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Anthelminthic Veterinary Medicines Interactions with the Soil Microbiota

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Abstract: Anthelminthics (AHs) are used to control gastrointestinal nematodes (GINs) in productive animals. They are rapidly excreted by animals, ending up in soil through direct deposition of animal dung or application of animal excreta as manures. Most environmental research on AHs has focused on their toxicity to aquatic organisms and soil fauna while their interactions with the soil microbiota, a key component of a functioning soil ecosystem, have been overlooked. In this article, we summarize current knowledge on the interactions of AHs with the soil (micro) biota, we highlight recent evidence for the toxicity of AHs on soil microorganisms and discuss those results in the frame of the current environmental risk assessment (ERA) of veterinary medicines.

Keywords: Anthelminthics · Ecotoxicity · Environmental risk assessment · Soil microbiota

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1. Introduction

Anthelminthics (AHs) are veterinary medicines used in livestock farming for the control of ecto- and endoparasites and mainly of gastrointestinal nematodes (GINs) that infest animals during grazing. GINs constitute a major threat for pasture grazing ruminants like cattle and small ruminants (sheep, goats) worldwide.[1] Helminthiases have an important economic impact on farmers' revenue.[2] Therefore, a systematic control of GIN infections is crucial for the productivity of livestock farms. For more than 50 years, this is achieved with the use of synthetic AHs.

AHs currently on the market can be classified in different chemical groups (Table 1). Benzimidazoles constitute the first and oldest class of synthetic AHs. Their activity is based on the multifunctional and reactive chemical skeleton of the benzimidazole ring which provides a multitude of biological activities.[3] They all act by binding to the β-tubulin leading to suppression of its polymerization and disruption of cell mitosis.[4] The first benzimidazole in the market was thiabendazole (TBZ), currently used mostly as a postharvest fungicide. This group was gradually populated with more compounds like albendazole (ABZ) and its oxidation derivative ricobendazole (RBZ), fenbendazole (FBZ) and its oxidation derivative oxfendazole (OXF), flubendazole (FLU), and mebendazole (MBZ).

Macrocyclic lactones are the other very important group of AHs. They can be further divided into avermectins and milbemycins. Ivermectin (IVM), eprinomectin (EPM) and doramectin (DOM) are the most important avermectins, and moxidectin (MOX) is the most important member of milbemycines.[5] Both avermectins and milbemycines were discovered as secondary metabolites of different soil strains of the genus *Streptomyces*.[6] Macrocyclic lactones act as allosteric antagonists for ligand-gated chloride channels, particularly those controlled by the neurotransmitters γ-aminobutyric acid (GABA) and glycine.[7] The chemical structure of all macrocyclic lactones is based on a 16-membered macrocyclic lactone ring which consists of four major (A1a, A2a, B1a, B2a) and four homologous minor components (A1b, A2b, B1b, B2b). All semi-synthetic avermectins are the result of mixtures of these homologous components and chemical modifications.[8]

Other chemical classes of AHs include (a) imidazothiazoles. (b) tetrahydropyrimidines, (c) pyrazinoisoquinolines, (d) salicylanilides (e) amino-acetonitrile derivatives (AADs), (f) spiroindoles and (g) cyclooctadepsipeptides. Levamisole (LVM) is the first and only member of the imidazolthiazoles, while tetrahydropyrimidines comprise pyrantel, morantel and oxantel. Members of these groups act as nicotinic acetylcholine receptor agonists.[9] Pyrazinoisoquinolines constitute an important group of AHs with its main member in the market being praziquantel (PZL) acting on the calcium ion channels.[10] Closantel, rafoxanide and oxyclozanide are the main members of the class of salicylanilides acting by decoupling oxidative phosphorylation.[4] The AADs are a new class of AHs with activity against GINs that are resistant to benzimidazoles and macrocyclic lactones.[11] Monepantel (MOP) is the first member of this class. Derquantel (DER) is the first commercial member of the spiroindoles which are used in combination with macrocyclic lactones and act as antagonist of the nicotinic acetylcholine receptor.[12] Emodepside (EMO) is the

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only member of the cyclic octadepsipeptides class which acts on the calcium-activated potassium channel (SLO-1).[13]

