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DOTTORATO DI RICERCA IN
SCIENZE DELLA TERRA

CICLO XXXIV

The potential of soil invertebrates for hydraulic characterization of low permeability media and optimization of soil and aquifer reclamation

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Abstract

The European Commission recognizes that groundwater cannot be considered independently from the surrounding environment, thus necessitating an interdisciplinary approach. Such an approach is needed to understand the consequences of natural and anthropic disturbance on groundwater and hydrogeologic dynamics, which become even more important when low permeability media, or alluvial aquifers, are considered. In these hydrogeological contexts, a close link between soil quality and water resources in proximity exists, in such a way that to improve and sustain water quality it becomes critical (i) to identify the consequences of disturbances on the unsaturated zone in the aquifer and (ii) to restore the quality of the water. Soil disturbances can be related to natural hazards or can be caused by anthropogenic activities: the former can either be triggered by, or can affect, infiltration and groundwater flow, while the latter is often linked to the release of contaminants into the terrestrial environment with associated leaching into groundwater, which are then transported far from their sources. Concerning the identification of the consequences of soil disturbances, recently edaphic fauna has been identified as a potential biological indicator of soil geotechnical and hydrogeological properties. Its use, however, should not be limited only to bioindication. Edaphic fauna plays an important role in maintaining soil quality and health and in providing ecosystem services, as it is involved in the translocation, breaking and decomposition of organic matter, nutrient cycling, soil structure formation and, consequently, water regulation. All these characteristics make edaphic fauna important for its potential application in bioremediation processes.

In the light of these considerations, research for the PhD had the following aims: (i) to identify new biological indicators of hydrogeological characteristics and dynamics in low permeability media, specifically to characterize landslide evolution and assess potential relationships between its hydrogeological features and soil fauna; and (ii) to develop a new approach for the bioremediation of the unsaturated zone of contaminated aquifers using soil fauna in all the bioremediation processes involved (from bioremediation to ecotoxicity monitoring).

From the investigations it emerged that (i) soil fauna abundance and composition (depending on the organisms considered) could be an indicator of hydraulic features in a low-permeability system and, in addition, (ii) soil fauna could contribute to the evaluation of soil quality to support the recovery of areas impacted by natural disturbances. As far as anthropic disturbances are concerned, soil fauna was efficiently involved in all bioremediation phases. Its use as an indicator of environmental contamination was thoroughly assessed considering different types of contamination, allowing the following conclusions to be drawn: (i) the need for toxicity-based approaches to supplement a chemical-based approach when running risk assessment, since bioindicators can provide information on compounds not looked for in chemical analyses; (ii) the importance of selecting different target organisms and end-points in soil-ecotoxicity monitoring, and (iii) the role of elutriation in ecotoxicity tests with soil fauna as a complement of the solid phase. The identification of resistant groups, able to survive in contaminated environments and, therefore, suitable for further investigation in the bioremediation of hydrocarbons, PCBs and PCDD/Fs, was successfully carried out and earthworms were selected as potential organisms of interest. From PCBs and PCDD/Fs bioremediation tests at microcosm scale it emerged that (i) earthworms could be an effective tool to reduce ecotoxicity of organically contaminated soils, (ii) with good possibilities for organic contaminants concentration reduction; (iii) although earthworms offer good possibilities as organic contaminant concentration reduction, their application should be carefully monitored, with the hydraulic characteristics of the contaminated media taken into consideration, since there is a chance that these organisms could enhance the percolation of contaminants and nutrients into groundwater. Such limitations further highlight the need for a multidisciplinary approach in bioremediation studies.

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PRELIMINARY REMARKS

Research activity during the PhD period aimed to investigate the link between soil quality and groundwater through the study of soil fauna. Specifically, soil fauna was studied as a key component within two lines of research: (i) the identification of new biological indicators of hydraulic characteristics and hydrogeological functioning in low-permeability media, and (ii) the development of novel approaches towards the monitoring and bioremediation of the unsaturated zone of contaminated aquifers.

With regard to the first line of research, a landslide area was selected as a study site to investigate soil fauna capabilities as an indicator not only of soil degradation but also of its hydraulic features.

In the second line of research, soil fauna application in all the phases of the remediation process was investigated – from contamination detection to land reclamation and the monitoring of the bioremediation process. In addition, the effect of vermiremediation in the groundwater contamination process and nutrients mobility was assessed.

In order to present the two lines of research and their results, the thesis was divided into three main chapters: (i) an introduction presenting the background leading to the research questions underpinning the aims of the study; (ii) the research outcomes, resulting in scientific papers that make up the sections of the second chapter, (iii) the conclusions, in which the main findings of the investigations and how they meet the goals of the research are reported.

Chapter One

INTRODUCTION

European Commission legislation increasingly recognises that groundwater cannot be considered independently from the surrounding environment and, therefore, needs to be managed through an interdisciplinary approach. Indeed, in analysing the consequences of natural and anthropogenic disturbances on groundwater systems and hydrological dynamics, it is important to understand the influence that these factors exert on the landscape above aquifers (Holman, 2006). This is essential when low permeability media are considered, since in this hydrogeological context the phreatic surface flows at a shallow depth below the ground, normally resulting in interaction between groundwater bodies and the soil.

Soil degradation and consequently soil quality produce a reduction in ecosystem functions and services (Lal, 2015) with four types of soil degradation being identified:

- Physical: this involves structural attributes, leading to a reduction in pore geometry and interconnectivity and thus increasing soil susceptibility to erosion, compaction, and desertification, finally reducing water infiltration and increasing surface runoff.
- Chemical: this includes acidification, salinization, nutrient depletion, or even contamination by industrial by-products or waste.
- Biological: this implies the reduction of soil organic matter content and biomass carbon, as well as a decrease in soil activity and biodiversity.
- Ecological: this is a combination of the previous three types of soil degradation, affecting ecosystem functions such as nutrient cycling, water infiltration and purification, producing perturbations of the hydrological cycle and a decline in biome productivity.

It is, therefore, clear that a close link exists between soil quality and water resources in proximity, so that identifying the consequences of soil disturbances and restoring soil quality is critical for improving and sustaining the associated water quality.

Soil disturbances can be related to natural hazards, which can be triggered by, or affect, infiltration and groundwater flow, or can be the result of anthropogenic activities that can shape ecosystem properties.

Section 1.1:
Natural hazards and biological indicators of soil degradation

1.1.1 Hydrological risk and natural hazards: Landslides

Among natural hazards that can be triggered by geologic and hydrologic dynamics, landslides are the most frequent globally; indeed Dilley et al. (2005) reported that, only in the year 2005, the global area exposed to landslides was around 3.7 million km^2, with 66 million people living in the 820,000 km^2 identified as high-risk zones. Landslides represent an issue of concern particularly in mountain environments, where they are frequently triggered by rainfall or rising groundwater that, by increasing pore water pressure, decreases the sheer strength of the soil which may lead to slope failure (Iverson, 2000; Petley, 2008; van Asch et al., 2007) (Figure 1).

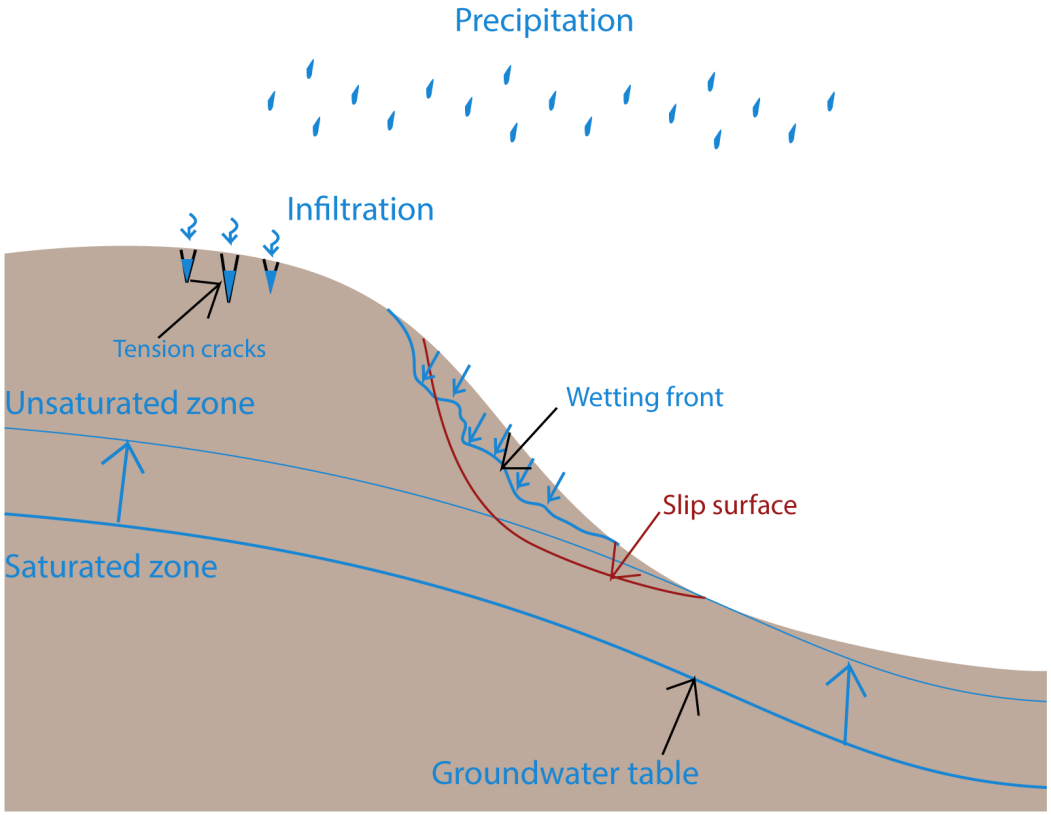

Figura 1. Mechanism of rainfall-induced slope failure (from Arairo et al., 2016; modified)

The occurrence of landslides modifies both the surface landscape and geomorphic conditions, altering topography, chemical and physical properties of the soil, and drainage (Geertsema and Pojar, 2007). The study of this particular natural hazard can, therefore, help in identifying weak zones and contribute to minimizing their impact.

For many years researchers have used chemical and isotopic tracers as indicators of hydrogeological dynamics (e.g. Andreo et al., 2004; Aquilina et al., 2006; Celle-Jeanton et al., 2003). However, recent studies have observed that microbial communities can be considered biological tracers of great potential in this field (Bucci et al., 2017). A study by Bordoni et al. (2019), which analysed the effects of different agricultural practices, suggested that soil geotechnical and hydrogeological properties can be linked to edaphic fauna.

1.1.2 Soil disturbances indicators: Edaphic fauna

According to the body size, edaphic fauna is categorized into microfauna (< 200 µm), mesofauna (0.2 - 2 mm) and macrofauna (> 2 mm - 20 mm), with functions ranging from physical effects to chemical and biological processes. Soil fauna plays an important role in maintaining soil quality and health and in providing ecosystem services. It is also involved in many processes such as organic matter translocation, breaking and decomposition, nutrient cycling, soil structure formation and, consequently, water regulation. In particular, through their burrowing and feeding activities, some of these organisms (e.g. earthworms, termites and ants) contribute to the improvement of aeration and water infiltration, the incorporation of organic matter into the soil and the stabilization of soil aggregates, leading to their designation as "ecosystem engineers" (Mekonen Ertiban, 2019). As a result, the reduction in diversity of soil organisms can cause ecosystem malfunction.

Edaphic fauna communities are highly sensitive to environmental variation and destabilization as they live, feed, and reproduce in the soil, making them a good bioindicator of soil conditions (Menta and Remelli, 2020). Moreover, as Mekonen Ertiban (2019) have observed, soil fauna has two advantages compared to microbial communities: first, as they are at a higher level in the food chain, they serve as integrators of physical, chemical, and biological properties related to feeding habits; and second, their generation time is longer than metabolically active microbes, making them more temporally stable. Since natural hazards change both the topography and soil properties, soil fauna can be a useful indicator for this type of ecosystem disturbance.

Section 1.2:
Anthropogenic disturbances and bioremediation

1.2.1 Land contamination and environmental sustainability

Apart from natural hazards, anthropogenic disturbance is one of the major drivers of terrestrial ecosystem dynamics globally. In fact, over the last few decades, increasing urbanization and industrialization have been accompanied by a constant release of potentially harmful chemical compounds into the terrestrial environment with associated leaching into groundwater, rivers, and lakes (Figure 2).

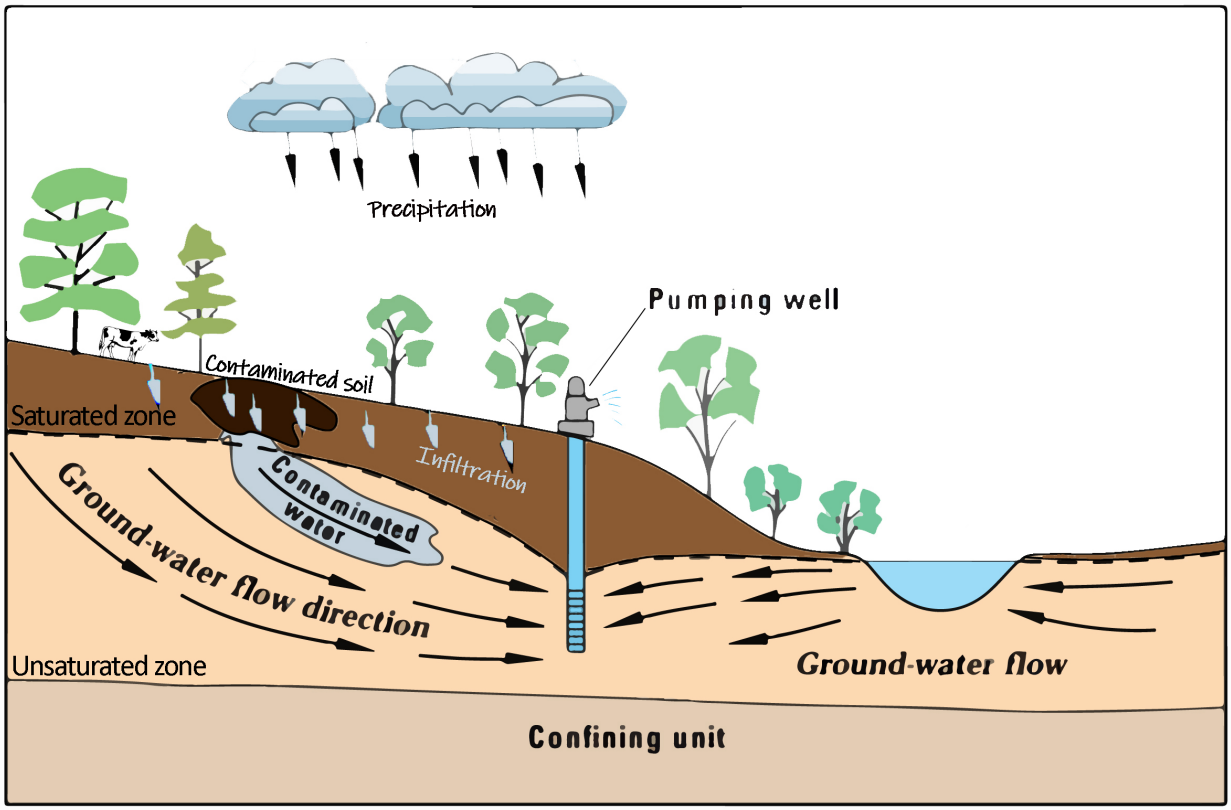

Figura 2. Cross-section of contaminant infiltration and transport through groundwater flow (from U.S. Geological Survey, 1998; modified)

The resulting transport of contaminants far from their sources makes polluted soils a widespread problem of global concern (Fontanetti et al., 2011).

However, due to the high cost of physicochemical techniques, together with the need to adopt more environmentally sustainable strategies, researchers have turned their attention to bioremediation, an eco-friendly alternative for soil remediation. Bioremediation techniques can be applied both in situ or ex situ, with the main purpose of reducing, detoxifying, degrading, mineralizing, or transforming more toxic pollutants into less toxic ones. The goal is the recovery of appropriate microbial, plant and animal communities to support ecosystem functioning (Majer, 1989). In bioremediation microbial communities have represented the main focus. Bacterial activity, however, may be limited by detrimental environmental conditions (e.g. anaerobic conditions, pH, nutrient levels, contaminant bioavailability, etc.) and an unfavourable energetic balance or reduced bioavailability (Campanella et al., 2002; Hickman and Reid, 2008).

Bioremediation efficiency, therefore, mainly depends on the following three variables (Sharma, 2020):

- The nature and concentration of pollutants. In addition to the concentration of the contaminant, the rate of degradation depends on the nature of the pollutant (e.g. agrochemicals, chlorinated compounds, dyes, greenhouse gases, heavy metals, hydrocarbons, nuclear waste, plastics, and sewage) and, consequently, on the amount of "catalyst" present, meaning the number of organisms able to metabolize the contaminant (Abatenh et al., 2017; Azubuike et al., 2016; Sharma, 2020).
- The physicochemical characteristics of the environment. Microorganism growth and activity are affected by pH, temperature, moisture, soil structure, solubility in water, nutrients, site characteristics, redox potential, and oxygen content (Abatenh et al., 2017; Sharma, 2020).
- Contaminant bioavailability. Physicochemical bioavailability of pollutants to the microbial population (i.e. contaminant concentration, type, solubility, chemical structure, and toxicity) (Abatenh et al., 2017; Sharma, 2020).

Given the proper environmental conditions, therefore, the success of bioremediation depends on the bioavailability of the contaminant to a microbial population capable of degrading it (Abatenh et al., 2017).

1.2.2 Bioremediation and monitoring: Edaphic fauna approach

Despite soil decomposer fauna being rarely considered in remediation processes on account of its scarce ability to degrade the chemicals themselves, this fauna can enhance microbial activity and also take part in the entire remediation process (Haimi, 2000). In particular, Haimi (2000) suggested two roles for soil fauna: first, it can be used in ecotoxicological tests to assess the toxicity of the contaminated matrix in pre- and post-remediation, and throughout the remediation process; and second, it can participate in the bioremediation process itself, either directly (depending on the type of contaminant, e.g. organic contaminants) or indirectly by enhancing site recovery and ecosystem functioning.

When risk assessments are carried out, it is important to note that only bioassays are able reflect the effects of bioavailable contaminants (Chapman and Long, 1983). In fact, when testing for toxicity, chemical analyses allow for the characterization of the contamination level of the medium (soil or water), but are inadequate when employed to assess its biological quality.

In the bioremediation process, organisms involved can be either autochthonous or allochthonous, with the former present in polluted environments being the most promising as one of the main problems in bioremediation is represented by the toxicity of the contaminants to organisms; polluted environments, in fact, have been found to be favourable for the growth and metabolism of autochthonous species (Azubuike et al., 2016; Verma and Jaiswal, 2016). Among soil fauna, the various groups exert their effects at spatial scales related to their size and activity ranges. One example is represented by earthworms, which are able to affect the hydraulic properties of soil by causing bypass flow (Edwards et al., 1990); as ecosystem engineers, earthworms can also create new habitats or modify ecosystem features (e.g. bioturbation), with their burrowing activity significantly altering topsoil porosity, leading to enhanced infiltration, water storage and moisture content control (Mando et al., 1996; Meysman et al., 2006). Since these are variables known to limit bioremediation, all modifications introduced by earthworms are crucial for the biological functioning of the soil as well as improving microbial metabolism and plants uptake of nutrients and water – all these factors make earthworms one of the most eligible candidates for soil restoration (Hickman and Reid, 2008). Not all earthworms species, however, have the same impact either on soil properties or on contaminants, since this depends on the functional group to which they belong (Sanchez-Hernandez, 2019): endogeic and anecic earthworms are soil feeders and display more intense burrowing activity than epigeic ones; in contrast, epigeic and anecic earthworms would be more suitable for degradation of contaminants accumulated in decaying plant debris, because they are closely linked to organic residues accumulated on the soil surface. It is necessary to take all these ecological characteristics into account when choosing the most suitable organisms, depending not only on their potential for soil remediation but also considering the fact that the tunnel network created by earthworms may favour the movement and leaching of contaminants in the soil. Since it is known that soil is an environmental sink for contaminants and acts as a secondary pollution source for other environmental compartments such as groundwater, choosing the right bioremediation strategy becomes even more relevant – in this process, a multidisciplinary approach that takes into account the nature of the contaminant, the type of organisms and soil properties is of fundamental importance (Sanchez-Hernandez, 2019).

Section 1.3:
Aims of the study

Given the previous considerations, research activity during PhD period aimed to investigate novel approaches to prevent deterioration of shallow groundwater bodies, related to both natural and anthropogenic causes, acting on the unsaturated zone, specifically through the monitoring and restoration of soil quality mediated by soil fauna. To achieve this, two parallel lines of research, strictly related to each other, were developed to address the following aims:

- Identify new biological indicators of hydrogeological characteristics and dynamics in low permeability media, where the water table flows at shallow depth, so that groundwater bodies interact with soil. Specifically, the aim of this study was to examine the evolution of a landslide situated in a low permeability media and assess potential relationships between its hydrogeological features and soil fauna.
- Develop a new approach for the bioremediation of the unsaturated zone of contaminated aquifers. The aim was to use soil fauna throughout the entire bioremediation process – from bioremediation processes to ecotoxicity monitoring – so that the following variables could be assessed:
  - □ the ecotoxicity of a shallow aquifer system utilized by the local population for domestic and agricultural purposes;
  - □ the ecotoxicity of an area characterized by active oil seepage, including the identification of the autochthonous soil faunal community;
  - □ the suitability of earthworm – alone and in association with plants – in the decontamination of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) and biphenyls (PCBs), together with the simulation of earthworm action in the groundwater contamination process and nutrient mobility;
  - □ the characteristics of the autochthonous arthropods community in PCBs and PCDD/Fs polluted soils, including the monitoring of the bioremediation process described in the point above using ecotoxicological tests on soil and water matrix.

Chapter Two

RESEARCH ACTIVITY

The activity carried out during this research led to the publication of three scientific papers in indexed journals, with two further articles under submission. For an overview of the results obtained, the correspondence between research aims and articles is reported in this section.

General aim: the identification of new biological indicators of hydrogeological characteristics and dynamics in low permeability media, where the water table flows at shallow depth, so that groundwater bodies interact with the soil.

1. Remelli, S.; Petrella, E.; Chelli, A.; Conti, F.D.; Lozano Fondón, C.; Celico, F.; Francese, R.; Menta, C., 2019. Hydrodynamic and Soil Biodiversity Characterization in an Active Landslide. Water 11, 1882. https://doi.org/10.3390/w11091882
   Specific aim: the characterization of the evolution of a landslide situated in a low permeability media, together with an assessment of the potential relationship between its hydrogeological features and soil fauna.

General aim: the development of a new approach for the bioremediation of the unsaturated zone of contaminated aquifers, using soil fauna throughout the entire bioremediation process.

2. Zanini, A.; Petrella, E.; Sanangelantoni, A.M.; Angelo, L.; Ventosi, B.; Viani, L.; Rizzo, P.; Remelli, S.; Bartoli, M.; Bolpagni, R.; Chelli, A.; Feo, A.; Francese, R.; Iacumin, P.; Menta, C.; Racchetti, E.; Selmo, E.M.; Tanda, M.G.; Ghirardi, M.; Boggio, P.; Pappalardo, F.; De Nardo, M.T.; Segadelli, S.; Celico, F., 2019. Groundwater characterization from an ecological and human perspective: an interdisciplinary approach in the Functional Urban Area of Parma, Italy. Rend. Fis. Acc. Lincei 30, 93–108. https://doi.org/10.1007/s12210-018-0748-x
   Specific aim: the evaluation of the ecotoxicity of a shallow aquifer system utilized by the local population for domestic and agricultural purposes.
3. Remelli, S.; Rizzo, P.; Celico, F.; Menta, C., 2020. Natural Surface Hydrocarbons and Soil Faunal Biodiversity: A Bioremediation Perspective. Water 12, 2358. https://doi.org/10.3390/w12092358
   Specific aim: the evaluation of the ecotoxicity and the identification of the autochthonous soil fauna community in an area characterized by active oil seepages.
4. Remelli, S.; Scibona, A., Nizzoli, D., Mantovani, L., Tribaudino, M., Celico, F., Menta, C. Vermiremediation applied to PCB and PCDD/F contaminated soils and its implications for percolating water (*submitted*)
   Specific aim: an assessment of earthworms' suitability – alone and in association with plants – in the decontamination of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) and biphenyls (PCBs), together with a simulation of earthworm action in the groundwater contamination process and nutrient mobility.
5. Remelli, S.; Celico, F., Menta, C. Ecotoxicity monitoring for vermiremediation assessment of PCB and PCDD/Fs contaminated soils (*submitted*)
   Specific aim: a characterization of the autochthonous arthropod community in PCBs and PCDD/Fs polluted soils, together with the monitoring of the bioremediation process described in the previous study using ecotoxicological tests on the soil and water matrix.

SECTION 2.1:
BIOLOGICAL INDICATORS OF HYDROGEOLOGICAL CHARACTERISTICS AND DYNAMICS

Article

Hydrodynamic and soil biodiversity characterization in an active landslide

Sara Remelli, Emma Petrella, Alessandro Chelli, Federica D. Conti, Carlos Lozano Fondón, Fulvio Celico, Roberto Francese, Cristina Menta

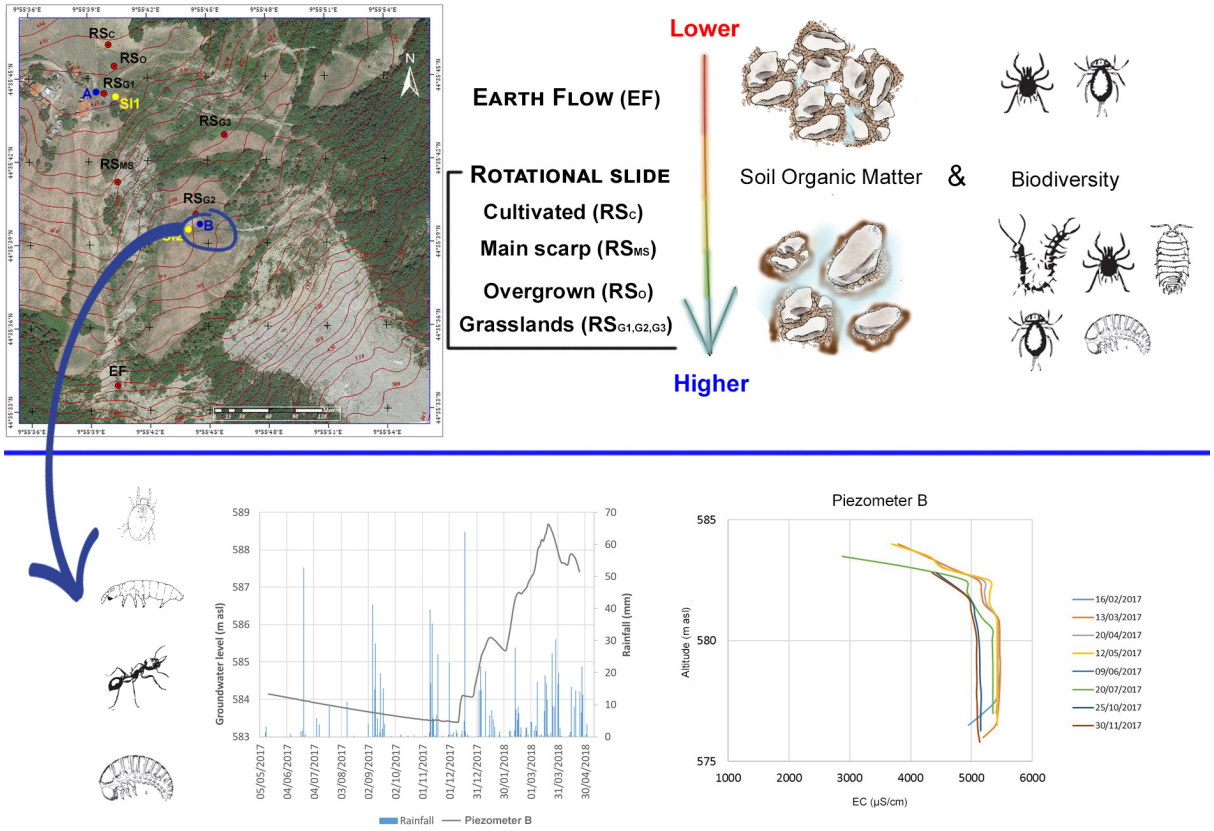

Figura 2. Graphical abstract from Remelli et al. 2019

water

MDPI

Article

Hydrodynamic and Soil Biodiversity Characterization in an Active Landslide

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Abstract: Landslides are common in the Northern Apennines (Italy) and their resulting changes in soil structure affect edaphic fauna biodiversity, whose activity has concurrent impacts on soil structural stability and water-holding capacity. The aim of this study was to characterise landslide evolution and assess potential relationships between its hydrogeological features and soil fauna. The landforms of the study area, located in the River Taro valley, were mapped and the hydraulic head fluctuations and groundwater electrical conductivity profiles were measured. The soil arthropod community was studied in seven sites, one subject to earth flow and six to rotational slide; the last ones were divided into the main scarp of the slide, and five sites characterized by different land use: three grassland, a wheat cultivated field and an overgrown area. Soil organic matter (SOM) and pH measurements were performed. Hydrogeological results suggest unexpected rapid percolation of relatively low-salinity waters through the unsaturated zone. Both lower SOM content and arthropod biodiversity were found in earth flow area, while higher values were found in grasslands. Fauna composition appears to be a good indicator of soil degradation processes, linked to the hydraulic features, and contributes to the evaluation of the soil condition in landslide areas for further agricultural purposes.

Keywords: microarthropods; soil fauna; low-permeability media; porosity; soil organic matter; Collembola; hydraulic features; aquifer heterogeneity

1. Introduction

Landslides are complex systems dependent on geological, geomorphological, hydrogeological and geotechnical factors, and represent worldwide danger for people, buildings and transportation infrastructures. They are common in the Northern Apennines (Italy), a folded and thrusted belt developed since the Cretaceous period [1–4], where different types of landslides can be detected and large earth flows are widespread [5]. In several areas, earth or rock rotational and/or translational slides evolve into earth flows [6,7]. These landslides can be huge [8], and the affected areas can reach up to 10^5 m^2 (with displaced volumes up to 10^8 m^3).

A major role is played by tectonics, which caused the development of faults and thrusts (representing weak zones with associated sets of fractures) controlling the recent uplift that conditioned most of the slopes' geomorphological evolution [6,9]. Nevertheless, rainfall is the triggering factor, especially during those seasons (usually, autumn and spring) that are characterised by precipitations lasting for several days [10]. The distribution of precipitation is influenced by the recent climate changes [11], and prolonged dry periods alternated with intense rainfalls are more and more frequent. The relationship between initial slope conditions and rainfall frequency/intensity, and the reactivation of large landslides is a major research challenge [12,13], and some authors have

attempted to find one-to-one correlation between rainfalls thresholds and slope failures [14], or to integrate methods [15].

Above- and belowground biodiversity is affected by landslides: changes in site condition lead to changes in soil physical and chemical composition and, consequently, in vegetation cover, contributing to increased habitat diversity [16]. The most obvious consequences of landslides are on topography, yet these processes also have a substantial impact on soil properties, by exposing parent material and removing organic matter and A horizons that lead to textural and chemical changes. Landslide processes often bear textural sorting; moreover, remoulding and liquefaction of clays and silts in earth flows reduce structure and porosity, and increase soil density [17]. Changes in soil density and porosity are consequences of erosion, which leads to changes in soil chemistry too [17]. Soil organic matter is mostly found in the soil surface, therefore decreases significantly through erosion; formation of soil aggregates is consequently disrupted, and soil porosity decreases. Reduced soil organic matter leads to overall diminishing of biomass and productivity [18] and sets back the ecosystem to the initial stages of soil development, with deep un-weathered parent material and rock exposure. Since soil biota is affected by the amount of organic matter, its reduction affects biodiversity [19]. Moreover, changes in topography and soil properties and the resultant changes in vegetation, shape a gap between landslides and the surrounding habitat, with chance of settlement for different soil communities. Age and position on the landslide also influence habitat and vegetation communities, as demonstrated by Smith et al. [20] when studying revegetation patterns on debris slides and flows. Changes in fauna community also occur because earth flows create ponds that predispose sites to beaver colonization and floristic explosions on landslide deposits support deciduous-dependent fauna, while soil cliffs related to rotational landslides provide a habitat for small mammals [16]. Since a landslide can be considered a disturbance agent that changes soil and vegetation patterns, changes in edaphic fauna are also expected because soil fauna is closely linked to soil properties and vegetation.

The aim of this research was: (i) to study the effects of a landslide complex on soil biodiversity, in terms of soil arthropod community, in different areas involved in the landslide process (agriculture ecosystems, abandoned farmlands, etc.), and (ii) connect geohydrological information with soil biodiversity in order to show how soil fauna can be used in landslide studies.

2. Materials and Methods

2.1. Study Area

The study area (Case Pennetta landslide) is located in the Taro River valley (Northern Apennines). During the past 25 years, the local climate condition has been characterized by annual mean precipitation ranging from 938 to 1230 mm, and annual mean temperature between 11.0–12.4 °C [10].

In the area, three tectonic units belonging to the Ligurian Domain of the Northern Apennines [21] outcrop. In detail, the geological sequence can be described as follows (from the top to the bottom of the slope): (i) *Arenarie di Scabiazza* (SCB), made up of thin layers of claystone alternating with sandstone layers, 15 to 20 cm thick, that gradually change from thin to very thin sandstone layers ending with marls; (ii) *Argille a Palombini di Monte Rizzone* (AMR), made up of claystones and intercalated decimetric limestone beds. Locally, AMR disappears and SCB lies directly on the rocks of the underlying *Ottone* tectonic unit, which is made of claystones containing clasts and blocks of limestone (*Argille a blocchi*, CCVb); (iii) these lie directly (tectonic boundary) on the underlying *Flysch of Monte Caio* (CAO), which is made up of thick beds of marly-limestones turbidites and intercalated thin-to-medium argillite beds. All the rocks are deeply tectonised and the layering is locally completely destroyed.

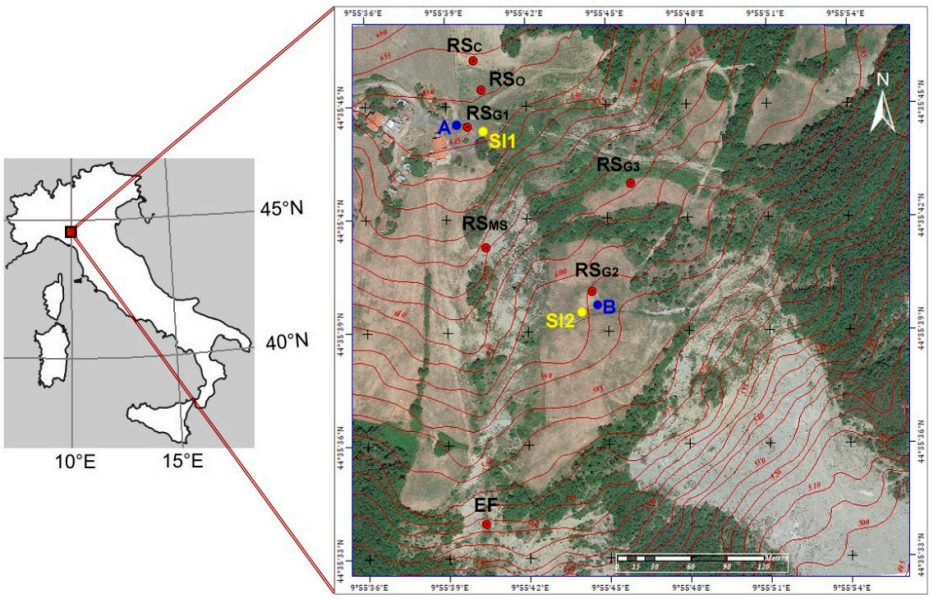
Steep surfaces and scarps usually characterize the lower portion of the studied slope, where the thick beds of marly-limestones turbidites (CAO) crop out. Conversely, milder and smooth landforms are usually found in the upper portion, due to prevailing rocks containing clay and claystone (AMR, CCVb), with the exclusion of the areas affected by landslides.

The main known reactivations of the Case Pennetta landslide occurred in 1916, 1927 and 2000–2001. During this last event, a state road and the Parma-La Spezia railway were involved in the mass movement. Moreover, new surficial effects of the slope's instability have been detected since 2010, both in the fields (cracks and changes in slope shape and dip), and on structures and houses (tilting and microcracks). The landslide's rate of movement was usually in the order of a few mm/year to a few cm/year (from extremely slow to very slow in the velocity scale by Cruden and Varnes [22]), even though some acceleration was observed during total reactivation events (e.g., in 1927 and in 2001). In the past, the landslide underwent a limited number of interventions, essentially in underground and surficial drainage systems.

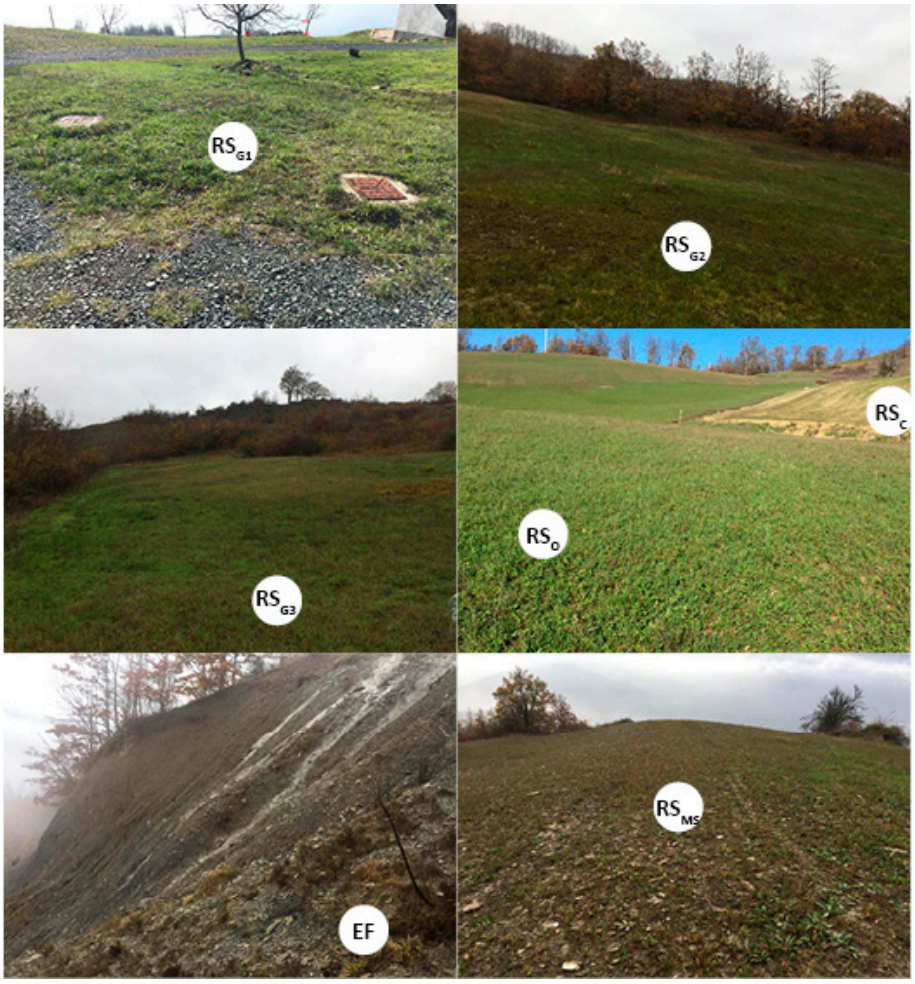
2.2. Geological and Geomorphological Survey

The landforms were mapped by merging geomorphological maps and different datasets [23]. A digital surface model (DSM) of the study site was constructed via photogrammetric mapping using an unmanned aerial vehicle (UAV). DSM was later converted onto a digital terrain model (DTM) voiding vegetation with terrain contours from available digital cartography. Differential GPS measurements provided ground control points for UAV images and also benchmarks for areas covered by vegetation. Elevation contour lines, available from Regional Technical Cartography at scale 1:5000 [23] were utilized to fill in data gaps. The different data types were finally interpolated on a 1 m aperture mesh using a kriging algorithm.

