Assessment of the Responsiveness to Different Stresses of the Microbial Community from Long-Term Hydrocarbon-Contaminated Soils

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Abstract Soils exposed to long-term contamination with hydrocarbons may present extreme challenges to maintain the biological resilience to the stress. To elucidate the relationships between the initial event of contamination and the responsiveness to the stress, we investigated the extent of the microbial resilience of biological functions from two contaminated soils sampled from a petrochemical area (S1, underwent diffuse hydrocarbon contamination, and S2, from a land farming unit where an alkaline petrochemical sludge was treated) after the Cd, saline, and acid stresses. Both contaminated soils were characterized by low organic matter content compared with a pristine soil. Although similar Shannon diversity index and heterotrophic bacterial count were observed, different bacterial community structures (PCR-DGGE) and less enzymatic activities characterized the contaminated soils. Particularly, functional diversity determined by Biolog EcoPlates™ was not detected in S2 soil. Only the S1 soil showed resilience of the enzymatic activities and functional diversity, suggesting the presence of a well-adapted microbial community able to face with the stresses.

The S2 was the most disturbed and less responsive soil. However, an increase in the functional diversity was evidenced after acidification, and it is possible to correlate this responsiveness with the sludge properties treated in the land farming unit. In addition, if the selected stress can reverse the soil condition provoked for the first disturbance, responsiveness could be expected.

Keywords Hydrocarbons · Soil resilience · Stress · Microbial activity

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1 Introduction

Different bacterial species that inhabit the soil environment can form a robust community and provide the soil with the capacity of performing a range of ecological functions. An important benefit of biological diversity to soil functioning may be to provide greater resistance to stress and disturbance. The microbial communities have an inherent capacity to resist stresses (resistance) and subsequently to recover (resilience). Resistance and recovery measurements are usually used to characterize the response of microbial activity when evaluating soil functional stability (Griffiths et al. 2001).

Soils exposed to long-term contamination with hydrocarbons may present extreme challenges to maintenance of diverse structural and functional microbial communities. Vivas et al. (2008) analyzed soils historically contaminated by different levels of heavy metals and hydrocarbons and observed that the pollution had caused a genetic and metabolic alteration in soil

microbial communities. Also, Thavamani et al. (2012) found that the enzymatic activities were severely inhibited in soils that were contaminated with both polycyclic aromatic hydrocarbons (PAHs) and heavy metals, together with a reduction in the microbial population diversity. A reduced metabolic diversity in highly hydrocarbon-contaminated soils was observed by Shi et al. (2005).

Although in situ bioremediation using indigenous microorganisms is by far the most widely used technique for decontamination of polluted sites (Pandey et al. 2009), such treatment generates a soil with a new microbial community (Viñas et al. 2005; Del Panno et al. 2005) where the capacity of the resulting biological community to perform a range of ecological functions is particularly important.

How the newly selected community established in the contaminated soil could respond to environmental stresses still remains not well understood. These aspects could be crucial to the successful development and use of bioremediation technologies.

Degens et al. (2001), in their study about the influence of soil catabolic diversity on the resistance to stress or disturbance, demonstrated that a decline in microbial diversity may reduce the soil communities' resilience toward other natural or man-made perturbations.

Monitoring the extension in which the soils have been affected can be achieved following different approaches. In several cases, variation in microbial diversity, community structure, and activity could function as a predictor of change in soil health (Nielsen et al. 2002).

In this context, we hypothesized that the soil microbial community from a hydrocarbon-contaminated soil has a particular responsiveness to stresses depending of its contamination history and the stressor applied. That is, the contaminated soils with reduced microbial functional diversity could show resilience to determined stresses. We propose to investigate the extent of the indigenous microbial resilience of biological functions from contaminated soil after different stresses, with the aim to find a relationship between the initial event of contamination and the responsiveness to the stress.

To test the hypothesis, we analyzed the functional response to stresses of the soil microbial community from two sites from a petrochemical industry in La Plata-Ensenada petrochemical polo.

Considering the origin of soils, accidents like spills from tanks, leaking pipelines, and overturning from stored sludges could happen impacting on the soil.

Thus, changes in pH, salinity, and heavy metal contamination were chosen as representative stresses and possible to occur into the petrochemical area.

To evaluate the biological response, determination of enzymatic activities, functional microbial diversity, and heterotrophic bacterial count were performed, before and after applying stress treatments to the soil microcosms.

2 Experimental Procedures

2.1 Soil Collection and Characterization

Two sites representing hydrocarbon-contaminated soils, S1 and S2, placed within a petrochemical industry belonging to La Plata-Ensenada petrochemical polo were selected. Soil samples from S1 site were taken from the field surrounding the petrochemical industry. This field was a uniform grass-covered area that underwent a diffuse hydrocarbon contamination.

Due to the industrial activity, the long-turn production of petrochemical sludges led to their accumulation and storage inside the facility handling them. The sludge was characterized by a high concentration of polycyclic aromatic hydrocarbons (50 g kg^{-1}) and alkaline pH (10.41). "Land farming" was the bioremediation process applied by the generator to reduce the hydrocarbon content from the sludge. Briefly, the sludge was incorporated into soil surface on a confined area and it was periodically tilled to aerate the mixture. Finally, after 2 years, the process was over, reducing the initial soil hydrocarbon concentration to an acceptable value to allow handling of the material within the petrochemical area. This material was selected and sampled as the S2 soil.

A third site, out of the petrochemical area influence, was selected as a site from it was taken reference information (baseline). The soil from this area was under long-term grass pasture; it was named P soil. In all the cases, composite soil samples were collected from the top soil, up to 20 cm.

