European Science Foundation

Standing Committee for Life, Earth and Environmental Sciences (LESC)

ESF LESC EXPLORATORY WORKSHOP

Non-molecular manipulation of soil microbial communities

SCIENTIFIC REPORT

University of Udine
Udine, Italy, 18-20 October 2004

Convened by:
Philip C. Brookes[1] and Maria De Nobili[2]

[1] Rothamsted Research, UK
[2] Dipartimento di Scienze Agrarie e Ambientali, Università degli studi di Udine, I

Non-molecular manipulation of soil microbial communities

Little is known of the physiological adaptations of soil micro-organisms (collectively the soil microbial biomass) which equip it for survival in the soil environment. While soil organic matter can provide sufficient energy for considerable basal metabolism of the soil micro-organisms for months or even years, it does not provide sufficient energy to increase, or even maintain, this biomass. In the field, inputs of fresh substrates enter the soil, but they are relatively small compared to the surprisingly large size of the microbial biomass. For example, the microbial biomass in the plough-layer of an arable soil at Rothamsted which has never been fertilized, but where wheat has been grown continuously for more than 150 years, contains about 10 tonnes of living microbial cells - or the weight of 100 live sheep - per hectare. This huge microbial population maintains itself upon a total annual organic input of about 1.2 t C ha^{-1}, little more than twice the amount of C in the standing crop of microbial biomass. Not surprisingly, therefore, this biomass shows many features typical of an extremely substrate limited, or dormant, population. These include a very slow rate of respiration, c.a. 10% of that of micro-organisms growing exponentially in vitro, about one cell division every 6 months on average and a turnover rate of about 1.5 y. Paradoxically, this biomass has an adenosine 5'-triphosphate (ATP) concentration (about 11 μmol ATP g^{-1} biomass C) and adenylate energy charge (AEC = 0.80 to 0.95) typical of exponentially growing micro-organisms in vitro. Both of these features remain unchanged during prolonged laboratory incubations, for months or even years, even without further substrate inputs. Why should this large, mainly dormant, population expend its scarce energy reserves to maintain both ATP and AEC at near theoretically maximal levels? Under such conditions a survival strategy based upon energy conservation, using options such as spores or resting cells with low ATP and AEC would apparently seem more logical. However, oligotrophic marine micro-organisms, which can also survive long periods with scant availability of energy-available substrates, maintain "expensive protein synthesizing machinery necessary for immediate use of any exogenous substrate..." . These apparently contradictory features of a dormant soil microbial biomass, living in a nutrient-poor environment, yet with high ATP and AEC, are identical evolutionary responses to the fact that substrates availability is scarce and discontinuous in both environments, and for this reason must be rapidly utilized whenever they become available. A survival strategy based upon

maintenance of a relatively active or 'alert' metabolic state, despite the expenditure of scarce energy reserves, may have better value, in evolutionary terms, than one based upon resting cells or spores. However, there must be some molecular signal to cause the microbial biomass to switch from an 'alert' to a fully metabolically active state. Otherwise, before dormant cells had increased their metabolism sufficiently to utilise a fresh substrate input, a cohort of more speculative micro-organisms could already have captured it. Even so, the next stage, involving the synthesis of the suites of enzymes involved in full metabolism, is a much more demanding process, in terms of expenditure of both energy and cellular reserves. If it was invoked at the wrong time, this would clearly lead to rapid starvation and death. It would therefore be logical for a cell to become activated only when it senses the proximity of a substrate, in sufficient quantities to support growth and cell division.

Recently it was shown that this activity shift could be initiated in soil by the cells sensing molecular signals or 'trigger molecules' derived from the substrate. These 'trigger molecules' are likely to be soluble, of low molecular weight, readily diffusible in soil solution and naturally derived from the organic residue involved. They are also likely be rapidly degradable so that they are present only in low concentrations in soil solution, declining as the substrate is decomposed. They would not be released from stabilised soil organic matter as false signals would be repeatedly given. The origin of the extra carbon mineralized after stimulation of the microbial biomass by ‘trigger molecules’is not known.

The fact that the activity of the microbial biomass could be manipulatable with molecular signals may open new strategies in the improvement of bioremediation techniques of organic polluted soils. This may be done, for example, by stimulating specific functional groups within the biomass or in developing cocktails of ‘trigger molecules’ to initiate microbes to degrade organic pollutants.

Since the ‘trigger molecules’ cause a greater expenditure of energy by the biomass than is actually obtained from them, it may also be possible to induce multiple stimulation, exhaustion and ultimate elimination of persistent and undesirable micro-organisms such as soil borne plant pathogens.

The successful and safe release of genetically engineered microrganisms (GEMs) in the environment cannot be implemented without a precise knowledge of their

possible survival in soil. The knowledge of the metabolic strategies adopted by native soil microrganisms is a fundamental step in assessing potential risks and benefits. It is possible that a direct manipulation of microbial communities, by means of molecular signals, could however lead to much safer and better results in bioremediation of contaminated sites and bio-augmentation of nutrient release. A safe, biological control of plant pathogens could also be easily achieved by inducing selective fungistasis and bacteriostasis.

The new possibilities opened up by recent results have roused great interest, but many issues remain controversial. In particular, it is still debatable whether molecular signals can alter the basal rate of soil organic matter decomposition. This can have important bearings on organic C sequestration in soil.

The aim of the Exploratory Workshop held at the University of Udine (I) from the 18th to the 20th of October 2004 was to investigate the possibility to manipulate soil microbial communities to our benefit with methods other than the release of genetically modified micro-organisms.

The workshop organization was aimed at giving participants the possibility to discuss the most problematic issues related to the topic. Sessions were started by invited speakers which gave invited lectures aimed at focussing the state of the art knowledge related to organization of microbial communities, survival strategies, limitations to organic matter turnover and new methodological approaches. A limited number of volunteered papers was also accepted and presented during the workshop to allow participants to give their direct contribution in highlighting contemporary research status. The final programme of the workshop is reported in appendix 1 and the abstracts of invited lectures and volunteered paper presented are reported in appendix 2 of this report. An abstract book was handed out at the participants at registration and is available on request to other scientists interested in this field of research.

Discussions saw the active participation of all attendees (Appendix 3) and allowed the exchange of experiences and points of view on controversial aspects, they not only made possible to delineate guidelines for future research but also increased the wish for future cooperation. During the final discussion it was decided that the scientific outcome of the workshop should be summarized in four main topics:

1) Manipulation of soil microbial communities by substrate and biomass addition (topic summarized by Claude Alabouvette)
2) Testing of Ecological Evolutionary Theories: The Soil Context (topic summarized by Jim Harris)
3) Microbial functions and energetics (topic summarized by Rainer Jorgensen and Marco Contin)
4) Priming effects and trigger molecules (topic summarized by Maria De Nobili)

Manipulation of soil microbial communities by substrate and biomass addition (topic summarized by Claude Alabouvette)

Can we manipulate the community structure of the soil microbial biomass was one of the questions raised by Phil Brookes in his introduction to the workshop.

Based on the communications and discussions during this workshop, we can respond positively to this question. Indeed several examples were given showing that, as a result of either organic matter addition or soil microbial inoculation, the structure of the microbial communities was changed. But another important question was raised: can we predict how these changes will affect the properties of the soil. In other words, are we able to modify the structure and activities of the microbial communities to reach a given objective such as: improving fertility, increasing the suppressive level to diseases, increasing the quantity of immobilized carbon, increasing the bio-remediation ability of the soil?

To be able to chose the most relevant ways of manipulating the soil microbial activities we need to re-address the methodological questions of: how to accurately measure the biomass, how to assess microbial activities, what is the time scale at which these parameters should be measured, how to take into account the spatial heterogeneity of microbial activities, how to assess the role of abiotic soil characteristics on microbial activity.

Most of these points have been discussed during the workshop and will be summarized by other co-ordinators. However, I would like to underline a few points

which are of the first importance: thanks to molecular techniques (presentation by A.G. O'Donnell), we possess new tools to characterize both the structure and the activities of the microbial communities. For the first time we can describe the microbial communities and observe the modifications of these communities induced by different practices without *a priori* hypotheses. Most of the techniques targeting the DNAs enable us to detect not only the cultivable but also the non cultivable populations, and now proteomics enables to better assess the activities of the microbial communities in situ. Microbial growth can be assessed by monitoring dsDNA which seems a more suitable method than measuring the ATP content (P. Nannipieri).

Thus we have new tools to address a few basic questions such as: which are the populations responsible for a given activity, for example:

1. What are their respective sizes?
2. How does a perturbation affect these different populations and their respective activity?
3. Is it important or not to lose a population without affecting microbial activity?
4. How can we enhance the populations responsible for a given activity of interest?
5. What is the spatial distribution of the populations?
6. How do the physic-chemical properties of soil affect the populations and their activities?

Regarding management of the microbial communities by addition of microbial biomass and organic matter, we have a few specific points to address using the available tools.

In the case of substrate addition, we should characterize the type of organic matter since we already know that different composts added to the same soil induced different changes in the structure and activity of the microbial communities. Therefore, we have to consider the interactions between the microbial communities of the organic matter and those of the soil itself.

The consequences of this addition of organic matter to a given soil could have contrasting effects when considering different properties of interest such as: carbon sequestration, soil fertility disease suppression towards several soil-borne diseases.

Regarding addition of a specific biomass (inoculant) we must follow the population dynamics in relation to the biotic and abiotic soil characteristics, but also assess the activity of the introduced population and for environmental purposes the side effects on the non-target organisms and soil functions.

Finally, if we want to choose among several practices which is the most suitable to reach a given objective we need make a prognostic of the changes that will affect the soil microbial activities. Obviously it will never be possible to make a decision based on a single parameter. Thus we should define among the measurable parameters those which can be used as indicators of the soil quality.

In conclusion, we have to clearly define the objectives for which we want to add substrate or biomass to the soil but also to remember that this addition will have consequences on other soil properties or functions. All currently available methodologies have their own advantages and limitations. Therefore, only a combination of methods can provide an overview of what is going on in the soil black box. Using this approach we can determine the space and time scale at which these microbial parameters have to be monitored to correlate changes affecting the structure and activities of the microbial communities. In this way we may ultimately be able to control the changes affecting the soil properties we desire to improve, defining a suite of suitable parameters which can be used as predictive indicators of the changes that will affect the soil activity under different agronomical practices.

There is a need to address these questions urgently since there is a social and political demand to recycle the organic wastes from agriculture, industry and municipal origin. These wastes can be composted then added to the soils. But we should be able to predict the consequences of this addition of large amounts of organic wastes on the soil functions to improve but not to damage the soil functioning in regard to environmental protection and global warming.

