Environmental Impact of Anthelmintics in Livestock and Alternatives to Minimize their Use

Guidelines for Environmental Sampling to Analyse Anthelmintic Residues in Farm

a living document

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Introduction

There are multiple ways to contribute samples to ENVIRANT, which are detailed below. However, participation does not have to involve all sample types (faeces, manure/bedding/slurry, soil, water) and the most intensive temporally-resolved approach, but can consist of samples potentially containing anthelmintics and controls taken at a single sampling event.

Therefore, there are different decision points to chose your approach. Please communicate with the experts that will later on handle the samples for anthelmintic and biodiversity analyses (Working Group 1 and 2 leaders, national contact points).

How to chose your approach:

Decision 1: determine the goal of your study

- **Approach 1: Spatial overview (Single/limited-Time Points)**
  - Goal: Map the current distribution of anthelmintics across a wider geographical landscape, on a pan-European scale, not at farm level.
  - Timing: A single sampling event (or very few, depending on the type of sample) conducted after anthelmintic administration.
  - Key Requirement: Include proper controls (i.e., samples from close-by non-treated areas) to provide a baseline for comparison.
- **Approach 2: Temporal (Time series after Anthelminthic Treatment)**
  - Goal: Track the occurrence, degradation, and fate of anthelmintics over a specific period in different environmental samples.
  - Timing: Multiple sampling events, specifically:
    1. Once before anthelmintic administration (Pre-sampling/Baseline).
    2. Several times after anthelmintic administration.

Decision 2: select samples (Applicable to Both Approaches)

Once your approach is chosen, select the types of samples needed and that can be obtained in your case. The chart prioritizes four key matrices:

- Faeces (Source)
- Bedding material, Manure and/or Slurry (Waste Management)

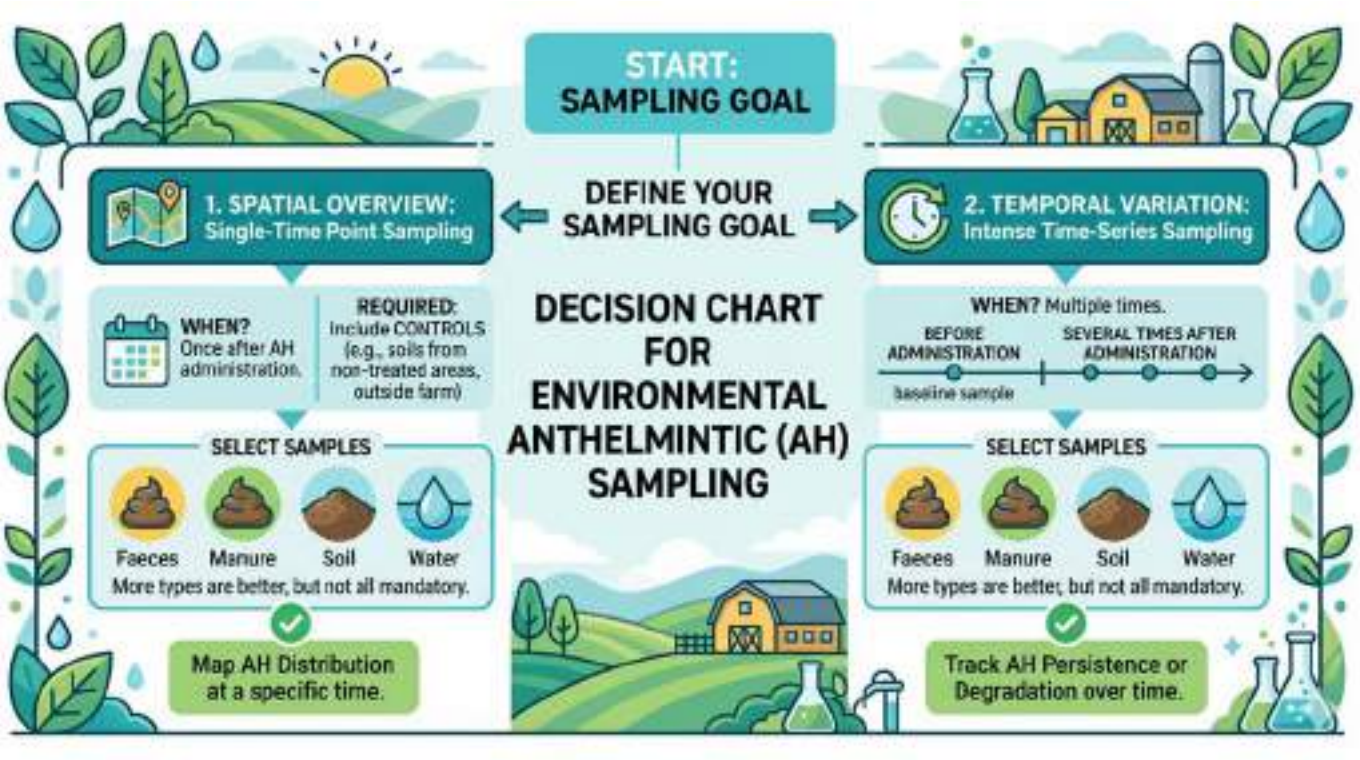
- Soil (Terrestrial Environment)
- Water (Aquatic Environment)

