Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

2023

Doctoral thesis

Integrated pest and disease management in aquaponics

Ewumi Azeez Folorunso

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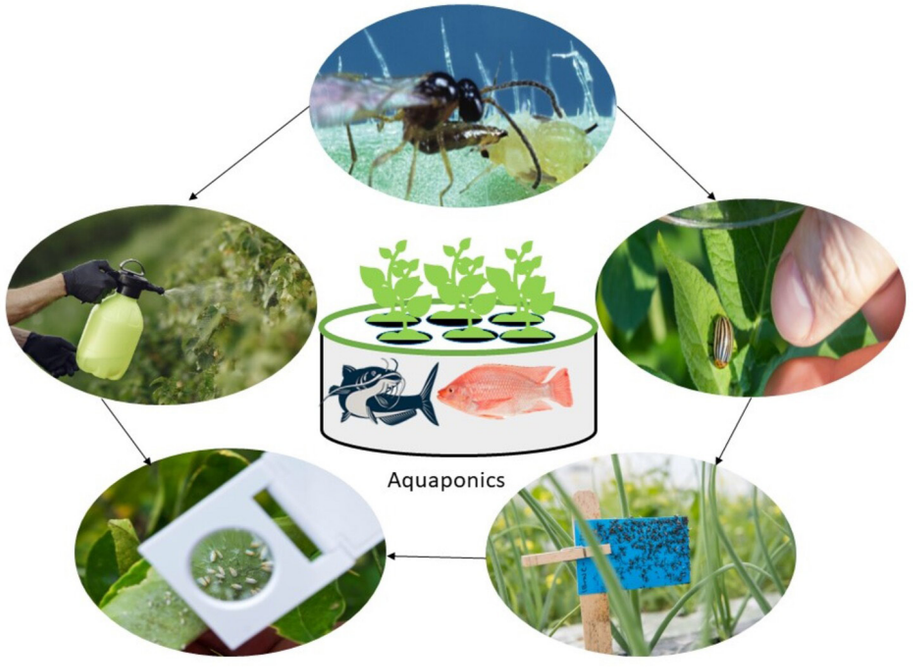

Doctoral thesis by
Ewumi Azeez Folorunso

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Title of thesis:
Integrated pest and disease management in aquaponics
Integrovaná ochrana proti škůdcům a chorobám v akvaponii

Doctoral study program:
Zootechnics / Fishery, full-time

Folorunso, E.A., 2023. Integrated pest and disease management in aquaponics. Doctoral thesis. University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Czech Republic, 134 pp. ISBN 978-80-7514-187-3 (with the summary in English and Czech)
Graphic design & technical realisation: JENA Šumperk, www.jenasumperk.cz

CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1. Introduction

According to the United Nations Department of Economic and Social Affairs, Population Division (2021), the global human population has risen from 2.5 billion in 1950 to 7.9 billion in 2021, with projections indicating a further increase to 11 billion by the end of the century. This population growth has led to an increased demand for food worldwide. However, the current global food production is constrained by the limitations set by our planetary boundary framework, which defines a safe environmental space within which humanity can operate without causing harm to Earth's systems (Rockström et al., 2009). Regrettably, the rate at which we are consuming and degrading natural resources surpasses their global regeneration rate, resulting in a decline in resources essential for food production, such as water, land, and minerals (Van Vuuren et al., 2010). This situation necessitates a shift from the current production methods towards waste-preventive or regenerative practices in order to meet the increasing food demands while minimizing resource consumption. Aquaponics has emerged as a promising food production system that can address the aforementioned challenges through nutrient and waste recycling.

Aquaponics is a food production system that combines fish farming with hydroponic crop cultivation. It utilizes beneficial bacteria to convert dissolved and suspended wastes from fish into absorbable nutrients for plants. This process enables the water to be reused effectively for the fish, creating a sustainable and symbiotic environment. In addition, aquaponics provides a medium for plants to reuse CO_2 produced by fish, and it also allows the reuse of waste heats generated from fish section or building exhausts to heat greenhouses (Biernatzki and Meinecke, 2014; Körner et al., 2021). The ability to reduce generated waste from aquaculture while providing essential nutrients for plant growth makes the food production method a vital tool for food sustainability (Goddek et al., 2015). Also, providing a reliable alternative source of plant nutrients reduces the pressure on mining natural minerals for agriculture. Furthermore, apart from its benefits in waste and nutrient reuse, aquaponics plays a crucial role in boosting urban food production by utilizing marginal lands in urban areas.

The emergence of modern aquaponics started in the USA in the 1970s, and several institutions with interest in more sustainable farming practices are reported to have been involved in aquaponics co-evolvement. Though several researchers have stated their significant history with aquaponics, modern aquaponics, starting in the early 1980s, is thought to be the work and the systems produced by James Rakocy and his team at the University of the Virgin Islands (UVI) (Lennard, 2017).

At inception, aquaponics was considered a 'backyard' farming that only fulfills the subsistence of family food demands. Over the years, it has progressed into an industrial-scale production phase with technical improvements in design and practice that favors increased production capacities and efficiencies. A significant evolution in aquaponics design is the emergence of decoupled aquaponics from the traditional one-loop aquaponics design (coupled). A traditional one-loop aquaponics system integrates aquaculture and hydroponics units, where water containing essential nutrients circulates continuously. The tradeoff interplay in this design is limited by the distinct differences in the optimal condition requirements of plants, fish, and beneficial bacteria. These variations explain the challenges in achieving optimal operation for all the three components (Goddek et al., 2016; Goddek and Keesman, 2020).

Decoupled aquaponics, on the other hand, constitute independent compartments of aquaculture and hydroponics. The dissolved wastewater from the aquaculture units is periodically fed to the hydroponics units. Consequently, the conditions within each compartment are fine-tuned to meet the specific needs of the organisms or crops involved. For instance, fish, which normally thrives in higher pH levels can be provided with water

that has a higher pH, while plants that flourish in lower pH and higher temperatures can benefit from heated and acidified water as they exit the RAS. In addition, due to variations in temperature in the temperate regions, water and air heating is required for fish and plant (respectively), to optimize their growth during cold seasons (Kyaw and Ng, 2017; Alkhalidi et al., 2020). Decoupled systems are categorized into a double-recirculating aquaponics systems (DRAPs), where the transferred RAS water is recirculated within the hydroponics, and SRAPS (single recirculating aquaponic system), where the water only makes a single pass through the plant component (Kloas et al., 2015). There have been additional advancements in the design aimed at enhancing its efficiency. Among these innovations are sludge digesters, which enhance the availability of bioavailable nutrients for plant utilization. Another notable addition is the incorporation of desalination units that trap undesirable minerals in the used hydroponics water. These minerals are then redirected from the water heading to the fish culture units and reintroduced into the hydroponics section, reducing reliance on external fertilizers (refer to Figure 1).

The growth and improvement of the designs and technology can be attributed to the rapidly emerging studies in this aspect in the last decade (Monsees et al., 2017; Goddek and Keesman, 2018, 2020; Baganz et al., 2022). Other aspects of aquaponics such as biology ((Schmautz et al., 2017; Bartelme et al., 2018; Eck et al., 2019), nutrition (Bittsanszky et al., 2017; Roy et al., 2022; Shaw et al., 2022) and economics (Castilho-Barros et al., 2018; Greenfeld et al., 2019, 2020) have also received increased attention in the same time-frame. On the other hand, areas, such as pest and disease management, have received little or no significant attention since inception. Pest and disease management are problematic due to the simultaneous existence of fish, plants, and beneficial bacteria in the same water loop (as in coupled aquaponics), limiting the available adoptable treatment options during pest/ diseases infestations in a hydroponic section or disease outbreaks in fish culture units.

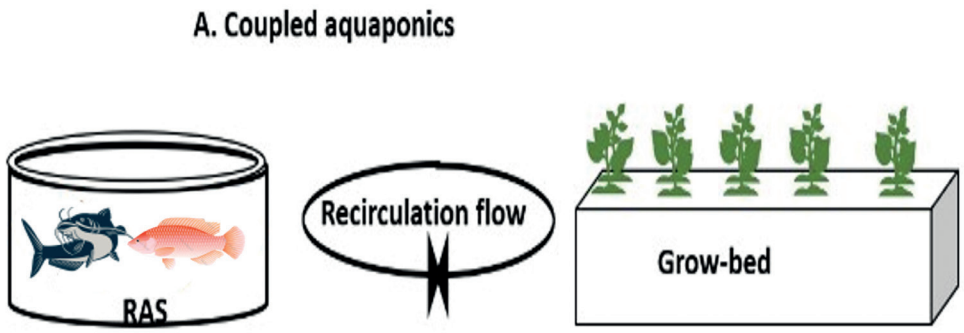

Figure 1a. *Schematic design of a typical coupled aquaponics systems constituting recirculating aquaculture system (RAS) and hydroponics grow bed with a close-loop water flow.*

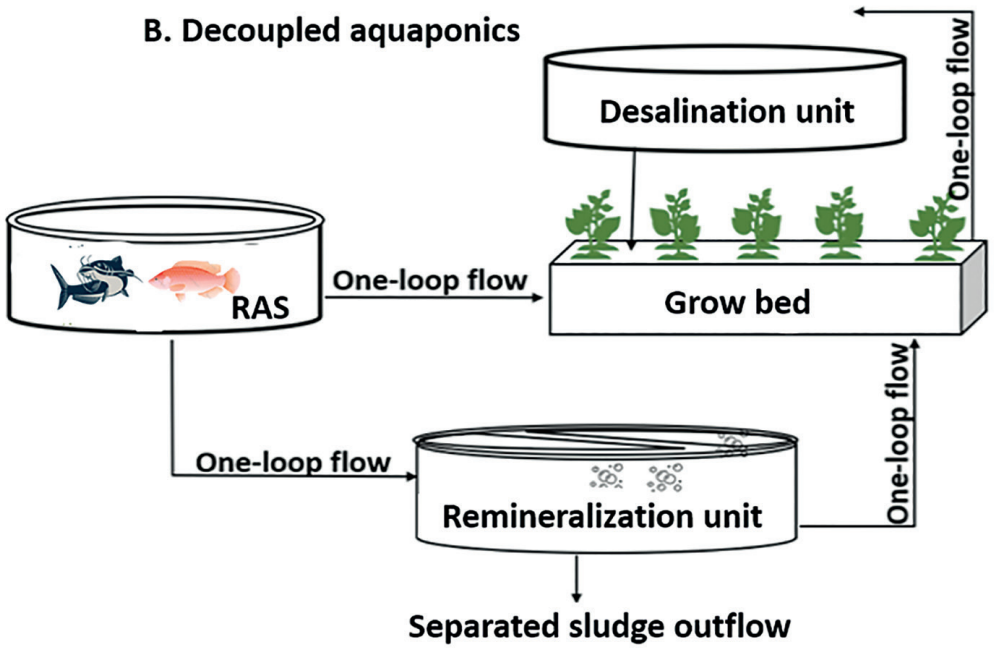

Figure 1b. *Schematic design of typical decoupled aquaponics systems constituting independent units of RAS, hydroponics, mineralization, and desalination connected by non-return valves.*

1.1. The significance of pests and diseases in aquaponics

In addition to the interconnectedness of aquaponics components, which restricts the available choices for pest and disease treatments, another unique aspect of dealing with pest and disease challenges in aquaponics is the convergence of interdisciplinary knowledge necessary for effectively managing both aquaculture and plant cultivation fields. Aquaculture practitioners are fish farmers and do not usually have expertise in addressing day-to-day challenges in plant cultivation. Also, plant experts/practitioners do not usually have expertise in addressing everyday challenges in fish farming. For example, in a survey conducted in South Africa, most aquaponics farmers (82 %) reported to not having any system for detecting and treating fish diseases, while only 23 % have the required skills to detect and treat diseases in fish and plants (Mchunu et al., 2018). Similarly, the aquaponics survey conducted by Love et al. (2014) in the United States also showed that about 41 % of aquaponics farmers had insufficient knowledge to treat diseases in plants and fish. In another survey conducted across 21 European countries, about 60 % of aquaponics farmers disagreed (or not sure) about having the knowledge required to address plant pests (Villarroel et al., 2016). Hence, identifying and diagnosing diseases or pests in aquaponics may be primarily complex for aquaponics practitioners. Therefore, quotient information on potent pests and diseases in aquaponics and their safe remedies are currently inadequate.

1.2. Pest and disease infestations in aquaponics

Pest infestations in fields and greenhouse or hydroponics cultivations are considered 'norms' and almost inevitable in commercial agriculture. While greenhouses are practically designed to optimize plant growth, they have become comfortable enclosures for pests and pathogens

due to the stable microclimatic conditions provided for the plants. In addition, unlike in open fields, natural regulators such as parasitoids and predators capable of controlling the damage of herbivorous pests are usually lacking in greenhouses (Knapp et al., 2020). Hence, once pests are introduced into aquaponics greenhouse, their infestations and further crop damage might be inevitable if not controlled.

Using Goddek et al. (2015) criteria, plant and fish pests and pathogens in aquaponics can be categorized into four groups based on specific alternative treatment solutions. These are (1) plant pests – mostly insects that damage the leaves and roots (e.g., whiteflies, aphids, spider mites, thrips); (2) plant diseases – microorganisms (e.g., bacteria, fungi, and viruses); (3) fish parasites (e.g., *Cichlidogyrus halli* and *Scutogyrus longicornis*); and (4) fish diseases caused by fungi, viruses, and bacteria (e.g., *Saprolegnia* spp.).

1.2.1. Fish diseases and parasites

According to Yildiz et al. (2017), fish pathogens typically cause diseases in conventional aquaculture settings when there are existing acute stressors that compromise the immunity of the cultured fish. However, fish possess natural mechanisms to maintain their homeostasis through physiological changes. Unfortunately, these mechanisms face challenges in traditional aquaponics systems due to the instability of water quality parameters and the microbial community within the system. Moreover, since aquaponics relies primarily on recirculating water supply, which creates an ideal environment for pathogen amplification, it becomes difficult to completely prevent the introduction of pathogens into the fish culture units (Yanong, 2019). Consequently, fish pathogens are naturally prevalent in the RAS (recirculating aquaculture system) compartment of aquaponics.

Common among these are fish obligate pathogens which require a fish host cell to replicate-including major fish viruses (e.g., Viral hemorrhagic septicemia viruses (VHSV), infectious hematopoietic necrosis Viruses (IHNV)) and some bacteria (e.g., *Renibacterium salmoninarum, Yersinia ruckeri, Candidatus Branchiomonas cisticola,* and *Ca. Piscichlamydia salmonis*) (Cherif and Hammami, 2012).

When multiple stressors such as temperature, dissolved oxygen, pH levels, and pathogen infestations coincide, fish disease outbreaks in aquaponics can lead to significant mortality rates. This combination, along with the substantial initial investment required for aquaponics systems, can potentially force the complete cessation of aquaponics operations if swift action is not taken to address the outbreaks (Love et al., 2015). For example, tanks inoculated with a bacterial fish pathogen, *Streptococcus iniae*, resulted in 40 % mortality of Barramundi (*Lates calcarifer*) within 48 h of exposure (Bromage et al., 1999). Similarly, in an investigation of the common diseases found in aquaponics, Chitmanat et al. (2015) recorded that *Aeromonas hydrophila* and *Flavobacterium columnare* infected catfish stocked at different stocking densities right at the beginning of the experiment. Albeit the absence of reports of fish parasites in aquaponics, many obligate fish dinoflagellate parasites such as *Amyloodinium ocellatum* have been identified to have capacity to infect freshwater and saltwater fish species in aquaponics systems (Nozzi et al., 2016). *Myxobolus cerebralis* is another fish parasite considered as obligate pathogens of fish in RAS systems.

Enhancing the sustainability of aquaponics relies on eradicating these pathogens within the food production system. In coupled aquaponics, the use of medicines and antibiotics registered for treating fish diseases is not feasible within the system. However, infected fish can be relocated to a separate fish hold where appropriate treatments can be administered.

1.2.2. Plant pests

Herbivorous insects in the greenhouse are favored by the abiotic (relative humidity and temperature) and biotic conditions (other existing live organisms), and they rapidly grow in population. Common groups of pests in aquaponics or stand-alone hydroponics are insects and mites. Insect pests are classified as insects that disrupt the overall well-being of plants through their destructive impact on various plant parts, including leaves, stems, roots, or fruits. Examples of insect pests are; whiteflies (e.g., sweet potato whitefly (*Bemisia tabaci,* Gennadius), greenhouse whitefly (*Trialeurodes vaporariorum*)), aphids (e.g., cotton aphids (*Aphis gossypii*), potato aphids (*Macrosiphum euphorbiae*) and rose aphids (*Macrosiphum rosae*)), scale insects (e.g., Citrus mealybug (*Planococcus citri*)), soft scale insects (e.g., Brown soft scale (*Coccus hesperidum*)), moths (e.g., cotton bollworm (*Helicoverpa armigera*), beet armyworm (*Spodoptera exigua*), cabbage moth (*Mamestra brassicae*), cabbage looper (*Trichoplusia ni*)), thrips (e.g., water flower (*Frankliniella tritici*), onion thrips (*Thrips tabaci*)), leafminers (e.g., tomato leafminers (*Tuta absoluta*), burgess (*Liriomyza trifolii*), Blanchard (*Liriomyza sativae Blanchard*)), sciarid flies (e.g., Fungus gnats (*Bradysia Coprophilia*), Tuomikoskija (*Pseudexechia Tuomikoski*), Johannsen (*Bradydia impatiens*)), and beetles (e.g., pepper weevils (*Anthonomus eugenii*), black vine weevil (*Otiorhynchus sulcatus*)) (Knapp et al., 2020).

On the other hand, mites are minuscule arachnids found in landscapes, gardens, and greenhouses. They thrive by feeding on a variety of vegetables, fruit trees, vines, berries, and ornamental plants (Smith et al., 2010). While they may be associated with insects, it's important to note that mites belong to the arachnid class, which also includes spiders and ticks, rather than being classified as insects themselves. Examples of mites are gall mites (e.g., tomato russet mite (*Aculops lycopersici*)), spider mites (e.g., two-spotted spider mites (*Tetranychus urticae*), tomato spider mites (*Tetranychus evansi*)), flat mites (e.g., red palm mites (*Raoiella indica*)), and tarsonemid mites (e.g., Broad mite (*Polyphagotarsonemus latus*), and Cyclamen mites (*Phytonemus pallidus*)).

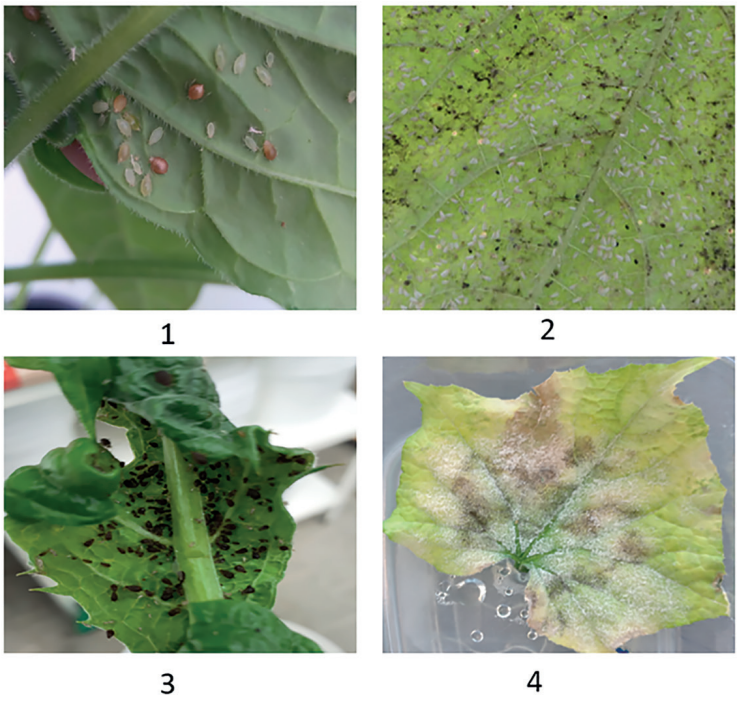

Figure 2. *Pictorial representation of plant pests and diseases taken from the aquaponic hall of the Faculty of Fisheries and Protection of Waters (FFPW). From the top left (1), greenpeach aphids and mummies covered the cucumber leaf's underleaf. Top right (2) was an underleaf of cucumber leaf densely infected by greenhouse whitefly with their honeydew. The bottom left (3) is a highly infested tomato leaf by black-bean aphids. The bottom right (4) is a highly infected cucumber leaf from a powdery mildew pathogen, Podosphaera xanthii.*

1.2.2.1. Pest damages

Insect pests cause significant damage to plants, resulting in reduced growth and yield, or serve as vectors for plant pathogens. The damages could be directly destructive or indirectly by predisposing infested plants to further damage (e.g., reduced photosynthetic abilities). Thrips have a range of host plants ranging from most vegetables to ornamentals and damage plants by feeding on plant aerials and further puncturing epidermal and parenchymal cells. Whiteflies are among the most problematic pests in hydroponic and aquaponics greenhouses due to their direct and indirect effects on vegetables. Directly, they extract phloem sap and produce honeydew, upon which sooty mold grows. Indirectly, they can transfer up to 300 virus species. By feeding, reducing the quality of produce and fiber, and lowering crop yields as a result of a virus, whiteflies in California and Western Arizona (United States) damaged cotton, sugarbeets, melons, and lettuce, causing economic losses worth millions of dollars (Johnson et al., 1982). Similarly, aphids have also been found to have direct and indirect effects on greenhouse crops (Figure 2). Direct effects through feeding on leaves may include loss of leaf saps, leaf deformation, production of honeydew, and the resulting sooty mold fungi. Aphids are also associated with the transfer of several virus species. Aphids caused direct damage in wheat, spring barley, sugar beet, potatoes, field beans, and peas, accounting for 12.5 %, 9 %, 6.5 %, 5.7 %, 46.3 %, and 15.8 % average percentage losses, respectively (Tatchell, 1989).

Mites are also responsible for a significant portion of the losses of crops in indoor and field agriculture. Gall mites damage plants by feeding on epidermal cells and curling leaves (gall mites) (Van Houten et al., 2013). Spider mites significantly reduce photosynthetic rates in plants by piercing the tissues with stylets and sucking epidermal and mesophyll cell contents (Park and Lee, 2002).

These crop damages amount to huge economic losses to greenhouse farmers in different parts of the world, reducing their profitability and financial stabilities. Though, there are limited information on the specific quantification of the financial losses incurred due to pest attacks in greenhouses, but experts have estimated that losses due to pest attacks can amount to about 10 % of the potential income in greenhouses (FarmBiosecurity, 2020). However, in general, there are several reports quantifying the financial losses resulting from pests and pathogens. In Brazil, which is one of the largest producers of agricultural products such as soybeans, sugarcane (sugar and ethanol), oranges, and greenhouse crops, aphids and whiteflies were reported to cause an average annual loss of 7.7 %, which amounts to approximately 25 million tons of food, and US$ 17.7 billion of economic losses (USDA, 2015). In the United State, Whitefly attacks in greenhouses is estimated to cause crop damages worth between US$ 100–500 million annually (Knapp et al. 2020). Also, parasitic nematodes caused damages worth of USD 40.3 million in India agriculture, which is about 0.03 % of the damages caused globally (Abad et al., 2008). Spider mites on the hand when investigated on their economic impacts on a hectare of soybean, showed that they would cost damages worth at least USD 20 per hectare in terms of economic injury level (the amount of pest damages that justifies the cost of control) (Padilha et al., 2020). On a global basis, stem-borers contribute to losses of approximately $334 million, while shoot-flies account for losses of around $274 million. According to Sharma (2006), insect pests in sorghum result in annual yield losses exceeding $1,079 billion globally. Economic losses due to crop-destroying arthropods globally exceed $470 billion annually, undoubtedly an underestimate, given the paucity of data from the developing world (Culliney, 2014).

To control or reduce the losses caused by pest attacks, the emergence of these pests is usually met with either chemical control or the use of natural enemies (biological control agents). Rising environmental issues such as resistance of pests to pesticides, carcinogenic effects of the chemicals on humans, and general environmental pollution are uproaring a condemnation of the use of chemicals. Moreover, pesticides are generally prohibited in aquaponics systems (especially in coupled aquaponics) due to their toxicity risks to fish and beneficial microorganisms. Though biological control methods have been severally suggested as safe control methods for aquaponics, there are currently no biological control agents approved for use in traditional aquaponics systems. Hence, increasing efforts are being channelled towards establishing safe and efficient control methods against these pests.

1.2.3. Plant pathogens

The humid aquatic condition of aquaponics is suited for root-borne pathogens such as pseudo-fungi belonging to the taxa of Oomycetes (e.g., root rot diseases caused by *Pythium* spp. and *Phytophthora* spp.), which are known to produce a motile form of locomotion called zoospores (Stouvenakers et al., 2019). They can spread quickly over an entire crop or system with the aid of the zoospores. Hence, they can shut down an aquaponics operation over a short infestation period if not swiftly addressed. For example, 100 % of cucumbers became infected with an oomycete, *Pythium aphanidermatum,* within three days of exposure to contaminated water (Goldberg et al., 1992). Other fungal genera detected in hydroponics or aquaponics are *Verticillium* and *Didymella*. Also, bacteria pathogens such as *Ralstonia*

sp., *Xanthomonas* sp., *Clavibacter* sp., *Erwinia* sp., and *Pseudomonas* sp., as well as viral pathogens, tomato mosaic (*Solanum lycopersicum*), cucumber *mosaic virus* (CMV), melon necrotic spot virus, lettuce infectious virus, and tobacco necrosis have been reported to have tendencies to be transmitted within hydroponics medium (Jarvis, 1989; Hong and Moorman, 2005). Other pathogens commonly found in aquaponics are listed in Stouvenakers et al. (2019). Recently, there have been fears of a few pathogens having properties to act as cross-contaminants in fish and plants (Mori and Smith, 2019). Though there have not been significant records of such situations, species such as *Gilbertella, Pythium,* and *Phytophthora* have been reported to have such properties. Hence, there is also a need to establish control methods with `amphibian-like` properties.

As mentioned above, there are also limited information on the specific quantification and valuation of the damages posed by the pathogens on greenhouse crops and vegetables. Different global or regional reports have however quantified some of the damages. In general, losses due to diseases amount to 25 % of world crop production per year (Dubey et al., 2016). Viruses pose a significant threat to global food security by causing epidemics in nearly all crucial agricultural crops. These outbreaks result in substantial yield losses, estimated to cost over 30 billion USD each year on a global scale (Nicaise, 2014). Similarly, bacterial diseases also cause devastating damages to crop and significant economic losses. Annually, they cause crop losses of over $1 billion dollars (Mansfield et al., 2012). Collectively, Plant diseases alone are estimated to cause annual losses of up to $10 billion USD in the global floriculture crop industry, according to a recent estimation (McGovern and Elmer, 2017).

1.3. Addressing pest and disease infestations in aquaponics

It has become established that control approaches in the fields and greenhouse hydroponics cannot be automatically adopted for use in aquaponics. While there may be a need to create aquaponics-tailored options, exploring the efficacies, mechanisms, life cycle, and mode of action of existing control methods may provide quicker solutions to pests and diseases in the food production system. Combining these existing methods may also be explored to achieve similar result. An integrated pest and disease management approach has been long established in greenhouse and field operations to combine sustainable control methods to address pest and disease infestations.

1.3.1. Integrated pest and disease management

Integrated pest and disease management (IPDM) is a combination of environmentally, toxicologically, and economically sustainable farming practices that prevent pest damage primarily through the use of natural factors limiting pest population growth and disease development, which resort only if needed to other, preferably non-chemical measures (Van Lenteren and Nicot, 2020). IPDM enables farmers to adopt an approach that considers the environment, economics, and the availability of resources when determining pest and disease management strategies. It incorporates various methods for controlling diseases and pests, aiming to minimize pest activities below the point where they cause economic damage. These methods encompass preventive measures, cultural practices, biological controls, mechanical interventions, physical barriers, and the judicious use of chemicals. The selection and combination of these control methods depend on several factors, including the specific pest or disease, the severity of the infestation, and the economic threshold. IPDM emphasizes the need for continuous adaptation to accommodate interactions between different methods and to stay abreast of the latest knowledge in the field. Typically, an IPDM plan commences

with preventive measures, such as choosing cultivars that are resistant to prevalent pests and pathogens in the region. If other methods prove ineffective, consultation regarding chemical control may be considered as an option.

In 2014, Bittsanszky et al. (2017) explored the possible use of integrated pest management in aquaponics by applying natural enemies; silverleaf whitefly (*Encarsia formosa*), Darwin wasps (*Ichneumons wasps*), and predaceous mite (*Phytoseiulus persimilis*), insecticides; Pegasus Syngenta®, Envidor® and Natural® in aquaponics and hydroponics systems. The authors reported no damage to fish, and the tomatoes cultivated had significantly improved quality. However, this study failed to report the parameters assessed to reach such generalized conclusion. Several other studies, such as Junge et al. (2017) and Merchant (2021), have suggested the adoption of IPDM in aquaponics systems, but none of the research studies have put forth an IPDM framework that comprehensively evaluates the opportunities and obstacles associated with fish, beneficial bacteria, and plants inhabiting in the water. Hence, designing an IPDM structure that assesses the risks associated in adopting different IPDM methods for plants and fish in aquaponics was one of the objectives of this thesis (see Chapter 2).

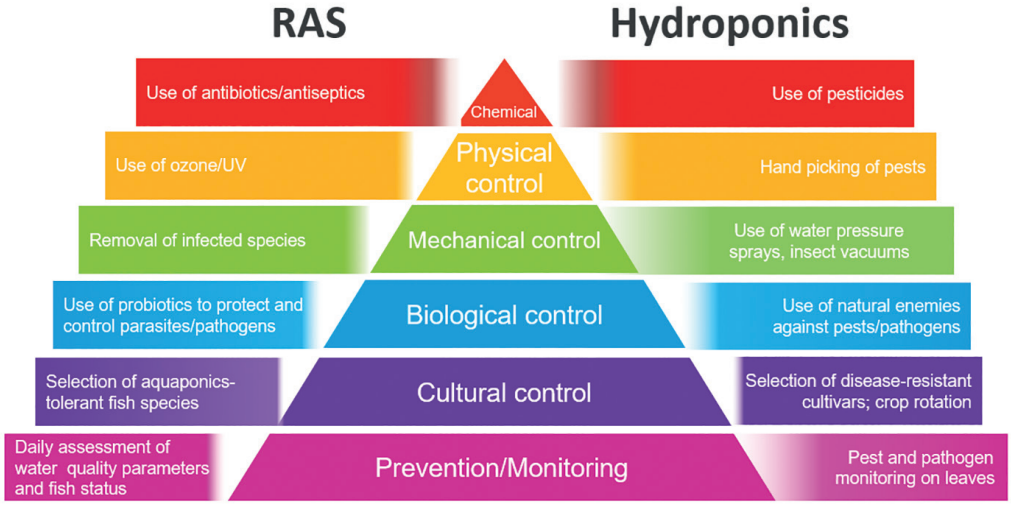

Figure 3. *A sustainable pyramid scheme on integrated pest and disease management designed for aquaponics.*

1.3.2. IPDM in Aquaponics

Despite IPDM schemes being globally accepted for safe agricultural operations in most agricultural food production systems, the IPDM principles do not directly answer aquaponics' safety questions (e.g., fish and nitrifying bacteria). The simultaneous presence of fish and beneficial bacteria in the same water loop as hydroponics plants would demand an exquisite review of the adoptable options for IPDM in aquaponics. In addition, a typical aquaponics IPDM would not only consider the pest and disease management of the hydroponics units, but it should provide a holistic operational procedure that also covers safety operations for fish and biofilter units in aquaponics. This proposed comprehensive aquaponics IPDM can apprehend the established principles of IPDM in greenhouse and field operations. For instance, IPDM generally combines environmentally and economically sustainable methods that prevent and control plant pests and diseases. This principle can be replicated in aquaponics

in a pyramidal-flow approach (as highlighted in Figure 3). The pyramidal-flow highlights a bottom-up approach that assigns preference to the adoption of a safer, environmentally, and economically sustainable control methods in the sequence; prevention→cultural→biological→mechanical→physical→chemical. While prevention and cultural methods are actively used as preventive approaches, biological control methods are suggested as a curative approach ahead of other methods in aquaponic systems (Stouvenakers et al., 2019; van Lenteren and Nicot, 2020). In general, biological control methods are perceived to be safe for fish and beneficial bacteria in aquaponics, but there is currently no biological control agent that has been examined (for safety) or considered efficient for aquaponics crops (Rivas-García et al., 2020). Therefore, one of the objectives of this thesis was to examine the efficacy of different biological control agents and establish their safety for aquaponic systems (See Chapter 3). Chemical control methods, on the other hand, are considered as 'last resort', and are only used when desired results are not achieved (Rakocy, 2012; Reinhardt et al., 2019). However, there is a huge knowledge gap on the specific influence of pesticides, such as runoff rates, and the effects of such on fish and beneficial bacteria inhabiting aquaponics' process water. Consequently, one of the primary goals of this thesis was to explore the runoff rates of various pesticides and assess their impact on aquaponics fish and beneficial bacteria. The aim was to identify pesticides that are safe for use in aquaponics systems (see Chapter 4 and 5).

1.3.2.2. Does fish and plant disease management carry similar risks in aquaponics?

The 'autonomy' the aquaponics farmers have to treat fish diseases away from aquaponics shared water-loop makes fish diseases management less risky. However, it is not practicable to transfer hydroponics crops during pest/disease outbreaks due to root attachments to the cultivating systems. Hence, plant-related pest and disease management problems are considered priorities. Therefore, the urgency required to establish safe pest and disease management measures in the hydroponics section of aquaponics forms the basis of our focus on plant perspectives of this topic.

1.4. Objectives of the thesis

The current study was aimed at a comprehensive investigation of adoptable IPDM methods for the aquaponics system, and this was achieved through the following specific objectives;

1. Assessment of the risks associated with the use of IPDM in different aquaponics designs (Paper 1)
2. Establishment of adoptable biological control for aquaponics pests and pathogens (Paper 1, 2 and 4)
3. Investigation of safe chemical control options adoptable for aquaponics (Paper 3 and 4)
4. Investigation of the specific effects of adoptable chemical controls on fish and nitrifying bacteria in aquaponics (Paper 3 and 4)

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CHAPTER 2

INTEGRATED PEST AND DISEASE MANAGEMENT IN AQUAPONICS: A METADATA-BASED REVIEW

Folorunso, E. A., Roy, K., Gebauer, R., Bohatá, A., Mraz, J., 2021. Integrated pest and disease management in aquaponics: A metadata-based review. Reviews in Aquaculture 13(2), 971–995.

My share on this work was about 50 %.

REVIEWS IN Aquaculture

Reviews in Aquaculture (2021) **13**, 971–995 doi: 10.1111/raq.12508

Integrated pest and disease management in aquaponics: A metadata-based review

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Received 9 June 2020; accepted 30 August 2020.

Abstract

Aquaponics has the potential to produce sustainable, high-quality food through integration of hydroponics and aquaculture, but its commercialization is stalled by bottlenecks in pest and disease management. We reviewed integrated pest and disease management steps and techniques in hydroponics to qualify as suitable techniques for different aquaponic designs. Non-chemical prophylactic measures are highly proficient for pest and disease prevention in all designs. Still, the use of chemical control methods remains highly complicated for all systems. We simulated 10–20% runoff concentrations of 9 pesticides in the common UVI design and compared them with NOEC, LC_{50} of fish. Endosulfan seems most toxic with runoff AI (20.7 μg L^{-1}) exceeding LC_{50} (10.2 μg L^{-1}) and NOEC (0.05 μg L^{-1}). At 20% runoff, most chemical pesticides pose risks in aquaponic systems. Natural pesticides were also discussed as potential alternatives with low acute toxicity to fish, but little is known about their effects on water and bacteria. While insecticides and herbicides are replaceable by well-established commercial biocontrol measures, fungicides and nematicides would still be relevant in aquaponics due to low efficiency of alternatives (e.g. natural enemies, entomopathogenic fungus). Monitoring and cultural control are the first approaches to contain pest population below the action threshold. Biological controls, in general, are adaptable to a larger extent. Further studies are required on how to utilize indigenous microbial community in aquaponics (dominated by Proteobacteria; effective at ~10^3–10^9 CFU mL^{-1}) as a frontline defence.

Key words: Aquaculture, aquaponics, diseases, hydroponics, pest.

Abbreviations
BCA: biological control agent
DAPs: decoupled aquaponics
IPDM: integrated pest and disease management
NOEC: no observed effect concentration
RAS: recirculating aquaculture system
UVI: University of the Virgin Island;
AI: active ingredient

Introduction

The world growing human population will need a further 50% increase in the current food production by 2050 (FAO, 2017). The intensification in food production has resulted in heavy pollution, destruction of habitats, loss of species and erosion of biodiversity (Tilman *et al.* 2002; FAO 2017). There is an eminent interest in shifting the current production model to a more balanced 'eco-economy' that recycles nutrients, prevents or reduces waste, and supports dietary changes (Conijn *et al.* 2018). In this context, sustainable aquaculture methods, which also include practices such as aquaponics, are viewed as an important tool (Tacon *et al.* 2009). Aquaponics is considered a sustainable system that integrates intensive fish culture with hydroponic plant cultivation (Rakocy *et al.* 2004). It allows wastewater from fish to be purified by plants, and then, the purified wastewater may be reused, leading up to >90% water reuse (Tyson *et al.* 2011; Dalsgaard *et al.* 2013; Zou

et al. 2016). It produces little or no pollution compared with conventional fish culture systems (Goddek *et al.* 2015). This further lowers the demand for industrial fertilizers (Rakocy 2007) as compared to agriculture or hydroponic plant cultivation. Despite these benefits, aquaponics still largely remains a 'backyard activity' rather than the desired commercialization (Monsees *et al.* 2017a; Mchunu *et al.* 2018). This is mainly because substantial doubts still exist, as many key questions about the overall feasibility of aquaponic production remain unanswered (Goddek *et al.* 2015; Short *et al.* 2017; Monsees *et al.* 2017a, b; Lunda *et al.* 2019). With only few published surveys available on ground-level realities (Love *et al.* 2014, 2015; Short *et al.* 2017; Mchunu *et al.* 2018), it is often difficult to assess the adoption or success of aquaponics in the commercial context (Monsees *et al.* 2017b).

One of the core bottlenecks hindering the commercialization of aquaponics is pest and disease management (Pilinszky *et al.* 2015; Junge *et al.* 2017; Goddek *et al.* 2019). Pests such as aphids, spider mites, whiteflies and fungus gnats and plant pathogens such as bacteria, fungi and viruses have been reported to cause severe damages to hydroponic plants and reduce yields (Rakocy *et al.* 2012; Goddek *et al.* 2019), simultaneously increasing the investments for pest management control. Depending on the design of aquaponics (coupled, decoupled, with/without mineralization unit; see supplementary material for more details), the use of existing approaches of pest and diseases management often faces restrictions in fear of the possible effects of pesticide, repellent or biological control agent on the non-targeted system components. There is an urgent need to establish pest and disease management that is accommodative for different aquaponic designs. To the best of our knowledge, despite having ample practical or theoretical literature on various aspects of aquaponics, technicalities on integrated pest and disease management (IPDM) 'tailor-made' for aquaponics have been somewhat overlooked for long.

In the last decade, IPDM has evolved to replace excessive use of pesticides for control of pests and pathogens in both field and indoor agriculture (Greenberg *et al.* 2012; Schnelle & Rebek 2013). As a sustainable approach, it combines or synergizes preventive, cultural, mechanical, physical, biological and chemical control methods in established steps to keep pest activities below economic losses (Soloneski & Larramendy 2012; Somerville *et al.* 2014). Like in field agriculture, IPDM principles in hydroponics are carried out in chronological steps that stretch from activities before an outbreak of pest/disease to an assessment of the control method applied. These steps include (i) prevention, (ii) identification of pest, (iii) monitoring of pest activity, (iv) determining and selecting control method(s) and (v) assessment of the method (Stein 2006). Hydroponics, being one of the major components of aquaponics, interacts with the rest of the aquaponic system, depending on the design of such a system (Monsees *et al.* 2016; Monsees *et al.* 2017a). This interaction would expose other components of the system to any IPDM selected in step 4 above. Hence, for effective IPDM implementation, IPDM steps and decision taken would have to consider the components of aquaponic design. This aspect of aquaponics remains completely unaddressed.

To address this knowledge gap, we made an exhaustive review of the existing tools of IPDM in aquaponics and discussed them in the context of different aquaponic designs (background information on aquaponic designs has been provided in the supplementary text). To the best of our knowledge, the present review is the first of its kind. Hence, we investigated the common preventive IPDM techniques and their possible adoption in aquaponics, suitable chronological IPDM steps and methods for different aquaponic designs, and further suggested alternatives for IPDM techniques that are found to be detrimental to aquaponic systems. We further provided an organized strategy inventory that provides technical information on the alternatives and relevant conclusive assessment of the control methods.

Materials and Methods

Collection of literature information

Available published data were collected and compiled using online databases, Google Scholar, ScienceDirect, nature, Scopus and Web of Science. Keywords such as 'Integrated Pest and Disease Management (IPDM)', 'hydroponics', 'pests', 'diseases', 'aquaponics' 'biocontrol', 'coupled' and 'decoupled' were used individually or in combinations to generate matches. Only peer-reviewed and published articles in the English language or with an English abstract were selected. Aside from the primary articles that fulfilled the search criteria, further articles were obtained scanning through the relevant cross-references contained therein.

Assumptions and interpretations

The terms or phrases such as 'microbial biocontrol agent and microbial inoculum', 'intensive aquaculture system and RAS', 'multi-loop aquaponics and decoupled aquaponics', 'coupled and conventional aquaponics', 'integrated pest and disease management and integrated pest management' and 'biological control agents and natural enemies' were used synonymously.

