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MARILIA deliverable: Description of the Use Cases and Test Experiments

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MARILIA			
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Summary

Abstract

D1.1 and D1.2 focused on the identification of the stakeholders and the interactions with the key players in the drinking water industry, with the aim to derive the main requirements and targets for the introduction of a new microbiological analysis method.

This deliverable dives into how labs actually perform the analysis, breaking the whole process into elementary steps.

This is crucial to understand the operative challenges, limits and constraints that users currently experience, in order to gather further insight on how to design the MARILIA assay.

The analysis was carried out through “Use Case” forms, which provide a narrative and graphic description of how the user executes the job.

The final aim of this Deliverable is setting the baseline for a full comparison between the gold standard methods currently adopted and MARILIA, paving the way to the definition of test experiments.

1. Definition of *Use Case*

A *Use Case*[1] is defined as a list of actions to describe the "step-by-step" interaction between a process actor and a system for achieving a goal.

Born in the field of software development, the use case has been progressively adopted in the field of product and process innovation, particularly when dealing with complex processes.

In fact, by breaking down a process into sub-processes, it is possible to analyze each step individually, but above all to create a basis for comparing different complex processes, as long as they are used to achieve the same purpose.

We will use this to define the individual actions through which the methods of microbiological analysis are performed, and then to report the necessary actions to be implemented in the new MARILIA method of analysis.

Each use case will be described according to the following scheme:

- **Introduction**, which gives an overview of the whole process;
- **Brief description**, which describes when this use case occurs;
- **Actors**, which lists the actors involved in the process;
- **Requirements**, which provides information on the equipment and all the material necessary to carry out the process;
- **Basic Flow**, which includes the step by step description of all the actions necessary to carry out the process.

2. The gold standard *Use Cases*

In this chapter, we describe the use cases of the two standard methods used for the microbiological analysis of drinking water. The description of the actions and equipment required to perform each method comes from the reference article *Water quality monitoring - A Practical Guide to the Design and Implementation of Freshwater Quality Studies and Monitoring Programmes (WHO)*[2] (always showed in Italic form), and has been confirmed by the laboratory technicians of the stakeholders interviewed (see D1.2).

1.1 Multiple Fermentation Tube Technique Use Case

Introduction

The *Multiple Fermentation Tube Technique* (or *Most Probable Number, MPN*) is a statistical method based on the random dispersion of microorganisms per volume in a sample. In this method, the

[1] I. Jacobson, 1992. Object-Oriented Software Engineering: A Use Case Driven Approach

[2] Water Quality Monitoring - A Practical Guide to the Design and Implementation of Freshwater Quality Studies and Monitoring Programmes Edited by Jamie Bartram and Richard Ballance Published on behalf of United Nations Environment Programme and the World Health Organization © 1996 UNEP/WHO

measured volumes of water are added to a series of tubes containing a liquid indicator growth medium. The media receiving one or more indicator bacteria show growth and characteristic color change. Color change is absent in those receiving an inoculum of water without the indicator bacteria. Once the distribution of positive and negative reactions becomes visible, the MPN of indicator organisms in the sample may be estimated by referring to statistical tables.

This test is rolled-out in three steps: presumptive, confirmatory stage and complete.

Brief description

Case 1: The water utility schedules the water analysis tests in a particular node of the water network, as required by law.

Case 2: The drinking water customer requests from the water utility, or private laboratory, to certify the quality of the water supplied.

This method is usually implemented to detect Coliforms*, E. Coli and Legionella*, and the required sample is 100 ml of water, 1000 ml for Legionella.

Actors

A sampler goes to the designated analysis point to collect the sample. Once in the laboratory, **one specialized technician** can carry out the whole process.

