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Population size of indigenous *Rhizobium leguminosarum* biovar *trifolii* in long-term field experiments with sewage sludge cake, metal-amended liquid sludge or metal salts: Effects of zinc, copper and cadmium

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ABSTRACT

There is conflicting evidence, and therefore continuing concern, as to whether metals in sewage sludge are deleterious to soil microbial processes and long-term agricultural productivity. Nine field experiments with sewage sludge cakes, three with metal-amended liquid sludges and three with inorganic metal salts were set up across Britain in 1994 to give individual metal dose–response treatments to try to answer this question. This study reports on the effects of Zn, Cu and Cd on the population size of *Rhizobium leguminosarum* biovar *trifolii*, a nitrogen fixing symbiont of white clover (*Trifolium repens*), in soils from these experiments over 11 years. Significant ($P < 0.05$) reductions in indigenous rhizobial numbers occurred on the Zn metal dose–response treatments at eight of the sludge cake sites in 2005, but few consistent effects were evident on the Cu or Cd metal dose–response treatments during the 11-year monitoring period. The soil total Zn concentrations where effects occurred were near to the UK statutory limit of 300 mg kg^{-1} for soils receiving sewage sludge. No significant reductions occurred in any treatments on the metal-amended liquid sludge or inorganic metal salt experiments in which the metals would be expected to be in a more bioavailable form, even after 11 years. The effects in the sludge cake experiments were related consistently with soil total Zn, with no recovery to date. The reductions in clover rhizobial numbers in the sludge cake experiments were due to Zn effects on free-living rhizobia in the soil, with gradual die-off over a long time with increasing soil total Zn concentrations. Currently, no consistent adverse effects on rhizobia have been seen at the UK limits for Cu and Cd of 135 and 3 mg kg^{-1}, respectively.

1. Introduction

Sewage sludge can be used as a soil amendment to provide organic matter and nutrients (MAFF, 1987). However, sewage sludge also contains heavy metals in amounts that are larger than those found in most soils. Long-term use on agricultural land will lead to increased soil metal concentrations and possible detrimental effects on soil microbial activity, and associated implications for agricultural productivity and soil fertility. For these reasons there is concern and on-going debate about the use of sewage sludge in agriculture (e.g. Smith, 1991, 1996; Smith and Giller, 1992; Brendecke et al., 1993; Witter et al., 1994a,b; Pepper et al., 1994; McBride, 1995). The debate has centred on whether metals are deleterious to soil fertility and if so, which metals and at what soil concentrations.

Research on this subject in the few previously available field experiments in Europe was confounded because they were set up with sludges from sewage treatment works for other aims and contained highly differing amounts of various heavy metals. It was therefore not possible to identify which range of heavy metals present in sewage sludge were responsible for detrimental effects on soil microbial processes (Brookes and McGrath, 1984; Chander and Brookes, 1991; Smith, 1991; Chaudri et al., 1993).

In previous studies Zn, Cu and Cd had been implicated as being detrimental to soil micro-organisms and microbial activity at

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concentrations near to or higher than the UK statutory limits for these metals in sludge treated soils (e.g. Brookes and McGrath, 1984; Chander and Brookes, 1991; Smith, 1991; Chaudri et al., 1993). Chromium, Pb and Hg were recognised as being largely insoluble, and therefore less bioavailable, in soils of near-neutral to neutral pH (McGrath, 1987), whereas Ni was not toxic to soil microbial biomass even at three times the UK limit of 75 mg kg^{-1} (Chander and Brookes, 1991). Zinc had been implicated as being detrimental to *Rhizobium* (Chaudri et al., 1993; McGrath et al., 1988, 1995). However, other work screening dedicated historical sludge field sites suggested Cd could be important in determining the presence of *Rhizobium* in soil (Smith and Giller, 1992; Smith, 1997).

The UK maximum permitted metal concentrations for Zn, Cu and Cd in soils of pH 6–7 are 300 mg Zn kg^{-1}, 135 mg Cu kg^{-1} and 3 mg Cd kg^{-1} (SI, 1989). As a result of recommendations from an Independent Scientific Committee Reviewing the "Soil Fertility Aspects of Sludge Applications to Agricultural Land" (MAFF/DoE, 1993), the advisory limit for total Zn for topsoils of pH 5–7 was reduced to 200 mg kg^{-1} (a reduction from 300 mg kg^{-1} at pH 6–7 to 250 mg kg^{-1} at pH 5.5 to $<$6) and the total Cd limit for grassland (0–7.5 cm) was reduced from 5 to 3 mg kg^{-1} (DoE, 1996). These reductions were seen as precautionary measures to protect soil fertility.

As a direct result of this debate about which metals from processed sewage sludge are toxic to rhizobia near to existing metal concentration limits in soil, nine field experimental sites were established across Britain to represent agricultural soils likely to be used for the recycling of sewage sludge (Gibbs et al., 2006a). From the above, Zn and Cd were important to investigate, and there is one report suggesting that *Rhizobium leguminosarum* biovar *trifolii* is sensitive to Cu at $>$135 mg kg^{-1} soil (Chaudri et al., 2000). No other field experiments have used the same sludges at each site, from selected municipal sewage treatment plants contaminated predominantly with one of these metals, to give individual metal dose–response treatments ranging from background to above the UK limit for soils receiving sewage sludge (Gibbs et al., 2006a). These field experiments are unique in that they represent individual metal dose–response treatments using the same ex-treatment works sludges on a wide geographical scale and range of soil types that are likely to affect the bioavailability of metals in soil. At three of the sites, there were two additional field experiments that had the same metal-amended liquid sludges or inorganic metal salt treatments added, using the same protocol, to give individual Zn, Cu and Cd dose–response treatments similar to those in the sludge cake experiments (Gibbs et al., 2006b; Defra, 2002). These were immediately adjacent to the cake experiments, and each other, at all three sites.

In this paper, we report on the effects of Zn, Cu and Cd in these field experiments on the agriculturally and ecologically important micro-organism *R. leguminosarum* biovar *trifolii*, a symbiont of white clover (*Trifolium repens*) over an 11-year period, from 1994 to 2005. The aim was to identify which metal, if any, was causing toxicity to this micro-organism, which was consistent over time and in what soil conditions, with an emphasis on metal concentrations. Concentrations in soil rather than loading were the focus because the exact exposure of microbes in our case is to the concentration in the top 25 cm soil, rather than the loading which was added 10 or more years ago. If only loadings are considered it will not be possible to compare results with published experiments where tillage could be deeper or shallower.

Further aims were to compare the toxicity of sludge cakes produced by municipal sewage treatment works with equivalent amounts of metals added to a low metal sludge or with metal salts. The latter two sources have often been used in the literature due to the ease of experimentation and represent less organic matter or zero added organic matter, respectively. The expectation was that metals may be increasingly toxic when applied with less organic matter. However, if the toxicity should prove to be the same in the long-term, this would pave the way for the use of similarly derived published data in sewage sludge risk assessment, and for more easily performed future experiments. A final aim was to test whether extractable metal concentrations would explain the differences in toxicity between sites and between the three sources of added metals better than total metal concentrations. The hypothesis being that extractable metals may be closer to the bioavailable pool, and a key test is whether they show a better relationship with biological effects than total metal concentrations (McLaughlin et al., 2000). The results for microbial biomass and soil respiration, which were also measured at the same times/treatments, will be the subject of further papers.

