 2011; Chen et al., 2014; Terayama et al., 2016). Animal carcinogenicity studies are more or less negative for acetamiprid, it is classified as a non-carcinogen to humans by the U.S. Environmental Protection Agency (McKelvey et al., 1988; Parks et al., 1988; Obata et al., 2001).

At various dose levels (regarding the LD_{50} value), acetamiprid causes a drop in body weight and clinical symptoms in mice, including respiratory depression, sitting in the corner, diarrhea, depression, dullness, etc. (Singh et al., 2021). Considering its physical and chemical properties (permanent in nature, water solubility), toxicity studies on organisms not targeted by this chemical substance have revealed worrying results and which warn of its widespread and high-intensity use on an international scale (Arican et al., 2019; Guedegba et al., 2019; Ewere et al., 2021).

An investigation of neonicotinoid levels in selected aquatic environments in approximately 9 different countries revealed that concentrations of this family of insecticides in several of the environments evaluated far exceeded the predicted short-term and long-term interim water quality thresholds of 0.2 g/L and 0.035 g/L, respectively (Morrissey et al., 2015). This implies that non-target organisms such as aquatic organisms (e.g., molluscs, fish, etc.) are not immune to potential damage from neonicotinoids.

It is common to find this group of pesticides, including acetamiprid, in water ecosystems around the world (You et al., 2019). Acetamiprid has potential toxic effects on fish that can cause human health hazards. When exposed to Acetamiprid, zebrafish embryos displayed severe developmental toxicity with a range of impacts on behavior, growth, morphology, hatchability, and mortality at high doses (You et al., 2019). According to Raj and Joseph (2015), fish exposed to this pesticide over time demonstrated lower protein and carbohydrate content in their liver, brain, and gill tissues during all exposure times. In a study examining the toxic effects of Acetamiprid in mosquito fish (*Gambusia halbrooki*), which is one of the non-target organisms, the LC_{50} values for 24 and 96 hours were estimated to be 75.9 mg/L and 42.2 mg/L. In addition, in this study, where biochemical parameters were also examined, it was observed that glutathione-S-transferase and lactate dehydrogenase (LDH) activities increased at the end of the 24th hour (Demirci and Güngördü, 2020).

Below is a table that summarizes some of the studies on the toxic effect of acetamiprid on aquatic organisms. This non-exhaustive list shows nevertheless that many researchers wonder about the toxicity of this molecule in several where it was probably detected.

Table 2.5. Potential effects of acetamiprid on aquatic organisms (CEAEQ 2020)

Organisms	Parameters	Duration of exposure	Value (mg/L)
Algae			
Anabaena flosaquae	EC_{50} (Growth)	5 j	> 1,3
Navicula pelliculosa			> 1,1
Skeletonema costatum			> 1
Aquatic invertebrates			
Aedes aegypti	LC_{50}	24 h	0.65
		48 h	0.329
	LC_{50}	24 h	1.11
Americamysis bahia	LC_{50}	24 h	0.066
Chironomus tepperi	LC_{50}	48 h	0.0022
Culex pipiens	LC_{50}	24 h	0.044
			0.167
			0.31
		48 h	0.454
			0.18
			0.054
			0.027
		72 h	0.296
			0.02
			0.047
			0.1
			0.026
Daphnia magna	EC_{50} (mobility)	24 h	50
Gammarus fasciatus	EC_{50} (mobility)	96 h	0.08
Gammarus pulex	LC_{50}	96 h	0.05
Hexagenia sp.	LC_{25}	96 h	0.055
	LC_{50}		0.78
	EC_{25} (behavior)	96 h	0.0022
	EC_{50} (behavior)		0.004
Simulium latigonium	LC_{50}	96 h	0.00373
Fish			
Cyprinodon variegatus	LC_{50}	96 h	100
Embryos of *Danio rerio*			13.33
Danio rerio juveniles			36.91
Larva's *Danio rerio*			15.52
Danio rerio adults			10.36
Lepomis macrochirus			> 119.3
Oncorhynchus mykiss			> 100

LC_{20}: lethal concentration of a substance to 20% of test organisms;
LC_{25}: the lethal concentration of a substance in 25% of the test organisms.

However, because of their recent market entry, there is currently little information on this class of insecticides' effects on non-target species, especially over the extended term (Main et al., 2018; Cossi et al., 2020)

2.2.3. Evaluation of the effects of lambda-cyhalothrin and acetamiprid on living organisms

Biomonitoring

To measure the effects of pollutants on living beings and ecosystems, tools such as chemical analyses are commonly used. These analyses reveal the presence of one or more compounds in a sample and, most often, measure their concentration (e.g., 0.3 µg/L, 0.5 µg/L, 1 µg/L of a pesticide in different lakes/river water samples). The progress made over the last decades now allows the detection and quantification of chemical substances present in trace amounts (µg/L, ng/L, or even lower), which were previously "invisible" because of detection limits that were too high. Nevertheless, these analyses do not allow: (1) to know if the analyzed pollutants are bio-available: i.e., assimilable by the organisms; (2) to know the toxicity of these pollutants towards the organisms; (3) to evaluate the interactions between the various pollutants (additive, synergistic or antagonistic effects).

Since physicochemical analyses are useful but insufficient to assess the environmental risk linked to pollutants, to assess the impact of substances on living organisms, it is necessary to use the "living". This approach, known as "biomonitoring", refers to the set of tools based on "the use of living organisms (organism or population/community at different levels of integration: organs, cells, biochemistry, physiology, tissues) to monitor the evolution of modifications and alterations, or the stability of the quality of an environment" (Gourlay et al., 2011). As detailed in the following schema (figure 2.4), its application can be made at different scales.

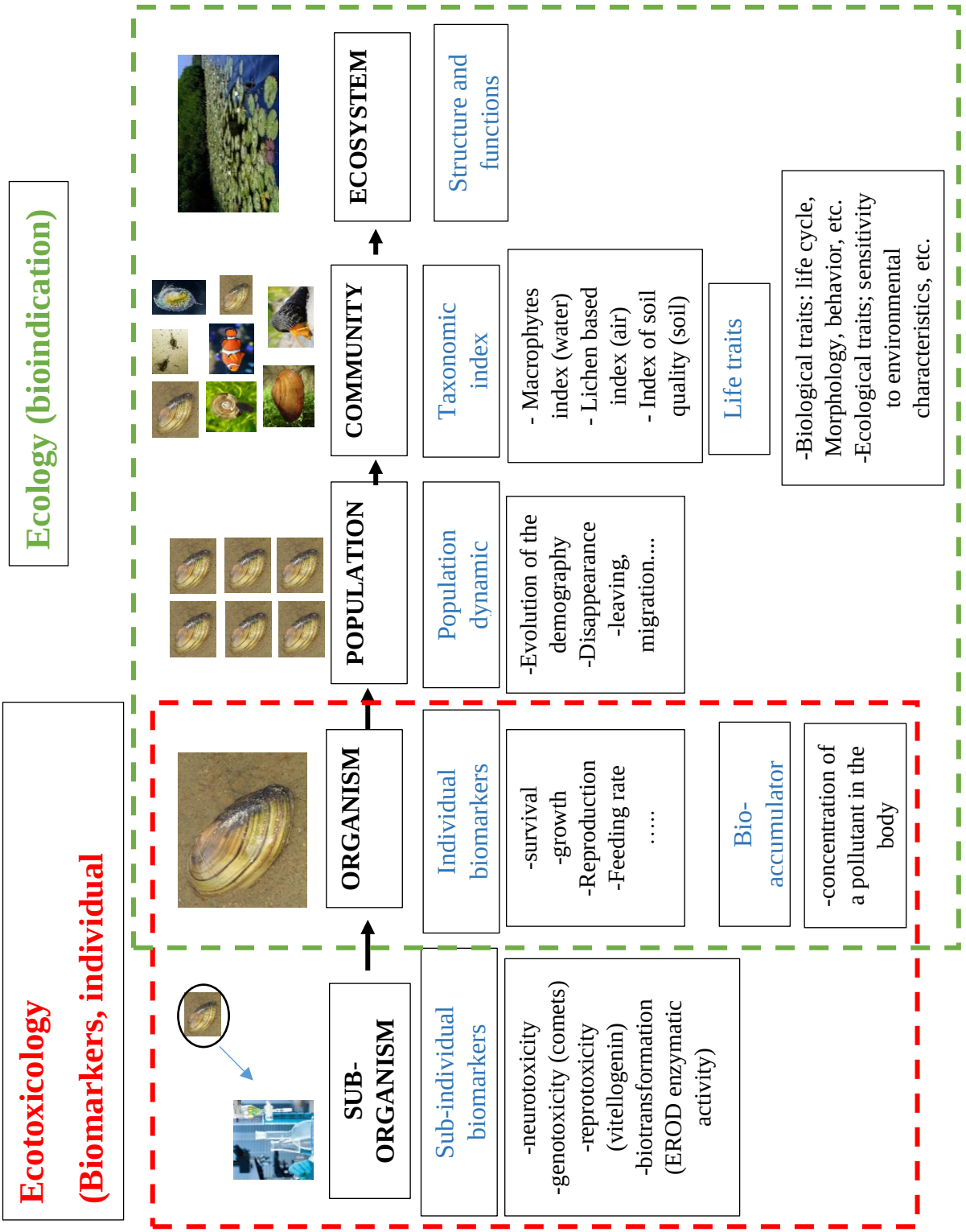


Figure 2.4. Biomonitoring: the different levels of observation (Geffard, 2009; Lamonica, 2016)
- In black capital bold: the levels of observation (sub-organism, organism, etc.) /
In blue: what is observed/measured.

In this thesis project, we seek to better understand the impacts of the two pesticides described above on bioindicator species, the case of mussels, by adopting an ecotoxicological approach based on biomarkers at the organism level.

Any organism exposed to chemical pollution implements a diversity of molecular and cellular responses that allow it to cope with the aggression of the pollutants (INERIS, 2008). The biomarker approach consists of studying these biological responses on laboratory organisms, indigenous organisms or organisms encased in the natural environment, in order to establish the diagnosis of pollution. Unlike bioindicators, biomarkers are therefore specifically designed to assess the risk associated with toxic pollutants (= ecotoxicological or ecotoxic risk).

Because of the variability of biological responses between species, biomarkers are evaluated on sentinel organisms for which a prior work of development/validation of the measure has been carried out (Gourlay et al., 2011).

Biomarkers, which are known as early indicators, are important tools for measuring sublethal effects caused by stress in organisms. Like an "alarm bell", they can detect pollution before the damaging effects on populations and communities are felt. They are physiological, biochemical or histological indicators of xenobiotic exposure or effects (Forbes et al., 2006). Biomarkers indicate dysfunction at the cellular, organ, or whole organism level and thus can provide a precursor signal for declining health and or performance of an organism, and thus have implications on a larger scale, such as the population level (Theuerkauff, 2019). They also have the advantage of being sensitive and specific. Indeed, the responses of an organism to pollution are based on mechanisms that may be specific to families of chemical compounds and/or a toxic mode of action (INERIS, 2009). The measurement of a biomarker can therefore prove invaluable in identifying the mechanisms at the origin of the effects of contaminants and in orienting the diagnosis of a pollution.

Biomarkers of antioxidant system

In order to limit or prevent cellular damage, organisms have developed defense systems called antioxidants in case of oxidative stress, a natural consequence of oxygen metabolism. This oxygen can be toxic when it is transformed into highly reactive radical species. Reactive

oxygen species (ROS) are generated endogenously in small quantities (2% to 5%) during cellular respiration, which involves electron transfer, and can also be generated following oxidative stress or during any reaction involving electron transfer (Dawson et al., 1993). All living organisms produce them via aerobic metabolism at different levels, primarily the mitochondria and the endoplasmic reticulum containing cytochromes P-450 (Lesser, 2006).

The three main mechanisms of antioxidant action developed by aerobic living organisms consist of (1) eliminating reactive species and the catalysts for their formation, (2) inducing antioxidant systems, and (3) increasing the activity of repair and removal systems for damaged molecules (Cossu-Leguille, 1996).

Under normal conditions, the ROS produced are detoxified into H_2O by defense systems (antioxidant molecules and enzymes, in particular) that allow a balance to be reached, called the oxidative balance.

These defense systems consist of antioxidant enzymes comprising mainly Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPX) and antioxidant molecules such as glutathione (GSH reduced form, GSSG oxidized form) and vitamins C and E (Theuerkauff, 2019).

SOD, CAT, and GPx which are enzymatic-type cellular antioxidants will be more specifically studied during the work of this thesis:

- SODs are metalloproteins (they contain a metal atom: copper, zinc, manganese, iron and/or nickel in their active site) with an enzymatic activity catalyzing the dismutation of superoxide anions ($O_2^{\bullet-}$) into oxygen and hydrogen peroxide (H_2O_2) according to the following reaction: $2\ O_2^{\bullet-} + 2H+ \rightarrow O_2 + H_2O_2$ (figure 2.5).

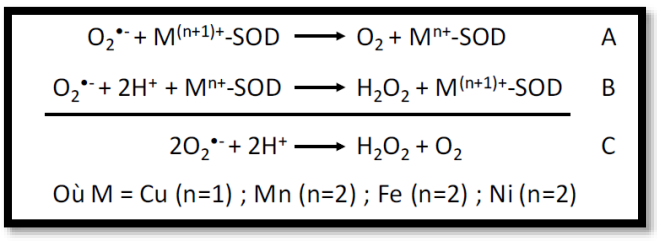


Figure 2.5. Reactions catalyzed by superoxide dismutase (SOD)

The different metal groups of superoxide dismutase's allow these enzymes to catalyze, through the redox couple of the metal ion, the reduction of two molecules of $O_2^{\cdot -}$ (A and B). The reaction balance of superoxide dismutase corresponds to the equation C; M= metal, n= electronic charge of the metal.

- GPx prevents the formation of free radicals. It can reduce H_2O_2 to H_2O and organic hydroperoxides (ROOH) to alcohol (ROH) (Favier, 2003).

- CAT is a homotetrameric enzyme consisting of four protein subunits each with a heme group with a Fe^{3+} molecule bound to the active site. It catalyzes the transformation of hydrogen peroxide (H_2O_2) into water and oxygen according to the following reaction: $2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$. (Figure 2.6).

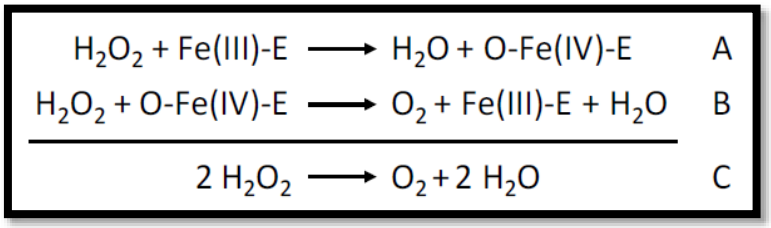


Figure 2.6. Reactions catalyzed by catalase.

The heme group of the catalase, in the form of ferric ion, allows the reduction of a first molecule of H_2O_2 (A). The oxidized ferryl ion reacts with a new molecule of H_2O_2 (B) to return in its ferric ion form. The reaction balance of catalase corresponds to equation C.

Use of bivalve molluscs (mussels) in ecotoxicology studies

Mussel interests

In ecotoxicological studies, the use of so-called sentinel or bioindicator species is common to ensure the ecological relevance of the results of the research to be conducted. According to Heink and Kowarik (2010), these types of indicators are used to describe the pressures that human activities exert on the environment. These bioindicator species, whether animal or plant, allow for a rigorous assessment of the potential toxic risk of a pollutant or a complex mixture of pollutants. They are selected on the basis of certain specific characteristics, including having a significant role in the functioning of their ecosystem, being sensitive to

the pollutants under consideration, being widely distributed in the area under study, having a long lifespan and finally being easily manipulated during laboratory or field experiments (Lagadic et al., 1997; Ramade, 2007)

According to the literature review of previous studies on the toxicity of these two substances, very little research has been carried out on aquatic invertebrates, in this case the phylum of Molluscs. This group of animals includes several genera of important species widely used to characterize the state of degradation of the aquatic environment because of their high sensitivity to environmental changes (Cossi et al., 2020). They include several genera of important species widely used to characterize the state of degradation of the aquatic environment because it is very sensitive to environmental changes (Cossi et al., 2020).

Bivalve molluscs are good bioindicator and sentinel organisms, because they are animals distributed all over the world, and adapted to many ecosystems: fresh, brackish, or salt water, arctic, temperate or tropical temperatures, etc. (Staichak et al., 2021) Examples include the class of bivalves, more specifically mussels, which are typically used in environmental toxicity studies to assess the degree of disturbance and adverse effects that xenobiotics (pesticides) may have on behavior, survival and alteration of the functioning of the organism of aquatic species (Milam et al. 2005; Beggel et al., 2017; Günal et al., 2018). The use of mussel species for this type of study is intrinsically related to some of their characteristics, such as being long-lived, sedentary, burrowing, filter feeding, and fairly large (mussels have a significant amount of soft (light) tissue for biochemical analysis; (vi) spent valves (dead mussels leave a historical record) (Grabarkiewicz et al., 2008; Hanana et al., 2017).

In addition, they filter feed and breathe large quantities of water (between 3 and 5 liters per hour in freshwater bivalves), and thus accumulate contaminants in the water and suspended matter in their tissues (Kryger and Riisgard, 1988). Furthermore, mussels are sessile organisms, which move very little. They are therefore dependent on variations in local environmental conditions, such as water and sediment quality.

Bivalves are known to be a good experimental model because their response to the presence of a contaminant is relatively constant. In addition to their accessibility in the field, it is possible to obtain individuals from aquaculturists. It is thus possible to have healthy organisms for different control studies and to transplant (embed) them in polluted sites.

These organisms can be used to evaluate the effect of different chemical compounds in the laboratory, either through in vivo or in vitro exposures (Zhou et al., 2008). Moreover, the immune system of bivalves is well known to be especially sensitive (to the presence of xenobiotics and to different environmental stresses (Hannam et al., 2009).

Description of the test organism: Unio terminalis

The bivalve considered in our study, *Unio terminalis* (Bourguignat, 1852), constitutes a bioindicator species particularly interesting for ecotoxicological studies thanks to its ecological and biological characteristics.

This bivalve mollusc belongs to the order Unionida, in the family Unionides of which all representatives live in freshwater. Widely distributed in Turkey and in several other countries of the sub-region (Israel, Iraq, Palestine, etc.), this species can be studied in ecotoxicology in various areas, with different levels of pollution. Its morphology is quite similar to other freshwater mussels (Image 2.2) but can vary considerably depending on the region and the watershed. The shell is marked by concentric growth streaks that are added each year, which allows the age of the individuals to be determined.

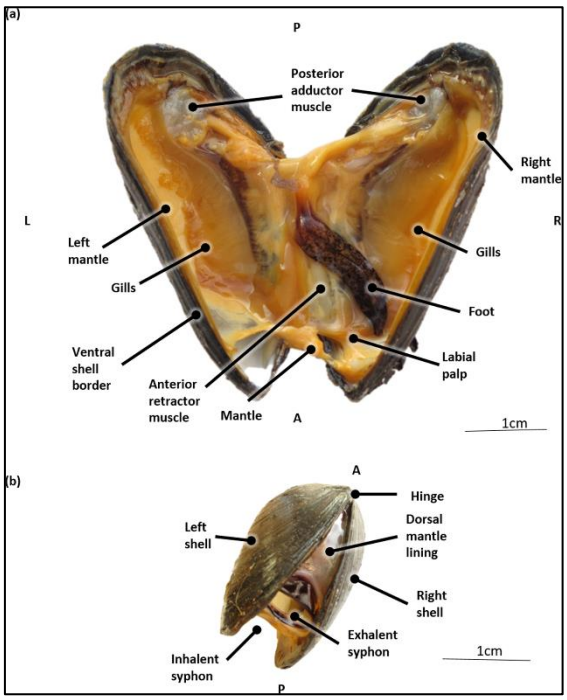


Image 2.2. General anatomy of the mussels (Unionidae) (Eggermont et al., 2020)

This mussel species, which is not endangered, lives in freshwater ecosystems and its vertical (burying) and horizontal movements are carried out by the movements of its foot.

Its mechanism of nourishment, which involves filtering enormous amounts of water, is crucial for maintaining the equilibrium of lake and river ecosystems. Indeed, by consuming detritus as well as invertebrates and planktonic algae, freshwater mussels participate in the cycle of organic matter in the water column. In addition, these animals are prey for many aquatic mammals such as otters and raccoons, and are therefore an important link in the food chain (Vaughn and Hoellein, 2018).

