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Trasporto ed attenuazione naturale di contaminanti emergenti nelle acque sotterranee

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PhD in
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Transport and Natural Attenuation of Emerging Contaminants in Groundwater

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1. Introduction

Emerging Contaminants (ECs) encompass a wide array of substances, including microplastics, pharmaceuticals and personal care products (PPCPs), pesticides, per- and polyfluoroalkyl substances (PFAS), and hydrocarbon additives such as MTBE and ETBE. These compounds, often originating from human activities, are of particular concern due to their persistence in the environment and potential adverse effects on human health and ecosystems; indeed, although the knowledge about their environmental concentrations and fate is still mostly unknown, some of these ECs have the potential for bioaccumulation and/or are bioactive substances that may compromise living organisms. The interaction between surface water and groundwater becomes increasingly critical when polluted surface waters contribute to aquifer recharge through lateral transfer or percolation processes. In this context, PPCPs present a notable challenge. Once released into wastewater, they are poorly removed during treatment, and their presence in groundwater can be exacerbated by sewer pipe failures that allow direct discharge of contaminants into the surrounding environment (Figure 1).

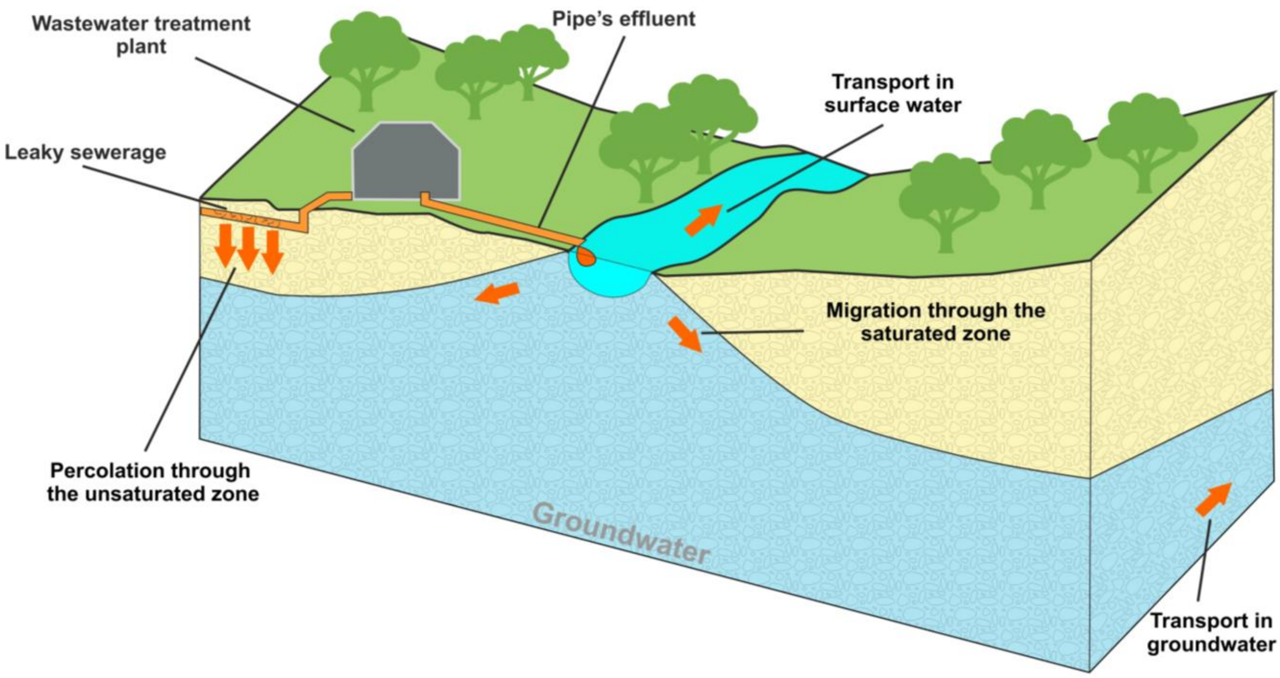

Figure 1. Groundwater vulnerability to contamination by Emerging Contaminants.

The project adopted a multidisciplinary approach, integrating fieldwork, laboratory analyses, and data evaluation.

The initial phase focused on analyzing microplastics in natural springs within the Parma aquifer. In paricular, microplastics are defined as solid and water-insoluble polymer-based particles smaller than 5 mm that can be typified as primary if they are originally produced as microplastics, such as fibres, and particles, particollarly used in cosmetics or as secondary, which instead result from plastic fragmentation by physical, biological, and chemical processes.

Due to the absence of a standardized protocol for microplastic extraction, a specific methodology was developed for groundwater samples. This approach enabled the identification and quantification of microplastics in groundwater, emphasizing how the hydrogeological characteristics of the region may act as key drivers of microplastic contamination.

Subsequently, attention shifted to personal care products (PCPs). PCPs belongs to the group of pharmaceuticals and personal care products (PPCPs), a diverse collection of chemicals that include all drugs (both prescription and over-the-counter medications) and non-medicinal consumer substances, such as the chemicals found in lotions, soaps, fragrances, body and sunscreen creams. This group deemed to be the markers of wastewater presence in the urban groundwater system because of their human-related use. This is because PPCPs generally are poured into wastewater by excretion from a citizen’s body and washed off with tap and sink water. The selection of contaminants for detailed study was based on factors such as industrial usage, persistence in the environment, and the scarcity of specific studies. Phenoxyethanol, Dimethicone, and Disodium EDTA were selected for further investigation both in laboratory and field settings.

(i) Phenoxyethanol (Phy-Et) is a bactericidal agent widely used in leave-on and rinse-off cosmetic cosmetics as a preservative due to its pure chemical form, pleasant smell, and colorless appearance. This compound has a large spectrum of antimicrobial activity and is effective against Gram-negative and Gram-positive bacteria, while it exerts only a mild inhibitory effect on the resident skin microbiot. Opinions on the safe concentration and use of this substance vary between regulatory bodies, with he European Scientific Committee on Consumer Safety approving its use up to 1% in cosmetics for all consumers, while the French National Agency advises against its use in products for infants' nappy areas.

(ii) Dimethicone is the most widely used silicone in cosmetics and is not subject to European regulations concerning its concentration in environmental matrices or in the consumer products for which its use is intended.

(iii) Disodium EDTA finds applications in several sectors, including (a) cosmetics and personal care products, (b) the food industry and (c) the pharmaceutical industry. The advantage of using this substance in different industrial contexts is due to its ability to chelate heavy metals, which predisposes this chemical to assist the conservation and stabilization activities of certain products. At the same time, the possible presence of Disodium EDTA in environmental matrices could lead to the mobilization and dispersion of heavy metals in soil and water systems, potentially damaging ecosystems and human health.

In the field, the research began by sampling and analyzing groundwater to detect potential contamination from sewer pipe leaks and to assess their impact on PCPs concentrations. This was followed by laboratory studies on the natural attenuation of PCPs. Through mesocosm experiments (Figure 2), biodegradation processes were simulated to evaluate the effectiveness of contaminant degradation under the specific environmental conditions of the studied aquifer. This approach facilitated the determination of how PCPs concentrations influence biodegradation processes and how these contaminants interact with the local microbial communities.

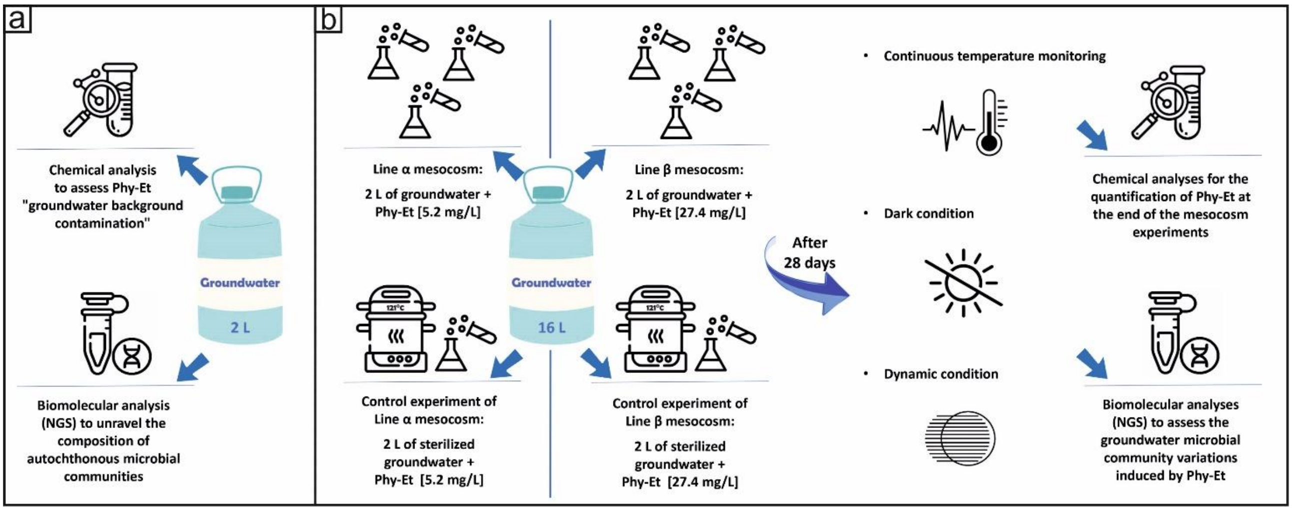

Figure 2. (a) Groundwater microbial community characterization and assessment of Phy-Et background contamination; (b) experimental protocol setup for mesocosms.

A second round of field activities was then conducted to monitor and verify the spatial distribution of PCPs in wastewater, expanding the monitoring network to analyze variations in PCPs concentrations in effluent from wastewater treatment plants.

During the third year of the PhD, a three-month research stay at the Instituto Politécnico de Castelo Branco (Portugal) provided an opportunity to analyze environmental data collected between 2010 and 2023 from an aquifer impacted by hydrocarbon, methyl tert-butyl ether (MTBE), and ethyl tert-butyl ether (ETBE) contamination. In particuar, MTBE is the most commonly used fuel additives, due to its high octane rating, low cost, and ease of production but despite the advantages of using MTBE, it has raised significant environmental concerns. For this reason, in response to environmental regulations in this regard, many markets began replacing MTBE with ETBE, an additive produced from bioethanol. However, similar concerns have also been raised regarding ETBE in terms of water quality when its concentrations are present. Using Compositional Data Analysis (CoDa), this extensive dataset enabled an in-depth investigation of contaminant concentrations in groundwater over time, as well as the study of temporal variations through statistical methods. This approach contributed to a better understanding of the interactions between different pollutants at the site scale and the effectiveness of hydraulic barriers.

The integration of data from all research phases has significantly advanced our understanding of the dynamics of groundwater contamination by emerging contaminants and their potential for biodegradation, addressing critical gaps in this emerging area of study.

2. Research Activities

The majority of the research activities were conducted in the alluvial aquifer of Parma, which was selected as an appropriate test site to achive the mentioned objectives. Exclusively the study related to the petroleum additives was conducted within an industrial area covered by confidentiality agreements. At the end of the doctoral program, the only unpublished research concerns this topic, with results presented in a simplified format that excludes specific details about the study area and the data. This section summarizes the main results achieved during the PhD, presented through the sequence of scientific articles published in open access journals indexed in Scopus and Web of Science. To facilitate comprehension and situate the results within the various topics addressed throughout the three-year program, the correspondence between the individual topics and the related publications is outlined below.

2.1 In-depth Analysis of Microplastic Contamination;

- Severini, E.; Ducci, L.; Sutti, A.; Robottom, S.; Sutti, S.; Celico, F. River–Groundwater Interaction and Recharge Effects on Microplastics Contamination of Groundwater in Confined Alluvial Aquifers. *Water* **2022**, 14, 1913. https://doi.org/10.3390/w14121913

2.2 Understanding the Transport and Natural Attenuation of Personal Care Products (PCPs);

- Ducci, L.; Rizzo, P.; Pinardi, R.; Solfrini, A.; Maggiali, A.; Pizzati, M.; Balsamo, F.; Celico, F. What Is the Impact of Leaky Sewers on Groundwater Contamination in Urban Semi-Confined Aquifers? A Test Study Related to Fecal Matter and Personal Care Products (PCPs). *Hydrology* **2023**, 10, 3. https://doi.org/10.3390/hydrology10010003

- Ducci, L.; Rizzo, P.; Bucci, A.; Pinardi, R.; Monaco, P.; Celico, F. The Challenge Posed by Emerging Environmental Contaminants: An Assessment of the Effectiveness of Phenoxyethanol Biological Removal from Groundwater through Mesocosm Experiments. *Sustainability* **2024**, 16, 2183. https://doi.org/10.3390/su16052183

- Ducci, L.; Rizzo, P.; Pinardi, R.; Celico, F. An Interdisciplinary Assessment of the Impact of Emerging Contaminants on Groundwater from Wastewater Containing Disodium EDTA. *Sustainability* **2024**, *16*, 8624. https://doi.org/10.3390/su16198624

In this PhD Thesis, a dedicated section for Materials and Methods is not included, as these are outlined in the respective scientific works. Similarly, for a more in-depth examination of the state of the art on the various topics discussed, the same researches are referenced.

Regarding the contributions provided within the each studies mentioned above, the details are outlined below in a schematic form:

- River–Groundwater Interaction and Recharge Effects on Microplastics Contamination of Groundwater in Confined Alluvial Aquifers
  - ❖ A large part of the experimental activities, particularly related to hydrogeological aspects and activities aimed at developing the microplastic extraction protocol
- What Is the Impact of Leaky Sewers on Groundwater Contamination in Urban Semi-Confined Aquifers? A Case Study Related to Fecal Matter and Personal Care Products (PCPs)
  - ❖ A large part of the experimental activities
  - ❖ Integrated data analysis, particularly focusing on hydrogeological, hydrochemical, and metagenomic data
  - ❖ Text writing
  - ❖ Review work during the peer review phases
- The Challenge Posed by Emerging Environmental Contaminants: An Assessment of the Effectiveness of Phenoxyethanol Biological Removal from Groundwater through Mesocosm Experiments
  - ❖ A large part of the experimental activities
  - ❖ Integrated data analysis
  - ❖ Text writing
  - ❖ Review work during the peer review phases
- An Interdisciplinary Assessment of the Impact of Emerging Contaminants on Groundwater from Wastewater Containing Disodium EDTA
  - ❖ A large part of the experimental activities
  - ❖ Integrated data analysis
  - ❖ Text writing
  - ❖ Review work during the peer review phases
- A first application of the compositional data analysis (CoDa) approach to refine the delimitation of capture zones around hydraulic barriers in contaminated sites
  - ❖ Data analysis and study
  - ❖ Text writing

2.1. In-depth Analysis of Microplastic Contamination

water

MDPI

Article

River–Groundwater Interaction and Recharge Effects on Microplastics Contamination of Groundwater in Confined Alluvial Aquifers

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Abstract: Literature provides only a few examples of contamination of groundwater with microplastics, mainly investigated using a chemical approach. Little importance is given to the hydrogeological processes able to affect the contamination, such as river–groundwater interactions. This study was carried out with two aims. The first aim is the formulation of a method with a high result-to-cost ratio, based on the hydrogeological aspects of the investigated area. Microplastics were extracted from samples through filtration and successively counted and characterized morphologically through analysis of optical microscopy images. The second aim is to evaluate the presence of microplastics in some portions of an alluvial aquifer using this methodology. Microplastics in groundwater showed a higher circularity and Feret diameter than those found in surface waters, indicating that in porous aquifers the transport is likely more influenced by the microplastics' shape than by their size. The aquifer recharge did not modify the microplastics' characteristics in groundwater, whereas in surface water the flood wave promoted the resuspension of microplastics with lower circularity. These findings provide new pieces of evidence on the presence and transport of microplastics in both groundwater and surface waters, underlining how the hydrogeological characteristics of the area can be one of the main drivers of microplastics' contamination.

Keywords: microplastics; river–groundwater interaction; microscopy; confined aquifer; aquifer recharge; transport

Citation: Severini, E.; Ducci, L.; Sutti, A.; Robottom, S.; Sutti, S.; Celico, F. River–Groundwater Interaction and Recharge Effects on Microplastics Contamination of Groundwater in Confined Alluvial Aquifers. *Water* **2022**, *14*, 1913. https://doi.org/10.3390/w14121913

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1. Introduction

Microplastics are intensively studied pollutants, defined as solid and water-insoluble polymer-based particles smaller than 5 mm [1,2] and larger than so-called nanoplastics, whose upper limit is usually set between 100 and 1000 nm [3]. They can be typified as primary if they are originally produced as microplastics, such as for nurdles, fibres, and particles used in cosmetics [4–6]; or as secondary, which instead result from plastic fragmentation by physical, biological, and chemical processes [7,8]. Their origin can be attributed to the enormous production of plastics worldwide, which according to Geyer et al. [9], reached 380 Mt in 2015. Most of the monomers used to produce the most common plastics (e.g., polyethylene, polypropylene, polystyrene, and polyethylene terephthalate) are derived from fossil hydrocarbons, resulting in non- or scarcely biodegradable final products [9,10]. Biodegradable plastics are also produced using both renewable and fossil sources, but in lower quantities than nonbiodegradable plastics, and they can nevertheless contribute to microplastic pollution, albeit in transient fashion [11]. Consequently, plastic waste accumulates in landfills, or worse, in the environment [12], where it takes centuries for breakdown and decomposition [12,13]. The presence of this pollutant in different environmental matrices worldwide has been ascertained by the scientific community, threatening both human health and the biosphere.

Plastic pollution was initially recognized and studied in marine environments, where investigations started in the 1970s, e.g., [14,15]. Comparatively, the study of microplastic pollution in freshwater ecosystems started much later and produced many fewer studies [16–18], possibly due to difficulty in sampling uniformity in non-large-surface water bodies. Paradoxically, other important aquatic compartments such as groundwater, which is the single most important supply of drinking water in many areas of the world [19], have received almost no attention to date. The first case of microplastics contamination in groundwater was reported in a fractured medium, with karst crevices and conduits reportedly allowing the transfer of microplastics [20]. In other aquifers, without conduits and/or with lower porosity, soil can be assumed to work as a barrier for microplastics, likely dissuading researchers from further investigations. This hypothesis has recently been challenged, with two articles reporting microplastic contamination in alluvial aquifers, raising new questions on microplastics contamination and migration processes, especially from a hydrogeological perspective. Goeppert and Goldscheider [21] demonstrated the possible microplastics transport in alluvial aquifer using tracer tests (uranine and microplastic tracer particles), thus invalidating the role of the aquifer (and soil) as *a priori* barrier for microplastics. Samandra et al. [22] were the first—and to the best of our knowledge so far, the only—to report on microplastics contamination in an alluvial aquifer. They detected eight different microplastic polymer types in groundwater and evaluated microplastics abundance.

Microplastics can contaminate groundwater through different contamination pathways, according to hydrogeological factors such as the groundwater recharge source (e.g., vadose zone or surface water) and timing (e.g., recharge or recession periods), the diffuse (e.g., losing river) or point (e.g., leaks of the drainage system) microplastic source, the aquifer characteristics (e.g., hydraulic conductivity), and the possible interactions between surface water and groundwater (e.g., gaining or losing river). Moreover, differently from solute contaminants, the percolation and transport of microplastics in the aquifer are also influenced by the relationships between pore diameters and microplastics' dimensions.

This work has two aims. The first aim is to set up a protocol for the separation, quantitative analysis, and geometrical characterization of microplastics in water samples. The second aim is to test the protocol in two subareas of a confined alluvial aquifer, verifying the presence of microplastics in groundwater and the possible effects given by a recharge event due to river–groundwater interactions. We hypothesize that the recharge and recession entity and timing of an area play a pivotal role in driving groundwater contamination by microplastics. Although this is easily expected, few qualitative and quantitative empirical data are available about these dynamics on microplastics. In addition, we hypothesize that microplastics in groundwater have different geometrical properties compared with microplastics from the feeding surface waters, due to different interactions between microplastics and the environment (river or aquifer) during the transport processes. Together with the particles' surface charge, we also hypothesize that the geometrical characteristics of microplastics are pivotal in controlling their distribution in porous media. Results from this work provide a method of operation based on a hydrogeological perspective. This, along with the chemical characterization of polymers of microplastics and their transport of pollutants, described in other literature, should be central in the process of standardization of microplastics-analysis methods, which are now strongly asked by policy makers, e.g., the new Directive (EU) 2020/2184 of the European Parliament and of the Council of 16 December 2020 on the quality of water intended for human consumption.

2. Materials and Methods

2.1. Study Area

This study was performed in an alluvial aquifer surrounding the city of Parma (Po Plain, Northern Italy, Figure 1). This area was selected due to its peculiar hydrogeological and anthropic characteristics. Intensive agriculture (mostly hay, tomatoes, and maize) results in numerous recognized sources of microplastics contamination [23]. The city of Parma is located in the center of the study area. It represents another possible important microplastics source for surface waters [24,25] and groundwater due to leaks in the drainage system [26,27]. Finally, the area has many large industrial complexes, another possible source of microplastics contamination [28], and is close to the so-called packaging valley, composed of more than 300 firms working in packaging and packaging machinery manufacturing and providing the biggest amount of industrial plastic wastes in the region [29]. All these factors can be expected to contribute to microplastic production and pollution [28]. Three rivers cross the area, namely the Taro, Baganza, and Parma River. The last two merge within the Parma city area and all of them flow into the River Po, the largest Italian river.

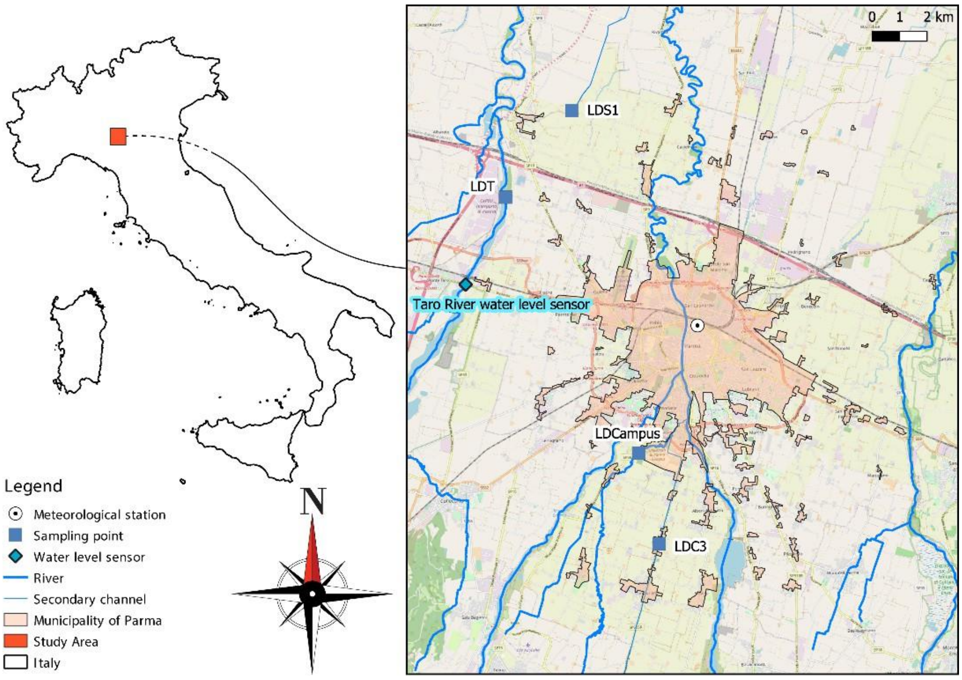

Figure 1. Study area. Base map and data from OpenStreetMap and OpenStreetMap Foundation (https://www.openstreetmap.org, accessed on 15 November 2021).

From the (hydro)geological perspective, the area has already been characterized by Zanini et al. [30], while several Master Theses of the University of Parma investigated some phenomena at small scale, e.g., [31,32]. The area is characterized by the Emilia-Romagna Supersynthem (Lower Pleistocene, about 800 ky BP to present), made up of fan and alluvial plain deposits, together with intravalley and terrace deposits. The grain size of the sediments is highly variable, resulting in the juxtaposition of more-permeable layers (gravel and sand) with low-permeability layers (clay and silt) [30]. The same authors tested the hydraulic conductivity of these layers in the wells of the Parma University Campus (which includes the piezometer LDCAMPUS used within this study). A pumping test performed in the more-permeable layer resulted in transmissivity and storativity of 3×10^{-4} m^2/s and 1.9×10^{-4}, respectively. In the low-permeability layer, the hydraulic conductivity was lower (in the order of $10^{-7}/10^{-9}$ m/s) and estimated using a Lefranc test. Granulometric data of a litholog near LDCAMPUS (3.8 km N-E, Figure S1, Tables S1 and S2) is reported in the supplementary material and provides another piece of evidence of the low permeability of the confining unit.

2.2. Data Acquisition and Microplastics Sampling

The geological contextualization performed by Zanini et al. [30] was spatially expanded using stratigraphic data from the Geognostic tests database of the Emilia-Romagna Region [33] and Petrucci et al. [34], together with outcropping data from the Geological map of the Emilia-Romagna Region, 1:25,000 scale [35]. Near the sampling points, a detailed reconstruction of outcropping materials and shallow aquifer architecture was performed using geophysical investigations (i.e., resistivity imaging) from Francese et al. [36]. These helped to verify the confined or unconfined conditions of the aquifer. Finally, these data were used to identify two subareas to evaluate the effect of river–groundwater interaction and aquifer recharge on microplastics contamination in groundwater.

Water samples were collected from four stations during a small recession phase (25 March 2021), interrupted by a recharge event (13 April 2021) (Figure S2). A complete representation of hydraulic head variations during the hydrologic year in LDCAMPUS is reported in Figure S3. During both sampling periods, two surface-water samples were collected, namely LDT (sampling point in the Taro River) and LDC3 (sampling point in the La Riana Channel). The other two samples were collected from groundwater, namely LDCAMPUS (piezometer) and LDS1 (natural spring). The sampling time was established using precipitation data (Parma meteorological station, Figure 1) and data of the Taro River flow (Taro River water-level sensor, Figure 1), obtained from the Regional Environmental Protection Agency of Emilia-Romagna (ARPAE) website [37]. In addition, piezometric heads in LDCAMPUS were measured using a pressure transducer with a data logger (STS DL.OCS/N/RS485). The LDCAMPUS piezometer is composed of a 3 in. polyvinyl chloride (PVC) tube reaching 30 m bgl (below ground level) and screened between 23.80 and 26.80 m bgl. The piezometer stratigraphy data begins with deposits made by silt and clay from 0 to 4.90 m bgl. Below this horizon lies the confined "shallow aquifer" (from 4.90 to 26.70 m bgl), which is mainly made of gravels with rare and thin clay lenses. From 26.70 m bgl, other deposits made by silt and clay are found and the piezometer ends at 30.00 m bgl.

All water samples were taken and processed with glass or metallic tools and instruments to avoid contamination, except for the hydrogen peroxide (H_2O_2) container which was made of plastic (High-density polyethylene, HD-PE). In surface (and spring) water, 5 L samples were taken using a glass beaker at different depths (surface, medium, bottom) near the riverbank and stored in glass bottles with cork tops. All material was rinsed with Milli-Q water before sampling. A metallic Bailer (250 mL) with a metallic wire was used in the piezometer. Groundwater was not purged before sampling to avoid contamination from the plastic tube of the water pump. The bailer sampler was used to sample groundwater at groundwater/air interface and at medium height, but not at the piezometer bottom to avoid contamination from the piezometer tube made of PVC, which fragments (with density up to 1.37 g/cm^3) fall to the bottom of the water column. A total of 5 L of groundwater was stored and processed in the same manner as for surface waters. The sample amount was decided based on different needs: The first is to maximize the number of possible microplastics found sampling a higher volume, which is usually between 0.5 and 2 L [20,22,38]. The second is to sample a sufficiently, yet easily transported and stored, large volume. Thus, the final sample volume was set to 5 L for both surface and groundwater, making them also more comparable. Although final values are reported as microplastics per liter (microplastics/L), the treatment of different volumes entails the use of different amounts of reagents, making the comparison between processed surface water, groundwater, and analytical blank samples (5 L of Milli-Q) meaningless.

Together with microplastics, other samples were taken for isotopic analysis (^{3}H, δD‰, and δ^{18}O‰), to evaluate the effect of a recharge event. The analyses for the determination of the tritium activity were carried out according to the procedures provided by the International Atomic Energy Agency [39] at the Isotope Geochemistry Laboratory at the University of Trieste (Italy). The analytical prediction uncertainty was ±0.5 TU. Stable isotope analyses (δD‰ and δ^{18}O‰) were performed at the Isotope Geochemistry Laboratory at the University of Parma (Italy), using a Delta Plus mass spectrometer (Thermo Fisher

Scientific, Waltham, MA, USA) coupled to an automatic HDO device preparation system. In the field, temperature, dissolved oxygen (O_2 mg/L and O_2%), electrical conductivity (EC), Oxidation Reduction Potential (ORP), and pH were measured using a multiparameter probe (HI9829 HANNA Instruments, Woonsocket, RI, USA), calibrated the day before sampling. These measurements were not performed in LDT (Taro River) due to logistic problems with the probe.

2.3. Microplastics Extraction and Processing

The 5 L samples were processed after 24 h, to allow the sedimentation of suspended materials. After that, the supernatant and suspended solids were filtered on polycarbonate track-etched (PCTE) filter membranes (47 mm diameter and 10 μm pore diameter, Steriltech Corporation). Although PC is a plastic material, the hydrophilic surface of the filters prevents its staining, also avoiding false positives from filter particles. Between 2 and 7 membranes were used per sample, according to the suspended solid load. Bottles and vacuuming gear were rinsed thoroughly with Milli-Q water (Millipore, Bedford, MA, USA) before and after every sample to avoid contamination. The filter membranes were left covered in an oven until dry [40]. The digestion of organic material was performed after drying by placing the membranes in 20 mL of 30% H_2O_2, followed by further oven drying [40]. Once dried, the membranes were carefully washed with Milli-Q water using in the same beaker and checked using backlighting for possible residuals. Filters were again placed in the oven until dry. After desiccation, the solid sample was rinsed with a high-density floatation solution (5.57 M) of K_2CO_3 (≥99.0%, VWR Chemicals) for microplastic isolation [41]. The samples were poured into density-separation glass funnels for the separation of sediments (denser) from microplastics (less dense). The lower-laying sediments were eliminated from the funnel and the K_2CO_3 solution was retrieved for future use [41]. The upper liquor was filtered over a single PCTE filter and Milli-Q water was used to carefully wash the remaining microplastics from the funnels. After that, the filter was dried at ambient temperature in a covered glass Petri dish.