Testing of Ecological Evolutionary Theories: The Soil Context (topic summarized by Jim Harris)

The following key conceptual areas can be selected for future investigation:

- Coupling between environment and community
  - Models encapsulating biotic and abiotic components calibrated to real-world and laboratory microcosm investigations
  - Feedback loops between biological communities and abiotic parameters
  - Are there diversity-function relationships and do they have thresholds?
  - Does the "hump-back" species distribution curve apply in soil ecosystems?
  - How do we measure, and what are the determinants of, resistance and resilience of soil properties and processes?
  - Can we develop indicators of ecosystem maturity based on measures of thermodynamic efficiency?
- Speciation
  - Is 'species' an appropriate concept amongst soil microbes?
  - At what rate does speciation occur?
  - Is this linked to isolation-reconnection cycles?
- Fungi
  - How do we develop a generalised theory of fungal community dynamics?
  - How do we characterise the individual for indeterminate organisms? Is this actually a tenable concept?
- Viruses
  - How prevalent are prokaryotic and eukaryotic viruses in soils; to what extent are they associated with hosts or in free form?
  - What is their biomass and mobility in structured soils?
  - How are we to characterise them?
  - What is their role in mediating soil functions in parasitic and predatory modes?
- Horizontal gene transfer
  - How does spatial context affect transfer rate?
  - When and where is the dynamics dominated by clonal reproduction and mutation?
  - What role does phage-mediated transfer play?
- Dispersal
  - What are the relative rates of dispersal of organisms via physical and biotic vectors?
  - What is the dependence of rate on spatial scale?
  - How does this affect speciation?
- Interactions
  - How do we characterise the nature of the interactions between individuals ?

  - How do we characterise the development and reinforcement of feedback loops by evolutionary mechanisms ?
  - Can we link diversity in biological communities and it connection to soil architecture to thermodynamic efficiency?
  - Can we test biodiversity-function interactions rapidly in soil microcosms?
  - Are microbial communities facilitating, inhibiting or following plant assemblages?
- Scaling
  - How do we identify the characteristic scales in the soil-microbe complex that delineate different levels of appropriate abstraction for application in models?
  - How do we scale these upwards to provide useful insight as to provision of ecosystem services?

Microbial functions and energetics (topic summarized by Rainer Jorgensen and Marco Contin)

This topic was discussed throughout several sessions of the meeting as microbial energetics play a central role in the explanation of microbial survival in soil, trigger responses of micro-organisms and priming effect in organic matter mineralization.

Microbial biomass is a fundamental measurement in quantitative soil microbial ecology. The size of the microbial biomass gives information on the amount of energy (carbon) and nutrients stored in this living soil organic matter pool. Concentrations of microbial biomass C, soil organic matter and rates of basal respiration are strongly correlated in soils under equilibrated environmental conditions. Basal respiration is commonly measured from the respiration of conditioned, i.e. sieved and pre-incubated soils at an optimum water content (roughly 50% water holding capacity) and a constant temperature of between 20 to 30 ºC. Changes in land use management and contamination by organic and inorganic pollutants may disturb the interrelationship between microbial biomass C, basal respiration and soil organic matter.

The majority of the soil microbial community is dormant under field conditions and probably the entire microbial community in conditioned soils. Dormant means that the organisms are not actively growing, i.e. increasing their biomass or number. The shift from dormancy to active growth is not reflected by changes in adenosine 5'-

triphosphate (ATP) concentration (ATP-to-microbial biomass C ratio) and adenylate energy charge (AEC) levels if growth is not limited by any nutrient, especially N. Under the conditions of balanced growth, soil micro-organisms maintain a roughly constant ATP-to-microbial biomass C ratio and AEC level, but also a roughly constant microbial biomass C-to-N ratio. In contrast to these ratios, the metabolic quotient qCO_2, i.e. the ratio of basal respiration to microbial biomass C is increased by growth processes due to their energy demand. Shifts in qCO_2 values indicate first of all shifts in the age structure of a soil microbial community, but may also reflect the efficiency of substrate use under specific (disturbing) environmental conditions. The knowledge about the relationships between anabolism and catabolism in soil micro-organisms is restricted, especially under C-limited conditions. The important question remains unsolved whether CO_2 evolution is always combined with the formation of microbial synthesis products or not. Also dormant, i.e. starving and non-growing micro-organisms need energy to maintain ATP concentrations and AEC levels and have to synthesis new microbial tissue for the reconstruction of damaged or senescent cell components and reproduction. Because soil organisms cannot persist indefinitely by only utilising their own energy reserves, they have to rely on the mineralization of soil organic matter and fresh substrates**.**

The part of the glucose-responsive microbial biomass, which is reflected by the ratio of SIR (substrate-induced respiration) to microbial biomass C by fumigation extraction, gives information on the energetic status of a soil microbial community. In the rhizosphere, a hot-spot of energy supply in soil, the glucose-responsive part of the microbial biomass is much larger than in the bulk soil indicating a more active microbial community. There is little information on the effects of the microbial community structure in terms of the ratio of fungal to bacterial biomass on microbial energetics and microbial function. Although molecular methods give information on the diversity of the microbial community structure, it is still an unsolved methodological problem to estimate the exact size of the two main groups of micro-organisms, i.e. the biomass of fungi and bacteria. Changes in the ratio of fungal to bacterial biomass may affect the energetic status of a soil microbial community, because bacteria are believed to have a more rapid (but less efficient) substrate and biomass turnover than fungi. However, this hypothesis can only be proved if quantitative methods are available to

estimate the biomass of these two groups. Starving fungi may behave completely different than starving bacteria in soil.

Often seasonal changes in microbial community structure are reported based on data by PLFA or molecular methods. However, it is uncertain if the amount of available energy is sufficient to support these large shifts in microbial community structure. In most agricultural soils, a relatively small C input by plant residues (roots and stubble) of 2 to 3 t C ha^{-1} maintains a relatively large microbial biomass of 1 t C ha^{-1}. This means the amount of energy to support strong shifts in microbial community structure is limited. Farmyard manure and compost add a large microbial community together with new micro-sites to soil and may have larger effects on the microbial community structure for this reason.

Some of the questions that were repeatedly discussed by participants during the meeting can be summarized as follows:

1. Are ATP concentrations and AEC appropriate indexes of the energetic status of soil micro-organisms (collectively the soil microbial biomass)?
2. Can we correlate microbial energetic status, again as indicated by ATP concentrations and AEC, to microbial activity? And if so, to what extent?
3. Can we improve our understanding of microbial energetic status by also considering other parameters?
4. Why do soil micro-organisms have such an high energetic status in the mainly resting soil populations?
5. Can we manipulate functions of soil microbial biomass by modifying its energetic status or its metabolism and, further more, can we predict the changes likely to be obtained?

Answers were offered for some but not all of these questions.

The AEC as an indicator of the energetic state of a living cell was postulated by Atkinsons (1971), but Chapman, Fall and Atkinson (1971) were the first to relate AEC to microbial survival, and no significant progress was made afterwards.

During the meeting there was considerable debate whether the large ATP concentration in microbial biomass and high AEC ratio was of genuine biological significance or not. Could it be, for some unknown reasons, simply a chance event due to, perhaps the way the microbial community is structured or is it a genuine

evolutionary response of the microbial biomass to equip it for survival in soil? Thus, an organism which exists as spores (low ATP and AEC) in its resting state could be at a serious selective disadvantage compared to a more speculative organism which is prepared to invest energy to maintain high ATP and AEC, to take immediate advantage of fresh substrate when it arrives. It is interesting that the same phenomenon is observed in both soil and deep marine microbial ecosystems.

The mean ATP concentration of the soil microbial biomass (around 10 - 12 µmol ATP g^{-1} biomass C) is remarkably similar to that of micro-organisms actively growing *in vitro* irrespective of global location, soil management, heavy metal contamination and soil amendment, but under some circumstances (e.g. soils subjected regularly to long periods of drought, saline soils, nitrogen limitations) this does not appear to hold. However, measurement of the ATP concentration of the soil microbial biomass is subject to considerable experimental uncertainty caused by the propagation of errors in both soil ATP and biomass C determinations. It is likely that even quite considerable measured differences (say < 20 %) are not statistically significant.

ATP represents only a small (about 1%) part of the total energy stored in a cell. It is certainly the part more quickly available to supply energy requested for example for substrate assimilation.

The main weakness of ATP and AEC measurements is that they give a static representations of adenylate pools but nothing is known relatively to their dynamic turnover. The total ATP pool of actively growing micro-organisms *in vitro* turns over every few seconds, but we do not know what happens to resting organisms within the soil microbial biomass. Could the turnover time of their ATP be much longer and, if so, how may it be measured? The turn-over time of total adenylates would probably better represent the metabolic status of the microbial cells.

Could we use other energy indices such as cyclic-AMP, GTP and other non adenine nucleotide triphosphates, NADH and NADPH, or others?

The ATP concentration and the AEC ratio of the soil microbial biomass represents a mean value for that population and can be very different from those of a single cell or even a single functional group, which could be actively growing, starving or dying, depending on the specific environmental conditions. However, in any case, since the ATP concentration and the AEC of the soil microbial biomass are near their

maximum theoretical limits, they must represent the concentrations of the majority of the population. The paradox of a dormant, or resting, soil microbial population maintaining a high energetic status still needs to be explained.

The possibility of being able to identify the species or group(s) that are doing each activity, is now provided by molecular technologies, that can give us answers that were not possible since some of the hypothesis on microbial energetics were formulated during 1970 – 80.

Priming effects and trigger molecules (topic summarized by Maria De Nobili)

Reports of priming effects, that is the increased (positive priming) or decreased (negative priming) mineralization of soil organic matter (measured as changes in the amounts of CO_2 evolved) are frequently reported after addition of easily decomposable organic substrates to soil. Their existence has long been a matter of debate and priming effects have been frequently considered to be artefacts caused by errors of methodology. Their elusive nature suggests that, if indeed they occur, they are the expression of complex phenomena whose mechanism or mechanisms are far from being understood. They occur after the addition of a very wide range of substrates types and concentrations, with no apparent relationship, in most cases, between the amount of substrate added and the measured priming effect. Moreover the same substrate additions may induce very different effects in different soils.

Discussion about the possible nature of priming effects was one of the main themes of the workshop. All participants, although expressing widely different points of view, shared the opinion that understanding the mechanism(s) that produce priming effects will reveal important new insights into the functioning of the soil microbial community, particularly in relation to the role of soils as carbon sinks or sources and their behaviour with respect to global warming issues.

Two of the key lectures dealt with priming effects. The first: “Role of dissolved organic carbon in soil organic matter turnover” by U. Hamer and B. Marchner, was given by Ute Hamer in the session devoted to “Limitations to organic matter turnover” and considered priming effects caused by addition of soluble substrates in different soils

and soil horizons. The second, by Yakov Kuzyakov examined factors and mechanisms of rhizosphere priming effects. Both lectures provided evidence that mineralization of soil organic matter is at least partially controlled by availability of water soluble easily degradable substances. Nevertheless the overall carbon balance shows that only very variable and relatively small amounts of soil organic C are actually mineralised by this mechanism.

The hypothesis that priming effects in the rhizosphere represent a strategy for plants to promote the release of nutrients by enhancing mineralization of soil organic matter is fascinating. It demands further investigation of the mechanisms involved before it can be fully accepted.

