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Characterization of soil health in an Italian polluted site by using microorganisms as bioindicators

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Abstract

The soil *status* inside and outside the chemical ACNA factory (Cengio, Italy) has been investigated by assessing chemical and biological parameters. Three sites, two (A and B) located inside the so-called high-risk zone and one (C) outside the factory were considered. Chemical analysis performed in the first metre of depth indicated the presence of inorganic pollution (copper and arsenic) in site A and organic (1,2,3,4-tetrachlorobenzene and 2,4-dichloroaniline) and inorganic (arsenic and mercury) contaminations in site B. Site C did not show any significant contamination and was considered as control. Biological analyses were performed by evaluating functional (substrate utilization pattern and enzymatic activities of the soil extracts) and structural (bacterial population density, composition and structure) diversity of the soil bacterial community as indicator of soil health. Moreover, 20 bacterial strains per site, able to grow on minimal medium added with 2,4-dichloroaniline as sole carbon source, were selected and identified.

The metabolic profile of soil extracts, characterized by using Biolog GN MicroPlates, coupled with hierarchical clustering, showed a high similarity between the microbial communities isolated from sites A and B. The highest amounts of enzymatic activities were recorded in site C. Bacterial density in all sites did not differ significantly and a prevalence of *r*-strategist (fast growing and copiotrophic) bacteria was observed. The composition of the bacterial community showed a higher similarity between sites A and B than with site C. Only few bacterial species were common to the three sites: as a consequence, a prevalence of bacterial species typically associated to each site was observed. The fraction of bacteria able to grow on 2,4-dichloroaniline as sole carbon source on the total culturable bacteria in each site was lower than 0.1%. Bacterial identifications were performed by using Biolog MicroPlates. By considering the bacterial strains able to grow on minimal medium added with 2,4-dichloroaniline, a higher similarity between sites A and C than with B site was recorded. In conclusion, the presence of different chemical pollutants did not affect the bacterial density but rather the community structure. The characterization of soil chemical properties, metabolic fingerprinting, enzymatic activities and bacterial community structure could be useful tools for the evaluation of the soil health.
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Keywords: Polluted soil; Metabolic profile; Enzymatic activities; Microbial community structure; 2,4-Dichloroaniline

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1. Introduction

Soil is a complex microhabitat regulating plant productivity and the maintenance of biogeochemical cycles by the activity of microorganisms able to degrade organic compounds including xenobiotics. Since air, groundwater and surface water could be adversely affected by soil deterioration, soil health, resulting from the combination of chemical, physical and biological components, become fundamental for the integrity of terrestrial ecosystem (Nannipieri et al., 2003). On the other side, it is well documented that human industrial activities strongly affect biological systems and, more in particular, the soil *status*. The concept of soil health has been introduced in the late 1980s. According to the recent definition of Doran and Safley (1997), the soil health is ''the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain biological productivity, promote the quality of air and water environments and maintain plant, animal and human health''.

Several bioindicators of soil health and quality have been developed and reviewed (Trasar-Cepeda et al., 2000; Van Bruggen and Semenov, 2000; Nielsen et al., 2002; Anderson, 2003). Among them, microorganisms, due to their capability to respond quickly to environmental changes, are expected to be efficient bioindicators. Microbial indicator has been defined as ''a microbial parameter that represents properties of the environment or impacts to the environment, which can be interpreted beyond the information that the measured or observed parameter represents itself'' (Nielsen et al., 2002). Microbial bioindicators could be based on functional and structural diversity of the bacterial community. Functional diversity can be defined as the number, type, activity and rate at which a set of substrate is utilized by a bacterial community (Zak et al., 1994). Among the functional diversity indicators, the carbon utilization pattern and the measurement of enzymatic activities expressed by the whole bacterial community have been suggested as useful tool to evaluate the soil *status* (Nielsen et al., 2002). The metabolic profile, obtained by Biolog assay, provides a qualitative physiological fingerprinting of the potential functions of the microbial community (Garland and Mills, 1991). Since enzymatic activities in soil are mainly of microbial and fungal origin, the characterization of soil enzyme pattern could improve our knowledge on microflora activity, soil productivity and impact of pollutants (Pankhurst et al., 1995).

Structural diversity is defined as the number of parts or elements within a system, indicated by such measures as the number of species, genes, communities or ecosystems. Several indices such as the species richness, diversity and evenness are used to describe the structural diversity of a community and to monitor changes in microbial diversity due to environmental fluctuations, land management practices and pollution (Øvreås, 2000). The structural diversity of a bacterial community has been found to be very sensitive to environmental changes reacting by shift in its composition (Kandeler et al., 1999). In several cases, variation in microbial population and activity could also function as a predictor of change in soil health (Nielsen et al., 2002). Even more, the ratio copiotrophs/oligotrophs has been proposed to reflect the nutrient stress tolerance of the species present in soil (Van Bruggen and Semenov, 2000). Therefore, the comparison of the density, activity and the composition of soil microbial community could be useful to evaluate the influence of the different environmental conditions on soil habitat.

Different works characterized the influence of artificial chemical contamination on functional and structural diversity of microbial community by using microcosms experimental setup (Wünsche et al., 1995; Frostegård et al., 1996; Müller et al., 2001a,b; Pozo et al., 2003). However, little is known about the structure, diversity and activity of microbial community, in soils with a long pollution history.

