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MOBILES

D3.1 - Sampling design and library preparation

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Deliverable D3.1 – Sampling design and library preparation

Short summary:	Document describing protocols, methods and initial results on how to collect soil samples and prepare genomic libraries to be used for sequencing analysis.
Due date:	M12
WP, leader:	[WP3, CNR-ISAFOM]
Authors:	[Anna Marzec-Grządziel, ISSPC] [Giuseppe Puglia, CNR-ISAFOM] [Angelo Ferraro, NTUA]

Dissemination Level

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List of participnts

Participant No	Participant organisation name	Country
1 Coordinator	ETHNICON METSOVION POLYTECHNION (NTUA)	GR
2	CONSIGLIO NAZIONALE DELLE RICERCHE (CNR)	IT
3	INSTITUT NATIONAL DE RECHERCHE POUR L'AGRICULTURE, L'ALIMENTATION ET L'ENVIRONNEMENT (INRAE)	FR
4	UNIVERSITA DEGLI STUDI DI ROMA LA SAPIENZA (UR)	IT
5	EDEN TECH (EDEN)	FR
6	UNIVERSIDAD PUBLICA DE NAVARRA (UPNA)	ES
7	INSTYTUT UPRAWY NAWOZENIA I GLEBOZNAWSTWA, PANSTWOWY INSTYTUT BADAWCZY (ISSPC)	PL
8	THE AGRICULTURAL RESEARCH ORGANISATION OF ISRAEL - THE VOLCANI CENTRE (ARO)	IL
9	UNIVERSITE DE BORDEAUX (UBx)	FR
10	TECHNOLOGIKO PANEPISTIMIO KYPROU (CUT)	CY
11	HEMIJSKI FAKULTET, UNIVERZITET U BEOGRADU (UBE)	RS
12	MAT4NRG-GESELLSCHAFT FUR MATERIALIEN UND ENERGIEANWENDUNGEN MBH (Mat)	DE
13	TECHNISCHE UNIVERSITAT CLAUSTHAL (TUC)	DE
14	GRANT GARANT SRO (GG)	CZ
15	CENTRUM BADAN I INNOWACJI PRO-AKADEMIA STOWARZYSZENIE (RICPA)	PL

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Content

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1. D3.1 SUMMARY

Soil biodiversity reflects the variability among living microorganisms which contribute a wide range of essential biochemical pathways to the sustainable function of all ecosystems. They act as the primary driving agents of nutrient cycling, regulating the dynamics of soil organic matter, soil carbon sequestration and greenhouse gas emission, modifying soil physical structure and water regimes, enhancing the amount and efficiency of nutrient acquisition by the vegetation thus enhancing plant health. Abiotic and biotic factors can change microbial community structure as well as their ecosystem function. Therefore, the soil condition from the moment of sampling to the end of the analysis process should be taken into account in terms of its impact on its microflora (species composition).
The protocol described in this deliverable provides guidance for MOBILES consortium members on how to take samples, how to handle them and under what conditions to store them for the microbial biodiversity assessment. This document provides the methodology for the correct collection of a soil sample for the assessment of soil microbial biodiversity within MOBILES. In the selected Experimental Places (EPs), level assessment of soil microbial biodiversity will be carried out. The protocol will provide detailed guidelines for soil sampling, storage and shipment procedure to ensure the quality and uniformity of samples. The second section describes how to process samples at the ISSPC laboratory, the RNA and DNA isolation procedures, how to store samples, and how to prepare them for subsequent sequencing.
No authorizations to access the selected EPs were required since such sites were public, overseen by other EU projects, or part of partner institutions.
In the next section a brief summary of MOBILES project is provide in order to frame the D3.1 work.

2. MOBILES project short description

The National Technical University of Athens (NTUA) is working with another 15 partners from academia, research, and industry to develop prototypes of electronic and organism-based biosensors to monitor organic chemicals, antimicrobial-resistant (AMR) bacteria, and pathogens in water, soil, and air.
The MOBILES project is studying and developing biosensors for detecting heavy metals, antibiotics, pesticides, arsenic, microplastics, and nanoplastics. It is also developing genetically modified plants and bacteria for detecting heavy metals, antibiotics, and pesticides, and the use of marine diatoms for monitoring bioplastic degradation.

