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Protocol

Study Protocol of Predictive Dynamics of Microbiological Contamination of Groundwater in the Earth Critical Zone and Impact on Human Health (DY.MI.CR.ON Project)

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Abstract: Groundwater is one of the major sources of water supply for human needs. But anthropic activities such as agriculture are causing significant volume depletion and quality deterioration, favoring microbial contamination that has a negative impact on human health. The geological characteristics of the ground can influence the transport of microorganisms, especially if made of permeable rock. Furthermore, irrigation with untreated or partially treated wastewater can represent an additional health risk due to the potential transmission of pathogens to food. The aim of our research is to provide an interdisciplinary perspective on this issue by integrating hygienic, geological, and agronomic skills. Water samplings are scheduled seasonally by four monitoring campaigns in five sampling points placed in two Southern Italy regions, Apulia (one point at the outlet and two wells near the wastewater plant at Carpignano Salentino, Lecce province, Italy) and Sicily (two wells at Scicli and Pozzallo, Ragusa province, Italy) Laboratory experiments of microorganism transport in permeable rocks will be carried out under saturated and unsaturated conditions. A mathematical model of transport through porous media will be implemented and validated with laboratory measurements. The model will be used to develop a monitoring tool to control sites in Apulia and Sicily where periodic cultural and molecular detection of pathogenic bacteria, viruses, and protozoa will also be taken. In addition, an analysis of the microbiological contamination of herbaceous crops due to the use of low-quality water will be conducted to assess the Quantitative Microbial Risk Assessment (QMRA). The project will provide methodological tools to evaluate anthropogenic pressures and their impact on environmental matrices. The results will allow these pressures to be modulated to minimize environmental and agri-food microbiological contamination and protect public health.

Keywords: microbiological contaminants; porous aquifer; microbial transport modeling; Earth critical zone; wastewater treatment plant; groundwater quality

1. Introduction

In recent years, many water-borne diseases from contaminated groundwater have been reported by several countries (i.e., Denmark, Finland, Sweden, Norway, and the USA) with varying levels of economic development [1,2]. These diseases are caused by pathogenic microorganisms of enteric origin which, once reaching the groundwater, pose a significant risk to public health. Groundwater contamination occurs as a result of point and diffuse sources, both natural and anthropogenic: for example, grazing can easily lead pathogens into water bodies, facilitated by rainfall events as well as the spreading of manure used as fertilizer, which is considered one of the main sources of environmental pollution when mismanaged [3]. Although wastewater reuse has been considered a water-saving strategy to manage water scarcity [4], on the other hand, several cases concerning the diffusion of pathogenic microorganisms have been reported due to uncontrolled disposal and inadequate treatment of wastewater [5–7].

The wide variety of pathogenic microorganisms that can reach groundwater includes bacteria such as *Escherichia coli*, *Enterococcus* spp., *Salmonella* spp., and *Clostridium perfringens*, known as indicators of fecal contamination [8]. These microorganisms can lead to gastrointestinal infections such as dysentery and diarrhoeal diseases [9–11]. In addition, verotoxigenic *Escherichia coli* (VTEC) infection comprises a wide range of symptoms from mild uncomplicated enteritis in healthy adults to fatal hemorrhagic diarrhea and colitis, including hemolytic uraemic syndrome, among vulnerable individuals [12].

Microbial contamination can be influenced by many interlinked risk factors, pre- and post-conditions, and source-specific characteristics. These risk factors, both individually and in combination, may show significant spatial and temporal variability [13].

Runoff processes mobilize these microorganisms, leading to potential groundwater contamination through infiltration into the unsaturated zone (also known as the vadose zone) which is the portion of the Earth between the land surface and the water table (top of the phreatic zone) [14] (Figure 1). It is widely believed that the groundwater quality, particularly for the deeper aquifers, is preserved by filtration processes through the subsoil or is protected by impermeable layers [15]. This perception is not fully correct [16]. As a matter of fact, microbiological contamination of groundwater can occur easily in aquifers characterized by fractures and karst phenomena even if they are deep [17]. In these aquifers, water can rapidly move, allowing the transport of microorganisms, with negligible interactions with rocks. In porous aquifers, instead, although the microorganisms can be easily transported through the voids of the rock [17], they also undergo interactions with the matrix, which can reduce pollution loads. Several factors need to be considered to understand the dynamics that control groundwater microbiological contamination. In this regard, several authors have developed predictive modeling systems to understand the mechanisms that control the transport of pathogens through porous media (particularly porous rocks) under varying conditions of velocity, saturation, and salinity [18–21].

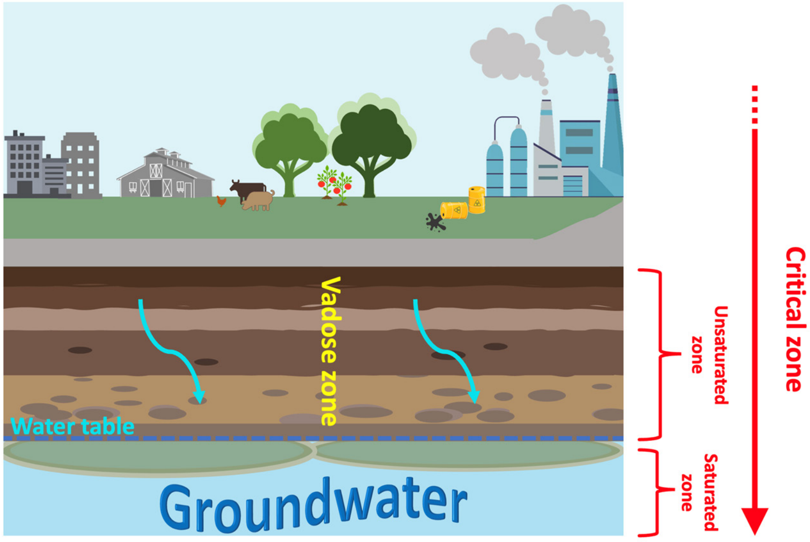

Figure 1. Schematic representation of microbial contaminant transport into the subsoil.

