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A soil microscale study to reveal the heterogeneity of Hg(II) impact on indigenous bacteria by quantification of adapted phenotypes and analysis of community DNA fingerprints

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Abstract

The short term impact of 50 μM Hg(II) on soil bacterial community structure was evaluated in different microenvironments of a silt loam soil in order to determine the contribution of bacteria located in these microenvironments to the overall bacterial response to mercury spiking. Microenvironments and associated bacteria, designated as bacterial pools, were obtained by successive soil washes to separate the outer fraction, containing loosely associated bacteria, and the inner fraction, containing bacteria retained into aggregates, followed by a physical fractionation of the inner fraction to separate aggregates according to their size (size fractions). Indirect enumerations of viable heterotrophic (VH) and resistant (Hg^R) bacteria were performed before and 30 days after mercury spiking. A ribosomal intergenic spacer analysis (RISA), combined with multivariate analysis, was used to compare modifications at the community level in the unfractionated soil and in the microenvironments. The spatial heterogeneity of the mercury impact was revealed by a higher increase of Hg^R numbers in the outer fraction and in the coarse size fractions. Furthermore, shifts in RISA patterns of total community DNA indicated changes in the composition of the dominant bacterial populations in response to Hg(II) stress in the outer and in the clay size fractions. The heterogeneity of metal impact on indigenous bacteria, observed at a microscale level, is related to both the physical and chemical characteristics of the soil microenvironments governing mercury bioavailability and to the bacterial composition present before spiking.

Keywords: Bacterial community; Mercury; Soil microenvironment; DNA fingerprint; RISA; Indirect enumeration

1. Introduction

Bacterial response to heavy metal contamination in soil provides a relevant model for ecological studies to assess the influence of environmental characteristics on the quantitative and qualitative modifications of soil bacterial communities induced by hydrosoluble toxicants. Heavy metals in soil are known to have a deleterious effect on the numbers of bacteria, microbial biomass and activities, and diversity within bacterial populations (for reviews see [1,2]). Several studies reported an impact of heavy metals at the community level using phenotypic or genetic fingerprinting techniques. Microbial community measurements based on phospholipid fatty acid (PLFA) composition allowed the detection of shifts in microbial composition in different soil types after short- and long-term metal exposures [3]. Smit et al. [4] used amplified ribosomal DNA restriction analysis (ARDRA) as a genetic fingerprinting tool to show modifications of the community structure in copper-contaminated soils. The shifts can reflect an increase in bacterial community metal tolerance as demonstrated by Bååth [5] who used the thymidine-incorporation method. The increase in the relative abundance of adapted phenotypes, generally evidenced in contaminated environments, could be the mechanism responsible for such an increased tolerance observed at the community level [6,7].

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When evaluating the bacterial response to heavy metal contamination in soil, environmental parameters must also be considered. Generally, more significant modifications of bacterial activities, cell numbers and biomass occur in light-textured soils than in soils rich in clay minerals and organic matter [7–11]. Such observations are explained by a reduced bioavailability of the metal bound by clay minerals and humic-like materials [12–14]. Metal accessibility to bacterial cells is also dependent on circulation and diffusion processes in soil pores. Ranjard et al. [11] have shown that the balance between macro- and microporosity in different soil types controlled the magnitude of metal impact.

Soil structural organization in aggregates of different size and stability defines a mosaic of microenvironments differing by their physical, chemical and structural properties [15–18]. Consequently, indigenous bacteria are subjected to heterogeneous conditions depending on their location. Some microenvironments are more favorable bacterial habitats due to a better nutrient and water status and to the absence of predation by protozoa [17,19–24]. In spite of the importance of cell location for the degree of impact by a contamination, only a few studies have assessed bacterial response at a microscale level [11,17,25]. The role of soil microenvironments in modulating quantitative (cell density) and qualitative (activity, diversity, community structure) bacterial responses and thus the overall impact of metals on soil bacterial communities has never been investigated.

In this study, the soil was fractionated to evaluate the response of indigenous bacteria to heavy metal contamination in various microenvironments. Our objectives were (1) to evaluate the contribution of bacteria associated to different microenvironments (designated hereafter as bacterial pools) to the overall response of the bacterial community and (2) to determine to what extent soil physical, chemical and microbiological characteristics modulate the impact of the metal on the soil microflora. These goals were assessed with a model system consisting of a silt-loam soil artificially polluted with 50 μM of Hg(II) as previously described [11].

