Carbon sequestration techniques in agricultural soils.

MSc. Internship report

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Abstract

Agricultural practices that enhance OM content and quality can play a central role in reducing green-house gases emissions and sequester C in soils. For this project 4 different soil treatments have been studied for their potential to sequester C in soils. Application of a new green-chemistry product, namely iron-porphyrin, at low and high doses, amendment with a mature compost and a combination of compost and low dose of iron-porphyrin have been studied in two different soil types. Iron-porphyrin is used as a biomimetic catalyst and is expected to enhance C stabilization through polymerization of humic substances in soils. Differently, the amendment of compost is intended to sequester C thanks to hydrophobic protection of labile C components in the more humified fraction of organic matter from compost. In order to study the effects of these methods, an experiment under laboratory controlled conditions experiment was carried out. Soil samples coming from two different pedogenic and climate regions in southern and northern Italy were pot in glass dishes. The four treatments mentioned above were done for each soil, moreover, controls of soil without any treatment were done. Triplicates were done for each sample. The soil dishes were incubated in the dark at room temperature for 3 months. The soils were subjected to wet-dry cycles, by adding DEMI water every 2 days. At the beginning and end of the incubation period humic (HA) and fulvic acids (FA) were extracted. Moreover, the C, H, N content of HA and FA fractions and of soils before and after the extraction were analysed. HA and FA from each soil sample were also characterized for their C distribution by ^{13}C-CPMAS-NMR spectroscopy. The data obtained about the chemical characteristics of HA and FA and soils were thereafter put in relation with the PLFA and NLFA content, analysed in some soil samples coming from a field experiment connected with the present project.

The results show that lower C mineralization occurred in the soils amended both with compost and iron-porphyrin plus compost. This suggest an effect on stabilization of added C in soil. Differently, the two types of soils treaded with only iron-porphyrin did not show any changes in their C mineralization rates. The NMR results showed significant variation in the C distribution among the phenolic and aromatic- C groups in the humic substances extracted from soils. However, no significant changes were found in their hydrophobicity over the hydrophilicity, nor in the C/N and H/C was observed.

The PLFA and NLFA analysis form the field soil show that the amendment with mature compost stimulated biological activity: in particular bacterial growth is enhanced in both low and high doses of compost. Also AM fungi colonization is enhanced be the soil addition of compost, especially if in high dose, as compared to the control. High dose of compost increases also the colonization of Gram+ bacteria over Gram-bacteria, while in low dose of compost the contrary is observed. Treatment with iron-porphyrin led to a reduction of fungal colonization as compared with the control. In particular AM fungi decline by the treated with only iron-porphyrin. Moreover, an increase of Gram- bacteria over the Gram+ is observed.

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1 Introduction

1.1 Background

1.1.1 Soil organic matter

Soil organic matter (SOM) is universally recognized as a key parameter of soil quality (Franzluebbers, 2003, Dumanski et. al, 2000). It affects several chemical, physical and biological properties of soils that strongly influence soils health and functioning (Plaza et al., 2013). For instance, SOM enhances the formation of stable aggregates, improving soil structure (Franzluebbers, 2003); nevertheless, it impacts nutrient cycling and influences water-holding capacity and cation-exchange capacity, playing a central role in assuring soil fertility and sustaining crop production (Lal & Kimble 1997). SOM also supports microbial activity and soil fauna, enhancing the biodiversity of soils. (Droogers, 1997; Lal & Kimble 1997; Reeves 1997). In figure 1 an overview of the most important roles of SOM for soil quality and biodiversity is shown.

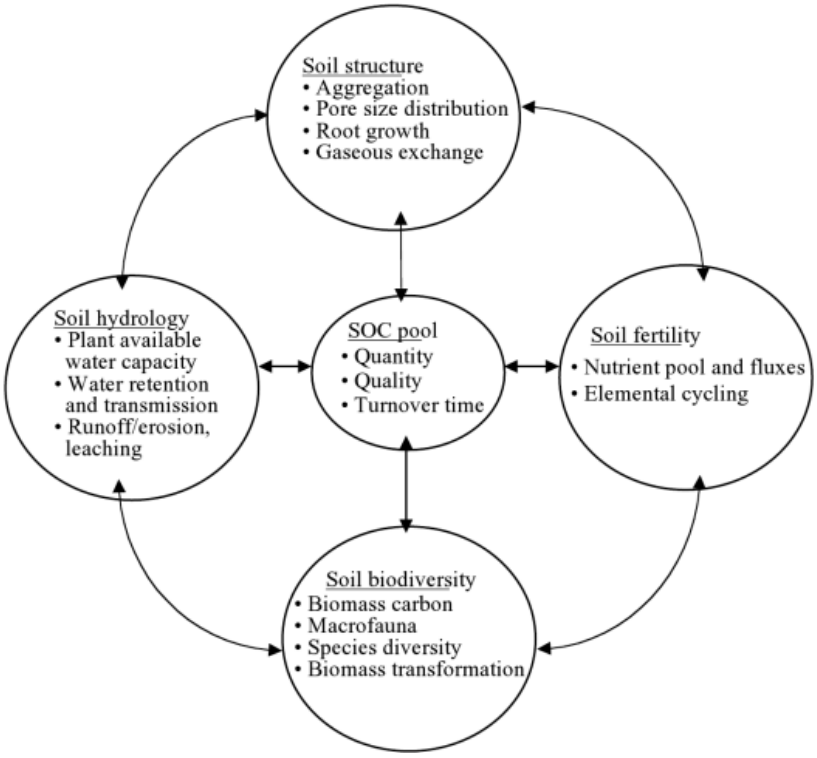


Figure 1: roles of SOM in soil quality (Lal, 2002).

The content of organic matter in the top 15 cm varies between 1-5% in most agricultural soils (Schnitzer, 1991). Although as shown above preserving, SOM is crucial for assuring soil functioning, in the latest years most agricultural soils have lost 50 to 70% of their original SOC pool (Lal,). This does not only imply a loss of fertility, but also a contribution to atmospheric CO_2 enrichment with an intensification of GHG effects.

Soil organic matter in fact can be seen as a sink but also a source of C. Approximately 80% of global terrestrial biosphere C is found in soils both as living biomass and soil organic matter (Simfukwe, 2011). Globally, the carbon pool in soils is equal to 2500 gigatonnes (Gt), made up by 1550 Gt of soil organic carbon (SOC) and 950 Gt of soil inorganic carbon (SIC). However, the C stock in soil is dynamic and subjected of losses and gaining (Lal, 2004; Bhattacharyya et al, 2011). The key process that determines the stabilization of OC in soils is the biodegradability of SOM (Ohno et al., 2014). If SOM is readily and easily biodegradable, part of it will be mineralized by microorganisms, and a transfer of C from the soil to the atmosphere as CO_2 will occur. On the contrary, a more recalcitrant and stable SOM will enhance the permanence of C in soil, lowering the emissions of CO_2 from soil. For this reason, maintaining or increasing the carbon held in soils will potentially have a significant impact on the global carbon cycle and budget.

1.1.2 Soil C sequestration

As mentioned above, the key process that influences SOM stability in soil is its biodegradability. Several factors influence the biodegradability of SOM, like origin and molecular structure of the SOM itself, climate, soil properties like textural composition, moisture content, level of aggregation and soil geochemistry (Plaza, 2013). Anthropogenic activities may affect the OC cycle by, either, accelerating the decline or, effectively preserve SOM through land management practices. For example, a way to maintain SOM content and prevent CO_2 emission is to adopt practices that allow soil carbon sequestration. Soil C sequestration implies a net removal of C from the atmosphere (CO_2) and its transfer to the soil, where it is stored as soil organic matter (Powlson *et al.*, 2011; Lal, 2003). Conservation tillage, application of manures and compost as well as crop rotations and winter cover crops are some of the land management practices that can be adopted to enhance carbon sinks (Dumanski J., 2004; Gobin et al., 2011).

Soil C sequestration in soil has been proposed by the Intergovernmental Panel on Climate Change (IPCC) as one of the possible measures through which greenhouse gas emissions can be mitigated (Follett, 2001). Also the Kyoto protocol and the United Nations Framework Convention on Climate Change (UNFCCC) identified opportunities of reducing atmospheric CO_2 removal through C sequestration in soils (De Neve, et al., 2003; Dumanski J., 2004). The adoption of practices that aim at C sequestration and SOM preservation can lead to a "win-win" situation that allows not only environmental advantages but also economic benefits (Lal, 2003). For these reasons in the latest years it has been nationally and internationally working to develop new policies that aim to improve the capacities of land uses and agricultural practices to enhance carbon sequestration and to increase the carbon stocks in soils. Many European projects have the goal to investigate which land management practices can effectively allow C sequestration in soils, being as well advantageous considering land productivity and economic feasibility. Among these studies there is the LIFE-CarbOnfarm project, described in the next section.

1.1.2 The LIFE-CarbOnfarm project

The European project "LIFE12 ENV/IT 719 - LIFE-CarbOnFarm" (www.carbonfarm.eu) aims at developing environmental sustainable land use practices that preserve soil organic matter (SOM) content in soils. The project is managed by a consortium made up by 7 partners (six public and one private): CERMANU Research Centre of University of Napoli, Agricultural Department of University of Torino, Department of European-Mediterranean Cultures of University of Basilicata, CREA Research Centre of Pontecagnano, Agricultural Agency of Regione Campania, Agricultural Agency of Regione Basilicata, Prima Luce Farmers co-operative association. The project is based on a 5 years long (2013-2018) field experiments in which different methods of SOM managements are investigated. Four typical regional farming systems are involved in the project. These farms are located in southern and northern Italy (two in Campania region and two in Piemonte region), in sites different from each other's for climate patterns and soil properties. The cultivations set in the experimental fields of these farms are maize, open field horticultural crops and commercial fruit tree orchards. The methods applied to preserve SOM quality and enhance C sequestration in soils are the same for all the farms and they include soil application of composts, from local organic sources, and the use of a green chemistry product, namely water soluble iron-porphyrins. In order to assess the efficiency of these methods on soil organic carbon stabilization and on the improvement of physical and biological soil properties, several monitor activities are carry out during the 5 years experiment. In fact, total organic carbon and nitrogen soil content are annually measured. These data are compared with the data of the CO_2 and NO_2 fluxes from soil, measured with a respiration static chamber system. These analysis are intended to enlighten weather the agricultural methods lead to changes in the C and N cycles. Moreover, analysis of the molecular characterization of OM are performed, like Fourier Transform Infrared spectroscopy, 13C solid state Nuclear Magnetic Resonance spectroscopy and off-line Thermochemolysis Gas Chromatography-Mass Spectrometry. This is done to give insight into possible structural changes of OM following stabilization practices of C in soils. To evaluate possible changes in the physical soil structure related to the SOM management methods, water stable aggregate distribution, aggregate stability and organic carbon content of soil aggregates are studied once every year. The practices to stabilize SOM are related also with the biological fertility of soil. In fact, in order to assess whether these SOM management methods affect the soil microflora is studied with phospholipid fatty acids (PLFA) and arbuscular mycorrhizal fungi (AMF) analysis are performed one per year. Lastly, crop productivity is evaluated by measuring the crop yield, plant biomass and plant N and P content.

1.2 Objectives of the internship

The internship was part of the European project "LIFE12 ENV/IT 719 - LIFE-CarbOnFarm". The objective of the project was to study the efficiency in soil C sequestration of the soil management practices adopted in the LIFE-CarbOnFarm project. Therefore, the practices applied in the field experiment were repeated in a second experiment under laboratory controlled conditions. Laboratories controlled conditions were preferred in order to decrease the number of uncertainties found in the field and to focus only on soil C sequestration. The efficiency of the followed management practices was investigated:

1. Soil application with mature humified manure compost.
2. Soil treatment with modified water soluble iron-porphyrin biomimetic catalyst in low and high concentration.
3. Soil application with both mature humified compost and iron-porphyrin in low concentration.

To be able to study the efficiency of these practices in SOC stabilization, the parameters that have been studied were:

- TOC content before and after the application of the above mentioned practiced;
- amount of C extractable content in isolated HA and FA, before and after the application of the above mentioned practices
- characterization of HA and FA fractions, to check changes in C distribution in their chemical structure
- C/N and H/C ratio in both isolated HA and FA fractions.