Nevertheless, an interdisciplinary approach to understanding the pathogen transport process through unsaturated zones is still rare and, even if there are studies at the laboratory scale, there is a lack of research on a field scale [22,23]. Numerical models, calibrated by integrating both laboratory and field experimental data, can represent efficient tools able to predict contaminant dynamics within different scenarios and are therefore useful in preventing groundwater contamination.

The major processes controlling the transport of microorganisms in the subsurface are attachment/detachment, straining, and/or inactivation. These processes are affected by several factors including the water saturation, the porosity, and the mineralogy of the porous medium as well as the chemical composition of the waters [24]. The survival and persistence of microorganisms in groundwater are significantly affected by the climate which regulates the precipitation and the surface and subsurface temperature. Moreover, the fate of microbiological contaminants is related to the hydraulic properties of the unsaturated zone, connecting the surface to the groundwater, and to the hydrogeological characteristics of the aquifer [17,25]. Phenomena such as the aquifer recharge, the runoff and infiltration processes, and the seawater intrusion influence groundwater pollution and make the management of groundwater quality difficult [26]. In addition, it is known that failure to protect groundwater sources, coupled with inadequate water treatment, are the main reasons for microbial contamination of drinking water [27].

Furthermore, due to the extensive use of antibiotics for clinical and veterinary purposes, wastewater is suspected to be a hot spot for antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARBs) [28]. Contaminated groundwater used for irrigation is a crucial issue for the safety of horticultural crops. Microbiologically contaminated water can contaminate the surfaces of leaves or microbes can be absorbed by radical roots directly from soil and concentrate into plant tissues, increasing the risk for consumers, for example, in the case of ready-to-eat salads. Nevertheless, the groundwater-derived contamination of vegetables is not currently well explored [29,30], as well as the evaluation of the health risk derived from the ingestion of such food.

In Italy, several studies have been carried out on the microbiological contamination of groundwater in order to understand the effects of groundwater quality in different aquifer characteristics [31–35]. Although public health authorities have strict regulations on the distances between human and animal waste disposal sites and drinking water wells, with the intent of protecting human health, they neglect any hydrogeological assessment [17].

In the context of global water scarcity due to increasing water demand and the effect of climate change, understanding the microbial transport process through the unsaturated porous media and their fate before reaching the groundwater is essential to prevent further contamination of the aquifer and is a crucial issue for the optimal management of unconventional water resources for irrigation and industrial use.

Increasing groundwater contamination suggests the identification of mechanisms that control the movement of contaminants through the unsaturated zone, an important portion of the critical zone (CZ) (Figure 1), a thin living layer that connects the atmosphere and geosphere, including aquifers, and where important physical, biological, and chemical processes occur at different space–time scales; studying the processes occurring in the CZ requires an interdisciplinary, multiscale, and integrated approach, including traditionally stand-alone disciplines.

The aim of the DY.MI.CR.ON Project (predictive dynamics of microbiological contamination of groundwater in the Earth critical zone and impact on human health) is to improve our understanding of groundwater contamination through an interdisciplinary approach in which geological, hydrogeological, hygienic, and sanitary knowledge converge synergistically. The geological characterization of the media through which the water with contaminants flows and the study of the impact of agricultural activities and sanitation risk on public health will allow the development of monitoring plans, mitigation strategies, and contamination risk forecasting with maps to identify priority areas and actions aimed at preserving groundwater quality [36,37]. These activities will improve planning capacity and overcome crucial challenges such as environmental quality, sustainable development, food security, and human safety.

2. Materials and Methods

2.1. Aim and Organization

Our project is included in Mission 4 "Instruction and Research" Component 2 of the NRRP (National Recovery and Resilience Plan)—Territory and water resource protection. This work, financed by "European Union-Next Generation EU", aims to improve the knowledge about groundwater contamination by a novel and unusual interdisciplinary approach, based on microbiological, geological, and agronomic aspects.

Several experimental techniques, developed at different scales (from laboratory scales to catchment scales through the field one) and environmental conditions, are applied to study microorganism transport in unsaturated porous media [21,38,39], specifically rocks. The major output of this project task is a model that can be used to describe and predict unidimensional (1D) transport of pathogens through porous rocks and that can be extended to account for more complex bio-geochemical systems and three-dimensional (3D) geometries.

The Research Units and Work Packages of the DY.MI.CR.ON Project are described in the GANTT chart (Figure S1).