The possible mechanisms involved are:

- Triggering (activation without growth)
- Preferential substrate utilisation
- Microbial activation (activation by growth)
- Competition between roots and micro-organisms for limiting elements (P or N).

The trigger molecules hypothesis was also much debated. In the trigger molecule response, the addition of very small quantities of substrate (typically 10 -15 μg C g^{-1} soil) causes up to several times more CO_2-C to be evolved than was contained in the added trigger molecules. In contrast, orders of magnitude more substrate - C is added in the case of priming effects but, typically only 10 -20 % more (or less) CO_2-C is evolved than was added. However, it was considered that both trigger molecule and priming effects are the expressions of different aspects of the same phenomenon. Trigger molecules therefore operate at an intermediate level between priming agents and signal molecules, which are much more specific in their action and act at a much lower concentration (10^{-10} molar).

Our limits in understanding the mechanisms involved are probably due to the fact that our observations are limited to changes in overall population levels and activity, but more information is needed about what happens at the community level. Further study must therefore combine traditional methodologies with community level physiological

analysis and molecular techniques which can nowadays allow the analysis of the structure and *in situ* activities of soil microbial populations.

Future Activities

During the final discussion, the issue of future activities was examined. Everyone felt that the workshop had contributed to create a clearer perception of future research needs in this field. Participants also wished to be able to continue to exchange ideas and experiences. It was decided that contacts should be maintained and every possible effort should be made to identify avenues for future funding of activities. These will include application for an ESF network as well as some form of European Commission funding.

Concerning the strategy of future actions, our first target is the 7th Frame Program of the European Union. At present they are currently collecting ideas and suggestions for thematic domains for future European support. Our research activity could for instance fit in the Environmental Technology Action Plan (ETAP), which was created by the Union, at the end January 2004, with the aim of stimulating both the development and deployment of technologies which, in the words of the Commission's Communication, "reduce pressures on our natural resources, improve the quality of life of European citizens, and stimulate economic growth". Environmental technologies are not only valid in meeting challenges facing the environment, but also represent a potential boon for EU competitiveness– giving the Union a leadership position in emerging and innovative fields.

Further action to be undertaken:

1. Circulate the ESF Workshop report to participants.
2. Write a proposal to ESF for a network by February 2005.
3. If the ESF proposal is successful, organise a new meeting by - possibly - October 2005, with the aim of getting to a well defined outline for a FP7 proposal.
4. Write the FP7 proposal by - likely - April 2006.

5. Together with these initiatives, we are launching a call for contribution for a special issue of Biology and Fertility of soils as workshop memoir, then representing the scientific output of the meeting.

Statistical information about the participants

The total number of workshop attendees was 32, 25 of these were founded by ESF. The participants came from 13 different countries (Figure 1), The composition reflects a well sorted mixture of young and experienced scientists (Figure 2) and a reasonable proportion of woman scientists (Figure 3).

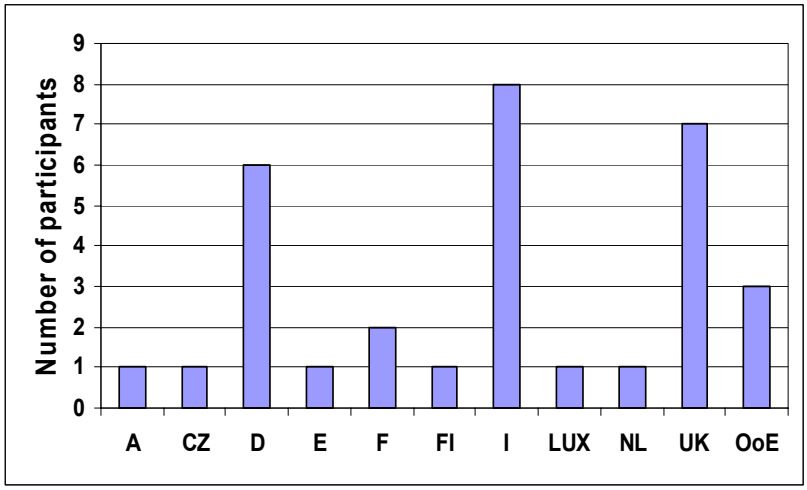

Figure 1. Countries of origin of participants. (OoE: out of Europe).

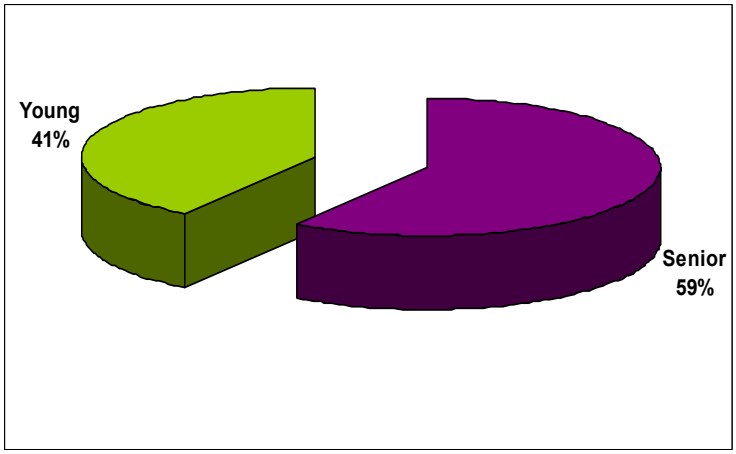

Figure 2: Age structure of participants.

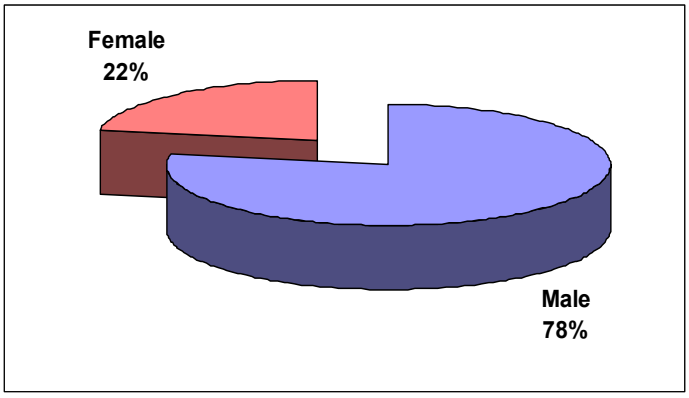

Figure 3: Gender structure of participants.

APPENDIX 1

FINAL PROGRAMME

Monday 18 October 2004

09:00-09:30 **Prof. M.A. D'Aronco - Vice Rector** of Udine University
Word of Welcome

Prof. Lucien Hoffmann
(Standing Committee for Life and Environmental Sciences)
Presentation of the European Science Foundation (ESF)

Profs. P.C. Brookes and M. De Nobili

Introduction to conference

Session 1: The organization of soil microbial communities
Chairpersons: **Prof. P. Nannipieri and Prof. P.C. Brookes**

09:30-11:00 Introductory keynotes

Prof. P. Nannipieri
Biological space, microbial diversity and the priming effect

Prof. H. Insam
Long term effects of soil compost amendment on microbial biomass activity and diversity

Prof. H. Van Veen
New perspectives on the mechanisms of soil fungistasis

11:00-11:20 *Coffee break*

11:20-12:00 Volunteered communications

Dr. O. Dilly
Activation of microbial communities by glucose in an agricultural and forest soil

Prof. R. Joergensen
Reasons for exceptional low ATP and AEC levels of microorganisms under specific soil conditions

12:00-13:00 Discussion and summary

Session 2: Survival strategies of the soil microbial biomass
Chairpersons: **Prof. H. Insam and Prof. R. Joergensen**

14:30- 15:30 Introductory keynotes

Prof. Claude Alabouvette
Biological control of soil borne plant pathogens

Prof. P.C. Brookes
The survival strategy of the soil microbial biomass

15:30-15:50 *Coffee break*

15:50-16:50 Volunteered communications

Dr. C. Mondini

Dynamics of soil microbial biomass activation by trace amounts of substrate

Dr. P. Hirsch

Survival of bacterial and fungal inoculants in soil

Prof. M. De Nobili
Changes in microbial biomass and activity during storage of air-dried soil for 100 years

16:50-18:00 Discussion and summary

18:00 Close of the meeting

Tuesday 19 October 2004

Session 3: Limitations to organic matter turnover
Chairpersons: **Dr. H. Fritze and Prof. D. Hopkins**

09:00-10:00 Introductory keynotes

Prof. U. Hamer and B. Marschner
Role of dissolved organic carbon in soil organic matter turnover

Prof. D. Hopkins
Carbon as substrate for soil microorganisms

10:00-10:40 Volunteered communications

Dr. S. Kemmitt
The paradox of soil organic matter mineralization

Dr. F. Hoyle
Microbial response to addition of glucose

10:40-11:00 *Coffee break*

11:00-11:20 Volunteered communications

Dr. G. Mühlbachovà

Microbial biomass and its activities under long-term heavy metal stress in soils near a lead smelter

11:20-13:00 Discussion and summary

13:00-14:30 *Lunch at the "Osteria Alla Ghiacciaia" Restaurant*

Session 4: Soil organic carbon dynamics and the rhizosphere
Chairpersons: **Dr P. Hirsch and Prof. M. De Nobili**

14:30-15:30 Introductory keynotes

Prof. K. Inubushi
Effect of increased CO_2 rates on microbial biomass metabolism

Prof. Y. Kuzyakov
Factors and mechanisms of rhizosphere priming effects

15:30-15:50 *Coffee break*

15:50-16:30 Volunteered communications

Dr. H. Fritze
Cadmium in upland forests after vitality fertilization with wood ash - a summary of soil microbiological studies into the potential risk of cadmium release

Dr. A. Mamilov
Shifts in microbial eco-physiology during decomposition of plant residue

16:30-18:00 Discussion and summary

18:00 Close of the meeting

Wednesday 20 October 2004

Session 5: New methodological approaches
Chairpersons: **Dr. O. Dilly and Prof A. O'Donnell**

09:00-10:00 Introductory keynotes

Prof A. O'Donnell
Twenty years of molecular analysis of bacterial communities in soils and what have we learned about function

Prof. R. Evershed
Probing soil microbial communities using compound-specific stable isotope approaches

10:00-10:40 Volunteered communications

Dr. M. Contin
Variability of ATP concentration in the soil microbial biomass: physiological changes or artefacts of the methods?

