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Long-term Impact of Sewage Sludge Application on *Rhizobium leguminosarum* biovar *trifolii*: An Evaluation Using Meta-Analysis

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Abstract

The Long-Term Sludge Experiment (LTSE) began in 1994 at nine UK field sites as part of continuing research into the effects of sludge-borne heavy metals on soil fertility. The long-term effects of Zn, Cu, and Cd on the most probable numbers of cells (MPN) of *Rhizobium leguminosarum* biovar *trifolii* were monitored for 8 yr in sludge-amended soils. To assess the statutory limits set by the UK Sludge (Use in Agriculture) Regulations, the experimental data were reviewed using statistical methods of meta-analysis. Previous LTSE studies have focused predominantly on statistical significance rather than effect size, whereas meta-analysis focuses on the magnitude and direction of an effect, i.e., the practical significance rather than its statistical significance. Results showed Zn to be the most toxic element causing an overall significant decrease in *Rhizobium* MPN of −26.6% during the LTSE. The effect of Cu showed no significant effect on *Rhizobium* MPN at concentrations below the UK limits, although a −5% decrease in *Rhizobium* MPN was observed in soils where total Cu ranged from 100 to <135 mg kg^{-1}. Overall, there was nothing to indicate that Cd had a significant effect on *Rhizobium* MPN below the current UK statutory limit. In summary, the UK statutory limit for Zn appears to be insufficient for protecting *Rhizobium* from Zn toxicity effects.

Core Ideas

- Meta-analysis provides effect size of Cd, Cu, and Zn in sludge on *Rhizobium* MPN in soils.
- Zn in sludge significantly decreased *Rhizobium* MPN in soil by −26.6%.
- Below UK limits, Cu in sludge decreased *Rhizobium* MPN in soil less than 5%.
- Cd in sludge had no significant effect on *Rhizobium* MPN in soils.

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Application of sewage sludge to agricultural land is currently seen within the UK as the best practical environmental option for recycling this material (Department for Environment, Food and Rural Affairs, 2007; Gendebien et al., 1999, 2010; Water UK, 2010). Previous work (Berrow and Webber, 1972; Smith, 1996; Thornton et al., 2001) has shown that, due to the domestic and industrial sources of wastewater and the nature of wastewater treatment processes themselves, sewage sludge frequently contains concentrations of potentially toxic heavy metals that are significantly greater than the background concentrations found in soils. This is problematic because heavy metals are extremely persistent and can contaminate soils for decades (Alloway and Jackson, 1991; McGrath, 1987). Although the quality of sludge materials has improved in recent years due to declining metal content, there remains an understandable concern that increasing the quantities of sewage sludge used in agriculture could potentially lead to an accumulation of heavy metals within the environment and a long-term reduction in the quality of agricultural soils.

To prevent a potentially hazardous accumulation of heavy metals in sludge-amended soils, the UK Sludge (Use In Agriculture) Regulations set statutory maximum limits (Table 1) for Cd, Cu, Pb, Hg, Ni, and Zn because these are considered to pose the greatest risk to soil and human health (UK Statutory Instrument, 1989). Following implementation of the statutory limits, two independent scientific reviews were conducted to determine possible risks to food safety, assess the potential long-term impacts of repeated sludge application to agricultural land, and confirm that the legislation put in place was sufficient to protect soil quality. These were performed by the Steering Group on Chemical Aspects of Food Surveillance (Ministry of Agriculture, Fisheries and Food, 1993a) and an independent scientific committee (Ministry of Agriculture, Fisheries and Food, 1993b), both commissioned on behalf of the UK Ministry for Agriculture, Fisheries and Food and the UK Department of Environment (now combined as the Department for Environment, Food and Rural Affairs). Overall, it was concluded that heavy metal uptake by plants was unlikely to pose a significant risk to food safety (Ministry of Agriculture, Fisheries and Food, 1993a); hence the limits proposed by the

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Abbreviations: CL, confidence limit; LOEC, lowest observed effect concentration; LTSE, Long-Term Sludge Experiments; MPN, most probable numbers.

Table 1. Maximum concentrations of heavy metals in sludge-amended soils and average annual rate of application during a 10-yr period permitted by the UK Sludge (Use In Agriculture) Regulations (UK Statutory Instrument, 1989).

Metal	Soil limit value				Max. permissible annual avg. over 10 yr
	pH 5.0 < 5.5	pH 5.5 < 6.0	pH 6.0–7.0	pH > 7.0	
	mg kg^{-1}				kg ha^{-1} yr^{-1}
Zn	200	250 (200)†	300 (200)	450 (300)	15.0
Cu	80	100	135	200	7.5
Ni	50	60	75	110	3.0
	pH 5.0 and above				
Cd	3				0.15
Pb	300				15.0
Hg	1				0.1

† Values in parentheses are UK advisory limits (Ministry of Agriculture, Fisheries and Food, 1993b).

UK Sludge (Use In Agriculture) Regulations were deemed sufficient to protect plants, animals, and humans from metal toxicity. However, this could not be said for soil microorganisms (Ministry of Agriculture, Fisheries and Food, 1993b).

Evidence for the potential impact of heavy metals on the soil microbial community was only beginning to emerge when the UK Sludge (Use In Agriculture) Regulations were first drafted, and therefore soil microorganisms were not considered when establishing the statutory limits. However, there was concern that a decrease in the diversity and activity of soil microorganisms due to heavy metal toxicity could disrupt biogeochemical processes essential to crop production (Ministry of Agriculture, Fisheries and Food, 1993b). The alarm was raised in 1984 when a marked decline in the health of white clover (*Trifolium repens* L.) growing on sludge-amended soils was observed at the Woburn Market Garden Experiment (Bedfordshire, UK). Investigations performed by McGrath et al. (1988) and Giller et al. (1989) subsequently confirmed that the observed decline in clover health was due to the ineffective fixation of atmospheric N_2. This was caused by the toxic effect of heavy metals on *Rhizobium leguminosarum* biovar *trifolii* in soil and was not a direct phytotoxic effect on the clover itself, as plant health was restored by the addition of inorganic N fertilizer (McGrath et al., 1988). To elucidate which metals were having an effect on *Rhizobium*, Chaudri et al. (1992) added metal salt solutions (Cd, Cu, Ni, and Zn), at six different rates, to soils previously amended with farmyard manure (i.e., with low metal content). After 18 months, no *Rhizobium* cells were found in soils treated with the Zn (≥385 mg kg^{-1}) and Cd (≥7.1 mg kg^{-1}) salt solutions, with reductions of 82 and 99% also observed in soils containing concentrations of Cu at 191 and 225 mg kg^{-1}, respectively; Ni appeared to have no effect on *Rhizobium* across the concentration range of 26 to 54 mg kg^{-1}.

To verify these results, Chaudri et al. (1993) performed an investigation at two long-term sludge experiment field sites in Brunswick (Lower Saxony, Germany). The number of *Rhizobium* cells (cells g^{-1} soil) found in arable and ex-woodland soils receiving inorganic fertilizers ranged from 4.2 to 9.3 × 10^3 and 9.3 × 10^3 to 4.0 × 10^4, respectively, with similar ranges seen in soils receiving a "low-metal" sludge treatment at a rate of 100 m^3 ha^{-1} yr^{-1}. At both sites, the number of *Rhizobium* cells in soils receiving 300 m^3 ha^{-1} yr^{-1} of a "high-metal" sludge treatment decreased dramatically where the total concentration of Zn exceeded the UK statutory limits—reaching no observable cells in some cases. Most alarming, however, was the decline in *Rhizobium* cell numbers seen in the arable soil receiving 300 m^3 ha^{-1} yr^{-1} of the "low-metal" sludge treatment, eventually reaching zero cells as the total concentration of Zn increased from 218 to 254 mg kg^{-1}. This decline occurred at concentrations below the UK statutory limit for Zn, although it was noted that the corresponding limit set by Germany (200 mg Zn kg^{-1} for soils with pH > 6) would prevent such an occurrence. These results were taken into account when reviewing the UK Sludge (Use In Agriculture) Regulations, prompting the recommendation that precautionary limits of 200 and 300 mg Zn kg^{-1} should be applied to sludge-amended soils with pH 5 to 7 and pH > 7, respectively (Ministry of Agriculture, Fisheries and Food, 1993b).