General Rule for Success:
The more sample types included, the better your overview of the environment.

Point to be considered:

- More than one anthelmintic residue can be analysed per sample depending on the analytic capacities of each analytical lab or national contact point.
- **Annex I** includes "**The Anthelmintic Treatment Sampling Schedule**" for Benzimidazoles and Macrocyclic Lactones.

You can use this flowchart to quickly determine your sampling strategy and ensure your study design meets your environmental monitoring goals.



Guidelines for Faecal Samples



- **Sample Amounts**
  For cattle, collect ~100 g wet weight per animal.
  For sheep or goats, collect ~10 g wet weight per animal.

For individual animal samples (best option), consider at least 5 animals.

Type of Approach

- **For Approach 1**, collect as fresh as possible from the ground, 2-4 weeks after anthelmintic treatment.
- **For tracking after anthelmintic treatment (Approach 2),** collect before anthelmintic treatment (1 day before or just before the administration) and 1, 2, 4, 7, 14 days after treatment. Monitoring can be extended up to 6–8 weeks, or longer, in the case of sustained-release (i.e. long acting) anthelmintic formulations.
- **How to Collect Samples**
  Collect the samples using a clean device (e.g., shovel), avoiding contact with the ground. Immediately transfer the sample into a zipped bag or a vacuum packed plastic bag to preserve anaerobic conditions during storage and transport.
  Protect from light and keep at ~4 °C until arrival at the laboratory. At this stage, subsamples can be prepared and transferred to a 50 ml polypropylene centrifuge tube with a screw cap.
- **Transport to the Laboratory**
  Place the samples in an insulated box with ice blocks to keep them cool during transport/shipment.
  The receiving lab should always check that samples arrive in good condition.
  If you are not the laboratory that will analyze the samples store the samples at -20°C until you can ship to the analytical lab.
- **Sample Preparation before Chromatographic Analysis**
  This procedure can be carried out by the laboratory collecting the samples or by the laboratory performing the chromatographic analysis.
  - Upon receipt, faecal samples should be inspected, non-representative material removed (e.g., stones, bedding, coarse debris), and manually mixed using a spoon. For large quantities, use a big bucket and blend

smooth with a paint paddle mixer fitted to a cordless drill at low speed to prevent spills.

- Split the samples into the following aliquots: 2 x 10 g (sheep or goats) or 2 x 50 g (cattle). Store them at -20°C until analysis.

- **Recommendations**

  Always label samples with the date, animal species, country, and farm/animal ID. If the animals received anthelmintic treatment, also note the drug name, dose, how it was given, and the date it was administered. If it is possible, take a photo at every step.

  Create a sample form and table, recording all information in a paper or digital lab notebook.

