Annex 1: *Information for the organisation of microbial interlaboratory studies for soil improvers and growing media*

1. Introduction

The goal of an interlaboratory study (ILS) is to determine and measure the accuracy of a specific test method.

Under the scope of CEN/TC 223/WG5 'Microbiology', the ILSs will test the accuracy of three methods developed to determine, according to the requirements for soil improvers and growing media in the Fertilising Products Regulation (FPR) [Regulation (EU) 2019/1009]:

- The absence of *Salmonella* spp. in 25 g or 25 ml of a growing medium or soil improver.
- The number of colony forming units (CFU) of *Escherichia coli* in 1 g or 1 ml of a growing medium or soil improver, which has to be lower than 1000 CFU.
- The number of colony forming units (CFU) of *Enterococcaceae* in 1 g or 1 ml of a growing medium or soil improver, which has to be lower than 1000 CFU.

Drafts of the methods to be followed are detailed in:

- *Salmonella* spp.: Annex 5
- *E. coli*: Annex 6
- *Enterococcaceae*: Annex 7

The organisation in charge of the ILSs will ship the samples to the participating labs. Shipments will be organised in batches that allow timely processing by the participating labs. Batches could potentially be structured around each material tested.

The samples shall be maintained under refrigeration (5 °C ± 3 °C) and protected from light until they are tested. The samples shall be analysed as soon as possible after reception by the participating laboratory, and no later than 5 days after shipment. Laboratories participating in the ILS shall record and report the time spent between sample reception, start and end of the analysis.

It is recommended that the samples are analysed by the same person on the same day, to minimise variability. If this is not possible, laboratories shall report on it. The concentrations of microorganisms on the spiked samples should be stable for approximately 5 days after inoculation at 5 °C ± 3 °C. Beyond that period of time, the results shall not be accepted.

Participating laboratories will be given one month from reception of the samples to submit the test results to the organiser of the ILS.

2. Design of the interlaboratory study

Each laboratory participating in the study shall receive samples of the following five different growing media and soil improvers (materials or matrices):

- Biowaste compost (Rottegrad 3)
- Green compost (Rottegrad 5)
- Solid digestate
- A blend of peat, green compost, wood fiber, liming material and inorganic fertilisers (NPK + micro elements)
- A blend of peat and 2% chicken manure

From these five materials, the organiser of the ILS shall prepare 3-4 laboratory samples, depending on whether the test will be for detection (*Salmonella* spp.) or for enumeration (*E. coli*, *Enterococcaceae*).

The participating labs will prepare 3 test portions from each laboratory sample for detection and 2 test portions for enumeration, or alternatively 3 test portions for both enumeration and detection, which will be analysed following the corresponding standard for each pathogen.

3. Preparation of the samples by the ILS organiser

3.1General

The organiser of the ILS shall be responsible for collecting the sample materials, gathering their corresponding documentation, and preparing them before shipment to the participating labs. Preparation of the samples by the ILS organiser shall be done according to the diagram shown in Figure 1, and as detailed below.