The aims of this work were: (i) to apply several biological and chemical parameters in a field trial analysing the soil health *status* of polluted sites and (ii) to study the soil microbial community structure in these polluted sites. The Associated National Chemical Companies (ACNA) factory, whose activity followed a long process – started in 1882 with the production of explosives (nitroglycerin, dynamite and trinitrotoluene), paints, nitric and sulphuric acids, amines and phenols and ended in 1999 – was considered. This kind of production involved strong occupational risks and induced a deep environmental impact on the health of the soil and on the quality of the water of the Bormida River (going through 17

towns of Piemonte and Liguria), whose bed was diverted inside the factory. Although no significant health risk related to environmental exposure have been recognized in the population living in the Bormida valley (Cadum et al., personal communication), epidemiological studies performed on dyestuff employed people pointed out a high incidence and mortality for bladder cancer related to aromatic amines (Puntoni et al., 1988) and polycyclic aromatic hydrocarbons exposures (Bonassi et al., 1989).

Three sites were characterized for their chemical pollution, microbial community structure and soil health by using several bioindicators such as enzymatic activities, carbon utilization pattern and copiotrophic/oligotrophic ratio. The advantages and disadvantages of each bioindicator will be also discussed.

2. Materials and methods

2.1. Soil sampling

Three different soil sites (A—sand 71.2%, loam 9.3%, clay 19.5%, pH 8.28, TOC 1.47%; B—sand 56.0%, loam 33.5%, clay 10.5% pH 8.26, TOC $< 0.1\%$; C—sand 88.1%, loam 5.7%, clay 6.2% pH 7.98, TOC $< 0.1\%$) located around the industrial area were considered (Fig. 1). Sites A and B were located inside the so-called ''high-risk zone'' on two little hills constituted by different wastes of the industrial activity. Several years ago, the hill corresponding to the B site was top-filled with 50 cm of not polluted soil. Site C, located uphill the industrial area, was considered as the control site.

For chemical analysis, soil cores corresponding to 25 cm and 1 m of depth were considered. For biological purposes, four samples per site, each one constituted by four, randomly placed, 25 cm soil cores were aseptically collected, maintained at 4 °C and processed within two days.

2.2. Chemical analysis

Determination of metal concentrations has been performed following the Environmental Protection Agency (EPA) method no. 6020a (http://www.epa.gov/epaoswer/hazwaste/test/pdfs/6020a.pdf) by using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS; Agilent Technologies Inc., Palo alto, CA, Mod. 4500) technique and terbium (^{159}Tb) and rhodium (^{103}Rh) as internal standard (100 μg/ml). Measurements of semivolatile organic compounds and polycyclic aromatic hydrocarbons (PAHs) were performed following the EPA methods nos. 8270d and 8280b



Fig. 1. Map of the ACNA factory area showing locations of sampling sites.

(http://www.epa.gov/epaoswer/ hazwaste/ test/ pdfs/ 8270d.pdf; http://www.epa.gov/epaoswer/hazwaste/ test/pdfs/8280b.pdf) by using high resolution gas chromatography–low resolution mass spectrometry (HRGC–LRMS, Agilent Technologies Inc., Mod. 5973).

2.3. Soil metabolic fingerprinting

Ten grams of fresh soil from each site have been suspended in 90 ml of 0.18% sodium pyrophosphate and vigorously shaken for 30 min. Serial dilutions were performed in $MgSO_4 \cdot 7H_2O$ buffer (0.1 M), the dilution 10^{-4} was centrifuged at 1000 rpm for 10 min and 150 μl of the supernatant were used to inoculate the wells of the Biolog GN MicroPlates. Three plates for each site were incubated at 25 °C for seven days, manually read and digitalized every day in order to follow microbial activity during the time course. The intensity of the colour developed in each well was expressed as numerical value: 2 for positive reaction, 1 for borderline reaction and 0 for no reaction.

2.4. Soil enzymatic activity characterization

Cellulase and xylanase activities on carboxymethyl-cellulose (CMC-Fluka) and birchwood xylan (Fluka), respectively, were evaluated following Schinner and von Mersi (1990): 2 g of fresh soil from each site were added to 3 ml of 2 M acetate buffer (pH 5.5) and to 3 ml of CMC (0.7%) or xylan (1.2%) and incubated at 35 °C for 24 h. The mixture was then filtered with Whatman no. 1 paper and the presence of reducing sugars measured at λ575 following the method of Miller (1959) using glucose (1 mg ml^{-1}) as standard.

β-Glucosidase activity was evaluated following Eivazi and Tabatabai (1988): 1 g of fresh soil from each site was added to a mixture containing 250 μl of toluene, 4 ml of modified universal buffer (MUB; pH 6) (Alef and Nannipieri, 1995) and 1 ml of 25 mM *p*-nitrophenyl-β-D-glucoside (PNG, Sigma) and incubated at 37 °C for 1 h. Then, 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.1 M Tris buffer (pH 12) were added and mixed. The suspension was filtered with Whatman no. 1 paper and the enzyme activity evaluated at λ400 using *p*-nitrophenol (1 g l^{-1}) as standard.

Acid phosphomonoesterase was measured following Eivazi and Tabatabai (1977): 1 g of fresh soil from each site was added to a mixture containing 250 μl of toluene, 4 ml of modified universal buffer (pH 6.5) (Alef and Nannipieri, 1995) and 1 ml of *p*-nitrophenyl phosphate (PNP; Sigma) and incubated at 37 °C for 1 h. Then, 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH were added and mixed. The suspension was filtered with Whatman no. 1 paper and the enzyme activity evaluated at λ400 using 1 g l^{-1} *p*-nitrophenol as standard.