Soil samples were placed in sealed plastic bags, placed in a cooled box, transported to the analytical laboratories unsieved, and kept at field moisture to preserve the microbial community. At the laboratory, the soils were sieved (2 mm) and the biological analyzes were done during the first 48 h. The sieved soils were

stored at 4 °C until the physicochemical analyses were finished.

Chemical and physical analyses were performed according to standard techniques (Sparks 1996). Heavy metals were determined by atomic adsorption spectroscopy (AAS) after acid digestion with H_2SO_4/HNO_3. Hydrocarbon concentration in soil samples was quantified as total GC-available hydrocarbons (GCAHC) (Del Panno et al. 2005).

The microbial community structure of S1, S2, and P soils were determined by PCR denaturing gradient gel electrophoresis (DGGE) (Ibarrolaza et al. 2009). The amounts of PCR products loaded in the DGGE gels were standardized by the Qubit® 2.0 fluorometer. The principal component analysis (PCA) was done taking into account the presence/absence of the bands from the gel to make a comparative analysis of the community's structure from the soils. Additionally, DGGE banding data were used to estimate the Shannon index of general diversity ($H' = -\Sigma \ (n_i/N) \ \ln \ (n_i/N)$). For this analysis, each band was treated as an individual operational taxonomic unit (OTU). The relative surface intensity of each band, expressed as peak height in the densitometric curve (n_i), and the sum of all peak heights in the curve of a given sample (N) were used as estimates of species abundance (Fromin et al. 2002).

Viable bacterial counts were determined in R2A-agar medium (Ibarrolaza et al. 2009). The agar plates were incubated at 24±2 °C for 10 days. The most probable number (MPN) of aromatic and aliphatic hydrocarbon-degrading bacteria was determined in 96-well microtiter plates (Wrenn and Venosa 1996), using mineral salts medium (LMM) (Vecchioli et al. 1990). The inoculated microtiter plates were incubated at 24±2 °C for 21 days. Fungi were enumerated on Cooke Rose Bengal agar (RB) supplemented with streptomycin (30 mg 100 ml^{-1}) in order to inhibit the bacteria growth. The agar plates were incubated at 24±2 °C for 7 days.

2.2 Stress Treatments and Evaluation

Three stress treatments were imposed on each of the three soil samples and consisted of either increase in soil Cd concentration, increase in electrical conductivity (EC), or decline in pH, in two levels of each stress treatment.

The samples from each soil were divided into 21 soil microcosms, each one equivalent to 100 g dry soil. The treatments were carried out on three replicate microcosms of 100 g dry soil, and three replicates were reserved without any treatment and used as control microcosms.

The following stress conditions were selected with the aim to produced significant effect on the soil microbial community from each soil studied. For Cd stress, the soil microcosms were treated with $CdSO_4$ at 140 and 400 mg Cd kg^{-1} dry soil.

The saline stress was provoked by the addition of 21.8 and 77.9 meq NaCl kg^{-1} dry soil.

For pH stress, the soil microcosms were adjusted to pH 4.5 or pH 5.5 with dilute HCl (1 mM).

Following imposition of the treatments, all soil microcosms were incubated at 24±2 °C and 70 % water holding capacity (WHC) for 2 weeks. This period of time was chosen to allow sufficient equilibration of the microbial communities after imposition of each stress treatment and for decomposition of organic C released from microorganisms killed by the treatments.

At the end of the incubation period, the Cd concentration was determined following the extraction protocol developed by Tessier et al. (1979) and modified by Smeda and Zyrnicki (2002). The following Cd fractions were studied: water-soluble forms removed by water (deionized); acid-soluble forms associated with carbonates; reducible forms associated with oxides and hydroxides of Al, Mn, and Fe; and oxidizable forms associated with organic matter. The Cd fractions were named F1, F2, F3, and F4, respectively. The concentrations of metals in the extracts were measured by atomic emission spectrometry using a Shimadzu 6650 AA spectrometer.

In order to evaluate the impact of the stresses on the microbial population, the bacteria heterotrophic counts in R2 agar were carried out before and 2 weeks after applying stresses.

Before and after 2 weeks from the stress treatment, the activities of alkaline (pH 11) and acid (pH 6.5) phosphatases (Verchot and Borelli 2005), lipase activity (Margesin et al. 2002), and urease activity (Kandeler and Gerber 1988) were determined.

Community-level physiological profiles (CLPPs) were assessed by the Biolog EcoPlate™ system (Biolog Inc., CA, USA), before and after 2 weeks from the stress treatment. Each 96-well plate consisted of three replicates, each one comprising 31 sole carbon sources and a water blank (Insam 1997). The plates were incubated at 24±2 °C, and the oxidation of 31 sole carbon sources was analyzed daily by measuring absorbance (reduction of the tetrazolium dye) at 590 nm using

an automatic plate reader, for 10 days. The multivariate dataset was subjected to PCA. The average well color development (AWCD=Σ ODi/31) was calculated from all of the net absorbance, where ODi is the optical density value from each well.

2.2.1 Statistical Analyses

All the data were the mean of three replicates. The significance of differences among mean values was determined using one-way analysis of variance (ANOVA) and Tukey's posttest. PCA was carried out using the XLSTAT software.

3 Results

Selected physical and chemical parameters of the loam soils analyzed are shown in Table 1. Similar aliphatic hydrocarbon concentrations were detected in S1 and S2 soil samples, but the highest PAH concentration was detected in S2 site. Determination of the metals Ni, Cu, Cr, Zn, and Cd was carried out obtaining values below or close to the limit of detection method (1 mg kg^{-1}) (data not shown). That implied that the industrial contamination mainly consisted of organic pollutants.