A 1 mg/L solution of Nile Red (Fisher Scientific) and methanol (≥99.8%, VWR Chemicals) was prepared just before the microscopic analysis, according to Erni-Cassola et al. [40]. After applying a few drops (3 to 4) to cover the filter, they were cut in half using a scalpel blade (previously rinsed with Milli-Q) to fit over standard microscope slides and stage, covered with coverslips, and fixed with tape. The samples were stored at 60 °C for 10 min in the dark. A stereomicroscope (Leica S8AP0) was equipped with a camera (Leica DFC295 with Leica Application Suite, version 4.2.0), an external fluorescence light source (excitation 470 nm, royal blue), and an orange photography filter [40,42]. Microscopy images were taken using exposure times between 0.7 s and 1 s. Several images were taken for every filter, usually overlapping by around 30% to enable reconstruction of the panoramic view of the filter, using the demo version of Autostitch [43]. A minimum background signal (e.g., fine sediments on the filter) must be provided, for the program to reconstruct the image correctly and to avoid distorted outputs. After the reconstruction, ImageJ (version 1.53, [44]), was used to perform automated particle recognition and quantification based on the fluorescent particles using parameters similar to Erni-Cassola et al. [40] and Prata et al. [45]. After the automated quantification of microplastics, an output is produced by ImageJ, in which the microplastics selected in the red channel are highlighted in yellow. This final image is inspected for false-positives or errors (see Section 4.1). The ImageJ script was tested using artificially made filter images (Figure S4). A more detailed description of the protocol and the specifications used in both Autostitch and ImageJ is reported in the Supplementary Material, together with QA/QC measures derived from Erni-Cassola et al. [40] and Koelmans et al. [46]. Simultaneously, three replicates of 5 L Milli-Q water were analyzed as control samples (blanks), using the same procedure described above. The resulting analytical contamination was used to set the ImageJ parameters for plastics identification. Since the control samples were contaminated by microplastics with lower area and higher circularity than those from the environmental samples, these were excluded by the ImageJ identification imposing those values as limits for the particle identification (see Sections 3.2 and 4.1). Thus, in the environmental sample, only microplastics with a lower circularity and higher area of the median value of those in the control samples were counted and characterized. Besides the count of the fluorescent microplastics, the used ImageJ protocol allows for the quantification of important features such as the microplastics area (μm^2), Feret diameter (μm), and circularity, which is expressed in a number between 0 (low) and 1 (high). Statistical analysis of these results was performed using the statistics software R [47]. The statistical approach was chosen to verify possible differences in the above-mentioned geometrical parameters between the samples and sampling time (i.e., after the recharge event). Differences among controls (Ctrl), groundwater (GW), and surface-water samples (SW) were tested using the Kruskal test [48] using the package "Rstatix" [47]. If significant, Dunn's post hoc test was performed [49] through the R package "FSA" [50], using the Benjamini–Hochberg method for adjusted p values. Differences among groundwater (GW) and surface-water samples (SW), together with differences among the sampling times, were tested using the Mann–Whitney U test [51]. On the contrary, since data were normally distributed, the abundances between the two sampling times were compared using the paired t-test [51]. Both statistical tests were chosen based on the sampling design, together with verified and nonverified assumptions.

3. Results

3.1. Hydrogeological Features of the Study Area and the Effects of Aquifer Recharge

A detailed hydrogeological characterization of the investigated area is presented in Figure 2. The outcropping sediments are related to the Ravenna Subsynthem (AES8) and the Modena Unit (AES8a) [52]. The Ravenna Subsynthem (originated between the upper Pleistocene and Holocene) primarily comprises sandy gravel, sand, and stratified silt, with a discontinuous cover of clayey silt. The Modena Unit (originated during the Holocene) primarily comprises sands with gravel lenses, covered by a discontinuous layer of clayey silt [52]. The outcropping materials were grouped based on their relative permeability. More permeable outcropping (gravel and sand) is found in the southwest part of Figure 2 (near the foothill) and nearby the main watercourses, while the less-permeable outcroppings (clay and silt) are reported in the northeast part of Figure 2. Thus, the aquifer is identified as unconfined at the southwest and near the Taro River, which dissected the clay and silt cover. On the contrary, it is identified as confined in the remaining area (northeast), where the confining (clay and silt) layer can reach 15 m of thickness (litholog W181120P501).

The points in the two subareas where sampling was performed are also reported in Figure 2. Each one is composed of one (or two) sampling points for surface waters and another one for groundwater. The first subarea is in the central part of Figure 2. It is composed of a losing channel (namely LDC3), a losing stream (LDB), and a piezometer downgradient (LDCAMPUS, Figure 3a). Within these points, the aquifer changes from unconfined to confined. The hydraulic head of the pressure transducer in the LDCAMPUS piezometer showed a recession period of groundwater interrupted by a recharge event (Figure S1). The aquifer recharge supplied by rain results in a small rise of the hydraulic head. This phenomenon, although of small entities, is appreciable also in the previous (small) recharge events (Figures S1 and S3). The fast response of the system provides other pieces of evidence to the high permeability of the surrounding area. Without the recharge event, the hydraulic head would keep lowering with a regression coefficient of 0.015 d^{-1}. The recharge resulted also in a variation of EC (Table 1). In both LDC3 and LDCAMPUS, the EC values were lower after the recharge given by precipitation. This evidence is consistent with the conceptualized river–groundwater interaction, with LDC3 feeding LDCAMPUS.

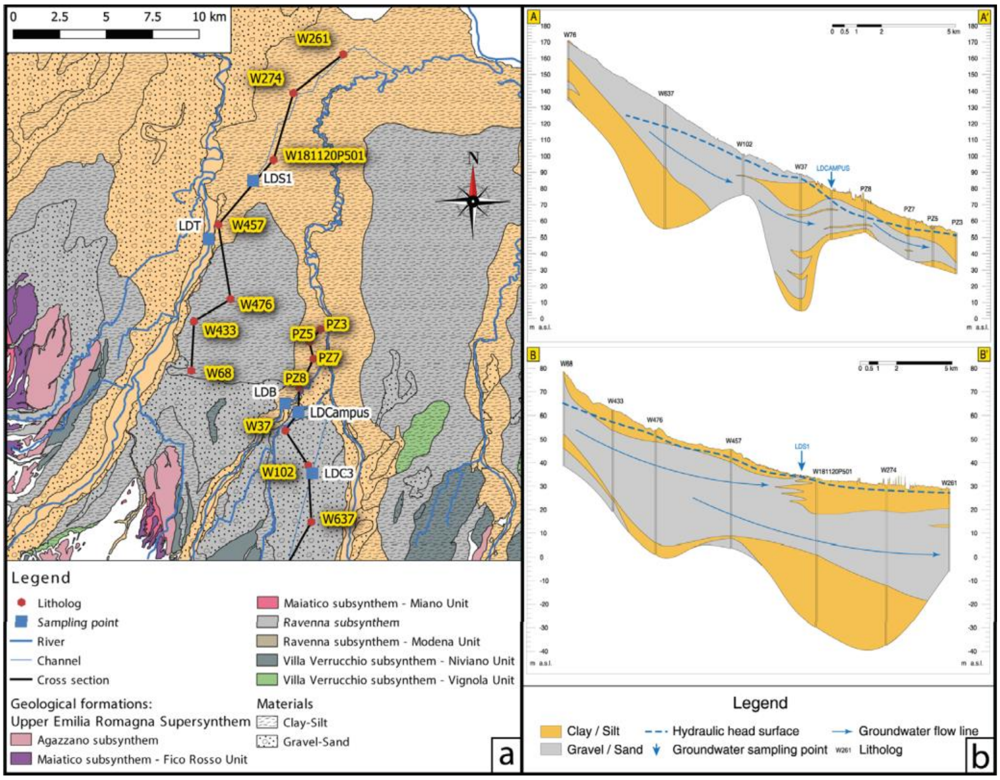

Figure 2. Geological and hydrogeological settings of the investigated area at large scale. (**a**) Geological chart of the region including the two subareas, with geological Formations and outcropping materials, modified from the Geological map of the Emilia-Romagna Region, 1:25.000 scale [35]. (**b**) Hydrogeological cross sections are reported in panel (**a**); the hydraulic head values were derived from Zanini et al. [30].

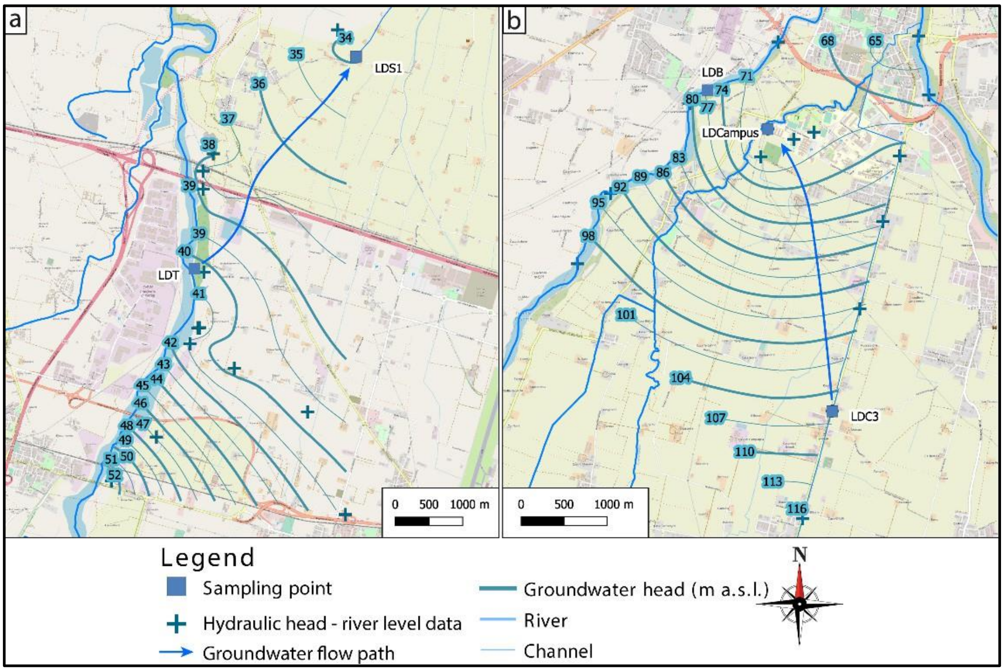

Figure 3. Groundwater net for the subareas. (**a**) Groundwater flowpath between LDT and LDS1, hydraulic head data from Lancini [32]; (**b**) groundwater flowpath between LDC3 and LDCAMPUS, hydraulic head data from Viani [31].

Table 1. Isotopic and field data. The dot represents not-analyzed samples.

Date	Sample	t (°C)	O_2 (mg/L)	O_2 (%)	EC (μS/cm)	ORP (mV)	pH	^{3}H (TU)	δD‰ (vs V-SMOW)	δ^{18}O‰ (vs V-SMOW)
25 March 2021	LDS1	14.0	2.99	29	586.1	89.8	7.6	7.06	−45.10	−7.44
25 March 2021	LDCAMPUS	16.0	8.51	87	893.0	201.5	7.3	-	-	-
25 March 2021	LDC3	6.4	8.96	73	608.7	143.1	8	-	-	-
13 April 2021	LDS1	14.0	2.08	20	577.5	154.9	7.3	9.30	−43.80	−7.42
13 April 2021	LDC3	7.7	9.22	77	467.7	139.5	8	-	-	-
13 April 2021	LDCAMPUS	14.0	7.67	81	308.4	190.2	6.9	-	-	-
13 April 2021	Precipitation	-	-	-	-	-	-	10.00	-	−13.2

The water level of the Taro River rapidly increased after rainy days. Therefore, the discharge of LDS1 increased, due to the high groundwater recharge provided by the Taro River (Figure 3b). The spring discharge was not measured during the first sampling due to the low flow and incompatibility with the current meter (Small Current Meter C2, OTT, Kempten, Germany). After the recharge event consequent to the flow rise in the Taro River, the spring discharge raised from nonmeasurable to 4.8 L/s. The recharge effect on LDS1 is evident also in tritium content (Table 1). After the recharge event, the tritium content of the spring increased by 31.7% (+2.24 TU) and was closer to the tritium content of precipitation. Thus, the tritium content of LDS1 over time is consistent with the proposed river–groundwater interaction, with LDT feeding LDS1. These data also testify that the groundwater flow nets reconstructed with data from [30–32] are still valid, at least in the investigated subareas. Unlike for surroundings of LDCAMPUS, no data about hydraulic conductivity and storativity are available for this subarea. Nevertheless, given the fast response of the spring to the recharge, and the comparable grain size of the aquifer, we assumed that hydraulic conductivity is like that of the area near LDCAMPUS, in the order of 10^{-4} m/s.

3.2. Microplastics Quantification and Geometric Characterization

To reconstruct the whole panoramic view of the filters, typically 45 ± 11.4 (average ± standard deviation) photos were taken for every microscope slide, i.e., half-filter. The maximum number of photos taken was 76, while the lowest was 29. In total, 989 photos were taken. The microplastics' presence in both surface and groundwater was highly heterogeneous. Surface-water samples showed a large range of values, with a mean concentration of 47.4 ± 51 microplastics/L, while the groundwater samples' mean was 26.88 ± 24.37 (Figure 4). The concentration of analytical and field samples is reported in Table S3. All the samples showed an increase in microplastics concentrations after the recharge event.

Regarding the geometrical characterization, control and environmental samples showed interesting and peculiar characteristics. A statistical difference was reported between microplastics in both environmental samples and analytical blanks, not only in the area (Chi square = 140.31, df = 2, p value = 2.2 × 10^{-16}), but also in circularity (Chi square = 74.415, df = 2, p value = 2.2 × 10^{-16}) and in the Feret diameter (Chi square = 152.86, df = 2, p value = 2.2 × 10^{-16}). These differences were further tested using the Dunn's test for pairwise comparison, showing that microplastics in control samples had a smaller area and Feret diameter than surface and groundwater samples, while the circularity was higher in control samples (Tables S3 and S4). This information justified the setting of an identification limit for environmental samples' microplastics equal to the median value of the analytical blanks' microplastics to avoid their overestimation. However, this is expected to result in an underestimation of sample microplastics with dimensions and characteristics in the range excluded during image analysis. While an underestimate is not ideal, it is acceptable, to account for the observed levels of contamination. After this, the environmental samples were analyzed again with the new parameters to exclude the laboratory contamination, and the differences between surface waters and groundwater were tested using Mann–

Whitney U test. The results revealed a higher circularity and Feret diameter in groundwater than in surface waters, whereas no difference was reported for the area (Table S3). The geometrical features of microplastics were also tested between the two sampling times (before and after the recharge event). As a result, the Mann–Whitney U test highlighted a significant difference between the sampling times in circularity for surface water samples (W= 149608, p value = 1.17×10^{-2}), whereas no other difference was statistically significant (Tables S3 and S4).

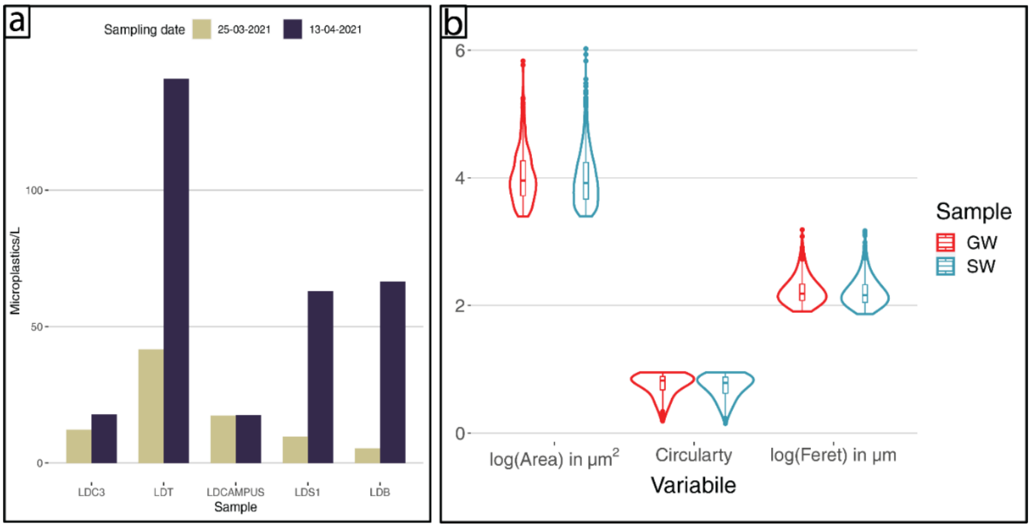

Figure 4. Microplastics characterization. (**a**) Microplastics occurrence (particles/L). (**b**) Microplastics' geometric attributes in surface (SW) and groundwater (GW) samples; inside the violins, boxplots indicate the minimum and maximum values, lower and upper quartile, and median. Area and Feret diameter were log-transformed for graphical reasons.

4. Discussion

4.1. Assets and Disadvantages of the Extraction Protocol

The proposed sampling strategy aimed to avoid contamination during sampling by using only metallic or glass tools. Sampling with the metallic Bailer can be more time-consuming than the direct sampling with a beaker. This is in any case dependent on the depth of piezometric level and the Bailer volume.

The methodology used for microplastics extraction, derived from the integration of previously reported methods, allowed a good characterization of the microplastics contamination through an inexpensive and easily performable procedure. Although microplastics analysis through fluorescence has already been reported as a successful method [53], some improvements were adopted. Starting from the protocol proposed in Erni-Cassola et al. [40], a higher number of filters was used for every sample during the first filtration step. This is due to the high load of suspended solids found in surface waters during the recharge event, which happened to obstruct the filter and possibly cover microplastics with silt and clay, which were difficult to remove from the final processed sample (Figure 5). The high amounts of suspended solids also made the use of a floatation solution for microplastic unavoidable. To also allow the isolation of denser microplastics such as PVC, a 5.57 M solution of potassium carbonate (K_2CO_3) was prepared, with a density of 1.54 g/cm^3. This particular floatation solution was preferred to others such as ZnCl and NaI, as K_2CO_3 is cheaper, recyclable, and more environmentally friendly [41], while having a higher density than the NaCl solutions (up to 1.2 g/cm^3).

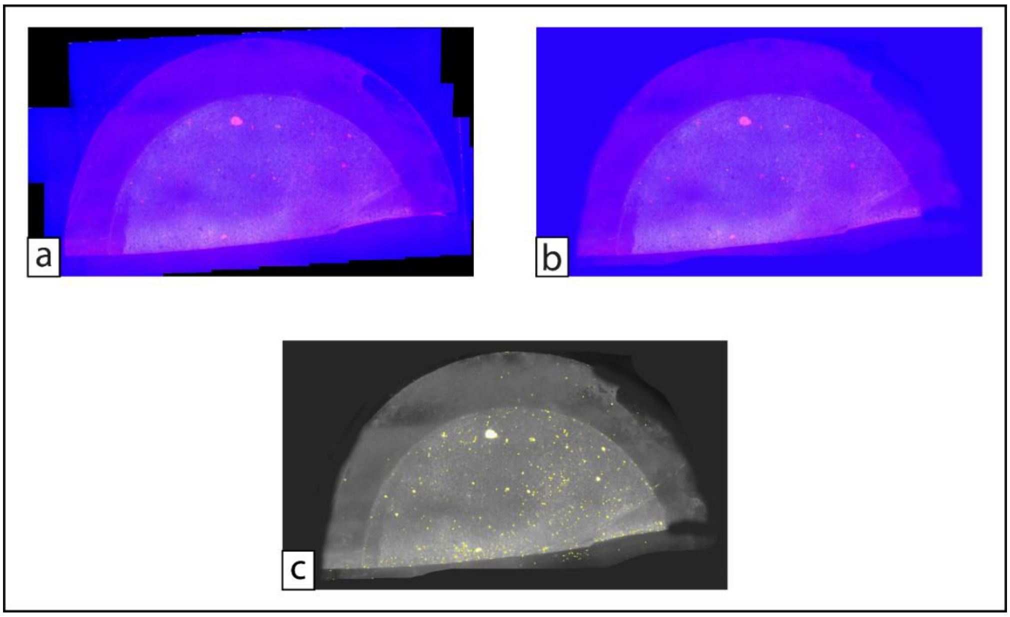

Figure 5. Filter membranes reconstructed and processed. (**a**) The image output from Autostitch. Only the central area of the filter is the actual filtering area, whose pink background could be due to the presence of silt and clay stained by the dye; on the top side of the image, it is still possible to see the original scale of the individual photos taken. (**b**) The brush tool with blue color was used to correct filter imperfections. (**c**) The final output from ImageJ (red channel), in which the identified microplastics are underlined in yellow, allows the postanalysis search of errors or false positives.

In addition, high-density solutions of K_2CO_3 can easily precipitate [41], forming crystals in the final processed sample. Despite that, these crystals had no consequences during fluorescence analyses (Figure 6), since they have no red color and were well-eliminated from the image during the color-splitting step in ImageJ (see detailed analysis protocol in the Supplementary Material). These macroscopic precipitates were associated with K_2CO_3 since they were not present in the previous stages of the analysis.

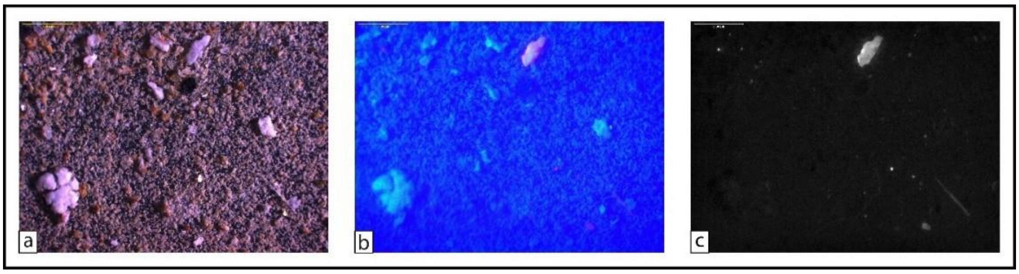

Figure 6. High-magnification image of an analyzed filter membrane. (**a**) Original image under normal light; the white object in the bottom-left corner is supposed to be K_2CO_3 precipitate. (**b**) Same filter portion under fluorescence conditions. (**c**) The final output from ImageJ (red channel).

The filters were clipped in half as in Erni-Cassola et al. [40], but early cutting attempts of dry filters resulted in the loss of material. As an adjustment, filters were cut immediately after the Nile red solution application, which wetted the material over the filter and avoided its loss during the cutting phase. If used in a larger volume than necessary, it was observed that the Nile red solutions could dye the filter membrane's borders. In this case, the filter image was modified in a raster graphics editor using a blue brush to correct these defects (Figure 7), which would otherwise be considered as microplastics by ImageJ. The blue color was preferred since it cannot result in false positives in ImageJ due to channel separation (see detailed analysis protocol in the Supplementary Material).

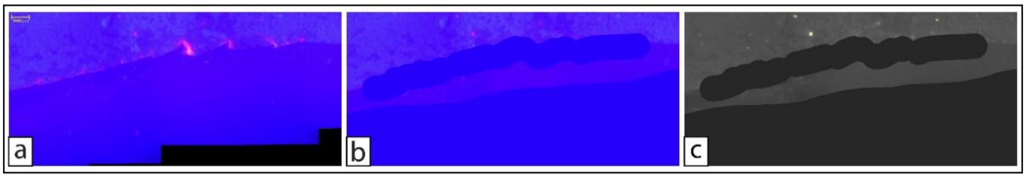

Figure 7. Filter correction using the brush tool. (**a**) Original image from Autostitch; the pink spots are filter portions accidentally dyed by the Nile red solutions and are quite common in the clipped segment of the filter. (**b**) Color image after correction. (**c**) The final output from ImageJ (red channel).

The use of microscope slides and coverslips was preferred to simply uncovered Petri dishes, for two reasons: firstly, they avoid laboratory contamination without lowering the image quality. Secondly, they allowed the inspection of the whole (half-)filter, conversely to Petri dishes whose edges would interfere with the microscope objective. The use of Petri dishes larger than the filter (47 mm diameter) was discouraged, as they could not steadily hold the filter during sample handling. The necessity to analyze the whole filter, instead of some random parts of it, is derived from the microplastics distribution in the filter, which is not homogeneous. In fact, during the filtration process, it was observed that microplastics did stick to the internal walls of the filtration unit. After rinsing, they were more concentrated on the external area of the filter. In addition, more microplastics were also found in the central part of the filter, where the first spilled sample was filtered. For these reasons, the use of randomly taken images was avoided and an accurate, albeit more time-consuming, reconstruction of the whole filter image was performed using the demo version of Autostitch, as reported in Maes et al. [43], using images taken through a microscope and resulting in a higher resolution with a theoretical smaller minimum dimension of particles for detection.

The microscope adaptations proposed by Labbe et al. [42] were successfully replicated and utilized for the analyses presented in this study. Although a high number of microplastics was detected, the comparison between normal light and the final output of the identification procedure (Figure 6) showed a lower sensitivity for microplastics of elongated and thin shape such as fibers, which were not detected for their whole length but only for portions of it. Lastly, the microscope adaptation was realized with a very low budget (<100 EUR). The main issue with microplastics analysis is often laboratory contamination and usually other studies focus on the extent of contamination alone [22,40]. On the contrary, in this method, the contamination was excluded by setting the ImageJ macro to identify and characterize microplastics different from those found in the analytical blanks, which were characterized by a (statistically significant) lower area and Feret diameter but a higher circularity than those in environmental samples. As a result, the laboratory contamination is no longer a major issue, although this implies the noncharacterization of smaller microplastics, and of those with size and geometries similar to those of analytical blanks, resulting in a likely underestimate. The possible sources of contamination were identified in unfiltered laboratory solutions and airflow (although AC was turned off or down during analyses). This was ascertained by testing the microplastics abundance in analytical blanks (5 L Milli-Q) analyzed using filtered (3x) and unfiltered (3x) reagents and Milli-Q. As a result, the analytical blanks analyzed using unfiltered reagents and Milli-Q had a mean contamination of 37.13 microplastics/L (respectively 35, 56.4, and 20 microplastics/L), whereas those analyzed using filtered reagents and Milli-Q showed significantly lower contamination (13.4, 6.4, and 35.6 microplastics/L, respectively), with a mean value of 18.4 microplastics/L. Moreover, the geometrical characteristics of microplastics were similar between the samples treated with filtered and unfiltered reagents and Milli-Q. This consideration supports the origin of microplastics' contamination in unfiltered laboratory solutions, which could contain smaller microplastics than those in environmental solutions [45,54], with a possible more rounded shape due to the abrasive and smoothing effect of the used salts (i.e., K_2CO_3) in plastics packaging. The material (high purity grade) bought for the laboratory solutions was sold in plastic packages, except for Nile red. Hence, a prefiltration of all the used solutions should be performed before use [22,43,45]. Since it was not present in Erni-Cassola et al. [40], the refiltration was not performed before the analysis of the presented samples.

4.2. Aquifer Recharge and River–Groundwater Interaction as Drivers of the Microplastic Contamination

Microplastics were present in all the analyzed samples, providing further evidence of microplastics contamination in groundwater, of which studies are still rare. Groundwater samples showed a large range of particle concentrations (26.88 ± 24.37 microplastics/L) but had a lower mean particle concentration than surface-water samples (47.4 ± 51 microplastics/L), which also showed a large range of values, although the aquifer in the investigated subareas is fed by losing streams and channels. Hence, a filtering effect of the aquifer can be ascertained and its effects on microplastics contamination can be better clarified by looking at the geometries of microplastics found in groundwater. These had a higher circularity and Feret diameter than those in surface water. According to Pirard [55], the circularity parameter "*lack[s] clear physical significance*", since it can be equal for very different shapes. Nevertheless, it is an optimal descriptor of the progressive change from any initial morphology, since it is the ratio of the microplastics' projected area to that of a sphere projecting the same perimeter length. As such, this ratio is an adequate measure of the extent of transport processes, in which particles are expected to degrade toward sphericity [56,57]. Moreover, a more rounded shape fosters the transport of particles in porous media such as alluvial aquifers, as reported by Keller et al. [58] and by further authors for bacteria [59] and other colloidal materials [60,61]. In other words, microplastics found in groundwater are a subsample of those in surface waters, where only those that underwent prolonged transport processes (higher circularity) can be transported also into the aquifer and groundwater. The remaining part of microplastics in surface waters, which contribute to the higher concentration in surface waters, is constituted likely by mostly secondary microplastics, which did not yet undergo transport processes. The higher Feret diameter for microplastics in groundwater compared to those in surface waters is an unexpected result. We speculate that it is related to the presence in groundwater of microplastics with a more simplified shape than those in surface waters, e.g., untangled fibers or nylon fragments, which can be easily crinkled during the transport processes in surface waters. Nevertheless, these are just hypotheses, establishing new questions and encouraging further investigations about the effect of microplastics' shapes during transport in porous media.