2. Materials and methods

2.1. Soil and microcosm set up

The soil used was collected from a cultivated silt-loam soil at La Côte Saint André (LCSAc, France). It was chosen because it had no previous exposure to mercury and contained a background level of 72.3 ng Hg g^{-1} dry weight. Soil sampling, storage and microcosm set up were previously described by Ranjard et al. [11]. Briefly, microcosms containing 10 g (dry weight) of soil were spiked with 50 μM of mercuric chloride (10 μg Hg(II) g^{-1}) and incubated at 22°C for 30 days. Unspiked control microcosms were incubated for 30 days under the same conditions. Characteristics of unfractionated soil, outer and inner soil fractions, and of various size fractions of the inner fraction are listed in Table 1.

2.2. Soil fractionation

The microenvironments were separated by using two soil fractionation procedures: successive soil washes to separate the outer and the inner soil fractions followed by a physical fractionation of the inner fraction to separate aggregates into size fractions. The fractionation was repeated twice using three microcosms for each incubation time. The soil washing procedure was performed with 10 g of soil as described by Ranjard et al. [11]. This procedure separated bacteria located in macropores, i.e., easily washed out from the surface of aggregates (outer fraction), from those located in micropores, i.e., retained in soil aggregates after washings (inner fraction). The supernatants containing microorganisms released from the outer fractions were pooled, centrifuged ($9800 \times g$, 20 min) and resuspended in 50 ml of sterile 0.8% NaCl solution. An aliquot was dried (105°C, 24 h) to determine the dry weight of the soil.

The remaining washed soil (inner fraction), pooled from three microcosms, was further fractionated to separate microenvironments based on the size of stable aggregates by the slightly modified procedure described by Kabir et al. [26]. Sand size fractions containing stable aggregates above 50 μm in size, including coarse and fine sand particles, fraction 250 to 2000 μm and 50 to 250 μm, respectively, were obtained by wet sieving using sterile cool water (< 10°C) to reduce bacterial growth. The soil suspension, containing aggregates and particles below 50 μm, was aseptically transferred into a sedimentation flask. The silt size fraction containing aggregates and coarse silt particles (20–50 μm) was obtained by gravity sedimentation. Aggregates and particles below 20 μm were withdrawn. The sedimentation step was repeated three times by resuspending the sedimentated soil in cool sterile water. Fine silt particles and 2–20 μm aggregates constituting the second silt size fraction, were pelleted from the supernatant by centrifugation at $90 \times g$ at 10°C in a swinging bucket rotor in 250-ml centrifuge tubes. The dispersible clay fraction (< 2 μm) was obtained by an overnight flocculation of the supernatant at 4°C after addition of $CaCl_2$ (50 mM final). Moist size-fractions 250–2000, 50–250 and 20–50 μm were weighed in tared receptacles. Aliquots were taken for (1) bacterial enumerations, (2) ribosomal intergenic spacer analysis (RISA) fingerprinting, (3) mercury content analysis, (4) organic C and clay content analysis by sulfochromic oxidation and textural analysis respectively and (5) the determination of the dry weight after a 24-h drying at 105°C. Size-fractions below 20 μm were resuspended in 200 ml of sterile cool water. Subsamples of 20 ml were used for all of the analyses mentioned above.