Requirements

Necessary **equipment** to perform the analysis is:

- *Incubator or water-bath capable of maintaining a temperature to within ± 0.5 ºC of 35 and 37 ºC and to within ± 0.25 ºC of 44 and 44.5 ºC. The choice of temperature depends on the indicator bacteria and the medium.*
- *Autoclave for sterilizing glassware and culture media. The actual required size depends on the volume of work to be undertaken.*
- *Distillation apparatus, with storage capacity for at least 20 liters of distilled water.*
- *Laboratory balance, accuracy ± 0.05 g, with weighing scoop.*
- *Racks for tubes and bottles of prepared culture media and dilution water. These must fit into the autoclave.*
- *Pipettes, reusable, glass, 10-ml capacity graduated in 0.1-ml divisions, and 1-ml capacity graduated in 0.01-ml divisions.*
- *Test-tubes, 20 × 150 mm for 10 ml of sample + 10 ml of culture medium, with metal slip-on caps.*
- *Bottles, with loose-fitting caps, calibrated at 50 and 100 ml, for 50 ml of sample + 50 ml of culture medium.*
- *Measuring cylinders, unbreakable plasticware or glass, capacity 100, 250, 500 and 1,000 ml.*
- *Test-tube racks to hold tubes in incubator and during storage.*

- *Thermometer for checking calibration of incubator or water-bath.*
- *Refrigerator for storage of prepared culture media.*
- *Hot-air sterilizer for sterilizing pipettes.*
- *Bunsen burner or alcohol lamp.*
- *Durham tubes, 6 × 30 mm.*
- *Pipette cans for sterilizing pipettes.*
- *Wash-bottle.*
- *Pipette bulbs.*
- *Wire loops for inoculating media, and spare wire.*
- *Spatulas.*
- *Container for used pipettes.*
- *Brushes for cleaning glassware (several sizes).*
- *Miscellaneous tools*.

Necessary **consumables** to do the analysis are:

- *Culture media: for example, lauryl tryptose broth, brilliant green lactose bile (BGLB) broth, and E. coli medium, Mac Conkey or Lactose broth, Eosin methylene blue (EMB) agar, nutrient agar, etc…*
- *Disinfectant for cleaning laboratory surfaces and a container for discarding pipettes.*
- *Detergent for cleaning glassware and equipment.*
- *Phosphate-buffered saline solution*
- *Autoclave tape*

Basic Flow

1. **Presumptive stage** (*Figure 1*). *Prepare media of single and double strength in test tubes with Durham's tube and autoclave it.*
2. *Take three sets of test tubes containing five tubes in each set, one set with 10 ml of double strength (DS) and other two containing 10 ml of single strengths (SS).*
3. *Using sterile pipettes, transfer 10 ml of water to each of DS broth tubes. Transfer 1 ml of water sample to each of 5 tubes of one set of SS broth and transfer 0.1 ml of water to five tubes of the remaining last set of SS broth tubes.*
4. *Incubate the tubes at 37°C for 24 hours.*
5. *After the incubation, observe the gas production in Durham's tube and color change of the media.*
6. *Record the number of positive results from each set and compare with standard chart to give presumptive coliform count per 100 ml water sample*.

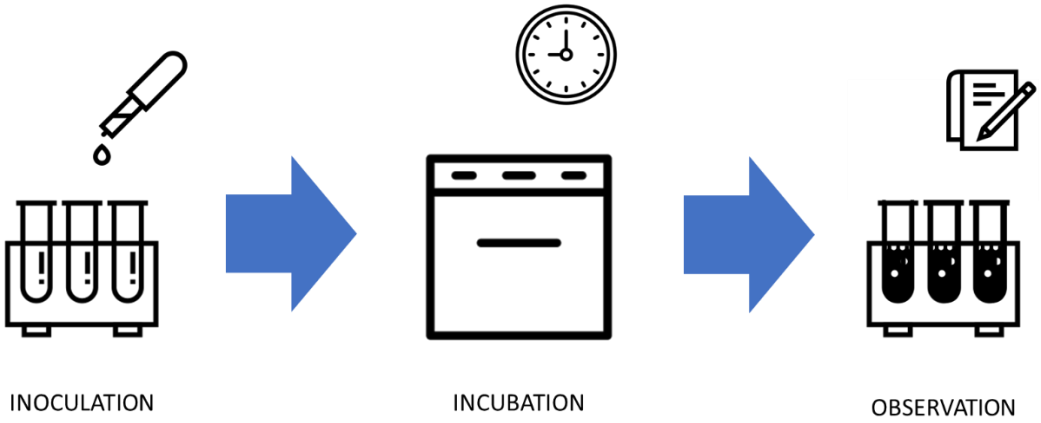

Figure 1. Presumptive Stage actions

7. **Confirmatory stage** (*Figure 2*). *Take the positive tube from the presumptive test and re-inoculate in duplicates in fresh media.*
8. *Incubate one plate at 37°C for 24 hours and another at 44.5°C for 24 hours.*
9. *Look for typical colonies in the media; blue back with green metallic sheen colonies are of E.coli (Coliform or Legionella) in agar.*

