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BIO-BIO PROJECT

BIODIVERSITY-BIOINDICATION TO EVALUATE SOIL HEALTH

Editors
R.M. Cenci and F. Sena

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Based on conclusions from the International Workshop on the

BIODIVERSITY – BIOINDICATION TO EVALUATE SOIL HEALTH

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Editors: **Roberto M. Cenci and Fabrizio Sena**

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Introduzione

Le parole Biodiversità e Bioindicazione, racchiudono significati profondi, a volte complessi e non sempre fruibili dal Cittadino della strada.
Televisioni, giornali e il complesso e variegato mondo mediatico parlano spesso di biodiversità, nuova scienza e parola che è stata coniata da poco più di venti anni per il volere del presidente degli Stati Uniti d'America J. Carter.
Dopo la conferenza di Rio, tenutasi nel 1992, parlare di Biodiversità è un obbligo, non si può scrivere un articolo che parli d'ambiente senza introdurre la parola magica, forse se ne parla troppo senza sapere l'esatto significato e sicuramente si fa poco per salvaguardare e proteggere la Biodiversità che giorno dopo giorno riduce il suo numero di specie sul pianeta Terra come neve al sole.
Per la Bioindicazione, la storia è totalmente opposta, la Bioindicazione nasce con l'uomo e da sempre l'uomo la utilizza spesso senza rendersene conto, è diventata come il nostro sistema nervoso vegetativo, respiriamo, il cuore pulsa senza che noi ce ne accorgiamo, senza la nostra volontà.
La vista di cannucce di lago è un segnale fornito da un bioindicatore, ci indica la presenza di un ambiente umido, quindi un luogo con la presenza di acqua. Il pettirosso che in autunno si affaccia alle nostre case ci dice che sta arrivando l'inverno, il freddo, la cattiva stagione. Gli esempi sarebbero molteplici e non vorrei annoiare il Lettore con una lista interminabile.
Un aspetto ancora poco usuale è quello di utilizzare i concetti di Biodiversità e Bioindicazione per valutazioni ambientali, comunemente vengono utilizzati uno, due bioindicatori e niente di più.
Nel caso del Progetto BIO-BIO si è voluto dare un segnale forte applicando un numero importante di Bioindicatori con aspetti che coprono il campo della Biodiversità per una lettura molto diversificata che copra le tre reti trofiche e che nel tempo stesso tenga in considerazione dell'aspetto temporale con le influenze stagionali che possono modificare le risposte dei bioindicatori, il tutto affiancato da una robusta analisi chimico-fisica dei suoli.
Al Progetto hanno partecipato esperti nazionali ed internazionali i quali hanno creduto in esso, la loro esperienza è stata fondamentale per armonizzare il Progetto, il loro impegno è stato continuo, instancabile permettendo la realizzazione di questo volume. I risultati ottenuti, unici nel loro genere, potranno servire ad altri esperti come esempio da imitare per valutare, in modo esaustivo e completo, la qualità e la salute dei suoli con particolare attenzione a quelle aree che hanno subito una importante pressione antropica.
Gli esperti dell'Istituto dell'Ambiente e della Sostenibilità hanno collaborato con entusiasmo per portare a termine il Progetto, così pure la struttura della Provincia di Pavia si è dimostrata instancabile nell'affiancare gli esperti e il personale del Nostro Istituto.
A tutti un grazie e un arrivederci al prossimo Progetto BIO-BIO 2.

R.M. Cenci

Introduction

The words 'biodiversity' and 'bio-indication' carry complex meanings that are often intricate and not always understandable to the average citizen.
The elaborate and diverse media-world of television, newspapers, magazines and scientific journals often speak of biodiversity, a new science and word that was coined twenty five years ago on the wishes of Jimmy Carter, the then President of the United States of America.
Since the Rio Conference of 1992, discussions on biodiversity are an obligation in several sectors. In many cases, it is not possible to write an article on the state of the environment without introducing this magical word. Perhaps it is a word that is used too easily or without knowing its exact meaning. Certainly, not enough effort is made to safeguard and protect the biodiversity of our world as day after day, the number of species on the planet diminishes like spring snow when exposed to the sun.
For bio-indication, the story is totally opposite. Bio-indication is a concept created by man and used unconsciously in the same manner as our nervous system or when we breathe or when our heart beats. It happens subconsciously and without us noticing it. The occurrence of reed beds around lake margins is a bio-indicator. The presence of these plants indicates a humid environment, therefore, water can be found nearby. Similarly, when certain birds, such as the Robin, approach our houses in the autumn, it is a sign that the cold, hard season of winter is arriving. The examples are endless and I do not want to bore the Reader with a long list!
The use of biodiversity and bio-indication for environmental assessments is relatively uncommon, limited often to one or two bio-indicators.
However, through the BIO-BIO Project, there was an intention to give a strong message that through the use of a significant number of bio-indicators, a robust chemical and physical analysis of soil could be provided. Uniquely, the bio-indicators adopted in the Project contained aspects that addressed the issue of biodiversity, provided an insight into the three major nutritional networks while at the same time took into consideration any temporal characteristics related to seasonal influences that can change the signal of specific bio-indicators.
Many national and international experts have participated and believed in the goals of the BIO-BIO Project. While their experience and knowledge was fundamental in addressing key issues within the Project, their effort was continuous and untiring, culminating eventually in the creation of this volume. The results obtained, unique in their character, will provide other experts with an exhaustive and complete example to appraise and follow for the assessment of soil quality and health, with special attention to those areas that are susceptible to human pressure.
My colleagues from the Institute of Environment and Sustainability have collaborated with their usual enthusiasm and, together with the expertise of the researchers of the Province Pavia, have helped me complete the Project.
To all, I convey my eternal thanks and a bid you farewell until the BIO-BIO 2 Project.

R.M. Cenci

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The importance of soil biodiversity and bio-indication within the EU Thematic Strategy for Soil Protection

LUCA MONTANARELLA

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The new EU Thematic Strategy for Soil Protection will include a strong reference to the pool of soil biodiversity as a key function of soils to be preserved. Since available knowledge on soil biodiversity is recognised as being very limited, main effort of the strategy will be in stimulating new research programmes in this field of science. Related to this will be the increased development of soil quality indicators taking into account the biological function of soils. A full range of potential bio-indicators for soil health and functioning is available but still needs to be fully explored for operation soil monitoring activities.

1. Introduction

Soil protection has never been ranking high among the priorities for environmental protection in Europe. Soils are commonly not well known by the European citizens, particularly since only a small fraction of the European population is currently living in rural areas and having a direct contact with soils.

The majority of the urban population in Europe has only little understanding for the features and functions of soils. The most common perception is usually that soils are a good dumping site for all kind of wastes and that soils can be quite useful as surfaces for building houses and infrastructure.

Only during the last 2-3 years the need for a coherent approach to soil protection has come on the political agenda in Europe and was therefore introduced as one of the thematic strategies to be developed within the Community's 6th Environment Action Programme (6th EAP). The rationale behind the development of a coherent approach to soil protection is based on the recognition of the multi-functionality of soils. Soils are not any more considered only as dumping sites, construction surfaces or means for production (agriculture) but also as a fundamental environmental compartment performing vital ecological, social and economic services for the European citizens: filtering and buffering of contaminants allowing us to have clean drinking water, pool of biodiversity, source of raw materials, sink for atmospheric carbon dioxide, archive of cultural heritage etc.. These functions are now recognised of equal importance as the traditional soil functions commonly attributed to soils: production of food, fibre and wood (agriculture and forestry) and surface for housing and infrastructure (spatial development).

In order to develop a soil protection policy it is important to recognise that soils have distinctive features that make them quite different from the other environmental compartments, like air and water. Soils are first of all highly diverse both in space and over time. Soil properties can be completely different for soils only at few meters distance one from the others. The development of a common soil map of Europe has helped describing the very high spatial variability of soils across the European continent (fig. 1). Soils are not static but develop over time. The timescale for these changes is usually very long (hundreds of years). Therefore, for policy making purposes, we consider soils as essentially a non renewable resource. The high variability of soils implies that any soil protection strategy needs to have a strong local element build in. It is at local level that we can act in specific ways that are appropriate to the features of these particular soil types. This of course brings up the important distinction that needs to be made in identifying the actors that must develop and implement soil protection measures. It should be recognised that, while there are important local elements that need to be build in any soil protection strategy, there are nevertheless, clearly identified off site effects of soil degradation that justify an European or even global approach to soil protection. Erosion, decline of organic matter, soil contamination, soil compaction, soil sealing, loss of biodiversity have very important off-site consequences, like silting of

hydropower stations, increase of atmospheric carbon dioxide, contamination of drinking and bathing waters, contamination of food, increased frequency of flooding and landslides, etc.. All these off-site effects seriously threaten human health and have substantial economic implications.

A key feature for developing a soil protection strategy is the recognition of the implications linked with the fact that soils in Europe are commonly submitted to property rights. The majority of soils are in private property and this brings up a series of environmental liability implications.

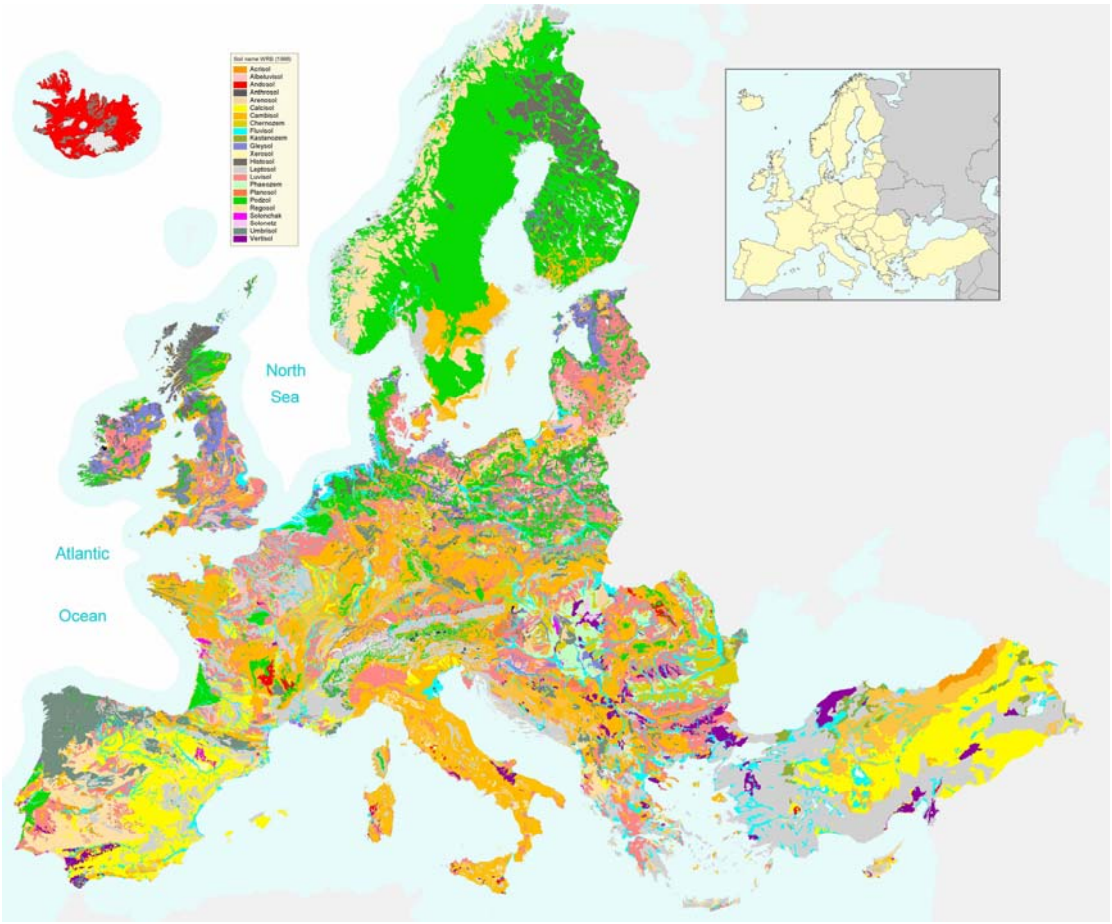


Figure 1: Soil map derived from the Soil Geographical Database of Europe at scale 1:1,000,000.

The EU soil protection strategy builds upon the recognition that the important functions of soils are threatened by severe degradation processes. The major threats identified so far are soil erosion, decline in organic matter content, loss of soil biodiversity, soil contamination, salinization, soil compaction, soil sealing and major hydro-geological risks (flood and landslides).

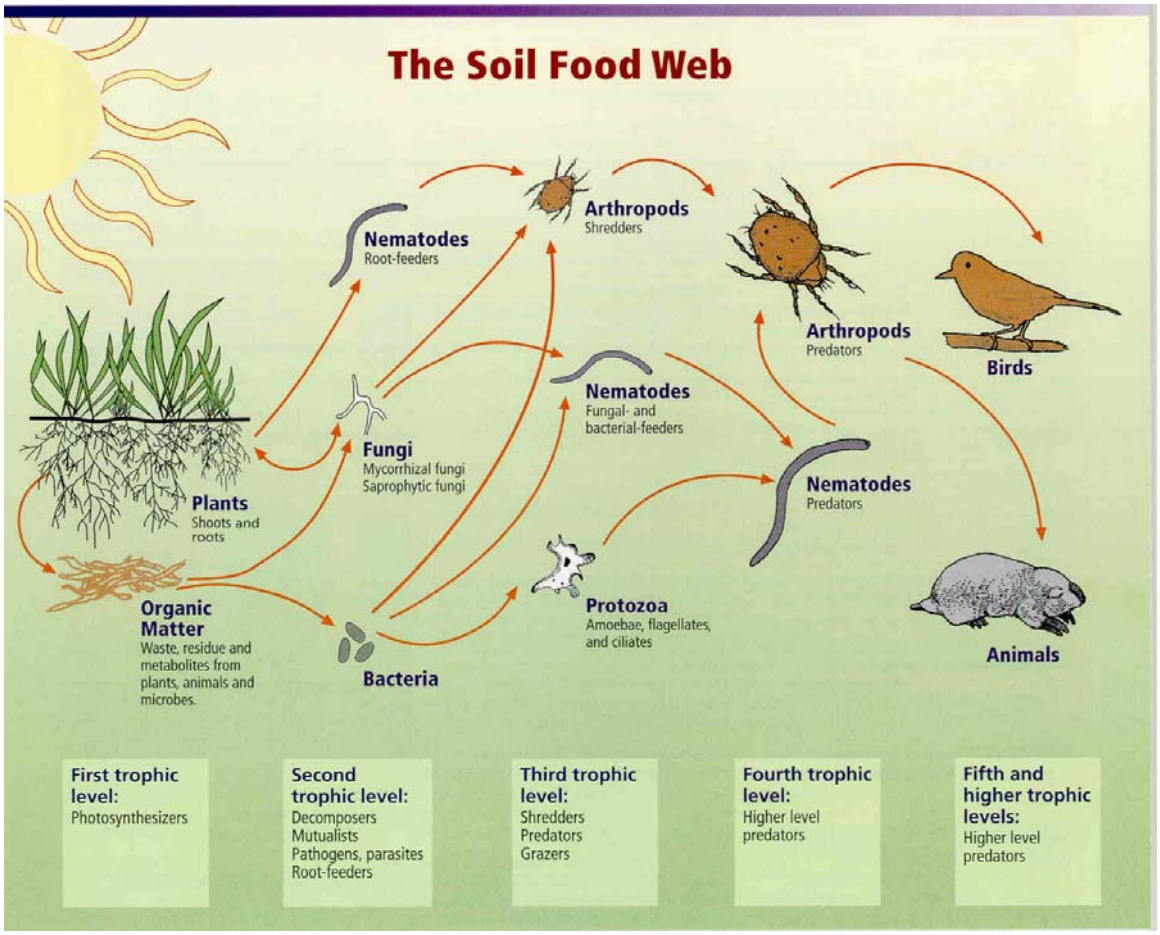
2. The importance of soil biodiversity

The decline of organic matter is closely linked to the loss of soil biodiversity. Soils are a major habitat for plants and animals. Millions of organisms can be present in just one teaspoon of soil. Fungi, bacteria, nematodes, earthworms and higher animals form a complex food web (fig. 2) that is still only partially known and understood. Many species still are waiting to be correctly identified and described. The increasing use of agro-chemicals and the rapid decline in organic matter content are threatening the diversity of organisms in soils. Only little is known on the impact of genetically modified crops on the gene pool in soils. Root residues from these new GMO's could affect the soil biodiversity. There is still a lot to be investigated in this respect.

Recognising that soils contain as much biodiversity as the above ground habitats requires to take steps towards protecting this precious resource from further degradation. This was also recognised by the Conference of Parties (COP) to the Convention on

Biological Diversity (CBD) at its 6th meeting in Nairobi April 2002 that decided (COP decision VI/5, paragraph 13) "…to establish an International Initiative for the Conservation and Sustainable Use of Soil Biodiversity as a cross-cutting initiative within the programme of work on agricultural biodiversity, and invites the Food and Agriculture Organization of the United Nations, and other relevant organizations, to facilitate and coordinate this initiative".

Protecting the soil habitat against the impact of human activities that could threaten the diversity of species should have the same importance as the protection of above ground natural habitats.



Source: USDA – NRCS Soil Quality Institute

Figure 2: Soil food web.

To protect soil biodiversity, the Commission will consider the extension of the annexes of the Habitats Directive to complete the so far limited list of soil-based habitats requiring special protection. Complementarily, the importance of soil in the management plans for designated Natura 2000 sites will be increased. A considerable amount of research will be required to establish more completely the biodiversity aspects of soil and the areas which might merit such designation.

Not enough is known about soil biodiversity. This will also be addressed in the Seventh Framework Programme with a view to gaining a better understanding of the function of biodiversity as an environmental service. This knowledge-building process will also be supported by ongoing initiatives under the Convention on Biological Diversity and the Forest Focus Programme.

The European Commission is committed within the Soil Thematic Strategy to develop calls for research projects to support policymaking in line with the objectives of the strategy and incorporate in decision-making any new knowledge acquired on soil biodiversity from 2006 onwards.

3. The importance of bio-indication for monitoring soil contamination

One of the main threats to soil biodiversity and soil health in general is contamination both by diffuse and local pollution. For this reason the use of bio-indicators for the detection of soil contamination is highly relevant for the correct assessment of this threat to soil health.

Diffuse pollution is generally associated with atmospheric deposition, certain farming practices and inadequate waste and wastewater recycling and treatment. Atmospheric deposition is due to emissions from industry, traffic and agriculture.

Deposition of airborne pollutants releases into soils acidifying contaminants (e.g. SO2, NOx), heavy metals (e.g. cadmium, lead arsenic, mercury), and several organic compounds (e.g. dioxins, PCBs, PAHs).

Acidifying contaminants gradually decrease the buffering capacity of soils leading them in some instances to surpass their critical load resulting in a sudden massive release of aluminium and other toxic metals into aquatic systems. In addition, acidification favours the leaching out of nutrients with subsequent loss of soil fertility and possible eutrophication problems in water and excess of nitrates in drinking water. Moreover it may damage beneficial soil micro-organisms, slowing down biological activity.

Ammonia and other nitrogen deposition (resulting from emissions from agriculture, traffic and industry) cause the unwanted enrichment of soils and subsequent decline of biodiversity of forests and of high nature value pastures. In some European forests the nitrogen input reaches extreme values of up to 60 kg N per hectare per year. Pre-industrial deposition was below 5 kg.

With regard to radioactive substances forest soils deserve particular attention. The characteristic cycling of nutrients in a forest ecosystem implies that for many radionuclides (e.g. caesium-134 and -137 as released by the Chernobyl accident) there is no elimination of radioactive substances (except by radioactive decay). Thus we are today still confronted with levels of radioactivity in forest produce above the maximum permitted levels, especially in wild mushrooms.

A number of farming practices can also be considered as a source of diffuse soil contamination, although their effects on water are better known than on soil.

Production systems where a balance between farm inputs and outputs is not achieved in relation to soil and land availability, leads to nutrient imbalances in soil, which frequently result in the contamination of ground- and surface water. The extent of nitrate problems in Europe underlines the seriousness of this imbalance.

An additional problem relates to heavy metals (e.g. cadmium, copper) in fertilisers and animal feed. Their effects on soil and soil organisms are not clear, although studies have shown the possible uptake of cadmium in the food chain. The effects on soil of antibiotics contained in animal feed are unknown.

Pesticides are toxic compounds deliberately released into the environment to fight plant pests and diseases. They can accumulate in the soil, leach to the groundwater and evaporate into the air from which further deposition onto soil can take place. They also may affect soil biodiversity and enter the food chain.

The current authorisation process of pesticides assesses inter alia the environmental risks of individual pesticides in the soil; however information on the combined effects remains limited. By this authorisation process pesticides with unacceptable risks are being eliminated. The volume of pesticide active ingredients sold across the 15 EU Member States reached 321,386 tonnes in 1998.

While the use of pesticides is regulated, and they should be only applied following Good Farming Practice, pesticides have been found to leach through the soil into groundwater and to be eroded with soil into surface water. Accumulation in soil occurs, in particular of those compounds now prohibited in the EU.

With regard to waste, sewage sludge, the final product of the treatment of wastewater, is also raising concern. It is potentially contaminated by a whole range of pollutants, such as heavy metals and poorly biodegradable trace organic compounds, what can result in an increase of the soil concentrations of these compounds. Some of them can be broken down to harmless molecules by soil micro-organisms whereas others are persistent including heavy metals. This may result in increasing levels in the soil with subsequent risk for soil micro-organisms, plants, fauna and human beings. Potentially pathogenic organisms like viruses and bacteria are also present. However sewage sludge contains organic matter and nutrients such as nitrogen, phosphorus and potassium, of value to the soil and the options for its use include application on agricultural land. Provided that contamination is prevented and monitored at source, the careful and monitored use of sewage sludge on soil should not cause a problem, and, indeed, on the contrary could be beneficial and contribute to an increase of soil organic matter content. 6.5 million Tonnes of sludge (dry matter) are produced every year in the EU. It is estimated that by 2005 there will be a 40% increase in the total quantity of sewage sludge available due to the progressive implementation of the Urban Wastewater Directive. A recent implementation report by the Commission on the latter indicates progress but also major delays in the implementation of that Directive in most Member States.

A more serious concern for human health is deriving from the large number of highly contaminated sites in Europe. These sites are particularly numerous in many of the EU candidate countries, where contamination associated with the 3000 former military facilities constitutes a major problem which is not yet fully evaluated.
Estimates of the number of contaminated sites in the EU range from 300 000 to 1.5 million. This wide range in estimations is due to the lack of a common definition for contaminated sites and relates to different approaches to acceptable risk levels, protection targets and exposure parameters.
Soil clean-up is a difficult operation with very high costs. Expenditure for decontamination of contaminated sites greatly varies between Member States. In 2000 the Netherlands invested EUR 550 m in decontamination, Austria 67 and Spain 14. Such disparities reflect different perceptions of the severity of the contamination, different remediation policies and targets, and different ways of estimating expenditure. The European Environment Agency has estimated the total costs for the clean-up of contaminated sites in Europe to be between EUR 59 and 109 billion.

4. Conclusions

The full understanding of the complex biological systems within the various soils of Europe is still in its infancy. Extensive basic research on soil ecosystems and their functioning is required before any clear conclusions can be derived on the importance of soil biodiversity and its protection. The future EU framework programme for research will take into account these research needs to the extend that is possible.
Full assessment of soil quality can not avoid addressing soil ecosystem functioning. In this respect, the use of bio-indication as an integrated monitoring tool for soil degradation by pollution is certainly a possible way forward. On-going research activities in Europe, particularly the exploratory research programme of the JRC on bio-indication and soil biodiversity will allow the full understanding of these various aspects, and eventually pave the way for the operational implementation of this soil monitoring technique.

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BIO-BIO Project

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The Pavia Project had as principal objective the evaluation of the quality and health of soil in Pavia Province and included a study to appraise the eventual differences in soil health, that have resulted from different management practices: organic farming, animal manure and mineral fertilizers and soil receiving sewage sludge.
Soil health was appraised by studying physical and chemical properties coupled with biodiversity and bio indication concepts, using some organisms and/or their "products" that are present under the three main management systems. Twelve international organizations participated in the BIO-BIO Project.

1. Introduction

The Pavia Project, completed a few months ago, had as its principal objective the evaluation of the quality and health of soil in Pavia Province, Lombardy, in northern Italy. A further objective was to adopt an innovative and multidisciplinary approach. The area under investigation area covered 3000 square kilometres. Taking account of the different uses of soil in Pavia Province, international standard methods were adopted for the identification of sampling points, the collection, treatment and analysis of the samples for heavy metals, macro-elements, dioxins, furans, soil acidity, physical properties (water retention, pore size, geochemical profile, etc.) and biological data (bacteria and terrestrial mosses).

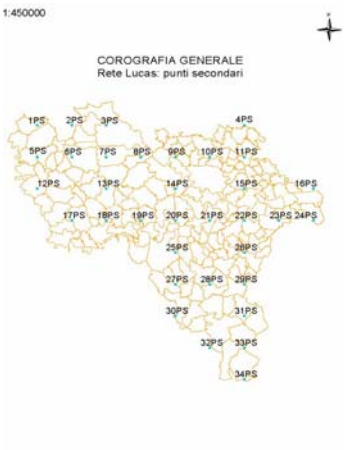

Figure 1-investigated are in the Pavia Project

The Pavia Project included a study to appraise the eventual differences in soil health, which have resulted from different management practices:

- Biological farming.
- Soil receiving animal manure and mineral fertilizers.
- Soil receiving with sewage sludge.

These three different ways of treatment-utilization of the soil are very common in Pavia Province, but also in Lombardy, elsewhere in Italy and in Europe as a whole.

Soil is a complex entity able to breathe, to assimilate carbon and nitrogen, to decompose and mineralize organic compounds of vegetable and animal origin, and to store reserves in the form of humus. These functions are enabled by the presence in the soil of organisms that intervene, with their metabolism, in the processes of transformation and regeneration of the soil components. Energy enters in the soil system mainly through the decomposition of organic residues, whose rate of degradation is regulated mainly by the microbial biomass. Another aspect to consider is the contamination of soil by inorganic elements and/or organic compounds that can significantly change manner the activity of the microbial pool and other indispensable organisms ensuring that soil remains a living ecosystem.

This is the background to undertaking this multidisciplinary study with four main aspects:

- Temporal and seasonal aspect (four samplings in one year.)
- Chemical analyses of different layers (0-5 cm; 0-15 cm and 15-30 cm) to establish the presence of organisms.
- Physical data.
- Biodiversity and Bio-indication, across an important pool of organisms to cover the three management practices.

2. Investigated areas

The Pavia Project concentrated studies in three areas: one used for biological cultivations in which the ground did not receive any type of manure over the last 10 years; the second where the soil received animal manure and 150 kg/ha/year of mineral manure (15N-15P-15K), during at least the 10 years; and the third where the soil received sewage sludge and NH_3 and H_2O treatments, for more of ten years.

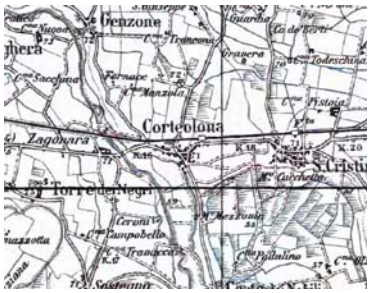

Figure 2-area Cascina Novella

Figure 3-area Cascina Orsine and Cascina Nuova

3. Biological methods to indicate the soil quality

Soil health was appraised by studying biodiversity and bio indication, using some organisms and/or their 'products' that are present under the three main management systems. The organisms considered include:

- Bacteria, fungi, nematodes, amoeboid, protozoides (micro-net).
- Mites and collembola (meso-net).
- Earthworms (macro-net).

Figure 4- Cascina Novella

The organisms listed are present in the soil, and comparison of the different methods has provided useful information on the level of soil health. In addition, the same soil has been used to cultivate clover to evaluate the degree to which the growth of the clover is inhibited by the presence of inorganic contaminants and/or organic composts. The potential genotoxicity of the soil was evaluated by the analysis of the DNA of the cultivated clover.

4. Participants in the Project

A large amount of information has been generated by the project and my sincere thanks go to all national and international institutions taking part for their enthusiasm and dedication to the work they have carried out during these past two years. I also wish to express my gratitude to these experts for their contributions that have provided information at European scale.

Figure 5-technicians at work

The organizations that have participated in the BIO-BIO Project, except those experts from the Province of Pavia who have helped us beyond all expectations, include:

University of the Sacro Cuore of Piacenza responsible for treatment and analysis of the soil samples to determine the concentration of the heavy metals;

ERSAF colleagues have analyzed soil profile.

Superior Institute of Health of Rome that prepared the analysis of the PCBs and appraised the Sanitary Hygienic Risk;

University of the Studies of Milan which participated with two groups, one that concentrated on nematodes, their identification and mass, the other a group of researchers that prepared the cultivation of the clover and the analysis of the DNA in the clover tissues;

Experimental Institute Nutrition of the Plants of Rome and the University of Wageningen (NL) Alterra Department of Soil Science that used the bacteria and their products to identify the main functional groups.

University of Turin, department of Clinical and Biological Sciences that used amoeboids;

University of Camerino, Molecular department of Biology, Cellular, Animal, which employed the protozoides to appraise the potential toxicity of the soil;

University of the Studies of Parma which employed the QBS Index Collembola and Arthropods (Biological Quality of the Soil);

University of the Piedmont Eastern which used earthworms to verify eventual variations in the DNA from the contaminants present in the soil;

University of Rennes (F) 1 UMR CNRS EcoBio used the earthworms in two ways: biodiversity researching the different species and as bio indicators for appraising their bio-mass;

Institute for Environment and Sustainability of Ispra, European Commission, which analyzed some chemical parameters, dioxins and furans.

Finally, it was my privilege and pleasure to coordinate the BIO-BIO Project.

5. Conclusions

All participants in the Project BIO-BIO are convinced by the usefulness of this research because the results produced fill a significant gap in the literature and provide the politicians, administrators and farmers vital information about the health of soil, particularly in those areas used for the disposal of sewage sludge.

It is our duty as environmental researchers to study and to understand whether current agricultural practices can maintain soil health such that the land can be left safe for future generations of citizens.

Modelling nutrient fate from agriculture: an integrated framework

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Many European countries face high nutrient loadings and the scientific community is asked to provide tool sand methodologies to alleviate the pressure on the environment originating from agriculture. This paper presents a tiered approach for addressing nutrient fate at various scales that makes best use of readily available data at EU level. A statistical approach is used at large-river basin to identify areas with highest nutrient losses. A physically-based model is then used to identify within those areas, the major processes and pathways controlling nutrient losses. Finally, a farm-scale model is used to elaborate appropriate farming practices.

1. Introduction

Although nutrients are essential for plant and animal populations, high concentrations can degrade water and soil quality. To control and reduce pollution coming from nutrients, the EC has been setting stringent regulations:

- The Nitrates Directive (91/676/EEC, 1991) requires Member States to designate Nitrate Vulnerable Zones (NVZ), and imposes to implement various action programs for reducing water pollution generated by agriculture;
- The Water Framework Directive (2000/60/EC, 2000) required Member States to identify sources of pollution, evaluate their impacts on the ecological status of surface and subsurface waters and implement river basins management plans

Agriculture is one of the main sources of nutrient loading to water bodies, with contribution to nitrogen loading ranging from more than 80% in Denmark to less than 30% in Finland (OECD, 2001). Italian's agriculture contributes to more than 60% of the nitrogen measured in the streams (OECD, 2001). Combating diffuse pollution from agriculture is complicated due to the temporal and spatial lag between the management actions taken at the farm level and the environmental response (Schröder et al., 2004). Appropriate farming practices are among the most efficient way of reducing nutrient losses. In Italy, the nitrogen efficiency (ratio of nitrogen application and nitrogen uptake) is slightly higher than 70% (OECD, 2001), indicating that better fertiliser use could be achieved through appropriate management. Nutrient management (single versus split application, timing), appropriate crop rotation (introducing nitrogen fixing crop in the crop sequence) are among the various options available to reduce the amount of nitrogen lost to the environment.

This paper presents a tiered approach for addressing nutrient fate at various scales that makes best use of readily available data at EU level taking into account the policy requirements of various Directives. Firstly (tier 1) a statistical approach (Grizzetti et al., 2005) is used at large-river basin to identify areas with highest nutrient losses. In a subsequent step (tier 2), the physically-based SWAT (Arnold et al., 1998) model is used to identify within those areas, the major processes and pathways controlling and contributing to nutrient losses. The third step (tier 3) involves the use of the farm-scale model EPIC (William, 1995) to elaborate appropriate farming practices that could reduce pollution load without endangering the farm economic sustainability. The last step consists in field measurement to validate the results of the farm scale model.

This paper will focus primarily on steps 1 and 3. The statistical tool is used to calculate the diffuse emission of nitrogen for Italy. EPIC is then used to illustrate the potential impact of nutrient management on nitrogen leaching. The field work was performed in the context of the Pavia Project (Cenci et al., 2006) in the area of Pavia to study the effect of various farming practices including traditional farming, organic farming, and sludge-application type of farming on nitrogen cycling

and leaching. However, the results are still under analysis. The paper will present only the modelling results.

2. Materials and methods

2.1 Modelling Approach

2.1.1 STATISTICAL MODEL

The statistical modelling approach consists of a simplified conceptual model, considering two different pathways in nutrient transfer from sources to the catchment outlet. Diffuse sources (DS), include applied fertiliser (artificial and manure), atmospheric deposition, and scattered dwellings, are first reduced in the soil and then retained partially in the streams, while point sources (PS) which include discharges from sewers, waste water treatment plants, industries, and paved areas are only retained in the streams (Grizzetti et al., 2005).

2.1.2 EPIC MODEL

The EPIC (Erosion-Productivity Impact Calculator; Williams et al., 1995) is a field scale model, originally developed to simulate the long-term effects of soil erosion on soil productivity. A nutrient cycling and pesticide fate routines were added later on. The various developments of EPIC are given by Gassman et al. (2005).

2.2 EU Database

The data used in this study was derived from a harmonized database developed for EU15. Three major data sets are being used which include a soil map, a land-cover land use map, and a climatic database. Pan-European soil data collected for this research includes the European Soil Bureau Database (ESBD) v1.0, which provides an important source of information for the EC in the monitoring of soil quality, soil organic matter, degradation, contamination, and for assistance in the formation and evaluation of policies towards sustainable agriculture (ESB, 1998). Landover data are available from the Corine (Coordination of information on the environment) database. Meteorological data for the years 1990 to 2003 were obtained from the Monitoring Agriculture and Regional Information Systems (MARS) Unit of the JRC. The MARS meteorological database contains daily data spatially interpolated on a 50 km x 50 km grid-cell.

In addition, atmospheric N deposition data was derived from the Precipitation Chemistry Database of the Co-operative Programme for the Monitoring and Evaluation of the Long-Range Transmission of Air Pollutants in Europe (EMEP). Nitrogen and phosphorous input data for both mineral and manure fertiliser was taken at NUTS level 2 from the Capri (Common Agricultural Policy Regional Impact Analysis economic) model (Heckelei and Britz, 2000).

3. Results

The tier 1 approach was applied to the whole Italian territory. The map of the diffuse emission is shown in *Figure 1*.

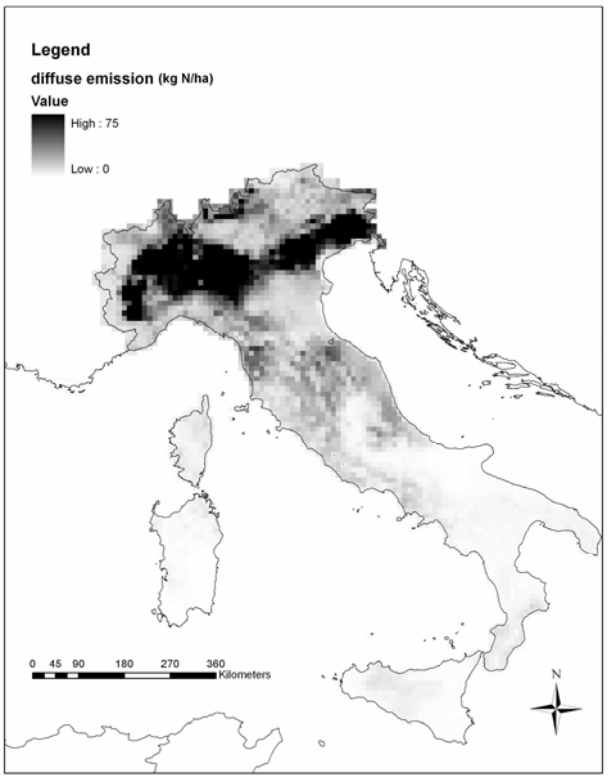

Figure 1. Calculated nitrogen diffuse emission (kg N/ha) for Italy.

The highest emission of nitrate occurs in the Po valley, the area of intensive agriculture in Italy. Agriculture is indeed predicted to be the major source of nitrate in the Po River (Figure 2) with local contribution ranging from 50 to 70% of the total nitrogen load. This value is in agreement with the OECD that estimates the contribution of agriculture to total nitrogen loading to be around 60% (OECD, 2001).

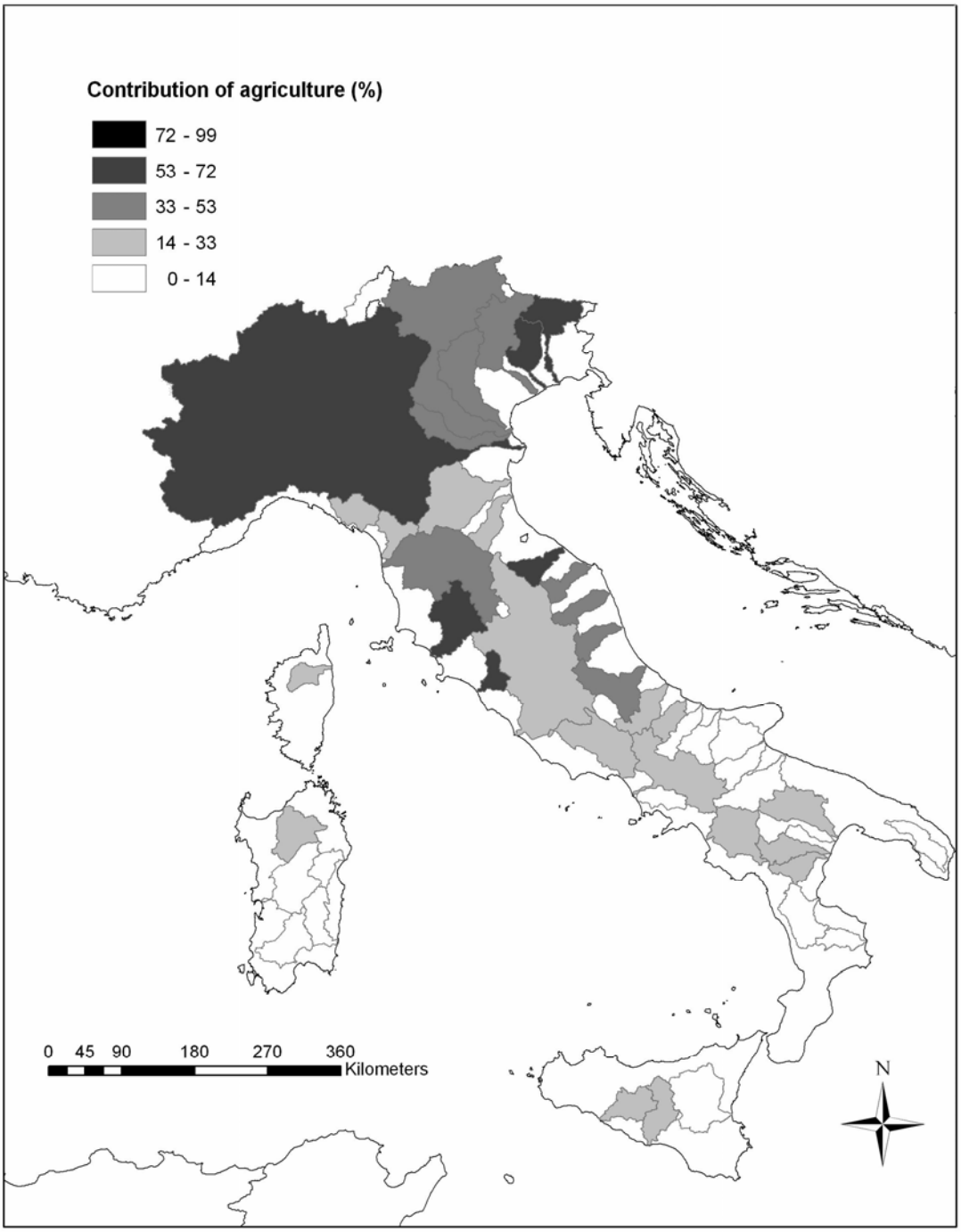

Figure 2. Calculated contribution (%) of agriculture to total nitrate load for the Italian catchments larger than 1000 km2.

