

# Harmonized procedures for soil, water and manure samples management and analysis

Deliverable D1.2

INNOVATIVE DECISION-MAKING TOOL FOR DEFINING THE MOST SUITABLE MANURE MANAGEMENT STRATEGIES TO ACHIEVE A SUSTAINABLE LIVESTOCK FARMING SYSTEM DURING THE WHOLE VALUE CHAIN

Proposal number: 101135400-2



Deliverable D1.2 – Harmonized procedures for soil, water, and manure samples management and analysis			
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## 1. INTRODUCTION

WP 1 covers the diversity in livestock and current practices. We'll obtain region-specific data on manure management from facilities across the EU and China, considering different animal categories (cow, pig, and poultry), climates (Atlantic, Mediterranean, alpine, and continental), and livestock types (intensive, semi-intensive, extensive, conventional, and organic). This information, completed with bibliographic data, will result in an up-to-date inventory regarding manure management practices already available and implemented, and associated pollutants affecting air, soil, water, and manure, that will feed WP3 modelling and LCA, and consequently, the DSS tool of WP4.

First, 32 case-study farms were carefully selected aiming to cover the different livestock farming typologies. In the different case studies, air, soil, water, and manure contaminants will be assessed. Regarding soil, water and manure pollutants, samples will be collected in two periods (dry and wet) and analyzed according to the protocol described in this document to ensure comparability. This protocol includes procedures for sampling, sample preparation, storage, shipment, and analysis. (Treated) manure samples and soils from different geographical locations using own (treated) manure for fertilization will be assessed in selected case studies. For water pollution assessment, the examination of pollutants occurrence will be conducted in near water receiving bodies associated with case study farms.

## 2. OBJECTIVES

To develop a protocol for soil, water and (treated) manure samples collection, pretreatment, storage, shipment and contaminant analyses, including physic-chemical parameters, antibiotic residues, antibiotic resistance genes, pathogens, antibiotic resistance bacteria and heavy metals.

## 3. TARGET POLLUTANTS

### 3.1. ANTIBIOTIC RESIDUES

**Nineteen  $\beta$ -lactam antibiotics** (amoxicillin, ampicillin, benzylpenicillin, cloxacillin, nafcillin, oxacillin, penicillin V, cefadroxil, cefapirin, cefalexin, cefalonium, cefazolin, cefoperazone, cefquinome, ceftiofur, cephradine, desacetylcephapirin, desfuroylceftiofur cysteine disulphide and dicloxacillin), **12 (fluoro)quinolones** (cinoxacin, ciprofloxacin, danofloxacin, difloxacin, enoxacin, enrofloxacin, flumequine, nalidixic acid, marbofloxacin, norfloxacin, oxolinic acid and sarafloxacin), **13 sulfonamides** (dapson, sulfachloropyridazine, sulfaclozine, sulfadimethoxine, sulfadoxine, sulfadiazine, sulfamethazine, sulfamerazine, sulfamethoxazole, sulfamethoxy-pyridazine, sulfapyridine, sulfaquinoxaline and sulfathiazole), **7 macrolides** (erythromycin, gamithromycin, spiramycin, tilmicosin, tulathromycin, tylosin and tylvalosin), **2 lincosamides** (lincomycin and pirlimycin), **3 amphenicols** (chloramphenicol, florphenicol and thiamphenicol), **4 tetracyclines** (chlortetracycline, doxycycline, oxytetracycline and tetracycline), **2 pleuromutulines** (tiamulin and valnemulin) and **1 diamino pyridine derivate** (trimethoprim) will be monitored.

### 3.2. ANTIBIOTIC RESISTANCE GENES (ARGs)

The selected antibiotic resistance genes to be monitored are listed in Table 1. Thirty-seven genes, expected in manure, soil and water, will be screened by means of Resistomap, covering 10 resistance mechanisms.

Table 1: Antibiotic resistance genes to be screened in NUTRITIVE project.