One of the major obstacles encountered when setting regulatory limits remains a lack of data available to establish dose–response curves, giving more accurate estimates of the minimum heavy metal concentrations having an adverse effect on soil microorganisms. The long-term impacts of heavy metal contamination on soil microorganisms are still not fully understood (Giller et al., 1998, 1999, 2009), and the need for long-term monitoring of soil microbial communities in contaminated soils has been recognized by several researchers (McBride, 2003; McGrath et al., 1994, 1995). Therefore, following the review of the UK Sludge (Use In Agriculture) Regulations, an investigation into the long-term impact of sewage sludge applications on soil microorganisms was established by the Department for Environment, Food and Rural Affairs as part of continuing research into the effects of heavy metals on soil fertility (Ministry of Agriculture, Fisheries and Food, 1993b).

Long-term Sludge Experiments

The Long-Term Sludge Experiments (LTSE), conducted by ADAS, Rothamsted Research, and WRc in England and Wales and the Macaulay Land Use Research Institute (now the James Hutton Institute) and SAC (now SRUC) in Scotland, began in 1994 at nine UK field sites (Fig. 1), chosen to provide a range of soil properties (Table 2) from various climatic regions (UK Water Industry Research Limited, 2002, 2007; Gibbs et al., 2006). Five sludge treatments were applied annually to experimental plots during the course of 4 yr (1994–1997). Three of the sludge treatments contained elevated concentrations of either Zn, Cu, or Cd and were applied in increasing quantities to establish dose–response curves for the three heavy metals (Table 3). The remaining "uncontaminated" treatments contained concentrations of heavy metals typical for sludge produced in the UK (Control 1 and Control 2; Table 4) and were applied at a single rate (Table 2) to control for the effect of applying organic C to soil; an untreated soil was also included

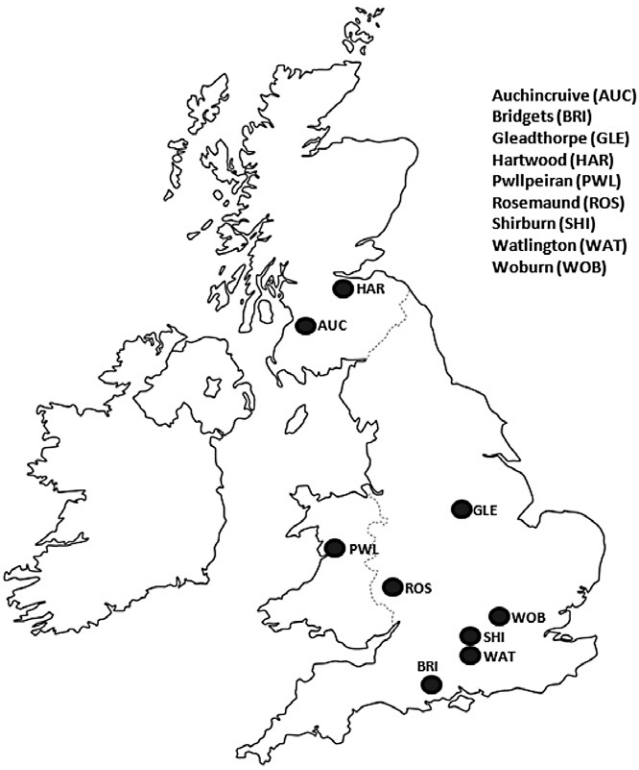

Fig. 1. Location of the Long-term Sludge Experiment field sites (adapted from Gibbs et al., 2006).

in the experimental design (the experimental design was described in detail by Gibbs et al. [2006] and UK Water Industry Research Limited [2002, 2007]). Application of the Zn, Cu, and Cd sludge treatments were supplemented with corresponding sludge material (i.e., digested [for Zn and Cd] and undigested [for Cu]; Table 4) from Controls 1 and 2 to ensure that uniform quantities of organic C were applied across all levels of the dose–response curves (Gibbs et al., 2006). Note that the rate of organic C application for digested and undigested sludge treatments varied among sites (Table 2). The same sludge cake materials were transported to each site to ensure that exactly the same treatments were applied using the same methods of application. Sludge treatments were replicated three times (n = 3) in fully randomized blocks consisting of 6- by 8-m plots and annually cultivated by spading machine to a depth of 25 cm (Gibbs et al., 2006). Each dose–response curve was comprised of four levels of increasing metal concentration, ranging from 150 to 450, 50 to 200, and 1 to 4 mg kg^{-1} for Zn, Cu, and Cd, respectively, although the target metal concentrations were not achieved at all of the sites (Table 5; Gibbs et al., 2006); an additional level was included for the Shirburn site due to the calcareous nature of the soil. Soil pH was kept constant at all sites for the duration of the experiment by the application of lime (with the exception of the calcareous site, Shirburn, which was left at the natural pH of 8). The sites in England and Wales were maintained at a pH of 6.5, whereas the soil pH at the Scottish sites, Auchincruive and Hartwood, were kept at 6.0 and 5.8, respectively.

In 1997 the most probable numbers of cells (MPN) of *Rhizobium* ($\log_{10}$ cells g^{-1} soil) observed in sludge-amended soils were slightly lower than in the untreated soil, with the exception of the Auchincruive site (Gibbs et al., 2006). Furthermore, at the Watlington field site, the reduction in *Rhizobium* MPN in soil receiving the Zn sludge treatment at Dose–Response Level 3 (where concentrations of Zn were closest to the UK statutory limit) was >1 $\log_{10}$ cells g^{-1} soil compared with the corresponding uncontaminated control (Control 1); however, the difference was not found to be statistically significant (Chaudri et al., 2008). Therefore, it was concluded that there was insufficient evidence to indicate an adverse effect of heavy metals on *Rhizobium* MPN in the short term.

Following the final applications of sludge in 1997, *Rhizobium* cell numbers continued to be monitored for 8 yr, with sampling events occurring in 1999, 2001, 2003, and 2005 (*Rhizobium* MPN was not measured at Auchincruive in 1999). Statistical analysis of the experimental data for each of the sampling events was done by analysis of variance (ANOVA) and multiple regression to

Table 2. Soil properties at each of the Long-Term Sludge Experiment field sites in 1994 prior to the application of sludge treatments. Application rates for digested and undigested sludge treatments applied from 1994 to 1997 are also given (adapted from Gibbs et al. 2006).

Property	Auchincruive	Bridgets	Gleadthorpe	Hartwood	Pwllpeiran	Rosemaund	Shirburn	Watlington	Woburn
Sand, %	51	10	71	59	24	8	44	56	80
Silt, %	29	60	22	20	53	67	36	28	12
Clay, %	20	30	7	21	23	25	20	16	8
Texture	sandy clay loam	silt clay loam	sandy loam	sand clay loam	clay loam	silt clay loam	clay loam (calcareous)	sandy loam	loamy sand
pH	6.0	6.8	7.1	5.8	5.4	7.0	8.0	7.4	7.2
Organic C, %	2.5	1.5	1.2	4.7	3.3	1.7	3.0	1.3	1.3
Fe_2O_3, %	4.24	4.00	1.66	3.32	5.69	4.77	3.25	3.71	2.90
Al_2O_3, %	3.54	5.10	1.51	7.87	4.97	5.77	2.45	2.25	1.13
MnO_2, %	0.12	0.23	0.06	0.96	0.15	0.16	0.12	0.09	0.03
Total Zn, mg kg^{-1}	82.4 (0.42)†	49.4 (0.61)	34.4 (0.92)	72.3 (1.87)	140 (4.59)	77.8 (0.98)	68.5 (0.94)	43.3 (0.49)	44.8 (1.06)
Total Cu, mg kg^{-1}	22.6 (0.14)	12.2 (0.24)	7.4 (0.28)	19.8 (0.33)	13.2 (0.74)	17.3 (0.09)	13.1 (0.12)	11.4 (0.11)	13.9 (0.36)
Total Cd, mg kg^{-1}	0.33 (<0.01)	0.95 (0.04)	0.17 (0.01)	0.23 (0.01)	0.15 (<0.01)	0.24 (0.01)	0.37 (0.01)	0.27 (0.01)	0.12 (<0.01)
Rhizobium MPN, $\log_{10}$ cells g^{-1} soil	4.6	4.8	4.9	4.8	2.8	5.0	4.6	4.9	4.7
Digested sludge, t C ha^{-1} yr^{-1}	17.25	12.00	17.25	18.50	8.25	14.25	9.25	15.75	20.25
Undigested sludge, t C ha^{-1} yr^{-1}	20.75	12.25	17.00	20.25	7.50	14.25	10.25	16.75	20.25