The EPIC model was then set-up on the Pavia region, to evaluate the impact of various management strategies to reduced nitrogen losses. Different approaches exits to limit nutrient losses. In this particular case, EPIC was used to evaluate the impact of the fractioning of the application of nitrogenous fertiliser. In the base run, mineral nitrogen was applied one week before planting for all areas under corn cultivation. In the scenario, the same amount of nitrogenous fertiliser was applied in the form of manure in two doses: 25% before planting and 75% one month after planting. The overall yield was very similar in both scenarios (a decrease of less than 5% is observed in the second scenario) while a dramatic decrease in nitrate leaching is observed. The spatial distribution of nitrate leaching for both scenarios is shown in *Figure 3*.

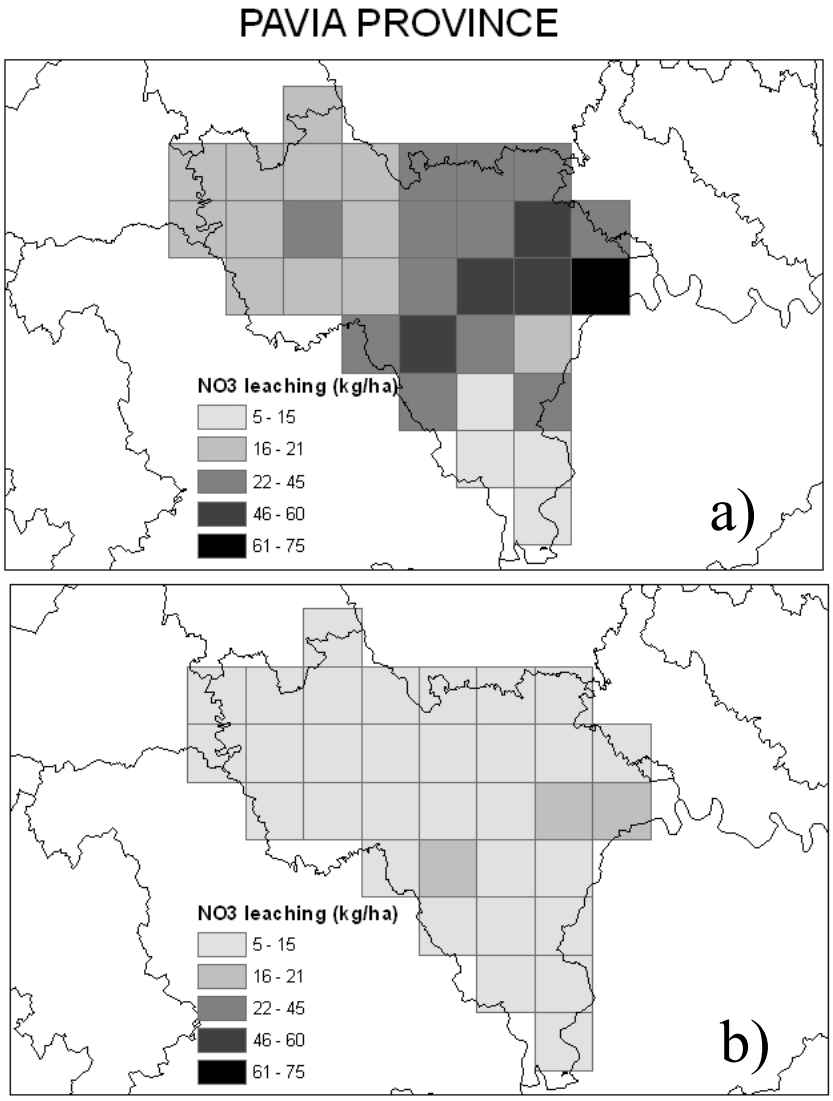

Figure 3. Nitrate leaching for the Pavia for the two nutrient management scenarios: a) one single application of mineral nitrogen, b) split applications of organic nitrogen.

4. Conclusions

A tiered integrated approach for addressing nutrient fate at various scales has been developed. The tier 1 statistical screening tool was used and identified the Po valley as a region with higher emission rate of nitrogen when compared with other regions. The EPIC model was then used to evaluate the impact of a split fertiliser application versus one single application in the Pavia region. It was predicted that a decrease by 60% of the nitrate leaching could be achieved by applying the same amount of mineral nitrogen currently used in two consecutive applications of manure while affecting the crop yield by less than 5%.

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Soils of the BIO-BIO fields

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For each BIO-BIO field, general soil properties and main soil processes are described schematically, which should help analysing inter-fields soil similarities and differences. Soil maps, as reference frame showing surrounding soil patterns, are reported.

1. Introduction

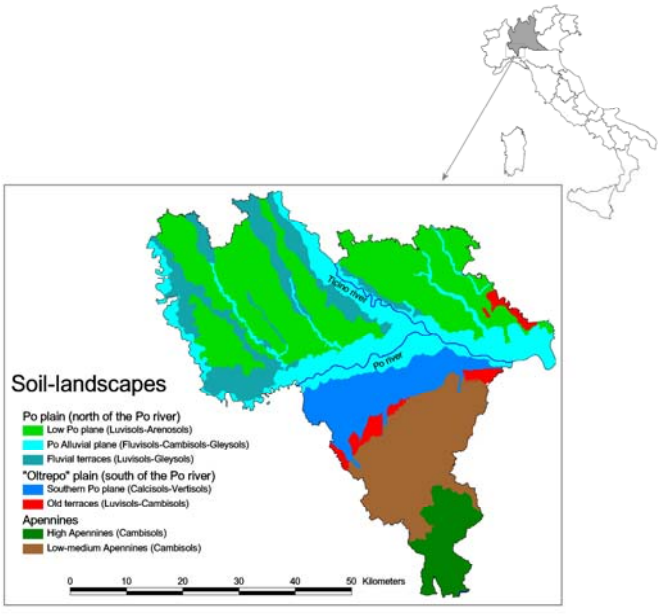
The bio-bio fields are in the Province of Pavia (Lombardy Region, Northern Italy) and are representative of soils and landscape occurring in the Po river plain. Soils formed in fluvial and alluvial deposits of both Pleistocene and Holocene age. They are very intensively cultivated (rice and maize are the main crops).

To describe the general properties of the soils in the three bio-bio fields, soil survey was curried out. Soil description and sampling methods were consistent with the ISO standard. As a reference framework, the 1:50,000 soil map and georeferenced database of the Regione Lombardia Agro-Forestry Development Agency (ERSAF) were applied.

Because of the structure of the sediments, the soils of the bio-bio fields have a high spatial variability. In spite of that, the main soil types that are shown by the ERSAF semi-detailed soil maps turned out to be representative also at the detailed field level. Therefore for each field:

- the description of the most representative soil profile is taken from the ERSAF georeferenced soil database;
- the ERSAF 1:50,000 soil map is reproduced to describe the general soil pattern.

For the representative soil type of each field, soil classification is reported according to Soil Taxonomy (Soil Survey Staff, USDA Handbook, NRCS, n. 436, 1999).



2. Soils of the "Cascina Nuova" field

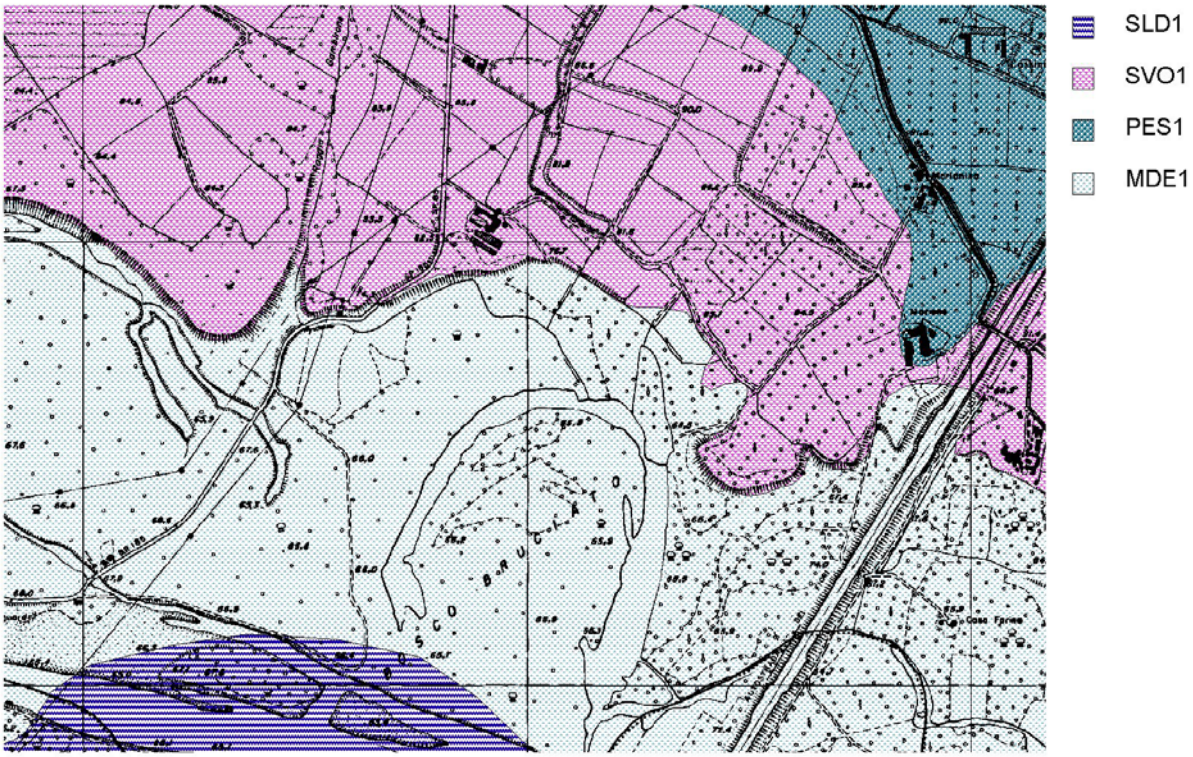
2.1 Description of the soils

According to the ERSAF georeferenced soil database the representative soil type is 'S. Varese O' (soil mapping symbol: **SVO**).
'S. Varese O' soil type consists of deep, well drained soils formed in fluvial deposits on nearly level fluvial plain. Taxonomic Class: Coarse loamy, mixed, superactive, mesic Typic Dystrustepts (Soil Taxonomy, 1999).

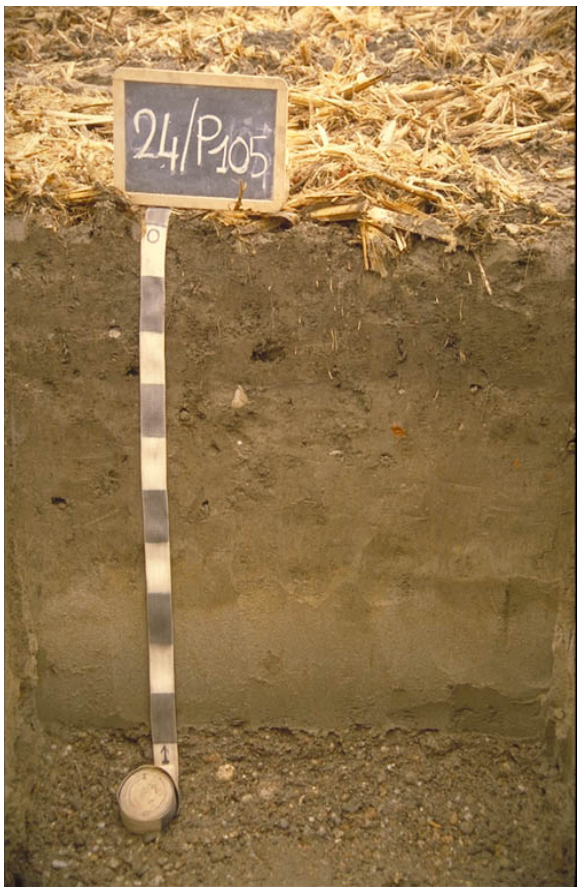
Soil profile description:
(colours are for moist soil unless otherwise stated)

- 0 to 30 cm: Ap horizon; grayish brown (10YR 5/2) slightly fine gravelly loamy sand; non calcareous;
- 30 to 50 cm: Bw1 horizon; brown (10YR 5/3) slightly fine gravelly loamy sand; non calcareous;
- 50 to 80 cm: Bw2 horizon; brown (10YR 4/3) slightly fine gravelly loamy sand; non calcareous;
- 80 to 100 cm: C layer; olive brown (2.5Y 4/3) slightly fine gravelly sand; non calcareous.

2.2 General soil pattern (from ERSAF 1:50,000 soil map)



3. Soils of the "Cascina Orsine" field



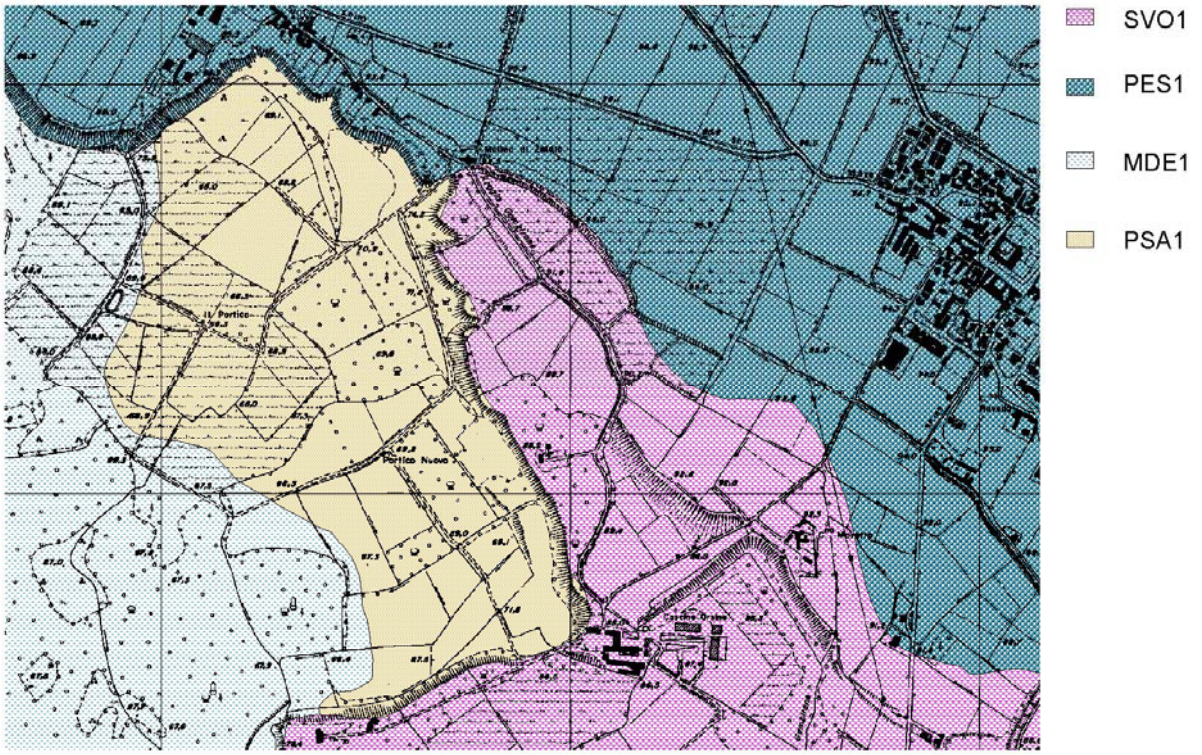
3.1 Description of the soils

According to the ERSAF georeferenced soil database the representative soil type is 'Parosacco' (soil mapping symbol: **PSA**).
'Parosacco' soil type consists of deep, poorly drained soils formed in fluvial deposits on nearly level fluvial plain. Taxonomic Class: Sandy, mixed, mesic Typic Humaquepts (Soil Taxonomy, 1999).

Soil profile description:
(colours are for moist soil unless otherwise stated)

- 0 to 35 cm: Ap horizon; very dark grayish brown (2.5 Y 3/2) slightly fine gravelly loamy sand; non calcareous;
- 35 to 50 cm: Apg horizon; dark gray (5Y 4/1) slightly fine gravelly loamy sand; common yellowish brown (10YR 5/4) mottles; non calcareous;
- 50 to 80 cm: CA horizon; grayish brown (2.5 Y 5/2) slightly fine gravelly loamy sand; common yellowish brown (10YR 5/4) mottles; non calcareous;
- 80 to 100 cm: C layer; gray (5 Y 6/1) slightly fine gravelly sand; few yellowish brown (10YR 5/4) mottles; non calcareous.

3.2 General soil pattern (from ERSAF 1:50,000 soil map)



4. Soils of the "Cascina Novella" field

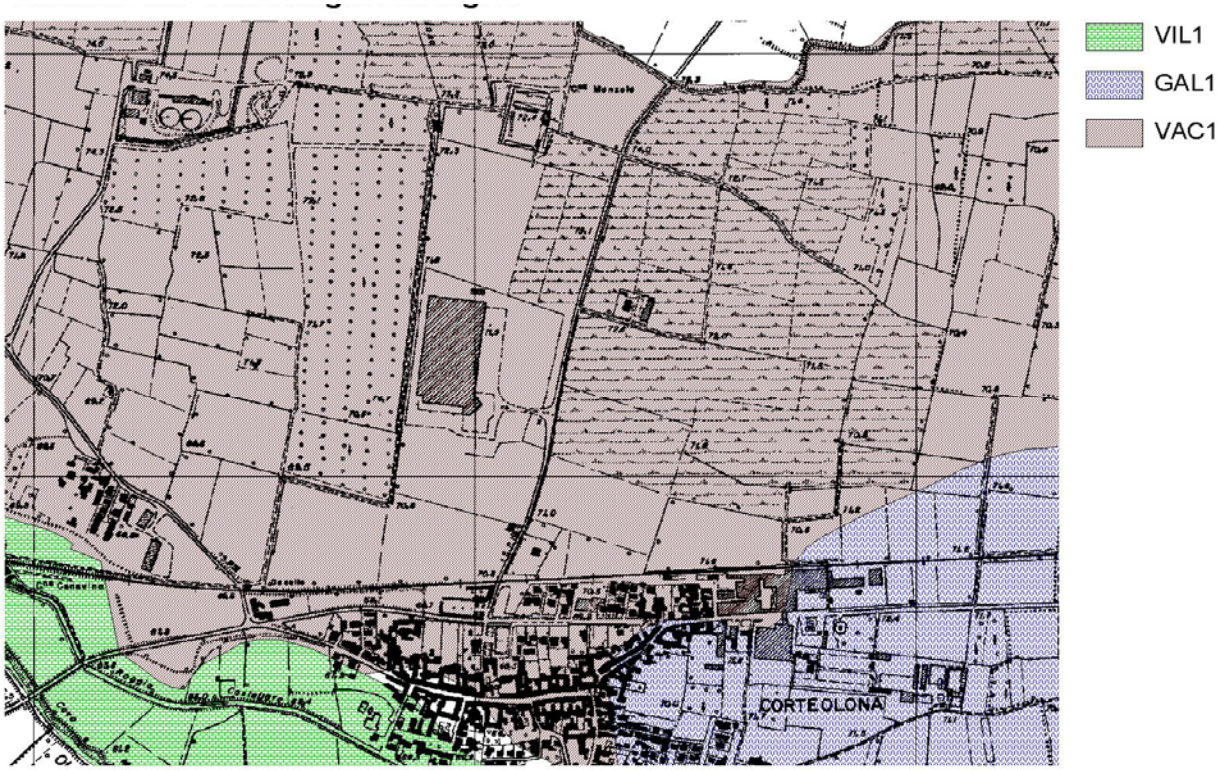
4.1 Description of the soils

According to the ERSAF georeferenced soil database the representative soil type is 'Valcova' (soil mapping symbol: **VAC**).
'Valcova' soil type consists of deep, moderately well drained soils formed in fluvial and glacio-fluvial deposits on nearly level fluvial plain. Taxonomic Class: Fine silty, mixed, superactive, mesic Aquultic Haplustafs (Soil Taxonomy, 1999).

Soil profile description:
(colours are for moist soil unless otherwise stated)

- 0 to 45 cm: Ap horizon; brown (10YR 4/3) silt loam; common dark gray (10YR 4/1) mottles; non calcareous;
- 45 to 90 cm: Bt horizon; yellowish brown (10YR 5/4) loam; many light brownish gray (2.5Y 6/2) mottles; non calcareous;
- 90 to 100 cm: 2Cg layer; yellowish brown (10YR 5/4) sandy loam; many gray (N 6/) mottles; non calcareous.

4.2 **General soil pattern** (from ERSAF 1:50,000 soil map)



Area description, soil sampling, physical and chemical analysis

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This report describes the sampling, geographic information and chemical analysis performed on soil samples taken in three areas of North Italy (Pavia Province, Lombardy). The study was performed in order to have analytical information about contaminants of soil in cultivated areas as well as to compare the effects on soil and life forms of three different agricultural practices.

1. Introduction

Life is organized into a feeding hierarchy from producers to consumers, ending with decomposers. Taken together, the soils, plants, animals and all abiotic components produce the ecosystems, generally grouped together in various biomes. Today we face crucial issues, principally the preservation of the diversity of life in the biosphere and the survival of the biosphere itself [1].

Analysis of soil is performed for a variety of environmental and geological purposes. For example, chemical data is used in the identification of various soil types; under certain conditions soil can release significantly higher concentrations of many metals than are found in the underground water. The importance of these types of data is well established and has been going on for decades.

Moreover, soil represents the interface with other compartments and environmental sectors, like air, water, wastes, agriculture, forests. On soil are distributed sludge, fertilizers, manure, pesticides; dispersed pollutants in the atmosphere are eventually deposited on the soil; the agricultural practices themselves involve, during time, a certain negative impact. Several anthropic pressure and the natural processes can determine, in different way, the degradation of the "soil resource", whose regeneration often requires very long time. The knowledge of soil is at the heart of agriculture and food.

2. Investigated area

2.1 Description

Our comparative study has been performed in three areas, of about one hectare each, where different methods of fertilization were used. The first area was manure with bovine manure, in the second sewage sludge and artificial fertilizers were used, while the third one was cultivated without any fertilizer.
The investigated sites are located in the village of Bereguardo (two areas) and the third in the village of Corte Olona.
The first area is placed near the Farmhouse Cascine Orsine and is used since 25 years for the biological cultivation. It doesn't receive any treatment but only water for the irrigation from the channel in the nearby. The culture was *polifita* meadow, as can be observed in *Figure 1*.
The second site is near the farm Cascina Nuova. It is also a polifita meadow but fertilized with manure and 150 kg/ha of mineral fertilizer (15N-15P-15K). The third area is located in the village of Corte Olona, inside the farmhouse Cascina Novella. The culture is corn and the field is receiving since 10 years sewage sludge essays with NH_3 and H_2O (about 360 q/ha). The soil is periodically bedewed with a liquid solution of herbicide.

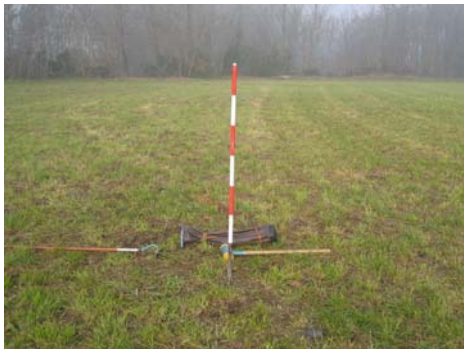
2.2 Geographical coordinates

In *Figure 1* are shown the areas where the soil sampling have been performed, together with their respective coordinates.

Cascina Orsine (biological site) UTM/UPS Map Datum: WGS 84
32T Lat. 0529517
Long. 5001289

Cascina Novella (amended site) UTM/UPS Map Datum: WGS 84
32T Lat. 0529517
Long. 5001289

Figure 1 – picture of the three areas with relative geographical coordinates



Cascina Nuova (manure site) UTM/UPS Map Datum: WGS 84
32T Lat. 0501619
Long. 5010492

3. Description of sampling

Just as a book cannot be judged by its cover, so soils cannot be evaluated at the surface only. Instead, a soil profile should be studied from the surface to the deepest extent of plant roots, or to where regolith or bedrock is encountered. In this work the soil profiles were dug out and then studied as described following.

Figure 2 and 3 - Profiles in the sampling areas Cascina Novella and Cascina Orsine

3.1 Sampling strategy

The sampling strategy has foreseen, for every investigated site, a sampling area of 20x20 meters. It

was derived from a French approach developed by INRA [2]. Every area was equally divided in 9 sub-units having 6.6 m side. Litter, roots, stones and other coarse material were removed before sampling. The different horizons have been sampled and the 9 sub samples of each horizon were mixed together in a homogeneous mean sample. Moreover, three different depths were sampled: 0-5 cm, 0-15 cm and 15-30 cm [3].

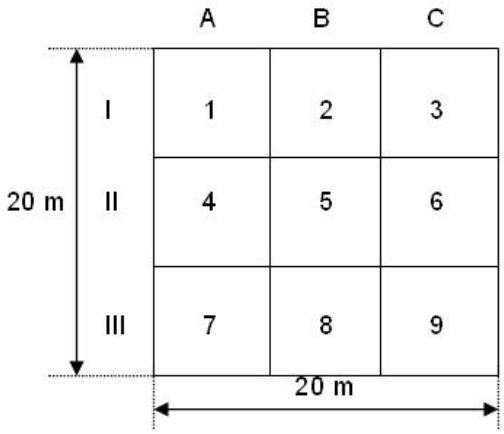


Figure 4 - Sampling scheme

Soil samples collection has been performed contemporarily with the AM-1 sampling campaign of the Pavia Project, by specialized technicians of the Soil & Waste Unit of JRC Ispra.

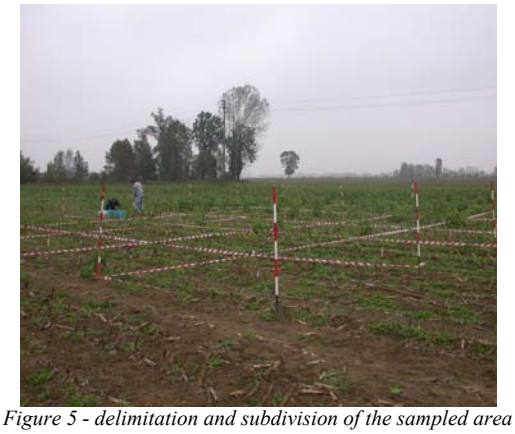


Figure 5 - delimitation and subdivision of the sampled area

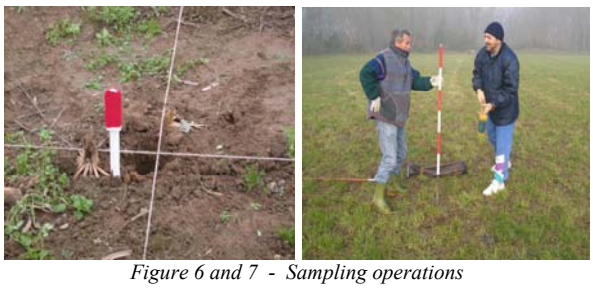


Figure 6 and 7 - Sampling operations

Each of the 9 soil samples (3 areas and 3 depths) was homogenized and then divided in 4 sub-samples. First aliquot was utilized for the determination of total mercury content, total and organic carbon, pH measurement and macro-elements determination. The second has been delivered to the technicians of the University Sacro Cuore in Piacenza for the heavy metals determination.
Another part, after air drying, was delivered to the Experimental Institute of Plants Nutrition in Rome for microbiological analysis and evaluation of PCBs concentration (analysis performed by the Health Superior Institute in Rome). The fourth fraction of soil, frozen and preserved in dark glass bottles, was used for the determination of dioxins' concentration.

3.2 Sampling

Based on the aforementioned profile information, the disturbed soil samples were obtained manually using an Auger device; an example can be seen in *Figures 8 and 9.*

Figure 8and 9 - Examples of an Auger sampling device

4. Analysis and results

4.1 Used methods

When possible, ISO procedures were applied to the samples for the requested analysis. In case no ISO procedures were available, internal methods of analysis were followed. These internal methods have been developed for similar sample types by our laboratories, both tested and proofed with Reference Materials.
Quality Control was ensured by the parallel analysis of several replicates of appropriate matrices-matching Certificate Reference Materials. The mercury analytical method was recently benchmarked on International scale [4] and proofed to be fully under control and traceable to the SI (IRMM 2003). For every single analysis the ISO procedure is hereby indicated, as well as a short resume of the procedure itself.

4.2 Sample pre treatment and preparation

According with ISO 11464 Procedure [5], after storage at 4°C and prior to analysis, samples were dried in

oven at 40° C, gently crushed using a mortar and pistil and passed over a nylon sieve with an aperture of 2 mm. The passing fraction was collected and unified in brown borosilicate bottles until the withdrawal for the analysis. For pH measurements the material was used in this form. In case of WDXRF, organic carbon, total carbon, C, H, N and Hg measurements the test portion was ground to a fine sample using a planetary ball mill, up to 250 µm particle size.

4.3 Texture and water content

In *Figure10, 11 and 12* are shown texture and humidity values for the three areas. The sandy fraction results dominant for the three sites, except the most superficial horizons of Cascina Novella where the loam fraction was predominant.

The water content results higher for the superficial layers. An exception is Cascina Novella where the most elevated value has been found in the layer between 30 and 50 cm in concomitance with more high values of clay and loam.

Globally these values are well representative of soil formed in the sediments of the plain of Po River.

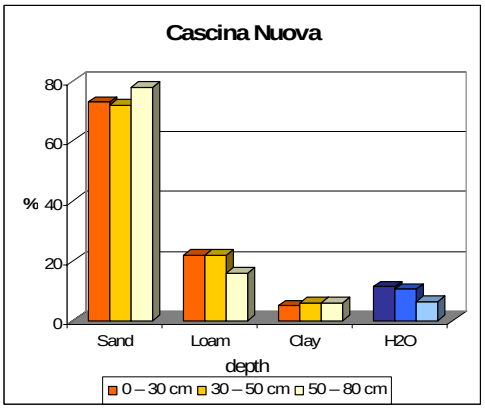


Figure 10 – texture and water content values (manure)

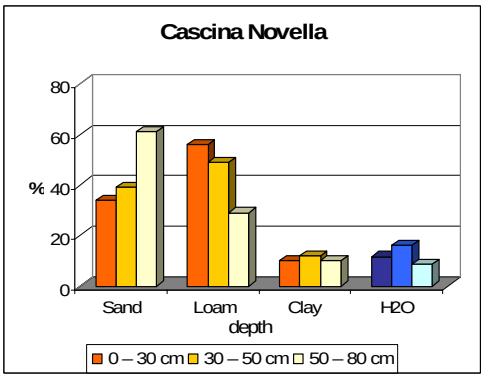


Figure 11 – texture and water content values (sludge)

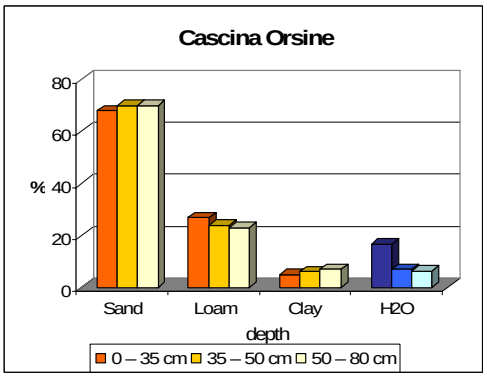


Figure 12 – texture and water content values (biological)

4.4 Soil water retention, bulk density, saturated water content

For every point of sampling and in the different horizons the samples were taken in double. Undisturbed soil samples were collected with manual sampler in steel cylinders of 53 mm diameter and 50 mm height. All samples were analyzed for:

- Bulk density, using ISO method 11272
- Water retention at different tensions.

These values were used for the calculation of soil porosity and saturated water content.

The water retention curves, bulk density and water content at saturation of the 3 areas are shown respectively in *Figures 13 – 15* and *Table 1*.

Regarding water retention curves, the three areas are generally characterized by coarse texture (from loamy sand to loam).

In detail, the amended area (CaNO), is characterized by medium texture (sandy loam), while manure and biological areas (CaNU, CaOR) are characterized by dominance of the sandy component.

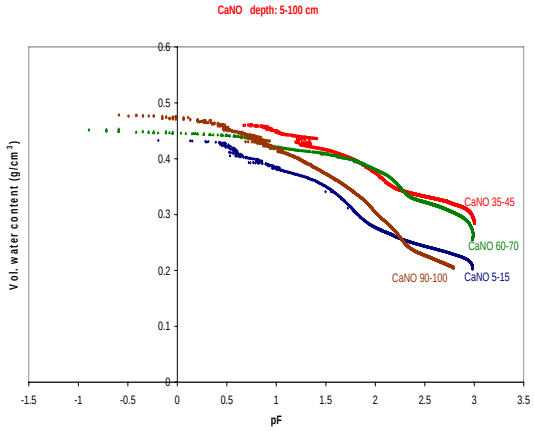


Figure 13 – soil water retention curve for sludge amended area

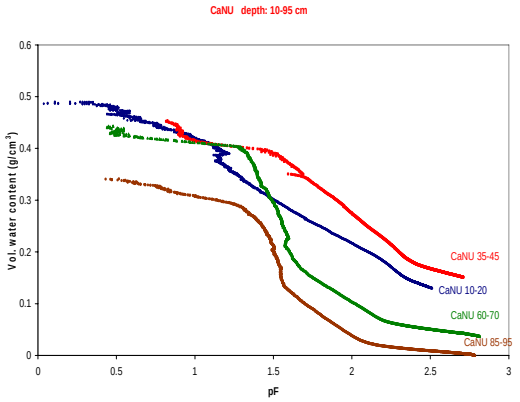


Figure 14 – soil water retention curve for manure area

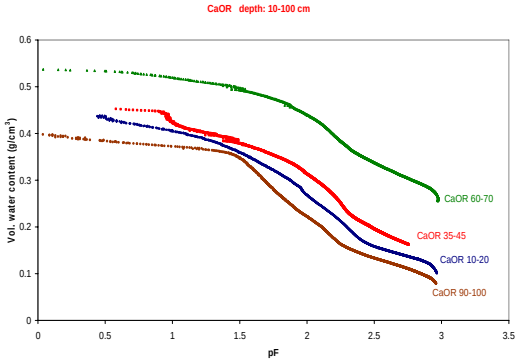


Figure 15 – soil water retention curve for biological area

*Table **1** – bulk density, saturated water content and porosity values*

Ca NO		0÷30 cm	30÷50 cm	50÷80 cm	80÷100 cm
Bulk density	g/cm^3	1.52	1.54	1.57	1.28
saturated water content	g/cm^3	0.43	0.46	0.45	0.48
Ca NU		0÷30 cm	30÷50 cm	50÷80 cm	80÷100 cm
Bulk density	g/cm^3	1.28	1.6	1.56	1.83
saturated water content	g/cm^3	0.49	0.45	0.44	0.34
Ca OR		0÷35 cm	35÷50 cm	50÷80 cm	80÷100 cm
Bulk density	g/cm^3	1.28	1.55	1.63	1.56
saturated water content	g/cm^3	0.45	0.45	0.54	0.4

The data confirm a moderate tendency to have high density, due to local agricultural practices. This is also confirmed at general level for soil of Padano-veneta Lowland.

4.5 pH

Soil pH is one of the most indicative measurements of the chemical properties of soil. Whether a soil is acid, neutral or basic has much to do with the solubility of various compounds, the relative bindings of ions to exchange sites, and the activity of various micro-organisms [6].

pH was measured according to 1M KCl method described in ISO 10390 Procedure [7]. The use of the soil/KCl-solution mask the differences in salt concentrations, displaces a high percentage of the exchangeable H^+ and Al^{3+} , and are thus more correlative with the pH values "in field".

The pH instrument was calibrated with a two point calibration using calibration standards traceable to NIST Standard Reference Material.

As shown in *Figure 16* there is a probable relation between the ratio acid oxides / alkali oxides present in the soil (sum of silica and aluminium oxides divided by sodium, potassium, calcium and magnesium oxide, from WDXRF analysis) and the respective pH value.

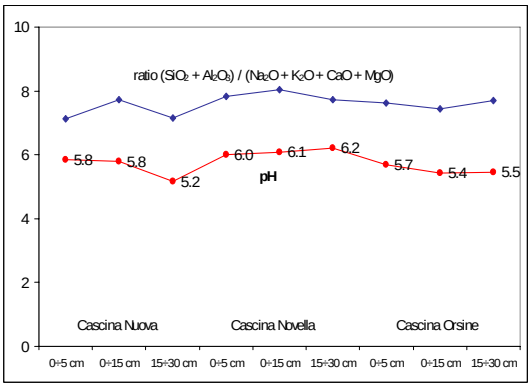


Figure 16 – relation between pH and oxides content

4.6 CHN measurements

Carbon, Nitrogen and Hydrogen have been analysed by both CHNS and Carbon analyzer instruments. For organic carbon content, the solid samples were acidified using 1% HCl in order to destroy inorganic carbon prior to measurements. The inorganic carbon has been calculated by difference between total and organic carbon.

The concentration distribution of the organic C is not homogeneous; as expected is observed a direct relationship with the land use. The variations of concentration are directly influenced by land use; the low values found in the most superficial horizons, are due to the type of crop (corn and grass in this specific case). The most elevated values, in the same horizon, are found in those areas where the "stress" on soil is smaller (biological or grassland). Respect to nitrogen, the general distribution results rather monotonous. Considering the relationship among organic C and nitrogen, as index of the humification processes, the

higher values have obviously been found in the biological area.

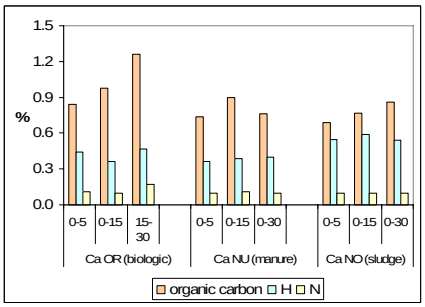


Figure 17 - Values of organic carbon, hydrogen and nitrogen in soil

4.7 Heavy metals

Heavy metals concentrations in soil at the three levels of depth are reported in *Table 2*.

Is evident the difference of concentration observed for all elements between the biological and manure areas compared with the amended area. This is also due, in first approximation, to the different soil typology.

It can be seen that the concentration of heavy metals in the site Cascina Novella are well in agreement with values found in all the soil of Pavia Province. On the other hand, in the Pavia Province only a small percentage of soils are amended with sewage sludge.

Table 2 - Concentration values of heavy metals and carbon in the 3 layers of soil

Site	Layer depth	Al	As	Cd	Cr	Cu	Hg	Ni	Pb	Zn
	cm	%	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Ca OR (biologic)	0-5	4.68	6.7	0.22	33	12.1	0.04	18.7	18.3	61
	0-15	4.55	6.4	0.27	32	12.2	0.04	19.4	18.5	61
	15-30	4.69	9.7	0.33	34	13.1	0.05	20.4	17.4	61
Ca NU (manure)	0-5	4.62	9.2	0.3	32	12.8	0.05	21.8	15.1	53
	0-15	4.07	7.5	0.24	31	11.2	0.04	18.2	16.9	57
	0-30	4.56	9.8	0.31	31	11.8	0.05	22.3	15.4	52
Ca NO (sludge)	0-5	7.32	20.6	0.84	58	28.5	0.08	34.5	29	88
	0-15	6.94	21.0	0.79	61	30.2	0.09	32	22.7	84
	0-30	7.13	22.4	0.79	59	30.8	0.08	34.4	24.6	95
AM3 Corte Olona	mean value	5.81	15.1	0.42	66	28.,0	0.08	42	22.3	84
% Uncertainty		3	10	15	8	8	24	8	9	7

The soils of the first two farmhouses are very similar. In fact the distance among the two sites is about one kilometre only, while the farmhouse Cascina Novella is located around 30 km south-east.

This is confirmed by the average vlues of heavy metals concentration found in the area of Corte Olona. In this big area, greater than 40 km^2, sludge or manure are both used. Several industries are also present and their influence in the environment, soil in our case, is important. The concentration values are similar to those found in Cascina Novella, with the exception of cadmium whose concentration was double of what found in the big area.

4.8 Dioxins

In *Figure 18* are shown values of PCDD/Fs for the three investigated areas, expressed in WHO-TEQ (World Health Organization – Toxic Equivalent Quantities). They were obtained by analysis of the three soil layers.

