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Changes in soil microbial properties as indicators of adverse effects of heavy metals **

INTRODUCTION

In high-productivity agricultural ecosystems, natural soil fertility is commonly supplemented by applications of nutrients, either as inorganic fertilizer or organic manures, and occasionally both. However, the activities of the soil micro-organisms (collectively the soil microbial biomass) in decomposing plant and animal residues and in the formation and mineralization of soil organic matter still underpins the fertility of these managed systems. In natural ecosystems these natural processes determine, almost entirely, the fertility of their soils. Any decline in natural soil fertility will therefore have disproportionately large effects in natural systems but still cannot be ignored in managed ones. The soil-plant ecosystem may be damaged, either in the long- or short-term, by agents that inhibit or stop the natural functioning of the soil micro-organisms.

The heavy metals, e.g. Cu, Ni, Cd, Cr, Zn, Pb, are by far the most important inorganic pollutants of soil. They differ from organic pollutants in that, once they have entered soil they persist, for all practical purposes, indefinitely. Currently, the only practical means of their removal is to remove the soil itself, hardly a practical proposition in most cases.

Mandatory European Union (EU) limits are designed to stop the accumulation of heavy metals above 'safe' soil metal concentrations. The limits are based upon known effects of heavy metals on plant and animal health. Until recently, they took no account of possible effects on soil micro-organisms or microbial actvities despite their essential role in maintaining soil fertility.

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Brookes and McGrath (1984) reported decreased total amounts of soil microbial biomasss in soils contaminated with heavy metals more than 20 years previously from past sewage-sludge applications (reviewed elsewhere in this paper). Since then a great deal of further research in this area has been carried out and new UK legislation is being drafted to decrease the current limit for Zn from 300 to 200 µg Zn g^{-1} soil, based on observed adverse effects of Zn on Rhizobium in agricultural soils (MAFF/DOE, 1993a and b). Even so, amounts of metals permitted in agricultural soils in Europe are still set at concentrations at, or only just below, those at which effects on the soil microbial ecosystem can be detected. This is in marked contrast to limits for some other pollutants (pesticides in drinking water, for example) which are many times lower than concentrations at which adverse biological effects have been demonstrated.

In the USA, maximum permitted metal concentrations in agricultural soils are, depending upon the metal, 3 to 10 times larger than in the EU, which involves markedly different philosophies (Table 1). Irreversible effects upon the soil microbial ecosystem have been consistently demonstrated at soil metal concentrations well below the USA limits (Brookes 1994). In Table 1 are given heavy metal concentrations at which significant (commonly 50% decreases) changes in soil ecosystem functioning can be demonstrated.

The European approach is based upon the view that, while there is inevitably some escape of metals into the environment in industrial societies, it is best to operate in ways which cause the minimal contamination that is compatible with modern life.

Here I review recent findings of the effects of heavy metals on the functioning of the soil microbial ecosystem. Several 'levels' of microbial ecosystem system struc-

Table 1. *Maximum concentrations of metals allowed in agricultural soils treated with sewage sludge.*

Country	Year	Total soil metal concentration, mg kg^{-1} soil						
		Cd	Cu	Cr	Ni	Pb	Zn	Hg
European Community	1986	1-3	50-140	100-150[a]	30-75	50-300	150-300	1-1.5
US[b]	1993	20	750	1500	210	150	1400	8

[a] Now withdrawn.
[b] Calculated from maximum cumulative pollutant loading limits, assuming incorporation to 15 cm depth and average soil bulk density of 1.33 g cm^{-3}, but not including the background concentration of these elements in soils.
(From McGrath *et al.*, 1995).

Table 2. *Soil heavy metal concentrations at which significant effects (1-5) on the microbial ecosystem were detected.*

Microbial indicator	Soil total metal concentration, mg kg^{-1} soil			
	Cu	Ni	Cd	Zn
Total microbial biomass[1]	45	nd	3-5	160
Heterotrophic N_2-fixation[2]	37	21	3.4	127
Autotrophic n_2-fixation[3]	20	2.5	3	50
Symbiotic *Rhizobium*-legume N_2-fixation[4]	99	27	10	334
Rhizobium leguminosarum bv. *trifolium*	27-48	11-15	0.8-1	130-200

nd - not determined.

Effect:
[1] 50% decrease (Tiwari *et al.*, 1997).
[2] Significant decrease (McGrath *et al.*, 1995).
[3] 50% decrease (Brookes *et al.*, 1993).
[4] 50% decrease (McGrath *et al.*, 1988).
[5] Several orders of magnitude (McGrath *et al.*, 1995).

ture are considered. These are (1) Microbial communities and activities, (2) Specific micro-organisms or functional groups, (3) Specific biochemical markers, and (4) Novel approaches. These include DNA technology and the use of *lux* genes. Where appropriate, the different methodologies are evaluated for their suitability as diagnostic tests for evaluating heavy metal effects on the soil microbial ecosystem

1. EFFECTS OF HEAVY METALS ON MICROBIAL COMMUNITIES AND THEIR ACTIVITIES

Soil microbial biomass

A full description of the soil microbial biomass concept, the method of measurement and its limitations, were first presented by Jenkinson and Powlson (1976). Instead of considering soil micro-organisms as seperate species or even classes (e.g. fungi and bacteria) the biomass was measured as a single unit, or pool, of the total mass of micro-organisms or the nutrients, initially C or N, immobilized within the microbial cells. This permitted, for the first time, the measurement of a single discreet pool of soil organic matter, the micro-organisms themselves. It is this pool that is responsible for the decomposition of plant and animal residues, the immobiliza-

tion and mineralization of the major plant nutrients (C, N, P, and S) and for the formation and degradation of soil organic matter. It is thus ultimately responsible for the maintenance of soil fertility and is indeed, as eloquently described by Jenkinson (1977), "the eye of the needle through which all organic matter must pass" as it is broken down into simple inorganic components, including water, carbon dioxide, nitrate, phosphate and sulphate, that plants can use again. By treating the micro-organisms as a defined and measureable pool, and by appropriate use of isotopically laballed substrates, fluxes of C and N (and later P) through the microbial population could be measured, leading to a new understanding of the importance and role of the biomass in the maintenance and regulation of soil fertility.

