UNIVERSITÀ DEGLI STUDI DI PADOVA

Dipartimento Territorio e Sistemi Agro-Forestali

CORSO DI LAUREA IN

SCIENZE FORESTALI E AMBIENTALI

Side effects of silicone-based monomolecular films for mosquito control on non-target organisms inhabiting freshwater wetlands

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ANNO ACCADEMICO 2024-2025

Summary

Abstract

Wetlands are among the most productive and important areas on the planet, providing numerous essential ecosystem services for both human and environmental health, and serving as valuable refuges for plant and animal species. The variety of species inhabiting wetlands, and their interactions contribute to the balance of the ecosystem itself; however, these species are often threatened by human activities, which lead to habitat loss and biodiversity decline. Not all species associated with wetlands are welcomed by humans. Mosquitoes are often feared because they are vectors of well-known diseases such as malaria, Zika virus, West Nile, Dengue, and Chikungunya. The risk of contracting these diseases has fueled an ambivalent relationship between humans and these ecosystems, leading to the development of mosquito control strategies long focused on the use of synthetic insecticides (larvicides and adulticides). However, growing environmental awareness has driven research towards identifying more ecological strategies. Silicone-based monomolecular films are newly discovered products; they create a barrier on the water's surface that prevents mosquito larvae and pupae from breathing and lowers surface tension, preventing adults from laying eggs. The aim of this study was to demonstrate that, although effective against mosquitoes, this strategy can have side effects on certain non-target model species of Hemiptera and Coleoptera. The insects were mainly collected from channels, ditches, lakes, and ponds, and immediately brought to the laboratory, where they were subjected to tests with varying dosages under standard conditions. The species considered include natural predators of mosquitoes. The results reveal that these insects are extremely sensitive to the silicone film; in fact, Gerridae, Corixidae, Dytiscidae, Notonectidae, and Gyrinidae showed 100% mortality within 24 hours of applying 1 ml/m^2 of the product. Their reduction could have repercussions on biodiversity and destabilize the food chain, leading to a paradoxical increase in mosquito populations. The use of monomolecular films and other mosquito control methods must therefore be carefully evaluated to balance the effectiveness in reducing the target species with the preservation of biodiversity in wetlands. The implementation of integrated management strategies, which consider the ecological importance and ecosystem services of wetlands, is crucial to ensuring the sustainability of these valuable ecosystems and maintaining their critical role in supporting life on Earth.

Riassunto

Le zone umide sono tra i luoghi più produttivi ed importanti del pianeta, offrendo molteplici servizi ecosistemici fondamentali per la salute umana e ambientale e rappresentando rifugi preziosi per specie sia vegetali che animali. La varietà di specie che abita le aree umide e le loro interazioni contribuiscono all'equilibrio dell'ecosistema stesso; tuttavia, tali specie sono spesso minacciate dalle attività umane, che causano perdita di habitat e biodiversità. Non tutte le specie legate alle zone umide sono però gradite dall'uomo. Le zanzare sono spesso temute perché vettori di note patologie per l'uomo (malaria, Zika vitus, West Nile, Dengue e Chikungunya). Il rischio di contrarre tali patologie ha alimentato una relazione ambivalente tra l'uomo e questi ecosistemi, portando allo sviluppo di strategie di controllo delle zanzare per lungo tempo incentrate sull'impiego di insetticidi di sintesi (larvicidi e adulticidi), tuttavia la crescente sensibilità nei confronti dell'ambiente ha indirizzato la ricerca all'individuazione di strategie maggiormente ecologiche. I film monomolecolari a base di silicone sono prodotti recentemente scoperti, essi creano una barriera sulla superficie dell'acqua che impedisce a larve e pupe di zanzara di respirare e abbassano la tensione superficiale, impedendo agli adulti di deporre. Obbiettivo del presente studio è stato quello di dimostrare che, sebbene efficacie contro le zanzare, questa strategia può avere effetti collaterali su alcune specie modello non target di Rincoti e Coleotteri. Gli insetti sono stati raccolti principalmente da canali, fossati, laghi e stagni, e portati subito in laboratorio, dove sono stati sottoposti a test con dosaggi variabili in condizioni standard. Le specie considerate comprendono predatori naturali di culicidi. I risultati dimostrano che questi insetti sono estremamente sensibili al film siliconico; Gerridi, Corissidi, Ditiscidi, Notonette e Girinidi hanno infatti mostrato una mortalità del 100% entro 24 ore dall'applicazione di 1 ml/m² del prodotto. La loro riduzione potrebbe avere ripercussioni sulla biodiversità e destabilizzare la catena trofica, portando ad un paradossale aumento nella popolazione delle zanzare. L'uso di film monomolecolari e altri metodi di controllo delle zanzare deve quindi essere attentamente valutato per bilanciare l'efficacia nella riduzione della specie target e la conservazione della biodiversità nelle zone umide. L'implementazione di strategie di gestione integrate, che considerino l'importanza ecologica e i servizi ecosistemici delle zone umide, è fondamentale per garantire la sostenibilità di questi preziosi ecosistemi e per mantenere il loro ruolo cruciale nel supportare la vita sulla Terra.

1. Introduction

1.1 Wetlands' ecosystem services

"Wetland" is any area either covered or saturated by water, which can be both permanently and seasonally wet. According to the Ramsar international wetland conservation treaty, wetlands are defined as "areas of marsh, fen, peatland or water, whether natural or artificial, permanent or temporary, with water that is static or flowing, fresh, brackish or salt, including areas of marine water the depth of which at low tide does not exceed six meters […] and may incorporate riparian and coastal zones adjacent to the wetlands, and islands or bodies of marine water deeper than six metres at low tide lying within the wetland".

There are different kinds of wetlands, and each of them is a unique ecosystem, with peculiar characteristics, like hydrology, soil composition, temperature, vegetation, and animals, typically adapted to live in these conditions.

Mediterranean wetlands are one of the most important hotspots of biodiversity in the world, and the support they provide to human well-being is much more than one might expect. In particular, wetlands can provide lots of ecosystem services from which people can benefit. For example, there are three different inter-related ways through which wetlands contributes to human health: by satisfying humans' social and cultural needs, simply thanks to its aesthetical appreciation or spiritual contemplation, by providing products, which can be used for pharmaceutical or some other medical aims, and by improving humans' socio-economic status, which can be also valued in economic terms (Horwitz et al, 2012, Galewski et al. 2012).

In general, ecosystem services can be classified in support services, regulatory services, supply services and cultural services (Galewski et al., 2012).

Supporting services are the most fundamental processes without which none of the other services could exist, such as photosynthesis, nutrient cycling, the creation of soils, and the water cycle.

Regulatory services are all the processes that moderate natural phenomena and participate in making ecosystem clean, sustainable, and resilient. Wetlands act as natural storage areas and can be considered as a natural cleaning system. For example, hydrophytes that live in those areas can purify water.

When chemicals from various sources like agriculture, human waste, and industries enter wetlands, they settle at the bottom where they are absorbed by plants and turned into nutrients, that support wetlands' wildlife fauna. By trapping sediment and controlling chemicals, wetlands prevent eutrophication of downstream water bodies which can lead to harmful algae blooms. Additionally, well-managed wetlands, can recharge groundwater and provide uncontaminated drinking water, reducing municipal budgets and dramatically reducing ill health and infant mortality (Global Wetland Outlook: Special Edition 2021).

Especially in dry regions like the Mediterranean, wetlands play a crucial role in managing water resources, supplying clean water that Mediterranean communities rely on for domestic uses, industrial purposes, energy generation, and agricultural irrigation (Ramsar Convention on Wetland 2018).

Mediterranean wetlands also help to manage extreme weather events and to contain natural disasters' risk (Convention on Wetlands 2021; Horwitz et al. 2012). In particular, coastal wetlands act as a buffer against storms and wind by absorbing their energy and reducing damage to inland areas. Moreover, during periods of heavy rainfall and flooding, the presence of vegetation slows down water's flow, allowing its absorption into the soil, releasing it gradually or retaining it on the surface (Krumar et al. 2017; Mediterranean Wetland Outlook 2018), whereas during droughts wetlands act as a water storage upholding coastal communities' survival. Vegetation also holds together the banks of lakes, rivers and beaches and prevent from soil erosion.

Furthermore, wetlands mitigate climate change by storing greenhouse gasses, they are one of the most important carbon sinks in the world, especially forested wetlands. Peatlands and vegetated coastal wetlands actually capture about as much carbon as the world's forests combined (Moomaw et al., 2018).

Provisioning services include any benefits people derive directly from nature, which are not only food (fish and shellfish, fruits, rice ext.) and drinkable water, but also timber, fuel wood, and lots of aquatic plants, fungi and bacteria traditionally used for their medicinal qualities to fight minor diseases, to alleviate pain (headaches, wounds, stomach cramps) and to form antibiotics (Horwitz et al. 2012; Mediterranean Wetlands Observatory 2012).

Provisioning services are closely related to important cultural services benefits. The beauty of water bodies, wild landscapes and the animal and plant diversity found in wetlands, enables many activities from fishing, bird watching and hunting; all of which contribute to the emotional and physical welfare of people and naturally make wetlands attractive tourist destinations. Furthermore, the inherent characteristics of wetlands and other ecosystems often hold cultural and spiritual significance, including contributing to regional identity. This cultural heritage encompasses traditional wisdom regarding the attributes, social meaning, and responsible management of wetland resources (Mediterranean Wetland Observatory 2012; Ramsar Convention on Wetlands 2018).

Many authors reported the significant role of wetlands in enhancing the psychological and social welfare of human societies through their spiritual, recreational, inspirational, and educational significance. Wetlands contribute to human well-being mainly by its recreational use for physical activities, although recent works documents substantial mental health benefits as well, which increase with the species richness of urban greenspaces (Fuller et al., 2007). The study conducted by Carter demonstrates that an appropriate access to green and blue spaces, with long-term planning involving local communities, promotes visiting, valuing, and caring for urban wetlands, enhancing community well-being (Carter, 2015). Additionally, the exposure to blue-green spaces helps people recover from stress. Those who are already under a lot of stress are more likely to develop health issues because of an increased physiological stress response in busy urban settings. However, these individuals tend to find relief from negative emotions when they spend time in green-blue spaces, which also boosts positive feelings in most people (Maund et al., 2019; Reeves et al., 2019).

1.2 Causes and costs of wetlands and biodiversity loss

Among the main drivers behind the loss of wetlands, climate changes are one of those with greatest effects. Climate change is profoundly affecting wetlands through rising temperatures, declining precipitation, sea level rise, and more frequent extreme weather events. This results in longer droughts and heavier downpours, with the latter increasing flood frequency (Mediterranean Wetlands Outlook 2018).

Climate changes and rising temperatures have an impact especially on wetlands' biodiversity by forcing animals and plants to move and migrate in areas where temperatures are lower and upsetting their biological processes, phenology, and behaviours. However, this migration may not be rapid enough (Galewski et al. 2012).

Urbanization is another big stressor that reduces wetland surface area and leads to a loss of biodiversity, because is linked to supplementary demographic pressures like excessive water withdrawals, the conversion of wetland ecosystems into urban areas and agricultural zones, a higher water demand for domestic, industrial, and energy purposes, eutrophication and pollution of aquatic ecosystems, an increasing of hunting, fishing and invasive species diffusion (Mediterranean Wetlands Outlook 2018; Global Wetland Outlook: Special Edition 2021; Martínez-Megías & Rico 2022). More polluted waters could be also the result of an ever-increasing lack of water, due to the decrease in rivers flow, which affect their pollutants' dilution levels. Additionally, deforestation of abandoned agricultural land, the construction of dams, and increased water withdrawal upstream, play significant roles in decreasing wetlands' surface area and water discharges, sometimes even locally preponderant. Dams, in particular, cause multiple impacts, including the fragmentation of rivers and aquatic animals' populations, a reduction in the water and sediments delivered to wetlands, and downstream coastal erosion.

Combined, these factors contribute to the loss and degradation of the habitats of thousands of plant and animal species and to the degradation of wetlands, exacerbating the water quality issues and increasing the economic and social costs for the affected populations; people who directly rely on wetlands for their living, often the very poor, are in fact driven into even deeper poverty and frequently have to face severe water stress and increasing water shortages (Mediterranean Wetland Outlook 2018).

The reduction in wetland surface area and biodiversity loss can lead to an increase in infectious diseases, by intensifying the exposure to microbial and chemical contaminants and to water-borne and vector-borne pathogens. Furthermore, the deterioration of wetlands weakens their ability to protect against water-related threats like floods, droughts, and storm surges (Krumar et al. 2017). Precisely, the loss of natural wetlands is reducing their ability to clean and filter water, leading to worsening water quality, with negative consequences on human wellness. This decline in wetland function exacerbates the already alarming trend of water quality deterioration projected to rapidly increase over the next several decades, which will, in turn, increase risks to human health, economic development, and ecosystems. As reported in the study conducted by the International Food Policy Research Institute and Veolia, by 2050, in a drier climate change scenario and a moderate upsurge of income and population, 1 in 3 people will be at high risk of nitrogen and phosphorus pollution, and 1 in 5 people will be at high risk of water pollution from BOD (Veolia, IFPRI 2015). Additionally, changes in wetlands can affect local environmental quality and negatively impact mental health, causing distress or illness due to the inability to find solace in one's home environment (Horwitz et al. 2012).

A reduction in the diversity of communities can endanger human's health by reducing the "dilution effect" and hence increasing the transmission of diseases of pathogenic agents to humans (Keesing & Ostfeld 2021; Kocher et al. 2023).
Some diseases are spread between different host species by a vector, which is a species that does not cause the disease itself but carries the pathogen from one host to another. Among the species infected by the vector, some cannot transmit the pathogen themselves and are referred to as "dead-end" species. More diverse communities have a higher number of these species, which decreases the chances of the pathogen being transmitted to humans by spreading the vectors among more hosts. Additionally, diverse communities help prevent the establishment and spread of invasive exotic species (Galewski et al., 2012).

The higher urban and suburban development has led to an increasingly close contact between people and wetlands, which often are not considered as natural methods of diseases prevention, but as sinks for the proliferation of harmful species. One of those species are mosquitoes. Citizens tent to misunderstand about wetlands and mosquitoes, assuming that all wetlands produce mosquitoes, and all mosquitoes are vectors of viruses that cause diseases such as West Nile virus (Ciota 2017; Mazzacano & Black 2013). Actually, one study conducted by Poulin demonstrated that in the Camargue the competition by some forty species of mosquitoes reduce the presence of non-indigenous mosquitoes, which are vectors of the West Nile. However, a mosquito eradication campaigns is conducted in the region, which could favour the proliferation of mosquitoes that transmit these diseases (Poulin, 2012).

1.3 Mosquito control methods and their effects on non-target organisms

The fight against mosquitoes has been closely tied to the need to reduce the spread of diseases and improve public health. Early organized mosquito control efforts were viewed as an eradication attempt, carried out through aggressive measures like draining and oiling wetlands and using highly toxic, broad-spectrum pesticides, the effect of which still persist in the landscape today (Dye-Braumuller et al. 2020; Mazzacano & Black 2013) . Many of the mosquito control agents used today are more specific and less toxic than those from the past. However, their use still significantly harms many aquatic invertebrates, along with the fish, birds and amphibians that inhabit and rely on wetlands.

Effects on non-target organisms exposed to these products might result from direct toxicity, but they can also be indirect, affecting the composition of wetland communities and food webs by disrupting the food supply for animals.

1.3.1 Chemical control methods

One of the oldest methods for mosquito control involves the use of petroleum-based oils (Lee et al. 2018). These oils are refined products derived from crude oil, consisting of complex mixtures of hydrocarbons, primarily paraffinic and naphthenic (Agnello, 2000).

The effectiveness and characteristics of these oils depend on their specific composition, which is influenced by the source of the crude oil and the refining process. They fight insects through physical actions such as suffocation, disruption of cellular membranes, interference with reproductive processes, and acting as physical barriers or repellents (Andrade-Ochoa et al., 2018; Da Silva et al., 2015; Iikura et al., 2020).

Petroleum-based oils have been employed as a pest control method since the late 19th century. Over time, more refined and targeted formulations, known as horticultural oils, were developed to be less phytotoxic and more effective against a broader range of pests (Agnello, 2000). However, the use of petroleum-based oils must be carefully managed to avoid environmental damage, including water pollution, soil contamination, toxicity to non-target organisms, and phytotoxicity (Mozley & Butler 1978; Najar-Rodríguez et al. 2008).

Subsequently, newer methods were developed. Since the 1950s, organophosphates (OPs) such as Malathion have been used for mosquito control and in agriculture (Dye-Braumuller et al. 2020). OPs are pesticides derived from phosphoric acid that disrupt cholinesterase enzymes (ChE), which regulate the neurotransmitter acetylcholine (Ach). This disruption causes muscular twitching, paralysis, and death in insects (Aroniadou-Anderjaska et al., 2023).

Despite their effectiveness as chemical control methods, organophosphates have considerable environmental and non-target impacts: they are highly to moderately toxic to humans and other animals, highly toxic to beneficial insects, like bees, moderately toxic to fish, and acutely toxic to aquatic invertebrates. This broad-spectrum toxicity represents a significant concern, despite their natural tendency to degrade quickly in the environment.

The persistence in the environment, however, can be prolonged because they strongly bind to soils and sediments. Additionally, OPs can seep into groundwater, posing risks of pollution and poisoning. (Dye-Braumuller et al. 2020; Mazzacano & Black 2013; Karthick Rajan et al. 2024; Rani et al. 2021).

Following the use of organophosphates, other chemical products were discovered. Pyrethroids are synthetic insecticides derived from pyrethrin compounds found in chrysanthemum flowers. They affect the insect nervous system, causing a rapid "knock-down" effect. This effect is enhanced by using a synergist like piperonyl butoxide (PBO), which prevents insects from detoxifying the pyrethroid.

Pyrethroids, such as permethrin, Sumithrin, prallethrin, etofenprox, and deltamethrin, are widely used in mosquito control, agriculture, and household pest products due to their low toxicity to mammals and birds and their effectiveness at low doses (Dye-Braumuller et al. 2020; Mazzacano & Black 2013). Their mechanism of action disrupts the normal function of nerve cells in insects, leading to paralysis and death.

Despite their effectiveness, pyrethroids pose significant risks to non-target organisms and the environment. They are highly toxic to aquatic invertebrates, crustaceans, fish, and beneficial insects like bees (Corcos et al., 2020). The inclusion of PBO in formulations increases the toxicity to fish, amphibians, and other aquatic organisms, and it is considered a possible human carcinogen (Mazzacano & Black 2013). Environmental concerns include the persistence of pyrethroids in soil and sediment, where they can contaminate water bodies through runoff, spray drift, and erosion (Amweg et al. 2005; Gan et al. 2005). This contamination affects non-target aquatic species such as Ephemeroptera, Plecoptera, Odonata, Hemiptera, Coleoptera, and Trichoptera (Antwi & Reddy 2015). Additionally, the continuous application of pyrethroids can disrupt the biodiversity and abundance of non-target species, impacting ecological balance and food webs. Overall, the widespread use of pyrethroids in urban and agricultural settings increases risks for aquatic ecosystems (Ranatunga et al. 2023).

Insect growth regulators (IGRs) are increasingly being used since the late 1980s as alternatives to synthetic insecticides due to their effectiveness at very low quantities, and they have been widely used in mosquito control.

IGRs come in two main forms: juvenile hormone analogues and chitin synthesis inhibitors (Ur Rahman et al., 2024). Juvenile hormone analogues, such as methoprene and pyriproxyfen, disrupt the normal growth and development of insects by mimicking natural hormones doses (Dye-Braumuller et al. 2020; Mazzacano & Black 2013). Methoprene, a terpenoid compound, prevents the transition from larval to adult stages by maintaining high levels of juvenile hormone, resulting in death during the molting process (Ansari et al. 2005). Pyriproxyfen, another juvenile hormone analogue, inhibits the emergence of adults from the pupal stage and disrupts mosquito development and reproduction (Hustedt et al., 2017). Chitin synthesis inhibitors, like novaluron, interfere with the production of chitin, a critical component for insect molting and exoskeleton formation. These IGRs prevent larvae from progressing to the next developmental stage, ultimately leading to death (Ur Rahman et al., 2024).

Despite their targeted action, IGRs can have significant side effects on the environment and non-target organisms, especially in aquatic ecosystems. Methoprene and other IGRs have been shown to reduce populations of non-target insects like chironomids and other dipterans, which can disrupt local biodiversity and food webs. The contamination of water bodies through runoff, spray drift, and erosion can further affect non-target aquatic species, including beneficial insects, crustaceans, and fish (Dye-Braumuller et al. 2020; Nelsen & Yee 2023).

1.3.2 Biocontrol methods

Another approach employed in mosquito control involves the introduction of mosquitoes' natural predators. Historically, one of the most used "traditional" biological control agents are larvivorous fishes. Among these, the mosquitofish species *Gambusia affinis* (Western mosquitofish) and *Gambusia holbrooki* (Eastern mosquitofish) are well-known. Originally native to the southeastern United States, they have been widely distributed worldwide for mosquito control due to their ease of breeding and handling, insatiable appetite, rapid reproduction rate, and ability to thrive in various environmental conditions. Their adaptability, prolific breeding, and voracious predation make them effective candidates for biocontrol (Benelli et al. 2016; Dye-Braumuller et al. 2020; Pyke 2008). However, Gambusia species are not effective against all mosquito species in every habitat, are not specific to mosquitoes, and may struggle to hunt in densely vegetated areas. Paradoxically, they may inadvertently increase mosquito numbers by consuming predatory aquatic insects that naturally prey on mosquito larvae (Mazzacano & Black 2013). Thus, the very traits that make mosquitofish effective for controlling mosquitoes across diverse habitats, also render them ideal invasive species (Walton, 2007).

A more recent strategy for mosquito control involves introducing pathogenic organisms, such as *Bacillus thuringiensis serovariety israelensis* (Bti) and *Lysinibacillus sphaericus*, which are key components of the well-known larvicide VectoMax G (Thierry et al., 2023).
Bti formulations are the main non-chemical method used to control mosquito larvae in the United States, Europe, and several other countries (Benelli et al. 2016; Lacey 2007). Bti produces a protoxin that is highly toxic when ingested by mosquito larvae. These activated toxins disrupt midgut cells, causing the larvae to stop feeding and die (Mazzacano & Black 2013). Bti does not affect all insects, but it is effective against various Diptera, particularly those in the suborder Nematocera (Floore 2006). The most susceptible families within this suborder are Culicidae (mosquitoes), Simuliidae (black flies), and Chironomidae (non-biting midges).