The enzymatic activities of lipase, urease, alkaline and acid phosphatases, and microbial counts determined from soils are shown in Table 1. S1 soil samples showed urease, lipase and phosphatase acid, and alkaline activity levels lower than P samples. With the exception of lipase activity, all the enzymatic activities were significantly lower in S2 than those in S1 soil samples. The heterotrophic bacterial density ranged from 10^7 to 10^8 CFU g^{-1} dry soil in all soil samples. In agreement with the highest PAH concentration, the highest values of PAH-degrading bacterial density were detected in S2 soil microcosm.

Although we have measured the DNA concentration from each sample to add equivalent PCR amplicon concentration into the wells, different intensity among the lines could be appreciated, included among the replicated sample of each soil (Fig. 1). Especially, that result revealed the contaminated soils heterogeneity. For these reasons, we considered applying the PCA, taking into account the presence/absence of the bands to make a comparative analysis of the soil community's structure. Additionally, we used the DGGE banding data to

estimate the Shannon index of general diversity ($H'=-\Sigma$ (n_i/N) ln (n_i/N)). The H′ indices were 3.38±0.12 (P); 3.25±0.23 (S1), and 3.06±0.31 (S2), indicating a similar diversity level among the samples sampled from contaminated soils and the pristine soil. The result of PCA analysis of DGGE bacterial banding patterns for the three soils is shown in Fig. 1.

The PC1 and PC2 accounted for 24.37 and 18.78 %, respectively, of the total variance. The three soils showed different bacterial community structure. The S1 and P soil microcosms were separated on the PC1 axis from S2 soil microcosm. This relative position on the PCA coordinates show that S1 and P shared more OTUs among them than with S2. The large statistical deviation obtained for S2 soil microcosms could be attributed to the variability of DNA among the replicates. However, it was clearly located toward the left on the PC1 axis, differing significantly from S1 soil microcosms.

The CLPP was tested to assess the potential metabolic diversity of microbial communities from the soils; in addition, the AWCD was also calculated. The AWCD values for S1 soil microcosms increased rapidly with the incubation, as it was observed in P soil microcosms, reaching peak color development in approximately 200 h. The AWCD for S2 soil microcosms did not exceed the minimum value detected at the start of incubation. With the aim to analyze comparatively the functional diversity among the sites, a PCA with the CLPP data were done (Fig. 2). The statistical analysis of the dataset indicated that the first two principal components accounted for 60 % of the total variability. The S2 soil samples were principally separated from the S1 soil samples on the PC1. The S1 soil samples were separated from P ones on the PC2 axis, corresponding to a relatively lower assimilation capacity to itaconic and α-cyclodextrin.

3.1 Stress Treatments

The responses from S1, S2, and P soil samples to the three different stress treatments (Cd contamination, salinity increase, and pH decrease) were determined in soil microcosms. Table 2 shows the values of Cd (from each fraction obtained by the sequential extraction: F1, F2, F3, and F4); EC and pH were determined in each microcosm, 15 days after applying the stresses.

As it could be observed in Table 2, the Cd distribution between the different fractions was different

Table 1 Physical, chemical, and biological properties of soils sampled from the hydrocarbon-contaminated (S1 and S2) and pristine (P) sites

Soil	P	S1	S2
Coordinates	34° 51′ 59″ S, 57°55′ 20″ W	34° 53′ 27″ S, 57° 55′ 42″ W	34° 53′ 19″ S, 57° 55′ 38″ W
Soil texture	Loam	Loam	Loam
pH	6.23 (0.08)[a]	7.34 (0.21)	7.71 (0.27)
WHC (%)	43.71	35.22	27.45
Carbon (%)	9.51 (2.44)	2.41 (0.30)	2.20 (0.88)
Organic matter (%)	16.35	4.15	3.78
Nitrogen (%)	2.69 (1.53)	0.22 (0.03)	0.20 (0.12)
Phosphorus (mg kg^{-1})	30.33 (3.04)	3.67 (0.58)	8.33 (0.63)
C/N	3.54	10.95	11
CEC (cmolc kg^{-1})	41.70 (2.07)	30.10 (2.67)	26.57 (1.27)
EC (mS cm^{-1})	2.66 (0.35)	0.31 (0.04)	2.72 (0.30)
Aliphatic hydrocarbons (AHs) (mg kg^{-1})	<1	189.72 (22.57)	194.77 (5.18)
Polycyclic aromatic hydrocarbons (PAHs) (mg kg^{-1})	<0.2	61.52 (12.51)	278.97 (20.00)
Enzymatic activity			
Lipase (µg pNP g^{-1})	613.41 (82.16) a	436.42 (42.56) b	559.15 (134.14) a,b
Urease (µg N–NH$_4^+$ g^{-1})	234.84 (17.20) a	104.98 (5.84) b	71.29 (13.35) c
Alkaline phosphatase (µg pNP g^{-1})	3871.15 (87.60) a	2086.49 (71.24) b	195.90 (22.52) c
Acid phosphatase (µg pNP g^{-1})	6072.33 (413.25) a	1364.42 (74.84) b	116.41 (50.99) c
Microbial population density			
Heterotrophic bacteria (log CFU g^{-1})	8.31 (0.07) a	7.16 (0.01) c	7.96 (0.14) b
Aliphatic hydrocarbon-degrading bacteria (AH) (log MPN g^{-1})	5.01 (0.19) a	5.141 (0.21) a	5.29 (0.17) a
Polycyclic aromatic hydrocarbon-degrading bacteria (PAH) (log MPN g^{-1})	3.90 (0.32) b	3.47 (0.12) b	5.59 (0.12) b
Fungi (log CFU g^{-1})	5.00 (0.01) a	3.88 (0.09) c	4.93 (0.05) b

Values with the same letter are not significantly different between treatments ($P < 0.05$)

WHC water holding capacity, *EC* electrical conductivity, *CEC* cation exchange capacity

[a] Values between parentheses are standard deviations

compared with the different soil microcosms. The S1 and P soil microcosms showed the highest Cd percentage in F3, after both Cd stresses were applied. A 54.11 % of Cd was determined in F2 from S2 soil microcosms, after the lowest Cd stress (140 mg kg^{-1}) and was mainly distributed between F2 and F3 after the highest Cd stress (400 mg kg^{-1}).