Alkaline and neutral phosphomonoesterases were evaluated following Alef and Nannipieri (1995): 5 g of fresh soil from each site were added to a mixture containing 750 μl of toluene, 5 ml of di-sodium phenylphosphate (27 g l^{-1}) and 10 ml of borate (12.4 g l^{-1}, pH 10) or citrate buffer (300 g l^{-1}; pH 7), respectively, and incubated at 37 °C for 3 h. The suspension was added of 30 ml of demineralised water and filtered with Whatman no. 1 paper. Then, 2.5 ml of the supernatant were added to 2.5 ml of borate or citrate buffer, 7.5 ml of demineralised water and 500 μl of 6.7 mM 2,6-dibromo-quinone-chlorimide solution and incubated at room temperature for 30 min. The suspension was then diluted to 50 ml with demineralised water and the enzyme activity evaluated at λ600 using phenol (10 μg ml^{-1}) as standard.

Urease was evaluated following Bremner and Mulvaney (1978): 2 g of fresh soil from each site were added to a mixture of 4 ml of Tris–maleic acid buffer (0.2 M; pH 6.7), 500 μl of toluene, 1 ml of urea (10%) and incubated at 37 °C for 1 h. The suspension was added of 1 ml of $AgSO_4$ (1.4 g l^{-1}) and 10 ml of KCl (2N) and filtered with Whatman no. 1 paper. Then, 1 ml of the supernatant was added to 2.5 ml of a solution containing phenol 10 g l^{-1}, sodium nitroprussiate 50 mg l^{-1}, 2.5 ml of 5% sodium hypochlorite and 4 ml of demineralised water and incubated at room temperature for 30 min. The enzyme activity was evaluated at λ650 using ammonium chloride (3.82 g l^{-1}) as standard.

All determinations of enzymatic activities were performed in triplicate and all values are averages of the three spectrophotometrical evaluations.

2.5. Bacterial density evaluation

Fifty grams of fresh soil from each site were added to 450 ml of sodium pyrophosphate (0.18%), shaken

at 25 °C, 180 rpm for 2 h and left to settle for 30 min. Serial dilutions were performed in sulphate buffer and 100 μl of each suspension was distributed in triplicate on 1/10 Trypticase Soy Agar (TSA-Fluka) added with 100 μg ml^{-1} of cycloheximide (ICN, Irvine, CA, USA). Bacterial density was recorded after two and seven days of incubation at 28 °C and expressed as cfu g^{-1} of fresh soil. The ratio between fast growing copiotrophs (*r*-strategist, two days) and slow growing oligotrophs (*k*-strategist, seven days) bacteria was determined.

Twenty-two colonies from a representative dilution were selected and isolated from 1/10 TSA plates. Cultures of bacterial cells, grown in Luria–Bertani (LB) broth medium (Miller, 1972) were stored at −80 °C in 50% glycerol.

2.6. Isolation of 2,4-di-chloroaniline degrading bacteria

In order to select strains able to use 2,4-di-chloroaniline as unique carbon source, 100 μl of each dilution, obtained as described above, were spread on M9 agar (Sambrook et al., 1989) added with the xenobiotic (1 g l^{-1}; Fluka) and cycloheximide. The density of 2,4-dichloroaniline degrading bacteria in each site was determined and colonies were then randomly selected, isolated and maintained on M9 agar plates added with the pollutant. Cultures of bacterial cells, grown in M9 broth supplemented with 2,4-di-chloroaniline were stored at −80 °C in 50% glycerol.

2.7. Bacterial strains identification

Bacterial identifications by metabolic fingerprinting analysis were performed by the Biolog® system (Biolog® Inc., Hayvard, CA, USA). Bacteria were grown on TSA, transferred twice on Biolog Universal Growth (BUG) agar and incubated at 28 °C for 24 h. Colonies were scraped from the medium, suspended in a 0.4% saline solution and the inoculum density adjusted to the specified turbidity range. One hundred and fifty microlitres of this suspension were inoculated in each well of a Biolog GN or GP MicroPlate. Microplates were incubated at 28 °C and manually read after 24 h. Data were collected and analysed by Biolog® Microlog 2 software.

2.8. Statistical analysis

Since bacterial population approximate an exponential normal distribution (Loper et al., 1985), bacterial density values were logarithmically transformed before analysis. Non-transformed and transformed values were submitted to analysis of variance and to Fisher's least significant test ($P < 0.05$) using Statview statistics package.

Correspondence analysis, analysing two-way contingency table of numerical variables, was performed on the bacterial identifications by xLSTAT 2.5 software.

Hierarchical clustering analysis, classifying a set of observations described by numerical variables into groups, was performed on enzyme activities, soil metabolic fingerprinting and on bacterial identifications by Biodiversity Pro software (http://www.sams.ac.uk/dml/projects/benthic/bdpro/downloads.htm) by using single linkage technique. The similarity among the sites was calculated by the same software as Jaccard index ($J_i = J/r \times 100$, where J is the number of species found in both the samples and r is the number of species found in one sample or in the other). Bacterial species richness was evaluated by Margalef's index ($D_{Mg} = S - 1/\log_2 N$, where S is the number of species and N is the number of strains) and bacterial biodiversity by Shannon–Wiener index ($H' = -^{S}\Sigma_{i=1}\, p_i \log_2 p_i$, where S is the total number of species and p_i is the relative abundance of the species). Both D_{Mg} and H' index were calculated by Biotools plug-ins for Excel.