Values with minor differences in concentration for dioxins and furans (PCDD/F) have been found in the biological and manure areas. As concern the vertical profiles of concentration, a pretty constant value is noticed through the soil column analyzed. This has been found despite the facts that for soils the main source of dioxins and furans is by atmospheric depositions. The phenomenon is easily explainable, because plowing in fact homogenizes soil until depth of about 50 cm.

Is interesting to see that in the area where sewage sludge is used, the concentration values are slightly higher than what is found in the other two areas.

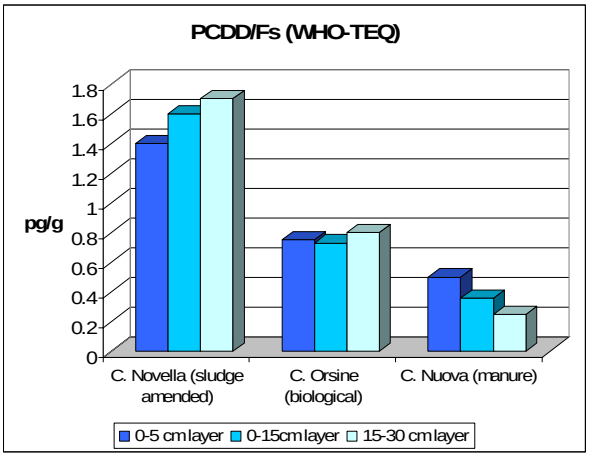


Figure 18 - Values of PCDD/Fs in the three investigated areas

The found concentration values are 10-20 times lower than limits of the D.M. Nr 471 - 1999[8] . For green or residential areas the foreseen value is 10 pg/g. German legislation sets a limit of 40 pg/g in soil for agricultural use. This limit is reduced to 5 pg/g in case of lawns for forage (Bund/Länder-Arbeitsgruppe DIOXINE, 2002).
To explain the difference of concentration in the amended area, we suppose an influence coming from the use of sewage sludge. Sludge quantity annually used is 5 tons pro hectare and involves a layer of 30 cm. We suppose a soil bulk density of 1.4 kg/dm^3, 20% of humidity and the dioxins concentration of 150 pg/g in the sludge mixed at 50%. Then we obtain an increase in soil equal to 0.05 pg/g for every year of sludge shedding. In our case the sewage sludge have been used for 15 years .That correspond therefore to an increase of 0.75 pg/g, in agreement with values found in this study.
The influence of sewage sludge in the increasing of dioxins' concentration values has been found in literature [9], [10]. For instance contribute of the dioxins and furans in soil as consequence of using sewage sludge in agriculture, represents in the UK 1.8% of all possible sources [11]. Also in Denmark the use of sewage sludge is considered a smaller source for dioxins [12]. In country like Finland, United Kingdom, Ireland, France, Denmark and Luxembourg, the recycling percentage of sewage sludge in agriculture overcomes 60% [13].
Remember that the chemical properties of this type of compounds, clearly hydrophobic, have the effect to stimulate a low bioavailability for plants.

4.9 PCBs

The concentration values of the 17 species of PCBs (HCB, PCB 28, 52, 77, 81, 101, 105, 118, 126, 128, 138, 153, 156, 169, 170, 180 and PCB 209) with high toxicity found in the three different soils are reported in *Figure 19*. These values are referred to the 0 - 15 cm layer. Concentrations are in agreement each other and low as absolute value. Similar concentrations are observed in agricultural, natural or forest areas [14].

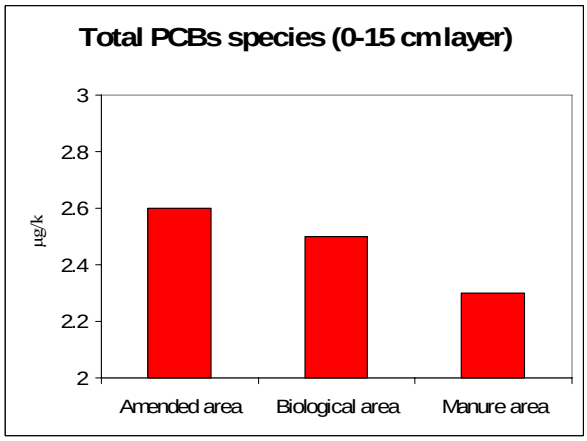


Figure 19 - concentration values of the 17 species of PCBs (µg/kg)

5. Conclusions

The general picture for the three areas is in good agreement with values found in the whole Pavia Province [15].
For what concern the concentrations of the heavy metals in the areas treated with sewage sludge, is not possible to find any increase in comparison to the other farms.
As it regards the PCBs, dioxins and furans, the found concentrations are always well below the limits. For the dioxins and furans, the use of sewage sludge has a modest influence to raise their concentration in soil.

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Microbial indicators for assessing biological fertility status of soils

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Micro organisms respond rapidly to changing environmental conditions so that they are sensitive indicators of soil health and commonly used for soil status monitoring. The aim of this study was the characterization of three differently managed agricultural soils by using microbial indicators to assess soil biological fertility status. The study was carried out in Pavia Province, in Italy. The managements involved a soil defined as biodynamic (Biodynamic); a soil characterized by periodic application of stable cattle sludge and chemical fertilizer (Manure); a soil characterized by ten years of depurated and stabilized organic sludge amendment (Sludge). Samples were taken four times during a year, at two depths: 0-15 and 15-30 cm. An extensive characterization of soil organic matter was carried out for all soils. Biochemical parameters included metabolic quotient, mineralization quotient and microbial quotient. Community level physiological profile analysis (CLPP) was used to investigate functional diversity of soil bacteria. Total amounts of fungi and bacteria were determined by direct microscopy. Indicators related to labile and humic organic matter fractions suggest significantly lower soil fertility and lower sustainability in the Sludge amended treatment. Differences between the Biodynamic treatment and Manure treatment were small.

1. Introduction

The concept of "soil quality" is generally understood as the capacity of soil to function as a living system able to fulfil all its function, to sustain biological productivity, to promote the quality of air and water environments and to maintain plants, animals and humans health (Doran and Parkin, 1994). In particular nowadays it's commonly accepted the concept of "soil fertility" as the capacity of soil to sustain biological production and all humans and natural factors affecting this production could be rightly considered as fertility factors (Sequi, 1989).

Being a very complex concept, attempts to carry a global evaluation of soil fertility result very hard and this is why fertility factors are generally included in three distinct categories on the base of their nature: physic, chemical and biological. Only the complex interaction of these three aspects makes up agronomic or integral fertility of soil, from which productivity depends.

Chemical fertility refers to the sum of available nutrients to plants while physical fertility concerned soil structure and texture. Biological fertility, instead, include metabolic expression of soils. Soil metabolic activity can be defined as the overall of reaction, both biotic and abiotic, that can ensure soil fertility. Since biotic reaction are essentially microbial ones, it's possible to confuse soil metabolic activity as soil microbial activity, but while microbial activity is a term to indicate the wide range of activities carried out by micro organism in soil, biological or metabolic activity of soil reflects not only microbial activities but also the activities of the other organisms in the soil, including for example plant roots (Nannipieri et al., 1990). Although the two terms are conceptually different they are often confused.

Microbial fraction represents a really important component in soil fertility whose failing could become soil as a simple mechanical support for plants. Micro organisms, more than other organisms, are highly adaptable to varying conditions and respond rapidly to them (Hargreaves et al., 2003). For this reason they can be considered as sensible indicators of soil health and this is why they are usually used for soil status monitoring (Yakovchenko et al., 1996). In particular, measurements of microbial activity are actually included as indicators in a lot of national and international monitoring programs on soil quality.

At the end it's likely to affirm that a better estimate of soil biological fertility is possible by using a lot of biological indicators. Usually an important criterion for an indicator is that it should respond promptly and

accurately to perturbations (Holloway and Stork, 1991). No individual measurement is enough as a single index of soil quality. However, examination of several (or even the ratios between them) may provide useful information as in this case, on management-induced effects on soil fertility.
The aim of this study was to characterize soil microbial activity of three differently managed soils by using microbial indicators in order to better understand soil biological fertility status.

2. Materials and Methods

2.1 Soils

Soil samples were taken in Lombardy region at the north of Italy, in the area of Pavia Province. The study sites have been identified in the district of Corteolona and Bereguardo (about 35 km of distance one to each other).
First site (Biodynamic) was in Bereguardo. It was a meadow grass cultivation characterized by 25 years of biodynamic management with no fertilizer or manure application on soil, no herbicides and pesticides use, and no ploughing since 2002.
Second site (Manure) was also in Bereguardo. It was again meadow grass cultivation but the difference was the periodic application on soil of stable cattle sludge (150 kg manure/year /hectare) and 15N-15P-15K fertilizer. It was no ploughing since 1999.
Third site (Sludge) was in Corteolona. It was a maize cultivation characterized by ten years of depurated and stabilized organic sludge amendment.

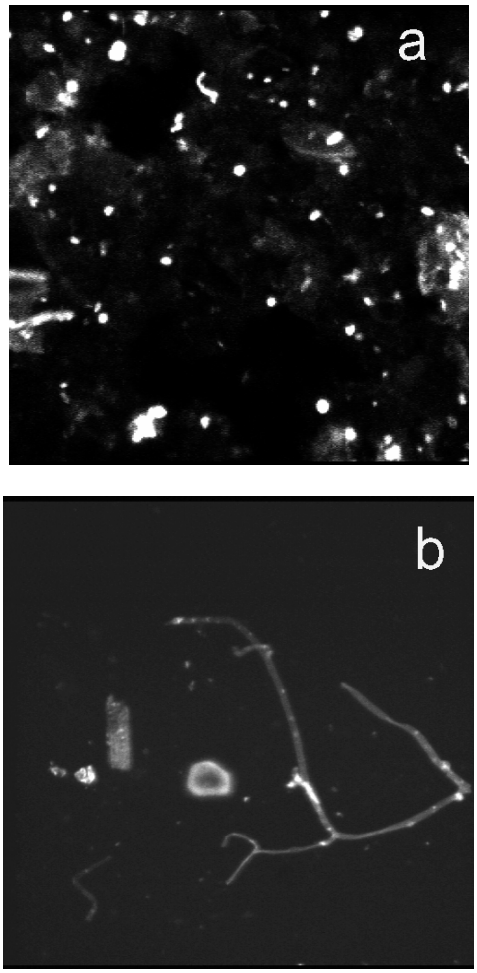
2.2 Sampling

Sampling took place four times during a year on September 2004, and January, March and July 2005 for microbial indicators and CLPP analysis. Biomass of fungi and bacteria, potentially mineralizable nitrogen and hot water extractable carbon were determined only in September and March, and only in the upper soil layer. In each site a study plot (20m x 20m) has been identified and sampling involved (0-15) cm and (15-30) cm layers considering that microbial biomass decrease according to available organic matter decreasing as depth increase.
Within each plot five bulked soil samples were collected. Since it is not desirable that natural field variations should influence the results of biological indicators interfering with effects of interests such as long-term agricultural practices, standardized environmental factors in laboratory tests have been carried out for biochemical measurements and CLPP analysis, as field variability, water tension and temperature, have the advantage to allow the comparison of soils (Schloter et al., 2003). For these reasons samples was stored at 4°C and pre-conditioned (60% of water holding capacity) at 30°C for a week until starting analyses according to indication for Mediterranean area.
In addition to the analyses by the Italian laboratory, a limited number of samples were analysed by Alterra (the Netherlands) for fungal and bacterial biomass, potentially mineralizable nitrogen and hot water extractable carbon. This was done only in the September and March samples and only in the upper soil layer. The samples were kept cool during transport by courier, and after receipt stored at 12°C for 1 (September) or 2 weeks (March).

2.3 Analytical Methods

Qualitative and quantitative characterization of soil organic matter has been carried out. Total organic carbon contents, C_{org}, were determined according to the Springer and Klee method (1954). Total extractable carbon, C_{ext}, humic and fulvic carbon fraction, C_{HAFA}, and humification parameters (DH, humification degree, and HR, humification rate) were determined by using Ciavatta et al. method (1990).
Extraction of the soil organic matter was carried out by 0.1N NaOH and 0.1N $Na_4P_2O_7$ at 65°C for 48 hrs in N_2 atmosphere. Humic acids (HA) were precipitated by acidification (pH<1.5) of the extract and fulvic acid (FA), which remained in solution, were purified on a polyvynilpyrrolidone column and then recollected to the humic portion. Total extractable carbon, C_{ext}, and humic plus fulvic acid carbon, C_{HAFA}, were determined by dichromate oxidation method, according to Ciavatta et al. (1990). Humification parameters were calculated according to Ciavatta et al. (1990), as follows:
HR (%) = 100 x C_{HAFA} / C_{org}
DH (%) = 100 x C_{HAFA} / C_{ext}.
Biochemical determinations concerned metabolic quotient (qCO_2), representing specific activity as CO_2 evolution per unit of microbial biomass (Anderson and Domsch, 1990) and mineralization quotient (qM), defining as total microbial activity as CO_2–C evolution respect to total organic carbon (Dommergues, 1960). They were determined by classical measures of total organic carbon, C_{org}, microbial biomass carbon, C_{mic} (Vance et al., 1985) and respiration of soil (Isermayer, 1952) considered as basal respiration at 7^{th} days, corresponding to carbon mineralization value in field condition.
Organic matter decomposition processes have been also investigated as cumulative mineralization curves, considering total amount of mineralized carbon in laboratory conditions, C_{cum}, as daily CO_2-C evolution during 14 days of analysis. A non-linear regression square analysis was used to calculate kinetic parameters as mineralization kinetic constant, k_{MIN}, and potentially mineralizable carbon, C_0, from average cumulative data of C-mineralization (Riffaldi et al., 1996). The first

order kinetic model of organic matter decomposition was $C_{cum}=C_0 (1-exp^{(-k_{MIN}*t)})$ (StaSoft Italia 6.0).
Since soil is generally substrate limited under natural conditions (Stotzky, 1997), community level physiological profile analysis, CLLP, (Garland and Mills, 1991) was used.
Potential soil microbial activity was investigated by integration (I_g) of typical logistic density-dependant equation as proposed in Guckert et al. (1996) by using $I_g=\int^t_0 AWCD_0/(1+exp^{-r(t-s)})dt$. The calculation needs kinetic parameters obtained from Lindstrom model (Lindstrom et al., 1998) as potential average wells colour development, $AWCD_0$, and potential rate of microbial communities increase, k_{CLPP} (StaSoft Italia 6.0).
Bacteria were measured by confocal laser scanning microscopy and automatic image analysis (Bloem et al., 1995), after staining of soil smears with DTAF, a fluorescent dye which binds to proteins (Bloem and Vos, 2004). From the number and cell volumes bacterial biomass was calculated and expressed as µg C/ gram soil.



Microscopic image of soil bacteria (a) and fungal hyphae (b) (from Bloem et al., 1997).

Fungi in soil smears were stained with differential fluorescent stain, a mixture of two stains: fluorescent brightener which binds to cell walls (polysaccharides) and europium chelate which binds to nucleic acids (DNA and RNA). The total amount of fungal hyphae in soil was determined by measuring hyphal length under the microscope. The total hyphal length was used to calculate fungal biomass in terms of µg C/ gram soil (Bloem and Vos, 2004).
Potentially mineralizable nitrogen was measured by incubation of a soil sample under water (in slurry) for 1 week at 40°C (Keeny en Nelson, 1982; Canali en Benedetti, 2006). These warm and anoxic conditions are optimal for a quick mineralization of organic matter by anaerobic bacteria. The lack of oxygen prevents conversion of released NH_4 to NO_3 (nitrification) and uncontrolled N losses by denitrification can not occur. The amount of mineral nitrogen (NH_4-N) released is a measure of the quality (N-content and decomposability) of the organic matter, and thus for biological soil fertility.
Hot water extractable carbon was determined as the amount of dissolved organic carbon that is released during incubation of a soil sample in hot water during 16 hours at 80°C (Gani et al, 2003). This is a measure of easily decomposable (labile) organic carbon. This fraction is important food for bacteria and fungi and is also correlated with soil aggregate stability (formation of clay-humus complexes).

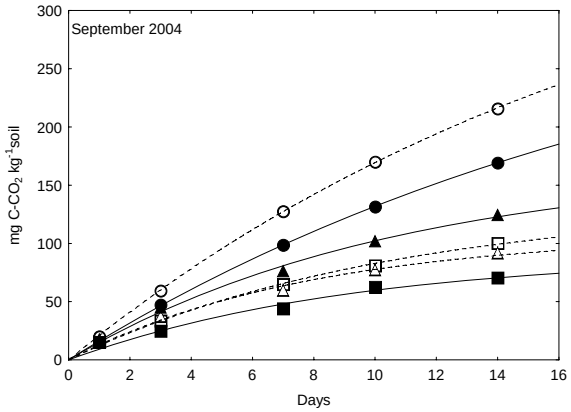
3. Results and Discussions

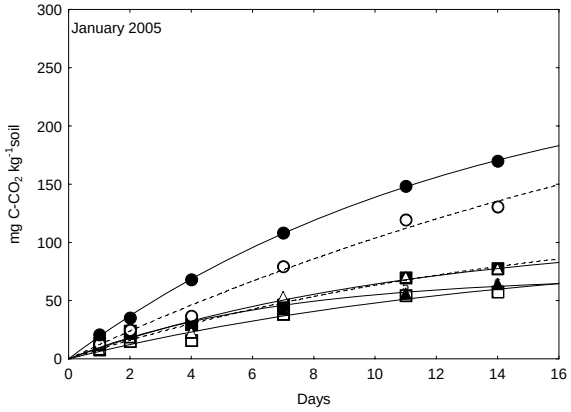
Results of organic matter characterization in soil are shown in *Table 1* as average content of all sampling because of no differences were found between them.
It's possible to notice a consistent similarity in total organic carbon values, C_{org}, for all managements while the extractable, C_{ext}, and humic and fulvic fraction, C_{HAFA}, of organic matter are significantly lower in both layer of Sludge treatment.

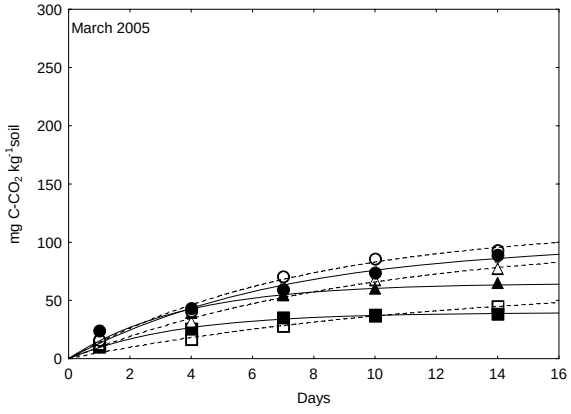
Table 1. Organic matter characterization. C_{org} = total organic carbon, g C 100g^{-1} soil; C_{ext} = total extractable carbon, g C 100g^{-1} soil; C_{HAFA} = humic and fulvic fraction of organic carbon g C 100g^{-1} soil; DH = humification degree, %; HR = humification rate, %. For each parameter different letter indicate significant differences (LSD test).

Site	Depth (cm)	C_{org}	C_{ext}	C_{HAFA}	DH	HR
Biodynamic	0-15	1.09	0.85	0.45	53.7	38.9
	15-30	1.05	0.81	0.46	56.1	45.3
Manure	0-15	1.14	0.87	0.45	47.0	45.5
	15-30	1.05	0.83	0.37	41.6[a]	40.2
Sludge	0-15	1.06	0.73[a]	0.33[a]	37.6[a]	38.0
	15-30	1.03	0.70[a]	0.34[a]	41.2[a]	40.7

Humification parameters indicate a better situation in Biodynamic and Manure treatments (0-15) cm layer compared to Manure (15-30) cm layer and Sludge treatment where values of humification degree (DH) are significantly lower in both layer then other soils. This fact can reveal a better conservation of organic matter in Biodynamic management and a sink function of soil. More intensive agricultural practices in Sludge management affect humification processes in soil. No significant differences were found in humification rate (HR).







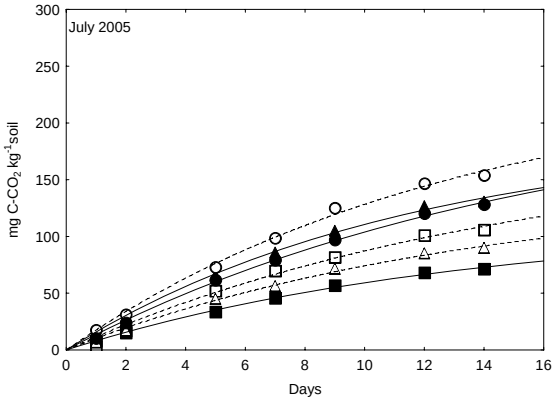


Figure 1. Soil respiration kinetic as cumulative CO_2-C evolution. (Ball = Biodynamic; Triangle = Manure; Square = Sludge. Full symbol and continue line indicate (0-15) cm layer; empty symbol and dotted line represent (15-30) cm layer). (StaSoft Italia 6.0)

Figure 1 shows soil respiration as CO_2-C evolution. Biodynamic management presents the maximum values of kinetic curves and maximum potentially mineralizable carbon (C_0 in *Table 2*) in all samplings respect to other managements. March 2005 sampling put in evidences the lowest curves and lowest microbial activity for all managements. Organic matter decomposition rates show low mineralization kinetic constant values, k_{MIN}, in Biodynamic management respect to Manure and Sludge treatments for all sampling, with the exception in July 2005 sampling. In the same time it's possible to observe in each treatment higher values of k_{MIN} in March sampling (*Table 2*).

Table 2. Kinetic parameters of organic matter decomposition processes: k_{MIN} = mineralization kinetic constant (1/days); C_0 = potentially mineralization carbon (mg CO_2-C kg^{-1} soil). (StaSoft Italia 6.0). For each parameter different letter indicate significant differences (LSD test).

Site	Depth	September 2004		January 2005		March 2005		July 2005	
	(cm)	k_{MIN}	C_0	k_{MIN}	C_0	k_{MIN}	C_0	k_{MIN}	C_0
Biodynamic	0-15	0.048[a]	344[a]	0.077	259[a]	0.223[a]	80	0.063	222[a]
	15-30	0.051[a]	424[a]	0.037[a]	334[a]	0.123	119[a]	0.076	242[a]
Manure	0-15	0.094	168	0.148[b]	71[b]	0.274[a]	64	0.087	190
	15-30	0.127	108	0.063	135	0.140	84	0.073	143
Sludge	0-15	0.107	91	0.088	106	0.266[a]	40[b]	0.075	112
	15-30	0.095	135	0.067	98	0.119	46[b]	0.065	182

Low mineralization curves in March 2005 sampling (*Figure 1*) could be explain considering natural competition for nutrients and energy substrate in spring months between micro organism and crops (grassland in Biodynamic and Manure treatments and maize crop in Sludge management). Besides, similarly between management, results indicate a brief and intensive activity of microbial populations in organic matter decomposition corresponding to the sampling of March 2005 as showed by high kinetic constants values, k_{MIN}. In January 2005 microbial activity is characterized by

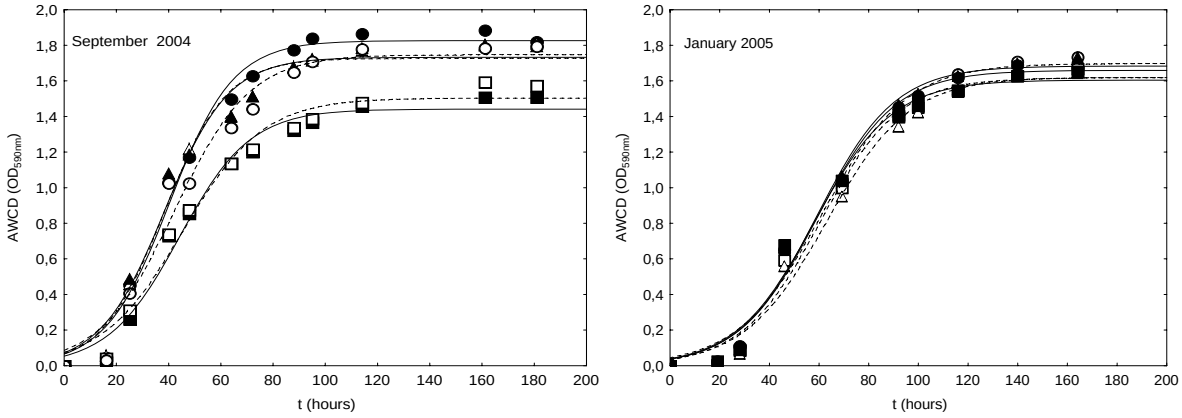
lower values of kMIN, according to very low temperature characterizing cold months.
On the contrary highest mineralization curves in September 2004 are well representative of typical situation in Mediterranean area, where maximum microbial activity is expected in mild autumn but not during hot-sultry summer (Gallardo et al., 1991). In the same time this fact explain also the high curves in July sampling when the irrigation generates dry-rewetting effect of soil with a characteristic flush in microbial activity (Riffaldi et al., 2003).

Values of qCO_2 are not too high (*Table 3*). In fact, metabolic quotient, qCO_2, shows an average good situation for all sampling and managements with the exception of March 2005, especially in deeper layer of Sludge treatment. This result, according to mineralization curves, can be due to the competition for nutrients during spring months and put in evidence a more stressed microbial community respect to other sampling.

Table 3. Eco-physiological indexes. qCO_2 = metabolic quotient, CO_2-C evolution per 100 g of microbial biomass (g CO_2-C 100 g^{-1} C_{mic} h^{-1}); qM = mineralization quotient, total microbial activity as CO_2-C evolution respect to total organic carbon (g CO_2-C 100 g^{-1} C_{org}). For each parameter different letter indicate significant differences (LSD test). No letter indicates similarity between values.

Site	Depth	September 2004		January 2005		March 2005		July 2005	
	(cm)	qCO_2	qM	qCO_2	qM	qCO_2	qM	qCO_2	qM
Biodynamic	0-15	0.26	1.23[a]	0.32	1.16	0.19	0.55	0.29	0.82
	15-30	0.19	1.44[a]	0.23	1.24	0.55[a]	0.95[a]	0.34	1.08[a]
Manure	0-15	0.10[a]	0.70	0.08[a]	0.36[a]	0.27	0.44	0.18[a]	0.81
	15-30	0.24	0.65	0.45[b]	1.11	2.00[b]	0.63	0.14[a]	0.55
Sludge	0-15	0.09[a]	0.46	0.15	0.58[a]	0.58[a]	0.37	0.27	0.51
	15-30	0.32	0.75	0.18	0.73	0.20	0.42	0.23[a]	0.79

On the contrary mineralization quotient values, qM in *Table 3*, show a good situation only in Biodynamic treatment and also in this case with the exception of March sampling. In fact, as reported in Dommergues (1960) organic matter addition on soil implies a decreasing of qM values because of the promotion of microbial activity, as can be observed in Manure and Sludge treatments where qM values put in evidence an elevated mineralization activity respect to total organic matter availability.
Scientific evidences demonstrated how the quality of organic matter added to soil can affect microbial C-use efficiency. In particular mineralization processes of high quality organic matter take more time (lower values of kMIN) respect to mineralization time requested for low quality organic matter (Benedetti and Sebastiani, 1996; Alianello and Benedetti, 1994). Infact too quickly mineralization processes (high kMIN values) could make available excessive amount of nitric and ammoniac nitrogen. Besides the application on soil of organic sludge causes a phenomenon named "priming effect" that result by stimulation of microbial activity processes. As consequence micro organisms consume more carbon than that one added on soil (Benedetti, 2004).



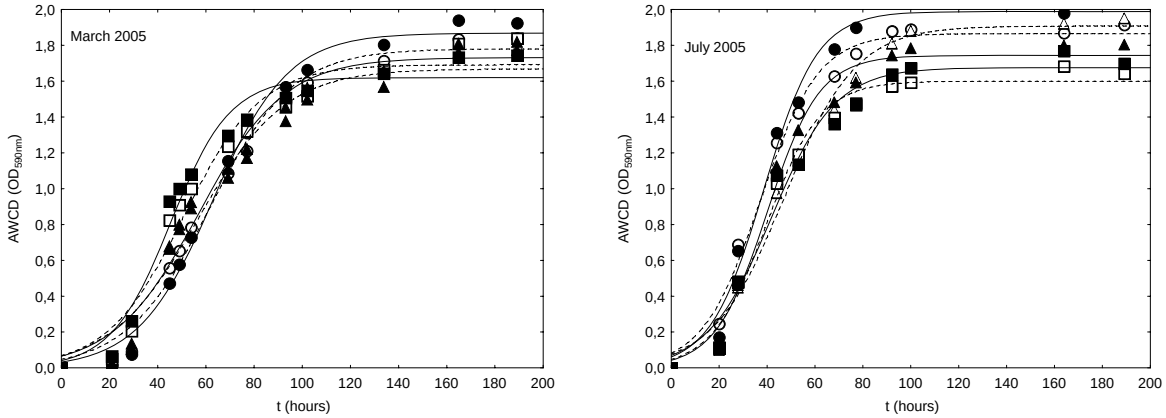


Figure 2. Community level physiological profile. (Ball = Biodynamic; Triangle = Manure; Square = Sludge. Full symbol and continue line indicate (0-15) cm layer; empty symbol and dotted line represent (15-30) cm layer). (StaSoft Italia 6.0)

Table 4. Kinetic parameters of microbial potential activity growing curves (community level physiological profile). kCLPP = potential rate of increase of growing curve (1/hours); Ig = potentially microbial activity as growing curve integration (OD_{590nm} h^{-1}). (StaSoft Italia 6.0). For each parameter different letter indicate significant differences (LSD test).

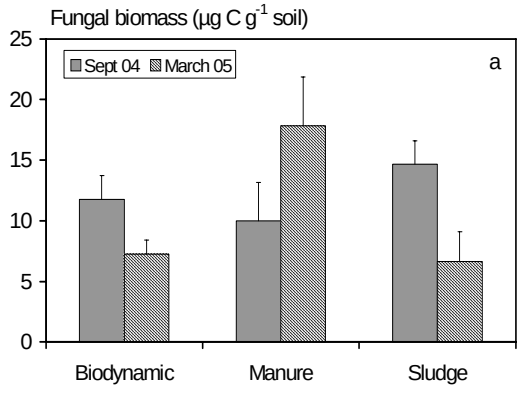
Site	Depth (cm)	September 2004		January 2005		March 2005		July 2005	
		kCLPP	Ig	kCLPP	Ig	kCLPP	Ig	kCLPP	Ig
Biodynamic	0-15	0.064	272.4	0.064	192.0	0.064	255.9	0.089	314.5
	15-30	0.059	261.9	0.059	194.9	0.058	248.3	0.083	298.8
Manure	0-15	0.064	211.0[a]	0.064	194.9	0.057	249.6	0.092	274.7[a]
	15-30	0.060	259.5	0.060	182.2	0.055	241.4	0.067	290.1
Sludge	0-15	0.063	260.6	0.063	187.8	0.081[a]	246.5	0.075	262.2[a]
	15-30	0.063	220.1[a]	0.063	185.2	0.063	252.4	0.088	251.6[a]

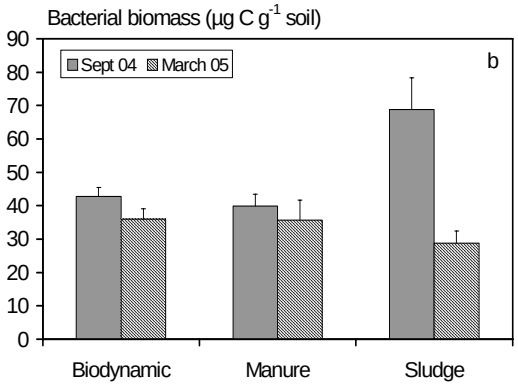
About community level physiological profile analysis, Biodynamic management presents high potential microbial activity in all sampling, Ig values in *Table 4*, while Manure and Sludge managements soils show always a lower potential microbial activity. In the same time it's possible to observe less potential activity in January 2005 for all sampling and managements. This evidence can be observed by equivalent values of Ig and kCLPP to indicate a similar behaviour of microbial communities in all managements during cold months.
These results fit very well with organic matter mineralization curves.
We conclude that functional diversity of microbial communities in the investigated soils is not affected by the different management practices. Probably, more information about genetic composition of microbial communities could reveal changing as reported in literature for heavy metals or human activity impact (Ovreas and Torsvik, 1998; Schloter et al., 2005).

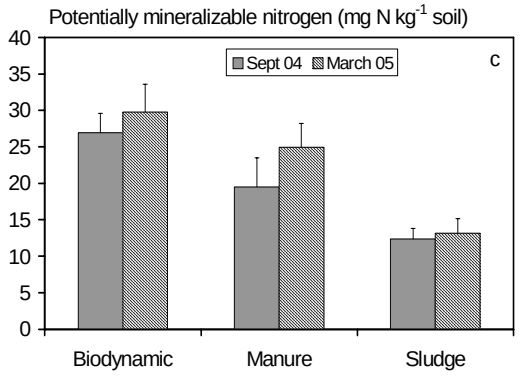
Although fungal biomass was significantly higher in the Manure treatment in March (*Figure 4a*), and bacterial biomass was significantly higher in the Sludge treatment in September (*Figure 4b*), there was not a consistent difference in fungal and bacterial biomass between the three sites. However, for a firm conclusion more than two sampling dates are needed.
In our experience nitrogen mineralization and hot water extractable carbon are less variable than amounts of bacteria and fungi. Both, potentially mineralizable N (labile N) and hot water extractable carbon (labile C) showed a consistent pattern with significantly lower values in the Sludge treatment (one way analysis of variance, $p < 0.05$). This was found on both dates with both parameters which are related to the availability of easily decomposable organic matter. Higher values are supposed to be more "sustainable" (Gani et al., 2003). A higher amount of nitrogen available for mineralization by soil microbes (mineralizable N) indicates higher biological soil fertility because nitrogen is usually the main limiting factor for crop production. A higher amount of hot water extractable carbon indicates a higher availability of food for micro organisms. Carbon is usually the growth limiting factor for soil micro organisms. More intensive land-use involving soil tillage, fertilization and grazing, stimulates microbial decomposition and tends to result in a net decrease in the labile carbon pool and ultimately in a decrease in total soil organic matter, aggregate stability and biodiversity.

The levels of fungal and bacterial biomass of 10 and 40 µg C/g soil, respectively, are relatively low and characteristic for regularly ploughed arable fields. Less tilled grassland soils usually contain at least two-fold higher levels around 20 µg fungal and 100 µg bacterial C per gram soil (Bloem et al., 2006). Also the levels of mineralizable N (10-30 mg N/kg soil) and hot water extractable carbon (200-700 µg C/ g soil) are relatively low. Considerably higher levels of 100-200 mg N/kg and 1000-3000 µg C/g are characteristic for grassland soils (Gani et al., 2003; Sparling et al., 2003; unpublished results of soil quality monitoring in the Netherlands)..
The significantly lower levels of mineralizable nitrogen and hot water extractable carbon in the Sludge treatment compared to the Biodynamic and Manure treatments are in agreement with significantly lower amount of total extractable carbon and the lower humic and fulvic acid fraction of organic carbon (*Table 1*).

The differences between the Biodynamic site and the Manure site are less consistent. Potentially mineralizable nitrogen tends to be higher in Biodynamic, but this is statistically not significant *(Figure 4c*). However, the potentially mineralizable carbon (C_0, *Table 2*) was significantly higher in the Biodynamic treatment.







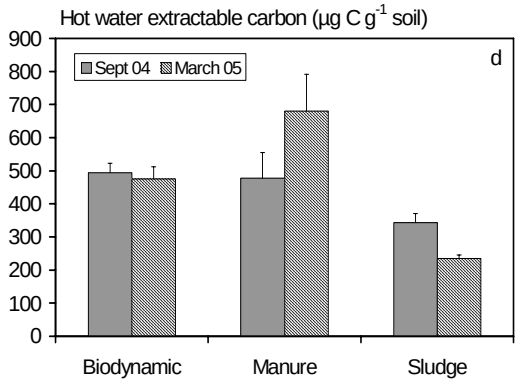


Figure N. 4. Biomass of fungi (a) and bacteria (b), potentially mineralizable nitrogen (c) and hot water extractable carbon (d) in soils with different management, sampled in September 2004 and March 2005

4. Conclusions

Microbial and biochemical indicators ware measured in samples of three agricultural fields on farms with different management:

- grassland under biodynamic management (Biodynamic)
- grassland with application of farm yard manure and mineral fertilizer (Manure)
- maize cultivation with sewage sludge amendment (Sludge)

There were no consistent differences in a range of microbial indicators between the three sites. Also the total soil organic carbon content did not show significant differences. However, potentially mineralizable N (labile N) and hot water extractable carbon (labile C) showed a consistent pattern with significantly lower values in the Sludge treatment. Soils with higher values are supposed to be more "sustainable". A higher amount of nitrogen available for mineralization by soil microbes (mineralizable N) indicates higher biological soil fertility. A higher amount of hot water extractable carbon indicates a higher availability of food for micro organisms. More

intensive land-use involving soil tillage, fertilization and grazing, stimulates microbial decomposition and tends to result in a net decrease in the labile carbon pool and ultimately in a decrease in total soil organic matter, aggregate stability and biodiversity.

The lower levels of mineralizable nitrogen and hot water extractable carbon are in agreement with the significantly lower amount of total extractable carbon and the lower humic and fulvic acid fraction of organic carbon found in the Sludge treatment compared to the Biodynamic and Manure treatments. The differences between the Biodynamic site and the Manure site are less consistent. Potentially mineralizable nitrogen tends to be higher in Biodynamic, but this is statistically not significant. However, the potentially mineralizable carbon (C_0) was significantly higher in the Biodynamic treatment.

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Evaluating soil quality in ecosystems based on modern respiratory approaches

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Soil respiration is a classical indicator for soil quality and a range of laboratory methods is available. This paper gives an overview on the techniques for measuring the soil CO_2 evolution rate or O_2 uptake rate in the laboratory, their advantages and disadvantages and possibilities for estimating simultaneously stable carbon isotopes in the respired CO_2-C. A simultaneous measurement of the CO_2 evolution rate or O_2 uptake rate, defined as respiratory quotient, seems relevant for studying humus preservation in soil, the 'soil energy-omics' and also to study positive and negative priming and triggering effects in soil in the presence of exogenous substrate. For ecological evaluation, a simplified classification of soil respiration data including the consideration of organic C content, bulk density and horizon thickness is given which need to explored by more detailed analysis of the extensive bibliography.

1. Introduction

The thematic strategy for soil protection (COM, 2002) expressed the need to care for soil since this earth constituent is a vital resource increasingly under pressure. With this strategy, the Commission of the European Communities stated the importance of soil protection and the increasing recognition internationally as soil performs a multitude of key environmental, economic, social and cultural functions that are essential for human life. The awareness to the state of our soils is also expressed at the regional level, e. g., in the Pavia province, Italia (Cenci et al., 2006).

Among the threats to soil are erosion, the decline in organic matter, local and diffuse contamination, sealing, compaction, the decrease in bio-diversity, salinisation and finally desertification. Desertification seems currently affect up to 50 % of the earth surface. The prevention of these threats is necessary to ensure the multi-functionality of soil. The soil protection strategy also includes also the removal of radiative forcing CO_2 from the atmosphere by sequestrating in soil organic matter. However, the efficiency of C sequestration in soil is mainly driven by soil respiration (Andrews and Schlesinger, 2001; Lal et al., 2004; Dilly et al., 2005).

Soil is a living medium hosting an extensive biodiversity which can consist of more than 1000 species within one gram of soil and individual species ('genomics', 'transcriptomics') in high or low abundance and active or resting stage (Torsvik and Øvreås, 2002). The diversity and the abundance of individual species and their respective physiological stage vary dependent on environmental conditions such as substrate accessibility, temperature and water availability (Dilly, 2006). It is generally agreed that the biotic consortium in soil responds sensitive to environmental conditions and human impact (Powlson, 1994).

Due to the sensitive response of soil microbial communities, a set of microbiological indicators have been identified for assessing soil quality in monitoring and research programmes and also for routine analysis (Bloem et al., 2006). Soil microbiological indicators should refer to the multivariate dimension of soil, should enable to check soil resilience and also should be measurable and non-redundant. The attributes are recognized for common soil properties such as pH values, organic carbon content, bulk density and nutrients controlling soil fertility (Yemefack et al., 2006).