† Values in parentheses are standard errors (n = 3).

determine the effect of each metal on *Rhizobium* at each of the field sites. During 1999 and 2001, cell numbers generally remained lower in sludge-amended soils than the untreated soil. This was thought to be due to unfavorable competition from other proliferating microorganisms because the soil microbial biomass C content was greater in the sludge-amended soils than the untreated soil at each of the LTSE field sites (UK Water Industry Research Limited, 2002, 2007). A number of these decreases, observed at six of the LTSE field sites (Gleadthorpe, Hartwood, Rosemaund, Shirburn, and Watlington) in 1999, were found to be statistically significant ($p < 0.05$) in soils receiving the Zn, Cu, and Cd sludge treatments. By 2001, however, significant differences ($p < 0.05$) were observed only in soils receiving the Zn and Cd sludge treatments, indicating that the *Rhizobium* populations in soils receiving the Cu sludge treatment were recovering (UK Water Industry Research Limited, 2002). No statistically significant differences in *Rhizobium* MPN between soils receiving contaminated and uncontaminated sludge treatments were reported for any of the nine field sites during 1999 or 2001. However, significant inverse relationships were found between *Rhizobium* MPN and the total concentrations of Zn and Cu at five of the LTSE field sites in 1999 (UK Water Industry Research Limited, 2002; Table 6). In 2001, no significant relationships were observed between total Cu concentration and *Rhizobium* MPN, whereas the linear relationship to total Zn was statistically significant at six of the field sites (UK Water Industry Research Limited, 2002; Table 6); significant inverse relationships were also seen for total Cd at Gleadthorpe and Shirburn.

During 2003, cell numbers in soils receiving the Cu and Cd sludge treatments (Dose–Response Level 4) at Auchincruive and Shirburn, respectively, were significantly ($p < 0.05$) lower in comparison to the corresponding uncontaminated controls (UK Water Industry Research Limited, 2007; Table 7). During 2005, however, significant ($p < 0.001$) reductions were observed in soils receiving the Zn sludge treatment at seven of the LTSE field sites (Table 7), particularly at Hartwood where *Rhizobium* cells were not detected at Levels 2 to 4 of the Zn dose–response curve. Similarly at Gleadthorpe and Pwllpeiran ($p < 0.01$), as well as Hartwood ($p < 0.05$), *Rhizobium* MPN were significantly lower in soils receiving the Cd sludge treatment at Level 4 of the dose–response curve. However, only at Hartwood were *Rhizobium* MPN in soils receiving the Cu sludge treatment (Level 4) significantly ($p < 0.05$) lower than in the soil receiving the uncontaminated control (Table 7). By 2005, *Rhizobium* MPN were now

Table 3. Target metal concentrations for dose–response curves at Long-Term Sludge Experiment sites.

Dose–response curve	Target metal concentration in soil		
	Zn	Cu	Cd
	mg kg^{-1}		
Level 1	150	50	1
Level 2	250	100	2
Level 3	350	150	3
Level 4	450	200	4
Level 5†	600	275	5

† Additional level applied only at the Shirburn site to account for potential calcareous soil–metal interactions.

Table 4. Properties of sludge treatments applied for 4 yr (1994–1997) at Long-Term Sludge Experiment sites.

Property	Control 1 (digested sludge)	Control 2 (undigested sludge)	Zn (digested sludge)	Cu (undigested sludge)	Cd (digested sludge)
Dry matter, %	18.3	36.7	23.5	18.0	67.8
Organic C, %	38.1	42.9	31.6	37.6	12.9
pH	7.3	7.3	7.5	5.2	6.8
Zn, mg kg^{-1}	560	490	**6000†**	550	1100
Cu, mg kg^{-1}	590	450	**1400**	**5050**	540
Cd, mg kg^{-1}	1.8	1.7	11.2	0.7	**44**

† Values in bold are above EU Sludge Directive lower limits for the respective metal concentrations in sludge: Zn, 2500–4000 mg kg^{-1}; Cu, 1000–1750 mg kg^{-1}; Cd, 20–40 mg kg^{-1} (CEC, 1986).

Table 5. Total metal concentrations measured for each of the Long-Term Sludge Experiment sites in 1997 (adapted from Gibbs et al. 2006).

Sludge treatment	Metal concentrations measured in 1997								
	Auchincruive	Bridgets	Gleadthorpe	Hartwood	Pwllpeiran	Rosemaund	Shirburn	Watlington	Woburn
	mg kg^{-1}								
Zn 1	147 (1.8)†	202 (27.7)	134 (6.6)	171 (16.8)	212 (8.3)	152 (5.3)	185 (5.9)	203 (14.0)	125 (14.1)
Zn 2	236 (10.2)	299 (17.9)	232 (49.0)	240 (11.6)	274 (2.4)	251 (4.5)	298 (20.4)	304 (35.5)	211 (8.1)
Zn 3	314 (8.1)	414 (9.3)	334 (44.4)	406 (30.9)	371 (17.1)	444 (8.0)	345 (5.4)	459 (21.6)	304 (24.9)
Zn 4‡	342 (30.3)	459 (51.0)	291 (29.4)	443 (4.2)	370 (20.5)	421 (34.7)	414 (17.5)	539 (10.6)	224 (16.4)
Cu 1	66 (4.2)	75 (2.7)	69 (6.8)	74 (8.4)	81 (2.9)	74 (5.5)	76 (7.2)	109 (0.3)	56 (3.3)
Cu 2	103 (9.1)	136 (2.4)	120 (18.3)	129 (6.6)	182 (13.7)	137 (17.7)	140 (5.9)	178 (28.1)	92 (10.3)
Cu 3	140 (17.0)	212 (1.0)	188 (39.4)	195 (15.7)	202 (15.3)	202 (38.7)	171 (2.1)	254 (14.1)	161 (15.0)
Cu 4‡	206 (31.6)	209 (3.8)	166 (23.6)	239 (17.1)	234 (37.9)	219 (21.8)	224 (12.6)	309 (26.4)	188 (37.6)
Cd 1	1.0 (<0.01)	1.8 (0.38)	1.0 (0.20)	1.2 (0.09)	1.1 (0.07)	1.0 (0.04)	1.3 (0.14)	1.3 (0.06)	0.8 (0.08)
Cd 2	2.6 (0.29)	2.5 (0.28)	1.9 (0.13)	2.0 (0.06)	2.3 (0.39)	1.7 (0.090	2.1 (0.23)	2.5 (0.17)	1.7 (0.22)
Cd 3	3.4 (0.39)	3.4 (0.21)	2.8 (0.17)	3.4 (0.10)	3.4 (0.14)	2.1 (0.85)	3.5 (0.20)	4.0 (0.11)	2.9 (0.17)
Cd 4‡	3.8 (0.22)	4.7 (0.42)	3.3 (0.21)	4.6 (0.13)	4.2 (0.56)	3.7 (0.02)	4.1 (0.31)	4.8 (0.16)	3.5 (0.37)

† Values in parentheses are standard errors ($n = 3$).

‡ Values of Zn, Cu, and Cd for dose–response Level 5 at Shirburn are 579 ± 20.3, 262 ± 12.6, and 5.0 ± 0.44 mg kg^{-1}, respectively.

significantly and inversely related with total Zn at eight of the nine LTSE field sites, with significant relationships also observed at four of the sites for both total Cu and total Cd (Table 6).