Recent studies have shown that Bti formulations like VectoMax G do not impact species richness or community diversity of macroinvertebrates and zooplankton. However, the continuous elimination of target organisms (anopheline and culicine larvae) could potentially reduce ecosystem diversity and richness over the long term, affecting the structure of the aquatic fauna that coexist with these larvae (Mazzacano & Black 2013; Thierry et al. 2023). This issue warrants further attention, especially in conservation units such as parks or protected areas.

Among the latest discoveries in mosquito control, the Sterile Insect Technique (SIT) and the use of *Wolbachia* bacteria are increasingly gaining attention and being adopted globally. Despite their effectiveness and minimal impact on the environment and non-target species, traditional methods remain more widely used. This preference is mainly due to the high costs associated with implementing these innovative techniques, which require infrastructure, laboratories, specific equipment, and specialized personnel. These expenses extend beyond the production of sterile insects or those infected with *Wolbachia* to include their release and long-term monitoring (Benedict, 2021), as these methods do not provide immediate results like conventional pesticides or oils. Additionally, being relatively new methods, they may encounter regulatory barriers and require further studies to be fully accepted, not only by health and environmental authorities but also by public opinion.

SIT involves releasing large numbers of sterile male insects that compete with wild males to mate. The offspring from these matings will be sterile, reducing the reproductive capacity of females and ultimately decreasing the target population (Alphey et al., 2010). One of the main advantages of SIT is its species specificity: released males only seek out females of the same species, making this technique environmentally friendly with minimal non-target effects. Since no toxic chemicals are used, the only expected impacts are indirect, resulting from the reduction or local elimination of the target vector or pest species (Alphey et al., 2010).

Wolbachia are common intracellular bacteria found in arthropods and nematodes. These alphaproteobacteria endosymbionts are transmitted vertically through host eggs and alter host biology in various ways, including inducing reproductive manipulations such as feminization, parthenogenesis, male killing, and sperm-egg incompatibility. They can also move horizontally across species boundaries, resulting in widespread and global distribution among diverse invertebrate hosts. In symbiotic relationships with arthropods, *Wolbachia* exhibit a range of phenotypic effects and generally act as reproductive parasites (Werren et al. 2008).
One of the key mechanisms by which *Wolbachia* controls mosquito populations is through cytoplasmic incompatibility (CI), that leads to early embryonic death when males infected with *Wolbachia* mate with females that are not infected, reducing the proportion of uninfected mosquitoes (Hoffmann et al. 2011).

The process of introducing *Wolbachia* into mosquito populations involves releasing large numbers of infected mosquitoes into the environment. However, this method faces challenges, particularly regarding the ecological impacts of releasing large numbers of modified mosquitoes (Popovici et al., 2010). Concerns include the potential for horizontal gene transfer between *Wolbachia* and other organisms, which can introduce unknown phenotypes into new hosts, affecting phylogenetically close or distant species (Ahmed et al. 2016; Werren et al. 2008). Additional research is ongoing to prevent the accidental release of female mosquitoes into the environment, due to the risk of unwanted population replacement and the establishment of *Wolbachia* bacteria in the environment, as well as the ability of female mosquitoes to bite and spread pathogens (Mains et al., 2016).

1.3.3 Monomolecular Surface Films

During the 1980s (Nayar & Ali 2003) a new type of chemical mosquitoes control method was developed as an upgrade of the already existing surface oils. Monomolecular surface films (MMFs) emerged as a safer and more efficient alternative, being less harmful to non-target organisms and needing lower application rates compared to petroleum oils. MMFs were introduced also to address issues associated with chemical insecticides, such as vector resistance, ecological harm, and environmental pollution.

Consequently, researchers and developers shifted their focus to non-insecticidal compounds, particularly those suitable for urban areas where indoor insecticide spraying is impractical, and vector control primarily relies on antilarval methods (Batra et al., 2006).

MMFs are alcohol ethoxylates (AEs), a type of non-ionic surfactant produced by adding ethylene oxide to an organic alcohol, typically in the presence of a catalyst.

When applied to water, MMFs form an invisible, one-molecule-thick layer on the surface. This layer kills mosquito larvae and pupae by reducing surface tension, making it difficult for them to attach their siphon tubes to the water surface for breathing. Wetting of tracheal structures can also occur, causing anoxia. Consequently, the immature stages of mosquitoes suffocate. Additionally, adult mosquitoes attempting to emerge from the pupal stage at the air-water interface become wetted with the MMF and drown. Drowning may also occur when adult mosquitoes that land on the water surface to lay eggs (Stark 2005).

Recently, a new MMF (Aquatain® Mosquito Formulation) has been developed. Like its predecessors, Aquatain AMF® forms a thin film on the water surface, disrupting surface tension and preventing mosquito larvae and pupae from breathing, thus acting as a larvicide, pupicide, and adulticide. According to its producers, Aquatain AMF® is suitable for both professional and domestic use in still and stagnant waters such as lakes, reservoirs, rice fields, sewers, drains, manholes, drainage channels, water tanks, drinking water, saucers, buckets, and gutters; and is effective against *Aedes albopictus*, *Culex pipiens* and other mosquito species (Dieng et al. 2022; Kavran et al. 2020; Mbare et al. 2014).

1.4. Aquatain

Aquatain is a silicone-based layer primarily composed of polydimethylsiloxane (PDMS). The exact content of PDMS in Aquatain can vary slightly depending on the manufacturer and the specific formulation. Aquatain AMF distributed in Italy by Bleu Line contains 89% PDMS. However, according to the label, Aquatain AMF manufactured by Aquatain Products Pty Ltd. contains 78% active ingredient (PDMS) and 22% inert ingredients. Its mode of action is based on lowering the water surface tension, classified as a physical mosquito control method, causing the drowning of eggs, suffocation of larvae and pupae, and prevention of adult emergence and oviposition, even in presence of wind and rain (Bukhari et al. 2011; Mbare et al. 2014; Dawood et al. 2020; Kavran et al. 2020).

1.4.1 PDMS' properties

PDMS is a polymer of the silicone-based elastomers, characterized by a repeating backbone of oxygen (O) and silicon (Si) atoms, which are bonded to two methyl groups (Ariati et al. 2021). This unique organic/inorganic structure imparts PDMS with outstanding physicochemical properties, including optical transparency, hydrophobicity, chemical inertness, permeability, non-toxicity, and excellent biocompatibility (Meng et al. 2020; Xia et al. 2019). The robust Si-O bond contributes significantly to PDMS's thermal stability, making it ideal for high-temperature applications such as heat-transfer agents and high-performance elastomers. The bonding characteristics and chemical composition of its side groups result in remarkably low surface free energy, leading to distinctive and desirable surface properties (Mark 2004). PDMS chains exhibit unparalleled flexibility and mobility compared to other polymers, enabling high permeability, low viscosity, and unique surface qualities across a broad temperature range. Furthermore, its chemical structure allows for modification with various chemical groups, making PDMS a versatile material used widely as a matrix in diverse applications (Mark 2004; Meng et al. 2020; Kong et al. 2023).

1.4.2 Product Technical Specifications

According to the technical data sheet, Aquatain AMF, available in a 50 ml domestic use package distributed in Italy by Bleuline, is composed of 89% polydimethylsiloxane (PDMS) and 11% inert ingredients and adjuvants. When applied to stagnant and still water, it creates a very thin, rapidly spreading film. This film works through a physico-mechanical action and remains effective for at least 4 weeks. Aquatain AMF is suitable for various still water environments, including lakes, reservoirs, rice paddies, ditches, manholes, drainage channels, water tanks, and flowerpot saucers. The recommended application rate is 1 ml per square meter of surface. PDMS does not affect key water parameters such as temperature, pH, dissolved oxygen, hardness, ammonia, or nitrogen compounds. The product is considered "eco-friendly" and environmentally safe.

For the variant distributed by AQUATAIN PRODUCTS PTY. LTD., there are no reported ecological impacts associated with its use. Aquatain AMF is a high molecular weight liquid polymer with a very low vapor pressure (<1 mm Hg), making it unlikely to become an atmospheric contaminant unless aerosolized (which is improbable since the product is self-spreading and does not require spray equipment). The non-volatile nature and strong binding affinity for particulate matter ensure that it adsorbs to particles and settles out, thereby not contaminating the water. Polydimethylsiloxane degrades abiotically in soil into smaller molecules, which are then either biodegraded or volatilized into the air, where they decompose under sunlight. In optimal conditions, the final degradation products are inorganic silica, carbon dioxide, and water vapor. The product is removed from wastewater by over 80% during treatment. Additionally, it is reported to have no adverse effects on soil microorganisms, earthworms, subsequent crops, or aquatic organisms.

1.4.3 Aquatain's effects on mosquitoes: a study rewiew

Several studies aimed at demonstrating the effects and efficacy of Aquatain on various mosquito species at different developmental stages have been conducted in natural, semi-natural, and laboratory environments. Kavran et al.'s study showed that in laboratory conditions, the product applied at a dose of 1ml/m² is particularly effective against *Aedes albopictus*, reducing the number of L1-L2 larvae by 72% just one day after application. The mortality rate of *Culex pipiens* larvae was much lower at one day post-application (17%) but reached 100% by the second day, whereas similar mortality levels (98.6%) in A. albopictus were achieved after ten days of exposure. Older larvae (L3-L4) and pupae were more susceptible to AMF than L1-L2 instars. This differential susceptibility is likely due to the reduced ability of late instars and pupae to utilize dissolved oxygen, in contrast to younger larvae that are less dependent on atmospheric oxygen. Similar results were observed under semi-natural conditions, with the mortality in an *A. albopictus* population reaching an average of 95% eight days after product application. However, such high mortality rates can be achieved in a shorter time when higher temperatures accelerate the development of juvenile stages, making them more susceptible (the relationship between higher temperatures and increased mortality was further confirmed by the study of Dawood et al.). The susceptibility of *C. pipiens* was also confirmed in field experiments, showing mortality rates of 99.55% and 88.76% in the two study areas, two days after application. Persistence times and long-term effects, however, were highly variable. One significant influencing factor was the presence of vegetation. Results indicated *"...a substantial reduction on day 21 of treatment and a lower reduction up to day 28 in a densely vegetated channel, whereas mosquito reduction persisted (up to 56 days) in channels without vegetation*", where the AMF layer remained undisturbed. In densely vegetated water bodies, females can find 'openings' on the water surface for oviposition, where AMF is unable to reduce surface tension. In such channels treated with AMF, females may start laying eggs as soon as these open patches form.

Similarly, Dawood et al. evaluated the effectiveness of Aquatain AMF against immature stages of *Culex pipiens*. They observed mortality in both pupal and larval stages at various Aquatain AMF doses (0.5, 1, and 2 ml/m²) and different temperatures. Pupal mortality (average %) reached 76.0%, 86.0%, and 98.2% 12 hours after treatment, compared to 0.2% mortality in untreated water, for doses of 0.5, 1.0, and 2.0 ml/m², respectively. Higher mortality rates were observed in 4th instar larvae across all Aquatain™ doses, with average mortality (%) reaching 62.9%, 80.4%, and 88.9% at 0.5, 1.0, and 2.0 ml/m², respectively, 24 hours after treatment. For 1st instar larvae, the average mortality (%) was 15.1%, 26.9%, and 38.2% at 0.5, 1.0, and 2.0 ml/m², respectively, after 24 hours post-treatment. At lower treatment doses (0.5 ml/m²), average mortality was 15.1% for 1st instars and 62.8% for 4th instars after 24 hours of exposure, and 76.0% for pupae after 12 hours of exposure.

A significant reduction in larvae and pupae population of *Aedes albopictus* and *Culex pipiens* were also measured by Drago et al., in northeastern Italy. Additionally, both species showed more than 90% inhibition of adult emergence for two weeks. A field study revealed that a similar level of emergence inhibition was observed also in *Anopheles gambiae* and *A. arabiensis*. Although this inhibition became evident only three weeks after treatment, it remained below 10% up to six weeks following the product application (Mbare et al., 2014). This study highlights the high susceptibility of these two species by applying half the recommended dosage (0.5 ml/m²), resulting in ≥90% larval mortality. When the sub-lethal dose was used, live larvae in treated ponds often exhibited signs of weakness, such as slow movement when disturbed on the water surface, in contrast to those in untreated ponds. Additionally, females that emerged from ponds treated with sub-lethal doses of AMF were 2.2 times less likely to lay eggs compared to females from untreated ponds.

Baz's study (Baz M.M. 2017) focused on the effects of applying doses of 0.5 ml/m² and 1 ml/m² of Aquatain AMF in abandoned wells, which had previously been used for irrigation but have now become ideal breeding grounds for species such as *Culiseta longiareolata*, *Culex antennatus*, and *C. univittatus*. Consistent with earlier research, the study demonstrated the product's effectiveness against mosquitoes, showing a mortality rate on *C. pipiens* larvae of 97.9% within three days when using a sub-lethal dose, and 100% when using the manufacturer's recommended dose. In the case of the sub-lethal dose, a mortality rate below 50% was observed 15 days after application, whereas with the recommended dose, this level was reached after 18 days.

A different study was conducted by Dieng et al. (2022) on female *A. aegypti*, offering them various combinations of sites for oviposition. When all sites contained untreated water, no significant preferential choice was recorded between the sites, with similar numbers of eggs laid per site. Specifically, out of the 2084 eggs deposited, 30.13% (628 eggs) were in W1, 20.49% (427 eggs) in W2, 22.69% (473 eggs) in W3, and 26.68% (556 eggs) in W4. However, when given a choice between two AMF-treated sites and two untreated water sites, significantly more eggs were laid in the untreated water sites. Of the 1185 eggs oviposited, 95.44% (1131/1185) were deposited in containers with water, and 4.56% (54/1185) deposited in containers with AMF-treated water. Similar results were observed in environments with more AMF-treated options. When only AMF-treated containers were available, *A. aegypti* still deposited eggs in all four containers, but the total number of deposited eggs was significantly lower (540). Additionally, female mortality rates were high in AMF-treated environments, increasing with the number of such sites. Thus, this study demonstrated that AMF served as a deterrent to ovipositing *A. aegypti* and indirectly acted as an adulticide.

Particularly interesting is the study by Bukhari et al. (2011) where twelve 0.5-acre rice paddies were selected for a field study. Six paddies were designated as control and six as treatment groups, ensuring that both groups were comparable in terms of mosquito larval densities and adult emergence, the number of each non-target species caught, and rice plant characteristics (variety, height, plant density, and tiller count). Aquatain was applied twice during the study. The first application of Aquatain caused a 93% reduction in the emergence of *anopheline* adults and a 70% reduction of *culicine* adults compared to the emergence in the control and treatment paddies before treatment. Similarly, there was an 88% reduction in the emergence of anopheline adults and an 82% reduction of culicine adults after the second Aquatain application. Additionally non-target organisms from various taxa were collected using area samplers and emergence traps to assess if Aquatain affected their abundance. The study found no significant difference in the densities of most non-target organisms between control and treatment paddies, indicating minimal adverse effects, except for backswimmers, which experienced a notable reduction in population density.

Numerous studies have been conducted to test both the efficacy and the side effects of MMFs, particularly Arosurf® MSF and Agnique® MMF; the first commercial MMFs (Table 1.1). As Stark (2005) noted, "The vast majority of studies indicate that MMFs have little effect on non-target organisms. The only species that may be vulnerable are those that make contact with the air-water interface to breathe or live on the water surface. However, even though a fairly large number of non-target species have been evaluated, there is still a lack of information concerning the potential long-term impacts of repeated applications to wetlands."

There are few studies that test the efficacy of Aquatain on non-target fauna. These studies were conducted under varying conditions, some in standard settings and others in semi-field or field environments. Consequently, the effects of Aquatain can vary significantly depending on the circumstances, potentially concealing negative effects on non-target organisms. For example, Bukhari et al. (2011) tested Aquatain's efficacy in rice paddies and found that "the presence of vegetation, both rice plants and algae, may have provided a substrate for broad-shouldered water striders to hold on to, preventing them from drowning. Similar to mosquito larvae, the water beetles and backswimmers may have utilized the untreated water surface in between the algal mass to pick up the air bubble they require for respiration." Additionally, "backswimmers are active predators and are known to detect their prey by the surface waves […] reduced surface tension of water might have affected their ability to forage for prey and therefore reduced their survival.".

Organism	Effect	Reference
Mosquito fish (*Gambusia affinis*)	No adverse effects	Levy et al. 1982
Aquatic snail (*Gyraulus* sp.)		
Tree frog (*Hyla cinerea*)	No adverse effects	Webber & Cochran 1984
Suckermouth catfish (*Hypostomus plecostomus*)		
Longnose killifish (*Fundulus similus*)	No adverse effects	Hester et al. 1991
Grass shrimp (*Palaemonetes pugio*)		
Freshwater shrimp (*P. paludosus*)		
Fiddler crab (*Uca* spp.)		
Crayfish (*Procambarus* spp.)		
Freshwater amphipod (*Gammarus* spp.)		
Freshwater isopod (*Asellus* spp.)		
Fairy shrimp (*Streptocephalus seali*)		
Polychaete (*Laeonereis culveri*)		
Mayfly naiads (*Callibaetis pacificus*)	No adverse effects	Mulla et al. 1983
Diving beetle adults (*Berosus metalliceps*)		
Corixids (*Corisella* spp.)	Acute lethal effects	Takahashi et al. 1984
Notonectids (*Notonecta unifasciata*)		
Clam shrimp (*Eulimnadia* sp.)		
Beetle adults (*Tropisternus lateralis*)		
Hemipterans		
Dytiscidae	No adverse effect	Karanja et al. 1994
Hydrophilidae		
Planorbidae		
Ampullaridae		
Corixidae (*Micronecta* spp.)		
Notonectidae (*Anisops* spp.)		
Nepidae		
Belostomatidae		
Glossiphonnidae		
Ranidae		
Notonectidae (*Anisops sardae*)	No adverse effects	Batra et al. 2006
Mosquito fish (*Gambusia affinis*)		
Dytiscidae	No adverse effects	White & Garret 1977
Gerridae	Acute lethal effects	
Dugesia dorotocephala	No adverse effects	Levy et al. 1981
Romanomermis culicivorax		
Tadpole shrimp (*Triops longicaudatus*)	Acute lethal effects	Su et al. 2014

Table 1.1: Side effects of MMFs on non-target organisms. Except for Batra et al. 2006, Su et al. 2014, other studies are reported in Nayar & Ali 2003. White & Garret 1977 is cited in Stark 2005.

1.5 Non-target organisms

In aquatic environments, invertebrates are generally categorized into two practical, non-taxonomic groups: microinvertebrates and macroinvertebrates. Microinvertebrates are very small, typically less than one millimeter in length, and include groups like Protozoa, Cnidaria, Rotifera, Nematoda, Gastrotricha, Tardigrada, and Hydrachnidia. In contrast, macroinvertebrates are larger, usually more than one millimeter in size by the end of their larval or adult stages, making them visible to the naked eye (Campanaro et al. 2005). Many freshwater macroinvertebrates belong to major taxonomic groups typical of terrestrial environments. Their terrestrial origin is evidenced by the fact that some, such as many Beetles and Heteroptera, complete their entire life cycle in water. These animals have developed specific morphological, behavioral, and physiological adaptations for aquatic environments, yet some continue to breathe atmospheric air through specialized mechanisms. Other groups, like Caddisflies and Diptera, maintain a closer connection to the terrestrial environment, as their life occurs both in water and in the air (Campaioli et al. 1994). Aquatic insects are crucial for freshwater ecosystems as they are involved in primary consumption, decomposition of organic matter, nutrients' recycling and predation. They play key roles in food chains, acting as both predators and prey, which supports various species such as fish, birds, and amphibians, and helps manage populations of harmful invertebrates like mosquitoes. Thus, these insects are essential for transferring energy and nutrients between aquatic and terrestrial environments, and their presence signals a well-functioning and balanced ecosystem (Santos & Fernandes 2021; Vörösmarty et al., 2010).

The non-target organisms used in this study are described below.

1.5.1. Dytiscidae

The order of beetles includes the largest number of insects present on Earth, with approximately 400,000 described species. Adults have a hard exoskeleton, elytra that cover functional wings, and a compact body. Although only 5% of beetle species are aquatic, they constitute one of the main groups of freshwater arthropods (Millan et al. 2024). Defining a beetle as aquatic is, however, an extremely complex operation; this depends on the amount of time spent in contact with water, the level of immersion, the degree of dependence on water, and the motivation to come into contact with water (Jach & Balke 2008). But for some lesser-known species, such information can be very difficult to obtain (Del Claro & Guillermo 2019). Among the most numerous families are: Dytiscidae, which includes approximately 4,000 species, Hydrophilidae, Hydraenidae, and Elmidae (Jach & Balke 2008).

Also called “water beetles” or “diving beetles”, dytiscids are one of the most feared predators in freshwater environments. Adults are distinguished from other aquatic beetle families by their highly specialized adaptations, including a rounded, dorsoventrally flattened body shape, evolved for optimal swimming stability and flow adaptation. They additionally possess large, oar-like hind legs with enlarged distal segments (tibia and tarsus) equipped with thrust-creating swimming hairs or platelets (Figure 1.1), as well as variable respiratory mechanisms that enable their survival in aquatic environments (Millan et al. 2024; Nachtigall 2009; Yee 2023).

Figure 1.1: Adult of Eretes griseus (Fabricius, 1781) at the tub's bottom

Adults are usually dark in color, brown or black, sometimes with yellowish margins or spots, but there are also species of other colors, including reddish, tawny, or pale with a dark-patterned dorsal side (such as *Eretes griseus*, observable in the figure). The cuticle of the elytra is usually smooth and glabrous, sometimes finely silky or heavily punctate. Many species possess large polygonal meshes impressed on the elytra (“reticulation”), which may sometimes occur in combination with finer reticulation (“micro-reticulation” or “secondary reticulation”), followed by coarser patterns ("macro-reticulation" or "primary reticulation") that can present different designs depending on the species. The head is recessed into the pronotum, with large compound eyes but absent ocelli (Yee et al. 2023). The dorsal surface of the body is usually sclerotized, with colors such as pale yellow, dark gray, dark brown, black, or greenish (Figure 1.2), while the ventral surface is primarily membranous, typically yellowish-white or transparent (Balke et al. 2005).

