CHARLES UNIVERSITY

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF BIOCHEMICAL SCIENCES

DISSERTATION

ANTHELMINTICS IN THE ENVIRONMENT: CIRCULATION, METABOLISM AND EFFECTS

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Hradec Králové 2024

STATEMENT OF AUTHORSHIP

I hereby declare that this thesis is my original work which I solely composed by myself under the supervision of Assoc. Prof. Ing. Petra Matoušková, Ph.D. and PharmDr. Ivan Vokřál, Ph.D. All used literature and other sources are summarised in the list of references and properly cited. This work has not been submitted for any different or equal degree.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně pod vedením své školitelky doc. Ing. Petry Matouškové, Ph.D. a konzultanta PharmDr. Ivana Vokřála Ph.D. a Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

In Hradec Králové Mgr. Martina Navrátilová

ACKNOWLEDGEMENTS

First and foremost, I extend my deepest gratitude to Assoc. Prof. Petra Matoušková, my dissertation supervisor, provided guidance and a warm approach throughout my PhD journey. My special thanks go to my consultant, Dr. Ivan Vokřál, for his steadfast support in research and life.

I am deeply thankful to Prof. Jan H. Christensen for welcoming me into the Department of Plant and Environmental Sciences at the University of Copenhagen. My internship there was transformative, thanks in large part to the encouragement and fresh perspectives provided by Nikoline J. Nielsen and Peter Christensen.

My sincere appreciation goes to all my colleagues and students, past and present, at the Department of Biochemical Sciences. Special thanks go to Dr. Lenka Applová, Dr. Diana Dimunová, and PhD candidates Magdaléna Kozlíková and Michaela Šadibolová for their camaraderie and support. Your friendship has made this challenging journey enjoyable and memorable.

I am grateful for the financial support provided by Charles University (SVV 260 550), the Charles University Grant Agency (GA UK 1136120), and the project “Grant Schemes at CU” (reg. no. CZ.02.2.69/0.0/0.0/19_073/0016935), and Erasmus+, which were crucial in facilitating my research and internship.

Heartfelt thanks to my family, especially my mother, for her enduring love, patience, and unwavering belief in my ability to complete my PhD studies.

Last but not least, I must express my gratitude to Alena Pakostová for her exceptional assistance. Our coffee breaks together were not only moments of relaxation but also times filled with valuable discussions.

ABSTRACT

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Title: Anthelmintics in the environment: circulation, metabolism and effects

This dissertation investigates the environmental impact of anthelmintics, specifically focusing on their circulation, metabolism, and effects in the environment. The primary objective was to explore the extent to which anthelmintics, particularly albendazole, affect plants under laboratory and field conditions and the potential for these effects to contribute to drug resistance in parasites.

The study used an analytical technique, UHPLC-MS/MS, to monitor the uptake and biotransformation of albendazole and biochemical assays to asses phytotoxicity in plants like alfalfa and clover. Field experiments demonstrated the circulation of albendazole and its metabolites from sheep dung to plants and back to the sheep, indicating continuous environmental exposure. Furthermore, the investigation into the parasitic nematode *Haemonchus contortus* revealed changes in the expression of drug-metabolizing enzymes and the nematode's capability to metabolize albendazole, suggesting the potential for developing drug resistance.

The findings highlight the need for a holistic approach to the use of anthelmintics in agriculture, considering not only their effectiveness in controlling parasites but also their environmental impact and the risk of inducing drug resistance. The dissertation contributes significantly to the understanding of the environmental dynamics of veterinary pharmaceuticals and underscores the importance of sustainable agricultural practices and improved drug management strategies.

ABSTRAKT

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Název: Anthelmintika v prostředí: cirkulace, metabolismus a jejich působení

Tato disertační práce se zabývá dopadem anthelmintik na životní prostředí, konkrétně se zaměřuje na jejich cirkulaci, metabolismus a účinky v životním prostředí. Primárním cílem bylo prozkoumat, do jaké míry anthelmintika, zejména albendazol, ovlivňují rostliny v laboratorních a terénních experimentech, a potenciál těchto účinků přispívat k lékové rezistenci parazitů.

Laboratorní studie využívala analytickou techniku UHPLC-MS/MS ke sledování příjmu a biotransformace albendazolu a biochemické testy k posouzení fytotoxicity v rostlinách jako vojtěška a jetel. Terénní experimenty prokázaly cirkulaci albendazolu a transformačních produktů z ovčího trusu do rostlin a zpět k ovcím, což ukazuje na kontinuální expozici v životním prostředí. Dále zkoumání parazitické hlístice *Haemonchus contortus* odhalilo změny v expresi enzymů metabolizujících léčiva a schopnost hlístice metabolizovat albendazol, což naznačuje potenciál pro rozvoj rezistence na léčivo.

Zjištění zdůrazňují potřebu holistického přístupu k používání anthelmintik v zemědělství, s ohledem nejen na jejich účinnost při léčbě parazitické infekce, ale také na jejich dopad na životní prostředí a riziko vyvolání rezistence na léčivo. Disertace významně přispívá k pochopení environmentální dynamiky veterinárních léčiv a zdůrazňuje význam udržitelných zemědělských postupů a zlepšených strategií v oblasti používání léčiv.

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LIST OF ABBREVIATIONS

ABC transporters	ATP-Binding Cassette Transporters
API	Active Pharmaceutical Ingredient
BZs	Benzimidazoles
dSPE	Dispersive solid-phase extraction
ERA	Environmental Risk Assessment
FDA	the United States Food and Drug Administration
GCB	Graphitized carbon black
HRMS	High-Resolution Mass Spectrometry
LC-MS	Liquid Chromatography-Mass Spectrometry
LLE	Liquid–Liquid Extraction
MATE transporters:	Multidrug and Toxin Extrusion Protein
MLs	Macrocyclic Lactones
OECD	Organisation for Economic Co-operation and Development
PAHs	Polycyclic Aromatic Hydrocarbons
PFAS	Per- and Polyfluoroalkyl Substances
PSA	Primary-Secondary Amine
PSs	Priority Substances
Q-TOF	Quadrupole Time-of-Flight mass spectrometer
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe extraction
QuPPE	Quick polar pesticide extraction
SLE	Supported Liquid Extraction
SPE	Solid-Phase Extraction
TPs	Transformation Products

UHPLC-MS/MS	Ultra-High Performance Liquid Chromatography-tandem Mass Spectrometry
WFD	Water Framework Directive
WHO	World Health Organization
WWTP	Wastewater Treatment Plant

1 INTRODUCTION

Pharmaceuticals have attracted significant attention among the various organic compounds that can enter the environment, owing to their biological effects and documented presence, although typically at low levels. Biological pharmaceuticals, including vitamins, vaccines, antibodies, insulin, and other protein-based drugs, are not generally regarded as environmental concerns. This is primarily due to their high likelihood of undergoing metabolism in humans or animals and their ability to readily degrade in the environment. However, non-biological pharmaceuticals are acknowledged by the European Commission to be an emerging environmental problem (EUROPEAN COMMISSION, 2019; González Canga et al., 2009). Non-biological pharmaceutical residues are ubiquitous contaminants with reported concentrations ranging from ng.L^{-1} to μg.L^{-1} in aquatic environments worldwide. However, this concentration range is considered to be directly non-toxic for humans (Miller et al., 2018).

The increasing demand for pharmaceuticals to address age-related and chronic diseases, combined with shifts in clinical approaches, has driven significant changes in human healthcare. For example, medications employed in managing specific chronic conditions - antihypertensives, lipid-modifying agents, antidiabetic agents, and antidepressants - have experienced a notable rise in occurrence throughout OECD nations over the past few decades (OECD, 2021).

Of paramount importance when discussing pharmaceuticals is the type of active pharmaceutical ingredients (APIs) involved, which encompass a wide range of drug classes, including antimicrobials, anticancer agents, and anti-inflammatory drugs, among others. Many APIs have been detected worldwide in various environmental compartments, including soils, biota, sediments, surface water, groundwater, and drinking water sources (OECD, 2019a).

Extensive research has been done on the occurrence and fate of APIs and their metabolites/transformation products within the water cycle. APIs can enter aquatic systems through various routes, such as wastewater discharge from manufacturing facilities and hospitals, households (both through patient use and improper disposal), (aqua)farming practices, landfills, and the disposal of expired and unused drugs. Once in aquatic environments, these compounds can undergo various processes, including dilution, degradation, and bioaccumulation, affecting both aquatic, e.g. praziquantel – planarians (Mathews et al., 2023) and terrestrial ecosystems, e.g. ivermectin – dung beetle (Ambrožová et al., 2021).

Over the past two decades, scientific circles and regulatory bodies have become increasingly aware of the potential of environmental risk in increasing global production and consumption of both veterinary and human pharmaceuticals (Busfield, 2015; Miller et al., 2018; Peake et al., 2015).

As global data on pharmaceutical exposure continues to expand, the German Environment Agency aims to collate this information into a publicly accessible database. In 2014, a database was established as part of the research project "Pharmaceuticals in the environment - occurrence, effects, and options for action," compiling worldwide environmental concentrations of human and veterinary pharmaceutical residues. Almost 1000 pharmaceutical residues were detected in 61 matrices across 89 countries in all five United Nations Regional Groups. The current update of this database consists of 2062 publications, including those published in peer-reviewed journals between 2017 and 2021.

European legislation, initiated in 2001 for veterinary medicines and extended to human medicines in 2006, mandates the inclusion of an Environmental Risk Assessment (ERA) in the Market Authorization process. This framework evaluates the environmental benefit/risk associated with pharmaceuticals, applicable primarily to new drug licenses after the specified dates. The ERA consists of two integral phases: the first assesses potential environmental exposure, necessitating the second phase, if required, which focuses on ecotoxicological effects. Despite these regulatory measures, there are no significant consequences associated with the quality of ERA submissions by pharmaceutical companies. While legal obligations to assess the potential environmental impacts of drugs have been in place for several years, the effectiveness of these regulations is limited (de la Casa-Resino et al., 2021).

Recognizing these shortcomings, the proposed revision of EU Pharmaceutical legislation aims to strengthen the regulatory framework. It introduces additional information requirements and responsibilities for pharmaceutical companies. Notably, this includes an expansion of the ERA scope to cover the entire lifecycle of medicinal products and addresses specific concerns related to antimicrobials, as outlined by the European Commission. This revision seeks to enhance the accountability of pharmaceutical companies in assessing and mitigating the environmental impact of their products, aligning regulations more closely with environmental protection goals (EU, 2019).

Antimicrobials include antibiotics, antivirals, antifungals and antiparasitics that could be used to treat or prevent infectious agents in animals, plants and humans (Picot et al., 2022). Antimicrobial resistance has garnered significant attention in recent years, emerging as a prominent global public health concern (WHO, 2014). Antimicrobial resistance constitutes an intrinsic biological process exacerbated by the excessive and improper use of antimicrobial agents. Initially susceptible to these agents, microorganisms undergo resistance development through genetic mutations or the acquisition of exogenous genetic material. The documented occurrence of over 100 distinct resistance mechanisms across numerous decades underscores the persistent likelihood of ongoing resistance evolution and emergence (WHO, 2019). Today, there is no doubt that human activities help accelerate this natural process, and the future of antimicrobials is under threat. Even though antimicrobials encompass four distinct groups, antibiotics have received predominant attention, while antiparasitic agents have been comparatively overlooked. However, antiparasitic resistance is just as serious as resistance to antibiotics.

2 THEORETICAL BACKGROUND

2.1 Definition of basic terms

This section below provides a brief overview of the three pharmaceutical terms.

A **medicinal product**, often referred to as a **pharmaceutical (product)**, **medication or drug,** is a substance or combination of substances which fulfils at least one of the following conditions. First, it is presented to treat or prevent disease in humans or animals. Second, the purpose is to be used with a view to restoring, correcting, and modifying physiological functions and exerting a pharmacological, immunological or metabolic function. (EU, 2019). There are two basic constituents of medicinal products: **active substances** and **excipients**.

The **active substance**, also known as the **Active Pharmaceutical Ingredient** (API), is any substance or mixture of substances intended to be used in the manufacture of a veterinary medicinal product that, when used in its production, becomes an active ingredient of that product (EU, 2019).

Excipients can be summarized as all constituents occurring in a medicinal product, not active substances or packaging material. From an environmental point of view, the excipients play a minor importance compared to APIs, and they will not be further discussed.

The active substance is formulated together with excipients into various **drug formulations**, such as tablets, capsules, injections, creams, or liquids, to make it convenient for patients to take or administer. In the formulation process, excipients play an essential role and may perform a variety of functional roles, e.g. drug solubility and permeability.

These definitions clearly show that pharmaceuticals and APIs are related but distinct terms. For instance, acetaminophen is an API known for its pain-relieving properties, whereas Paralen® is a pharmaceutical product containing acetaminophen along with excipients. These terms should not be used interchangeably by health professionals. However, when discussing environmental contamination by pharmaceuticals, monitoring primarily focuses on the detection/quantification of individual APIs. More often, we meet with the use of terms pharmaceutical residues or drug **residues** or just **pharmaceuticals** instead. Until now, there has been no nomenclature, so these terms and their variations seem to be used interchangeably.

The monitoring efforts aim to quantify the levels of APIs/pharmaceutical residues present in various environmental matrices, such as water, sediment, soil, and biosolids, to assess their potential impact on ecosystems and human health.

2.2 The challenge of managing active pharmaceutical ingredients (APIs) in the environment

APIs present significant challenges regarding their environmental impact, which are rooted in several critical factors. Understanding these challenges is crucial for addressing the environmental concerns associated with pharmaceutical residues (OECD, 2019a, 2019b). The concentration and impact of pharmaceutical residues on the environment depend on several factors, which are listed in Table 1.