Dr. G. Renella

Use of reporter genes for monitoring heavy metal bioavailability and concentration of organic molecules including signals in soil

10:40-11:00 *Coffee break*

11:00-12:30 Discussion and summary

12:30-13:30 Closing remarks and general conclusions

13:30 End of workshop

15:00-16:30 *Supplementary discussion on ATP concentration of the soil microbial biomass*

APPENDIX 2

ABSTRACTS OF KEYNOTE LECTURES AND VOLUNTEERED PAPERS

BIOLOGICAL SPACE, MICROBIAL DIVERSITY AND PRIMING EFFECT

P. Nannipieri

Dipartimento della Scienza del Suolo e Nutrizione della Pianta
Università di Firenze
P.le delle Cascine 28, 50144 Firenze, Italy

Soil is a complex microhabitat for the presence of a great microbial diversity, the discontinuity and heterogeneity of the system, where nutrients and energy sources are scarce, and the capacity of the solid phase to adsorb important molecules, such as proteins and nucleic acids, and to carry out enzyme-like reactions. Torsvik, *et al.,* (1996) calculated the presence of about 6000 different bacterial genomes g^{-1} soil by taking genome size of *Escherichia. coli* as a unit. This diversity should be huge if microbial species inhabiting soil are not ubiquitous but locally native (Tiedje et al 2001). Microbial biomass is large but it is not uniformily distributed, being mainly localised in "hot spots", such as aggregates with better nutrient and living conditions than the bulk soil, zones with accumulated particulate organic matter or animal manures and the rhizosphere. Even if the available space is extensive in soil, the biological space, that is, the space occupied by living microorganisms, represents a small proportion, generally less than 5% of the overall available space. The fact that microbial biomass and enzyme activities increased after the addition to soil of energy sources and then they returned to the original level once the source is exhausted, has conducted to hypothesise a possible relationship between available biological space, microbial biomass and enzyme activities (Nannipieri et al 1983).
The links between microbial diversity and soil functioning are not always clear. An example is given by the several theories which try to interpret the priming effect, that is, the effect of fresh organic matter inputs to soil on the decomposition of soil organic matter. A recent theory by Fontaine et al (2003) hypothesised that the priming effect results from the competition for energy and nutrient acquisition between the microorganisms specialised in the decomposition of fresh organic matter and those feeding on polymerised soil organic matter. We have shown that low molecular weight root exudates such as glucose, oxalic acid and glutamic acid, can have different effects on bacterial diversity and on the priming effect (Falchini et al 2003).

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LONG TERM EFFECTS OF SOIL COMPOST AMENDMENT ON MICROBIAL BIOMASS, ACTIVITY AND DIVERSITY

M. Ros*, K. Aichberger, G. Desalegn, B. Knapp, G. Innerebner, H. Insam

University of Innsbruck, Institut für Mikrobiologie, Technikerstraße 25, 6020 Innsbruck, Austria
*E-mail marga.ros@uibk.ac.at

The virtue of compost for agricultural and horticultural use has been shown previously, but long-term studies comparing different types of compost amendment on soil quality and crop yield are needed. Chemical and physical characteristics, as well as bulk microbial parameters such as biomass and activity are important; however, these parameters do not reveal effects on structure or function of the microbial community.

In the present project we study a long-term (10 year) crop rotation experiment. The plots are annually treated with inorganic fertilisers at different levels, as well as with composts of sewage sludge, organic wastes, green (yard) wastes and manure. We determine structural components of the compost and the soil microflora with 16s rDNA based PCR followed by DGGE, as well as changes of soil functional abilities by community level physiological profiling (CLPPing) using Biolog^{TM} (GN)-plates.

The preliminary results indicate effects of the different composts not only on chemical, physical and biological bulk parameters, but also on the microbial functional and structural traits. As far as can be concluded from the data available to-date, the different composts leave a microbial imprint in the soils.

NEW PERSPECTIVES ON THE MECHANISMS OF SOIL FUNGISTASIS; THE CLASH BETWEEN BACTERIA AND FUNGI

J.A. Van Veen

Netherlands Institute of Ecology (NIOO-KNAW)
Heteren
The Netherlands

Soil fungistasis has been a widely interpreted phenomenon, originally related towards the inhibition of fungal spore germination by soil factors. An evenly wide variety of mechanisms has been proposed to explain the process.

A slightly deviating definition of fungistasis is the inhibition of fungal network development in soil by a complex of biological and abiotic factors. Most popular putative mechanisms of the inhibition of fungal development in soil are competition for substrate and the production of anti-fungal compounds by other microbes in soil.

I will provide information on the relative importance of both proposed mechanisms derived from experiments with different complex soil microbial communities.

This work has led to questions on the importance of bacteria-fungi interactions in soil for the diversity and dynamics of soil microbial communities. Often, bacteria and fungi are considered separated from each other in terrestrial ecosystem studies. However, in soil there is a huge variety of interactions among those biota which are the dominant inhabitants of soil. These interactions strongly determine the size, diversity and activity of both groups. In connection to the aforementioned inhibitory factors of fungal development, I will also deal with recent information on the importance and on the mechanisms of bacterial mycophagy, *i.e.* the utilization of fungal hyphal material as substrate by bacteria.

ACTIVATION OF MICROBIAL COMMUNITIES BY GLUCOSE IN AN AGRICULTURAL AND A FOREST SOIL

O. Dilly[1] and A. Zyakun[2]

[1] Lehrstuhl für Bodenökologie, Technische Universität München, 85764 Neuherberg & Institut für Bodenkunde, Universität Hamburg, Allende-Platz 2, 20146 Hamburg, Germany
Email o.dilly@ifb.uni-hamburg.de
[2] Skryabin Institute of Biochemistry and Physiology of Microorganisms RAS, 142290 Pushchino, Moscow Region, Russia

The effect of the addition of glucose enriched in ^{13}C on soil respiration, soil respiratory quotient and $^{13}C/^{12}C$ isotope ratio was studied for an eutric and a dystric Arenosols under agricultural and forest land use respectively. The two soils were previously already investigated by Dilly (2001a; 2003b; 2003) without considering modern ^{13}C isotope analysis. The experiments were done in a closed system at 22 °C

at 40 to 70 % WHC using a Sapromat respirometer that continuously reproduce oxygen consumed as carbon dioxide is fixed in sodium hydroxide (Dilly, 2001a). After addition of barium chloride and titration for the estimation of carbon dioxide evolution the C precipitated was washed, centrifuged and dried for ^{13}C isotope analysis (Kristiansen et al., 2004). The $\delta^{13}C$ values which relates $^{13}C/^{12}C$ isotope ratio in the sample to an international standard remained longer on a higher level in comparison to respiration rate and respiratory quotient suggesting that glucose was initially transformed to exo-metabolites and later successively degraded. When soil was preconditioned with glucose the maximal initial respiratory response was reduced, the respiratory quotient lowered and $\delta^{13}C$ values increased indicating that glucose is oxidised to a higher extent and priming effect on soil organic matter degradation became lower. When 0.5 mg C was added to one gram soil, the $\delta^{13}C$ values stayed for more than 4 weeks at higher level although the respiration rates were not affected and respiratory quotient showed values typical for soil basal metabolism. This explained low recovery of added glucose in respired, microbial and available C after 6 days in these soils (Dilly, 2001b). The presentation draws attention to measured and modelled data on soil respiration and furthermore the interactions between glucose and organic matter degradation on soil respiratory processes.

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REASONS FOR EXCEPTIONAL LOW ATP AND AEC LEVELS OF MICROORGANISMS UNDER SPECIFIC SOIL CONDITIONS

R.G. Joergensen and M. Raubuch

Department of Soil Biology and Plant Nutrition, University of Kassel at Witzenhausen

In most soils, microorganisms maintain high ATP-to-microbial biomass ratios and AEC levels. These features are characteristic, but surprising in an energy-limited environment. However, some interesting exceptions exist. It was repeatedly shown that the ATP-to-microbial biomass C ratio is lowered under N limited conditions, e.g. after adding glucose without an additional nitrogen source, leading to significant negative relationships with the microbial biomass C-to-N ratio. Nutrient limitations might be also the reason for low ATP-microbial C ratios in the organic layers of forest soils. However, in tropical forest soils, large microbial biomass C-to-N ratios are also combined with very low ATP-to-microbial biomass C ratios, although no indications exist for nutrient limitations or other stress factors such as salinity or high Cu concentrations. A decline in the ATP-to-microbial biomass C and in AEC levels was also observed with increasing salinity in combination with marked changes of the microbial community structure. In contrast, high Cu concentrations lead to decreased AEC levels but not to decreased ATP-to-microbial biomass C ratios indicating another pathway for affecting the physiology of soil microorganisms.

We draw from our observations and from literature data the hypothesis that high ATP and AEC levels are necessary to maintain cell integrity and thus survival in highly competitive soils, otherwise these organisms would be decomposed by their starving neighbours. In soils, where different stress factors, energy or nutrient limitations reduce the microbial diversity, soil microorganisms are able to lower their ATP-to-microbial biomass ratios and their AEC levels.

BIOLOGICAL CONTROL OF SOIL BORNE PLANT PATHOGENS

C. Alabouvette and C. Steinberg

INRA, UMR Microbiologie Géochimie des Sols BP 86510 F-21065 DIJON Cedex

A plant disease results from the intimate interaction between a plant and a pathogen, and today there is a great effort of research devoted to the study of plant-pathogen interactions at the cellular and molecular level. The importance of these direct interactions should not hide the role of environmental factors that influence disease severity. These indirect interactions are particularly important in the case of diseases induced by soilborne pathogens. The existence of soils that suppress diseases provides an example of biotic and abiotic factors affecting the pathogen, the plant or the interaction between the plant and the pathogen. Indeed in suppressive soils disease incidence or severity remains low in spite of the presence of a virulent pathogen, a susceptible host plant and climatic conditions favorable for disease development.

Soils suppressive to diseases caused by a range of important soil-borne pathogens have been described; they include fungal and bacterial pathogens but also nematodes. The large diversity of pathogens controlled by suppressive soils shows that soil suppressiveness is not a rare phenomenon. On the contrary, every soil has some potential of disease suppression, leading to the concept of soil receptivity to diseases.

In order to develop biological strategies of diseases control it is important to understand the mechanisms of soil suppressiveness. From a theoretical point of view, both the abiotic characteristics of a soil and its biological properties can be responsible for disease suppression. To evaluate the respective role of biotic and abiotic factors, one can eliminate organisms by biocidal treatments such as steam or methyl bromide. In most cases, as in the case of Fusarium wilt suppressive soils, suppressiveness is fundamentally microbial in nature, it is destroyed by biocidal treatments and can be restored in the disinfested soil by mixing a small quantity of suppressive soil or by re-introduction of a mixture of microorganisms previously isolated from the suppressive soil. This demonstration of the essential role of the saprophytic microflora does not establish that soil physical and chemical properties play any role in the mechanisms of suppressiveness. On the contrary, in the case of *R. solani* diseases, the steamed disinfested soils exhibit similar or higher level of suppressiveness than that of the natural soils This indicates that the abiotic characteristics of the soils, play a more important role in restraining the pathogen activity of *R. solani* than the indigenous soil microflora.

As it is difficult to modify the soil abiotic characteristics, the main approach towards biological control of soilborne diseases consists in modifying the microbiological balance of the soil. This can be achieve by either introduction of antagonistic microorganisms or cultural practices, such as organic amendments. In the later case, a greater biomass can enhance the general suppression phenomenon which contributes to the control of some diseases such as Fusarium wilts. Specific suppression will be enhanced by application of strains of microorganisms selected for their antagonistic capacity towards the target pathogen. The modes of action of the biological control agents are very diverse and well studied. They involved both microbial antagonism: parasitism, competition, antibiosis and induction of resitance within the plant. In general, a single biological control agent possesses more than one mode of action. The different modes of action can be expressed simultaneously or successively and contribute together to the efficacy of biological control.