Moreover, the microbial community structure of microbes in the soil from the experimental field amended with different treatments was done. This was done to assess whether the different land management practices affected the microbial community of soil. The cycle of C is mediated by communities of microorganisms and in turn the different forms as C is available for microorganisms can affect their community, favouring some species to the detriment of some others. Therefore, the study of the microbial community structure in relation with the soil treatments is expected to give more insight into soil C sequestration dynamics and its consequences in soils.

1.2.1 Research questions

The main research question of the present internship project was:

"Can OC be stabilized in soil through land use practices like mature compost application, treatment with water soluble iron porphyrin and a combination of the two?"

To be able to answer this question, the sub-request questions were:

1. Does the amount of TOC and extractable OC changes before and after the application of the treatments?
2. Is there any changes in the C distribution among the C functional groups found in FA and HA structures before and after the application of the methods mentioned above?
3. Does the elemental content, and in particular the C/N and H/C change after the application of the methods?
4. Does the use of these methods affect the soil microbial structure?
5. Do the treatments have different outcome if applied in different soil type?

We hypothesized that the management practices used for the LIFE-CarbOnFarm project, namely manure compost application, treatment with water soluble iron porphyrin and the combination of the two would have enhance the C stabilization in soils. Therefore less extractable C is expected in the treated soils as compared with the control. Moreover, it is hypothesized that in the FA and HA extracted from the soil amended with

mature compost as well as with iron-porphyrin would have shown an enhancement of the hydrophobic C groups in theirs structure. This would led to higher C/H ratio and C/N at the end of the experimental period. Moreover, we hypothesized that the less availability of labile C would have been reflected in differences in the microbial structure of the treated soils.

2 Materials and Methods

In order to be able to answer the research questions, an experiment under laboratory controlled conditions was performed. Mature compost, water soluble iron porphyrin and a combination of the two were applied on two different soil types. Details on the experimental set-up, soil types and the characteristic of the mature compost and iron-porphyrin used are find in section 2.1. In section 2.2 the analytical methods are reported. After 3 months of incubation in the dark the soils were analysed for their C and N content (section 2.3.2) , thereafter the HA and FA were extracted. Details on the extraction procedure are in section 2.2. ^{13}C-CPMAS NMR and elemental (C,N,H) analysis were performed on the HA and FA fractions to see possible differences among the humic substances from different soils treatments, an overview on the method applied for these analysis are in section 2.3.1 and 2.3.2. Moreover, PLFA and NLFA analysis performed on the soil samples coming from the field of CarbOnfarm project, where the same C stabilization methods were applied, were done to answer the research question 4. Section 2.3.3 reports how PLFA and NLFA analysis has been done.

2.1 Experimental set-up

Soil samples coming from 2 different experimental fields involved in the CarbOnFarm project were used for the experiment; description of the soil types used are in section 2.1.1. For each type of soil 4 treatments were applied, as follow:

- Soil application with mature compost: 5mg compost/g soil (corresponding to a field dose of about 20ntons ha^{-1}).
- Iron-porphyrin at low dose: -1.8 ug/g soil (corresponding to a field dose of about 5 kg ha^{-1})
- Iron-porphyrin at high dose: 3.6 ug/g soil (corresponding to a field dose of about 10 kg ha^{-1})
- Mature compost (5mg compost/g soil) and iron-porphyrin at low dose (1.8 ug/g soil)

Moreover, controls of soil without any treatment were done. For each treatment, as well as for the controls, triplicates were done. 50 gr of each type of soil were weighed in a glass dish and kept in a dark room for 16 weeks. In total 15 dishes were prepared for each soil type (fig. 2).

The treatments were applied only once, at the beginning of the experiment. The amount of mature compost and iron-porphyrin applied to the soil dishes was the same used for the field experiment, calculation to get the doses are reported in Annex 1. Moreover, since during the hot, dry summers, irrigated soils in the experimental fields are subjected to frequent wet–dry cycles, also the soils in the dishes were subjected to wet-dry conditions. Therefore, 15 ml and 10 ml of DEMI water was added to the soil every 2 days respectively for the Castelvolturno and Tetto Frati soils. This amount of water was calculated based on the field capacity and moisture content of the two soils, measured in the fields and reported in the next section (2.1.1). Except for the addition of water, no other operations have been done during the experiment to the soil dishes.

Figure 2: experimental soil dishes in the incubation room.

2.1.1 Soil characteristics

One soil sample used for the experiment came from the Po River Valley, in the province of Torino in northern Italy. In this report, we refer to this soil type with the name of "Tetto Frati" (TF). TF originated in a temperate climate region (type F climate), in an area characterized by recent alluvial materials calcareous. This soil type has a sandy loam texture with a low clay content (around 7%) and a SOM content equal to 1.7-1.8%. It is classified as Typic Ustifluvent according the USDA soil taxonomy (Grignani et al., 2012). The soil moisture calculated based on the field capacity of this soil type was equal to 17% and this same moisture level was kept in the laboratory experiment.

The other soil, named Castelvolturno (CV) was sampled in the coastal plains in Napoli, in southern Italy. The soil originated in an area with Mediterranean climate (type S). The soil was a silty clay loam with 30-33% of clay and 1.2% of SOM content. It is defined as a Vertic Haploxeralf using the USDA soil taxonomy. The soil moisture of this soil type was equal to 23%. In table 1 an overview of the soils characteristics is reported.

Table 1. Textural composition (%), bulk density and TOC (g/kg) content of soils types used for the experiment (Spaccini et al., 2012). "Torino" refers to Tetto Frati soil, while "Napoli" to Castelvolturno Soil.

Field sites	Soil type	Sand	Silt	Clay	Bulk density	TOC
Torino	Typic Ustifluvent	36.9	56.2	6.9	1.50	11.5
Napoli	Vertic Haploxeralf	47.0	20.1	32.9	1.40	10.5

2.1.2 Manure compost

The compost used for the experiment derived from cow manure mixed with corn straw and wood pellet. The material was composted in an aerated static pile system for 90 days. The maximum temperature reached was 70°C. At the end of the composting process the compost was air dried. It contained 28% C and 2% N. It mainly contained O-alkyl C groups. The C distribution within the organic molecules of compost is shown in annex 2. Compost was added to the experimental soil plaques with a ratio of 5mg/g soil both for the treatment of compost alone and together with iron-porphyrin. This was done to reproduce the field treatment which is equal to 20 ton compost/ha soil. Calculation of the amount of compost for the laboratory experiment are reported in Annex 1.a.

The addition of humified compost is expected to sequester C by hydrophobic protection. In the studies of Spaccini *et al.* (2002) and Piccolo (2004,) humic acids extracted from compost showed to form close associations with the fresh organic compounds present in soil after 3 months of incubation. The introduction of easily degradable C into the hydrophobic domains of HS is thought this hamper microbial mineralization of this

labile organic matter. Moreover, both Spaccini (2002) and Piccolo (2004) observed a higher OC sequestration in the silt- and clay-sized fractions of soil. This suggests that HA from compost not only tend to form associations with the more labile C, but they also promote stable organo-mineral association with finer soil particles (Spaccini et al., 2002; Piccolo, 2004), enhancing a further C sequestration in soils. The mature compost used for the present experiment is constituted largely by biologically stabilized, "humified" organic matter (mainly O-alkyl C) groups with a high degree of hydrophobicity, therefore is expected to give similar results of the HA extracted from compost used by Spaccini and Piccolo.

2.1.3 Water soluble iron-porphyrin

Iron-porphyrins are nontoxic compounds which mimic the activity of the heme prosthetic group of oxidative enzymes. In industry, synthetic metal-porphyrins are often applied to catalyse the oxidation of various hydrocarbons, such as polychlorinated aromatics and other pollutants, drugs, various lignin models (Piemonte et al., 2013). Recent studies (Piccolo, 2005; Smejkalova et al., 2006) show that in the presence of a singlet oxygen donors, such as hydrogen peroxide, or under UV radiation, iron-porphyrin can enhance the oxidative coupling of the phenolic components in humic matter. This happens because in the presence of hydrogen peroxide or UV light, iron porphyrins produce the highly reactive oxoiron(IV)-porphyrin radical cations, which show strong oxidizing ability. Organic substrate can therefore undergo an oxidation giving unstable free radicals that are stabilized by spontaneous mutual coupling, and, as a consequence, oligomerization occurs. As a results of the oxidative oligomerization of the phenolic compounds, an increase in molecular size of humic matter can occur. In fact, Piccolo (2005) observed an increase of apparent weight-average molecular weight (MWA) of sodium humate solutions by the 10.7% and 11.5% after 5.5 and 13h of UV irradiation at both pH 7 and 3.5. An increase in MWA values was also found when samples were kept in the dark for 8 and 11 days after the end of irradiation. The implement of molecular weight of HS suggests that iron-porphyrins can turn weakly associated humic superstructures into more stable covalently bound oligomers or polymers (Smejkalova et al., 2006). This implies an increase of the chemical energy of the intermolecular bonds in SOM humic fractions, which can result in a higher stability of the humic matter towards microbial degradation, eventually enhancing OC sequestration.

Iron-porphyrins are made by a tetra-pyrrole ring to which an iron atom is coordinated and diverse lateral components (fig.3). For this experiment a synthetic modified iron-porphyrin was used. The basic porphyrin has been modified by connecting the active porphyrin ring to clay surfaces in order to improve the persistence by decreasing the solubility and allowing a close interaction with soil clay minerals(Nuzzo and Piccolo, 2013).

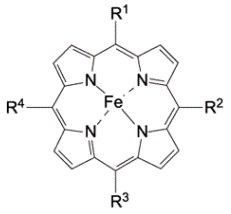


Figure 3: general structure of iron-porphyrins.

2.2 Humic and Fulvic Acids extraction

At the beginning and at the end of the experiment HA and FA fractions were extracted and isolated from the experimental soils. 50 g of soil was weighed and extracted with 400 ml of NaOH (0.1 M) and $Na_4P_2O_7*10H_2O$ (0.01M) 1:8 extraction (soil:solution). In the headspace of the bottles N_2 was flushed before closing them to avoid CO_2 to dissolve in NaOH. Thereafter, the bottles were put in a horizontal shaker at 120 rpm for 24 hours. The solution was centrifuged at 7000 rpm for 20 minutes to separate better the solution from the soil fractions. Subsequently, the supernatant solution was filtered with glass fibres and it was acidified at pH 1 with HCL (12M) to promote the precipitation of HA, since they are insoluble at pH <2. The acidified solution was allowed to stand on a bench overnight, then it was centrifuged at 7000 rpm for 20 minutes to recover the HA fraction. The supernatant containing FA was sampled and put in a baker while the HA were treated with HF and HCl to purified them from the silicates. In order to do that a solution was prepared adding 2.5 ml of HCL (37%) and 2.5 ml of HF (40%) in 1L of DEMI water. The HA were incubated with 200 ml of the solution for 24 hours in a horizontal shaker at 120 rpm. Thereafter, the solution was centrifuged at 7000 rpm for 20 min: the supernatant was thrown away, while the HA precipitated was re-dissolved in DEMI water. At this point the both solutions of

HA and FA were transferred into dialysis membranes (Spectrapore 3, 3500 Mw cutoff) and put in DEMI water until the dialysis water gives an electrical conductivity of 15 µS/cm. Once the dialysis was finished the sample were put in a freezer at -20 °C and subsequently freeze-dried.