Investigations were performed to acquire the subsurface geological features. Two corings (SI1 and SI2) were drilled within the study site at different depths beneath the field surface (Figure 1a).



(**a**)



(**b**)

Figure 1. (**a**) Location of the piezometers (blue points), the corings (yellow points) and the soil sampling sites (red points) (the contour lines show the altitude in meters above sea level); (**b**) Sampling sites: RS$_{G1}$: Rotational Slide-Grassland 1; RS$_{G2}$: Rotational Slide-Grassland 2; RS$_{G3}$: Rotational Slide-Grassland 3; RS$_{O}$: Rotational Slide-Overgrown; RS$_{C}$: and Rotational Slide-Cultivated; EF: Earth Flow; RS$_{MS}$: Rotational Slide-Main Scarp.

2.3. Hydrogeological Investigations

Two piezometers were drilled to measure the hydraulic head fluctuations and the groundwater electrical conductivity (EC) profiles within the investigated groundwater (Figure 1a). Piezometer A is 25-m deep and screened between 1 and 25 m below ground (623 to 648 m a.s.l.). Piezometer B is 15m deep and screened between 3 and 15 m below ground (576 to 588 m a.s.l.). Both piezometers were drilled very close to the cores SI1 and SI2 (Figure 1a).

In each of the two piezometers, a pressure transducer with data-logger (STS DL.OCS/N/RS485) was installed to monitor the hydraulic head on an hourly basis, from May 2017 to May 2018. The same piezometers were used to measure the hydraulic head through a water level meter, on a monthly basis from April 2017 onwards.

The EC of groundwater was measured inside the screened interval of each piezometer, in order to analyse possible haloclines and use the groundwater EC to investigate the hydrogeological behaviour of the studied system, according to previous studies [24–27]. EC vertical profiles were measured on a monthly basis with a borehole probe (SOLINST TLC), from April 2017 to May 2018. Measurements were carried out at 1-m-depth intervals. The effectiveness of EC values was always verified through laboratory analyses.

2.4. Soil Samples and Chemical Analysis

Soil samples were collected in seven sites located in the study area: six of those were taken from the area affected by the rotational slide and one from the earth flow (Table 1; Figure 1a,b). The sites were selected within the rotational slide to analyse differences (i) among the land use types (cultivated, overgrown, grassland) observed at the study area, and (ii) between the main scarp and the flat areas.

Table 1. Sampling sites characteristics.

Site Code	Movement Type	Land Use	Coordinates (UTM)
EF	Earth flow	Eroded zone belonging to the active earth flow area	32N 573645 4938131
RS_C	Rotational slide	Wheat cultivated field	32N 573634 4938510
RS_{G1}	Rotational slide	Permanent grassland	32N 573628 4938442
RS_{G2}	Rotational slide	Permanent grassland	32N 573732 4938321
RS_{G3}	Rotational slide	Permanent grassland	32N 573764 4938410
RS_{MS}	Rotational slide	Main scarp with scarce vegetation cover	32N 573645 4938357
RS_O	Rotational slide	Overgrown field	32N 573641 4938486

Soil samples were collected for chemical analysis according to FAO recommendations [28]. Nine soil subsamples were used to perform pH and soil organic matter (SOM) analyses. pH analysis was conducted by placing a pH meter in a soil:distilled water liquid mixture in ratio 1:2.5 [29]; SOM was determined by using LOI-Loss on Ignition according to Ball's method [30], and igniting 1 g of dried soil at 450 °C for 4 hours.

2.5. Soil Arthropod Investigation

Three soil samples ($10 \times 10 \times 10$ cm^3) were collected from each site in November 2018 for soil microarthropod extraction. Arthropod extraction was performed via the Berlese–Tüllgren funnel (2 mm sieve mesh; extraction time 10 days), and the specimens were placed in a preservative solution (75% ethyl alcohol and 25% glycerol by volume). Class taxonomic level for Myriapoda, and order level for Hexapoda, Chelicerata and Crustacea [31] were considered. Collembola and Acari are generally the two most present groups, in terms of species diversity and abundance, so these two groups were studied more in-depth. Collembola were classified at family level, and Acari were divided between Oribatida and other Acari. The number of specimens present for each group was counted and their abundance (ind/m^2) registered. Number of taxa (NT) and Acari-to-Collembola ratio (A/C) [32] were calculated on the basis of this classification level.

2.6. Statistical Analysis

The Pearson correlation coefficient was used to determine if there was a relation between pH and SOM. The Kruskal–Wallis test for multiple comparison was performed to establish if the study sites differed in pH and SOM. Where significance was found ($p \leq 0.05$), pairwise multiple comparisons between sites according to Conover were made as post-hoc.

Total abundance, number of taxa as well as abundance of taxa always present in the study sites and Acari/Collembola ratio were compared by using the Conover test as done for environmental

parameters. The Akaike's Information Criterion (AIC) was used to establish the best model to study soil fauna community data matrix. The R package "vegan" was used for arthropod communities to observe variance in community composition depending on the study site and its environmental characteristics. The Bray–Curtis distance was calculated as a measure of the dissimilarity between sites in the composition of the edaphic community, considering both the presence and the abundance of the different groups found; true abundances were log-transformed according to Borcard [33], in order to avoid placing the same importance on absolute differences in abundance without considering their order of magnitude. The same tests performed to study total community composition were conducted for Collembola communities. All data were analysed using R version 3.5.2 (R Core Team, Vienna, Austria)[34].

3. Results

3.1. Geological and Geomorphological Features of the Landslide

The Case Pennetta landslide extends from 650 m a.s.l. to the Taro riverbed (265 m a.s.l.). Its length is about 1600 m, and its maximum width is approximately 130 m. The geomorphological map (Figure 2) shows the landslide's main landforms, with emphasis on the portions analysed within this study (rotational slide and upper part of the earth flow). The well-developed main scarp, characterised by a concave longitudinal topographic profile, and the head surface (in the counter-slope) account for a typical rotational movement.

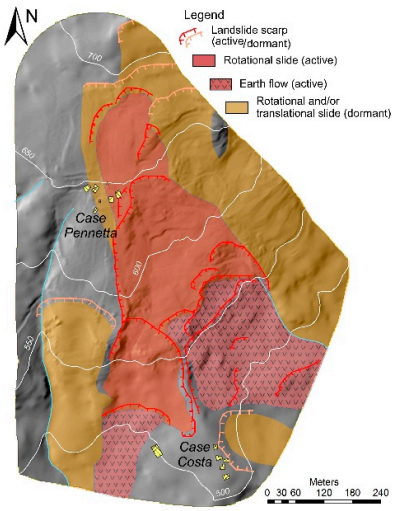


Figure 2. Geomorphological map. The contour lines show the altitude in meters above sea level.

This kinematic type pertains to the depleted mass of the landslide that extends down to 510 m a.s.l., where the toe of the failure surface is masked by the materials accumulated. Earth flows start from the front of this depleted mass and reach the Taro River, representing the foot of the landslide.

The rotational part of the landslide affects SCB and marginally AMR. Core SI1 (Figure 1a) shows, down to 30–31 m below ground (b.g.; 616–615 m a.s.l.), a matrix supported deposit characterized by a clay-silty matrix, clasts of different size (0.5 to 12 cm). From 31 m to 34 m b.g. the deposit is clast-supported and poorer in matrix compared to the upper portion. The top of the bedrock was detected at 35 m b.g. (612 m a.s.l.). Between 6–9 m and 11–14 m b.g., there is an alternation of compact and loose levels with a certain degree of disaggregation. Between 21 m and 23 m b.g., the rocks are more disaggregated. Core SI2 (Figure 1a) shows clasts and blocks of different size (from grains to 12 cm) with abundant silty-clay matrix, from the ground surface to 20 m b.g. A level richer in clay was observed at 14–15 m b.g. Dispersed organic material has also been found in the first 20 m of coring.

Between 20 and 23 m b.g., the deposit is clast-supported, with clasts up to 10 cm in size. Between 21 and 21.50 m b.g. the matrix is more abundant than in the other portion of this depth interval. At 23 m b.g. (561 m a.s.l.), the rock is made of thin layers of claystone and sandstones (SCB).

3.2. Hydrogeological Behaviour

Hydraulic head measurements show several peaks during infiltration events (Figure 3), therefore suggesting rapid percolation of water from the ground surface towards the saturated zone. The groundwater EC varies significantly with depth, and the haloclines show variations over time, strictly related to local precipitations (see examples in Figure 3). Therefore, according to the results obtained in other sites [26,35], these EC variations depend on the effective infiltration of local rainwater that determines the mixing between lower-salinity fresh infiltration waters and higher-salinity pre-event groundwater. In both piezometers A and B, the EC in groundwater varies significantly with depth (Figure 3), with a great difference between the bottom (e.g., >4000 µS/cm in piezometer A) and the top value (e.g., around 1000 µS/cm in piezometer A). A step-like shape is observed in both wells. In each of the wells, the EC steps were always detected at the same depth.

On the whole, the hydrogeological investigations depict (i) a near-surface medium that is characterised by both porosity and permeability higher than those expected in clay-rich sediments (see rapid effective infiltration and arrival of fresh-infiltration waters at the groundwater table), and (ii) relatively low salinity of waters percolating through the unsaturated zone.

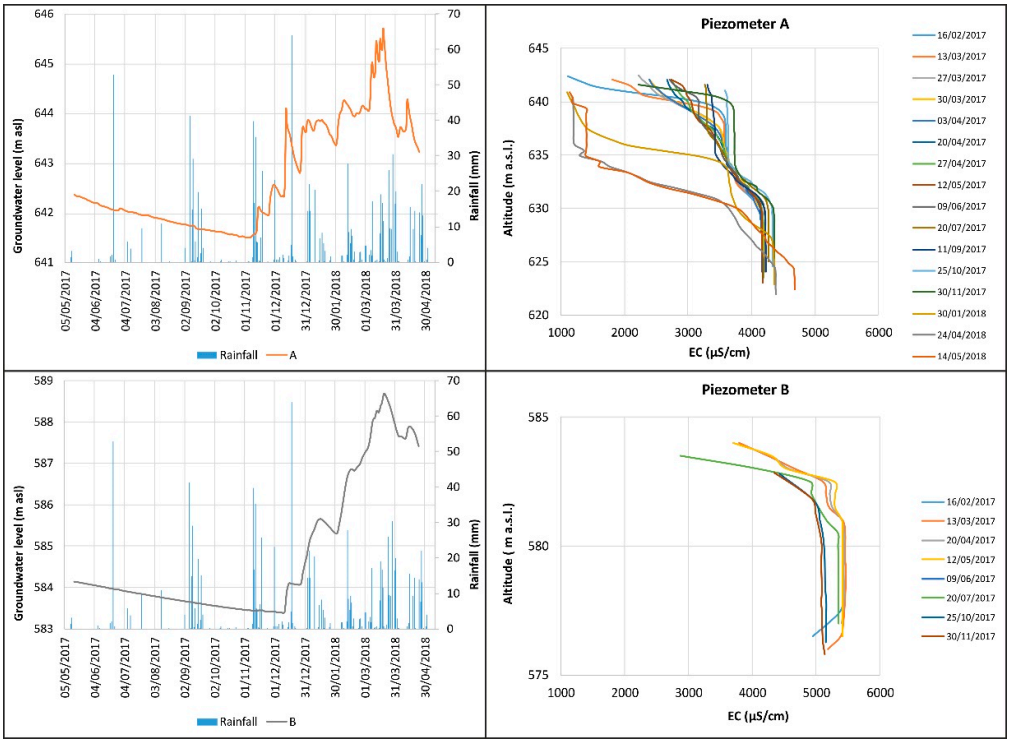


Figure 3. Hydraulic head fluctuations measured in piezometers A and B, and daily precipitation recorded at Mormorola station and the vertical profile of electrical conductivity (EC) in piezometers A and B.

3.3. Soil Characterization

Results of pH and SOM are shown in Figure 4. The different pH values, from neutral to moderately alkaline, were not significantly distinct from each other, while percentages of SOM depended on the sampling sites ($p < 0.05$). In the RS_{G1}, SOM appeared to be higher than in the RS_C, RS_{MS} ($p < 0.05$) and EF areas ($p < 0.01$), in which it was significantly lower than in RS_{G2} and RS_{G3} ($p \leq$

0.05; $p < 0.01$). According to the Pearson correlation, there was no relation between SOM and pH found in this study area.

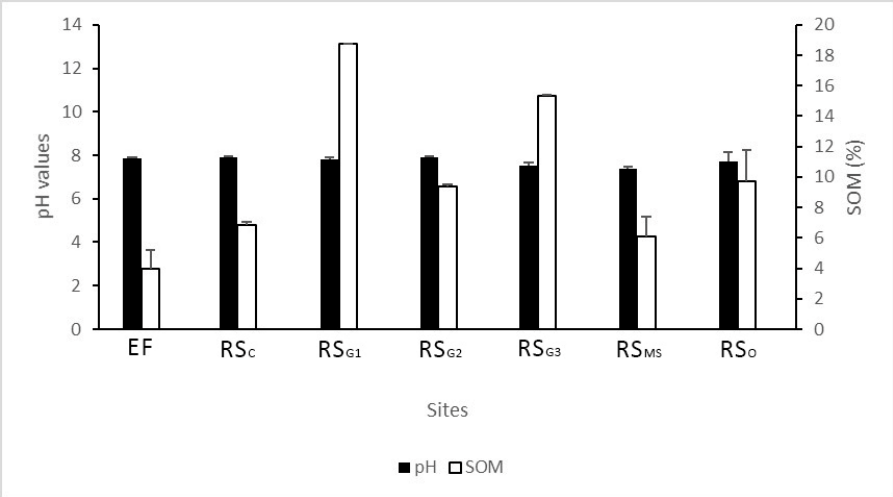


Figure 4. Average and standard error of the pH and SOM found in each site.

3.4. Soil Microarthropod Community

Table 2 shows the results of soil microarthropod community. Overall, 17 taxa were found in the study area, five of which found in all sites: Acari, Coleoptera, Collembola, Diptera and Hemiptera. The greatest total abundance per m² was found in RS_{G2}, statistically higher than RS_C, RS_{MS}, RS_{G3} ($p \leq 0.05$) and EF ($p < 0.01$); EF showed the lowest abundance, less than in RS_O and RS_{G1} ($p < 0.05$). RS_C and EF areas revealed a significantly lower number of groups than the other sites ($p \leq 0.05$), except for the comparison between RS_C and $RS_{G}1$, and between both RS_C and EF, and RS_{MS}. The best model to study soil fauna community data matrix was considering site and SOM as independent variables, with the result that each one contributed significantly to edaphic community composition ($p < 0.01$ and $p \leq 0.05$ respectively). The Bray–Curtis distance denoted the greatest dissimilarity (from 38% to 50%) between EF and the other sites, particularly RS_{G2}, while fauna composition appeared to be similar for over 80% between RS_{G2}, RS_{G3} and RS_O (Figure 5).

Table 2. Average ± standard error of the number of individuals (ind./m²) for each faunal group, the number of groups, the total abundance (ind./m²) and the ratio Acari/Collembola found in the seven sites studied.

Faunal group	EF	RS_C	RS_{G1}	RS_{G2}	RS_{G3}	RS_{MS}	RS_O
Acari	311 ± 79	7268 ± 5867	7844 ± 447	27588 ± 2654	4681 ± 313	6008 ± 3273	4897 ± 1345
Araneidae	14 ± 7		21 ± 0	42 ± 12	42 ± 12	64 ± 37	-
Chilopoda	-	14 ± 7	11 ± 6	42 ± 25	11 ± 6	-	7 ± 7
Coleoptera	14 ± 7	255 ± 131	573 ± 123	499 ± 67	796 ± 190	594 ± 294	566 ± 209
Larvae	-	255 ± 161	541 ± 244	403 ± 74	786 ± 196	531 ± 305	559 ± 203
Collembola	870 ± 492	1755 ± 494	1040 ± 123	10870 ± 650	3917 ± 1122	2420 ± 735	3276 ± 1769
Diplopoda	28 ± 19	-	-	-	-	-	-
Diplura	-	7 ± 7	53 ± 18	202 ± 80	21 ± 12	74 ± 18	64 ± 25
Diptera	50 ± 7	78 ± 58	159 ± 43	74 ± 18	74 ± 6	21 ± 7	120 ± 57
Larvae	14 ± 7	64 ± 44	42 ± 21	64 ± 25	11 ± 6	11 ± 7	64 ± 12
Hemiptera	142 ± 131	28 ± 14	276 ± 86	393 ± 153	902 ± 337	21 ± 12	248 ± 146
Hymenoptera	7 ± 7	-	85 ± 37	510 ± 270	510 ± 221	42 ± 25	1720 ± 1481
Isopoda	-	-	-	21 ± 12	11 ± 6	-	-
Lepidoptera	-	-	-	-	11 ± 6	-	-
Larvae	-	-	-	-	11 ± 6	-	-
Pauropoda	-	28 ± 19	-	21 ± 12	11 ± 6	-	28 ± 14
Protura	-	14 ± 14	-	-	-	-	-
Psocoptera	-	-	-	-	11 ± 6	11 ± 6	-
Symphyla	-	-	6496 ± 2415	-	42 ± 12	11 ± 6	21 ± 12
Thysanoptera	-	7 ± 7	-	117 ± 6	106 ± 61	53 ± 31	14 ± 7
Number of groups	8 ± 1	10 ± 0	10 ± 0	11 ± 1	13 ± 1	10 ± 1	10 ± 0
Total abundance	1437 ± 709	9469 ± 5699	16559 ± 2674	40379 ± 3711	11146 ± 1243	9320 ± 4380	10962 ± 3910
Acari/Collembola	2 ± 1	6 ± 5	8 ± 1	3 ± 0	1 ± 0	2 ± 1	2 ± 1

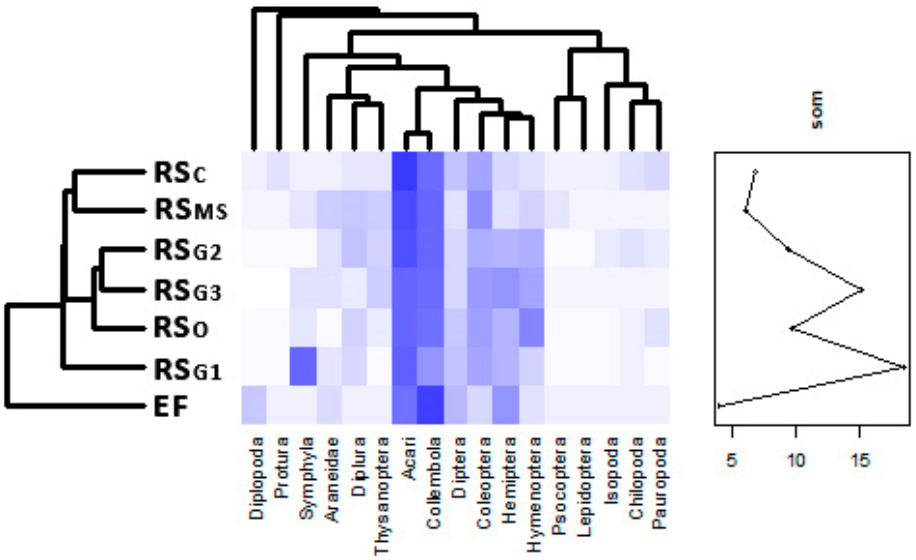


Figure 5. Heat map of the community composition, with colour intensities proportional to taxa abundance, and UPGMA (unweighted pair-group method using arithmetic averages) clustering of a matrix of Bray–Curtis distance among sites (on the left side) and of the taxa that occur more often together (on the top side); on the right side, the SOM values are displayed.

Greater Acari abundance was found in RS_{G2}, significantly higher than in RS_C ($p < 0.05$) and EF sites ($p < 0.01$); in EF their presence was lower than RS_O and RS_{G1} ($p < 0.05$). Among them, a distinction was made between those belonging to Oribatida and to other Acari (Figure 6). The Acari community strongly depends on the site ($p \leq 0.01$); with EF abundance of Oribatida significantly lower than that of the other sites ($p < 0.05$) except for RS_C (lower than RS_{G2}, $p < 0.05$) and RS_{MS}. The abundance of Oribatida was greater than other Acari in RS_{G3} and RS_O only.

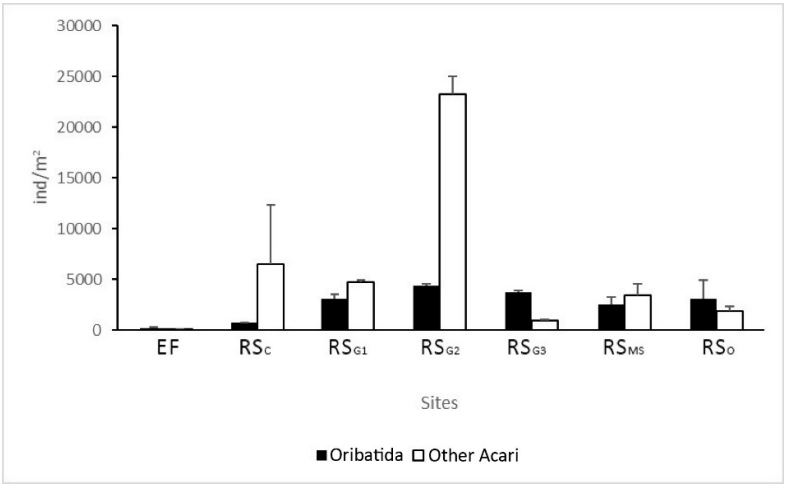


Figure 6. Average and standard error of the number of Oribatida and others Acari (ind./m²) found in each site.

Like Acari, less abundant Coleoptera and Collembola were found in EF. The number of Coleoptera in EF was lower than in RS_{G3} and in RS_O ($p < 0.05$). The abundance of Collembola observed in RS_{G2} was statistically greater than in RS_{G1} and in EF ($p < 0.05$, $p < 0.01$). Ten Collembola families

were identified (Table 3), with four present in all sites: Entomobryidae, Hypogastruridae, Isotomidae and Onychiuridae/Tullbergiidae.

Table 3. Average ± standard error of the number of Collembola per m².

Collembola family	EF	RS_C	RS_{G1}	RS_{G2}	RS_{G3}	RS_{MS}	RS_O
Arrhopalitidae	-	7 ± 7	-	11 ± 6	-	-	-
Entomobryidae	623 ± 429	934 ± 532	563 ± 67	393 ± 80	955 ± 110	1306 ± 215	205 ± 51
Hypogastruridae	71 ± 51	14 ± 7	21 ± 12	138 ± 18	1337 ± 735	106 ± 61	1076 ± 416
Isotomidae	85 ± 65	425 ± 170	96 ± 31	10243 ± 766	329 ± 116	616 ± 282	1592 ± 1392
Neanuridae	21 ± 12	7 ± 7	-	-	-	-	-
Onychiuridae/ Tullbergiidae	64 ± 64	361 ± 165	350 ± 67	42 ± 12	1136 ± 313	265 ± 104	255 ± 44
Odontellidae	-	-	-	11 ± 6	-	-	-
Katiannidae	-	7 ± 7	-	-	-	-	42 ± 21
Sminthuridae	-	-	11 ± 6	32 ± 6	117 ± 55	127 ± 74	21 ± 12
Sminthurididae	7 ± 7	-	-	-	42 ± 25	-	85 ± 56

The whole Collembola community composition depended on the site ($p < 0.01$), with EF and RS_O showing the highest dissimilarity, RS_{G1} and RS_C resulting more similar to each other than the other sites, and Entomobryidae and Onychiuridae/Tullbergiidae as families that occurred together more often (Figure 7). Through multiple comparison between the families always present RS_O proved to be the site with significantly fewer Entomobryidae than RS_{MS} and RS_{G3} ($p < 0.01$; $p < 0.05$), and more Hypogastruridae than RS_C, RS_{G1} and EF ($p < 0.01$, $p < 0.01$, and $p < 0.05$ respectively). The Hypogastruridae found in RS_C were lower than those in RS_{G3}, too ($p < 0.05$). Most Isotomidae were in RS_{G2}, more than in RS_C and EF ($p < 0.01$). The greater presence of Onychiuridae/Tullbergiidae was in RS_{G3}, more than RS_{G2} and EF ($p < 0.01$), whose number was lower than in RS_C and RS_{G1}, too ($p < 0.05$).

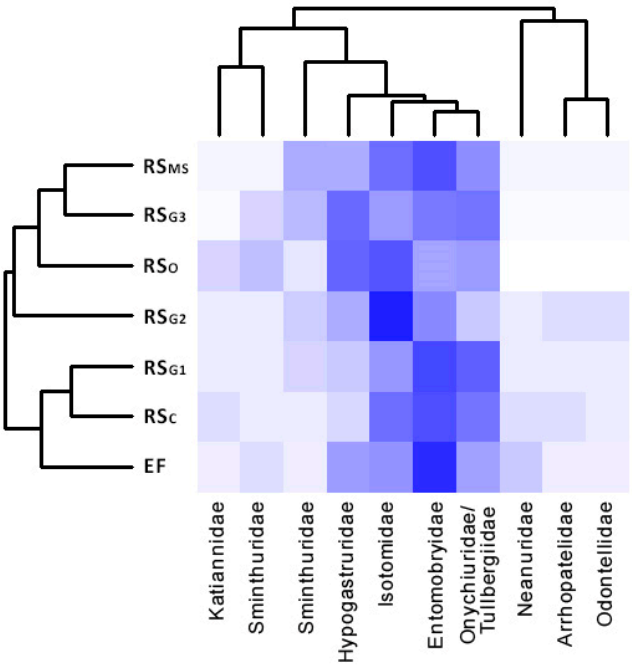


Figure 7. Heat map of the Collembola community composition, with colour intensities proportional to family abundance, and UPGMA (unweighted pair-group method using arithmetic averages) clustering of a matrix of Bray–Curtis distance among sites (on the left side) and of the families that occur more often together (on the top side).

The lowest abundance of Diptera was in the RS_{MS}, significantly different from RS_{G1} and RS_O ($p < 0.05$). The only other group found in all sites was Hemiptera, which showed a higher number in RS_{G3}

than in RS_C, EF and RS_{MS} ($p < 0.05$). Acari/Collembola ratio, being higher in RS_{G1}, did not differ significantly between sites.

4. Discussion

In this research, the hydrodynamic characterization of a complex landslide was carried out to evaluate the effects of this system type on soil biodiversity, in terms of soil arthropod community, with the aim to highlight if soil fauna can be a useful tool in landslide studies.

No difference was found in soil pH, similarly to other results obtained from landslide areas by Wilcke [36]. Instead, organic matter content (SOM) varied significantly between sites. As known, soil pH and SOM alteration can highlight a soil disturbance condition and contribute to altering ecosystem biodiversity.

Not only does SOM influence nutrient availability, it also promotes soil aggregation and improves water infiltration, and its content is known to decline from grassland to cultivated areas [37].

In this study, the lowest amount of SOM was detected in the earth flow area (EF), followed by the main scarp of the rotational slide (RS_{MS}), and both sites were similar to the cultivated site within the rotational slide (RS_C). The differences observed between earth flow (EF) and grasslands (RS_{G1}, RS_{G2}, RS_{G3}) show the surface heterogeneity created after slope failure.

It is known that the slip face at the upper edge of a landslide is more unstable, subject to erosion and not readily colonized [38]. Moreover, as suggested by some authors [36], fertility reduction caused by landslides, can involve the whole area. Usually, SOM content is an important feature in driving soil faunal community, and to confirm this, our study shows an evident relationship between SOM content and soil microarthropods. On the other hand, since arthropod faeces are involved in humus production and soil aggregation, soil arthropod abundance and diversity contribute to incrementing soil structure and, consequently, soil stability. In this study, earth flow (EF) showed the poorest soil faunal community, in terms of both number of groups and abundance. Moreover, as for SOM content, soil arthropod abundance in the earth flow (EF) proved to be similar to the main scarp of the rotational slide (RS_{MS}) and cultivated area sampled within the rotational slide (RS_C). This result confirms that the whole landslide area (EF + RS) is involved in a soil degradation process. Indeed, the soil arthropod abundance detected in RS_{G1}, being similar to RS_C and lower than a typical grassland condition [39], suggests that a stressful action is affecting the area dramatically. The Collembola community supports this observation since, despite its abundance in the EF being lower than the other sites, the community structure in this site was similar to RS_{G1}, RS_C and RS_{MS} (79%, 72% and 69%, respectively). Entomobryidae was the most abundant family found in these sites and the specimens found for this family are almost all epigeic. This result suggests that these specimens were less affected by belowground disturbance as reported in previous studies [40,41]. As known, Collembola have a leading role in the soil structure making; indeed, their faeces contribute to the water-holding capacity and soil aggregation [42]. In this study, the greatest abundance of this group was found in RS_{G2}, whose community structure was richer than the other sites, overall for the greatest presence of Isotomidae. RS_{G3} showed a high number of Onychiuridae/Tullbergiidae. As reported by Rusek [43], Onychiuridae, rare in clay soils, are known for their ability to make "microtunnels" in the soil matrix, ability shared with Oribatida Acari, thus increasing aeration and drainage.

As for the Collembola, even for Acari the abundance of this taxon was very low in EF, confirming the degradation condition of this site. Moreover, no difference was detected between Oribatida and others Acari in terms of abundance in this site. On the other hand, RS_{G3}, showing the greatest abundance of Oribatida, confirms the importance of a grass cover in soil biodiversity maintenance. Indeed, Oribatida are typical of stable and humid soils, rich in organic matter, since they can survive in submerged condition even over long periods of time, while they are susceptible to drought and their densities decline in man-modified areas subjected to agricultural treatments [44–46]. Centipedes (Chilopoda), which have a thinner cuticle and are sensitive to water stresses [47], were also found in all sites except EF, confirming the degradation condition of this site.

Contributing to SOM contents and consequently to soil resistance to erosion, organisms involved in litter breakdown can play a key role in soil structure maintenance. Coleoptera and Diptera larvae, Symphyla, Diplopoda, Isopoda and Thysanoptera were observed among the main arthropod groups found in this study that take part in this process. Abundance of Coleoptera such as Oribatida was greater in RS_O and in RS_{G3} than in the other sites; both groups can affect soil structure while moving into the pores and pushing particles aside, contributing to soil porosity. Coleoptera larvae, rarely observed in EF, are good indicators of soil water content, as their permeable cuticle makes them susceptible to desiccation [48].

Considering that ants build complicated burrows deep into the ground, forming a network of macropores [49], the greater abundance of this group in RS_{G2}, RS_{G3}, and RS_O when compared to the four other sites, suggests a greater infiltration capacity of these soils. Differently from ants, abundance of Diptera was greater in RS_{G1}. Their presence could be related to the occasional presence of goats, factor that produces a high manure content, increasing the abundance of soil dwelling Diptera [50]. This idea is supported by the high number of Symphyla present in this site, whose abundance strongly depends on SOM content [51]. Moreover, Symphyla can give information about soil structure, since they cannot dig tunnels actively in soil and they use existing pores to move in the soil.

Isopoda is a group susceptible to water loss by evapotranspiration and live in moist habitats where they feed mainly on dead and decaying plant material. These considerations highlight that these properties are typical of RS_{G2} and RS_{G3}, where this group was observed. Thysanoptera showed similar behaviour, having the greatest abundance in the two sites.

These results point out that soil arthropod community can influence and/or can be influenced by the hydraulic features of a low-permeability system in a landslide area. Firstly, arthropods such as Onychiuridae, Oribatida, Coleoptera, and ants are able to increase both the effective porosity and the permeability of the upper "aquifer" medium, therefore enhancing the effective infiltration of rainwater in a porous low-permeability system. Since these arthropods can increase porosity and permeability in several tens of centimetres below ground, their ability to create macropore (mm in dimension) networks causes the vertical permeability to decrease significantly with depth. Due to this vertical heterogeneity, further emphasised by plausible rock weathering, a significant contrast in permeability is expected within the shallow aquifer medium, and temporary perched groundwater could be found within the landslide area during the main rainwater events. Taking into consideration the heterogeneous distributions of the arthropods mentioned above, as well as the geological heterogeneity of the medium studied, temporary perched groundwater could be also discontinuous within the study site. This hypothesis is in agreement with the existence of several temporary springs observed at different altitudes during wintertime (data not shown).

In a wider context, the higher permeability of the shallow "aquifer" system enhances the rapid recharge of the groundwater intercepted and monitored through piezometers A and B, in agreement with the "nervous" hydraulic head fluctuation observed by means of the pressure transducers. The slight difference in time lag between rain event and hydraulic head rise observed between piezometers A and B is also in agreement with the system's heterogeneity.

As for the bioindicator role of some arthropods, Isopoda seem to be a good indicator to detect the areas where the groundwater head comes frequently close to the ground (see examples at RS_{G2} and RS_{G3}), due to their ability to live in moist habitat. They were actually identified in RS_{G2} and RS_{G3} only, where the groundwater often flows out diffusely, during the main rainwater events. On the whole, these results further emphasize the effectiveness of coupled hydrogeological–biological approaches [52].

5. Conclusions

The study suggests that the earth flow, differing from the other sites analysed within the rotational slide for its lower arthropod biodiversity and abundance, appears to be the most stressed area, exposed to erosion, and susceptible to extreme conditions, such as drought. The cultivated area sampled within the rotational slide fits with the many conventional agricultural systems, as reported

in other studies, showing low arthropod biodiversity and abundance. The overgrown site instead showed a community composition more similar to RS_{G3}, and only partially different from RS_{G2}, mainly in total abundance, determined above all by Acari and Collembola densities. These three sites are characterized by good conditions in terms of grass presence and consequently rhizosphere, SOM content, water retention and porosity—all of which are important factors in supporting a complex and structured soil arthropod community. RS_{G1} appears to have an intermediate situation, showing great arthropod abundance, mainly due to the high arthropod density strongly related to high SOM contents, yet, on the other hand, showing characteristics observed in disturbed and degraded soils, as the Collembola community suggested, being very similar to those observed in stressed sites.

From the methodological point of view, this study suggests the possibility to effectively merge soil fauna and hydrogeological investigations to better understand the hydraulic features and behaviour of low-permeability systems. At the same time, the approach applied is efficient to distinguish degraded soils, often associated with types of landslides, such as earth flows, that cause partial or complete soil removal, from more preserved soils. The latter are often associated with types of landslide, such as rotational or translational slides, that are characterized by movement in depth, along the sliding surface, while the upper portion of the depleted mass can remain untouched.

The use of the soil fauna as indicator of the health state of the soil in landslide areas may contribute to the evaluation of the potentiality of these surfaces for agricultural purposes. For instance, plants (like maize) or trees (like apple orchards) that need a consistent rate of water would represent kind of cultivation that could be irrigated by extracting the waters from the landslides themselves once the paths of these have been identified within the body of the landslide.

The surfaces of landslides, which are obviously not active or characterized by very slow rate of movement like in the case studied, may represent for some portions within mountain chains, such as the Italian Apennines, the only areas suitable for cultivation. In the Apennines, this fact occurred in the past when many large landslides were exploited with these purposes, and for settlements, but even today they could represent the way to restoration and re-use of these surfaces, contributing to the maintenance and care of the territory and the prevention of landslide movement.

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Section 2.2:

A new approach for the monitoring and bioremediation of the unsaturated zone of contaminated aquifers

Article

Groundwater characterisation from an ecological and human perspective: an interdisciplinary approach in the Functional Urban Area of Parma, Italy

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FORESEEING GROUNDWATER RESOURCES

Groundwater characterization from an ecological and human perspective: an interdisciplinary approach in the Functional Urban Area of Parma, Italy

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Abstract
In the Parma Functional Urban Area, hydro-geo-ecology was investigated through an interdisciplinary approach, with emphasis on the shallow aquifer system. The study pointed out that domestic wells and *fontanili* are both fed by shallow groundwater affected by PCE and nitrate contamination, upgradient of the rural area located north of Parma City. Moreover, *Folsomia candida* tests suggested the possibility that other types of contaminants (not analysed in this study) can affect the shallow groundwater. Nowadays, PCE concentrations in the city centre are slightly higher than the limit set by law. Moreover, PCE aerobic biodegradation can be due to the local microbial community and then an effective natural attenuation can be expected along the groundwater flow pathway. These results suggest a very low risk for human health, linked to the groundwater consumption in the rural area north of Parma City. Conversely, no forecasts can be made at present about the possible impact of low PCE concentrations on the aquatic ecosystem observed at the *fontanili*. Concerning nitrate contamination, the higher concentrations detected in some wells and *fontanili* suggest a high risk for both human health and aquatic ecosystems. In a wider context, thanks to the interdisciplinary approach that combines successfully well-established investigation methods, the present study allows a better knowledge of the hydro-geo-ecological behaviour of groundwater-dependent ecosystems. At the same time, through purpose-designed experimental investigations and simulation models, this approach could be used as a sort of guideline useful in studying such complex environmental systems.

Keywords Groundwater characterization · Alluvial plain · Shallow aquifer system · Interdisciplinary approach · Functional Urban Area

1 Introduction

Within the AMIIGA project (INTERREG Central Europe), the city of Parma (Italy) became a pilot site with a contamination of chlorinated hydrocarbons in the shallow aquifer. To study the evolution of the contamination and to identify

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the possible sources, it was necessary to widen the limits of the focus area to a larger territory. In particular, we studied the shallow aquifer at the Functional Urban Area (FUA) scale of Parma (the city plus its commuting zone), sensu EU-Organisation for Economic Co-operation and Development.

The main drinking water and industrial wells pump groundwater from relatively deep aquifers (from fifty to several hundreds of meters below the ground surface). The actual monitoring wells and the main hydrogeological studies focused on these deeper horizons, whereas little information exists concerning the shallow aquifer that is utilized by the local population for domestic and agricultural purposes. Moreover, the shallow groundwater feeds the so-called *risorgive* or *fontanili* that in turn feed a complex system of channels and ditches (Rossetti et al. 2005; Bonaposta et al. 2011). The *risorgive* or *fontanili* are small, semi-artificial, aquatic ecosystems sensu Kløve et al. (2011) that are typical of the Po River Basin, the largest Italian watershed (about 71,000 km^2). Urban and agricultural pressures cause widespread nitrate contamination of the shallow groundwater in the Po plain (e.g. Cinnirella et al. 2005; Bassanino et al. 2011). The same contamination has been detected in most of the *fontanili* (e.g. Rossetti et al. 2005; Laini et al. 2011; Abdelahad et al. 2015).

Therefore, the anthropic pressure on this shallow aquifer can cause a negative impact on (i) human health [directly (drinking water for private uses) or indirectly [water used for irrigation or cattle breeding)] and (ii) peculiar (*fontanili*) groundwater-dependent ecosystems (GDE).

Taking into consideration the environmental scenario, an interdisciplinary work was necessary to have a complete overview of the hydro-geochemical and hydro-ecological processes taking part in a such complex ecosystem.

The main goal of this manuscript is to present the state of the art of this interdisciplinary and holistic research, as well as to show how different disciplines are necessary to analyse the aquatic environment. Therefore, this work is also a sort of guideline related to an effective interdisciplinary approach necessary to characterize groundwater bodies from both the ecological and human perspective.