Fifteen days after the saline stress, different final conductivities were obtained in the microcosms, getting the highest EC in S2 soil microcosm.

Fifteen days after the acid stress, an increase in the pH values was observed in the contaminated soil microcosms, in comparison to those adjusted at the beginning of the acid stress experiment, pH 5 and pH 4.

Clearly, the original physicochemical properties of each soil microcosm have conditioned the Cd distribution and the final values of EC and pH.

3.2 Effect of Stresses on Microbial Activity and Heterotrophic Bacterial Count

To analyze the stress effects, each enzyme activity value and bacterial heterotrophic count was expressed as the ratio of the value after the stress to the value before the stress. Figure 3 shows the rate values of lipase, urease, phosphatase activities, and heterotrophic bacterial counts from hydrocarbon-contaminated and the pristine soil microcosms.
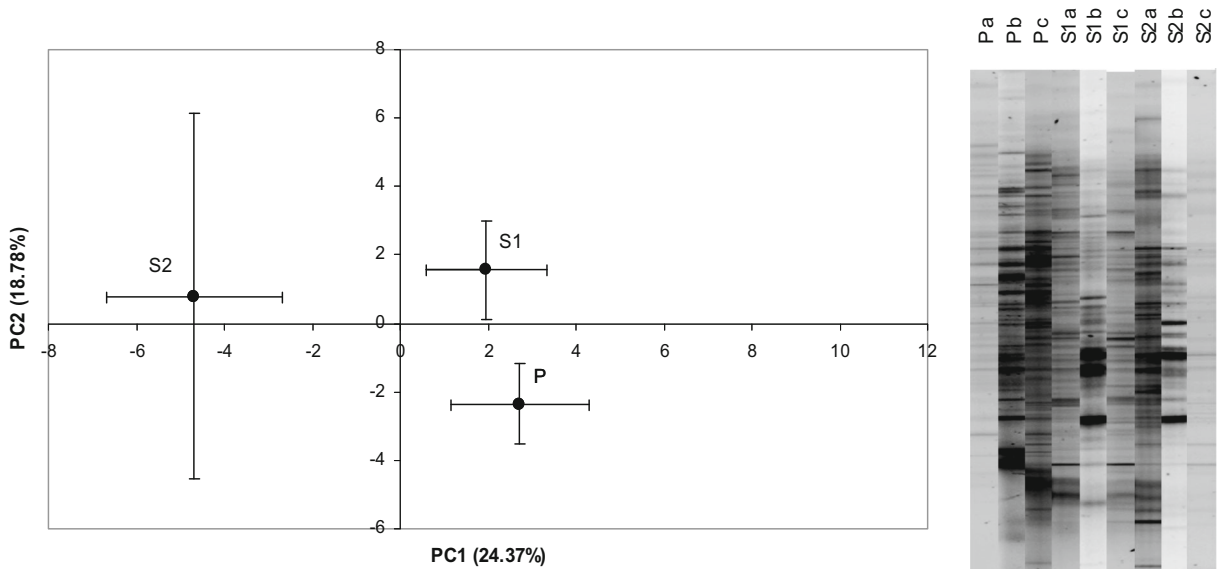
Fig. 1 Principal component analysis of soil microcosms P, S1, and S2 based on PCR-amplified 16S rDNA DGGE community fingerprint profiles. Data represent the average obtained from three replicates of each soil microcosm. *Bars* are the standard deviations along both axes

3.2.1 Cd Stress

The effect of the Cd addition in S1, S2, and P soil microcosms is shown in Fig. 3a. The lipase activity was significantly diminished in S2 soil microcosm after Cd addition, and the alkaline phosphatase activity was the only enzyme activity significantly diminished in the hydrocarbon-contaminated soils after Cd addition in both levels. The pristine soil microcosm shows resilience of all enzymatic activities. Resilience of bacterial

heterotrophic count was observed from the contaminated and pristine soil microcosms.