From this pioneering work was also developed the concept of the biomass as an 'early warning' of changing soil conditions and as an indicator of the direction of change. For example, on changing from forest or grassland to arable, microbial biomass decreased much more rapidly than total soil organic matter (Ayanaba *et al.*, 1976). Similarly, Powlson *et al.* (1987) found no significant increase in total soil organic matter following 18 years of straw incorporation in two Danish soils. In contrast, the total amount of biomass had increased by nearly 50% over the same period, compared to soils where the straw had been burnt.

Brookes and McGrath (1984) used the biomass concept to investigate the residual effects of heavy metals from past applications of sewage-sludge on microbial and soil organic matter dynamics. The experiment they studied was the Market Garden Experiment at Woburn, a sandy loam of about 10% clay and a pH of 6.5 which had received annual applications of sewage sludge or sludge compost (high-metal soils) from 1942 to 1961 or farmyard manure or inorganic fertilizer (low-metal soils) from 1942 to 1967. All plots received inorganic fertilizer annually since the applications of organic manures ceased. In 1984, the high-metal soils contained Cu, Ni and Zn at up to about current European Union (EU) permitted limits and Cd at up to three times the current permitted limit.

In the low-metal soils there was a reasonably close linear relationship between soil biomass content and total soil organic matter, with the biomass C comprising about 1 to 2% of total soil organic C (Brookes and McGrath, 1984). This was within the range typically reported for sandy soils in temperate regions (Jenkinson and Ladd, 1981). In contrast, in the sludge-treated high-metal soils the amounts of biomass were only about half those in the low-metal manured soils and some were lower than in the soils given inorganic fertilizer. Equally surprisingly, there was no relationship between amounts of biomass and amounts of organic matter in the high-metal soils, unlike the low-metal soils. This was despite (1) the comparatively small amounts of heavy metals in the sludge-treated soils and (2) at that time, the last sludge applications were more than 20 years ago. Because total concentration of Cu, Ni, Cd and Zn were very closely correlated it was not possible to determine which metals or combinations of metals were producing these effects.

Brookes *et al.* (1997) measured heavy metals, biomass C and biomass specific

respiration (see later) along a gradient obtained by sampling along the middle of adjacent plots of the Market Garden Experiment which had previously received continuous inorganic fertilizer (NPK), farmyard manure (FYM) or sewage-sludge, as described above (Figs. 1-3). A very smooth gradient of heavy metals was measured (Fig 1) and microbial biomass C (Fig 2) increased linearly between the NPK and FYM plots, in line with increasing soil organic matter concentrations. However, after 120 μg Zn g^{-1} soil, obtained from the second sludge soil sample, there was a smooth decline in biomass nearly to the level in the soil given inorganic fertilizer. I attribute this to heavy metals. It is exceedingly unlikely that organic pollutants, if present in the sludges initially, would have persisted for so long. Similarly, biomass C as a percentage of soil organic C declined sharply at about the same soil Zn concentration (Fig. 3).

Chander and Brookes (1991a) reported biomass and organic C measurements in field experiments at Luddington (sandy loam, 15% clay) and Lee Valley (silt loam, 21% clay) experimental farms. Both soils received half- and full-rate dressings of metal- contaminated sludge and full rates of uncontaminated sludges. Both soils had similar pH's (5.6 to 5.9) and were under grass. The major findings, summarised, were:
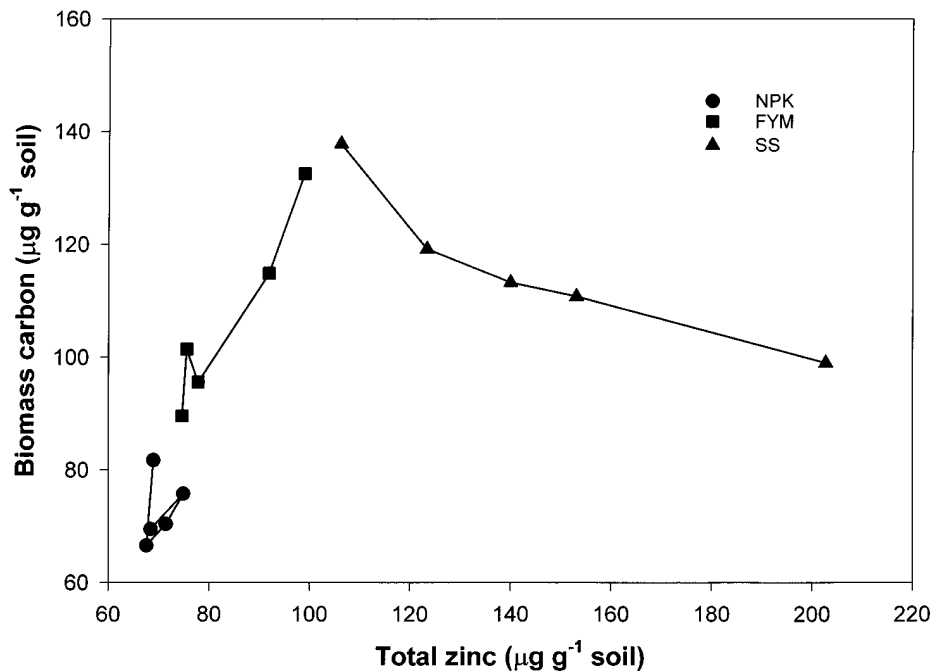