1. **Diversity and quantity of APIs and their metabolites and transformation products (TPs):**

   One of the key challenges arises from the sheer diversity and quantity of over 3,000 APIs used in human and veterinary medicine in the EU (BIO Intelligence Service 2013; aus der Beek et al., 2016). This vast production amounts to approximately 100,000 tons annually, as reported by Weber et al. in 2014. Another significant challenge arises from the metabolic processes that APIs undergo within the human or animal body, leading to the formation of metabolites. Lastly, after excretion into the environment, both the parent compounds and their metabolites can undergo further transformation via biotic and abiotic processes, giving rise to several or many TPs formed based on one parent API (Kümmerer, 2009). The relation between API and its transformation is summarized in Figure 1.

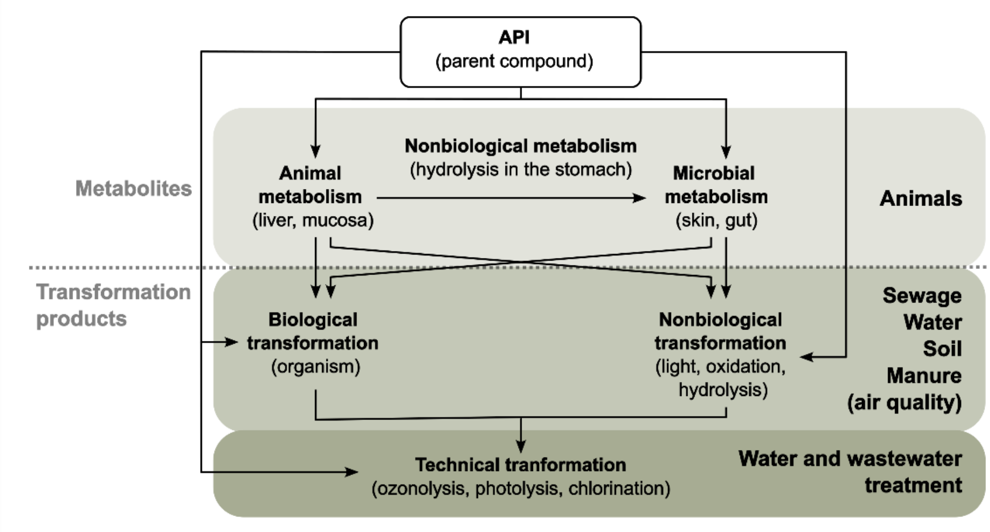


Figure 1 Relation among active pharmaceutical ingredients (API), metabolites and transformation products. Based on Kümmerer2009 (Kümmerer, 2009)

2. **Pharmacological activity at low concentrations:**

APIs are designed to have specific pharmacological effects in organisms at low concentrations. This characteristic means that even trace amounts of pharmaceutical residues in the environment can have biological impacts on non-target organisms in aquatic or terrestrial ecosystems and disrupt their normal physiological processes (Parezanović et al., 2019).

3. **Stability and continuous release:**

APIs are designed to be stable to reach and interact with target molecules in the organism. This stability often translates to slow biodegradation in the environment. Additionally, continuous use of pharmaceuticals by humans and animals leads to a constant release into the environment, which can exceed natural biodegradation rates. This continuous discharge can result in a quasi-persistent presence of APIs in ecosystems (Moermond et al., 2022).

4. **Limited removal by conventional wastewater treatment:**

Conventional wastewater treatment plants (WWTPs) are not explicitly designed to remove API compounds from wastewater. While they can remove some compounds to varying degrees, many compounds can pass through these treatment processes largely unchanged, ultimately entering surface waters (Su et al., 2021).

5. Lack of WWTPs of veterinary pharmaceuticals in agriculture and aquaculture:

The absence of WWTPs, especially in large-scale farming, presents a significant challenge in managing large volumes of organic waste. Manure lagoons or agricultural lagoons serve as efficient solutions for waste management, nutrient recycling-fertilization, and biogas production. However, they pose significant environmental risks. These risks include potential groundwater contamination, air quality degradation, greenhouse gas production, and the risk of transfer of antibiotic resistance genes to bacteria in the environment, thus, antibiotic resistance development in human pathogens (Van Epps and Blaney, 2016) (Oliver et al., 2020).

Heavy rains or floods can lead to the leaking or overflowing of lagoons, exacerbating the risk of environmental contamination. However, these lagoons can pose serious threats to the environment in cases of collapse or leakage (Agga et al., 2022).

WWTPs situated in animal farms have demonstrated effective removal of total antibiotic residues from wastewater, as reported by Zhang et al. (2018). Additionally, solid waste forms, such as manure or dewatered sludge, tend to exhibit significantly higher concentrations of these antibiotic residues. In fact, the concentration in these solid wastes can be 80 to 250 times greater compared to the treated liquid waste (Zhang et al., 2018). This finding is particularly significant considering that such solid material is commonly used as fertilizer in fields, which could potentially lead to the spread of antibiotic residues in agricultural lands (Zhang et al., 2018) (Van Epps and Blaney, 2016).

6. Long-term and multi-route exposure of non-target organisms:

The presence of APIs in various environments could significantly affect non-target organisms. Aquatic organisms are impacted by antidepressants, altering fish and crustacean numbers of total eggs (Lopes et al., 2020). Vultures in South Asia experienced near-extinction due to renal failure caused by consuming diclofenac-treated livestock carcasses (Oaks et al., 2004). Diclofenac was, therefore, banned in veterinary medicine. Similarly, other non-steroidal non-steroid inflammatory drugs, such as nimesulide, show the same toxicity in Gyps vultures (Mathesh et al., 2023). Tamschick et al. (2016) tested susceptibility to contraceptive 17α-ethinylestradiol in three different frog species. The occurrence of sex reversal (male-to-female) in these frogs varied significantly across species and was dependent on the concentration of 17α-ethinylestradiol (Tamschick et al., 2016). Fish in aquatic environments suffer from haematological changes, metabolic disorders, immunosuppression, and other disorders due to antibiotic oxytetracycline (Bojarski et al., 2020). Heneberg et al. (2018) bring evidence that benzimidazole fungicides – albendazole and thiabendazole (also used as anthelmintics), could have significant detrimental effects on ant *Myrmica rubra* queens (Heneberg et al., 2018).

Table 1 Typology of pharmaceutical residues in the environment (OECD, 2019b)

Sources	Pathways	Concentration patterns	Pharmaceutical properties	Receiving environment type (sinks)	Concentration, context-dependent factors
Pharmaceutical manufacturing plants WWTPs - Municipal - Hospitals - Industry Agriculture (particularly intensive livestock farming) Aquaculture Septic tanks Waste management facilities (landfills)	**Point source** (WWTP discharge) **Diffuse source** (i.e. agricultural runoff, leaching of septic tanks to groundwater)	**Continuous** (e.g. WWTPs) **Seasonal** (linked with farming practices and with seasonal influenza and allergies, water flow and temperature) **Intermittent** (linked with rainfall events, stormwater overflow, irrigation patterns and pandemics)	**Persistence** - Half-life - Solubility - Metabolites -Transformation products **Bioaccumulation** **Toxicity** - Individual effects - Population effects - Additive effects - Mixture effects **Mobility**	Rivers Lakes Groundwater Soil Sediment Coastal zones Oceans	Medical, agriculture and veterinary practices Illicit drug use Consumption rates Pharmaceutical properties Disposal and waste management practices WWTP technology, operation and removal efficiency Receiving environment type Climate Drainage characteristics Water flow variations Sunlight, temperature Presence of other pollutants Exposure history

WWTPs - waste water treatment plants

2.3 The sources of APIs in the environment and their fate

The dissemination of APIs like anthelmintic drugs in the environment presents a multifaceted problem. These substances, common in human and veterinary medicine, enter the environment through various channels, each bearing distinct implications for both ecosystems and human health. Key sources include pharmaceutical manufacturing, consumer usage, and improper disposal listed in Table 1 (Bártíková et al., 2016a; Díaz-Cruz et al., 2003; Miettinen and Khan, 2022).

In response to this growing concern, in 2014, the German environment agency embarked on an ambitious project to track the environmental presence of pharmaceuticals, leading to the creation of the 'Pharmaceuticals in the Environment' database: https://www.umweltbundesamt.de/en/database-pharmaceuticals-in-the-environment-0. This valuable resource, encompassing 2,062 publications and 276,895 entries, provides an in-depth look at pharmaceutical residues. The latest update focuses on worldwide measured environmental concentrations published in peer-reviewed journals from 2017 to 2020 (Dusi et al., 2019). It reveals that 47% of these residues are in surface waters, with groundwater and wastewater accounting for 8% and 40%, respectively. However, the database also highlights a research gap: veterinary pharmaceuticals in manure, dung, soil, and sediments are significantly underrepresented, making up only 5% of the total entries, indicating a critical area for future study (aus der Beek et al., 2016; Dusi et al., 2019).

2.3.1 Metabolites and transformation products of APIs; focus on veterinary anthelmintics

A significant percentage (30-90 %) of these APIs are excreted as parent compounds from the body through urine and faeces as parent compounds (BIO Intelligence Service 2013). It's important to note that the exact percentage of excretion can vary widely depending on several factors, including the specific drug, its formulation, and individual variations in metabolism (Baggot, 1992). Certainly, in the context of antiparasitic drugs used in animals, there are examples of APIs that are primarily excreted as parent compounds. These include macrocyclic lactones like ivermectin and moxidectin (González Canga et al., 2009), salicylanilides including closantel (González Canga et al., 2009; Michiels et al., 1987; Page, 2008).

However, not all antiparasitic drugs are excreted in this manner. Some antiparasitic drugs undergo significant metabolism within the animal's body, leading to the formation of several metabolites. Examples of these include imidazothiazoles like levamisole, benzimidazoles like albendazole (Myers et al., 2021), quinoline derivatives such as praziquantel (Qian et al., 2017). Some benzimidazoles are interesting because they are prodrugs, initially inactive in their administered form but later undergo metabolic activation in the body, transforming into active anthelmintic agents. For instance, febantel is converted by cyclization into fenbendazole and further oxidized to fenbendazole-sulfoxide, which plays an important role in its anthelmintic activity (Myers et al., 2021; Pattanayak et al., 2024).

This metabolic transformation can generate a multitude of new chemical entities, some of which may be more persistent or have different toxicological properties than the parent compounds (Maculewicz et al., 2022). Consequently, even if a pharmaceutical is designed to be biodegradable, its metabolites may not be, thus increasing the number of potentially problematic compounds released into the environment (Puhlmann et al., 2021). Furthermore, the extent of metabolism can vary widely from one individual to another and between species, making it challenging to predict the exact composition of pharmaceutical residues that will end up in the environment (Martignoni et al., 2006). This variability in metabolism, coupled with the vast number of APIs in use, adds to the complexity of managing and mitigating their environmental impact.

2.4 ANTHELMINTIC DRUGS IN THE ENVIRONMENT

Anthelmintic drugs are a useful chemotherapeutic tool for global parasite control in animals and humans. Represent about one-third of all veterinary drugs used yearly, and predictions estimate their consumption will grow in future years worldwide (AnimalhealthEurope, 2023). Despite the fact anthelmintics are valuable drugs that improve the health of livestock, pet animals and people, their use also brings severe problems in the form of environmental pollution and the affection of non-target species, including both fauna and flora (Bártíková et al., 2016b; de Souza and Guimarães, 2022; Jacobs and Scholtz, 2015).

The most used anthelmintic drug classes were introduced to the market before the year 2000. Anthelmintics from different classes are often, mainly in veterinary medicine, combined to increase their effectiveness. The main classes and their representatives are listed here: benzimidazoles (albendazole, fenbendazole), imidazothiazoles (levamisole), tetrahydro pyrimidines (morantel, pyrantel), macrocyclic lactones (ivermectin, doramectin, eprinomectin), salicylanilides (closantel), pyrazinoiso-quinoline derivatives (praziquantel). Since 2000, only three new anthelmintic drug classes have entered the market: amino-acetonitrile derivatives (monepantel), spiroindoles (derquantel), cyclic octadepsipeptides (emodepside). However, the last three groups are not available in any formulation in the Czech Republic's market. The following sections briefly overview the two most used anthelmintics classes in the Czech Republic.

2.4.1 Benzimidazole anthelmintics

Benzimidazoles (BZs) are heterocyclic aromatic compounds containing fused benzene and imidazole rings. Benzimidazole derivatives have many therapeutic applications, including antiparasitic, antifungal, antibacterial, anti-inflammatory, antihypertensive, and more. Besides, a number of benzimidazole derivatives play an important role in livestock gastrointestinal parasite elimination.

Benzimidazole anthelmintics constitute a significant class of anthelmintics used to treat infections caused by nematodes (*Haemonchus spp.*), cestodes (*Moniezia spp.*), and trematodes (*Fasciola hepatica*). The most common representatives are thiabendazole, albendazole, mebendazole, fenbendazole, oxfendazole, triclabendazole and flubendazole (Figure 2).

The mode of action of BZs is based on the inhibition of microtubule polymerization by pseudo-irreversible binding to the parasite's cellular beta-tubulin, causing cell death. Although BZs also bind to the mammalian beta-tubulin, the affinity for parasite beta-tubulin is significantly higher; therefore, side effects are not observed in the host (Köhler, 2001; Lacey, 1988).