2. Materials and methods

2.1. Experimental field sites and treatments

In 1994, nine field experiments with sewage sludge cake were established in Britain on sites representing a range of soil physical and chemical properties. Six of the field experiments were in England (Gleadthorpe, Woburn, Watlington, Rosemaund, Bridgets and Shirburn), two in Scotland (Auchincruive and Hartwood) and one in Wales (Pwllpeiran) and are described in detail elsewhere (Gibbs et al., 2006a). All field experiments had the same experimental design, with completely randomised block treatments at each site, and all treatments were replicated three times. Individual metal dose–response treatments were created at each site from 1994 to 1997 by annually applying sewage sludge cakes (at four metal rates) which were predominantly enriched in either Zn, Cu or Cd relative to the other metals. Details of the treatments and sludges applied are given in Table 1. Furthermore, to achieve as near as practically possible constant organic matter inputs on all the metal dose–response treatments, low metal content sludge cakes were also applied, at the same time. Control treatments which

Table 1
Treatments and total additions of zinc, copper and cadmium (kg ha^{-1} 1994–1997)

	Metal source		
	Cake[a]	Liquid[b]	Salts
Treatment			
No sludge	0	0	–
Low metal sludge	88[c]	28	0
Zn rate 1	254	–	–
Zn rate 2	514	348	399
Zn rate 3	784	696	845
Zn rate 4	1065	1094	1303
No sludge	0	0	–
Low metal sludge	66[d]	24	0
Cu rate 1	125	–	–
Cu rate 2	245	181	205
Cu rate 3	375	351	424
Cu rate 4	500	523	593
No sludge	0	0	–
Low metal sludge	0.33[c]	0.08	0
Cd rate 1	2.15	–	–
Cd rate 2	4.64	5.02	6.00
Cd rate 3	7.22	7.80	9.87
Cd rate 4	9.72	10.58	13.01

[a] Includes metals added in the low metal sludge on treatment Rates 1–4, where low metal sludge was also added to achieve constant organic matter additions in all treatments (104 t ha^{-1}, split over four applications 1994–1997).
[b] Includes metals added in the unamended low metal sludge on treatment Rates 2–4, where the same low metal sludge was also added to achieve constant organic matter additions in all treatments (30 t ha^{-1}, split over four applications 1994–1997).
[c] Digested sludge to match the processing of the Zn- and Cd-rich sludges.
[d] Undigested sludge, to match the processing of the Cu-rich sludge.

received either no sludge or low metal content sludge cakes at the same organic matter addition as the dose–response treatments were also included (Table 1).

Two additional sets of field experiments, similar in design and metal dose–response treatments to the sludge cake experiments (Table 1), were set up in the 3 years from 1995 to 1997 at three of the sites (Woburn, Watlington and Rosemaund). One set of these experiments received metal-amended liquid sludges and the other inorganic metal salts (as Zn, Cu or Cd carbonates) (Gibbs et al., 2006b; Defra, 2002) giving a total of six additional field experiments (Table 1).

All sites were thoroughly cultivated annually within each plot, using a spading machine to incorporate and mix the metals into a 0–25 cm soil depth. Fertilisers (N, P, K, Mg and S) were added to individual plots at all the sites when required at recommended rates (Anon., 2000), as was lime to equalise soil pH levels at each site, with the exception of Shirburn, which was a calcareous site. From 1994 to 1997, all experiments were managed under Italian rye grass, after which wheat or Italian rye grass was grown in alternate years, except for the Welsh site (Pwllpeiran) and the two Scottish sites (Auchincruive and Hartwood) which were always managed under Italian rye grass as appropriate to their land capability.

In 1994 and 1997 the untreated control, low metal sludge control, and the rate 3 Zn, Cu and Cd treatments at all the sludge cake, metal-amended liquid sludge and metal salt field experiments were sampled in spring and analysed. The rate 3 metal treatments were chosen because they were designed to represent individual soil total metal concentrations as near as possible to the UK limits for soils receiving sewage sludge (Zn 300, Cu 135, Cd 3 mg kg^{-1}), once the final additions had ceased in 1997. Then in 1999, 2001, 2003 and 2005, soils from all treatments at the nine sites, including all the sludge cake, metal-amended liquid sludge and metal salt experiments, were sampled in spring and analysed. Twenty-five soil cores were collected from each plot to a depth of 25 cm using an auger made of tempered steel, and bulked in the field to give representative samples of each plot. Augers were cleaned of any adherent soil and sterilised using 70% ethanol inbetween plots to avoid any cross contamination with microbial cells.

2.2. Soil preparation and chemical analysis

Field moist soil samples from each plot were thoroughly mixed, sieved to <3 mm before dividing into two portions. One portion was stored at 4 °C for microbial work and the other air-dried. A subsample of the air-dried portion was ground to <0.5 mm in an agate ball mill and used for the determination of 'total' metal concentrations using aqua regia, exchangeable K and Mg, bicarbonate extractable P, organic C and total N (MAFF, 1986). A further subsample was used to determine ammonium nitrate (NH_4NO_3) extractable metal concentrations (Deutsche Institute für Normung, 1997) and soil pH (in water 1:2.5 w/v).

The metal concentrations in the aqua regia and NH_4NO_3 extracts were determined in the laboratories of the four participants. Different instrumentation (electrothermal vaporisation atomic absorption spectrometry, inductively coupled plasma atomic emission spectrometry and inductively coupled plasma mass spectrometry) was used for the analyses and for this reason it was essential that rigorous quality control procedures were put in place. Each participating laboratory applied in-house quality control procedures, including the running of standards in each batch of analyses. In addition, inter-laboratory tests were undertaken regularly with the same samples being analysed in each of the four laboratories.

2.3. Enumeration of indigenous R. leguminosarum bv. trifolii

The most probable number (MPN) method was used to estimate the number of indigenous clover rhizobia in the bulked soil from each plot, using a 10-fold dilution series (Vincent, 1970), and *T. repens*, cv. Menna, as the trap host. Briefly, moist soil equivalent to 10 g on an oven dry basis were added to 90 ml of sterile deionised water and shaken on a rotary shaker for 30 min to give a soil suspension. From this, a further seven 10-fold dilution steps in triplicate were carried out and three replicate plant infection tubes were inoculated with 1 ml aliquots of each of the dilution triplicates. The tubes were then placed in a controlled environment growth cabinet with 14 h days at 20 °C, light intensity 350 μmol m^2 s^{-1} and 16 °C nights. All tubes were examined for nodulation after 3 weeks and the most probable number of rhizobia calculated using the MPNES computer program (Woomer et al., 1990). Inter-laboratory sample analysis exchanges were undertaken between the three laboratories undertaking the rhizobia MPN analyses, with good agreement found across a range of MPN values (nil to $>10^4$ cells g^{-1} soil).