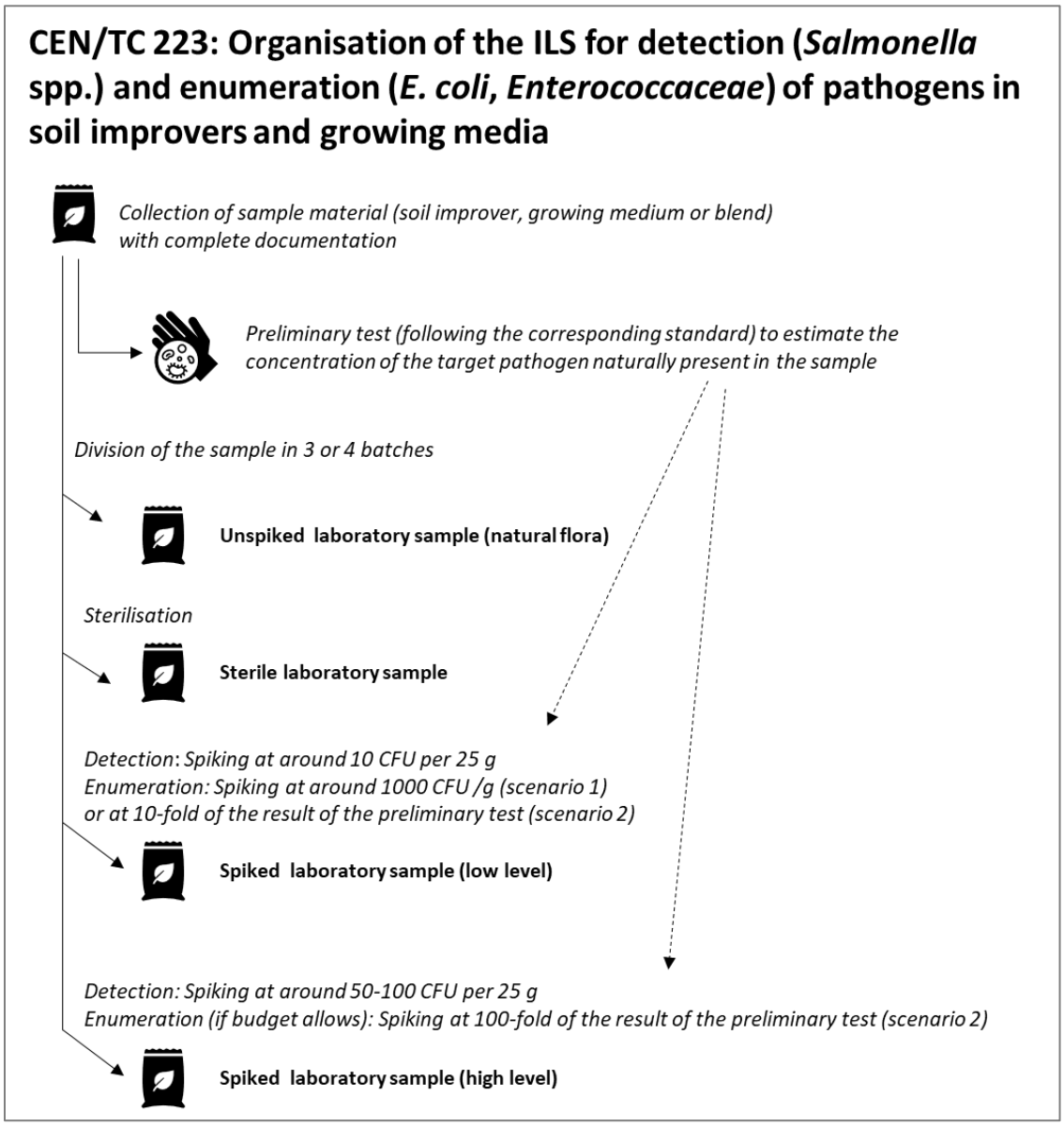

Figure 1: Diagram of the sample preparation to be done by the ILS organiser for the detection and enumeration of pathogens in soil improvers and growing media. The results of the preliminary test shall be used to estimate the level of spiking needed.

3.2Sterilised laboratory sample

For each of the five matrices to be tested, the organiser of the ILS shall sterilise three laboratory samples (one per pathogen) in an autoclave at 121 °C ± 3 °C for 15 min. The sterile laboratory samples will serve as a negative control (i.e., no bacteria should be observed on the selective medium at the end of the test).

3.3Unspiked laboratory sample (for enumeration)

For each of the matrices to be tested, the organiser of the ILS shall prepare at least two unspiked laboratory samples (for *E. coli* and *Enterococcaceae*). These samples will show whether the methods allow for the enumeration of the target pathogenic bacteria at the concentrations naturally found in soil improvers and growing media.

3.4Spiked laboratory samples

In order to determine the level of spiking needed, the organiser of the ILS shall conduct a preliminary test to estimate the concentration of each of the target pathogens naturally present in the samples.