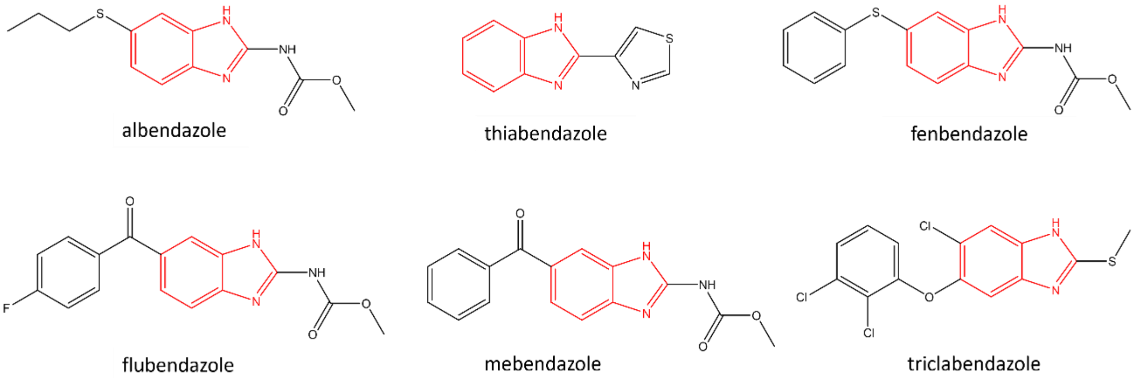
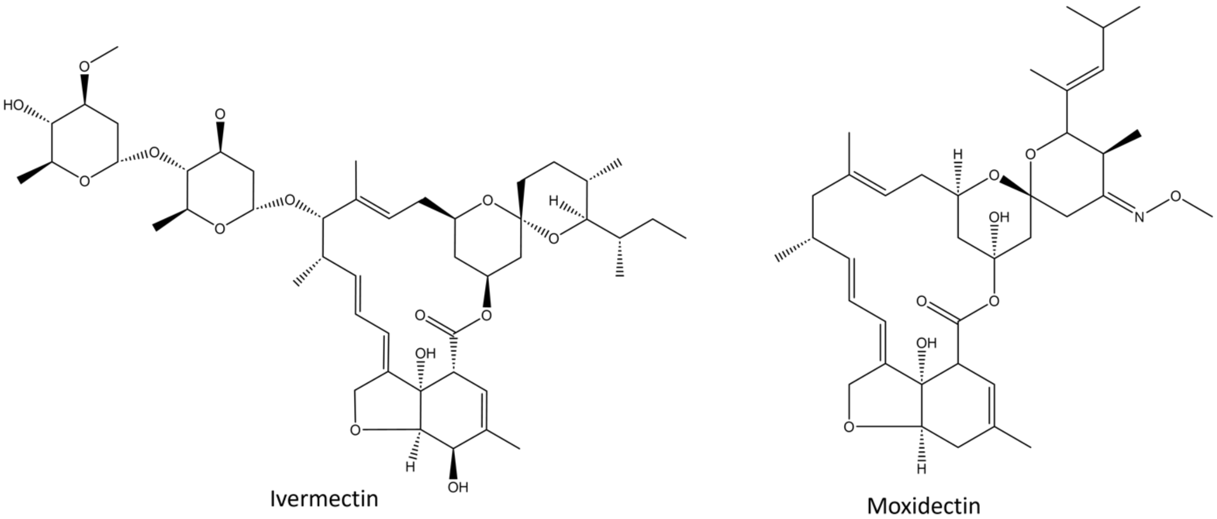


Figure 2 Chemical structures of typical representatives of benzimidazole anthelmintics. The benzimidazole core is marked red.

2.4.2 *Macrocyclic lactone anthelmintics*

Macrocyclic lactones (MLs) are compounds of biosynthetic origin formed during the fermentation process of Streptomyces spp. They can be categorized into two groups: avermectins and milbemycins (Figure 3) (Vardanyan and Hruby 2016). Avermectines consist of a 16-membered pentacyclic macrocyclic ring containing spiroketal moiety, benzofuran ring, and disaccharide or monosaccharide. Milbemycins are structurally similar but differ in the absence of saccharide moieties. Compared to benzimidazoles, MLs do not act on trematodes and cestodes, but in addition to nematodes, they act against a number of ectoparasites. Often, they are referred to as endectocides because of their activity against endo- and ecto-parasites (Prichard et al., 2012). At the same time, MLs,


Ivermectin

Moxidectin

Figure 3 Representatives of each group: Avermectins- Ivermectin, Milbemycins-Moxidectin

e.g. abamectin, are commonly used as insecticides in agriculture and horticulture (Stevens et al., 2010).

The first member of the avermectin subgroup, ivermectin, was introduced in 1981 and continues to play a crucial role in livestock anthelmintic therapy worldwide. Other members of the avermectin subfamily include abamectin, doramectin, eprinomectin, and selamectin. On the other hand, the milbemycin subfamily consists of moxidectin, nemadectin, milbemectin and milbemycin oxime (Laing et al., 2017; Prichard et al., 2012).

MLs function as allosteric modulators of glutamate-gated chloride ion channels, which results in the opening of these channels in various parasite tissues. Nematodes are affected in their pharyngeal muscles, motor nerves, female reproductive tracts, and excretory/secretory vesicles (Martin et al., 2021). Excretory/secretory vesicles help modulate the host immune system, thereby increasing parasite survival. It was proved that ivermectin reduces the release of immune modulation proteins from excretory/secretory vesicles in filarial nematode *Brugia malayi, Dirofilaria immitis* and ascaris nematode *Ascaris suum* (Harischandra et al., 2018; Loghry et al., 2020; Moreno et al., 2010).

2.4.3 The sources of veterinary anthelmintics in the environment

From 2022 to 2030, the global anthelmintic drugs market is poised for substantial growth, with a projected compound annual growth rate of 4.5%, culminating in a market increase of 42.21% by 2030, to reach an estimated value of USD 3.2 billion (2021). This notable expansion is driven primarily by the escalating prevalence of parasitic infections in developing countries and increased awareness of treatment options in developed nations (Selzer and Epe, 2021).

The environmental impact of anthelmintic residues, particularly from veterinary sources, remains a pressing concern. Anthelmintics are used in livestock farming, which often bypasses WWTP, which is typical for human pharmaceuticals or pharmaceutical manufacturing emissions. These substances, including metabolites and TPs, enter the soil environment through animal waste or processed fertilizers, leading to potential soil absorption or runoff into groundwater systems or directly to water systems by treating fish in aquaculture or by effluent from manure lagoon (Díaz-Cruz et al., 2003).

Highlighted by studies, the quasi-persistent nature of these pollutants, e.g. ivermectin in the environment, underscores the urgent need for comprehensive understanding and mitigation of their effects on both terrestrial and aquatic ecosystems (Boxall et al., 2004; Heinrich et al., 2021).

Veterinary anthelmintics can enter the environment through various pathways, each with distinct implications:

Animal Waste and Manure:

The administration of veterinary drugs, particularly anthelmintics, to animals results in both metabolized and unmetabolized drug residues being excreted through faeces and urine. In agricultural contexts, animal waste and manure, which are rich in these drug residues (during a short time period), are commonly utilized as fertilizers (Heinrich et al., 2021; Pan and Chu, 2017). The European Commission's new regulations promote the use of organic and waste-based fertilizers in the EU, aiming to reduce environmental impact and health risks and lessen reliance on imported fertilizers. This change, aligning with the Green Deal's objectives, facilitates a shift towards sustainable agriculture by enabling organic fertilizers to replace up to 30% of mined fertilizers (Commission, 2020). This practice leads to a complex interaction between agricultural activities and environmental health.

However, when animal waste after anthelmintics treatment is applied to fields, the residues of anthelmintic drugs within it can be transferred into the soil (Lagos et al., 2023). This transfer is facilitated by natural processes such as rainwater percolation or irrigation systems. As these drug residues infiltrate the soil, they can either be absorbed by it or, more concerningly, leach groundwater systems. Additionally, surface runoff during heavy rains or excessive irrigation can carry these residues to nearby water bodies. This leaching poses a significant risk for water bodies and negatively impacts the aquatic biota (Obimakinde et al., 2017; Vokřál et al., 2023). Various factors, including the type of soil, land management practices, and climate, can influence the degree of runoff. The amount and prevalence of anthelmintics released by treated animals are influenced by various factors, such as the anthelmintic class, target species, dose, route of administration, and treatment frequency (Beynon, 2012b; McKellar, 1997). As many studies proved, the presence of anthelmintic residues in animal waste can have detrimental effects on invertebrates and overall ecosystem health (Vokřál et al., 2023).

For instance, ivermectin, which is excreted in faeces largely unchanged, accumulates in the environment and decreases species richness. Studies in the Czech Republic (Ambrožová et al., 2021) or Brazil (Correa et al., 2022) have shown reduced biodiversity due to ivermectin contamination. Dung beetles, crucial for dung degradation, are significantly impacted by ivermectin, which affects reproduction, motility, etc. Dung beetles have a significant ecological role and potential in the biological control of gastrointestinal nematodes, although their effectiveness can vary based on several factors, including the beetle species, climate conditions, and ecological dynamics (Szewc et al., 2021).

Benzimidazoles also pose environmental risks and have been detected in various water systems, posing risks to the environment. Particularly, albendazole exhibits acute toxicity to the freshwater invertebrate *Daphnia magna*, with an LC_{50} of 16.5 $\mu g.L^{-1}$, a concentration also found in environmental samples. Similarly, albendazole at concentrations of 1–12 $mg.kg^{-1}$ dung dry weight can adversely affect the reproduction of the earthworm *Eisenia fetida*. It is proven that earthworms are able to reduce the number of gastrointestinal nematodes, such as *H. contortus* or *Trichostrongylus colubriformis* (d’Alexis et al., 2009). This highlights a broader ecological concern, as the widespread use of albendazole might indirectly affect these beneficial soil organisms, thereby impacting the natural control of these nematodes. Altogether, these examples demonstrate the importance of implementing sustainable agricultural practices and strategic land-use planning to minimize the adverse environmental impact of veterinary drugs (Boxall et al., 2004)

Aquaculture:

The rapidly growing industry, which was responsible for 46% of total fish production in 2018, is essential in meeting global dietary needs. By 2030, it's projected to account for 62% of fish consumption due to increasing population demands and declining capture production. However, this growth is not without environmental costs. The use of veterinary drugs like anthelmintics in aquaculture plays a crucial role in maintaining fish health. However, their use can lead to the release of pharmaceutical residues into water directly or to surrounding waters through effluent discharge.

Attention should be paid, for example, to praziquantel, a widely used anthelmintic in aquaculture, which is particularly effective against flatworms (Bader et al., 2019; Morales-Serna et al., 2018). Its application varies globally; for example, in Japan, praziquantel is used for treating blood fluke in cultured Pacific blue tuna (Ishimaru et al., 2013), while in Norway, it targets tapeworms in Salmonidae (Geitung et al., 2021). The effectiveness of treatment depends on various factors delivery, including parasite type, host species, and environmental conditions (water temperature) (Norbury et al., 2022). Cases of reduced susceptibility to praziquantel, for example, resistance in *Eubothrium sp.* infecting Atlantic salmon, have been observed in Norway and have had detrimental effects on industry (Geitung et al., 2021). This is a significant issue where proper dosing of drugs cannot be reached, e.g. oral administration (Bader et al., 2019; Mathews et al., 2023; Norbury et al., 2022).

Studies on praziquantel's persistence in sediments show risks to free-living turbellarians and bottom-dwelling organisms. Its disposal into waste treatment systems might not eliminate the drug completely, leading to environmental release (Barton et al., 2021). It disrupts the regeneration of planarians and may affect protozoans, molluscs, arthropods, and plants (Mathews et al., 2023). While praziquantel is generally safe for fish and mammals, its broader ecological impacts require further research. In conclusion, the use of praziquantel in aquaculture is crucial for controlling parasites, but it raises significant environmental concerns. The development of resistance, especially under subtherapeutic doses, and the potential ecological consequences on various non-platyhelminth organisms highlight the need for responsible drug use.

2.5 Anthelmintic drugs in plant species

Referring back to the earlier chapter that discusses the prevalence of veterinary anthelmintics in the environment, it's crucial to explore and understand their interactions with plant systems, highlighting the environmental implications of such interactions. Plants are known to uptake and bioaccumulate both organic and inorganic compounds, referred to as xenobiotics. Biotransformation is a key process in reducing the potential toxicity of xenobiotics in plants, a mechanism integral to phytoremediation practices.

Over the past decade, there has been a significant surge in research related to the uptake and translocation of antibiotics by various plant species (Keerthanan et al., 2022; Tai et al., 2019; Zhang et al., 2019). This growing interest aligns with the One Health approach, which emphasizes an integrated effort to combat antimicrobial resistance, mitigate environmental contamination, and enhance food security (Centers for Disease Control and Prevention 28.08.2023). On the other hand, research on the theme of anthelminticsin plants is scarce. Even though antiparasitic resistance has been recognized by authorities such as WHO and FDA (Picot et al., 2022).

Anthelmintics used in veterinary medicine often enter plant systems through environmental exposure, such as the irrigation of contaminated water from manure lagoons or through the application of sludge from manure lagoons, WWTPs, and other organic wastes from livestock production.

Plants have a sophisticated biotransformation system to process xenobiotics, involving a multi-phase mechanism. In Phase I, xenobiotics undergo initial chemical modifications, such as oxidation, reduction, or hydrolysis, primarily catalyzed by enzymes such as cytochromes P450 monooxygenases, peroxidases and aldo-keto reductases (Minerdi et al., 2023). This phase aims to increase the compounds' reactivity. Phase II involves the conjugation of these modified xenobiotics with endogenous substances, including glutathione, amino acids, or saccharides, significantly increasing their solubility (Bártíková et al., 2016a). A notable pathway in this phase is the formation of malonyl glycosides through the reaction of glycosylated metabolites with malonyl-CoA, leading to the stabilization of these compounds and enhanced water solubility. The final stage, Phase III, reaction specific to plants, involves the sequestration and transport of these conjugated compounds. Since plants cannot excrete xenobiotics like animals, they rely on this phase for the transport of detoxified xenobiotics into the vacuole or incorporation into the cell wall, utilizing transporters such as ABC transporters (ATP-binding cassette) or MATE transporters (multidrug and toxin extrusion protein) (Zhang and Yang, 2021).

This complex process is vital for plant survival in diverse environments and has significant ecological and environmental implications, especially in understanding plant interactions with pollutants and their role in phytoremediation.

In recent years, detailed research on the uptake and metabolism of specific anthelmintics, including benzimidazoles - albendazole, fenbendazole, flubendazole and macrocyclic lactones - ivermectin, as well as amino-acetonitrile derivatives - monepantel, has provided insights into their diverse metabolic pathways. These pathways include oxidation, hydroxylation, carbonyl reduction, and Phase II processes such as N-glycosylation, O-glycosidation, sulfation, and acetylation. In contrast, the highly lipophilic ivermectin tends to accumulate in plant parts instead of forming extensive metabolites, underscoring significant interspecies differences in plant responses to these substances.