2.3 Analytical methods

2.3.1 ^{13}C-CPMAS-NMR analysis

The chemical structure of humic samples was investigated using cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance spectroscopy (CPMAS-13C-NMR). This technique is a non-destructive spectroscopic methods widely used to characterize natural organic matter (De Marco et ., 2012). The isolated humic and fulvic acids samples, after freeze-drying were weighted and finely powdered within a quartz agate mortar. Thereafter, they were packed in 4-mm zirconia rotors with Kel-F caps. Spectra were obtained with a Bruker AVANCE^{TM} 300 instrument (Bruker, Rheinstetten, GE) equipped with a 4-mm-wide bore MAS probe, operating at a 13C resonating frequency of 75 MHz and a rotor spin rate of 5000 Hz. 1510 data points were collected over an acquisition time of 20 ms, a recycle delay of 2.0 s, and 4000 scans. CPMAS-NMR spectra were done on triplicates for each sample. The obtained spectra were split into six main spectral regions: 0–45 ppm representing the unsubstituted saturated alkyl carbons (C); 45–60 ppm for methoxyl-C and N-alkyl C; 60–110 ppm alkyl-C singly bonded to one oxygen atom, such as ring C in carbohydrates or bonded to two oxygen atoms, such as anomeric C of carbohydrates; 110–145 ppm for proton- and alkyl-substituted aromatic C; 145–160 ppm for phenol-C; 165–200 ppm for carboxyl-C, including carboxyl, ester and amide C and 190–200 ppm carbonyl C of aldehydes and ketones. The estimative of integrated areas of each spectral region were determined by using the MestRe-C software package. The amount of each C group was expressed in percentage. Moreover, the percentage of alkyl (0-45ppm), aromatic (145-110ppm) and phenolic (160-145ppm) C were summed together to represent the hydrophobic components of humic substances, while the relative areas of carboxylic (200-160ppm), m methoxyl- and alkyl (60-45) and O-alkyl (110-60) C were summed as index of hydrophilicity. The ratio between the hydrophobic and hydrophilic parts (HB/HI), was therefore calculated.

2.3.2 Elemental analysis

The soil samples before and after the treatments and before and after the extraction of HA and FA were characterized for their C and N content. Moreover, the HA and FA were analysed for their content of C, N and H. For these measurements a Fisons EA 1108 Elemental Analyzer (Fisons Instruments S.p.A., Rodana, MI, Italy) was used. The soil samples were firstly dried in a hoven at 40 °C and ground to a fine powder using a quartz agate mortar and pestle. In the case of extracted HA and FA, they were only ground after the freeze-drying step. Consequently, approximately 22-25 mg of soil sample and around 2,5 of HA and FA was weighed to a tin aluminium capsule. The exact weight was recorded and the capsule was folded and wrapped before being placed into the auto sampler. For each sample triplicates were done. In the case of extracted HA and FA, they were only ground after the freeze-drying step. The analysis was done in order to detect possible changes in the C content, C/N and H/C ratio in the soil amended with different treatments. C/N ratio have been calculated by first dividing the mg C and N/g soil by respectively 12 and 14 (their molecular weight) and then putting them in ratio. For the H/C was done the same, dividing mg H and C/g soil by 1 and 12.

2.3.3 NLFA and PLFA analysis

For the internship neutral and phospholipid fatty acid (NLFA and PLFA) analysis was used to study microbial biomass and community structure of soil samples collected in the experimental field. This analytical method is based on the identification of fatty acids as biomarkers to determine the presence and abundance of broad functional microbial groups such as fungi, gram positive (+) and negative (-) bacteria, actinomyces, etc (Zelles, 1994). This method is often used to determine gross changes in the microbial community associated with different environmental conditions (Hills et al., 2000). In our case, NLFA and PLFA analysis was performed to assess if different soil managements affected the soil microbial community composition and structure.

The analysis were performed using soil samples coming from the experimental field of Castelvolturno. This was preferred upon the soil used for the laboratory experiment for the absence of plants in the latter and the fact that it was kept in dark for all the experimental period. These factors flattened the microbial community, making less meaningful the analysis. Moreover, the PLFA and NLFA analysis is performed once a year as part of

the monitoring activity of the CarbOnfarm project (see section 1.1.2), therefore it was an opportunity for me to acquire practice with this method, as well as to contribute to finalize the annual activities for the project.

For the analysis, 50 gr of soil was collected from the rhizosphere (of the maize plants) grown in the control plots (CT) and in the plots amended with compost at low (MC L 10 tons ha^{-1}) and high (MC H 20 tons ha^{-1}) concentration and with iron-porphyrin (FePh 5 kg ha^{-1}). The soil sampling was performed during crop cycle about 70 days after sowing. The PLFA and NLFA were extracted following the modified Blight and Dyer (1959) technique described by Bardgett et al. (1996). The detailed procedure is reported in Annex 2. Briefly, 1 gram of frozen soil was extracted for 2 hours with a mixture of chloroform/methanol/citrate buffer at pH 4 (1:2:0.8 v/v). After centrifugation, the upper phase was collected and split into two phases by adding chloroform and citrate buffer. The lower phase was recovered and dried with N flux. Thereafter, the lipids are fractionated into neutral, glycol- and phospho- lipids on a silica gel column by elution with chloroform, acetone and methanol, respectively. The neutral and phospholipids were dried under N2 flux at 37 °C and stored at -20 °C. Neutral and phospholipids were hydrolysed to free fatty acids by alkalization and consequently derivatized into fatty acids methyl esters. In this step methyl nonadecanoate fatty acid (19:0) was added to the sample as internal standard. The FAME are then separated from the head groups by using n-hexane and analysed by GC/MS. A PerkinElmer Autosystem XL (GC) equipped with a PE Turbomass-Gold quadrupole mass spectrometer was used.

In Annex 3 the list of the PLFA used as biomarkers and the related microbial group which they were representative of, is found. The relative area of the chromatographic peak obtained for each PLFA and NLFA was divided by that of the internal standard (19:0). Each PLFA and NLFA content was expressed as nmol of PLFA per gram of wet soil. The calculation done are reported in Annex 4 The concentration of PLFA relative to the same microbial group (Gram+ bacteria, Gram– bacteria, fungi and actinomycetes) were summed together. Microbial community indices such as the ratio of Gram+/Gram– bacteria and the ratio of arbuscular mycorrhiza fungal hyphae to bacteria were calculated. In the case of arbuscular mycorrhiza (AM) fungi, C16:1ω5 NLFA were used as indicators for arbuscular mycorrhiza (AM) fungi (Cozzolino, 2012). In particular, this NLFA was taken into account as representative of spores and propagules of AM fungi, reflecting therefore the AM fungal growth (Ngosong, 2012). As marker of the AM fungi biomass the C16:1ω5 PLFA can be considered. However, it is not entirely specific only for AMF, but it can also be representative of for some Gram- bacteria (Cozzolino, 2012). To assess whether this PLFA indicates the presence of AM fungi or Gram- bacteria, the ratio between the NLFA and PLFA was done. According to Olsson (199) if the NFLA/PLFA ratio is between 1 and 200 the PLFA is representing AM fungi, while if the ratio is lower than 1 it must be considered biomarkers of Gram- bacteria.

3. Results

3.1 Yield of extraction

3.1.1 Castelvolturno soil

In figure 4 the C content in the non-extracted soils (in blue), the C retained in the soil after extraction (in green), and the C content of FA and HA fractions (in violet and light-blue, respectively) is shown. Moreover, in red it is reported the loss of C, calculated by subtracting the C content in the soil after extraction and in HA and FA to C measured in the non-extracted soil. CT1 and CT2 refer respectively to the control soils at the beginning and the end of the experiment. FePh H and FePh L to the soils treated with iron-porphyrin at high and low doses, MC represents the soil amended with mature compost, while MC+FePh L is the soil treated both with mature compost and low dose of iron-porphyrin.

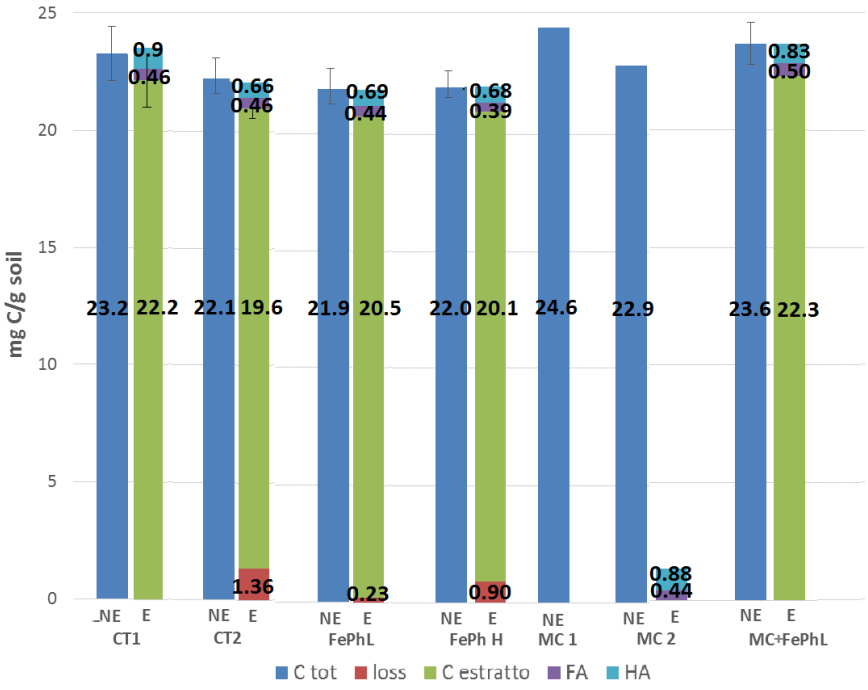


Figure 4: carbon content in non-extracted soils ("NE", in blue), the soil after extraction ("E", in green), and in the extracted fractions of FA (violet) and HA (light-blue) in Castelvolturno soils. In red is shown the loss of C after extraction. CT 1 and 2 are respectivetely control soil at the beginning and at the end of the experiment. FePh L represents the soil treated with iron-porphyrin in low dose, FePh H the treatment with high dose of iron-porphyrin. MC 1 and 2 are respectevely the soil amended with compost at the beginning and at the end of the experiment, while MC-FePh L is the soil with the addition of both compost and iron-porphyrin in low dose.

At the beginning of the experiment the C content of the control soil (CT1) was 23.2 mg/g soil. At the end of the experiment the amount of C in the control soil (CT2 in blue) decreased to 22.1mg/g soil, suggesting a mineralization rate of 1.1mg C/g soil within 3 month. The soils treated with iron-porphyrin at high (FePh H) and low (FePh L) doses show similar C content at the end of the experiment: 21.9 mg/g soil in FePh L and 22.0 mg/g soil in FePh H. This implies that the rate of C mineralization does not change with the application of the treatment. In CT1 the non-extractable C (bar in green) was equal to 22.2 mg/g soil, while the C contained in HA and FA was respectively 0.9 and 0.46 mg C /g soil. Differently, in CT2 the C retained in soil after extraction was 19.6 mg C /g soil, while the amount of C in HA and FA extracted was respectively of 0.66 and 0.45 mg/g soil. The less amount of C in HA from CT2 in comparison with in those from CT1 could suggest that this fraction is preferentially utilized by microorganisms while the FA fraction seem not degraded by microorganism. It is also noticed that the amount of C retained in soil in CT2 is lower than in CT1. However, the C loss after extraction in CT 2 is significant, equal to 1.36 mgC/g soil. In FePh L the C content retained in the soil after extraction of humic substances was equal to 20.5. In FePh H the value was slightly smaller (20.1 mg/g soil),

however, it must be considered that this sample underwent a loss of C of 0.90 mg/g soil, which might explain this difference. The HA and FA of these soils have similar content of C to that of HA and FA from CT2: 0.68-0.69 mg C/g soil in HA, and 0.44 and 0.38 mg/g soil in FA from FePh L and FePh H, respectively. In the case of soil amended with manure compost (MC), at the beginning of the experiment the soil contained 24.6 mgC/g soil. The C content was higher than in the control due to addition of the OM of the compost. At the end of the experiment the C content decreased to 22.9 mgC/g soil. Unfortunately, due to a technical drawback implying the sample loss, the C in the soil after extraction has not been analysed. It is known however than the C contained in the extracted HA was 0.88 mg/g soil, therefore higher than those extracted in the soils non-amended with compost, while in FA it was 0.44 mg/g soil. The soil amended with both compost and iron-porphyrin (MC+FePh L) shows higher C content at the end of the experiment than the soil amended only with compost (23.6 mgC/g soil). This suggests lower C mineralization led by the presence of iron-porphyrin. The C content in HA and FA is similar to that in MC, with a slightly higher C content in the FA (0.5 mg/g soil) and a lower C in HA (0.83 mg/g soil).

