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Evaluation of bioventing on a gasoline–ethanol contaminated undisturbed residual soil

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Abstract

Remediation methods for environmental contamination problems based on physical or chemical processes frequently present low efficiency and/or high costs. On the other hand, biological treatment is being proved to be an accessible alternative for soil and water remediation. Bioventing is commonly used for petroleum hydrocarbon (PHC) spills. This process provides better subsurface oxygenation, thus stimulating degradation by indigenous microorganisms. In Brazil, gasoline and ethanol are routinely mixed; some authors suggest that despite gasoline high degradability, its degradation in the aquifer is hindered by the presence of much rapidly degrading ethanol. The present study evaluates a bioventing treatment of a gasoline–ethanol contaminated undisturbed residual soil from Rio de Janeiro. Contamination and treatment effects were monitored by conventional microbiology methods, chemical analysis, and ground penetrating radar (GPR) measurements. Results of culturable bacterial population counts show the effect of contamination and bioventing on the microbiota of gasoline and gasoline–ethanol containing soils; however, GPR responses to these variations are not conclusive and still need to be assessed.

Keywords: Gasoline–ethanol contamination; Bioremediation; Residual soil; Ground penetrating radar; Culturable microbiota

1. Introduction

Environmental contamination problems receive great attention nowadays because of the direct threat they represent for human health. Biodegradation may occur naturally, and no intervention is necessary to clean up the contaminated area. In other cases, engineering measures are essential to accelerate natural attenuation by creating selective conditions to stimulate the activity of specific microbial consortia (generally by nutrient or oxygen injection into the subsurface) [1,2]. If monitoring of contaminants or degradation by-products suggests that biodegradation has occurred, it cannot evaluate whether it is still happening nor if it will continue until the mineralisation of contaminants [1]. As microorganisms are the key factor in the degradation of natural and xenobiotic molecules, a need emerges to understand responses of the microbial population to pollution [3]. Environmental contamination has led to decreased biodiversity, extinction of sensitive species, and an artificial selection of better adapted ones. Therefore, monitoring of microbial populations may provide important information on those events, as microorganisms are the first to suffer environmental impacts and exhibit its effects.

Biological treatment of pollution is an accessible and efficient alternative for soil and water remediation. Petroleum hydrocarbons (PHC) have a natural origin and have always been ubiquitous in the environment; consequently, many microorganisms have a natural ability to degrade them very easily [4–7]. Most studies deal with ground water contamination and the literature supports that PHC biodegradation

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is widespread in the saturated subsoil. However, processes in unsaturated soils are less understood. Limited availability of oxygen in the subsurface may hinder microbial metabolic processes leading to biodegradation [2].

Bioventing is a commonly used technique to remediate PHC contaminated soils, as it enhances the ability of soil microbiota to degrade natural and xenobiotic compounds. Air injected in the unsaturated zone provides microorganisms with adequate oxygen conditions so that degradation may continue efficiently for longer periods of time [8,9].

In Brazil, gasoline is currently mixed to 26% (v/v) ethanol. When spills occur, ethanol reaches subsurface waters much more rapidly than gasoline components [10]. In gasoline–ethanol contaminated aquifers, ethanol is preferentially utilised, and therefore degraded, over other persistent gasoline compounds, mainly benzene, toluene, ethyl-benzene, and xylenes (BTEX). BTEX compounds are degraded by inducible enzymes that can be repressed when easily degradable substrates are present at high concentrations [11]. Also, ethanol degradation leads to a depletion of electron acceptors in soil for BTEX degradation. Poor oxygenation conditions may thus prevail in the aquifer, delaying BTEX degradation, supposedly leading to longer plumes [12]. This situation is of special concern for benzene that degrades rather slowly in anaerobiosis [13].

The relationship between structure and function has been examined in recent literature [14–17] for the evaluation of environmental contamination, but a wide-ranging concept for has not emerged so far. A need to conciliate experimental possibilities and field reality has led to the use of microcosms and lysimeters, which consist of intact or reconstituted soil columns kept under controlled conditions. Those systems allow for several measurements such as distribution and microbiological processes [18,19]. PHC biodegradation rates obtained from experiments in laboratory conditions are comparable to those from field measurements; thus, predictions made from microcosms studies data can be good indicators in practice [20].

Contaminant distribution and degradation in the subsurface can be monitored by several physical, chemical, and microbiological methods. A multiple-parameter, multi-disciplinary approach provides the most useful information on impact and fate of contaminants in the environment [21,22]. A multi-disciplinary research programme under way at the Pontifical Catholic University of Rio de Janeiro (PUC-Rio), aims at the understanding of effects of PHC contamination in tropical soils, as well as the evaluation of monitoring and remediation techniques potentially applicable for PHC contaminated sites. This paper presents some results of a study with an unsaturated residual soil in which PHC contamination and its effects were monitored under laboratory conditions.

Traditional microbiology techniques were used to evaluate total culturable heterotrophic bacterial populations. Although this methodology presents the disadvantage of looking only into culturable microbial populations, it has been used to assess soil toxicity in a rapid and cost-effective manner. Additionally, enzymatic activity generally correlates well with bacterial abundance estimations in subsoils [23,24]. Chemical analyses by gas chromatography determined PHC amounts in soil samples during the experiment. Additionally, ground penetrating radar (GPR) was used to monitor contaminant fate in soil. GPR sends electro-magnetic waves (EMW) into the subsurface aiming to detect differences in soil electric properties [25]. The use of this technology is based on recent research performed in sandy soils of temperate and cold climates [26–30]. During biodegradation, uncharacteristic high conductivities have been attributed to microbial activity in PHC-contaminated sediments, because of acid and bio surfactant production by indigenous microorganisms. When in contact with soil water, these compounds increase soil conductivity causing an attenuation of the EMW [29]. Also, some authors consider that the occupation of soil voids by the contaminant causes a loss in contrast that would decrease or prevent the EMW reflection at the contact of two soil layers. Effects of ethanol presence on BTEX persistence as well as the possibility of GPR measurements still have to be assessed in unsaturated soil.

The main purposes of this study were (1) to determine the influence of bioventing on the biodegradation of gasoline and gasoline–ethanol mixture in unsaturated soil; (2) to evaluate changes in microbiota as an indicator of soil toxicity; (3) to evaluate the effect of ethanol on gasoline components degradation; and (4) to determine whether GPR and culturable bacterial counts can be useful tools for monitoring distribution and degradation of PHC in unsaturated residual soil.

2. Material and methods

2.1. Soil

The material used in the present investigation is a natural, unsaturated soil, taken from a depth of circa 2.5 m of a typical weathering profile of kinzigitic gneissic rocks found in the metropolitan area of Rio de Janeiro, Brazil. At this depth in the site, the material presents macroscopic features found in its mother rock belonging, therefore, to the pedological horizon C. From the geotechnical point of view, the material comprises a young or saprolitic gneissic residual soil.

