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FUNCTIONAL IMPACT OF GENETICALLY MODIFIED MICRO-ORGANISMS ON THE SOIL ECOSYSTEM.

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Abstract.

Most attempts to monitor the effects of introductions of Genetically Modified Micro-organisms (GMMs) have centred on the enumeration of specific populations. However for a significant perturbation to be measured, changes of between 100% and 300% (0.3 and 0.5 on a log scale) are necessary for the impact to be significant. Standard population measurements, assessing the survival, dissemination and effect on total indigenous populations do not give an indication of the functioning of the ecosystem. There is very little literature regarding the functional impact of GMMs, for instance the effect upon nutrient cycling or functionally important groups of organisms. Through out this review a number of methods for the detection of perturbation in the rhizosphere/soil ecosystem are assessed. A range of non functional methods are summarised first, including molecular and non molecular genetic population diversity studies. Functional methodology, for example nutrient cycling, is then assessed for application as indicators of impact in the soil ecosystem and the significance of measuring functional impact is highlighted. These functional methods are classified into those relying upon the culturability of the target organisms and those that do not. Non culture methods discussed include biomass, respiration, nutrient cycling, mRNA studies and soil enzyme assays. Recently a range of soil enzyme assays have been used as alternatives to population measurements. The impact of a chromosomally marked *Pseudomonas fluorescens* (SBW25), on soil chitobiosidase, N-acetyl glucosaminidase, acid and alkaline phosphatases, phosphodiesterase, aryl sulphatase and urease were studied. Using these enzyme assays impacts of less than 20% could be detected. A series of interactions were observed which depended on whether a mixture of soil enzyme substrates were added to soil. Generally, microbial inoculation increased the enzyme activity of the biomass, but effects are likely to be

dependent upon the nature of the genetic modification. The environmental implications of these effects are discussed.

1. Introduction

Modern molecular biological techniques provide the potential to manipulate (add, delete, change) the genetic make up of plants, animals and micro-organisms to the benefit of the human population. To maximise these benefits and minimise harm, both to the human populations (physically and conceptually) and to the environment, studies into the ecology and the effects of Genetically Modified Organisms (GMOs) opon other organisms must be conducted.

At present there are strict guidelines regarding the release or the marketing of genetically modified organisms, requiring extensive evaluation of the organism itself and the effects of the genetic modification prior to the environmental application of such organisms (European Comunity (EC) directives 90/220/EEC and 94/15/EC(e), British Department of the Environment (DoE) Genetically Modified Organisms (deliberate release) regulations 1992,1993 and 1995). Thus a thorough analysis of such organisms is required, and both the EC and the DoE have provided substancial funding for such work. IMPACT is one of the projects funded by the EC Biotechnology programme, running between 1993 and 1996, involving 17 European partners in the area of ecological implications of biotechnology and incorporates an industrial platform. The word IMPACT is an acronym for **I**nteractions between **M**icrobial inoculants and resident **P**opulations in the rhizosphere of **A**gronomically important **C**rops in **T**ypical soils. The primary goal of the project is to understand the molecular ecology of genetically engineered bacteria in rhizosphere ecosystems. It is designed to “examine key areas for example: the effect of inoculation on soil fertility; the potential benefits (plant health and yeald) that can be achieved with these inoculants, both wild type

and modified and the interactions of such inoculants on selected components of the rhizosphere/soil flora." It is hoped that at the completion of the project a considerable amount of valuable information and technology will be attained that will be of great value for the commercial use of microbes in various areas of microbiology. These will include improved inoculum technology, detection methodology and tools for the study of microbial ecology.

A major goal of research in the wide range of possible applications of genetically modified organisms is in the production of improved microbial biological control agents antagonistic to plant diseases. Annual losses of economic crops world wide caused by plant diseases was estimated to be between 13 and 20% of total crop yeilds. This represents losses of over 5 X 10^{10} US dollars per annum (James 1981). The agrochemical market in 1991 was estimated at 26800 million US dollars, however biological control products accounted for less than 0.5% of the total (Powell and Jusum, 1993).

Currently the only commercially available genetically modified biocontrol agent on a large scale is the bacterium *Agrobacterium radiobacter* K1026 (Kerr 1989, Jones and Kerr 1989). This is a strain modified from a wild type biocontrol agent (*Agrobacterium radiobacter* K84) of crown gall in rose trees, stone fruit trees and nut trees caused by *Agrobacterium tumefaciens*. The metabolite that confers the biocontrol activity is the antibiotic agrocin 84, which is encoded on a plasmid along with resistance genes to agrocin 84. It was found that the biocontrol agent was unstable as the plasmid could transfer to pathogenic species. This problem was overcome by the deletion of the region of the plasmid that encoded the transfer gene, Tra, rendering the biocontrol agent incapable of conjugative

transfer to the pathogen. The manipulated strain is now commercially available throughout the world under the marketing name "No Gall". Even this genetically modified strain which did not have additional genetic information inserted, was greeted with great scepticism by the general public and the media, irrespective of the proven (and increased!) safety of the strain and the subsequent benefits of increased crop yields. This should serve as a sharp reminder of the stumbling blocks that can be encountered with the use of genetically modified organisms and encourage thorough investigation of every aspect of each individual strain/organism which is genetically modified. Release of environmentally harmful GMOs would probably result in a ban of other, potentially beneficial, GMOs.

Assessment of potentially harmful effects of genetically modified micro-organisms (GMMs) on the soil and especially rhizosphere ecosystems is therefore essential (Smit *et al* 1992). Previously, most attempts to monitor the effects of microbial introductions to the rhizosphere have centred on microbial enumeration of specific populations, often aided by molecular techniques (for example Tsushima *et al* 1995). These methods rely on large changes, as microbial numbers, measured on a log scale, require differences of between 100 and 300% (0.3 and 0.5 on a log scale) to be significant, and do not provide a wide picture of the overall effect of the introduced microbe on the whole ecosystem. De Leij *et al* (1993a) used colony development for the quantitative assessment of r and K strategists from different habitats and formulated an ecophysiological index (EPI), but these methods also rely on culturable micro-organisms. Molecular detection methods do no more than monitor or detect specific micro-organisms and do not give any indication of the actual effect of the inoculum on the ecosystem (Pickup, 1991, Van Elsas and Waalwijk 1991, Tsushima *et al* 1995 and for a review see Morgan 1991). Marker genes can be deployed to track GMMs *in situ* using potential luminescence (Meikle *et al*, 1994), or to reisolate and enumerate the introduced

micro-organisms. For example De Leij *et al* (1993b) used a most probable number technique to recover a genetically modified *Pseudomonas* from soil. Such measurements only provide information about the presence and survival of a specific micro-organism in soil.

Previous work using the genetically modified *Pseudomonas fluorescens* SBW25 strain carrying the *lacZY* and *xylE* functions has concentrated on the ecology of the released organism (De Leij *et al,* 1994a), and has relied upon culturable methods to assess impacts on specific microbial groups (De Leij *et al,* 1994b). This, however, does not provide an overall view of the impact of the GMM upon the functioning of the soil ecosystem as a whole.