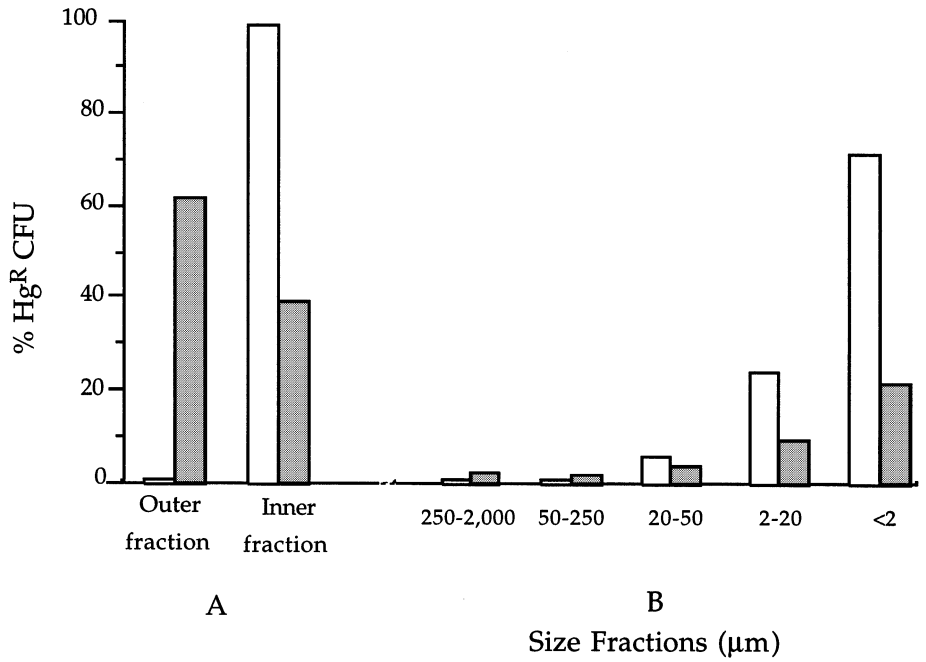

Fig. 1. Comparison of the distribution of mercury resistant bacteria (Hg^R) in the outer and inner fractions (A) and the different size fractions (B) of LCSAc soil before ($t = 0$, white bars) and after 30 days of soil exposure ($t = 30$, gray bars) with 50 μM of Hg(II). Distribution of Hg^R was calculated as followed:

$$\%\ Hg^R \frac{Hg^R\ g^{-1}\ \text{fraction} \times \%\ \text{weight distribution of fraction}}{(Hg^R\ g^{-1}\ \text{outer fraction} \times \%\ \text{weight distribution of outer fraction}) + (Hg^R\ g^{-1}\ \text{size fractions} \times \%\ \text{weight distribution of size fractions})} \times 100$$

2.3. Bacterial counts

Microorganisms were extracted by blending soil samples with 50 ml of a 0.8% (w/v) sterile NaCl solution for 90 s in a Waring Blender (Eberbach Corporation, New Hartford, USA). The homogeneous soil suspension was serially diluted 10-fold in sterile saline solution. Indirect counts of mercury resistant (Hg^R) and viable heterotrophic (VH) bacteria were carried out by spreading 100 μl of appropriate dilutions on plate count agar media (PCA media) supplemented with or without 50 μM of $HgCl_2$ [11]. Enumerations were performed just before soil spiking ($t = 0$) and after 30 days of incubation for spiked and control microcosms ($t = 30$). Three plates were inoculated per dilution. Cycloheximide was used as an anti-fungal agent (200 μg ml^{-1}, final concentration). Bacterial colonies were counted after 4 days of incubation at 28°C.

2.4. Measurement of mercury content in soil samples

Total mercury content analysis was performed on 200 mg of each soil sample after 30 days of incubation with Hg(II). Mercury content was determined by atomic absorption spectrometry after thermal decomposition of the sample using an AMA 254 spectrometer (ALTECH, Pragues, Czech Rep.). Calibration was made with fresh solutions with known $HgCl_2$ concentrations. The detection limit was 0.5 μg of mercury and the coefficient of variation of the standard curve was 2%.

2.5. DNA fingerprinting of bacterial communities

Bacterial DNA was extracted, purified and quantified from unfractionated soil and from various soil microenvironments following a protocol presented in a previous study [27]. The intergenic spacer region between the large and the small subunit of ribosomal sequences was amplified by PCR using 100 ng of purified template DNA with the primers FGPS1490-72 and FGPL132-38 [28]. Amplification reactions were performed in a final volume of 50 μl containing 5 μl of 10× dilution buffer, 15 mM $MgCl_2$, 200 μM of each dNTP, 25 pmol of each primer, 1 μg T4 gene 32 protein and 2.0 units-Expand™ High Fidelity *Taq* polymerase (Boehringer Mannheim, Meylan, France). Amplification was performed after a hot start at 94°C for 3 min, followed by 25 cycles consisting of 94°C for 1 min, annealing at 55°C for 30 s and elongation at 72°C for 1 min in a thermocycler (Perkin-Elmer Cetus 2400, Norwalk, USA). A final elongation step at 72°C for 5 min preceeded cooling at 4°C.

PCR products (20 μl) were loaded on a 5% non denaturing acrylamide gel (acrylamide-N, *N*-methylenebisacryl-

amide, 29:1, Bio-Rad, Ivry sur Seine, France) and separated by electrophoresis for 12 h at 60 V and 5 mA (DSG200-02, C.B.S. Scientific, Del Mar, USA). Gels were stained with SYBR green I (FMC Bioproducts, Le Perray en Yvelines, France) according to the manufacturer's instructions. The banding patterns were photographed using Ilford FP4 film and a 302-nm UV source. Reproducibility of fingerprint profiles was checked for each incubation time using bacterial pool DNA obtained from independent fractionation experiments.

2.6. Statistical analysis

Significant differences ($P < 0.05$) in VH and Hg^R numbers were determined by the Student's *t*-test (Statview-SE). Correlation coefficients between soil characteristics were calculated with Statview-SE software.