Metadata analyses and simulations on pesticide runoff

Data (n = 236) from 168 research articles about hydroponics and intensive fish culture systems published between

1975 and 2020, covering 14 pests and 23 pathogens, were analysed. To estimate pesticide effects on nitrification and P solubility, data on initial (control) and final values of N mineralization/ P solubility after pesticide application were extracted from studies. Percentage changes were computed (*i.e.* increase or decrease relative to the study controls). To estimate the effective dosage of microbial biological control agents, inhibition concentration (CFU mL^{-1} or CFU g^{-1}) of the potential agents was extracted from hydroponic studies (*in vitro* and *in vivo*). This concentration was compared with the concentration specifications of the corresponding commercial products as obtained from major commercial websites of the biopesticide products. To estimate the phyla of the microbial community in aquaponics (and relative dominance), the percentage proportion of phyla in different studies were grouped accordingly and interquartile ranges were calculated. In cases of data unavailability, inferences were drawn from intradisciplinary studies on hydroponics and intensive aquaculture.

To investigate the effects of pesticides on fish in aquaponics, application doses, NOEC (no observed effect concentration for fish), LC_{50} (data on *Oreochromis niloticus* only) and values of 9 pesticides (cypermethrin, deltamethrin, actara, mancozeb, glyphosate, carbofuran, methomyl, endosulfan and fenvalerate) were extracted from studies and/or commercial specifications. We simulated 10% and 20% runoff of active ingredient in the recommended application rate (formula, runoff AI = percentage runoff × percentage of the active ingredient in product × application dose per hectare) in the most common, standardized and widely described aquaponic system design, that is UVI aquaponic model. The area–volume specification of UVI (University of Virginia Island) systems that we considered during simulation is as follows: 111 196 L total system water volume; 219.6 m^2 (plant) grow bed area; and available dilution water 506.4 L m^{-2} of plant area (Rakocy *et al.* 2004; Bailey & Ferrarezi 2017). A runoff of 10–20% was considered as a reasonable assumption and practically reachable in hydroponic greenhouse activities. All data were analysed in R (R Development Core Team 2015). Graphical modelling (jitter box plot, LOESS plot) was performed using the 'ggplot2' package in R (Wickham 2016).

Results and Discussions

System-specific dilemmas in employing IPDM procedures

Background information on coupled and decoupled aquaponic systems (DAPs), in general, has been provided in the supplementary text. Scanning the available literature, comparative advantages and disadvantages of IPDM between coupled and decoupled aquaponic systems are summarized in Table 1 (*data* from Jarvis 1989; Stanghellini 1993; Stanghellini & Rasmussen 1994; Ehret *et al.* 2001; Song *et al.* 2004; Tyson *et al.* 2004, 2011; Date *et al.* 2005; Sommerset *et al.* 2005; Rakocy 2007; Appels *et al.* 2008; Pedersen *et al.* 2009, 2010; Rakocy *et al.* 2012; Goddek *et al.* 2015; Kloas *et al.* 2015; Jones 2016; Sirakov *et al.* 2016; Monsees *et al.* 2017a, b; Schmautz *et al.* 2017; Yavuzcan Yildiz *et al.* 2017, 2019; Bartelme *et al.* 2018; Goddek & Keesman 2018; Roh *et al.* 2018; Delaide *et al.* 2019; Kotzen *et al.* 2019; Mori & Smith 2019; Stouvenakers *et al.* 2019). We encountered some system-specific dilemmas (*i.e.* incompatibilities) in the type of pest and disease control methods that can be used.

Coupled aquaponics

The pesticides, repellents and biological control used during pest and disease infestation of plants in hydroponic units may affect the biofilter bacteria and fish (Rakocy 2007; Rakocy *et al.* 2012; Stouvenaker *et al.* 2019). The nutrient recovery in mineralization unit carried out either by aerobic or by anaerobic microbial digestion of fish sludge can be sensitive to pesticides (Goddek *et al.* 2015; Stouvenaker *et al.* 2019), thus affecting the overall nutrient mobilization of the aquaponic system. On the other hand, fish antibiotic/therapeutic residues used in the rearing unit can be taken up by the plants which can further be transferred to human (Rakocy *et al.* 2012). All these can culminate in the end impact, which majorly is the effects on the safety of the final harvests meant for human consumption.

Decoupled aquaponics

In elementary DAPs, the plant water is not reused for fish production. Therefore, the water (if laden with pesticides, repellents or biological controls) cannot enter the fish culture and mineralization unit either. This design could accommodate more approaches to pest and disease management without much interference with other compartments. Recent designs of DAPs, however, allow to reuse the evapotranspiration water from plants via condensation in cooling traps (Kloas *et al.* 2015; Monsees *et al.* 2017a), or to treat the hydroponic solution in desalination units (Goddek *et al.* 2018) before passing on to the fish unit. However, the transfer of pesticide/repellent via these 'water recovery' options is presumably minimized or nullified (Reinhardt *et al.* 2019). Future research should focus on the residual chemicals (from pesticides/repellents) in the condensate (for cooling traps) or filtrate (for desalinization units) to reinforce the safety claims of DAPs over coupled aquaponics.

Prevention

The rationale behind 'prevention in aquaponics' is clarified in the supplementary text. Below, using peer-reviewed articles, we highlighted the potential effects of various prophylactic measures on different aquaponic components.

Table 1 Checklist of advantages of pest and disease management procedures in coupled and decoupled aquaponic systems

IPM steps	Pest and disease management procedure			CAP	DAP (without water reuse)	DAP (with water reuse)
Prevention	The direct addition of chemical sanitary is destructive to fish and beneficial bacteria.			✗	✓	✗
Pest detection and identification	No possible effect.			✓	✓	✓
Monitoring of pest	No possible effect.			✓	✓	✓
Reviewing and selection of control methods.	No possible effect.			✓	✓	✓
a.	Cultural control	No possible effects.		✓	✓	✓
b.	Physical control	UV irradiation	Nitrifying bacteria are photosensitive but are shielded in the media bed.	✓	✓	✓
		Blue-light emitting diodes	Non-detrimental.	✓	✓	✓
		Heat	Non-detrimental.	✗	✓	✓
		Sonication	Effects yet unknown.	✗	✓	✓
	Filtration	Media	Non-detrimental.	✓	✓	✓
		Slow	Non-detrimental.	✓	✓	✓
c.	Mechanical control	Picking/ blasting	Non-detrimental.	✓	✓	✓
		Traps	Non-detrimental.	✓	✓	✓
		Density manipulation	Non-detrimental.	✓	**NA**	**NA**
d.	Biological control	Parasitoids	Non-detrimental	✓	✓	✓
		Predators	Non-detrimental	✓	✓	✓
		Microbial inoculant	Capable of initiating competition with beneficial bacteria.	✗	✓	✗
e.	Chemical control	Pesticides/antibiotics in coupled aquaponics can cause: • Destruction of beneficial bacteria • Alteration of biofilter efficiency • Residual effect on plant/vegetable • Residual effect on fish The multi-loop capacity of decoupled systems allows optimization of the system, and thus, the chemicals can be contained at the applied unit.		✗	✓	✗
Assessment of the selected method.	No possible effect			✓	✓	✓

Present gaps in knowledge/ impact unknown (future research areas)

- Residual effects of fish antibiotics and fish vaccines on vegetables or fruits.
- Effects of probiotics and microbial biological agents on the beneficial bacteria.
- Effects of natural pesticides on fish and beneficial bacteria.

Symbols: ✓ = 'safe'; ✗ = 'threat', 'CAP' indicates 'coupled aquaponics', 'DAP' indicates 'decoupled aquaponics'.

Replicability issues of general prophylactics in aquaponics

Various prophylactic measures are carried out separately in hydroponics and RAS systems to avoid the infestation of pests, pathogens or the occurrence of diseases (Stouvenakers *et al.* 2019). Most of these measures are usually put in place before the emergence of diseases and pests. Their replicability (from hydroponics to RAS or vice versa) in aquaponic systems could be determined by the administration procedure, the nature of the measure and the type of aquaponics. In this sight, some major replicability issues are highlighted in Table 2.

Plant compartment → Fish compartment

Hydroponic farmers have developed practices that are regularly taken to prevent pests and disease outbreaks in the system (Goddek *et al.* 2016). These include (i) general sanitation routine, (ii) direct treatments and (iii) environmental manipulation. General sanitation routine includes the use of disinfection mats, specific protective clothes, room sanitization, barrier netting or planting measures such as seasonal fallow period and use of disease-resistant plant cultivars (Jarvis 1989; Stanghellini 1993; Albajes *et al.* 1999). Direct treatments include

Table 2 Bottlenecks of common prophylactic measures of hydroponics or intensive aquaculture domains for application in aquaponic systems

Category	Measures	Bottleneck/ risk	References
Environmental manipulation	• Increasing temperature (heating nutrient solution)	• Alteration of optimal nitrification and mineralization in CAP. • Indirect effects on fish growth.	Emparanza (2009), Somerville *et al*. (2014)
	• Lowering pH	• Alteration of optimal nitrification and mineralization in CAP. • Competition among nitrifying bacteria. • Indirect negative effects on fish growth.	Tyson *et al*. (2004), Zou *et al*. (2016), Rakocy (2007), Hüpeden *et al*. (2016)
	• Lowering humidity	• No reported effects on fish and beneficial bacteria. • Unknown effects on evapotranspiration and condensate in decoupled systems. • Possible effects on physiological activities on crops.	Jarvis (1989), Stouvenakers *et al*. (2019), Stanghellini (1993), Xu *et al*. (2007)
Hydroponic measures	• Fallow period. • Planting certified seeds. • Disinfecting tools. • Sanitizing room. • Wearing specific body wears. • Barrier netting. • Planting sacrificial plants. • Using chemical sanitaries.	• No reported effects on fish and beneficial bacteria on the rest of the measures except chemical sanitaries, which can kill or reduce the beneficial bacteria and fish in CAP. • Fallow periods would simply lower or stop culture activities for the designated period and subsequently reduce yield.	Date *et al*. (2005), Jones (2016), Song *et al*. (2004), Stanghellini and Rasmussen (1994)
RAS measures	• Using pathogen-free water. • Using dietary additives. • Using disease-resistant strains. • Lowering stocking density. • Disinfection of working tools. • Quarantining.	• Use of chemical disinfectants (e.g. hydrogen peroxide), prophylactic antibiotics and dietary immunostimulants in coupled aquaponics are capable of impairing nitrification processes and residual effects on vegetables.	Cabello (2006), Pedersen *et al*. 2009

treatment towards the standing population, for example water treatments with ultraviolet radiation (UV), heating, slow filtration techniques or use of chemical sanitary such as cyromazine, chloramines, humic acid and prochloraz, which are not recommended in coupled aquaponics (Song *et al*. 2004; Date *et al*. 2005; Jones 2016). In environmental manipulation, farmers usually manipulate environmental variables, temperature, pH, humidity, water vapour density and their interaction. Disease prevalence is generally dependent on these factors (Jarvis 1989). A case example is provided in the supplementary text. This implies that solutions would have to be selected based on the aquaponic design at hand.

Those measures which do not involve direct application into the common nutrient water are usually replicable across all aquaponic systems. In contrast, prophylactic measures needing direct application into process water pose risk, especially in coupled aquaponics. The measures involving environmental manipulation to eliminate target pest or pathogen pose can be overcomplicated at times. The manipulated environmental conditions might be antagonistic to optimum conditions required by fish, biofilter bacteria or the plants itself.

Fish compartment → Plant compartment

Similarly, prophylactic measures in recirculating aquaculture system (RAS) include general culture measures (e.g. use of pathogen-free water, tools disinfection, quarantining, use of pathogen-resistant strains and stocking at low density) and substance-based measures (e.g. probiotics, bioremediators, anti-parasitic substances) (reviewed in Assefa & Abunna 2018; Dawood *et al*. 2019; Lieke *et al*. 2019).

The substance-based measures might be detrimental in conventional aquaponics due to the residual effects (residues) they could pose a threat to plants, rhizosphere microbiota, final uptake into fruits or leafy vegetables, or mineralization units itself (Rakocy 2007; Rakocy *et al*. 2012). Research on this aspect has been meagre, and existing knowledge is mostly presumptive or qualitative.

Pest monitoring

Despite several preventive measures underlying, pest and pathogen outbreak might still be inevitable. Hence, farmers would have to be prepared to carry out further steps of IPM. Pest and disease monitoring are the first steps in IPM. In general, pest location and identification require frequent

plant inspection (mainly leaves) (Boissard *et al.* 2008). One of the most common methods for pest monitoring in the greenhouse is conventional sticky traps (Pinto-Zevallos & Vänninen 2013). The traditional pest scouting, identification and counting on sticky cards might be exhausting for a large-scale aquaponics (Xia *et al.* 2015). There are ample studies carried out on the use of automated systems for this exercise (*see* Cho *et al.* 2007; López-Morales *et al.* 2008; Xia *et al.* 2015). On the other hand, the aquatic medium of aquaponics creates more room for pathogens such as *Pythium, Fusarium* and *Phytophthora* species (Jarvis *et al.* 1993; Goddek *et al.* 2018). Hence, more extensive and frequent plant inspection would be required to early detect potential pests and pathogens in the system. Farmers should frequently observe for bloom disease symptoms and monitor flies (e.g. fungus gnats, mosquitoes), which might be vectors of plant pathogens (virus, fungi and bacteria). Besides, seedlings and portions of nutrient solution exposed to light also develop algae, which not only competes for nutrients but also serves as food for shore flies and fungus gnats.

On sighting 'actual' pest, correct identification of such pests is the most important step to controlling it (Norris *et al.* 2003). The correct 'current' pest identification technique is the most important step in pest control (Norris *et al.* 2003). Ease of identification of pests might be associated with farmer's experience or access to the consultation (internet or experts). There are numerous photographic guides on the identification of different pests (Jepson 1987; Blackman & Eastop 2000; Zhang 2003).

Disease monitoring in rearing unit

Furthermore, to establish the health status of an entire aquaponic system, pathogen detection and identification would have to be further extended to the RAS unit of the system. In this respect, the measurement of ammonia and nitrite of water should be carried out at least once a week to establish the efficiency of the biofilter at converting ammonia to nitrate. Also, fish behaviours such as swimming and response to feeding should be observed daily for unusual behaviour. Common places of infections such as eyes, gill filaments, caudal peduncle and distal ends of caudal and dorsal fins should be frequently observed for signs of diseases. Besides, key water quality parameters such as dissolved oxygen, pH and temperature should be tested daily. All these practices are necessary for all designs of aquaponics, and they are of utmost importance to the early detection of pathogens and pests and are not perceived to create any possible negative feedback.

Monitoring pest activity (population level of pest)

After detection and proper identification of the pest, a farmer would need to establish surveillance to determine the level of the detected pest population, and further assess the potential for economic loss (Norris *et al.* 2003; Abrol & Shankar 2012). This is usually carried out with the use of sticky card traps, light traps, sex pheromone traps, etc. All these methods have been severally reviewed and discussed in Abrol and Shankar (2012) and Miller *et al.* (2015). The results from the monitoring activities would inform the farmers on when to initiate a control strategy (if needed), at a point (action/economic threshold), where the cost of yield loss exceeds the cost of given management (Hallett *et al.* 2014). It is usually expressed as a ratio of 'cost of control' to the product of 'price of produce, loss per yield and reduction in pest attack (Yencho *et al.* 1986). Several studies have established an action threshold for pests and different plants (Nault & Shelton, 2010; Ramsden *et al.* 2017). Hence, the monitoring of pests in aquaponics using the above method(s) is not perceived to pose any further negative effects on other components of the system and would fit-in into the IPDM of any design.

Reviewing and selecting a control strategy

The rationale behind reviewing and selecting a control strategy is clarified in the supplementary text.

a. Cultural control

This is a common, proven approach for agriculture–horticulture yet overlooked in aquaponics so far. Cultural control is practices that are employed before, during or after planting to prevent pests and diseases (Rodríguez-kábana & Canullo 1992). These practices range from selection of disease-resistant crop variety or less succulent plants to crop rotation (Somerville *et al.* 2014). Other practices include spacing, companion planting, trap cropping and fertilization (Somerville *et al.* 2014; Jones 2016). Furthermore, stunted growth of plants or yellowing of leaves in conventional aquaponics might be attributed to the imbalances in the fish density: plant area ratio. Thus, the numbers of fish in the rearing unit are usually increased to obtain an improved result in the system or vice versa (Somerville *et al.* 2014). All these practices (summarized in Tables 2 and S3 in supplementary material) are directly non-detrimental to any units of aquaponics, and their clinical adoption would reduce the cost for other control methods. Some key bottlenecks or disadvantages of cultural controls that can indirectly affect aquaponics are summarized in Table 2, and supporting information is provided in the supplementary text.

Cultural and pest monitoring methods are the best first approach to contain pest infestation or disease after they have been detected, because they can be used to keep the pest population below the action threshold (economy injury level), with little or no cost. Risks of economic loss

can be reduced when they are effectively combined with preventive measures. However, since these methods are manipulative strategies, farmers require a good understanding of the system and culture organisms/plants to plan an effective cultural control method. Otherwise, farmers might have to seek other control methods.

b. Physical and mechanical control measures

Jet streaming with water (for plant pests). Being the most basic one, it involves actively removing the pests away from the plants by using high-pressurized 'jet stream' of water to wash off the pests on leaves or plants, to minimize their infestation or kill them (Somerville *et al.* 2014). But the limited penetration of water jets deeper into the canopy to eradicate most of the insect pests is questionable. Besides, fetching bulk quantity of water for jetting might be cumbersome for the time taken and expensive especially for large-scale aquaponics (Sakthivel *et al.* 2011). However, this method is generally adaptable in all aquaponic designs with no foreseeable threat.

Ultraviolet (UV) irradiation (for water-borne pathogens). UV irradiation which is commonly effective at a wavelength of 200 to 280 nm (Van Os 2009) produces detrimental effects on microorganisms by damaging their DNA and consequently reducing microbial loads by up to 99% (Elumalai *et al.* 2017; Xu *et al.* 2018). Mori and Smith (2019) study found that there is a wide variation in the sensitivity of fish and plant pathogens to UV doses. An example is furnished in the supplementary text. To optimize UV treatment, the recommended turbidity is < 2 NTU (Zheng *et al.* 2014). Hence, the prefiltration of nutrient water is usually carried out before the use of UV irradiation to remove the suspended solids. In aquaponics, however, turbidity can be manually reduced by pre-treating the water in gravel or sand-bed unit, where protozoa and algae can also be removed (Bennet 2008). However, any beneficial bacteria or microbe in the process water will most likely be neutralized as well (Mori & Smith 2019), indicating overall effects this could have in coupled systems. Results of some specific studies using UV on aquaponic water are compiled in the supplementary text. The technical use of UV sterilizers in aquaponics can be restricted to treating incoming freshwater (water source) to avoid immeasurable effects on the beneficial bacteria and rhizosphere community.

However, the use of UV in aquaponics has not been largely reported to creating a significant problem in the system designs of aquaponics, but their use might be restricted to only large-scale aquaponics which could also be increasing cost at large. Alternatively, an influx of pathogenic organisms in irrigation water sources is sources of pathogens in RAS systems; hence, it would be more cost-effective for farmers (especially small-scale farmers) to use disease-free water sources. Groundwater sources such as borehole and well water or rainwater have been reported to be more pathogen-free than surface waters such as river or lake water which contain more pathogenic organisms (Steele & Odumeru 2004; Bregnballe 2015).

Ozonation (for water-borne pathogens). Ozone application is highly effective for the control of microbial and chemical contamination in hydroponics and highly efficient at inactivating pathogens such as *Fusarium* sp., *Phytophthora* sp. and *Pythium* sp. in nutrient solutions (Igura *et al.* 2004; Schnitzler 2004). However, it produces oxidative by-products (e.g. reactive oxygen species, free radicals) and a significant amount of residual oxidants (e.g. brominated compound and haloxy anions, OH^-) that are toxic to fish (Igura *et al.* 2004; Gonçalves & Gagnon 2011; Graham *et al.* 2011). Ozone decomposition which is initiated by pH and temperature leads to the formation of OH^- and reactions of compounds such as sulphite, nitrite, olefinic aliphatic hydrocarbons, phenols, polyaromatic hydrocarbons, organic amines and sulphides (Hoigné 1988). The oxidative property and the resulting reactions or effects of the above-listed compounds on the standard water quality in aquaponics make the use of ozone in aquaponics dangerous as it poses risk to fish and beneficial bacteria and even human (Pattillo 2017). Farmers might be compelled to work with experts to develop an ozonation process that fits specifically for a certain design to ensure a high level of safety.

Filtration (for water-borne pathogens). Filtration in hydroponics involves filtering incoming water or effluent water from particulates such as microorganisms through a granulated or fibrous material (Berkelmann *et al.* 1995; Boller & Kavanaugh 1995). Filtration techniques majorly used in hydroponics are membrane and slow sand filtration (Ehret *et al.* 2001). Water flow rate, sand/grain size and genus of pathogen determine the effectiveness of slow sand filtration at removing pathogens (van Os *et al.* 1999; Deniel *et al.* 2006). Results of some specific studies using slow filtration on aquaponic water are compiled in Table S2 and discussed in the supplementary text. Fine sand and common grain size of 0.15–0.3 mm might be perceived to be impractical with large capacity aquaponics, but farmers could rather substitute with larger media such as gravel or less fine sand to increase flow rate, but the better result is reportedly obtained with finer sand size. The peculiarity of slow sand filtration is that it is highly cost-effective for the removal of pathogens (Bennett 2008), making it affordable for use in small-scale aquaponic systems. Filtration techniques are non-selective; hence, coupled aquaponic farmers should preferably install them at the inlet of the biofilter to treat

freshwater from the water source. Though slow sand filtration does not eliminate all pathogens (van Os *et al.* 2001), it can easily 'fit-in' into any aquaponic design, when it is aimed for removing pathogens from water source right at the inlet of the biofilter. Besides, since this technique automatically removes particles from water, reducing turbidity, its combination with UV improves the overall quality of water.

c. Biological control measures

Entomopathogenic microorganisms (for plant pest). Entomopathogenic microorganisms are considered the most important group of microorganisms for controlling greenhouse pests (Osborne *et al.* 2004). The common among these are entomopathogenic bacteria, *Bacillus thuringiensis*, entomopathogenic fungi and entomopathogenic nematodes (Osborne *et al.* 2004; Khan *et al.* 2012). Entomopathogenic nematodes are commonly used against soil-dwelling insect pest; hence, their use in the soilless system is limited (Osborne *et al.* 2004). However, many entomopathogenic nematodes used in greenhouses are commercially available (e.g. NemaShield, Nemasys, Scanmask, Nemaflor, Nemycel and Entonem) (Kaya & Koppenhöfer 1996; Koppenhöfer *et al.* 2000). Entomopathogenic bacteria attack host via ingestion (per os), making them effective against pest larvae. *Bacillus thuringiensis* subs. *kurstaki* have been found effective against *Tuta absoluta* which causes serious damages in tomatoes (Giustolin *et al.* 2001; González-Cabrera *et al.* 2011).

On the other hand, entomopathogenic fungi parasites directly breach the cuticle to enter the insect haemocoel, causing infections in many insect species (Khan *et al.* 2012). They have been found effective against many insect species belonging to the orders Hemiptera, Orthoptera, Thysanoptera, Homoptera, Coleoptera, Diptera and Lepidoptera. Also, their acaripathogenic characteristics make them a potential biocontrol for a broad range of mites and ticks (Zimmermann 2007; De Faria & Wraight 2007). Some bioinsecticides are based on entomopathogenic fungi (Ascomycota: Hypocreales) commonly used in protected cultures against sucking pests such as whiteflies, thrips, aphids, mealybugs and scales (Inglis *et al.* 2001; Osborne *et al.* 2004). In Table S6, we presented some of the approved entomopathogenic microorganisms in use in the EU and United States.

Microorganisms as biocontrol agents (for fish). Inactivated and attenuated microorganisms or their derivatives commonly referred to as vaccines have been used against bacterial, fungal or viral fish diseases in intensive aquaculture systems (*reviewed in* Assefa & Abunna 2018). They are usually administered orally, through bath or injection (Sommerset *et al.* 2005). Some options are much more 'applied' than vaccination in intensive fish culture units, owing to their broad-spectrum effect and flexibility in the application (through feed or in water directly). Beneficial live microorganisms called probiotics, their growth substrates called prebiotics (in a combination called 'symbiotic') or simply immunostimulants, and herbal extracts through feed have much wider application in fish disease management (*reviewed in* Dawood *et al.* 2019; Soltani *et al.* 2019). Most biological controls designated for fish pest and disease management are usually advocated as 'fish-friendly' choices, albeit their slow mode of action than chemical therapeutics (some of which might sooner or later face ban in Europe; Lieke *et al.* 2019).

Non-targeted effects of microorganisms as biocontrol agents (BCAs) in aquaponic set-ups. Studies analysing the non-targeted effects of biological control agents in the aquaponic system are limited and often contradictory. At least the ones having direct application in water (or systems) might have some effect, positive or negative, which requires further clarification (Stouvenakers *et al.* 2019). There are few reasonable risks associated with the inoculation of foreign microbial BCA in coupled aquaponics (elaborated in the supplementary text).

For plant compartment – *Pseudomonas fluorescens* and related species are known to colonize the rhizosphere aggressively and establish competition with root pathogens for nutrients (Couillerot *et al.* 2009). Such competition may concern the acquisition of organic substrates released by seeds and roots (Kamilova *et al.* 2005), as well as micronutrients such as soluble iron, which is often in limited amounts in aquaponics (Eck *et al.* 2019; Robaina *et al.* 2019). Also, microbial communities can produce multiple modulatory effects on plant physiology (Joyce *et al.* 2019). *Microbacterium oxydans, Pseudomonas thivervalensis* and *Burkholderia cepacia* tested as plant growth-promoting bacteria affected the cultivation-dependent and cultivation-independent bacterial communities in the root endosphere and rhizosphere of *Brassica napus* (Ren *et al.* 2019), which can further reduce plant immunity to diseases.

For fish compartment – Only a few studies have investigated the effects of entomopathogenic microorganisms on fish, which might be due to their low adoption in pest and disease management or safety perception of the public on the products. The results from such studies are compiled and provided in the supplementary text. On the other hand, there are also reported effects of microbial biocontrol agent (probiotic) use in RAS systems. Phaeobacter probiotics grown primarily against pathogens of the family *Vibrioniaonaceae* in RAS biofilter limit the colonization of the pathogen, but further competes with nitrifying bacteria for oxygen, nutrients and space in the biofilm which would have led to reduced nitrification recorded (Prol-García &

Pintado 2013). These studies showed probiotics added to an established biofilter can endanger the beneficial biofilter population or reduce the efficiency of the unit. Existing knowledge and the gaps in between are mentioned in the supplementary text.

Potential of aquaponic microbial community as biological control. As discussed above, the use of external microbial biocontrol in aquaponics is limited by their potential effects on fish and beneficial bacteria. Hence, it is important to explore the potential of the indigenous microbial community for disease management. Recent studies have explored the potential of aquaponic microbial community at disease control (Schreier *et al.* 2010; Schmautz *et al.* 2017; Wongkiew *et al.* 2018; Bartelme *et al.* 2018; Eck *et al.* 2019). About 13 to 15 phyla have been reported in different compartments of the system, but the dominant genera are usually 6–7 (Figure S4). Approximately, proteobacteria (42%), bacteroides (15%) and actinomycetes (13%) form the dominant bacterial consortium in most aquaponic systems. The average CFU in biological filter is about 7.3×10^6 per gram of media, and total concentration of bacteria on biofilter media ranges between 5.1×10^6 and $1.1 \times 108 \times 10^7$ (Munguia-Fragozo *et al.* 2015). Proteobacteria (e.g. *Pseudomonas*) and Bacteroidetes (e.g. *Bacillus*) species have been used successfully as biological agents in hydroponics and aquaculture (probiotics). Additional information in Figure S2 in supplementary material shows that microbial inoculants majorly tested in hydroponics are dominated by heterotrophic bacteria (e.g. *Pseudomonas* and *Bacillus*) (68%), due to their broad-spectrum efficiency over several pathogens. They are most effective between 10^3 and 10^9 CFU mL^{-1} (*see* Table 3 and Figure S3 in the supplementary material). Though they seem to hold good potential for disease control and prevention in aquaponics (Montagne *et al.* 2017; Stouvenakers *et al.* 2019), but there is currently limited information on the specific taxonomic identification of the microbial phyla and the possible usage characteristics. Moreover, Figure S1 (supplementary material) shows that *Bacillus* sp. (38%), *Trichoderma* sp. (19%) and *Burkholderia* sp. (14.3%) are relatively more available in commercial biopesticides than *Pseudomonas* sp. (4.8%). Common genera and effective dosages of common microbial biological agents against plant pathogens in hydroponic studies are presented in Table 3, and the common biopesticides approved for use in the EU and United States are provided in Tables S1 and S6 in the supplementary material.

Table 3 Common genera and effective dosages of common microbial biological agents against plant pathogens tested in hydroponics

Microbiological control agents	Pathogens	Effective spore concentration	References
Pseudomonas spp.	*Pythium aphanidermatum* and two strains of *Rhizoctonia solani*	10^5 CFU mL^{-1}	Moruzzi *et al.* (2017)
Fusarium spp.	*Curvularia lunata, Fusarium semitectum, F. oxysporum* f. sp. *lactucae, Rhizoctonia solani*	1.2×10^5–1.6×10^5 CFU mL^{-1}	Thongkamngam and Jaenaksorn (2017)
Pseudomonas spp.	Deleterious rhizosphere microflora	1.05×10^3 CFU mL^{-1}	Peer and Schippers (1989)
Rhizosphere microbiota	*Fusarium oxysporum*	10^5 CFU mL^{-1}	Fujiwara *et al.* (2013)
Lysobacter spp.	*Pythium aphanidermatum*	10^{5-6} CFU g^{-1}	Folman *et al.* (2004)
Rhizosphere microbiota	*Pythium aphanidermatum*	10^5 CFU mL^{-1}	Postma *et al.* (2000)
Pseudomonas spp.	*Pythium aphanidermatum, P. dissotocum*	10^7 CFU mL^{-1} (3×10^5 CFU g^{-1})	Chatterton *et al.* (2004)
Pseudomonas spp.	*Fusarium oxysporum*	10^9 CFU mL^{-1}	Duffy and Defago (1997)
Rhizosphere microbiota	*Pythium aphanidermatum*	10^5 CFU mL^{-1}	Postma *et al.* (2000)
Pseudomonas spp.	*Fusarium solani*	0.6–1.8×10^5 CFU g^{-1}	Anderson and Guerra (1985)
Pseudomonas spp.	*Pythium aphernidermatum*	10^7 CFU mL^{-1}	Sopher and Sutton (2011)
Rhizosphere microbiota	*Pseudomonas corrugate*	10^8 CFU mL^{-1}	Lee *et al.* (2010)
Bacillus spp.	*Pythium aphernidermatum*	1×10^9 CFU mL^{-1}	Utkhede *et al.* (2009)
Trichoderma spp.	*Pythium aphernidermatum, P. cryptogea*	10^8, 10^9 CFU g^{-1}	Khalil *et al.* (2009)
Fusarium spp.	*Fusarium oxysporum*	10^6 CFU mL^{-1}	Eparvier *et al.* (1991)
Pseudomonas spp.	*Fusarium oxysporum*	10^8 CFU mL^{-1}	Eparvier *et al.* (1991)
Bacillus spp.	*Phytophthora capsica*	10^6 CFU mL^{-1}	Li *et al.* (2020)
Pseudomonas koreensis 2.74	*Pythium* spp.	$10^{7.5}$ CFU mL^{-1}	Hultberg *et al.* (2011)
Lysobacter	*Pythium aphernidermatum*	10^4 CFU mL^{-1} (log 5–6 CFU g^{-1} root)	Folman *et al.* (2004)
Pseudomonas spp.	Pathogen prevention	10^5 CFU mL^{-1}	van Os and Postma (2000)

Pseudomonas, Bacillus, Trichoderma and *Fusarium* are effective against root pathogens at microbial load ranging from 10^4–10^9, 10^6–10^9, 10^7–10^9 and 10^5–10^7 CFU mL^{-1}, respectively.

Macro-organisms as biocontrol agents (for plants only). The use of natural enemies against pests, as an option, is available for greenhouse pests (Paulitz & Bélanger 2001). A prerequisite for the release of natural enemies is that natural enemies immediately suppress the pest populations and due to their reproduction can manage several pest generations (van Driesche & Heinz 2004; Hajek & Eilenberg 2018). The most common macro-organisms used as biological control agents in hydroponics are summarized in Table 4 and the supplementary text. These natural enemies are either predators or parasitoids. Predatory mites (Acari) such as *Amblyseius swirskii* and *Phytoseiulus persimilis* are highly efficient due to the wide range of pests such as whiteflies, thrips, phytophagous mites and dipterans they can attack (Navarro-Campos *et al.* 2020). However, despite their reported success in many crops, the sticky hairs on tomato plants have reduced their performances on tomato pests (Gullino *et al.* 2020). Common predatory ladybirds (Coleoptera) such as *Adalia bipunctata, Cryptolaemus montrouzieri* and *Dephalstus catalinae* are used on greenhouse aphids, mealybugs, scales and whiteflies (Gullino *et al.*, 2020). Other predatory biocontrol agents include Hemipterans (e.g. *Macrolophus pygmaeus*), Nematodes (e.g. *Heterorhabditis bacteriophora*) and Neuropterans (*Chrysoperla carnea*), which primarily predate on thrips, shore flies and aphids, respectively (McEwen *et al.* 2007). In contrast, parasitoids such as *Aphidius colemani* (for aphids) and *Encarsia formosa* (for whiteflies) are more specific in the pest they attack, making them more sustainably compatible with other biocontrol agents. However, they are highly prone to attacks from hyperparasitoids such as *Alloxysta victrix* and *Asaphes lucens*, rendering them less effective at controlling large pest infestations (Sullivan 2009).

Non-targeted effects of macroscopic biocontrol agents (BCAs) in aquaponic set-ups. In terms of non-targeted impacts, the common presumption is that the parasitoids and predators would eventually be consumed by fish if they accidentally drop in water (Somerville *et al.* 2014). Lee and Welander (1994) investigated the influence of predators (e.g. rotifers

Table 4 Most common macro-biological control agents in hydroponics

Macro-biological control agents	Pathogen	Release rate (per m^2)	Price ($)
Aphidius colemani	Cotton aphids (*Aphis gossypii*), green peach aphids (*Myzus persicae*) and tobacco aphid	1–1.5 mummy	0.054/ mummy
Aphidius ervi	Potato aphids (*Macrosiphum euphorbiae*), rose aphid (*Macrosiphum rosea*)	1–1.5 mummy	0.15/ mummy
Aphidius matricariae	Potato aphids (*Macrosiphum euphorbiae*) and rose aphids (*Macrosiphum rosea*)	1–1.5 mummy	0.054/ mummy
Aphelinus abdominalis	Green peach aphid (*Myzus persicae*)	1–1.5 mummy	0.054/ mummy
Parasitic wasps mix	Aphid species	1–1.2 mummy	–
Adalia bipunctata	Aphid species	10–20 individuals/ plant	0.2/ individuals
Encarsia formosa	Greenhouse whitefly (*Trialeurodes vaporariorum*), tobacco whitefly (*Bemisia tabaci)*	2.15–2.5 parasitoid pupae	0.032/ parasitoid pupae
Chrysoperla rufilabris, Chrysoperla carnea	Aphids, spider mites, thrips, whitefly	4–9 larvae	0.028/ larvae
Trichogramma pretiosum	Genus *Heliothis* sp. and other Lepidoptera pests	1 tab for 27.9 m^2 (60000 parasite eggs/tab)	30/ tab
Macrolophus pygmaeus	Whitefly (eggs, larvae and pupae), thrips and aphids	10 individuals	–
Orius laevigatus	Thrips (sometimes spider mites, aphids and moth eggs)	3–5 individuals	0.08/ individual
Stratiolaelaps scimitus	Fungus gnat (*Sciaridae*)	54–108 individuals	0.012/ individual
Amblyseius swirskii	Thrips, whitefly and mite species	50 individuals	0.0021/ individual
Amblyseius (Neoseiulus) californicus	Broad mites and cyclamen mites.	22 individuals	0.035/ individual
Phytoseiulus persimilis	Two-spotted spider mites (*Tetranychus urticae*)	54 individuals	0.01/ individual
Mesoseiulus longipes	Spider mites	32 individuals	0.04/ individual
Phytoseiulus persimilis	Two-spotted spider mites	54 individuals	0.01/ individual
Amblyseius (Neoseiulus) cucumeris	Thrips	100–200 individuals	0.0006/ individual
Iphiseius (Amblyseius) degenerans	Thrips	0.2 individual	5.9/ individual
Typhlodromips montdorensis	Whitefly, mites, small arthropods	50–150 individuals	0.0025/ individual
Steinernema carpocapsae	Shore flies (*Scatella stagnalis*) and caterpillars.	500 000 infective juveniles	0.5/ 100000 juveniles

The rate at which the natural enemies are released varies with the pest, natural enemies and area. For aphids, an average of 1-1.5 mummy of aphid parasites is released per square metre area.*Sources*: Royal Brinkman, Koppert, Bugs for Bugs, Green methods, Biobest.

and nematodes) on nitrification in aerobic biofilm processes and found biofilm predators reduced nitrate production rate (from 4 mg N L^{-1} $hour^{-1}$ to 3 mg N L^{-1} $hour^{-1}$) in 2 weeks – indicating a strong negative effect on nitrification. However, these are not common macro-organisms as BCAs for aquaponics. Most likely, the macro-organisms as BCAs pose negligible interferences or risk than the microbial BCAs in aquaponic system functioning (e.g. negative interferences with a biofilter, mineralization unit, plant microbiota). Their influences on the overall microbial community structure or parasitism on fish are still unknown (Schmautz *et al.* 2017). To avoid a backflow effect of the macro-organisms (as BCA) on the crop in absence of enough prey (*i.e.* after successful elimination of target pests), provisions of alternative food or hosts would have to be provided (Bennison 1992; Frank 2010). Further information on this aspect is provided in the supplementary text.

d. Chemical control

The use of pesticides (insecticides, fungicides, herbicides, acaricides, nematicides) is considered 'last resort' in IPDM, owing to their detrimental effects on non-target organisms and persistence (Fournier & Brodeur 2000; Stouvenaker *et al.* 2019). They, however, comparatively facilitate mass production of high-quality crops and are inexpensive (van Lenteren 2000; Ikeura *et al.* 2011). This makes them quite inevitable. The system-specific dilemmas associated with chemical control have already been discussed above. Additional clarity on the precautious approach to be used for chemical control in aquaponics is elaborated in the supplementary text.

Insecticides. Insecticides are highly effective emergency action chemicals that control macro-insect vectors and insect pest populations when it exceeds economic thresholds (Ascough *et al.* 2008; Morand & Lajaunie 2018). Insecticides can be divided into organochlorine, organophosphorus and carbamate compounds, where pesticides in each group have similar characteristics (Gerba 2019). Aside from the persistence issues associated with these chemicals, their use in aquaponics can be directly deleterious to fish and beneficial bacteria in coupled aquaponics or make reuse of water difficult for decoupled aquaponic farmers. Hence, aquaponic reliance on insecticides has continued to raise questions on its products (Reinhardt *et al.* 2019). However, there are available alternatives that are highly adaptable to completely replace the use of insecticide in hydroponics and aquaponics (further elaborated in the supplementary text).

Algaecides. Although the problem of weeds and the related use of herbicides in aquaponic set-ups seem mostly irrelevant, there can be concerns with algae. Since optimum growth conditions for hydroponic crops and algae are the same, the latter is always an integral part of hydroponic culture media if left unmanaged (Coosemans 1995). They compete with hydroponic crops for nutrients; hence, their control is eminent for optimal growth of the desired crops (Masser *et al.* 2013). Algaecides are chemicals used to keep algae from interfering with the growth of hydroponic crops (Sene *et al.* 2010). Algaecides might not be toxic to fish when applied according to manufacturer's instruction (Masser *et al.* 2013), but they can disrupt the overall behavioural response of fish coupled with their phytotoxic characteristics (Hostovsky *et al.* 2014). However, algae presence in aquaponics can be associated with 'sub-par' management. The use of herbicide in aquaponics might be avoided if adequate measures are taken. Algae growth in aquaponics is initiated by access to light; hence, if nutrient solutions and fish tanks are either shaded or covered with a dark material, the growth of algae would be completely controlled (Schwarz & Gross 2004; Somerville *et al.* 2014). Hence, the use of algicides could be avoided in most aquaponic production.

Fungicides. The warm, high relative humidity and wind-free condition in greenhouse support fungal growth on leaves and dispersal in the air (Halaši *et al.* 2008). Hence, farmers need to prevent an outbreak of fungal diseases. There are existing measures that are taken to prevent a fungal outbreak, including the planting of fungi-resistant seeds, frequent sanitization of tools, environmental condition manipulations such as increased temperature (they barely survive at 30°C), reduced relative humidity (below 85%) through the diffused fresh warm air and adjusted moisture level.

When a fungi disease is identified, farmers should immediately remove affected plants and discard all debris in the greenhouse to reduce its spread. Microbial biological agents from *Bacillus, Trichoderma* and *Pseudomonas* species have all been identified to significantly reduce fungal growth (*see* Table S1 and S6 in supplementary material). However, their unimpressive results due to variable performances under different environmental conditions have reduced their use (Weltzien 1991; Heydari & Pessarakli 2010). The inevitability of fungi attacks is further mentioned in the supplementary text.

Repeated foliar application of fungicides is usually adopted to control a fungal outbreak in both field and indoor agriculture. Chemicals such as phosphate, potassium bicarbonate, surfactants and foliar nutrients have also been reported as good remedies against fungal attacks (Crisp *et al.* 2006). Fungicides are destructive to fish and beneficial bacteria in coupled systems, but their use could be adopted in decoupled systems where nutrient solutions

are not reused in the RAS unit. Sulphur, which is either applied as a spray or via vaporization under high temperature, is considered an effective organic substance against powdery mildew (Crisp *et al.* 2006). However, side effects such as toxicity to beneficial mites and insects (Calvert & Huffaker 1974), transmission of off-flavours to crops (Martin & Salmon 1931; Gubler *et al.* 1996), contribution to environmental pollution (Hofstein *et al.* 1996) and health concerns for human (Mehler 2003) have reduced their use. Alternatives to synthetic pesticides are discussed in the following chapter.

