BIOREM biochemical monitoring manual

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Produced by **ISE-CNR**

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1. Introduction

Although degradation processes in Europe vary considerably from Member State to Member State, with different threats having different degrees of severity, soil degradation is an issue all over the Union. An estimated 115 million hectares of Europe's total land area are subjected to water erosion, and 42 million hectares are affected by wind erosion.

The erosion processes are a consequence of land mismanagement, such as overgrazing, excessive land clearing for extensive agriculture and fuelwood; these practices can also lead to the formation of bare soils that became encrusted and unproductive.

The depletion of organic matter due to loss of vegetation cover leads to the destruction of soil aggregates and to the dispersion and redistribution of soil particles by erosion (Casenave and Valentin, 1989). These processes have ultimately resulted in surface crusting and the compaction of a thin surface layer that prevents the natural regeneration of vegetation (Valentin and Bresson, 1992).

It is generally recognized that land degradation leads to losses of soil organic C and N stocks (Dlamini et al., 2014), and thereby to the further loss of productivity and of the ability to provide several other ecosystem services (UNEP, 2012).

An estimated 45% of European soils have low organic matter content, principally in southern Europe but also in areas of France, the UK and Germany. Below a certain level of soil organic matter, the levels of production fall, either directly, because of a deficiency in nutrients of organic origin, or indirectly, because of a degradation of the structure of the soil. According to Loveland and Webb (2003), a threshold of 3,4% of soil organic matter (2 % soil organic carbon) is the level below which potentially serious decline in soil quality may occur. Le Villio et al. (2001) consider a threshold value between 2 or 3 % of soil organic matter in a silty soil. From an environmental and economic point of view, several regions in southern Europe have a critical level of soil organic matter below which the agricultural production could be sharply decreased. In Mediterranean regions, the low levels of soil organic matter of certain densely populated regions are already producing alarming consequences on the levels of production.

Soil depletion and impoverishment cause three main environment-related problems and human level: (1) soil erosion of farming areas, (2) marginalization and abandonment of agricultural land, and (3)soil sealing.

(1) Soil erosion by water is a widespread problem throughout Europe. Physical factors like climate, topography and soil characteristics are important in the process of soil erosion. The Mediterranean region is particularly prone to erosion because it is subject to long dry periods, followed by heavy bursts of erosive

rain, falling on steep slopes with fragile soils. With a very slow rate of soil formation, any soil loss of more than 1 t/ha/year can be considered as irreversible within a time span of 50-100 years. The level of soil erosion in the involved regions is between 1 and 10 t/ha/ye (data from IRENA - PESERA project).

(2) The marginalization of farming areas is driven by a combination of social, economic, political and environmental factors. Marginalization makes farming less viable over time, leading to the eventual abandonment of agricultural land (within farms or as a whole farm). The environmental effects of marginalization are linked to the abandonment of agricultural land, which may lead to a loss of landscape diversity and related loss in biodiversity, and to an increasing vulnerability to fires, and in some cases, soil erosion. This arises from a re-growth of different shrubs and eventually woodland vegetation on abandoned agricultural land, which suppresses biodiversity-rich grasslands and leads to an increased fire risk in Mediterranean areas.

(3) The conversion of agricultural land to artificial surfaces, which is also known as soil sealing, can impact on soil, water and biodiversity resources. Sealing increases the risks of soil erosion and water pollution, disturbs agricultural habitats, impacts on animal migration patterns and affects the hydrological cycle (increased water runoff and decreased water retention) leading to an increased risk of floods. In addition, it affects the esthetical value of agricultural landscapes, and increases their fragmentation, which can result in more noise and emissions because of increased traffic levels.

Currently soil management techniques are being developed to limit the problems of erosion and decreases of the quantities of organic matter within the soil. In some environments reduced tillage or no-tillage are considered to improve soil properties, and these techniques have been shown in some environments to be a way to conserve and improve soil organic matter quantity and quality (Tebrügge and During, 1999). In some areas there is considerably less deep ploughing and some farmers are increasingly using conservation tillage. The practices of conservation tillage and conservation farming in general are developing rapidly, in some systems there is no disruption of the soil and direct drilling in to the soil surface. These techniques must be used correctly and appropriately and are not universally applicable. Most of the new techniques are developed in USA, Canada, Australia, and to a lesser degree, in Europe, where actually, only 1 million hectares are cultivated in this manner (less than 1 % of UAA).