This work was carried out in synergy between the University of Parma and other public authorities, within a framework agreement devoted to protect and manage both the groundwater and the groundwater-dependent ecosystems. Recently, within the same agreement, some interdisciplinary studies have been carried out in the Apennine chain (Cantonati et al. 2016; Chelli et al. 2016; Segadelli et al. 2017a, b).

2 Study area

In the Po plain sedimentary basin, the sedimentation processes have been marked by transgressive–regressive cycles (e.g. Ricci Lucchi et al. 1992). On the basis of these discontinuities, sediments may be grouped into supersynthems, synthems and minor rank geological bodies (Di Dio 2005). The Quaternary Marine Supersynthem (Upper Pliocene to Lower Pleistocene) consists of sediments of continental shelf, prodelta, delta front and fan-delta resting on Pliocene clays. The bottom of this supersynthem is defined by an evident sub-aerial surface of erosion and/or absence of deposition related to tectonic regional uplifting and tilting of the southern margin of the Po plain sedimentary basin. As documented by seismic lines and stratigraphic data, in the area of interest this supersynthem is up to 1600 m thick. The Emilia–Romagna Supersynthem (Lower Pleistocene, about 800 ky BP to present) consists of alluvial plain and fan deposits and of intra-valley and terrace deposits. This supersynthem is subdivided in two synthems separated by a stratigraphic discontinuity at about 450 ky (Di Dio 2005).

The grain size of the sediments is largely variable (from gravel to silt–clay) and frequently dependent on the eustatic–climatic oscillations at the scale of 20–40 ky. This heterogeneity causes the aquifer system to be made up of several permeable horizons intercalated by low-permeability clays and silts. The present work is focused mainly on the shallow permeable horizon (the upper 30 m below ground) that directly interacts with the urbanized territory of the City of Parma (Fig. 1), including the historical downtown and recent settlements. The area is densely urbanized, with a concentration of residential built-up areas, trading and services. The mean ground elevation is about 55 m above sea level (m asl).

The first information about groundwater contamination at the pilot site (hydrocarbons, MTBE, BTEX) was collected in 2002 during a gas station decommissioning. At the end of the procedure, chemical analysis showed also PCE concentrations in groundwater higher than the legal limits, even in piezometers upgradient with reference to the gas station. During the last few years, the PCE concentration has been up to 24 µg/L (Parma Municipality, unpublished data). At the present state, the sources of this pollution are still unknown. In 2013, a historical analysis, just on the surrounding of the pilot site of the commercial activities, which potentially used PCE, was carried out (not reported here for brevity). However, it did not allowed the identification of the responsibilities. For this reason it was necessary to design and improve the environmental investigations.

A second, but not less important, contamination that was found in the shallow aquifer regarded the nitrates; unfortunately, only a small amount of information was available and essentially related to the *fontanili*. These data show nitrate concentration up to 83 mg/L south of Parma City (Emilia Romagna Region, unpublished data), therefore suggesting a serious contamination and negative impacts on both the human health and the GDEs.

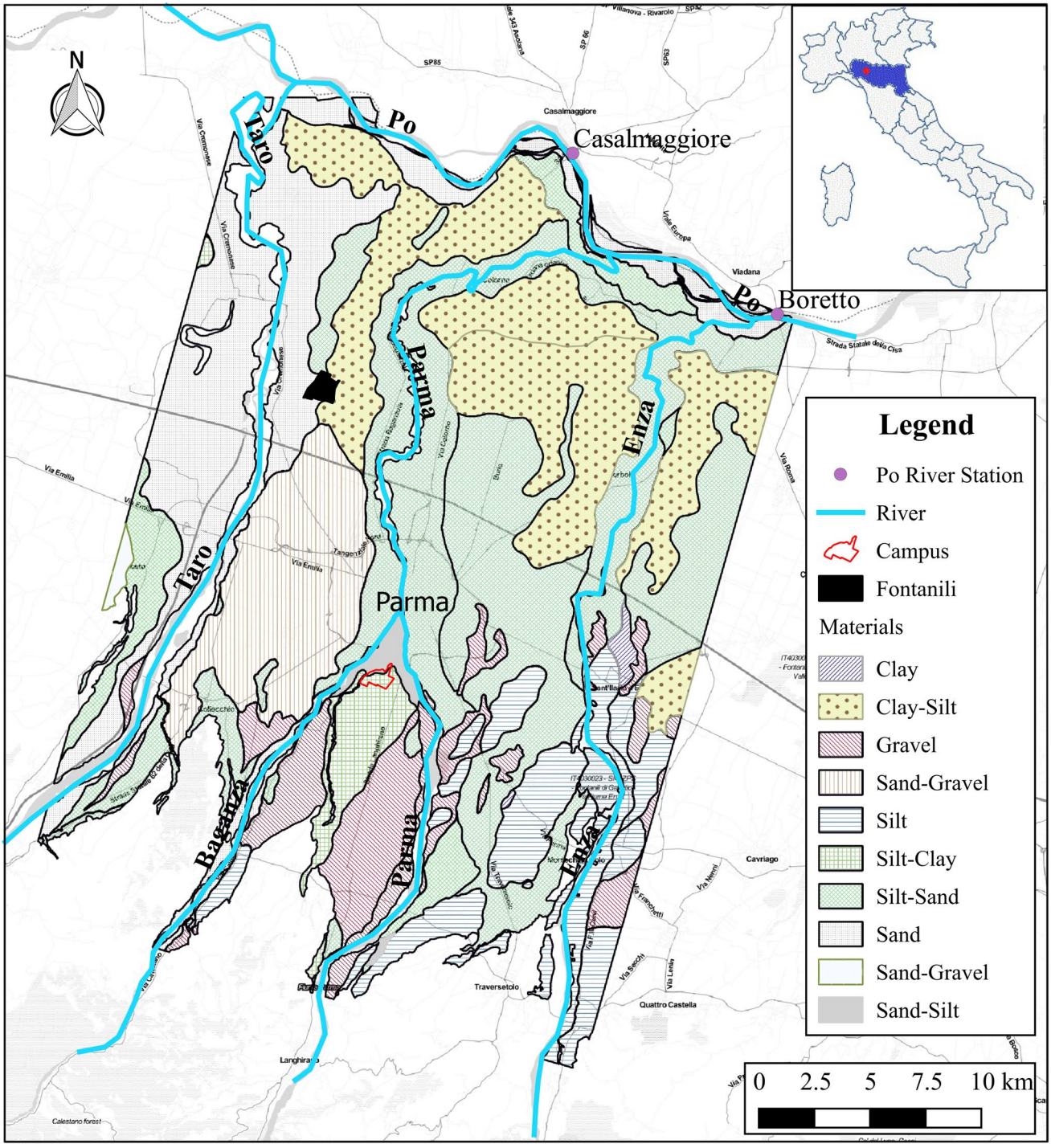

Fig. 1 Schematic map of the study area

3 Materials and methods

3.1 Geological, hydrogeological and geophysical investigations

Nineteen boreholes were drilled within the study area (Fig. 2a), to reconstruct the lithostratigraphic sequence of the shallow alluvial aquifer (Fig. 2b). Two of these boreholes (one cluster made of two wells each) were drilled at the university campus, to analyse possible variations in hydraulic head with depth, due to the vertical heterogeneity of the medium. The cluster is made of one well 27 m deep (P8d; screened from 24 to 27 m below ground) and one well 11 m deep (P8 s; screened from 8 to 11 m below ground). The

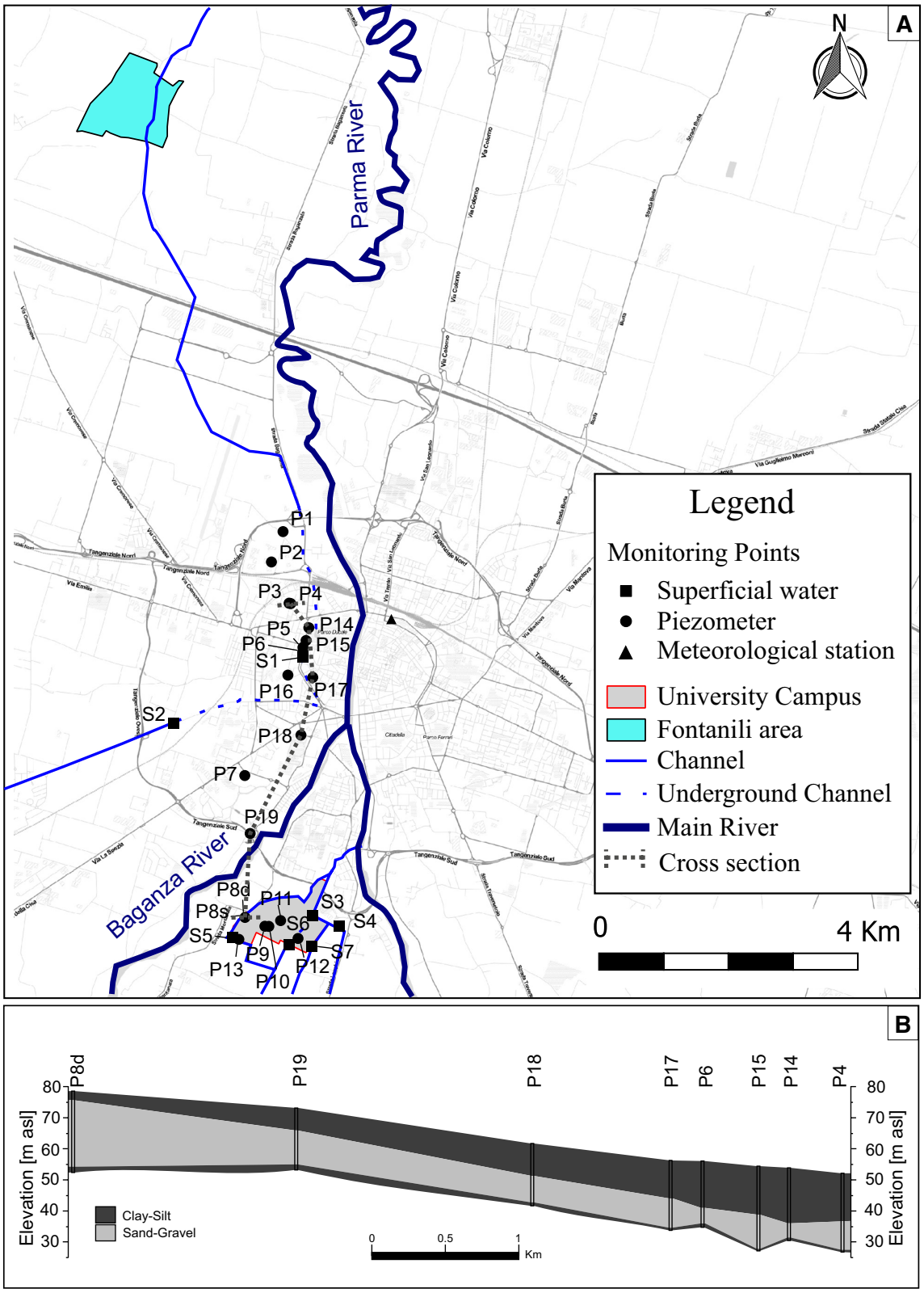

Fig. 2 **a** Location of monitoring points in the study area. **b** Geological cross section

other piezometer was drilled along a transect parallel to the groundwater flow line reconstructed at basin scale (approximately, from south to north), throughout the Parma urban area and passing from a site where the shallow groundwater was polluted by chlorinated solvents. These wells are 25–30 m deep and screened within the coarser-grained sediments (Fig. 2b).

The groundwater head was measured through a water level meter in all the available shallow wells (Fig. 2a) to reconstruct the groundwater flow net at the FUA scale. The hydraulic head was measured on an hourly basis (through pressure transducer with data-logger) in three wells to analyse the relationships between precipitations and groundwater head fluctuations. The precipitations were monitored on an hourly basis at a meteorological station located within the study area.

One pumping test was carried out in P8d. The hydraulic heads were monitored continuously (through pressure transducers with data-logger) in the pumping as well as in some monitoring wells. Three slug tests and five Lefranc tests were carried to calculate the hydraulic conductivity of the coarser and the finer sediments, respectively.

Resistivity data were collected at the university campus using a commercial georesistivimeter, to understand how continuous the confining silty-clay horizon at a scale larger than that of a single borehole was. The measurements were inverted to a true resistivity volume using a finite elements approach on a tetrahedral grid. Inversion was fully 3D and topography was also modelled to account for electrical field distortion due to morphology gradients. This inversion algorithm is well described in the literature (e.g. Cardarelli and Fischanger 2006) and it is generally indicated as the smoothness-constrained approach under Occam assumptions. The misfit between measured and calculated values was lower than 5% for most of the collected data points.

3.2 Isotopic and chemical investigations

The sampling campaigns for chemical, stable isotope ($\delta^{18}O$, $\delta^{2}H$) analyses and metagenomic analyses were carried out from March 2017 to February 2018 every 3 months, approximately. The rainfall at the Parma Campus station was collected on a monthly basis using 10-l polyethylene bottles containing about 300 ml of vaseline oil to prevent the evaporation processes. Oil contamination was carefully avoided by syringing the water samples out of the bottle.

pH measurements were performed in field with multiparametric probe. Alkalinity was determined by Gran titration with HCl. Main anion concentrations were determined by ion chromatography at the Aquatic Ecological Laboratory of the University of Parma, Italy. Samples for cation analysis were acidified at pH < 3 with concentrated HNO_3. Global analytical accuracy was evaluated by ionic balance. The main contaminants (hydrocarbons, chlorinated solvents, etc.) were analysed in a certified private laboratory.

Stable isotope analyses ($\delta^{18}O$, $\delta^{2}H$) were carried out at the Isotope Geochemistry Laboratory of the University of Parma, Italy. The analytical prediction uncertainty was better than 0.1‰ for $\delta^{18}O$ and about 1.0‰ for $\delta^{2}H$. The compositions of $\delta^{18}O$ and $\delta^{2}H$ are reported as $10^3 \times \delta^{18}O$ (V-SMOW, Vienna Standard Mean Ocean Water).

3.3 Microbiological and metagenomic investigations

Several studies demonstrated the possibility of using microbial communities as natural tracers of subsurface dynamics (e.g. Naclerio et al. 2009; Celico et al. 2010; Bucci et al. 2017; Farnleitner et al. 2005; Mayer et al. 2016). In this case, microbiological and metagenomic analyses were carried out to verify the existence or not of an autochthonous microbial community able to degrade chlorinated solvents.

Water samples for metagenomic analyses were collected in sterile 1000 ml bottles and transported in a refrigerated box. Filtration processes were completed within 2 h after sampling. The profile of bacterial populations thriving in groundwater partially contaminated by organic chlorinated solvents, of the DNPLs type, was analysed. DNA was extracted from groundwater and NGS 16SrDNA profiling was obtained at the Genprobio Srl Laboratory.

Partial 16S rRNA gene sequences were amplified from extracted DNA Amplifications were carried out and PCR products were purified by the magnetic purification step. Sequencing was performed using an Illumina MiSeqsequencer with MiSeq Reagent Kit v3 chemicals. The fastq files were processed using QIIME (Caporaso et al. 2010). To calculate downstream diversity measures, operational taxonomic units (OTUs) were defined at 97% sequence homology (Edgar 2010). All reads were classified to the lowest possible taxonomic rank using QIIME (Caporaso et al. 2010) and a reference dataset from the SILVA database (Quast et al. 2013). Similarities between samples (beta-diversity) were calculated by Jaccard index using a Dendro-UPGMA program available at http://genomes.urv.es/UPGMA/.

For growth and isolation of the methylotrophic bacteria detected with metagenomic analysis, two selective media were prepared, HYP (Kanamaru et al. 1982) and NMS (https://www.dsmz.de/catalogues/catalogue-microorganisms.html), while TSB medium (tryptic soy broth) was used for the propagation of the bacterial isolates.

Five enrichment cycles were carried out on the two selective culture media HYP and NMS, by adding methanol (1% v/v), as the sole source of carbon, to the single tubes in two parallel enrichment lines. Once the five enrichment cycles have been completed, the bacteria were inoculated on HYP and NMS media solidified with agar. The 25 colonies

isolated by repeated streaks on the selective media were then checked for growth with and without methanol to exclude the presence of autotrophs

As the enzyme methane monoxygenase, possessed by some methylotrophic bacteria, is known to be able to catalyse a fortuitous reaction of dehalogenation on organic halogenate compounds, a dechlorination assay was performed for detecting the chlorine ions released from a halogenated test molecule by the selected bacteria (Song et al. 2004; Bergmann and Sanik 1957).

3.4 *Folsomia candida* tests

Water collected in nine water wells was tested (P3, P5, P6, P14, P15, P16, P17, P18, P19 in Fig. 2a). Water samples for *Folsomia candida* tests were collected in 1000 ml bottles and transported in a refrigerated box to the laboratory. The springtails *F. candida* used in the test were sourced from laboratory cultures at the Parma University, previously synchronized removing the eggs from the culture, and, after the start of hatching, putting in Petri dishes with the breeding substrate. After 10 days, the juveniles were ready for the test to be performed. The experiment considered the springtails' survival. Five replicates for each water sample were prepared adding water to reach 60% of WHC in Petri dishes containing filter papers. Ten specimens from synchronized breeding cultures were transferred into each. 2 mg of pulverized cereal mix was given as food. The Petri dishes were incubated for 14 days in a temperature conditioned room at 20 ± 2 °C. Once a week, the dishes were aerated, the corresponding water was added to reach 60% of WHC, and organisms were fed with 2 mg of cereal mix. At the end of the test, adults were counted under a stereomicroscope after floatation.

Folsomia candida tests were carried out to evaluate the potential groundwater contamination, because this Collembola is an effective bioindicator often used in ecotoxicological studies.

3.5 Plant diversity analysis

Plant diversity was explored at the heads and the first sectors of fed channels (about 50–100 m in length) of five *fontanili* flowing out at the study area, in October 2016 by a whole flora meandering searches exploring the entire habitat under investigation. At each site, the presence of all the visible macrophytes was assessed using an aquascope (i.e. a bucket with a transparent bottom). In addition, several vascular specimens were collected for laboratory identification.

The plant diversity analysis was carried out to characterize the GDEs (*fontanili*) from the biological point of view.

3.6 Numerical flow model

Numerical groundwater modelling is widely used for environmental applications for: (i) investigation of the aquifer properties (e.g. Fienen et al. 2009; Zanini et al. 2017; D'Oria et al. 2018), (ii) prediction of the pollution spread and strength at a site (e.g. Gzyl et al. 2014; Chen et al. 2016), (iii) identification of pollution sources (e.g. Neupauer and Lin 2006; Cupola et al. 2015; Zanini and Woodbury 2016), (iv) design and test of reclamation actions (e.g. Xu et al. 2012) and (v) evaluation of well-capture areas (e.g. Paradis et al. 2007; Chelli et al. 2018; Feo et al. 2018) for developing hydraulic barriers. With the aim of reconstructing the groundwater flow net and the possible effects of groundwater contamination within the rural area, a numerical flow model was implemented at FUA scale. All the computations were performed by MODFLOW 2005 (Harbaugh 2005; Harbaugh et al. 2017). The boundaries of the model are: Po River at north (hydrogeologically downstream), Enza River at east, Taro River at West and the Apennine piedmont south of the Parma town. Concerning the relationships between rivers and shallow groundwater, only the Po River plays an important role as boundary conditions, because it is the only river interacting directly with the shallow aquifer at FUA scale.

The grid frame covers an area of 25×40 km^2. The FUA is represented through a finite difference grid of 200×200 m^2 with 200 rows and 125 columns (25,000 cells per layer). Considering the boundary conditions, only 15,467 cells per layer are active. The active area is about 627 km^2.

The FUA area was represented with two layers: the first one represents the shallow clayey material and the second one the investigated aquifer.

4 Results

4.1 Geological and hydrogeological setting

The reconstruction of the lithostratigraphic sequence takes advantage of the results of stratigraphic and *facies* analysis, using the information from the 14 boreholes drilled within this work. The lithostratigraphic sections allowed the reconstruction of a reliable conceptual model. Beneath the ground surface, the stratigraphic sequence begins with deposits made by silt and clay, whose thickness progressively increases from 1 to 19 m moving northward. Below this horizon, the confined "shallow aquifer" (mainly made of gravels and sands with discontinuous clay lenses) whose thickness is some tens of meters, at least can be found. It is continuous throughout the whole investigated stratigraphic sequence, while it is not detected in the southern end of the FUA where the shallow aquifer is unconfined (Di Dio

2005). Beneath the "shallow aquifer", a continuous bed of fine-grain-sized deposits has been found.

This lithostratigraphic model was further supported by the results of the geophysical investigations. A resistivity profile was extracted from the 3D resistivity volume along the section with higher sensitivity and data were interpretable down to a depth of about 30 m below the surface. The resistivity distribution in depth well shows the presence of a continuous aquifer (300 Ωm, up to 15 m thick at the University Campus), confined between two silty-clay continuous horizons. The upper horizon (50 Ωm) is up to 3 m thick, according to the boreholes stratigraphies, while the lowest one (30 Ωm) is at least 10 m thick.

The hydraulic parameters (transmissivity and storativity) of the shallow aquifer, estimated by means of the pumping test that was carried out at the university campus well field, were 3×10^{-4} m^2/s and 1.9×10^{-4}, respectively. These values are in agreement with the hydraulic conductivity values ($1.0–2.0 \times 10^{-5}$ m/s) calculated through the slug tests performed in the other purpose-drilled boreholes. The hydraulic conductivity (in the order of $10^{-7}/10^{-9}$ m/s) of the finer-grained deposits (aquitards) was calculated by means of Lefranc tests.

On the whole, the shallow groundwater flows from south to north. The groundwater head is not strictly characterized by rapid fluctuations during rainfall events (Fig. 3). This observation is in agreement with the existence of a continuous confining horizon throughout the study area, which does not allow local recharge due to effective infiltration of rainwater and leakage from surface channels. Therefore, the shallow groundwater is recharged upgradient of the urbanized area, where (i) the shallow aquifer crops out, (ii) the rivers are hydraulically linked to the shallow groundwater and (iii) the nearby relieves (Apennines) laterally feed the alluvial aquifer.

The Local Meteoric Water Line (LMWL) was defined using data from 2008 to 2015. The resulting major axis regression line is the following (Fig. 4):

$$\delta^2 H = 7.67\,(\pm 0.07)\,\delta^{18}O + 7.56\,(\pm 0.43) permil.$$

This equation is far from the meteoric water line found by Longinelli and Selmo (2003) for the Northern Italy precipitation (δ^2H = 7.71 δ^{18}O + 9.40). This is not surprising because the thermal features of the perturbations are largely variable in Northern Italy. The annual weighted δ^{18}O mean values of precipitation change largely (from − 10.0 to − 7.6‰, from 2008 to 2015): the average value is − 8.86 ± 0.74 (standard deviation). On the other hand, the Parma, Enza, and Baganza rivers for the period February 2004–August 2006 gave an average value of − 8.79 ‰ (Iacumin et al. 2009), whereas wells of the Parma area gave an average value of − 8.51‰; moreover, recent data on wells in the Parma area give, on

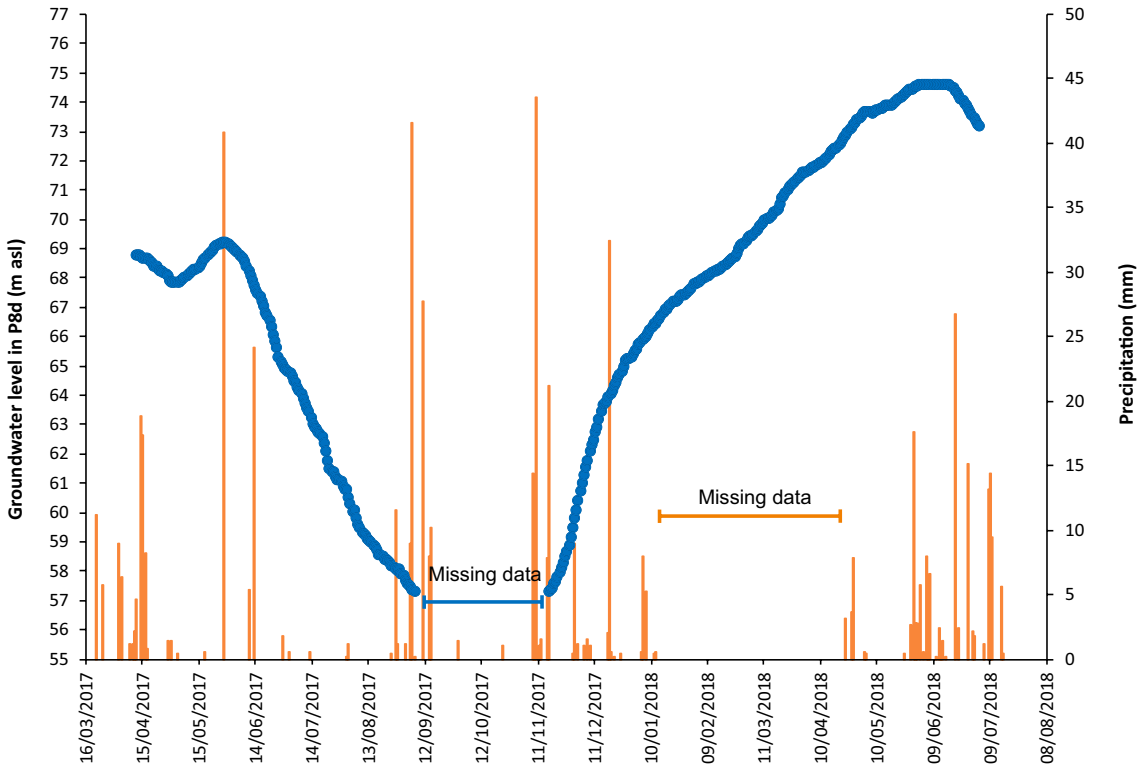

Fig. 3 Groundwater level fluctuations in P8d (dotted line) and daily rainfall in Parma meteorological station (bars)

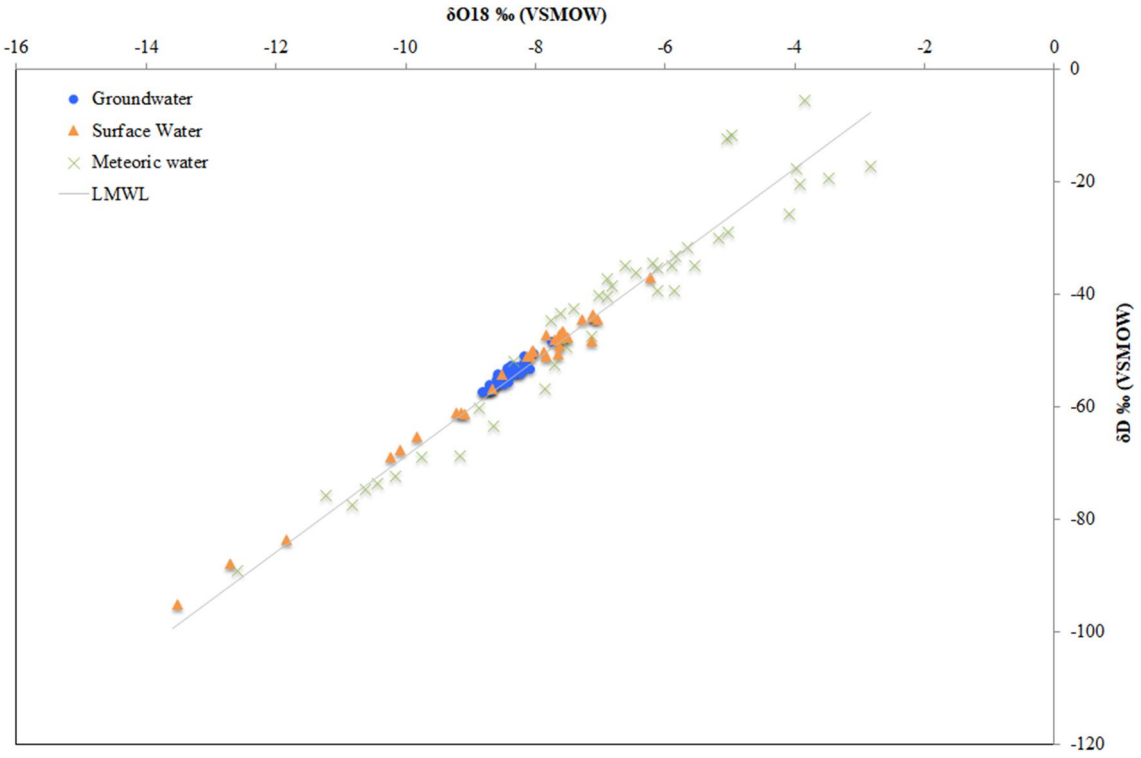

Fig. 4 $\delta^{18}O$ vs $\delta^{2}H$ relationship in rainfall surface and groundwater samples

average, − 8.41‰. Practically, all these values are the same, indicating that rivers and groundwater from the Apennines feed the shallow aquifers within the Parma plain. This data also confirm the finding of the shifted answer of the piezometric fluctuation compared with the local precipitation.

4.2 Groundwater contamination

Nitrate concentrations in groundwater varied over space and time, during the observation period. Concentration was lower in summer and autumn, and rapidly increased in winter. For example, in well P5 the NO_3 concentrations changed from less than 10 mg/L (in July and September 2017; see example in Fig. 5) to 48 mg/L (in December 2017 and February 2018; see example in Fig. 5). Moreover, the concentrations seem to increase northwards, along the groundwater flow lines. For example, in December 2018, concentration in groundwater increased from less than 20 mg/L to up to 30 mg/L from south to north. The exception was the well P5 (located in the city centre) where the highest nitrate concentration was detected (Fig. 5). On the whole, this preliminary investigation suggests the existence of two main nitrate sources. The first source is located in the southern part of the studied FUA and coincides with the agricultural lands in the upper plain, where the shallow aquifer is unconfined. According to observations made worldwide, agricultural lands suffer excess nitrogen application that is not assimilated by crops and, thus, pollutes surface and groundwater (e.g. Hu et al. 2010; Bartoli et al. 2012). In irrigated areas, nitrate is involved in fast transfer towards water bodies (Hu et al. 2010; Sutton et al. 2011; Savci 2012). During the summer period, water of irrigation flows into the surface channels with relatively high nitrate concentration (see for example the sampling campaign made in July 2017; Fig. 5) and migrates easily trough permeable soils, thus creating a major risk of groundwater contamination. Taking also into consideration the transfer time of nitrates in the saturated zone, the easy migration is in agreement with the seasonal variations of nitrate concentrations at the study site. At the same time, the coexistence of high nitrate concentration in surface channels close to the university campus (more than 50 mg/L in S3 in Fig. 5) and the very low concentrations in the local groundwater (less than 10 mg/L in all the piezometers drilled at the Campus area) further supports the absence of hydraulic interaction between surface- and groundwater within the studied portion of the urban plain. The results are in agreement with the finding of groundwater response to precipitation and the isotopic analyses. The second source of nitrates is seepage from the sewer systems in the urban area, according to the highest value detected in piezometer P5.

Chlorinated solvents in concentration higher (PCE up to 15 µg/L) than the legal limits (PCE up to 1.1 µg/L) occurred in groundwater in some observation piezometers. The highest concentrations were detected in a known contaminated

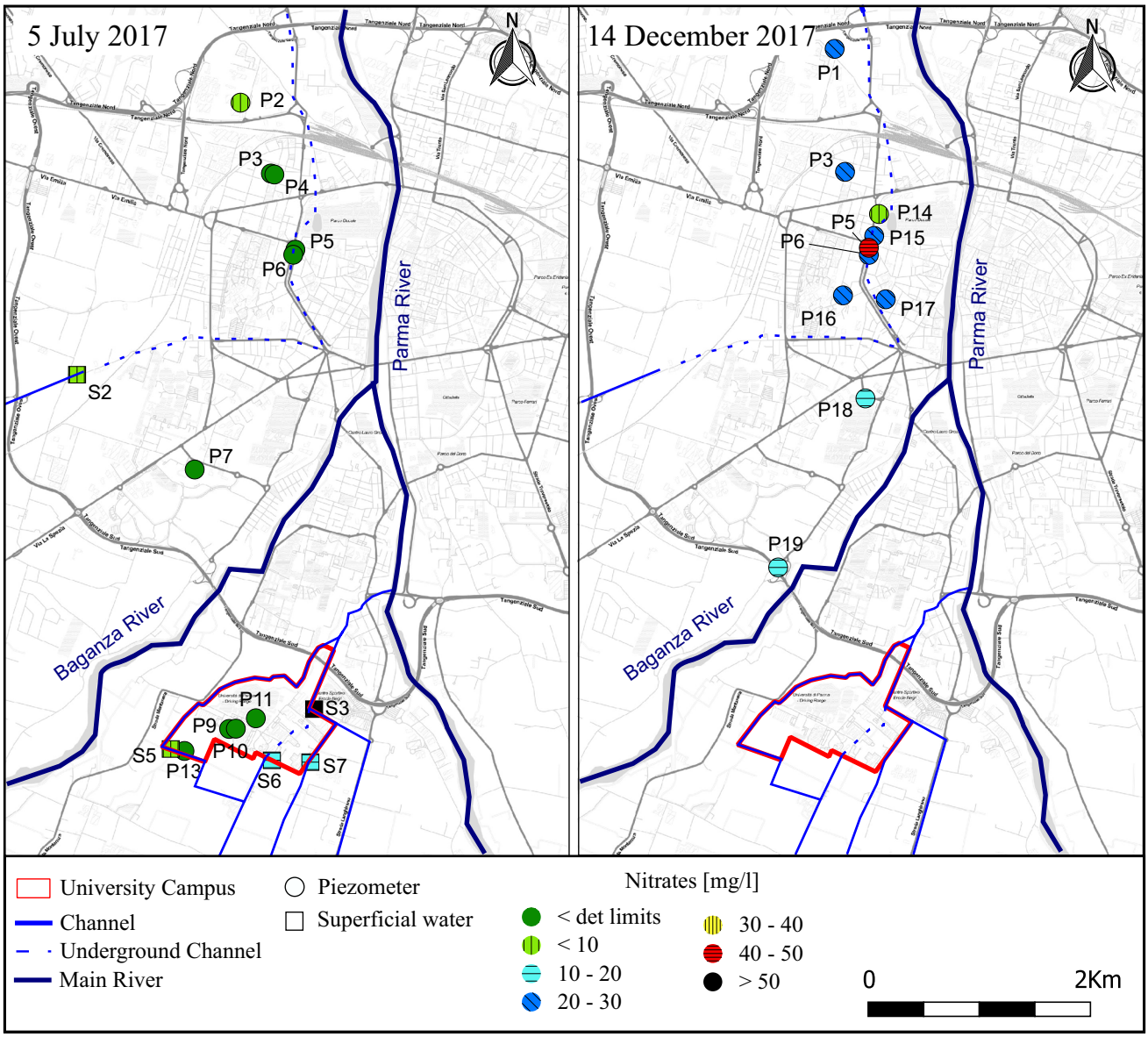

Fig. 5 Nitrate distribution in the shallow groundwater

site of the city centre (e.g. P5 in Fig. 5), as well as downgradient starting from this site (e.g. P3 in Fig. 5). In single observation wells, the PCE concentration varied over time, therefore suggesting possible PCE pools located in the transition zone between the lowest and the highest hydraulic head measured during the observation period. The spatial distribution of PCE suggests the existence of multiple pools, because the highest concentrations cannot be explained as part of a unique plume. At the same time, the rapid decrease of concentration downgradient starting from the most polluted site suggests effective dilution due to dispersion and/or attenuation due to microbial degradation. The latest hypothesis was further investigated through metagenomic analyses (see hereafter).

4.3 Microbial communities

The results obtained with the molecular analyses (community profiling by NGS sequencing) highlighted that the bacterial community present in the groundwater collected in the various piezometers along the groundwater pathway is mainly composed of aerobic bacteria.

Analysis of the samples from the most contaminated piezometers unveiled a community containing a higher percentage of methophiles belonging to different genera (*Methylobacter*, *Methylocella*, *Methylococcus*, *Crenothrix*), all known for being endowed with methane monooxygenase. The presence of these bacterial genera suggested that the most probable pathway of biodegradation of the chlorinated solvents

(e.g. PCE) could be an oxidative (co-metabolic) dehalogenation by means of methane monooxygenase. These bacterial genera were present only in the water from piezometers closer to the focus of PCE contamination (P3, P5 and P6). Bacteria obtained by enrichment, selection and isolation procedures were able to grow with methanol as the sole carbon source. In addition, through the test of the dehalogenase activity, it was shown that some of these isolated bacterial strains have indeed the capacity to dehalogenate chlorinated organic compounds. The isolated strains have been identified as *Proteobacteria* of the *Methylophilaceae* family. Moreover, the samples from wells P5 and P6 contained a number of sequences belonging to the *Rhodoferax* genus known to be a vinyl chloride (VC) utilizing bacterium (Paes et al. 2015) This may indicate a possible reductive pathway in anaerobic niches of the plume leading to incomplete degradation and transport of the contaminant in the aerobic zone where *Rhodoferax* can use it. VC was never found among contaminants.

4.4 *Folsomia candida* tests

The results obtained in this study highlight some differences in *F. candida* survival between water samples. The percentage of surviving specimens comprised between 47% and 83% after 14 days of the test (Fig. 6). P6 and P15 showed the highest percentage of survival with a springtail mortality of 17 and 23%, respectively, followed by P18, showing a mortality of 30%. P3 reported the smallest number of survivors, 47%. P6 and P18 showed the highest variability of results (Fig. 6). However, despite the brief period of the test, P3, P14, P15, P18 and P19 showed eggs and juvenile springtails in the test containers. This suggests that also for water samples where the mortality was higher, the reproduction was not completely inhibited. In a study aimed to evaluate biochar toxicity on *F. candida*, Conti et al. (2018) reported that the reproduction proved to be a more sensitive end point in comparison to survival.

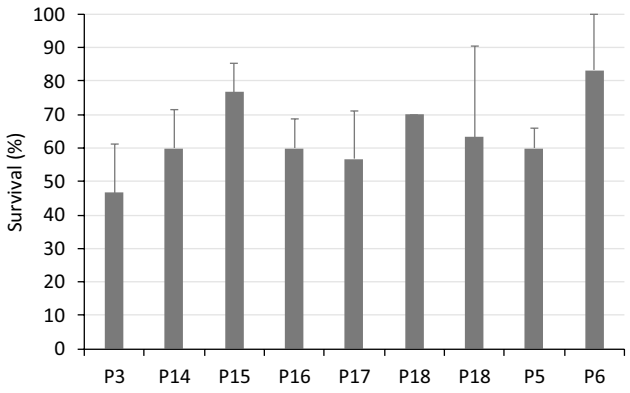

Fig. 6 Survival (in %) of *F. candida* during the test. The error bars correspond to the standard error

On the whole, the results of these tests cannot be completely explained considering only PCE and nitrate contamination, therefore suggesting the possibility of the existence of other types of contaminants not analysed in this study.

4.5 Plant diversity

A total of 38 vascular plants were recorded, with an overall mean number of 21.4 ± 6.7 taxa per site (Table 1). Two additional macroalgal taxa were also recorded: *Chara vulgaris* Linnaeus 1753 and *Nitella mucronata* (A. Braun) F. Miquel 1840 for a total of 40 species were recognized. The most widespread plant taxa, recorded in all the habitats investigated, were amphibian species typical of ecotonal belts (i.e. marginal ecotones): *Lycopus europaeus* L. subsp. *europaeus*, *Lythrum salicaria* L., *Mentha aquatica* L., *Ranunculus repens* L., and *Symphytum officinale* L. subsp. *officinale*. On the contrary, these aquatic species were rather less represented, with exclusively three species present in at least three (*Sparganium erectum* L. subsp. *erectum*) or two sites (*Alisma plantago-aquatica* L. and *Callitriche stagnalis* Scop.), respectively. However, despite the extremely reduced area occupied by *fontanili* (in the order of 300 m^2), a total of 13 hydrophytes were recorded (including the two macroalgae). This is a relevant output compared to data available for the main large lakes of North Italy (up to 370 km^2 for the Garda Lake) that indicate a hydrophyte diversity in the range of 11–26 species (Bolpagni et al. 2013a, 2017).