AHs are administered to animals in various ways (oral, subcutaneous, topical, *etc.*) and their mode of application, their physicochemical properties and the type of animal treated are key determinants of their level of excretion but also on their mode of excretion either in feces or in urine.[14] AHs are released in animal excreta (mostly in feces) at levels ranging from 50–90%, with macrocyclic lactones being on the higher part of this range.[15] Excretion dynamics of AHs vary but most of the administered amount is excreted during the first 4–10 d.[16] AHs are excreted either intact or in the form of their metabolites produced mainly through the action of flavin monooxygenases and cytochrome P450 oxidases leading to oxidative derivatives that often carry anthelminthic activity as well.[17] It should be noted that AHs derivatives are present in animal excreta along with the parent compounds and are characterized by higher polarity that often makes them more mobile in the environment.[18]

AHs can be released in soil *via* different routes depending on the livestock farming system employed: (a) directly *via* grazing of pasture-reared animals (*e.g.* small ruminants) or (b) indirectly through the application in soils of manures derived from farms of intensively reared animals (Fig. 1). The first route is important for the contamination of grasslands while the second route is the main route of entrance of AHs in agricultural soils.

Most of the synthetic AHs currently available were introduced in the market before 2000. This might have led us to believe that several studies would be available regarding their environmental fate. However, there is surprisingly little knowledge on the environmental fate of AHs compared to other organic pollutants like pesticides or antibiotics. Most of the available studies have focused on benzimidazoles like ABZ, FEN and FLU,[19,20] and macrocyclic lactones like IVM, EPM[21,22] while much less is known about the other classes.

In soil AHs could interact with the soil fauna and soil microbiota with the outcome of this interaction being either detrimental, neutral or beneficial. The vast majority of studies have looked at the effects of AHs on dung arthropods and soil macroorganisms (*e.g.* earthworms, collembola, nematodes) while little attention has been given to their interactions with the soil microbiota. Here, we (a) summarize current knowledge regarding the presence of AHs in the environment; (b) further focus on the interactions of AHs with the soil microbiota, both their microbial degradation but also their toxicity to the soil microbiota; (c) we provide an overview of the current environmental risk assessment (ERA) for AHs, highlight its limitations and needs for improvement with particular focus on the assessment of their microbial toxicity and (d) we discuss future perspectives on AH environmental research.

2. AHs in the Environment: Distribution and Environmental Fate

Monitoring studies have identified AHs as common contaminants of natural water bodies. AHs end up in surface water systems through discharges from wastewater treatment plants which fail to remove AHs effectively.[23] Alternatively AHs could be released in surface water systems through runoff from agricultural fields or grasslands amended with animal feces.[19,24] Monitoring studies have detected residues of AHs in surface waters (up to 200 ng L^{-1}), in marine waters (up to 42 ng L^{-1}) and in sediments (up to 700 ng L^{-1}).[25,26] Recent monitoring studies in China showed that benzimidazoles were the most commonly detected AHs, followed by macrocyclic lactones, tetrahydropyrimidines and diphenylsulfides.[27] Benzimidazole residues reached a maximum of 61 ng L^{-1} and were mostly accounted to ALB and RBZ. The distribution pattern of AHs in the riverine water suggested a higher contribution of AHs from non-agricultural areas through the wastewater treatment systems rather than by agricultural activities. AHs are

Table 1. A list of the most used AHs grouped by chemical class. The IUPAC chemical names are given in Table 1 of the Supplementary Information.