Soil restoration through the application of organic matter and tree plantation are currently used to contrast soil erosion and to improve soil quality. Organic amendments (compost, green manure, animal manure, etc.) applied to soil have long been employed for enhancing favourable soil conditions (Sebastia et al., 2007) and for recovering degraded soils in semiarid regions (Garcia et al., 1998). The addition of organic

materials can increase soil organic matter content and contribute to C fixation in soil, thus decreasing the greenhouse effect derived from CO_2 emissions (West and Post 2002). Loss of SOC can be reversed by ceasing cultivation and returning to the original land cover or other perennial vegetation. The effectiveness of soil organic matter application to promote the soil colonization by natural spontaneous plant species has been previously reported (Ros et al., 2003). The maintenance of this vegetation cover is essential for sustaining microbial activity and growth, thus enhancing biogeochemical nutrient cycles (Bouwman and Vangronsveld, 2004). Ross et al. (2002) report the re-establishment of soil equilibrium after the first year onwards from the application of fresh or stabilized organic waste to a semiarid soil, favored not only by the increased organic matter content but also by the increase in microbial biomass, the maintenance of the soil's biochemical cycles and the establishment of a plant cover.

2. Ojective

The environmental management processes typically involve site characterization, remediation and monitoring. A variety of chemical and biological analyses have been developed and are widely accepted as the means to understand environmental and biological conditions at each of these stages. However, characterization and restoration evaluation is mainly based on converging lines of chemical, geochemical, and microbiological evidence. BIOREM methodology provides an useful new analytical system intended to complement those already in use. For example, traditional physical-chemical analyses of soil coupled with biological analyses provide a faster, more thorough, dynamic monitoring of soil conditions.

The BIOREM methodology can be used to identify and quantify key microorganisms, enzymes, and/or genes involved in specific biogeochemical processes without growing microorganisms in the laboratory. These analyses can therefore answer previously unanswerable questions about the potential of sites to support biological restoration processes and the effectiveness of the restoration approaches. While traditional techniques provide a "static" physical, chemical and biological information about the status of soil in a given moment, BIOREM methodology can provide a dynamic monitoring with high level of reliability, efficiency, and precision.

The BIOREM results provide consultants, regulators, and stakeholders with knowledge and information to make informed decisions.

3. Soil characterization methodology

The biotic fraction of organic matter, which is made up of live organisms, plays a fundamental role in soil since it is ultimately responsible for the state of the organic matter and, in general terms, for the development and functioning of a land ecosystem.

The capability of soil microbial communities to mineralize and stabilize fresh organic matter is, in fact, a key point for soil fertility since they help set in motion biogeochemical cycles and decide their structure. Microorganisms have a great influence on many organic matter oxidation, hydrolysis and degradation reactions, and these in turn, are reflected in the natural cycles of carbon, nitrogen, phosphorus and other elements, thus establishing (or not) the ideal conditions for the development of a stable plant covering. This is essential if a soil is to be of sufficient quality to maintain a suitable degree of natural fertility. However, such biological activity is influenced by many factors, which in the long run, depend on soil structure, such as moisture content, temperature, clay and humus content.

In particular, the soil enzymatically active humus content, which is the stable fraction of soil organic matter able to protect microorganisms and enzymes fro degradation, plays an important role in the functioning of soil ecosystems. Its degradation, resulting in diminished soil microbial and physical quality, has been considered as the first step towards the irreversible degradation, called desertification. Humus is, in fact a key attribute of soil quality in that it promotes infiltration of air and water, holds water and nutrients, helps establish and maintain a strong soil physical structure, provides nutrients (N and P), buffers the effects of contaminants and entraps enzymes. Enzymes binding humic substances are more stable to some environmental stresses such as temperature extremes, digestion by proteases, etc., than free enzymes and can persist for extended periods, thereby providing a long-term perspectives in indicating the history of the sample and not just a snapshot of the time of sampling. These enzymes remain at a constant and stable level of activity in the soil regardless of microbial proliferation and are not influenced by the usual forms of regulation which affect enzyme synthesis and secretion. For these reasons, humo-enzyme complexes are universally considered of great importance in soil biological fertility and soil resilience. They are also of scientific interest, since they represent a “crossing-point” between mineral and organic reactions in soil, that is between chemical and microbiological soil processes. Due to these ecological properties, humo-