2.1.1. Geotechnical, physical, chemical, and mineralogical characteristics

Undisturbed and remoulded samples were taken for soil characterization, comprising standard geotechnical, mineralogical, and geochemical testing. The material presents randomly distributed grey and white facies, the first one prevailing at the sampling depth. Fig. 1 shows grain size distribution curves for both facies and Tables 1–3 present a summary of geotechnical, physical, mineralogical, and chemical properties of the material.

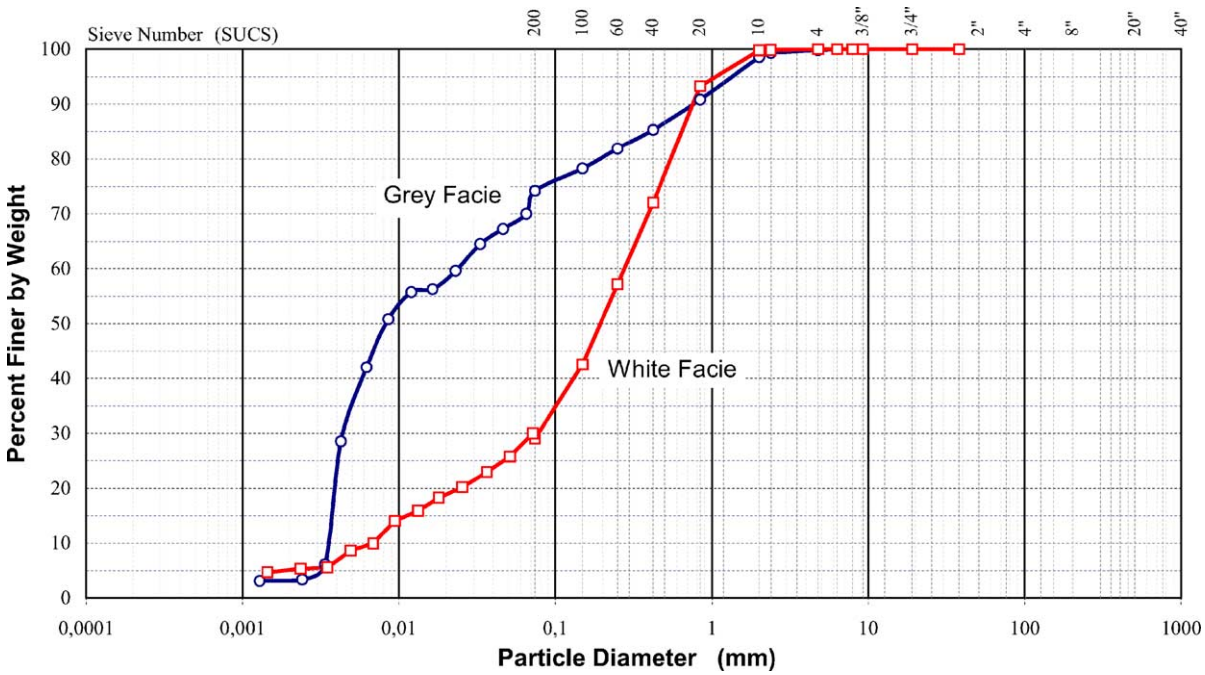

Fig. 1. Grain-size distribution curves of white and grey facies.

The grey facie of the soil that predominates in the weathering profile at the sampling depth, comprises a sandy silt material with quartz and mica biotite as dominant minerals. The white facie is a silty sand, with quartz and potassic feldspar as main minerals. The clay fraction of both facies is quite low, the mineral gibbsite being dominant in the white facie while kaolinite is dominant in the grey one. The values of both specific density of the grains (G_s) shown in Table 2, and weathering parameters K_i and K_r, shown in Table 3, are in agreement with such mineralogical composition. Facies of the soil with greater mica content present G_s values as high as 2.80 while those with larger feldspar content show G_s values down to 2.60; the values indicated in Table 2 represent average ones. The low values of K_i and K_r as well as

Table 1
Geotechnical characterization

Facie	Grain size distribution (%)				Consistency limits (%)			Colloidal activity
	Gravel	Sand	Silt	Clay	W_L	W_P	PI	
White	0–7	63–74	23–25	4–5	43.7–61.4	30.4–47.4	13.3–14.0	0.30–0.36
Grey	1–2	30–33	58–66	3–6	58.3–62.1	37.3–38.2	21.0–23.9	0.14–0.25

Table 2
Physical and mineralogical characterisation

Facie	Physical indexes						Mineralogy	
	e	n	W (%)	S (%)	ρ_d (g/cm^3)	G_s	Coarse fraction	Fine fraction
White	0.82	44.9	7	30	1.45	2.63	Quartz; potassic feldspar	Kaolinite; gibbisite
Grey	0.93	48.0	13	35	1.41	2.72	Quartz; biotite	Kaolinite

e: voids ratio; n: porosity; W: gravimetric moisture content; S: degree of saturation; ρ_d: dry specific weight; and G_s: relative density of soil grains. Mineralogical data obtained through X-ray diffraction and optical microscopy.

Table 3
Chemical characterization

Facie	pH	CEC (meq./100 g)	K_i	K_r	C_{org} (%)	N (g/kg)	P (mg/kg)
White	4.9	1.8	0.54	0.51	0.5	0.1	1
Grey	4.6	6.3	1.30	1.20	1.3	–	14

CEC: cationic exchange capacity; K_i: SiO_2/Al_2O_3; K_r: $SiO_2/(Al_2O_3 + Fe_2O_3)$; C_{org}: organic matter content; N: nitrogen content; and P: phosphorous content.

the low soil pH, regardless of the facie under consideration, indicate a high weathering degree for the soil, with leaching of silicium and occurrence of iron oxides in the soil fine fraction. There are evidences that as the weathering degree in residual soil profiles increases, there is a trend for homogenization of their properties [31,32]. This may explain the relatively low difference observed between the average physical indexes of the two facies of the material here considered, particularly in what refers to their porosity and dry specific weight (Table 2).

2.1.2. Microbiological characteristics

For microbiological evaluation, soil samples were aseptically collected when the blocks were extracted, at the same location, and kept refrigerated until analysed, up to 4 h later. Average total heterotrophic bacterial populations were determined at 10^4 colony forming units per gram of soil (CFU g^{-1}); the presence of culturable gasoline degraders in the indigenous microbiota was detected by growth on minimum mineral medium (0.5 g $(NH_4)_2HPO_4$, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.001 g $FeSO_4{\cdot}7H_2O$, 0.1 g K_2HPO_4, 0.01 g $Ca(NO_3)_2$, 1000 mL distilled water) amended with gasoline (20 mL L^{-1}).