- **Equipment**

  Use gloves, mask, and other Personal Protective Equipment (PPE), shovel, stainless steel spoon, wide-mouth sterile container, pole, bucket (polyethylene or steel), , paint paddle mixer, small blender or a cordless drill.

Guidelines for Manure, Bedding Material & Slurry



Select the type of sample to be analysed based on the aim of the study:

(a) Bedding material accumulating at the surface of the inner site of the unit (farm or fence).

(b) Manure samples stored or piled outside the unit.

(c) Slurry samples taken from an underground tank beneath slatted floors.

Definitions

Bedding material should be the target samples if the purpose of the study is to define the amount of veterinary drugs (i.e. anthelmintics) that is accumulated inside the veterinary unit (farm) and it can be potentially transported through surface runoff (or leaching depending on the type of surface) to compartments outside the farm.

Manure samples should be taken if the goal of the study is to define the concentrations of veterinary drugs (i.e. anthelmintics) in the manure before application in the agricultural field which could give a measure of how much anthelmintics are released in agricultural soils.

Slurry is a liquid or semi-liquid mixture of faeces, urine, feed residues and water collected in an underground tank beneath slatted floors, mainly in cattle farms. Unlike solid manure, it contains little or no bedding material. The tank must be agitated before sampling to ensure a representative sample. Moisture content determination is essential to standardise results and express analyte concentrations on a dry weight basis.

Type of Approach

Here both sampling Approaches, 1 and 2, could be implemented depending on the goal of the study.

- **Sample Amounts**

  Bedding material or manure: 50-100 g per sample to make sure you have enough for reliable testing.

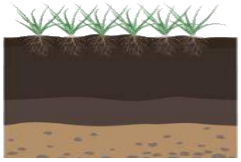
  Slurry: 150 – 200 mL per sample, or tank.

- **Where to Collect Samples**
  Bedding material: take samples using the W method (collect a portion of material from each point on a 'W' shape, avoiding field margins). That will cover the surface of the unit (farm or fence).
  Manure: take from the inner part of the manure pile or the center of the animal fence area to avoid outer dry bits.
  Slurry: take the sample from the tank beneath slatted floors.
- **When to Collect Samples**
  Sampling Approach 1
    Bedding material or Slurry: collect a few days (2-4 weeks) after anthelminthic application
    Manure samples or Slurry: collect samples prior to application of manure/slurry in the agricultural field.
  Sampling Approach 2
    Bedding material or Slurry: collect before anthelmintic treatment (1 day before or just before the administration), and on days 1, 7, 14, 28 and 56 days if possible.
    Manure samples or Slurry: collect a few days before manure/slurry is going to be applied in the agricultural field.
- **How to Collect Samples**
  Bedding material: choose 1-3 points per site and collect samples (0-10 cm depth) following the W-shaped random pattern. From each point take 5 sub-samples and homogenize immediately in the same bag.
    Approach 1 the number of samples should be 3.
    Approach 2 the number of samples could be limited to just 1.
  Manure: from the pile, dig into the depths while skipping dried edges, bottom, and side walls. Inside the fence, shovel from the middle to dodge fence dirt—check if the floor was cleaned that day so it's fresh manure. Take 3 sub-samples (50 g each) per manure pile (minimum 20 cm depth).
    For sampling approach 1 keep and handle sub-samples separately.
    For sampling approach 2 homogenize the samples to have one homogenized sample per manure pile.
  Slurry: take the sample directly from the tank after agitating it beforehand (the most appropriate). If no prior agitation is carried out, three samples (at least 50

mL each) must be collected at three different depths (surface, mid-water and bottom), which are then combined into a single sample.

