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POSTGRADUATE STUDIES PROGRAMME

Host and Microbe Interactions

"*In vitro* assessment of the toxicity of anthelmintics on ammonia-oxidizing archaea"

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Abstract

Anthelmintics play a crucial role in managing helminth infections in ruminants within the livestock industry. However, these substances undergo partial metabolism in gastrointestinal system of animals, leading to a significant release into the environment through excretions. Subsequently, they permeate various environmental compartments, with agricultural soils being the primary recipient. Once in the soil, anthelmintics interact with essential soil microbiota including ammonia-oxidizing microorganisms (AOM). Numerus studies indicate the sensitivity of AOM to xenobiotic substances with recent data highlighting the substantial impact of anthelmintics on the activity and abundance of soil ammonia-oxidizing bacteria (AOB) and archaea (AOA). This sensitivity underscores their role as indicators for assessing the impact of abiotic stresses on soil microbiota in environmental risk assessment schemes. The current thesis focuses on evaluating the toxicity of commonly used anthelmintics, specifically albendazole and its sulfoxide derivative ricobendazole, along with fenbendazole, ivermectin, and eprinomectin on the soil microbial community. Our main objective was to assess the adverse effects of these substances on two strains of AOA, namely *Ca.* Nitrosocosmicus frankladianus and *Ca.* Nitrosotalea sinensis. The impact of each compound on the activity of AOA was evaluated in liquid cultures over a range of concentrations by monitoring nitrite production. The stability of anthelmintics in the liquid cultures of AOA was assessed in parallel. Benzimidazoles-albendazole, ricobendazole, and fenbendazole- demonstrated no discernible effect on the activity of *Ca.* Nitrosocosmicus frankladianus. However, *Ca.* Nitrosotalea sinensis exhibited higher sensitivity to these compounds. Macrocyclic lactones- ivermectin and eprinomectin- significantly affected the activity of both AOA strains, with *Ca.* Nitrosocosmicus franklandianus showing lower sensitivity to ivermectin. Regarding the stability of anthelmintics in the liquid cultures of the AOA strains, no generalized pattern of drug degradation was observed. In conclusion, the findings of this study provide preliminary evidence for the toxicity of eprinomectin and ivermectin, towards *Ca.* Nitrosocosmicus franklandianus and *Ca.* Nitrosotalea sinensis. Further

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investigations are expected to explore other soil isolated AOA strains of the genus *Nitrososphaera*. To overcome identified issues associated with the bioavailability of anthelmintic drugs in the liquid cultures of AOA strains, future studies will delve into the solubility of selected compounds using the TWEEN 20 solvent, along with its potential toxicity in a wider range of nitrifying microorganisms (both bacteria and archaea).

Key words: anthelmintics, ammonia-oxidizing archaea (AOA), benzimidazoles,

macrocyclic lactones

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1. Introduction

1.1 Anthelmintics as environmental pollutants

Anthelmintics are drugs used to treat infections in animals, caused by endoparasites, known as helminths. These parasites include nematodes, trematodes, and cestodes. The primary purpose of anthelmintics is to kill or expel these worms from the body without posing a serious risk to the host (Beynon, 2012; Abongwa et al., 2017).

Anthelmintics can classified into several categories including: benzimidazoles, imidazothiazoles, tetrahydopyrimidines, macrocyclic lactones, amino-acetonitrile derivatives, spiroindoles and cyclic octadespipeprides (Abongwa et al., 2017).

Anthelmintics target metabolic pathways that are essential to the parasite but not to the host, making them selectively toxic to the parasites. This ensures that the medication effectively eliminates the parasites while minimizing harm to the host (Aiello & Moses, 2016). Many anthelmintics prevent parasites from using carbohydrates as an energy source. By disrupting the ability of parasites to utilize carbohydrates, their energy production is hindered, leading to a decrease in their activity. This approach specifically targets the enzymes of the parasites, ensuring minimal impact on the host's energy metabolism. Additionally, this strategy can help weaken the overall survival and reproductive capabilities of the parasites, ultimately aiding in their elimination from the host's system. One example of interfering with carbohydrate utilization is by targeting the enzymes involved in glycolysis, a crucial pathway for energy production in parasites. By selectively inhibiting these enzymes, the ability of parasites to generate energy is disrupted, leading to their decreased activity (Sheth, 1975).

These substances, after being administered to animals, can enter the environment through various pathways. They can be released either as the parent compound or as their byproducts (Horvat et al., 2012). One of the primary paths by which anthelmintics enter the environment is through direct excretion. Animal excretions, including urine and feces, can contain anthelmintics, as some of these compounds are not effectively absorbed by the gut (Boxall et al., 2004). In some cases, the application of manure or slurry from animals treated with anthelmintics to soil can lead to the spread of these drugs into the environment and to the contamination of surface water through runoff or leaching (Kools et al., 2008; Kolodziejska et al., 2013).

Following their discharge into the ecosystem, these compounds can reach different environmental niches and be distributed into soil, water, and biota. Under certain circumstances, these substances can persist in the environment for days and depending on the physicochemical properties of each compound, its degradability, and the characteristics of the receiving environment, they can endure for years (Boxall et al., 2004). It has been demonstrated that ivermectin may linger in the soil for extended periods of time and negatively impact soil fauna, especially the fungus *Fusarium oxysporum*, whose capacity to reproduce was hampered by presence of the drug (Beynon, 2012).

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The persistence of these molecules in the environment may have significant impacts on biota, including plants, animals, and microorganisms. Their accumulation in the food chain can lead to bioaccumulation and biomagnification, posing potential risks to the overall health of the ecosystem. Several studies have demonstrated the impact of anthelmintics on non-target microorganisms. Analyzed water sediments, in particular, have been shown to contain anthelminthic residues, especially macrocyclic lactones, which are toxic to a broad range of aquatic microorganisms (Beynon, 2012). In addition to affecting aquatic microorganisms, macrocyclic lactones have been shown to have negative effects on dung beetle populations (Mooney et al., 2021). It has also been found that doramectin, another anthelmintic, is highly toxic and inhibits the growth of the aquatic crustacean *Daphnia magna* (Kołodziejska et al., 2013).

These findings highlight the importance of considering the broader ecological impacts of anthelmintic use and implementing strategies to mitigate these risks. Therefore, it is crucial to study the occurrence and behavior of anthelmintics once they are released into the environment.

1.1.1 Macrocyclic Lactones

Macrocyclic lactones are chemical substances produced by the fermentation of soil Actinomycetota, specifically *Streptomyces avermitilis*. Since their introduction in 1980, they have exhibited antiparasitic action against both nematodes and arthropods at low dose levels (Merola & Eubig, 2012; Abongwa et al., 2017). There are two main categories of macrocyclic lactones: avermectins and milbemycins. The first category includes compounds such as ivermectin, eprinomectin, emamectin, selamectin, abamectin, and doramectin. Milbemycin oxime and moxidectin represent the second category of macrocyclic lactones (Romero-González et al., 2014; Aiello & Moses, 2016)

Avermectins consist of four complexes: A1, A2, B1, and B2. Each complex includes two homologues, represented by the letters "a" and "b." The numbers "1" and "2" indicate the presence of a double bond between C22 and C23 or the presence of hydrogen at C22 and a hydroxy group at C23, respectively. The designations "A" and "B" relate to the presence of methoxy or hydroxy groups at position C5 (Souza et al., 2022).

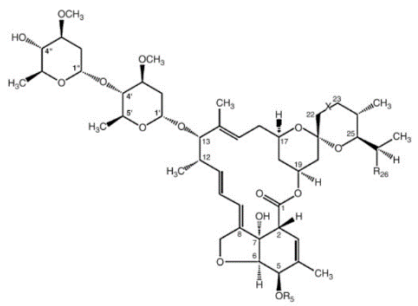

Figure 1*. Chemical structure of avermectins.*

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Both categories share the same antiparasitic mechanisms, targeting mutations in GluCls (Glutamate-gated Chloride Channel) receptor genes, mutations in GABA (4-aminobutyric acid) receptor genes, and decreased GluCl receptor gene expression (Riviere & Papich, 2018). Specifically, they bind to ligand-gated chloride channels, such as glutamate-gated chloride channels (GluCls), present in neurons and pharyngeal muscles of nematodes and arthropods (Merola & Eubig, 2012; Abongwa et al., 2017). This mechanism induces an influx of chloride ions into the parasitic neurons, leading to hyperpolarization, paralysis, and death. In addition to their effects on GluCls, avermectins also act as antagonists of 4-aminobutyric acid (GABA) and nicotinic receptors. By interfering with gamma-aminobutyric acid-mediated neurotransmission, avermectins further contribute to the paralysis and death of the parasite (Romero-González et al., 2014).

1.1.1.1 Ivermectin

Ivermectin, widely used as an anthelmintic agent (Riviere & Papich, 2018), is derived from the chemical alteration of avermectin. Initially released by Merck in the 1980s, ivermectin is the 22,23-dihydro derivative of avermectin B1. Specifically, it consists of at least 80% 22,23-dihydro-avermectin B1a and 20% 22,23-dihydro-avermectin B1b (Campbell et al., 1983; Crump, 2017; Souza & Guimarães, 2022).

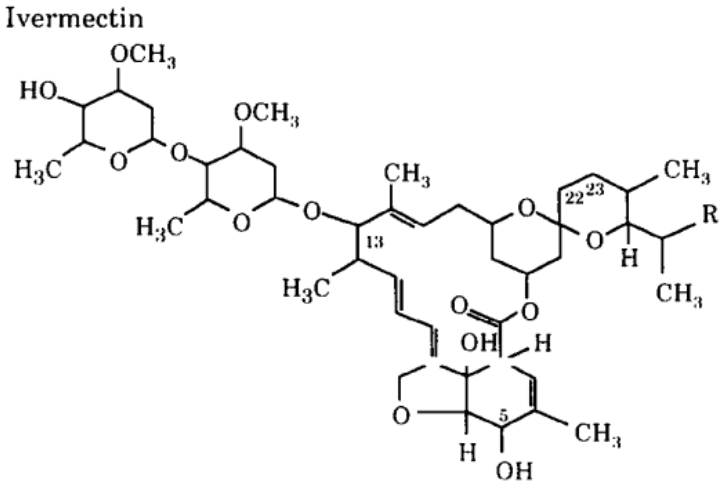

Figure 2*. Chemical structure of ivermectin*

Ivermectin has demonstrated efficacy against nemathelminths and Arthropoda, while its effectiveness against Platyhelminthes is uncertain. It has an extensive

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spectrum of antinematodal effects, specifically targeting many superfamilies of nematodes including *Trichostrongyloidea, Strongyloidea, Metastrongyloidea, Rhabditoidea, Ascaridoidea, Oxyuroidea, Spiruroidea, Filaroidea, and Trichuroidea* (Campbell et al., 1983).

Similar to other compounds in the avermectin category, ivermectin acts by influencing GluCl channels and GABA-activated channels. In a more precise manner, it functions as a positive allosteric modulator (PAM) of the GluCl ion channels. Ivermectin is situated within the outer membrane of the phospholipid bilayer, enabling the substance to exert its effects on the chloride channels (Martin et al., 2021). Furthermore, ivermectin also affects somatic muscle, facilitates the opening of non-GABA activated channels, and inhibits GABA-activated channels (Martin, 1997).

The sensitivity to ivermectin differs between the different homomeric and heteromeric GluCl channels. Notable variations in the impact of ivermectin have been documented among closely related species. This phenomenon can be attributed to the potential impact of minimal alterations in the amino acid sequence of any of the subunits, resulting in a modification of the sensitivity of the channel to ivermectin (Martin et al., 2021).

Ivermectin has also demonstrated an antiviral effect against many RNA viruses through its ability to interfere with the nuclear translocation of viral proteins. Consequently, it has been used in preventing several single-strain RNA viruses in humans (Chacca et al., 2022). Furthermore, it has been used in the field of human medicine against a range of parasitic infections and infestations, such as onchocerciasis, lymphatic filariasis, strongyloidiasis, and scabies (Crump, 2017). Additionally, it has been used against COVID-19. However, conflicting findings exist regarding the efficacy of these medications against COVID-19 (Chacca et al., 2022).

The widespread utilization of ivermectin in the livestock industry and its limited degradation through metabolism, resulting in a substance mostly unchanged in secretions (Jensen & Scott-Fordsmand, 2012), combined with the low volatility and the low water solubility of the substance, have led to major concerns regarding its environmental impact (Souza et al., 2022).

Ivermectin has a strong effect on aquatic organisms due to its capacity to cause oxidative damage, including cytotoxicity and genotoxicity, through direct DNA damage. Significant impacts on aquatic invertebrate species have been documented in relation to the administration of ivermectin. Studies have also demonstrated significant toxicity to the reproductive and growth rates of invertebrate species, even when exposed to lower concentrations. Amphipods, specifically *Gammarus pulex* and *Gammarus fossarum*, also have a considerable amount of sensitivity towards ivermectin (Mancini et al., 2020; Chacca et al., 2022).

Terrestrial species are also negatively affected by ivermectin. It can negatively affect invertebrate species and organisms that are responsible for the decomposition of manure, with dung beetles and fly larvae to be the most sensitive (Õmura, 2008; Chacca et al., 2022)

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In conclusion, while the utilization of ivermectin in livestock offers certain advantages, it is important to acknowledge that this substance raises significant environmental concerns and exhibits negative impacts on surface water, sediment, soil, and dung.

1.1.1.2 Eprinomectin

Eprinomectin, a semisynthetic avermectin compound, was developed by subsequent modifications of avermectin B1, two decades after the initial introduction of ivermectin into the veterinary industry. Eprinomectin is a hydrophobic 16-membered macrocyclic lactone, containing 90% of the B1a and 10% of the B1b component. More specifically eprinomectin contains 90% of 4′-epi-acetylamino-4″-deoxyavermectin B1a and 10% of 4′-epi-acetylamino-4″-deoxyavermectin B1b (Luis & Chávez, 2011; Riviere & Papich, 2018; Mao et al., 2019).

