Methods for the characterization of faecal sludge in Vietnam

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Abbreviations and acronyms

BOD_5	Biochemical oxygen demand
Cd	Cadmium
COD	Chemical oxygen demand
E. Coli	Escherichia coli
EAWAG	Swiss Federal Institute of Aquatic Science and Technology
EPFL	Swiss Federal Institute of Technology of Lausanne
Fe	Iron
H. eggs	Helminth eggs
NH_4^+	Ammonium
Ni	Nickel
Pb	Lead
PO_4	Phosphate
QA/QC	Quality Assurance/Quality Control
rpm	round per minute
SANDEC	Department of Water and Sanitation in Developing Countries
Std	Standard
TN	Total nitrogen
TP	Total phosphorus
TS	Total solid
TSS	Total suspended solid
URENCO	Urban Environmental Company
VFA	Volatile fatty acid
VS	Volatile solid
VSS	Volatile suspended solid
WWTP	Wastewater treatment plant
Zn	Zinc

* The two authors contributed equally to the preparation of the document.

1. Introduction

Worldwide, 2.7 billion people rely on onsite sanitation technologies (Strande, 2014). It is therefore of great importance to be able to accurately determine the characteristics of the resulting faecal sludge in order to develop appropriate faecal sludge management plans, and to design adequate treatment technologies. Yet no standard reference exists of methods for the analyses of faecal sludge as there are for other environmental fields, such as *Standard Methods for the Examination of Water and Wastewater* (Baird et al., 2012) or *Methods of Soil Analysis* (Dane and Topp, 2012). Methods to characterize wastewater and soils are frequently adapted to faecal sludge. However, faecal sludge characteristics are much more variable, and sampling methodologies are not established. The result is that methods have to be adapted on an individual project or research basis.

Faecal sludge is defined by Strande (2014) as follows:

> *«Faecal sludge comes from onsite sanitation technologies, and has not been transported through a sewer. It is raw or partially digested, a slurry or semisolid, and results from the collection, storage or treatment of combinations of excreta and blackwater, with or without greywater. Examples of onsite technologies include pit latrines, unsewered public ablution blocks, septic tanks, aqua privies, and dry toilets. FSM includes the storage, collection, transport, treatment and safe enduse or disposal of FS. FS is highly variable in consistency, quantity, and concentration.»*

The goal of the PURR project (Partnership for Urban Resource Recovery) is to assess the potential of anaerobic digestion as a treatment technology for faecal sludge in Vietnam. In urban areas of Vietnam, the majority of households rely on septic tanks, which are emptied by private and public companies.

This document presents the methodologies that were developed and used for the sampling procedures and laboratory analyses of faecal sludge during the PURR study in Hanoi, Vietnam. Some aspects of protocols were adapted specifically to the local context, but in general methods should also be readily adaptable to other locations and contexts.

Schematized in Figure 1 are the main steps that were taken during the characterization of faecal sludge. These steps are further developed in sections 2, including information on sampling; 3, on laboratory organization; and 4, on preparation and preservation of samples. All the methods are provided in the Annexes.

Annex 12 presents the synthetic sludge recipe that was developed for laboratory tests with an anaerobic reactor. This recipe can also be used or adapted for other tests concerning biological treatment options.

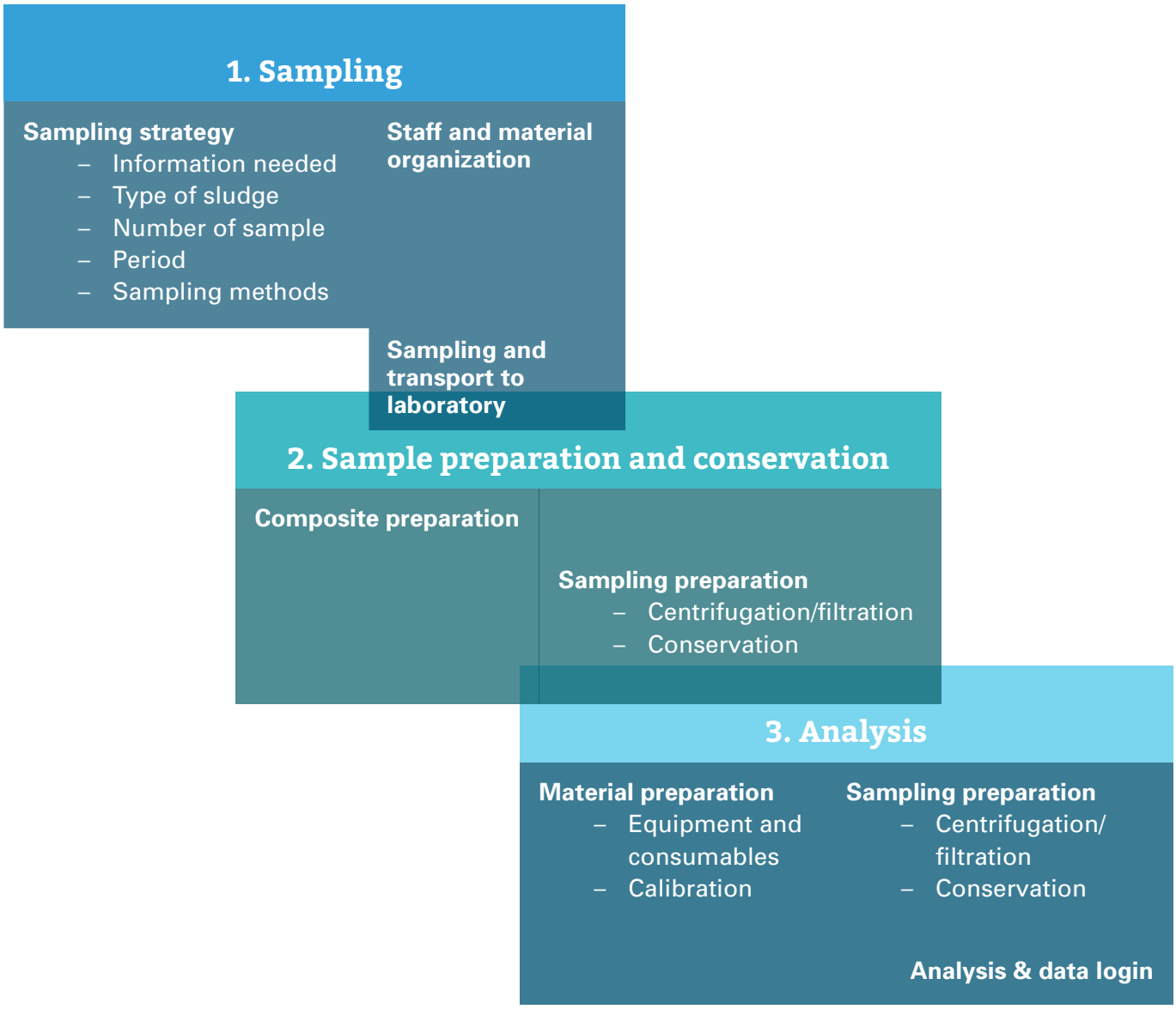

Figure 1: Steps required for reliable characterization study of sludge

2 Sampling

When designing a sampling campaign, it is necessary to determine a sampling strategy, identify relevant sampling locations, select methods, properly train employees, organize materials, and ensure a safety plan is followed.

2.1 Sampling strategy

First, decisions need to be made on the type of information that will be required, and the level of detail. For example, is the study for design of a new, or optimization of an existing treatment plant? To monitor an existing treatment plant? Or for research to understand faecal sludge characteristics and rates of stabilization?
In most cases it will be essential to estimate quantity in addition to characteristics (Strande et al., 2016). The number and different types of systems will also influence decisions, as well local discharge standards. The sampling strategy includes the following:

Type of information required – This includes the desired output of the campaign, for example understanding variability of faecal sludge, or characterization on a regional scale for a treatment plant. This will influence the extent, duration and spatial distribution of the sampling campaign.

Level of required accuracy – This will determine the number and location of samples.

Type and number of sanitation systems – This is determined by the existing sanitation infrastructure, including:

- Onsite systems (e. g. lined / unlined latrine, septic tank, public toilet, community ablution blocks)
- Wastewater treatment plants (e. g. primary, secondary, post-treatment)

Geographic scope – The physical boundaries of the sampling campaign need to be defined.

Analytical parameters – The type of parameters that will be analysed will influence required sample volumes, preparation, storage, and required laboratory capabilities.

Material and means for sampling – This will impact the planning of safety, transport, and required staff.

When beginning a new study, the following aspects that influence the characteristics of faecal sludge need to be considered:

Physical context: Climate, hydrology and hydrogeology

Usage of onsite system: Number of users, additives, inputs to system (e.g. blackwater from toilet, greywater from kitchen)

Design of sanitation system: Type of technology (e.g., pit latrine or septic tank), number of chambers, storage duration and conditions (temperature, humidity, etc.), volume of storage, percolation or groundwater intrusion

State of onsite system: Construction quality, size, demographic data, building usage

Collection and transport: Manual / mechanical emptying, pump capacity, sludge dilution during emptying, addition of chemicals to remove odours

Collecting preliminary information is of outmost importance in developing a sampling plan. To ensure the accuracy of sampling, a quality assurance and quality control (QA/QC) procedure needs to be implemented, this is further explained in section 3.1.

2.2 Sampling location and method

Sampling locations will be directly influenced by the goal of the study. For example, if the goal is to design pumping technologies, faecal sludge should be sampled from onsite systems. If the goal is to design a treatment plant for the existing situation, then faecal sludge being transported, or discharged from collection and transport trucks should be assessed. In Vietnam, most of the faecal sludge collected is discharged illegally. Therefore, collection and transport companies are generally reticent to sample at discharge sites, as it draws attention of authorities. Samples can thus be taken individually for each septic tank at the household, just after pumping from truck.

Collection methods also directly influence the results, as faecal sludge is generally not homogenous. A sampling method has to be defined to be representative of the sludge.

Sample locations included in PURR studies included the following, as presented in (Bassan et al., to be published):

Household faecal sludge

In general, when faecal sludge is emptied from septic tanks, 100 % of the tank is not collected, and emptiers frequently add water to thick sludge to aid in collection. In the study conducted in Hanoi, samples were taken directly from access ports on the top of the trucks immediately following collection to get a representative sample of faecal sludge that would be delivered to a treatment plant.