Dytiscid larvae are campodeiform with a heavily sclerotized cephalic capsule and prognathous mouthparts with well-developed mandibles, optimized for predation and external digestion; they

Figure 1.2: Specimens of Hydaticus leander (Rossi, 1790; "a"), Eretes griseus (Fabricius, 1781; "b"), Agabus didymus (Olivier, 1795; "c")

are indeed large and curved, equipped with ducts through which they inject digestive enzymes into their prey. The body has varied shapes, usually elongated and fusiform, generally wider at the metathorax or mid-abdomen, terminating in an elongated segment with a respiratory siphon. Like adults, larvae vary in size among species. Larvae have well-developed legs and a pre-tarsus equipped with claws, which ensure high predatory capacity (Yee et al. 2023); they are indeed fierce and voracious predators, even dangerous to adult specimens.

When mature, larvae leave the water to pupate in the soil, inside self-constructed pupal chambers. In pupae, the cuticle color is whitish or slightly yellow, orange, or brown. The pupation time varies widely from a few weeks to several months, although such information is lacking for most species (Yee et al. 2023).

Adults require more frequent contact with the atmosphere than juvenile stages in order to breathe. For this reason, they are equipped with an air reserve beneath the elytra, which must be regularly renewed by breaking the surface of the water with the tip of the abdomen. The duration of immersion varies depending on the species, temperature, and activity (Calosi et al. 2007). The sub-elytral cavity is connected to thoracic and abdominal spiracles from which the tracheal system branches throughout the entire body. When the beetle submerges, the oxygen in the bubble is progressively consumed, and the carbon dioxide produced by metabolism is released into the water. This air reserve, retained in the sub-elytral cavity, can also be expelled and retained by hydrophobic hairs at the tip of the abdomen (Larson et al. 2000), thus functioning as a physical gill (Balke et al. 2004), which, during inactivity, allows the beetle to remain submerged for a longer period of time (Figure 1.3; Figure 1.4). The dissolved oxygen in the water diffuses into the bubble, while nitrogen slowly diffuses out, causing a decrease in the size of the bubble over time. As the surface area of the bubble decreases, the gas exchange rate decreases, and the beetles must resurface (Yee et al. 2023). Dytiscids can be found in a wide range of environments, such as groundwater, moist soils, steppe lakes, ponds, forest puddles, large lakes, rivers, streams, springs, and even high-altitude alpine lakes. These beetles hide among emergent or submerged vegetation, along the banks or shores of aquatic habitats. Most species prefer habitats with abundant aquatic vegetation, and as a result, nutrient-rich areas tend to host a diverse community of Dytiscids (Balke et al. 2005).

Figure 1.3: Adult of Hydaticus Leander (Rossi, 1790) while restoring its air reserves

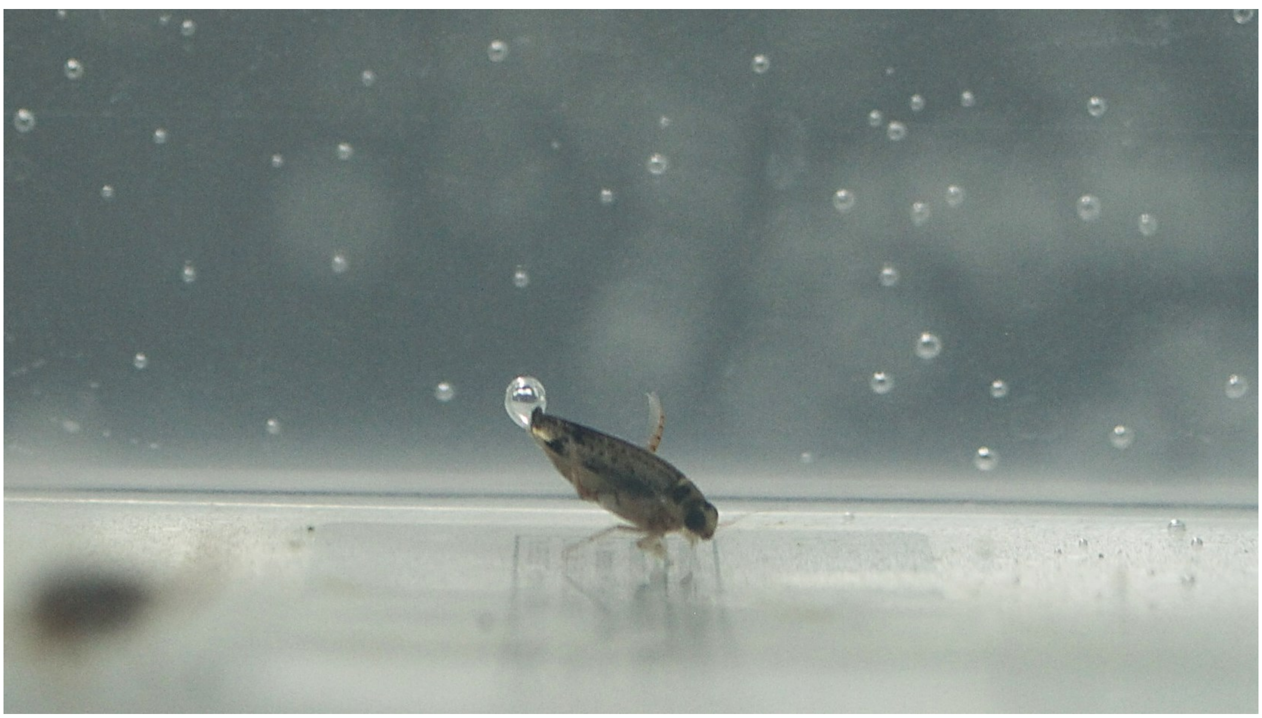

Figure 1.4: Particular of the air bubble at the top of the abdomen of a Eretes griseus (Fabricius, 1781)

1.5.2. Gyrinidae

Gyrinidae, also known as whirligig beetles, are small insects that, in some geographical areas, can reach up to 17 mm in length (Stals 2007). Dark brown or black in color, they are excellent swimmers and have specialized adaptations for life on the water's surface. On sunny days, they move in circles on the surface of streams or small ponds, with movements that resemble a kind of dance. On cloudy days or when they feel threatened, they retreat to the shore or dive underwater (Stals 2007). These beetles often move in groups composed of hundreds of individuals, sometimes even belonging to different species (Balke et al. 2005; Kriska 2022; Stals 2007).

The body of the whirligig beetle is oval in shape, with a highly convex dorsal side and a slightly flattened ventral side, surrounded by a sharp edge that separates the hydrophobic dorsal surface from the wettable ventral surface (Stals 2007). The elytra are marked by rows of small depressions and are shorter than the abdomen, which remains visible to the naked eye (Figure 1.5).

The head has compound eyes, divided by the sharp lateral edge: the upper eyes face upwards, while the lower ones face downwards. Between the two groups of eyes are the antennae, which detect vibrations that help locate prey, such as dead or weakened insects floating on the surface. Gyrinidae

Figure 1.5: Particular of the dorsal surface of a Gyrinus substriatus (Stephens, 1829)

are predators, though they sometimes behave as scavengers. Their elongated front legs are used to capture prey and cling to substrates when diving, while the shorter, flattened middle and hind legs, shaped like paddles, enable them to swim swiftly both on the surface and underwater (Balke et al. 2005; Kriska 2022; Stals 2007). When submerged, the beetles breathe using air trapped between the abdominal hairs under the elytra, forming a visible bubble at the tip of the abdomen (Balke et al. 2005; Kriska 2022).

Females lay their eggs on submerged or floating vegetation, often in rows or clusters. The larvae, which are elongated and flat, crawl on vegetation or the substrate, but they are also capable of swimming using undulating body movements. Apneustic, they breathe through their thin body cuticle and lateral tracheal gills. They spend their entire development underwater, unlike the adults that remain mostly on the surface. The larvae are carnivorous and predatory, digesting their prey externally using mandibles that inject digestive enzymes. In the autumn, mature larvae build cocoons from debris, mud, and an adhesive substance secreted by the larvae themselves, attaching them to plants or surfaces. Inside these chambers, the larvae pupate until emergence, which occurs after about 8-14 days (Nilsson 1996 in Kriska 2022).

Gyrinidae inhabit nearly all freshwater environments, though some species can also be found in brackish waters. They can adapt to various habitats, from stagnant waters to slow-flowing rivers, and even in calm inlets of fast-flowing streams (Kriska 2022; Stals 2007).

1.5.3. Gerridae

Called "water striders," "pond skaters," or "wherrymen," the Gerridae family includes 63 genera and around 530 species, distributed worldwide (Aukema 1995). With few exceptions, they spend almost their entire life cycle on the water's surface, and over time, they have undergone specific morphological adaptations to suit this lifestyle. Depending on the species, they range in length from 1.6 mm to 36 mm. They have long legs and bodies that vary from an elongated to globular shape (Henry 2017). The entire body and appendages are covered with a layer of micro- and macro-hairs, which provide water repellency and prevent sinking (Kriska 2022). Their heads, which lack ocelli, extend beyond the anterior edge of their compound eyes. The front legs are relatively short and robust, while the middle and hind legs are slender and elongated, with femurs and tibiae generally of similar length, enabling them to "glide" on the water's surface (Kriska 2022). The claws of the middle and hind legs are typically smaller than those of the front legs (Figure 1.6).

Figure 1.6: Ventral view of a Gerridae spp.

Wing polymorphism is common (Henry 2017; Aukema 1995), with macropterous and apterous morphs being predominant.

The most distinctive feature of the Gerridae, developed to adapt better to life on the water surface and unique among Heteroptera, is the modification of the thorax: it is greatly elongated, with the middle and hind coxae oriented on a horizontal plane. Most species of the genus *Gerris* have black backs, while the middle and posterior parts of the pronotum and sometimes the elytra may be yellowish, reddish, or brown in some species (Kanyukova 2023).

Adults overwinter on land, but their development cycle, including egg-laying—on floating or submerged plants (Aukema 1995)—and nymph growth, is associated with water. Water striders are polyphagous predators that feed on planktonic arthropods and those fallen on the water's surface, and they may act as pest control agents in aquatic environments (Aukema 1995; Kanyukova 2023;

Kriska 2022). They capture insects fallen into the water with their front legs and then suck the body fluids (Kriska 2022).

Water striders inhabit diverse aquatic environments, forming large colonies or groups of varying sizes. Some species are found only in still waters, such as lakes, ponds, marshes, and calm parts of streams and rivers, usually avoiding surfaces with extensive vegetation, while others live in flowing waters, coastal marine habitats, and even the open ocean, such as the species *Halobates*, known as "marine striders" (Aukema 1995).

1.5.4. Corixidae and Micronectidae

Corixidae, commonly known as "water boatmen" due to their characteristic swimming style, are insects with an elongated oval body, flattened dorsoventrally. Their length can vary from 1.5 to 16 mm, and their back is decorated with transverse stripes and bands (Henry 2017; Kriska 2022. Figure 1.7).

Figure 1.7: Dorsal view of a Sigara nigrolineata (Fieber, 1848)

They have wings that allow for short movements, which are used by adults of univoltine species to migrate to larger habitats where they will spend the overwintering period, while many multivoltine species overwinter in the egg stage (Thorp & Covich 1991). They possess a short, broad, unsegmented triangular rostrum. The tarsi of the front legs are paddle-shaped and are used for gathering food, while the hind legs, highly developed and equipped with long swimming hairs, are used like oars for underwater propulsion. The shape of the front legs allows these insects to collect algae or other debris from the substrate; however, there are some predatory species that primarily feed on mosquito larvae and chironomids. Most species live in freshwater, whether still or slow-flowing, but they are not found in fast-flowing waters. They are typical of permanent aquatic habitats but can easily colonize temporary ones as well. A few species have adapted to living in brackish environments (Scudder 1976 in Cheng 1976).

The respiration of Corixidae occurs through air bubbles carried on their bodies, which function as both physical gills, facilitating gas exchange with dissolved oxygen in the water, and gas reserves. They have spiracles located ventrally in the thoracic and abdominal sections, from which atmospheric air accumulated in the bubbles enters during inspiration, and these bubbles must be periodically renewed by returning to the surface. When submerged, the insect releases gas from the front thoracic spiracles into the space between the pro-thorax and ptero-thorax. The hind legs, equipped with special water-repellent hairs, then push this gas backwards across the body's surface. The abdomen, flattened from top to bottom, helps with gas exchange between the stored air and surrounding water. Oxygenated gas is drawn in through the first pair of abdominal spiracles and moves forward through the thorax (Popham 1959). This same strategy is used by Micronectidae (Parsons 1976).

Previously, Micronectidae were considered a subcategory of Corixidae due to their similar morphological characteristics, such as the unsegmented triangular rostrum; however, they are now regarded by many researchers as a separate family. They are very small, ranging from less than a millimeter to up to 5 mm. They are characterized by an exposed scutellum, three-segmented antennae, the absence of ocelli (Figure 1.8), and a male genital apparatus that allows for stridulation, which has garnered significant attention from experts (Reid et al. 2018).

Figure 1.8: Dorsal view of a Micronectidae spp

1.5.5. Notonectidae

The Notonectidae, or "backswimmers," are medium-sized insects ranging from 5 to 15 mm in length, with a flat belly and a strongly convex back (Henry 2017; Kriska 2022). Their coloration varies from black to brown or yellowish, often with distinctive patterns that give their elytra a marbled appearance in shades of orange and black (Berchi 2013), as seen in *Notonecta maculata* (Figure 1.9).

Figure 1.9: Lateral and dorsal view of an adult of Notonecta maculata (Fabricius, 1794)

Their hind legs resemble oars, fringed with long swimming hairs (Kriska 2022). While swimming, they orient their ventral side towards the water's surface, and their dorsal side faces downward (Figure 1.10), which is why they are commonly known as backswimmers (Nelson 2023).

Figure 1.10: An adult of Notonecta maculata (Fabricius, 1794) while swimming upside-down

When disturbed, they quickly dive down but can also exit the water and fly, producing a loud buzzing sound (Del Claro & Guillermo 2019; Nelson 2023). These polyphagous predators feed on small prey like amphipods and aquatic arthropods, but they can also capture larger prey, such as small fish and tadpoles (Aukema 1995; Nelson 2023). Additionally, it has been shown that *Notonecta maculata* releases substances that deter mosquitoes like *Culiseta longiareolata* from laying eggs (Silberbush et al. 2010). In Europe, adult Notonecta species are typically visible in summer, with some species overwintering as adults and reproducing in spring, while *Notonecta maculata* may overwinter as either eggs or adults (Savage 1989 in Nelson 2023). Eggs are usually laid on aquatic plants or debris (Aukema 1995). Most species live in lentic habitats or slow-flowing river sections, avoiding fast-flowing waters. They tend to prefer open areas with limited aquatic vegetation, as too much plant matter can restrict air supply.

Their respiration is facilitated by hydrophobic microstructures (microtrichia and setae) on their body surface, particularly on their elytra, which allow the formation and maintenance of an air film, even during dynamic activities like swimming. This air film not only aids in breathing by retaining a reserve of air for extended periods but also reduces drag as they move through the water (Ditsche-Kuru et al. 2011). This air reserve is periodically renewed at the surface (Figure 1.11), allowing the insect to exploit dissolved oxygen in the water that diffuses into the air bubble, thus extending the time they can remain submerged.

Figure 1.11: A backswimmer while breathing from the atmosphere

1.6 Study aim

The implementation of the biocide regulation has significantly reduced the number of products available on the market while also driving up the costs of registering new ones. This stricter regulatory framework has presented considerable challenges for companies in the industry, forcing them to seek alternative solutions to remain compliant. To navigate the restrictions imposed by the biocide regulation, many companies have shifted toward developing products that eliminate insects through mechanical, or physical, rather than chemical means. Since these products are not classified as biocides, they are exempt from the rigorous registration process required for chemical products, enabling companies to avoid the associated costs and complexities.

The development of products like Aquatain is a clear example of how companies have adapted to the challenges presented by European biocide regulations. This approach not only ensures compliance with current laws but also addresses the growing consumer demand for more eco-friendly and sustainable pest control options. However, the absence of strict regulations for mechanical methods introduces potential risks.

Because Aquatain is not subject to the same level of scrutiny as chemical biocides, there is concern about the lack of data on its potential side effects on non-target species. Therefore, the aim of this study is to evaluate whether Aquatain might have unintended consequences on non-target organisms, contributing to a more thorough understanding of the potential risks associated with products that, while not classified as biocides, could still have an environmental impact.

2. Material and methods

2.3. Sampling design

The aim of this study was to investigate any potential side effects of Aquatain on insects that rely on atmospheric oxygen or water surface tension for survival. Due to the limited knowledge about the presence of organisms potentially susceptible to the product in the area surrounding the city of Padua, as well as the scarcity of studies addressing the side effects of Aquatain on aquatic macroinvertebrates, the sampling phase began with a comprehensive bibliographic analysis. This analysis involved consulting checklists of Italian fauna at national, regional, and, where possible, local levels, along with articles and studies on species abundance and richness in Italian wetlands.

This preliminary work facilitated the selection of the organisms studied, and the identification of habitats most favorable for their development. The insects examined in this study belong to two orders: Coleoptera and Hemiptera. From the former, specimens of various species within the families Dytiscidae and Gyrinidae were sampled, while from the latter, species from the families Gerridae, Notonectidae, and Corixidae were collected.

In the first half of June, surveys were conducted in various wetlands around the city of Padua to identify the most suitable areas in which to find specimens of Gerridae, Corixidae, Micronectidae, Dytiscidae, Notonectidae and Gyrinidae. Site selection was primarily based on three factors: species presence, species abundance, in order to avoid compromising ecosystem stability, and proximity, to minimize potential transport-related damage. Once the most suitable sites were identified, the final sampling phase, aimed at collecting specimens to verify Aquatain's effects, could begin. This sampling phase, along with laboratory tests, was conducted from July to September. Final sampling areas, and groups found in them, are listed in Table 2.1 and shown in Figure 2.10.

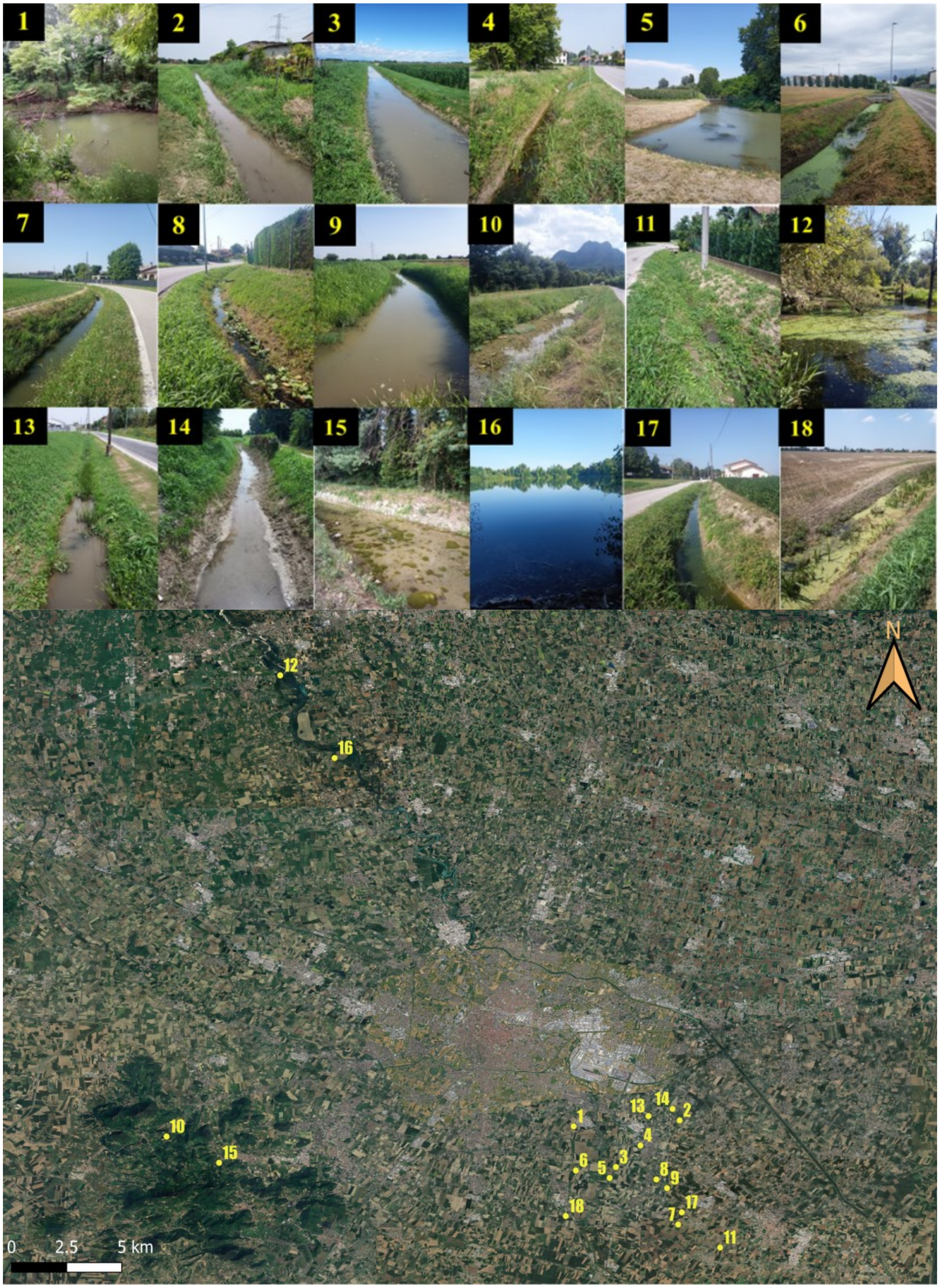

Figure 2.10: Sampling sites and their locations around Padua, shown at a scale of 1:10,000.

