Environmental Fate of Ivermectin and its biological metabolites in Soils: Potential implications for the Environmental Impact of Ivermectin Mass Drug Administration for Malaria Control

Gerald Enos Shija

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in
Crop and Soil Environmental Sciences

Kang Xia, Chair
Cassidy Rist
Roger Schürch
Issa Lyimo

December 06th, 2022
Blacksburg, Virginia

Keywords: Ivermectin, 3″-O-demethylivermectin, 24-Hydroxylmethylivermectin, degradation, temperate, tropical regions, soil, manure, pats, first-order kinetics.

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Academic Abstract

Despite significant vector control advancements in the past years, the current malaria trends suggest that new control strategies are urgently required. These new approaches should address the current frontline intervention challenges like increasing insecticide resistance in mosquitoes and residual transmission issues. Insecticide-treated livestock (ITL) is one of the novel potential strategies to overcome the above challenges. ITL involves treating livestock near humans with an insecticide like ivermectin (IVM) to kill zoophagic malaria vectors. However, ivermectin pharmacokinetics data suggests that most IVM-administered drugs remain intact, and more than 90 % of this drug is eliminated in feces. Biological metabolites: 3″-O-demethylivermectin (3DI) and 24-hydroxymethyl ivermectin (24OHI) are also excreted in feces. Therefore, using manure from treated cattle as fertilizers contaminates the soil, ground, and surface water with IVM or its metabolites through leaching and hydraulic water flow affecting the soil and aquatic ecosystems. Contemplating the contamination impacts, these drugs' environmental fate and effects could be regarded before massive IVM applications. Many researchers have tried to address this subject in temperate regions compared to the tropics, where IVM is urgently needed. Regional discrepancies such as soil types and climate can independently and dependently determine the fate and impact of ivermectin. Our research investigates the environmental fate of IVM and its primary biological metabolites. Laboratory and field studies in Tanzania and Virginia were conducted to simulate the difference between tropical and temperate climates. Soil and soil-manure mixture spiked with IVM were layered into two 5 mm layers in columns exposed to natural sunlight. The remaining IVM and its primary metabolite were quantified using Liquid Chromatography with a tandem mass spectrometry detector (LC-MS/MS. These compounds degraded up to 1.5 times faster in Tanzania than in Virginia, depending on temperature, soil depths and type, organic matter, and soil moisture. When IVM is subcutaneously injected into cattle, drug residues and metabolites: 3DI and 24OHI are excreted in feces following a positive skewed Poisson distribution profile. IVM, 3DI, and 24OH were found to degrade rapidly when cattle pats when exposed to the field. Since we conducted our study in the Summer, no IVM or its metabolites leached into the soil beneath. The obtained half-lives suggest that ivermectin’s massive drug administration has little to worry about, primarily when the dung from treated cattle is spread into the field in thin layers in the Summer before farm application.

Environmental Fate of Ivermectin and its biological metabolites in Soils: Potential implications for the Environmental Impact of Ivermectin Mass Drug Administration for Malaria Control

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General Audience Abstract

Despite significant vector control advancements in the past years, the current malaria trends suggest that new control strategies are urgently required. These new approaches should address the current frontline intervention challenges like increasing drug resistance in mosquitoes and residual transmission issues. Treating cattle with ivermectin is one of the novel potential strategies to overcome the above challenges. This strategy is effective because the amount of ivermectin (IVM) found in the blood of treated cattle is enough to kill malaria vectors feeding on them. However, the literature suggests that most IVM-administered drugs remain intact, and more than 90 % of this drug is eliminated in feces. Metabolite bioproducts: 3″-O-demethylivermectin (3DI) and 24-hydroxymethyl ivermectin (24OHI) are also excreted in feces. Therefore, using manure from treated cattle as fertilizers contaminates the soil, ground, and surface water with IVM or its metabolites through leaching and hydraulic water flow affecting the soil and aqua ecosystems. Contemplating the contamination impacts, these drugs' environmental fate and effects could be regarded before massive IVM applications. Many researchers have tried to address this subject in temperate regions compared to the tropics, where IVM is more needed. Regional discrepancies such as soil types and climate can independently and dependently determine the fate and impact of ivermectin. Our research investigates the environmental fate of IVM and its primary bioproducts. Laboratory and field studies in Tanzania and Virginia were conducted to simulate the difference between tropical and temperate climates. Soil and soil-manure mixture spiked with IVM were layered into two 5 mm layers in columns exposed to natural sunlight. The remaining IVM and its primary metabolite were quantified on the appropriate instrument. These compounds degraded up to 1.5 times faster in Tanzania than in Virginia, depending on temperature, soil depths and type, organic matter, and soil moisture. When IVM drug is injected into cattle, the intact drug and its bioproducts: 3DI and 24OHI, are eliminated in feces following a favorable skewed normal distribution profile. IVM, 3DI, and 24OH were found to degrade rapidly when cattle pats when exposed to the field. Since we conducted our study in the Summer, no IVM or its bioproducts leached into the soil beneath. The obtained data suggest that ivermectin’s massive drug administration has little to worry about, primarily when the dung from treated cattle is spread into the field in thin layers in the Summer before farm application.

Acknowledgments

Foremost, I thank almighty God, as everything in my life, including my existence, is due to him. I always trust my **Lord Jesus Christ** with all my heart and am not confident in my grasp. Secondly, I am deeply grateful to my advisor Dr. Kang Xia for her confidence in me and invaluable tutelage during my Ph.D. study and thesis writing. I thank her for her patience, positive motivation, enthusiasm, and knowledge. Through her top-notch guidance, I could do my Ph.D. exploration and write this Thesis. I could not have envisioned getting a better advisor and mentor for my Ph.D. research. Thirdly, besides my supervisor, I would like to convey my sincere gratitude to the rest of my thesis committee: Dr. Cassidy Rist, Dr. Issa Lyimo, Dr. Roger Schürch, and Dr. Chao Shang for their inspiration, perceptive comments, and challenging questions. Fourth, I want to offer special thanks to my wife: Beatrice Baltazary Mtenga, and my daughter Rita Gerald Shija for their devotion and love. You were always on my side, encouraging me to work hard and believe even when it seemed impossible. Your support was pure, and I will never forget that and work hard for you and other coming family members. My gratitude extends to Fr. Erick Wanyonyi Simiyu, Sister Florenciana, Pastor John, Pastor David Makwenda, Fr. Patrick, Disciple Godfrey, and Fr. Lado for their prayers and guidance. This group of servants of God has been crucial for how I approached and conducted my Ph.D. study.

I thank my fellow current and former laboratory mates in Dr. Kang Xia's research group: Dr. Sheldon, Dr. Aihua, Amina, Brandon, Chaoqi Chen, McAlister Council-Troche, and Dr. Thomas Dick, for their valuable contributions to method development, instruments operation, training, and sample preparations. As lab mates, we had stimulating discussions and sleepless nights working together towards deadlines, and we had fun for the past three years. I also thank Miriam Ruhinda, Alphonse Assenga, Ally Daraja, Fecilian Meza, Cassian, Ngunda, Leticia Makwara, Sangiwa, Nuru, Yusta, Bona, Omary, and Dora Meta for their valuable contributions to the field works, laboratory organizations in Tanzania, and prayers. Last but not least, I would like to thank my mother: Leah Joseph Mchembe, Uncle: Gideon and John, and my late father, B. Shija, for their support. Thanks to Mzee Elia and Massunga for Cattle volunteering in Ifakara, Tanzania, and SPES, USA.

'My wish is that you should have at least form 4 education level' Francisco J. Maige (R.I.P)

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1.1 Chemistry, Usage, and Biological Metabolism of Ivermectin

1.1.1 Chemistry

The antiparasitic agent, ivermectin, **Figure 1.1**, is a semi-synthetic drug obtained from fermentation by selective reduction of olefins [1]. Structurally, ivermectin component compounds (≥ 80 % H_2B_{1a} and < 20 % H_2B_{1b}) are made of a dihydroxycyclohexene ring fused to a tetrahydrofuran moiety [1]. It is because of its structural arrangements and being among members of the avermectin family that H_2B_{1a} and its homology H_2B_{1b} are called 5-O-demethyl-22,23- dihydroavermectin A_{1a} and is 5-O-demethyl-25-des(1-methyl propyl)-22,23-dihydro-25-(1-methyl ethyl) avermectin A_{1b} respectively [1]. Ivermectin, as its composition, has a variable molecular weight ranging from 872.21 to 875.10 calculated from the percentage composition and empirical formula of H_2B_{1a} and H_2B_{1b} [1].

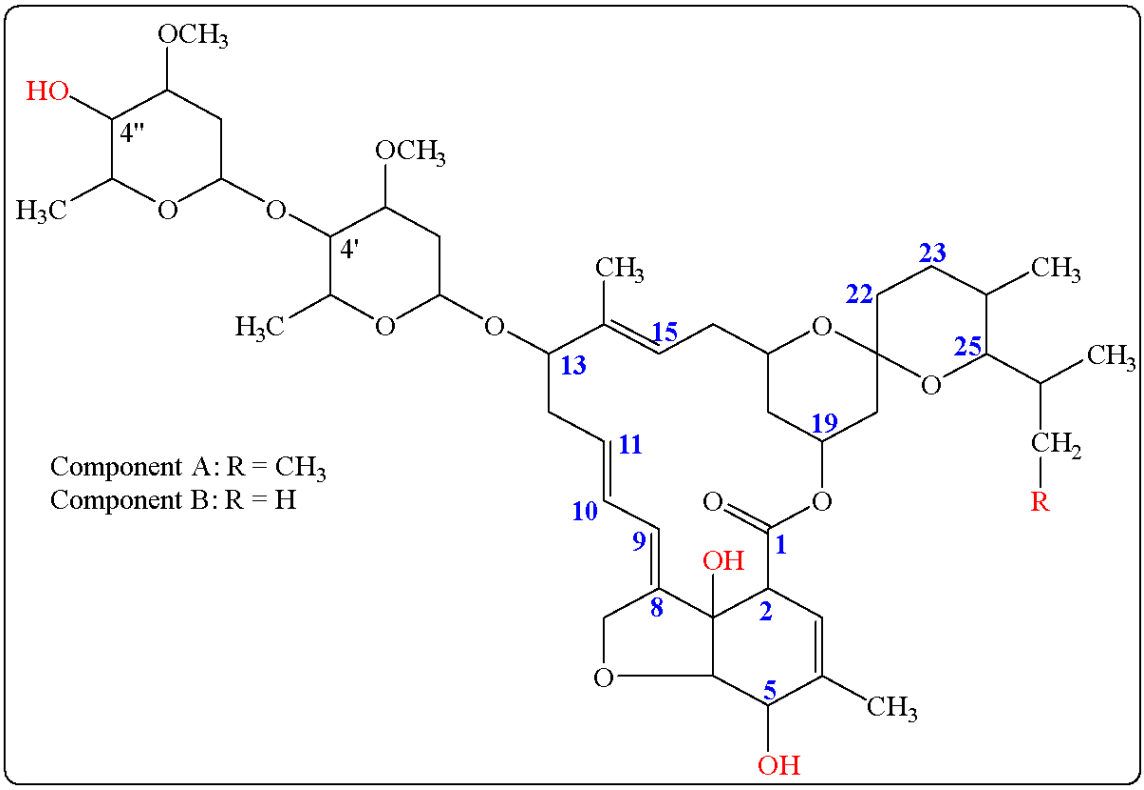

Figure 1.1: Ivermectin structure showing the two components H2B1a and H2B1b

Although ivermectin contains two hydroxyl groups that can form hydrogen bonding with a polar solvent, the hydrocarbon part controls its solubility [1]. Thus, ivermectin solubility in water at room temperature is about $1\,\mu g/cm^3$ or less [1]. Though it is nevertheless insoluble in water, it does dissolve in other protic solvents like butane1-ol, methanol, and hexan-1-ol. The insolubility properties of ivermectin in water should not be considered divergent from its lipophilicity nature [1]. There is no significant relationship between solvent polarity and solubility except in hydrocarbons which rank solubility to dipole moments [1]. That is why some nonpolar solvents confirm less ivermectin solubility in these solvents regardless of their polarity [1]. Although the solubility properties of ivermectin lack a clear trend and are difficult to predict, this partition behavior among solvents affords extensive solvent combinations for sample preparations and instrumental analysis [1].

Ivermectin is very stable in a solid powdered state, especially when there are no extraneous reactants or impurities to react with [1]. However, its stability decreases significantly when in solutions because this drug has many functional groups prone to participation in various reactions [1]. Some of these reactions are pH-dependent, as indicated by a change in stability with a shift in pH leading to acidic or basic hydrolysis. For example, at pH = 6.3, ivermectin does not undergo significant degradation [1]. However, a noticeable acceleration of ivermectin degradation in solution is revealed at other pH values [1]. The pH-reaction rate profile well depicts pH = 6.3 as the optimum value, with the rest of the pH values falling into either acidic or basic hydrolysis categories. Under acidic hydrolysis (pH less than 6.3), ivermectin degradation by hydrolysis of its two

sugar rings forming monosaccharide and aglycone degrades as main products. However, at pH above 6.3, ivermectin undergoes isomerization reactions or basic hydrolysis [1].

Light and the nature of the solvent play a key role in ivermectin degradation in solutions. While ivermectin is stable in the dark in aliphatic hydroxylic solvents, it is photolabile in aromatic solvents under the influence of fluorescent and UV radiations [1]. Moreover, under nonpolar solvents, ivermectin undergoes an oxidative degradation reaction at the 8a-methylene site, leading to the formation of 8a-hydroxy, 8a-aldehyde, and other oxidation products [1]. Another crucial structural aspect of ivermectin controlling its properties is the presence of a pharmacophore made of a 16-membered macrocyclic backbone [2]. This structure backbone is responsible for various properties combating endo- and ectoparasite-like nematode and arthropod parasites in animals and humans[3].

1.1.2 Usage

The veterinary industry was the first to utilize ivermectin for its antiparasitic properties in the early 1980s for combating horse nematode *Onchocerca cervicalis* [4]. The considerable achievement of this “wonder drug” in the veterinary industry caused its extended use to humans in 1987, treating river blindness and reducing its clinical symptoms, which usually overwhelmed poor rural communities, especially in America and Africa. Due to its success, ivermectin was used in onchocerciasis elimination programs in Africa and other places worldwide[4]. The usage of ivermectin has increased in the past 30 years, especially after the introduction of mass drug administration (MDA) to battle many tropical neglected diseases (TNDs). More than 600 million doses have been approved since the first MDA, with around 70 million people taking this drug[5]. However,

these numbers are expected to inflate in the coming years because of the application of ivermectin in controlling many diseases, including malaria.

Malaria is an essential vector-borne parasitic disease responsible for significant human morbidity and mortality of many people worldwide, significantly less than five years of children and pregnant women[6]. Although malaria cases are reported in about 90 countries worldwide, there is a significant difference in the number of reported cases and deaths[6]. For example, World Health Organization (WHO) approved China to be a malaria-free country in 2020; in the same year, tropical sub-Saharan Africa reported more than 95 % of malaria affliction[6]. On the way to be more precise, even though the problem perseveres mainly in the tropics, malaria can be understood as principally an African problem. The good news for Africa is that malaria-transmitting mosquitoes are susceptible to ivermectin even at low concentrations compared to other tested vectors offering an auspicious tool for malaria control [7-9]. Laboratory and field studies indicate that *Anopheles gambiae s.s.* may die due to the consumption of the blood of ivermectin-treated cattle. This implies that after a standard dose administration, mosquitoes can be destroyed by ivermectin concentrations in human and animal blood [10]. During MDA programs under clinical trials, ivermectin is administered orally, poured on, or through subcutaneous injection in livestock, depending on the animal type [11]. In humans, the oral administration route is the only approved ivermectin formulation.

1.1.3 Biological Metabolism in Livestock

Ivermectin metabolism by livestock is one of the most crucial determinants of the pharmacokinetic profile of this drug in a particular species [12]. Species with rapid ivermectin metabolic activities usually lead to inadequate bioavailability and high drug

elimination as metabolites or low residues parent drug eliminations [13]. In some cases, the increase in metabolic product elimination may change the potential environmental toxicological implications of ivermectin parent drug or its metabolites. Therefore, excellent knowledge of the ivermectin metabolism in cattle, which is expected to be used for malaria vector control, is crucial in the drug fate and impacts studies.

The pharmacokinetic studies performed in cattle indicate that the ivermectin administration route affects the distribution and rate of metabolization in the body [12]. While subcutaneous injection attained the highest plasma bioavailability compared to the self-release bolus and pour-on administration route, pour-on recorded the highest fecal concentration compared to the other two formulations [11]. Similarly, compared to the single bolus study above and oral administration, subcutaneous injection substantially boosted the area under the plasma concentration-time curve or systemic availability of ivermectin [11]. Bioavailability is another factor that is determined by the route of administration. While subcutaneous injection demonstrates the highest bioavailability, the topical administration route has the lowest bioavailability, with oral administration sitting between the two formulations [13]. Moreover, although parenteral administration retards drug absorption compared to the oral route, it is the one that leads to higher bioavailability, prolonged activity period, and hence excellent efficacy against parasites [14].

Another factor that affects ivermectin distribution and bioavailability is its chemistry (**see section 1.1.2**). This drug is said to be lipophilic in nature, therefore, has a high affinity to fats, oils, lipids, and organic matter [13]. That is why it is unsurprising to accumulate in fatty tissues of the target organism, which acts like ivermectin's reservoir. On this basis, the highest amount of ivermectin is recorded in the liver, followed by fat, and the least

amount is reported in brain tissues [13]. It is through accumulation that ivermectin perseveres in the body for a prolonged period, experiencing low plasma elimination at the trend pigs > goats > sheep > cattle [13].

The ivermectin accumulation and persistence in the edible tissues has emerged as a safety concern to the meat-consuming public [15-17]. To address this challenge, ivermectin metabolism studies have been carried out in rats, cattle, sheep, and pigs [13, 18-21]. These studies aimed and identifying these metabolites, determining their amount, distribution, and elimination profiles. Moreover, studies addressed the excretion profile of ivermectin residues and the withdrawal period in different species. Findings from these studies suggest that most of the administered drug remains unchanged, producing tiny quantities of both significant and minor non-polar metabolites depending on the studied species (see **Figure 1.2**). The 24-desmethyl-24-hydroxymethyl-H_2B_{1a} and 24-desmethyl-24-hydroxymethyl-H_2B_{1b} are the primary ivermectin metabolites isolated in vivo in all the above species[22]. These metabolites are reported to be more water soluble than ivermectin with less environmental toxic effects. Apart from the above metabolites, pigs' and goats' metabolic activities on ivermectin produce 3″-O-desmethyl-H_2B_{1a} and 3″-O-desmethyl-$H_2B_{1b,}$ respectively [23]. Moreover, metabolites with lower polarity than those mentioned above have been reported in cattle and sheep fat tissue, suggesting that a small number of metabolites produced in these species undergo an esterification reaction to less polar minor metabolites [24]. Although ivermectin undergoes metabolism into different polar and non-polar, including minor products, the metabolite accounts for less than 10% of the administered ivermectin regardless of the animal species and the mode

of administration[13]. Therefore, more than 90% of parent drugs plus the produced metabolites are discharged into the environmental compartments.

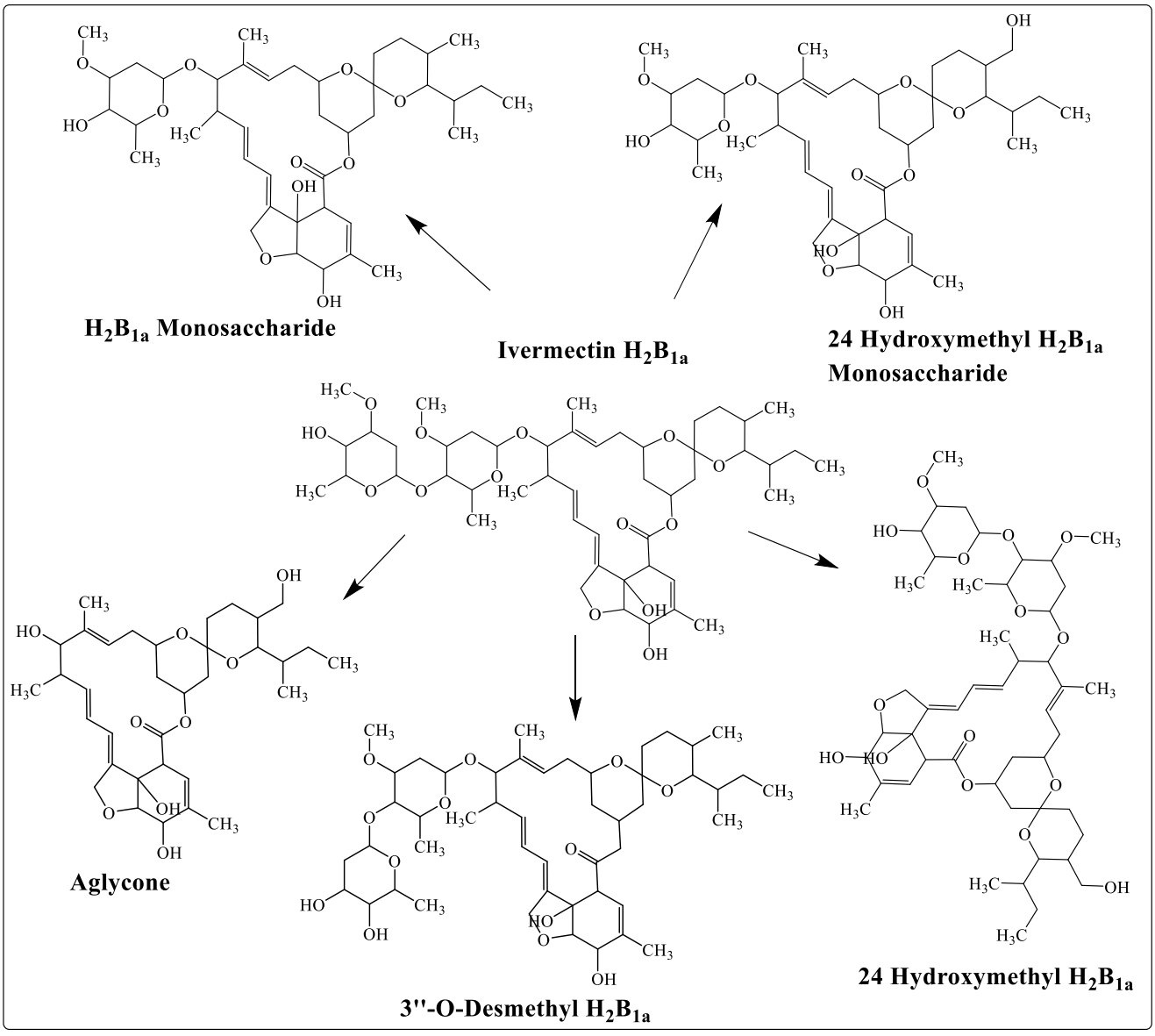

Figure 1.2: Ivermectin metabolic pathways lead to the formation of polar and non-polar metabolites. This diagram was drawn from the literature of Miwa and coworkers[22]

1.2 Pathways of Ivermectin and its Metabolites in the Environment

As pointed out, most of the administered ivermectin still needs to be metabolized regardless of the animal species and mode of administration. In cattle, for example, more

than 40 % of the administered dose remains as an active parent drug. Most of these remaining ivermectin residues and metabolites are eliminated into feces, with less than 2 % excreted in the urine [24, 25]. A small percentage (about 1 %) of ivermectin residues are eliminated in milk in humans, dairy cows, ewes, goats, and camels[26-28]. The ivermectin excretion data are consistent with its high lipid solubility [24, 26, 29].

Table 1.1 below summarizes some of the reported ivermectin in feces for some routes of administration.

Table 1.1: Ivermectin fecal excretion

Reference	**Administration**	**Cattle(kg)**	**C_{max} (ppm)**
[30]	0.2 mg per kg bw (SCI)	-	3.9
	0.5 mg per kg bw (PO)	-	8
[31]	12 mg day^{-1} for 135 days (SRB)	300	1.4
[32]	0.5mg per kg bw Topical	361	8
[33]	12 mg day^{-1} for 135 days (SRB)	220	4
[11]	0.2 mg per kg bw (SCI)	400	1.2
	0.5 mg per kg bw (PO)	400	18.5
	12.7 mg day^{-1} for 135 days (SRB)	400	4
[34]	0.2 mg per kg bw (SCI)	276	0.4

SCI = Subcutaneous injection, PO = Pour-on, SRB = Self-release bolus, C_{max} = Maximum concentration recorded in the dungs.

These significant values reported in a unit kilogram of the dry weight of feces suggest that bile is the main ivermectin elimination route [11]. The ivermectin elimination rate varies with time, with the maximum excreted amount (C_{max}) reported on day 2 to day five in most studies [30-34].

The data in **Table 1.1** indicate that the route of administration and difference in animal body weight plays a crucial role in determining the amount of excreted ivermectin residues [33, 35]. As the general rule of thumb, ivermectin's pour-on administration results in higher drug residues in manure compared to subcutaneous injection, and oral administration leads to rapid drug elimination. The diet of animal species also plays a crucial part in the excretion profile of ivermectin in feces [36]. For example, Cook et al. [36] reported that in grazing cattle, ivermectin excreted in feces was far less (0.09 ppm) than in grain-fed cattle (0.36 ppm).

The ivermectin residues and metabolites may enter the terrestrial compartment via the spreading of manure from intensively reared animals on arable land or by excretion of dung by animals on pastures [37]. Similarly, ivermectin residues can directly contaminate surface water via treated cattle standing in shallow water bodies [37]. In addition, indirect release into the water could arise through leaching from polluted soil or manure into groundwater or via run from grazing or arable land after the application of dung from treated animals [37].

The sediment compartment may be contaminated via transfer from surface waters into sediments or sedimentation of eroded material from pastures or arable land [37]. Because of its high affinity for soil and particulate matter, neither leaching nor runoff is assumed to be a significant source for contamination of freshwater ecosystems with ivermectin [38].

However, transporting absorbed ivermectin residue with eroded soil might be possible. The risk of soil translocation from erosion is highest when crop coverage is lowest, i.e., in the fall after harvesting or in spring before seeding [38]. The postharvest period with a high erosion risk coincides with when large animals are treated with ivermectin, and farmers can spread manure [38]. It may, in some regions, also coincide with the time of intensive rainfall events, initiating soil erosion [39]. **Figure 1.3** below summarizes the ivermectin and its metabolite's pathways into environmental compartments.

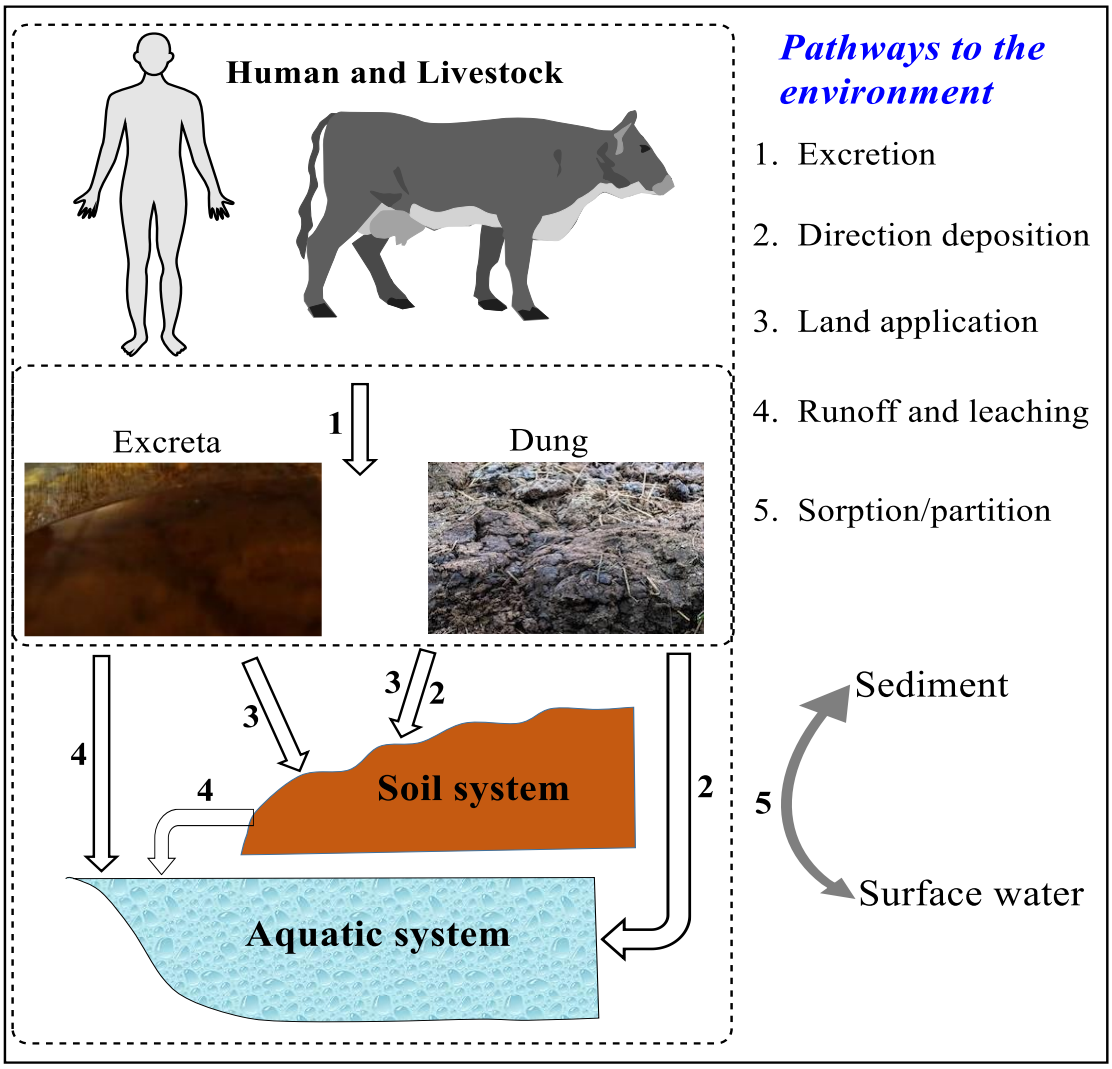

Figure 1.3: Ivermectin and its metabolite's pathways into different environmental compartments

1.3 Environmental Fate and Impacts of Ivermectin and its Metabolites

1.3.1 Environmental Fate in Manure and Soil

As highlighted in Section 1.2, ivermectin and its metabolites in a terrestrial environment is either transformed, distributed to other environmental compartments, or absorbed in plant and animal tissues [40]. The distribution of ivermectin and its metabolites to different ecological compartments occurs through leaching or hydraulics runoff [40]. The magnitude of these three pathways depends on the solubility properties of these drugs. While under the leaching process, these drugs percolate through the soil contaminating groundwater; hydraulic runoff involves the surface and subsurface flow of the medicines in aqueous or particulate phases [40]. The degree to which these paths transfer ivermectin and its metabolites will be firmly influenced by the compound's sorption properties, season, and the nature of the hosting terrestrial matrix (pH and cation exchange capacity affect ionic binding, the potential of ivermectin)[41]. Since 3″-O-demethylivermectin and other ivermectin metabolites are more polar than ivermectin[42], it is more likely to leach with water through the soil or manure matrix. Halley's column experiment well documented the leaching difference between ivermectin and its metabolite [42]. Under this experiment, it was found that while metabolites and degradation products leached through the soil column, ivermectin was tightly bound to the soil at the top of the column [42].

Ivermectin leaching is proportional to the water movement rate [43]. Thus, this drug residue's ultimate leaching and subsurface runoff occur in highly procurable, sandy soils that are also low in organic matter. Soil organic matter affects leaching and subsurface runoff by providing more surface area for adsorption and altering soil pH [44]. Moreover, while more clayed soil holds more water and absorbs chemicals, highly weathered soil

with more Al and Fe-oxide content at low pH provides positively charged sites for the sorption of deprotonated ivermectin and 3″-O-demethylivermectin functional groups, slowing leaching [44].