MOBILES aims are to tackle chemicals, including persistent and mobile pollutants (PMPs) and contaminants of emerging concern (CECs), that degrade the environment. Another severe global health risk is associated with increasing antimicrobial resistance (AMR) in bacteria. Foodborne pathogens, including Listeria, Salmonella, and Campylobacter, pose significant public health risks and are already monitored. However, current bacterial detection methods for environmental control are slow and require specialized laboratories with trained personnel. Similarly, conventional pollutant detection methods, such as chromatography and mass spectrometry, are accurate but time-consuming and require specialized equipment. State-of-the-art detection methods are unsuitable for

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constant on-site and real-time monitoring. The long time between sampling and detection reduces the efficiency of public health and environmental protection authorities in implementing effective countermeasures. To tackle this problem, several electrochemical biosensors will be developed within the MOBILES project.

Biosensors are devices that combine biological elements with electronic systems to detect specific pollutants. The MOBILES project enhances these sensors with advanced nanomaterials, significantly improving their sensitivity and reliability. All biosensors will have common basic electronics and functional principles (e.g., an organic ligand able to recognize target pollutants), but they will differ in the biological element employed: (i) aptasensors based on aptamers that recognize bacterial cells or spore surfaces, (ii) electronic noses for detecting and quantifying volatile organic compounds (VOCs) produced by bacteria, (iii) genosensors for detecting genes involved in antibiotic resistance, and (iv) interdigital capacitors functionalized with aptamers for estradiol, a member of CECs family.

Continual threats (such as industrial pollution and the overuse of drugs and pesticides) to sources of drinking water require real-time solutions for wide-ranging water monitoring systems to detect toxicants such as heavy metals, pesticides, and antibiotics. Conventional methods are limited in their ability to detect sub-lethal concentrations of active antibacterial compounds. The damage caused by the activity of an antibacterial agent or pesticide may stimulate different biological mechanisms of bacterial repair. Each antibiotic and/or pesticide triggers specific cellular pathways, mechanisms, and targets within the bacterial cell. This specific biological response, enabling the detection of antibiotics and pesticides using microorganisms, is being investigated in the MOBILES project through the use of genetically modified bacteria to detect toxic pollutants in water. For detecting heavy metals (cadmium, chromium, lead, mercury) in water, MOBILES is developing a flow-through device for continuous monitoring using biological systems (genetically modified bacteria) combined with an optical sensor and flow unit.

Highly toxic arsenic pollution can come from various sources, including industrial activities, mining, and even natural processes. Water and food contaminated by arsenic can cause serious health problems, including cancer and heart disease. For detecting arsenic pollution in soil and groundwater, the MOBILES project is developing genetically modified plants that change colour when arsenic is present in the soil or water used to grow them. The project will conduct safety evaluations to ensure that the genetically modified organisms and developed devices have minimal environmental impact. A specific work package (WP4) is dedicated to evaluate the effects of genetically modified organisms on other organisms and the environment. Safety tests and environmental impact of MOBILES organisms are performed in laboratory using EFSA guidelines.

Microplastic and nanoplastic pollution is raising concerns about its potential impact on human health. The transfer of very small plastics through the trophic chain is a potential source of contamination at all trophic levels. Understanding the distribution, degradation, and life cycle of micro- and nanoplastics in the marine environment is limited by the intrinsic difficulties of current techniques for detecting, quantifying, and chemically identifying small particles in liquids. The MOBILES project is

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addressing this challenge by utilizing marine diatoms. Diatoms are known for their resilience and adaptability, making them ideal candidates for studying the biodegradation of bioplastics in marine environments. Preliminary studies have shown promising results, indicating that diatoms not only survive in environments containing bioplastics but also contribute to their biodegradation.

In addition to the development of sensors, MOBILES will undertake comprehensive metagenomic analysis, profiling the microbiota of polluted areas across Europe. This work will uncover gene clusters and reveal genetic diversity, enabling a deeper understanding of microbial functions. These insights will provide genetic markers to facilitate rapid evaluation of soil and land health. Two annual sampling rounds are planned for at least two years, and sample collection will be conducted at different locations to target microbiota related to specific pollution types: Greece for urban wastewater contamination, Poland for heavy metal pollution, Cyprus for microplastics and plastics, France for agriculture and animal farming, Italy for arsenic, and Germany for chemicals and heavy metals from former mining activities. Genomic and transcriptomic data will be analysed, visualized, and interpreted using bioinformatic tools and soil metagenomic web-based platform specifically realized by MOBILES partners. The project's data storage, located in Spain, will be connected to other well-known genomic databases in order to provide a wide range of information.

The biosensors will be rigorously tested with real-world samples from polluted sites to validate their environmental performance.