3. Results

3.1. Chemical soil pollutant

Chemical analysis performed in the first metre of soil depth showed different type of pollutant in each site. Site A was characterized by an inorganic pollution with arsenic (121.2 mg kg^{-1} of soil) and total copper (690.0 mg kg^{-1} of soil) concentrations both overcoming the guideline value for an industrial zone. These results were in agreement with the analysis performed at 25 cm of depth showing copper (790 mg kg^{-1}) and arsenic (180 mg kg^{-1}) as main

Table 1
Quantitative evaluation of chemical pollutant (mg kg^{-1}) in each soil site at 1 m and at 25 cm of depth compared to the guideline value

Site	Pollutant	Concentration in a 1 m soil core (mg kg^{-1})	Concentration in a 25 cm soil core (mg kg^{-1})	Guideline value (mg kg^{-1})
A	Total copper	690.0	790	600
	Arsenic	121.2	180	50
B	Arsenic	112.0	24	50
	Mercure	12.5	4.2	5
	Tetrachlorobenzene	30.4	9.1	25
	2,4-Dichloroaniline	5.8	2.4	5
C	[a]	–	–	–

[a] No pollutant was detected in soil site C.

pollutants. The analysis performed on the first metre of soil from B site showed both inorganic and organic pollution with mercure (12.5 mg kg^{-1} of soil), arsenic (112.0 mg kg^{-1} of soil), 2,4-dichloroaniline (5.8 mg kg^{-1} of soil) and 1,2,3,4-tetrachlorobenzene (30.4 mg kg^{-1} of soil) overcoming the guideline values for an industrial area. The same pollutants were recorded at 25 cm of depth (arsenic 24 mg kg^{-1}, mercure 4.2 mg kg^{-1}, 1,2,3,4-tetrachlorobenzene 9.1 mg kg^{-1} and 2,4-dichloroaniline 2.4 mg kg^{-1} of soil), but their concentration did not overcome the guideline values. Site C, located uphill the area, did not show any significant pollution and was therefore used as control site (Table 1).

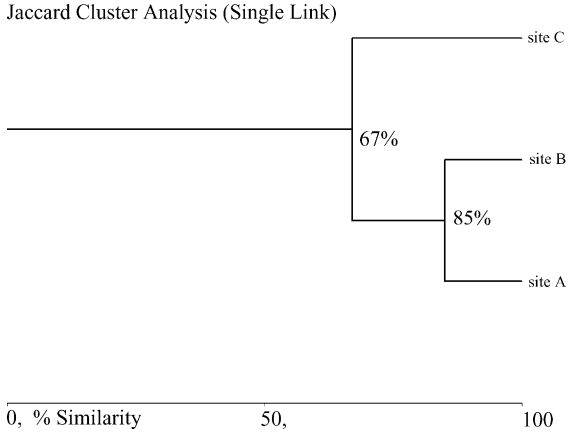


Fig. 2. Dendrogram indicating relationship among soil samples according to substrate utilization patterns obtained by using Biolog GN MicroPlates. Soil sites were compared by using Biodiversity Pro software. The tree was constructed by using Jaccard distance equation and single linkage method.

3.2. Substrate utilization patterns of soil microbial populations

The metabolic profile of the soil extracts from each site was followed for seven days and hierarchical clustering was performed each day. A higher metabolic versatility was observed in A (73 positive wells) and B (70 positive wells) sites than in C site (53 positive wells). The comparison of the substrate utilization pattern of each site at seven days showed a high similarity (J_i = 85%) between the sites A and B. As a consequence, site C clustered alone having a 67% of similarity (J_i) with the cluster including A and B sites (Fig. 2). No variations in the clustering were recorded during the time lapse (data not shown).

3.3. Soil enzymatic activities

Cellulase and β-glucosidase activities were present in all the sites, while xylanase was found only in site C. Site B showed the highest amount of cellulase activity, while β-glucosidase activity was significantly higher in site C (Table 2). Phosphomonoesterase activities were detected in all the sites. The activities of acid, alkaline and neutral phosphomonoesterase were significantly higher in site C than in sites A and B. In particular, in site C, alkaline phosphomonoesterase activity was more than eight times higher than that observed in site A and twice that measured in site B (Table 2). Neutral phosphomonoesterase activity in site C was five times higher than that measured in site A and three times higher than that measured in site B (Table 2). In general, site C showed the highest amount of enzymatic activities (Table 2).

Table 2
Quantitative analysis of soil enzymatic activities

	Site A	Site B	Site C
Cellulase[a]	19.63 ± 0.24 a	25.13 ± 0.13 c	20.95 ± 0.10 b
Xylanase[a]	0.00 ± 0.00 a	0.00 ± 0.00 a	14.66 ± 0.19 b
β-Glucosidase[b]	79.25 ± 0.23 b	48.58 ± 0.41 a	84.72 ± 0.51 c
Acid phosphomonoesterase[b]	26.36 ± 0.89 b	24.36 ± 0.14 a	38.31 ± 0.16 c
Alkaline phosphomonoesterase[c]	168.24 ± 17.11 a	675.20 ± 10.02 b	1414.60 ± 25.50 c
Neutral phosphomonoesterase[c]	60.75 ± 8.13 a	103.23 ± 10.33 b	336.83 ± 23.44 c
Urease[d]	472.35 ± 25.17 b	0.00 ± 0.00 a	0.00 ± 0.00 a

Values (mean value ± standard deviation) designated with same letters (a–c) are not significantly different ($P \leq 0.05$) according to Fisher's least significant difference test.
[a] Measured as μg glucose ml^{-1} h^{-1}.
[b] Measured as μg *p*-nitrophenol ml^{-1} h^{-1}.
[c] Measured as μg phenol ml^{-1} h^{-1}.
[d] Measured as μg ammonium ml^{-1} h^{-1}.