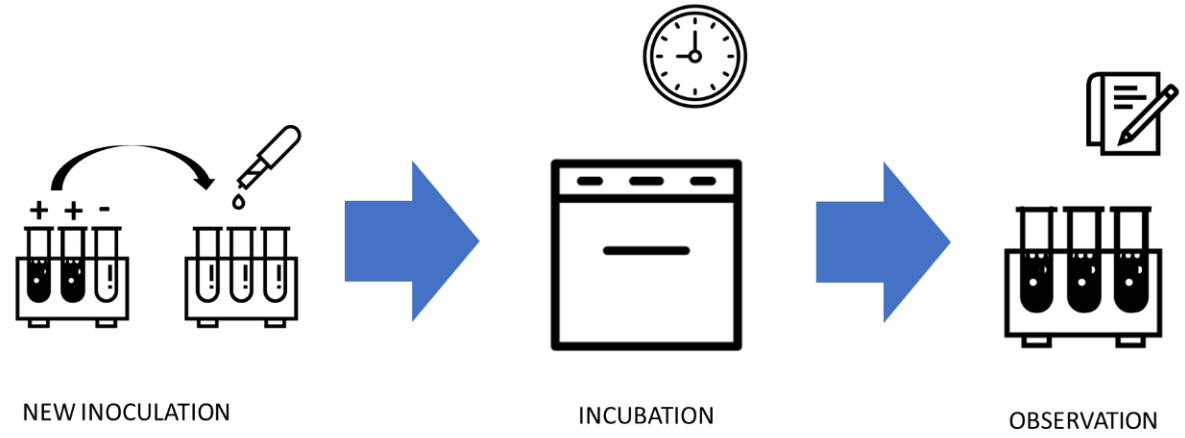

Figure 2. Confirmatory Stage actions

10. **Completed stage**. *Inoculate the colony into a tube of Lactose broth containing a Durham's tube.*
11. *Subculture the colony on Nutrient agar plate. This subculture is considered optional.*
12. *Incubate the broth cultures at 37°C and 44.5°C and Nutrient agar at 37°C.*
13. *Examine for acid and gas production in Lactose broth. The nutrient agar is used for Gram staining and for IMViC tests.*

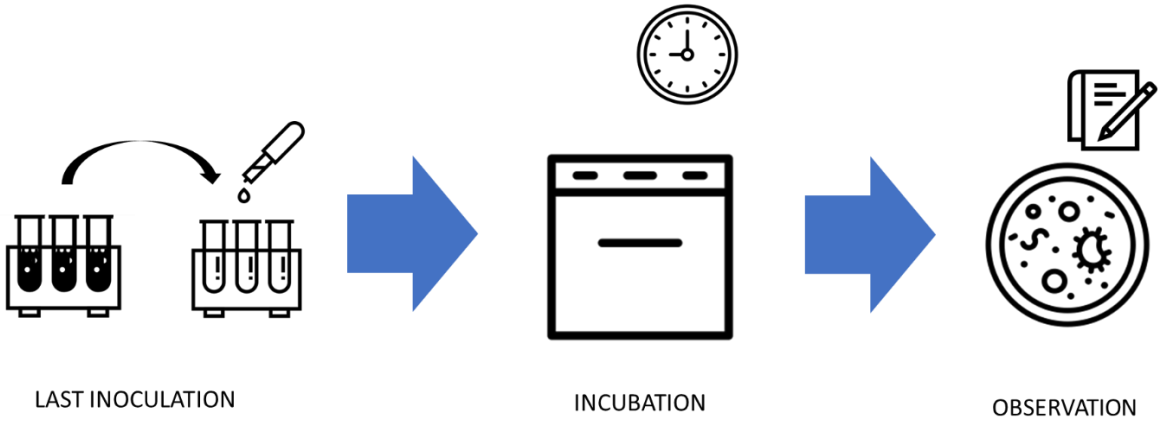

Figure 3. Completed stage actions

1.2 Membrane Filter Technique Use Case

Introduction

During this process, water is passed through a membrane filter with a pore size of 0.45 µm. After that, the membrane is transferred onto an agar plate and incubated. Bacterial cells trapped on the membrane will grow into colonies that can be counted.