Nematicides. Plant-parasitic nematodes feed on plants or seeds and rapidly spread in the circulation of nutrient solution in hydroponics. The common nematode species associated with the greenhouse include root-knot (*Meloidogyne*), lesion (*Pratylenchus*), burrowing (*Radopholus*) and leaf stem, or foliar nematodes (Aphelenchoides or Ditylenchus) (Moens & Hendrick 1992; Giannakou & Anastasiadis 2005; Hugo & Malan 2010). Some alternatives (weak) to potential nematicide applications are presented in the supplementary text. Nematode infestation and outbreak are not so common in the greenhouse (especially when there are good hygiene routines), but on their outbreak, farmers might have to trust chemical control to curtail the outbreak. Hence, this group of pesticides is also still relevant to the outbreak of nematodes in soilless systems.

Non-targeted effects of pesticides in aquaponic set-ups. Effects on fish: The rationale and introductory background are presented in the supplementary text. The amount of active ingredient in sprayed pesticide solution that escapes or drift into nutrient solution is generally unknown. We investigated ten common pesticides by simulating runoff of 10% and 20% of active ingredient (AI) from the commercial application rate diluting into the nutrient solution of a standard UVI aquaponic system with 506.4L of available water per m^2 of plant sprayed. Resulting concentrations were compared with the corresponding NOEC (fish) and LC_{50} (*Oreochromis niloticus*) values of the pesticides (Figure 1). At 10% runoff concentration, endosulfan is the most toxic with value (20.7 μg L^{-1}) highly greater than the corresponding NOEC (0.05 μg L^{-1}) and LC_{50} (10.2 μg L^{-1}) concentrations. Carbofuran, cypermethrin and deltamethrin also show potential toxicity with values greater than NOEC (40 μg L^{-1}, 2 μg L^{-1} and 0.3 μg L^{-1}, respectively). Expectedly, all pesticides become more toxic at 20% runoff concentrations compared with 10% runoff. Based on the results, we urge the system managers to adopt precautions to keep runoff thresholds below 10-15% – the lower the safer. The pesticides such as actara, glyphosate, mancozeb and methomyl appear comparatively less risky. However, their application should not overlook effects on microbe-mediated nutrient solubilization processes in aquaponic systems. Their biodegradation over time can alter overall water quality in aquaponic systems. As an example, the effects on nitrification and phosphorus solubility can be considered (Figs 2, 3).

In general, the result from this study indicates that higher runoff of pesticides would pose more threat to aquaponics (especially coupled aquaponics). In other studies, high residues of endosulfan, cypermethrin and deltamethrin were reported in hydroponically grown vegetables, with effects increasing by dosage concentration (Hatzilazarou *et al.* 2004). In a recent study (Hong *et al.* 2020), imidacloprid (IMI) caused significant alterations in microbial communities and induced sub-lethal acute stress in cultured animals. Beneficial bacteria were decreased, while pathogenic forms increased after exposure to IMI. Some additional studies in this regard have been compiled in the supplementary text. These results have shown that apart from the pesticide chemical components (which a farmer cannot alter), the volume of application dosage and application technique might increase the amount of pesticide solution drifting into the water. We further generated 'trigger' percentage runoff (*see* Table S5 in supplementary material) from corresponding pesticides' LC_{50} and calculated runoff concentrations. These values will help farmers to have an idea of the runoff percentage that will trigger havoc in the system, and further help them in selecting safer pesticides, which are pesticides with higher 'trigger' percentage runoff.

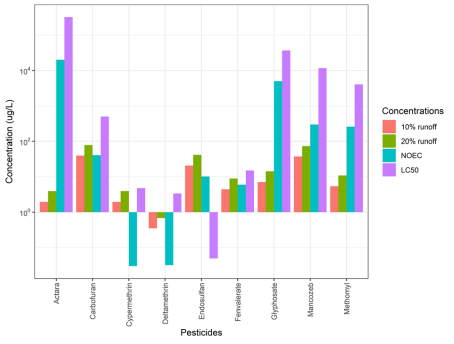

Figure 1 Comparing no observe effect concentrations (NOEC) (fish) and LC_{50} with simulated 10% and 20% runoff of application doses obtained from established application doses of pesticides (n = 30). At 10% runoff concentrations, deltamethrin, cypermethrin and endosulfan values are greater than their corresponding NOEC values. Endosulfan is the most toxic to fish with concentration (20.7 μg L^{-1}) greater than corresponding NOEC and LC_{50} concentrations. References are provided in the supplementary bibliography.

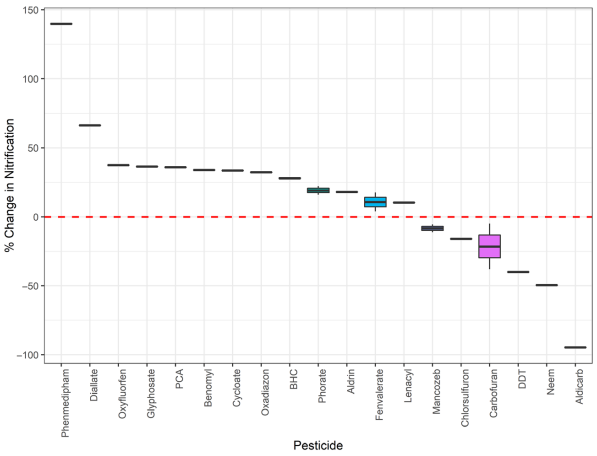

Figure 2 Effects of different pesticides on the percentage increase or decrease in nitrification in water. The percentage change in the initial (before pesticide application) and final (after pesticide application) nitrification-N levels was computed from data extracted from studies (15 articles, references provided in supplementary bibliography). Narrow boxes or heavy dashes are indicative of limited data for the selected pesticides

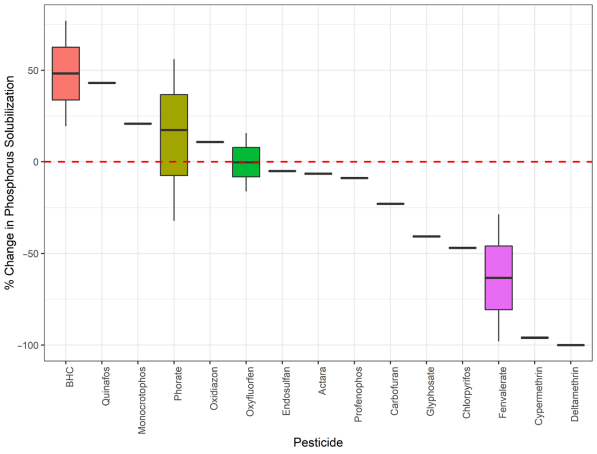

Figure 3 Effects of different pesticides on the percentage change in phosphorus solubility in water. The percentage change in the initial (before pesticide application) and final (after pesticide application) P-solubility levels was computed from data extracted from studies (10 articles, references provided in supplementary bibliography). Narrow boxes or heavy dashes are indicative of limited data for the selected pesticides

However, reducing pesticide solution runoff is a prominent exercise to minimize pesticide active ingredient ending up in the water. A prior covering of openings leading to the nutrient solution would minimize the quantity of the pesticide solution and subsequently the active ingredient drifting into the nutrient solution. It might be difficult to completely 'shut out' drifting of pesticide solution into the nutrient solution, but a dilution of the nutrient solution through the addition of freshwater would dilute the active ingredient into more folds, reducing their effects on fish, rhizosphere community and beneficial bacteria. Pesticides are quite prone to evaporation (Sanusi *et al.* 1999). Using cooling traps to rapidly capture pesticide-laden water as condensate and discarding it is worth exploring for smaller systems. Caution should be exercised in not returning the untreated condensate. In this perspective, the flexibility of the decoupled aquaponics allows the manager to reuse or discard such condensate. Few advanced, practical techniques to reduce influx/drifting away from pesticides to system water are discussed in the supplementary text.

Effects on Nitrification – N mineralization: Nitrification, as one of the key processes in aquaponics, converts ammonia and provide nitrate through metabolic activities of chemoautotrophic bacteria (e.g. *Nitrosomonas*, *Nitrobacter* and *Nitrospira*) (Monsees *et al.* 2017b). This process requires among other factors, oxygen for ammonia and nitrite oxidation (3.43 mg for the oxidation of 1 mg NH_3–N and 1.14 mg for the oxidation of 1 mg NO_2–N) (Chen *et al.* 2006; Suhr & Pedersen 2010). Pesticide biodegradation in water is associated with carbon dioxide evolution and oxygen uptake; hence, standard water quality parameters can be altered (Teater *et al.* 1958; Wainwright & Pugh 1973; Parr 1974). We surveyed existing literature to investigate the positive and negative effects of selected pesticides on nitrification (Figure 2). The effect on nitrification was measured by the percentage change towards or against the nitrification processes. The interquartile range of change is between −7% and + 33.6%. Aldicarb, carbofuran, chlorsulphuron, DDT, mancozeb and neem showed negative changes (below 0%) on nitrification. Aldrin, benomyl, BHC, cycloate, fenvalerate, glyphosate, lenacil, oxadiazon, oxyfluorfen, PCA and phorate produced positive change ranging between 0 and 100%, while phenmedipham produced positive change> 100%. These varying effects would have originated from the disruption or stimulation of the growth of nitrifying bacteria or the processes involved in nitrification. Reduction in nitrifying bacteria biomass (Widenfalk *et al.* 2009), phototrophic carbon assimilation (Downing *et al.* 2004), oxygen depletion (Downing *et al.* 2008) and reduced diversity of microbial structure (Muturi *et al.* 2017) are some of common specific effects of pesticides on nitrification.

Effects on Phosphorus solubility activities: In modern aquaponics, pH in the mineralization unit is lowered (<6) to improve phosphorus solubility (along with other nutrients), for plant optimal requirement (Goddek *et al.* 2018). With pesticide biodegradation reactions strongly connected with pH (and temperature) changes (Siddique *et al.* 2002; Al-shaalan *et al.* 2019), they are presumed to have effects on phosphorus solubility in aquaponics. This study generated data on the effects of different pesticides on phosphorus solubilization (Figure 3). The interquartile range of change is between −34.3% and + 17.8%. BHC, monocrotophos, oxadiazon, profenophos and quinalphos have positive changes on P solubility from 0 to 75%. Actara, carbofuran, chlorpyrifos, cypermethrin, deltamethrin, endosulfan, fenvalerate, glyphosate and profenophos show negative changes (below 0%) to phosphate solubility. Possible effects of pesticides on condensate and desalination units in decoupled aquaponics are summarized in the supplementary text.

Alternatives to synthetic pesticides – natural pesticides: Organic pesticides are mostly essential plant oils and extracts such as extracts of neem oil, pyrethrum oil, soya bean lecithin, clove oil, thyme oil, cinnamon oil, rosemary oil, tea tree, garlic oil and peppermint oil. They are considered an alternative to synthetic pesticides because they are less or non-persistent in the environment, less toxic and produce little or no residual effects (Schmutterer 1990; Mfarrej & Rara 2019). Coupled with their antimicrobial effects, they have been reported in many studies as effective against many plant pathogens and pests (*see* Table 5). There is also growing interested in the use of plant extracts and oils to replacing fish antibiotics. Some of the studies in this regard and common modes of action of natural pesticides are detailed in the supplementary text.

There is, however, little or no knowledge of how the mechanisms of actions would affect non-target organisms (including fish and beneficial bacteria) or disrupt biological or chemical processes. There are no NOEC values established for essential oils; thus, we compared a simulated 10% and 20% runoff concentration of 7 natural pesticides (clove, garlic, cottonseed, pyrethrum, rosemary, neem and thyme oils) with their corresponding lethal concentrations (LC_{50}) to fish (Figure 4). Comparatively, concentrations of natural pesticides are lower than the corresponding LC_{50} at both runoff concentrations, with pyrethrum having the lowest value (0.67 μg L^{-1}), making natural pesticides expectedly safer than synthetic pesticides. Expectedly, all pesticides reach towards their corresponding lethal concentrations, when runoff increases from 10% to 20%. However, the unavailability of NOEC values indicates that the effects of the pesticides on fish behaviour, biology, water chemistry and beneficial bacteria may still be unknown. Moreover, some studies have identified significant effects of essential oils on fish, water chemistry and microorganisms. The chemical instabilities that could emerge from both natural and synthetic pesticide degradation can also be associated with nitrogen and phosphorus availability in water (*see* Figures 2 and 3). Hence, coupled aquaponic farmers should rather rely on prophylactic measures, cultural control and biological control methods. Decoupled aquaponic farmers should invariably adopt natural pesticides as 'last resort' ahead of synthetic pesticides.

Other chemicals: In hydroponics, pathogen contamination arises from many sources, including infested rainwater, surface water, growth media and infected plant material (Ehret *et al.* 2001). Hence, frequent disinfection of working tools and nutrient solutions are reliable 'exercises' to eliminate pathogen infestation. Common chemicals used for disinfection in hydroponics and possible bottlenecks of their use in aquaponics are provided in Table S4 in the supplementary material. Additional technicalities, risks surrounding their use, are summarized in the supplementary text. However, they can reduce microbial populations to near zero when directly applied to water (Barta 2000), making them unsuitable for coupled aquaponics.

Table 5 Commercial natural pesticides

Natural pesticides	Pathogens/ pests	Effective dose for pest (mg L^{-1} or indicated otherwise)	LC_{50} for fish (mg L^{-1} or indicated otherwise)	Half-life in water (days)	References
Clove oil	*Rhizoctonia* spp.	100%	14.1	3.27	Aye and Matsumoto (2011), Velíšek *et al*. (2005)
	Pseudaletia unipuncta	0.04–0.69%			Akhtar *et al*. (2008)
Thyme oil	*Botrytis* spp,	1	6.6	15	Combrinck *et al*. (2011), Tab arraei *et al*. (2019)
	Aspergillus flavus	350–500			Omidbeygi *et al*. (2007)
Garlic	*Phytophthora infestans*	55–110	6.19%	15	Portz *et al*. (2008), Abd El-Galil and Aboelhadid (2012)
	Fusarium oxysporum	50%			Tariq and Magee (1990)
Caraway	*Penicillium* spp.	2	14		Combrinck *et al*. (2011), Tab arraei *et al*. (2019)
Rosemary	Spider mite	7.5–10%	3.4	37.5	Miresmailli and Isman (2006), Baker and Grant (n.d.)
Cinnamon	*Penicillium* spp.	3	–	15	Combrinck *et al*. (2011), Baker (2010)
Lecithins	*Sphaerotheca fuliginea*	0.2%			Homma *et al*. (1992)
Citronella	*Hyadaphis foeniculi*	0.53–0.56	17.3	30	Abramson *et al*. (2006), Baker *et al*. (2016)
Oregano	*Botrytis cinerea*	1	5	–	Combrinck *et al*. (2011), Merchan-Arenas *et al*. (2018)
	Penicillium spp.	2–3			Combrinck *et al*. (2011)
Lavender	*Botrytis cinerea*	25.6	99.7	–	Soylu *et al*. (2010), Beheshti *et al*. (2018)
Neem	Mustard aphids	5%	4	0.03–4	Biswas (2013)
	Rhizoctonia spp.	100			Aye and Matsumoto (2011)
Peppermint	*A. flavus* strains	0.05	38 (4 hours)	9	Kumar *et al*. (2007)
Citrus	*Penicillium* spp.	3	0.7	0.167	Combrinck *et al*. (2011)
	Aspergillus flavus	1.6%			Velázquez-Nuñez *et al*. (2013)

Effective doses and toxicity of the natural pesticides to fish depending on the structure of the compounds which vary among the natural pesticides. Lavender, citronella and clove are less toxic to fish at 99.7 mg L^{-1}, 17.3 mg L^{-1}, and 14.1 mg L^{-1}, respectively.

These chemicals naturally react with water molecules and other components to produce reactions that would largely be toxic to either fish or beneficial bacteria or both. Hence, their direct application into nutrient solution in coupled aquaponics might be destructive to the entire system. Decoupled aquaponic farmers would have the advantage to discard such nutrient solution or be left to neutralize (depending on the type of chemical) before being used in the RAS unit. For disinfection of working tools, such as pruning shears, containers, pipes and hoses, they should be left to dry after disinfection, before their further use. Where they are used to disinfect rock wools and growth media, the rock wools or growth media should either be autoclaved or left to be completely dry before being put back to use.

Natural elements: Some naturally existing minerals such as copper, sulphur, zinc and iodine have been found effective in controlling pests and diseases in hydroponics. Their use in aquaponics has not been much reported, but the possible effects are summarized in Table S4 of the supplementary material. Copper, zinc and iodine uses have majorly been adopted to eliminate root pathogens (*Fusarium, Pythium*) and necrosis by direct addition to the nutrient solution (Duffy and Défago, 1997; Runia 1994). Sulphur granules or micronized sulphur spray is used as fungicides (Crisp *et al*. 2006). Few risks associated with the use of sulphur are highlighted in the supplementary text. Generally, the direct addition of elements in a common nutrient solution can create loads of additional minerals being transported to the RAS unit, which would exceed the maximum nutrient tolerance for fish and biofilter bacteria. However, the farmer can dilute the nutrient solution with fresh water to reduce the possible aftermath effects.

Strategy inventory for IPDM in aquaponics. Keeping the space size limitations in mind, a brief executive summary of aquaponic IPDM arsenal for the farmers is provided below, further elaborated in the supplementary text. The potential alternatives to specific scenarios are briefly outlined below.

Alternatives to insecticides: The use of natural enemies, which predate or live as a parasite on pests, has been severally identified as an existing considerable alternative with a high level of success. The use of biological control in agriculture has long emerged, and the use in indoor systems

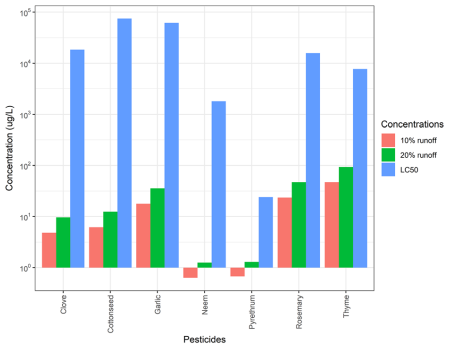

Figure 4 Comparison of the lethal concentrations (LC_{50}) of natural pesticides with corresponding simulated 10% and 20% active ingredient runoff concentration in a typical UVI hydroponics (n = 15 toxicity studies; 6 commercial natural pesticides). References are provided in the supplementary bibliography. At simulated 10% runoff concentrations, all pesticides are comparatively less than corresponding lethal concentrations.

such as greenhouse is more effective because the farmers can optimize the efficiency of the natural enemies in the 'mini' ecosystem of the greenhouse than in the field (Van Lenteren & Woets 1988; Vincent *et al.* 2007). There is the existing large commercial availability of larvae, pupae, nymph, eggs and adults of the common natural enemies of pests available around the world (*see* Table 4); almost all insects and pests have commercial biological control solution (Vincent *et al.* 2007). The predatorial and parasitic activities of the natural enemies are not perceived to create any negative feedback on any aquaponic design. Also, the use of barrier netting, screening of openings and manipulation of temperature and other cultural methods in IPDM mentioned above are safety measures to shut out the insect pests from greenhouse or production enclosures. These measures and the alternative stated above if well implemented in the IPDM steps can address insect pest infestation in aquaponics; hence, the use of insecticides in aquaponics can be of little or no relevance in successful aquaponic production.

Plant insect pests: Adequate alternatives in the form of biocontrol agents are available (entomopathogenic bacteria + fungus, or, natural enemies with banker plant system, or, organic derived/natural pesticides, etc.). The use of chemical pesticides is avoidable.

Plant fungus: Chemical fungicides remain the most reliable option yet. Sulphur fumigation or spraying with natural elements is safer than fungicides. Better is to avoid fungal outbreak at all costs by routine environmental manipulation (temperature, humidity, ventilation control). There is limited scope for biocontrol. A future alternative needs to be developed.

Plant pathogenic microbes: The use of non-discriminative chemical antimicrobials must be avoided. Combined usage of slow filtration techniques + disinfection of incoming freshwater (with UV) + encouraging aquaponic microbial community itself (selective inoculants of proteobacteria, bacteriodetes, trichoderma, etc., referred as biopesticides) offers excellent plant biosecurity. For acute cases, natural elements or organic/natural pesticides may be used.

Plant nematode infestation: Chemical nematicides, sanitizers, remain the most reliable control. Organic/natural pesticides (herbal, essential oil extracts) are safer, but less efficient alternatives. Filtration of incoming water, screening of stocking material and periodic sanitization of units ensure enough biosecurity against nematodes.

Nutrient solution algae bloom: Application of algaecides can be avoided completely with physical barriers (covering reservoirs/ black coloration of reservoirs to avoid light), routine cleaning and periodic sanitization.

Pathogenic microbes for fish: Antibiotics/ medicated feed can be avoided. The use of UV and/or ozonation (carefully) was integrated with filtration units to screen incoming freshwater. Encouragement of endemic probiotics and bioremediators may be considered through inoculation.

Fungus and parasites for fish: Chemical therapeutics should be preferably applied through bath treatment in quarantine tanks and thus avoiding contamination of the system water. Medicated feed (herbal extracts) provide a safer, yet less efficient alternative. Lesser stocking density, good prophylactics and biosecurity screening will most likely avoid occurrence.

Conclusive assessments of control methods. Conclusion on biological control: The use of microbial inoculants as biological control agents might be a great potential in aquaponics, but the potential influence they can have on beneficial bacteria and their activities raises questions about their use especially in coupled aquaponics. There seems to be good potential in the microbial community of aquaponics as biocontrol agents, but there are needs for further studies on taxonomic identification and usage characteristics. On the other hand, the natural enemies (predators and parasites) are not perceived to create any problem in all designs. However, the periodic cost of acquiring the natural enemies for the augmentative release can reduce farmer's profit in the long run. Hence, rapid pest detection, identification and subsequent monitoring can simply keep the pest population below the economic threshold (action threshold). Also, effective barrier netting and screening of openings in

the greenhouse are preventive measures against pest infestation that can reduce the frequency of pest attacks.

Conclusion on physical control: Physical control adoption in aquaponics might be considered complicated, and farmers would have to strictly consider the level of interaction between the units of the aquaponic design before positioning or location of UV, filtration and ozonation. Coupled aquaponic farmers should only adopt slow filtration, ozone and UV sterilizers as water treatments for freshwater (water source) right before impounding the biofilter, because of the possible deleterious effects on the beneficial bacteria. On the other hand, decoupled aquaponic farmers might want to use them between the units (especially to control algae growth), but a well-planned preventive and cultural control would rather curb existence or reduce pathogens and algae in the system.

Conclusion on chemical control: To control fungi and other pathogens, coupled aquaponic farmers would have to completely rely on preventive approaches and other IPDM methods other than chemical control, as effects of pesticides can be destructive to the system and make aquaponic products unhealthy for human consumption. On the other hand, decoupled aquaponic farmers should also rather explore the possibilities of controlling pathogen attacks with cultural, physical and biological control alternatives. However, if desired results are not obtained, farmers should cautiously use natural pesticides with adequate assessment of the nutrient solution to investigate pesticide compounds before reusing in the RAS unit; otherwise, the nutrient solution should be discarded.

Conclusion

For the first time, we have reviewed the existing IPDM methods in hydroponics for adoption in different aquaponic designs. Prophylactic measures (except chemical sanitary) such as tool disinfection, general sanitation routines, barrier nettings and environmental condition manipulations such as increasing temperature and lowering of relative humidity are not found to create problems for any aquaponic design. The use of physical control methods, UV, ozone and slow sand filtration should be limited to treating water sources right before impounding the biofilter due to the possible deleterious effects on beneficial bacteria in the system. On the other hand, chemical control methods are highly complicated for all systems. While insecticides and herbicides are completely replaceable by well-established commercial biocontrol and prophylactic measures, fungicides and nematicides would still be relevant in aquaponics due to low-efficiency levels of alternative IPDM methods. We investigated possible effects of 9 pesticide runoff in aquaponics and found endosulfan showing the highest toxicity followed by cypermethrin, deltamethrin and carbofuran. All pesticides influence phosphorus and nitrogen availability in water. Natural pesticides show no acute toxicity to fish at runoff concentrations, but they should be avoided in coupled systems – future researches are needed to evaluate their side effects on non-target components of the system (such as biofilter-rhizosphere community). Similarly, synthetic pesticides in which runoff concentrations are higher than corresponding NOECs cannot be guaranteed for use, as they are capable of disrupting nitrogen and phosphorus availability, among other possible effects. In biological control, except microbial inoculants, natural enemies of pests (predators and parasites) are mostly safe for any aquaponic design. The microbial community of aquaponics itself, dominated by Proteobacteria, shows great potential for biological control – effective at microbial load 10^3-10^9 CFU mL^{-1}. The prophylactic measures involving little or no physical application into the nutrient solution are highly recommendable approaches for all aquaponic designs.

Acknowledgement

The authors would like to thank the Ministry of Education, Youth and Sports of the Czech Republic – the CENAKVA project (LM2018099), CENAKVA Center Development (CZ.1.05/2.1.00/19.0380) and the project Reproductive and Genetic Procedures for Preserving Fish Biodiversity and Aquaculture (CZ.02.1.01/0.0/0.0/16_025/0007370). The authors greatly appreciate the help of the anonymous reviewers in improving this article.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Material. Supplementary text, Figures and Tables.

CHAPTER 3

POTENTIAL USE OF ENTOMOPATHOGENIC AND MYCOPARASITIC FUNGI AGAINST POWDERY MILDEW IN AQUAPONICS

Folorunso, E. A., Bohatá, A., Kavkova, M., Gebauer, R., Mraz, J., 2022. Potential use of entomopathogenic and mycoparasitic fungi against powdery mildew in aquaponics. Frontiers in Marine Science, 9, 992715.

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frontiers | Frontiers in Marine Science

TYPE Original Research
PUBLISHED 07 November 2022
DOI 10.3389/fmars.2022.992715

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

RECEIVED 12 July 2022
ACCEPTED 13 October 2022
PUBLISHED 07 November 2022

CITATION
Folorunso EA, Bohatá A, Kavkova M, Gebauer R and Mraz J (2022) Potential use of entomopathogenic and mycoparasitic fungi against powdery mildew in aquaponics. *Front. Mar. Sci.* 9:992715. doi: 10.3389/fmars.2022.992715

Potential use of entomopathogenic and mycoparasitic fungi against powdery mildew in aquaponics

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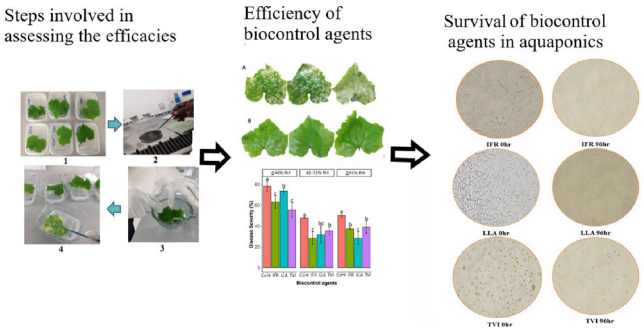
Aquaponics has the potential to produce sustainable and accessible quality food through the integration of hydroponics and aquaculture. Plants take up dissolved nutrients in fish wastewater, allowing water reuse for fish. However, the simultaneous presence of fish and plants in the same water loop has made phytosanitary treatments of diseases such as powdery mildew problematic due to risks of toxicity for fish and beneficial bacteria, limiting its commercialization. Entomopathogenic and mycoparasitic fungi have been identified as safe biological control agents for a broad range of pests. This study aimed to investigate the efficacy of entomopathogenic fungi, *Lecanicillium attenuatum* (LLA), *Isaria fumosorosea* (IFR), and mycoparasitic fungus *Trichoderma virens* (TVI) against *Podosphaera xanthii.* Also, we investigated the possible harmful effects of the three fungal biocontrol agents in aquaponics by inoculating them in aquaponics water and monitoring their survival and growth. The findings showed that the three biocontrol agents significantly suppressed the powdery mildew at 10^7 CFU/ml concentration. Under greenhouse conditions (65-73% relative humidity (RH)), a significant disease reduction percentage of 85% was recorded in *L. attenuatum*-pretreated leaves. IFR-treated leaves had the least AUDPC (area under disease progress curve) of ~434.2 and disease severity of 32% under 65-73% RH. In addition, *L. attenuatum* spores were the most persistent on the leaves, the spores population increased to 9.54 x 10^3 CFUmm^{-2} from the initial 7.3 CFUmm^{-2} under 65-73%. In contrast, in hydroponics water, the LLA, IFR, and TVI spores significantly reduced by more than 99% after 96 hrs. Initial spore concentrations of LLA of 10^7 CFU/ml spores were reduced to 4 x 10^3 CFU after 96 hrs. Though the results from this study were intended for aquaponics systems, relevance of the results to other cultivation systems are discussed.

KEYWORDS

aquaponics, *Lecanicillium attenuatum*, *Isaria fumosorosea*, *Trichoderma virens*, powdery mildew, biological control, Aquaponics, hydroponics

10.3389/fmars.2022.992715



GRAPHICAL ABSTRACT
The general flow of the study is shown starting with the set-up of the leaves in growth chambers, treatment with entomopathogenic fungi and inoculation of P. xanthii. In the middle of the flow is a comparisons of untreated leaves **(A)** with LLA-treated leaves **(B)** after 20 days. The graph below the leaves shows the disease severities under different relative humidities. On the right are time-based pictures showing the survival of the entomopathogenic fungi in aquaponics water at 0hr and after 96hr.

1 Introduction

The continuous growth of the global population demands increased food production, depleting natural resources such as land, water, and nutrients. Hence, there is an urgent need to adopt sustainable food production systems that ensure a reliable and healthy food supply. Aquaponics is a food production method that uses wastewater from fish culture to cultivate plants in hydroponics; in a coupled or decoupled system (Monsees et al., 2017; Lennard and Goddek, 2019). The wastewater from the fish culture unit, carrying dissolved fish wastes, is constantly reclaimed between the recirculating aquaculture unit (RAS) and the hydroponics unit (coupled) or fed periodically to the hydroponics unit in a separate compartment (decoupled). The dissolved fish wastes are converted into plant essential nutrients by beneficial bacteria (Rakocy, 2012). The high water reuse capacity (up to 90%) and the conversion of dissolved fish wastes into plant essential nutrients (among other benefits) make aquaponics approaches a potentially sustainable food production system (Goddek et al., 2019).

Despite these benefits, the simultaneous presence of fish, plant, and beneficial bacteria in the same water loop (especially in coupled aquaponics), has made pest and disease management a pressing challenge in aquaponics, limiting its commercialization (Stouvenakers et al., 2020). Therefore, there is a need to establish control methods with little or no negative influence on plants, fish, and beneficial bacteria. In previous studies focused on identifying an integrated pest and disease management suitable for aquaponics (Folorunso et al., 2021), it was found that existing commercial biological controls are natural remedies for aquaponics pests and could pose little or no harm to non-target organisms. On the other hand, pathogen management still relies on chemical controls, which can pose high toxicity risk to fish and beneficial bacteria. Moreover, plant-pathogen proliferation is more problematic because of the conducive aquaponics/hydroponics ambient environment for fungi (Stouvenakers et al., 2019).

Powdery mildew, caused by *Podosphaera xanthii*, is one of the most severe plant diseases in greenhouse hydroponics/aquaponics cucumbers and other crops (Schuerger and Hammer, 2003; Savvas et al., 2009; Pollastro et al., 2022). Chemical fungicides have primarily been most widely used control mechanisms in stand-alone hydroponics or field agriculture. But, as stated, spray drifts of fungicides in hydroponics units can cause harmful effects to fish and beneficial bacteria in coupled aquaponics where the water is recircled back to the fish and the biofilter or limit the water reuse capacity of decoupled aquaponics systems (Folorunso et al., 2021; Rašković et al., 2021). Thus, consensus efforts are currently being channeled towards developing control approaches with little or no effects on non-target organisms. Though biological control agents (BCAs) have been identified as sustainable alternatives (Rivas-García et al., 2020), there are currently no BCAs certified for use in multitrophic or integrated systems such as aquaponics.

Additionally, despite the successes reported in many field trials, inconsistencies and mismatches in the transition of results

from laboratory trials to the field have made the adoption of many BCAs less attractive (Sawant et al., 2017; Ni and Punja, 2021). Fungal biological control agents are comparatively more tolerant to varying environmental conditions than other microbial biocontrol; hence, they are considered as better alternatives against powdery mildew pathogens (Tesfagiorgis et al., 2014; Gafni et al., 2015). *Ampelomyces quisqualis, Trichoderma afroharzianum, T. asperellum, T. aspelloides, T. harzianum*, *T. viride*, and *Clonostachys rosea f. catenulata* (= *Gliocladium catenulatum*) have been tested against powdery mildew under laboratory, field, and greenhouse conditions (Gafni et al., 2015; Hafez et al., 2018; Ni and Punja, 2021). However, desired efficacy levels are not achieved when these species are often applied alone (without fungicides) (Sawant et al., 2017; Sarhan et al., 2020; Ni and Punja, 2021). Insufficient growth of the BCAs and varying environmental conditions were identified by Carbó et al. (2020) and Giotis et al. (2012) as limiting factors. Therefore, currently, there are interests in fungal biocontrol agents that are solely efficient and adaptable to varying environmental conditions. Some entomopathogenic and mycoparasitic fungi are more persistent and adaptable to varying environmental conditions (Rivas et al., 2014; Xie et al., 2015; Carbó et al., 2020). *Lecanicillium* and *Isaria* species are among the most commonly used entomopathogenic fungi against greenhouse pests.

Lecanicillium species are entomopathogenic fungi that have been commercialized as biopesticides for whitefly control and have a dual role against aphids and pathogens (e.g., Mycotal® and Vertalec®). However, there are conflicting reports on their efficacy towards control of powdery mildew pathogens. Kim et al. (2007); Kim et al. (2008) found no significant differences in the activity of *L. muscarium* and *L. longisporium* against cucumber powdery mildew due to varying environmental factors. *L. attenuatum* have been identified as a highly persistent entomopathogenic fungus with a rapid germination rate (Wang et al., 2017). It acts as mycoparasites, producing compounds such as chitinase, allowing penetration into pests and other fungi (Askary et al., 1998; Kim et al., 2007). Studies such as Askary et al. (1998); Kim et al. (2008), and Goettel et al. (2008), among others, have identified its potential against powdery mildew. There has been a limited number of studies (Drummond et al., 1987) focusing on the efficacy of *L. lecanii* in optimal and suboptimal conditions.

Isaria fumosorosea is another entomopathogenic fungus with high efficacies against pests such as whiteflies, aphids, thrips, citrus psyllid, and spider mites (Zimmermann, 2008; Avery et al., 2011). Though *I. fumosorosea* strains can be isolated from powdery mildew-infested plants (Kavkova and Curn, 2005), only one study has investigated its potential against powdery mildew pathogens. Kavkova and Curn (2005) found that cucumber plants pretreated with *I. fumosorosea* in high relative humidity conditions were not different in their susceptibility to powdery mildew than untreated controls. Thus, knowledge gaps in their use as a biological control against powdery mildew partially anchor the basis for conducting this study.

Trichoderma spp. are mycoparasitic fungi considered the most versatile biocontrol agents due to the secretion of bioreactive compounds that hinder the growth of fungal pathogens. Several *Trichoderma* species have been tested against powdery mildew with certain levels of success (Chet and Inbar, 1994; Elad, 2000; Woo et al., 2014). However, their efficacy in controlling powdery mildew declines as the disease spreads (Elad, 2000; Liu et al., 2020). Susceptibilities to temperature, relative humidity, and UV irradiation are the major ecological factors determining the effectiveness of entomopathogenic and mycoparasitic fungi (Henis and Chet, 1975; Devi et al., 2005; Abbaszadeh et al., 2011). However, in many instances, relative humidity often exacerbates effects due to its influence on the intensity of temperature and UV irradiation (Abbaszadeh et al., 2011). *T. virens* is a soil-based species that is highly useful for controlling *Pythium ultimum* and *Rhizoctonia* spp. in field and greenhouse crops (Rubin, 2010). It has a considerable high tolerance to varying environmental conditions (Anand et al., 2006). Yet, there is no study on their efficacy in controlling cucumber powdery mildew. The existing knowledge gap on the optimal physiological conditions and efficiency has limited their usage and commercialization. Additionally, since it has been reported that foliar applications in aquaponics drift to aquaponics water (Rašković et al., 2021), it is imperative to investigate the natural capability of the potential fungal biocontrol agents to survive or reproduce in aquaponics. This is because their survival in aquaponics water may be harmful to the fish and beneficial bacteria in coupled aquaponics, where there is a simultaneous presence of fish and nitrifying bacteria in the same loop as plants.

Thus, the present study sought to investigate the efficacy of *Lecanicillium attenuatum* (LLA), *Isaria fumosorosea* (IFR), and mycoparasitic fungus *Trichoderma virens* (TVI) against cucumber powdery mildew under different relative humidity conditions. This was achieved by assessing; (a.) the disease progress and severity, (b.) the disease reduction capacity of the BCAs, (c.) the growth and persistence of the BCAs, and (d.) their survival in the aquaponics medium.

2 Materials and methods

2.1 Propagation of cucumber powdery mildew

The pathogen of cucumber powdery mildew, *Podosphaera xanthii*, identified through a diagnostic guide (Kristkova et al., 2009), was collected from the greenhouse of the Faculty of Fisheries and Protection of Waters (FFPW) in the Czech Republic, where the natural infestation was recorded in August 2020. The pathogen was cultured on leaves of healthy cucumber plants (variety Superstar F1, Semo a.s., Czech Republic) and

grown for two weeks in an MLR-352 growth chamber (25 ± 1°C; 16h of light, 60-65% RH (Akribis, UK). Fresh cultures of the pathogen were made every two weeks simply by tapping the old infected leaves with new potted cucumber leaves.

2.2 Strains of entomopathogenic and mycoparasitic fungi

Lecanicillium attenuatum, strain CCM 9195 (a new strain, sequence of strain CCM 9195 have been submitted to the NCBI database, barcoding database website https://www.ncbi.nlm.nih.gov, accession No. of the strain is OP503941), was isolated in 2008 from bark beetle adults in NP Sumava, Czech Republic. The strain was identified as *L. attenuatum* based on microscopic observation and cytochemical analysis using Deng et al. (2010), and Simmons (2007) approaches. The strains of *I. fumosorosea*-based product, PFR-97 20% WDG, and *T. virens*-based product, SoilGard®, used in this study were procured from Certis USA LLC., USA. Bioproduct PFR-97 20% WDG, was based on the blastospores of the naturally occurring strain Apopka 97 of the fungus, *Isaria fumosorosea*, while bioproduct SoilGard® was based on the blastospores of the naturally occurring strain GL-21 of the fungus, *Trichoderma virens*. Both strains were re-isolated from the bioproducts, and pure cultures were used for the experiments.

2.3 Propagation of suspensions of biological control agents

All three strains were cultivated in potato dextrose agar (PDA, Difco Laboratories, Sparks, MD, USA) at 25 ± 1°C. Conidial suspensions of each strain were prepared by scraping off conidia (using an inoculating loop) into sterile 0.05% Tween 80 solution. Suspensions were then filtered through a cheesecloth to remove mycelia debris, and the conidia concentration was determined by a Neubauer hemocytometer (Bright-Line™, Sigma-Aldrich, Germany). The suspensions were subsequently adjusted to $1.0x10^7$ conidia/ml.

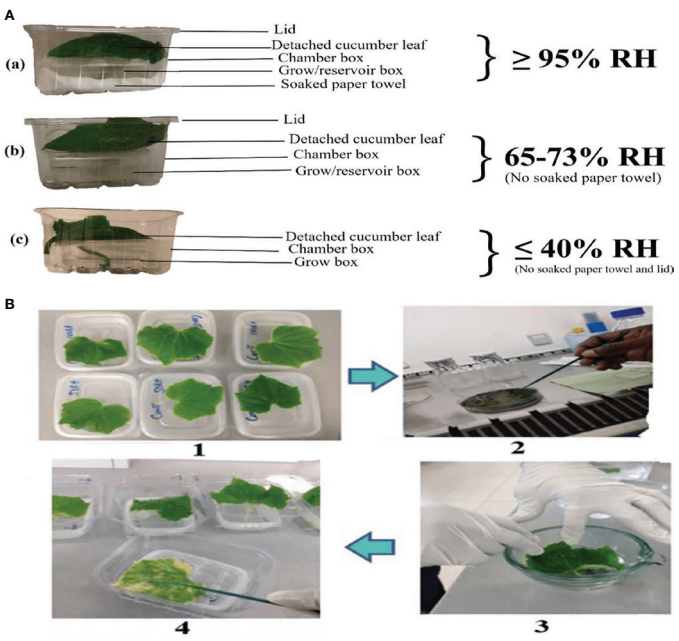


FIGURE 1
(A) shows the regulating features of the three relative humidity conditions of the growth chambers, ≥ 95% RH (a), 65-73% RH (b), and ≥ 40% RH. **(B)** shows the flow of activities, treatment setup (1), suspension preparation (2), pretreatment of leaves with isolates (3), and the inoculation of the *P. xanthii* (4).

2.4 Growth chamber

Experiments were conducted under three relative humidity conditions, ≥ 95%, 65-73%, and ≤40% RH. These relative humidity conditions were regulated in experimental growth chambers (Figure 1A). Each growth chamber contained a smaller grow box (110 × 85× 30 mm) enclosed in a chamber box (145 × 120 × 65 mm). The leaf was placed on the water-filled smaller grow box so that the leaf's petiole was embedded in the water through a hole driven on the lid. This was then placed in the bigger grow box (chamber box). Relative humidity, ≥ 95%, was regulated using Kim et al. (2007) approach. A soaked paper towel was placed at the bottom of the bigger grow box before the chamber was covered with the lid (Figure 1Aa). Without the soaked paper towels, the relative humidity ranged between 65-73% (Figure 1Ab). When the growth chambers were opened to the ambient temperature of the laboratory (21-24 ^{0}C) and not laid with the soaked paper towel, the relative humidity was ≤40%. The relative humidity in each growth chamber was measured using an electronic thermo-hygrometer (TFA Dostmann GmbH & Co.KG, Germany). Each treatment had six replicates (Fungi isolates and relative humidity conditions).