The project enrolls five Research Units (RUs):

1. RU1 (Department of Biology—Hygiene and Environmental Virology, University of Pisa, Tuscany, Italy—Principal Investigator)
2. RU2 (Interdisciplinary Department of Medicine, Hygiene Section, University of Bari Aldo Moro, Apulia, Italy)
3. RU3 (National Research Council, CNR—Water Research Institute, IRSA, Apulia, Italy)
4. RU4 (Department of Geosciences, University of Padua, Veneto, Italy)

5. RU5 (Department of Agriculture, Food and Environment, University of Catania, Sicily, Italy)

The project is organized into seven Work Packages (WPs):

1. WP1—project coordination.
2. WP2—involvement of the main stakeholders in the project activities and realization of collaboration agreements.
3. WP3—environmental monitoring of microbiological and physico-chemical main features of water/soil/rock system as a result of contamination effects from anthropic activities at field and catchment scale.
4. WP4—analysis of the microbiological contamination of herbaceous crops due to the use of low-quality water and Quantitative Microbial Risk Assessment (QMRA).
5. WP5—hydraulic characterization of porous media and study of the transport processes of microorganisms on a laboratory scale.
6. WP6—modeling of the transport processes of microbiological contaminants at different application scales.
7. WP7—dissemination and communication of scientific results.

The methodological approach of the project is summarized in the following diagram (Figure 2):

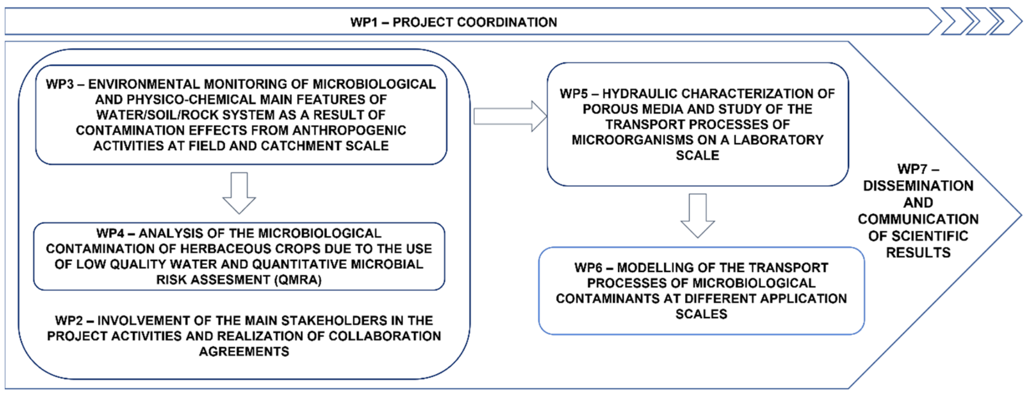

Figure 2. Diagram of Work Packages (WPs) in DY.MI.CR.ON Project (September 2023–September 2025).

2.2. Study Cases

Two different case studies have been selected from RU2 and RU5 in their own regions. The managers of the area under study are involved in analyzing the contamination process at two different scales: at the field scale in the area downstream of the wastewater treatment plant (WWTP) in the Salento peninsula (Southern Apulia, Italy), and in the Ragusa area of Sicily (Italy), at the catchment scale.

The Apulian study case is located near the town of Carpignano Salentino (Figure 3), about 20 km far from the city of Lecce, where a civil WWTP discharges about 1680 m^3/d of effluent into a system of infiltration ponds, which infiltrate into the unsaturated zone, using their natural filtering capacity against microbiological and chemical contaminants. The Carpignano plant is 1 of about 30 WWTPs in the Apulia Region that are connected to infiltration ponds, which form a spreading system for the infiltration of wastewater into the unsaturated zone [31,40], in accordance with the current regulation Legislative Decree 3 April 2006 no. 152 [41]. The Carpignano WWTP carries out primary treatments, including grilling and sand separation, followed by denitrification, oxidation, and secondary sedimentation. In addition, the effluent is disinfected with sodium hypochlorite before being discharged into the subsoil through the infiltration ponds.

Figure 3. Apulian study case (Carpignano Salentino, Lecce province, Italy): wastewater treatment plant and its infiltration ponds.

The ponds at the Carpignano Salentino plant are dug into Pliocenic carbonate porous rock belonging to the Uggiano la Chiesa Formation. The unsaturated zone below the ponds, which is about 60 m thick, consists of a porous rock that belongs to the Calcareniti di Andrano and Pietra Leccese Formations, both of Miocene age [42] (Figure 4).

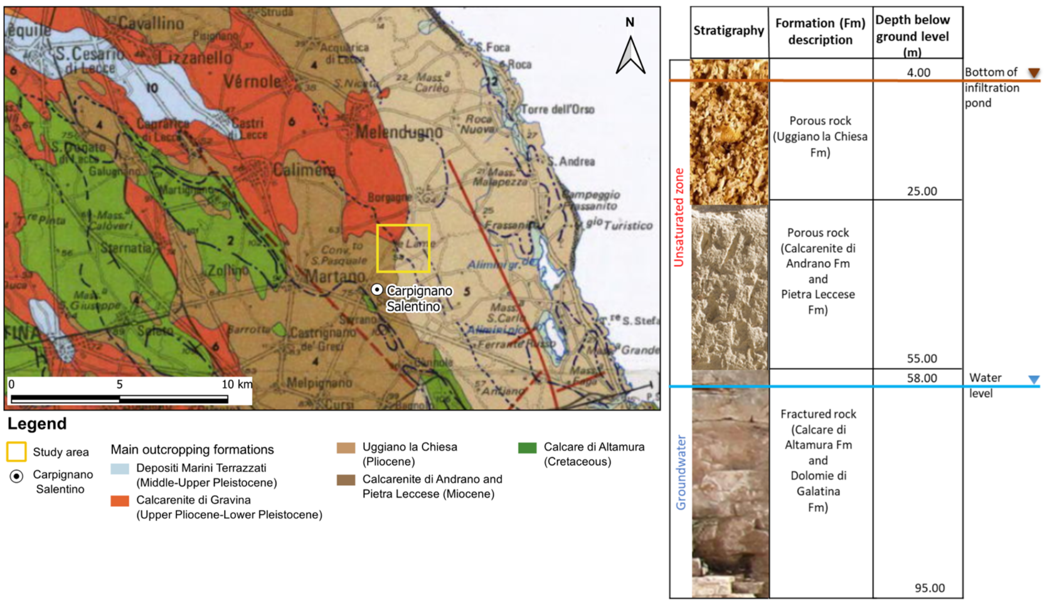

Figure 4. Geological map with the main outcropping formations and stratigraphy of the study area in Carpignano Salentino, Lecce province, Italy.