- **Transport to the Laboratory**
  Place the samples in a cold box for storage at ~4°C during transportation to the laboratory which was collecting the samples. Ship the samples as soon as possible in a cold box with plenty of ice blocks to keep them cool during shipment. Protect samples from light.
  If you are not the laboratory that will analyze the samples store the samples at -20°C until you can ship to the analytical laboratory. The receiving lab should always check that samples arrive in good condition.
- **Sample Preparation before Chromatographic Analysis**
  This procedure can be carried out by the laboratory collecting the samples or by the laboratory performing the chromatographic analysis.
  1. Bedding material or manure samples are mixed thoroughly using a spoon and to remove non-representative materials (e.g., stones or large debris), as well as to ensure an even distribution of organic fractions.
  2. Subsamples (2 g of manure or bedding material; 20 mL of slurry) are then taken from the homogenised material for extraction and subsequent chromatographic analysis.
  3. Samples are then placed in a wide-necked polypropylene plastic bottle and stored at -20ºC until analysis.
- **Recommendations**
  Always label samples with the date, animal species, country, and farm/animal ID. Indicate the type of sample (bedding/manure/slurry). If the animals received anthelmintic treatment, also note the drug name, dose, how it was given, and the date it was administered. If it is possible, take a photo at every step. Create a sample form and table, recording all information in a paper or digital lab notebook.
- **Equipment**
  Use gloves, mask, and other PPE, stainless steel spoon, shovel, bucket (polyethylene or steel), drill whisk or small blender, and sterile containers.

Guidelines for Soil Samples



- **Where to Collect Samples**
  There are two areas where sampling should focus
  - High deposition area: just outside the fence, where animals are walking and drink water etc. selecting points where animals spend most of their time as hotspots of faecal deposition.
  - Reference area: zones with low or minimal faecal deposition, representative of background conditions.
- **When to Collect Samples**
  Sampling Approach 1: sample both areas, high deposition and reference areas at a single time point after treatment (i.e. 2-4 weeks post application)
  Sampling Approach 2: sample both areas, high deposition and reference areas before anthelmintic treatment and at 7, 14, 28 and 56 days after administration of drugs. This will allow differentiation between residue levels associated with faecal deposition and background concentrations.
- **How to collect the sample**
  For both sampling approaches, collect soil samples underneath faeces/bedding material, but avoid contamination with faeces residues, removing the top layer of vegetation or litter. Transfer 5 independent top soil (0-10 cm) samples with an auger, corer or shovel to a plastic bag from the **exact same location** as the faecal sample to ensure the two samples can be directly linked. Even in the days that follow, always take the samples from the same spot.
  Keep a very good track of the placement of each sample and keep notes or photos (i.e. coordinates, photos of the sampling place and if easily available meteo data and soil properties). That will help very much the interpretation of the data.
- **Transport to the Laboratory**
  Place the samples in a cold box with ice blocks for storage at ~4°C during transportation to the laboratory that will collect the samples. Protect samples from light.

If you are not the laboratory that will analyze the samples, store the samples at ~4 °C for soil nematodes analysis (150 g) or at -20°C for the other analyses until you can ship to the analytical laboratory, see below.
The receiving laboratory should always check that samples arrive in good condition.

- **Sample Preparation before Chromatographic Analysis**
  Soil samples are first manually homogenised using a spoon or a spatula. Then, remove plant debris, like roots and plant tissues, but also stones, from the samples.
  Analyses include:
    - 2 g for microbiome analysis and DNA extraction. Storage at -20°C. Ship the samples in small plastic tubes or bags to the analysing lab.
    - 100 g for physicochemical analysis (at least: pH, soil organic C, soil texture, CEC, N, P, K content). Samples should be air-dried or dried at ≤40 °C immediately (or kept at 4°C, for 48-72 hours, maximum 7 days), homogenised and sieved (typically <2 mm), and then they can be stored in dry, dark conditions at room temperature until analysis.
    - 150 g for soil nematode analysis. It is recommended that the analysis is carried out as soon as possible, keeping the sample at 4°C for a maximum of 4 weeks. Ship the samples in plastic bags to the analysing lab.
    - 10 g for chromatographic analysis (e.g. HPLC), if not done immediately then store at -20°C.