Regarding the effects of the recharge event, although a general increase in microplastics concentrations was found after precipitations, this difference was not statistically significant (Table S3). In the Taro River (LDT) and spring (LDS1) subarea, the increase in microplastics in groundwater is undeniable (Figure 4). On the other hand, the subarea with LDC3, LDCAMPUS, and LDB showed a lower increase in microplastics contamination after the recharge event. Hence, the effects of the recharge event can be detected only under certain circumstances. In both subareas, a higher microplastics contamination was expected due to the higher hydraulic head given by aquifer recharge. This process was investigated at laboratory scale in porous media by Bizmark et al. [62], who found that a higher pressure in the medium is responsible for an enhanced transport of colloidal particles such as microplastics. In addition, the higher discharge of the river and channel after precipitation leads to a highly unsteady flow regime, which promotes the resuspension and transport of deposited bottom sediments (and microplastics), the release of contaminants from the interstitial water of the sediments, and causes land erosion [63]. In the investigated area, only the Taro River (LDT) experienced a higher microplastics contamination after precipitation, whereas the channel (LDC3) experienced a smaller increase in microplastics contamination. The groundwater fed by these surface waters responded accordingly, and only in LDS1 was the microplastics concentration higher after the aquifer recharge. Due to the heterogeneity of surface-water contamination and relative recharge of groundwater, the difference between the sampling periods was not statistically significant. Nevertheless, these data prove that the processes described in Bizmark et al. [62] can be upscaled to a km scale and that the different entities of groundwater contamination should be compared only between the same groundwater flow path and systems when possible. The precipitation and consequent aquifer recharge did not promote any change in the geometrical parameters of microplastics, except for circularity in surface waters, characterized by a lower value after the recharge event. This fact is consistent with the above-mentioned dynamics and testifies that a phase of high river discharge fosters the formation of new or the resuspension of sedimented secondary microplastics, which are typified by a lower circularity value since they did not yet undergo prolonged transport processes. This does result in a rise in microplastics concentration, but this is not statistically significant (Table S3; Figure 4). It is in any case sufficient to lower the circularity median value of microplastics in surface waters.

5. Conclusions

The investigation was performed using a protocol based on a self-made fluorescence microscopy setup and an open-source image-processing program, resulting in a high result-to-cost ratio. Such a technique allows even those who lack access to a specialized analytical chemistry laboratory and high levels of contamination control measures to conduct research in this field. This will be particularly useful in developing countries, where the research in this field is less advanced than that in developed countries and complicated by potentially limited access to expensive equipment, e.g., micro-FTIR or micro-Raman [64].

In addition, the presented results shed new light on the interaction of microplastics' abundance and geometrical attributes with the hydrogeological characteristics of the aquifer. Although not yet characterized from a chemical perspective, the microplastics observed in the samples provide valuable information regarding their presence and transport in a porous material, i.e., alluvial aquifer. Furthermore, these findings raise new and urgent questions about microplastics in groundwater. For example, which mechanisms influence the transport of microplastics in alluvial aquifers? How do the geometrical features of microplastics, coupled with their electrochemical characteristics, affect their transport in the aquifer? Extensive research is still required in this field, which is poorly explored and demands further attention by the scientific community.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14121913/s1, The sampling, extraction, and analyses protocol of microplastics, with the ImageJ macros and QA/QC measures; Figure S1: Location of granulometric data; Figure S2: Hydrological data measured in surface and groundwater; Figure S3: Hydraulic head and precipitation during 2021 in LDCAMPUS; Figure S4: Artificial filter images; Table S1: Granulometric composition (ASTM D422–63) between 1–1.60 (m bgl); Table S2: Granulometric composition (ASTM D422–63) between 5–5.7 (m bgl); Table S3: Microplastics abundance and statistical analyses results; Table S4: Microplastics geometrical characteristics.

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2.2. Understanding the Transport and Natural Attenuation of Personal Care Products (PCPs)

hydrology

MDPI

Article

What Is the Impact of Leaky Sewers on Groundwater Contamination in Urban Semi-Confined Aquifers? A Test Study Related to Fecal Matter and Personal Care Products (PCPs)

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Abstract: Urban areas exercise numerous and strong pressures on water bodies, implying that different external anthropogenic factors also stress groundwater. Sewerage networks play an important role, being the place of wastewater flow. When sewerage deterioration conditions occur, aquifers can be contaminated by contaminants contained within wastewater. The study aims to verify the impact of sewerage leaks in urban semi-confined aquifers through a multidisciplinary approach. Geological, hydrogeological, hydrochemical, microbiological, and biomolecular investigations are carried out in a test site close to a sewer pipe, from February to October 2022. Microbiological analyses are carried out on a monthly basis, contextually to hydraulic head measurements in purpose-drilled piezometers. The presence of sandy intercalations and the prevalence of silt within the outcropping (about 10 m thick) aquitard makes the aquifer vulnerable to percolation from leaky sewers, therefore causing persistent microbial contamination in groundwater. The presence of fecal indicators (including pathogenic genera), corrosive and human-associated bacteria markers, is detected. The magnitude of microbiological impact varies over time, depending on hydrogeological factors such as dilution, hydrodynamic dispersion, and variation of the groundwater flow pathway at the site scale. As for personal care products, only Disodium EDTA is detected in wastewater, while in groundwater the concentrations of all the analyzed substances are lower than the instrumental detection limit.

Keywords: sewer; leakage; urban semi-confined groundwater; personal care products; microbiological pollution

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1. Introduction

Over the years, anthropic pressure has heavily impacted the aquatic environment and resources: land-use modifications, environmental planning, and the development of industry and agriculture are just a few examples of polluting human activities. In particular, in highly populated areas, growing urbanization implies an increase in water demand, changes in water uses, and degradation of water quality [1] since human activity also causes the release of anthropogenic contaminants into water bodies. [2–5]. Because of these strong and numerous pressures, urban areas have the potential to pollute groundwater bodies in many ways, and in Italy, national and regional legislations are contemplating the necessity to improve plans for the management and remediation of the most industrialized urban areas damaged by groundwater contamination [6]. Groundwater and surface water can be contaminated by pollutants such as hydrocarbons, nitrates, phosphorus, chlorinated solvents, heavy metals, fecal bacteria, emerging contaminants, and many others. In particular, unconfined aquifers, where only the lower limit is an impermeable layer while the upper limit is characterized by high permeability, are more vulnerable to anthropogenic pollution than confined aquifers. In fact, pollutants can more easily cross the permeable rock and contaminate groundwater [7].

In this regard, it is essential to underline sewer networks' role in making confined or semi-confined aquifers vulnerable. Sewer networks are deemed among the most important urban infrastructure systems, as they play an essential role in protecting the urban water environment, providing public health and safety, preventing the transmission of waterborne infection, and reducing the risk of urban floods [8–10]. When sewerage is subject to wear and tear through exfiltration from a leaky sewerage system, groundwater may be contaminated by wastewater [11,12]. In addition to the pipes' ages, deterioration is the other main reason for the leaks in sewerage networks. Several causes lead to pipeline structural weakening: (i) wastewater chemical characteristics, (ii) sewerage's construction features (e.g. pipe material, installation depth, method, and size), and (iii) geological events, such as landslides and/or seismic events [13–21]. The vulnerability triggered within a confined or semi-confined aquifer occurs when sewer pipes are constructed at depths greater than the low permeability horizon. With the occurrence of sewer leaks, the effluents can cross the rock easily, being within a horizon with no negligible permeability.

In particular, PPCPs (pharmaceuticals and personal care products) are a unique group of emerging environmental contaminants deemed to be the markers of wastewater presence in the urban groundwater system because of their human-related use [18,22–25]. This is because PPCPs generally are poured into wastewater by excretion from a citizen's body and washed off with tap and sink water [26]. Moreover, in sewage treatment plants, PPCPs can still be detected in the effluents since conventional treatment processes cannot remove the PPCPs from wastewater altogether [27]. For this reason, wastewater plant effluents are also inferred as a source of PPCPs, since an important fraction of them may be released into the aquatic environment through the effluents [28] and reach the aquifer through interaction with surface water courses. PPCPs have been detected in groundwater, showing that the PPCP contaminants and the following ecological risks in groundwater and surface water may be of major concern [29], generating potential risks for public health and environmental safety. Although concentrations of PPCPs in the environment are low, continuous exposure to these compounds is a critical concern with unknown long-term impacts [27].

To sum up, urban areas exercise numerous and strong pressures on water bodies, implying that different external anthropogenic factors also stress groundwater. However, there are limited data and studies on monitoring indicators of contamination from sewerage leaks.

This type of monitoring can be carried out through chemical and microbiological analysis of groundwaters. In particular, as mentioned above, the class of PPCP contaminants is considered an indicator of leaky sewers because of their domestic consumption. In addition to this, microbiological groundwater pollution is closely connected with the presence of a leaky sewerage system. Wastewaters are characterized by high bacterial loads with possible pathogens and viral agents. The microbiological water quality is then examined through the microbial groups' identification of more straightforward determination: the indicators of fecal contamination. These microorganisms (e.g., fecal coliforms and intestinal streptococci), usually present in human feces and warm-blooded animals, are closely related to the degree of pollution from urban wastewater and their presence in groundwater. The migration of bacterial cells through natural clayey media or other low-permeability rocks has been studied mainly at the laboratory scale through column tests [30–33] and subordinately at the site scale [34,35]. In particular, Rizzo et al. (2020) showed that the porosity of clay minerals could vary from micropores (<0.002 μm diameter) to mesopores (0.002–0.05 μm diameter). Aggregation of clay particles forms pores >0.1 μm (macropores). The coexistence of micro-, meso-, and macropores leads to a nonuniform path within the clayey medium, and the migration of bacterial cells could be selectively concentrated in certain sediment volumes. Consequently, it is essential to study such phenomena at the site scale.

The main goal of this work is to present the first results of a multidisciplinary and holistic research designed through different disciplines necessary to analyze the possible impact of leaky sewers on groundwater quality in urban semi-confined aquifers, from a phenomenological point of view. Geological and hydrogeological investigations and chemical and microbiological (culture-based and biomolecular) analyses were carried out. Since chlorinated solvents and microplastics have already been detected in the Parma aquifer [36,37], testifying to the anthropic pressure exerted on groundwater, this study area was selected as a test site.

2. Materials and Methods

2.1. Study Area

The site was selected because of the different types of urban anthropic pressures it is subjected to. Parma is the second town by population in the Emilia Romagna region (northern Italy), representing a critical anthropogenic contamination source for surface waters and groundwater from leaks in the urban sewerage system. The area also has multiple large industrial districts, and intensive agriculture is also performed on this site. All these factors are intrinsically bound to the water body's pollution.

From a geological point of view, this research was carried out in the alluvial Parma aquifer (Po Plain), where the groundwater flows from southwest to northeast at a basin scale [36]. The map (Figure 1) and section (Figure 2), the latest based on the interpretation of stratigraphic profiles derived from published databases by the Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA) and Regione Emilia-Romagna, are in agreement with existing stratigraphic models [38] and reveal a sequence of geological units (or synthems) characterized by basal unconformities caused by the tectonic action of buried Apennine thrust fronts. Sedimentation in the study area derives from the alluvial dynamics of the Apennine streams that filled the Po basin during the Quaternary [39–42]. Overall, the system is made up of an alternation of fine-grained (clays and silts) and coarse-grained bodies (gravels and sands), generated by climatic cycles and shifting riverbeds that lead to a multilayered aquifer system at a basin scale. This study involves only the shallowest aquifer (a few tens of meters thick) of the whole system, characterized by post-LGM (last glacial maximum) sedimentation.

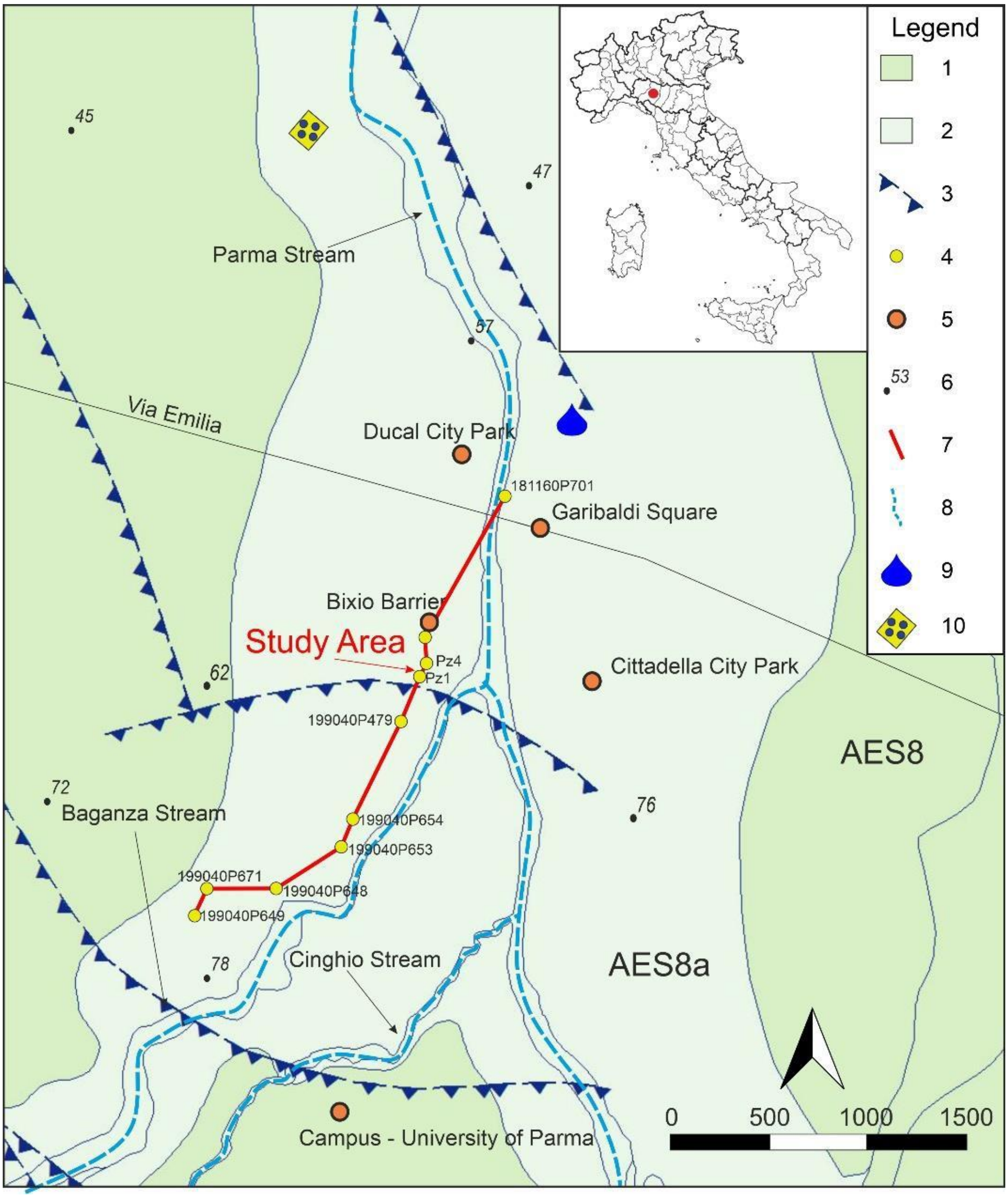

Figure 1. Geological map and section at basin scale; (1) AES8: Emiliano-Romagnolo superiore synthem – Ravenna subsynthem; (2) AES8a: Emiliano-Romagnolo superiore synthem – Modena subsynthem; (3) main thrust.; (4) borehole; (5) toponym; (6) quoted point; (7) geological section's trace; (8) stream; (9) meteorological station; (10) wastewaters treatment plant "Parma Ovest".

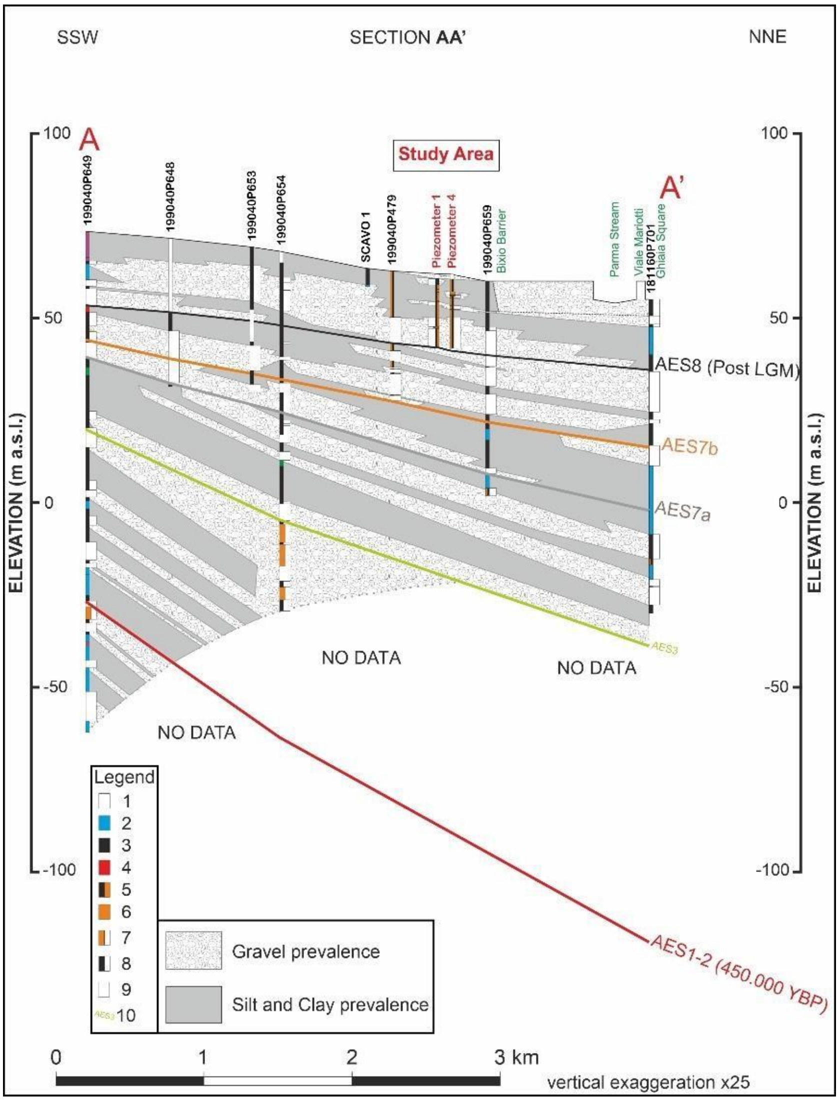

Figure 2. Geological section at basin scale (the trace is shown in Figure 1): (1) fill material, (2) grey/light blue clay and silt; (3) yellow/brown clay and silt; (4) red clay and silt; (5) silt; (6) sand; (7) gravel and sand; (8) gravel and clay/silt; (9) gravel; (10) Code of Geological Unit [*sensu* 37]: AES1: Emiliano-Romagnolo superiore synthem – Monterlinzana subsynthem; AES2: Emiliano-Romagnolo superiore synthem – Maiatico subsynthem; AES7a: Emiliano-Romagnolo superiore synthem – Villa Verucchio subsynthem – Niviano unit; AES7b: Emiliano-Romagnolo superiore synthem – Villa Verucchio subsynthem – Vignola unit; AES8: Emiliano-Romagnolo superiore synthem – Ravenna subsynthem.

Geological and Hydrogeological Investigations

Geological investigations were carried out to (i) reconstruct in detail the geological features of both the unsaturated and the saturated media within the shallowest aquifer that directly interacts with the underground sewer systems and to (ii) quantitatively detect, at the site scale, the grain size of fine-grained layers to define the integrity [43] of the unsaturated aquitard to fecal bacteria and PCPs migration.

Geological characterization of the study area involved 30 continuous core borings (Figure 3) with a drilling diameter of 4 inches and depths varying between 8 and 20 meters below the ground surface. Extracted core samples have been stored in dedicated cataloging boxes, and an accurate lithological description was made for each drill hole. These on-site descriptions were taken during drilling and included grain size and chromatic variety recognition. Subsequently, the rock samples were studied by particle size analysis in the laboratory.

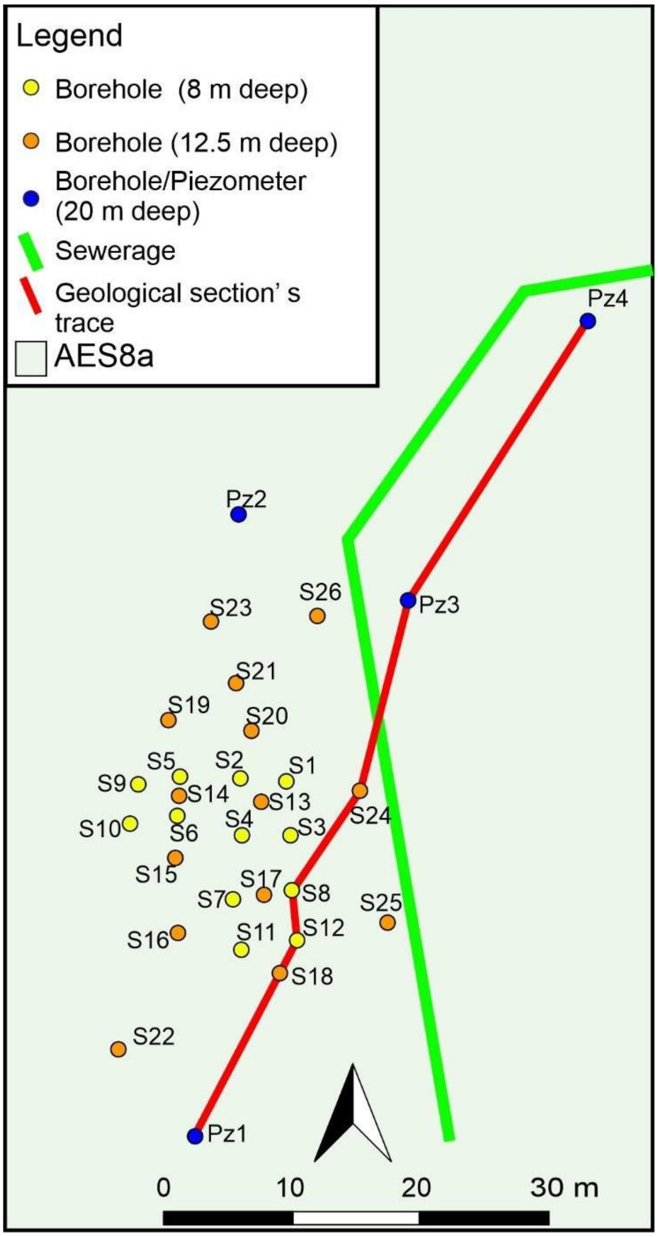

Figure 3. Location of boreholes and piezometers drilled at the test site (the red segment is related to Figure 4).

A total of six samples collected from core plugs were dedicated to grain size analysis. Small amounts of granular material were carefully extracted with a precision chisel from the middle portion of the plugs to avoid alteration along the side walls. All samples were dried in an oven at the controlled temperature of 35 °C for two days to remove most of the moisture and to ease disaggregation. Sample masses collected from the core plugs varied from 0.15 to 0.32 g. Prior to the analysis, samples were disaggregated in tap water via mechanical stirring for two minutes; then, disaggregated materials were left resting for 30 minutes. Grain size analyses were carried out with a Mastersizer 3000 (Malvern Panalytical®) laser diffraction granulometer. The laser granulometer calculates the grain size distribution of particles having an equivalent diameter ranging from 10 nm to 3500 μm. The instrument was equipped with a Hydro EV liquid dispersion unit, suitable for analyzing previously disaggregated low cohesion samples. Following previous work [44], a specific standard operating procedure was developed to analyze all samples efficiently and to minimize the alteration during the analysis. To allow the disaggregation of the fine-grained, clayey-silt samples, a preliminary phase of ultrasonication was needed before the analysis. Analyses were replicated with 25 successive runs for each sample, and the grain size distribution was defined by an average particle diameter, modal value, and span (sorting degree) with associated standard deviation. Data regarding sand, silt, and clay fractions were obtained after the conversion to the Udden–Wentworth standard grain size classes. Details of the analytical parameters adopted during grain size analysis and information regarding the standard operating procedure are provided in the Supplementary Materials (SM.pdf).

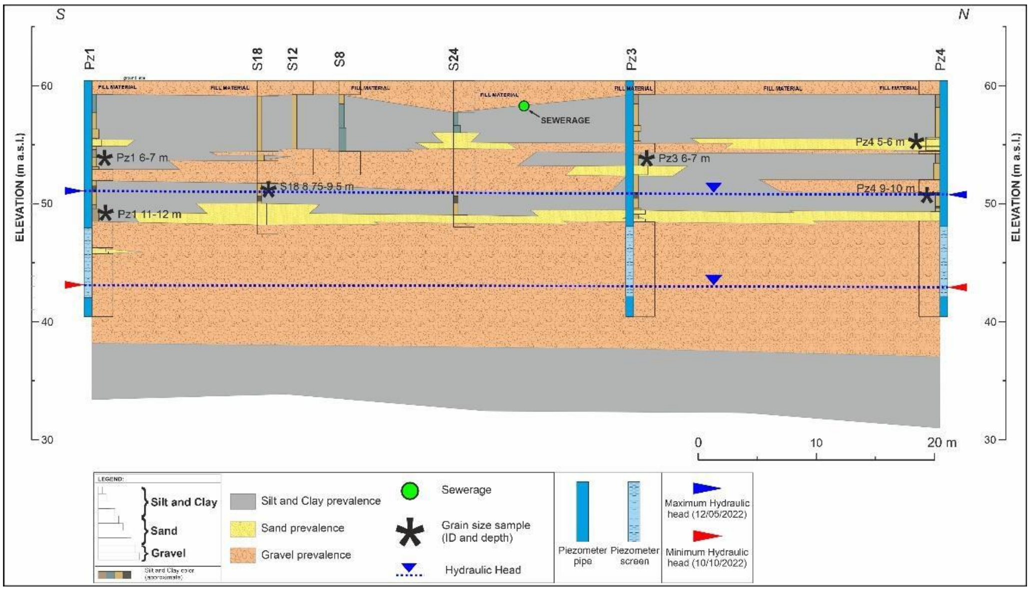

Figure 4. Geological section at site scale (the trace is shown in Figure 3).

Four of the boreholes (Pz1 to Pz4 in Figure 3) were equipped with 20 m deep piezometers screened from 12.60 to 18.90 m below ground. Hydraulic head measurements were carried out in all piezometers with a water level meter from March to October 2022, monthly. The daily rainfall data to be compared to hydraulic head fluctuations were acquired by the Regional Agency for Prevention, Environment, and Energy of Emilia-Romagna (Arpae). The rainfall monitoring station is located in Parma (Figure 1).

Contextually, physico-chemical parameters such as temperature, pH, electrical conductivity (EC), redox potential, and total dissolved solids (TDS) were measured in situ with the multiparameter HANNA probe (mod. HI9828, HANNA Instruments, Villafranca Padovana, Italy).

2.2. Water Sampling and Analyses

Water-sampling activities related to chemical–physical parameters and piezometric monitoring were carried out monthly (from February 2022 to October 2022) on the four piezometers drilled within the test site (Pz1, Pz2, Pz3 and Pz4). In parallel to these sampling activities, microbiological monitoring of fecal indicators was carried out monthly in Pz3, which is the piezometer drilled closest to the sewer system, within the test site (Figure 3). In the same piezometer (Pz3), chemical (PCPs) and biomolecular (bacterial community) analyses were also carried out only once, in June 2022, and the same analyses were also performed in the incoming wastewater of the sewage treatment plant (Parma Ovest).

2.3. Chemical Analyses

Three substances commonly used in cosmetics were selected for the chemical analyses: (i) 2-phenoxyethanol, which is a bactericidal agent widely used in cosmetics as a preservative, (ii) dimethicone, which is the most widely used silicone in cosmetics and is not subject to European regulations, (iii) disodium EDTA, which is a chelating agent: it reacts and forms complexes with metal ions that can affect the stability and/or appearance of cosmetic products; the Organization for Economic Cooperation and Development (OECD) classifies disodium EDTA as a remarkably persistent and not biodegradable substance in the environment.

Three groundwater samples were collected in Pz3 to analyze the content of 2-phenoxyethanol (CAS number: 122-99-6), dimethicone, and disodium EDTA (CAS number: 139-33-3); 50 milliliter (mL), 1 liter (1L), and 250 milliliter (mL) ambered glass bottles were used for 2-phenoxyethanol, dimethicone, and disodium EDTA analyses, respectively. In addition, three samples were collected at the entrance of the "Parma Ovest" wastewater treatment plant to verify if wastewater, which flows into the pipes close to Pz3, was characterized by the presence of these three contaminants.

All samples were stored in a refrigerated box and transported to the laboratory. The analyses were performed at Analytice s.a.r.l. (société à responsabilité limitée), a laboratory with ISO 17025 accreditation recognized by the French Accreditation Committee (ILAC full members). In particular, GC/MS was performed to analyze 2-phenoxyethanol. The samples were analyzed according to an accredited in-house method. After the addition of potassium carbonate, the samples were extracted with ethanol. The extracts were then injected into gas chromatography (column RTX-WAX) coupled to a mass spectrometer detector (GC-MS). GC/MS after derivatization was also used to quantify disodium EDTA. Samples were analyzed versus EDTA, and values were then calculated into disodium EDTA. The samples were analyzed according to EN ISO 16588. The samples were derivatized with isopropanol/acetylchlorid and extracted with hexane. The extracts were then injected into gas chromatography (column DB-XLB) coupled to a mass spectrometer detector (GC-MS). Dimethicone was analyzed according to an in-house method. First, the samples were extracted with hexane. Next, the extracts were injected into high-pressure liquid chromatography coupled to a refractive index detector (HPLC-RI). Samples were determined as PDMS (CAS 63148-62-9) and calculated as polydimethylsiloxane with a viscosity of 1000 mPa.s with HPLC/RI after hexane extraction.

2.4. Next-Generation Sequencing (NGS) for Bacterial Community Analyses

NGS (next-generation sequencing) analyses were carried out to verify whether (i) the bacterial community in groundwater is characterized by pathogenic bacteria and/or human-associated bacteria markers and/or bacteria potentially capable of degrading PCPs (or xenobiotics in general) and (ii) the bacterial community in wastewaters is characterized by microorganisms potentially capable of enhancing the corrosion of pipelines. More generally, microbial community characterization in heterogeneous and complex hydrogeological systems will be used as an effective natural tracer to refine knowledge about (i) hydrodynamic and hydrochemical processes, as well as (ii) recharge and/or contaminant origin [35,45–47].