The amount of water in the soil affects the chemical leaching process, too [43]. Water encourages leaching compared to dry soil because when water is added to dry soil, it just fills the soil pores slowing its rate of moving to the subsurface and probably reaching the water table [44].

Although leaching and hydraulic runoff play a part in ivermectin distribution, researchers report that ivermectin is rarely mobile in the soil and does not translocate readily into the groundwater [42, 45]. Thanks to its low solubility in water and high affinity for soil and manure, as indicated by its high octanol-water partition coefficient (K_{ow} = 1651) and organic-carbon binding constant (K_{oc} = 12600-15700) values [45]. Therefore, ivermectin has low water solubility and vapor pressure and is firmly bound to the soil and manure particles. This means it cannot evaporate and is retained much in soil and manure particles. Consequently, the ivermectin contained in the soil and manure is subjected to absorption by terrestrial flora and fauna and degradation/transformation [46, 47]. Ivermectin fate absorbed in plants and animal tissues depends on several factors, including the species type. For example, ivermectin absorbed in the ribwort plantain is subject to transformation via demethylation and hydroxylation [48].

Although leaching, hydraulic runoff, and absorption play some role in ivermectin and terrestrial fate, degradation plays the most crucial role in this regard. However, a different pattern might be predicted for 3″-O-demethylivermectin and other metabolites, which are more soluble than the parent drug. Therefore, depending on soil properties and moisture

contents, 3″-O-demethylivermcetin, and other ivermectin metabolites are more prone to leaching and percolation to lower soil horizons and contaminate ground water [42]. However, if 3″-O-desmethlyivermectin is applied in dry soil with limited leaching/percolation, it remains on the surface and can undergo photodegradation. Conversely, ivermectin is lipophilic and remains at the soil or dung surface and, therefore, is more prone to degradation even under leaching favorable environments.

Ivermectin degradation is divided into two main parts: aerobic degradation and photodegradation [45]. Under photodegradation, ivermectin residues trapped within the manure, soil, or soil manure mixture have been reported to undergo degradation to polar products, with their polarity and solubility in water increasing with time [42]. For example, according to Halley et al., ivermectin dosed in soil and steer feces/soil samples within two weeks degrades to aglycone and monosaccharides of the ivermectin components as major degradation products [42]. The produced degradation products are unstable as they further degraded to more polar products within eight weeks [42]. The persistence of ivermectin residues in these matrices will depend on many factors, including climatic conditions, season, and location, sorption capacity, oxygen availability, and matrix depth [49]. **Table 1.2** below summarizes some findings on photodegradation. A close look into the above results make it very clear that factors like the type of soil, the ratio of manure and soil, and temperature play a crucial role in the photodegradation rate. While there was no significant photodegradation of ivermectin in manure only[50], manure combined with soil enhances degradation [42, 51].

Table 1.2: Half-life of ivermectin photodegradation in different experimental settings

Reference	Experiment	Half-life, $t_{\frac{1}{2}}$ (d)
[42, 51].	Degradation of ivermectin in the soil-manure mixture during the summer	$7 \leq \mathbf{t_{\frac{1}{2}}} \leq 14$
	Degradation of ivermectin in the soil-manure mixture during the winter	$91 \leq \mathbf{t_{\frac{1}{2}}} \leq 217$
	Outdoor, thin, and dry film experiments	3 h
[30].	Degradation of ivermectin in manure	$\ll$ after 5 d
[52].	Degradation of ivermectin manure	3.5
[53].	Photodegradation on thin, dry film	2-3 h

The half-lives are reported in days except stated otherwise.

The temperature has also considerably influenced a compound's photodegradation rate. For example, while the half-life of 91–217 d was documented for ivermectin in a soil-manure mixture through winter weather environments at 22 ^{o}C, ivermectin photodegraded much more rapidly in the same mix during the summer period at an average of 31 ^{o}C, with a half-life of 7–14 d. These findings suggest that the application of manure from ivermectin-treated animals should be made during the summer to minimize its impact through fast degradation [42, 51]. Aerobic degradation is another [54] pathway that eliminates ivermectin from terrestrial environments through microbial activities. This degradation process leads to the formation of more water-soluble compounds than ivermectin. The rate of aerobic degradation depends on temperature. Thus, ivermectin degrades faster in the summer compared to its degradation rate in winter [42]. The higher

ivermectin degradation rate in the summer can be associated with reduced ivermectin-matrix attraction potential at low moisture contents [55].

Although more degradation studies are conducted, and the kinetics of these reactions are well documented, especially in temperate regions, there needs to be more information on tropical areas. Moreover, identifying degradation products in terrestrial environments is still a work in progress. For example, Krogh's [49] research group isolated and quantified 22,23-dihydroavermectin B_{1a} monosaccharide and 22,23-dihydroavermectin B_{1a} aglycone as ivermectin transformation products in the soil. However, these two products were less than 10% of the parent drug; therefore, it was logically concluded that no transformation products were observed.

1.3.2 Environmental Fate in Surface Water

When ivermectin and its biological metabolites enter the aquatic system, several mechanisms include partitioning between water and sediments, absorption by plants and animals, and degradation occurs [1]. The extent of partition between solid and water compartments depends on the amount of organic matter in the soil and the water pH [1]. Although there is no information partition and distribution of ivermectin biological metabolites in the aquatic system, it is expected to remain more in the aqueous phase than ivermectin due to polarity factors [1]. The extra OH group, which forms hydrogen bonding with polar solvents, causes the improved solubility of 3″-O-demethylivermectin in this substance compared to ivermectin. On the other hand, generally, ivermectin undergoes hydrolysis reaction in solution at a pH greater or less than 6.3, although it is very stable at a pH of 6.3 [1]. Moreover, although at moderate acidity (pH between 4 to 5), ivermectin is subjected to acidic hydrolysis, the drug itself is neutral. This neutrality

behavior further reduces ivermectin affinity with water; therefore, hydrophobic interaction between ivermectin and soil increases [1]. However, the ivermectin disappearance from the aqueous phase cannot be explained by partitioning to the sediment and organisms' absorption without considering degradation reactions. A variable half-life is reported in aqueous media as applied to terrestrial environments. Halley et al. reported on degradation via photolysis in the terrestrial environment that ivermectin decreased to half its initial concentration in 12 h [51]. This finding differed from his other study conducted in dry, thin film settings [42]. Under the dry, light film experiment, a half-life of about four-fold less than the previous study was reported [42]. Although these findings differ in half-lives, they do not rule out that ivermectin degrades significantly in water than it does in soil or manure by far. However, in the aquatic and terrestrial environments, photodegradation determines the accumulation, final products' persistence, and nature of the absolute stances. Where photodegradation is low, expansion and persistence of ivermectin will increase. On the contrary, when photodegradation is significantly high, accumulation and endurance in those environmental compartments will be reduced. This further suggests that reactions impact the fate of ivermectin in aquatic and terrestrial environments.

1.3.3 Environmental Impacts on Aquatic Ecosystems

The ivermectin impact on aquatic ecosystems has been studied on different species in both freshwater and oceans. Similarly, 3″-O-demethylivermectin impact on *Daphnia magna* is well documented in the literature [42]. Ivermectin impact assessment on *Daphnia magna* is the most widely used methodology for toxicity tests because it allows for distinctive variables to be measured and used for gauging toxicity. Currently, toxicity

tests in Daphnia dogma evaluate factors like restriction in mobility and lethality and acute immobilization assessment.

All the findings speculate that one must investigate drug input and output into those aquatic systems to understand its impact. As reported in the previous section, ivermectin contaminates the marine system through hydraulic runoff and treating animals in shallow water. Likewise, 3″-O-demethylivermectin is more likely to percolate and leach into the aquatic systems [42]. But when these drugs enter the water systems, distribution depends on many factors, including solubility and sediments. The rapid sorption of ivermectin to deposits may cause ivermectin accumulation in sediments, thus attaining levels that affect benthic organisms, particularly after continual exposure [42]. The opposite is likely for 3″-O-demethylivermectin, which is more soluble than ivermectin and will be much more in water than segments [42]. Degradation under oxygen or sunlight and absorption by aquatic plants and animals will determine ivermectin persistence. However, impacts will depend on species and other factors. The following studies have tried to address the ivermectin impacts on aquatic ecosystems. Halley and co-workers found that *Daphnia magna* was very sensitive to ivermectin, with LC_{50} reported around 25 ppt [42]. However, the sensitivity of the same species to ivermectin decrease by 100-fold in the presence of sediments. Contrary to *Daphnia magna*, bluegill sunfish and rainbow trout were not sensitive, as indicated by LC_{50} being above 4800 and 3000 ppt, respectively [56]. The higher tolerance for fish is attributed to the fact that ivermectin does not bioconcentrate and is not retained in fish, i.e., bioconcentration value of 52 and half-lives of elimination from fat and liver is 4.3 and 4.7 days, respectively [23]. However, the findings in fish are sometimes different. The study on salmon indicated that ivermectin at 0.178 ppm was

fatal to this species [57]. Similarly, Domingues *et al.* noticed the behavior change of adult *Danio rerio*, a small freshwater fish, induced by chronic exposure to ivermectin at 0.25 to 25 µg/L concentrations range. At 0.25 µg/L, the fish spent most of their time at the bottom of the tank and eventually lost weight [57]. At 25 µg/L, the fish lacked energy and enthusiasm and developed dark coloration and mild curvature of the spine with severe effects in males [57]. Like the bluegill sunfish and rainbow trout, no toxicity to the growth of algae exposed to ivermectin at 9 ppm was observed [57]. Another study reported contrasting findings indicating that while benthic microcrustaceans (i.e., cladocerans, ostracods) and nematodes were negatively impacted by ivermectin, tardigrades benefitted from the presence of the drug, which reduced their predators [57].

Although there is no clear trend on ivermectin toxicity to aquatic organisms, factors like the type of the species, presence, or absence of sediments in water, sex of the species, the concentration of the drug, and time of exposure play a crucial role. That means findings from one species cannot necessarily be extrapolated to another.

1.3.4 Environmental Impact on Terrestrial Organisms

Ivermectin is reported to affect both target and non-target organisms. While affecting targeted organisms is the primary chemotherapeutical objective, damaging non-target organisms is the leading environmental concern. **Table 1.3** summarizes some of the impacts of target and non-target ivermectin on terrestrial organisms.

It is essential to note that while some studies provide supporting findings, others contradict each other. The impact on terrestrial organisms varies depending on species, sex, age, climate, and drug administration mode.

Table 1.3: Selected ivermectin impacts on terrestrial

Organism	Impact	Ref.
Microbes	Ivermectin showed no impacts on fungus or bacteria, even at higher concentrations of up to 2 g/kg. Furthermore, ivermectin and its metabolites show no effects on soil-dwelling bacteria metabolic activities at 0.03 µg/kg or higher.	[58] [42]
Earthworms	Twenty-eight days of exposure to 315 ppm of ivermectin is lethal to about 50 % of earthworms in the soil. No ivermectin toxicity to earthworms was recorded in cow dung collected after intraluminal bolus delivering 12 mg of this drug daily.	[42] [54]
Diptera, Muscidae	Although ivermectin prevented the development of adult flies from eggs, it had no appreciable impacts on pupa development.	[59]
Dung beetles	Ivermectin does not impact the survival of dung beetles or their larvae dwelling in the dung from treated cattle.	[60]
	A study on the physiology and behavior of dung beetles collected from ivermectin-treated cattle reported sub-lethal and lethal impacts of ivermectin residue on mature dung beetles. Ivermectin residues in dung, even at low concentrations, reduced the sensory capacity of dung beetles, restricting their locomotion and sensitivity to food.	[61]
	Massive usage of ivermectin negatively impacts environmental biodiversity and functionality, affecting dung beetles' richness and abundance.	[62]
	González-Tokman group reported that ivermectin compromises the reproductive success, physiological conditions, and sexual traits of dung beetles.	[63]

	Ivermectin residues cause a 0.05 mm mean reduction of dung beetles' horn length, compromising their probability of winning an intrasexual competition by 30%.	[64]
Nematodes	No adverse impact was recorded on nematodes	[65]
Mosquitoes	Ivermectin applied under mass drug administration diminishes the survival and population of *Anopheles* mosquitoes.	[66]
	Ivermectin suppressed An. arabiensis intestinal blood meal digestion, affecting their egg production and survival over time after feeding on the blood of ivermectin-treated cattle.	[67]
White mustard	Ivermectin at 50 nM concentration inhibited root growth in white mustard.	[68]
Soybeans	Ivermectin somehow reduces the weight and number of beans. Moreover, it induced alteration in soybeans antioxidant enzymic activities, reducing activities and content of isoflavones aglycones which threaten the soybean's therapeutic values.	[69]
A. thaliana	Ivermectin did not induce any antioxidant enzyme alteration, but it significantly negatively impacts gene expression.	[70]

1.5 Analytical Methods for Ivermectin and its Metabolites

To have an accurate picture of the environmental impact of substances like ivermectin and its metabolites, one must be able to record the actual concentrations of these species about the observed effects. This, among other things, requires having verified analytical methods and instrumentations. The analytical science community has dramatically

improved recently, especially by developing modern sensitivity instruments [71]. Yet sample preparation remains a crucial step in the whole analytical process. It is well known that sample matrices may interfere with the qualitative and quantitative analysis of target analytes by chromatographic techniques, even under a mass spectrophotometer detector. Therefore, a sufficient sample treatment should be performed before the final qualitative and quantitative analysis. Sample preparation always aims to isolate target analytes from the matrix substances in a suitable form for chromatographic separation and detection [72]. Ivermectin and its metabolites have been reported in environmental and biological samples [73-79]. Moreover, our project targets some of this environmental sample. Therefore, the following sections will highlight some methods for ecological sample preparations concerning our analytes.

1.5.1. Environmental Samples

The choice of sample extraction and clean-up techniques depends on several factors, including the type of the matrix substance, interactions between analyte and matrix, nature, and levels of the analyte in the samples, and measurement methods. For example, environmental samples always contain ivermectin or its metabolites at low concentrations ranging from ppm to ppt scale. Therefore, extraction and clean-up methods deployed should not interfere with or destroy the analyte lather, being able to concentrate it to detectable and quantifiable levels. Several analytical techniques have recently been used to prepare environmental samples like water, soil, manure, and sediments for quantitative analysis of ivermectin and metabolites. **Table 1.4** summarizes some of the preparation techniques used for environmental samples.

Table 1.4: Selected sample preparation techniques used for environmental samples

Sample	Extraction	Clean-up	% Recovery	Ref.
Soil, sediment, and water	PLE (methanol + water)	Oasis SPE C18	73-81	[41].
Cattle feces	SLE (acetone + isooctane)	SPE C18	84	[80].
Reindeer feces	SLE (acetone + isooctane)	SPE C18	95-116	[81].
Cattle dung	SLE (acetone + water)	-	-	[42].
Dung and soil	SLE (acetonitrile + water)	SPE cartridges	93	[32].
Tap and surface Waters	HF-SLM(methanol+ acetonitrile) Methanol	-	80	[74].
Water		SPE	-	[78].

From **Table 1.4**, it is essential to note that solid-liquid extraction (SLE) dominates ivermectin sample preparation methods with hollow-fiber supported liquid membranes (HL-SLM) have been rarely used. Published methods using SLE report that they are simple and achieved recoveries of approximately 80-100 %.

1.5.2 Biological Samples

In analytical chemistry, good sample preparation and clean-up are essential tools for reducing sample complexity and enhancing a combination between biological samples and new solvents [82]. The reduced complexity will also improve analytical method selectivity and sensitivity [82, 83]. Moreover, reduced interference from matrix compounds may enhance the method's robustness and serve the instrument from matrix contaminations[84-86]. Enriching analytes in a new medium may increase sensitivity and ensure compatibility with an analytical tool [87, 88]. This implies that poor sample preparation can quickly become a bottleneck for the quality of an analytical method. This is crucial for biological samples because analytes are usually found in abundant complex

matrices at low concentrations [89]. For example, ivermectin is administered at low concentrations producing metabolites at 10 %. With a large percentage of ivermectin remaining unmetabolized and eliminated in feces, plus its lipophilic nature, a small portion will remain unmetabolized and probably will be combined with peptides and proteins present in the biological fluids [90, 91].

Therefore, proper sample preparation is always necessary to safeguard a high enrichment of analytes to reach the detection limit by removing matrix components that otherwise might interfere with the detection of the target analytes.

Several methods are deployed to extract and clean up ivermectin residues and metabolites from biological samples like liver, plasma, milk, and muscle. These samples have different sample preparation considerations because they might involve the removal of proteins and fats. **Table 1.5** summarizes some of the techniques used for biological samples.

Table 1.5: Examples of Extraction techniques used for biological sample preparations

Sample preparation	**Author's comments**	**Ref.**
Solvent extraction followed by continuous solid phase extraction (SPE) of **ivermectin** from post-mortem and in vivo tissues of dung beetles.	The method was good enough in terms of precision and accuracy.	[92]
Liquid-liquid extraction (LLE) of ivermectin from milk.	The method experiences no matrix effects and can be extended to other macrolide endectocides in milk while serving money and time.	[93]
Ivermectin extraction from human plasma by using Oasis HLB solid phase extraction cartridges.	The method was very robust and suitable for clinical pharmacokinetics studies.	[94]

Solid-liquid extraction of **ivermectin** and **its metabolites** from swine tissues	The method was able to extract ivermectin and two metabolites from swine tissue.	[24]
Solid-phase extraction of **ivermectin** and unidentified **metabolites** from plasma.	The method was sufficient to extract the expected analytes	[95]
Ivermectin extraction from meat using QuEChERS methodology	Simple sample preparation method	[96]
Temperature purification (LTP) ivermectin extraction from edible oils	The first method to be used in edible oil with a similar outcome to that of SPE	[97]

1.5.3 Separation and Detection Methods

Several separations and detection methods have been deployed to analyze ivermectin and its metabolites for qualitative and quantitative purposes. Floate et al. used thin-layer chromatography for screening dung samples for ivermectin analysis [98]. However, although this method was rapid and easy to use but was reported to have poor selectivity and sensitivity [98], to overcome those two analytical challenges, researchers prefer to use Liquid chromatography-tandem mass spectrometry (LC-MS/MS) for ivermectin and its metabolites plus degradation products analysis [99]. LC-MS/MS uses ionization sources like atmospheric pressure chemical ionization, particle beam, thermospray, electrospray, and atmospheric pressure photoionization for qualification and quantification. For example, researchers have used electrospray for quantification, and authors have used particle beam and thermospray for ivermectin and its metabolites qualitative analysis [100].

Although many scientists have reported using these methods utilizing quadrupole, triple quadrupole, time-of-flight (TOF), and ion trap fragmentation techniques, some challenges

have also been reported. An excellent example of the difficulties encountered is the matrix effect on ionization efficiency, which leads to signal suppression or enhancement [101]. High or ultra-performance liquid chromatography (H/UPLC) has been extensively used to determine ivermectin residue and its metabolites in various matrices. The U/HPLC method uses either UV, fluorescence, or Mass spectrophotometer detection. **Table 1.6** summarizes some of the methods used for ivermectin and metabolite quantification.

Ivermectin and its metabolites contain four double bonds, with only two of these bonds falling into conjugated as a diene function at carbon 8,9,10, and 11 [102]. This structural arrangement is essential for UV VIS spectrophotometric analytical analysis as it causes a strong UV absorption band at 245 nm. However, this conjugation system is not enough for fluorescent detectors, and a derivatization process must occur for detection under this instrument.

Table 1.6: Analytical methods that have been used for ivermectin and metabolites detection

Analyte	Instrumentation	Extraction	Clean-up	% Recovery	Ref.
Ivermectin	HPLC-FLD	Acetonitrile	SPE alumina	70-80	[103]
Ivermectin	HPLC-FLD	Acetonitrile	SPE alumina + C_{18}	73-97	[35]
Ivermectin	HPLC-FLD	Acetone+ isooctane	SPE C_{18}	95-116	[104]
Ivermectin	HPLC-FLD	Acetone+ isooctane	SPE C_{18}	84	[80]
Ivermectin	HPLC-FLD	Acetonitrile	LLE	63≤	[105]
Ivermectin	HPLC-FLD	Acetonitrile + water	SPE	86	[94]
Ivermectin	HPLC-FLD	Acetonitrile + ethyl acetate water	LLE	85≤	[106]
Ivermectin	LC-FLD	Acetonitrile + water	LTP	89-100.7	[107]
Ivermectin	LC/ESI-MS/MS	Ethanol	LLE	NA	[108]
Ivermectin+Metabolites	HPLC-RIDA	Acetone + water	LLE	NA	[42]
Ivermectin	HPLC-UV	Methanol +ethyl acetate	Florisil SPE	78<	[34]
Ivermectin	HPLC-UV	Methanol	SALE	90<	[109]
Ivermectin	HPLC-FLD	Methanol	LLE	94<	[110]
Ivermectin+Metabolites	HPLC-MS/MS	Methanol +ethyl acetate	LLE	NA	[70]
Ivermectin+Metabolites	UHPLC-MS/MS	80 % Methanol	LLE	NA	[69]

Ivermectin+Metabolites	UHPLC-MS/MS	Acetonitrile	LLE	NA	[111]
Ivermectin	UHPLC-MS/MS	Water +ethyl acetate	LLE	NA	[68]
Ivermectin	HPLC-FLD	Acetonitrile	LLE	75-100	[112]

During derivatization, ivermectin loses both hydroxyl groups in the benzofurane structure via trifluoro acetylation, **Figure 1.4**.

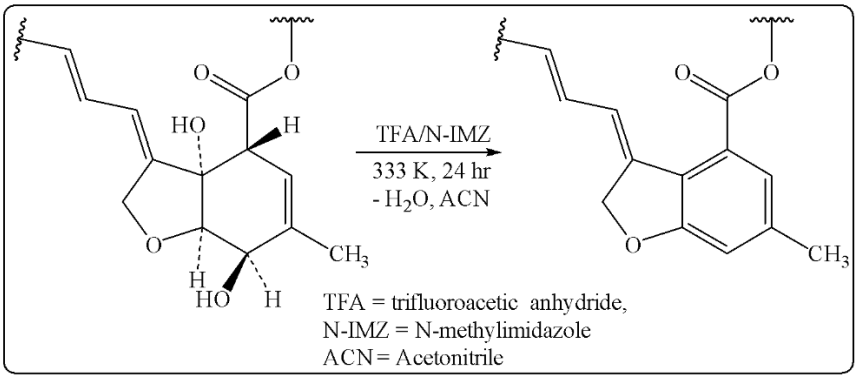

Figure 1.4: Ivermectin derivatization scheme showing the formation of two double bonds in the hexagonal ring, which saves as a chromophore for fluorescent detectors.

This reaction develops a fluorescent π-bond and accounts for a peculiar absorption maximum and an emission maximum of 364 nm and 463 nm, respectively[102], as shown in the chromatogram, **Figure 1.5**.

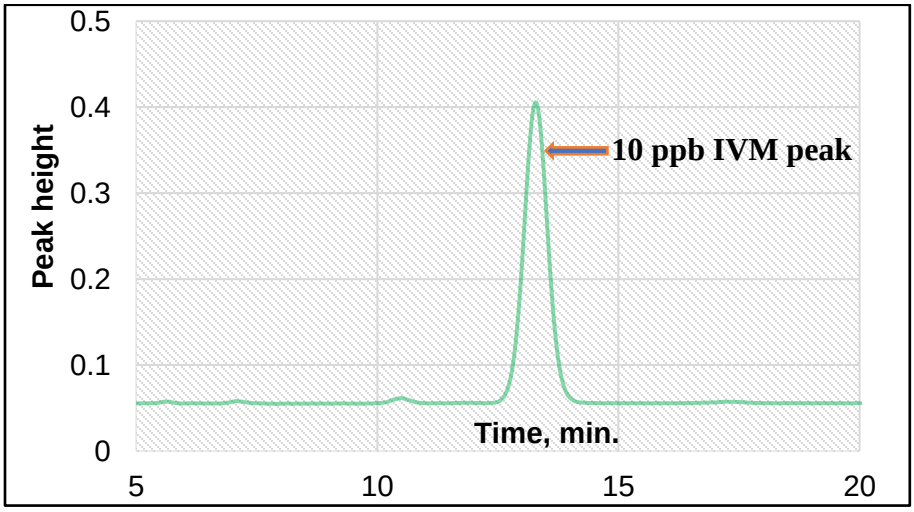

Figure 1.5: 10 ppb ivermectin (IVM) water sample extract chromatogram with IVM peak (t_R = 13 minutes). This peak was obtained after sample extraction and IVM derivatization.

1.6 Summary

Ivermectin discovery and approval for veterinary and human use divide the scientific world into two parts. First is its application for the treatment of animal and human diseases. In cattle, for example, 200 µg/kg dose is said to be effective against not less than seven gastrointestinal nematode species, arthropod, lice, and ticks' parasites [113]. Oral administration in sheep eradicates at least 93 % of endoparasites [113]. Furthermore, 300 µg/kg of subcutaneous injection on swine is highly efficacious against the porcine mange mite and sucking louse and much more in horses, dogs, and goats [113]. In humans, it has been used against a broad range of nematodes and arthropods, including Strongyloidiasis stercoralis, tissue microfilaria of Onchocerca volvulus, and lymphatic filariasis, gastric nematodes, and ectoparasites such as lice, scabies, mites, and botflies[25, 113]. There is a new paradigm of ivermectin application, shifting to more applications for controlling malaria under MDA. Ivermectin has shown high efficacy in killing vectors of disease-causing parasites such as mosquitoes, sandflies, and tsetse flies and reducing malaria transmission [113, 114]. Trials for malaria prevention are promising because of ivermectin's unique mode of action compared to currently used insecticides for malaria vector control. Thus, it likely could thwart the issue of emerging insecticide resistance [115-117]. Moreover, ivermectin can target indoor and outdoor-biting mosquitoes, including those in crepuscular activity [118]. Furthermore, ivermectin activity targets four out of the five variables of vectorial capacity: human biting rates, the mosquitoes-to-human ratios, mosquito survival through one day, and the extrinsic incubation period of malaria parasites [119, 120]. Therefore, the application of ivermectin, especially in tropical countries like Tanzania, will increase. The second part is on

pharmacokinetics data which reports that only a tiny amount of the administered drug remains in the body, with about 90 % of the residue drug and metabolites discharged into the environment via feces and manure, as discussed in previous sections [56]. This is the standard practice for most rural communities where cattle dung is used as farm manure instead of industrial fertilizers [121]. Contamination of the soil with cattle dung from ivermectin treated cattle can lead to contamination of surface and ground waters through runoff and hydraulic water flows. Terrestrial, aqueous flora and fauna toxicity caused by ivermectin contamination has been reported [122-124]. These impacts are huge on soil properties, including fertility, nitrogen cycle, and hence low productivity. The compromised agricultural productivity may mean a threat to food security, although studies must conduct in this regard. Therefore, if ivermectin is used to fight malaria, it will lead to food insecurity; it will be like solving one problem by creating the other. This is where the dilemma between avoiding ivermectin with all its efficacy against many parasites, including malaria vectors, and serving the environment, continues using it, and facing hunger after ivermectin administration comes.

Scientific communities have tried to investigate this issue by conducting studies on the environmental fate and impacts of ivermectin, and factors determining its persistence and effects are being established. Most studies agree that photodegradation is among the crucial determining factors of ivermectin environmental persistence. Unfortunately, most of these studies are conducted in temperate climates. Halley et al. reported that climatic conditions determine ivermectin residues persistence, differing locations and seasons, and affect chemical and dung degradation. The group recorded that degradation of ivermectin would be in the order of 7 to 14 days under summer conditions and 91 to 217

days in winter. These two pieces of information suggest that findings on ivermectin fate from one climate or season cannot be extrapolated to another environment or season[42]. Thus, results in spring in temperate climates cannot be used to predict what will happen in the summer in tropical climates. Therefore, more similar studies in tropical countries are required before decision-making on ivermectin mass drug administration for malaria control.

1.7 Study Aims and Hypotheses

This study's main aim is to investigate the environmental fate of ivermectin, 3″-O-desmethylivermectin, and 24-hydroxymethylivermectin in soils and manure in tropical and temperate regions in both field and laboratory settings. This study will be conducted in Ifakara, Tanzania, and Blacksburg, Virginia, United States of America, aiming at generating data for environmental implications on ivermectin MDA for malaria control. This project will be accomplished under the following objectives:

Objective 1: To understand the sorption performance of *sol-gel-poly-THF* turned cellulose substrate for extraction and clean-up of ivermectin in soil and water samples.

Hypotheses -Compared to other analytical methods, the fabric phase solid extraction (FPSE) media method is simple, low-cost, and fast compared to the traditional solid phase extraction method with similar or better results and can be used for our field setting in Tanzania.

Objective 2: To conduct laboratory studies to investigate the transformation of ivermectin and 3″-O-demethylivermectin in soils and soil-manure mixtures from temperate and tropical regions.

Hypotheses-Ivermectin and its 3″-O-demethylivermectin will have different transformation pathways and rates; in these two regions because they experience different temperatures, annual rainfall distribution, and have different soil characteristics.

Objective 3: To perform a field-scale investigation of the environmental fate of ivermectin and 3″-O-demethylivermectin in cattle manure and soil in Tanzania.

Hypotheses -Cattle metabolic activities will be affected by environmental conditions and diet leading to different ivermectin residue concentrations and metabolites. Furthermore, excreted ivermectin residues and metabolites entrapped in the cattle dungs or pats-soil mixture will undergo rapid transformation compared to what is reported in the literature due to other environmental conditions in tropical regions.

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Chapter 2: Understanding the sorption performance of *sol-gel-poly-THF* turned cellulose substrate for pre-concentration of ivermectin in soil and water samples

Gerald Enos Shija and Kang Xia

(To be submitted to the Journal of Chemosphere)

Abstract

Ivermectin, a member of the macrocyclic lactone family, proved to be a panacea for many veterinary and human diseases. Although the health benefits are crucial, ivermectin poses some serious environmental concerns. Therefore, developing an analytical method for infield ivermectin extraction becomes equally important. Under this study, the cellulose substrate was turned with sol-gel-poly-THF to create a selective and sensitive FPSE-HPLC-FLD method to quantify ivermectin in soil and water samples. Ivermectin extracted by FPSE media from the soil, environmental, and Millipore water samples were derivative and instrumental analyses. The parameters like FPSE media size, sorption solvent, sorption, desorption times, mixing type, and speed directly affecting the extraction process were optimized. Under the optimal conditions, we detected ivermectin at a concentration as low as 0.23 ppb and precisely quantified it at up to 0.75 ppb. When this

method was used, satisfactory recoveries above 80 % were obtained for all tested samples. The percentage recoveries were similar to or better than those obtained with traditional SPE-HPLC-FLD. Considering that SPE-HPLC-FLD has many challenges and results accepted, this method can be preferred to monitor ivermectin in soil and water.

2.1 Introduction

Ivermectin is a member of the naturally occurring or semisynthetic macrocyclic lactone avermectin family produced through fermentation reaction in the soil-dwelling bacteria, *Streptomyces avermitilis*. For a long time, ivermectin has been successfully used as a broad-spectrum therapeutic drug for the treatment of diseases like sarcoptic mange in cats [1, 2], dogs[1, 3, 4], foxes [5], pigs [6-8], wild boars [9], and camels [10, 11]. However, ivermectin therapy potentially grew when promising results were observed in treating human diseases.