3. MATERIAL AND METHODS

3.1. Material

3.1.1. Biological material (test organism)

The freshwater mussels, *Unio terminalis* (Bourguignat, 1852), used as living material in the study were purchased from a local supplier. Before starting the experiments, the mussels were acclimatized in a ventilated environment with the required water quality for 21 days (Image 3.1). During the 21 days of the acclimation process, mussels were fed every day using phytoplankton (*Spirulina* sp.) and maintained at the appropriate temperature in the laboratory conditions.

Prior to receiving the mussels, aquariums of 15 to 20 liters were carefully cleaned and disinfected. They were filled with water and provided with a functional aeration system.

Image 3.1. Stock of mussels in acclimatization phase

3.1.2. Chemicals and stock solution preparation

The active substances, lambda-cyhalotrin (CAS No.91465-08-6; technical grade 97%) and acetamiprid (CAS No. 160430-64-8; technical grade 96%) were obtained from Shenzen Agro Hunter Co Ltd. and Hebei Veyong Bio-Chemical Co. Ltd., China, respectively.

Stock solutions were prepared for each of the substances by dissolution in the solvent dimethyl sulfoxide (DMSO). The solutions obtained were stored, in the refrigerator while waiting for their use in the various tests carried out.

3.1.3. Other equipment

Apart from the living organisms (mussels) and the active substances, various other materials and equipment were used during the different tests until the final results were obtained: precision balances; micropipette, Eppendorf tubes, centrifuge, freezer with cooling function at -80 °C, measuring devices, microplate readers, etc. Their different characteristics and uses will be described in the methodology section.

3.2. Methods

3.2.1. Experimental design

The toxicity tests were carried out according to the standards and recommendations of the OECD (1993) and APHA (1998) with modifications to freshwater mussels.

In order to evaluate the effects of the two active substances on individual mussels, preliminary experiments were carried out to calculate their acute toxicity values (LC_{50}) for the organism of interest in this study (*Unio terminalis*). (APHA, 1998).

Acute toxicity tests

Preliminary experiments: In order to calculate the baseline values that can be used in the determination of the concentrations of the active substances for the main experiment, a preliminary experiment was set up for 24 hours: Wide ranges of concentrations were used, including 1, 1/10, 1/100, and 1/1000. We then determined the range between the lowest concentration resulting in 100% mortality and the highest concentration resulting in 0% mortality (no death).

With the results of the preliminary experiments, the nominal concentrations used for the two pesticides are, respectively, for acetamiprid (0.01 mg/L; 0.1 mg/L; 1 mg/L; 10 mg/L; 25 mg/L and 100 mg/L; mg/L=ppm); and for lambda-cyhalothrin (0.1 mg/L; 0.5 mg/L; 1 mg/L; 5 mg/L; 10 mg/L; 20 mg/L).

Main experiments: Two days before the start of the experiment and during it, the mussels were not fed. The experiments were carried out, one after the other, for each pesticide. It has been prepared for the acute toxicity test of each pesticide; Seven aquariums filled with 15 L of tap water. As a prelude to the tests, the biochemical properties of the water contained in the aquariums were evaluated and determined. After the acclimatization period in the laboratory, the mussels were randomly selected and distributed in each aquarium, at the rate of 10 individuals per aquarium. In the experiment, each concentration was performed in triplicate to determine the 95% confidence limits. These experiments lasted for 96 hours under static conditions. During the experimental phase, the physicochemical analyses of the water in the aquariums were carried out and noted.

After the acute tests, mussels were exposed to sublethal concentrations during the 48 hours, 7 days and 21days periods. For the sublethal study, a total of 4 groups of mussels were used: one control group (freshwater without any chemicals), second control (DMSO solution), and two groups with two different doses of the pesticides in two replicates (Image 3.2). In order to keep the concentration of chemicals in the water constant, the test environment will be renewed periodically. These in vivo bioassays were performed under semi-static conditions (APHA, 1975). During the experimental phase, the physicochemical analyses of the water in the aquariums were carried out and noted (Table 3.1).

Table 3.1. Water quality parameters for acute toxicity tests conducted with the mussels (from January to June 2021)

Test organism	Toxicant	Water quality parameters			
		pH	Temperature ^{o}C	Dissolved oxygen (mg/L)	Conductivity µS/cm
Mussels (*Unio terminalis*)	Acetamiprid	7.92	21	6,8	298
	Lambda-cyhalothrin	7.98	21	7,1	312

At the end of each experiment, all mussel individuals sampled for analysis were dried with a paper towel and excess water removed. Then, they were weighed with a precision scale 0.01 g. In addition to the weight, different measurements were taken on each individual: height, length and width with calipers (precision of 0.01 mm).

Image 3.2. Example of the experimental set-up of the chronic exposure of mussels

Methods for studying the organism's response to pesticide chronic exposure

Our work on the species *Unio terminalis*, an indicator species and ecosystem engineer, and on the effects of its exposure to active substances (lambda-cyhalothrin and acetamiprid) on its physiology has allowed us to acquire a lot of knowledge, mainly on the level of immune defense and the functioning of key organs such as gills and digestive glands, in order to have a global vision at the organism level. Markers of antioxidant defense (CAT, SOD, GPx), oxidative stress, analysis of hemolymph and histological analysis have been performed to improve the understanding of the response of these organs and the animal's body to these xenobiotics. The summary of the methodologies is presented in Table 3.2.

Table 3.2. Summary of the methodologies and markers deployed to meet the three objectives of the thesis

Experimental conditions	Exposure time	Level of Analysis – (Organ/Organism)	Biomarkers	Methodology
-Freshwater (no chemicals added) -Dimethylsulfoxide (DMSO) -Lambda-cyhalothrin	48 hours 7 days 21 days	Hemolymph	- Hemocytes - Oxidative stress markers	Hemolymph sampling -Total Hemocyte Counts (THCs) Total antioxidant status (TAS) Total oxidative stress level (TOS)
		- Gills - Digestive glands	Activities of antioxidant enzymes	Activity Catalase (CAT), Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx)
		All tissues	Histology	Histopathological evaluation and illustration
-Freshwater (no chemicals added) -Dimethylsulfoxide (DMSO) -Acetamiprid	48 hours 7 days 21 days	Hemolymph	- Hemocytes - Oxidative stress markers	Hemolymph sampling -Total Hemocyte Counts (THCs) - Total antioxidant level (TAS) - Total oxidative stress level (TOS)
		- Gills - Digestive glands	Activities of antioxidant enzymes	Activity Catalase (CAT), Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx)
		All tissues	Histology	Histopathological evaluation and illustration

3.2.2. Hemolymph analysis

Determination of total hemocyte counts (THCs)

Total Hemocyte Counts (THCs) in mussels are a health indicator and a stress indicator. Changes in hemocyte counts occur in bivalve species exposed to toxic substances, causing weakening of the immune system power (Auffet et al., 2006).

At the end of each exposure time (48 hours, 7 and 21 days) to both pesticides, hemolymph samples were collected from each mussel individual (Image 3.3). Approximately 0.5 mL of

hemolymph was collected using a sterile 1 mL plastic syringe with a needle that was pre-filled with 0.5 mL formalin (4%) (to avoid hemocyte aggregation). For each individual hemolymph sample, a hemolymph fraction was examined by light microscopy to determine the total number of hemocytes in each sample using a hemocytometer following the method described by Yavuzcan and Benli (2004).

Image 3.3. Collection of hemolymph from the mussel using a sterile syringe

Total antioxidant level (TAS) and total oxidative stress level (TOS)

In the perspective of determining the values of Total Antioxidant Status (TAS) and Total Oxidative Stress (TOS) in hemolymph, samples of hemolymph were collected, put in Eppendorf tubes, and stored at -80˚C pending the analysis.

To perform the analyses, we used commercial kits, in this case The Rel Assay (TAS Rel Assay Diagnostics kit, Lot MS22128A, Ref RL0017, Gaziantep, Turkey; TOS Rel Assay Diagnostics kit, Lot MS22142O, Ref RL0024, Gaziantep, Turkey). The use of this method and this kit is due to the fact that the values obtained at the end of the test have accuracy above 97%.

Total Antioxidant Status Determination (TAS): The principle of the assay is based on the incubation of the ferrylmyoglobin radical with 2,2'-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS) generated by peroxidase activation with H_2O_2 to produce the ABTS+ radical cation. It results in a proportionally stable blue-green color that can be measured at

660 nm. The concentration of antioxidant levels in the added samples is directly proportional to the concentration of the color formed (Miller et al., 1993).

Determination of Total Oxidative Stress Level (TOS): It is based on the conversion of N, N-dimethyl-p-phenylenediamine (DMPD) into its radicals form depending on the oxidative stress level of the environment, and the color radical product (DMPD+) is determined colorimetrically. 20 µL of DMPD (100 mM) was added to 10 µl of hemolymph samples taken in 2 ml of acetate buffer (0.1 M, pH: 4.8) and incubated at 37°C for 75 minutes and DMPD+ formation was determined photometrically at 505 nm. The results are calculated from the calibration graph obtained using 2.52 mM $FeCl_2$ as standard and hydrogen peroxide (H_2O_2) solution prepared in PBS (Verde et al., 2002).

3.2.3. Procedures for histopathological examination

Immediately after the collection of hemolymph samples from mussel individuals subjected to pesticide exposure, all tissues of mussels were dissected for histological studies (Image 3.4). For rapid fixation and better preservation, they were placed in Davidson's solution (330 ml of 95% ethanol; 220 ml of formalin; 115 ml of glacial acetic acid; 335 ml of distilled water). They were kept in this fixative solution for 24 hours before being placed in a 70% ethanol solution.

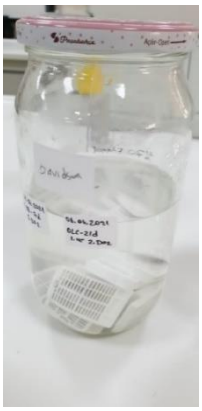


Image 3.4. Mussel tissue cassettes placed in Davidson's solution

The tissues were then exposed to a series of 70%, 80%, 90%, 96% absolute ethyl alcohols, in which they remained for 2 hours each, for dehydration. After dehydration, they were passed through xylol for 2 hours for alcohol removal and polishing. The tissue pieces were

embedded in special molds filled with liquid paraffin. Paraffin blocks were kept in the refrigerator until cutting. For histopathological examination, 5-6 microns thick sections were taken with a rotary ThermoShandon microtome. These sections were then placed on a heated slide in a 60°C water bath overnight. The slides were kept in an oven set at 50-60°C for 1 night to ensure that the paraffinic sections adhered well to the slide. The slides were then stained with Hematoxylin and Eosin according to the Presnell and Schreibman (1997) procedures and fixed with Entellan or Canada Balm (Luna, 1968). Tissue sections made into fixed preparations for histopathological evaluation were examined under a light microscope, Zeiss Primo Star, and illustrated with Zeiss camera Zen microscopy software. “The criterion of the histopathological alteration of the tissues was scored according to the intensity of the lesions as* (-) none (no histological alterations), which represents normal histological structure; (+) histopathology in > 20% of fields (mild); (++) histopathology in 20-60 % of fields (moderate) and (+++) histopathology in < 60% of fields (severe)” (Benli et al., 2008).

3.2.4. Biochemistry analysis methods

After the dissection of the mussels in order to evaluate the effects of pesticides on enzyme levels, the gills and digestive glands were wrapped separately in aluminum foil and placed in the liquid nitrogen container (Images 3.5-3.7). They were stored in a freezer at -85˚C until biochemical analysis.

Image 3.5. Collection of mussel target organs after opening

Image 3.6. Packing of gills (left) and digestive glands (right) in the folio

Image 3.7. Packages of mussel tissues to be preserved in liquid nitrogen

Superoxide dismutase (SOD)

The basis of the method is the elimination of the superoxide formed in the environment with the enzyme superoxide dismutase (SOD) and the formation of O_2 by xanthine oxidase (XO), which is based on the coloring of the remaining amount by staining, which in turn forms a colored compound with NBT (Sun et al., 1988; Karasu Benli et al., 2012). This color intensity measurement is made by the microplate equipment.

Superoxide dismutase activity assay (SOD): Around 100 mg of tissue samples (gills, digestive glands) were diluted in 900 µL buffer made of 1 mM EDTA, 210 mM Mannitol, and 70 mM sucrose at pH = 7.2.

Superoxide dismutase (SOD), activities were measured spectrophotometrically using commercial kits (Cayman Chemical, USA). The amount of tissue enzyme was determined

in a microplate reader using the Cayman SOD assay 706002 kit (Batch: 0628264, USA) for SOD activities with the results given as nmol/min/ml.

Catalase activity (CAT)

The method is based on the destruction of hydrogen peroxide (Johansson and Borg, 1988; Karasu Benli et al., 2012).

Determination of Catalase Activity (CAT): The method is based on the breakdown of hydrogen peroxide: Around 100 mg of tissue samples (gills, digestive glands) were diluted in 900 µL buffer made of 50 mM potassium phosphate, and 1 mM EDTA, pH 7.0.

Catalase (CAT) activities were measured spectrophotometrically using commercial kits (Cayman Chemical, USA). The amount of tissue enzyme was determined in a microplate reader using the Cayman KAT assay 707002 kit (Batch: 0625062, USA) for CAT activities with the results given as nmol/min/ml.

Glutathione peroxidase (GPx)

Glutathione Peroxidase Determination (GPx): This double enzyme method includes the reduction of hydrogen peroxide (H_2O_2) with GPx and the oxidation of NADPH to NADP with glutathione reductase. Enzyme activity is determined by the reduction in optical density of the reaction content as a result of the oxidation of NADPH to NADP at 37°C. In commercial kits, it indirectly measures the activity of GPx, which double reacts with glutathione reductase. Oxidized glutathione produced by hydroperoxide reduction by GPx is recovered as GR and NADPH in a reduced state. Around 100 mg of tissue samples (gills, digestive glands) were diluted in 900 microL buffer made of 50 mM Tris-HCL and 1 mM DTT at pH 7.5 with 5 mM EDTA. The method used is based on the one developed by Paglia & Valentine (1967) and Ursini et al. (1985). The homogenized tissue was centrifuged at 10,000 RPM for 15 min at +4°C for SOD, CAT, GPx samples. After centrifugation, the supernatant was collected and stored in Eppendorf tubes in a cooler until the next experiment or at -85°C for the next day's experiments.

Glutathione peroxidase (GPx) activities were measured spectrophotometrically using commercial kits (Cayman Chemical, USA). The amount of tissue enzyme was determined in a microplate reader using the Cayman GPx assay 703102 (Batch: 0628144, USA) for GPx activities with the results given as nmol/min/ml. Protein determination in tissues was studied using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

3.3. Statistical Analysis

The LC_{50} value was calculated with Finney's Probit Analysis using a computer program (CEAM, 1999) with a 95% confidence interval. Data were analyzed for normality and homogeneity of variance using the Shapiro-Wilks and Levene Test. Two-way ANOVA test was used to compare continuous variables among multiple outcome variables. The Bonferroni test was used for multiple comparisons between groups. The statistical threshold (significance) applied has been 0.05. The entire statistical analysis was carried out using the GraphPad Prism 9.5.0 software. Results are shown as mean ± standard error (SE).

4. RESULTS

4.1. Acute Toxicity of the Active Substances (Lambda-cyhalothrin and Acetamiprid) on the Freshwater Mussel *Unio terminalis*

During each of the experiments, mortality at the level of each aquarium was evaluated every 24 hours with the systematic removal of dead mussels (those whose shells are open with the inability for the individual to close them). Once removed, measurements are taken on each dead individual: weight, height, length, and width (average weight = 41.098 ± 1.655 g; average length = 5.34± 0.0987 cm; average height = 2.547 ± 0.028 cm; average width = 1.927 ± 0.049 cm).

The water's physicochemical characteristics meet the OECD's (Organization for Economic Co-operation and Development) requirements for the validity of toxicity testing (OECD, 1993). After the period of 96 hours of exposure, the acute toxicity of each pesticide was statistically determined using Probit software with a 95% confidence interval.

At the end of the 96 hours of testing, no dead mussels were counted in the control groups (water without toxicants and DMSO). The lethal concentrations (LC_{50}) of each pesticide are respectively 33.52 mg/L for acetamiprid and 1.1 mg/L for lambda-cyhalothrin. In both cases of pesticide tested, in comparison to the control and DMSO groups, the acute toxicity results revealed an increase in mortality with increasing concentrations of acetamiprid and lambda-cyhalothrin (Figure 4.1).

After 3 days of exposure, the lowest concentration of lambda-cyhalothrin causing mussel mortality was 0.1 mg/L resulting in a mortality rate of about 10% (with 1 mg/L, the mortality rate is 40%). While for the experiment with acetamiprid, the mortality rate noted was about 30% for a dose equivalent to 25 mg/L. This trend is even more noticeable in Figure 1, which illustrates the relationship between nominal pesticide concentrations (log10) and reported cumulative mortality rates. While low concentrations of lambda-cyhalothrin are already toxic to mussels (e.g., 0.5 mg/L for a 10% mortality rate), a 20-fold higher dosage of Acetamiprid was required to cause the same toxic effect (10 mg/L for a 10% mortality rate). Comparing the values of the two pesticides obtained after the 96-h toxicity tests, it is obvious

to conclude that lambda-cyhalothrin needs a low acute concentration to cause a toxic effect in this species and is therefore more toxic than acetamiprid for this organism.

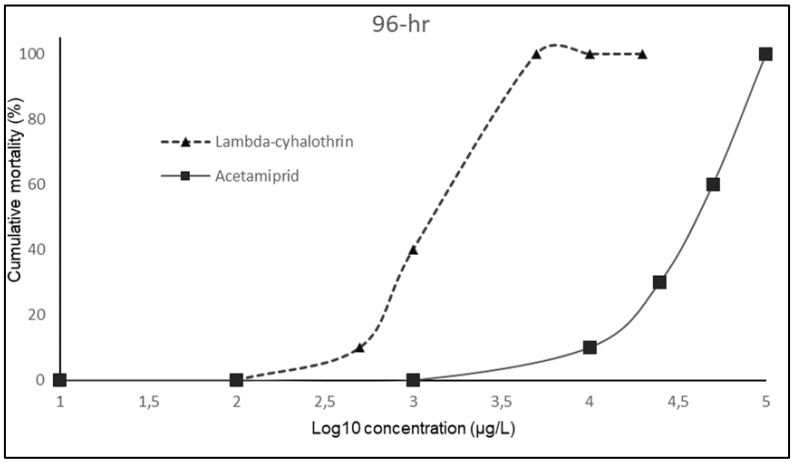


Figure 4.1. Relative mortalities (%) of the mussel versus pesticide concentration (96-h bioassay)

4.2. The Sublethal Impact of LCH on the Freshwater Mussel *Unio Terminalis*

4.2.1. Histology analysis

For examination and evaluation of the tissue preparations, the published research articles about histopathology of mussels in the open literature were used (Sunila, 1988; Arockia et al., 2012; Kumar et al., 2012; Lima et al., 2012; Moezzi et al., 2013; McElwain et al., 2014; Faggio et al., 2018).

Table 4.1. Histopathological alterations of the freshwater mussel (*Unio terminalis*) after exposure to LCH

Groups	Exposure time	Tissue/ Histopathology			
		Gills		Digestive glands	
		Deformations of the lamella	Hemocytic infiltration	Degenerations of the tubules	Tubule loss
Control	48h	-*	-	-	-
	7 d	-	-	-	-
	21 d	-	-	-	-
Control (DMSO)	48h	-	-	-	-
	7 d	-	-	-	-
	21 d	-	-	-	-
0.11 mg/L	48h	-	-	-	-
	7 d	+	+	+	+
	21 d	++	++	++	+
0.22 mg/L LCH	48h	-	-	++	-
	7 d	++	+	+++	++
	21 d	+++	++	+++	+++

* The histopathological alterations were scored as "(-) none (no histopathological alterations), which represents normal histological structure; (+) histopathology in > 20% of fields (mild); (++) histopathology in 20-60 % of fields (moderate), and (+++) histopathology in < 60% of fields (severe)

The histology of gill lamellas of the control groups' mussels shows a normal appearance, with well-preserved lamellae formed by a single layer of epithelial cells with a normal distribution of lateral frontal cilia (Image 4.1). When compared to control groups, exposure to lambda-cyhalothrin resulted with hemocytic infiltration (Image 4.2) and degeneration of the gill tissues after exposure to different durations. The control groups' digestive gland tissues were a normal appearance with tubules (Image 4.4). The LCH exposed mussel's digestive gland tissues exhibited severe degeneration (Image 4.4) and tubule loss of the digestive tubules (Image 4.5).