3.2.2 Saline Stress

The saline stress effect in S1, S2, and P soil microcosms are shown in Fig. 3b. Resilience of all enzymatic activities and heterotrophic bacterial count was detected in hydrocarbon-contaminated soil microcosms. The higher saline stress produced a relative increase in the lipase
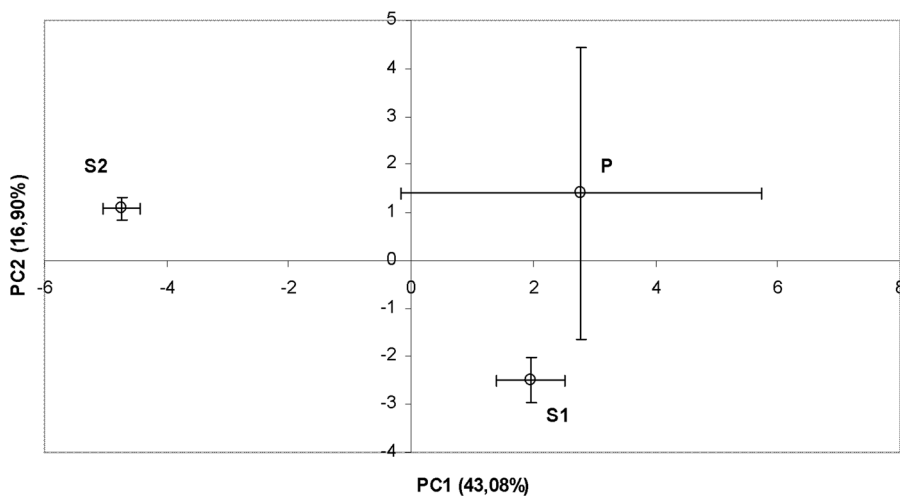
Fig. 2 Principal component analysis of CLPP data after 256-h incubation in Biolog EcoPlate™ from the soil microcosms P, S1, and S2. Data represent the average obtained from three replicates of each soil microcosm. *Bars* are the standard deviations along both axes

Table 2 Values of available Cd(II), electrical conductivity, and pH determined in P, S1, and S2 soils microcosms before (control treatment) and after 15 days from the stresses, in the two levels of treatment

Treatments		Soils		
		P	S1	S2
	Fraction[a]	Cd (percent value respect total Cd extracted)		
	F1	1.83 (0.16)[b]	No detection	No detection
After Cd stress 140 mg kg^{-1} soil	F2	12.05 (0.82)	22.31 (0.45)	54.11 (9.67)
	F3	65.40 (3.24)	73.11 (4.08)	23.57 (6.72)
	F4	20.73 (2.86)	4.91 (3.26)	22.31 (2.95)
After Cd stress 400 mg kg^{-1} soil	F1	0.91 (0.03)	0.30 (0.01)	No detection
	F2	17.80 (5.19)	25.71 (2.75)	41.47 (1.69)
	F3	67.72 (3.22)	61.77 (2.00)	45.37 (1.02)
	F4	15.57 (2.00)	12.21 (0.89)	13.15 (1.10)
		Electrical conductivity (mS cm^{-1})		
Control		2.70 (0.35)	0.31 (0.09)	2.75 (1.22)
After salinity stress 21.8 meq NaCl kg^{-1} soil		3.27 (0.14)	1.45 (0.28)	5.10 (1.51)
After salinity stress 77.9 meq NaCl kg^{-1} soil		5.02 (0.60)	4.44 (0.73)	10.24 (1.53)
		pH		
Control		6.23 (0.29)	7.43 (0.05)	7.84 (0.06)
After acid stresses pH 5		4.89 (0.08)	6.16 (0.02)	6.33 (0.15)
After acid stresses pH 4		4.52 (0.03)	5.14 (0.05)	5.38 (0.18)

The value of Cd was determined in each fraction obtained by the application of sequential extraction protocol, and was expressed as percentage of the total Cd extracted

[a] Cd fractions: F1, water-soluble forms removed by water; F2, acid-soluble forms associated with carbonates; F3, reducible forms associated with oxides and hydroxides of Al, Mn, and Fe; and F4, oxidizable forms associated with organic matter

[b] Values between parentheses are standard deviations ($P<0.05$)

activity from the P soil microcosm, and resilience of the rest of enzyme and heterotrophic count were also observed. In comparison with the other soil microcosms, an increase in acid phosphatase was observed in most contaminated soil microcosms after the higher saline stress.

3.2.3 Acid Stress

The acidification effect is shown in Fig. 3c. Resilience of lipase, urease, and acid phosphatase activity was observed in S1 soil microcosm. Only resilience of acid phosphatase was observed in S2 soil microcosms. The acidification produced a significant decrease of lipase activity and an increase in urease activity in S2 soil microcosm. The alkaline phosphatase was significantly diminished in both hydrocarbon-contaminated soil microcosms after the two acidification levels. Resilience

was observed in P soil microcosm for all enzymatic activities after soil acidification in the two levels.

The acid stress was the only stress that provoked a significant effect on the soil heterotrophic bacterial population of all soil microcosms. A significant decrease was observed in S1 and P soil microcosms only after pH 4 ($7.0.10^6$ and $8.4.10^7$ CFU/g dry soil, respectively). Both levels of acid stress produced significant decrease on bacterial population in S2 soil microcosm ($2.3.10^5$ and $4.5.10^6$ CFU/g dry soil after pH 5 and pH 4, respectively).