Since 1987, ivermectin has been used extensively in humans to control endemic pathogens. For example, ivermectin is used for preventing and treating onchocerciasis, lymphatic filariasis, strongyloidiasis, scabies, and head lice in Asia, Africa, and Latin American countries [12]. Some findings suggest that ivermectin administered alone or in combination with partner drugs can be used to manage neglected tropical diseases and malaria [13, 14]. Thanks to its ability to remain in the human bloodstream following a

standard recommended dose, ivermectin can kill blood-feeding *Anopheline* vector mosquitoes [15-19] and malarial parasites [20]. Therefore, it is not surprising for UNITAID to sponsor community-wide ivermectin mass drug administration (CMDA) to conquer malaria and potentially boost other immeasurable health and socio-economic conditions. The efficacy of the ivermectin drug is uncontested. Yet, at the same time, the pharmacokinetics studies report that at least 90 % of its administered dose is excreted in feces and urine [21-23], which can lead to ivermectin contamination of environmental compartments through direct deposit of excreta and urine or disposal latrines through leaching and runoff [24]. Contamination of ecological compartments has lethal and sub-lethal effects on beneficial non-target microorganisms [25-28]. Therefore, evaluating the safety of ivermectin in the aquatic and soil ecosystem is crucial, especially after CMDA. To achieve this, a selective, cost-effective, and clog-free water sampling, extraction, clean-up, and analysis technique is required. Moreover, the method must be applicable in field settings to support testing during mass drug administrations and to avoid sample transportation.

Several analytical methods dealing with ivermectin analysis in different samples have been developed, most involving solid-phase extraction cartridges [27, 29, 30]. However, these methods, particularly solid-phase extraction, are laborious, time-consuming, subject to cartridge clogging, and cannot be used in the field. To address these challenges and simplify the analyte extraction, Kabir and Fortun [31] developed a fabric phase sorptive extraction method by turning the sorption properties of fabric substrate with selected sol-gel precursor and sol-gel active inorganic/organic polymer. This method positively addressed most of the drawbacks commonly encountered in traditional sorbent-

based extraction techniques, including solid-phase extraction, and can be used in the field [32]. The most crucial step in this method is selecting the appropriate sorbent using an available second-order mathematical model. These models allow calculating an analyte's complete recovery (%) using K_{ow} values. The valid K_{ow} values for these models are those between 2 and 11500[33]. Unfortunately, as far as we know, no published studies have used this method to determine ivermectin (K_{ow} = 1651) in environmental water samples [34].

Therefore, this study explores the potential of a two-step FPSE enrichment/clean-up technique to extract ivermectin from small, complex environmental samples, followed by HPLC-FLD detection after derivatization. This method will optimize in-field sample handling, achieve higher enrichment factors, and enhance sensitivity. The method will be validated using soil, Millipore Mill-Q waters, and complex environmental waters. Moreover, the method's performance for ivermectin will be compared with solid-phase extraction (SPE).

2.2 Material and Methods

2. 2.1 Instrumentation

The ivermectin chromatograms, **Figure 2.1** were recorded on an 1100 Series HPLC instrument (Agilent Technologies, Inc., Palo Alto, CA, USA) consisting of an autosampler, an LC-10AT pump, a SIL-20A automatic injector, and an RF-10A XL fluorescence detector. A C-18 ZOBRAX extended analytical 4.6 mm × 12.5 mm column with a guard column (Waters, Milford, MA, USA) and 20 µL injection volume was employed. The column temperature was kept constant at 30 ^{o}C to obtain the reproducible retention time of ivermectin detected at 364 nm and 463 nm excitation and emission wavelengths,

respectively. Water adjusted with formic acid (99.9 % v/v water) was used as mobile phase A, and 100 % acetonitrile was used as mobile phase B at a 1.2 mL/min flow rate. The dualistic isocratic elution was used for the separation, the data were collected using Analyst® 1.4.2 software (MDS Scitex, Concord, ON, Canada), and ivermectin peak, **Figure 2.1** was consistently recorded at a 13-minute retention time.

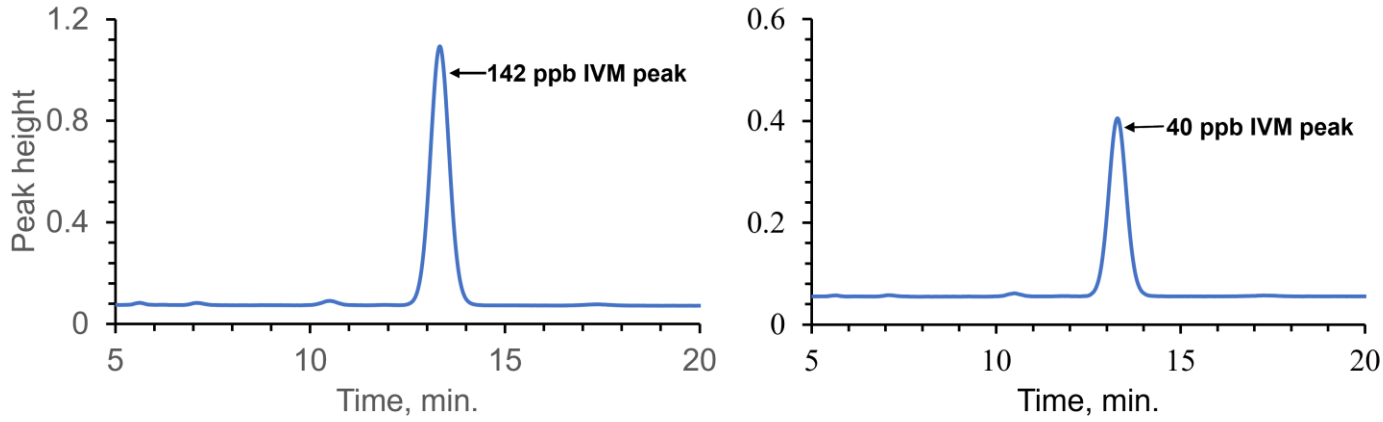

Figure 2. 1: The ivermectin chromatogram (t_R= 13 min.) recorded on ivermectin samples under optimized chromatographic conditions after a black sample was spiked with the analyte to make 142 ppb(left) and 40 ppb (right) ivermectin samples.

2.2.2 Chemicals and Reagents

Formic acid, Methanol, Dichloromethane, Acetone, Water Optima (HPLC-grade), N-methylimidazole (99 %), Tetramethoxysilane (97 %), Triethylamine (99.7 %), Trifluoroacetic acid (99 %), Trifluoroacetic anhydride (99 %+), and Vortex mixer were procured from Thermo Fisher Scientific (Reagent Lane, Fair Lawn, New Jersey, 07410 USA). Tetrahydrofuran (HPLC-grade), α-hydro-*w*-hydroxy poly-(oxy-1,4-butanediol) or poly (tetrahydrofuran (Average Mn ≈ 250) and ivermectin (purity 94 % H_2B_{1a} and 2.8 % H_2B_{1b}) were purchased from Sigma-Aldrich (3050 Space Street St. Louis MO 63103 USA). Oasis HLB Extraction Cartridge (3 mL-60 mg) was bought from Waters Cooperation (Milford, Massachusetts, USA).

2.2.3 Preparation of Fabric Substrate

In most cases, commercially available cellulose fabric substrates retain finishing chemicals residues on their surface, attracting other contaminants and interfering with the smearing process. In addition, their surface hydroxyl groups require activations for perfect sol-gel coating[33]. Therefore, cellulose fabric substrates were subjected to a pretreatment regimen developed and reported in the literature [32, 35]. In summary, a 10 cm × 10 cm (100 cm^2) fabric material was soaked in deionized water for 15 minutes under continuous sonication before being cleaned in a large volume of deionized water. The above fabric was soaked in 1 N NaOH solution for 60 minutes under sonication, followed by washing the alkaline treated fabric with ample deionized water five times. The cleaned fabric material was then treated with 0.1 N HCl solution for 60 minutes under sonication before being washed with 30 mL of deionized water and finally dried overnight in an

incubator at 303 K. Subsequently, the dried fabric was stored in a desiccator waiting for sorbent coating.

2.2.4 Preparation of Sol-gel Poly-THF Coated FPSE Media

The preparation of sol-gel poly-THF coated media is accomplished through slow immersion polymeric coating technology. Like other polymeric reactions, this coating technology has three sequential reactions: Initiation, propagation, and termination. Three hydroxyl groups are formed on the methyltrimethoxysilane (MTMS) via catalytic hydrolysis of its methoxy groups during the initiation step. The above step is followed by creating 3D networks by polycondensation reaction of the precursors utilizing the formed hydroxyl groups included in the initiation step. Finally, the termination step comes into play. During this step, random incorporation of sol-gel-active polymer into the glowing sol-gel network in the fabric substrate surface via polycondensation reaction [33]. Considering the above chemistry and the literature reports, we modified what is reported in Kabir [36] to fit our laboratory setting. Briefly, a sol-gel solution designed for creating the sol-gel poly-THF coating was prepared by dissolving 10 g of poly-THF polymer and 10 mL sol-gel precursor methyltrimethoxysilane (MTMS) in 20 mL of 50:50 v/v methylene chloride: acetone and mixed 4 mL of 95:5 v/v trifluoroacetic-water as the sol-gel catalyst. The mixture was then vortexed for 180 seconds, centrifuged for 300 seconds, sonicated for 120 seconds, and finally, the clear supernatant part of the sol solution was transferred to a clean 150 mL amber-colored reaction glass bottle. The coating process was done by slowly immersing the pretreated cellulose fabric substrate into the sol-gel solution and left there for 4 hours for the complete formation of sol–gel coating. Then, the sol-gel poly-THF coated substrate was removed from the sol solution, kept in the desiccator overnight,

and incubated at 348 K for 12 hours for solvent evaporation and aging of the sol-gel coating. After that, the coated FPSE media was rinsed with dichloromethane followed by methanol to remove unreacted and unbounded residual sol solution ingredients from the painted surface, followed by dried and stored in air-tight containers to prevent the accumulation of unwanted analytes from the environment.

2.2.5 Standard Ivermectin Solution and Reagents Preparations

The reagent solutions were prepared separately by mixing trifluoroacetic anhydride and N-methylimidazole in acetonitrile ($50{:}50\ \mathrm{v/v}$). Both solutions were freshly prepared on a 9-day basis and well stored to avoid contamination and any form of transformation. An 18.7 ppm of the reference standard stock solution was prepared by dissolving ivermectin in acetonitrile and stored in the fridge. The 250, 142, 100, 40, 10, and 1 ppb of working solutions were prepared daily by diluting the stock reference standard solution with acetonitrile. All standards were designed using glass vessels to avoid loss due to sorption and stored in amber glass vessels to minimize photodegradation.

2.2.6 Sample Preparation

Environmental water was collected from the nearby pond and transported to our research laboratory in a Pyrex borosilicate glass bottle. The soil was collected in nearby farms in Blacksburg, VA. To know the level of contamination of the environmental water used for this study, we did only routine characterization and used them as collected without further filtration. Our analysis also included Millipore water for comparison purposes. During water sample preparation, 10 mL of either environmental or Millipore water was measured in a 50 mL vial and spiked with 700 µL of 10, 40, or 142 ppb of IVM standards. The resulting solution was mixed with the aid of vortexing for 60 seconds. For soil sample

preparation, 1g of soil was mixed with 1.7 mL of water, then spiked with 166.1, 46.8, and 11.7 IVM standards at the above concentrations, vortexed for 1 minute, and equilibrated in the fridge for 24 hrs. For repeatability purposes, each sample was analyzed in a triplet.

2.2.7 Statistical Analyses

JMP Pro 16 (SAS Institute Inc, 2019) and Origin 9.1® software were used for data analysis in this study. Analysis of Cook's distance (D) was done to identify outliers. The results from this analysis confirmed that no data should be considered outliers. To compare percentage recoveries with the analytical method used or any variable in question, regarded as an independent replicate ($n = 3$). For percentage recoveries, the sample mean, and standard deviations were entered in Origin 9.1® software for graphical analysis.

2.3 Results and Discussion

2.3.1 Ivermectin Analyte Extraction Procedures

2.3.1.1 Water samples Fabric Phase Sorptive Extraction (FPSE)

Before sample extraction, a predetermined size FPSE media (section 2.3.4.4) was conditioned in 6 mL of a 1:1 (v/v) mixture of methanol-acetonitrile for 5 minutes. After conditioning, the media was washed in a large volume of Millipore water for complete cleaning and removing excess solvent absorbed in the FPSE media. During sample extraction, a conditioned and cleaned sol-gel poly-THF coated FPSE medium was paced inside the 50 mL vial containing a 10 mL water sample. Afterward, the vial was sealed and vortexed the sample at 800 rpm for a predetermined time (section 2.3.4.2). After the sorption, the FPSE media was removed from the vial, made free from excess water, and

transferred to the clean test tube for desorption. The desorption process was done by vortexing twice the media in 4 mL methanol at 800 rpm for a predetermined time (section 2.3.4.3). The obtained eluent (8 mL) was dried in the Labconco Rapidvap, and the residue was reconstituted in 700 µL ACN.

2.3.1.2 Soil Samples Fabric Phase Sorptive Extraction (FPSE)

The soil sample was extracted by mixing 2g of soil sample with 5 mL of methanol in a 50 mL polypropylene copolymer centrifuge tube with a lid. The sample was vortexed at 800 rpm for 10 minutes and centrifuged at 3500 ppm for 5 minutes. Immediately after centrifuging the samples without disturbing the sediment, the supernatant was transferred into a vial. Then another 5 mL methanol was added into the tube containing the deposit and vortexed at 800 rpm for 10 min, followed by centrifuging the sample was centrifuged at 3500 ppm for 5 min. Immediately after centrifugation of the samples, the obtained supernatant was transferred to a vial containing the supernatant above and subjected to a clean-up process without disturbing the sediment. During sample clean-up, 10 mL of the above supernatant was transferred into the vial and dried in the Labconco Rapidvap. The obtained crude residues were dissolved in 10 mL of water and cleaned up using the FPSE method described in 2.3.1.1. The obtained clean residues were subjected to derivatization and instrumental analysis, and the percentage recoveries were determined.

2. 3.1.3 Water Samples Solid-phase Extraction (SPE)

SPE Oasis HLB cartridges were conditioned with 3 mL methanol and equilibrated with 3 mL Millipore Milli-Q water. Then 100 mL water samples were loaded on a cartridge with a vacuum pump at the rate of 0.5 mL/ min. After sample loading, cartridges were washed with 3 mL (1:1, *v/v*) methanol-water and dried under a vacuum, followed by elution of the

target analyte with 4 mL ACN at 0.5 mL min^{-1}. Finally, the extract was gently evaporated to dry the Labconco Rapidvap and reconstituted into 700 µL ACN.

2. 3.1.4 Soil Samples Solid-phase Extraction (SPE)

During IVM extraction, 5 mL of ACN was added to the fortified soil sample, followed by mixing with a vortex mixer set at 800 rpm for a minute, then shaken in the shaker for an hour. The sample was then centrifuged at 4000 rpm for 5 minutes, and without disturbing the sample, the supernatant was removed and reserved. Next, the extraction procedure was repeated, and the obtained supernatant was combined and mixed in a vial. Finally, 10 mL of the well-mixed supernatant was dissolved in 100 ml of water and subjected to the Oasis HLB cartridges clean-up procedure, as explained in section 2.3.1.3 above.

2.3.2 Analyte Derivatization and HPLC Procedure

Since using a fluorescence detector for ivermectin analysis requires the generation of fluorogenic moiety within the molecule via derivatization, we adopted the method explained in the pieces of literature[37],[38] with slight modification for that purpose. Briefly: 700 µL of the reconstituted sample was mixed with 100 µL of a prepared mixture of *N*-methylimidazole and acetonitrile (50:50, *v/v*) in the vial. The resulting mixture in the vial was mixed by adding 50 µL of triethylamine and 100 µL of a prepared mixture of trifluoroacetic anhydride/acetonitrile (50:50*, v/v*). Finally, 50 µL of tetrahydrofuran was added. To attain through mixing the added reagent with our sample, the vial closed and vortexed for 10 seconds before another reagent was added. The mixture was obtained after adding all reagents and vortexing for 3 minutes. The reaction mixture was incubated at 333 K for 30 minutes under constant slow shaking to complete the derivatization reaction. The total content of the vial was quickly transferred to a 2 mL quartz cell to avoid

evaporation of the solvent. The mixture was then cooled to room temperature and immediately analyzed with HPLC-FLD under conditions stated in *section 2.2.1*.

2.3.3 Optimization of FPSE Media Parameters

2.3.3.1 Desorption solvent

A known concentration of ivermectin was spiked in an equal volume of chosen solvents like methanol, acetone, acetonitrile, and 1:1 acetonitrile/methanol mixture based on our analyte solubility properties. The solutions were vortexed with the 30 cm^2 pre-conditioned and equilibrated FPSE media for 10 minutes. Then the media is removed, the solution reconstituted at 700 µL acetonitrile, derivatized, and analyzed. The obtained peak areas were used to calculate the average percentage (*n = 3*) of ivermectin retained in solutions. **Figure 2.2** shows that methanol has a sizeable average rate contained in solution after extraction at all tested concentration levels. Therefore, it was deployed as a desorption solvent for our work.

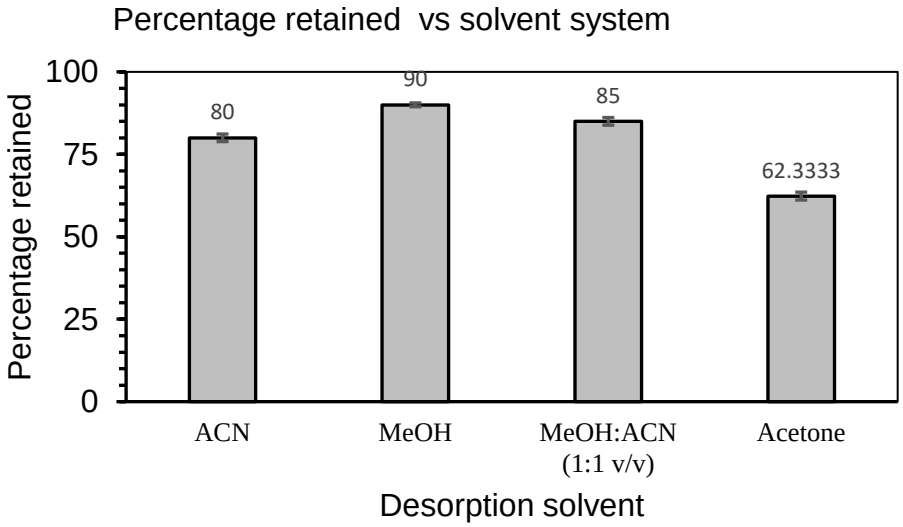

Figure 2. 2: Effect of desorption solvent on extraction efficiency. The error bars represent the standard errors of the means (n =3).

2.3.3.2 Sorption time

The sorption profile was investigated using an aqueous sample prepared by spiking 10 mL of water with 700 µL of 40 ppb ivermectin crude soil extracts or standards to optimize the sorption time. During this investigation, the prepared samples were extracted with a 30 cm^2 FPSE media by varying the exposure time from 3 to 12 minutes. The FPSE media was well prepared by conditioning in the predetermined solvent mixture and followed by washing in the deionized water to remove unreacted materials and activate their active sites. Methanol was used as the desorption solvent, and desorption was done by vortexing the FPSE media at 800 rpm twice for 4 minutes each turn. These percentage recoveries were taken as the average of three samples per time interval. The extracted analytes were then used to calculate the percentage recoveries at each run plotted against exposure time. **Figure 2.3** below represents the percentage recoveries per media exposure time in the sample.

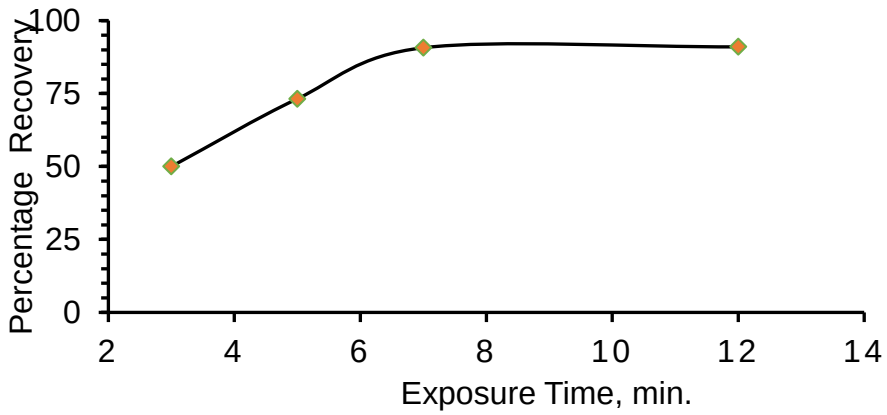

Figure 2. 3 : A typical sorption time profile for FPSE media ivermectin sorption from aqueous samples spiked with 700 µL of 40 ppb ivermectin standard or crude soil extract vortexed at 800 rpm.

From **Figure 2.3,** it is clear that there is a significant increase in ivermectin sorption from samples between 3 and 7 minutes, where it seems to attain equilibrium. Therefore, a 7-minutes sorption time was used for all the subsequent experiments.

2.3.3.3 Desorption time optimization

2.62 ppb water samples were prepared for desorption experiments and subject to the sorption process using the procedure explained in section 2.3.3.2 at 7 minutes sorption time. After sorption, the desorption process was performed by vortexing twice the FPSE media in ACN between 3 minutes per turn (6 minutes in total) to 6 minutes per turn (12 minutes in full). The desorption time profile, **Figure 2.4**, was generated by plotting average percentage recoveries ($n = 3$) against whole exposure time.

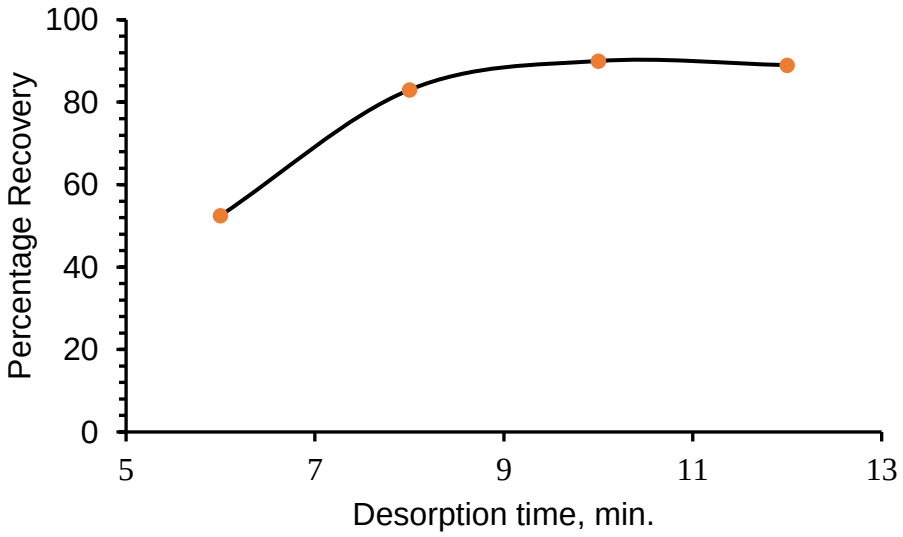

Figure 2. 4: Desorption Time Optimization.

Figure 2.4 indicates an increase in percentage recovery as the desorption time increases, with more increase experienced between 6 and 8 minutes. The average percentage almost doubled with the addition of only two minutes. The average percentage recovery

seems to remain constant after 10 minutes. Apart from the fact that a 10-minute sorption time provided maximum percentage recovery, we opted to use 8 minutes to fit our research targets.

2.3.3.4 FPSE media size

To avoid wastage of FPSE media and optimize extraction efficiencies, we conducted experiments to determine our project's proper media size. These experiments were conducted by varying the media size from 6.25 to 30 cm^{-2} and exposing the media to a 2.62 ppb water sample at optimized times for the sorption and desorption conditions. The obtained percentage of recoveries was plotted against the FPSE media size, **Figure 2.5**

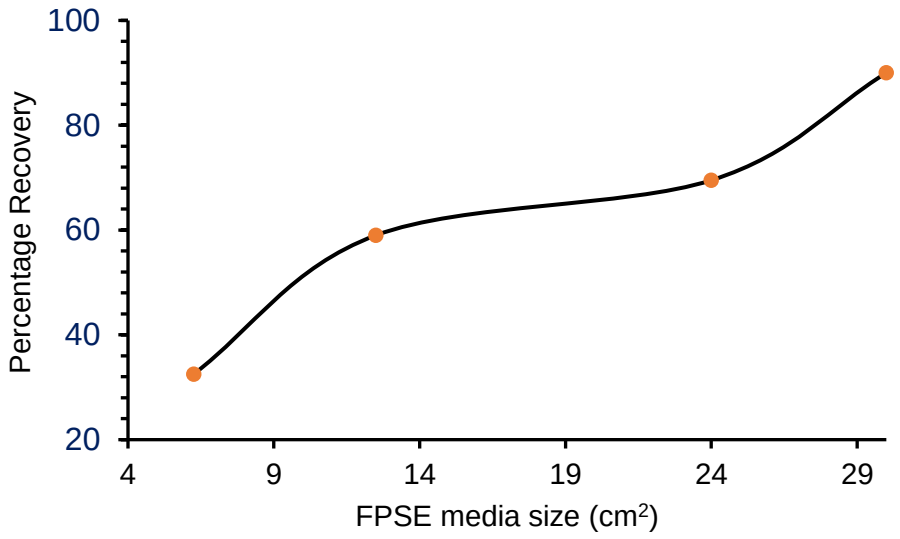

Figure 2. 5: The graph average percentage recoveries against FPSE media size obtained at 7- and 8-minute sorption and desorption time in methanol.

Figure 2.5 illustrates an increase in percentage recoveries as the FPSE media size increases significant increase in percentage recovery is observed at the lower surface area. Although we did not reach the flat side of the curve, the approximately 90 % recovery fits our research purpose.

2.3.3.5 Ivermectin stability

2.3.3.5.1 Ivermectin extract stability

Since the developed method will be applied for infield extraction, it can be anticipated that ivermectin extract will be stored before instrumental analysis. Therefore, ivermectin extract stability for the estimated storage time should be carefully studied to avoid compromising recoveries. Samples were prepared by spiking a known ivermectin concentration in the environmental waters in Tanzania. Then the samples were subjected to infield FPSE sorption and desorption following the optimized procedures. The vials containing ivermectin extracts were closed and wrapped in aluminum foil to avoid direct contact with sunlight. They were packed in the box and transported at average temperature to the laboratory in the USA for instrumental analysis. In the laboratory, the samples were stored in the fridge (set at 5^{o}C) for six weeks before the first batch ($n = 3\ randomly\ selected$) was subjected to derivatization and instrumental analysis following the optimized procedures. Under the same conditions, the second, third, and fourth batches were derivatized and analyzed after 8, 10, and 14 weeks of laboratory storage. The percentage recoveries were calculated based on the tested extract's peak areas about the peak area recorded for the expected ivermectin concentration in the final section. **Figure 2.6** represents the average percentage recoveries ($n = 3$) for each analysis 14 weeks after extraction. It is clear from **Figure 2.6** that there is no clear trend of change in percentage recoveries for more than three months to suggest that ivermectin was not degrading with time. Therefore, it was concluded that ivermectin was stable under these storage conditions.

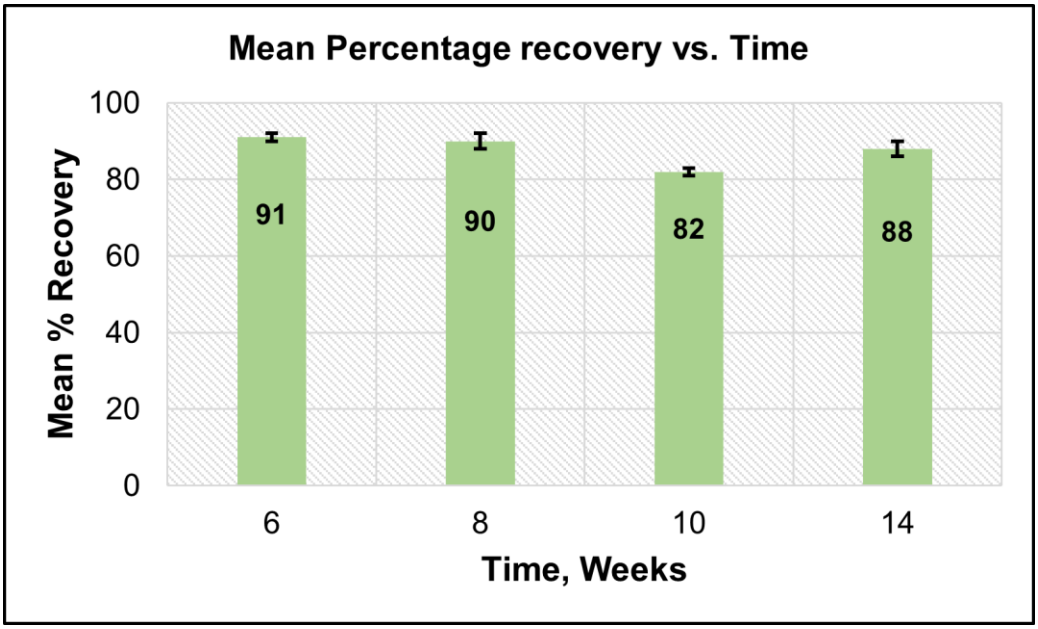

Figure 2. 6: The average percentage of recoveries recorded for fourteen weeks storage time. Error bars represents the standard deviation from the mean (n = 3).

2.3.3.5.2 Underivatized standard solution stability

The ivermectin standards stock solution stability data was generated to justify the storage conditions and the period over which the solutions will be user generated. The primary standard solution was prepared and stored away from heat and sunlight (in the fridge at 5^{o}C. To determine the stability of this standard at these storage conditions, we designed three operational standards (40 ppb) from the prepared and well-stored primary bar. These operating standards were subjected to derivatization, instrumental analysis, and recorded peak areas. Finally, the experiment was repeated by preparing the working standard in triplicate and doing the study as above for 18 weeks at a 3-week time interval. The obtained peak area per analysis was averaged and plotted against time, **Figure 2.7**.

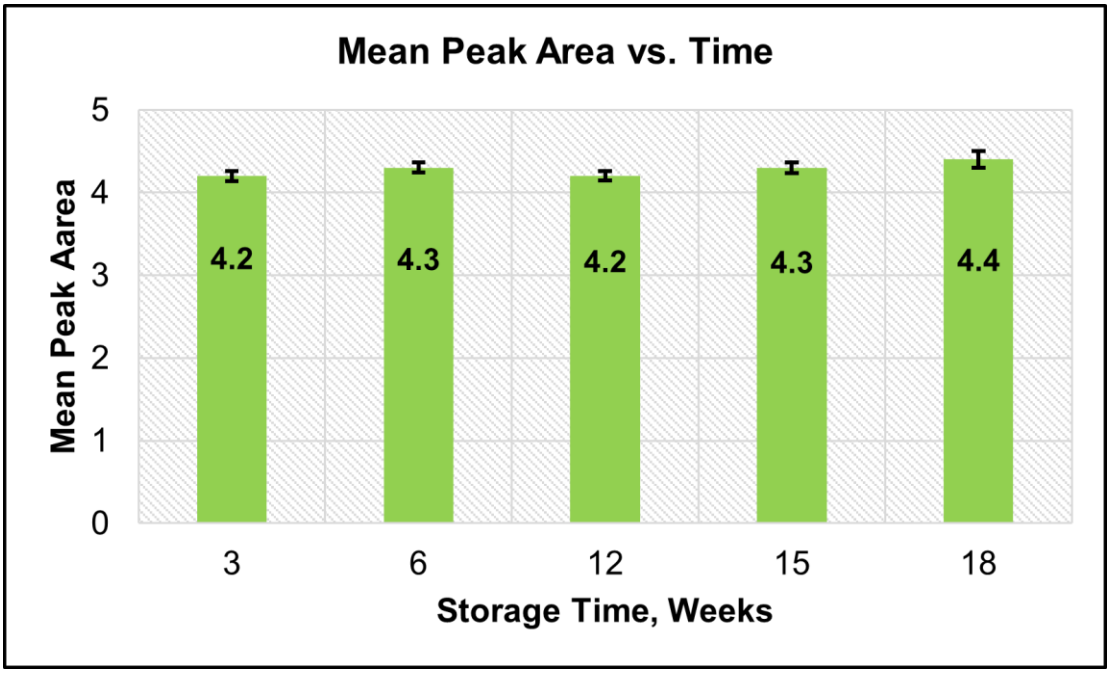

Figure 2. 7: Average peak areas ($n = 3$) for 40 ppb ivermectin standard for 18 weeks storage time. The error bars represent the standard deviation from the mean peak areas.