3.1.2 Tetto Frati soil

In Tetto frati soil it is generally seen a lower content of C in comparison with Castelvolturno soil. At the beginning of the experiment the C content in TF soil was 12.3 mg/g soil. At the end of the experiment it is observed that C content does not vary neither in the control soil (12.4 mg/g soil) nor in the soils treated with iron-porphyrin, where it is around 12.4 and 12.6 mg/g soil in FePh L and H respectively. The slightly higher values are non-statistically significant in respect to that of CT1. The loss of C in CT1 and CT2 after the extraction of HS is very high: 2.2 and 1.8 mgC/g soil. This could be caused by the omission of organic carbon present in the lighter and bigger OM fractions that were excluded from the measurements due to filtration of the samples during the extraction of HA and FA. The amount C retained in the CT1 after extraction was 8.51 mg/g soil, while the HA and FA have 0.87 and 0.72 mgC/g soil. In CT2 the C measured in the soil after extraction was 9.45 mg/g soil, while the C in HA and FA decreased at 0.75 and 0.43 mg C/ g soil, respectively. FePh L and H soils show similar amount of C retained in the HA and FA as compared to CT2. FePh L have slightly less C contained in HA fraction (0.69 mg(g), while FePh L shows lower amount of C as FA fraction (0.36 mg/g soil). The C retained in these soils after extraction is higher than in CT2. In fact in these samples the loss of C after extraction is less significant than in CT1 and CT2 (it is 0.46 mgC/g soil in FePh H and no loss in FePh L). On the contrary, an overestimation of C in FePh L may occur, as the content of C in the extracted soil plus measured in HA and FA exceed the C in the non-extracted soil.

In the soil amended with mature compost the C content does not decreases within 3 month: at the beginning of the experiment it was 14.15, at the end it was 14.11 mg/g soil, suggesting no C mineralization by microorganisms. Moreover, the amount of C contained in HA and FA does not change from that of the humic substances (HS) extracted from CT2 (0.69 and 0.42 mgC/g soil). However, an important C loss is seen for this sample after extraction. In the soil treated with both compost and iron-porphyrin the overall C content decreases to 12.5 mgC/g soil after 3 month, suggesting more mineralization. After the extraction a loss of C similar to that for MC is seen. The C found in FA extracted from this soil sample is similar to that of the other soil samples (0.39 mg/g soil), while that in HA is much higher (0.91 mg/g soil).

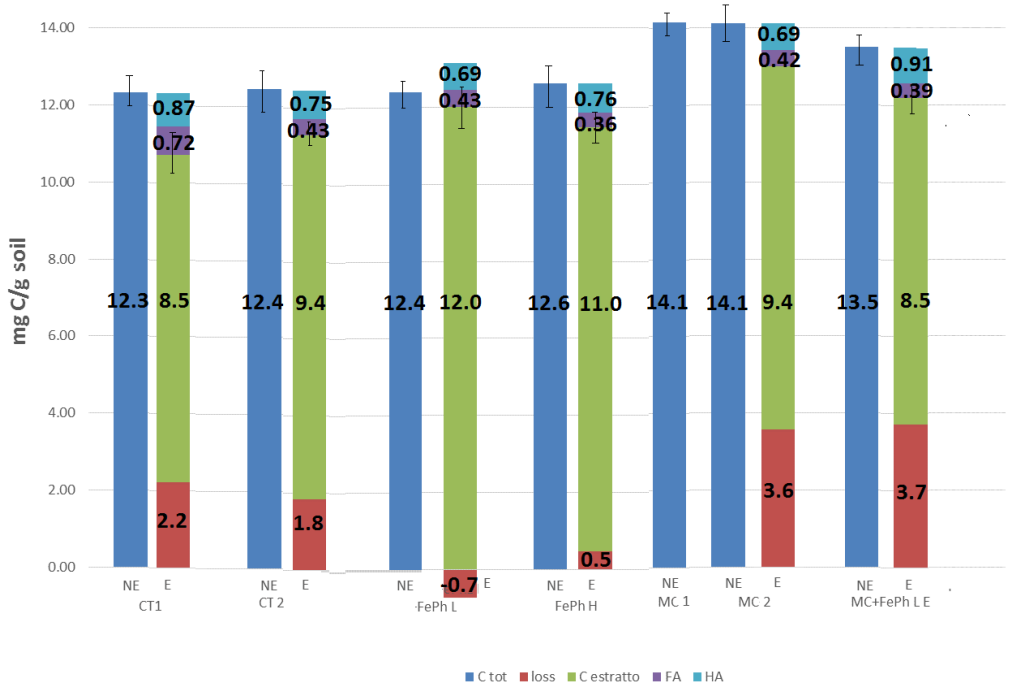


Figure 5: : carbon content in non-extracted soils ("NE", in blue), the soil after extraction ("E", in green), and in the extracted fractions of FA (violet) and HA (light-blue) in Tetto Frati soils. . In red is shown the loss of C after extraction. CT 1 and 2 are respectivetely control soil at the beginning and at the end of the experiment. FePh L represents the soil treated with iron-porphyrin in low dose, FePh H the treatment with high dose of iron-porphyrin. MC 1 and 2 are respectevely the soil amended with compost at the beginning and at the end of the experiment, while MC-FePh L is the soil with the addition of both compost and iron-porphyrin in low dose.

3.2 HA and FA Characterization

3.2.1 Castelvolturno samples

3.2.1a Fulvic acid fraction

In table 2 the C distribution among the 6 functional groups defined in the NMR spectra are given for each FA sample. CT1 and CT2 refer respectively to the FA from the control soils at the beginning and at the end of the experiment. MC represent the FA extracted from the soil amended with mature compost, FePh L and FePh H the FA from the soil treated with low and high dose of iron-porphyrin and MC+FePh L refer to the treatment with both mature compost and iron-porphyrin. The table shows an overall similar molecular composition for all the FA samples. All the FA samples reveal a predominance of O-alkyl C (53.3-58.9%), followed by alkyl-C (16.6-20.7%).

Considering FA from control soils, a slightly higher percentage of O-alkyl carbon is seen in CT2 (58.5%) in comparison to CT1 (55.5%). This is balanced by a lower percentage of alkyl carbon (19.2% in CT1 and 17.2% in CT2) and methoxyl C (12.9 in CT1 and 11.5% in CT2). These small variations only slightly change the HB/BI ratio, which is slightly lower in CT2 (0.30) than in CT1 (0.35). FA from MC follows have the same trend of C distribution of CT2, showing the same HB/BI ratio (0.29). Also the FA from MC+FePh L show the same value of HB/HI ratio (0.30). However, these FA have a lower percentage of phenolic (0.6%), aromatic (1.6%) and O-alkyl C (56.6%) as compared to CT2. These lower proportion are balanced with relative accumulation of alkyl C (20.7%) and methoxyl C (10.8%) higher than in CT2, where it was 17.2% and 11.5% respectively.

The FA from FePh H and L show the same HB/HI of CT1 (0.35), which is slightly higher than that of CT2 (0.30). In these FA samples in fact the C distribution is very similar to CT1, except for a slightly lower relative amount of phenolic C (0.6% for FePh L and 1.7% for FePh H). In comparison with CT2 these FA samples show a lower

accumulation of O-alkyl C (55.1 and 53.6%), compensated by a higher percentage of alkyl C (around 20% in both).

Table 2: C distribution in FA extracted from Castelvolturno soils.

	CT1	CT2	FEPH L	FEPH H	MC	MC+FEPH L
CARBOXYLIC C (%)	7.9	7.1	7.9	7.4	7.8	6.7
PHENOLIC C (%)	2.7	1.6	0.6	1.7	1.8	0.6
AROMATIC C (%)	4.8	4.1	5.2	3.9	4.2	1.6
O-ALKYL-C (%)	55.5	58.5	55.1	53.6	58.9	56.6
METHOXYL/ (%) N-ALKYL	12.9	11.5	11.9	12.9	10.8	13.8
ALKYL-C (%)	19.2	17.2	19.4	20.6	16.6	20.7
HB/HI	0.35	0.30	0.34	0.35	0.29	0.30

The similar distribution of C in the FA samples is clearly visible also from the NMR spectra, reported in figure 6. It is in fact seen that all the spectra show the same main peaks: the peak at 71 ppm is the most intense in all the samples and it can be attributed the overlapping carbon signals at 2, 3, and 5 positions in cellulose and hemicellulose glucopyranose units (Dou, 2008). Still in the region of O-alkyl C there is another sharp peak at 100 ppm, probably related to the anomeric C of cellulose and hemicellulose glucopyranose. Another important peak is at 173 ppm, in the region of phenolic C that denotes the presence of CO_2H groups (or amides and esters). Within the alkyl-C region (0-45 ppm) an overlapping of several peaks is seen, in particular, at 19 and 21 ppm there are 2 peaks that can be attributed to CH_3 and CH_2 groups in different positions in long alkyl chains (Piccolo, 1990; Schnitzer, 1983). Also from 55 to 63 ppm there is a large resonance, probably due to C-N bonds in amino acids (56ppm) and $-CH_2(OH)-$ in carbohydrate (around 63 ppm)

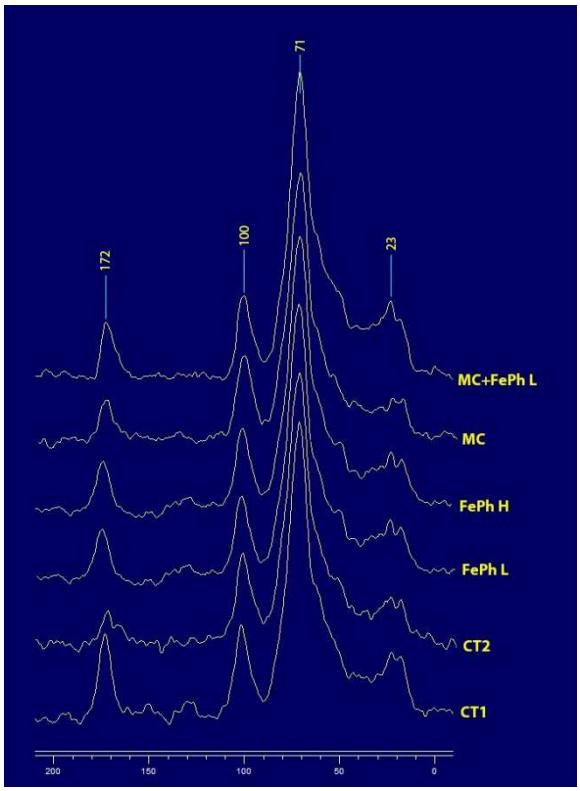


Figure 6: NMR spectra of FA extracted from Castelvolturno soils

In table 3 the C/N and C/H ratios of FA extracted from CV soils are shown. It is seen that the C/N ratio does not change in the control soils (CT1-CT2) within 3 months, whereas it increases in all the soils treated with iron-porphyrin (12.97 and 11.35 for respectively FePh L and H). The increasing of the ratio can suggest more consumption of N from the free amino acids and/or proteins in these soils than in the control or less C mineralization. In the soils amended with compost the starting C/N is unknown, however at the end of the experiment the MC and MC+FePh L have similar C/N ratio value (12.46-12.8). The higher ratio value as compared to CT2 is probably due to the addition of humified OM, that increased the amount of hydrophobic C groups in the soils, decreasing the relative amount of short C molecules containing N. the H/C ratio decreases during the experiment in all the soils as compared with CT1. This suggests a slight increase of aromatic-C groups over the aliphatic groups. However, the changes in H/C are not significantly big, confirming the low incidence of treatments in increasing hydrophobicity of FA, already suggested by NMR results (table 2).

Table 3: C/N and H/C ratios of FA extracted from Castelvolturno soils.