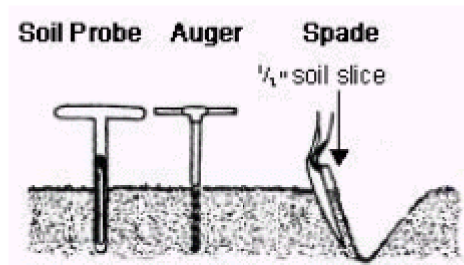
enzyme complexes may be extremely useful to indicate the rate of biological degradation of the entire soil ecosystem. In addition, their measurement can provide information on soil resistance to adverse degradative factors and also to assess the capability of a soil ecosystem to be regenerated through ecologically sound practices.

3.1 Soil sampling

Beneficial results of a soil test depend on the availability of a good sample. The sample should represent the area from which it is taken from. A soil sample must be taken at the right time and in the right way. The tools used, area sampled, depth and uniformity of the sampling, information provided, and packaging are all factors influencing the quality of the sample.

3.2 How to Take Samples

- Use a soil-sampling probe, an auger, a spade or shovel.



- Tools should be either stainless steel or chrome-plated. Do not use brass, bronze, or galvanized tools because they will contaminate samples with copper and/or zinc.
- Mix soil cores for each sample in a clean, plastic bucket. If the bucket has been used to hold fertilizer or other chemicals, wash it thoroughly before using it for soil samples.

In the BIOREM project the soil samples have been taken by an environmentally friendly soil sampling probe (core sampler 15 cm diameter, 30 cm length). The advantage of the use of the corer is the fact that the user can sample a representative composite soil sample with a minimum soil disturbance. The user applies a

pressure to the corer lower handle which penetrates the soil profile and upon collection; the removal of the core sample is done by pushing the plunger, releasing the content of the corer into the sampling bag.

This coring device allows to collect soil samples at various depths in sufficient quantity to perform an adequate analysis (a quantity varying between 1 and 2 kg is recommended).

The corer is almost mandatory in vegetation covered soils, as the spoons are highly not recommended in such situations. The corer cuts through the vegetation and reaches the soil with a minimum vegetation cover disturbance.

<u>Sampling Depth</u>

Collect samples to the same depth considered for plowed (usually about 30 cm) because this is the zone in which fertilizers have been incorporated.

3.3 Where to Take Samples

When collecting samples, avoid small areas where the soil conditions are obviously different from those in the rest of the field—for example, wet spots, old manure and urine spots, places where wood piles have been burned, severely eroded areas, old building sites, fence rows, spoil banks, and burn-row areas. Also the fertilizer bands in fields where row crops have been grown should be avoided. Because of the samples taken from these locations could not be typical of the soil in the rest of the field, including them could produce misleading results.

- Areas within a field where different crops have been grown in the past should be sampled separately, even if you now plan to grow the same crop in the whole field. Areas that have been limed and fertilized differently from the rest of the field should also be sampled separately. Each sample should represent only one soil type or area—for example, bare or vegetated area. For each unique area, take at least six to eight samples. Place all the samples for one unique area in a plastic bucket and mix thoroughly. Use the mixture in the bucket to fill a soil sample bag about two-thirds full.
- If the field you are sampling contains areas that are obviously different in slope, color, drainage, and texture, etc. submit separate samples for each area.

Within any soil there is an inherent variability in physical-chemical and biological properties. The degree of variability differs according to numerous factors, including the size of the area, the management and the soil type. These factors can produce spatial variability that is considerably larger than that encountered in other media.