These data reinforce previous evidences highlighting the pivotal role of GDEs in supporting biodiversity at the local scale, especially in lowlands affected by huge human-driven alterations (Kuglerová et al. 2014; Bolpagni and Laini 2016; Bolpagni et al. 2016). The present results are also in strong agreement with those obtained by Bolpagni et al. (2013b) and Bolpagni and Piotti (2015, 2016) for a series of aquatic habitats placed along the Oglio River, a left tributary of the Po River located about thirty linear kilometres far from the area under analysis. More in detail, the semi-natural lotic habitats in analysis exhibit a relatively high plant diversity in line with that of lentic habitats (6.5 ± 4.2 taxa per site, respectively). This is probably due to the high water level stability of *fontanili*, thanks to their stable underground feeding throughout the year. In fact, this type of water feeding guarantees minimum fluctuations in the hydrometric levels of *fontanili*, as verified by Bolpagni et al. (2013b) for the marginal habitats of the Oglio River. Generally, *fontanili* act actively as refugee for a very rich aquatic and amphibian flora suggesting a key role in local to global strategies for plant conservation (Bolpagni et al. 2018). However, as widely discussed in the present paper, groundwater is largely impacted by multiple stressors that can heavily affect its quality, and cascading the quality of the biocoenoses it sustains. Accordingly, further investigations are needed to better

Table 1 List of the plant species recognized in the five investigated *fontanili*

LF	Species	F1	F2	F3	F4	F5
I rad	*Alisma plantago-aquatica* L.				x	x
G rhiz	*Berula erecta* (Huds.) Coville		x	x	x	x
I rad	*Callitriche stagnalis* Scop.		x	x		
H scap	*Cardamine amara* L. subsp. amara		x	x		x
He	*Carex acutiformis* Ehrh.		x	x		
He	*Carex riparia* Curtis	x	x		x	x
G rhiz	*Eleocharis palustris* (L.) Roem. & Schult.				x	x
I rad	*Elodea canadensis* Michx.					x
G rhiz	*Equisetum ramosissimum* Desf.	x		x	x	x
H scap	*Galium palustre* L. subsp. elongatum (C. Presl) Lange	x	x	x		x
I rad	*Glyceria maxima* (Hartm.) Holmb.					x
I rad	*Groenlandia densa* (L.) Fourr.		x			
G rhiz	*Iris pseudacorus* L.			x	x	x
G rhiz	*Juncus articulatus* L.			x		x
G rhiz	*Juncus subnodulosus* Schrank		x	x		
H scap	*Lycopus europaeus* L. subsp. europaeus	x	x	x	x	x
He	*Lythrum salicaria* L.	x	x	x	x	x
H scap	*Lysimachia nummularia* L.		x	x	x	x
H scap	*Lysimachia vulgaris* L.	x	x	x	x	
H scap	*Mentha aquatica* L.	x	x	x	x	x
H scap	*Myosotis scorpioides* L.		x	x	x	x
I rad	*Myriophyllum spicatum* L.			x		
H scap	*Nasturtium officinale* R. Br.		x	x		
He	*Phragmites australis* (Cav.) Trin. ex Steud. s.l.		x	x	x	x
I rad	*Potamogeton acutifolius* Link		x			
I rad	*Potamogeton crispus* L.			x		
I rad	*Potamogeton friesii* Rupr.		x			
H rept	*Ranunculus repens* L.	x	x	x	x	x
H scap	*Rorippa amphibia* (L.) Besser			x		x
H scap	*Scrophularia auriculata* L.	x		x		x
NP	*Solanum dulcamara* L.			x	x	x
H scap	*Stachys palustris* L.		x	x	x	x
I rad	*Stuckenia pectinata* (L.) Börner			x		
I rad	*Sparganium erectum* L. subsp. erectum			x	x	x
H scap	*Symphytum officinale* L. subsp. officinale	x	x	x	x	x
G rhiz	*Typha latifolia* L.				x	x
H rept	*Veronica beccabunga* L.		x			
H scap	*Veronica anagallis-aquatica* L.	x	x	x		x

For each taxon, we reported the presence/absence datum, and the life form (LF) as follows: *G rhiz* rhizomatous geophyte, *H rept* reptant hemicryptophyte, *H scap* scapose hemicryptophyte, *He* helophyte, *Hy* hydrophyte, *NP* nano-phanerophyte

clarify the interplay between groundwater resurgence and biotic diversity across multiple spatial and temporal scales, especially in lowland human-driven plains.

4.6 Numerical flow model

Since the shallow groundwater flows in confined conditions within the most part of the modelled area, the recharge of the aquifer was represented through a boundary condition at south as the general head boundary. The shallow aquifer at the study area is essentially recharged by rainwater infiltrating within the southern part of the plain, as well as by nearby aquifers belonging to the Apennine Chain, in agreement with findings in other sites (e.g. Aquino et al. 2015; Petrella and Celico 2009) and in agreement with the results of isotopic investigations.

Once the model was completed, it was calibrated at first in steady state using the hydraulic heads monitored

by the Emilia Romagna Environmental Protection Agency (ARPAe; data available at https://www.arpae.it/elenchi_dinamici.asp?tipo=dati_acqua&idlivello=2020).

Taking into account the small amount of information available about the hydraulic features of the shallow aquifer, an estimation of the hydraulic conductivity field was obtained through the software PEST (Doherty and Hunt 2010). In particular, the pilot points procedure was considered. The hydraulic conductivity of the outcropping low-permeability horizon was set to 5×10^{-7} m/s.

The hydraulic conductivity of the aquifer medium (second layer) was estimated through a calibration performed at first in steady state to evaluate a starting solution of the hydraulic conductivities and then in transient conditions using the hydraulic heads measured in the ARPAe wells. Figure 7 shows the location of the pilot points used in the

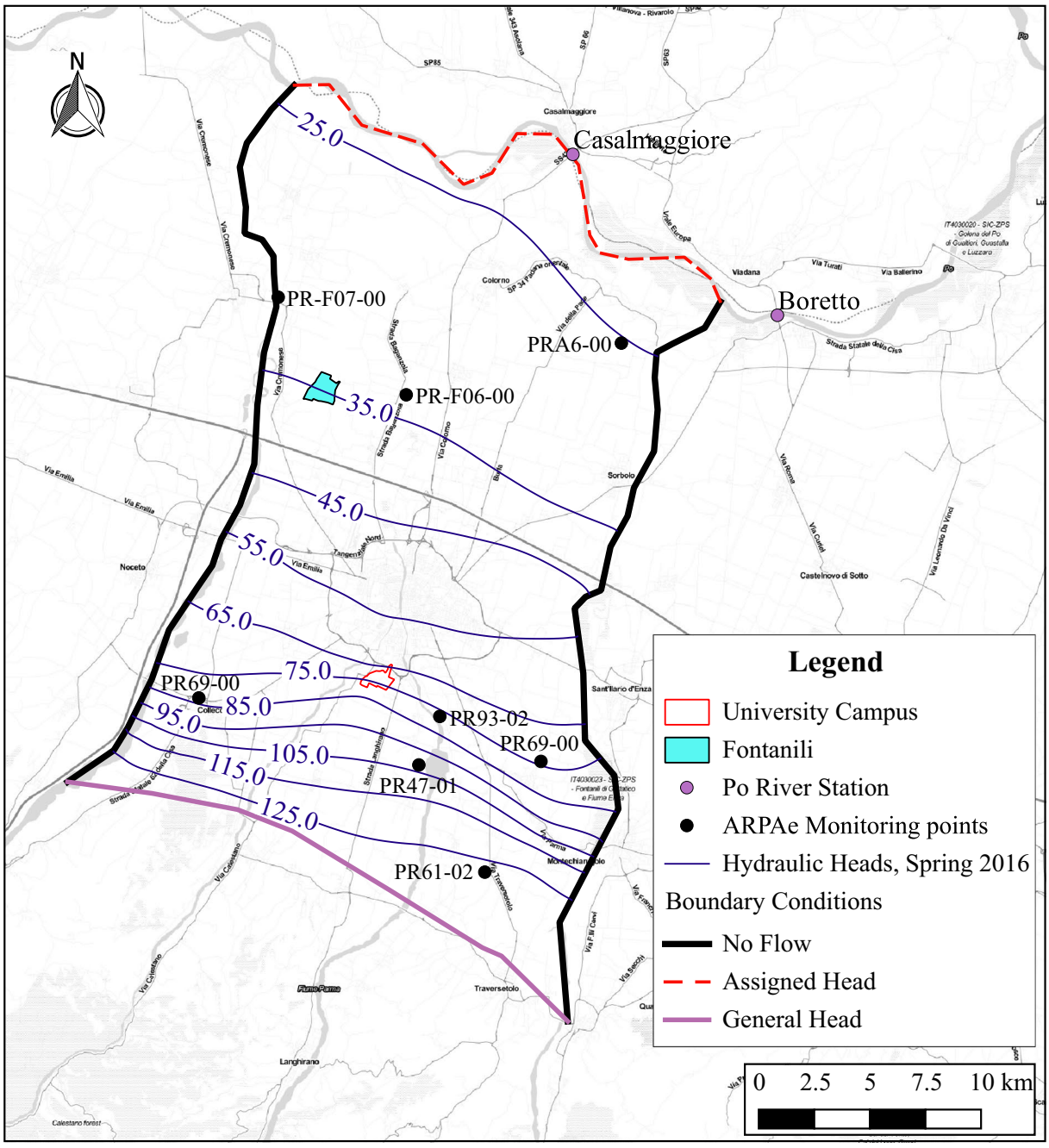

Fig. 7 Modelled groundwater flow net

estimation procedure. An exponential variogram with a small variance (0.2) and a range of 5 km was assumed.

The transient model reproduces 2400 days from April 1, 2010 to October 26, 2016 through 80 stress periods of 30 days each. Stress periods are subdivided into 30 time steps of 1 day. The upstream and downstream boundary conditions (BC) were set up according to observations. In particular, the downstream BC was set up according to the Po River daily level measurement at two monitoring stations (Fig. 7). The upstream boundary condition was set up according to piezometric contour heads. The observations at the monitoring wells showed a seasonal variability. Considering the available data at the ARPAE wells for the period 2010–2016, it was noticed that the average difference between maximum and minimum was about 4 m. The mean estimated level at the upstream boundary conditions was 128 m a.s.l.. For this reason, the upstream boundary condition was set up as a sinusoidal wave with a period of 360 days and a variation of ± 2 m with respect to the average level.

The widely accepted measure of model calibration (Anderson and Woessner 1992) is the normalized root mean square error (nRMSE). If nRMSE is below 10% (ASTM 2006), the model calibration is acceptable.

The following statistics are considered to evaluate the calibration results:

$$\text{Mean error (ME)} : \frac{1}{N}\sum_{i=1}^{N} \text{Hc}_i - \text{Ho}_i,$$

$$\text{Mean absolute error (MAE)} : \frac{1}{N}\sum_{i=1}^{N} \left|\text{Hc}_i - \text{Ho}_i\right|,$$

$$\text{Root mean square error (RMSE)} : \sqrt{\frac{1}{N}\sum_{i=1}^{N} \left(\text{Hc}_i - \text{Ho}_i\right)^2},$$

$$\text{Normalized root mean square error (nRMSE)} : \frac{\sqrt{\frac{1}{N}\sum_{i=1}^{N} \left(\text{Hc}_i - \text{Ho}_i\right)^2}}{H_0\text{MAX} - H_0\text{MIN}},$$

where N è is the number of observations, Hc_i is the computed hydraulic head level at the monitoring point i, Ho_i is the observed hydraulic head level, and H_0MAX and H_0MIN are the maximum and minimum observed hydraulic head.

Figure 8 shows the good agreement between the computed and observed hydraulic heads. Considering the statistics and in particular the nRMSE value, the capability of the numerical model of reproducing the observed values is clear. In this case, 111 observations were used.

Figure 9 shows, as example, the computed and observed hydraulic head levels at the monitoring point PR-F07-00. It is possible to see the good agreement between the computed and observed values and the dependence on the boundary conditions.

5 Parma FUA in a hydro-geo-ecological perspective

The Parma FUA hydro-geo-ecology (sensu Hanckock et al. 2009) was investigated through an interdisciplinary approach, with emphasis on the shallow aquifer system. This approach was carried out because there is an increasing recognition that groundwater is essential not only for human uses (domestic and agricultural, at the study site), but also for many ecological communities. As a matter of fact, when the groundwater flows out or comes close to the ground surface, the contribution of water and nutrients influences both the type and the persistence of aquatic ecosystems. At the same time, groundwater contaminants can have a negative impact on human health, as well as on aquatic communities.

The study pointed out that domestic wells and *fontanili* are both fed by shallow groundwater at least affected by PCE and nitrate contamination, upgradient of the rural area located north of Parma city. Moreover, *Folsomia candida* tests suggested the possibility that other types of contaminants (not analysed in this study) can affect the shallow groundwater.

Nowadays, PCE concentrations in the city centre are slightly higher than the legal limit. Moreover, PCE aerobic biodegradation can be due to the local microbial community and then an effective natural attenuation can be expected along the groundwater flow pathway. These results suggest a very low risk for human health, linked to the groundwater consumption in the rural area north of Parma City. Conversely, no forecasts can be made at present about the possible impact of low PCE concentrations on the aquatic ecosystem observed at the *fontanili*, because no information exists about the effects of a prolonged interaction between low-level chlorinated solvents and local biological species. Concerning nitrate contamination, the higher concentrations detected in some wells and *fontanili* suggest a high risk for both human health and aquatic ecosystems.

Taking into consideration the main findings of this work and the existence of other contamination sources not involved in this preliminary investigation (e.g. a

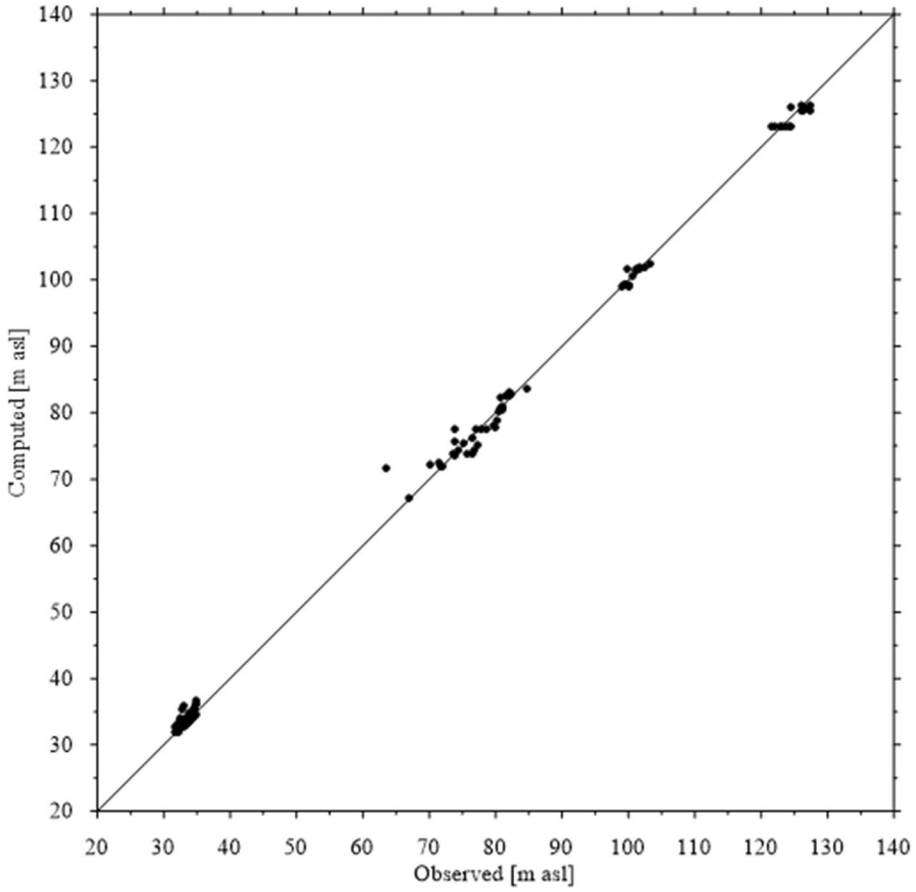

Fig. 8 Computed vs observed hydraulic head levels at PR-F07-00

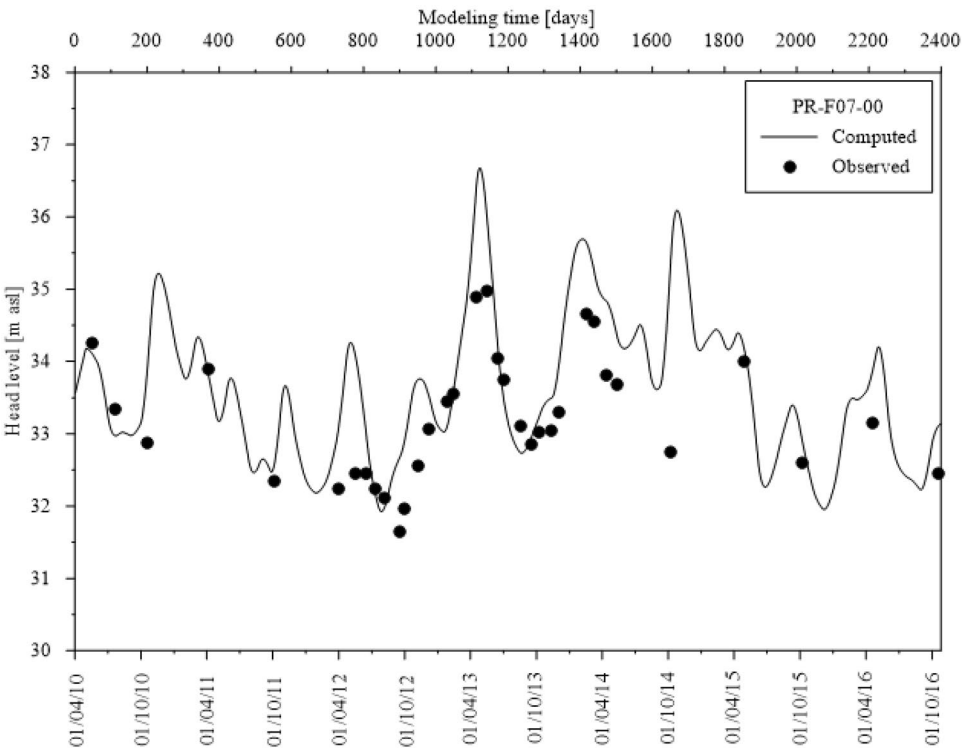

Fig. 9 Observed and modelled hydraulic head levels vs time at PR-F07-00

decommissioned municipal landfill), a long-term hydro-geo-ecological study has been planned. This study will also check possible progressive changes in groundwater temperature due to the increasing number of shallow geothermal boreholes within the FUA. Actually, these boreholes could alter the thermal equilibrium and, thus, make the environment incompatible with the existing biotic species at the *fontanili*. In fact, it is known that the persistence of some organisms in specific aquatic environments is more driven by water temperature than, for example, the hydrochemical features (e.g. Bolpagni and Laini 2016).

In a wider context, thanks to the interdisciplinary approach that combines successfully well-established investigation methods, the present study allows a better knowledge of the hydro-geo-ecological behaviour of GDEs. At the same time, through purpose-designed experimental investigations and simulation models, this approach could be used as a sort of guideline useful in studying such complex environmental systems.

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Section 2.2:

A new approach towards the monitoring and bioremediation of the unsaturated zone of contaminated aquifers

Article

Natural surface hydrocarbons and soil faunal biodiversity: a bioremediation perspective

Sara Remelli, Pietro Rizzo, Fulvio Celico and Cristina Menta

water

MDPI

Article

Natural Surface Hydrocarbons and Soil Faunal Biodiversity: A Bioremediation Perspective

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Abstract: Hydrocarbon pollution threatens aquatic and terrestrial ecosystems globally, but soil fauna in oil-polluted soils has been insufficiently studied. In this research, soil hydrocarbon toxicity was investigated in two natural oil seepage soils in Val D'Agri (Italy) using two different approaches: (i) toxicological tests with *Folsomia candida* (Collembola) and *Eisenia fetida* (Oligochaeta) and (ii) analysis of abundance and composition of micro- and meso-fauna. Soil sampling was done along 20 m-transepts starting from the natural oil seepages. Toxicological testing revealed that no exemplars of *F. candida* survived, whereas specimens of *E. fetida* not only survived but also increased in weight in soils with higher PAH concentrations, although no reproduction was observed. Analysis on microfauna showed that Nematoda was the most abundant group, with distance from seepages not affecting its abundance. Arthropoda results showed that Acarina, Collembola and Diptera larvae represented the most abundant taxa. The highest divergence in community composition was found between soils situated near seepages and at 5 m and 10 m distance. Arthropoda taxa numbers, total abundance and Acarina were lower in soils with high PAH concentration, while Diptera larvae were not significantly affected. Earthworms, together with Nematoda and Diptera larvae, could therefore represent ideal candidates in PAH degradation studies.

Keywords: soil arthropods; soil microfauna; bioremediation; bioindicators; natural oil seepages

1. Introduction

Over the years, the development of the global economy has led to a growing demand for oil products. Activities related to oil extraction and use, however, are among the leading factors determining a variety of environmental issues. Among these, leaking underground and aboveground storage tanks, improper disposal of petroleum waste and accidental spills are the main cause of soil and groundwater contamination [1]. Extensive damage involving food webs, thus affecting human health, can result from such hydrocarbon contamination through a series of bioaccumulation events, as well as the percolation and transportation of contaminants.

Despite this, not all hydrocarbon-contaminated soils are a consequence of human activities, since about 80% of the total production of crude oil derives from natural terrestrial fields [2], which allows the effects of continuous discharges in natural seeps to be studied. Hydrocarbons are naturally found in underground geological formations, where they are produced from abundant organic matter and its chemical alteration, slowly migrating as a result of lithostatic pressure and tectonic activity, finally producing spontaneous hydrocarbon emissions that can be detected on the Earth's surface [3]. These seepages can activate oxidation-reduction processes that cause changes in soil chemical and mineralogical composition. In addition, the gases that seep onto the surface partly displace soil air. This, together with bacterial oxidation of light hydrocarbons, generates an anaerobic environment that can directly or indirectly induce significant changes in the pH and oxidation-reduction potential

of the surrounding environment [4,5]. An oxygen-poor environment, combined with changes in the solubility of macro- and micro-nutrients, is a well-known stress factor for plants [6]. Furthermore, accumulation of pollutants in animals and plant tissues may induce mutations, even causing death [7].

Such consequences of hydrocarbon contamination, however, are not exclusively confined to local systems: soil micro-biota diversity loss often results from the spread of a limited number of fast-growing hydrocarbon degraders, which are dominant in contaminated conditions [8]. Such a phenomenon is also to be seen among soil fauna [9,10], which is well known to be involved in key processes such as organic matter decomposition, nutrient mineralization and microbial spore dispersal. Considering that several species of invertebrates are negatively affected by hydrocarbons, the consequent loss of biodiversity can affect the entire ecosystem functioning [11]. Some studies have shown that the soil faunal community observed in a contaminated area differs from uncontaminated areas. For instance, [12] observed that communities in polluted soils exhibited the dominance of different groups such as Collembola, Protura and Diplura, which were suggested to positively correlate with major detected contaminants (Pb, Sb). At the same time, Symphyla showed a negative correlation with these pollutants [12]. Another study, on the other hand, revealed that Isopoda and Hymenoptera abundances in petrochemical-contaminated sites were higher when compared with uncontaminated areas [13]. Thus, not all terrestrial invertebrates show high sensitivity to hydrocarbon contamination, and it is possible that organisms living in seepage areas find a suitable environment that can contribute additional organic carbon [14]. In addition, the ability of soil fauna to live in association with microbial communities should be taken into account, since some of these microbes utilize hydrocarbons as an energy source and are responsible for the breakdown of organic pollutants [10,15].

This study combines two different approaches, both aimed at quantifying soil surface hydrocarbon effects on soil communities, with a particular focus on soil fauna. First, the effects of natural hydrocarbons in soils are assessed on two species—*Folsomia candida* Willem, 1902 (Collembola: Isotomidae) and *Eisenia fetida* (Savigny, 1826; Oligochaeta: Lumbricidae), both extensively used in ecotoxicological testing—using a lab approach. Second, the responses of the soil faunal community to hydrocarbons naturally present on the surface of an area characterised by active oil seepages are analysed with regard to community abundance and composition. If soil fauna survives in such a stressful environment, we can hypothesise that it has the ability to take advantage of the presence of hydrocarbons. Consequently, the identification of the most resistant groups may be the goal for further studies aimed at deepening the potential of specific soil fauna to accelerate decontamination processes in bioremediation systems, either directly or indirectly, through symbiotic relationships that could enhance microbial degradation.

2. Materials and Methods

2.1. Area of Study

In Italy, natural hydrocarbon emissions are mainly located along a strip of land parallel to the Apennine chain: The Southern Apennine Trust-belt, known as Val d'Agri, ranks among the largest onshore petroleum deposits in Europe, the source rocks of which are marine anoxic carbonates facies containing sulphur [16]. Val d'Agri, located near the town of Potenza, is a 12 km-wide and 30 km-long intermontane basin, covering the upstream extent of the entire Agri River drainage basin, oriented NW-SE at about 600 m a.s.l. and filled with alluvial and lacustrine deposits hundreds of meters thick [17].

In Val d'Agri, the main oilfield is located within fractured carbonate rocks attributable to the Apennine Platform [18–20]. Oil and gas are stored within limestone and dolomite from the Miocene to Cretaceous period [21]. The reservoir is under a Pliocene siliciclastic succession and a thick layer of mélange.

Hydrocarbon extraction dates from the 1990s, through wells placed between 1800 m and 3500 m below sea level. Spontaneous gas (mainly H_2S) and hydrocarbon emissions, however, have occurred in

Val d'Agri since the 19th century, particularly in the Tramutola municipality and, from the 20th century, have led to the drilling of many exploration and production wells near the hydrocarbon seepages; these were decommissioned and have been closed for several decades.

The study area is located about 2 km west of Tramutola, in the Rio Cavolo valley (a tributary of the Agri River), where plant cover is mainly represented by oak, hygrophilous formations and thermophilic shrubs. Within this area, two sites characterised by the presence of oil seepages were selected for soil sampling in March 2019: site A, close to a hydrocarbon spring located at 636 m a.s.l., and site B, close to a diffuse hydrocarbon spring located at 643 m a.s.l. (Figure 1).

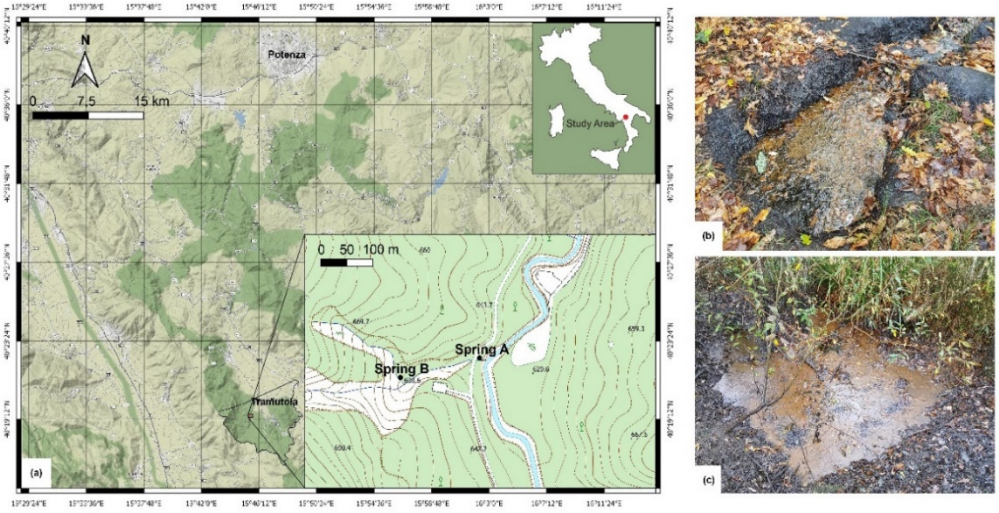

Figure 1. Area of study: (**a**) location of the springs; (**b**) seepage A0 and (**c**) seepage B0 [22,23].

Both hydrocarbon springs are situated over an E-W tectonic fault zone [24]. From the hydraulic point of view, this fault zone acts as a conduit, as in classic karst systems [25] or as a conduit-barrier system (e.g., in carbonate aquifers of southern Italy and in other similar systems abroad; [26–34]), therefore enhancing the local upflow of fluids. Perennial springs are mainly fed by the shallow groundwater flowing within the nearby carbonate aquifer (Figure 2), with a possible contribution from a deeper confined aquifer [35]. In the wider hydrogeological context, the carbonate system belongs to the so-called Mesozoic carbonate platform complex, characterised by very high permeability due to a well-developed fracture network and karst conduits [36]. In contrast, the deeper confined system belongs to the so-called syn-orogenic turbidite complex and the outer/inner basins complex, characterized by a lower permeability [36].

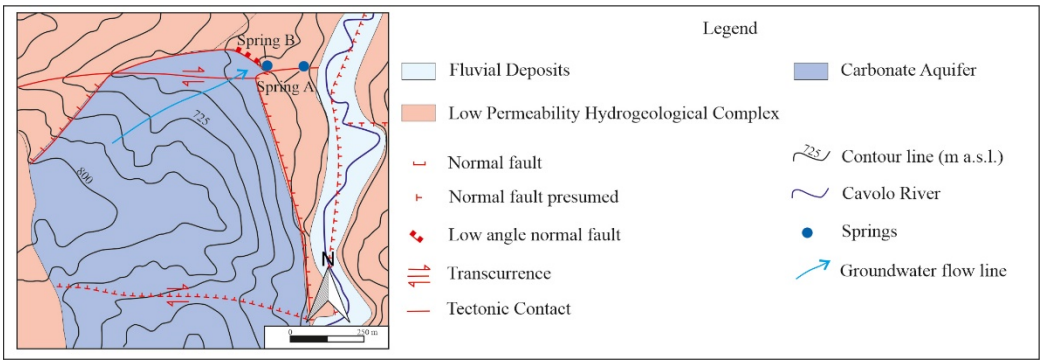

Figure 2. Hydrogeological map of the study area (from Rizzo et al., 2020 modified) [35]; the blue points show the location of the investigated springs A and B (the geological sketch is taken from Olita, 2018, modified) [24].

2.2. Chemical Analysis on Soil

BTEX (Benzene, Toluene, Ethylbenzene and Xylene) and PAH (Polycyclic Aromatic Hydrocarbons) concentrations in soils with hydrocarbon seepages (called A0 and B0, Figure 1b,c), collected near springs A and B, were determined. The analyses were performed by Biochemie Lab S.r.l. following the EPA 5030C 2003 + EPA 8015D 2003 protocol for BTEX and the EPA3510C 1996 + EPA 8270E 2018 protocol for PAH.

The other soil samples collected for zoological purposes along the transects starting from A0 (called A5 and A10) and from B0 (called B5, B10, B15 and B20) were not analysed in the present study, since no detectable hydrocarbons were reported by previous observations at these sites.

2.3. Ecotoxicological Tests on F. candida and E. fetida

The springtail *F. candida* (Exapoda: Collembola) and the earthworm *E. fetida* (Oligochaeta: Lumbricidae) were used to test soil toxicity in A0 and B0.

F. candida came from laboratory cultures at Parma University. Growth, survival and reproduction tests were carried out according to [37]. Individual specimens were maintained at 20 ± 2 °C (with 50–55% RH) and fed weekly on a pulverized mixture of dried organic cereals (20% wheat, 20% oats, 20% rye, 20% spelt, and 20% rice). Specimens used for egg deposition (aimed to obtain age-synchronized juveniles to be used in the test) were collected from breeding containers and mixed to prevent them originating from a single breeding line. All springtails used for testing were 10 days old and age-synchronized by removing eggs from the deposition cultures and, once hatched, inserting juveniles into Petri dishes with moistened breeding substrate with a ratio of 8:1 (w/w) plaster of Paris and activated carbon powder.

For survival and reproduction tests, Petri dishes were filled with 0.5 cm of testing soil, wetted with deionized water to reach 40–60 % of the total water holding capacity (WHC). Five replicates were set up for both soil A0 and B0. Ten *F. candida* specimens aged 10 days were added to each Petri dish using an exhauster, checking that none of the exemplars died during the process. Springtails were maintained at 20 ± 2 °C with 70–80% relative humidity (RH) and fed with the same mixture of cereals used during the breeding. The Petri dishes were incubated for 28 days, aerated once a week and watered when water loss exceeded 2% of the initial WHC. At the end of this period, the number of surviving adults and new-born springtails (when present) were recorded using a stereomicroscope with floatation technique. In order to assess the validity of the test, the same procedure was applied using a control soil consisting of a standard substrate: 70% quartz sand, 20% kaolinite clay, 10% peat and calcium carbonate to adjust the pH to 6.0 ± 0.5.

The sexually mature *E. fetida* were supplied by a worm breeding company. Survival and reproduction tests were carried out according to [38]. Test containers were filled with 500 g of testing soil, to which deionised water was added to achieve a soil moisture of 40–60% of the WHC. Five replicates were prepared for both soil A0 and B0. Ten earthworms were washed with distilled water, dried and weighed, then placed in the container and maintained at 20 ± 2 °C with 80–85% RH for 28 days. During the test period, earthworms were fed weekly with cattle manure, and water was added when water loss > 2% of the initial WHC. At the end of the test, surviving earthworms and cocoons (when present) were counted. Surviving specimens were washed with distilled water and weighed. To assess the validity of the test, the same procedure applied to *F. candida* was followed, with standard substrate as a control soil.

2.4. Soil Fauna Extraction

For soil fauna extraction, soil sampling spots were selected by gradually moving away from seepages A0 and B0 at intervals of 5 m along transect A and B, respectively. Based on the results obtained from BTEX and PAH analysis, it was decided to select additional spots where there was a major concentration of hydrocarbon. Consequently, three spots along the transect starting from

A0 (named A0, A5 and A10) and five spots starting from B0 (named B0, B5, B10, B15 and B20) were selected. For each spot, three soil samples (replicates) of 10 × 10 × 10 cm were collected using a spade.

Regarding microfauna extraction (fauna < 200 μm), 10 g of soil from each replicate were placed in a modified Baermann funnel. The sample, wrapped in a piece of muslin and supported by a metal gauze, was placed into a Petri dish, partially covered with water and left for 24 h. Microfauna leaving the soil sample during this period and falling through the gauze was collected in the Petri dish. It was then examined using a stereomicroscope and a microscope and identified at the phylum taxonomic level (i.e., Ciliophora, Nematoda, Rotifera, Sarcomastigophora and Tardigrada).

Soil microarthropods (200 μm–2 mm) were extracted from each soil replicate using a Berlese–Tüllgren funnel for 10 days. The extracted soil arthropods were collected and preserved in a solution consisting of 75% ethyl alcohol and 25% glycerol by volume. Identification was carried out at different taxonomic levels (i.e., class for Myriapoda, order for Hexapoda, Chelicerata, and Crustacea), each of them counted using a stereomicroscope.

2.5. Data Analysis

In order to analyse ecotoxicological differences between soil A0 and B0, Student's *t*-test was performed on the proportion of survivors, new-borns per survivor and, for *E. fetida*, survivors' rate of growth. To meet the assumptions of parametric statistical tests, arcsine transformation of the proportion of survivors and log-transformation of the weight values were applied [39].

To evaluate the effects of natural hydrocarbon presence on microfauna, the number of organisms extracted for each phylum was considered as the dependent variable, whereas for arthropod communities, the number of observed taxa and their total abundance, together with the abundance of each Arthropoda taxon, were set as the response variables. Data between equally spaced spots from seepages A and B were compared using Student's *t*-test. Within every site, data obtained along the transect were compared using one-way ANOVA and Tukey test as a post-hoc. To meet the assumptions of parametric statistical tests, log-transformation was applied both to the number of observed phyla/taxa and their abundance (using log(x + 1) to avoid zeros) [39]. For each spot, the Simpson Index of Diversity (1-D) and the Shannon Diversity Index (H) were applied to the Arthropoda community.

As far as the differences in arthropod taxa assemblages among the sample sites were concerned, these were studied through square root transformation of the community matrix to minimize the influence of the most abundant groups, after which the Bray–Curtis dissimilarity index was calculated. On the dissimilarity matrix obtained, a permutational multivariate analysis of variance (PERMANOVA) was conducted. Site and distance from the seepage were considered as independent variables, and only distances up to 10 m were included to avoid unbalanced models. In the event of a significant result, pairwise comparisons were performed using the R package "RVAideMemoire" and dissimilarities in data were visualized with a principal coordinate analysis (PCoA) [40]. An analysis of similarity percentages (SIMPER) was then performed to test which arthropod groups were driving the differences in assemblages. Ordination, PERMANOVA and SIMPER were all performed using the R package "vegan" [41]. A $p \leq 0.05$ was considered significant. Statistical analyses were performed using R v.3.6.3 (R Core Team, Vienna, Austria) [42].

3. Results

3.1. Chemical Analysis on Soil

No BTEX were found in any of the analysed soil samples. Several PAHs, however, were present (Table 1). Benzo(g,h,i)perylene was the PAH with the highest concentration in both A0 and B0 soils, watered by springs A and B, followed by Benzo(a)anthracene, Benzo(b)fluoranthene and Benzo(k)fluoranthene in soil B0.

Table 1. Chemical analysis on soils A0 and B0.

	Soil A0 (mg/kg)	Soil B0 (mg/kg)
Dry weight content at 105 °C (%)	63.5	43.3
Skeleton between 2 cm and 2 mm (%)	56.3	1.0
Benzene	<0.01	<0.01
Etilbenzene	<0.05	<0.05
Styrene	<0.05	<0.05
Toluene	<0.05	<0.05
Xylenes	<0.05	<0.05
o-Xylene	<0.05	<0.05
p,m-Xilenes	<0.05	<0.05
Total aromatic compounds (D. Lgs.152/06)	<0.10	<0.10
Benzo(a)anthracene	0.16	0.95
Benzo(a)pyrene	<0.01	<0.01
Benzo(b)fluoranthene	<0.05	3.93
Benzo(k)fluoranthene	<0.05	0.58
Benzo(g,h,i)perylene	0.28	1.45
Chrysene	0.21	1.23
Dibenzo(a,e)pyrene	<0.01	<0.01
Dibenzo(a,l)pyrene	<0.01	0.10
Dibenzo(a,i)pyrene	<0.01	<0.01
Dibenzo(a,h)pyrene	<0.01	<0.01
Dibenzo(a,h)anthracene	<0.01	0.34
Indeno(1,2,3-c,d)pyrene	0.05	0.19
Pyrene	<0.05	0.61
Total IPA (D.Lgs. 152/06 All.5 Table 1)	<1.0	8.25

3.2. *Ecotoxicological Tests on* F. candida *and* E. fetida

In the control soil, the conditions of validity for the tests were met for both *F. candida* and *E. fetida* [37,38].

Neither vital Collembola nor new-borns were observed after 28 days in either soil A0 or B0.

In tests with *E. fetida*, a significant difference between A0 and B0, both for survival and rate of growth, was observed ($p \leq 0.05$ for both), with a loss of weight in earthworms after 28 days in soil A0 compared with a weight gain in those in soil B0 (Figure 3). No cocoons were found, either in A0 or B0.