The measurement of perturbations with soil biochemical factors, such as enzyme activities, may be an alternative way of monitoring effects of the introduced GMM on the ecosystem, in a more sensitive and comprehensive way. Soil phosphatase activity has been shown to be an important indicator of the impact of soil management systems and of the organic matter content of the soil (Jordan *et al*, 1995). Doyle and Stotzky (1993) found no difference in enzyme activities (aryl sulfatase, phosphatases and dehydrogenase) when an *E coli* strain was introduced into soil. In contrast, Mawdsley and Burns (1994) successfully used soil enzyme measurements to detect perturbations caused by a *Flavobacterium* inoculation onto wheat seedlings, finding increased α -galactosidase, β-galactosidase, α-glucosidase and β-glucosidase activities.

2. Non-functional indicators of perturbation

In soils one of the major factors to consider is perturbation caused by soil treatments, and implicit in this is that the baseline ecology is well understood. Several methods are now available for the measurement of the diversity of microbial populations, some of which are therefore applicable for the detection of perturbations caused by soil treatments (Table 1). These methods are effective in assessing the diversity of micro-organisms within or between environmental samples.

There are several other DNA/molecular based methods for identifying micro-organisms and/or measuring their diversity, these techniques are highlighted in Table 2. These methods are often highly sensitive, specific and are therefore powerful tools in the identification, quantification and monitoring or detection of specific micro-organisms in soil. However species concepts of micro-organisms are much less definitive than species differentiation for larger organisms. It, therefore, seems more favourable to consider the biodiversity of micro-organisms in terms of genetic diversity. Genetic diversity in itself cannot wholly be described as definitive considering the known genetic promiscuity of bacteria, where functional elements can be exchanged through the community rapidly. In this way, the community as a whole and the commutative expression of the gene pool and possibly the potential cumulative expression of the gene pool can be considered as one. Gene expression is measured by inducing specific elements of the gene pool or alternatively by probing for specific genetic elements which encode targeted.

All the methods described so far are of importance in the study of microbial population dynamics, taxonomy and diversity within the populations under study, but the sum of the parts using these methods never makes the answer to the whole question. Furthermore they do not in themselves give any indication of the actual functioning of the community, and therefore can not give an indication of the impact of any perturbing agent on the ecological functioning of the ecosystem. To overcome this problem, methods of looking at the dynamics and functioning of the community and ecosystem as a whole are required.

Functional analysis should be considered in terms of benefits and detriments to man or the environment. A proportion of the biota will carry out beneficial functions, for example promoting crop growth, whereas others such as pathogens are harmful. A reduction in biodiversity is likely to be useful only if the harmful components are eliminated. Beneficial features could be enhanced by their improvement, phenotypically or genotypically for example to reduce farming inputs. This is especially important in areas like crop rhizosphere studies where nutrient dynamics can greatly influence crop yields. Functional diversity, should therefore, concentrate on keystone populationswhose loss would be detrimental to the overall functioning of the ecosystem (keystone processes). The loss of diversity in terms of a loss a species may not be of significance in terms of the effect on the processes it was involved in, if the functional gaps left are filled by other resident species, then the overall effect will be cancelled out.

Microbial, biochemical and metabolic diversity may be increasing as a consequence of manipulation of the environment. The microbial communities adapt rapidly to the extrinsic agrochemicals and industrial pollutants, developing novel metabolic pathways to detoxify or resist or even to assimilate the chemicals. This has been of great concern to the agrochemical

and pharmaceutical industries, where microbial resistance to pesticides and antibiotics is of considerable economic importance. There are a number of key areas in the soil ecosystem function that should be considered in any functional assessment of impact of treatments, these key areas are summarised in Figure 1. Methods of measuring perturbation in the different components of the system are subsequently assessed through the review.

3. Functional indicators of perturbation

Methods of assessing functional diversity can be divided in two halves, those requiring culture techniques and those that do not.

a. Culture methods:

i. Using selective media.

Selective media can be used for the isolation and enumeration of bacteria able to utilise specific components of the media on which they were isolated on, for example using chitin as a sole carbon source (Inbar and Chet 1991a and b). Chitin utilisation (chitinase production) may be a key component in the antifungal capacity of many micro-organisms (Inbar and Chet 1991a and b) and its decomposition is a key soil process in nutrient cycling (Gould *et al* 1981). Other substrate examples include cellulose (Lynch *et al* 1981), starch and acetate (Mills and Wasel 1980). Doyle and Stotzky, (1993) used soil extract media, basal media and variations including growth factors and amino acids to isolate a range of micro-organisms with different phenotypes. Janzen *et al* (1995) directed their isolation techniques to select for micro-organisms from specific communities involved in key areas of nutrient cycling (sulphate reducers, denitrifiers nitrogen fixers and phosphorus mobilises). They found a wide range of changes in the dynamics of different functional groups with the addition of a compost extract.

ii. Characterisation of microbial communities by colony development.

De Leij *et al* (1993) characterised soil and rhizosphere communities by classifying colonies by age (speed of appearance on agar plates over a ten day period). The colonies were then classified into r and K strategists, r strategists are fast growing, opportunistic organisms that predominate in nutrient rich environments, but are not competitive when nutrient sources are recalcitrant or become scarce. K strategists are much slower growing organisms that compete well when an environment reaches its carrying capacity and nutrient sources are scarce or recalcitrant. K strategists have a more efficient metabolism and are more abundant in crowded and well established niches, they tend to be less affected by toxins and perturbation than the sensitive r strategists (Andrews and Harris 1986). Ability to withstand toxic or changing conditions is an important functional attribute and thus this kind study is useful in diversity and perturbation analysis.

iii. The BIOLOG system.

BIOLOG relies on the redox dye tetrazolium violet to detect NADH formation by active microbes utilising sole carbon sources. This system was originally designed as a taxonomic guide for the identification of bacteria, but it has been used as a method of assessing the functional diversity of microbial communities (Garland and Mills 1991, Zac *et al* 1994). BIOLOG has the advantage that up to 95 different sole carbon sources can be tested for the growth of bacteria from an environmental sample. It can be considered a semi-quantitative method as rate of colour development over time can be assessed (up to 72 hours) but unlike specific culture media it does not allow enumeration of the organisms using the carbon source and taxonomic identities cannot be evaluated. The problem that, like other culture techniques,

only organisms that can grow in the substrate will give results also exists with BIOLOG. As the system is based on sole carbon source utilisation, only functional groups of carbon utilisation can be identified, interference by fungal growth can also be a problem. The use of this system in the assessment of functional diversity or the effect of a treatment (a perturbation) on functional diversity produces a very large data set and thus is a sensitive method of detecting a perturbation or differences between samples, in an ecologically relevant manor.

b. Non-culture methods:

i. Microbial biomass and respiration (activity) measurements.

There are various methods of performing biomass measurements, which provide an assessment of the whole microbial population without the necessity of culturing the micro-organisms. This is important because of the problems that culturability presents (for instance the high proportion of non culturable cells in soil (Colwell *et al* 1985)).