Fig. 1. Heavy metals along a soil transect of Woburn Market Garden Experiment.
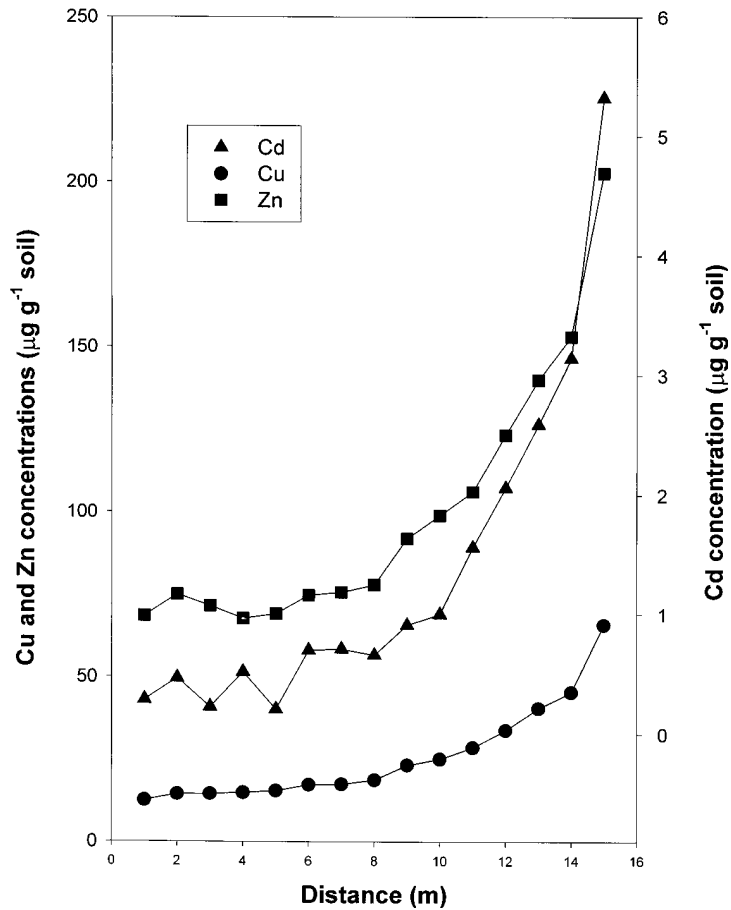
Fig. 2. Microbial biomass and biomass specific respiration (●) along a soil transect of Woburn Market Garden Experiment.

1. Both Cu and Zn at about 2-3 times current EU permitted limits decreased soil biomass in both the sandy loam (15%) clay and silt loam (21% clay) soil.

2. At these soil concentrations, Cu at both sites decreased the biomass by about 40% compared to soils given uncontaminated sludge. Zinc decreased the biomass by about 40% in the sandy loam and 30% in the silty loam soils.

3. Nickel at about 2 to 3 times current EU limits did not affect microbial biomass in either soil. Similarly, Cd at twice current limits had no effect in the silt loam soil.

In a further field experiment, sewage-sludges, enriched with different rates of single metals (Zn, Cu and Ni) and metal combinations, were applied to a sandy

loam soil (9% clay, pH around 6.5) in 1982 at Gleadthorpe Experimental Husbandry Farm. This experiment was used to investigate effects of single metals and combinations of metals on the biomass and on relationships between biomass and soil organic matter (Chander and Brookes, 1993). Main findings included:

1. Zinc at about 2.3 and Cu at about 4.9 times current permitted EU limits decreased soil biomass by about 40 and 50% respectively compared to soils receiving uncontaminated sludges.

2. A combination of Cu and Zn, each at about 1.5 times current EU limits decreased biomass by about 60% compared to soils given sludge. This suggests synergistic interactions between metals and biomass.

Other work has shown decreased microbial biomass formed during sludge decomposition after incorporation to soil. Two field experiments were established at Braunschweig, Germany with (in brief) the following treatments: inorganic fertilizer; uncontaminated sludge; metal-contaminated sludge – both at two rates (5 or 16 t ha^{-1} dry weight). Two sites were used, an old arable (pH 6.1 to 6.8) or ex-woodland (pH 5.3 to 5.7) of the same soil type. After 9 years, total metal concentrations in some of the sludge-amended soils exceeded the German limits (mg kg^{-1}) of 300 Zn, 100 Cu, and 3 Cd, but not for Ni. On both soils, the biomass was increased each year with either rate of uncontaminated sludge. However there was less, or even no, increase in biomass following addition of metal-contaminated sludges, even at the higher rate (Fliessbach *et al.*, 1994).

In a laboratory experiment, Chander *et al.* (1993) added sewage sludges enriched, or not, with the single metals Cu, Ni, Cd or Zn, to a sandy loam soil of the Woburn Market Garden Experiment. The unenriched (low metal) and metal-enriched (high metal) soils were added separately at four rates so that total soil metal concentrations were between 1-4 times the European Union (EU) current permitted limits. The main aim was to determine the maximum individual soil metal concentrations which decreased either the amount or activity of the biomass.

Sludge addition increased biomass C by about 30% at the lowest rate of application (40 t ha^{-1} dry weight) and by about 4.5 fold at the highest (160 t ha^{-1} dry weight) rate after four weeks, with no effects attributable to the metals. (It should be noted that these are much higher sludge rates than in the previous experiment). However, during the longer 64 week incubation, the biomass declined exponentially in all treatments. Larger applications of high metal sludges caused final biomasses which were smaller than those given low metal sludge and no sludge. None of the single metals at the current EU permitted limits showed any adverse effects on biomass. However, Zn, Cu, or Cd, individually at about twice the EU limit, decreased biomass C by about 20% whereas Ni at four times the limit decreased the biomass by about 15%. Thus this suggests that although toxic effects of heavy metals may be delayed by the antagonistic effect of enhanced substrate availability via the sludge in the short-term, once the sludge has been decomposed the metals exert their negative effects upon the soil microbial biomass.

MICROBIAL ACTIVITY MEASUREMENTS

The fertility of all natural ecosystems depends upon the mineralization and immobilization of soil organic C, N, P and S and on the decomposition of plant and animal residues that enter soil. These processes are all mediated by a suite of complex metabolic process provided by the soil microbial biomass and higher soil organisms. Since heavy metals are proven inhibitors of most enzymic reaction in soil (e.g. Tyler, 1981) it is essential that heavy metals are not permitted to accumulate in soils to concentrations at which these processes are inhibited or suppressed.

Microbial activities (e.g. respiration and N mineralization) can fluctuate enormously, even over a few days, under field conditions, even in pristine soils – see Brookes (1994) for a discussion of this. However, under controlled laboratory conditions of suitable moisture (usually between 40 to 50% WHC) and temperature (usually between 15 to 25°C) the microbial mineralization of both C and N in sieved (2 to 6.25 mm) proceed practically linearly for long periods and can be determined accurately and precisely (Jenkinson and Powlson, 1976).