The pairwise visual comparisons of the band patterns were performed using the negative of the gel photo. A data matrix, taking into account presence/absence and relative intensity, from 0: absence, to 4: maximum intensity, of each band in a given profile, was constructed with bacterial pools as rows and bands as columns. This matrix was subjected to a principal component analysis (PCA) on a covariance matrix using ADE-4 software [29]. This method enabled variations to be studied within the community RISA profiles. It also provided an ordination of bacterial communities plotted in two dimensions on the first two principal components. The proportion of the data variance explained by each principal component was calculated.

3. Results

3.1. Weight distribution and characteristics of soil microenvironments

The soil fractionation procedure did not result in a significant loss of material since mass recovery was more than 95% of unfractionated soil. The inner fraction of the soil represented 92.1% of total soil weight (Table 1). Sand size fractions contributed for half of the weight of the inner fraction, 29.7% and 20.7% for 250–2000 μm and 50–250 μm, respectively, whereas the dispersible clay size fraction (< 2 μm) contributed the least (8.2%).

A strong positive correlation was found between organic C and clay contents in the various microenvironments ($r = 0.95$, $P < 0.05$). The outer fraction had twice as much organic C content than the inner one (Table 1). In the latter, the highest organic C and clay contents were recovered in the finest size fractions (< 20 μm).

Total mercury content was measured 30 days after soil spiking with 10 μg Hg g^{-1} of soil (50 μM of Hg(II)) in unfractionated soil and soil fractions. After incubation, 98% of introduced mercury was still retained into the soil matrix. Furthermore, the soil fractionation procedures did not induce any mercury loss since mercury content in soil fractions represented 99% of mercury content in the unfractionated soil. The highest mercury concentrations were found in the outer fraction (41.5 μg Hg g^{-1}) and in the finest size fractions of the inner fraction, 20.8 and 25.3 μg Hg g^{-1} in the 2–20 μm and < 2 μm size fractions, respectively (Table 1). A positive correlation with both clay ($r = 0.88$, $P < 0.05$) and organic C ($r = 0.98$, $P < 0.05$) contents was noticed.

3.2. Impact of Hg(II) on the distribution of viable heterotrophic and mercury resistant bacteria in soil microenvironments

Counts of VH and Hg^R bacteria are presented in Table 2. Numbers of VH bacteria at $t = 0$ and $t = 30$ in unfractionated soil and various microenvironments were not significantly different ($P < 0.05$) and remained similar to the numbers obtained in control microcosms during the same period of incubation. Similar observations were obtained with acridine orange direct counts (data not shown). The

Table 1
Weight distribution, organic C, clay and mercury contents of unfractionated soil and soil fractions

Soil sample	Weight distribution* (%)	Organic C content** (mg g^{-1} sample)	Clay content** (mg g^{-1} sample)	Hg content*** (mg 10^{-3} g^{-1} sample)
Unfractionated soil	100	14.5 ± 0.2	212.0	9.8 ± 0.23
Outer fraction	7.9	20.5 ± 0.26	nd	41.5 ± 1.74
Inner fraction	92.1	10.3 ± 0.17	230.0	5.3 ± 0.25
Sub-fractionated inner fraction:				
Coarse sand size fraction: 250–2 000 μm	29	4.4 ± 0.14	91.0	1.67 ± 0.14
Fine sand size fraction: 50–250 μm	20.7	5.1 ± 0.08	100.0	1.4 ± 0.18
Coarse silt size fraction: 20–50 μm	19.5	5.8 ± 0.08	112.0	3.14 ± 0.35
Fine silt size fraction: 2–20 μm	14.7	22.6 ± 0.22	344.0	20.8 ± 0.31
Dispersible clay fraction < 2 μm	8.2	34.1 ± 0.18	nd****	25.3 ± 0.19

nd: not determined
*results expressed as % of the unfractionated soil dry weight
**values are the mean of duplicates ± S.D.
***Hg content was determined 30 days after soil spiking with 50 μM Hg(II); values are the mean of duplicate ± S.D.
****the low quantity of material did not allow textural analysis but it is likely that this fraction would be one of the most clay-concentrated

Table 2
VH and Hg^R CFU per gram in unfractionated soil and soil fractions before soil spiking with 50 μM Hg(II) ($t=0$) and after 30 days of incubation with Hg(II) or in control unspiked soil ($t=30$)