Brief description

Case 1: The water utility schedules the water analysis tests in a particular node of the water network as required by law.

Case 2: A drinking water customer requests from the water utility, or private laboratory, to certify the quality of the water supplied.

This method is usually used to detect *Enterococci, Clostridium Perfringens and Pseudomonas Aeruginosa*, and the required sample is 100 ml of water.

Actors

A sampler who collects the sample at the selected point. Once in the laboratory, **one specialized technician** can carry out the whole process.

Requirements

The necessary equipment to perform the analysis is:

- *Incubator or water-bath.*
- *Membrane filtration apparatus, complete with vacuum source (electrically operated pump, hand-pump or aspirator) and suction flask.*
- *Autoclave for sterilizing the prepared culture media.*
- *Boiling-pan or bath.*
- *Laboratory balance.*

- *Racks for bottles of prepared culture media and dilution water. These must fit into the autoclave.*
- *Distilling apparatus with storage capacity for at least 5 liters of distilled water.*
- *Refrigerator for storage of the prepared culture media.*
- *Hot-air sterilizer for sterilizing pipettes and glass or metal Petri dishes.*
- *Thermometer for checking calibration of incubator or water-bath.*
- *Pipette cans for sterilizing pipettes.*
- *Boxes for Petri dishes to use in hot-air sterilizer.*
- *Reusable bottles for culture media.*
- *Measuring cylinders, capacity 100 ml and 250 ml.*
- *Reusable pipettes, glass, capacity 1 ml and 10 ml.*
- *Bottles to contain 9 ml volumes of solution.*
- *Flasks for preparation of culture media.*
- *Wash-bottle.*
- *Blunt-edged forceps.*
- *Pipette bulbs.*
- *Spatulas.*
- *Container for used pipettes.*
- *Brushes for cleaning glassware (several sizes).*
- *Fire extinguisher and first-aid kit.*
- *Miscellaneous tools.*

The necessary consumables to perform the analysis are:

- *Methanol for disinfecting filtration apparatus using formaldehyde gas (unnecessary in the laboratory, but essential if analyses are done in the field). It is essential to use methanol. Ethanol or methylated spirits cannot be used as substitutes.*
- *Membrane filters, 0.45 µm pore size and of diameter appropriate for the filtration apparatus being used and complete with absorbent pads.*
- *Disinfectant for cleaning laboratory surfaces and a container for discarded pipettes.*
- *Culture media (mEndo Agar LES and mFC Agar).*
- *Phosphate-buffered saline solution.*
- *Petri dishes, glass or aluminum (reusable) or plastic (disposable).*
- *Polyethylene bags for wrapping Petri dishes if dry incubator is used.*
- *Magnifying lens (as an aid to counting colonies after filters are incubated).*
- *Wax pencils for labelling Petri dishes.*
- *Autoclave tape.*
- *Detergent for cleaning glassware and equipment.*

Basic Flow

1. **Preparatory stage.** *Sterilize the tips of the blunt-ended forceps in a flame and allow them to cool.*
2. *Remove a sterile membrane filter from its package, holding it with the sterile forceps only by its edge.*
3. *Place the membrane filter in the filter apparatus and clamp it in place.*
4. *Mix the sample by inverting its container several times. Pour or pipette the desired volume of sample into the filter funnel.*