As shown in Figure 2, a simple core sampling device was constructed to obtain a representative sample of the truck contents. Sampling devices should have a sufficient diameter to allow collection of thick sludge with coarse particles (at least 5 cm internal diameter), and have a height of at least 150 cm to sample the entire depth.

Larger and denser particles settle very rapidly in the truck, while the upper layer in the truck is less dense. The core device is essential to obtain a representative sample of faecal sludge, including all the layers of faecal sludge, from settled sludge that is at the bottom of the tank to supernatant at the top. It prevents sample bias due to more obtaining supernatant or thickened sludge.

To take a sample, the access port of the truck (i.e., manhole) was opened immediately following collection of faecal sludge from the septic tank. The sample device was inserted until it touched the bottom of the tank with the end open, so that the sludge can enter the tube, and was then closed to remove the core sample. The core sample was then collected in a bucket, and sent to laboratory. When several trucks are required to empty an onsite system, the same number of core should be taken from each truck to collect a representative sample of the entire volume of faecal sludge pumped out.

In addition, a questionnaire should be used to collect information such as the inputs to the septic tank, frequency of sludge collection, age of system, and design of the onsite system (see questionnaire in annex 14). This provides useful information to understand and predict faecal sludge characteristics. It will also help in the management of potential future treatment plants.

Public toilet faecal sludge

In Hanoi, is a legal discharge site for public toilet faecal sludge exists, and these trucks did not allow core sampling. Therefore, samples were collected during truck discharge. As shown in Figure 3, a "bucket" collector was used to collect known volumes of sludge during discharge. The bucket needs to be mounted on the end of a bar that is at least 1m long to obtain faecal sludge from the discharge valve, and to avoid being splashed by sludge. Composite samples were taken, consisting of four samples of equal volume. One sample was taken at the beginning (i.e., at valve opening), two samples in the middle and

one sample at the end of the discharging period. At least a 1 L volume is recommended for the sub-samples to ensure representativeness. This method can also be used for sampling of any type of faecal sludge, if access to the discharge point can be arranged.

Wastewater sludge

For this study, samples were also obtained from wastewater treatment plants, for which methods are better established, but sampling still needs to be considered carefully. Depending on the goal of the sludge characterization, wastewater sludge can be sampled from different locations.

For example, if the goal is to monitor the operation of the sludge treatment unit, the defined volume of wastewater sludge should be collected before and after the sludge treatment unit. If the tanks are well-mixed, the sludge is considered homogeneous, and can be collected directly below the surface with a bucket. Otherwise, collecting in pipes before and after treatment is a possibility, or a tube-like sampling device can also be used (see picture 2). Sampling in wastewater treatment plants also needs to consider weather (e. g. heavy rains) and diurnal variations of flows, and should be collected over a period of time.

When samples are collected, they should be placed in clean bottles that are filled completely to the top, and stored on ice during transport to the laboratory. Temperature and pH should be measured immediately on site from sample bottles, and transport to the laboratory should be as rapid as possible.

Figure 2: Bottom of the core sampling device

Figure 3: Sampling with a bucket mounted on a bar

2.3 Sampling staff

People collecting samples need to be adequately trained, as the handling of faecal sludge and wastewater sludge involves health risks from pathogens, and other potentially harmful components. In addition, they should be informed about the goal of the sampling campaign, and types of sanitation systems.

The number of people required for sampling events will depend on the sampling method and strategy. During PURR characterization studies, when samples were taken from household septic tanks, three people were requires for sampling. One person was in charge of obtaining information from the household and emptiers (e.g., questionnaire, observations, monitoring, and pictures). Two people were responsible for collecting sludge from the truck (i.e., core sampling from access port), measuring temperature and pH with portable probes, cleaning sampling materials onsite, and transport to the laboratory.

If samples are collected at legal discharge locations, sampling can be carried out with two people.

2.4 Sampling materials

As shown in Figure 4, required materials include safety equipment, sampling, transport and cleaning materials. An example of a sampling kit includes the following:

- Thick protective clothing, including pants, long-sleeve jacket, and rubber boots
- Protective equipment including laboratory gloves, long rubber gloves, protective glasses and mask with activated carbon filter
- Sampling device (core sample, or bucket mounted on a bar)
- Bucket of 10 L with graduations of 1 L
- Container of 1 L with a spout to transfer sludge to storage containers
- Funnel
- Multiple containers with variable sizes (e.g., 0.5 L and 1 L), depending on sample volume needed
- Labels and pens to identify samples in bottles (i.e., date, location, the type of onsite system, and other important information)
- Cold box with ice
- Portable pH meter and thermometer
- Water and wash paper for cleaning instruments
- Water for cleaning if there is no available water at the sampling site, at least 4 L
- Soap and javel (bleach) for rapid cleaning of materials on site
- Plastic bags to store personal belongings (e.g., shoes, clothes, wallet, mobile phone)
- Flashlight
- Measuring stick (steel rule)
- Tools to open the access port
- Camera
- Questionnaires and pen to record information on the sampling site
- Car or motorbike to transport material

Figure 4: Material used for the sampling during the characterization study in Hanoi

Safety equipment is described in the following section. The number of containers required, and the size of the sampling devices will vary based on the volume of sludge that is sampled. This volume is determined by the number of parameters that will be analysed, and should be at least 1 L per sample.

2.5 Safety during sampling

In order to ensure safety and health while sampling, it is important to avoid direct contact between sludge and skin. To ensure this, the following procedures need to be followed:

- Wear protective sampling clothes (pants, jacket, and boots) which are only worn at the sampling site; store clean clothes and shoes in a closed plastic bag in a clean environment.
- Wear protective eyewear.
- Always wear two pairs of gloves (laboratory gloves covered by long rubber gloves).
- Once all samples are taken and onsite parameters analysed, clean all materials and devices with water and bleach, directly on the sampling site, taking care not to spread faecal sludge in the environment.
- Once all material and device are properly cleaned, and all containers are in the cold box, take off the long rubber gloves.
- Take off sampling shoes and your sampling clothes, put all sampling clothes and safety gears in a closed bag.
- Take off the laboratory gloves.

Wash the sampling area with clean water and make sure to leave a clean environment around the sampling site before leaving.

3 Laboratory organization

Sample preservation and preparation will depend on the parameters that are to be analysed, and available time. In order to ensure reliable and accurate results, the following aspects need to be planned in advance, and are discussed below:

- Develop quality assurance and quality control procedures (QA/QC), including blanks, duplicates and calibration.
- Determine parameters and number of analyses.
- Determine required materials and laboratory equipment.
- Determine consumables, including laboratory supplies, chemical reagents and preservation methods.
- Outline laboratory safety measures for analyses.
- Outline time and logistics required to perform all analyses.

3.1 Quality assurance and quality control

To ensure the quality of analyses and accuracy of results, a QA/QC program must be followed. Quality Assurance (QA) entails following a set of operating principles during sample collection and analyses. Quality Control (QC) entails incorporating methods to verify reliability of results. If inaccurate or non-reproducible results are obtained, then the source of error can be identified, and methods and procedures adjusted. The following strategy was applied during the PURR characterization study, and can be followed in general to ensure reliable results, and more information on QA / QC can be found in (Rice et al., 2012) and on *https://www.epa.gov/measurements*:

- Calibration of all laboratory instruments before and during use, including standards, dilution curves and blanks (see below).
- Duplicate field samples every 10 sample events to ensure reproducibility of sampling method (i. e., two samples are taken at the same location, from the same sanitation technology, at the same time and processed blind in parallel).
- Duplicate analysis every eight samples for all parameters to ensure reproducibility of analytical methods (i. e., larger sample volumes need to be taken, so that it is possible for each parameter to be analysed two times and results compared).
- It is recommended that the relative error does not exceed 10 %. If difference between duplicate analyses performed on the same sample is greater than 10 %, trouble shooting is required to determine the source of error in the analytical method.

Standards are solutions of known concentrations that are used to calibrate analytical machines, and ensure accuracy during analysis. Before running a series of analyses, standards of different known concentrations that extend below and above the expected concentration of samples are analysed. This allows verifying or adjusting the equipment to ensure accurate results. Standards can also be spiked into samples to test recovery, accuracy in different matrices, and overall accuracy of laboratory results. It is also recommended that one designated person in the laboratory is overall responsible for each analytical machine.

A solution was made with distilled water, sodium oxalate, ammonium sulfate and disodium hydrogen phosphate to prepare standard for PURR study (for solution preparation, see Annex 10).

3.2 Analytical parameters

Analytical parameters are determined based on the objectives of the characterization study. Physical, chemical and biological parameters all influence the design and on-going operation of treatment plants. Basic information on each parameter used in PURR characterization studies is presented for staff collecting samples below, and more information can be found in (Niwagaba et al., 2014). Analytical methods are presented in the annexes 1 to 9. The methods used were adapted from and based on Standard Methods for the Examination of Water and Wastewater (Rice et al., 2012).

3.2.1. Onsite measures

It is important to take onsite measure rapidly onsite upon sampling.

Temperature is an important parameter in understanding and predicting rates of biological activity, treatment processes, and pathogen die-off. Temperature is measured with a probe immediately at the sampling point.

pH (potential hydrogen) is a measure of the acidity (< 7) or alkalinity (> 7) of sludge based on the chemical activity of hydrogen ions in solution. pH also has a strong influence on biological processes, including pathogen inactivation. The pH is measured with a probe immediately at the sampling point.

3.2.2. Solid and organic content

Solid and organic content analyses are conducted in the laboratory. Methods are presented in Annexes 1 and 2.

Total solids (TS) is a measure of the solid fraction of sludge, and greatly influences the solid/liquid separation or dewatering technologies.
TS is quantified as the matter remaining after heating the sample at 105 °C for 24 h (or until weight no longer changes). Results are reported as mg/L or g/L (see annex 1).

Volatile solids (VS) is a measure of the solid organic matter in sludge, and is an indicator of potential biodegradability.
VS is quantified as the matter that is volatilized during heating at 550 °C. Results are reported as mg/L or g/L (see annex 1).

Total suspended solids (TSS) is a measure of organic and mineral matter that is suspended in sludge. This is the part of solid fraction that settles faster, for example in settling-thickening tank or be retained in drying beds.
TSS is quantified as the sludge that does not pass through a 0.45 µm filter after heating the sample at 105 °C for 24 h. Results are reported as mg/L or g/L (see annex 1).