The majority of this research has been spearheaded by our research group. We conducted the first *in vitro* metabolism study with albendazole and flubendazole in common reed cells in 2013 (Podlipná et al., 2013). Subsequent in vitro studies involved the meadow plant harebell (*Campanula rotundifolia*) (Stuchlíková et al., 2016), ribwort plantain (*Plantago lanceolata*) (Stuchlíková Raisová et al., 2017), and greenhouse experiments with soybean (*Glycine max*) (Podlipná et al., 2021), *Arabidpsis thaliana* (Syslova et al., 2019) alfalfa (*Medicago sativa*) (Stuchlíková et al., 2019) and the ribwort plantain (*Plantago lanceolata*) (Stuchlíková et al., 2018). In these studies, anthelmintics were either added to the irrigation water or released from sheep faeces.

Moving to the field experiments with alfalfa and clover (*Trifolium pratense*), they played a pivotal role, particularly regarding the role of faeces from albendazole-treated sheep. We monitored the transfer of albendazole and its main transformation products, albendazole sulfoxide and albendazole sulfone, from the faeces into the adjacent soil and plants. During a 12-week experiment, these compounds were detected in both plants and the soil up to 20 cm distance, and the drug residues were not completely removed from the faeces. This research is critical for understanding the ecological impacts of anthelmintics and the role of various plant species in environmental remediation.

For the untargeted metabolism studies, we employed LC-MS hybrid quadrupole-time-of-flight mass analyzers, such as the micrOTOF (Bruker Daltonics, Germany) and Q-TOF Synapt G2-Si (Waters, Inc., Milford, MA, USA). Targeted analysis was conducted using the LCMS-8030 triple quadrupole mass detector (Shimadzu, Kyoto, Japan). This advanced analytical approach has been instrumental in revealing the intricate details of anthelmintic metabolism in plant systems.

Phytotoxic effects like inhibited root growth or germination of seeds (Raisová Stuchlíková et al., 2020) and variations in the production of secondary metabolites, including isoflavones, have been noted in research (Podlipná et al., 2021). Additionally, changes in oxidative stress markers, such as the activity of biotransformation enzymes like superoxide dismutase and peroxidase (Navrátilová et al., 2020; Stuchlikova et al., 2018), and variations in proline content (Podlipná et al., 2021; Raisová Stuchlíková et al., 2020; Stuchlikova et al., 2018) have been observed. However, these effects do not show a consistent trend across different plant species, which makes it challenging to conclude a significant overall effect of these substances on the plants studied.

3 ANALYSES OF CONTAMINANTS IN ENVIRONMENTAL MATRICES

Since its establishment in 2000, the Water Framework Directive (WFD) has served as the fundamental framework for freshwater conservation laws in Europe. European Union Directive 2013/39/EU mandates the monitoring of certain Priority Substances (PSs) (Directive, 2013). These PSs are well-known pollutants for which regulations and limits are already in place, and they include categories like metals, polycyclic aromatic hydrocarbons (PAHs), pesticides, biocides, and per- and polyfluoroalkyl substances (PFAS). Moreover, Contaminants of Emerging Concern have been drawing attention due to the growing evidence of their presence and potential harm and are currently under early phases of research and regulatory scrutiny, which could potentially be classified as PSs in the future.

The European Commission initiated the first Contaminants of Emerging Concern watch list in 2015, which is periodically updated to mirror the progressive understanding of environmental risks and the emergence of novel contaminants (Commission, 2015). The most recent update is contained in Decision (EU) 2022/1307, which, besides other things, covers eight antimicrobial pharmaceuticals used in both veterinary and human medicine, listed in Table 2 (Union, 2022). Of these, fipronil, a massively used insecticide, has raised concerns due to its persistence in the environment and potential human health risks (Chen et al., 2022). Perhaps in the future, there will also be some representative anthelmintics that are used in veterinary medicine or human medicine, e.g. compounds such as closantel and ivermectin are known to cause both acute and chronic toxicity in aquatic environments. Moreover, there have been reports indicating the persistence of ivermectin in the environment, along with evidence of its detrimental impacts on soil and dung biota. (Adeleye et al., 2023; Liebig et al., 2010; Zortéa et al., 2017)

Table 2 Watch list of substances for Union-wide monitoring ***(COMMISSION IMPLEMENTING DECISION 22.7.2022)***

Category	Compounds
Antibiotics	sulfamethoxazole, trimethoprim, clindamycin, ofloxacin
Antidepressants and diabetes medication	venlafaxine and its metabolite O-desmethylvenlafaxine, metformin and its metabolite guanylurea
Azole compounds - pharmaceuticals	clotrimazole, fluconazole, miconazole
Azole compounds - pesticides	imazalil, ipconazole, metconazole, penconazole, prochloraz, tebuconazole, tetraconazole
Fungicides	famoxadone, dimoxystrobin, azoxystrobin
Herbicides	diflufenican
Insecticides and veterinary pharmaceuticals	fipronil
Sunscreen agents	butyl methoxydibenzoylmethane, octocrylene, benzophenone-3

Analytical chemistry plays a pivotal role in understanding and thereby helping to mitigate the contamination of the environment by various pollutant sources, including veterinary drugs (Patnaik, 2017). The development and application of analytical techniques have significantly enhanced our ability to detect and quantify trace amounts of a wide array of organic pollutants in various matrices during the last decade (Mofijur et al., 2024).

Advanced analytical techniques, such as liquid and gas chromatography coupled with mass spectrometry, have become indispensable tools in this endeavour. However, the diverse nature and low concentration levels of these pollutants and different matrices pose unique challenges, necessitating continuous advancement in analytical methodologies, including sample collection, sample preparation and instrumentation (Azizi and Bottaro, 2020; Mofijur et al., 2024; Patnaik, 2017).

3.1 From the field to the lab

In the next sections, I will focus mainly on sample collection and sample pretreatment, as establishing a pipeline for the collection and handling of different environmental matrices was an important aspect of my work. In addition, I will explore sample extraction methods, specifically LLE and QuEChERS, which were my main additions to our experiments and are described in more detail. The optimization and validation of these extraction techniques were crucial elements of my work, reflecting their significance in the broader context of environmental analysis.

3.1.1 Samples collection

Environmental sampling often involves the collection of non-living samples using methods like air samplers, water samplers (Figure 4), soil corers (Figure 5), and sediment traps. The techniques are designed to avoid altering the physical, chemical, or biological properties of the samples. Choosing the right combination of sampling methods and samplers is key to gathering accurate information and answering our research questions correctly. If we don't choose wisely, we might risk the integrity of our study from the start. In the environmental analysis, researchers are facing false negative results resulting from the analytical method detection limit, as can be seen in Figure 6.

Grab sampling is a fundamental method in environmental sampling where a single sample is collected from a specific point at a certain time. This method is

Figure 4 Water samplers (WinLab®)

Figure 5 Soil corers

straightforward, quick, and often used for immediate assessments of environmental conditions at a particular location. It is commonly employed in identifying trends in water or soil samples. A notable limitation of grab sampling is its susceptibility to variability and potential bias (Lee et al., 2019; Mooney et al., 2021; Tisler et al., 2022; Zrnčić et al., 2014).

Composite sampling involves combining multiple grab samples taken from different locations or times. This method provides a more representative analysis of the area or time period being studied. Composite sampling effectively addresses the limitations of grab sampling by integrating a broader range of data points. This method was involved in our field monitoring studies (Navrátilová et al., 2023).

Passive sampling uses devices placed in the environment for an extended period and is typically used in water or air quality monitoring. It's effective for monitoring low-level contaminants over time or cases of episodic contamination, which could be missed by spot sampling. It allows complementary knowledge of system contamination (Guibal et al., 2017; Mathon et al., 2022). The primary benefit of passive sampling in rivers is the acquisition of time-weighted data on pollution levels (Figure 6).

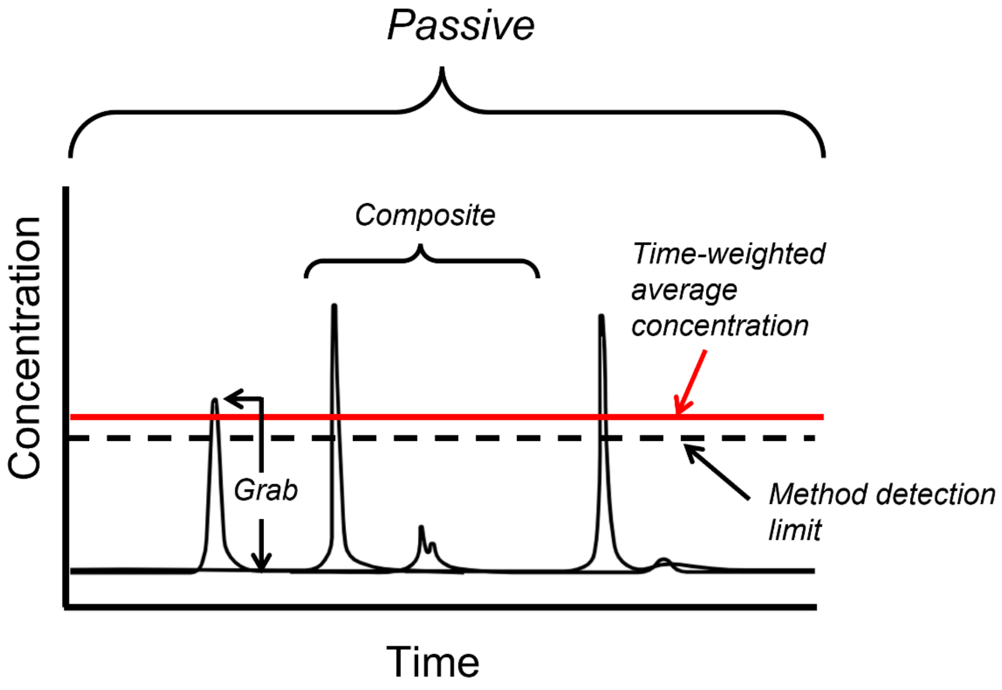


Figure 6 Comparison of different sampling techniques. Based on the work of David Alvarez, PhD, Columbia Environmental Research Center

Real-time sampling (monitoring)

This new approach aims to expand the portfolio of mobile sensors, which are very common for air monitoring. In 2017, the Eawag - Swiss Federal Institute of Aquatic Science and Technology, led by Heinz Singer, initiated a highly innovative project that resulted in the development of a novel transportable platform for real-time water-quality monitoring. The MS^{2}field platform combines sample preparation, liquid chromatography–high-resolution mass spectrometry (HRMS) measurement, automated data processing, and remote control, demonstrating its capability to quantify pollutants at high sensitivity and frequency. Its field applications provided significant insights into pollutant dynamics in various water bodies, revealing concentration peaks and patterns that traditional methods might miss. The technique, for example, was applied in monitoring pesticide dynamics in an agricultural region, where it collected over 3000 data points across 41 days, with samples taken at intervals of every 20 minutes, which is highly impressive compared to other sampling techniques available.

This groundbreaking approach offers immense potential for environmental monitoring and management, expanding the scope of real-time control in water management and environmental toxicology, which is the main benefit compared to passive sampling. The MS^{2}field's innovative design and successful field applications mark a significant advancement in the field of environmental science (Stravs et al., 2021).

3.1.2 Sample pretreatment

In environmental analysis, the pretreatment is a critical step to ensure the accuracy and reliability of the analytical results. Some of the initial and essential pretreatment steps are filtration, drying, homogenization, and sieving.

Filtration is the most commonly used pretreatment method for water analysis. There is a variety of materials, e.g. glass or cellulose, and pore sizes, e.g. 0.45 µm, on the market. The material needs to be considered according to the nature of the analyte to avoid its loss (Molnarova et al., 2023).

Reducing or removing water content from solid samples such as soil or plants is crucial and can be achieved through various methods. Oven drying involves heating samples at 30°C to 60°C, which is suitable for compounds not sensitive to heat but may result in the loss of volatile compounds (Białk-Bielińska et al., 2016; O'Kelly, 2004). Air-drying is a simpler approach where samples are exposed to room temperature but slower and potentially less consistent. Freeze drying, also known as lyophilization, involves freezing the samples at around -50°C under low pressure. This method is ideal for preserving heat-sensitive and volatile compounds, although it requires specialized equipment (Białk-Bielińska et al., 2016).

The benefits of the freeze-drying process include reducing the final water content to a range of 1-4%. This significant reduction in moisture simplifies subsequent procedures for sample analysis. In contrast, for samples dried using oven or air-drying methods, there's a need to evaluate the residual water content further, as these methods might not be as effective in reducing moisture to such low levels (O'Kelly, 2004).

Homogenization is a critical step aimed at achieving a uniform composition of the sample matrix, especially vital in environmental analysis where samples are often collected in large volumes. In the context of soil analysis, homogenization becomes essential due to the inherently heterogeneous nature of soil, which includes elements like grains, rocks, and plant roots. The process typically involves grinding, sometimes manually using a mortar and pestle or using automatic mill systems to break down large aggregates. Sieving, on the other hand, is employed to separate soil particles based on size; various sieve mesh size is involved, but typically, the desired particle size is <2 mm (De Mastro et al., 2022; García Valverde et al., 2021; Kumirska et al., 2019; Salvia et al., 2012).

3.1.3 Sample extraction

Sample preparation is a fundamental aspect of analytical chemistry, crucial for the accurate and reliable analysis of various sample matrices, including environmental samples. The complexity and diversity of these samples often require a well-considered approach to sample preparation, which directly influences the quality of the analytical results (Ribeiro et al., 2014; Soares da Silva Burato et al., 2020).