Adequate and environmentally friendly planning of the sampling program has been done in the BIOREM project in order to assure that samples represent the areas and depths desired, that sampling variability is properly determined and accounted for, and that there are sufficient numbers of samples at the appropriate locations to fulfill the purposes of the sampling.

3.4 When to Take Samples

Collect samples three to six months before remediation time. You will then have the test report in time to plan your remediation program. If you submit samples immediately after harvest in the fall, you are likely to receive the results promptly because the laboratory workload is lighter at that time than in the spring. If possible, try to collect your samples at the same times every year, or if the samples are taken in different period of the year, please take into account the season effect.

Do not collect samples when the soil is too wet because it will be difficult to mix the cores. As a rule, if the soil is too wet to plow, it is also too wet to sample.

3.5 How Often to Sample

Six months are necessary to soil for reaching a new state of equilibrium after a remediation strategy application, so that after the characterization sampling, the first monitoring sampling should be done at least after six months from the beginning of the experimentation.

3.6 Submitting the Sample

Procedures for sampling operations will be carefully documented. Careful documentation during sampling is required so that all relevant information on the nature of the sample (when it was taken, where it was taken and under what conditions it was taken) will be clearly recorded on site at the time of sampling by the person conducting the sampling. This is necessary because variations in sampling procedures can have a marked effect on the results of analysis.

3.7 Sample receipt, storage and disposal

Almost as important as proper sampling, is the proper storage of samples prior to analysis. It is important to ensure that the passage of a sample through the laboratory's analytical systems is fully documented, and corresponds to the practices laid down in the relevant Standard Operating Procedures. Equally important are the arrangements for disposal of samples. The sample should be logged in and stored in such a way as to minimize its deterioration. The condition of each sample and its storage location should be recorded and, where appropriate, the analyses to which it is to be subjected should also be recorded.

The BIOREM samples were sieved (2 mm) and stored dried at room temperature until chemical and biochemical analysis, and stored at -80°C until biological analysis.

4. Analytical methods

A variety of different analytical methods are usually available for determining the concentration of any variable in a soil sample. The choice of method is critical for ensuring that the results of the analysis meet the laboratory's requirements, because different methods have different precisions and sensitivities and are subjected to different potential interferences. Consideration must be given to these parameters before choosing a method, although the technical literature does not always provide adequate information. Nevertheless, a number of standard methods which have procedures described in sufficient details are available for most of the analytical determinations involved in soil quality monitoring.

BIOREM methodology

4.1 Chemical characterization

The chemical parameters and methods used in the BIOREM methodology are showed in the following table.

Analysis	Method	References
Total organic C and Total N	Determination with a Thermo Finnigan Flash 1112 elemental analyzer	
Macro and micronutrients	The total contents of P, K, Na, Ca, in the soils are determined, after nitricperchloric acid digestion, by ICP spectrometry	UNE-EN ISO 11885:1998
Heavy metals	Atomic absorption in the suitable dilutions of a nitric-perchloric extract from the soil samples	UNE-EN ISO 11885:1998
pH and electrical conductivity	Electrical conductivity and pH are measured in a 1/5 (w/v) aqueous extract, in a Crison conductivimeter and pH-meter	
Cationic exchange Capacity (CEC)	**CEC** is calculated as the sum of concentrations of cations analysed by ICP.	
Water-holding capacity	Calculated from the amount of water retained by the saturated soil, with no drainage, at 1/3 atmospheric pressure	Analytical Standardization Methods of the Spanish Group

pH and electrical conductivity (EC). pH and EC of the different soils are measured in a pH-meter (Crison GLP21+) and EC-meter (Crison GLP31+, cell conductivity Pt 1000) respectively. pH measure is done with raw soil:water extract (1:5) and EC after centrifuging and filtering through ash-less filter paper (Albet 145 110).

Total organic C and N. The Corg and N concentrations in the soils and soil particle-size fractions are determined with a Thermo Finnigan Flash 1112 elemental analyzer (Franklin, MA, USA), after hydrochloric acid had been used to eliminate carbonates.

Macro and microelements. 1 g of soil is digested in microwave digestor with perchloride, then the sample is filtered and analyzed using an Optical ICP Thermo ICAP 6500 duo.