The one-way ANOVA p-values = 0.22 was obtained in JMP indicates that there was no significant change in peaks areas for the 18-week storage period. Since there was no significant change in peak area obtained for those 18-week storage times, it was concluded that there was no substantial change in ivermectin concentration over time (degradation). Therefore, we stored our standards under these conditions.

2.3.4 Method Performance

2.3.4.1 Method validation

Apart from the fact that we managed to develop a method for ivermectin detection at very acceptable recoveries, the performance characteristics of the method to ensure the adequate identification and quantification of the target analyte in our laboratory setting were not fully known. Several parameters such as linearity, accuracy and precision, trueness (expressed as recovery), and limits of detection (LODs) and quantification (LOQ)

were studied. A linearity study to verify the relationship between the analyte signal's concentration in the sample was conducted by preparing seven levels of mixed standard (1, 10, 50, 100, 142, 200, and 250 ppb) reconstituted to 700 µL each with anhydrous ACN. These solutions were subjected to derivatization, as explained in section 3.2, followed by instrumental analysis. The obtained mean peak areas ($n = 3$) of these standard concentrations were used to obtain analytical curves using the least-squares linear regression method. As shown in **Figure 2.8**, a calibration gave a higher level of linearity within the tested range with a correlation coefficient (R^2) of 0.9998, demonstrating the excellent relationship between peak area and ivermectin concentration.

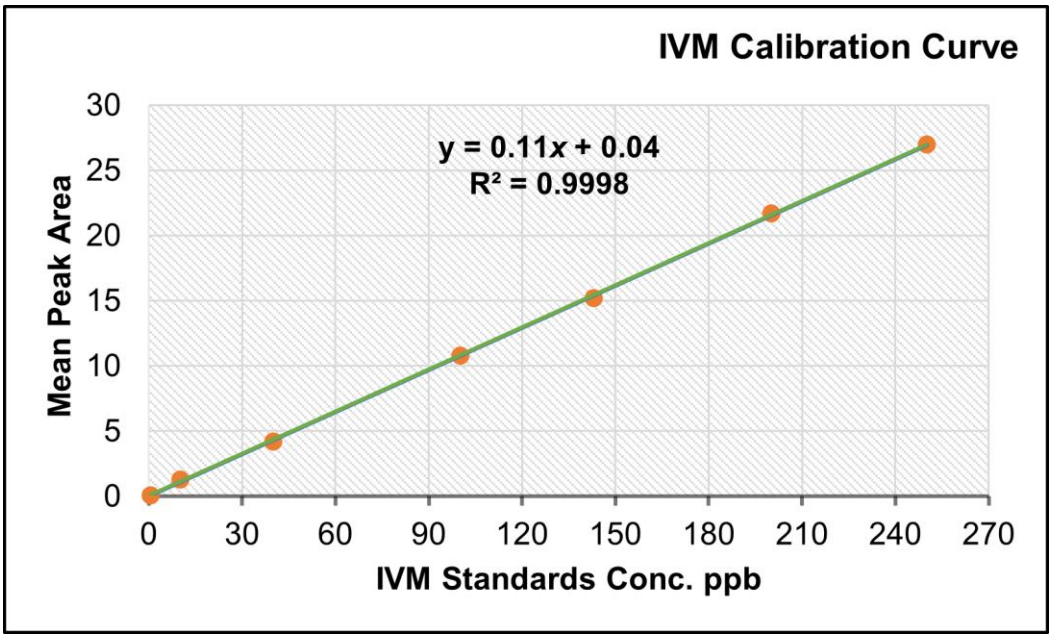

Figure 2. 8: The graph indicating the relationship between the average peak area (n = 3) and ivermectin concentration during instrument calibration.

The lower limit of detection (LOD) was defined as the signal corresponding to three times the signal-to-noise ratio ($3S_o/b$). While the lower limit of quantification (LOQ), defined as the lowest ivermectin concentration that can be analyzed with precision and accuracy,

was calculated as ten times the baseline noise ($10S_o/b$). The coefficient of instrumental sensitivity, *b,* is the slope of the linear portion of the calibration curve for the set of standards with concentrations ranging from 1 to 250 ppb; and S_o was estimated as the standard deviations of peak areas obtained after ten subsequent injections of standard the lowest concentration (0.*8* ppb). The results obtained from those ten subsequent injections are presented in **Table 2.1.** Using the results depicted in **Table 2.1**, we calculated the So; hence, LOD and LOQ were computed and found to be 0.23 and 0.75 ppb, respectively.

Table 2.1: Determination of LOD and LOQ

Injection Number	Peak area	Injection Number	Peak area
1	0.077	6	0.082
2	0.078	7	0.075
3	0.098	8	0.09
4	0.082	9	0.093
5	0.082	10	0.098

Accuracy was determined based on the recovery of known amounts of analytes. Water samples were prepared by spiking a known dose of ivermectin (10, 40, and 142 ppb) in blank environmental or Millipore Mill-Q waters. The spiked water samples were prepared in triplicate and subjected to FPSE and SPE extraction procedures, and the percent recoveries for each method were calculated using equation 1. The results of accuracy studies are shown in **Table 2.2**, and it is evident that the method is accurate within the

desired recovery range. In addition, the percentage recoveries resulting from three individual sample preparations and analyses agree with a method target range.

$$Percentage\ recovery = \frac{Peak\ area\ of\ the\ sample\ extract}{peak\ area\ of\ the\ standard\ sample} \times 100\% \qquad (1)$$

The method's precision was evaluated through the repeatability of the technique (intra-assay accuracy) by assaying ten replicate injections of a known concentration of ivermectin standard during the same day under the same experimental conditions. The percent relative standard deviation (RSD) values of the retention time, area, and height of the ivermectin peak are presented in the Supporting information. Our method is of acceptable precision[39].

Table 2.2: Mean peak area ($n = 3$), CV, and percentage recovery of ivermectin by FPSE and SPE from Millipore water (MilliQ Water), soil, and Environmental water samples.

Extraction method	Sample	Expected recovery(ppb)	Av. sample peak area	Standard peak area	Coefficient of variation, %	Mean % recovery
FPSE	***Env. Water***	10	0.92	1.1	3.1	83.3
		40	4.04	4.6	7.5	87.8
		142	12.2	12.6	0.8	96.8
	MilliQ Water	10	1.22	1.30	0.8	93.8
		40	3.70	4.10	4.2	89.8
		142	12.10	12.10	0.9	100
	Soil	10	1.20	1.23	1.7	97.6
		40	4.0	4.2	1.7	95.2
		142	11.9	12.1	1.7	98.3
SPE	***Env. Water***	10	1.12	1.2	5.4	93.3
		40	3.91	4.20	1.0	93.1
		142	14.6	15.9	1.4	91.8
	MilliQ Water	10	1.28	1.5	11.7	85.3
		40	3.89	4.5	2.3	86.4
		142	14.4	15.9	2.5	90.6
	Soil	10	1.3	1.5	5.0	86.7
		40	4.3	4.7	4.8	91.5
		142	12.5	13.1	4.6	95.4

2.3.4.2 How do our FPSE and SPE compare?

For comparison purposes, we analyzed simultaneous soil, Millipore, and environmental water samples spiked with 142, 40, and 10 ppb ivermectin in triplicate by both FPSE-HPLC-FLD and traditional SPE-HPLC-FLD. First, the obtained peak areas were compared with freshly prepared standards to calculate percentage recoveries. The percentage recoveries averaged ($n = 3$) are depicted in **Figure 2.9**. Data from the **Figure 2.9** suggest that there was a variation in percentage recoveries between methods and concentrations. To confirm the significance' difference, we performed a two-way ANOVA (full factorial) statistical test in JMP Pro 16. During modeling, methods (FPSE and SPE) and concentration levels were considered as independent (categorical) variable. The means and standard deviations of percentage recoveries ions were computed from all three samples. The obtained means were used for statistical analyses. The p-values obtained for methods, concentration and concertation method interaction terms are 0.39, 0.22, and 0.22, respectively. These findings suggest that there was no significant difference between the two methods and that the concentration of samples did not affect our percentage recoveries. LS can further confirm this suggestion Means difference Turkey HSD analysis (α=0.05)

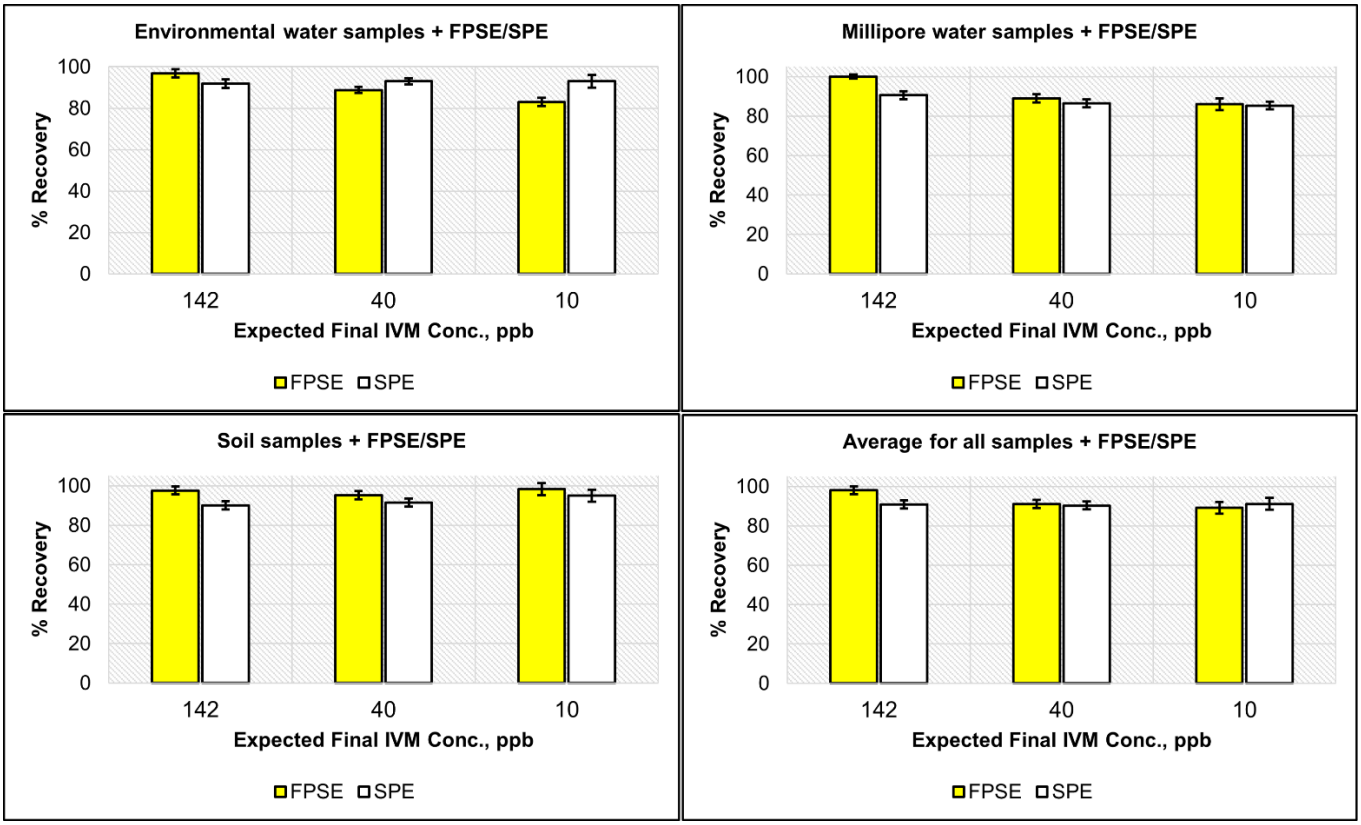

Figure 2. 9: Compared average percentage recoveries obtained with FPSE and SPE for soil, environmental, and Millipore water ivermectin samples at three expected recovery concentrations. The error bars in this diagram represent the standard deviation from the mean percentage recoveries (n = 3).

Although no substantial differences in percentage recoveries were found between the results of these two methods, FPSE provides similar recoveries to SPEs with added advantages. FPSE is found to be about 6 times more rapid because it removes the exhaustive labor-intensive of the SPE method. The FPSE method can be employed for environmental water samples, avoiding the problems of the C-18 cartridges clogging experienced for SPE. Moreover, the FPSE method can be used for infield sample analysis at a low cost compared to SPE.

2.4 Conclusion

Under this research, an FPSE-HPLC-FLD for determining the ivermectin concentrations in soil, Millipore, and environmental water samples was developed and validated. The FPSE media was synthesized by coating poly (tetrahydrofuran (average $M_n \approx 250$) on cellulose material. The method's optimum extraction parameters, like FPSE media size, desorption solvent, sorption, and desorption time, were determined. The method's applicability was tested on both Millipore and environmental samples and different ivermectin concentrations. An excellent percentage of recoveries were obtained and compared with those obtained with traditional SPE-HPLC-FLD. Although there were no significant differences in percentage recoveries between these two methods, FPSE-HPLC-FLD has the advantage of overcoming more challenges experienced when using SPE-HPLC-FLD. Therefore, FPSE-HPLC-FLD can be preferred as an efficient analytical method for infield ivermectin concentration monitoring in environmental water and soil.

2.5 Acknowledgments

We are grateful for the financial support from ISGlobal (Barcelona Institute for Global Health), Virginia polytechnic institute and state university, and Ifakara health institute. In addition, we thank the University of Dodoma for the study leave of Gerald Shija.

2.6 Conflict of Interest declaration

The authors proclaim no conflicting financial interests.

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Chapter 3. Transformation of Ivermectin and 3″-O-demethylivermectin in Soils from Temperate and Tropical regions

Abstract

Ivermectin (IVM) is a broad-range anti-parasitic drug usually employed to treat and control veterinary and human parasites. Because of its efficiency in the medical field, its use has been increasing. However, most of the administered IVM remains intact and is eliminated with metabolites (3″-O-demethylivermectin:3DI included), primarily in feces and to some degree (~ 2%) in urine. This means that greater use of IVM may lead to environmental contamination in areas where manure and urine can enter the environment. Furthermore, the discharged ivermectin residues have been reported to be toxic to non-target soil and manure-dwelling organisms, destroying the ecosystem and compromising soil productivity due to conceded nutrient recycling. Therefore, the transformation study of IVM and 3DI under different environmental conditions becomes crucial. Researchers have tried to address this subject. However, most of their findings are generated in temperate climates. Regional discrepancies such as soil types and environment can independently and dependently determine the fate and impact of ivermectin. Photodegradation investigation of ivermectin and its primary biological metabolite, 3″-O-desmethylivermectin, was conducted in Tanzania and Virginia to investigate the differences between tropical and temperate climates. Ivermectin and its metabolite were spiked at 1 ppm into the soil from these regions. These soils were layered into two 5 mm layers in the column and exposed to natural sunlight for up to 100 h at different temperatures and 20, 15, and 10 % soil moisture contents. The top and lower soil layers were collected at various predetermined time intervals during this study. The remaining ivermectin and 3″-O-demethylivermectin were quantified using Liquid Chromatography-tandem Mass Spectrometry. The results suggest that 3″-O-demethylivermectin and ivermectin experienced rapid degradation in the upper layer, fitting into simple first-order

kinetics. The obtained half-lives in this study indicate that photodegradation was about 1.5 times faster in Tanzania compared to Virginia. Due to photonic depth issues, ivermectin and 3″-O-demethylivermectin in the lower layer experienced retarded degradation with not more than 60 % degradation after 100 h. The obtained half-lives suggest that mass administration of ivermectin would not result in its environmental accumulation, primarily when the dung from treated cattle is spread into the field in thin layers in the summer before planting.

3.1 Introduction

Ivermectin, a member of the macrocyclic lactone family, was semi-synthesized by Satoshi Omura in the early 1970s, marketed as a veterinary drug, and became a best seller in 1981 [1]. Since its discovery, this “wonder drug” has demonstrated an unprecedented broad therapeutic index against both endo-and ectoparasites [2-6]. Administered orally, topically, or parenterally, ivermectin is toxic to parasites and insect pests [7]. In its early years, ivermectin dominated the veterinary industry showing its efficacy against parasites like lungworms, mites, lice, and horn flies [8-10]. Additionally, ivermectin demonstrated unparalleled effectiveness against the ixodid tick, *Rhipicephalus (Boophilus) microplus* [7]. These ticks parasitize cattle, causing economic damage in most tropical countries [11]. Currently, ivermectin is used for parasite control in billions of livestock and pet worldwide, helping to enhance food and leather product production and keeping companion animals, particularly dogs and horses, healthy [7].

Unmatched success in the animal health market led to ivermectin approval as a human chemotherapeutic after a large-scale community trial by Merck, WHO, and other stakeholders on human Onchocerciasis [7]. Since its approval for human use, ivermectin has become a cornerstone component of mass drug administration (MDA) programs to eliminate onchocerciasis (river blindness) and lymphatic filariasis (elephantiasis) in bucolic areas of Africa and Latin America [2]. For instance, in 2012, 99.3 and 105 million Africans received annual ivermectin doses for onchocerciasis and lymphatic filariasis elimination campaigns, respectively. Furthermore, due to its extended therapeutic application for neglected tropical diseases like malaria, ivermectin consumption is expected to increase in the following decades, especially in low-income countries.

Although Ivermectin has many positives regarding animal and human cures, its residues have negative environmental impacts. Ivermectin and its metabolites excreted in feces are reported to be lethal for dung-dwelling invertebrates, threatening nutrient availability[12, 13]. Similarly, ivermectin residues in the manure can be toxic to important decomposers such as dung beetles and flies [14-16]. Furthermore, increased ivermectin demands and supply will increase its discharge to the environment, which potentially means more toxicity to dung beetles and other non-target organisms in the background. For example, the toxicity of dung beetles has many implications for the terrestrial ecosystem. Firstly, it affects the activities of other organisms like earthworms, soil bacteria, and coprophilous fungi because dung beetles' action on dung creates a conducive environment for these organisms [17]. Secondly, decreased populations of dung beetles affect methane release from the soil, resource circulation between plants and animals, and hence low soil fertility[18-21]. Loss of soil fertility could result in loss of food and pasture productivity, leading to famine and loss of livestock health and yield [22]. Therefore, with the increasing awareness of food and environmental security, public concern, persistence, and the fate of ivermectin and its metabolites must be prioritized to avoid these impacts.

Photo transformation is the primary environmental process that determines ivermectin and metabolites persistent in the soil/manure compartments. The concentration of ivermectin residues trapped in soil/manure mixture has decreased due to exposure to sunlight [23]. The rate of photo transformation reactions could be affected by many factors, including temperature, climate, and soil type[24]. In feces/soil mixtures, ivermectin transforms more rapidly in summer than in winter in the same

environments[12]. Similarly, Bull and co-workers report that ivermectin degrades more slowly in sandy soil, followed by clay soil, with sand-loamy soil showing a rapid rate of degradation [25]. However, most of these studies are conducted in temperate geographical zones, with information lacking in tropical regions where most of the ivermectin is used to control neglected diseases. Different degradation rates can be expected because tropical regions' soils are highly weathered due to intense rainfall, experiencing high temperatures, and low soil organic matter [17].

This study reports the photo transformation study of ivermectin and its primary metabolites in the soil and soil/manure mixture performed in temperate and tropical climates. Since sunlight cannot penetrate beyond 5 mm in the soil, our experimental setting will include layering the soil into two layers 5 mm thick each. The soil layers above the screen (upper layers) only receive natural sunlight [26, 27].

3.2 Materials and Methodology

3.2.1 Materials

3.2.1.1 Substrates

Two agricultural soils used under this photodegradation study were 0-15 cm topsoil collected from Madison, Virginia, United States, and Ifakara, Morogoro, Tanzania. The selected physiochemical properties are presented in **Table 3.1** below.

Table 3.1: Selected physicochemical properties of the soil used in this study

Soil properties	Virginia	Ifakara
pH	5.60	6.80
Soil organic matter (%)	1.21	0.46
Soil type	Clay loam	Sandy loam
Soil color	Light brown	Reddish brown

In Tanzania, fresh manure was donated by local farmers, and in the United States, fresh dairy manure was collected from a local dairy farm in Blacksburg, Virginia. All accumulated soil and manure samples were used as substrates for this study. Before the sample dosing, gravimetrical moisture contents for both substrates were determined in the laboratory. Except for manure sampled in Blacksburg, whose moisture content was determined by freeze-drying method at -40 ^{o}C for two days, soils and manure in Tanzania were air-dried in the dark at room temperature. The weight of wet soil or manure was recorded on an analytical balance. The manure in the United States was freeze-dried for two days and weighed again, and the difference in weight between wet and dried manure was recorded as gravimetrical moisture content for the manure. For all soil substrates and manure in Tanzania, wet substrates, after recording the weight, were spread on aluminum foil on top of the bench and allowed to dry under the influence of air. The weight of the soil was recorded daily until the constant weight was recorded for five consecutive days. As for the manure substrate above, the difference between the weight of wet and dry substrate was considered substrate gravimetric moisture content. All substrate background analyses (data provided in the supporting information) indicated they were free from ivermectin or 3″-O-demethylivermectin contaminations. Dry soil was passed through a 2 mm sieve to remove gemstones and large flora fragments and homogenized. Some of the homogenized soil was packed in falcon paper and stored for laboratory experiments, and the rest was used for making a soil manure mixture. Soil manure mixture was made by mixing 10 % of manure and 90 % soil, and the heterogeneous mixture was ground while mixing until a homogeneous mixture was obtained. The

obtained soil manure homogenous mixture was packed and stored for incubation experiments.

3.2.1.2 Chemicals and materials

The chemicals used in this photodegradation study were ivermectin (IVM, CAS MKCC9996 purity 95 ≥ 80 % H_2B_{1a} and < 20 % H_2B_{1b}) and 3″-O-demethylivermectin (3DI) procured from Sigma-Aldrich Co. (3050 St Luis MO, USA). Since they were of high purity and suited the analytical grade, they were used without further purification. LC-MS grade solvents, methanol, acetonitrile, and ultrapure pure water were purchased from Fisher Scientific (1 Reagent Lane, Fair Lawn, New Jersey, USA). Formic acid, ammonium formate, 0.2 µm filters, syringe, and needles were bought from Fisher Scientific (1 Reagent Lane, Fair Lawn, New Jersey, USA). Water free from IVM, 3DI, or any other contaminants used for moisture balance during photodegradation exposure was produced in our laboratory using the Milli Q-gradient system (Millipore, Bedford, MA). Glassware was soaked, cleaned, rinsed, and dried according to our laboratory-approved protocols. To overcome the solubility issues and have similar preparation between IVM and 3DI, 1000 ppm stock solutions of these chemicals were prepared in HPLC grade methanol and stored at -20 ^{o}C for over two months. All spiking dosages were prepared by serial dilution method of these stock solutions in methanol. The calibration curve concentrations standards were designed by diluting the respective stock standards in a 1:4 *v/v* water-methanol mixture.

3.2.2 Experimental Design

Transformation of ivermectin and its metabolite is one of the main determinants of its persistence in the soil or manure hence the drug's impacts on non-target organisms.

Many factors affect the ivermectin degradation process, including sunlight temperature, the nature of the soil, and the amount of organic matter. We studied the effects of temperature, sunlight, the nature of the soil, and moisture contents on the degradation of ivermectin and 3″-O-demethylivermectin. Soil and 10 % manure soil samples from Ifakara, Tanzania, and Virginia, United States of America, were used. Virginia soil is highly organic compared to the soil in Ifakara, which has highly weathered. To mimic the different climatic conditions, the incubation was done in Ifakara and Virginia. Soil or soil-manure samples were prepared by spiking 1 mL of 10 ppm of 3DI or 1 mL of 10 or 100 ppm dry and homogenized soil and soil-manure mixture prepared in section 2.3.1.1 above. After complete evaporation of methanol, a certain amount of water was added to make a 10, 15, or 20 % and well-mixed to distribute the analyte into the substrate. Then, the IVM and 3DI spiked soil or soil-manure column were parked into two layers separated by a screen, and the weights of the samples were recorded. Samples were prepared in triplicates (to study the rate of drug degradation), and the mean values were calculated from these samples. The model packed 5 mm below in the column was termed the lower layers, and the top 5 mm above the lower layer's column was termed the upper layer. After that, the samples were exposed to direct sunlight at a specific time interval from 0 to 100 accumulated hours in July to August 2021 and April 2022 in Virginia and December 2021 and June to August 2022 in Ifakara, **Figure 3.1**. During the exposure period, the temperature ranged from 16 to 28 ^{o}C in most of the afternoon to evening in Virginia, and 22 ^{o}C to 30 ^{o}C was the temperature range in Ifakara. There was no rainfall during the incubation period. To keep 10, 15, and 20 % of soil moisture contents during the experiment, samples were weighed at 3-h intervals to determine the loss in weight, which

was considered loss due to water evaporation. Therefore, the amount of water equal to that loss in weight was added. To stop degradation when the samples are not exposed to natural sunlight.

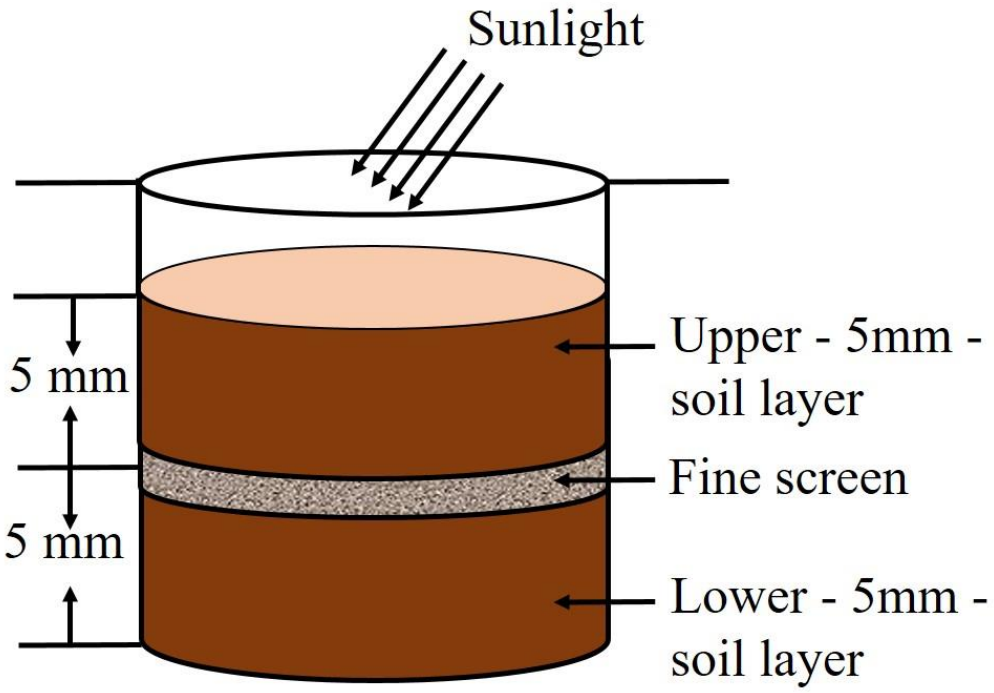

Figure 3.1: Representation diagram of the compartment used in the laboratory degradation study. The column was made of Pyrex glass and covered with aluminum on the side to allow sunlight to enter only through the top of the column.

The experiment kept factors like moisture content and soil organic matter constant. Samples were brought indoors, covered, held in a particular container, and refrigerated at night or during inclement weather. After each time interval, three replicate samples were removed from the incubation settings, the exemplary screen was lifted at the end of a photodegradation period, and the top 5 mm soil on top of the screen (upper layer) was transferred into a storage container for storage at -20 ^{o}C until further extraction, cleanup, and analysis. Likewise, the soil below the fine screen (lower layer) was collected into a separate storage container at -20 ^{o}C until further extraction, cleanup, and analysis.

Samples from Tanzania were extracted at Ifakara Health Institute (IHI) and shipped to Blacksburg as dry residues in well-packed vials. The transportation and procedures were in line with the method established in **section 2.3.3.5** to avoid further degradation. After each incubation period, the upper and lower layers were processed and analyzed for ivermectin and its metabolites.

3.2.3 Sample Extraction and Clean-up

The soil or soil-manure samples' layers were ground and mixed well after sunlight exposure for 0 to 100 hours. Approximately 1.00 g (exactly recorded weights) were weighed and mixed with 2 mL methanol in a 10 mL polypropylene copolymer centrifuge tube and covered tightly with a lid. The sample was vortexed at 800 rpm for 10 minutes and centrifuged at 3500 ppm for 5 minutes. Immediately after the centrifugation of samples and without disturbing the sediment, the supernatant was transferred into a vial. Then another exactly 2 mL methanol was added into the tube containing the deposit and vortexed at 800 rpm for 10 min, followed by centrifuging the sample at 3500 ppm for 5 min. Immediately after centrifugation of the pieces, the obtained supernatant was transferred to a vial above without disturbing the sediment. The obtained supernatant was mixed (via vortexing for 5 seconds) for samples extracted, evaded solvent to dryness for samples extracted at Ifakara in the predetermined conditions. No degradation was expected because our stability test in chapter 2 indicated that IVM was stable for more than 21 weeks under those conditions. Upon arrival in our laboratory in the US, the residues were dissolved in 4mL methanol. During analysis, 2 mL was pipetted into the 2 mL HPLC vial and centrifuged at 10000 rpm for 10 min. After centrifuging, 100 uL of the supernatant was pipetted and diluted to 1000 uL with LC/QQQ mobile phase (0.1% FA

+20% H2O + 80% MeOH). The obtained solution was filtered through a 0.20 µm syringe filter and analyzed on the LC-MS/MS as per the instrumental setting shown in **Tables 3.2** and **3.3**

3.2.4 Instrumental Analysis

IVM and 3DI were analyzed on a 1290 Infinity LC System connected to a 6490 triple quad LC-MS (Agilent Technologies, Santa Clara, CA, USA). The sample injection and temperature maintenance at 4 ^{o}C were done by autosampler Thermostat (1290 series, Agilent Technologies). Fragment-specific mass spectrometry settings optimized by direct infusion of 1 µg cm^{-3} IVM or 3DI prepared in 1:4 v/v water-methanol mixture and other general locations are summarized in **Table 3.2**

Table 3.2: LC-mass spectrometry setting

Parameters	Qualifier **IVM** (892.6–569.4) and **3DI** (878.6 – 569.0)
Gas temperature	523 K
Gas flow rate	14 l/min
Nebulizer	45 psi
Sheath gas temperature	473 K
Sheath gas flow rate	8 L/min
Capillary potential	3.5 kV
Collision energy	10 kV
Dwell	150 kV
Cell accelerator potential	4 kV
Fragmentor potential	380 kV

Waters UPLC HSS T3 (2.1 x 100 mm, 1.8 mm particle size, 34 Maple St., Milford, MA 01757, USA) column was used for liquid chromatography separation. Mobile phase A (0.1 % v/v formic acid prepared in LC-grade water) and mobile phase B (0.1 % v/v formic acid prepared in methanol and 20 mM ammonium formate were freshly prepared in every

instrumental run. The analytes were eluted by a mobile gradient phase, as shown in **Table 3.3,** flowing at 0.5 and 0.6 cm^3/ min flow rates. The obtained data were analyzed by using Agilent MassHunter Quantitative Analysis software.