Based on this preliminary test, the organiser of the ILS will spike a set of laboratory samples with the target pathogenic bacteria from suspensions at a known concentration before sending these samples to the participating labs.

The following strains of the target pathogenic bacteria have been proposed for spiking in this ILS:

- *E. coli*: wild-type strain (not *E. coli* O157:H7, as this is a pathogenic strain, and the method is not tailored for this strain).
- *Salmonella* spp.: wild-type H_2S-positive strain. For example, *Salmonella enterica* serovar Enteretidis may be used.
- *Enterococcaceae*: wild-type strain. For example, *Enterococcus faecalis* WDCM 00009, *E. faecalis* WDCM 00087 or *E. faecalis* WDCM 00176.

3.4.1 *Spiked laboratory samples (for detection)*

Considering the results of the preliminary test, the organiser of the ILS will spike two batches of samples:

- 1 laboratory sample near the limit of detection (meaning <10 CFU per 25 g)
- 1 laboratory sample at a higher level (meaning 50-100 CFU per 25 g)

3.4.2 *Spiked laboratory samples (for enumeration)*

Considering the results of the preliminary test, the organiser of the ILS will spike one or two batches of samples for the enumeration tests, based on two possible scenarios (organisations applying for the ILS need to include both scenarios and their associated costs):

a. Scenario 1:
- 1 laboratory sample so that the end concentration of *E. coli* / *Enterococcaceae* is around the limit in the Fertilising Products Regulation (1000 CFU/g).

b. Scenario 2 (if budget allows):
- 1 laboratory sample so that the end concentration of *E. coli* / *Enterococcaceae* is 10-fold above the result of the preliminary test.
- 1 laboratory sample so that the end concentration of *E. coli* / *Enterococcaceae* is 100-fold above the result of the preliminary test.

Alternatively, the organiser of the ILS may spike 2-3 laboratory samples with the three target pathogens at the same time, with the concentrations specified above.

4. Testing

4.1 Detection of *Salmonella* spp.

Laboratories participating in the ILS for the detection of *Salmonella* spp. (Figure 2) will receive three (blind) laboratory samples per material that correspond to:

- One sterilised laboratory sample to serve as the negative control.
- One laboratory sample spiked at the detection limit (<10 CFU per 25 g).
- One laboratory sample spiked at a higher level (50-100 CFU per 25 g).

Each participating laboratory shall obtain three test portions from each laboratory sample (3 replicates) according to prEN 13040-2 and conduct three independent detections following the steps in the *Salmonella* spp. standard (WI 00223121).