Sample preparation is critical for separating the analytes of interest from the matrix components that may interfere with the analysis. It is often the most time-consuming part of the analytical process, yet it is essential for achieving the desired sensitivity, selectivity, and accuracy in measurement (Soares da Silva Burato et al., 2020). The main objectives of sample preparation include increasing the concentration of the analytes, removing interfering substances, and converting the analytes into a suitable form for analysis (Moldoveanu and David, 2021).

3.1.4 Liquid–Liquid Extraction (LLE)

In environmental analysis, Liquid-Liquid Extraction (LLE) is a critical step to ensure the accuracy and reliability of analytical results. This extraction method, one of the oldest and most widely used, is based on the separation of components in a homogeneous solution, distributing analytes between two mutually immiscible or partially miscible phases, typically water and a non-polar organic solvent like diethyl ether, hexane, or ethyl acetate (Kim et al., 2012). The efficiency of LLE hinges on the analyte's solubility in the organic solvent and the partition coefficient (K), with the aim being to adjust the pH of the aqueous matrix to optimize the extraction of acidic or basic compounds (Patnaik, 2017) (Kim et al., 2012).

Despite its advantages, LLE presents certain drawbacks, such as the formation of turbidity and emulsions, along with high consumption of organic solvents. This often necessitates the evaporation of the solvent before its injection into the analytical system. Moreover, it is known that repeated extraction can enhance extraction efficiency compared to a single-step process, but this also leads to increased consumption of organic solvents (Patnaik, 2017).

Supported liquid extraction (SLE) offers a modern alternative. In this method, an inert diatomaceous earth is used as support for the aqueous phase. Unlike in LLE, where two immiscible phases are vigorously shaken together, SLE involves the adsorption of the aqueous sample onto the diatomaceous earth surface, followed by passing an immiscible organic solvent through a cartridge (Meunier et al., 2015). This process results in very efficient extraction without the formation of emulsions increased extraction recovery, is easier to automate, and does not require a manifold.

Concurrently, the growing need for rapid and efficient sample preparation in routine analytical chemistry has shifted focus towards high-throughput liquid phase microextraction techniques. These include dispersive liquid-liquid microextraction, single-drop microextraction, and hollow fibre liquid-phase microextraction. The advancement in automation and robotics has further facilitated the adoption of these methods in routine laboratories, making them an essential tool for high-throughput analysis. This evolution reflects the ongoing quest for more efficient, time-saving, and automated methods in the field of analytical chemistry (Carasek et al., 2023; Ju et al., 2023).

Yet, the simplicity of LLE, which requires no complex instrumentation and its broad applicability to a wide range of analytes maintains its enduring value in research labs where routine analysis may not be practical or possible. This method continues to be relevant, especially in scenarios requiring a straightforward approach.

3.1.5 QuEChERS dispersive SPE

QuEChERS, which stands for Quick, Easy, Cheap, Effective, Rugged, and Safe, is a nowadays widely recognized sample preparation method for the extraction of various residues and contaminants in food, crops, environmental matrices, and biological fluids. Developed by Michelangelo Anastassiades and Steven J Lehotay, initially for pesticide residue analysis in fruits and vegetables (Anastassiades et al., 2003), QuEChERS has been expanded to a variety of other applications due to its simplicity, efficiency, and adaptability as it is proved by 3500 publication reporting QuEChERS as extraction method according to Web of Science (Knowledge, 22.01.2024).

The method has been used effectively to extract a variety of pharmaceutical residues from the soil. These include anthelmintics like albendazole (Navrátilová et al., 2023), the antiparasitic compound dicyclanil (Salvia et al., 2012) and other pharmaceutical residues such as antiepileptics, β-blockers, and antidepressants (Kumirska et al., 2019). Additionally, it has been used for extracting antibiotics and analgesics (García Valverde et al., 2021), as well as insecticides (Bueno et al., 2022), mycotoxins (Pantano et al., 2021), and environmental contaminants such as PAHS.

However, it is not appropriate for highly polar analytes with a log K_{ow} -2 (Rejczak and Tuzimski, 2015). However, to address this limitation, Michelangelo Anastassiades et al. 2013 have introduced a novel technique called quick polar pesticide extraction (QuPPE) specifically designed for extracting anionic polar compounds, e.g. glyphosate or streptomycin, facing this challenge (Anastassiades et al., 2013).

The original QuEChERS method, when first developed, did not incorporate any buffering agents. This absence could lead to stability issues and reduced extraction recovery for pH-sensitive compounds, such as thiabendazole. Recognizing this limitation, subsequent modifications were introduced to enhance the method's efficiency.

To address this, citrate buffer at pH 5-5.5, which has a relatively low buffering capacity, was introduced by Anastassiades et al. in 2007 (Anastassiades et al., 2007). This buffer aimed to improve the stability and extraction recovery of pH-sensitive analytes. Additionally, Lehotay suggested the use of an acetate buffer at pH 4.75, known for its strong buffering capacity (Lehotay, 2007). The inclusion of these buffers in the QuEChERS method significantly increased the extraction recovery of pH-sensitive compounds.

These enhancements in the QuEChERS method have not gone unrecognized. Modified versions of QuEChERS, incorporating these buffering strategies, have been officially recognized and accepted by authoritative bodies in the field of analytical chemistry. The Association of Official Analytical Chemists (AOAC) acknowledged this method as the AOAC Official Method 2007.01. Furthermore, the European Committee for Standardization (EN) also recognized it, specifically under the standard EN 15662, for the analysis of pesticide residues. This acknowledgement, as highlighted in the work of Perestrelo et al. (2019), reflects the method's improved reliability and effectiveness in analyzing pesticide residues, particularly in addressing the challenges associated with pH-sensitive compounds (Perestrelo et al., 2019).

The key advantages:

- Simple and fast: The method is straightforward, making it easy to use and quick to execute.
- Cost-effective: It requires less solvent and no special equipment. Extraction salts and cleanup mixtures could be bought pre-weighed or prepared within a laboratory.
- Safety and environmental friendliness: QuEChERS uses less toxic solvents and generates less waste than traditional methods.
- Versatility: It is adaptable to various matrices and a wide range of analytes. Various modifications can be made:
    - Na_2EDTA chelation of Mg^{2+} complexes Ca^{2+}
    - Acidification of acetonitrile
    - Addition of different organic solvents e.g. methanol
    - Different ratios of extraction salts

Limitations:

- Molecular polarity: not so effective for very polar/anionic and non-polar compounds:
- High-fat content matrices: could be solved within the cleanup step, e.g. freezing out the lipids

Extraction process

The core of the QuEChERS method lies in its solvent extraction phase, typically using acetonitrile, followed by a liquid–liquid partition induced by the addition of salts, and a subsequent dispersive solid-phase extraction (d-SPE) – cleanup procedure (Ajibola et al., 2020). The scheme of the three main QuEChERS methods is shown in Figure 7.

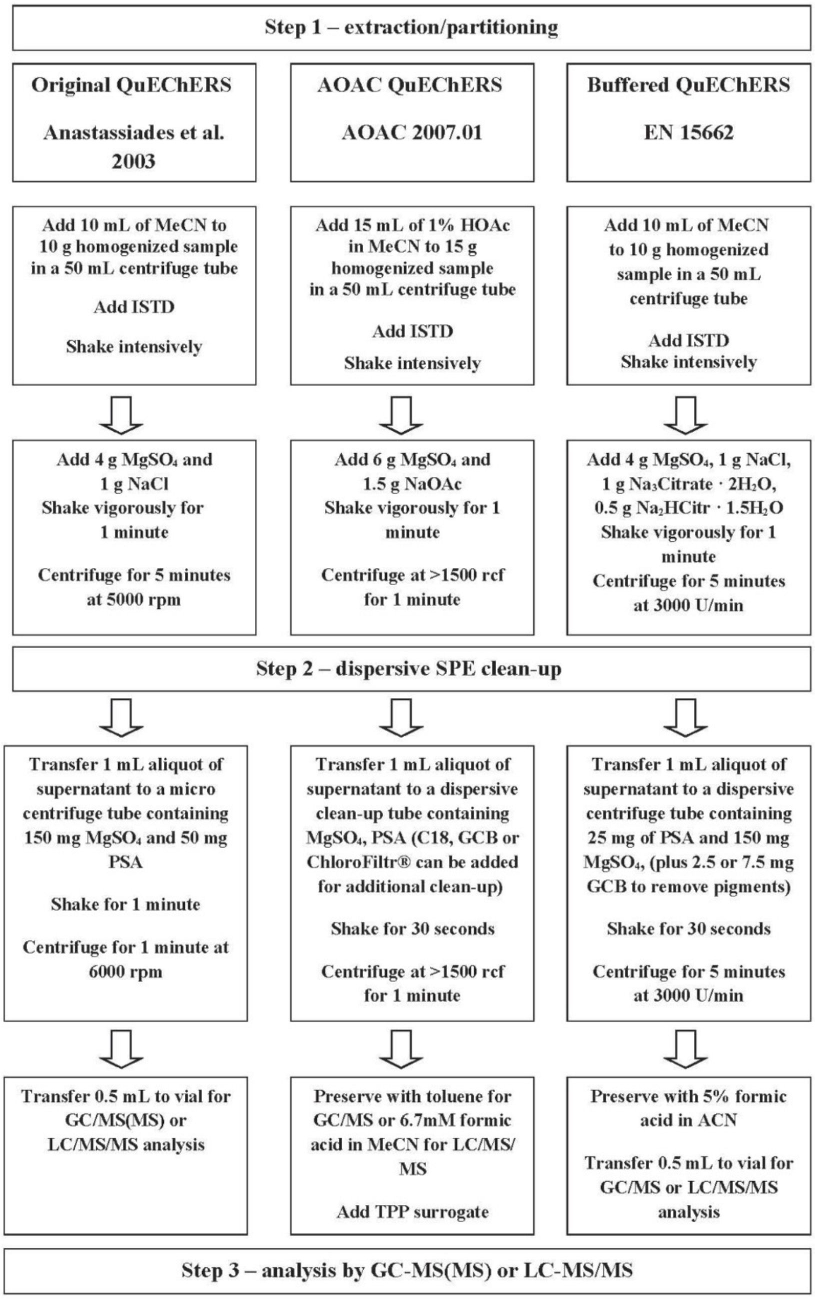


Figure 7 Schematic layout of three main QuEChERS methods: Original QuEChERS method, AOAC 2007.01 Official Method and The European Official Method EN 15662. Abbreviations: MeCN -acetonitrile; ISTD- internal standard; GCB -graphitized carbon black; TPP- triphenyl phosphate; NaOAc - sodium acetate; HOAc - acetic acid; MgSO4 - magnesium sulphate anhydrous; NaCl - sodium chloride; PSA - primary secondary amine; Na3Citrate - sodium citrate tribasic dihydrate; and Na2HCitr -sodium citrate dibasic sesquihydrate. Reproduced from (Rejczak and Tuzimski, 2015)

Extraction process

In the initial phase of the QuEChERS extraction method, a variety of salts are utilized to facilitate the phase separation between acetonitrile and water. This includes the use of drying salts like magnesium sulfate ($MgSO_4$) and sodium sulfate (Na_2SO_4), which are particularly effective in enhancing the recoveries of polar compounds. They achieve this by reducing the water content in the sample, thereby promoting the partitioning of these compounds into the acetonitrile phase (Anastassiades et al., 2003; Perestrelo et al., 2019).

To further refine the process, especially for pH-sensitive compounds, buffered salts could be introduced. These include sodium acetate (NaOAc), disodium hydrogen citrate sesquihydrate ($C_6H_8Na_2O_8$), and trisodium citrate dihydrate ($C_6H_9Na_3O_9$). The addition of these buffered salts is crucial as they help maintain a stable pH, ensuring the stability and integrity of pH-sensitive analytes.

Moreover, the incorporation of sodium chloride (NaCl) plays a significant role in enhancing the selectivity of the extraction. By adjusting the polarity of the extraction solvents through NaCl addition, the solvent becomes less polar. This reduction in polarity is beneficial as it minimizes the likelihood of polar matrix interferences, thereby refining the extraction process. NaCl also contributes to the efficient phase separation (Anastassiades et al., 2003).

Cleanup process- dispersive SPE (d-SPE)

This step aims to purify the final extract by removing undesirable co-extracted polar interferences from the matrix obtained in the extraction step. The method is grounded in classical SPE principles; however, unlike SPE, an aliquot of the sample extract is added to the loose dSPE material and mixed. This leads to increased interaction between the extract and the sorbent, enhancing the efficiency of removing matrix interferences. Moreover, it is more eco-friendly because it uses a smaller amount of organic solvents (Geis-Asteggiante et al., 2012; Rejczak and Tuzimski, 2015). For example, with 1 mL of extract, this method avoids the additional waste of organic solvents typically seen in classical SPE, which requires at least 1 mL each for conditioning and elution steps. Additionally, the amount of sorbent used is comparable to that in classical SPE.

Primary Secondary Amine (PSA) is a widely used sorbent for removing a variety of substances. As outlined by González-Curbelo, et al. (2015), PSA is particularly effective in extracting sugars, fatty acids, organic acids, lipids, and some pigments. This versatility makes it a staple in many purification processes (González-Curbelo et al., 2015).

In the area of pigment extraction, graphitized carbon black (GCB) is notably efficient, especially for carotenoids and chlorophyll. However, GCB's effectiveness diminishes with planar compounds (Rejczak and Tuzimski, 2015). Addressing this shortcoming, Agilent has developed the Carbon-S® sorbent. This advanced hybrid carbon material, with its optimized content and pore structure, significantly enhances the recoveries and relative standard deviations of planar pesticide compounds like thiabendazole, offering a superior alternative to standard GCB (Zou et al., 2023).

Another innovation in sorbent technology is Chlorfilter®, a specialized product developed by United Chemical Laboratories. Based on a polymeric material, Chlorfilter® is particularly selective for chlorophyll, making it a targeted solution for specific purification needs (Walorczyk et al., 2015).

Octadecyl Silica (C18) also plays a crucial role in purification, primarily in extracting fats, waxes, and non-polar interferences. However, its effectiveness is somewhat limited with more lipophilic compounds (González-Curbelo et al., 2015). This specificity highlights the need for choosing the right sorbent based on the nature of the compounds involved.