MOBILES workplan is organized in 6 WP listed in Table 1. Management actions and collaboration with other EU funded projects are implemented within WP6 while disseminations, exploitation and communication (DEC) activities are grouped within WP5. WP1-WP2 deal with interconnected scientific and technological activities to develop electrochemical biosensors to detect specific pollutants, and organism-based biosensor to monitor other typology of selected pollutants. In WP3 an extensive metagenomic analysis will be performed in order to enable searches for diverse functionalities across multiple gene clusters in polluted and not polluted areas across Europe. In WP4 the safety of all genetically modified bacteria and plants will be tested using standard EU procedures (e.g., EFSA guidelines for genetically modified organisms), and a pre-industrial design of the various biosensors will be provided along with stability (shelf-life) tests.

Table 1. MOBILES WPs list

WP	Work Package Title	Lead Name	Start Month	End month
1	Electronic biosensors for environmental monitoring	INRAE	1	36
2	Detection of pollutants via biotic sensors	UR	1	36
3	Metagenomics database and fully-sequenced polluted soil microbiota	CNR-ISAFOM	1	42
4	Environmental performance and safety of developed organisms, and packaging of sensor devices	RICPA	10	42
5	Dissemination, exploitation and communication of project outcomes	GG	1	42
6	Project Management and Coordination	NTUA	1	42

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3. AIMS AND HYPOTHESES

The Task D3.1 “Sampling design and library preparation” is part of WP3 and aims to identify the changes of microbial diversity of soil microorganisms under the influence of different pollutions.

THE SOIL SAMPLING PROTOCOL. Guidance on the collection, handling and storage of soil for the assessment of microbial processes in laboratory are provided in order to ensure the consistency of sample data across various European areas. The protocol aims to present a short procedure for collecting a soil sample for the subsequent assessment of soil microbial diversity. The protocol is intended to provide information on how to properly collect a soil sample for testing in order to later assess the microbiological quality of the soil in laboratory.

3.1. SELECTION OF THE SAMPLING SITE

The choice of the areas (Figure 1) from which samples were taken resulted from a careful evaluation of research purposes and scopes: provide a picture of the microbiota communities in contaminated fields across European countries. Each place was identified and recorded on a map with reference to the location of easily recognizable fixed objects. Widely available, free software was used to determine the exact GPS location of sampling (e.g., Google Maps). Where possible, these locations were marked so that comparative testing or re-sampling could be done.