ID	Address	City	Latitude	Longitude	Type	Organisms
1	Boschetto Legambiente	Saonara	45.353456	11.920959	Pond	Gerridae, Corixidae
2	Via Granzetta	Saonara	45.356832	11.982262	Canal	Corixidae
3	Via Orsaretto	Polverara	45.330447	11.945418	Canal	Corixidae
4	Via Orsaretto	Legnaro	45.342642	11.959577	Ditch	Gerridae
5	Via L. Da Vinci	Polverara	45.324259	11.941777	Lock pool	Corixidae
6	Via Gruato	Gruato	45.328472	11.92227	Ditch	Dytiscidae
7	Via Palù inferiore	Brugine	45.297948	11.981372	Ditch	Dytiscidae, Corixidae
8	Via N.Sauro	Legnaro	45.323434	11.968797	Ditch	Dytiscidae
9	Via N.Sauro	Legnaro	45.318490	11.974963	Canal	Gerridae
10	Via Calti Pendice	Teolo	45.347713	11.685665	Canal	Dytiscidae, Notonectidae
11	Via Buffa	Campagnola	45.284897	12.0056514	Ditch	Culicidae
12	Area naturale del fiume Brenta	Grantorto	45.6086028	11.7513495	Oxbow lake	Gyrinidae, Gerridae
13	Via XI Febbraio	Legnaro	45.359255	11.964207	Ditch	Dytiscidae
14	Via Grolli	Saonara	45.363338	11.978144	Canal	Corixidae
15	Via J. Facciolati	Torreglia	45.332843	11.716043	Slackwater pool	Gerridae
16	Via Storara	Piazzola sul Brenta	45.561836	11.782789	Lake	Corixidae
17	Via G. Puccini	Brugine	45.304801	11.983505	Ditch	Dytiscidae
18	Via Ca' di Bosco	Ca' di Bosco	45.3027182	11.9164133	Ditch	Dytiscidae

Table 2.2: Location, type and organisms sampled for each study site

2.3.1. Sampling method

Depending on the species to be sampled and the conditions of the sampling area, different tools and capture techniques were used. The tools are listed in the following table (Table 2.3).

Tool	Description	Figure
Sampling Net	Water net with a triangular frame measuring 35 cm per side, equipped with a fine mesh white transparent net (1x1 mm) with a depth of 40 cm. It has a laminated handle 75 cm long.	
Dipper	Equipped with a telescopic aluminium handle, allowing the operator to reach greater depths, it features a circular frame made of stainless steel or aluminium, to which a graduated beaker made of rigid plastic with a capacity of 500 ml is attached.	

Tub	Rigid plastic tub with handles, white in colour, with a capacity of 50 Liters and dimensions of 50 cm in diameter and 20 cm in height.	
Sieve	Small kitchen sieve with a plastic frame and fine aluminium mesh measuring 0.5 x 0.5 mm.	
Pasteur pipette	Disposable 3ml plastic pipette	
Transport containers	Containers of varying sizes and capacities selected based on the species to be sampled. Among the most commonly used are 1-liter glass jars, originally intended for food storage, and 11-liter Ikea Samla containers, with dimensions of 39x28x14 cm.	

Table 2.3: Sampling tools and their features

Upon reaching the sampling area, a net was used to perform "sweeps" along the vegetation on the bank; the collected contents were then gently transferred into a circular plastic basin, previously filled with water from the site. From the basin, the species of interest were extracted using a small sieve for larger specimens, such as Gerridae, Dytiscidae, Notonectidae, and Gyrinidae, or with a plastic Pasteur pipette with the tip cut off (to enlarge the opening) in the case of Corixidae. Specimens of *Micronecta sholtzi*, due to their small size and tendency to remain on the bottom rather than near the bank vegetation, could not be collected with the net; therefore, a dipper was used, performing scoops similar to those recommended for collecting mosquito larvae (Figure 2.8). After collection, the insects were placed in containers filled with water from the site and immediately transported to the laboratory, taking care to secure the containers in the vehicle to avoid sudden movements and damage to the specimens.

2.4. Experimental settings

After capture, the specimens were immediately transferred to the laboratory, where they were subjected to tests in a closed and protected environment, at a constant temperature between 27 and 30 °C, to avoid the influence of external agents on the results. The tests were conducted in Ikea Samla containers, with a capacity of 5 Liters and dimensions of 28x20x14 cm, filled with 2 Liters of dechlorinated water that had been prepared in the preceding days. To ensure the insects could breathe, the lids of the containers were modified by cutting a 22x14 cm window in the centre, which was then covered with a fine mesh tulle netting of 0.5x0.5 mm, secured with hot glue (Figure 2.11).

Figure 2.11: Liters Ikea Samla container used for tests

Before proceeding with the tests, the salinity, temperature, and pH characteristics of the water in each container were measured and recorded using a water quality meter produced by Ueomul. Subsequently, the specimens were placed in each container, with a variable number depending on the species of interest, using a kitchen sieve and tools such as tweezers, spoons, and pipettes. The containers were then labelled with a number and a letter to distinguish the treated environment from the control. Following this, the product was applied. Aquatain AMF® was chosen, in a 50 mL domestic package distributed by Bleuline (Figure 2.12).

Figure 2.12: Aquatain AMF® package used for tests

Initially, the tests were based on the recommended dose of 1 mL/m². Given that the surface area of the containers used for the tests measured 28x20 cm, the dose to be applied was determined to be 56 µL. Subsequently, the dose was reduced to 28 µL, and finally to 14 µL. The different amounts of product were taken from the bottle using a micropipette. Mortality was monitored at intervals of 1 hour, 2 hours, and 24 hours, calculating the ratio between the total number of organisms and those that survived at the end of each interval. The individual species were identified using both dichotomous keys and DNA analysis in cases where identification was too complex. To this end, samples were prepared in test tubes containing absolute alcohol for use in barcoding. After 24 hours, the surviving organisms were returned to their original habitat, the treated water was collected in appropriate containers for disposal, and the containers were washed and prepared for future use. The following table summarizes the characteristics of the study.

2.5. DNA analysis

After the tests were completed, the captured specimens were identified to determine their species. The process began with an analysis of morphometric traits. When it was not possible to confidently identify a species based on these observations, molecular analysis was conducted. Representative specimens from the populations in the trays after treatment were sampled, as identification using dichotomous keys (Elliott & Humpesch 1983; Tamanini 1979) was considered insufficient. The 10 samples selected for barcoding were distributed as follows: Gerridae (5), Corixidae (3), Dytiscidae (1), and Gyrinidae (1).

DNA barcoding (Jinbo et al., 2011) relies on the amplification, sequencing, and comparison of DNA segments with standard sequences stored in databases.

For animal cells, two regions of DNA are used: the cytochrome c oxidase subunit I (COI) and cytochrome c oxidase subunit II (COII) genes, which are highly conserved within the same species but show interspecific variability, making them valuable markers for taxonomic and population genetics studies. Universal primers are used to amplify the target regions of these fragments (Ratnasingham et al., 2007).

The molecular analysis was conducted according to the protocols described in the "DNeasy Blood & Tissue Handbook", a technical manual provided by Qiagen, a leading biotechnology company, which details the procedures required for DNA extraction and purification from blood and animal tissue samples.

The steps followed include:

- Sample Preparation: Small tissue fragments were taken from sampled animals, specifically from the upper part of the body, to reduce the risk of DNA contamination from the insect's intestine or stomach (Figure 2.13a). The collected fragments were preserved in absolute alcohol at -50°C.
- Cell Lysis: The sample fragments, after mechanical mincing in a TissueLyser II at 30 Hz for one minute, were lysed by adding proteinase K (Figure 2.13b and 2.13c). This step was performed at 50°C for several hours, allowing the protease to effectively degrade proteins.
- DNA Purification: The DNA was inserted into a DNeasy Mini spin column and treated with a buffer solution. During centrifugation, the silica-based membrane in the DNeasy Mini spin

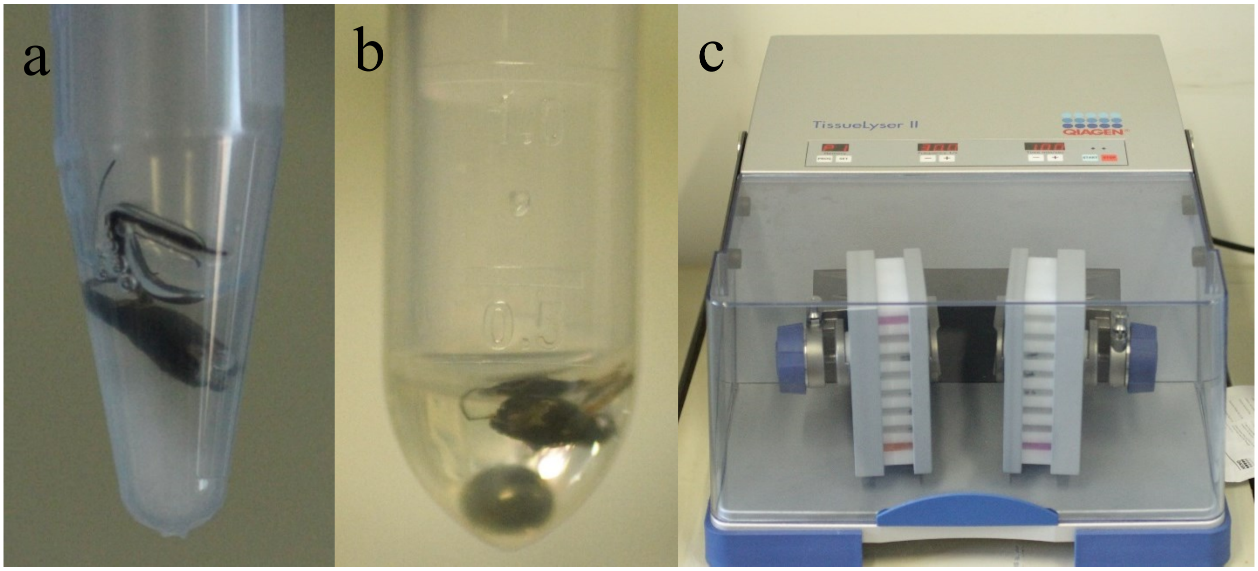

Figure 2.13: a) Piece of Gerris thoracicus (Schummel, 1832). b) Piece of Gyrinus substriatus (Stephens, 1829) before mincing. c) TissueLyser II used for samples' fragmentation.

column selectively bound the DNA, while contaminants passed through and were removed. Finally, the DNA was eluted in AE buffer.

- Amplification: Following extraction, a PCR was conducted to amplify the target fragment of mitochondrial DNA using universal primers LCO-1490/HCO-2198 (Folmer et al., 1994) and C1J-1718 and C1N-2191 (Simon et al., 1994).

  The polymerase chain reaction (PCR) was carried out in a total volume of 20 µL per sample using GoTaq ® Green Master Mix (Promega, USA). Samples were then amplified under the following conditions: 5 cycles at 95°C for 5 minutes, 95°C for 1 minute (denaturation), 47°C for 50 seconds (annealing), 72°C for 1 minute (extension) and 40 cycles at 95°C for 1 minute (denaturation), 50°C for 1 minute (annealing), 72°C for 1 minute (extension), and 72°C for 5 minutes (final extension). Subsequently, the amplified products were separated using an agarose gel and observed under a UV transilluminator (Figure 2.14; Figure 2.15).

The obtained material was sent to BMR Genomics for sequencing. The sequences were then processed using the MEGA XI software (Koichiro et al., 2021) and used to query online databases, such as the Barcode of Life Database (BOLDSYSTEMS), accessible at www.boldsystems.org, and NCBI (National Center for Biotechnology Information), to achieve greater precision in identification.

Figura 2.14: Thermal cycler used for PCR

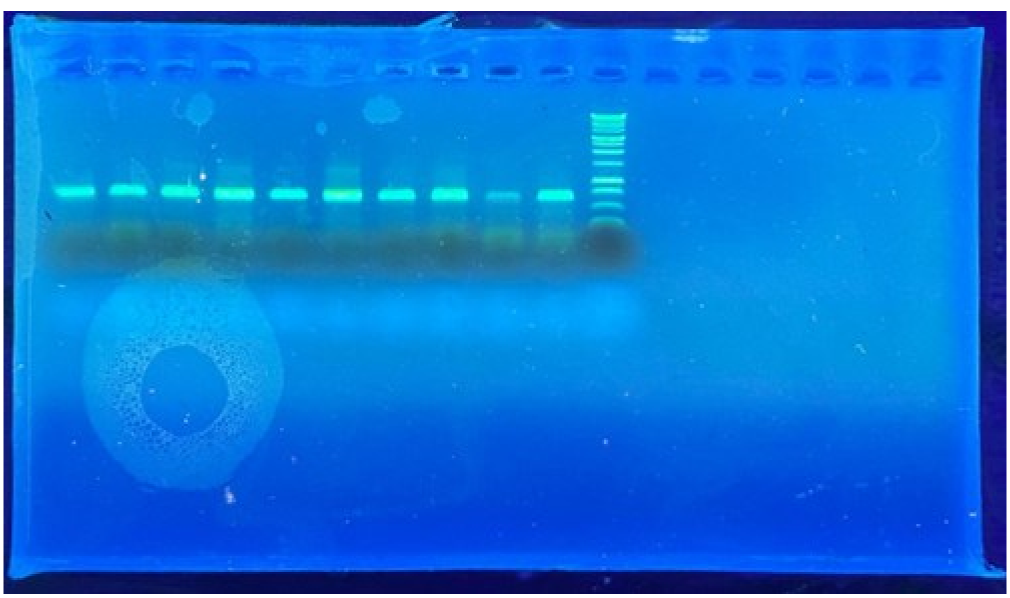

Figura 2.15: Agarose gel containing wells filled with amplified DNA

2.6. Data analysis

After applying the product in the previously indicated doses — specifically, the manufacturer's recommended dose (1 mL/m²), half of the recommended dose, and a quarter of the recommended dose — and adjusting the quantities according to the surface area of the trays used in the study (56 µL, 28 µL, and 14 µL, respectively), the treated and control groups were monitored at intervals of one hour, two hours, and 24 hours post-application, recording the number of individuals that had died in each tray.

The collected data were then analyzed using R, a software and programming language commonly used for statistical analysis and data visualization. In R, the data were processed to obtain essential descriptive measures, such as the median, minimum, and maximum, which helped to better understand the distribution and variability of the observed data.

Additionally, the results from the qualitative analysis of the water contained in the trays were used to perform a correlation analysis between the water characteristics and mortality rates (as the ratio between deceased individuals and the total number of individuals within the individual containers, both treated and untreated, after 1 hour, 2 hours, and 24 hours) in order to identify any potential influence of the water on mortality. The correlation analyses were conducted in R using Spearman's correlation coefficient, a statistical method that measures the strength and direction of the relationship between two variables. This method is ideal for data that do not follow a normal distribution or that show nonlinear relationships. Spearman's coefficient, which ranges from -1 to 1, indicates a positive or negative correlation depending on whether the relationship between the variables is direct or inverse, while values close to zero suggest no significant correlation.

2.6.1. Cox proportional hazards model

The effectiveness of Aquatain AMF® doses was assessed through a survival analysis. Survival curves were estimated by the Kaplan-Meier method (Klein and Moeschberger, 2006). To account for possible insect intra-cluster dependence due to containers, a marginal Cox proportional-hazards model was applied where robust standard errors were obtained to adjust for such dependence (Therneau and Grambsch, 2000; Martinussen and Scheike, 2007). The Cox model was validated by checking the proportional hazards assumptions with a Schoenfeld residual analysis (Klein and Moeschberger, 2006). One Cox model was built for each insect family. The dependent variable was the lifetime of each insect; the categorical explanatory variable was the dose (four levels: one untreated control and three doses, i.e., 0.25, 0.5 and 1 ml/m^2); the cluster factor was the container identity. Pairwise comparisons among untreated control and doses were performed using adjusted *p*-values (Tukey correction). All analyses were run in R (R Core Team, 2021). Cox models were developed and validated using the 'survival' package (Therneau and Lumley, 2020). Pairwise comparisons were run using the 'emmeans' package (Lenth *et al.*, 2020). Kaplan-Meier curves were plotted using the 'survminer' package (Kassambara *et al.*, 2020).

3. Results

3.1. Barcoding results

The individuals identified exclusively using dichotomous keys belonged to the species summarized in Table 3.1. From the tissue fragments collected from the 10 insect samples where identification based on morphometric traits was not entirely certain, 10 sequences were obtained. These sequences, queried against the NCBI and Bold System databases, matched the species summarized in Table 3.2.

Family	Species
Gerridae	*Aquarius najas (De Geer, 1773)*
Corixidae	*Hesperocorixa sahlbergi (Fieber, 1848)*
	Sigara lateralis (Leach, 1818)
	Micronecta scholtzi (Fieber, 1860)
Dytiscidae	*Hydaticus leander (Rossi, 1790)*
	Eretes griseus (Fabricius, 1781)
Notonectidae	*Notonecta maculata (Fabricius, 1794)*

Table 3.1: Species determined with dichotomous keys method

Family	Species	Sequence length (n. of nucleotides)	NCBI correspondence	Bold System correspondence
Gerridae	*Gerris thoracicus (Schummel, 1832)*	615	100%	100%
	Gerris thoracicus (Schummel, 1832)	627	100%	100%
	Aquarius paludum (Fabricius, 1794)	613	100%	100%
	Aquarius paludum (Fabricius, 1794)	580	100%	100%
	Gerris lacustris (Linnaeus, 1758)	461	98.64%	99.17%
Corixidae	*Sigara nigrolineata (Fieber, 1848)*	571	100%	100%
	Sigara nigrolineata (Fieber, 1848)	621	99.84%	100%
	Sigara striata (Linnaeus, 1758)	610	100%	100%
Dytiscidae	*Agabus dydimus (Olivier, 1795)*	621	99.67%	99.83%
Gyrinidae	*Gyrinus substriatus (Stephens, 1828)*	611	97.65%	99.66%

Table 3.2: Species resulting from barcoding analysis and relative correspondence found in NCBI and Bold System data

3.2. Mortality rate

Observed mortality percentages were calculated with the formula:

$$Mortality\ Percentage = \left(\frac{Deceased\ Individuals}{Total\ Individuals}\right) \times 100$$

Mortality was recorded after 1 hour, 2 hours, and 24 hours for each of the three doses and for the control group. The following graphs show the observed mortality percentages for each group.

In Gerridae (Figure 3.1), when applying 56 microliters of product, the recorded mortality rates were 95.56% within the first hour, 0% between 1 and 2 hours, and 4.44% between 2 and 24 hours after application. Using half of the recommended dose, i.e., 28 microliters (0.5 mm/m² proportional to the tray surface), mortality reached a maximum of 100% within one hour of application. With 14 microliters, the recorded mortality rates were 65% in the first hour, 15% in the second hour, and 20% between 2 and 24 hours. In the control group, mortality remained at 0% after 1 and 2 hours, but slightly increased to 8.42% after 24 hours. Overall, mortality reached its peak within one day of product application at all doses considered (56 µL, 28 µL, and 14 µL).

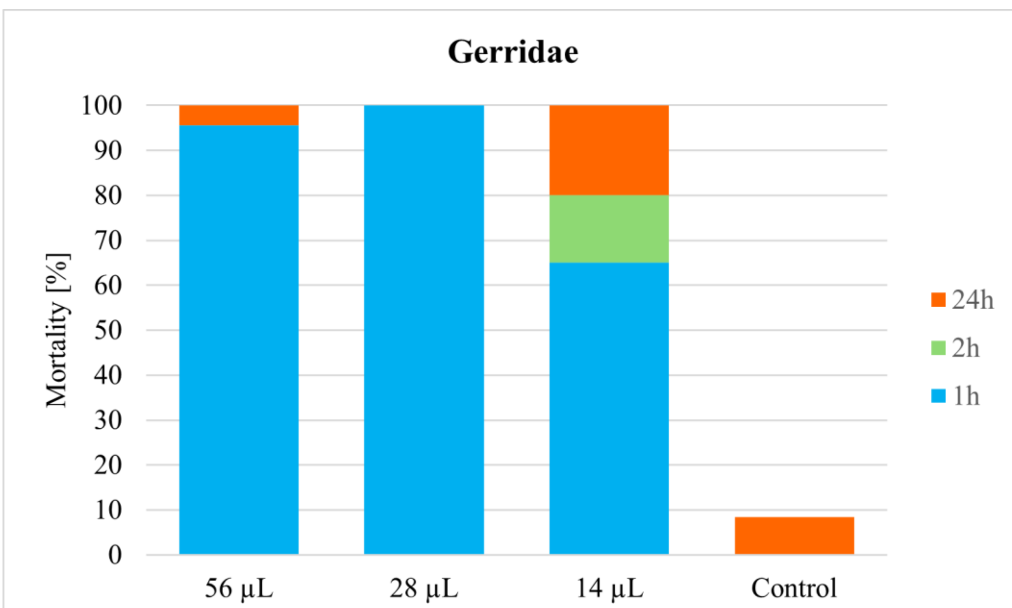

Figure 3.1: Mortality rate in Gerridae

In the Corixidae group (Figure 3.2), with a dose of 56 µL, mortality was 31.11% after 1 hour, 53.33% between 1 and 2 hours, and 15.56% between 2 and 24 hours. With 28 microliters, mortality remained at 0% after 1 hour but increased to 100% within 2 hours. Even with 14 microliters, mortality remained at 0% after 1 hour, increased to 6.67% between 1 and 2 hours, and reached 56.67% between 2 and 24 hours. In the control group, no mortality was observed after 1 and 2 hours (0%), while it increased to 3.81% after 24 hours. Mortality reached its peak within 24 hours only when the product was applied at doses of 56 and 28 µL, while at a dose of 14 µL, mortality after one day of exposure was 63.33%.