The last set of noteworthy sorbents are Z-Sep and Z-Sep+, both zirconia-coated silica products provided by Supelco. Z-Sep+, which additionally includes bonded C18, is not typically recommended for the extraction of hydrophobic analytes as it might reduce their recovery, as noted by Geis-Asteggianteet al. (2012), with a negative impact on avermectins (e.g., ivermectin) and β-lactams (e.g., amoxicillin) (Geis-Asteggiante et al., 2012). These sorbents are designed to replace the commonly used combination of PSA/C18, with the aim of achieving superior results in removing matrix interferences. Both Z-Sep and Z-Sep+ are specifically engineered for extracting carboxylic acids, proteins, pigments, fats, waxes, and other non-polar to moderately polar interferences. They are particularly effective in samples with varying lipid content: Z-Sep is optimal for samples with less than 15% fat, while Z-Sep+ is more suited for those with more than 15% fat content (Rajski et al., 2013).

4 OBJECTIVES OF THE DISSERTATION THESIS

This thesis intends to determine the extent to which anthelmintics affect plants in laboratory conditions and whether some effect could be observed in field experiments.

Specific aims of this dissertation thesis included:

1. investigation of the biotransformation pathway of albendazole and assess its potential phytotoxicity in alfalfa seeds and *in vitro* leaf and root explants;
2. monitoring the uptake and phytotoxicity effect of albendazole anthelmintics from sheep manure on clover in a greenhouse experiment, and in case of positive findings, conducting further studies in field experiments;
3. monitoring of the transfer and persistence of albendazole and its transformation products from sheep dung to soil and plants in real agricultural conditions;
4. investigation of the effect of albendazole residues in plants that were administered to sheep and the presence of these residues in biological samples of sheep;
5. evaluation of the influence of albendazole residues from sheep feed on the expression of resistance-related genes and production of metabolites in adults of *Haemonchus contortus*.

5 CANDIDATE'S CONTRIBUTION TO PUBLICATIONS INCLUDED IN THE DISSERTATION THESIS

This doctoral thesis is composed of a commented collection of peer-reviewed scientific publications published in impacted international scientific journals. Information about the journal impact factor (IF), article influence score (AIS) and quartile (Q) are assessed for the year of publication.

5.1 Publication I

Raisová Stuchlíková, L., **Navrátilová, M.,** Langhansová, L., Moťková, K., Podlipná, R., Szotáková, B., & Skálová, L. (2020). The Identification of Metabolites and Effects of Albendazole in Alfalfa (*Medicago sativa*). *International journal of molecular sciences*, *21*(16), 5943. **(IF20: 5.924 Q1Q2; AIS20: 1.123 Q2Q2)**

Candidate's contribution

- LC-MS methodology and validation
- sample preparation, LC-MS analysis, data analysis
- revising the manuscript

5.2 Publication II

Langhansová, L., **Navrátilová, M.,** Skálová, L., Moťková, K., & Podlipná, R. (2021). The Effect of the Manure from Sheep Treated with Anthelmintics on Clover (*Trifolium pratense*). *Agronomy-Basel,*, 11(9), 1892. **(IF21: 3.949 Q1Q1; AIS21: 0.503 Q2Q2)**

Candidate's contribution

- LC-MS methodology and validation
- sample preparation, LC-MS analysis, data analysis
- writing and revision of the manuscript

5.3 Publication III

Navrátilová, M., Vokřál, I., Krátký, J., Matoušková, P., Sochová, A., Vrábľová, D., Szotáková, B., & Skálová, L. (2023). Albendazole from ovine excrements in soil and plants under real agricultural conditions: Distribution, persistence, and effects. *Chemosphere*, 324, 138343. **(IF22: 8.8 Q1 AIS22: 1.138 Q2)**

Candidate's contribution

- experimental design and performance of field experiments
- sample preparation, LC-MS analysis,
- data analysis, interpretation of results and data visualization
- writing and revision of the manuscript

5.4 Publication IV

Navrátilová, M., Raisová Stuchlíková, L., Matoušková, P., Ambrož, M., Lamka, J., Vokřál, I., Szotáková, B., & Skálová, L. (2021). Proof of the environmental circulation of veterinary drug albendazole in real farm conditions. *Environmental pollution (Barking, Essex: 1987)*, *286*, 117590. **(IF21: 9.988 Q1; AIS21: 1.398 Q1)**

Candidate's contribution

- design and performance of the experiments
- optimization and validation of LC-MS analysis,
- data analysis, interpretation of results and data visualization
- writing and revision of the manuscript

5.5 Publication V

Dimunová, D., Matoušková, P., **Navrátilová, M.,** Nguyen, L. T., Ambrož, M., Vokřál, I., Szotáková, B., & Skálová, L. (2022). Environmental circulation of the anthelmintic drug albendazole affects expression and activity of resistance-related genes in the parasitic nematode *Haemonchus contortus*. *Science of the Total*

Environment, 822, 153527 **(IF22: 9.8 Q1 first decile; AIS22: 1.432 Q1 first decile)**

Candidate's contribution

- sample preparation, LC-MS analysis, data analysis
- data analysis, interpretation of results and data visualization
- revision of the manuscript

6 RESULTS

6.1 Monitoring of phytotoxicity effect of albendazole and its biotransformation in alfalfa plant

Publication I: Raisová Stuchlíková, L., **Navrátilová, M.,** Langhansová, L., Moťková, K., Podlipná, R., Szotáková, B., & Skálová, L. (2020). The Identification of Metabolites and Effects of Albendazole in Alfalfa (*Medicago sativa*). *International journal of molecular sciences*, *21*(16), 5943. **(IF20: 5.924 Q1Q2; AIS20: 1.123 Q2Q2)**

Albendazole is a widely employed broad-spectrum deworming drug. Its widespread application necessitates a thorough understanding of its behaviour in the environment, particularly its uptake, phytotoxicity, and biotransformation in plants. Currently, there is a gap in comprehensive knowledge regarding these aspects of albendazole.

This research aims to bridge this gap by investigating the biotransformation pathway of albendazole and assessing its potential phytotoxicity in alfalfa seeds and *in vitro* leaf and root explants. Alfalfa, chosen as a representative fodder plant, could be exposed to albendazole through animal excrements during grazing or the application of manure for field fertilization. The concentration range of albendazole 0.01-10 μM used in Murashige and Skoog medium corresponds to estimated albendazole concentrations in the soil near faeces from sheep treated with albendazole (Prchal et al., 2016). A systematical UHPLC-MS/MS-based analytical approach was used for the biotransformation study. Phytotoxicity was assessed through seed germination, root elongation test, and proline content.

The study findings indicated that albendazole did not inhibit seed germination and seedling growth in alfalfa. However, albendazole decreased the amount of net proline, a stress marker, demonstrating its lower synthesis or covering energy-demanding processes (Mattioli et al., 2009).

Both root and leaf explants could uptake albendazole and transform it into 21 and 12 metabolites, respectively. The scheme of the proposed biotransformation pathway of albendazole in alfalfa roots is depicted in Figure 8. Albendazole sulfoxide, together with albendazole sulfone, represents the primary metabolites of albendazole biotransformation throughout the tested species, e.g. helminths (McCarthy and Moore, 2015), plants (Podlipná et al., 2013; Stuchlíková et al., 2016; Stuchlíková Raisová et al., 2017), animals (Blanco-Paniagua et al., 2022) and humans (Lanchote et al., 1998). These two metabolites were present even at the lowest tested concentration of 0.01 μM. The remaining metabolites were mostly present at 10 μM, as they are less abundant, and we encounter the detection limit of the UHPLC-MS/MS instrumentation here. The presence of albendazole and its metabolites in alfalfa leaves suggests that if fodder plants containing traces of these substances are consumed by helminth-infected animals, it may contribute to the development of resistant strains of helminths in livestock.

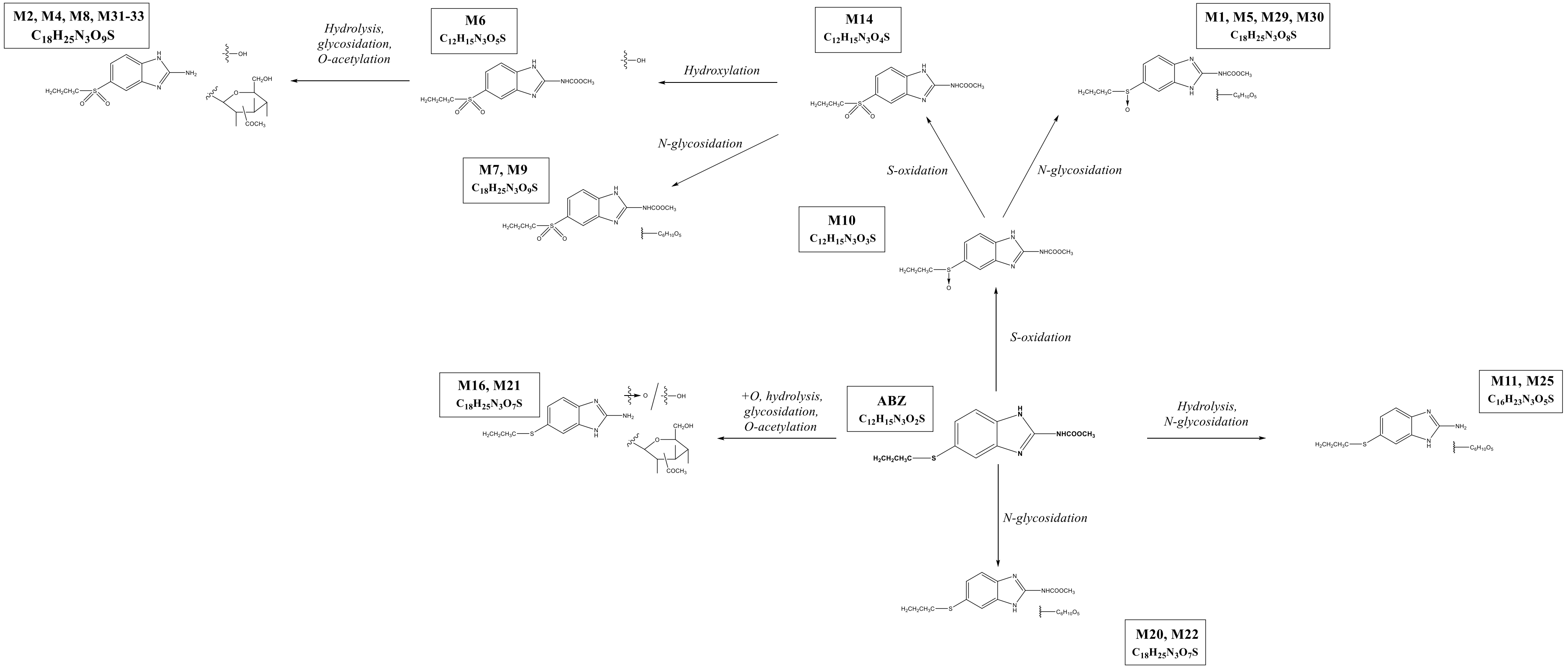


Figure 8 The scheme of the proposed biotransformation pathway of albendazole in alfalfa roots (Raisová Stuchlíková et al., 2020)

6.2 Monitoring of phytotoxicity effect of three anthelmintics – albendazole, monepantel, and ivermectin – from sheep dung on clover (*Trifolium Pratense*)

> **Publication II:** Langhansová, L., **Navrátilová, M.,** Skálová, L., Moťková, K., & Podlipná, R. (2021). The Effect of the Manure from Sheep Treated with Anthelmintics on Clover (Trifolium pratense). *Agronomy*, 11(9), 1892. **(IF21: 3.949 Q1Q1; AIS21: 0.503 Q2Q2)**

Our previous research focused on albendazole uptake, biotransformation, and phytotoxicity in alfalfa explants exposed to anthelmintics in growth medium. The current study set out to explore the influence of the effects on the clover that was cultivated in soil fertilized with dung from sheep treated with recommended doses of albendazole (10 $mg.kg^{-1}$ b.w.), ivermectin (0.2 $mg.kg^{-1}$ b.w.), or monepantel (2.5 $mg.kg^{-1}$ b.w.). The uptake and biotransformation of these drugs in clover were monitored over six weeks using UHPLC-MS/MS. Additionally, several stress indicators (such as proline accumulation, lipid peroxidation, and antioxidant enzyme activities) were assessed.

The findings revealed that clover was able to uptake and transport albendazole, monepantel and their transformation products to the plants' shoots, whereas ivermectin was not detected. Possible explanations for the lack of ivermectin could be attributed to either its physico-chemical properties, which hinder ivermectin uptake by plant tissues, or the possibility that the limit of detection of UHPLC-MS/MS used in the study was not sensitive enough to detect trace amounts of the drug.

Minimal or no drug-induced responses were observed in the stress markers examined, with only a temporary elevation observed in certain antioxidative enzyme activities. Overall, the dung from anthelmintic-treated sheep did not cause chronic stress in clover; however, it could facilitate the entry of anthelmintics into other organisms and the food chain.

6.3 Distribution, persistence, and effects of albendazole from sheep dung in soil and plants in agricultural conditions

Publication III: **Navrátilová, M.,** Vokřál, I., Krátký, J., Matoušková, P., Sochová, A., Vrábľová, D., Szotáková, B., & Skálová, L. (2023). Albendazole from ovine excrements in soil and plants under real agricultural conditions: Distribution, persistence, and effects. *Chemosphere*, 324, 138343. **(IF22: 8.8 Q1 AIS22: 1.138 Q2)**

Following the completion of experimental studies in greenhouses, we shifted our focus to field experiments. As mentioned earlier, albendazole is employed in treating parasitic infections in animal husbandry, and humans primarily enter the environment through the excreta of treated animals left on pastures or used as organic fertilizers. Efforts to enhance the utilization of organic fertilizers instead of mineral fertilizers have gained importance in recent years, but this shift brings the risk of transferring veterinary drugs, including anthelmintic residues, to the field.