Soil sample	$t=0$		$t=30$				Hg^R enrichment factors between $t=0$ and $t=30$
			control		Spiked soil		
	$VH\times10^7$ CFU g^{-1} sample*	$Hg^R\times10^4$ CFU g^{-1} sample*	$VH\times10^7$ CFU g^{-1} sample*	$Hg^R\times10^4$ CFU g^{-1} sample*	$VH\times10^7$ CFU g^{-1} sample*	$Hg^R\times10^4$ CFU g^{-1} sample*	
Unfractionated soil	$3.30\pm0.7^{a,b}$	4.5 ± 0.5^{d}	$3.4\pm0.5^{a,b}$	6.15 ± 1.1^{d}	1.65 ± 0.32^{b}	353 ± 25^{h}	79
Outer fraction	1.00 ± 0.30^{b}	0.22 ± 0.09^{e}	1.54 ± 0.2^{b}	0.31 ± 0.02^{e}	$2.4\pm0.5^{a,b}$	583 ± 78^{h}	2650
Inner fraction	3.71 ± 0.57^{a}	4.27 ± 0.35^{d}	$2.99\pm0.9^{a,b}$	5.9 ± 0.16^{d}	1.37 ± 0.53^{b}	46 ± 13^{g}	11
Sub-fractionated Inner fraction:							
250–2 000 μm	0.26 ± 0.04^{b}	0.01 ± 0.001^{f}	nd	nd	0.43 ± 0.05^{b}	5.8 ± 1.4^{d}	580
50–250 μm	0.27 ± 0.11^{b}	0.03 ± 0.01^{f}	nd	nd	0.39 ± 0.1^{b}	4.6 ± 3^{d}	153
20–50 μm	0.55 ± 0.15^{b}	$1.23\pm0.17^{d,e}$	nd	nd	0.58 ± 0.6^{b}	22.4 ± 1^{g}	18
2–20 μm	$5.38\pm0.75^{a,c}$	6.95 ± 0.5^{d}	nd	nd	$4.57\pm0.37^{a,c}$	69.5 ± 23.2^{g}	10
<2 μm	$6.07\pm0.5^{a,c}$	37.5 ± 4.1^{g}	nd	nd	8.1 ± 1.6^{c}	$184\pm43^{h,g}$	5

Letters in superscript indicated statistical differences ($P<0.05$).
*Mean counts ± S.D.

highest densities of VH bacteria were found in the finest size fractions (<20 μm) representing about 80% of the VH microflora of the inner fraction.

At $t=0$, Hg^R bacteria were present in LCSAc soil (4.5×10^4 CFU g^{-1} of unfractionated soil) and were detected in all fractions at levels ranging from 1.0×10^2 to 3.75×10^5 CFU g^{-1} sample (Table 2). The highest Hg^R densities were observed in the 2–20-μm and <2-μm size fractions where they represented 23% and 71% of total recovered Hg^R bacteria, respectively, (Fig. 1). After 30 days of incubation with mercury, numbers of Hg^R bacteria significantly increased ($P<0.05$) in unfractionated soil (about 80-fold) and in all soil fractions (Table 2). During the same period, Hg^R bacterial numbers remained unchanged in the control. The highest increase occurred in the outer fraction (about 2650-fold). In the size fractions, enrichment factors varied from five-fold in the dispersible clay size fraction to 580-fold in the coarse sand size fraction. The relative distribution pattern of Hg^R bacteria was altered only in the outer and in the inner soil fractions between $t=0$ and $t=30$ while it remained similar in the various size fractions (Fig. 1).

Between $t=0$ and $t=30$, the percent of Hg^R bacteria among the VH community (% Hg^R/VH) in the unfractionated soil increased from 0.13 to more than 20. In the outer fraction, the % Hg^R/VH increased from 0.02 to 24, while it reached only 3 in the inner fraction at $t=30$. Regarding size fractions, % Hg^R/VH ranged from 0.0025 in sand size fractions to 0.62 in the clay size fraction at $t=0$. At $t=30$, it reached 1.2 and 2.3 in these fractions, respectively.

