MOLECULAR CHARACTERIZATION OF THE MICROBIAL COMMUNITY INVOLVED IN THE CARBON CYCLE IN DIFFERENT AREAS AND TYPES OF SOIL MANAGEMENT

CARACTÉRISATION MOLÉCULAIRE DE LA COMMUNAUTÉ MICROBIOLOGIQUE IMPLIQUÉE DANS LE CYCLE DU CARBONE PROVENANT DE ZONES ET DE SYSTÈMES DE GESTION DE SOLS VARIÉS

CARATTERIZZAZIONE MOLECOLARE DELLA COMUNITÀ MICROBICA COINVOLTA NEL CICLO DEL CARBONIO IN AREE E SISTEMI GESTIONALI DEL SUOLO DIVERSI

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Summary

This paper addresses the diversity of two soil bacterial groups involved in the biogeochemical carbon cycle: bacteria implicated in chitin degradation and methanotrophs. To evaluate the influence of soil physico-chemical and anthropic characteristics on the diversity of these microbial groups, total DNA was directly extracted from soils differently managed and sampled in central and south Italy. PCR-Denaturing Gradient Gel Electrophoresis (DGGE) analyses targeting genes coding for chitinase (*chiA*), particulate methane monooxygenase (*pmoA*) and 16S rRNA from bacteria, actinomycetes and type I or II methanotrophs were used to fingerprint the soil bacterial communities. DGGE cluster analysis showed a clear separation of the bacterial communities on the basis of the sampling sites. The Canonical Corrispondance Analysis (CCA) suggests that the edaphic factors such as granulometry and pH, could be responsible for determining the composition of these bacterial groups.

Keywords: *metanotrophs, chitinase, DGGE.*

Résumé

Cet article concerne deux groupes microbiotiens clés impliqués dans le cycle du carbone: ceux qui dégradent de la chitine et les méthanotrophes. Les expériences, effectuées par l' extraction directe de l'ADN et par l'analyse DGGE (électrophorèse sur gel en gradient dénaturant) se sont déroulées sur divers systèmes de sols sujets à différents modes de gestion et répartis dans plusieurs zones de l'Italie centrale et

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méridionale avec l'objectif de comprendre l'influence des facteurs environnementaux sur la structure de ces groupes microbioens. Ont été étudiés les gènes 16S rARN de bactéries, actinomycètes et méthanotrophes des types I et II et les gènes fonctionnels qui codent pour la chitinase (*chiA*) et pour la sous-unité catalytique de la méthano-monooxygénase insoluble (*pmoA*). Le site d'échantillonnage a montré une plus grande influence que le mode de gestion des sols. L'analyse canonique des correspondances (ACC) a mis en évidence une corrélation significative de ces groupes microbiens avec la granulométrie et le pH des sols.
Mots-clés: *méthanotrophes, chitinase, DGGE.*

Riassunto

Si riporta lo studio di due gruppi microbici chiave del ciclo del carbonio: i degradatori della chitina e i metanotrofi. Allo scopo di comprendere l'influenza dei fattori ambientali e antropici sulla diversità di questi gruppi, sono state effettuate prove, mediante l'estrazione diretta del DNA dal suolo e l'analisi DGGE (Denaturing Gradient Gel Electrophoresis), prendendo in esame suoli distribuiti in Italia centrale e meridionale e soggetti a vari sistemi gestionali. Sono stati considerati i geni codificanti per l'enzima chitinasi (*chiA*), la metano monossigenasi insolubile (*pmoA*) e l'rRNA 16S di batteri, attinomiceti e metanotrofi di tipo I e II. Il sito di campionamento mostra una maggiore influenza sulla diversità delle comunità batteriche rispetto al tipo di gestione, mentre i fattori edafici quali la granulometria ed il pH del suolo sono responsabili della composizione e diversità di queste comunità.
Parole chiave: *metanotrofi, chitinasi, DGGE.*