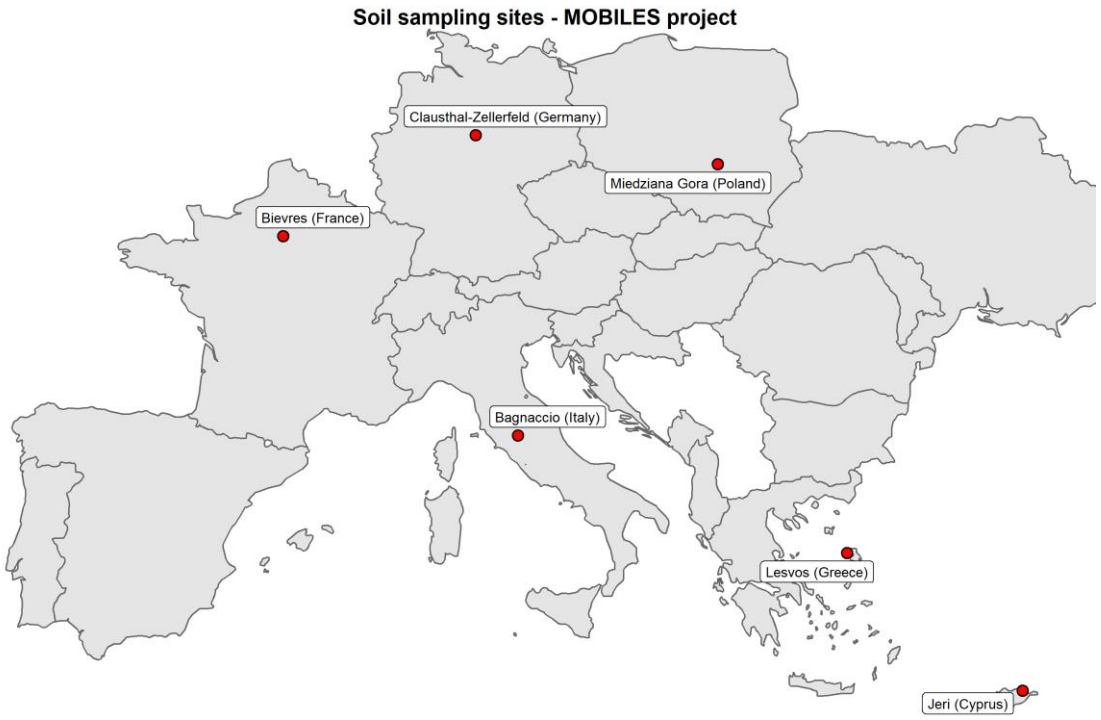

*Figure 1. MOBILES Soil sampling sites in Europe. **Germany**: Clausthal-Zellerfeld, 51°48'16.1"N 10°20'27.8"E; **Poland**: Miedziana Gora, 20°33'E 50°56'N; **France:** Bièvres, 48°46'07.0"N 2°11'31.8"E; **Italy**: Bagnaccio, 42°45’82.53’’ N 12°06’56.90’’ E; **Greece**: Lesvos, 39°14'19.6"N 25°59'48.1"E; **Cyprus**: Jeri, 35.10349° N, 33.39940° E*

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3.2. SAMPLING CONDITIONS

Soil needed for laboratory tests were taken from the sites at a moisture content that facilitates its subsequent screening. Sampling during or after long periods (>30 days) of drought, frost or flooding was avoided.

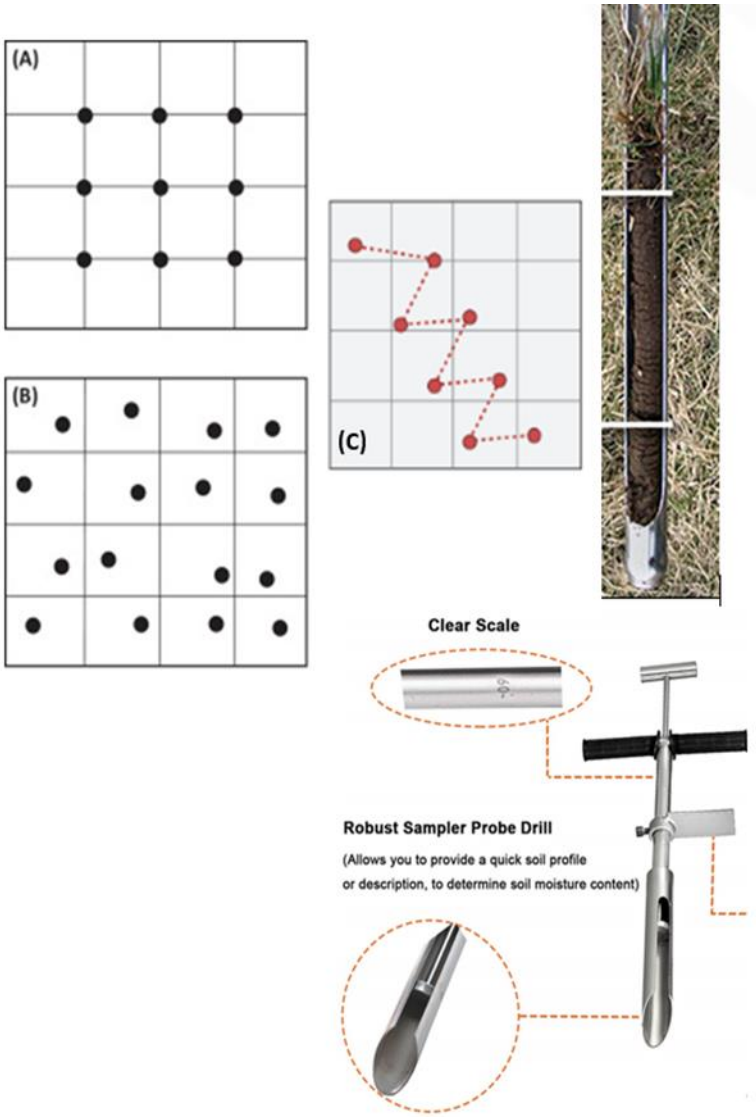

Figure 2. Grid example for soil collection (A – regular, systematic grid sampling method, B – random sampling method, C – zig-zag method)

3.3. SAMPLING RULES

In the case of taking soil samples in aerobic conditions (e.g. from the field), a depth not exceeding 20 cm was usually used. In order to reduce the amount of fresh organic carbon entering the soil, the plant cover, visible parts of roots and above-ground parts of plants and soil fauna were removed. Indeed, organic components from root residues and other sources of organic matter can cause unforeseen changes in the activity and composition of soil microorganisms.