2.4. Statistical analysis

Soil total metal concentrations and NH_4NO_3 extractable soil metal concentrations are expressed on an oven dry weight basis. GENSTAT 9 for Windows (VSN International, Hemel Hempstead, UK) was used for all statistical analyses. Initial analysis showed the distribution of soil chemical and rhizobial MPN data for all sites and years to be skewed. Hence, all data, except soil pH, were $\log_{10}$-transformed to achieve normality and homogeneity of variances for statistical analysis. One-way ANOVA was used to test important treatment effects. Regression and correlation analyses were used to investigate the relationships between soil chemical properties and clover rhizobia MPNs. The RSEARCH procedure in GENSTAT was used as a multiple regression approach to evaluate the effects of soil properties other than metal concentrations on the number of rhizobia (the selection of variates is detailed below).

3. Results

3.1. Soil chemical analysis

Tables 2 and 3 show the soil physico-chemical properties of the field experiments in 2005, which is 7–8 years after additions of the sludge cake, and the liquid sludge and metal salts ceased. Across the nine sludge cake field experiments, soil pH ranged from 5.3 to 8.1, soil organic carbon 0.74–8.13% and soil total nitrogen 0.10–0.61% (Table 2).

Tables 2 and 3 show that the three cake, liquid and salt experiments had the same soil type and similar background metal concentrations at each site. The soil pH was similar in all the liquid sludge and metal salt experiments, with only the Rosemaund metal salt experiment having a slightly lower pH (Table 2). In these six experiments, Rosemaund had the highest amount of organic carbon and nitrogen (Table 2). There was more variation in soil pH, soil organic carbon and soil total nitrogen in the Woburn, Watlington and Rosemaund sludge cake experiments compared to their respective liquid sludge and metal salt experiments (Table 2). Generally, soil organic carbon and total nitrogen contents were lowest in the metal salt experiments and highest in the sludge cake experiments, as would be expected since the former had received no organic matter inputs.

Soil total metal concentrations were generally higher in the sludge cake and metal salt experiments than the corresponding metal-amended liquid sludge experiments (Table 3). The extractability of Zn in NH_4NO_3 in soils from the sludge cake experiments decreased in the order Hartwood > Gleadthorpe > Auchincruive > Pwllpeiran > Rosemaund ≥ Woburn > Watlington > Bridgets > Shirburn. Extractable Zn concentrations at Hartwood were *c.* 90-times greater than at Shirburn (*c.* 57 mg kg^{-1} compared with 0.61 mg kg^{-1}) for plots receiving the highest rate of Zn-rich sludge

Table 2
Summary of soil properties in the long-term field experiments in 2005

Site	Top soil texture[a]	Clay (%)	Soil pH (H_2O) range	Soil Org. C range (%)	Soil total N range (%)
Sludge cake experiments					
Gleadthorpe	Sandy loam	7	5.7–6.3	0.96–3.10	0.16–0.35
Woburn	Loamy sand	8	6.5–7.3	0.74–2.05	0.10–0.33
Watlington	Sandy loam	16	6.4–7.4	1.00–3.83	0.16–0.61
Pwllpeiran	Clay loam	23	5.9–6.8	3.25–5.18	0.38–0.61
Rosemaund	Silty clay loam	25	5.6–7.1	1.35–2.86	0.23–0.40
Bridgets	Silty clay loam	30	5.8–7.2	1.49–3.42	0.21–0.42
Auchincruive	Sandy clay loam	20	6.0–6.5	2.45–4.72	0.18–0.35
Hartwood	Sandy clay loam	21	5.3–6.5	2.78–8.13	0.19–0.46
Shirburn	Calc. Clay loam	20	7.4–8.1	2.15–3.41	0.34–0.54
Metal – amended liquid experiments					
Woburn	Loamy sand	9	6.5–7.1	0.31–1.61	0.11–0.21
Watlington	Sandy loam	18	6.6–7.5	1.12–1.91	0.18–0.25
Rosemaund	Silty clay loam	23	6.4–7.0	1.28–2.36	0.24–0.29
Metal salt experiments					
Woburn	Loamy sand	9	6.5–7.1	0.43–1.30	0.13–0.17
Watlington	Sandy loam	18	6.5–7.3	0.93–1.36	0.20–0.29
Rosemaund	Silty clay loam	23	6.3–6.8	0.88–2.12	0.20–0.26

[a] Avery (1980) soil texture and % clay measured in 1994 before sludge or metal salt applications.

(Table 3). Extractable Cu concentrations were highest at Watlington and Pwllpeiran and lowest at Bridgets. Extractable Cd concentrations were highest at Rosemaund (0.29 mg kg^{-1}) compared with the other sites (0.03–0.18 mg kg^{-1}) (Table 3). For the metal-amended liquid sludge and metal salt experiments Zn, Cu and Cd extractability was generally highest at Woburn followed by Watlington and Rosemaund (Table 3). Also, in the metal-amended liquid sludge and metal salt experiments Zn extractability was higher than in the cake experiments at these sites. The heavier soils with higher clay contents (e.g. Hartwood, Auchincruive, Pwllpeiran and Rosemaund) generally had the highest extractable metal concentrations, the exception being Bridgets (Tables 2 and 3).

3.2. *Effects on indigenous populations of* R. leguminosarum *bv.* trifolii

Fig. 1 shows the population of indigenous clover rhizobia over time in soils from three treatments: the untreated control plots, low metal sludge control plots and Zn-rich sludge cake plots, with soil total Zn concentrations near to the UK statutory limit of 300 mg kg^{-1} (i.e. the rate 3 application, Table 1). For the sake of clarity, not all Zn treatments are shown in Fig. 1. Over an 11-year period (1994–2005) clover rhizobial numbers in the untreated control and low metal sludge control plots remained at about 10^4–10^5 cells g^{-1} soil at the Gleadthorpe, Woburn, Watlington, Rosemaund, Bridgets and Shirburn field experiments (Fig. 1). In the continuous grass experiments at Pwllpeiran, Auchincruive and Hartwood, rhizobial numbers in both control plots were more variable over time (Fig. 1). To explore the effect of Zn we first used ANOVA. This demonstrated that, in 1997, following the fourth and final sludge cake applications, no statistically significant differences ($P > 0.05$) were found in rhizobial numbers between the untreated controls, the low metal sludge controls, and the rate 3 Zn, Cu or Cd treatments at all the sludge cake, metal-amended liquid sludge and metal salt sites (Fig. 1; data for sludge cake Cu and Cd, and for metal-amended liquid sludge and metal salt experiments not shown, but are discussed below). Generally, rhizobial numbers were slightly lower in the low metal sludge cake plots compared with the untreated controls, although statistically there was no significant difference between these two treatments at all the sludge cake sites over time (Fig. 1). However, after 1997 in soils

Table 3
Summary of soil metal concentrations in the long-term field experiments in 2005