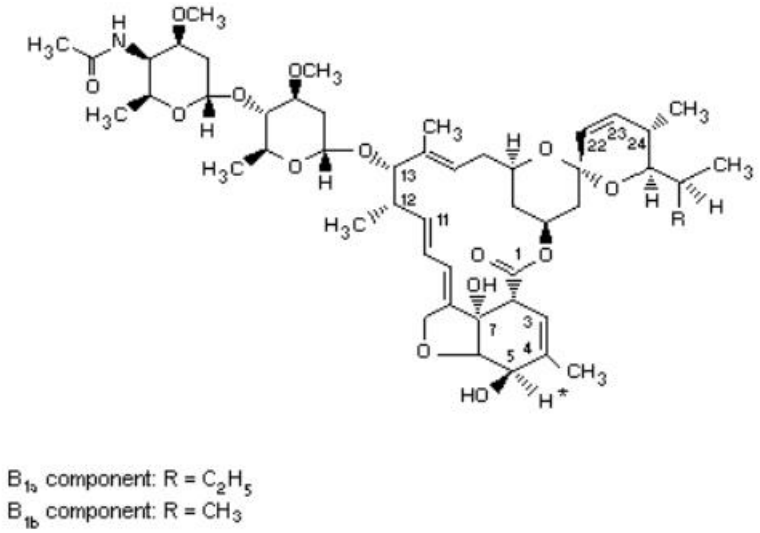

Figure 3. *Chemical structure of eprinomectin.*

Originally, eprinomectin was applied for the treatment of both endo- and ectoparasites in cattle, specifically targeting gastrointestinal nematodes, horn flies, and lice (Riviere & Papich, 2018; Mao et al., 2019). Studies have demonstrated its significant efficacy against various parasites, including *Haemonchus placei, Ostertagia ostertagi, Trichostrongylus axei, T. colubriformis, Cooperia oncophora, C. punctata, C. surnabada, Nematodirus helvetianus, Oesophagostom radiatum, Bunostomum phlebotomum, Tichuris* spp., and *Dictyocaulus viviparous* (Luis & Chávez, 2011).

Eprinomectin holds particular importance in the livestock industry due to its suitability for treating dairy cattle. Its properties, including extensive tissue distribution and extended plasma retention make it suitable for use during lactation when other

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anthelmintics like ivermectin, is contraindicated due to high transfer rates into the milk. Eprinomectin exhibits reduced distribution in milk, eliminating the need for a withdrawal milk period (Romero-González et al., 2014; Riviere & Papich, 2018).

Eprinomectin has limited solubility in animals, with only 10% undergoing metabolic processes within the organism leading to substantial accumulation in the soil (Souza et al., 2022). While considered less toxic than other avermectin-based anthelmintics like ivermectin, eprinomectin disperses significantly in soil, potentially contaminating non-target organisms (Litskas et al., 2013). A study on the *Allium cepa* assessed the phytotoxicity and genotoxicity of eprinomectin. Results indicated no significant impact on seed germination but the growth of the roots of the plant was diminished. The diminished rate of growth was ascribed to the potential impact of the chemical on the plant's mitochondria, as well as its influence on the cellular division process. Phytotoxic and genotoxic effects were observed affecting the growth of *Allium cepa* interacting with the DNA of the plant (Souza et al., 2022).

Research has highlighted the significance of soil composition, especially the presence of clay, organic matter, and cation exchange capacity, in relation to the impact of eprinomectin. In a separate study, eprinomectin exhibited mortality, diminished reproductive capabilities, and avoidance behaviors in *Folsomia candida*. Its toxicity was lower in soil with enhanced substance retention, associated with higher levels of clay, organic matter, and cation exchange capacity. This emphasizes the potential consequences of diminishing microorganisms in the soil, leading to an imbalance within the ecosystem. The potential for an extended lifespan of the substance or its metabolites in soil arises from the possibility of diminished detoxification and xenobiotic degradation processes, which can be attributed to the intricate interplay between the soil environment and microorganisms. The degradation of eprinomectin is restricted in the absence of microbial activity, potentially causing significant environmental consequences (Serafini et al., 2019).

1.1.2 Benzimidazoles

Benzimidazoles constitute an additional classification of anthelmintic drugs. The compound known as benzimidazole was initially identified within the molecular framework of vitamin B12 during the 1950s (Kamanna, 2019). Thiabendazole was the basic substance of the first anthelmintic in this category, which was introduced to the market a decade later, in 1961 (Deokate et al., 2014). A number of other compounds belonging to this classification were subsequently released as broad-spectrum anthelmintics. These include thiabendazole, cambendazole, albendazole, fenbendazole, mebendazole, oxfendazole, oxibendazole, and parbendazole (Baggot & Mckellar, 1994).

Benzimidazole is a heteroaromatic nitrogen-containing compound. It is also a bicyclic compound having an imidazole ring fused with benzene. Simultaneously, it

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exhibits amphoteric properties and has the capacity to function as either an acid or a base (Kumar et al., 2017; Veerasamy et al., 2021).

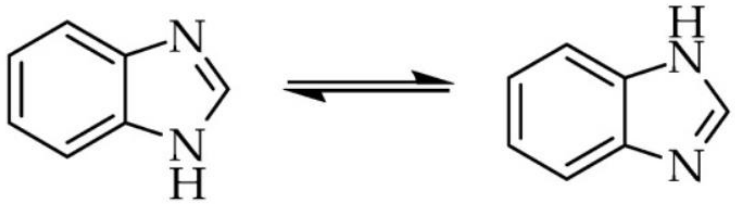

Figure 4. *Chemical structure of benzimidazole.*

Initially, benzimidazoles were used as fungicides for plants before being introduced into the field of animal health (Kamanna, 2019). The utilization of these anthelmintics, compared to previous ones, offered numerous benefits. These included a broader spectrum of activity and enhanced efficacy against the early developmental phases of parasites while ensuring the safety of the animal (Riviere & Papich, 2018). Benzimidazoles have been employed in the treatment of gastrointestinal nematodes in both cattle and domestic animals, including dogs and cats. Substances within this classification have demonstrated efficiency against nematodes, cestodes, and liver flukes. Notably, these substances have exhibited considerable effectiveness in combating the intestinal parasite *Ascaris*, the roundworm *Enterobius*, and *Trichuris* (McCracken & Lipkowitz, 1990; Riviere & Papich, 2018).

The application of benzimidazoles extends beyond veterinary use; they are also utilized in the agricultural industry to combat insects and fungi. Additionally, these substances have been used against protozoa, mycobacteria, and viruses. In the field of human healthcare, previous studies have demonstrated the potential utility of these compounds in the treatment of various conditions such as epilepsy, diabetes, cancer, inflammation, and as antioxidants (McCracken & Stillwell, 1991; Kopel et al., 2015; Kumar et al., 2017; Veerasamy et al., 2021).

The mode of action of benzimidazoles involves the modulation of nematodes' motility, nutritional absorption, and enzymatic activity (Deokate et al., 2014; Clare Vinaud & de Souza Lino Junior, 2017). The primary mechanism of action centers mostly on the impact on the cytoskeleton of nematodes, although reported cases also indicate an influence on glucose uptake and the inhibition of ATP levels through oxidative phosphorylation (Kumar et al., 2017). The disruption of nematode cell structure is attributed to the impact of benzimidazoles on the process of tubulin polymerization. Tubulin, a protein found in eukaryotic cells, plays a crucial role in the cytoskeleton. Benzimidazoles, disrupt the formation and polymerization of microtubules by targeting tubulin. Microtubules, composed of α-tubulin and β-tubulin, are responsible for the movement of chromosomes during cell division and provide structural support to the cell. Consequently, the disruption of microtubules leads to the loss of cytoskeletal integrity in parasites, ultimately resulting in their demise (Martin,

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1997; Deokate et al., 2014; Abongwa et al., 2017; Clare Vinaud & de Souza Lino Junior, 2017).

Due to the extensive utilization of benzimidazole in both veterinary and human health care, a significant quantity of these compounds is detected in the environment. Approximately 95% of the primary compound, can be excreted into the environment, either in its original form or as its metabolites. It has been observed that these metabolites can exhibit higher levels of activity compared to the original compound in certain instances. Simultaneously, the metabolism of mammals is extensive, leading to the presence of benzimidazole metabolites in various animal tissues, milk, and secretions. Due to their persistent nature, a significant proportion of these substances can persist in manure for extended periods ranging from several days to months. Therefore, the utilization of manure results in the dispersion of this chemical within the soil as well as other environmental compartments (Porto et al., 2019; Porto et al., 2021). Residues of benzimidazole have been detected in several aquatic environments, including surface and ocean waters, as well as in drinking water sources (Lagos et al., 2021).

Carbendazim, a fungicide classified as a member of the benzimidazole category, has been detected in many environmental compartments, including soil, water, and air. This substance exhibits toxicity against various species, including animals, as well as soil organisms such as earthworms, fungi, and azotobacteria (Zhou et al., 2023). A study conducted in the aquatic environment also demonstrated that there was evidence of toxicity in the invertebrate species *Daphnia magna*, with fenbendazole identified as the primary and most potent toxicant (Oh et al., 2006).

1.1.2.1 Albendazole and Ricobendazole

Albendazole is a broadly utilized benzimidazole derivative. Both the parent compound and its sulfoxide derivatives are frequently employed as anthelminthic agents in the field of livestock agriculture (Riviere & Papich, 2018). It is administered to sheep and cattle for the treatment of gastrointestinal nematodes, tapeworms, lungworms, and liver flukes. It possesses the capability to target the parasite at many developmental stages, including early stages, such as egg cells and larval stages, as well as later stages. Evidence has demonstrated its significant efficacy against *Taenia saginata*, *Dictyocaulus* spp., *Moniezia* spp., and *Fasciola hepatica*, as well as *Paragonimus kellicotti* in cats and *Filaroides hirthi* in dogs (Gyurik et al., 1981; Dayan, 2003; Vardanyan & Hruby, 2006; Puspitasari et al., 2016). Albendazole functions by binding to the tubulin of the parasite, resulting in cellular failure.

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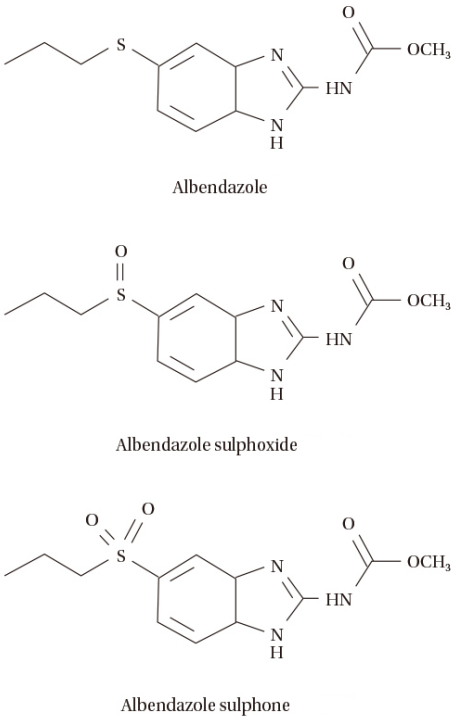

Figure 5. *Chemical structure of albendazole and its metabolites albendazole sulphoxide and albendazole sulphone.*

In 1982, albendazole received approval for use in human healthcare (Dayan, 2003) to combat infections caused by parasite species such as *Ascaris lumbricoides*, *Enterobius Vermicularis*, *Ancylostoma duodenale*, and *Necator americanus*. Additionally, it can be applied against the cestodes responsible for echinococcosis cysticercosis, as well as the nematodes responsible for filariasis (Horton, 2000; Vardanyan & Hruby, 2006).

Upon entering the body, albendazole undergoes sulfide oxidation, leading to the formation of albendazole sulfoxide, commonly known as ricobendazole (ABZ-SO). Subsequently, the sulfoxide derivatives undergo a second oxidative step, resulting in the formation of albendazole sulfones (ABZ-SO_2). This oxidation process forms a derivative that possesses anthelmintic properties similar to those of the parent compound. The effectiveness of albendazole is believed to be due to the presence of these metabolites (Horvat et al., 2012; Riviere & Papich, 2018). The oxidation of albendazole into its metabolites takes place in the liver and is facilitated by the protein family known as flavin-containing monooxygenase (FMO) and the cytochromes P450 (Dayan, 2003; Molina et al., 2007; Capece et al., 2009).

Albendazole and its metabolites are frequently detected as residues in the environment. Both albendazole and its metabolite ricobendazole, have been found in plants, rumen content, manure, and feces. The concentration of albendazole sulfoxide is consistently higher than that of the parent compound and its sulfone (Navrátilová et al., 2021). These compounds have been discovered to be hazardous to a wide range of soil and aquatic species. It has been determined that this substance is harmful to the earthworm *Eisenia fetida*, resulting in a decrease in its growth rate and enzymatic

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activity. Additionally, it produces dysfunctions and deformations in zebrafish and poses a threat to the crustacean *Daphnia magna*. Simultaneously, research has revealed that albendazole and its metabolites exhibit toxicity towards specific types of yeast *(Saccharomyces cerevisiae*), the gram-negative bacterium *Vibrio fischeri*, and microalga *Raphidocelis subcapita*. Studies conducted on two plants belonging to the *Fabaceae* family, specifically *Medicago sativa* and *Trifolium*, have revealed that these plants assimilated the compound, which was then detected in their leaves. Notably, albendazole sulfoxide was found to be present in higher proportions. The presence of albendazole in plant leaves, along with the abiotic stress it causes, raises concerns about its consumption by animals and its potential impact on the environment and the food chain (Belew et al., 2021; Belew et al., 2021; Belew et al., 2021; Navrátilová et al., 2022)

1.1.2.2 Fenbendazole

Fenbendazole is an additional medication that belongs within the class of benzimidazoles methylcarbamate. Fenbendazole and its derivative, oxfendazole, in combination with albendazole are among the most used pharmaceuticals within this classification (Riviere & Papich, 2018).