The bacterial community characterization was carried out in Pz3 groundwater and in wastewater samples collected at the entrance of the "Parma Ovest" treatment plant (IN sample). Both ground- and wastewater samples (2 L) were collected in sterile bottles. All samples were stored in a refrigerated box and transported to the laboratory. The samples were filtered through sterile mixed esters of cellulose filters (S-PakTM Membrane Filters, 47 mm diameter, 0.22 μm pore size, Millipore Corporation, Billerica, MA, USA) within 24 h of collection. Bacterial DNA extraction from filters and soils was performed using the commercial kit FastDNA SPIN Kit for soil (MP Biomedicals, LLC, Solon, OH, USA) and FastPrep® (MP Biomedicals, LLC, Solon, OH, USA). After the extraction, DNA integrity and quantity were evaluated by electrophoresis in 0.8% agarose gel containing 1μg/mL of Gel-RedTM (Biotium, Inc., Fremont, CA, USA). The bacterial community profiles in the samples were generated by next-generation sequencing (NGS) technologies at the Genprobio Srl Laboratory. Partial 16S rRNA gene sequences were obtained from the extracted DNA by polymerase chain reaction (PCR) using the primer pair Probio_Uni and Probio_Rev, targeting the V3 region of the bacterial 16S rRNA gene [48]. Amplifications were carried out using a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA), and PCR products were purified by the magnetic purification step involving Agencourt AMPure

XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) to remove primer dimers. Amplicon checks were carried out as previously described [47]. Sequencing was performed using an Illumina MiSeq sequencer (Illumina, Hayward, CA, USA) with MiSeq Reagent Kit v3 chemicals. The fastq files were processed using a custom script based on the QIIME software suite [49]. Paired-end read pairs were assembled to reconstruct the complete Probio_Uni/Probio_Rev amplicons. Quality control retained sequences with a length between 140 and 400 bp and a mean sequence quality score >20, while sequences with homopolymers >7 bp and mismatched primers were omitted. To calculate downstream diversity measures, operational taxonomic units (OTUs) were defined at 100% sequence homology using DADA2 [50]; OTUs not encompassing at least two sequences of the same sample were removed. All reads were classified to the lowest possible taxonomic rank using QIIME2 [49,51] and a reference dataset from the SILVA database v132 [52]. The biodiversity of the samples (alpha diversity) was calculated with the Shannon index. Similarities between samples (beta diversity) were calculated by weighted uniFrac. The range of similarities is calculated between values 0 and 1. Principal coordinate analysis (PCoA) representations of beta diversity were performed using QIIME2. In the PCoA, each dot represents a sample distributed in tridimensional space according to its bacterial composition.

2.5. Microbiological Investigations (Fecal Indicators)

For microbiological analyses (fecal indicators), groundwater samples (1 L) were collected in sterile bottles. All samples were stored in a refrigerated box and transported to the laboratory. Filtration processes were carried out within 2 h after collection. Indicators of microbial contamination were determined in groundwater samples using classic methods of water filtration (1000 mL) on sterile membrane filters (GN-6 Metricel, pore size 0.45 µm, Pall), with incubation on: (1) m-FC Agar for 24 h at 44 °C, for fecal coliforms and (2) Slanetz–Bartley agar for 48 h at 37 °C for intestinal enterococci.

3. Results

3.1. Geological, Hydrogeological, and Hydrochemical Investigations

The shallowest part of the sedimentary succession shows alternating coarse-grained layers, composed of gravel and sand, and fine-grained layers, such as silts and clays (Figure 4). In addition, a cover of anthropogenic fill material consisting of gravel and bricks was found over the entire area approximately 1 meter below ground. Below the fill material, the geological setting at site scale can be summarized as follows: (i) a first layer composed of silt and clay with thickness varying from 5 to 7 meters, (ii) a second layer composed of gravel and sand varying in thickness from 1 to 5 meters and characterized by a separation into two distinct elements proceeding northward in the proximity of Pz3, (iii) a third layer composed of silt and clay with thickness varying from 2 to 3 meters, and (iv) a fourth layer of gravel and sand (the shallowest alluvial aquifer at the test site).

Fine strata can be interpreted as the stratigraphic record of periods (or areas) of low-energy depositional systems, and for this reason, evidence of paleosols has been found diffusely in all investigations. In contrast, coarser erosive levels may be associated with periods of higher river energy or paleochannel areas.

Laser particle analysis conducted on six selected samples, collected at different depths, allowed to constrain the grain size distribution of the two silt-dominated layers characterizing the study area. The coarse-grained gravel bodies in the middle and at the bottom of the stratigraphic interval were not considered for grain size analysis because of the instrumental upper limit of 3.5 mm of the laser granulometer. Altogether, the average grain size varies from 16.2 to 62.4 µm, with the modal peak shifting from 7.2 to 78.6 µm (Table 1). Silt is the dominant fraction of all the analyzed samples, with a volume percentage spanning from 56.6 to 71%, with the sand percentage falling between 7.2 and 37.3% and clay in the 5.9–21.7% range. Samples collected along Pz1 and Pz3 boreholes display a significantly lower sandy and higher clayish fractions than Pz4 and S18. The shape of the granulometric distribution is highly asymmetric (left-skewed) for all samples but is particularly pronounced in samples characterized by relatively high sand contents (Pz4 and S18).

Table 1. Summary of laser diffraction grain size analysis (± values refer to standard deviation).

Sample Name	Mean Diameter (µm)	Mode (µm)	Span	Clay (%)	Silt (%)	Sand (%)
PZ1 6–7 m	31.99 ± 1.97	28.32 ± 14.03	5.44 ± 0.16	18.09	64.9	17.01
PZ1 11–12 m	20.81 ± 0.79	8.62 ± 0.04	4.73 ± 0.1	21.7	71.04	7.26
PZ3 6–7 m	16.18 ± 0.61	7.18 ± 0.02	4.82 ± 0.13	28.1	67.08	4.82
PZ4 5–6 m	57.06 ± 2.05	75.2 ± 4.15	2.69 ± 0.05	5.97	56.67	37.36
PZ4 9–10 m	48.68 ± 2.74	57.51 ± 5.55	3.97 ± 0.07	10.46	60.69	28.85
S18 8.75–9.5 m	62.43 ± 3.91	78.62 ± 5.22	2.83 ± 0.03	5.98	54.32	39.7

Conversely, clayey silt samples show less asymmetric distributions with platykurtic shape (Pz1 and Pz3). The span (sorting degree) is higher in samples showing a higher clay fraction (wider grain size distributions), while it is lower in sandy silts (narrower and more leptokurtic grain size distributions) (Table 1). Figure 5 shows the comparison of the average grain size distribution curves. More details regarding the grain size distribution curves are provided in the Supplementary Materials (SM.pdf; Figure S1).

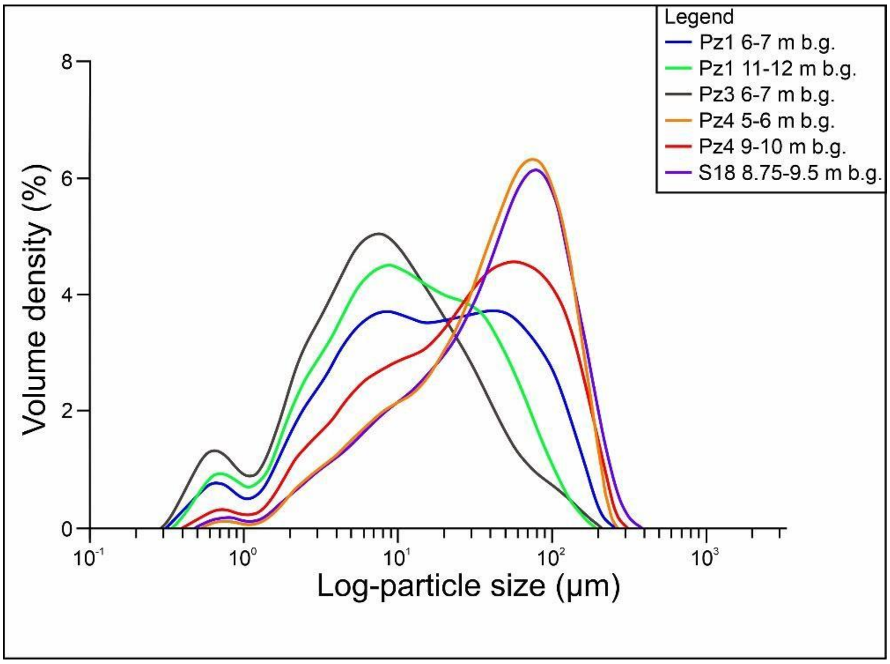

Figure 5. Comparison of the six average grain-size distribution curves.

During the observation period, the hydraulic head varied from 43 to 51 m a.s.l. (Figure 6). The recession period started in June, with negligible variations in the head during the precipitations observed in summer and autumn. The only effect of rainfall on groundwater recharge during the recession was observed from September to October 2022, when a slight recharge caused the decrease in head to slow down (as testified by analyzing the recession phase in a classic semi-logarithmic plot). Differently, precipitations in winter caused the hydraulic head to rise, therefore suggesting a significant and relatively rapid recharge, in agreement with previous findings obtained a few kilometers upgradient regarding the test site [36].

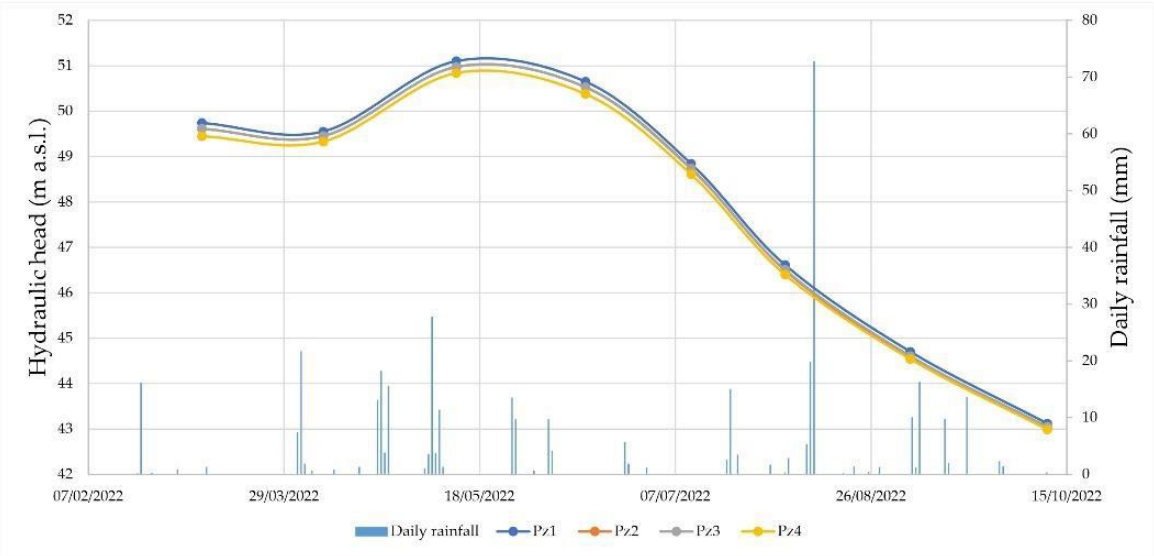

Figure 6. Hydraulic head fluctuations vs. daily rainfall (dates are given in day/month/year).

Significant modifications clearly characterize the groundwater flow net in the shallowest aquifer over time at the test site scale, therefore suggesting significant variations in terms of groundwater flow directions (and contaminant dispersion) at the site scale during the hydrologic year (see examples in Figure 7).

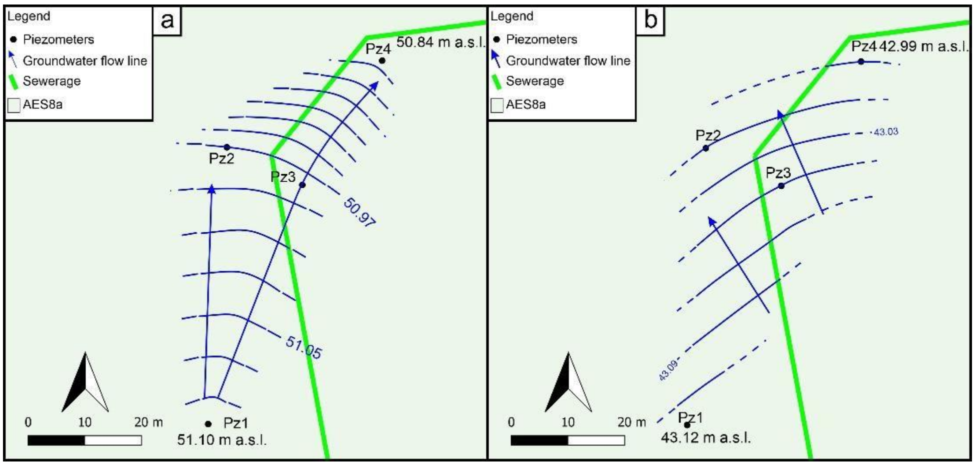

Figure 7. (**a**) Groundwater flow net in May 2022; (**b**) groundwater flow net in October 2022.

The groundwater EC in Pz3 varied significantly over the research period, from 363 to 764 μS/cm. This variation can be due to different factors. Overall, the highest EC values were measured in high flow, with a general decrease during the recession (Figure 8). However, a sporadic increase in EC was observed at the beginning of the recession period (June 2022) and at the end of the same phase (October 2022). At the end of the recession period, the increase in EC can be correlated to the slight recharge documented through the analysis of the hydraulic head hydrograph, therefore confirming an overall direct relationship between groundwater EC and head. This relationship is not surprising in a semi-confined aquifer system, where the effective local infiltration of rainfall is expected to be negligible and very slow, and the head rise is mainly due to recharge in the upgradient and unconfined alluvial aquifer. Therefore, contrary to observations made in unconfined aquifers [53–56], this recharge cannot generate temporary or relatively prolonged haloclines within the local groundwater, usually characterized by the lowest EC values into the shallowest saturated zone. In confined and semi-confined aquifers, the recharge (and the resulting hydraulic head rise) can cause the mobilization of higher-EC groundwaters flowing in those portions of the heterogeneous systems where (i) higher mean residence time, and/or (ii) higher water-rock interactions, and/or (iii) higher mineral dissolution can be observed. At the site scale, a possible additional factor influencing the variations over time in terms of groundwater EC at the observation point (Pz3) is the variation of the groundwater flow net mentioned above. This factor could be linked, at the study site, to surface–groundwater interactions [37] because the variations in terms of stream heads and groundwater flow directions can modify the local impact of stream recharge on groundwater, as well as in terms of physico-chemical features.

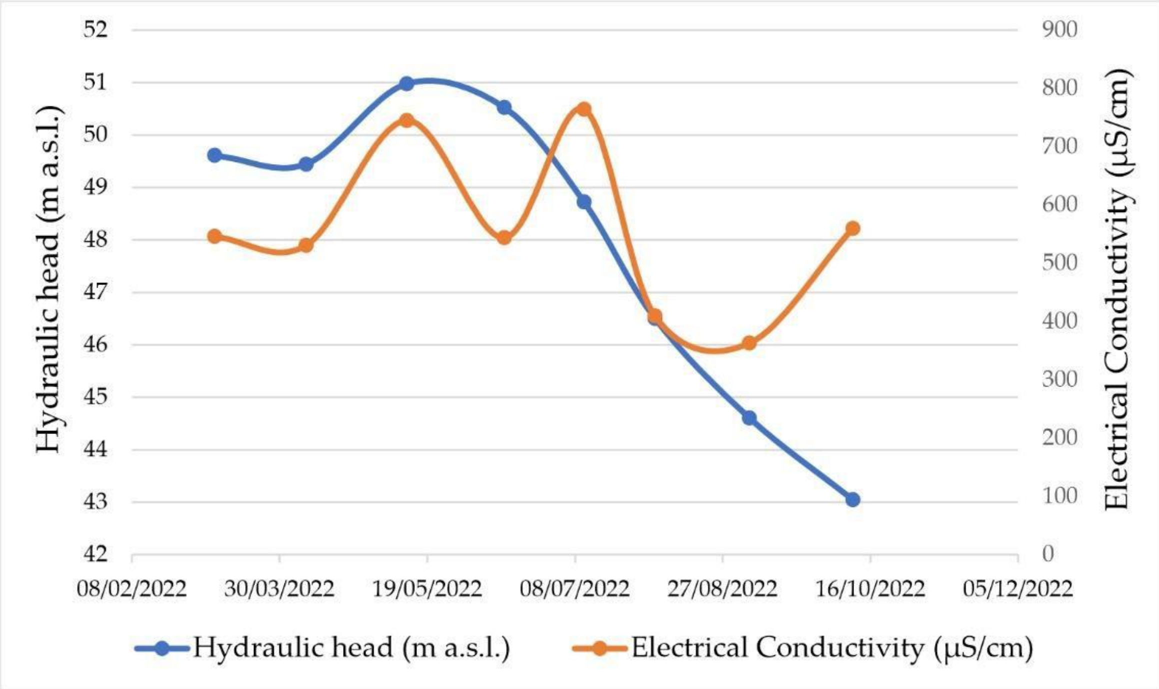

Figure 8. Hydraulic head fluctuations vs. groundwater EC in Pz3 (dates are given in day/month/year).

The groundwater temperature in Pz3 groundwater varied between 13.7 °C and 16.1 °C. Overall, the groundwater temperature variation over the observation period seems to be correlated to the groundwater EC (Figure 9), suggesting that similar factors mainly influence both parameters. The groundwater temperature at the test site is not significantly influenced by the atmospheric one. In the atmosphere, the highest temperature was recorded during summer, from late July to early August 2022, while the highest groundwater temperature was measured at the end of the recharge (May 2022).

Dissolved oxygen in groundwater is around 60%, pH ranged from 6.9 to 7.8, and redox potential between −84 and 330 mV, with the highest values detected during recharge and the negative one, detected only in October 2022, in late recession.

As for PCPs, only disodium EDTA was detected in wastewater, while in groundwater, the concentrations of all the analyzed substances were lower than the instrumental detection limit (Table 2).

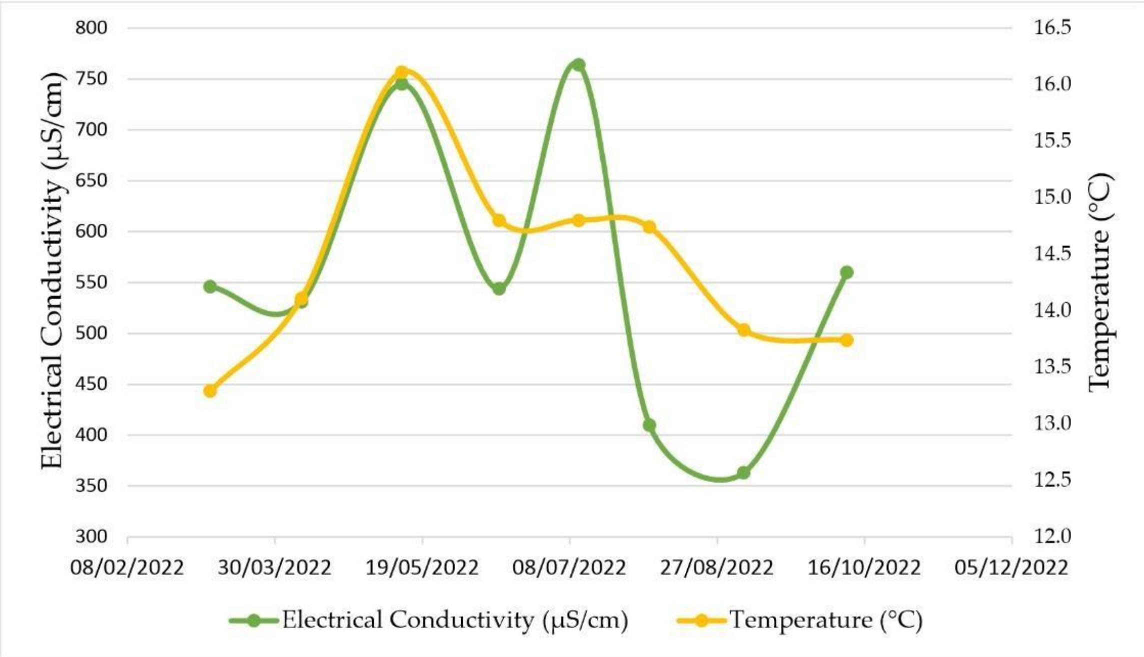

Figure 9. Groundwater EC vs. groundwater temperature in Pz3 (dates are given in day/month/year).

Table 2. Results of PCP analyses.

Sample	Parameter (CAS)	Technique	Analytical Method	Detection Limit	Results	Unit
IN wastewater	2-Phenoxyethanol (122-99-6)	GC-MS	In-house	0.2	<0.2	mg/L
	Disodium EDTA (139-33-3)	GC-MS	ISO 16588	1	**53**	µg/L
	PDMS calculated as polydimethylsiloxane with a viscosity of 1000 mPa.s (63148-62-9)	HPLC-RI	In-house	100	<100	mg/L
Pz3 groundwater	2-Phenoxyethanol (122-99-6)	GC-MS	In-house	0.2	<0.2	mg/L
	Disodium EDTA (139-33-3)	GC-MS	ISO 16588	1	<1	µg/L
	PDMS calculated as polydimethylsiloxane with a viscosity of 1000 mPa.s (63148-62-9)	HPLC-RI	In-house	100	<100	mg/L

3.2. Microbiological and Biomolecular Investigations

The isolation and counting of fecal coliforms and enterococci made it possible to ascertain that continuous and constant leaks from the local sewers compromised the microbial quality of groundwater. The monthly monitoring results show that Pz3 groundwater is always characterized by fecal contamination (Table S1), even though intestinal enterococci were an indicator more reliable than fecal coliforms, in agreement with findings in other hydrogeological settings [57–59]. As a matter of fact, the concentration of fecal indicators in Pz3 groundwater ranged from 0 to 303 colony-forming units (CFU)/L of fecal coliforms and from 10 to 98 CFU/L of intestinal enterococci.

Concerning the results of biomolecular investigations, all the 16S rRNA gene sequences obtained within this study have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under the accession number PRJNA897727.

Proteobacteria, *Actinobacteria, and Bacteroidetes* represented the three major phyla in the groundwater sample (Pz3), accounting for, on average, 64%, 16%, and 12%, respectively (Figure S2). Differently, in wastewaters, a dominance of *Epsilonbacteraeota*, *Proteobacteria,* and *Firmicutes* was observed, accounting for 37%, 3%, and 13%, respectively (Figure S2).

The analysis of bacterial community composition at the family level (Figure S3) revealed a predominance of *Burkholderiaceae* (20%), *Sphingomonadaceae* (18%), *Microbacteriaceae* (4%), *Solimonadaceae* (4%), and *Spirosomaceae* (4%) in Pz3 groundwater, while in wastewaters, high percentages of *Arcobacteraceae* (37%), *Pseudomonadaceae* (11%), *Burkholderiaceae* (8%), *Moraxellaceae* (4%), and *Aeromonadaceae* (3%) were detected.

When analyzing the groundwater microbial communities at the genus level (Figure S4), it emerged that *Novosphingobium* reached the highest percentage in the Pz3 groundwater (14%). Among the most abundant genus, *Limnohabitans* (5%) *Sphingomonas* (4%), and *Massilia* (3%) were also detected. Overall, NGS results showed that the bacterial community consists of genera belonging to aerobic, facultatively anaerobic, chemo-organotrophic, and oxidase bacteria [60–63]. The genus *Novosphingobium* accommodates Gram-negative, aerobic, nonsporulating, chemoorganotrophic, and rod-shaped bacteria that are metabolically versatile [60,64] and are found in a wide range of ecological habitats, such as agricultural soil [65], pesticide-contaminated soil [66,67], plant surfaces [68], and aquatic environments [69]. In addition, different species of *Novosphingobium* appear to have the potential to degrade different xenobiotics and recalcitrant compounds, such as, for example, polycyclic aromatic hydrocarbons [70–73] and sulfanilic acid, widely used in PPCPs [74]. *Sphingomonas* are opportunistic pathogens that take advantage of underlying conditions and diseases for humans and can generate infections, including bacteremia/septicemia [75].

On the other hand, wastewaters were characterized by a bacterial community characterized by Gram-negative, aerobic, facultatively anaerobic ubiquitous, and pathogen genus [76–79], where the main genera are *Arcobacter* (37%), *Pseudomonas* (11%), *Bacteroides* (3%), and *Acinetobacter* (3%). *Arcobacter* has emerged as an important food-borne zoonotic pathogen, causing sometimes severe infections in humans and animals [80]. At the same time, associated with human infections are also the *Acinetobacter (3%)*, *Streptococcus* (2%), and *Comamonas* (2%) genera [81–83]. In addition to this, the *Streptomyces* genus has also been detected in wastewaters (2%), known for having steel's corrosive capacities [84].

4. Discussion

Sewer pipeline ruptures are a severe risk to groundwater quality. When sewerage deterioration conditions occur, aquifers can be contaminated by contaminants contained within sewer water. The exposure to the risk of wastewater contamination in the study area is also increased by the presence of bacteria with corrosive capacities that were detected in wastewaters. Previous studies show how much biological corrosion of sewers and sewage treatment plants constitutes a severe problem, resulting in the loss of billions of dollars a year [85].

In this specific case, detailed granulometric analyses made it possible to understand that the outcropping aquitard consists of clayey/sandy silts. Therefore, this is characterized by a not-negligible permeability that makes the aquifer vulnerable to percolations from damaged pipelines. As a result, mechanical filtration is not entirely effective, and microbial contamination (including pathogens) of groundwater can also occur.

The microbiological quality of water is generally assessed by monitoring fecal indicator bacteria (e.g., intestinal enterococci and coliforms). However, it must be noted that many alternative parameters exist [86]. It has been proposed that the members of the *Bacteroides* genus hold promise as alternative indicators of fecal pollution [87] owing to several advantages, including short survival rates outside the hosts, exclusivity to the gut of warm-blooded animals, and constituents of a larger portion of fecal bacteria compared with fecal coliforms or enterococci [88]. In this regard, both *Bacteroidetes bacterium OLB11* and *Faecalibacterium* were detected in Pz3. Members of *Faecalibacterium* are commensal bacteria, ubiquitous in the gastrointestinal tracts of animals and humans [89].

Although at low concentrations, the presence of these bacteria further demonstrates the negative impact of sewers' loss on groundwater microbial quality (and human health). The results obtained at the study site further confirmed that intestinal enterococci are better indicators than fecal coliforms to detect microbial contamination of groundwater. As a matter of fact, in groundwater, intestinal enterococci generally persist for more extended periods, rarely multiply, and are more resistant to different environmental stresses [46]. Furthermore, enterococci were always detected during the monthly monitoring, with a narrower range of contamination than coliforms. In particular, Figure 10 shows the variation of microbial contamination indicators (C.F.U./L) over time as a function of the variation of the hydraulic head (m a.s.l.) in Pz3.

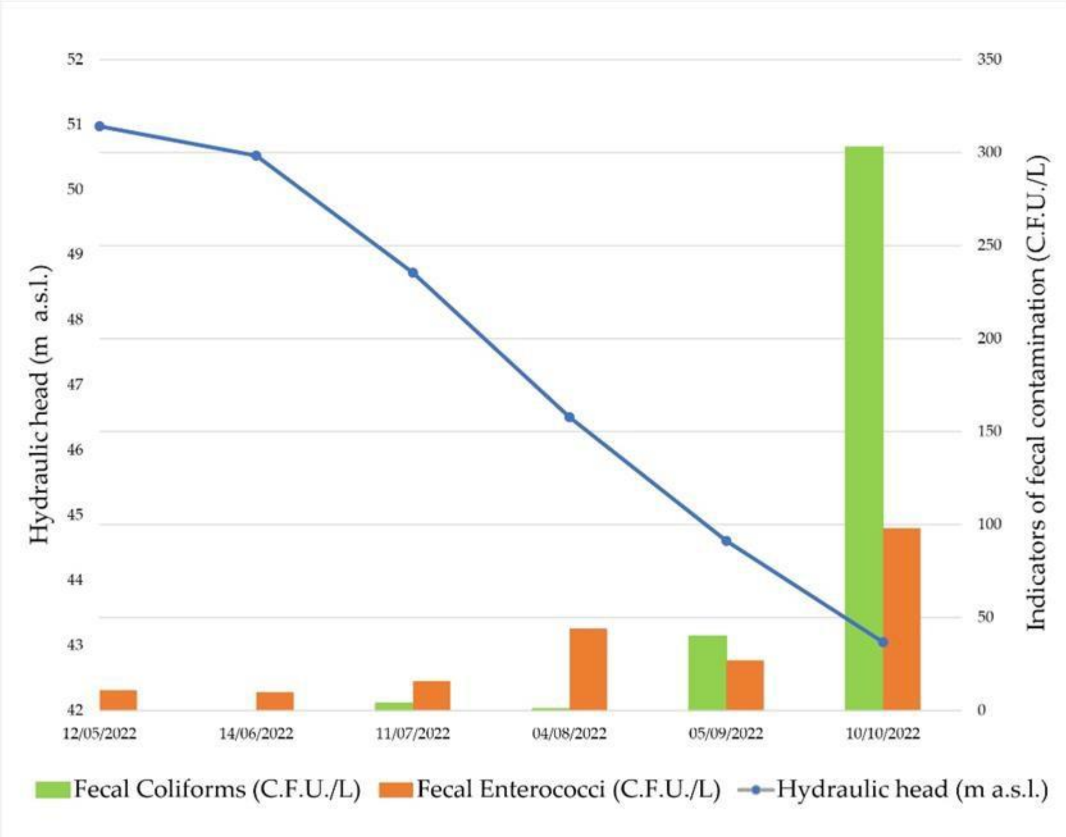

Figure 10. Indicators of fecal contamination (C.F.U./L) in the function of the hydraulic head variation in Pz3 (dates are given in day/month/year).