There are methods of distinguishing between different aspects of the soil biomass, for example the fungal and bacterial biomass. This is important when considering the bacterial biomass, as the fungal component is in general much larger than the bacterial component and, therefore, the bacterial biomass is overshadowed in total biomass measurements. Muramic acid is a molecule specific to bacteria and cyanobacteria and has been successfully estimated in sediments (Moriarty 1977, King and White 1977). There have been problems with muramic acid assays as biomass measures in soil, as the quantities found were much higher than the amount anticipated by pure culture methods (Miller and Casida 1970). This does not present a problem in perturbation studies, where

shifts in the amount of muramic acid are the important parameters rather than validation as an accurate biomass measure.

Measurements of fungal biomass were traditionally based on chitin contents (Frankland *et al* 1978). More recently assays have been developed for the measurement of ergosterol content of soil, which has been suggested as a better index of fungal biomass (Newell *et al* 1987). West *et al* (1987) recommended the measurement of ergosterol content to assess changes in soil fungal communities. Fritz and Baath (1993) found that soil ergosterol content increased by 9% in forest soil polluted by alkaline dust deposition whilst phospholipid fatty acid 18:2ω6 (another proposed fungal biomass measure) decreased by 23%.

One method of using microbial biomass to assess functional diversity or to direct biomass measurements at specific functional groups is to measure microbial activity as an indirect measure of biomass and rates of growth or activity. This objective can be attained by substrate induced respiration (SIR) or substrate induced growth response (SIGR) which is similar to SIR but is based on the concentration of substrate addition required to cause growth of the standing population (Schmidt 1992 after the models of Simkins and Alexander 1984) measured by CO_2 accumulation. SIR (and hence SIGR) tend only to measure specific groups, i.e.: those that can utilise the added substrate and thus can be directed at specific functional groups by varying the carbon source added. In respiration/biomass measurements it is assumed that the microbial community can utilise glucose, for example Schmidt *et al* (1992) 2,4-dinitrophenol as the inducer substrate. SIGR is a similar method to estimating the biomass from ecophysiological maintenance carbon requirements, (Anderson and Domsch 1985a and b) which is also measured by

CO_2 evolution, using varying carbon sources. Maintenance carbon requirement data could be used as a method of determining the effect of a treatment on an ecosystem, as in natural field conditions these values can be considered stable as a yearly mean (Lynch and Panting 1980, Jenkinson and Ladd 1981), a change can be expressed as a shift in the ratio of dormant to active portions of the soil biomass (Anderson and Domsch 1985b). This means that this method can be used to identify lasting or long term significant effects as a change in the maintenance carbon requirement (which may be a shift in the community or the diversity, but such a level of specificity would not be identified by this method, unless targeted carbon sources are used).

Microbial changes in the field can often be related to soil moisture contents and fluctuations there of (Van Gestel *et al* 1992). Therefore any treatment that causes changes in soil moisture holding capacity, or a microbial inoculant with significant properties related to soil moisture content (e.g. good survivability under desiccation) will have a significant effect on biomass related to fluctuations in field moisture content. Community diversity and available nutrients may thus be affected. Van Gestel *et al* (1992) and Botner (1985) found significant responses of microbial biomass (measured by a fumigation extraction procedure) to alternate wetting and drying regimes. Drying and rewetting is a fairly drastic treatment to the soil biomass and would thus be expected that perturbation would show in the biomass measurements, but it is a condition that occurs regularly under natural field conditions and so must be considered in any complete assessment of impact or risk. Using similar methods to those above, with the addition of labelled carbon (^{14}C), Botner (1985) and Van Gestel *et al* (1993) could distinguish the effect of wetting and drying on different microbial groups, i.e.: the fast and slow growing microbes, which in ecological theory can be distinguished as r and K strategists (as described earlier).

Another method of using respiration to produce ecologically relevant data is to evaluate the metabolic quotient (qCO_2) of the soil. Metabolic quotients (CO_2 production per unit biomass C) could be expected to be higher in younger ecological systems (Ross and Sparling 1993) which will contain a relatively high proportion of r strategists. This suggests a combination of respiration and other biomass measurements to evaluate the metabolic quotient of soil could also be an important indicator of perturbation. Higher qCO_2 could be an indicator of larger proportions of r strategists which may be present as a result of a perturbation providing a flush of available nutrients. Changes in soil respiration are a measure of changes in soil microbial activity, increases in soil microbial respiration are expected as a consequence of global warming (Jenkinson *et al* 1991). Gallardo and Shelsinger (1994) evaluated the response of soil micro-organisms to changing environmental factors, focusing on atmospheric nitrogen deposition, by measuring soil respiration as well as biomass nitrogen. They found that changes caused by various treatments (ammonium-nitrate, phosphate and sucrose additions) in soil microbial biomass and respiration could be distinguished, showing that respiration as well as biomass N can be used in assessment of such perturbation.

Nitrogen in the form of microbial biomass N may be the most important pool of nitrogen in terms of plant uptake because of its high turnover through the mineral pool compared to other nitrogen pools (Stockdale and Rees 1994). This means that biomass N will have a large influence on plant nutrition, acting as a pool of relatively available nutrients, and is especially important in stress conditions where nutrients are released from dying biomass. The biomass also releases nitrogen from other N pools making it available through the biomass N pool, depending on the nitrogen status of the microbial

biomass, more of the nitrogen (which may not be needed by the biomass) could be mineralised to the mineral pool. Therefore perturbation to the biomass N (and possibly other elements like phosphorus and sulphur) could influence crop production, and could thus act as an important indicator of perturbation in a functional sense with a relationship with plant growth and plant nitrogen uptake/content, which would then be important factors to assess.

ATP relationships with biomass have been shown to be correlated (Jenkinson 1988). In some circumstances this relationship can break down, with effects of soil organic matter and season seen (Wardle and Parkinson 1991). The fact that some factors do affect ATP levels in relation to biomass, indicates that in some circumstances it can be used as a functional measure (activity measure) of perturbation in combination with other biomass measures. The physiological state of the soil flora can also be assessed by measuring the adenylate energy charge of the system (Ciardi *et al* 1991).

Other biomass measures may indicate a perturbation in the biomass caused by a treatment, but do not give an indication of what aspect of the biomass (the nature of the changes) is affected, unless they are used in combination (as described earlier with the metabolic quotients) with other biomass measurements, or with other soil properties (e.g. soil enzymes).

ii. mRNA studies.

Messenger RNA is the intermediary step between DNA and functional products (e.g. enzymes). Measurement of mRNA has a distinct advantage over DNA measurement as it is only produced in response to ecological conditions resulting in a functional product and is

therefore more of a definitive indicator of functional impact. It thus indicates activity with respect to the gene (i.e. it is a measure of activity not just the presence of the gene) and the physiological condition of the target organism.

Geremia *et al* (1993) used Northern analysis to show that the induction of an antifungal protease from *Trichoderma harianum* is due to an increase in the corresponding mRNA level. Honelage *et al* (1995) developed methods to detect the mRNA encoding an extracellular protease of *Bacillus megaterium* in soil. They demonstrated the applicability of mRNA probing techniques to localise microbial processes in soil. Lambais and Mehdy (1993) using mRNA techniques found suppressed plant defence response (β glucosidase, endochitinase and chalcone isomerase production) during VA mycorrhiza development. They also found evidence that these responses were linked to phosphate deficiency. The work of Lambais and Mehdy was vindicated by Franklin and Gnadinger (1994) who also found changes in mRNA synthesis in arbuscular endomycorrhiza infected parsley roots. This work was followed by Mcgarl *et al* (1995) who demonstrated increased mRNA levels encoding protease inhibitors in alfalfa in response to wounding and also to micro-organisms present in soil.