Introduction

Soil is a large sink for organic carbon within the terrestrial biosphere. Practices which cause a decline in soil organic matter are also responsible of damages to soil resilience and, often, to agricultural productivity (Powlson *et al*., 2001). The soil microorganisms are the principal agents of transformation of soil organic matter and their activities are greatly influenced by soil physico-chemical properties and ecological interactions (Powlson *et al*., 2001; Terahara *et al*., 2009).
In present study we selected soils under different managements (intensive tree plantations, lands fit for seed, pasture and forests) distributed in some areas of central and south Italy to investigate the influence of soil physico-chemical characteristics on richness and diversity of soil bacteria and, in particular, of two bacterial groups involved in carbon cycle: the bacteria implicated in chitin degradation and the methanotrophs. Chitin is the second most abundant polysaccharide in nature and its degradation represents a key step of carbon cycle in soil ecosystems and contributes to the atmospheric release of CO_2, implicate in global warming (Terahara *et al*., 2009). Methane oxidation plays an important role

in reducing the atmospheric CH_4 emission, an other potent greenhouse gas (Henckel *et al*., 1999).
Total DNA was directly extracted from soil and a molecular approach, based on PCR-DGGE targeting genes for chitinase (*chiA*), particulate methane monooxygenase (*pmoA*) and 16S rDNA from bacteria, actinomycetes, and type I or II methanotrophs, was performed to fingerprint the soil bacterial communities.

Materials and Methods

Agugliano (AN) experimental area was set up in 1994 at the farm "Pasquale Rosati" of the Faculty of Agriculture of the Polytechnic University of Marche, on a hillside (central Italy, 100 msl). The site was managed under a *Triticum durum* and *Zea mais* rotation. A split-plot with randomized blocks was designed to compare two tillage techniques (conventional 40 cm deep ploughing; no tillage with chemical desiccation and chopping) and two fertilizer treatments (0 Kg/ha NH_4NO_3 – 70 Kg/ha P_2O_5; 90 Kg/ha NH_4NO_3 – 70 Kg/ha P_2O_5). Berchidda (OT) experimental area was located in a hilly basin (south Italy, 275-300 msl). In the area different soil managements lead to the creation of an ecological progression from an intensive agricultural practice (vineyard with conventional tillage, TV) to a conservative condition (Mediterrean forest with cork oak, CO), throw intermediate steps (grass-covered vineyard, GV; temporary grassland, TG; pasture, PA). TV was established in 1994, cv Vermentino, trained as single guyot (5-7 buds/plant) and manured with organic fertilizer. GV was established in 1985, cv. Vermentino, trained as purred cordon (12-15 buds/plant), no tillage, foliar fertilization and the grass covered with freely self-sown. TG, PA and CO were established 30 years ago. At the time of sampling TG have been planted with common oat, Italian ryegrass and clover. In PA, were present mainly *Hordeum leporinum, Trifolium michelianum, T. subterraneu*, and *T. resupinatum.* TG and PA were pastured with sheep. CO was dominated by shrub cover and distributed *Quercus suber* L. trees. Viggiano (PZ) experimental area (south Italy, 970 msl) consisted of two farms cultivated with barley and temporary grassland (*Trifolium* with sorghum and vetch). Organic plots were fertilized by spreading liquid manure and conventional plots by chemical fertilizers. Montecuccioli (FI) experimental area (central Italy, 150 msl) was located in a farm managed under olive grove. The randomize blocks were designed to compare two different managements (conventional tilled; grass covered with freely self-sown), all plots were treated with mineral fertilization. Fagna (FI) experimental area (central Italy, 285 msl) was set up in 1994 at the farm of the Research Centre for Agrobiology and Pedology (CRA-ABP). The site was managed under *Zea mais* as monoculture (conventional 40 cm deep ploughing; fertilization N 150Kg/ha, P_2O_5 and K_2O 125Kg/ha) or *Triticum aestivum* and *Vicia faba* rotation (conventional 40 cm deep ploughing; fertilization N 100Kg/ha, P_2O_5 150 Kg/ha). In May 2007 from bulk soil of each managements three-five soil

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replicates were collected at 0-20 cm depth. The soil physical and chemical characteristics of the experimental areas are reported in Table 1.