A common tendency of primary studies is to focus predominantly on statistical significance rather than effect size. It is often assumed that the absence of statistical significance provides evidence for the null hypothesis, hence the total number of significant results is compared with that of nonsignificant results to determine whether an effect exists or not (Hedges and Olkin, 1980). In the case of the LTSE, from a total of 459 measurements of *Rhizobium* MPN, during the course of 8 yr, only 23 were reported to be significantly lower in soils treated with contaminated sludge than in soils receiving the corresponding uncontaminated controls (Table 7). Similarly, less than half (n = 40) of a possible 105 linear relationships were reported to be statistically significant (Table 6). However, nonsignificant results can simply be due to low statistical power where small sample sizes have been used in primary studies (Borenstein, 2000). In this

Table 6. A summary of statistically significant inverse relationships found between *Rhizobium* cell numbers (log10 cells g^{-1} soil) and total metal concentration (mg kg^{-1}) during the course of the Long-Term Sludge Experiment (1999–2005) (data compiled from Gibbs et al., 2006; UK Water Industry Research Limited, 2002, 2007).

Sludge Treatment	Auchincruive	Bridgets	Gleadthorpe	Hartwood	Pwllpeiran	Rosemaund	Shirburn	Watlington	Woburn
					1999				
Zn	–	–	$p < 0.001$ $R^2 = 59\%$	–	–	–	–	$p < 0.05$ $R^2 = 34\%$	–
Cu	–	–	$p < 0.001$ $R^2 = 62\%$	$p < 0.01$ $R^2 = 50\%$	–	$p < 0.05$ $R^2 = 35\%$	–	$p < 0.01$ $R^2 = 57\%$	$p < 0.05$ $R^2 = 34\%$
					2001				
Zn	–	$p < 0.01$ $R^2 = 52\%$	$p < 0.001$ $R^2 = 73\%$	$p < 0.01$ $R^2 = 44\%$	$p < 0.001$ $R^2 = 73\%$	$p < 0.001$ $R^2 = 59\%$	–	$p < 0.01$ $R^2 = 47\%$	–
Cu	–	–	–	–	–	–	–	–	–
Cd	–	–	$p < 0.01$ $R^2 = 54\%$	–	–	–	$p < 0.05$ $R^2 = 35\%$	–	–
					2003				
Zn	–	$p < 0.05$ $R^2 = 22\%$	$p < 0.001$ $R^2 = 39\%$	$p < 0.001$ $R^2 = 78\%$	–	$p < 0.05$ $R^2 = 28\%$	–	$p < 0.01$ $R^2 = 19\%$	$p < 0.01$ $R^2 = 37\%$
Cu	$p < 0.001$ $R^2 = 14\%$	–	–	–	–	–	–	–	–
Cd	–	–	–	–	–	$p < 0.001$ $R^2 = 27\%$	$p < 0.01$ $R^2 = 25\%$	–	–
					2005				
Zn	$p < 0.001$ $R^2 = 32\%$	$p < 0.01$ $R^2 = 13\%$	$p < 0.01$ $R^2 = 33\%$	$p < 0.01$ $R^2 = 100\%$	$p < 0.05$ $R^2 = 43\%$	$p < 0.001$ $R^2 = 47\%$	–	$p < 0.001$ $R^2 = 27\%$	$p < 0.001$ $R^2 = 59\%$
Cu	–	$p < 0.05$ $R^2 = 5\%$	–	$p < 0.001$ $R^2 = 26\%$	$p < 0.05$ $R^2 = 16\%$	$p < 0.05$ $R^2 = 7\%$	–	–	–
Cd	–	–	$p < 0.01$ $R^2 = 21\%$	$p < 0.05$ $R^2 = 47\%$	$p < 0.01$ $R^2 = 20\%$	–	–	–	$p < 0.05$ $R^2 = 18\%$

Table 7. A summary of instances where *Rhizobium* cell numbers (log10 cells g^{-1} soil) were significantly lower in soils treated with contaminated sludge than in soils receiving corresponding "uncontaminated" sludge treatments during the course of the Long-Term Sludge Experiment (1999–2005) (data compiled from UK Water Industry Research Limited, 2002, 2007).

Dose–response level	Auchincruive	Bridgets	Gleadthorpe	Hartwood	Pwllpeiran	Rosemaund	Shirburn	Watlington	Woburn
					2003				
Cu (4)	$p < 0.05$ (32)†	–	–	–	–	–	–	–	–
Cd (4)	–	–	–	–	–	–	$p < 0.05$ (30)	–	–
					2005				
Zn (1)	–	–	–	$p < 0.001$ (71)	–	–	–	–	–
Zn (2)	–	$p < 0.001$ (22)	$p < 0.001$ (44)	$p < 0.001$ (100)	–	–	–	–	$p < 0.001$ (52)
Zn (3)	–	$p < 0.001$ (21)	$p < 0.001$ (36)	$p < 0.001$ (100)	–	$p < 0.001$ (42)	–	$p < 0.001$ (27)	$p < 0.001$ (45)
Zn (4)	–	–	$p < 0.001$ (61)	$p < 0.001$ (100)	$p < 0.001$ (46)	$p < 0.001$ (52)	–	$p < 0.001$ (33)	$p < 0.001$ (85)
Cu (4)	–	–	–	$p < 0.05$ (37)	–	–	–	–	–
Cd (4)	–	–	$p < 0.05$ (30)	$p < 0.05$ (74)	$p < 0.05$ (33)	–	–	–	–

† Observed decreases (%) in soils receiving Zn, Cu, or Cd sludge in comparison to uncontaminated controls are shown in parentheses.

case, the contaminated and uncontaminated sludge treatments were applied in triplicate, which may not be sufficient to overcome the natural variation in *Rhizobium* cell numbers observed within the soil environment. Therefore it could be that the effect of applying contaminated sewage sludge was consistently negative across all field sites (i.e., *Rhizobium* MPN were lower in contaminated soils) but produced nonsignificant results due to a low sample size, hence the overall effect may have been overlooked.

Meta-analysis approaches this ambiguity by focusing on the magnitude and direction of an effect, i.e., the practical significance, rather than its statistical significance (Borenstein, 2000; Borenstein et al., 2009). The goal of meta-analysis is also to test the null hypothesis of no effect; however, this is achieved by establishing a common framework in which direct comparisons can be made between independent primary studies (i). The observed effects (R_i) are then combined to give an overall summary effect (M) across a number of primary studies (k), thus giving a more precise estimation of effect size. Combining studies in this way markedly increases statistical power by increasing the overall sample size and can help reduce the "noise" created by sampling error within each study (Hedges and Pigott, 2001). Meta-analysis is becoming increasingly applied within the environmental sciences to help understand a wide range of research questions. It was the aim of this study to provide an additional review of the LTSE, using the statistical methods of meta-analysis, to give a clear overview of the experimental data. This allows an evaluation of previous conclusions and an assessment of the statutory limits set by the UK Sludge (Use In Agriculture) Regulations by assessing the long-term impact of heavy metals on *Rhizobium* populations in contaminated soils.

Methods

Data Sources and Treatment

Data were used from previously published results. For each of the LTSE field sites, *Rhizobium* MPN across all dose–response levels were reported for 1999 (with the exception of Auchincruive), 2001, 2003, and 2005 by UK Water Industry Research Limited (2002, 2007). Gibbs et al. (2006) reported *Rhizobium* MPN for each of the LTSE field sites measured during 1997 at Dose–Response Level 3; however, no standard errors (or standard deviations) were reported for the data and hence effect sizes could not be calculated. Where standard errors have been reported as 0.00, these values were set equal to 0.001. In addition, values reported as $<x$ (i.e., lower than the limit of detection) have been set equal to x; this was necessary for some of the data from Hartwood. In all cases, *Rhizobium* MPN were determined using the methods described by Vincent (1970) and Woomer et al. (1990) and reported as the arithmetic mean ($\bar{x}$) of three replicates ($n = 3$) with standard error (SE); all standard errors were converted to standard deviations (SD = SE × $\sqrt{n}$). Total concentrations (mg kg^{-1}) of Zn, Cu, and Cd, were also recorded as covariables. All data included in the meta-analysis are presented in the supplemental material (Supplemental Table S1). Analysis of *Rhizobium* MPN was performed by three laboratories at separate institutions: Rothamsted Research (for all sites in England and Wales), the Macaulay Institute (for Hartwood), and SAC (for Auchincruive). Therefore a quality assurance program was implemented to determine inter-laboratory precision and variability. No significant differences in *Rhizobium* MPN data were observed among the three laboratories, none of which could detect *Rhizobium* cells in the Hartwood soil sampled from plots receiving the Zn sludge treatment at Dose–Response Level 4 (UK Water Industry Research Limited, 2007). Hence the LTSE data set, as a whole, allows comparisons to be made among sites with a considerable degree of confidence, which would not necessarily be the case if a data set for meta-analysis was compiled from a number of independent, and international, long-term sludge experiments with various experimental designs.