<u>Gene</u>	<u>Target Class</u>	<u>Gene</u>	<u>Target Class</u>
<i>16srRNA</i>		-	
<i>aacC</i>	Aminoglycosides	<i>ermC</i>	MLS
<i>aadA1</i>	Aminoglycosides	<i>ermF</i>	MLS
<i>strA</i>	Aminoglycosides	<i>florR</i>	Phenicols
<i>strB</i>	Aminoglycosides	<i>fexA</i>	Phenicols
<i>vanA</i>	Vancomycin	<i>cfr</i>	Phenicols
<i>ampC</i>	$\beta$ -lactams	<i>qnrS</i>	Quinolones
<i>blaCMY_2</i>	$\beta$ -lactams	<i>qnrB</i>	Quinolones
<i>blaCTX-M</i>	$\beta$ -lactams	<i>sul1</i>	Sulfonamides
<i>blaIMP</i>	$\beta$ -lactams	<i>sul2</i>	Sulfonamides
<i>blaNDM</i>	$\beta$ -lactams	<i>tetB</i>	Tetracyclines
<i>blaOXA</i>	$\beta$ -lactams	<i>tetC</i>	Tetracyclines
<i>blaSHV</i>	$\beta$ -lactams	<i>tetG</i>	Tetracyclines
<i>blaTEM</i>	$\beta$ -lactams	<i>tetH</i>	Tetracyclines
<i>blaVIM</i>	$\beta$ -lactams	<i>tetM</i>	Tetracyclines
<i>mecA</i>	$\beta$ -lactams	<i>tetO</i>	Tetracyclines
<i>mcr</i>	Colistin	<i>tetQ</i>	Tetracyclines
<i>Int1</i>	MGE	<i>tetW</i>	Tetracyclines
<i>ermA</i>	MLS	<i>tetX</i>	Tetracyclines
<i>ermB</i>	MLS	<i>dfrA1</i>	Trimethoprim

### 3.3. PATHOGENS

The selected pathogens to be monitored are: *Salmonella* and *Campylobacter*.

### 3.4. ANTIBIOTIC RESISTANCE BACTERIA (ARBs)

The selected antibiotic resistance bacteria to be monitored are: ESBL *E. coli* and vancomycin resistant *Enterococcus faecalis*.

### 3.5. HEAVY METALS

The selected heavy metals to be monitored are: manganese (Mn), Cadmium (Cd), Copper (Cu), Lead (Pb), Chromium (Cr), Nickel (Ni) and Zinc (Zn).

## 4. SAMPLING

### 4.1. MANURE

The manure used for fertilization, either non-treated or treated, will be sampled. Manure sampling will depend on the nature of the manure itself:

- liquid manure should be stirred before taking the sample; also sampling the top and the bottom of the storage should be avoided, since stratification could influence the composition of the sample; it would be useful taking the samples from different points of the storage and at various depths to obtain the whole picture of the manure composition. For this purpose, a tube sampler with a valve is required; if not a simple bottle tied to a stick can be used, with a loss in accuracy. The samples collected must be transferred to a larger container and manually mixed to obtain a homogeneous “composite sample”. From the composite sample must derive a final subsample of at least 1 kg, collected in a sealed container (e.g. bottles, double-sealed, if necessary) to avoid leakage and correctly labelled (see label specifics below).

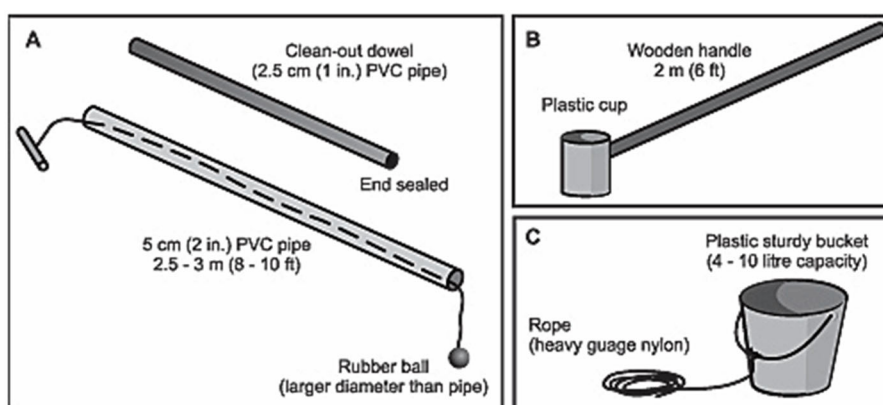


Figure 1. Liquid manure sampling devices: (A) composite, (B) pole-and-cup, (C) bucket and rope

- solid manure samples should be collected with a shovel at least 40-50 cm below the pile surface and away from edges, since the contact with air may alter the composition. If the sample is taken directly from a poultry building or bedpack area, it is important to collect at least 10 subsamples from various areas to obtain a composite sample representative of the whole livestock. The composite sample is collected in a container big enough (e.g. sacks, etc.) where it is mixed to obtain a homogeneous final product from where a final subsample

is obtained and stored in a sealed container (e.g. plastic bags). Since it is usually more difficult to homogenise solid manure, a larger volume than liquid may be needed, so a sample volume of at least 1 L (1 kg) is recommended.