But success of biological control requires a good establishment of the biological control agents in the soil and in the rhizosphere where it has to interact with the plant and the pathogen. Ecological fitness of biological control agents is a field of research that has been neglected and needs more attention.

THE SURVIVAL STRATEGY OF THE SOIL MICROBIAL BIOMASS

P.C. Brookes[1], M. De Nobili[2], M. Contin[2], C. Mondini[3]

[1]Rothamsted Research, Agriculture and Environment Division, Harpenden, UK
[2]Dipartimento Di Produzione Vegetale E Tecnologie Agrarie,
University of Udine, Via Delle Scienze, Udine, Italy
[3]Istituto Sperimentale Per La Nutrizione Delle Piante, Via Trieste, Gorizia, Italy
E-mail: philip.brookes@bbsrc.ac.uk

The soil microbial biomass, (the sum of the masses of all soil micro-organisms with a volume less than about 5 x 10^3 μm^3) can be considered as the living fraction of soil organic matter. Living in a generally substrate-poor environment, the biomass exhibits many features typical of a dormant population, e.g. very slow turnover time, long mean rate of cell division etc. Yet, paradoxically, it has an ATP concentration (typically 10-12 μmol ATP μg^{-1} biomass C) and adenylate energy charge (AEC = {(ATP + 0.5 ADP)/(ATP + ADP + AMP)}) typical of micro-organisms in exponential growth. (Energy considerations indicate that only a small fraction can be in this state at any time). We have long considered that these are evolutionary responses to low substrate availability, a hypothesis supported by our recent discovery of a hitherto unknown mechanism in soil micro-organisms. Thus, the soil microbial biomass, detecting traces of substrate, expends more energy than the substrate contains. Why should it adopt this strategy in the substrate-scarce soil environment? We hypothesise that this shift from dormancy to activity is initiated by it sensing 'trigger molecules' derived from the substrate. It thus invests more energy than is in the 'trigger molecules', anticipating a genuine 'food event' that will provide sufficient energy to support growth. We now plan to investigate this phenomenon further, by combining techniques from soil biochemistry, molecular biology and isotopic studies. This research may improve our ability to manipulate the community structure of the soil microbial biomass.

DYNAMICS OF SOIL MICROBIAL BIOMASS ACTIVATION BY TRACE AMOUNTS OF SUBSTRATE

C. Mondini[1], M.L. Cayuela[2], M.A. Sanchez-Monedero[2] and A. Roig[2]

[1] Istituto Sperimentale per la Nutrizione delle Piante, sez. di Gorizia,
via Trieste 23, 34170 Gorizia, Italy
[2] CEBAS-CSIC, Campus Universitario de Espinardo, Apartado de Correos 164,
30100, Espinardo, Murcia, Spain

Soil represents a nutrient-poor environment for microorganisms in which substrate availability is both scarce and infrequent. So far very little is known about the physiological modification adopted by microorganisms to survive in the unfavourable soil environment. Nevertheless, it is suggested that, to be able to rapidly utilize new

substrate that becomes available, the microbial biomass maintains a relatively active or alert metabolic state and that the shift to the fully metabolically active state is elicited by it detecting simple and easily degradable molecules derived from the substrate (trigger molecules) (De Nobili et al., 2001). It was shown that trace concentrations (about 11 μg C g^{-1}dry soil) of several trigger solutions (glucose, amino acids and root exudates) caused the biomass to evolve more CO_2-C than was contained in the trigger solution (De Nobili et al., 2001). However, the activation of the microbial biomass was obtained after a lag phase, the duration of which was extremely variable. Therefore, the aim of our research was to investigate the dynamics of soil microbial biomass activation caused by trace amounts of trigger molecules.

Trigger molecules solutions (amino acids, glucose, compost aqueous extract, protein hydrolyzate) were added (15 μg C g^{-1} dry soil) to a moist (40% WHC) grassland soil (soil organic C 25 mg kg^{-1}, microbial biomass C 750 μg g^{-1}) previously conditioned for 5 days at 25°. The respiratory response of soil microbial biomass to different rates (5-10-15 μg C g^{-1} dry soil) of glutamic acid was also measured. The rate of CO_2 production was measured every 85 min by gas chromatography during the incubation of the samples at 25 °C. The cumulative CO_2-C evolved from soil amended with trigger molecules, after subtraction of CO_2-C evolved from the control, showed a huge priming effect with 5-15 times more CO_2-C evolved respect to that added. The greatest amount of extra CO_2-C evolved was obtained with a solution of amino acids (250 μg C g^{-1} dry soil in 3 days of incubation). Addition of different amounts (5-10-15 μg C g^{-1} dry soil) of glutamic acid showed significant differences among the treatments as a function of the C added.

Dynamics of the extra CO_2-C evolved from amended soil depended on the trigger molecules added, but, unlike De Nobili et al. (2001), in all the cases no lag-phase was recorded. The maximum CO_2 respiration was always measured immediately after the addition of the trigger molecules and from this point onwards the rate of respiration was always decreasing towards stable values that were achieved from 1 to 3 days after the addition.

The results of the present work were therefore only partially in agreement with those of De Nobili et al. (2001), confirming the stimulating effect of the addition of trace amounts of substrates on microbial biomass activity. However, unlike De Nobili et al. (2001), we found that the respiratory response of microbial biomass to the addition of trigger molecules was practically instantaneous. We do net yet understand the reasons for the differences in the lag-phase between our work and that of De Nobili et al. (2001). Nevertheless, the immediate activation of microbial biomass following addition of trace amounts of readily available substrate supports the hypothesis of soil microbial biomass maintaining always an alert or active metabolic state, as an evolutionary adaptation in order to survive in the extremely nutrient-poor soil environment.

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SURVIVAL OF BACTERIAL AND FUNGAL INOCULANTS IN SOIL

P.R. Hirsch

Plant Pathogen Interactions Division, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK

Beneficial bacteria and fungi are applied to soil or plants for several reasons: to improve plant nutrition; to enhance plant growth; or to suppress plant pests and diseases. Well-known examples of the first group are rhizobia, the symbiotic nitrogen fixing bacteria of leguminous plants, which are applied as soil or seed inoculants. Some other bacteria such as *Azospirillum spp*. can fix nitrogen but their primary impact on plant growth is thought to be due to their production of phytohormones that stimulate root growth. Biological control agents that protect plants from pests and diseases include strains of *Pseudomonas fluorescens* that secrete the antifungal metabolites phenazine or phloroglucinol, and filamentous fungi such as *Pochonia chlamydosporia* and *Paecilomyces lilacinus* that parasitize plant parasitic nematodes.

Most microbial inoculants flourish in the relatively nutrient-rich rhizosphere where they colonise the surface of roots and exert their beneficial effects. However, they may not compete with indigenous strains, nor survive long enough to have an impact. Similarly, they may not persist in the absence of host plants in nutrient poor soil, especially if there is strong competition from the indigenous microbiota. The variable performance and unreliability of many biological control agents is probably due, in part, to these unpredictable factors.

The survival of inoculant bacteria and fungi has been studied on roots and in soil using both selective plate culture and also PCR-based methods, which avoid the need for culture. The methods and their limitations, and their utilization in glasshouse and field studies, will be discussed. Results include the finding that rhizobial inoculants can survive for many years in the absence of their host legume; and that some isolates of the nematode biological control fungus, *Pochonia chlamydosporia,* do not proliferate in the absence of a plant host.

CHANGES IN MICROBIAL BIOMASS AND ACTIVITY DURING SOIL STORAGE OF AIR-DRIED SOIL FOR 100 YEARS

M. De Nobili and P.C. Brookes

[1]*Rothamsted Research, Agriculture and Environment Division, Harpenden, UK*
[2]*Dipartimento di Scienze Agrarie e Ambientali, University of Udine, Italy*

Soils from the Long-Term or Classical Field Experiments at Rothamsted Research, UK have been routinely sampled and stored air-dry for more than 150 years. While the soil chemical and physical properties have remained largely unchanged, we know nothing about changes in soil microbiological properties which may have occurred over the same period. We recently investigated changes in microbial biomass carbon

(C), ATP and C mineralization and activity which occur during the rewetting of such soils and compared them to the same measurements in freshly sampled soils. The % organic C, total N and pH in a Rothamsted grassland soil were almost identical whether freshly sampled or stored air-dry for 100 years. However, about twice as much water extractable C was measured in the stored compared to freshly air-dried sample. When the same soils were rewetted and incubated for 12 days, about 4 times more CO_2-C was evolved from the stored soil.

Soil microbial biomass ATP concentrations in a range of freshly sampled moist Rothamsted soils ranged from about 9.5 to 14.5 μmol ATP g^{-1} biomass C, typical of much previously published data. Following rewetting and a 12 day incubation at 25 ^{o}C, the mean biomass ATP concentrations in a Rothamsted arable soil (measured in samples taken from 1913 to 1982 and given inorganic fertilizer) was 4.4 μmol ATP g^{-1} biomass C. However, the mean biomass ATP concentration in a similar Rothamsted soil given farmyard manure (FYM) since 1852 and then treated identically, was 12.9 μmol ATP g^{-1} biomass C i.e. the same as in fresh moist soils. The FYM-derived soil organic matter in the long-term stored soil therefore preserved both a larger biomass and its ATP concentration. However, during the 12 day incubation, there was a significant decrease in both biomass C and ATP in the rewetted and long-term stored soils, suggesting that the biomass was damaged in some way during long-term storage. These results indicate that soil organic matter exerts a protective effect on the soil microbial biomass when soils are stored air-dry. This effect is removed when the previously dried soils are rewetted.

ROLE OF DISSOLVED ORGANIC SUBSTRATES IN SOIL ORGANIC MATTER TURNOVER

U. Hamer and B. Marschner

Geographical Institute, Ruhr-University Bochum, 44780 Bochum, Germany
Ute.Hamer@rub.de

Dissolved organic matter (DOM) may be the most important microbial substrate in soils since all microbial uptake mechanisms require an aqueous environment. Therefore, the amount and composition of DOM in the soil solution strongly influences microbial activity. Low molecular weight organic substances that are easily available for microorganisms are continuously entering the soil solution and thus have a high potential in accelerating or retarding the mineralization of soil organic matter (SOM), which is termed as positive or negative priming effect. Based on incubation experiments in which we examined the influence of different uniformly ^{14}C-labeled sugars, amino acids, organic acids and catechol on the mineralisation of organic matter (lignin, peat, 11 soil samples, charred maize, rye and wood residues, particle size fractions) we will discuss the importance of priming effects on SOM turnover.