Site	Soil total metal range (mg kg^{-1})			NH_4NO_3 extract. soil metal range (mg kg^{-1})		
	Zn	Cu	Cd	Zn	Cu	Cd
Sludge cake experiments						
Gleadthorpe	30–435	8.0–253	0.20–4.9	0.10–32	0.10–1.5	0.10
Woburn	42–425	12–202	0.50–3.8	0.10–8.2	0.10–1.4	0.10–0.15
Watlington	32–390	9.0–160	0.23–3.8	0.15–6.8	0.08–2.4	0.01–0.08
Pwllpeiran	87–283	12–201	0.25–4.5	0.05–14	0.11–2.3	0.01–0.16
Rosemaund	68–339	18–154	0.27–2.5	0.04–8.7	0.03–1.4	0.01–0.29
Bridgets	46–400	10–195	0.42–4.3	0.02–2.8	0.08–0.77	< 0.001–0.03
Auchincruive	18–491	1.0–248	0.70–2.6	0.02–29	0.003–0.94	0.013–0.18
Hartwood	70–545	17–207	0.26–4.3	0.18–57	0.014–0.87	0.004–0.16
Shirburn	57–656	13–198	0.36–4.6	0.01–0.61	0.10–1.4	0.01–0.16
Metal-amended liquid sludge experiments						
Woburn	44–374	10–182	0.20–4.5	0.37–24	0.10–1.3	0.10
Watlington	44–255	12–104	0.30–2.9	0.01–4.4	0.10–1.9	0.01–0.11
Rosemaund	41–200	11–91	0.14–2.2	0.05–13	0.05–0.75	0.01–0.03
Metal salt experiments						
Woburn	44–466	8.6–192	0.20–4.9	0.04–53	0.01–0.96	0.01–0.12
Watlington	38–460	9.0–160	0.39–5.2	0.14–48	0.15–13	0.02–1.6
Rosemaund	62–339	15–144	0.25–2.7	0.10–14	0.10–0.57	0.01–0.08

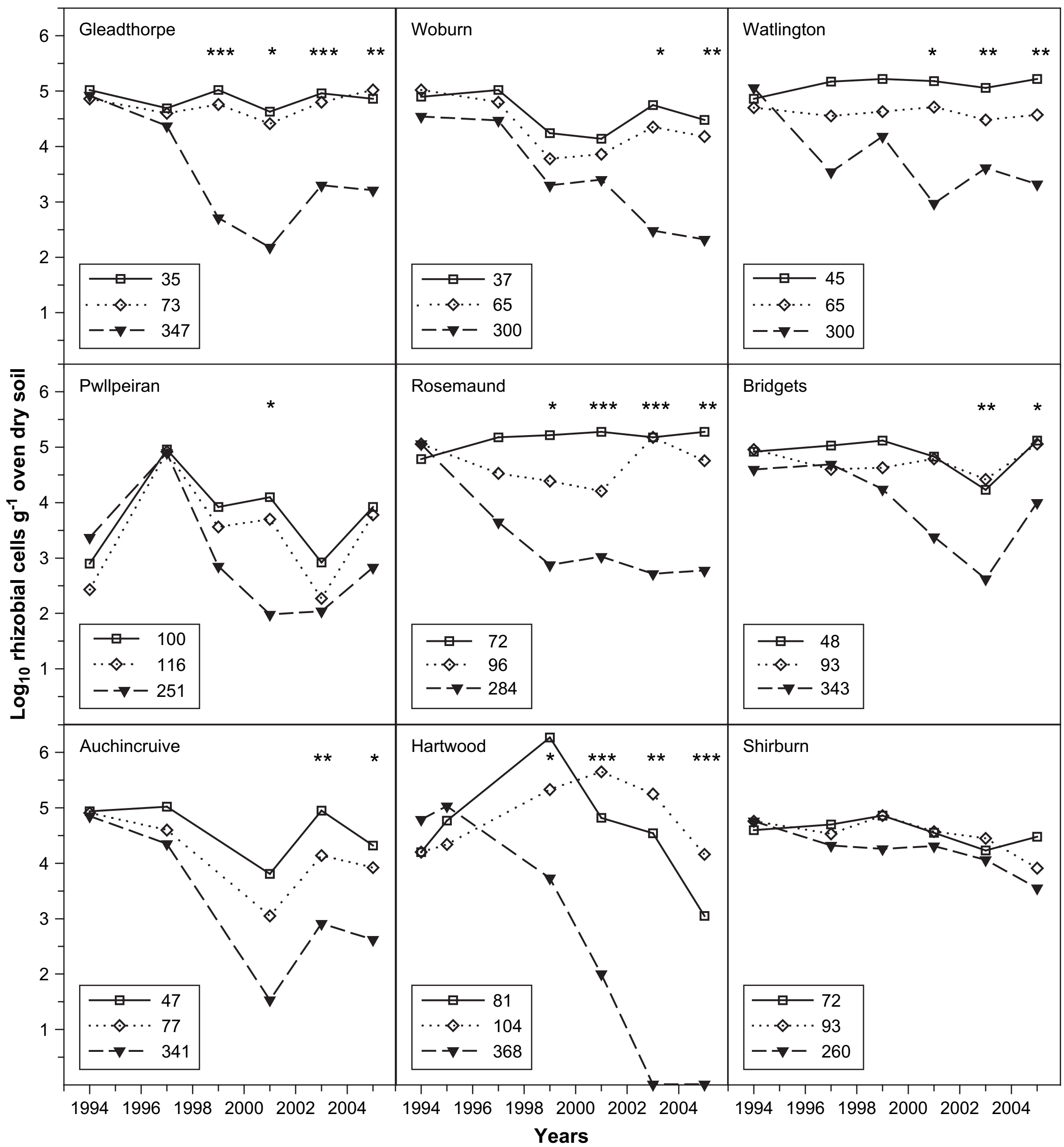

Fig. 1. Numbers of indigenous clover rhizobia in soil over time. Treatments: □ Untreated control; ◇ Low metal sludge cake control; ▼ Zn-rich sludge rate 3; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ denote the levels of significance based on F ratios from one-way ANOVA of these treatments. Where no significance is shown there was no significant difference between treatments. Values shown in the key on each graph are the concentrations of total Zn (mg kg^{-1}) in the soils of the above treatments in 2005.

treated with the rate 3 Zn sludge cake, rhizobial numbers were lower than both control treatments in all years and at all sludge cake sites (Fig. 1). For example, in 1999, 2001, 2003 and 2005 rhizobial numbers were significantly ($P<0.05$ to <0.001) lower in the Zn rate 3 treatments at Gleadthorpe, Rosemaund and Hartwood compared to the untreated control plots and low metal sludge cake control treated soils (Fig. 1) at Watlington in 2001, 2003 and 2005 and at Woburn, Bridgets and Auchincruive in 2003 and 2005 ($P<0.05$ to <0.01) (Fig. 1). By 2005 the differences in rhizobial numbers between the low metal sludge cake control and the Zn rate 3 treatments were $\geq 1 \log_{10}$ at all sites, except the calcareous site at Shirburn. At Hartwood, no rhizobia could be detected in sampling years 2003 and 2005 in most of the Zn-rich sludge cake treatment plots but there were the usual numbers of rhizobia in the control treatments (Figs. 1 and 2). At Shirburn, the difference in numbers was not significant in any year, nor was there any order of magnitude difference between the low metal sludge cake control treatment and any of the Zn-rich treatments (Figs. 1 and 2).

Because the effects of Zn have been shown above to increase with time, we then used data from the most recent year (2005) and from all Zn and appropriate control treatments in a linear regression approach at each of the nine sludge cake sites, in order to explore the relationship between the actual soil Zn concentrations in each plot and the number of rhizobia (Fig. 2). At all eight non-calcareous sites, clover rhizobial numbers decreased significantly ($P<0.01$–0.001) with increasing soil total Zn concentrations. The exception was the calcareous Shirburn site, where there were no significant differences (Fig. 2).