3.3. Impact of Hg(II) on the RISA profiles of bacterial pools associated with the various microenvironments

Fingerprint analysis was performed on unfractionated

Table 3
Comparison of RISA profiles of bacterial pools associated with unfractionated soil and soil fractions before ($t=0$) and 30 days ($t=30$) after spiking with 50 μM (Hg(II))

	Number of bands on RISA profile							
	Unfractionated soil	Outer fraction	Inner fraction	250–2 000 μm	50–250 μm	20–50 μm	2–20 μm	<2 μm
Total number of bands:								
at $t=0$	40	32	40	46	45	47	44	32
at $t=30$	40	44	44	45	45	47	43	39
Changes in RISA profiles between $t=0$ and $t=30$	19	27	16	3	5	3	5	19
Number of new bands*	4	13	5	0	0	0	0	9
Number of lost bands	4	1	1	1	0	0	1	2
Number of intensified bands	8	9	8	0	2	2	2	7
Number of weakened bands	3	4	2	2	3	1	2	1

*bands visualized only in the profiles obtained at $t=30$

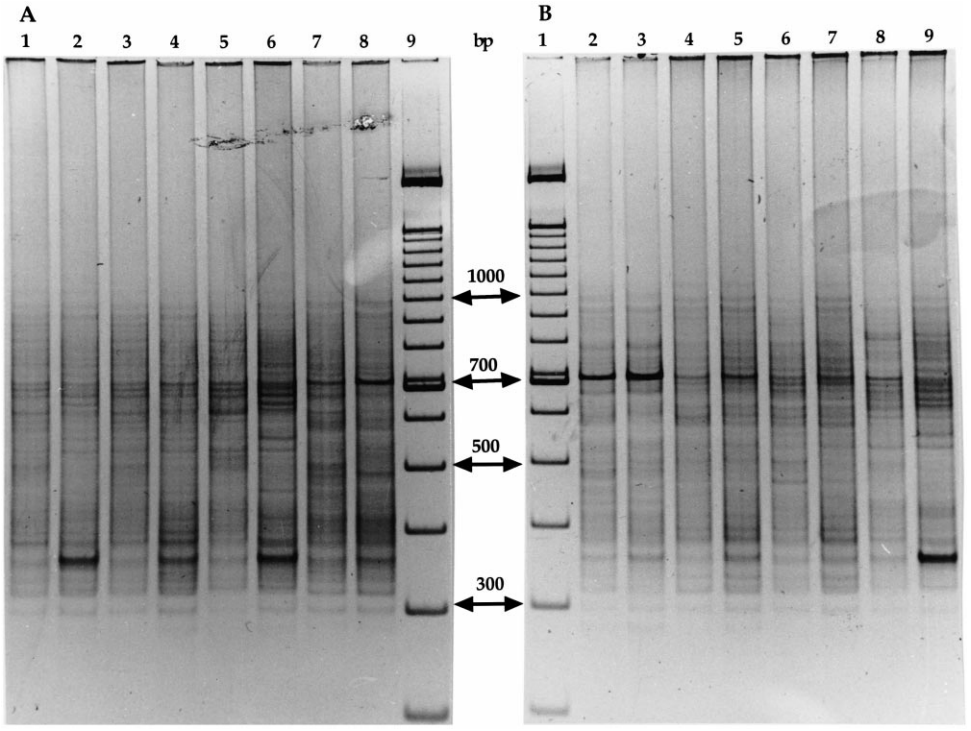

Fig. 2. Electrophoresis in 5% acrylamide gel of amplified eubacterial intergenic spacer between 16S and 23S rDNA from DNA extracted from unfractionated soil and microenvironments before (−) and after 30 days of Hg(II) spiking (+). A: lane 1: unfractionated soil (−), lane 2: unfractionated soil (+), lane 3: inner fraction (−), lane 4: inner fraction (+), lane 5: outer fraction (−), lane 6: outer fraction (+), lane 7: size fraction 250–2000 μm (−), lane 8: 250–2000 μm (+), lane 9: 100-bp DNA ladder. (B) lane 1: 100-bp DNA ladder, lane 2: size fraction 50–250 μm (−), lane 3: 50–250 μm (+), lane 4: size fraction 20–50 μm (−), lane 5: 20–50 μm (+), lane 6: size fraction 2–20 μm (−), lane 7: 2–20 μm (+), lane 8: size fraction <2 μm (−), lane 9: <2 μm (+).

soil, on inner and outer fractions and on size fractions at $t=0$ and $t=30$ (Fig. 2). RISA profiles obtained at $t=0$ exhibited from 32 to 47 bands (Table 3) and were similar to the profiles obtained from unspiked soil after 30 days of incubation (data not shown). Hg(II) spiking induced modifications in the profiles for the unfractionated soil as well as for the various microenvironments (Fig. 2). Changes were mainly due to the appearance of new bands and to an increase of relative intensity of previously existing bands (Table 3).