The similarity (J_i) of the soil sites, by considering the hierarchical clustering of soil enzymatic activity profiles, was 83.3% both between sites A and B, and B and C, whereas between A and C sites was 71.4% (data not shown).

3.4. Bacterial density

The bacterial densities evaluated as log cfu g^{-1} of fresh soil did not differ significantly in each site (6.75 in site A, 6.70 in site B and 6.70 in site C). In all the sites, a prevalence of *r*-strategist, fast growing strains, was observed: 91.82% in site A, 87.41% in site B and 79.59% in site C. The density of 2,4-dichloroaniline degrading bacteria among the sites did not differ significantly (3.70 in site A, 3.48 in site B and 3.43 in site C) and was about 3 log order lower than that of the whole bacterial density. The percentage of 2,4-dichloroaniline degrading bacteria on the whole aerobic culturable population was 0.09% in site A, 0.06% in site B and 0.05% in site C (data not shown).

3.5. Identification of bacterial strains and community structure

The bacterial community of site A was composed by 49% Gram-negative non-enteric, 51% of Gram-positive (46% spore-forming and 5% non-spore-forming) bacteria. The richness and the diversity of bacterial species evaluated as D_{Mg} and H' index were 2.73 and 2.45, respectively. Among non-enteric Gram-negative, pseudomonads group was the most abundant with five species identified (*Pseudomonas maculicola* 5%, *P. fluorescens biotype F* 5%, *P. fluorescens* 5%, *P. corrugata* 5% and *P. alcaligenes* 9%). Among non-enteric Gram-negative bacteria, two strains of *Sinorhizobium meliloti* (10%) and *Sphingobacterium spiritivorum* (10%) were identified. The genus *Bacillus* was the 32% of the population with a prevalence of *Bacillus cereus* (18%), followed by *B. mycoides* (9%) and *B. subtilis* (5%). Among the other Gram-positive spore-forming bacteria, one strain of *Brevibacillus brevis* (5%) and two strains of *Paenibacillus pabuli* (9%) were identified. Only one strain of Gram-positive non-spore-forming bacteria was found and identified as *Arthrobacter histidinolovorans* (5%) (Table 3).

The bacterial community of site B was composed by 74% of Gram-negative (69% Gram-negative non-enteric and 5% of Gram-negative enteric), and 26% Gram-positive bacteria (13% spore-forming and 13% non-spore-forming). The richness and the diversity of bacterial species evaluated as D_{Mg} and H' index were 2.91 and 2.54, respectively. The pseudomonads group was the most abundant with nine species identified (*P. syringae pv. aptata* 9%, *P. putida biotype B* 5%, *P. marginalis* 5%, *P. maculicola* 13%, *P. fluorescens biotype F* 13%, *P. corrugata* 5%, *P. auriantica* 9%, *Xanthomonas campestris pv. malvacearum* 5% and *Stenotrophomonas maltophilia* 5%). Only one strain of Gram-negative enteric bacteria were isolated and identified as *Serratia fonticola* (5%). Among Gram-positive spore-forming bacteria, two strains of *Bacillus megaterium* (8%)

Table 3
Relative abundance of microbial species in each site

	Site A (%)	Site B (%)	Site C (%)
Arthrobacter histidinolovorans	5		
Aureobacterium saperdae		8	
Cellulomonas cellulans		5	
Bacillus amyloliquefaciens			10
Bacillus cereus	18		
Bacillus circulans			10
Bacillus megaterium		8	
Bacillus mycoides	9		5
Bacillus pumilus			18
Bacillus subtilis	5		
Brevibacillus brevis	5		
Paenibacillus pabuli	9	5	
Pseudomonas alcaligenes	9		
Pseudomonas auriantica		9	
Pseudomonas corrugata	5	5	
Pseudomonas fluorescens	5		
Pseudomonas fluorescens biotype A			5
Pseudomonas fluorescens biotype F	5	13	
Pseudomonas maculicola	5	13	
Pseudomonas marginalis		5	
Pseudomonas putida biotype A			5
Pseudomonas putida biotype B		5	
Pseudomonas syringae pv. aptata		9	10
Stenotrophomonas maltophilia		5	
Xanthomonas campestris pv. malvacearum		5	13
Xanthomonas campestris pv. poinsetticola			14
Agrobacterium rhizogenes			5
Sinorhizobium meliloti	10		
Sphingobacterium spiritivorum	10		
Klebsiella planticola			5
Serratia fonticola		5	

and one of *P. pabuli* (5%) were identified, while among Gram-positive non-spore-forming bacteria, two strains of *Aureobacterium saperdae* (8%) and one of *Cellulomonas cellulans* (5%) were found (Table 3).

The bacterial community of site C was composed by 57% of Gram-negative (52% Gram-negative non-enteric and 5% of Gram-negative enteric), and 43% Gram-positive spore-forming bacteria. The richness and the diversity of bacterial species evaluated as D_{Mg} and H' index were 2.28 and 2.27, respectively. The pseudomonads group was the more abundant (47%) with five species identified (*P. syringae pv. aptata* 10%, *P. putida biotype A* 5%, *P. fluorescens biotype A* 5%, *X. campestris pv. poinsetticola* 14% and *X. campestris pv. malvacearum* 13%). Among non-enteric Gram-negative, one strain of *Agrobacterium rhizogenes* (5%) was identified. Only one strain belonging to the enteric group was identified as *Klebsiella planticola* (5%). The *Bacillus* genus was well represented (43%) by five species (*B. amyloliquefaciens* 10%, *B. circulans* 10%, *B. subtilis* 5%, *B. mycoides* 5% and *B. pumilus* 18%; Table 3).