Both fecal indicators show a similar trend. Observing intestinal enterococci, a progressive increase in concentrations was found following the progressive lowering of the hydraulic head during the recession period. This overall inverse relationship suggests a significant reduction in the dilution in groundwater. In fact, during the depletion period, the saturated medium is progressively and significantly thinned (Figure 4); consequently, the contaminants turn out to be more concentrated than in high flow.

However, when analyzing the same graph in a shorter timescale, short-term variations in fecal indicators are observed (on a monthly basis, from July to September 2022). This variation is probably due to the variations in terms of groundwater pathway and to the effect of these variations on contaminant dispersion within the saturated medium. As conceptually described in Figure 11, where a segment of the sewer was hypothesized to be damaged (segment in red), the modification of the groundwater flow direction over time can cause the contamination plume to oscillate between two end positions (full and dashed red plumes in Figure 11), therefore emphasizing the transverse mechanical dispersion and the impact of the same sewer segment on Pz3.

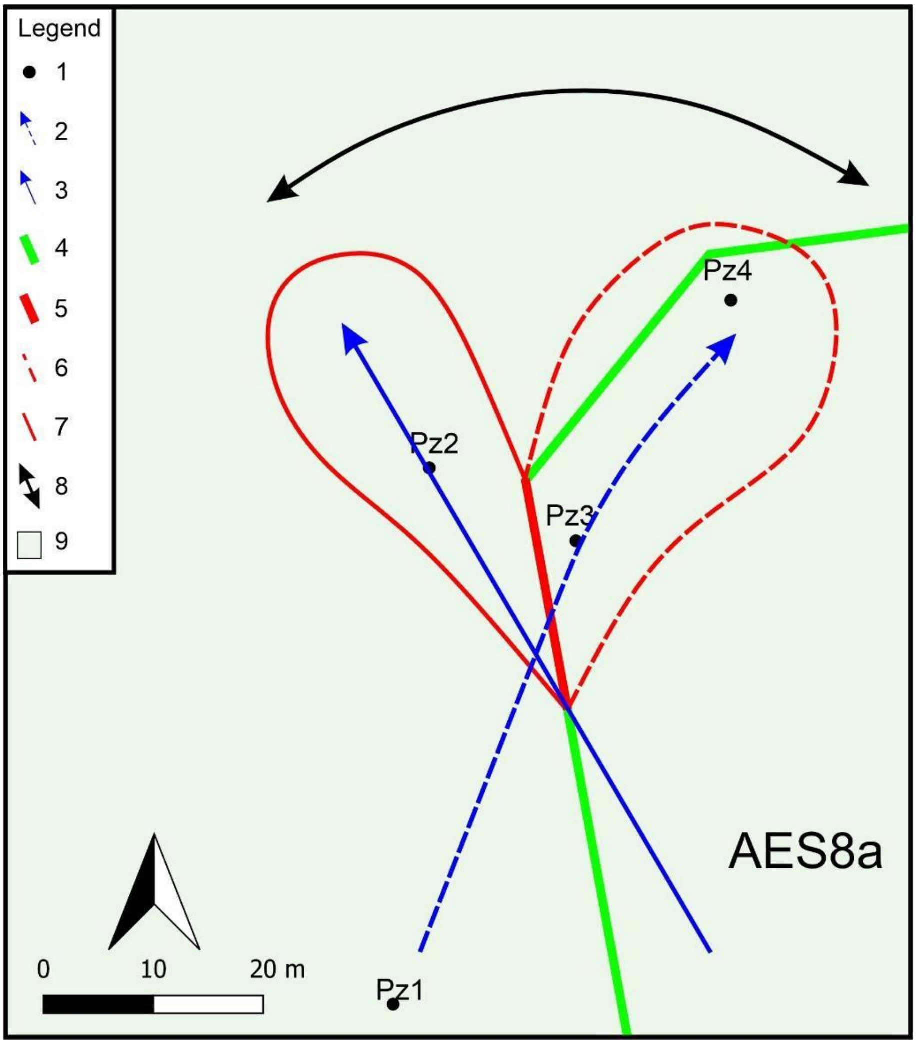

Figure 11. Conceptual oscillation of a contamination plume over time that is due to variation in the groundwater flow direction: (1) piezometer; (2) groundwater flow direction in May 2022; (3) groundwater flow direction in October 2022; (4) sewerage; (5) conceptual leaks; (6) conceptual plume theoretically linked to the groundwater flow direction in May 2022; (7) conceptual plume theoretically linked to the groundwater flow direction in October 2022; (8) variation of groundwater flow direction over time; (9) outcropping geological unit (AES8a).

As for PCPs, to the authors' knowledge, it is the first time that 2-phenoxyethanol, disodium EDTA, and dimethicone have been analyzed in groundwater. These first results testify that groundwater is not affected by cosmetic pollution, although the presence of disodium-EDTA has been detected in wastewaters, and at the same time, the impact of sewerage leaks has been ascertained from the microbiological point of view. Therefore, it is possible to deduce that cosmetics have not been detected in the aquifer because of hydrodynamic dispersion, as illustrated above for fecal indicators and/or biodegradation.

Concerning microbial communities with biodegradative capacity against substances used for PCP production, data in the literature do not provide detailed indications. However, the biomolecular analyses in this study showed the presence of bacteria able to degrade xenobiotics and recalcitrant compounds in groundwater and wastewater. It would be interesting to check whether these microorganisms, having recalcitrant-substance degrading capabilities, may also be able to degrade recalcitrant chemicals contained in cosmetics, such as disodium EDTA.

Merkova et al. (2016) proved that the biodegradation of CAPB (cocamidopropyl betaine), an amphiphilic surfactant commonly used in various personal care products, is ensured by two Gram-negative bacteria of the widespread genera *Pseudomonas* and *Rhizobium* [90]. The present study is one of the few ones to date that has investigated the presence of bacteria potentially capable of biodegrading this class of substances. Both the *Pseudomonas* and *Rhizobium* genera were detected in both waste- and groundwater samples, accounting for about 6% and 1%, respectively.

Moreover, the presence of oxidizing bacteria detected in groundwater and wastewater facilitates the triggering of Fenton reactions, one of the most commonly used advanced oxidation processes (AOPs) [91], which was efficient at eliminating illicit drugs and pharmaceuticals from wastewaters [92]. Nevertheless, as discussed before, during the hydrological year, positive and negative redox potential alternation occurs in groundwater. Therefore, some biodegradative processes can temporarily inhibit, and the natural attenuation of PCPs could be discontinuously allowed.

Through OECD guidelines, EDTA is considered persistent and nonbiodegradable. The OECD Guidelines for the Testing of Chemicals are a unique tool for assessing the potential effects of chemicals on human health and the environment. Accepted internationally as standard methods for safety testing, the guidelines are used by industry, academic, and government professionals involved in the testing and assessment of chemicals (industrial chemicals, pesticides, personal care products, etc.). However, OECD tests are conducted on a deductive basis and not through laboratory tests that simulate specific hydrogeological settings. Therefore, it would be appropriate to verify whether and how much these compounds are biodegradable through purpose-designed degradation tests. This would make it possible to verify if EDTA is somehow biodegradable or recalcitrant in groundwater environments. If, as reported by the OECD tests, EDTA turns out to be persistent even from simulations test, then it could be inferred that the lack of EDTA detection in groundwater is the result of the only hydrodynamic dispersion processes that make the concentrations of the substance lower than the instrumental detection limits now available.

5. Conclusions

The multidisciplinary approach used in this study allowed the demonstration that leaky sewers can cause significant microbial pollution of groundwater in semi-confined aquifer systems, whose semi-confining aquitards are mainly made of silts and characterized by granulometric and hydraulic layered heterogeneity. At the same time, this study pointed out that (i) the impact of leaky sewers on groundwater microbial quality may vary over time, depending on both the hydraulic regime and the changing groundwater pathway, and (ii) intestinal enterococci are indicators more reliable than fecal coliforms to detect microbial contamination in such hydrogeological and urban settings.

As for PCPs, no negative impacts were observed on groundwater at the test site, despite the presence of detectable EDTA in wastewater. However, at this stage, there is not enough information to adequately discriminate among the possible causes (e.g., hydrodinamic dispersion, biodegradation, too high instrumental detection limits, etc.) of these results. At the same time, the same results allow the designing of effective supplementary investigations for the near future.

On a wider and methodological perspective, this research demonstrates that (i) multidisciplinary approaches are the most effective way to study the possible impacts of leaky sewers in semi-confined urban aquifers, and (ii) a prolonged monitoring of both hydraulic heads and qualitative groundwater features is fundamental to correctly analyze the causes and the possible variations over time of contamination phenomena induced by leaky sewers.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/hydrology10010003/s1: Test: SM.pdf; Figures: Figure S1 Grain size distributions with an average curve in black and variability range in light gray. Grain size distribution curves for PZ1 6–7 m (A), PZ1 11–12 m (B), PZ3 6–7 m (C), PZ 5–6 m (D), PZ4 9–10 m (E) and S18 8.75–9.5 m (F). Φ, equivalent mean diameter; m, modal value of the grain size distribution; S, span (sorting) of grain size distribution; n, number of measurements; Figure S2 Phylum level microbial community composition in samples collected from groundwater and wastewater; Figure S3 Family level microbial community composition in samples collected from groundwater and wastewater; Figure S4 Genus level microbial community composition in samples collected from groundwater and wastewater. Table: Table S1 Monthly monitoring of fecal contamination indicators. Reference [93] is cited in the supplementary materials.

Author Contributions: Conceptualization, P.R. and F.C.; methodology, L.D., P.R., F.B. and F.C.; software, L.D., P.R., R.P. and M.P.; validation, L.D.; formal analysis, L.D., P.R., R.P. and M.P.; investigation L.D., P.R., R.P., A.S., A.M. and M.P.; resources, F.C.; data curation, L.D., P.R., R.P. and M.P.; writing—original draft preparation, L.D., P.R., R.P. and M.P.; writing—review and editing, L.D., P.R. and F.C.; visualization, L.D., P.R., R.P. and M.P.; supervision, P.R., F.B. and F.C.; project administration, F.C.; funding acquisition, F.C. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are all the available data; biomolecular data (fastq format) are available at the following link: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA897727/ (accessed on 14 November 2022).

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Conflicts of Interest: There are no conflicts of interest related to this paper.

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The Challenge Posed by Emerging Environmental Contaminants: An Assessment of the Effectiveness of Phenoxyethanol Biological Removal from Groundwater through Mesocosm Experiments

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Abstract: The occurrence of emerging pollutants (EPs) such as pharmaceuticals and personal care products (PPCPs) has raised serious concerns about the possible adverse effects on ecosystem integrity and human health. Wastewater treatment facilities appear to be the main sources of PPCPs released in aquatic environments. This research examines the effectiveness of groundwater microbial community activities to remove phenoxyethanol (Phy-Et), currently exploited as a preservative in many cosmetic formulations at a maximum concentration of 1% but which has shown, at higher levels of exposure, adverse systemic effects on animals. Mesocosm experiments were carried out for 28 days using two different concentrations of the substance (5.2 mg/L and 27.4 mg/L). The main results obtained through chemical and microbiological investigations revealed a significant Phy-Et reduction (≈100% when added at a concentration of 5.2 mg/L and ≈84% when added at a concentration of 27.4 mg/L), demonstrating that some autochthonous microorganisms in the analyzed samples played a "key role" in removing this compound, despite its proven antimicrobial activity. Nevertheless, the decrease in the "natural attenuation" efficacy (≈16%) when using higher concentrations of the chemical suggests the existence of a "dose-dependent effect" of Phy-Et on the process of biodegradation. Biomolecular investigations carried out through next-generation sequencing (NGS) revealed (i) the presence of a significant fraction of hidden microbial diversity to unravel, (ii) variations of the composition and species abundance of the groundwater microbial communities induced by Phy-Et, and (iii) a biodiversity reduction trend correlated to the increase of Phy-Et concentrations. Overall, the preliminary information obtained from the experiments carried out at the laboratory scale appears encouraging, although it reflects only partially the complexity of the phenomena that occur in natural environments and influences their "auto-purification capability". Accordingly, this research paves the way for more in-depth investigations to develop appropriate tools and protocols to evaluate the occurrence and fate of Phy-Et in nature and assess the impact of its release and the effects of long-term exposure (even at low concentrations) on ecosystems and health.

Keywords: emerging environmental contaminants; phenoxyethanol; mesocosms; natural attenuation; groundwater microbial communities

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1. Introduction

Environmental pollution threatens human and planetary health, jeopardizing modern societies' sustainability [1]. In 2015, diseases attributable to pollution caused 9 million premature deaths, accounting for 16% of all deaths worldwide, a number three times higher compared to deaths for AIDS, tuberculosis, and malaria combined and 15 times higher compared to deaths for all wars and other forms of violence [2]. Unfortunately, estimates updated to 2019 still confirmed this number of deaths per year [1]. Overall, ambient air pollution and toxic chemical pollution, which are consequences of industrialization and urbanization, have caused many deaths, rising by 7% since 2015 and over 66% since 2000 [1]. Among the various categories of pollutants, there has been a growing interest in the so-called emerging pollutants (EPs) in the last decades. This category includes all the substances that could be pollutants that are not currently covered by controls but that could be covered in the future; this will depend on future ecotoxicity studies and concentrations found [3]. More than 700 compounds grouped in 20 classes of EPs are present in the NORMAN database (www.norman-network.net) [4] and include surfactants, antibiotics and other pharmaceuticals, steroid hormones and other endocrine-disrupting compounds (EDCs), fire retardants, sunscreens, disinfection by-products, new pesticides and pesticide metabolites, naturally occurring algal toxins, etc. [5,6]. In addition, another group of pollutants denoted as "contaminants of emerging concern" (CECs) chemical substances used for long periods in various anthropic sectors and released into the environment in the absence of control, and by-products of these, which are now detected by analysis of soils, surface waters, and groundwater. The term "contaminants of emerging concern" is usually used when very little information is available about the magnitude and frequency of risks posed by this category of substances in the environment and human health [7–9].

Contamination caused by urbanization, fast population growth, agricultural activities, and industrial development has drastically affected water resource quality [10]. The discovery of numerous new compounds in drinking, ground, and surface water has alarmed the public, mainly when human health-based guidelines are unavailable [11,12]. One of the largest sources of EPs is represented by wastewater treatment plants (WWTPs) [13] since wastewater treatments are not designed to treat these compounds and, thus, high amounts are released into the environment through effluents [14].

The main aim of this research was to investigate the effectiveness of biological removal of (Phy-Et) from groundwater through mesocosm experiments to assess the contribution of autochthonous microbial communities in the natural attenuation process of this chemical substance.

Phy-Et is an aromatic glycol ether that occurs naturally in green tea or can be produced in the laboratory due to its commercial importance [15] It is widely found in a large range of leave-on and rinse-off cosmetic products such as moisturizers, hand disinfectants, soaps, serums, sunscreen creams, mascaras, eyeliners, eye balms, and perfumes due to its pure chemical form, pleasant smell, and colorless appearance [16,17]. Other uses include shampoos, shaving creams, ultrasound gels, insect repellents, antiseptics, solvents, anesthetics, cellulose acetate solvents, dyes, and ink and ink manufacture [15]. This compound has a large spectrum of antimicrobial activity and is effective against Gram-negative and Gram-positive bacteria, such as *Escherichia coli* and *Staphylococcus aureus*, and yeasts, such as *Candida* [15,18,19], although it has only a weak inhibitory effect on resident skin flora [20].

The European Scientific Committee on Consumer Safety considers Phy-Et safe for all consumers (including children of all ages) when used as a preservative in cosmetic products up to a concentration of 1%. However, the French National Agency for the Safety of Medicines and Health Products (ANSM) advised against using Phy-Et as a preservative in cosmetic products intended for application to the nappy area of infants and children under three years [20]. Nevertheless, toxicological studies revealed adverse systemic effects in animals at levels of exposure many magnitudes higher (around 200-fold higher) than those to which consumers are exposed [20]. Accordingly, in light of the vast increase in personal care product consumption and their significant release in aquatic ecosystems, it is important to assess the fate of Phy-Et in the environment and the microorganisms' ability to degrade.

2. Materials and Methods

2.1. Study Area

Groundwater samples for mesocosm experiments have been collected from a piezometer (Pz3) located within the alluvial Parma aquifer (northern Italy; Figure 1). Parma is the second town by population of the Emilia Romagna region and represents a critical contamination source of surface waters and groundwater from leaks in the urban sewerage system [21]. The area is characterized by large industrial districts and intensive agriculture practices both in urban and suburban areas [21]. The piezometer Pz3 is very close to a pipeline of a wastewater treatment plant called "Parma Ovest", in whose waters has been ascertained a continuous and constant leak from the local sewers demonstrated by the detection of fecal coliforms and enterococci [21]. Accordingly, for assessing the biological removal of Phy-Et, groundwater microbial communities have most likely been shaped by a selective pressure caused by frequent contact with sewage and, thus, emerging environmental contaminants.

The aquifer system of the Parma urban area is represented by an alternation of fine-grained (clays and silts) and coarse-grained bodies (gravels and sands) belonging to the alluvial geological units (or synthems) that filled the Po River basin during the Pleistocene–Holocene period (Figure 1). Specifically, the sedimentation processes in this area primarily result from the sedimentary dynamics originating from Apennine streams [22–25]. Groundwater in this aquifer system exhibits a predominant southwest-to-northeast flow at the basin scale, from the Apennine to the central Po Plain and consequently toward the Adriatic Sea [25]. The hydraulic conductivity of the studied aquifer is frequently variable in the three dimensions due to the wide heterogeneity of the geological medium. Zanini et al. [26] calculated a hydraulic conductivity varying from 1.2×10^{-5} to 4.9×10^{-5} m/s (mean 2.3×10^{-5} m/s; median 1.7×10^{-5} m/s) in coarse-grained horizons and from 9.3×10^{-9} to 1.3×10^{-7} m/s (mean 1.6×10^{-7} m/s; median 9.7×10^{-8} m/s) in fine-grained layers.

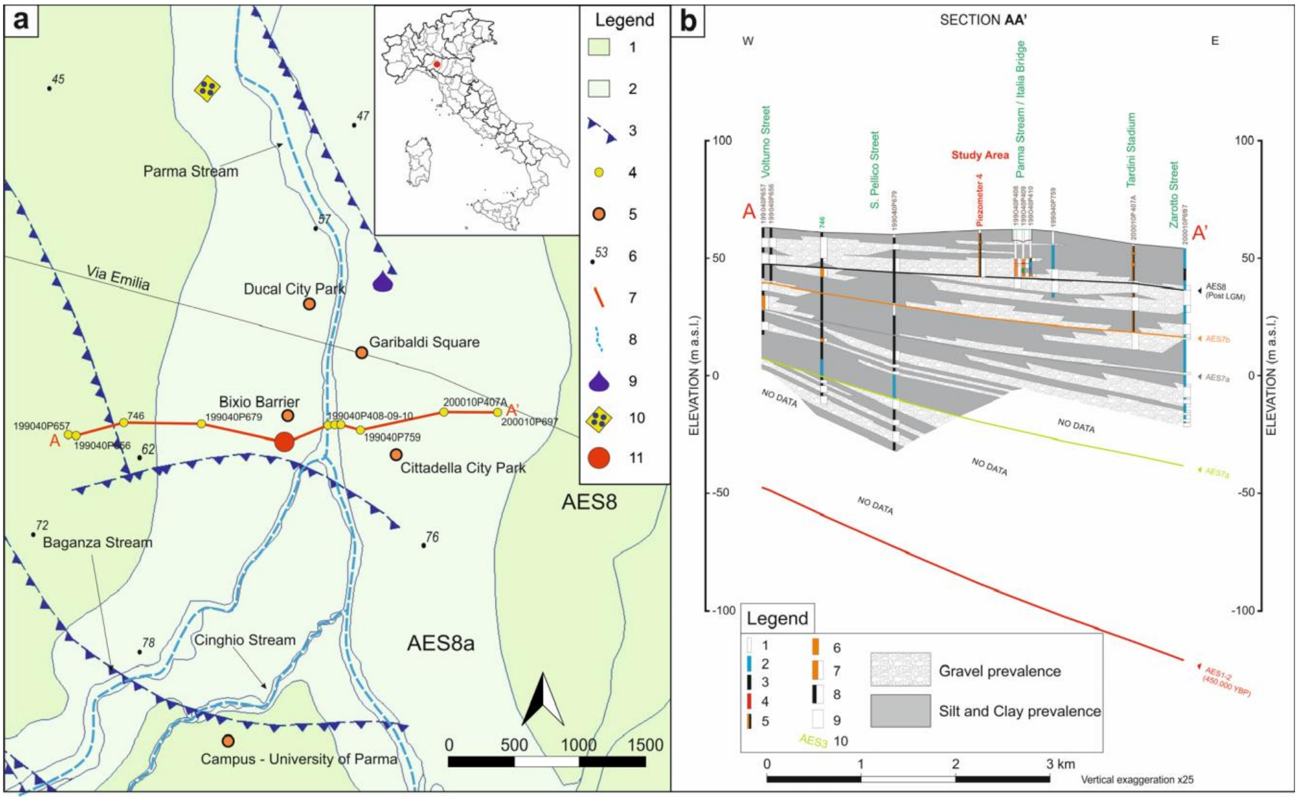

Figure 1. (**a**) Geological map and section at basin scale (from Ducci et al., 2022, modified [21]); (1) AES8: Emiliano–Romagnolo superiore synthem—Ravenna subsynthem; (2) AES8a: Emiliano–Romagnolo

superiore synthem—Modena subsynthem; (3) main thrust; (4) borehole; (5) toponym; (6) quoted point; (7) geological section's trace; (8) stream; (9) meteorological station; (10) wastewaters treatment plant "Parma Ovest"; (11) study area; (from Ducci et al., 2022, modified [21]). (**b**) Geological section at basin scale (the trace is shown in (**a**)): (1) fill material, (2) grey/light blue clay and silt; (3) yellow/brown clay and silt; (4) red clay and silt; (5) silt; (6) sand; (7) gravel and sand; (8) gravel and clay/silt; (9) gravel; (10) Code of Geological Unit (sensu Di Dio et al., 2005 [27]): AES1: Emiliano–Romagnolo superiore synthem—Monterlinzana subsynthem; AES2: Emiliano–Romagnolo superiore synthem—Maiatico subsynthem; AES7a: Emiliano–Romagnolo superiore synthem—Villa Verucchio subsynthem—Niviano unit; AES7b: Emiliano–Romagnolo superiore synthem—Villa Verucchio subsynthem—Vignola unit; AES8: Emiliano–Romagnolo synthem—Ravenna subsynthem.

The presence of samples derived from 30 core borings made it possible to reconstruct the local stratigraphy at the site scale [21]. Excluding the anthropogenic material (gravel and bricks) that fill the entire study area about 1 m thick, founded succession can be summarized as follows: (i) the first layer consists of silt and clay with a thickness ranging from 5 to 7 m; (ii) the second layer is primarily composed of gravel and sand, with a thickness varying between 1 and 5 m. Particularly, it exhibits a clear separation into two distinct elements as it extends northward, near Pz3; (iii) the third layer comprises silt and clay, with a thickness ranging from 2 to 3 m; and (iv) the fourth layer represents the shallowest alluvial aquifer at the test site and is composed of gravel and sand (Figure 2). These sedimentary bodies result from ancient alluvial dynamics associated with the paleo-river systems of the Parma and Baganza streams.

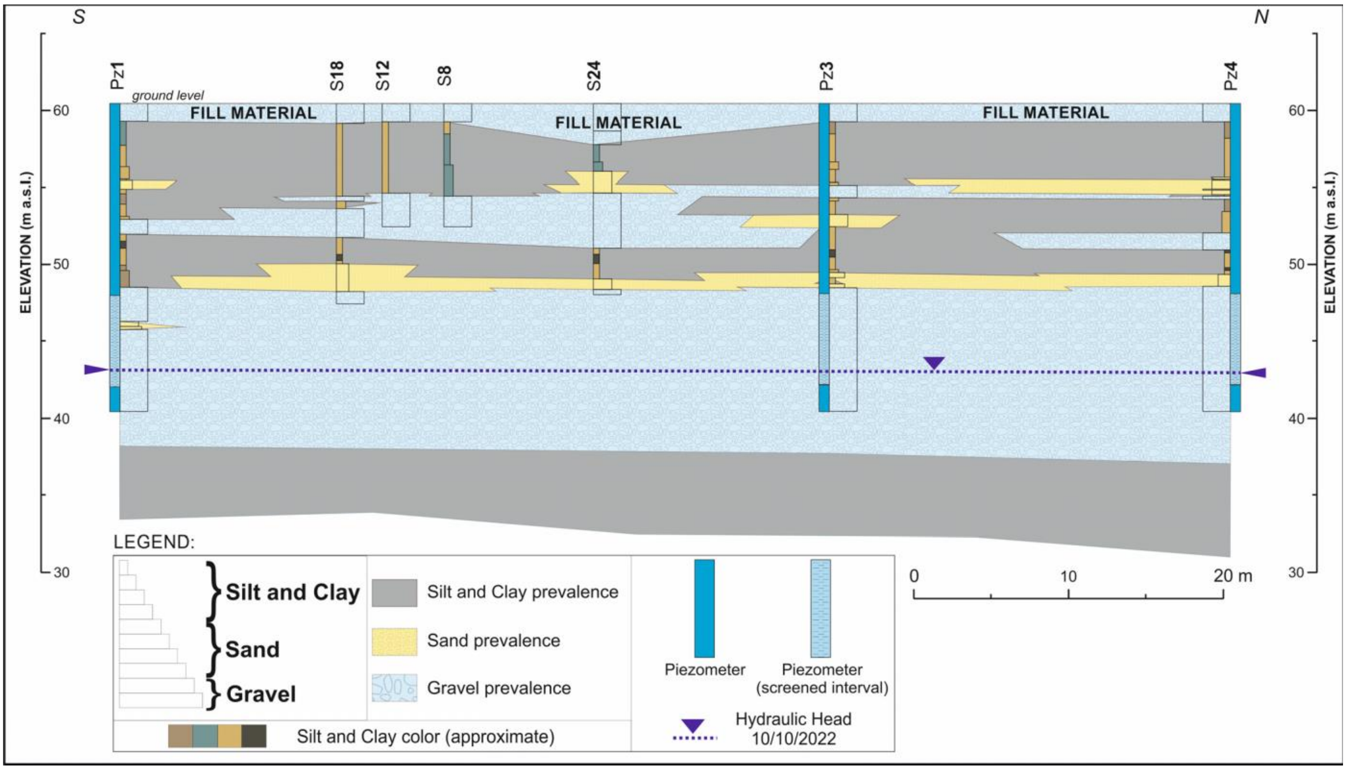

Figure 2. Geological section at site scale (from Ducci et al., 2022, modified [21]).

The groundwater flow net reconstructed in the study area is characterized by seasonal variations during the hydrologic year, even though the overall flow is always directed from South to North (Figure 3).

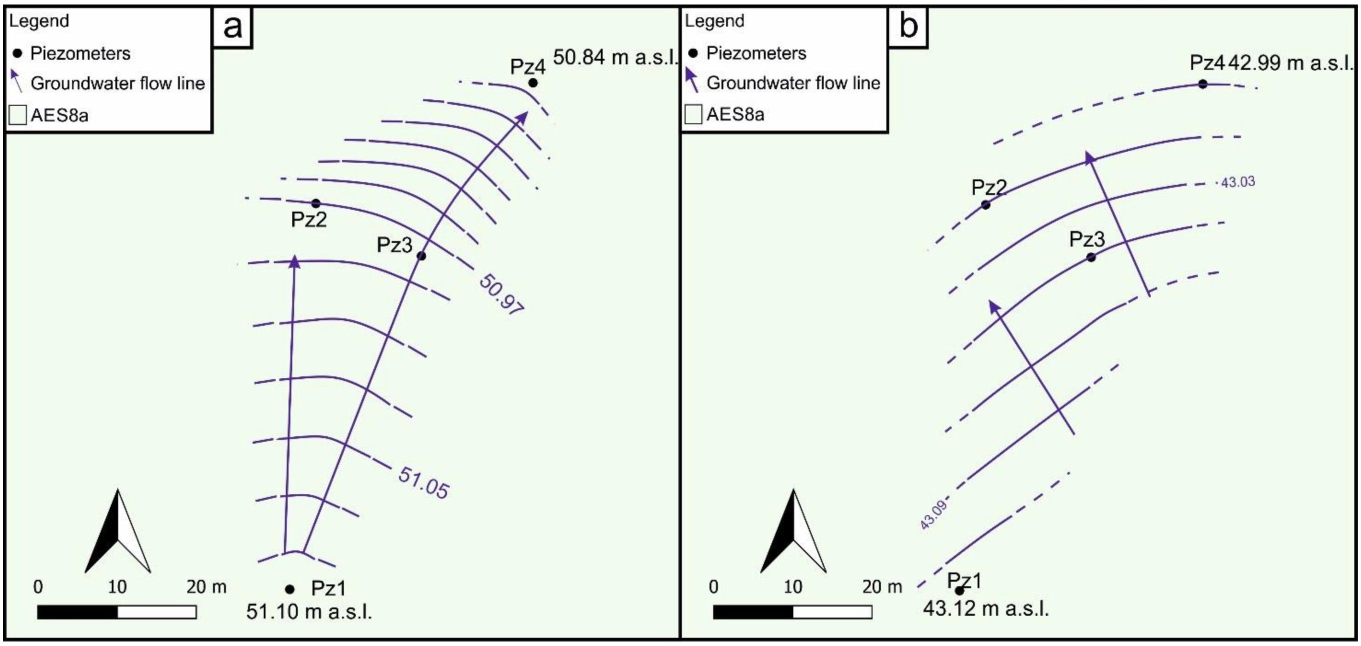

Figure 3. (**a**) Groundwater flow net in May 2022; (**b**) groundwater flow net in October 2022 (from Ducci et al., 2022, modified [21]).