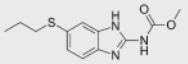
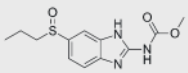
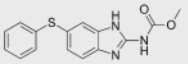
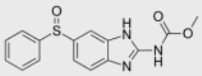
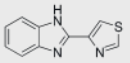
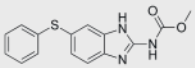
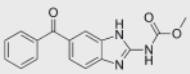
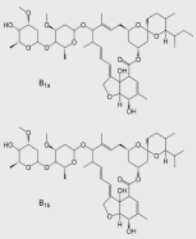
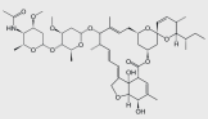
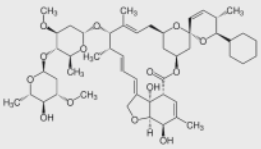
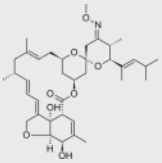
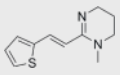
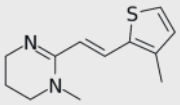
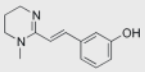
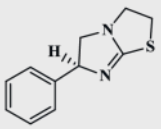
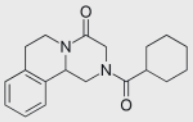
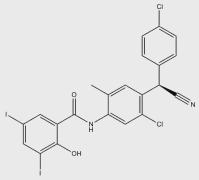
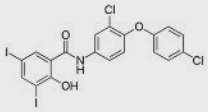
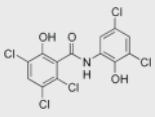
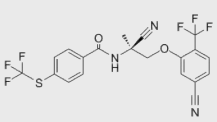
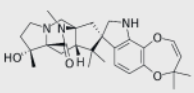
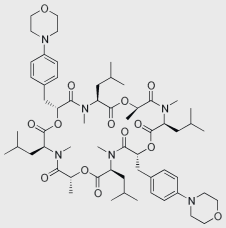
	Compound name	Chemical structure	Mode of action
Benzimidazoles	albendazole		Bind to the β-tubulin leading to suppression of its polymerization and disruption of cell mitosis
	ricobendazole		
	fenbendazole		
	oxfendazole		
	thiabendazole		
	flubendazole		
	mebendazole		
Macrocyclic Lactones	ivermectin		Allosteric antagonists for ligand-gated chloride channels, particularly those controlled by the neurotransmitters γ-aminobutyric acid (GABA) and glycine
	eprinomectin		
	doramectin		
	moxidectin		

Table 1. Continued

Tetrahydropyrimidines	pyrantel		Agonists of nicotinic acetylcholine receptor (nAChR)
	morantel		
	oxantel		
Imidazothiazoles	levamizole		Nicotinic acetylcholine receptor (nAChR) agonist
Pyrazinoisoquinolines	praziquantel		Acts on the calcium ion channels
salicylanilides	closantel		Decoupling oxidative phosphorylation amd inhibiting the production of ATP
	rafoxanide		
	oxyclozanide		
amino-acetonitrile derivatives (AADs)	monepantel		Agonist of nematode specific MPTL-1 & ACR-20 receptors
spiroindoles	derquantel		Antagonist of the nicotinic acetylcholine receptor
cyclooctadepsipeptides	emodepside		Acts on calcium-activated potassium channel (SLO-1)

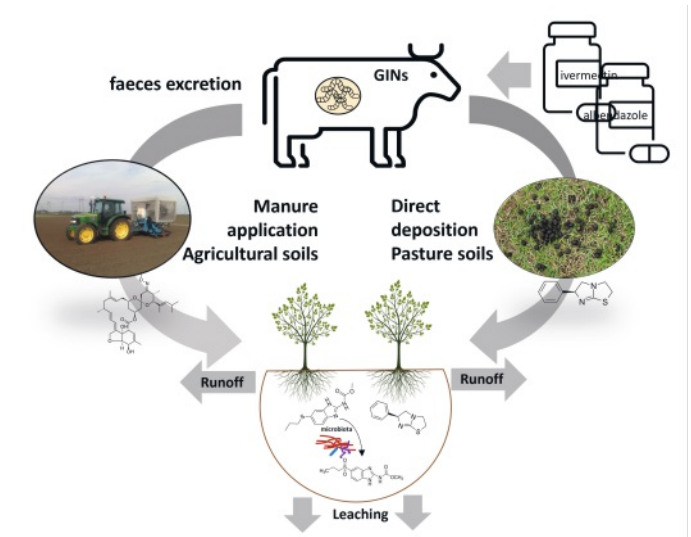


Fig. 1. The route of anthelminthics from livestock farms to soil and other environmental compartments.

also present in groundwater systems. A recent study of Mooney *et al.*,[28] in groundwater systems in Ireland showed that the levels of AHs ranged from 1 to 41 ng L^{-1}. Benzimidazoles were again the most frequently detected group with ABZ and FBZ and their oxidation transformation products being the most frequently detected products. The temporal patterns of detection were associated with land-spreading of manure and grazing of sheep/cattle.