Spatial distribution of species, based on their frequency, obtained by correspondence analysis showed that four species (*P. fluorescens biotype F*, *P. corrugata*, *P. maculicola*, *P. pabuli*) were shared between A and B sites, two species (*P. syringae pv. aptata* and *X. campestris pv. malvacearum*) between B and C sites and only one specie (*B. mycoides*) between A and C sites (Fig. 3A). Cluster analysis showed a higher similarity between A and B sites ($J_i = 17.4\%$) than with the site C ($J_i = 8.7\%$) (Fig. 3B).

3.6. Identification of 2,4-di-chloroaniline degrading bacteria

The selection of bacterial strains able to grow on 2,4-di-chloroaniline as sole carbon source was performed on M9 medium added with the pollutant. The *Bacillus* genus was predominant in site A with three species identified (*B. circulans* 23.6%, *B. maroccanus* 17.6% and *B. megaterium* 17.6%). Strains belonging to the species *P. pabuli* (17.6%) and *Rhodococcus erythropolis* (23.6%) were also detected in site A. Site B showed the lowest richness in species being composed by *Corynebacterium liquefaciens* (50%) and two species of *Rhodococcus* (*R. erytropolis* and *R. fascians* with a frequency of 35% and 15%, respectively). Bacterial strains able to grow on the pollutant recorded in site C were *B. maroccanus* (41.2%), *B. megaterium* (17.6%), *Cellulomonas cellulans* (17.6%) and *P. fluorescens biotype A* (23.5%) (Table 4).

Cluster analysis (Fig. 4) showed a higher similarity between A and C sites ($J_i = 28.4\%$) than with B site. Site B clustered alone with 14.1% of similarity (J_i) with A and C sites.

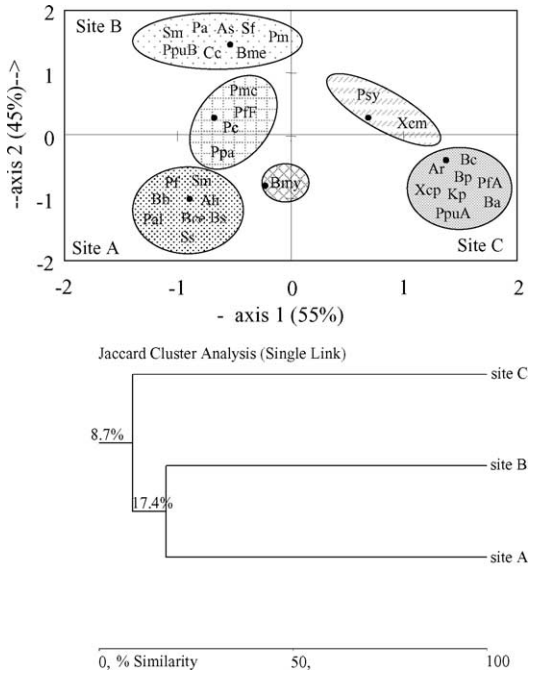


Fig. 3. Correspondence analysis performed on the bacterial identifications by xLSTAT 2.5 software, indicating the bacterial species shared among the three sites. Abbreviation used: Ah, *Arthrobacter histidinolovorans*; As, *Aureobacterium saperdae*; Cc, *Cellulomonas cellulans*; Ba, *Bacillus amyloliquefaciens*; Bce, *Bacillus cereus*; Bci, *Bacillus circulans*; Bme, *Bacillus megaterium*; Bmy, *Bacillus mycoides*; Bp, *Bacillus pumilus*; Bs, *Bacillus subtilis*; Bb, *Brevibacillus brevis*; Pca, *Paenibacillus pabuli*; Pal, *Pseudomonas alcaligenes*; Pa, *Pseudomonas auriantica*; Pc, *Pseudomonas corrugata*; Pf, *Pseudomonas fluorescens*; Pfa, *P. fluorescens biotype A*; PfF, *P. fluorescens biotype F*; Pmc, *Pseudomonas maculicola*; Pmr, *Pseudomonas marginalis*; PpuA, *Pseudomonas putida biotype A*; PpuB, *P. putida biotype B*; Psy, *Pseudomonas syringae pv. aptata*; Sml, *Stenotrophomonas maltophilia*; Xcm, *Xanthomonas campestris pv. malvacearum*; Xcp, *X. campestris pv. poinsetticola*; Ar, *Agrobacterium rhizogenes*; Sme, *Sinorhizobium meliloti*; Ss, *Sphingobacterium spiritivorum*; Kp, *Klebsiella planticola*; Sf, *Serratia fonticola* (a). Dendrogram indicating relationship among soil sample according to bacterial identification; soil sites were compared by using Biodiversity Pro software; the tree was constructed by using Jaccard distance equation and single linkage method (b).