2.2. Groundwater Sampling and Mesocosm Setup

Groundwater samples were collected from the piezometer Pz3, immediately below the hydraulic head, with a sterile bailer in October 2022.

The groundwater temperature has been monitored monthly at the piezometer Pz3 from March 2022 to October 2022 using the multiparameter HANNA probe (model HI9828, HANNA Instruments, Villafranca Padovana, Italy).

In detail, for mesocosm experiments, 16 L of water was sampled. In addition, another 2 L of water was used to carry out biomolecular and chemical analyses aimed at characterizing autochthonous microbial communities and determining the "background contamination" by Phy-Et (Figure 4a).

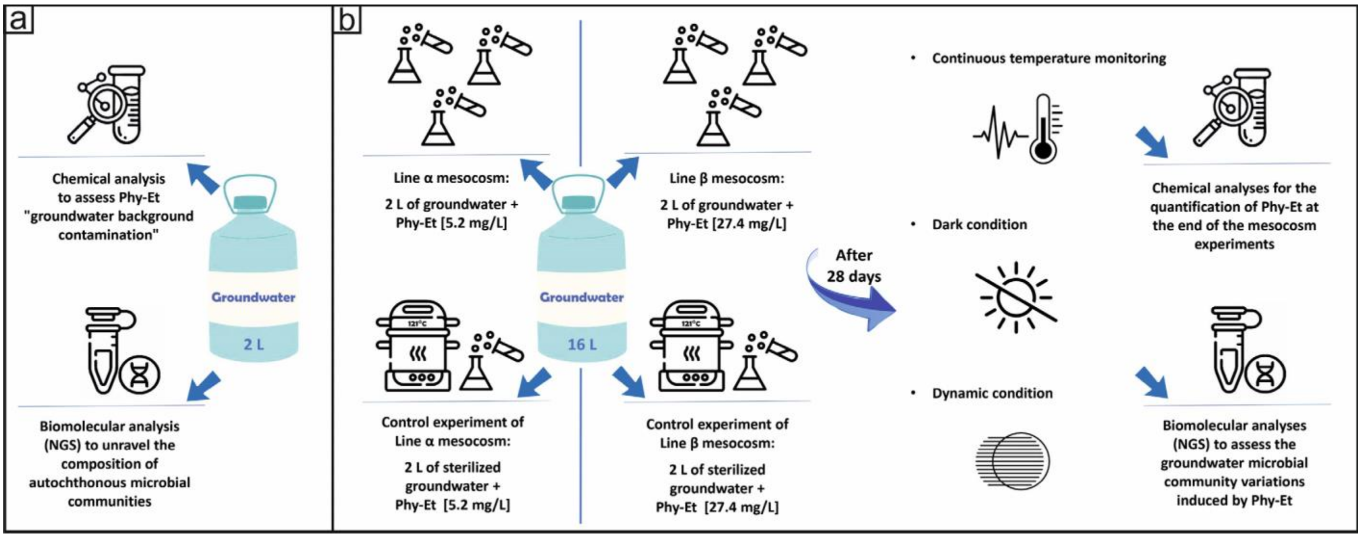

Figure 4. (**a**) Groundwater microbial community characterization and assessment of Phy-Et background contamination; (**b**) experimental protocol setup of mesocosms.

Mesocosms were prepared by transferring 2 L of groundwater in sterile glass Erlenmeyer flasks and by adding Phy-Et (BiOrigins, Fordingbridge, United Kingdom) at two different concentrations: 5.2 mg/L and 27.4 mg/L. The experiments carried out with Phy-Et at a concentration of 5.2 mg/L will be henceforth also indicated as Line α, whereas the ones carried out with Phy-Et at a concentration of 27.4 mg/L will be reported as Line β. For each Line, three different mesocosms were set up (Figure 4b). The Phy-Et concentrations arbitrarily chosen for the experiments were more than 20 and 100 times higher than the instrumental detection limit for this compound (0.2 mg/L).

Afterward, the mesocosms were sealed and incubated at room temperature for 28 days, in the dark, with agitation (140 rpm), using the OrbitalShaker—OHAUS® (Model: SHHD6825DG, S/N: 210331001, Nänikon, Zürich, Switzerland).

In addition, the experimental plan has included two controls, consisting of sterilized groundwater samples added with Phy-Et at the concentrations of 5.2 mg/L and 27.4 mg/L, to highlight a possible removal of this compound from groundwater not attributable to the microbial activities (Figure 4b).

2.3. Temperature Monitoring and Chemical Analyses for Phy-Et Detection

For the entire duration of the mesocosm experiments (28 days), the temperature was monitored every 5 min through an immersion thermometer with a data logger (Elitech model: GSP-6 Serial No. EFG218100242, London, UK).

Chemical analyses for Phy-Et detection were performed both on the original groundwater and samples collected at the end of the mesocosm experiments. A total of 50 mL of water was poured into amber glass bottles and transported to the laboratory in a refrigerated box. The analyses were performed at Abich S.r.l., an Italian company with UNI EN ISO 9001:2015 [28] and UNI CEI EN ISO 13485:2016 [29] accreditations. According to an accredited in-house method, they were based on the ultra-high-performance liquid chromatographic-diode array detection method (UHPLC/DAD) (Rif. HPLC n.0018).

2.4. Next-Generation Sequencing (NGS) for Bacterial Community Analyses

Next-generation sequencing (NGS) analyses were carried out firstly to characterize autochthonous groundwater microbial communities in groundwater samples collected at the piezometer (Pz3) and then to assess those variations in their composition after the addition of Phy-Et to the mesocosms at two different concentrations (5.2 mg/L and 27.4 mg/L).

Groundwater samples (1.95 L) were filtered through sterile mixed esters of cellulose filters (S-Pak™ Membrane Filters, 47 mm diameter, 0.22 μm pore size, Millipore Corporation, Billerica, MA, USA) within 24 h from the collection. Bacterial DNA extraction from filters was performed using the commercial kit FastDNA SPIN Kit for soil (MP Biomedicals, LLC, Solon, OH, USA) and FastPrep® Instrument (MP Biomedicals, LLC, Solon, OH, USA). After the extraction, DNA integrity and quantity were evaluated using electrophoresis in 0.8% agarose gel containing 1 μg/mL of Gel-Red™ (Biotium, Inc., Fremont, CA, USA). Next-generation sequencing (NGS) technologies generated the bacterial community profiles in the samples at the Genprobio Srl Laboratory following the protocol reported by Ducci et al. [21].

The 16S rRNA gene sequences obtained in this study were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under the accession number PRJNA977861.

3. Results

3.1. Microbiological and Physico-Chemical Characterization of Groundwater

Autochthonous bacterial communities of groundwater collected from the piezometer Pz3 were characterized by a predominance of *Proteobacteria* (relative abundance 64.14%), followed by *Bacteroidetes* (relative abundance 8.34%), *Chloroflexi* (relative abundance 5.23%), *Verrucomicrobia* (relative abundance 3.91%), *Actinobacteria* (relative abundance 3.38%), *Aci-*

dobacteria (relative abundance 3.07%), and other phyla, which were detected with relative abundance values lower than 3% (Figure 5). At the genus level, the five major taxa were *Methylotenera*, *Pseudomonas*, unclassified microorganisms of the *Burkholderiaceae* family, *Massilia*, and *Cavicella*, which were retrieved at percentages of 8.71%, 7.27%, 4.36%, 2.18%, and 2.00%, respectively (Figure 5).

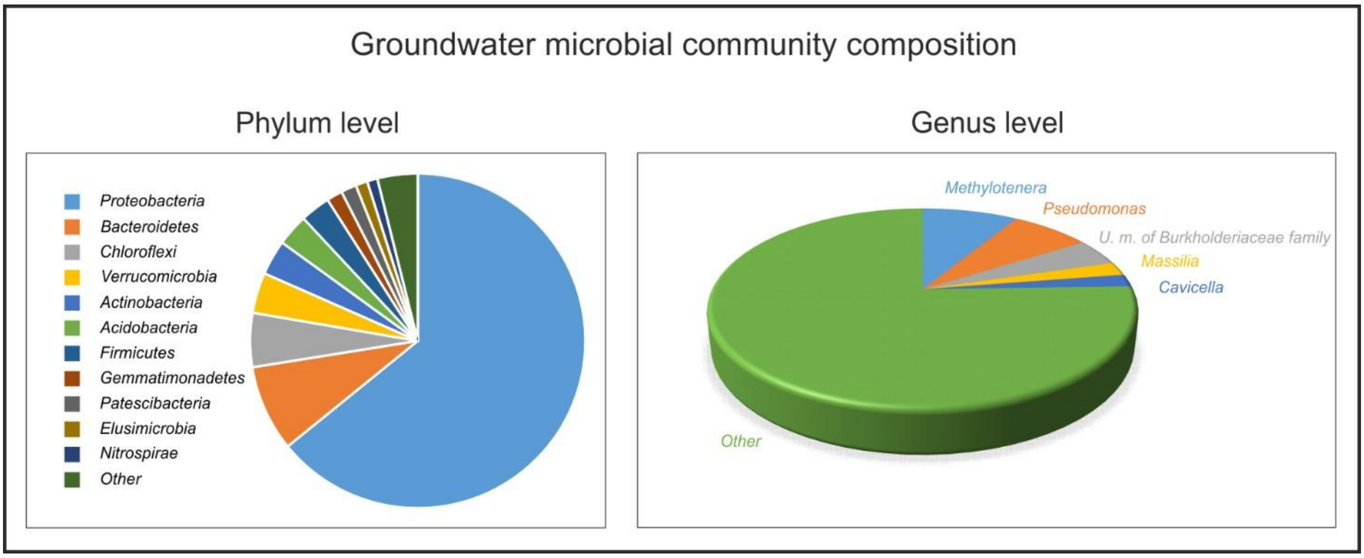

Figure 5. Microbial community composition at phylum (**on the left**) and genus (**on the right**) levels of groundwater collected from the piezometer Pz3. Taxa with relative abundance values below 1% and 2% for phylum and genus levels, respectively, are labeled "Other". U. m. refers to unclassified microorganisms.

Chemical analyses for determining Phy-Et revealed that its concentration was lower than the instrumental detection limit (0.2 mg/L). Groundwater temperature during the observation period ranged from 13.2 °C to 16.1 °C, with an average of 14.4 °C.

3.2. Results of Physico-Chemical and Microbiological Analyses for the Mesocosm Experiments

At the end of the 28-day experimental period, water samples were collected from the mesocosms in which Phy-Et was initially added at two different concentrations and were subjected to chemical analyses to assess whether there had been a reduction.

The same analyses were also performed on the two controls, consisting of sterilized groundwater added with Phy-Et, to detect any losses of this compound from the systems, not due to biodegradation processes.

The results obtained clearly showed the removal of the compound of about 100% in Line α mesocosms (from 5.2 mg/L to below the detection limit [0.2 mg/L]). On the other hand, in the Line β mesocosms, a reduction of about 84% was detected (from 27.4 mg/L to concentrations ranging between 3.21 ± 0.01 mg/L and 5.24 ± 0.02 mg/L). No significant variations in the concentration of the chemical were recorded in the controls, evidence which demonstrates that microorganisms were the "key player" in the degradation process.

The continuous monitoring of water temperature in the mesocosms allowed the detection of fluctuations varying from a minimum of 17.9 °C to a maximum of 24.2 °C, with an average of 21.0 °C.

On the whole, the results of the biomolecular investigations carried out to unravel the composition of bacterial communities exposed to the two concentrations of Phy-Et in the mesocosm experiments revealed, at the phylum level, the predominance of *Proteobacteria*, followed by *Bacteroidetes*, *Verrucomicrobia*, *Acidobacteria*, *Actinobacteria*, and *Patescibacteria* (Figure 6).

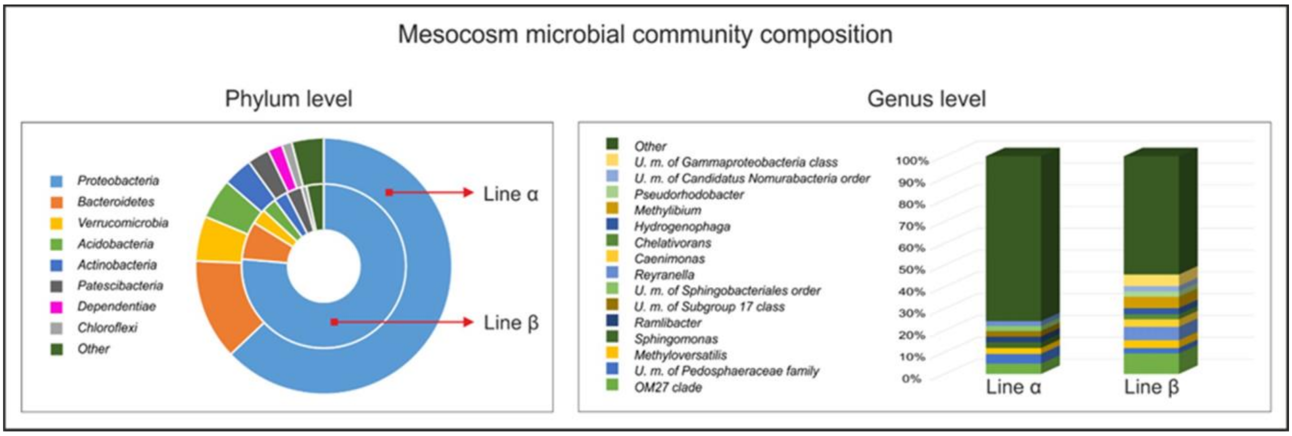

Figure 6. Mesocosm microbial community composition at phylum (**on the left**) and genus (**on the right**) levels of Lines α and β. Taxa with relative abundance values below 1% and 2% for phylum and genus levels, respectively, are labeled "Other". U. m. refers to unclassified microorganisms. The relative abundance of each taxon has been calculated as the mean of values of the triplicate experiments.

However, differences emerged when comparing the percentages of these taxa with the relative abundance of the same phyla retrieved in the original groundwater sample. For example, the abundance of *Proteobacteria* went from 64.14% to 62.92% (on average) in Line α mesocosms and 76.36% (on average) in Line β mesocosms. Moreover, the same goes for the other phyla, as reported in Table 1.

Table 1. Variations of the relative abundance average values of the main phyla in groundwater samples.

Taxonomy	Pz3	Line α	Line β
Proteobacteria	64.14%	62.92%	76.36%
Bacteroidetes	8.34%	12.71%	7.51%
Chloroflexi	5.23%	1.27%	1.02%
Verrucomicrobia	3.91%	5.68%	3.03%
Actinobacteria	3.38%	3.69%	2.70%
Acidobacteria	3.07%	5.08%	2.92%
Firmicutes	2.90%	0.22%	0.32%
Gemmatimonadetes	1.53%	0.65%	0.23%
Patescibacteria	1.49%	2.78%	3.08%

In addition, the phylum *Chloroflexi*, which was among the major three phyla in the original groundwater sample (5.23%), showed a decrease in the mesocosms to percentages of 1.27% and 1.02% when Phy-Et was added at 5.2 mg/L and 27.4 mg/L, respectively.

When analyzing the mesocosm microbial communities at the genus taxonomic level (Figure 6), the top six taxa retrieved in the Line α experiments were: microorganisms of the clade *OM27* (average relative abundance 4.77%), unclassified microorganisms of *Pedosphaeraceae* family (average relative abundance 4.46%), *Methyloversatilis* (average relative abundance 2.72%), *Sphingomonas* (average relative abundance 2.64%), *Ramlibacter* (average relative abundance 2.61%), and unclassified microorganisms of Subgroup 17 class (*Acidobacteria* phylum; average relative abundance 2.51%). On the other hand, the top six genera found in the Line β mesocosms were microorganisms of the clade *OM27* (average relative abundance 9.49%), *Reyranella* (average relative abundance 6.14%), unclassified microorganisms of *Gammaproteobacteria* class (average relative abundance 5.35%), *Methylibium* (average relative abundance 5.18%), *Methyloversatilis* (average relative abundance 3.57%), and *Caenimonas* (average relative abundance 3.47%).

These results suggest that Phy-Et has modified the composition and species abundance of the groundwater microbial communities, most likely inhibiting the growth of cells sensitive to its action and favoring the survival of those tolerant or able to metabolize it.

In addition, another important aspect that emerged is the presence of diverse unclassified microorganisms at the genus level, accounting on average for 41.58% and 29.14% in Line α Line β mesocosms, respectively. This observation demonstrates that a large portion of microbial diversity in the analyzed systems remained hidden.

The rarefaction analysis, a measure used to estimate the alpha diversity in samples and gauge whether sequencing efforts captured the microbial diversity (Supplementary Materials Figure S1), revealed a biodiversity reduction trend correlated to the increase of Phy-Et concentrations.

4. Discussion

On a global scale, pharmaceuticals and personal care products (PPCPs) have risen in the last decade due to advances in research and development, increased world population, and accessibility to healthcare [30,31]. Given their known or suspected adverse ecological or human health effects, PPCPs are considered two of the most abundant classes of emerging contaminants in the environment [32,33]. Among the most dramatic effects on living organisms are the feminization of fishes [10,34], alterations in the reproduction and development of some fish species caused by very low concentrations of environmental estrogens [35], and increased antibiotic resistance of pathogenic microorganisms [36]. Their presence in aquatic and terrestrial ecosystems is associated with their occurrence at influents and effluents of wastewater treatment plants [33], whose removal capacity strongly depends on the features of the pollutant and the technologies used for wastewater treatments.

In this research, the authors have focused on Phy-Et, a compound present as a preservative in many cosmetics. According to the currently available safety data, this substance is considered safe when used at a concentration of up to 1%, is a rare sensitizer, and is one of the most well-tolerated preservatives [20]. Nevertheless, animal studies revealed different adverse systemic effects at higher levels of exposure than consumers when using Phy-Et-containing products [20]. In addition, other authors who analyzed the toxic effects of this compound in an eukaryotic model organism, *Allium cepa*, claimed that, in the light of their data, "it should be ensured that the use of Phy-Et should be limited, if not preferred, or if it is absolutely necessary to use it in doses that do not have toxic effects on the organisms" [15]. Accordingly, the main aim of this work was to analyze the capacity of microbial communities to remove Phy-Et (supplied at two different concentrations) from groundwater through mesocosm experiments designed to represent and simulate, on a laboratory scale, the conditions of shallow groundwater flowing in the daily heterothermic zone, characterized by significant and frequent temperature fluctuations closely tied to atmospheric conditions. Additionally, in the piezometer Pz3, the recorded temperature values ranged from 13.2 °C to 16.1 °C, with an average value of 14.4 °C whereas the temperature of the mesocosms ranged from a minimum of 17.9 °C to a maximum of 24.2 °C, with an average value of 21.0 °C. Even though the water temperatures in the lab-scale experiments were higher than those measured in the field during the observation period, it is worth noting that many of the genera detected in the mesocosms include species characterized by a wide range of growth temperatures. Accordingly, the authors are convinced that the observed microbial community variations, induced by the presence of Phy-Et, nevertheless provide an accurate representation of the events that would occur in nature.

However, in a wider context, similar temperatures to those measured in the mesocosms have also been found in other hydrogeological systems [37], with values reaching 24.0 °C during summer. In a future perspective, further studies will be carried out with the use of a refrigerated thermostat (e.g., [38]) to control the temperatures to which these systems are exposed, to subject the autochthonous microbial communities to the same fluctuations observed during the hydrological year in the field.

In the frame time considered, autochthonous microorganisms (i) were able to reduce by ≈100% and ≈84% the amount of Phy-Et present in the systems with higher removal effectiveness when the concentration of the contaminant was lower (5.2 mg/L), and (ii) represented the major contributors to the natural attenuation process. Additionally, these particularly encouraging data also suggest the existence of a "dose-dependent effect" of Phy-Et on the process of biodegradation, given the observed decrease of the "natural attenuation efficacy" (≈16%) with the increasing contaminant concentrations. The analysis of bacterial communities through NGS technologies revealed drastic changes in their composition determined by the presence of Phy-Et, evidenced by a reduction in biodiversity linked to increasing Phy-Et concentrations. It is known that Phy-Et exerts its antimicrobial activity by uncoupling oxidative phosphorylation from respiration and by competitively inhibiting malate dehydrogenase [39]. Moreover, it also acts as a bactericidal agent by increasing the permeability of the cell membrane to potassium ions and exerts a direct inhibitory effect on microbial DNA and RNA synthesis [39].

Therefore, it is not surprising that the addition of the contaminant has shaped microbial communities so that the more sensitive microorganisms were under-represented in the mesocosm experiments compared to the original groundwater.

On the other hand, the presence of microorganisms of the clade *OM27* as dominant components of the "contaminated" samples is noteworthy. *OM27* clade is a cluster of unculturable bacteria, phylogenetically related to the predatory deltaproteobacterial genus *Bdellovibrio* [40,41], which have a geographically wide distribution [40] but of which very little is known [42]. Their potential predatory "attitude" could confer an advantage under the selective pressure of Phy-Et. Conversely, the biodegradation potentials of some members belonging to the bacterial genera such as, for example, *Methyloversatilis*, *Methylibium*, *Ramlibacter*, *Reyranella,* and *Sphingomonas*, are known, and this could explain why they were found as the main representatives in the mesocosm experiments.

Interestingly, there is a large portion of microbial biodiversity that remains hidden, as demonstrated by the relatively high percentages of unclassified microorganisms at the genus level, and that should be uncovered through the development of appropriate research tools, given the importance of acquiring knowledge on the main processes underlying the bacterial biodegradation of contaminants in nature.

Several thousands of PPCPs are produced globally every year, and their release into the environment remains an unavoidable by-product of a modernized lifestyle [43–46]. PPCPs may enter the environment as components of human or animal waste after incomplete absorption and excretion from the body or may result from medical, industrial, agricultural, or household waste emissions [47–50]. Trace amounts of PPCP-related compounds have been found in waste, aquatic ecosystems, or finished drinking water [48,51–58], suggesting widespread contamination and highlighting the importance of monitoring these compounds once they are released into the environment.

This research has demonstrated that microorganisms have a high potential for removing the largely used preservative Phy-Et from groundwater, at least in an artificial laboratory system. However, knowledge about the extent of their degradative capabilities in natural contexts still remains scarce. Accordingly, this topic should be further deepened, for this and many other contaminants of emerging concern, through the improvement of tools and protocols to evaluate their occurrence and fate in nature (for example, by promoting the execution of several large-scale monitoring campaigns in different environmental contexts and developing of different analytical methods for CEC detection, together with extensive analyses aimed at unraveling microbe potentiality for biodegradation, based on traditional and molecular microbiological methods) and the assessment of the impact of their release and the effects of long-term exposure (even at low concentrations) on ecosystems and health.

5. Conclusions

In light of the extensive global production of pharmaceuticals and personal care products (PPCPs), their inevitable release into the environment represents a salient implication of contemporary lifestyles. Consequently, persistent endeavors are required to evaluate these compounds' occurrence and subsequent fate in the natural environment and understand the potential long-term effects on ecosystems and human health.

This research's focal point revolved around examining Phy-Et, a common preservative in cosmetic products. Although considered safe at concentrations up to 1% based on existing data, studies have raised concerns about its adverse systemic effects at higher exposure levels.

This work sought to understand the potential of microbial communities in removing Phy-Et from groundwater through specifically designed mesocosm experiments, which aimed to simulate shallow groundwater conditions.

While this research has demonstrated encouraging results, including significant reductions in Phy-Et concentrations and the pivotal role played by autochthonous microorganisms, it has also revealed the existence of a dose-dependent effect. The composition of bacterial communities was notably altered by Phy-Et, which determined a decrease in biodiversity. These findings underscore the significance of Phy-Et's impact on microbial ecosystems.

In addition, the presence of both unculturable microorganisms, such as the *OM27* clade, and a significant fraction of "hidden" microbial diversity to unravel raises intriguing possibilities and challenges for future explorations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su16052183/s1, Figure S1: Rarefaction curves of the samples. The alpha diversity plots were obtained by using the Shannon index.

Author Contributions: Conceptualization, L.D., P.R. and F.C.; methodology, L.D. and P.R.; software, R.P. and P.M.; validation, A.B. and F.C.; formal analysis, L.D., P.R., A.B., R.P. and F.C.; investigation, L.D. and P.R.; resources, F.C.; data curation, L.D., P.R., A.B., R.P. and P.M.; writing—original draft preparation, L.D., P.R., A.B., R.P. and F.C.; writing—review and editing, L.D., P.R., A.B. and F.C.; visualization, L.D., P.R., R.P. and P.M.; supervision, A.B. and F.C.; project administration, F.C.; funding acquisition, F.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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An Interdisciplinary Assessment of the Impact of Emerging Contaminants on Groundwater from Wastewater Containing Disodium EDTA

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Abstract: In recent years, there has been a surge in interest concerning emerging contaminants, also known as contaminants of emerging concern (CECs), due to their presence in environmental matrices. Despite lacking regulation, these chemicals pose potential health and environmental safety risks. Disodium EDTA, a widely utilized chelating agent, has raised concerns regarding its environmental impact. The present work aimed to verify the presence of Disodium EDTA at the exit of eight wastewater treatment plants discharging into some losing streams flowing within a large alluvial aquifer. Conducted in the Province of Parma (Northern Italy), the research employs a multidisciplinary approach, incorporating geological, hydrogeological, chemical, and microbial community analyses. Following a territorial analysis to assess industries in the region, through the use of ATECO codes (a classification system for economic activities), the study investigated the concentration of Disodium EDTA in effluents from eight diverse wastewater treatment plants, noting that all discharges originate from an activated sludge treatment plant, released into surface water courses feeding the alluvial aquifer. Results revealed detectable levels of Disodium EDTA in all samples, indicating its persistence post-treatment. Concentrations ranged from 80 to 980 μg/L, highlighting the need for further research on its environmental fate and potential mitigation strategies. Additionally, the microbial communities naturally occurring in shallow groundwater were analyzed from a hydrogeological perspective. The widespread presence of a bacterial community predominantly composed of aerobic bacteria further confirmed that the studied aquifer is diffusely unconfined or semi-confined and/or diffusely fed by surface water sources. Furthermore, the presence of fecal bacteria served as a marker of diffuse leakage from sewage networks, which contain pre-treated wastewater. Although concentrations of Disodium EDTA above the instrumental quantification limit have not been found in groundwater to date, this research highlights the significant vulnerability of aquifers to Disodium EDTA. It reveals the critical link between surface waters, which receive treated wastewaters impacted by Disodium EDTA, and groundwater, emphasizing how this connection can expose aquifers to potential contamination. At this stage of the research, dilution of wastewaters in surface- and groundwater, as well as hydrodynamic dispersion within the alluvial aquifer, seem to be the main factors influencing the decrease in Disodium EDTA concentration in the subsurface below the actual quantification limit. Consequently, there is a pressing need to enhance methodologies to lower the instrumental quantification limit within aqueous matrices. In a broader context, urgent measures are needed to address the risk of diffuse transport of CECs contaminants like Disodium EDTA and safeguard the integrity of surface and groundwater resources, which are essential for sustaining ecosystems and human health.

Keywords: emerging environmental contaminants; Disodium EDTA; surface–groundwater interaction; wastewater; groundwater microbial communities

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1. Introduction

Between the 1960s and 1970s, the adverse effects of some new synthetic chemical products on the environment and human health became apparent soon after their synthesis and subsequent widespread release into environmental matrices. Among the most significant examples are organic pollutants (POPs), including organochlorine pesticides such as dichlorodiphenyltrichloroethane, commonly known as DDT, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) [1]. This scenario has, therefore, highly influenced legislation and the assessment of the impact of chemical pollution substances that are subject to monitoring for environmental protection and safeguarding.

In recent decades, there has also been a growing interest in the study and research into environmental matrices of new chemical substances that are not included in current environmental legislation and regulatory programs. These chemicals, known as contaminants of emerging concern (CECs), are not yet regulated under current environmental regulations or laws but are found in numerous and spread environmental matrices, such as rivers [2–5], lakes [6], soils [7], wastewater [8–10], biosolids [11], and even groundwater bodies [12–16].

In particular, urban wastewater treatment plants (WWTPs) cannot remove CECs from sewage using conventional treatments [17,18], such as filtration [19], activated sludge [20,21], and disinfection [22]. Based on the above information, greener technologies are urgently needed to effectively remove these contaminants in wastewater treatment plants without negatively impacting the environment [23]. For this reason, wastewater plant effluents are also inferred as a source of these chemicals since an important fraction of them may be released into the aquatic environment through the effluents [24–26] and reach the aquifer through interaction with surface water courses, generating potential risks for public health and environmental safety. In addition to this, due to their direct exposure to anthropogenic impacts, surface waters are vulnerable to becoming important basins for these micropollutants [27–32]. This, on the one hand, entails adverse effects on both wildlife and humans [33–35], restraining the use of water for consumption, irrigation, and recreation purposes [31,36], and, on the other hand, can generate groundwater pollution, through the feeding of the aquifer by impacted surface water courses.

CECs include over 3000 types of compounds and their derivatives, such as pesticides, fertilizers, microplastics, heavy metals, pharmaceutical and personal care products (PPCPs), including fragrances, UV filters, insect repellents, and antimicrobials [37,38]. Concerning risks to human health, CECs can harm human health and wildlife, resulting in reproductive and endocrinal disabilities [39,40]. The EU Water Framework Directive (WFD) was created in 2000 to avoid additional deterioration of the water status. The WFD specifies regulatory measures to prevent the deterioration of the ecological and chemical status of water bodies within the European Union [41]. Since 2000, it has been Europe's leading law for water protection. It applies to inland, transitional, and coastal surface waters and groundwater and aims to achieve a good chemical status for all water bodies [41]. The WFD has given significant impetus in this direction, but achieving objectives for the protection of water resources involves the development of complex policies. It is necessary to affect a heterogeneous set of phenomena that compromise the ecological water status, adopting a multidisciplinary method through a holistic approach, which guarantees an accurate overview of the problem. The European Commission, to improve the application and implementation of chemical monitoring programs by the Member States, established in 2010 an expert group, the Chemical Monitoring and Emerging Pollutants (CMEP), to deepen the scope of emerging pollutants, including analytical methods, information on hazards, levels in the environment and patterns of use [42].