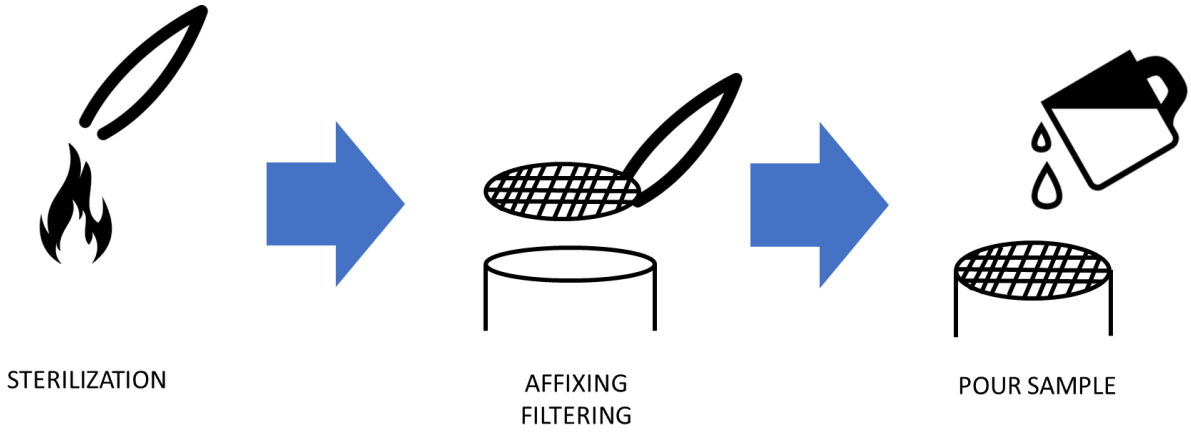

Figure 4. Membrane filter technique preparatory steps

5. *Apply a vacuum to the suction flask and draw the sample through the filter; disconnect the vacuum.*
6. *Dismantle the filtration apparatus and remove the membrane filter using the sterile forceps, taking care to touch only the edge of the filter.*
7. *Remove the lid of a previously prepared mENDO agar LES plate and place the membrane, grid side uppermost, onto the agar. Lower the membrane, starting at one edge in order to avoid trapping air bubbles between the membrane and agar.*

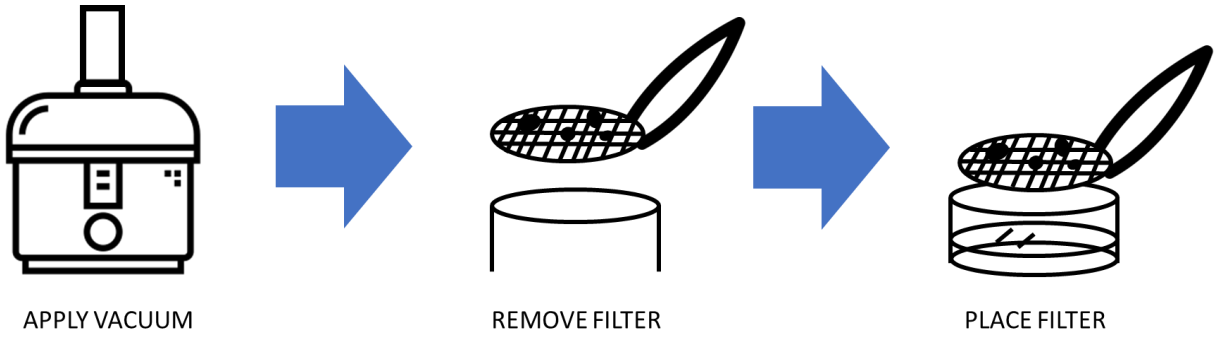

Figure 5. Membrane filter technique preparatory steps (2)

8. **Two-steps enrichment stage.** *Mark the petri dish with the sample number or other identification. The sample volume should also be recorded.*
9. *Repeat the procedure (1 to 8) with the same volume but place the membranes on mFC plates.*
10. *Incubate mEndo agar LES plates at 35 ± 0.5°C for 22 – 24 hours and mFC plates at 44.5 ± 0.2°C for 22 to 26 hours, all lid side down. In order to maintain the temperature within such*

a narrow range, a water bath is typically used for incubation of the mFC agar plates. These plates are placed in watertight plastic bags and then submerged in the water bath.