Replicates: Each bulk sample consisted of at least 10 pooled subsamples from a 0-20 cm deep layer (Figure 2). First, the top layer of the soil was removed. The soil samples were collected from the 0–20 cm layer and transferred to bags and then stored at 4 °C, e.g. refrigerator, until they were sent to the laboratory. In ISSPC laboratory, pooled samples were sieved and divided into 3 replicates. Thus, prepared samples were subjected to DNA and RNA isolation (as soon as possible) and were stored at -80°C.

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Soil samples were taken (Spring 2025) from 6 countries: Poland, Figure 3; France, Figure 4; Italy, Figure 5; Greece, Figure 6; Germany and Cyprus

Figure 3. Soil sampling in Poland

Figure 4. Soil sampling in France

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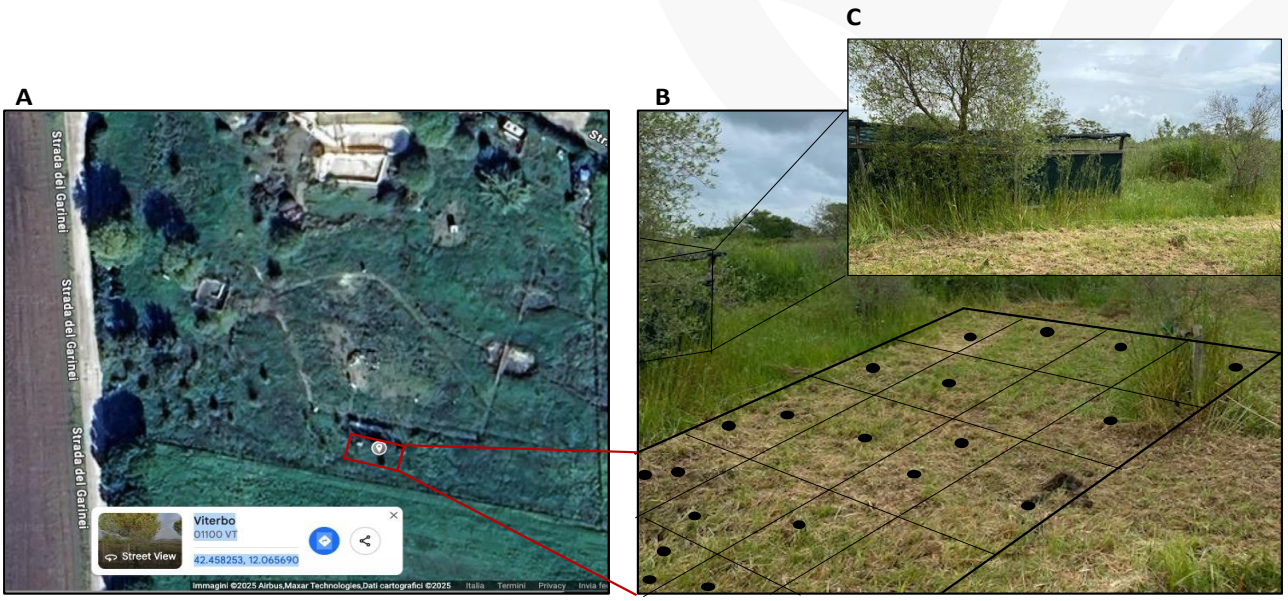

Figure 5 Soil sampling in Italy (A – the red tag in the spring thermal area represents the plot used for soil sampling, B – magnification of the area (7m × 4m) where the soil was collected using a random sampling, C – magnification of the experimental field adjacent to the selected area

Figure 6 Soil sampling in Greece

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3.4. LABELLING OF SAMPLES (SAMPLES ID)

Containers or plastic bags for samples were clearly and unambiguously labelled so that each sample is assigned to the place from which it was collected. Appropriate abbreviations were used for each country: PL (Poland), GER (Germany), GR (Greece), CYP (Cyprus), FR (France), IT (Italy). Each sample name contained S, as an abbreviation for spring sampling. In Greece, two samples typology were taken and labelled 1 and 2. Each technical repetition was labelled 1-3.

3.5. SAMPLE TRANSPORT CONDITIONS

Collected soil samples were transported to the local laboratory as soon as possible in refrigerated conditions. Samples shipped to ISSPC laboratory were transported in dry ice within 1 or 2 days.