Most work indicates that mineralisation of both C and N are little, if at all, affected at soil metal concentrations at around maximum current EU limits. Thus, Tyler (1981) reported that microbial respiration was not depressed below about 1000 μg Cu or Zn g^{-1} soil in forest soils. Similarly, there was no apparent change in soil C or N mineralisation in arable soils containing heavy metals from past sewage-sludge applications compared to similar 'low-metal' control soils (Brookes and McGrath, 1984; Brookes et al., 1984). This was despite the fact that the biomasses in the high-metal soils were often only half the size of the biomasses in the low-metal soils. Thus these much smaller microbial populations in the high-metal soils were able to mineralise soil organic matter and decompose crop and animal residues to the same extent and at the same rate as the much larger populations in the low-metal soils. Whether the heavy metals had produced a smaller population with the same, or a different, community structure is currently being determined (D. Ayabe, personal communication).

N$_2$ fixation

Biological N$_2$ fixation requires the presence of the enzyme nitrogenase, which occurs only in micro-organisms. The three main types of N$_2$-fixing micro-organisms, autotrophs, heterotrophs and symbionts, vary in the amounts of N$_2$ they can fix in temperate regions. In order to directly convert the nitrogenase activity to N$_2$-fixed requires ^{15}N analytical techniques which may not always be feasible. However, the conversion of acetylene to ethylene and subsequent chromatographic analysis provides a simple and sensitive test for nitrogenase activity provided the results are not extrapolated too far (Giller and Day, 1985). There is certainly some data to suggest that both heterotropic and non-symbiotic N$_2$-fixation could be suitable tests for soil pollution by heavy metals. This is discussed below.

Heterotrophic N₂ fixation

Free-living heterotrophs have very slow rates of N_2-fixation in most soils which are difficult to measure. Fixation can also be variable, developing measureably in some but not in other apparently similar soils. This lack of reproduciblity convinced Lorenz *et al.* (1992) that difficulties in optimising and standardising incubation conditions presently effectively prevents the use of heterotrophic microbial activity measurements as indicators of effects of heavy metals on soil ecosystems.

However, this was on unamended soils. Following amendment with up to 5000 μg glucose g^{-1} soil, both Brookes *et al.* (1984) and Lorenz *et al.* (1992) found that heavy metals close to, or less than, current EU permitted limits decreased heterotrophic N_2-fixation by up to 90%. More work is certainly required to standardise operating conditions if heterotrophic N_2 fixation is to have any value as a biological indicator in metal-contaminated soils.

Autotrophic N₂-fixation

Field measurements of autotrophic N_2-fixation by Cyanobacteria are extremely variable. For example, Witty *et al.* (1979) estimated that up to 28 kg N ha^{-1} y^{-1} could be fixed on soils of the Broadbalk Continuous Wheat Experiment. However, amounts varied fivefold in their work. In contrast, when I tried to measure nitrogenase activity *in situ* over a year, I found nothing at any time.

However, measurement of nitrogenase activity as an indicator of N_2-fixation by Cyanobacteria under standard laboratory conditions could be a possible indicator of heavy metal effects. However, the measurements would reflect the potential of contaminated and uncontaminated soils for N_2-fixation rather than fixation in the field.

Brookes *et al.* (1986) incubated moist, fresh low- and high-metal soil from the Woburn Market Garden Experiment under laboratory conditions of 20°C day, 16°C night, 16 h day and 50% Water Holding Capacity. In the low-metal soil there was an initial lag-period of about 14 d, then the rate of acetylene reduction increased rapidly, peaking on day 28 then declining slowly until day 118. In contrast, acetylene reduction had barely commenced by day 50 in the high-metal soil. It then increased regularly but much more slowly than in the low-metal soil, and until the experiment ended. There was about three times more acetylene reduction in the low- than high-metal soil by day 118. Similarly, the low-metal soil fixed about ten times more $^{15}N_2$ in 24 h than did the high-metal soil. A thick crust of Cyanobacteria formed on the surface of the low-metal soil by day 25 but hardly any was obvious on the high-metal soil by day 118.

In a further experiment (Brookes *et al.*, 1986), soil was sampled at 40 cm intervals along the middle of a low- and high-metal plot. Concentrations of EDTA-extractable Zn, Cu, Ni and Cd increased in a curvilinear manner between the low- and high-metal plots. In contrast, total acetylene-reduction decreased linearly with

increasing soil metal concentration during the 60 d experimental period. It was halved at about 50 µg total Zn, 20 µg Cu, 2.5 µg Ni and 3 µg Cd. Because the soils contained all these metals, in closely correlated concentrations, it is not known which metal or combination of metals induced these effects. However, apart from Cd, the maximum concentrations of individual metals were well within individual EU maximum permitted limits (about 30% of the soils contained above 3 µg Cd g^{-1} soil).

Lorenz *et al.* (1992) obtained a metal gradient by mixing different proportions of a low- and a high-metal Woburn soil. They also reported inhibition of the growth of Cyanobacteria and decreased acetylene reduction in the high-metal soils but only at the maximum concentrations (a mixture of 83% sludge and 17% FYM soil or 100% sludge soil).

The lag phase of 14 d that Lorenz *et al.* (1992) determined for the high-metal soil was much shorter than the 50 d reported by Brookes *et al.* (1986). It seems likely that this was because, however careful the mixing was done, particles – or islands – of uncontaminated soils would exist side by side with contaminated soils. In contrast the metals would be very much more homogenously distributed when sampled along a natural gradient in the field.

Cyanobacteria also appear very sensitive to incubation conditions in other ways. It is interesting that Lorenz *et al.* (1992) failed to get them to grow even on uncontaminated Luddington (UK) soil (similar to Woburn soil in most respects). They also reported that in other Swedish experiments Cyanobacteria failed to grow even when uncontaminated soils were incubated under apparently ideal conditions. If Cyanobacteria and autotrophic N_2 fixation are to be used as indicators of metal-contamination in soil we need to know more about how to culture them on soils under laboratory conditions.