Although the knowledge about their environmental concentrations and fate is still mostly unknown, some of these CECs have the potential for bioaccumulation and/or are bioactive substances that may compromise living organisms. For example, some UV filters, such as 4-methylbenzylidene camphor (4-MBC), are suspected of presenting estrogenic activity [39].

Within this ongoing research, Ducci et al. [43] detected the presence of Disodium EDTA at the inlet of a wastewater treatment plant in Italy, while this was not detected in the groundwater. Disodium EDTA (CAS: 139-33-3), also known as sodium ethylenediaminetetraacetate, is a widely used chemical compound in various sectors due to its chelating properties and ability to bind metal ions. Disodium EDTA has the chemical formula $C_{10}H_{14}N_2Na_2O_8$ and features a complex molecular structure. It is a polyaminocarboxylic acid renowned for its capacity to form stable complexes with metal ions, particularly heavy metal ions like calcium and magnesium. Its water solubility contributes to its versatility in various applications. Disodium EDTA finds applications in several sectors, including (i) the food industry (it is used as a chelating agent to prevent food oxidation and improve the stability of food colorants), (ii) cosmetics and personal care products (it stabilizes products like creams and lotions, preventing the formation of undesired precipitates), and (iii) the pharmaceutical industry (it is employed as a chelating agent in pharmaceutical formulations to enhance drug stability).

Despite its numerous uses, Disodium EDTA has raised concerns regarding its potential environmental impact. As described in the previous lines, the advantage of using Disodium EDTA in different industrial contexts is due to its ability to chelate heavy metals, which predisposes this chemical to assist the conservation and stabilization activities of certain products. At the same time, the possible presence of this substance in environmental matrices could lead to the mobilization and dispersion of heavy metals in soil and water systems, potentially damaging ecosystems and human health.

The present work aimed to verify the presence of Disodium EDTA at the exit of eight wastewater treatment plants discharging into some losing streams flowing within a large alluvial aquifer. The choice of analyzing wastewaters instead of groundwater in terms of Disodium EDTA concentrations is because the current chemical analyses still have high detection limits that do not allow the detection of this compound in saturated aquifers to date [43]. From this perspective, the link between wastewater treatment plants and groundwater was obtained by analyzing the aquifer properties in detail from a hydraulic point of view. The autochthonous microbial community in groundwater was also characterized in order to check the existence of possible natural attenuation. Microbial communities were also used as natural tracers to refine knowledge about local hydrodynamic processes and interaction between surface and groundwater.

2. Study Area

The Po Valley is largely the most urbanized and industrialized area in the Italian peninsula, and the possible sources of Disodium EDTA contamination are numerous and widespread. The research was carried out in different geographic and geologic contexts of Parma province, partly placed in the Northern Apennine valley floor, on the south, and partly in the Parma plain to the North, both located in this highly industrialized sector of Italy (Figure 1).

In the valley floor contexts (LDCA and LDLA wastewater treatment plants), the geological unit is deposited to the sides of the current riverbeds by composing small intravalley plains, covering the Apennine bedrock with thicknesses less than 10 m and characterized with very coarse lithologies (gravel in prevalence). In the plain (LDTR, LDCO, LDFE, LDPA, LDFO, and LDME wastewater treatment plants), the same geological unit consists of several hundred meters thick of alternating fine (silts and clays) and coarse (gravels and sands) layers resulting from the alluvial dynamics of the Apennine rivers and streams that filled the basin (Figure 1).

From a hydrogeological point of view, groundwater flows in this unit according to the topographic gradient from S–SE to N–NE [44]. The hydrogeologic macro asset can be defined as a multilayer aquifer where the highest permeability bodies consist of gravels and sands (hydraulic conductivity varying from 1.2×10^{-5} to 4.9×10^{-5} m/s from Zanini et al. [45]) with decreasing grain size toward the north, and silty–clayey interposed aquitards (hydraulic conductivity varying from 9.3×10^{-9} to 1.3×10^{-7} m/s from Zanini et al. [45]). As demonstrated by Ducci et al. [43], locally, these aquitards do not have sufficiently low permeability to allow total hydraulic isolation of the different high-permeability bodies. At the bottom of the entire system, Pliocene clays are considered as a regional-scale diffuse aquiclude. The hydraulic relationships between the aquifer and surface water courses have been investigated for specific tracts of several streams in the Parma Plain area [46,47], also through isotopic investigations, confirming the existence of losing streams [48]. The interaction between surface and groundwater was further analyzed at the site scale in the present work. In some cases, the sedimentary geometries of river or stream incisions that deposit coarser sediments (known as channel belt geometry, see Amorosi et al. [49] and Bruno et al. [50]) could be able to hydraulically connect the surface and the underlying aquifers; on the contrary, where riverbeds stand over continuous fine deposits bodies, the direct connection is absent or inconsiderable.

The hydraulic heterogeneity of aquifer bodies in the plain can lead to the presence of local barriers in groundwater flow proceeding downstream. This setting could cause the outcrop of groundwater originating the known "fontanili" and "risorgive" (e.g., [51,52]), which has also been the subject of analysis in the present work (Figure 1). From the ecological point of view, the risorgive or fontanili are small, semi-artificial, aquatic ecosystems (see Kløve et al. [53]) that are usually detected within the Po River Basin, the largest watershed in Italy (more than 70,000 km^2).

Figure 1. Hydrogeological map (the hydrogeological sections are shown in Figure 2).

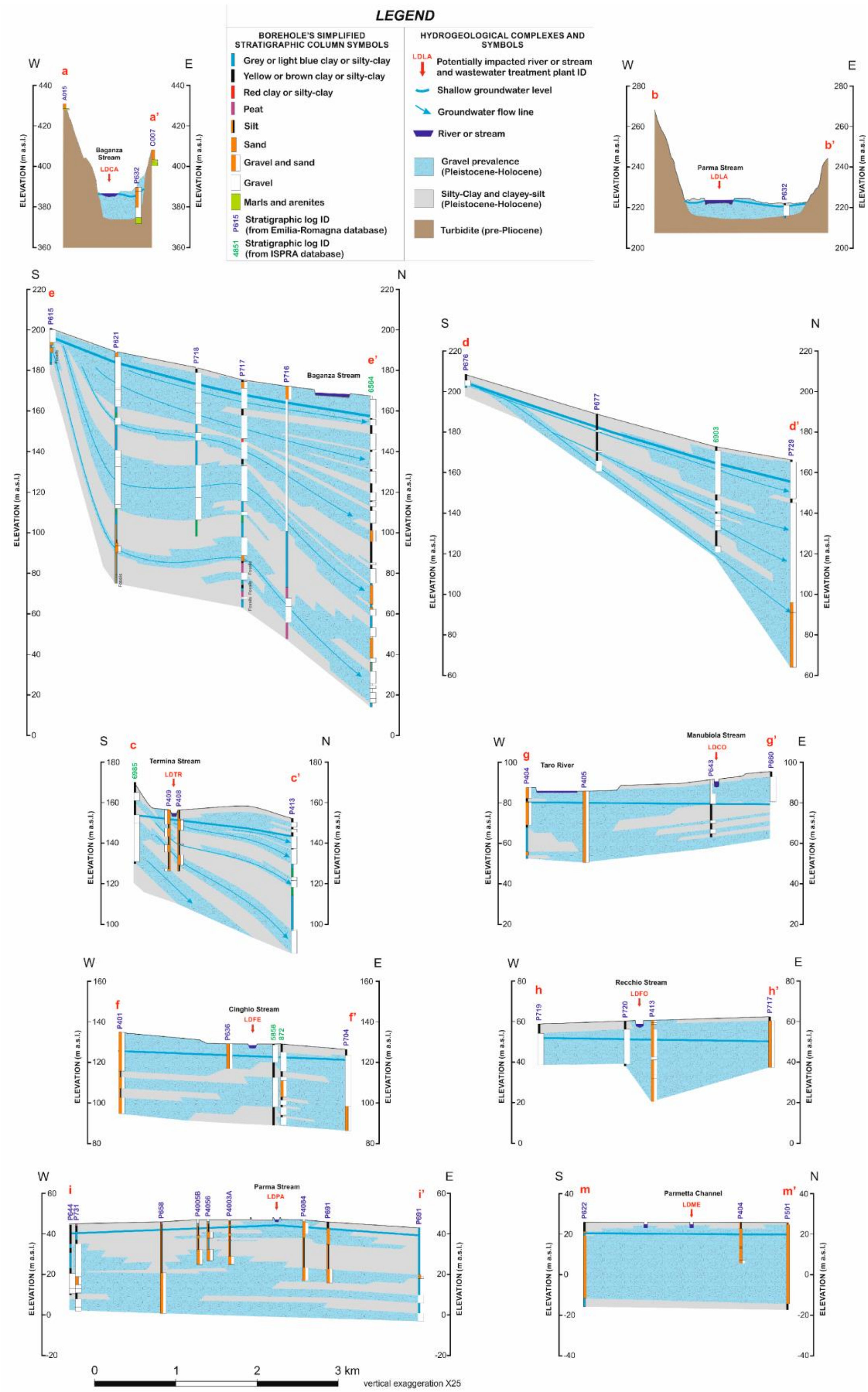

Figure 2. Hydrogeological sections (section traces are shown in Figure 1).

The test area is a built environment with several agricultural (intensive models prevailing; e.g., [54]) and industrial activities, the latest dominated by the following sectors (i) agro-alimentary; (ii) production of food machinery, preservation equipment, machines, and packaging; (iii) pharmaceuticals and perfumery; and (iv) personal care and well-being [55].

Due to the human activities described above, different kinds of contamination were already detected and studied within the shallow aquifer system. Agricultural pressures cause widespread nitrate contamination of groundwater and groundwater-dependent ecosystems (GDEs) in the study area (e.g., [51,56]), as well as within the wider Po plain (e.g., [57–62]). Differently, urban and industrial activities were linked to microplastics [47], chlorinated solvents [45,46,63], fecal matter, and personal care products [43,64].

3. Materials and Methods

3.1. Geological Elaborations

Considering that Disodium EDTA discharges flow into local canals, streams, and rivers, the relationship between watercourses and aquifer bodies assumes high importance in understanding the possible infiltration of this substance into groundwater. For this purpose, ten hydrogeological sections orthogonal to the riverbed were designed and constructed downstream of each wastewater treatment plant's release point. The geological and stratigraphic data on which these reconstructions were derived from the scientific literature [44] and subsurface investigation (by ISPRA and Emilia-Romagna Region boreholes public databases). The elaboration focused on reconstructing the physical (and consequently hydraulic) connection between the existing incisions of the surface runoff reticulum and the aquifer layers in the first tens of meters of the subsurface. The piezometric level refers to the shallow aquifer layer and was included based on the groundwater flow net reported in [44].

3.2. Examination of Productive Activities in the Territory of Parma

A preliminary examination of productive activities, identified by the ATECO (short for 'ATtività ECOnomiche', an Italian classification system for economic activities) code, was conducted within the territory of the Province of Parma. The ATECO code consists of an alphanumeric sequence designed to identify a specific economic activity. In this sequence, the letters in the code delineate the macroeconomic sector, while the numbers, ranging from two to six digits, provide details at various levels, specifying the articulations and subcategories within the sectors themselves. ATECO serves as the classification system for economic activities in Italy and is used by ISTAT (in Italian: Istituto Nazionale di Statistica) for statistical purposes in collaboration with other institutions, ministries, and business associations involved in statistics. This assessment aimed to determine which business activities could be involved in the use of Disodium EDTA, considering the substance's specified applications in the current regulations of the European territory.

3.3. Wastewater Discharge Selection and Sampling

Following the identification of companies potentially involved in the use of Disodium EDTA, the next step involved selecting eight wastewater treatment plants in Parma territory to sample their effluents.

To diversify the types of sampled discharges, the selection parameters for wastewater treatment plants to be characterized were: (i) plant capacity expressed with (*a*) the average daily volume of treated effluents (m^3/d) and (*b*) the population equivalent units, (ii) the number of inhabitants in the municipality where the plant is located (from ISTAT database), (iii) the number of industrial activities discharging into the wastewater treatment plant, and (iv) the number of industrial activities potentially involved in the use of Disodium EDTA discharging into the wastewater treatment plant while maintaining the fact that all discharges originate from an activated sludge treatment plant, released into surface water courses flowing within the alluvial aquifer. Among the eight plants, two were selected to analyze the potential impact (in terms of Disodium EDTA) of the only civil wastewater since the industrial wastewater treated at the same plants originates from industrial activities that do not utilize the studied emerging contaminant.

To ensure the comparability of the analytical results and minimize the dilution due to surface runoff, wastewaters were simultaneously sampled in each of the eight plants through 24 h composite sampling on the same day in April 2023, during a prolonged no-rain period.

3.4. Chemical Analysis

In accordance with other authors (e.g., [43,65]), wastewater sample collection took place once in April 2023, during dry weather conditions (defined as no rain in the previous 24 h and <2 mm in the previous 48 h [66]).

Water samples were collected from eight wastewater treatment plants in Parma. The selection of these plants is based on the ATECO code for recognizing which business activities could be involved in the use of disodium EDTA.

All wastewater samples (250 mL) were collected in amber glass bottles, stored in a refrigerated box, and transported to the laboratory.

The analyses were performed at Analytice s.a.r.l. (société à responsabilité limitée), a laboratory with ISO 17025 [67] accreditation recognized by the French Accreditation Committee (ILAC full members). In particular, gas chromatography combined with mass spectrometry (GC/MS) was performed to analyze Disodium EDTA. Samples were analyzed versus EDTA, and values were then calculated into Disodium EDTA. The samples were analyzed according to EN ISO 16588 [68]. The samples were derivatized with isopropanol/acetylchlorid and extracted with hexane. The extracts were then injected using gas chromatography (column DB-XLB) coupled to a mass spectrometer detector (GC-MS).

3.5. Next-Generation Sequencing (NGS) for Bacterial Community Analyses

According to several authors (e.g., [69–76]), molecular approaches can help understand hydrogeological settings and dynamics. With this aim, the present study employed next-generation sequencing (NGS) techniques to analyze microbial communities naturally occurring in shallow groundwater and use them as indicators of surface–ground-water interactions and markers of diffuse leakage from sewage networks. Groundwater was sampled from 11 piezometers (Pz1S, Pz2S, Pz3S, Pz4S, Pz3A, Pz4A, Pz5A, Pz6A, Pz7A, Pz8A, Pz3C; Figure 1), and 3 springs (LDF1, LDF2, LDS1; Figure 1).

These groundwater sample points were selected based on their interception of the shallow aquifer, which is directly fed from surface watercourses flowing into the unconfined portion of the aquifer, rendering it the most susceptible to contamination from Disodium EDTA. In particular, the area depicted in Figure 1 includes 11 piezometers drilled to depths ranging from 20 to 27 m and screened within the shallowest gravel and sand layer.

Groundwater samples (2 L) were passed through sterile mixed esters of cellulose filters (S-PakTM Membrane Filters, 47 mm diameter, 0.22 μm pore size, Millipore Corporation, Billerica, MA, USA) within 24 h of collection. Bacterial DNA extraction from these filters was executed using the commercial FastDNA SPIN Kit for soil (MP Biomedicals, LLC, Solon, OH, USA) along with the FastPrep® Instrument (MP Biomedicals, LLC, Solon, OH, USA). Post-extraction, DNA integrity and quantity were assessed via electrophoresis in 0.8% agarose gel containing 1 μg/mL of Gel-RedTM (Biotium, Inc., Fremont, CA, USA). The bacterial community profiles in the samples were then generated using next-generation sequencing (NGS) technologies at the Genprobio Srl Laboratory, adhering to the protocol outlined by Ducci et al. [64].

4. Results

4.1. Hydrogeological Model

Starting from the south, in the intravalley lowland (LDCA and LDLA wastewater treatment plants), groundwater flows within the unconfined coarse deposits according to the topographic gradient. Recharge, in addition to local precipitation, could derive from the Apennine aquifers located on the valley slopes and from the Baganza and Parma streams waterflow. The presence of fine sediment in or on top of the most superficial aquifer is so minimal as never to support shallow groundwater confinement conditions. Low permeability rock units (turbidites) or aquiclude marly-clay units can be found at the base of the alluvial gravel aquifer, as well as the edges (sections aa' and bb' in Figure 2).

In the foothill fluvial terracing contexts (LDTR, LDFE, and LDCO wastewater treatment plants), groundwater flows under unconfined conditions and within an aquifer in hydraulic continuity with the riverbeds, respectively, of Termina Stream, Cinghio Stream, Manubiola Stream, and Taro River. In these cases, the frequent top lap stratigraphic geometries (see Mitchum [77]) of the higher-permeability strata could facilitate the downflow of groundwater and, consequently, of the released substance to the deeper portion of the multilayer aquifer (sections cc', dd', ee', gg' and ff' in Figure 2).

Proceeding to the north, toward the plain (location of the remaining wastewater treatment plants and sampling points shown in Figure 1), an increase in thickness and frequency of the finer layers is known to promote confinement of the more superficial aquifer; however, this condition may disappear where the integrity of aquitards is disrupted by the incision of current riverbeds or their past record. Recharge derives mostly from upstream and from losing riverbeds. Down valley to all the analyzed wastewater treatment plant's release points, this condition has been verified from the hydrogeological setting to result in the correspondence of Recchio, Parma, and Parmetta streams (sections hh', ii' and mm' in Figure 2).

All the reconstructed scenarios show frequent interaction relationships between surface water and groundwater despite the substantial difference in geographical and geological location of the analyzed sections (Figure 2). Therefore, canals, rivers, streams, and their riverbeds composed mainly of highly permeable sediments (sand and gravel) diffusely favor the migration of Disodium EDTA towards the groundwater within the whole study area.

The hydrogeological findings provide a crucial understanding of the aquifer's vulnerability and its interaction with surface water, establishing the foundation for the subsequent examination of industrial activities in the region (see paragraph below 4.2).

4.2. Productive Activities and Wastewater Treatment Plants

In the study area, there are a total of 316 industrial activities that collectively impact the 8 selected wastewater treatment plants, and among them, 25 may use Disodium EDTA. Industrial activities attributed to the use of Disodium EDTA fall within the following production categories (ATECO): (i) production of ready meals and dishes from other food products, (ii) manufacture of basic pharmaceutical products, (iii) manufacture of toiletry products: perfumes, cosmetics, soaps, and similar items, (iv) production of other food products not elsewhere classified (n.e.c.), and (v) packaging and wrapping of food products (see Table 1) [78,79].

Table 1. Complete inventory of industrial activities in the study area that, according to the ATECO description of their production activities, could potentially use Disodium EDTA.

ATECO Code	ATECO Description of Economic Activities	Number of Industrial Activities in the Study Area
10.85.09	Production of ready meals and dishes from other food products	7
21.10.00	Manufacture of basic pharmaceutical products	2
20.42.00	Manufacture of toiletry products: perfumes, cosmetics, soaps, and similar items	3
10.89.09	Production of other food products not elsewhere classified (n.e.c.)	2
82.92.10	Packaging and wrapping of food products	3

Diverse features characterize the eight selected wastewater treatment plants. The range of average daily volume varies from a minimum of 450 to a maximum of 24,000 (m^3/d). The population equivalent units range from a minimum of 4000 to a maximum of 168,000, while the number of inhabitants ranges from a minimum of 2062 to a maximum of 100,015 citizens. Table 2 summarizes the most significant characteristics of each plant, including the total number of industrial activities that discharge effluents into the individual wastewater treatment plant and those attributed to the use of Disodium EDTA.

Table 2. Sampled wastewater treatment plant characteristics.

	Plant Capacity				
Wastewater Treatment Plant	**Average Daily Volume (m^3/d)**	**Population Equivalent Units (n)**	**Inhabitants (n)**	**Industrial Activities (n)**	**Industrial Activities that Can Use Disodium EDTA (n)**
LDCA	450	4000	2062	1	0
LDME	450	9600	3168	4	0
LDCO	3000	20,000	14,711	22	1
LDFE	6700	50,000	9168	56	4
LDFO	3800	16,000	5543	15	1
LDLA	10,500	25,000	10,801	126	1
LDPO	24,000	168,000	100,015	61	8
LDTR	1800	9900	9591	31	2

The preliminary assessment of industrial activities using ATECO codes allowed for the identification of potential sources of Disodium EDTA, justifying the selection of effluent samples from the associated treatment plants, whose discharges are released into portions of the aquifer where interaction has been confirmed, as discussed in Section 4.1.

4.3. Chemical Results

Disodium EDTA was detected in all wastewater samples collected within the present study, with concentrations ranging from 80 to 980 µg/L and an average value of 343 µg/L (Table 3).

Table 3. Results of Disodium EDTA analyses.

Sample	Parameter (CAS)	Technique	Analytical Method	Disodium EDTA (µg/L)
LDCA	Disodium EDTA (139-33-3)	GC-MS	EN ISO 16588 [68]	83
LDME	Disodium EDTA (139-33-3)	GC-MS	EN ISO 16588 [68]	80
LDCO	Disodium EDTA (139-33-3)	GC-MS	EN ISO 16588 [68]	340
LDFE	Disodium EDTA (139-33-3)	GC-MS	EN ISO 16588 [68]	350
LDFO	Disodium EDTA (139-33-3)	GC-MS	EN ISO 16588 [68]	160
LDLA	Disodium EDTA (139-33-3)	GC-MS	EN ISO 16588 [68]	480
LDPO	Disodium EDTA (139-33-3)	GC-MS	EN ISO 16588 [68]	270
LDTR	Disodium EDTA (139-33-3)	GC-MS	EN ISO 16588 [68]	980

The chemical analysis confirms detectable levels of Disodium EDTA in the sampled effluents, illustrating its persistence after the treatment and potential impact on the aquifer.

4.4. Biomolecular Investigations

The MiSeq sequencing runs produced an average of 55,033 sequences for the samples collected at piezometer Pz1S, Pz2S, Pz3S, Pz4S, PZ3A, Pz4A, Pz5A, Pz6A, Pz7A, Pz8A, and Pz3C. It should be noted that the final read number reported for the Pz3C point fell below 30,000 (refer to Table 4), which may not be sufficiently high for a thorough examination of the microbial community. Regarding spring samples, the amount of DNA extracted was so low that the PCR failed and did not allow the microbial community analysis to be performed.

Table 4. Number of 16S rDNA sequences obtained after NGS analysis.

Groundwater Sample	Final Read Number
Pz1S	45,846
Pz2S	39,521
Pz3S	54,855
Pz4S	54,741
Pz3A	69,745
Pz4A	51,091
Pz5A	77,817
Pz6A	73,062
Pz7A	59,631
Pz8A	44,792
Pz3C	23,600

The 16S rRNA gene sequences generated in this study have been archived in the NCBI Sequence Read Archive under accession number PRJNA1117376.

The analysis of the groundwater microbial community revealed a high degree of heterogeneity.

Proteobacteria, *Bacteroidetes*, *Firmicutes,* and *Actinobacteria*, accounting for average values ranging from 9.21% to 55.31%, were the four major phyla in the groundwater samples. *Proteobacteria* was the dominant phylum in most samples, except for Pz4A, where *Bacteroidetes* was the most abundant phylum (51.11%), while for Pz6A and Pz8A, the highest concentrations were achieved by *Firmicutes*, with concentrations of 40.41% and 50.39%, respectively.

The most abundant families in groundwater samples were *Burkholderiaceae*, *Comamonadaceae*, *Sphingomonadaceae*, *Flavobacteriaceae*, *Lachnospiraceae*, *Oxalobacteraceae*, *Xanthomonadaceae*, and *Acetobacteraceae*, with a range of average abundance between 1.97% and 8.26%. The *Burkholderiaceae* family shows significant variation in abundance levels across the different sampling points. At the Pz1S, Pz2S, Pz3S, and Pz4S points, the abundance was, respectively, 23.91%, 21.67%, 20.13%, and 13.31%. However, this abundance decreased significantly at Pz3A, Pz4A, Pz5A, Pz6A, Pz7A, and Pz8A, ranging between 0.11% and 1.50%. A similar trend can be observed for the *Sphingomonadaceae* family, with abundance ranging from 13.77% to 18.03% at Pz2S, Pz3S, and Pz4S. The abundance was minimal in the remaining sampling points, ranging from 0.17% to 2.75%. The families *Xanthomonadaceae* and *Acetobacteraceae* exhibited significant presence exclusively at Pz1S, with values of 20.90% and17.72%, respectively, while in the remaining sampling points, the abundance oscillated between 0.03% and 1.13%. The *Flavobacteriaceae* family was particularly abundant at the Pz4A and Pz4S points, with 45.39% and 6.32%, respectively. The abundance was very low in the remaining sampling points, ranging from 0.06% to 1.23%. The *Comamonadaceae* family showed significant abundance at sampling points Pz3A, Pz4A, Pz5A, and Pz7A, with percentages ranging from 3.12% to 27.75%. *Lachnospiraceae* dominated at Pz6A and

Pz8A with concentrations of 15.07% and 19.91%; *Oxalobacteraceae* dominated at Pz7A with 23.03%, while the concentration was strongly limited at the other points.

At the genus level, the most abundant bacteria found in all groundwater samples were *Flavobacterium*, *Limnohabitans*, *Novosphingobium*, *Pseudomonas*, *Hydrogenophaga*, *Herminiimonas*, *Silanimonas*, and *Roseococcus*; the concentrations averaged between 1.82% and 5.46%. In particular, the average prevalence of each bacterial genus provides an overview of the distribution of bacterial genera across the entire dataset of this study; however, each sample has a unique bacterial composition, with a distinct combination of dominant bacterial genera (Table 5). Various bacterial genera are more prevalent than others in different samples, indicating significant variations in their prevalence among the different samples. For example, it can be observed that sample Pz4A is mainly composed of *Flavobacterium* at 44.74%, while *Limnohabitans* is the predominant genus at 22.31% in sample Pz7A and *Hydrogenophaga* in Pz1S, with a concentration of 22.01%.

Table 5. Percentage distribution of the top 3 bacterial genera for the different sampling points (Pz).

Pz3A		Pz4A		Pz5A		Pz6A		Pz7A	
Methylobacter	5.03%	Flavobacterium	44.74%	Pseudomonas	6.75%	Bacteroides	8.15%	Limnohabitans	22.31%
Methylotenera	4.68%	Limnohabitans	22.92%	Lactobacillus	3.97%	Escherichia-Shigella	6.06%	Herminiimonas	22.01%
Methylomonas	4.46%	Pseudarcicella	4.28%	Streptococcus	2.90%	Bifidobacterium	3.61%	Pseudomonas	11.69%
Pz1S		Pz2S		Pz3S		Pz4S		Pz8S	
Hydrogenophaga	22.01%	Novosphingobium	12.14%	Novosphingobium	14.26%	Novosphingobium	8.00%	Bacteroides	7.21%
Silanimonas	20.81%	Azospirillum	6.00%	Limnohabitans	5.17%	Azospirillum	7.47%	Faecalibaculum	6.88%
Roseococcus	17.60%	Sphaerotilus	5.93%	Sphingomonas	3.60%	Flavobacterium	6.26%	Lactobacillus	6.57%

To sum up, the groundwater community is significantly heterogeneous; however, it is emphasized that, at the lower level, the shallow aquifer is characterized by a bacterial community predominantly composed of Gram-negative, aerobic, chemoorganotrophic bacteria, with wide ranges of growth such as *Flavobacterium* [80], *Limnohabitans* [81], *Novosphingobium* [82], *Pseudomonas* [83], and *Hydrogenophaga* [84]. Furthermore, it is noteworthy that among the top 3 abundant genera detected in Pz5A, Pz6A, and Pz8A, bacterial genera belong to the mammalian intestinal microbiome (e.g., *Bacteroides, Escherichia-Shigella, Bifidobacterium, Lactobacillus, and Faecalibaculum*) [85–89].

The dominance of aerobes confirms that the studied aquifer is diffusely unconfined or semi-confined and/or diffusely fed by surface water courses. Additionally, the presence of fecal bacteria indicates diffuse leakage from sewage networks, which contain pre-treated wastewater and, consequently, Disodium EDTA [43]. These data confirm further vulnerability for the shallow aquifer; in addition to its interaction with surface waters, losses from the sewage system, including Disodium EDTA, can potentially impact groundwater quality [43].

From the perspective of potential genera capable of degrading Disodium EDTA, organisms including representatives of the genera *Methylobacterium, Variovorax,* and *Bacillus* were isolated through the use of a mixed culture utilizing EDTA as the sole carbon source from an effluent treatment plant [90]. In the analyzed samples, the genus *Methylobacterium* was detected in groundwater samples Pz3A, Pz5A, Pz6A, Pz7A, Pz8A, Pz2S, Pz3S and Pz4S with an average of 0.19%. Additionally, the genus *Variovorax* was found with an average concentration of 0.20% in Pz3A, Pz4A, Pz5A, Pz6A, Pz7A, Pz8A, Pz2S, Pz3S and Pz4S, while *Bacillus* was found in Pz3A, Pz4A, Pz5A, Pz6A, Pz7A, Pz8A and Pz3S with a concentration of 0.27%. These data show that, based on the literature available so far, the bacterial community is not significantly characterized by microorganisms with degradative potential for Disodium EDTA.

5. Discussion

In summary, this study highlights the critical importance of interdisciplinarity in understanding and managing the potential contamination of groundwater by Disodium EDTA and, more widely, of CECs. The combined analysis of the aquifer's hydraulic properties and the autochthonous microbial community provided valuable insights into local hydrodynamic processes and the interaction between surface water bodies, where Disodium-EDTA-impacted effluents are discharged, and groundwater. This integrated approach offers a more comprehensive and refined assessment of contamination risks, as current detection techniques for groundwater are still not sensitive enough to directly identify this compound in aquifers [43].