Table 3.3: Gradient elution spectrometry settings

Time, min	Mobile phase B%	Flow rates, cm^3 /min.	Maximum Pressure Limits, bar
0	90	0.5	1000
0.25	90	0.5	1000
0.5	100	0.5	1000
2.75	100	0.6	1000
2.76	90	0.6	1000
4.74	90	0.5	1000

3.2.5 Statistical Analyses

The statistical summary (mean, standard deviations, data distribution, and outliners identification) was done on JMP Pro 16 (SAS Institute Inc, 2019). The obtained mean and standard deviation was used to calculate the percentage of ivermectin and 3DI in the upper and lower layers at each time interval. The average (n = 3) of IVM and 3DI concentrations recorded on samples without exposure to sunlight were termed C_o, and those registered after exposures for a predetermined time were termed C_t. These two variables were used to calculate the percentage of ivermectin, or its metabolite, left in the sample after sunlight exposure for time, t, by using the equation:

$$\text{Percentge IVM 3DI in the sample after time, t} = \frac{C_t}{C_o} \times 100 \qquad (1)$$

The obtained values using equation 1 from each layer and samples were plotted against the incubation period. The significance of the half-lives was calculated by fitting the

percentage of IVM and 3DI in the upper layer into a simple first-order kinetics in Origin 9.1® software.

3.3 Results and Discussion

3.3.1 The transformation of IVM and 3DI in the Soil

When the percentage of 3DI and IVM remaining in the upper and lower layer are plotted against the incubation period, graphs similar to those presented in **Figures 3.2** and **3.3** are obtained. These figures indicate that the concentration of 3DI and IVM in the soil and soil-manure layers decrease with the increase of the incubation period. IVM and 3DI have very high vapor pressures suggesting that they cannot evaporate into the air in our experimental setting[23]. Moreover, our practical set-up rules leaching or any forms of analyte loss. Therefore, the decrease in concentration suggests that IVM and 3DI under our experimental settings were subject to transformation. These transformation processes may include biotic and abiotic elimination and hydrolysis under both acidic and basic conditions. Our findings align with the literature on the environmental fate of IVM, alluding that this drug is prone to change, and several conditions affect its persistence in the soil[28-30].

Although the literature agrees on the transformation reactions, variable information is reported on the transformation products. For example, *Tremblay* and *Wratten* wrote that IVM degradation results in more polar and less toxic degradation products than the parent drug[31]. To some existent, *Tremblay* and *Wratten's* findings agree with Krogh et al., where two IVM transformation products were isolated and structurally identified[24].

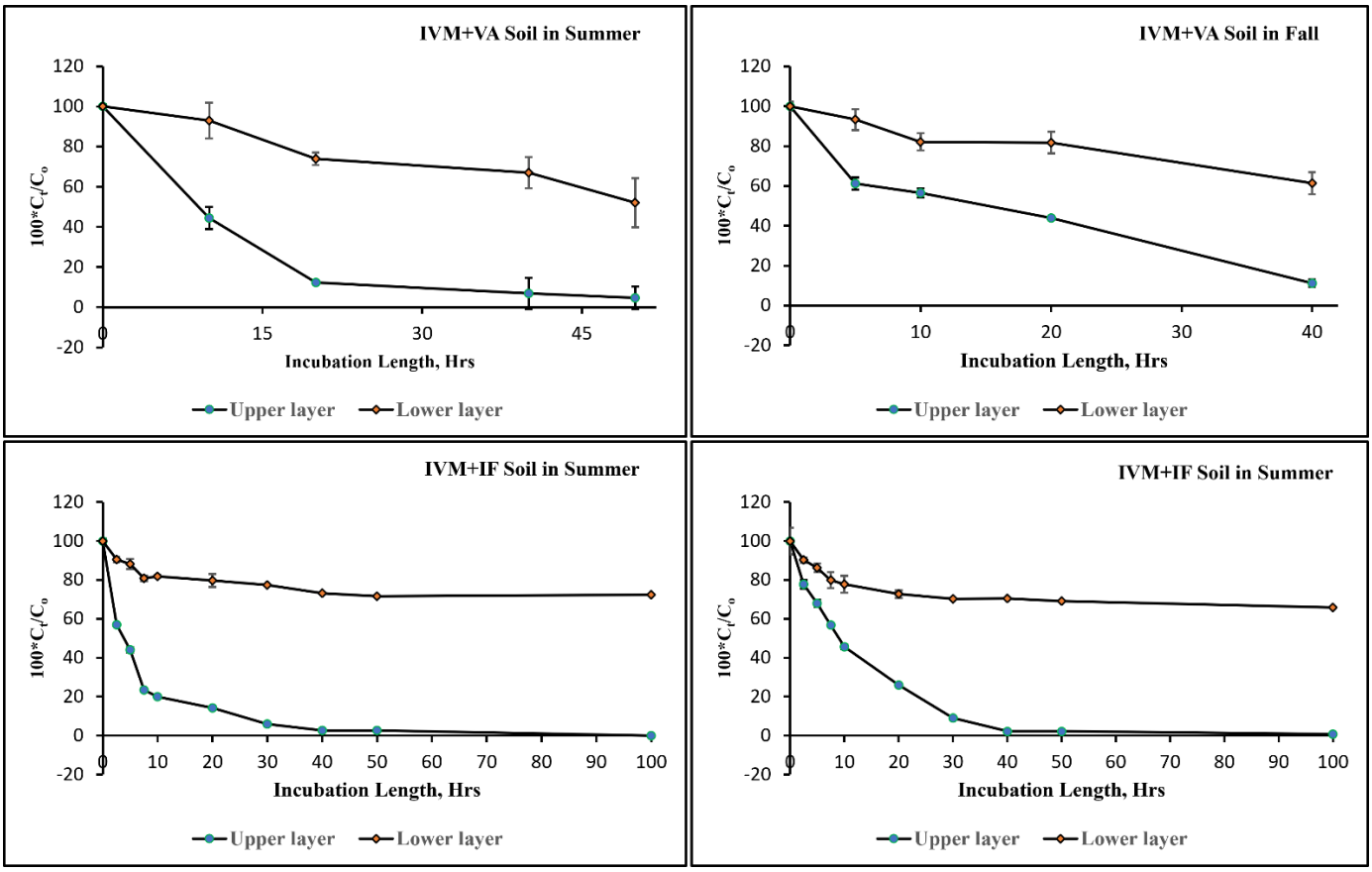

Figure 3.2: IVM transformation profile showing the percentage of analyte remaining in the soil after exposure to sunlight at a particular time interval. The soil incubation laboratory experiments were conducted in the Summer and Fall in Virginia (VA) and Summer at Ifakara (IF) in Tanzania at 10 and 20 % soil moisture content. The error bars under these diagrams represent the standard deviation from the mean percentage.
(n = 3).

However, Krogh et al. acknowledged that the amount of the degradation products was too small (less than 10 % of the parent drug) to conclude that they could identify any degrades[24].

Although 3DI and IVM are subject to transformation, results indicate that these two analytes' transformation is not unformed in both layers.

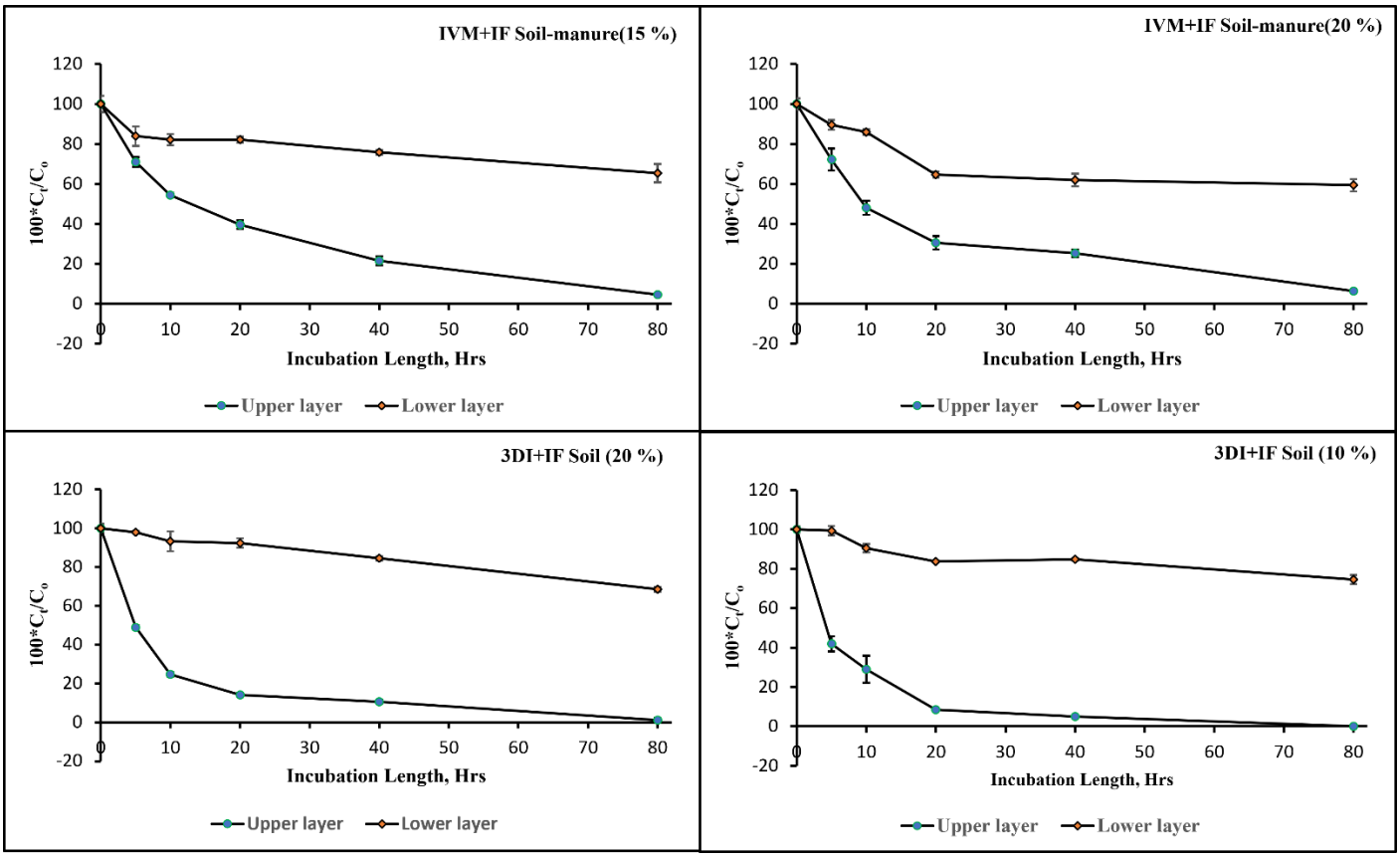

Figure 3.3: The average percentage (n = 3) of IVM and 3DI concentration remaining in soil top 5mm and bottom 5 mm as upper and lower layers after exposure to natural sunlight. Laboratory studies at Ifakara (**IF**) were performed in the Summer at 10 and 15 and 20 % soil moisture content. Error bars represent $\pm SD$.

Figures 3.2 and 3.3 show variations in transformation rate with soil depths. Moreover, soil moisture, organic matter, and temperature affect the degradation rate. The following subsections will represent the results and discuss this study.

3.3.2 The Degradation Profile of IVM and 3DI in Different Soil Depths

Figures 3.2-3.5 below indicate that IVM or 3DI in the soil or soil-manure mixture packed 5 mm below the surface transforms slowly to less than 40 % of the initial concentration after 100 h of exposure to sunlight. But a significant decrease in IVM and 3DI was observed in the top 5 mm soil or soil-manure layer when exposed to natural sunlight. For

example, 3DI in the soil incubation experiment conducted at Ifakara indicates that keeping moisture contents at 15 % leads to only a 3 % decrease of the analyte in the lower layer. Contrary to what is observed in the lower layer, the top 5 mm soil experienced more than a 55 % decrease in the analyte in the same experimental setting during that period.

Similarly, the data shows that keeping moisture content at 10 % and exposure to IVM soil sample in Virginia leads to 56 % in the upper layer compared 8 % decrease observed in the lower layer in 10 h. incubation length. This decreasing trend is similar in all incubation experiments in Ifakara and Virginia. While there is limited information on the degradation profile of 3DI, IVM degradation in a similar environment has been reported. Crouch *et al.* said that ivermectin in a thin film exposed to natural sunlight is subjected to

rapid transformation [32]. This transformation reaction results in polar compounds with mostly different physical and chemical properties compared to the parent drug.

Similarly, Halley et al. stated that ivermectin in soil or soil-manure mixture decomposes by the action of sunlight via a photodegradation reaction, decreasing the concentration of ivermectin in the matrix due to the formation of more polar degrades compared to the parent drug [12]. Although we could not find literature on 3DI degradation, it is clear that it follows a similar trend. Since sunlight plays a crucial role in IVM and 3DI transformation, it is unsurprising to observe the limited transformation of these compounds at depths > 5 mm. The change in transformation rate with depth was reported on endocrine-disrupting 4-nonylphenol (4-NP) research in our laboratory. In that study, a rapid 4-NP transformation rate was observed in the upper 5 mm of soil compared to the rate recorded in the lower layer during the same period [33]. The observed difference in the transformation rate of substances with depth is attributed to the photic depth factor. The

photic depth of light into the soil or soil manure is known to be very limited to about the top 5 mm of the soil surface. Therefore, its influence is considerably different in the top 5 mm soil layer than in the bulk soil or soil manure matrices [34]. For example, the chemical environment of the top 5 mm of the soil layer is conspicuously distinct from that of the rest of the soil. Because under this soil layer, the solid, solution, and vapor phases are all near the soil-atmosphere interface and are subject to sunlight irradiation. In addition, sunlight heating the soil also alters the surface and the soil properties like moisture content and temperature [27, 35]. Since factors like moisture content and temperature affect degradation, it is not surprising that most photochemical transformations occur in this layer [36, 37].

3.3.3 Transformation Kinetics of IVM and 3DI in the Soil

Kinetics consideration of this type of transformation reaction is critical because they generate information that predicts self-life, fate, and magnitudes of environmental impacts of substances [38-40]. Under this study, the term kinetics refers to the study of the rate of IVM and 3DI photo- and aerobic transformation reactions. IVM and 3DI are very sensitive to sunlight; therefore, they undergo rapid photo transformation on the matrix surface. The photo transformation rates reported range from less than 0.5 a day to 21 h, depending on the temperature and nature of sample matrices. The studies conducted in water at 41 ^{o}N in the temperate region reported photo transformation half-lives of about 12 h or less. Four times smaller photodegradation half-life was written when the experiment was conducted in the summer sunlight in a thin, dry film on glass outdoors. However, a 142 times slow photodegradation rate was reported in 41 ^{o}N USA on soil TLC plates when the experiment was conducted in the Fall (September). Another pathway for eliminating IVM

and 3DI is the action of microbes under aerobic or anaerobic conditions. This form of degradation depends on temperature and the ability of oxygen to diffuse into the soil particles. That is why it is not surprising that rapid degradation is reported in the summer compared to the winter. In most of the winter season, soil or soil-manure mixtures will be water saturated. The oxygen diffusion into subsoil is limited, and soil temperature is low. Therefore, the rate of microbial activities, including degradation reactions, may be lowered. Under low oxygen contents, only anaerobic degradation can take place. This reaction is minor and has a negligible effect on drug concentration.

Although transformation can be either biotic or abiotic, kinetics information plays a crucial role in suggesting factors that determine the fate and impacts of chemical substances. Kinetics studies generate data that help to predict the degradation mechanism and offer methods for detoxifying contaminated soil or manure. That is why it is common to find persistence and fate studies expressed in this aspect. Under this aspect, the concentration of the analyte is determined as the exposure period progresses. The obtained concentration is fitted into the model, and half-lives are calculated from those models. Halley et al. calculated the ivermectin half-lives by subjecting the observed remaining IVM concentration in the soil to the simple first-order model[41]. Other studies have reported that IVM follows this model under different environmental settings[42, 43]. The IVM and 3DI degradation reaction rates and pathways may be affected by many factors.

$$\frac{C_t}{C_0} \times 100\ (y) = \exp^{(-kt(x))} \qquad (2)$$

C_o and C_t are IVM or 3DI in the soil or soil manure matrices at zero and t exposure length, respectively, and *k* is the rate constant for photodegradation reaction in the upper layer. Therefore, the simple first-order photodegradation rate constants, k, were established from the decay curve like those presented in **Figure 3.4**.

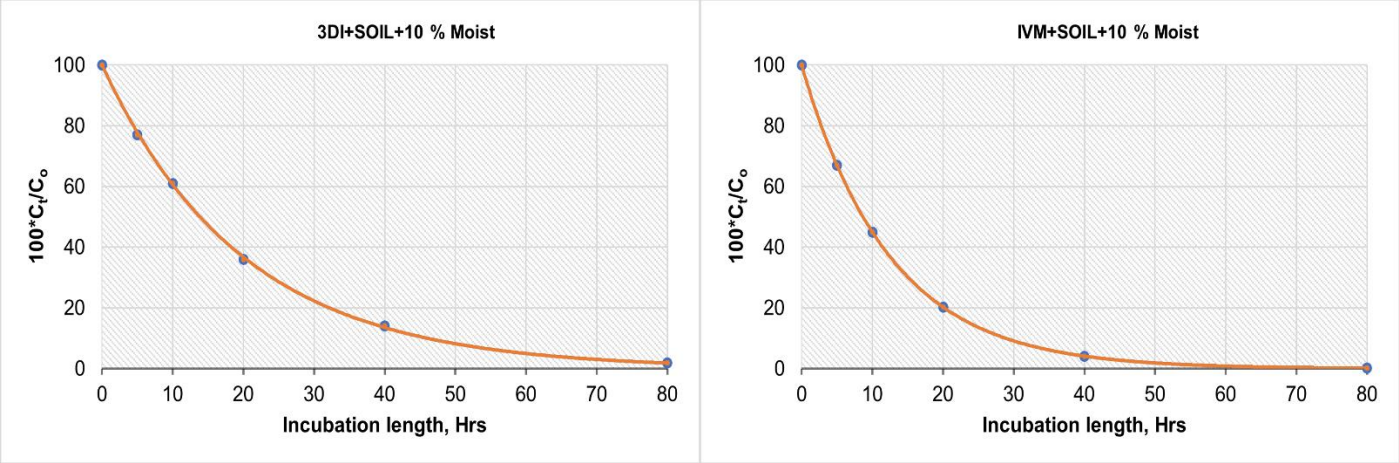

Figure 3.4: Representative simple first-order model (SFM) curves of 1 ppm 3DI and 1 ppm IVM soil extracts after incubation at Ifakara, Tanzania.

The photo transformation half-lives ($t_{1/2}$) were calculated using equation 3:

$$t_{\frac{1}{2}} = \frac{0.693}{k} \qquad (3)$$

and obtained transformation half-lives are listed in Table 3.4(**a** and **b**).

Table 3.4a: Summary of transformation kinetics half-lives obtained in Virginia, USA

		Sample moisture contents (%)	
		10	**15**
Sample matrix	**Analyte**	$t_{1/2}$(hrs.)	$t_{1/2}$(hrs.)
Soil	IVM	12 ± 1.2	20 ± 1.8
	3DI	-	17 ± 2.0
Soil-manure	IVM	23 ± 3.0	58 ± 2.0
	3DI	30 ± 2.0	58 ± 3.0

Table 3.4b: Summary of transformation kinetics half-lives obtained in Ifakara, Tanzania

		Sample moisture contents (%)		
		10	**15**	**20**
Sample matrix	**Analyte**	$t_{1/2}$(hrs.)	$t_{1/2}$(hrs.)	$t_{1/2}$(hrs.)
Soil	IVM	8.9 ± 1.0	13 ± 2.1	14 ± 1.2
	3DI	14 ± 3.0	24 ± 5.0	5 ± 2.0
Soil-manure	IVM	15 ± 2.2	19 ± 2.0	22 ± 3.0
	3DI	23 ± 4.0	17 ± 4.1	26 ± 2.5

The transformation half-lives data in Table 3.4(**a** and **b**) demonstrates that 3DI and IVM transformed differently between tropics and temperate regions. Moreover, moisture content and organic matter have impacted the transformation rate. The following subsections will discuss the correlation between these factors and observed half-lives.

3.3.3.1 Effect of climate on degradation kinetics of IVM and 3DI

The obtained half-lives summarized in Table 3.4(**a** and **b**) suggest that IVM and 3DI in soil or soil manure mixture undergo more rapid degradation in Ifakara than in Virginia under all experimental settings. **Figure 3.5** illustrates the trend in **Table 3.4**(**a** and **b**). For example, 1 ppm 3DI in the soil-manure at 15 % moisture content took only about 17 h for its concentration to reduce to 0.5 ppm compared to nearly 58 h spent to attain the same concentration reduction in Virginia. Similarly, IVM in the soil at 15 % moisture content degraded approximately 1.5 times slower in Virginia compared to the degradation rate obtained in Ifakara. To test the significance of these differences in transformation rate, we performed a one-way ANOVA test whereby half-lives were considered continuous variables, and study sites were treated as categorical variables. The statistical test confirmed that the difference was significant at p-values < 0.05.

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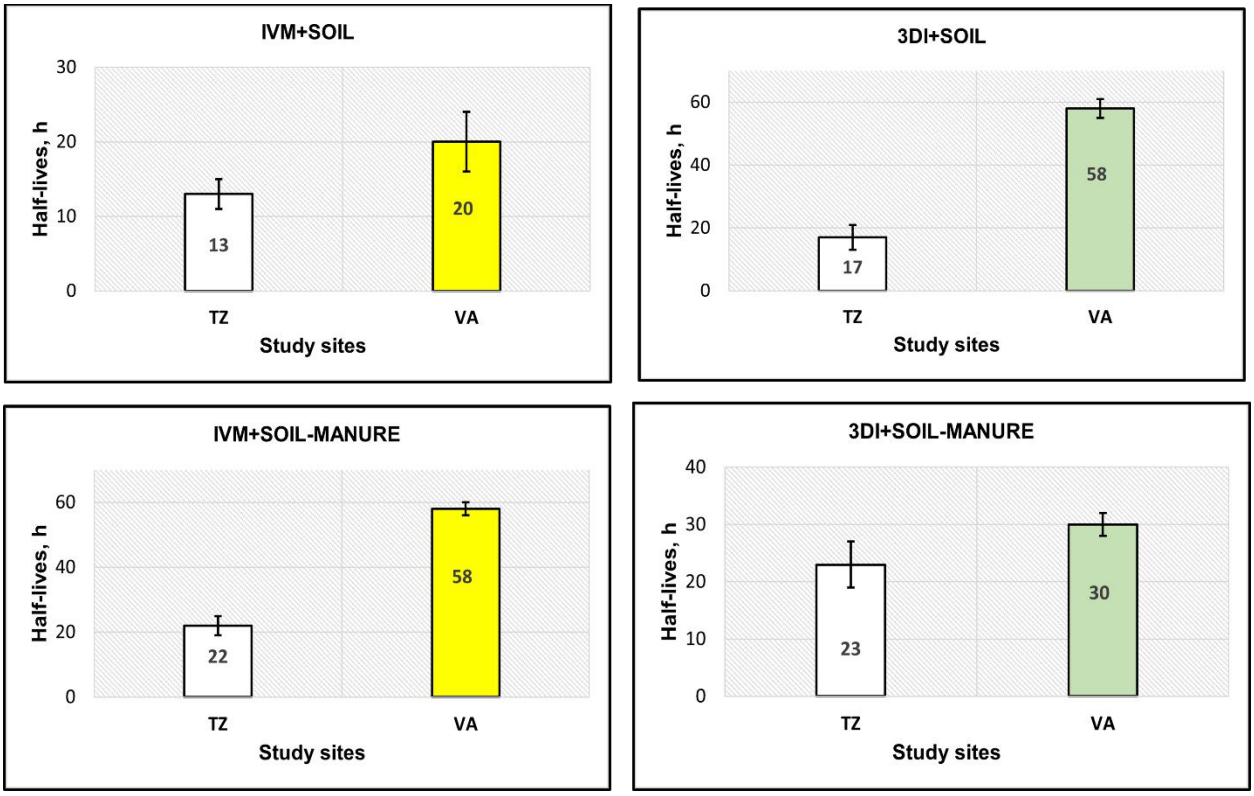

Figure 3.5: Summary of the obtained transformation trend of IVM and 3DI in Virginia (VA) and Tanzania (TZ).

This variation in transformation rates may be attributed to the difference in the environmental temperature and soil properties between the two study sites. The transformation study in Tanzania was conducted in the 22 to 30 ^{o}C temperature range compared to Virginia, where the temperature ranged from 16 to 28 ^{o}C. This means that Tanzania's studies were conducted in warmer environments in average terms compared to those in Virginia. The variation of the transformation rate trend between warm and cold climates is not unique to our study. Halley et al. reported a similar variation in transformation rate. In their laboratory sunlight exposure experiment, IVM in soil/manure mixture degraded faster with short half-lives between 7 and 14 days in the summer. The

retarded trend was recorded in the winter, where IVM degraded slowly, with a half-life ranging between 90 to 240 days [12]. Another factor contributing to the difference in photo transformation rates recorded in Ifakara and Virginia is the soil composition difference between the two study sites. Ifakara is in the tropical region, which experiences heavy seasonal rainfall and low humidity in most seasons. Therefore, its soil is eroded and comminated Fe and Al oxides, compared to Virginia soil which is more clayed and has high organic matter contents compared to Ifakara soil. The impacts of organic matter on degradation rate will be disseminated more in the following subsection when we discuss how the addition of manure impacted our results.

3.3.3.2 Effect of manure addition on degradation kinetics of IVM and 3DI

The impacts of addiction organic matter on the transformation rate were examined by adding 10 % of manure to the soil. This addition proved crucial to the transformation rate, regardless of climate and moisture content. In both cases, the retarded degradation was observed compared to pure soil. **Figure 3.6** depicts the selected representatives' examples in this regard. **Figure 3.6** reports that 3DI was found to transform 1.6 times slowly when 10 % of manure was added compared to the half-life recorded without manure addition. The impacts were more significant in cold temperatures compared to warm environments. For example, IVM in soil manure matrix degraded three times slower in Virginia than 1.31 times observed in Tanzania. Similarly, 3DI transformed 3.4 times in Virginia compared to 1.9 times retardation recorded in Tanzania with the addition of the same amount of manure.

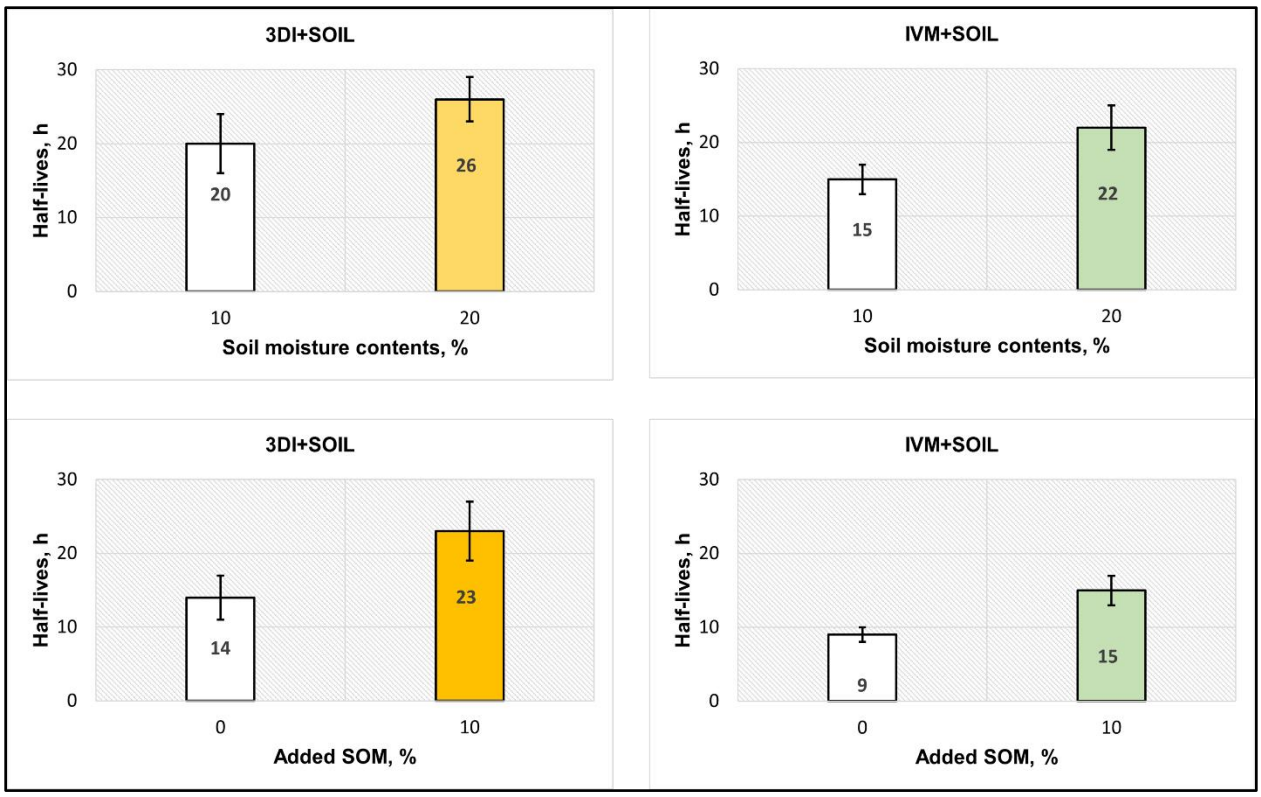

Figure 3.6: Variation of transformation rate with adding soil organic matter (SOM) and moisture content.

Although we were able to record consistency transformation retardation with organic matter addition, the impacts of soil organic matter on the degradation of pharmaceutical drugs are controversial. Some studies report that the presence of organic matter in the soil retains pharmaceuticals creating conducive transformation environments in the soil. However, the ivermectin trapped in manure degradation trend contradicts this narrative. Åsbakk et al. reported that ivermectin entrapped in the pasture could persist for more than two grazing seasons [44]. Similarly, Sommer et al. didn't observe any changes in ivermectin concentration levels in cow dung collected from dosed cattle for 45 days between the Fall and Summer seasons [45]. Our results under this study somehow agree with the findings that the presence of organic matter retard the ivermectin and 3DI degradation rate. IVM has a high affinity to organic matter, and since the addition of

manure increases the organic matter, this drug will be much more entrapped in this matrix than in soil. Moreover, Manure added to the soil may alter the physicochemical properties of ivermectin and 3DI in the soil. Findings from Boxall et al. [46], Thiele-Bruhn [47], and Aust have proved that adding manure to soil affects the sorptive properties of veterinary drugs like ivermectin and probably their metabolites. The altered sorptive properties may affect their persistence and transformation because ivermectin residues are strongly sorbed or susceptible to photodegradation. Furthermore, IVM and 3DI entrapped in the soil-manure mixture may experience reduced sunlight intensity due to manure matrix screening or shielding effect and hence a lower degradation rate [48].

Another impact of adding organic matter to the soil is on the light transmittance of the soil. The color of the soil particles and the presence of organic matter would intuitively be expected to affect the light transmittance of soils; *Woolley* and *Stoller* observed that, when the diameter of soil particles is held constant, the lighter-colored soil allowed light to penetrate further than the darker ones. This phenomenon was explained based on increased sunlight absorption on dark soil with high organic matter contents [49]. The reduced light transmittance affects its penetration depth and hence recorded degradation rate.