	C/N	H/C
CT1	9.6	1.96
CT 2	9.61±0.2	1.75 ± 0.02
FEPH L	12.97 ±0.03	1.83 ±0.001
FEPH H	11.35 ±0.9	1.74 ± 0.02
MC	12.46 ±0.7	1.74 ± 0.07
MC+FEPH L	12.80	1.86 ± 0.04

3.2.1b Humic acid fraction

Also in the case of HA the C distribution is very similar in all the samples (table 4). As well as it was seen for FA the main C groups present in HA are O-alkyl C groups and alkyl-C. However, the HA show to be richer in Alkyl C and less in O-alkyl C. HA from CT2 show a relative amount of O-alkyl C (28.9%) slightly lower than HA from CT1 (31.4%). In turn, the percentage of alkyl-C (35%) in CT2 is significantly higher than in CT1 (30.5%). As a consequence, the HB/HI ratio increases throughout the experimental period (from 0.77 to 0.88). In the case of HA from MC, although the percentage of phenolic (2.1%) and aromatic (12.3%) C is slightly higher than CT2, there is an accumulation of O-alkyl C (32.1%), while the relative proportion of alkyl C is lower (29%). This results in a reduction of the HB/HI ratio value, which is 0.77, similarly to that of CT1. The HA samples from the iron-porphyrin treated soils (FePh L and H and MC+FePh L) have HB/HI ratio value around 0.8, similarly to that of CT2. In fact, these samples differ from CT2 for very minor variances. For example, FePh L has lower relative amount of alkyl C than CT1 (31.2%), however it is compensated by slightly higher percentages of aromatic C (12.2%), phenolic C (2.4%) and O-alkyl (12.2%). Also MC+FePh L has lower relative portion of alkyl C than CT1 (30.), balanced by higher percentages of aromatic C (13.2%). HA from FePh H have only minor differences with CT2, like a little lower relative amount of aromatic C (8.8%) and higher O-alkyl C (30.5%).

Table 4: C distribution in HA extracted from Castelvolturno soils.

	CT1	CT2	FEPH L	FEPH H	MC	MC+FEPH L
CARBOXYLIC C (%)	10.9	9.6	8.8	9.0	10.0	10.0
PHENOLIC C (%)	1.5	1.4	2.4	2.3	2.1	2.2
AROMATIC C (%)	11.5	10.6	12.2	8.8	12.3	13.2
O-ALKYL-C (%)	31.4	28.9	30.8	30.5	32.1	29.6
METHOXYL/ (%) N-ALKYL	14.1	14.6	14.7	15.4	14.5	14.9
ALKYL-C (%)	30.5	35.0	31.2	34.0	29.0	30.1
HB/HI	0.77	0.88	0.84	0.82	0.77	0.84

The NMR spectra of HA samples show that the major peaks are in the alkyl (40-0 ppm) and O-alkyl C (60-110ppm) regions. The peaks at 24 and 29 ppm correspond to alkyl C in long-chain polymethylene structures (e.g. fatty acids, waxes, and resins). The sharp peak at 71 that was seen also in the FA samples, can be attributed to the C-O groups in the C_2, C_3, and C_5 carbons in carbohydrates. Nevertheless, the side peak at 56 ppm can be assigned to CH_3 groups of aromatic ethers (Piccolo, 2990). At 102 ppm is found a peak related to the anomeric C, as seen in FA. Moreover, a broad resonance at 125-130 ppm, which was not observed in FA spectra, indicates the presence of unsubstituted aromatic rings. Finally, at 172 ppm denotes the presence of CO and CO_2H groups.

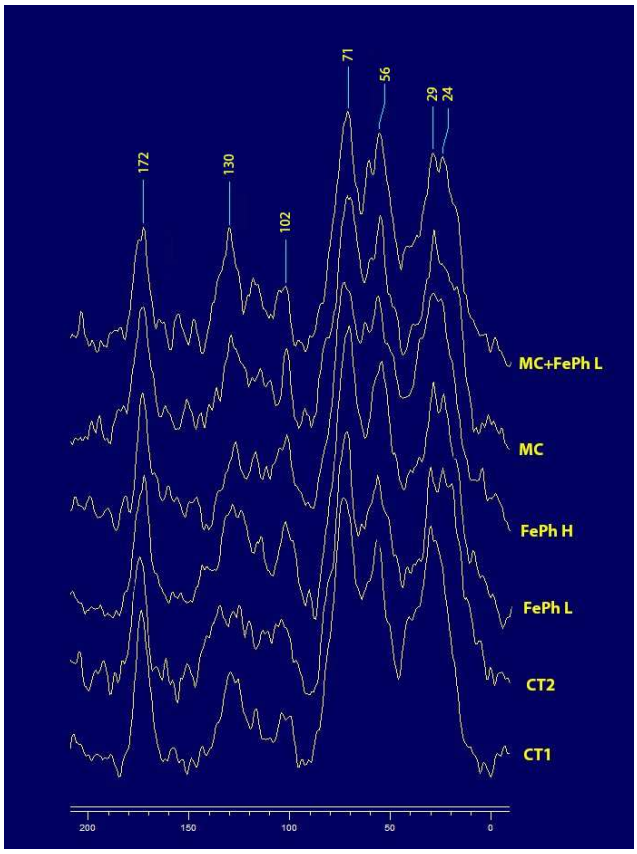


Figure 7: NMR spectra of HA extracted from Castelvolturno soils

In table 5 it is seen that C/N of HA extracted from CV soils slightly lower than that of FA of the same soil. Only negligible differences are seen among the HA samples. The value in fact only slightly decreases in CT2 and FePhL (9.06-9.13) as compared with CT1 (9.6), while in and FePh H in is higher (10.4). This suggests an higher C consumption of C over that of N contained in CT2 and FePh L and a contrary trend in FePh H. The HA from MC and MC+FePh L show similar value among each other's (9.69 and 9.99), which are higher than CT2.

The H/C ratio is constant during all the experiment for all the HA samples, staying around 1.42 and 1.53. It shows to be slightly lower than the H/C of FA fraction of the same soil type, suggesting a higher content of aromatic components in HA, as table 4 shows.

Table 5: C/N and H/C ratios of HA extracted from Castelvolturno soils.

	C/N	H/C
CT1	9.6	1.44
CT 2	9.06 ±0.22	1.53 ±0.014
FEPH L	9.13 ±0.20	1.42 ±0.01
FEPH H	10.41 ±0.18	1.52 ±0.04
MC	9.70 ±0.11	1.51 ± 0.05
MC+FEPH L	9.99 ±0.006	1.45 ±0.05

3.2.2 Tetto Frati samples

3.2.2a Fulvic acid fraction

The FA from Tetto Frati soil, as well as thos from Castelvolturno, are mainly made up by O-alkyl groups (46.1-56,9%) as reported in table 6. FA from control soil at the beginning (CT1) and at the end of the experiment (CT2) show small variations in their C distribution: for example, the relative content of carboxylic C decreases throughout the experimental period (from 11.9% in CT1 to 7.7% in CT2). Also the percentage of phenolic and aromatic C decrease in CT2 (1.9 and 4.9% respectively, against the 3 and 7.3% of the CT1). On the other hand, the proportion of O-alkyl C (49%), methoxyl C (12.5%) and alkyl C (22.8%) are higher than in CT2 (where they are 46.1%, 12,6% and 19.1%). This variations do not lead to any variation of the HB/HI, which stays equal to 0.42 both for CT1 and CT2. In the case of FA from MC, FePh L and FePh H, the HB/HI ratio decreases in comparison to that of CT2. In fact, although there is a little relative accumulation of aromatic C (5.5-6.8%), in these samples the percentage of O-alkyl C is significantly higher (54.3-56.9%) than in CT2 (49.1%). This causes a reduction of the HB/HI ratio value that is around 0.3 for all these samples. Finally, the FA from MC+FePh L shows HB/HI ratio similar to that of CT2. This FA sample in fact show C distribution analogous to CT2 FA, except for minor differences like a higher percentage of alkylyc C (24.7%), balanced by a slightly lower relative amount of aromatic, O-alkylyc and methoxyl C (3.9, 48.2 and 14.7%)

Table 6: C distribution in FA extracted from Tetto Frati soils.

	CT1	CT2	FEPH L	FEPH H	MC	MC+FEPH L
CARBOXYLIC C (%)	11.9	7.7	12.9	7.8	7.4	6.9
PHENOLIC C (%)	3.0	1.9	1.1	2.1	1.9	1.5
AROMATIC C (%)	7.3	4.9	6.8	5.4	5.9	3.9
O-ALKYL-C (%)	46.1	49.1	54.5	56.9	54.3	48.2
METHOXYL/ (%)	12.6	13.5	10.1	10.9	12.3	14.7
N-ALKYL (%)						
ALKYL-C (%)	19.1	22.8	14.5	17.0	18.1	24.7
HB/HI	0.42	0.42	0.29	0.32	0.35	0.43

The trend of C distribution of C in the FA samples from Tetto Frati soil is similar to that of Castel volturno soils. The NMR spectra are in fact very similar, showing the same peaks. Also in these spectra a sharp resonance at 70 ppm denotes the -CH (OH)- in carbohydrate (Dou, 2008), while broad resonance between 51 and 56 indicates the presence of aliphatic methyl esters and amino acids (Piccolo, 1990). Moreover, also in these FA samples, the CH_2 groups of long alkyl chains result in an overlapping of peaks between 17 and 23 ppm. As it was seen in the FA from CV soils, at 100 ppm a peak representing anomeric C of cellulose and hemicellulose is seen. Nevertheless, an important peak is observed at 173 ppm, denoting the presence of CO_2H groups (or amides and esters). This peak is particularly high in the FePh L sample, and in fact this sample showed the higher relative amount of carboxylic C (table ..) In MC FA, there is another small peak at 150 ppm due to the phenolic C and N-substituted aromatic C. However, this does not result in a higher relative amount in comparison with the other samples.

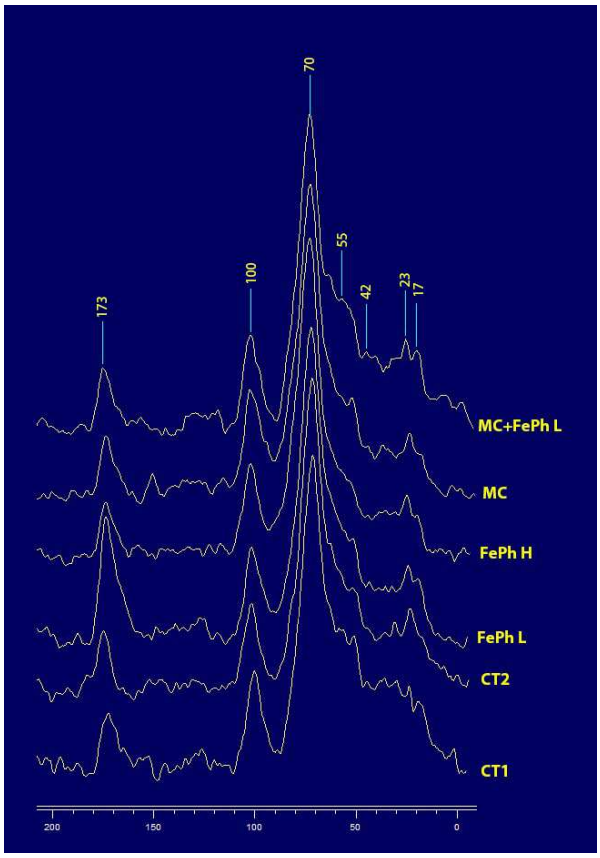


Figure 8: : NMR spectra of FA extracted from Tetto Frati soils.

Table 7 shows the C/N and C/H ratios of FA extracted from TF soils. The C/N ratio increases all the FA samples as compared to the control CT1. There is not significant difference between the soils treated with iron porphyrin and the control soil at the end of the experiment. In the soils samples amended with compost alone the FA show a C/N higher than those in the soil treated with both compost and iron-porphyrin. Anyway, also these samples do not differ significantly from CT1. Also the H/C ratio is very similar for all the FA samples, being around 2 for all of them.

Table 7: C/N and H/C ratios of FA extracted from Tetto Frati soils.