Table 1 - *Physical and chemical soil characteristics. T, conventional tillage; S, no tillage; 0, without nitrogen fertilization; 90, with nitrogen fertilization; TV, vineyard with conventional tillage; GV, grass-covered vineyard; TG, temporary grassland; PA, pasture; CO, cork oak forest; OB, organic barley; OC, organic clover; TB, traditional barley, TC, traditional clover; TO, conventional tilled olive-grove; GO, grass covered olive-grove; BB, broad bean; W, wheat; M, mais.*

Site	Management	Sand	Silt	Clay	Texture	pH
Agugliano	Wheat-mais rotation (S0)	10,5	51,2	38,3	Silty-clay	8,4
	Wheat-mais rotation (S90)	10,3	40,3	49,4	Clay	8,4
	Wheat-mais rotation (T0)	24,8	37,3	37,9	Clay loam	8,1
	Wheat-mais rotation (T90)	24,9	35,3	39,8	Clay	8,1
Berchidda	Tilled vineyard (TV)	83,0	4,8	12,2	Loamy -sand	5,1
	Grass covered vineyard (GV)	80,2	8,4	11,4	Loamy -sand	6,2
	Temporary grassland (TG)	73,4	13,8	12,8	Loamy -sand	5,6
	Pasture (PA)	73,2	13,2	13,6	Loamy -sand	5,4
	Cork oak forest (CO)	73,6	13,4	13,0	Loamy -sand	5,9
Viggiano	Traditional barley (TB)	35,9	30,9	33,2	Clay loam	7,5
	Traditional clover (TC)	35,9	29,4	34.7	Clay loam	7,5
	Organic barley (OB)	25,1	38,8	36,1	Clay loam	7,5
	Organic clover (OC)	27,2	34,0	38,8	Clay loam	7,5
Montecuccioli	Tilled olive-grove (TO)	18,4	46,8	34,9	Silty clay loam	7,4
	Grass covered olive-grove (GO)	21,1	40,1	38,8	Clay	7,3
Fagna	Broad bean (BB)	48,5	37,4	14,2	Loam	8,4
	Wheat (W)	50,3	35,3	14,5	Loam	8,4
	Mais (M)	49,6	34,7	15,7	Loam	7,2

DNA was extracted from soil samples using the DNA Spin Kit for Soil (BIO 101 System Q-Biogene) following the manufacturer's instructions. PCR amplifications were performed with primers targeting genes for bacterial 16S rRNA (986f and Uni1401r; Felske *et al*., 1998); actinomycetal 16S rRNA (P243f and P518r; Ørreas et al., 1997; Heuer et al., 1997); methanotroph type I 16S rRNA (MB10γ and 533R; Henckel *et al*., 1999); methanotroph type II 16S rRNA (MB9α and 533R; Henckel *et al*., 1999); chitinase, *chiA* (GA1F and GASQr; Williamson *et al*., 2000); catalytic subunit of particulate methane monossigenase, *pmoA* (A184 and MB661; Costello *et al*., 1999; Krief *et al*., 2005). DGGE analysis were carried out using Ingeny phorU-2 System (Ingeny International BV) on a 6% polyacrylamide gel (acrylamide/bis ratio, 37.5:1), under denaturation conditions, urea 7 M and 40% formamide, with denaturing gradient ranging from 46 to 58% and 40-70 for 16S rRNA and functional genes, respectively. Actinomycetal 16S rDNA DGGE was carried out on a 8% polyacrylamide gel with a denaturing gradient ranging from 40 to 70%. Gel runs were carried out as previously described (Pastorelli *et al*., 2009).
DGGE profiles were analysed by Gel Compar II Software v 4.6 (Applied Maths). Differences of richness (number of bands), Shannon-Weiner index and Simpson index were statistically validated using the two-ways ANOVA and averages were compared with Tukey-Kramer test ($P<0.01$; $P<0.05$). Genetic similarities of the