Cationic Exchange Capacity. The CEC is the number of positive charges that a soil can contain. An equivalent is defined by the number of charges in terms of a given number of hydrogen ions. From the results obtained by ICP (mg/k), CEC is calculated as the sum of concentrations of cations displaced (Na, Ca, K, Mg) in meq/100g.

4.2 Soil proteomic methods

Microorganisms are isolated from 60g of soil of the duplicates incubation for further proteome characterization by using the density-gradient method of Taylor & Williams (2010) based on Nycodenz and quantified by fluorescence microscopy staining with SYBR Green I (Invitrogen). For protein extraction, cell pellets (2 x 109 cells) are subjected to a phenol extraction method followed by 0.1M ammonium-acetate in methanol and acetone precipitation. Proteins extracted from soil cells are separated by SDS-PAGE and stained by colloidal Coomassie-Brilliant-Blue G-250 (Roth, Kassel, Germany).

All bands are cut and subjected to tryptic hydrolysis prior liquid chromatography coupled to tandem mass spectrometry analysis (LC-MS/MS; nanoESI-Q-TOF mass spectrometer, QSTAR-XL, Applied Biosystems). Protein identification is performed by database comparison against NCBI bacteria and fungi via Mascot Daemon v2.2.2. An additional search is performed against the complete metagenome of soils (Minnesotta farm and FACE/OTC soils) at the Integrated-Microbial-genomes with Microbiome Samples (img/m) from the DOE-Joint-Genome-Institute. Peptides are considered to be identified when a probability <0.05 is found. Proteins identified by only one peptide are included in the identification list if the expected value of the peptide is <0.07. Functional information of proteins is obtained according to cluster orthologue groups from the Kyoto-Encyclopedia of Genes and Genomes (KEEG).

4.3 Genomic methods

DNA isolation and amplification

DNA is isolated from 1.0 g of each homogenized sample using the BIO101 FastDNA kit (La Jolla, CA, USA). DNA concentrations are determined using a NanoDrop 2000c (Thermo Scientific, Wilmington, USA). Recovered DNA is amplified using barcoded 16S rDNA and 18S rDNA pyrosequencing tags. A 16S rDNA gene fragment is amplified using the primers BSF8 and USR515 and conditions established by Bibby et al. (2010). A 18S rDNA gene fragment is amplified using the primer set nu-SSU-0817-59 and nu-SSU-1196-39 and conditions established by Borneman and Hartin (2000). All amplicons are cleaned and pooled in equimolar concentrations into a single tube before sequencing on a Roche 454 GS FLX using titanium chemistry.

Processing of pyrosequencing data

Raw read output is quality filtered by discarding reads with < 200 bp and average quality score < 25. Filter-pass reads are parsed into their respective sample-specific barcode bins only if they matched the entire forward primer and barcoded sequence. Forward and reverse primers and barcodes are removed after binning. After the quality filtering, all samples are rarefied to 1200 sequences per sample (in the case of 16S rDNA) and 7300 sequences per sample (in the case of 18S rDNA gene). All sequence analyses are conducted using the QUIIME pipeline (Caporaso et al., 2010). Phylotypes are selected at the 97% sequence similarity level and the taxonomic identity is determined using the RDP scheme. Rarefaction curves are constructed using the Rarefaction tool from the RDP pipeline.

Barcoded pyrosequencing of 16S and 18S rRNA genes, community structure and diversity

4.4 Chemical and biochemical characterization of soil humic matter

Extraction and purification are essential steps to study humic carbon and active humic-enzyme compounds. These compounds are characterized from chemical and biochemical point of view.

The chemical and biochemical properties of humic carbon are studied on soil extracts obtained using a pyrophosphate solution 0.1M, pH 7 and membrane ultrafiltration (cut off 10.000 dalton).

On soil humic extracts, are carried out the following analyses:

•*Enzymatic tests* linked to main nutrients cycles (hydrolitic enzymes: phosphatase and β-glucosidase) representing the level of extracellular enzymes.