Microbiological determinations were performed at the Environmental Microbiology Laboratory of the Civil Engineering Department (PUC-Rio).

2.2. Study design

Preliminary studies were conducted to determine the study design. Two culture media and two bacteria counting methodologies were evaluated; contamination, sampling, bioventing, and GPR measurements procedures were also set according to that preliminary assay [33]. Water, gasoline, and gasoline–ethanol mixtures penetration in soil, their retention and volatilization were also evaluated in undisturbed soil columns. Such information will be published elsewhere.

2.2.1. Soil blocks

Undisturbed cubic soil blocks (30 cm side) were collected for the experiment. Soil blocks were covered with aluminium foil, plastic film and paraffin, to avoid moisture changes, and left in a constant humidity room until required for testing.

Six structured soil blocks were used (refer to Table 4): block 1 was a control, receiving neither contamination nor treatment; block 2 was ventilated; blocks 3 and 4 were contaminated with gasoline and gasoline–ethanol mixture, respectively, receiving no treatment; blocks 5 and 6 were both ventilated after being contaminated with gasoline and gasoline–ethanol mixture, respectively.

2.2.2. Bioventing

A constant airflow of 2 psi was injected by a compressor into the core of blocks 2, 5, and 6, to provide constant oxygenation of the soil. Bioventing started 10 days after contamination.

Table 4
Soil blocks contamination and treatment applied

Blocks	Name	Contamination	Bioventing
1	Control (Ctl)	None	No
2	Bioventing control (Ctl Bv)	None	Yes
3	Gasoline control (Ctl G)	Gasoline	No
4	Gasoline–ethanol control (Ctl Get)	Gasoline–ethanol mixture	No
5	Gasoline–ethanol bioventilated (G Bv)	Gasoline	Yes
6	Gasoline–ethanol bioventilated (Get Bv)	Gasoline–ethanol mixture	Yes

2.2.3. Contamination

Ten litres of contaminant (regular gasoline for blocks 3 and 5, ethanol-amended gasoline, 22% (v/v), for blocks 4 and 6) were poured onto the surface of the blocks, enough to soak the soil and percolate. Contaminants were applied in the center of the blocks upper surface so as to avoid sideways preferential flow. To minimise moisture loss from the soil samples, all blocks were covered with a 3 cm coarse sand layer. This layer was kept moisten by regular water sprinkling over a top layer of cotton fibers. Water sprinkling started with bioventing and was applied one to three times a week according to the ambient temperature.

2.2.4. GPR acquisition

For geophysical monitoring, a Radan GPR unit (Sir 2000 system-GSSI) was used with a 900 MHz shielded antenna (model no 3101D). A 20 cm section was sampled at the center of the block, one signal registered per millimeter. Data were acquired at 512 samples per scan, 16 bits per sample and a 16 ns range.

Data were analysed and processed with the Radan for Windows 2000® software from GSSI, the same processing parameters being applied at each step, for all blocks. The first step in data processing was to detach the most significant part of the acquired section, selecting scans corresponding to the central 20 cm to avoid boundary effects on radar reflection data. Data were processed with an IIR Filter in the 450–1800 MHz band, restoration gain, then amplified five times so as to obtain more contrast for better imaging.

The antenna was passed on the block surface prior to and after gasoline contamination (Fig. 2). Data were acquired four times during the assay: before contamination, 12 h, 30, and 100 days after contamination.

2.2.5. Culturable populations monitoring

Bacterial culturable heterotrophic populations were evaluated prior to and after contamination and bioventing. At each sampling, three samples were taken from the same collecting holes drilled horizontally on the side faces of the blocks at three different depths from the surface (8, 15, and 20 cm deep, approximately) to form a composite sample.

Fig. 2. Acquisition with GPR: the 900 MHz shielded antenna was passed on the soil blocks surface. The unit control from GSSI can be seen at the back.

Samples were taken on a different side of the soil blocks each time. Soil samples were collected before contamination, 5 and 20 h later, 2 and 10 days after contamination. Bioventing started 10 days after contamination; soil samples were then collected 5 and 24 h later, and regularly afterwards; the assay was run for 141 days. Soil samples were diluted in sterile distilled water and total culturable heterotrophic bacterial populations were enumerated by colony forming units counting in tryptone soy agar (TSA) (Oxoid) 1:10, by pour plating [34,35].

2.2.6. Chemical monitoring

Gas chromatography analyses were performed at the Fuel Laboratory at the Chemistry Department of the Pontifical Catholic University of Rio de Janeiro. Soil samples were collected with microbiology samples and kept frozen until analyzed, except for samples from 2 months after the end of the assay, which were kept refrigerated.

Contaminants were extracted from soil samples by orbital shaking in methanol.

Gasoline content in soil was determined using a Shimadzu chromatographer equipped with a flame ionization detector and an AT-Wax capillary column (25 m, 0.25 i.d.), temperature programmed from 35 °C (5 min) to 230 °C (2 min), at a rate of 20 °C/min. Detection limit: 100 ppm.

3. Results

3.1. GPR measurements

Results from GPR measurements are shown in radargrams in Figs. 3–8. Radargrams are shown for each block with the central scan corresponding to the signal amplitude variation.

Data from before contamination present a strong reflection close to the 7.50 ns value (double time), between 0.20 and 0.30 m deep that correspond to the bottom of the block.

After contamination, no perceptible change was seen in data from gasoline-contaminated soils compared to non-contaminated soils (Figs. 3–6). However, in gasoline–ethanol containing soils, electrical variations become apparent (Figs. 7 and 8).

A decrease in EMW velocity as well as amplitude losses were seen in data from days 30 and 100, characterizing a retardation effect. It is also visible that in ventilated soils these 30- and 100-day variations are smaller (Figs. 4, 6, and 8).

Variations of soil dielectric constant *K*, calculated from GPR measurements, are shown in Table 5. Increases in *K* values correspond to the attenuation seen in radargrams. *K* values 20 h after contamination are higher in soils containing ethanol than in gasoline-contaminated ones. In data from 30 and 100 days after contamination, *K* values increased less in ventilated soils than in non-ventilated ones, in both gasoline and gasoline–ethanol contaminated blocks (Table 5).

3.2. Microbial counts

Results from CFU counts showed a decrease in culturable bacterial populations a few hours after contaminants addition, followed by an increase 24 h after contamination (Fig. 10). Initial populations, before contamination, varied from 1.8×10^3 to 1.06×10^4 CFU g^{-1} soil. From the 2nd day on, bacterial populations remained stable around 10^2 CFU g^{-1} soil (gasoline–ethanol containing soils) and 10^3 CFU g^{-1} soil (non-contaminated and gasoline-contaminated soils) until day 10, when bioventing started (Fig. 9).