Quantification of mRNA by reverse transcription PCR in soil extracts has been used to study Manganese peroxidase gene expression in sterilised, polycyclic aromatic hydrocarbon contaminated soil (Bogan *et al*, 1996). Transcripts of mRNA and extracted enzyme levels were temporarily correlated, but separated by a short (1-2 day) lag period. However 3 different primers were required for 3 different manganese peroxidase gene transcripts. This highlights the specificity of the mRNA methodology and may allow targeting of specific organisms or groups of organisms, which may be important in the study of GMMs. The specificity of this method may raise problems in the study of ecosystem functional processes

as a whole as a number of mRNA primers may be needed for a group of enzymes serving the same function. This is especially important with genetic variation in the same enzyme between different organisms. Quantification of mRNA is often much more difficult to measure than their products, for example, soil enzyme activities especially if a whole suite of enzymes is measured as a large range of expensive primers may be required. Another disadvantage to soil enzyme measurements is that mRNA measurements is separated from the actual activity of the product. As with enzyme activities mRNA is only a measure of the potential functional activity in soil and is detached further step from the actual activity.

iii. Soil nutrient cycles.

Nutrient cycles are the result of the functional activities of the microbiota and plant roots and thus studies of such cycles are ultimately indicators of cumulative function. Nutrient cycles are of great importance to agriculture as perturbations in nutrient cycling could influence crop production. The advantage of nutrient cycle measurements is that they can be conducted *in situ* as opposed to the potential activities of the enzyme measurements. Nutrient cycle measurements have the disadvantage that they are much more difficult to measure than enzyme activities.

One method to study a key nutrient cycle is to model the flow of an element through its respective cycles and look for differences between treatments. The identification of relative bottle necks or relative flow rates in such systems would be of prime importance as these would be the keystone features of the cycle. Janzen *et al* (1995) looked at the effect of compost extract on the communities involved in element cycling (including sulphate reduction, P mobilisation and denitrification) found a number of perturbations to

these processes. The best method to study nutrient cycling systems in this context is by the addition of labelled substrates by spiking, dilution or enrichment techniques, for instance Barraclough and Puri (1995) used ^{15}N dilution and enrichment techniques to separate heterotrophic and autotrophic pathways of nitrification. Similar techniques could be applicable to the study of nutrient assimilation by different components of the biomass or a crop plant. Tate and Mills (1983) used $^{14}CO_2$ evolution from glucose and succinate in the presence of protein synthesis inhibitors to evaluate the diversity and function of bacteria in a pahokee muck soil.

Another method of utilising nutrient cycle models is to incorporate variations into the models. For instance Goncalves and Caryle (1994) modelled the influence of moisture and temperature on net nitrogen mineralisation in a forested sandy soil, other variations caused by soil treatments could easily be incorporated into such models as an indicator of the functional influence of perturbing treatments. Schipper *et al* (1994) studied the relative competition between different pathways of degradation or assimilation of a single substrate, in this case nitrate and its dissimilatory reduction to ammonium or the second, the denitrification pathway. Differences in the relative prevalence of the two pathways with a soil treatment could indicate a shift in a key functional microbial community, which in this case may influence nitrogen sources available to the plant.

A key issue in nutrient cycling is its interaction with the soil microbial biomass. Nutrients pass through the microbial biomass, for instance the soil internal nitrogen cycle is largely comprised of two independent processes, mineralisation and immobilisation which are both under microbial control, but are temporally and physically separate. For example Drury (1991) looked at the availability of ammonium to the microbial biomass and at the internal nitrogen cycle, showing the separation of the two processes. The two

processes are separated by the inorganic nitrogen pool and thus availability of different nitrogen sources depends upon the rate of the two processes mediated by the microbial biomass, shifts in the nitrogen cycling microbial communities may show as differences in the abundance of nitrogen in different forms. The availability of the nitrogen depends upon the needs of the microbial community, if sufficient nitrogen is available to the biomass then more nitrogen may become available in the inorganic pool primarily as nitrate (Jansson 1958).

Carbon and nitrogen ratios in the biomass are extremely important in the cycling of nutrients. Models of carbon and nitrogen mineralisation in relation to the biomass of the respective element can be a useful tool in assessing ecosystem function. For example Hassink (1994a) used a food web model to assess the effects of soil texture on the size of the microbial biomass and on the amount of carbon and nitrogen mineralised per unit microbial biomass, finding that coarse textured soil had a smaller biomass but a higher mineralisation rate per unit biomass. Hassink (1994b) also looked at the effect of grassland management on soil organic C and N and rates of C and N mineralisation. The rates were much higher in old grassland (10 years) than young grassland (1-3 years). Shifts in components of such well modelled systems would be a good indicator of the functional impact of treatment.

A perturbation to a nutrient cycle may manifest itself as an effect on the crop plant which will be extremely important in the human perspective but this is a cumulative effect and is not always evident. Tied in with this are the indigenous soil enzymes, which have the added advantage as a perturbation measure of each enzyme being a small part of the whole picture which may not be seen at the end of a whole nutrient cycle.

iv. Soil enzyme measurements.

These are extremely important in a functional sense in the soil environment. Soil enzymes are key to the cycling of nutrients in soil and are thus critical to the availability of nutrients to both microbiota and plants. Enzymes are secreted as a response to exogenous conditions, such as phosphatase secretion in response to phosphate deficiency or plant chitinase in response to fungal/insect attack, and are thus a good indicator of change or perturbation. They are thus an indicator of the functional status of the biota not just the chemical environment as measured by nutrient cycle measurements. They are especially effective as enzymes are adsorbed to or entrapped in the soil and thus can provide an indication to the history of the sample and not just a snap shot from the time of sampling. Many soil enzymes are secreted into the soil environment by soil microbes and by plant roots to make available the nutrients others are released into the soil with the lysis of microbial and plant cells. These extracellular enzymes are often immobilised in the soil environment and thus protected from degradation. There are several mechanisms by which soil enzymes can become immobilised in the soil, these include adsorption to soil particles (especially clay particles) and organic matter, immobilisation into the organic matter or microbial polymer (entrapment) or between soil particles (Weetall 1975). Once immobilised the enzymes may still be active in the soil, but at variably reduced rates caused by factors such as steric hindrance, diffusional resistance, blocking of active sites, electrostatic effects or reversible denaturation (Haska, 1981).

If soil enzymes can be assessed and shown to vary with different soil treatments, then such measurements could be a useful indicator of perturbation and of changes functional

diversity. Land management practices have been shown to have a significant influence on soil enzyme parameters, for example Farrel *et al* (1994) showed that cultivation can have a large effect on the soil aryl sulphatase activity. Long term grassland soil cultivation (69 years) caused a 66% reduction in aryl sulphatase activity, whilst cultivation of a forest soil for 40 years resulted in a 88% decrease in activity. Large differences in sulphatase activity were found between a cultivated forest soil (63% decrease in activity) and a similar forest soil that had been left fallow for five years (30% decrease in activity).