3.3 Effect of Stresses on Community-Level Physiological Profiling

The CLPP was tested to assess the potential metabolic diversity of soil microbial communities after the stress imposition.
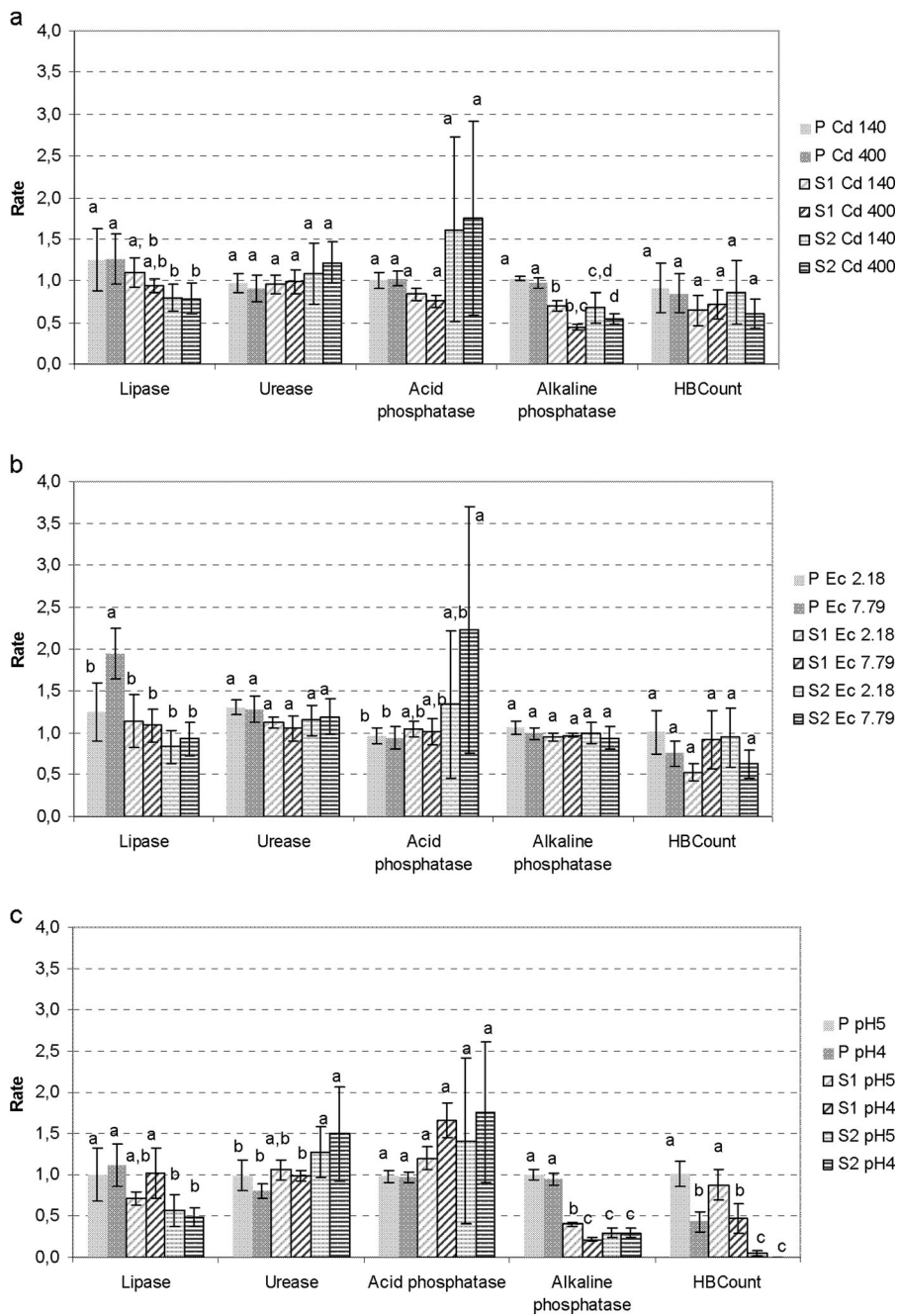
Fig. 3 Ratio of the enzyme activity values and bacterial hetero-trophic counts after the stress to their respective values before the stress. The rates obtained from each variable for the three soils in the two stress levels were statistically analyzed. Each variable was separately analyzed; *different letters on the bars* indicate significant differences between the soil microcosms for each variable. **a** After Cd contamination; **b** after salinity stress, and **c** after acid stress

No significant changes were observed after the Cd and saline stresses in S1 and P soil microcosms (Fig 4a, b, respectively).

As well as was observed in enzymatic activity, the acidification produced the most significant changes on the metabolic diversity in the most contaminated soil.

Figure 4c shows the PCA obtained by CLPP data for the soil microcosms before and after acid stresses were applied. The two first principal components accounted for 43.48 % of the total variability. No significant changes were observed in S1 and P soil microcosm after acid stress. Instead, a significant shift to the positive PC1 axis was observed in S2 soil microcosm after the acid stress in the two levels.

4 Discussion

There are no many studies about the resilience to stresses from hydrocarbon-contaminated soils (Griffiths and Philippot 2013). Studies of this type could give a comprehensive knowledge about the soil functionality of contaminated soils and postremediation treatment. In this work, we analyzed soil samples of two different sites inside a field of a petrochemical industry operating for 40 years; the S1 soil samples from a uniform grass-covered area underwent a diffuse hydrocarbon contamination while the S2 soil samples taken from a land farming unit, where an alkaline and highly PAH-contaminated sludge had been treated.

We discussed the responsiveness from the two contaminated soils, taken as reference the behavior of a pristine soil. The contaminated soils were characterized by low OM content and CEC value and less enzymatic activity compared with the pristine soil.

The greatest effect of hydrocarbon contamination was observed on S2 soil microcosm, where the highest reduction on the substrate assimilation capacity and enzymatic activities were detected. Shi et al. (2005) observed a significant reduction in the functional diversity by the CLPP analysis in highly contaminated soils.

The bacterial community structure was also different between the contaminated soils, in agreement with their previous history. As a routine practice in land farming treatment, the tillage could sharpen the shift in S2 microbial community structure. Steenwerth et al. (2005) observed that tillage caused a greater shift in microbial community structure after a dry/wet event, compared with grassland soil.

Although similar bacterial diversity and heterotrophic bacterial counts were observed among the soils, the lowest microbial activity was observed in S2 soil, suggesting that the residual S2 hydrocarbon concentration was too high to hold an active soil microbial community, even though the hydrocarbon value would indicate the end of land farming process.