*Figure 2. Mixing of the collected solid manure in a large container (bucket) to obtain a final subsample stored in a smaller sealed container (plastic bag)*

#### 4.2. SOIL

Each field is sampled three times: before (T1), immediately after (T2), and 2 to 3 weeks after fertilization (T3). T2 should be taken immediately after the fertilizer has been incorporated into the field, i.e. on the same day as the manure application. If the manure is first spread on the fields and only a few hours later mixed with the soil, then T2 is when the manure is mixed with the soil. Ideally, at the moment of fertilization, a homogenized sample of manure used as fertilizer is also taken.

In each field, the same 4 plots of 10 m x 10 m should be sampled at a depth of 0 - 30 cm at the 3 time points. There should be a distance of 10 m from every border to the plots. Within each plot, fifteen individual samples are taken in diagonal lines using a probe (preferably), auger or shovel (Figure 3). In case a shovel is used, a V-shape is created in the soil (depth 30 cm) and a slice (1 cm) of soil is obtained (Figure 4). Each individual sample will weigh around 1 kg. Later, a mixed soil sample for every plot is prepared from these 15 samples. The total amount of mixed sample for each plot should be 1 kg. The remaining sample is returned to the plot.

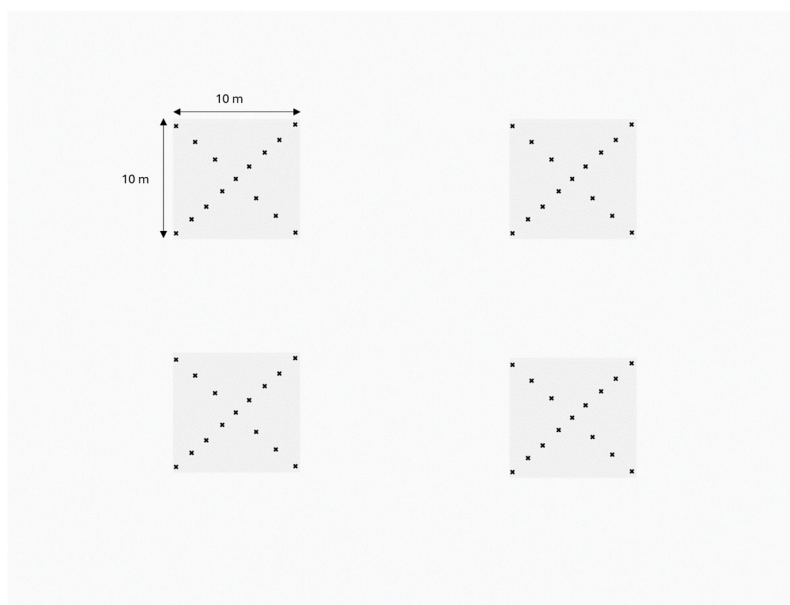


Figure 3. Four plots are chosen on the field. Within each plot, 15 points are selected, spread diagonally across the subplot, and combined at the end.

Fields must meet the following criteria: 1) manure is applied to the field (preferably from the farm's animals) and 2) the field has not been fertilized with fresh animal manure for at least 5 months before the start of the first sampling (T1).

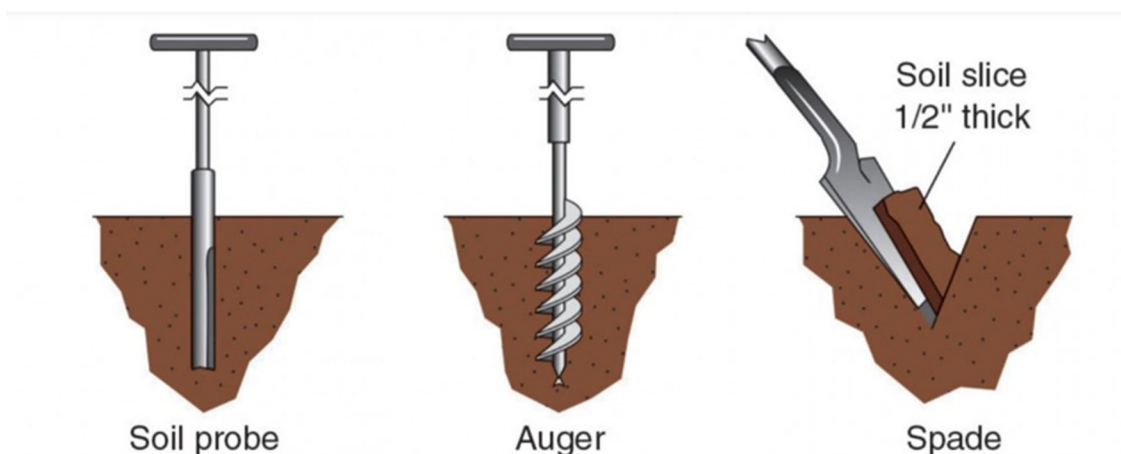


Figure 4. Illustration of sampling with a probe, auger or shovel (Fernandez and Hoefl, 2012).