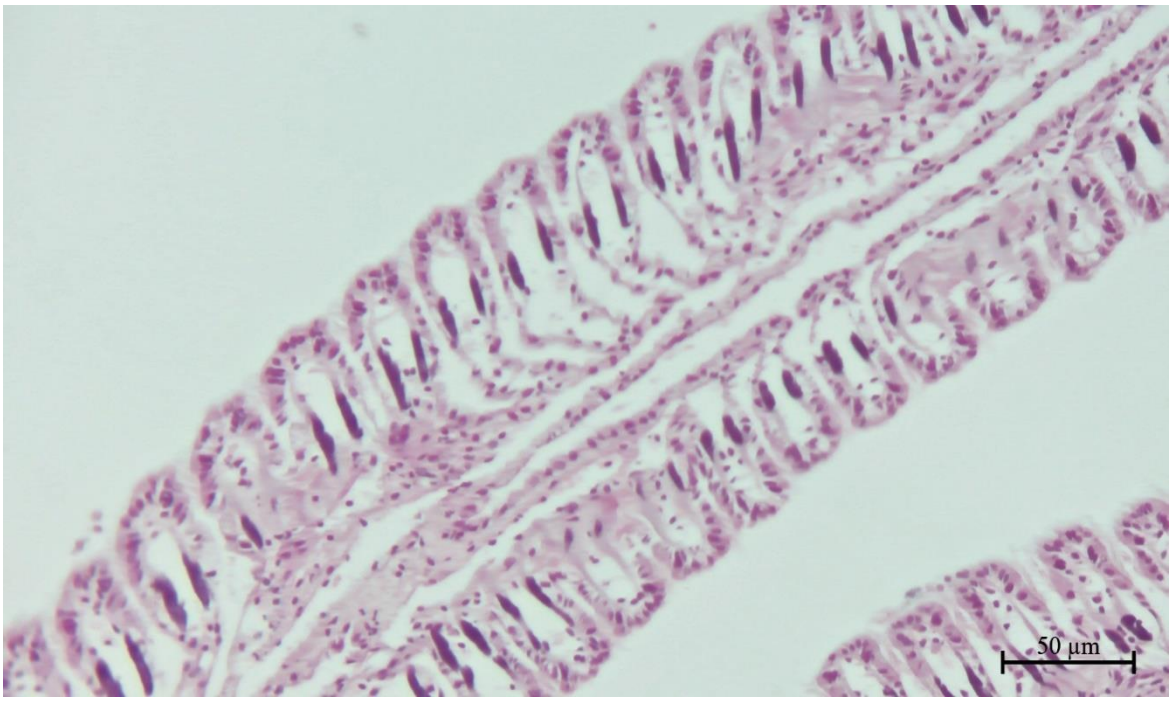


Image 4.1. Control group of gill tissues of *U. terminalis* (H&E)

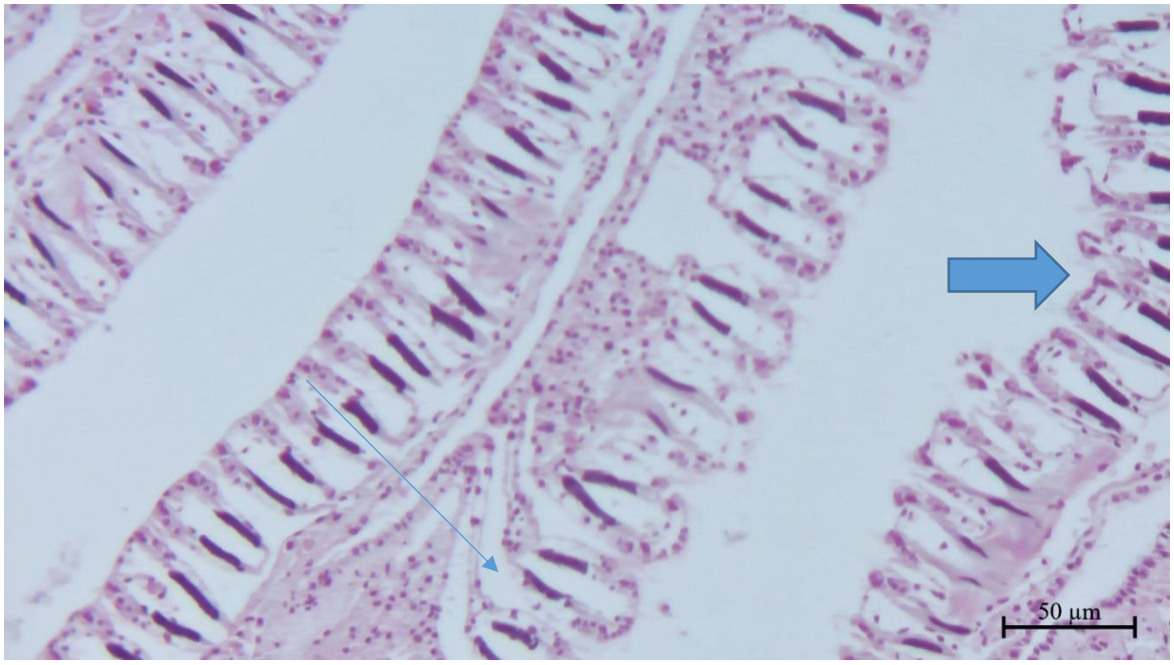


Image 4.2. Deformations of gill tissues after exposure to 0.22 mg/L LCH for 21-d (H&E)

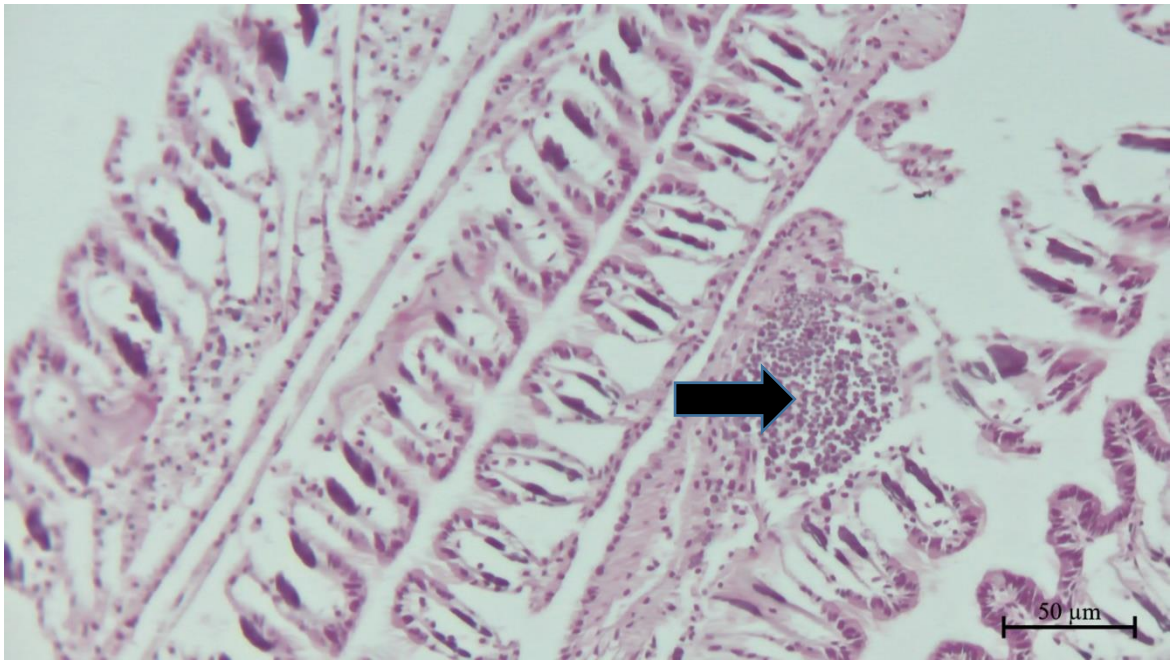


Image 4.3. Hemocytic infiltration (black arrow) of gill tissues after exposure to 0.22 mg/L LCH for 7-d (H&E)

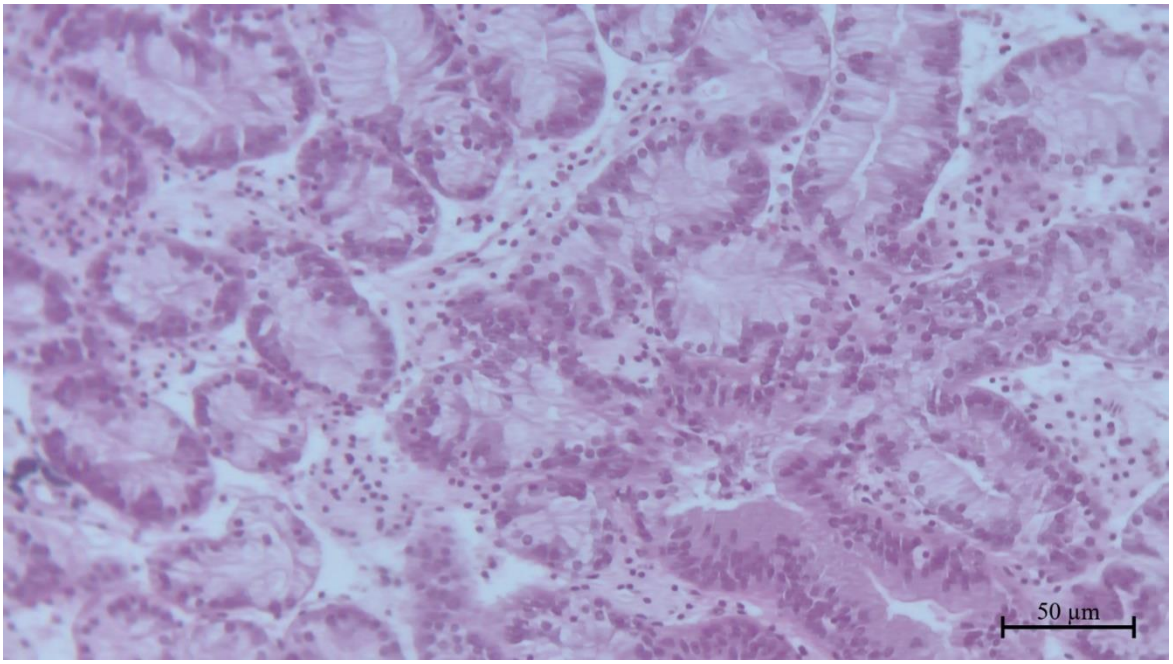


Image 4.4. Control group of digestive gland tissues of *U. terminalis* (H&E)

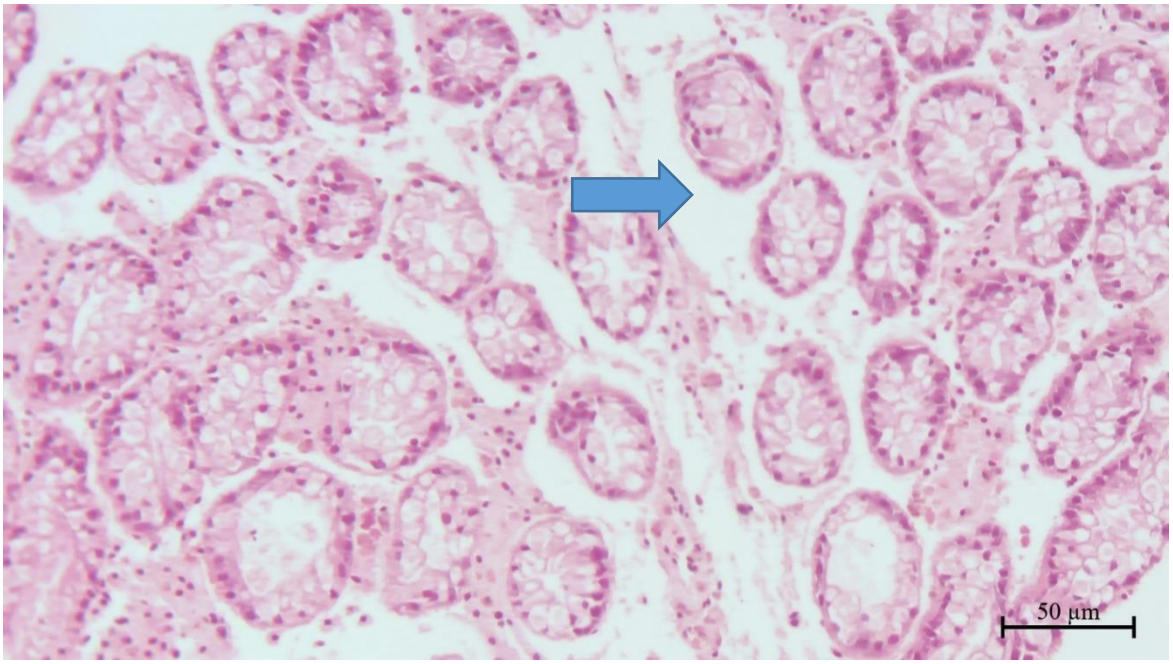


Image 4.5. Degeneration and loss of the tubules (blue arrow) after exposure to 0.22 mg/L LCH for 21-d (H&E)

4.2.2. Outcomes from hemolymph analysis

Total hemocyte counts results

As a result of the experiments, Total Hemocyte Counts of freshwater mussel *Unio terminalis* exposed to Lambda-cyhalothrin at three different time periods were performed. The results of Total Hemocyte Counts (THCs) are reported in Table 4.2 and presented in Figure 4.2.

Table 4.2. Total hemocyte counts (THCsx10^3/mL)

Pesticide	Trial Groups	Exposure time		
		48 hours	7 days	21 days
Lambda-cyhalothrin	Control	64.400±3.251a	59.600±2.414a	65.600±1.949a
	Control (DMSO)	63.667±2.940a	59.667±2.352a	57.933±1.706a
	0.11 mg/L	49.667±2.642b	44.800±1.184b	34.000±1.583c
	0.22 mg/L	41.533±2.077b	36.067±1.614c	35.067±1.933c

*Small letters (a,b,c) in the column refer to significance differences between different groups of the same exposure time: Same letter = no difference, different letter = difference ($p< 0.05$)

The graphical representation of the results obtained for the THCs values is given in the following figure 4.2.

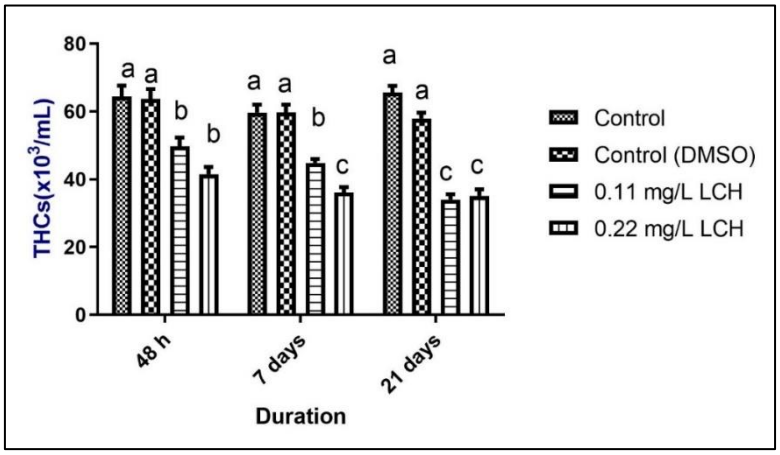


Figure 4.2. Total hemocyte counts (THCs) in *Unio terminalis* organisms after exposure to lambda-cyhalothrin (LCH)

After analysis of Table 4.2 and Figure 4.2, we notice that the THC values of *Unio terminalis* hemolymph exposed to 0.11 mg/L and 0.22 mg/L of LCH decreased after 48h, 7 days and

21 days of exposure compared to the control groups with a significant difference between the different groups ($p<0.05$). The statistical difference between the control groups was not significant considering all exposure times, while it was significant after 7 days of exposure between the two doses of LCH.

Total antioxidant status (TAS) levels

The TAS values (mmol/L) for freshwater mussels (*Unio terminalis*) exposed to 0.11 and 0.22 mg/L TCL for 48 hours, 7 and 21 days are reported in Table 4.3.

Table 4.3. Hemolymph TAS values in freshwater mussel (*Unio terminalis*) exposed to LCH

Pesticide (TAS)	Trial Groups	Exposure time		
		48 h	7 days	21 days
Lambda-cyhalothrin	Control	0.620±0.004a	0.624±0.013a	0.636±0.014a
	Control (DMSO)	0.607±0.007a	0.613±0.009a	0.621±0.028a
	0.11 mg/L	1.308±0.348c	0.859±0.199b	0.954±0.299b
	0.22 mg/L	1.274±0.159c	1.259±0.369c	0.995±0.241b

*Small letters (a,b,c) in the column refer to significance differences between different groups of the same exposure time: Same letter = no difference, different letter = difference ($p< 0.05$)

The graphical representation of the results obtained for the TAS values is given in Figure 4.3.

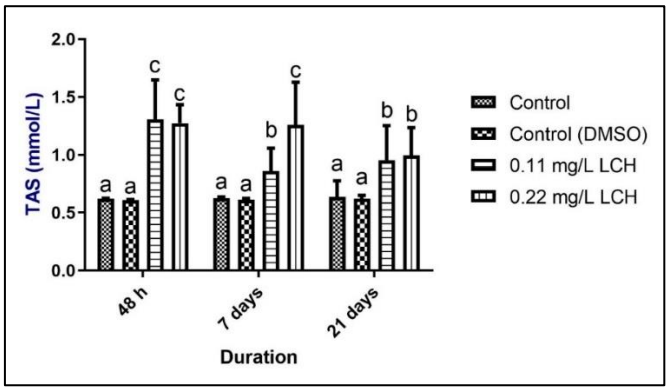


Figure 4.3. Total antioxidant status (TAS) levels in *Unio terminalis* organisms after exposure to lambda-cyhalothrin (LCH)

After analysis of Table 4.3 and figure 4.3, we notice that the TAS values of *Unio terminalis* organisms exposed to 0.11 mg/L and 0.22 mg/L of LCH increased after 48h, 7 days and 21 days of exposure compared to the control groups with a significant difference between the

different groups ($p<0.05$). The statistical difference between the control groups was not significant considering all the exposure times, while it was significant after 7 days of exposure between the two doses of LCH. The highest TAS value was obtained after 48 hours of exposure at a concentration of 0.11 mg/L: TAS (mmol/L) = 1.308±0.348.

Total oxidative stress (TOS) levels

The TOS values (mmol/L) for freshwater mussels (*Unio terminalis*) exposed to 0.11 mg/L and 0.22 mg/L LCH for 48 hours, 7 and 21 days are reported in Table 4.4.

Table 4.4. Hemolymph TOS values in freshwater mussel (*Unio terminalis*) exposed to LCH

Pesticide (TOS)	Trial Groups	Exposure time		
		48 h	7 days	21 days
Lambda-cyhalothrin	Control	2.288±0.187a*	2.129±0.228a	2.101±0.348a
	Control (DMSO)	2.015±0.271a	2.203±0.140a	2.169±0.038a
	0.11 mg/L	3.196±0.786b	3.422±0.729b	2.836±0.186b
	0.22 mg/L	3.029±0.531b	3.412±0.629b	3.158±0.289b

*Small letters (a,b,c) in the column refer to significance differences between different groups of the same exposure time: Same letter = no difference, different letter = difference ($p< 0.05$)

The graphical representation of the results obtained for the TOS values is given in Figure 4.4

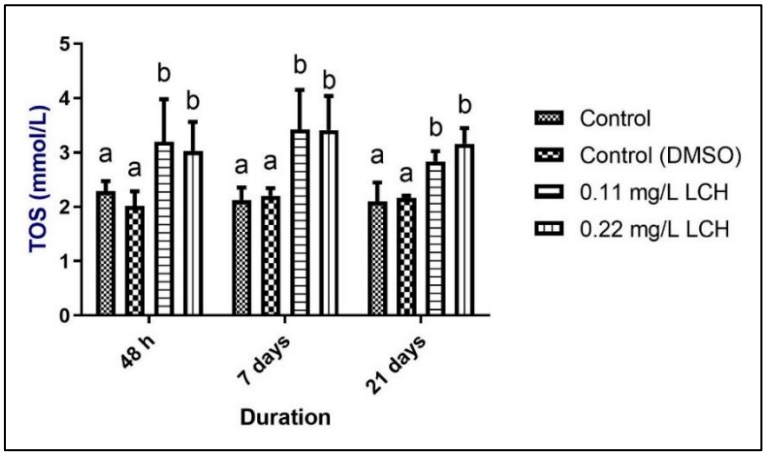


Figure 4.4. Total oxidative stress (TOS) levels in *Unio terminalis* organisms after exposure to lambda-cyhalothrin (LCH)

After analysis of Table 4.5 and Figure 4.4, we noticed that the TOS values of *Unio terminalis* organisms exposed to 0.11 mg/L and 0.22 mg/L of LCH increased after 48h, 7 days and 21 days of exposure compared to the control groups with a significant difference between the

different groups ($p<0.05$). The statistical difference between the control groups was not significant considering all exposure times. The highest TOS value was obtained after 7 days of exposure at the dose of 0.11 mg/L: TOS (mmol/L) = 3.422±0.729.