In the same study Farell *et al* (1994) looked at changes in the kinetic properties of the soil sulphatases. After cultivation they found a reduction in the V_{max} (74% in the grassland soil and 90% in the forest soil) and the Michaelis constant (K_m), which decreased with the duration (years) of cultivation. Variations in K_m values obtained from the forest and grassland soils indicate that the origin of the enzymes were different in the two soils. Such kinetic studies can therefore be a good indicator of differences in soil enzymes with soil type and of soil management methods, and may be sensitive enough to be applied to assessing the perturbations caused by less drastic soil treatments. The measurement of the soil sulphatase was extremely sensitive to the soil treatment (soil management system) showing extremely large differences, this makes it a prime candidate as an indicator of small scale perturbation than the kinetic parameters, with simpler methodology required.

A second example of a study on the effect of land management practices on soil enzyme activities is the work of Jordan *et al* (1995). They evaluated microbial methods (including soil acid and alkaline phosphatase activities) as indicators of soil quality in long term cropping practices in historical fields. Differences in phosphatase activities

among soils could be related to land management practices and soil properties (especially organic matter), for example acid phosphatase activity was 150% higher in soil under continuous corn with no till receiving full fertility treatment than under conventional tillage with no fertility treatment. Alkaline phosphatase activity was 50% higher in soil under continuous corn with full fertility than without the fertility treatment. This study again shows that soil enzyme activities can be extremely sensitive to the large perturbations caused by soil management practices and thus have great potential as a general indicator of smaller scale perturbation.

In another study involving the study of crop and land management systems on soil enzyme activities, Bopaiah and Shetty (1991) found decreases in urease and an unspecified phosphatase (but not dehydrogenase) with increasing depth in rhizosphere soil from a coconut based multistoried cropping system. Differences were also found in urease and phosphatase with the different crops grown, cocoa and pineapple producing contrasting results to the coconut rhizosphere (higher phosphatase activity in the coconut rhizosphere and higher urease activity with multistoried systems over a coconut monocrop). Depth and crop system had a large influence on the soil enzyme activities.

The effect of pesticides on the activity of various soil enzymes has been investigated by a number of authors (Endo *et al* 1982, Mishra *et al* 1987). A prime example of this is the work of Satpathy and Behera (1993) in an examination of the effect of malathon on cellulase, protease, urease and phosphatase activities in a tropical grassland soil. They found that cellulase and protease levels of activity recovered to almost the same level as the control 21 days after malathon application, whereas urease and, to a larger extent, phosphatase activities did not show such a recovery in the same time period. The work of

Satpathy and Behera can be regarded as a successful use of soil enzymes as an indicator of perturbation, which in this case was caused by the addition of a pesticide.

A similar situation to the addition of pesticides to soil is the input of pollutants, which can also have major effects on the whole soil ecosystem. Ohtonen *et al* (1994) found that cellulase activity decreased with increasing pollution along an industrial pollution gradient of sulphur, nitrogen and heavy metals. They also found that they could correlate the trend in cellulase activity with a decrease in respiration along the same pollution gradient. On comparison they found that cellulase activity was in fact a better indicator of pollution than the respiration measurements, correlating well with the gradient of pollution.

A proposed target for genetic manipulation is the insertion or enhancement of genes encoding specific enzymes, prime candidates for this are the chitinase enzyme production because of its speculated role in the biocontrol of fungal crop pathogens. Ridout *et al* (1986) looked at the protein production induced in a *Trichoderma* species when cell wall fragments of the crop plant pathogen *Rhizoctonia solani* (to which the *Trichoderma* species was an antagonist) was used as the sole carbon source. Both β-glucanase and chitinase, from the chitin degradation chain, were found to be important components of the inducible extracellular proteins analysed by electrophoretic profiles. The system of degradation was extremely complex with several other inducible proteins found that were probably important components in the system and thus in the biocontrol of the pathogen.

The situation where genes encoding enzymes (especially extracellular) are the target of manipulation give an added emphasis to the use of soil enzyme activities as a measure of

perturbations caused by the introduction of such organisms into the soil and rhizosphere. In an attempt to understand the effect of manipulated enzyme production on a soil micro-organism, Williamson and Hartel (1991) studied the rhizosphere growth of *Pseudomonas solanacearum* which was genetically altered in extracellular enzyme production (endopolygalactouronase A and endoglucanase). They found that the strains that had been enhanced in terms of enzyme production had a greatly reduced fitness in the rhizosphere. The authors did not use the opportunity to study the effect on soil extracellular enzyme activity with the inoculation of strains with such functional modifications.

There is little data regarding the use of soil enzymes as a measure of perturbations caused by the introduction of extraneous micro-organisms into the soil or rhizosphere ecosystems, and the information that is available is contradictory. Doyle and Stotzky (1993) looked at methods, including arylsulphatase, dehydrogenase, acid and alkaline phosphatase, for the detection of changes in the microbial ecology of the soil caused by the introduction of micro-organisms. They found no consistent significant differences in any of the enzyme activities caused by the inoculation of *E. coli* strains. It must be noted however that this experiment did not reflect ecologically relevant conditions, as the strains used were not natural soil organisms and the experiment was conducted in bulk soil rather than in crop rhizospheres where commercial innoculants are often targeted and where nutrient sources allowing enhanced activity and proliferation are available this is perhaps not surprising as the work was not conducted under relevant conditions for the release of GMMs into soil. The metabolic activity of the *E coli* strain was likely to be low since no substrate, be it rhizodeposition or soil amendments, were present that could be used as a substrate source. Furthermore *E. coli* is not a soil organism, and is unlikely to establish a viable population after introduction into soil. In contrast to the work of Doyle and

Stotzky, Mawdsley and Burns (1994) introduced a *Flavobacterium* species into the rhizosphere of wheat. They found that the microbial inoculant caused increased α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase activities in the more ecologically and agriculturally relevant conditions of the wheat rhizosphere.

Naseby and Lynch (1996) used a number of simple enzyme assays for the detection of perturbations resulting from different soil treatments, including the introduction of a modified *Pseudomonas fluorescens* SBW25 strain and substrate amendments. The aim of the experiment was to deduce whether these assays were sensitive enough to measure perturbations caused by microbial inoculation, and to uncover the extent of any perturbation. Specific attention was paid to the validation of soil biochemical techniques as a method of monitoring the effects of inoculation. Differences in rhizosphere soil biomass (measured by ATP content) and several key soil enzyme activities with microbial inoculation and/or in exaggerated conditions (addition of the enzyme substrates, chitin, urea and glycerophosphate, to soil) were measured.

Substrate addition significantly decreased biomass as measured by ATP (Fig.2) content of the soil. It was expected that an input of nutrients would increase the size of the rhizosphere community. A possible reason for this anomaly is that substrate additions caused a change in the physiological state or the constituents of the microbial community, with a higher proportion of microbes with a smaller ATP content in the substrate treatments than in the treatments without substrate addition. This explanation is made plausible by the observations of Karl (1980) who compiled data of the ATP content of several micro-organisms and found a range of 0.5 to 18 ηmol ATP/mg dry weight organism.