2.5 Application of biocontrol agents and pathogen

To assess the efficacy of the fungal biocontrol agents, detached cucumber leaves were pretreated with the prepared suspension of the fungal isolates (Figures 1B1, 1B2). The pretreatment was carried out by dipping the detached leaves into a suspension of 1.0 x 10^{7} spores/ml for 30 seconds (Figure 1B3). Then, the leaves were left to dry freely on a tabletop for 30 minutes before being placed in the growth chamber. The control leaves were dipped in 0.05% aqueous Tween 80 solution. After 48 hours, powdery mildew pathogen, *P. xanthii* isolates were inoculated on the treated leaves by scrapping off the conidia from the highly infected leaves with a loop and touching the healthy leaves at 3 points (Figure 1B4). The growth chamber units within treatments were kept 50-60 cm from each other, and the treatments were kept two meters apart to reduce inter-plot interference.

2.6 Disease assessment

The domination and inhibition capacity of the BCAs was assessed by estimating the area of the leaves covered by the *P. xanthii*. This was done by estimating the percentage of powdery mildew coverage on the leaves. Disease severity was scored using a 12-grade scale described by Horsfall and Barratt (1945) with minor modifications: 0 = 0%, 1 = 0–3%, 2 = 3–6%, 3 = 6–12%, 4 = 12–25%, 5 = 25–50%, 6 = 50–75%, 7 = 75–87%, 8 = 87–94%, 9 = 94–97%, 10 = 97–100%, 11 = 100% disease. A mean disease severity (DS) was calculated for each treatment by adding the products of the number of infected leaves and their corresponding ratings, thereby dividing the sum by the product of maximum rating value (12), the number of leaves in that entire observation and 100. The formula was expressed as follows;

$$\mathbf{DS}\ (\%) = \sum(N_{grp}\ X\ R)/12N_{obs}\ X\ 100 \qquad (1)$$

where N_{grp} is the number of leaves in a group, R is the rating value, and $N_{obs}$is the number of leaves in the observation. Disease severity assessment was further complemented by evaluating the final disease levels (FDL), the percentage of the leaf area covered by powdery mildew on day 20 after inoculating the BCAs.

To assess the efficacy of the BCAs and the percentage reduction of the disease, the area under the disease progress curve (AUDPC) was calculated using the percentage of the leaf covered by powdery mildew during the experiment. It summarizes the disease intensity over a certain period. A mean AUDPC value was calculated for each treatment at each corresponding relative humidity by adding up the average percentage of the disease (percentage of leaves covered by powdery mildew) in previous and current situations. It is then multiplied by the time (days) in-between differences. The formula was expressed as follows;

$$\mathbf{AUDPC} = \sum_{i}^{n}\left\{\left[\left(\frac{Y_i + Y_{i-1}}{2}\right)\right]\ (X_i - X_{i-1})\right\} \qquad (2)$$

where Y_i is the percentage of the leaves covered by powdery mildew (Yi/100) at the ith observation and X_i is the day of the ith observation, while n indicates the total number of observations [modified from Shaner and Finney (1977)].

2.7 Determination of the persistence of entomopathogenic and mycoparasitic fungi

To evaluate the persistence of the BCAs on leaves, three leaves were randomly selected from each treatment on the first day and after 20 days of inoculation. Leaf discs (25 mm in diameter) were collected from each leaf and suspended in a 100 ml 0.05% Tween 80 solution in Erlenmeyer flasks. The flasks were subjected to an orbital shaker (150 rpm) for 30 minutes. The resulting leachate solutions were diluted to obtain a countable number of colony-forming units (CFU). Next, aliquots were spread on plates containing PDA (39 g/l PDA) with 0.25 g of antibiotic chloramphenicol (Sigma-Aldrich, Germany). 0.5 ml was inoculated from each dilution on the growing medium (PDA) surface and cultured at 25°C and 18 h/6

h lighting. The number of CFU of *T. virens* was counted after two days (48 hrs), while the CFU of *I. fumosorosea* and *L. attenuatum* were counted after five days (120 hrs).

2.8 Effects of fungal isolates in aquaponics

2.8.1 Survival of the fungal isolates in an aquaponics water

In order to investigate the possible effects of the fungal isolates on fish and microbial communities in a typical aquaponics system, it is essential to know if the fungal isolates have the natural capability to germinate in the aquaponics water. Hence, we inoculated them into the aquaponics water and observed their spores germination and sporulation over a stipulated period. This was carried out in two phases; a germination test to observe the spores structures under the microscope at different timepoints and a further quantification of the colonies at the timepoints.

Twelve 200 ml of water were collected in a 250 ml conical flask from the mineralization tank of the FFPW tilapia-based aquaponics system. The water samples were subsequently aerated on a rotary shaker (Sheller, Korea) at 200 rpm. After 48 hours, prepared suspension of the isolates containing $1.0x10^7$ conidia/ml was inoculated into the aerated mineralized water. For comparison, the isolates were inoculated into 200 ml 0.05% tween 80 solutions as control. For the germination test, the RAS-inoculated fungal isolates were cultured in a PDA just after the inoculation in the water. The mycellium and possible sporulation patterns were observed under the microscope after 24 hours. Then, for quantification, in a separate assay, they were cultivated in PDA by adding 0.5ml of the solution to PDA in separate Petri dishes, 90 by 15 mm. The isolate cultivations were carried out at 0, 1, 6, 12, 24, 48, and 96hr from when the water samples were collected. The water samples were left in a shaker for the entirety of the experiment. They were cultivated for 24 hours under room temperature and >70% relative humidity. The CFUs of the isolates were counted after 24 to 120 hrs of cultivation. Prior to counting, the structures of the isolates were first observed under a light microscope to compare their physiology with the control inoculated in tween solution.

2.9 Statistical analysis

The experimental efficacy design was in a randomized complete block design, with each treatment having six replicates. Data were collected from each replicate on days 10, 15, and 20. To assess the impact of the relative humidity on the disease severity and AUDPC, the data were subjected to a two-way analysis of variance (ANOVA) using R statistical software (Wickham, 2016). The comparisons between treatments were performed using Fisher's protected Least Significant Difference (LSD). We set the statistical significance at the conventional $p< 0.05$ level. The break-in y-scale bars followed the approach of Xu et al. (2021). To assess the performance of each treatment to the control, we calculated the disease percentage reduction (DPR) for each treatment using the formula below:

$$\mathbf{DPR}\ (\%) = \left(\frac{X_c - X_t}{X_c}\right) \times 100 \qquad (3)$$

X_c is the % of leaf area covered by powdery mildew (for control), and X_t is the corresponding value of the treatments.

3 Result

3.1 The disease progress

On day 20 of inoculation of *P. xanthii*, leaves in the control group (under ≤40% RH) were covered by the pathogen CFUs. This was used as a sign to mark the end of the experiment. The efficacy of microbiological agents on the progress of the disease considering the assessment of AUDPC was statistically significant (P<0.05) under ≥95% relative humidity (≥95% RH) and 65-73% relative humidity (65-73% RH) (Figure 2). The AUDPC value of the LLA-treated leaves in ≥95% was the lowest (319.6) over the given study period (20 days). The AUDPC of the untreated cucumber leaves (control) grown under ≥ 95% relative humidity was significantly higher (930) than the AUDPC of IFR (529.6) and TVI (606.7) (Figure 2). Similarly, under the 65-73% RH condition, the AUDPC values of the leaves treated with BCAs, LLA (486.7), IFR (434.2), and TVI (545.8), were significantly different from the control. Nevertheless, at ≤ 40% RH, the AUDPC of IFR and TVI treated cucumber leaves values were high, and both were significantly different from the control and LLA.

3.2 The effects of the BCAs on disease severity

The result indicated that all BCAs tested reduced disease severity (DS %) as compared to control untreated leaves. Considering the analysis for disease severity, at a ≥95% relative humidity condition, both LLA and IFR-treated leaves showed significantly less severity than the control and TVI-treated cucumber leaves. Under 65-73% relative humidity conditions, leaves treated with IFR had low severity rates (32.8%) (Figure 3). Under ≤40% relative humidity condition, there was no significant difference between the disease severity of the control and BCAs (Figure 3).

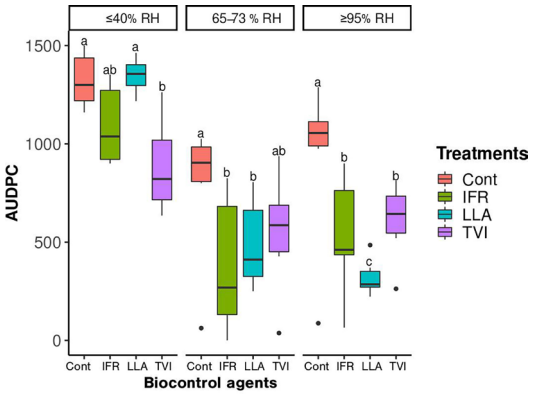


FIGURE 2
The disease progress of powdery mildew on cucumber leaves treated with microbiological agents, *Lecanicillium attenuatum* (LLA), *Trichoderma virens* (TVI), and *Isaria fumosorosea* (IFR), and control (Cont). The detached cucumber leaves were grown for 20 days under different relative humidity conditions, ≥95% RH, 65-73% RH, and ≤40% RH. The boxes are the mean ± SE (n = 6). The different letters indicate that values are significantly different at *P< 0.05* according to the Least Significance Difference test.

After 20 days of inoculation with *P. xanthii*, the final disease level, measured by the percentage of the leaf area covered by powdery mildew, was recorded. The leaves treated with LLA had a significant 11% and 9% of the leaf area covered by powdery mildew under 65-73% RH and ≥95% RH, respectively (Table 2, Figure 4). Under ≥95% RH condition, IFR and TVI were statistically significant from the control with FDL of 18.5% and 25.8%, respectively (Table 1). The FDL leaves treated with the BCAs under 65-73RH significantly differed from the control. It stagnated between 58% and 60% under 65-73 and ≥95% RH,

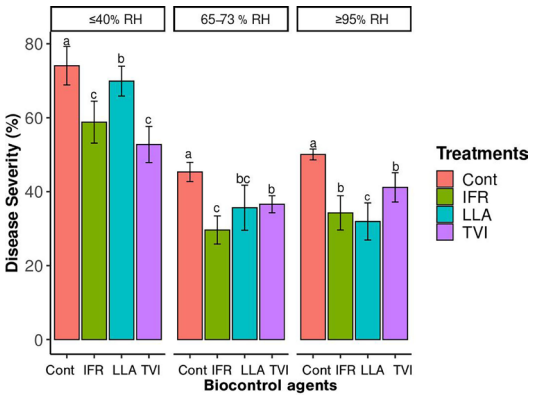


FIGURE 3
The disease severity (DS) curves for powdery mildew development on cucumber plants when treated with *Lecanicillium attenuatum* (LLA), *Trichoderma virens* (TVI), and *Isaria fumosorosea* (IFR), and untreated control (Cont). The data represent the disease severity (%) for each week's treatment under the designed relative humidity conditions, ≥95% RH, 65-73% RH, and ≤40% RH. All the leaves in the treatments were pretreated 72 hours before inoculating *P. xanthii*. The vertical bars are the mean ± SE (n = 6). The letters indicate that values are significantly different at *P< 0.05* according to the Least Significance Difference test.

FIGURE 4
Efficacy of microbiological agents, *L. attenuatum* on cucumber leaves artificially infected with powdery mildew. **(A)** leaves treated with 0.05% tween 80 solution (control) after 20 days of the treatment, and **(B)** leaves treated with *L. attenuatum* after 20 days. Both treatments were applied 48 hours before the inoculation of *P. xanthii*. Other pictorial presentation showing a comparisons of TVI and IFR-treated leaves can be found in the Figure 1 of the supplementary material.

respectively (Table 1). Similarly, the final disease of the cucumber leaves grown under a relative humidity condition of ≤40% showed no significant difference between the treatments and the control.

3.3 The efficiency of the BCAs on disease reduction

The potency of the microbiological agents at disease reduction varied with relative humidity conditions. All the microbial agents were most efficient at controlling the powdery mildew under the ≥95% and 65-73 relative humidity conditions. LLA was the most efficient, with a disease percentage reduction of 85% under 65-73% RH conditions (Figure 5). The disease reduction of IFR and TVI under the same condition was 68.6% and 56%, respectively (Figure 5). In contrast, all the BCAs were largely ineffective under ≤40% RH conditions.

Nonetheless, there was no significant difference between the mean FDL of the BCAs. In contrast, at low humidity (≤40%), only TVI-treated leaves showed a disease severity (85%) significantly different from the control. The mean FDL of LLA-treated and IFR-treated leaves were insignificant to the control.

3.4 Persistence of the BCAs

A significant reduction in fungal populations were observed in the low relative humidity condition (≤40% RH). TVI significantly reduced from 1.0×10^2 CFU per mm^2 of disc area on the first day to 4.97 on day 20. Similarly, the 7.9 × 10 and 7.3 × 10 CFU of *I. fumosorosea* and *T. virens* per mm^2 of disc area before the inoculation of *P. xanthii* spores (respectively), significantly reduced to 3.42 × 10 and 1.17× 10 at day 20 respectively (Table 2). In contrast, all the fungal isolates significantly increased under the higher relative humidity conditions, 65-73% and ≥95%. *L. attenuatum* spores significantly increased to 9.54 × 10^3 $CFUmm^{-2}$ in 65-73% RH and 5.51 × 10^3 $CFUmm^{-2}$ in ≥95% RH growth chamber. On the other hand, *I. fumosorosea* spores significantly increased from 7.9 × 10 before the inoculation of *P. xanthii* spores to 1.60 × 10^4 $CFUmm^{-2}$ in 65-73% RH and 5.78 × 10^3 $CFUmm^{-2}$ in ≥95% RH growth chamber (Table 2).

TABLE 1 The final disease level (FDL) of the cucumber leaves treated with microbiological control agents, *Lecanicillium attenuatum* (LLA), *Isaria fumosorosea* (IFR), and *Trichoderma virens* (TVI), and the untreated control (control) on the 20th day.

Treatments	FDL (%)		
	≤ 40% RH	65-73% RH	≥ 95% RH
Control	96.67[a]	59.67[a]	58.83[a]
L. attenuatum	97.00[a]	9.00[b]	11.17[b]
I. fumosorosea	91.83[ab]	20.33[b]	18.50[b]
T. virens	85.83[b]	28.33[b]	25.83[b]
P-value (0.05)	0.028	0.0037	0.002

Values are presented as mean. Means in columns with different letters are significantly different (P< 0.05 according to the Least Significance Difference test).

TABLE 2 The mean CFU of fungi isolates found per unit area (mm^2) of leaf discs of cucumber leaves treated with biological agents, *Lecanicillium attenuatum* (LLA), *Trichoderma virens* (TVI), and *Isaria fumosorosea* (IFR).

Treatments	Day 1	Day 20		
		≤40% RH	65-73% RH	≥95% RH
L. attenuatum ($CFUmm^{-2}$)	7.3×10	1.17×10^{c}	9.54×10^{3a}	5.51×10^{3b}
I. fumosorosea ($CFUmm^{-2}$)	7.9×10	3.42×10^{c}	1.60×10^{4a}	5.78×10^{3b}
T. virens ($CFUmm^{-2}$)	1.0×10^{2}	4.97×10^{c}	1.43×10^{3a}	2.72×10^{2b}

The leaf disc samples were randomly taken from each treatment on day 20 of inoculation. Values are presented as mean. Means in a row with different letters are significantly different ($P< 0.05$ according to the Least Significance Difference test).

3.5 Survival of BCAs in aquaponics medium

L. attenuatum germinated the most after 24 hrs with a fungal load of about 10^6 ($p<0.05$). The fungal load of the LLA, IFR, and TVI significantly reduced from 0 hr to more than 99% after 96 hrs. *L. attenuatum* fungal load reduced to about 4 x 10^3 CFU after 96 hrs (Figure 6). On the other hand, IFR fungal load sharply reduced from 4.5 x 10^5 in 0 hr to 1.8 x 10^3 in 96 hrs. Similarly, the variation in the structures of the microbes over the 96 hours showed that the organisms could not sporulate in this medium (Figure 7).

4 Discussion

Several studies have identified biological controls as possible alternatives to fungicides against pathogens in aquaponics. Nonetheless, there is currently no biological control treatment approved for use against powdery mildew in aquaponics systems. In addition, there is still a paucity of information on favorable environmental conditions that optimize the efficacy of the BCAs in field and greenhouse crops. Therefore, we investigated the effectiveness of two naturally occurring entomopathogenic fungi and mycoparasitic fungus against *P. xanthii* under different relative humidity conditions. The efficacy of the BCAs varied depending on the relative humidity of the growth chambers.

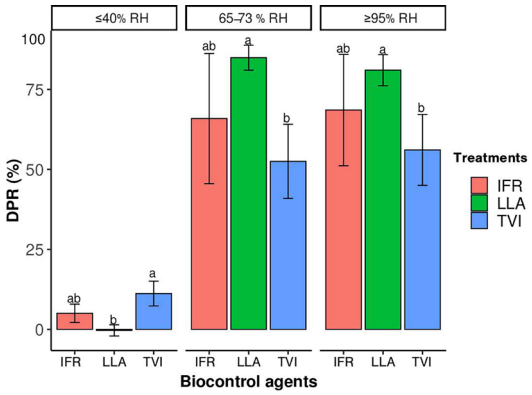


FIGURE 5
The efficacy of the BCAs: *Lecanicillium attenuatum* (LLA), *Isaria fumosorosea* (IFR), and *Trichoderma virens* (TVI) measured by disease reduction percentage (DPR) of powdery mildew pathogen, *Podosphaera xanthii*, on cucumber leave grown for 20 days under different relative humidity conditions. The vertical bars are the mean ± SE (n = 6). The different letters indicate that values are significantly different at $P< 0.05$ according to the Least Significance Difference test.

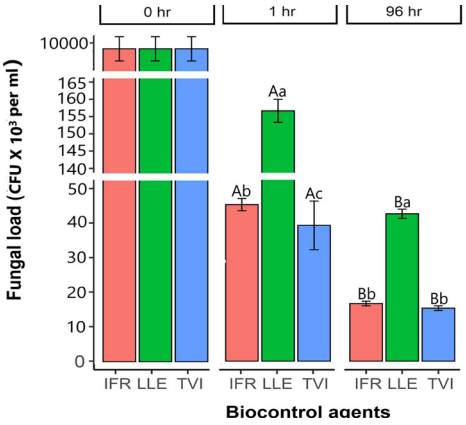


FIGURE 6
The comparisons of colony-forming units of the BCAs, *L. attenuatum* (LLA), *I. Fumosorosea* (IFR), and *T. virens* (TVI), inoculated into aquaponics water in decoupled aquaponics and 0.05% tween 80 solution. The CFUs per culture were counted after 24 to120 hrs of cultivation. Capital letters indicate a significant difference between 1 and 96 hrs, while the small letters indicate significant differences within the time points (1 and 96 hrs). The vertical bars are the mean ± SE (n = 3). The different letters indicate that values are significantly different at $P< 0.05$ according to the Least Significance Difference test.

4.1 The efficacy of the entomopathogenic fungi

All the BCAs investigated in this study showed significant effects against the powdery mildew pathogen, *P. xanthii., L. attenuatum* was the most efficient, reducing the disease severity by 85% and having a final disease level of 11%. Furthermore, we found that at a relative humidity condition of 65-73%, all the three BCAs showed a strong efficacy against the powdery mildew. Though the result from this study was aimed at providing in-depth information on adopting these fungal biocontrol agents for aquaponics, the results from our study are however, relevant for all cultivation systems. Our findings are consistent with the greenhouse results of Kim et al. (2007). Only a few studies have investigated the potential of *L. attenuatum* against powdery mildew, but there are results from related species, *L. lecanii*. Romero et al. (2007) investigated the efficacy of *Ampelomyces quisqualis, L. lecanii*, and *Bacillus substilis* against melon powdery mildew under two relative humidity conditions, 75–80% and 90–95%. After three weeks, the authors recorded 61% and 81% disease reduction in leaves treated with *L. lecanii* under 75-85% and 90–95% RH, respectively. In contrast, Kim et al. (2008) reported no significant differences in the activity of two isolates of *L. lecanii* against cucumber powdery mildew under RH conditions ≥95%. The discrepancy in the results could be associated with the biological and physical states of the microbiological control agents. Also, it could be related to differences in the strains of *L. attenuatum*. On the other hand, the focus of this study did not cover the mechanism of action of the fungi isolates against *P. xanthii*. The effectiveness of *L. lecanii* against several plant diseases is associated with its antagonistic, parasitic, and disease resistance-inducing characteristics (Goettel et al., 2008).

Isaria fumosorosea has severally been developed as mycopesticides for a broad range of pests, including whiteflies (Faria & Wraight, 2007), mites and ticks (Pena et al., 1996), termites (Yanagawa et al., 2008), thrips (Panyasiri et al., 2007) and aphids (Yeo et al., 2003). However, aside from the study by Kavkova and Curn (2005), which investigated the development and survival of *Sphaerotheca fuliginea* after treatment with *I. fumosorosea*, none of the existing studies have investigated its potential as a biocontrol agent against powdery mildew pathogen. Our study found that *I. fumosorosea* significantly reduced the disease intensity and spread under 65-73% and ≥95% RH conditions. Similarly, the disease severity of leaves treated with IFR under 65-73% RH condition was slightly less than disease severity under ≥95% RH. This could be partly associated with the preference of the powdery mildew spores to the drier condition in the 65-73RH growth chamber. Therefore, favoring an increased mycoparasitism and sporulation of *I. fumosorosea*. Here, the present study did not investigate the mechanisms of the fungus against the pathogen. But, other studies have identified mycotoxins such as

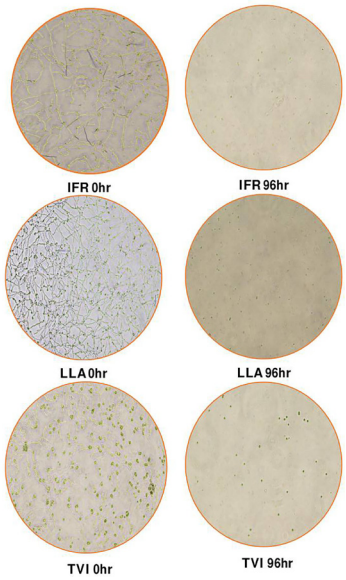


FIGURE 7
The structures of the fungal biocontrol agents, *Isaria fumosorosea* (IFR), *Lecanicillium attenuatum* (LLA), and *Trichoderma virens* (TVI) under an electron microscope, 96 hrs after inoculation in the aquaponics water.

Beauvericin, pyridine-2,6-dicarboxylic acid, and dipicolinic acid found in *Isaria fumosorosea* (Zimmermann, 2008).

4.2 The efficacy of the myco parasitic fungus

Our study found that TVI was the least efficient BCAs under 65-73% and ≥95% relative humidity conditions. However, its final disease levels significantly differed from the controls under the three relative humidity conditions. Currently, no study has delved into the efficacy of the *T. virens* against *P. xanthii.* In contrast, similar results were reported with other species such as *Trichoderma harzianum, T. album, T. viride*, and *T. hamatum* (Mmbaga et al., 2008; Elsisi, 2019). The relatively low-efficiency level of Trichoderma in the soilless medium could be associated with its preference for soil medium. Even though a comparatively higher population of TVI spores was found on the leaf hours after inoculation, the population of TVI spores was the least under the three relative humidity conditions on day 20. This could be associated with the fact that *T. virens* are natural soil-based microbes (Rubin, 2010). Thus, its low survival on a non-soil growing medium may make the beneficial microbial biocontrol agent less suitable for non-soil systems such as aquaponics. Like *L. attenuatum* and *I. fumosorosea*, few studies have delved into the mode of action of *T.virens.*

4.3 Persistence of the BCA spores

We also demonstrated that the population of the BCA spores differs at different relative humidities. The population of *L. attenuatum* and *I. fumosorosea* spores were significantly higher under 65-73% RH than in ≥95% RH conditions. This may be associated with the fact that *P. xanthii* has an affinity for drier conditions, and spores of *L. attenuatum* does not germinate in the absence of *P. xanthii* (Miller et al., 2004; Rennberger et al., 2018). But, the persistence of the spores under optimal relative humidity conditions (≥95%), where there are few powdery spores, shows that *L. attenuatum* and *I. fumosorosea* spores sporulate survive on the leaves' wax. However, these growth stages cannot protect the plants against further infestation of *P. xanthii* (Miller et al., 2004). As a consequence, a repeated application would be necessary to replace the spores and protect the plant. Miller et al. (2004) reported that spores of *L. lecanii* applied did not germinate except in the presence of

powdery mildew (or some other fungus). In addition, the applied spores were found in ungerminated form, except when they were within immediate (B/100 mm) proximity to *S. macularis* f. sp. *fragariae* spores or colonies. Despite this, there are arguments that the spores population of heterotrophic microorganisms such as these fungi can be manipulated in aquaponics systems (Stouvenakers et al., 2019).

4.4 Survival of the biological control agents in aquaponics medium

Despite the observed growth of the BCAs in the aquaponics water, the chains of their mycelia were found to 'shrink' with time. All the fungal loads of the BCAs inoculated in the aquaponics water were reduced by over 99% after 96 hrs of inoculation. This indicates that after 6-7 days of application, there may not be any traces of the BCAs in a typical coupled aquaponics system In addition, a 10% runoff of spraying solution ending up in hydroponics/aquaponics suggested by Folorunso et al. (2021), would arithmetically mean that a less significant population of the fungi would be left after 96 hrs. Though, no study has evaluated the surviving abilities of these BCAs in aquaponics, their sharp loss over the short timeframe found in our study could be associated with certain factors including; unfavourable water quality parameters (e.g. temperature, dissolve oxygen, pH); biotic factors (such as antagonistic aquatic microbes) or water chemical parameters (e.g. C:N ratio, biochemical oxygen demand, chemical oxygen demand) (Stouvenakers et al., 2020; Mann and Davis, 2021; Sharma et al., 2021). This partly explains why entomopathogenic fungi have not been reported as an integral part of the microbial communities in aquaponics. (Eck et al., 2019; Schmautz et al., 2022). In addition, the inability of these BCAs to survive over time in a typical aquaponics system could also be associated with the absence of organic carbon sources, essential for entomopathogenic cell divisions and further survival (Stouvenakers et al., 2019). Therefore, if these BCAs drift off to an aquaponic nutrient medium during spraying, their survival in such medium may depend (over time) on the availability of sufficient carbon. In another study (Leonard et al., 2002), the survival of BCAs in water was reported to vary due to the absence or presence of an inadvertent supply of carbon sources.

Stouvenakers et al. (2019) reviewed the possibility of using indigenous heterotrophic microorganisms in aquaponics as plant protection against pathogens. The authors suggested that the multiplication of heterotrophic microbes in aquaponics can be harnessed by adding organic compounds such as humic substances. Though, unregulated multiplication of the heterotrophic microorganisms in coupled aquaponics systems can establish a competitive environment with the nitrifying bacteria (Leonard et al., 2002; Stouvenakers et al., 2019). Thus, this can alter the optimization of the biofilter. Therefore, if a disease reduction level achieved with the first dose application is still below the economic threshold, a second application should be withheld and applied later to avoid possible effects on the natural microbial load of the aquaponics systems. It is noteworthy to state that the significant drop in the fungi population does not significantly translate to biological significant, as none of the fungi used in this study at these concentrations has been associated with any human health defects.

5 Conclusion

Aside from Sirakov et al. (2016) study, which investigated the efficacy of indigenous bacterial isolates of aquaponics against *Pythium ultimum in vitro*, there are currently no research studies on the use of BCAs adoptable for aquaponics. The present study showed a remarkable result in controlling and suppressing *P. xanthii* in cucumber leaves pretreated with entomopathogenic and mycoparasitic fungi. Findings show that at a 10^7 $CFUml^{-1}$ concentration, *L. attenuatum* was the most efficient under 65-73% relative humidity, a practically sustainable condition in the greenhouse. The overall AUDPC of the treated leaves over the given period (20 days) and disease severity showed that the leaves treated with *L. attenuatum, I. fumosorosea*, and *T. virens* were significantly the least infected by *P. xanthii*. So, *L. attenuatum, I. fumosorosea*, and *T. virens* efficiently controlled powdery mildew under high relative humidity conditions. An accidental drift or runoff of the fungal isolates into the aquaponics system during foliar application may not generate a significant problem, as their survival in aquaponics was non-significant. Their low survival in water could make them a sustainable and selective option for controlling fish pathogens at higher concentrations and stipulated timeframe. Hence, future research should focus on the dual role of the fungal isolates against both plant and fish pathogens in aquaponics.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

EAF, AB, RG, MK, and JM contributed to the conception and design of the study. EAF wrote the first draft of the manuscript and performed the statistical analysis. EAF, AB, RG, MK, and JM contributed to the data interpretation and revision of the manuscript. EAF, AB, JM, MK, and RG wrote sections of the manuscript. All authors contributed to manuscript revision, and approved the submitted version.

Funding

The study was financed by the Grant Agency of the University of South Bohemia (GAJU), No. 053/2021/Z, Ministry of Education, Youth and Sports of the Czech Republic (MSMT) under project CENAKVA (LM2018099), and the Ministry of Agriculture of the Czech Republic, National Agency for agricultural Research (NAZV), No. QK 22010255.

Acknowledgments

The technical assistance of the laboratory member, Mrs. Olga Divisova, is gratefully appreciated. The authors greatly appreciate the help of the reviewers in improving this article.

Conflict of interest

Author MK was employed by Dairy Research Institute, Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.992715/full#supplementary-material

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CHAPTER 4

BOTANICAL AND MICROBIAL INSECTICIDES APPLICATION IN AQUAPONICS – IS THERE A RISK FOR BIOFILTER BACTERIA AND FISH?

Rašković, B., Gebauer, R., Folorunso, E. A., Božić, G., Velíšek, J., Dvořák, P., Bořík, A., Grabic, R., Mráz, J., 2022. Botanical and microbial insecticides application in aquaponics – Is there a risk for biofilter bacteria and fish? Frontiers in Marine Science, 9. 1055560.

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My share on this work was about 15 %.

frontiers | Frontiers in Marine Science

TYPE Original Research
PUBLISHED 22 November 2022
DOI 10.3389/fmars.2022.1055560

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

RECEIVED 28 September 2022
ACCEPTED 31 October 2022
PUBLISHED 22 November 2022

CITATION
Rašković B, Gebauer R, Folorunso EA, Božić G, Velíšek J, Dvořák P, Bořík A, Grabic R and Mráz J (2022) Botanical and microbial insecticides application in aquaponics - is there a risk for biofilter bacteria and fish? *Front. Mar. Sci.* 9:1055560. doi: 10.3389/fmars.2022.1055560

Botanical and microbial insecticides application in aquaponics - is there a risk for biofilter bacteria and fish?

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Aquaponics is a food production system that combines aquaculture with hydroponics. The simultaneous existence of fish, beneficial bacteria and plants in the same water loop predisposes the fish and bacteria to a possible detrimental effect of plant protection products. Additionally, there is an inadequate exploration of scientific studies on the impact of pesticides on fish and bacteria in aquaponics systems. This study investigated the effects of three commercial insecticides based on the following active ingredients: pyrethrum, azadirachtin, and spinosad, on aquaponics systems. Due to ethical concerns in animal testing, applying insecticides directly to aquaponics setup was impossible. Therefore, three separate experiments were conducted: (1) Pesticide runoff rate – in which insecticides were applied to basil (*Ocimum basilicum*) plants grown in two hydroponic systems: media bed and floating raft. The concentrations of applied insecticides were measured in the water of nutrient solutions of the hydroponics after 1, 3, 6, 12, 24, 36, 48, 72 and 96h to establish a pattern of degradation of insecticides. The result from this experiment showed that pyrethrum and spinosad were detected in unquantifiable concentrations in the nutrient solutions. Hence, further experiments were conducted only with azadirachtin. In a biofilter trial (2) – azadirachtin, at three concentrations (1.5 μg L^{-1}; 7.5 μg L^{-1}; and 15 μg L^{-1}), was added to a running biofilter to investigate the effects on nitrifying bacteria. Mild effects were recorded in the nitrification and bacteria microbiome. In the third trial (3) – Nile tilapia (*Oreochromis niloticus*) were exposed to similar concentrations of azadirachtin for seven days (and the same period for recovery) to investigate effects on fish hematology, blood biochemistry, antioxidative enzymes in the brain, gills, muscle, liver and intestine and histopathology of gills and liver. Results showed mild effects in

hematology and biochemistry profile in fish and higher levels of lipid peroxidation in the liver during the exposure. The results indicate a safe use of pyrethrum and spinosad in aquaponics setup, while azadirachtin has to be used with care, especially in coupled aquaponics systems.

KEYWORDS

azadirachtin, spinosad, pyrethrum, Nile tilapia, biofilter bacteria, fish exposure, degradation rate

1 Introduction

Aquaponics is a sustainable food production system that integrates the simultaneous culture of plants and fish. Modern aquaponics started in the 1970s mainly as a hobby and backyard activity, but it advanced in recent years, and it is "on the brink of commercialization" (Palm et al., 2018). Increased interest in production was accompanied by the growth of published research papers covering this topic, which increased almost exponentially until 2019 (Yep & Zheng, 2019). Aquaponics combines well-established practices transferred from both plant and animal sciences. This includes plant pest management, as the main recommendation is to use integrated pest management (IPM) for pest control in aquaponics (Bittsanszky et al., 2017). This is because aquaponics is regarded as eco-friendly food production, so chemical plant protection products must be considered the last resort for treating pests. A comprehensive review paper was recently published by Folorunso et al. (2021) in which several recommendations were given prior to the use of chemical agents: (a) culture control; (b) physical and mechanical control measures (such as UV irradiation, ozonation, and filtration) and (c) biological control measures. Chemical control practices were mainly used from the knowledge gained in hydroponic systems (Stouvenakers et al., 2019), with the major difference being that in aquaponics, fish (or other aquatic organisms) is added to the hydroponics system. This means that fish is regarded as a "non-targeted organism" in aquaponics, as unwished effects of plant chemical treatment could have adverse effects on fish (Yavuzcan Yildiz et al., 2019; Folorunso et al., 2021).

Moreover, in aquaponics, between plant and animal components of the system, there is also a microbial component populating the biofilter, which acts as biological water treatment (Yang et al., 2012). Using chemical treatment in aquaponics can also modulate the bacterial population in the biofilter, which could subsequently lower the water nitrification rate (Rašković et al., 2021). The easiest way to avoid the risk of applied chemicals affecting fish is to adopt decoupled aquaponic systems, which can physically separate water from the plant and fish components of the system (Stouvenakers et al., 2019; Baganz et al., 2022), but this is not always feasible. Moreover, coupled (one-loop) systems in which water flows in all compartments of aquaponics are the most frequently utilized by practitioners around the globe (Palm et al., 2019).

In aquaponic and hydroponic setups, plants are susceptible to different kinds of pests and diseases. Greenhouses carry even higher risks due to the specific environment in which plants are grown, characterized by high humidity, temperature, and plant density (Reddy, 2016). Plant pests include various groups of organisms such as fungi, viruses, bacteria, insects, and nematodes, among others (Jensen, 1997), but the presence of pest insects is of particular importance because, apart from the direct impact they will have on plant, they can also serve as a vector for other types of diseases (Wisler & Norris, 2005). As already mentioned, insects in aquaponics are usually treated with biological and biodegradable insecticides. These natural products are shown to be effective against various insects in a range of hydroponic facilities and setups across the World, such as in Egypt, Greece, and Thailand (Saleem et al., 2019; Lykogianni et al., 2021; Thaochan et al., 2021) or even Antarctica (Bergstrom et al., 2018), while similar scientific studies in aquaponics are lacking. To the authors` knowledge, there is not a single research paper or grey literature findings that focus on effects of insecticides to biofilter bacteria and fish. There is also a lack of information on the specific amount of biological insecticides used in aquaponics or hydroponics (Isman, 2020), as the Food and Agriculture Organization of the United Nations does not provide detailed statistics. Ujváry (2010) recognized three large groups of natural agents used worldwide for insect control: botanical insecticides, microbial insecticides, and semiochemicals. The present study aimed to test the impact of three insecticides: two botanical insecticides (pyrethrum and azadirachtin) and one microbial (spinosad). They were chosen due to their availability and presence in stores, primarily in the Czech Republic, where this study was conducted. Pyrethrum and azadirachtin are extracts from chrysanthemum (*Chrysanthemum cinerariifolium*) plant and neem tree (*Azadirachta indica*) seeds, respectively, while spinosad is the fermentation product of aerobic soil bacterium (*Saccharopolyspora spinosa*). All three insecticides have different properties and modes of action: spinosad is a systemic insecticide (van Leeuwen et al., 2005), meaning that it is soluble in water and has fast access to the

plant vascular system; azadirachtin is weakly systemic through the leaves and systemic in the root (Kreutzweiser et al., 2011), while pyrethrum is non-systemic insecticide. These properties of insecticides are essential since, together with accidental drift, they can enter the water in which fish are reared. The concentrations of insecticides in the water are extremely important due to the possible acute or chronic effect on fish, and one of the measurements is lethal concentration LC_{50} – the concentration of toxicant which will lead to death of 50% of exposed fish within certain time frame (usually 96h). The review of LC_{50} for all three insecticides and several important aquatic species can be found elsewhere (Ujváry, 2010; Rašković et al., 2021).

The present study aimed to: (1) assess the risk of commercial formulations of insecticides mentioned above by applying them to basil plants (*Ocimum basilicum*) and monitoring their concentrations in the water for 3-4 days; (2) apply detected concentrations of the selected insecticides on working biofilter in order to assess possible effects on nitrification; (3) apply measured concentrations of the selected insecticide on Nile tilapia (*Oreochromis niloticus*) in order to investigate possible subacute toxicity to fish. The study is conceived as a simulation of a real-life scenario that can later be extrapolated to small aquaponics units and more comprehensive production systems.

2 Material and methods

2.1 Pesticide formulations

In order to investigate a real-life scenario, commercial products of insecticides were purchased from a local shop in České Budějovice (Czech Republic), and the following formulations were used: ND Spruzit AF (W Neudorff, Germany), with pyrethrum as an active ingredient (1.8% of Pyrethrum); Neem Azal - T/S (Biocont Laboratory, Czech Republic), with azadirachtin as an active ingredient (1.2% of azadirachtin); Spintor (AgroBio Opava, Czech Republic), with spinosad as an active ingredient (22.8% of spinosyn A and D). Neem Azal was applied at the rate of 0.3 mL m^{-2} of plant area. Spruzit, on the other hand, was applied at the rate of 60 mL m^{-2} while Spintor was applied at the rate of 0.04 mL m^{-2} area. These application doses were manufacturers' recommended dosages for greenhouse vegetables. 300 mL of spraying solutions were prepared per each treatment, hence, each experimental unit was sprayed with 100 mL of insecticide solution.

2.2 Study design

The major obstacle during the planning phase of the study was that it was not possible to obtain ethical permission to assess the toxicity of insecticides to fish in an aquaponics setup. Fish toxicity tests are allowed only in laboratory conditions in line with specific guidelines provided by OECD, US EPA or similar national guidelines. Moreover, due to the complexity of the aquaponics system and the fact that it is challenging to manage fish, plants, water quality and biofilter at the same time, the authors decided that instead of investigating the effects of the insecticides in one aquaponics setup, it would be more precise and methodologically exact to conduct the experiments in three phases. The first experiment was conducted to investigate the concentrations of insecticides ending up in different aquaponics systems following foliar application on the basil plant. Using the basis of information on concentrations obtained from the first experiment, a potential risk assessment for biofilter bacteria and fish was investigated only with azadirachtin. The decision to exclude pyrethrum and spinosad from these experiments was because we could not detect quantifiable concentrations of the insecticides in the hydroponics solution (more data and rationale for this decision can be found in the result section); Therefore, the second experiment was conducted with three chosen concentrations of azadirachtin (1.5 μg L^{-1}, 7.5 μg L^{-1}, 15 μg L^{-1}), in a dose-response manner. These concentrations were chosen considering the multiple application of the insecticide in hydroponic/aquaponics practices. In order to test the possible effects of the insecticide runoff on the nitrifying bacteria, these three concentrations were subsequently applied to the water of a working biofilter; the third experiment was a subacute toxicity test of azadirachtin to the Nile tilapia using the same concentrations as in the second experiment with a biofilter.

2.3 Experiment 1 – pesticide runoff in water

Two hydroponic systems were assembled at the experimental facility based at the Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice (Czech Republic): media bed and floating raft systems. Both systems were assembled inside an experimental greenhouse equipped with automatic temperature, lighting and humidity regulation. (1) each experimental unit consisted of 12 polyethylene grow boxes (60 cm× 40 cm × 28 cm) filled with commercially available expanded clay pebbles (hydroton), filled up to 85% (51 cm) of the grow box and 12 polyethylene sump buckets with the volume of 40 L that served as a reservoir of water for each container (Figure 1). Water was pumped to grow box using an electric pump (6000 SOBO, 85W), regulated with the sensor, and the water returned to the plastic tank *via* the force of gravity through a bell siphon outlet. 8 plants of basil with an average height of 15.1 ± 2.4 cm were transplanted to each grow box at 15 cm spacing. The plants were placed in the net pots so that the tips of the roots pass through the perforated net pots filled with the same hydroton pebbles to ensure an easy passage of water between the plant root and the expanded clay pebbles. Plants were left to acclimatize in the experimental units

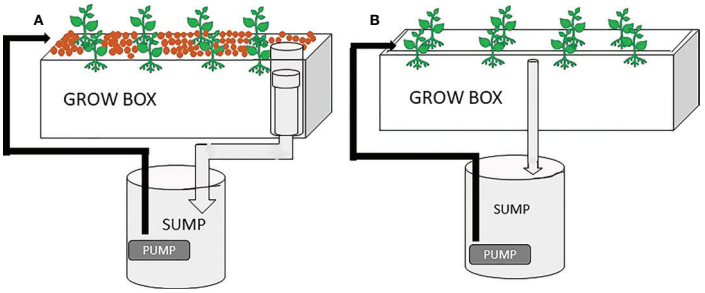

FIGURE 1
The schematic design showing the sample media bed system **(A)**, consisting of grow pebbles, bell siphon outlet, 0.2 m^2 grow box, 40 L sump bucket, waterflow pipes, submersible pump and basil plants; and the raft system **(B)**, consisting of 0.2 m^2 grow box, polystyrene grow foam, 40 L sump bucket, submersible pump, waterflow pipes, and basil plants.

for one week, during which the nutrients solutions were prepared from commercial Flora (General Hydroponics, California, USA) using the manufacturer's guidelines for leafy vegetables (FloraGro 2.5 mL L^{-1}, FloraMicro 2 mL L^{-1}, and Florabloom 1 mL L^{-1}). The trial commenced at 18:00 hr on the 16th of July, 2019. A foliar application of the three insecticides was carried out by spraying each unit with 100 mL of the prepared insecticide solution, while three control replicates were sprayed with water of same volume. During the foliar application, precautions were taken to avoid contamination of different treatments by demarcating each experimental unit with cardboard before the application. Before the foliar application, pesticides were thoroughly dissolved in water by mixing, using the manual provided by the manufacturer. Water samples for determining the concentration of insecticides were taken after 3, 6, 12, 24, 48, 72, and 96h post application. The plant growth was monitored for adverse effects of insecticides 7 and 14 days after the beginning of the trial, while basic water parameters were measured using a multimeter, HI9849 (Hanna, Romania). Dissolve oxygen (DO), pH, and electrical conductivity (EC) were kept at >5 mg L^{-1}, 5.5-6.5, and >1, respectively, throughout the entire duration of the experiment (Table 1).