bacterial communities in the different soils were determined with UPGMA (Unweighted Pair Group Mathematical Average) using Dice coefficient.
A canonical correspondence analysis (CCA) using direct gradient analysis was done by using PAST program software (http://folk.uio.no/ohammer/past). Measures of populations (richness values, Shannon-Weiner index and Simpson index) were treated as species variables and compared with measures of soil variables (texture, soil pH). The statistical significance of the relationship between species and soil variables was assessed by permutation test of both the first ordination axis and the combination of both the first and second axes.

Results and discussion

Richness, Shannon-Weiner index and Simpson index derived from DGGE profiles and their statistical analyses are reported in figure 1.
The mean number of bands in 16S rDNA-DGGE analyses of total bacterial communities were from 6.2±0.95 (Agugliano) to 10±1 (Montecuccioli) and no significant differences resulted among the different experimental areas for any species variable considered. The mean number of bands of actinomycetal 16S rDNA-DGGE profiles were from 5.8±0.49 (Berchidda) to 10.5±0.65 (Viggiano) and highly significant differences ($P<0.01$) could be observed in richness and diversity (Shannon-Weiner and Simpson indices) between soil sampled in the different experimental areas. Methanotrophs type II, resulted more abundant in our soils than type I, according to statements from Rastogi *et al*. (2009) and Abell *et al*. (2009). The mean number of bands of methanotrophs 16SrDNA-DGGE profiles ranged from 2.8±0.37 (Berchidda) to 11.67±1.67 (Fagna) and from 1±0 (Montecuccioli) to 3.5±0.29 (Agugliano) for type II and type I, respectively. Highly significant differences were obtained among soils from the different experimental areas both for richness and diversity. DGGE profiles obtained with partial fragments of the functional genes *chiA* and *pmoA* showed a lower number of bands (ranged from 1 to 3 and 0-6, respectively). No significant differences were found among the different soil managements, while significant differences were obtained among the different experimental areas. The mean number of bands varying from 1.33±0.33 (Fagna) to 2.5±0.29 (Viggiano) for *chiA* gene and from 0.67±0.33 (Fagna) to 4.75±0.48 (Viggiano) for *pmoA*. *pmoA* gene showed an highly significant difference for richness and Shannon-Weiner index.
UPGMA analysis of DGGE patterns derived from bacterial 16S rDNA, generates separate clusters depending on sampling site and not on the management (Fig. 2A). In actinomycetal 16S rDNA dendrograms (Fig. 2B) separate clusters could be observed only for Berchidda and Agugliano experimental areas while methanotrophs type II made up separate clusters for Agugliano, Berchidda and Fagna (Fig. 2D) sites. Functional genes did not generate any separate cluster depending on sampling site or soil management (Fig. 2E and F).

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Figure 1 - *Richness, Shannon-Weiner index, and Simpson index bacterial communities according to sampling site. Letters (a, b) stand for statistically significant differences (Tukey-Kramer test p<0.05).*