  For HPLC analysis, subsamples are then taken from the homogenised material for extraction and subsequent chromatographic analysis. Samples are then placed in a wide-necked polypropylene plastic bottle and stored at -20ºC until analysis.
- **Recommendations**
  Label each sample with the date and exact sampling location. Also note the weather conditions during sampling, such as temperature and humidity. Take a photo at every step.
  Record any other anthelmintic treatments previously administered to animals that have grazed or otherwise used the sampled area.
- **Equipment**
  Gloves, PPE, shovel/corer/auger, sterilized glass jars, plastic bags, plastic tubes.

Guidelines for Water Samples



- **Sample Amounts**
  Fill up 2.5 liters to cover all tests needed from wells or surface spots.
  The volume can go from 100-250 mL for surface water or 500 mL for groundwater until 500 – 1,000 mL for surfacewater or 2.5 L for groundwater, depending the equipment to analyse the samples:

  Note: The required sample volume depends on the sensitivity of the analytical instrument and the target quantitation limits. For example, the 2.5 L volume above reflects instrumentation approximately 20 years old. State-of-the-art LC-MS/MS systems — such as the Agilent 6495, Waters Xevo TQ Absolute, SCIEX 6500+/7500+, and Thermo Scientific Orbitrap platforms — offer substantially improved sensitivity and may achieve the same detection limits with smaller volumes (e.g., 100 mL or less), while also reducing the risk of SPE cartridge blocking. Confirm the appropriate volume with your analytical laboratory before sampling.
- **Where to Collect Samples**
  Collect water samples downstream from livestock farming areas, at least at one of these points:
  - Small streams, natural or artificial (surface water).
  - Large streams where the wider area of livestock farms are draining (surface water).
  - Well taps in the livestock farming area (groundwater).

  As control a second sample, upstream of the farm, can be collected from streams.
- **When to Collect Samples (for tracking after anthelmintic treatment)**
  For tracking after anthelmintic treatment: take samples 60 days after administration of anthelmintics to check for runoff impacts. In case there is an intense precipitation event before the 60 days, take the sample 3-7 days after the event.
  Sampling can also be carried out for point-in-time assessments.
  Under Approach 2, samples shall be collected on a monthly basis for a duration of one year.
- **How to Collect Samples**
  Wash the bottles before the sampling with a EDTA solution.

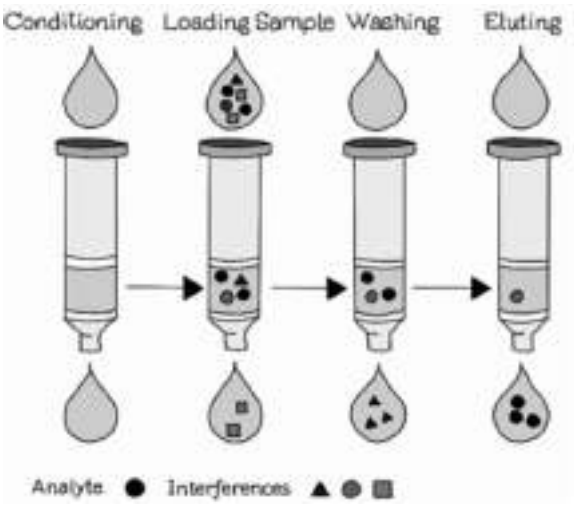
Procedure for collecting surface water:

1. Rinse borosilicate glass amber bottle three times with source water first.
2. For surface water, grab from at least 0.5 m deep if possible.
3. Groundwater: Purge the well with several volumes of water to remove stagnant water. Then, collect the water into the bottle, use a pump to fill the bottle.