(2) In the experiment above, pyrethrum was not detected at a quantifiable concentration in the water samples. This forms a basis for conducting a similar trial in raft systems to attest to the hypothesis that runoff concentrations might defer in different hydroponics systems. The floating raft system was assembled in 12 identical plastic grow box connected to a sump (Figure 1B). At the top of each grow box was a 3" thick polystyrene foam sheet having 8 holes in which 4"-diameter net pots can fit in. Plants were placed in the net pots in a way that the roots were always in contact with water on which polystyrene foam was floating, while stem and leaves were above the sheet. The same protocol was followed in the media bed for the application of insecticides and trial started at 18:00 hr on the 10th of August, 2019. The only difference was that water samples were taken after 1, 3, 6, 12, 24, 48 and 72h, because in the media bed trial, pyrethrum concentration was below the limit of quantification at all sample times (see the chapter "Results"), so we hypothesized that it would be found in the water of hydroponic system after 1h. The average basil plant height (± SD) used in this experimental setup at the start of the experiment was 30.0 ± 0.8 cm.

2.3.1 Determination of the pesticides in water

A combination of online solid-phase extraction, liquid chromatography, and mass spectrometer (LC-LC-MS) was used to quantify pesticide concentrations in water samples. The developed analytical method was based on a work by Khan et al. (2012). HTS XT-CTC auto-sampler (CTC Analytics AG, Zwingen, Switzerland), Accela 1250, and Accela 600 LC pumps (Thermo Fisher Scientific, San Jose, CA, USA) were components of the LC-LC system together with Hypersil Gold aQ column 20 x 2.1 mm, 12 µm (SPE) and Hypersil Gold Phenyl 50 x 2.1 mm, 5 µm as analytical column (Thermo Fisher Scientific, San Jose, CA, USA). Gradient elution for sample extraction (injected sample volume 1 mL) and chromatographic separation is further described in Table 1 of the Supplementary Material. Solvent A represents ultra-pure water prepared with AquaMax Basic 360 Series and Ultra 370 Series (Young Lin Instruments, purchased from Labicom, Czech Republic) and Solvent B methanol (Merck, Germany, LC grade).

Compounds were detected with triple quadrupole mass spectrometer TSQ Quantiva and HESI ion source (Thermo Fisher Scientific, San Jose, CA, USA). The instrument operated in negative and positive ion mode and selected reaction monitoring data acquisition mode. Detailed instrument setup is concluded in Table 3 of the Supplementary Material. The methods of evaluating parameters such as linearity of a

TABLE 1 The physico-chemical parameters (mean values ± SD) of the nutrient solutions during the raft and the media bed experiment.

Parameters	Groups			
	Control	Pyrethrum	Azadirachtin	Spinosad
Temperature (°C)	27.3 ± 0.5	26.8 ± 0.8	27.6 ± 0.4	26.9 ± 0.3
pH	6.18 ± 0.07	6.11 ± 0.04	6.31 ± 0.13	6.22 ± 0.11
Dissolved oxygen (mg L^{-1})	10.2 ± 1.2	8.69 ± 1.57	10.24 ± 1.72	8.14 ± 2.0
Electrical conductivity (µS cm^{-1})	3572 ± 336	3580 ± 223	3599 ± 169	3496 ± 70

No significant differences was noticed between groups (one-way ANOVA followed by Tukey's HSD post-hoc test, P>0.05).

calibration curve, the limit of quantification (LOQ) (Solliec et al., 2014), accuracy, and precision (Kruve et al., 2015) were evaluated prior to sample analysis. The results are summarized in Table 4 of the Supplementary Material. Sample matrix used for all evaluation parameters was identical to the experimental samples.

The results were calculated with an average response factor with internal standard calibration when each sample was spiked with 20 ng of isotopically labeled standard (Borik et al., 2020). Analytical data postprocessing and reporting were performed with TraceFinder 4.1 (Thermo Fisher Scientific, San Jose, CA, USA). Analytical standard of azadirachtin used for method evaluation and preparation of calibration curve was purchased from Sigma-Aldrich (Czech Republic). Stock solutions of native and internal standards were prepared at 1 mg mL^{-1} in methanol (Merck, Germany, LC grade) and stored at −20°C.

2.4 Experiment II - biofilter trial

The biofilter study is conceived on the design of an already published trial study (Rašković et al., 2021). The biofilter trial was run in 12 polyethylene circular tanks with a net volume of 35 L each. The tanks were filled with 12 L of dechlorinated tap water, 3 L of RAS water, and 3 L of biofilter media BT10 (Ratz Aqua and Polymer Technik, Germany) from a running RAS. The system was placed indoors in an air-conditioned room. All buckets were equipped with two round air stones (5 cm diameter, Hailea, China), delivering air and mixing the bio media-water solution to mimic the conditions of a biofilter. Air was supplied with a central air blower (Secoh JDK-50, Japan). During the stabilization period, the bacteria consortium was fed 10 mg L^{-1} of NH_4-N twice daily using NH_4Cl (Penta, Czech Republic) stock solution (1.5 mg L^{-1} of NH_4-N). Temperature, oxygen saturation, and pH were measured twice daily (mean ± SD; pooled data for all buckets, no significant differences $p > 0.05$; t = 22.4 ± 0.1°C; O_2 = 90.4 ± 4.0% and pH was kept between 7 and 8 using 10% $NaHCO_3$ solution (Penta, Czech Republic) with a handheld multimeter (HI9829, Hanna Instruments, Romania). Before the experiment's commencement, the water was well mixed between treatments and control to ensure homogeneity of the water parameters.

After the stabilization period, the azadirachtin-based pesticide (10.6 g L^{-1} of azadirachtin; Neem Azal T/S, Biocont, Czech Republic) was applied from a stock solution with a concentration of 1.5 µg L^{-1} azadirachtin. The stock solution was prepared right before the application by mixing 2.1226 mL of the pesticide and 997.9 of ultra-pure water and shook vigorously to ensure proper mixing (calculation based on declared azadirachtin concentration in the pesticide and its density of 0.98 g mL^{-1}). The azadirachtin was applied in three concentrations, while the control was left untreated. The concentrations were as follows: 1.5 µg L^{-1}; 7.5 µg L^{-1}; and 15 µg L^{-1}. The lowest concentration (1.5 µg L^{-1}) mimicked the highest concentration detected in the plant trial, while the others were 5 and 10 times higher, respectively, mimicking possible accumulations in other hydroponic systems such as nutrient film technique and drip irrigation which use lower water volumes compared to rafts systems used in this experiment. To measure the azadirachtin concentrations, 15 minutes after the application, 10 mL of water was sampled from each unit, filtered into a glass vials through 0.2 µm PVDF syringe filter (Whatman, Germany). The water samples were frozen until further analyses (described below). Samples of the stock solution and tap water were also taken (Table 1 in the Supplementary Material).

2.4.1 Determination of nitrogen species in water

During the next 72 hours, the NH_4-N, NO_2-N and NO_3-N were measured twice daily using standard spectrophotometric method (APHA, 1989). Temperature, oxygen saturation and pH were measured twice daily (HI9829, Hanna Instruments, Romania). pH was maintained in a range of 7 to 8 using 10% $NaHCO_3$ solution (Penta, Czech Republic). The systems were fed with 10 mg L^{-1} and 15 mg L^{-1} of NH4-N daily using NH_4Cl (Penta, Czech Republic) in the morning and evening, respectively. In order to investigate the possible effects of these pesticide concentrations on the nitrifying bacteria, 25 media elements were collected from each unit after 6 hours of application for DNA analysis.

The bacteria load in the elements was extracted 6 hours after application of the pesticide. Bacteria were obtained by adding 50 mL of ultrapure water to a 100 mL falcon tube containing the elements and vigorously vortexed for two minutes. This was followed by placing the tube in an ultrasonic bath (Sonorex; Baudelin) for 5 minutes. Afterwards, the media were removed and the biofilm was centrifuged (5000 rpm, 10 min). The pellet was used for DNA extraction (DNEasy, Quiagen, Germany). Sequencing on an Illumina MiSeq (2 × 300 bp) was done by GATC AG (Konstanz, D) according to the InView™ Microbiome Profiling protocol (see Schmautz et al., 2017 for details). Data have been made available under the study accession PRJEB56899 at EBI.

2.5 Experiment III – fish exposure trial

2.5.1 Description of semi-static exposure assay

180 individuals of Nile tilapia (*Oreochromis niloticus*) with average body mass of 141 ± 23 g (mean ± SD) were purchased from Kirschauer Aquakulturen GmbH fish farm (Schirgiswalde - Kirschau, Germany). Fish were transported to the Laboratory of Nutrition (Institute of Aquaculture and Protection of Waters, University of South Bohemia in České Budějovice), where they were kept in plastic tanks and fed daily with Skretting T3 tilapia feed (3 mm floating pellets; 44% crude protein, 10% lipid, 25% carbohydrate and 11.5% ash; Skretting, Czech Republic), at a ratio of 2.5% body weight. Prior to the beginning of the experimental trial, fish were transferred to Laboratory of Aquatic Toxicology and Ichtyopathology (Research Institute of Fish Culture and Hydrobiology, University of South Bohemia in České Budějovice) and randomly allocated to 12 glass aquaria (15 fish in each aquaria) with following dimensions (L x W x H): 65 x 45 x 40 cm, and total volume of 100 L. Fish were placed in the aquaria for 10 days of acclimatization period, while subsequent semi-static exposure assay was conducted for 7 days. In addition, recovery period of another 7 days was given to the same batch of fish. Fish were exposed to the following nominal concentrations of azadirachtin-based commercial product AZA in triplicates: 1.5 μg L^{-1}, 7.5 μg L^{-1}, 15 μg L^{-1} and control group, which contained only water. During the course of the experiment, actual concentration of azadirachtin was determined in the water using spektrometr TSQ Quantiva Triple-Stage Quadrupole (Thermo Scientific) on day 1 (after addition of water), 3, 5 (both before and after exchange of water) and 7 (before termination of the experiment) of the trial. Actual concentrations differed more than 20% comparing to nominal concentrations, so we decided to use actual concentrations in the text of this manuscript, as recommended by OECD guidelines (OECD, 2019). Concentrations were measured as following: group A (nominal - 1.5 μg L^{-1}): 2.04 ± 0.99 μg L^{-1}; group B (nominal 7.5 μg L^{-1}): 6.52 ± 2.83 μg L^{-1}; group C (nominal 15 μg L^{-1}): 7.93 ± 2.91 μg L^{-1}, while in control group, no trace of AZA was discovered in the sampled water; thus, experimental groups will be named as Group 0, Group 2, Group 6.5 and Group 8 μg L^{-1}. During semi-static exposure and recovery period, total volume of dechlorinated tap water was exchanged on every second day (days 1, 3 and 5), while basic water parameters were measured using HI 98194 (Hanna Instruments) device. Following values are recorded during (1) exposure assay: temperature: 26 ± 1°C, pH value: 7.8 ± 0.5, oxygen saturation: 90-99%; total ammonium 0.02 mg L^{-1} and (2) recovery period: temperature: 26 ± 1°C; pH value: 7.8 ± 0.5; oxygen saturation: 90-99%; total ammonium: 0.02 mg L^{-1}.

2.5.2 Fish sampling

At the end of the exposure period (day 7) and at the end of the recovery period (day 14), three fish per aquarium was randomly picked and anaesthetized with a solution of buffered ethyl 3-aminobenzoatemethanesulfonic acid (MS 222) (Sigma-Aldrich, Czech Republic). Blood was sampled using heparinized syringe and needle (5000 IU heparin sodium salt in 1 mL), with insertion of needle in the caudal vein. The blood samples were later stabilized with an aqueous solution of heparin sodium salt in the rate of 0.01 mL L^{-1} and were immediately processed. Second portion of blood was used for biochemical analyses and was centrifuged at 1500 ×g for 10 min in a microcentrifuge (MPW 55, MPW Instruments, Poland). Supernatant, containing blood plasma was collected, transferred into tubes on ice and stored at -80°C until subsequent analysis. After blood sampling, fish were carefully dissected and second gill arch from the right side of every fish, and part of liver were taken for histological assessment and placed in 4% formalin (Sigma-Aldrich, Czech Republic) for fixation, while samples of gills, brain, kidney, muscle, intestine and liver were taken for determination of concentrations of antioxidative enzymes in mentioned fish organs. These samples were snap frozen in liquid nitrogen and kept at -80°C until further processing. Frozen tissues were later weighted and homogenized in 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA (1:10, w/v) using an Ultra Turrax homogenizer (Ika, Germany) and divided in two parts – one for measuring TBARS and other was subjected to centrifugation at 12,000×*g* for 30 min at 4°C and supernatant is used for determining of antioxidant parameters (SOD, GPx and GR).

2.5.2.1 Heamatological and biochemical blood plasma parameters

Several heamatological parameters were determined from sampled blood and analyzed using protocol by Svobodova et al. (1991): number of red blood cells (RBC), concentrations of heamatocrit (Ht) and haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), number of leukocytes and leukogram (lymphocytes (%), monocytes (%),

neutrophil segments (%), neutrophil bands (%), myeloid sequence (%)).

Concerning plasma biochemical parameters, following one were measured: total protein (TP), albumin (ALB), globulin (GLB), glucose (GLU), triglyceride (TG), phosphorous (P), magnesium (Mg), creatinine (CREA), lactate (LACT) and ammonia (NH3) concentrations, and activities of alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and creatinine kinase (CK). They were determined using a blood analyser VETTEST 8008 (IDEXX Laboratories Inc., USA) according to already established protocol (Kolarova and Velisek, 2012).

2.5.2.2 The antioxidant parameters and lipid peroxidation of tissues

Three antioxidant parameters were determined spectrophotometrically from the tissues of sampled fish: (1) glutathione peroxidase (GPx; EC 1.11.1.9), by determining the rate of NADPH oxidation at 340 nm by the reaction with glutathione reductase (GR; EC 1.6.4.2) The specific activity was determined using the extinction coefficient 6.22 mM cm^{-1} (Lawrence and Burk, 1976); (2) GR, by measuring rate of NADPH oxidation at 340 nm (Carlberg and Mannervik, 1975). For both GPx and GR activity - one unit was defined as the quantity of enzyme that consumes 1 mol mL^{-1} of substrate or generates 1 mol mL^{-1} of product per minute and is expressed in IU per mg of protein; (3) total superoxide dismutase (SOD; EC 1.15.1.1) activity was detected using the method developed by Marklund and Marklund (1974), based on autoxidation of pyrogallol. SOD activity was assessed at 420 nm and expressed as the amount of enzyme per milligram of protein. For lipid peroxidation of sampled tissues, the TBARS assay was employed using methodology described by Luschak et al. (2005). The TBARS concentration was calculated by the absorption at 535 nm and a molar extinction coefficient of 156 mM cm^{-1}. The value was expressed as nmol of TBARS g^{-1} wet weight tissue.

2.5.2.3 Histological processing and assessment

After 24 hours of fixation samples were transferred to 70% ethanol and stored for further processing. Later, they were placed in tissue processor (Leica TP 1020, Nussloch, Germany), dehydrated, cleared using xylene and embedded in paraffin. Paraffin blocks were cut on 5 µm thickness using microtome and mounted on glass slides, which are further stained using automated staining device (Leica ST 4040, Nussloch, Germany). At the end, cover slides are mounted and slides were assessed for the presence of histopathological alterations using semiquantitative scoring system. Each alteration that was present in the slides of gills and liver was given one of the following scores: 1 (mild), 2 (moderate) or 3 (severe), depending on amount of altered tissue.

2.6 Statistics

Prior to statistical analysis, all data sets were tested for normality and homoscedasticity using Shapiro-Wilk's and Levene's test, respectively. If data set passed both assumptions, then ANOVA followed by Tukey's HSD *post-hoc* test was used, and if not, non-parametric Kruskal-Wallis H test was used, while difference between experimental groups was tested using Mann Whitney U test. The significance level (α) was set at 5% while all values are presented as means ± standard deviation (SD). For all statistical analysis PAST software, version 4.06b (Hammer et al., 2001) was used.

3 Results

3.1 Experiment I – pesticide runoff and degradation rate

Measurements of concentration of insecticides in the water after application on plants showed distinctive patterns in both tested systems during time points (Figure 2). Concentrations of pyrethrum were lower than the limit of quantification (50 ng L^{-1}) in all sampling points in both systems. Spinosad showed lower concentration in the water when applied to plants in media bed system, comparing to floating raft system.

The highest concentration of spinosad sampled in the water from single replicate was 13 ng L^{-1} at media bed system and 230 ng L^{-1} at floating raft system, while mean concentrations of spinosad in both systems peaked early, between 6 and 24 h after application and gradually declined afterwards. Mean concentrations of azadirachtin were also higher in floating raft system at each sampling point, similar to spinosad. Maximal concentration in single replicate was 1.3 µg L^{-1} at media bed system and 1.4 µg L^{-1} at floating raft system, but peaks were measured at different sampling points: when applied at media bed system, peak concentrations were established after 24 h and were gradually lowered after 48 h, while concentration in the water from floating raft system peaked later, after 48 h and started to decline afterwards. The percentage of these detected concentrations were <0.01% of the applied concentration of the active ingredient per treatment.

3.2 Experiment II - biofilter trial

Biofilter trial showed no significant differences between control and buckets supplemented with azadirachtin at any concentration for all nitrogen compounds (NH_4-N, NO_2-N, and NO_3-N). However, mean values of NH_4-N in group AZA 15 was significantly higher comparing to AZA 1.5 after 12 hours

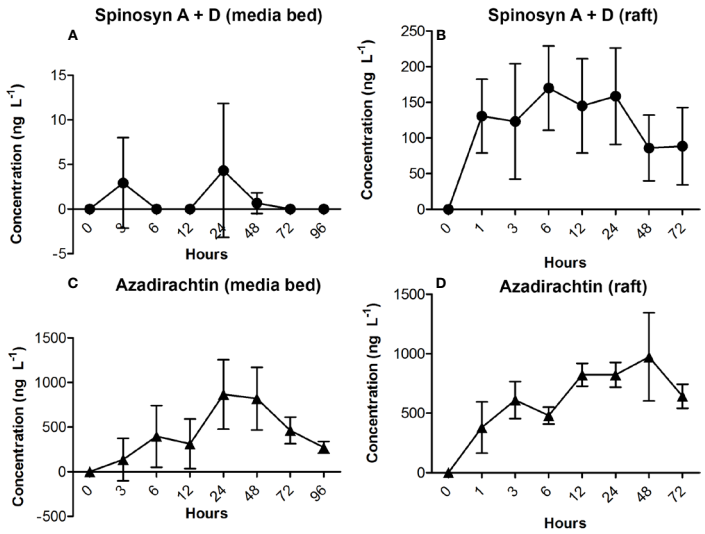

FIGURE 2
Aqueous concentrations of two insecticides in relation to time points at two different systems for growing basil in hydroponics: **(A)** concentrations of spinosyn A + spinosyn D at media bed system during 96h; **(B)** concentration of spinosyn A + spinosyn D at floating raft system during 96h; **(C)** concentration of azadirachtin at media bed system during 96h; **(D)** concentration of azadirachtin at floating raft system during 96h.

of the trial (Figure 3). From the DNA result of the bacteria consortium in the biofilter to show community compositions, the largest bacteria phylum in all the treatments and control was Proteobacteria, with a percentage proportion >65% in all the treatments. Other major phyla were group after the end; Bacteroidetes (7-10%), Gemmatimonadetes (1-3%), Nitrospirae (4-16%), and Acidobacteria (2-3%) (Figure 4). Temporal community changes were found in the percentage proportions of Nitrospirae in the treatments and control. The average percentage proportion of Nitrospirae in control (16%) is significantly higher than the proportion in the 1.5 μg L^{-1} (4.1%), 7.5 μg L^{-1} (6.5%), and 15 μg L^{-1} (5.8%).

3.3 Experiment III – fish exposure trial

The average body mass and length of experimental fish in each treatment did not show a statistical difference from the control in both sampling points (7 and 14 days; Table 2). Hematological analysis revealed that majority of parameters did not significantly differ from the control group (Table 3). However, lower values were established between group AZA 8 compared to control for MCV after the exposure period ($P < 0.05$), but the same parameter was very similar among the groups at the end of the recovery period. MCHC showed a contrasting pattern, as groups AZA 6.5 and AZA 8 were higher compared to the control ($P < 0.05$) only at the end of the recovery period and not after the exposure. A Higher number of erythrocytes was recorded in fish from AZA 8 group compared for AZA 2 group after the end of exposure (and not recovery) period ($P < 0.05$). On the other hand, the number of leukocytes was lower in groups AZA 2 and AZA 8 compared to the control after the exposure period and remained lower in groups AZA 8 and AZA 6.5 after recovery.

Similar to the hematology results, only a few parameters were significantly altered in the blood biochemistry of the experimental animals after the exposure period (Table 4), but those changes ameliorated and no differences were established after the recovery period. The concentration of glucose and ammonia in the blood of animals from groups AZA 6.5 and AZA 8 were increased compared to both the control group and AZA 2 ($P < 0.05$), while creatine kinase was increased in group AZA 8, compared to all groups ($P < 0.05$). The dose-dependent response was established for the lactate concentration, as it increased in AZA 6.5 and AZA 8 groups ($P < 0.05$).

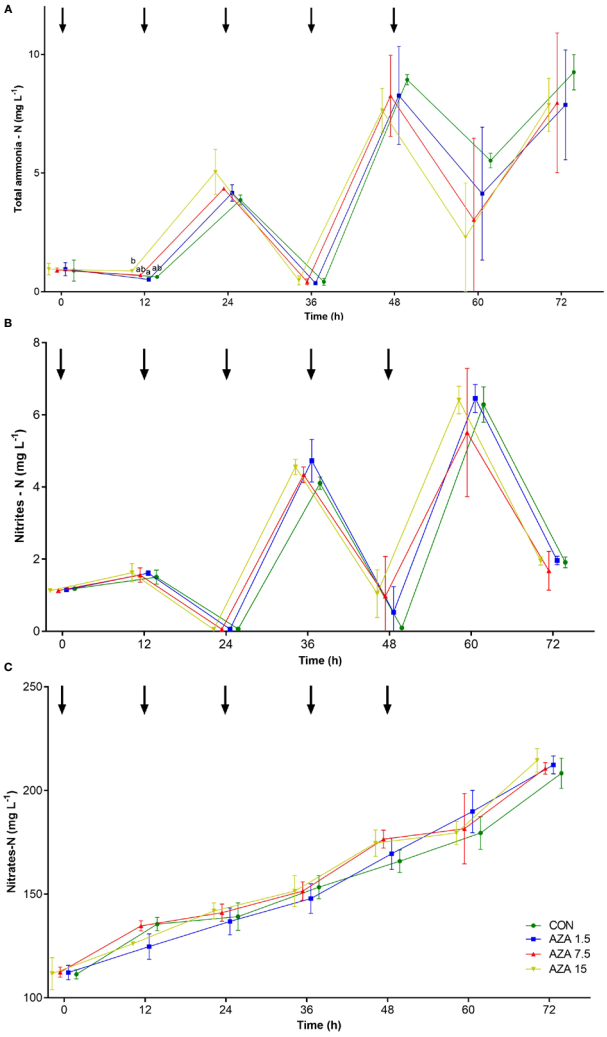

FIGURE 3
Concentrations (mean ± SD) of nitrogen compounds in the water from working biofilter exposed to varying concentrations of azadirachtin during 72h: **(A)** total ammonia – nitrogen; **(B)** nitrites – nitrogen; **(C)** nitrates – nitrogen; lines with different colors and symbols represent control group (CON), and concentration of azadirachtin insecticide (AZA 1.5; AZA 7.5 and AZA 15 μg L^{-1}) added to the working biofilter; points are shifted to the left or to the right in order for easier comparison among groups; arrows show time when ammonium chloride was added to the buckets; different letters represent significant differences between groups within the same sampling point (one-way ANOVA followed by Tukey's HSD posthoc test, P<0.05).

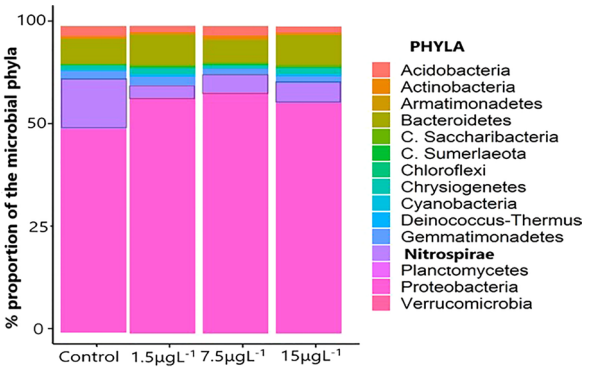

FIGURE 4
The percentage proportion of the bacteria consortium in the biomedia samples (n=3) from the biofilter treatments, 1.5 μgL^{-1}, 7.5 μgL^{-1}, 15 μgL^{-1}, and control. Samples were taken 6 hours after the application of azadirachtin in the biofilter.

Concentrations of three antioxidant enzymes (GR, GPx, SOD) from five different tissues showed a mild effect from azadirachtin (Figures 5A–C). The concentrations of GR in any tissue did not differ between groups, while in GPx, only AZA 6.5 was lower compared to control in the sampled intestine ($P < 0.05$) after the exposure period. SOD concentration in the fish brain from the highest exposure group (AZA 8) was significantly increased after the recovery period compared to control fish ($P < 0.05$). On the other hand, lipid peroxidation measured by malondialdehyde concentration in the liver (TBARS assay) showed that all three exposed groups had higher values compared to the control ($P < 0.05$) after the exposure period. However, all values dropped after the recovery period (Figure 5D).

Histology of hepatopancreas showed no signs of significant alterations (Supplementary Table 2) after 7 days of exposure and 7 days of recovery. The majority of fish in all groups had some mild changes in the pancreas and they included degranulation of eosinophilic granulocytes, cellular inclusions, and vacuolated cytoplasm of hepatocytes. However, they were present in all groups and did not show statistical significance among groups ($P > 0.05$). Similar results were recorded in the gills (Supplementary Table 2), where higher semiquantitative scores were noted for infiltration of leukocytes in branchial tissue and for the proliferation of interlamellar cell mass in the primary epithelium. However, neither histopathological alteration in gills showed statistical significance among groups ($P > 0.05$).

4 Discussion

4.1 Insecticides runoff and degradation in nutrient solution

After applying three different insecticides to basil plants, it was obvious that pyrethrum possessed the fastest degradable properties, as there were no pyrethrum concentrations above the quantification limit (50 ng L^{-1}) in any tested water sample, even one hour after application. Pyrethrum is composed of six esters, commonly known as pyrethrins which act as active substances (Zhu et al., 2020), but pyrethrin I and pyrethrin II account for approximately 73% of total amount of natural esters in

TABLE 2 Mean ± SD body mass (g) and total length (mm) of experimental fish measured after exposure (7 days) and recovery period (14 days).

Parameter	Sampling time	Control	AZA 2	AZA 6.5	AZA 8
Fish body mass (g)	7 days	144.9 ± 20.3	137.7 ± 32.5	135.2 ± 31.4	132.6 ± 30.4
	14 days	142.4 ± 27.0	113.8 ± 26.8	118.0 ± 35.5	114.2 ± 35.4
Fish total length (mm)	7 days	159.8 ± 7.9	154.6 ± 14.4	153.4 ± 18.4	157.1 ± 14.6
	14 days	157.2 ± 11.9	144.9 ± 12.8	152.8 ± 20.5	148.1 ± 18.0

No significant differences was noticed between groups (one-way ANOVA followed by Tukey's HSD post-hoc test, P>0.05).

TABLE 3 Hematology values and leukogram (mean ± SD) measured from the blood of Nile tilapia kept in control and exposed to different concentrations of azadirachtin (AZA 2 μg L^{-1}; AZA 6.5 μg L^{-1} and AZA 8 μg L^{-1}) for 7 days and after recovery period (14 days).

Parameter	Sampling time	Control	AZA 2	AZA 6.5	AZA 8
Haematocrit PCV (L L^{-1})	7 days	0.19 ± 0.02	0.18 ± 0.02	0.20 ± 0.03	0.18 ± 0.03
	14 days	0.19 ± 0.02	0.18 ± 0.02	0.17 ± 0.04	0.18 ± 0.04
Haemoglobin Hb (g L^{-1})	7 days	65.7 ± 8.8	60.8 ± 6.7	64.8 ± 9.3	64.1 ± 8.7
	14 days	51.6 ± 11.6	56.9 ± 6.9	54.1 ± 10.7	62.4 ± 17.6
Erythrocyte count RBC (T L^{-1})	**7 days**	**1.72 ± 0.22^{ab}**	**1.63 ± 0.21^{a}**	**1.74 ± 0.31^{ab}**	**1.95 ± 0.12^{b}**
	14 days	1.53 ± 0.34	1.56 ± 0.28	1.50 ± 0.60	1.56 ± 0.41
MCH (pg)	7 days	38.6 ± 5.7	37.6 ± 4.6	37.7 ± 5.6	32.8 ± 4.2
	14 days	34.2 ± 6.3	37.5 ± 9.4	39.1 ± 9.8	40.1 ± 4.3
MCV (fl)	**7 days**	**114 ± 15^{a}**	**113 ± 19^{ab}**	**116 ± 22^{ab}**	**91 ± 16^{b}**
	14 days	127 ± 14	118 ± 22	122 ± 33	122 ± 24
MCHC (g L^{-1})	7 days	339 ± 21	336 ± 29	330 ± 36	365 ± 41
	14 days	**270 ± 49^{a}**	**318 ± 42^{ab}**	**323 ± 20^{b}**	**335 ± 46^{b}**
Leukocyte count (G L^{-1})	**7 days**	**5.67 ± 1.89^{a}**	**3.10 ± 0.73^{b}**	**3.64 ± 1.51^{ab}**	**3.00 ± 2.15^{b}**
	14 days	**4.93 ± 1.60^{a}**	**3.81 ± 1.21^{ab}**	**2.32 ± 1.09^{bc}**	**2.09 ± 0.69^{c}**
Lymphocytes (%)	7 days	89.5 ± 5.5	91.1 ± 4.0	90.3 ± 5.8	89.6 ± 7.8
	14 days	90.2 ± 10.0	88.3 ± 6.3	90.0 ± 6.0	95.7 ± 4.5
Monocytes (%)	7 days	4.86 ± 2.77	3.59 ± 2.35	3.02 ± 1.90	4.30 ± 4.00
	14 days	3.02 ± 3.42	5.42 ± 3.94	4.59 ± 1.74	2.21 ± 1.89
Neutrophil segments (%)	7 days	3.07 ± 1.17	2.54 ± 1.33	2.34 ± 2.05	1.69 ± 0.99
	14 days	1.00 ± 1.29	2.68 ± 1.51	1.97 ± 0.95	1.30 ± 1.71
Neutrophil bands (%)	7 days	0.80 ± 0.83	0.50 ± 0.42	0.79 ± 0.97	0.70 ± 0.70
	14 days	0.69 ± 0.79	0.59 ± 0.87	0.31 ± 0.56	0.32 ± 0.51
Developmental phases – myeloid sequence (%)	7 days	1.82 ± 2.22	2.27 ± 1.23	3.52 ± 3.25	3.69 ± 2.91
	14 days	5.08 ± 5.78	2.99 ± 2.19	3.17 ± 3.96	0.48 ± 0.83

Values represent mean ± standard deviation; Different letters in superscripts in the same row represent statistical differences between groups (ANOVA, followed by Tukey post-hoc test, $p < 0.05$). The rows with bold values are the statistically significant variables.

pyrethrum (Ujváry, 2010). Moreover, in studies where concentrations of pesticides were assessed in soil and runoff water, pyrethrin II was determined in 10 to 100 times higher concentrations compared to pyrethrin I (Antonious et al., 1997) and this is the main reason why pyrethrin II was chosen as a focus molecule in testing water samples from the present study. The biodegradation of pyrethrum is extremely fast and the half-life of this compound strongly depends on sunlight, with a half-life ranging between 10-12 minutes (Ujváry, 2010). Since the foliar application of the insecticides was conducted in July and August, a reaction with the sun could be the probable reason why the pyrethrin was not detected at a quantifiable concentration in the nutrient solution.

In contrast to pyrethrum, two other insecticides were detected in the water in both tested systems (media bed and floating raft) (Table 5). The fact that water from the media bed system contained lower concentrations of insecticides is probably due to the adsorbent properties of expanded clay pebbles. Some types of clay show excellent removal efficiency of pesticides in the water (Cosgrove et al., 2019), which are, for particular substances, even comparable with active carbon. For example, for pesticide diuron, removal efficiency of expanded clay is 98%, surpassing 92% when active carbon is used (Tahar et al., 2014), but percentages are highly dependent on the type of adsorbent and pesticide/toxicant. Mean and maximal concentrations of spinosad in the water sampled from both tested systems should not be considered as a risk for fish in the aquaponics system. NOEC (No Observed Effect Concentration) of spinosad for fish such as Common carp *(Cyprinus carpio)*, Common minnow *(Phoxinus phoxinu)* and rainbow trout *(Oncorhynchus mykiss)* have been reported to range between 0.7 and 5.2 mg L^{-1} (Barden, 1998). Also, while chronic NOEC for aquatic invertebrates is 0.0012 mg L^{-1}, the compound is considered unharmful to microorganisms. In addition, 96h LC_{50} concentrations for spinosad are reported as > 202 mg L^{-1} for guppies (*Poecilia reticulata*) and platys (*Xiphophorus maculatus*) (Pereira et al., 2016) and > 500 mg L^{-1} for juvenile Coho salmon (*Oncorhynchus kisutch*) (Deardorff & Stark, 2009). These concentrations are thousand times higher than the highest concentration (1.3 ng L^{-1}) reported in the present study, hence,

TABLE 4 Blood biochemistry values (mean ± SD) measured from the blood of Nile tilapia kept in control and exposed to different concentrations of azadirachtin (AZA 2 μg L^{-1}; AZA 6.5 μg L^{-1} and AZA 8 μg L^{-1}) for 7 days and after recovery period (14 days).

Parameter	Sampling time	Control	AZA 2	AZA 6.5	AZA 8
Albumin (g L^{-1})	7 days	4.22 ± 1.39	4.56 ± 1.33	4.33 ± 1.12	4.67 ± 1.80
	14 days	4.11 ± 1.27	4.44 ± 1.24	4.78 ± 1.48	4.67 ± 1.58
Total globulins (g L^{-1})	7 days	25.7 ± 2.6	24.6 ± 3.4	25.3 ± 4.0	25.0 ± 4.8
	14 days	27.4 ± 2.9	26.6 ± 5.9	28.0 ± 3.5	27.4 ± 2.2
Alkaline phosphatase (μkat L^{-1})	7 days	0.13 ± 0.03	0.12 ± 0.02	0.14 ± 0.07	0.13 ± 0.03
	14 days	0.10 ± 0.04	0.14 ± 0.05	0.13 ± 0.06	0.12 ± 0.04
Alanine aminotransferase (μkat L^{-1})	7 days	0.77 ± 0.45	0.70 ± 0.36	0.66 ± 0.07	0.85 ± 0.30
	14 days	0.65 ± 0.23	0.86 ± 0.27	0.87 ± 0.30	0.76 ± 0.21
Aspartate aminotransferase (μkat L^{-1})	7 days	1.21 ± 0.28	1.26 ± 0.32	1.36 ± 0.21	1.42 ± 0.17
	14 days	0.93 ± 0.17	1.09 ± 0.35	1.13 ± 0.25	1.17 ± 0.21
Inorganic phosphate (mmol L^{-1})	7 days	1.38 ± 0.29	1.44 ± 0.29	1.39 ± 0.31	1.41 ± 0.34
	14 days	1.46 ± 0.34	1.53 ± 0.26	1.53 ± 0.26	1.54 ± 0.30
Total protein (g L^{-1})	7 days	29.9 ± 3.0	29.1 ± 3.1	29.9 ± 3.6	29.7 ± 3.9
	14 days	32.1 ± 2.9	31.0 ± 4.9	32.8 ± 3.8	32.1 ± 2.6
Glucose (mmol L^{-1})	**7 days**	**4.26 ± 0.69^{a}**	**4.67 ± 0.75^{a}**	**5.88 ± 1.09^{b}**	**6.85 ± 0.72^{b}**
	14 days	3.88 ± 0.40	3.74 ± 0.72	3.37 ± 0.48	3.99 ± 0.82
Ammonia (μmol L^{-1})	**7 days**	**282 ± 58^{a}**	**289 ± 59^{a}**	**387 ± 49^{b}**	**460 ± 92^{b}**
	14 days	322 ± 47	292 ± 39	317 ± 42	319 ± 54
Magnesium (mmol L^{-1})	7 days	0.93 ± 0.10	0.98 ± 0.07	0.94 ± 0.11	0.92 ± 0.14
	14 days	0.99 ± 0.91	0.91 ± 0.16	0.95 ± 0.12	0.95 ± 0.15
Triacylglycerol (mmol L^{-1})	7 days	3.38 ± 0.82	3.47 ± 0.68	3.40 ± 0.50	3.48 ± 0.40
	14 days	3.58 ± 0.40	3.48 ± 0.53	3.65 ± 0.35	3.69 ± 0.50
Creatine kinase (μkat L^{-1})	**7 days**	**16.1 ± 1.4^{a}**	**16.6 ± 0.8^{a}**	**16.9 ± 0.9^{a}**	**18.3 ± 1.0^{b}**
	14 days	14.7 ± 1.7	14.9 ± 1.3	15.9 ± 1,3	15.8 ± 1.1
Lactate dehydrogenase (μkat L^{-1})	7 days	21.9 ± 3.5	21.2 ± 4.3	22.0 ± 2.4	22.9 ± 3.4
	14 days	21.0 ± 1.3	21.1 ± 1.3	20.0 ± 1.6	20.6 ± 1.4
Lactate (mmol L^{-1})	**7 days**	**1.58 ± 0.40^{a}**	**1.64 ± 0.27^{a}**	**2.19 ± 0.28^{b}**	**2.71 ± 0.47^{c}**
	14 days	1.88 ± 0.45	1.82 ± 0.22	1.56 ± 0.34	1.80 ± 0.38

Values represent mean ± standard deviation; Different letters in superscripts in the same row represent statistical differences between groups (ANOVA, followed by Tukey post-hoc test, $p < 0.05$).
The rows with bold values are the statistically significant variables.

no risk is perceived. Moreover, it was impossible to determine the exact analyte concentration presented in the sample during sampling time due to the high degradation rates of spinosyn in the water matrix under low temperature (4°C) and complete dark conditions. Although it was unable to properly quantify concentrations of spinosyn, the estimated concentration values also seem to be orders of magnitude lower than reported LOEC. In contrast, the persistence of azadirachtin in the water was longer compared to both pyrethrum and spinosad and could be considered a risk, as it is common practice to use it in multiple applications (Pavela & Benelli, 2016).

It is noteworthy to state that, the low percentage runoff (< 0.001%) of pyrethrum and azadirachtin detected in the nutrient solution is an indication of the mildness of their could-be-effects when applied in aquaponics systems. This result is in line with the meta-analysis based review by Folorunso et al. (2021). In the study, using the established NOEC and LC_{50} of commonly used pesticides, the authors found that only a runoff >10% of these pesticides could cause detrimental effects in aquaponics systems.