### 4.3. WATER

Grab samples will be taken in each sampling site 3 to 4 weeks after fertilisation. 4 L sterile polypropylene bottles will be filled with water from the sampling sites for physic-chemical, heavy metals and pathogens analyses. Two 1 L amber glass/aluminium bottles will be (almost) filled with 2 L of water from the sampling sites for antibiotic residues analysis. Four 1 L sterile polypropylene bottles will be filled with 4 L of water from the sampling sites for antibiotic resistance genes determination.

#### 4.4. LABELLING

Sample label must indicate:

- Type of sample
  - Soil
  - Water
  - Manure (non-treated or treated)
- Case study
- Sampling date (dd/mm/yyyy)

There will be also a specific template to insert the other additional information that is not reported on the sample's label.

### 5. PRETREATMENT AND STORAGE

All samples must be appropriately stored for a maximum storage time of 24 h before shipment.

#### 5.1. MANURE

Upon arrival in the lab, 5 x 100 g subsamples are stored at -20 °C for the analysis of the antibiotic residues and DNA extraction. Next, 5 x 100 g are stored at 4 °C for bacteriological analysis.

#### 5.2. SOIL

A mixed soil sample will be produced on the field by mixing the 4 x 1 kg soil samples in a clean bucket<sup>1</sup>. After vigorous shaking and rotating the sample (manually, with gloves), a subsample of at least 1 kg is produced. Only for T1, 1.6 kg is needed. The remaining fraction is returned to the soil. Transport to the lab should happen in a cooler. Upon arrival in the lab, 5 x 100 g subsamples are stored at -20 °C for the analysis of the antibiotic residues and DNA extraction. Next, 5 x 100 g are stored at 4 °C for bacteriological analysis. For T1, an additional 300 g is stored in duplicate at 4 °C for the analysis of heavy metals.

#### 5.3. WATER

All water samples must be stored at 4°C.

### 6. SHIPMENT

At least one-week notice will be given to ILVO ([laurens.tuts@ilvo.vlaanderen.be](mailto:laurens.tuts@ilvo.vlaanderen.be)) to plan the soil and manure analyses and to USC ([monica.dosil@usc.es](mailto:monica.dosil@usc.es)) to plan the water analyses.

From each soil sample, 3 x 100 g in a frozen state and 2 x 100 g in a refrigerated state are required for the analysis at ILVO. Only for T1, an additional 300 g (refrigerated) is required for heavy metal

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<sup>1</sup> NOTE: The combination of these 4 plots is still under consideration. It is also possible that only 2 plots can be combined, meaning there will be 2 samples at every T for each field.

analyses. In both cases, cooled, express shipment is necessary to avoid the degradation of antibiotic residues and the survival of pathogens. Therefore, enough cooling elements will accompany the contents of the package making sure the frozen content remains frozen and the cooled content remains at refrigerated temperature in an isolated box. T1 and T2 can be shipped together. T3 should be sent separately. To avoid the rise of the temperature above 5 °C during all the shipment phase, the samples must be placed in boxes of thermal insulating materials (e.g. polystyrene) full of dry ice. What is left of the sample is stored at the appropriate conditions (2 x 100 g at -18 °C, and 3 x 100 g and 1 x 300 g at 4 °C) in case the shipment is unsuccessful.

From each water sample, one amber glass/aluminium bottle and two polypropylene bottles are required for the analysis at USC. Cooled, express shipment is necessary to avoid the degradation of antibiotic residues and antibiotic resistance genes. To avoid the rise of the temperature above 5 °C during all the shipment phase, the samples must be placed in boxes of thermal insulating materials (e.g. polystyrene) full of dry ice or with enough cooling elements. The remaining sample (one amber glass/aluminium bottle and two polypropylene bottles) is stored at -20°C in case the shipment is unsuccessful.

From each manure sample, 3 x 100 g subsamples in a frozen state and 2 x 100 g subsamples in a refrigerated state will be shipped to the laboratory that will make the analysis. What is left of the sample (5 x 100 g subsamples) is stored at the appropriate conditions in case the shipment is unsuccessful.