Symbiotic N_2-fixation

Symbiotic N_2 fixation by *Rhizobium leguminosarum* biovar *trifoli* in symbiotic association with *Trifolium repens* (white clover), along a transect of soils of the Woburn Market Garden Experiment, was decreased by 50% or more in pots of soil containing above 334 µg Zn, 99 µg Cu, 27 µg Ni and 10 µg Cd g^{-1} soil (McGrath *et al.*, 1988). Yields of clover of the high-metal soils were restored to those of the low-metal soils by applying inorganic N, i.e. the effects were not caused by phytotoxicity. Rather, McGrath *et al.* (1998) proved that the decreased clover yields and N_2-fixation were because the clover root nodules were ineffective in fixing N_2, although nodulation did occur in the high-metal soil. Giller *et al.* (1989) showed that the ineffectiveness of the *Rhizobium* sp. in fixing N_2 was not directly due to metal toxicity. Instead, the metals had selected for the survival of a single *Rhizobium* sp. genotype, which was ineffective in N_2-fixation.

An understanding of the interactions between heavy metals and the legume-Rhizobium symbiosis is clearly important. However, due to the long bioassay peri-

ods required, it seems unlikely that the symbiosis will be developed as a routine indicator of soil pollution by heavy metals. While the sampling, preparation and measurement of clover dry matter yields, total %N and total plant ^{15}N are feasible, the work involved is considerable.

MICROBIAL SPECIFIC ACTIVITY MEASUREMENTS

From the above, measurements of microbial biomass appear useful indicators of environmental stress due to heavy metals, while microbial activity measurements at soil metal concentrations commonly occuring in agricultural soils may not. However, combining the two measurements, to give rates of activities per unit of biomass (biomass specific activities), has been shown to be a much more subtle indicator of environmental stress. In summary, evidence is accumulating that environmental stress such as that produced by heavy metals causes a diversion of energy from biosynthesis to microbial activity in the soil microbial biomass.

Thus, rates of CO_2 evolution (μg CO_2-C g^{-1} soil) from both low- and high-metal soils from the Woburn Market Garden Experiment were not significantly different during laboratory incubations. However, rates of biomass specific respiration, (measured as μg C respired g^{-1} biomass C d^{-1}) was twice the rate in the high-metal soils than in the low-metal ones (Brookes and McGrath, 1984). Similarly, the biomass specific respiration (Fig. 3) increased markedly with increasing soil metal concentration along a gradient while biomass C declined, in line with the above observation.

From such observations, Killham (1985) developed a simple bioassay procedure based on proportionating ^{14}C-labelled glucose between biomass-^{14}C and ^{14}C evolved. He showed, for a given increase in stress, that the ratio: [(respired ^{14}C):(biomass ^{14}C)] was, on average, twice as great as the magnitude of the decrease in either respiration or dehydrogenase activity.

Similarly, about 10% more total and 20% more ^{14}C-labelled CO_2 were evolved from a high-metal than a low-metal Woburn soil during the first five days following addition of ^{14}C-labelled glucose and maize (Chander and Brookes, 1991b). In contrast, about 30% less ^{14}C-labelled biomass was synthesised per unit of added substrate, which is in line with the findings of Killham (1985). Similarly, Chander and Brookes (1991c) showed that plant-derived inputs of organic ^{14}C were about 20% less in the high-metal than low-metal soil. Also, the biomasses in the high-metal soil contained about 30% less of this ^{14}C-labelled organic C than in the low-metal soil. These results suggest that two mechanisms operate in causing smaller biomasses in metal-contaminated soils. These are (1) decreased C inputs from growing plants and (2) decreased efficiency of conversion of this C into new biomass. The latter mechanism appears to be the more important.

Results from our laboratory and elsewhere indicate that measurements of linked parameters such as microbial biomass and soil respiration, giving microbial
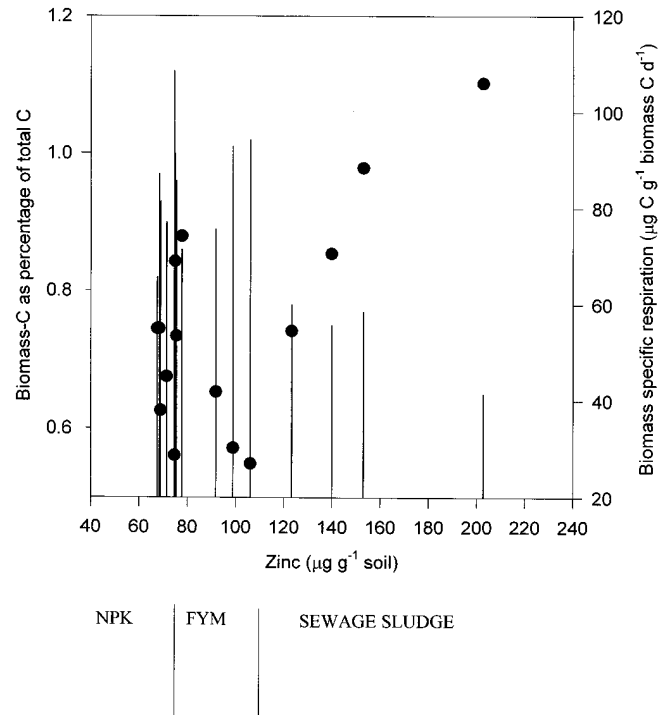
Fig. 3. Biomass specific respiration and biomass-C as percent of total C against metal gradient.

specific respiration, are much more useful than either measurement standing alone. Indeed, it could be argued that 'stand alone' measurements can only really be interpreted when dealing with well-designed field experiments with proper 'control' plots. Non-experimental field data, be it from agricultural or unmanaged ecosystems is usually difficult to interpretate because of lack of suitable 'controls' with which to compare it. To overcome this problem, Brookes (1994) suggested that linked parameters such as microbial specific respiration (or the link between biomass C and total soil organic C, as discussed above) may itself constitute an internal control. Thus, when soils deviate much from biomass specific activity or biomass specific C ratios perceived as normal for the particular management, soil type or climate, it may provide an 'early warning' that the soil ecosystem is under stress and that more research is needed.