Substrate addition caused significant increases in soil urease (Fig.3), chitobiosidase (Fig.5). It is possible that the addition of substrates for urease and chitobiosidase would have a direct effect on the activities of these enzymes which are induced in the presence of their respective substrates The substrate / depth interaction in chitobiosidase activity can also be attributed to the presence of chitin predominantly in the upper soil regions. Inoculation with bacteria (without substrate addition) had a significant effect on chitobiosidase, urease, and alkaline phosphatase (Fig.4) at depth interval 1 only (where activity is at its highest). There was an apparent reduction in alkaline phosphatase activity, which may be attributed to an direct or indirect effect of the inoculum, resulting in a displacement of the rhizosphere communities that produce larger amounts of this enzyme. At this stage however, there is nothing to indicate that the GMM and wild-type bacteria would cause different effects.

Microbial inoculation in the presence of the substrate additions, showed significant differences in chitobiosidase, alkaline phosphatase and urease activities, as well as in the biomass. All of the effects on the enzymes are the opposite to those seen with microbial inoculation without the substrate addition, i.e.: chitobiosidase and urease activities are significantly lower with the inoculation in the presence of substrate additions than without inoculation; alkaline phosphatase activity was significantly higher. These results suggest that the microbial inoculation had a ‘buffering effect’ on the rhizosphere ecosystem, i.e. there was a reduction in the response of enzyme activities to the change in conditions presented by the substrate additions.

There are several possible ways in which the microbial inoculation could orchestrate changes in the microbial community. The first possibility is, that the inoculant is competitively excluding certain microbial populations. This would be a direct effect of the large inoculum size, giving the introduced microbe a ‘head start’ over the soils resident microflora. Another direct effect of the inoculant could be in the strain’s metabolic activity, i.e. in its metabolic by-products or other products (antibiotics or siderophores) that might have directly affected the indigenous microbial community. The second set of possibilities are the indirect effects of the inoculant, where the introduced organism affects either root secretions or some rhizosphere organisms which in turn affects other rhizosphere organisms and thus affect the soil enzymes.

4. Conclusions.

Throughout this review the significance of the measurement of the functional perturbations caused by GMMs has been highlighted. It is important to realise the potential applications and limitations of different methods of measuring the impact of GMMs, and that no single method will prove to be the best. Methodology must be assessed for each particular case, and the mostly likely out-come will be the selection of a number of inter-related methods (e.g. gene probing and soil enzyme measurements). Figure 6 illustrates the line of succession that the impact of introducing a genetically modified micro-organism may take, with the first functional product of the DNA that can be monitored being messenger RNA, which then proves to produce the gene products (enzymes and metabolites). The ultimate assay of the effect of the gene products is the plant bioassay, especially in the human perspective, which includes productivity, health and ion uptake (nutrient concentrations or ratios).

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METHOD	REFERENCES
Fatty Acid Methyl Ester (FAME) analysis	Gumstead *et al* 1988, O'Donnell *et al* 1994 and Rainey *et al* 1994
Organic pyrophosphate content (ms pyrolysis)	Rainey *et al* 1994
Immunofluorescence	van Elsas and Waalwijk 1991, Morgan 1991
Other immunological techniques	Carter and Lynch 1993
Total cellular protein profiles	Rainey *et al* 1994

Table 1: non-nucleic acid techniques for the measurement of the diversity of microbial populations and perturbations.

METHOD	REFERENCES
Restriction Fragment Length Polymorphism (RFLP)	Mills 1994
DNA finger printing	Mills 1994
Polymerase Chain Reaction (PCR)	Mills 1994, van Elsas and Waalwijk 1991, Morgan 1991, Seal 1994
DNA/RNA sequence analysis	Mills 1994, Morgan 1991
DNA/RNA probes (hybridisation)	Christienson and Poulsen 1994, van Elsas and Waalwijk 1991, Morgan 1991, Seal 1994
Community hybridisation techniques using the reanealation time of DNA samples	Ritz and Griffiths 1994 using the methods of De Ley 1970

Table 2:Nucleic acid based methods of identifying micro-organisms and/or measuring diversity.

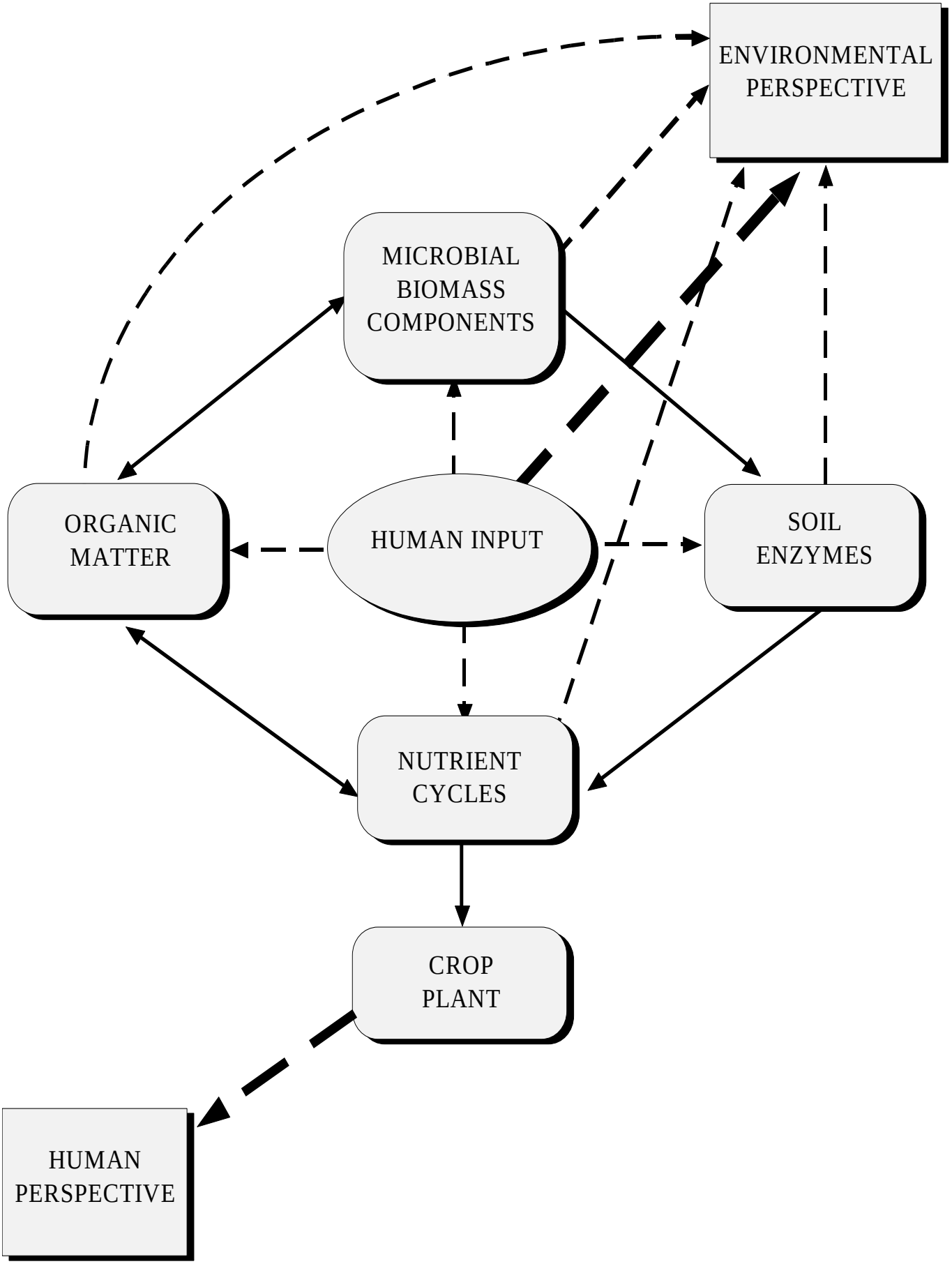

Figure 1: The relationship between key areas of ecosystem function that should be considered in perturbation studies.