## 6.1. MANURE

The analyses of physic-chemical parameters, heavy metals, pathogens and antibiotic resistance bacteria will be done by the case-study responsible. For antibiotic residues and antibiotic resistance genes, samples must be sent to:

Laurens Tuts Brusselsesteenweg 370 9090 Melle Belgium
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## 6.2. SOIL

All the analyses (physico-chemical, heavy metals, antibiotic residues, antibiotic resistance genes, pathogens and antibiotic resistance bacteria) will be done by EV ILVO. Therefore, samples must be sent to:

Laurens Tuts Brusselsesteenweg 370 9090 Melle Belgium
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### 6.3. WATER

The analyses of physico-chemical parameters, heavy metals, pathogens and antibiotic resistance bacteria will be done by the case-study responsible. For antibiotic residues and antibiotic resistance genes, samples must be sent to:

Mónica Dosil Pías  
ETSE. University of Santiago de Compostela  
Rúa Lope Gómez de Marzoa s/n.  
15782. Santiago de Compostela. Spain

## 7. ANALYSIS

### 7.1. PHYSIC-CHEMICAL ANALYSES

#### 7.1.1. Manure samples

Manure characteristics (organic matter, N and P compounds, pH, etc.) will be carried according to ISO reference methods.

#### 7.1.2. Soil samples

Soil characteristics (total organic carbon (TOC), nutrients (N and P compounds), pH-KCl) will be measured according to ISO and other reference methods (e.g., ISO10694, BAM, ISO 13878; ISO 10390). Only the soil before fertilization (T1) will be analyzed.

#### 7.1.3. Water samples

Water characteristics (pH, total and volatile solids (TS, VS), total and volatile suspended solids (TSS, VSS), total organic carbon (TOC), ammonium ( $\text{NH}_4^+$ ), nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ )) will be determined according to the Standard Methods (APHA, 2017).

### 7.2. ANTIBIOTIC RESIDUES

#### 7.2.1. Manure samples

Manure samples were analyzed with a multi-residue method for 63 antibiotics from 10 classes as described in Rasschaert et al. (2020) and Huygens et al. (2021). After addition of 2 mL MeCN + 5% TCA to 2 g of sample, the sample was homogenized for 1 min and shaken for 30 min. Next, this is centrifuged for 15 min at 4000 g. The supernatant is evaporated under nitrogen in a water bath at 40 °C after which it is sonicated and reconstituted in 1 mL of  $\text{H}_2\text{O}/\text{ACN}/\text{MeOH}$  (50/25/25) + 0.05% acetic acid (AA). The filtered (0.22  $\mu\text{m}$ ) extract can be diluted and injected into the UHPLC-MS/MS system (Acquity UHPLC, column: BEH C18 (100 mm $\times$ 2.1 mm i.d., 1.7  $\mu\text{m}$ , solvent A: water + 0.05%

AA, solvent B: ACN/MeOH (50/50) + 0.05% AA, Xevo TQ-XS mass spectrometer (Waters Corporation)).

### 7.2.2. Soil samples

A multi-residue method is followed. Soil samples are extracted according to Huygens et al (2022). To 2 g of a soil sample, 4 mL of 0.125% (v/v) trifluoroacetic acid (TFA) in acetonitrile is added and shaken for 10 min at 250 rpm and centrifuged for 15 min at 3500 g. Thereafter, 5 mL Mcllvaine-EDTA buffer (pH 4) is added. The Mcllvaine-EDTA buffer is prepared by adding 5 mL 1M citrate solution, 28 mL 0.2M Na<sub>2</sub>HPO<sub>4</sub>-solution and 7.44 g Na<sub>2</sub>EDTA.2H<sub>2</sub>O to 100 mL HPLC grade H<sub>2</sub>O. Next, this is shaken for 20 minutes at 250 rotations per minute (rpm) to homogenize the mixture and centrifuged at the end. This supernatant is evaporated in a water bath (40 °C, under N<sub>2</sub>). The obtained extract is purified using tandem solid phase extraction (SPE) where a hydrophilic-lipophilic balance (HLB) column is combined with a mode cation exchange column. The resulting extract is evaporated under N<sub>2</sub> at 40°C, redissolved in 1 mL of H<sub>2</sub>O/ACN/MeOH (50/25/25), sonicated and filtered. Finally, UHPLC-MS/MS (Acquity UHPLC, column: BEH C18 (100 mm×2.1 mmi.d., 1.7µm, solvent A: water + 0.05% acetic acid, solvent B: ACN/MeOH (50/50) + 0.05% acetic acid, Xevo TQ-XS mass spectrometer (Waters Corporation)) is used for separation and quantification.