Volatile suspended solids (VSS) is a measure of the organic fraction of the TSS, and are an indicator of what can be degraded during aerobic or anaerobic processes.
VSS is quantified as the amount of TSS volatilized during heating at 550°C. Results are reported as mg/L or g/L (see annex 1).

Chemical Oxygen Demand (COD) is a measure of total organic compounds that can be degraded by biological and chemical processes. It is commonly used as a design value, and tests are relatively easy to implement and reproduce (more than Biological Oxygen Demand – BOD).
COD is quantified as the oxygen needed to oxidize all organic matter in a sample. Results are reported as mg/L or g/L (see annex 2).

Soluble Chemical Oxygen Demand (SCOD) is a measure of the soluble organic fraction of COD, and are generally more readily degradable than non-soluble COD.
SCOD is quantified as the COD in the fraction following filtration at 0.45 µm. Results are reported as mg/L or g/L (see annex 2).

3.2.3. Nutrients content

Nutrients content analyses are conducted in the laboratory. Methods are further presented in Annexes 3 and 4.

Total nitrogen (TN) is a measure of the total nitrogen in the sludge (organic and inorganic), and is an important nutrient for growth, inclu-

ding organisms in treatment processes, enduse in agriculture, and also negative environmental impacts.
Total nitrogen is quantified as the sum of total kjeldahl nitrogen (ammonia, organic and reduced nitrogen), NO_3 and NO_2.
NO_3 and NO_2 are expected to be at very low concentrations in anaerobic conditions of septic tanks. Results are reported as mg/L or g/L of total N (see annex 3).

Ammonium (NH_4^+) is a measure of the bioavailable form of N in anaerobic sludge, and is important for growth, but also high concentrations can be toxic.
It is quantified as nitrogen in ammonium (N–$NH4^+$) after a reaction in alkaline solution by colorimetry. Results are reported as mg/L or g/L as N (see annex 3).

Total phosphorus (TP) is a measure of the sum of all P (dissolved form – orthophosphate, inorganic and organic). Similarly to nitrogen, phosphorus is an important source of nutrients.
It is quantified by colorimetry after acid hydrolysis. Results are reported as mg/L or g/L as P (see annex 4).

Ortho-phosphate (PO_4^{3-}) is the dissolved form of phosphorous, and is the bioavailable form which is important for growth.
It is quantified after reaction in an acidic reaction by colorimetry as phosphorus in PO_4^{3-} ($P\text{-}PO_4^{3-}$). Results are reported as mg/L or g/L as P (see annex 4).

3.2.4. Parameters influencing anaerobic digestion

In addition to the above parameters, Volatile Fatty Acids (VFA) and heavy metals are used to monitor digester operation (see Annexes 5 and 6).

Volatile Fatty Acids (VFA) are short chain fatty acids that originate from biodegradation of organic matter and are an important metric to ensure stable operation of anaerobic digesters. VFA accumulation causes a pH decrease that is inhibitory for anaerobic digestion.
It is quantified as equivalent acetate content after esterification. Results are reported as mg/L or g/L (see annex 5).

Heavy metals (Iron (Fe), Zinc (Zn), Nickel (Ni) and Lead (Pb)) are important for microbial growth at low concentrations, but can inhibit biological processes at high concentrations.
They are quantified after a first step of digestion in acid that allow releasing all metals into measurable form. Results are reported as mg/L or g/L see annex 6).

3.2.5. Pathogens

Pathogens are excreted with faeces and are responsible for the spread of human diseases. Indicator organisms are used as a metric of pathogen concentrations (see Annexes 7, 8 and 9).

***Salmonella* spp.** are used as an indicator for bacterial contamination.
They are quantified by plating methods with a differential media. Results are reported as number of colony forming unit per litre.

Ascaris lumbricoides are used as an indicator for faecal contamination of water. They are widely used due to their high resistance, and ability to remain for months to even years in sludge or soils.
They are extracted through various steps or washing and filtration, and enumareted with microscope. Results are reported in number of eggs per litre.

Table 1: Timing for analytical procedures in the laboratory.

Parameter	Maximum time for analysis following collection	Total time required to complete analysis	Time for digestion / mineralization
VFA	As quickly as possible	15 min	10 min
NH_4^+	2 days	25 min	No
PO4	2 days	25 min	No
TN	2 days	1 h 40 min	1 h
TP	2 days	1 h 40 min	1 h
COD	2 days	2 h 30 min	2 h
Soluble COD	2 days	2 h 30 min	2 h
TS-VS	7 days	27 h 20 min	24 h + 3 h
TSS-VSS	7 days	27 h 20 min	24 h + 3 h
Metals	-	1 h 50 min (Fe, Pb, Ni, Zn)	1 h

3.3 Laboratory timing

A laboratory plan needs to be developed to optimize time during sampling, transportation, preservation and analyses (including availability of machines). It is essential to analyze samples in a timely fashion to ensure accurate results, and prevent changes due to factors such as degradation or volatilization. Reasonable time frames for analyses, if samples are prepared and stored properly, are presented in Table 1. These timings are general recommendations, and changes can be made within reason, especially if it allows for several samples to be analyzed for all parameters at the same time, for example in order of SCOD, COD, TP, TN, TS, TSS, NH_4^+, $P\text{-}PO_4^{3-}$.

When several people are involved in sampling and analysing the samples, it is useful to have a table in the lab where all tasks are recorded, with the responsible person for each task, and their contacts. This type of checklist facilitates the organization of the analytical activities (see exampling in annex 12). A standard operating procedure (SOP) should also include a log book where all analytical activities, responsible parties, use of machines and calibrations including dates and comments.

Each person working in the laboratory also needs to maintain their own laboratory log book to record all analyses, samples, experimental procedures, QA/QC, information on problems or uncertainties during the analyses, etc. Lab-books need to be written in pen, cannot have removable pages, and should be kept in the laboratory at all times. Information on how to keep a lab book can be found *http://www.ruf.rice.edu/~bioslabs/tools/notebook/notebook.html.*

3.4 Laboratory materials and equipment

The following equipment is required to perform the analyses of the parameters described in section 3.2:

- Oven that can maintain 105 °C
- Muffle furnace that can maintain 550 °C
- Spectrophotometer for reading test tube kit results. For example, Hach provides test kit tubes of 16 mm diameter, and Hach-Lange 13 mm diameter
- Scale with 0.1 mg accuracy
- Pipette 10 ml
- Pipette 1 ml
- Digester to hold tubes required for analyses, with thermostat that can be set at 100 °C (TN and TP analyses), and 148 °C (SCOD and COD analyses)
- Centrifuge with a speed of 5000 rpm (e.g., rcf = 3820 × g, rotor 220.97 V 01)
- Vacuum pump
- Hood to manipulate hazardous and volatile substances
- Glasses, ladle, and other current laboratory consumables

The required chemicals for the analyses are described with the protocols for each parameter in annexes.

3.5 Laboratory safety

In order to work in safe conditions, direct contact is to be avoided with sludge and reagents. Sludge should be assumed to contain pathogens such as virus, bacteria, protozoan, parasite, and some hazardous chemicals for human health might be used during analysis (e.g., acids). Therefore, the following minimum precautions are to be strictly respected. More complete information can be found on *https://sis.nlm.nih.gov/enviro/labsafety.html*:

- Always read safety related indications on the chemical boxes and bottles prior to use.
- Always wear laboratory jacket, closed shoes, long wears.
- Always wear laboratory glasses when manipulating sludge and chemicals.
- Always wear vinyl laboratory gloves when manipulating sludge and chemicals.
- Wear active carbon mask when manipulating sludge and chemicals.
- If you get your skin in contact with chemicals, clean with detergent and rinse thoroughly with water.
- Always clean the working space and your hand with detergent and ethanol at the end of analysis.
- Ensure that chemicals and products are stored in a closed and safe location.
- Never eat, drink or smoke in the laboratory, never bring food or drink into the laboratory.
- Sulfuric acid is capable of causing very severe burns.

4 Preparation and preservation of the samples

Required sample volumes are determined by the number and type of analytical procedures to be carried out, for more information refer to annex 11. As described above, as soon as samples are taken, they should be stored on ice and transported to the laboratory as soon as possible.

4.1 Composite preparation

Following collection and transportation to the laboratory, upon arrival composite samples need to be prepared. All aliquots from one sampling point need to be combined and stored in one container (except for duplicates that are done in parallel).

Once the sample has been combined, then it needs to be homogenized and sub-samples prepared in volumes relevant to the selected analytical procedures. To homogenize samples, stir rapidly with a ladle and then immediately distribute to sub-sample containers. In order to ensure the representativeness of the sub-samples, be sure to include equal and representative quantities of liquid and solids in all jars). Leave approximately 2 cm of headspace at the top of the jar. All containers are to be labelled with the date and sample code and stored at 4 °C in the fridge.

The following distribution of samples was used during the characterization study in Hanoi, and was for analyses of the presented parameters. For design studies or monitoring, the number of bottles can be reduced according to the number of parameters analyzed.

- 0.35 L plastic bottle for *E. coli* and *Salmonella* analyses in external laboratory
- 0.35 L plastic bottle for Helminth eggs analysis in external laboratory
- 0.35 L plastic bottle for preservation (see details below)
- 1 L (2 × 0.5 L plastic bottle) for laboratory analysis (i.e. TS, VS, TSS, VSS, COD, TN, TP, protein, lipid, carbohydrate, Ni, Zn, Fe, Pb)
- 4 × 50 ml (Falcon tubes filled to the top) for centrifugation for the soluble parameters (i. e. SCOD, NH_4^+, PO_4, SO_4, VFA) (i. e., 6 × 50ml if duplicate analysis)
- 800 ml for drying at 105 °C for total fibers and heavy metals in crucible or for short storage in plastic bottle

In addition, at least one aliquot of sample should be stored to repeat tests in case of errors or uncertainties.