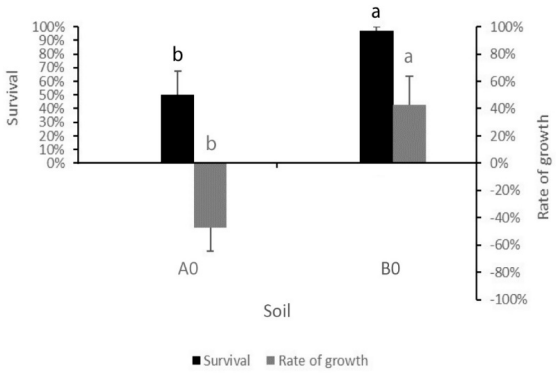

Figure 3. Average and Standard Error of the percentage of surviving earthworms and of the rate of growth in soil A and B after 28 days. Different letters above bars mean significant differences between A0 and B0 ($p \leq 0.05$).

3.3. *Soil Fauna Characterization*

3.3.1. Soil Microfauna

Results from microfauna extraction are reported in Table 2.

Table 2. Average ± Standard Error of the number of individuals for each faunal group (microfauna per 10 g and Arthropoda per m^2) found along transects in study sites, together with the Simpson Index of Diversity (1-D) and the Shannon Diversity Index (H) calculated for Arthropoda.

	A									B										
	0 m			5 m			10 m			0 m			5 m			10 m			15 m			20 m		
Microfauna (<200 μm)																								
Ciliophora	7.33	±	6.36	3.33	±	3.33	8.67	±	4.67		-		2.00	±	1.15	1.33	±	0.67		-		0.67	±	0.67
Nematoda	16.00	±	13.11	36.67	±	15.68	74.00	±	22.72	9.33	±	3.53	67.33	±	40.16	38.00	±	20.53	4.67	±	2.91	18.00	±	11.02
Rotifera		-		1.33	±	1.33	5.33	±	0.67		-			-			-			-			-	
Sarcomastigophora	0.67	±	0.67	1.33	±	1.33		-			-			-		0.67	±	0.67		-		2.00	±	1.15
Tardigrada		-			-		0.67	±	0.67		-		4.67	±	2.91	2.67	±	1.76		-		0.67	±	0.67
Microarthropods (200 μm–2 mm)																								
Acarina	92.00	±	81.61	481.21	±	353.83	608.59	±	314.97		-		4415.84	±	3220.18	304.30	±	128.75	7.08	±	7.08	311.37	±	290.14
Araneidae		-			-			-			-			-			-		7.08	±	7.08		-	
Chilopoda		-			-		56.61	±	37.45		-			-			-			-			-	
Coleoptera	7.08	±	7.08	49.54	±	25.52	35.38	±	18.72		-		49.54	±	18.72	14.15	±	14.15	7.08	±	7.08	28.31	±	14.15
larvae		-		49.54	±	25.52	21.23	±	12.28		-		35.38	±	14.15	7.08	±	7.08		-		7.08	±	7.08
Collembola	3651.56	±	3588.06	226.45	±	183.99	750.13	±	504.98	28.31	±	28.31	643.98	±	396.10	169.84	±	53.43		-		707.67	±	580.80
Diplura		-			-			-			-		14.15	±	14.15		-			-			-	
Diptera	7.08	±	7.08	14.15	±	14.15	42.46	±	32.43	42.46	±	42.46	70.77	±	30.85	84.92	±	44.19	42.46	±	12.26	183.99	±	60.46
larvae	7.08	±	7.08	14.15	±	14.15	42.46	±	32.43	35.38	±	35.38	42.46	±	24.51	77.84	±	46.40	28.31	±	28.31	162.76	±	51.03
Hemiptera		-			-		7.08	±	7.08	7.08	±	7.08	42.46	±	42.46	21.23	±	21.23		-		92.00	±	46.40
Hymenoptera	14.15	±	14.15	7.08	±	7.08	14.15	±	14.15		-		42.46	±	24.51	21.23	±	0.00		-		70.77	±	60.46
larvae		-			-		14.15	±	14.15		-			-			-			-			-	
Isopoda	7.08	±	7.08	14.15	±	14.15	14.15	±	7.08		-		35.38	±	18.72		-			-		106.15	±	76.55
Lepidoptera	7.08	±	7.08		-			-			-			-			-			-			-	
larvae	7.08	±	7.08		-			-			-			-			-			-			-	
Pauropoda		-			-			-			-		155.69	±	123.99		-			-			-	
Pseudoscorpionida		-			-			-			-		7.08	±	7.08		-			-			-	
Psocoptera		-			-		7.08	±	7.08		-		7.08	±	7.08	7.08	±	7.08		-			-	
Symphyla		-			-		28.31	±	28.31		-		35.38	±	35.38	7.08	±	7.08		-			-	
1-D	0.19	±	0.16	0.40	±	0.11	0.64	±	0.09	0.49	±	0.29	0.44	±	0.09	0.67	±	0.07	0.35	±	0.17	0.67	±	0.05
H	0.38	±	0.31	0.73	±	0.14	1.25	±	0.29	0.22	±	0.22	0.95	±	0.19	1.31	±	0.19	0.54	±	0.28	1.31	±	0.15

In site A, microfauna was mostly represented by Nematoda (82%), Ciliophora (12%) and Rotifera (4%), while Sarcomastigophora and Tardigrada together accounted for less than 2% of the microfauna found. In site B, Nematoda accounted for 90% of the microfauna, followed by Tardigrada (5%), Ciliophora (3%) and Sarcomastigophora (2%), while no Rotifera were found.

Ciliophora and Nematoda were mostly found at 5 m and 10 m from the seepages, both in site A and B, but their abundance did not differ significantly either between equally spaced spots from the seepages or along the transects (Figure 4a,b).

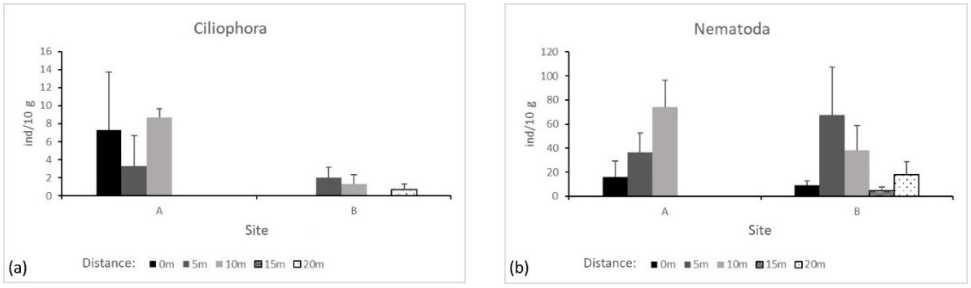

Figure 4. Average and Standard Error of phyla accounting for at least 90% of total microfauna abundance. (**a**) Ciliophora and (**b**) Nematoda abundance per 10 g of soil. No letters above bars mean no significant differences within transects ($p > 0.05$).

There were also no differences observed for the other microfauna, with the exception of Rotifera along transect A (absent in B), where they resulted to be more abundant at 10 m from the seepage ($p \leq 0.01$).

3.3.2. Soil Arthropods

Results from microarthropods extractions are reported in Table 2. A total of 15 groups of Arthropoda was found: Acarina and Collembola were the most abundant taxa, together representing 89% of the total arthropods extracted; only Diptera (3%), specifically larvae, were present in all soils; Coleoptera, mostly larvae, Isopoda, Hymenoptera, Hemiptera and Pauropoda together represented 6% of the total; each of the remaining taxa accounted for less than 1%.

Along transect A, Collembola represented 75% of the specimens extracted, followed by Acarina (19%), Coleoptera (2%) and Diptera (1%). The other groups together accounted for 3% of the arthropods found, with Araneidae, Diplura, Pauropoda and Pseudoscorpionida being absent. Along transect B, Acarina, Collembola and Diptera represented 65%, 20% and 5%, respectively, of the total arthropod abundance. Hemiptera, Pauropoda, Isopoda and Hymenoptera each accounted for 2%, with Coleoptera accounting for 1% and a further 1% made up of the remaining groups. Chilopoda and Lepidoptera larvae were absent.

PERMANOVA analysis revealed no site-dependent differences between arthropods assemblages up to 10 m, whereas the distance from the seepage resulted as a significant factor ($p \leq 0.01$; Figure 5).

Pairwise comparisons revealed a difference between 0 m and further distances ($p < 0.05$ for both comparisons). Following this result, SIMPER analysis was conducted using distance as a grouping factor. Faunal assemblages differed for >78% in contrasts between samples collected near the seepage (i.e., at 0 m) and the other distances, while 5 m differed from 10 m assemblages for <50% (Table 3).

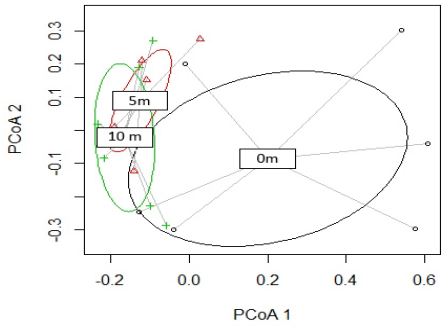

Figure 5. Principal coordinate analysis (PCoA) ordination plot with the dissimilarity in arthropods communities according to their distance from the seepage.

Table 3. Results of similarity percentages (SIMPER) analysis. Most influential arthropod groups are shown, accounting for a cumulative dissimilarity between distances from the seepages of 70%. Overall (%): average contrast dissimilarity; Ratio: average contribution to overall dissimilarity to sd ratio; Cum. (%): ordered cumulative contribution of each arthropod group.

Contrasts between Distances			Overall %	Most Influential Groups	Ratio	Cum. %
0 m	-	5 m	82.82	Acarina	2.08	36.54
				Collembola	1.25	63.01
				Coleoptera larvae	0.88	72.03
	-	10 m	78.10	Collembola	1.56	30.51
				Acarina	1.75	59.00
				Diptera larvae	1.11	68.63
				Hymenoptera	1.14	75.04
5 m	-	10 m	48.50	Acarina	1.41	28.47
				Collembola	1.43	45.67
				Diptera larvae	1.07	54.55
				Coleoptera larvae	1.02	61.87
				Hymenoptera	1.14	67.29
				Pauropoda	0.89	72.51

A separate analysis of the two sites revealed an increase in both Simpson and Shannon indexes with greater distance from the springs, with the exception of B15. Diversity was greater in transect B compared to transect A (Table 2).

The number of taxa and abundances (both total abundance and abundances of each taxon) did not differ significantly either between equally spaced spots in A and B nor along transect A (Figure 6). On the contrary, both number of groups and total abundance differed significantly between distances on the transect from spring B ($p \leq 0.001$ and $p < 0.01$. respectively) (Figure 7). Both data were higher in B5 than in B0 and B15. The number of taxa in B0 were also lower than those found in the other soils along the transect, and the total abundance was higher at a distance of 20 m from the seepage.

Acarina abundance did not differ along transect A, in contrast to transect B (where $p < 0.01$ among distances in transect B). In site B, Acarina were mostly found at 5 m from the seepage. Here their abundance was significantly higher than that found in B0 and at 15 m (Figure 8a).

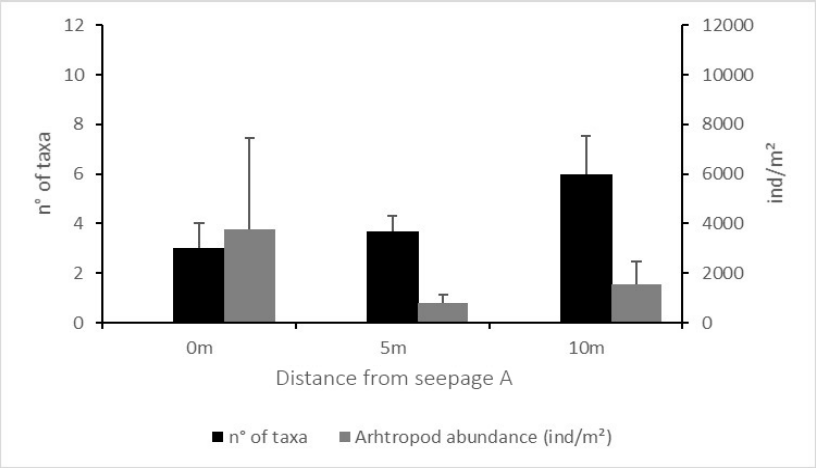

Figure 6. Average and Standard Error of the number of taxa and the number of individuals per m^2 found along the transect at 0 m (soil surrounding seepage A), 5 m and 10 m from seepage A. No letters above bars mean no significant differences within transects ($p > 0.05$).

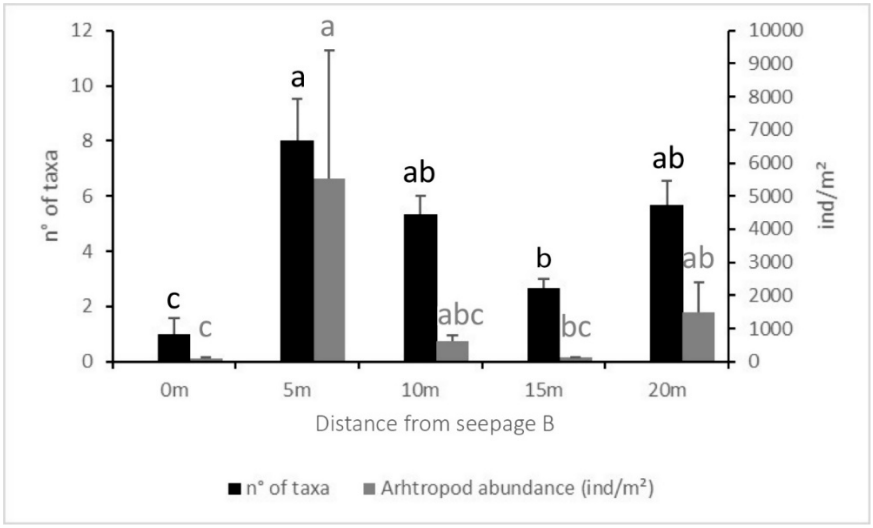

Figure 7. Average and Standard Error of the number of taxa and the number of individuals per m^2 found along the transect at 0 m (soil surrounding seepage B), 5 m, 10 m, 15 m and 20 m from seepage B. Different letters above bars mean significant differences within transects ($p \leq 0.01$).

A higher abundance of Collembola was found in site A, but no significant differences were found along the transect (Figure 8b). In site B, their abundance was lower than in site A but with significant differences among the transect ($p \leq 0.01$): more Collembola were found at 5 m and 20 m than at 15 m from B0.

Diptera, mostly larvae, were present in lower numbers, and their abundance did not differ significantly along either transect A or B. Nevertheless, a gradual increase was found with greater distance from the springs (Figure 8c). Among the remaining taxa, only Pauropoda abundance differed significantly along transect B ($p \leq 0.01$), as they were only found at 5 m from spring B.

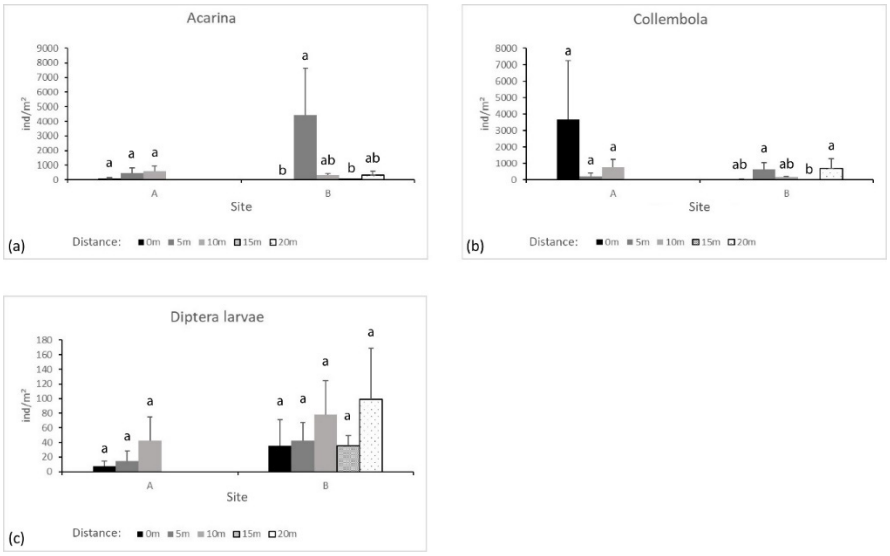

Figure 8. Average and Standard Error of groups accounting for at least 90% of total arthropod abundance. (**a**) Acarina abundance per m^2; (**b**) Collembola abundance per m^2; (**c**) Diptera larvae abundance per m^2. Different letters above bars mean significant differences within transects ($p \leq 0.01$).

4. Discussion

Soil fauna plays a key role in soil ecosystem functioning, since it is involved in the decomposition of organic matter, regulation of the microbial community and nutrient cycle. This key role, together with the stability of community composition in an undisturbed environment, makes soil fauna a widely used tool for the bio-indication of changes in soil properties [43]. Species such as *F. candida* and *E. fetida* are, therefore, frequently used as indicators on account of their sensitivity to soil contamination. Our study revealed that *F. candida* was negatively affected by the presence of hydrocarbons, so much so that no springtails survived after 28 days and no reproduction was observed, confirming the toxic impact of these compounds for some organisms. However, ecotoxicological tests on *E. fetida* highlighted the fact that other organisms, such as earthworms, which are generally sensitive to contaminants, were able to survive in soils containing hydrocarbons. Results in soil B0, when compared to A0, suggested that organisms such as these are able to take advantage of higher concentrations of these toxic compounds, confirming observations made by García-Segura et al. [44]. The lower survival of earthworms in soil A0, however, might also be related to the physical properties of the soil (i.e., high skeleton proportion). The absence of cocoons, however, suggested that *E. fetida* was also negatively affected by the presence of hydrocarbons, even if it is possible that reproductive activity was only slowed down. Since earthworms are under investigation for their crude-oil decontamination potential [45], the results of our study point to *E. fetida* as a potential species of interest for bioremediation, especially as some studies have found this species to be not only more resistant than others to this contaminant, but also to possess an effective ability to breakdown crude oil [46–48].

Erstfeld and Snow-Ashbrooky [9] have suggested that some invertebrate communities are favourably impacted by the presence of hydrocarbons, since carbon represent a food resource. In light of this and considering the strong symbiotic relationship with the final element in the chain of organic pollutant breakdown (i.e., microflora) [10], hydrocarbon-resistant soil fauna has been proposed as a bioremediation agent. In our study, microfauna was mostly represented by nematodes as in other similar studies, which found higher abundances of deposit feeders (especially nematodes) near active springs which, by feeding on the same bacteria, caused a reduction in the amount of other microfauna through competitive exclusion [14,49–51]. In addition, the abundance of Nematoda was not significantly affected by the proximity of the seepages, either in A or B. Such a result contrasts with Blakely et al. [52], who recommend nematodes as good indicators for some contaminants, such as PAHs, because their

permeable cuticle leads to a direct exposure to soil particles and contamination, thus increasing their sensitivity to pollutant compounds. Other studies, on the other hand, highlight the fact that nematodes are petroleum fuel tolerant and are able to co-operate with bacteria to promote the biodegradation rate of petroleum hydrocarbons [51,53,54]. In fact, in a recent study on bacterial communities carried out in the same study area, several bacterial strains capable of degrading hydrocarbons were isolated and screened, such as those belonging to genera *Achromobacter* and *Pseudomonas* [55].

With regard to Simpson and Shannon indexes calculated on arthropods in the present study, these generally increased as the distance from the springs became greater. Higher values, however, were found along transect B, even though B0 had a higher concentration of PAHs. This result is in line with García-Segura et al. [44], who noted that diversity indices were generally higher in contaminated sites. Nevertheless, the extreme conditions near the springs can affect soil fauna biodiversity, together with the microbial community, by stimulating hydrocarbon degraders while, at the same time, rendering other organisms inactive [10].

In our study, higher densities of arthropods were found in A0 and B5. Since PAH concentration in B0 was higher, it is possible that arthropod abundance, mainly driven by a greater abundance of Collembola and Acarina, increased with the presence of hydrocarbons at low concentrations. Together with nematodes, Erstfeld and Snow-Ashbrook [9] noted that the abundance of collembolans also increased with some hydrocarbon contaminants, such as PAHs, a result also confirmed by Migliorini et al. [12], who found that Collembola exhibited higher densities in contaminated sites. Like nematodes, a higher abundance of Collembola could be linked to changes in microbial dynamics [56]. Finally, even though Acarina was one of the most abundant taxa in this study, this taxon was absent in the soil surrounding spring B (i.e., the one with higher hydrocarbon concentration). This represents an interesting result, considering the fact that Acarina, together with Coleoptera and Hymenoptera, are generally thought to be more tolerant than other organisms to hydrocarbons, due to their lower permeable cuticle [52]. In contrast, other taxa were confined to one spot: Diplura, Pauropoda and Pseudoscorpionida were only found in B5, a result in line with García-Segura et al. [44], who found the presence of these taxa only in moderately contaminated sites; Chilopoda was found only in A10, with a progressive reduction in soil concentration of hydrocarbons moving away from the seepage, suggesting they are to be considered pioneers in site rehabilitation [57]. Diptera was the only mesofaunal group found in all spots, where its larvae did not seem to be strongly affected by hydrocarbons, even if their abundance generally increased with greater distance from the springs; their presence in sites with a low number of groups could be due to the reduction of competition and/or predation, as well as changes in the microbial community [58].

To conclude, it is to be noted that differences in arthropod assemblages in the first 10 m from the seepages were driven by the distance from the seepage rather than the sample area (A or B), even if B was characterized by higher PAH concentrations. In fact, community assemblages from soils collected further away from seepages were similar for almost 50% of the population, independent of whether they came from site A or B, while there was a significant difference between them and assemblages from soils collected near the seepages. Apart from the presence of hydrocarbons, sample areas share similar characteristics, so it is possible to speculate that soil populations further from the seepages consist of primary edaphic zoocoenoses that, nearer the seepage, are gradually substituted and dominated by more resilient organisms. These results could be compared with those of Melekhina [59], who found that the proportion of microfaunal taxa in an oil polluted area in the Subarctic of European Russia changed during a seven years remediation study, with some groups of microarthropods acting as biomarkers of succession stages. In Melekhina's study, the highest relative abundance of dipterous larvae was observed at the first stage of zoocoenosis recovery, but then, their proportion decreased with the increasing of collembolans, similarly to what observed along transects in our study.

As a final observation, even if the site did not significantly affect community structure, nevertheless, a higher variability was observed within communities near seepages; this was probably due to different

concentrations of hydrocarbons. As suggested by Migliorini et al. [60], even small differences in environmental characteristics can result in distinct edaphic populations.

5. Conclusions

Results obtained with *E. fetida* suggest that this particular earthworm could benefit from the presence of some of the compounds found in these soils, thus responding to the need for organisms which are both easy to breed and able to accelerate bioremediation processes in contaminated industrial sites.

Moreover, this study reveals that some soil organisms, specifically Collembola and Acarina, even if negatively affected by the higher oil concentration near the seepages, are still able to increase their abundance at lower levels of contaminant pollution. Other soil fauna, on the other hand, such as Nematoda and Diptera larvae, seem not to be affected by the presence of PAHs and are able to live at higher concentrations of hydrocarbons, some of which having carcinogenic properties (e.g., Benzo(b)fluoranthene). Therefore, taxa such as these could be suitable for study as potential candidates for incorporation in PAH degradation processes.

In a wider perspective, purpose-designed experiments are being planned with the aim of exploring the degrading potential of consortia made up of soil fauna and bacterial strains isolated at the study site.

Author Contributions: Conceptualization, C.M., F.C. and S.R.; methodology, C.M. and S.R.; validation, C.M. and S.R.; formal analysis, S.R.; investigation, P.R. and S.R.; resources, C.M.; data curation, S.R.; writing—original draft preparation, C.M. and S.R.; writing—review and editing, C.M., F.C. and S.R.; visualization, S.R.; supervision, C.M. 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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SECTION 2.2:

A NEW APPROACH TOWARDS THE MONITORING AND BIOREMEDIATION OF THE UNSATURATED ZONE OF CONTAMINATED AQUIFERS

Article (submitted)

Vermiremediation applied to PCB and PCDD/F contaminated soils and its implications for percolating water

Sara Remelli, Alessandro Scibona, Daniele Nizzoli, Luciana Mantovani, Mario Tribaudino, Fulvio Celico, Cristina Menta

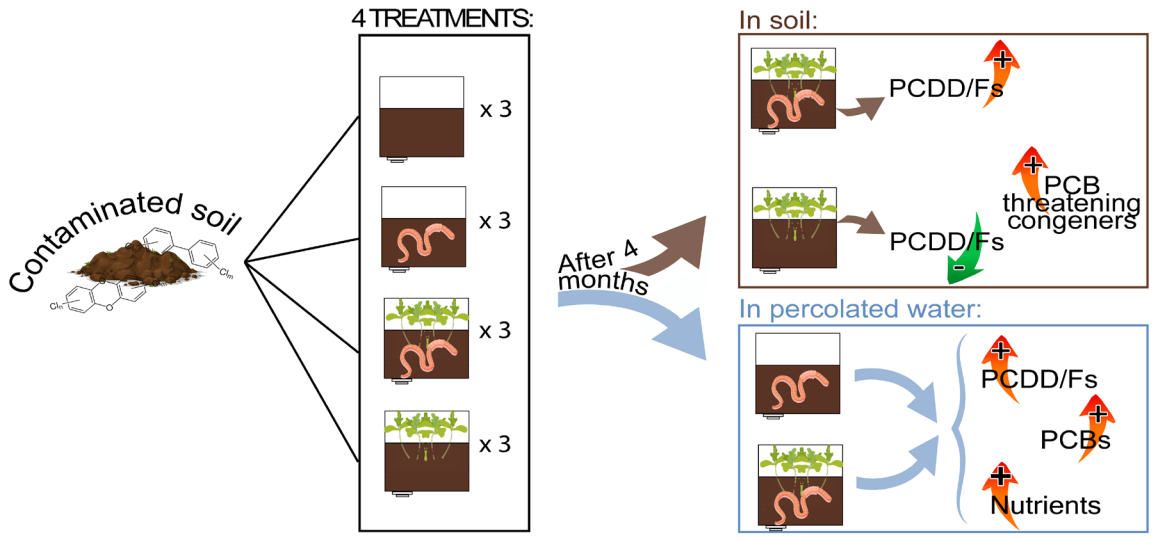

Figura 3. Graphical abstract from Remelli et al. (*submitted*)

Vermiremediation applied to PCB and PCDD/F contaminated soils and its implications for percolating water

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Abstract: PCDD/Fs (polychlorinated dibenzo-p-dioxins/dibenzofurans) and PCBs (polychlorinated biphenyls) are ubiquitous persistent pollutants with reduced bioavailability, which bioremediation using soil fauna is still managed to treat. This research set out to: (i) study the suitability of earthworms (*Eisenia fetida*), alone and associated with plants (*Lepidium sativum*), for the decontamination of PCDD/F and PCB polluted soils in Brescia-Caffaro (Italy), at total and congener concentration levels; (ii) simulate the action of earthworms in groundwater contamination process and nutrient mobility. Five treatments were set up: (i) uncontaminated soil with *E. fetida* (NC); (ii) contaminated soil (C); (iii) contaminated soil with *E. fetida* (CEf); (iv) contaminated soil with *L. sativum* (CLs)*;* (v) contaminated soil with *E. fetida* and *L. sativum* (CEfLs). PCBs and PCDD/Fs in the soil prior to testing were measured. Analysis was repeated in soil treatments and percolating water at the end of the test period (4 months). Dissolved nutrient concentrations were measured in percolated water.
PCB and PCDD/F were significantly reduced after 4 months in all treatments. Treatments did not differ in total PCBs, but CEfLs congeners were less environmentally threatening; CEf and CLs resulted in lower PCDD/Fs. The action of earthworms could enhance contaminants and soluble reactive phosphorous content in percolating water.

Keywords: Earthworms; Persistent Organic Pollutants (POPs); bioremediation; groundwater contamination; nutrients mobility

1. Introduction

Industrialisation is often accompanied by the release of chemical compounds into the environment and their leaching into groundwater, making polluted soils a problem of global concern (Fontanetti et al., 2011). A classification of priority Persistent Organic Pollutants (POPs) was drawn up in the Stockholm Convention (2001) to define persistent and bioaccumulative chemicals, these included polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) (Lallas, 2001).

Due to their chemical stability and hydrophobicity, PCDD/Fs and PCBs have been detected in various compartments of the environment, including water and biota (Castro-Jiménez et al., 2008; Weber et al., 2008). Both chemical classes are associated with anthropogenic activities, and can persist for periods ranging from decades to centuries (Weber et al., 2008). Once emitted, they are preferentially bound to organic

matter; then, through ingestion, they can be biomagnified in the trophic chain from animals to humans (Pereira, 2004). Their removal is therefore a worldwide issue, requiring the study of remediation techniques.

In recent years, due to the high cost of physico-chemical techniques and the need to adopt environmentally sustainable strategies, interest has turned to bioremediation. Most attention was paid to microbial communities, however the degradation of PCDDs and PCBs can occur at a very slow rate because of an unfavourable energetic balance and reduced bioavailability (Campanella et al., 2002; Hickman and Reid, 2008).

Other bioremediation tools are plants, which have been shown to improve PCDD/F and PCB degradation by breaking down organic contaminants, improving microbial activity through roots exudates and allowing aerobic conditions, taking pollutants directly from the soil (van Aken et al., 2009; Campanella et al., 2002; Toussaint et al., 2011). Among plants, *Lepidium sativum* L. has proved to be useful in the phytoremediation of metal contaminated soils (Mojiri et al., 2013; Smolińska, 2020). However, as far as microbial communities are concerned, phytoremediation is often hindered by the low bioavailability of contaminants (van Aken et al., 2009; Campanella et al., 2002).

Within the sphere of bioremediation strategies, little is still known about the potential role of soil fauna, even though vermiremediation seems to be a reliable tool, overcoming both microbial and plant limits (Haimi, 2000; Rodriguez-Campos et al., 2014; Zeb et al., 2020). Through bioturbation, earthworms can change soil properties, altering bioavailability and the distribution of soil pollutants and nutrients (Curry and Schmidt, 2007; Hickman and Reid, 2008; Schaefer and Juliane, 2007). All these activities can improve the dispersal of PCB-degrading microorganisms and increase contact between them and contaminants (Hickman and Reid, 2008; Singer et al., 2001). Moreover, earthworms can absorb contaminants through nutritional uptake, and degradation can be enhanced by their gut microbes (Banerjee et al., 2019; Zeb et al., 2020). Finally, it has been observed that metabolic substances, mucus and amino acids can promote plant growth and phytoremediation (Banerjee et al., 2019).

Among earthworms species *Eisenia fetida* has been put forward as useful in introducing bacteria into soils, therefore promoting PCB degradation (Li et al., 2015).

This research aimed to study soil fauna suitability for the decontamination of PCB- and PCDD/F- polluted soil by performing vermiremediation tests in microcosms, and simulating the consequences in groundwater contaminant and dissolved nutrient mobility. In greater detail, this study sought to highlight: i) the suitability of *E. fetida* in removing POPs from soil; ii) the usefulness of this species, and consequently of vermiremediation techniques, in supporting and enhancing phytoremediation; iii) the biological significance of these techniques, integrating the conventional quantification of total PCB and PCDD/F concentrations with a congener-specific investigation; iv) the potential limitation of conducting in-field vermiremediation, considering that the digging action of earthworms can enhance lying contaminant and increase pollutant

concentrations in groundwater; v) the potential relationship between earthworm-based techniques and the mobilization and loss into groundwater of biologically available nutrients.

2. Materials and methods

2.1. Study area

One of the most important PCB and PCDD contaminated sites in Europe is the so-called national priority site ("SIN") of Brescia-Caffaro, located in the city of Brescia (northern Italy, Lombardy Region; Figure 1), caused by the industrial activities of the Caffaro S.p.A. chemical factory, which produced PCBs and PCB mixtures, such as Fenclor and Apirolio, from 1930 to 1984. During that period contaminated surface water, outflowing from the Caffaro factory, was used to irrigate adjacent agricultural areas. The result was that more than 100 ha of agricultural land, located south of the Caffaro factory, were found to be contaminated by heavy metals, metalloids (Hg, As) and persistent chlorinated organic pollutants, with a prevalence of PCBs, PCDDs and PCDFs that often exceed both residential (0.06 mg/kg) and industrial (5 mg/kg) legal limits (di Guardo et al., 2017). Moreover, the presence of these contaminants has been ascertained not only in soil but also in the groundwater, with a constantly evolving PCB plume (Pili et al., 2017). The study site, covering an area of about 460 m^2, lies in a green area outside the SIN, about 5 km south of the Caffaro factory (Figure 1).

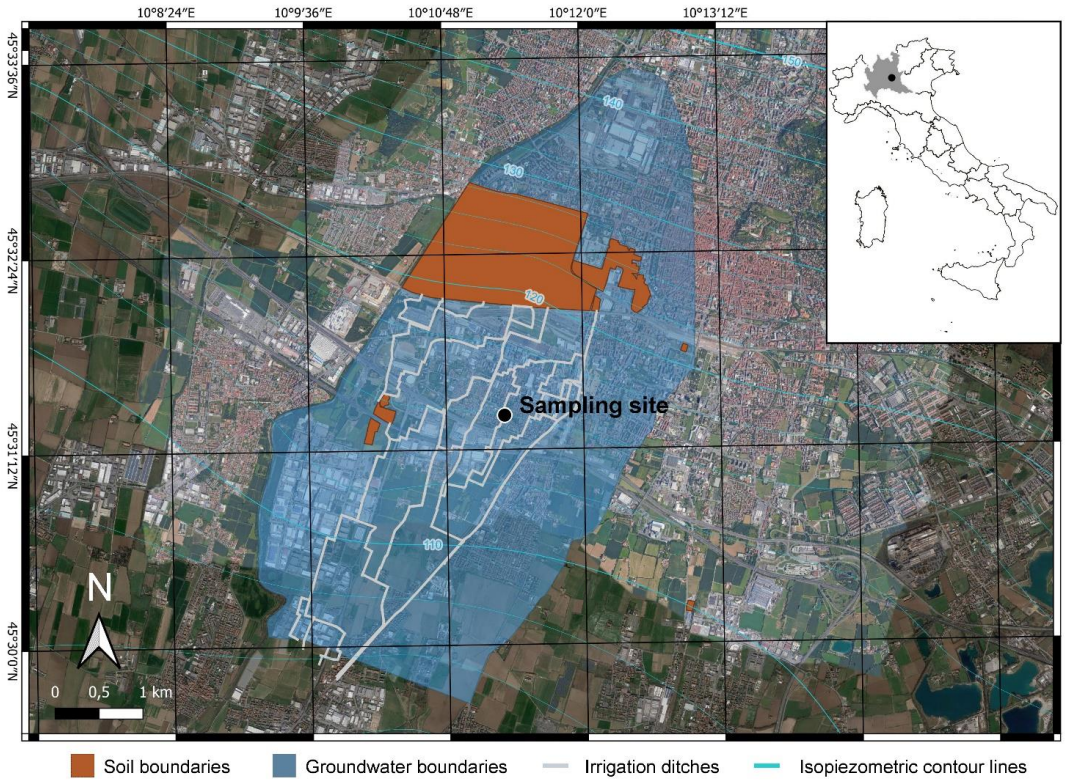

Figure 1. Area of study, with sampling site, Caffaro contaminated soil and groundwater boundaries, irrigation ditches and isopiezometric contour lines of shallow groundwater (m asl; updated 2014) (Ministero della Transizione Ecologica, 2021; QGIS.org, 2021; Regione Lombardia, 2021).

From a lithostratigraphic perspective, three lithological units were identified (up to about 200m): (i) the sandy gravel Unit (Würm Auct., Holocene - Middle-Upper Pleistocene), (ii) the conglomeratic Unit (Ceppo Lombardo Auct., Middle Pleistocene), (iii) the silty clay Unit (Villafranchiane Auct., Middle-Lower Pleistocene) (Pili et al., 2017).

Soil classification of the area has been included in Luvisols based on the World Reference Base for Soil Resources (Chesworth et al., 2008); soil at a depth of between 0 and 0.2 m consists of a silty topsoil with gravel containing plant frustules and root systems.

2.2. Bioremediation experimental design

About 0.25 m^3 of contaminated topsoil (C) was sampled from the study site (no more than 20 cm deep). To reduce the heterogeneity of the naturally weathered soil, the contaminated soil used in this experiment was air-dried and mechanically homogenized, through several mixing steps following the "one-dimensional Japanese Slab-Cake" (JSC) technique (Low et al., 2010), applied at microcosms preparation time and at microcosms sampling time for the analysis. To verify the validity of experimental conditions, a biological, non-contaminated potting soil (no-contaminated soil - NC) was used.

2.3. Microcosm setup

For the microcosm setup, 15 polypropylene tanks (base = 24.5x29.5 cm), with cut-off bottom were overturned and filled with soil (soil height = 23 cm). The experimental design comprised 5 different treatments (3 replicates for each one): 1) no-contaminated soil with *E. fetida* (NC), 2) contaminated soil without *E. fetida* (C), 3) contaminated soil with *E. fetida* (CEf), 4) contaminated soil with *Lepidium sativum* (CLs), 5) contaminated soil with *E. fetida* + *L. sativum* (CEfLs).

In order to confirm the viability of *E. fetida* and *L. sativum* in C, a preliminary test on their mortality and seedling emergence was carried out before microcosm setup, according to ISO (ISO 11268-2:2012, 2012; ISO 17126:2005, 2005).

For treatments with *E. fetida*, sexually mature earthworms were supplied by a worm breeding company. Earthworms were washed with deionized water, towelled and weighed. Fifteen specimens (of 4.27 ± 0.16 g) were placed in each microcosm and fed weekly with 20 g of air-dried cattle manure. At the end of the experiment, the number of earthworms in each microcosm was recorded.

For treatments with *L. sativum*, 0.5 g of seeds was added to each microcosm.

The experiment lasted 112 days (from February 23 to June 15, 2021). Two sampling times were considered: before the microcosm setup (T0) and after 112 days (T112). At each sampling time, soil was collected using a brass drilling machine (Ø = 1.90 cm, height = 9.00 cm) for physico-chemical analysis.

During the test period microcosms were kept at 20 ± 2°C with 80-85% relative humidity (RH), and deionised water was added when water loss > 2% of the initial water holding capacity (WHC).

2.4. Mineralogical and physico-chemical analysis

X-Ray powder Diffraction (XRD) was performed on C at T0 and on contaminated microcosms at T112. XRD analyses were performed with a Bruker D2 Phaser powder diffractometer with the following specifications: Cu Kα (λ = 1.54178 Å) radiation, 30 kV and 10 mA, Ni filtered, 2θ between 5° and 70°, steps of 0.02°, and a sampling time of 1 s. The geometry of the diffractometer was θ-θ, and a solid-state detector was used. Orientation effects were minimized with a sample rotation of 30 rpm. The mineralogical phases were identified using the Bruker software EVA and the Crystallography Open Database (COD).

At the beginning of the test pH and soil organic matter (SOM) were measured on three replicates of C. After, they were measured at T112 in each microcosm. The pH analysis was conducted on a soil distilled water liquid mixture (1:2.5 w/v) using, besides pH electrode, a temperature probe to achieve automatic temperature compensation (Società Italiana della Scienza del Suolo, 1986). SOM was determined by using LOI - Loss on Ignition, i.e. the ignition of 1 g of dried soil at 550°C for 4 hours (Heiri et al., 2001).