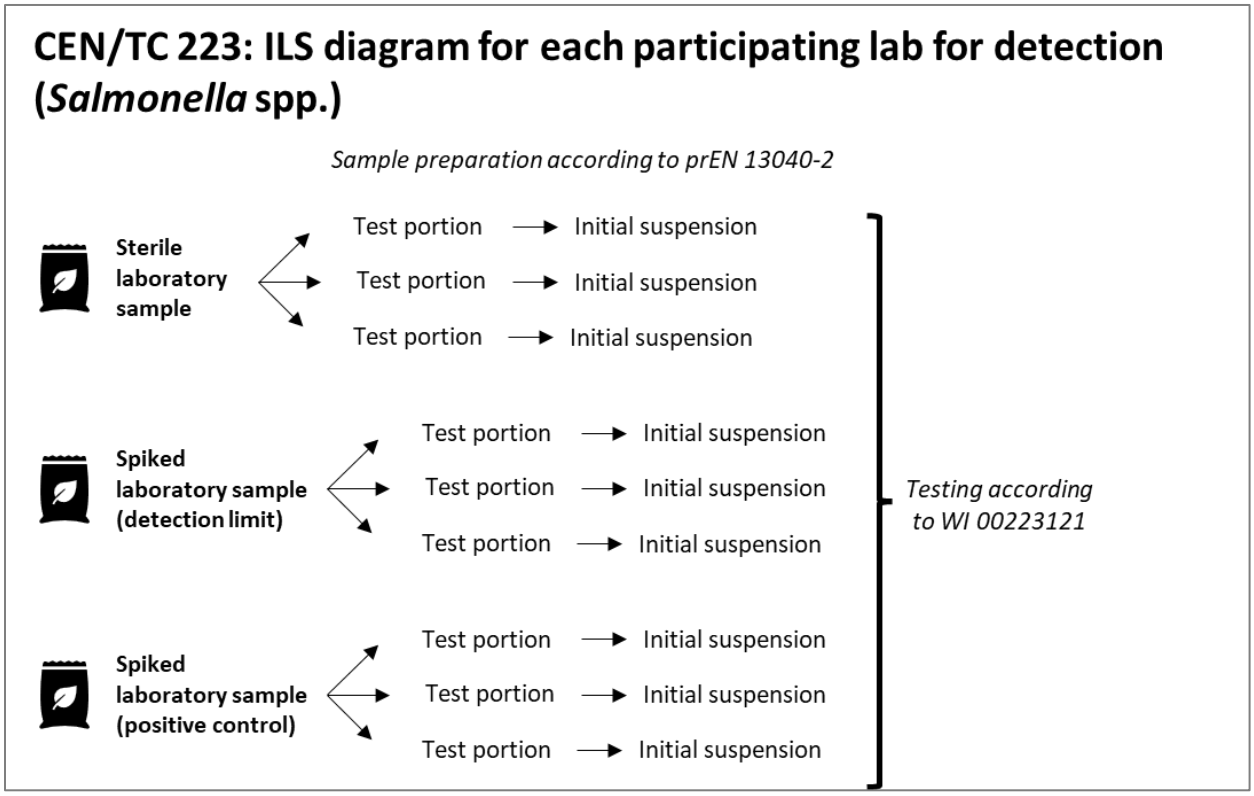

Figure 2: Diagram of the ILS for the detection of Salmonella spp. in soil improvers and growing media.

4.2 Enumeration of *E. coli* or *Enterococcaceae*

Laboratories participating in the ILS for the enumeration of *E. coli* or *Enterococcaceae* (Figure 3) will receive three (blind) laboratory samples (scenario 1) or four (blind) laboratory samples per material (scenario 2) that correspond to:

- One sterilised laboratory sample to serve as the negative control.
- One unspiked laboratory sample (natural flora).

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- One laboratory sample spiked at 1000 CFU/g (scenario 1) or 10-fold of the result of the preliminary test (scenario 2).
- (if budget allows) one laboratory sample spiked at 100-fold of the result of the preliminary test (scenario 2).

Each participating laboratory shall obtain two test portions from each laboratory sample (2 replicates) according to prEN 13040-2 and conduct two independent enumerations following the steps in the *E. coli* standard (WI 00223114*)* or the *Enterococcaceae* standard (WI 00223119).