4.2 Filtration for soluble parameters and suspended solids

The following procedure is recommended for filtration of septic tank, public toilet sludge (for characteristics similar to faecal sludge in Hanoi), and wastewater sludge, and was adapted from Standard Methods (Rice et al., 2012). Faecal sludge from public toilets in this study refers to sludge collected from storage tanks that are collecting waste, and are emptied frequently (e.g. days to weeks). For thicker sludge, a preliminary dilution might be required (e. g. for unlined pit latrines). To analyze soluble parameters (i.e. VS, VSS, SCOD, NH_4^+, PO_4, SO_4, VFA, ripley ratio, and other soluble compounds), filtration should be completed within 24h of sampling.

As faecal sludge and wastewater sludge tends to have higher total solids than wastewater, samples can be centrifuged at 6000 rpm for 10 to 20 min (rcf = 3820 × g, rotor 220.97 V01). Then, the supernatant can be filtered with 1.5 µm porosity filters (Whatman 1827-110 Grade 934-AH), followed by 0.45 µm porosity with a Büchner funnel and glass Büchner flask with vacuum pump. If filters clog, replace them with new ones. It might require multiple filters for the entire sample volume. Following filtration, the supernatant is stored in labelled 50 ml Falcon tubes. One of the tubes should be set aside for preservation, as discussed below.

4.3 Preservation procedure

Preservation of samples is crucial to allow reliable analytical results. Sludge composition changes with the time, depending on factors such as light, oxygen, temperature and microbial activity. Analyses should only be done on well preserved samples, and within the period in which the results will be representative of the initial sludge composition (see Table 1). Depending on the analysis, to extend the storage time sub-samples can be preserved by the addition of preservatives such as H_2SO_4 or HNO_3 (Centre d'expertise en analyse environnementale du Québec, 2012). These include the following analyses:

Total COD, total nitrogen, total phosphorus can be analysed within 28 days if the samples are preserved with sulphuric acid. 1 ml of sulphuric acid H_2SO_4 (97 % pure) should be used for each 100 ml of sample. Because the reaction of sulphuric acid with water is highly exothermic, it is recommended to slowly add sulphuric acid to the sample. In addition, to preserve the sample composition, it is important to for the suspended solid to settle at the bottom, to avoid sulphuric acid directly reacting with the coarse solids.

Soluble COD and NH_4^+ can be analysed within 28 days if the samples are preserved with sulphuric acid. In this case, 1 ml of sulphuric acid H_2SO_4 (97 % pure) should be used for each 100 ml of sample, following sample filtration (i. e., addition of H_2SO_4 to the supernatant after filtration).

Soluble metals can be analysed within 180 days by adding nitric acid HNO_3 to lower the pH < 2.

The other parameters cannot be analysed on samples following preservation with acid. They must be analysed on the collected or filtered samples. Samples should always be stored at a maximum temperature of 4 °C, and for a minimum period of time, to limit biologically induced changes. If external laboratories are responsible for conducting analyses, samples should be delivered on ice directly to that laboratory on the day of sampling.

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Figure 5:On-site measure of pH and temperature with probes. Photo: Sandec

Annex
Laboratory protocols

Annex 1

Analysis protocol for TS/VS and TSS/VSS

Substrate

Total sample for TS-VS and supernatant after filtration at 1.5 and 0.45 µm for TSS-VSS

Time

27 hours (24 h in oven + 2 hours in furnace)

Materials

- 100 ml of feacal sludge (200 ml if duplicate)
- 2 50 ml Falcon tubes
- 2 crucibles (4 if duplicate)
- 50 ml of Sterile distilled water (SDW)
- 1.5 and 0.45 µm porosity Whatman filters
- 110 mm diameter Büchner funnel
- Glass Büchner flask
- A ladle to homogenize sample

Note

For faecal sludge from septic tanks, it is assumed that sludge density is equal to water density, based on laboratory evaluation conducted during this study. The volume V is determined by weight (1 L = 1kg).

Protocol TS & VS (Adapted from Standard methods 2540 B and 2540 E)

1. Put the crucible for 24 h in the oven at 105 °C (if volatile solids are to be measured, ignite a clean evaporating dish at 550 °C for 1 h)
2. Cool down the crucible 15 min in a **desiccator**
3. Weigh the crucible, $\mathbf{P_1}$. Do not use hands, use tongs/pliers
4. Homogenize the sample with a ladle and immediately fill the crucible with the volume **V** of sample. Use a **ladle** (the ladle should be always emptied to keep all particles for analyzing), and an **accurate scale** 0.1 mg to measure the volume of the sludge. sludge density can be considered equivalent to water density
5. Weigh the crucible + sample, $\mathbf{P_2}$. Do not use hands.
6. Put it in the oven **24 h at 105 °C**
7. Cool down the crucible 15 min in a **desiccator**
8. Weigh crucible + dry sample, $\mathbf{P_3}$. Do not use hands.
9. Put it in the muffle furnace **2 h at 550 °C**
10. Wait for the furnace to cool down to 100–200 °C before opening (rapid change in temperature could damage the sample)
11. Weigh crucible + sample, $\mathbf{P_4}$. Do not use hands.

$$TS\ [g/l] = \frac{P_3 - P_1}{V} \times 1000 \quad ; V\ [ml];\ P\ [g]$$

$$VS\ [g/l] = \frac{P_3 - P_4}{V} \times 1000 \quad ; V\ [ml]$$

Protocol

TSS & VSS (Adapted from Standard method 2540 D and 2540 E)

1. Put the crucible and the filter 24 h in the oven at 105 °C (if volatile solids have to be measured, ignite a clean evaporating dish at 550 °C for 1 h)
2. Cool down the crucible + filter 15 min in a **desiccator**
3. Weigh the crucible + filter, $\mathbf{P_1}$. Do not use hands.
4. Weigh a empty Falcon tube of 50 ml (make sure that the tube is dry), $\mathbf{P_2}$
5. Homogenize the sample with a ladle and immediately fill the crucible with the volume V of sample. Use a **ladle** (the ladle should be always emptied to keep all particles for analyzing), and an **accurate scale** 0.1 mg to measure the volume of the sludge. sludge density can be considered equivalent to water density
6. Weigh the Falcon tube + raw sample $\mathbf{P_3}$;
   $V = P_3 - P_2$ = (Falcon tube + raw sample) – (empty Falcon tube)
7. Centrifuge at **6000 rpm during 20 min (rcf = 3820 × g, rotor 220.97 V01)**.
   Equilibrate the tubes at **0.01 g** before centrifuging
8. Carefully filter the supernatant with a **1.5 µm** porosity filter.
9. Recover the solid part in the tube with SDW and dispense it in the crucible with the filter
10. Put it in the oven **24 h at 105 °C**
11. Cool down the crucible 15 min in a **desiccator**
12. Weigh crucible + filter + dry sample, $\mathbf{P_4}$. Do not use hands.
13. Burn it in the furnace **2 h at 550 °C**
14. Wait the furnace cooling down to 100–200 °C before opening (rapid change in temperature could damage the sample
15. Weigh crucible + Filter + burned sample, $\mathbf{P_5}$. Glass filter does not lose weight while burning at 550°C. Do not use hands.

$$\text{TSS [g/l]} = \frac{P_4 - P_1}{V} \times 1000 \quad ; \text{P [g]} \ , \text{V [ml]}$$

$$\text{VSS [g/l]} = \frac{P_4 - P_5}{V} \times 1000$$

Annex 2

Analysis protocol for Total COD and Soluble COD

Dichromate method according to standard ISO 6060-1989, DIN 38409-H41-H44

Time

2 h 30 min for each of the methods (the samples can be put to mineralize at the same time)

Substrate

total sample for Total COD, supernatant after filtration at 1.5 and 0.45 µm; if necessary, dilute the sample to allow filtration. 10 ml of faecal sludge is enough to analyze both parameters

Note

Always use a new pipette if you have to pipette faecal sludge several times. If sample too thick, cut the extremity of the tip to allow pipetting

Protocol

Total Chemical Oxygen Demand (COD): Hach Kit LCK 014 (ISO 6060-1989)

Materials

- 10 ml of faecal sludge
- 100 ml of SDW (Sterile distilled water)
- 1 50 ml volumetric flask (depends on how many dilutions)
- 1 reagent reservoir (depends on how many dilutions)
- 1 1–10 ml pipette
- 1 100–1000 µl pipette

Note

- Measuring range: 1000–10,000 mg/l COD
- Storage conditions: 15 - 25 °C (protect from light)
- **Factor dilution of 10** tends to be a reasonable range for faecal sludge from septic tanks in Hanoi.
- If sample is too thick, cut the very end of the pipette tip to allow pipetting.

1. Preheat the thermostat to **148 °C**
2. **Homogenize** the sample, therefore, gently mix with a ladle and pipette immediately
3. **Perform analyses as indicated in the kit notice, ensuring a fix time of cooling after heating in the thermostat, and cleaning the test tube before measuring in a calibrated photometer.**

Protocol

Soluble COD: Hach Kit LCK 514

Materials

- 10 ml of faecal sludge
- 100 ml of SDW (Sterile distilled water)
- 1 50 ml Falcon tube
- 1 25 ml volumetric flask (depends on how many dilutions)
- 2 reagent reservoirs (depends on how many dilutions)
- 1 1–10 ml pipette
- 1 100–1000 µl pipette
- 1.5 and 0.45 µm porosity Whatman filters

Note

- Measuring range: 100–2000 mg/l COD
- Store the Reagent in a fridge
- **No dilution** is recommended for faecal sludge from septic tanks in Hanoi, unless it is required to allow filtration.

1. Preheat the thermostat at **148 °C**
2. **Use a ladle to fill the Falcon tubes in a homogenize way and then centrifuge, 6000 rpm during 20 min (rcf = 3820 × g, rotor 220.97 V01)**
3. **Filter** the supernatant with a filter of **1.5 µm**, and then with **0.45 filter**
4. **Perform analyses as indicated in the kit notice, ensuring a fix time of cooling after heating in the thermostat, and cleaning the test tube before measuring in a calibrated photometer.**

Annex 3

Analysis protocol for TN and NH_4^+

Substrate used

Total sample for TN; supernatant after filtration at 1.5 and 0.45 µm for NH_4^+. If necessary, dilute the sample to allow filtration.