Although the magnitude of the residual amounts of AHs in feces have been extensively monitored,[29] only a few studies have followed the distribution of AH compounds from animal dung pats to soil and from there to other environmental compartments. Navratilova *et al.*,[30,31] showed that the deposition of animal dung from ABZ-treated animals in grasslands leads to a slow diffusion of ABZ and mostly of its sulfonated derivatives to soil. These are further taken up by fodder plants which upon grazing by small ruminants led to the recirculation of low levels of AHs favoring the development of resistance but also raising concerns about the introduction of residues of AHs in the food chain. Similar results were observed for IVM from cattle dung.[32]

3. Interactions of Anthelminthics with Soil Biota

Most studies on AHs in soil have focused on its effect on soil fauna while little is known about the interaction of AHs with the soil microbiota. Guided by the similar interactions of other biocides like pesticides and antibiotics with the soil microbiota there are two potential outcomes: (i) the soil microbiota could co-metabolize or become acclimated to rapidly degrade AHs or (ii) the soil microbiota is poisoned by the AHs.

3.1 *Anthelminthics Soil Microbial Degradation*

Degradation is the main dissipation process of AHs in soil. Although we have a rather clear picture of the persistence of the different AHs in soil, the role of microorganisms in the dissipation process has been assumed but strong data to support this are scarce. Mougin *et al*.,[33] showed that ivermectin dissipation was slower in sterilized compared to non-sterilized soils. The first direct evidence for the involvement of the soil microbiota in the degradation of AHs were provided by Lagos *et al*.,[23] who noted a substantial retardation in the dissipation of ABZ, IVM and EPM in fumigated compared to non-fumigated soils. In a following study the same authors showed that under laboratory conditions repeated applications of ABZ in a soil originated from a livestock farm resulted in an accelerated degradation of ABZ.[34] The authors suggested that this was first evidence for the acclimation of the soil microbiota to degrade AHs and use them as an energy source.

Further evidence for the involvement of soil microorganisms in the degradation of AHs were given through the isolation of soil microorganisms able to actively transform AHs. Ali *et al*.,[35] first reported the isolation of a *Burkholderia cepacia* strain able to rapidly degrade abamectin, followed by Wang *et al*.,[36] who isolated a *Stenotrophomonas maltophilia* abamectin-degrading strain. The same authors later isolated an IVM-degrading *Aeromonas taiwanensis* strain.[37] Regarding benzimidazoles, Perruchon *et al*.,[38] reported the isolation of a bacterial consortium that was able to rapidly degrade TBZ. Further studies revealed that the active degrading member of the consortium, a *Sphingomonas* strain, depended on a *Hydrogenophaga* strain for supplementation of B12.[39] Using this consortium Lagos *et al*., showed that bioaugmentation of feces could be an effective mitigation measure to remove ABZ, FBZ and TBZ from feces and reduce their potential dispersal in soil.[40] Recently Lagos *et al*., isolated two *Acinetobacter* rapidly degrading ABZ.[41]

3.2 *AHs Toxicity on the Soil (Micro)biota*

Recent methodological progress in soil microbiology has advanced our understanding of the role of soil microorganisms in ecosystem functioning. Soil microorganisms contribute to a series of major ecosystemic functions and services including soil fertility, plant productivity, attenuation of pollution, carbon storage and soil structuring, production and consumption of greenhouse gases.[42] In light of these advances, EU regulatory bodies like EFSA are revising their procedures for assessing the potential risk of pesticides for soil microorganisms and exploring new testing procedures.[43] Until now they have relied on the OECD 216 N transformation test, an outdated and crude test, to assess the toxicity of pesticides on the soil microbiota. The same test is currently required at Phase II of the risk assessment of veterinary medicines to assess their potential toxicity to soil microorganisms.[44]

What do we know about the effects of AHs on the soil microbiota? The answer is, very little. A quick literature search, without further refinement of the content of the hits, using the terms 'anthelminthic' and 'toxicity' and 'soil microorganisms' identifies just seven articles, while a similar search for 'pesticides' and 'veterinary antibiotics' returned 5750 and 261 hits respectively.