### 7.2.3. Water samples

The multiresidue method developed by Tuts et al. (2024) will be applied. Briefly, to 100 mL water sample, 3 mL Na<sub>2</sub>EDTA (0.1 M) is added before adjusting the pH to 3 with HCl (3.5 M). Internal standards (cefotaxime, ceftiofur-D3, clindamycin, metacycline, piperacillin, roxithromycin, sulfadimethoxine 13C6, threo-chloramphenicol-D5 and trimethoprim-D9) are added. OASIS HLB solid-phase extraction (SPE) cartridges (6 cc barrel size, 500 mg sorbent weight, 60 µm particle size and 80 Å pore size; Waters, Milford USA), previously conditioned with 5 mL MeOH and 5 mL H<sub>2</sub>O, are used for the clean-up of the sample. After passing 100 mL of the sample through the column, a washing step with 5 mL H<sub>2</sub>O and subsequent drying of the column is needed. The retained antibiotics are eluted with 5 mL MeOH and the obtained extract is evaporated under N<sub>2</sub> at 40°C until full dryness. The residues are then resolved in 1 mL of the reconstitution liquid (50:25:25 v/v H<sub>2</sub>O:MeCN: MeOH + 0.05% acetic acid) and filtered through a 0.22 µm PVDF filter (Merck) into a vial. Finally, UHPLC-MS/MS (Acquity UHPLC, column: BEH C18 (100 mm×2.1 mmi.d., 1.7µm, solvent A: water + 0.05% acetic acid, solvent B: ACN/MeOH (50/50) + 0.05% acetic acid, Xevo TQ-XS mass spectrometer (Waters Corporation)) is used for separation and quantification.

## 7.3. ANTIBIOTIC RESISTANCE GENES

### 7.3.1. Manure samples

The same protocol as next described for soil samples will be used for manure samples.

### 7.3.2. Soil samples

At first, DNA extraction is performed using the PowerSoil DNA Isolation Kit (Qiagen). From each sample 0.25 g is extracted according to the manufacturer's protocol. The DNA yield is measured using a NanoPhotometer (Implen, München, Germany) and Quantus™ fluorometer (Promega, Madison, WI, USA) in order to determine the DNA quality and quantity of the obtained extract. Next, a screening (Resistomap) will be carried out on the DNA extracted from the soil samples to detect the relevant antibiotic resistance genes (Table 1).

### 7.3.3. Water samples

The concentration of the samples will be performed using Rexeed 25A filtration units (Asahi Kasei Medical) following a protocol adapted from <http://aem.asm.org/content/75/16/5284.long> with modifications described in Rivadulla et al. (2024). Prior the filtration, the filtration unit will be flushed using the red port with 1L of sterile reagent grade water at a rate of 2900 ml/min. Two liters of each sample will be pumped through the filter unit at a rate of 2900 ml/min. Once filtered, the pressure of the system should be discharged by releasing the tubing from the peristaltic pump. To elute the sample, the filter unit should be inverted (blue port up) in a way that the blue port will be used as the influent for the elution buffer and the red port will be used as collector. The elution buffer will be flushed through at a rate of 650 ml/min. Because of the dead volume of the filters and the tubing, use 150 mL buffer. The sample will be subsequently filtered through 3 nitrocellulose sterile membrane filters of 0.22 µm pore (50 ml each) to retain the biomass. The filters can be storage at -20°C until DNA extraction.

DNA will be extracted by triplicate from each solid sample using the Nucleospin Microbial DNA extraction kit (Macherey Nagel). Filters retaining biomass were cut into pieces, added directly to the bead beating tubes and DNA was extracted using the same kit. DNA concentration and purity will be determined using Qubit fluorometer and Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA).

A representative arrange of different ARGs and MGEs families will be carried out using a SmartChip by high-throughput qPCR (HT-qPCR) in Resistomap (Helsinki, Finland) to detect the relevant antibiotic resistance genes (Table 1).

## 7.4. PATHOGENS

### 7.4.1. Manure samples

The same protocol as next described for soil samples will be used for manure samples.

### 7.4.2. Soil samples

A suspension ( $10^0$ ) and further sample dilutions ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ) are needed. Therefore, 99 mL of buffered peptone water (CM0509, Oxoid) is added to 11 g of soil. After homogenization in a stomacher for 1 min, three serial dilutions (1:10) of samples are prepared in buffer peptone water (BPW).