For the purposes of meta-analysis, an experimental treatment (E) must be compared with a suitable control (C). Hence the *Rhizobium* cell numbers in soils receiving the Zn and Cd sludge treatments were compared with those in soils receiving the uncontaminated digested sludge (Control 1; Table 4), whereas *Rhizobium* cell numbers in soils receiving the Cu sludge treatment were compared with that in soils receiving the uncontaminated undigested sludge (Control 2; Table 4). Each dose–response level (Levels 1–4, plus Level 5 only at Shirburn) at each of the nine LTSE field sites was considered as an independent primary "study," giving a total of $k = 37$ studies for the three contaminated sludge treatments following each of the sampling events (except for 1999, when $k = 33$). Individual meta-analyses were performed for each sludge treatment, grouping the data according to soil texture and total metal concentration. Finally, the data for each sludge treatment were grouped according to the date of each sampling event (1999, 2001, 2003, and 2005) to investigate the changes in effect with time; a cumulative effect was also calculated by combining data for successive sampling events.

Due to variations in soil type and climate, and discrepancies between the target metal concentrations and those actually achieved (UK Water Industry Research Limited, 2002, 2007; Department for Environment, Food and Rural Affairs, 2008), it could not be assumed that the overall effect on *Rhizobium* cell numbers would be the same for each of the nine field sites, although the experimental design and applied sludge treatments were identical. Therefore a random effect model was chosen when calculating summary effects for the meta-analysis overall (Borenstein et al., 2009; Hedges and Vevea, 1998), with the data from each site considered to be an individual sample from a population of possible effects. This model assumes that the true effect sizes for each individual study are normally distributed about a mean value and takes into account both sampling error within each study and the true variation in effects observed among sites.

Meta-Analysis

All calculations were performed according to Borenstein et al. (2009). Effects were calculated for each independent study as the log response ratio (Hedges et al., 1999):

$$R_i = \frac{\bar{x}_E}{\bar{x}_C} \quad [1]$$

$$\ln(R_i) = \ln\left(\frac{\bar{x}_E}{\bar{x}_C}\right) = \ln \bar{x}_E - \ln \bar{x}_C \quad [2]$$

where $\bar{x}_E$ and $\bar{x}_C$ are the respective means of the experimental and control groups. The within-study variance (V_i) and standard error (SE_i) of each effect were then calculated as

$$V_i = \left(\frac{\mathrm{SD}_\mathrm{E}^2}{n_\mathrm{E}\bar{x}_\mathrm{E}^2}\right) + \left(\frac{\mathrm{SD}_\mathrm{C}^2}{n_\mathrm{C}\bar{x}_C^2}\right) \quad [3]$$

$$\mathrm{SE}_i = \sqrt{V_i} \quad [4]$$

where SD_E and n_E and SD_C and n_C are the standard deviation and sample size of the experimental and control groups, respectively. Except when the data were grouped by year, the effect sizes calculated for each sampling event (j) were combined to give an average effect for each independent study during the experiment:

$$\bar{R}_{ij} = \frac{1}{m}\left(\sum_j^m R_{ij}\right) \quad [5]$$

where m is the total number of sampling events (m = 4 [1999, 2001, 2003, and 2005]; for Auchincruive, m = 3 [2001, 2003, and 2005]); mean within-study variances ($\bar{V}_{ij}$) and standard errors ($\overline{\mathrm{SE}}_{ij}$) were also calculated in the same way. Effects were then weighted (W_{ij}), using the reciprocal of Eq. [3] and substituting the mean variance for each independent study ($1/\bar{V}_{ij}$). Lower (LL) and upper (UL) confidence limits were determined as

$$\mathrm{LL}_i = \bar{R}_{ij} - 1.96 \times \overline{\mathrm{SE}}_{ij} \quad [6]$$

$$\mathrm{UL}_i = \bar{R}_{ij} + 1.96 \times \overline{\mathrm{SE}}_{ij} \quad [7]$$

where 1.96 is the Z value corresponding to a confidence limit of 95%. A two-tailed test of the null hypothesis ($\alpha = 0.05$) was then performed for each individual study:

$$\mathrm{Z}_i = \frac{\bar{R}_{ij}}{\overline{\mathrm{SE}}_{ij}} \quad [8]$$

As mentioned above, the overall variance for each individual study in the random effects model ($\bar{V}_{ij}{}^*$) is the sum of the within-study variance plus the between-study variance (T^2):

$$\bar{V}_{ij}{}^* = \bar{V}_{ij} + T^2 \quad [9]$$

An estimate of the between-study variance was therefore determined by calculating the Q statistic: the weighted sum of squares across all studies in the meta-analysis, representing the total observed variation (Borenstein et al., 2009):

$$Q = \sum_{i=1}^k W_{ij}\bar{R}_{ij}^2 - \frac{\left(\sum_{i=1}^k W_{ij}\bar{R}_{ij}\right)^2}{\sum_{i=1}^k W_{ij}} \quad [10]$$

The difference between Q and the degrees of freedom (df = $k - 1$), assuming a fixed effect size for all studies (i.e., $T^2 = 0$), then represents the true variation between effect sizes:

$$T^2 = \frac{(Q - \mathrm{df})}{C} \quad [11]$$

where C is a quantity used to give units of the original metric:

$$C = \sum_{i=1}^k W_{ij} - \frac{\sum_{i=1}^k W_{ij}^2}{\sum_{i=1}^k W_{ij}} \quad [12]$$

Because it was not assumed that the between-study variance would be the same for each subgroup, separate values of T^2 were calculated in each case. Finally, summary effect sizes were calculated as the weighted mean across all studies within each group:

$$M = \frac{\sum_{i=1}^k \bar{W}_{ij}{}^*\bar{R}_{ij}}{\sum_{i=1}^k \bar{W}_{ij}{}^*} \quad [13]$$

where $\bar{W}_{ij}{}^*$ is the weight assigned to each individual study based on the overall mean variance (Eq. [9]) for each study. The variance (V_M) of the summary effect was then calculated as the reciprocal of $\bar{W}_{ij}{}^*$, using the sum of weights:

$$V_M = \frac{1}{\sum_{i=1}^k \bar{W}_{ij}{}^*} \quad [14]$$

The standard error (SE_M) was calculated as above (Eq. [3]) using the summary variance, following which 95% confidence limits and statistical significance were determined by substituting M and SE_M into Eq. [6], [7], and [8], respectively. The summary effect and 95% confidence limits (LL_M and UL_M) were then converted back into the original metric (i.e., $\log_{10}$ cells g^{-1} soil) and expressed as a percentage of the overall increase or decrease:

$$M(\%) = 100 - [\exp(M) \times 100] = 100 - \left(\frac{M_\mathrm{E}}{M_\mathrm{C}}100\right) \quad [15]$$

Calculated effect sizes represent the percentage difference in the $\log_{10}$ of *Rhizobium* cell numbers in soils receiving contaminated sludge treatments compared with soils receiving uncontaminated sludge treatments. Therefore the actual percentage difference in *Rhizobium* cell numbers will be much greater, particularly if *Rhizobium* MPN is a low value. The results for each meta-analysis are presented as "forest plots," where each point represents the overall effect size for an individual subgroup. For clarity, only summary effect sizes are shown; effects calculated for individual studies are given in the supplemental material (Supplemental Table S1). The 95% confidence limits for each effect size are represented as horizontal lines; the observed effect is not statistically significant if the 95% confidence limit spans the center line, at which point the effect size is equal to 0 ($\bar{x}_\mathrm{E} = \bar{x}_\mathrm{C}$).