Next, to compare sites, and test the maximum concentration limits in soil, we selected the range of metal concentrations from the low metal sludge cake up to 300 mg kg^{-1} Zn, 135 mg kg^{-1} Cu and 3 mg kg^{-1} Cd i.e. the maximum UK statutory soil metal limits (SI, 1989). Table 4 shows the calculated percentage reductions in

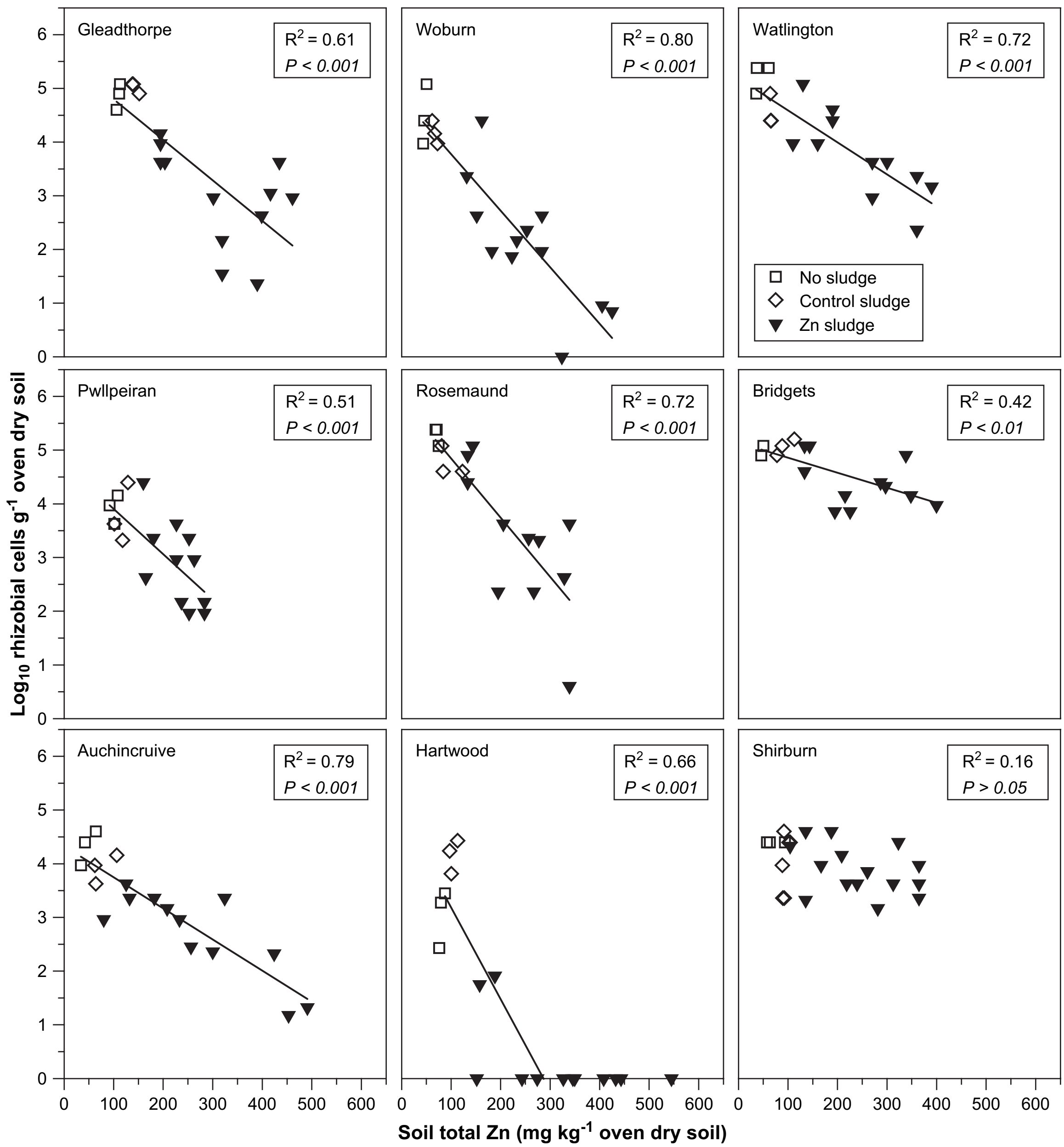

Fig. 2. Relationship between soil total Zn and indigenous populations of clover rhizobia present in soils from all Zn treatments and relevant controls in the sludge cake experiments in 2005. Lines indicate linear regressions of all data points (except Hartwood where the Zn rate 3 and 4 treatments were excluded from the regression). Treatments: □ Untreated control (included in regression statistics); ◇ Low metal sludge cake control; ▼ Zn-rich sludge cake rates 1–4.

$\log_{10}$ clover rhizobial numbers by regression analysis on the metal dose–response treatments up to the UK soil metal limits, calculated as:

$$\text{Percentage reduction} = 100 - [(\log_{10}\text{ MPN in soil at the UK metal limit}/\log_{10}\text{ MPN in low metal sludge treated soil})100]$$

Values for $\log_{10}$ MPNs in the above equation were obtained by solving regression equations (see Fig. 2 for examples) at metal concentrations representing the low metal sludge cake control soils and those representing the respective Zn, Cu and Cd UK limits. In all 4 years (1999, 2001, 2003 and 2005) at Gleadthorpe, Watlington and Hartwood significant percentage reductions (i.e. slope of linear regressions not equal to zero, at $P < 0.05$ to <0.001) in rhizobial numbers occurred at soil concentrations representing the UK statutory soil Zn limit of 300 mg kg^{-1}, compared to the low metal sludge cake control soils (Table 4). In addition, significant ($P < 0.05$ to <0.001) reductions in rhizobial numbers occurred at Rosemaund and Bridgets in 2001, 2003 and 2005, at Woburn in 2003 and 2005, Pwllpeiran in 2001 and 2005, and Auchincruive in 2005. On average, the percent reductions in rhizobial numbers were in the order: Hartwood > Gleadthorpe > Woburn > Pwllpeiran = Rosemaund > Watlington > Bridgets > Auchincruive > Shirburn (Fig. 2 and Table 4).

Table 4
Calculated percentage reduction in $\log_{10}$ clover rhizobial numbers by regression analysis on metal dose–response treatments up to the UK metal limits for soils

Site	Zn (300 mg kg^{-1})				Cu (135 mg kg^{-1})				Cd (3 mg kg^{-1})			
	1999 (%)	2001 (%)	2003 (%)	2005 (%)	1999 (%)	2001 (%)	2003 (%)	2005 (%)	1999 (%)	2001 (%)	2003 (%)	2005 (%)
Sludge cake experiments												
Gleadthorpe	38***	45***	39***	33**	31***	NS	NS	NS	NS	(36)**	NS	21**
Woburn	NS	NS	37**	59***	17*	NS	NS	NS	NS	NS	NS	18*
Watlington	11*	25**	19**	27***	13**	NS	NS	NS	NS	NS	NS	NS
Pwllpeiran	NS	52**	NS	43*	NS	NS	NS	16*	NS	NS	NS	(20)**
Rosemaund	NS	20***	28**	47***	5*	NS	NS	7*	NS	NS	(27)***	NS
Bridgets	NS	21**	22*	13**	NS	NS	NS	5*	NS	NS	NS	NS
Auchincruive	ND	NS	NS	32***	ND	NS	14***	NS	ND	NS	NS	NS
Hartwood	>2 log[a]	39**	78***	100**	5**	NS	NS	26**	NS	NS	NS	47*
Shirburn	NS	NS	NS	NS	NS	NS	NS	NS	NS	21*	(25)**	NS
Metal-amended liquid sludge experiments												
Woburn	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Watlington	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Rosemaund	NS	NS	21**	NS	NS	NS	NS	NS	NS	NS	NS	NS
Metal salt experiments												
Woburn	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Watlington	NS	NS	NS	NS	NS	NS	7*	NS	NS	NS	NS	NS
Rosemaund	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

ND = Not determined; NS = Not significant by regression analysis; *$P<0.05$; **$P<0.01$; ***$P<0.001$.
Brackets indicate regressions with soil total Zn in the cadmium plots.
Percentage reduction in MPN = 100 − [($\log_{10}$ MPNs in low metal sludge soil/$\log_{10}$ MPNs in soil at UK metal limit) × 100%].
[a] Maximum reduction > 2 $\log_{10}$ on metal dose–response treatments.