#### Enumeration of *Salmonella* spp.

Minor modifications are made to ISO 6579-1:2017E. Samples (100) and dilutions ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ) are incubated at  $37 \text{ }^\circ\text{C}$  for  $18 \pm 2$  h. Subsequently, 3 drops of the pre-enrichment culture and dilutions are inoculated on MSR/V agar plates (CM0910, Oxoid) supplemented with MSR/V selective supplement (SR0161E, Oxoid) and incubated at  $41.5 \pm 1 \text{ }^\circ\text{C}$  for  $24 \pm 3$  h. Negative plates are incubated for an additional  $24 \pm 3$  h. From the positive plates, a 10  $\mu\text{L}$  loop from the edge of the migration zone is inoculated on XLD (CM0469, Oxoid) and incubated at  $37 \pm 1 \text{ }^\circ\text{C}$  for  $24 \pm 3$  h. Presumptive *Salmonella* colonies are further confirmed through a multiplex *Salmonella* and *Salmonella* Typhimurium PCR as described by Aabo et al. (1993) and Lin and Tsen (1999) respectively. Isolates can be stored in BHI + glycerol at  $-18 \text{ }^\circ\text{C}$ .

#### Enumeration of *Campylobacter* spp.

From a soil sample, 99 ml of Bolton Broth (CM 0983, Oxoid) is added to 11 g of a sample. After homogenization for 1 min, three consecutive 1:10 dilutions of the samples are prepared in Bolton Broth. The samples and the dilutions are then incubated under microaerobic conditions (85%  $\text{N}_2$ , 10%  $\text{CO}_2$ , 5%  $\text{O}_2$ ) in a Forma Series II 3110 Water-Jacketed  $\text{CO}_2$  incubator (Thermo Scientific, Waltham, MA) at  $41.5 \text{ }^\circ\text{C}$  for 24 and 48 h. After 24 h of enrichment, 10  $\mu\text{L}$  of the culture is plated on CCDA plates (CM0739, Oxoid) supplemented with CCDA selective supplement (mCCDA, Oxoid, SR0155E). The plates are incubated under microaerobic conditions at  $41.5 \text{ }^\circ\text{C}$  for 24 and 48 h. If no growth of *Campylobacter* is observed after 24 h, 10  $\mu\text{L}$  of the enrichment incubated for 48 h is plated on mCCDA plates. These plates are also incubated under microaerobic conditions at  $41.5 \text{ }^\circ\text{C}$  for 24 and 48 h. Colonies of presumptive *Campylobacter* will be confirmed with a multiplex *Campylobacter jejuni* and *Campylobacter coli* PCR as described by Linton et al. (1997). Isolates can be stored in BHI + glycerol at  $-18 \text{ }^\circ\text{C}$ .

#### Enumeration of *E. coli*

The sample is screened for the presence of generic *E. coli* by plating 100  $\mu\text{L}$  of the dilutions (Preparation) on Rapid'E. coli 2 (3564024, BioRad) (incubation at  $44 \text{ }^\circ\text{C}$  for 24 h).

#### Enumeration of *Enterococcus*

Inoculation (100  $\mu\text{L}$ ) of the serial dilutions (see Preparation) will be performed on Slanetz-Bartley agar. Suspected *Enterococci faecalis/faecium* isolates are identified by MALDI-TOF and can be stored in BHI + glycerol at  $-18 \text{ }^\circ\text{C}$ .

### 7.4.3. Water samples

#### Enumeration of *Salmonella* spp.

Salmonella detection will be performed following ISO 19250:2010 with some minor modifications. 100 mL to 500 mL of surface water and 1 L of groundwater will be filtered through a cellulose ester membrane (142 mm Ø) with a pore size of 0.45 µm due to potential turbidity. The membrane will be placed in 100 mL sterile 0.1% (w/v) peptone water and homogenized for 1 min. Subsequently, an aliquot of the homogenized material will be mixed with a selective enrichment medium, Rappaport-Vassiliadis Broth (RV). After incubation for 24 h at 35°C, 100 µL will be subcultured on xylose lysine deoxycholate agar and incubated for another 24 h at 35°C.

Presumptive *Salmonella* colonies are further confirmed through a multiplex *Salmonella* and *Salmonella* Typhimurium PCR as described by Aabo et al. (1993) and Lin and Tsen (1999), respectively. Isolates will be stored in BHI + glycerol at -80 °C.