4.2.3. Outcomes from biochemical analysis

The changes in the levels of SOD, CAT and GPx enzymes in the digestive glands and gill tissues of the freshwater fish *Unio terminalis* exposed to different doses of Lambda-cyhalothrin were studied and the results obtained are reported in the following tables (Table 4.5-4.7)

Superoxide dismutase (SOD) activity

The changes in the levels of SOD enzymes in the digestive glands and gill tissues of the freshwater fish *Unio terminalis* exposed to different doses of lambda-cyhalothrin were studied and the results are displayed in Table 4.5.

Table 4.5. SOD activities (Mean±SE) in *Unio terminalis* organs exposed to lambda-cyhalothrin

Antioxidant parameters	Organs	Trial Groups	Exposure time		
			48 h	7 days	21 days
SOD (U/mg protein)	Gills	Control	183.293±31.073a	178.807±17.865a	183.518±30.715a
		Control (DMSO)	173.579±13.753a	165.938±11.036a	185.649±8.146a
		0.11 mg/L LCH	205.742±17.566a	185.814±21.222a	155.876±7.146b
		0.22 mg/L LCH	206.133±25.039a	189.950±23.037a	152.983±15.952b
	Digestive glands	Control	167.187±23.948a	168.051±18.833a	182.613±24.313a
		Control (DMSO)	173.566±26.610a	188.448±10.404a	173.128±22.725a
		0.11 mg/L LCH	421.876±83.895c	203.515±54.226b	162.402±25.277a
		0.22 mg/L LCH	484.259±96.231c	282.770±36.265b	156.739±18.995a

*Small letters (a,b,c) in the column refer to significance differences between different groups of the same exposure time: Same letter = no difference, different letter = difference ($p< 0.05$)

Gills

The graphical representation of the results obtained for the enzyme SOD activities in the gills of *Unio terminalis* is given in the following figure (Figure 4.5).

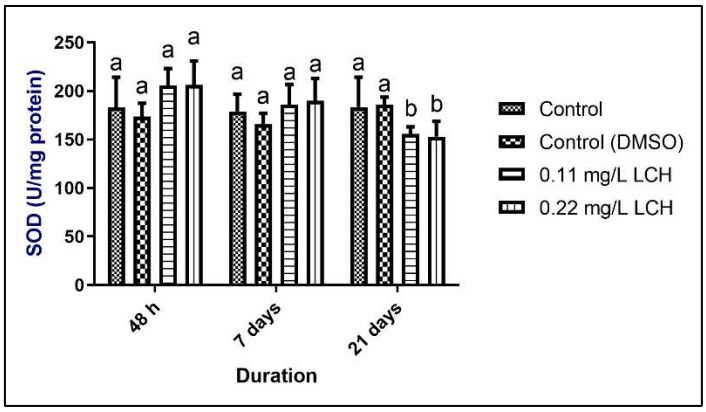


Figure 4.5. SOD activities in the gills of *Unio terminalis* after exposure to lambda-cyhalothrin (LCH)

From the analysis of the table and the graphical representation, it can be concluded that there is a significant stimulation and decrease of the SOD enzyme activity in the gills of *Unio terminalis* organisms exposed to 0.11 mg/L and 0.22 mg/L of lambda-cyhalothrin after 21 days of exposure. However, there was no significant difference between the SOD values obtained in the different groups after 48 hours and 7 days of exposure to LCH.

Digestive glands

The graphical representation of the results obtained for the enzyme SOD activities in the digestive glands of *Unio terminalis* is given in the following figure (Figure 4.6).

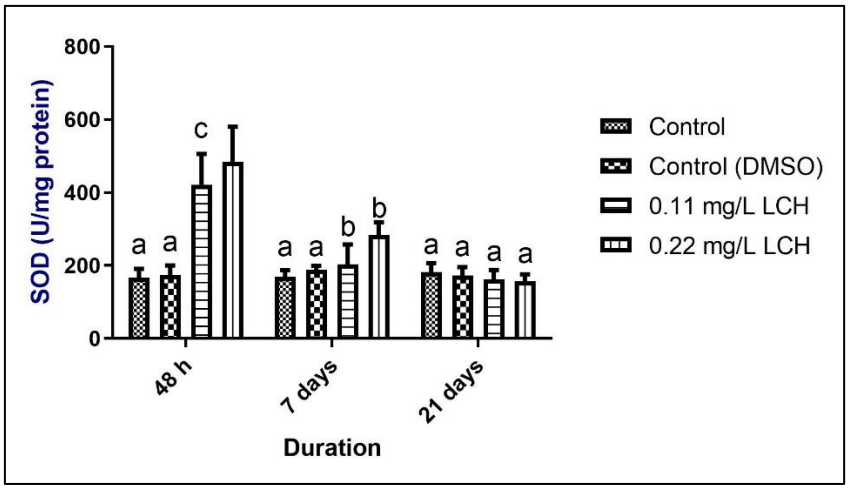


Figure 4.6. SOD activities in *Unio terminalis* organisms (digestive glands) after exposure to lambda-cyhalothrin (LCH)

From the analysis of the table and the graphical representation, it can be concluded that there are some stimulation and a significant increase of the SOD enzyme activity in the digestive glands of *Unio terminalis* organisms exposed to 0.11 mg/L and 0.22 mg/L of lambda-cyhalothrin after 48 hours and 7 days of exposure. However, there was no significant difference in SOD values between the two groups after 21 days of exposure to LCH.

Catalase (CAT) activity

The changes in the levels of CAT enzyme in the digestive glands and gill tissues of the freshwater fish *Unio terminalis* exposed to different doses of lambda-cyhalothrin were studied and the results obtained are reported in the following table (Table 4.6).

Table 4.6. CAT activities (Mean±SE) in *Unio terminalis* organs exposed to lambda-cyhalothrin

Antioxidant parameters	Organs	Trial Groups	Exposure time		
			48 h	7 days	21 days
CAT (mmol/min/ mg protein)	Gills	Control	14.088±1.344b	13.694±1.452b	14.869±2.098b
		Control (DMSO)	14.516±1.630b	14.224±0.880b	15.978±1.839b
		0.11 mg/L LCH	9.600±0.738a	14.018±2.071b	15.649±1.139b
		0.22 mg/L LCH	9.911±1.045a	15.602±1.641b	16.049±1.108b
	Digestive glands	Control	43.794±8.169b	48.198±6.323b	47.707±6.930b
		Control (DMSO)	45.126±8.620b	48.164±7.720b	45.679±7.835b
		0.11 mg/L LCH	26.298±0.047a	49.372±2.191b	49.973±8.442b
		0.22 mg/L LCH	22.389±2.679a	44.013±4.665b	58.015±12.729b

*Small letters (a,b,c) in the column refer to significance differences between different groups of the same exposure time: Same letter = no difference, different letter = difference ($p< 0.05$)

Gills

The graphical representation of the results obtained for the enzyme CAT activities in the gills of *Unio terminalis* is given in the following Figure (Figure 4.7).

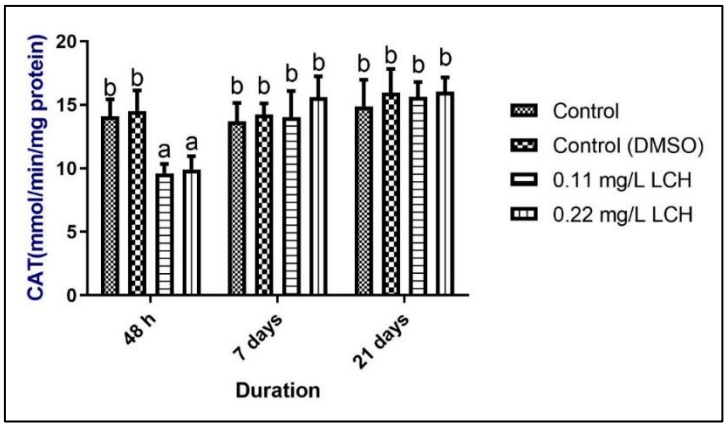


Figure 4.7. CAT activities in the gills of *Unio terminalis* after exposure to lambda-cyhalothrin (LCH)

After analysis of the data obtained, a significant decrease in CAT enzyme activities was noted after 48 hours of exposure to 0.11 mg/L and 0.22 mg/L of Acetamiprid compared to the control groups. Nevertheless, after 7 and 21 days of exposure to LCH doses, there was

no difference between the obtained CAT values, nor a real and differential stimulation of the enzyme in the gills of *Unio terminalis* organisms of the different groups submitted to the experiment.

Digestive glands

The graphical representation of the results obtained for the enzyme CAT activities in the digestive glands of *Unio terminalis* is given in the following Figure (Figure 4.8).

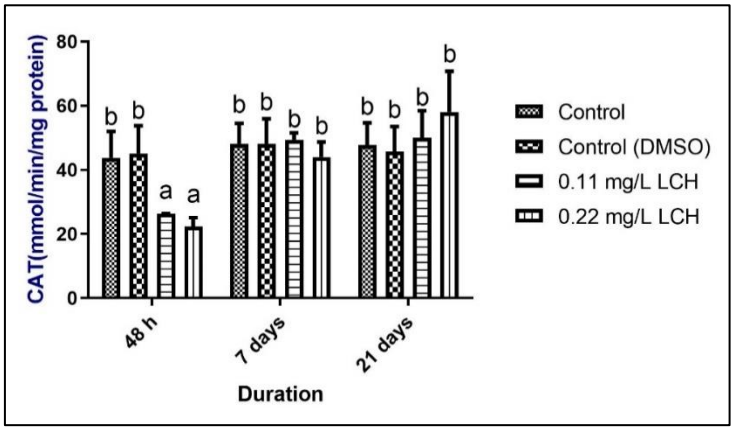


Figure 4.8. CAT activities in the digestive glands of *Unio terminalis* after exposure to Lambda-cyhalothrin (LCH)

After analysis of the data obtained, a significant decrease in CAT enzyme activities was noted after 48 hours of exposure to 0.11 mg/L and 0.22/L of Acetamiprid compared to the control groups ($p<0.05$). Nevertheless, after 7 and 21 days of exposure to LCH doses, there was no difference between the obtained CAT values, nor a real and differential stimulation of the enzyme in the gills of *Unio terminalis* organisms of the different groups submitted to the experiment.

Glutathione peroxidase (GPx) activity

The changes in the levels of GPx enzymes in the digestive glands and gill tissues of the freshwater fish *Unio terminalis* exposed to different doses of Lambda-cyhalothrin were studied and the results are displayed in the table below (Table 4.7).

Table 4.7. GPx activities (mean±SE) in *Unio terminalis* organs exposed to lambda-cyhalothrin

Antioxidant parameters	Organs	Trial Groups	Exposure time		
			48 h	7 days	21 days
GPx (nmol/min/mg protein)	Gills	Control	1.383±0.219a	1.546±0.267a	1.559±0.218a
		Control (DMSO)	1.387±0.295a	1.442±0.234a	1.491±0.181a
		0.11 mg/L LCH	2.462±0.958b	2.865±0.634b	2.589±1.01b
		0.22 mg/L LCH	2.324±0.646b	2.928±0.669b	2.756±0.571b
	Digestive glands	Control	0.549±0.224a	0.624±0.289a	0.744±0.058a
		Control (DMSO)	0.619±0.242a	0.673±0.229a	0.755±0.031a
		0.11 mg/L LCH	1.114±0.446b	1.327±0.211b	1.294±0.228b
		0.22 mg/L LCH	2.929±0.833c	3.368±1.528c	1.280±0.198b

*Small letters (a,b,c) in the column refer to significance differences between different groups of the same exposure time: Same letter = no difference, different letter = difference ($p< 0.05$)

Gills

The graphical representation of the results obtained for the enzyme GPx activities in the gills of *Unio terminalis* is given in Figure 4.9.

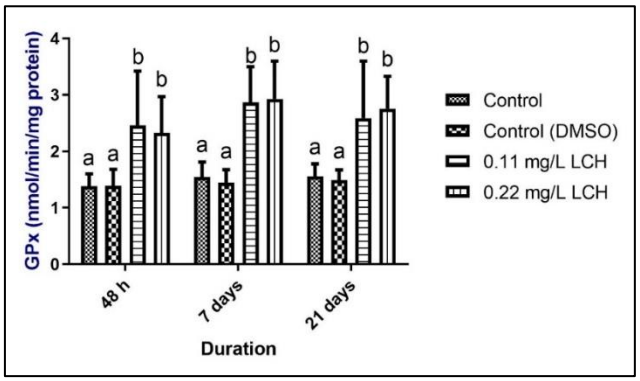


Figure 4.9. GPx activities in *Unio terminalis* organisms (Gills) after exposure to lambda-cyhalothrin (LCH)

After analysis of the data obtained, a significant increase in GPx enzyme activities in the gills of *Unio terminalis* was noted after 48 hours, 7 days and 21 days of exposure to 0.11 mg/L and 0.22 mg/L Lambda-cyhalothrin (LCH) compared to the control groups ($p<0.05$).

Digestive glands

The graphical representation of the results obtained for the enzyme GPx activities in the digestive glands of *Unio terminalis* is given in the following figure (Fig 4.10).

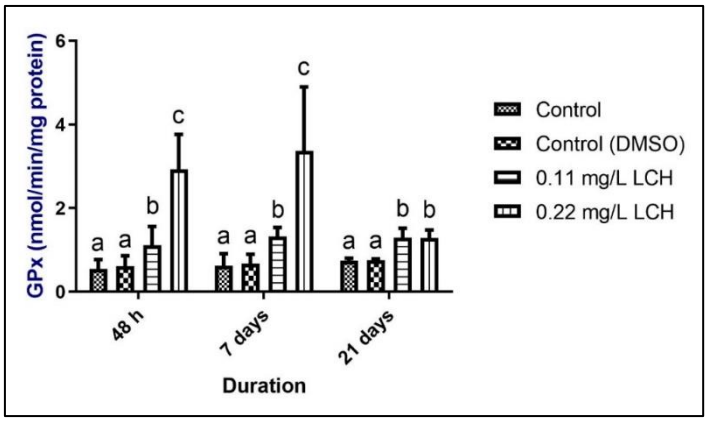


Figure 4.10. GPx activities in the digestive glands of *Unio terminalis* after exposure to lambda-cyhalothrin (LCH)

After analysis of the data obtained, a significant increase in GPx enzyme activities in the digestive glands of *Unio terminalis* was noted after 48 hours, 7 days and 21 days of exposure to 0.11 mg/L and 0.22 mg/L of Lambda-cyhalothrin (LCH) compared to the control groups ($p<0.05$) . A significant difference was also found between the two doses of LCH after 48 hours and 7 days of exposure ($p<0.05$).

4.3. The Sublethal Effects of Acetamiprid on the Freshwater Mussel *Unio Terminalis*

4.3.1. Histology analysis

For examination and evaluation of the tissue preparations, the published research articles about the histopathology of mussels in the open literature were used (Sunila, 1988; Arockia et al., 2012; Kumar et al., 2012; Lima et al., 2012; Moezzi et al., 2013; McElwain et al., 2014, Faggio et al., 2018). According to examination of the tissues mantle, foot, intestine, nephridium, gonads, and the adductor muscle did not reveal any histopathological alterations following exposure to sublethal acetamiprid when compared to control and DMSO control groups. The histopathological results of gills and digestive gland tissues of the freshwater mussel exposure to acetamiprid are summarized in Table 4.8.

Table 4.8. Histopathological alterations of the freshwater mussel (*Unio terminalis*) after exposure to acetamiprid

Groups	Exposure time	Tissue/ Histopathology				
		Gills		Digestive glands		
		Deformations of the lamella	Hemocytic infiltration	Degenerations of the tubules	Lipofuscin aggregates	Tubule loss
Control	48h	-*	-	-	-	-
	7 d	-	-	-	-	-
	21 d	-	-	-	-	-
Control (DMSO)	48h	-	-	-	-	-
	7 d	-	-	-	-	-
	21 d	-	-	-	-	-
3.35 mg/L ACE	48h	-	-	-	-	-
	7 d	-	-	+	+	-
	21 d	-	-	++	+	-
6.70 mg/L ACE	48h	-	-	++	+	-
	7 d	++	+	++	++	++
	21 d	++	+++	+++	++	+++

* The histopathological alterations were scored as "(-) none (no histopathological alterations), which represents normal histological structure; (+) histopathology in > 20% of fields (mild); (++) histopathology in 20-60 % of fields (moderate), and (+++) histopathology in < 60% of fields (severe)"

The histology of the gill lamellas of the control groups' mussels shows a normal appearance, with well-preserved lamellae formed by a single layer of epithelial cells with a normal distribution of lateral frontal cilia (Image 4.6). When compared to control groups, exposure to acetamiprid resulted with lipofuscin aggregates (Image 4.7) and degeneration (Image 4.8) of the gill tissues after exposure to different durations. The control groups' digestive gland tissues were normal appearance with tubules (Image 4.9). The ACE exposed mussel's digestive gland tissues exhibited severe degeneration (Image 4.10), lipofuscin aggregates and tubule loss of the digestive tubules (Image 4.11).