2. EFFECTS OF HEAVY METALS ON SPECIFIC MICRO-ORGANISMS OR FUNCTIONAL GROUPS

Soil is a complex material with, for example, cation exchange, buffering properties and chelation reactions which may be quite different to those in simple

media. These soil properties will have very different effects on metal bioavailability than would occur in less complex systems. Thus, while there has been a great deal of research into the effects of heavy metals on soil micro-organisms grown in aqueous or solid culture media, it is impossible to extrapolate the results to the soil environment with any certainty. In most cases, it is equally difficult to study single species *in vivo* and their use in this field is therefore limited. In addition most important soil processes, for example the mineralization or formation of soil organic matter, depend upon the functioning of the microbial community as a whole rather than the activity of individual species. At present, there seem few possibilities to use single microbial genuses or species as biological indicators, as discussed below.

Legume-Rhizobium symbiosis

The ability of various *Rhizobium* species to infect legumes and to fix atmospheric nitrogen is well known. McGrath *et al.* (1988) reported that clover grown on high-metal soils of the Woburn Market Garden Experiment had root nodules but these were unable to fix N_2. The nodules were small and white and easily distinguishable from the much larger, pink nodules found on clover grown in low-metal soils, which were actively-fixing N_2. Free-living *Rhizobium* sp. added to soil were also less able to survive in high-metal than low-metal soils from the same experiment (Giller *et al.*, 1993). Since these results were all obtained at soil metal concentrations at around current EU limits it might seem that *Rhizobium* and symbiotic N_2 fixation is a good indicator of heavy metal effects on the soil indicator. The problem is that most tests for identification of actively-fixing *Rhizobium* strains in soil use sterile host plants grown in sterile nitrogen-free media for two to four weeks, within which time nodules should be produced and the presence or absence of N_2-fixing *Rhizobium* detected (e.g. Hirsch and Skinner, 1992). While probably not a problem for general research, the extended bioassay period and the need for sterility makes *Rhizobium* a rather unattractive proposition as a bioindicator of effects of heavy metals on the soil-microbial ecosystem.

Mycorrhizae

There is evidence for depressed rates of mycorrhizal infection in metal-contaminated soils at 'agricultural' concentrations (e.g. Koomen *et al.*, 1990). However they also sugested that the metals may have encouraged the proliferation of metal-resistant mychorrhizae already present. As McGrath *et al.* (1995) pointed out, sludge applications invariably increase soluble P concentrations in soil which may confound the results by also suppressing mycorrhizal infection. The analytical difficulties and skill required in measuring mycorrhizal infection in roots, both in pots and field soils would probably preclude mycorrhizae as a bioindicator. It can also take a considerable time for measureable mycorrhizal infection to occur in pot experiments, which may also cause analytical problems.

In summary, there are problems in monitoring and interpreting effects of heavy metals on single microbial species or microbial groups and their use as bio-indicators of heavy metal pollution appears negligible at this stage.

3. BIOCHEMICAL MARKERS AS BIOLOGICAL INDICATORS OF EFFECTS OF HEAVY METALS

Markers for whole biomass

Specific biochemicals extracted from the cells of the soil micro-organisms can be used theoretically to investigate the effects of heavy metals on soil microbial ecosystem functioning. Some constituents can be used to gauge heavy metal effects upon the whole biomass. Jenkinson and Ladd (1981) laid down stringent criteria to be met:

1. The constituent must be present in the same concentration in the biomass in different soils.
2. It must be present only in living organisms, i.e. it must not occur exocellularly.
3. It must be capable of being extracted quantitatively from soil.
4. There must be an accurate and precise method(s) to estimate it.

While no cell constituent fully meets these conditions, several have been tried. These include adenosine 5′ mono-, di- and triphosphate, muramic acid, N-acetylglucosamine and the nucleic acids. The above, with the exception of adenosine 5′ triphosphate (ATP), all occur exocellularly in soil in sufficient quantities to violate the above criteria (Jenkinson and Ladd, 1981). Soil ATP analyses however have proved very useful in assessing heavy metal effects on the soil ecosystem.

Adenosine 5′ triphosphate

Adenosine 5′ triphosphate occurs in all living cells but has only a transitory existence in dead cells or exocellularly. Once extracted from soil it may be analysed with high accuracy and sensitivity by the fire-fly luciferin-luciferase enzyme system. A sucessful method of extracting ATP from soil must (1) release all ATP from the microbial cells, and (2) inactivate ATPases and phosphatases, so preventing ATP hydrolyis. We use a reagent based on a mixture of trichloroacetic acid (TCA), paraquat and phosphate (P), developed and described by Jenkinson and Oades (1979). The phosphate and paraquat prevent sorption of the ATP by positive and negative sites respectively on soil surfaces and the TCA provides maximum inhibition of the enzymic hydrolysis of ATP. Other extraction reagents have been proposed, for example the sulphuric acid-phosphate based reagent of Eiland (1983) which gives similar results. However, it is essential that neutral or alkaline reagents are not used under any circumstances, (for example the sodium bicarbonate-chloroform based reagent of Paul and Johnston, 1977) as they do not inhibit enzymic dephosphorylation of ATP and give incorrect, low values (Brookes *et al.*, 1987).

Due to its unique role in cellular energetics, it would seem likely that ATP would provide a better indication of microbial activity than total biomass. However, for reasons that we do not yet understand, soil ATP content is very closely correlated with total soil biomass content in soils, *not* with activity (Jenkinson, 1988), in soils incubated with and without substrates such as straw (Ocio and Brookes, 1990) or glucose and ryegrass (Chander and Brookes, 1991b). In the latter two cases the addition of the substrates caused several-fold increases in rates of CO_2 evolution, a reliable indicator of metabolic activity. Addition of the substrates also caused both biomass and ATP to increase by up to two-fold but the concentrations of ATP in the biomass remained constant and at the same concentration as in unamended soils (about 11 μmol ATP g^{-1} soil).

Brookes and McGrath (1984) reported that heavy metals from past sludge additions in the Woburn Market Garden Experiment caused biomass decreases of up to 50% compared to similar soils given FYM (see above). Similar decreases in ATP in the same experiment were also measured so that the biomasses in the low- and high-metal soils had the same ATP concentrations (again around 10 to 12 μmol ATP g^{-1} soil) despite decreases in biomass and increases in the specific respiration of this biomass in the high-metal soils.