11. *After 22 – 24 hours, remove the mEndo agar LES plates from the 35°C incubator and count the colonies that are dark red, mucoid, have a dark center or (more typically) produce a metallic sheen. These are considered to be total coliform colonies.*

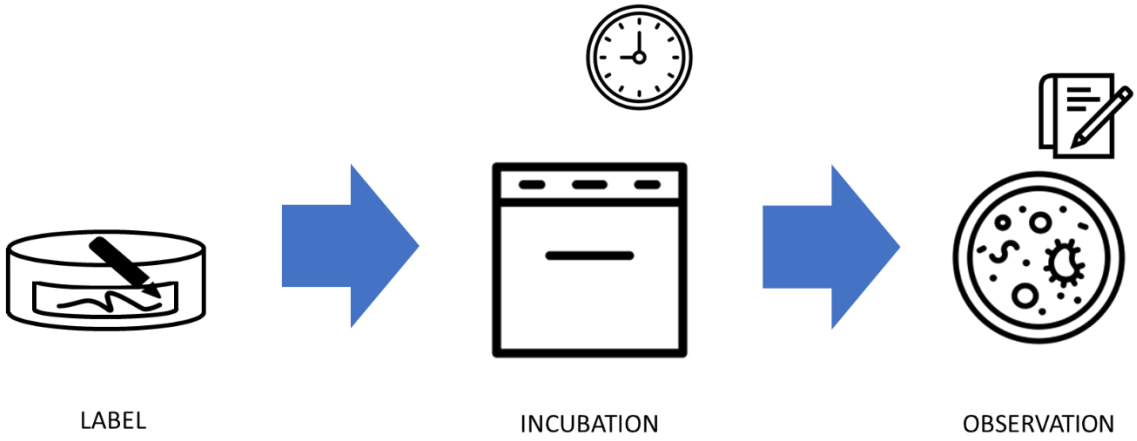

Figure 6. Membrane filter technique last steps

12. *From the mEndo agar LES plates, choose two total coliform colonies that are isolated on the membrane and confirm that they are Gram-negative rods and non-spore formers.*
13. *After 22 to 26 hours, remove the mFC agar plates from the 44.5°C water bath and count the colonies that have any blue color. These are considered to be fecal coliform colonies.*
14. *From the mFC agar plates, choose two fecal coliform colonies that are isolated on the membrane. Confirm that they are Gram-negative rods and non-spore formers.*
15. *Calculate the total bacteria CFU per 100 ml for each sample.*
16. *The original density is estimated from the volume of sample filtered and the number of colonies counted on the membrane.*

3. The MARILIA Use Case

Introduction

Since MARILIA is still in the development phase, the actual analysis process and use case are still to be defined. This will be done in the course of the development work, with the aim of improving the user experience and minimizing the “learning curve” for operators.

With MARILIA, we will develop peptide-based peroxidase components and conjugate them to bacteria-binding proteins (BBP) that target pathogens.

The initially separated peroxidase components will form an active enzyme and, thus, produce a signal when brought together during the detection process. Unlike most molecular techniques which require several expensive reagents (fluorophores, buffers, enzymes and antibodies) in

combination with sophisticated instruments for sample processing and analysis, our approach aims to reduce the reagent, personnel, and instrument costs for pathogen identification.

Brief description

Case 1: The water utility schedules the water analysis tests in a particular node of the water network as required by law.

Case 2: The drinking water customer requests from the water utility, or private laboratory, to certify the quality of the water supplied.

This method can be used to detect *Enterococci, Clostridium Perfringens and Pseudomonas Aeruginosa*, *Coliforms, E. Coli and Legionella*, and the required sample is 100 ml of water, 1000 ml for Legionella.

Actors

A sampler who collects the sample at the selected point. Once in the laboratory, **one specialized technician** can carry out the whole process.

Requirements

The laboratory must have equipment, platform and consumables not completely defined yet, but certainly including:

- Test tubes
- Sampling bottle, sterile
- Filters
- Pump

Basic Flow

1. Water sample is Incubated with DNA-tagged proteins for several minutes.

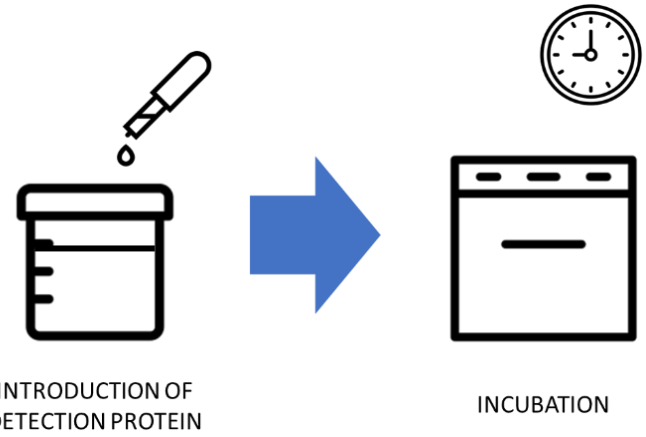

Figure 7. The first steps of MARILIA process[3]