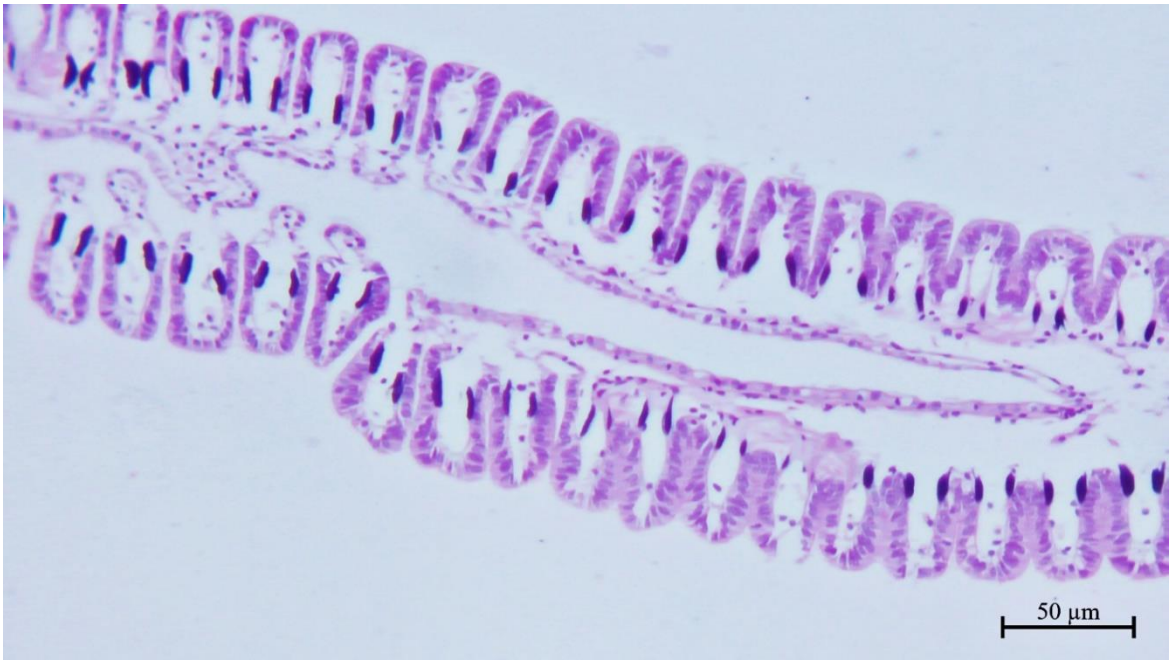


Image 4.6. Control group of gill tissues of *Unio terminalis* (H&E)

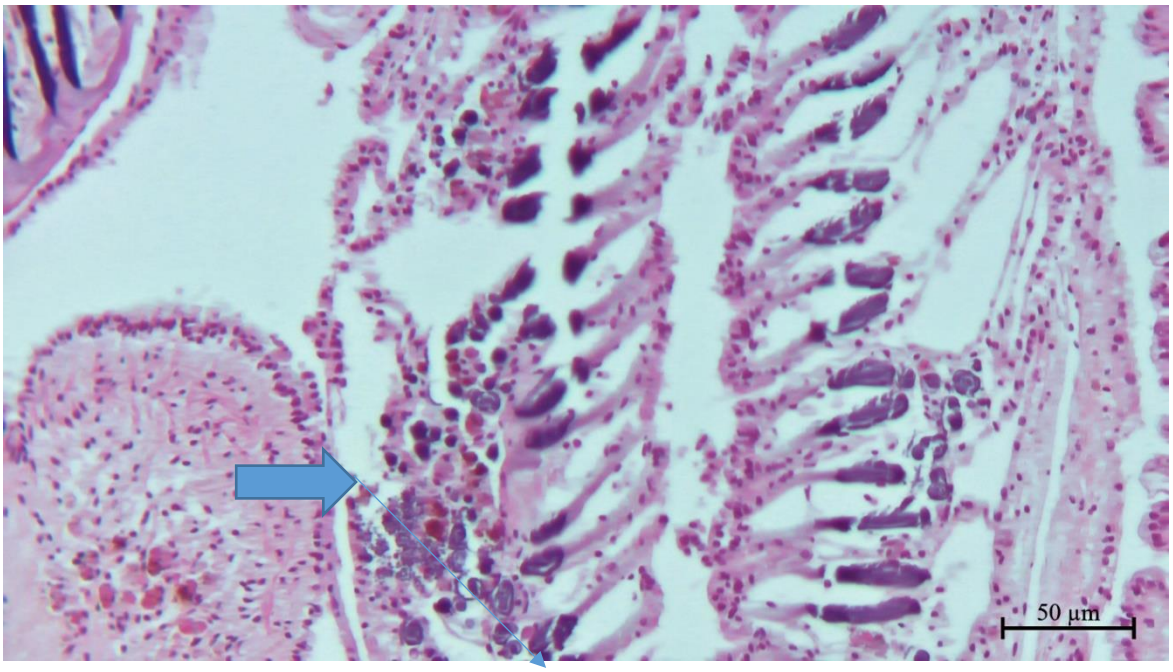


Image 4.7. Lipofuscin aggregates of gill tissues after exposure to 6.65 mg/L ACE for 21-d (H&E)

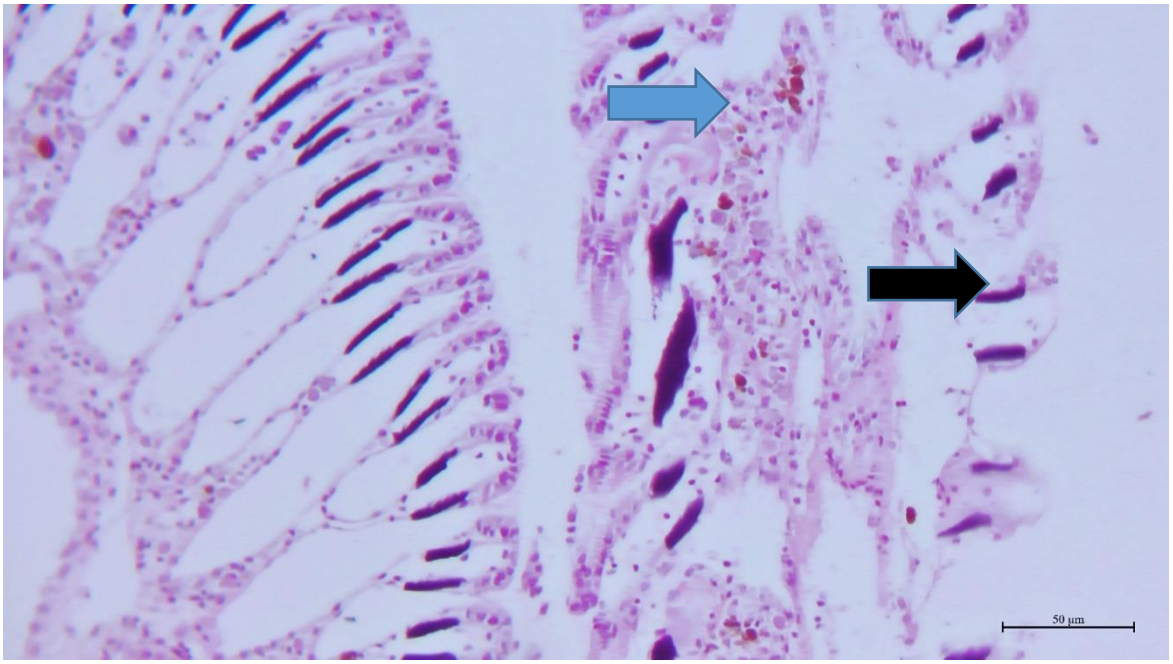


Image 4.8. Degeneration (black arrow) and lipofuscin aggregates (blue arrow) of gill tissues after exposure to 6.65 mg/L ACE for 7-d (H&E)

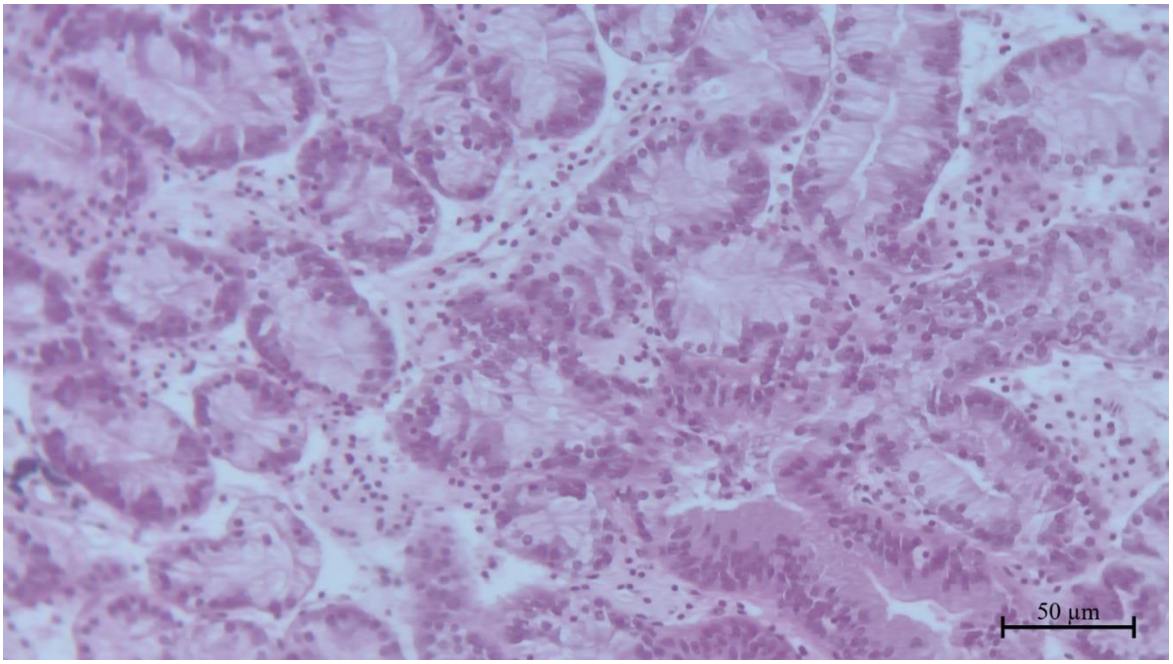


Image 4.9. Control group of digestive gland tissues of *Unio terminalis* (H&E)

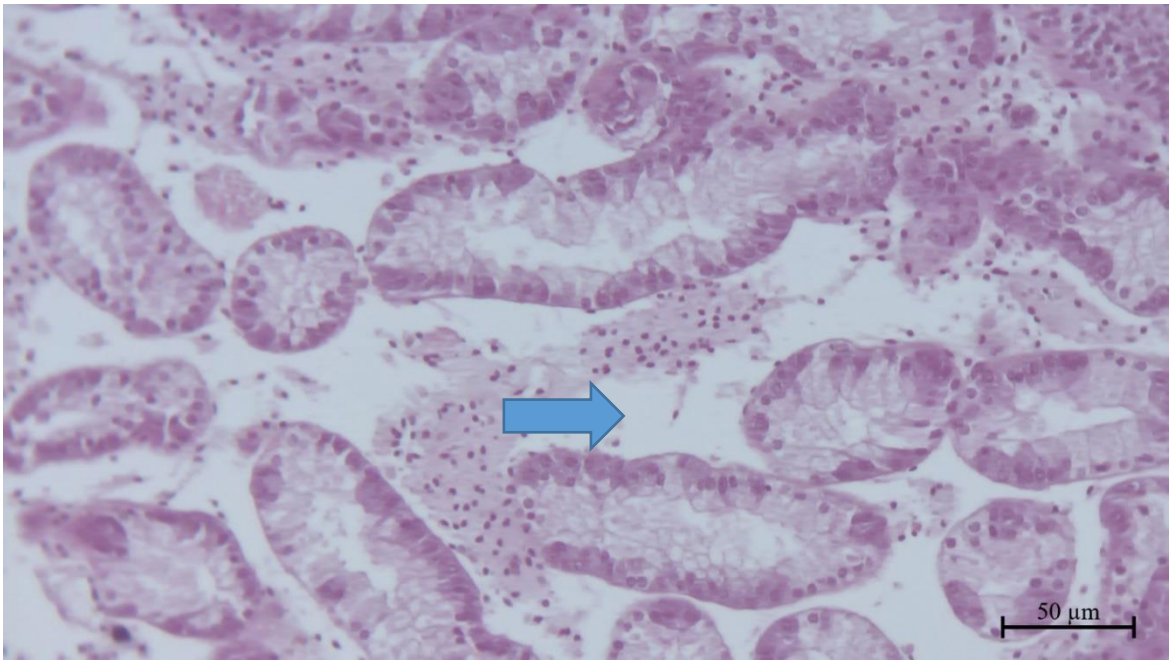


Image 4.10. Degeneration and loss of the tubules (blue arrow) after exposure to 6.65 mg/L ACE for 21-d (H&E)

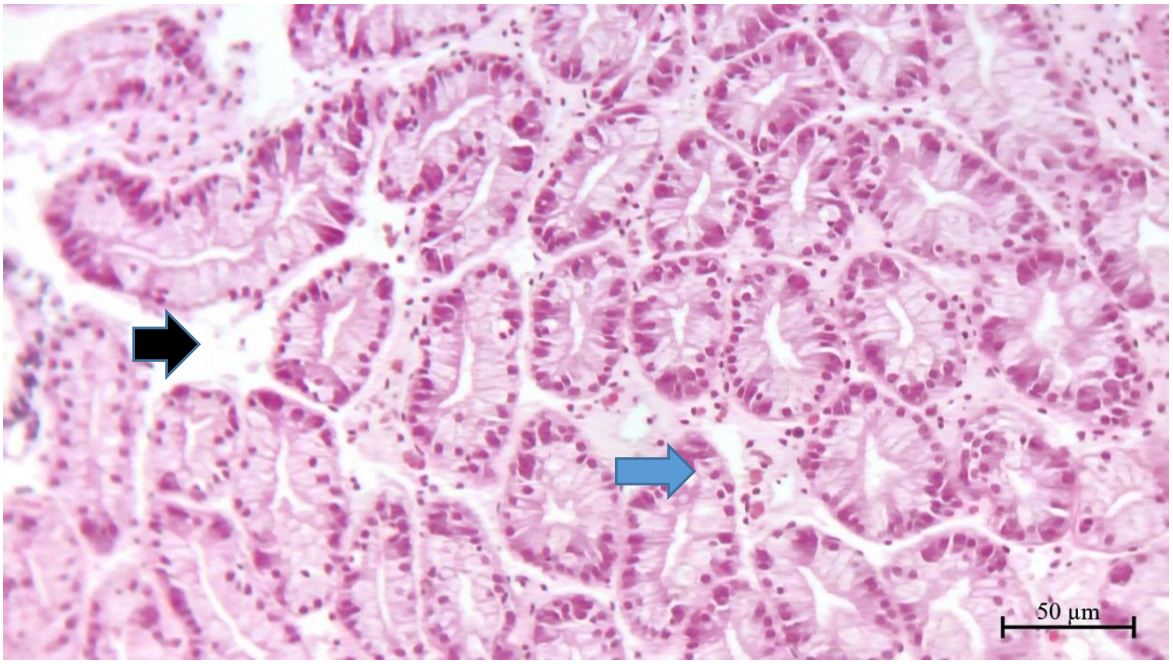


Image 4.11. Loss of the tubules (black arrow) and lipofuscin aggregates (blue arrow) after exposure to 6.65 mg/L ACE for 7-d (H&E)

4.3.2. Findings related to hemolemph analysis

Total hemocyte counts (THCs)

As a result of the experiments, Total Hemocyte Counts of freshwater mussel *Unio terminalis* exposed to Acetamiprid at three different time periods were performed. The results of Total Hemocyte Counts (THCs) are reported in Table 4.9 and presented in Figure 4.11.

Table 4.9. Total hemocyte counts (THCsx10^3/mL)

Pesticide	Trial Groups	Exposure time		
		48 h	7 days	21 days
Acetamiprid	Control	67.933±2.497a	62.133±2.463a	66.000±2.261a
	Control (DMSO)	67.000±2.513a	62.867±2.175a	60.733±2.273a
	3.35 mg/L	54.533±2.257b	47.667±1.594b	42.800±1.730b
	6.70 mg/L	44.133±1.913b	41.467±1.597b	37.533±2.102c

*Small letters (a,b,c) in the column refer to significance differences between different groups of the same exposure time: Same letter = no difference, different letter = difference ($p< 0.05$)

The graphical representation of the results obtained for the THCs values is given in the following figure (Figure 4.11).

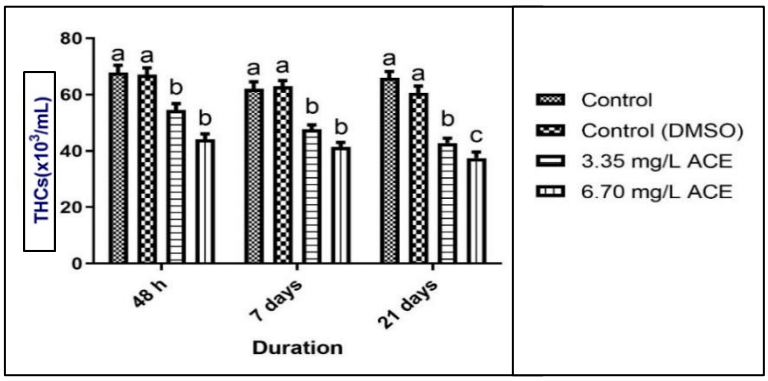


Figure 4.11. Total hemocyte counts (THCs) in *Unio terminalis* organisms after exposure to acetamiprid

After analysis of Table 4.10 and Figure 4.11, we notice that the THC values of *Unio terminalis* hemolymph exposed to 3.35 mg/L and 6.70 mg/L of ACE decreased after 48h, 7

days and 21 days of exposure compared to the control groups with a significant difference between the different groups ($p<0.05$). The statistical difference between the control groups was not significant considering all the exposure times, while it was significant after 21 days of exposure between the two doses of ACE.

Total antioxidant status (TAS) levels

The TAS values (mmol/L) for freshwater mussels (*Unio terminalis*) exposed to 3.35 mg/L and 6.70 mg/L ACT for 48 hours, 7 and 21 days are reported in Table 4.10 and shown in Figure 4.12.

Table 4.10. Hemolymph TAS values in freshwater mussel (*Unio terminalis*) exposed to ACE.

Pesticide (TAS)	Trial Groups	Exposure time		
		48 h	7 days	21 days
Acetamiprid	Control	0.621±0.005	0.629±0.013	0.635±0.015
	Control (DMSO)	0.608±0.008	0.609±0.009	0.620±0.027
	3.35 mg/L	0.618±0.027	0.605±0.005	0.606±0.010
	6.70 mg/L	0.617±0.068	0.610±0.006	0.639±0.019

The graphical representation of the results obtained for the TAS values is given in the Figure 4.12.

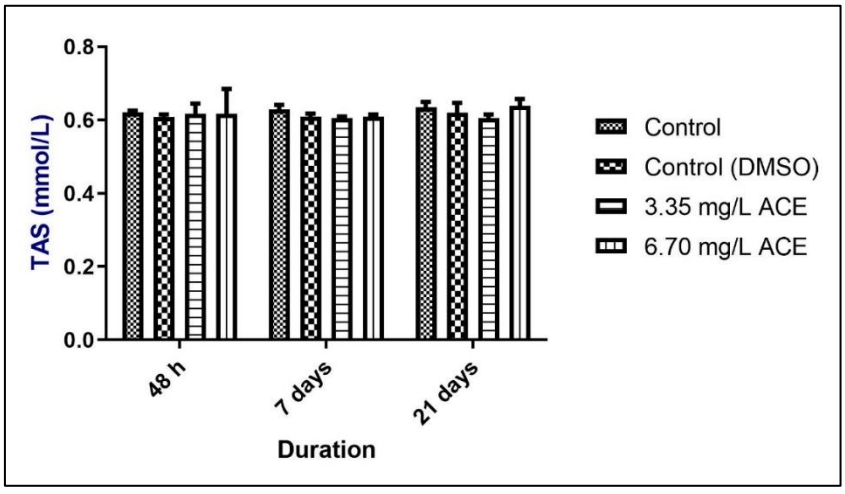


Figure 4.12. Total antioxidant status (TAS) levels in Unio terminalis organisms after exposure to acetamiprid

According to Table 4.10 and Figure 4.12, the TAS values of the freshwater *Unio terminalis* organisms exposed to 3.35 mg/L and 6.70 mg/L of ACE for 48 hours, 7 and 21 days are not significantly different compared to the control groups ($p>0.05$).

Total oxidative stress (TOS) levels

The TOS values (mmol/L) for freshwater mussels (*Unio terminalis*) exposed to 3.35 mg/L and 6.70 mg/L ACE for 48 hours, 7 and 21 days are reported in Table 4.11 and Figure 4.13.

Table 4.11. Hemolymph TOS values in freshwater mussel (*Unio terminalis*) exposed to ACE

Pesticide (TOS)	Trial Groups	Exposure time		
		48 h	7 days	21 days
Acetamiprid	Control	2.363±0.209	2.129±0.227	2.055±0.429
	Control (DMSO)	2.036±0.331	2.203±0.139	2.236±0.089
	3.35 mg/L	2.291±0.529	2.636±0.369	3.045±0.239b
	6.70 mg/L	2.091±0.505	2.681±0.531	3.636±0.459b

*Small letter (b) in the column refer to significance differences between different groups of the same exposure time: Same letter = no difference, different letter = difference ($p< 0.05$)

The graphical representation of the results obtained for the TOS values is given in the following figure (Figure 4.13).

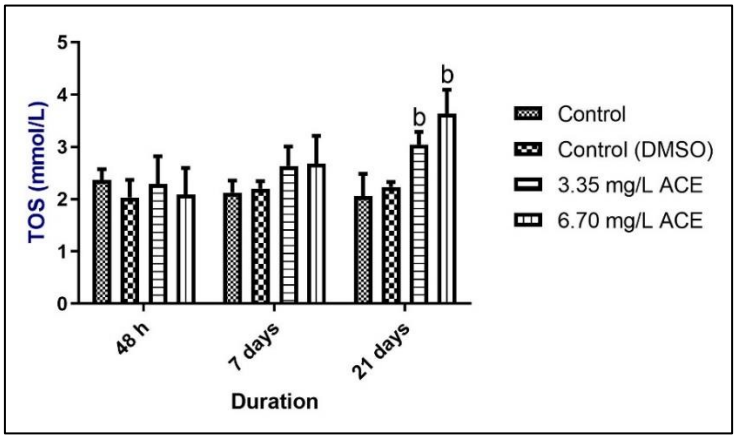


Figure 4.13. Total oxidative stress levels in *Unio terminalis* organisms after exposure to acetamiprid (ACE)

According to Table 4.11 and Figure 4.13, the TOS values of the freshwater *Unio terminalis* organisms exposed to 3.35 mg/L and 6.70 mg/L of ACE for 48 hours, 7 days are not

significantly different compared to the control groups (p>0.05). However, after 21 days of exposure, there is an increase in TOS values with a significant difference between the groups exposed to different doses of ACE and the control. The highest TOS value was obtained after 21 days of exposure at a concentration of 6.70 mg/L: TOS (mmol/L) = 3.636±0.459

4.3.3. Findings related to biochemical analysis

The changes in the levels of SOD, CAT and GPx enzymes in the digestive glands and gill tissues of the freshwater fish *Unio terminalis* exposed to different doses of Acetamiprid were studied.