Note

- **Always use a new pipette if you have to pipette faecal sludge several times. If sample too thick, cut the extremity of the tip to allow pipetting.**
- Measure each sample 2 times in the photometer and use the average.
- **[NH_4^+] < [TN]**

Protocol

Total Nitrogen
Kit Total N: Hach Kit, LCK 338 (EN ISO 11905-1)

Standard method

EN ISO 11905-1 Digestion with Peroxodisulphate (Koroleff Digestion (Peroxodisulphate), and Photometric Detection with 2.6-Dimethylphenol)

Time

1 h 40 min

Materials

- 10 ml of faecal sludge
- 100 ml of SDW (Sterile distilled water)
- 1 20 ml reaction tube
- 1 50 ml volumetric flasks (depends on how many dilutions)
- 1 reagent reservoirs (depends on how many dilutions)
- 1 1–10 ml pipette
- 1 100–1000 µl pipette

Note

- Measuring range: 20–100 mg/l of N
- pH must be within the range 3–12 (adjust, if necessary)
- Temperature of the sample: 15–25 °C
- **Dilution factor of 25** seems to be well adapted for faecal sludge from septic tanks in Hanoi
- If sample too thick, cut the very end of the tip to allow pipetting

1. Preheat the thermostat at **100 °C**
2. **Homogenize** the sample, therefore, gently mix with a ladle and immediately pipette
3. **Perform analyses as indicated in the kit notice, ensuring a fix time of cooling after heating in the thermostat, and cleaning the test tube before measuring in a calibrated photometer.**

Protocol

Ammonium-Nitrogen, NH4+
Kit NH4+: Hach Kit, LCK 303 (ISO 7150-1, DIN 38406 E5-1)

Method
Indophenol Blue

Time
25 min

Materials

- 10 ml of faecal sludge
- 100 ml of SDW
- 1 50 ml Falcon tubes
- 1 25 ml volumetric flasks (depends on how many dilutions)
- 1 reagent reservoirs (depends on how many dilutions)
- 1 1–10 ml pipette
- 1 100–1000 µl pipette
- A ladle
- 1.5 and 0.45 µm porosity Whatman filters

Note

- Measuring range: 2–47 mg/l of NH_4^+ (2.5–60 mg/l NH_4^+)
- pH must be within the range 4–9 (adjust, if necessary with sodium hydroxide solution or sulfuric acid)
- Temperature of the sample: 20 °C
- Store reagents in the fridge at 4 °C
- Factor dilution of 25 to be tested for faecal sludge samples from septic tanks in Hanoi.

1. **Use a ladle to fill the Falcon tubes in a homogenize way and then centrifuge, 6000 rpm during 10 min (rcf = 3820 × g, rotor 220.97 V01)**
2. **Filter** the supernatant with a filter of **1.5 µm**, and then with a **0.45** porosity
3. **Perform analyses as indicated in the kit notice, ensuring a fix time of cooling after heating in the thermostat, and cleaning the test tube before measuring in a calibrated photometer.**

$$X\ [mg/l]\ of\ NH_4^+\text{-}N = X * 18/14\ [mg/l]\ of\ NH_4^+$$

$$; M_N = 14g/mol\ ;\ M_{NH4+} = 18g/mol$$

Annex 4

Analysis protocol for TP and PO_4^{3-}

Substrate used

Total sample for TP; supernatant filtered at 1.5 µm and 0.45 µm for PO_4, if necessary, dilute the sample to allow filtration.

Note

- **Always use a new pipette if you have to pipette faecal sludge several times. If sample too thick, cut the very end of the tip to allow pipetting.**
- Measure each sample 2 times in the photometer and calculate the average
- **[N-PO_4] < [TP]**

Protocol

Total Phosphorus: Hach kit LCK 350 (EN ISO 6878-1-1986, DIN 38405 D11-4)

Method

Phosphormolybdenum Blue

Time

1 h 40 min

Materials

- 10 ml faecal sludge
- 100 ml of SDW
- 1 50 ml volumetric flasks (depends on how many dilutions)
- 1 reagent reservoirs (depends on how many dilutions)
- 1 1–10 ml pipette
- 1 100–1000 µl pipette

Note

- **Use only phosphate-free detergents to rinse glassware. Otherwise fill with hydrochloric acid (approx. 10 %) and leave to stand several hours**
- Measuring range: 2.0–20.0 mg/l of PO_4–P
- Without hydrolysis, only the (dissolved) orthophosphate is measured
- pH sample: 2–10
- Temperature sample/reagent: 15–25°C
- Inverting the cuvette after hydrolysis improves the reliability of the result
- **Factor dilution** of **20** seems to be well adapted for faecal sludge from septic tanks in Hanoi

1. Preheat the thermostat at **100°C**
2. **Perform analyses as indicated in the kit notice, ensuring a fix time of cooling after heating in the thermostat, and cleaning the test tube before measuring in a calibrated photometer.**

Protocol

PO43-, Test Orthophosphate: Hach Kit LCK 049 (Adapted from EN ISO 6878-1-1986)

Method

Vanadate-Molybdate method

Time

25 min

Materials

- 10 ml faecal sludge
- 100 ml of SDW
- 1 50 ml Falcon tube
- 1 25 ml volumetric flasks (depends on how many dilutions)
- 1 reagent reservoirs (depends on how many dilutions)
- 1 1–10 ml pipette
- 1 100–1000 µl pipette
- A ladle
- 1.5 µm and 0.45 porosity Whatman filter

Note

- **Use only phosphate-free detergents to rinse glassware. Otherwise fill with hydrochloric acid (approx. 10 %) and leave to stand several hours**
- Measuring range: 1.6–30.0 mg/l of PO_{4-}-P (5–90mg/l PO_{4-})
- pH sample: 3–10
- Temperature sample/reagent: 15–25°C
- **Factor dilution** of **2** seems to be well adapted for faecal sludge from septic tanks in Hanoi.

1. Use a ladle to fill the Falcon tubes in a homogenize way and then centrifuge, 6000 rpm during 10min (rcf = 3820 × g, rotor 220.97 V01)
2. **Filter** the supernatant with a filter of 1.5 µm porosity, and then with a 0.45 porosity filter
3. **Perform analyses as indicated in the kit notice, cleaning the test tube before measuring in a calibrated photometer.**
4. Reading is in P inside orthophosphate. To convert to orthophosphate concentration:

   X [mg/l] of PO_{4-}-P $\longrightarrow$ X * 95/31 [mg/l] of PO_{4-} ; M_P = 31g/mol ; M_{PO4-}= 95g/mol

Annex 5

Analysis protocol for VFA

Substrate

Faecal sludge – analyses on supernatant filtered at 0.45µm. if required, preliminarily centrifuge and filter at 1.5 µm, after dilution or not.

Protocol

Volatile fatty acid (Organic acids): Hach Kit LCK 365 (Adapted from Standard Method 5560)

Method

Esterification method

Time

15 min

Materials

- 10 ml of faecal sludge
- 100 ml of SDW (Sterile distilled water)
- 1 50 ml Falcon tubes
- 1 beaker
- 1 1–10 ml pipette
- 1 100–1000 µl pipette
- 1 syringe for filtration
- 0.45 µm porosity Whatman filter
- A ladle

Note

- Measuring range: 50–2500 mg/l CH_3COOH (Acetic Acid)
- 3 < pH < 9
- T °C sample/reagent: 15–25°C
- The time to perform the analysis should be as short as possible to avoid volatizing VFA in air. Reduce as much as possible the transfer of solution from tube to tube, and always close tubes to avoid volatilization. When centrifuging, fill the Falcon tube with the sample to the top.
- After reaction, the time to read should be always the same for each sample and analysis, < 15 min.

1. Preheat the thermostat at **100 °C**
2. Use a ladle to fill the Falcon tubes in a **homogenize** way and then **centrifuge, 6000 rpm during 10 min (rcf = 3820 × g, rotor 220.97 V01)**. Always fill the Falcon tube to **top** (the less air is inside the better it is)
3. **Perform analyses as indicated in the kit notice, ensuring a fix time of cooling after heating in the thermostat, and cleaning the test tube before measuring in a calibrated photometer.**

Annex 6

Analysis protocol for Metals (Ni, Zn, Fe, Pb)

Substrate used

Faecal sludge (total sample for Crack-Set; Digested sample filtered at 15–20 µm for all metals)

Note

- **Always use a new pipette if you have to pipette faecal sludge several times. If sample too thick, cut the extremity of the tip to allow pipetting.**
- Measure each sample 2 times in the photometer and calculate the average.

Protocol

Protocol for digestion of samples: Hach Crack-Set Kit LCW 902

Method

Acid digestion with oxidizing agent

Time

1 h 50 min

Materials

- 20 ml of faecal sludge
- 2 20 ml reaction tube
- 1 1–10 ml pipette
- 1 100–1000 µl pipette
- 15–20 µm porosity filter

Note

This analysis allows digesting the sample. Therefore the dissolved and non-complexed ions heavy metals as well as undissolved and complexly bound heavy metals can be analyzed.
If the sample exhibits turbidity after the Crack-Set has been used, this must be eliminated by filtration.
Temperature of the sample: 15–25°C.
7 ml to 17 ml of digested sample are required to analyze all metals content (2 or 5 ml for Ni, 5 or 10 ml for Pb, 2 ml for Fe, 2 ml for Zn).

1. Preheat the thermostat at **100 °C**
2. **Homogenize** the sample, therefore, gently mix with a ladle
3. **Perform analyses as indicated in the kit notice, ensuring a fix time of cooling after heating in the thermostat, and cleaning the test tube before measuring in a calibrated photometer.**

Protocol Fe: Hach Kit LCK 321 (ISO 6332-1988)

Time
25 min

Materials

- 2 ml of digested sample (Crack-Set)
- 30 ml of SDW
- 1 25 ml volumetric flasks (depends how many dilution)
- 1 reagent reservoirs (depends how many dilution)
- 1 1–10 ml pipette
- 1 100–1000 µl pipette

Note

- Measuring range: 0.24–7.2 mg/l of Fe
- pH must be within the range 3–10 (adjust, if necessary with sodium hydroxide solution or sulfuric acid)
- Temperature of the sample: 20 °C
- **Store reagents in the fridge at 4 °C**
- Factor **dilution** between **20 and 40** should be tested for faecal sludge samples from septic tanks in Hanoi.