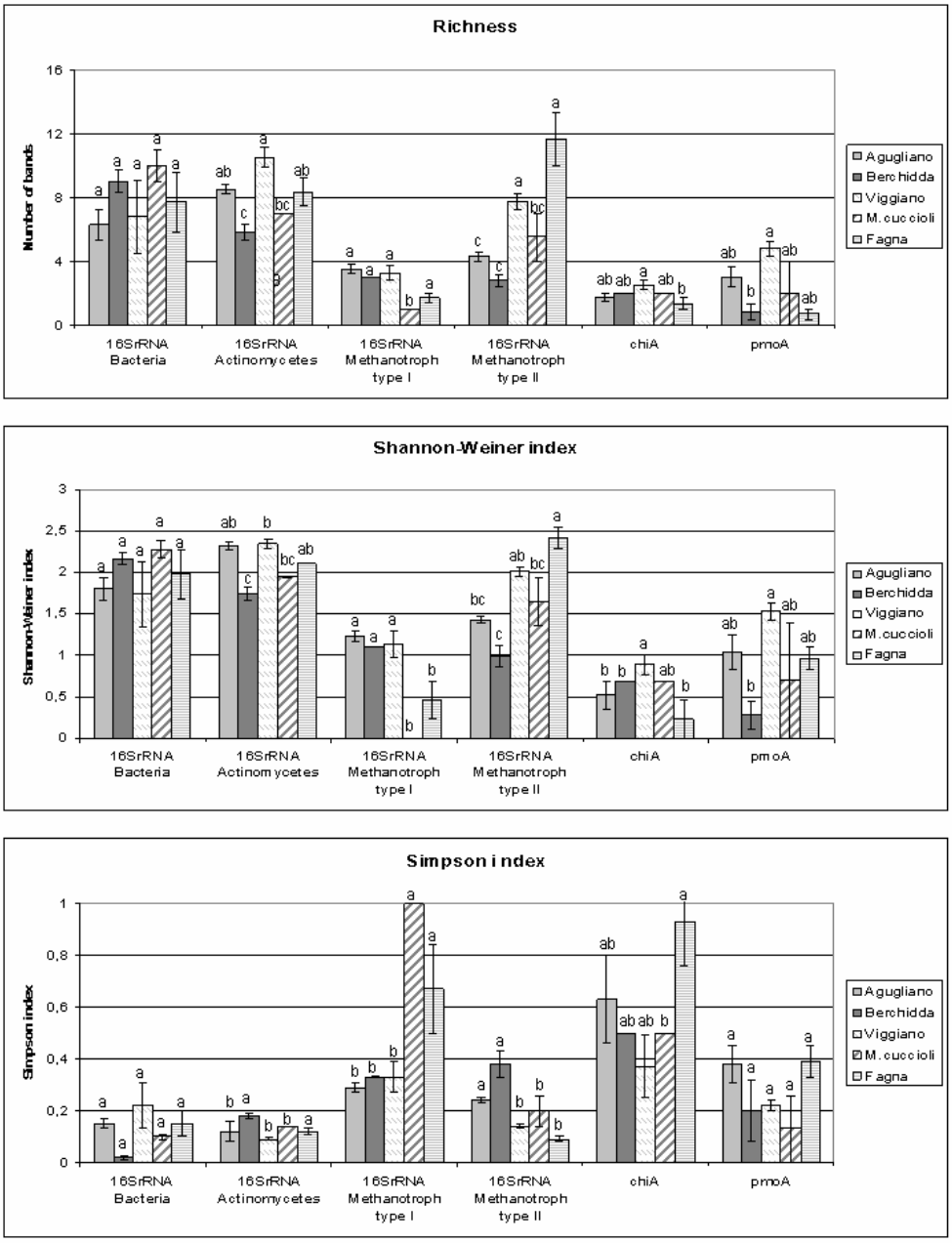