Our study aimed to investigate whether albendazole could be transferred to soil and plants following field fertilization, with the findings offering insights into the connection between the presence of anthelmintic drugs in the environment and the prevalence of resistant helminths. To gather information regarding the subsequent fate of albendazole from sheep faeces, we monitored the distribution of albendazole and its two primary metabolites, albendazole sulfoxide and albendazole sulfone, in the soil surrounding the faeces, as well as their uptake and effects on plants in the field experiment (Figure 9).

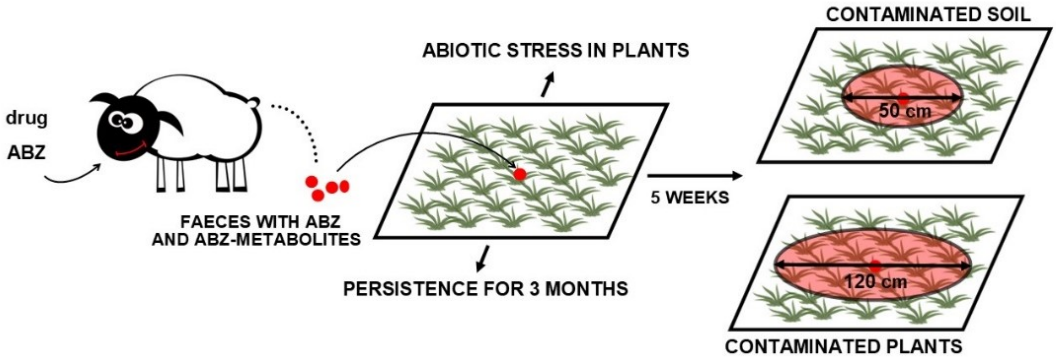


Figure 9 Scheme of field experiment (Navrátilová et al., 2023)

Faecal samples were collected from a flock of sheep 24 hours after the deworming procedure with Aldifal (p.o., albendazole 10 mg.kg^{-1}). The faecal material was thoroughly mixed, and representative samples weighing 100g each were prepared for manual application onto central plants. To ensure proper sampling, we established a circle of 75 cm around each central plant. Soil samples were taken at two different depths, and samples of two plant species, red clover and alfalfa, were collected at distances ranging from 0 to 75 cm from the faecal material. This sampling was conducted for a period of 13 weeks following fertilization. For the extraction of environmental samples, the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) and LLE (Liquid-Liquid Extraction) sample preparation procedures were employed. A validated UHPLC-MS/MS method was utilized for the targeted analysis of albendazole and its transformation products.

The results showed that two transformation products of albendazole, albendazole sulfoxide (which retains anthelmintically active) and albendazole sulfone (which is inactive), remained present in the soil up to a distance of 25 cm from the faecal material. These transformation products were consistently detectable in plants throughout the entire 13-week duration of the experiment. Surprisingly, the albendazole transformation products were even detected in plants located as far as 60 cm away from the faeces. Furthermore, the central plants displayed signs of abiotic stress. This extensive distribution and persistence of albendazole transformation products in both soil and plants accentuate the negative environmental impact of albendazole, as previously observed in other studies.

6.4 Evidence of the environmental circulation of anthelmintic drug albendazole in a field experiment

Publication IV: <u>Navrátilová, M.,</u> Raisová Stuchlíková, L., Matoušková, P., Ambrož, M., Lamka, J., Vokřál, I., Szotáková, B., & Skálová, L. (2021). Proof of the environmental circulation of veterinary drug albendazole in real farm conditions. *Environmental pollution (Barking, Essex: 1987)*, *286*, 117590. **(IF21: 9.988 Q1; AIS21: 1.398 Q1)**

In the context of modern livestock farming, the challenge of combating resistant parasitic worms is increasingly significant. To address this pressing issue, the present study was conducted to investigate the potential circulation of albendazole and its transformation products (TPs) in a field study. The study involved treating sheep with the recommended dose of albendazole (p.o.,5 mg.kg^{-1} b.w.) and then utilizing the collected faeces as fertilizer for an experimental field planted with fodder plants like alfalfa and clover. These plants were subsequently consumed by sheep from a different farm, as depicted in Figure 10.

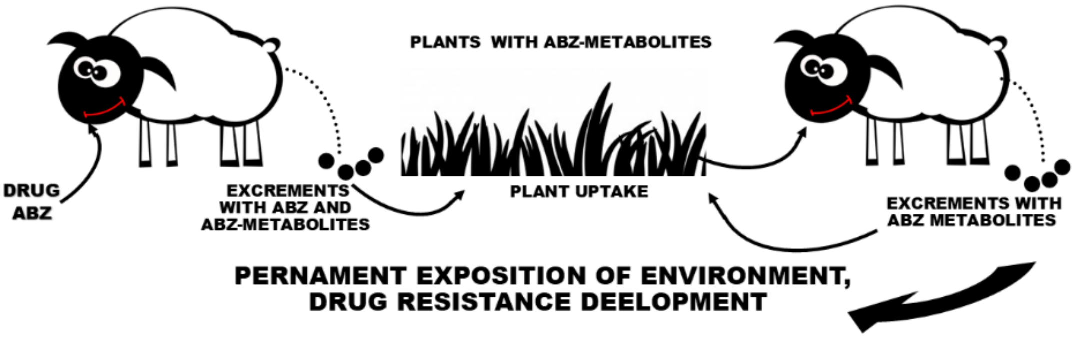


Figure 10 The overview of the field experiment (Navrátilová et al., 2021)

In this research work, albendazole and its TPs, namely albendazole sulfoxide and albendazole sulfone, were determined by UHPLC-MS/MS. We found remarkably high concentrations of the active albendazole sulfoxide in all samples analyzed, including dung, plants, ovine plasma, rumen content, and faeces. This finding provides the first evidence of an undesired circulation of albendazole metabolites from sheep dung into plants used as fodder. As a result, ecosystems and the food chain could be constantly exposed to the active drug metabolite, potentially promoting the development of drug resistance in helminths.

In summary, this field study demonstrates the concerning circulation of albendazole and its transformation products from sheep dung to plants and onward to other sheep. This continuous exposure poses a permanent risk to ecosystems and the food chain, potentially contributing to the emergence of drug-resistant helminths.

6.5 Effect of environmental circulation of albendazole on the parasitic nematode *Haemonchus contortus.*

Publication V: Dimunová, D., Matoušková, P., **Navrátilová, M.,** Nguyen, L. T., Ambrož, M., Vokřál, I., Szotáková, B., & Skálová, L. (2022). Environmental circulation of the anthelmintic drug albendazole affects expression and activity of resistance-related genes in the parasitic nematode *Haemonchus contortus. Sci Total Environ, 822*, 153527 **(IF22: 9.8 Q1 first decile; AIS22: 1.432 Q1 first decile)**

This study provided an extension of knowledge from our last research, which proved the circulation of albendazole and its transformation products from sheep dung to plants used as feed to other sheep. This study was designed primarily to investigate the potential impact of the aforementioned environmental circulation on parasitic nematodes, specifically focusing on *Haemonchus contortus*. *H. contortus* is a common blood-feeding gastrointestinal parasitic worm of small ruminants, which has been shown to possess great ability for drug resistance development.

Two fields were used (both with alfalfa and clover), one fertilized with dung from sheep treated with albendazole at the recommended dosage (p.o.,5 mg.kg^{-1} b.w.) and the other with dung from untreated sheep (control group). After a 10-week growth period, the fresh fodder from both fields was fed to two groups of sheep. The groups of sheep differed in whether they were infected with *H. contortus* before (group I) or after (group II) the start of forage consumption. The overview of the field experiment is depicted in Figure 11.

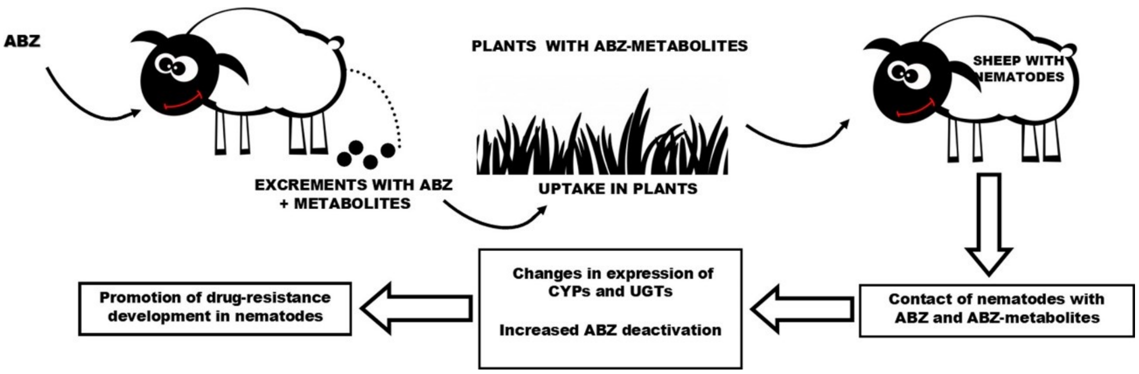


Figure 11 The overview of the field experiment (Dimunová et al., 2022).

Nematode eggs and adults were isolated from both groups, and various parameters were compared to assess the defence system against the effects of albendazole and albendazole sulfoxide. There was no difference in the number of eggs and adult parasites between groups I and II on any diet. Also, no significant variations in sensitivity to albendazole were observed across sheep groups. Another parameter tested was the change in expression of drug-metabolizing enzymes, in particular, isoforms of cytochrome P450, UDP-glycosyltransferases and efflux transporters P-glycoproteins in *H. contortus* adults presented in the sheep abomasum during the feeding.

The exposed nematodes exhibited significant upregulation (cyp-6,cyp-7 and cyp-8) and downregulation (cyp-2) of certain cytochromes P450 across the two groups. Also, significant upregulation of certain UDP-glycosyltransferases (UGT365B1 and UGT368B2) were observed; however, no changes were observed in the expression of P-glycoproteins compared to the control group. These changes in the gene expression profiles of drug-metabolizing enzymes in *H. contortus* after exposition to sub-lethal dosages present in plants may also represent some advantage in the metabolism of the albendazole-related compounds when the recommended dose is used.

We monitored the most abundant metabolites in the metabolism of two substrates, albendazole and albendazole sulfoxide, in *H. contortus* adults. We focused on albendazole sulfoxide, albendazole sulfone, N-glycosides of albendazole, N-glycosides of hydrolyzed albendazole, and N-glycosides of albendazole sulfoxide. Adult worms from both groups (I and II) generally showed greater capability in metabolizing both substrates. While the variances were not extensive (though still statistically significant), it is important to consider that our experiment involved nematodes being exposed to albendazole-transformation products for a limited duration. In contrast, typical grazing animals may repeatedly consume plants containing traces of albendazole albendazole-transformation products over an extended period. Consequently, we could anticipate more significant alterations in expression and function, potentially leading to the nematodes developing increased adaptation to albendazole-related drugs.

7 DISCUSSION

The extensive use of veterinary drugs in the agriculture and aquaculture industry has led to significant environmental pollution (Boxall et al., 2004). The 2022 European Animal medicines industry report indicates the market distribution as follows: vaccines at 32.5%, antiparasitics at 29.2%, antibiotics at 9.5%, and other products at 28.8% of total sales (AnimalhealthEurope, 2023). The majority of these drugs are used extensively in livestock treatment, which subsequently leads to their introduction to the environment in large amounts. For some of the drug classes, e.g., antibiotics or nonsteroidal anti-inflammatory drugs, negative effects on the environment have already been proven, and they are currently recognized as important environmental pollutants (Klampfl, 2019). Despite their necessity in managing parasitic infections in livestock, anthelmintics are often overlooked as environmental pollutants (Beynon, 2012a). Anthelmintics are regularly given to livestock to combat parasitic worm infections. The drug residues, including both the parent drug and its metabolites, are eliminated from the body through faeces or urine and subsequently introduced into the environment. As a result, fields might become contaminated through the application of manure, while grasslands or meadows are directly affected by the excrement of livestock that graze on them. This contamination can potentially adversely affect non-target organisms, including plants.

There is limited understanding of the environmental fate and impact of anthelmintic residues in ecosystems. Recognizing this knowledge gap, we aimed to investigate how these residues, particularly albendazole, influence non-target plants such as clover and alfalfa and if the circulation of these residues in the environment can influence the behaviour of the target parasitic nematode *H. contortus* towards the development of anthelmintic resistance.

As plants are one of the first organisms exposed to the residues of anthelmintics in the environment, the first step in our investigations was to evaluate if and how plants are affected by anthelmintic residues. For the pilot experiments, we employed the common anthelmintic drug albendazole and plant alfalfa (seeds and explants), an important plant for agriculture. To bring the experiments closer to the real field conditions, the concentration range of used albendazole was extrapolated based on the levels observed in sheep faeces 0.01 to 10 µM (Prchal et al., 2016). The results proved that albendazole decreased the amount of net proline, a stress marker, demonstrating its lower synthesis or covering energy-demanding processes (Mattioli et al., 2009), while seed germination was not affected. As our group proved in previous research with common reed (*Phragmites australis*), plants can uptake and metabolize anthelmintics (Podlipna et al., 2013). Also, alfalfa has been shown to uptake and biotransform albendazole, primarily to albendazole sulfoxide and albendazole sulfone, the two metabolites that can also be found in mammals (Publication I).

Experiments with the alfalfa were performed in a strictly controlled laboratory environment where the albendazole was supplied to the plant explants in the cultivation media. As this can be seen as a limitation of the study, we decided to perform an experimental setup closer to the field conditions. Moreover, we employed two other anthelmintics, ivermectin and monepantel, that represent important members of anthelmintic from a group of macrocyclic lactones and aminoacetonitrile derivatives, respectively. This time, clover was used as a target plant, as this is a common species located in pastures or agricultural lands. Clover was grown in soil enriched with sheep dung containing albendazole, ivermectin, or monepantel. This allowed us to observe if drugs can be absorbed by roots and distributed to the rest of the plant. From the soil, only albendazole and monepantel were absorbed by the plant and distributed to the stems and leaves. Ivermectin, on the other hand, was not detected, suggesting its unique physico-chemical properties or limitations in our detection capabilities. The important finding of the study is that by the absorption from the soil to the plant body, the albendazole and monepantel enter back into the food chain, which is a critical concern (Publicsion II).