The groundwater is located in a layered aquifer system, characterized by an NW-SE-oriented fault system that separates two different aquifers: the shallower one is located in the Calcarenite di Andrano Formation while the deeper one is located in the fractured limestone belonging to the Dolomie di Galatina Formation, the oldest Cretaceous formation in the Salento peninsula [43].

The microbiological quality of the groundwater downstream of the WWTP is monitored at field-scale sampling monitoring wells. Specifically, these wells have been chosen after water table reconstruction which allows us to clearly identify the groundwater flow direction.

The Sicilian study case is located in the south-eastern part of Sicily, in the western sector of the Hyblaean Promontory, where the soils are characterized by a sequence of pelagic variations from calcareous to calcareous–marly, starting from the alternation of gray-greenish marl and limestone marl belonging to the Hybla Formation of the upper Cretaceous period (Figure 5) [44]. The natural environment of the land within the Ragusa area has changed substantially in recent years, due to the following phenomena: the development of residential and tourist accommodation activities (sometimes unplanned and abusive), the development of agro-industrial activities, and the significant increase in greenhouse cultivations. In particular, over 80% of the available water resources are currently used for the irrigation of agricultural crops. The production and processing of agricultural and livestock products represent a further factor in the degradation of surface and underground water resources due to the use of nitrogen fertilizers and pesticides, as well as the spreading of livestock and agri-food waste on agricultural soils. The nature of the production activities, the methods of release and propagation of pollutants in the groundwater, and the qualitative characterization of the water from the various sectors (agricultural, civil, and industrial), together with the hydrogeological characteristics of the aquifers, are essential aspects for defining a finalized survey plan for the identification of the qualitative and quantitative status of the groundwater of the territory. For this reason, in 2020, the province of Ragusa (Sicily, Italy), as part of the Mo.Ri.So. project ("Monitoraggio Risorse Idriche Sotterranee"), in collaboration with the University of Catania (Sicilia, Italy), implemented the existing monitoring network, consisting of 11 instrumented wells, by adding 13 more wells, in order to thicken the network.

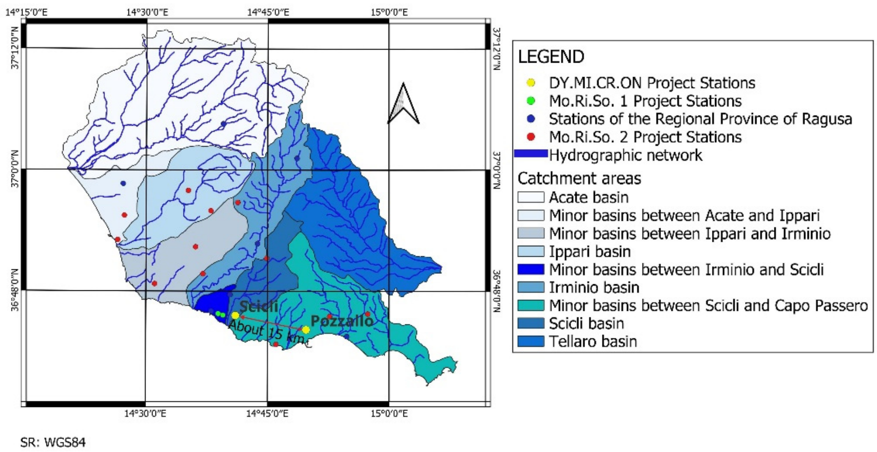

Figure 5. Sicilian case study: hydrological catchments and monitoring of well network in Ragusa area, Sicily, Italy.

The Mo.Ri.So project identified the "minor basin between Scicli and Capopassero" as the basin in the province of Ragusa with the highest level of microbiological pollution. In this basin, two wells were selected (Figure 5): "Scicli" (36°45′46″ N 14°40′34″ E; approximately 70 m deep) and "Pozzallo" (36°44′20″ N 14°49′01″ E; approximately 120 m deep). Both wells are subjected to systematic sampling and monitoring to assess the chemical–physical and microbiological quality of the groundwater.

At the catchment scale, the chemical–physical quality (pH, water temperature, electrical conductivity, dissolved oxygen, and redox potential) of groundwater affected by intensive agriculture is regularly monitored in the extensive Mo.Ri.So. well network. The monitoring could provide data to calibrate and validate transport models, which may be used to predict travel times through the unsaturated zone under the groundwater.

2.3. Sampling and Concentration for the Research of Bacteria

In this study, for monitoring in the Carpignano Salentino area, one point at the exit of the wastewater treatment plant (WWTP), for the effluent discharge directly on the soil, and two wells, in the proximity of the WWTP, were selected, which were chosen for previous characterization from a geological and biological point of view. For the Ragusa province area, two monitoring wells were selected, according to their qualitative and quantitative characteristics. The sampling strategy consists of four seasonal campaigns during 2024–2025, for a total of 20 samples.