At this point, we could appreciate two different sceneries from contaminated soils. One represented by the grassland soil underwent a diffuse hydrocarbon contamination (S1) with a relatively reduced microbial activity and the other one represented by the material sampled from the land farming unit (S2) with a significantly reduced microbial activity. Then, the stresses were the subsequent disturbances applied to these chronically contaminated soils.

Cadmium is considered to be the metal with the most adverse effects on microbial biomass and activity in heavy-metal-contaminated soils (Khan et al. 2007), as it is not required for any known biological process. Its behavior is related to the chemical associations that occur in soils. After 15 days of Cd addition, very low values of Cd were determined in F1 fraction, corresponding to water-soluble fraction extracted from the soils. It constitutes the most mobile and potentially the most available metal, but usually negligible (Chaignon et al. 2003). The S1 and P soil microcosms showed the highest Cd percentage in the F3 fraction.

However, the resilience in the extracellular enzyme activities and metabolic diversity were detected in both S1 and P soil microcosms.

Due to the large sorption capacity of the OM toward metals (Yin et al. 2002), it is possible to attribute this compound the resilience observed in P soil microcosm. This result was similar to those shown by Degens et al. (2001) from pasture soil microcosms under Cu stress. The authors attributed the catabolic resistance to high catabolic evenness and to the physicochemical properties of the soil, like organic matter, CEC, and biomass. Despite the unfavorable physicochemical properties of S1 soil microcosms, only the alkaline phosphatase was inhibited by the Cd addition. Also, resilience of the enzymatic activities after saline stress was similar in S1 and P soil microcosms, suggesting the presence of a well-adapted microbial community able to face with stresses.

The apparent resilience of the enzymatic activities in the most contaminated soil microcosm, S2, after Cd and saline stresses, could be the result of the lack of metabolic response of some enzymes due to the significantly reduced metabolic status before the stresses. However, the Cd addition reduced significantly the lipase and even
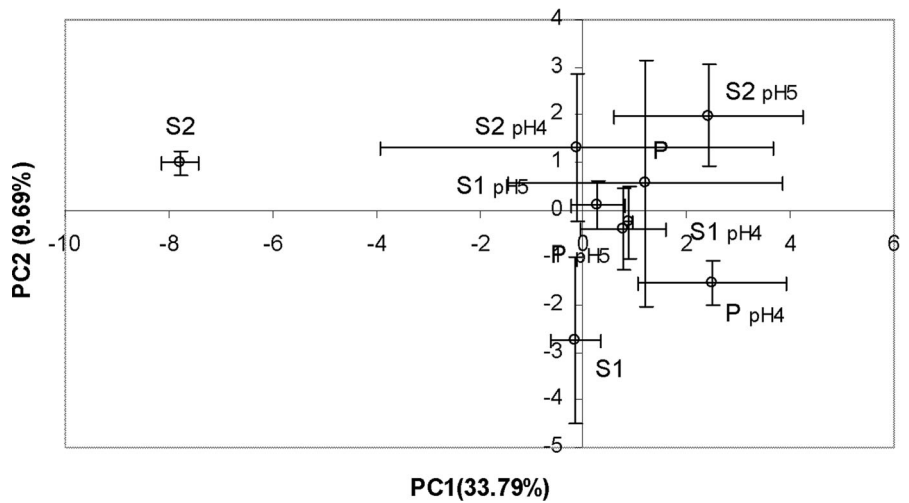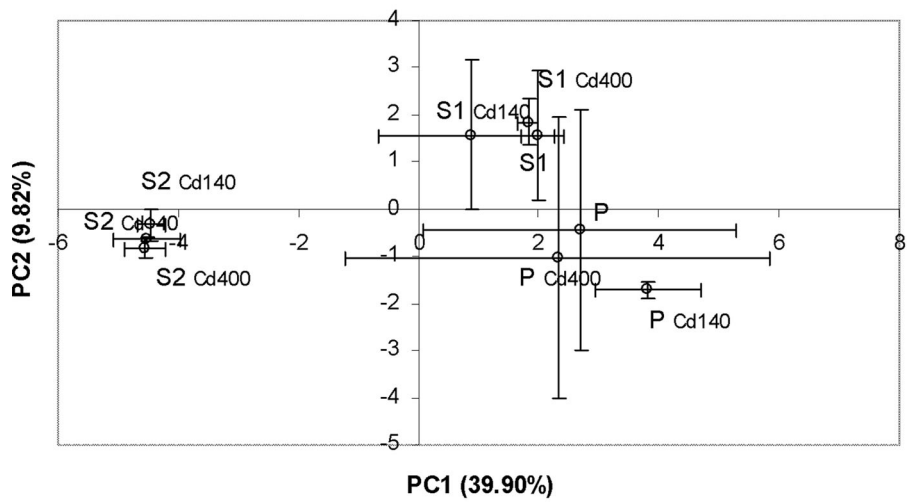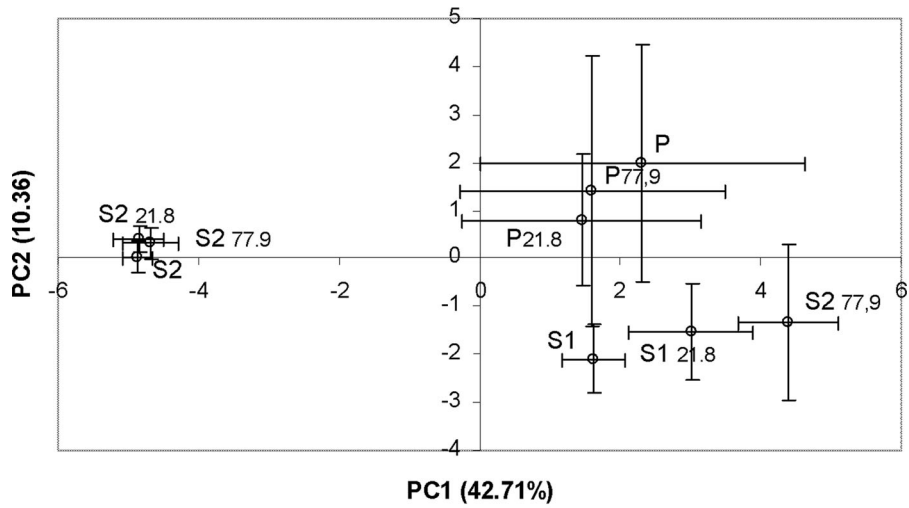
◀ **Fig. 4** Principal component analysis of CLPP data after 256-h incubation in Biolog EcoPlate™ from the soil microcosms P, S1, and S2 before and after 15 days of the **a** Cd contamination (140 and 400 mg kg$_{-1}$ dry soil), **b** salinity stresses (21.8 and 77.9 meq NaCl kg$_{-1}$ dry soil), and **c** acid stresses (PH 4 and pH 5). Data represent the average obtained from three replicates of each soil microcosm. *Bars* are the standard deviations along both axes