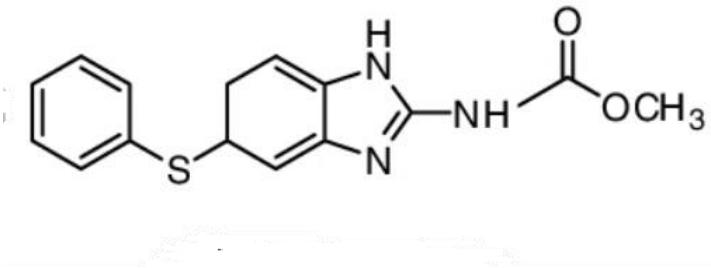

Figure 6. *Chemical structure of fenbendazole.*

Fenbendazole is administered orally and is used in the treatment of cattle, domestic animals, and humans. This substance can be employed to treat gastrointestinal parasites and lungworms in ruminants, horses, and pigs, as well as cats and dogs (Papich, 2015; Riviere & Papich, 2018). It is effective against various types of roundworms, whipworms, hookworms, tapeworms, and trematodes, including species of *Toxascaris, Ancylostoma, Filaroides*, and *Strongyloides*. Additionally, it has demonstrated efficacy against anaerobic flagellated protozoa such as *Giardia* spp. (Papich, 2015; Sykes, 2022). Simultaneously, current studies in the field of human therapeutics have investigated the potential of fenbendazole to influence human cancer cells and exhibit an anticancer effect (Dogra et al., 2018).

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Fenbendazole is primarily metabolized into oxfendazole in the liver. Furthermore, oxfendazole is converted into sulfone by oxidation. Sulfone is less potent than oxfendazole, which serves as the active compound and can be utilized as the primary component. The metabolism of fenbendazole, similar to albendazole, occurs through the action of FMO enzymes (Short et al., 1988; Riviere & Papich, 2018).

Febantel is a compound that undergoes metabolism to produce fenbendazole once it enters the body. Fenbendazole is a prodrug that is metabolized in the liver. It goes through a process of hydrolysis and removal of the methoxyacetyl group, followed by cyclization, resulting in the formation of fenbendazole. Additionally, it is used as a treatment for hookworms, whipworms, tapeworms, and strongyles (Papich, 2015; Riviere & Papich, 2018).

From an environmental point of view, studies on *Plantago lanceolate* and *Arabidopsis thaliana* have shown that the presence of these substances in these plants leads to oxidative stress, impacting plant development, metabolic activities, and photosynthesis. Elevated levels of proline, a crucial amino acid that aids plants in coping with stressors, suggest that the drug impeded the capacity of the plant to effectively respond to stress. Fenbendazole also affects the synthesis of antioxidant enzymes and secondary metabolites, such as polyphenols. (Syslová et al., 2019; Podlipná, 2022). Although plants have the ability to convert the substance into inactive metabolites, it can be transformed back into its active form, leading to the buildup of the substance in the tissues of vegetarian animals. Simultaneously, studies have shown that fenbendazole is a chemical that is resistant to degradation by microbiological agents and undergoes a very slow rate of breakdown in the environment (Wagil et al., 2015). The results obtained indicate that fenbendazole has the potential to be a pollutant that can negatively affect the environment.

1.2 Soil microbiota

Soil microorganisms constitute a highly intricate and dynamic ecosystem, playing a crucial role in maintaining soil health. These microorganisms are responsible for approximately 90% of soil processes (Islam et al., 2020; Jools et al., 2022). The bulk soil and the soil surrounding plants, referred to as the rhizosphere, contain diverse microorganisms, including bacteria, fungi, archaea, protozoa, and viruses. The rhizosphere, due to the excretions from plant roots, exhibits higher levels of microbial diversity and abundance compared to the surrounding soil. This is because the phenolic compounds, amino acids, and sugars released by roots promote the growth and establishment of a microbiome. In contrast, the microbial content in the bulk soil is influenced by factors such as pH, organic matter, moisture, and soil structure (Jools et al., 2020; Chauhan et al., 2023; Sapkota, 2023).

Soil microorganisms serve a vital role in nutrient cycling and various bio-geochemical cycles. They contribute to soil fertility, aid in the decomposition of organic matter, and assist plants in nutrient uptake, growth, and resilience against both abiotic

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and biotic stressors. Microorganisms enhance the nitrogen availability in the soil by either increasing its source or directly supplying it to the plants, as they possess the capability to extract and fix nitrogen from the atmosphere. Also, the presence of microorganisms in the soil leads to an enhanced availability of phosphorus. Enzymatic activities and other components facilitate the conversion of insoluble phosphorus into accessible forms for the plants (Islam et al., 2020; Chauhan et al., 2023).

The rhizosphere is inhabited by various microorganisms, including plant growth-promoting bacteria, mycorrhizal fungi, nematodes, protozoa, actinomycetes, and blue-green algae. These organisms play a crucial role in enhancing plant growth and overall health (Chauhan et al., 2023). Bacteria are more abundant in these regions compared to bulk soil due to the presence of high phosphorus amounts and iron (Islam et al., 2020). Biodiversity varies across plants due to the production of diverse chemicals by their roots, which serve to attract various microbes (Chauhan et al., 2023).

These microbes are vital and perform diverse functions in the soil. Bacteria play a crucial role in soil by enhancing soil structure through the production of compounds, breaking down complex organic compounds through biodegradation, and aiding in the availability of nitrogen through nitrogen fixation (Islam et al., 2020; Sapkota, 2023). Acidobacteria also play an essential role in the breakdown of carbon. Cyanobacteria possess the capacity to perform nitrogen and carbon fixation, hence enhancing soil fertility and soil porosity (Sapkota, 2023).

Fungi can also have a positive impact on the soil and plants. Bacteria and fungi have the ability to break down complex chemical substances into simpler forms that can be readily absorbed by plants. Fungi secrete enzymes capable of catalyzing the conversion of organic materials, thereby preserving the equilibrium of the soil. Additionally, they possess the capacity to assimilate heavy metals and contribute to the control of diseases (Islam et al., 2020; Jools et al., 2022; Sapkota, 2023).

Mycorrhizal fungi may reach plant roots intracellularly, specifically in the arbuscular region, or penetrate the roots externally and form a symbiotic relationship with plants, playing a critical role in their development and growth. They facilitate the nutrients uptake by plants and simultaneously aid in protecting against stresses (Islam et al., 2020; Chauhan et al., 2023; Sapkota, 2023).

Archaea are crucial in the nitrogen cycle and the cycling of other essential nutrients like carbon and sulfur. They undergo oxidation of sulfur compounds, subsequently releasing them into the environment, rendering them accessible to other microbes and plants. In addition, they have the ability to produce and oxidize methane (Islam et al., 2020; Odelade & Babalola, 2019).

Protozoa are a significant class of microorganisms that play a vital part in soil ecosystems. The group of soil protists includes testate amoebae, naked amoebae, ciliates, flagellates, microsporidia, and sporozoans (Foissner, 2014). They contribute to maintaining the equilibrium of the soil food chain as they consume bacteria, yeast, algae, and small protozoa (Sapkota, 2023). Flagellates are the primary consumers of bacteria in soil, playing a crucial role in soil fertility. Through bacterial feeding, they

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release significant quantities of nitrite, phosphorus, and other nutrients into the surrounding environment (Hillel & Hatfield, 2004; Sofos, 2014).

1.3 The biogeochemical cycle of nitrogen

Biogeochemical cycles, or nutrient cycles, describe the transfer of chemical elements between several environmental compartments, including the atmosphere, soil, water, and living organisms (Brusseau et al., 2019). These cycles are crucial as they ensure the availability of vital nutrients to organisms and facilitate the proper functioning of ecosystems. Biogeochemical cycles include a combination of biological, geological, and chemical processes involving vital elements like carbon, oxygen, hydrogen, phosphorus, nitrogen, and sulfur (Encyclopedia Britannica, 2023).

Nitrogen is a crucial element that plays a vital role in microorganisms, functioning as an essential component of nucleic acids, proteins, and other organic molecules (Ntougias et al., 2012). Approximately 78% of the atmosphere consists of nitrogen (Falkowski, 2001), however, despite its high availability, eukaryotic microorganisms and eukaryotic cells are unable to break down and use nitrogen due to the high energy required for the breakdown of its triple bond (N≡N) (Ntougias et al., 2012). A limited number of microbes possess the capability to directly utilize nitrogen and transform it into biologically available nitrogen through a process known as nitrogen fixation (Madigan et al., 2003).

Nitrification is a vital step within the nitrogen cycle. Nitrifying microorganisms, which are strictly prokaryotic, perform the oxidation of ammonia to nitrite under aerobic conditions. Next, nitrite undergoes oxidation to form nitrate (Madigan et al., 2003; Bernhard, 2010; Ntougias et al., 2012).

Denitrification is the ultimate stage of the nitrogen cycle, where bacteria and archaea transform the oxidized forms of nitrogen back into N_2 under anaerobic conditions (Falkowski, 2001).

In addition to the process of nitrification and denitrification, two other processes, namely ammonification and assimilation, are also recognized as part of the nitrogen cycle. Bacteria and fungi generate ammonia by decomposing plant and animal waste as well as dead tissues through ammonification. In addition, during the process of assimilation, plants utilize ammonium and nitrates directly to synthesize organic nitrogen (Bernhard, 2010).

1.3.1 The role of soil microorganisms in the biogeochemical cycle of nitrogen

Soil microorganisms are of great importance for the nitrogen cycle, as they are necessary for the execution of the nitrogen cycle processes. A number of

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microorganisms participate in nitrogen fixation, whereas others contribute to nitrification and denitrification. Significant contributors to the nitrogen cycle encompass various microorganism with specific roles such as denitrifying fungus, nitrifying archaea, anammox bacteria, aerobic denitrifying bacteria, and nitrifying bacteria (Hayatsu et al., 2008).

Nitrogen fixation, the conversion of atmospheric nitrogen to ammonia or ammonium ions, is exclusively performed by prokaryotic microorganisms, particularly by either free-living or symbiotic bacteria. Various free-living bacteria have the ability to do nitrogen fixation. These microorganisms can be classified as either aerobic, such as *Azotobacter* spp., *Azospirillum* spp., and cyanobacteria, or anaerobic, such as *Clostridiaceae* (Madigan et al., 2003; Ntougias et al., 2012).

Symbiotic nitrogen-fixing bacteria, forming mutualistic relationships with leguminous plants, reside in the root systems of these plants and transform the atmospheric nitrogen into NH_4^+ while simultaneously obtaining nutrients from the plant in return. The symbiotic nitrogen-fixing bacteria include members of the genus *Rhizobium*, *Bradyrhizobium*, and *Frankia* (Ntougias et al., 2012). The host plant in this symbiotic connection provides sugars to the symbiont, which utilizes them as an energy source for nitrogen fixation. In contrast free-living bacteria, like *Azotobacter*, are chemolithotrophs and utilize inorganic molecules as an energy source (Wagner, 2011).

The ability of these microorganisms to carry out the nitrogen fixation process is related to the presence of the enzyme nitrogenase, which enables them to combine atmospheric nitrogen with hydrogen to generate ammonia (Ntougias et al., 2012).

Microorganisms, decay bacteria, and fungi, play a crucial role in ammonification, as they are responsible for recycling nitrogen through the decomposition of plant and animal waste. Then ammonium ions are generated and subsequently used by nitrifiers (Bernhard, 2010).

Nitrification is carried out by microorganisms known as nitrifiers. Ammonia-oxidizing bacteria or archaea are responsible for the first stage of nitrification, whereas nitrite-oxidizing bacteria complete the subsequent and final stage of the process. Furthermore, bacteria belonging to the genus *Nitrospira* have the capability to carry out the entire process of ammonia oxidation in a single cell (Daims et al., 2015; Van Kessel et al., 2015). Some chemolitho-autotrophic bacteria, have also been found to perform the oxidation of ammonia in anaerobic conditions (Madigan et al., 2003).

Denitrifying microorganisms exhibit variability including bacteria, archaea, and fungi. Microorganisms such as *Micrococcus denitrificans*, *Thiobacillus denitrificans*, certain *Pseudomonas* and *Paracoccus* species, archaea such as *Haloferax denitrificans* and *Pyrobaculum aerophilum*, and soil fungi like F*usarium oxysporum* and *Fusariom solani* have demonstrated the capability to oxidize nitrate (Hayatsu et al., 2008; de Sousa et al., 2012).

Four enzymes are required for denitrification to take place. The process begins with the oxidation of nitrate to nitrite by the enzyme nitrate reductase (Nar). Next,

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nitrite is further oxidized to nitric oxide by the enzyme nitrite reductase (Nir). Following this reaction, nitric oxide is then oxidized to nitrous oxide by the enzyme nitric oxide reductase (Nor). Finally, nitrous oxide is ultimately oxidized to dinitrogen, the final form of nitrogen, by the enzyme nitrous oxide reductase (Nos) (Ntougias et al., 2012).

1.4 Nitrification

Nitrification is the biological process of converting ammonia into nitrite and nitrate through oxidation. However, until the late nineteenth century, the process was considered as chemical rather than biological. It was not until 1862 that Pasteur postulated the biological origin of nitrification (Koops et al., 2006). The procedure involves two steps and is executed by distinct groups of prokaryotic microorganisms (Madigan et al., 2003). Nitrification typically occurs in aerobic conditions, and various parameters such as salinity, temperature, pH, oxygen levels, availability of organic carbon, and C:N ratio influence the rate of nitrification in addition to substrate availability (Jorgensen et al., 2008; Hayatsu et al., 2021; Ayiti et al., 2022 Norton et al., 2023).

The oxidation of ammonia is of great significance, especially for the agricultural industry. It serves as a dominant pathway for the flow of nitrogen in soils and plays a crucial role in determining the availability of nitrates (Jorgensen et al., 2008; Rao et al., 2017). Nitrogen is crucial for plants as it serves as a nutrient, making it highly significant for their growth and development. Simultaneously, the nitrification process converts excess ammonia, which adversely affects plants and hinders nutrient absorption and other vital functions, into more beneficial nitrogen forms that can be utilized by plants (Ayiti et al., 2022). Humans have also utilized the process of nitrification to eliminate surplus nitrogen from wastewater treatment plants (Jorgensen et al., 2008).