PCB and PCDD/PCDF concentrations were measured on three replicates of C at T0 and on soil and percolating water of the contaminated microcosms at T112. For each sample measured, 50 g of soil and 2 L of water were collected in glass vials for the analyses, which were performed at Biochemie Lab S.r.l. following the EPA 1668C 2010 protocol for PCB and the EPA 1613B 1994 protocol for PCDD/PCDF (U.S. EPA, 2010, 1994). At T112, soil for chemical analysis was taken after water collection, in order to keep earthworm tunnels intact and be able to assess their effect on water percolation.

Percolated water samples were immediately filtered (Whatman GF/F) for the determination of inorganic nutrients at the end of the test. An aliquot was stored in polyethylene vials for dissolved silica (DSi), ammonium ($N\text{-}NH_4^+$) and nitrate + nitrite ($N\text{-}NO_x$) analyses and in glass vials for soluble reactive phosphorus (SRP). $N\text{-}NH_4^+$ (Koroleff, 1970), $N\text{-}NO_x$ (APHA et al., 1998), SRP(Valderrama, 1981) and DSi (Golterman et al., 1978) in percolating water were determined with standard spectrophotometric methods (Perkin Elmer, Lambda 35). The same procedure was carried out with an analytical blank to ensure that environmental samples were not contaminated during the data-collection process.

2.5. Statistical analysis

A distance matrix based on Bray-Curtis dissimilarity was calculated, using a “vegan” package (Oksanen et al., 2020), on the contaminant congeners matrix (square-root transformed in order to minimize the influence of the most abundant groups). Then, Non-metric MultiDimensional Scaling (NMDS) was performed in order to observe whether the congener grouping depended on soil treatment. PERmutational Multivariate ANalysis Of VAriance (PERMANOVA) was used to test for differences in congener assemblages among the different treatments visualized with NMDS.

ANOVA assumptions were met for both pH and SOM, contaminant congeners and total concentrations, and for water dissolved nutrients, which were used as a response variable. Dunnett’s test was used to

compare concentrations from treatments at T112 against those from contaminated soil at T0. One-Way ANOVA, followed by Tukey test, was used to compare differences in response variables (contaminant congeners and total concentration, both in soil and water, and water dissolved nutrients) using treatments at T112 as factor.

Due to the large number of congeners, PCB and PCDD/Fs were assigned to different groups. For PCB, groups were based on their potential toxicity, estimated on the basis of their environmental threaten and structural specificity for microsomal enzyme induction, as suggested by McFarland and Clarke (McFarland and Clarke, 1989), and divided into: (i) G1A, the three most potent (inducers of mixed-function oxidase, MFO, of pure 3-methylcholanthrene-type) congeners, (ii) G1B, MFO mixed-type inducers frequently found in environmental samples, (iii) G2, MFO phenobarbital-type inducers that are also prevalent in the environment, (iv) G3, weak or not related to MFO induction, but frequently occurring in the environment or in high concentrations in animal tissues compared to other PCB congeners, thus being an issue of concern, (v) G4, mixed-type inducers that have been reported infrequently in biota and in very low tissue concentrations, (vi) LT, the remaining and lower environmentally threatening congeners. For PCDD/Fs, congeners were assigned to groups based on their Toxic Equivalency Factor (TEF), which is their relative toxicity coefficient used to express the toxicity of mixtures of PCDD/Fs in toxin equivalents of 2,3,7,8-TCDD (WHO-TEF) (van den Berg et al., 1998), the most toxic congener catalogued by the World Health Organization (WHO) (Srogi, 2008).

To understand relations between treatments and congener groups (at T112, or water dissolved nutrients), considering that our dataset contains both quantitative and qualitative variables, Factor Analysis of Mixed Data (FAMD) was run. Computation and visualization of FAMD data was conducted using “FactoMineR” (Husson et al., 2020) and “factoextra” (Kassambara and Mundt, 2016) packages respectively.

Congener-specific analysis (ANOVA and its follow-up tests, as described above) on soil and percolating water were conducted only on contaminant congeners exceeding soil and groundwater concentration limits (pursuant to Leg. Dec. 152/2006).

A p-value ≤ 0.05 was considered significant. All analyses were performed using R (version 4.0.5) (R Core Team, 2021).

3. Results and Discussion

At T0, the mineral composition of C was dominated by quartz as the main mineralogical phase, together with feldspar plagioclase, muscovite and their alteration, represented by chlorite group minerals (Figure 2a).

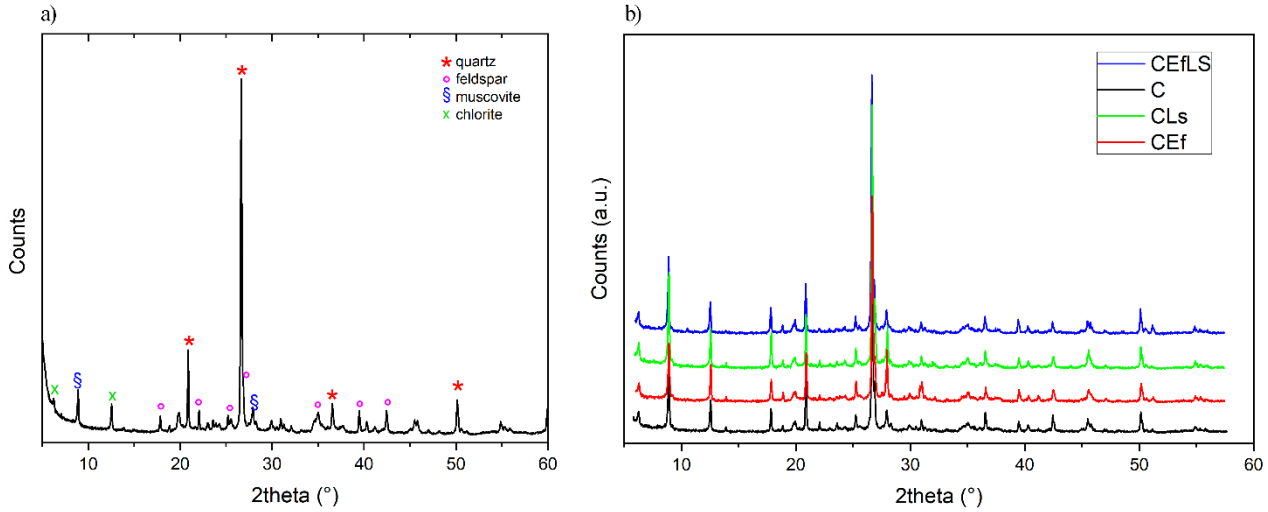

Figure 2. X-ray diffraction pattern of: a) the soil prior to testing (C at T0), and b) the soil belonging to the contaminated microcosms at the end of the bioremediation test (T112).

C mineralogical composition did not change over time and, at the end of the test, no differences were found between treatments (Figure 2a-b).

pH and SOM values too did not change significantly, with values ranging between 7.44 and 7.88 for pH and 7.60% and 8.47% for SOM (Table S1), meaning that the addition of manure as the earthworms' food supply did not introduce variability between treatments.

3.1. Total PCB and PCDD/Fs

PCB and PCDD/F total concentrations exceeded Leg. Dec. 152/2006 limits for public, private and residential soil (PCB: 60000 ng/kg, and PCDD: 10 ngTE/kg), at both T0 and T112 (in all treatments). Limits for groundwater were exceeded too (PCB: 0.01 µg/l, and PCDD/Fs: 4 x 10^{-6}µg TE /l). Only in T0 did PCDD/Fs exceed limits for commercial and industrial use (PCDD: 100 ngTE/kg).

A significant reduction in PCB and PCDD/F soil concentrations was observed from T0 to T112 in all treatments, C included ($p < 0.001$; Figures 3a-b).

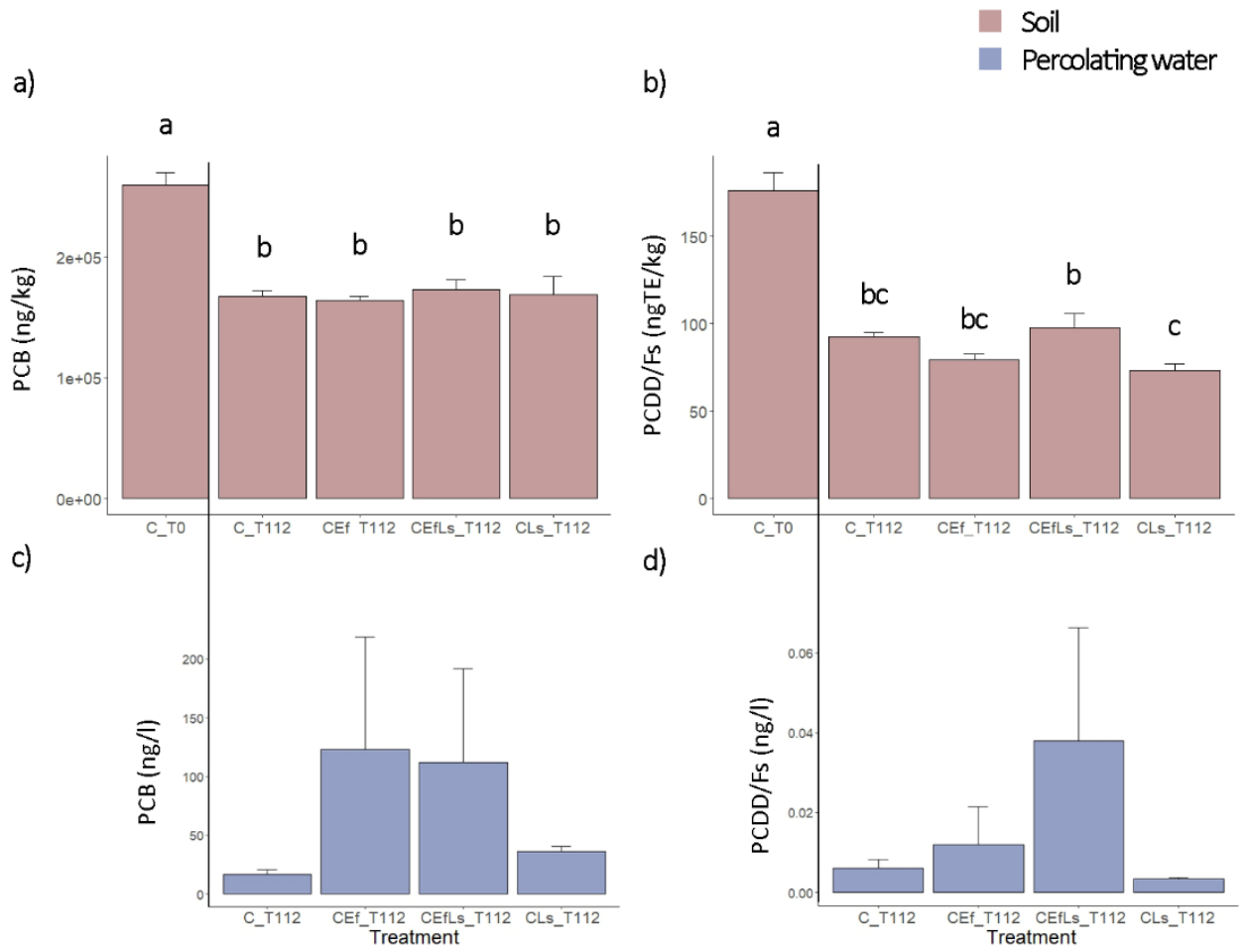

Figure 3. (a) PCB (ng/kg) and **(b)** PCDD/F (ngTE/kg;WHO-TEF conversion) total concentration in contaminated soil at the beginning (C_T0) and at the end ("Treatment"_T112) of the experiment. **(c)** PCB (ng/l) and **(d)** PCDD/F (ng/l; WHO-TEF conversion) total concentration in percolating water from contaminated soil at the end ("Treatment"_T112) of the experiment. Different letters mean significant differences ($p \leq 0.05$).

It has been observed that percolation is a factor assisting the disappearance of chemicals in soil (Chen et al., 2013). In our study the addition of water to simulate percolation contributed to the reduction of contaminant concentration in the soil. Nevertheless, total contaminant concentrations (Figure 3) in soils after percolation and in water were often higher in microcosms with bioremediation treatments than in C, suggesting a baseline biodegradation by means of autochthonous microorganisms, in addition to the percolation effect. This is in accordance with Chen et al. (2013) who observed, in a laboratory experiment with aged soil, that PCDF and OCDF concentrations can decrease by up to 99% in 12 weeks due to volatilisation, infiltration, runoff and biological degradation by means of autochthonous microorganisms. Moreover, in the present experiment, autochthonous biodegradation could have been biostimulated by the increase in aeration associated with soil manipulation during the microcosms setup, as well as by irrigation adjustments to achieve a comparable water content in all treatments (Terzaghi et al., 2020).

At the end of the test, the soil concentration of PCB did not differ between treatments, while PCDD/Fs showed the highest concentrations in CEfLs treatment and the lowest in CLs (ANOVA: $p < 0.05$; Figure 3b).

In percolating water, the two treatments with earthworms showed higher PCB and PCDD/Fs means when compared to the other treatments, however no significant differences were observed between treatments (Figures 3c-d). Soil results combined with percolating water concentrations suggest that the presence of earthworms seem to have little bioremediation effect when compared to C, while a slow bioremediation effect is observable in CLs after 4 months. Earthworms have been used for bioremediation for other contaminants, for example in a three-month hydrocarbon bioremediation experiment by Ceccanti et al. (2006), however it is possible that for POPs more time was needed to see the positive effects of vermiremediation. Indeed, in addition to the lower bioavailability of POPs, a slowdown effect on earthworm reproduction (suggested by NC higher number of earthworms after 4 months, as reported below) should be considered.

Whereas CLs results support the hypothesis that plants can improve the degradation rate despite not being able to absorb PCB and PCDD/Fs, due to the weak solubility and high lipophilicity that bind those pollutants to the soil (Campanella et al., 2002). Indeed, Campanella et al. (2002) highlighted that PCB and PCDD/Fs could be bioremediated by treatments that involved two phases, anaerobic and aerobic, and that plant roots, mostly involved in the aerobic step, can promote the establishment of new bacterial strains. Surprisingly, plants benefits were not observed in CEfLs, indeed PCDD/F concentrations were significantly higher than in CLs. Our results suggested that in CEfLs contaminant bioavailability was reduced by earthworms and plant interaction, thus hindering natural attenuation. This contrasts with previous studies, which found that the combination of vermiremediation with phytoremediation can be successfully used for the remediation of soils contaminated with organic contaminants (Zeb et al., 2020). However, Lacalle et al. (2020) pointed out that factors such as type of soil, chemical properties of the contaminants and the biological species selected for the remediation can strongly affect the outcome of the remediation process. Moreover, it is known that the effect of earthworms on soil bacterial community composition could be dependent upon the type of substrate under study, as nutrient dynamics were modified (de Menezes et al., 2018; Koubová et al., 2015; Medina-Sauza et al., 2019). In our study, in CEfLs, the association of earthworms with a plant-roots system could have led to significant changes in the composition of the autochthonous microbial community, with a shift in keystone taxa and a reduction in natural attenuation.

In both earthworm treatments, contaminant concentrations in percolated water were higher than the treatments where earthworms were absent, suggesting that when water was added to simulate percolation, a portion of the large amount of non-bioavailable contaminants migrated in percolating water, facilitated by tunnels created by earthworm activity. This is in agreement with Jarvis et al. (2008), who found that preferential water flow occurred in macropores created by earthworms in temperate clay soils, increasing the risk of leaching and subsequent contamination of subsurface and groundwater (Bertrand et al., 2015).

3.2. PCB and PCDD/Fs congeners

Concentrations of all contaminant congeners analysed are reported in Table S2 and Table S3.

It emerged from PERMANOVA that the treatments differed in soil contaminant composition ($p < 0.01$), with a higher variability within CEfLs (Figure 4a).

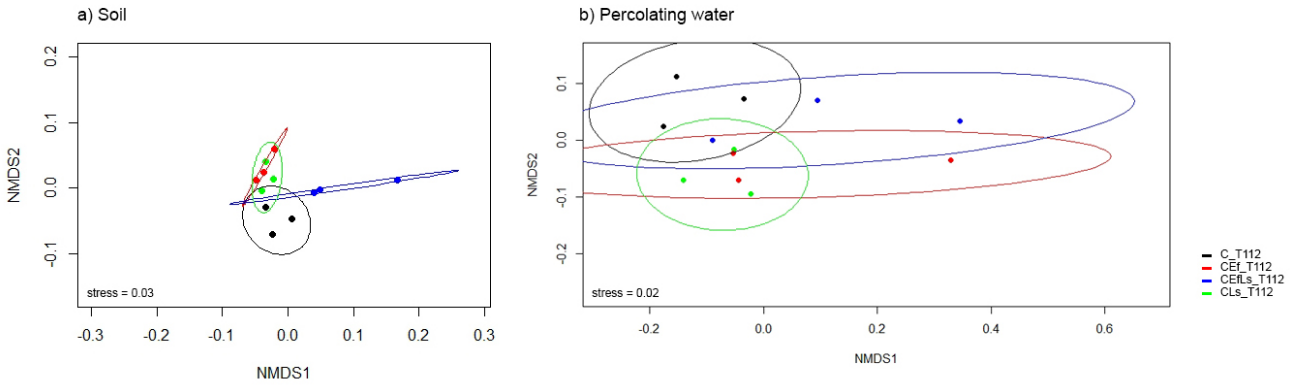

Figure 4. NMDS displaying differences in PCB and PCDD/Fs congener assemblages among the different treatments in: **a)** contaminated soil and **b)** percolating water, at the end of the experiment.

On the contrary, no differences between treatments were found in percolating water, where earthworm activity resulted in a great similarity between treatments, and a high variability within them (Figure 4b).

PCB congener groups in soil, assigned based on environmental threatening potential, showed a similar pattern in CEf and CLs treatments (Figure 5a).

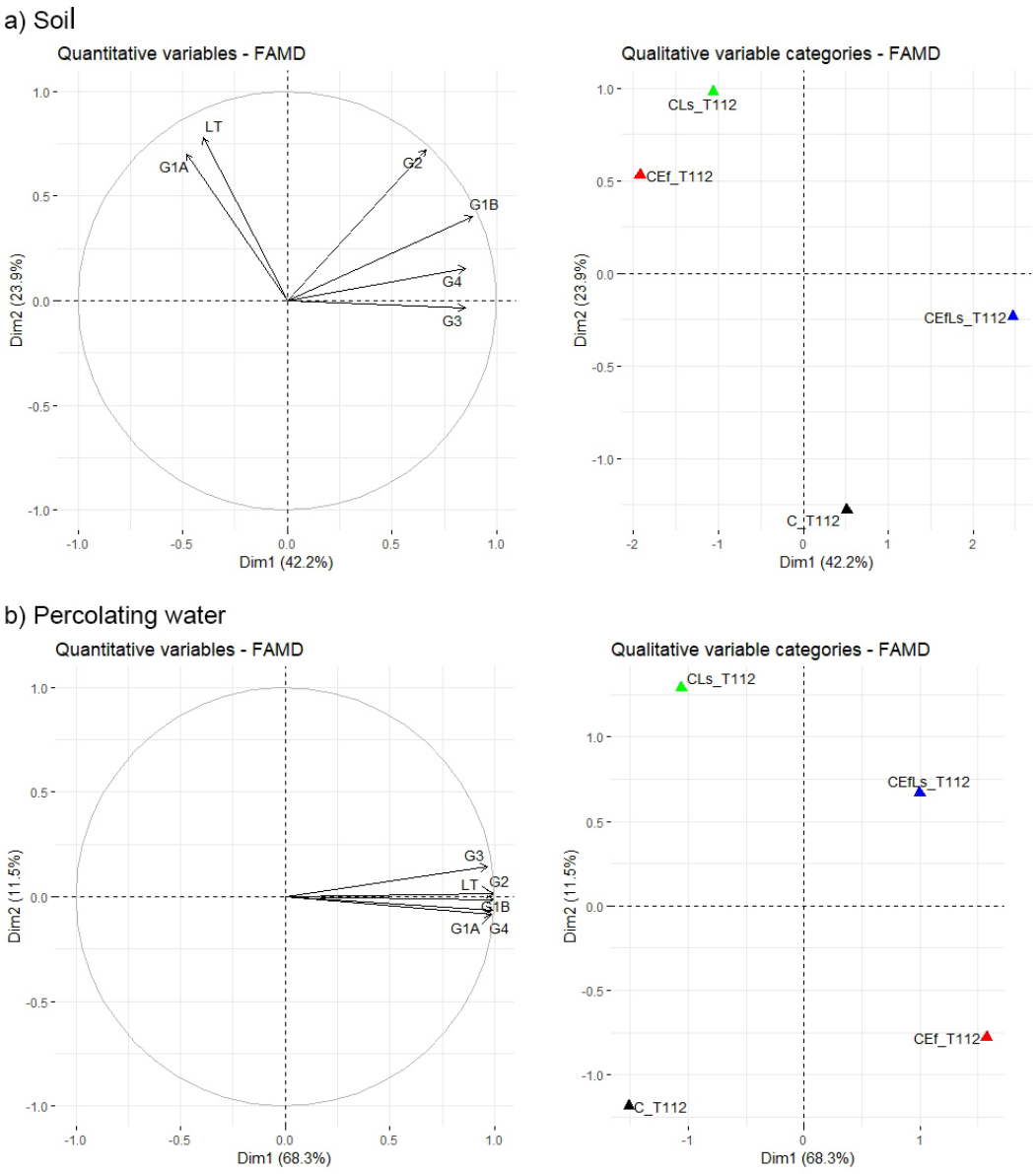

Figure 5. FAMD output for quantitative (PCB congener group concentrations) and qualitative (treatments) variables and their contribution to the dimensions 1 and 2 in **a)** soil and **b)** percolating water, at the end of the experiment.

Above all, G1A and LT were predominant in CLs. This could be attributed in the first case to an enhancement of PCB 77 (Table S2), possibly due to the transformation of higher-level PCB congeners, and in the second case to the presence in LT of high-chlorinated congeners, which are less often taken up and transported inside the plants (thereby experiencing limited metabolism), and tend to be released into the environment when plant decomposition occurs (Jing et al., 2018). In the G1A group, congener concentration never exceeded legal limits, confirming that a qualitative approach is needed, since these congeners, despite being little reported in environmental samples, have a very high toxicity (McFarland and Clarke, 1989). On the other hand, G3 was the prevailing group in CEfLs. This treatment was characterized by a higher presence of congeners that, although weaker, frequently occur in the environment, and may be of concern, since they are generally found in high concentrations in animal tissues (McFarland and Clarke, 1989). In percolating

water, all groups increased for the two earthworm treatments, with G1A, G1B and G4 showing a decrease in CLs (Figure 5b).

For PCDD/F congeners in soil, CEf and CLs treatments showed a similar pattern, opposite to C, with a reduction in higher TEF congeners (except TEF=1, which was similar in all treatments, Figure 6a).

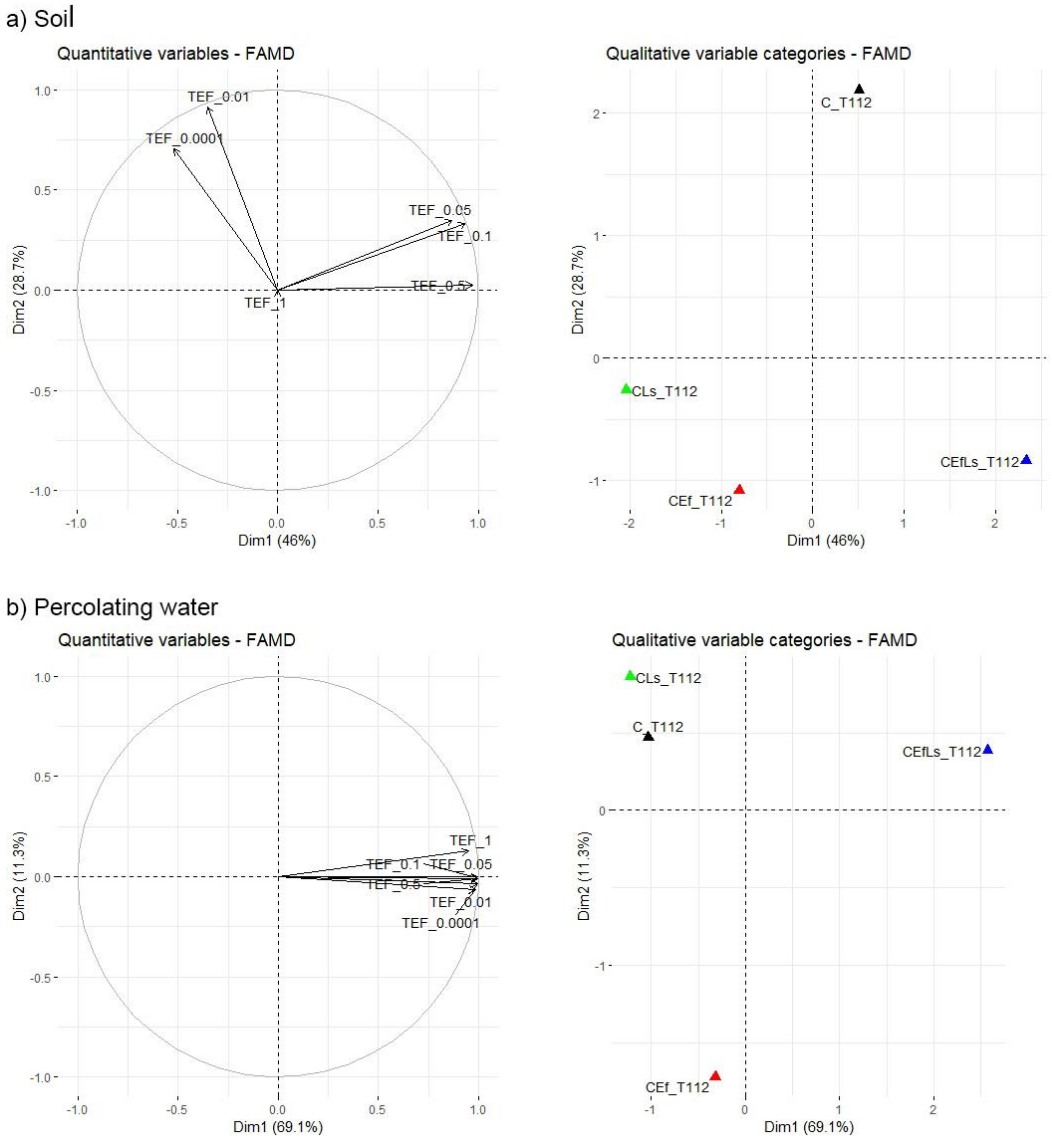

Figure 6. FAMD output for quantitative (PCDD/F congener group concentrations) and qualitative (treatments) variables and their contribution to the dimensions 1 and 2 in **a)** soil and **b)** percolating water, at the end of the experiment.

Higher TEF PCDD/Fs tended to decrease in CLs and, since the ability of plants to uptake those contaminants from soil is very limited, it is likely they boost indigenous soil microorganisms, which can biodegrade PCDDs/Fs (Urbaniak et al., 2019). On the one hand, CLs was the most beneficial treatment for PCDD/F decontamination, but the worst for PCB bioremediation, since it was related to the group of congeners with the highest toxicity. In percolating water, groups with high TEF (0.1 and 1) increased in CEfLs (Figure 6b). This could be because, just as for total PCB and PCDD/F concentrations, casts and burrows may

have contributed to contaminant losses through soil lixiviation; a process that can also govern some nutrient dynamics (le Bayon and Milleret, 2009).

After 112 days 7 congeners reported differences ($p \leq 0.05$) between C_T0 and at least one of the treatments at T112 (Table 1).

Table 1. PCB and PCDD/F congener concentrations (ng/kg) in contaminated soil at the beginning (C_T0) and at the end (T112) of the experiment. Bold type means a significant difference ($p \leq 0.05$) between the C_T0 and the treatments. Different letters mean significant differences ($p \leq 0.05$) between treatments at T112. Only congeners reporting differences between C_T0 and _T112 or within treatments at T112 were reported, for all congener concentrations see Table S2.

	C_T0	C_T112	CEf_T112	CEfLs_T112	CLs_T112
PCB					
PCB101	5146.67±1344.24	4646.67±485.29^{b}	6606.67±383.03^{ab}	3203.33±469.41^{c}	7406.67±658.04^{a}
PCB110	7853.33±1510.91	12666.67±1466.67^{bc}	**19133.33±1281.06^{ab}**	8810.00±1863.18^{c}	**20800.00±1517.67^{a}**
PCB118	7110.00±1682.88	**2826.67±161.90**	**2536.67±130.43**	**2833.33±116.81**	**2470.00±251.20**
PCB138	29033.33±2026.77	**15900.00±945.16**	**15500.00±503.32**	**20300.00±1159.02**	**15466.67±1650.59**
PCB149	14600.00±1582.19	**7516.67±759.26**	**7513.33±239.88**	**9796.67±359.64**	**7683.33±1000.11**
PCB153	28466.67±1779.83	**10750.00±563.47^{b}**	**11066.67±317.98^{ab}**	**14400.00±781.02^{a}**	**10923.33±1154.07^{b}**
PCB170	21666.67±3435.27	**13500.00±585.95**	**11500.00±435.89**	**13633.33±952.77**	**12000.00±953.94**
PCB177	9386.67±1413.95	**5503.33±423.57**	**4233.33±197.01**	**5133.33±443.82**	**4566.67±356.20**
PCB180	51100.00±7824.96	**25533.33±833.33**	**22866.67±674.12**	**28200.00±1709.78**	**23333.33±2049.66**
PCB183	6026.67±872.72	4260.00±409.51	**3683.33±140.51**	4363.33±273.03	**3926.67±457.98**
PCB187	23633.33±2425.79	**16400.00±1053.57**	**13600.00±1410.67**	17733.33±1020.35	**15233.33±1329.58**
PCB205	528.33±37.03	**6410.00±496.02**	**5163.33±189.06**	**6690.00±55.68**	**5456.67±664.19**
PCB209	14233.33±2444.27	10836.67±578.51	10170.00±750.27	10513.33±1085.44	**7500.00±1129.70**
PCB95	3883.33±706.76	4456.67±374.89^{ab}	**6770.00±559.40^{ab}**	4050.00±841.61^{b}	**7440.00±859.21^{a}**
PCDD/Fs					
1,2,3,4,6,7,8-HpCDD	84.30±9.36	**16.90±0.59**	**15.27±0.66**	**9.02±4.40**	**14.10±0.70**
1,2,3,4,6,7,8-HpCDF	176.33±13.42	**127.00±6.43^{a}**	**74.83±10.55^{b}**	**74.00±1.21^{b}**	**103.47±18.56^{ab}**
1,2,3,4,7,8-HxCDF	229.33±21.28	**140.00±7.00^{ab}**	**134.67±5.78^{b}**	**175.67±10.99^{a}**	**136.00±8.50^{b}**
1,2,3,6,7,8-HxCDD	79.80±7.69	**4.56±0.41**	**4.76±0.91**	**7.14±0.75**	**4.77±0.41**
1,2,3,6,7,8-HxCDF	71.80±10.60	**145.33±7.22^{a}**	**40.43±2.00^{b}**	**46.07±1.68^{b}**	**38.93±2.41^{b}**
1,2,3,7,8-PeCDF	196.00±16.29	**115.33±4.37**	**100.70±3.73**	**117.27±11.05**	**97.30±6.09**
2,3,4,6,7,8-HxCDF	53.07±3.90	**29.33±0.07^{ab}**	**30.83±1.27^{ab}**	49.87±10.45^{a}	**26.23±2.22^{b}**
2,3,4,7,8-PeCDF	175.00±8.33	**101.87±3.32**	**90.80±5.10**	**116.33±15.33**	**82.50±4.76**
2,3,7,8-TCDD	29.77±0.80	**0.05±0.00**	**0.05±0.00**	**0.05±0.00**	**0.05±0.00**
2,3,7,8-TCDF	357.67±26.72	**210.33±2.73**	**220.33±11.46**	**269.00±36.14**	**200.33±8.45**
OCDF	210.00±31.79	**124.67±4.10**	**120.67±1.33**	156.67±17.07	**122.33±11.84**

At T112, differences between treatments were observed for 4 PCB and 4 PCDD/F congeners: in most cases CLs and CEfLs showed the highest and the lowest concentrations of PCB respectively. In most cases C had the highest concentrations of PCDD/Fs, while CEf and CLs had the lowest. This confirms that CLs could be a suitable treatment for PCDD/Fs, but less effective for PCB bioremediation.

Analysis of single congeners in percolating water did not show significant differences between treatments (Table S3). Indeed, high variability was observed, suggesting that many factors might play a hand in contaminant percolation, confirming that soil contamination poses an unpredictable threat for groundwater.

3.3. Water dissolved nutrients

In this study, attention was also paid to the effect of bioremediation techniques on dissolved nutrients. Results suggested that earthworm activity affected phosphorous (P) and, to a lesser extent, silica (Si) mobility in soils. Of the water dissolved nutrients analysed, N-NO_x was mainly linked to CLs, even if its contribution to overall nutrient variability was weak. SRP and DSi concentrations were mostly related to earthworm treatments, while NH_4^+ tended to decrease mainly in CEfLs (Figure 7).

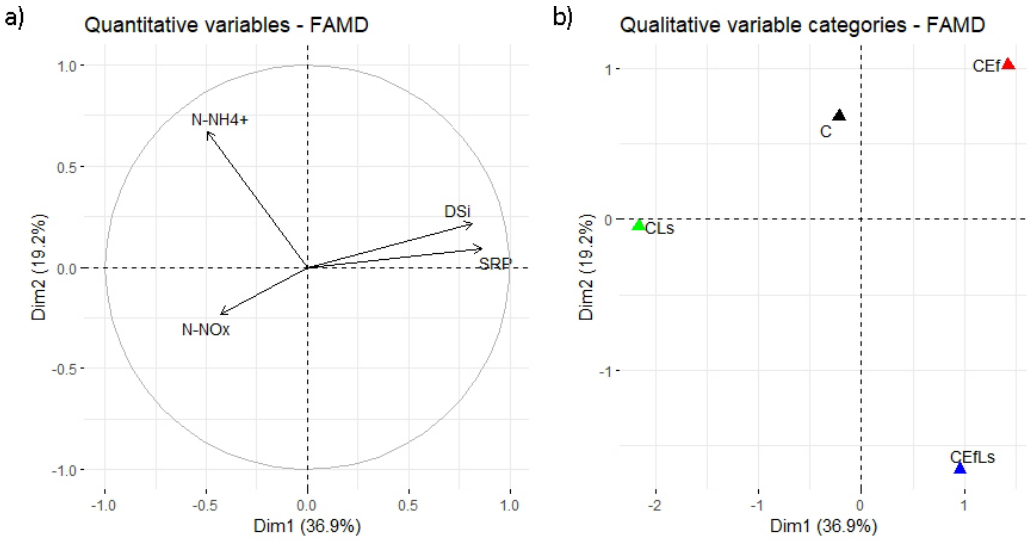

Figure 7. FAMD output for quantitative (water dissolved nutrient concentrations) and qualitative (treatments) variables and their contribution to the dimensions 1 and 2 in a) soil and b) percolating water, at the end of the experiment.

Only the SRP concentration differed among the treatments, being higher in CEf (Table 2).

Table 2. Nutrient concentrations (µg/l) in contaminated treatments at the end of the experiment. Different letters mean significant differences (p≤0.05) between treatments.

	C_T112	CEf_T112	CEfLs_T112	CLs_T112
SRP	196.98 ± 110.64^{ab}	485.73 ± 58.83^{a}	375.05 ± 102.13^{ab}	60.42 ± 19.68^{b}
N-NO_x	64601.47 ± 1441.88	65114.04 ± 5056.21	65768.70 ± 3473.84	65885.42 ± 3704.85
N-NH_4^+	552.80 ± 79.46	528.97 ± 85.42	385.46 ± 12.34	596.84 ± 148.88
DSi	2858.68 ± 139.12	3070.87 ± 412.16	2829.88 ± 280.33	2190.27 ± 145.75

This could be due to earthworm gut enzymes, i.e. acid phosphatases and alkaline phosphatases, and phosphorus-solubilizing microorganisms present in earthworms' casts, indeed they are able to enhance phosphorus content in vermicomposting (le Bayon and Binet, 2006; Prakash and Karmegam, 2010; Sangwan et al., 2010). Furthermore, without plant mediation, earthworm burrows might have facilitated its leaching into percolating water. The same applies to dissolved silica, the solubility of which in earthworm treatments could have been enhanced by bacteria living in the earthworm gut. In CEfLs this was counterbalanced by making the nutrient more bioavailable for plants (Bityutskii et al., 2016; Georgiadis et al., 2019; Hu et al., 2018). Water percolation may be a relevant issue that should be taken into account when vermiremediation techniques are applied, mainly when no plants able to uptake nutrients are present. The leaching and transfer of some of them, especially P and nitrogen (N), from soils to groundwater is a primary factor to consider when evaluating the risk of eutrophication in continental waters (Glibert, 2017; Sharpley, 1993).

3.4. Preliminary tests and E. fetida abundance at the end of the experiment

Both *E. fetida* and *L. sativum* showed their viability for helping with the treatment of PCB and PCDD/F contaminated soils. Preliminary tests showed 100% *E. fetida* survival one month after exposure to C, while *L. sativum* seedling emergence was 87.78±3.24%. This points to the possible inclusion of both earthworms and cress in the bioremediation tests.

After 4 months, the mean ± standard error of the earthworm count in the treatments were: CEf: 120.67±2.03, CEfLs: 106±20.30, and NC: 538.67±24.69; this suggests a slowdown effect of contaminated soil for earthworm reproduction.

4. Conclusions

The development of autochthonous biodegradators in PCB and PCDD/F aged contaminated soils is a well-known process that, in this study, might have been enhanced by aeration and laboratory-controlled conditions, determining a baseline biodegradation of total contaminant concentration. After 4 months, no evidence of PCB biodegradation promoted by earthworms and/or cress was observed for total contaminant concentration, but at the congener level the interaction of earthworms and cress seems to be useful in terms of the environmental threat. For PCDD/Fs, on the other hand, the CEf and CLs treatments gave the best results. Since PCB and PCDD/Fs often occur together, and considering both contaminant concentration and toxicity, the use of earthworms seems to be the most promising treatment among those considered in this study. However, due to the low bioavailability of those contaminants, together with their slowdown effect on earthworm reproduction, it may be assumed that more time, and a higher starting number of earthworms, are needed to observe clearer results about vermiremediation efficiency in soil. Since many factors can affect bioremediation responses, further research to establish the optimum species and combinations thereof is needed. Moreover, bioremediation effects on contaminant bioavailability highlight the importance of

assessing the efficiency of remediation methods not only in terms of a reduction in contaminant concentrations but also the recovery of soil health. Finally, data suggest that attention must be paid to the consequences of earthworm action on percolating water content, both for contaminants discussed here and, more broadly, for dissolved nutrients.

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SUPPLEMENTAL MATERIAL

Table S1. pH and SOM (%) in soil at the beginning (T0) and at the end (T112) of the experiment.

	pH		SOM (%)	
	T0	T112	T0	T112
C	7.75 ± 0.06	7.57 ± 0.01	8.41 ± 0.18	8.13 ± 0.26
CEf		7.50 ± 0.06		8.48 ± 0.37
CEfLs		7.52 ± 0.03		7.60 ± 0.17
CLs		7.63 ± 0.02		8.02 ± 0.13

Table S2. PCB and PCDD/Fs congeners concentrations (ng/kg) in contaminated soil at the beginning (C_T0) and at the end ("Treatment"_T112) of the experiment.