3.3.3.3 Effect of moisture content on degradation kinetics of IVM and 3DI

Table 3.4 and Figure 3.6 indicates that soil moisture contents also played a crucial role in IVM and 3DI transformation kinetics. In all the samples, IVM or 3DI entrapped in the soil or recorded at 20 % soil-manure mixture degraded rapidly at 10 % but slowly. Our findings are in line with the sunlight penetration factor. There is a direct correlation between the moisture contents of the soil and light penetration [50]. The moisture in the soil tends to

close up the crevices between soil particles, limiting sunlight penetration through the soil. For example, Michael et al. reported that moist soil at 0.5 -1.0 mm depth showed the same level of light transmittance in dry soil at 1.5 - 2.0 mm deep [50]. The limited penetration means less light intensity reaches the 3DI or IVM in the matrix, hence a low degradation rate. In summary, at higher soil moisture, the photodegradation rates decrease. Soil moisture, especially at saturation point, decreases sunlight penetration depth due to reduced sunlight reflection on the soil particles [51].

3.4 Conclusion

The transformation of IVM and its metabolites determines the persistence and impact on the non-target organism. Under this study, IVM and 3DI were found to degrade under the influence of sunlight. Due to sunlight penetration issues, photodegradation was experienced on the top 5 mm soil and soil manure layer. In all laboratories, and experimental settings, IVM and 3DI concentration in the top 5 mm soil layer decreased by 99 % compared to only around 50 % decrease in the 5 mm lower layer with 80 to 100 h of outdoor exposure. Kinetics parameters of degradation reaction determined on pseudo-first-order model suggest that 3DI and IVM degraded faster in Ifakara compared to Virginia under all experimental settings. Moreover, degradation parameters indicated that moisture content, presence or absence of manure in the soil, and environmental temperature correlate to the photodegradation rate. However, optimum manure and soil moisture contents for fast degradation have yet to be discovered. Therefore, more research should be conducted on this topic because soil moisture is one of the vital soil parameters, and manure is always added to soil to improve soil productivity. Otherwise, our research suggests that photodegradation can be used to remove IVM and 3DI from

the soil. For a tropical country like Tanzania, the removal can take up to 10 days to reach negligible concentrations, mainly when it is spread in less than 5 mm layers outdoors in the summer under dry conditions.

3.5 References

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Chapter 4. Field-scale investigation of the environmental fate of ivermectin and its metabolites in cattle manure and soil in Tanzania

Abstract

Ivermectin has unprecedented efficacy against many ecto- and endoparasites and is among the leading malaria vector control drug under clinical trial. However, when administered to livestock, around 90 % is eliminated from the body as residue drugs and metabolites reach the soil. For this reason, in this study, the environmental fate of ivermectin and its metabolites was investigated at the field scale. Bovine cattle were subcutaneously injected with recommended therapeutic ivermectin dose (1.0 ml/50kg body weight). Some of the collected cattle dung were analyzed, some were placed in the field, and the degradation profile of the ivermectin and its metabolites were followed. LC-MS/MS analysis indicated that the collected manure contained ivermectin residues, 3″-O-demethylivermectin(3DI), and 24-OH-ivermectin(24OHI) as metabolites. All three substances followed the normal and the same excretion profile, with the highest elimination rate recorded on the second-day post-treatment. No ivermectin or its metabolite was recorded 40 days post-treatment. The control dungs did not contain ivermectin or its metabolites. When the pats and pats-soil mixture from treated cattle collected on the second day was placed in the field, ivermectin and its metabolites were found to degrade. The degradation rate depended on pats and pats-soil depths, with the 0-5 mm layer experiencing more significant degradation than the layer below that depth. Ivermectin in the pat's upper layer was found to degrade three times more rapidly than in the pats-soil mixture. Due to limited leaching or hydraulic runoff, there is no contamination of the soil beneath the pats or pats-soil mixture by ivermectin or its metabolites. The above findings suggest that exposure to pats or pats-soil mixture from ivermectin-treated animals in the summer may be an IVM and its metabolites decontamination method before taking manure to agricultural land.

4.1 Introduction

Malaria is a mosquito-borne infectious and life-threatening global disease accountable for a staggering number of deaths of humans, especially children, and pregnant women. According to the World malaria report, in 2020, the disease caused about 0.6 million deaths, representing a 12 % increase compared to the previous year [1, 2]. This means that if there are no substantial efforts to overcome the 2019-2020 trend, the number of deaths is expected double in the next five years.

Although malaria is a global disease, its burden is unevenly distributed, with tropical countries suffering the most. For example, in 2020, Africa reported nearly 95 % of malaria incidents and 96 % of deaths worldwide, with 80 % of all deaths in children under five years [2]. The uneven distribution of the malaria burden is associated with climatic factors such as rainfall, humidity, and, more predominantly, seasonal temperature variation[3-6]. Malaria parasites stop growth entirely at temperatures below 16 ^{o}C, and many vector mosquitoes suspend gnawing activity at low temperatures. This accounts for the malaria burden difference between tropical and temperate regions [3].

Malaria elimination strategies that worked in temperate countries need to be better to address the problem in tropical countries, as indicated by current disease burden statistics. For example, frontline insecticide-treated nets and indoor residual spraying strategies are limited by increasing drug resistance in mosquitos and residual transmission [7, 8]. Consequently, pioneering and comparable techniques are urgently required to widen the malaria control device cabinet[9].

Among the upcoming and promising potential strategies targeting areas with moderate to high malaria mosquitoes is Insecticide-treated livestock (ITL) [10]. Under ITL, livestock in

nearby proximity to humans is treated with insecticides. Zoophagic mosquitos die or experience diminished reproductivity after feeding upon animals treated with an insecticide leading, reducing malaria transmission [10].

Ivermectin (IVM) is among the potential existing candidate for ITL because it uniquely addresses challenges in mosquito vector control. Among IVM's advantages is its ability to deal with drug resistance, may be used to target both outdoor and indoor mosquitoes, and can target 80 % of variables of vectorial capacity [11, 12]. Moreover, IVM may be used independently or integrated with other tools or ITL candidates [13]. Further, IVM has unmatched records in veterinary and human health parasites and vectors. For example, a Fritz *et al.* semi-field work recorded that feeding on cattle treated with IVM was lethal to 90 % of fed *Anopheles gambiae s.s.* [14]. Similarly, *Anopheles farauti* mosquitos feeding on 0.6 mg/kg ivermectin-dosed pigs died within 2-4 days up to a week after treatment, and lethal impacts persisted for 14 days [15].

However, ivermectin's ITL candidacy is surrounded by environmental toxicity issues. Although IVM has protracted blood plasma concentrations and lipophilic characteristics that guarantee pharmacological efficacy, most of this drug's residue and metabolites are excreted in feces [16, 17]. Therefore, cattle feces could contain a mixture of intact ivermectin residues and metabolites.

IVM and its metabolites may enter the environment by applying manure from treated cattle. Once in the terrestrial environment, IVM and metabolites are subject to sorption into soil particles and transformation. Photodegradation is the primary mechanism determining IVM and its metabolite fate in the soil or soil manure mixture compared to acidic or basic hydrolysis and partition to another environmental compartment[18]. The

transformation rate ranges from hours to years depending on environmental conditions such as temperature and soil acidity or alkalinity [19]. Moreover, ivermectin, metabolites, and degrades (formed on storage) are subjected to leaching and hydraulic runoff, contaminating surface and groundwater [20]. Although IVM fate and toxic pathways in aquatic environments and its environmental fate in the underwater system are still unclear, literature has essential information on terrestrial environments in temperate regions [21-27]. For example, Sommer et al. reported retarded dung degradation from IVM-treated cattle, threatening nutrient recycling and food security [21].

Even though IVM and its metabolite's fate are documented in temperate regions, the same bundle of information is lacking in tropical countries where IVM is highly needed if it passes as an ITL candidate. Moreover, there is a variability of details on the IVM, and its metabolites discharged in cattle feces. Iglesias et al. Reported 0.771 ppm as the maximum IVM concentration in cow dung on day three post-treatment [28]. Iglesias said the drug's concentration was around 67 % of that reported by the Suarez group using the same administration methodology [29]. Significant differences in climatic conditions, sex, age, diet, and cattle body weight play a crucial role in ivermectin pharmacokinetics affecting the excreted amount of ivermectin residue and metabolites [30, 31]. For example, Cook et al. reported that 5-fold greater ivermectin concentrations were eliminated in the feces of grain-fed cattle compared with pasture-fed (grazing) cattle[32]. Moreover, diet affected the extraction trend as the peak excretion levels of 0.36 ppm (grain-fed and 0.09 ppm (pasture-fed) of ivermectin were recorded at 6- and eight dpi, respectively [32]. This implies that finding from different climatic zones, especially with other cattle species and diets, cannot be extrapolated to another area. Therefore, as

ivermectin is being considered for ITL strategies, research should be conducted in widespread malaria regions and within the perspective of inhabitants-level administration where regional variants (vegetation, climate) might alter the amount in soil and water successive environmental effects. This study aims to illustrate the elimination profile of ivermectin and its metabolite in bovine cattle following subcutaneous administration and investigate their ecological fate in cattle manure and soil in Tanzania.

4.2 Materials and Methods

4.2.1 Materials

Ivermectin (IVM, CAS# MKCC9996 purity 95 ≥ 80 % H_2B_{1a} and < 20 % H_2B_{1b}) and 3-O-demethylivermectin (3DI) standards were procured from Sigma-Aldrich Co. (3050 St Luis MO, USA). Since they were of high purity and suited the analytical grade, they were used without further purification. LC-MS grade solvent, methanol, acetonitrile, and ultrapure pure water were purchased from Fisher Scientific (1 Reagent Lane, Fair Lawn, New Jersey, USA). Formic acid, ammonium formate, 0.2 µm filters, syringe, and needles were bought from Fisher Scientific (1 Reagent Lane, Fair Lawn, New Jersey, USA). Water free from IVM, 3DI, or any other contaminants produced in our laboratory using the Milli Q-gradient system (Millipore, Bedford, MA).

4.2.2 Treatments and Animals

Before the field trial, the health of the cattle (bovine: indigenous breed-Tanzania short horn zebu breed) used for this study was investigated by healthy personnel from Ifakara Health Institute. The weight of cattle was measured for seven continuous days before the trial. Their medication records were received from owners. The pre-trial information indicated that cattle enrolled in this study were healthy and free from animal diseases.

They have not received antibacterial or antifungal medications in the past six months. During the investigation, two families volunteered the cattle, forming a herd of cattle shown in **Figure 4.1**.

Figure 4. 1: Herd of Cattle where study animals were randomly selected.

Two groups of 11 cattle each (1-3 years old) were randomly selected from this group and located into two paddocks separated 500 m apart. The selected cattle were labeled with a permanent mark and allowed to graze with the group except during sample collection, where they were separated from other group members and tied according to their numbers. The cattle in the treated group received 200 µg/kg IVM (IVOMEC, Merial)

subcutaneously injected in the lateral midline of the neck as a single dose per live weight on 09 July 2022. The animals in the control group endured untreated.

4.2.3 Faecal Collection and Pat Preparations

Twenty-four hours after IVM dosing, cattle were allocated to their respective prepared paddocks according to their number. Dung was allowed to accumulate for 12 hours before the cattle were moved out or released for grazing. Sampling was done by randomly selecting three paddocks from the treated and control group and collecting equal weights of dung from these paddocks. The three dungs collected from each group were well-mixed to form one composite piece. This sampling process will be repeated until enough representatives from each treatment group are obtained. This procedure was repeated on days 2, 5, 10, 15, and 45 post-IVM administration. Three of the composite dung samples randomly selected from the samples above on the 2nd-day post-treatment were used to make a soil-manure mixture sample. These mixtures were made by mixing the three obtained composite dung samples with 10 % soil at three different field locations and flagged for 70 days. Similarly, three pats from treated cattle brought on 2nd-day post-treatment were equally spaced from each other in the field. These pats were marked and stayed monitored for 45 days post-exposure day.

4.2.4 Pat and Soil-manure Sampling

Sampling was conducted by collecting samples at 0-2 cm and 2-10 cm for depth for cattle dung-soil mixture, a surface (0-5 mm), and a subsurface (several locations from below the 5 mm depth for pats. The samples were collected on days 0, 1, 5, 15, and 30 after the pat was laid out on the field and on days 0, 3, 8, 15, and 30 for the cattle dung-soil mixture. The soil immediately beneath each pat and cattle dung mixture was also

sampled to a depth of about 2 cm. The obtained samples were held at -18 ^{o}C in insulated containers containing icepacks or dry ice and transported to Ifakara Health Institute. The samples were stored in the freezer at -20 ^{o}C until sample extraction. Each sample collected was analyzed for IVM, 3DI, and 24OHI.

4.2.5 Sample Extraction, Clean-up, and Instrumental Analysis

Samples were extracted by mixing about 1.0 g of dung, cattle soil-manure mixture, or pats with 2 mL methanol in a 10 mL polypropylene copolymer centrifuge tube. The tube was covered tightly with a lid, the sample vortexed for 5 seconds, and the solvent was allowed to digest the sample for 1 h. Then the sample was vortexed at 800 rpm for 10 minutes and centrifuged at 3500 rpm for 5 minutes. Immediately after the centrifugation of samples and without disturbing the sediment, the supernatant was transferred into a vial. Then another precisely 2 mL of methanol was added into the tube containing the deposit and vortexed at 800 rpm for 10 minutes, followed by centrifuging the sample at 3500 rpm for 5 minutes. Immediately after centrifuging the samples, the supernatant was transferred to a vial above without disturbing the sediment. The obtained supernatant was mixed (via vortexing for 5 seconds). The solvent was evaporated to dryness and shipped to the laboratory in the USA. Upon arrival, the dry residues were dissolved in 4 mL of methanol for analysis. During analysis, 2 mL was pipetted into the 2 mL HPLC vial and centrifuged at 10000 rpm for 10 min. After centrifuging, the supernatant was diluted in was LC/QQQ mobile phase (0.1 % FA +20 % H_2O + 80 % methanol). The solution was filtered through a 0.20 µm syringe filter and analyzed on the LC-MS/MS.

4.2.6 Weather

Daily precipitation, temperature, and humidity were recorded from Tanzania Meteorology Agency (MTA) headquarters, Ubungo, Dar es Salaam.

4.2.7 Statistical Analyses

The statistical summary (mean, standard deviations) was done on JMP Pro 16 (SAS Institute Inc, 2019). Outlier data normality distribution (test for normality), normal quality plots, and Generalized Linear Model (GLM) was done on JMP Pro 16 (SAS Institute Inc, 2019). The obtained mean and standard deviation for degradation experiments was used to calculate the percentage of ivermectin and 3DI in the upper and lower layers at each time interval. The average (n = 3) of IVM, 24OHI, and 3DI concentrations recorded on cattle-pats or cattle-pats mixture without exposure to sunlight were termed C_o, and those registered after exposures for a predetermined time were termed C_t. These two variables were used to calculate the percentage of ivermectin, or its metabolite, left in the sample after sunlight exposure for time, t, by using the equation:

$$\text{Percentge remaining in the sample after time, t} = \frac{C_t}{C_O} \times 100 \qquad (4.1)$$

The obtained values using equation 4.1 from each layer and samples were plotted against the incubation period. The transformation half-lives were calculated by fitting the percentage of IVM, 24OHI, and 3DI in the upper layer into a simple first-order kinetics in Origin 9.1® software.

4.3 Results and Discussion

4.3.1 Weather

No rainfall was recorded during the study except for some cloudy days for a few hours. The minimum temperature (17 ^oC) was recorded during the night, and the maximum

temperature (30 ºC) was observed in the afternoon. The daily mean temperature during our fieldwork is presented in **Figure 4.2**.

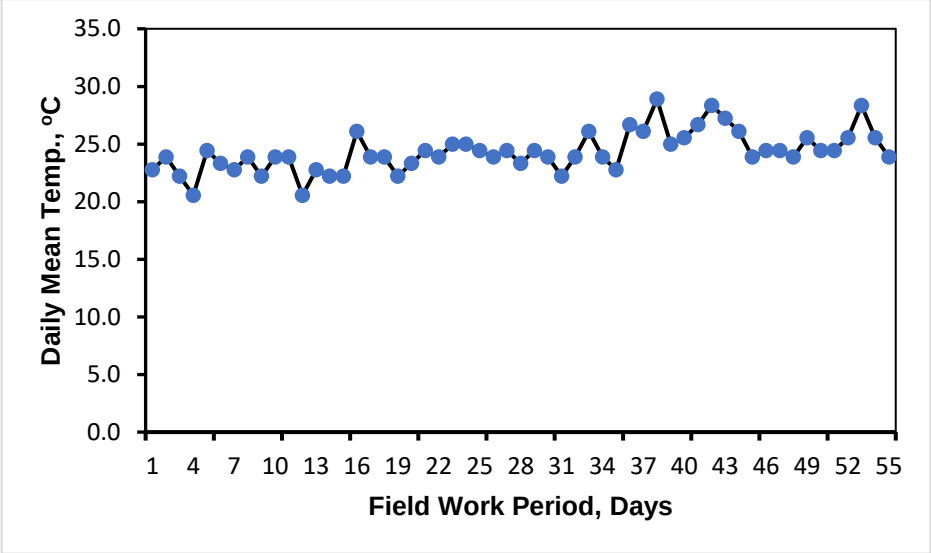

Figure 4.2: The average field temperature recorded during the fieldwork in July and August 2022.

4.3.2 IVM Fecal Excretion Profile

IVM was detected in feces for all the subcutaneously treated cattle collected from 1 to 30 post-treatment days. Before ivermectin administration, no ivermectin residues were detected in feces or the control group. IVM unmetabolized residues elimination profiles in feces resemble the positively skewed Poisson distribution P >0.05, which may be considered normal distribution. The recorded IVM concentration statistically differed significantly between the day-point (P< 0.05). The post hoc Turkey analysis tests differed between day one and day 45.

Figure 4.3 depicts that the highest IVM residue concentrations expressed per dung dry weight of 0.89 ppm were detected in feces. This maximum ivermectin residue concentration was excreted in feces on the 2nd-day post-treatment. Compared with its

metabolites discussed in the following subsection, IVM was almost 100-fold more elevated in the cattle dung than 3DI, with 24OHI sitting between the two compounds. The concentration of both drugs decreased with time, and 3DI was below detection limits from day ten post-treatment. However, IVM was detected in feaces for more than 30 days but less than 45 days post-treatment.

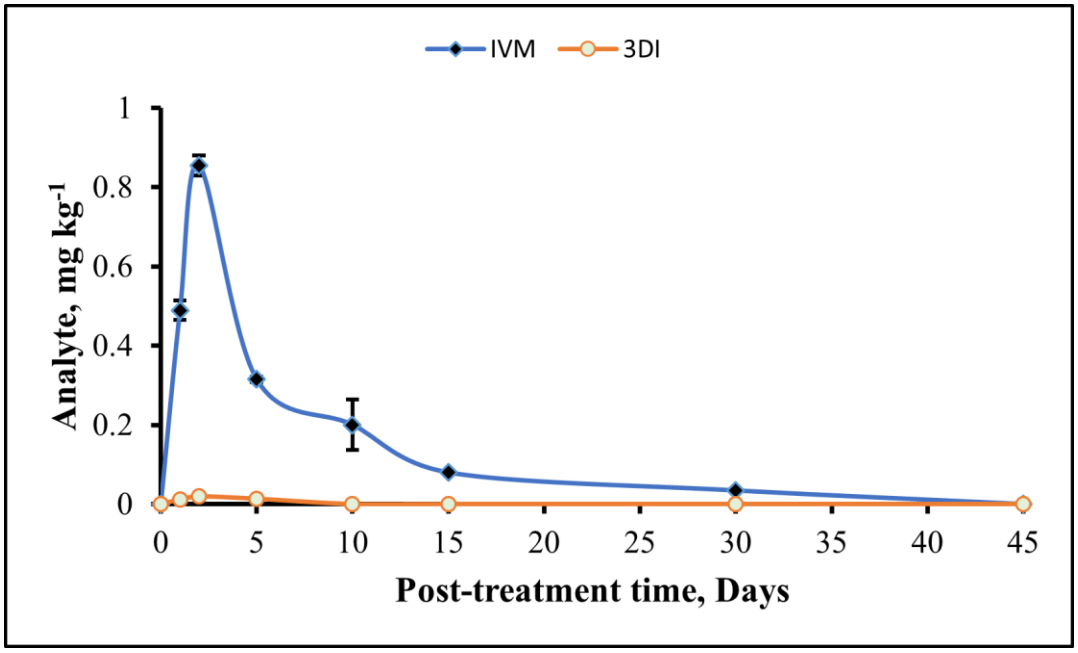

Figure 4.3: IVM and 3DI concentrations-time profile in cattle dung (mg kg^{-1} of dry weight) after 0.2 mg kg^{-1} of cattle body weight subcutaneous injection. Vertical bars represent standard deviations.

The obtained IVM and 3DI concentration-time profile presented in **Figure 4.3** indicates that neither IVM nor 3DI was observed before drug administration. The first detection of IVM and its metabolites took place 24 hours after drug administration. While IVM and 24OHI remained detectable for 42 days, 3DI was below the detection limit within 15 days. The ivermectin excretion profile in feces reported in this field study is like those reported in previous studies [17, 25, 33-35]. This profile may be divided into three main stages: the

emergent stage, which always occurs on day one after subcutaneous injection, the climax concentration stage, and the stage where minimum ivermectin concentration is detected. While the emergent stage is known to appear on day one post-treatment, the stage with the highest elimination and low detection stages differs. Similarly, in all cases, the ivermectin concentration reported in all three steps varies among the studies. For example, Lifschitz et al. studied the ivermectin elimination profile of ivermectin and found 1.2 ppm as a maximum concentration in feces [36]. Their reported concentration is almost 30 % higher than what we obtained in our studies. However, Lifschitz et al. could not report any of the metabolites found in our study [36].

Sommer et al. [33] said 2.8 ppm IVM in the dung on the 1st day, followed by 3.9 ppm on the 2nd day. The IVM fecal concentration decreased by 13-fold about maximum fecal concentration within two weeks. Sommer's findings are similar to our findings regarding days for emergent and climax stages, and even the concentration ratios between the climax stage and emergent stage are identical (1.4 vs 1.7) [33]. However, we report a longer IVM fecal excretion period compared to Sommer et al.($13.5\ vs \approx 40\ days$) [33]. Similarly, the IVM excretion profile reported by Fernandez and co-workers[34] has a maximum equal concentration with our findings (0.85 ppm); however, our maximum concentration comes on day two post-treatment. In Fernandez's study, a 0.85 ppm IVM level was reported on day 4, with this concentration dropping to 0.27 on day 14 and 0.011 on day 31 post-treatment [34]. Moreover, in Lumaret and coworkers' field study under Spanish conditions, the IVM elimination profile indicated that the drug residue in cattle is rapidly eliminated with a pinnacle concentration of 0.42 ppm reported on day five post-treatment. No IVM was detected in the dung further than 12 days. Lumaret group findings

differ in climax IVM concentration almost twice as compared to their results, and our climax concentration comes nearly 2.5 earlier than their findings. Moreover, although we started detecting IVM on day one post-treatment, we could see it almost four times longer than their findings. Although the maximum concentration eliminated in feces varies, all researchers agree that bile and feces are the main routes for ivermectin residue elimination. The question remains, why those variations in terms of maximum concentration and elimination periods?

There are some attempts to explain this variability in the IVM elimination profile, especially on maximum concentrations detected in feces plus the persistence time [32, 37]. For example, Taylor et al. hinted that the higher initial IVM fecal concentration in grazing cattle compared to their grain-fed counterparts resulted from less drug absorption in the former group [37]. On the other hand, in grain-fed animals, due to the longer dwelling time of digesta in the intestinal tract, there is a superior anthelmintic drug absorption compared with grazing animals. Therefore, if factors like the volume of feces are consistent in both groups, an opposite trend would be observed. This argument is supported by cook et al., who reported up to a five-times increase in IVM fecal concentration in grain-fed cattle compared with grazing animals [32]. The opposite trend in drug elimination due to diet differences can be attributed to the fact that drug absorption is not only altered by food, but other mechanisms, such as enhanced bile acid activity by dietary fats, play a crucial role. This is believed to be the mechanism that explains the boosted absorption of the lipid-soluble drug, like IVM, with a meal containing fat [38]. Moreover, food may also act as a physical impediment to avoiding drug access to the abdominal tract's mucosal exterior, affecting both actively and passively absorbed drug compounds [39].

Furthermore, types of macro- and micronutrients affect drugs' metabolic activities and hence elimination rate [40]. While carbohydrates appear to have minimal effect on drug metabolism, high-protein, low-carbohydrate diets can quicken the metabolism of drugs by the liver due to improved mixed-function oxidase system activity [41, 42]. Therefore, more studies in this area under the IVM context should be conducted to enhance the drug metabolism and elimination database.

4.3.3 Fecal Excretion Profile of IVM Metabolites in Cattle

Apart from IVM detection and quantification in feaces, we could detect 3DI and 24OHI metabolites in feces. The concentration of 3DI was determined throughout the study and protected in **Figures 4.3 and 4.4**. The elimination of these metabolites in feces follows similar trends to that of the parent IVM drug residues. However, some differences between the eliminations of these compounds are observed. While IVM and its metabolite's maximum elimination in feces were recorded on the second day post-drug administration, IVM recorded a 100-fold concentration. The IVM concentration reported on concentration on the second day was 25 times compared to that of 24OHI. The registered peak areas of 24OHI compared to that of 3DI indicates 24OHI was eliminated at large quantities in feces compared to 3DI. For example, on day 2nd after IVM administration, 24OHI was four times higher than 3DI but 25 less than IVM. In all cases, the metabolites followed a similar trend to the parent drug, except they were slowly eliminated in the dung compared to the parent drug. Our results agree with those previously published by Chiu et al. [43].

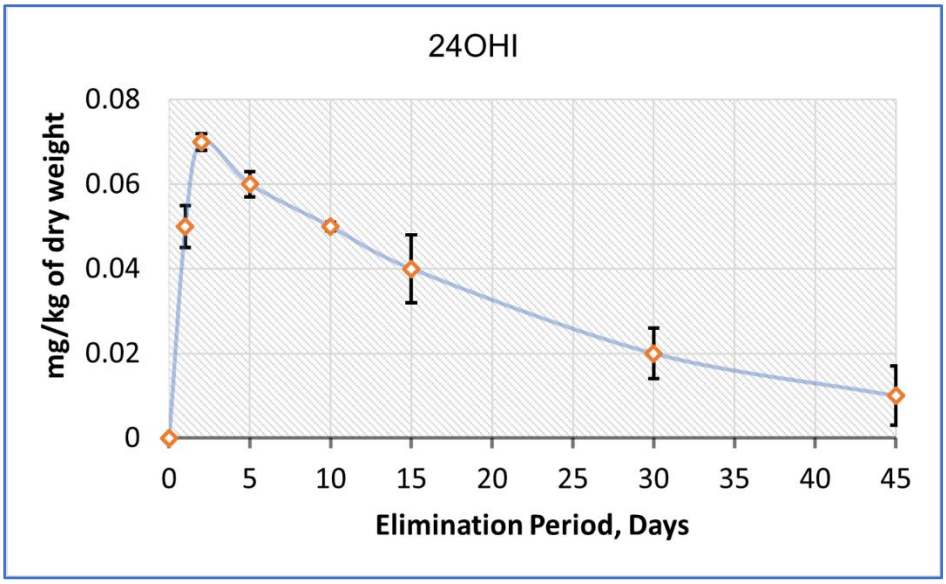

Figure 4.4: 24OHI elimination profile in cattle dung. The error bars represent $\pm SD$

Under Chiu et al. study, these metabolites accounted for not more than 2 % of the total residues in all three studied species (cattle, sheep, and rats). Similarly, Fink et al. have reported these metabolites and suggested that they are more polar than parent IVM. This may be why these metabolites were eluted faster from the column than the parent drug, confirming the suggestion that these compounds are more polar than IVM [16]. Moreover, an unspecified amount of ivermectin metabolites were reported by Wolde et al. Although, under this study, cattle manure was exposed in the field before analysis; therefore, it needs to be clarified whether they were biological metabolites or degradation products.

4.3.4 Pats and Soil-pats Mixture in the Field

Manure is the most crucial source of biofertilizers, maintaining and enhancing soil fertility by providing nitrogen, phosphorus, and potassium (NPK). Moreover, cattle manure offers many essential nutrients such as calcium, magnesium, sulfur, zinc, boron, copper, and manganese. Furthermore, the application of cattle dung manure increases soil organic matter contents. As a result, water infiltration, water holding capacity, and increased cation exchange capacity are improved [44]. Due to these applications, cattle dung

(manures) mainly ends up on farms. Given the environmental impacts of ivermectin, especially on decomposers, the application of manure from treated cattle can have unexpected outcomes for farmers. We studied the fate of 3DI, 24OH, and IVM from treated cattle by placing the pats and soil-pats mixture in the field. **Figures 4.5** and **4.6** illustrate the change in IVM concentrations after samples were placed in the area, with **Figures 4.7** and **4.8** showing the field-persistent profile of 3DI and 24OHI, respectively.

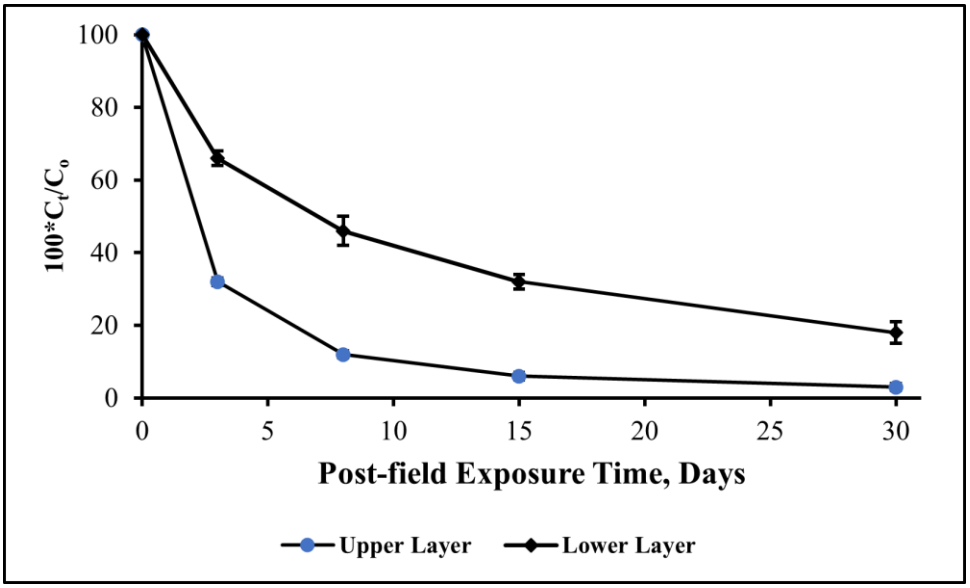

Figure 4.5: Percentage of IVM remaining in pats samples from treated cattle. The pats collected on 2nd day after treatment were placed in the field for 45 days, and the 0-5 mm depth (upper layer) and 5 mm depth below the upper layer were sampled and analyzed.

Our findings indicate that the concentrations of 24OHI, 3DI, and IVM residues in the pat and soil manure mixture decreased significantly in both the upper and lower layer with field exposure time. The percentage decrease for both analytes concentrations depends on the layer, sample matrix, and type of analyte. **Figure 4.5-4.8** indicates that the upper layer for both analytes experienced a much decrease in analyte concentrations compared to lower layers, with 3DI falling below detection limits in the upper layer within a week of field exposure.