•*Isoelectric focusing* of extracted humic substances to have a chemical and biochemical characterization of organic matter and purified enzymes

IEF electrophoretically separates amphoteric molecules according to their net surface charge density or isoelectric points (pI); pI is the pH value at which the net surface electrical charge is “zero”. Compounds with a comparable molecular weight and electrophoretic mobility, such as those in humic or fulvic fractions of soils or other organic compounds, but differing slightly in their electrical surface charge, may be sharply separated by IEF. The surface electrical charge of an organic compound is of particular importance when predicting its stability or movement (solubility) in a given physical environment characterised by an amount of electrical charges similar to that existing in many soils.

The parameters and methods used in the BIOREM methodology are showed in the following table.

Analysis	Method	References
β-glucosidase activity assay	determined using the 4-Methylumbelliferyl B-glucosidase as substrate. The product of the reaction 4-Methylumbelliferone is measured fluorimetrically (excitation 360 nm; emission 450 nm)	Marx et al., 2001; Vepsäläinen et al., 2001
Phosphatase activity assay	determined using the 4-Methylumbelliferyl phosphate as substrate. The product of the reaction 4-Methylumbelliferone is measured fluorimetrically (excitation 360 nm; emission 450 nm)	Marx et al., 2001; Vepsäläinen et al., 2001
Isoelectric focusing (IEF)	Cylindrical gel of polyacrylamide and ampholines in the pH range 4-6	Ceccanti et al., 1986
β-glucosidase activity on humic bands	After IEF, the humic bands were cut; the activity in each band was measured using the PNG (para nitrophenyl-β-D-glucopyranoside) as substrate. The product of the reaction para-nitrophenol (PNP) is measured spectrophotometrically at 398 nm	Ceccanti et al., 1989
Dehydrogenase activity assay	determined using in the INT (P-Iodio-Nitro-Tetrazolium-chloride) as substrate. The product of the reaction (INTF) is measured spectrophotometrically at 490 nm	Masciandaro et al., 2000

4.5 β-glucosidase activity assay

β-glucosidase activity is determined using the 4-Methylumbelliferyl β-glucosidase as substrate. A moist sample (equivalent weight to 2 g oven-dry material) is weighed into a sterile jar and 50 ml of water are added. A homogenous suspension is obtained by homogenising with sonication for 1 min. Aliquots of 100 μl are withdrawn and dispensed into a 96 well microplate (three analytical replicates sample). Finally, 100 μl of 1 mM substrate solution are added. Fluorescence (excitation 360 nm; emission 450 nm) of the product 4-Methylumbelliferone is measured with an automated fluorimetric plate-reader (Infinite® F200PRO Tecan) after 0, 30, 60, 120, 180 min of incubation at 30 °C.

4.6 Phosphatase activity assay

Phosphatase activity is determined using the 4-Methylumbelliferyl phosphate as substrate. A moist sample (equivalent weight to 2 g oven-dry material) is weighed into a sterile jar and 50 ml of water are added. A homogenous suspension is obtained by homogenising with sonication for 1 min. Aliquots of 100 μl are withdrawn and dispensed into a 96 well microplate (three analytical replicates sample). Finally, 100 μl of 1 mM substrate solution are added giving a final substrate concentration of 500 μM. Fluorescence (excitation 360 nm; emission 450 nm) of the product 4-Methylumbelliferone is measured with an automated fluorimetric plate-reader (Infinite® F200PRO Tecan) after 0, 30, 60, 120, 180 min of incubation at 30 °C.

4.7 Isoelectric focusing

Isoelectric focusing is carried out in cylindrical gel rods (0.5×8 cm) containing polyacrylamide gel (5% w/v) and carrier ampholines in the pH 4–6 range (Bio-Rad Laboratories, Richmond, California, USA) at a final concentration of 2% (Ceccanti et al. 1986). N,N,N′,N′-Tetramethyl- 1,2-diaminomethane and ammoniumperoxy-disulfate are also added in gel solution at 0.03%. Organic material (100 μl of pyrophosphate extract derived fraction >104 Da) at 4.4% of glycerine is applied at the top of the gel rod (cathode). A little amount (5 μl) of glycerine at 2.2% is added on the top of the sample to avoid the mixing with the cathodic solution (NaOH 0.02N); 0.01 M H3PO4 is used for the anodic cell. A pre-run of 1 h at the same current intensity and voltage used for the samples run is carried out for each gel tube (1.5 mA for each tube, 100–800 V); subsequently, the samples run is carried out for 2 h or longer until a stable IEF banding is reached. The electrophoretic bands are scanned by a Bio-Rad GS 800 densitometer, obtaining a typical IEF profile for each soil investigated. The IEF peak area is determined for each soil IEF profile, assuming as 100% the area under the entire IEF profiles (representative of the total loaded C). Gel pH is