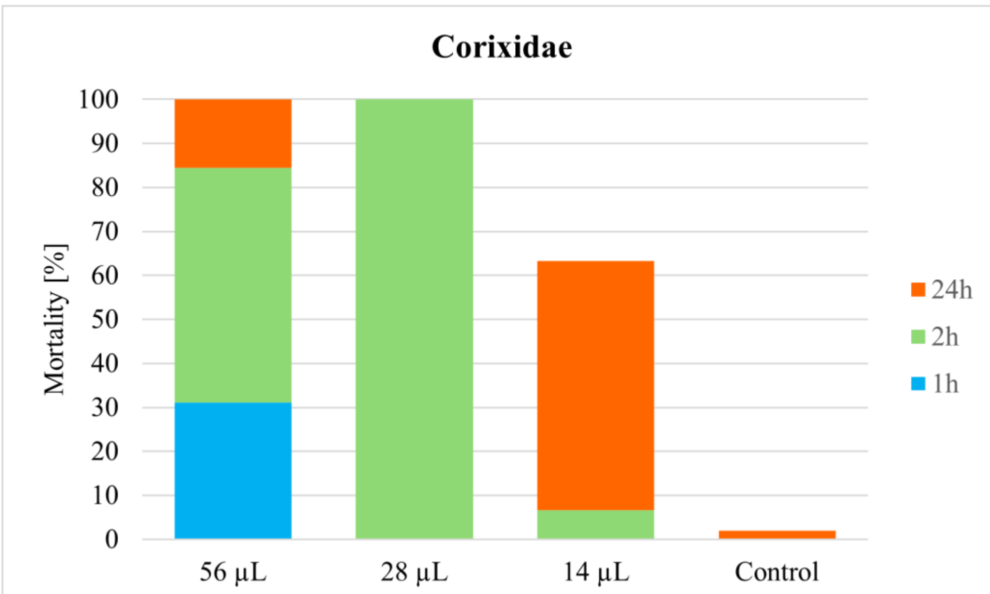

Figure 3.2: Mortality rate in Corixidae

In the Micronecta group, a dose of 56 microliters resulted in mortality of 8.89% within the first hour, 2.22% between 1 and 2 hours, and 80% between 2 and 24 hours after application. It is the only organism that did not reach 100% mortality, but 91.11%, after 24 hours of exposure to 56 microliters of product. With 28 microliters, mortality was 0% both after 1 hour and 2 hours but rose to 36% after 24 hours. With 14 microliters, mortality was 0% across all time intervals. In the control group, mortality was 1.11% after 1 hour, 0% between 1 and 2 hours, and 6.67% between 2 and 24 hours. Overall, mortality within 24 hours of exposure was 91.11%, 36%, and 0%, respectively, for 56, 28, and 14 µL of product (Figure 3.3)

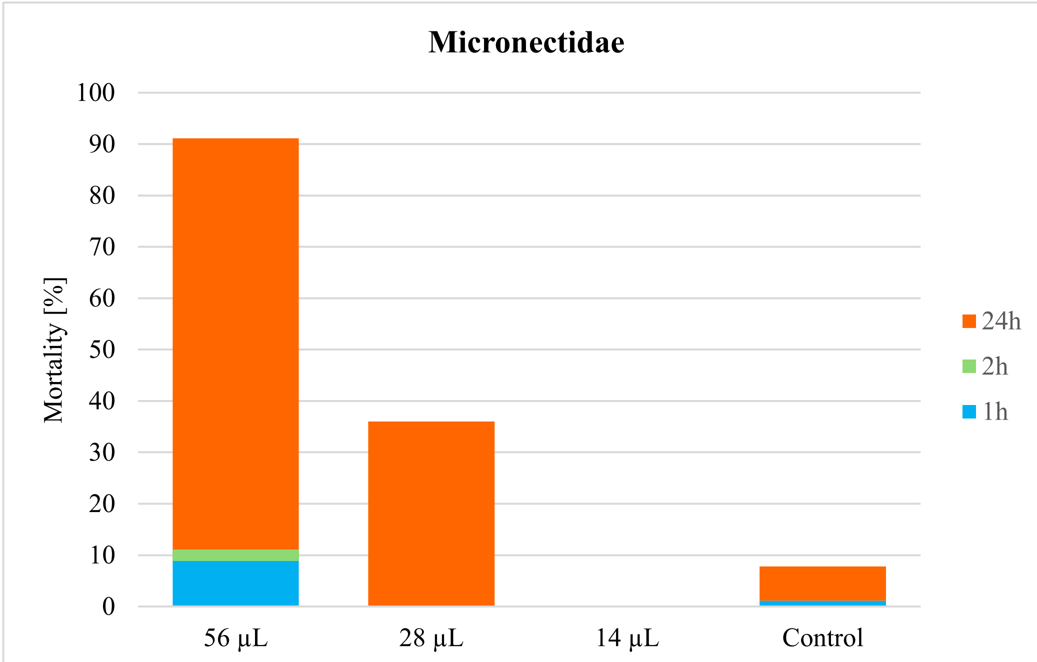

Figure 3.4: Mortality rate in Micronectidae

For Dytiscidae, using 56 microliters of product, mortality was 8.33% in the first hour, 75% in the second hour, and 16.67% between 2 and 24 hours. With 28 microliters, mortality was 0% in the first two intervals but increased to 91.67% within 24 hours. With 14 microliters, no mortality was observed in the first hour; between 1 and 2 hours, mortality reached 22.22%, and between 2 and 24 hours, it increased to 66.67%. Within 24 hours of application, the recorded mortality rates were 100%, 91.76%, and 88.89%, respectively, for doses of 56 µL, 28 µL, and 14 µL. In the control group, no mortality events were recorded (Figure 3.5).

The Notonecta showed 26.67% mortality in the first hour when the recommended dose was applied, followed by 60% between 1 and 2 hours and 13.33% between 2 and 24 hours. With 28 microliters, no mortality events were observed in the first hour, while mortality was 41.67% in the second hour and 50% between 2 and 24 hours. At the minimum dose and in the control group, no mortality was observed. The mortality rates reached were 100% when 56 µL of product was applied, 91.67% when half the recommended dose was applied, and 0% when the dose applied was 14 µL (Figure 3.6).

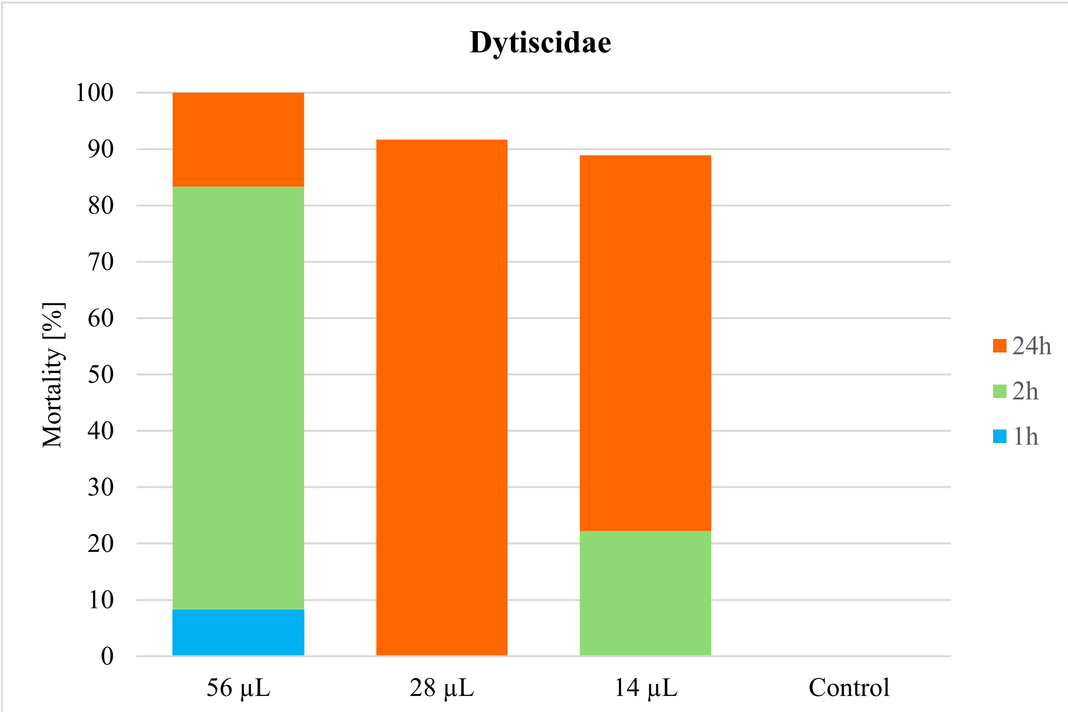

Figure 3.5: Mortality rate in Dytiscidae

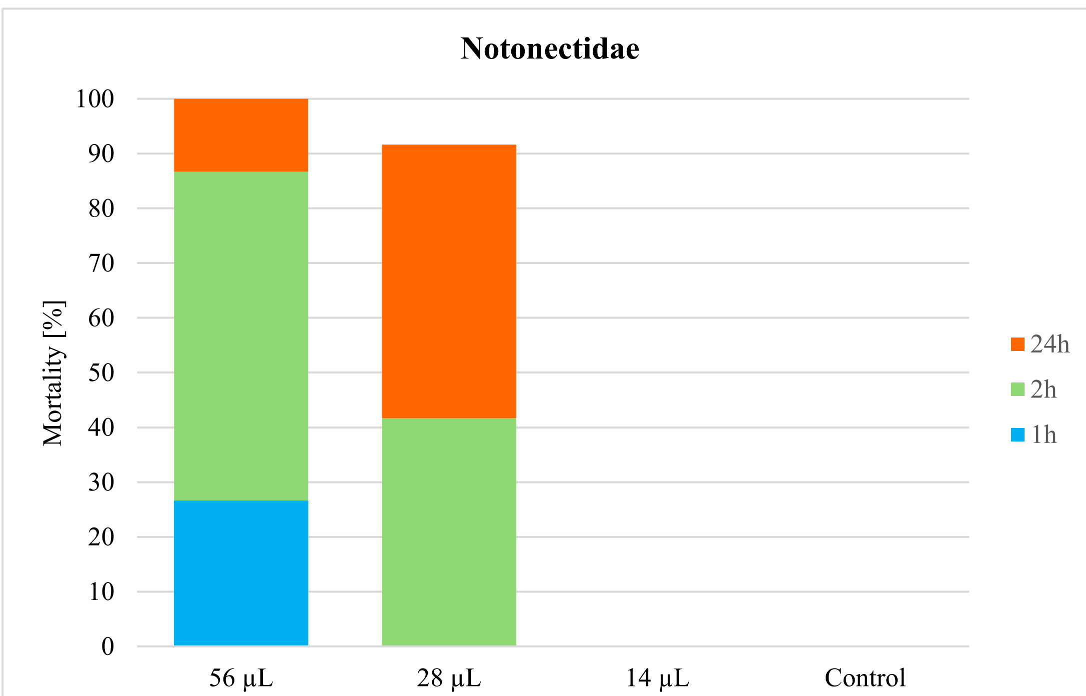

Figure 3.6: Mortality rate in Notonectidae

In the Gyrinidae (Figure 3.7) group, mortality in the first hour was 100% with both the recommended dose and 28 microliters. The mortality after 24 hours using 14 µL of product was 85% (65% after 1 hour, 15% between 1 and 2 hours, and 5% between 2 and 24 hours). In the control group, no mortality events were observed.

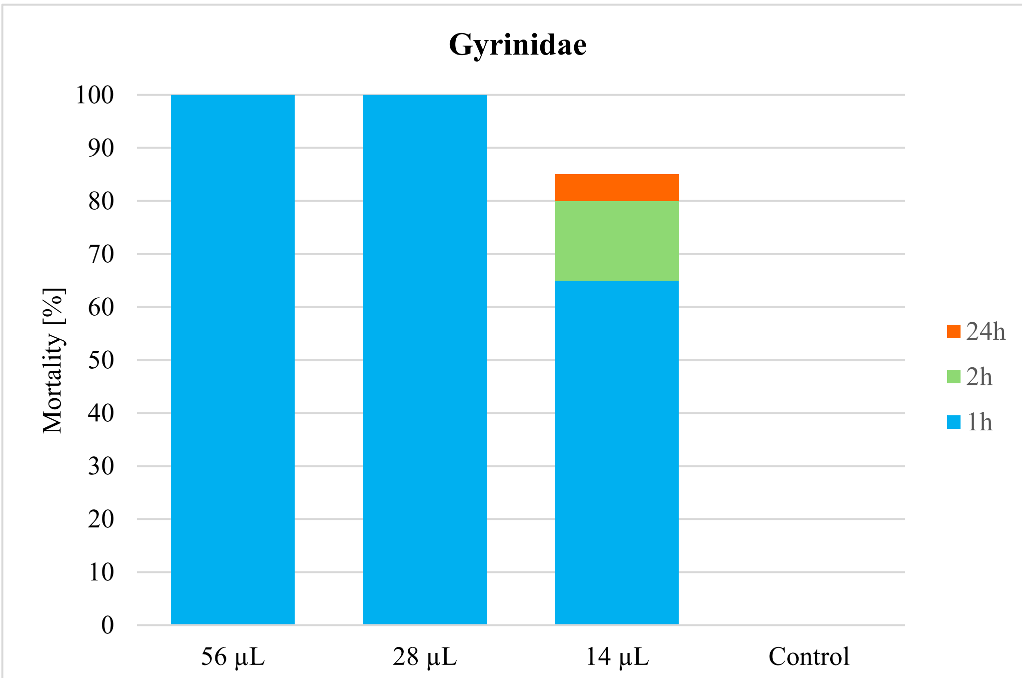

Figure 4: Mortality rate in Gyrinidae

Finally, for mosquito larvae in the L3-L4 stage, mortality was 56.67% in the first hour with 56 microliters of product, 40% between 1 and 2 hours, and 3.33% between 2 and 24 hours. With 28 microliters, mortality was 30% in the first hour, 10% in the second hour, and 60% between 2 and 24 hours. So, mortality after one day of exposure to 56 and 28 microliters of product was 100%. With 14 microliters, mortality remained 0% in the first and second hours but reached 36.67% between 2 and 24 hours. In the control group, mortality was 0% in the first two intervals and 2.22% between 2 and 24 hours.

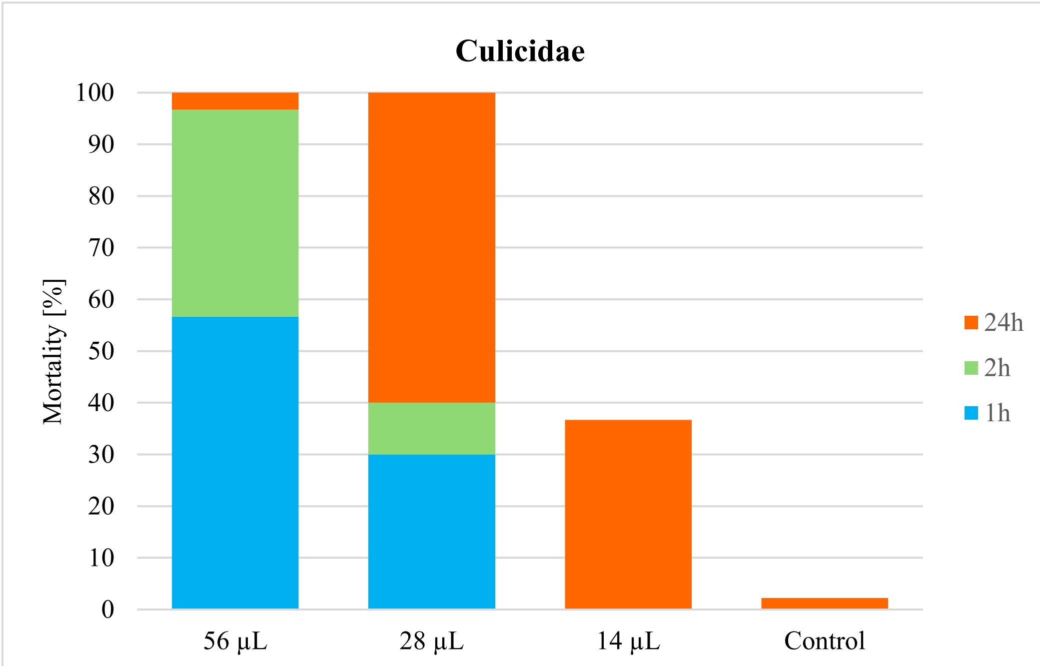

Figure 3.8: Mortality rate in mosquitoes' larvae

3.3. Descriptive Statistical Analysis

The descriptive statistical analyses concerning observed mortality in the three-time intervals reported the following results (Figures 3.9-3.12).

Gerridae

Treated

```
  mortality1h        mortality2h        mortality24h
 Min.   : 80.00    Min.   : 80.00    Min.    :100
 1st Qu.:100.00    1st Qu.:100.00    1st Qu.:100
 Median :100.00    Median :100.00    Median  :100
 Mean   : 95.56    Mean   : 95.56    Mean    :100
 3rd Qu.:100.00    3rd Qu.:100.00    3rd Qu.:100
 Max.   :100.00    Max.   :100.00    Max.    :100
```

Control

```
  mortality1h  mortality2h  mortality24h
 Min.   :0    Min.   :0    Min.   : 0.000
 1st Qu.:0    1st Qu.:0    1st Qu.: 0.000
 Median :0    Median :0    Median : 0.000
 Mean   :0    Mean   :0    Mean   : 4.444
 3rd Qu.:0    3rd Qu.:0    3rd Qu.: 0.000
 Max.   :0    Max.   :0    Max.   :20.000
```

Corixidae

Treated

```
  mortality1h        mortality2h        mortality24h
 Min.   :  0.00    Min.   : 40.00    Min.    :100
 1st Qu.:  0.00    1st Qu.: 60.00    1st Qu.:100
 Median : 20.00    Median :100.00    Median  :100
 Mean   : 31.11    Mean   : 84.44    Mean    :100
 3rd Qu.: 60.00    3rd Qu.:100.00    3rd Qu.:100
 Max.   :100.00    Max.   :100.00    Max.    :100
```

Control

```
  mortality1h  mortality2h  mortality24h
 Min.   :0    Min.   :0    Min.   : 0.000
 1st Qu.:0    1st Qu.:0    1st Qu.: 0.000
 Median :0    Median :0    Median : 0.000
 Mean   :0    Mean   :0    Mean   : 4.444
 3rd Qu.:0    3rd Qu.:0    3rd Qu.: 0.000
 Max.   :0    Max.   :0    Max.   :40.000
```

Figure 3.9: Descriptive statistics of mortality for treatment and control groups in Gerridae and Corixidae

Micronectidae

Treated

```
  mortality1h       mortality2h      mortality24h
 Min.   : 0.000   Min.   : 0.00   Min.   : 60.00
 1st Qu.: 0.000   1st Qu.: 0.00   1st Qu.: 80.00
 Median : 0.000   Median : 0.00   Median :100.00
 Mean   : 8.889   Mean   :11.11   Mean   : 88.89
 3rd Qu.: 0.000   3rd Qu.:20.00   3rd Qu.:100.00
 Max.   :40.000   Max.   :40.00   Max.   :100.00
```

Control

```
  mortality1h       mortality2h       mortality24h
 Min.   : 0.000   Min.   : 0.000   Min.   : 0.00
 1st Qu.: 0.000   1st Qu.: 0.000   1st Qu.: 0.00
 Median : 0.000   Median : 0.000   Median : 0.00
 Mean   : 2.222   Mean   : 2.222   Mean   :13.33
 3rd Qu.: 0.000   3rd Qu.: 0.000   3rd Qu.:20.00
 Max.   :20.000   Max.   :20.000   Max.   :40.00
```

Dytiscidae

Treated

```
  mortality1h       mortality2h       mortality24h
 Min.   : 0.000   Min.   : 33.33   Min.   :100
 1st Qu.: 0.000   1st Qu.: 83.33   1st Qu.:100
 Median : 0.000   Median :100.00   Median :100
 Mean   : 8.333   Mean   : 83.33   Mean   :100
 3rd Qu.: 8.333   3rd Qu.:100.00   3rd Qu.:100
 Max.   :33.333   Max.   :100.00   Max.   :100
```

Control

```
  mortality1h   mortality2h   mortality24h
 Min.   :0     Min.   :0     Min.   :0
 1st Qu.:0     1st Qu.:0     1st Qu.:0
 Median :0     Median :0     Median :0
 Mean   :0     Mean   :0     Mean   :0
 3rd Qu.:0     3rd Qu.:0     3rd Qu.:0
 Max.   :0     Max.   :0     Max.   :0
```

Figure 3.10: Descriptive statistics of mortality for treatment and control groups in Micronectidae and Dytiscidae

Gyrinidae

Treated

```
  mortality1h   mortality2h   mortality24h
 Min.   :100   Min.   :100   Min.   :100
 1st Qu.:100   1st Qu.:100   1st Qu.:100
 Median :100   Median :100   Median :100
 Mean   :100   Mean   :100   Mean   :100
 3rd Qu.:100   3rd Qu.:100   3rd Qu.:100
 Max.   :100   Max.   :100   Max.   :100
```

Control

```
  mortality1h   mortality2h   mortality24h
 Min.   :0     Min.   :0     Min.   :0
 1st Qu.:0     1st Qu.:0     1st Qu.:0
 Median :0     Median :0     Median :0
 Mean   :0     Mean   :0     Mean   :0
 3rd Qu.:0     3rd Qu.:0     3rd Qu.:0
 Max.   :0     Max.   :0     Max.   :0
```

Notonectidae

Treated

```
  mortality1h      mortality2h       mortality24h
 Min.   : 0.00   Min.   : 33.33   Min.   :100
 1st Qu.: 0.00   1st Qu.:100.00   1st Qu.:100
 Median :33.33   Median :100.00   Median :100
 Mean   :26.67   Mean   : 86.67   Mean   :100
 3rd Qu.:33.33   3rd Qu.:100.00   3rd Qu.:100
 Max.   :66.67   Max.   :100.00   Max.   :100
```

Control

```
  mortality1h   mortality2h   mortality24h
 Min.   :0     Min.   :0     Min.   :0
 1st Qu.:0     1st Qu.:0     1st Qu.:0
 Median :0     Median :0     Median :0
 Mean   :0     Mean   :0     Mean   :0
 3rd Qu.:0     3rd Qu.:0     3rd Qu.:0
 Max.   :0     Max.   :0     Max.   :0
```

Figure 3.11: Descriptive statistics of mortality for treatment and control groups in Gyrinidae and Notonectidae

Culicidae

Treated

	mortality1h		mortality2h		mortality24h
Min.	: 0.00	Min.	: 33.33	Min.	:100
1st Qu.	: 0.00	1st Qu.	:100.00	1st Qu.	:100
Median	:33.33	Median	:100.00	Median	:100
Mean	:26.67	Mean	: 86.67	Mean	:100
3rd Qu.	:33.33	3rd Qu.	:100.00	3rd Qu.	:100
Max.	:66.67	Max.	:100.00	Max.	:100

Control

	mortality1h		mortality2h		mortality24h
Min.	:0	Min.	:0	Min.	:0
1st Qu.	:0	1st Qu.	:0	1st Qu.	:0
Median	:0	Median	:0	Median	:0
Mean	:0	Mean	:0	Mean	:0
3rd Qu.	:0	3rd Qu.	:0	3rd Qu.	:0
Max.	:0	Max.	:0	Max.	:0

Figure 3.12: Descriptive statistics of mortality for treatment and control groups in Culicidae

3.4. Water quality-mortality correlations

The potential influence of water characteristics on the mortality of each examined group was assessed using Spearman's correlation method. With a water quality meter, parameters were measured for total dissolved solids (TDS), which include mineral salts, metals, and other inorganic and organic particles, as well as electrical conductivity (EC), temperature, and pH. The correlation coefficients and p-values from the correlation analysis conducted between the characteristics mentioned above and the percentage mortality, observed at intervals of one hour, two hours, and twenty-four hours for each analyzed group, are reported below. Correlations are considered significant at P< 0.05, so results indicate that there is no statistical significance (Figures 3.13-3.19).