Bioventing did not introduce any changes in contaminated soil populations, which presented very low culturable

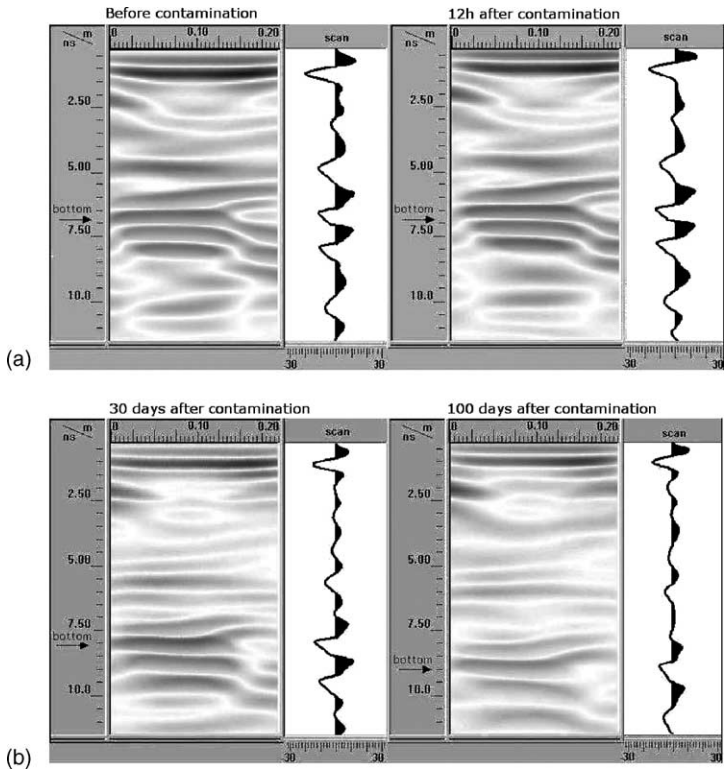

Fig. 3. Radargrams from untreated non-contaminated soil before contamination, approximately 20 h, 30 days, and 100 days after contamination. The central scan is shown for each radargram (amplitude × time).

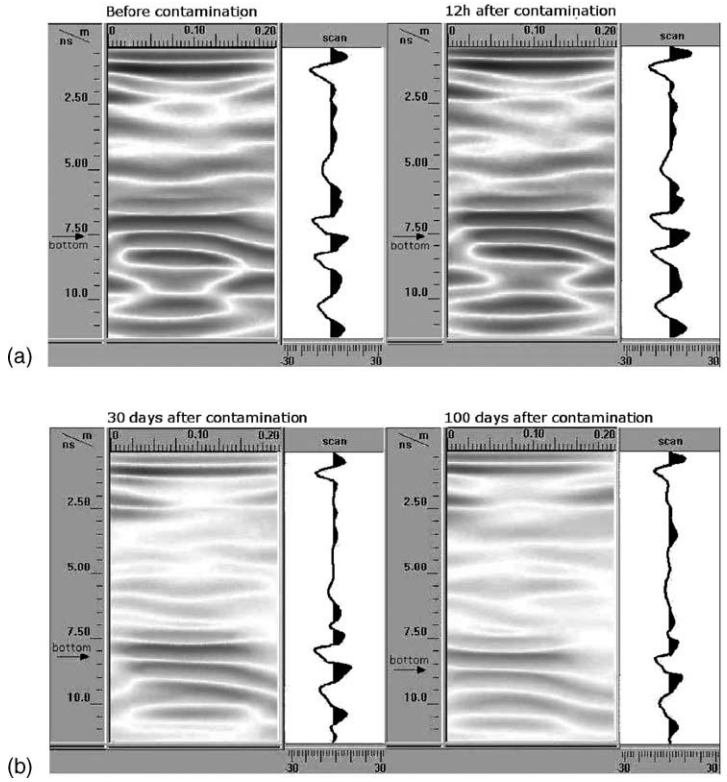

Fig. 4. Radargrams from non-contaminated ventilated soil before contamination and approximately 20 h, 30 days, and 100 days after contamination. The central scan is shown for each radargram (amplitude × time).

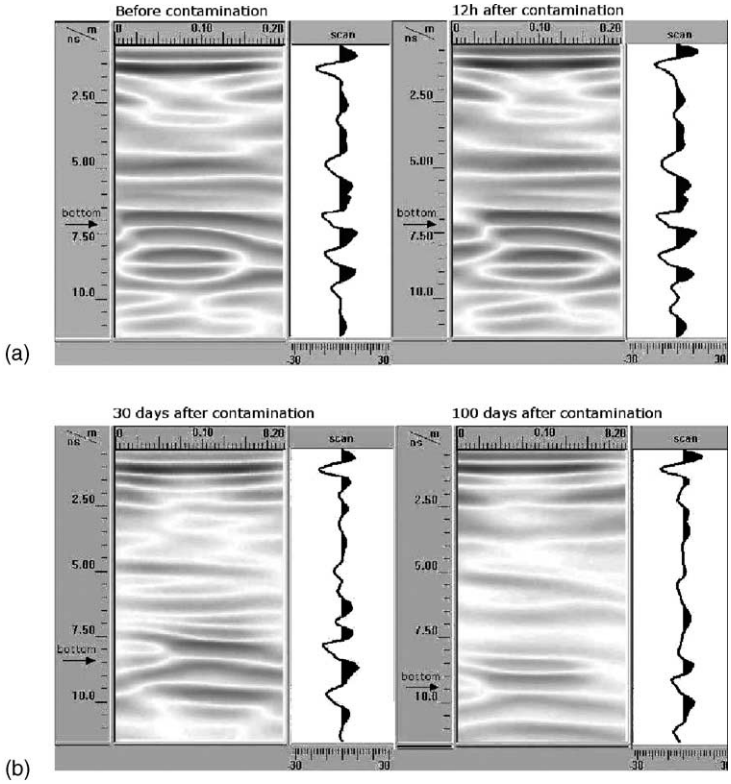

Fig. 5. Radargrams from non-ventilated gasoline-contaminated soil, before contamination and approximately 20 h, 30 days, and 100 days after contamination. The central scan is shown for each radargram (amplitude × time).