The analysis for the detection of bacterial targets focuses on the search for *Escherichia coli*, *Salmonella* spp., *Enterococcus* spp., *Sulfite-reducing Clostridia*, *Clostridium perfringens*, and *E. coli* VTEC on outgoing wastewater and monitoring wells (3L).

E. coli: The detection in outgoing wastewater follows the procedure described in APAT CNR IRSA 7030 Manual and Guidelines 29/2003—Method E [45]. For groundwater monitoring wells, water is analysed according to the standard UNI EN ISO 9308-1:2017 [46].

Salmonella spp.: The detection in outgoing wastewater and in groundwater monitoring of well water follows the APAT CNR IRSA 7080 Manual and Guidelines 29/2003 [47].

Enterococcus spp.: For outgoing wastewater, the procedure is described in APAT CNR IRSA 7040 Manual and Guidelines 29/2003—Method C [48]. For groundwater monitoring wells, water is analysed according to the standard EN ISO 7899-2:2003 [49].

Sulfite-reducing Clostridia: The research of spores of *Sulfite-reducing Clostridia* follows the standard APAT CNR IRSA 7060 Manual and Guidelines 29/2003—Method B for both outgoing wastewater and well water [50].

Clostridium perfringens: For *Clostridium perfringens* counts in wells, water is analysed according to the standard UNI EN ISO 14189:2016 [51].

E. coli VTEC (verocytotoxin-producing *E. coli*): The detection of *E. coli* VTEC is carried out with filtration followed by cultivation in BPW (Buffer Peptone Water). Then, this is followed by an extraction of DNA which is tested with real-time PCR for genes of Shiga toxins (stx 1 and stx 2) and for the *eae* gene (intimine) (Iq-Check STEC VirX, Bio-Rad, Hercules, CA, USA). If positive, the sample requires additional molecular tests (Iq-Check STEC SerO, Bio-Rad, Hercules, CA, USA) to identify the serogroup (O157, O26, O45, O103, O111, O121, and O145). An aliquot of BPW is cultured on *CHROMagar^{TM} STEC* (CHROMagar, Roubaix, France) to isolate *E. coli* O157; they can be recognized from non-O157 from their non-fluorescence under UV lights. Further confirmation is carried out with the same real-time PCR. This procedure is carried out for both outgoing water and well water.

2.4. Research of Human Pathogenic Viruses, Protozoa, and Bacteriophages in Groundwater

The primary field filtration is executed in two different procedures based on the different types of water. For well water, a volume of 1000 L is field-filtered with *Nanoceram* electropositive cartridges (Argonide Corporation, Sanford, FL, USA) placed in the appropriate housing and connected to a tap of the well and a vacuum pump. Furthermore, it is necessary to additionally take 10 L of the water sample to be analyzed which must be contaminated in the laboratory with 1 mL of *Murine Norovirus* process control. For the outgoing water, a volume of 50 L is field-filtered as described above. After completing this part, elution is carried out with a beef extract concentration of 3% and then a secondary concentration is executed with PEG *(polyethylene glycol)*–NaCl.

Samples are transported through couriers in heat-insulating containers containing dry ice to the laboratories where the following analyses are conducted.

The presence of bacteriophages is determined by the double-agar-layer method by following the standard BS EN ISO 10705-2:2001 [52] with the automatized kit (Bluephage S.L., Barcelona, Spain).

For viral detection, the extraction of nucleic acids is carried out with the kit of extraction "*Nuclisens System* (bioMérieux, Marcy-l'Étoile, France)" which involves the use of magnetic silica beads that can bind both DNA and RNA molecules due to charge interaction. The obtained extract is purified to remove the main PCR inhibitors with the kit "*OneStep PCR Inhibitor Removal Kit* (Zymo Research, Irvine, CA, USA)". At the end of the procedure, the purified extracts are stored at −80 °C.

For protozoan detection, the extraction for the study of *Giardia* spp. and *Cryptosporidium parvum* is executed with the kit "*QIAamp DNA Mini Kit* (QIAGEN, Venlo, The Netherlands)". This kit uses silica gel membranes that bind DNA molecules. The obtained extract is purified to remove the main PCR inhibitors with the kit "*OneStep PCR Inhibitor Removal Kit* (Zymo Research; Irvine, CA, USA), and then all extracts are stored at −80 °C.

The nucleic acids extracted are then analyzed with real-time (RT)-qPCR for the detection of Human Adenovirus (HAdV), Enterovirus, Norovirus GII, Hepatitis A virus (HAV), Hepatitis E virus (HEV), and Rotavirus. The protocols are summarized in Table S1 [53–57] and the quantification of the viral genome is reported as GC/liters. *Giardia* spp. and *Cryptosporidium parvum* are searched through qualitative PCR. Samples are analyzed with horizontal agarose gel electrophoresis and visualized with UV light after staining with ethidium bromide. The details of the protocols are summarized in Table S2 [58,59].

If the amplification of viral genomes is positive for Human Adenovirus and Enterovirus, infectivity is tested by seeding on permissive cell cultures. After the sample was previously decontaminated with chloroform, the suspension containing the virus is sown onto the cell monolayer and everything is incubated in a thermostat at 37 °C with 5% CO_2. They are then observed daily with an optical microscope for a week until the cytopathic effects (which represent evidence of viral infectivity) are detected. In the event of a cytopathic effect and therefore infectious viral particles, the viral titer is determined using the micromethod. A microplate is prepared into which serial dilutions of the virus are seeded on permissive cells. Plate readings are taken after 5 days of incubation in a thermostat. At the time of reading, the wells are observed under an optical microscope, and by comparison with the control ones, it is possible to identify where the cytopathic effect occurs. To quantify viral infectivity, the value DCP50/mL is obtained using the Spearman–Karber method [60].