	C/N	H/C
CT 1	10.0 ±0.01	2.00 ± 0.07
CT 2	11.87 ± 0.63	1.96 ±0.19
FEPH L	11.36 ±0.18	2.13 ±0.15
FEPH H	10.95 ±0.18	2.05 ±0.11
MC	11.36 ±0.17	2.09 ±0.16
MC+FEPH L	9.5	2.05

3.2.2b Humic acid fraction

The HA of Tetto Frati control soil show a constant distribution of C during the experiment. The relative amount of O- alkyl C slightly decreases at the end of the experiment (29.6% in CT1 and 30.7% in CT2) but this is the only minor difference between the samples. Their HB/HI in fact stays constant at 0.85-0.86. This ratio decreases in the HA from MC, where it is 0.76. In fact in this FA sample, the polar C groups (9.6 % of

carboxylic and 32.8% O-alkyl C) are higher in percentage as compared to CT2, while the alkyl-C decreases (24.3%), leading to a lower NB/HI ratio. The samples containing iron-porphyrin (FePh L, FePh H and MC+FePh L) have similar HB/HI values to the CT1 and CT2 (0.81-0.86). FePH L shows a relative amount of alkyl C (24.4%) lower than CT2, balanced by a higher amount of aromatic (33%) and O-alkyl C (17%). FePh H has lower percentage of carboxylic components in comparison with the other samples (6.1%), but a higher relative amount of allylic C (32.1%). The HA from MC+FePh L have the highest relative accumulation of carboxylic (12.2%), compensated by a lower percentage of O-alkyl C (27.4%) as compared with the other HA samples. In comparison with CT2 HA from this soil have higher phenolic (4.7%) and aromatic C (16.2), as it was seen for the HA from MC and FePh L.

Table 8: C distribution in HA extracted from Tetto Frati soils.

	CT1	CT2	FEPH L	FEPH H	MC	MC+FEPH L
CARBOXYLIC C (%)	8.7	8.3	7.9	6.1	9.6	12.2
PHENOLIC C (%)	1.7	2.4	3.4	1.6	3.1	4.7
AROMATIC C (%)	13.8	13.9	17.0	12.0	15.8	16.2
O-ALKYL-C (%)	29.6	30.7	33.4	31.5	32.8	27.4
METHOXYL/ N-ALKYL (%)	15.5	15.0	13.8	16.7	14.3	14.1
ALKYL-C (%)	30.6	29.6	24.4	32.1	24.3	25.3
HB/HI	0.86	0.85	0.81	0.84	0.76	0.86

The NMR spectra of HA samples are similar to those of CT soil. Also in these HA samples spectra the major peaks are in the alkyl (40-0 ppm) and O-alkyl C (60-110ppm) regions. The peaks at 24 and 30 ppm corresponds to alkyl C in long-chain polymethylene structures (e.g. fatty acids, waxes, and resins). The peaks at 71 and 56 ppm can be attributed to the C-O groups in the C_2, C_3, and C_5 carbons in carbohydrates and to CH3 groups of aromatic ethers, respectively. In comparison with HA from CV soils, here the spectra reveal higher resonance at 56 ppm. Similarly to CV HA, at 102 ppm is found a peak related to the anomeric C. Moreover, a large resonance at 110-135 ppm is observed, representing the protonated aromatic rings. Within this overlapping of signals, a sharper peak at 130 ppm and weaker resonance at 116ppm are found in all the samples. In MC+Fe and CT2 is seen broader resonance between 152 and 145 ppm as compared with the other samples, indicating a slightly higher amount of phenolic C. Lastly, all the samples show a high peak at 172 ppm, which denotes the presence of carbonyl and carboxyl carbons in fatty acids, amino acids, acetyl groups etc..

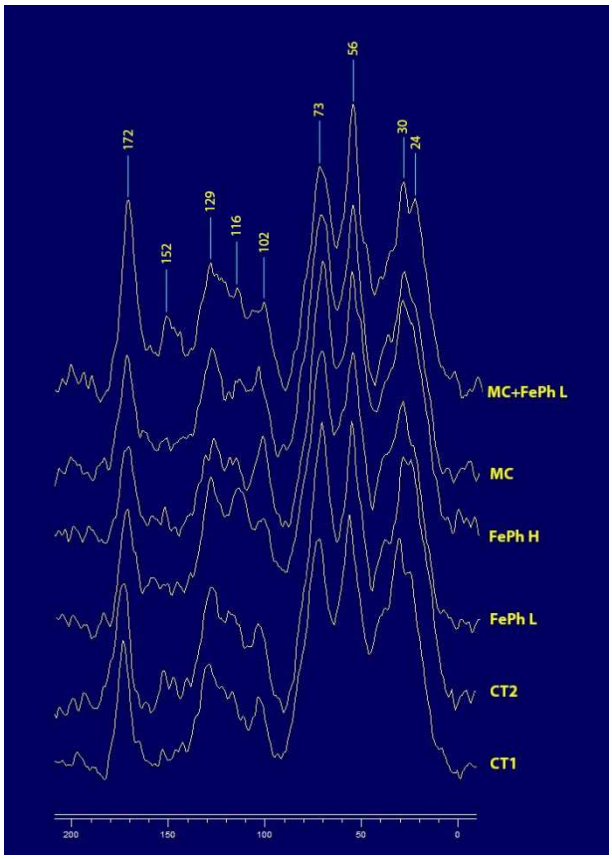


Figure 9: : NMR spectra of HA extracted from Tetto Frati soils.

Table 9 displays the C/N and H/C ratio of HA extracted from TF soils. The C/N ratio at the end of the experiment increases in CT2 to 11.10 and in MC+FePf L to 11.87. In all the other samples it stays constant at around 10, with only negligible differences. This is in line with the NMR results, that show no significant changes in the chemical structure of the HA samples. This is confirmed also by the H/C ratio, which is similar for all the HA, around 1.3. Only HA MC shows a slight higher H/C ratio (1.68).

Table 9: C/N and H/C ratios of HA extracted from Tetto Frati soils.

	C/N	H/C
CT 1	10.04 ±0.04	1.32 ±0.02
CT 2	11.10 ± 0.03	1.31 ±0.02
FEPH L	10.00 ± 0.04	1.25 ± 0.01
FEPH H	10.02 ± 0.10	1.28 ±0.01
MC	9.30 ± 0.11	1.68 ± 0.05
MC+FEPH L	11.87 ±0.04	1.34 ± 0.06

3.3 PLFA and NLFA analysis

In table 2 the total amount of PLFA, the amount of PLFA representative of fungi, Gram+ and Gram– bacteria, as well as the sum of bacterial PLFA is shown. It can be observed that the total microbial biomass is enhanced by the soil amendment compost in both low and high dose (MC L and MC H). In fact, while in the control soil the total amount of PLFA is around 43.4 nmol/g soil, in the soil amended with low and high dose of compost it increases up to 67.3 and 58.7 nmol/g soil respectively. This enhancement of total PLFA is given mostly by the increase of bacterial PLFA. In fact, it is observe that in MC L and H the fungal PLFA only slightly increase as compared to the control soil, while the bacteria biomass undergo a significant enhancement (from 26.8 to 29 and 39 nmol/g soil). In particular, the Gram – bacteria growth is higher in comparison with Gram + growth. Especially in MC L the Gram- PLFA are the double (30 nmol/g) than in CT (15nmol/g) and are higher than in MC H (21 nmol/g)

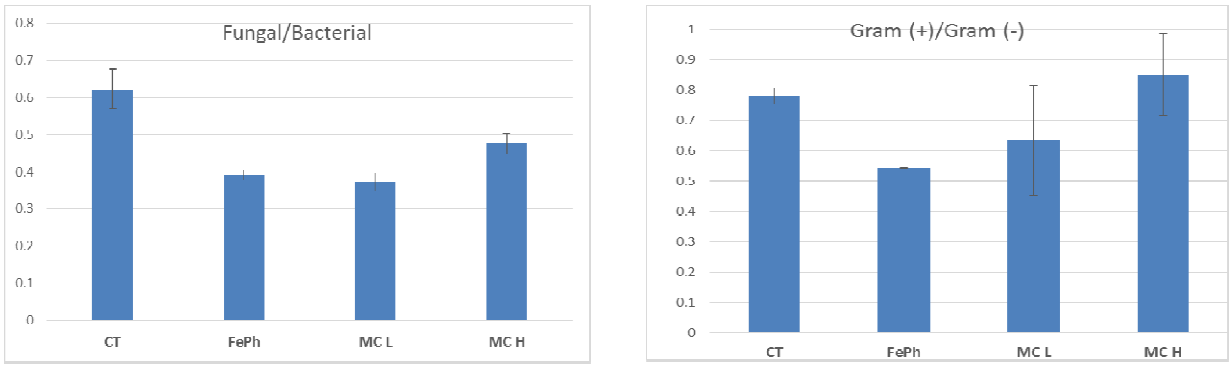
Differently, the soil treatments with iron-porphyrin seem to reduce the total microbial biomass (38.5 nmol PLFA/g soil) as compared with the control. The content of fungal PLFA is in fact decreased: 10.8 nmol/g soil against the 16.5 nmol/g of the control. Even though the amount of Bacterial Gram+ PLFA slightly decreases in this soil, however, the Gram- bacteria are marginally enhanced by the treatment with the iron-porphyrin. As a result, the total amount of bacterial PLFA (27.7 nmol/g) is similar to that of the control (26.8 nmol/g).

Table 10: PLFA content in control soil (CT), soil treated with iron-porphyrin (FEPH) and soil amended with low and high dose of mature compost (MC L and MC H, respectively) from the experimental field in Castelvolturno.

PLFA (nmol/g soil)	CT	FEPH	MC L	MC H
TOTAL PLFA	43.4 ± 3.9	38.5 ± 2.7	67.3 ± 1.9	58.7 ± 0.7
FUNGAL PLFA	16.5 ± 0.6	10.8 ± 1.1	18.1 ± 1.4	18.9 ± 0.5
BACTERIAL PLFA	26.8 ± 3.3	27.7 ± 1.7	49.2 ± 0.6	39.8 ± 1.3
BACTERIAL GRAM (+)	11.7 ± 1.2	9.7 ± 0.6	18.7 ±3.5	18.2 ± 2.2
BACTERIAL GRAM (-)	15.1 ± 2.1	17.9 ± 1.1	30.4 ± 3.0	21.6 ± 0.8

In figure 10.a the ratio of fungal PLFA over the bacterial PLFA content is shown. It is observed that the ratio value is greater in the control soil (0.62) than in the treated soils. In the other soils the lower ratio value suggests a greater bacterial growth over the fungal growth. In fact, as it was seen in table 9 in the treated soils the fungal PLFA tend to decrease (in FePh) or are similar to the control soils (MC L and H). On the contrary, the bacterial PLFA are equal to the control in FePh, while they significantly increase in MC L and MC H. In all the treated soil the fungal/bacterial is similar, ranging between 0.39 and 0.47.

Figure 10 b displays the ratio between Gram+ and Gram- biomass. In the control soil has the ratio value is 0.78. The value slightly increases in MC H (0.85), suggesting a slightly higher growth of Gram+ over the Gram- . Differently, in FePh and MC L the Gram+/Gram- value drops to 0.54 and 0.63 respectively. In these soils the Gram- colonization is more enhanced in comparison to that of Gram+ and to the control soils. However, the figure also shows that in the soils amended with compost (MC L and H) the wide variability between replicates leads to large error bars.

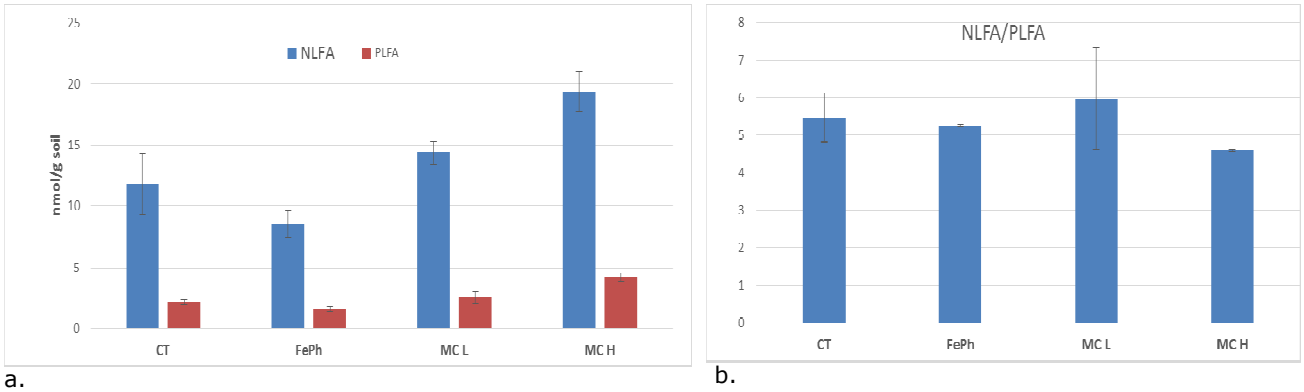


a. b.