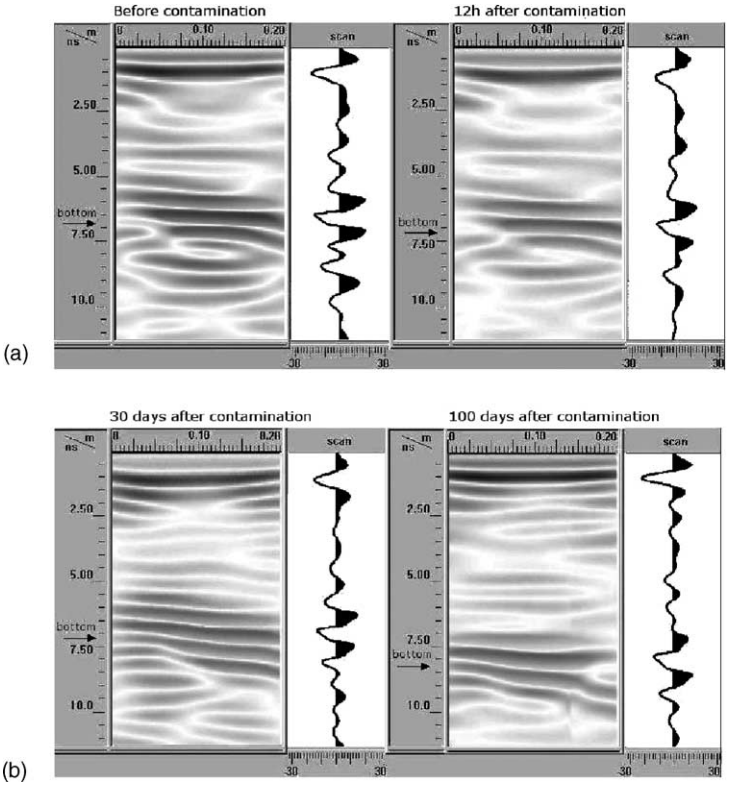

Fig. 6. Radargrams from ventilated gasoline-contaminated soil before contamination and approximately 20 h, 30 days, and 100 days after contamination. The central scan is shown for each radargram (amplitude × time).

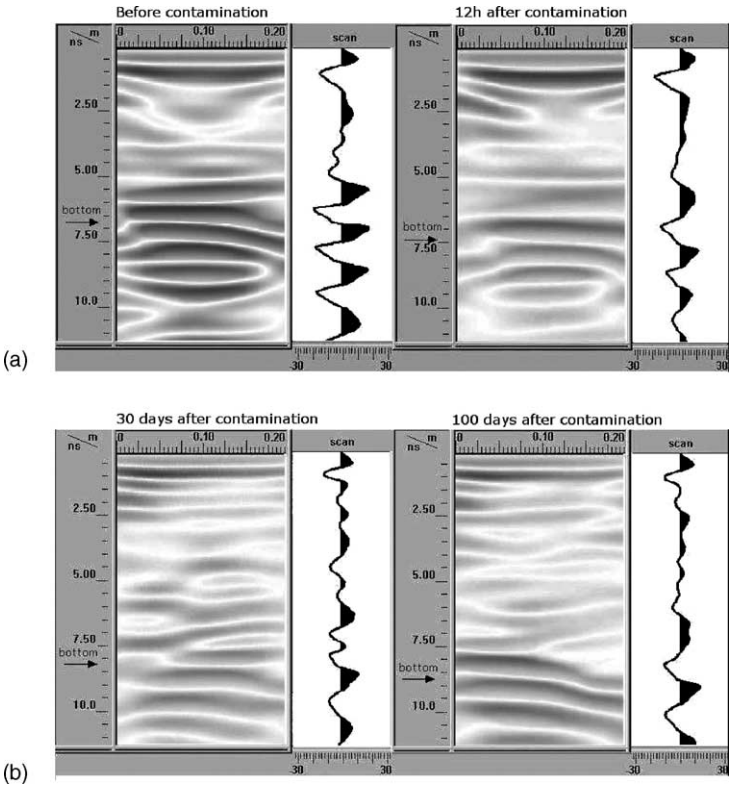

Fig. 7. Radargrams from non-ventilated gasoline–ethanol contaminated soil before contamination and approximately 20 h, 30 days, and 100 days after contamination. The central scan is shown for each radargram (amplitude × time).

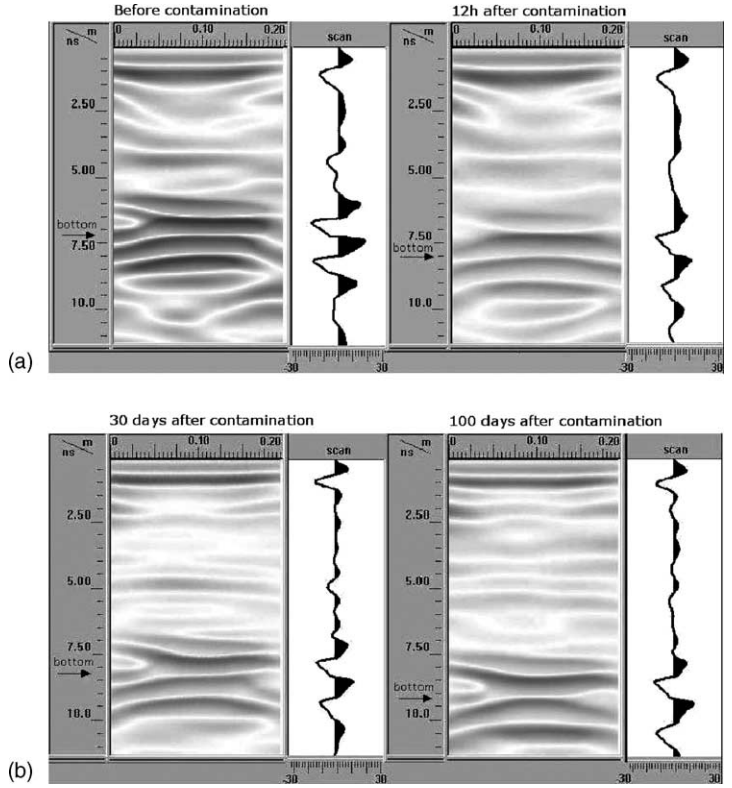

Fig. 8. Radargrams from ventilated gasoline–ethanol contaminated soil before contamination and approximately 20 h, 30 days, and 100 days after contamination. The central scan is shown for each radargram (amplitude × time).