2.5. Physicochemical Characterization in Groundwater

Temperature (°C), pH, and electrical conductivity (E.C., µS/cm) are measured in situ using a multiparameter probe (Hanna Instruments HI-9828).

Chemical Oxygen Demand (COD) is determined through colorimetric analysis, following the ISPRA 5135 Manual and Guidelines 117/2014 [61].

Total nitrogen and total phosphorus are measured photometrically using a UV-Vis spectrophotometer according to the APAT CNR IRSA Method 4060 Manual and Guidelines 29/2003 [62].

Anions (fluorides, chlorides, bromides, nitrates, and sulfates) and cations (sodium, potassium, magnesium, and calcium) are determined by ion-exchange chromatography according to the APAT CNR IRSA Method 4020 Manual and Guidelines 29/2003 and the APAT CNR IRSA Method 3030 Manual and Guidelines 29/2003, respectively [63,64].

2.6. Research on Human Pathogenic Viruses in Vegetables

Vegetables collected from Sicilian soil are placed in test tubes and shipped by couriers in heat-insulating containers filled with dry ice to the laboratory where the analyses take place. The leaves are then shredded, eluted, shaken with a small steel ball, incubated overnight, and filtrated. This is followed by decontamination with chloroform and concentration experiments with PEG and NaCl. The extraction is carried out with the kit of extraction "*QIAamp Viral RNA Minikit* (Qiagen, Hilden, Germany)" and with "*QIAamp DNA Minikit* (Qiagen, Hilden, Germany)". This protocol is taken from Carducci's publication [29].

Viral identification and quantification are executed for the detection of Human Adenovirus, Enterovirus, Norovirus GII, Hepatitis A virus, Hepatitis E virus, and Rotavirus. The protocols followed are the same as those previously used for viral detection in groundwater. If the amplification and quantification reactions of Human Adenovirus and Enterovirus give a positive result, the samples are shown on permissive cell cultures. The protocol to follow is the same as previously indicated for cell cultures carried out on positive groundwater samples.

A QMRA is performed to assess gastrointestinal illness resulting from the ingestion of contaminated raw products [65]. The QMRA framework includes five steps: (i) selection of index pathogens, on the basis of their occurrence in the field study; (ii) quantification of index pathogen concentrations in raw products (field study); (iii) estimation of exposure level through one-year ingestion of contaminated raw food, using literature data on the consumption of vegetables; (iv) evaluation of dose–response relationships for each index pathogen based on current scientific knowledge; and (v) calculation of cumulative annual risk of gastrointestinal infection/illness derived from the modeled exposure scenario. Moreover, the collected information and data will be used to develop a reverse QMRA to establish risk-based thresholds (critical limits) of index pathogens deriving from exposure to raw products. Critical limits are developed considering a tolerable risk of gastrointestinal illness (e.g., 1 illness case/1000 exposed) through different exposure scenarios, such as the ingestion of plant tissues with microbial internalization and the accidental (or voluntary) ingestion of well waters impacted by sewage. The methodology will identify the best practices to reduce the risk of microbiological contamination while favoring its natural decay.

2.7. Laboratory-Scale Hydraulic Characterization of Rocks and Hydrogeophysical Monitoring of Flow and Transport Experiments

Laboratory-scale transport experiments are carried out to study the interaction of bacteria and bacteriophages with the rocks under variable saturation conditions using columns made of carbonate porous rock cores. As the unsaturated zone controls water movement from the land surface to the aquifer, an understanding of unsaturated-zone flow processes is crucial in determining the amount and quality of groundwater [14]. Particularly, unsaturated hydraulic properties, represented by water retention and hydraulic conductivity functions, are essential in modeling flow and solute transport in the vadose zone. These properties are determined for the rock core samples drilled in the Apulian study area by using different methods, including the Quasi-Steady Centrifuge (QSC) method [66], which allows for the measurement of hydraulic properties from saturated to very dry conditions.

In addition, rock core samples will be used to set up laboratory-scale transport experiments under variable saturation conditions [67,68]. Porous rock columns, about 10 cm in diameter and 50 cm in height, are used to study, at the pore scale, the interaction of the microorganisms with the rock and their travel time through the column.

Tensiometers and solution samplers are installed at different depths along the columns for monitoring pressure head and microbial concentrations, respectively. A fractional collector of the outflow is installed at the bottom of the columns [24]. Microbiological analysis of the sampled solution is carried out to evaluate infectivity abatement.

Hydrogeophysical monitoring plays a key role in characterizing flow and transport in the columns used for laboratory-scale experiments. Electrical Resistivity Tomography (ERT) is applied to detect and quantify the processes of microbial migration through unsaturated and saturated media, with particular reference to preferential pathways.

2.8. Field-Scale Hydrogeophysical Monitoring of Flow and Transport

The model parameterization is tested by non-invasive geophysical measurements able to rapidly characterize flow and transport processes in the bedrock at the field scale. Particularly, time-lapse electrical and/or electromagnetic surveys are carried out to image the electrical conductivity distribution below the infiltration ponds in order to monitor the waterfront dynamics [69,70].