Soil respiration is a classic and integrative indicator reflecting the soil energetics and current soil 'carbon currency' (Dilly, 2005). The soil respiration is usually to be estimated in the field. However, soil respiration rates in the field include plant roots and refer to factors affecting microbial metabolism ('metabolomics') such as temperature, water availability and short-term carbon

availability. Therefore, soil respiration data from the field can hardly be used as an indicator to compare soils from different locations, land use types and management practices. Consequently, soil respiration as soil quality indicator is here addressed without the presence of active roots, and also without the variation in temperature and water level affecting microbial catabolism. In general, soil respiration can be correlated to organic carbon and to microbial biomass but this is associated to the number and the nature of the considered dataset (Dilly and Munch, 1995; Dilly, 2006). Thus, soil respiration can not be considered as redundant. More information referring to specific environmental conditions can be indicated when correlation factor is smaller than 1. Then, multivariate correlations with the influence of several factors should be considered.

The aim of this paper is to give information on mechanisms behind the respiration values, an overview on the methods which can be applied in the modern laboratory and a simplified procedure on the evaluation of soil quality based on the respiration values. This helps for the development for the new field 'soil energ-omics', defined here as the custom or law on the energetic values in soil.

2. Respiratory processes

Respiration occurs within living cells by which the chemical energy of organic molecules is released in a series of metabolic steps involving the consumption of O_2 and the liberation of CO_2 and water. The release of CO_2 is also named C mineralization. The most well know respiration formula is most likely the glucose oxidation by heterotrophic organisms according to $C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ H_2O + 6\ CO_2$. This formula gives a respiratory quotient (RQ), defined as the ratio of mol CO_2 evolution per mol O_2 uptake, of 1. However, substrates differing with reference to carbon and oxygen content and nutrients like nitrogen and also the involvement of electron acceptors alternative to molecular oxygen such as nitrate, manganese, iron, sulphate and organic acids induce respiration quotients unequal from 1 during complete oxidation (*Figure 1*).

Due to the availability of the substrates and the preference for incomplete versus complete degradation, the intensity of respiration may vary largely. Organic acids such as oxalic acids and citric acid are abundant substrate released by roots and stimulate the soil respiratory metabolism similarly than glucose (Degens et al., 2001) but their complete oxidation generally increase respiratory quotient toward values higher than 1 (*Figure 1*, for oxalic acid). In contrast, refractory and complex compounds such as lignin, fats, oil and proteins may induce small response in respiration but their complete oxidation expects respiratory quotients lower than 1 (*Figure 1*, for palmitic acid), dependent to their nature (Dilly, 2001; 2003). E.g., the addition of humic acids was found to have a small effect on soil respiration (Dilly, 2004) and thus the respective respiratory quotient was only slightly modified in contrast to the expectation.

Substrate	Acceptors	Products	Respiratory quotient
$C_{15}H_{31}COOH$	$23\ O_2$	$16\ H_2O + 16\ CO_2$	RQ < 1.0
$C_6H_{12}O_6$	$6\ O_6$	$6\ H_2O + 6\ CO_2$	RQ = 1.0
$C_2H_2O_4$	$0.5\ O_6$	$H_2O + 2\ CO_2$	RQ > 1.0
	NO_3^-	N_2O, N_2	
	SO_4^{2-}	S	
	Fe^{3+}	Fe^{3+}	

Figure 1 Respiratory formula and the respective respiratory quotient for microbial oxidation of palmitic acid, glucose and oxalic acids with the use of molecular oxygen and some alternative electron acceptors

The composition of the organic matter in soil has been estimated to be equivalent to $C_{308}H_{328}O_{90}N_5$ (Schulten 1993) and also $C_{6932}H_{7662}O_{1970}N_{110}$ (Schulten and Leinweber, 2000). The complete oxidation refers to the adjusted O_2 uptake and CO_2 evolution and the overall RQ values between 0.29 and 0.906 (Dilly, 2001). In addition, the composition of soil organic matter may be variable dependent on the composition of the input and transformation stage which is in turn dependent the consortium and the physiology of the organisms in soil.

Glucose is important in life cycle and an easily decomposable compound leading to several fold increase of metabolic activity when added to soil (Stout and Dutsch, 1968). It is therefore not surprising, that glucose is frequently applied to soil for studying the response of the soil microbial biomass. The substrate-induced respiration after optimal glucose addition was found to be correlated to the amount of soil microbial biomass which was estimated with fumigation techniques earlier (Anderson and Domsch, 1978). After glucose addition, the respiratory quotient should essentially stay at 1 but values drastically exceeding 1 can already be observed within the first day after substrate addition (Dilly, 2001). This may be related to either biomass growth or to a larger extend of glycolysis, the hexose monophosphate shunt, and Entner-Doudoroff pathway for biosynthetic purpose, the pyruvate decarboxylation and tricarboxylic acid cycle to obtain precursor metabolites (Stryer, 1995; Perry and Staley, 1997). The involvement of anaerobic processes cannot be excluded but seems unlikely since properties such as soil texture and water content did not show apparent affects on respiratory quotient during this period for a range of soils (Dilly, 2001).

3. Techniques for the quantification of soil respiration

Respiration can be estimated by the CO_2 evolution and O_2 uptake. The measurements of CO_2 evolution is more sensitive since the atmospheric background of 0.04 % is low and changes can be estimated more precisely. In contrast, sensitive O_2 sensors against the air background of approximately 21 % are hardly to develop and thus indirect methods for the estimation of O_2 uptake are used. Indirect methods for the estimation of O_2 uptake rely on the determination of changes in pressure in a closed system.

Eight open and closed systems for measuring soil respiration in the laboratory were distinguished in *Table 1*. In contrast to closed systems, open techniques do not currently allow the simultaneous estimation of CO_2 evolution and O_2 uptake due to the lack of sensitive O_2 sensor. However, closed systems are limited when soil respiration is estimated at high water content and in the presence of lime due to the absorption of CO_2 and the spontaneous release of abiotic CO_2. A continuous purging of CO_2 is essential to ensure gas exchange equilibrium and to refer to CO_2 evolution related to active soil metabolism. Thus, soil preconditioning in the presence of CO_2 absorbent or the use of a continuous flow system is essential before respiration can be determined exactly. Of course, it is necessary to compare data from contrasting respirometry techniques and laboratories. Kaiser et al. (1992) found that values were reduced by 25 % after changing from the closed system No 3 to the open system No 1.

Table 1. Methodological principles for the estimation of respiration with reference to CO_2 evolution and O_2 uptake; […] indicate that changes of the respective gas can not be estimated

No	Principle	CO_2	O_2	Name or Reference
1	Open - air with CO_2	Purged	[Continuous supply]	Heinemeyer et al. (1989)
2	Open – CO_2-free air	Purged (trapped)	[Continuous supply]	Bayer AG (Anderson pers. Comm. 1991)
3	Closed temporarily[1]	Enrichment	[Depletion]	Wösthöff (Anderson and Domsch 1978)
4	Closed temporarily[1]	Enrichment	Depletion	Columbus Inc., Ohio
5	Closed[1]	Enrichment	Depletion	Gaschromatography
6	Closed[1]	Enrichment	Depletion	BaPS - Barometric Process Separation
7	Closed	Purged (trapped)	Depletion	‘Weckglas‘/Aqualytic
8	Closed	Purged (trapped)	Continuous supply	Sapromat Voith / IBUK / MP686 Maynard Project Cambridge

[1] This technique is limited when analysing soils with high pH values since CO_2 is dissolved in H_2O and absorbed by $CaCO_3$ leading to the formation of $Ca(HCO_3)_2$

When CO_2 is trapped in NaOH, the addition of $BaCl_2$ induces the precipitation of $BaCO_3$. The washed and dried precipitate can be analysed with an elementary analyser followed by an isotopic mass spectrometer. The composition of the isotopic composition of the precipitate helps to separate the origin of the CO_2 derived from inorganic C, soil organic matter or substrate added with a specific signature (Zyakun and Dilly, 2005). Soils under C3 plants such as wheat or beech and with a pH value of 4 to 6 show usually ^{13}C-CO_2 signatures ($\delta^{13}C$) of approximately -26 ‰, in contrast to those under C4 plants such as mays or sugar cane with -12 ‰. When relatively ‘heavy’ glucose from C4 plants is applied to two soils with similar basal respiratory activity, both below C3 plants, the values of -14 ‰ and -16 ‰ respectively indicate that the soil microbiota respond to a smaller extent to glucose in the later case. The mass isotopic balance

linking the ^{13}C-CO_2 signature and the respiratory activity after the glucose addition indicates if priming effect may have been induced by the application of the readily available substrate. The priming effect is defined as any strong positive or negative short-term changes in the turnover of soil organic matter induced by moderate treatments of the soil (Kuzyakov et al., 2000). If positive priming effects occurred in the two soils, the soil with lower δ13C values showed higher priming. All over, stable carbon and nitrogen isotopes in soils, vegetation and invertebrates can contribute to understanding landscape processes (Cook and Dawes-Gromadzki, 2005).

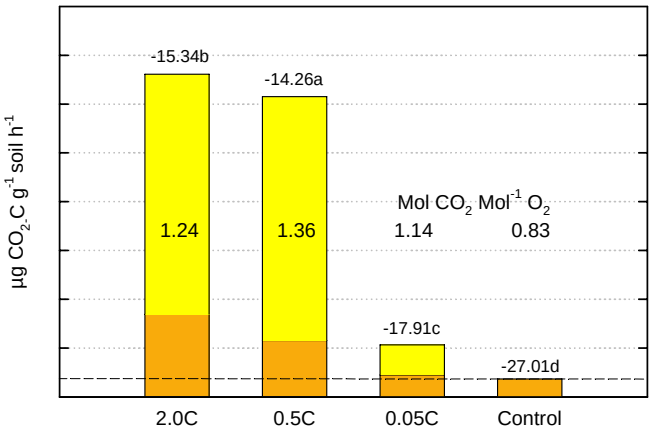

Figure 2. Respiration, respiratory quotient and $\delta^{13}C$ in arable soil within 24 hours after addition of 2.0, 0.5, and 0.05 mg C per g soil; more details in Zyakun and Dilly (2005) The respiratory quotient [Mol CO_2 Mol^{-1} O_2] is given in the centre of the Figure.

Figure 2 shows that increase microbial respiration with increasing substrate rate. The $\delta^{13}C$ value of respired CO_2 was lower at the high substrate rate of 2 mg glucose-C g^{-1} than at 0.5 C indicating a higher mineralization of indigenous soil organic matter with high glucose. The respiratory quotient was also higher at higher input of exogenous glucose. This is an example for studying priming and triggering effects and thus the possibility in C sequestration of atmospheric CO_2 in soil using ^{13}C (or ^{15}N) labelled substrates such as litter and glucose.

4. Classification of data on soil respiration

Soil has to be sampled for horizons indicative for the site-specific biogeochemical cycles (e.g., A horizon), at least in triplicates at independent field plots. Sampling at a specific soil depth should bear this in mind.
Then, soil basal respiration (BAS) has to be done at 22 °C and at 40 to 70 % water holding capacity firstly (Pell et al., 2006). Afterwards, glucose should be added to induce the maximal initial respiratory response (MIRR). The substrate-induced response (SIR) of soil estimated during the first 4 to 6 hours after substrate addition can be converted to microbial biomass values when soil meets specific pre-requisites such as aerobic conditions, no recent substrate amendment and bacterial-fungal-ratio of approximately 1 to 3 (Anderson and Domsch 1978; Höper 2006). The original factor for the conversion of SIR to microbial carbon is 40 mg C per ml CO_2 and h (Anderson and Domsch, 1978), the modified factor for the apparatus after Heinemeyer et al (1989) 30 mg C ml^{-1} CO_2 h^{-1}.

Table 2. Simplified classification of respiration and microbial biomass data into low, typical and high values according to some internal screening (unpublished results);respiration and biomass data are related to soil weight, organic C content, microbial respiration was derived by microbial biomass to determine the respiratory quotient .

Class	µg CO_2-C g^{-1} soil h^{-1}	mg CO_2-C g^{-1} C_{org} h^{-1}	µg C_{mic} g^{-1} soil	mg C_{mic} g^{-1} C_{org}	µg CO_2-C g^{-1} C_{mic} h^{-1}
Low	< 1	< 20	< 100	< 5	< 1.0
Typical	1 to 10	20 to 100	100 to 1000	5 to 20	1.0 to 2.0
High	> 10	> 100	> 1000	> 20	> 2.2

It is essential to determined soil water content since the data are expressed at first on the basis of gram soil weight. After drying and weighing, the soil should be combusted for approximately 3 hours at 500 °C for the determination of loss on ignition. Loss on ignition can be used as an estimate of soil organic carbon when dividing by 2 and for soils with no lime and low clay content. *Table 2* shows a simplified classification of

respiratory and biomass data separating low, typical and high values. This classification assumes a specific amount of organic C and a specific soil bulk density but needs some subtraction or addition otherwise (*Table 3*). The exact estimation of bulk density requires the careful determination with cylinders of 100 m³ or higher.

Table 3. Essential adjustment of dry-soil-weight related respiratory and biomass data when bulk density and organic matter were exceptional (unpublished results).

Respiratory values	Bulk density [g cm^{-1}]	Organic C [mg C g^{-1} soil]	Adjustment
Exceptional	< 0.8	> 50	Lowering of values / class
Typical	0.8 to 1.6	9 to 50	No adjustment
Exceptional	> 1.6	< 9	Increase of values / class

The mass-related data multiplied with horizon thickness and bulk density gives values related to soil area. For one soil with all relevant horizons, values of 500 kg C ha^{-1} is typical which represents about 10 cows or 100 sheep per ha on the basis of 500 and 50 kg for the cow and the sheep biomass respectively with 20 % dry matter and 10 % C content. These values are comparable to those reported by Smith and Paul (1992). From an ecological viewpoint the values per horizon and area, or per unit soil volume should be considered for the comparison of different sites.

Irrespective of bulk density but adjusted to horizon thickness across the soil profile, physiological properties can be used for the evaluation of soil quality. Examples of eco-physiological properties are the microbial, metabolic and respiratory quotient that relate microbial C to total organic C, current microbial mineralization related to microbial C (which is a measure for C use efficiency of the microbial biomass) and the ratio of CO_2 evolution per O_2 uptake (difference between two respiration measures and current microbial anabolic and catabolic property and also substrate property) respectively.

5. Conclusions

The modern evaluation of soil quality includes respiratory indicators that are sensitive to human and environmental impact. Respiratory indicators are both sensitive and integrative for the response of soil microbial communities in comparison to specific tools such as molecular techniques. In addition, soil respiration values are directly related to important soil conservation issues such as humus conservation. The extensive data base on soil respiration achieved during the last decades enables the general soil quality evaluation with reference values. This needs to be explored in-dept despite different techniques may have been applied. Serious efforts should be done to relate data to soil surface area by the consideration of bulk soil and organic carbon content. The knowledge on bulk density is not necessary when using respiration values in eco-physiological indicators. Modern respiratory approaches combine the estimation of the respiratory indicators and isotopic signature of respired carbon since giving detailed information of the current microbial physiology and used substrate respectively.

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Pavia's Province Project: evaluating soil bio-hazards on *Dictyostelium* development

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The low eukaryote *Dictyostelium discoideum* was recently characterized as a new sensitive biosensor organism for soil contamination. The bioassay, based on the evaluation of the inhibition of *Dictyostelium* developmental rate was applied to three Pavia's Province farmlands which have been subjected to different fertilization treatments. The biologically treated soil was non toxic, while low toxicity was found in the soil fertilized with manure and mineral compounds. Higher but dubious toxicity was observed in the soil treated with depuration in both the sampling.

1. Introduction

The soil amoeba *Dictyostelium discoideum* is a low eukaryote, which has been widely studied for the investigation of several cellular processes such as cell motility, cell adhesion, development, chemotaxis and lately also to study the molecular mechanisms underlying drug resistance [1,2]. *Dictyostelium* cells live and proliferate as solitary amoebae, feeding on bacteria by phagocytosis and dividing by binary fission. In nature, they live in the forest wood, decaying leaves and humid soil. Under laboratory condition, cells can be cultured on agar plates or in shaken liquid medium or in combination with bacteria. Depletion of food triggers a developmental program, whereby cells cluster together by chemotaxis giving rise to aggregates of approximately 10^5 cells. Each aggregate undergoes differentiation in at least two cell type and engages in a sequence of morphogenetic changes typical of a multicellular organism. The aggregates develop into fruiting bodies consisting of a "sorus", containing spores, hold by a slender stalk. The whole developmental program is accomplished in approximately 24 hours. Moreover the two phases of *Dictyostelium* life cycle -growth and development- are temporally separate and mutually exclusive. (*Figure 1*) [1, 3].

By contrast to bacteria and plants and similar to animals, the *Dictyostelium* cell, is not protected by a cell wall, thus conferring to the amoebae high sensitivity to environmental stressors.

These cellular and developmental properties and the rather unique capacity of the cells to develop both under submerged condition and on solid surface render

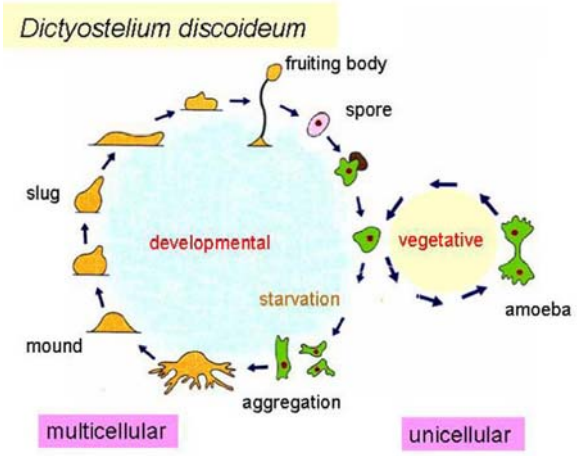

Figure 1. Dictyostelium discoideum life cycle
On the right is depicted the vegetative phase of Dictyostelium life cycle in which unicellular amoebae divide by binary fission. On the left the developmental stage, wherein cells depleted of food aggregate, by chemotaxis to form multicellular organisms of 10^5 cells. Each aggregate, containing two different cells types, organizes into a structure named fruiting body consisting of spores resting atop a cellular stalk. The spores will germinate in the presence of nutrients producing mitotically dividing cells.

Dictyostelium a potentially attractive biosensor to detect the presence of bio-hazardous compounds in the extracellular environment, both on soil or water. By exploiting all these characteristics, we have developed

an easy, cheap and quick bioassay which allows to evaluate the influence of toxic substances on the rate of fruiting bodies formation. The assay detects biological effects of heavy metal on soil with varying sensitivity, as high as 0,1 and 1375 mg/kg air dried for Hg and Cd respectively or as low as 6390 and 2556 mg/kg air dried soil for Cu and Zn, respectively. The sensitivity of the cells for all the above mentioned heavy metals under submerged condition is higher of a factor 10 to 100.

By comparing the *Dictyostelium* with other commonly used bioassay in a series of contaminated soils, the *Dictyostelium* sensitivity is comparable to that of other biosensor organisms such as *Collembola* and *Earthworm* [4, 5].

Here we present some results obtained with the *Dictyostelium* assay applied to three different farmlands of the Pavia's province. We report that the bio-hazard for untreated soil is comparable to that of control soil in two samples collected in different seasons. By contrast the soil fertilized with manure and mineral compound results weakly toxic and toxicity varies in the course of the year. The soil fertilized with depuration mud results toxic, independently of the season.

2. Material and methods

2.1 Cell culture

Dictyostelium cells of the parental strain, referred as AX2-214, were grown in axenic medium in shaken suspension at 150 rpm and at a temperature of 23°C [6,7].

For development, amoebae were harvested during exponential growth, washed twice with water and then resuspended in water at the density of $1x10^8$ cells per ml. Afterwards, $2x10^7$ cells were spread on soil and allowed to develop at 23°C.

2.2 Dictyostelium bioassay

Each soil sample (approximately 6g) was moisturized with sterile water, aliquoted in 6 wells of a 24well plate and levelled with a weight. Upon the surface, in an area of about 1 cm^2, a drop of approx. 2 x 10^7 cells was spread on. The plates were incubated at 23°C [7].

The developmental rate was evaluated after 24-26 hours

as the number of fully developed fruiting bodies, which were counted by an operator with the support of a stereo-microscope and a grid.

Three independent assays were performed. All counts were processed by parametric statistical analysis using Excel statistical function to calculate means and standard deviation and the raw values significance was statistically analysed with the t-test (1 tail, pvalue: $p<0.05$) (*Figure 2*).

3. Results

3.1 Soil samples

Soil samples were collected from three different areas of the Pavia's province (Cascina Orsine, Cascina Nuova and Cascina Novella) to a maximal deepness of 1 m. The three fields were differently fertilized in the past years. Cascina Orsine was biologically treated, neither pesticides nor weed killer had been used; Cascina Nuova was fertilized with manure and mineral fertilizer (15N, 15P, 15K 150 kg/ha) and Cascina Novella was manure with depuration mud treated with NH_3 and H_2O (360 q/ha).

Soils were collected at two different seasonal times: the first at the beginning of November 2004 and the second in July 2005 [8].

After the treatments performed by the Alessandria Unit the soil samples were supplied to our lab for the assay.

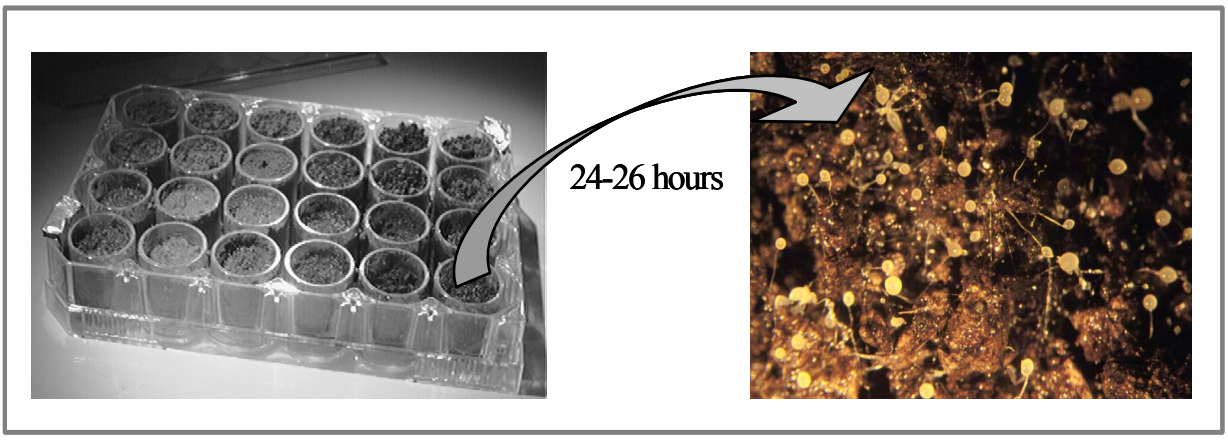

Figure 2. Schematic representation of Dictyostelium bioassay.
About $2x10^7$ Dictyostelium cells were spread on the levelled surface of the soil (6 samples for each assay). After 24-26 hours the number of fruiting bodies formed was counted at a stereo-microscope using a grid

A total of three assays were performed for each soil sample.
As control, a standard soil previously used for analysis of contaminated sites was employed [9].
A toxicity index is quantified in relation to the inhibition of *Dictyostelium* development. A reduction from 0 to 25% in the number of fruiting bodies formed is considered non-toxic, between 25 and 50% is considered slightly toxic, between 50 and 75% toxic and from 75 to 100% very toxic.

3.2 First sampling – November 2004

The soils of the three farmlands of Pavia's Province were analyzed within two months after sampling. C. Orsine didn't affect the development of *Dictyostelium*, behaving like control soil. Instead, the number of formed fruiting bodies was reduced to different degree by both C. Nuova and C. Novella (*Figure 3*).

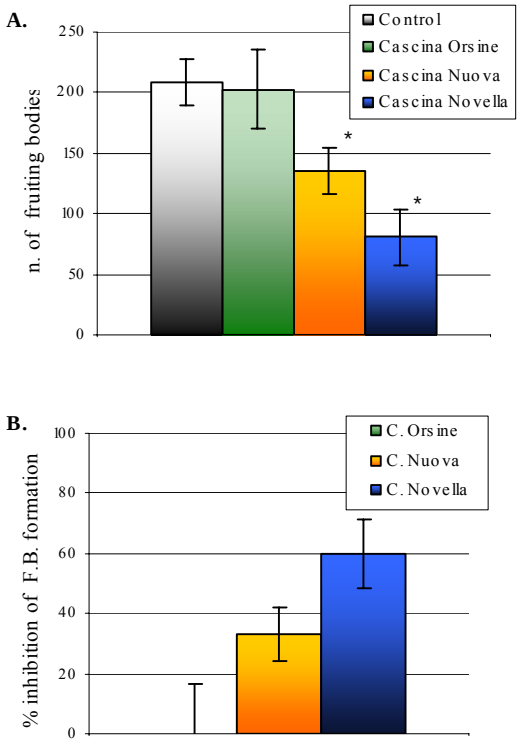

Figure 3. Analysis of the first sampling of Pavia's soils with Dictyostelium test.
Panel A. Soil samples from the three farmlands collected on November 2004 were examined with the bioassay. Histograms display the mean values of the fruiting bodies formed in three independent assays whereas the error bars indicate the standard deviations.
The asterisk * indicates significant differences between raw data from control soil and each Cascina's sample (p value $p<0.05$).
Panel B. Data are expressed as % inhibition of all the three farmlands compared to control soil.
F.B., Fruiting Bodies

3.3 Second sampling – July 2005

An identical analysis was carried-out on the soils collected in the second sampling.
Compared to the first one only a slight, but not statistically significant, inhibition was detected for the C. Orsine soil. The C. Novella soil was slightly less inhibitory compared to previous analysis. In contrast a stronger inhibitory effect was observed for C. Nuova (*Figure 4*).

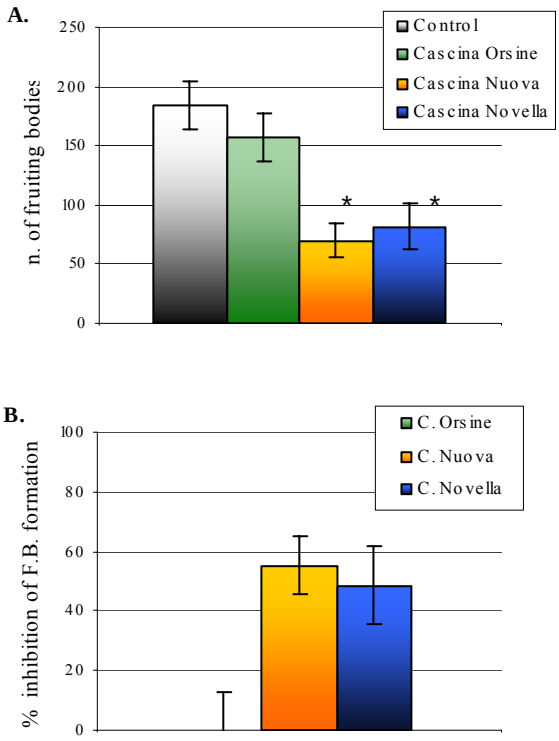

Figure 4. Analysis of the second sampling of Pavia's soils with Dictyostelium test.
Panel A Soil samples from the three farmlands collected on July 2005 were examined with the bioassay. Histograms display the mean values of the fruiting bodies formed in three independent assays whereas the error bars indicate the standard deviations.
The asterisk * indicates significant differences between raw data from control soil and each Cascina's sample (p value $p<0.05$).
Panel B. Data are expressed as % inhibition of all the three farmlands compared to control soil.
F.B., Fruiting Bodies

4. Discussion

The analysis of the Pavia's soil samples with *Dictyostelium* test highlight a difference between the two seasonal sampling. In particular, the C. Nuova soil, fertilized with mineral compounds and manure, displays an increase in fruiting bodies inhibition from 33% (November 2004) to 55 % (July 2005), ranging from slightly toxic to toxic.

C. Orsine soil results non toxic.
The C. Novella soil appears more toxic (inhibition varies between 60% and 49%); this robust reduction of fruiting bodies formation, couldn't be univocally attributed to the presence of bio-hazardous compounds in the soil. Most likely the impaired development also results from the physical features of this soil that is very limey and clayey. The soil fails to absorb water, creating condition for the development of the cells that cannot be compared with the other soils. These results highlight, in our opinion, the necessity to create a bank of standard soils with different physicochemical properties to be used as control soils in bioassays. In the absence of such controls the apparent toxicity of a given soil cannot be evaluated conclusively.
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Development and application of whole cell biosensors based on recombinant cell lines of the ciliated protozoan *Tetrahymena thermophila* for ecotoxicity screening

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This report describes the development and the application in ecotoxicity screening of whole cell biosensors (WCB) based on recombinant cell lines of the ciliated protozoan *Tetrahymena thermophila*. Cells of this worldwide distributed, freshwater, eukaryotic microorganism were transfected with the expression vector pD5H8, containing the coding sequence of the reporter gene for the "Green Fluorescent Protein" (GFP), under the control of a homologous, stress inducible *hsp70* promoter. By this method a fluorescent bioreporter strain able to detect general toxicity was obtained. The toxicity assay was performed by exposing the cells to various dilutions of environmental relevant pure compounds or more complex environmental (field) samples (effluent discharges, soil elutriates etc..) and the fluorescent emission was easily detected by means of fluorescence microscopy.
In this study, the Tetrahymena whole cell biosensors were used in the frame of the BIO-BIO project (sponsored from the "Provincia di Pavia") in order to assess the potential toxicity of soil elutriates coming from three farms managed using different agricultural systems (conventional, organic and threaded with sewage sludge). In parallel to the *Tetrahymena* bioreporter assay, also classic lethality assays were performed. The results showed that the bioreporter assay allows a better evaluation of the toxicity displayed by the elutriate samples with respect to the lethality, assay in the presence of low levels of toxicity.

1. Introduction

Environmental pollution caused by industrialization represents a serious problem that threatens the existence of every life forms and natural habitats to a greater extent, day by day.
The analysis of complex environmental samples is primarily based on chemical analytical methodologies which, although accurate and sensitive, fail to provide data on bioavailability, potential synergistic/antagonist effects of the various toxicants on living organisms, as well as on the potential effect of unknown or chemically undetected substances.
Thus, methods able to fulfils these critical requirements and in the meanwhile able to reveals sub-lethal levels of toxicants in the real environment, are urgently needed. Several recent reports showed that bioreporter assays based on genetically modified cells (whole cell biosensors, WCB), represent a rapid, inexpensive and efficient alternative method for environmental monitoring [1,2,7,8,11]. These particular biosensors, that use whole cells as biosensing elements, instead of specific molecular entities (enzyme, antibodies, DNA), are able to provide an integrate view of the global cellular processes in response to noxious substances. The general approach for producing a biosensor using intact cells consists in fusing a stress-inducible specific promoter-DNA sequence from a well characterized gene regulation system to a reporter gene. The final genetic construct is inserted into the selected host cell. When the noxious substance is present, the expression of the reporter gene is induced, producing a signal that can be measured.
In this work, WCB biosensors have been obtained by transfecting cells of the protozoan ciliate *Tetrahymena thermophila* with a plasmid containing the coding sequence of the reporter gene *Green Fluorescent Protein* (*GFP*) [3] under the control of the stress inducible *hsp70* promoter [Barchetta S, La Terza A. Ballarini P. and C.Miceli, manuscript in preparation], to generate a fluorescent bioreporter strain able to reveal a general condition of stress.
For the construction of the WCB**,** ciliates and, in particular *Tetrahymena* species, represent an ideal bio-material, since they offer a numbers of suitable

characteristics to be used as biosensing elements of environmental sensors: a) they occupy the first trophic levels and consequently are early warning indicators of cellular suffering; b) they are available for most of the newly developed molecular genetic techniques; c) they can be easily cultured and maintained in small volumes; d) cell lines can be frozen and maintained in liquid nitrogen. Moreover, the analysis of the recently sequenced macronuclear genome of *Tetrahymena thermophila* has revealed that this ciliate shares a degree of sequence conservation with human genes higher than that showed by other single-celled eukaryotic model organisms [6], yeast included. These considerations make *Tetrahymena* an appealing bio-system for toxicity assessment, since it can provide information of direct relevance to human health and thus represent a valid alternative to the use of vertebrates in biomedical research. *Tetrahymena* is already widely used as a bioindicator: a database named TETRATOX has been established as a collection of toxic potency data for more than 2,400 industrial organic compounds [13]. For TETRATOX, the assay is a short-term, static protocol in which the 50% impairment growth concentration (IGC_{50}) is the recorded endpoint. In many other cases lethality assays or inhibition of chemotaxis assays are also used [4].

In the *Tetrahymena* biosensor assay here established, the fluorescence emission represents the toxicity endpoint. By this assay, simple fluorescence microscopy techniques allow the real time and *in vivo* detection of fluorescence, without cell fixation requirement. This makes collection of experimental data easy and rapid, if compared with the classical physiological endpoint measurements such as loss of mobility, lower proliferation rate, etc.

These *Tetrahymena* biosensors were specifically developed and successfully tested in the frame of the following research projects*:* 1) Environmental monitoring of the industrial site of Acna di Cengio (CN), Italy (sponsor: Ministero dell'Ambiente), 2) Environmental biotechnology (sponsor: CNR). Here we report the use of this rapid and sensitive bioassay, assessed in comparison with the classic lethality test for the evaluation of the potential toxicity of soil elutriates coming from three agricultural farms under different agricultural management systems. This monitoring plan has been performed in the frame of the Biodiversity and Bioindication (BIO-BIO) project (sponsor: "Provincia di Pavia") under the supervision of Dr. Roberto Cenci, European Commission, Joint Research Centre, Institute for Environment and Sustainability at Ispra , Italy.

2. Materials and methods

2.1 Environmental samples

The elutriates samples prepared from the soil collected in the three farms following the procedure US-EPA (1991), were kindly provided by the laboratory of the Prof. A. Viarengo from the University of Piemonte Orientale at Alessandria.

2.2 Tetrahymena Strains and Culture Conditions

Strains Cu 428.2 VII, Mpr/Mpr [6-methylpurine-sensitive (6-mps)], and SB1969 II ChxI-I/ChxI-I [cycloheximide-sensitive (cys)], were kindly provided by Professor E. Orias (University of Santa Barbara). Cells were grown routinely in 2% PPY (2% proteose peptone, 0.2% yeast extract and 10 um FeCl36H2O) at 30 °C with moderate shaking. To prevent bacterial and fungal growth, the medium was enriched with penicillin G (100 units/ml), streptomycin (100 ug/ml) and amphotericin B (0,025 ug/ml).

2.3 Construction of recombinant *Tetrahymena* biosensors cell lines

The *Tetrahymena thermophila* biosensor cell lines were obtained from the wild type Cu428 strain. Cells were electroporated with the expression vector WT-GFP-pD5H8, which was appropriately prepared to contain the *GFP* reporter gene under the transcriptional control of the 5' and 3' flanking sequences of a *T. thermophila* inducible *hsp 70* gene. The derived recombinant cells were stably transfected since this type of plasmid is arranged to be integrated in the endogenous rDNA. When a stress condition occurs, the GFP transcription is activated *via* the promoter region, which is recognized by the endogenous transcription factors. The fluorescence emission can be easily quantified by a microscope or in terms of protein produced, by western blot analysis.

2.4 *Tetrahymena* biosensors assay and lethality assay protocols

Fluorescence and lethality tests were performed in BD Falcon™ 96-well Optilux Microplates with crystal clear polystyrene for easy microscopic viewing, by exposing a fixed number of cells (usually 100) to various dilutions of each soil elutriate sample in a final volume of 250 □l of TRIS-Cl 10 mM pH 7.5 in a dark moist chamber at 30±1 °C. The cells were observed at fixed intervals of time for 24 hours. The observation of the fluorescent cells was carried out with a 20× objective on a Nikon Diaphoto TMD inverted microscope with attached digital camera. GFP fluorescence was detected by using a filter set with an excitation wavelength of 470- 490 nm and an emission wavelength of 520 nm.

Moreover, in order to facilitate fluorescence detection, the cells were immobilized by adding dibucaine [12] at the final concentration of 0.5 mM or by using one drop of "Protoslow" solution (www.blades-bio.co.uk). The results of the assays are expressed as LC_{20} or EC_{20},

which represent the effective elutriate concentrations causing in 20% of cells a lethal effect or the *hsp70* gene expression (revealed as fluorescence emission), respectively. For each experiment a control sample of cells treated and analyzed in the same way, but with the addition of buffer instead of soil elutriate, was carried out. Lethal effects or fluorescence emission were never detected in control samples.
Data were obtained and reported in graphs essentially according to standard procedures described by Stephan [14] and Harumoto *et al.* [9]. Each value is the mean (± SE) of three replicated independent experiments.

3. Results and discussion

In this study, WCB biosensors have been obtained by transfecting cells of *Tetrahymena thermophila* with the circular vector pD5H8 containing, as reporter gene, the coding sequence of the GFP [3] under the control of a homologous, stress inducible *hsp70* promoter. By this way, a fluorescent bioreporter cell line able to reveal a general condition of stress was obtained. The choice of the *hsp70* promoter is sustained by the consideration that this protein represents a key regulator of the universal cellular process known as heat shock response and its expression is promptly induced by cells in response to various stress signals [10]. Hence, *hsp* gene expression represents a rapid, sensitive and prognostic marker of the presence of even sub-lethal levels of chemical and/or physical environmental stressors. As diagrammed in *Figure 1*, following exposure to a toxic agent(s), the recombinant *Tetrahymena* cells become increasingly fluorescent as GFP accumulates.

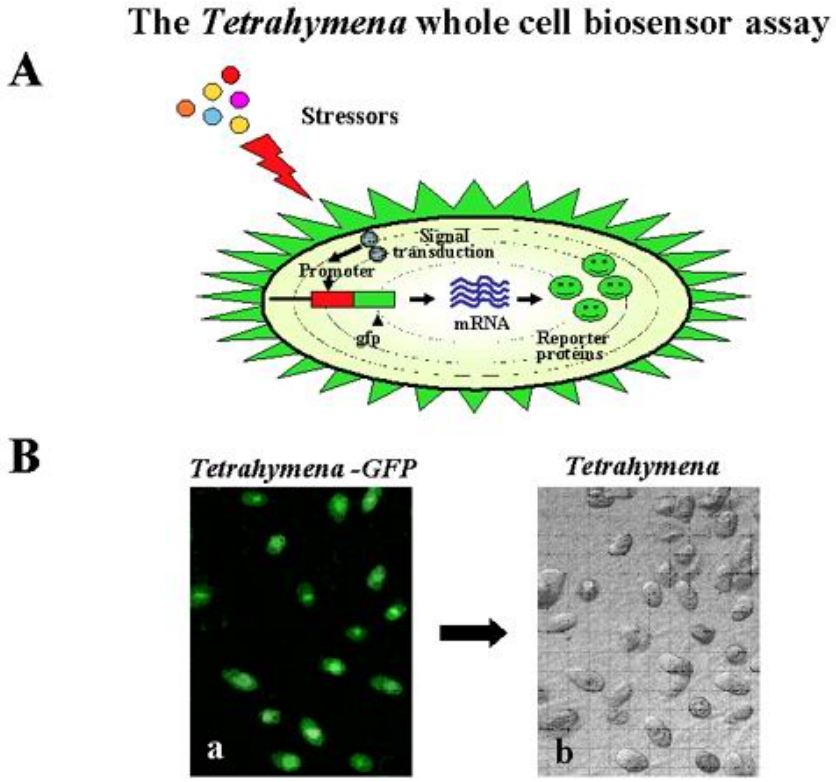

Fig.1 The *Tetrahymena* whole cell biosensor assay.
A) Representation by cartoon of the molecular events that elicit the *hsp70-gfp* gene induction following environmental stress exposure.
B) Recombinant *Tetrahymena* cells observed at the microscope after the exposure to stress: in a), cells showing *gfp* induction following stress and in b), the corresponding image in bright field.

In general, this protein is an ideal reporter for whole cell biosensing systems since it is autofluorescent; it has high stability and quantum yield [3]. In this particular case, the codon usage of the *GFP* gene has been optimised for *Tetrahymena* by site directed mutagenesis (kindly provided by Turkewitz A., from University of Chicago). Thus, in this assay the GFP fluorescence emission represents the toxicity endpoint.
In this study, the *T. thermophila* biosensor assay, as well as the more classic lethality test performed with the same organism, were used for the assessment of the potential toxicity of soil elutriates coming from three agricultural farms located in the province of Pavia in Lombardia (Italy). The selected farms, known

as Cascina Orsini, Cascina Nuova and Cascina Novella, are under different agricultural management systems. In particular, Cascina Orsini is an organic farm, Cascina Nuova employs conventional agricultural practices and animal manures as amendants, and finally, the agricultural fields of the farm indicated as Cascina Novella are mainly amended with treated sewage sludge. For the toxicity analysis, we have used two sets of soil elutriates collected from each selected sampling site during different period of the year. Two separate sampling were performed in different seasons and a set of elutriates was obtained for each sampling. The first sampling was realized during autumn and, the second one during summer. The results of the toxicity assays for each set of elutriates, obtained by means of both *Tetrahymena* biosensors (EC_{20} fluorescence) and lethality tests (LC_{20}) are reported on the graphs of *Figure 2* where, the upper and bottom panels show the toxicity data for the autumn 2004 and the summer 2005 sets, respectively.