more the alkaline phosphatase in this microcosm. The enzymatic responsiveness of S2 soil microcosm would be consequence of the land farming process. The acute contamination plus the periodical tillage could produce the selection in a relatively short time of a specialized hydrocarbon degrader community but unable to respond to stresses. The exception was the increase of acid phosphatase produced by the highest saline stress.

In accordance with their original metabolic status, resilience was only observed in CLPP patterns from the less contaminated as well as from P soil microcosms. Several authors observed that the metal stresses did not necessary lead to a distinguishable CLPP pattern (Knight et al. 1997; Kelly et al. 1999). In this point, it is necessary to take into account that the CLPP determination shows the potential of only the culturable fraction of the microbial community to respond to substrates, while enzyme analyses reveal the status of the whole microbial community (Classen et al. 2003).

Similarly, resilience of bacterial heterotrophic counts was observed in pristine as well as in the hydrocarbon-contaminated soils after Cd and saline stresses, suggesting the presence of tolerant populations in the soils. Due to the low OM content in the contaminated soils, it is possible assume the presence of more tolerant bacterial populations in the contaminated soils than in the pristine one.

Although the significant reduction in bacterial heterotrophic counts after the lowest pH in S1 soil microcosm, resilience of enzymatic activity, with the exception of alkaline phosphatase, and resilience of functional diversity were observed at the two levels of acid pH. A similar behavior was observed in pristine soil microcosm. Our results suggest that the diffuse hydrocarbon contamination allowed a degrading microbial community be selected, preserving a relative functional stability based on their generalist catabolic capacity to face to the stresses.

The acid stress produced acute changes in S2 soil microcosm. In addition to the inhibition of lipase and alkaline phosphatase activities, the heterotrophic bacterial counts were significantly diminished after both

levels of acid stress. However, an increase in the functional diversity was evidenced. In this point, it is important to note the sludge properties treated by land farming which influenced the S2 soil properties. The sludge (RC90), extensively analyzed as previous condition to decide the remediation treatment (Morelli et al. 1995), revealed a high pH (10.41). We could suggest that during the land farming process, the alkaline sludge selected a degrading microbial community adapted to a pH higher than the original soil pH. When this soil (S2) was subjected to acid stress, the dead biomass resulted in an input of carbon source easily available for the surviving population. The recovery of functional diversity suggested that the surviving populations were potentially active under the relatively lower pH. Furthermore, the addition of acid certainly modified the interaction between the different compounds of the contaminated soil, modifying their bioavailability and consequently allowing the increase of the microbial activity. Therefore, the potential functional diversity of the metabolically inactive S2 soil could be restored by acidification. Tobor-Kaplon et al. (2006) suggested that the soil stability depends on the mechanisms of adaptation developed during the first disturbance and whether a subsequent disturbance is similar to the first in terms of the mechanisms with which the organisms deal with the disturbance. In this sense, it can be assumed that the surviving populations would have been present in a dormant state before the acidification was done and after acid stress become potentially active.

The more disturbances happen multiple factors influence the responsiveness to a subsequent disturbance, certainly reducing the possibility to predict any response. Griffiths and Philippot (2013), in their recently published review on resistance and resilience of the soil microbial community, suggested that there is no general response to disturbance because stability is particular to the disturbance and soil history. From our results, a hydrocarbon diffuse soil contamination, even in a soil with low organic matter content, could be quite stable to cope with Cd, saline, and acid stresses. Instead, it could be expected a particular responsiveness to stress from a bioremediated soil, in relation with the previous history (the sludge properties). In agreement with Griffiths and Philippot (2013), although having analyzed the responsiveness only through the biological resilience, our results reflected the relevance of the previous disturbance nature and the stress selected to

assess the resilience of the soils, especially in hydrocarbon-contaminated soils.

5 Conclusion

The previous pollution history is one determining factor of the soil responsiveness to stress. A hydrocarbon diffuse soil contamination could influence as little that it be quite stable to cope with the stress. Instead, the soil from a land farming unit represent a more disturbing situation resulting in a lack of response to some stresses. However, whether the stress can reverse the soil condition provoked for the first disturbance, a recovery of the soil activity could be expected. From our results, a controlled acidification could improve the functional diversity of the bioremediated soil. It could be interesting to include the assessment of responsiveness to diverse stresses to know the extent of the soil stability after a bioremediation treatment.

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