Superoxide dismutase (SOD) activity

The changes in the levels of SOD enzyme in the digestive glands and gill tissues of the freshwater fish *Unio terminalis* exposed to different doses of Acetamiprid were studied and the results obtained are reported in the following table (Table 4.12).

Table 4.12. SOD enzyme activities (X±SE) in *Unio terminalis* organs exposed to acetamiprid

Antioxidant parameter	Organs	Trial Groups	Exposure time		
			48 h	7 days	21 days
SOD (U/mg protein)	Gills	Control	175.032±22.611a	185.546±19.576a	183.519±30.715a
		Control (DMSO)	184.292±15.129a	170.077±12.461a	191.067±13.641a
		3.35 mg/L ACE	367.519±33.109c	266.069±39.985b	161.498±16.393a
		6.70 mg/L ACE	356.418±55.778c	284.651±56.590b	172.099±19.971a
	Digestive glands	Control	183.853±25.823a	188.051±35.238a	187.340±29.209a
		Control (DMSO)	190.232±29.362a	182.594±10.534a	175.959±27.615a
		3.35 mg/L ACE	462.629±76.053b	196.696±36.997a	187.580±40.323a
		6.70 mg/L ACE	454.570±93.524b	199.695±50.707a	182.723±24.949a

*Small letters (a,b,c) in the column refer to significance differences between different groups of the same exposure time: Same letter = no difference, different letter = difference ($p< 0.05$)

Gills

The graphical representation of the results obtained for the enzyme SOD activities in the gills of *Unio terminalis* is given in the following figure (Figure 4.14).

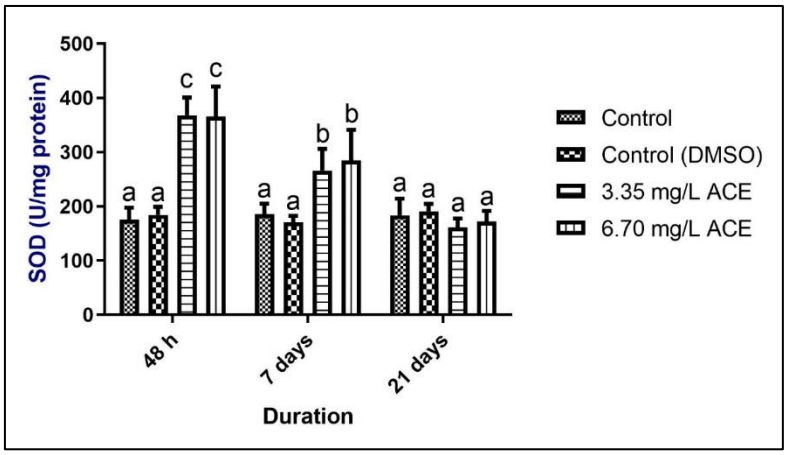


Figure 4.14. SOD activities in the gills of *Unio terminalis* after exposure to Acetamiprid

From the analysis of the table and the graphical representation, it can be concluded that there was some stimulation and a significant increase of the SOD enzyme activity in the gills of *Unio terminalis* organisms exposed to 3.35 mg/L and 6.70 mg/L of Acetamiprid after 48 hours and 7 days of exposure ($p<0.05$). However, there was no significant difference between the SOD values obtained in the different groups after 21 days of exposure to ACE.

Digestive glands

The graphical representation of the results obtained for the SOD enzyme activities in the digestive glans of *Unio terminalis* is given in Figure 4.15.

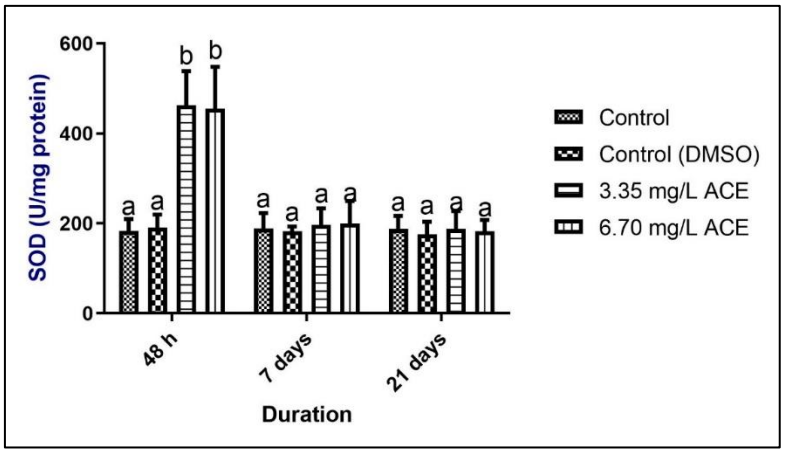


Figure 4.15. SOD activities in the digestive glands of *Unio terminalis* organisms after exposure to acetamiprid (ACE)

After the analysis of the data obtained, it is noted a significant increase of the the SOD enzyme activities after 48h of exposure to the doses 3.35 mg/L and 6.70 mg/L of Acetamiprid compared to the control groups ($p<0.05$). Nevertheless, after 7 and 21 days of exposure to ACE doses, there was neither difference between the obtained SOD values, nor a real and differential stimulation of the enzyme in the digestive glands of *Unio terminalis* organisms of the different groups submitted to the experiment.

Catalase (CAT) activity

The changes in the levels of CAT enzyme in the digestive glands and gill tissues of the freshwater fish *Unio terminalis* exposed to different doses of acetamiprid were studied and the results are displayed in the Table below (Table 4.13).

Table 4.13. CAT activities (mean±SD) in *Unio terminalis* organs exposed to acetamiprid

Antioxidant parameter	Organs	Trial Groups	Exposure time		
			48 h	7 days	21 days
CAT (mmol/min/ mg protein)	Gills	Control	13.591±1.478	13.367±1.229	14.537±2.012
		Control (DMSO)	14.010±1.896	13.912±0.783	15.327±1.627
		3.35 mg/L ACE	14.010±1.896	14.943±1.707	16.585±1.296
		6.70 mg/L ACE	12.512±1.653	16.401±1.312	15.025±1.013
	Digestive glands	Control	45.460±9.698	48.425±7.739	47.834±8.486
		Control (DMSO)	47.126±8.750	48.383±9.452	45.400±9.590
		3.35 mg/L ACE	34.640±4.594	54.346±7.817	44.452±13.284
		6.70 mg/L ACE	38.509±8.200	55.880±3.771	48.799±5.169

Gills

The graphical representation of the results obtained for the enzyme CAT activities in the gills of *Unio terminalis* is given in the following figure (Figure 4.16).

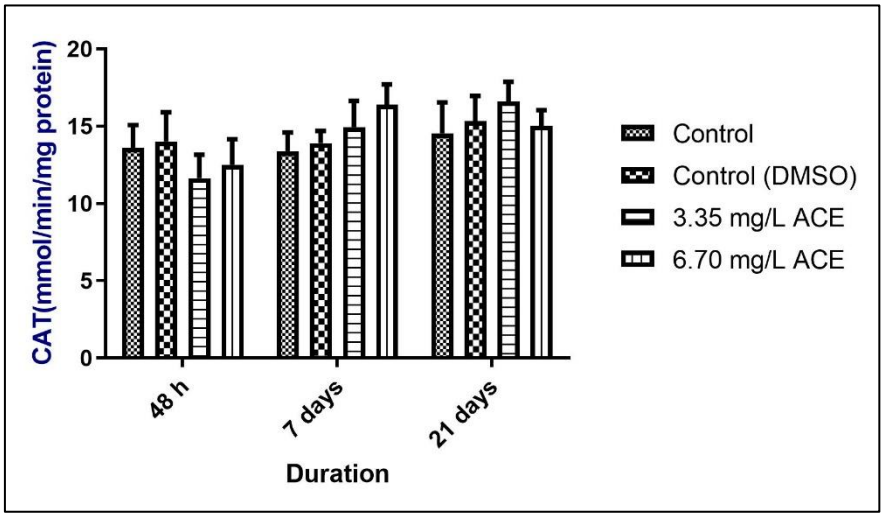


Figure 4.16. CAT activities in the gills of *Unio terminalis* after exposure to acetamiprid

After analysis of the results, it is evident to conclude that there is no significant difference between the CAT values obtained in the different groups after 48 hours, 7 days and 21 days of exposure to the concentrations of 3.35 mg/L and 6.70 mg/L of Acetamiprid.

Digestive glands

The graphical representation of the results obtained for the enzyme CAT activities in the digestive glands of *Unio terminalis* is given in the following figure (Figure 4.17).

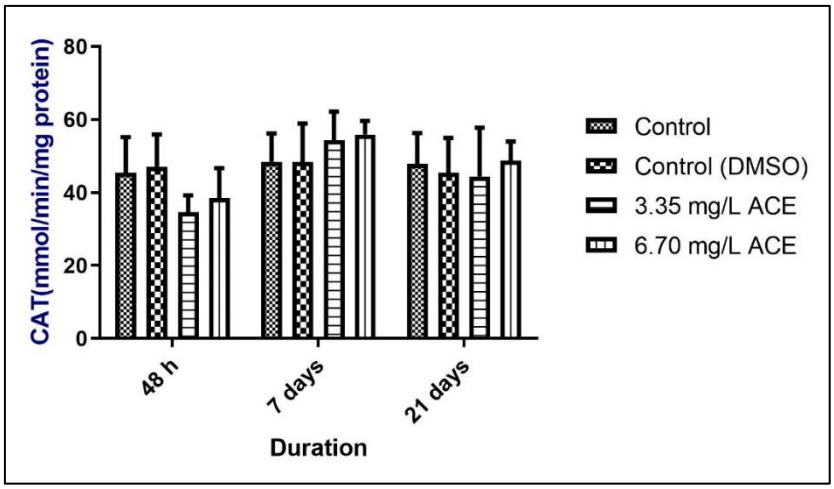


Figure 4.17. CAT activities in the digestive glands of *Unio terminalis* after exposure to acetamiprid

After analysis of the results, it is evident to conclude that there is no significant difference between the CAT values obtained in the different groups after 48 hours, 7 days and 21 days of exposure to the concentrations of 3.35 mg/L and 6.70 mg/L of Acetamiprid.

Glutathione peroxidase (GPx) activity

The changes in the levels of GPx enzyme in the digestive glands and gill tissues of the freshwater fish *Unio terminalis* exposed to different doses of Acetamiprid were studied and the results are displayed in Table 4.14.

Table 4.14. GPx activities (mean±SE) in *Unio terminalis* organs exposed to acetamiprid (ACE)

Antioxidant parameters	Organs	Trial Groups	Exposure time		
			48 h	7 days	21 days
GPx (nmol/min/mg protein)	Gills	Control	1.373±0.045a	1.289±0.089a	1.359±0.098a
		Control (DMSO)	1.394±0.040a	1.242±0.071a	1.491±0.181a
		3.35 mg/L ACE	1.424±0.800a	2.263±0.201b	2.203±0.429b
		6.70 mg/L ACE	1.322±0.206a	2.151±0.383b	2.186±0.439b
	Digestive glands	Control	0.642±0.282a	0.633±0207a	0.723±0.053a
		Control (DMSO)	0.667±0.236a	0.673±0.229a	0.761±0.027a
		3.35 mg/L ACE	1.634±0.281b	1.513±0.598b	1.460±0.251b
		6.70 mg/L ACE	1.552±0.554b	1.515±0.614b	1.589±0.301b

*Small letters (a,b) in the column refer to significance differences between different groups of the same exposure time: Same letter = no difference, different letter = difference ($p< 0.05$)

Gills

The graphical representation of the results obtained for the enzyme GPx activities in the gills of *Unio terminalis* is given in the following figure (Figure 4.18).

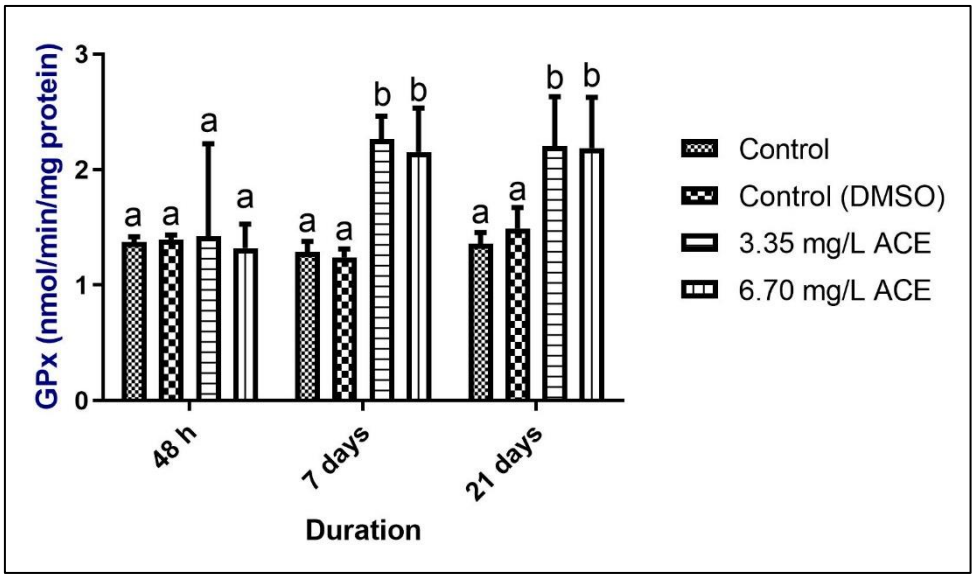


Figure 4.18. GPx activities in the gills of *Unio terminalis* after exposure to acetamiprid

After analysis of the data obtained, a significant increase in GPx enzyme activities in the gills of *Unio terminalis* was noted after 7 days and 21 days of exposure to 3.35 mg/L and 6.70 mg/L of Acetamiprid compared to the control groups ($p<0.05$). Nevertheless, after 48 hours of exposure to ACE doses, there was neither difference between the GPx values obtained, nor a real and differential stimulation of the enzyme in the digestive glands of *Unio terminalis* organisms from the different groups submitted to the experiment.

Digestive glands

The graphical representation of the results obtained for the enzyme GPx activities in the digestive glands of *Unio terminalis* is given in the figure below (Figure 4.19).

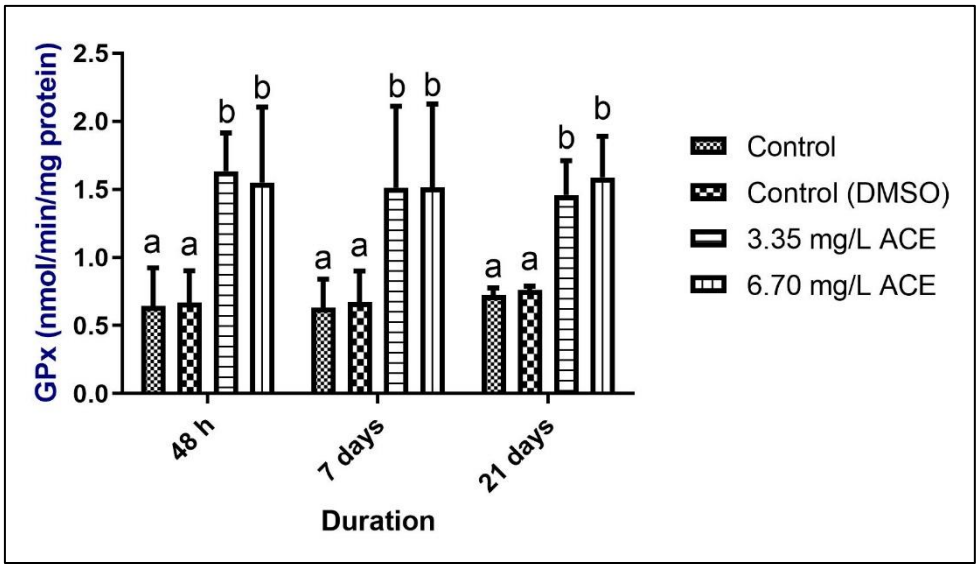


Figure 4.19. GPx activities in the digestive glands of *Unio terminalis* after exposure to acetamiprid (ACE)

After analysis of the data obtained, we can conclude that there is a significant increase in GPx enzyme activities in the digestive glands of *Unio terminalis* organisms after two days, 7 days and 21 days of exposure to 3.35 mg/L and 6.70 mg/L of Acetamiprid compared to the control groups.

Out of the different findings, we can retain the important points summarized in tables 4.15 and 46.

Table 4.15. Summary of acute and chronic toxicity effects of lambda-cyhalothrin on *Unio terminalis* mussels

<table>
<tr><td rowspan="3">Acute Toxicity (96h-LC_{50})</td><td colspan="4">Chronic toxicity</td></tr>
<tr><td rowspan="2">Experimental conditions</td><td rowspan="2">Exposure time</td><td colspan="2">Biomarkers</td></tr>
<tr><td>Hemolynph</td><td>Histology</td></tr>
<tr><td rowspan="3">1.1 mg/L</td><td rowspan="3">*Exposition group*:
-Freshwater (no chemicals added)
-Dimethylsulfoxide (DMSO)
-Lambda-cyhalothrin (0.11 mg/L and 0.22 mg/L)

Test organism:
Unio terminalis</td><td rowspan="3">48 hours
7 days
21 days</td><td>- Significant decrease of THC values
- Significant increase of TOS values
- Significant increase of TAS values</td><td>-Deformations of gills tissues (after exposure to 0.22 mg/L of LCH for 21 days)
-Hemocytic infiltration (after exposure to 0.22 mg/L of LCH for 7 days)
-Degeneration and loss of the tubules (after exposure to 0.22 mg/L of LCH for 21days</td></tr>
<tr><td colspan="2">Antioxidant (SOD, CAT, GPx)</td></tr>
<tr><td colspan="2">A significant decrease of the SOD enzyme activity exposed to 0.11 mg/L and 0.22 mg/L of after 21 days of exposure
A significant increase of the SOD enzyme activity in the digestive glands exposed to 0.11 mg/L and 0.22 mg/L after 48 hours and 7 days of exposure

A significant decrease in CAT enzyme activities was noted after 48 hours of exposure to 0.11 mg/L and 0.22 mg/L

A significant decrease in CAT enzyme activities was noted after 48 hours of exposure to 0.11 mg/L and 0.22/L

A significant increase in GPx enzyme activities in the gills after 48 hours, 7 days and 21 days of exposure to 0.11 mg/L and 0.22 mg/L

A significant increase in GPx enzyme activities in the digestive glands of Unio terminalis was noted after 48 hours, 7 days and 21 days of exposure to 0.11 mg/L and 0.22 mg/L</td></tr>
</table>

Table 4.16. Summary of acute and chronic toxicity effects of acetamiprid on *Unio terminalis* mussels

<table>
<tr><td rowspan="3">Acute Toxicity (96h-LC_{50})</td><td colspan="4">Chronic toxicity</td></tr>
<tr><td rowspan="2">Experimental conditions</td><td rowspan="2">Exposure time</td><td colspan="2">Biomarkers</td></tr>
<tr><td>Hemolynph</td><td>Histology</td></tr>
<tr><td rowspan="3">33.52 mg/L</td><td rowspan="3">*Exposition group:*
-Freshwater (no chemicals added)
-Dimethylsulfoxide (DMSO)
-Acetamiprid (33.52 mg/L and 6.70 mg/L)

Test organism:
Unio terminalis</td><td rowspan="3">48 hours
7 days
21 days</td><td>- Significant decrease of THC values
- Significant increase of TOS values
- Significant increase of TAS values</td><td>- Lipofuscin aggregates of gill tissues after exposure to 6.65 mg/L ACE for 21-d (H&E)
- Degeneration (black arrow) and lipofuscin aggregates (blue arrow) of gill tissues after exposure to 6.65 mg/L ACE for 7 day

-Degeneration and loss of the tubules (blue arrow) after exposure to 6.65 mg/L ACE for 21-d (H&E)
-Loss of the tubules and lipofuscin aggregates after exposure to 6.65 mg/L ACE for 7 days</td></tr>
<tr><td colspan="2">Antioxidant (SOD, CAT, GPx)</td></tr>
<tr><td colspan="2">-A significant increase of the SOD enzyme activity in the gills after 48 hours and 7 days of exposure to the doses of 3.35 mg/L and 6.70 mg/L of ACE
-A significant increase of the SOD enzyme activities in the digestive glands after two days of exposure to the doses 3.35 mg/L and 6.70 mg/L of ACE
-No significant difference between the CAT values obtained in the different groups after 48 hours, 7 days and 21 days of exposure to both concentrations of ACE.70
-A significant increase in GPx enzyme activities in the gills was noted after 7 days and 21 days of exposure to 3.35 mg/L and 6.70 mg/L of ACE
-A significant increase in GPx enzyme activities in the digestive after two days, 7 days and 21 days of exposure to 3.35 mg/L and 6.70 mg/L of ACE</td></tr>
</table>

5. DISCUSSION

Lambda-cyhalothrin (LCH) and Acetamiprid (ACE) are toxic substances that often accumulate in aquatic ecosystems, but little is known about the response of natural communities in this ecosystem to this contamination. This is especially true for mussel communities, which play a major ecological role in this ecosystem and are also species capable of bio-accumulating contaminants and therefore likely to become a danger to humans through the food chain and the biomagnification process.