#### Enumeration of *Campylobacter* spp.

ISO 17995:2019 is used as a reference method for the isolation of campylobacters from wastewater and surface water samples. 100 mL to 500 mL of surface water and 1 L of groundwater will be filtered through a cellulose ester membrane (142 mm Ø) with a pore size of 0.45 µm due to potential turbidity. The filters will be transferred into Bolton broth for enrichment and incubated at 44°C in an anaerostat (AnaeroJar, Oxoid, Basingstoke, UK) under a microaerobic atmosphere (CampyGen 3.5 L, Oxoid, Basingstoke, UK). After 24 and 48 h, 100 µL of the culture will be spread on CCDA plates (CM0739, Oxoid) supplemented with CCDA selective supplement (mCCDA, Oxoid, SR0155E). These plates are also incubated under microaerobic conditions at 41.5°C for 24 and 48 h. Colonies of presumptive *Campylobacter* will be confirmed with a multiplex *Campylobacter jejuni* and *Campylobacter coli* PCR as described by Linton et al. (1997). Isolates will be stored in BHI + glycerol at -80 °C.

#### Enumeration of *E. coli*

100 mL to 1000 mL of surface water and 1 L of groundwater will be filtered through a cellulose ester membrane (142 mm Ø) with a pore size of 0.45 µm due to potential turbidity. The membrane will be plated on Chromogenic Coliform Agar medium (P05318A, Thermo Scientific) as described in UNE-EN ISO 9308-1 and incubated at 37°C for 24 h. Isolates will be stored in BHI + glycerol at -80 °C.

#### Enumeration of *Enterococcus*

100 mL to 1000 mL of surface water and 1 L of groundwater will be filtered through a cellulose ester membrane (142 mm Ø) with a pore size of 0.45 µm due to potential turbidity. The membrane will be placed on Slanetz-Bartley agar (CM0377, Oxoid) and incubated at 44°C for 48 h. Isolates will be stored in BHI + glycerol at -80 °C.

## 7.5. ANTIBIOTIC RESISTANCE BACTERIA

### 7.5.1. Manure samples

The same protocol as next described for soil samples will be used for manure samples.

### 7.5.2. Soil samples

A suspension ( $10^0$ ) and further sample dilutions ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ) are needed. Therefore, 99 mL of buffered peptone water (CM0509, Oxoid) is added to 11 g of soil. After homogenization in a stomacher for 1 min, three serial dilutions (1:10) of samples are prepared in buffer peptone water (BPW).

#### Enumeration of ESBL *E. coli*

To detect ESBL *E. coli*, 100  $\mu$ L of consecutive dilutions in BPW (following Section Preparation) is plated on *Brilliance* ESBL medium (P05302A, Oxoid) and incubated at 44 °C for 24 h.

#### Enumeration of *Enterococcus* resistant to vancomycin

To detect vancomycin-resistant *Enterococcus*, 100  $\mu$ L of the serial dilutions in BPW are plated on Slanetz-Bartley agar (CM0377, Oxoid) containing vancomycin with a concentration of 4  $\mu$ g/mL and incubated at 44 °C for 48 h. Suspected *Enterococci faecalis/faecium* isolates are identified by MALDI-TOF and can be stored in BHI + glycerol at -18 °C.

### 7.5.3. Water samples

#### Enumeration of ESBL *E. coli*

100 mL to 1000 mL of surface water and 1 L of groundwater will be filtered through a cellulose ester membrane (142 mm  $\varnothing$ ) with a pore size of 0.45  $\mu$ m due to potential turbidity. The membrane will be plated on *Brilliance* ESBL medium (P05302A, Thermo Scientific) and incubated at 44°C for 24 h. Isolates will be stored in BHI + glycerol at -80 °C.

#### Enumeration of *Enterococcus* resistant to vancomycin

100 mL to 1000 mL of surface water and 1 L of groundwater will be filtered through a cellulose ester membrane (142 mm  $\varnothing$ ) with a pore size of 0.45  $\mu$ m due to potential turbidity. The membrane will be placed on Slanetz-Bartley agar (CM0377, Oxoid) containing vancomycin with a concentration of 4  $\mu$ g/mL and incubated at 44°C for 48 h. Isolates will be stored in BHI + glycerol at -80 °C.

## 7.6. HEAVY METALS

### 7.6.1. Manure samples

Heavy metals (Mn, Cd, Cu, Pb, Cr, Ni, Zn) will be determined by means of inductively coupled plasma atomic emission spectroscopy (ICP-OES).

### 7.6.2. Soil samples

Heavy metals (Mn, Cd, Cu, Pb, Cr, Ni, Zn) will be determined by means of inductively coupled plasma atomic emission spectroscopy (ICP-OES).

### 7.6.3. Water samples

Heavy metals (Mn, Cd, Cu, Pb, Cr, Ni, Zn) will be determined by means of inductively coupled plasma atomic emission spectroscopy (ICP-MS).

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