Consistent trends with time were not seen on the Cu- or Cd-rich treatments either in the sludge cake, metal-amended liquid sludge or metal salt experiments (Table 4). However, in 1999 on the Cu-rich sludge cake treatments there were significant ($P<0.05$ to <0.001) percentage reductions in $\log_{10}$ clover rhizobial numbers at Gleadthorpe, Woburn, Watlington, Rosemaund and Hartwood (Table 4). Similarly, in 2003 at Auchincruive there was a reduction, and in 2005 at Pwllpeiran, Rosemaund, Bridgets and Hartwood there were reductions in the Cu-rich sludge cake treatments (Table 4). For the Cd-rich sludge cake, significant ($P<0.05$ to <0.01) reductions in $\log_{10}$ clover rhizobial numbers occurred at Gleadthorpe and Shirburn in 2001, at Rosemaund and Shirburn in 2003, and at Gleadthorpe, Woburn, Pwllpeiran and Hartwood in 2005 (Table 4). However, in the sludge cake experiments, there were many more significant reductions (slope not equal to zero) in Zn treatments (23/36 observations) than Cu (10/36) and Cd (4/36 related to Zn).

None of the metal-amended liquid sludge and metal salt experiments showed significant reductions in $\log_{10}$ clover rhizobial numbers in any year and for any metal at their respective UK soil limits, except for Zn in 2003 (21%; $P<0.01$) at the Rosemaund metal-amended liquid sludge experiment and Cu in 2003 (7%; $P<0.05$) at the Watlington metal salt experiment (Table 4).

Linear regression analysis was carried out on the 2005 soil physico-chemical and rhizobia MPN data from the sludge cake sites, but excluding Bridgets and Shirburn, because these sites showed the least effects (Table 4 and Fig. 2). Soil total Zn was found to account for 52% of the variance in the data set ($P<0.001$; based on variance ratio), whilst extractable Zn accounted for only 38% of the variance ($P<0.001$) (Table 5). Despite the similar probability values, the large difference between the % variance accounted for indicates that extractable Zn is not a better indicator of the likely effects than total Zn. Even when further soil parameters such as organic C and pH were added in multiple regressions with extractable Zn, the % variance accounted for was only 44% ($P<0.001$) after including organic C, demonstrating that the extra analysis involved in the extraction is of no benefit. Again using linear regressions, total Cu and Cd also accounted for lesser proportions (29 and 22%, respectively; $P<0.001$) of the variance, most likely due to the inevitable small amounts of Cu and Cd in the Zn-rich sludge (Gibbs et al., 2006a). The regression with soil organic carbon explained only 16% of the variance ($P<0.001$), and soil pH and extractable K and Cd all showed much weaker regressions. Extractable Cu showed no fit, and neither did extractable Mg, P or total N (Table 5).

A number of best multiple regression models can be selected using the above variates. However, when an increased number of parameters are chosen, spuriously high explanatory power is often observed. Therefore, individual parameters were not selected on the basis of such statistical models alone. Rather, following the results of the above analysis, we chose total soil Zn concentration as most important explanatory variable, and then chose soil pH and organic carbon as the key soil factors which affect the expression of toxicity of metal cations in aerobic soils (McLaughlin et al., 2000), and evaluated the resulting models in terms of the effect on the percent variance accounted for. The model statement included the number of rhizobia as the response variate, a constant and total soil Zn concentration as forced terms, then soil pH and organic carbon as free terms in a generalized linear model. The effects of including pH and organic C in the model separately were to increase the % variance accounted for from 52 to 55% and 56%, respectively ($P<0.001$), and adding both 56% ($P<0.001$). However, it was noted that in the model with three parameters the pH term was not significant. It is therefore concluded that the best explanatory factor for the decrease in rhizobia was total Zn, modified only to a small extent by soil organic C. It is worth re-emphasising that in

Table 5
Linear regressions between soil analyses and most probable numbers of *Rhizobium leguminosarum* biovar *trifolii*

Variate	% Variance accounted for by regression	P	Slope
Total Zn	52	<0.001	−3.39
Extractable Zn	38	<0.001	−1.15
Total Cu	29	<0.001	−2.15
Total Cd	22	<0.001	−3.04
Organic C	16	<0.001	−2.93
Soil pH	7	<0.01	0.99
Extractable K	3	<0.05	1.53
Extractable Cd	3	<0.05	−0.66

Note: no fit was observed for extractable Cu, Mg, P, or total N.

these experiments the additions of organic matter were controlled, as was soil pH within a relatively small range, with the exception of Shirburn (high pH due to $CaCO_3$).

4. Discussion

In our study, adverse effects on the population size of indigenous free-living clover rhizobia were only observed in soils from the sludge cake field experiments, and not in the metal-amended liquid sludge or metal salt experiments, even after 11 years (Table 4). Broos et al. (2004) made similar observations and postulated that factors other than metals, such as other contaminants or excess N-supply, may have been responsible for these effects. Mineralisation of added sludge organic matter does produce appreciable amounts of N in soil. It is known that high concentrations of soil nitrogen can inhibit nodulation, which may be mistakenly interpreted as reductions in, or elimination of, rhizobia from a soil. However, in these experiments, to avoid these possible confounding effects, low metal content sludge cakes were applied to act as organic matter control plots and all the plots received metal-rich sludge cakes at all experimental sites at the same time. This achieved as near as practically possible constant organic matter inputs and mineral N in all the treatments, and this did not reduce rhizobia numbers, as shown in the low metal sludge cake control treatments (Fig. 1). Furthermore, Italian rye grass was grown on all the field experiments every year during the period 1994–1997 when sludges were being applied to remove excess mineralised N (Gibbs et al., 2006a,b). Following this period, mineral N in all the plots decreased and N fertiliser was required and applied according to standard farm practice (Anon., 2000). In addition, it is notable that the effects of Zn became stronger with time, when mineralisation of sludge N would be decreasing and at its lowest in 2005. Therefore, the comparison between the low metal sludge cake control plots and their respective metal-rich sludge cake treatment plots in our experiments disproves the conjecture of Broos et al. (2004) concerning N.