However, the process of nitrification results in the production of nitrous oxide, a greenhouse gas that contributes to air pollution (Jorgensen et al., 2008; Hayatsu et al., 2021; Norton et al., 2023). Simultaneously, the process of leaching has led to the contamination of groundwater with nitrate (Hayatsu et al., 2021), while the rapid conversion of NH_4^+ to NO_3^- has caused an accumulation of NO_3^- in the soil (Norton et al., 2019). At the same time, the loss of nitrate from the soil through denitrification or leaching out of the root zone has led to human-induced nitrogen input into the soil. This excessive input has resulted in increased nitrogen leaching into other environmental compartments and higher greenhouse gas emissions (Norton et al., 2019; Ayiti et al., 2022).

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1.4.1 Microorganisms that participate in nitrification

Microorganisms play a crucial role in the nitrification process, as the conversion of ammonia to nitrite and then to nitrate is exclusively carried out by a group of prokaryotic microorganisms known as nitrifiers (Van Kessel et al., 2015).

Nitrifying microorganisms can be classified into two groups: ammonia-oxidizers and nitrate-oxidizers. In the initial stage of nitrification, the process involves the conversion of ammonia to nitrite by either ammonia-oxidizing bacteria (AOB) or ammonia-oxidizing archaea (AOA). Subsequently, nitrite-oxidizing bacteria (NOB) are responsible for the oxidation of nitrite to nitrate (Van Kessel et al., 2015). Recent studies have shown that some bacteria from the *Nitrospira* genus can fully facilitate the process of converting ammonia to nitrate, known as Comammox (Norto et al., 2019). The abundance of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) is controlled by the levels of ammonium in the environment, with AOA being more prevalent in habitats with low ammonium concentrations (Li et al., 2015).

The capacity of AOB and AOA to oxidize ammonia is attributed to the ammonia monooxygenase (AMO), an enzyme enhanced by the *amoA* gene (Li et al., 2015; Norton et al., 2019). AMO is a membrane- bound enzyme that uses copper as a cofactor in its catalysis and it is responsible for the oxidation of ammonia to hydroxylamine (NH_2OH), the first step in the process of the oxidation of ammonia to nitrite (Rotthauwe et al., 1997; Musiani et al., 2020). The oxidation of hydroxylamine to nitrite was initially thought to occur through the use of hydroxylamine oxidoreductase (HAO). Recent findings have revealed that the output of HAO is not nitrite, but rather nitric oxide. This nitric oxide is then converted into nitrite by an unidentified enzyme (Kuypers et al., 2018)

1.4.2.1 Ammonia-Oxidizing Bacteria (AOB)

The majority of AOB are obligate aerobes and chemolitho-autotrophs, belonging to the phylum Proteobacteria, specifically in the classes Betaproteobacteria and Gammaproteobacteria. They participate in the initial stage of the nitrification process by catalyzing the oxidation of ammonia to nitrite through the AMO enzyme. They use the ammonia present in the environment as an energy source, and they obtain carbon from CO_2 through the Calvin cycle (Kowalchuk & Stephen, 2001; Sihna & Annachhatre, 2007).

The contribution to nitrification varies among AOB, and they can be found in various environmental compartments (Hayatsu et al., 2021). They are primarily found in aerobic environments with a high concentration of ammonia, such as agricultural soil, forest soil, and grasslands. They can also be found in various aquatic environments, ranging from the ocean to salt lagoons and salt lakes (Kowalchuk & Stephen, 2001; Madigan et al., 2003; Koops et al., 2006; Hayatsu et al., 2021).

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Nitrosomonas, a Gram-negative betaproteobacterium, is a common type of AOB found in both terrestrial and aquatic environments, as well as in wastewater treatment plants (Madigan et al., 2003). The *Nitrosomonas* genus can be classified into six distinct lineages. All *Nitrosomonas* species possess the ability to perform nitrification and can be found in various biological niches (Koops et al., 2006).

Nitrosospira spp., a member of the Betaprotebacteria class, is frequently found in agricultural soils and soils fertilized with urea. These species are prevalent in acidic soil environments (Koops et al., 2006; Hayatsu et al., 2021). *Nitrosolobus* spp. and *Nitrosovibrio* spp. are also recognized as soil ammonia oxidizers (Madigan et al., 2003).

Nitrosococcus spp., a genus of Gammaproteobacteria, is predominantly found in marine environments due to its salt requirements. *Nitrosococcus* spp. exhibits a preference for saline or hypersaline environments, such as oceans and salt lakes (Koops et al., 2006; Campbell et al., 2011; Hayatsu et al., 2021).

AOB play a crucial role in the conversion of ammonia into nitrite in wastewater treatment facilities. Wastewater treatment plants (WWTP) have a low carbon-to-nitrogen (C:N) ratio, providing an optimal environment for the growth and proliferation of ammonia oxidizers (Kowalchuk & Stephen, 2001). *Nitrosomonas* spp. are frequently found in WWTP (Koops et al., 2006).

1.4.2.2 Ammonia-Oxidizing Archaea (AOA)

The initial discovery of AOB occurred almost a century ago, and initially, they were considered to be the sole microorganisms with the ability to oxidize ammonium. The discovery of the role of archaea in ammonia oxidation occurred almost 20 years ago (Francis et al., 2007). The initial identification of the *amoA* gene occurred within the phylum Crenarchaeota, which was subsequently reclassified as Thaumarchaeota. The very first example of ammonia-oxidizing archaea was *Nitrosopumilus maritimus*, which demonstrated the ability to develop chemolithoautotrophically by utilizing ammonia as an energy source and contain ammonia monooxygenase genes (Francis et al., 2007; Hayatsu et al., 2008).

Following the successful cultivation of *N. maritimus*, the cultivation of other AOA followed, resulting in the emergence of five primary clusters. *Nitrososphaera, Nitrosocosmicus, Nitrosotalea, Nitrossocaldus* and *Nitrosopumilus* (Lehtovirta-Morley,2018).

Both AOA and AOB share comparable genes responsible for producing the key AMO enzyme, which facilitates the process of ammonia oxidation. Nevertheless, they differ in their genetic composition and metabolic processes (Fisk et al., 2022). AOB utilize hydroxylamine oxidoreductase to catalyze the oxidation of hydroxylamine to nitrite. In contrast, AOA do not possess hydroxylamine oxidoreductase homologues. Recent studies suggest the existence of a new enzyme in AOA that is responsible for the conversion of hydroxylamine to nitrite. Every AOA that has been studied so far has

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been shown to possess both glutamate dehydrogenase and glutamine synthetase. Glutamate dehydrogenase plays a crucial role in the process of assimilating ammonia (Lehtovirta-Morley, 2018; Taylor & Kurtz, 2020; Wright & Lehtovirta-Morley, 2023).

At the same time, ammonia- oxidizing archaea are also autotrophic; however, they assimilate inorganic carbon via hydroxybutyrate cycle, while AOB use the Calvin Cycle to fix CO_2 (Lehtovirta-Morley, 2018).

AOA like AOB can be found in both terrestrial and aquatic environments. However, studies have shown that AOA are more abundant than AOB (Taylor & Kurtz, 2020). Soil pH and NH_4^+ availability are two main factors that influence the composition of AOA and AOB (Fisk et al., 2022). AOA are most prevalent in environments characterized by low NH_4^+ concentrations and oligotrophic conditions. They are particularly abundant in acidic soil, where *Nitrososphaera* and *Nitrosotalea* are the predominant AOA genera. On the other hand, AOB are more dominant in alkaline soils and thrive in eutrophic environments with high ammonia concentrations, such as fertilized soils and wastewater treatment plants. In maritime ecosystems with limited ammonia supply, the growth of AOA is more advantageous compared to AOB, particularly for *Nitrosoarchaeum* and *Nitrosotenuis* species (He et al., 2018; Lehtovirta-Morley, 2018; Taylor & Kurtz, 2020; Jung et al., 2022; Zou et al., 2022).

1.4.2.3 Anaerobic Ammonia Oxidizers (Anammox)

Traditionally ammonia oxidation has been primarily carried out in aerobic environments; however, it can also be achieved under anaerobic conditions. The process is referred to as anammox, which stands for anaerobic ammonia oxidation (Ntougias et al., 2012). The anaerobic oxidation of ammonia is an exergonic reaction in which nitrite serves as the electron acceptor and N_2 gas is produced as the final product. The origin of NO_2^- is the end result of the oxidation of ammonia by ammonia oxidizing microorganisms (Madigan et al., 2003; Shively et al., 2009).

The annamox process is carried out by chemolitho-autotrophic bacteria that are classified in the phylum *Planctomycetes* (Madigan et al., 2003). The anammox bacteria consist of seven genera, namely *Candidatus* Brocardia, *Candidatus* Scalindua, *Candidatus* Kuenenia, *Candidatus* Brasilis, *Candidatus* Jettenia, *Candidatus* Anammoximicrobium, and *Candidatus* Anammoxoglobus (Anjali & Sabumoun, 2022).

The capacity of these bacteria to carry out the oxidation of ammonia in the absence of oxygen is attributed to a distinct cellular structure known as the anammoxosome (Madigan et al., 2003; Ntougias et al., 2012). The primary constituents of the annamoxosome membrane are predominantly ladderane lipids (Ntougias et al., 2012). In order for ammonium to undergo oxidation, it must first be transported into the annamoxosome, where the oxidation occurs (Holmes et al., 2019).

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Although anammox bacteria are discovered in engineered environments, they can also be found in natural habitats that lack oxygen, including marine environments, brackish and fresh waters, sediments, and certain terrestrial environments (Sonhiphand et al., 2014; Holmes et al., 2019). The anammox process plays a crucial role in the nitrogen cycle by efficiently removing fixed nitrogen from engineered and natural systems. This advantageous process is extensively applied in wastewater treatment systems and obviates the necessity for aeration or organic carbon inputs. Furthermore, it reduces greenhouse gas emissions when compared to conventional denitrification techniques (Shively et al., 2009; Sonhiphand et al., 2014).

1.4.2.4 Nitrite Oxidizing Bacteria (NOB)

Nitrite-oxidizing bacteria (NOB) are responsible for the oxidation of nitrite, generated by AOM, and they possess the capability of transforming it into nitrate (Picone et al., 2021). Nitrate is a crucial nitrogen source for numerous microorganisms, with nitrite oxidation serving as its primary source (Sorokin et al., 2012).

NOB are classified into six genera, which include Chloroflexi and Nitrospirae phyla, alpha, beta, gamma, and delta Proteobacteria (Daims et al., 2016; Leung et al., 2022). As of now, *Nitrobacter*, *Nitrospira*, *Nitrotoga*, *Nitrococcus*, *Nitrospina*, and *Nitrolancetus* are the six recognized genera (Daims et al., 2016).

NOB derive their energy from the oxidation of nitrite (Abeliovich, 2006), and they fix carbon dioxide for growth (Prosser, 2005; Spieck & Lipski, 2011). However, in contrast to AOM, NOB exhibit the ability to develop in a heterotrophic or mixotrophic way, like some certain strains of *Nitrobacter* and *Nitrospira* (Prosser, 2005; Myrold, 2021). The nitrite oxidoreductase, an essential enzyme found in all NOB, is responsible for the oxidation of nitrite by these bacteria (Daims et al., 2016). Although nitrate oxidoreductase is a complex of three structural subunits associated with a membrane, its precise composition varies among the six genera of NOB. For example, the nitrate oxidoreductase is situated at the cytoplasmic site of the cell membrane in *Nitrobacter*, rather than the outside site of the cell membrane in *Nitrospira* (Abeliovich, 2006).

It has also been proven that NOB may form colonies with AOM. AOM obtain additional nutrients generated by NOB while serving as an energy source for NOB within these aggregates (Daims et al., 2016). Particularly susceptible to aggregation are *Nitrospira* and *Nitrotoga* species (Spieck & Lipski, 2011).

NOB have a broad range of pH and salinity tolerances and are very prevalent in both natural and engineered environments (Abeliovich, 2006). The *Nitrobacter* and *Nitrospira* genera play a crucial role in terrestrial environments, whereas the *Nitrospina* genus is prominent in marine environments such as sea waters and sediments (Daims et al., 2016). The genus *Nitrospira* is extremely diverse and common among NOB. It is prevalent in many natural environments, as well as in wastewater treatment plants

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and drinking water treatment systems (Leung et al., 2022). *Nitrospina* may also be identified in oxygen minimum zones and anoxic marine sediments (Davis et al., 2009).

NOB exhibit variations in their nitrite affinity. The genus *Nitrospira* has a competitive edge in environments with low nitrite availability. They are classified as k-strategists, characterized by a low maximal activity level yet a high substrate affinity. In contrast, r-strategists such as *Nitrobacter* have a high level of nitrite oxidation activity but low substrate affinity (Daims et al., 2016).

1.4.2.5 Complete Ammonia Oxidizers (Comammox)

The process of nitrification was previously believed to occur in two steps. First, AOM would convert ammonia to nitrite. Then, NOB would convert nitrite to nitrate. However, in 2015, bacteria from the *Nitrospira* genus were discovered to be capable of completely oxidizing ammonia to nitrate, challenging the previous understanding of nitrification (Daims et al., 2015; van Kessel et al., 2015). The genus was formerly classified as NOB (Lehtovirta-Morley, 2018; Hu et al., 2021). The initial nitrifier to be isolated in a pure culture was *Nitrospira inopita*. Its genome contained the genetic information for both ammonia and nitrite oxidation pathways (Daims et al., 2015). *Candidatus* Nitrospira nitrosa and *Candidatus* Nitrospira nitrificans were also found to encode all the enzymes and oxidize ammonium completely to nitrate (van Kessel et al., 205). Prior to that point, none of the nitrifying microorganisms had the capability to oxidize both ammonia and nitrite substrates (Daims et al., 2015). Two clades of comammox *Nitrospira* have been described, clade A and clade B, based on *amoA* gene phylogeny, all of them belonging to lineage II (Koch et al., 2018).

Comammox bacteria have been identified in various soil habitats, coexisting with ammonia-oxidizing microbes (Lehtovirta- Morley, 2018; Hu et al., 2021). Existing information regarding the prevalence and spatial arrangement of comammox bacteria indicates that these microorganisms are adapted to low levels of ammonia, confirming their oligotrophic lifestyle in aquatic and engineering environments. However, more recent research has revealed that in terrestrial ecosystems, comammox *Nitrospira* are not only oligotrophic but also copiotrophic, with a broader ecological niche range than previously assumed (Li et al., 2023).