The experimental approach used in this thesis is that of in vivo bioassays carried out in the laboratory with the exposure of *Unio terminalis* organisms to different concentrations of Lambda-cyhalothrin and Acetamiprid for several duration of time.

We started with acute toxicity bioassays (96-hour duration) for both active substances. Then, we carried out chronic toxicity bioassays (exposure duration: 48 hours, 7 days and 21 days), which allowed us to evaluate the general activity, as well as the capacity of defense and adaptation of the mussels facing this chronic exposure, whose organisms were exposed to the contaminants LCH and ACE. We evaluated some markers of oxidative stress, in order to determine the sensitivity of the different organs of mussels (gills and digestive glands), involved in various functions.

5.1. Acute Toxicity

In the field of environmental management, several problems are raised regarding the abusive use of pesticides which are regularly found in several ecosystems (terrestrial, aquatic) thus inevitably threatening non-target organisms. Through this study, it has been estimated, under laboratory conditions, the LC_{50} values for two commonly found pesticides on the market and in the field, in order to inform future researchers on their lethal doses on mussel species. The probit data of LCH and ACE on the freshwater mussel *Unio terminalis* are reported in Annex 1 and Annex 2, respectively. Based on those values and the international classification of toxicity based on LC_{50} (Houndji et al., 2020), it appears that lambda-cyhalothrin is highly toxic, whereas acetamiprid is moderately toxic for the species *Unio terminalis* [(LC_{50}, very highly toxic LC_{50} < 0.1 ppm; LC_{50}, highly toxic = 0.1–1 ppm; LC_{50} moderately toxic >1–10

ppm; slightly toxic >10–100 ppm; and practically nontoxic >100 ppm) (Zucker 1985a, 1985b; Paul and Simonin, 2006).

This conclusion, on their degree of toxicity, is similar to that formulated by Guedegba et al. (2019) through their toxicity studies conducted on juveniles of Nile Tilapia with these same two substances. This could be explained by the physico-chemical properties of lambda-cyhalothrin. Indeed, as pyrethroids, it has a "low water solubility" and "high lipophilicity" which lead to a faster and more rigid absorption by living organisms and abiotic elements (Hamer et al., 1994). According to this analysis, this greater sensitivity of mussels to the chemical lambda-cyhalothrin is certainly due to the fact that after the rapid dissolution of the chemical, resulting in a more homogeneous solution, the high absorption leads to a severe toxicity revealed by the LC_{50} values obtained. In the control of crop pests and other target organisms, these pesticides are highly valued because of their rapid decomposition and absorption. Nevertheless, this is not without risk for aquatic organisms that also ingest it more rapidly with consequences in their biological development process, with the possibility of accumulation of residues of these toxic substances in their tissues, ultimately leading to death.

Our results are consistent with the experiments which showed that, based on acute toxicity, nicotinoid insecticides are less toxic than pyrethroid insecticides with regard to aquatic organisms (Barbee and Stout, 2009; Morrissey et al., 2015; Guedegba et al., 2019).

The 96h-LC_{50} of acetamiprid obtained in our study is higher than that reported by other authors compared to its toxicity on other aquatic species such as fish and other molluscs. Indeed, this value is of the order of 5.66 ppm for the fish *Oreochromis mossambicus* (Arican et al., 2019); while it is 182.9 ppm for the species of the same genus, *Oreochromis niloticus* (Guedegba et al., 2019). On the African catfish (*Clarias gariepinus*) juveniles, its acute toxicity value was evaluated to 0.0033 ppm (Houndji et al., 2020).

The value of lambda-cyhalothrin 96h- LC_{50}, which is 1.1 mg/L, is higher than those evaluated for other invertebrate aquatic organisms. Indeed, the values of Lambda-cyhalothrin 96h-LC_{50} are 0.84 ng/L and 0.207 mg/L respectively for juveniles and adults of *Astacus leptodactylus* (Berber et al., 2013); 0.00021 mg/L for the bluegill sunfish species (*Lepomis macrochirus*); 0.00024 mg/L for the Rainbow trout species (*Oncorhynchus mykiss*)

and 0.00036 mg/L for the species water flea (*Daphnia magna*) (Barbee et al., 2010). These results show that this insecticide has a low toxicity to mussels compared to these other organisms; and then mussels are less sensitive to acute lambda-cyhaloothrin exposure in comparison to the cited species.

As reported by Oribhabor et al. (2007), in summary, the diverse sensitivity of organisms to chemical compounds is a function of elements including cuticular disposition to toxicant penetration, rate of enzymatic breakdown, compound excretion, and availability of physiological storage mechanisms in the organisms (Rand, 1995). However, since the values of 96h - LC_{50} are not zero, these are organisms that can be endangered by this substance when it is present in their living environment, even if they are not the direct targets of its use. Since pesticides are often applied in combination with others, it is likely that both substances are found in the same ecosystem. Studies on the toxicity of their mixture show equally serious results in terms of their acute toxicity. Indeed, Guedegba et al. (2019) and Houndji et al. (2020) found out that the acute toxicity of the mixture of acetamiprid and Lambda-cyhalothrin on fishes presents a greater toxicity than that of lambda-cyhalothrin on these same species.

Assuming that the experimental conditions in our case study are semi-static, the toxicity of these compounds may differ and be somewhat dangerous in field conditions when several factors are effective. For example, because of the properties of pyrethroids (low water solubility and resilience; significant adsorption on particles) (Rasmussen et al., 2008; Bajet et al., 2012), their toxicity in the natural environment may be lessened.

5.2. Evaluation of Findings on Hemolymph Parameters in Freshwater Mussel *Unio Terminalis*

Bivalve molluscs have a circulatory system consisting of a dorsal posterior heart, from which an anterior and a posterior aorta branch out into all tissues. This circulatory system, composed of hemolymph, is said to be "open" because the smallest vessels open directly into sinuses, or lacunae, directly bathing the organs and cells. The colorless hemolymph is composed of circulating cells, the hemocytes, which infiltrate all the animal's tissues. These hemocytes occupy essential functions in several physiological processes: gas exchange, osmoregulation, nutrient transport and digestion, waste elimination, shell repair and wound

healing, and immune defense (Cheng, 1984; Gosling, 2003). These cells generally have a key role in maintaining homeostasis.

Some hemocyte parameters have the ability to indicate if the components of the immune system (e.g. total number of hemocytes) are structurally competent to perform their immune roles (oxidative activity, etc.) (Auffret, 2005; Sepici-Dincel et al., 2013). These cellular descriptors can be measured from easily collected non-lethal hemolymph samples and can be used to determine the health status of individuals.

The elevation of hemocyte density in the circulatory system and the infiltration of hemocytes into tissues are the two immediate responses generally observed following a disturbance (infection, injury, contamination) (Mayrand et al., 2005).

The use of this particular cell type in this species constitutes a support of choice for ecotoxicological studies because of their implications described previously in the physiology of molluscs. Indeed, an alteration of the hemocyte functions can result in an impairment of the different major physiological functions of the individuals and have repercussions at the population level.

5.2.1. Total hemocyte counts (THCs)

For the case of Lambda-cyhalothrin experiments and after analyzing the results obtained, we can conclude that the values of THCs in *Unio terminalis* decreased by 15-20% by comparison to the control group ($P<0.05$), after 48 hours, 7 days and 21 days of exposure to LCH. The lowest value of THCs was obtained after 21 days of exposure at the dose of 0.11 mg/L ($THcsx10^3/mL = 34.000 \pm 1.583$). By considering each concentration of LCH, THCs were affected.

For the case of Acetamiprid experiments and after analyzing the results obtained, we can conclude that the values of THCs in *Unio terminalis* decreased by 8-20% by comparison to the control group ($P<0.05$), after 48 hours, 7 days and 21 days of exposure to ACE. The lowest THC values are obtained after 21 days of exposure at the dose of 6.70 mg/L ($THCsx10^3/mL = 37.533 \pm 2.102$).

Lambda-cyhalothrin and Acetamiprid, knowing that the different concentrations (doses) used are lower than their LC_{50}, cause a decrease in the abundance of hemocytes in *Unio terminalis* confirmed by the THCs results.

Similar observations of decreases in THCs values were made on *Unio mancus* mussels exposed for 144 h to Bisphenol A (BPA) contaminants (Yildirim et al. 2016). Other cases in which crustaceans were exposed to chemical compounds showed similar results: example of freshwater crabs (*S. henanense*) exposed for 96 h to varying concentrations (58 mg/L and 116 mg/L) of Cd (Qin et al. 2012); common shrimp *Crangon crangon* exposed to PCB (Smith and Johnston, 1992)); *Litopenaus vannamei* exposed for 96 h to different concentrations of Sirius® 250 SC herbicide (0.1 µg L/1 to 1000 µg L/1) (de Mello et al., 2011)

Considered relevant indicators of stress and disturbance in crustaceans, hemocyte concentration data obtained by counting are non-specific and highly dependent on physiological alterations in relation to physicochemical factors of the environment (Auffret et al., 2006).

The effects of contaminants on the immune response of bivalves have been measured mainly on marine species (e.g. blue mussel or oyster) and freshwater species such as the Elliptio mussel. It appears from these studies that chemicals present in the environment can affect the immune system of mussels by acting on hemocytes (Mydlarz et al., 2006). Examples of chemical compounds capable of disrupting one or more immune parameters in bivalves are: Lead, Arsenic (metals), Benzene (solvent), Silica (particles), Aminofluorene acetyl (aromatic amines), phenyltins (organotins), Chlordane and Malathion (pesticides) (Galloway and Depledge, 2001).

The differences observed between the different values of THCs in our experiments are coherent with the observations of Sauve et al. (2002) who found that the hemocytes of freshwater mussels and marine bivalve species are very sensitive to environmental stressors. Crustacean hemocytes are known to have a crucial function in immune systems (Johansson et al., 2000) and the total number of hemocytes in the hemolymph is an indication (or indicator) of the immunological condition of an organism. Hemocytes participate in the healing mechanisms of traumatized tissues and the shell, as well as in the transport and

digestion of nutrients. They are also involved in the internal defense processes of the organism and in the excretion of foreign bodies. Since the immunity of bivalves is mainly based on cellular mechanisms, this decrease in the concentration of immune cells may decrease the efficiency of the fight against infectious agents, especially pathogenic bacteria present in their ecosystem (Akaishi et al., 2007). According to Jussila et al. (1997), xenobiotics or stressors can have inhibited hemocyte mobilization, which explains the decrease in THC levels.

But given their key role in the immune system, we would have thought that this mussel would respond to the stress caused by these pesticides by an overproduction of hemocytes, necessary for their elimination from its body. This would involve internal regulatory mechanisms of xenobiotics in bivalves (Giamberini, 1993). However, it is likely that when the detoxification capacities of the organism are exceeded, the contaminants can produce toxic effects leading to the reduction of the hemocyte pool as observed after 21 days of exposure to these pesticides. Decreases in total hemocyte counts were less consistent after 48 hours but more pronounced after 7 and 21 days of experimentation.

Günal et al. (2018) describe THCs as a simple, rapid, and relevant biomarker for showing the effect of biocidal use on marine and freshwater ecosystems, which can be used to examine the effects of xenobiotics on non-target organisms. Our results also allow us to conclude that the determination of THCs in mussel organisms may be an important biomarker for examining cases of environmental pesticide pollution of water ecosystems supporting these species.

5.2.2. Total antixodant status (TAS)

During our experiments, we measured the total antioxidant capacity (also known as Total Antixodant Status, TAS values) of the species *Unio terminalis* with hemolymph samples, in order to assess their organisms' abilities to neutralize the harmful pesticides (LCH and ACE), which can cause damage their cells/tissues and contribute to the development of diseases. The greatest advantage of this assay (test) is the possibility to quantify the antioxidant potential of all antioxidants present in a given biological material and not merely the antioxidant capacity of a particular compound (Yağan et al., 2014).

Antioxidants are molecules that protect cells and tissues from oxidative stress (example of ROS = Reactive Oxygen Species which are generated as a derivative product of normal cell metabolism).

In mussels, antioxidants are important for protecting the mussel's tissues from damage caused by environmental contaminants, including heavy metals and other toxic substances. However, it is known that mussels, like other marine organisms, contain high levels of antioxidants, which may provide health benefits to humans who consume them. Thus, several studies and investigations have shown that TAS values in the hemolymph of aquatic ecosystem entities can be considered as biomarkers of the response of their immune defense system to any stressor (Sepici Dinçel et al., 2009).

Case of exposure of *Unio terminalis* to LCH

After performing the chemical test using the methods based on chemical indicators, we found that LCH affected the TAS values in the mussel organisms after the different exposure times. There was an increase in the TAS values for each of the LCH doses used compared to the control groups.

Although the highest TAS value was obtained after 48 hours of exposure at the 0.11 mg/L dose, it showed no significant difference from the TAS value obtained after the same time period for the 0.22 mg/L dose. With the increase in the number of days of exposure, we observe a decrease in the TAS values obtained after a short period of exposure compared to those obtained after long periods of exposure for both doses of LCH. This would imply that mussel organisms have increasing difficulty in producing antioxidants to cope with this pesticide but still have high TAS values to survive for several days in spite of the contamination of their environment.

This finding shows the fact that antioxidant defenses/protections had been exhausted, most likely because of oxidative stress that happened post exposure and that is seemingly to be time-dependent (Kaloyianni et al., 2009; Selvi et al., 2011). In some other studies, the same increase was observed, for example in the gills of *Mytilus galloprovincialis* mussels, exposed to different heavy metals (Pb, Cd) although the results were not statistically significant (Franco-Martinez et al., 2016). However, Kocamaz et al. (2018) found out that Lambda-

cyhalothrin induced the decrease of TAC in tissues (gonad, brain, liver) of Tilapia, after exposure to 2.901 µg/L of LCH during 7 and 15 days.

Case of exposure of *Unio terminalis* to ACE

The global antioxidant activity of *Unio terminalis*, as assessed by TAS determination through chemical kits, was not altered by Acetamiprid. Similar to our results, Cossi et al. (2020) found out that the total antioxidant capacity (TAC) of the gastropod *Biomphalaria straminea* was not influenced either by ACE as substance active, nor by Assail® 70 (that is a commercial formulation of the acetamiprid). The author explained this by the interaction of several antioxidants that can be individually initiated or inhibited by ROS and resulting in an unchanging TAC. Zhou et al. (2008) suggest that this may be due to a greater tolerance to this pesticide by *Unio terminalis* mussels, which therefore have a lower or no antioxidant response.

Mussels compared to rats seem to defend themselves better after exposure to acetamiprid for one week. Indeed, the results of Khovarnagh and Seyedalipour's (2021) experiments on rats confirmed by the observations of Toghan et al. (2022), showed that ACE caused a decrease in TAS values in the livers and brains of this species. This indicates a high production of ROS by ACE, a high oxidative stress that may be the result of an elevated lipid peroxidation.

In summary, the increase in TAS values is considered by some authors as a compensatory balance system to oxidative stress caused by LCH (Patetsini et al. 2013), hence the lack of variation could imply a better regulation of the toxicant (ACE) by mussel organisms *Unio terminalis* (Franco-Martinez et al., 2016).

5.2.3. Total oxidative stress (TOS)

Total oxidative stress (TOS) is intended to measure the overall level of oxidative stress in *Unio terminalis*, meaning to measure the concentration of ROS in hemolymph samples from these organisms.

Case of exposure of *Unio terminalis* to LCH

We remarked a significant increase in TOS values between the different treatments compared to the control groups. This implies a high rate of oxidative stress caused by this chemical (LCH) and an elevated risk of diseases (structural or biochemical changes in the organism) which are linked to oxidative stress. In fish, the same increase of TOS was observed by Kocamaz et al. (2018) who subjected the fish species Tilapia to a dose of 2.901 µg/L of LCH, for 7 and 15 days, and then evaluated the TOS in tissues namely gonad, brain, liver of the organisms in the experiment.

Case of exposure of *Unio terminalis* to ACE

Overall we noticed no significant difference in TOS values in mussels exposed to ACE. The only difference was observed in mussels that were exposed for 21 days to ACE at 3.35 mg/L and 6.70 mg/L. It can be said that following reduced exposure times (e.g. 48 hours, 7 days) the levels of oxidative stress are constant with almost no risk of disease. On the other hand, when we tend toward 21 days of exposure to ACE, the mussels are more prone to suffer from diseases and cellular or even tissue disruptions due to oxidative stress which becomes threatening.

Comparing our results with that of Cossi et al. (2020), we can say that ACE has different effects on TOS depending on the organisms that face the exposure. Indeed, in the case of gastropod *B. straminea* that was subjected, for 14 days, to different concentrations of ACE, either through the active substance itself (1500 µg/L) or through a commercial formulation (1500 µg/L of Assail® 70), showed a significant reduction in ROS generation (or production).

5.3. Evaluation of Histological Findings in Freshwater Mussel *Unio Terminalis*

Histopathological studies in tissues of aquatic animals are the gold standard, reliable and direct indicators of environmental stressors. Histological testing is often impractical in human subjects, using biomarkers with a known histological distribution may fill the need to localizing toxic injury to distinct organs or tissues (Reddy and Rawat, 2013). There is no published study found in the open literature related to the histopathological effects of ACE

and LCH on freshwater mussels. The gills of freshwater mussels exposed to sublethal ACE revealed lipofuscin aggregates and degeneration, hemocytic infiltration and degeneration after exposure to LCH. Similar to our findings, Kuzukiran et al. (2022) determined severe histopathological alterations such as "lipofuscin aggregates, haemocytic infiltration, hyperplasia, loss of cilia, enlarged haemolymphatic sinus and fusion of gill filaments in the gill tissues" of *Unio sp.* after exposure to sublethal concentrations of phthalate esters for 96h. Same histological alterations were also reported with different toxicants in the literature for different mussel species as haemocytic infiltration and lipofuscin-like structure in gill tissues of *Unio crassus* after exposure to copper and zinc pyrithione (Tresnakova et al., 2020). Marine mussel organisms also showed lipofuscin in the gill tissues in the field studies after exposure to environmental stressors as metals with anoxic conditions (Sarasquete et al., 1992). Alalibo et al. (2019) observed cellular infiltrations, fusion of the lamellar, hyperplasia, tissue inflammations, vacuolation, tissue degeneration and necrosis of the gill tissues on *Sarotherodon melanotheron* exposure to 7-11 g/L LCH for 96 h.

The digestive gland tissue of the control group organisms showed normal structure with digestive tubules composed of epithelial cells. Exposure to sublethal ACE resulted in degeneration of the tubules, lipofuscin aggregates and tubule loss of the digestive gland according to duration and the concentration of the neonicotinoid pesticides. For sublethal LCH exposure digestive gland revealed tubule degeneration and loss. Digestive gland tissues are mostly used for assessing the effects of xenobiotics on mussels' health (Faggio et al. 2018). Digestive tubule degeneration of the digestive gland of the mussels was reported by different authors following exposure to different toxicants (Lowe and Clarke, 1989, Balamurugan and Subramanian, 2021). Tresnakova et al. (2020) determined deformations and loss of digestive tubules of *Unio crassus* after exposure to antifouling compounds for 2 and 7 days. Similarly, Stara et al. (2020) reported lipofuscin aggregates in the digestive gland tissue of *Mytilus galloprovincialis* after exposure for 20 days of neonicotinoid pesticide. Jiao et al. (2023) reported histopathological damages in the liver tissues of *Xenopus laevis* exposure to 45 mg/L ACE for 14 d.