Soil organic carbon and pH were only weakly related to the number of indigenous clover rhizobia at any of the sampling times in any of our experiments, and total N was unrelated (Table 5). It is known that the survival of *R. leguminosarum* biovar *trifolii* can be affected at soil $pH < 5.5$, with severe reductions in numbers at $pH < 4.5$ (Richardson and Simpson, 1988), although others have reported survival in soils of pH 4.7 (Hirsch, 1996). Soil pH was >5.3 in all the field experiments over the 11-year period and, therefore, is unlikely to have markedly affected the survival of clover rhizobia. For example, at Hartwood in 2005, although soil pH in one of the low metal sludge cake control plots was 5.3, it still had 4.24 $\log_{10}$ rhizobial cells g^{-1} soil. Broos et al. (2005) also found no significant correlation between the number of rhizobia and soil pH in survival experiments, using soils from field experiments previously treated with either metal-contaminated sewage sludges or metal salts.

It is also unlikely that the effects can be attributed to organic contaminants in the applied sludge cakes. Chaudri et al. (1996) added 10 organic compounds frequently occurring in sewage sludge to soil (without the sludge) at worst case concentrations and reported an adverse effect of only pentachlorophenol, which significantly reduced the population of indigenous *R. leguminosarum* biovar *trifolii*. Furthermore, in our study no reductions in rhizobial numbers occurred in the low metal sludge cake control plots which had received the same amount of digested sludge cake organic matter as the Zn treatment plots. Therefore, the reductions in clover rhizobial numbers on metal-rich sludge cake treatments cannot be attributed to soil pH, organic carbon or total nitrogen, and they are unlikely to be due to organic contaminants.

For Zn, the reductions were progressive over time, with no recovery in numbers to date (Fig. 1 and Table 4). At Hartwood, where we report a lack of rhizobia by the MPN method, no rhizobia were detected even when 'bait' clover plants were grown directly in soil from these treatments. Plants grown in soil from the low metal sludge control treatment had nodules present on their root systems and were healthy and green, indicating that they were fixing atmospheric N_2. On the other hand, plants grown in soil from the Zn-rich sludge cake rate 3 treatment at Hartwood were stunted in growth and chlorotic. This was due to the absence of nodules on the root systems and, therefore, a lack of atmospheric N_2-fixation resulting in no supply of N to the plant after soil N had been exhausted.

It appears from Fig. 2 that at the responsive sites there may be no clear threshold for the effect of Zn. It is not possible, therefore, to set a limit based on no effects. It is perhaps more acceptable to base a limit on a given percentage reduction, remembering that loss of function (in this case lack of symbiotic nitrogen fixation with clover) occurs when very few cells remain (likely when population size is <1 on a log scale). Any chosen percentage (on a log scale) can be read from the linear relationships shown on Fig. 2.

For Cu and Cd, the effects with time were inconsistent (see Table 4). The effects seen on the Cu-rich sludge cake treatments in 1999 at Gleadthorpe, Woburn, Watlington, Rosemaund and Hartwood were not strong, and in the 2001 and 2003 sampling years were not significant ($P > 0.05$) (Table 4). The effects of Cu occurred again in 2005 at Pwllpeiran, Rosemaund, Bridgets and Hartwood, and although significant, were weak, except at Hartwood.

The effects on rhizobia seen in the Cd-rich sludge cake treatments at Gleadthorpe and Shirburn in 2001, Rosemaund and Shirburn in 2003, and Gleadthorpe, Woburn, Pwllpeiran and Hartwood in 2005 also resulted in significant negative relationships when regressed against soil total Zn concentrations on these plots (Table 4). For example, the soil total Zn concentrations in 2005 in the Cd-rich sludge cake dose–response treatments at Gleadthorpe, Pwllpeiran and Hartwood on average ranged from 192 to 209, 118–175 and 122–177 mg kg^{-1}, respectively. Soil total Zn and Cd concentrations were also found to be significantly correlated with each other on the Cd-rich sludge cake treatments at these sites ($r^2 = 0.61$–0.98), making it difficult to unequivocally identify the metal causing toxicity. However, the consistent evidence from the Zn-rich sludge cake treatments at these sites suggests that Zn in the Cd-rich sludge cake treatments at some of the sites was most likely to be responsible for the reduction in rhizobial numbers (see Table 4).

In two long-term field experiments at Braunschweig, in Germany, to which metal-contaminated liquid sludges had been added in the past, reductions in clover rhizobial numbers were attributed to increasing soil total Zn concentrations, even though the treatments applied contained a mixture of metals (Chaudri et al., 1993). With the exception of Zn, all metal concentrations in these experiments were at levels below the UK soil limits. Similarly, in a metal-contaminated sludge cake experiment at Gleadthorpe, in England, significant reductions in numbers of clover rhizobia and pea rhizobia with increasing soil total Zn, and to some extent Cu, were reported (Chaudri et al., 2000). In that study, Cu concentrations where effects occurred were above the UK soil limit of 135 mg kg^{-1}, whereas corresponding Cd concentrations were below the limit of 3 mg kg^{-1} and generally had no effect. Contrary to this, some workers have suggested that Cd may be more toxic to rhizobia than other metals (Smith and Giller, 1992; Smith, 1997). Our study to date points to Zn as the metal consistently causing toxicity to rhizobia, and not to Cu or Cd at the concentrations in our experiments. In a recent study, Broos et al. (2005) also concluded that Zn and not Cd was the metal most toxic to free-living rhizobia in soils in the absence of the host plant. They assessed the survival in the laboratory of *R. leguminosarum* bv. *trifolii* that they inoculated into soils from field experiments previously receiving either sewage sludge or Cd salts, whereas our study looked at the effects of metals added in either sludge cake, metal-amended liquid sludge or metal salts on the *indigenous* populations of *R. leguminosarum* bv. *trifolii* under field conditions.

In our study, soil total Zn alone was found to account for 52% of the variance in the clover rhizobial MPN data from the sludge cake experiments, excluding Bridgets and Shirburn, whereas extractable Zn only accounted for 38% of the variance. To be of practical or scientific use, extractable Zn would have to be a closer indicator of bioavailable Zn than total metal concentrations. The inclusion of either soil pH or organic carbon, or both, with total Zn only slightly increased the variance accounted for in the MPN data. The fact that 52% of the variance in the clover rhizobial MPN data from the seven sludge cake field experiments is accounted for by soil total Zn is remarkable. These field experiments are geographically and climatically different and represent a range of soil physical and chemical properties, although the experimental design and treatments applied were the same, and all received the same low metal and metal-rich sludge cakes. It should be noted that although pH had little effect on the relationship with Zn, this may be due to the fact that in these experiments the soil pHs were controlled in a relatively narrow range (the majority of plots were between 6 and 7 by 2005), apart from the calcareous site, which showed no effect. If soil pHs fall, it is likely that pH may become an important factor along with Zn concentrations.

The presence of the host plant is known to confer protection on rhizobia against metal toxicity (Giller et al., 1998; Broos et al., 2004). In our study, the host clover plant, *T. repens*, was not present on any of the plots at any field site. The exclusion of clover was deliberate, since the aim of the study was to identify which metal, if any, was responsible for toxicity to the free-living rhizobia in the soil. The presence of the host plant would have offered protection to the *Rhizobium*, therefore, confounding this aim.