The overwhelming majority of articles regarding the ecotoxicity of AHs focus on aquatic organisms, soil macro fauna and dung arthropods. Generally, AHs are particularly toxic to aquatic organisms like planktonic crustaceans (*Daphnia magna*), fish species (*Danio rerio*) copepods and cladocerans.[15] AHs could also pose a threat to earthworms.[45] AHs are particularly toxic to dung-dwelling flies and beetles.[16] In addition several studies have suggested adverse effects of AHs on plants.[46]

Based on their biochemical mode of action, AHs are not expected to have undesirable effects on the soil microbiota. However, evidence to the contrary has begun to appear in the literature. AVM showed no unacceptable risk for the soil microbiota as determined by the OECD 216 N transformation test.[47] More recent studies using shotgun metagenomic analysis revealed that AVM at the concentration of 1 mg/kg increased the abundance of antibiotic resistance genes but overall had a temporal effect on the microbial diversity and metabolic functioning.[48] Hentz *et al*.,[49] showed that MOX released in soil from animal dung imposed strong inhibitory effects on soil microbial activity, microbial biomass carbon and N transformation even at concentrations of 1.9 ng/kg. Several tests have been performed to test the potential toxicity of AHs to nematophagous fungi, in an effort to put forward integrated strategies for the control helminthiases. FEN, triclabendazole and IVM showed *in vitro* EC_{50} values in the range of 7 to 47.2 μg ml^{-1} for a range of nematophagous fungi, whereas LVM was not toxic with EC_{50} values > 546.5 μg ml^{-1}.[50]

Further tests have focused on the toxicity of AHs on key soil functional microbial groups like arbuscular mycorrhizal fungi (AMF) and ammonia-oxidizing microorganisms (AOM). These groups were identified as potential indicators of the toxicity of pesticides on soil microorganisms.[51] Gibrixi *et al*.,[52] showed in a gnotobiotic system composed of the legume *Lotus japonicus* and the AMF *Rhizophagus irregularis,* that the application of ABZ, but not of IVM, inhibited the development and functionality of arbuscules, the symbiotic organelle of AMF, at a concentration of 0.75 μg g^{-1}. Regarding AOM, earlier studies by Konopka *et al*.,[53] showed that field applications of IVM at concentrations of 1 and 10 mg kg^{-1} did not have an effect on nitrification rates and the abundance of AOA and AOB, although when mixed with zinc bacitracin and monensin inhibitory effects on the abundance of AOB were evident. A more recent study looked at the impact of laboratory-scale repeated applications of ABZ, IVM and EPM on the soil microbiota.[54] The authors noted a consistent and long-lasting inhibitory effect by all AHs, but primarily by ABZ, on nitrification and the abundance of ammonia-oxidizing bacteria and ammonia-oxidizing archaea while comammox (complete ammonia oxidation) bacteria were less responsive. In addition, the authors observed dose-dependent shifts in the composition of the community of bacteria, fungi and protists. The inhibitory effects of AHs at concentration levels which are encountered in agricultural soils regularly receiving manures on key functional microbial groups involved in C, N and P cycling is alarming and should be considered in ERA of AHs, although further tests are needed to establish the mechanisms of the toxicity observed.

4. Environmental Risk Assessment (ERA) of Anthelminthics: Soil Microbiota as a Key Protection Goal

We will focus on the ERA of AHs in Europe, while differences to other jurisdictions could apply. The ERA of AHs in Europe was until recently based on Directive 2001/82/EEC. This was later formalized and harmonized with the publication of two guidelines on how to perform ERA in Phase I and Phase II, VICH GL6[55]) and GL38,[44] respectively. Further supporting documents were issued by the EMA to provide tools and procedures for exposure assessment.[56] Based on Directive 2001/82/ECC it was possible to ask for re-evaluation (referral procedure) of the risk associated with the use of specific products if new data raise concerns for human health and the environment. To date five AH products containing IVM, DOR, EPM and MOX went through a referral procedure and they were granted authorization based on a positive benefit/risk balance.[57]

A new Regulation 2019/6, which came into force in January 2022, repeals Directive 2001/82/EEC, and regulates veterinary medicine authorization in EU. The general tiered ERA is maintained, however there are certain provisions that would reduce the environmental risk associated with veterinary medicines like AHs: (a) all products given authorization before the implementation of VICH GL38 should go through a full ERA, (b) a substance-

based ERA, instead of product-based currently in place would be explored. This will facilitate consistency with other regulatory frameworks, increase consistency of ERA and reduce administrative burden (c) products that fulfil the criteria of PBT (Persistent, Bioaccumulative, Toxic) or very PBT would not be granted authorization.