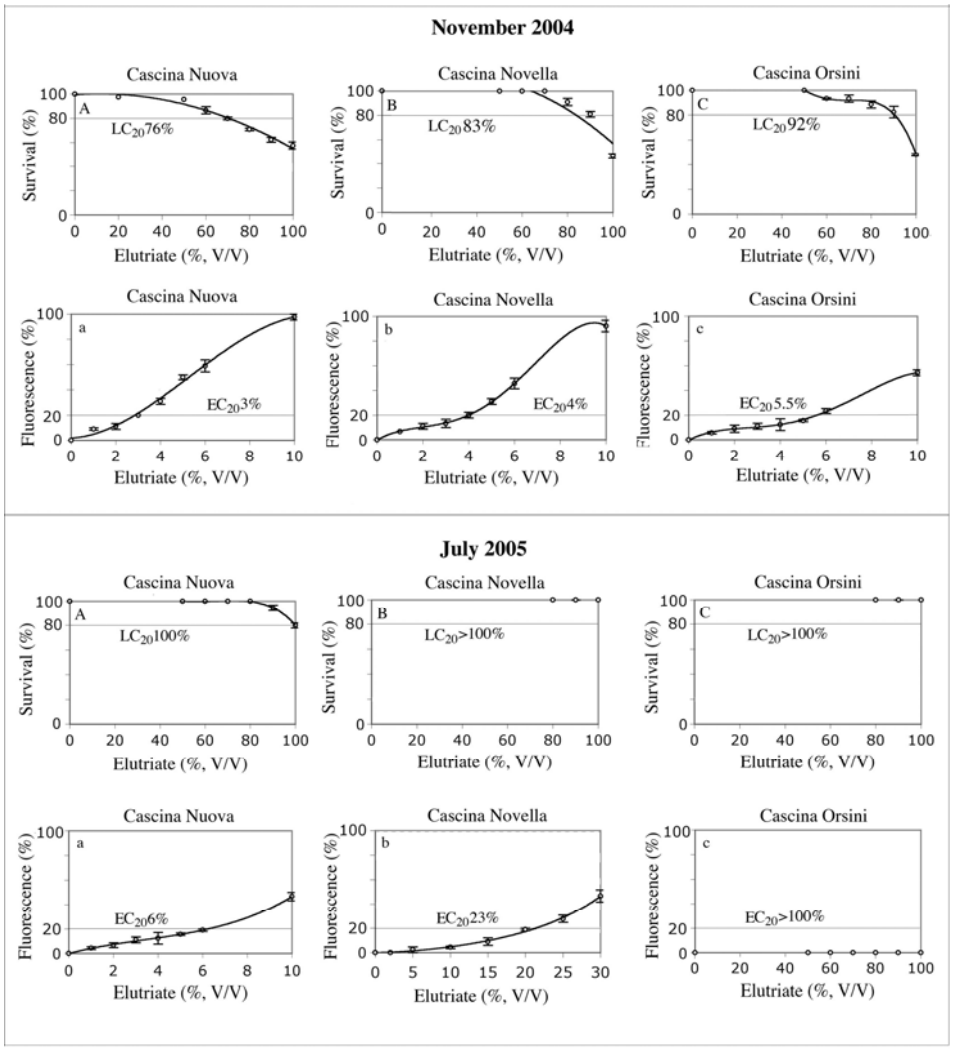

Fig.2 Toxic effects of elutriates on *T. thermophila* cells are expressed as LC_{20} in (A, B and C), and as EC_{20} in (a, b and c). The upper panel shows the toxicity data on the elutriates collected in November 2004; the bottom panel shows the toxicity data on the elutriates collected in July 2005. Each value is the mean (±SE) of three replicated experiments.

The data were expressed as LC_{20} and EC_{20} since the more conventional LC_{50} (50 % lethality concentrations) and the EC_{50} (50% fluorescence concentrations) values often resulted > = 100 (% V/V of elutriate), particularly in lethality tests. Therefore, the choice of LC_{20} and EC_{20}, that are more sensitive parameters, allowed us to more accurately evaluate the toxicity levels of the three type of soil elutriates and to make possible their relative classification.

At a first glance of the result analysis, the following

observations came out :1) moderate, low levels of toxicity were determined for almost all assayed soil elutriates by the use of both methods and, 2) differences in the toxicity levels between the two sets of elutriates clearly appeared.
Regarding the last observation, the toxicity level of the elutriate samples belonging to the autumn collection (November 2004) resulted to be higher than that showed by the summer samples (July 2005). This finding is not surprising since other authors [5] found a similar seasonal variation of soil toxicity; for one year they monitored soil toxicity of agricultural fields in the area of Mainz (Germany), a region highly charged by anthropogenic air pollution; they found the presence of low levels of toxicity in late summer that increased during autumn, reaching a peak in late winter that subsequently decreased again, during spring and summer. These authors developed a hypothesis of an airborne origin of soil pollutants that increased in the raining season and was then transformed into not harmful compounds by soil microorganisms.
The seasonal variations recorded by our assays might be related also in this case, to the location of the three monitored farms in Pianura Padana, which is a region that from the point of view of the air pollution very closely resembles to that of Mainz.
Lethality and biosensor assays showed a clear divergence in their respective sensitivities in most of the set analysis. As summarized in *Table 1*, the more toxic autumn set displayed LC_{20} values ranging from 76% of elutriate concentration for Cascina Nuova samples to 92% for Cascina Orsini samples, whereas the EC_{20} values obtained by means of the *Tetrahymena* biosensor assay range between 3% of elutriate concentration for Cascina Nuova and the 5.5% for Cascina Orsini samples. In the less toxic summer set, the LC20 value results to be 100 % for Cascina Nuova and higher than 100% for the other farms. For the same set of sample the EC_{20} values results to be 6% and 23% for Cascina Nuova and Cascina Novella respectively and higher than 100 % for Cascina Orsini. The LC_{20}/EC_{20} ratio highlights the higher sensitivity of the biosensor test over the lethality tests in revealing sub-lethal concentration of toxicants. The LC_{20}/EC_{20} ratio ranges from about 25 to 16.7 for both set of elutriates and was not measurable (nc) for the Summer elutriates of Cascina Novella and Cascina Orsini since both samples showed LC_{20} values higher than 100 and in the case of Cascina Orsini also EC_{20} values were higher than 100.
All together these data suggest that in the presence of low levels of toxicity, the bioreporter assay allows a better evaluation of the toxicity displayed by the different elutriate samples with respect to the lethality assay.

Table1 - Comparison of the LC_{20} and EC_{20} values obtained for the November 2004 and July 2005 elutriates. The LC_{20}/EC_{20} ratio highlights the higher sensitivity of the fluorescence (EC_{20}) over the lethality (LC_{20}) tests.

November 2004 Elutriates	LC_{20} (% V/V)	EC_{20} (% V/V)	LC_{20}/EC_{20}	July 2005 Elutriates	LC_{20} (% V/V)	EC_{20} (% V/V)	LC_{20}/EC_{20}
Cascina Nuova	76	3	25.30	Cascina Nuova	100	6	16.67
Cascina Novella	83	4	20.75	Cascina Novella	>100	23	nc
Cascina Orsini	92	5.50	16.73	Cascina Orsini	>100	>100	nc

To conclude, independently from the sampling periods, the descending order of toxicity revealed by both assays in the three farms was the following:

Cascina Nuova > Cascina Novella > Cascina Orsini

Also, the toxicity of the samples appears very low and totally absent in the summer elutriates of Cascina Orsini.

4. Concluding remarks

In this study, we developed and showed the validity of the *Tetrahymena* bioreporter assay for the assessment of the toxicity of soil elutriates coming from three agricultural farms under different agricultural management systems. Our genetically modified cells are capable to produce a fluorescent signal at concentrations significatively lower than those detected by means of the more conventional lethality tests performed with the same organism. Thus, this assay is particularly suited to unveil sub-lethal concentrations of toxicants even in complex environmental samples such as soil elutriates and, consequently to furnish early warning data. Moreover, further advantages are offered by the fact that this assay allows the real time and *in-vivo* detection of the fluorescence**.**
We are still working in order to optimize the assay, mainly in terms of reduction of the processing time and to obtain a better quantification of the fluorescent signal. In particular, the simple use of a microtiterplate-fluorimeter could increase the technology of the system and facilitate the processing of larger numbers of replica samples within a single run and to allow the monitoring of the kinetic of the stress responses. With further refinements, we are confident that this rapid and cheap bioassay could be applied to routine monitoring of a large number of complex environmental samples.

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Nematode communities in three differently managed agricultural fields

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We sampled three sites, located in Pavia Province (northern Italy), under different agricultural practices, named "biological", "sewage" and "manure". The aim of this study was to value the soil health using nematode communities as bioindicators. The community of the biological-managed site community had the highest taxonomic and trophic diversity and maturity. The food web resulted fairly good structured and this site was in better conditions in comparison with the others. The sewage-managed site had the highest enrichment condition, but the lowest nematode density. Finally, the community of the manure-managed site was the worse and the least diversified and structured, being dominated by the plant feeders, especially by genus *Paratylenchus*, whose high density can be related to ecological degradation.

1. Introduction

Soil biocoenosis includes a vast diversity of organisms depending on each other for carbon and energy. Among these organisms, microbes, mainly bacteria and fungi, are directly involved in organic matter decomposition and nutrient cycling. Other organisms, nevertheless, are also important in soil ecosystems, because they significantly affect microbial activity through trophic relationships [60] and control populations of the lower trophic levels. The organisms grazing on bacteria and fungi contribute to increase the availability of nutrients for plants, otherwise immobilized in the microbial biomass [20, 39]. This function, carried out mainly by protozoa and nematodes, is crucial for plant production and, thus, for the development of sustainable agriculture and forestry [55]. Many environmental factors and human disturbances affect soil ecosystems [30, 12, 25, 54, 56, 41]; particularly in agricultural systems these disturbances are tillage, amendments and pesticides [25, 44, 41]. Intensive agricultural practices can have local negative consequences, such as a lower soil fertility and a reduced biodiversity. These changes mean also a profound alteration of biological regulation and nutrient availability [40, 26]. To preserve the soil health, practices to achieve a sustainable agriculture were developed. Substitution of synthetic compounds with organic matter in agricultural management led to better soil properties [57, 2, 49].

Many researches used soil organism as bioindicators of soil quality [65, 9, 1, 36, 51]. Among soil organisms nematodes have propitious characteristics to monitor the environmental conditions [8, 63] even better than indices based on microbes, Collembola and mites [45].

Our work is part of the "BIO-BIO Project", whose aim is to value the soil health in three agricultural fields differently managed. This valuation would be principally done on the basis of the presence/absence of the inputs and the amendment type. In our case we used nematodes to value the effect of these practices on food web structure and soil processes.

2. Materials and methods

2.1 Study sites

We sampled three sites located in Pavia Province (northern Italy): two in Municipality of Bereguardo (Cascina Orsine and Cascina Nuova), 10 km north-west from Pavia, and the third in Municipality of Corteolona (Cascina Novella), 17 km south-east from

Pavia. In each sites one field was selected for different agricultural managements, as shown in *Table 1*.
Onwards, the fields of Cascina Orsine, Cascina Nuova, and Cascina Novella will be named "biological", "manure" and "sewage", respectively.

2.2 Sampling design

Soil samples for nematode analysis were collected on 21 September 2004, and 18 January, 22 March and 3 July 2005. For maize fields these samplings correspond to the period before harvest, after harvest, before sowing and growing respectively. Other fields were grown with grasses. In particular, at the "manure treated" site (Cascina Nuova) organic and mineral fertilizers were added before the samplings of 18 January and 22 March, respectively. In each field one representative plot (20 m x 20 m) was selected. From each plot 12 cores 0-15 cm depth were collected by a 2.5 cm diameter corer following a random pattern. These cores were mixed and then divided in three composite samples. Exactly 50 g of fresh soil from each composite sample was processed for successive nematode community analysis.

Table 1. Agricultural practices in the three different sites.

	Site		
	Bereguardo (biological)	Bereguardo (manure)	Corteolona (sewage)
Cultivation	2 yrs meadow	5 yrs meadow	maize
Ploughing	2 yrs before	5 yrs before	every year
Manure/ amendments	-	manure and mineral fertilizer	sewage sludge treated with NH_3
Pesticides	-	herbicide every 5 yrs	herbicide

2.3 Soil analyses

Moisture and pH of all soil samples were measured. Moreover, 50 g of soil (fresh weight) were air-dried at room temperature for a week and then moisture was determined (percentage) by difference in weight between before and after drying. The pH was measured in soil: distilled water (ratio of 1:2,5 w/v) suspension after drying and sieving soil sample through a 2 mm mesh sieve.

2.4 Nematode analysis

Nematodes were extracted from 50 g of soil (fresh weight) of each composite sample using modified Bearmann method [33] for 48 h, then were fixed with 2% formaldehyde. Nematodes were counted at stereoscopic microscope (30X magnification). Their density was expressed as individuals per 100 g dry soil (nematodes x 100 g ds). About 100 randomly chosen specimens were isolated, mounted on slides and identified usually to genus but in a few cases to family level. Nematodes were separated into feeding groups according to Yeates et al. [67] and c-p groups according to Bongers [4, 8]. In this system the c-p classification is based on ecological characteristics of taxa, such as life cycle, reproductive rate, colonization ability and tolerance to disturbance. Taxa are scaled 1-5 with colonizer and persistent at the extreme points. Bongers et al. [6] also distinguished two types of opportunistic nematodes: enrichment opportunists (c-p 1), which only develop under food-rich conditions and form dauerlarvae, and general opportunists (c-p 2), which prefer food-poor condition and are unable to form dauerlarvae.
Several ecological indices were calculated to describe diversity and structure of nematode community: Margalef species richness (d), Simpson's dominance index (λ), Shannon's diversity index (H') calculated on base *e*, Pielou's evenness (J'), Trophic diversity (T) [25], Nematode Channel Ratio (NCR) [70], Maturity Index (MI) [4], modified maturity indices ΣMI [68] and MI2-5 [5], Plant Parasite Index (PPI) [4], Enrichment Index (EI) [21], Structure Index (SI) [21], Bacterivore Index (BaI) [22], Basal Index (BI) [21] and Channel Index (CI) [21].
Indices T and NCR refer to the community trophic composition; in particular NCR allows for only bacterial-feeding and fungal-feeding nematodes to give an index of relative contribution of the two channels to overall decomposition.
The maturity indices are based on c-p scale of nematode taxa. These values are the weighted means of the c-p value of all nematodes present in community (ΣMI) or of only free-living (MI) or of only plant feeding (PPI) or of free-living excluding enrichment opportunists c-p 1 (MI2-5).
The last five indices are based on classification into functional guilds [7] combining feeding groups [67] and c-p scaling [4]. The EI and SI provide location of the food web along an enrichment and structure trajectory respectively [21]. Precisely EI reflects eutrophication, while SI is linked to the food web complexity. They are independent one another and both contribute to define the faunal profile that can be represented in a two dimensional graph, in which horizontal axis is structure trajectory and vertical axis is enrichment [21]. The BaI expresses weighted abundance of general opportunist with respect to overall bacterial feeding opportunists (c-p 1 and 2) [22]. The CI is the weighted abundance of fungal feeders among the opportunistic nematode grazers on fungi and bacteria (Fu_2 and Ba_1 guilds) [21].

2.5 Statistical analyses

Soil parameters measures, nematode densities, trophic groups' frequencies and indices values were examined

by multifactor analysis of variance (ANOVA) to prove influences of sampling date and type management. Furthermore, interaction between two factors was investigated. Differences with P<0.05 were considered significant. One-way ANOVA was used to test differences among soil parameters, nematode densities and trophic group frequencies of the samples from three plots in each single sampling. When P<0.05, differences were considered significant and Duncan's multiple range test was applied to highlight these differences. For trophic group analysis, when ANOVA was not possible, Kruskall-Wallis test was used.

The similarity level among all samples was evaluated by Bray-Curtis coefficient [10] on the basis of taxa composition. Then Cluster Analysis was carried out by PRIMER software [15]. Frequencies of the taxa were square root transformed. Appling a descriptive factor to samples, an Analysis of Similarity (ANOSIM) [13] was carried out on Cluster Analysis result to examine if differences among samples characterized by different factor were significant. This analysis gives an R value in Global test and an R value for each pair groups comparison in Pairwise test. The R values range from 0 to 1, so the more values are close to 1, the more significant are differences. By the Similarity Percentages Analysis (SIMPER) [14, 15] it is possible compute the average similarity between all pairs of intra-group samples and discriminate the species more responsible of similarity within cluster.

Furthermore, Principal Component Analysis (PCA) was performed with Statgraphics Plus version 5.1 software [47] to ordinate samples on the one hand on the base of taxa composition and on the other hand on the base of indices values. The sample points' positions of three different groups were compared by ANOVA test and Duncan's multiple range tests, if it is possible, or by Kruskall-Wallis test.

3. Results

3.1 Soil pH and moisture

Both parameters were influenced by two factors, namely sampling and agricultural type management. In *Table 2* results of multifactor ANOVA are reported. The factors strongly interact to one another.

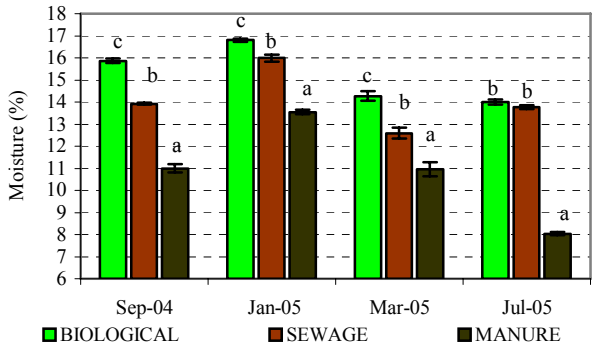
Regarding mean values during overall sampling period, biological-managed and manure-managed plots had the highest (15,2% ±0,4 SE) and the lowest (10,9% ±0,6 SE) values of moisture, respectively (Duncan's multiple range test, P<0.05). Instead biological-managed plot had the lowest values of pH (5.7), while the sewage-managed had the highest (6.4) (Duncan's multiple range test, P<0.05).

Table 2. Multifactor ANOVA for soil parameters in three different-managed plots and for four sampling. Stars beside F-test values means significant factor effect (=P<0.001; **= P<0.0001).*

	F-test		
Parameters	Sampling	Management type	Factors interaction
pH	16.6**	55.0**	8.2*
moisture	252.5**	731.1**	33.0**

In the *Figure 1a and 1b* the trends of two soil parameters are depicted. The values measured at each sampling in the differently-managed plots were compared by means of Duncan's multiple range test; the results are reported in *Figure 1a and 1b*. Differences among moisture (*Figure 1a*) values in the different plots remained in the same arrangement in all samplings.

a)



b)

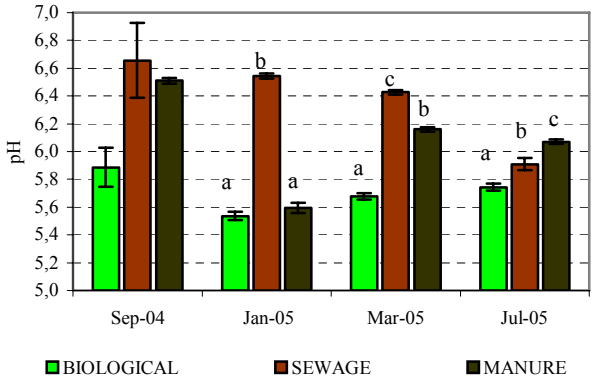


Figure 1. Mean values and relative standard error of soil parameters: a) moisture and b) pH. Different letters on the columns mean significant differences according to Duncan's test (P<0.05).

The pH was rather steady in biological-managed plot, while it changed a lot in other plots. In manure-managed plot it sharply decreased from September 2004 to January 2005 after manure application.

3.2 Nematodes

3.2.1 Density and taxa composition

Both sampling and agricultural type management factors had influenced nematode densities (ANOVA F=14.9, P<0.0001 for sampling; F=109.9, P<0.0001 for agricultural management). Furthermore, the effect of agricultural practices was different for each plot in the same sampling date (factor interaction in ANOVA F=3.8, P<0.01). Nematode density was low in the sewage-managed plot and high in the manure-managed plot, except in the first sampling (Duncan's test P<0.05 or Kruskall-Wallis P<0,05) (*Figure 2*). Annual mean densities were 861(±42 SE) in the biological-managed plot, 341(±68 SE) in the sewage-managed plot and 1069(±150 SE) in the manure-managed plot.

During the whole sampling period, the nematode abundances had small fluctuations in the biological-managed plot. Instead, these changed significantly in the sewage-managed plot (ANOVA F=58.8, P<0.0001). The maximum number was reached in January (*Figure 2*). Density in the manure-managed plot showed a considerable increase from September to January, then had remained steady (*Figure 2*).

Forty genera were identified during study. This number also includes rare genera present at only one sampling date. Thirty-one genera belonging to 24 families and 8 orders were extracted from samples of biologic-managed plot, 32 belonging to 27 families and 9 orders from those of sewage-managed plot and 30 belonging to 22 families and 9 orders from those of manure-managed plot. Twenty genera only were common to all three plots. Considering common genera only (frequency over 5%) for each plot, there were 8 genera in biologic-managed plot, 6 in the sewage-managed and 4 in the managed manure, which form 73%, 72% and 70% of the whole community, respectively. These common genera are shown in Table 3.

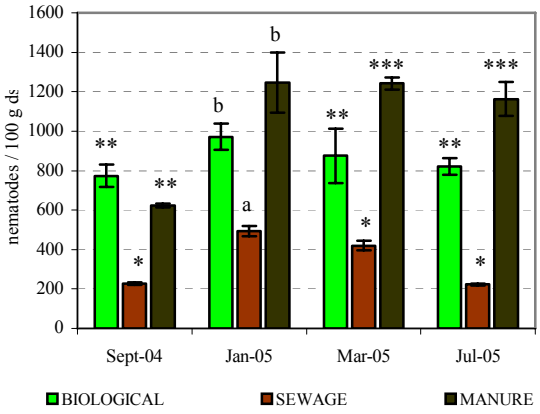


Figure 2. Nematode densities (nematodes/100 g dry soil) in the three plots for all samplings date. Columns show mean values and relative standard errors. Distinct number of stars or letters on the columns mean significant differences according to Duncan's test (P<0.05) or Kruskall-Wallis test (P<0,05) respectively.

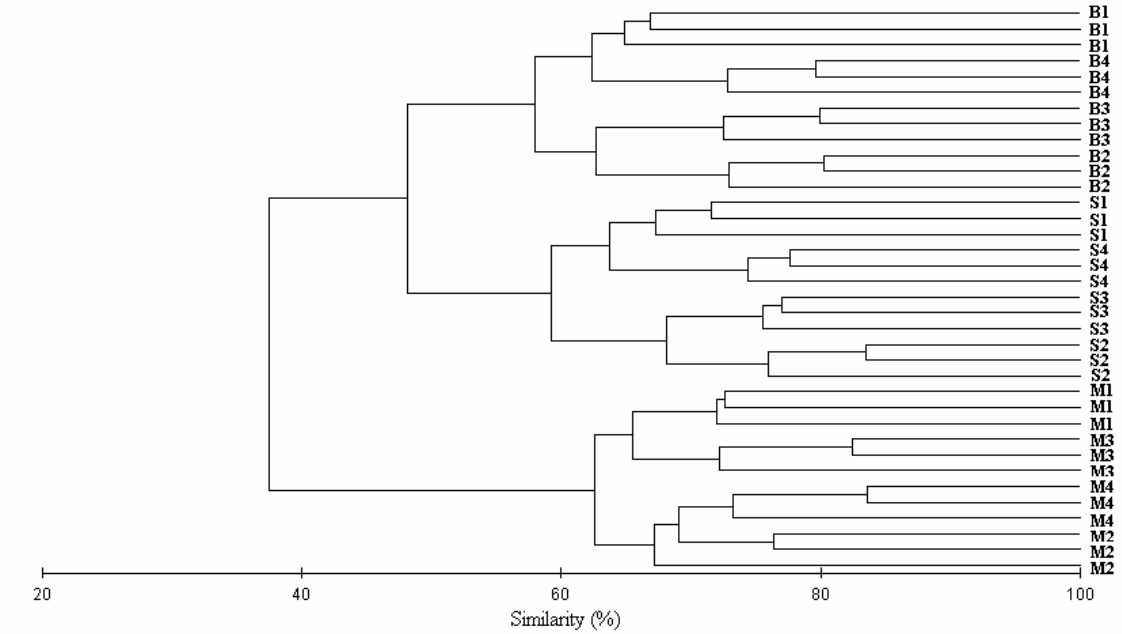


Figure 3. Cluster Analysis using group-average linking on Bray-Curtis genera similarity. Labels: B = samples from the biological-managed plot; S = samples from the sewage-managed plot; M = samples from the manure-managed plot. Numbers after label letters: 1= September 2004; 2 = January 2005; 3 = March 2005 and 4 = July 2005.

The cluster analysis is reported in *Figure 3*. All samples collected from the same plot during the whole sampling period are grouped into one cluster, so in the dendrogram three well-defined clusters are evident. According to the similarity level, samples from manure-managed plot are more different from the others. This first division takes place at similarity level lower than 40%. Instead, the other two groups have a similarity around 50%. The sample collected at the same sampling are combined in the same sub-cluster.
The ANOSIM showed the effective separation of management types (Global test R=0.94). In pair wise comparisons the samples from manure-managed and from sewage-managed plots were the least similar (R=1,00), while the samples from biologic-managed and from sewage-managed plots were the most similar (R=0.81).

Table 3. Mean relative abundances of the main genera in the three plots. In bold the > 5% values.

taxa	Biological	Sewage	Manure
Acrobeloides	**6.3**	1.9	4.1
Aphelenchoides	**7.0**	**7.1**	2.8
Cephalobus	**5.1**	**7.9**	1.7
Ditylenchus	3.7	**11.1**	1.7
Filenchus	**7.2**	**8.5**	1.1
Geomonhystera	0.6	0.0	**9.0**
Helicotylenchus	**8.5**	2.5	0.1
Mesorhabditidae	3.6	**5.2**	0.7
Panagrolaimus	**7.8**	0.9	**8.6**
Paratylenchus	**15.7**	0.7	**45.8**
Prismatolaimus	**15.5**	3.9	0.1
Rhabditidae	2.0	**32.2**	**6.6**

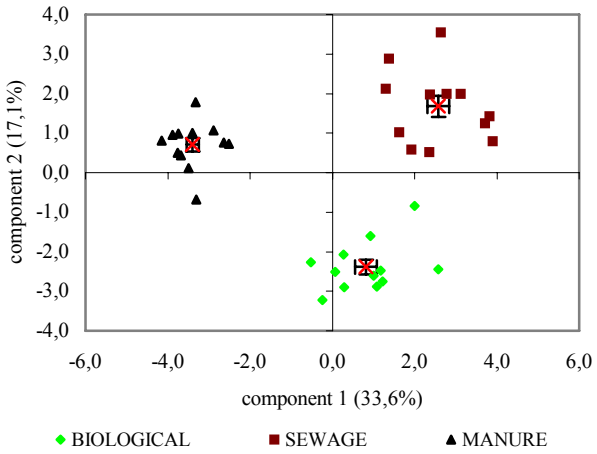


Figure 4. Scatter plot for first two principal components, which encompass 57% of the total variation in the sample points of the three different plots. Red crosses and error bars are the centroids and standard errors of the three sample groups.

By SIMPER analysis, similarity between samples within same cluster amounted to 62% for biological-managed plot, 66% for sewage-managed plot and 64% for manure-managed plot. The genera more responsible of these similarities were in order of importance: *Prismatolaimus*, *Helicotylenchus* and *Filenchus*, to which traced back 34% of similarity of the samples in biological cluster. Rhabditidae, *Ditylenchus*, *Cephalobus* and *Filenchus* were responsive of 56% of samples similarity in sewage cluster. Finally, *Paratylenchus* and *Geomonhystera* were responsive of 38% of samples similarity in manure cluster.
The PCA confirmed the differences among the three plots according to the taxa composition. In two dimensions scatter plot for first two principal components the samples from the same plot resulted much closer together than each other sample (*Figure 4*). For the three groups centroid was calculated and is shown in *Figure 4*.
Successive statistical analysis highlighted significant differences among all three groups along both axis (ANOVA F= 176.6, P<0.0001 and Duncan's test P<0.05 for first component and ANOVA F= 99.3, P<0.0001 and Duncan's test P<0.05).

3.2.2 Trophic composition

The abundances of the trophic groups, except for those of predators, were affected by both sampling date and management type (*Table 4*). The dynamics of the trophic groups was quite different in the three plots as significant interaction between factors proves (*Table 4*).

Table 4. Two-factor analysis of variance (ANOVA) for trophic groups (BF=Bacterial feeders; HF=Hyphal feeders; PF= Plant feeders; OM= Omnivorous; PR=Predators) in three different-managed plots and for four sampling. Stars mean significant factor effect (= P<0.05; **= P<0.01; ***=P<0.001; ****= P<0.0001).*

	F-test		
Trophic group	Sampling	Management type	Factors interaction
BF	696.0****	1566.0****	16.6****
HF	6.0***	14.3***	4.3**
PF	29.9****	176.7****	26.7****
OM	3.6*	11.8***	4.6**
PR	0.3	4.1*	0.4

In the three plots nematode fauna fundamentally consists of bacterial, hyphal and plant feeders. Predators were absolutely rare during the whole period (*Table 5*). Bacterial feeders peaked in the sewage-managed plot with the exception of the last sampling date, when they were more abundant in biological-managed plot (*Table 5*). In sewage-managed plot the number peaked in January; this increase was related to

Rhabditidae. In the biological-managed plot bacterial feeders were mainly *Prismatolaimus* and *Panagrolaimus*, two of the most abundant genera in this plot as described above (Table 3). The second was responsible of the final peak.
The manure-managed plot had the lowest frequencies of bacterial feeders (mainly *Geomonhystera* and *Panagrolaimus*), while had the highest of the plant feeders (almost only *Paratylenchus*). *Paratylenchus* was well represented also in the biological-managed plot, but it was not so dominant (Table 3). The sewage-managed plot had the lowest frequencies of the plant feeders, but had the highest of the hyphal feeders (*Aphelenchoides* and *Ditylenchus*). At the last sampling there was an increase of relative abundances of *Filenchus* and *Heterodera* juveniles, moreover *Psilenchus* and *Paratylenchus* were detected.
Omnivorous were abundant only in the biological-managed plot in January.

Table 5. Mean relative abundances of the trophic groups in the three plots. Significant P-values for ANOVA or Kruskal-Wallis test. Values followed by a different letter within a row differ significantly in Duncan's test (P<0.05) and those followed by a different numbers of stars differ significantly in Kruskal-Wallis test.

		Plot			
Trophic group	Sampling date	Biological	Sewage	Manure	P-value
Bacterial feeders	Sep-04	35.4**	60.6***	16.3*	0.027
	Jan-05	40.8^{a}	73.6^{b}	61.2^{b}	0.006
	Mar-05	50.7^{b}	61.8^{c}	40.8^{a}	0.006
	Jul-05	67.5^{c}	43.7^{b}	30.1^{a}	0.000
Hyphal feeders	Sep-04	24.0	17.2	8.6	-
	Jan-05	5.3*	17.2**	10.3*	0.039
	Mar-05	21.2**	28.8***	8.1*	0.027
	Jul-05	6.0^{a}	18.0^{b}	8.3^{a}	0.002
Plant feeders	Sep-04	39.1^{b}	18.2^{a}	73.4^{c}	0.000
	Jan-05	45.4^{c}	4.5^{a}	26.2^{b}	0.000
	Mar-05	25.7^{b}	7.3^{a}	49.3^{c}	0.000
	Jul-05	24.3^{a}	33.1^{a}	64.2^{b}	0.001
Omnivorous	Sep-04	1.0	3.5	0.9	-
	Jan-05	8.5^{b}	3.8^{a}	0.9^{a}	0.006
	Mar-05	5.6	1.1	1.1	-
	Jul-05	1.9^{ab}	4.3^{b}	0.0^{a}	0.044
Predators	Sep-04	0.5	1.1	0.8	-
	Jan-05	0.0^{a}	0.9^{ab}	1.4^{b}	0.024
	Mar-05	0.0	1.0	0.7	-
	Jul-05	0.3	0.9	1.7	-

3.2.3 Ecological indices

The indices were significantly different in the three plots, except *d* and NCR, as highlighted by ANOVA for management type factor (*Table 6*). The sampling factor affected 9 of the 15 selected indices and, in more pronounced way, H', λ and J', all based on the community structure (Table 6). The interaction between two factors was significant for indices, apart of PPI and SI (*Table 6*). Since the values of these indices were not affected by sampling factor, they were somewhat constant during the whole period.
Duncan's test calculated on indices mean values of all sampling dates highlighted that H', λ, J', ΣMI, MI2-5 and BI values differ among the three plots. The biological-managed plot has the highest values of MI and T. The sewage-managed plot had the highest of EI and the lowest of BaI and CI and that manure-managed plot had the lowest of PPI and SI (*Table 6*).
Thus almost all indices were able to point out differences between plots. According to these result it was difficult to rank indices in order of importance. Therefore an empirical approach for comparing the ability of each index to point out the differences between values obtained from each plot was attempted. The discriminating factor was calculated for each index summing the products of the number of the pair wise significant differences (obtained by Duncan's test) among plots at each sampling date and a correction

factor referred to ANOVA P-value in the same comparison. These factors are reported in Table 6. The order that achieved is the following: d< CI < SI < BI < PPI < NCR = EI < MI2-5 = BaI < T < MI = ΣMI < H' < λ < J'.

In the faunal profile (Ferris et al. 2001) the samples from three plots were located in the two upper quadrants (*Figure 6*). In detail, samples from manure-managed plot were located in the left quadrant, while the other in the right one. The points of the biological-managed plot were more scattered than the others. The sample points of the manure-managed plot were significantly different along structural trajectory (*Table 6*), while the sample points of the sewage-managed plot were significantly different along enrichment one (*Table 6*).

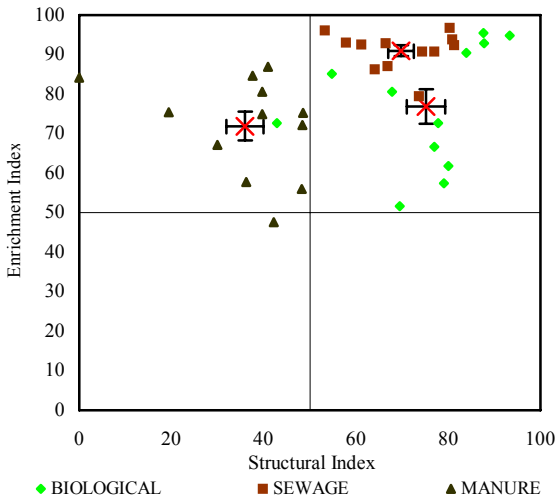


Figure 6. Faunal profile of three plots. Red crosses and error bars are the centroids and standard errors of the three sample groups.

4. Discussion

The soil pH and moisture are known to influence nematode community [16, 37, 53]. Although in this study they were different in the three plots, the pH and moisture values did not explain the different community structures. The main factors which affected nematode communities were, in fact, agricultural management practices. Many studies [66, 25, 19, 42, 44, 43, 11] already proved that cultivation, chemical application, tillage and amendment type influence structure and dynamics of nematode community. In this study all these conditions changed in each plot.

Nematode densities in biological and manure-managed plots were comparable to those of other agro ecosystems [25, 44, 46], but they were lower than those of natural undisturbed grasslands [17]. Instead, the sewage-managed plot has a small population. In another field, in Pavia province too, managed with sewage sludge and monitored in 2004 (unpublished study) density was five times higher. It was not possible to know if this depends on treatment with NH_3 subjected to sewage sludge, or its original characteristics, or on the background conditions in whole area. The NH_3 sewage concentration was unknown by us, so we could not know if this low density was related to a toxic effect as reported by Rodriguez-Kábana [50]. Furthermore, Šály [52] proved that some herbicides can reduce nematode population density.

Communities of the three plots had a similar number of genera, two third of which were in common, but the relative abundances were fundamentally dissimilar. This explains the assemblage in three well-defined groups of the samples collected from same plot by the Cluster Analysis and PCA. Both analyses showed a major similarity among biologic-managed and sewage-managed plots. The taxa more responsive of this result were also the most abundant in each plot.

The more representative genera in the biological-managed and manure-managed plot were the bacterial and plant feeders, while in the sewage-managed plot were the bacterial and hyphal feeders. In this last plot, the predominance of these trophic groups, in particular of the Ba_1 (bacterial feeders c-p 1) and Fu_2 (hyphal feeders c-p 2) guilds [7], was related to organic matter in soil through grazing on primary decomposers [32, 24, 59, 29]. However, the Ba_1 nematodes were more numerous than the Fu_2 in all sampling dates (dates not shown). Therefore, although the hyphal feeders were higher in the sewage-managed plot in comparison with the other plots, the CI value was the lowest (Table 6). This means that bacterial decomposition is higher in this plot, the resources are more easily available and N-rich [59] and the microbial activity are enhanced [64, 28, 27]. Ferris et al. [19] considered that the greater abundance of Ba_1 nematodes was to be related to soil fertility. Ferris and Matute [22] advised also that, for management of soil fertility in systems driven by organic input, the enrichment opportunistic bacterial feeders must be maintained at high levels by frequent supply of labile organic matter. In case of the sewage plot these considerations must be well-pondered because, although Ba_1 is the predominant guild, these nematodes are scarce in number.

The plant feeders were scarce in this plot. Many researches reported an inhibitory effect of organic amendment on the phytophagous nematodes [35, 23, 50]. The C:N ratio, soil environment and decomposer organisms are the main factors which determine the effectiveness of amendments in lowering the plant feeders' population [50]. Low C:N ratios appear to lower the plant feeders' density [58].

Table 6. Two-factor ANOVA for ecological indices value in three type-managed plots and for four sampling dates. Stars point out significant factor effect and factors interaction (= P<0.05; **= P<0.01; ***=P<0.001; ****= P<0.0001). In table are reported mean values on whole sampling period of ecological indices (d = Margalef's index; H' = Shannon's index; λ = Simpson's index; J'=Pielou's evenness; MI = Maturity Index; ΣMI = MI including plant feeders; MI2-5 = MI without holding c-p 1 class; PPI = MI for only plant feeders; EI = Enrichment Index; SI = Structural Index; BaI = Bacterivore Index; B = Basal Index; CI = Channel Index; T = Trophic diversity; NCR = Nematode Channel Ratio).Different letters beside mean values mean significant differences (Duncan's multiple range test P<0,05) In the second column there are discriminating factor values.*

		Plot			F-test		
Indices	Discriminating	Biological	Sewage	Manure	Sampling	Management type	Factors interaction
d	1	3,39	3,26	3,18	3,4*	0,8	4,0**
H'	21	$2,34^{c}$	$2,15^{b}$	$1,87^{a}$	8,1***	29,6****	17,5****
λ	25	$0,12^{a}$	$0,18^{b}$	$0,27^{c}$	9,1***	65,2****	37,8****
J'	27	$0,84^{c}$	$0,79^{b}$	$0,69^{a}$	9,18***	62,6****	30,5****
MI	14	$2,20^{b}$	$1,77^{a}$	$1,85^{a}$	0,9	35,3****	11,0****
ΣMI	14	$2,23^{c}$	$1,83^{a}$	$1,95^{b}$	6,0**	44,1****	8,7****
MI2-5	12	$2,57^{c}$	$2,38^{b}$	$2,19^{a}$	1,8	26,2****	5,2**
PPI	10	$2,31^{b}$	$2,26^{b}$	$2,08^{a}$	2,4	11,4***	0,9
EI	11	$76,8^{a}$	$91,0^{b}$	$71,9^{a}$	2,6	24,2****	10,2****
SI	7	$75,1^{b}$	$69,8^{b}$	$35,9^{a}$	0,1	34,7****	1,7
BaI	12	$28,8^{b}$	$10,3^{a}$	$33,8^{b}$	4,6*	21,3****	10,8****
BI	8	$22,5^{ab}$	$17,7^{a}$	$24,7^{b}$	4,3*	4,1*	2,8*
CI	6	$22,3^{b}$	$12,5^{a}$	$20,3^{b}$	10,7***	3,7*	6,4***
T	13	$2,5^{b}$	$2,3^{a}$	$2,1^{a}$	2,8	10,7***	15,9****
NCR	11	0,78	0,75	0,79	7,6**	0,8	4,1**

The biologic and manure-managed plots can be assimilated to low and high disturbed grasslands respectively, and to agricultural systems cultivated with grasses. Bacterial feeders and plant feeders are the predominant trophic groups both in natural or managed grasslands and in perennial herbaceous cropped systems [61, 25, 18].