Gerridae

Spearman's Correlation Coefficients

	TDS	EC	T	pH	mortality1h	mortality2h	mortality24h
TDS	1.000	0.9900	-0.34600	-0.3880	0.13400	0.1060	0.1940
EC	0.990	1.0000	-0.35900	-0.4230	0.11500	0.0863	0.1810
T	-0.346	-0.3590	1.00000	0.2890	0.00132	-0.0219	-0.2610
pH	-0.388	-0.4230	0.28900	1.0000	-0.14500	-0.1030	-0.0756
mortality1h	0.134	0.1150	0.00132	-0.1450	1.00000	0.9840	0.8950
mortality2h	0.106	0.0863	-0.02190	-0.1030	0.98400	1.0000	0.9010
mortality24h	0.194	0.1810	-0.26100	-0.0756	0.89500	0.9010	1.0000

p-values

	TDS	EC	T	pH	mortality1h	mortality2h	mortality24h
TDS	NA	0.00000	0.0332	0.01620	4.21e-01	5.27e-01	2.43e-01
EC	0.0000	NA	0.0269	0.00807	4.92e-01	6.07e-01	2.76e-01
T	0.0332	0.02690	NA	0.07800	9.94e-01	8.96e-01	1.13e-01
pH	0.0162	0.00807	0.0780	NA	3.84e-01	5.36e-01	6.52e-01
mortality1h	0.4210	0.49200	0.9940	0.38400	NA	0.00e+00	3.33e-14
mortality2h	0.5270	0.60700	0.8960	0.53600	0.00e+00	NA	1.33e-14
mortality24h	0.2430	0.27600	0.1130	0.65200	3.33e-14	1.33e-14	NA

Figure 3.13: Spearman Correlation Analysis Results between Water Characteristics and Gerridae Mortality

Corixidae

Spearman's Correlation Coefficients

	TDS	EC	T	pH	mortality1h	mortality2h	mortality24h
TDS	1.000	0.9950	0.154	-0.299	0.326	0.0740	0.141
EC	0.995	1.0000	0.151	-0.293	0.327	0.0739	0.125
T	0.154	0.1510	1.000	-0.242	0.215	-0.1630	-0.233
pH	-0.299	-0.2930	-0.242	1.000	-0.197	-0.2000	-0.376
mortality1h	0.326	0.3270	0.215	-0.197	1.000	0.5640	0.428
mortality2h	0.074	0.0739	-0.163	-0.200	0.564	1.0000	0.766
mortality24h	0.141	0.1250	-0.233	-0.376	0.428	0.7660	1.000

p-values

	TDS	EC	T	pH	mortality1h	mortality2h	mortality24h
TDS	NA	0.0000	0.417	0.1080	0.07880	6.98e-01	4.59e-01
EC	0.0000	NA	0.427	0.1160	0.07730	6.98e-01	5.11e-01
T	0.4170	0.4270	NA	0.1970	0.25400	3.91e-01	2.16e-01
pH	0.1080	0.1160	0.197	NA	0.29600	2.90e-01	4.04e-02
mortality1h	0.0788	0.0773	0.254	0.2960	NA	1.17e-03	1.83e-02
mortality2h	0.6980	0.6980	0.391	0.2900	0.00117	NA	8.04e-07
mortality24h	0.4590	0.5110	0.216	0.0404	0.01830	8.04e-07	NA

Figure 3.14: Spearman Correlation Analysis Results between Water Characteristics and Corixidae Mortality

Micronectidae

Spearman's Correlation Coefficients

	TDS	EC	T	pH	mortality1h	mortality2h	mortality24h
TDS	1.000000	0.9750	-0.0725	-0.64800	0.0385	-0.000473	0.40800
EC	0.975000	1.0000	-0.1060	-0.69700	0.0804	0.064700	0.38300
T	-0.072500	-0.1060	1.0000	0.21700	0.4080	0.362000	0.24700
pH	-0.648000	-0.6970	0.2170	1.00000	0.2860	0.193000	-0.00104
mortality1h	0.038500	0.0804	0.4080	0.28600	1.0000	0.870000	0.33700
mortality2h	-0.000473	0.0647	0.3620	0.19300	0.8700	1.000000	0.40200
mortality24h	0.408000	0.3830	0.2470	-0.00104	0.3370	0.402000	1.00000

p-values

	TDS	EC	T	pH	mortality1h	mortality2h	mortality24h
TDS	NA	0.00e+00	0.6750	1.92e-05	8.24e-01	9.98e-01	0.0134
EC	0.00e+00	NA	0.5390	2.36e-06	6.41e-01	7.08e-01	0.0212
T	6.75e-01	5.39e-01	NA	2.04e-01	1.35e-02	3.03e-02	0.1470
pH	1.92e-05	2.36e-06	0.2040	NA	9.06e-02	2.59e-01	0.9950
mortality1h	8.24e-01	6.41e-01	0.0135	9.06e-02	NA	5.40e-12	0.0445
mortality2h	9.98e-01	7.08e-01	0.0303	2.59e-01	5.40e-12	NA	0.0151
mortality24h	1.34e-02	2.12e-02	0.1470	9.95e-01	4.45e-02	1.51e-02	NA

Figure 3.15: Spearman Correlation Analysis Results between Water Characteristics and Micronectidae Mortality

Dytiscidae

Spearman's Correlation Coefficients

	TDS	EC	T	pH	mortality1h	mortality2h	mortality24h
TDS	1.0000	0.9930	-0.1110	-0.7070	-0.0517	-0.1830	0.0605
EC	0.9930	1.0000	-0.1260	-0.6960	-0.0516	-0.1530	0.0987
T	-0.1110	-0.1260	1.0000	-0.1560	0.1210	-0.0333	-0.0516
pH	-0.7070	-0.6960	-0.1560	1.0000	0.3310	0.0878	-0.0407
mortality1h	-0.0517	-0.0516	0.1210	0.3310	1.0000	0.2860	0.2490
mortality2h	-0.1830	-0.1530	-0.0333	0.0878	0.2860	1.0000	0.6050
mortality24h	0.0605	0.0987	-0.0516	-0.0407	0.2490	0.6050	1.0000

p-values

	TDS	EC	T	pH	mortality1h	mortality2h	mortality24h
TDS	NA	0.000000	0.622	0.000233	0.819	0.41500	0.78900
EC	0.000000	NA	0.578	0.000322	0.820	0.49600	0.66200
T	0.622000	0.578000	NA	0.489000	0.593	0.88300	0.82000
pH	0.000233	0.000322	0.489	NA	0.132	0.69800	0.85700
mortality1h	0.819000	0.820000	0.593	0.132000	NA	0.19700	0.26400
mortality2h	0.415000	0.496000	0.883	0.698000	0.197	NA	0.00283
mortality24h	0.789000	0.662000	0.820	0.857000	0.264	0.00283	NA

Figure 3.16: Spearman Correlation Analysis Results between Water Characteristics and Dytiscidae Mortality

Notonectidae

Spearman's Correlation Coefficients

	TDS	EC	T	pH	mortality1h	mortality2h	mortality24h
TDS	1.0000	0.9790	0.1500	-0.7120	0.0575	0.310	0.381
EC	0.9790	1.0000	0.1940	-0.6720	0.0661	0.310	0.385
T	0.1500	0.1940	1.0000	0.0797	0.0540	0.193	0.272
pH	-0.7120	-0.6720	0.0797	1.0000	-0.1670	-0.335	-0.330
mortality1h	0.0575	0.0661	0.0540	-0.1670	1.0000	0.649	0.545
mortality2h	0.3100	0.3100	0.1930	-0.3350	0.6490	1.000	0.944
mortality24h	0.3810	0.3850	0.2720	-0.3300	0.5450	0.944	1.000

p-values

	TDS	EC	T	pH	mortality1h	mortality2h	mortality24h
TDS	NA	0.000000	0.464	4.59e-05	0.780000	1.23e-01	5.46e-02
EC	0.00e+00	NA	0.342	1.71e-04	0.748000	1.23e-01	5.23e-02
T	4.64e-01	0.342000	NA	6.99e-01	0.793000	3.44e-01	1.79e-01
pH	4.59e-05	0.000171	0.699	NA	0.414000	9.39e-02	1.00e-01
mortality1h	7.80e-01	0.748000	0.793	4.14e-01	NA	3.38e-04	3.97e-03
mortality2h	1.23e-01	0.123000	0.344	9.39e-02	0.000338	NA	4.43e-13
mortality24h	5.46e-02	0.052300	0.179	1.00e-01	0.003970	4.43e-13	NA

Figure 3.17: Spearman Correlation Analysis Results between Water Characteristics and Notonectidae Mortality

Gyrinidae

Spearman's Correlation Coefficients

	TDS	EC	T	pH	mortality1h	mortality2h	mortality24h
TDS	1.0000	0.9430	-0.7470	-0.848	-0.0622	-0.0164	-0.0319
EC	0.9430	1.0000	-0.6930	-0.812	-0.1090	-0.0684	-0.0829
T	-0.7470	-0.6930	1.0000	0.683	-0.0219	-0.0444	-0.0395
pH	-0.8480	-0.8120	0.6830	1.000	-0.1410	-0.2570	-0.2390
mortality1h	-0.0622	-0.1090	-0.0219	-0.141	1.0000	0.9590	0.9580
mortality2h	-0.0164	-0.0684	-0.0444	-0.257	0.9590	1.0000	0.9990
mortality24h	-0.0319	-0.0829	-0.0395	-0.239	0.9580	0.9990	1.0000

p-values

	TDS	EC	T	pH	mortality1h	mortality2h	mortality24h
TDS	NA	4.33e-08	0.000892	3.32e-05	8.19e-01	9.52e-01	9.07e-01
EC	4.33e-08	NA	0.002910	1.35e-04	6.87e-01	8.01e-01	7.60e-01
T	8.92e-04	2.91e-03	NA	3.54e-03	9.36e-01	8.70e-01	8.85e-01
pH	3.32e-05	1.35e-04	0.003540	NA	6.03e-01	3.37e-01	3.73e-01
mortality1h	8.19e-01	6.87e-01	0.936000	6.03e-01	NA	4.95e-09	5.70e-09
mortality2h	9.52e-01	8.01e-01	0.870000	3.37e-01	4.95e-09	NA	0.00e+00
mortality24h	9.07e-01	7.60e-01	0.885000	3.73e-01	5.70e-09	0.00e+00	NA

Figure 3.18: Spearman Correlation Analysis Results between Water Characteristics and Gyrinidae Mortality

Culicidae

Spearman's Correlation Coefficients

	TDS	EC	T	pH	mortality1h	mortality2h	mortality24h
TDS	1.000	0.996	0.111	-0.927	-0.429	-0.427	-0.132
EC	0.996	1.000	0.110	-0.934	-0.439	-0.436	-0.132
T	0.111	0.110	1.000	-0.187	-0.246	-0.271	-0.163
pH	-0.927	-0.934	-0.187	1.000	0.519	0.520	0.270
mortality1h	-0.429	-0.439	-0.246	0.519	1.000	0.990	0.838
mortality2h	-0.427	-0.436	-0.271	0.520	0.990	1.000	0.841
mortality24h	-0.132	-0.132	-0.163	0.270	0.838	0.841	1.000

p-values

	TDS	EC	T	pH	mortality1h	mortality2h	mortality24h
TDS	NA	0.00e+00	0.660	3.16e-08	7.54e-02	7.72e-02	6.03e-01
EC	0.00e+00	NA	0.664	1.46e-08	6.83e-02	7.08e-02	6.00e-01
T	6.60e-01	6.64e-01	NA	4.58e-01	3.24e-01	2.76e-01	5.19e-01
pH	3.16e-08	1.46e-08	0.458	NA	2.73e-02	2.68e-02	2.79e-01
mortality1h	7.54e-02	6.83e-02	0.324	2.73e-02	NA	3.55e-15	1.39e-05
mortality2h	7.72e-02	7.08e-02	0.276	2.68e-02	3.55e-15	NA	1.24e-05
mortality24h	6.03e-01	6.00e-01	0.519	2.79e-01	1.39e-05	1.24e-05	NA

Figure 3.19: Spearman Correlation Analysis Results between Water Characteristics and Culex pipiens larvae Mortality

3.5. Cox proportional hazards model

Below are the survival curves for each group of tested organisms and the results of the Cox analyses, which will be discussed in the following chapter.

Gerridae showed a very low survival probability from the first hour of exposure to the product, both at the recommended dose (56 µL) and at half the recommended dose (28 µL). One hour after applying 14 µL, the survival probability was between 50% and 25%, decreasing slightly in the next hour and reaching 0% after 24 hours. In the control groups, the survival probability remained high (Figure 3.20).

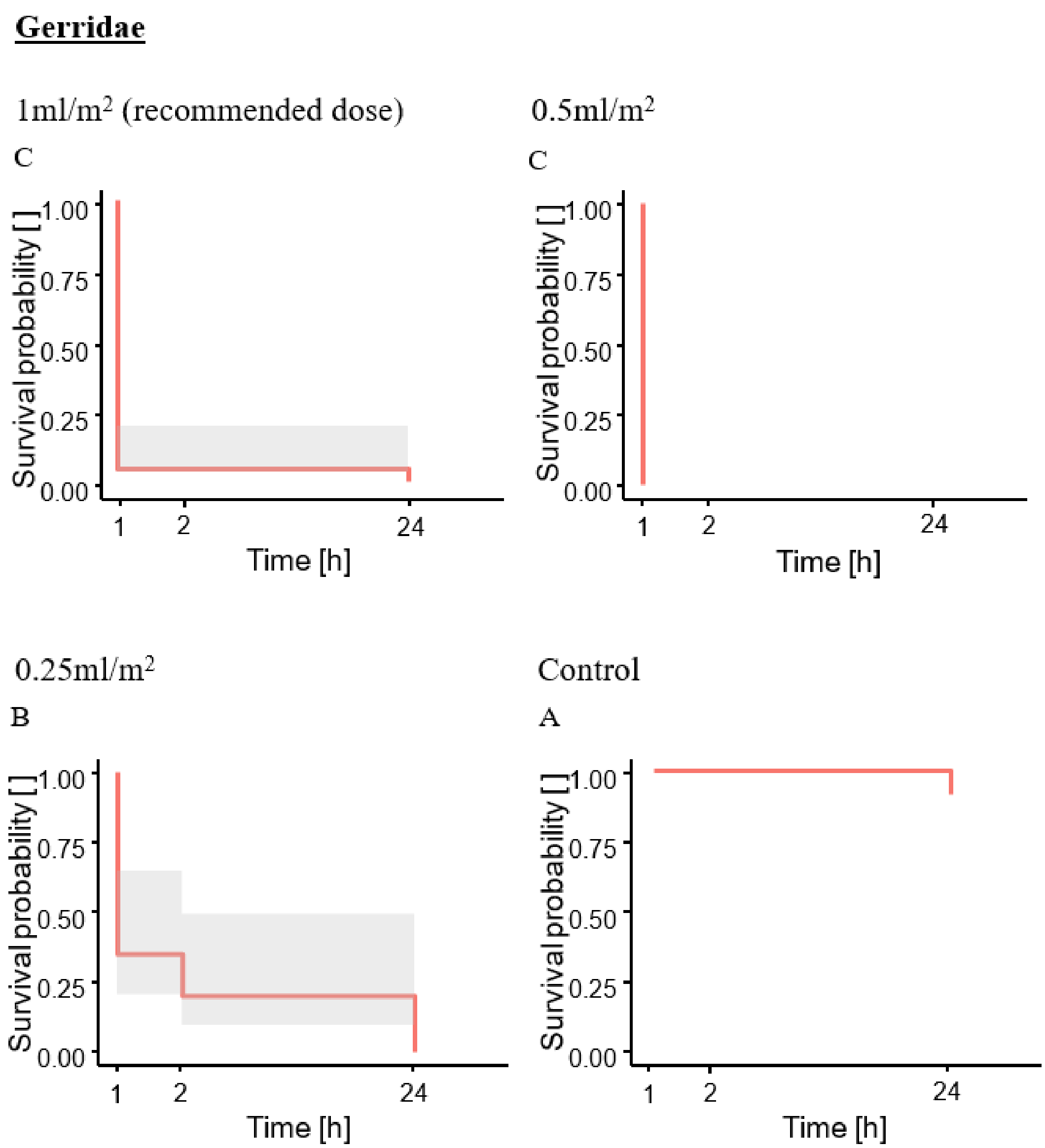

Figure 3.20: Comparison of Kaplan-Meier survival curves between treatment doses and control in Gerridae

Corixidae, at the recommended dose, showed a survival probability between 50% and 75% in the first hour, dropping to 0-25% after two hours and reaching 0% after 24 hours. With half dose (28 µL), no reduction in survival probability was observed after one hour; however, after two hours, it decreased to 50%, reaching 0% within 24 hours. With 14 µL, the survival probability remained at 100% for one hour, slightly decreasing (to 75-100%) after the second hour and stabilizing between 25% and 50% after 24 hours. In the controls, the survival probability only slightly decreased, remaining just below 100% within 24 hours (Figure 3.21).

Corixidae

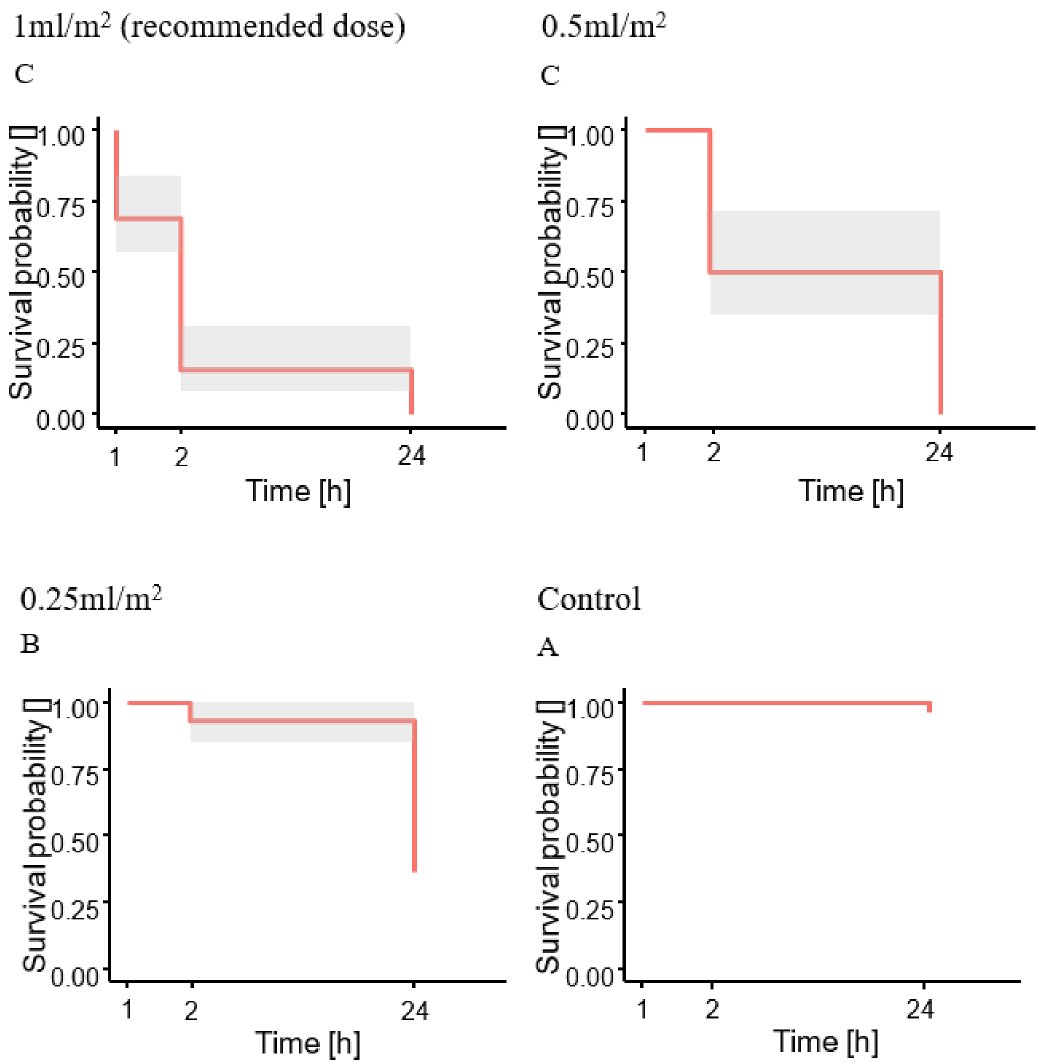

Figure 3.21: Comparison of Kaplan-Meier survival curves between treatment doses and control in Corixidae

For Micronetidae, with 56 µL the survival probability remained between 75% and 100% in the first two hours, dropping to 0-25% after 24 hours. With half dose, the survival probability remained constant in the first two hours, decreasing to between 50% and 75% at the end of 24 hours. With 14 µL, no reductions were observed, and the survival probability remained at 100% even after 24 hours. In the controls, the survival probability was stable for the first two hours, dropping only slightly after 24 hours (Figure 3.22).