The mineralisation of all examined organic materials (lignin, peat, SOM, black carbon) was influenced by the addition of at least one of the tested substrates. The data clearly indicated that not only SOM of arable soils is affected, but also SOM of

surface and subsoil horizons of forest soils. The acceleration of the mineralisation of organic carbon is a common process immediately after the addition of water soluble organic substrates that are easily available for microorganisms. This may suggest that stabilisation of organic materials in soils is at least partially controlled by the lack of easily available organic substrates. Some data indicate that labile as well as stable pools of SOM are affected by priming. Negative priming effects were sometimes observed after catechol or oxalic acid additions. Results with repeated substrate additions suggest that the composition of the microbial community and structural changes induced by the respective substrate contribute to the observed priming effects. Nevertheless, in most cases the C-input from the ^{14}C-labeled substrates was higher than the loss due to positive priming.

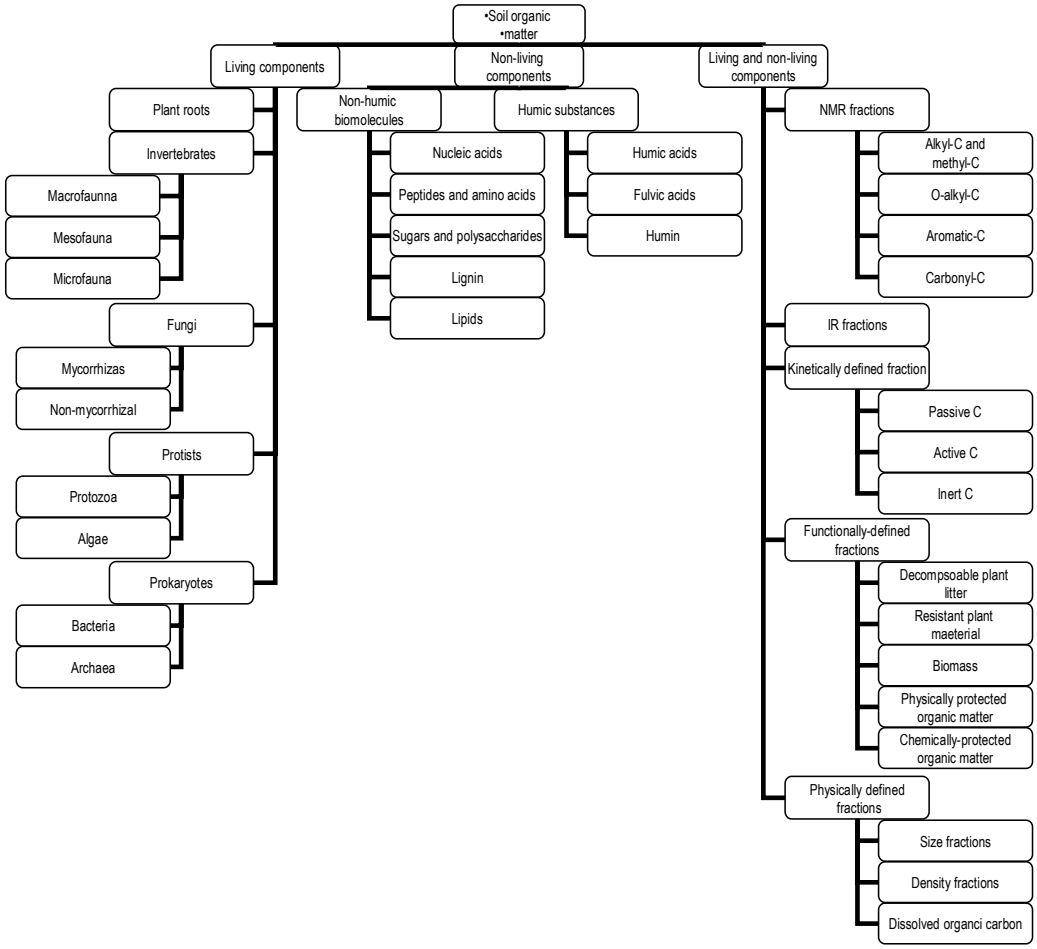
CARBON AS A SUBSTRATE FOR SOIL ORGANISMS

D.W. Hopkins

School of Biological and Environmental Sciences, University of Stirling, Stirling FK9 4LA, Scotland UK [d.w.hopkins@stir.ac.uk]

Humans view soil C in various physical (e.g. aggregates, density fractions), chemical (e.g. carbohydrates, aromatic compounds), biological (e.g. microbial biomass), and even economic (e.g. dollars per tonne or C credits) ways which are not usually ecological. Indeed, even amongst environmental scientists, the application of increasingly sophisticated analytical approaches for characterising soil C has probably contributed to the ecological relevance of soil C becoming obscured. The soil organisms do not, however, have these perspectives, but regard organic compounds simply as substrates supplying resources and energy. Chemical characterization of soil organic matter has been an objective of soil scientists for many centuries and has led to a bewildering array of fractions. This means, for example, that the C in recently arrived plant litter may simultaneously appear in the particulate matter, any of the non-humic biomolecules, the dissolved organic matter and in any of the various kinetically-defined fractions. In the figure, some of the different fractions of soil organic matter are summarised to illustrate the lack of a common framework and the difficulties of working with highly complex mixtures. The ecological relevance of these fractions has been questioned by soil science "dissidents", but deeply cherished terms such as humic acid have, like some of the molecules it purports to represent, proved remarkably resistant to decay. Understanding the chemical composition of the different fractions of soil C varies enormously with context. In relation to soil biodiversity, two components of the non-humic biomolecules, lipids and nucleic acids, are receiving particular attention because of the taxonomic information they carry, with the result that detailed molecular structures have been resolved. The substrate quality of soil organic matter can be regarded as the combined properties that influence the supply of C and energy to the heterotrophic soil organisms. Although this is a simple concept, the ability to assess substrate quality is not easy. Early studies recognised that different components of plant litter were lost at different rates, a reflection of their

resource value to the decomposer organisms, and more recently theoretical approaches to consider detritus as a continuum of molecules from the recalcitrant to the highly labile have been developed. However, for empirical purposes, what is required are analytical that can be applied across a wide range of soils to assess the proportions of different groups of organic in the soil organic matter (i.e. a "soil metabolomic" approach to include the full complement of metabolites in the soil), or indicators of these compounds which reflect their relative stability. Substantial progress has been made with infrared spectroscopy, pyrolysis-GC-MS or pyrolysis-MS and nuclear magnetic resonance and Raman spectroscopy techniques. However, we are still a long way from being able to characterise all potential biological substrates in soil organic matter, and for key groups of organic compounds, most notably those containing N, the biochemical characteristics remain remarkably sparse even with the application of modern spectroscopic techniques and the refinement of classical wet chemical techniques.



Summary of different soil organic matter fractions. The different compartments arise from a variety of different schemes for fractionation and approaches to analysis. The different compartments are not mutually exclusive and the divisions between different branches cannot therefore be regarded as rigid.

THE PARADOX OF SOIL ORGANIC MATTER MINERALIZATION

S. Kemmitt[1], P.C. Brookes[1], T. O'Donnell[2], I. Waite[2]

[1]*Agriculture and Environment Division, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ sarah.kemmitt@bbsrc.ac.uk, philip.brookes@bbsrc.ac.uk*
[2]*School of Biology, University of Newcastle, University of Newcastle-Upon-Tyne, NE1 7RU tony.odonnell@ncl.ac.uk, I.S.Waite@newcastle.ac.uk*

The mineralization of soil organic matter is a basic and central feature of the carbon cycle. However, several key questions remain unanswered. Firstly, soil micro-organisms (collectively the soil microbial biomass), can only mineralise this humified material at very slow rate. Why have some organisms not evolved an ability to mineralise it faster, and so obtain an evolutionary advantage over less successful ones? Secondly, even when more than 90 % of the original microbial biomass is killed by fumigation with $CHCl_3$, the mineralization of soil organic matter proceeds for long periods(i.e.>100 days) at the same rate as that in a similar, but non-fumigated soil. How can the much smaller and much less diverse microbial population in the fumigated soil, which is believed to mainly comprise fast–growing Pseudomonads, mineralise the large humifed, recalcitrant soil organic matter pool at the same rate as the larger, complex, population in the non-fumigated soil? This is a phenomenon that has baffled soil microbial ecologists ever since its discovery, more than 4 decades ago.

To study this phenomenon further, we incubated an arable and grassland soil for 62 days with and without an initial fumigation. Even thirty days after chloroform fumigation, long after the 'flush' of mineralization of the fumigant-killed biomass had passed, the biomass had only recovered to 20-30% of that in the unfumigated control, whether measured using biomass C or ATP concentration. However, the rates of CO_2 evolved over days 30-62 were very similar in the fumigated and unfumigated treatments: around 2 µg CO_2-C in the arable and 12-13 µg CO_2-C g^{-1} d^{-1} in the grassland soil.

Thus, the large intact biomass in the unfumigated soil and the depleted biomass in the fumigated soil were both mineralizing soil organic matter at the same rate.

It is usually assumed that the rate limiting step in the mineralization of soil organic matter is biological. However, this data suggests that the rate controlling step may not be regulated by the size or activity of the biomass. For example it may be regulated by physical/chemical factors (e.g. diffusion, desorption, oxidation, free radical activity, hydrolysis, etc.).

We are investigating the community structure and activity of the microbial populations in the fumigated and non-fumigated soils using microbial (biomass C and CO2-C), biochemical (PLFAs) and molecular techniques (16S and 18S rRNA) to explore the link between microbial community structure and it's activity in an attempt to resolve the paradox of soil organic matter mineralization.

MICROBIAL RESPONSE TO ADDITION OF GLUCOSE

F.C. Hoyle[a, b], D.V. Murphy[a], P.C. Brookes[c]

[a]Centre for Land Rehabilitation, School of Earth and Geographical Sciences, Faculty of Natural and Agricultural Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, WA6009. [b]Department of Agriculture Western Australia, Centre for Cropping Systems, PO Box 483, Northam, WA6151. [c]Agriculture and the Environment Division, Rothamsted Research, Harpenden, Hertfordshire, AL52JQ, United Kingdom

Evolution of CO_2-C is reported following the addition of glucose (10-50 mg C kg^{-1} soil) to arable soils, either amended, or not with cellulose. An immediate CO_2-C release (equivalent to a maximum 60% of glucose-C applied) resulted 4-69 h after application, and was attributable to microbial decomposition of applied glucose-C. Previously, De Nobili et al. (2001) proposed that additions (11.3-34 mg C kg^{-1} soil) of 'trigger molecules' (e.g. glucose, amino acids, root exudates) caused the soil microbial biomass to become activated in anticipation of a significant 'food event'. They measured up to several times more CO_2-C evolved than applied in their solutions, and suggest this 'extra' C was derived from endocellular reserves. In this study however, we found no evidence of an initial response on application of glucose-C to soils that would suggest use of endocellular C. In our study, two further phases of microbial activity were however observed in cellulose-amended soils, from 69-300 h during which CO_2-C rates declined significantly to below those in comparable control soils and a further, variable phase of CO_2-C accumulation from 300 h. We propose this pattern of CO_2-C evolution was attributable to either the activation of different microbial populations in cellulose amended soils on addition of glucose-C, or end-product inhibition of cellulase activity.