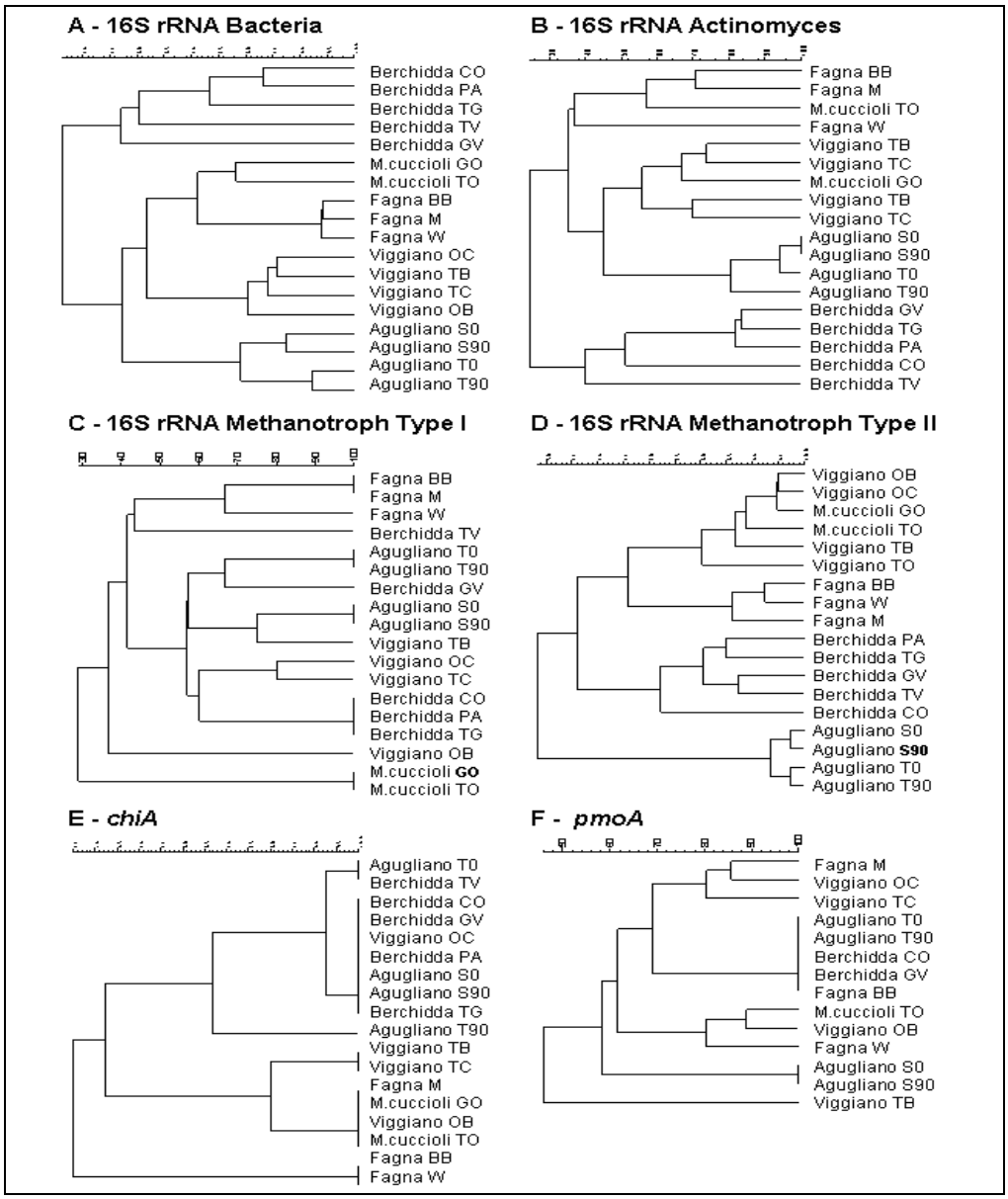