Specifically, non-invasive geophysical measurements able to spatially characterize flow by highlighting possible preferential paths and transport processes in the unsaturated zone will be carried out at the field scale in the Apulian study case [70]. In addition, hydrogeophysical measurements will provide information on the hydrological and hydraulic characteristics of the subsurface, as well as on the presence and motion of fluids and solutes in the unsaturated and root zone useful for model parametrization [71].

2.9. Modeling of Pathogen Transport in Porous Rocks at Different Scales

The modeling of virus and bacteria transport in porous media is a key component of the proposed project, as it will help bring together all information coming from hydraulic tests, column transport experiments, and field evidence. Following earlier works [18–21], we are developing a numerical model that combines one-dimensional (1D) mass conservation laws for solute and pathogen transport in porous media coupled with kinetic laws of attachment/attachment, straining, agglomeration, and growth (Monod Growth Kinetics). The equations are discretized with a finite difference method and solved explicitly. The model is implemented in the programming language MATLAB [72] and validated with laboratory tests. Measurements taken in the laboratory include flow, pathogen concentration at the core outlet, and solution composition. These measurements are used to estimate the parameters within the kinetic laws of attachment/attachment, straining, agglomeration, and growth and validate model predictions.

The approach is illustrated schematically in Figure 6.

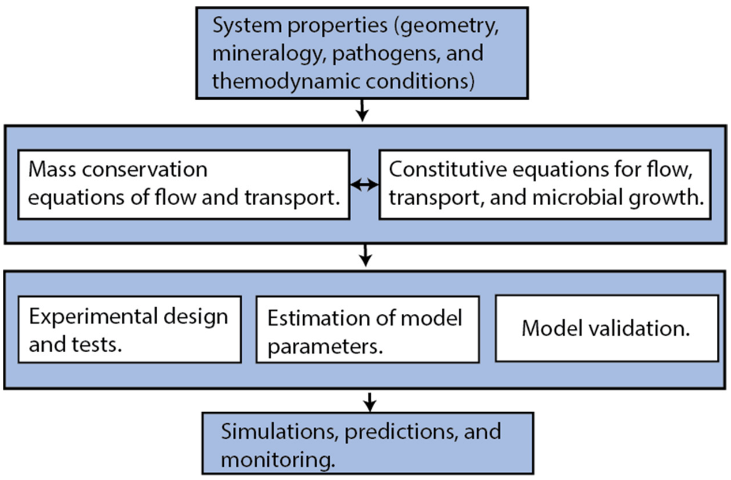

Figure 6. Conceptual flowchart of the reactive transport model in porous media for pathogens and solutes developed in this work.

2.10. Quality Assurance and Quality Control

All the laboratory methodologies of the project provide the use of internal and external quality control. In particular, for bacterial detection in water samples, ISO (International Standard Organizations) methods are already standardized with specific controls. For viral detection in water samples and herbaceous crops, concentration/elution methods include a process control based on the artificial contamination of the sample by a non-human virus at a known concentration (Murine Norovirus) to evaluate the recovery efficiency, while for molecular detection for both matrices (PCR and real-time quantitative PCR), commonly negative and positive controls are used, aiming to also estimate a possible inhibition of the amplicon.

3. Results and Conclusions

3.1. Dissemination and Communication of Scientific Results

During the entire duration of the project, a direct interaction with the stakeholders is guaranteed using a communication strategy based on the following:

1. Publication of scientific papers in international journals and participation in national/international conferences and other relevant events;
2. Organization of at least one workshop with the stakeholders and one public lecture for each RU and invitation of scientific experts, water quality managers, and policymakers as well as local authorities;
3. Communication to the stakeholders and the civil society through the project website will be used in order to communicate, to an external wide audience, the outcomes of the study.

3.2. Possible Application Potentialities and Scientific and/or Technological and/or Social and/or Economic Impact

Groundwater represents the main source of water supply for drinking purposes, as well as for agricultural and livestock needs [73]. This resource becomes essential in areas where surface water resources are limited or insufficient to meet local needs. It is estimated that around 75% of the population in the European Union depends on groundwater for water supply.

The use of groundwater is conditioned by its chemical and microbiological quality, which in turn is impacted by anthropic activities and by the geological characteristics of the area [74]. Compared to other water bodies, groundwater is considered a high-quality water resource because the unsaturated zone warrants protection against pollution brought by infiltrating water, acting as a physical filter but also delaying travel time and thus allowing biodegradation and other attenuation processes to take place [75]. In fact, aquifer vulnerability is generally assessed in terms of the characteristics of the overlying unsaturated zone. Particular geologic features can affect aquifer vulnerability such as in the case of an unsaturated zone composed of karstic and/or fissured rocks [70,76] or highly permeable layers that activate preferential flow, thus increasing the groundwater contamination risk.

In recent decades, the increasing groundwater contamination caused by industrial, agricultural, and anthropic activities called for better identification of mechanisms controlling contaminant movement through the unsaturated zone and the development of the capability to characterize and predict the contaminant movement in space and time with acceptable accuracy. This is particularly true for the presence of microorganisms, potentially causing human diseases, originating from several different sources, including landfills, treated wastewater infiltration, septic tanks, and recharge basins. This might have serious impacts on the risk for human health and consequently have negative implications for the suitability of groundwater especially for drinking and crop irrigation uses [77].

Whatever the microorganisms' origin, they would travel through the unsaturated zone before reaching the groundwater. Once contamination of groundwater occurs, the damage is often long-term, and the remediation cost is prohibitive. Furthermore, the contamination of groundwater involves considerable social inconvenience, for instance, caused by water unavailability for drinking purposes, as well as economic damage for all the activities related to the use of water of a certain chemical and microbiological quality, such as agricultural or livestock activities.