1. **Perform analyses as indicated in the kit notice, cleaning the test tube before measuring in a calibrated photometer.**

Protocol Zn: Hach Kit LCK 360

Time
10 min

Materials

- 0.2 ml of digested sample (Crack-Set)
- 30 ml of SDW
- 1 25 ml volumetric flasks (depends how many dilution)
- 1 reagent reservoirs (depends how many dilution)
- 1 1–10 ml pipette
- 1 100–1000 µl pipette

Note

- Measuring range: 0.24–7.2 mg/l of Zn
- pH must be within the range 3–10 (adjust, if necessary with sodium hydroxide solution or sulfuric acid).
- Factor **dilution** between **20 and 40** should be tested for faecal sludge samples from septic tanks in Hanoi.

1. **Perform analyses as indicated in the kit notice, cleaning the test tube before measuring in a calibrated photometer.**

Protocol

Ni: Hach Kit LCK 337 (DIN 38406-E11)

Time
10 min

Materials

- 2 ml of digested sample (Crack-Set)
- 30 ml of SDW
- 1 25 ml volumetric flasks (depends how many dilution)
- 1 reagent reservoirs (depends how many dilution)
- 1 1–10 ml pipette
- 1 100–1000 µl pipette

Note

- Measuring range: 0.12–7.2 mg/l of Ni
- pH must be within the range 3–10 (adjust, if necessary with sodium hydroxide solution or sulfuric acid).
- Factor **dilution** of **5** should be tested for faecal sludge samples from septic tanks in Hanoi.

1. **Perform analyses as indicated in the kit notice, cleaning the test tube before measuring in a calibrated photometer.**

Protocol

Pb: Hach Kit LCK 306

Time
15 min

Materials

- 10 ml of digested sample (Crack-Set)
- 30 ml of SDW
- 1 20 ml reaction tube
- 1 25 ml volumetric flasks(depends how many dilution)
- 1 reagent reservoirs (depends how many dilution)
- 1 1–10 ml pipette
- 1 100–1000 µl pipette

Note

- Measuring range: 0.12–2.40 mg/l of Pb
- pH must be within the range 3–10 (adjust, if necessary with sodium hydroxide solution or sulfuric acid).
- Factor **dilution** of **1 to 5** should be tested for faecal sludge samples from septic tanks in Hanoi.
- Do not forget to insert the reaction tube in the photometer to read the blank before adding the reagent C.

1. **Perform analyses as indicated in the kit notice, cleaning the test tube before measuring in a calibrated photometer.**

Annex 7

Protocol for *Ascaris* eggs extraction and decortication

Adapted from (Moodley et al., 2008).

Materials

- 50 ml Falcon tube (several per sample)
- Centrifuge with a swing-out rotor and buckets that takes 15 ml and/or 50 ml plastic conical test tubes
- Metallic sieves: 1 × 100µm and 1 × 20 µm
- 2 100 ml beaker
- Vortex
- Pasteur pipettes
- At least 3 glass (Schott) bottles of 1 L
- Glass microscope slides (76 × 26 × 1.2 mm)
- Square and rectangular cover-slips (22 × 22mm and 22 × 40 mm)
- A binocular compound microscope with a 10× and a 40× objective
- 1 pipette 10–100 µl
- 1 pipette tips 10–100 µl
- Nail polish
- 4 Falcon tubes 15 ml

Reagent solutions

- 10 ml faecal sludge
- AmBic solution
- $ZnSO_4$ solution
- 0.1 N H_2SO_4 solution
- Trigene for desinfection

Protocol

1. Weight one empty Falcon tube per sample.
2. Mix the sample and put 10ml of the sludge in each Falcon tube.
3. Weight again the filled Falcon tubes to know the weight/volume of the sludge.
4. Add AmBic solution little by little and mix the sample with a vortex each time after adding a bit of the solution until a volume of around 50 ml is reached. To enhance the mixing put a glass stick in the Falcon tube before vortexing the first time. If no vortex is available fill up the falcon tube at once until 50 ml, shake the tube by hand for 5 minutes.
5. Prepare the 20 µm sieve with on top of it the 100 µm sieve, next to the sink.
6. Vortex the sample or shake it and pour it on the 100 µm filter while the 20 µm sieve is attached underneath.
7. Rinse the Falcon tubes and their lids several times with water and pour it also on the filter to recover all the eggs.
8. Rinse the 100 µm filter with water; use a lot of water under pressure to force the eggs to pass through the sieve until only deposits are left. Pay attention that the 20 µm sieve underneath does not overflow!.
9. Put the 100 µm in the sink and rinse the 20 µm sieve as well with pressurized water.
10. When only the particles which do not pass the 20 µm sieve remain, put some water in the sieve and transfer it with the remaining particles in a clean FT. Repeat the operation until no particles are left over on the sieve. To facilitate the handling, incline and shake the sieve slightly. In case the recovered volume exceeds 50 ml, distribute it in several FTs. In the case when only one tube is necessary, prepare another one with water having the same weight. When several tubes are used be sure that they have the same weight as they will be centrifuged. Verify their weights with a balance.

11. Rinse the filters upside-down with water.

12. Centrifuge the sample for 5 minutes at 3000 rpm. Pay attention to the symmetrical arrangement of the tubes in the centrifuge.

13. Discard the supernatant with a sterile plastic Pasteur pipette until a volume of around 5 ml is left over. In general the content of 2 Falcon tubes can be put together.

14. Add $ZnSO_4$ solution little by little and mix the sample with a vortex each time after adding a bit of the solution until a volume of around 35–40ml is reached, if not vortex is available add the $ZnSO_4$ solution at once and shake the FT by hand for 5 minutes.

15. Weight the tubes and adjust their weight until they are the same or fill one tube with water (same weight) in order to have a symmetrical arrangement in the centrifuge.

16. Centrifuge for 5 minutes at 2000 rpm.

17. Recover the supernatant only with a sterile Pasteur pipette and put it on the 20 µm sieve which is in the sink. Do this for all FTs in case more than one was used.

18. Rinse the eggs and particles on the sieve with a lot of water to get rid of the $ZnSO_4$.

19. Recover again with water what is left over on the sieve by inclining the sieve. Pour the remains of the sieve in a clean Falcon tube.

20. Rinse the filter upside-down to clean it.

21. Prepare again the Falcon tubes of equal weight by adding water if necessary.

22. Centrifuge for 5 minutes at 2500rpm.

23. Discard the supernatant with a sterile Pasteur pipette. If several tubes are present for the same sample put the deposits together and centrifuge it again in case the deposit is big.

24. Count the number of Pasteur pipette drops, put them into a microtube 2 ml and write down the number of drops on the tube. If more than 25 drops are counted, distribute them equally in two tubes. Label the tube(s) with sample name, date, number of drops, Name of operator.

25. Add 500 µl H_2SO_4 in the 2 ml micro tube and mark the level of the tube content. Put the sample in an incubator for 14 days at 26 °C.

26. Clean everything with ethanol.

Annex 8

Protocol for Decortication and Enumeration of *Ascaris* eggs

Note

If the eggs were shed from female worm in faeces, it is very difficult to distinguish development stages under microscope. In this case, it is recommended to decorticate the eggs prior to enumeration with microscope. This allows to remove the outer layer of the egg and determining the development stage. This step should be done just before reading the samples viability.

Protocol

Preliminary decortication method

(Adapted from Pecson and Nelson, 2005; Brownell and Nelson, 2006; Kim et al, 2012; Masure et al; 2013)

Materials and reagent

- 1 50 ml Falcon tube per sample
- Vortex
- Chronometer
- 1 pipette 100–1000 µl
- Javel 1% concentration
- Distilled water

1. Pipette 1ml from the tube of extracted eggs sample into a 50 ml Falcon tube.
2. Add up to 10 ml 1% Javel (1ml egg solution/all the deposits + 9ml Javel).
3. Wait 3 minutes for the reaction to happen.
4. Add 30 ml distilled water.
5. Centrifuge for 5 min at 2500 rpm.
6. Let the sediments settle for around 2 minutes in case the centrifuge is inclined.
7. Discard the supernatant with a sterile Pasteur pipette.
8. Count the number of drops and put them into a microtube 2 ml and write down the number of drops on the tube. If more than 25 drops are counted, distribute them equally in two tubes. Label the tube(s) with sample name, date, number of drops, name of operator.
9. Enumerate Helminth Eggs as explained in following.

Protocol

Enumeration method

Materials

- Glass microscope slides (76×26×1.2 mm)
- Square and rectangular cover-slips (22 × 22 mm and 22 × 40 mm)
- A binocular compound microscope with a 10× and a 40× objective
- 1 pipette 10–100 µl
- Nail polish

1. Draw a grid on a microscope slide or glue a microscope slide grid and write the sample's name down.
2. Pipette 70 µl of the solution on the slide, put a cover slide (22 × 50) on it and seal it with transparent nail polish.
3. Read the slide to enumerate and determine the development stage of the eggs.