measured at 0.5 cm intervals with an Orion microprocessor (model 901, Orion research) connected to a microelectrode gel-pHiler (Bio-Rad Laboratories, Richmond, California, USA).

Enzyme activities were tested also on bands obtained by IEF.

4.8 β-glucosidase activity assay on bumic bands

To determine β-glucosidase activity of the humic bands obtained by IEF, the gel is gently removed from the glass tubes. The bands are cut and pre-washed for 1 h with 2 ml 0.1 M phosphate buffer, pH 6.4, at 37°C. Pre-washing removes the carrier ampholytes, salts, and other impurities from the gel, without releasing the gel-trapped humic matter (Ceccanti et al. 1989). After removal of the buffer, 2 ml of fresh 0.1 M phosphate buffer, pH 6.4, and 0.5 ml 0.05 M PNG are added and the mixture is incubated at 37°C under shaking for 17 h. The β-glucosidase activity is expressed as mg PNP $kg_{dry\ soil}^{-1} h^{-1}$.

4.9 Dehydrogenase activity assay

Dehydrogenase activity is determined by the method of Masciandaro et al. 2000. Soil is mixed with 0.2 ml of 0.4% INT solution (in distilled water, w/v). The control is the soil treated with distilled water (0.2 ml), instead of INT. Soils are incubated for 20 h at room temperature in darkness. The iodonitrotetrazolium formazan (INTF) formed by the reduction of INT is extracted by adding 10 ml of a mixture of 1:1.5 ethylene chloride and acetone; then the soil mixture is vigorously shaken by vortex for 1 min and finally centrifuged. The INTF concentration is measured spectrophotometrically at 490 nm, and the results are expressed as mg INTF $kg_{dry\ soil}^{-1} h^{-1}$.

The methods followed the requirement of:

- Linearity
- Limit of Detection
- Precision
- Accuracy

4.10 Stable Isotope Probing

Isotopic analysis is carried out by Elemental Analysis - Isotope Ratio Mass Spectrometry (EA-IRMS). Tin capsules containing reference or sample material are loaded into an automatic sampler. From where they are dropped, in sequence, into a furnace held at 1080 °C and combusted in the presence of oxygen. Tin capsules flash combust, raising the temperature in the region of the sample to ~ 1700 °C. The combusted gases are then swept in a helium stream over combustion catalysts (tungstic oxide/zirconium oxide) and through a reduction stage of high purity copper wires to produce SO_2, N_2, CO_2, and water. Water is removed using a Nafion™ membrane. Sulphur dioxide is resolved from N_2 and CO_2 on a packed GC column at a temperature of 32 °C. The resultant CO_2 peak enters the ion source of the IRMS where upon it is ionized and accelerated. Gas species of different mass are separated in a magnetic field then simultaneously measured on a Faraday cup universal collector array. Both references and samples are converted to CO_2 and analysed using this method. The analysis proceeds in a batch process by which a reference is analysed followed by a number of samples and then another reference. For what concern δ15N the samples are introduced into a Thermo- Flash EA, Elemental Analyser where N_2 gas is produced by combustion at 1020 °C. The gases, moved along in a continuous flow of helium, are separated by a GC column, and introduced into a continuous flow gas source mass spectrometer for nitrogen isotopic analysis. Repeated measurements took place for each of the samples. Analytical precision is 0.2‰ for both δ13C and δ15N values.

5 Result elaboration

Duplicate analysis are used as a method of precision checking, while internal standard and external reference materials are used as methods of accuracy checking.

Statistical analysis was used to accept the validity of the results.