4.2 Effects of Azadirachtin on biofilter bacteria

The proper functioning of biofilter, inhabited by bacteria, is essential for aquaponic setup (Wongkiew et al., 2018). Bacteria in the biofilter are transforming ammonia (a toxic excretion towards fish) to nitrates in one step (COMMAMOX) or in two-step reaction (first, ammonia-oxidizing bacteria (AOB) are transforming ammonia to nitrites, and then nitrite-oxidizing bacteria (NOB) are transforming nitrites to nitrates) (van Kessel et al., 2015; Kasozi et al., 2021). The monitoring of ammonia concentration is essential for fish production in recirculating aquaculture systems (Becke et al., 2019), and

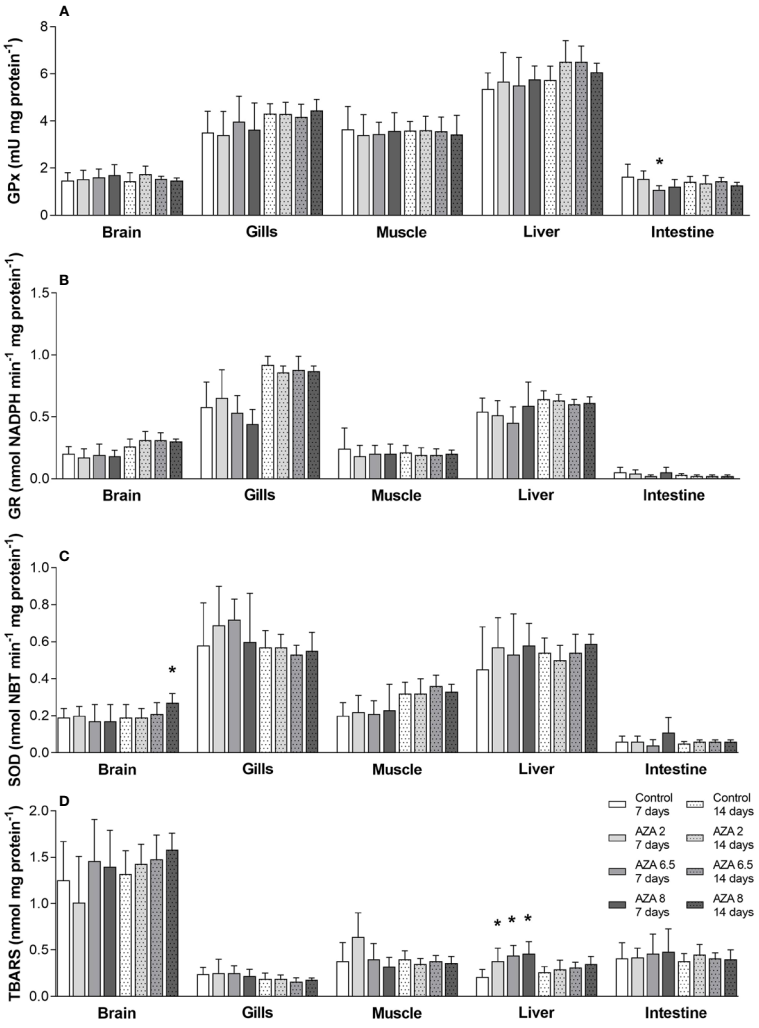

FIGURE 5
The mean ± SD concentrations of: **(A)** glutathione peroxidase; **(B)** glutathione reductase; **(C)** superoxide dismutase; **(D)** thiobarbituric acid reactive substances in the brain, gills, muscle, liver, and intestine of Nile tilapia exposed to 0, 2, 6.5, and 8 mg L^{-1} after 7 days and after recovery period (at the end of 14 days); asterisk denotes statistically significant difference (one way ANOVA followed by Tukey's *post hoc* test; $P < 0.05$) between control and exposed group.

the high concentrations could be detrimental to fish (Becke et al., 2019), so it is of utmost importance not to disrupt nitrifying processes that are ongoing in the biofilter. The addition of azadirachtin to buckets with fully operating biofilter did not show any adverse effects on the nitrification process of biofilter bacteria, except at the sampling point after 6h of exposure. This is in contrast with the pilot study that a similar group of authors already published using the same

TABLE 5 The calculated percentage runoffs of the insecticides generated from the detected maximum concentrations of the insecticides in the nutrient solution and the concentrations of the applied active ingredients (AI) per treatment.

Active Ingredients	Maximum conc.	AI applied	Percentage of runoff
Spinosad	1.3 ng L^{-1}	0.0792 g	< 0.001
Azadirachtin	1.5 µg L^{-1}	0.03 g	< 0.001

experimental design, in which buckets supplied with azadirachtin constantly showed higher concentrations of ammonia during the first step of nitrification compared to control (Rašković et al., 2021). The probable reason for the conflicting result is the concentration of the azadirachtin since, in the mentioned study; the nominal concentration was 20 µg L^{-1}, higher than in the present study. However, a slight reduction in Nitrospirae levels (an essential bacteria phylum in the nitrite-oxidizing process) was noted six hours after azadirachtin application in all the treatments. Thus, azadirachtin reduced nitrifying bacteria but not enough to stop nitrification and collapse the system. There are currently no studies on the influence of azadirachtin on nitrifying bacteria in water or nutrient solutions; however, similar effects have been reported in soil nitrification and nitrogen fixation studies. Singh et al. (2015) identified azadirachtin as the major contributor to the suppression of the growth of 49-99% of the nitrogen fixers in the soil after treating 1kg of soil with 1.13 mg of azadirachtin for 30, 50, and 80 days.

Neem tree seeds (from which azadirachtin is obtained by extraction) are known for their bactericidal properties, and it is already shown that in a culture of rohu carp (*Labeo rohita*) fed with neem seed cake, the concentrations of ammonia, nitrites and nitrates in the water increased compared to control (Das et al., 2018). This is confirmed by quantifying the number of nitrifying bacteria, which also dropped, at least during the first 60 days of feeding fish with neem seed cake. In the same study, the dose-dependent effect of toxicity to bacteria is shown since fish fed with higher incorporation of neem seed in the diet lived in water with increased concentrations of ammonia, nitrites, and nitrates (Das et al., 2018), and this is the probable answer for no effects shown in the present study. To the authors' knowledge, there were no more studies on the effect of azadirachtin on the biofilter bacteria, but the effects of azadirachtin were tested to nitrifying soil bacteria, and published results were also in discrepancy. Some reported a robust inhibitory effect of azadirachtin on an abundance of AOB soil communities (Gopal et al., 2007; Singh et al., 2015), while others are even reporting the stimulatory effect of azadirachtin on both microbial abundance and diversity for AOA and AOB soil microflora (Suciu et al., 2019). The differences in soil were explained mostly by different soil niches (Suciu et al., 2019), which is not applicable to biofilter bacterial communities. Moreover, biofilter communities in aquaponics are well known and described (Eck et al., 2019; Kasozi et al., 2021); and the microbial diversities vary in different compartments of the aquaponics system (Schmautz et al., 2017; Schmautz et al., 2022).

4.3 Effects of azadirachtin on Nile tilapia

One of the known adverse effects of pesticides during chronic exposures is in reducing weight of fish (Stanley & Preetha, 2016). Even though the aim of exposure was not to evaluate weight gain during 14 days of the fish trial, we have to emphasize that fish exposed to insecticide experienced reduced average body mass by approximately 20% compared to the control group after a recovery period. Average body mass did not differ significantly from the control group due to the large variability, but future studies should be focused on this fact since aquaponics aim to obtain optimal fish growth for commercial reasons.

The mechanism of toxicity of azadirachtin in animals is well established: it reduces RNA synthesis and cell proliferation by blocking the formation of microtubules in insects and mammals (Salehzadeh et al., 2003; Morgan, 2009). It is also proven that azadirachtin have genotoxic effects in the Mozambique tilapia (*Oreochromis mossambicus*) (Chandra & Khuda-Bukhsh, 2004) and that it modulates hormonal status in common carp (Korkmaz & Örün, 2022). Concerning effects on the hematological status of different fish exposed to azadirachtin in the present study, increased number of RBC and decreased values of MCV and leukocytes after exposure in the highest concentration (AZA 8) is mostly in line with other studies. Common carp exposed in acute (96h) test to 40 and 60 µL L^{-1} of azadirachtin showed decreased values of MCV (Murussi et al., 2016a), and the same was shown in goldfish (*Carassius auratus*) after using high concentrations of azadirachtin solution (10-20 mg L^{-1}) as antiparasitic agent (Kumar et al., 2013). A decrease in MCV and higher levels of RBC indicate impaired oxygen transport functions. However, the levels of MCV and RBC were back to normal after the 14 days of recovery, indicating the mildness of the effect. On the other hand, leukocyte levels showed decreased values even in the recovery period, which can impair the immune system in the long-term

period (Yang et al., 2021), even after exposure to 6.5 μg L^{-1} of azadirachtin.

In the blood biochemistry analysis, blood glucose, plasma ammonia, creatine kinase and lactate levels were significantly elevated compared to the control group after 7 days of exposure, but no significant differences were recorded after recovery. This result is in line with Oyoo-Okoth et al. (2011), where authors exposed Nile tilapia (*Oreochromis niloticus*) to high concentrations of azadirachtin ranging from 500 to 3600 mg L^{-1}. Blood glucose and plasma ammonia increased throughout the entire duration of the experiment (96h). This phenomenon is a typical response to pesticide exposure in fish, such as common carp exposure to simazine (Velisek et al., 2009), metribuzin (Velisek et al., 2009) or formulation containing a mixture of terbuthylazine and S-metolachlor (Dobšíková et al., 2012). Creatine kinase is already proposed as an alternative biomarker of pesticide toxicity in mammals (Bhattacharyya et al., 2011) and is known to be involved in the metabolism of nitrogen, more exact, in the excretion of nitrogen waste, apart from dominant ammonia (Randall, 2011). Hyperammonemia in fish blood results from altered physiological processes in the liver, which fails to metabolize ammonia (Dobšíková et al., 2012)

Concentrations of antioxidant enzymes are typically altered upon fish exposure to azadirachtin, and they depend on fish species, used concentration, and trial duration (Winkaler et al., 2007; Plhalova et al., 2018). However, in the present study, the response was minimal and the only significant and consistent parameter was lipid peroxidation in liver in all three used insecticide concentrations after 7 days of exposure. This points out to cell injury in hepatocytes caused by free radicals and toxicity of azadirachtin, which is already showed in acute and chronic exposures to rainbow trout (Alak et al., 2017) and neotropical fish - piava (*Megaleporinus obtusidens*) (Glusczak et al., 2011).

Studies on effects of azadirachtin on fish has shown that it induces histopathological alterations in liver of the stinging catfish (*Heteropneustes fossilis*) (Kumar et al., 2013), as well as in gills of the common carp (Murussi et al., 2016b) and *Prochilodus lineatus* (Winkaler et al., 2007), which is in contrast with findings in the present study. However, in all mentioned studies, concentrations azadirachtin used for exposure were higher: (1) 10.47 mg L^{-1} in chronic, 4 weeks trial; (2) 40-60 μL L^{-1} in acute, 96h test; (3) 2.5-7.5 g L^{-1} in acute, 24h trial, which can explain observed histopathology in target organs.

5 Conclusion

To the author's knowledge, this is the first study that aimed to assess risk of use of botanical/microbial insecticides to all three components of an aquaponics system. Although these insecticides are commonly used, it is not easy to find any guidance to aquaponics setup, even though there is a substantial difference between hydroponic and aquaponics systems. Evaluation from the present study showed that the use of pyrethrum and spinosad do not pose a risk affecting the fish in the aquaponics setup, at least not in the chosen combination of plant/fish species (basil/Nile tilapia). However, slight caution has to be taken into consideration, as both insecticides are very unstable and it is very hard to measure their concentrations in the water properly. On the other hand, azadirachtin showed mild adverse effects on fish at the highest measured concentration after one foliar application. Even on the lowest tested concentration (relevant concentration, determined in the real-life scenario), lipid peroxidation in liver and drop of leukocytes in the fish blood were detected. Therefore, caution when azadirachtin is used and more studies on this topic are recommended in the future.

Data availability statement

The data presented in the study are deposited in the EBI repository, accession number PRJEB56899.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of the University of South Bohemia based on the EU harmonized Animal Welfare Act of the Czech Republic and approved by the Departmental Expert Committee for Authorization of Experimental Projects of the Ministry of Education, Youth, and Sports of the Czech Republic (permit MSMT-6744/2018-4).

Author contributions

Conceptualization – BR, JM; Formal analysis – BR, RGe, JV, AB, RGr, JM; Funding acquisition – BR, JM; Investigation – BR, RGe, EF, GB, JV, PD, AB, RGr; Project administration – JM; Resources – JV, RGr, JM; Visualization – BR, RGe, EF; Writing – BR, EF; Writing - review & editing – JV, RGe, JM. All authors contributed to the article and approved the submitted version.

Funding

This research has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 652831 (AQUAEXCEL^{2020}). This output reflects only the author's view and the European Union

cannot be held responsible for any use that may be made of the information contained therein. Additional support for the research was received by the agreement between University of Belgrade - Faculty of Agriculture and Ministry of Education, Science and Technological Development of Serbia [grant number 451-03-68/2022-14/200116] and CENAKVA VVI Research Infrastructure (ID LM2018099, Ministry of Education, Youth and Sports of the Czech Republic, 2019–2022).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.1055560/full#supplementary-material

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CHAPTER 5

USE OF FUNGICIDES AND BIOCONTROL IN AQUAPONICS; IMPLICATIONS FOR FISH AND NITRIFYING BACTERIA

Folorunso, E., Gebauer, R., Bohata, A., Tomčala, A., Velisek., Dvořák, P., Kuebutornye, F., Franta, P., Mráz, J., 2022. Use of fungicides and biocontrol in aquaponics; implications for fish and nitrifying bacteria. Manuscript.

My share on this work was about 50 %.

Use of fungicides and biocontrol in aquaponics; implications for fish and nitrifying bacteria

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Abstract

Aquaponics is a method of producing food in a sustainable manner through the integration of aquaculture and hydroponics, which allows simultaneous cultivation of fish and economic crops. Disease infestation is a critical challenge in aquaponics due to the limited, available safe curative methods. Biological control and natural fungicides can be crucial to the sustainable control of diseases in aquaponics. Therefore, we examined the use of entomopathogenic fungi (*Isaria fumosorosea* and *Lecanicillium attenuatum*), mycoparasitic fungus (*Trichoderma virens*), and the risks associated with the use of natural (clove oil and lecithin) and synthetic (tebuconazole) fungicides on a biofilter and Nile tilapia, *Oreochromis niloticus,* in aquaponics. Our study identified that *T. virens*, besides its biocontrol properties, can improve the growth of basil plants in aquaponics at a concentration of 1×10^7 spores per ml. The foliar application of clove oil, lecithin, and tebuconazole spray-drifted, and were detected in aquaponics water at a percentage runoff rate of 0.3 %, 2.3 %, and 0.3–0.8 % respectively. In the biofilter, tebuconazole and clove oil at the maximum runoff concentrations, showed no significant effects on the nitrification processes over a 96 hr exposure period. In contrast, lecithin altered the ammonium and nitrite levels by increasing ammonium-nitrogen levels from an initial 5 mg L^{-1} at the 1st hour to ~13 mg L^{-1} at the 6th hour post application. These runoff concentrations were further evaluated on the physiology of *O. niloticus* in a 28-day semi-acute toxicity test. The tebuconazole-treated group showed a significant effect on hematological, biochemical, and antioxidative parameters. Eugenol, on the other hand, had no significant effect on the fish physiology, indicating its suitability for all aquaponics systems. The use of lecithin and tebuconazole should only be limited to decoupled aquaponics.

Keywords: *Aquaponics, tilapia, fungicides, biocontrol, entomopathogenic fungi, Trichoderma*

1. Introduction

Aquaponics is a sustainable food production system that integrates hydroponics with recirculating aquaculture system (RAS) to simultaneously produce economic crops and fish. The toxic fish wastes (ammonia and nitrite) in fish wastewater are converted to plant-absorbable nutrients by nitrifying bacteria, allowing water reuse (Rakocy, 2012). The provision of alternative sources of nutrients to grow economic plants reduces the pressure on the mining of minerals for commercial fertilizers, and the reuse of water (up to 90 %) implies that the food production system can be practiced in arid regions and marginal lands in urbans (Junge et al., 2017). Aquaponics can either be coupled, when the wastewater is constantly recirculated between a RAS and hydroponics unit in a closed-loop, or decoupled (on-demand

coupled), when both the RAS and the hydroponics units are independent systems, with RAS water periodically fed to the hydroponics unit (Goddek et al., 2016; Monsees et al., 2016; Baganz et al., 2022).

However, the interactions between these subsystems predispose the units to associated problems in other units, limiting the optimal functionality of the entire system. Managing pests and diseases in aquaponics has emerged as a highly significant obstacle, primarily because any applied treatment can have adverse impacts on the fish, plants, and beneficial bacteria cohabiting the shared water-loop (Stouvenakers et al., 2019; Folorunso et al., 2021). Aquaponic crops are particularly susceptible to varying pests and diseases due to the characterized high humidity, temperature, and the absence of pests' natural enemies in aquaponics greenhouses (Mori Smith, 2019). With chemical control methods constantly `frowned` upon in aquaponics, biological control has been severally suggested as the safe and sustainable alternative.

However, there are currently no biocontrol agent that has been approved for aquaponics. Folorunso et al. (2021) and Rivas-García et al. (2014), in their reviews, identified the potential of indigenous microbes of aquaponics as biological control agents, using microbial consortia information provided in Eck et al. (2019, 2021) and Schmautz et al. (2017, 2022). Sirakov et al. (2016) isolated microbes from different compartments of aquaponics to exert an inhibitory effect on both plant (*Pythium ultimum*) and fish pathogen (*Saprolegnia parasitica*). Forty-two of the 924 isolates inhibited the growth of both fungi in an *in vitro* trial. In another study, Stouvenakers et al. (2022) used eight efficacious microbes isolated from the rhizoplane of aquaponically-grown lettuce to suppress damping-off caused by *Pythium aphanidermatum* in lettuce seedlings. Although these studies identified indigenous microbial consortia as potential biocontrol agents, but the insufficient information on the proteomic identification of these isolates, and their exact location in aquaponics may limit their use at commercial scales. Folorunso et al. (2022) on the other hand, explored the potential of commercial entomopathogenic and mycoparasitic fungi against powdery mildew in detached cucumber leaves. In a separate experiment, authors also investigated their survival in RAS water to establish their risks to fish and beneficial bacteria in aquaponics. The authors found that, all the investigated biocontrol agents, *Lecanicillium attenuatum, Isaria fumosorosea,* and *Trichoderma virens,* were highly efficient against the disease, and the inability of their spores to survive beyond 96 hr in RAS confirmed their suitability for aquaponics. However, since these experiments were conducted separately, they might not have captured a real-life scenario, such as their effects on plant growth.

Using fungicides has been considered as `last resort` in aquaponics (Stouvenakers et al., 2019; Folorunso et al., 2021). However, there is still high reliance on fungicides, due to the inconsistencies in the efficiency of available biocontrol agents stemming from varying biotic and abiotic factors (Stouvenakers et al., 2019; Rivas-García et al., 2020). To identify safer pesticides group for aquaponics, Folorunso et al. (2021), using meta-analysis, simulated a percentage runoff of 1%, 10%, and 20% for different synthetic and natural pesticides and further compared the resulting concentrations with their corresponding NOEC (No observed effect concentration) and LC_{50} for aquatic organisms. The authors found that most synthetic pesticides would be toxic to fish and nitrifying bacteria if 1–10% of their active ingredients runoff or spray-drifts into the aquaponics water during foliar application. In contrast, natural pesticides were found to be non-toxic at a 20% runoff rate, indicating their suitability for aquaponics systems.

Clove oil and lecithin are natural essential oils that have been severally reported to have antifungal properties (Sukatta et al., 2008; Thabet Khalifa, 2018). Both compounds are considered environmentally friendly and readily available at low cost and in large amounts. For

example, lecithin is commonly used as a food additive and has been constantly reported to be virtually non-toxic to mammals (Misato et al., 1977). Thabet and Khalifa (2018) investigated the use of clove oil against *Fusarium oxysporum, F. solani, F. semitectum,* and *Rhizoctonia solani* at 0, 0.5, 1, 2, and 4 % v/v. At 4 %, clove oil showed complete growth inhibition towards all the pathogens in vitro and a significant decrease in disease incidence and severity *in vivo.* Other studies have reported similar results against *Botrytis cinerea* (Siripornvisal et al., 2009) and *Aspergillus* species (Hu et al., 2019). Similarly, cucumber leaves pre-treated with lecithin at 2000 mg L^{-1} partially inhibited the powdery mildew pathogen, *Sphaerotheca fuliginea* (Homma et al., 1977). In another study, soy lecithin applied at > 5000 mg L^{-1} inhibited up to 85.6 % of rice blast fungus, *Pyricularia oryzae*. Though these studies established the compounds' antifungal properties, there is a complete knowledge gap on their runoff rate and their risks in integrated systems such as aquaponics.

Synthetic fungicides are considered less sustainable due to their harmful effects on the environment, but their rapid actions make them the most frequently adopted option during fungi infestations (Lukens, 2013; Macirella et al., 2022). Tebuconazole is a systemic fungicide widely and frequently applied against a broad spectrum of pathogens (Othmène et al., 2020). The antifungal property of tebuconazole, as with other azoles, is related to the cell wall integrity disruption and reduction of ergosterol by interacting with a cytochrome P450 (CYP) enzyme, sterol 14-demethylase (Youness et al., 2018). Tebuconazole being a systemic fungicide, implies that the active ingredient translocates to the plants tissue, reducing their chances of drifting off to aquaponics water. However, there is a knowledge gap on the runoff rate of tebuconazole in soilless systems and the potential impact of the runoffs on fish and microbes.

Therefore, this present study aimed to (1) monitor the runoffs of natural (clove oil and lecithin) and synthetic (Tebuconazole) fungicides applied in decoupled aquaponics systems at different timepoints; (2) investigate the effects of entomopathogenic fungi, *Lecanicillium attenuatum, Isaria fumosorosea,* and mycoparasitic fungus, *Trichoderma virens* in aquaponics; (3) investigate the effects of runoff concentrations of the fungicides on nitrification in a mature biofilter and; (4) investigate the impact of runoff concentrations of foliar fungicides on the hematology, biochemical, and antioxidative parameters of Nile tilapia (*Oreochromis niloticus*). The aim is to explore the potential subacute toxicity to tilapia. The study is designed as a simulation of a real-life scenario, which results can be applicable and relevant to small aquaponics setups and more extensive production systems.

2. Materials and methodology

2.1. Experimental plan

For ethical concerns regarding the unmeasured effects of fungicides on fish, three experiments were conducted separately. The first, a pre-requisite experiment, was conducted on monitoring the runoff of the active ingredients of applied fungicides in the water of decoupled aquaponics over 72 hr timepoints. Using the concentrations detected in the aquaponics water in the first experiment, we exposed a matured biofilter to the maximum concentrations of the active ingredients to investigate their effects on nitrification. In the third experiment, *Oreochromis niloticus* were exposed to the maximum concentrations in a semi-acute toxicity test over 28 days to determine subacute toxicity.

2.2. Fungicides

The natural fungicides, clove oil and lecithin, and the synthetic fungicide, tebuconazole, were obtained in the Czech Republic. Clove oil, constituting 86 % of eugenol was obtained from Dr. popov Co., Czech Republic. Lecithin (LECID), constituting 100 % lecithin, was obtained from AgroProtec s.r.o, Czech Republic. Magnicur fungimat, on the other hand, constituting 2.5 % of tebuconazole, was obtained from SBM Life Science s.r.o, Czech Republic.

2.3. Entomopathogenic and mycoparasitic fungi

Lecanicillium attenuatum (strain CCM 9195), was isolated from adult bark beetles in NP Sumava, Czech Republic. The identification of this strain was determined through microscopic observation and cytochemical analysis using the methods described by Deng et al. (2010) and Simmons (2007). *Isaria fumosorosea*-based product, PFR-97 20% WDG, and the *Trichoderma virens*-based product, SoilGard®, were obtained from Certis USA LLC., USA. The bioproduct PFR-97 20% WDG consisted of blastospores from the naturally occurring strain Apopka 97 of the fungus *I. fumosorosea.* SoilGard® on the other hand was composed of blastospores from the naturally occurring strain GL-21 of *T. virens.* The pure cultures used for the experiments were created by re-isolating the organisms from the bioproducts.

2.4. Experiment 1: Fungicide runoffs in aquaponics

2.4.1. Operation of the aquaponics system

This experiment was conducted in October 2021 in the aquaponics hall of the Faculty of Fisheries and Protection of Waters (FFPW), University of South Bohemia. The aquaponics was decoupled, constituting independent RAS, mineralization unit, and recirculating raft hydroponics units. The RAS comprised four 630 L fish tanks, an overhead 2500 L biofilter, a drum filter (DVS, Netherlands) that periodically flushes into a 70 L cone vortex sludge collector, and a 750 L water retention tank. The outlets of the fish tanks and the vortex empty into a 2500 L underground aerated sludge separator. The fish tanks were stocked with tilapia, *Oreochromis niloticus* (185 g ± 23 g) at a stocking density of 40 kg m^{-3}. The fish were fed with Skretting TI-4.5 tilapia feed (floating pellets measuring 4.9 mm) twice a day. The feed composition included 30% crude protein, 6% lipid, 5.2% carbohydrate, and 6% ash. The fish were fed at 2.5 % body weight.

The wastewater in the sludge separator was transferred to a 2500 L mineralization tank via a submersible pump (DWO 150 400V). After approximately 40 days, the supernatant layer in the mineralized water was transferred via gravity to independent raft hydroponics units connected via a non-return valve. The hydroponics section comprised 21 units, six treatments (3 fungicides and 3 EPFs), and a control group, with three replicates. Each experimental setup consisted of a polyethylene grow box (60 cm × 40 cm × 28 cm) and a 26 L cylindrical sump bucket serving as a reservoir (Figure 1). An electric pump (6000 SOBO, 85W) was used to pump water from the sump to the grow box, and the water flowed back to the sump by gravity through an outlet in the grow box. Eight basil plants, with an average height of 30.1 ± 2.4 cm, were transferred to each grow box, maintaining a spacing of 15 cm between each plant.

Prior to the experiment, Genovese basil seedlings were planted in rockwool cubes and irrigated only with tap water (until both cotyledons of the seedlings had completely opened) and after that with fertilizer solution (1500 µS cm^{-1}, Yara®, Prague, Czech Republic). Once the roots were long enough (ca. 5 cm, an 8-leaf stage), the plants were transplanted to the

aquaponic system into a styrofoam floating raft. The nutrient scale of the mineralized water was determined using the standard operating procedures for water analysis in accordance with ČSN ISO 7150-1 (ISO, 1984). The missing or insufficient minerals/nutrients were supplemented with commercial fertilizer based on the Resh recommendations (Resh, 2022). The concentrations of nutrients in the mineralized water and their corresponding targeted values are presented in Table S1 of the supplementary material. Plants were left to acclimatize in the experimental units for one week before the experiment commenced.

2.4.2. Treatment preparation and foliar application

Prior to the experiment, both clove oil and lecithin were pre-tested on basil leaves at reported concentrations in the literature to observe their physical effects on the leaves. Clove oil was reportedly efficient against plant fungi at 0.25–5 % (v/v) (Walter et al., 1997; Sharma et al., 2017; Thabet Khalifa, 2018). However, when applied at doses ranging from 1 to 5 % (v/v), the compound burnt basil leaves. Hence, it was applied at 0.5 % in the experiment. Lecithin, on the other hand, which was reported to be efficient when applied at a rate ranging from 0.5 to 4 % (Hoa and Ducamp, 2008; Schirra et al., 2009; Bohinc et al., 2016), showed no observable physical effects at the maximum concentration, 4 % (v/v). Hence, it was applied at this concentration. Magnicur fungimat, however, is a commercial fungicide, therefore, it was applied at the recommended dosage of 60 ml L^{-1}. A volume of 300 mL of spraying solutions was prepared for each treatment, indicating an application of 100 mL of the solution to each experimental unit. The essential oils (clove oil and lecithin) were prepared by dissolving the products in water with two drops of Tween 20 (Sigma Aldrich, Czech Republic) solution to improve their solubility in water.

The suspensions of the fungal biocontrol agents (BCAs) were prepared using Folorunso et al. (2022) approach. The conidial concentrations in the suspension of *L. attenuatum, I. fumosorosea,* and *T. virens* were determined by a Neubauer hemocytometer (Bright-Line™, Sigma-Aldrich, Germany), and the suspensions were subsequently adjusted to 1.0 $x10^7$ conidia mL^{-1}.

The foliar application was conducted at 18:00 hr. In order to prevent cross-contamination between different treatments, measures were implemented by marking off each experimental unit using cardboard prior to the application of the foliar treatment. To investigate the runoff of the fungicides in water, water samples were taken at time points 0, 1, 3, 12, 24, 48, and 72 h post application. The water samples were taken by directly filtering the water into a 10 ml glass vial (Merci, Czech Republic) via a 0.45 µm syringe filter and syringe (Macherey-nagel, Germany). Also, 10 ml of the sprayed solution was collected to determine the percentage of runoff from the spraying solution. The samples were stored under -20 °C until GC-MS analysis. The plant growth was monitored for adverse effects of the treatments by measuring the heights of each plant weekly and the fresh weight of each experimental unit after four weeks. The water parameters were assessed on a weekly basis using a multimeter (HI9849, Hanna, Romania). Throughout the entire experimental duration, the levels of dissolved oxygen (DO), pH, and electrical conductivity (EC) were maintained above 5 mg L^{-1}, within the range of 5.5–6.5, and between 1000–1500 µS cm^{-1}, respectively.

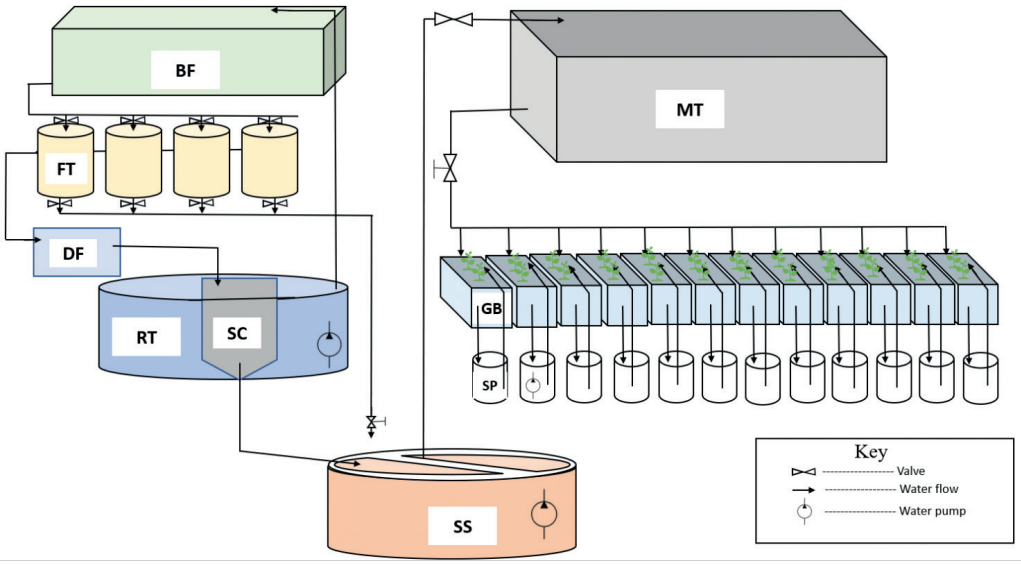

Figure 1. *A schematic design of DSAP (decoupled recirculating aquaponics systems) at the aquaponics hall of FFPW. "BF" indicates Biofilter, "MT" indicates Mineralization tank, "SC" indicates Sludge collector, "FT" indicates Fish tank, "RT" indicates Retention tank, "DF" indicates Drum filter, "SS" indicates Sludge separator, "GB" indicates Grow bed, and "SP" indicates sump. There was a pump in each of the sump buckets of the hydroponics unit.*

2.4.3. Determination of the fungicides in water

Extraction of lecithin, phosphatidylcholine (PC), from the water samples was carried out using the modified approach of Folch et al. (1957). 10 ml of the water sample and 50 ml of chloroform stabilized by ethanol (Sigma-Aldrich, Czech Republic) were added to a 150 ml separatory funnel. The solution was vortexed and placed in a shaker (IKA 8000500, Cole Parmer, Canada) for 30 minutes at 200 rpm. The solvent was evaporated to dryness in a Biobase nitrogen evaporator (Super 96-Hole, China) at 100 °C. A 500 µl of hexane (Sigma-Aldrich, Czech Republic) was added to the evaporated tubes, vortexed and stored in 1 ml glass vial. 250 µl of the stored sample was used for further analysis. Methylation of lipids was induced with boron trifluoride-methanol complex solution and NaOH as described by Appelqvist (1968). The FAME C 23:0 was used as an internal standard. FA composition was analysed by gas chromatography (GC) (Trace Ultra FID; Thermo Scientific, Milan, Italy) using a BPX-70 50m fused silica capillary column (id. 0.22 mm, 0.25 µm film thickness, SGE, USA). The temperature gradient starts at 70 °C and was held for 30 seconds. Then the temperature increased by 30 °C per minute until it reached 150 °C. After that, the temperature increased to 220 °C at a rate of 1.5 °C per minute and was held for 11 minutes. The whole analysis was completed in 60 minutes. The temperature of the PVT injector was 170 °C and that of the detector was 260 °C. The peaks were identified, and quantification was achieved in Thermo Xcalibur 3.0.63 (Thermo Fisher Scientific Inc.) by comparing sample retention times and peak areas to retention times and peak area in 7 levels (1000–15 $\mu g ml^{-1}$) of the standard mixture Supleco 37 Component FAME mix (Sigma-Aldrich).

To measure the amount in the water samples of the clove oil treatment, samples were first extracted using chloroform: dichloromethane 50:50 (v:v). 2 ml of the sample and 2 ml of chloroform: dichloromethane were added into a 20 ml glass test tube. The solution was then

vortexed for 30 seconds. The lower layer was collected into a clean glass tube. The samples underwent analysis using a GC MS/MS system, which consisted of the following components: a TriPlus autosampler, a Trace GC Ultra gas chromatograph equipped with a TG-5MS fused silica capillary column (30 m × 0.25 mm × 0.25 µm), and a mass spectrometer TSQ Quantum XLS (Thermo Fischer Scientific, USA). Helium was used as a carrier gas at a flow rate of 1.0 ml/min. A volume of 1 µL of the sample was injected into the SSL injector in the spitless mode, set at a temperature of 280 °C. The oven temperature was programmed in a way that it initially started at 40 °C and was maintained for 5 minutes, then increased to 150 °C at a rate of 3 °C/min and held for 0.5 minutes, followed by an increase to 250 °C at a rate of 10 °C/min, further increased to 290 °C at a rate of 25 °C, and finally maintained at 290 °C for 10 minutes. The temperature of the transfer line was held at 250 °C, while the ion source operated at 200 °C. The Total Ion Chromatogram (TIC) mode was performed on Q1 at an ionization energy of 70 eV and a mass range of 50–450 mz^{-1}. To prevent detector congestion, scanning was initiated 6 minutes after injection. The data were processed using Thermo Xcalibur 3.0.63 software (Thermo Fisher). Component identification was conducted by comparing it with the NIST Mass Spectral Search Program library v 2.0 f (Thermo Fisher). Quantification was achieved through the Q3 SIM mode, which focused on the fragmentation ions of the target compounds, as well as an external calibration curve. Thujone served as both an internal and external standard.

In the tebuconazole sample preparation, 2.5 ml of water samples were added into a 50 ml teflon centrifuge tube, and then 12.5 ml dichloromethane was added. The tube was tightly capped and vigorously mixed for 15 seconds using a vortex mixer. It was then placed in an ultrasonic bath for 10 minutes. 1.5 g of anhydrous sodium chloride and 6 g sodium sulphate were added to the tube and immediately mixed in a vortex mixer for another 15 seconds. The tube was further placed in an ultrasonic bath for 5 minutes. The tube was centrifuged for 5 min at 4000 rpm at room temperature using Megafuge (Thermo) centrifuge. The organic layer was transferred into a clean glass tube using a Pasteur pipette. The extracts were evaporated to dryness using a Biobase nitrogen evaporator. 1 ml of acetone was then added to the empty evaporated tubes and vortexed vigorously for 10 seconds. 1 µL of the solution was used for GC-MS/MS analysis, while the rest was stored at -20 °C C/-80 °C. The settings and configuration of GC – MS/MS were the same as for clove oil. Calibration curve was created using commercial tebuconazole (Magnicur fungimat) used in this study as external standard.

2.6. Experiment iii: Semi-acute-toxicity trial

To investigate the effects of the runoffs on fish, Nile tilapia were exposed to degrading concentrations of the fungicides (according to the result from experiment 1) for 14 days, using a semi-static toxicity procedure (OECD, 2000). One hundred eighty individuals of *O. niloticus* with an average body mass of 168 ± 14 g (mean ± SD) were obtained from the RAS systems of the aquaponics hall of FFPW. The fish were relocated to the Laboratory of Aquatic Toxicology and Ichthyopathology (part of the Research Institute of Fish Culture and Hydrobiology at the University of South Bohemia in České Budějovice). The toxicity trial took place from January 17 to February 28, 2023, within this facility. The fish were randomly selected and stocked at a density of 15 fish per aquarium (65 x 45 x 40 cm). There were three treatments (three fungicides) and one control with three replicates. The fish were acclimatized for ten days and fed daily with Skretting TI-4.5 tilapia feed (4.9 mm floating pellets; 30 % crude protein, 6 % lipid, 5.2 % carbohydrate, and 6 % ash; Skretting, Czech Republic) at the rate of 2 % body weight. Each aquarium had two air stones to aerate the systems constantly, and 70 % of the water was exchanged daily to neutralize the ammonia build-up in the system. Temperature: 26 ± 1 °C, pH: 7.8 ± 0.5, oxygen saturation: 90–99 %; and total ammonium 0.02 mg L^{-1} were regulated daily.

On day 11, the maximum concentrations of the fungicide, eugenol (0.0125 mg L^{-1}) and tebuconazole (0.39 mg L^{-1}), were applied into the aquarium to mark the beginning of the exposure phase. For tebuconazole, the LoD value, 0.39 mg L^{-1}, was used because it was the consistently detected concentration in the samples. However, only 1 % (2.04 mg L^{-1}) of the maximum concentration of PC was applied. The rationale behind this stemmed from the result of the biofilter experiment where the compound substantially increased ammonium-nitrogen to 13 mg L^{-1}, which is higher than the reported LC_{50} for tilapia (2.46 mg L^{-1}) (Redner and Stickney, 1979). Prior to the semi-acute toxicity test, a pre-trial of the toxicity test was conducted to determine lecithin's acute toxicity on a smaller sample size of tilapia. Thirty tilapia (168 ± 14 g) were randomly selected and stocked at a density of 5 fish per tank (120 m^3). Water quality parameters, pH, dissolved oxygen, and temperature were measured and regulated as above, and the fish were acclimatized for 48 hours. Stock solutions of 100 % (204 mg L^{-1}), 50 % (102 mg L^{-1}), 25 % (51 mg L^{-1}), 10 % (20.4 mg L^{-1}) and 5 % (10.2 mg L^{-1}) of the detected lecithin concentrations were prepared and applied to the tanks corresponding to different concentrations. One tank was untreated to serve as a control group. After 24 hrs, 100 % mortality was recorded in the 100 %, 50 %, 25 % and 10 % groups. In the 5 % group, 60 % (3 fish) mortality was recorded, while no mortality was recorded in the control group. Despite establishing the toxicity levels of lecithin concentration in the pre-trial, we guessed it is crucial to know the potential mild effects of the compound on fish to account for a lower application rate. Hence, 1 % of the maximum detected concentration was used in a major trial along with other fungicides.

On days 12, 13, 14, and 15, equivalent concentrations detected at time points 24, 48, 72, and 96h were applied in the aquaria after exchanging 70 % of the water to mimic a life scenario. Since detected concentrations at 72 and 96 hr were stable, the fish were further exposed to the 96h concentrations on days 16 and 17 to make it a week. To imitate the repeated applications usually practiced in the field, this sequence was repeated for another week, starting with the maximum concentrations on day 18. Water samples were taken on days 11, 17, 18, and 24 to confirm the actual concentrations of the active ingredients.

2.6.1. Fish sampling

At the conclusion of the exposure period on day 14, three fish were randomly selected from each aquarium and anaesthetized using buffered ethyl 3-aminobenzoatemethanesulfonic acid (MS 222) (Sigma-Aldrich, Czech Republic). Blood samples were taken by a heparinized syringe and needle containing 5000 IU heparin sodium salt in 1 mL. Blood was drawn through insertion into the caudal vein. The collected blood samples were immediately stabilized using an aqueous solution of heparin sodium salt at a concentration of 0.01 mL L^{-1}. The second portion of the blood was subjected to biochemical analyses, which involved centrifugation in a microcentrifuge (MIKRO 185, Beverly, USA) at 1500 ×g for 10 minutes. The resulting blood plasma was then collected, transferred into tubes kept on ice, and stored at -80 °C until subsequent analysis.

The fish were carefully dissected, and samples of various tissues (gills, brain, kidney, and liver) were collected for the purpose of evaluating the concentrations of oxidative stress and antioxidative enzymes present in the fish tissues. These tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C until further processing. The frozen tissues were subsequently weighed and homogenized in a 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA at a ratio of 1:10 (weight/volume) using an Ultra Turrax homogenizer (Ika, Germany). The homogenized samples were divided into two portions – one for measuring TBARS and the other was subjected to centrifugation at 12000×g for 30 minutes at 4 °C, with

the resulting supernatant used to determine the antioxidant parameters (SOD, GPx, and GR). Prior to blood sampling, batch weight and individual weight of 5 randomly selected fish were taken per aquarium.

2.6.2. Hematological and biochemical analysis

The hematological parameters assessed in the sampled blood include; the count of red blood cells (RBC), packed cell volume (PCV), concentrations of hematocrit (Ht), white blood cells (WBC), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), the count of leukocytes, and leukogram analysis (lymphocytes (%), monocytes (%), neutrophil segments (%), neutrophil bands (%), and developmental phases – myeloid sequence (DPMS) (%)). The protocol described by Svobodova et al. (1991) was followed to determine these hematological parameters.

Concentrations of various components including total protein (TP), albumin (ALB), globulin (GLB), glucose (GLU), triglyceride (TG), phosphorous (P), magnesium (Mg), creatinine (CREA), lactate (LACT), and ammonia (NH_3) were analyzed. In addition, the activities of alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and creatinine kinase (CK) were also assessed. These analyses were carried out using the Catalys One blood analyzer from IDEXX Laboratories Inc., USA, following an established protocol described by Kolářová and Velíšek (2012).