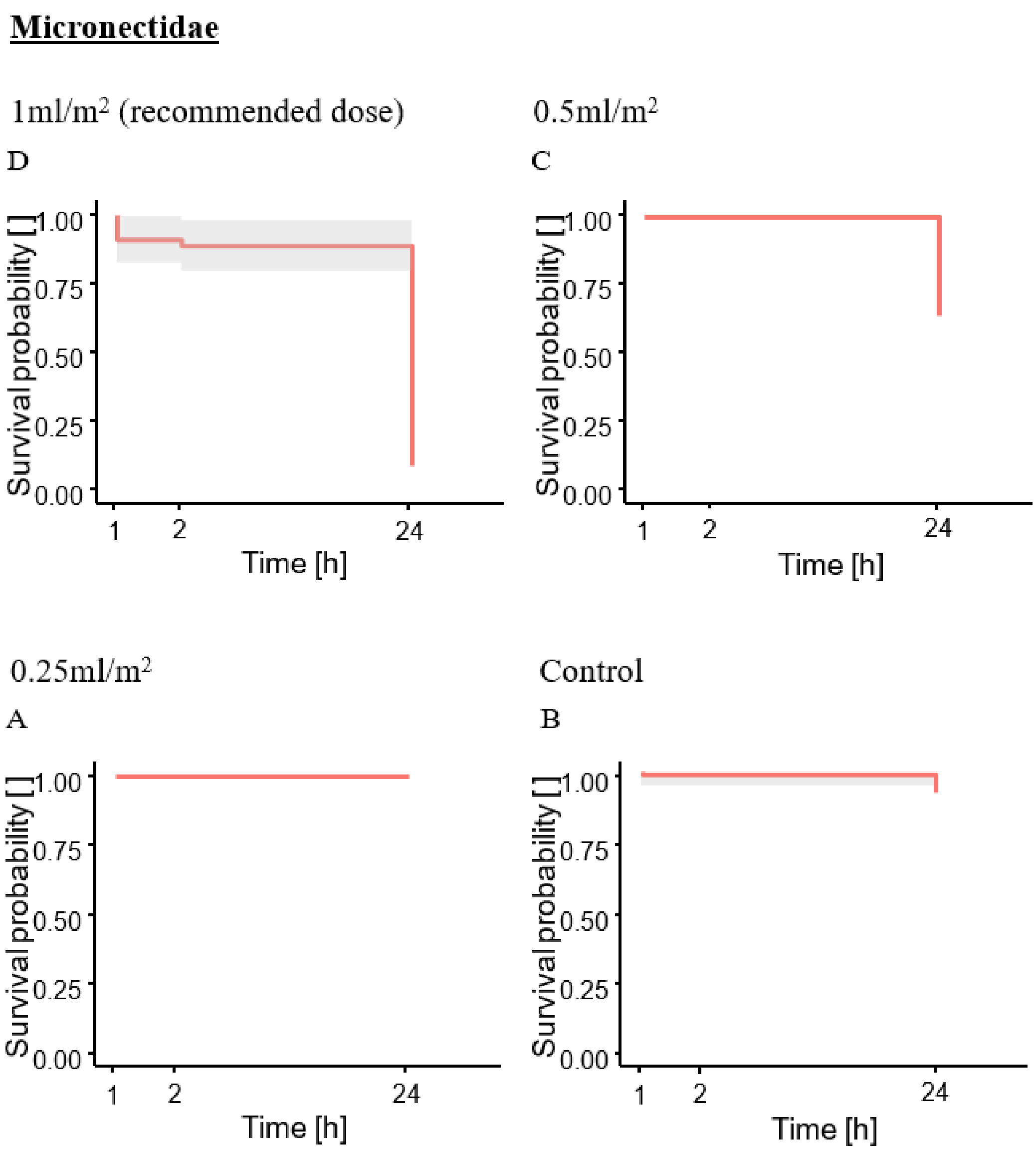

Figure 3.22: Comparison of Kaplan-Meier survival curves between treatment doses and control in Micronectidae

In Dytiscidae, the recommended dose reduced the survival probability to 75-100% after one hour and to 0-25% after two hours, reaching 0% after 24 hours. With half dose, the survival probability remained stable for the first two hours, decreasing to 0-25% after 24 hours. With 14 µL, the survival probability remained unchanged after one hour but dropped to 75% after the second hour and to 0-25% after 24 hours. In the control groups, no reductions in survival probability were observed (Figure 3.23).

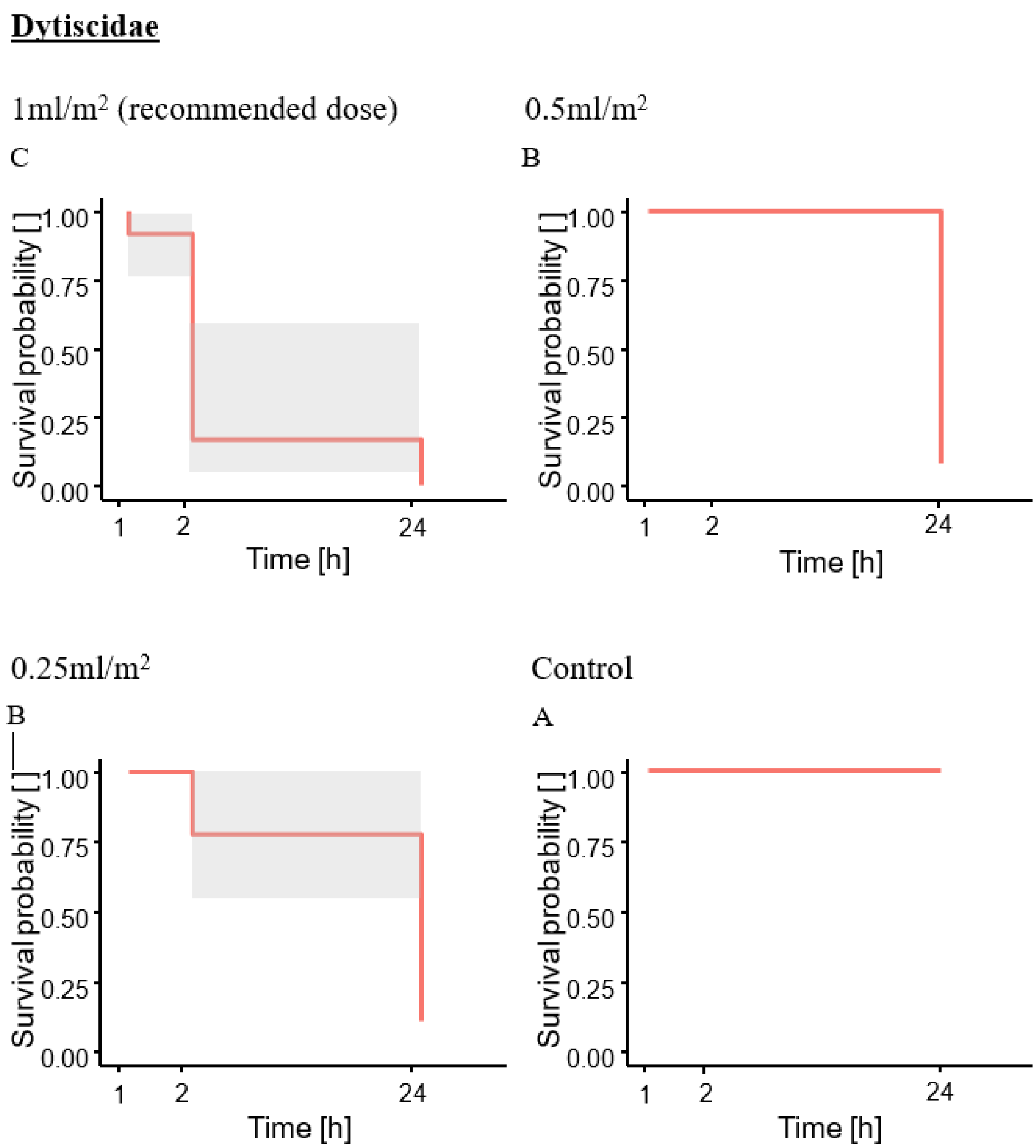

Figure 3.23: Comparison of Kaplan-Meier survival curves between treatment doses and control in Dytiscidae

Notonectidae had a reduced survival probability of 75% in the first hour with the recommended dose, decreasing to 0-25% after two hours and to 0% after 24 hours. With 28 µL, no reductions in survival probability were observed in the first hour, but it dropped to 50-75% after two hours and to 0-25% after 24 hours. With 14 µL, the survival probability did not decrease, nor did it in the control group (Figure 3.24).

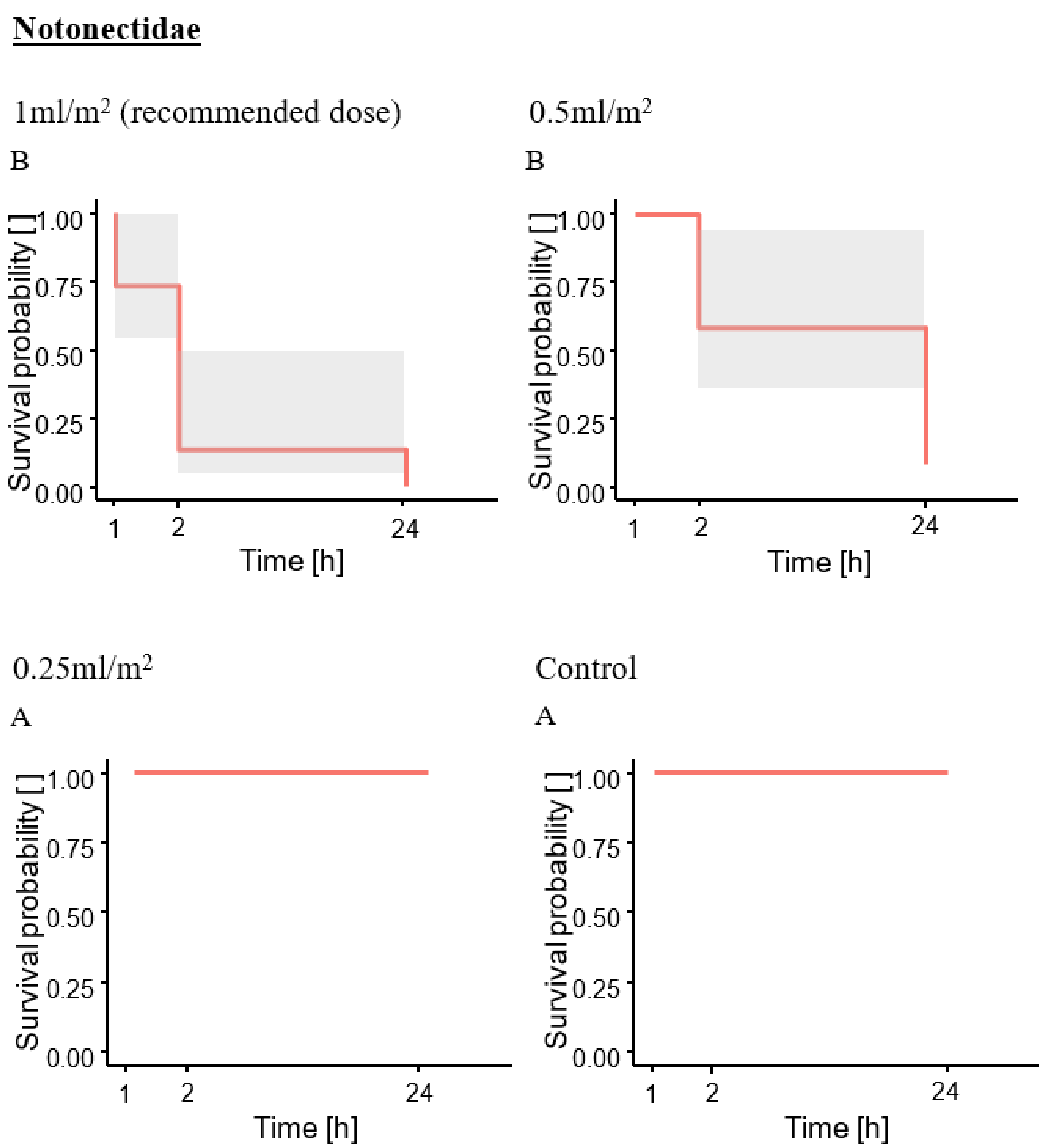

Figure 3.24: Comparison of Kaplan-Meier survival curves between treatment doses and control in Notonectidae

In Gyrinidae, treatment with 56 µL and 28 µL reduced the survival probability to 0% in the first hour of exposure. With 14 µL, the survival probability decreased to 25-50% in the first hour and further reduced to 0-25% in the following hours. In the controls, no reductions in survival probability were observed (Figure 3.25).

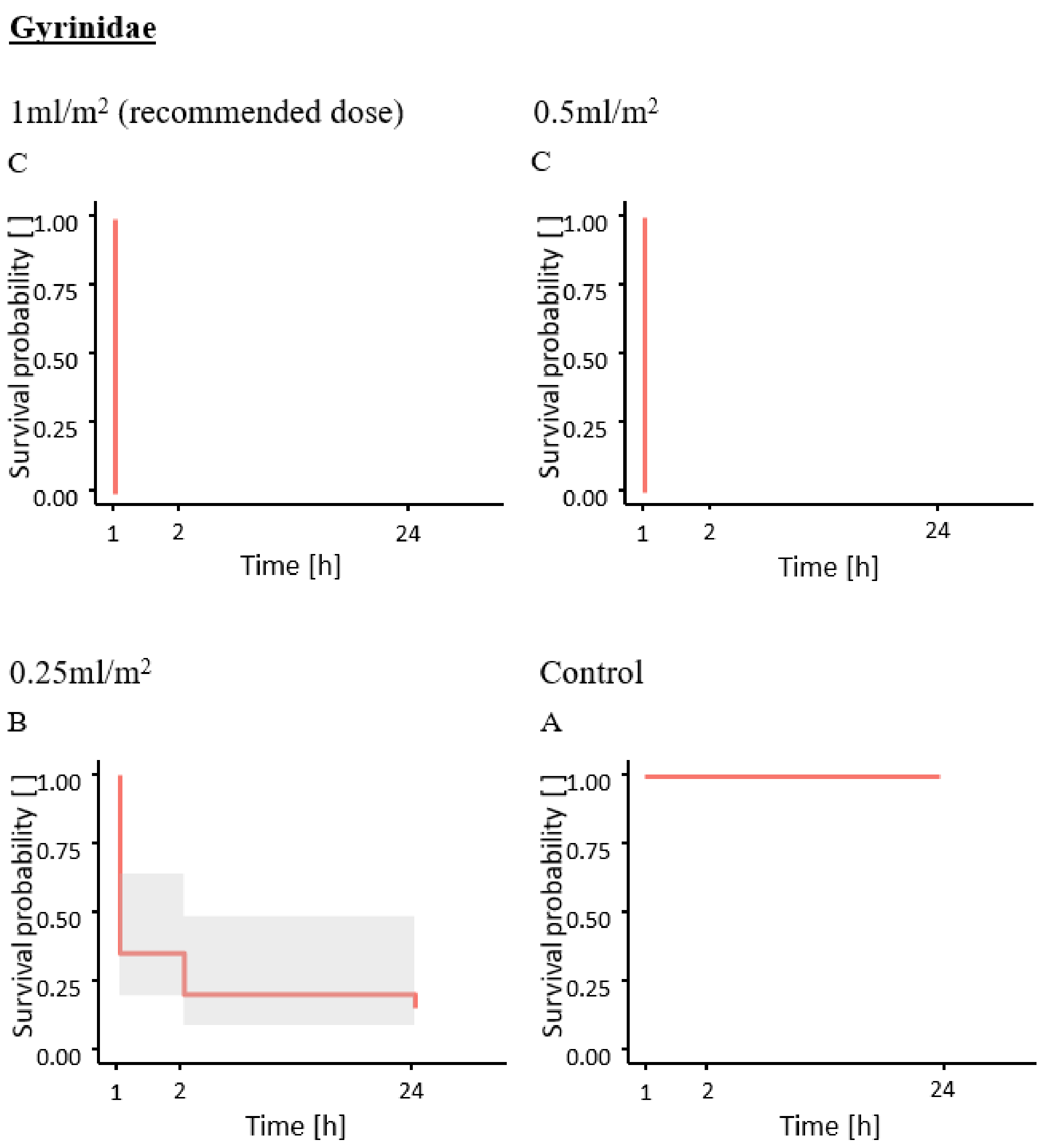

Figure 3.25: Comparison of Kaplan-Meier survival curves between treatment doses and control in Gyrinidae

Finally, in mosquito larvae, the recommended dose reduced the survival probability to 25-50% in the first hour, decreasing to 0-25% after two hours and reaching 0% within 24 hours. With half dose, the survival probability dropped to 50-75% in the first two hours, reaching 0% after 24 hours. With 14 µL, the survival probability remained unchanged for the first two hours, decreasing to 25-50% after 24 hours. In the controls, the survival probability slightly decreased after 24 hours, stabilizing between 75% and 100% (Figure 3.26).

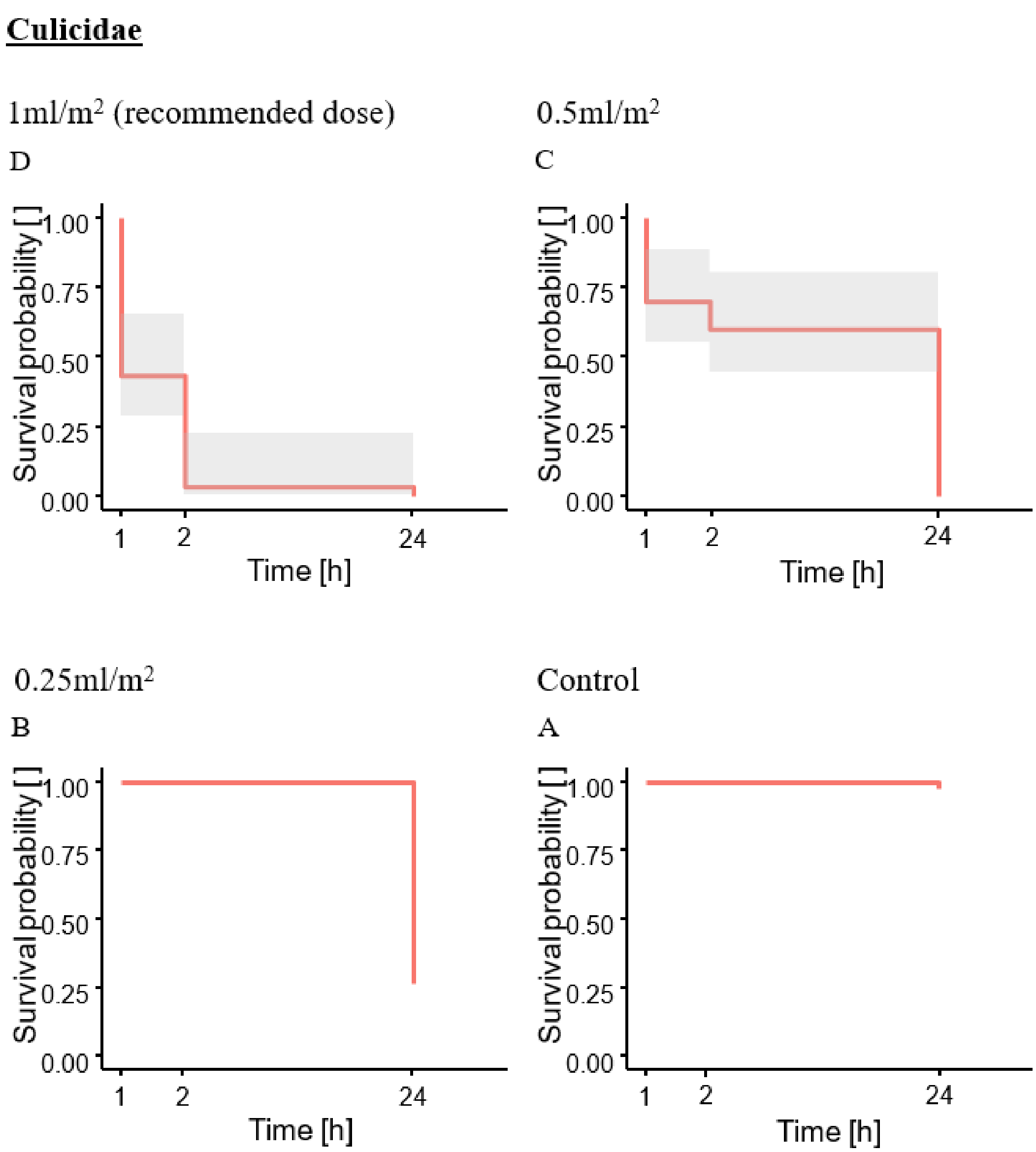

Figure 3.26: Comparison of Kaplan-Meier survival curves between treatment doses and control in Culicidae

4. Discussion

4.1. Mortality rate

The results obtained in this study have demonstrated that Aquatain, when applied at the recommended dose in open waters and wetlands with high biodiversity, can indeed cause harm to non-target species, many of which are predators that, by nature, play an extremely important role in maintaining the ecological balance of the ecosystem. Among the six non-target organisms examined, five (Gerridae, Corixidae, Dytiscidae, Notonectidae, and Gyrinidae) showed 100% mortality after 24 hours of exposure to 56 microliters of the product, similar to what was observed in mosquito larval tests. The effects of the silicone film were particularly evident on these organisms, which shortly after application showed "cleaning" behavior of their siphons from the product (Figure 4.1).

Figure 4.1: Culex pipiens larva while "cleaning" its siphon

The effects of Aquatain were also visible, especially in Gerridae, Dytiscidae, Notonectidae, and Gyrinidae. The former use the water's surface tension to move, which, along with the insect's unique morphology, prevents drowning. The monomolecular film is specifically designed to reduce the water's surface tension, preventing adult mosquitoes from resting on the water's surface and consequently laying eggs. However, the same principle causes rapid drowning in Gerridae, which, lacking swimming structures, can be seen struggling to reach the surface before sinking to the bottom (Figure 4.2). Gerridae, along with Gyrinidae, were the organisms most affected by the product, showing high mortality rates from the first hours of exposure. Only two out of 45 Gerridae individuals survived more than two hours of treatment.