Table 5
Soil dielectric constant (*K*) measured by the GPR, and microbial counts (CFU g^{-1}) of culturable bacterial populations

Soils	Initial soil		Twenty hours after contamination		Thirty days after contamination		Hundred days after contamination	
	K	CFU g^{-1}	*K*	CFU g^{-1}	*K*	CFU g^{-1}	*K*	CFU g^{-1}
Non-treated control	12	1.85×10^3	12	3.27×10^3	16	1.30×10^5	21	1.12×10^5
Ventilated control	12	1.06×10^4	12	6.40×10^3	15	1.40×10^5	17	1.73×10^5
Gasoline-contaminated	12	2.70×10^3	12	1.60×10^3	16	5.50×10^1	21	1.12×10^5
Gasoline–ethanol contaminated	12	2.60×10^3	17	5.50×10^2	21	4.00×10^1	23	1.27×10^2
Ventilated gasoline-contaminated	12	1.06×10^4	14	9.50×10^2	14	9.50×10^1	18	5.61×10^3
Ventilated gasoline–ethanol contaminated	12	8.07×10^3	16	1.20×10^3	18	1.20×10^2	22	3.90×10^4

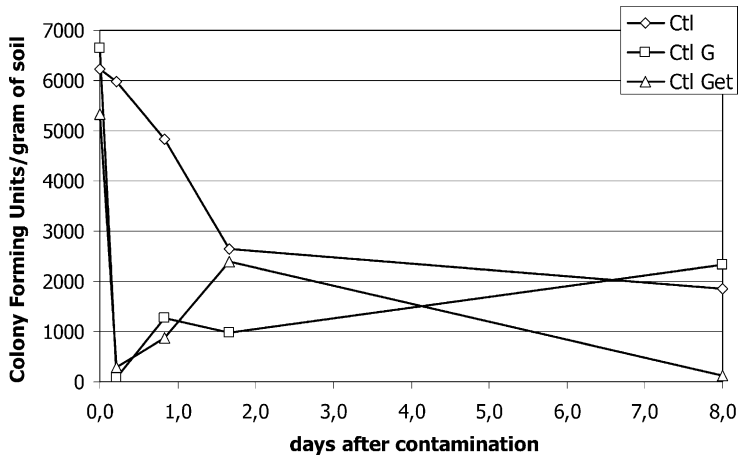

Fig. 9. Total culturable heterotrophic bacterial population after soil contamination. (□) Ctl non-contaminated control; □ CtlG gasoline-contaminated control; and ■ CtlGet gasoline–ethanol contaminated control.

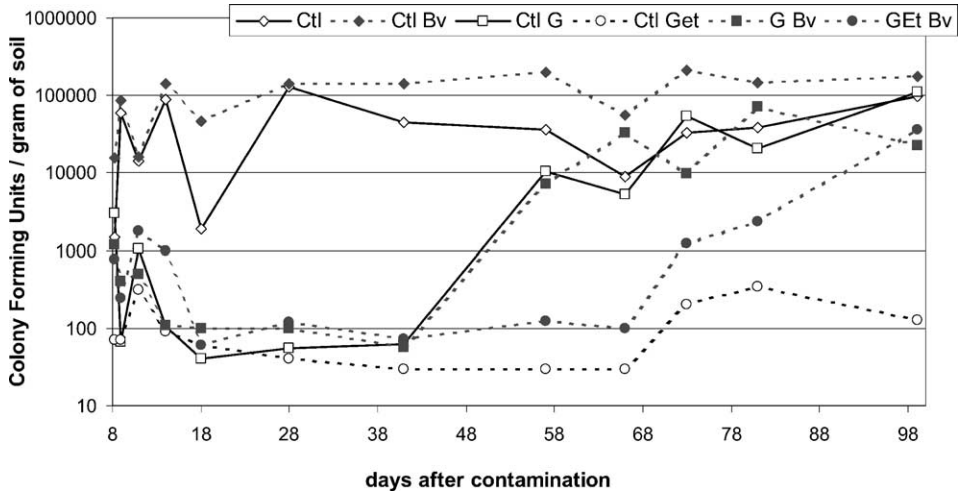

Fig. 10. Total culturable heterotrophic bacterial population after soil bioventing (colony forming units per gram of soil), (□) Ctl non-contaminated control; ◆ CtlBv bioventilation control; □ CtlG gasoline-contaminated control; ■ CtlGet gasoline–ethanol contaminated control; (○) GBv gasoline-contaminated ventilated soil; and (●) GetBv gasoline–ethanol contaminated ventilated soil).

populations (10 to 10^2 CFU g^{-1}) for 40 days. Non-contaminated soils showed an increase in culturable bacterial population which stabilised around 1.5×10^5 CFU g^{-1} soil for bioventilated soils and around 5×10^4 CFU g^{-1} soil, 20 h after bioventing was introduced (Fig. 10).

In gasoline-contaminated soils bacterial populations resumed growth 40 days after contamination, in both ventilated and non-ventilated blocks with no marked difference between them; both soils attained around 10^5 CFU g^{-1} soil. Resuming of culturable bacteria growth occurred with a

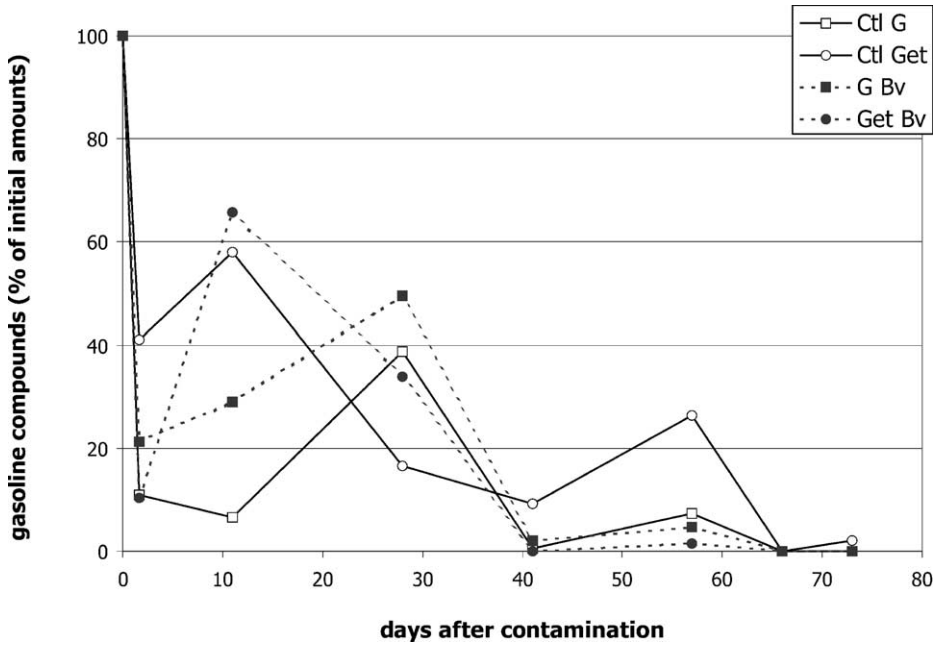

Fig. 11. Percentage of initial gasoline amounts remaining in contaminated soil samples from right after to 7 months after contamination, determined by GC analysis (bioventing was interrupted after 141 days).

30-day delay in soils with gasoline and ethanol. Before the recovering of its culturable bacteria, the bioventilated soil presented a slightly higher population than non-ventilated soil; once recovery started, the bioventilated soil showed a rapidly increasing bacterial population while in the non-ventilated one it remained around 10^2 CFU g^{-1} soil (Fig. 10).