Figure 10: : Fungal/Bacterial PLFA ratio (a) and Gram+/Gram- ratio (b) in control soil (CT), soil treated with iron-porphyrin (FePh) and soil amended with low and high dose of mature compost (MC L and MC H, respectively) from the experimental field in Castelvolturno.

As reported in section 2.3.3 the C16:1ω5 NLFA is a biomarker of AM fungi. Differently, the C16:1ω5 PLFA can represent the AM fungi or the Gram- bacteria. In figure 11.b the NLFA/PLFA ratio for each soil is shown. It is noted that the value is higher than 1 for all the soil sample. This implies that in these soils the C16:1ω5 PLFA can be considered representative of the AM fungi group instead of the Gram- bacteria (Olsson, 1999). In figure 11.a it is seen that the amount of C16:1ω5 NLFA and PLFA increase in the soil amended with low and high dose of mature compost as compared with the control. In control soil the NFLA and PLFA amount is respectively 11.8 and 2.1 nmol/g soil. In MC L they increase to 14.4 nmol NLFA/g soil and 2.5 nmol PLFA/g soil, while in MC H there are 19.3 nmol NLFA/g and 4.2 nmol PLFA/g soil. In the soil treated with iron-porphyrin both the C16:1ω5 NLFA and PLFA decrease as compared to the control (8.5 and 1.6 nmol of respectively NLFA and PFLA per gram soil).
Whereas variations are observed among the content of C16:1ω5 NLFA and PLFA in the different soil samples, the NLFA/PLFA ratio is similar for all the samples. The ratio indicates the proportion of C allocated to storage structures is similar for all the samples.

In fact, the ratio value ranged between 4.5 and 6. The control and the soil treated with iron-porphyrin have very similar ratio values (5.5 and 5.3 respectively); in the soil with the addition of high dose of compost the ratio slightly decrease (4.6), while with low dose of compost the ratio value is higher, equal to 5.9. A large error bar is seen for this latter soil sample (MC L), caused by high variation between the replicates.



a. b.

Figure 11: C16:1ω5 NLFA (in blue) and PLFA (in red) amount (a) and C16:1ω5 NLFA/PLFA ratio (b) in control soil (CT), soil treated with iron-porphyrin (FePh) and soil amended with low and high dose of mature compost (MC L and MC H, respectively) from the experimental field in Castelvolturno.

Figure 12 shows the ratio between the AM fungi biomass and the saprotrophic fungi biomass. The first is given by the C16:1ω5 PLFA, while the saprotrophic fungi biomass is calculated by the sum of C18:2ω6,9 and C18:1ω9 PLFA. The results show that the ratio slightly increase in the soil with iron-porphyrin and low dose of compost (both around 0.17) as compared with the control (0.15). In the case of soil amended with high dose of

compost the ratio value is the highest, increasing up to 0.29 and reflecting the high growth of AM fungi in this soil, observed in figure 11.a.

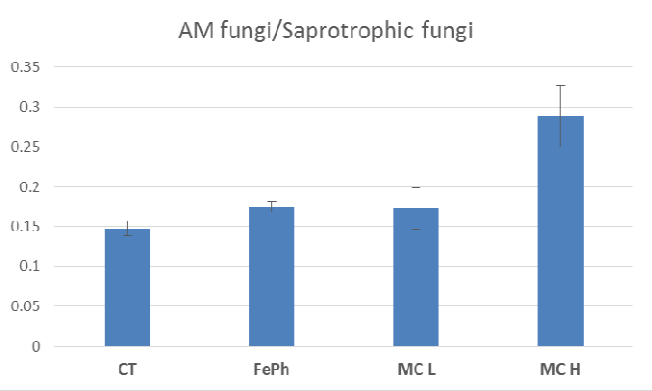


Figure 12: AM fungi/Saprotrophic fungi ratio in control soil (CT), soil treated with iron-porphyrin (FePh) and soil amended with low and high dose of mature compost (MC L and MC H, respectively) from the experimental field in Castelvolturno.

4 Discussion

4.1 Yield of extraction

The results of C content analysis do not confirm that treatment with iron-porphyrin enhance C stabilization in Castelvolturno soil within 3 months. In fact, in the soil samples treated with the biomimetic catalyst the mineralization rate is the same as the control. A lower mineralization is expected in the case of C sequestration is soils, followed by an increase of C in soils. Not only in these soils the C content before extraction is the same, but also the extractable amount of C (contained in HA and FA) does not change as compared to the soil. This seems to suggest that the treatments with iron-porphyrin does not enhance sequestration of the endogenic C of this soil type. However, it must be considered that the control soil at the end of the experiment underwent a significant loss of C after extraction. This could be due to a loss of humic material during the procedure of extraction, alternatively an analytical error could have occurred while analysing the C retained in the soil. Since it is unknown the origin of this loss, it is difficult to compare the C content in the control soils and in the treated soil after extraction. Regarding the amount of C retained by soils after extraction, this value seems to be higher in the soils treated with iron-porphyrin than in the control soils at the end of the experiment. This could suggest that in these soil samples C is more bond in the mineral particles, becoming more difficult to extract and potentially less bioavailable, implying C sequestration after iron-porphyrin treatment. However, this difference in the C retained in the soil can be explained with the loss of C both in the control soil and in the FePh H. This C loss that cannot be clarified makes difficult to assess whether C sequestration actually occurred.

In the case of iron porphyrin added together with compost (MC+FePh L), a lower mineralization in comparison with the soil amended only with compost (MC) is noticed in CV soil. The C content at the end of the experiment was in fact higher in MC+FePh L soil than in MC. This can suggest that in the presence of exogenous C iron-porphyrin reduce C removal from soil, enhancing therefore C sequestration. The amount of C in HA and FA fractions is slightly lower in HA and little higher in FA as compared with the soil treated only with compost. This can suggest a harder extractability of the former an easier extractability of the latter in MC+FePh L than in MC. Alternatively, it can suggest that in MC+FePh L the HA are used by microorganisms without being mineralized. Rather, HA are broke down in smaller molecules that go replacing FA fractions. Unfortunately, is not possible to compare the C retained in the C after extraction in MC+FePh L and MC soils, to see weather more C is retained in soil in the presence of iron-porphyrin. This would have confirmed the hypothesis that in the presence of exogenous C iron-porphyrin can enhance the sequestration of C in CV soil.

In Tetto Frati soil no C mineralization is registered in the control soils as well as in the soils treated with iron-porphyrin. Also the C contained in HA and FA fractions in the treated soils is very similar to that in the control. Nevertheless, it is interesting to notice that in the soil samples treated with iron-porphyrin the C losses after extraction are lower than in the control soils. This was seen in lower proportion in CV soil. This could suggest a higher retention of C in the treated soils, possibly due to more polymerization of OM. However, this is not seen in the TF soils amended with compost. In fact, the C losses in the soil with only compost (MC) and with compost and iron-porphyrin (MC+FePh L) are equal. Moreover, in MC+FePh L soil more mineralization of C is hypothesized, since the amount of total C in this soil is lower than in MC at the end of the experiment. This is in contrast with the results from CV soils, where less mineralization was seen in MC+FePh L.

Considering these results it can be concluded that addition of iron-porphyrin show to effectively reduce C mineralization and enhance C sequestration only in the presence of extraneous C (coming in this case from compost) in Castelvolturno soil. Catelvolturno soil in comparison with Tetto Frati soil has higher clay content (table 1), which can enhance the interaction between OM and iron-porphyrin, promoting therefore the stabilization of C. Qualche referenza??

4.2 Humic Substances Characterization

The NMR results show that of the soil treatment do not lead to significant changes in the C distribution of HA and FA within 3 months in both TF and CV soils. Iron-porphyrin was expected to promote the polymerization of organic matter in soil (Piccolo et. al, 2004). This would have enhanced the content of aromatic and phenolic compounds in HA and FA, increasing the HB/HI ratio. Also the addition of compost was thought to reduce the relative amount of labile C and increase the overall hydrophobicity of HA and FA, thanks to the addition of humified C molecules in the soil and their sequestration of labile C into their hydrophobic protection of labile C components (Spaccini, 2012). However, both the NMR results as well as the C/N and H/C ratios show not significant changes in the C distribution within the humic substances of the soils. In the previous section (4.1.)

it was seen that iron-porphyrin could possibly enhance carbon sequestration in MC+FePh L. The HA from MC+FePh L are however very similar for their C distribution to HA from MC. While their H/C ratio is very similar, the HB/HI ratio value in HA from MC+FePh L is slightly higher than MC samples, due to slightly lower relative amount of O-alkyl C. It could be hypothesized that this C groups was preferentially used by microorganisms, since the amount of C in HA in this soil sample is lower than in HA from MC. Regarding the FA of MC+FePh L, which contain more C than FA from MC, they showed a lower phenolic and aromatic C relative content as compared with MC, which can become less easily extractable by the presence of iron-porphyrin, since this is observed also in the FePh L FA samples. In turn, it contained more alkyl-C. As result the HB/HI ratio does not vary from the value of MC. The H/C ratio is higher than the MC and other treated samples, suggesting less utilization of recalcitrant C by microbes.

In Tetto frati soil the addition of iron-porphyrin seems to decrease the HB/HI in FA of the soil, while with the addition of both iron-porphyrin and compost it seems to increase the relative amount of alkyl-C, leading to a slight increase of the HB/HI ratio(table 6). However, these changes are not significant, as shown by the similar H/C ratio and no effects are caused in the extractability of FA C (figure 5). In the case of HA it was seen that more C was extracted as HA in the soil treated with both compost and porphyrin. The HA extracted from this soil showed a higher HB/HI ratio as compared to the soil treaded with only compost, due to a decrease of the relative amount of O-alkyl C groups.

The results of this project are not in line the findings of Piccolo (2004) that suggested polymerization of humic substances promoted by iron-porphyrin. However, the authors worked in a much simplifies system, adding the iron-porphyrin not directly to the soil but to a sodium humate solution. In the present project the effects of iron-porphyrin were studied in HA and FA, which are much more complexed systems than the sodium humate solution used by Piccolo. Moreover, Piccolo did not the interaction between OM and the soil mineral fraction, which can have an influence in iron-porphyrin efficiency. In a so complex system as soil, it is thought that 3 month are a too short period time to be able to observe the effects of iron-porphyrin (as well as compost) in C sequestration soils. Therefore, the efficiency of these methods cannot be excluded within a longer period of time.

4.3 PLFA and NLFA Analysis

It is seen that after 1 year of soil treatments, the amendment with mature compost stimulated biological activities. It is interesting to observe that especially in the soil with low dose of compost, instead of high dose, the microbial growth is enhanced (table 9). In particular bacteria are fostered to growth in these soils (figure 10.a). The amount of Gram – increases both in MC H and L in comparison with the control. However, the Gram+/Gram- ratio in MC H only slightly increases as compared with the control, while in MC L its value is lower (figure 10.b). Gram+ bacteria are able to degrade more aromatic and recalcitrant C compounds, while Gram- usually prefer easily degradable C sources (Cozzolino et al., 2015). This suggests that in MC H there is a lower bioavailability of labile C sources as compared with MC L and the control soil. As a consequence of this, Gram- bacteria prevail upon Gram+. This could be the effect of more hydrophobic protection of labile C due to more humified OM present in MC H (Spaccini, 2012). Less availability of easily degradable C is confirmed also by figure 12, where is it observed that in MC H the ratio between AM and saprophytic fungi is the highest. In fact, saprophytic fungi as well as Gram- bacteria prefer the utilization of short C chain and in MC H its relative amount decreases. The amendment of high dose of mature compost seems therefore to change the quality of OM in soils, enhancing the presence of aromatic, recalcitrant C components. In MC L the contrary situation is observed: the bacteria that use labile C growth more than those degrading more recalcitrant C. The lower Gram+/Gram- as compared to the control suggest that the amendment of compost does not only add humified C substances to the soil but also more easily degradable C compounds that are in this case preferentially degraded by microorganisms.