[3] The images used to represent the various steps in MARILIA's Use Case are for illustrative purposes only and do not reflect, as for the other two methods, exactly the actions that are expected to be performed. At this stage we are not yet able to provide an accurate representation of the metho

2. Water is filtered
3. Cells are recovered from filter by reverse elution

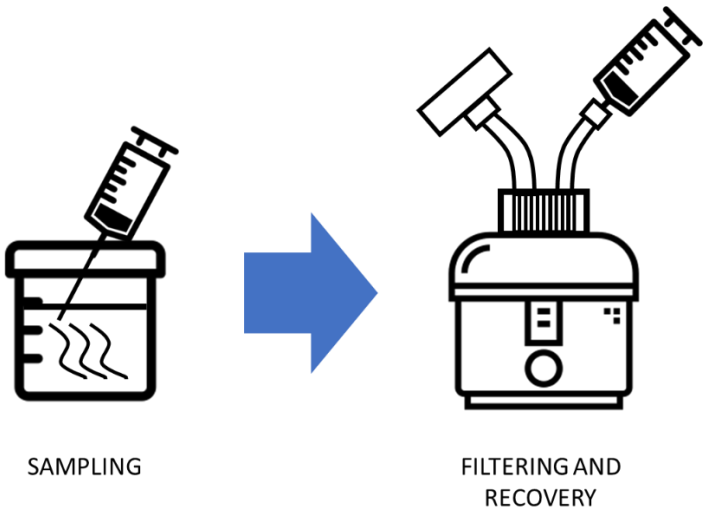

Figure 8. Filtering steps of MARILIA process[3]

4. Capturing of bacteria on detection platform for several minutes
5. Washing of detection platform
6. Incubation with HRP protein for several minutes

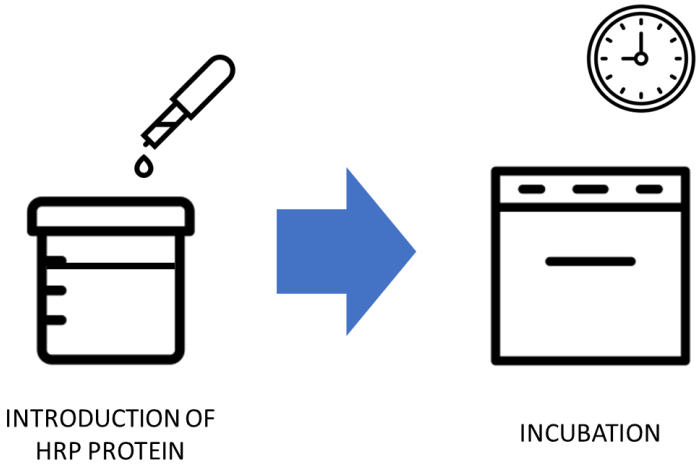

Figure 9. HRP protein steps of MARILIA process[3]

7. Washing of detection platform
8. Incubation with chromogenic substrate for several minutes

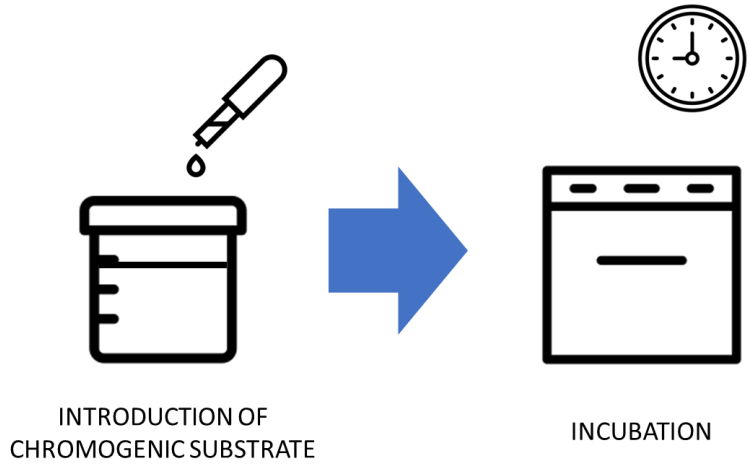

Figure 10. HRP chromogenic substrate steps of MARILIA process[3]