Necessary checks to be carried out when a problem is detected with an analytical method

Item	Checks
Calculations and records	Check calculations for a transposition of digits or arithmetic errors. Confirm

	that results have been recorded in the proper units and that any transfer of data from one record to another has been made correctly.
Standard solutions	Check the standard solutions that are used for calibrating equipment. Old solutions may have deteriorated and errors may have occurred in the preparation of new ones. Check on storage conditions, the age of solutions and their expected shelf-life.
Reagents	Check whether old reagents have deteriorated. Check fresh reagents to ensure that they have been properly prepared. Check the storage conditions of reagents, especially those that must be stored away from the light or at a controlled temperature. Check the shelf-life of reagents, discarding any that are outdated or have been improperly stored.
Equipment	Check calibration records and maintenance records for all reagent dispensers and measuring equipment used for the analysis of the variable where the method is out of control. Items such as automatic pipettes, balances and spectrophotometers should be checked and recalibrated if appropriate. Ascertain that equipment is being properly used.
Quality control materials	Check on the storage conditions of quality control materials, ensuring that bottles are tightly sealed and that they are not being subjected to extremes of temperature. Run analyses on several aliquots to determine whether the concentration of the variable remains within two standard deviations of the target value and close to the mean of the last 20 determinations.

6 Conclusions

Chemical and physical analysis have been carried out in the BIOREM project in order to evaluate soil quality and restoration. This set of parameters are easy and fast to determine and also quite economic. The cost is about 15-30 € for soil sample in triplicate. However, chemico-physical parameters are not sensitive enough and they change very slowly. In contrast, biological indicators offer certain advantages over physicochemical methods, since are very sensitive even to small changes occurring in soil and they offered a high resolution picture of changes in soil under BIOREM remediation strategy.

Among the various biological indicators that have been proposed to monitor soil health, soil enzyme activities have great potential to provide a unique integrative biological assessment of soils and the possibility of assessing the health of the soil biota. Besides, soil enzyme activities provide an easy, relatively rapid, and low cost procedure to monitor soil health. The cost of enzyme activities carried out on soil (total enzyme activity) are similar to that of chemico-physical analysis, averagely 30€ for soil sample in triplicate, slightly higher, about 35€, the extracellular enzyme activities conducted on soil extract that need a further step consisting in the extraction of the enzyme from the soil.

The humic-bound enzymes represent another paramount parameters useful for soil monitoring, since they are considered a constitutive fraction of SOM capable of conferring resilience and resistance to stressed soils in extreme environments. This biochemical component of SOM was used also to assess soil response to different management practices. The methodology to isolate, purify and characterise these enzymatically active fractions of SOM (extracellular humic–enzyme complexes) is based on three steps: (1) pyrophosphate extraction of humic matter, (2) ultrafiltration (UF) of the various components of the organic extracts on molecular mass exclusion membranes, followed by (3) the analytical isoelectric focussing technique (IEF). Mainly due to the long procedure, the cost of this analysis is about 80 € for each sample resulting more expensive with respect to the previous analyses.

Moreover, new "OMICs" technologies have been applied in this project and benefit the understanding of the processes taking place at the microbial community level after remediation strategy application. Genomic tools based on bacterial (16S) and fungal (18S) gene pyrosequencing

(DNA and RNA methods) have been successfully used to evaluate the effects of compost and plants on the structure of the microbial community.

However, these technologies need long procedures for DNA-RNA extraction and quantification and sophisticated and expensive instrument for microbial identification, making them more expensive of the previous biochemical tests with a cost ranging around 200 € for sample.

In addition, BIOREM explored the frontiers of environmental metaproteomics with the aim to understand the functional response of specific microbial populations. Metaproteomics is an appropriate approach for unraveling functional insights within the microbial community. For this purpose, proteins were categorized in functional groups related to cellular metabolism. However, proteomic represents an expensive approach, in terms of both time and cost due to the long procedure and expensive apparatus necessary (400-500 € for sample). Still in its infancy, proteomics is starting to provide insights into this functionality, coupled to microbial phylogeny, thanks to the application of state-of-the-art mass spectrometers.

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