The incapacity of water treatment plants to remove chemicals from the class of CECs [17,18] endangers surface water and poses a significant threat to groundwater quality [91–93]. In areas where surface and groundwater interact, such as the study area in this research, wastewater pollutants can contaminate aquifers.

In this specific case, the study area is characterized by high industrial activity, totaling 316 industrial activities. Of these, 25 may use Disodium EDTA. The daily volume of wastewater varies widely between different plants, ranging from 450 to 40,000 (m^3/d). Population equivalent units fluctuate between 4000 and 180,000, while the population residing in the area ranges from 2062 to 100,015 inhabitants. Within this heavily industrialized area, the aim of this study was to diversify, as much as possible, the wastewater treatment plants from which effluents were sampled while keeping constant the fact that discharges were released into portions of the unconfined aquifer, which, through dispersion, feeds the underlying shallow aquifer. The risk of contamination from wastewater in the study area is confirmed by the detection of Disodium EDTA in all wastewater samples collected within the present study, with concentrations ranging from 80 to 980 μg/L, with an average value of 348 μg/L. In particular, as shown in Figure 3, no association was found between the number of industries potentially using Disodium EDTA, according to the ATECO code, discharging wastewater into each treatment plant and the concentration of Disodium EDTA detected in the plant's effluents. Despite variations in the presence of industries utilizing Disodium EDTA among different treatment plants, the concentrations of the substance fluctuate without a discernible pattern. For example, LDPO, with eight industries using Disodium EDTA, exhibits concentrations of 270 μg/L, while LDTR, with only two industries utilizing Disodium EDTA, records the highest concentration of 980 μg/L. LDCA and LDME, which have no industries using Disodium EDTA, show overlapping concentrations of 83 μg/L and 80 μg/L, respectively. Furthermore, considering the characteristics of the plants in terms of size and treated volumes, this lack of direct association is emphasized, challenging conventional assumptions and highlighting the need for further research to clarify the complex factors influencing the presence of Disodium EDTA in wastewater treatment plants. Additionally, the overlapping concentrations between LDCO and LDME suggest the possibility of "background contamination" from domestic effluents [94].

However, despite (a) the widespread detection of Disodium EDTA in wastewaters, (b) the hydraulic interconnection between surface- and groundwater, and (c) the losses from the sewage system, Disodium EDTA has never been detected in the studied groundwater [43]. This result can be due to one or more of the following factors: (i) the relatively high quantification limit of the available analytical techniques; (ii) the dilution of Disodium EDTA, also due to hydrodynamic dispersion within the aquifer system; and (iii) the possible microbial natural attenuation (Figure 4).

The results of the microbial community suggest that the vulnerability of the shallow aquifer remains high, as evidenced by the presence of aerobic bacteria that confirm the aquifer is diffusely unconfined or semi-confined and/or widely fed by surface water courses, along with the presence of genera belonging to the mammalian intestinal microbiome, indicating leaks from sewer networks. Both of these factors expose the shallow aquifer to pollution from Disodium EDTA, highlighting its vulnerability; on the one hand, through effective infiltration from surface water bodies impacted by contaminated effluents, and, on the other hand, through leaks from sewage systems [43] (as shown in Figure 4). Furthermore, although some microbial genera with potential degradative capabilities for EDTA, such as *Methylobacterium*, *Variovorax*, and *Bacillus*, have been detected in groundwater, their concentrations appear to be low, suggesting that the possible contribution of biodegradation to the fate of Disodium EDTA in the studied aquifer is limited. It is important to note that this assessment of low capacity is exclusively based on bacteria whose physiology is known and for which the degradative potential towards compounds like EDTA has been tested; in particular, for uncultured organisms, their degradative capabilities are unknown, and they represent a significant portion of the microbial community in the analyzed samples, with percentages ranging from 3.63% to 49.70%, and an average of 20.40%.

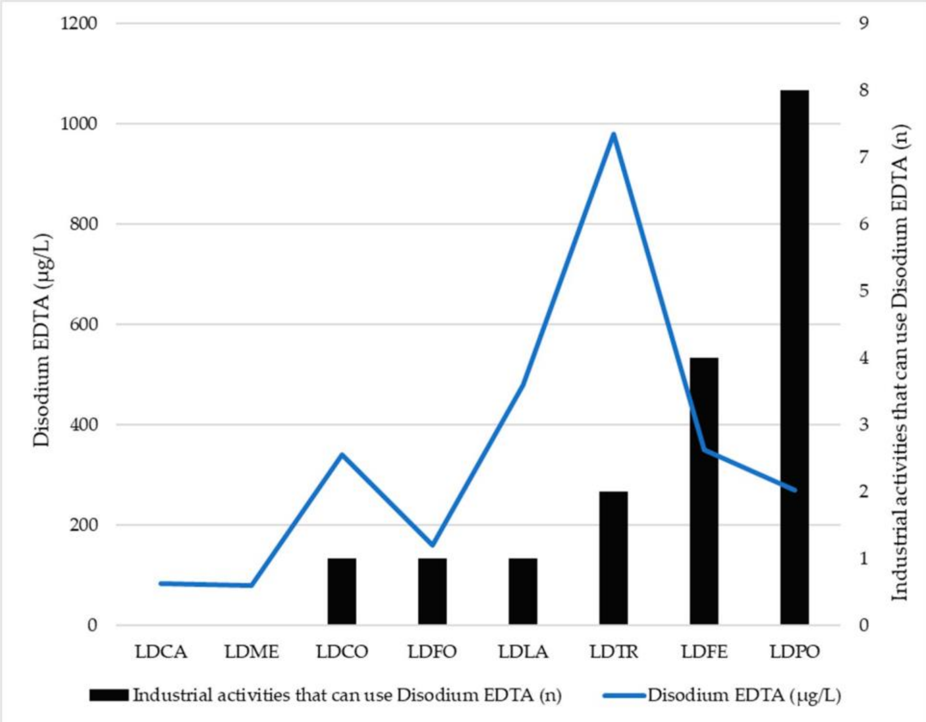

Figure 3. Comparison between the number of industries potentially using Disodium EDTA and the concentration of Disodium EDTA in wastewater effluents. The bar chart (black bars) represents the number of industrial activities categorized by the ATECO code that can use Disodium EDTA. The line graph (blue line) shows the concentration of Disodium EDTA (μg/L) detected in the effluents of each corresponding treatment plants.

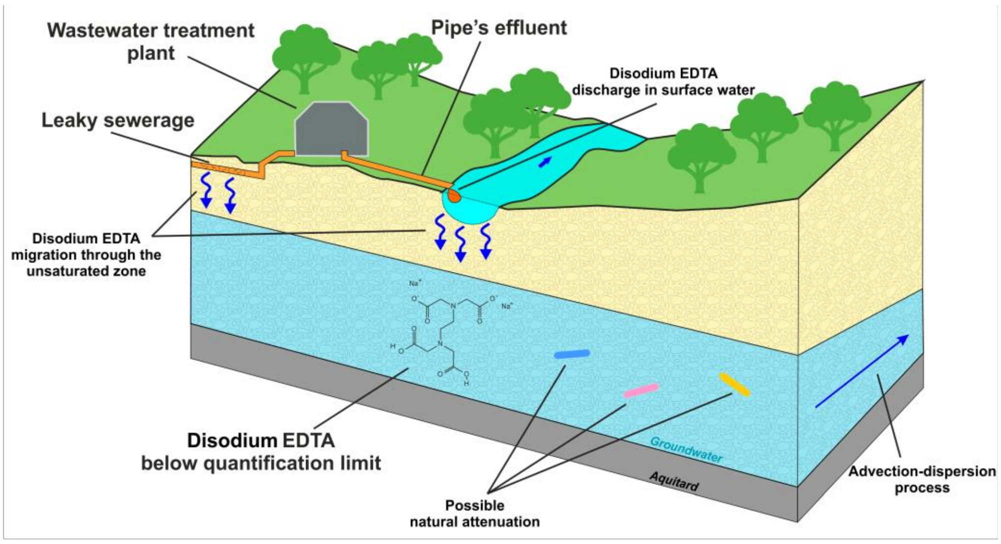

Figure 4. Conceptual model.

6. Conclusions

In conclusion, the findings of this study highlight the significant challenges posed by the presence of Disodium EDTA in wastewater and its potential impact on both surface water and groundwater quality. The inability of conventional water treatment plants to effectively remove such chemicals increases the risk of contamination, particularly in regions characterized by high industrial activity, including those involved in food production and packaging as well as PPCPs production. Despite the absence of detectable levels of Disodium EDTA in groundwater thus far, the hydrogeological dynamics elucidated in this interdisciplinary research underscore the interconnectivity of surface and subsurface water systems, thereby expanding the scope of potential environmental repercussions. Consequently, there is a pressing need to enhance methodologies to lower the instrumental quantification limit within aqueous matrices. In a broader context, urgent measures are needed to address the risk of diffuse transport of CECs contaminants like Disodium EDTA and safeguard the integrity of surface and groundwater resources, which are essential for sustaining ecosystems and human health.

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2.3. *A first application of the compositional data analysis (CoDa) approach to refine the delimitation of capture zones around hydraulic barriers in contaminated sites*

1. Introduction

Groundwater pollution from petroleum products is a significant threat in many industrialised areas [1]. Pollution accidents can occur in any oil storage, utilisation or refining context and vary in severity from minor events, such as those under storage tanks, to major accidents at refineries. Another contributing factor to this contamination is pipeline leakage [2; 3], which may arise from construction defects [4], third-party damage [5; 6], or natural events such as landslides and earthquakes [7; 8], as well as corrosion induced by the transported fluid [9; 10]. In any case, petroleum hydrocarbons (PH), due to their lower octanol-water partition coefficient, percolate through soil columns and reach groundwater, contaminating it [11]. Approximately 5.74 million tonnes of oil were lost of tanker accidents in the range of 1970 to 2017 [12] and the total volume of oil lost to the environment from tanker spills in 2023 was approximately 2,000 tonnes [13]. PH are considered emerging pollutants due to their recalcitrance [14]. Petroleum is in fact a complex mixture of gaseous, liquid and solid hydrocarbons with minor quantities of nitrogen, oxygen, and sulphur containing compounds, trace levels of metallic constituents [15 – 17] to which various components and additives are added and combined during the refining and fuel formulation process, with the aim of improving combustion properties.

Oxidants are a category of fuel additives that gained prominence in Europe and the United States in the late 1970s due to the necessity of enhancing the octane rating of gasoline following the removal of tetraethyl lead. In the United States, the addition of oxidants to gasoline increased significantly after the Clean Air Act (CAAA) amendments of 1990, which mandated the use of reformulated and oxygenated gasoline in certain urban areas to reduce air pollution caused by motor vehicles [18]. Fuel oxidants are primarily classified into two chemical categories: ethers and alcohols [19]. The ethers recognised by the U.S. EPA include methyl tert-butyl ether (MTBE), ethyl tert-butyl ether (ETBE), tert-amyl methyl ether (TAME) and diisopropyl ether (DIPE). With regard to oxidising alcohols, the most prominent examples are ethanol (EtOH), tert-butyl alcohol (TBA) and methanol (MeOH). Of these oxidants, methyl tert-butyl ether (MTBE) is the most commonly used in petrol formulations, due to a number of favourable characteristics, including high octane power, low cost, ease of production and favourable transfer and mixing characteristics [20]. Despite the advantages of using MTBE, it has raised significant environmental concerns. Between 1996 and 2002, this compound was detected in over 1,500 public water supply systems across 28 states in the United States [21], raising alarms about human health, particularly regarding potential carcinogenic effects and risks to reproduction and development [22]. In response to environmental regulations, many markets began replacing MTBE with ETBE, an additive produced from bioethanol [23]. However, despite ETBE's solubility in water and its relatively persistent nature in the environment, it raises similar concerns regarding water quality [19].

In light of the environmental concerns associated with this type of pollution, Feo et al. have developed a three-dimensional model that can be used to accurately analyse and predict the migration of contamination plumes to mitigate the spread of such contaminants into groundwater [24]. More generally, barrier wells are constructed at contaminated sites as containment systems designed to prevent the migration of contaminants. Their effectiveness is monitored through the analysis of water flow and water quality downstream of the barriers. Hydraulic barriers can vary in terms of design and implementation, and their effectiveness depends on factors such as site geology, the quantity and type of contaminant, and local hydrogeological conditions. Verifying the functionality of barrier wells is crucial to ensure that these containment measures are truly effective [25]. To evaluate the efficacy of hydraulic barriers and provide management strategies, several research studies and protocols have been established, including the coupled experimental-modeling procedure [26], a numerical study examining the effects of various barrier configurations on groundwater flow [27], and an analytical model for analyzing groundwater flow in the presence of an impermeable barrier [28]. However, to our knowledge, the effectiveness of barrier wells has never been statistically evaluated. Considering the data´s compositional nature [29] a Compositional Principal Component Analysis (CoDa-PCA) was applied as an exploratory approach [30], aiming to analyze samples' variability; and at the same time allowing a dimensionality reduction. The CoDa biplot worked as a tool to investigate if and how much this statistical approach can be used to check the effectiveness of the functionality of barrier wells in a study area affected by spill events, through the 2019-2023 timeline. The main challenge when dealing with compositional data such as the chemical content of water samples, is that traditional statistical analyses, based on the computation of correlations or covariances, can lead to misleading results. Aitchison's groundbreaking work [29] laid the foundation for compositional data analysis (CoDa), which has since been developed further by many others [e.g. 31 – 34]. The CODA approach has been widely applied in various fields, including geochemistry [35 – 38], where issues are addressed using standard compositional techniques [30]. In this study, the first step was to define a Pollution Chemical Indicator, using the geometric mean of the considered contaminants (MTBE, ETBE, and TPH) in each sampled point (A, B, C, D, E, F, WX, WY, WZ and WW), to approach pollution characterization and handle zero replacement. The second step involved the CoDa-PCA computation to evaluate the space-time impacts in the studied area, with an expert-based validation.

This research is the result of the work carried out entirely at the Polytechnic Institute of Castelo Branco, where all the activities necessary for its development have been conducted. The data used in this study is private and under industrial confidentiality, therefore, cannot be shared with the scientific community, and no details will be given concerning the study site and the raw data acquired through hydrogeological, hydrochemical, and isotopic investigations.

2. Study Area

The study area has been operational since 1970, serving as a site for the distribution, intermediate storage, and pipeline transfer of different petroleum products. More recently, an environmental remediation process has been initiated, leading to the development and implementation of a groundwater remediation system involving the extraction of contaminated water through dedicated pumping wells. As part of the ongoing environmental procedure and the remediation activities currently in progress, groundwater level measurements and sampling for chemical analyses are carried out on a quarterly schedule. In particular, groundwater quality assessments have reported elevated ETBE concentrations in barrier wells WZ and WW, as well as in piezometers B and E.

3. Materials and Methods

3.1. Experimental investigations

The hydraulic head was measured once (in March 2022) in six piezometers (A, B, C, D, E, and F) drilled within the study area, using a water level meter, as to reconstruct the groundwater flow net and check the effect of the hydraulic barrier from the hydraulic point of view. Piezometers are up to 90 meters deep and fully screened, while pumping wells are about 80 meter deep and fully screened. Wells WX and WY pump a negligible discharge, while wells WZ and WW pump up to about 15 L/min each.

A pumping test was conducted in order to characterize the aquifer properties close to the hydraulic barriers. The test was conducted pumping a constant flow rate of 33.4 L/min for about 10 hours from well WW and observing drawdown in monitoring well WZ, as well as in piezometers B and E. The distance between the well WW and the observation points range from 9 to 32 meters.

In March 2022, groundwater samples were taken from four piezometers (A, C, D and E) and two wells (WZ and WW) to conduct stable isotope analyses ($\delta^{18}O$, $\delta^{2}H$). No water was sampled in wells WX and WY for chemical analyses due to the presence of LNAPLs (Light Non-Aqueous Phase Liquids) in the wells. All samples were stored in a refrigerated box and transported to the laboratory. Stable isotope analyses were performed using a Delta Plus mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) coupled to an automatic HDO device preparation system. The technique consists of bringing the liquid sample into an isotopic equilibrium, at a controlled temperature of 18 °C, with a pure gas (CO_2 in the case of oxygen and H_2 in the case of hydrogen). The analytical prediction uncertainty was ± 0.1‰ for $\delta^{18}O$, ± 1‰ for $\delta^{2}H$.

3.2. Statistical investigations

The statistical analysis was conducted based on a multi-year dataset, spanning from 2019 to 2023, of Total Petroleum Hydrocarbons (TPH), MTBE, and ETBE concentrations in A, C, D, E, WZ and WW.

This study revisits the 2019-2023 dataset (n. 306 chemical analyses), utilizing a comprehensive and robust compositional exploratory approach. The objective is to delve into the compositional characteristics of the data, with a particular focus on identifying and analyzing space-time associations. By leveraging the available wells and piezometers, this approach aims to provide deeper insights into how environmental variables evolve over time and across different spatial locations. This refined understanding will contribute to more informed decision-making regarding the sustainable management of resources in the region.

3.2.1. The zeros problem

Compositional data, often referred to as compositions, consists of vectors of positive values that quantify the relative contributions of different components within a whole. These data inherently convey only relative information, meaning that the absolute values of the components are less important than their ratios to one another [29; 39; 40]. This unique structure is defined by the Aitchison geometry, a framework developed by Aitchison [29]. which introduced the log-ratio methodology to handle the complexities of compositional data. However, one of the primary challenges with using the log-ratio approach arises when zero values are present in the dataset, as they render the log-ratio calculations impossible, thus complicating compositional data analysis (CODA). To overcome this issue in the current study, the geometric mean of three key components—MTBE, ETBE, and TPH—was computed at each sampled point. This approach served two purposes: first, to derive a singular, representative pollution indicator, and second, to ensure that the resulting data matrix was free of zeros, enabling a coherent and robust analysis. By addressing the zero-value problem, this method allowed for a more accurate and meaningful interpretation of the compositional data, particularly in the context of environmental pollution.

3.2.2. Covariance biplot

Principal Component Analysis (PCA) is a widely used method for multivariate analysis of real-valued data [41]. However, it cannot be directly applied to Compositional Data Analysis (CoDA) because the variables involved are not standard real random variables [30]. In CoDA, the sample space is a restricted subset of the real space. To accommodate this, the CoDa package was used to compute a version of PCA adapted for compositional data, known as CoDa-PCA, which accounts for the unique characteristics of compositional data [29]. CoDa-PCA provides three key tools for compositional data analysis [30]: 1. It uses orthogonal coordinates—such as ilr (isometric log-ratio) and olr (orthonormal log-ratio)—as the principal components; 2. It allows for analysis of sample variability; 3. It facilitates dimension reduction while maintaining the integrity of the compositional structure. Figure 1 shows the covariance biplot [42], where the key vectors in the CoDa-biplot are represented by segments connecting two vertices of rays. In the covariance biplot, longer links represent the primary sources of variation in the samples.

4. Results

The drawdown observed at the pumping well and at the monitoring points during the pumping test revealed maximum drawdowns of approximately 7.25 m at the pumping well, 2.0 m at B, and 1.2 m at WZ. These data were processed using Neuman's [43] solution for unconfined aquifers. It was estimated a transmissivity in the order of $5.\cdot10^{-5}$ m^2/s and a storage coefficient in the order of $1.\cdot10^{-3}$. No significant variations were observed using the data measured in the different observation points, therefore suggesting a continuous and nearly homogeneous aquifer medium at the local scale.

The hydraulic head is located several tens of meters below the ground surface and the groundwater flow net clearly shows a large well-capture area around the hydraulic barrier and then its efficacy in capturing the contaminated groundwater.

The $\delta^{18}O$ values range from -5.8‰ to -5.4‰, while the $\delta^{2}H$ values vary between -35.6‰ and -31.9‰. Considering the analytical uncertainty of ± 0.1‰ for $\delta^{18}O$ and ± 1‰ for $\delta^{2}H$, the isotopic data exhibit overlapping $\delta^{18}O$ and $\delta^{2}H$ values across all sampling locations, indicating a high degree of similarity in the isotopic content among the study area. Therefore, the isotopic signature of groundwater further confirms the existence of a nearly homogenous groundwater flowing through a nearly homogeneous carbonate aquifer at site scale.

The concentrations of TPH, MTBE, and ETBE detected in piezometers (A, C, D and E) and barrier wells (WZ and WW) during the observation period often exceeded the regulatory limit value "CSC" (in Italian: *Concentrazione Soglia di Contaminazione*) for each contaminant. For example, in Italy, the CSC value is established by *Decreto Legislativo 152/2006* and its subsequent amendments, and defines the regulatory limit, above which potential environmental contamination is identified [44]. The threshold proposed for TPH is reported in *Decreto Legislativo 152/2006 – Allegato 5 - Parte IV - Tabella 2* and is set at 487 μg/l [44], while for MTBE and ETBE, the limit is set at 40 μg/L by the ISS (in Italian: *Isituto superiore di Sanità)* with *Parere del 12/09/2006 n. 45848*. In more details, piezometer E was characterized by up to ~500 μg/l of TPH, ~90 μg/l of MTBE and ~6100 μg/l of ETBE, while in barrier wells the maximum concentrations were ~900 μg/l, ~120 μg/l and ~18000 μg/l for TPH, MTBE and ETBE, respectively [45]. From the statistical analysis conducted, considering the concentrations of MTBE, ETBE, and TPH, synthesized by the corresponding geometrical mean in each observed point, A, B, C, D, E, WZ, and WW, and working as a Pollution Chemical Indicator, over the period from 2019 to 2023. Significant associations emerge, as shown in Figure 1.

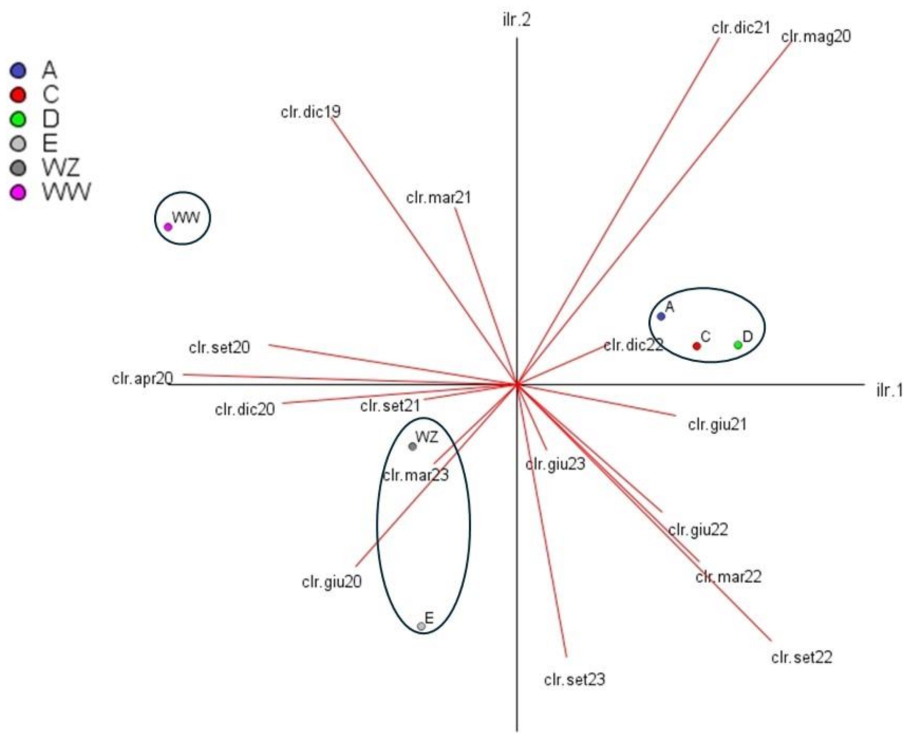

Figure 1. Covariance bi-plot of observed data (Pollution Chemical Indicator), during the period 2019-2023.

Specifically, A, C, and D, as well as WZ and E exhibit noteworthy associations, while WW shows an independent behavior.

5. Discussion

The groundwater flow net clearly shows the efficacy of the hydraulic barrier at the study site. The effect of pumping in terms of interception of the contamination plume is maximized by the hydraulic continuity of the carbonate aquifer, as well as by its nearly homogeneous behavior at site scale. The homogeneous behavior at site scale is further confirmed by the isotopic signature of groundwater, which is characterized by very high similarity within the whole investigated area.

At the same time, the nearly homogenous isotopic content of groundwater does not allow using this natural tracer to define the well-capture zone of each barrier well. Conversely, the statistical analyses of chemical data revealed that E-waters (those characterized by the highest contaminants concentrations among the observation piezometers) are statistically associated with WZ-waters and not with WW-waters, therefore refining knowledge about the extension of the single well-capture zones. These relationships suggest that the well-capture zone related to WZ intersects the contamination plume migrating within the area where E was drilled, in accordance with the groundwater flow net. Furthermore, A, C, and D are represented as an independent group. None of these piezometers indicate the presence of a contamination plume, as the monitoring data for the three analytes in each of the three piezometers are below the CSC thresholds. Therefore, all the piezometers are drilled in portions of the aquifer that are not influenced by the contamination plume. Although A, C, and D are not hydraulically connected, they all represent a portion of the aquifer that, from a chemical standpoint, does not intersect the plume. Furthermore, no association is observed between A, C, and D and wells WZ and WW. This lack of association can be attributed to the spatial proximity of C and D with wells WX and WY. In contrast, although A is close to WW, it does not exhibit hydraulic affinity with it, likely due to the presence of a low-permeability fault zone that compartimentalise the aquifer system, causing that the groundwater intercepted by piezometer A is not involved in the well WW capture zone [46].

6. Conclusion

In conclusion, the multidisciplinary approach adopted has yielded significant results in the study area, providing a valuable foundation for optimizing the operation of barrier systems and safeguarding water resources. The homogeneity of the aquifer at site scale has been confirmed through data obtained from pumping tests and isotopic analyses. Additionally, the exploratory CoDa-PCA allowed a historical overview of the groundwater's dataset concerning the concentrations of TPH, MTBE, and ETBE (synthesized as a Chemical Pollution Indicator by the corresponding geometrical mean), which led to the evidence of association between E and WZ, compatible with the effectiveness of the hydraulic barrier. These findings not only advance our comprehension of pollutant dynamics within the study area through the application of a statistical approach but also provide a foundation for targeted remediation strategies that can be implemented to ensure the ongoing protection of groundwater resources.

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3. Conclusions

The activities carried out during this research project have contributed new insights into the understanding of the dynamics that influence and determine the contamination of groundwater by emerging contaminants. The extraction protocol and methodology developed for microplastic detection in groundwater allowed for the identification and evaluation of the hydraulic dynamics involved in aquifer contamination, with particular attention to the influence of particle characteristics. Microplastics found in groundwater, in particular, show higher circularity compared to those in surface waters, suggesting that particles with more spherical shapes are more easily transported through porous environments, such as alluvial aquifers.

The analysis then focused on personal care products (PCPs), selected based on criteria related to industrial use and chemical properties, including persistence in the environment and potential effects on human health. The field research involved investigations and sampling of groundwater to identify potential contamination from sewer pipe leaks, using the analysis of chemicals (PCPs) and microbial communities as indicators of the presence of domestic wastewater.

A crucial aspect of the project was the evaluation of the potential natural attenuation of phenoxyethanol (Phy-Et), a chemical compound commonly used as a preservative in cosmetic products. In particular, 28-day mesocosm experiments were conducted using two different concentrations (5.2 mg/L and 27.4 mg/L), simulating the reference environmental context. Biomolecular investigations through next-generation sequencing (NGS) revealed the presence of a significant fraction of "hidden" microbial diversity, variations in the composition and abundance of microbial communities in groundwater, and a trend toward reduced biodiversity correlated with increasing Phy-Et concentrations.

Simultaneously, after conducting a territorial analysis to assess the industries present in the study area using ATECO codes (the classification system for economic activities), the study proceeded by investigating the concentration of Disodium EDTA, a synthetic chemical compound used as a chelating agent in cosmetic products and as a food preservative, in the effluents of eight different wastewater treatment plants. All discharges came from activated sludge treatment plants, released into surface watercourses that recharge the alluvial aquifer. The results showed detectable levels of Disodium EDTA in all samples, indicating its persistence after treatment, with concentrations ranging from 80 to 980 μg/L. However, despite (a) the detection of Disodium EDTA in wastewater, (b) the hydraulic interconnection between surface and groundwater, and (c) leaks from the sewer system, Disodium EDTA was never detected in the studied groundwater. The outcome of this phase of the research suggests that the causes may be attributed to one or more of the following factors: (i) the relatively high detection limit of the available analytical techniques; (ii) the dilution of Disodium EDTA, possibly due to hydrodynamic dispersion within the aquifer system; and (iii) the potential microbial natural attenuation.

Finally, data analysis was conducted using a robust Compositional Principal Components Analysis (CoDa-PCA), which gave interesting evidences about the possibility of using, in a near future, statistical approaches to understand the effectiveness of hydraulic barriers in contaminated sites.

In conclusion, the integration of data from different phases of the project has led to significant progress in understanding the dynamics influencing groundwater contamination by emerging contaminants. The interaction between surface and groundwater, particularly in the context of surface waters vulnerable to anthropogenic pollution, proves to be a crucial factor, as these waters can influence groundwater through lateral transfer and/or infiltration processes. In this framework, both microplastics and personal care products (PCPs) present significant challenges, as wastewater treatment plants have proven ineffective in removing them. Moreover, the domestic use of these compounds exacerbates the contamination risk in case of sewer pipe failures, leading to direct release into the soil. Both the discharge of wastewater into surface water bodies and sewer pipe failures are therefore determining factors in the risk of groundwater contamination. The study of the biodegradability of these substances remains an evolving field of research, requiring further studies and in-depth exploration. The conclusions of this work underscore the urgency of addressing, from multiple fronts, the challenges posed by emerging contaminants, emphasizing the increasing need to develop analytical techniques that lower the detection limits for these substances, as well as effective methodologies for their removal from wastewater, in order to implement more effective monitoring and management strategies for the protection of groundwater.