2.6.3. The antioxidant and oxidative stress parameters

Four antioxidant and oxidative stress parameters were assessed using spectrophotometric methods in the sampled tissues of *O. niloticus*. These parameters include: (1) glutathione peroxidase (GPx; EC 1.11.1.9), (2) glutathione reductase (GR, EC 1.6.4.2), (3) superoxide dismutase (SOD), and (4) thiobarbituric acid reactive substances (TBARS). GPx was determined by measuring the rate of NADPH oxidation at 340 nm by the reaction with glutathione reductase (Carlberg and Mannervik, 1975). The specific activity of the enzymes was determined using established methods: (1) GPx activity was assessed by measuring the consumption of substrate or generation of product per minute, utilizing an extinction coefficient of 6.22 mM cm^{-1} (Lawrence and Burk, 1976). (2) GR activity, on the other hand, was determined by measuring the rate of NADPH oxidation at 340 nm (Carlberg and Mannervik, 1975). The activity of both GPx and GR enzymes was expressed in International Units (IU) per milligram of protein, with one unit defined as the quantity of enzyme that consumes 1 mol mL^{-1} of substrate or generates 1 mol mL^{-1} of product per minute. (3) The total SOD activity was detected by observing the autoxidation of pyrogallol, following the approach by Marklund and Marklund (1974). The SOD activity was measured at 420 nm and expressed as enzyme quantity per milligram of protein. (4) For the assessment of lipid peroxidation in the sampled tissues, the TBARS assay was employed, following the methodology described by Lushchak et al. (2005). The concentration of TBARS was calculated using an absorption value at 535 nm and a molar extinction coefficient of 156 mM cm^{-1}. The TBARS value was expressed as nanomoles of TBARS per gram of tissue protein. Protein levels were estimated spectrophotometrically using the Bradford method (1976). bovine serum albumin serving as the standard.

2.7. Statistical analysis

The data from each experiment were presented as means ± standard deviations (SD) of three replicates. Differences were determined by one-way analysis of variance, and significant results ($p < 0.05$) were compared using the Tukey post-hoc test. All the results were analyzed using the R statistical software (version 4.1.3) (R Core Team, 2016).

3. Result

3.1. The runoff of fungicides in aquaponics

The active ingredients of clove oil (eugenol), lecithin (PC), and tebuconazole (tebuconazole) were all detected in the water samples at different time points. Maximum concentrations of eugenol (0.0125 mg L^{-1}) and PC (204 mg L^{-1}) were detected in the early hours (1–3 hours) post-application (Figure 2). Tebuconazole, in contrast, was detected between detection limit (LoD) of 0.39 mg L^{-1} and quantifiable limit (LoQ) of 1 mg L^{-1} in all the timepoint samples, but the levels were unquantifiable. On the other hand, the maximum concentration of eugenol, 0.0125 mg L^{-1}, detected at 1 hr, degraded to 0.0123 mg L^{-1} at 96 hr, indicating a degradation rate of 1.6 % in 96 hours. PC, however, degraded to 132 mg L^{-1} at 96 hr, from 204 mg L^{-1} recorded in the first hour, indicating a degradation rate of 64.7 % in 96 hrs.

Regarding the runoff rate, the runoff of the active ingredients ranged between 0.3–2.3 %. Eugenol, constituting 4300 mg L^{-1} of the sprayed solution, and being detected in water at a maximum concentration of 0.0125 mg L^{-1}, dissipated at a percentage runoff of 0.3 % (Table 1). Similarly, tebuconazole showed a runoff rate of 0.3–0.8 % from the 125 mg L^{-1} sprayed solution. However, lecithin had the highest runoff rate of 2.3 % from the spraying solution of 8545 mg L^{-1}.

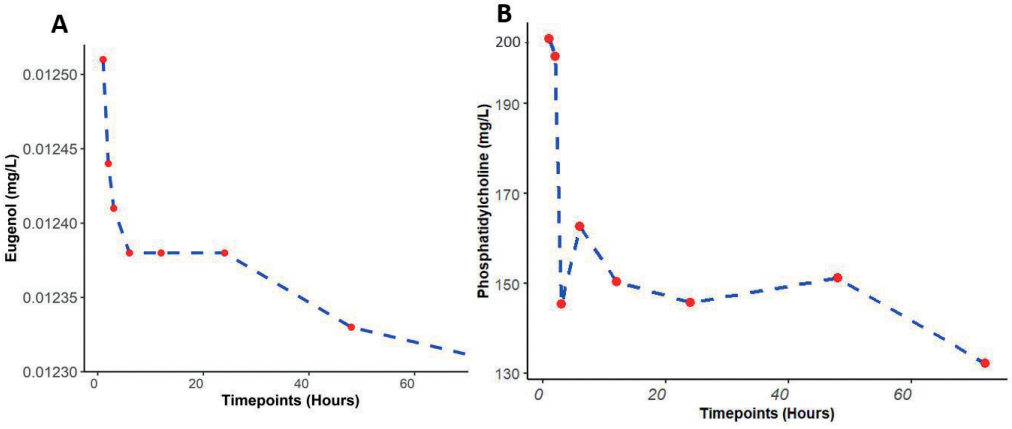

Figure 2. *Graphs showing the degradation pattern of eugenol (A) and Phosphatidylcholine (B), respectively, over 96 hours. Maximum concentrations of eugenol (0.0125 mg L^{-1}) and PC (204 mg L^{-1}) were detected in the early hours (1–3 hours) of post-application.*

Table 1. *The percentage runoff values of the fungicides, which were calculated based on the maximum concentrations of the insecticides found in the nutrient solution and the concentrations of the active ingredients (a.i) in the sprayed solution.*

Fungicides (a.i.)	a.i. in application solution (mg L^{-1})	Max. conc. detected (mg L^{-1})	Percentage runoff
Clove oil (Eugenol)	4.177	0.0125	0.3
Lecithin (PC)	8,545	204	2.3
Tebuconazole (Teb)	125	0.39–1	0.3–0.8

3.1.1. Effects of treatments on basil growth

Over the four weeks, the effects of the treatments (fungicides and biocontrol) on basil growth were measured. The essential oils and the synthetic fungicide showed no effect on the growth of basils (Figure 3). However, *T. virens*-treated basils were significantly higher than the other treatments and the EPF-treated groups (Figure 3B). However, there was no significant difference in the yield and the root (*data not shown*) within the fungicide and the EPF groups. From the transplanted mean height of 30.88 ± 4.66 cm, the average height of basils increased to 68.8 ± 5.98 cm in *T. virens*-treated basils, which was significantly higher than in *I. fumosorosea* (61.95 ± 2.14 cm), *L. attenuatum* (62.67 ± 3.16 cm) and the control group (61.09 ± 5.38 cm).

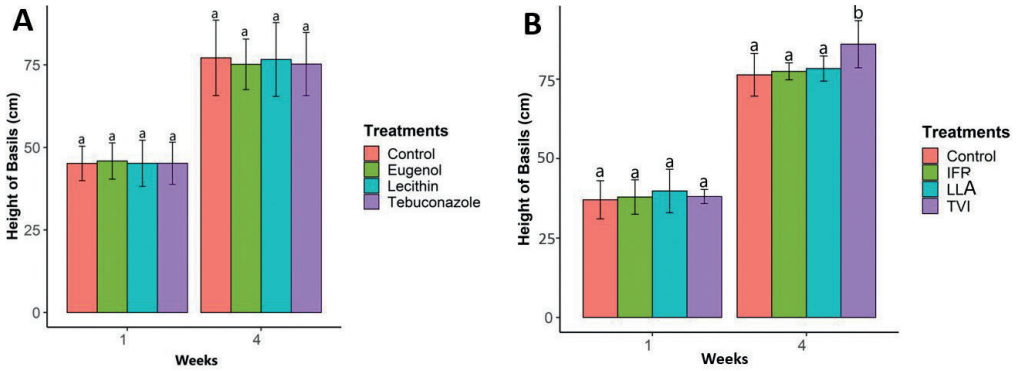

Figure 3. *Graphs A and B show the growth of basil treated with the fungicides (eugenol, lecithin, and tebuconazole) and EPFs (I. fumosorosea (IFR), L. attenuatum (LLA), and T. virens (TVI)) respectively in aquaponics for four weeks.*

3.2. Effects of fungicide runoffs on biofilter and nitrification

In the biofilter experiment, lecithin showed significant effects on nitrification in the biofilter. The ammonium level in the biofilter treated with PC spiked to 13 mg L^{-1} from the initially added 5 mg L^{-1} after 6 hours (Figure 3). However, this value dropped to 10 mg L^{-1} at the 12th hour and further to 4 mg L^{-1} after 24 hours. In addition, from 24 hours onward, the ammonium levels and metabolism improved in the PC treatment. In contrast, eugenol and PC-treated biofilters were non-significant from the control, with the NH_3-N levels dropping to zero after 24 hours in eugenol, tebuconazole, and control. Similarly, in the second nitrification phase, the mean NO_2-N in the PC treatment was highly significant compared to other treatments, reaching highest (27 mg L^{-1}) at the 18 hr time point before gradually decreasing back to zero at the 60 hr time point. In contrast, eugenol, PC, and tebuconazole-treated biofilters increased NO_2-N in 12 hr (~ 3 mg L^{-1}), which dropped to 0 mg L^{-1} at the 18 hr timepoint.

In the last phase of the nitrification, mean nitrate-nitrogen gradually increased in eugenol, tebuconazole, and control biofilter from the zero hours (38.6, 40.7, and 44.85 mg L^{-1}, respectively) to the 72 hr post application (Figure 4). After 72 hours of post application of the fungicides, peak nitrate level was recorded in control (76.8 mg L^{-1}), followed by eugenol (72 mg L^{-1}) and tebuconazole (68 mg L^{-1}). However, in the PC-treated biofilter, we recorded a highly significant difference in nitrate-nitrogen levels after the 0 hr timepoint, decreasing at other time points and reaching the lowest level (10 mg L^{-1}) at 72 hr.

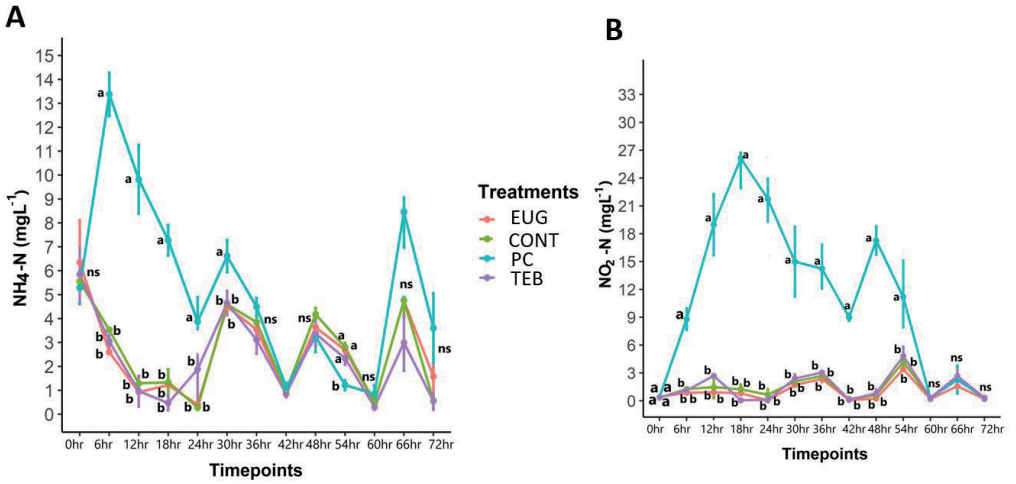

Figure 4. *The concentrations (mean ± SD) of ammonium nitrogen (A) and nitrite nitrogen (B) over 72 hours in biofilters treated with active ingredients, eugenol (EUG), PC, and tebuconazole (TEB). "ns" indicates "not significant".*

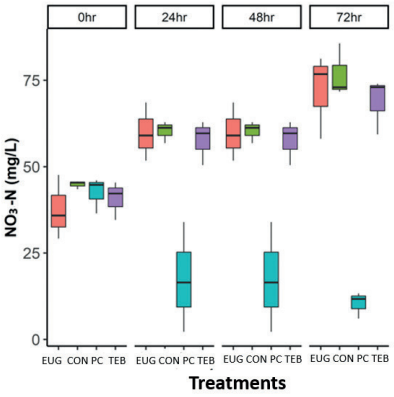

Figure 5. *The concentrations (mean ± SD) of nitrate nitrogen in biofilters treated with active ingredients, eugenol (EUG), PC, and tebuconazole (TEB) over 72 hours post-fungicide application.*

3.3. Effects of fungicide runoffs on tilapia hematology

In the present study, hematological variations in the *O. niloticus* were evaluated after exposure to a sub-lethal concentration of lecithin, clove oil, and tebuconazole for fourteen days and after depuration/recovery (fourteen days in the clean water). The effect of the fungicides on blood parameters, RBCs, WBCs, Hb, MCV, MCH, MCHC, and Leukogram of *O. niloticus* are

presented in Table. 2. A time-dependent significant decrease in Hb, MCH, and lymphocyte values was recorded in the blood of *O. niloticus* between 14 days of exposure and 14 days of recovery (Table 2). RBCs were significantly decreased after 14th-day exposure to lecithin, clove oil, and tebuconazole. RBC values, however, dropped in all the treatments after the recovery period, reaching non-significant values with the control group. MCH values were significantly higher than the control in the blood of *O. niloticus* exposed to PC and tebuconazole after the exposure on day 14. However, on the 28th-day sampling, the MCH values returned to normal and were non-significant from the control. Similarly, MCV values were insignificantly higher in fish blood treated with lecithin, clove oil, and tebuconazole compared to the control group after the exposure period. These values significantly dropped to control levels after recovery, except PC, which significantly differed from the treatments and control. Monocyte values were significantly higher than the control in *O. niloticus* treated tebuconazole after the recovery period. Also, lymphocyte values in tebuconazole-treated groups were significantly lower than the control and other treatments after the recovery period. In contrast, the values of both parameters in clove oil and lecithin-treated groups were non-significant with the control after exposure and recovery periods.

Table 2. *Alterations in blood serum of hematological parameters of O. niloticus exposed to runoff concentrations of lecithin, clove oil, and tebuconazole over a period of 28 days.*

Parameters	Days	Control	Eugenol	PC	Tebuconazole
PCV (L L^{-1})	14	0.31 ± 0.04^{a}	0.29 ± 0.03^{a}	0.29 ± 0.04^{a}	0.28±0.04^{a}
	28	0.29 ± 0.05^{a}	0.31 ± 0.03^{a}	0.29 ± 0.03^{a}	0.29 ± 0.03^{a}
Hb (g L^{-1})	14	98.47 ± 17.35^{a}	95.4 ± 18.94^{a}	95.17 ± 20.21^{a}	98.47 ± 16.77^{a}
	28	**97.35 ± 14.93^{a}**	**95.13 ± 6.26^{a}**	**91.83 ± 9.59^{b}**	**91.27 ± 12.07^{b}**
RBC (T L^{-1})	14	**2.2 ± 0.24^{a}**	**1.9 ± 0.34^{b}**	**2.0 ± 0.44^{ab}**	**1.8±0.29^{b}**
	28	2.08 ± 0.33^{a}	2.19 ± 0.24^{a}	2.21 ± 0.26^{a}	2.10 ± 0.31^{a}
WBC (G L^{-1})	14	9.1 ± 1.93^{a}	7.83 ± 2.55^{a}	9.39 ± 3.41^{a}	7.83 ± 4.31^{a}
	28	9.44 ± 2.21^{a}	8.56 ± 2.66^{a}	8.78 ± 2.35^{a}	8.33 ± 3.08^{a}
MCH (pg)	14	**44.95 ± 5.79^{b}**	**51.29 ± 4.48^{a}**	**47.49 ± 5.36^{ab}**	**49.44 ± 5.19^{ab}**
	28	41.46 ± 5.92^{a}	43.82 ± 4.81^{a}	41.92 ± 5.49^{a}	43.77 ± 4.6^{a}
MCV (fl)	14	140.24 ± 15.44^{a}	155.9 ± 16.3^{a}	152.8 ± 30.5^{a}	153.55 ± 12.47^{a}
	28	**142.57 ± 20.2^{a}**	**141.05 ± 14.6^{a}**	**134.75 ± 15.06^{b}**	**144 ± 22.22^{a}**
MCHC (g/l)	14	322.6 ± 43.05^{a}	331 ± 35.22^{a}	316.96 ± 41.96^{a}	322.69 ± 32.46^{a}
	28	291.33 ± 18.23^{a}	311.64 ± 27.64^{a}	310.93 ± 18.4^{a}	305.98 ± 22.62^{a}
Lymphocyte (%)	14	97.61 ± 1.37^{a}	97.47 ± 2.5^{a}	98.06 ± 1.00^{a}	96.28 ± 3.24^{a}
	28	**96.96 ± 1.83^{a}**	**95.71 ± 2.19^{a}**	**97.44 ± 1.48^{a}**	**92.93 ± 3.56^{b}**
Monocytes (%)	14	0.74 ± 0.64^{a}	1.88 ± 1.77^{a}	1.06 ± 0.59^{a}	1.71 ± 1.45^{a}
	28	**1.04 ± 1.02^{b}**	**1.02 ± 0.63^{b}**	**1.27 ± 0.72^{b}**	**2.22 ± 0.93^{a}**
Neutrophil segments (%)	14	0.47 ± 0.62^{a}	0.77 ± 0.25^{a}	0.72 ± 0.93^{a}	0.61 ± 0.5^{a}
	28	1.17 ± 0.82^{a}	1.2 ± 1.03^{a}	0.59 ± 0.81^{a}	1.11 ± 0.51^{a}
Neutrophil bands (%)	14	**0.61 ± 0.45^{a}**	**0.04 ± 0.13^{b}**	**0.12 ± 0.19^{b}**	**0.67 ± 0.63^{a}**
	28	**0.22 ± 0.24^{b}**	**1.2 ± 0.82^{a}**	**0.26 ± 0.47^{b}**	**0.71 ± 0.48^{ab}**
DPMS (%)	14	0.57 ± 0.73^{a}	0.44 ± 0.83^{a}	0.04 ± 0.13^{a}	0.72 ± 1.34^{a}
	28	**0.6 ± 0.84^{b}**	**0.86 ± 0.71^{b}**	**0.44 ± 0.43^{b}**	**3.02 ± 3.07^{a}**

The different superscript lowercase letters in the rows indicate the significant level. The abbreviated parameters are: the packed cell volume (PCV), haemoglobin (Hb), number of red blood cells (RBC), white blood cells (WBC), mean corpuscular haemoglobin (MCH), concentrations of heamatocrit (Ht), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), the number of leukocytes and leukogram (lymphocytes (%)), and Developmental phases – myeloid sequence (DPMS) (%)).

3.4. Effects of fungicide runoffs on tilapia's biochemical parameters

Regarding the plasma biochemical results, the LAC and LDH levels were significantly ($P < 0.05$) affected by eugenol, lecithin, and tebuconazole after exposure and recovery period (Figure 6A and G). The values of LAC in *O. niloticus* exposed to eugenol, lecithin, and tebuconazole significantly increased after exposure. These values significantly decreased further after the recovery period. GLU levels, which were non-significant in all the treatments and control after the exposure period, significantly decreased in groups exposed to lecithin and tebuconazole after the recovery period. On the other hand, TP, ALB, GLOB, and TAG values in the tilapia exposed to these chemicals were primarily non-significant to the control group after the exposure but became significantly higher than the control group after the recovery period (Figure 6E, C, D, and F). TP and ALB values significantly increased in *O. niloticus* exposed to lecithin (PC) and tebuconazole from their non-significant values after exposure to significantly higher values after recovery. Regarding the plasma transaminases (ALT and AST), we found no significant differences in serum ALT and AST levels among groups (Figure S1, supplementary material).

3.5. Effects of fungicide runoffs on tilapia's oxidative stress and antioxidant activities

The effects of the runoff concentrations of lecithin, clove oil, and tebuconazole were observed in the tissue of *O. niloticus* brain, gill, kidney, and liver. The tilapia group exposed to tebuconazole showed significantly increased GPx levels in the gill and liver after the 14 days exposure period (Figure 7A). In the gill, GPx values were non-significant with the control after the 14-day recovery period. In contrast, while the GPx levels in the liver of the control group remained significantly unchanged over the entire exposure and recovery period, the GPx levels in the *O. niloticus* exposed to clove oil, lecithin, and tebuconazole increased after the exposure and further after the recovery period. Also, the GPx values recorded in the kidney of the tebuconazole group significantly decreased after the exposure period but increased and became non-significant with the control after the recovery period.

However, no significant effect was recorded in the GR levels in the kidney and liver of the *O. niloticus* exposed to the runoff concentrations of lecithin, clove oil, and tebuconazole (Figure 6B). In contrast, the GR levels in the brain of the lecithin and tebuconazole-treated group significantly increased after the 14 days exposure period. While the GR levels in the brain of those exposed to clove oil became insignificant with the control after the recovery period, lecithin and tebuconazole-treated groups were significantly higher than the control. There was, however, no statistical significance in the SOD and TBAR values recorded in the brain, liver, gill, and kidney of the treatments and the control group (Figure S2, supplementary material).

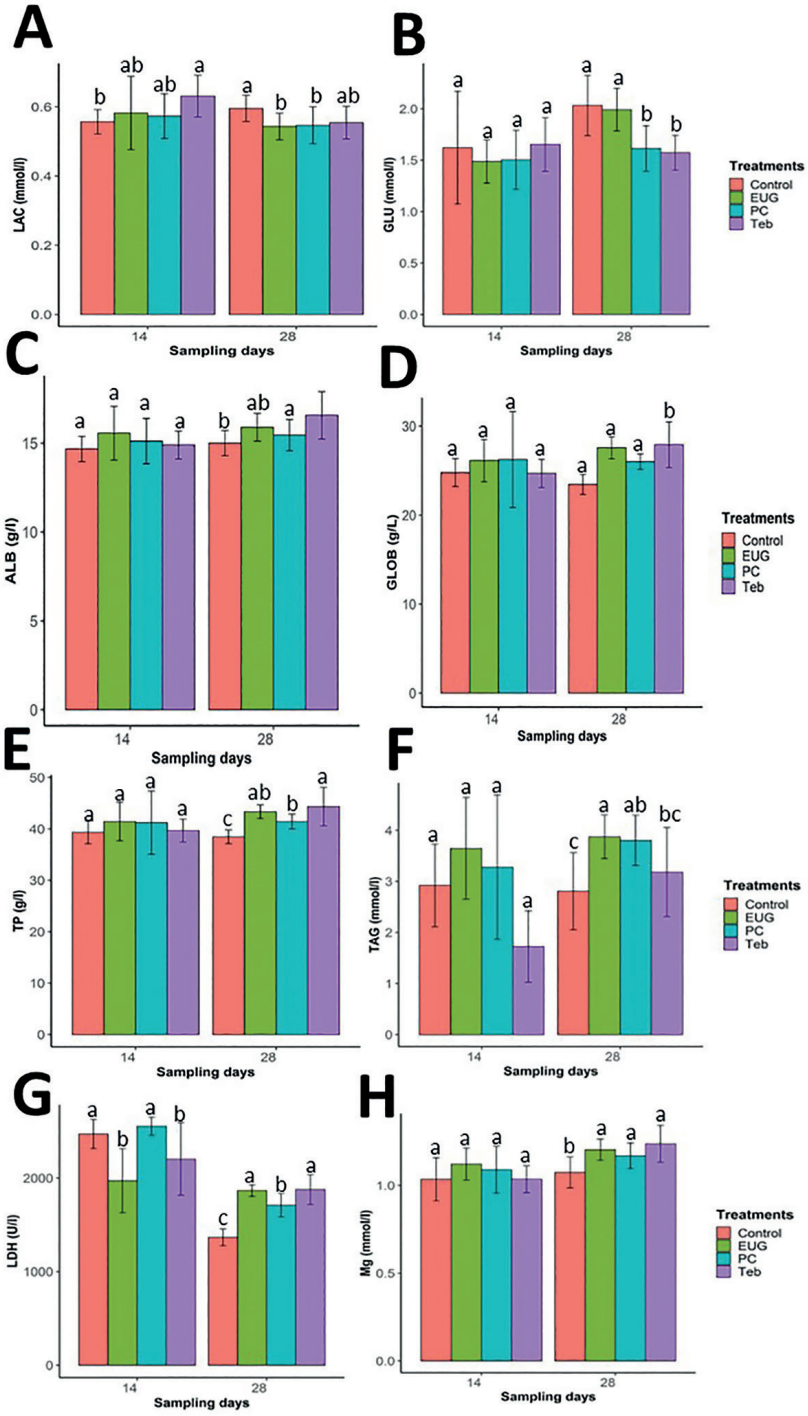

Figure 6. *Plasma biochemical parameter (LAC, GLU, ALB, GLOB, TP, TAG, LDH, and Mg) levels of O. niloticus after clove oil (EUG), lecithin (PC), and tebuconazole (Teb) exposure. Values are expressed as mean ± SD from triplicate groups. Bars with different letters indicate significant differences.*

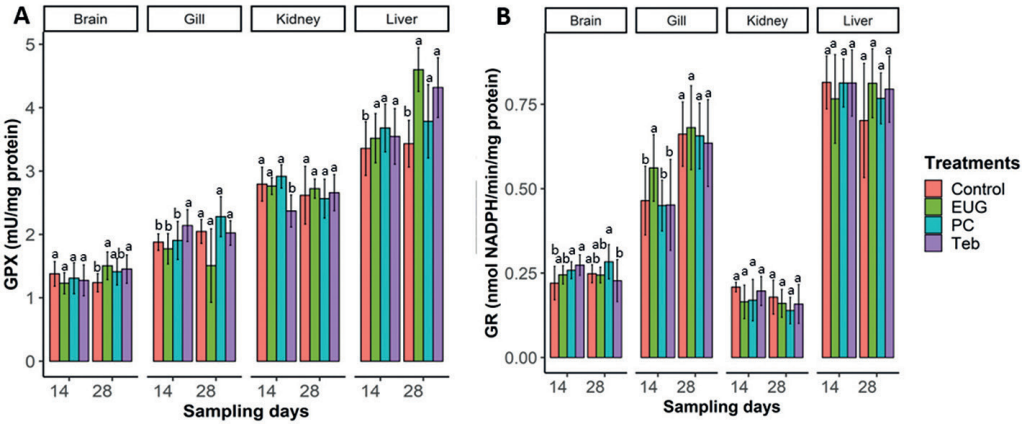

Figure 7. *Effects of the runoff concentrations of lecithin (PC), clove oil (EUG), and Tebuconazole (Teb) on levels of GPX (A) and GR (B) in Nile tilapia (O. niloticus) brain, gill, kidney, and liver tissues. Bars with different letters indicate significant differences.*

4. Discussion

As there are emerging novel approaches to control pests and diseases in aquaponics, it is essential to identify and assess their potential impacts on fish and beneficial bacteria inhabiting the same water loop. We examined the risks associated with the potential usage of biocontrol agents, natural and synthetic fungicides in aquaponics. Our results identified the potential effect of *T. virens* on basil growth in aquaponics. We investigated the runoff rates of natural and synthetic fungicides applied at recommended dosages, and the influence of these thresholds on fish and biofilters, examining their suitability for aquaponics.

4.1. Effects of entomopathogenic and mycoparasitic fungi on basil growth

In the current study, *T. virens* significantly improved the growth of basil over a period of four weeks in aquaponics. The positive influence of *T. virens* on the growth of plants has been severally reported in field studies. Thale Cress inoculated with *T. virens* in a petri dish for four days showed an increase in the shoot and root on day 5 (Contreras-Cornejo et al., 2009). In another study, the inoculation of *T. virens* into the seedlings of Scots pine resulted in significantly high seedling height and biomass (Halifu et al., 2019). In the current study, the influence of *T. virens* was only observed at the height of basil, and this could be because *T. virens* has more affinity for soil medium, where it strives better (Trushina et al., 2013). However, Yedidia et al. (2001) reported that *T. virens* improved the degradation and absorption of nutrients, such as P, Fe, Mn, Zn, Cu, and Na, in hydroponics, thus, promoting cucumber growth and yield.

4.2. Fungicide runoff and rate of degradation

Runoff rates of the selected fungicides showed a range of 0.3–2.3 %. The runoff rates cannot be compared due to the differences in the inclusion rates of their active ingredients and their recommended application doses. No study has examined the runoff rates of these fungicides in water or hydroponics; however, few studies have investigated their runoff rates in agricultural fields. Potter et al. (2014), in the United States, compared the runoff rates of tebuconazole

and metolachlor and recorded 9.8 % of the annual application dose of tebuconazole as runoffs in the soil after 24 hours. However, the study did not examine the degradation rate over a significant timeframe. In the current study, the recorded runoff rate was lower (0.3–0.8 %), but the compound was significantly persistent over 96 hrs. This result differs from similar studies carried out on soil medium. Potter et al. (2005) had earlier recorded a dissipation runoff rate of 5.5 ± 2.7 % in peanuts after four repeated applications. The lower runoff rate reported in the current study could be attributed to the 'one-off' foliar application compared to the repeated applications observed in the studies above. In addition, other factors, such as the cultivating medium and abiotic factors, such as temperature, persistence, oxygen levels, and soil moisture, could also affect the runoff rate (Bromilow et al., 1999).

The highest runoff concentration recorded in the nutrient solution was lecithin (PC). However, the active ingredients of lecithin dissipated faster than eugenol and tebuconazole in 96 hrs. This is because PC is water-soluble and easily hydrated (Li et al., 2018). While there are no studies on the degradation of lecithin in hydroponics or aquaponics, the hydrolytic degradation of PC into other molecular components depends on factors such as temperature, pH, and dissolved oxygen. In Subramanian et al. (2014), a heated lecithin solution was hydrolytically degraded into trimethylamine and further into dimethylamine under a model system of pH 5.6. As a natural product, lecithin does not have legislative restrictions for human/animal consumption or exposure, but its impact is unresearched.

Though the lowest runoff rate was recorded in clove oil treatment, the maximum concentration of its active ingredient, however, only dissipated 1.6 % over the entire 96 hrs. This could be associated with its weak water-solubility property (Baker et al., 2018). Moreover, eugenol does not readily hydrolyze in water but volatilizes over time through microbial degradation (Mohammadi et al., 2017). Hence, the absence or the limited amount of organic matter or sediments in the decoupled aquaponics could have partly contributed to its low degradation over the experimental period. In aquaculture, several studies have delved into examining the use of eugenol as fish anesthetics and the impact of the aftermath, but there is no study on their degradation and the effects of their derivatives in water (Ulanowska and Olas, 2021; Dable-Tupas et al., 2023). In the current study, eugenol's maximum concentration detected in aquaponics was far lower than its corresponding 24h LC_{50} for tilapia (16.98 mg L^{-1}) and NOEC (6.25 mg L^{-1}). Hence, it is not perceived to cause any havoc in aquaponics design at an application rate of 0.5 % (Charoendat et al., 2009; Gueretz et al., 2017).

4.3. Effects of fungicide runoff on biofilter's nitrification

Biofilter is an essential component of RAS, converting fish toxic waste (ammonia and nitrite) into a non-toxic waste (nitrate) in one or two-steps reactions enacted by nitrifying bacteria (nitrification) (Monsees et al., 2017). Hence, monitoring the influence of runoff concentrations of the selected fungicides on ammonia, nitrite, and nitrate levels in biofilters at different time points would give an insight into the risk of adopting these fungicides in aquaponics systems. In the current study, the maximum runoff concentrations of clove oil and tebuconazole were not found to significantly affect the nitrification activities in the biofilter over 96 hours of post-application. Since biofilter naturally constitutes autotrophic microbial load, microbial degradation of clove oil could have degraded the low runoff concentration, incapacitating the compound to cause a significant problem in the biofilter.

Lecithin, however, substantially increased NH_4^-N and NO_2^-N levels in the biofilter. Lecithin on hydrolysis yield choline and amino ethyl alcohol (MacArthur et al., 1917). These compounds are water-soluble, nitrogen-containing alcohols, and their dissociation and hydrolysis could be highly attributed to the observed increase of the initial 5 mg L^{-1} of NH_4^-N applied in the water.

In the current study, at 6 hr post-application, the mean NH_4^-N in lecithin treatment reached ~10 mg L^{-1}, significantly higher than the total ammoniacal nitrogen (TAN) recommended for warm water biofilters (Timmons et al., 2002). Simultaneously, the mean dissolved oxygen saturation in the biofilter gradually decreased to less than 20 % until 18 hr, indicating the lecithin compounds' degradation. We guess the increased ammonium levels (until 18 hr) affected the nitrification rate of the biofilters, deoptimizing the ammonium and nitrite oxidizing bacteria, and resulting in the further increase of NO_2^-N and significantly lower NO_3^-N. A similar result was reported by Ling and Chen (2005). The authors found that at the total ammonia nitrogen (TAN) concentration of 10 mg L^{-1}, an exponential decrease of nitrification with an increased nitrite level was observed in the floating bead filter, fluidized sand filter, and submerged bio-cube filter. This result is also in line with Puznava et al. (2001). In the current study, albeit the resurfaced nitrification activities observed in the lecithin treatment after 24 hr, lecithin's chemical capacity to significantly alter nitrification processes (even at a lower application rate of 0.1 %) make it highly unsuitable for coupled aquaponics systems.

4.4. Effects of fungicide runoffs on fish hematology

Water environmental disturbances can be a potential source of stress for fish, and this can be detected by measuring the changes in the plasma substrate concentrations or erythrocytes parameters such as cell volume and enzyme activities (Palawski and Knowles, 1986). Also, plasma-biochemical parameters and enzyme activity levels are known to reveal stress indicators in fish. RBC and Hb are responsible for the transport and excretion of nutrients, oxygen, body wastes, and carbonic acid gas; hence, it is used to measure anemia (Kim et al., 2004). In this paper, the decreased levels of RBC observed after exposure to PC and tebuconazole indicate that the compound might induce anemia in *O. niloticus* due to the decreased synthesis of erythrocytes in the bone marrow equivalents. After the recovery period, the RBC recovered and was non-significant with the control. Hb was, however, highly significant. This result is in line with Lutnicka et al. (2016). Authors found that *Cyprinus carpio* exposed to tebuconazole concentrations ranging from 1 to 2.5 mg L^{-1} for 14 days showed reduced erythrocytes and hemoglobin levels. Similarly, the authors also found that RBC recovered to the control value after the recovery period.

The lymphocyte and monocyte analysis also showed the persistence of tebuconazole effects on *O. niloticus*. Lymphocytes are responsible for secreting specific antibodies in response to antigenic stimuli (Kaattari, 1992). Therefore, the significant reduction observed in the lymphocyte percentage of *O. niloticus* exposed to tebuconazole indicates its long-term influence on the reduction of fish immunity. This result is in line with Lutnicka et al. (2016). *C. carpio* exposed to tebuconazole had significantly reduced lymphocyte level (86.6 %) compared with the control (94 %) after detox. However, Osman et al. (2019) reported an opposite observation with *Tilapia zilli*. Authors found that *T. zilli* exposed to 0.8–1.6 µg L^{-1} of Penconazole (a fungicide in the same category with tebuconazole) for three months showed a significantly increased lymphocyte (60.7–63 %) compared to the control (37.7–39 %). The discrepancy in the result could be associated with the exposure timeframe, chemical composition, or exposure conditions. In the current study, tebuconazole showed long-term damage and higher significant effects on the hematology of *O. niloticus*. On the other hand, except in RBC, where the mean value of clove oil-treated group was lower than the control after exposure, the clove oil was non-significant with control in the rest of the parameters, indicating its safety in aquaponics. Lecithin (PC), at 1 % of its maximum runoff concentration, returned to normal after the recovery period in most of the parameters, but its not recommended for coupled aquaponics.

4.5. Effects of fungicide runoffs on fish biochemical parameters

Due to the exposure to the fungicides, the intermediary or long-term metabolism of *O. niloticus* was altered as a physiological strategy displayed by fish to contain the toxicants. The increased levels of LAC observed in the tebuconazole-exposed group after recovery correlate with enhanced levels of glycemia required in fish exposed to stress (Heath, 1995). Similarly, increased LDH levels indicate an enhancement of anaerobic metabolism, a reaction to energy depletion caused by a lack of oxygen (Sancho et al., 2010). The fulcrum between balanced catabolism and anabolism found in fish is LDH. Hence, the raised LDH recorded in the PC and tebuconazole-treated group is a sign of the physiological response of the fish to neutralize the toxicological influence of the fungicides. This result is in line with Nur et al. (2017), Sancho et al. (2010), and Yeltekin et al. (2020). Zebrafish exposed to 230 µg L^{-1} of tebuconazole for 14 days showed increased levels of LAC and LDH after exposure and after the 14 days recovery period (Sancho et al., 2010). In this paper, the non-significant levels of these parameters in clove oil with control indicate that the compound are safer in this perspective.

Total protein, albumin, and globulin are protein molecules used to monitor fish disorder in the immune system, and they are also used to transport substances such as lipids, hormones, and inorganic ions (Narra et al., 2017). Since the levels of albumin and globulin had no significant changes after the exposure of all the chemicals in this study, it could imply that no haemodilution occurred in all the treated fish. However, increased albumin and globulin levels were recorded in tebuconazole-treated groups after recovery. On the other hand, several studies have reported reduced globulin and albumin levels in fish following recovery from pesticide exposures (Banace et al., 2008; Girón-Pérez et al., 2007). However, some globulins, such as lysozyme, immunoglobulin, and complement proteins, are immune-related and could increase or decrease under stress conditions (Ghelichpour et al., 2017). The biochemical parameters showed significant traces of stress only from tebuconazole and lecithin treatments.

4.6 Effects of fungicide runoffs on fish antioxidative activities

GPx and GR are modulators of brain function and signalling (Tabassum et al., 2016). Therefore, irregularities shown in their values may indicate that the activities of the fish nervous system were altered by the fungicide exposures (Ufer and Wang, 2011; Vieira et al., 2022). Tebuconazole-treated groups showed a slight increase of the GPx on the gill after exposure and the brain after recovery. This result differs from Yeltekin (2022) and Tabassum et al. (2016). Van fish exposed to 2.5 mg L^{-1} of tebuconazole for 96 hours showed increased levels of GPx after the exposure. Similarly, *Channa punctata* exposed to 0.5 ppm of propiconazole for 96 hr showed a significantly decreased in the GPx value (6.97) from that recorded in the gill of the untreated control (8.59). The differences could be associated with the concentrations and the exposure timeframe.

Conclusion

For the first time, we have examined the risks associated with adopting biocontrol and fungicides (natural and synthetic) in aquaponics. Our study identified that *T. virens,* besides its biocontrol property, can improve the growth of basil plants in aquaponics at a concentration of 1 x 10^7 spores per ml. The foliar application of clove oil (eugenol), lecithin, and tebuconazole at recommended dosages, spray-drifted, and were detected in aquaponics water at a percentage

runoff rate of 0. 3%, 2.3% and 0.3–0.8% respectively. In the biofilter, tebuconazole and clove oil at the maximum runoff concentration showed no significant effects on the nitrification processes during a 96 hr exposure period. In contrast, lecithin altered the ammonium and nitrite levels by substantially increasing ammonium-nitrogen levels from an initial 5 mg L^{-1} at the 1st hour to ~13 mg L^{-1} at the 6th-hour post application. These runoff concentrations were further evaluated on the physiology of Nile tilapia in a 28-day semi-acute toxicity test. The tebuconazole-treated group showed a significant effect on hematological (haemoglobin, red blood cell, MCH, etc.), biochemical (total protein, albumin, globulin, etc.), and antioxidative (glutathione peroxidase and glutathione reductase) parameters. Eugenol, on the other hand, showed no significant effects on the fish physiology, indicating its suitability for all aquaponics systems. Lecithin and tebuconazole, due to their effects on the biofilter and fish, respectively, their use should only be limited to decoupled aquaponics.

Funding

The study was financed by the Grant Agency of the University of South Bohemia (GAJU), No. 053/2021/Z, Ministry of Education, Youth and Sports of the Czech Republic (MSMT) under project CENAKVA (LM2023038).

Ethical approval

The fish trial experiment was carried out in compliance with the guidelines set by the Institutional Animal Care and Use Committee (IACUC) of the University of South Bohemia, adhering to the EU harmonized Animal Welfare Act of the Czech Republic. The experiment was also approved by the Departmental Expert Committee for Authorization of Experimental Projects of the Ministry of Education, Youth, and Sports of the Czech Republic (permit MSMT-6744/2018-4).

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CHAPTER 6

6. General discussion

Despite the apparent challenges surrounding the limited and safe options available to address pests and diseases in aquaponics, there has been little, or no study establishing safe control measures and approaches for evolving pests and disease outbreaks in aquaponics systems (Rivas et al., 2014). This has been severally reported to be a major threat to aquaponics success and its commercialization (Stouvenakers et al., 2019). Hence, the basis for establishing safe options for disease and pests in aquaponics was well justified. In paper 1, we exhaustively reviewed existing approaches in hydroponics and aquaculture and their associated risks for aquaponics. This was followed up by papers 2, 3, and 4 where we investigated safe and sustainable disease control options for pests and diseases in aquaponics. In addition, their associated risks for other aquaponics compartments were equally investigated.

6.1. Critical and step-wise adoption of IPDM in aquaponics

6.1.1. Prophylactic/Preventive measures

Paper 1 identified and assessed commonly used prophylactic or preventive measures established in IPDM in greenhouse and field operations. We found that, either passively or actively, preventive measures are taken as necessities against pest and disease outbreaks in indoor agriculture. They play a crucial role in integrated systems like aquaponics due to the economic advantage of disease and pest prevention compared to controlling outbreaks. We have classified these measures into two categories: safe prophylactic measures and risk-associated measures. Safe prophylactic measures refer to those that are considered to have minimal risks in various aquaponics designs. On the other hand, risk-associated measures are those that are perceived to carry observable risks in different aquaponics designs. We identified tools such as sanitation/disinfection mats, protective clothes, room sanitizers, maintenance of relative humidities, water filtration, barrier netting; and planting measures such as seasonal fallow period and use of disease-resistant plant cultivars as safe preventive measures usually observed in the IPDM schemes of hydroponics in greenhouses.