In the manure-managed plot community was dominated by the plant feeders. The combined fertilization of manure and mineral amendments influenced their population dynamics. On one hand the application of manure can enhance the bacterial populations, and consequently the bacterial feeding nematodes [32, 25] to the detriment of phytophagous populations [35, 23, 50]. On the other hand the use of the mineral fertilizer is related sometimes with increase in the number of phytophages, following Kozłowska and Domurat [38], and sometimes with decrease of bacterivores [44]. We noted both dynamics. The manure was added just before the second sampling (January), when we noted a tremendous increase of nematode density and bacterial feeder's relative abundance. This pattern was more apparent considering their absolute number, increased from 102 to 760 nematodes per100 g dry soil. Simultaneously, plant feeders decreased from 457 to 327 nematodes per 100 g dry soil. Instead, at the third sampling (March), just following mineral fertilizer application, the plant feeders increased again to detriment of the bacterial feeders which decreased (Table 5). The increase of bacterial feeders was due mainly to Ba_1 nematodes. At the following sampling (March) general opportunist bacterivores were already more than enrichment opportunist.

In this plot the plant feeders belonged almost only to genus *Paratylenchus*. This genus can be super dominants in meadows undergoing degradation [63] and they may also indicate an altered status of vegetation cover [61]. Also Yeates [68] claimed that nematode fauna in droughty soils is often dominated by *Paratylenchus*.

In the biological-managed plot community had the highest trophic diversity (Table 6). Only in the last two samplings bacterivores significantly increased as regards phytophagous. In the last sampling date growth was due in particular manner to *Panagrolaimus*. That enhanced from 1,5% in March to 23,2% in July. Since *Panagrolaimus* belongs to Ba_1 guild [7, 21], the peak should be indicative of an increase of bacterial biomass related to an organic matter addition unknown for us. Excluding the last sampling, the Ba_2 guild was the most abundant during whole sampling period. This can

mean a low amount of available [7, 21] resource for the most part of year. This situation promoted the growth of *Prismatolaimus*, namely of persisted bacterial feeders [4, 8].
Among plant feeders in biological-managed plot there was *Paratylenchus* that seemed to characterize these grasslands. This genus was abundant also in abandoned grassland in two sites of Germany [18]. While in the manure-managed plot it included the almost all phytophagous, in biological-managed plot there were other two well-represented genera: *Helicotylenchus*, indicating a more stable habitat, [4] and *Filenchus* common in every soil sample [3].
Omnivores and predators had small percentage in all plots; however they were sensitive regarding pollutants and other disturbances [7].
The project wanted to value soil quality status of the three selected fields. Although taxonomic and, especially, trophic structure give respectively good information about diversity and status of soil processes, such as decomposition and nutrient mineralization [45], ecological synthetic indices seem to be more suitable tools to compare communities and express a judgment [63].
A discriminating factor was calculated to rank our indices. This approach was similar to that used by Ravera [48] to compare the ability of six diversity indices to discriminate between the stations and the sampling dates in a river monitoring. The first indices in our rank were those of diversity (namely H', λ, J') confirming that the differences in taxonomic structure among the three plots were strong.
Although the diversity indices had a major discriminating power, they do not weight taxa according their qualitative characteristics as indices specific for nematode community do [45]. Among these, MI and ΣMI were the first for importance. They showed a more mature community in the biological-managed plot; ΣMI distinguished also the manure-managed plot from the sewage-managed. Bulluck III et al. [11] observed that ΣMI expressed better than PPI or MI the differences in nematode communities of differently fertilized. They inferred that ΣMI was more sensitive to changes in trophic structure.
The sewage-managed plot had the less mature community; this was related to greatest relative abundance of c-p 1 class. This nematodes were responsible also of EI and BaI values significantly higher and lower respectively than those in others plots (Table 6); these mean a greater enrichment status of the food web [21]. The mean MI value on the one hand was comparable to those calculated for different treatments of annual crops in Michigan [25]; on the other hand it was less than those obtained for four crop management systems in Nebraska [44]. The mean MI values calculated for biological and manure-managed plots were less than those obtained in three Welsh grasslands [69] under conventional and organic regimes. The values were much more different than those calculated for nine years undisturbed grassland in South Bohemia, Czech Republic [31].
Excluding enrichment opportunists, the MI2-5 referred to a stressed nematode community in our manure-managed plot, while in the sewage-managed plot community was more stable. This index is useful to value the effect of enrichment [37]. Furthermore, Wasilewska [63] claimed that MI2-5 does not record short-term changes like those due to addition of nitrogen fertilizer and/or organic matter. So it can be an indicator of long-term ecosystem conditions.
According to Bongers [4] the PPI values seem to be positively related with plant primary production and, hence, with soil fertility. On this basis, our manure-managed plot was the plot in the worse condition. This was confirmed by the dominance of generalist opportunists (c-p 2) over enrichment opportunists among bacterivores.
Although many conclusions about soil ecosystem condition can be drawn, ecological indices do not provide an absolute measure, but they require a reference system represented by the community of a putatively undisturbed site [34]. Even if a reference system was at our disposal, its validity should be limited, because indices values can change with geographic regions and ecosystems [46]. Therefore, in our case, to express a quality judgment for the plots, we should have at our disposal a reference community of plain soils of northern Italy. Such a reference is lacking.
So we limited to compare our studied plots. The guidelines for specific diagnostics and expected condition of soil food webs suggested by Ferris et al. [21], based on sample point position in the faunal profile, can help to draw the conclusions. In our study, the sample point of manure-managed plot was located in upper left quadrant (Quadrant A according to Ferris et al [21]), while other two groups in the upper right (Quadrant B). Therefore, the manure-managed and the biological-managed plots can be assimilated to manage grassland disturbed and structured, respectively, and the sewage-managed plot to annual crop agriculture structured. All plots are N-enriched [21].

5. Conclusions

Summarizing in this study we observed that:

1. All three plots had N-enriched resources and bacterial-dominated decomposition channels, especially the sewage-managed plot.
2. In the sewage-managed plot the community was dominated by bacterial feeders, mainly Ba_1 guild. This seems to indicate soil fertility. Therefore, this

positive aspect must be pondered on the basis of the scarce nematodes density. The community has also a fairly good structure.

3. In the manure-managed plot, chemical fertilization and organic matter addition seemed to have an opposite effect on community dynamics. The excess of *Paratylenchus* among plant feeders seems to indicate degradation [63]. Also the PPI value can be related to lower fertility in comparison with the other two plots. Moreover, the community was little mature and structured.
4. Finally, in the biological-managed plot the community had the highest taxonomic and trophic diversity and maturity and had a fairly good structure.

In conclusion, according to our considerations on nematode community status, our plots can be arranged in this order: Biological > Sewage > Manure.

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Soil Nematodes as Bio-Indicators of Soil State: Limits and Prospects

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This report focuses on the often overlooked, but fundamental difference between (1) proving "forward" the effects of environmental or anthropogenic factors on soil populations, and (2) inferring "backward" the operation of such factors in the soil from changes in soil populations. Numerical simulations are used to illustrate that the forward detection of impacts on soil populations can be achieved with a standard sampling design, whereas the backward prediction of impacts from soil populations requires extraordinary effort in order to obtain a reasonable level of accuracy. The nematological analysis of soil health in differently managed agricultural fields by B. Biagini and A. Zullini is briefly discussed as an excellent example of forward bio-indication. Suggestions are made how to handle the almost inevitable lack of replicated management regimes on different sites.

1. Introduction

Amongst soil organisms, nematodes are seen as the most promising candidates for bio-indication of soil status (Cortet et al., 1999; Achazi, 2002). Using the well established classifications of nematode feeding types and cp-groups as well as various indices of nematode community structure (Yeates et al., 1993; Bongers and Bongers, 1998; Ferris et al., 2001), researchers have consistently exploited nematodes to investigate the propagation of broadly defined disturbance effects and fertilization effects through the soil ecosystem (Freckman and Ettema, 1993; Villenave et al., 2001). In addition, it has repeatedly been shown that soil nematodes respond differentially to xenobiotic substances (Bongers et al., 2001; De Nardo and Grewal, 2003; Jonker et al., 2004; Ekschmitt and Korthals, in press). During the last decade, approximately 170 papers were published in international journals, where nematode indices were used to evaluate and indicate the status of soils and sediments.

In this report, two aspects of soil bio-indication shall be illustrated, which emerged from the wealth of previous work on nematode bio-indication, namely (1) the discrepancy between "*forward*" and "*backward*" bio-indication, and (2) the quantitative accuracy achievable in soil bio-indication. The nematological investigation on soil health in differently managed agricultural fields by B. Biagini and A. Zullini is then briefly reviewed within this framework.

2. Forward and backward bio-indication

Expressed in the most general terms, bio-indication is based on a simple reverse conclusion. Because, e.g., some nematode groups show a negative numerical response to a specific pollution, it is concluded that conversely, a decline of susceptible nematode populations is indicative of this pollution. Empirical evidence has made it clear, that this reverse conclusion is not as straightforward as it may appear on first glance. One difficulty comes from the fact that the nematode populations under consideration will, most likely, be simultaneously subjected to other impacts than pollution, such as soil conditions, resource availability, disturbance, and interactions with other organisms. These "other" impacts may obscure or even overrule the pollution effects (Schratzberger et al., 2000; Kelaher et al., 2003). A second difficulty arises because, over time, nematode populations may be selected for tolerance towards repeated or permanent stresses, and may therefore change their response to such stresses (Millward and Grant, 2000). While pollution induced community tolerance (PICT) can be used to reveal previous exposure to pollutants, a PICT analysis always requires thorough calibration and comparison against unaffected control sites (Blanck, 2002). A third problem arises because some of the forces driving nematode populations may relate to events in the past, or to spontaneous intrinsic dynamics of the populations themselves, or may in some other

way withdraw themselves from analysis by the investigator. Therefore a relevant proportion of population variation may be left unexplained (Ekschmitt et al., 2003). As a consequence, the reverse conclusion, i.e., the indication of soil impacts from observations on nematodes, is generally less unequivocal than desired, and this is perfectly congruent with the rule of Aristotelian propositional logic that reverse conclusions do not generally hold.

To avoid possible conceptual confusion, it is proposed here to discriminate (1) proving *forward* the effects of environmental or anthropogenic factors on soil populations, from (2) inferring *backward* the operation of such factors in the soil from observations on soil populations. The next section provides a quantitative illustration of the difference between *forward* and *backward* bio-indication.

3. Quantitative accuracy of bio indication

It is a classic notion in the ecological literature that soil animals show an aggregated pattern in space. As a broadly generalised rule of thumb the spatial variance of soil animal species can be predicted according to the geometric distribution, which is a special case of the negative binomial distribution (Ekschmitt et al., 1997). This general rule, together with the observation that data of species groups tend to exhibit lower spatial variance due to stochastic compensation of the distribution patterns of individual species (Ekschmitt 1998), is used here to imitate bio-indication through numerical simulation. It is assumed that some kind of impact, which is scaled from 0 to 1, linearly reduces nematode abundance to 1/10 of its original value. 50 soil samples are taken along an impact gradient and the linear regression of impact strength versus nematode abundance is evaluated. *Figure 1* shows typical simulation results for three sampling scenarios: (a) impact on a single species, (b) impact on an ensemble of 10 species with identical response, and (c) impact on 10 species obtained from bulk samples composed of 10 sub-samples each. *Forward* and *backward* bio-indication is illustrated for each scenario.

The simplest scenario with a single species and no sub-sampling suffices already to statistically prove the impact effect ($p = 0.04$). The statistical proof is substantially strengthened in scenario (b) where 10 species are evaluated ($p < 0.001$). However, in both scenarios (a) and (b) the prediction of impact strength from nematode abundance is far beyond acceptable levels of accuracy. The 95% confidence range of prediction amounts to approximately ±0.5, which means that prediction uncertainty, is as large as the entire impact gradient, i.e. ranging from 0 to 1. Only if the sampling effort is radically increased does the uncertainty of *backward* bio-indication narrow down to a practically useful level. In scenario (c) with ten-fold sub-sampling, the 95% confidence range of prediction is reduced to ±0.2, which still means that only two or three levels of impact intensity can be discriminated along the impact gradient from 0 to 1.

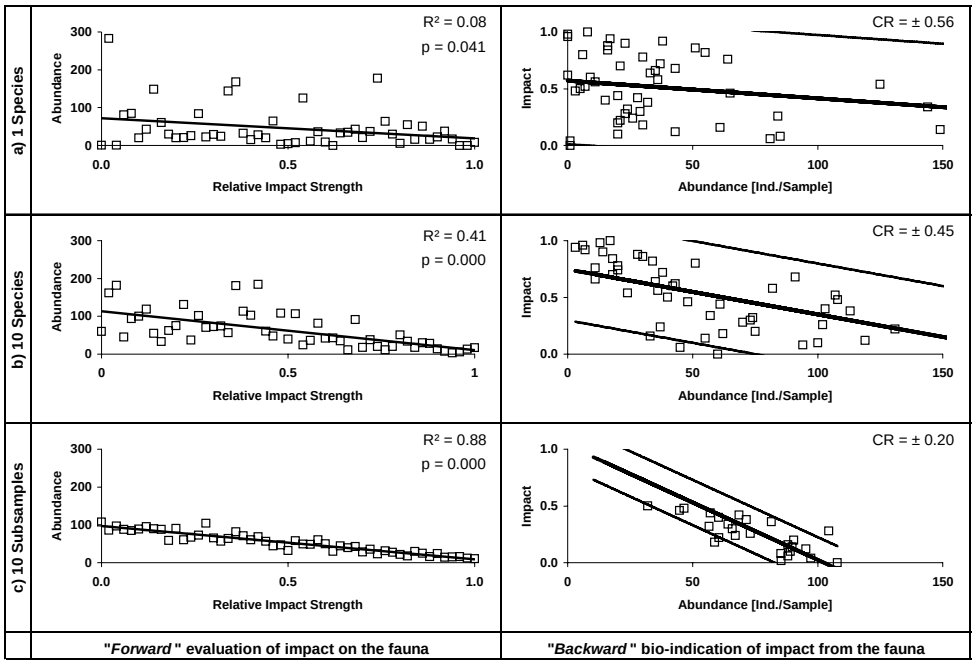

Figure 1. Examples of simulated bio-indication a) Evaluation of a single species, b) evaluation of an ensemble of ten species with coherent response to the impact, c) evaluation of ten species with ten-fold subsampling

In summary, the simulations depicted in *Figure 1* illustrate that the *forward* detection of impacts on soil populations can be achieved with a standard sampling design, whereas the *backward* prediction of impacts from soil populations requires extraordinary effort in order to obtain a reasonable level of accuracy. And it should be mentioned here that these results are equally valid for positive impacts, and for other soil organisms than nematodes.

4. The nematode project on soil health

It is therefore an excellent decision of the soil health project not to advocate a nematode index of soil health status, and instead to consistently stay in the *forward* mode of argument. The investigation on soil health in three differently managed agricultural fields exploits the functional diversity of nematodes together with the functional linkages of nematodes to other soil compartments to evaluate the impacts of agricultural practice on the soil ecosystem. A rich palette of nematode community parameters is evaluated to account for the multi-dimensionality of management effects, thereby creating a detailed picture of the differences between management types. A substantial difficulty seems less related to this nematological analysis than to the experimental design established in the research programme: there are no replicates of the same management regime on different sites. Obviously, a full factorial design of managements and sites is almost impossible to find in a real landscape. Nevertheless, the statistical consequences are severe because the experimental design itself does not enable a strict separation of management effects from site effects. To remedy this deficiency, the authors should clearly illustrate that the investigated sites were carefully selected to represent similar soils and climates, and the authors should consistently purify management effects from site effects by means of multivariate statistical analysis.

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Evaluation of the environmental impact of agricultural management practices using soil microarthropods

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The QBS indexes (QBS-ar and QBS-c) and the *Folsomia candida* soil test have been applied in order to evaluate the biological soil quality of three experimental sites, characterized by different agricultural regimen. The experimental sites were chosen in order to test the effects of a gradient of environmental pressure on the soil biological status (from biodynamic agriculture to conventional agriculture). The results however have probably been affected by a significant difference in soil characteristics among the sites and by the limited number of investigated sites; consequently, from the experimental data, it is difficult to rank the experimental sites in terms of biological soil quality.

1. Introduction

Soil fauna is an important component of soil systems because of its involvement in many aspects of organic matter decomposition, partial regulation of microbial activities, nutrient cycles and granular structure. Pollutants and other degradation factors can cause both quantitative and qualitative changes in fauna, which affect soil functioning (Bruce et al., 1997; Chauvat & Ponge, 2002; Gillet & Ponge, 2003). Use of soil bioindicators and test organisms may be helpful to detect environmental changes. Van Straalen (1998), in a review related to soil arthropod communities, specified that such bioindicators may play a role in soil monitoring measures.

The types of invertebrate soil fauna used in monitoring pollutant effects include nematodes, enchytraeids and other oligochaetes, gastropods, springtails, isopods, arachnids (Cortet et al, 2000; Parisi et al., 2005; van Straalen, 2004). Some species in a single taxon may be specified as indicators of soil quality or as test organisms and used in toxicology tests. In the collembolan taxon, *Folsomia candida* is the most frequently used species in both sub-lethal and lethal testing (et al., 2000; Crommentuijn et al., 1993; Crommentuijn et al., 1995; Hopkin, 1997; Trublaevich & Semenova, 1997; van Gestel & Mol, 2003). *Onychiurus armatus* (Bengtsson et al., 1985; Tranvik et al., 1993), *Orchesella cincta* (Joosse & Buker, 1979; Nottrot et al., 1987; Posthuma et al., 1992; van Straalen et al., 1987; van Straalen et al., 1989), *Isotoma notabilis* (Tranvik et al., 1993), *Sinella communis* (Greenslade & Vaughan, 2003), *Tetrodontophora bielanensis* (Gräff et al., 1997) and other collembolan species (Chauvat & Ponge, 2002) have been used in laboratory tests but have not reached the same level of routine use as has *F. candida.* Because of the species-specific differences in responses to contaminants, the tests conducted on *F. candida* provide partial indications as to the effects provoked by these substances on the collembolans; this information has also been useful to calibrate experiments on other species. Some collembolan species like *Folsomia quadrioculata, F. fimetariodes, Isotoma minor* and others species have been used to evaluate the effects of chemicals on collembola in field (e.g. Hopkin, 1997).

As it is known, changes in the concentration of some metals in the soil or food can modify the species diversity and the density of the Collembola. When not altering the density, they can still influence the biology and reduce the survival potential, the rate of growth and the reproduction of species more sensitive to these elements. In many cases this effect is dose-dependent. The growth rate and the survival of *O. armatus*

decreased significantly with increasing Cu and Zn concentrations (Tranvik et al., 1993); its survival was also lower when raised on a diet of fungus contaminated with Pb (Bengtsson et al., 1985). The growth response of *O. cincta* to zinc differed between the sexes (Posthuma, 1990). Growth appeared to be significantly slower in *O. cincta* fed with food containing lead (Joosse & Verhoef, 1983) or iron (Nottrot et al., 1987; van Straalen et al., 1987) and the duration of the moulting interval was found to be shorter in lead contaminated conditions (Joosse & Verhoef, 1983). In addition, variations in trace element concentrations in the soil can provoke effects on the fecundity of individuals. Tranvik et al. (1993) reported that reproduction in terms of eggs production in *O. armatus* was reduced by the presence of Cu and Zn.
The aims of this study were to evaluate the effects of three different agronomic management, (meadow under biodynamic agriculture, meadow under conventional agriculture, corn under conventional agriculture) on the soil microarthropods communities, by using the QBS approach (QBS: Biological Soil Quality). The effects of soils on the survival and reproduction of euedaphic *Folsomia candida* have also been evaluated.

2. Materials and methods

2.1 QBS-ar

The QBS-ar index is based on the following concept: the higher soil quality, the higher will be the number of microarthropod groups well adapted to soil habitats. QBS is applied to soil microarthropods, separated according to the biological form approach (sensu Sacchi and Testard, 1971), with the intention of: 1) evaluating the microarthropods' level of adaptation to the soil environment life (Parisi, 1974), and 2) overcoming the well-known difficulties of taxonomic analysis to species level for edaphic mesofauna. Edaphic microarthropods show morphological characters that reveal adaptation to soil environments, such as: reduction or loss of pigmentation and visual apparatus; streamlined body form, with reduced and more compact appendages (hairs, antennae, legs); reduction or loss of flying, jumping or running adaptations; reduced water-retention capacity – e.g. thinner cuticle, lack of hydrophobic compounds on the outer surface (Parisi, 1974).
Focusing on the presence of these characters, and not requiring the complex taxonomic identification to the species level, means that QBS analysis can be used also by non-specialists.
The main phases for obtaining QBS values are: 1) sampling; 2) microarthropods' extraction; 3) preserving the collected specimens; 4) determination of biological forms; 5) calculation of QBS index (Parisi, 2001; Parisi et al., 2005).

2.1.1 SAMPLING

In the study site, a representative area for soil sampling, homogeneous for slope and plant vegetation (if present), is delimited. It is recommended that the pedological profile be defined and to collect soil samples for chemical and physical analyses. Samples for QBS calculation have to be collected when soil moisture ranges between 40 and 80 % of field capacity. Above ground plant cover and the litter has to be removed; the soil is sampled within a 10 x 10 cm area, which is excavated to 10 cm depth. A square soil corer can be used if soil structure and tree roots allow this. The sample is placed in a plastic bag.

2.1.2 EXTRACTION OF MICROARTHROPODS

Soil samples are transported to the laboratory protected from thermal shock. A simple and cheap Berlese-Tullgren funnel (*Figure 1*) can be used for extraction. The soil core is carefully placed on the mesh above the funnel together with all the soil lost from sample during handling before inserting a bottle filled with preservative liquid (2 parts 75% ethanol and 1 part glycerol) beneath the funnel. The extraction system should be kept free from vibrations and other disturbance.

Figure 1--Berlese-Tullgren funnel, used for the soil microarthropods extraction

Extraction duration (never less than 5 days) will be proportionate to the soil sample water content, as determined by appropriate curve (Parisi, 1974). It will be slightly shorter for litter.

2.1.3 SPECIMEN PRESERVATION AND THE OBSERVATION

Extracted specimens are observed under a stereomicroscope at low magnification (range 5-100x; usually 20-40 x is sufficient) in the same preservative liquid (*Figure 2*).

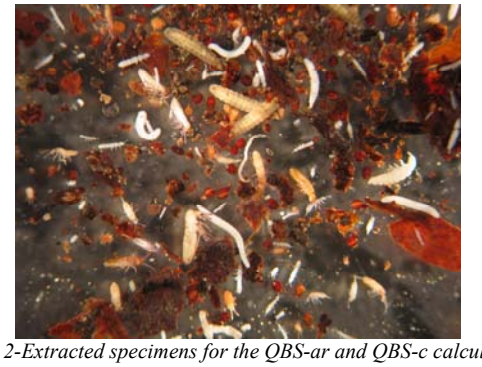

Figure 2-Extracted specimens for the QBS-ar and QBS-c calculation.

2.1.4 DETERMINATION OF BIOLOGICAL FORMS AND CALCULATION OF QBS INDEX

To define biological forms present in a sample means to recognize the different adaptation levels to soil environment for every systematic group. Within each higher taxon, QBS method requires searching for the biological form (morpho-type) that is most adapted to soil. This type will receive an Eco-Morphological score (EMI), proportionate to its adaptation level (Parisi et al., 2005). As a general rule, eu-edaphic (i.e., deep soil-living) forms score an EMI=20, hemi-edaphic (i.e., intermediate) forms are given an index rating proportionate to their degree of specialization, while epi-edaphic (surface-living) forms score EMI=1. Some groups have a single EMI value: e.g. Pauropoda (*Figure 3*), pseudoscorpion (*Figure 4*) and Diplura (*Figure 5*) EMI=20, because all species belonging to these groups show a similar level adaptation to soil. Other groups display a range of EMI values (e.g., for Collembola and Coleoptera, EMI=1-20), because these groups have species with different soil adaptation levels. Whenever two eco-morphological forms are present in the same group, the final score is determined by the higher EMI. In other words, the most highly adapted microarthropods belonging to a group determine the overall EMI score for that group.

To calculate the QBS score of a sample, it is sufficient to sum up the EMIs of all collected groups (Parisi, 2001; Parisi et al., 2005).

Figure 3 - Pauropoda

Figure 4 - Diplura

Figure 5 - Pseudoscorpion

2.2 QBS-c

QBS-c (Parisi, 2001) is a biological index similar to QBS-ar, but based only on collembola community.

Collembola present high density and great species difference in soil environment. Adaptation to edaphic life determined in collembola morphological changes (loss of pigment, reduction in the number of the eyes, development of particular sensory hair) that, according to their adaptation degree to the hypogeal environment, allows to distinguish typical biological forms. The QBS-c index is based on the attribution of a numerical value to any collembola biological form present in the sample. This index, which is not substitutive of the QBS-ar but complementary, allows increasing the information obtained studying the edaphic fauna and it results particularly sensitive to parameters such as the organic substance availability and the water rate stability, both related to climatic variations.

2.3 *Folsomia candida* Test

2.3.1 THE SPECIES

Folsomia candida (Willem, 1902) (*Figure 6*) originates from the Isotomidae family, whose representatives have been found all over the world. *F. candida* reaches 3 mm in length, it is completely depigmented, anophthalmic and it has a well developed post-antennal organ. It is a parthenogenetic species and its eggs are often deposited in easily identifiable groups under laboratory conditions. It is a species typical to soils rich in organic matter and it lives in the deeper strata of litter and soil surfaces. It is one of the collembolan species that has been most intensively studied. Cultures

of this springtail are very easy to maintain. Their short reproductive cycle at 20°C makes them ideal for conducting laboratory experiments (Fountain & Hopkin, 2001; Janssen & Bergema, 1991; Sandifer & Hopkin, 1997).

Figure 6 – Folsomia candida

2.3.2 COLLEMBOLAN CULTURES

The collembolans used in the tests were obtained from synchronous egg hatchings deposited by adult populations raised in the laboratories at the University of Parma. To obtain synchronous aged individuals, the adults were put in plastic containers with filter paper, maintained humid, and removed after three days (Hopkin, 1997). The obtained neanids were maintained at a temperature of 20° C, a light/darkness cycle 12/12 h, an air humidity level of 50% and were fed on a pulverized mix of wheat, oats, rye, soy and rice. After 12 days the individuals were used in the tests.

2.3.3 SOIL

Artificial soil for control condition was prepared according to the ISO 11267 (ISO 11267/99) guidelines. The soil contained 10% *Sphagnum* peat, 20% kaolinite clay and 70% quartz sand. The pH was adjusted to 6 by the addition of calcium carbonate, pH values were verified (1 M KCl) as optimum for the performance of *F. candida* (Crommentuijn et al., 1997; Sandifer & Hopkin, 1996). Natural soil was frozen for 24 hours. The natural and artificial soils were kept damp at a 50% of the water holding capacity with deionised water. After three days, 12 individuals taken from the synchronous hatchings previously described were introduced into each plastic container that contained the soil and the humidity was maintained relatively constant with the periodic addition of deionised water. Five control repetitions were performed with artificial and natural soil. At the end of the experiment (28 days), the soil was watered and the collembolan that floated on the surface were counted (procedure ISO 11267/99).

3. Results and discussion

3.1 QBS indexes

The QBS-ar index showed the highest values, for all the sampling date, in Santagostino site, even if they were characterized by large seasonal variability. Cascina Orsine showed intermediate values for the September and January samples, while in March and in July this site had the lowest values; the behaviour of Cascina Novella was specular, with the lowest values in September and January and the intermediate values in March and July (*Figure 7*). Important taxa for a well structured soil microarthropds community, such as Symphila, were present in Cascina Orsine only in the first sampling date.

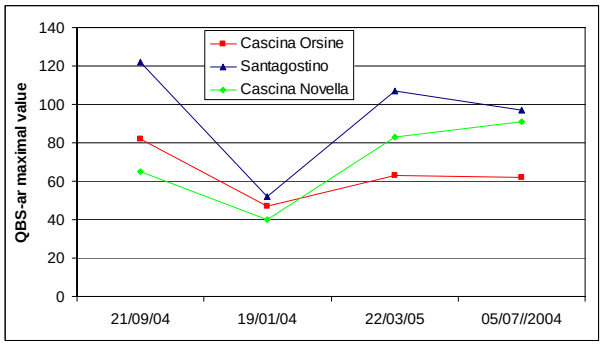

Figure 7 – QBS-ar maximal values for the different sites and different sampling dates

In all the studied sites the higher QBS-c values were observed in the samples of March 2005 (fig. 8). At that time the sites of Cascina Novella and Sant'Agostino showed QBS-c index values quite similar, whereas Cascina Orsine site had a 70 points lower index value as compared to both the others.

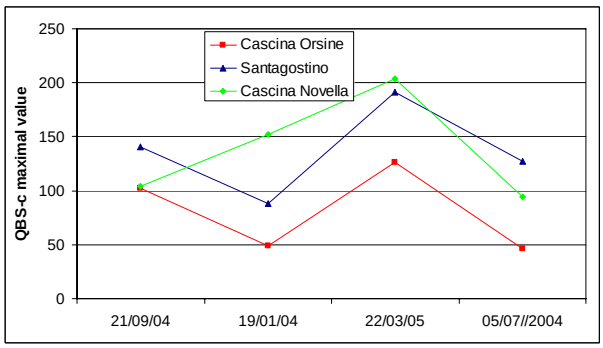

Figure 8 – QBS-c maximal values for the different sites and different sampling dates

The Cascina Orsine site shows high seasonal variation, in fact in January and July samples were not present forms well adapted to soil life such as onychiurid, that were present in March and September ones

The QBS-c index values of the Cascina Novella site increased substantially since the beginning of study, reaching in March 2005 the higher value of 2004. In these samples specimens of folsomid with 31 EMI value were always present and, have been noted also specimens of neelid and onychiurid, both forms highly sensitive to soil environmental changes.

The seasonal variations in QBS-c index of Sant'Agostino site are similar to those showed by Cascina Orsine site, even though the values are higher. In samples of Sant'Agostino site, were present collembola biological forms well adapted to soil life such as folsomid with 31 EMI value and two types of onychiurid (37 EMI and 40 EMI).

The general seasonal trend of QBS-ar showed a regular variation, characterized by the highest values during the middle season, when the temperature and soil water content in the upper 10 cm of soil are not limiting factors for the microartropods communities. It is necessary to consider however, the important differences in both soil characteristics and agronomic management, in the three sampling sites.

Cascina Orsine is characterized by biodynamic agriculture system that could represent a positive factor for the soil biological quality, but the land use is a recent established meadow and the soil has very high sand content. These soil properties could have determined during the spring and summer season, unfavourable life condition for some soil microarthropods life forms.

Santagostino is a long term established meadow, heavily manure, with a sandy loam soil with some skeleton content; in this case the biological soil quality should be even better than Cascina Orsine, due to the high organic matter input and to the lack of any chemical input; in fact even if in this farm the agricultural system is "conventional", the agronomic management of permanent meadows does not require any pesticides input. The physical characteristics of this soil are not optimal, but this is probably compensated by the high organic matter input.

Cascina Novella, on the other hand, is a corn monocropped field, where sewage sludge are applied and where the chemical inputs are quite high; the soil however is characterized by better physical parameters and this can compensate the effects of chemical input on soil microarthropods communities.

3.2 *Folsomia* test

The experiment showed that the survival of collembola adults was similar in the control condition and S. Agostino and Cascina Novella sites; Cascina Orsine site showed a lower value. Three natural soils showed wide differences in respect to artificial soil in the juvenile production. In effect, the number of young individuals was lower that that obtained in natural soils conditions.

	Adults	Young individuals
Control soil	11 ± 1	385 ± 86
Cascina Orsine	6 ± 3	97 ± 62
S. Agostino	11 ± 2	93 ± 43
Cascina Novella	11 ± 1	97 ± 14

4. Conclusions

The bioindicators used in this research has shown to be enough sensitive to detect the important seasonal variation in soil conditions and the effects of the main agronomic practices.

However, due to large differences of the three sites, not only in term of agricultural regimen, but also in terms of agronomic history and soil characteristics, the experimental data obtained are not able to express a clear gradient of biological soil quality among the three investigated sites.

Further investigation should consider a more accurate experimental design, aimed to minimize the effect of factors not relevant to the objective of the investigation. In the present research the main objective was to assess the effects of different agricultural systems (biodynamic, conventional with manure, conventional with sewage sludge) on soil biological quality, but the errors induced by difference in soil characteristics, type of crop, agronomic history has been probably to high.

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Microarthropods of the soil: convergence phenomena and evaluation of soil quality using QBS

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Traditional approaches to soil quality evaluation were based on the use of physical, chemical and microbiological indicators. Recently, new methods, based on soil microarthropods have been proposed for soil quality evaluation. Soil microarthropods have been shown to respond sensitively to land management practices and to be correlated with beneficial soil functions. In Italy, a new approach (called QBS-ar index) based on the types of edaphic microarthropods has been proposed to assess soil biological quality. The QBS-ar is based on microarthropod community present in a soil sample. Each type found in the sample receives a score from 1 to 20 (ecomorphological index, EMI), according to its adaptation to soil environment. The QBS-ar index sums up these scores, thereby characterizing the microarthropod community of the sample being studied. QBS has been applied on a range of soil types and land uses in Italy, its validity evaluated for assessing biological quality of soil in different situations.

The extraordinary biodiversity present in the soil poses many taxonomic problems, but is of great interest for environmental research, both basic and applied.

Among the various groups that have colonized the soil – or rather 'soils', given their great diversity – the microarthropods of the atmobios are a material that is proving to be more and more important for understanding how the basal strata of the earth's ecosystems function.

There are many extremely old groups of microarthropods, dating from the Devonian (more than 350 million years ago), such as the Collembola, in the soils. Over the lengthy period of adjustment to the hypogean life the euedaphic microarthropods have accumulated a body of characteristics that bear witness to their invasion of these particular habitats.

It is an impressive convergence and many of these characteristics are morphological, easily understood, such as reduction of the visual apparatus, loss of pigmentation or cryptal coloration, reduction of appendices and the acquiring of special structures, essential for hypogean life.

The confining of these groups in the soils, that is, the groups' incapacity to leave them, is due to the stability of these habitats. Actually, various factors such as water, temperature, organic substance vary only slightly over the short and medium term. In addition there is, obviously, no light. As a result, the euedaphic microarthropods are stenoeic and unable to survive abrupt variations in environmental factors. They are particularly sensitive to soil degradation and to disturbances caused, for example, by agricultural cultivation and trampling.

Years ago I asked myself how to quantify the level of convergence existing in the communities of edaphic microarthropods. Returning to the old concept of "biological form", I set up (Parisi, 2001) a table in which each characteristic, significant in terms of adaptation to life in the soils (both as an active process and as a consequence of this process, for example anophthalmia) is weighed and expressed with a synthetic value: EMI (ecomorphological index). This index then makes it possible to characterize the various systematic groups, in terms of their confinement in the soil. The sum of the EMI of the various groups (indicated by the acronym QBS, biological quality of the soil) is a measure of the degree of the community's overall convergence to edaphic life.

To evaluate the reliability of this computation I found the QBS-ar (the index relating to the entire community of microarthropods) in soils in various stages of degradation, starting from the hypothesis that the more a soil is disturbed and degraded, the lower the QBS-ar value of its community of microarthropods will be. In fact, the forms most bound to the stability of the soils will be those that disappear first.

The data gathered confirmed that the hypothesis was correct. This gave rise to the growing use of the QBS-

ar for evaluating the biological soil quality. This index has proved to be useful for an initial description of the soil's state of conservation, since it is able to characterize effectively the state of the microarthropod community as a whole and, indirectly, the condition of the soil. It has been seen that this may also be used for purposes other than the original ones (degradation, disturbance), for instance in evaluating soil pollution by certain polluting agents. But a wider survey is needed in this regard.

It isn't necessary to determine population density nor is a detailed taxonomic analysis required for calculating the QBS-ar, which is easy and fast to utilize, and therefore helpful in mapping biological soil quality.

A way is presently being studied for facilitating use of a particular form of QBS, the QBS-C, regarding only Collembola: this index should be particularly useful in cases where the object of investigation is above all the hydric balance of the soil.

Evaluation of soil toxicity using a battery of stress biomarkers on the earthworm *Eisenia andrei*

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Earthworms (*Eisenia andrei*) were utilised in Biodiversity-Bioindication project, and exposed in climate chambers for 10 days to three different agricultural soils sampled at two different seasons (fall and summer). The three soils were subject to different treatments: soil from Cascina Nuova was treated with a traditional approach, soil from Cascine Orsine was subject to biological treatment, whereas soil from Cascina Novella was treated with biological muds.
The stress syndrome in adult *E. andrei* was investigated with a set of four biomarkers of stress: lysosomal membrane stability, lysosomal accumulation of lipofuscin and neutral lipids, and Ca^{2+}-ATPase activity, and one biomarker of exposure, AChE activity. Data were compared with the results obtained in a parallel study, where earthworms were exposed to soil collected from an industrial area.
The results demonstrated that mortality of earthworms was not affected in individuals exposed to the three soils sampled in two seasons and only a minimal level of oxidative stress as sublethal physiological impairment (with statistical significant change but lower than 20% and therefore of minimal biological injury in the animals exposed to Ca` Novella and Ca` Orsine soils).

1. Introduction

Among soil organisms, earthworms such as *Lumbricus* and *Eisenia* spp. (Anellida, Oligochaeta), are considered to be of particular interest to evaluate adverse effects of contaminants.
Earthworms possess a number of qualities required in animals used for bio monitoring of terrestrial ecosystems. They are numerous, easy to sample, widely distributed and relatively immobile; they are in full contact with the substrate in which they live and consume large volumes of this substrate.
On these organisms, we developed a battery of stress biomarkers (i.e. parameters able to evidentiate the biological effects of the total charge of pollutants present in the environment) to detect the pollutant stress syndrome induced on worms by exposure to contaminants.
For Biodiversity-Bioindication project, earthworms (*Eisenia andrei*) were exposed in climate chamber for 10 days to three natural soils sampled in two different seasons (fall and summer).
E. andrei adults stress syndrome was investigated using a set of biomarkers of stress, such as lysosomal membrane stability, lysosomal accumulation of lipofuscin and neutral lipids, and Ca^{2+}-ATPase activity, and a biomarker of exposure (AChE activity) suitable to evidentiate toxic effects due to pesticides such as carbamate and organophosphorus compounds.
Lysosomal membrane stability is recognized as an extremely sensitive indicator of cellular effects of pollutants in different species such as molluscs and fishes (Lowe et al., 1992; Moore et al., 1996). Lysosomal accumulation of lipofuscin was utilized because lipofuscin represents a lipid peroxidation end-product and its increase is related to the oxidative stress induced by pollutants. The lysosomal accumulation of neutral lipids is a useful indicator of alteration of lipid metabolism.
Ca^{2+}-ATPase activity plays a fundamental role in regulation of Ca^{2+} homeostasis and different toxic chemicals, that are able to produce oxidative stress in the cells as well as heavy metal ions, can affect the function of Ca^{2+}-ATPase by acting on SH-residues.

2. Methods

2.1 Lysosomal membrane stability

Lysosomal membrane stability was assessed using the neutral red retention ability (NRR) of coelomocytes as follows.

2.2 Coelomocyte preparation

Coelomic fluid was obtained from the gastrointestinal/coelomic cavity of adult earthworms by the extrusion method (Eyambe et al., 1991; Fugère et al., 1996).

Animals were rinsed in saline solution, containing 0.85 mg/mL NaCl at 10°C, and one-fourth of the posterior part of the body was massaged to expel the content of the lower gut. They were then transferred to the saline extrusion medium consisting of 5% ethanol, 2.5 mg/mL of the mucolytic agent guaiacol glycerol ether, adjusted to pH 7,3 with 1 N NaOH (Brousseau et al., 1997), for 3 min at 4°C. The extrusion medium, containing the extruded coelomocytes, was transferred to a centrifuge tube and centrifuged for 10 min at 150 x g, and then resuspended in 1.0 mL of Ca^{2+}free-LBBS. Ca^{2+}Free-LBSS (Ca^{2+}Free Lumbricus basal salt solution) solution contains 71,5 mM NaCl, 4,8 mM KCl, 0,4 mM KH_2PO_4, 0,3 mM $Na_2HPO_4.7H_2O$, 1,1 mM $MgSO_4.7H_2O$, 4,2 mM $NaHCO_3$, adjusted to pH 7.3 and 300 mosM osmolarity (Brousseau et al., 1997).