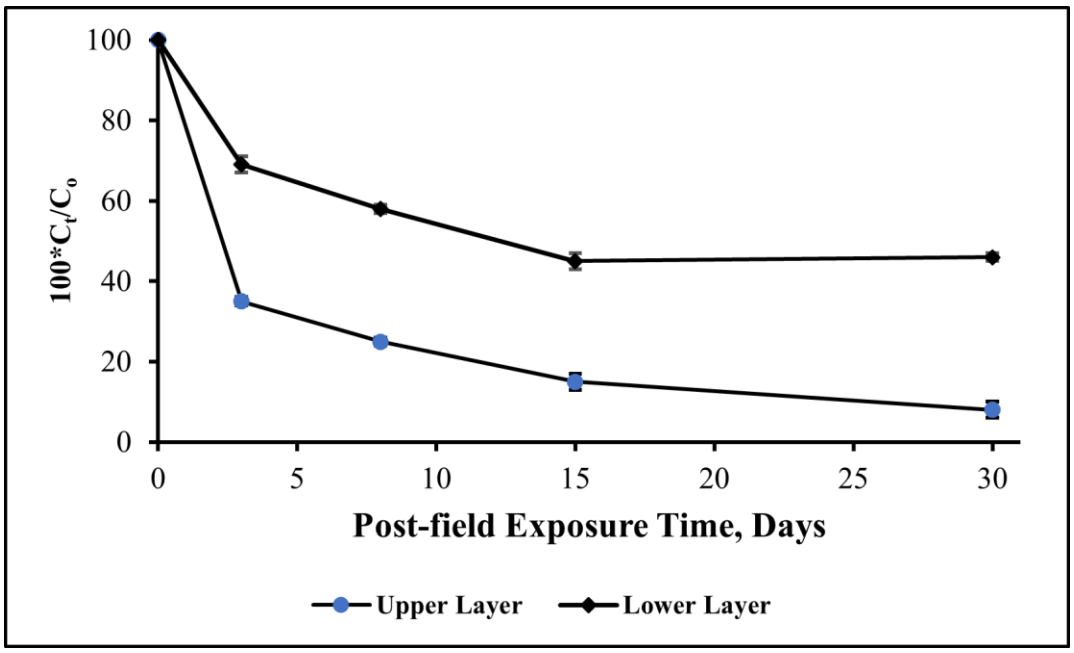

Figure 4. 6: Percentage of IVM remaining in pats-soil mixture samples from treated cattle. The cattle dung collected on 2nd day after treatment was mixed with soil and placed in the field for 45 days. The error bars represent $\pm SD$.

IVM is said to be poorly metabolized in cattle resulting in more of its residue and a small number of metabolites entering the environment via the excretion application of manure from IVM-treated cattle [45, 46]. The application of manure, recommended for sustainable yield farming [47], has many benefits regarding nutrient or nitrogen sequestration. Still, it threatens to spread veterinary drugs into farmland [45]. Eventually, IVM residue and its metabolites are applied to farm animals contaminating soil, surface, and groundwater[48]. Therefore, it is unsurprising that manure from treated cattle has been reported to affect terrestrial flora and fauna [49-51] and threaten soil productivity [48]. Moreover, using this drug to compact malaria will reduce ivermectin-free manure; therefore, there is a need to understand the fate of these three substances in the farms. This study highlights the potential for on-farm field experiments to improve understanding of the fate and effects of these compounds.

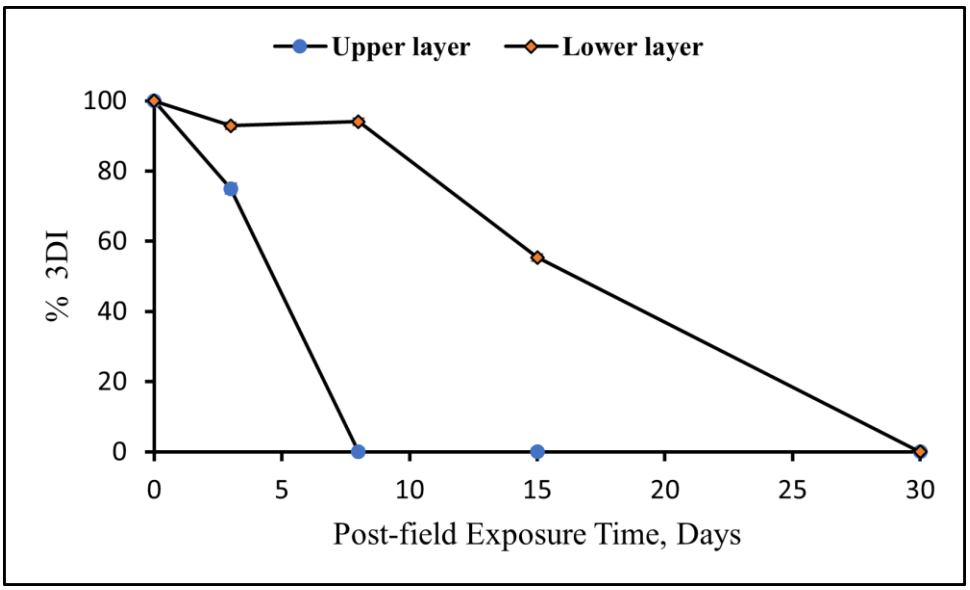

Figure 4.7: Percentage of 3DI remaining in the pats-soil mixture after placing the samples in the field for 45 days.

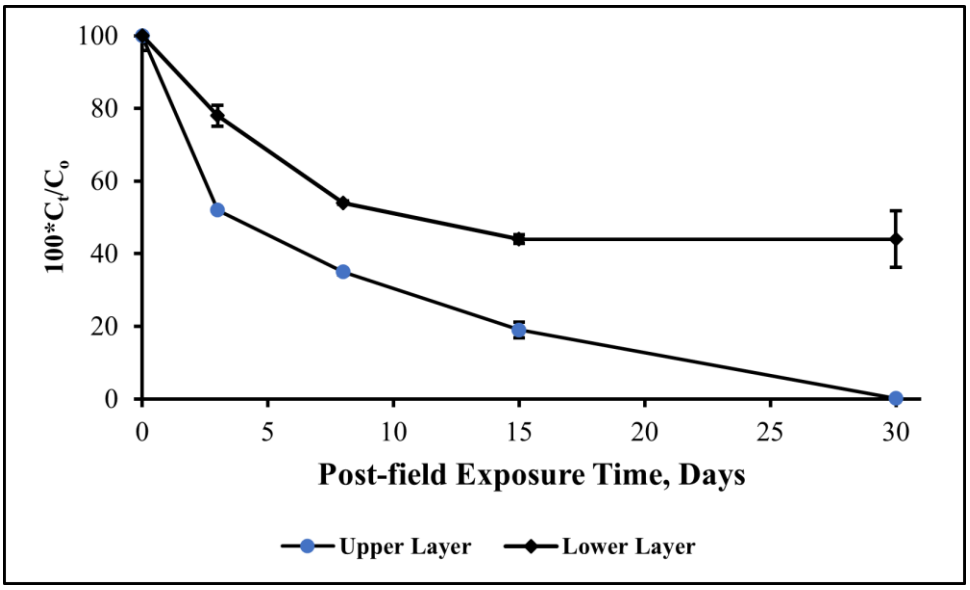

Figure 4.8: Average percentage of 24OHI remaining in Pat-soil mixture after field exposure. The error bars represent $\pm SD$.

Our results suggest that IVM and its metabolites extracted in feces and placed in the field are prone to degradation. The rate of the gradation of these three substances depends on sunlight and hence the depth of the pats or pat-soil mixture. Although the literature needs more information on photodegradation aspects with depth consideration, it may

have affected the observed results. Variable data is reported on the fate of IVM and 3DI entrapped in cattle dung or soil manure mixture when manure is placed in the field. Sommer et al. highlighted that no degradation of ivermectin was revealed in dung pat set in the area for up to six weeks [33]. The above observation was contrary to what Halley et al. observed [16]. Halley et al. observed that IVM is degraded depending on the environmental conditions. Under exposure to sunlight in the summer, IVM in 2:1 soil: manure mixture was found to degrade rapidly, with half-lives ranging between 1 to 2 weeks. A slow degradation rate was observed in the winter when the same experiment was conducted and half-lives of about 13 to 31 weeks. The worst-case scenario was observed when the same investigation was born in the dark at room temperature, where the half-live was up to 17 times compared to those reported in the summer[16]. Furthermore, IVM is rapidly decomposed when exposed to sunlight as a thin, dry film on glass with a half-life of less than a week. Similarly, rapid degradation of IVM in pats exposed in the field under higher soil temperature Spring has been reported indicating that finding found in the laboratory can be extrapolated to the area [52]. Our results align with Lumaret et al. findings, where IVM and 3DI concentration reported on the 2nd-day post-treatment decreased with field-exposure time. However, our findings suggested that the IVM and 3DI degradation rates depend on the depth of the pats or soil-manure mixture (**Figure 4.5-4.7**). IVM in pats decreased by 65 % in the upper layer compared to only 31% in the lower layer in the first three days after field exposure. Similarly, 3DI in soil manure mixture upper layer fell to zero concentration in 8 days compared to less than 20 % decrease observed in the lower layer. Since all samples were subject to the same environmental conditions, with the only difference being sunlight contacts,

photodegradation plays a vital role in the observed decrease in the upper layer can be associated with photodegradation. However, we can rule out other forms of degradation. In the previous study, we observed IVM entrapped in the soil, or soil manure lower layer degraded not more than 60 % after 100 h of sunlight exposure. Contrary to the field study, IVM was found to degrade by more than 80 % lower layer. Similarly, 3DI fell below detection limits within 6 days in the upper layer and took only 30 days to drop below detection limits. The rapid degradation rate observed in this study can be attributed to the fact that other forms of degradation, like aerobic and anaerobic, were more feasible in this study. Microbes control these forms of degradation, therefore, can take place even during the night. The opposite was confirmed in the laboratory set-up where during the night, samples were frozen, slowing down microbial activities (degradation).

4.3.5 Kinetics Consideration of Degradation of IVM and its Metabolites

To put our findings into a clear picture, the obtained data upper layer data were kinetically modeled into simple first-order degradation kinetics. This model presumes that the change in chemical concentration against time (dA/dt) is related to its concentration at that time and a degradation rate constant *k*. Thus, the concentration at time t after field exposure A_t can be expressed as:

$$\ln\left(\frac{A_t}{A_0}\right) = -kt_{\frac{1}{2}} \qquad (1)$$

Whereby *k* is the degradation rate constant and A_t and A_0 are observed analyte at t and 0, respectively. The representative graphs are shown in **Figures 4.8-4.10** below. The excellent fitting (R^2 > 97 %) of our data into this model confirms that we have only one

degradation pool; thus, all ivermectin and metabolites are equally subjected to degradation.

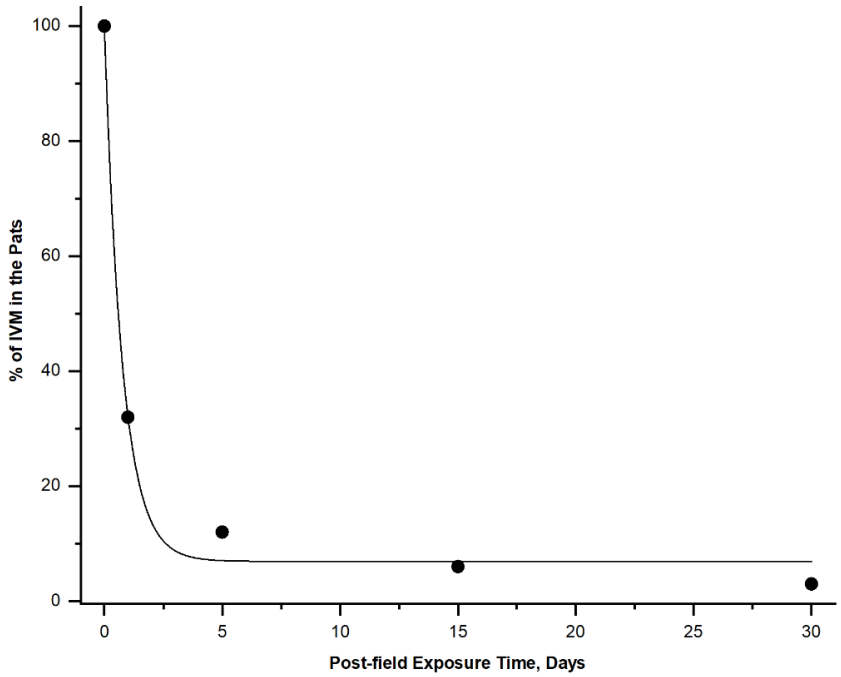

Figure 4.8: Simple first-order Kinetics model fitting for ivermectin in the pats.

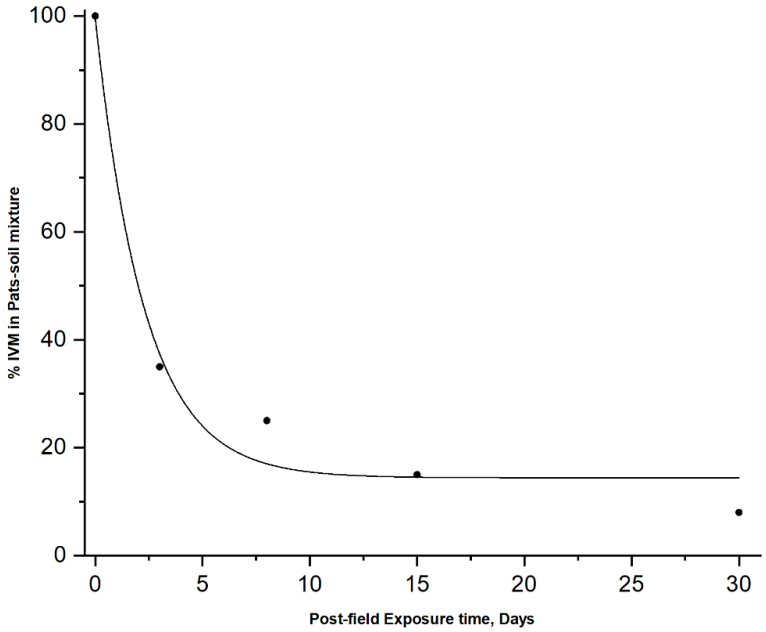

Figure 4.9: The percentage of ivermectin in the pats-soil mixture fitted into a simple first-order model.

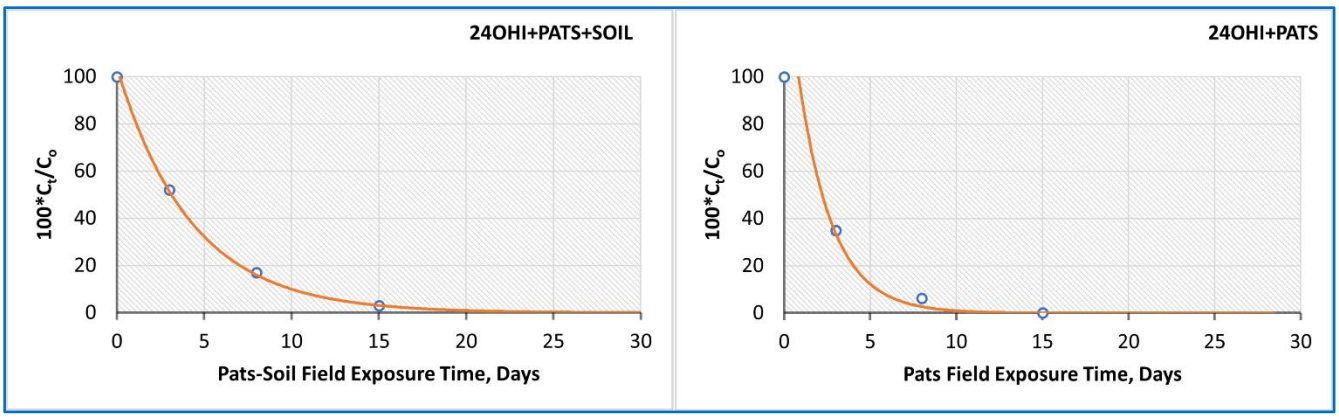

Figure 4. 10: Percentage of 24OHI remaining in the pats and pats-soil mixture fitted into the simple first-order kinetics model.

Unlike in Krogh and co-workers, where bi-exponential first-order was reported. Under this model written by Krogh, IVM initially present in the matrix is subjected to two degradation pools [53]. The minor degradation fraction of ivermectin was assumed to be strongly sorbed or desorption on the sample matrix and therefore is kinetically hindered and does not undergo degradation easily [53].

Table 4.1a: Field ivermectin degradation half-lives in pats and pats-soil mixture

Field Sample	R^2	Half-life, d
Pats	0.99	0.8 ± 0.11
Pats-soil mixture	0.98	2.2 ± 0.66

Table 4.1b: Field 24OHI degradation half-lives in pats and pats-soil mixture

Pats-soil mixture	0.99	3.62 ± 1.22
Pats	0.97	2.01 ± 0.82

The half-life computed from the simple first-order kinetics of the degradation reactions and findings presented in **Table 4.1** further confirms that ivermectin in the cattle-pats and pats-soil manure is subject to degradations. These findings are similar to Halley et al. and contrary to Sommer et al. findings [16]. However, our data suggest that the degradation under our experimental setting was about 7 to rapid compared to what was reported by

Halley in the summer[16]. The observed differences can be attributed to other environmental factors like moisture, humidity, soil and pat composition, and experimental settings. Half-live differences between cattle-pats and soil-parts mixture indicate an excellent example of practical setting effects. During the testing stage, we expected that adding soil to pats would decrease the organic matter contents, decreasing sunlight shielding and fastening the degradation reaction process. However, the obtained half-live suggest that ivermectin entrapped in pats degraded almost three times more rapidly than in pats with 10 % soil. This trend is different from what we reported in the previous chapter. This study found IVM to degrade slowly in soil manure mixture compared to pure soil. This finding further cements the impact of depth when considering half-lives calculation under degradation. Because the upper layer in pats was within 5 mm compared to the 2 cm sampled in the cattle-pats and pats-soil mixture. Given the limitations of sunlight penetration, the pats-soil combination used most samples with low sunlight contact, slow degradation, and higher concentrations with time compared to pats.

4.3.6 Toxification of the Soil Under Pats and Pat-soil Mixture

To find the best way to detoxify dung and use them as manure safely, it is essential to consider the possibility of contaminating soils where the pats will be laid. To investigate the transport of IVM and its metabolites from cattle-pats or cattle-pats mixture, the soil beneath the replicates samples was sampled and analyzed for IVM, 3DI, and 3OHI.

We analyzed these samples and found that the soil beneath the pats and soil-manure mixture had no ivermectin or metabolites. This finding differs from Iglesias et al. [54], who reported soil contamination below the pats. However, our results were expected because, during the field, no rainfall was observed. Therefore, leaching, or hydraulic runoff, the

main pathways of ivermectin and metabolites from manure to the soil, was very unlikely to occur. However, if this study was conducted during the rainy season, leaching could be expected, though to a low extent, because of the lipophilic nature of ivermectin. IVM movement through water runoff is expected to be minimum in most agricultural areas because most of the lands are flat and covered with either crop, glass, or both. However, the investigation could be done in the rainy season to confirm these hypotheses.

4.3.7 IVM and its Biological Metabolites Potential Environmental Impacts

The environmental impact of ivermectin measures how this drug, its metabolites, or degradation products (formed on storage) interfere with biological activities and biodiversity in environmental compartments. Harmful ecological impacts can be predicted by measurement or using the established LC_{50} or EC_{50} values [55]. These parameters provide an estimated concentration of either ivermectin, its biological metabolites, or degrades that would cause mortality or any other significant inhibition or immobilization of around half of a population of organisms [55]. Currently, ivermectin's natural metabolites and degrades have not been reported to cause harm to any flora or fauna in aquatic or terrestrial environments[16]. Studies on the environmental impacts of 24OHI, 3DI, and other metabolites say that these substances do not harm even the organisms that are sensitive to ivermectin, even at high concentrations[16]. Therefore, there are no established LC_{50} values for these substances. However, studies on the fate and impact on aquatic and terrestrial organisms have been conducted for ivermectin, and many of these organisms' LC_{50} and EC_{50} values have been documented[16, 34, 56, 57]. **Table 4.2** presents some of these LC values, which will be used to predict the potential environmental impacts based on ivermectin excretion and the ecological transformation

profile under our study. These organisms are considered representative based on their sensitivity to ivermectin and ecological importance. For example, dung beetles are responsible for breaking down and recycling cattle dung into the soil, hence recycling the nutrients from the manure through the ecosystem[58].

Table 4.2: Examining the environmental impact of ivermectin

Terrestrial Organisms	**LC_{50}, ppm**	**Time interval, Days**	**Ref.**
Earthworm (*Eisenia fetida*)	15.8	14	[56]
Earthworm (Eisenia foetida)	315	28	[16]
Springtail (*Folsomia fimetaria*)	8.4	28	[57]
Dung fly (*Scathophaga stercoraria*)	0.04	> 30	[59]
Dung fly (*Neomyia cornicina*)	0.13	> 30	[60]
Dung fly (*Musca autumnalis*)	~4.4	> 30	[33]
Dung fly (*Haematobia irritants*)	~4.4	> 30	[33]
Pot worm (*Enchytraeus crypticus*)	> 300	28	[57]
Collembolan (*Folsomia candida*)	0.3	28	[61]
Collembolan (*Folsomia fimetaria*)	8.4	28	[57]
Dung beetle (*Aphodius constans*)	0.9	21	[62]
Dung beetle (*Aphodius constans*)	0.60	21	[63]
Dung beetle (*Onthophagus taunts*)	0.1	> 30	[64]
Aquatic Organisms			
Freshwater crustacean (*Daphnia magna*)	5.7 ng/L	48	[16]
Green alga (*Chlorella pyrenoidosa*)	9	14	[55]
Catadromous fish (*Salmo salar*)	0.02	96	[65]

On the contrary, Halley et al. have reported that *Daphnia magna* are among the most easily affected aquatic organisms[16]. Although we didn't observe any leaching or runoff in our study because it was conducted during the summer, it is essential to consider if our research was to be done in the winter.

The data in Table 1 can be compared with the ivermectin fecal excretion profile and data showing how ivermectin change with time when exposed to the field. **Figure 4.11** below summarises cattle's ivermectin fecal excretion profile after a subcutaneous ivermectin injection of 0.2 mg/kg of cattle body weight dose.

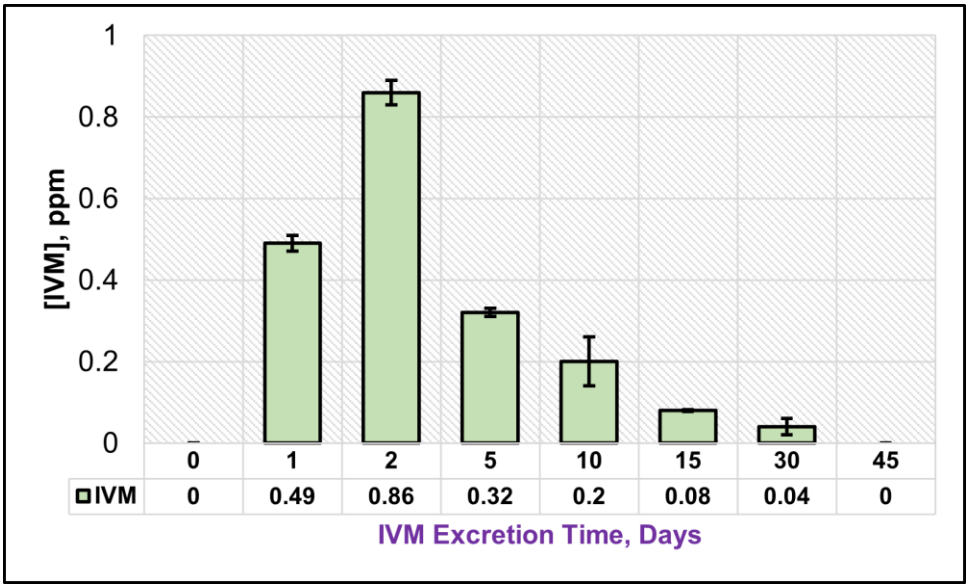

Figure 4.11: Ivermectin fecal excretion profile in cattle. Error bars represent $\pm SD$.

The comparison of ivermectin excretory data under our study and the LC_{50} data in **Table 4.11** suggests the following. The ivermectin residues excreted in feces under our investigation are below LC_{50} values for most terrestrial organisms, with some exceptions. Some terrestrial organisms like dung beetle (*Aphodius constans*) can be impacted by colonizing dungs excreted on certain days (day 2 only for *Aphodius constans)* and not from other days. However, looking into transformation data, ivermectin in pats has 0.8 days half-life implying that ivermectin from day 2 will fall below 0.004 ppm within 21 days

of field exposure. Suggesting that, while 21 exposure days are required for dung beetles to experience impact, the ivermectin concentration will fall far below the LC_{50} value within that period. Similarly, dungs ivermectin residues excreted on day 10 may affect dung beetle (Onthophagus taunts) will drop below 0.06 within three days of pats field exposure, which is below the LC_{50} value for these species. This transformation trend can be extrapolated to both for other detected residues, especially for the first 10 days post-drug administration. On the other hand, aquatic species are said to be the most ivermectin-impacted species; however, given the lipophilic nature of this drug and the land setting, leaching, and hydraulic runoff are more unlikely. Therefore, spreading manure into the field and managing the ivermectin transformation process within a week helps ivermectin concentration to fall below LC_{50} in most non-target organisms. With these manure management techniques, low ivermectin elimination rate for most days, and fast degradation rate, MDA is an environmentally friendly malaria control technique in Africa.

4.5 Conclusion

IVM subcutaneous injection to cattle is eliminated in the dung, mainly as an unmetabolized drug with a small amount of 3DI and 24OHI. The rate of elimination changes with time, with the second day experiencing the highest IVM residues and metabolites. When the parts are placed in the field, IVM and its metabolites are subjected to degradation. The degradation rate depends on environmental conditions and the depth of the pats or soil manure matrix. Under our study, we experienced a rapid degradation rate for IVM and 3DI located in the upper layer compared to the lower layer. However, with time concentrations of this substance decreased substantially, even on the lower layer. Since no IVM or its metabolites were detected in the soil beneath the cattle pats, it

is fair to conclude that no leaching occurred. Compared to the lather and sublethal impacts concentrations reported in the literature, IVM concentrations quantified in this study do not affect some terrestrial organisms. For example, earthworms are said to be affected by 28 days of exposure to 15.8 ppm of IVM, and springtails are sensitive to exposure to 8.4 ppm in the same period[55]. Therefore, concentration levels reported in this study appear less toxic based on high impacts, apparent effects, and potential impacts basis. Although more studies on the degradation products should be conducted to identify them and suggest their environmental impacts, exposing pats and pats-soil mixture with shallow depth can be used IVM,3DI, and 24OHI detoxification without contaminating soil through leaching or hydraulic water flow.

4.5 References

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Chapter 5: Conclusion and Recommendations

Results from this research indicate that ivermectin and its major metabolite entrapped in the soil undergo degradation regardless of climate conditions. The rate of degradation of these substances depends on the temperature, sunlight, soil depth, soil organic matter, and moisture contents. The general trend indicates that ivermectin in the soil degrades very rapidly at higher temperatures, sunlight, and shallow soil (depths of not more than 5 mm). On the other hand, organic matter and high moisture contents lower the degradation rate of ivermectin and its metabolites. While aerobic and anaerobic forms of degradation may have taken place, photodegradation reactions dominate the degradation process. The degradation rate observed in the upper 5mm layer, regardless of the study site, is at least three times higher than that observed in the lower layer. This difference between the 5 mm and below layer is due to light penetration issues. Sunlight does not penetrate beyond a 5 mm layer, with less penetration depth expected in the presence of soil organic matter and soil moisture beyond field capacity. Soil moisture beyond soil field capacity saturates the soil, fills the soil macropores, and slows oxygen diffusion. The hindered oxygen diffusion affects the aerobic degradation contribution to the overall degradation rate. Therefore, it is unsurprising that all substances experienced slow degradation with increased soil moisture. Comparing the two study sites, it is evident that all cases degraded to about 1.5 faster in Tanzania than in Virginia to the magnitude. Tanzania, which is in a tropical region, experienced higher temperatures during our study compared to Virginia, in a temperate area. IVM and 3DI degraded following simple first-order kinetics; using these models, we calculated the half-lives for the degradation reaction.

In the fieldwork conducted at Ifakara, Tanzania, we detected IVM residue and its two metabolites: 3DI and 24OHI, after 200 micro-gram/kg of live weight subcutaneous injection dose in cattle. The excretion profile of all isolated compounds in feces followed a favorable skew Poisson distribution which is approximately normal ($P > 0.05$). The amount of ivermectin excreted in feces differed significantly between the first 30 days ($P < 0.005$). However, the difference decreased in the later day points. The maximum concentration of ivermectin (0.9 ppm) and its metabolites was recorded on day two post-drug administration.

The cattle pats collected on day two post ivermectin administration (with higher concentration) were mixed with soil, and some were grouped without mixing with the soil. All these samples were placed in the field at Ifakara and followed the kinetics of the ivermectin and metabolites degradation reaction. The result from this study indicates that ivermectin in cattle pats and cattle pats soil mixture degradation follows the same trends as that observed in the laboratory experiments. Thus, IVM, 3DI, and 24OHI in the upper layer experienced rapid degradation compared to the lower layers. The degradation half-lives were calculated by fitting the remaining concentration in the samples suggesting the trend: 3DI> 24OHI>IVM. Overall, IVM and 3DI in the field experiment experienced faster degradation than our sunlight exposure laboratory experiment.

Since we conducted our study in the Summer, no IVM or its bioproducts leached into the soil beneath. The obtained data suggest that ivermectin's massive drug administration has little to worry about, primarily when the dung from treated cattle is spread into the field in thin layers in the Summer before farm application.

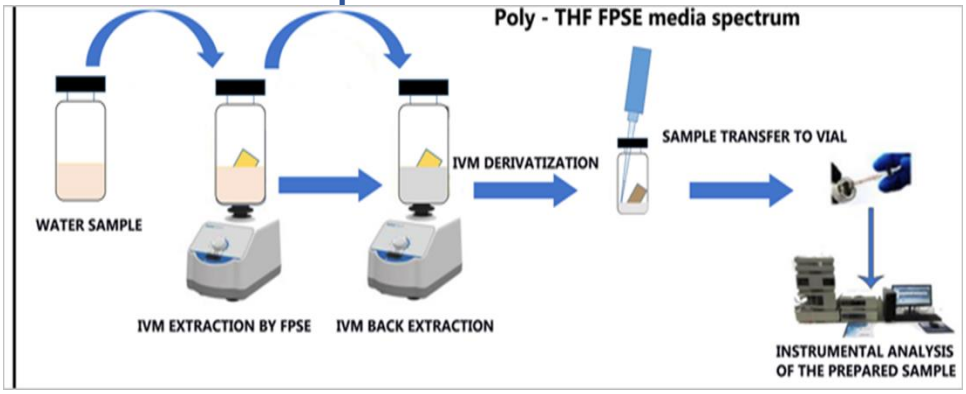
Through this project, we were able to investigate and develop an analytical method and use part of it for ivermectin extraction and clean-up. This method proved simple and cheap compared to the traditional SPE method. We also investigated the transformation of ivermectin under different environmental settings in temperate and tropical areas. Finally, we performed environmental fate investigation studies of ivermectin and its metabolites and used the obtained data plus literature to predict the ecological impacts of mass drug administration. Through this study, we can recommend the following:

1. To conduct laboratory and field study of FPSE method performance for ivermectin metabolites so that this method can be used for extraction and clean up these substances from environmental samples for instrumental analysis.
2. Since the field study was conducted in the summer, we could not determine some aspects like hydraulic runoff and leaching. Furthermore, we need more information on the transformation rate in winter seasons. Therefore, it is essential to conduct similar studies during the rainy season to investigate those missing aspects that determine ivermectin and its metabolite's fate. Moreover, it will help us to generate data and compare what happens in the summer and rainy seasons.
3. Under our study, we reported the transformation process but could not extract the degrades and study their fate and impacts. Therefore, although it seems very difficult to isolate these products, it is essential to establish experimental settings for isolation and analysis of these compounds.
4. Under this study, we used existing data on LC_{50} to predict the potential impacts; these data are primarily generated from temperate regions; given the ecological

differences between tropical and temperate regions, it is imperative to conduct environmental impact studies in a temperate area.