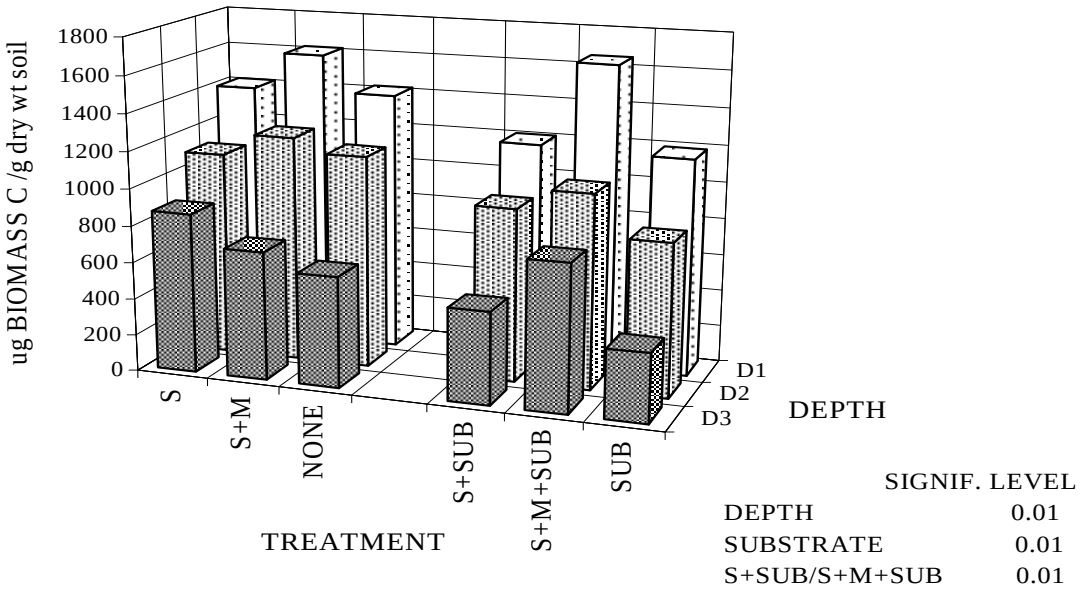

Figure 2: Total biomass carbon of a silty loam soil as influenced by depth (D1, 5-20cm; D2, 20-35cm; D3, 25-50cm) and six treatments: S, presence of seedling; M, microbial inoculation; SUB, substrate addition; NONE, none of the former added. Statistical significant results shown bottom right (after Naseby and Lynch, 1996).

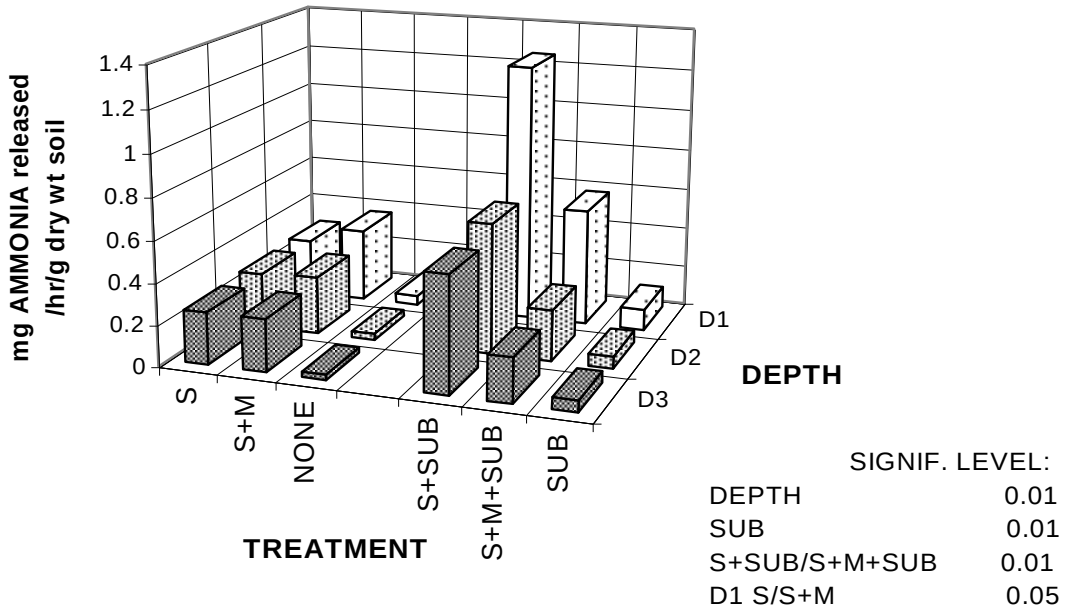

Figure 3: Urease activity in a silty loam soil as influenced by depth (D1, 5-20cm; D2, 20-35cm; D3, 25-50cm) and six treatments: S, presence of seedling; M, microbial inoculation; SUB, substrate addition; NONE, none of the former added. Statistical significant results shown bottom right (after Naseby and Lynch, 1996).

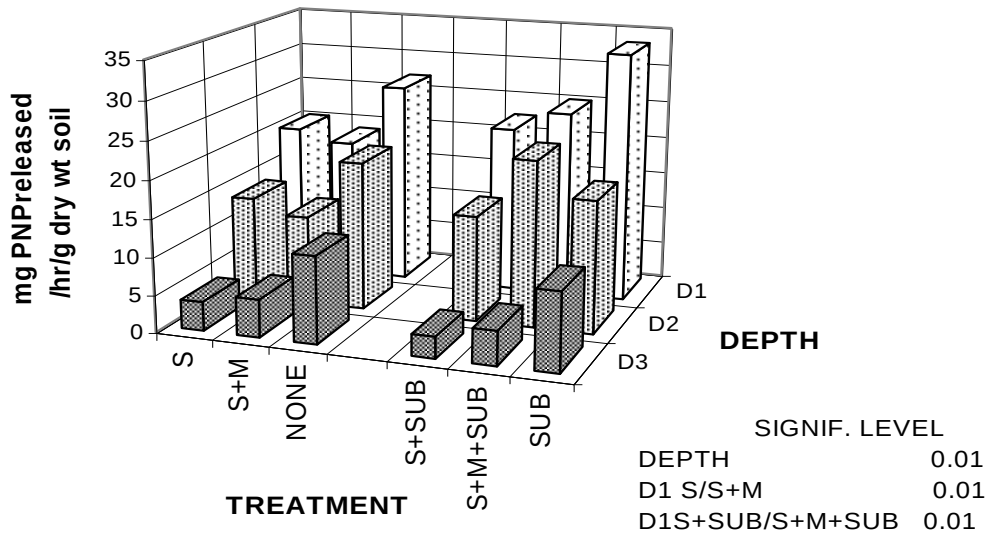

Figure 4: Alkaline phosphatase activity in a silty loam soil as influenced by depth (D1, 5-20cm; D2, 20-35cm; D3, 25-50cm) and six treatments: S, presence of seedling; M, microbial inoculation; SUB, substrate addition; NONE, none of the former added. Statistical significant results shown in bottom right (after Naseby and Lynch, 1996).