The fact that no adverse effects were seen in the metal-amended liquid sludge and metal salt experiments was unexpected and surprising. Conventional wisdom would suggest that metals added as salts to soils might be the most toxic to micro-organisms, with the metal-amended liquid sludge next and the sludge cake showing the least toxicity because the added organic matter in the sludge cake experiments would ameliorate metal toxicity to micro-organisms by binding metals, thus reducing bioavailability, and also through 'physical' protection (Babich and Stotzky, 1980). Certainly, extractable soil Zn, Cu and Cd concentrations were highest in the metal salt experiments and lowest in their respective sludge cake experiments (Table 3), thus partly supporting the above hypothesis. However, in our study the Zn added to soil in sludge cake had the most adverse effect on rhizobia MPNs, compared to little or no measurable effects of adding Zn to soil in either liquid sludge or as a carbonate metal salt (Table 4). Consequently, we require an alternative hypothesis to explain our data.

It is well established that micro-organisms in soils are largely associated with organic matter, which they use as a substrate for energy and reproduction. Numerous studies have documented increases in microbial biomass with organic matter additions (e.g. Jenkinson and Ladd, 1981). On a microscopic scale, the incorporated sludge organic matter particles are likely to have attracted most of the soil microbes, as a substrate. These particles in the Zn-rich sludge cake treated soils will have high concentrations of Zn (approx. 6000 mg kg^{-1}) reflecting concentrations in the applied sludge cake (Gibbs et al., 2006a). Therefore, we propose that Zn toxicity to the free-living rhizobia seen in the sludge cake experiments is due to direct toxicity through exposure to very high concentrations of Zn in and around the sludge cake particle 'hot spots' in the soil, and poisoning at a cellular level when mineralising this organic matter for cell maintenance and reproduction, leading to cell disruption. However, it is clear that the run-down in population takes time (years). In our opinion, this is a plausible hypothesis for reductions in numbers of free-living rhizobia, with possible gradual extinction over time in the Zn-rich treatments of the sludge cake experiments as observed at the Hartwood site. A possible reason for seeing inconsistent effects on indigenous rhizobia in either the Cu-rich or Cd-rich sludge cake treatments may be that these metals were not toxic at the concentrations achieved in the sludge cake treated soils.

Furthermore, it is possible that no detrimental effects of Zn were seen in either the metal-amended liquid sludge or metal salt experiments, because the metals were not associated with organic matter. The Zn would be much more evenly incorporated throughout the soil and not be found as 'hot spots' of very high concentrations associated with organic matter like in the sludge cake experiments. We suggest that in the metal-amended liquid sludge and metal salt experiments micro-organisms are mostly associated with uncontaminated organic matter from root exudates and from root and crop residues left after harvest, and incorporated into the soil during cultivation. Therefore, in these experiments the micro-organisms are not exposed to the 'hot spots' of high Zn concentrations and associated organic matter as they would be in the sludge cake experiments. The probable interaction between organic matter and zinc observed in this study may have implications beyond the recycling of sewage sludge to agricultural land, as there are large amounts of other organic materials (e.g. farm manures, composts and paper mill sludges) recycled to land that also contain zinc.

The field experiments in our study have been running for 11 years and it is possible that effects of either Cu or Cd in the sludge cake experiments, or Zn, or Cu, or Cd in the metal-amended liquid sludge and metal salt experiments may occur in future. Therefore, further monitoring of rhizobial numbers in all plots of these experiments needs to be carried out. It is important to determine whether: (1) the detrimental effects in the Zn-rich sludge cake treatments increase with time and/or occur at lower soil Zn concentrations; (2) rhizobial numbers in the Zn-rich sludge cake treatments recover to those in the untreated control and low metal control treatments; (3) Cu and Cd become detrimental with time in the sludge cake experiments; and (4) effects are seen in the metal-amended liquid sludge and metal salt experiments with time. Moreover, future research needs to examine and understand the intimate relationship between relatively uncontaminated and metal-contaminated sludge organic matter and micro-organisms at the appropriate physical scale. For example, determining the spatial distribution of Zn and micro-organisms in relation to sludge organic matter in soils treated with low metal and Zn-rich sludge cakes may lead to a better understanding of the mechanisms of effects observed in this study. Future research should also focus on whether Zn toxicity has affected the genetic diversity of populations of *R. leguminosarum* bv. *trifolii* within each site and between sites where effects are seen. Previous studies have shown that metals not only decrease the *Rhizobium* population size, but also alter genetic diversity of the surviving pollution (Giller et al., 1998; Hirsch et al., 1993; Lakzian et al., 2002). We also need to consider possible interactions with other components of the microbial community. We know, for example, that the structure of the microbial community changes in response to both metals (Bååth et al., 1998; Campbell et al., 2003) and organic matter 'hotspots' (Frostegård et al., 1997) so that competition and predator–prey interactions may also play a part in rhizobia survival. However, because we have low metal sludge cake control treatments with no effects observed, these effects would also be due to metals, but indirectly.

Trying to explain differences in the size of the effects between sites based on bulk soil properties is a conundrum and important if we are to protect all of our soils. No single soil property measured at the macro scale, other than Zn, was shown to have a large impact in these experiments; so further work on these aspects is needed.

Sludges currently being recycled to agricultural land in the UK generally have much lower metal contents (Water UK, 2006) than those applied in our field experiments, so these should be regarded as "worst case". Similarly, the sludges used in our study would not meet the criterion for 'clean' sludge under the USEPA-503 regulations, which are based on maximum cumulative loading rates rather than soil metal concentrations (USEPA, 1993). Under USEPA-503, 'clean' sludges can be applied to land over longer periods of time until the US maximum loading rates are met. So in our study, we are currently investigating whether gradually building up soil metal concentrations over a long time period (decades) using both metal-rich and low metal sludge cakes will result in greater metal binding and lower bioavailabilities to soil micro-organisms and crops (Gibbs et al., 2006a).

5. Conclusions

Adverse effects on the population of indigenous free-living clover rhizobia were only observed in soils from the sludge cake field experiments, but surprisingly not in the metal-amended liquid sludge or metal salt experiments in which the metals would be expected to be in a more bioavailable form, even after 11 years (Table 4). Reductions in indigenous clover rhizobial numbers occurred consistently at eight sites in the Zn-rich sludge cake treated soils, and not in the Cu- or Cd-rich sludge cake treated soils. These effects were not due to soil pH, or organic carbon, or total nitrogen. We conclude that, with the exception of the calcareous site Shirburn, there is robust evidence from the sludge cake field experiments that Zn, and not Cu or Cd, is the metal most likely to be detrimental to the survival of free-living clover rhizobia in soils. Furthermore, there has been no significant recovery in numbers to date in the Zn-rich sludge cake treated soils at all sites where effects have occurred compared to untreated control and low metal control soils. The metal-amended liquid sludge and metal salt experiments have shown no consistent effects on numbers of free-living clover rhizobia to date.

No consistent adverse effects were seen in our study at the current UK soil limits for Cu and Cd of 135 and 3 mg kg^{-1}, respectively. Time will tell whether Zn becomes even more detrimental, or if there is a recovery in rhizobial numbers in the Zn-rich sludge cake treatments. Similarly, whether Cu- and/or Cd-rich sludge cake treatments, or the Zn, Cu and Cd treatments in the metal-amended liquid sludge and metal salt experiments begin to show impacts on rhizobia over time, again, remains to be seen.

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