- **Transport to the Laboratory**
  Transport the samples to the lab within 24 hours, keeping them closed, into portable coolers at 4°C and in the dark to stay fresh until testing.
  If the analytical laboratory is located elsewhere, the samples may be filtered through the appropriate cartridges before shipment. In this case, the cartridges should be stored at 4°C, protected from light, and shipped to the analytical laboratory within 48 hours.
- **Sample Preparation Before HPLC or LC-MS/MS Analysis**
  This procedure can be carried out by the laboratory collecting the samples or by the laboratory performing the analysis. Upon arrival at the laboratory, water samples should be kept under chilled conditions and stored at 4 °C in amber glass bottles until analysis. Samples should be analysed as soon as possible, preferably within 48–72 hours, and, within a maximum of 7 days after collection. If this is not possible, samples can be frozen until they are analysed.
  The advice is to send the water sample to a the analytical laboratory, or, alternatively, concentrate the sample on the Solid Phase Extraction (SPE) cartridge and send this to the laboratory according to the following procedure.
  The following procedure is described for an analysis by LC-MS/MS. If a different HPLC method is to be used, compatibility of the final solvent composition and analyte sensitivity should be verified during method validation (Mooney et al., 2019).

  1. Equilibration and homogenisation. Before extraction, samples should be allowed to equilibrate to room temperature and be pre-shaken to ensure homogeneity.
  2. Aliquot for extraction. Transfer 500 ± 0.1 mL of water sample into a 1 L amber glass bottle.
  3. Sample modification prior to extraction. Add 100 mL methanol to the 500 mL sample, shake, and then adjust the sample to pH 7.0 ± 0.05 with HCl (0.1 M) or NH4OH (0.1 M).

4. Solid-phase extraction. Extract the sample using polymeric DVB SPE cartridges (Enviro-Clean® HL DVB PE Frits 200 mg 6 mL). Before loading, cartridges should be: conditioned with 5 mL methanol:acetone (50:50, v/v), then 5 mL methanol, and equilibrated with 5 mL ultrapure water at pH 7. Load the sample at approximately 3 mL/min under vacuum.



5. Bottle rinse and cartridge wash. Rinse the sample bottle with 10 mL water:methanol (90:10, v/v) and pass this rinse through the cartridge. Then wash the cartridge with a further 5 mL water:methanol (90:10, v/v).
6. Drying and elution. Dry the SPE cartridge under vacuum for 30 min. Elute the retained analytes with 10 mL methanol:acetone (50:50, v/v) into suitable collection tubes. At this point, the tubes can be sent to the laboratory for their analysis or continue on to the completion of the procedure.
7. Concentration of the extract. Add 500 µL DMSO as a keeper solvent. Then evaporate the eluate under nitrogen at 50 °C until only 500 µL remains. This is the main concentration step prior to instrumental analysis.
8. Final preparation for injection. Sonicate the concentrated extract for 2 min and vortex for 30 s. Filter through a 0.22 µm syringe filter into amber glass HPLC vials before analysis.

- **Recommendations**

  Label each bottle with the date, exact location, and collection method for easy tracking. Take a photo at every step if it is possible.

- **Equipment**
  Gloves, borosilicate glass amber bottle, bucket (polyethylene or steel).
- **Reference**
  Mooney, D.; Coxon, C.; Richards, K.G.; Gill, L.; Mellander, P.-E.; Danaher, M. Development and Optimisation of a Multiresidue Method for the Determination of 40 Anthelmintic Compounds in Environmental Water Samples by Solid Phase Extraction (SPE) with LC-MS/MS Detection. Molecules 2019, 24, 1978.

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ANNEX

Approach 1 (Spatial Overview)

While the primary objective of Approach 1 (Spatial Overview) is to map the current distribution of anthelmintic residues across Europe, the operational execution depends on country specific conditions, target study areas, the number of pilot farms, and the available budget/funding of each partner.

To maximize participation and sample acquisition while preserving data integrity, this Annex outlines the flexible sampling protocol when farm visits cannot be strictly synchronized within the standard post-treatment window.