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4. SOIL SAMPLES PROCESSING AT THE ISSPC LABORATORY

4.1 PREPARATION OF SOIL SAMPLES FOR TESTING

The soil samples were prepared for testing as soon as possible after receiving them by the ISSPC laboratory. Plants, stones, root remains were removed before the soil was sifted through sieves with 2 mm holes (Figure 7A). Sieving the soil through 2 mm sieves facilitates gas exchange between particles since it is recommended to maintain aerobic conditions in the soil. This also makes possible to remove small stones, remnants of plant debris and fauna (Figure 7B). In case a soil sample was too wet, it was sprinkled on a tray before sowing and dried in a gentle stream of air. After sieving, the samples were weighed and divided into three technical replicates (Figure 7C-D). Each replicate was placed in three types of containers: a zip-lock bag (chemical analysis), a 5 ml test tube (DNA and RNA isolation), and a 50 ml backup test tube (sample for storage at ISSPC) (Figure 7E).

Figure 7. Soil samples preparation (A – sieving of soil sample, B –sieved soil sample, C – weighting of soil sample, D – soil samples divided into three technical replicates, E – soil samples in three type of containers)

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4.2 SAMPLE STORAGE CONDITIONS

The sieved samples were either directly analysed or stored under specific conditions depending on analysis. Samples for sequencing and chemical analyses were stored in the dark at -80℃ with free access to air.

4.3 NUCLEIC ACIDS ISOLATION PROTOCOL

DNA and RNA were isolated from soil using commercially available kits. DNA was isolated using the FastDNA™ SPIN Kit for Soil (Mp Biomedicals) according to the manufacturer's instructions. RNA was isolated using the RNeasy PowerSoil Total RNA Kit (Qiagen). The concentration of the obtained DNA and RNA isolates was checked using a fluorometer and QuantiFluor® ONE dsDNA System and QuantiFluor® RNA System reagents. The stability and quality (RIN – RNA integrity number) of RNA was checked using the 4150 TapeStation System and RNA ScreenTape Assay for TapeStation Systems. Results of concentration and quality of RNA and DNA are listed in Table 2. Due to the low RNA concentration obtained for samples from Poland, the isolation was repeated. The DNA and RNA have been divided and split in two aliquots. One half will remain at the ISSPC laboratory as a bank sample. The other half will be sent for sequencing.

Table 2. Concentration and quality of obtained DNA and RNA

Sample	DNA	RNA	
	Concentration [ng/µl]	Concentration [ng/µl]	RIN
PL_S_1	37,0	20,0	8,1
PL_S_1	34,0	14,0	-
PL_S_1	40,0	11,0	-
PL_S_1		16,0	8,7
PL_S_1		12,0	-
PL_S_1		14,0	-
GER_S_1	89,0	42,7	8,5
GER_S_2	84,0	55,4	8,7
GER_S_2	92,0	50,6	8,7
GR_S_1_1	30,0	48,3	8,1
GR_S_1_2	29,0	58,3	8,5
GR_S_1_3	34,0	61,6	8,7
GR_S_2_1	34,0	71,9	8,6
GR_S_2_2	62,0	84,1	8,8
GR_S_2_3	65,0	84,3	8,8
CYP_S_1	59,0	150,0	7,9
CYP_S_2	58,0	83,5	7,8
CYP_S_3	60,0	75,2	8,1
FR_S_1	>200	184,0	9,0
FR_S_2	>200	186,0	9,1
FR_S_3	>200	200,0	8,9
IT_S_1	28,0	44,1	8,0
IT_S_2	31,0	61,2	7,9
IT_S_3	30,0	46,1	-

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5. List of Figures

6. List of Tables

7. List of Abbreviations

Abbreviation	Abbreviation for
AMR	Antimicrobial-Resistant
CECs	Contaminants of Emerging Concern
DEC	Disseminations, Exploitation and Communication
EFSA	European Food Safety Authority
EPs	Experimental Places
GPS	Global Positioning System
PMPs	Persistent and Mobile Pollutants
VOCs	Volatile Organic Compounds
WP	Work Package

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8. Project Consortium

	www.ntua.gr/en/
Italian National Research Council	www.cnr.it/en
INRAE	www.inrae.fr/en
SAPIENZA UNIVERSITÀ DI ROMA	www.uniroma1.it/en/pagina-strutturale/home
eden tech	www.eden-microfluidics.com/
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université de BORDEAUX	www.u-bordeaux.fr/en
Cyprus University of Technology	www.cut.ac.cy/?languageId=1
University of Belgrade Faculty of Chemistry	www.chem.bg.ac.rs/index-en.html
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