These bacteria have a significant role in the process of nitrification in wastewater treatment plants and are the predominant species in activated sludge systems (Mehrani et al., 2021). Within engineered systems such as biofilters and ground water-fed sand filters, the abundance of microorganisms surpasses that of other ammonia oxidizers (Koch et al., 2018).

1.5 Ammonia-oxidizing microorganisms as bioindicators for assessing the toxicity of xenobiotics

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Soil microorganisms play a pivotal role in the efficient functioning of soil processes, as mentioned earlier. However, their dynamics are significantly influenced by various human activities, including global warming, climate change, and contamination from chemical substances like pesticides (Thiele-Bhuhn et al., 2020). The alteration of soil biological diversity is identified as one of the key risks to soil ecology (Wessen & Hallin, 2011).

Several microorganisms have been proposed as potential biological indicators for assessing the toxicity of xenobiotic compounds in soils. AOM are particularly recommended as highly effective bioindicators for assessing the potential toxicity of xenobiotic substances on soil microbiota. They are commonly employed to gauge soil contamination (Wessen & Hallin, 2011).

AOM are well-suited for toxicity evaluation assessment due to their sensitivity to xenobiotic substances. Given their crucial role in the nitrogen cycle, their physiology, biochemistry, and life cycle have undergone extensive study. Moreover, established standard methods exist for monitoring these microorganisms (Karpouzas et al., 2022).

1.6 Ammonia-oxidizing microorganisms used in the current study

The current study was focused on the examination of two types of AOA: *Candidatus* Nitrosocosmicus franklandianus (C13) and *Candidatus* Nitrosotalea sinensis (Nd2). *Candidatus* Nitrosocosmicus franklandianus belonging to the lineage of the order *Nitrososphaerales* demonstrates remarkable tolerance to high levels of ammonia and nitrite (Lehtovirta et al., 2016). Thriving within a temperature range of 30-45 °C, with an optimum growth temperature of 37 °C, and preferring a pH range of 6–8.5, it engages in autotrophic growth utilizing HCO_3 as its carbon source (Lehtovirta et al., 2016; Nicol et al., 2019).

Ca. Nitrosotalea sinensis is a chemolithoautotrophic, obligate acidophile microorganism, particularly successful in acidic environments. With a temperature range spanning from 20 to 42 °C, and an optimal temperature of 35 °C, it thrives within a pH range of 4 to 6.1, with an optimal pH of 5.2 (Lehtovirta-Morley et al., 2011; Herbold et al., 2017). *Ca.* Nitrosotalea sinensis has small, rod-shaped cells with angular and electron-dense poles, measuring 0.2 × 0.7 μm in size. In contrast, *Ca.* Nitrosocosmicus franklandianus has a larger diameter of 1.1 μm. *Ca.* Nitrosocosmicus franklandianus and *Ca.* Nitrosotalea sinensis both possess the *amoA*, *amoB*, and *amoC* genes (Lehtovirta-Morley et al., 2011; Lehtovirta et al., 2016).

1.7 Purpose of this study

The overarching objective of this study was to assess the impact of specific synthetic anthelmintics on soil microorganisms. Within this context AOM serve as valuable microbial indicators to gauge the ecotoxicity of these drugs. Their selection is

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based on distinctive characteristics, including their significant functional role, sensitivity to xenobiotic substances, and the availability of suitable research tools for their in-depth study, as elucidated in the preceding introductory part.

The primary aim of this thesis was to conduct an in *vitro* assessment of the impact of selected anthelmintics, specifically those belonging to the benzimidazole group (like albendazole) and the group of macrocyclic lactones (like ivermectin and eprinomectin), on the activity of two distinct ammonia-oxidizing archaeal strains. These strains, *Candidatus* Nitrosocosmicus franklandianus and *Candidatus* Nitrosotalea sinensis, were chosen for their ecological, physiological, and phylogenetic diversity, making them representative of AOA commonly found in soil environments. Simultaneously, the study investigated the degradation of the examined substances in liquid cultures to determine their stability under aseptic, *in vitro* conditions.

2. Materials and Methods

2.1 Cultivation of ammonia-oxidizing archaea

2.1.1 *Candidatus* Nitrosocosmicus franklandianus

Candidatus Nitrosocosmicus franklandianus was cultured in Modified Fresh Water medium and incubated in the dark at a constant temperature of 35 °C without agitation. The pH of the medium was adjusted to 7.5 by adding a 1M HEPES. The medium was supplemented with NH_4^+ at a concentration of 1mM.

For the preparation of the medium, four distinct solutions were used:

1. Basal Salts 10X Solution
2. Modified trace elements
3. Modified vitamin solution
4. Selenite-tungstate solution

The composition of each individual solution is outlined in the respective tables below. These solutions prepared in 1L of ddH_2O, underwent sterilization via autoclaving and were subsequently stored at 4 °C.

Table 1. Materials and concentrations *used* for the preparation of Basal Salts 10x Solution.

Materials	Final Concentrations
NaCl	10 g L^{-1}
$MgCl_2 \cdot 6H_2O$	4 g L^{-1}

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$CaCl_2 \cdot 2H_2O$	1 g L^{-1}
KH_2PO_4	2 g L^{-1}
KCl	5 g L^{-1}

Table 2. *Materials and concentrations used for the preparation of Modified Trace Element Solution.*

Materials	**Final Concentrations**
H_3BO_3	30 mg L^{-1} (0.5 mM)
$MnCl_2 \cdot 4H_2O$	100 mg L^{-1} (0.5 mM)
$CoCl_2 \cdot 6\ H_2O$	190 mg L^{-1} (0.8 mM)
$NiCl_2 \cdot 6\ H_2O$	24 mg L^{-1} (0.1 mM)
$CuCl_2 \cdot 2\ H_2O$	2 mg L^{-1} (0.01 mM)
$ZnSO_4 \cdot 7\ H_2O$	144 mg L^{-1} (0.5 mM)
$Na_2MoO_4 \cdot\ H_2O$	36 mg L^{-1} (0.15 mM)
HCl (12.5 M)	8 ml L^{-1} (100 mM)

Table 3. Materials and concentrations used for the preparation of Modified Vitamin Solution.

Materials	**Final Concentrations**
Biotin	0.02 g L^{-1}
Pyrodixine HCL	0.05 g L^{-1}
Thiamine HCL	0.05 g L^{-1}
Nicotinic acid	0.05 g L^{-1}
Calcium pantothenate	0.05 g L^{-1}
P Aminobenzoic Acid	0.05 g L^{-1}
Vitamin B12	0.01 g L^{-1}

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Table 4. *Materials and concentrations used for the preparation of selenite-tungstate solution.*

Materials	Final Concentrations
NaOH	4 g L^{-1}
$Na_2SeO_3 \cdot 5H_2O$	0.06 g L^{-1}
$Na_2WO_4 \cdot 2H_2O$	0.08 g L^{-1}

Subsequently, the components listed in Table 5 were incorporated into the Modified Fresh Water medium at the specified concentrations per liter of the Basal Salts 1X solution.

Table 5. *Materials and concentrations used for the preparation of Modified Fresh Water Medium.*

Materials	Final Concentrations
HEPES buffer (1 M)	10 ml L^{-1}
$NaHCO_3$ (1 M)	2 ml L^{-1}
FeNaEDTA (7.5 mM)	1 ml L^{-1}
Modified trace elements	1 ml L^{-1}
NH_4Cl (1 M)	1 ml L^{-1}
Modified Vitamin solution	1 ml L^{-1}
Selenite-tungstate solution	0.1 ml L^{-1}

2.1.2 *Candidatus* Nitrosotalea sinensis

Candidatus Nitrosotalea sinensis was cultured in a MES-buffered liquid Fresh Water medium (pH 5.3) and maintained at a temperature of 35 °C without agitation. Additionally, the medium was supplemented with NH_4^+ at a concentration of 1mM.

The Fresh Water medium was formulated using the following solutions:

1. Basal Salts 10X Solution
2. Modified Trace elements

Subsequently, the ingredients outlined in Table 6 were introduced per liter of the Basal Salts 1X Solution to complete the preparation of the final Fresh Water medium.

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Table 6. *Materials and Concentrations used for the preparation of Fresh Water Medium.*

Materials	Final Concentrations
$NaHCO_3$ (1 M)	2 ml L^{-1}
FeNaEDTA (7.5 mM)	1 ml L^{-1}
Modified Trace Elements	1 ml L^{-1}
NH_4Cl (1 M)	0.5 ml L^{-1}

2.2 Experimental design

For each studied strain, 2 liters of Modified Fresh Water and Fresh Water medium, respectively, were prepared and stored in Duran glass bottles (5 liters) at a temperature of 35°C one day prior to inoculation. Once the medium reached the optimal temperature, a 2% (v/v) fresh culture of the strains in the exponential growth phase was added. Subsequently, at the onset of the exponential phase, the 2 L culture was distributed into individual Duran bottles (100 mL each), resulting in a total of 30 mL cultures to which the tested compounds were added. Three biological replicates were used for each treatment.

Anthelmintics exhibited low solubility; hence, working solutions were prepared and applied at different concentrations. The addition to liquid cultures was performed in 0.1% (v/v) dimethylsulfoxide (DMSO), which was sterilized by filtration with a specialized 0.22 mm PTFE sterile filter. Control cultures, were supplemented with a respective volume of DMSO.

Details on the anthelmintics and the range of concentrations used are provided in Table 7.

Table 7. *Concentrations of anthelmintics used in the current study.*

Albendazole	Ricobendazole	Fenbendazole	Ivermectin	Eprinomectin
190 μM (50 mg/L)	178 μM (50 mg/L)	334 μM (100 mg/L)	57 μM (50 mg/L)	109 μM (100 mg/L)
95 μM (25 mg/L)	89 μM (25 mg/L)	84 μM (25 mg/L)	29 μM (25 mg/L)	27 μM (25 mg/L)
19 μM (5 mg/L)	18 μM (5 mg/L)	34 μM (10 mg/L)	6 μM (5 mg/L)	11 μM (10 mg/L)
2 μM (0.5 mg/L)	2 μM (0.5 mg/L)	3.4 μM (1 mg/L)	0.6 μM (0.5 mg/L)	1.1 μM (1 mg/L)

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2.2.3. Assessment of the impact of anthelmintics on the activity of AOA

The influence of anthelmintics on the activity of the tested AOA strains was systematically monitored over a two-week period following the introduction of the drugs into the cultures. Activity was quantified by assessing the concentration of NO_2^- measured colorimetrically at 540 nm. To achieve this, 100 μL of each sample was added to individual wells of a microplate (96-well plate). Subsequently, 20 μL of diazotizing (0.5 g sulfanilamide in 100 mL 2.4 M HCl) and coupling (0.3g N-(1-napthyl)-ethylenediamine HCl in 100 mL 0.12 M HCl) reagents were introduced. The concentrations of the generated nitrite were determined using standard curves constructed from known concentrations of $NaNO_2$ (ranging from 0 to 100 μM).

2.2.3.1 Assessment of the impact of anthelmintics on the growth of AOA

Throughout the experimental duration, a lower production of NO_2^- was observed in the liquid cultures of the *Ca.* Nitrosocosmicus franklandianus strain upon the addition of albendazole at the lowest tested concentration of 2 μM compared to the higher tested concentrations (19-190 μM) of this drug and the control samples. A similar pattern in NO_2^- production was observed when the lowest tested concentrations of albendazole (2 μM), ricobendazole (2μM), and fenbendazole (3.4 μM) were introduced in the liquid cultures of *Ca.* Nitrosotalea sinensis strain. In such cases, real-time quantitative polymerase chain reaction (qPCR) was employed to monitor the growth of the AOA, comparing the abundance of the *amoA* gene between treated and control cultures.

2.2.3.1.1 DNA extraction

DNA extraction was performed on C13 strain encompassing both the control culture and cultures enriched with varying concentrations of albendazole (2 μM, 19 μM, 95 μM, and 190 μM). Similarly, DNA extraction was conducted on strain Nd2 including the control culture and cultures enriched with different concentrations of albendazole (2μM, 19μM, 95μM, and 190μM), ricobendazole (2μM, 18μM, 89μM, and 178μM), and fenbendazole (3.4μM, 34μM, 84μM, and 334μM).

The sample volumes were standardized at 2 ml, and DNA extraction was performed by using the kit 'Tissue DNA Extraction Kit' (Macherey-Nagel, Germany) following the manufacturer's provided instructions.

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2.2.3.1.2 Quantitative Polymerase Chain Reaction

To amplify *amoA* gene, Arch-amoAF and Arch-amoAR primers as described by Francis et al. (2005) were employed. The primer sequences are as follows:

- Arch-amoAF: 5'- STAATGGTCTGGCTTAGACG-3'
- Arch-amoAR: 5' - GCGGCCATCCATCTGTATGT-3'

The thermal profile of the reaction comprised the following steps:

1. 95°C for 3 mins
2. 95°C for 15 sec
3. 53 °C for 30 sec
4. 72°C for 45 sec

Steps 2 to 4 were repeated for 45 cycles.

Details of each reagent used for the qPCR are provided in Table 8.

Table 8*. Reagents used in qPCR for the amplification of the amoA gene.*

Master Mix	V (μL)	Final Concentration
KAPA SYBR FAST qPCR Master Mix (2x) Universal	5	1X
Arch-amoAF (20pmol/μL)	0.1	0.2 μM
Arch-amoAR (20pmol/μL)	0.1	0.2 μM
BSA (20μg/μL)	0.2	400 ng/μL
ddH_2O	2.6	-
DNA	2	-
V_{total}	**10 μL**	-

A standard curve, generated from known diluted (10^9-10^1) plasmid concentrations containing the target gene was employed. The qPCR efficiency for strain C13 was 87% with R^2 =0.994 and for strain Nd2 was 78.5% with R^2= 0.991.

2.2.4 Stability of anthelmintics in the liquid cultures of AOA

The concentrations of anthelmintics in the liquid cultures were determined using High Performance Liquid Chromatography (SHIMADZU LC-20AD). The system was equipped with a UV/VIS PD detector and comprises a SHIMADZU VP-ODs column

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(150 mm x 4.6 mm) and a SHIMADZU GVP-ODs precolumn (10 mm x 4.6 mm). Injection volume was set at 20 μL, and the mobile phase flow rate was 1 mL min^{-1}.