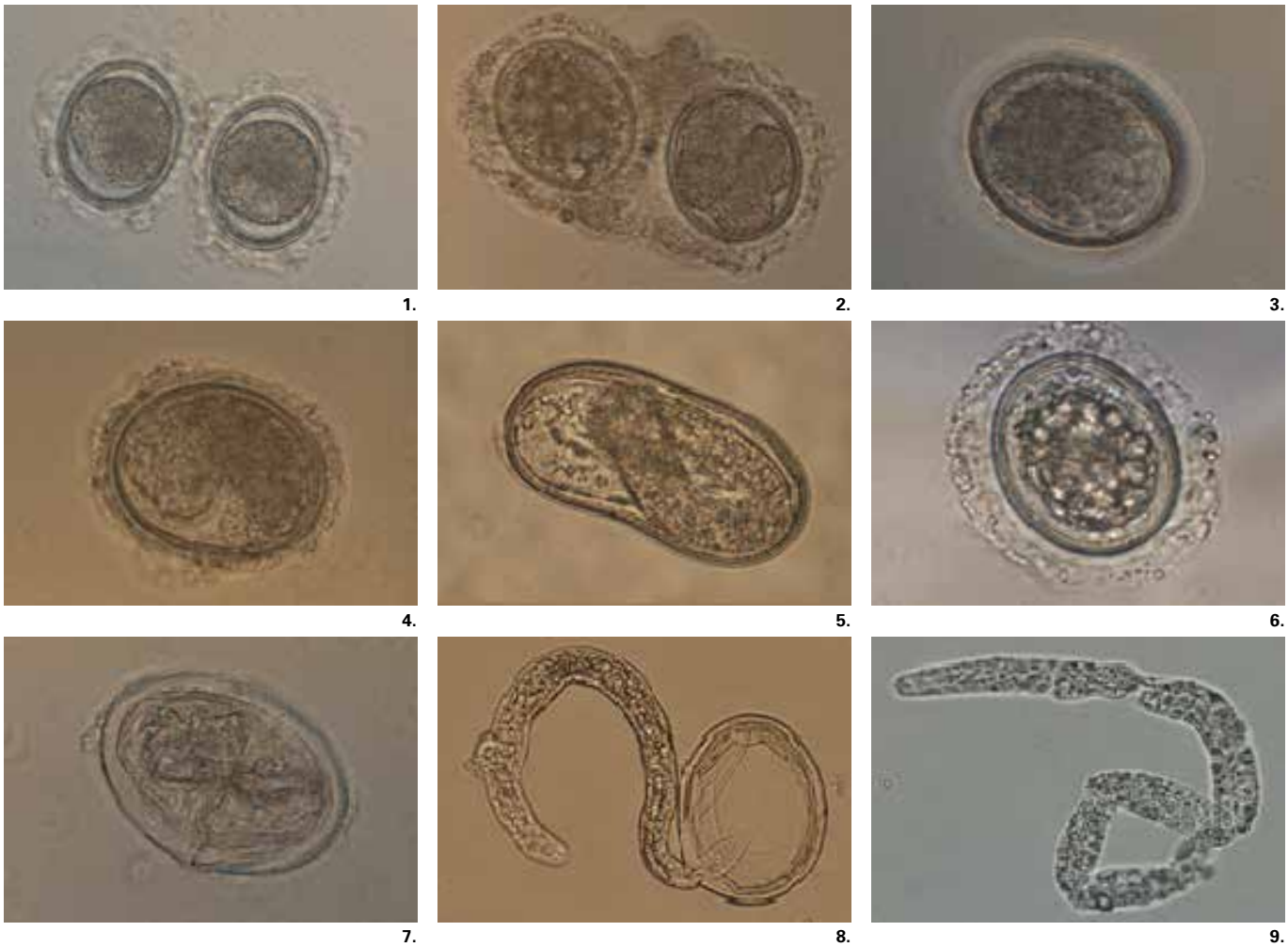


Figure A: 1. Two non-developed eggs. 2. Two developing eggs. The right egg has four cells. 3. One fully developing egg with an uncountable number of cells. 4. One pre-larval egg. 5. One egg in the larval stage. 6. One dead egg identifiable thanks to its "oil drops". 7. One dead egg identified because of its bad shape. 8. Hatching larva. 9. Larva moving freely in the solution. Pictures taken with a 40× objective by Livia Jost and Léa Randin.

The following development stages are used to classify the eggs. (See Figure A). Only the larval eggs and eggs developed into larvae have been considered as viable after incubation during 14 days in H_2SO_4 at 26 °C. Only viable eggs are considered infectious.

Undeveloped or developing: Undeveloped eggs are characterized by a monocellular embryo. This embryo is round-shaped and touches the surface of the lipid layer at some places only as illustrated in Figure A.1. It is otherwise surrounded by an "empty space". Developing eggs at their early stages can be recognized by observing the number of cells inside the shell as shown in Figure A.2. At a later development, the inside of the shell is just filled up with cells as presented in Figure A.3.

Pre-larval: The pre-larval stage can be identified when the cells are forming a crescent shape as shown in Figure A.4.

Larval: The larval stage is clearly identifiable as the larva is fully developed inside the egg-shell as illustrated in Figure A.5. Sometimes the larva is even motile.

Dead: Dead eggs are recognizable by the fact that some kind of "oil drops" appear as shown in Figure A.6 or because they are not in a good shape as presented in Figure A.7.

Larvae: Larvae can be observed during hatching as illustrated in Figure A.8 or free in the solution as shown in Figure A.9.

Annex 9

Protocol for plating and enumeration of *Salmonella*

Time

Approximatively 45 minutes for Agar preparation and 45 minutes for plate spreading (depending on the dilutions).

Note

Before working, clean the table and material to be used well with ethanol. If possible, this should be in a closed, air-conditioned room, without fan.

Materials

- Balance
- Bec bunsen (flame)
- Incubator at 37 °C
- 100 ml graduated flask
- 400 ml beaker
- Magnetic stirrer
- 10–100 µl pipet with sterile tips
- Petri dish (depending on number of samples)
- Approx. 12 Tubes Eppendorf (sterile) with support
- 100–1000 µl Pipet with sterile tips
- 200 ml Beaker (for ethanol)
- Glass sticks for plate spreading

Consumables

For 20–25 Petri (2 series of 3 dilutions):

20.8 g	BSA powder (Bismuth Sulfite Agar) (add 5.2 g / 100 ml)
400 ml	Ultrapure water
100 ml	Ethanol
	0.9 % saline isotonic water for dilution

Protocol	Agar preparation
1.	For 100 ml agar (approx. 5 Petri), weight 5.2 g BSA (Bismuth Sulfite Agar) → For 400ml, weight 20.8 g.
2.	Prepare 100 ml ultrapure water in a graduated flask.
3.	Mix the water and the BSA in a beaker on a heating magnetic stirrer until it boils. Let it boil for 1 min.
4.	Let the mixture cool down by stirring it on a second, cool magnetic stirrer.
5.	When agar mixture can be hold by hand, pour it in Petri dish (20–25 ml per dish).
6.	Wait for solidification. Make sure that humidity is not kept in the dishes (let it open near to the flame for approx. 15 min).
7.	Once the agar is solidified (after approx. 20–30min) turn the dishes upside-down.
8.	Let aside at least one dish as sterile control.
9.	Agar needs to be used before 24 hours after preparation. If necessary, store it in the fridge, at 4 °C.
	After 24 to 48 hours, the plates can be read, counting the black-brownish colonies that are surrounded by a metallic shade.

Protocol	Plating
10.	Prepare saline isotonic solution (0,9 %) for dilution (approx.15 ml saline isotonic solution (15 ml distilled water with 0.135 g NaCl)
11.	Choose dilution to obtain between 10 and 300 Unit Forming Colony (UFC). For example, test 1/10, 1/100, 1/1000, and 1/10'000 dilutions). Test at least three dilutions, and do triplicates for each dilution.
12.	Note the dilution and samples on the dishes.
13.	Clean well the working area, light up the flame and work near it. Prepare the dilutions in sterile Eppendorf tubes with the saline isotonic solution (each time 100 µl of sample with 900 ml of saline isotonic solution).
14.	Prepare one beaker with ethanol, and two glass sticks.
15.	Pipet 20 µl of sample in each dish, and spread it with the glass stick. Before spreading, dig the glass stick in ethanol, put it on the flame, and wait for cooling down (two glass sticks can be used to reduce waiting time). Spread the sample regularly all over the surface of the agar, until the sample has dried (some resistance).
16.	Turn the petri dish upside down, and incubate at 37 °C for 24 to 48 h. The number of colonies can be counted!

Annex 10

Protocol for preparation of standard solutions

Parameters to analyze: COD, NH_4^+, TN, PO_4^{3-}, TP

- Sodium Oxalate $C_2O_4Na_2$ used to analyze COD
- Ammonium sulphate $(NH_4^+)_2SO_4$ used to analyze TN and NH_4^+
- Disodium hydrogen phosphate Na_2HPO_4 used to analyze PO_4^{3-} and TP

All these 3 chemicals can be mixed all together with distilled water. They should be kept in dry conditions prior to tests.

Reactions

$2\ H_2O + 2\ C_2O_4Na_2 + 1\ O_2 \rightarrow 4\ CO_2 + 4\ NaOH$

$(NH_4)_2SO_4 + H_2O \rightarrow 2\ NH_4^+ + SO_{42-} + H_2O$

$2\ Na_2HPO_4 + 4\ H_2O = 3\ H_2 + 4\ NaOH + 2\ PO_4$

Element	Molar Mass [g·mol$_{-1}$]
C	12
O	16
Na	23
N	14
S	32.1
P	31
H	1

If 100 ml volumetric flasks are used to make the dilution, add 800 mg of $C_2O_4Na_2$, 26.4 mg of $(NH_4^+)_2SO_4$ and 14.2 mg of Na_2HPO_4.

Parameter	Dilution	Method	Concentration [mg·L-1]	Measuring range [mg·L-1]
COD	1/1	Kit Hach LCK 514	952	100–2000
NH_4^+	1/2	Kit Hach LCK 303	28 (N- NH_4^+)	2–47
TN	1/1	Kit Hach LCK 338	56 (N- NH_4^+)	20–100
PO_4^{3-}	1/2	Kit Hach LCK 049	15.5 (P- PO_4^{3-})	1.6–30
TP	1/2	Kit Hach LCK 350	15.5 (P- PO_4^{3-})	2–20

Parameter	Dilution	Method	Concentration [mg·L-1]	Measuring range [mg·L-1]
COD	1/10	Kit Hach LCI 500	952	100–2000
NH_4^+	1/50	Kit Merck 1.14752.0001	1.12 (N- NH_4^+)	0.05–3.00
TN	1/10	Kit Merck 1.00613.0001	5.6 (N- NH_4^+)	0.5–15.0
PO_4^{3-}	1/20	Kit Merck 1.14848.0001	1.55 (P- PO_4^{3-})	0.05–5.00
TP	1/20	Kit Merck 1.14848.0001 + Kit Merck Crack set 10	1.55 (P- PO_4^{3-})	0.05–5.00

Transport

Ammonium sulphate: no recommendation;
Sodium Oxalate and Disodium hydrogen phosphate: Use hermetic Eppendorf tube in polypropylene. Fill it to the top.

Note

If Sodium Oxalate and Disodium hydrogen phosphate is not anhydre anymore, dry it 24h in oven. It is always better to use hydrate chemicals will not oxidize at the air contact (but concentration should be adapted due to the higher molar mass).
Measuring range of the analysis methods should be taken into account to set the concentration.

Annex 11

Protocol for composite preparation and sample preservation

Substrate used

Faecal sludge

Notes

Immediately after sampling, samples should be transported with ice in cold box to the laboratory. Filtration should be achieved where necessary within 24h after sampling.