3.3. Chemical analysis

Results from CG analysis, in Table 6, showed some variability in contaminants content in soil right after contamination (samples taken when percolation of contaminants stopped). From day 60 on, a decrease is observed in all soil samples, from ventilated and non-ventilated blocks, with standard gasoline and ethanol-amended gasoline. Four months after contamination, gasoline components amounts in soil samples were under the detection limit, being no longer detected 7 months after contamination. Important oscillations in GC results reflect structured soil heterogeneity, sampling having been done on different sides of the blocks. In spite of this variation, results from 2 weeks after contamination show higher amounts of gasoline components in soils originally contaminated with gasoline–ethanol mixture (Fig. 11), in ventilated and non-ventilated soils.

Fig. 11 shows the decrease in gasoline amounts related to its initial concentration in soils.

4. Discussion

Soil type and depth seem to be the main factor controlling physical and biological properties in the subsurface and distribution of microbiological properties is related to that of chemical and physical properties in the environment; it is well known that geochemical, hydrologic, microbiological, and geological properties are all dependent on each other. The nature of the subsurface environment causes a great heterogeneity in physical and chemical properties, and these strong contrasts originate the high microbiological

Table 6
Amounts of gasoline components in soils measured by gas chromatography from right after to 7 months after contamination (gram per gram of soil)

Soil blocks	Right after	After 5 h	After 2 days	After 11 days	After 28 days	After 41 days	After 60 days	After 66 days	After 74 days	After 83 days	After 4.5 months	After 7 months
Gasoline control (Ctl G)	0.182	0.047	0.02	0.012	0.0705	0.00115	0.0132	<d.l.	<d.l.	<d.l.	<d.l.	n.d.
Gasoline–ethanol control (Ctl Get)	0.0755	0.026	0.031	0.438	0.0125	0.00703	0.0199	<d.l.	0.0015	0.104	<d.l.	n.d.
Gasoline bioventilated (G Bv)	0.117	0.02	0.025	0.339	0.058	0.00233	0.0054	<d.l.	<d.l.	0.274	<d.l.	n.d.
Gasoline–ethanol bioventilated (Get Bv)	0.1445	0.033	0.015	0.095	0.049	0	0.00216	<d.l.	<d.l.	0.037	<d.l.	n.d.

n.d.: non-detected and <d.l.: under detection limit.

heterogeneity found in the subsurface [36,37]. Differently from surface soils, subsurface systems receive very little nutrient input from the surface and microbiota often depends on nutrients that were present since soil formation or sedimentation, resulting in a much lower microbial biomass and activity than in surface soils. Flux, distribution, and availability of microbial nutrients are controlled by factors like: climate, or the amount of entering water and the quality of the nutrients input, which is poor in subsurface environments; soil organic and inorganic chemical properties, providing alternative sources of nutrients; and physical properties, like porosity, saturation degree, and hydraulic conductivity [36]. Microbial activity correlates positively to clay content and negatively with sand content because of the protective effect of clays on enzymes [24]. Soil fractionation studies also showed that microbial biomass is strongly associated with clays [41].

Studies with sediments showed that bacteria are preferentially located in soil micropores, within micro-aggregates themselves, only 4–10% of pore space in aggregates being colonised [38]. These populations are not similarly exposed to contaminants or nutrients as those living in macropores; on the other hand, they are less subjected to drastic changes in water availability leading to alternation of desiccation and wetting. Thus, their survival and the intensity of their metabolic degradation activity depend less on their ability to adapt rapidly [39,40]. However, microbial occupation of residual soils is still unknown.

Because of weathering, the residual soil in this study presents certain homogeneity in its properties, in spite of the presence of two different facies. Therefore, the undisturbed soil blocks used in the experiment can then be considered as representative of the natural environment.

As indicated in Tables 1 and 3, both facies of the soil have a low cation exchange capacity, a low colloidal activity and low organic matter content. Therefore, a fairly little physical–chemical interaction (e.g. sorption) between the soil and the contaminants considered in this work is expected. Residual gasoline saturation seems to be much lower in soils with residual water saturation; little or no interaction due to contact is left for gasoline because of residual water being in direct contact with the surface of soil particles; gasoline remains as a continuous phase forming thin films on the water-wet soil grains, films that drain very easily. In soils with no significant organic matter content, such as the one in this study, residual water and gasoline saturations are lower than in soils with higher organic matter contents [42]. These are the same soils that present less favorable conditions for bacterial growth [40]. In this experiment, chemical analysis showed a great variation in residual gasoline remaining in the soil blocks after contamination, probably because of differences in the structure and properties of soil blocks. That is to be expected when studying undisturbed soil lysimeters.

Results showed that the addition of pure gasoline to soil, even in great amounts, did not cause enough changes in its conductivity and dielectric permitivity to allow for gasoline detection by GPR, at least in the frequency used in this research. Conversely, gasoline amended with 22% ethanol caused much more changes in soil electrical properties, probably due to the difference between ethanol and gasoline dielectric constant (24.3 for ethanol, between 2.0 and 2.6 for BTEX); these differences showed in GPR results and soil dielectric constant (K) values. As ethanol has a higher dielectric constant than gasoline components and most geological materials, this characteristic should allow it to be detected in the subsurface. However, its kinetic behavior in the subsurface, high volatility and degradability may not allow differentiating it precisely and for a long time span.

All blocks, independently of contamination and bioventing, showed similar shifts in their electro-magnetic properties. Changes observed in GPR radargrams and dielectric constants of all blocks 30 and 100 days after contamination reflect variations in soil conductivity that may be the result of a water content increase and/or bacterial higher metabolic activity in soil. Highly active culturable and non-culturable bacteria probably present in contaminated soils, and greater culturable populations observed in non-contaminated soils might be responsible for the formation of acid residues that increased soil conductivity.

However, higher culturable bacterial populations cannot be accountable for increases in K values in this experiment. Data from day 30 show a decrease in culturable populations while K values increased, in all contaminated soils (Table 5). One-hundred days after contamination, populations were higher in the ventilated gasoline–ethanol contaminated soil than in the non-ventilated one; still, K values kept increasing in both blocks. Water seems to be the major factor in GPR signal attenuation, even in the small amounts added to maintain soil natural moisture. Interaction with soil clay-mineral salts may have increased conductivity and dielectric permitivity significantly enough to alter the velocity and the power of the reflected signal, thus overshadowing the influence of microbial activity on conductivity, even in soils with higher populations [43–45]. Preliminary results with the same undisturbed soil in saturated conditions suggest that mostly water is accountable for the signal attenuation observed in the GPR signal (unpublished data).

It is also visible in radargrams and K values that these 30- and 100-day variations are smaller in ventilated soils. That difference may be attributed to the treatment applied, which tends to decrease soil conductivity because of the drying effects of bioventing.