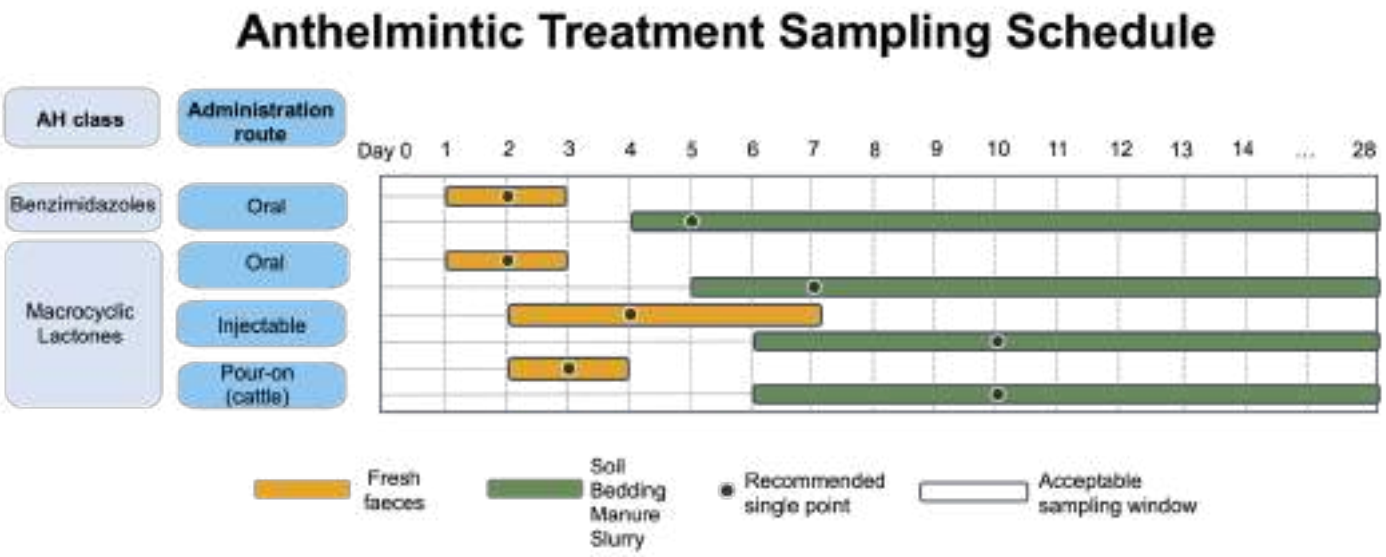
Scenario 1: Maximizing residue detection potential

Objective: Capture peak residue levels and baseline excretion profiles under ideal temporal conditions (common sampling windows across countries).

Sampling Window: From the day of administration up to 2 to 4 weeks post anthelmintic treatment.

Target Matrices: All 4 environmental matrices (Fresh Faeces, Bedding/Manure/Slurry, Soil and Water). In this scenario, fresh faeces must be collected because the sampling event falls strictly within the 2-4 weeks post-treatment window, corresponding to the timeframe of maximum residue excretion for target active substances (Benzimidazoles and Macrocyclic Lactones) (see graph below - Anthelmintic Treatment Sampling Schedule).

Key Requirement: Synchronized sampling visits scheduled within the narrow post-treatment window across participating countries. Record for each farm the anthelmintic used, route of application and treatment time.



Scenario 2: Assessing Real-World Residue Occurrence (Field Conditions)

Objective: Evaluate background environmental presence, long-term accumulation and practical contamination levels across randomly selected farms under routine management practices.

Sampling Window: > 4 weeks post anthelmintic treatment (or unconstrained timing).

Target Matrices: Bedding/Manure/Slurry, Soil, and Water (EXCLUDE Fresh Faeces). Do not collect fresh faecal samples due to rapid excretion dynamics and fast degradation kinetics, which risk false negatives.

Key Requirement: Record for each farm the anthelmintic used, route of application and treatment time.

Priority Target Analytes

Given the widespread veterinary usage and environmental persistence profiles of active substances, analytical efforts in both standard and extended sampling protocols should prioritize two main anthelmintic classes:

Benzimidazoles: (e.g., albendazole, fenbendazole, oxfendazole and their metabolites).

Macrocyclic Lactones: (e.g., ivermectin, moxidectin, eprinomectin).

Additional anthelmintic classes (e.g., imidazothiazoles like levamisole, or tetrahydropyrimidines) may be included depending on local treatment records and specific laboratory capacities.