Regarding AM fungi, its colonization is fostered in MC H than in MC L. Among the different mycorrhizal types, arbuscular fungi that form symbiosis with the roots of about 80% of all vascular plants are the dominant fungal symbionts that support plant growth, by providing more N and P (Ngosong, 2012). AM fungi play in fact a central role in soil nutrient cycling. AM fungi may occur naturally in arable soils, but their density and diversity may be increased by farm management practices such as fertilization or crop types. It can be hypothesized that plant growing in MC L and especially in MC H soil take are advanced by the presence of more AM fungi than in the control soils.

The results got on the PLFA and NLA analysis for the soils amended with compost are in line with what is found in literature is found: many articles show in fact that compost can positively or negatively affect the AMF

population, but it generally enhances bacterial biomass (Cozzolino et al., 2015, Aleklett et al., 2012). This can also be related with the results of C content in soils, in section 3.1.2. In figure 4 it was in fact observed an increase of C mineralization within 3 months in the soil of CV amended with compost in comparison with the control. This could be due to the addition of C substrate with the compost that in turn can promote bacterial growth. However, after 3 month experiment the humic substances from the soil amended with compost did not show any significant change in the C distribution as compared with the control soil.

The treatments with iron-porphyrin lead to different effects on microbial community. First of all the total PLFA content in this soil decreased (table 9). In fact, the fungal colonization was reduced as compared with the control. This could be caused by the decline of AM fungi in this soil as suggested in figure 11.a. As result to it the fungal/bacterial ratio dropped down as compared with the control soil (figure 10 a). The AM/saprotrophic fungi is similar to the control, suggesting also saprotrophic fungi decreases as well as AM fungi. Nevertheless, a shift in bacterial community is observed in this soil. The iron-porphyrin treatment leads to an increase of Gram-bacteria over the Gram+, resulting in an Gram+/Gram- ratio which is the lowest as compared with the other soils (figure 10b). This is not expected. In fact it is hypothesized that iron porphyrin would lead to a polymerization of OM, followed by an increase of the more aromatic C components over the easily degradable C compounds. This should lead to an enhancement of slow-growing microflora, able to degrade these compounds, like Gram+ bacteria. On the contrary, the actual results suggest a higher availability of labile C substrate.

5 Conclusions

- Treatments with only iron-porphyrin did not have important effects on soil respiration within 3 months in none of the soil used for the experiment. Some variation in C mineralization are however observed in the treatment with both compost and iron-porphyrin as compared to the amendment with only compost. In the soils with higher content of clay a lower mineralization in soils amended in the presence of iron-porphyrin, while in the sandy-loam soil the porphyrin leads to higher mineralization of C as compared with the soil treated only with compost.

- The treatments with iron-porphyrin at high and low doses, mature compost and a combination of compost and iron-porphyrin at low dose did not lead to significant changes in the C distribution of HA and FA fractions. The relative amount of carboxylic-, phenolic-, aromatic-, O-alkyl-, methoxyl-/ N-alkyl, Alkyl- C was the same for all the HA and FA samples. As a results, no important changes in their hydrophobicity over the hydrophilicity, nor in the C/N and H/C was observed.

- The amendment with mature compost stimulated biological activity: in particular bacterial growth is enhanced in both low and high doses of compost. However, also AM fungi colonization is enhanced be the soil addition of compost, especially if in high dose, as compared to the control. High dose of compost increases the colonization of Gram+ bacteria over Gram-bacteria, while in low dose of compost the contrary is observed.

- Treatment with iron-porphyrin led to a reduction of fungal colonization as compared with the control. In particular AM fungi decline by the treated with only iron-porphyrin. Moreover, an increase of Gram-bacteria over the Gram+ is observed.

- Even though treatments with iron-porphyrin and compost did not have important effects on soil respiration and OM quality in the short-term, the efficiency of these methods cannot be excluded within a longer period of time. In fact, the shift in the microbial community structure seen with the PLFA and NLFA analysis done in soil samples from an experimental field after 1 year of treatments, suggests that a change in OM quality did occur both in the soil amended with compost and that treated with iron-porphyrin.

6 Recommendation

Further research has to be conducted to obtain more insight on the efficiency of iron-porphyrin and compost treatments in soils to sequester C. Some suggestions for the future research are:

- To run the experiment for longer time, since within a 3 months period almost no effects have been observed;
- HA and FA could be extracted with a less aggressive extraction solution, like with water or CaCl2, in order to see some differences in the HA and FA fractions readily available for microorganisms.
- NMR analysis should be done also to the HA and FA the isolated from the compost and the soil amended with compost at the beginning of the experiment. This would make the comparison with the HA FA

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Annex

Annex 1: Calculation for the soil treatments within the laborator experiment

A. Calculation to assess the amount of dried compost to apply to the soil dishes.

The purpose of the laboratory experiment was to reproduce the soil treatments done in the field experiment. Therefore the same ratio compost/soil that was applied to the field was used also for the laboratory experiment. In the field 20 tons of compost/he soil were applied. In order to calculate the respective amount for the soil dishes, firstly the volume of the soil amended with compost was calculated. Thereafter the volume was converted to weight in order to have a ratio compost/soil based on the weight. The same ratio was therefore used for the experiment under controlled laboratory conditions.

Soil volume (m^3) = *surface soil (m^2) * soil depth (m)*

Soil weight (tons) = *soil volume (m^3) *density (ton/m^3)*

Ratio used for the lab experiment: $$\frac{weight\ of\ compost\ applied\ (ton)}{soil\ weight\ \ (ton)}$$

Example:

1 ha soil: 10000 m^2 soil

Depth of soil: 0.3 m

Density soil: 1.4 ton/m^3

Amount compost applied in the field: 20 tons/ha soil

soil volume (m^3): *(10000 m^2) * (0.3 m)= 3000* m^3

soil weight (tons): *(3000 m^3) * (1.4 ton/m^3)=4200*

ratio used for the lab experiment: $$\frac{20\ (ton)}{4200\ (ton)} = 4.76*10^{-3}$$

B Calculation to assess the concentration of iron-porphyrin to apply to the soil dishes.

To obtain the concentration of iron-porphyrin to be applied to the soil dishes, the same calculations made for the compost have been done. In the field 5 kg of iron-porphyrin are applied per hectare soil. This corresponded to the low dose of iron-porphyrin. Therefore the calculation were:

Soil volume (m^3) = *surface soil (m^2) * soil depth (m)*

Soil weight (tons) = *soil volume (m^3) *density (ton/m^3)*

Ratio used for the lab experiment: $$\frac{weight\ of\ iron{-}porphyrin\ applied\ (ton)}{soil\ weight\ \ (ton)}$$

Example:

1 ha soil: 10000 m^2 soil

Depth of soil: 0.1 m

Density soil: 1.4 ton/m^3

Amount iron-porphyrin applied in the field: 5 kg/ha soil

soil volume (m^3): *(10000 m^2) * (0.1 m)= 1000* m^3

soil weight (tons): *(1000 m^3) * (1.4 ton/m^3)=4200*

ratio used for the lab experiment: $\frac{5\ (kg)}{1400\ (ton)}$ = *3.6*10^{-3} g/kg*

The high dose of iron-porphyrin corresponded to the double concentration of the low dose of iron-porphyrin.

Annex 2: Characterization of mature compost

^{13}C-CPMAS-NMR analysis were performed on a sample of mature compost in order to characterize its C components and deep the knowledge into the C groups added to the soils through compost amendement. In table.. the relative percentage of each C groups identified in the NMR spectra is shown. Moreover, the HB/HI ratio is reported.

	COMPOST
CARBOXYLIC C	9%
PHENOLIC C	5%
AROMATIC C	8%
O-ALKYL-C	41%
METHOXYL/N-ALKYL	14%
ALKYL-C	24%
HB/HI	0.58

Annex 3: PLFA and NLFA extraction procedure

For the PLFA and NLFA extraction the following protocol has been used:

1) EXTRACTION:
   - weight 1 gr of soil inside a centrifuge tube
   - inside each tube add in sequence:

   1.5 ml citrate buffer ph 4 (0.15M sodium citrate, 0.15M citric acid;
   1.9 ml chloroform;
   3.8 ml methanol
   2 ml of blight and dyer solution (chloroform-methanol-citrate buffer in ratio of 1:2:0.8)
   - vortex and shake for 2 hours horizontally at 150 rpm
   - centrifuge at 2500rpm x 15min then transfer the upper phase in a clean centrifuge tubes.
   - the tubes with the soil are filled with 2.5ml of blight and dyer solution and then centrifuged for 15min at 2500, then combining the upper phase with the previous one.
   - add to the upper phase 1.55 ml of chloroform and 1.55 ml of citrate buffer, vortex and then centrifuge at 2500rpm for 15min.
   - in the tubes we will have two phases: recover 1.5 ml from the lower phase with a Pasteur and transfer to graduated tubes
   - dry under nitrogen flux

- storage in the freezer

2) SEPARATION:
   - condition the SPE columns with 2x 1ml of chloroform
   - dissolve the sample in 0.3 ml of chloroform and add to column
   - add other two times 0.3 ml of chloroform
   - add step by step to the column 5ml of chloroform and recover the neutral lipids downside in graduated tubes
   - add step by step to the column 20ml of acetone to recover glycolipids in plastic tube (throw away the eluted)
   - finally add step by step 5ml of methanol recovering phospholipids in graduated tubes.
   - dry under nitrogen flux at 40°c
3) TRANSESTERIFICATION:
   - add to each sample:

   15 microliter of standard c19;
   1ml toluene-methanol solution (1:1ratio);
   1ml koh-methanol 0.2m
   - incubate (water-bath) at 37°c x 15min
   - add to each sample:

   2ml hexane-chloroform solution (4:1ratio);
   30microliter acetic acid,
   2ml deionized water
   - vortex and centrifuge at 2500rpm for 5min
   - recover 3ml of the upper phase and transfer to new graduated tubes
   - add 2ml hexane-chloroform
   - vortex and centrifuge at 2500 rpm for 5min
   - recover the upper phase and combine with the previous
   - dry the sample with the upper phase at 40° under nitrogen flux
   - now the sample can be stocked in the freezer or directly analysed through GC-MS
   - to analyse: dissolve the sample in 0.2ml hexane and transfer in the vial ready for GS-MS injection .

Annex 4: PLFA biomarkers

The biomarkers representative of broad microbial groups were chosen based on literature review. In particular, the article of Cozzolino et al. was used. In the table below the fatty acids used as biomarkers and the relative microbial group are reported.

GRAM+	a15:0
GRAM+	15:00
GRAM+	i16:0
GRAM+	a16:0
GRAM-/FUNGHI	16:1w7c
GRAM-/FMA	16:1w5c
GRAM-	cy16:0
ATTINOMICETI	10Me16:0
GRAM+	i17:0

GRAM+	a17:0
GRAM-	cy 17:0
GRAM+	17:00
ATTINOMICETI	10 Me17:0
FUNGHI	18:2w6,9
FUNGHI	18:1w9c
GRAM -	18:1w7c
GRAM -	18:1w5
ATTINOMICETI	10Me18:0
GRAM-	cy19:0

The fatty acids are named as follows: *total number of carbons : number of double bonds*; the symbol ω; and the position of the first double bond from the methyl end of the molecule. Cis- and trans-configurations are indicated by c and t, respectively; iso and anteiso forms of methyl-branched fatty acids are indicated by i- and a-, respectively. 10Me indicates a methyl group placed on the tenth C atom from the carboxyl end of the molecule; cy refers to cyclopropane fatty acids (Cozzolino et al., 2015).

Annex 5: Calculation to convert the PLFA and NLFA value in nmol/g soil

The area of each PLFA was firstly divided by the area of the standard (C19). The result was multiplied by the real amount of standard in µl (0.034) and by the 200, which is the dilution automatically made by the instrument before the injection for the GC-MAS analysis. The result is the PLFA content expressed as µg/g soil. To express it in nmol/g soil the obtained value was divided by the molecular weight of the standard (284), multiplied by 2 (because during the PLFA extraction only half of the polar solution has been samples) and multiplied by 1000 to have nmol.

Standard (C19) stock solution was done by diluting 230.08 µg in 1000 µl. The volume of standard added in the sample solution was equal to 15µl, therefore the actual amount of standard added in each sample is equal to:

$$\frac{230.08\ \mu g * 15\mu l}{1000\mu l} = 0.034\ \mu g$$