Based on our laboratory studies, we designed several field experiments in real agricultural conditions using dung from albendazole-treated sheep. The field studies corroborated the laboratory findings, with albendazole and its transformation products detectable in soil and alfalfa and clover at substantial distances of 25 cm from applied sheep dung. This demonstrates albendazole's potential for wide environmental dissemination and persistence (Publication III). Consequently, the cycle of albendazole in the environment, especially from plants fertilized with sheep dung consumed by sheep (Publication IV), presents a tangible risk of perpetuating anthelmintic exposure and resistance in helminths, as our research on *Haemonchus contortus* revealed changes in drug-metabolizing enzyme expression related to albendazole exposure and greater ability to metabolize albendazole and albendazole sulfoxide, main transformation product. (Publication V).

The collective data from these studies demand a reassessment of anthelmintic use in agriculture, underscoring the need for sustainable practices and robust management to mitigate the risks of environmental contamination and resistance development. As we progress towards more sustainable agricultural practices, it is imperative to balance effective parasite control in livestock with environmental conservation and public health considerations.

8 CONCLUSIONS

This dissertation has systematically investigated the environmental impact of the anthelmintic drug albendazole, especially focusing on its effects on non-target plant species alfalfa and clover and parasite nematode *Haemonchus contortus*. Through a series of detailed studies, this research has illuminated the multifaceted nature of albendazole's environmental interactions, ranging from its uptake and biotransformation in plants to its role in the potential development of drug resistance in parasitic nematodes. Key findings from this research include:

Phytotoxicity and biotransformation in plants:

The studies demonstrated that while albendazole did not inhibit seed germination in alfalfa, it exhibited toxicity in leaf explants, highlighting the risk to plant health during later developmental stages. Both alfalfa and clover were capable of uptaking and biotransformating albendazole and its transformation products, raising concerns about the bioaccumulation of these compounds in the food chain.

Environmental persistence:

The persistence of albendazole and its transformation products in soil and plants, observed in field experiments, points to significant environmental contamination. The distribution of these compounds, detectable at considerable distances from the faeces pat, underscores their potential long-term ecological impacts.

The cycle of albendazole in farm ecosystems:

The research revealed a continuous cycle of albendazole within farm ecosystems, moving from sheep dung to plants and back to sheep. This cycle suggests ongoing exposure to albendazole and its transformation products, raising concerns about the development of drug resistance in helminths.

Impact on parasitic nematodes:

The study focusing on *Haemonchus contortus* demonstrated that sub-lethal doses of albendazole in the environment could lead to adaptations in parasites, potentially reducing the drug's effectiveness over time.

9 DISSEMINATION OF RESEARCH FINDINGS

9.1 Oral presentations

Navrátilová, M., Dimunová, D.,Vokřál, I., Matoušková, P., Skálová, L., (Sept 2021). Environmental circulation of albendazole and its *in vivo* effect of sublethal doses on *Heamonchus contortus. 26th Helminthological days,* Deštné in Orlické hory, Czechia

Navrátilová, M., Raisová Stuchlíková, L., Skálová, L., (Jan 2020). Circulation of anthelmintics in the environment.10th *Postgraduate and 8th Postdoc Conference*, Hradec Králové, Czechia

Navrátilová, M., Raisová Stuchlíková, L., Matoušková, P., (May 2019). Monitoring of albendazole anthelmintic drug in the environment. *25th Helminthological days,* Rejčkov, Czechia

9.2 Poster presentations

Navrátilová M., Sochová A., Vrábľová D., Vokřál, I., Matoušková, P., Skálová L., (Aug 2022). Monitoring of albendazole transfer from sheep dung to soil and fodder plants. 24th *International Mass Spectrometry Conference 2022.* Maastricht, The Netherlands

- Nico Nibering Student Travel Awards

Navrátilová M., Sochová A., Vrábľová D., Vokřál, I., Matoušková, P., Skálová L., (Apr 2022). Is there a possibility of albendazole residues circulation in ovine pastures? *5th International Mass Spectrometry School 2022.* Belfast, UK

- Nico Nibering Student Travel Awards

Navrátilová M., Sochová A., Vrábľová D., Skálová L., (Oct 2021). Monitoring of albendazole transfer from sheep dung to fodders plants and adjacent soil by UHPLC-MS. *22nd International Science Conference "EcoBalt 2021".* Riga, Latvia- online

Navrátilová M., Sochová A., Vrábľová D., Skálová L., (Sept 2021). Monitoring of albendazole transfer from ovine faeces to fodders plants and connected soil by UHPLC-MS/MS. *8th Czech chromatographic school,* Zaječí, Czechia

Navrátilová, M., Raisová Stuchlíková, L., Skálová, L., (Feb 2020). Circulation of anthelmintic drug albendazole in the environment. *Anthelmintics IV: From Discovery to Resistance*, Santa Monica Bay, California, USA

10 PUBLICATIONS UNRELATED TO THE TOPIC OF THE DISSERTATION THESIS

Most of the publications are related to the topic of *H. contortus*, e.g., biotransformation of anthelmintics.

1. Dimunová, D., **Navrátilová, M.,** Kellerová, P., Ambrož, M., Skálová, L., & Matoušková, P. (2022). The induction and inhibition of UDP-glycosyltransferases in *Haemonchus contortus* and their role in the metabolism of albendazole. *International journal for parasitology. Drugs and drug resistance*, *19*, 56–64. **(IF22: 4.0 Q1Q2; AIS22: 0.835 Q1Q2)**

2. Kellerová, P., **Navrátilová, M.,** Nguyen, L. T., Dimunová, D., Raisová Stuchlíková, L., Štěrbová, K., Skálová, L., & Matoušková, P. (2020). UDP-Glycosyltransferases and Albendazole Metabolism in the Juvenile Stages of *Haemonchus contortus*. *Frontiers in physiology*, *11*, 594116. **(IF20: 4.566 Q1 AIS20: 1.135 Q1)**

3. Kellerová, P., Raisová Stuchlíková, L., Matoušková, P., Štěrbová, K., Lamka, J., **Navrátilová, M.,** Vokřál, I., Szotáková, B., & Skálová, L. (2020). Sub-lethal doses of albendazole induce drug metabolizing enzymes and increase albendazole deactivation in *Haemonchus contortus* adults. *Veterinary research*, *51*(1), 94. **(IF20: 3.699 Q1; AIS20: 0.896 Q1)**

4. **Navrátilová, M.,** Raisová Stuchlíková, L., Moťková, K., Szotáková, B., Skálová, L., Langhansová, L., & Podlipná, R. (2020). The Uptake of Ivermectin and Its Effects in Roots, Leaves and Seeds of Soybean (*Glycine max*). *Molecules (Basel, Switzerland)*, *25*(16), 3655. **(IF20: 4.412 Q2Q2; AIS20: 0.694 Q3Q2)**
5. **Navrátilová, M.,** Raisová Stuchlíková, L., Skálová, L., Szotáková, B., Langhansová, L., & Podlipná, R. (2020). Pharmaceuticals in environment: the effect of ivermectin on ribwort plantain (Plantago lanceolata L.). *Environmental science and pollution research international*, *27*(25), 31202–31210. **(IF20: 4.223 Q2; AIS20: 0.603 Q3)**

6. Podlipná, R., **Navrátilová, M.,** Raisová Stuchlíková, L., Moťková, K., Langhansová, L., Skálová, L., & Szotáková, B. (2021). Soybean (*Glycine max*) Is Able to Absorb, Metabolize and Accumulate Fenbendazole in All Organs Including Beans. *International journal of molecular sciences*, *22*(13), 6647. **(IF21: 6.208 Q1Q2; AIS21: 1.064 Q2Q2)**

7. Stuchlíková, L. R., Jakubec, P., Langhansová, L., Podlipná, R., **Navrátilová, M.,** Szotáková, B., & Skálová, L. (2019). The uptake, effects and biotransformation of monepantel in meadow plants used as a livestock feed. *Chemosphere*, *237*, 124434. **(IF19: 5•778 Q1; AIS19: 0•913 Q1)**

8. Syslová, E., Landa, P., **Navrátilová, M.,** Stuchlíková, L. R., Matoušková, P., Skálová, L., Szotáková, B., Vaněk, T., & Podlipná, R. (2019). Ivermectin biotransformation and impact on transcriptome in Arabidopsis thaliana. *Chemosphere*, *234*, 528–535. **(IF19: 5•778 Q1; AIS19: 0•913 Q1)**

9. Syslová, E., Landa, P., Stuchlíková, L. R., Matoušková, P., Skálová, L., Szotáková, B., **Navrátilová, M.,** Vaněk, T., & Podlipná, R. (2019). Metabolism of the anthelmintic drug fenbendazole in Arabidopsis thaliana and its effect on transcriptome and proteome. *Chemosphere*, *218*, 662–669. **(IF19: 5•778 Q1; AIS19: 0•913 Q1)**

10. Zajíčková, M., Prchal, L., **Navrátilová, M.,** Vodvárková, N., Matoušková, P., Vokřál, I., Nguyen, L. T., & Skálová, L. (2021). Sertraline as a new potential anthelmintic against Haemonchus contortus: toxicity, efficacy, and biotransformation. *Veterinary research*, *52*(1), 143. **(IF21: 3.829 Q1; AIS21: 0.767 Q1)**

11.

11 ADDRESSED GRANTS

11.1 Principal investigator

2021–2023 START/MED/052 Anthelmintics in the environment – detection, consequences, a possible solution
2020-2023 Grant Agency of Charles University No. 1136120 Monitoring of anthelmintic drugs in the environment

11.2 Member of the research team

2023-2023 Technology Agency of the Czech Republic No. SS06020173 Methods reducing the risks of circulation of veterinary drugs in the environment
2019-2022 Grant Agency of Charles University No. 1568519 "Metabolic pathways of new potential anthelmintics in *Haemonchus contortus* and its host"
2018-2020 Czech Science Foundation No. 18-08452S Anthelmintics in plants – interactions with polyphenols biosynthesis and antioxidant defence

12 INTERNATIONAL SCIENTIFIC EXPERIENCE

11/2022 – 5/2023- 6-month internship at the Faculty of Sciences, the Department of Plant and Environmental Science, University of Copenhagen, Denmark, at Analytical Chemistry research group.

Supervisor: Prof. Jan H. Christensen

Aim of the internship:

Non-target determination of volatile organic compounds associated with the wheat phyllosphere microbiome by using GC-MS

13 TEACHING EXPERIENCE

Consultant of undergraduate students:

- Brodská, D., (2023). Optimalizace extrakce fenbendazolu z enviromentálních vzorků. Bachelor thesis
- Salvová, M., (2023). Změny v metabolismu flubendazolu během životního cyklu hlístice. Diploma thesis
- Kohoutová, E., (2023). Inhibitory enzymů redukujících anthelmintikum flubendazol u vlasovky slezové (Haemonchus contortus). Diploma thesis
- Sochová, A., (2021). Sledování přestupu albendazolu z trusu ovce domácí do pícnin pomocí LC-MS. Diploma thesis
- Vrábľová, D., (2021). Monitorovanie rozšírenia albendazolu z trusu ovce domácej v poľnohospodárskej pôde s pomocou LC-MS. Diploma thesis

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Ambrožová, L., Sládeček, F.X.J., Zítek, T., Perlík, M., Kozel, P., Jirků, M., Čížek, L., 2021. Lasting decrease in functionality and richness: Effects of ivermectin use on dung beetle communities. Agriculture, Ecosystems & Environment 321, 107634.

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15 APPENDICES

Copies of articles related to the topic of this dissertation thesis

1. Raisová Stuchlíková, L., **Navrátilová,** M., Langhansová, L., Moťková, K., Podlipná, R., Szotáková, B., & Skálová, L. (2020). The Identification of Metabolites and Effects of Albendazole in Alfalfa (Medicago sativa). International journal of molecular sciences, 21(16), 5943. (IF20: 5.924 Q1Q2; AIS20: 1.123 Q2Q2)
2. Langhansová, L., **Navrátilová, M.,** Skálová, L., Moťková, K., & Podlipná, R. (2021). The Effect of the Manure from Sheep Treated with Anthelmintics on Clover (Trifolium pratense). *Agronomy*, 11(9), 1892. **(IF21: 3.949 Q1Q1; AIS21: 0.503 Q2Q2)**
3. **Navrátilová, M.,** Vokřál, I., Krátký, J., Matoušková, P., Sochová, A., Vrábľová, D., Szotáková, B., & Skálová, L. (2023). Albendazole from ovine excrements in soil and plants under real agricultural conditions: Distribution, persistence, and effects. *Chemosphere*, 324, 138343. **(IF22: 8.8 Q1 AIS22: 1.138 Q2)**
4. **Navrátilová, M.,** Raisová Stuchlíková, L., Matoušková, P., Ambrož, M., Lamka, J., Vokřál, I., Szotáková, B., & Skálová, L. (2021). Proof of the environmental circulation of veterinary drug albendazole in real farm conditions. *Environmental pollution (Barking, Essex: 1987)*, *286*, 117590. **(IF21: 9.988 Q1; AIS21: 1.398 Q1)**
5. Dimunová, D., Matoušková, P., **Navrátilová, M.,** Nguyen, L. T., Ambrož, M., Vokřál, I., Szotáková, B., & Skálová, L. (2022). Environmental circulation of the anthelmintic drug albendazole affects expression and activity of resistance-related genes in the parasitic nematode *Haemonchus contortus. Sci Total Environ, 822*, 153527 **(IF22: 9.8 Q1 first decile; AIS22: 1.432 Q1 first decile)**