Sampling was conducted at two distinct time points. The initial timepoint coincided with the introduction of substances into the liquid cultures, at the onset of the exponential phase of the strain. The second sampling occurred at the end of the experiment when the strain reached the stationary phase. Anthelmintic compound concentrations were determined using a standard curve established from standard solutions within a concentration range of 0.01mg L^{-1} to 10mg L^{-1}. For preparing working solutions, tested compounds were added to organic solvents, acetonitrile (MeCN) (albendazole, ricobendazole, ivermectin), and methanol (MeOH) (fenbendazole, eprinomectin). The analysis parameters for anthelmintics are presented in Table 9.

Table 9. *Parameters of anthelmintics HPLC analysis.*

Anthelmintics	Mobile Phase	Solvents Ratio	Wavelength (nm)	Retention Time
Ivermectin	MeOH: H_2O	90:10	245	8.5
Eprinomectin	MeOH: H_2O	85:15	245	11.4
Albendazole	MeCN:H_3PO_4 (0.1%)	15:85	205	22.7
Ricobendazole	MeCN:H_3PO_4 (0.1%)	15:85	220	8.8
Fenbendazole	MeCN:H_3PO_4 (0.1%)	35:65	245	9.1

A drug recovery study was also conducted using two distinct culture media: one acidic (freshwater medium, pH = 5.3) and one alkaline (Skinner & Walker medium, pH = 7.5) matching the pH of the medium used for *Ca.* Nitrosotalea sinensis and *Ca.* Nitrosocosmicus franklandianus, respectively. The study encompassed four different concentration levels with three different dilutions of organic solvent (MeOH or MeCN). Compounds under testing were diluted in ratios of: 1:2, 1:5, and 1:10. The recovery rates for over eighty percent of all compounds were achieved. Each concentration of each substance, along with the chosen solvent and drug ratio for each concentration and medium, is detailed in the following table.

Table 10. *Culture to solvent ratio used for the extraction of anthelmintics from the AOA liquid cultures.*

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Substance	Concentration(μM)	Culture/ Solvent ratio	
		FW	MFW
Albendazole	190	1:10	1:10
	95	1:10	1:2
	19	1:2	1:2
	2	1:2	1:2
Ricobendazole	178	1:5	1:10
	89	1:5	1:10
	18	1:2	1:10
	2	1:2	1:10
Fenbendazole	334	1:10	1:10
	84	1:5	1:10
	34	1:5	1:10
	3.4	1:5	1:10
Ivermectin	57	1:10	1:10
	29	1:10	1:10
	6	1:2	1:2
	0.6	1:2	1:2
Eprinomectin	109	1:10	1:10
	27	1:10	1:10
	11	1:2	1:2
	1.1	1:2	1:2

2.2.5 Statistical Analysis and Calculation of the EC_{50}

Statistical analysis was conducted using IBM.SPSS v.29.0. The results from the nitrite production measurements were subjected to two-way ANOVA to assess the impact of treatment, time, and/or their interaction. Tuckey's post-hoc test was applied when significant interactions ($p < 0.05$) occurred between the two main factors, facilitating the identification of significant differences between individual treatments at each time point.

Subsequently, EC_{50} values were determined, representing the average concentration of each substance resulting in a 50% reduction in the activity (nitrite accumulation) of AOA. The dose-response model was employed, dividing the concentrations of produced nitrite by the mean value of the corresponding nitrite from the control culture. R software and the dose-response curve package v3.0-1 were utilized for the analyses. Initially, an empirical modelling approach was employed to select the best fitting model, followed by the four-parameter log-logistic model identified as the optimal compromise among tested models for comparing endpoint values.

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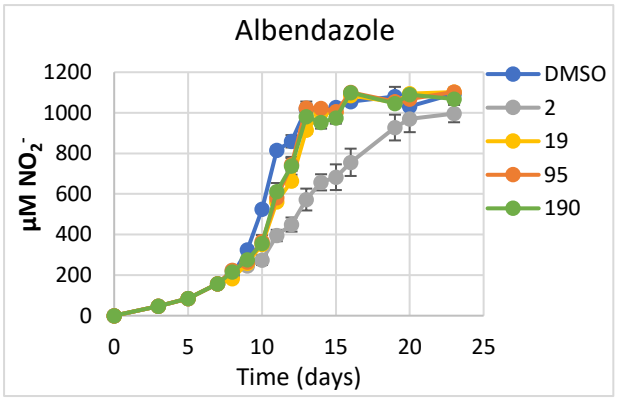
3. Results

The following figures depict the impact of anthelmintics on the activity and growth of AOA strains, each compound being presented individually. The plots showcase the nitrite concentrations recorded during the experimental period for each concentration of every anthelmintic drug. The bars illustrate the standard error among the three biological replicates.

3.1 Impact of anthelmintics on *Candidatus Nitrosocosmicus franklandianus*

3.1.1 Impact of Benzimidazoles on *Candidatus* Nitrosocosmicus franklandianus

(a)



(b)

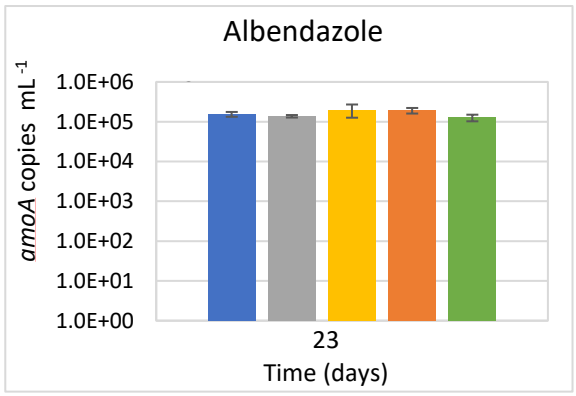


Figure 7. *Impact of albendazole on the activity (a) and growth (b) of Ca. N. franklandianus.*

The impact of albendazole on either the activity or the growth of the C13 strain was examined. Figure 7 suggests a significant impact ($p < 0.05$) on the activity of the AOA strain only for the lowest tested concentration of 2 µM. However, a comprehensive analysis using qPCR and statistical methods, as presented in Figure 7b, reveals no discernible differences ($p > 0.05$) in the abundance of *amoA* gene copies among the various samples, leading to the conclusion that none of the albendazole tested concentrations had a significant effect on the growth of the C13 strain.

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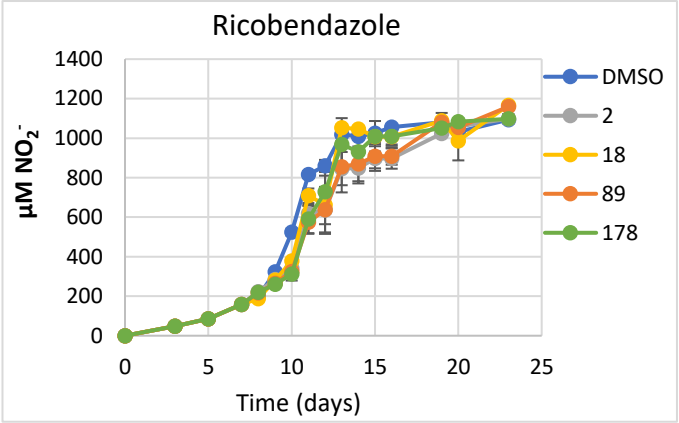

Figure 8. *Impact of ricobendazole on the activity of Ca. N. franklandianus.*

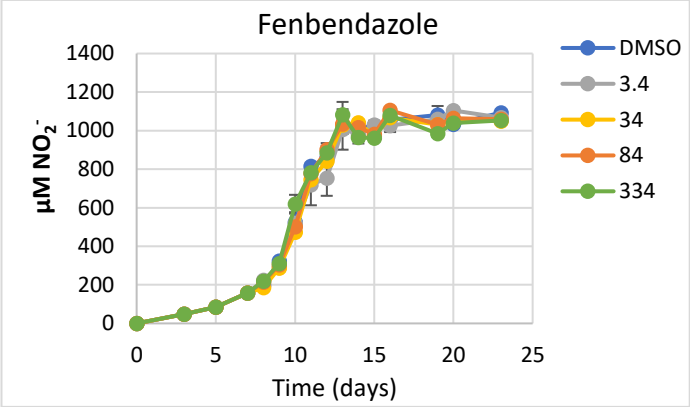

Figure 9. *Impact of fenbendazole on the activity of Ca. N. franklandianus.*

In addition, as illustrated in Figures 8 and 9, the introduction of ricobendazole and fenbendazole in the liquid cultures of *Ca.* N. franklandianus C13 strain exhibited no significant ($p > 0.05$) impact on the accumulation of nitrites compared to the control.

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3.1.2 Impact of Macrocyclic Lactones on *Candidatus* Nitrosocosmicus franklandianus

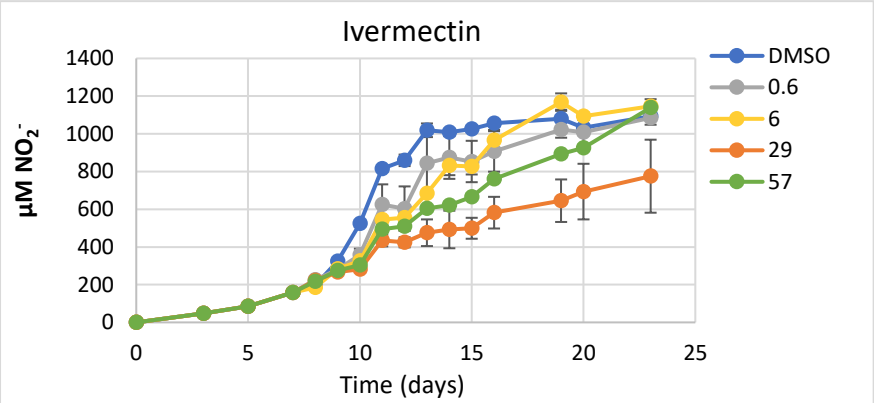

Figure 10. *Impact of ivermectin on the activity of Ca. N. franklandianus.*

As depicted in Figure 10, the activity of *Ca.* N. franklandianus was not significantly affected by the two lowest doses of ivermectin, 0.6 µM and 6 µM, respectively. In contrast, the two highest tested concentrations of 29 µM and 57 µM, significantly reduced ($p < 0.05$) the production of nitrites compared to the control. The concentration of 29 µM, although lower than the concentration of 57 µM, imposed a higher inhibition on nitrite formation by *Ca.* N. franklandianus. Subsequent HPLC analysis presented below (Figure 23) suggests that the highest tested concentration of 57 µM had not discernible impact due to low solubility and unavailability in the liquid cultures of the AOA strain. On the contrary, the 29 µM concentration appeared to affect nitrite production immediately after its introduction to the culture.

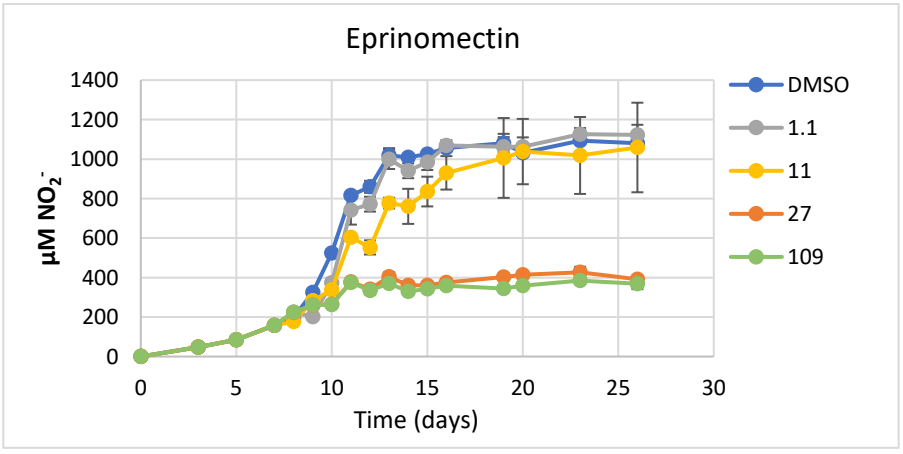

Figure 11. *Impact of eprinomectin on the activity of Ca. N. franklandianusC13 strain.*

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Eprinomectin exhibited the most pronounced impact on the activity of *Ca.* N. franklandianus, compared to the other tested compounds, as depicted in Figure 11. The doses of 27 μM and 109 μM, representing the highest tested concentrations, immediately affected the culture upon administration. The presence of these concentrations significantly impeded ($p < 0.05$) the activity of the AOA strain throughout the entire experimental procedure.

Table 11 presents the EC_{50} values (μM) for anthelmintics calculated based on their inhibitory activity on the ammonia oxidation capacity of *Ca.* Nitrosocosmicus franklandianus, with standard error provided in brackets. An asterisk indicates the highest concentration investigated, pointing out instances where deriving EC_{50} values through statistical analysis was not feasible.