Figure 2 - *Cluster analysis based on UPGMA of DGGE profiles. T, conventional tillage; S, no tillage; 0, without nitrogen fertilization; 90, with nitrogen fertilization; TV, vineyard with conventional tillage; GV, grass-covered vineyard; TG, temporary grassland; PA, pasture; CO, cork oak forest; OB, organic barley; OC, organic clover; TB, traditional barley, TC, traditional clover; TO, conventional tilled olive-grove; GO, grass covered olive-grove; BB, broad bean; W, wheat; M, mais.*



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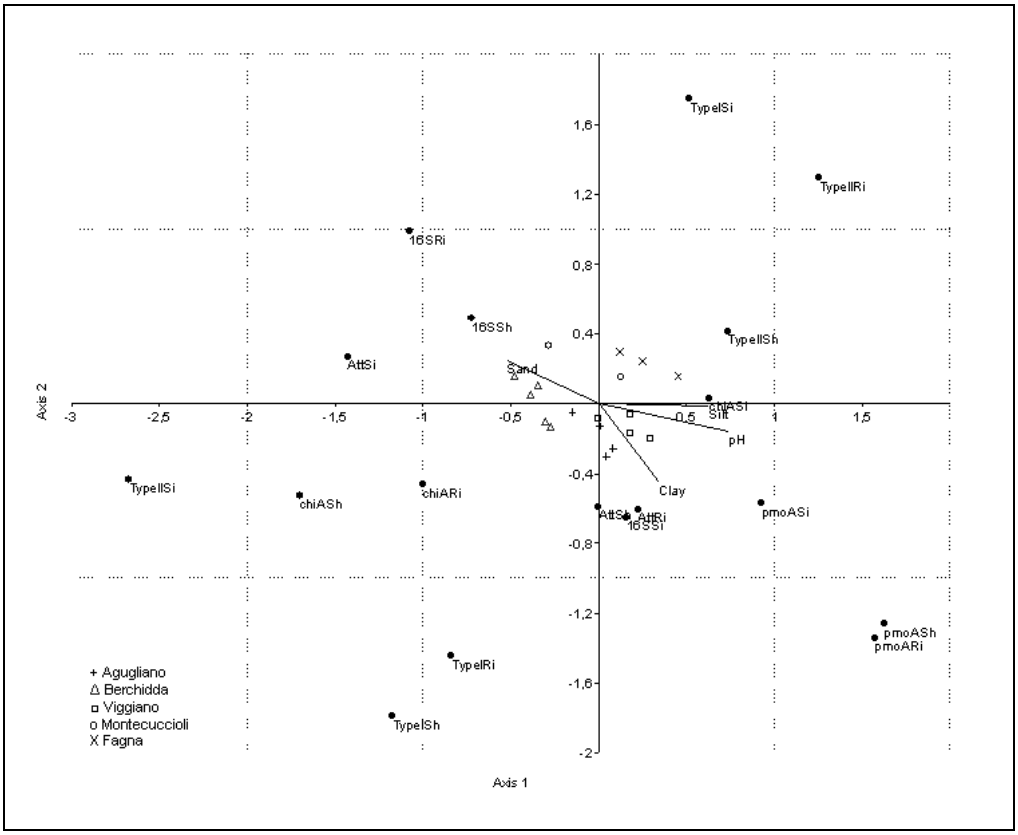
Canonical Correspondence Analysis (CCA) was used to detect a correlation between the analysis of the gene pools and edaphic variables. In CCA (Fig. 3) the first canonical axis explained 56,9% of the total variation, was statistically significant at P=0,02 and was dominated by the environmental variables pH (intraset correlation with axis of 0,7) and silt (0,6).

Figure 3 - *CCA ordination diagram of bacterial communities defined by the first and second axes. The plot was generated by richness, Shannon-Weiner index and Simpson index of soil bacterial communities estimated from DNA-DGGE profiles. Vectors represent the soil variables. Rh, richness; Sh, Shannon-Weiner index; Si, Simpson index.*



The second canonical axis explained an additional 35,7% of the total variation, was statistically significant at P=0,001 and was dominated by clay (0,45). In all examined soil bacterial communities (total bacteria community, Actinomycetes, methanotrophos, and chitin degrading bacteria) the richness and diversity resulted influenced by soil variables. Shannon-Weiner index and richness of bacterial 16S rRNA were significantly correlated with clay. Species variables derived from 16S rDNA-DGGE profiles of type I and type II methanotrophs were highly significant correlated with texture, but in opposition to each other. On the contrary as sustained Terahara *et al*. (2009) *chiA* functional gene resulted significantly

correlated with clay but not with pH. While *pmoA* functional gene resulted significantly correlated with clay and sand and pH according to statement from Abell *et al*. (2009).

Conclusion

Our results revealed a great bacterial diversity and the presence of several bacterial groups involved in carbon cycle in the bulk soil of the experimental areas considered. The major differences were observed among the different sites rather than among the different soil managements. The environmental factors soil type and pH would be responsible for shaping the composition of these bacterial groups.

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