Further statistical pairwise analysis of RISA profiles by principal component analysis (PCA), allowed (1) the mathematical ordination of bacterial pools associated with the various microenvironments on the two first principal components and (2) the comparison of the magnitude of changes induced by mercury spiking (Fig. 3). The first principal component explained 31% of the data variance and 21% was explained by the second component. At $t=0$, PCA ordination demonstrated the close structure of bacterial pools associated with (1) the unfractionated soil and the inner fraction, (2) the sand size fractions (>50 μm), (3) the silt size fractions (2–50 μm) and (4) the clay size (<2 μm) and the outer fractions. Mercury spiking resulted in score variations concerning the whole bacterial community and the bacterial pools associated with the inner, the outer and the clay size fractions. All these variations were explained by the second principal component.

4. Discussion

Soil structural organization defined microhabitats in which bacteria are subjected to various surrounding conditions in terms of structural and physico-chemical characteristics. A microscale approach was adopted in order to estimate the relative contribution of bacterial pools in the overall response to a short-term mercury spiking according to cell location and characteristics of microbial habitats. We used the quantification of adapted culturable phenotypes, i.e. Hg^R bacteria, as an indication of biologically available mercury [6,7,11,30–32]. The impact of exposure to Hg(II) on the bacterial community structure was studied using a genetic fingerprinting method (RISA) which has previously been demonstrated to be relevant and sensitive by Borneman and Triplett [33]. This genetic fingerprint is based on the length polymorphism of the amplified intergenic spacer between *rrs* and *rrl*. Since this approach, without further characterization, is limited to a comparative analysis of the community structure, we applied a multivariate analysis of RISA profiles to compare

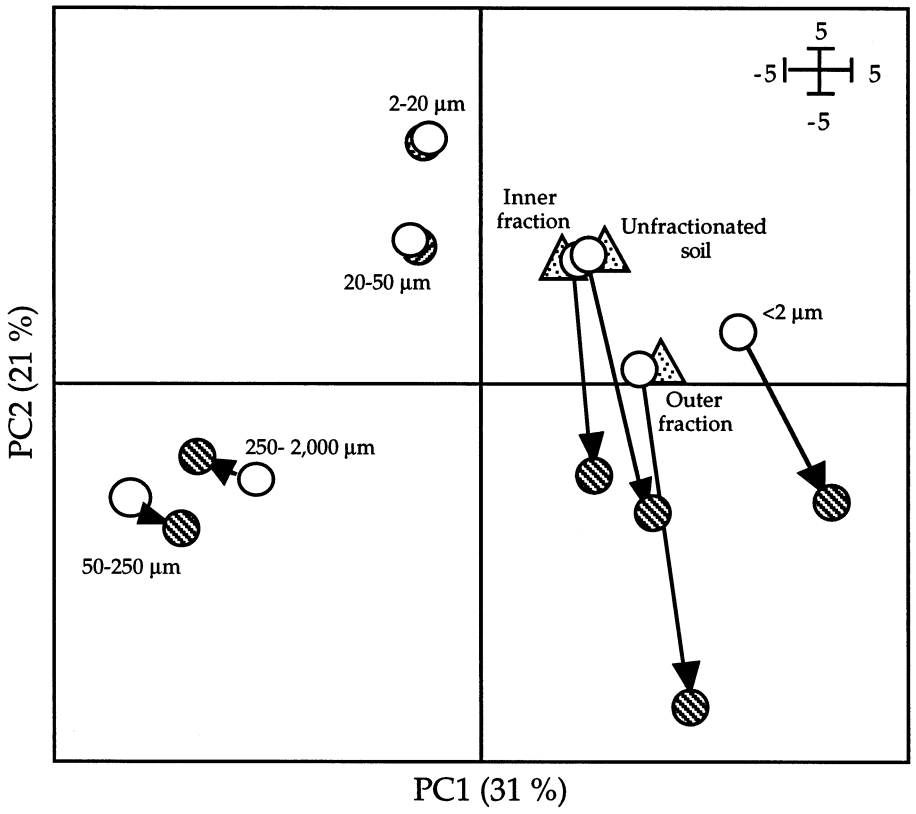

Fig. 3. PCA ordination of the genetic structure of the unfractionated soil bacterial community and the bacterial pools associated with soil microenvironments. △ (dotted) represent the bacterial pools after 30 days of incubation in unspiked soil. ● and ○ (dotted) the bacterial pools at $t=0$ and 30 days after soil spiking with 50 µM of Hg(II), respectively, in the unfractionated soil and in the microenvironments. Arrows indicate the magnitude of shifts.

the magnitude of the shifts in the different microenvironments due to mercury spiking.