4. Discussion

The ACNA site, developed in an area of 55 ha, represented a big environmental and social problem having caused pollution of air, surface water (Bormida River), ground water and soil other than heavy health effects and death among the factory workers. In particular, 2,500,000 m^3 of wastes and contaminated materials and 13 lagoons containing 300,000 m^3 of saline substances are contributing to the pollution of soil and water. This polluted site, officially declared ''polluted site of national importance'', is characterized by: (i) temporal variability: different kind of production activities related to different chemical pollutants occurred during the time and (ii) spatial variability: the industrial area was subdivided in sectors each one specialized in a different production and therefore being characterized by a different kind of pollutant. Therefore, the peculiarity of this area is

Table 4
Relative abundance of microbial species able to grow on 2,4-dichloroaniline in each site

	Site A (%)	Site B (%)	Site C (%)
Bacillus circulans	23.6		
Bacillus maroccanus	17.6		41.3
Bacillus megaterium	17.6		17.6
Paenibacillus pabuli	17.6		
Rhodococcus erytropolis	23.6	35	
Rhodococcus fascians		15	
Cellulomonas cellulans			17.6
Corynebacterium liquefaciens		50	
Pseudomonas fluorescens biotype A			23.6

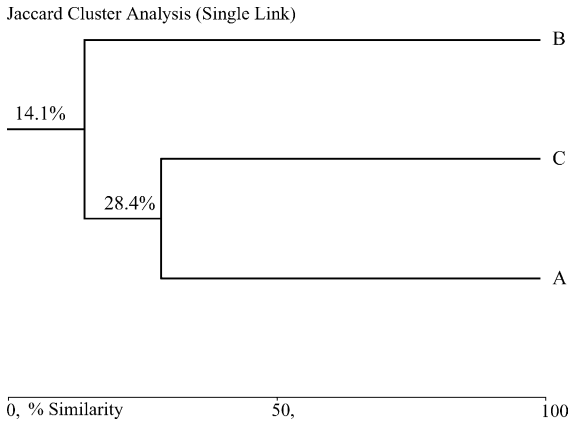


Fig. 4. Dendrogram indicating relationship among soil samples according to identification of bacterial strains able to grow on mineral medium added with 2,4-dichloroaniline as sole carbon source. Soil sites were compared by using Biodiversity Pro software. The tree was constructed by using Jaccard distance equation and single linkage method.

represented by its long pollution history and by the wide range of available sites, each one characterized by the presence of different classes of pollutants.

As pointed out by chemical analysis performed at 25 cm and 1 m of depth, site A was mainly polluted by arsenic and copper, while site C did not show any contamination. More complex was the situation of site B where soil chemical analysis at 1 m of depth pointed out high amount of arsenic, mercury, 2,4-dichloroaniline and 1,2,3,4-tetrachlorobenzene, while these pollutants were found at 25 cm of depth at a lower concentration than the guideline values for industrial area. This is related to an awkward tentative to stock the factory wastes, performed in this site several years ago, by top filling the pile with 50 cm of non-polluted soil. At the moment, the sites A and B have been dismantled and the wastes cemented.

In order to characterize the soil health *status* of these three sites, chemical analysis was coupled to biological analysis through the comparison of the soil metabolic and enzymatic profiles, the bacterial densities, the copiotrophic/oligotrophic ratio and the species richness and diversity.

The metabolic pattern of the soil extract from each site has been characterized by using carbon metabolic pattern (Biolog GN MicroPlates). The polluted sites A and B showed a wider versatility than the control site C. In agreement with this result, a higher percentage of oligotrophic bacteria, indicating a higher stability, was observed in site C. Even more, the presence of inorganic and/or organic pollutant seems to induce shifts in the bacterial community structure by selecting specific bacterial group more adapted to environmental stress or variability. This finding agree with the results of Müller et al. (2001a) showing an adaptation of the soil community related to the mercury exposure level in soil, due to an increased tolerance to this heavy metal. On the contrary, Knight et al. (1997) observed a reduction in carbon source utilization in soils characterized by low pH and amended with Zn or Cu compared to non-polluted soil. Moreover, shifts of carbon substrate utilization patterns were recorded also in soils contaminated by spindle oil and related to the development of a hydrocarbon utilizing bacterial community (Wünsche et al., 1995). Different enzymatic activities involved in C, P and N cycle were evaluated by in vitro quantitative assays on soil extract. Modifications in soil enzyme activity pattern mirror alterations of microbial activity, microbial community structure and environmental conditions. Therefore, the characterization of some enzymatic activities has been proposed as soil health indicator (Tate, 1995). In general, the highest values of enzymatic activities were recorded in the control site C indicating the presence of a more active microflora, including both bacteria and fungi, in this site than in the polluted ones. Reductions or inhibition of dehydrogenase and phosphatase activity in soils contaminated by polycyclic aromatic hydrocarbon and/or by heavy metals have also been reported by Maliszewska-Kordybach and Smreczak (2003). On the contrary, Pozo et al. (2003) showed that the addition of 3,3′-diaminobenzidine to soil increased both numbers of culturable bacteria and acid and alkaline phosphatase, arylsulphatase and dehydrogenase activities. However, these works, based one the use of carbon metabolic profile and enzymatic activity and performed on soil microcosm conditions could not fully reflect the real situation typical of a polluted soil in which a whole of chemical, physical and biological parameters could affect the ecosystem. Although the density of culturable bacteria did not vary in the three sites, differences in the structure of the bacterial community were observed. This result indicates that the presence of different chemical pollutants does not affect the bacterial density but rather influences the structure of the bacterial community. Decreases of the bacterial density in polluted soils have been reported many times (Müller et al., 2001a,b; Ellis et al., 2002; Yao et al., 2003). In fact, the presence of a pollutant induce an immediate tolerance increase due to its initial toxicity, followed by a more gradual enhancement due to the different competitive capability of surviving bacteria and to their physiological and genetic adaptation (Van der Meer, 1994; Müller et al., 2001a,b; Díaz-Raviña and Bååth, 2001). Alterations of microbial community structure were recorded by comparing the phospholipid fatty acid profile in heavy metals polluted and non-polluted soils (Frostegård et al., 1996; Bååth et al., 1998). However, referring to the long life history of the ACNA factory, we can speculate that an evolution of bacterial species adapted to those particular environmental conditions occurred.