Results

Soil Texture

Zinc

Figure 2a shows the summary effect sizes calculated for the Zn sludge treatment grouped according to soil texture. In each case, the observed effects were negative and statistically significant ($p < 0.001$). The Zn sludge treatment appeared to have the least effect on *Rhizobium* MPN in the calcareous clay loam soil at Shirburn (−12.1%; confidence limit $\mathrm{CL}_{95\%}$ = −17.0 to −6.0%). For the silty clay loam, clay loam, and sandy loam soils, the Zn sludge treatment appeared to have a similar effect on *Rhizobium* MPN, with overall effects ranging from −24.0 to −21.8%, while the greatest effect (−48.0%; $\mathrm{CL}_{95\%}$ = −64.4 to −23.8%) observed was in the sandy clay loam soils of the Scottish field sites (Auchincruive and Hartwood). This was largely due to the *Rhizobium* MPN being below the detection limit at Dose–Response Levels 3 and

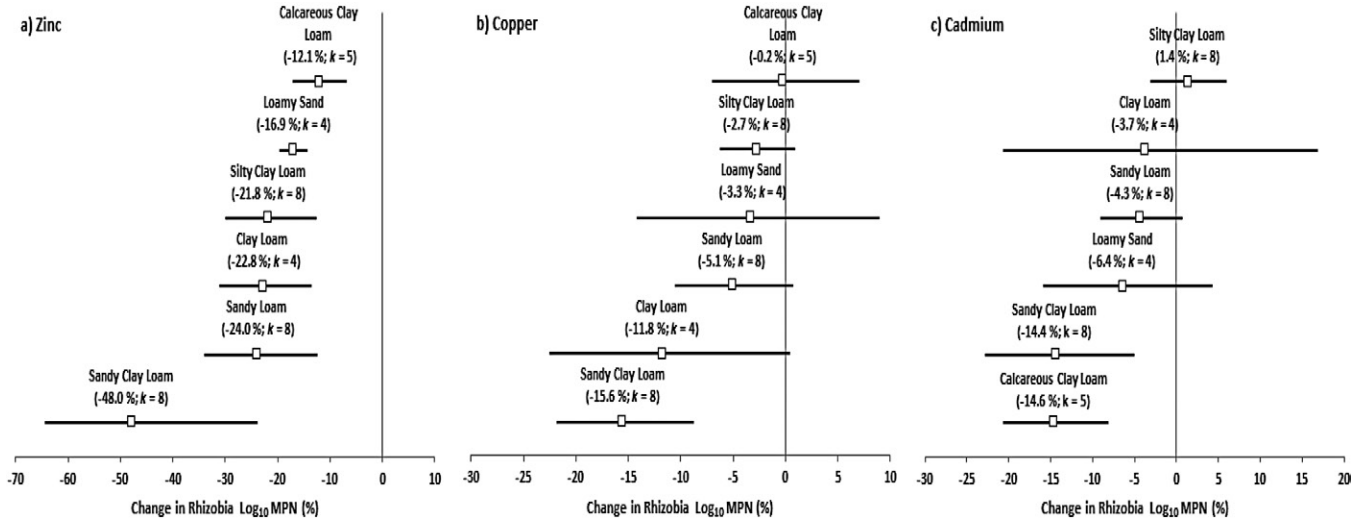

Fig. 2. Forest plots showing effects of (a) Zn, (b) Cu, and (c) Cd sludge treatments on *Rhizobium* most probable number of cells (MPN) grouped according to soil texture. Each point (□) represents the summary effect size for each soil type included in the Long-Term Sludge Experiment. The number of primary studies (*k*) used to calculate each effect size is indicated. Horizontal lines represent 95% confidence limits. Effects are not statistically significant if 95% confidence limit crosses the zero line.

4 during 2003 and 2005. Calculating separate effects (across Levels 1–4) for the two Scottish field sites clearly demonstrated this, as the overall effect observed at Hartwood (−63.8%; $CL_{95\%}$ = −70.3 to −55.9%; $p < 0.001$) was more than five times greater than the effect seen at Auchincruive (−12.0%; $CL_{95\%}$ = −28.8 to 8.7%; $p = 0.236$); the overall effect at Auchincruive alone was not statistically significant (data not shown). The most likely reason for the dramatic effect observed at Hartwood is the low soil pH (5.8), which would increase the solubility, and hence bioavailability, of heavy metal contamination. In comparison with the other field sites, concentrations of Zn extractable by NH_4NO_3 were consistently greater at Hartwood during the course of the LTSE (UK Water Industry Research Limited, 2002, 2007; Gibbs et al., 2006). In 1997, Gibbs et al. (2006) found the concentrations of Zn extractable by NH_4NO_3 to be inversely proportional (R^2 = 92%; $p < 0.001$) to the soil pH, which would also explain why the effect of the Zn sludge treatment was lowest at Shirburn.

Copper

The effect of the Cu sludge treatment in the different soil types was lower than that of Zn. In each case, the observed effects were negative; however, only in the sandy clay loam soils was the observed effect statistically significant (Fig. 2b). As observed for the Zn sludge treatment, Cu also had the greatest impact in the Scottish sandy clay loam soils (−15.6%; $CL_{95\%}$ = −21.8 to −8.8; $p < 0.001$). However, in this case, the individual effects of Cu on *Rhizobium* MPN seen at Auchincruive (−14.9%; $CL_{95\%}$ = −29.3 to 2.4%; $p = 0.088$) and Hartwood (−15.7%; $CL_{95\%}$ = −22.5 to −8.2; $p < 0.001$) were approximately equal, although again the effect at Auchincruive was not statistically significant (data not shown). It is unclear why Cu would have such a significant effect at the Hartwood site because Cu is readily immobilized by soil organic matter, which was greatest at the Hartwood site (Table 2). Gibbs et al. (2006) observed no significant relationship between soil pH and the NH_4NO_3–extractable Cu in 1997 but did find extractable Cu to be inversely proportional ($p < 0.001$) to soil Fe content, accounting for 64% of the observed variation, although the lowest Fe content was observed at the Gleadthorpe site (Table 2). In contrast, a slight positive relationship ($p < 0.001$) was observed between extractable Cu and soil Mn content, which accounted for an additional 14% of the observed variation when included in the original regression model. In this case, the greatest Mn content was seen at Hartwood (Table 2).

Cadmium

Only in the calcareous clay loam and sandy clay loam soils were the overall effects of the Cd sludge treatment statistically significant. In general, the effect of the Cd sludge treatment appeared to be similar in magnitude to that of Cu; however, in contrast to both the Zn and Cu sludge treatments, the greatest impact on *Rhizobium* MPN was seen in the calcareous clay loam (−14.6%; $CL_{95\%}$ = −20.7 to −8.1%; $p = < 0.001$) soil at Shirburn (Fig. 2c), although, in agreement with the results for the previous two sludge treatments, an effect similar in magnitude (−14.4%; $CL_{95\%}$ = −22.9 to −5.0%; $p < 0.01$) was also seen at the Scottish field sites. In this case, the overall effect at Hartwood (−22.0%; $CL_{95\%}$ = −36.7 to −3.9%; $p < 0.05$) was twice that observed at Auchincruive (−11.7%; $CL_{95\%}$ = −21.7 to −0.4%; $p < 0.05$), with significant effects seen at both sites (data not shown). Gibbs et al. (2006) also reported an inverse linear relationship (R^2 = 72%; $p < 0.001$) between NH_4NO_3–extractable Cd and soil pH, hence this is also likely to be due to low soil pH (6 and 5.8 for Auchincruive and Hartwood, respectively). However, the overall effect observed at Shirburn is anomalous to this trend. During the course of the LTSE, the mean total concentration of Zn in soils receiving the Cd sludge treatment at Shirburn ranged from 98.3 to 202.7 mg kg^{-1}; hence this effect is likely to have been due to confounding metal contamination (see below).