2.3 Neutral red retention assay

A stock solution of neutral red was prepared by dissolving 20 mg of neutral red in 1 mL of dimethyl sulfoxide (DMSO) A working solution was prepared by diluting 10 µL of the stock solution with 990 µL of Ca^{2+}free-LBBS. Coelomocytes (40 µL) were placed on polylysinated slides and the cells were allowed to adhere to slides for 15 min in a humidity chamber at 18 ± 1°C, before application of the neutral red working solution (40 µL). After 15 min, excess dye was eliminated and 40 µL of Ca^{2+}free-LBBS added. Images were recorded every 15 min for 1 h using a DM RB Leitz microscope (Leitz Wetzlar, Germany) equipped with a Dage MTI camera and analogue-digital converter (DAGE-MTI Inc., Michigan City, IN). Digitalisation was carried out by an image analysis system (NIH Image-Scion Image software, version 1.59). The rate of lysosomal neutral red leakage was measured as a variation of lysosomal optical density (OD).

2.4 Tissue sampling for lipofuscin and neutral lipid Analysis

Earthworms were cut into cross-sectional pieces and were flash-frozen for 40 seconds in N-hexane chilled with liquid N_2 and stored at -80°C. Cross sections (10 µM) were obtained at -27°C in a Leica cryostat.

2.5 Lipofuscin lysosomal accumulation

The lipofuscin content of chloragogenous tissue surrounding the earthworm intestine was evaluated using Schorml's Ferric Ferricyanide method (Pearse, 1972). Frozen samples were sectioned (10 µM), transferred onto glass slides, fixed in 10% formal-calcium at 4°C for 15 min, rinsed in water and immersed in reaction medium. This contained 1% ferric chloride and 1% potassium ferricyanide in a ratio of 3:1. Slides were stained for 5 min in the reaction medium, rinsed in 1% acetic acid for 1 min, washed in water and mounted with glycerine jelly. Lipofuscin accumulation was quantified by image analysis as previously described for Ca^{2+}-ATPase activity and expressed as percentage optical density.

2.6 Unsaturated neutral lipid lysosomal accumulation

Neutral lipid content was obtained from frozen tissue sections stained with the oil-soluble dye, Oil Red O (Moore, 1985).

Cryostat sections (10 µM) were fixed in 10% formal-calcium at 4°C for 15 min. Slides were washed in water and rinsed in 60% TEP (triethyl phosphate), stained with a solution of filtered Oil Red O 1% in TEP at 20°C for 15 min. Slides were then washed in 60% TEP for 30 sec and rinsed in water and mounted with glycerine jelly. Unsaturated neutral lipid content was quantified by image analysis as previously described for Ca^{2+}-ATPase activity and expressed in rate of optical density.

2.7 Cytochemical assay for Ca2+-ATPase activity

In order to detect Ca^{2+}-ATPase activity we followed a slightly modified version of a procedure for the cytochemical detection of Ca^{2+}-ATPase in the digestive gland cells of molluscs (Pons et al., 2002) concentrating on the post-clitellum intestinal tract.

Earthworms were cut into cross-sectional pieces, washed in 0.05 M cacodylate, fixed in 1% pFA in 0.05 M cacodylate, (pH 7.4) for 30 min at 4°C and rewashed in 0.05 M cacodylate. The samples were then dehydrated in acetone crescent concentrations at 4°C and embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany).

From the embedded samples, serial cross sections (2 □m) were cut using a HM350 Microm microtome (Walldorf, Germany), transferred onto glass slides and incubated for 6 h at room temperature in a medium containing 2.4 mM disodium salt ATP, 18 mM $CaCl_2$, 8 mM levamisole, 0.2 mM ouabain, 1 mM $Pb(NO_3)_2$, and 20 mM sodium barbiturate. Control samples were incubated in a calcium-free medium containing 2 mM EGTA.

After incubation, the medium was removed and slides washed in water and rinsed in an ammonium sulfide-saturated water solution (3 min) to reveal the brown lead sulfide precipitation. $Pb_3(PO4)_2$ precipitates stained with ammonium sulfide was quantified on sections by digital imaging as described previously by Pons et al. (2002).

2.8 AChE evaluation

Acetylcholinesterase is based on the reliable chemistry of Ellman, in which the thio-ester substrate acetylthiocholine (AchSC) is hydrolysed by the enzyme, releasing a sulfhydrylic group able to react with Ellman's reagent. The reaction is then followed with the use of absorption of 2-nitro-5-thiobenzoate anion formed from reaction.

One g of earthworm tissues was homogenized in five volumes (5 mL) of tissue extraction buffer (Tris-HCl buffer, pH 7.6, 0.1% Triton X-100) and then centrifuged at 10000 x g for 20 min.

AChE was evaluated in S10 homogenate using acetylthiocholine substrate following reaction for 10 min (at 412 nm) and expressed as nmol/min/mg protein.

3. Results

To demonstrate the sensitivity of the biomarker battery utilized, we also compared the results with the data obtained in a site exposed to industrial emissions investigated in a parallel study.

We did not observe effects on mortality rate (data not shown) of earthworms maintained for 10 days on Bio-Bio project soils sampled in the two different seasons and in the LINFA-site.

We evaluated in the coelomocytes, by neutral red retention assay, the lysosomal membrane stability. We have not found any differences in this biomarker due to the effects of the soil sampled in the different area in the two seasons, while it is possible to observe a significant decrease in the polluted site (*Figure 1*).

- *Lipofuscin lysosomal accumulation* in *Eisenia andrei* chloragogenous tissue, evaluated in cryostat cross sections by Schorml reaction, shows no significant differences on earthworms exposed to project soils sampled in fall and summer respect to the increase of the industrial site (*Figure 2*).
- *Neutral lipid lysosomal accumulation***,** tested in cryostat cross-sections of worm coelomic cavity cells using oil red staining, shows a weak increase in Ca' Nuova and Ca' Novella sampled soils in both seasons (*Figure 3*).
- *Ca^{2+}-ATPase activity*, evaluated on resin-embedded sections of *Eisenia andrei* intestinal epithelium after cytochemical staining, is a very sensitive parameter to detect biological effects due to exposure to soil pollutants (e.g. the decrease in the LINFA-polluted site), for its direct contact to soil ingested. Ca^{2+}-ATPase activity is not decreased after earthworm exposure to the soils sampled in fall and summer (*Figure 4*).
- *Acetylcholinesterase activity*, performed on S10 homogenate, is a sensitive biomarker of exposure to carbamate and organophosphate compounds.

For AChE activity, no characteristic trend was found. Given that natural soils were utilized in this study, the presence of pesticides with an inhibiting effect on AChE activity was not detectable on earthworms.

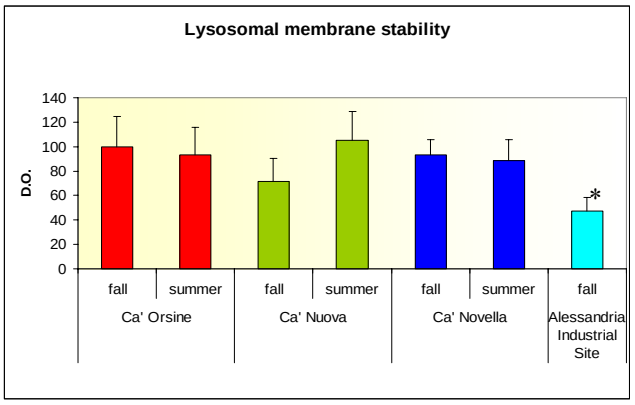

Figure 1. Lysosomal membrane stability in coelomocytes of Eisenia andrei

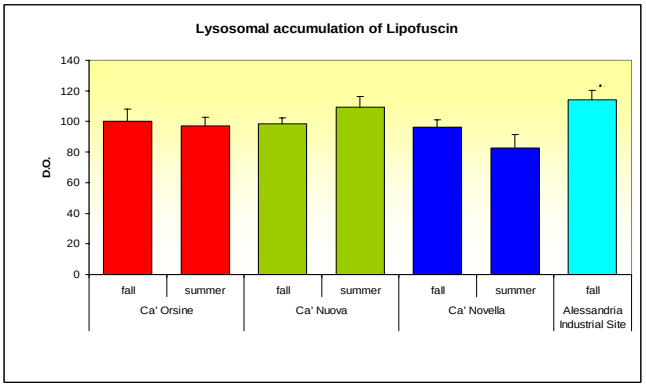

Figure 2. Lipofuscin lysosomal accumulation in Eisenia andrei chloragogenous tissue

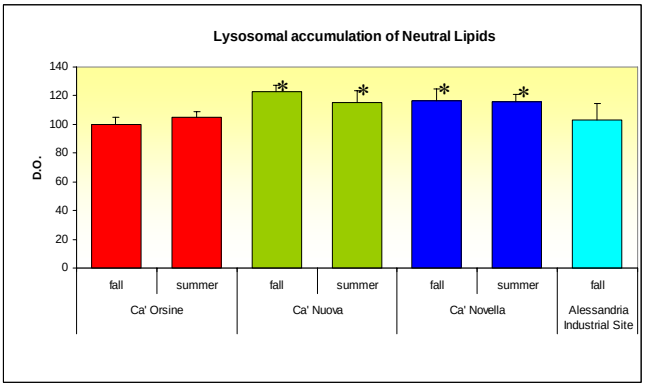

Figure 3. Neutral lipid accumulation in cryostat sections of Eisenia andrei coelomic cavity cells

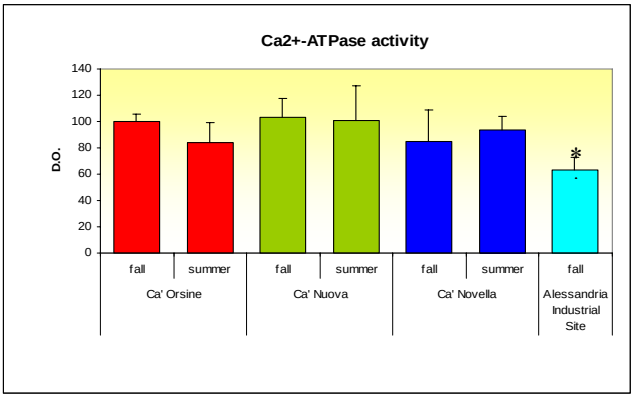

Figure 4. Ca^{2+}-ATPase activity evaluated Eisenia andrei intestinal epithelium

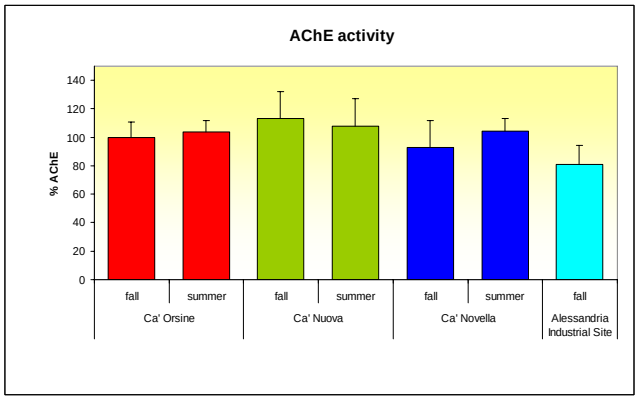

Figure 5. AChE activity performed on earthworm homogenate

4. Conclusion

The battery of biomarkers utilized in the project comprehends end-points at different levels of biological organisation, allowing a screening of biological effects both at organism (mortality) and cell (lysosomal membrane stability, lysosomal accumulation of lipofuscin and neutral lipids, and Ca^{2+}-ATPase activity) level, and a biomarker of exposure (AChE activity).

The approach based on the integrated study of a battery of biomarkers has been validated in several laboratory experiments and field trials and it represents a valid screening tool in soil ecological risk assessment and an early warning index of soil pollution.

Results demonstrate that the earthworms exposed to the soil sampled in fall and summer seasons show no effects in terms of mortality and only a minimal level of oxidative stress as sub lethal physiological impairment. This suggests a minimal level of contaminants in the bioavailable form in the soil samples not able to induce stress at cell and organism level (statistical significant change but lower than 20% and therefore of minimal biological injury).

Therefore the 3 sets of soil samples utilized in the analysis may be considered of good quality, being not able to induce sub lethal toxic effects on *E. andrei*, utilized in the experiments.

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Earthworms used as indicators of agricultural managements

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Earthworms, known as "ecosystem engineer", are also strongly influenced by the environmental conditions, especially human pressures. This study assessed the state of bio indicator played by earthworms in relation to three agricultural managements. It appeared that (i) earthworms were more abundant under organic and traditional agro systems compared to fertilized one with sewage sludge, (2) traditional agro system allowed the highest specific richness. However, it appeared difficult to compared different agricultural managements without taking into account the land uses and the pedological context. We proposed to increase the number of study sites, in order to asses the relevance of these biological parameters as bio indicators of agricultural managements.

1. Introduction

Soil is a major interface between the lithosphere and the atmosphere. It could be regarded as an interactive system in which the physical, chemical and biological characteristics (soil structure, organic matter, soil solution, fauna & flora) are strongly related (Coleman & Odum, 1992). In order to understand soil functioning, it is necessary to assess the place and the role of each one of its components (physical, chemical, biological) as well as the interactions between these components. Moreover, soil characteristics are strongly influenced by environmental conditions (mesological and human pressures). In that way, the agricultural soils which permit the vegetable production are submitted to anthropic constraints (mechanical or chemical). In occidental Europe, these constraints have increased significantly since the beginning of industrial era and more recently in relation to the development of an agriculture which had to answer to social waiting of the post-war period: how to adapt agriculture and its practices so as to make the countries self-sufficing in terms of food resource? If the practices carried out then allowed the increase of the outputs, they were also associated with (i) the degradation of the soil quality, in relation to the process of run-off or compaction, (ii) the decrease of soil biodiversity, in relation to several pressures as soil contamination and decline in soil organic matter. This last point was underlined during Rio and Kyoto conferences. In order to understand and manage this environmental degradation, it thus advisable to better know and understand the functioning of soil and also to develop some tools accounting for the deterioration of the systems. Research on the quality of air and water had allowed creating tools evaluating the degree of deterioration of the ecosystems. However, soil considered as a support of biodiversity, does not have any tool to assess the biological quality. In order to improve this, some national project (GESSOL, RMQS, French project) or international one have tried to create relevant biological indicator of soil quality.

In the European program Bio-Bio (Biodiversity-bioindicator), several protocols are used in order to test and validate a global indicator of the biological quality of soil. For thus, each trophic level is taken into account and evaluated. These trophic levels include at the same time the micro organisms (bacteria, protozoa, fuggy...) and all of the soil fauna (meso and macro fauna).

In temperate regions, the earthworms in term of biomass constitute the principal component of the total faunal biomass (Lee, 1985). They have a large influence on soil physical, chemical and biological properties and thus are considered as "ecosystem engineers" (*sensu* Jones *et al.,* 1994). In agro ecosystems, as in many other environments, their role in promoting soil fertility is important (Lee, 1985; Werner and Dindal, 1989). Furthermore, because of their strong interaction with soil, earthworm populations are also profoundly affected by (i) agricultural practices, such as soil tillage, crop residues, the use of fertilizers and pesticides, etc. (Edwards, 1983; Lofs-Holmin, 1983; Daugbjerg et al., 1988; Paoletti et al., 1998; Chan, 2001) and also by (ii) mesological conditions (Pérès, 2003). So, earthworms may be used as bio indicators of soil because they are easy to rear and classify and are very sensitive to both chemical and physical soil parameters (Paoletti et al., 1991).

Within the Bio-BIO project, we investigated the response of earthworms to different agricultural managements identified in the project: organic farming, traditional and fertilized managements.

The Bio-Bio project proposes to compare the agricultural managements by studying three experimental sites: a temporary pasture for the organic farming, a permanent pasture for the traditional management and maize for the fertilized management. However, concerning the earthworms, we have to take into account the potential influence of the different agricultural uses and also the mesological characteristics (Chan, 2001). Thus, we have proposed to increase the number of studied sites, in order to identify the major parameter which influences earthworm communities. So, we have combined the agricultural management to different land uses (pasture, crop) and different pedological conditions.

Thus, in order to assess the suitability of earthworms as bio indicators of soil uses, soil management and soil states, we have tried to answer to different questions:

- Is there an influence of the agricultural management, whatever the agricultural use? For this question we have studied the Bio-Bio sites.
- Within a specific agricultural management, is there an influence of the land use?
- Within a specific agricultural management and a land use, is there an influence of the pedological context?

2. Materials and methods

2.1 Sites

The three experimental sites were in the Pavia region, Italy (*Figure 1*).

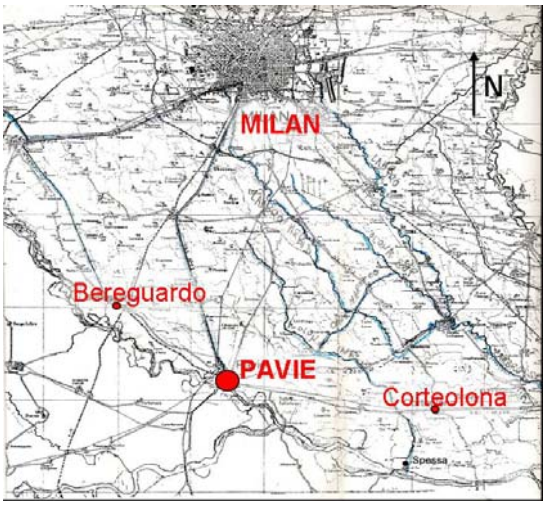

Figure 1. Localisation of the experimental sites

Site 1 "Orsine" was near Bereguardo (10 km West far from Pavia) and presented an organic farming management. Three agricultural land uses were studied: a temporary pasture (BdP, which was the Bio-bio site), maize (BdM) and a cereal/legume rotation (barley/pea) (BdC). Cropping histories of fields were, till 20 years: no pesticide, organic in-puts realised as cow manure or cow slurry (BdM, BdC), and a plough (20 cm deep) each year.

Site 2 "Nuova" was near Corteolona (20 km East far from Pavia), and presented traditional agricultural management: use of pesticides (herbicides), but no plough till 8 years. Only an agricultural land use was studied: a permanent pasture (TdP, which was the Bio-bio site).

Site 3 "Novella" was near Bereguardo and presented a fertilized agricultural management with high in-put of organic matter. Three agricultural land uses were studied: maize (BeM), crop (wheat) (BeC), rice (BeR). In order to assess the influence of pedological context, two rice fields different in terms of hydromorphy properties were studied (BeR1 and BeR2). The cropping histories of fields were till 20 years: spread of sewage sludge (BeM, BeC) or manure (BeR1, BeR2), use of pesticides and plough (30-40 cm). All informations concerning the management of the different treatments are given in *Figure 2*.

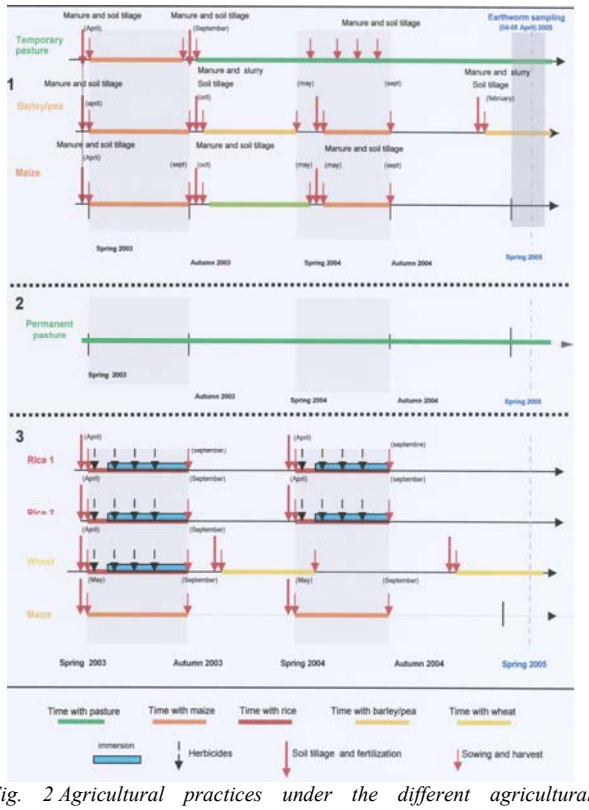

Fig. 2 Agricultural practices under the different agricultural managements (1: organic farming, 2: traditional, 3: fertilized) and land uses

In order to assess the influence of agricultural management, agricultural use or pedological conditions, we compared different groups of sites (*Table 1*).

Table 1 Codification and agricultural interventions of the studied sites. Crosses inform on the sites used for the different approaches (management influence, agricultural use influence, pedological influence). The crosses in management approach correspond to the Bio-Bio sites.

Agricultural management	Land use	Codification	Different approaches			Agricultural interventions		
			Management influence	Agricultural use influence	Pedological influence	Fertilization	Plough (depth)	Pesticides
1.Orsine Organic farming (Bd)	Maize (M)	BdM		X		-300q/Ha cow manure	20 cm	no
	Pois/Orge (C)	BdC		X		-300q/Ha cow manure -150q/Ha cow slurry	20 cm	no
	Temporary Pasture (P)	BdP	**X**	X		no till 2 years	20 cm	no
2. Nuova Traditional (Td)	Permanent Pasture (P)	TdP	**X**			-200q/Ha cow manure -15 (N, P, K) or 10 (N, P, K)	No	4L/Ha Asulox once/5 years x
3. Novella Fertilized (Be)	Maize (M)	BeM	**X**	XX		-20% boues d STEP (360q/Ha) -80% manure	30-40 cm	4L/Ha in 400-600L primagold water (312,5g//L S-metoclor and 187,5g/L terabutilazine pure)
	Froment (C)	BeC		XX		-20%boues (360q/Ha) d STEP (360q/Ha) -80% manure	30-40 cm	No data
	Rize 1 (R1)	BeR1		XX	X	- manure	30-40 cm	No data
	Rize 2 (R2)	BeR2			X	- manure	30-40 cm	No data

(Note: concerning the maize in the site 3, when we went to sample the earthworms, the farmer had just ploughed 3 days before. So we could not sample in this site. We proposed to change and sampled the earthworms in another site just nearby the ploughed one.)

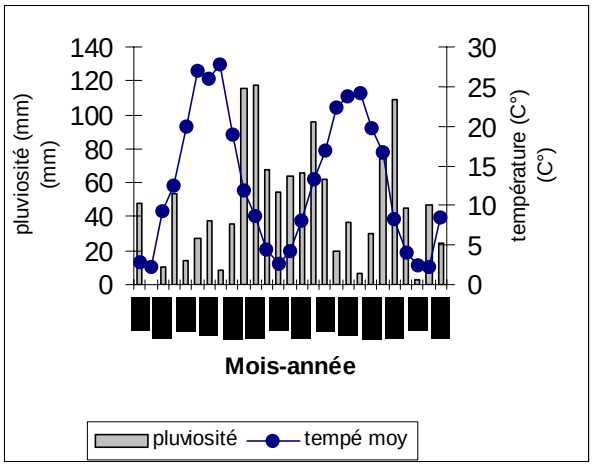

Figure 3. Mean monthly rainfalls and temperatures (climate station of Spessa, ERSAF)

The climate in the region is sub-continental, with influences of Mediterranean Sea. The mean monthly rainfalls range from 750 to 950 mm and the mean annual temperature is 10°C with large variations: from 3 ◦C in January to 25◦C in August (data from climate station of Spessa, ERSAF located at 5 km from Corteolona) (*Figure 3*).

Each site was situated on soils classified as Brunisol or as Reductisol (FAO). The soil characterisations were realised at two different moments: (i) on May 2006 a first characterization of physical and chemical properties was realised on all the 8 studied sites, (ii) on autumn 2006 a more precise analyse was realised on the 3 bio-bio sites (*Table 2*).

Table 2 Pedological and physico-chemical characteristics of the different sites

Agricultural management	Land use	Texture	CEC (meq/100 g)	Soil classification	pH	C % (0-30 cm)	C org % (0-30 cm)	Al mg/kg (0-30 cm)	Cd mg/kg (0-30 cm)	Cu mg/kg (0-30 cm)	Pb mg/kg (0-30 cm)	% Sand (0-30 cm)	% Loam (0-30 cm)	% Clay (0-30 cm)
1.Orsine *Organic farming (Bd)*	Maize (M)	Loam-sandy	13,2±1,2	Brunisol (with some feature of hydromorphy)	6,1									
	Barley/Pea (C)	Loam-sandy	11,4±1,9	Brunisol	6,2									
	Temporary Pasture (P)	Loamy	12,5±0,4	Brunisol	6,2	1,32	1,02	46400	0,27	12,4	18,06	68	27	5
2. Nuova Traditional (Td)	**Permanent Pasture (P)**	Sandy	15,8±0,5	Brunisol	6,6	1,05	0,9	44167	0,29	11,10	15,9	73	22	5
3. Novella *Fertilized (Be)*	**Maize (M)**	Loam-clay	18,7±0,9	Brunisol (with some feature of hydromorphy)	6,9	0,92	0,7	71300	0,80	29,8	25,4	34	56	10
	Rice 1 (R1)	Loam-sandy	20,6±3,3	Reductisol	7,1									
	Rice 2 (R2)	Loamy-clay	16,2±1,7	Brunisol	6,7									
	Wheat (C)	Loamy-clay	18,7±0,9	Brunisol	6,9									6,9

The sites were very different in terms of texture (site 1 and 2 have a sandy-loam texture, compared to the site 3 which presents a loamy-sandy-clay texture), pH (site 1 presents the lowest pH), and soil element as Pb, Cu, Zn (site 3 presents the highest values).

2.2 Earthworm sampling and identification

Earthworms were sampled in April (the end of the wet season). Earthworms were extracted using the formaldehyde method (Bouché, 1972; Cluzeau et al., 1999): after three sprayings of formaldehyde solution on a 1 m² (10 l per spraying with different concentrations: 0.25%, 0.25%, 0.4%), earthworms were collected at the soil surface. Soil samples (25 x 25 x 25 cm^3) were taken from the centre of the extraction areas and hand sorted to account for any earthworms not emerging. Three replicates (formol and hand-sorting methods) were performed per plot.

Note: because of the dryness of the soil, 10 l of water were spread the day before the earthworms sampling in order to improve the infiltration of the formaldehyde solution during the fauna extraction.

Earthworms were preserved in 4% formaldehyde solution and transported to the laboratory. Species were then identified by external characteristics using the key of Cluzeau (1996) based on Bouché work (1972), and weighed. Earthworm communities were characterised by their abundance (number collected per m²) and biomass (g per m²), their specific structure (based on earthworms' species) and their ecological structure. This last parameter is based on earthworm morphology and behaviour (localisation in soil, feeding behaviour), and corresponds to three ecological groups (Lee, 1959; Bouché, 1972, 1979): epigeic species (range 1 to 2.5 mm in diameter) live near the surface, feed on surface litter and create no or few burrows); anecic species (deep burrowing species, range 4 to 8 mm in diameter) also feed at the ground surface, live in semi-permanent burrows, more or less verticals and opened to the soil surface; endogeic species (range 2 to 4.5 mm in diameter) feed on mineral and humus particles within the soil, dig extensive systems of temporary burrows that they immediately refill with their casts, the burrows are mostly subhorizontal oriented and very ramified through the soil but rarely open to the surface).

Between-site differences were compared statistically using the Mann-Whitney rank test, a non-parametric method (P=0.05) to assess the impact of (i) the agricultural managements, (ii) the land uses, (iii) the pedological constraints.

The changes in earthworm communities along the anthropic gradient were investigated using a principal component analysis (ACP). The ACP was performed using ADE-4 software, a package for multivariate analysis and graphical display.

3. Results

3.2 Influence of agricultural managements (Bio-Bio sites)

Earthworm populations in the organic farming (BdP) and traditional managements (TdP) were significantly ($P < 0.05$) larger than those in the fertilized management, both in terms of earthworm abundance and biomass (*Figure 4*). Populations under organic farming pasture were somewhat smaller than those under traditional pasture, but this difference was not significant ($P > 0.05$). The overall (mean, n = 3) earthworm abundance in organic farming pasture, traditional pasture and fertilized maize were 32.6, 46.5 and 9.6 individuals m−2, and the corresponding earthworm biomass was 9.1, 3.5 and 1.2 gm−2.

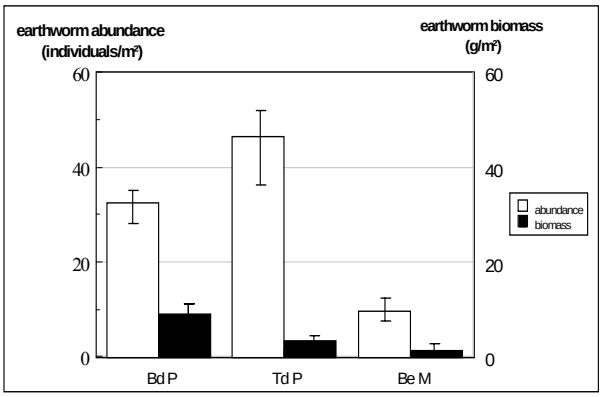

Figure 4 Earthworm abundance and biomass in three agricultural managements (mean+S.E., n = 3).

The earthworm ecological structure was different depending on the agricultural management (*Table 3*). Anecic species missed whatever the management. Endogeic species were most abundant in pasture, accounting for 100% of total abundance in organic farming and for about 99% of total abundance in traditional management. In fertilized management, epigeic species were three times more important than endogeic species.

Table 3 Mean abundance (individuals m−2) of earthworm ecological groups in three agricultural managements (mean, n = 3)

Management	Abundance of ecological groups (individuals.m-2)		
	Epigeic	Endogeic	anecic
BdP	0	32,6	0
TdP	0,25	46,25	0
BeM	7,3	2,3	0

Table 4 Mean abundance (individuals m−2) and biomass (g m-2) of earthworm species in three agricultural managements (mean, n = 3)

Management	Abundance of earthworm species (individuals m-2)					
	O. transpadanus	*E. tetraedra*	*A. a. antipai*	A. c. *paratypicus*	Lumbricus sp.	*M. dubius*
BdP	**32,7**	0	0	0	0	0
TdP	0	0	**24**	0,3	0,3	**22**
BeM	2,3	**7,3**	0	0	0	0
	Biomass of earthworm species (g m-2)					
BdP	9,2	0	0	0	0	0
TdP	0	0	2,5	0,2	0,5	1,5
BeM	0,5	0,7	0	0	0	0

The earthworm species composition was also affected by management (*Table 4*). Of the six species recorded, individuals *Eiseniella tetraedra* (Et, epigeic species) were recorded only in the fertilized maize. Individuals *Octodrilus transpadanus* (endogeic species) the most abundant species, were recorded in fertilized and organic farming managements. Individuals *Microscolex dubius* (endogeic species) and *Allolobophora antipai antipai* were recorded in traditional pasture and were the most dominant species of this management; however, *Allolobophora antipai antipai* were sampled only by hand sorting, thus limits the analyse of the species distribution. The other earthworm species, as Aporrectodea *caliginosa paratypicus* (endogeic) and Lumbricus sp. were considered as rare species accounting for their low abundances.

3.2 Influence of land uses and pedological contexts

For this part of the study, data were analysed using three groups of data (i) variability within organic farming, (ii) variability within fertilized management, (iii) variability within rice agro systems.

Organic farming management

Within organic farming management, land uses had significant effects on earthworm abundance or biomass (*Figure 5*). Earthworm populations were significantly ($P < 0.05$) larger in pasture and in maize than in crop rotation both in terms of earthworm abundance and biomass (*Figure 5*). Populations in pasture were about three times more abundant than those in maize, and in opposite, earthworm population biomass was greater in maize than in pasture, but these differences were not significant ($P > 0.05$). The overall (mean, n = 3) earthworm abundance in temporary pasture, maize and crop rotation were 32.6, 12 and 4.3 individuals m−2, and the corresponding earthworm biomass was 9.1, 13.8 and 3.5 gm−2.

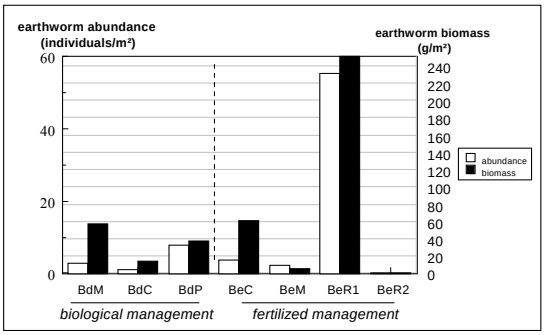

Figure 5 Earthworm abundance and biomass in the different land uses within two agricultural managements (mean+S.E., n = 3).

The earthworm ecological structure was different depending on the land uses (*Table 5*). Epigeic species missed whatever the land use. Endogeic group was the most abundant ecological group, especially in pasture, accounting for 100% of total abundance. Anecic species were recorded only in maize and crop rotation, but they were significantly lower abundant than endogeic.

Table 5 Mean abundance (individuals m−2) of earthworm ecological groups in three agricultural uses in organic farming management (mean, n = 3)

Land use	Ecological groups (individuals.m-2)		
	Epigeic	endogeic	Anecic
Maize (BdM)	0	10,3	1,7
Crop rotation (BdC)	0	4,0	0,3
Temporary pasture (BdP)	0	32,6	0

The earthworm species composition was also affected by land uses (*Table 6*). Of the three species recorded, individuals *Octodrilus transpadanus* (endogeic species) were the most abundant, and were recorded in all the land uses. This species was the only one sampled in pasture. Two anecic species were recorded: individuals Aporrectodea *caliginosa meridionalis* were recorded in maize and crop rotation, whereas Lumbricus *rubellus rubellus* was sampled only under maize.

Table 6 Mean abundance (individuals m−2) and biomass (g m-2) of earthworm species in three land uses within organic farming management (mean, n = 3)

Land use	Abundance of earthworm species (individuals m-2)		
	Oc tr	Acm	Lrr
Maize (BdM)	10,3	1,3	0,3
Crop rotation (BdC)	4	0,3	0
Temporary pasture (BdP)	32,7	0	0
	Biomass of earthworm species (g m-2)		
Maize (BdM)	12,6	1	0,3
Crop rotation (BdC)	3,3	0,3	0
Temporary pasture (BdP)	9,2	0	0

Fertilized management

Within fertilized management, land uses had significant effects on earthworm abundance or biomass, especially concerning the rice agrosystem. Earthworm populations were significantly ($P < 0.05$) larger in rice 1 than in cereal and maize both in terms of earthworm abundance and biomass (*Figure 5*). Populations in cereal were more abundant and the biomass was more important than those in maize (14.6 *vs* 1.2 individuals m−2; 16 vs 9.6 gm−2) but these differences were not significant ($P > 0.05$).

Within rice agro system, abundance and biomass of earthworms populations were significantly different ($P<0.05$): the pedological conditions observed in rice 2 (R2) strongly altered earthworm population, both in terms of abundance and biomass. The overall (mean, n = 3) earthworm abundance in rice1 and rice2 were 81.1 and 0.1 individuals m−2, and the corresponding earthworm biomass was 231 and 0.3 gm−2.

The earthworm ecological structure was different depending on the land uses, and only one earthworm species was recorded per ecological group (*Table 7*). Endogeic group, represented by *Octodrilus transpadanus*, was the most abundant ecological group (63% of overall population abundance), especially in cereal accounting for 100% of total abundance, and in rice1 where 143 individuals were recorded. Anecic species, represented by *Aporrectodea caliginosa meridionalis* (34% of overall population abundance) were recorded only in rice1, but they were significantly lower abundant than endogeic. Epigeic species, represented by *Eisenielle tetraedra* (3% of overall population abundance), were recorded accounting for 75 % of total abundance, and in rice2 where they were the only ecological group. These results suggested that constraints in cereal crop depressed earthworm biodiversity.

Within rice agro system, the pedological constraints observed in rice 2 (R2) altered earthworm population biodiversity: only one epigeic individual was recorded, while two species were sampled in Rice 1.

Table 7 Mean abundance (individuals m−2) and biomass of earthworm ecological groups and earthworm species in different agricultural uses within fertilized management (mean, n = 3)

Land use	Abundance of ecological groups (individuals m-2)		
	Epigeic *(E.tetraedra*	Endogeic *(O.transpadanus)*	Anecic *(A.caliginosa meridionalis)*
Cereal crop (BeC)	0	16	0
Maize (BeM)	7,3	2,3	0
Rice 1 (BeR1)	0	143	88
Rice 2 (BeR 2)	0,3	0	0
	Biomass of ecological groups (g m-2)		
Cereal crop (BeC)	0	14,7	0
Maize (BeM)	0,7	0,5	0
Rice 1 (BeR1)	0	37,8	43,3
Rice 2 (BeR 2)	0,1	0	0

Analyse of the communities structures

The first axis (axis 1) of the Principal Component Analysis was mainly defined by *O. transpadanus*, while *M. dubius, Lumbricus sp.* and *A. c. paratypicus* defined the second axis (axis 2) (*Figure 4a*). The projection on the first factorial plane (*Figure 4b*) showed that within organic farmer management, the land uses were similar even if BdM and BdP were marginally different from BdC. In contrary, no similarities were observed within fertilized management (site 3). This result could be explained by the high pedological heterogeneity observed in the site 3.

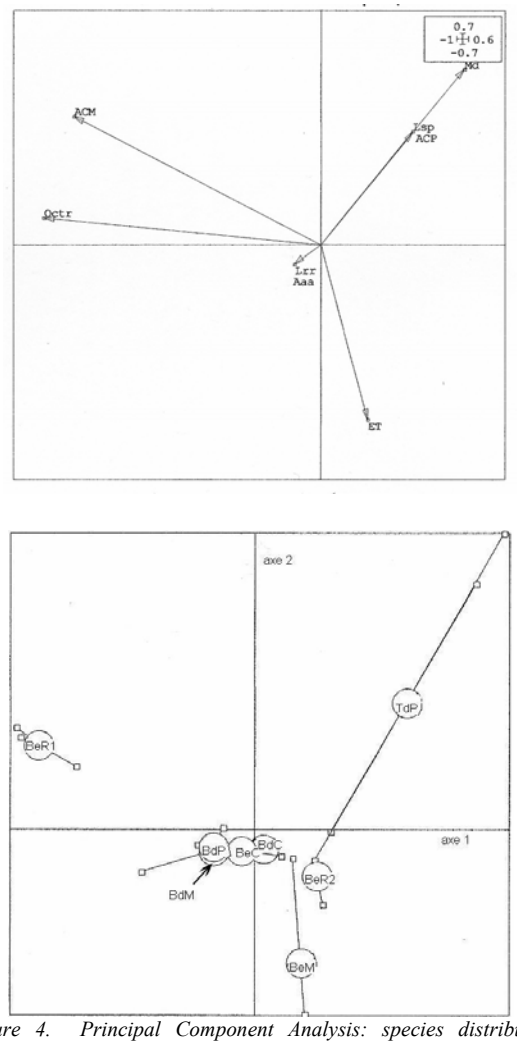

Figure 4. Principal Component Analysis: species distribution (Figure 4a) and projection of variables (Figure 4b).

4. Discussion

Earthworm biomasses in cultivated soils are usually lower than 50 individuals.m-2 (Gerard et Hay, 1979; Andersen, 1980; Edwards, 1983). In cereal agro system, their abundance can be lower than 10 ind. m-2

until disappearing, whereas in pasture they can reach 400 ind. m-² (Bachelier, 1976). Concerning the three agricultural managements studied in this work, the earthworm population parameters as abundance and biomass were very low, especially under pastures: 32.6 ind.m-2 under temporary pasture in organic farming management despite the fact that there is no tillage since September 2003, and no use of pesticide; 46.5 ind.m-2 under permanent pasture in traditional management despite the no tillage and the fertilisation. This result suggests that the constraints of these sites strongly altered earthworm population. This observation is reinforced by the distribution of the ecological groups: in most cases, anecic and epigeic species missed. However, the three ecological groups which characterized earthworm populations have complementary function in soil. Thus, the unbalance noted in the different study sites underlines the strong actual or former anthropic constraints. Moreover, only few earthworm species were recorded (5) and most of them were rare. These species are characteristic of the wet soils observed in Pavie region, resulting from the formation of the alluvial plain and its strong anthropization.