Bacterial identifications showed that the polluted sites A and B were characterized by higher species richness and diversity compared to control site C. This result is supported by the fact that any disturbance of soil will lead to an initial decrease of the biodiversity followed by an increase of the biodiversity due to the achievement of a new dynamic equilibrium (Van Bruggen and Semenov, 2000). Even more, a higher amount of *k*-strategist bacteria, typical of stable environmental situation and characterized by a lower adaptability (Panikov, 1999), was recorded in site C than in A and B. Although the Pseudomonadaceae and Bacillaceae families were prevalent in each soil site, only few bacterial species were shared among the sites. In fact, as shown also by the hierarchical clustering analysis, the control site C clustered alone suggesting that the presence of organic and/or inorganic chemical pollutant could select specific bacterial groups characterized by a high fitness with the environment in which they live (Van der Meer, 1994; Müller et al., 2001a,b; Díaz-Raviña and Bååth, 2001; Top and Springael, 2003). Altogether, our results (soil metabolic profiles, enzymatic activities and bacterial community structure) pointed out a higher similarity between A and B sites than with C site. This is consistent with the hypothesis that all the bioindicators used in this study could be affected by the presence of pollutants even if their concentration is under the guideline values. Even more, synergistic effects of the different kind of pollutants on the biological parameters analyzed cannot be ruled out.

Anilines and their derivatives, involved in the production of dyes, plastics, herbicides and pesticides, are increasingly released into the environment and are known to be toxic and carcinogenic to living organisms. Among the other detected pollutants, aromatic amines were the most spread inside the ACNA area and the most damaging for human health having caused the death of about 15 workers due to bladder cancer (Puntoni et al., 1988). In order to select microorganisms able to use 2,4-dichloroaniline as sole carbon source and therefore potentially useful for a future bioremediation process, bacterial strains from each site were isolated on minimal medium added with the xenobiotic. As shown by hierarchical analysis, site B, polluted by heavy metals, tetrachlorobenzene and 2,4-dichloroaniline, clustered alone, having no species in common with A and C sites. These results indicate that the presence of 2,4-dichloroaniline could select specific bacterial groups able to use this xenobiotic as carbon source. In particular, two species of *Rhodococcus* and one of *Corynebacterium*, belonging both to the order Actinomycetales, were isolated in the site B. These two genus are well known under a biotechnological point of view to have wide broad metabolic diversity and array of unique enzymatic capabilities (Warhurst and Fewson, 1994; Jung-Hyun et al., 2001), making them suited for bioremediation due to their capacity for long term survival in soil, their exceptional ability to degrade pollutants even in the presence of more readily assimilable carbon sources (Plette et al., 1996; Suzuki et al., 1996). In fact, different Authors underlined the degradative activity of Rhodococci and Corynebacteria against different classes of anilines (Travkin et al., 2002; Fuchs et al., 1991; Suzuki et al., 1996). Our preliminary results showed that *C. liquefaciens* and the two *Rhodococcus* strains tolerate concentration of 2,4-dichloroaniline of 4 and 3.5 g l^{-1} in the growth medium, respectively. Further experiments are in progress in order to obtain more information on the degradative capability of 2,4-dichloroaniline by bacterial strains isolated by site B.

The biological parameters analyzed suggested, in agreement to chemical analysis, that A and B sites are stressed environment and not healthy soils. Each considered bioindicator implies characteristic advantages and disadvantages. Several limitations on the utilization of carbon metabolic profile obtained by Biolog assay both as indicator of soil health and as identification system, due to its low reproducibility, culture and inoculum density dependency and the difficult result interpretation, have been underlined by several Authors (Haack et al., 1995; Smalla et al., 1998; Preston-Mafham et al., 2002). However, this assay is not time-consuming and is characterized by relatively low cost and could provide useful quali-quantitative information when a high number of samples are screened. Moreover, the bacterial identification database has been recently improved and enriched. Therefore, this rapid identification system seems to be a useful tool for bacterial identification and comparison of microbial community structure.

Studying soils with different chemico-physical properties and pollution, the response variability of

soil enzyme use of as indicator of soil health, has to be considered (Trasar-Cepeda et al., 2000). Since the involvement of fungal enzymatic activities, this indicator is not fully comparable with carbon metabolic pattern obtained by Biolog system in which the fungal response is ruled out due to their inability to oxidize tetrazolium salt. On the other side, considering both microbial and fungal enzymatic activities, the analysis of this indicator could provide more complete information on soil quality and health. Finally, the choice of the right set of enzymatic activities is required in order to improve the knowledge on the real soil *status*.

In conclusion, the use of different biological indicators, such as soil metabolic profile and enzymatic activity pattern, bacterial density, copiotrophic/oligotrophic ratio, bacterial species diversity and richness, seems to be more efficient and informative than the analysis of single parameters. Therefore, the combination of different indicators of the soil *status* could be a useful tool providing an integrated measure of soil health. However, further studies on the standardization of the methods would be advisable to improve the interpretation of each soil health indicator.

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