Despite the fact that soil microbiota generally possesses the necessary enzymes for BTEX degradation, an adaptation phase is necessary for those populations to adjust to the presence of contaminants and start their degradation. Ethanol, on the other hand, is constitutively degraded by microorganisms as it enters living organisms basal biochemical cycles. However, those compounds may be toxic to soil microorganisms when in high concentrations and a certain amount of time may be necessary for the microbiota to recover. When exposed to a contaminant, indigenous

microbiota may even enter and remain in a dormant state; another strategy is to maintain its metabolic rates in detriment of cellular multiplication [46]. Those strategies turn microbiota into non-culturable. These populations can show their reaction potential when adequately stimulated or when the toxicity of the medium decreases [22], and this response may be a valuable assessment tool.

Amounts of gasoline components decreased in soils of all blocks during the experiment. The ventilated soil with gasoline and ethanol presented the most important decrease (>98%), while in the non-ventilated one the reduction in contaminants quantities was the smallest (74%). Soils with gasoline presented an intermediate reduction in contamination, the ventilated soil attaining a slightly smaller amount in 60 days than the non-ventilated one (respectively, 4.6 and 7.3% remaining in soil). Consequently, bioventing seemed to have a more important effect in ethanol-containing soils than in gasoline-containing ones. Higher amounts of gasoline components in gasoline–ethanol contaminated soils in the first 2 weeks after contamination suggest a slower degradation of those compounds in the presence of ethanol. From the 3rd week on, this difference is no longer visible because of the important reduction of contamination in all soils. Also, ethanol being more volatile and more rapidly degraded, it may possibly be no longer present.

This study showed that culturable bacterial populations decreased significantly when great amounts of gasoline and gasoline–ethanol mixture are added to the soil. Also, it was visible that their recovery in gasoline–ethanol contaminated soils took place much later than in gasoline-contaminated ones, which suggests that soil toxicity lasts longer when ethanol is present. As shown by Corseuil et al. [13], ethanol leads to a depletion of electron acceptors in saturated environments; oxygen-limited conditions then predominate in ethanol-contaminated aquifers, delaying and even preventing BTEX degradation [47]; therefore, toxicity due to contamination lasts longer than in gasoline-contaminated environments. However, although culturable bacterial counts suggest a similar situation in our study conditions, chemical analyses do not allow for a conclusion about preferential degradation of ethanol leading to a longer persistence of BTEX in unsaturated soil.

Once culturable bacteria resumed growth, results do not show a significant difference between populations of ventilated and non-ventilated gasoline-contaminated soils. However, this difference is visible in gasoline–ethanol contaminated soils, even before the recovery of culturable populations. Microbiological data correlate with the decrease in gasoline amounts but not with absolute numbers of contaminant residue in soil. If electron acceptors depletion caused by ethanol degradation occurs in unsaturated soil as it does in saturated ones, aeration may have allowed for BTEX degradation to persist. CG results show that microbial recovery in gasoline-contaminated soils occurs when gasoline compounds in soils drop below 1% (after day 40); as for gasoline–ethanol contaminated soils, this recovery occurs only when contaminants amounts are no longer detected by the adopted methodology (68 days after contamination). Still, no differences are visible between gasoline and gasoline–ethanol contaminated blocks relating microbial recovery to gasoline amounts in soils.

Those more active/numerous populations would be expected to increase soil conductivity because of a higher production of metabolic products. However, GPR results showed a decrease in conductivity in ventilated soils, even in gasoline-contaminated ones where no differences in culturable bacterial numbers were found. Even though degrading activities from soil microbiota may alter GPR measurements, it seems that increasing soil water content prevented the detection of that activity in our experimental conditions. Therefore, further investigation with GPR and contaminated soils is necessary to clear all ambiguity.

Because of their dimensions and morphological simplicity, microorganisms are very difficult to study; that is why culturing has been used to examine them. This approach led to the present situation where a few strains, generally of medical interest, have already been extensively studied while the vast majority of them, mostly of environmental origin, remains unknown. Most environmental microorganisms are unable to grow on culture media; according to data found in literature, unculturable organisms would represent between 90 and 99.9% of total populations [48]. Present attempts to describe and understand microbial diversity aim to overcome culture shortcomings in order to obtain a more accurate image of what really occurs in the environment. Molecular biology studies revealed that the diversity of soil microorganisms reaches far beyond what can be analysed or visualised; it is important to determine now how important this diversity really is for the functioning and stability of ecosystems and the recovery of contaminated environments. Molecular approaches will hopefully integrate this recently acquired knowledge to that of soil processes studies, to obtain a better understanding of microbial diversity and its functional importance in ecosystems [49].

In this experiment, only culturable bacteria have been assessed so far, but unculturable populations must be considered. Contaminant-induced selection probably prevented culturable bacterial populations to expand while allowing the survival of highly specialised organisms, which would very unlikely, be able to grow on culture media. As degradation occurs and contaminants amounts decrease, soil toxicity also declines. More flexible bacterial populations, less specialised, found space to grow and compete; these strains are much more likely to grow in laboratory conditions, which explains the resuming of growth of culturable bacteria 40 and 70 days after contamination. As observed by da Silva and Alvarez [50], fewer BTEX degrading species were found in saturated soil contaminated with BTEX and ethanol than in BTEX-containing soils. Duarte et al. [51] showed an important shift in non-culturable bacteria in oil-contaminated soil. In the next steps of the present study, molecular tools combined with culture-dependent approaches may allow a

better understanding of total bacterial populations shifts due to contamination, bioventing and contaminants degradation, and a better evaluation of microbiota's capacity to return to its original state and profile [52].

5. Conclusions

The results presented here showed the possibility of evaluating contamination impact as well as bioventing stimulation of soil microbiota by counting culturable heterotrophic bacterial populations. Bacterial counts showed a delay in soil populations reaction to contamination in gasoline–ethanol contaminated soil when compared to gasoline-contaminated ones, which indicates a possible preferential degradation of ethanol on BTEX. However, additional and more accurate chemical analyses are necessary to better evaluate contaminant residue in soil and its effect on culturable bacterial populations.

Bioventing appears as an interesting tool in regenerating gasoline–ethanol contaminated soils, as it seems to accelerate soil detoxification and microbiota recovery. However, our study showed a lesser effect in gasoline-contaminated soil.

Despite the fact that contaminant presence and degradation in soil was not detectable by GPR in the conditions of this study, its use in conjunction with other assessment techniques may still be of great value and should be further evaluated for this particular use in contaminated soil. Additional studies with GPR are necessary to understand changes in radar signals after soil contamination; those studies may elucidate the issues of changes in contaminated soil conductivity.

Molecular biology studies will allow a better understanding of shifts in soil microbial populations, as they will show the molecular profile of both culturable and non-culturable populations.

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