In the context of global water scarcity due to increasing water demand and the climate change effects, a better understanding of microbial transport and fate processes through the unsaturated zone before reaching the groundwater is essential to prevent further contamination of aquifers which is a crucial issue for the optimal management of unconventional water resource for civil (potable and agricultural) and industrial use. Among other issues, the irrigation of high-value horticultural crops with scarce quality water (i.e., contaminated groundwaters or low-quality reused wastewater) could represent a health problem due to the presence of pathogenic microorganisms, especially in the case of raw consumption. To guarantee the safety of such foodstuffs, current regulations and guidelines rely on the assessment of the microbiological quality of irrigation water [78], focusing on bacterial fecal indicators (e.g., *Escherichia coli*). Enteric viruses are not considered although they are responsible for gastrointestinal illnesses associated with the consumption of such food and their relationships with bacterial indicators are often unreliable.

Given these considerations, it is clear that the assessment of health risks posed by microorganisms is a necessary step to be implemented to tackle practical problems. This is true at a variety of physical scales. For instance, at the larger scale of aquifer vulnerability, the risk assessment shall be based on an estimation of hazards, intrinsic aquifer vulnerability, and identification of critical areas, while the smaller scale of agricultural soil risk is linked to the transfer mechanisms from contaminated waters to the soil matrix, both of which affect the plant living tissues.

In all cases, the key component of this project is a better understanding of the fate of the microorganisms that migrate into the subsoil, with reference to variably saturated porous media. To this purpose, the main goal of this project is to develop, validate, and calibrate an experimental model of microbial contamination processes within the Earth's critical zone. The approach is interdisciplinary, starting from the validation of the model both in the laboratory and on the territory. The results of the experiments can be used in broader and geologically different scenarios, in accordance with the policies for the protection of the territory and water resources, according to Mission 2, Component 4 of the NRRP—Protection of the Territory and Water Resources and the recent European strategies for water protection. Specifically, the project will provide methodological tools that, applied in different fields and at different scales, will help to assess anthropic pressures and their impacts on environmental matrices, groundwater, soils, rocks, and plants.

All of this information and the corresponding model represent the scientific and technological results expected from the project and, consequently, its impact in terms of research.

The social and economic impact will be used to modulate anthropic activities by considering the specific geological and hydraulic characteristics of the soils and aquifers, in order to minimize the impacts of environmental contamination. It is known, in fact, that the quality of the available water resources affects human and animal health, including agricultural activities.

Specifically, the results of this project will produce the following:

(a) Enhancement of interdisciplinary research, scientific impact, and related co-benefits aimed at the following:
    (1) Addressing the knowledge gap on the transport and fate of microorganisms in porous media with variable saturation, through the study of the distribution in the soil and subsoil of various types of microorganisms, and their potential transfer to vegetables after spray irrigation or by sub-irrigation with contaminated water. In particular, since viruses are not included among the parameters for expressing a judgment on the quality of irrigation water, viral contamination will be studied along the entire agricultural supply chain (from contaminated irrigation water to the possible absorption of microorganisms by the root of the plants).
    (2) Assessing the health risks of consumers using monitoring data from feed water, soil, and herbaceous crops. In particular, QMRA will be carried out to evaluate the probability of gastrointestinal diseases deriving from the ingestion of contaminated raw products and the critical limits deriving from exposure to these products. The application of this methodology will also make it possible to select the best practices to reduce the contamination risk.
(b) Economic and environmental impacts:
    (1) Modulate anthropic activities, including the design of WWTP and the modality of effluent discharging, considering the geological and hydraulic characteristics of the unsaturated zone that affect the vulnerability of groundwater.
    (2) Limit anthropic pressures, through the application of more stringent containment systems based on the vulnerability of the territory in which these activities are present.
    (3) Propose guidelines and/or protocols introducing the analysis of new microbiological parameters (bacteriophages and viruses) in groundwater and WWTP effluents.
    (4) Introduce more restrictive WWTP effluent emission limits in areas where the risk of contamination of deep aquifers is greatest.

3.3. Limitation of the Study

This study is designed to provide a multidisciplinary and standardized approach that can explain the microbial dynamics of transport and interaction with the aquifer.

Despite this, it presents some limitations to be considered. It would be desirable to increase the sample number and extend the monitoring for a longer period of time to have a better understanding of the phenomenon in various environmental and atmospheric conditions.

It is also necessary to consider that any negative results in the detection of microorganisms may be due to the decay of biological structures and not necessarily to their retention in the porous rock.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/w17030294/s1: Table S1: Real-time PCR protocols used for the detection of viral targets; Table S2: PCR protocols used for detection of protozoan targets; Figure S1: GANTT chart of DY.MI.CR.ON Project. References [53–59] are cited in the Supplementary Materials.

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data curation, M.V., I.F., A.P., A.A., O.D.G., M.C.C., V.P., and M.M.; writing—original draft preparation, M.V., A.A., O.D.G., M.C.C., F.T., A.F.S., and M.T.M.; writing—review and editing, M.V., A.A., O.D.G., F.B. (Francesco Bagordo), D.C., F.B. (Federica Bivona), M.T.M., M.C.C., M.M., A.C., L.D.C., and M.A.D.D.; supervision, M.V., and O.D.G.; project administration, M.V.; funding acquisition, M.V., O.D.G., M.C.C., G.C., and M.M. All authors have read and agreed to the published version of the manuscript.

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