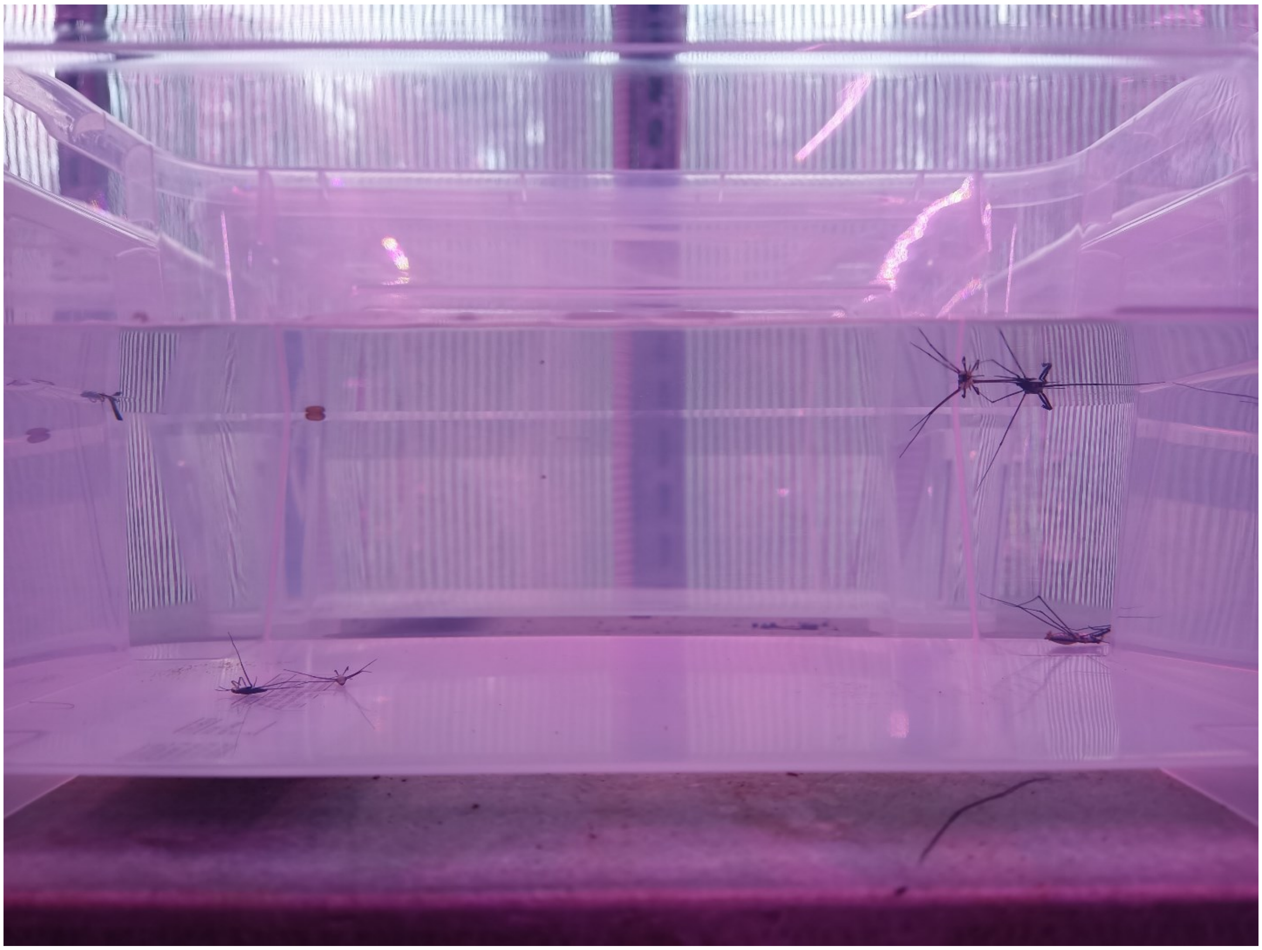

Figure 4.2: Individuals of Gerridae spp*. drowned in a treated container*

When observing the behavior of Dytiscidae individuals exposed to the treatment compared to controls, it is evident that the treated individuals attempted to climb the walls of the test containers and take flight. Often, they were observed with their wings spread, but rarely managed to fly. It could therefore be hypothesized that the film interferes not only with the respiratory system but also with the flying capability (Figure 4.3). However, there are no studies that could confirm this hypothesis.

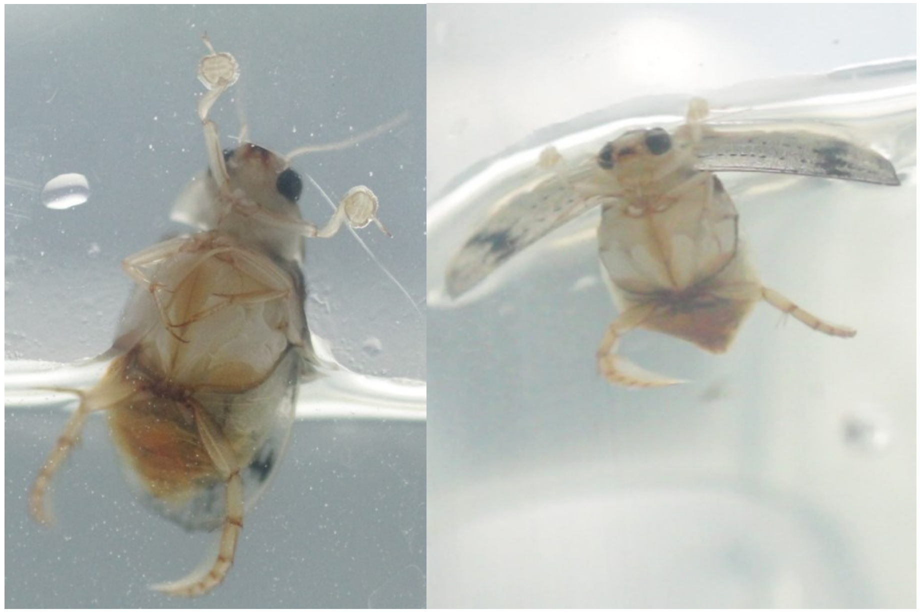

Figure 4.3: Adult of Eretes griseus exhibiting abnormal behaviors such as climbing the walls (left) or spreading their wings (right)

Notonectidae, like mosquito larvae, exhibited "cleaning" behaviors, often rubbing their abdomen with the last pair of hind legs.

The response of Gyrinidae individuals exposed to the product was extremely rapid, unlike the previous examples. Immediately after product application, Gyrinidae stopped their typical "dance" on the surface, quickly moving to the bottom of the containers and resurfacing only for short periods. This behavior could be seen as an attempt to find untreated areas large enough to return to the surface. For the same reason some individuals were observed attempting to climb containers walls, probably to escape the treated surface (Figure 4.4)

Corixidae did not exhibit abnormal behaviors, although they showed high mortality after just two hours of exposure (31.11% in the first hour and 53.33% in the second hour).

Micronectidae was the only organism tested that did not reach the maximum mortality levels within 24 hours of exposure to 56 microliters of product. From the first hours, lower mortality was observed compared to other organisms, indicating a greater resistance to the silicone film. After the first hour, mortality was recorded at 8.89%, 2.22% in the second hour, and 80% in the following period, leading to a 91.11% mortality rate after 24 hours of treatment. Notably, some Micronectidae individuals were observed 'resting' on air bubbles in the trays, which may have provided them with the means for prolonged survival (Figure 4.5).

Figure 4.4: An adult of Gyrinus substriatus while trying to climb the container's walls

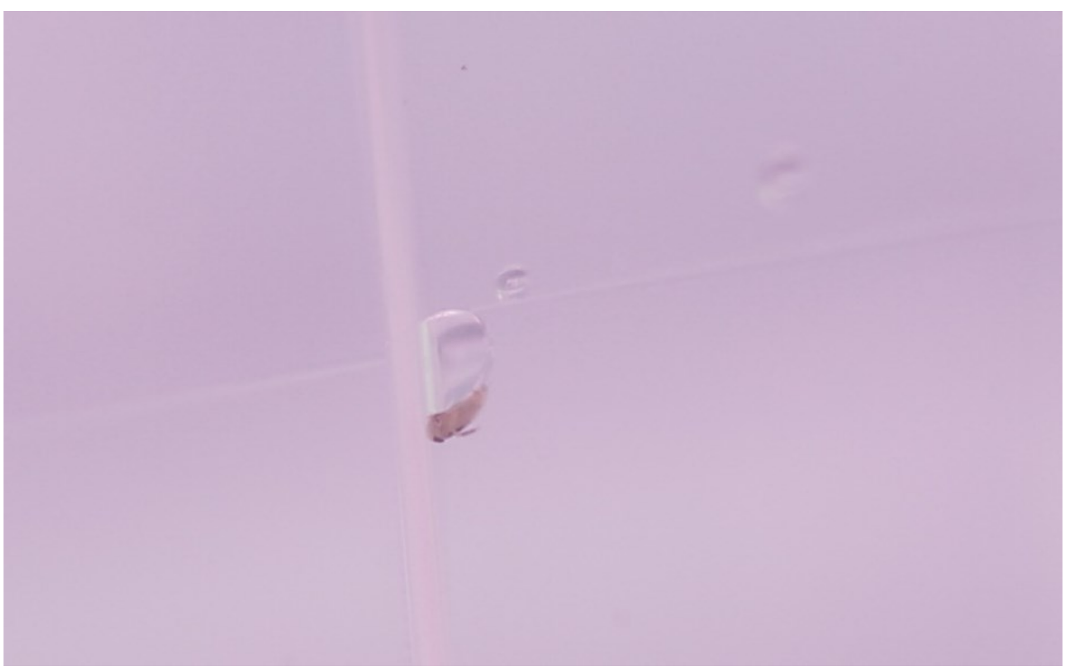

Figure 4.5: A Micronecta scholtzi breathing through an air bubble along the container's wall

When applying half the recommended dose (28 microliters of Aquatain), the organisms that experienced 100% mortality within 24 hours were Gerridae, Corixidae, Gyrinidae, and mosquito larvae. Half of the non-target organisms tested showed lower mortality within 24 hours compared to mosquitoes, although, except for Micronectidae (36%), the mortality was still close to the maximum levels (91.67% in Dytiscidae and Notonectidae). One of the main differences observed between the full-dose and half-dose tests is the significant reduction in "quick" mortality, i.e., that observed within the first hour of exposure. In 4 of the 6 non-target groups tested (Corixidae, Dytiscidae, Micronectidae, and Notonectidae), mortality after one hour was zero, unlike what was observed in Gerridae and Gyrinidae. Half-dose tests confirmed the high susceptibility of these two groups, which had been observed in full-dose tests: every individual from these organisms died within the first hour. Mortality after two hours was also generally reduced, with four of the groups tested showing mortality below 50% after two hours (Micronectidae: 0%; Dytiscidae: 0%; Notonectidae: 41.67%; Culicidae: 40%).

As expected, reducing the amount of Aquatain to 14 µL the levels of mortality reached are lower. The only group that still showed 100% mortality after 24 hours was Gerridae. This confirms what ecotoxicologist J.D. Stark reported in his Report for the Ministry of Health, citing studies by White & Garrett, who were the first to observe signs of drowning in the insect when in contact with oil-based products. Unlike Gerridae, tests on Micronectidae and Notonectidae individuals did not show any deaths, making them the only non-target organisms to show mortality lower than that of mosquito larvae (36.67% after 24 hours of treatment). The remaining groups showed higher mortality rates than the target organism for which Aquatain was designed. In Dytiscidae, mortality reached a maximum of 88.89% after 24 hours, while in Corixidae, it reached 63.33%. Gyrinidae showed higher mortality than mosquito larvae as early as the first hour of application, with a rate of 65%, which increased to 80% after two hours and finally to 85% after 24 hours. In summary, even with the recommended dose reduced by 75%, four of the six non-target organisms tested showed higher susceptibility than mosquito larvae.

Among the vulnerable non-target organisms, Gerridae require particular attention, not only because of the high mortality rates but also because they are one of the main predators of mosquitoes. According to Bukhari et al., vegetation would create interruptions in the silicone film, which organisms relying on the water's surface tension or atmospheric oxygen would exploit to prevent drowning. However, by applying smaller doses than recommended, only part of the container surface is covered by the product, leaving untreated areas where the organisms could survive. The high mortality of Gerridae, Gyrinidae, and Dytiscidae in tests with one-quarter of the recommended dose (100%, 85%, and 88.89% in 24 hours, respectively) demonstrates that even in the presence of untreated areas, contact with the product leads irreversibly to drowning (Figure 4.6).

Figure 4.6: limited spreading of Aquatain AMF layer at 28 µL dose

Field tests using emergence traps can be subject to variability, which might influence the results and underestimate the effect of Aquatain. One of the most important factors is the mobility of the insects, which may lead to migration and affect the results.

While it is easy to hypothesize the cause of such high mortality in Gerridae, the lack of prior studies makes it more difficult to identify the cause of mortality in other insects.

Notonectidae and Dytiscidae breathe using a plastron, a mechanism that uses body hairs to trap air bubbles beneath the elytra, which are used as physical gills underwater, allowing the insect to stay submerged for extended periods by diffusing dissolved oxygen from the bubble. Notonectidae use their hind legs to create water currents over their physical gills, irrigating them with oxygen-rich water. This ability may have provided them with better survival compared to other non-target organisms when exposed to lower doses of Aquatain, using untreated water currents. Dytiscidae, although better swimmers than Notonectidae and Corixidae, tolerate less density with their conspecifics (Šigutová et al. 2022), which could have caused stress, leading to increased movement, more surface breaks, and consequently a higher chance of coming into contact with the product. Many studies, including Bukhari's, do not specify the species of Dytiscidae observed, which could lead to underestimating the product's effect, as different Dytiscidae species have different submersion tolerances. Some species can remain submerged for longer, while others for less time depending on the water conditions they live in. Additionally, species like *Deronectes aubei* have tracheal setae on the pronotum, ventral side, and elytra that allow for cuticular respiration and oxygen acquisition where contact with the surface is more difficult (Yee et al. 2023; Kehl & Dettner 2009).

Micronectidae and Corixidae tend to stay on the bottom. Their adaptations for life on the bottom have led to the development of an advanced respiratory system, making them less dependent on atmospheric oxygen compared to other aquatic heteropterans (Popham 1959).

The study by Lee et al. (2018) showed that when mosquito larvae come into contact with the water-oil interface, tracheal flooding occurs. The oil adheres to the internal walls of the siphon and spiracles, causing suffocation. This phenomenon supports the hypothesis of siphon cleaning behavior. If the oil can adhere to the walls of the spiracles in mosquitoes, it is likely that the same happens to other non-target organisms. However, these organisms require more surface contact with the atmosphere to renew their water reserves, which increases the risk of coming into contact with the oil.

4.2. Cox proportional hazards model

The application of the Cox model enabled the evaluation of the effect of three doses (1mL/m², 0.5mL/m², and 0.25mL/m²) on the survival of the tested insects over time. This effect is quantified by the hazard ratio (HR) relative to the control group. A positive hazard ratio indicates an increase in mortality risk. Across nearly all tested organisms and doses, an increase in mortality probability was observed with increasing dose levels, though this increase was not always proportional.

Survival analysis of Corixidae showed increasingly high Cox coefficients with dose (3.22, 4.45, and 5.2 for 14, 28, and 56 µL of product), confirming a dose-dependent rise in mortality. Hazard ratio values indicate a mortality risk increase of 25 times with 14 µL compared to control, 85 times with half the recommended dose, and approximately 181 times with the recommended dose. The increase in risk is statistically significant, as indicated by low p-values (< 0.001). The emmeans analysis (Estimated Marginal Means) helped interpret the dose differences in terms of estimated log-risk, confirming the increased risk level, and grouped the four variables (the control and three doses) into three distinct groups: "a" (control), "b" (14 µL), and "c" (28 and 56 µL, grouped together as they showed no significant difference between them despite further log-risk increases).

In Gerridae, the estimated Cox hazard ratios similarly confirmed that mortality risk rose with dose. Specifically, a 14µL dose raised event risk about 49 times relative to control. The 28µL dose had a hazard ratio approximately 123 times greater than control, while 56µL was nearly 99 times greater. All findings were highly significant, though surprisingly, the 28µL dose resulted in a higher probability of death than the 56µL dose. This is likely due to individual survival variability at 56µL, with some individuals surviving longer, possibly due to inherent resistance, sampling variability, or error in attributing mortality.

In Micronectidae, hazard ratio values differed from other organisms, with higher mortality in the control group compared to the 14µL treatment group. Mortality of seven specimens in the untreated containers, versus no mortality in containers treated with a quarter dose, resulted in a negative hazard ratio for 14µL, suggesting a lower death probability than control. This anomaly might be due to external, random factors, such as transport-related harm or sampling of older or weaker individuals. Hazard ratios for 28µL and 56µL doses, however, align with predictions, with 28µL raising death risk ~5.5 times compared to control, while the recommended 56µL dose increased it 26 times. Emmeans analysis confirmed these findings, with generally lower log-risk values compared to other organisms, dividing all four conditions into distinct groups.

In survival analysis of Dytiscidae at escalating doses, the hazard ratios revealed a massive increase in mortality probability. The 14µL dose raised the mortality risk by over two billion times compared to control, while with 28µL the mortality increases by approximately 1.8 billion times, and 56µL raised risk almost eight billion times. This extremely high mortality is consistent with previous observations and is highly significant. The emmeans analysis gave marginal mean log-risk values of 21.3 and 21.4 for the 14µL and 28µL doses, respectively, which were thus grouped into "b", while the 56µL dose, with a log-risk of 22.8, fell into group "c," indicating a significant increase compared to lower doses.

In survival analyses of Notonectidae, results for 14µL showed no significant effect on mortality risk (hazard ratio of about 1), indicating it has a negligible effect. In contrast, doses of 28µL and 56µL showed sharply higher death risk. The increase in hazard ratios from 28µL (~7 billion times greater than control) to 56µL (~19 billion times greater) confirmed a dose-response relationship, with risk increasing alongside dose. Emmeans confirmed the null effect of 14µL, placing it in group "a" with control due to similar effects, while 28 and 56µL were grouped in "b" with estimated log-risk values of 22.7 and 23.7, respectively.

In Gyrinidae, survival analysis showed extremely high hazard ratios, similar to those for Dytiscidae (both aquatic beetle genera). The 14µL dose increased the event risk by about 1.8 billion times compared to control, while 28µL and 56µL doses resulted in even higher hazard ratios, increasing the risk by over 4.7 billion times compared to control. This extreme sensitivity of Gyrinidae to the product is consistent with observed rapid mortality, reaching near-maximal levels within an hour at both half and full recommended doses. The emmeans analysis confirmed this, with marginal mean log-risk values corrected at 21.3 for the 14µL dose (as seen in Dytiscidae) and 22.3 for higher doses, grouped under "c".

Analyses on *Culex pipiens* larvae also confirmed a dose-dependent increase in mortality probability. The Cox model reported progressively higher hazard ratios with higher doses: 14µL increased mortality risk by over 46 times compared to control, 28µL increased it by about 133 times, and 56µL raised it to 510 times. All dose effects were highly significant. The log-risk estimated by emmeans for the 14µL dose was 3.84, with a confidence interval of 2.61 to 5.08, showing a significantly higher risk than control. The group letter "b" indicates that 14µL represents a significantly different risk level from control ("a") but lower than higher doses. The 28µL dose further increased the risk (estimated log-risk of 4.9, marked as "c"), and the 56µL dose yielded a log-risk of 6.24, marked as "d," signifying a significantly higher risk than 14µL and 28µL.

4.3. Study limitations

Some of the organisms tested were collected in smaller numbers due to their limited populations in the wild, to avoid risking the balance of the ecosystem in which they live. In particular, the Gyrinidae were found in only one of the 18 sampling areas.

Although limited, cases of mortality were observed in the control groups, specifically in Gerridae (8 deaths within 24 hours), Corixidae (4 deaths within 24 hours), Micronecta (1 death within 1 hour of application and 6 within 24 hours), and mosquito larvae (2 deaths within 24 hours). Possible causes may include transportation-related damage or random, unmeasurable factors. In Gerridae, in particular, due to their predatory nature, cannibalism may have occurred, potentially explaining some deaths in the control groups. Dytiscidae and Notonecta are also predators; the former are especially known for their voracity, including intraspecific predation. However, as no deaths were observed in the control group, it is considered that this trait did not affect mortality in the treatment groups.

5. Conclusions

The aim of the present study is to demonstrate the presence of side effects associated with the use of one of the most employed mosquito control products on non-target organisms collected from freshwater wetlands near the city of Padua in Veneto, Italy. The product in question is a silicone-based monomolecular film called Aquatain AMF, distributed in Italy by Bleuline. Its mechanism of action depends on reducing the surface tension of water and interrupting gas exchange between water and the atmosphere, causing adverse consequences against mosquitoes such as larvae and pupae suffocation, adult drowning, and preventing egg deposition.

Tests and data analysis revealed that the product also affects organisms from other insect families, specifically Gerridae, Corixidae, Micronectidae, Dytiscidae, Notonectidae, and Gyrinidae. Applying 1mL/m², the dose recommended by the manufacturer, which corresponds to 56 µL proportionally to the surface of the test tanks, only one organism (*Micronecta scholtzi* Fieber, 1860) did not show 100% mortality within 24 hours but still exhibited very high mortality (91.11%). Even when half the recommended dose was applied, the families showing lower mortality rates within 24 hours compared to mosquito larvae were Micronectidae (36%), Dytiscidae (91.67%), and Notonectidae (91.67%).

The higher susceptibility of certain non-target organisms compared to mosquito larvae was further confirmed using 14 µL of the product, equivalent to one-quarter of the recommended dose. After 24 hours of application, mosquito larvae exhibited 36.67% mortality, while Corixidae, Gerridae, Dytiscidae, and Gyrinidae reached mortality rates of 63.33%, 100%, 88.89%, and 85%, respectively.

This study thus demonstrated that even mechanically acting products, which are subject to less stringent controls than biocides, can cause harmful effects on the environment. Despite the absence of harmful active ingredients, low toxicity, and a persistence in the environment of no more than four weeks, Aquatain AMF can negatively impact organisms whose presence is crucial for ecosystem balance and which are still poorly understood.

The only study available in the literature that analyzed the side effects of Aquatain on non-target organisms, particularly aquatic macroarthropods, is the one conducted in rice fields by Bukhari et al. (2011). As reported in paragraph 1.4.3, the only tested organisms that were affected by the presence of the monomolecular film were backswimmers, due to the reduction of surface tension, a characteristic essential for their survival. This study, however, demonstrated that in a controlled environment less influenced by environmental variables, the organisms susceptible to the product include, in addition to backswimmers, also water striders, diving beetles, water boatmen, pygmy water boatmen, and whirligig beetles. These findings highlight the importance of conducting further research, first in the laboratory to enhance knowledge of potentially vulnerable organisms, and subsequently in the field, to evaluate the influence of multiple environmental variables on the product's side effects on non-target fauna.

The present study does not aim to eliminate the use of Aquatain AMF for mosquito control but to restrict its application to sewers, drains, and other urbanized areas with low biodiversity, where it is highly effective for its intended purpose. Biodiverse wetland ecosystems are naturally balanced due to the presence of the organisms tested in this study, which, as predators, naturally regulate mosquito populations.

6. References

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