MICROBIAL BIOMASS AND ITS ACTIVITIES UNDER LONG-TERM HEAVY METAL STRESS IN SOILS NEAR A LEAD SMELTER

G. Mühlbachová[1], P. Tlustos[2]

1 Research Institute of Crop Production, Drnovska 507, 161 06 Prague 6 – Ruzyne, muhlbachova@vurv.cz
2 Czech Agricultural University in Prague, Kamycka 129, 165 21 Prague 6 - Suchdol

The soil microbial biomass, respiratory and dehydrogenase activity, the metabolic quotient (qCO_2 = mg CO_2-C evolved g^{-1} biomass C day^{-1}) and the ratio microbial biomass/organic carbon (B_c/C_{org}) were determined in arable and grassland soils with different level of heavy metal contamination (particularly Pb, Cd and As) near a lead smelter in Czech republic. The smelter has been in operation for over 200 years until the present. The working of lead ores ceased in 1972, but the secondary smelter is still

in operation. The 150 m high stack with more than 99% efficient ash filters was built in 1982.

Currently soil metal concentrations are up to about 2500 mg Pb kg^{-1} soil, 9 mg Cd kg^{-1} soil, 300 mg Zn kg^{-1} soil and 200 mg As kg^{-1} soil.

Significant relationships between soil organic C and microbial biomass ($r^2 = 0.64$), microbial biomass and dehydrogenase activity ($r^2 = 0.75$) and microbial biomass and respiratory activity ($r^2 = 0.45$) were obtained irrespective of the level of the contamination, whereas no significant relationships were found between microbial activities and the concentrations of heavy metals. Despite higher metal concentrations in grassland soils, microbial biomass C and B_c/C_{org} were larger than in arable soils, whereas qCO_2 in grassland soils tended to decrease.

A ten days incubation experiment with glucose added to contaminated and uncontaminated arable soils showed that the microbial biomass in contaminated soil after 24 h incubation was larger than in uncontaminated soil. However, lower consumption of added glucose was observed in the contaminated soil. In consequence, lower respiratory rates and qCO_2 were also found in the contaminated soil.

In order to examine the abilities of microbial communities to mineralize added substrate, the soils with different heavy metal concentrations were amended with glucose and then incubated for 48 hours. The K_2SO_4-extractable carbon and respiratory activity were periodically determined during the incubation. The results showed that if soils of similar organic matter content and of similar management were compared, the contaminated soils generally had slower decreases in added carbon and lower respiratory activity in comparison to soils having lower metal concentrations.

The simple monitoring of soils in Pribram area may not detect the direct influence of metal contamination on the microbial activities. Greater organic matter contents in grassland soils had positive effects on microbial communities despite to the level of metal contamination.

However, the capacity of soil micro-organisms to mineralize fresh organic substrates seems to be affected by heavy metals.

EFFECT OF INCREASED CO_2 RATES ON MICROBIAL BIOMASS METABOLISM

K. Inubushi

Chiba Univ., Japan

Atmospheric CO_2 concentration is increasing gradually and this will induce significant impacts on ecosystems, their components and interactions. In paddy fields, there are active sites of microbial processes related to N_2-fixation, methane and nitrous oxide gas formation and/or oxidation such as flooded water, oxidized or reduced soil layers and rhizosphere. A Rice FACE (Free -Air CO_2 Enrichment) experiment was conducted in paddy fields in Iwate Prefecture, Japan to study the effects of elevated CO_2 on various processes in the paddy soil-floodwater ecosystem. Effects of elevated CO_2 on soil microbial biomass, methane and nitrous oxide

formation/oxidation and N_2-fixation activity were investigated in FACE field as well as chamber experiment. In FACE experiment, rice plants were grown under ambient or elevated (ambient + 200ppmv) CO_2 throughout the growing season for three years. The pure CO_2 was released into the rice canopy in the FACE plot through octagonal-plastic CO_2 emitting tubes. Surface- and subsurface layer soils (0-1 and 1-10 cm depth, respectively) were sampled periodically, then microbial biomass C, N and N_2-fixation activity in both soil samples were quantified by chloroform-fumigation extraction method and acetylene reduction method, respectively. Methane and nitrous oxide (N_2O) gas emissions through rice plant were measured by cylindrical chamber. Growth chambers with ambient or elevated CO_2 concentration were also used for N_2-fixation activity of both surface and subsurface soil samples. PLFA analysis was also carried out.

During the first growing season, FACE did not cause any significant difference in microbial biomass C and N, compared to those in ambient CO_2 treatment. However in the second season, FACE caused a significant increase in biomass N in the upper soil layer at harvest, compared to those of ambient CO_2. Furthermore, FACE significantly increased the biomass C in both soil layers, from mid plant growth until harvest. Compared to the ambient CO_2 treatment, the FACE treatment significantly increased the N_2-fixation activity in both the upper and lower soil layers at harvest. In chamber experiment under elevated atmospheric CO_2 concentration, N_2-fixation activity also significantly increased in the surface soil layer during the early cultivation stages and in the sub-surface soil layer during the latter part of cultivation. Methane emission was slightly suppressed by elevated CO_2 in the first season, but enhanced significantly in the second and third seasons, and so did as N_2O, indicating positive feedback of global warming. From these results, elevated CO_2 concentration influenced these microbial processes significantly in submerged soil.

FACTORS AND MECHANISMS OF RHIZOSPHERE PRIMING EFFECTS

Y. Kuzyakov

University of Hohenheim, Germany, kuzyakov@uni-hohenheim.de

Living plants change the local environment in the rhizosphere and consequently affect the rate of soil organic matter (SOM) decomposition. The SOM decomposition rate may increase for up to 3- to 5-folds, or decrease by 10% to 30% by plant cultivation. Such short-term changes of the rate of SOM decomposition are caused by priming effects. Priming effects (= short term changes of SOM decomposition intensity) were frequently measured after addition of mineral or organic fertilizers, plant residues, as well as low molecular weight organic substances to soil. In the presence of plants, priming effects occur in the direct vicinity of the living roots and they can by called rhizosphere priming effect (RPE). Plant-mediated and environmental factors, such as, plant species, development stage, soil organic matter content, photosynthesis intensity, and N fertilization which affect RPE will be reviewed and discussed. Among other plant-mediated factors affecting RPE, root

growth dynamics and photosynthesis intensity are the most important. Soil related factors such as amount of decomposable C in SOM and N_{min} content are responsible for the switch between following mechanisms of RPE:

1) strong concurrence for N_{min} between roots and microorganisms, on the background of low level of decomposable C in soil leads to retardation of SOM decomposition,
2) activation of microbial biomass by easily available root exudates for faster SOM decomposition, or
3) preferential utilization of root exudates instead of SOM.

Activation of microbial biomass by root exudates for faster SOM decomposition is the most probable mechanism of RPE in natural and grassland soils having usually a high level of C_{org} and low level of N_{min}. In contrast, in arable soils having generally low level of C_{org} and high N_{min} content, preferential substrate utilization is expected to be the dominating mechanism of changes (decrease) of SOM decomposition in the presence of growing roots.

Hypothesis of succession of mechanisms of RPE along the growing root in accordance with the rhizodeposition types will be suggested. Different possibilities for mechanisms of filling up the C amount loss by RPE will be discussed.

The ecosystematic relevance of priming effects induced by plant rhizodeposition relates to the connection between exudation of organic substances by roots, the increase of microbial activity in the rhizosphere through utilization of these additional easily available C sources, and the subsequent intensive microbial mobilization of nutrients from the soil organic matter.

CADMIUM IN UPLAND FORESTS AFTER VITALITY FERTILIZATION WITH WOOD ASH - A SUMMARY OF SOIL MICROBIOLOGICAL STUDIES INTO THE POTENTIAL RISK OF CADMIUM RELEASE

J. Perkiömäki, H. Fritze

Finnish Forest Research Institute, P.O. Box 18, 01301 Vantaa, Finland

The use of wood ash in forestry has been questioned because the potential risk associated with its cadmium (Cd) content (1-30 mg kg^{-1}). In agriculture, wood ash is only allowed for use as fertilizer when its Cd content is below 3 mg kg^{-1}. This restriction has not been applied to forest soils and there is a lack of knowledge about the potential harmful effects of the Cd in wood ash on forest ecosystems. This paper summarizes our recent studies on the microbial communities of boreal coniferous forest humus exposed to Cd-containing wood ash treatment. The main objectives of our studies were to test if the Cd in wood ash has the potential to affect the humus layer microflora of coniferous upland forests and if the Cd has the potential to enter the human food chain. These objectives were tested both in laboratory and field experiments with ash and ash spiked with Cd (in laboratory 400 or 1000 mg Cd kg^{-1} as CdO or $CdCl_2$; in field 400 mg Cd kg^{-1} as CdO). In one study the dissolution of ash was accelerated by irrigating it with simulated acid rain (SAR).

Wood ash increased humus layer pH and microbial activities (respiration or thymidine incorporation rates) and changed its microfloral community structure (Biolog®, PLFA, 16S or 18S rDNA PCR-DGGE) in both laboratory and field and experiments. Spiking ash with Cd induced no further changes in the above-mentioned variables as ash alone. The Cd added with wood ash did not become bioavailable as detected with a bacterial biosensor *Bacillus subtilis* BR151(pTOO24). The form and level of Cd added in the ash had no further effect on the microbiological variables studied. Irrigation of ash with SAR did not increase the amount of bioavailable Cd, although the dissolution rate of the ash was increased. The concentration of Cd in soil water and in the berries of *Vaccinium uliginosum* and *V. vitis-idaea*, and the amount of humus bioavailable Cd did not increase with the ash or ash spiked with Cd although the ash spiked with Cd increased the amount of humus total and extractable Cd in the 4 year field study. Only the ash spiked with Cd and not the unspiked "normal" wood ash resulted in significantly higher Cd concentrations in the mushroom *Lactarius rufus* and a slight increase in the berries of *Empetrum nigrum* (first year only).