Total Metal Concentration

Zinc

A clear decline in *Rhizobium* MPN can be seen with increasing total Zn concentration, reaching an overall effect of −40.3% ($CL_{95\%}$ = −54.9 to −21.1%; $p < 0.001$), where the mean total Zn during the course of the LTSE exceeded 350 mg kg^{-1} (Fig. 3a);

this was predominantly in soils receiving the Zn sludge treatment at Dose–Response Level 4 (450 mg Zn kg^{-1}). However, more concerning is the negative effects observed in soils where the total concentration of Zn was below the UK statutory and advisory limit for Zn in sludge-amended soils. *Rhizobium* MPN were reduced by approximately 20% ($CL_{95\%}$ = −27.2 to −11.4%; $p < 0.001$) in soils where total Zn ranged from 200 to <300 mg kg^{-1}, with a decrease of −13.9% ($CL_{95\%}$ = −23.0 to −3.7%; $p < 0.01$) also observed in soils where total Zn ranged from 100 to <200 mg kg^{-1}. This would suggest that even the proposed advisory limit of 200 mg kg^{-1} (for soils with pH 5–7) may not be sufficient to protect *Rhizobium* populations.

Copper

Below 100 mg kg^{-1}, predominantly at Dose–Response Level 1 (50 mg Cd kg^{-1}), the Cu sludge treatment had no overall effect (−0.2%; $CL_{95\%}$ = −4.2 to 3.9%; $p = 0.910$) on *Rhizobium* MPN (Fig. 3b). However, as the total concentration of Cu increased, significant reductions in *Rhizobium* cell numbers were observed. For soils where the mean total Cu ranged from 170 to <200 mg kg^{-1} or was >200 mg kg^{-1}, the overall effect of the Cu sludge treatment was approximately −10%.

Cadmium

No significant effects were observed in soils where total Cd ranged from 0.5 to <1 mg kg^{-1} (−0.9%; $CL_{95\%}$ = −6.5 to 5.1%; $p = 0.772$); with the exception of the Bridgets and Woburn sites, this was also predominantly in soils receiving the Cd sludge treatment at Dose–Response Level 1. In soils where the mean total Cd ranged from 1.5 to <2 mg kg^{-1} (−6.2%; $CL_{95\%}$ = −13.8 to 2.2%; $p = 0.142$) and 2 to <3 mg kg^{-1} (−4.6%; $CL_{95\%}$ = −8.9 to −0.2%; $p < 0.05$), below the UK statutory limit, the overall effect size was approximately −5%, although only between 2 to <3 mg kg^{-1} was the effect on *Rhizobium* MPN statistically significant (Fig. 3c). Above the UK limit of 3 mg kg^{-1}, the effect of the Cd sludge treatment caused a significant decrease in *Rhizobium* MPN of approximately −10% (−9.6%; $CL_{95\%}$ = −15.3 to −3.6%; $p < 0.01$).

Change in Effect with Time

Zinc

A clear decrease in *Rhizobium* MPN can be seen in soils receiving the Zn sludge treatment during the course of the LTSE, with the overall effect following each sampling event decreasing from −19.0% ($CL_{95\%}$ = −21.8 to −16.0%; $p < 0.001$) in 1999 to −35.7% ($CL_{95\%}$ = −46.2 to −23.2%; $p < 0.001$) in 2005 (Fig. 4a). A steady decline was also seen in the cumulative effect with time, which decreased from that observed in 1999 (−19.0%) to an overall mean effect of −26.6% (Fig. 4b). In this case, the 95% confidence limits for the 1999 sampling event overlap with those for 2005 ($CL_{95\%}$ = −32.7 to −19.9%; $p < 0.001$); hence it cannot be said for certain whether the change in effect size corresponds to a significant change in *Rhizobium* MPN with time. Excluding data from the meta-analysis where the total concentration of Zn exceeded the UK statutory limits still gave a negative, and significant, cumulative effect of greater than −20% (−21.1%; $CL_{95\%}$ = −32.7 to −19.9%; $p < 0.001$), indicating an adverse effect on *Rhizobium* MPN at concentrations of Zn below the current UK limits (data not shown). In general, this decrease was seen for each level of the dose–response curve. For soils where the total Zn was below the UK advisory limit (100 to <200 mg kg^{-1}), the overall effect decreased from −13.2% ($CL_{95\%}$ = −24.7 to 0.1%; $p = 0.052$) in 1999 to −17.5% ($CL_{95\%}$ = −28.2 to −5.3%; $p < 0.01$) in 2005, with significant decreases in *Rhizobium* MPN observed for the 2001, 2003, and 2005 sampling events (Fig. 4c). Similarly, below the UK statutory limit for soils with pH 6 to 7 (200 to <300 mg kg^{-1}), the observed effects were statistically significant for each of the sampling events (Fig. 4c). The lowest effect size observed was −14.5% ($CL_{95\%}$ = −21.0 to −7.4%; $p < 0.001$), seen in 2001, after which the effect size increased steadily to −34.7% ($CL_{95\%}$ = −50.4 to −14.1%; $p < 0.01$), although for both concentration ranges there is considerable overlap between the 95% confidence limits for each of the sampling events. Above 300 mg kg^{-1}, all of the effects observed for each sampling event were statistically significant, with a general increase in effect size observed for consecutive sampling events (Fig. 4c). A dramatic decrease in *Rhizobium* MPN (−60.5%;

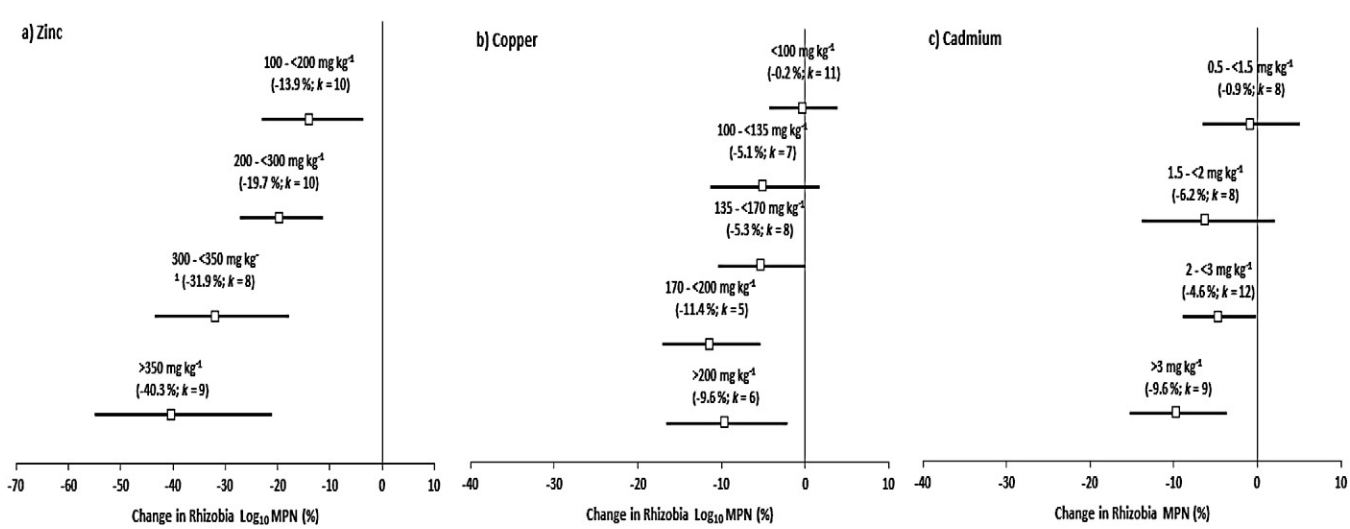

Fig. 3. Forest plots showing the effect of increasing total metal concentration on *Rhizobium* most probable number of cells (MPN) in soils receiving (a) Zn, (b) Cu, and (c) Cd sludge treatments. Each point (□) represents the summary effect size for data within specified concentration ranges. The number of primary studies (*k*) used to calculate each effect size is indicated. Horizontal lines represent 95% confidence limits. Effects are not statistically significant if 95% confidence limit crosses the zero line.