The difference of earthworm abundance and biomass between organic farming and traditional managements, even if it was not significant (32.6 ind.m-2 vs. 46.5 ind.m-2) could be explained by the fertilization in traditional management and also by the no plough of soil. In both pastures, epigeic species miss (in traditional management, Acp was rare). This finding contrasts with several studies which showed that epigeic species are very important in pasture (Bachelier, 1978; Pérès, 2003). In temporary pasture in organic farming management, this miss could be explained by the high predation in alluvial plain. In permanent pasture in traditional management, it was probably a result of the cow trampling. Moreover, anecic species also miss in both pasture sites. In organic farming, this result could be explained by the fact that this pasture is a temporary one included in a rotation: in September 2003, the plough could have strongly decreased the earthworm population as several studies have showed (Evans & Guild, 1948; Curry *et al.,* 1995; Curry et al., 2002). Anecic are very sensitive to soil tillage, because of their large size, they are more affected by the plough than the endogeic species (Rovira *et al.,*1987; Wyss & Glasstetter, 1992); moreover, the annual destruction of their burrow disturbs these species which live in sub-vertical permanent burrows (Lee and Foster, 1991). Furthermore, these anecic species recolonize very slowly a site because of their speed of reproduction and their moving (Pérès et al., 2006). The absence of anecic species in permanent pasture in traditional management could not be explained by the management which should be benefit for anecic species (enough food resource, no soil tillage); only the cow trampling could explain this absence. In the other hand, Nuova (traditional) site presents a more sandy soil texture than Orsine site (organic), which could have a bad influence on earthworm population: sand has a direct bad effect because of the abrasive properties, and has an indirect bad effect by creating a filter soil (Guild, 1948; El-Duweini and Ghabbour, 1965; Edwards and Lofty, 1972; Binet, 1993; Pérès *et al.,* 1998).

Concerning the structure of earthworm population in maize in fertilized management, the low values of abundance, biomass and species richness could be explained by several parameters. As we have observed for the temporary pasture, the soil tillage is well known to markedly decrease earthworm population. So the plough realised each year depressed the fauna population. Moreover, the use of some pesticides could have negative impact on the development of some earthworm species (abundance, biomass, reproduction) that influenced the specific structure of the population (Duddington, 1961; Cluzeau *et al.,* 1987; Cluzeau & Fayolle, 1988; Texier *et al.,* 1995; Tebrüge & Düring, 1999; Ablain, 2002). Furthermore, the chemical soil analysis of this site (table 2) shows that soil contents high values of element as Cu, Zn, Pb. A part of those elements could come from the pesticides and also from the sewage sludge. Earthworms are particularly sensitive to copper. Malecki et al. (1982) studied the effect of different heavy metals on *Eisenia foetida*; copper, given as nitrate, reduced reproductive rates at 100 mg/g. Van Rhee (1967, 1969, 1975, 1976) found that if copper concentration is >80 mg/g, earthworms are almost completely eradicated from orchards. Paoletti (1988) and Paoletti et al., 1988, 1995 observed a negative correlation between copper and earthworms in vineyards of north-eastern Italy. So, most of the characteristics of the fertilized management site (land uses, pedological constraints) could explain the earthworm population structure recorded in maize.

The study of the specific structure of earthworm population showed some differences related to the agricultural management. *Octodrilus transpadanus,* which is the most abundant species recorded in our study, presents a large distribution in central Europe, and is recorded in all the different soil types (Rosa, 1884; Bouché, 1978); its abundance is especially large in wet soils as marsh or banks. Observed in both organic farming management and fertilized management, this species missed in traditional management. In this last site, the sandy texture generates very important variations of soil water content that could explain the absence of this endogeic species. Even if this pasture is permanent, it seems that the embankment and the pedological conditions involved a decrease of the earthworm population and allowed the installation of small less vulnerable

species. So, the agricultural management could partly explain the results observed, however the land uses and the mesological and pedological constraints explained in addition the differences. Thus, the earthworm species should be an indicator, but in our study more an indicator of mesological conditions than of agricultural management.

The earthworm population recorded within a same agricultural management, appeared to be strongly influenced by the land uses and pedological context. Within the organic farming management, the plough realised each year in the crop rotation, appeared to have been unfavourable for earthworm populations in terms of abundance (4.3 individuals m−2), and biomass (3.5 gm−2). This bad effect was not balanced by the organic input (cow manure) realised at the same time. This finding confirms the major negative impact of soil tillage on earthworm population. Furthermore, several studies have described the toxicity of slurry, depending on the ammoniac content. In maize, the absence of plough and the presence of cultural residues on soil surface (food resource and protection from predation and climate constraints) explained the values of fauna. This confirms the need to protect soil surface in order to improve biological soil quality. The global characteristics of the temporary pasture (grass cover, organic input, no tillage) explained the highest values of earthworm population (even if these values are not as large as those found in the literature), and confirmed that within a same agro-pedological context, the pasture system is the most favourable for earthworm population (Pérès et al., 2006). Three species were record, but only *Octodrilus transpadanus* was not a rare species. The low value of endogeic species in temporary pasture was explained by the large quantity of juveniles, and thus a growth ratio (juvenils/adultes) which showed the restoration of the earthworm population. The large biomass and low abundance values for maize (BdM) and crop rotation (BdC) were linked to the presence of adults and also the presence of *Aporrectodea caliginosa meridionalis* (BdM and BdC) et *Lumbricus rubellus rubellus* (BdM).

Within the fertilized management, the earthworm populations were strongly affected by the pedological conditions: the hydomorphic and anoxic conditions observed in rice 2, explained the so low abundance of earthworms. This reductisol presented chemical and physical conditions that only epigeic species (E*iseniella tetraedra)*, because they always stay at the soil surface, could accept. The earthworm population was marginal in rice2, compared to earthworm population in Rice1 (231 ind. m-2), where the soil was a Brunisol. This finding suggests that pedological constraints could alter significantly greater earthworm population than land uses.

Moreover, spread of sewage sludge explained as well the low values observed in maize and cereal crop. The well known toxicity of Cu, Pb and Zn on earthworms combined to the recent soil tillage (BeC) and to the late harvest of maize in autumn (that compacted soil surface) could inform on the absence of anecic species. However, the study of the toxicity is not easy, because accumulation and toxicity of element are very variable depending on the earthworm species (Zusuki et al, 1980; Kruse et Barett, 1985; Barrera et al, 2001) and the ecological groups (Ireland, 1979; Ash et Lee, 1980; Ireland et Richards, 1981). *Octodrilus transpadanus* which was the most common species in our study, was not present in Rice2, confirming that the agro-pedological conditions were too much selectif for this species. E*iseniella tetraedra,* which was recorded under rice2, was also observed under maize. This finding suggests that soil maize is frequently saturated. This is confirmed by the hydromorphic features observed in the soil sample. The earthworm species appeared to be good indicator of soil characteristics.

In vineyards agrosystem, Cluzeau et al. (1998) demonstrated that earthworm biomass and abundance were correlated to microbiological biomass, and that, comparing conventional management to integrated management; these two biological components could reveal the anthropic constraints. The use of earthworms and micro organisms as indicators of agricultural management is thus possible. However, the results observed in our study demonstrated that parameters as abundance, biomass, species structure of earthworm population are strongly influenced by the agricultural practices (soil tillage, organic input ...) and the pedological context (physical and chemical characteristics). Thus in order to assess the relevance of these biological population parameters as bioindicators of agricultural management, it would be necessary to compare different agricultural managements by maintaining in addition the other things equal (land uses and pedological context).

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Impact of different agricultural practices on soil genotoxicity

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The presence of genotoxic chemicals in soil can negatively affect crop yield and human health. In this study *Trifolium repens* was used as bio-indicator to assess the impact of three different agriculture practices on soil genotoxicity. Three separate sites representative of the three different agricultural management systems were selected inside Pavia Province, Italy. Two biomonitoring experiments were performed in autumn 2004, after plant harvest and in summer 2005, just after soil preparation. Genotoxicity was evaluated with AFLP molecular markers. Results showed that all the three soils induced DNA damage in the indicator-plants. Nevertheless, on the base of the present findings, biodynamic agricultural management system seems the best farming approach to maintain soil quality with regard to genotoxicity.

1. Background

In the recent past, soil quality has attracted special attention the world over. A good soil quality is in fact fundamental to protect and improve long-term agricultural productivity, water quality, and habitats of all organisms including people.

Because of its high retention capacity, soil is very vulnerable to contaminant accumulation. Agriculture practices can introduce an abundance of substances into soils reducing their quality. Among these substances genotoxic compounds are of great concern. Genotoxicity is in fact one of the most dangerous effects of contaminated soil, since many xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs), heavy metals, and pesticides, are demonstrated to be DNA damage inducers (Klassen, 1995). Genotoxic compounds in soil can reduce crop productivity, can induce the build-up of resistance plant species and can negatively affect living organism health.

For this reason it is important to evaluate the impact of various agricultural management systems on soil genotoxicity.

At this regard physical and chemical methods for soil analysis do not provide sufficient information, since most soil genotoxics are unknown and the standard chemical analyses can assess the dangerousness of pollutants only in relation to the concentration of major contaminants and not also to the exposition time and to their bioavailability. Moreover soil pollutants can induce additive, antagonistic or synergistic effects and soil microflora can convert non-genotoxic compounds to genotoxic derivatives (Watanabe and Hirayama, 2001). In contrast, biological methods allow a direct assessment of genotoxic potential of soil stressors. Biological data can be used to estimate the environmental impact on ecosystem and individual organisms, including humans.

Higher plants can be considered sensitive and efficient bio-indicators of genotoxicity. They can be exposed for periods of few minutes to days or weeks. They are easy to handle, inexpensive and although the genotoxic effects observed in plants can not be extrapolated directly to human populations, the finding of plant bioassays may be taken into account for these purposes (Guimarães, 2000).

The present report examines agricultural activity in an environmental context and focuses on farming systems as the main vehicle for maintaining or improving soil and living organism health. In particular, the impact on soil genotoxicity of the three following different agricultural management systems were investigated: (1) biodynamic ecological farming system (Ali and Ismail, 2003), (2) traditional agriculture system using manure, dilute liquid sewage and mineral fertilizer (3) agriculture system using stabilized sewage sludge.

Soil genotoxicity was assessed by using the plant bio-indicator *Trifolium repens* L. cv Regal, since its documented sensitivity to organic and inorganic compounds (Young et al., 1995; Dueck et al., 2003). DNA damage induced by agricultural soils in the test-plant was detected with Amplified Fragment Length

Polymorphism (AFLP), which is a very sensitive molecular tool allowing the detection of DNA fragmentation and uniform or chromosomal mutations (Bagley et al., 2001; Citterio et al., 2002). Results obtained for the three different systems were analysed and compared.

2. Experimental section

2.1 Study area

Three separate sites representative of the following three different agricultural management systems were selected inside Pavia Province (North Italy, *Figure1*).
The first site, inside the "Cascina Orsine" farm, is located near the urban centre of Bereguardo. In this

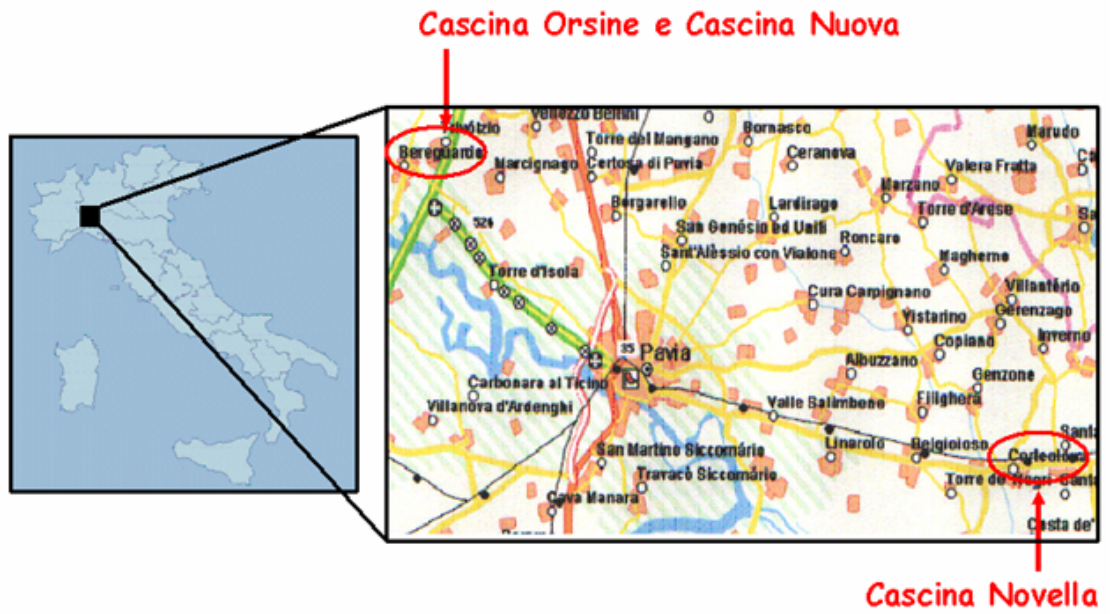


Figure 1 Map showing the location of the 3 study sites, C. Orsine, C. Nuova and C. Novella farms, representative of the three different agricultural management systems examined in this work.

area a biodynamic agriculture (BD) has been practiced for 25 years. Biodynamic agriculture is an advanced organic farming system that relies heavily on compost as a fertilizer. This is a system which is gaining increased attention for its emphasis on food quality and soil health.
The second site, inside the "Cascina Nuova" farm, is also located closed to Bereguardo. Agriculture practices consist of soil treatments with manure, dilute liquid sewage and mineral fertilizer 15N, 15P, 15K (150 Kg/ha).
The last site, inside the "Cascina Novella" farm, is located near Corteolona. This field has been treated with stabilized sewage sludge (about 360 q/ha) for 10 years.

2.2 Bio-monitoring experiment: soil sampling and test-plant exposition

T. repens L. seeds cv. Huianz (Ingegnoli, Milan, Italy) were directly grown in 3% organic matter soil for 15 days. The nearly 5-cm high plantlets were used as indicator-plants. Nine soil samples (0-30 cm) from each site were collected according to Jolivet sampling method (2003). Two sampling campaigns were carried out during September 2004 and July 2005 before and just after soil preparation, respectively. For each site, the collected soil samples were carefully mixed and six pots were prepared. Two-week-old test-plants were transferred to the 18 pots containing agricultural soils (30 plants per pot) and to other 6 pots filled with non-contaminated soil (control pots). Pots were placed in a growth room (25°C; 10 h dark/ 14 h light; 150 µmol m^{-2} sec^{-1}) for 15 days. At the end of exposition time, the percentage of plant survival and the shoot and root growth (fresh and dry weight) of healthy plantlets were measured and AFLP analyses was carried out.

2.3 AFLP (Amplified Fragment Length Polymorphism)

AFLP was used to quantify DNA damage in the white clover plants exposed to agricultural soils. The DNA was isolated using DNeasy isolation and purification kit (Qiagen, Italy) to obtain high quality DNA, free of polysaccharides or other metabolites which might

interfere with restriction endonucleases. The analysis was based on the principles described by Vos et al.(1995) and performed as described in the European Patent 0534858 (Keygene, Belgium) except that genomic DNA (200 ng) was digested (3 h) with EcoRI (6-base cutter) and MseI (4-base cutter) and legated with EcoRI adapter (5 pmol) and MseI adapter (50 pmol). The sequences of adapters, of primer pairs used in the pre-amplification reaction (M01 and E01) and the seven pairs of primers used for the amplification reaction were reported in *Table 1*.

Tale.1. Sequences of adapters and primers used for AFLP analysis. The following seven pairs of primers were used for amplification reactions: E31-M32, E39-M39, E35-M35, E35-M31, E39-M35, E31-M34 and E31-M39.

Adapters and primers	***DNA sequence***
EcoRI adapter	5'-CTCGTAGACTGCGTACC-3' 3'-CTGACGCATGGTTAA-5'
EcoRI + 1 primer (E01)	5'-GACTGCGTACCAATTCA-3'
EcoRI + 3 primer (E31)	5'-GACTGCGTACCAATTCAAA-3'
EcoRI + 3 primer (E35)	5'-GACTGCGTACCAATTCATA-3'
EcoRI + 3 primer (E39)	5'-GATGAGTCCTGAGTAAAGA-3'
MseI adapter	5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
MseI + 1 primer (M01)	5'-GATGAGTCCTGAGTAA A-3'
MseI + 3 primer (M31)	5'-GATGAGTCCTGAGTAAAAA -3'
MseI + 3 primer (M32)	5'-GATGAGTCCTGAGTAAAAC -3'
MseI + 3 primer (M34)	5'-GATGAGTCCTGAGTAAAAT-3'
MseI + 3 primer (M35)	5'-GATGAGTCCTGAGTAAACA-3'
MseI + 3 primer (M39)	5'-GATGAGTCCTGAGTAAAGA-3'

The analysis of the DNA amplification products was carried out as follows: 1.5 µl of the PCR-amplified mixture was added to an equal volume of loading buffer (80% formamide, 0.01% xylene cyanol FF, 0.01% bromophenol blue, 10 mM EDTA, pH 8.0), denatured for 5 min at 92°C, loaded onto a 4.5% sequencing polyacrylamide gel and electrophoresed in TBE electrophoresis buffer for 3 h at 80 Watt. The gel was then fixed in 10% acetic acid and exposed to an X-ray film (Kodak, Rochester, NY, USA). Visual inspection of the resulting autoradiograms allowed scoring of polymorphic bands. For statistical analysis, each AFLP band detected after electrophoresis of the amplification DNA products was scored as a binary character for its absence (0) or presence (1). The percentage of polymorphism (P %), that represents the ratio between the number of polymorphic bands and total detected bands x 100, were determined and data were analysed using the software program Statgraphics plus for Windows version 4.0 (Manugistic, Maryland USA). The statistically significant differences between each treated sample and the control were obtained by applying the comparison of proportion analysis.

3. Results

3.1 Indicator-plant development was not different in the three farming soils

The effect of soils from the three farms on plant development was evaluated by measuring plant survival and organ fresh and dry weight (FW and DW) after 15 days of clover plantlet exposure to soils.
Shoot and root FWs of indicator-plants are reported in *Figure 2*.

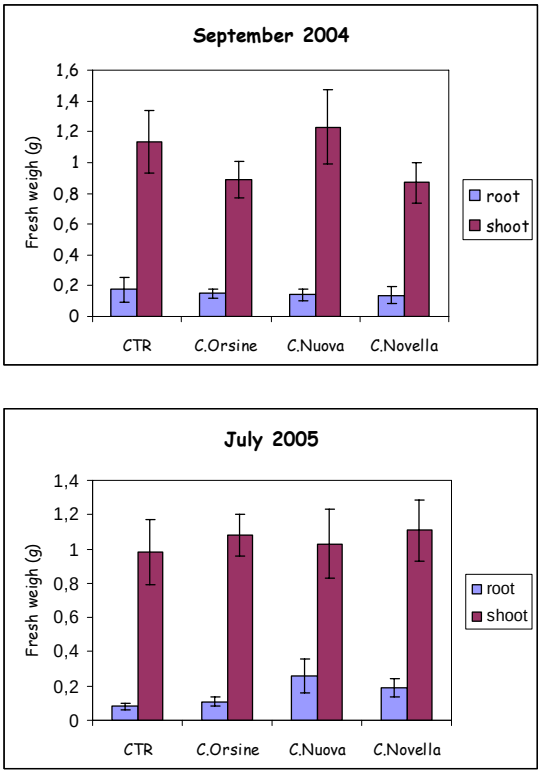


Figure 2 Fresh weigh (mean±standard deviation) of test plants grown in the differently treated farming soils. No significant difference among plants grown in the three agricultural soils and no difference between September and July bio-indication experiments can be observed (ANOVA, P<0.05).

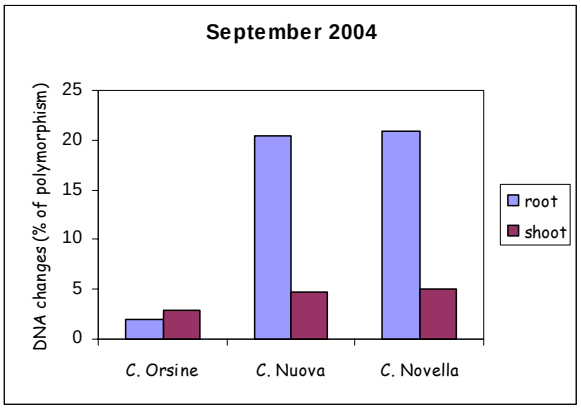
On average, FW and DW of the three groups of plants exposed to the agricultural soils were not statistically different and were not different from control values (ANOVA P<0.05). Moreover no statistical differences were detected between September and July bio-indication experiments.

3.2 Significant DNA changes were induced by farming soils

DNA damage was evaluated by AFLP technique, which detects alterations at the restriction sites of *MseI* and *EcoRI* enzymes and between the two restriction sites (i.e. deletions or insertions). Seven primer combinations were applied for root and shoot analysis. A total of 225 and 218 reproducible bands and a total of 211 and 210 bands were analyzed for root and shoot during September and July experiments, respectively. During September approximately 0,87% and 1.57% of analyzed bands and during July 1,06% and 1,47% were polymorphic among control roots and shoots.
These values were considered the basal polymorphic levels among *Trifolium repens* L. plants (i.e. intra-species variability) in the two bio-monitoring experiments. *Figure 3* presents an example of AFLP analysis where root DNA from plants exposed to different farming soils was compared with root DNA from control plants. Some polymorphisms were evident and were related to the changes in DNA sequence caused by genotoxic compounds present in the soil.

Figure 3. an example of AFLP analysis of root DNA from plants exposed to control and to farming soils. Arrowheads indicate some polymorphic bands.

Taking into account all the independent repetitions, DNA damage induced by each of the three differently treated soils were calculated as the percentage of polymorphism (P %) of exposed plants respect to control plants and reported in *Figure 4*.
In September experiment, soil from Cascina Orsine did not affect test-plant DNA, whereas soils from Cascina Nuova and Cascina Novella induced a percentage of polymorphisms in shoot and root statistically higher than that of the control. Both for Cascina Nuova and Cascina Novella, plant damage was about 4 fold greater in root than in shoot.



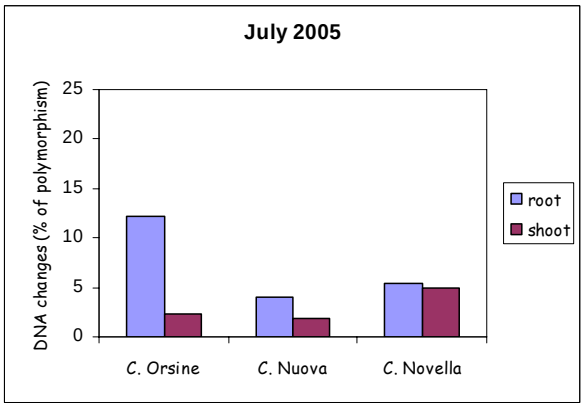


Figure 4. analysis of the percentage of polymorphism (P% = number of polymorphic loci/number of total loci) detected by AFLP in the DNA from the shoots and the roots of plants exposed to farming soils. Asterisks (*) indicate statistically significant differences as compared to controls (P<0,05).

The effect of soils collected in July, just after soil preparation was different: DNA damage was greatest in root of test-plants grown in Cascina Orsine soil although the percentage of polymorphism was restricted to roots and was approximately one half than that calculated for roots of plants grown in Cascina Nuova and Cascina Novella soils collected in September. Cascina Nuova soil induced also a significant DNA damage, but it was less extended. A

similar level of polymorphism was detected in plants grown in Cascina Nuova soil although the damage was not restricted to root but interested also plant shoot.

4. Discussion

Soil is a fundamental natural resource for agriculture. Successful farmers recognize that preservation of healthy, high-quality soils is essential to profitable and sustainable crop production (Kleinhenz and Bierman, 2001). One aspect of soil quality is related to the presence of genotoxic chemicals which can reduce crop yield and negatively affect human health. In farming soils, this type of xenobiotics can essentially originate from atmosphere deposition, from irrigation water and/or from agriculture practices. The chemical analyses, performed by Ispra researchers revealed that in the present study-case atmosphere and fertilizers were two of the genotoxic chemical sources. For example the considerable amount of polychlorinated dibenzodioxins and dibenzofurans (PCDD/F) found in all the three soils are likely ascribed to atmospheric depositions, whereas the higher amount of heavy metals detected in Cascina Novella soil is likely due to the recurring application of sewage sludge. Nevertheless, as explained in the introduction chemical analytical tools are not sufficient to establish the genotoxic potential of a soil and only the combination with a bio-indication system can help to assess the impact of different farming practices on soil genotoxicity. Experimental results showed that after plant harvest (September 2004 experiment) the only soil which did not induced any significant alteration in test-plant DNA was that from Cascina Orsine, where a biodynamic agriculture (BD) has been practiced for 25 years. However the same soil just after preparation for the new sowing (July 2005 experiment) induced DNA changes only in the roots. This suggested that Cascina Orsine BD practices introduced in the soil genotoxic substances or compounds that soil microrganisms and plants converted to genotoxic derivatives. A change in bioavailability of genotoxic substances should be also considered, although soil pH did not significantly change from September 2004 to July 2005. It is likely that these genotoxics were organic compounds because no DNA damage was detected in the test-plant shoots. Usually, for their chemical properties, organic substances such as PAHs are most retained in the root whereas heavy metals are transported to shoot inducing DNA damage also in this organ. The other two farming soils induced DNA damage in both September and July experiments. After plant harvest (September 2004) the "genotoxic activity" of the two soils was very high and both test-plant roots and shoots were affected. This genotoxicity levels were higher than those assessed just after soil preparation (July 2005). It means that, in spite of the introduction of fertilizers, at least part of the genotoxic compounds detected in September 2004 experiment were degraded or make less available or eliminated from the first 0-30 cm of soils before July 2005 sampling. This result needs a discussion considering that no increase in soil pH was detected after soil preparation and that the AFLP data were reliable because many repetitions were carried out. We can make different hypotheses. We can suppose that the previous soil treatments (July 2004) introduced in the soils more genotoxic chemicals than that performed in July 2005 or that Cascina Nuova and Cascina Novella practices introduced in the soil non genotoxic substances which need time to be converted in genotoxic derivatives by soil microrganisms. A further hypothesis is that irrigation water used during summer 2004 plant cultivation contained genotoxics. Only additional experiments will help to clarify this issue and to assess the real impact of the three type of farming practices on soil genotoxicity. On the base of the present findings, Cascina Orsine agricultural management system seems the best farming approach to maintain soil quality with regard to genotoxicity.

5. Acknowledgments

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Sanitary Risk assessment for specific areas in the Province of Pavia

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In the characterization study of the soils in the Province of Pavia three agricultural areas, using different manure methods have been considered. The three sites have been selected in order to assess, by computerized models of absolute health risk analysis, the effects on the environment and the human health coming from a continuous agricultural activity with amendant The assessment of the three agricultural areas in the Province of Pavia shows, that there is not any risk situation also in the "Worst-case" conditions, i.e. in a multiple and contemporaneous exposure for soil ingestion and soil dermal contact, and besides, for ingestion of the same soil vegetables.

1. Introduction

The presence of inorganic and organic pollutants in soil and subsoil over specific acceptability levels, in national and international guidelines provided, can have a negative effect for all the ecosystems and natural resources and, consequently, for the human health.
Therefore, it is very important to know the soil quality, also because the soil is used for agriculture and, consequently, the inorganic and organic contaminants can enter in the food chain [1].
In the characterization study of the soils in the Province of Pavia three agricultural areas, using different manure methods have been considered.
Two areas are situated in the Commune of Bereguardo and one in the Commune of Corte Olona. The areas situated in the Commune of Bereguardo are one near the farm Cascine Orsine, the other near the farm Cascina Nuova; the third, (Commune of Corte Olona) is in the Cascina Novella.
The three sites have been selected in order to assess, by computerized models of absolute health risk analysis, the effects on the environment and the human health coming from a continuous agricultural activity with amendant [2].
The non-carcinogenic effects are quantified by risk analysis models, by means of the Chronic Risk Index assess HQ (Hazard Quotient). The parameter HQ indicates, how many times the daily average dose, calculated on the real exposure, exceeds the reference dose [3-9].
A value of HQ<1 indicates, that there is not risk, whereas a value of HQ>1 indicates, that the most sensible population can have pathological effects. If the exposure route for one or more chemical pollutants are more than one (except synergistic or antagonistic effects), the additive property counts, i.e. HQ_{tot} is equal to the summation of all the HQ of every exposure route [3-9].
Today, the more used risk analysis software, that utilize the Chronic Risk Index as base criterion (HQ), are four and precisely two are Italian, ROME 2.1 version (APAT) [10], GIUDITTA 3.1 version (Province of Milan) [11], one is English, RISC 4.0 version (British Petroleum) [12] and one is American, RBCA Tool Kit 1.3 version (ASTM-EPA) marketed by GSI Inc. [13].
All four models are generally applied, in order to assess the risk related to the different exposures in contaminated sites under decontamination. The RISC has been used for the study in object, because only this model, among the different exposure scenarios, provides the risk probability calculation for the vegetables human consumption.
At the moment, there is not any regulation, defining the agricultural soil quality; therefore, for the acceptable limit concentrations in soil, it is possible to refer to the Table 1 column A of the Ministerial Decree (MD) 471/99 related to the public, green and residential soil, as proposed by the National Institute of Health [14].

The analysis has been carried out in accordance with the "Methodological criteria for the absolute risk analysis application to the contaminated sites" Rev. 0 June, 2005 – APAT, ARPA, ISS, ISPESL and ICRAM Edition.
The values of the chemical-physical and toxicological parameters used for the inorganic and organic substances selected, are reported in the database ISPESL-ISS, already processed for the above-mentioned document.

2. Conceptual model of the site and health risk scenarios

The conceptual model of the three studied sites considers [15]:

- the source of potential contamination;
- the route of identified contaminants (up to exposure point);
- the targets.

As "contamination source" only the surface sampled and analysed soil has been considered.
As "contaminant route" in this case the leaching in ground waters risk has not been considered, because it has been assessed non-significant in the studied areas; but, the potential risk of contaminant migration from plants to soil, by radical uptake, has been considered.
As "targets" only the adult resident has been considered, both for direct exposure by dermal contact and soil ingestion and for indirect exposure by food ingestion, coming from soils in object (vegetables as roots or whole plant) [16] [17].
The risk analysis software has been used on the basis of the studied substances, and precisely the metallic micro pollutants as Arsenic, Cadmium, total Chromium (III), Copper, Lead, Mercury, Nickel and Zinc, and organic micro pollutants as total PCB summation.
The maximum concentration values of the substances have been entered in the software, because the available values were insufficient for a suitable statistic processing, and, in order to adopt the "protective" concept, the worst case has been selected [18].
In *Table 1* the analytical results for every studied micro pollutant are reported: the values are expressed in dry substance (d.s.) mg/kg. These maximum concentrations have been used as "source" in the software, as above mentioned.

Table 1. Maximum concentrations in the three studied sub areas.

Metals	Concentrations expressed	Site Cascine Orsine	Site Cascina Nuova	Site Cascina Novella
As	mg/kg	9,7	9,8	22,4
Cd	mg/kg	0,33	0,31	0,84
Cr	mg/kg	34	32	61
Cu	mg/kg	13,1	12,8	30,8
Hg	mg/kg	0,005	0,05	0,09
Ni	mg/kg	20,4	22,3	34,5
Pb	mg/kg	18,5	16,9	29
PCBs	mg/kg	0.0046	0.0041	0.0032
Zn	mg/kg	61	57	95

In the tables x-y the "input" values used in the model RISC 4.0 version, in the study adopted, are reported in *Table 2* [19].

Table 2. Summary of input parameters

LIFETIME AND BODY WEIGHT	
Body Weight (kg)	70
Lifetime (years)	70
INGESTION OF SOIL	
Soil Ingestion Rate (mg/day)	1.00E+02
Exp. Frequency Soil (events/year)	3.50E+02
Exp. Duration Soil (years)	30

Absorption Adjustment Factor for			
Ingestion of Soil (-)		Soil Bioavailability (-)	
Arsenic	1.0	Arsenic	1.0
Cadmium	1.0	Cadmium	1.0
Chromium (III)	1.0	Chromium (III)	1.0
Copper	1.0	Copper	1.0
Lead	1.0	Lead	1.0
Mercury	1.0	Mercury	1.0
Nickel	1.0	Nickel	1.0
PCBs	1.0	PCBs	1.0
Zinc	1.0	Zinc	1.0

DERMAL CONTACT WITH SOIL			
Total Skin Surface Area (cm^2)			23.0E+03
Fraction Skin Exposed to Soil (-)			0.25
Adherence Factor for Soil (mg/cm^2)			1.0
Exposure Freq. Soil (events/year)			3.50E+02
Exposure Duration Soil (years)			30
Absorption Adjustment Factor for			
Dermal Expos. to Soil (-)		Soil Bioavailability (-)	
Arsenic	3.00E-02	Arsenic	1.0
Cadmium	1.00E-02	Cadmium	1.0
Chromium (III)	1.00E-02	Chromium (III)	1.0
Copper	1.00E-02	Copper	1.0
Lead	1.00E-02	Lead	1.0
Mercury	0.10	Mercury	1.0
Nickel	1.00E-02	Nickel	1.0
PCBs	0.10	PCBs	1.0
Zinc	1.00E-02	Zinc	1.0

INGESTION OF ROOT VEGETABLES INGESTION OF ABOVE GROUND VEGETABLES			
Root Veg. Ingestion Rate (g/day)			88
Above Ground Veg. Ing. Rate(g/day)			1.27E+02
Fraction Organic Carbon in Soil g/g			5.00E+02
Frequency Veg. (events/year)			3.50E+02
Exp. Duration Veg. Intake (years)			30
Fraction grown in home garden (-)			0.25
Koc (mg/l / mg/l)		log Kow	
Arsenic	N.D	Arsenic	N.D
Cadmium	N.D	Cadmium	N.D
Chromium (III)	N.D	Chromium (III)	N.D
Copper	N.D	Copper	N.D
Lead	N.D	Lead	N.D
Mercury	N.D	Mercury	N.D
Nickel	N.D	Nickel	N.D
PCBs	1.58E+0.5	PCBs	6.3
Zinc	N.D	Zinc	0.0
Vegetab.UptakeFactor[-] (from chemical database)		Kd [(mg/L)/(mg/kg)] (from chemical database)	
Arsenic	4.00E-02	Arsenic	29
Cadmium	0.55	Cadmium	37
Chromium (III)	N.D	Chromium (III)	2.00E+05
Copper	0.40	Copper	35
Lead	N.D	Lead	55
Mercury	0.90	Mercury	82
Nickel	4.00E-02	Nickel	88
PCBs	N.D	PCBs	N.D
Zinc	1.5	Zinc	0.0

3. Results and discussion

The inorganic micro pollutants concentrations in the three studied areas are fully under the MD 471/99 limits for the residential/green/public areas, that, as above mentioned, have been equalized to an agricultural soil.

The organic micro pollutants concentrations (PCB_s) in the three studied areas exceed the MD 471/99 limit (Table 1, column A), 0.001 mg/kg d.s.

It is important to observe, that this MD limit value for the parameter PCB_s does not consider the anthropic value of today.

It is knowledge, that the PCB_s concentrations in an anthropic area are generally included between 0.01 and 0.05 mg/kg d.s.; therefore, the value of 0.001 mg/kg d.s. (MD 471/99) is wrong and the new environmental regulation (Legislative Decree 152/2006) has changed the limit value from 0.001 to 0.060 mg/kg d.s.

Even though the inorganic parameters concentrations in the three studied areas are always under the decree limit values, the risk analysis has been carried out, because the MD 471/99 values have been calculated on the basis of a direct exposure, as ingestion, dermal contact, steam inhalation from soil, but the indirect exposure, as ingestion of contaminated soil vegetables, and, therefore, the risk of contaminants input in the soil-plant system have not been considered.

The risk analysis, as above mentioned, has been carried out by assessing the cumulative chronic index risk (Hazard Quotient) for all the substances, i.e. by summing all the chronic risk index of every considered substance.

In *Tables 3-4-5* the results for the three studied areas are reported: for every area, the chronic risk index, both for every substance, and for all the substances (cumulative risk) is fully lower than 1; therefore it is possible to conclude, that there is not any hygienic-health risk situation.

Table 3. Summary of Hazard Quotients (Cascina Novella)

	Ingestion of soil	Dermal contact of soil	Roots ingestion	Above ground vegetables ingestion	Total
Arsenic	1.00E-01	1.80E-01	1.30E-01	1.90E-01	6.10E-01
Cadmium	2.30E-03	1.30E-04	4.20E-02	6.00E-02	1.00E-01
Chromium (III)	5.60E-05	3.20E-05	0.00E+00	0.00E+00	8.80E-05
Copper	1.10E-03	6.60E-04	1.50E-02	2.20E-02	3.80E-02
Lead	1.10E-02	6.30E-03	0.00E+00	0.00E+00	1.70E-02
Mercury	4.10E-04	2.40E-03	1.20E-02	1.80E-02	3.30E-02
Nickel	2.40E-03	1.40E-03	3.10E-03	4.50E-03	1.10E-02
PCBs	2.20E-04	1.30E-03	1.40E-04	9.40E-05	1.70E-03
Zinc	4.30E-04	2.50E-04	2.10E-02	3.10E-02	5.30E-02
Total	1.20E-01	1.90E-01	2.30E-01	3.30E-01	8.60E-01

Table 4. Summary of Hazard Quotients (Cascina Nuova)

	Ingestion of soil	Dermal contact of soil	Roots ingestion	Above ground vegetables ingestion	Total
Arsenic	4.50E-02	7.70E+00	5.90E-02	8.50E-02	2.70E-01
Cadmium	8.50E-04	4.90E-05	1.50E-02	2.20E-02	3.80E-02
Chromium (III)	2.90E-05	1.70E-05	0.00E+00	0.00E+00	4.60E-05
Copper	4.70E-04	2.70E-04	6.20E-03	9.00E-03	1.60E-02
Lead	6.40E-03	3.70E-03	0.00E+00	0.00E+00	1.00E-02
Mercury	2.30E-04	1.30E-03	6.70E-03	9.80E-03	1.80E-02
Nickel	1.50E-03	8.80E-04	2.00E-03	2.90E-03	7.30E-03
PCBs	2.80E-04	1.60E-03	1.80E-04	1.20E-04	2.2E-0.3
Zinc	2.60E-04	1.50E-04	1.30E-02	1.90E-02	3.20E-02
Total	5.50E-02	8.40E-02	1.00E-01	1.50E-01	3.90E-01

Table 5. Summary of Hazard Quotients (Cascina Orsina)

	Ingestion of soil	Dermal contact of soil	Roots ingestion	Above ground vegetables ingestion	Total
Arsenic	4.40E-02	7.60E-02	5.80E-02	8.40E-02	2.60E-01
Cadmium	9.00E-04	5.20E-05	1.60E-02	2.40E-02	4.10E-02
Chromium (III)	3.10E-05	1.80E-05	0.00E+00	0.00E+00	4.90E-05
Copper	4.90E-04	2.80E-04	6.40E-03	9.20E-03	1.60E-02
Lead	7.00E-03	4.00E-03	0.00E+00	0.00E+00	1.10E-02
Mercury	2.30E-04	1.30E-03	6.70E-03	9.80E-03	1.80E-02
Nickel	1.40E-03	8.00E-04	1.80E-03	2.70E-03	6.70E-03
PCBs	3.20E-04	1.80E-03	2.10E-04	1.30E-04	2.50E-03
Zinc	2.80E-04	1.60E-04	1.40E-02	2.00E-02	3.40E-02
Total	5.50E-02	8.30E-02	1.00E-01	1.50E-01	3.90E-01

4. Conclusions

This procedure, especially the mathematical models utilization (software) is still "young", because it has been put to point the first time in the United States ten years ago. Today it is applied especially to assess, whether a soil contaminated by inorganic and/or organic substances, exceeding the provided quality standard, has or has not to be cleared.

In this paper, it has been applied to an agricultural soil, by using market risk analysis software, allowing foreseeing also the risk related to vegetables ingestion (radical and apical parts). Unfortunately, for this exposure scenario, there are not national reference standards (Italian average consumption of vegetables, etc.); therefore, the procedure adopted in this paper is still at the experimental stage and is a first application try to this case.

As above mentioned, at national level there are not specific quality standards for agricultural soils (standards for residential/public soils are temporarily adopted); therefore, the hygienic-health risk assessment (specific-site) for agricultural soils is helpful, to determine, whether the substances concentrations in these soils are acceptable or unacceptable.

The assessment of the three agricultural areas in the Province of Pavia shows, that there is not any risk situation also in the "Worst-case" conditions, i.e. in a multiple and contemporaneous exposure for soil ingestion and soil dermal contact, and besides, for ingestion of the same soil vegetables. Therefore, this assessment can be considered very protective for the human health to evaluate if concentrations of substances detected in these soils are acceptable or not.

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