Though no study has identified the beneficial influence of these measures in aquaponics, there are existing studies on their influences on hydroponic systems. To control tipburns in lettuce, Vanhassel et al. (2015) used relative humidity ranging between 95 and 100 % to reduce tipburn by 3–50 % in hydroponics systems. Authors further reported that raising the relative humidity beyond 95 % also improved Ca^{+2} transportation to the leaf margins of the lettuce. In another study, to prevent the emergence of *Fusarium solani* in hydroponically-grown Eustoma (*Eustoma grandiflorum*), Onozaki et al. (2020) tested twenty-nine cultivars to assess their resistance to two isolates of the pathogen. One cultivar, 'Papillon Pink Flash' was reported to be highly resistant to both isolates with no disease symptoms in four tests. Also, fine sand, glass wool, rock wool, granulate, and polyurethane foam, and slow filtration techniques can be used to prevent the attack of pathogens, *Fusarium oxysporum* f.sp. *lycopersici, F. oxysporum* f.sp. *cyclaminis* and *Xanthomonas campestris* in hydroponics systems at the flow rate of 100–300 $Lm^{-2}h^{-1}$ (van Os et al., 2001). Though the studies above were not conducted in aquaponics setups, there are no perceived adverse effects of these practices on fish or nitrifying bacteria. Hence, these practices are adoptable for all aquaponics systems.

We identified measures such as established use of phytosanitaries (e.g. cyromazine, hypochlorites, chloramines, humic acid, and prochloraz) as risk-associated and could pose significant havoc in coupled aquaponics or limit the reuse of water in decoupled aquaponics.

Greenhouse sanitizers, Zerotol (rate/contact time; 5% / 10 min), SaniDate12.0 (200 ppm / 5 min), Virkon (1% / 10 min), KleenGrow (2% / 10min), and GreenShield (5% / 10 min) were used to disinfect nutrient solutions, rockwools and plants to inhibit the growth of *Listeria monocytogenes* and *Salmonella Typhimurium* in a nutrient solution of hydroponics (Moodispaw, 2022). The authors reported a significant influence of the sanitizers against the pathogens, but there was no reports of their potential effects on plant rhizosphere or water biofilm. On the other hand, sodium hypochlorite, chlorine dioxide, and copper-silver ionization have been associated with negative outcomes. These include elevated levels of Na^{+} and Cl^{-} in nutrient solutions, the production of harmful by-products such as trihalomethanes, the formation of chlorates, and an increase in Cu^{+2} levels in nutrient solutions. (van Os, 2009; Allende and Monaghan, 2015). These aftermath effects condemn their use as phytosanitaries in coupled aquaponics, but they can be adopted in decoupled aquaponics. Some alternative chemicals suitable for coupled-aquaponics include; decreasing the temperature of nutrient solutions (Albright et al., 2007), sand-based grow substrates to reduce bacteria transmission (McVicar and White, 1982), and constant vacuuming of tank bottoms (Shinn et al., 2009; Mori and Smith, 2019). In addition, media filtration techniques such as membrane filtration and soil-based filtration have been reported to increase the amount of dissolved nitrogen and phosphorus after filtration (Hatt et al., 2008; Mayhead et al., 2018; Reed et al., 2023). Membrane filtration enhanced the uptake of 94.2% of ammonium and 97.7% of ortho-phosphate to increase the productivity of *Chlorella vulgaris* (Mayhead et al., 2018). Similarly, the protection of plant seeds with minerals like silicon and salicylic acid, which are used to protect hydroponics plants against pathogens, cannot be considered harmful since they are usually only applied on seeds prior to seeding (Saikia et al., 2003; Schuerger and Hammer, 2003; He et al., 2015).

Conclusively, preventive measures that do not necessarily require direct application into the common water-loop are considered replicable across all aquaponic systems. On the other hand, prophylactic measures requiring direct application into the common nutrient solution or sprayed on leaf surfaces may pose risks to aquaponics sustainability (especially in coupled aquaponics) and must be used cautiously (Figure 1).

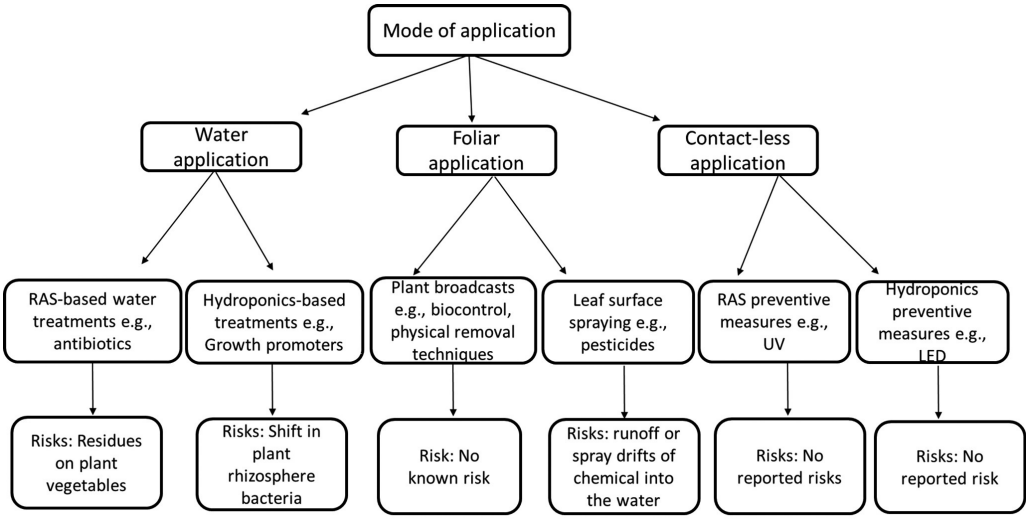

Figure 1. *A flow chart of pest and disease treatment flow from their mode of application to the possible risks in aquaponics systems.*

6.1.2. Revision and selection of control method

In the advent of pest or disease outbreaks, the plan or choice of a control approach a farmer would adopt depends on certain factors. While in hydroponics, farmers can 'swiftly' adopt cheaper and effective methods after considering the regulatory policies on the available methods, aquaponics farmers would have to further refer to the aquaponics design at hand and review the mode of application and level of interactions between the aquaponics units to ascertain the associated risks (Figure 1). The possible mode of application may include water application (direct input into the water), foliar application (applications of plant or leaf surfaces), and contact-less application. In addition, the selection of sustainable methods that would pose little or no negative effect on aquaponics would also have to be financially affordable regarding the scale of operation. Therefore, we reviewed and experimented with different IPDM methods commonly used in hydroponics in the order of cultural, biological, physical and mechanical, and chemical controls considering the components of different designs.

6.2.3. Cultural control methods

Cultural control methods are usually the measures taken before, during, and after planting seasons to avert diseases and pest infestations (Rodríguez-kábana and Canullo, 1992). In paper 1, we identified cultural measures such as insect screens, greenhouse climate control, fertilization, UV irradiations, light emitting diodes (LEDs), irrigation, ozone, removal of non-crop plants, crop rotation, composting, and pruning are common traditional planting practices adopted in greenhouse or field operations (Kruidhof and Elmer, 2020). Some practices like; constant removal of non-crop residues, composting, and pruning are equally not considered to cause havoc in any aquaponics design. We suggested using LED lights in hydroponics or UVs in RAS should be done cautiously. UV-LED lights are used in hydroponics to eliminate pathogens such as *Escherichia coli, Clavibacter michiganensis,* and *Fusarium oxysporum*; and in RAS systems to also eliminate pathogens in the water source or recirculating water, usually at an efficient dose ranging from 200–280 nm (Rurangwa and Verdegem, 2015; Kim et al., 2020). UV-LED lights use energy doses to inactivate pathogens in target water by damaging their DNAs to disrupt replication and damage to mRNA (Mori and Smith, 2019). Though there are fears that UV and LED lights installed between subsystems of coupled aquaponics may affect nitrifying bacteria in the biofilter, there are currently no studies that have proven such. In addition, nitrifying bacteria in biofilter are usually protected in a media (e.g. plastic elements) that shield them from UV energy doses.

However, this could be different in the rhizosphere (root zone) of independent hydroponics or aquaponics-connected hydroponics, where there are beneficial bacteria that support plant growth. In a study involving an investigation of the effects of beneficial bacteria, *Pseudomonas chlororaphis*, and UV-irradiation on the microbial diversity of windowfarm hydroponics, authors found that the diversity of bacteria and fungi in the water column was significantly decreased, and bacteria community structure was altered by UV irradiation (Lee et al., 2016). However, this effect was not found around the root areas. Similarly, Moriarty et al. (2018), which investigated the impact of UV treatment on a microbial load at an inlet and outlet of aquaponics systems, found that bacterial counts reduced by approximately 1.5 and 3.0 $\log_{10}$ CFU ml^{-1} on a 3-M Petri film and m-Endo agar, respectively. Though UV-LED lights are widely used to help reduce coliforms and potential pathogens in aquaponics, their influence on the rhizosphere of hydroponics plants is largely unknown. To limit its risks in coupled aquaponics, its use can be limited to treating incoming water sources prior to impounding in an aquaponics system.

6.3. Biological control

Biological control or biocontrol is the method of controlling pests or pathogens using their established natural enemies (other organisms) from the wild. In other words, biocontrol was defined as the use of living organisms to reduce the population of another organism (van Lenteren et al., 2018). In aquaponics, using IPDM perspective, biological control would mean using natural enemies to control or suppress fish or plant pathogens/pests below the economic threshold (a pest density/population that does not interfere with farmers' economic return). Several studies have suggested biological control methods as a safe and sustainable tool to address pests and diseases in aquaponics.

6.3.1. Biological control in hydroponics

In paper 1, we reviewed existing biocontrol agents in hydroponics and field agriculture to assess their direct or manipulative adaptation in aquaponics systems (Table 1). We found that common macro pests such as whiteflies, aphids, and spider mites (among others) have established commercial biocontrol agents that can be obtained globally. They are available in forms that can be hung or spread on plant shoots. Hence, they are not perceived to cause significant problems in aquaponics. We, however, found that the lack of reliability and low efficacy of biocontrol has been major problems.

Similarly, we found that commercial biocontrol agents for aquaponics pathogens are primarily inefficient, indicating a substantial reliance on chemical control methods. Their efficiencies are affected by complex factors such as; unstable environmental conditions in the microclimatic conditions of greenhouses, compatibility with other control methods, plant fertirrigation, quality of the product, mode of application, and mechanism of actions (Bardin and Pugliese, 2020). In addition, the needed time for the development of parasites or parasitoids (which varies among the biocontrol agents) could also cause a delay in their parasitic or prey activities, limiting their general efficacy at a given time. For example, *Aphidius colemani*, a common parasitic wasp for greenpeach aphids, takes about seven days (depending on the temperature) to hatch into eggs, after which they become adults and lay their eggs in the host. Therefore, these factors are limiting the efficiencies of the commercial BCAs, indicating the need to optimize their mechanism and biology.

To assess the use of biological control in aquaponics, several studies have investigated the use of indigenous microorganisms in aquaponics as biocontrol agents for plant and fish diseases (Figure 2). Rivas-García et al. (2020) reviewed the existing microbial communities in aquaponics to assess their potential to control or suppress aquaponics diseases. Using existing information in studies such as Eck et al. (2019, 2021a,b) and Schmautz et al. (2017, 2022), authors concluded that there is a lack of adequate information on the proof that aquaculture-based microbes can control plants diseases or improve plant growth (and vice versa). In addition, the lack of information on the exact location of a beneficial inoculum (e.g., fish tank, plant roots) in aquaponics has made using indigenous microorganisms as biocontrol agents largely irrelevant. Sirakov et al. (2016) investigated the potential of isolated microbes from different compartments of aquaponics to exert an inhibitory effect on both plant (*Pythium ultimum*) and fish pathogens (*Saprolegnia parasitica*). The authors obtained 924 isolates from different compartments of aquaponics and evaluated them for antagonism against the pathogens above. Fourty-two isolates from the entire isolates could inhibit both fungi in an *in vitro* trial. Although this study identified the new option of using biological control agents to control aquaponics diseases, but there was insufficient information on the proteomic classification of these microbes and their exact location in the aquaponics compartment. In

addition, as reported in many studies, in vitro outcomes are not usually directly transferable to the field, mainly due to varying microclimates in greenhouses (Folorunso et al., 2022). On the other hand, we could further investigate the potential of existing biocontrol agents by assessing their potential against aquaponic pests and diseases. The criteria for replicability of such biocontrol agents in aquaponics would have to focus on two primary objectives; the efficiency of such biocontrol, and their safety for fish and nitrifying bacteria (Folorunso et al., 2021).

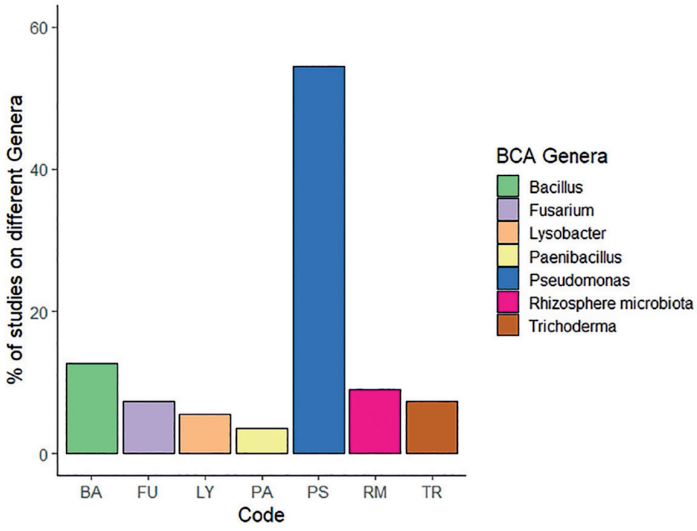

Figure 2. *Data (n=48) on successfully tested microbial biological agents in hydroponics (in vitro and in vivo) (1990–2020, references provided in supplementary bibliography). About 9% of the studies did not specify the genera but rather measured the efficiency of the entire rhizosphere microbiota at controlling diseases. "BCA" indicate "Biological control agents". (Source: Folorunso et al., 2021)*

Hence, in paper 2, we investigated the efficacy of entomopathogenic fungi (EPF), *Lecanicillium attenuatum* (LLA), *Isaria fumosorosea* (IFR), and mycoparasitic fungus, *Trichoderma virens* (TVI) against powdery mildew pathogen, *Podosphaera xanthii*. All the tested EPFs were efficient under high relative humidity conditions. Our findings showed that the three biocontrol agents significantly suppressed the powdery mildew at 10^7 CFU ml^{-1} concentration. Under greenhouse conditions (65–73% relative humidity (RH), a significant disease reduction percentage of 85% was recorded in *L. attenuatum*-pretreated leaves. IFR-treated leaves had the least AUDPC (area under disease progress curve) of ~434.2 and disease severity of 32% under 65–73% RH. In addition, *L. attenuatum* spores were the most persistent on the leaves; the spores population increased to 9.54×10^3 CFU mm^{-2} from the initial 7.3 CFU mm^{-2} under 65–73%. Though *T. virens* was the least efficient in this study, in paper 4, where in vivo assessment of the EPFs was investigated in basil grown in decoupled aquaponics. *T. virens* significantly improved the total height of basil plants over six weeks compared to the control and other EPFs. This result is in line with Hirano et al. (2008). The authors assessed the potential of *L. attenuatum* to protect the cucumber plant against the powdery mildew pathogen, *Sphaerotheca fuliginea.* The isolates inoculated in the plant roots reduced disease severity compared with non-inoculated plants and showed high colonizing ability on cucumber rhizoplane and inside root tissues. In order to assess their safety for fish and nitrifying bacteria, the EPFs were inoculated in an aquaponics water medium. Their

survival was impeded after 96 hrs, indicating low risks to fish and beneficial bacteria. The efficiency and survival experiment were conducted separately in different segments, which might not have fully captured a real-life scenario. Hence, there are further needs to investigate their effects on other parameters such as plant growth, rhizospheres, and aquaponics food quality.

Table 1. *Common commercial biopesticide-based bacteria approved for use in the EU and US. (according to Frederiks and Wesseler, 2019[1]; Matyjaszczyk, 2015[2]; Certis USA LIc[3].; Certis Europe, UK[4]; European commission, 2020[5]; Bayer Cropscience[6]; Valent Biosciences cop[7]; Bioworks, In., US[8]; University of Herfordshire[9]; Arista Life science[10]; Novozymes Biologicals, France[11]; Summit Agro, US[11]).*

Biopesticide brand*	Active ingredient	Strain	Spore concentration**	Formulation type***	Target pest	Company
			Diseases			
Serenade Max[1,2]	*Bacillus amyloliquefaciens* Subsp. *plantarum*	QST 713	1.31×10^{10} CFU/g	WP	*Podosphaera xanthii, Botrytis spp., Sclerotinia spp., Pseudomonas syringae, Xanthomonas* spp., *Corynespora cassiicola*	Bayer Crop Science, Global
Double Nickel 55[1,2,3]	*Bacillus amyloliquefaciens* Subsp. *plantarum*	D747	5×10^{10} CFU/g	WG	*Podosphaera xanthii, Peronosclerospora sorghi, Pythium* spp., *Botrytis* spp., *Rhizoctonia spp., Fusarium spp., Phytophthora spp.*	Certis USA LLC., US
Amylo-X[4]	*Bacillus amyloliquefaciens* Subsp. *plantarum*	D747	5×10^{10} CFU/g	WG	*Podosphaera xanthii, Botrytis spp., other vegetable diseases*	Certis Europe, UK
BioNem[5]	*Bacillus firmus*	GB-126		WP	plant-parasitic nematode	Bayer Crop Science, Global
LifeGard[2]	*Bacillus mycoides*	BmJ	3×10^{10} CFU/g	WG	*Podosphaera xanthii, Botrytis, Alternaria, Xanthomonas* spp., *Pseudomonas syringae*	Certis USA LLC., US
SONATA[5,6]	*Bacillus pumilus*	QST 2808	1×10^{9} CFU/L	Fermentation residues and water	*Podosphaera xanthii*	Bayer Crop Science, Global
XenTari[2,7]	*Bacillus thuringiensis Subsp. aizawai*	ABTS-1857		DF and WPG	*Spodoptera frugiperda*	Valent Biosciences, Global
OSTRINIL[9,10]	*Beauveria bassiana*	Cepa 147	5×10^{8} CFU/g	MG	*Ostrinia nubilalis*	Arista life science, France
CEASE[8]	*Bacillus subtilis*	QST 713	1×10^{9} CFU/g		*Botrytis cinerea*	Bio Works, Inc., US
Taegro[9,11]	*Bacillus amyloliquefaciens*	FZB24	1×10^{10} CFU/g	WP	*Peronosclerospora sorghi*	Syngenta and Novozymes Biologicals, Global
Aviv[12]	*Bacillus subtilis*	IAB/BS03	1×10^{7} CFU/ml	SC	*Botrytis, Sclerotinia* and *Podosphaera xanthii*	Summit Agro, USA
SUBTILEX[6,9]	*Bacillus amyloliquefaciens*	MBI 600	5.5×10^{10} CFU/g	WP	*Botrytis* spp.	Bayer Crop Science, Global
Proradix Plus[2]	*Pseudomonas* sp.	DSMZ 13134	$6,6 \times 10^{10}$ CFU/g		*Rhizoctonia* sp.	Sourcon Padena

**Spore concentration: CFU – colony forming units.
***Formulation type: WP- Wettable powder, WDG – Water-dispersible granule, SC – Suspension concentrate, WG – Granules, DF – Dry Flowables, MG – Microgranule.
[1] Frederiks and Wesseler, 2019
[2] Matyjaszczyk, 2015[2]
[3] Certis USA Llc (https://www.certisusa.com/).
[4] Certis Europe, UK (https://www.certiseurope.co.uk/).
[5] European commission (https://ec.europa.eu/).
[6] Bayer Crop Science (https://www.cropscience.bayer.com/).
[7] Valent Bioscience corporation (https://www.valentbiosciences.com/ /).
[8] Bioworks Inc. (https://www.bioworksinc.com//).
[9] University of Herdfordshire (https://sitem.herts.ac.uk/aeru/bpdb/Reports/57.htm).
[10] Arista Life science (http://www.arystalifescience.fr/#inline-auto152).
[11] Syngenta and Novozymes Biologicals, Global https://www.syngenta.com/protecting-crops/products-list).
[12] summit Agro, US.(https://summitagro-usa.com/products/aviv/).
(Source: Folorunso et al., 2021)

6.4. Physical and mechanical control

In paper 1, we identified physical control methods, such as water pressure equipment (e.g., jet-stream water to displace pests), to eliminate pathogens in nutrient solutions. High-pressurized jet streams remove or disperse target pests away from the plants by using a high pressurized jet stream' of water to minimize their infestation or kill them (Somerville et al., 2014). Though this practice is not perceived to aggravate any aquaponics design, the limited penetration of water jets deeper into the canopy to eradicate significant pest populations might be ineffective. Moreover, using high water volume for jetting might be cumbersome for the time taken and expensive in large-scale aquaponics (Sakthivel et al., 2011). Safe mechanical control methods we identified in paper 1 are trap cards, insect vacuums, fences, or electronic wires. None of these methods are perceived to cause a significant problem in aquaponics.

6.5. Chemical control methods

6.5.1. Chemical control in a plant context

The use of chemical control methods has been consistently frowned upon in aquaponics (Rakocy, 2012; Goddek et al., 2015; Stouvenakers et al., 2019; Folorunso et al., 2021). Moreover, the use of pesticides in the field is considered a 'last resort' because of their detrimental effects on the environment and the resistance of pests to their active ingredients. In paper 1, we, however, revealed that that there is still substantial reliance on chemical control due to several reports on the inconsistencies in the efficacy of commercial biocontrol agents. Moreover, there is currently no information on the specificity of the magnitude of damages different pesticides can cause regarding different aquaponics systems. In addition, there are established active ingredients that have minimal effects on the environment and are either non-toxic or less toxic to fish at high concentrations. For example, natural pesticides such as lecithin have no established LC_{50} for fish. Hence, the reasons above form the basis for investigating the possible use of safe pesticides for aquaponics.

In paper 1, which was a meta-analysis-based review, we assumed the toxicity levels of common pesticides by comparing different assumed percentage runoffs, 1 %, 10 %, and 20 % (using their recommended dose rate) with the established lethal concentrations (LC_{50}) and No Observe Effect Concentrations (NOEC) for aquatic organisms. Most synthetic pesticides would be toxic to fish and the beneficial bacteria at 10–20 % runoff concentrations, making them largely unsuitable for coupled aquaponics systems. On the other hand, natural pesticides were non-toxic at 20 % runoff. Though this could not only be used as a 'yardstick' to measure the safety of these pesticides in aquaponics, but the results gave us an overview of the magnitude of the expected effects of the investigated pesticides in aquaponics. However, there would be a need to identify safer options for 'last resort' IPDM practices and investigate their specific effects on nitrifying bacteria and aquaponics fish.

Therefore, in paper 3, we investigated the use of natural pesticides (azadirachtin and pyrethrum) and microbial pesticide (spinosad) in raft hydroponics systems to determine their runoff rate. The result revealed that only spinosad and azadirachtin were detected in water at a maximum concentration of 1.3 ng L^{-1} and 1.5 µg $L^{-1,}$ respectively. Since the maximum detected concentration for spinosad was less than the NOEC concentration for fish (1.15 mg L^{-1}), biofilter and fish were only exposed to azadirachtin (Cleveland et al., 2002). Artificial biofilter and fish were exposed to the detected maximum concentration and higher concentrations (7.5 µg L^{-1} and 15 µg L^{-1}) to account for multiple or repeated applications. Results showed mild effects in the haematology and biochemistry profile of Nile tilapia (*Oreochromis niloticus*) and higher levels of lipid peroxidation in the liver during the exposure. Conclusively, spinosad is safe for all aquaponics designs, while azadirachtin would have to be used cautiously, especially in coupled aquaponics systems. However, pyrethrum, which was not detected in water due to its low persistence, is highly toxic to fish and other aquatic life forms (Mauck et al., 1976); hence, its use should be prohibited in coupled aquaponics. These results are in line with Rašković et al. (2021). The authors investigated the impact of chlorpyrifos and two botanical insecticides (azadirachtin and pyrethrin) on a 250 L biofilter at a concentration of 0.7 µg L^{-1}, 7 µg $L^{-1,}$ and 20 µg $L^{-1,}$ respectively. Azadirachtin had adverse effects on the first step of nitrification (ammonia oxidation), as the concentration of ammonia was higher compared to other groups at all the sampled time points (0 h, 5 h, 13 h, 21 h, 29 h, 37 h, 45 h, and 53 h) post application.

In paper 4, we investigated the use of natural (clove oil and lecithin) and synthetic (tebuconazole) fungicides along with biocontrol agents, *L. attenuatum, I. fumosorosea,* and *T. virens.* We found that *T. virens* improved basil growth over a period of four weeks. In contrast, the natural (eugenol and lecithin) and the synthetic pesticide (tebuconazole) had no effect on basil growth, but all active ingredients were detected in the aquaponics water medium. Lecithin, eugenol, and tebuconazole were detected at a maximum concentration of 204 mg L^{-1}, 0. 3 %, and 0.3–0.8 % mg L^{-1} respectively. Furthermore, the influence of tebuconazole, eugenol, and lecithin on biofilters and fish was investigated. Lecithin sporadically increased ammonium and nitrite levels in the water while limiting the nitrate conversion phase, indicating a significant influence of the compound on nitrite-oxidizing bacteria (NOB). The effect of this compound on nitrification could be attributed to the dissociation of a nitrogen molecule present in the choline residue of the chemical structure of lecithin. The nitrogen molecule (ion) dissociated from lecithin and instantaneously increased the ammonium levels in the water, altering the optimal metabolism of ammonium and its further oxidation to nitrite. However, we could not deduce if this effect directly altered the efficiency of the nitrifying bacteria or if the recorded high ammonium level was due to the high ratio of NH_4 to ammonium oxidizing bacteria (AOB).

Similarly, the result from our semi-acute toxicity trial of the runoff concentrations on *O. niloticus* shows that lecithin and tebuconazole had significant effects on the fish after exposure and recovery period respectively. Tebuconazole, being more persistent in water, was

found to significantly alter haematological parameters (RBC and Hb), biochemical parameters (LAC, LDH, globulin, albumin), and antioxidative parameters (GPx and GR), making it unsuitable for coupled aquaponics. Lecithin, on the other hand, despite exposing the fish to less than 1% of the runoff concentration, also showed significant effects on the fish after exposure period. Eugenol in contrast were non-significant with the control making it suitable for all aquaponics systems.

These results have shown that the use of pesticides in aquaponics may not be completely 'shut out', as some candidates are either non-toxic or mildly detrimental to fish and biofilter systems. However, these studies did not investigate the residues of these compounds in plants. In addition, the common tradition of combining pesticides with biocontrol agents in field agriculture could also be explored to reduce the resistance of pests to pesticides while reducing their toxicity. Also, the influence of indigenous microbial consortia and the varying factor responsible for their diversity should be further explored to channel them into improving the health of aquaponics.

6.6. Conclusions

In reference to our assessment of the risks associated with the use of IPDM in aquaponics, we found that pyramidal-flow Integrated pest and disease management can be cautiously adopted in aquaponics, with further consideration of aquaponics design and its components.

We identified micro-pests or pathogens as a priority over macro-pests due to insufficient and inefficient commercial biocontrol agents, hence, reliance on chemical controls.

We found that entomopathogenic fungi, *Lecanicillium attenuatum* and *Isaria fumosorosea,* and mycoparasitic fungus, *Trichoderma virens* are safe biological control agents for all aquaponics designs.

We also found that *T. virens* significantly improved basil growth in aquaponics over a period of six weeks.

Our studies confirmed that foliar applications of pesticides in aquaponics can runoff or spray drift into nutrient solutions at a percentage ranging from 0.1–2.3%.

While spinosad pesticide is also safe for all aquaponics designs, use of azadirachtin should only be limited to decoupled aquaponics systems due to their negative effects on nitrification and mild effects on fish physiology.

We found that lecithin, which has been considered safe for food production systems, could create havoc in aquaponics due to its chemical ability to spike ammonia levels in water. Hence, its use should be prohibited in coupled aquaponics designs.

Eugenol, at 0.5% foliar application rate, had no adverse effects on nitrification activities in the biofilter (and fish); hence, its safe for use for pest and disease control in aquaponics.

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English summary

Integrated pest and disease management in aquaponics

This thesis has laid an essential foundation for developing aquaponics-safe approaches to address pest and disease outbreaks in aquaponics. Our review on adopting integrated pest and disease management in aquaponics not only customized pyramid-flow IPDMs for aquaponics but also inclusively initiated essential fish management pathways for aquaponics farmers who are primarily not fish farm experts. Furthermore, our review identified pathogen infestation challenges as priorities over macro-pests and diseases in fish culture units and the urgent need to establish safe phytopathogen management options. These conclusions were followed up by investigating safe biological control agents, where the efficacy of entomopathogenic and mycoparasitic fungi was investigated against powdery mildew, and their low-survival chance in RAS water was determined to affirm their suitability for different aquaponics designs (coupled and decoupled).

Amidst the doubts about the reliability of biological control, we explored potentially safe chemical control options that could be adopted for aquaponics systems, and investigated their possible negative impacts on aquaponics. The natural (azadirachtin, lecithin, and clove oi), microbial (spinosad) and synthetic (tebuconazole) pesticides investigated, runoff into nutrient solutions after foliar application, and were detected in significant concentrations at different time points post applications. Their percentage runoff in regard to spray solution varies significantly (0.1–2.3 %), owing to differences in the proportion of the active ingredients, recommended dosages and the properties of the compound. The percentage runoff of azadirachtin, eugenol, spinosad and tebuconazole ranged between 0.1 and 0.8 % of the sprayed active ingredients. On the other hand, 2.3 % of the sprayed lecithin were detected in the aquaponics water. Since eugenol and spinosad were detected at concentrations lower than their corresponding NOEC and LC_{50}, they are considered safe for all aquaponics systems. Pyrethrum, on the other hand, was not detected in the nutrient solution, which could be due to its non-persistence and fast degradation in water. However, its active ingredient (pyrethrin) is highly toxic to fish and other aquatic organisms, hence its usage should only be limited to decoupled aquaponics systems.

Regarding their effects on fish and biofilter, tebuconazole had a significantly persistent effects on fish hematology, biochemical and antioxidative activities over a 28-day semi-static period, indicating its unsuitability for coupled aquaponics designs. However, tebuconazole did not have significant effects on nitrification processes in the biofilter at the maximum runoff concentration. Lecithin, on the other hand, altered and spiked ammonium and nitrite levels in biofilter at its maximum runoff concentration, making the compound unsuitable for coupled aquaponics. In contrast, only mild non-significant effects of azadirachtin and eugenol were seen in biofilter nitrification, fish hematology, and biochemical parameters, indicating their low risks for all aquaponics systems (when applied according to the manufacturer's instruction).

Lastly, we explored the influence of biological control and fungicides on running aquaponics systems. *T. virens, I. fumosorosea* and *L. attenuatum,* controlled and suppressed powdery mildew over a period of four weeks. In addition, *T. virens* was able to improve the growth of the plant. The fungicides (clove oil, lecithin, and tebuconazole), on the other hand, did not show any influence on the basil growth.

Czech summary

Integrovaná ochrana proti škůdcům a chorobám v akvaponii

Tato práce přináší zásadní znalosti pro vývoj ověřených přístupů řešení problematiky škůdců a chorob v akvaponii. Naše hodnocení rizik spojených s využitím integrované ochrany proti škůdcům a chorobám (IPM) v akvaponii pomohlo nejen při kustomizaci pyramidového schématu IPM pro akvaponii, ale také zavedlo zásadní postupy IPM v chovných částech akvaponického systému napomáhajícího akvaponickým farmářům, kteří často nejsou odborníky v chovu ryb. Kromě toho náš literární přehled identifikoval zamoření rostlinnými patogeny jako prioritní výzvu ve srovnání s infestací bezobratlými škůdci a chorobami v chovných jednotkách ryb, a tudíž naléhavou potřebu zavést bezpečné přístupy managementu fytopatogenů. Na tyto závěry navazoval výzkum bezpečných agens biologické kontroly, kde byla zkoumána účinnost entomopatogenních a mykoparazitických hub proti padlí okurkovému a byla prokázána jejich nízká šance na přežití ve vodě v recirkulačních akvakulturních systémech (RAS), což potvrdilo jejich vhodnost pro různé designy akvaponie (jednosmyčkové i dvousmyčkové).

S nastalými otázkami o spolehlivosti biologické kontroly v akvaponii, jsme zkoumali potenciální možnosti chemické kontroly, které by mohly být využity v akvaponických systémech, přičemž jsme zkoumali jejich možné negativní dopady na akvaponický systém. Zkoumané přírodní (azadirachtin, lecitin a hřebíčkový olej), mikrobiální (spinosad) a syntetické (tebukonazol) pesticidy aplikované foliárně byly ve významných koncentracích detekovány v živném roztoku, a to v různých časových bodech po aplikaci. Jejich procentuální vyplavení v živném roztoku vztažené k aplikované dávce v postřiku se výrazně lišilo mezi fungicidy (0,1–2,3 %) v důsledku rozdílů v podílu účinných látek, doporučených dávkách a vlastnostech aktivní látky. Procentuální vyplavení azadirachtinu, eugenolu, spinosadu a tebukonazolu v živinovém roztoku se pohybovalo mezi 0,1–0,8 % aplikovaných účinných látek. Naproti tomu, lecitinu bylo v akvaponické vodě detekováno 2,3 % aplikované dávky. Protože eugenol a spinosad byly detekovány v koncentracích nižších než jejich odpovídající hodnoty NOEC a LC_{50}, jsou tyto látky považovány za bezpečné pro všechny akvaponické systémy. Pyrethrum v živném roztoku zjištěno nebylo, což mohlo být způsobeno jeho nízkou perzistencí a rychlou degradací ve vodě. Jeho účinná látka (pyrethrin) je však vysoce toxická pro ryby a další vodní organismy a její použití by mělo být omezeno pouze na dvousmyčkové akvaponické systémy.

Pokud jde o účinky těchto látek na ryby a biofiltr, měl tebukonazol významně přetrvávající účinky na hematologické ukazatele u ryb, ale také biochemické a antioxidační aktivity po 28denním semistatickém testu, což ukazuje na jeho nevhodnost pro všechny typy akvaponie. Tebukonazol však neměl významné účinky na nitrifikační procesy v biofiltru ani při maximální koncentraci naměřené v živném roztoku. Naproti tomu lecitin při maximální koncentraci zvýšil hladiny amoniaku a dusitanů v biofiltru, tzn. že tato látka není vhodná pro jednosmyčkové akvaponické systémy. Změny v nitrifikaci, hematologii ryb a biochemických parametrech po aplikaci azadirachtinu a eugenolu byly pouze nevýznamné, což ukazuje na jejich nízká rizika pro všechny typy akvaponie (při aplikaci podle pokynů výrobce).

Nakonec jsme zkoumali vliv biologické ochrany a fungicidů na běžící akvaponické systémy. Mikrobiální přípravky s *T. virens*, *I. fumosorosea* a *L. attenuatum* potlačovaly rozvoj padlí v průběhu čtyřtýdenní kultivace. Navíc aplikace *T. virens* zlepšila růst rostlin. Naproti tomu fungicidy (hřebíčkový olej, lecitin a tebukonazol) neovlivnily růst bazalky, ale byly detekovány v akvaponické vodě.

Acknowledgements

Foremost, I would like to express my sincere appreciation to my supervisor, Assoc. Prof. Jan Mráz, for the opportunity, unwavering supports, and constructive criticisms during the course of my study. I would like to extend this sincere gratitude to my consultant, Dr Radek Gebauer, for his enlightening guidance, insightful advice, and constructive criticisms, over the course of my study. I am also indebted to Dr. Bohatá Andrea, for her valuable transfer of knowledge, skills and scintillating discussions that grounded my understanding in the plant part of my research. It was a great honour and privilege to have worked under their supervisions.

I would like to thank all the staff of the Laboratory of Nutrition and other laboratories of the Faculty of Fisheries and Protection of Waters, who had contributed to successful completion of my experiments and lab works. In particular, my gratitude goes to Dr. Pavel Franta, Mr. Karel Procházka, Mrs. Zdeňka Machová, Dr. Aleš Tomčala, and Mr. Vít Náhlík for their unending and tremendous help on technical complications during my experiments, providing all the required helps on aquaponics hall and laboratory facilities for my experiments.

My warm appreciation also goes to my amiable past and present colleagues in persons of Mr. Felix Kofi Agbeko Kuebutornye, Dr. Ramin Bakht Shah, Dr. Koushik Roy, Mr. Mario Prečanica, Miss Lenka Kajgrová, Miss Hui Jia, Mr. Anil Axel Tellbüscher, and Dr. Petr Dvořák for their help during experiments and motivative discussions that lightens the heart during difficult moments. I would like to also appreciate Ms. Kačerová Lucie, and Ms. Tesařová Petra for their administrative and academic supports. I would like to also thank my friends, Abass Hammed, Lawal Kafayat, Laurine Mule, Dominika Sulakova, Salaudeen Ruqayat, Ojeyinka Akeem, Mebawondu Akindele, Olowe Olumide and Olayiwola lukman.

Last but not the least, I would like to pay my tremendous tribute to my mum, Alirat Ewumi, and my siblings, Abdul Fatai Ewumi, Muh. Jamiu Ewumi, Wasilat Zakariyah, Khadijat Adekoya, and Ibrahim Oluranti for being my support system and stronghold throughout the course of my study.

I also appreciate the financial supports from the following projects that funded parts of the research discussed in this dissertation:

- GAJU (053/2021/Z), Customization of integrated pest and disease management for aquaponics (2020–2021, PI: Ewumi Azeez Folorunso)
- CENAKVA VVI Research Infrastructure (ID LM2023038, Ministry of Education, Youth and Sports of the Czech Republic, 2019–2022)

List of publications

Peer-reviewed journals with IF

Gebauer, R., Brügmann, A., **Folorunso**, **E. A**., Goldhammer, T., Gebauer, T., Schöning, V., Bittmann, S., Knopf, K., Mráz, J., Kloas, W., 2023. Species and diet-specific aquaculture wastewater nutrient profile: Implications for aquaponics and development of sustainable aquaponics diet. Aquaculture, 739307. (IF 2022 = 5.135, AIS 2022 = 0.635)

Kuebutornye, F. K. A., Koushik, R., **Folorunso, E. A**., Mraz, J., 2023. Plant-based feed additives in *Cyprinus carpio* aquaculture. Reviews in Aquaculture, doi: 10.1111/raq.12840. (IF 2022 =10.400, AIS 2022 = 1.762)

Folorunso, E. A., Bohatá, A., Kavkova, M., Gebauer, R., Mraz, J., 2022. Potential use of entomopathogenic and mycoparasitic fungi against powdery mildew in aquaponics. Frontiers in Marine Science, 9, 992715. (IF 2021 = 5.247, AIS 2021 = 1.340)

Rašković, B., Gebauer, R., **Folorunso, E. A**., Božić, G., Velíšek, J., Dvořák, P., Bořík, A., Grabic, R., Mráz, J., 2022. Botanical and microbial insecticides application in aquaponics – Is there a risk for biofilter bacteria and fish? Frontiers in Marine Science, 9, 1055560. (IF 2021 = 5.247, AIS 2021 = 1.340)

Sarfo, I., Shuoben, B., Otchwemah, H. B., Darko, G., Kedjanyi, E. A. G., Oduro, C., **Folorunso, E. A**., Alriah, M. A. A., Amankwah, S. O. Y., Ndafira, G. C., 2022. Validating local drivers influencing land use cover change in Southwestern Ghana: A mixed-method approach. Environmental Earth Sciences, 81, 367. (IF 2021 = 3.119, AIS 2021 = 0.467)

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Folorunso, E. A., Rahman, M. A., Olowe, O. S., Sarfo, I., 2021. Influence of socio-economic factors and environmental hazards on technical efficiency of shrimp farms: A stochastic frontier production analysis. Aquaculture Research, 52, 3280–3290. (IF 2020 = 2.184, AIS = 0.324)

Folorunso, E. A., Rahman, M. A., Sarfo, I., Darko, G., Olowe, O. S., 2021. Catfish farming: A sustainability study at Eriwe fish farming village in southwest Nigeria. Aquaculture International, 29, 827–843. (IF 2020 = 2.953, AIS = 0.439)

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Abstracts and conference proceedings

Bamidele, N. A., Ikenweiwe, N. B., Alimi, A. A., **Ewumi,** A. O., Dairo, J., Akinnubi, I. A., Otubusin, S. O., 2015. Status of physical, chemical and biological activities of Isheri-Ogun River in relation to the surrounding anthropogenic activities. International Journal of Animal and Veterinary Sciences 9:6.

Ikenweiwe, N. B., Alimi, A. A., Bamidele, N. A., **Ewumi**, A. O., Fasina, K., Otubusin, S. O., 2015. Human activities damaging the ecosystem of Isheri Ogun River, South west, Nigeria. International Journal of Animal and Veterinary Sciences 9:6.

Folorunso, E., Roy, K., Bohatá, A., Gebauer, R., Mraz, J., 2021. Integrated pest and disease management in aquaponics. In: Book of abstracts. Aquaculture Europe 2021, October 12–15th, Funchal, pp. 425–426.

Training and supervision plan during study

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Basic of scientific communication	2020
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Applied hydrobiology	2020
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Czech language	2021

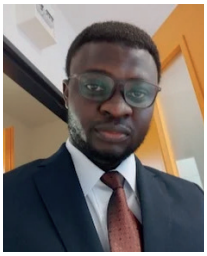
Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2020
Seminar days of RIFCH and FFPW	2021
Seminar days of RIFCH and FFPW	2022
University seminar/conference	2022

International conferences	**Year**
Folorunso, E., Roy, K., Bohatá, A., Gebauer, R., Mraz, J., 2021. Integrated pest and disease management in aquaponics. In: Book of abstracts. Aquaculture Europe 2021, October 12–15th, Funchal, pp. 425–426.	2021

Foreign stays during Ph.D. study	**Year**
Zurich University of Applied Sciences (90 days, practical work in a hydroponics climate chamber conducting experiments on the efficiency of different growth substrates).	2022

Pedagogical activities	**Year**
Leading of summer school project entitled proficiency of probiotics in aquaponics.	2022
Announcing the project entitled assessing the influence of cropping system on microbiota of aquaponics and hydroponics.	2023
Lecturing of interns on laboratory analysis of nitrogen species	2020
Training of interns on the daily routines of hydroponics and RAS operations	2021

Curriculum vitae



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