Groups	Congeners	C_T0	C_T112	CEf_T112	CEfLs_T112	CLs_T112
	PCB					
G1A	PCB126	29.53 ± 3.92	19.67 ± 0.42	290.00 ± 19.31	110.27 ± 88.37	241.90 ± 113.28
	PCB169	549.33 ± 6.44	5.17 ± 0.75	3.44 ± 1.06	4.89 ± 0.77	4.95 ± 0.53
	PCB77	28.57 ± 4.42	269.20 ± 162.24	368.33 ± 197.67	59.13 ± 6.31	389.67 ± 12.67
G1B	PCB105	3956.67 ± 586.52	1820.00 ± 25.17	1683.33 ± 69.36	1690.00 ± 115.04	1663.33 ± 137.76
	PCB118	7110.00 ± 1682.88	2826.67 ± 161.90	2536.67 ± 130.43	2833.33 ± 116.81	2470.00 ± 251.20
	PCB128	2194.33 ± 910.78	4223.33 ± 213.02	4106.67 ± 620.71	3633.33 ± 535.73	5020.00 ± 511.60
	PCB138	29033.33 ± 2026.77	15900.00 ± 945.16	15500.00 ± 503.32	20300.00 ± 1159.02	15466.67 ± 1650.59
	PCB156	2383.33 ± 123.47	1286.67 ± 26.67	1176.67 ± 69.36	1296.67 ± 50.44	1236.67 ± 127.06
	PCB170	21666.67 ± 3435.27	13500.00 ± 585.95	11500.00 ± 435.89	13633.33 ± 952.77	12000.00 ± 953.94
G2	PCB101	5146.67 ± 1344.24	4646.67 ± 485.29	6606.67 ± 383.03	3203.33 ± 469.41	7406.67 ± 658.04
	PCB153	28466.67 ± 1779.83	10750.00 ± 563.47	11066.67 ± 317.98	14400.00 ± 781.02	10923.33 ± 1154.07
	PCB180	51100.00 ± 7824.96	25533.33 ± 833.33	22866.67 ± 674.12	28200.00 ± 1709.78	23333.33 ± 2049.66
	PCB183	6026.67 ± 872.72	4260.00 ± 409.51	3683.33 ± 140.51	4363.33 ± 273.03	3926.67 ± 457.98
	PCB99	3033.33 ± 1155.95	1993.33 ± 258.28	3006.67 ± 198.10	1362.33 ± 353.52	3310.00 ± 301.39
G3	PCB151	5666.67 ± 540.41	1979.00 ± 589.49	2433.33 ± 112.60	3340.00 ± 174.74	2456.67 ± 430.56
	PCB177	9386.67 ± 1413.95	5503.33 ± 423.57	4233.33 ± 197.01	5133.33 ± 443.82	4566.67 ± 356.20
	PCB187	23633.33 ± 2425.79	16400.00 ± 1053.57	13600.00 ± 1410.67	17733.33 ± 1020.35	15233.33 ± 1329.58
	PCB52	1794.67 ± 656.08	4163.33 ± 1953.05	788.33 ± 112.19	674.00 ± 57.00	1133.67 ± 173.58
G4	PCB114	95.10 ± 12.40	125.67 ± 12.73	120.33 ± 3.18	149.67 ± 7.88	120.40 ± 14.69
	PCB123	558.00 ± 49.70	186.67 ± 14.11	264.33 ± 19.92	242.67 ± 38.74	176.67 ± 11.41
	PCB157	853.33 ± 106.98	533.00 ± 16.26	432.00 ± 26.56	503.00 ± 31.05	500.00 ± 32.58
	PCB167	909.00 ± 66.46	656.33 ± 39.62	609.33 ± 87.06	1349.67 ± 390.87	630.33 ± 36.50
	PCB189	542.00 ± 44.75	298.33 ± 10.84	254.33 ± 19.64	252.33 ± 21.79	269.33 ± 23.10
	PCB37	65.81 ± 33.24	64.63 ± 19.51	46.17 ± 18.09	42.93 ± 7.95	95.10 ± 11.44
	PCB81	26.63 ± 6.90	8.97 ± 1.09	12.17 ± 0.47	10.97 ± 0.12	10.38 ± 1.67
LT	PCB1	446.00 ± 169.54	738.00 ± 130.98	1010.67 ± 198.13	367.33 ± 88.88	529.67 ± 235.36
	PCB104	0.50 ± 0.00	1.97 ± 0.47	2.93 ± 1.04	1.83 ± 0.36	2.67 ± 0.41
	PCB110	7853.33 ± 1510.91	12666.67 ± 1466.67	19133.33 ± 1281.06	8810.00 ± 1863.18	20800.00 ± 1517.67
	PCB146	3680.00 ± 321.87	2013.33 ± 153.01	1896.67 ± 56.96	2350.00 ± 92.92	1956.67 ± 222.81
	PCB149	14600.00 ± 1582.19	7516.67 ± 759.26	7513.33 ± 239.88	9796.67 ± 359.64	7683.33 ± 1000.11
	PCB15	1290.00 ± 197.32	810.33 ± 36.67	699.00 ± 16.80	910.67 ± 39.98	714.33 ± 86.39
	PCB155	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00
	PCB188	8.26 ± 1.48	3.48 ± 0.12	3.41 ± 0.12	3.94 ± 0.23	3.60 ± 0.29
	PCB19	0.50 ± 0.00	7.00 ± 2.92	1.37 ± 0.44	13.13 ± 1.45	10.39 ± 4.48
	PCB202	1653.33 ± 135.44	1105.00 ± 79.74	870.33 ± 39.93	1007.33 ± 44.20	946.33 ± 94.94
	PCB205	528.33 ± 37.03	6410.00 ± 496.02	5163.33 ± 189.06	6690.00 ± 55.68	5456.67 ± 664.19
	PCB206	4493.33 ± 534.83	2806.67 ± 151.03	2406.67 ± 107.29	2570.00 ± 170.88	2503.33 ± 213.10
	PCB208	970.67 ± 133.56	482.33 ± 111.18	443.00 ± 23.52	472.67 ± 32.22	544.00 ± 16.26
	PCB209	14233.33 ± 2444.27	10836.67 ± 578.51	10170.00 ± 750.27	10513.33 ± 1085.44	7500.00 ± 1129.70
	PCB28	413.63 ± 202.48	155.43 ± 61.88	173.33 ± 33.39	608.00 ± 182.79	353.67 ± 132.18
	PCB3	1257.00 ± 437.31	352.33 ± 12.68	403.33 ± 157.32	218.33 ± 23.24	239.33 ± 22.88
	PCB4	210.00 ± 57.13	126.67 ± 6.84	140.67 ± 15.30	160.67 ± 15.84	114.97 ± 35.15
	PCB54	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00
	PCB95	3883.33 ± 706.76	4456.67 ± 374.89	6770.00 ± 559.40	4050.00 ± 841.61	7440.00 ± 859.21
	PCDD/PCDF					
TEF_1	1,2,3,7,8- PeCDD	0.25 ± 0.00	0.25 ± 0.00	0.25 ± 0.00	0.25 ± 0.00	0.25 ± 0.00
	2,3,7,8- TCDD	29.77 ± 0.80	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
TEF_0.5	2,3,4,7,8- PeCDF	175.00 ± 8.33	101.87 ± 3.32	90.80 ± 5.10	116.33 ± 15.33	82.50 ± 4.76
TEF_0.1	1,2,3,4,7,8- HxCDD	0.25 ± 0.00	2.93 ± 0.15	2.24 ± 0.56	1.07 ± 0.42	2.40 ± 0.60
	1,2,3,4,7,8- HxCDF	229.33 ± 21.28	140.00 ± 7.00	134.67 ± 5.78	175.67 ± 10.99	136.00 ± 8.50
	1,2,3,6,7,8- HxCDD	79.80 ± 7.69	4.56 ± 0.41	4.76 ± 0.91	7.14 ± 0.75	4.77 ± 0.41
	1,2,3,6,7,8- HxCDF	71.80 ± 10.60	145.33 ± 7.22	40.43 ± 2.00	46.07 ± 1.68	38.93 ± 2.41
	1,2,3,7,8,9- HxCDD	1.15 ± 0.90	5.71 ± 0.33	5.88 ± 0.56	7.39 ± 0.33	6.39 ± 0.50
	1,2,3,7,8,9- HxCDF	49.37 ± 5.84	25.47 ± 2.17	39.23 ± 3.02	25.08 ± 12.53	22.43 ± 3.65
	2,3,4,6,7,8- HxCDF	53.07 ± 3.90	29.33 ± 0.07	30.83 ± 1.27	49.87 ± 10.45	26.23 ± 2.22
	2,3,7,8- TCDF	357.67 ± 26.72	210.33 ± 2.73	220.33 ± 11.46	269.00 ± 36.14	200.33 ± 8.45
TEF_0.05	1,2,3,7,8- PeCDF	196.00 ± 16.29	115.33 ± 4.37	100.70 ± 3.73	117.27 ± 11.05	97.30 ± 6.09
TEF_0.01	1,2,3,4,6,7,8- HpCDD	84.30 ± 9.36	16.90 ± 0.59	15.27 ± 0.66	9.02 ± 4.40	14.10 ± 0.70
	1,2,3,4,6,7,8- HpCDF	176.33 ± 13.42	127.00 ± 6.43	74.83 ± 10.55	74.00 ± 1.21	103.47 ± 18.56
	1,2,3,4,7,8,9- HpCDF	39.57 ± 5.79	26.90 ± 0.97	26.17 ± 2.15	21.05 ± 9.51	26.03 ± 2.46
TEF_0.0001	OCDD	1922.00 ± 861.93	731.67 ± 57.17	641.00 ± 69.09	517.33 ± 143.88	689.00 ± 58.81
	OCDF	210.00 ± 31.79	124.67 ± 4.10	120.67 ± 1.33	156.67 ± 17.07	122.33 ± 11.84

Table S3. PCB and PCDD/Fs congeners concentrations (ng/l) in percolating water from contaminated soil at the end ("Treatment"_T112) of the experiment.

Groups	Congeners	C_T112	CEf_T112	CEfLs_T112	CLs_T112
	PCB				
G1A	PCB126*	0.002 ± 0.000	0.009 ± 0.007	0.008 ± 0.005	0.003 ± 0.000
	PCB169*	0.002 ± 0.000	0.002 ± 0.000	0.003 ± 0.001	0.002 ± 0.000
	PCB77	0.004 ± 0.000	0.014 ± 0.010	0.011 ± 0.005	0.005 ± 0.001
G1B	PCB105	0.129 ± 0.024	0.788 ± 0.601	0.623 ± 0.406	0.185 ± 0.029
	PCB118	0.213 ± 0.055	1.203 ± 0.934	0.929 ± 0.610	0.277 ± 0.056
	PCB128	0.413 ± 0.081	2.274 ± 1.704	2.445 ± 1.629	0.577 ± 0.037
	PCB138	1.537 ± 0.303	9.737 ± 7.482	8.183 ± 5.094	2.397 ± 0.374
	PCB156	0.087 ± 0.017	0.621 ± 0.485	0.486 ± 0.319	0.141 ± 0.017
	PCB170	1.254 ± 0.264	10.127 ± 8.037	8.120 ± 5.947	2.347 ± 0.217
G2	PCB101	0.409 ± 0.070	2.287 ± 1.752	2.649 ± 2.041	1.014 ± 0.228
	PCB153	1.392 ± 0.295	8.227 ± 6.137	7.123 ± 4.273	2.223 ± 0.431
	PCB180	2.403 ± 0.788	16.747 ± 12.827	15.087 ± 10.492	4.217 ± 0.864
	PCB183	0.322 ± 0.068	2.684 ± 2.158	2.068 ± 1.393	0.545 ± 0.053
	PCB99	0.224 ± 0.044	1.104 ± 0.848	1.249 ± 0.958	0.576 ± 0.112
G3	PCB151	0.065 ± 0.016	0.322 ± 0.250	0.237 ± 0.139	0.121 ± 0.056
	PCB177	0.450 ± 0.085	3.692 ± 2.909	2.635 ± 1.791	0.807 ± 0.077
	PCB187	1.483 ± 0.292	11.823 ± 9.338	9.607 ± 6.377	2.603 ± 0.279
	PCB52	1.182 ± 0.210	4.276 ± 2.366	6.396 ± 5.067	7.912 ± 4.017
G4	PCB114	0.015 ± 0.002	0.083 ± 0.062	0.070 ± 0.048	0.022 ± 0.002
	PCB123	0.002 ± 0.000	0.052 ± 0.030	0.037 ± 0.022	0.008 ± 0.003
	PCB157	0.037 ± 0.007	0.259 ± 0.198	0.213 ± 0.137	0.062 ± 0.007
	PCB167	0.117 ± 0.064	0.350 ± 0.277	0.281 ± 0.187	0.078 ± 0.014
	PCB189	0.026 ± 0.003	0.210 ± 0.128	0.146 ± 0.092	0.043 ± 0.004
	PCB37	0.009 ± 0.002	0.045 ± 0.029	0.030 ± 0.012	0.007 ± 0.001
	PCB81	0.003 ± 0.000	0.010 ± 0.007	0.007 ± 0.003	0.003 ± 0.000
LT	PCB1	0.002 ± 0.000	3.040 ± 1.552	11.321 ± 10.202	0.232 ± 0.230
	PCB104*	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000
	PCB110	1.323 ± 0.266	8.607 ± 6.797	9.337 ± 7.148	4.053 ± 1.216
	PCB146	0.171 ± 0.036	1.156 ± 0.882	1.023 ± 0.642	0.285 ± 0.051
	PCB149	0.681 ± 0.163	5.006 ± 3.848	4.220 ± 2.522	1.167 ± 0.263
	PCB15	0.047 ± 0.007	0.189 ± 0.135	0.215 ± 0.142	0.169 ± 0.063
	PCB155*	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000
	PCB188*	0.002 ± 0.000	0.004 ± 0.002	0.003 ± 0.001	0.002 ± 0.000
	PCB19*	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000
	PCB202	0.085 ± 0.015	0.602 ± 0.459	0.532 ± 0.341	0.142 ± 0.011
	PCB205	0.022 ± 0.004	0.128 ± 0.090	0.153 ± 0.097	0.040 ± 0.006
	PCB206	0.227 ± 0.082	1.682 ± 1.309	1.452 ± 0.955	0.380 ± 0.063
	PCB208	0.036 ± 0.005	0.235 ± 0.174	0.188 ± 0.113	0.065 ± 0.009
	PCB209	0.532 ± 0.259	19.393 ± 17.655	10.246 ± 8.163	1.528 ± 0.429
	PCB28	0.019 ± 0.005	0.093 ± 0.054	0.048 ± 0.007	0.012 ± 0.005
	PCB3	1.319 ± 0.951	3.335 ± 2.825	1.809 ± 0.695	0.730 ± 0.685
	PCB4	0.002 ± 0.000	0.081 ± 0.040	0.150 ± 0.148	0.052 ± 0.050
	PCB54*	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000
	PCB95	0.346 ± 0.060	2.263 ± 1.713	2.437 ± 1.747	0.781 ± 0.135
	PCDD/PCDF				
TEF_1	1,2,3,7,8- PeCDD	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000
	2,3,7,8- TCDD	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000
TEF_0.5	2,3,4,7,8- PeCDF	0.008 ± 0.003	0.015 ± 0.012	0.047 ± 0.034	0.004 ± 0.001
TEF_0.1	1,2,3,4,7,8- HxCDD	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000
	1,2,3,4,7,8- HxCDF	0.009 ± 0.004	0.021 ± 0.016	0.070 ± 0.054	0.005 ± 0.000
	1,2,3,6,7,8- HxCDD	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.001	0.001 ± 0.000
	1,2,3,6,7,8- HxCDF	0.003 ± 0.001	0.007 ± 0.005	0.017 ± 0.013	0.002 ± 0.000
	1,2,3,7,8,9- HxCDD	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.001	0.001 ± 0.000
	1,2,3,7,8,9- HxCDF	0.001 ± 0.001	0.004 ± 0.003	0.011 ± 0.008	0.001 ± 0.000
	2,3,4,6,7,8- HxCDF	0.003 ± 0.001	0.005 ± 0.004	0.017 ± 0.012	0.001 ± 0.000
	2,3,7,8- TCDF	0.014 ± 0.005	0.028 ± 0.021	0.088 ± 0.066	0.008 ± 0.001
TEF_0.05	1,2,3,4,6,7,8- HpCDD	0.003 ± 0.002	0.007 ± 0.005	0.011 ± 0.006	0.002 ± 0.000
TEF_0.01	1,2,3,4,6,7,8- HpCDF	0.006 ± 0.003	0.017 ± 0.013	0.054 ± 0.039	0.004 ± 0.000
	1,2,3,4,7,8,9- HpCDF	0.002 ± 0.001	0.004 ± 0.003	0.013 ± 0.009	0.001 ± 0.000
	1,2,3,7,8- PeCDF	0.007 ± 0.002	0.016 ± 0.012	0.053 ± 0.041	0.004 ± 0.001
TEF_0.0001	OCDD	0.076 ± 0.045	0.267 ± 0.231	0.594 ± 0.486	0.055 ± 0.005
	OCDF	0.008 ± 0.003	0.037 ± 0.029	0.168 ± 0.125	0.005 ± 0.000

* Concentrations (ng/l) not exceeding Leg. Dec. 152/2006 groundwater limits

Section 2.2:

A new approach towards the monitoring and bioremediation of the unsaturated zone of contaminated aquifers

Article (submitted)

The ecotoxicity approach as a tool for assessing vermiremediation effectiveness in PCB and PCDD/F contaminated soils

Sara Remelli, Fulvio Celico and Cristina Menta

The ecotoxicity approach as a tool for assessing vermiremediation effectiveness in PCB and PCDD/F contaminated soils

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Abstract: Chemical analyses are inadequate for assessing soil biological quality. Instead, the soil living community can be used both for monitoring and restoring soil health.

The aim of this research was to verify vermiremediation efficiency in PCDD/F and PCB contaminated soils from Brescia-Caffaro (Italy), using an ecotoxicity approach. To gauge whether Caffaro soil could sustain a living community, a characterization of the arthropod community was conducted. Earthworms' suitability for soil bioremediation was assessed applying ecotoxicity tests. Five treatments were set up: (i) contaminated soil; (ii) contaminated soil + *Eisenia fetida*; (iii) contaminated soil + *Lepidium sativum;* (iv) contaminated soil + *E. fetida* + *L. sativum*, (v) uncontaminated soil + *E. fetida*. The ecotoxicity tests were: *L. sativum* germination index and root elongation inhibition, and *Folsomia candida* survival and reproduction, applied on soil and elutriate on: starter soil (T0), after 56 and 112 days (T56 and T112), the last after water percolation.

Soil arthropod community was dominated by Hypogastruridae, Oribatida and, to a lesser degree, Formicidae and Coleoptera larvae. Ecotoxicity tests showed that *F. candida* reproduction and *L. sativum* root elongation were more adversely affected by pollutants than survival and germination. The higher soil ecotoxicity at T112 than at T56, suggested higher contaminant bioavailability after water addition. *F. candida* showed more variability between soil and elutriate than *L. sativum*. Both bioassays suggested earthworm treatment as the most promising. The importance of selecting different organisms in soil ecotoxicity monitoring, and the role of elutriate like a solid phase complement, was highlighted.

Keywords: Persistent Organic Pollutants; bioremediation assessment; earthworms; ecotoxicity tests; soil living community

1. Introduction

Polychlorobiphenyls (PCBs), polychlorodibenzo-p-dioxins and furans (PCDDs and PCDFs) are representative of a group called "Persistent Organic Pollutants (POPs)" that threaten human health and the environment (Campanella et al., 2002). They are regarded as ubiquitously persistent environmental pollutants in the ecosystem (Rathna et al., 2018). Once emitted, they tend to bind to the organic matter in the upper soil surface, where they can transfer to animals by ingestion and, through biomagnification along the trophic chain, to humans (Pereira, 2004).

Nowadays, numerous removal technologies are commercially available, but the effectiveness of these technologies depends on investment cost and operating expenses, which in turn depend on the national

economy. Against this background, bioremediation has proved to be an inexpensive and effective tool, with the potential to respond to the remediation need of restoring a functioning ecosystem (Majer, 1989).

Soil fauna have been considered not only a reliable tool in the bioremediation process but also in monitoring action to assess toxicity and the risk of contaminated soil before and after bioremediation (Haimi, 2000). In fact, soil biota is very dynamic and susceptible to soil disturbance, unlike most chemical and physical properties, which take longer to change; edaphic organisms are thus considered good indicators of soil health (Cardoso et al., 2013).

Diversity and abundance of soil fauna has been used as an indicator of anthropogenic impacts on terrestrial ecosystems because they are strictly correlated with physical, chemical, and microbiological soil attributes (Decaëns et al., 2004; Eggleton et al., 2005). Among the most representative organisms generally used as soil health indicators, mesofauna, being involved in many processes, such as organic matter translocation, breaking and decomposition, nutrient cycling, soil structure formation and water regulation, plays a key role in monitoring plans (Lavelle & Spain, 2001; Menta & Remelli, 2020). In aged contaminated sites, the number of tolerant specimens to be increased and the replacement of pollution-sensitive species with less sensitive ones can lead to a different species assemblage inside the community (Salminen et al., 2001). These changes are not only indicators of potentially contaminated soils but also could be useful for assessing the rate of bioremediation, both natural and supported (Rusin & Gospodarek, 2016). Among bioremediation techniques, vermiremediation is found to be a reliable tool, enhancing microbial activity and assisting phytoremediation, which are often adversely affected by low PCDD/F and PCB bioavailability, and supporting plant growth (Aken et al., 2009; Campanella et al., 2002; Haimi, 2000; Rodriguez-Campos et al., 2014).

When assessing the validity of remediation techniques, chemical analyses characterize the contamination level of the medium (soil or water), but are inadequate in assessing its biological quality. Combining such testing with bioassays, which can reflect the effects of bioavailable contaminants, can help to highlight the link between actual contamination levels and adverse effects on biota (Chapman & Long, 1983). The bioavailability of contaminants depends on several factors, such as physico-chemical properties of the contaminant itself, the characteristics of the environment and the organisms used in the bioassay (Aqeel et al., 2014). Thus, the use of multiple target species, especially if representative of different trophic levels and having different degrees of sensitivity toward toxicants, is highly recommended, since it allows a more complete evaluation of potential contaminant toxicity by considering different exposure routes and endpoints (e.g. survival, reproduction, growth and development) (Burton et al., 2002; Picone et al., 2016).

More than 1,800,000 potentially contaminated sites have been identified in Europe and, among them, 39 are in Italy (“SIN - Siti di Interesse Nazionale”, or National Priority Sites) (EEA, 2007). The SIN Brescia-Caffaro, deriving from the activities of the former Caffaro S.p.A. chemical factory, is one of the most polluted sites in Italy. Caffaro produced PCBs and PCB mixtures, such as Fenclor and Apirolio, from 1930 to 1984. During those

years contaminated surface water went into the soil (as irrigation water) of adjacent agricultural areas. This resulted in significant concentrations of persistent chlorinated organic pollutants, with a prevalence of PCBs and PCDDs/Fs exceeding residential (0.001 mg/Kg) and industrial (5 mg/Kg) legal limits respectively (di Guardo et al., 2017).

In the present study, we sought to: (i) characterize the soil arthropod community in order to assess whether highly PCB and PCDD/F contaminated soils, like those in Caffaro, can support a well-structured soil living community; (ii) monitor the effectiveness of vermiremediation and/or phytoremediation treatments (reported in a previous paper) in PCB and PCDD/F contaminated soil by using an ecotoxicological test. Since the determination of contaminants using traditional chemical analyses cannot predict their impact on living organisms, to obtain an ecosystemic approach PCDD and PCB biodegradation in the soil was monitored, both for soil and soil-elutriate, using a multi-organism and multi-endpoint approach in which the bioindicators belonged to different taxonomic groups: *Lepidium sativum* for higher plants, and *Folsomia candida* for mesofauna.

2. Materials and methods

The study site (45°31’24.75’’N, 10°11’18.60’’E), covering a surface area of about 460 m2, lies in a green area outside the National Priority Site (SIN) Brescia-Caffaro, about 5 km south of the Brescia-Caffaro factory (Northern Italy). The SIN is the result of activity performed at the Caffaro factory, and includes soils polluted by PCBs and PCDD/Fs that often exceed both residential (0.001 mg/Kg) and industrial (5 mg/Kg) legal limits (Vergani et al., 2019).

From Brescia municipality data, the stratigraphy of the study area is thus represented: 0 – 0.2 m silty topsoil with gravel, containing plant frustules and root systems, 0.2 – 0.8 m slightly silty to brown silty sand with gravel and root systems and heterometric, polygenic, from sub-angular to sub-rounded clasts, 0.8 – 1.7 fine and brown sandy silt with spread gravel and decimetre sandy gravel lens and heterometric, polygenic, from sub-angular to sub-rounded clasts, 1.7 – 2.0 slightly silty to brown silty sand with gravel and heterometric, polygenic, from sub-angular to sub-rounded clasts.

2.1. Soil arthropod characterization

To characterize the arthropod community at the study site, five soil samples (10 x 10 x 10 cm) about 5 meters from each other were collected using a spade. Soil arthropods (200 µm–2 mm) were extracted from each soil sample using a Berlese–Tüllgren funnel for 10 days. The extracted specimens were collected and preserved in a solution consisting of 75% ethyl alcohol and 25% glycerol by volume. Specimens were identified and counted using a stereomicroscope. Different taxonomic levels were considered: class for Myriapoda and, among them, order for Chilopoda, and order for Chelicerata, Crustacea and Hexapoda. We decided to maintain this different taxonomic level to obtain to a wider vision of the edaphic fauna community living in such a contaminated site. Within some groups, it was decided to consider a lower taxonomic level:

i) Coleoptera indicates many kinds of alteration in the environment, such as pollution, but beetles' different ecological requirements require that they be distinguished at least at family level (Menta & Remelli, 2020); ii) like Coleoptera, Collembola too have been seen to be useful in the assessment of bioremediation rates when considering at least family level (Rusin & Gospodarek, 2016); iii) among mites, a distinction was made between Oribatida and other Acarina, considering the close relationship between Oribatida and soil organic matter. Within holometabolous insects, adults and larvae were separated.

2.2. Bioremediation experimental design

About 0.25 m3 of silty contaminated topsoil (C) was sampled (no more than 20 cm deep), air-dried and mechanically homogenized following the "one-dimensional Japanese Slab-Cake" (JSC) technique (Low et al., 2010). As uncontaminated soil (control), biological potting soil was used.

For the microcosm setup, 15 polypropylene tanks, with a cut-off bottom, were overturned and filled with soil following this experimental design (3 replicates for each one): i) uncontaminated soil + *Eisenia fetida* (control, NCEf), ii) contaminated soil (C), iii) contaminated soil + *Eisenia fetida* (CEf), iv) contaminated soil + *Lepidium sativum* (CLs), v) contaminated soil + *E. fetida* + *L. sativum* (CEfLs).

E. fetida was supplied by a worm breeding company. For treatments with earthworms, 15 sexually mature individuals were placed in each microcosm, after being washed with deionized water, dried, and weighed. Food, consisting of 20 g of air-dried cattle manure, was supplied weekly. At the end of the experiment, the number of earthworms in each microcosm was reported, considering both adults and juveniles. For treatments with L. sativum, 0.5 g of seeds were added to each microcosm.

During the test period, microcosms were maintained at 20 ± 2°C with 80-85% RH, and deionized water was added when water loss > 2% of the initial WHC.

The experiment lasted 112 days (from February 23, 2021 to June 15, 2021). Three sampling times were considered: before microcosms setup (T0), after 56 days (T56), and after 112 days (T112). At each sampling time, soil needed for ecotoxicological tests was collected using a brass drilling machine (Ø = 1.90 cm, height = 9.00 cm). At T0 and T112, pH and soil organic matter (SOM) analyses were carried out, together with the measurement of PCB and PCDD/F total and congener concentrations (discussed in detail in a previous work) (Unpublished results). At T112, soil was taken after having percolated 2L of water (needed to simulate bioremediation impact on contaminants' mobility into groundwater, see the work mentioned above) (Unpublished results), in order to keep earthworm tunnels intact and be able to assess their effect on water percolation.

2.3. Ecotoxicological tests

Before the microcosm setup, a T0 test on contaminated soil from the Caffaro factory was carried out for *E. fetida* to determine its suitability for use in the bioremediation test. The sexually mature *E. fetida* belonged to the same breeding subsequently used for earthworm treatments applied to microcosms. Survival and

reproduction tests were carried out according to standard ISO 11268 (ISO 11268-2:2012, 2012). Test containers (three replicates) were filled with 500g of Caffaro soil, to which deionised water was added to achieve a soil moisture of 40–60% of the WHC. Ten earthworms were cleaned with distilled water, dried and placed in the container and kept at 20 ± 2°C with 80 – 85% RH for 28 days. During the test period, earthworms were fed weekly with cattle manure, and water was added when water loss > 2% of the initial WHC. After 28 days, surviving earthworms were counted; containers with (possible) cocoons were incubated for a further 28 days under the previous conditions, except that food was administered only once, after the removal of adults. At the end of the test (56 days), newborn juveniles were counted.

F. candida (Collembola: Isotomidae) for soil invertebrates and *L. sativum* (Brassicales: Brassicaceae) for plants were used to test soil and elutriate toxicity at T0, T56 and T112. Three replicates for each microcosm, for both test organisms and for both soil and elutriate, were carried out in the three trials. Before running the test procedure, these soil samples were dried at 50°C for 16h, sieved at 2 mm, and homogenised. To test soil toxicity, Petri dishes were filled with 30g of testing soil, wetted with deionized water to reach 40–60% of the total water holding capacity (WHC). To test elutriate toxicity, Petri dishes containing 5mL of an elutriate solution on filter paper were used. The elutriate was the liquid phase, obtained by shaking a solution of testing soil:water (1:4 w/v) for 30 min, and then allowing it to sediment at room temperature for 24 h.

The *F. candida* specimens used in the test came from laboratory cultures at Parma University. Growth, survival and reproduction tests were carried out according to standard ISO 11267 (ISO 11267:1999, 1999). Individual specimens were kept at 20 ± 2°C (with 50 – 55% RH) and fed weekly on dry yeast. Specimens used for egg deposition (aimed to obtain age-synchronized juveniles to be used in the test) were collected from breeding containers and mixed to prevent them originating from a single breeding line. All springtails used for testing were 10 days old and age-synchronized by removing eggs from the deposition cultures and, once hatched, inserting juveniles into Petri dishes with a moistened breeding substrate of plaster of Paris:activated carbon powder (8:1 w/w).

Ten *F. candida* specimens aged 10 days were added to each Petri dish using an exhauster, ensuring that none of the exemplars died during the process. Springtails were kept at 20 ± 2°C with 70 – 80% relative humidity (RH) and fed with the same dry yeast used during the breeding phase. The Petri dishes were incubated for 28 days, aerated once a week and watered when water loss exceeded 2% of the initial WHC. At the end of this period, adults and juveniles (when present) were euthanized by freezing. Vessels were filled with water and gently stirred with a spatula, allowing the animals to float on the water surface (flotation technique). A small amount, approx. 0.5 ml, of black ink was added to the water to increase the contrast between the water and the white Collembola. Then a digital photograph was taken, and the number of surviving adults and new-born springtails were counted using image analysis software provided by ImageJ (version 1.53). The same procedure was applied to both contaminated and control soil.

The survival rate (SR%) was calculated as follows:

$$SR\% = (Sn - Sc) / Sc * 100$$

where Sc and Sn are the number of survivors in the control sample and in soil (or elutriate), respectively.

The reproduction rate (RR%) was calculated as follows:

$$RR\% = (Rn - Rc) / Rc * 100$$

where Rc and Rn are the number of juveniles in the control sample and in soil (or elutriate), respectively.

Non pesticide-treated *L. sativum* seeds were used to test germination and root elongation, in accordance with standard ISO 17126 (ISO 17126:2005, 2005). As for *E. fetida*, tests at T0 were conducted before the microcosm setup. To test soil toxicity, Petri dishes containing a filter paper onto which 5g of soil saturated with deionised water were used; to test elutriate toxicity, Petri dishes with filter paper were prepared, as previously described for *F. candida* tests on elutriate. Ten seeds were added to each Petri dish; all dishes were wrapped in ParafilmTM to avoid cross contamination and incubated at 25 ± 1°C in a dark incubation chamber for 72 h. At the end of this period, germinated seeds were counted and their root elongation (the length from the tip of the root to the radicle) was measured. To assess the validity of the test, the same procedure was applied for the control soil.

The elongation inhibition rate (EI%) was calculated as follows:

$$EI\% = (Lc - Ln) / Lc * 100$$

where Lc and Ln are the mean values of root length in the control sample and in soil (or elutriate), respectively.

The germination index was calculated using the formula:

$$GI\% = (Gn * Ln) / (Gc * Lc) * 100$$

where Gn and Ln are the mean values of germinated seeds and root in soil (or elutriate), respectively, and Gc and Lc are the mean values of germinated seeds and root in the control sample, respectively.

2.4. Statistical analysis

One-Way ANOVA, followed by the Tukey test, was used to evaluate differences in earthworm numbers (total abundance, adults and juveniles) between treatments and the control sample after 112 days. Since ANOVA assumptions were not met, Box Cox transformation was applied.

To understand relations between treatments, PCB and PCDD/F total concentrations in soil (at T112) and ecotoxicological results, considering the fact that the dataset contains both quantitative and qualitative variables, a Factor Analysis of Mixed Data (FAMD) was run (Pagès, 2004). Computation and visualization of FAMD data was carried out using “FactoMineR” and “factoextra” packages respectively (Husson et al., 2020; Kassambara & Mundt, 2016).

For ecotoxicological tests, *F. candida* survival and reproduction rates and *L. sativum* elongation inhibition rate and germination index were considered as dependent variables. ANOVA assumptions were not met, and the “lme4” package was used to estimate a mixed effects logistic regression model with treatments and time

as predictors, a random intercept by microcosm, and family gaussian (Bates et al., 2014). The “emmeans” package was used for pairwise post hoc multiple comparisons with Bonferroni adjustment (Lenth et al., 2017).

A p-value ≤ 0.05 was considered significant. All analyses were performed using R (version 4.0.5) (R Core Team, 2021).

3. Results

Data on pH and SOM, as well as PCB and PCDD/F total and congeners concentration in the starter soil and in microcosms after 112 days of bioremediation test are reported in the Supplemental materials (Table A.1-2). Briefly, pH and SOM content did not change significantly from T0 to T112, and no differences were observed between treatments. Indeed, PCB and PCDD/F concentrations fell significantly after 112 days in all treatments. Moreover, even if treatments did not differ in terms of total PCBs, CEfLs congeners were less environmentally threatening. Finally, among the treatments, CEf and CLs resulted in lower PCDD/F total concentrations, with a reduction in higher TEF congeners too.

3.1. Arthropod community

Arthropods extracted in the sampling area totalled 7570.62 ± 1688.19 ind./m2, most were Collembola (46%) and Acarina (30%) (Acarina/Collembola ratio: 0.9 ± 0.5), followed by Hymenoptera Formicidae (12%), Coleoptera (5%), Diptera (larvae, 4%) and Hemiptera (2%) (Table 1). Isopoda, Araneae, Pauropoda, Protura and Thysanoptera accounted for the remaining 1%. Seven families of Collembola were identified, the most present was Hypogastruridae (80%), followed by Isotomidae (10%) and Sminthuridae (9%); the remaining 1% consisted of Entomobryidae, Bourletiellidae and Onychiuridae. Acarina consisted mainly of Oribatida (68%) and Coleptera in larvae (83%). Among Coleoptera adults three families were identified, the most abundant belonging to Carabidae (53%), followed by Staphilinidae (35%) and Curculionidae (12%).

Table 1. Soil arthropods (ind./m^2) extracted from Caffaro sampling site.

Group	Mean ± St.err.
Hexapoda Entognatha	
Protura	4.25 ± 4.25
Collembola	3490.21 ± 712.16
Bourletiellidae	8.49 ± 5.20
Entomobryidae	8.49 ± 5.20
Hypogastruridae	2776.88 ± 698.99
Isotomidae	360.91 ± 78.00
Onychiuridae	4.25 ± 4.25
Sminthuridae	326.94 ± 102.61
Tullbergiidae	4.25 ± 4.25
Hexapoda Insecta	
Coleoptera	365.16 ± 106.87
Carabidae	16.98 ± 7.94
Curculionidae	4.25 ± 4.25
Staphilinidae	12.74 ± 8.49
larvae	165.59 ± 45.24
Hemiptera	118.89 ± 56.81
Thysanoptera	4.25 ± 4.25
Hymenoptera (Formicidae)	900.15 ± 549.34
Diptera larvae	297.22 ± 111.53
Arachnida	
Acarina	2250.38 ± 719.10
Oribatida	1532.81 ± 590.31
Others	717.57 ± 206.64
Araneae	8.49 ± 8.49
Myriapoda	
Chilopoda	4.25 ± 4.25
Pauropoda	8.49 ± 5.20
Symphyla	4.25 ± 4.25

3.2. Eisenia fetida

Preliminary tests on *E. fetida* resulted in a survival rate of 100% after two months in contaminated soil, with no reproduction in any replicate.

Inside the microcosms, earthworms differed in terms of total abundance, adults and juvenile presence after 112 days of the experiment ($p < 0.001$, all). CEf and CEfLs differed from NCEf for all the parameters evaluated (Figure 1a-b), while CEf and CEfLs differed from each other only in terms of the number of adults (Figure 1a).

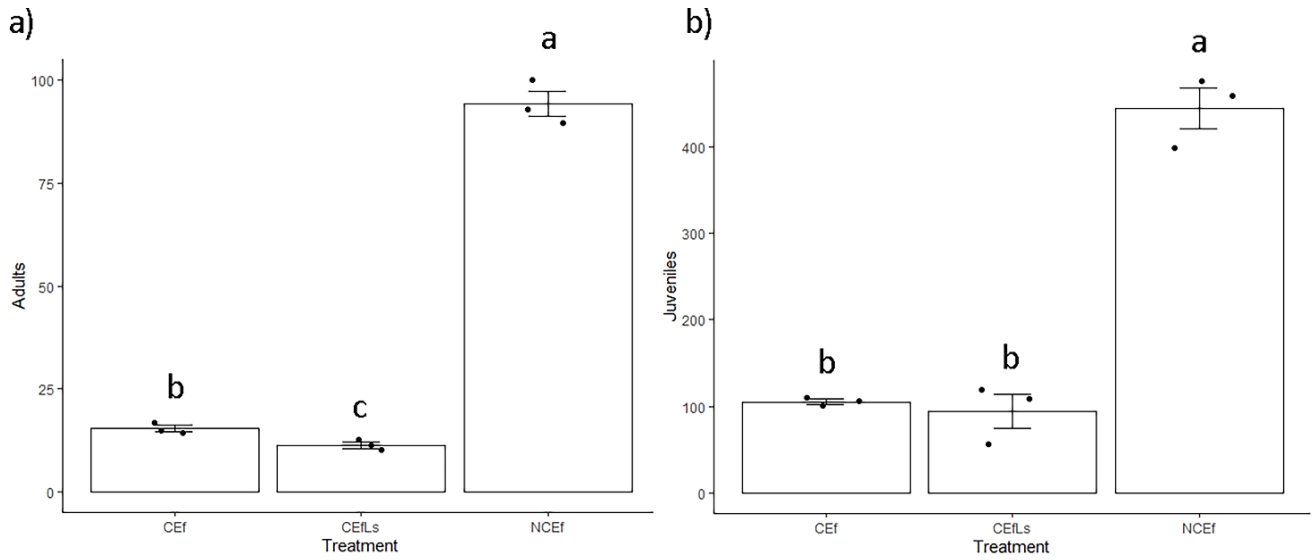

Figure 1. (a) Adults and **(b)** juveniles of *E. fetida* in contaminated soils (CEf and CEfLs) and control sample (NCEf) at the end of the experiment. Different letters correspond to significant differences (p≤0.05).