Chander and Brookes (1991b) incubated both low- and high-metal soils from the same experiment at 25°C and 40% WHC with and without separate additions of 5000 μg C g^{-1} soil as ryegrass or glucose for up to 50 days. Again, remarkably close linear corrrelations between biomass and ATP were found, irrespective of metal concentrations or amendment with the two very different substrates, ryegrass and glucose.

The value of ATP analyses in this work is that it is a completely independent estimate of biomass yet correlates remarkably closely with biomass C and other biomass estimates by fumigation-extraction (see above). This gives considerable confidence in both types of analyses in research into effects of heavy metals on soil microbial ecosystem functioning.

The finding of identical biomass ATP concentrations in unamended and substrate-amended soils, despite huge differences in activities, 'biomass standing crops' and soil metal concentrations ranging from background to above current EU permitted limits was unexpected. The true biological significance is currently unknown. What these methods cannot do is to shed light upon possible differences in the community structure of the biomasses in high- and low-metal soils.

Adenine nucleotide and adenylate energy charge in metal-contaminated soils

The adenylate energy charge {AEC = [(ATP) + (0.5ADP)] / [(ATP) + (0.5ADP) + (AMP)]} is defined as a linear measure of the metabolic energy stored in the adenine nucleotide pool of ATP, adenosine 5′ diphosphate (ADP) and adenosine 5′ monophosphate (AMP) (Atkinson 1977). Most data has come from estimates obtained *in vitro* and AEC's between about 0.95 to 0.80 indicate a highly

metabolically-active population undergoing rapid cellular division and biosynthesis. Values of AEC between about 0.8 to 0.4 indicate a stressed population with a low metabolic rate and incapable of much cellular biosynthesis. Adenylate energy charges lower than about 0.4 indicate a moribund or dying population, although microbial spores may have an AEC lower than 0.1. The potential value of AEC measurements in the work described here is that it is a ratio and thus detailed knowlege of past site history may not be critical, which is a limitation of many other approaches.

So far, most AEC measurements have been made on organisms grown *in vitro.* Brookes and McGrath (1987) measured the AEC in a low- and high-metal soil of the Woburn Market Garden Experiment. The soil ATP concentration, and total adenine nucleotide pool were significantly lower in the high-metal soil, indicating, as found previously, a smaller total soil microbial biomass. However, the AEC's of the low- and high-metal soil were both high (0.85 and 0.89 respectively) and comparable to others reported previously for moist soils extracted with acidic reagents (e.g. Tateno, 1985). Therefore, although, as discussed previously, several indices of microbial activity are considerably decreased in high-metal soils, this is not reflected in a lower AEC. This suggests that the magnitude of soil AEC's may not be a valid indicator of environmental stress.

Markers for bacteria and fungal biomass

The above methods refer to biological markers for the entire microbial biomass. Some progress has also been made in splitting the biomass at least into its fungal and bacterial components. Some approaches relevant to determining the effects of heavy metals on the soil fungal and bacterial communities are discussed below.

Direct microscopy

Total microbial biomass may be differentiated into spherical and cylindrical forms (fungi and actinomycetes) and the spherical further subdivided into a bacterial size class and an above-bacterial size class by visual or automated counting of suitably stained organisms in appropriately prepared soil suspensions in agar (e.g. Jenkinson *et al*., 1976). The technique is tedious, requires considerable skill and (often subjective) judgement and is not generally very popular. Nevertheless it does have the huge advantage that it is a direct measurement of the entire biomass and also reveals something of its complexity, unlike most indirect methods. Brookes *et al*. (1986) found, as previously, about twice as much total biomass in a low- than high-metal Woburn soil measured by microscopy. However the ratio of fungal to bacterial biomass were very similar in both cases (6.4 and 5.4 respectively). Therefore, although the heavy metal decreased the biomass 'standing crop' they did not alter bacterial/fungal ratios. Of course it is quite conceivable that the metals caused other changes to the microbial community structure which were not detected.

Phospholipid fatty acids

The ester-linked fatty acids in the phospholipids (PLFAs) are considered the most sensitive and useful chemical measures of microbial community structure. The fungal and bacterial components of the microbial biomass can be determined by specific 'signature' PLFAs. For example, bacteria characteristically contain odd-chain, methyl-branched and cyclopropane fatty acids. The PLFAs in fungi are typically saturated, even-chained, polyenoic fatty acids. Many actinomycetes contain methyl-branched tuberculostearic acid (Tunlid and White, 1992).

In both a forest and arable soil, the double-unsaturated 18.2ω6 PLFA increased proportionately, indicating a shift to fungi two weeks following Zn addition. There were also indications of changes in the proportions of several individual bacterial PLFAs, indicating shifts within the bacterial communities following Zn amendment of the soils (Frostegård *et al.*, 1996).

Much more complex changes in PLFAs were found by Frostegård *et al.* (1993) at six months after separate additions of Cd, Cu, Ni or Zn at different concentrations to a forest humus soil and an arable soil. In summary, PLFAs indicative of actinomycetes increased in the forest soil but tended to decrease in the arable soil. Various types of bacterial PLFAs increased in all metal-contaminated arable soils but were unaffected by metals in the forest soil. The fungal fatty acid, 18:2ω6, generally increased in response to increasing metal concentrations in the arable soils except following Cu amendment, where it decreased. Effects on PLFA patterns occurred at metal concentrations similar to, or lower than, those at which effects on ATP, respiration or total PLFAs occurred.