Table 11. *Average EC_{50} values (μM) for the anthelmintics investigated in this study, on the AOA strain Ca. N. franklandianus.*

Anthelmintic	***Ca.* Nitrosocosmicus franklandianus**
Albendazole	>190*
Ricobendazole	>178*
Fenbendazole	>334*
Ivermectin	34.38 (± 1.65)
Eprinomectin	12.37 (± 0.6)

3.2 Impact of Anthelmintics on *Candidatus* Nitrosotalea sinensis

3.2.1 Impact of Benzimidazoles on *Candidatus* Nitrosotalea sinensis

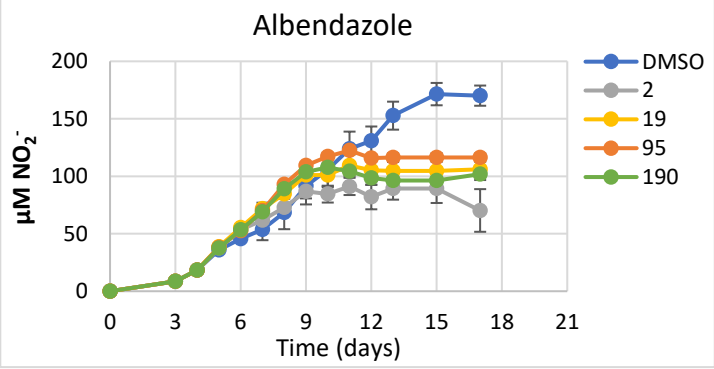

Figure 12. *Impact of albendazole on the activity of Ca. N. sinensis.*

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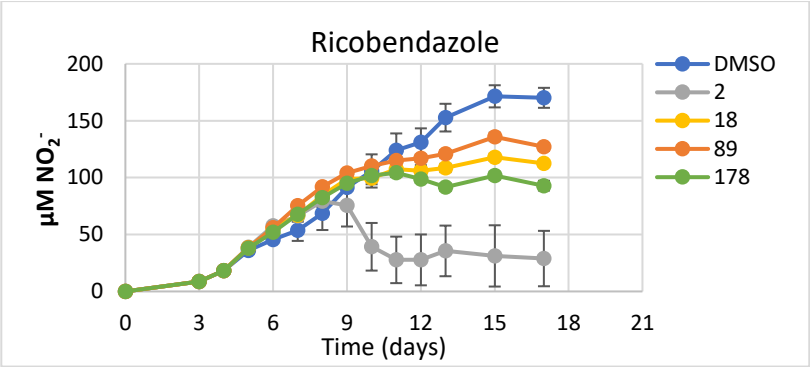

Figure 13. *Impact of ricobendazole on the activity of Ca. N. sinensis*

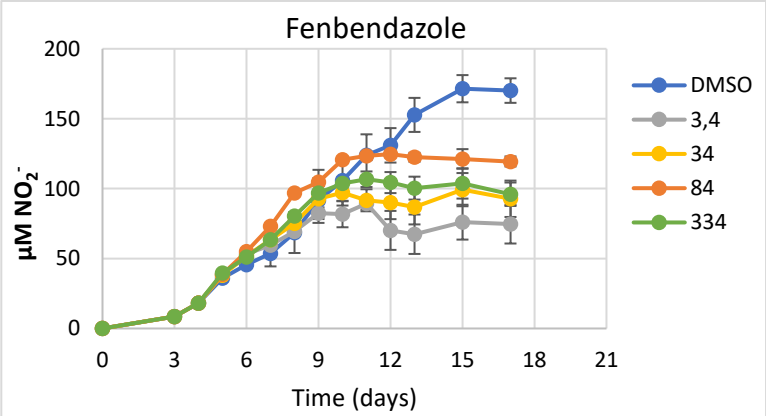

Figure 14. *Impact of fenbendazole on the activity of Ca. N. sinensis.*

Anthelmintics belonging to the benzimidazole class, including albendazole, ricobendazole, and fenbendazole, demonstrated similar effects on the activity of *Ca.* N. sinensis inhibiting almost completely the nitrite production by the AOA strain (Figures 12, 13 and 14). The results of qPCR analysis and statistical tests revealed a significant difference ($p < 0.05$) in *amoA* gene abundance between the control culture and cultures treated with albendazole and ricobendazole. This confirms that the presence of these compounds at all tested concentrations influenced both the activity and growth of this AOA strain. However, no variations in gene abundance were observed among different concentrations within each treatment.

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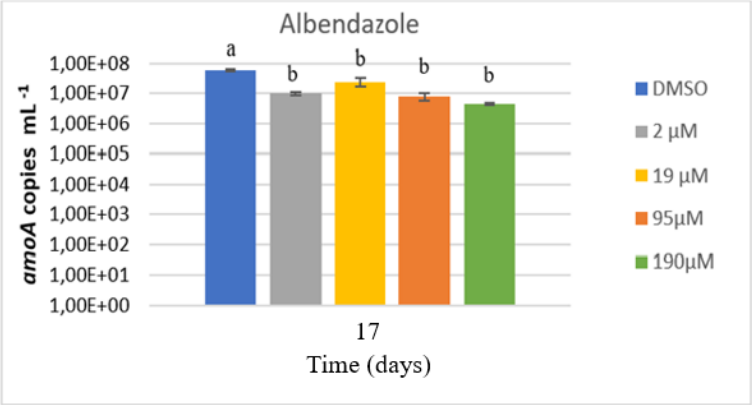

Figure 15. *The impact of albendazole on the growth of Ca. N. sinensis.*

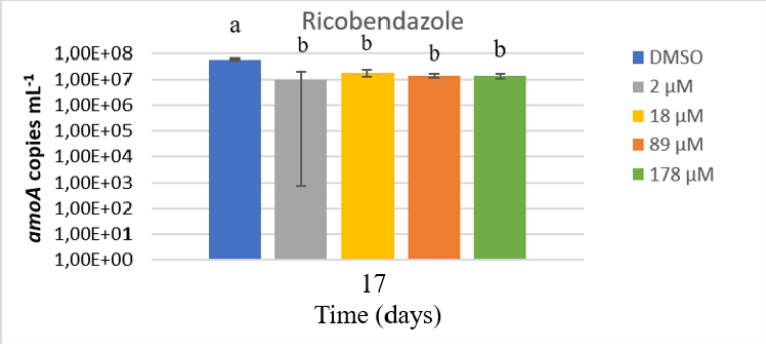

Figure 16. *Impact of ricobendazole on the growth of Ca. N. sinensis.*

Furthermore, the qPCR analysis and statistical examination conducted on the cultures treated with fenbendazole showed no significant difference ($p > 0.05$) between the control and the treated cultures. This observation contrasts with the results obtained from the measurement of nitrite production (Figure 14).

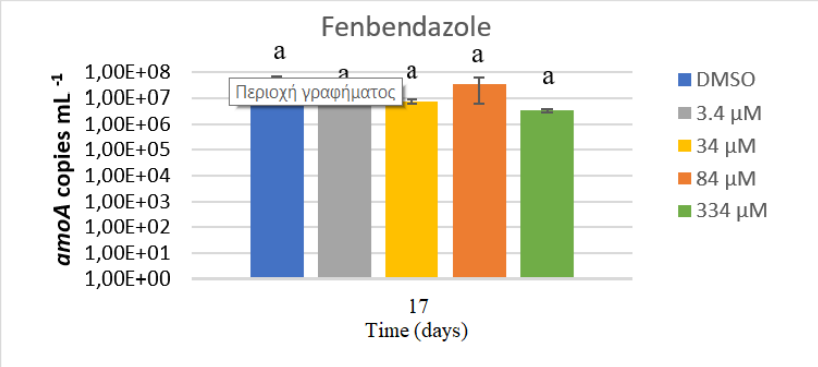

Figure 17. *Impact of fenbendazole on the growth of Ca. N. sinensis.*

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The decrease in nitrite production is not attributed to a decrease in strain growth but rather to the interaction between benzimidazoles and nitrites. This reduction in nitrite production is particularly pronounced at lower concentrations, influenced by two distinct factors: (i) the enhanced solubility of lower concentrations and (ii) the limited amount of nitrite formed before the introduction of benzimidazoles.

3.1.2 Impact of Macrocyclic Lactones on *Ca.* Nitrosotalea sinensis

Ivermectin and eprinomectin exerted substantial impact on the activity of *Ca.* N. sinensis. Even at the lowest tested concentrations of 0.6 µM and 1.1 µM, respectively, both compounds significantly inhibited ($p < 0.05$) the production of nitrite by the AOA strain.

Figures 17 and 18 illustrate that the three highest concentrations of the tested macrocyclic lactones had a notable effect on nitrite production. While the culture with the lowest concentration showed less inhibition than the culture with the highest concentrations, the production of nitrites was still reduced at the lowest concentration when compared to the control culture.

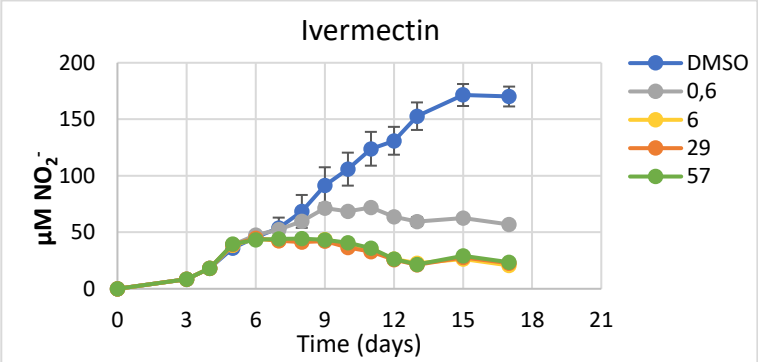

Figure 18. *The impact of ivermectin on the activity of Ca. N. sinensis.*

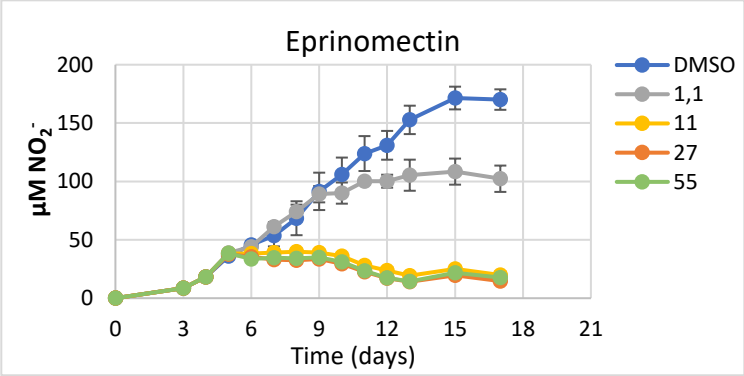

Figure 19. *Impact of eprinomectin on the activity of Ca. N. sinensis. strain*

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Table 12 presents the EC_{50} values (μM) for anthelmintics calculated based on their inhibitory activity on the ammonia oxidation capacity of *Ca. Nitrosotalea sinensis*. Standard errors are provided in brackets. An asterisk denotes the highest concentration examined and signifies cases where EC_{50} values could not be derived through statistical analysis.

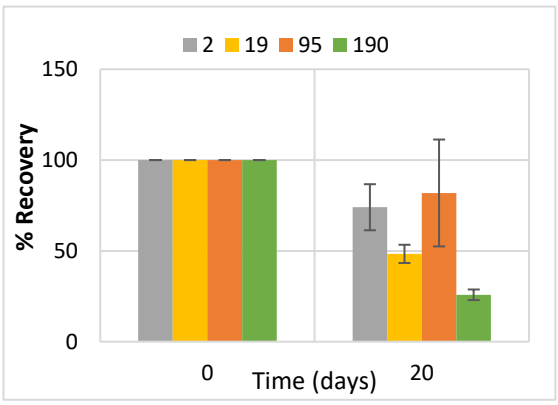
Table 12. *Average EC_{50} values (μM) for the anthelmintics investigated in this study, on the AOA strain Ca. N. sinensis.*

Anthelmintic	*Ca.* Nitrosotalea sinensis
Albendazole	<19
Ricobendazole	42.66 (± 15.67)[1]
Fenbendazole	<3.4
Ivermectin	0.43 (± 0.03)
Eprinomectin	1.81 (± 0.19)

3.3 Stability of anthelmintics in the liquid cultures of the AOA strains

The subsequent figures depict the outcomes of the HPLC analysis, conducted to assess the stability of anthelmintics in the liquid cultures of *Ca.* Nitrosocosmicus franklandianus and *Ca.* Nitrosotalea sinensis. The bars illustrate the standard error among the three biological replicates.

(a)



(b)

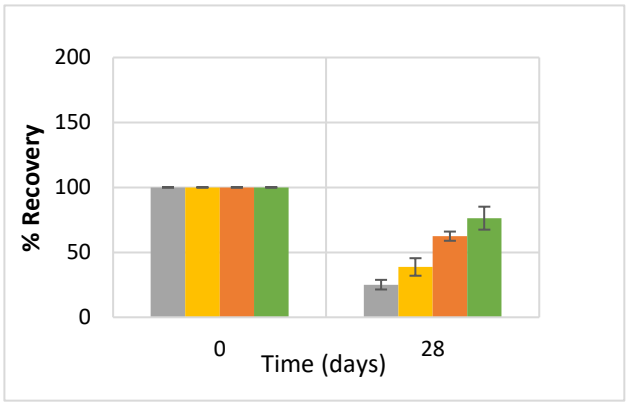


Figure 20. *Degradation of albendazole in the liquid cultures of (a) Ca. Nitrosocosmicus franklandianus and (b) Ca. Nitrosotalea sinensis.*

[1] The lowest tested concentration of 2 μM was not included in the EC_{50} calculation for ricobendazole

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The recovery of albendazole after 20 days in liquid cultures of *Ca.* N. franklandianus ranged from 26% for a 190 μM concentration to 82% for a 95 μM concentration.

In the case of *Ca.* N. sinensis, the degradation pattern of albendazole differed revealing an inverse relationship between the concentration of the compound in the culture and the percentage of degradation. The degradation of albendazole in the liquid cultures showed a progressive decrease from the lowest concentration (2 μM) to the highest concentration (190 μM), with degradation rates of 76%, 61%, 38%, and 24%, respectively.