3.3. Ecotoxicological tests on Folsomia candida and Lepidium sativum

From the FAMD a weak susceptibility to soil PCB and PCDD/F concentrations (which were mainly linked to CEfLs treatment) could be observed on the survival and reproduction rates of *F. candida* in elutriate, unlike what happened in soil (Figure 2).

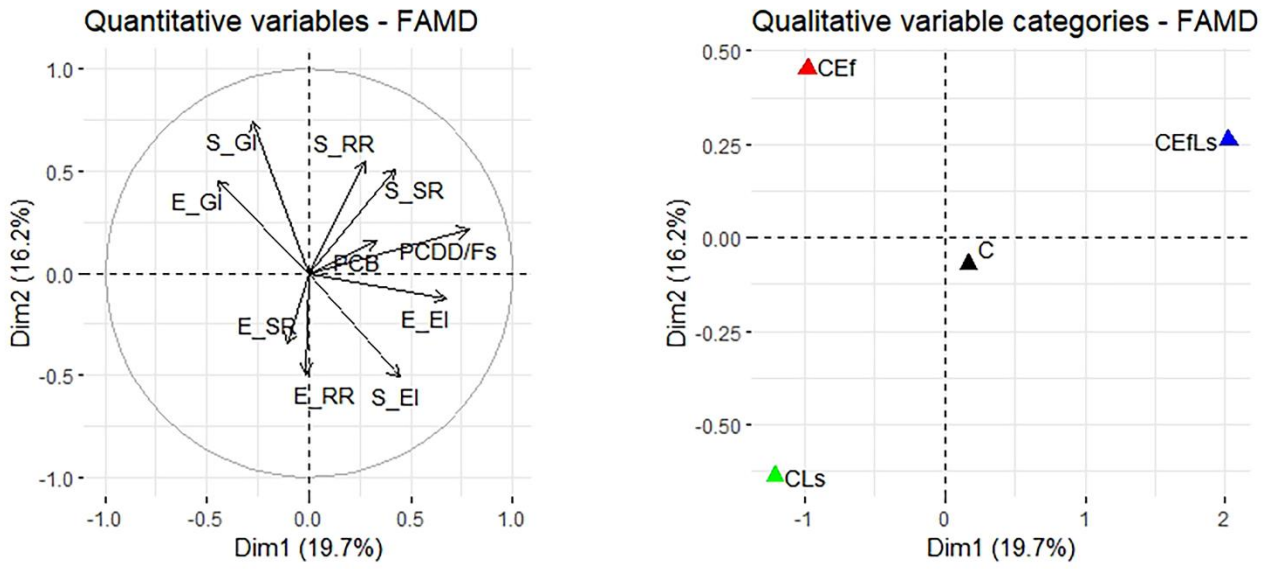

Figure 2. FAMD output for quantitative (ecotoxicological indexes) and qualitative (treatments) variables and their contribution to the dimensions 1 and 2 in soil. *F. candida* indicators were: S_SR and E_SR = survival rate in soil and elutriate respectively, S_RR and E_RR = reproduction rate in soil and elutriate respectively; *L. sativum* indicators were: S_GI and E_GI = germination index in soil and elutriate respectively, S_EI and E_EI = elongation inhibition in soil and elutriate respectively.

On the other hand, *L. sativum* results showed a higher germination index related to CEf treatment and an increased elongation inhibition linked to C.

3.3.1. *Folsomia candida*

The *F. candida* survival rate in soil (Figure 3a) depended on time ($p < 0.001$) and on the interaction between time and treatment ($p < 0.01$).

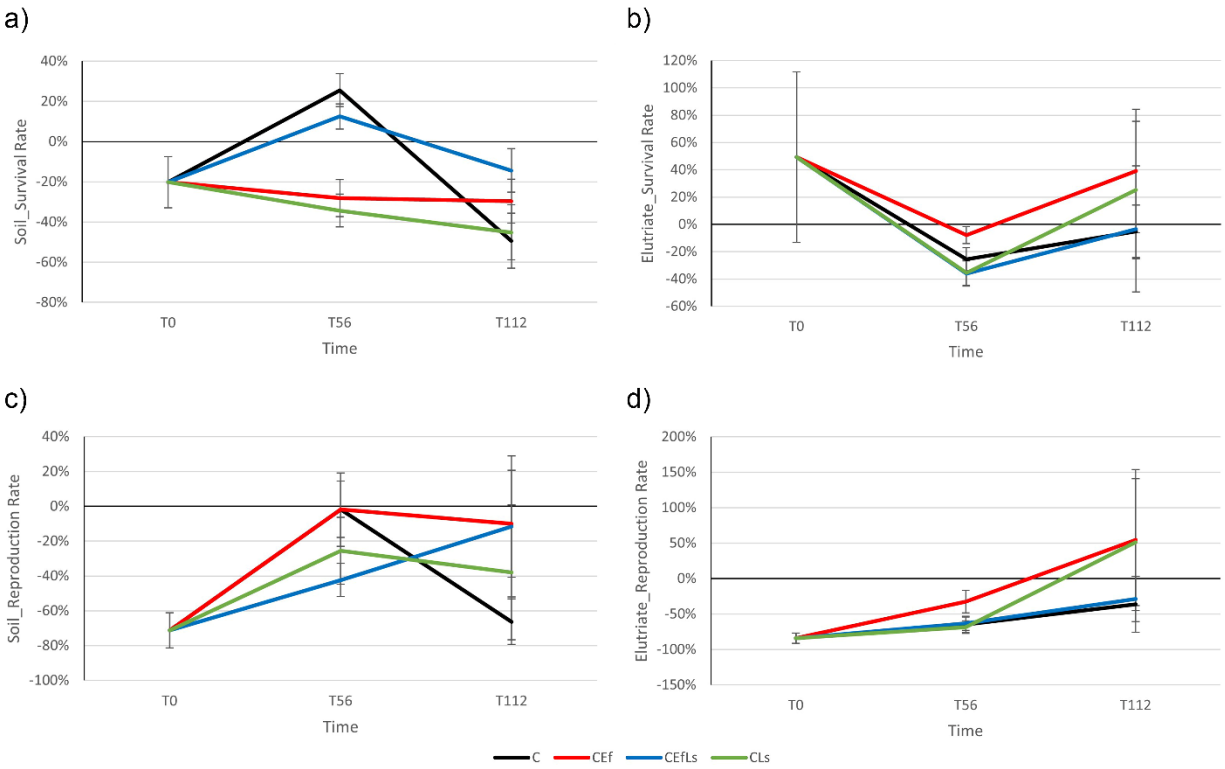

Figure 3. *F. candida* survival and reproduction rates in **a,c)** soil and **b,d)** elutriate respectively. The horizontal bar, corresponding to 0%, indicates results equal to the control sample, values above and below that bar indicate an increase and decrease, respectively, in treatment parameters compared to the control sample.

Differences were observed between T56 and T112 ($p \leq 0.001$). Within C, T56 posted higher values than T0 and T112 ($p \leq 0.01$ and $p < 0.001$, respectively). Within T56, differences were observed between C and CEf ($p \leq 0.01$), C and CLs ($p < 0.01$), and CEfLs and CLs ($p < 0.05$), C and CEfLs having the higher survival rate. The reproduction rate (Figure 3c) was significantly lower than those of the control sample since T0 (intercept: $p \leq 0.001$), with differences attributable to time, and lower values in T0 than T56 and T112 ($p < 0.01$ and $p < 0.05$).

For elutriate, neither survival nor reproduction rates showed patterns depending on treatment or time (Figure 3b-d), however at T0 contaminated soil resulted in higher survival than the control sample ($p < 0.05$).

3.3.2. *Lepidium sativum*

In soil, neither the germination index nor elongation inhibition showed a dependence on treatment or time (Figure 4a-c).

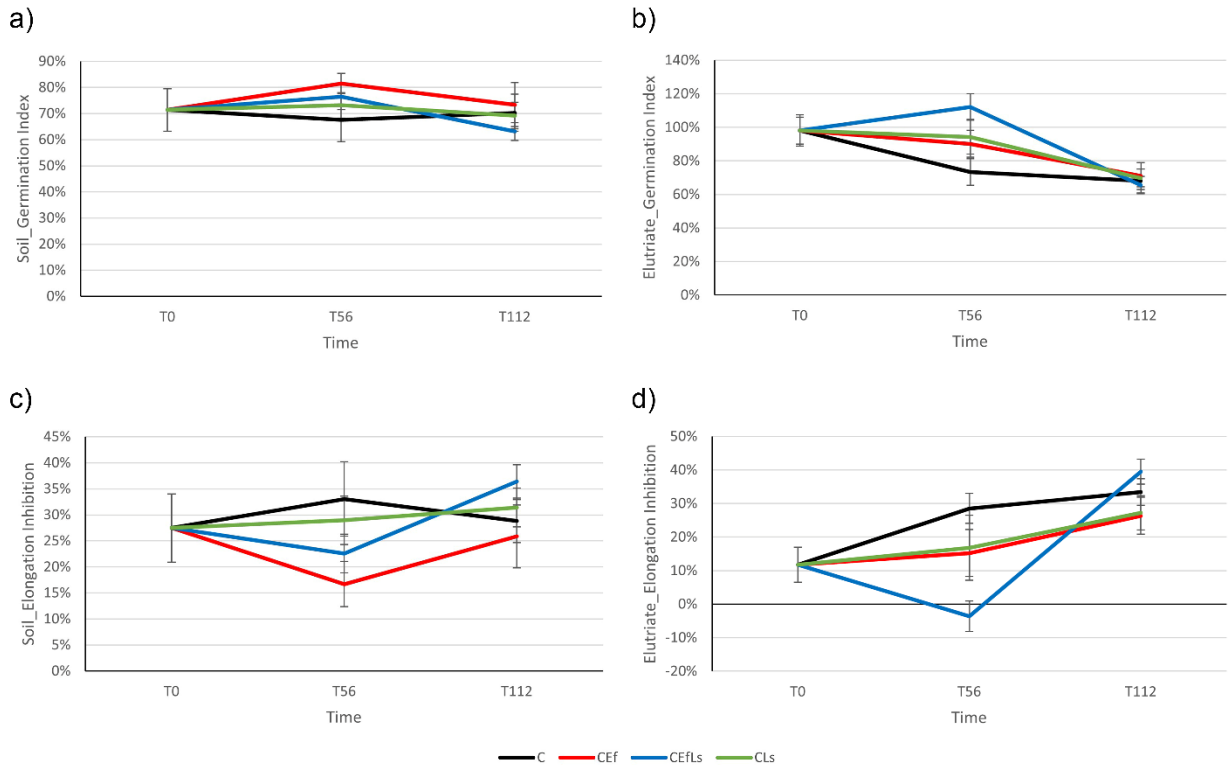

Figure 4. *L. sativum* germination index and elongation inhibition in **a,c)** soil and **b,d)** elutriate respectively. The horizontal bar, corresponding to 0%, indicates results equal to the control sample, values above and below that bar indicate an increase and decrease, respectively, in treatment parameters compared to the control sample.

For elutriate, a time effect was observed in the germination index ($p < 0.05$), which was lower at T112 than at T0 and T56 ($p < 0.001$, both; Figure 4b). Elongation inhibition not only increased with time ($p \leq 0.01$), but was also affected by the interaction of time and treatment ($p < 0.01$; Figure 4d). Root elongation was more inhibited at T112 than at T0 and T56 ($p < 0.001$, both), with a treatment difference in C (with T112 inhibition higher than T0, $p < 0.05$) and CEfLs (with T112 inhibition higher than T0 and T56, $p \leq 0.001$); moreover, a difference was observed between C and CEfLs in T56 ($p < 0.01$).

4. Discussion

4.1. Arthropod community

Soils from Caffaro were collected in order to extract and characterize the arthropod community and assess whether highly PCB and PCDD/F contaminated soils could sustain a soil living community, and of what kind, before setting up vermiremediation tests.

Most arthropods extracted belonged to the Collembola and Acarina groups, with low values of the ratio Acarina/Collembola, typical of low-quality soils, confirming that in degraded soils Acarina numbers decrease (Visioli et al., 2013).

Within Collembola, Hypogastruridae was the dominant family, suggesting it was capable of withstanding some contaminants, as observed by García-Segura et al. (2018) in PAH contaminated sites. On the other

hand, Rusin & Gospodarek (2016) found that Hypogastruridae and Isotomidae were the first families to recolonize soil contaminated by petroleum-derived substances after bioremediation, suggesting that in-field natural attenuation in Caffaro soils could be taking place, thus supporting results observed by the authors on soil contaminant concentration at the beginning and end of the experiment (Unpublished results).

With regard to Acarina, Oribatida were the most abundant mites, supporting the assumption of Iloba & Jarrett (2007) that adult Oribatid mites, having a stronger exoskeleton, could be consistently more tolerant or even active in extreme environments. Due to their lower permeable cuticle, the same applies to Coleoptera and Hymenoptera (Blakely et al., 2002). This is of particular interest since (Shen et al., 2021) observed that the use of beetle larvae in soil POP decontamination is feasible; while with ants being authentic ecosystem engineers that can affect the physical (soil porosity), chemical (nutrient content or pH), and biological (acceleration of decomposition rates) properties of soil (Frouz & Jilková, 2008), their influence on the redistribution of nutrients and even contaminants should be considered more carefully. Since invertebrate potential as bioreactors to treat environmental pollutants is considered promising, these results provide additional information about tolerant soil faunal groups that could be candidates for bioremediation studies (Remelli et al., 2020; Shen et al., 2021).

4.2. Bioremediation ecotoxicological effects

It is well established that over time a greater proportion of compound in soil becomes less extractable and less available for uptake or degradation than freshly added compound, reducing exposure and thus toxicity and risk (Alexander, 2000; Morrison et al., 2000). This has been demonstrated for plants, microorganisms and earthworms (Wilson & Naidu, 2008). This is the case for Caffaro soil, where the process of aging was supported by preliminary tests on *E. fetida*, with earthworm survival not being affected by the presence of PCB and PCDD/F. However, the absence of cocoons showed that *E. fetida* reproductive activity was damaged by contaminants, as already observed by (Remelli et al., 2020) at crude-oil contaminated sites. Nevertheless, even if in lower numbers than in the control soil, juvenile earthworms were observed after 4 months of bioremediation tests, supporting the hypothesis that reproductive activity was only slowed down. However, the difference in adult abundance observed between CEf and CEfLs suggested a negative impact of the presence of *L. sativum* on earthworm growth. Indeed, CEfLs was the treatment having overall most PCDD/F concentrations; moreover, this treatment was characterized by a higher presence of congeners frequently occurring in the environment and that are generally found in high concentrations in animal tissues (McFarland & Clarke, 1989; Unpublished results). CEfLs toxicity could in part be related to phytoremediation limits: even if CLs was shown to be the better bioremediation technique as regards dioxin content in our experiment, remediation by plants is often incomplete, since plants usually lack the biochemical pathways necessary to achieve the total mineralization of recalcitrant pollutants, leading therefore to undesirable

effects, such as the accumulation of toxic metabolites that may be released into the soil and enter the food chain (Aken et al., 2009).

The toxicity effects of PCB and PCDD/F contaminated soils on *F. candida* and *L. sativum* has not been studied in depth, and there is little information in literature, especially for the effect of PCDDs on terrestrial invertebrates. In the present study, ecotoxicological tests with both bioassays confirmed that soil contaminants affected more negatively *F. candida* reproduction and *L. sativum* development than their survival and germination respectively. This is in accordance with (Domene et al., 2007), who observed that *F. candida* reproduction is generally affected at lower waste concentrations than survival, showing that this is a more sensitive parameter.

In our study, the results obtained for *F. candida* in the soil matrix suggested a significant effect of time, with evidence of lower toxicity at T56. This reduction, compared to T0, might be explained by the higher aeration linked to the starter soil manipulation, which might have enhanced aerobic degradation by autochthonous microorganisms in the early stages of the experiment. On the other hand, lower toxicity in T56 compared to T112 suggests that toxicity was not related to contaminant concentrations, since they should have been reduced by water percolation in T112. Water percolation thus seems to enhance pollutants' mobility and bioavailability, inducing a higher toxic effect on Collembola. Nevertheless, as time progressed, treatments involving earthworms were seen to be the better solution for lowering soil toxicity both for *F. candida* survival and reproduction, confirming that vermiremediation can improve soil quality and biological activity (Sinha et al., 2008). On the other hand, CLs probably caused a toxic effect relating to the presence of high hazardous congeners. Generally, the measurement of toxicity through solid phase tests is considered the most relevant way of estimating its ecotoxicological potential, as it is closer to real-life situations (Bakir et al., 2014). However, elutriate represents a useful tool in ecotoxicity assessment as it gives information on pollutants' instantaneous bioavailability (McMillen et al., 2003). In this study, elutriate showed nonlinear results, suggesting that this parameter was more susceptible to PCB and PCDD/F total content, supporting the hypothesis that water enhanced contaminant mobility, disrupting their bindings with organic matter, and thus enhancing their bioavailability.

In this study a negative effect of time was observed on *L. sativum*, mostly in elutriate, with T112 soil more toxic than the starter soil. This result confirmed the suggestion for *F. candida* that the passage of water enhanced pollutants' mobility and bioavailability, thus inducing a toxic effect on *L. sativum*. It has already been observed, by Vašíčková et al. (2016) for heavy metals, that composting and vermicomposting reduce contaminants' mobility, regardless of changes in total concentration, thus reducing ecotoxicity on soil organisms which are mainly exposed to contaminants via pore water fraction. Indeed, at T56 less toxicity was observed in treatments with earthworms (probably because of the organic matter masking effect previously cited); while in T112, regardless of lower contaminant concentrations, water addition might have mobilized previously non-bioavailable contaminants bound to organic matter particles, especially humic substances,

thus increasing ecotoxicity. This toxic effect was particularly evident in CEfLs, characterized by a high presence of dioxins. Moreover, while for *F. candida* at T112 CEfLs toxicity was noticeable only in elutriate, *L. sativum* sensibility was observable in soil too, confirming that plant uptake is not always necessarily directly related to that part of the compound available to soil fauna (Wilson & Naidu, 2008).

5. Conclusion

At the present time little is known about the ecotoxicity of PCB and PCDD/F contaminated soil on terrestrial organisms and on the consequences of bioremediation techniques adopted to treat the problem.

This study provides new insights on the soil fauna community in PCB and PCDD/F aged contaminated sites, which could be used to monitor Caffaro soil health in future. Moreover, results on tolerant soil faunal groups could be of interest for studies on invertebrate application in bioremediation systems.

A battery of bioassays was also conducted to monitor the ecotoxicity of PCB and PCDD/F contaminated soils during a bioremediation experiment lasting 112 days involving vermiremediation and/or phytoremediation treatments. Variable results were observed for different experiment times and treatments, both for *F. candida* and *L. sativum* bioindicators, but it may be necessary to take the soil type into consideration in order to understand ecotoxicological dynamics and determine the bioavailability of contaminants for certain terrestrial organisms. Against this background, elutriate could help to complement soil testing, giving information about contaminant mobilization by means of water, responding to the several drawbacks associated with the organic matter matrix in solid-phase assays. Moreover, the varying sensitivity of *F. candida* and *L. sativum* highlighted the importance of selecting different organisms when testing soil ecotoxicity. On the one hand, the soil matrix impact on springtails, together with bioassay toxicity enhancement after water percolation, cannot be explained by contaminant chemistry data. On the other, both bioassays confirmed earthworms as the most promising treatment among those considered in this study. This study thus supports the need to consider bioassays as relevant tools for the assessment of remediation techniques.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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APPENDIX

Table A.1. pH and SOM (%) in soil at the beginning (T0) and at the end (T112) of the experiment.

	pH		SOM (%)	
	T0	T112	T0	T112
C	7.75 ± 0.06	7.57 ± 0.01	8.41 ± 0.18	8.13 ± 0.26
CEf		7.50 ± 0.06		8.48 ± 0.37
CEfLs		7.52 ± 0.03		7.60 ± 0.17
CLs		7.63 ± 0.02		8.02 ± 0.13

Table A.2. PCB and PCDD/Fs total and congeners concentrations (ng/kg) in contaminated soil at the beginning (C_T0) and at the end ("Treatment"_T112) of the experiment.

Congeners	C_T0	C_T112	CEf_T112	CEfLs_T112	CLs_T112
PCB					
PCB1	446.00 ± 169.54	738.00 ± 130.98	1010.67 ± 198.13	367.33 ± 88.88	529.67 ± 235.36
PCB101	5146.67 ± 1344.24	4646.67 ± 485.29	6606.67 ± 383.03	3203.33 ± 469.41	7406.67 ± 658.04
PCB104	0.50 ± 0.00	1.97 ± 0.47	2.93 ± 1.04	1.83 ± 0.36	2.67 ± 0.41
PCB105	3956.67 ± 586.52	1820.00 ± 25.17	1683.33 ± 69.36	1690.00 ± 115.04	1663.33 ± 137.76
PCB110	7853.33 ± 1510.91	12666.67 ± 1466.67	19133.33 ± 1281.06	8810.00 ± 1863.18	20800.00 ± 1517.67
PCB114	95.10 ± 12.40	125.67 ± 12.73	120.33 ± 3.18	149.67 ± 7.88	120.40 ± 14.69
PCB118	7110.00 ± 1682.88	2826.67 ± 161.90	2536.67 ± 130.43	2833.33 ± 116.81	2470.00 ± 251.20
PCB123	558.00 ± 49.70	186.67 ± 14.11	264.33 ± 19.92	242.67 ± 38.74	176.67 ± 11.41
PCB126	29.53 ± 3.92	19.67 ± 0.42	290.00 ± 19.31	110.27 ± 88.37	241.90 ± 113.28
PCB128	2194.33 ± 910.78	4223.33 ± 213.02	4106.67 ± 620.71	3633.33 ± 535.73	5020.00 ± 511.60
PCB138	29033.33 ± 2026.77	15900.00 ± 945.16	15500.00 ± 503.32	20300.00 ± 1159.02	15466.67 ± 1650.59
PCB146	3680.00 ± 321.87	2013.33 ± 153.01	1896.67 ± 56.96	2350.00 ± 92.92	1956.67 ± 222.81
PCB149	14600.00 ± 1582.19	7516.67 ± 759.26	7513.33 ± 239.88	9796.67 ± 359.64	7683.33 ± 1000.11
PCB15	1290.00 ± 197.32	810.33 ± 36.67	699.00 ± 16.80	910.67 ± 39.98	714.33 ± 86.39
PCB151	5666.67 ± 540.41	1979.00 ± 589.49	2433.33 ± 112.60	3340.00 ± 174.74	2456.67 ± 430.56
PCB153	28466.67 ± 1779.83	10750.00 ± 563.47	11066.67 ± 317.98	14400.00 ± 781.02	10923.33 ± 1154.07
PCB155	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00
PCB156	2383.33 ± 123.47	1286.67 ± 26.67	1176.67 ± 69.36	1296.67 ± 50.44	1236.67 ± 127.06
PCB157	853.33 ± 106.98	533.00 ± 16.26	432.00 ± 26.56	503.00 ± 31.05	500.00 ± 32.58
PCB167	909.00 ± 66.46	656.33 ± 39.62	609.33 ± 87.06	1349.67 ± 390.87	630.33 ± 36.50
PCB169	549.33 ± 6.44	5.17 ± 0.75	3.44 ± 1.06	4.89 ± 0.77	4.95 ± 0.53
PCB170	21666.67 ± 3435.27	13500.00 ± 585.95	11500.00 ± 435.89	13633.33 ± 952.77	12000.00 ± 953.94
PCB177	9386.67 ± 1413.95	5503.33 ± 423.57	4233.33 ± 197.01	5133.33 ± 443.82	4566.67 ± 356.20
PCB180	51100.00 ± 7824.96	25533.33 ± 833.33	22866.67 ± 674.12	28200.00 ± 1709.78	23333.33 ± 2049.66
PCB183	6026.67 ± 872.72	4260.00 ± 409.51	3683.33 ± 140.51	4363.33 ± 273.03	3926.67 ± 457.98
PCB187	23633.33 ± 2425.79	16400.00 ± 1053.57	13600.00 ± 1410.67	17733.33 ± 1020.35	15233.33 ± 1329.58
PCB188	8.26 ± 1.48	3.48 ± 0.12	3.41 ± 0.12	3.94 ± 0.23	3.60 ± 0.29
PCB189	542.00 ± 44.75	298.33 ± 10.84	254.33 ± 19.64	252.33 ± 21.79	269.33 ± 23.10
PCB19	0.50 ± 0.00	7.00 ± 2.92	1.37 ± 0.44	13.13 ± 1.45	10.39 ± 4.48
PCB202	1653.33 ± 135.44	1105.00 ± 79.74	870.33 ± 39.93	1007.33 ± 44.20	946.33 ± 94.94
PCB205	528.33 ± 37.03	6410.00 ± 496.02	5163.33 ± 189.06	6690.00 ± 55.68	5456.67 ± 664.19
PCB206	4493.33 ± 534.83	2806.67 ± 151.03	2406.67 ± 107.29	2570.00 ± 170.88	2503.33 ± 213.10
PCB208	970.67 ± 133.56	482.33 ± 111.18	443.00 ± 23.52	472.67 ± 32.22	544.00 ± 16.26
PCB209	14233.33 ± 2444.27	10836.67 ± 578.51	10170.00 ± 750.27	10513.33 ± 1085.44	7500.00 ± 1129.70
PCB28	413.63 ± 202.48	155.43 ± 61.88	173.33 ± 33.39	608.00 ± 182.79	353.67 ± 132.18
PCB3	1257.00 ± 437.31	352.33 ± 12.68	403.33 ± 157.32	218.33 ± 23.24	239.33 ± 22.88
PCB37	65.81 ± 33.24	64.63 ± 19.51	46.17 ± 18.09	42.93 ± 7.95	95.10 ± 11.44
PCB4	210.00 ± 57.13	126.67 ± 6.84	140.67 ± 15.30	160.67 ± 15.84	114.97 ± 35.15
PCB52	1794.67 ± 656.08	4163.33 ± 1953.05	788.33 ± 112.19	674.00 ± 57.00	1133.67 ± 173.58
PCB54	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00
PCB77	28.57 ± 4.42	269.20 ± 162.24	368.33 ± 197.67	59.13 ± 6.31	389.67 ± 12.67
PCB81	26.63 ± 6.90	8.97 ± 1.09	12.17 ± 0.47	10.97 ± 0.12	10.38 ± 1.67
PCB95	3883.33 ± 706.76	4456.67 ± 374.89	6770.00 ± 559.40	4050.00 ± 841.61	7440.00 ± 859.21
PCB99	3033.33 ± 1155.95	1993.33 ± 258.28	3006.67 ± 198.10	1362.33 ± 353.52	3310.00 ± 301.39
Total PCB	259333.33 ± 10867.89	167333.33 ± 4666.67	164333.33 ± 3382.96	173000.00 ± 8326.66	169000.00 ± 15044.38
PCDD/PCDF					
OCDD	1922.00 ± 861.93	731.67 ± 57.17	641.00 ± 69.09	517.33 ± 143.88	689.00 ± 58.81
OCDF	210.00 ± 31.79	124.67 ± 4.10	120.67 ± 1.33	156.67 ± 17.07	122.33 ± 11.84
1,2,3,7,8- PeCDD	0.25 ± 0.00	0.25 ± 0.00	0.25 ± 0.00	0.25 ± 0.00	0.25 ± 0.00
2,3,7,8- TCDD	29.77 ± 0.80	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
2,3,7,8- TCDF	357.67 ± 26.72	210.33 ± 2.73	220.33 ± 11.46	269.00 ± 36.14	200.33 ± 8.45
1,2,3,7,8- PeCDF	196.00 ± 16.29	115.33 ± 4.37	100.70 ± 3.73	117.27 ± 11.05	97.30 ± 6.09
2,3,4,7,8- PeCDF	175.00 ± 8.33	101.87 ± 3.32	90.80 ± 5.10	116.33 ± 15.33	82.50 ± 4.76
1,2,3,4,7,8- HxCDD	0.25 ± 0.00	2.93 ± 0.15	2.24 ± 0.56	1.07 ± 0.42	2.40 ± 0.60
1,2,3,4,7,8- HxCDF	229.33 ± 21.28	140.00 ± 7.00	134.67 ± 5.78	175.67 ± 10.99	136.00 ± 8.50
1,2,3,6,7,8- HxCDD	79.80 ± 7.69	4.56 ± 0.41	4.76 ± 0.91	7.14 ± 0.75	4.77 ± 0.41
1,2,3,6,7,8- HxCDF	71.80 ± 10.60	145.33 ± 7.22	40.43 ± 2.00	46.07 ± 1.68	38.93 ± 2.41
2,3,4,6,7,8- HxCDF	53.07 ± 3.90	29.33 ± 0.07	30.83 ± 1.27	49.87 ± 10.45	26.23 ± 2.22
1,2,3,4,6,7,8- HpCDD	84.30 ± 9.36	16.90 ± 0.59	15.27 ± 0.66	9.02 ± 4.40	14.10 ± 0.70
1,2,3,4,6,7,8- HpCDF	176.33 ± 13.42	127.00 ± 6.43	74.83 ± 10.55	74.00 ± 1.21	103.47 ± 18.56
1,2,3,7,8,9- HxCDD	1.15 ± 0.90	5.71 ± 0.33	5.88 ± 0.56	7.39 ± 0.33	6.39 ± 0.50
1,2,3,7,8,9- HxCDF	49.37 ± 5.84	25.47 ± 2.17	39.23 ± 3.02	25.08 ± 12.53	22.43 ± 3.65
1,2,3,4,7,8,9- HpCDF	39.57 ± 5.79	26.90 ± 0.97	26.17 ± 2.15	21.05 ± 9.51	26.03 ± 2.46
Total PCDD/Fs	176.00 ± 10.69	92.40 ± 2.40	79.43 ± 3.34	97.80 ± 8.11	73.03 ± 4.09

Chapter Three

CONCLUSIONS

The aim of this thesis was to investigate the link between soil and water matrix dynamics in order to develop new approaches that, through investigation of the unsaturated zone, could lead not only to a better understanding of the hydrogeological functioning of low permeability media but also to the development of innovative bioremediation techniques. Given the important role of soil fauna in soil functioning and water regulation, this was selected as the connection between the soil matrix and groundwater. In particular, the potential of soil fauna as both a bioindicator and promoter of soil health restoration was investigated to provide new insights on the consequences of soil disturbances on groundwater systems and hydrological dynamics, and to suggest sustainable management solutions to some natural and anthropogenic hazards.

Section 3.1:
Biological indicators of hydrogeological characteristics and dynamics

From investigations carried out during the PhD it emerged that soil fauna abundance and composition, depending on the taxa considered, was a good indicator of hydraulic features in a low-permeability system and, in addition, could contribute to the evaluation of soil quality in order to support the recovering of areas impacted by natural disturbances. This hypothesis was investigated in an experimental site located in the Taro River valley, in the Northern Apennines. The study produced the following conclusions:

- The soil arthropod community can influence the hydraulic features of a low-permeability system in a landslide area. Some groups found in this area (e.g. Onychiuridae, Oribatida, Coleoptera, and ants) are able to increase both the effective porosity and permeability of the upper “aquifer” medium. However, due to the depth at which their activity takes place and the heterogeneity of their distribution, it was expected that vertical heterogeneity in permeability and temporary perched groundwater would also be found, which led to several temporary springs being observed at different altitudes during wintertime.
- The soil arthropod community can be influenced by the hydraulic features of a low-permeability system in a landslide area. Some groups found in this area (e.g. Isopoda) are susceptible to water loss by evapotranspiration, thus representing a good indicator for detecting the areas where the groundwater head frequently comes close to ground level.
- The soil arthropod community is, in a wider context, an indicator of soil health, efficient for the detection of degraded soils. Landslides, such as earth flows causing partial or complete soil removal, can thus be differentiated from more well-preserved soils through the study of their soil fauna, contributing to the design of potential ways to restore and re-use these areas.

Section 3.2:
A new approach towards the monitoring and bioremediation of the unsaturated zone of contaminated aquifers

Regarding the development of a new approach towards the bioremediation of the unsaturated zone of contaminated aquifers, the application of soil fauna in all phases of the bioremediation process was investigated.

3.2.1 Biological indicators of land contamination: the role of edaphic fauna

Soil fauna use as an indicator of environmental contamination was examined in three experimental sites characterized by three different types of contamination, resulting in the following conclusions for each site:

- The Parma Functional Urban Area (domestic wells and *fontanili* fed by shallow groundwater affected by PCE and nitrate contamination). The study revealed that domestic wells and *fontanili* upgradient of the rural area located north of Parma city and fed by shallow groundwater are the least affected by PCE and nitrate contamination. Results of ecotoxicological tests with the Collembola *F. candida*, however, cannot be completely explained by considering only PCE and nitrate contamination. It seemed likely, therefore, that other types of contaminants (not analysed in this study) could affect the shallow groundwater. These results confirm the need for toxicity-based approaches to supplement a chemical-based approach when running risk assessments, since bioindicators are able to provide information on compounds not looked for in chemical analyses.
- Val d'Agri (natural oil seepages). In this study site, species such as *F. candida* (arthropod) and *E. fetida* (earthworm) are frequently used as indicators on account of their sensitivity to soil contamination. However, in this study only F. candida was negatively affected by the presence of hydrocarbons, confirming the toxic impact of these compounds on only some organisms. On the other hand, ecotoxicological tests on *E. fetida* highlighted that other organisms, such as *E. fetida*, which are generally sensitive to contaminants, were able to survive in soils containing hydrocarbons, even where there were detrimental effects on reproduction. These results highlight the necessity of using a variety of organisms as bioindicators in risk assessment as well as different end-points to properly evaluate the toxicity levels of an environmental matrix.
- Brescia-Caffaro (PCDD/F and PCB polluted soils from the industrial activities of the Caffaro S.p.A. chemical factory). In this study, results showed the same pattern observed in Val d'Agri for hydrocarbons. The arthropod *F. candida*, even if less affected by PCBs and PCDD/Fs than by hydrocarbons, was found to be more susceptible to the presence of contaminant than *E. fetida*, whose survival was not affected. However, as in the above-mentioned study, earthworm reproduction resulted as being a more sensitive endpoint as the reproduction rate was reduced by contaminants.

To conclude, both the Val d'Agri and Brescia-Caffaro studies revealed that not only are some organisms more sensitive than others, but that earthworms investigated for their decontamination potential represent potential organisms of interest for bioremediation of both hydrocarbons, PCBs and PCDD/Fs.

3.2.2 Identification of contamination-resistant groups belonging to edaphic fauna

The identification of resistant groups, i.e. those able to survive in contaminated environments and thus suitable for further investigation in the bioremediation of certain pollutants, was carried out in two sites: Val d'Agri, where analysis was conducted on soils contaminated by hydrocarbons; and Brescia-Caffaro, where investigations were done on soils contaminated by PCDD/Fs and PCBs. The following conclusions were drawn from each site:

- Val d'Agri. The hydrogeologic and isotopic surveys revealed that fluids, emerging from the two natural hydrocarbon seepages considered in the study area, supply the surrounding soil with a constant intake of PAHs, thus demonstrating that the study area is suitable for the selection of resistant invertebrates which could be potentially eligible for use in hydrocarbon bioremediation processes. In fact, autochthonous it was found that edaphic fauna underwent selective pressure, with communities near the seepages exhibiting structural differences when compared with those at a greater distance. It was hypothesised that this result was related to the presence of hydrocarbon resistant organisms found in these environments, and that these organisms could even benefit from the presence of the contaminant, either directly or indirectly (through symbiosis with microbial communities able to degrade hydrocarbons). In this study both micro- and mesofauna were considered, with Nematoda, Diptera larvae, Acarina and Collembola being identified as the most promising groups, since they were either slightly affected, or even unaffected, by the presence of hydrocarbons.
- Brescia-Caffaro. Chemical analysis confirmed that soils in this area were highly contaminated by both PCBs and PCDD/Fs and thus suitable for the selection of tolerant groups that could be involved in PCBs and PCDD/Fs bioremediation systems. Specifically, Collembola and Acarina were the most abundant groups, followed by Coleoptera and Hymenoptera Formicidae, both considered ecosystem engineers, which can affect physical (soil porosity), chemical (nutrient content or pH) and biological (acceleration of decomposition rates) properties of the soil. These characteristics make them of particular interest for inclusion in bioremediation processes.

3.2.3 Edaphic fauna ability in bioremediation and monitoring processes

The bioremediation of the unsaturated zone of contaminated aquifers through the use of soil fauna throughout the entire bioremediation process (from bioremediation processes to ecotoxicity monitoring) was investigated in PCDD/F and PCB contaminated soils from the Brescia-Caffaro area. Specifically, this involved:

- A microcosm experiment involving the contaminated soils to investigate the efficiency of earthworms (identified as suitable organisms in the above-mentioned studies) in remediation and reclamation processes. In particular, earthworms' (*Eisenia fetida*) suitability, alone or in association with grass (*Lepidium sativum*), for the remediation of PCB and PCDD/Fs contaminated soils was examined. In addition to total contaminant concentration, congeners were analysed and the consequences of earthworm action on contaminants and nutrient mobility into groundwater were simulated. Results showed a time-related reduction of contaminant concentration. Less threatening PCBs congeners were found when earthworms and grass were tested together, while a lower concentration of PCDD/Fs was observed in treatments with only grass. Moreover, a relation between earthworm activity, contaminant and nutrient mobility in percolated water was detected. The results highlighted the fact that since PCB and PCDD/Fs often occur together, and considering both contaminant concentration and toxicity, the use of earthworms seems to be the most promising treatment among those tested. Their application should, however, be carefully monitored, since there is a possibility that these organisms might enhance contaminant percolation into groundwater. It is, therefore, necessary to consider the hydraulic characteristics of the contaminated media when selecting *ex situ* or *in situ* vermiremediation. The former is preferable when unconfined aquifers are present, with an assessment of the application needing to be carried out, whereas the latter is more advisable when considering confined aquifers and low permeability media, where the low permeability thickness exceed earthworms digging depth.
- Ecotoxicological tests were used to evaluate the monitoring of the above-mentioned experiment; these tests represent a reliable tool not only to detect the presence of toxic compounds in both soil and water as a supplement to chemical-based approaches, but also to determine the efficiency of remediation techniques in terms of ecological site rehabilitation. Two main aspects were highlighted by the results: (i) earthworm treatment could be the most promising method since it tends to be the less toxic, confirming the fact that earthworms are promoters of soil health; and (ii) water addition in sufficient quantities to saturate the media seems to increase contaminants mobility and bioavailability, thus increasing soil toxicity for both tested organisms (collembolans: *F. candida*, and cress: *L. sativum*). In addition, the different sensitivity of the tested organisms were found to depend on the matrix considered (i.e. soil or elutriate). This evidenced that not only was it important to select different target organisms and end-points in soil ecotoxicity monitoring, but it was also necessary, as a complement of the solid phase, to evaluate the elutriate role in ecotoxicity tests with soil fauna.

To summarize, it was found that soil fauna can be efficiently involved in all phases of bioremediation. Earthworms resulted as being an effective tool with which to reduce the ecotoxicity of organically contaminated soils and, in addition exhibited a potential for use in the reduction of organic contaminant concentration. However, long-term studies are needed before such a potential role can be fully ascertained.

In conclusion, it is necessary to underline the importance of a multidisciplinary approach since the efficiency of some techniques is likely to be strongly related to the system analysed, presenting limitations for other systems. In order to identify critical issues and overcome limitations, therefore, the application of interdisciplinary methods is strongly recommended, so that the environment can be analysed from a broader, more effective perspective.

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