Soil ergosterol content

Ergosterol (ergosta-5,7,22-trien-3B-ol) is the predominant sterol in most fungi (Tundlin and White, 1992). Methods to measure soil ergosterol have been developed (e.g. Grant and West, 1986) and proposed as a way to estimate the soil fungal biomass content. The basic procedure involves extraction of the ergosterol from soil with methanol, followed by saponification and then re-extraction with hexane. The ergosterol is then determined by HPLC using a UV detector. It is not currently known if the fungal biomass in different soils has a very constant ergosterol content, as with ATP. Certainly, *in vitro*, ergosterol contents can vary at least threefold, depending upon species and growth conditions. West *et al.* (1987) considered that ergosterol analyses were most useful "to quantify *changes* in the fungal populations of soils". Frostegård and Båth (1996), however, showed that the PLFA 18:2ω6 (see above) was closely correlated (r=0.92) with soil ergosterol content, which indicates that both components are measuring the soil fungal biomass. Three independent biomass measurements (biomass C by fumigation-extraction, substrate-induced respiration and ATP) closely followed decreases in soil ergosterol content along a heavy metal gradient from a Finnish Cu-Ni smelter (Fritze *et al.*, 1989). Soil ergos-

terol may therefore have potential as an indicator of fungal biomass in metal-contaminated soils but this requires further evaluation.

Soil enzymes

If enzymes are to be used as bioindicators it may be important to differentiate between exocellular and endocellular enzymes. For example, Brookes (1994) reported less dehydrogenase activity in metal- contaminated soils of the Woburn experiment than in similar uncontaminated soils. In contrast, soil phosphatase activity was unaffected by the metals. Soil phosphatase activity can occur exo- and endocellularly while dehydrogenase only functions within the living cell in soil. These results, at face value, suggest therefore that dehydrogenase is a more reliable indicator than phosphatase of effects of metals on soil microbial activity. However, Chander and Brookes (1991d) showed that the dehydrogenase assay is sensitive to interference from Cu in soil because Cu stops the red colour developing of the artificial end-product (triphenyl formazan). Thus, when Cu is added to the soil in sludges, or in ionic form in solution, this abiological reaction can be incorrectly interpreted as decreased dehydrogenase activity caused by Cu. Other common heavy metals e.g. Ni, Cd or Zn do not cause this effect. As the interference is specific to Cu among the metals tested, dehydrogenase activity may be useful as an indicator of other heavy metals.

4. NOVEL APPROACHES

DNA technology

DNA, or gene, technology is proving to be an awesomely powerful new science with vast and unknown potential. The ability to transfer genetic material between species, even between the Plant and Animal Kingdoms, gives rise to enormous potential benefits in many areas such as agriculture, drug manufacture and medicine. Many would also argue that the risks that accompany such procedures are as massive as the potential benefits.

Recently, Dolly the sheep, the world's first mammal to be cloned from a somatic cell, was announced by the Roslin Institute, UK, and many countries are currently drafting legislation to prevent similar technology being used on humans. However, the progress with these methodologies in soil science has been desperately slow by comparision. This is not merely a reflection of too little research money being available. In fact, research into soil applications of DNA technology, for example to detect different genetic diversities in soil, has attracted a large amount of funding, sometimes by diverting money from other, apparently less exciting but potentially more productive, areas of soil science.

The major problems which are currently limiting progress are the nature both of soil and of the soil microbial biomass itself. Soil is an extremely heterogenous

material, containing both negatively and positively charged surfaces capable of absorbing DNA, enzymes and co-factors. Soil also contains large amounts of humic substances which are powerful inhibitors of many enzymes such as restriction enymes and polymerases and of nucleic hybridisation reactions, all of which are required to function at high efficiency.

Moreover, soil microbial populations are also relatively large and heterogenous. Most of the new methods only work well with cultures of single species, or, at best, simple mixtures of species. The base sequence ratio of guanosine to cytosine in different soil organisms also varies widely, from about 40 to 80% which causes problems in optimising analytical procedures (P.R. Hirsch, pers. comm.). As yet, there are no known published reports of the successful application of DNA technologies to studies of, for example, differences in genetic diversity of the microbial biomass between metal-contaminated and uncontaminated soils.

Lux genes

Bioluminescence-based biosensors are being developed as indicators of soil pollution. Paton *et al.* (1997) described an experiment where a soil isolate of *Rhizobium leguminosarum* bv. *trifoili* was marked with a *lux* gene cassette to enable the expression of bioluminescence. They found that bacterial bioluminescence responded sensitively and negatively to increasing heavy metal concentration in solution, in the order Cd > Ni > = Zn > Cu in an acute test and in the order Cd > Ni = Zn = Cu in a chronic test. On the basis of this work they considered that it may be possible to develop a 'microbial battery test system' to assess both chronic and acute responses of bacteria from different ecological niches to pollution in the soil system. So far, however, the difficulty of detecting bacterial bioluminescence in intact soil has not been overcome.

BIOLOG ASSESSMENTS OF SUBSTRATE UTILIZATION

Microbial communities which differ phenotypically may also differ in the range of carbon substrates which they are capable of utilizing (Garland and Mills, 1991). The Biolog microtiter plate system for identifying micro-organisms offers a simple and fast potential method to face the soil microbial community with up to 95 separate substrates. Knight *et al.* (1997) tested if the Biolog approach could show differences in the ranges of substrates which could be metabolised by the micro-organisms from uncontaminated and metal-contaminated soils. The soils were obtained by adding Cu, Cd or Zn at around current maximum permitted EU concentrations for agricultural soils. After a 3 year equilibration, microbial biomass was measured and compared with substrate utilization patterns of micro-organisms using the Biolog approach. The metabolic potential of the extracted microbial populations were decreased by both Cu and Zn, and also generally in lower pH soils. In contrast, total microbial biomass was unaffected except for a significant decrease at the lowest pH (4.1) with Cu.

This work needs to be repeated with freshly sampled field soils given long-term sludge applications rather than single doses of simple metallic salts. In principle, the use of Biolog microtitre plates is rapid and could offer a fast screening technique to detect stressed populations in situations where an appropriate control soil is available. However the relations between Biolog plate results and microbial ecosystem functioning in metal-contaminated and uncontaminated awaits evaluation.

CONCLUSIONS

1. There is no single microbiological property that is ideal for monitoring soil pollution.

2. Problems in interpretation of environmental measurements are common because of lack of suitable control, or baseline, measurements.

3. There are advantages in using measurements that have some form of internal control e.g. biomass as a percentage of total soil organic matter, as it helps side-step the lack of environmental control data.

4. A "watching brief" should be maintained on the newer methods of molecular biology as applied to soil microbial ecology. They have great potential in monitoring soil pollution. However their application requires considerable further developments before they can be used in soil.

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