Protocol

1. **Composite preparation**
   Dispense all the samples (bottles) in a bucket of 10 L (in 2 different buckets if duplicate analysis), then gently mix with a ladle to **homogenize** the sample and fill the different containers to the top (except for *E. coli, Salmonella* and Helminth eggs) and store at **4 °C** in the fridge.

2. **Distribution of sample in tubes and bottles for analyses and preservation**
   Distribution is presented as was done for the characterization study performed during PURR project.

   - **0.35 L** plastic bottle for *E. coli* and *Salmonella* analysis, leave 2.5 cm of air on the top of the bottle
   - **0.35 L** plastic bottle for Helminth eggs analysis, leave 2.5 cm of air on the top of the bottle
   - **0.5 L** plastic bottle for BOD_5 (filled to the top)
   - **Around 0.8 L** for drying at 105 °C in case of further analysis on dry sludge (use a **volumetric flask of 1 L** and insert it in the oven).
   - **0.35 L** plastic bottle for preservation (leave 2 cm on the top to add H_2SO_4)
   - **1 L** (2 × 0.5l plastic bottle filled to the top) for laboratory analysis
   - **4 × 50 ml** (Falcon tubes filled to the top) for centrifuging. **6 × 50 ml** if duplicate analysis

3. **Centrifuge**
   Centrifuge the 4 Falcon tubes at **5000 rpm** during **10 min** (rcf = 3820 × g, rotor 220.97 V01)

4. **Analyze VFA**
   Analyze **VFA**: **filter** 3 ml with a syringe at **0.45 µm** porosity. Analysis takes around 15 min.

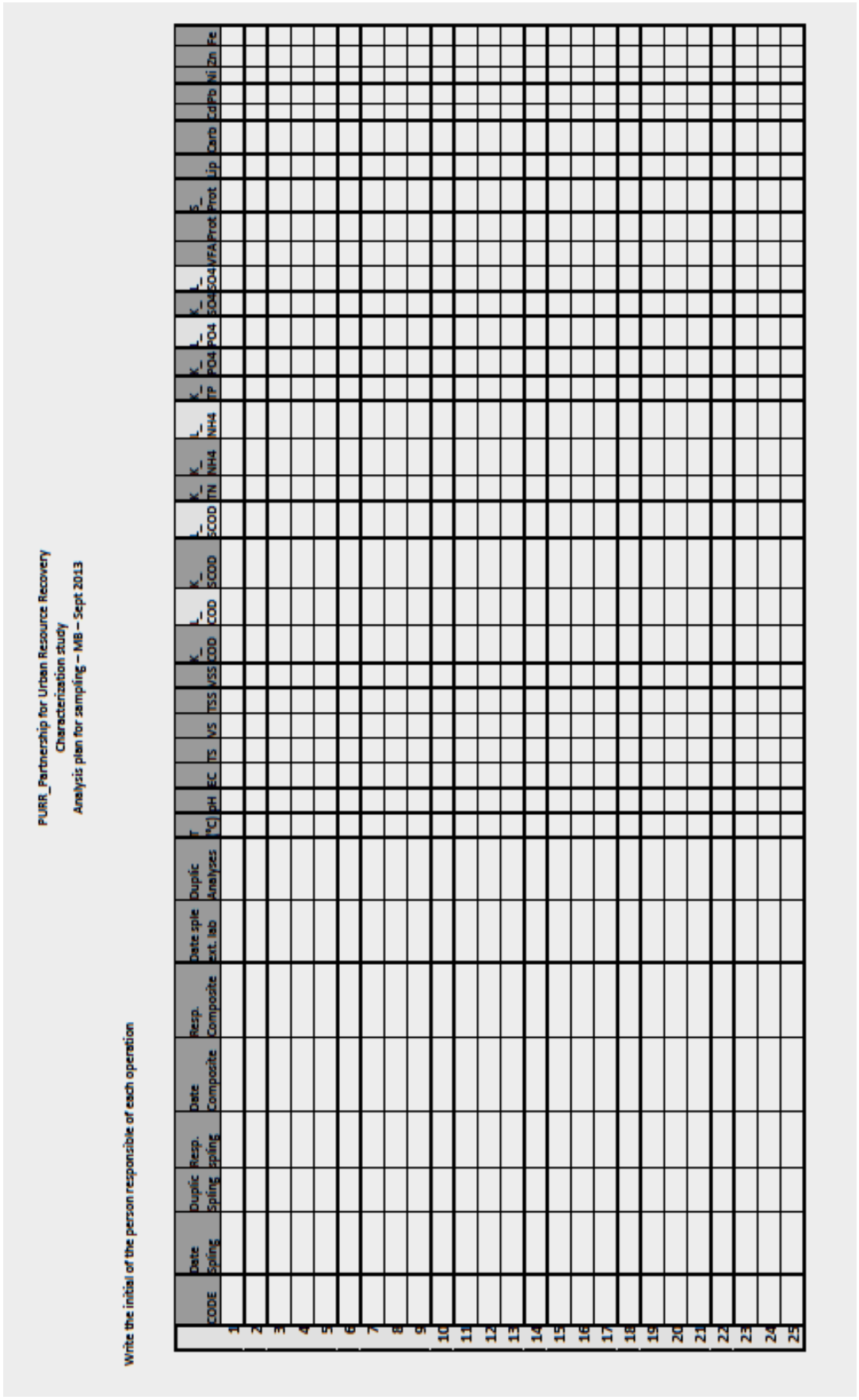
5. **Filter**
   Filter the 4 Falcon tubes with a **1.5 µm** porosity filter (Filter Whatman 1827-110 Grade 934-AH) with the vacuum pump. Then dispense the supernatant into 3 different 50 ml Falcon tubes. One of the 3 will be used for conservation.

6. **Preservation**
   Add 1 ml per 100 ml of sample. (1 %)
   Therefore slowly pipette 3.5 ml of sulphuric acid H_2SO_4 into the 0.35 L sample used for conservation. Slowly pipette 500 µl of sulphuric acid H_2SO_4 into the Falcon tube used for conservation. Note: If the sample is very thick, leave the bottle a few hours in the fridge to allow coarse particles settling, then add the sulfuric acid. This is to avoid burning the sample and trigger an exothermic reaction.

7. **Deliver samples to other laboratories**
   Samples should be delivered with ice in cold box during the sampling day or one day after to the other laboratories.

Annex 12

Example of recapitulative table for laboratory organization



FURR_Partnership for Urban Resource Recovery
Characterization study
Analysis plan for sampling – MB – Sept 2013

Write the initial of the person responsible of each operation

	CODE	Date Spling	Duplic Spling	Resp. spling	Date Composite	Resp. Composite	Date sple ext. lab	Duplic Analyzes	T (°C)	pH	EC	TS	VS	TSS	VSS	K_ COD	L_ COD	K_ SCOD	L_ SCOD	K_ TN	K_ NH4	L_ NH4	K_ TP	K_ PO4	L_ PO4	K_ SO4	L_ SO4	L_ PO4	VFA	Prot	S_ Prot	Lip	Carb	Cd	Pb	Ni	Zn	Fe
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Annex 13

Synthetic sludge recipe for experiments on anaerobic reactors

The recipe is based on the assessment of the faecal sludge characterization study done in Vietnam (Bassan et al, to be published). It aims to mimic the average characteristics of faecal sludge in terms of total solids (TS), volatile solids (VS), chemical oxygen demand (COD), soluble COD, total nitrogen (TN) , ammonium (NH_4^+) and phosphate (PO_4^{3-}). The trace element solution can be prepared in advance and stored at 4°C for one month.

Ingredient	Unit	Quantity
Walnuts	g	17
Straw flour	g	5
Kaolinite ($Al_2O_2Si_2 \cdot 2H_2O$)	g	1/1
NH_4HCO_2, anhydrous	g	2
Na_2HPO_4, anhydrous	mg	20
Trace Elements solution	**ml**	**10**
Industrial water	L	0.99
$FeCl_2 \cdot 4\ H_2O$	g/L	2
$MnCl_2 \cdot 4\ H_2O$	g/L	0.1
$CoCl2 \cdot 6\ H_2O$	g/L	0.19
$CuCl_2 \cdot 2\ H_2O$	g/L	0.00255
$ZnCl_2$	g/L	0.07
$AlCl_3 \cdot 6\ H_2O$	g/L	0.00552
H_3BO_3	g/L	0.006
$Na_2MoO_4 \cdot 2\ H_2O$	g/L	0.0414
$NiCl_2 \cdot 6\ H_2O$	g/L	0.024
EDTA (0.018 M)	g/L	0.5

Annex 14

Questionnaire on sludge origin for sampling

Sample code : ……..
Date:……Responsible: ……
Household address: No……
Street:……
District :……
Observation on household :
……
Observation on sampling :
……

I. QUESTIONS TO HOUSEHOLD

1. ***Number of persons living in the household / using the tank :*** Total: ……..…
2. ***Wastewater received in septic tank is from :***
   1❑ Toilet
   2❑ Bathing, Washing, Cleaning
   3❑ Kitchen
   4❑ Other, Specify : ……..
3. ***When did you build the septic tank ?***
   1❑ < 5 years
   2❑ 5 – 10 years
   3❑ 10 – 20 years
   4❑ > 20 years
   Exact year : ……..…
4. ***Information on the septic tank :***
   Volume :…….. (m^3)
   Chamber :…….. (chamber)
5. ***Bottom sealed :***
   1❑ Yes
   2❑ No
6. ***When did you empty the tank last time ?***
   1❑ < 5 years
   2❑ 5 – 10 years
   3❑ 10 – 20 years
   4❑ > 20 years
   Exact year : ……..…
7. ***Do you add chemicals to improve degradation in it?***
   1❑Yes, specify :……..……
   2❑ No

II. QUESTIONS TO THE TECHNICIAN

1. ***The volume of the truck (m^3)*** : ……..
2. ***The volume pumped (m^3)*** : ……..
3. ***The amount of water added (m^3)*** : ……..
4. ***Have you emptied the whole tank?***
   1❑ Yes
   2❑ No, what part?.....

III. OBSERVATIONS OF SAMPLING TEAM

1. ***Was the septic tank content stirred for emptying*** :
   1❑ Yes
   2❑ No
2. ***Number of truck emptied for one tank*** : ……..
3. ***Emptying method***:
   1❑ First supernatant, then sludge
   2❑ Only supernatant
   3❑ Other, specify : ……

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