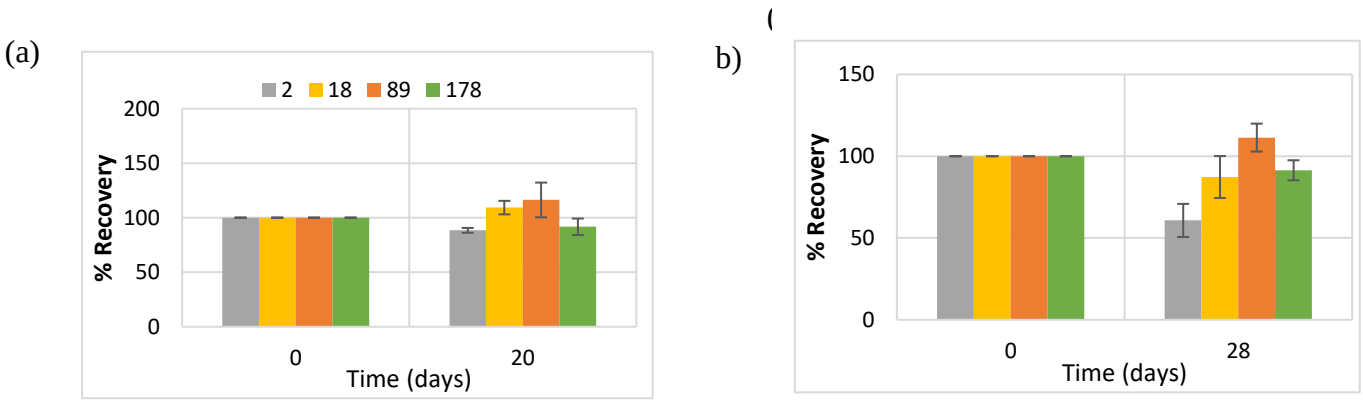

Figure 21. *Degradation of ricobendazole in liquid cultures of (a) Ca. Nitrosocosmicus franklandianus and (b) Ca. Nitrosotalea sinensis.*

Ricobendazole demonstrated a notable recovery percentage as depicted in Figure 21. This was observed in both liquid cultures of *Ca.* Nitrosocosmicus franklandianus and *Ca.* Nitrosotalea sinensis, with the lowest recovery values recorded at the 2 μM concentration, reaching 88% and 60%, respectively.

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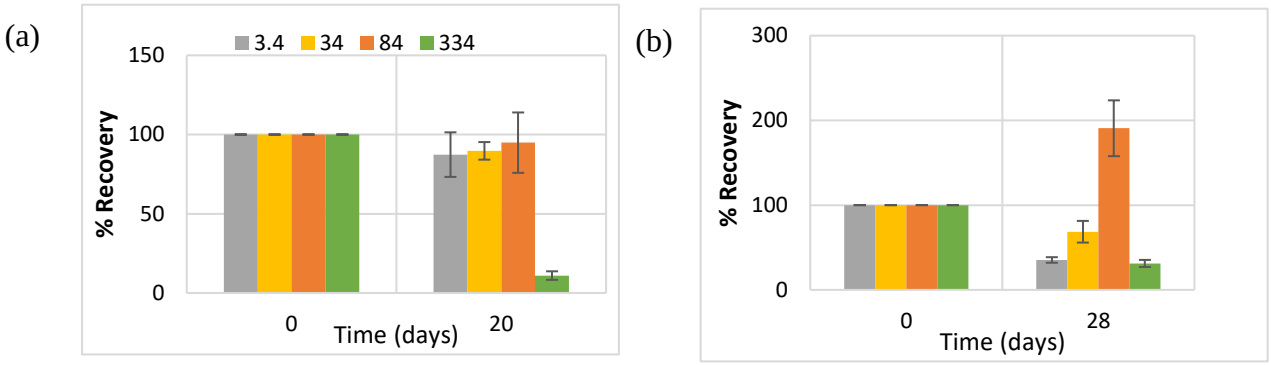

Figure 22. *Degradation of fenbendazole in liquid cultures of (a) Ca. Nitrosocosmicus franklandianus and (b) Ca. Nitrosotalea sinensis.*

Fenbendazole exhibited distinct degradation pathways in the two different AOA cultures. In the cultures of *Ca.* N. franklandianus, the 3 lowest concentrations- 3.4μM, 34μM, and 84μM- demonstrated high recovery percentages, with values of 87%, 89%, and 84%, respectively. Conversely, the highest concentration (334 μM) exhibited a lower recovery percentage, with the degradation rate reaching 89% (Figure 22a).

In the liquid cultures of *Ca.* N. sinensis, fenbendazole did not exhibit a high recovery percentage, with degradation percentages of 64% for the 3.4 μM concentration, 35% for the 34 μM concentration, and 69% for the 178 μM concentration. However, the 84μM concentration showed a remarkable 298% recovery percentage by day 28 of the experiment. These findings suggest potential solubility and bioavailability issues for the highest concentration of the tested compounds.

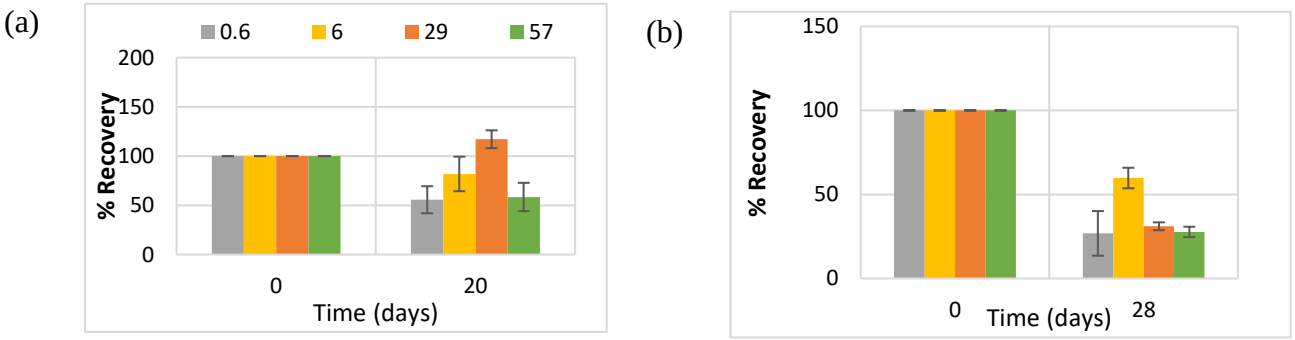

Figure 23. *Degradation of ivermectin in the liquid cultures of (a) Ca. Nitrosocosmicus franklandianu and (b) Ca. Nitrosotalea sinensis.*

Ivermectin exhibited a significant recovery rate in the liquid cultures of *Ca.* Nitrosocosmicus franklandianus throughout the experimental process. The lowest recovery percentage (56%) was observed at the concentration of 0.6 μM, while the maximum recovery percentage (117%) was observed at a concentration of 29 μM.

In contrast, in the cultures of *Ca.* Nitrosotalea sinensis ivermectin showed lower recovery, with the majority of concentrations exhibiting a degradation percentage of

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approximately 70%. The only exception was observed at the concentration of 6 μM, where 60% of the initial concentration was recovered.

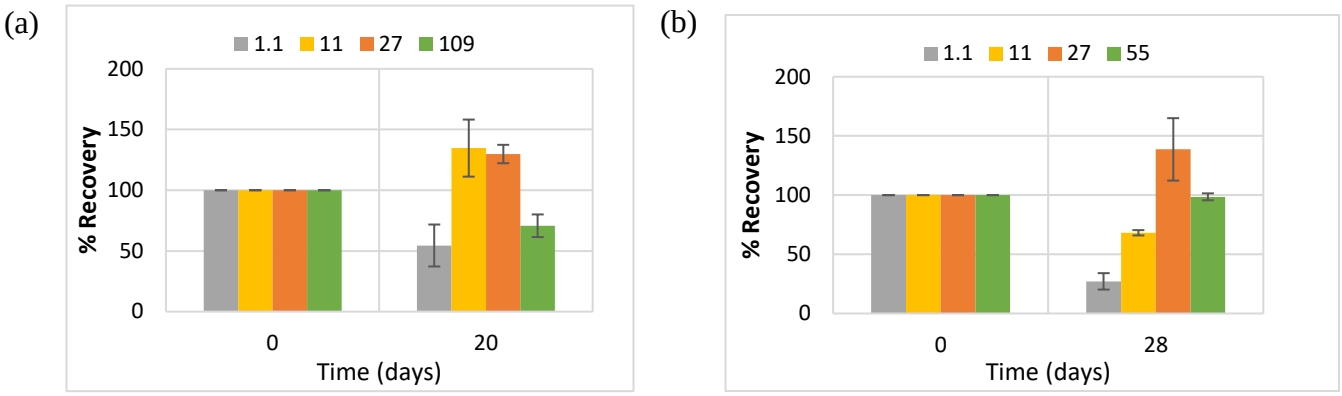

Figure 24. *Degradation of eprinomectin in liquid cultures of (a) Ca. Nitrosocosmicus franklandianus and (b) Ca. Nitrosotalea sinensis.*

Eprinomectin did not show a clear degradation pattern in the cultures of the two AOA strains. The cultures of *Ca.* Nitrosocosmicus franklandianus demonstrated a high recovery percentage at concentrations of 11 μM and 27μM, with the degradation of the compound peaking at the lowest tested concentration of 1.1 μM. Similarly, in the cultures of *Ca.* Nitrosotalea sinensis, the highest degradation of eprinomectin was observed at the lowest concentration of 1.1 μM, reaching to 74%.

4. Discussion

This research explores the toxicity of anthelmintics, specifically albendazole, ricobendazole, fenbendazole, and ivermectin, eprinomectin, categorized as benzimidazoles and macrocyclic lactones, respectively. Initially, the study aimed to determine the adverse effects of each of these anthelmintic classes on the activity of certain AOA strains in *in vitro* experiments. The impact of anthelmintics was evaluated by monitoring the production of NO_2^- in the liquid cultures of the selected AOA strains, while high-pressure liquid chromatography (HPLC) was employed to analyze the degradation and stability of the compounds in the liquid cultures. *In vitro* tests play a crucial role in assessing the toxicity of xenobiotic compounds, providing an accurate evaluation of their impact on AOM. These tests also allow for the identification of variations in toxicity among different microbial groups involved in the ammonia oxidation process, such as AOB and AOA. Additionally, *in vitro* tests serve as valuable tools for studying the mechanisms of xenobiotics toxicity.

The findings indicate that benzimidazole anthelmintics including albendazole, ricobendazole, and fenbendazole, did not significantly inhibit the activity of *Ca.* Nitrosocosmicus franklandianus within the tested concentration range. However,

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contradictory effects were observed in the case of *Ca.* Nitrosotalea sinensis. Despite the strong inhibition of the activity of this AOA strain by the tested benzimidazoles, its growth was only slightly influenced, as indicated by qPCR analysis showing minor variations in *amoA* gene abundance. These results contradict prior research suggesting the toxic impact of albendazole and its derivatives on the activity of soil AOM. Notably, earlier soil studies demonstrated the inhibitory effect of albendazole and its derivatives (albendazole sulfoxide and albendazole sulfone) on both AOA and AOB at certain concentrations (2 and 20 mg kg^{-1}) (Lagos et al., 2023). However, extrapolating from pure culture tests to predict effects in soil should be performed with caution. Additionally, contrasting findings from a study on thiabendazole, another benzimidazole used in fruit packaging plants, and its impact on the abundance of AOA at a dosage of 50 mg kg^{-1} soil, indicate the complexity of the observed effects, potentially influenced by parameters such as diffusion and degradation in the soil environment.

In contrast, macrocyclic lactones, eprinomectin and ivermectin significantly influenced the activity of both *Ca.* Nitrosocosmicus franklandianus and *Ca.* Nitrosotalea sinensis, even at lower concentrations. Nitrite production by *Ca.* Nitrosocosmicus franklandianus was notably affected by the highest eprinomectin concentrations, while *Ca.* Nitrosotalea sinensis exhibited reduced nitrite production when exposed to all tested concentrations of both eprinomectin and ivermectin. These results align with previous soil studies indicating decreased *amoA* gene abundance in AOA exposed to ivermectin and eprinomectin (Lagos, 2023). In contrast to a prior long-term on-field experiment that aimed to illustrate the impact of veterinary pharmaceuticals, such as ivermectin, momensin, and zinc bacitracin, on AOM, no significant influence of these substances on the nitrification rate or the abundance of AOM was observed under laboratory conditions. Nevertheless, tests conducted on treated soils did reveal an increase in the abundance of AOA compared to AOB, suggesting a potentially greater sensitivity of AOB to these treatments than AOA (Konopka et al., 2015).

Despite a limited body of research on the toxicity of anthelmintics to AOA, several studies have consistently indicated that *Ca.* Nitrosotalea sinensis is more susceptible to xenobiotic compounds compared to *Ca.* Nitrosocosmicus franklandianus. These investigations specifically explored the effects of pesticides such as iprodione and 3,5-dichloroaniline on AOM, as well as the impacts of nitrification inhibitors like dicyandiamide and 3,4-dimethylpyrazole phosphate. In both instances, *Ca.* Nitrosocosmicus franklandianus exhibited lower susceptibility to these substances, while *Ca.* Nitrosotalea sinensis was more adversely affected. (Vasileiadis et al., 2018; Papadopoulou et al., 2020).

In the analysis of the stability and degradation of anthelmintics in the liquid cultures of the AOA strains, no universal degradation pattern was identified. Notably, distinct degradation patterns were observed between the cultures of the two AOA strains. The variance in pH between the two culture media (alkaline vs. acidic) used for cultivating the selected AOA may contribute to these differences. Specifically, *Ca.* Nitrosocosmicus franklandianus was cultured at a pH of 7.5, whereas *Ca.* Nitrosotalea

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sinensis was cultivated at a pH of 5.3. Another potential explanation for the observed variations could be the limited solubility of the tested anthelmintics despite using DMSO as a solvent. This solubility issue has been examined in anthelmintic toxicity experiments in other model organisms (Oh et al., 2006; Puckowski et al., 2017). Previous research utilized TWEEN 20 as a solvent to enhance the solubility of anthelmintics and improve their bioavailability in axenic cultures of bacteria capable of degrading benzimidazoles (Lagos, 2023). Future experiments are anticipated to investigate whether the use of this substance will enhance the solubility and bioavailability of anthelmintics in liquid cultures of nitrifying strains.

5. Conclusions

In conclusion, this study marks the first *in vitro* exploration of potential adverse effects of anthelmintics on the activity of AOA. The findings of this research provide valuable insights into the toxicity of eprinomectin and ivermectin on two specific types of AOA, namely *Ca.* Nitrosocosmicus franklandianus and *Ca.* Nitrosotalea sinensis. Future investigations are expected to extend these effects to other soil AOA isolates within the *Nitrososphaera* genus, such as *N. viennensis*. Additionally, subsequent research will delve deeper into assessing the solubility and bioavailability of anthelmintics, utilizing the solvent TWEEN 20, while also exploring its potential toxicity in liquid cultures of diverse nitrifying microorganisms, including both bacteria and archaea.

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