4.1. Impact of Hg(II) spiking on bacteria in unfractionated soil

Before spiking, Hg^R bacteria represented about 0.1% of the VH bacteria in LCSAc soil corresponding to the range of values mentioned in the literature for microbial communities of uncontaminated natural ecosystems [31,34–36]. The short term incubation of soil with Hg(II) did not result in a change in the numbers of VH bacteria nor in the numbers of total bacteria determined by direct microscopy (data not shown) but induced an increase in Hg^R colonies (Table 2). Such an enrichment of resistant strains is a common observation in metal-contaminated soils or aquatic systems [6,7,11,30,31].

The DNA fingerprint of the soil community revealed modifications induced by mercury spiking (Fig. 2, Table 3). Similar results have already been reported for soils spiked with other heavy metal or organic toxicants by genetic or phenotypic fingerprinting methods [4,37,38]. Such an observation might be explained by the disappearance of sensitive populations and the enrichment of well-adapted ones [1,39] leading to a bacterial community more tolerant to the stressor [3,6].

4.2. Impact of Hg(II) spiking on bacteria in the inner and in the outer fractions

The most pronounced effect of Hg(II) spiking regarding Hg^R enrichment, distribution and shifts in DNA fingerprints, was found in the outer soil fraction (Tables 2 and 3, Figs. 1 and 3). The difference noted in the magnitude of changes could be explained by a higher mercury concentration in the outer fraction than in the inner one (Table 1). These results reflected the higher bioaccessibility of introduced soluble substances to bacteria located in macropores (in the outer fraction) [17,40]. Populations inhabiting macropores were directly affected by solutes by convection processes whereas populations inhabiting the micropores of the inner fraction were only affected by diffusion processes [11,41]. Consequently, bacteria located in the outer fraction were less protected from the toxicant input and contributed the most to the overall bacterial response.

4.3. Impact of Hg(II) spiking on bacteria in the size fractions of the inner soil fraction

The inner soil fraction is comprised of a mosaic of aggregates differing by their structural, chemical and physical characteristics. The separation of stable aggregates

according to their size allowed demonstration of the strong contribution of bacteria located in the sand size fractions (>50 μm) to the enrichment of Hg^R bacteria observed in the inner fraction (Table 2). The high Hg^R enrichment observed in the sand size fractions could reflect the higher bioaccessibility of mercury due to the predominance of large diameter pores which facilitate the circulation of solutes as shown in sandy soil compared to loamy and clay soils [18]. However, the low impact of mercury, revealed by the weak Hg^R enrichment in the finest fractions, could not be explained by the low diffusion in micropores alone since high concentrations of mercury were detected in these fractions (Table 1). These results suggested that the immobilization of metal on reactive organic and mineral surfaces, strongly represented in silt and clay size fractions, led to a decreased bioavailability [9,11,12–14].

Before mercury spiking, PCA of DNA fingerprints showed the relatedness between the population structure of bacterial pools associated with the sand size fractions, between pools associated with the silt size fractions and between pools associated with the clay size fraction and the outer soil fraction (Fig. 3). After 30 days of soil incubation with mercury, the shifts observed in the inner soil fraction mainly resulted from modifications occurring in the clay size fraction (<2 μm) whereas minor or no changes were detected in sand (>50 μm) and silt size (2–50 μm) fractions (Fig. 3). The increase of Hg^R numbers in these fractions, without shifts of the pool composition, could be explained by a low contribution of culturable Hg^R phenotypes to the RISA profiles. A similar conclusion was reported by other authors who observed discrepancies between the composition of the culturable bacterial populations and the composition of the whole community assessed by genetic fingerprints [4,42].

PCA also evidenced that closely related pools before spiking, exhibited similar magnitudes of shifts (Fig. 3). These results might indicate the influence of the bacterial pool composition before spiking in the response to metal stress [1,39,43]. In LCSAc soil, the occurrence of more sensitive pools in the outer and clay size fractions could be explained by a high proportion of Gram-positive cells among culturable [11] and total (Richaume, unpublished data) populations. Gram-positive cells are known to be less tolerant to heavy metals than Gram-negative cells [44,45].

5. Conclusions

The microscale approach has demonstrated the heterogeneous impact of mercury spiking in soil and revealed that the magnitude of mercury-induced modifications differed by several orders in microenvironments compared to unfractionated soil. The combined use of the adapted phenotype quantification and the RISA fingerprinting method pointed out the respective involvement of physico-chemical and microbiological properties of the microenvironments. The bacterial populations located at the aggregate surface were clearly the most affected by mercury spiking and contributed the most to the overall soil bacterial response. Our results emphasize the need for an increase in knowledge on the determinism of cell distribution and diversity in soil.

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