A two-phase ERA is currently applied to all veterinary medicines, including AHs. Phase I is composed of a list of 19 questions in the form of a decision tree. It assumes that if environmental exposure is below a certain threshold (<100 μg kg^{-1} for soil) no appreciable environmental risk is expected. The predicted environmental concentrations (PECs) of compounds are calculated based on the assumption that the whole amount of AHs is excreted in feces and urine, while no degradation occurs in excreta or soil. Certain groups of veterinary medicines, like AHs, regardless of the outcome of Phase I, should undergo a Phase II ERA. This involves three tiers which starts with Tier IIA (acute effects). If a risk is identified a refined ERA is performed at Tier IIB (chronic effects) and eventually at Tier IIC (field monitoring). ERA in Phase II is based on the comparison of PECs with Predicted Non Effect Concentrations (PNEC) derived from standardized toxicity tests with surrogate aquatic (*e.g*. algae, fish and *D. magna*) and terrestrial organisms (*e.g*. terrestrial plants, earthworms and dung flies/beetles). Certain refinements of PEC_{soil} could be implemented at this stage considering AHs degradation in feces and in soil. If the ratio of PEC/PNEC, termed Risk Quotient, is >1 then Tier IIB is triggered involving further ecotoxicity testing for chronic effects but also environmental fate studies. ERA exercises for IVM and ABZ showed that both compounds pose unacceptable risk (RQ >1) for aquatic organisms and certain mitigation measures should be taken to address the risk.[58,59]

Within the toxicity endpoints of Tier IIA the outcome of the OECD 216 N transformation test is required to show no unacceptable toxicity to soil microorganisms. As mentioned before this test is also the sole requirement for assessing the toxicity of pesticides on the soil microbiota. However there are growing concerns and criticism about the use of such an outdated test in ERA of pesticides[60] and veterinary drugs in view of the recent methodological advances in soil microbiology and the long list of high resolution standardized methods that could be used, instead of the OECD 216 test, to assess the toxicity of pollutants on the soil microbiota.[53] Considering recent evidence for the adverse effects of AHs on key functional microbial groups like AMF and AOM, we advocate for a revision of the ERA of AHs towards a more microbial-centric approach.

5. Conclusions and Future Research Priorities

The widespread occurrence of AHs in nature and their undesirable effects on aquatic organisms and soil fauna are facts. However, recent evidence suggest that AHs interact with the soil microbiota with the outcome of this interaction being often detrimental for soil microorganisms threatening ecosystem functioning. The current ERA for veterinary medicines, in accord with other regulatory frameworks (*e.g*. pesticides), overlooks the potential effects of AHs on soil microorganisms. In light of the One Health concept, we advocate for a multidisciplinary effort to discern the level and the extent of toxicity of AHs on the soil microbiota. Research priorities in this area are proposed:

- Monitoring at national or EU scale (*e.g* LUCAS database,[61]) would determine the exact exposure levels of soils to AHs.
- Implementation of well-designed soil studies to assess the effects of AHs on the soil microbiota at a range of concentrations (including always environmentally relevant concentrations or PEC_{soil}) combined with the use of functional and diversity endpoints, advanced molecular tools and proper bioindicators (AMF, AOM).
- Considering the effects of AHs on soil organisms from different trophic levels, we anticipate that beyond direct toxic effects on individuals further indirect effects across the soil food web are expected. Studies using holistic approaches that could disentangle the origin of the effects seen on the soil microbiota are very much required.
- Identify toxicity mechanisms of certain AHs using standardized and sensitive to abiotic stressors *in vitro* microbial systems (*e.g*. AOM) and advanced omic tools (proteomics and metabolomics).
- Holistic One Health-based exposure studies should define the route of AHs from veterinary farms to environment and from there to their trophic chain (*e.g*. plants). This will allow us to assess the potential risk for grazing animals and consumers.
- AHs in agricultural soils co-occur with other pollutants (metals, pesticides, microplastics, antibiotics). The effect of AHs' interaction with those pollutants on their fate and toxicity are unknown and should be explored assuming that increasing the number of stressors reduces soil ecosystem services.[62]

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Supplementary Information

The full list of the most used AHs grouped by chemical class, including the IUPAC chemical name, can be accessed at https://www.chimia.ch/chimia/article/view/2023_777.

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