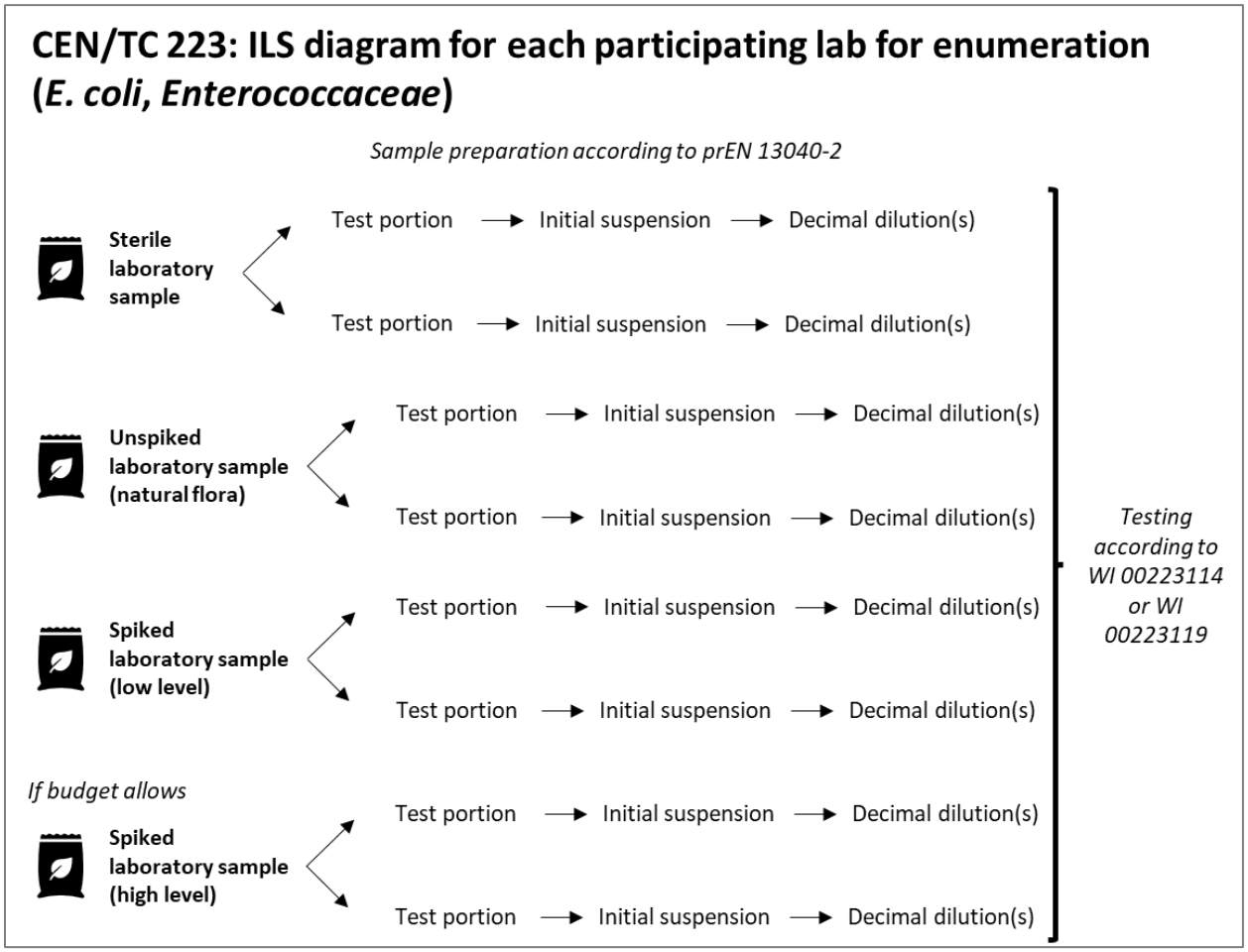

Figure 3: Diagram of the ILS for the enumeration of Enterococcaceae and E. coli in soil improvers and growing media.

4.3 Detection and enumeration

Alternatively, laboratories participating in the ILS for both detection and enumeration may receive three or four (blind) laboratory samples per material, from which they shall obtain three test portions according to prEN 13040-2 and follow the steps in the *Salmonella* spp. standard (WI 00223121), the *E. coli* standard (WI 00223114*)* and the *Enterococcaceae* standard (WI 00223119).

4.4General

The time between the end of the preparation of the pre-enrichment medium (detection) or the initial suspension (enumeration) and the moment when the inoculum comes into contact with the

final culture medium (as specified in the respective draft standards) shall not exceed 45 min. In addition, the time between the preparation of the initial suspension and the beginning of the preparation of the further dilutions shall not exceed 30 min.

If the ambient temperature of the laboratory is outside the recommended range (> 27 °C), these two times periods shall be reduced to minimise the risk of microbial growth and therefore of inaccurate results.

5. Reporting of results

The results of the tests shall be expressed according to the instructions provided in each draft standard and reported to the organisation coordinating the ILS within one month of the reception of the samples.

For the detection test, the output of the ILS is expected to be the LOD_{50}, sensitivity and specificity.

For the enumeration tests, the output of the ILS is expected to be the repeatability and reproducibility.

The organiser of the ILS is expected to produce an ILS report based on the results submitted by the participating laboratories.