SHIFTS IN MICROBIAL ECO-PHYSIOLOGY DURING DECOMPOSITION OF PLANT RESIDUES

A.S. Mamilov, O.M. Dilly

Institute of Microbiology and Virology, Bogenbai batyra 103, 480100, Almaty, Kazakhstan
mamilov@nursat.kz
Institut für Bodenkunde, Universität Hamburg, Allende-Platz-2, 20146 Hamburg, Germany
o.dilly@ifb.uni-hamburg.de

The mechanisms driving decomposition process are of great interest for decades. In out study litterbags with oat straw were incubated for 52 days in soil under beach forest and recovering cropland to follow the shifts in microbial eco-physiology. Microbial biomass measured by fumigation-extraction (FE) decreased continuously after 7 days of incubation, while substrate-induced respiration (SIR) peaked on day 17 in the two ecosystems. Comparing the values of glucose responsive (SIR) and chloroform sensitive (FE) biomass gives information about metabolic responsive component in total microbial biomass, i.e. the amount of microorganisms responsive to glucose addition. Metabolic-responsive biomass decreased after 17 days in both treatments indicating the increasing portion of dormant microorganisms with proceeding decomposition. The basal respiration (BAS) increased for 32 day and then decreased again. Considering the values to which extent SIR exceeds basal respiration may reflect requirements of soil microbiota in available carbon and may be expressed as carbon demand index. The quotient significantly decreased after 17 days of incubation. Decreasing carbon demands of microbiota developed on decomposed oat

straw during the period 17-32 days was probably caused by lowering portion of microorganisms responsive to glucose addition.
Reduction of microbial biomass and decomposition rate seems to be caused by a lowering capability of microorganisms to synthesise biomass due to increasing portion of metabolically irresponsive component rather than depletion of available carbon sources. Similar dynamics of microbial biomass, respiration rate, metabolic responsive component and carbon demands in contrasting ecosystems suggest a common character of physiological shifts during decomposition of incorporated plant residues.

TWENTY YEARS OF MOLECULAR ANALYSIS OF BACTERIAL COMMUNITIES IN SOILS AND WHAT HAVE WE LEARNED ABOUT FUNCTION

A.G. O'Donnell, S.R. Colvan, R. Husband

Institute for Research on Environment and Sustainability University of Newcastle upon Tyne, NE1 7RU, UK
tony.odonnell@ncl.ac.uk

Soils support a taxonomically and physiologically diverse biota widely regarded as more extensive than that of any other group of organisms. However, the limits of this diversity and its importance in delivering soil function remain unclear. One of the key considerations in relating microbial diversity to function is the ability to determine accurately and reproducibly the size, activity and diversity of the soil microbial biomass. However, prior to the introduction of molecular techniques, reliably measuring these parameters and relating them to individual organisms or taxa was virtually impossible with a clear mismatch between our ability to make process measurements such as respiration and soil enzyme activity and our ability to identify which organisms or groups of organisms were involved in these processes. With the introduction of molecular methods however, microbiologists have for the first time been able to open the microbial 'black box' in soils. The justification often given for opening this 'black box' is that the diversity of the contents therein is vitally important to the maintenance and sustainability of the biosphere since it is argued that by knowing what is in the box we will be able to manipulate its contents more effectively. However, despite almost 20 years of detailed sifting through the box contents, there is little evidence to support this suggesting that many of the thousands of microorganisms in soils are functionally redundant and that many of the major functions of the microbial biomass are unaffected by its exact species composition. This paper looks at some of the many approaches used to open the microbial black box and discusses whether the introduction of such techniques means that we are any closer to understanding the relationship between diversity and function in soils.

PROBING SOIL MICROBIAL COMMUNITIES USING COMPOUND-SPECIFIC STABLE ISOTOPES APPROACHES

R.P. Evershed

Organic Geochemistry Unit, Biogeochemistry Research Centre, School of Chemistry, Cantock's Close, Bristol BS8 1TS, UK

The complex nature of the soil microbial community coupled with their intractability to conventional *in vitro* culturing techniques makes their presence and specific roles difficult to detect and/or differentiate. Compound-specific stable isotope techniques, based on gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), offer a powerful new means of probing the soil ecosystem using either natural abundance or stable isotopically enriched substrates as tracers. This paper will provide an overview of the instrumental analytical techniques and experimental approaches that we are using to provide new insights into the activities of soil micro-organisms. Examples drawn from our recent and current work will be used to illustrate the possibilities that exist for the application of stable isotopically labelled tracers and the GC-C-IRMS technique in this area.

VARIABILITY OF ATP CONCENTRATION IN THE SOIL MICROBIAL BIOMASS: PHYSIOLOGICAL CHANGES OR ARTEFACTS OF THE METHODS?

M. Contin

Dipartimento di Scienze Agrarie e Ambientali – University of Udine, via delle scienze, 208 – 33100 Udine – contin@uniud.it

ATP has been proposed as a general indicator of life and an alternative measure of microbial biomass in aquatic environments, sediments and soils since 1966 (Holm-Hansen and Booth), but several methodological aspects and theoretical assumptions are still controversial.

Initially, great effort was devoted to develop efficient extraction reagents and an universally applicable methodology to extract ATP from soil micro-organisms. Many papers focused on that but there is still scientific debate. ATP concentration in microbial biomass and adenylate energy charge (AEC) are often indicated as the best indexes of ATP extraction efficiency (Karl, 1980), but in some instances (Webster et al., 1984, Bai, 1988) high recovery of internal standard (the spike) have erroneously been considered to assess the complete extraction of microbial ATP. I will show that this is incorrect.

The use of a strongly acidic extractant is the only guarantee of rapid and complete inactivation of the enzymes involved in hydrolyses of ATP during cellules disruption. It has been established that ATP added as an internal standard (the spike) behaves similarly to native ATP only in certain extractants (e.g. TCAPP) but differently in the

presence of DMSO (Contin et al., 1995), neutral or alkaline buffers (Brookes et al., 1997) or in recently glucose-amended soil (De Nobili et al., 1996).
A second important problem in using ATP as a measure of microbial biomass is the choice of the conversion factor. Is the ATP concentration of the microbial biomass constant or influenced by its activity? The literature is still unclear on this apparently trivial question. But, rather than rely on the mean value of ATP concentration of microbial biomass (ATP/Bc) it is interesting to examine its coefficient of variation (CV) to provide an indication of the accuracy and reliability of mean values.
Theoretical calculations, based on error propagation theory of ATP and Bc analytical variability, determine a CV for ATP/Bc of 11.6%. It means that all experimental values of ATP/Bc, based on 4 replicate measurements, falling into the range 11±3.7 belong to the same population, i.e. are not statistically different (P>95%; n=4). Thereafter variations within this range could not attributed to physiological changes of microbial populations but rather to imprecision of methodology. It is often possible to demonstrate that values of soil ATP/Bc exceeding this interval are attributable to flaws in the methodology. Moreover, the inherent variability of current methodology could be reduced increasing the number of replicates, imposing strictly standardized incubation conditions of soils and adopting very accurate analytical methods and protocols.

A constant ATP concentration in soil microbial biomass like any other process of scientific reduction is always difficult to determine whether it is a useful unifying hypothesis or whether it is an unrealistic oversimplification.

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USE OF REPORTER GENES FOR MONITORING HEAVY METAL BIOAVAILABILITY AND CONCENTRATION OF ORGANIC MOLECULES INCLUDING SIGNALS IN SOIL

G. Renella

Department of Soil Science and Plant Nutrition, University of Florence, Florence, Italy

Reporter genes are nucleic acid sequences encoding easily detectable proteins, like enzymes, light emitting molecules such as luciferin-luciferase system, green fluorescent protein (GFP) which fluoresces under UV light. Reporter bacteria contain genes attached to specific nucleic acid sequences so that only the reporter protein is

produced, or the reporter gene is under the control of other genes, so that both transcription and translation continues from the inserted gene directly into the reporter gene product, resulting in the synthesis of hybrid proteins (protein fusion).

Progresses in the knowledge of genes involved in different cellular events (growth, protein synthesis, nutrient aquisition and limitation, responses to red-ox conditions and stress), have allowed to project gene fusions for monitoring different cellular events such as induction, gene translation, metabolite and protein turnover, efficiency of molecular cloning, in microrganisms, but also in plants and animals.

Recent developments of this approach have led to progresses in our understanding of how soil microrganisms interact with their natural environments and perceive toxic organic and inorganic pollutants. Particularly, the *lux*-based whole-cell bacterial biosensors seem a promising application to soil toxicology for the detection of the so called 'bioavailable' fraction of heavy metals in contaminated soils.

Two main strategies have been followed for preparing soil toxicology tests based on production of light by *lux*-containing soil bacteria:

i) the reporter gene is inserted in sensitive soil bacteria (e.g *P. fluorescens* and *P. putida*, *Rhizobium* sp. and *Burkholderia* sp.), so that a reduced bioluminescence can be attributed to a negative effect on metabolic energy flow or to a reduction enzyme activity by soil pollutants (Paton et al., 1995)

ii) the reporter gene is inserted in resistant soil bacteria (e.g. *R. eutropha*) where its expression is under control of an inducible promoter, the light signal is specifically triggered by inductive stimulus (e.g. heavy metal) (Collard et al., 1994).

The use of *lux*-based 'metal-sensitive' and 'metal-resistant' reporter bacteria available for assessing heavy metal bioavailability in different heavy metal-contaminated soils is discussed, also considering their potential applications and limitations.

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APPENDIX 3

List of participants

Nr	Name		Country	e-mail address
1	Alabouvette	Claude	F	ala@dijon.inra.fr
2	Badalucco	Luigi	I	badalucc@unipa.it
3	Brookes	Phil	UK	philip.brookes@bbsrc.ac.uk
4	Contin	Marco	I	marco.contin@uniud.it
5	Crecchio	Carmine	I	crecchio@agr.uniba.it
6	De Nobili	Maria	I	maria.denobili@uniud.it
7	Dilly	Oliver	D	oliver@ecology.uni-kiel.de
8	Evershed	Richard	UK	r.p.evershed@bristol.ac.uk
9	Fritze	Hannu	FI	hannu.fritze@metla.fi
10	Hamer	Ute	D	ute.hamer@ruhr-uni-bochum.de
11	Harris	Jim	UK	j.a.harris@cranfield.ac.uk
12	Hirsch	Penny	UK	penny.hirsch@bbsrc.ac.uk
13	Hoffmann	Lucien	LUX	hoffmann@crpgl.lu
14	Hopkins	David	UK	d.w.hopkings@stir.ac.uk
15	Hoyle	Frances	AU	fhoyle@agric.uwa.edu.au
16	Insam	Eribert	A	heribert.insam@uibk.ac.at
17	Inubushi	Kazuyuki	J	inubushi@midori.h.chiba-u.ac.jp
18	Joergensen	Rainer	D	joerge@wiz.uni-kassel.de
19	Kemmitt	Sarah	UK	sarah.kemmitt@bbsrc.ac.uk
20	Kuzyakov	Yakov	D	kuzyacov@uni-hohenheim.de
21	Leita	Liviana	I	leita@isnp.it
22	Mamilov	Anvar	KZ	mamilov@nursat.kz
23	Mondini	Claudio	I	mondini@isnp.it
24	Muhlbachova	Gabriela	CZ	muhlbachova@vurv.cz
25	Nannipieri	Paolo	I	paolo.nannipieri@unifi.it
26	O'Donnell	Tony	UK	tony.odonnell@ncl.ac.uk
27	Raubuch	Markus	D	raubuch@wiz.uni-kassel.de
28	Renella	Giancarlo	I	giancarlo.renella@unifi.it
29	Sanchez-Monedero	Miguel	E	monedero@cebas.csic.es
30	Steinberg	Christian	F	steinber@dijon.inra.fr
31	VanVeen	Hans	NL	h.vanveen@nioo.knaw.nl
32	Wichern	Florian	D	fwichern@wiz.uni-kassel.de