9. Quantitative optical readout

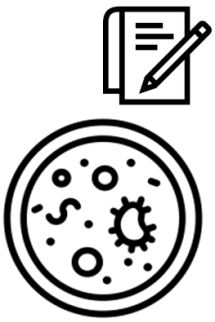

Figure 11. Final observation step of MARILIA process[3]

4. *Use Case* assessment

The description of the analysis processes through the *Use Case* approach allows us to highlight some limits and constraints of the two gold standard methods.

The ***Multiple Fermentation Tube Technique*** requires several pieces of equipment, although it uses a relatively small number of consumables. 13 steps are required before obtaining the result, with strong involvement of the technician during the sample processing phase.

The analysis has a high rate of subjectivity, both during the execution of the single steps and in the observation phase of the results, which is based on statistical assumptions. This cannot rule out mistakes, especially when hundreds of analyses are performed in a single day.

The other weakness of this method is certainly the time it takes to achieve the results. In fact, two distinct incubation phases are necessary, which extend the total required time up to 72 hours (depending on the bacterium sought).

Regarding the ***Membrane Filter Technique***, both the number of necessary pieces of equipment and the quantity of consumables used are high.

There are 16 steps required to carry out the entire process and some of them, as we reported during the interviews (see D1.2), are very expensive in terms of time and cost of personnel, such as the filtering step. Once again, the sample processing time represents a crucial limitation.

For what concerns the total incubation time, although it is generally shorter than for the Multiple Fermentation Tube Technique, it can reach up to 48 hours.

Since the observation of the colonies is done by visual inspection, this method has a strong subjective component in the interpretation of the results.

Both methods require fully equipped laboratory and controlled environment to be performed and large number of steps with high risk of external contamination. This leads us to think that it would be extremely difficult that they could be carried out directly in the field, even if the incubation times were reduced to a minimum.

Regarding the **MARILIA** *Use Case*, although there is no detailed information about the whole process yet, we can already notice some significant differences.

First of all, it is expected that the number of pieces of equipment required, as well as the number/quantity of consumables is significantly less than the other two methods.

The number of steps required will also be reduced, but above all the activities appear simpler and less subjective, decreasing the risk of possible external contamination.

In general, with MARILIA the sample processing time will be more than halved.

Besides, the MARILIA method requires two incubation phases, although much shorter than the other two methods. The duration of such phases is not yet clear but an achievable target (see D1.2) has been set at 4 hours.

Finally, MARILIA foresees a more accurate presentation of the results, which excludes the subjective component of the operator through the use of digitalized equipment.

5. Conclusions

In D1.1, the key stakeholders and the methods of analysis used were identified, while in D1.2 the information collected from some of these stakeholders are processed to compare the existing methods by giving us targets for our new method.

The purpose of this deliverable was to investigate the reasons behind the performances of the currently adopted gold standard methods, thus paving the way for the definition of the characteristics on which to focus our development efforts.

The description of the current analysis methods in the form of *Use Case* allowed us to make a comparison between these three very different methods and the results confirm what already emerged from the analysis carried out using the *QFD* (see D1.2).

The *Multiple Fermentation Tube Technique* and the *Membrane Filter Technique*, despite being effective in terms of accuracy of the results, can be improved for what concerns the processing time. Both require a long and demanding sample processing phase to be carried out by expert technicians; also, they provide subjective results. In addition, they are severely limited in the possibility of reducing times to results due to the various incubation phases.

A comparison with the preliminary description of the new MARILIA method of analysis shows that these limitations can be improved and suggests that development efforts should be concentrated on: 1) reducing and simplifying sample processing and 2) eliminating as much as possible the subjective aspects of this processing.

The preliminary analysis done during these first months highlights that an improvement of these features would bring a significant competitive advantage to the MARILIA solution, provided that the costs of equipment and consumables as well as the accuracy of the analysis remain at the level of the two gold standard methods.