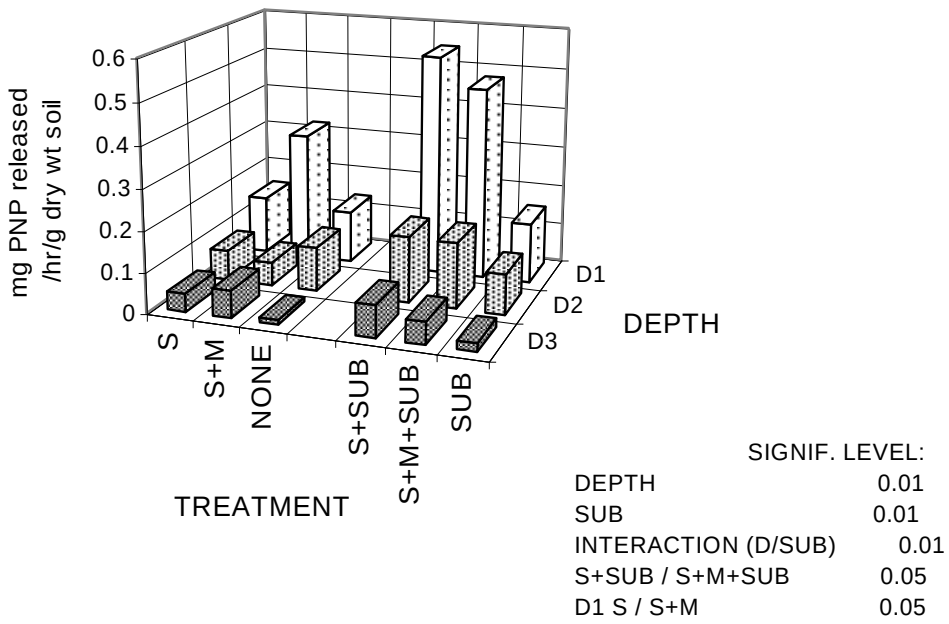

Figure 5: Chitobiosidase activity in a silty loam soil as influenced by depth (D1, 5-20cm; D2, 20-35cm; D3, 25-50cm) and six treatments: S, presence of seedling; M, microbial inoculation; SUB, substrate addition; NONE, none of the former added. Statistical significant results shown bottom right. (after Naseby and Lynch, 1996).

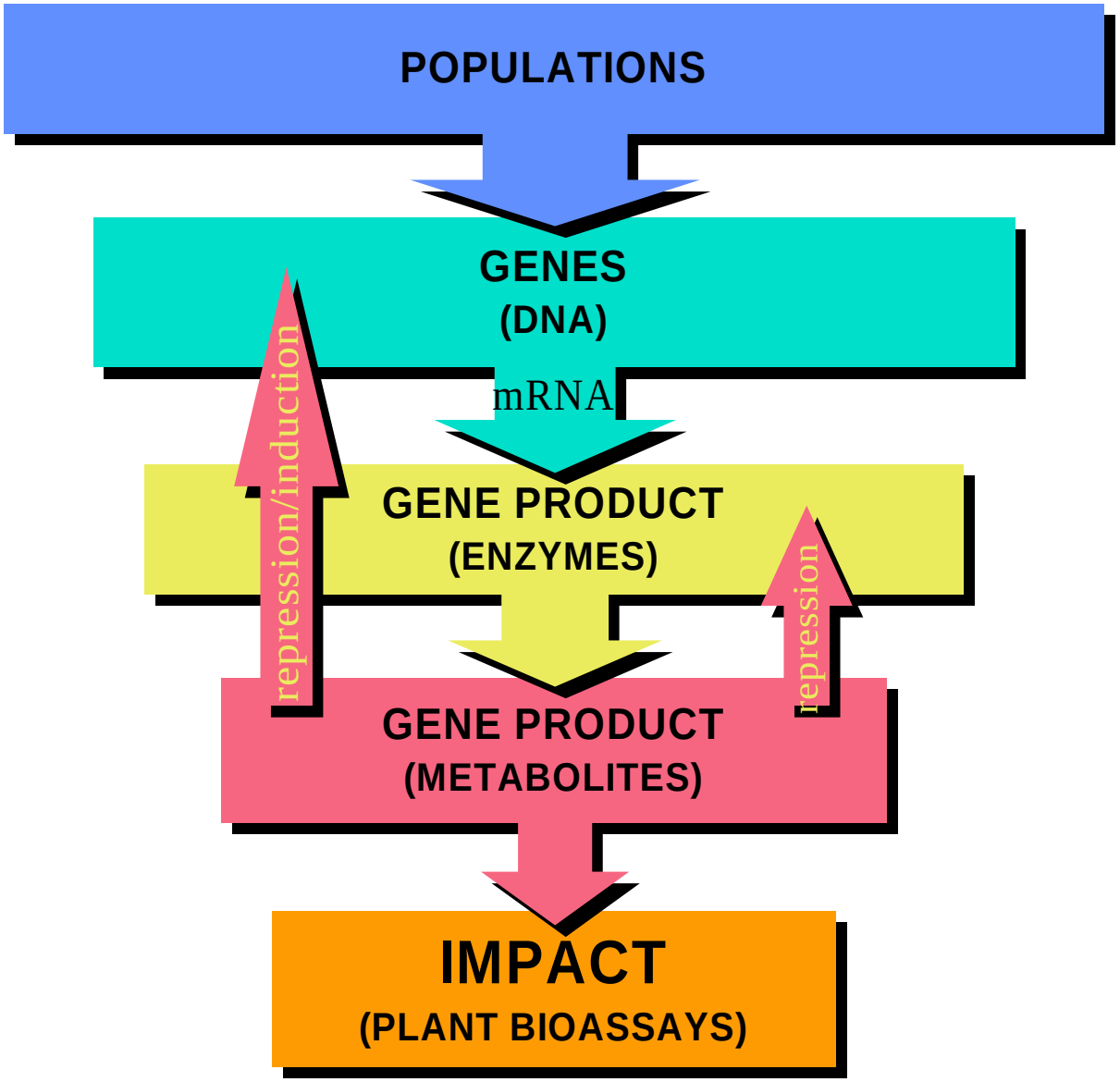

Figure 6: levels of perturbation measurement