5.4. Evaluation of Findings on Biochemistry Parameters in Freshwater Mussel *Unio Terminalis*

In mussel organisms, the gills have many functions (gas exchange (respiration), feeding (nutrient absorption), osmoregulation). These different roles are subject to the regulation of a neuronal system (neurotransmissions) (De marco, 2022).

The digestive glands of molluscs have a significant function in the digestion of food, synthesizing and secreting digestive enzymes (Ortega et al., 2014). They are often equated with the following topic: gastric glands, digestive organs, hepatopancreas, and perigastric organ, etc. They are also the target of chemical contaminants that are transported there for detoxification, through the ingestion of leaves, sediments, biofilm or water. Consequently, it is one of the primary locations where pollutants accumulate in molluscs and aquatic crustaceans (crayfish, crabs, oysters, etc.). It performs functions that in vertebrates are assigned to the intestine, liver, and exocrine pancreas (Vogt, 2019). The major role of the digestive glands both in digestion and in the storage and detoxification of toxic compounds makes it important to know the eventual effects of contaminants on the functioning of digestive and antioxidant enzymes.

When exposed to contaminants or chemicals, mussel species undergo several deformations or alterations in important tissues, from the digestive glands (Gornati et al., 2019) to the gills (Maisano et al., 2017) and gonads (Koagouw et al., 2021). These different lesions or tissue alterations can be revealed by histopathological studies or ultrastructure.

For these reasons, we also assessed the digestive gland responses, in addition to the gills in order to obtain a more integrative view of the effects of contaminants at the organism scale.

Within regular (normal) physiological states, there exists an equilibrium amidst the synthesis and removal of ROS. As we have seen, ROS, in small and controlled quantities, are needed for the correct working of cells and are implicated in cell signaling processes. In excessive quantities, they are harmful to the cell and participate in the process of cellular aging and certain pathologies (Ames et al. 1993; Simonian and Coyle, 1996; Madamanchi et al., 2005). Fine regulation of oxidative metabolism is therefore necessary for proper cell function and ultimately for the survival of organisms. Certain conditions can lead to an increase in the

intracellular production of ROS. These can be biotic stresses such as parasitism, attack by other organisms, attack by pathogens or abiotic stresses such as thermal shock, exposure to ultraviolet (UV) light or variations in oxygen availability. Oxidative stress is generally defined as a situation of imbalance in which pro-oxidants are in a large majority compared to antioxidants (Opara, 2006). This may be due to an overproduction of ROS and/or a decrease in antioxidant capacity. Therefore, the analysis of oxidative stress is done by measuring the species participating on both sides of the balance.

Antioxidant systems are responsible for protecting tissues, cells or organs from adverse effects by keeping ROS at tolerable levels and mitigating the harm associated with their elevated sensitivity. (Van der Oost et al., 2003.) A number of antioxidant defense systems are available in bivalve molluscs. Enzymatic components such as SOD, CAT and GPX are expressed by bivalve species to ensure cell defense against ROS that are of both endogenous and exogenous origin.

Differences in the responses of enzyme activities assessed at the level of each specific tissue (organs) can be interpreted by the existence of different metabolic activities of these tissues and dissimilar reactions to environmental factors. (Borkovic et al., 2008).

5.4.1. Exposure to lambda-cyhalothrin

Overall, the results obtained made it appear that the antioxidant defenses of *Unio terminalis* were meaningfully impacted by exposure to Lambda-cyhalothrin concentrations during the experiments.

Considering the gills and digestive glands of *Unio terminalis* organisms, LCH caused the stimulation of SOD enzymatic activity but with different responses. Indeed, a decrease in SOD values was observed in the gills after 21 days of exposure while a decrease was observed after exposure to LCH concentrations of 0.11mg/L and 0.22 mg/L.

As a reminder, Superoxide dismutase (SOD) is an enzyme that is found in many organisms, including mussels. These enzymes are expressed in virtually all living cells exposed to oxygen and constitute a multigene family of enzymes. It plays a crucial role in protecting cells from damage by neutralizing a type of harmful molecule called superoxide (the

superoxide anion O_2^-). (Fridovich, 1986; Yao et al., 2006). Superoxide is a byproduct of normal cellular metabolism, and it can damage cells and tissues if it is not neutralized. SOD converts superoxide into hydrogen peroxide, which is less harmful, and then into water, which is harmless. (Dringen et al., 2005)

In the gills and digestive glands of *Unio terminalis* organisms, LCH induced the stimulation of CAT enzyme activity with a decrease in its values after 48 hours of exposure at 0.11 mg/L and 0.22 mg/L chemical concentrations compared to the control groups. But no differences were found in either gills or digestive glands after 7 and 21 days of LCH exposure.

Catalase as for it, is an enzyme that is found in many organisms, including mussels. It is a vital part of preventing cells from damage by neutralizing a type of harmful molecule called hydrogen peroxide. It is a by-product of normal cellular metabolism that can damage cells and tissues if not neutralized. Catalase converts hydrogen peroxide into water and oxygen, which are harmless. (Toner et al., 2007.)

The observed decrease in these two enzymes, instead of their increase may mean that neither CAT nor SOD participated effectively in the cellular detoxification of the gills and digestive glands of *Unio terminalis* mussels during their exposure to LCH. There was thus an inhibition of the antioxidant protection mechanism that should be activated by the two enzymes in the specific organs studied in the experiments.

In comparison to CAT and SOD activities, LCH induced an increase of GPx activities in the gills and digestive glands of *Unio terminalis* organisms during the different exposure times. Glutathione peroxidase is an enzyme found in most tissues, including those of mussels. It is involved in protecting the organism from oxidative stress by the conversion of ROS, examples of hydrogen peroxide, into less harmful substances. This prevents oxidative damage to cells and tissues, which can lead to a number of health problems. In mussels, glutathione peroxidase may also play a role in detoxification, as well as in protecting mussel tissues from damage caused by environmental pollutants.

GPx acts more slowly than catalase but has a better affinity for H_2O_2 than the CAT. GPx is therefore essential for the breakdown of H_2O_2 produced continuously and at physiological levels in the cell. High levels of intracellular H_2O_2 result in preferential activation of catalase

while lower levels would be preferentially handled by GPx. These redundant mechanisms thus become complementary (Pamplona and Costantini, 2011).

According to our results, the expression of GPx activity is more apparent than that of the other two enzymes, despite the fact that catalase also has an affinity for H_2O_2. But it should be emphasized that the difference in regulation observed between these enzymes is probably due to their different properties. Indeed, CAT is more active when there is a peak in H_2O_2 generation (production), while the GPx is more effective for low quantities of H_2O_2 generated continually (constantly/ continuously) during cellular metabolic functioning (Pamplona and Costantini, 2011).

The increase in GPx values, in both gills and digestive glands, showed that LCH caused the production of free radicals and face somehow the defense of this enzyme.

There are many studies on the response of aquatic invertebrates to pyrethroid pesticides, but none refers to the effects of LCH on *Unio terminalis* organisms. Furthermore, it is presently inconsistent to find any studies reporting on the response of mussels to the effects of LCH in the scientific literature consulted.

Organisms of *Oreochromis mossambicus*, exposed to LCH at concentrations of g 0.3 μg/L and 1.1 μg/L for 15 days, showed decreasing enzyme activities (SOD, CAT, GPx, GST) in the liver tissues of this freshwater tilapia (Parthasarathy and Joseph, 2011). This author points out that inhibition of these enzymes can induce an accumulation of these oxidants and increase the sensitivity to oxidative degradation of hepatic cell membranes. In the liver of *Perccottus glenni* (the Chinese sleeper), LCH caused a reduction of SOD activity with the conclusion of a concentration-dependent induction after exposure to 1.50 μg/L and 2.00 μg/L of LCH. (Zhu et al., 2015).

However, it can cause in some cases, an increase in antioxidant enzyme activity. Chronic exposure of *Clarias gariepinus* to LCH concentrations (2.5×10^{-4} μg/L, 5.0×10^{-4} μg/L, and 6.25×10^{-4} μg/L) showed an increase after consecutively 7 days, 14 days and 28 days of experiments. (Ezenwosu et al., 2021).

Fluctuations in the activities of SOD, CAT and GPx which are major enzymes in mussel organisms, prove the effective production of dangerous molecules (free radicals), in the tissues (gills, digestive glands) of *Unio terminalis* organisms by Lambda-cyhalothrin, in proportions greater than the antioxidant defense capacity of the species. All these results imply that Lambda-cyhalothrin is harmful to the mussel *Unio terminalis*.

For additional aquatic organisms such as cyanobacteria, LCH induced a change in SOD enzyme activity in *Arthrospira platensis* at a dose of 11.94 µg/ml, corresponding to the EC50 value of LCH for this species. (Tunca, 2020). In other invertebrates such as snails, e.g., *Helix aspersa*, oxidative stress induced after exposure to LCH doses (10, 20 and 40 µM/L) revealed a decrease in Catalase (CAT) activity in the hepatopancreas of organisms of this species. (Bouaricha et al., 2022)

But it is not only in aquatic organisms that LCH causes tissue or organ damage. Indeed, experiments conducted on mammals have shown that LCH caused a consequent decrease in enzyme activities (SOD, CAT) in the livers of rats dosed at 10 mg/kg (Obulor et al., 2021); In *Oryctolagus cuniculus*, a significant dose-dependent increase in catalase (CAT) and superoxide dismutase (SOD) activities were noted in the ovaries of females exposed to 4.16 mg/kg and 8.32 mg/kg of LCH (Adam et al., 2020).

5.4.2. Exposure to acetamiprid

Oxidative disequilibrium may occur as a consequence of elevated reactive species, diminished scrubber antioxidant enzymes and molecules, or a mixture of these phenomena. We assessed the influence of acetamiprid on these inherent antioxidant defenses by examining the activities of GPx, CAT, and SOD, which are important enzymes for redox protection.

From our results, it can be highlighted that SOD enzymes actively participated in the detoxification of reactivated species from oxygen, both in the gills and in the digestive glands, during the 48 hours of exposure of mussel organisms to concentrations of 3.35 mg/L and 6.70 mg/L. This involvement in the maintaining of oxidative balance continued in the gills beyond 7 days of exposure.

It should be noted that according to our literature review, no study has really evaluated the toxicity of ACE on mussels, especially on their biochemical toxic impact. Our comparisons will therefore be made with other mammalian and invertebrate aquatic organisms that have been exposed to this chemical. The assessment of acetamiprid toxicity on amphibian species, such as the tadpoles of *Rana nigromaculata,* showed significant impairment of antioxidant enzymes with increases in SOD values for groups subjected to concentrations of 0.185 mg/L and 1.85 mg/L for 7 , 14, 21, and 28 days. (Guo et al., 2002). These observations are also corroborated by XIAO-HUA et al. (2006) who pointed out that the SOD activities of bacteria (*B. subtilis* and Pse. FH2) in response to 100 mg/L ACE exposure for 0.5 h reached significantly high values compared to the control group. Assessment of acetamiprid toxicity on amphibian species, such as the tadpoles of *Rana nigromaculata,* showed significant alteration of SOD enzymes (Guo et al., 2002).

However, the snail *Biomphalaria straminea* exposed to the lowest concentration of ACE (150 µg/L), showed a decrease in SOD activities (Cossi et al., 2020). Similarly, in another freshwater mussel species, *Anodonta cygnea*, ACE caused a decrease in SOD enzyme activity in the gills (Mishchuk et al., 2008). This difference can be explained by the applied dose, the exposure time and the physicochemical conditions of the experimental medium solutions. This is also the case in rat liver and kidney where these scavenger enzymes showed a decrease after exposure to different doses of CEA (110 mg/kg body weight; 55 mg/kg body weight; 27.5 mg/kg body weight) for 90 days. (Devan et al., 2015).

In contrast to the enzyme SOD, no effect was observed in the activity of the CAT enzyme. These results are similar to those obtained by Cossi et al (2020), after 14 days of exposure of the mollusc species, *Biomphalaria straminea* (gastropod) to 1.5 mg/L of the active substance Acetamiprid. (Guo et al., 2002). The same finding was made for amphibian species, for example *Rana nigromaculata tetrads,* whose CAT values remained unchanged after 7 and 14 days of exposure to 0.185 mg/L ACE (Guo et al., 2002).

But this chemical compound can induce CAT enzyme activity in some species under other conditions as observed in the digestive glands of freshwater mussels *Anodonta cygnea* with a decrease in the values of this enzyme (Mishchuk et al., 2008); bacteria (Guo et al., 2002), male reproductive rats (Zhang et al., 2011).

After exposure to various ACE doses (0.5 – 1 mg/L), a reduction of SOD and CAT activities was noticed in the gills of *Catla catla* fish (Veedu et al., 2022).

According to our results, we have identified an increase in GPx activity, both in the gills and in the digestive glands of mussels subjected to the different treatments. No inhibition was observed. Thus, this enzyme is highly involved in the detoxification of reactive species, notably through the reduction of hydrogen peroxide to water in the presence of glutathione (Lu and Holmgrn, 2013).

Several researchers have suggested that ROS may influence enzymatic activity, either by induction or inhibition (Xu et al., 2009, Cossi et al., 2020). In addition, the antioxidant enzymes present differences, which are located at the level of substrates, functioning of their mechanisms, gene regulation, mode of conversion, and therefore by consequence, differences in inactivation, regulation, activity (Cossi et al., 2020). These elements could at best explain the different variations observed in the enzymatic activities of SOD, CAT and GPx in response to the stimulation caused after Acetamiprid as also pointed out by Cossi et al. (2020).

Through our results, we were able to provide a better understanding of the fate of Acetamiprid and lambda-cyhalothrin in freshwater mussel organisms as well as the risks to them in case of contamination of their ecosystems.

On the basis of the pattern of outcomes obtained, it cannot be assumed that one organ (gills and digestive glands) is more sensitive than the other. Each of these organs may show different responses to toxicity. This theory is supported by other researchers who have conducted studies on mussels such as Cossu et al. (2000). According to this author, the reaction to chemical stress will be a function of the nature and level of pollution, the bioavailability of the contaminant and their diffusion in the respective cells based on the pathway of entry. Following exposure, severe tissue damage may be observed in one tissue, while the other shows no effect. It is therefore preferable to consider that both tissues are of major interest in the case of impact studies of contamination or pollution on freshwater species, with the use of sentinel species such as mussels.

The exposure time (up to 21 days of exposure) considered in our study was relevant and is a requirement for a good biomonitoring bioassay on mussel species. Indeed, some studies have shown that bivalves were able to recover even after 30 days of exposure to polluted sediments at downstream or lower reaches of the river (Cossu et al., 1997).

6. CONCLUSION AND RECOMMENDATIONS

This thesis project is a contribution to eco-toxicological studies in the field of aquatic pollution through the evaluation of the toxic effects of the exposure of freshwater mussels to the active substances of the world's most frequently applied pesticides, namely Lambda-cyhalothrin and Acetamiprid. Although there have been numerous studies on the relative toxicity of these two phytosanitary pesticides on aquatic organisms, information on the class of mussels is lacking. Most studies in ecotoxicology base their research on certain taxonomic groups such as poisons by shedding less light on the sensitivity of aquatic invertebrates compared to pesticides regularly detected in their living environment.

In conclusion, we can say that the experiments carried out during this thesis project have contributed to reaching our initial objectives. We were able to determine the acute toxicity of LCH and ACE on the mussel *Unio terminalis* and prove that these chemical substances, following chronic exposure of the organisms of this species have negative repercussions on the enzymatic activities and with the appearance of tissue damage at the level of important organs (gills, digestive glands). The data acquired during this thesis project will fill the existing gap in the scientific results of toxicity tests of these molecules on freshwater mussels and in general on invertebrates of aquatic ecosystems. The data obtained and the analyses carried out on this species could be very useful for any future study concerning the impact of anthropic activities (with a particular emphasis on the case of agricultural activities and the abusive use of pesticides) on freshwater ecosystems.

The analysis of enzymatic responses of mussel organisms showed diverse responses which prove that these parameters can be used in cases of contamination of non-target species of these pesticides. From the environmental point of view, our results tend to show that the possibility of contamination of humans through the food chain as the organisms studied showed signs of damage. The toxicity is therefore real and the impact observed on these organisms may be more serious in the natural environment. We can notice that the concentrations used in our research are relatively low. In natural conditions, these doses can be multiplied by 10 or even 100 due to the anarchic use and application of insecticides in agricultural fields. It is very important to remember here that the doses prescribed in the manuals of use of these products are based on ecotoxicological studies similar to ours and

these doses must be effectively respected to avoid cases of intoxication of living beings and pollution of the ecosystems adjacent to their area of use.

However, these results must be nuanced considering the complexity of natural ecosystems and the involvement of other environmental factors that can act on these molecules in the field. Thus, new and more dangerous molecules can be generated and provoke more damage at organic, tissue, cellular levels, etc. their doses can also be lower in the natural environment compared to the concentrations considered in our study, but no contamination is to be taken lightly especially as these chemical molecules are sensitive to biological agents and physicochemical factors of the environment of utilization. Field investigations have already revealed the presence of traces of these two pesticides on regularly consumed products (fruits, vegetables, etc.).

The responsible use of phytosanitary products in agriculture must become a major issue of environmental protection, and the different actors in the field must play their part so that our environment is healthier and that, at the same time, we can avoid health risks linked to the abusive use of these toxic compounds for all living organisms in the short and long term. It is also worth noting that alternatives to chemical pesticides currently exist on the market and that their adoption would be a major benefit for the good health of everyone, both producers and consumers.

Knowing that these pesticides are also dependent on the biological properties or capacities of the soils to degrade and mineralize them, the perspectives for this research work are then multiple.

Beyond these simple pesticides, their degradation also leads to changes in their molecular structure forming metabolites that are, through toxicity studies, equally dangerous. Scientific studies must continue to assess the toxic effects of the derivatives of these two pesticides studied on other organisms in order to substantiate the risks involved in the continued use of these chemicals.

The current trends of increasing global sales of pesticides show that the contamination of land and various ecosystems will continue for a long time. So, it becomes necessary to conduct field studies to increase awareness.

It would also be interesting to evaluate in a direct and precise way the quantities of these pesticides which dissipate after their application in the cotton fields in Benin, and to study their transfer in the various compartments of the immediate environment of the agricultural grounds, their fate in the ground namely adsorption/desorption, degradation and the transfer in the ground and water, to better appreciate the risk of contamination of the medium by these chemical compounds and their derivatives in the short and medium term.

Many other perspectives are also opening up in the wake of this study. The use of other species of bivalves specific to the ecosystems of the environments where these pesticides are used to set up other biological models based on the same biomarkers in order to alert in time in case of risk of contamination and to take adequate measures.

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ANNEXES

Annex-1. Lambda-cyhalothrin 96-hour LC estimations and confidence ranges

Table 1.1. Lambda-cyhalothrin (LCH) 96-hour LC estimations and confidence ranges

Values	Concentrations (%)	95% Confidence Limits
LC_1	0.2894	0.0124 - 0.4975
LC_5	0.4289	0.0535 - 0.6503
LC_{10}	0.5291	0.1141 - 0.7671
LC_{15}	0.6096	0.1865 - 0.8757
LC_{50}	1.1094	0.7648 - 2.9747
LC_{85}	2.0189	1.2703 - 24.9522
LC_{90}	2.3261	1.3969 - 42.3195
LC_{95}	2.8693	1.5991 - 93.0774
LC_{99}	4.2534	2.0405 - 412.2308

Annex-2. Acetamiprid (ACE) 96-hour LC estimations and confidence ranges

Table 2.1. Acetamiprid (ACE) 96-hour LC estimations and confidence ranges

Values	Concentrations (%)	95% Confidence Limits
LC_1	5.8179	0.8141 - 11.4582
LC_5	9.7170	2.2802 - 16.4835
LC_{10}	12.7733	3.9149 - 20.1828
LC_{15}	15.3626	5.6033 - 23.2833
LC_{50}	33.5185	21.6846 - 50.1039
LC_{85}	73.1317	49.1407 - 184.1258
LC_{90}	87.9563	56.8989 - 262.5671
LC_{95}	115.6211	69.8989 - 449.3217
LC_{99}	193.1085	100.8569- 1254.7150

Gazili olmak ayrıcalıktır