Appendices

Appendix A- Understanding the Sorption performance of *sol-gel-poly-THF* turned cellulose substrate for pre-concentration of ivermectin in soil and water samples



***Appendix* Figure A1:** Schematic presentation of the main steps involved in the FPSE process

Table A1: Effects of the desorption solvent on ivermectin percentage recovery from the soil, water, and manure samples

Desorption solvent	Average percentage recovery
Methanol	90 ± 5
Acetonitrile	80 ± 3
1:1 v/v acetonitrile: methanol	85 ± 5
Acetone	62 ± 4

Table A2: Effects of the sorption time on ivermectin percentage recovery from the soil, water, and manure samples

Sorption time, min	Average percentage recovery
3	50 ± 3
5	70 ± 2
7	91 ± 2
12	91 ± 1

Table A3: Effects of the desorption time on ivermectin percentage recovery from the soil, water, and manure samples

Desorption time, min	Average percentage recovery
6	53 ± 2
8	83 ± 3
10	90 ± 4
12	89 ± 2

Table A4: The average percentage recoveries against FPSE media size obtained at 7- and 8-minute sorption and desorption time in methanol

FPSE media size, cm^2	Average percentage recovery
6.25	33 ± 1
12.5	59 ± 2
24	70 ± 2
30	90 ± 1

Table A5: The average percentage recoveries for 14 weeks of ivermectin extracts stability test

Time, weeks	Average percentage recovery
6	90 ± 2
8	91 ± 2
10	77 ± 3
14	88 ± 1

Table A6: The obtained peak areas used to generate a calibration curve

IVM conc., ppb	Recorded peak area
0.5	0.11 ± 0.02
10	1.30 ± 0.04
40	4.20 ± 0.30
100	10.8 ± 0.80
143	15.2 ± 0.90
200	21.7 ± 1.20
250	27.0 ± 1.30

Table A7: Average percentage recoveries obtained with FPSE and SPE for soil, environment, and Millipore waters at three different expected recovery concentrations

	Millipore water samples					
Expected [IVM], ppb	142		40		10	
Method	SPE	FPSE	SPE	FPSE	SPE	FPSE
% Recoveries	91 ± 4	100 ± 3	87 ± 3	90 ± 2	86 ± 2	87 ± 3
	Environmental water samples					
	92 ± 3	97 ± 1	93 ± 2	88 ± 5	93 ± 2	84 ± 3
	Soil samples					
	96 ± 2	99 ± 2	92 ± 2	96 ± 2	91 ± 3	97 ± 2

Appendix B- Transformation of Ivermectin and 3″-O-demethylivermectin in Soils from temperate and tropical regions

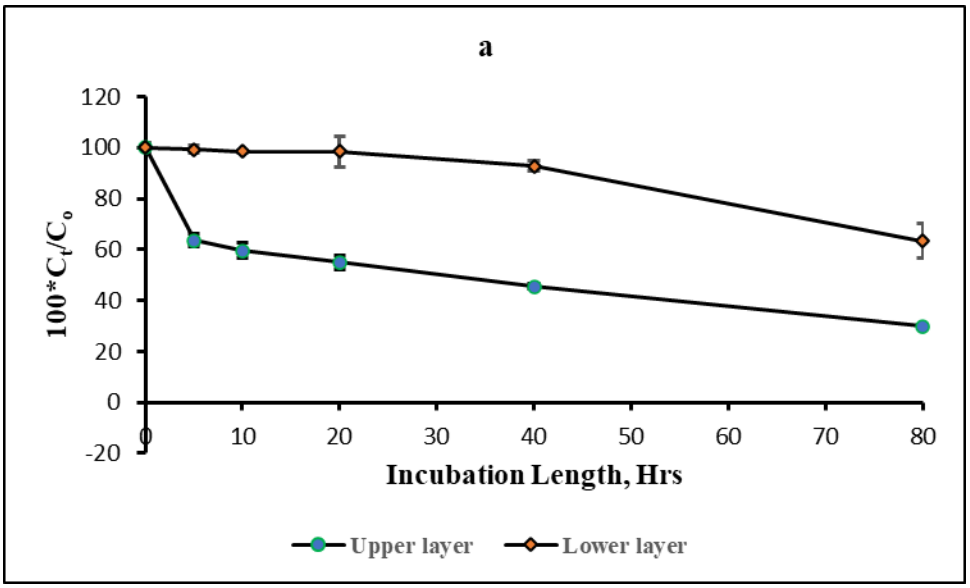


Figure B1: IVM remaining in the soil-manure mixture vs. time during sunlight exposure in Virginia, USA.

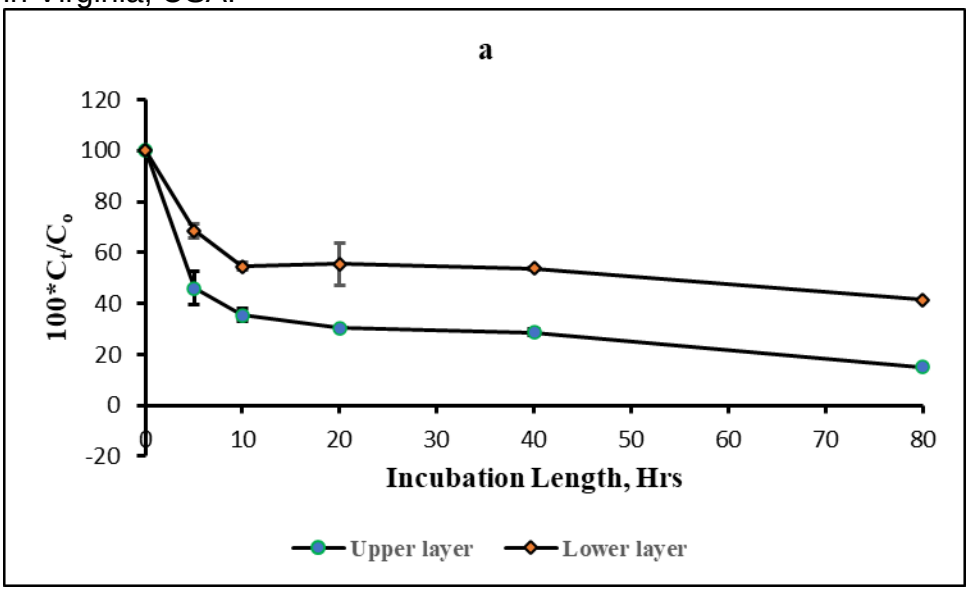


Figure B2: 3DI remaining in the soil-manure at 15% moisture contents vs time during exposure in Virginia, USA.

Table B1: The amount of IVM remaining in the soil during field incubation laboratory experiment in Virginia, USA.

Exposure Time, h	% IVM in the upper layer	% IVM in the lower layer
0	100 ± 1.31	100 ± 1.31
10	44.4 ± 5.47	92 ± 8.89
20	12.4 ± 0.71	74 ± 3.09
40	6.9 ± 1.30	67 ± 6.91
50	4.7 ± 0.56	52 ± 4.73

Table B2: The amount of IVM remaining in the soil-manure mixture during field incubation laboratory experiment in Virginia, USA.

Exposure Time, h	% IVM in the upper layer	% IVM in the lower layer
0	100 ± 1.80	100 ± 1.81
5	63.5 ± 2.50	99.4 ± 1.50
10	59.6 ± 2.90	98.4 ± 0.70
20	54.9 ± 2.90	98.4 ± 6.00
40	45.4 ± 1.00	92.7 ± 2.00
80	29.8 ± 0.69	63.4 ± 6.90

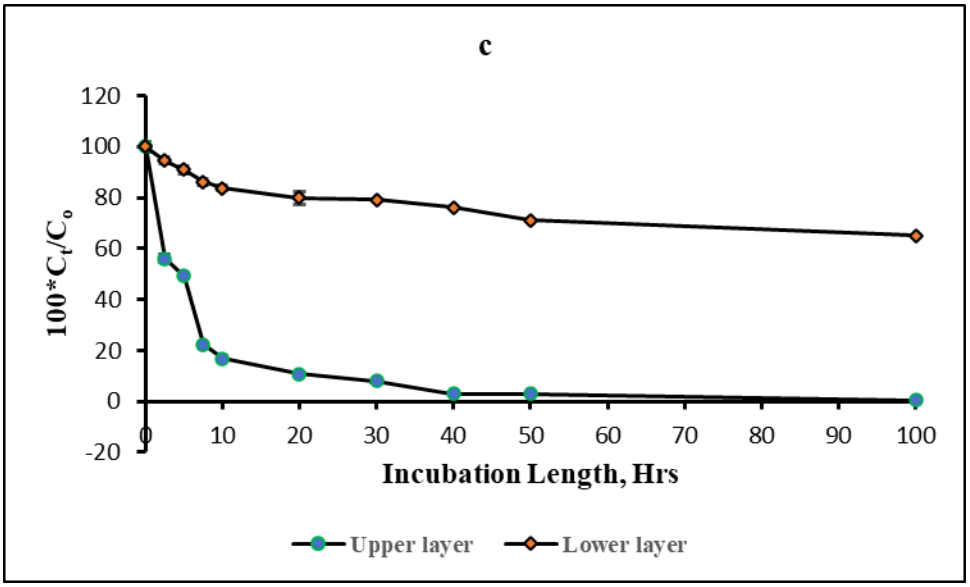


Figure B3: IVM remaining in the soil at 20 % moisture contents vs time during exposure in Ifakara, Tanzania.

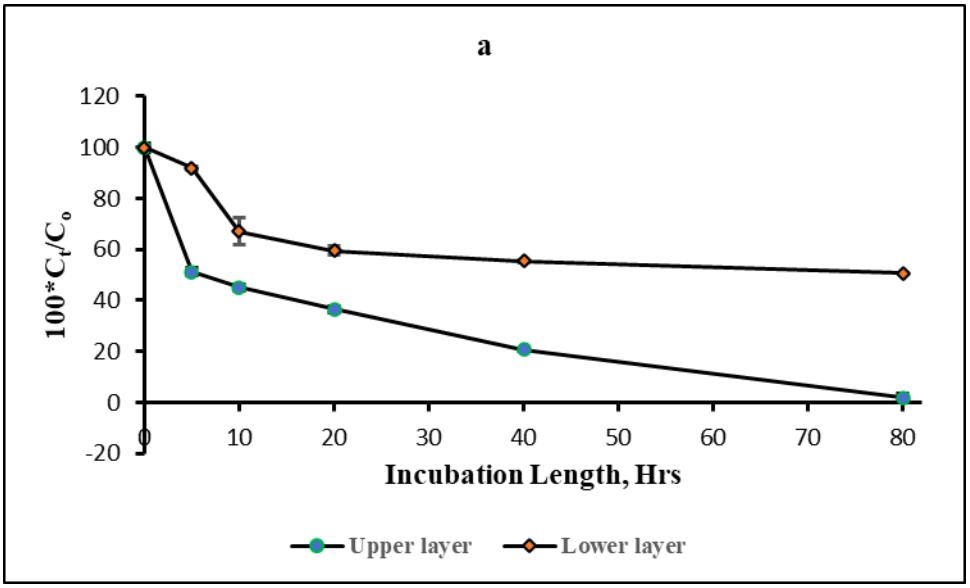


Figure B4: IVM remaining in the soil-manure mixture at 10 % moisture contents vs time during exposure in Ifakara, Tanzania.

Table B3: The amount of IVM remaining in the soil at 20% moisture content during an incubation laboratory experiment in Ifakara, Tanzania.

Exposure Time, h	% IVM in the upper layer	% IVM in the lower layer
0	100 ± 2.0	100 ± 2.0
2.5	56.1 ± 1.30	94.4 ± 1.30
5	49.3 ± 2.90	91.0 ± 1.60
7.5	22.3 ± 0.80	86.3 ± 1.30
10	16.7 ± 1.00	83.6 ± 1.00
20	10.7 ± 0.20	79.7 ± 2.60
30	8.0 ± 0.10	79.3 ± 0.70
40	2.9 ± 0.50	76.3 ± 2.60
50	2.9 ± 0.10	71.0 ± 1.4
100	0.5 ± 0.10	65.0 ± 1.40

Table B4: The amount of IVM remaining in the soil-manure mixture at 10 % moisture contents during field incubation laboratory experiment in Ifakara, Tanzania.

Exposure Time, h	% IVM in the upper layer	% IVM in the lower layer
0	100 ± 1.70	100 ± 1.70
5	51.2 ± 1.60	92.0 ± 0.80
10	45.0 ± 1.10	67.1 ± 5.20
20	36.5 ± 1.10	59.5 ± 1.80
40	20.7 ± 0.10	55.4 ± 0.50
80	1.9 ± 1.4	50.7 ± 0.00

Table B5: The amount of IVM remaining in the soil at 10 % moisture content during an incubation laboratory experiment in Ifakara, Tanzania.

Exposure Time, h	% IVM in the upper layer	% IVM in the lower layer
0	100 ± 1.50	100 ± 1.50
2.5	56.9 ± 1.30	88.2 ± 1.40
5	44.0 ± 1.40	80.9 ± 2.60
7.5	23.5 ± 0.50	81.8 ± 0.70
10	20.1 ± 1.00	79.8 ± 3.40
20	14.3 ± 0.60	79.7 ± 2.60
30	5.9 ± 0.80	77.4 ± 1.80
40	2.7 ± 0.20	73.1 ± 3.50
50	2.7 ± 0.10	71.5 ± 1.2
100	0.02 ± 0.00	72.3 ± 1.50

Table B6: The amount of IVM remaining in the soil at 15 % moisture content during an incubation laboratory experiment in Ifakara, Tanzania.

Exposure Time, h	% IVM in the upper layer	% IVM in the lower layer
0	100 ± 6.80	100 ± 6.80
2.5	77.8 ± 2.20	90.2 ± 1.40
5	68.0 ± 1.80	86.3 ± 2.20
7.5	56.9 ± 0.50	79.9 ± 4.10
10	45.7 ± 1.10	77.8 ± 4.30
20	26.0 ± 0.70	72.7 ± 1.90
30	5.9 ± 0.80	70.3 ± 2.00
40	9.0 ± 0.11	70.4 ± 0.90
50	2.2 ± 0.30	69.1 ± 2.10
100	0.7 ± 0.01	65.9 ± 0.60

Table B7: The amount of IVM remaining in the soil-manure mixture at 15 % moisture contents during field incubation laboratory experiment in Ifakara, Tanzania.

Exposure Time, h	% IVM in the upper layer	% IVM in the lower layer
0	100 ± 4.10	100 ± 4.10
5	71.0 ± 2.40	83.9 ± 4.90
10	54.0 ± 1.10	82.2 ± 2.80
20	39.6 ± 2.20	82.2 ± 1.50
40	21.5 ± 2.20	75.8 ± 1.10
80	4.6 ± 0.5	65.4 ± 4.60

Table B8: The amount of 3DI remaining in the soil-manure mixture at 10 % moisture contents during field incubation laboratory experiment in Ifakara, Tanzania.

Exposure Time, h	% 3DI in the upper layer	% 3DI in the lower layer
0	100 ± 3.00	100 ± 3.00
5	43.3 ± 1.80	96.9 ± 1.60
10	23.8 ± 0.50	86.0 ± 6.40
20	8.2 ± 0.30	79.1 ± 2.80
40	6.8 ± 0.40	78.5 ± 2.90
80	5.9 ± 0.20	69.7 ± 2.00

Table B9: The amount of 3DI remaining in the soil-manure mixture at 15 % moisture contents during field incubation laboratory experiment in Ifakara, Tanzania.

Exposure Time, h	% 3DI in the upper layer	%3DI in the lower layer
0	100 ± 2.20	100 ± 2.20
5	45.1 ± 4.30	97.0 ± 1.40
10	23.7 ± 1.20	90.0 ± 4.90
20	12.1 ± 0.70	89.3 ± 1.60
40	6.0 ± 0.30	81.2 ± 8.10
80	2.7 ± 0.40	73.1 ± 2.70

Table B10: The amount of 3DI remaining in the soil-manure mixture at 20 % moisture contents during field incubation laboratory experiment in Ifakara, Tanzania.

Exposure Time, h	% 3DI in the upper layer	% 3DI in the lower layer
0	100 ± 2.70	100 ± 2.70
5	65.8 ± 0.50	96.6 ± 1.70
10	40.3 ± 1.70	93.6 ± 5.10
20	39.4 ± 1.30	86.3 ± 1.00
40	29.4 ± 1.00	79.5 ± 6.80
80	8.5 ± 0.50	75.7 ± 2.00

Table B11: The amount of IVM remaining in the soil-manure mixture at 200 % moisture contents during field incubation laboratory experiment in Ifakara, Tanzania.

Exposure Time, h	% IVM in the upper layer	% IVM in the lower layer
0	100 ± 2.80	100 ± 2.80
5	72.3 ± 5.50	89.5 ± 2.40
10	48.1 ± 3.50	85.9 ± 1.40
20	30.6 ± 3.30	64.6 ± 1.50
40	25.3 ± 1.90	61.9 ± 3.10
80	6.4 ± 0.70	59.4 ± 3.10

Table B12: The amount of 3DI remaining in the soil at 10 % moisture contents during field incubation laboratory experiment in Ifakara, Tanzania.

Exposure Time, h	% 3DI in the upper layer	% 3DI in the lower layer
0	100 ± 2.20	100 ± 2.20
5	48.9 ± 1.00	98.0 ± 0.50
10	24.7 ± 1.20	93.2 ± 5.10
20	14.1 ± 1.00	92.4 ± 2.40
40	10.6 ± 0.90	84.4 ± 1.10
80	1.2 ± 0.50	68.7 ± 1.20

Table B13: The amount of 3DI remaining in the soil at 15 % moisture contents during field incubation laboratory experiment in Ifakara, Tanzania.

Exposure Time, h	% 3DI in the upper layer	% 3DI in the lower layer
0	100 ± 2.30	100 ± 2.30
5	71.4 ± 0.40	99.3 ± 1.00
10	54.9 ± 2.40	96.3 ± 3.40
20	33.7 ± 0.60	91.3 ± 0.40
40	29.7 ± 1.80	89.0 ± 4.90
80	9.0 ± 0.60	81.8 ± 4.30

Table B14: The amount of 3DI remaining in the soil at 20 % moisture contents during field incubation laboratory experiment in Ifakara, Tanzania.

Exposure Time, h	% 3DI in the upper layer	% 3DI in the lower layer
0	100 ± 1.50	100 ± 1.50
5	41.9 ± 3.90	99.3 ± 2.40
10	29.0 ± 6.90	90.5 ± 2.10
20	85.7 ± 0.20	83.7 ± 0.50
40	5.1 ± 0.50	84.8 ± 0.60
80	0.01 ± 0.00	74.5 ± 2.30

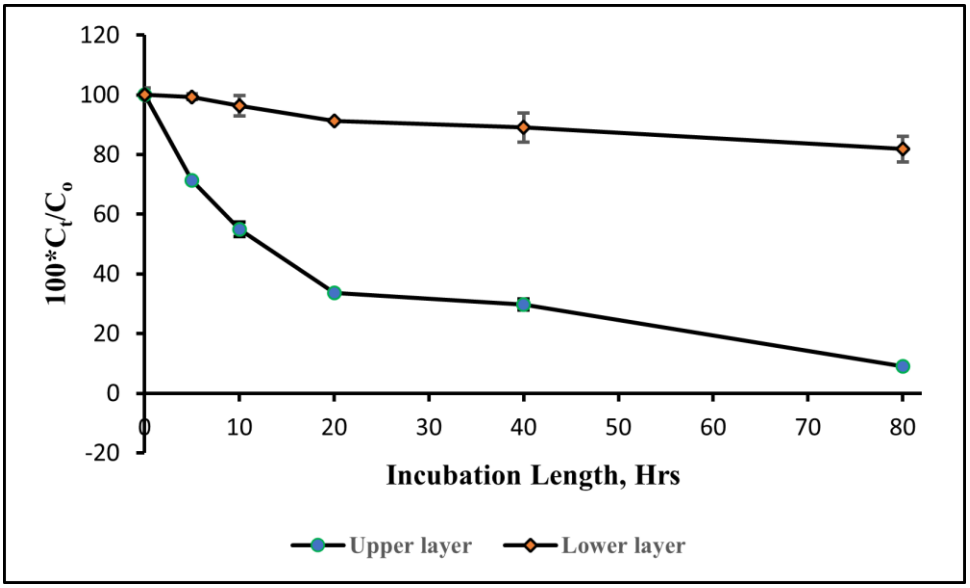


Figure B5: Concentration of 3DI in soil top 5mm and bottom 5 mm as upper and lower layers, respectively. 1 ppm 3DI soil samples at 15 % moisture content were incubated in sunlight at Ifakara, Tanzania.

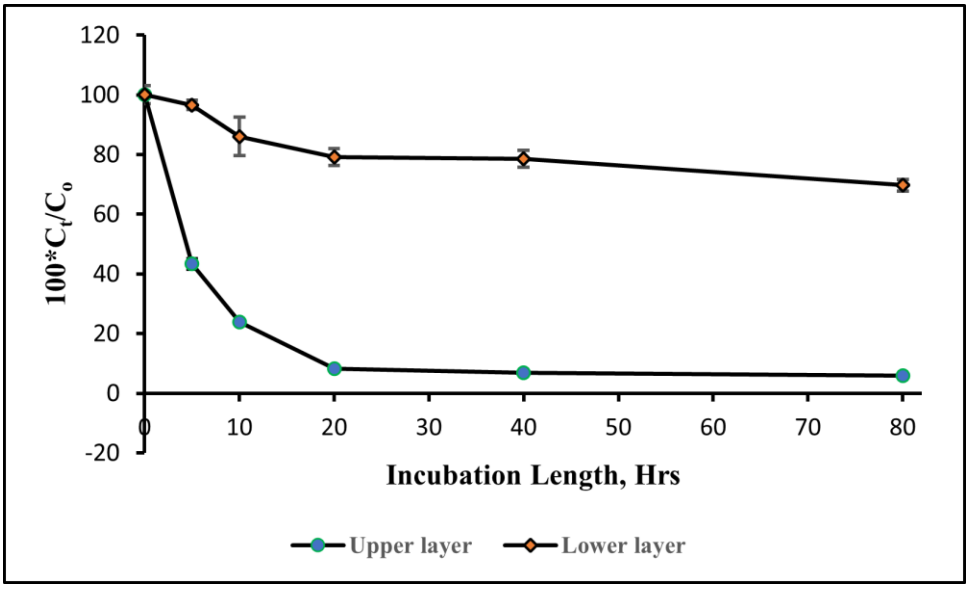


Figure B6: Concentration of 3DI in soil-manure top 5mm and bottom 5 mm as upper and lower layers, respectively. 1 ppm 3DI soil-manure samples at 10 % moisture content were incubated in sunlight at Ifakara, Tanzania.

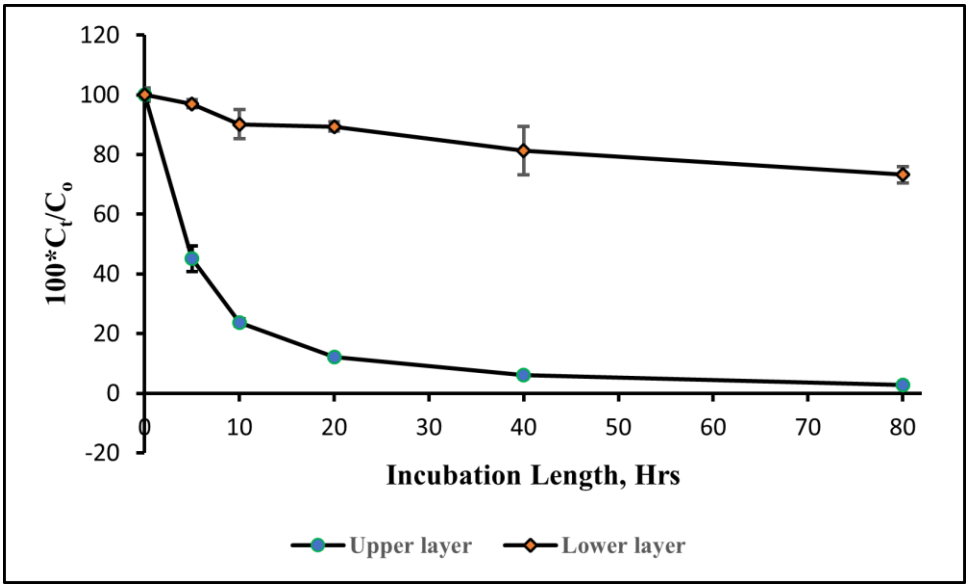


Figure B7: Concentration of 3DI in soil-manure top 5mm and bottom 5 mm as upper and lower layers, respectively. 1 ppm 3DI soil-manure samples at 15 % moisture content were incubated in sunlight at Ifakara, Tanzania.

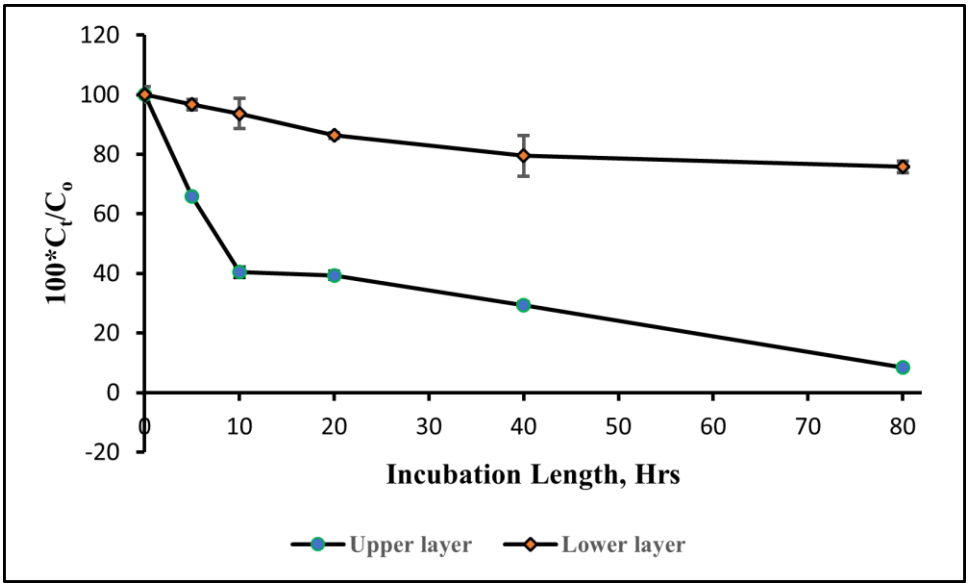


Figure B8: Concentration of 3DI in soil-manure top 5mm and bottom 5 mm as upper and lower layers, respectively. 1 ppm 3DI soil-manure samples at 20 % moisture content were incubated in sunlight at Ifakara, Tanzania.

Appendix C-Field-scale investigation of the environmental fate of ivermectin, 24-OH-ivermectin, and 3″-O-demethylivermectin in cattle manure and soil in Tanzania

Table C1: Ivermectin and 3″-O-demethylivermectin excretion profile of treated cattle. Samples were collected the before treatment, d (0), then d (1) after treatment etc. The concentration of ivermectin and 3″-O-demethyl ivermectin are reported on dry weight of the dung.

Time, d	0	1	2	5	10	15	30	45
[IVM], mg/kg	0	0.49 ± 0.02	0.86 ± 0.03	0.32 ± 0.01	0.20 ± 0.06	0.08 ± 0.002	0.04 ± 0.002	ND
3DI, mg/kg	0	0.01 ± 0.0002	0.02 ± 0.002	0.01 ± 0.0004	ND	ND	ND	ND

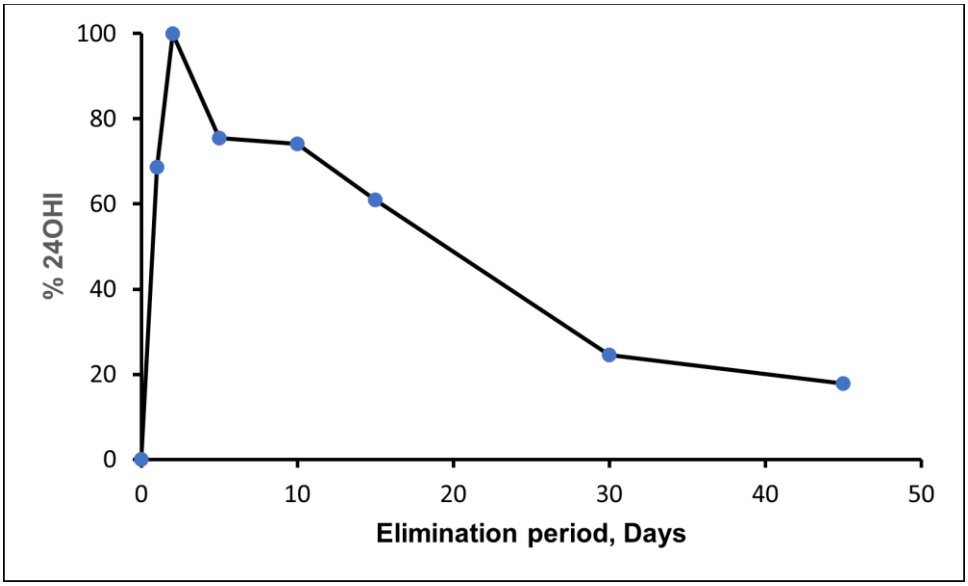


Figure C1: The change of 24OHI concentration in the dungs post IVM administration. These percentage values were calculated based on the maximum recorded 24OHI concentration in dungs.

Table C2: The amount of 24OHI remaining in the pats-soil mixture during field exposure experiment in Ifakara, Tanzania.

Exposure Time, h	% 24OHI in the upper layer	% 24OHI in the lower layer
0	100 ± 4.00	100 ± 4.00
3	52 ± 0.40	78 ± 2.90
8	35 ± 0.80	54 ± 0.50
15	19 ± 2.2	44 ± 1.20
30	ND	44 ± 7.80

Table C3: The change of 24OHI concentration in the dungs post IVM administration. These percentage values were calculated based on the maximum recorded 24OHI concentration in dungs

Elimination time, d	% 24OHI
0	0
1	67 ± 0.47
2	100 ± 0.20
5	75 ± 0.30
10	74 ± 1.00
15	61 ± 0.70
30	25 ± 0.60
45	18 ± 0.80

Table C4: The percentage of 3DI remained in pats-soil mixture upon samples exposure in the field for 30 days

Field exposure time, d	% 3DI in the upper layer	% 3DI in the Lower layer
0	100 ± 2	100 ± 2
3	75 ± 2	93 ± 2
8	ND	94 ± 1
15	ND	55 ± 8
30	ND	ND

Table C5: The percentage of IVM remained in pats-soil mixture upon samples exposure in the field for 30 days

Field exposure time, d	% IVM in the upper layer	% IVM in the Lower layer
0	100 ± 1	100 ± 1
3	35 ± 1	69 ± 2
8	25 ± 1	58 ± 1
15	15 ± 2	45 ± 2
30	8 ± 2	46 ± 1

Table C6: The percentage of IVM remained in pats upon samples exposure in the field for 30 days

Field exposure time, d	% IVM in the upper layer	% IVM in the lower layer
0	100 ± 3	100 ± 3
3	32 ± 1	66 ± 2
8	12 ± 1	46 ± 4
15	6 ± 1	32 ± 2
30	3 ± 1	18 ± 3