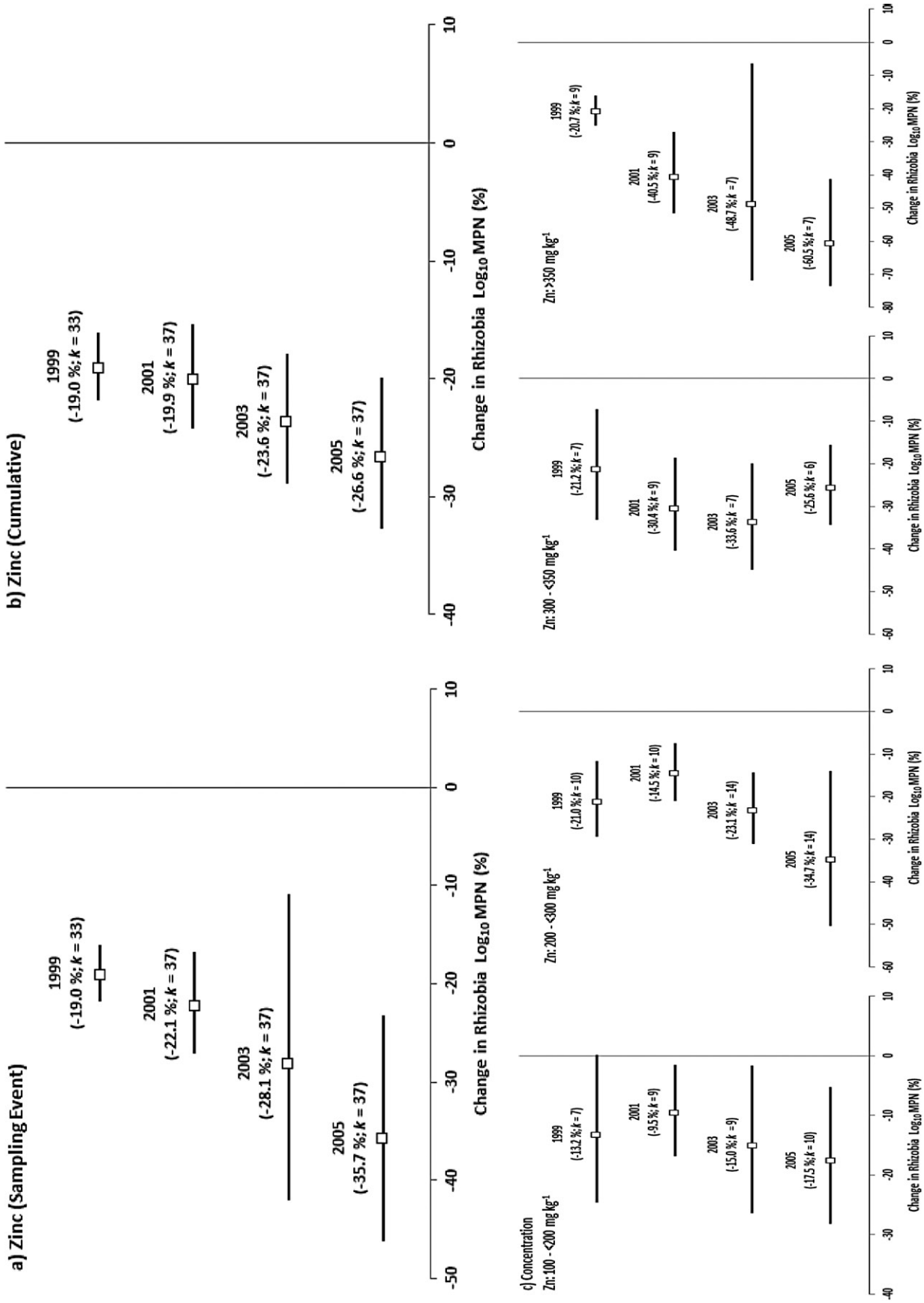

Fig. 4. Forest plots showing the change in effect of the Zn sludge treatment on *Rhizobium* most probable number of cells (MPN) with time: (a) after each sampling event, (b) cumulative effect with time, and (c) the change in effect with time and increasing concentrations of total Zn. Each point (□) represents the summary effect size for specified sampling events, with the exception of the cumulative effect, which represents the mean effect over consecutive years. The number of primary studies (*k*) used to calculate each effect size is indicated. Horizontal lines represent 95% confidence limits. Effects are not statistically significant if 95% confidence limit crosses the zero line.

$CL_{95\%}$ = −73.5 to −41.3%; $p < 0.001$) was observed for the 2005 sampling event in soils where total Zn remained >350 mg kg^{-1}. In this case, the effect observed after 8 yr was significantly different from that seen in 1999 (−20.7%; $CL_{95\%}$ = −25.2 to −16.0%; $p < 0.001$). Clearly, at this level, the Zn sludge treatment has had a marked and lasting effect on *Rhizobium* populations in the receiving soils.

Copper

For the Cu sludge treatment, with the exception of 2001, a significant overall effect was seen for each of the sampling events (Fig. 5a). For the 1999 sampling event, an effect of −7.8% ($CL_{95\%}$ = −13.3 to −1.4%; $p < 0.05$) was observed, following which the effects were −5% or less (Fig. 5a). No significant differences were observed among the effects for each sampling event. Additionally, no significant change in the cumulative effect was observed after 2001, which remained at −4.7% for 2003 and 2005 (Fig. 5b). Removing data where Cu exceeded the UK limit decreased the cumulative effect to −0.8% ($CL_{95\%}$ = −3.5 to −2.0%; $p = 0.570$), indicating that the Cu sludge treatment has had no overall effect on *Rhizobium* MPN at concentrations below the UK limit (data not shown). No statistically significant effects were observed for any of the individual sampling events below the UK limit (for soils with pH 6–7) of 135 mg kg^{-1}, nor were any significant changes observed with time (Fig. 5c). A marked decrease in *Rhizobium* MPN was seen in 1999 (−17.0%; $CL_{95\%}$ = −22.8 to −10.7%; $p < 0.001$), when total Cu ranged between 170 and <200 mg kg^{-1}. However, in this instance the subgroup was comprised of only $k = 2$ studies and consisted of data for sandy clay loam (Hartwood) and clay loam (Pwllpeiran) soils, for which the effect of the Cu sludge treatment was greatest (Fig. 2b and 5c). Similarly, above 200 mg kg^{-1}, a decrease of −24.3% ($CL_{95\%}$ = −33.5 to −13.9%; $p < 0.001$) was observed for the 2003 sampling event; again, the subgroup was predominantly comprised of data from sandy clay loam and clay loam soils. Even so, although the effect of the Cu sludge treatment was much lower than that of the Zn sludge treatment, the effects for soils where total Cu ranged from 135 to <170 mg kg^{-1} (−10.5%; $CL_{95\%}$ = −14.6 to −6.2%; $p < 0.001$) and 170 to <200 mg kg^{-1} (−7.1%; $CL_{95\%}$ = −12.9 to −0.9%; $p < 0.05$) do indicate that, in some cases, *Rhizobium* MPN had still not fully recovered by 2005 (Fig. 5c).

Cadmium

The overall effect of the Cd sludge treatment for the 1999 and 2001 sampling events was approximately −4% in each case (Fig. 6a), neither of which was statistically significant. Nevertheless, the overall effect of the Cd sludge treatment was negative for the duration of the LTSE (Fig. 6a and 6b). Significant effects were observed in 2003 (−10.9%; $CL_{95\%}$ = −14.5 to −7.1; $p < 0.001$) and 2005 (−7.9%; $CL_{95\%}$ = −11.0 to −4.7; $p < 0.001$), although in both cases the 95% confidence limits overlapped with those for 1999 and 2001. Little change was seen in the cumulative effect with time (Fig. 6b); however, excluding data where the total Cd exceeded the UK limit caused an increase in the overall effect for 2005, from −4.6% ($CL_{95\%}$ = −7.2 to −1.8%; $p < 0.01$) to −6.0% ($CL_{95\%}$ = −9.4 to −2.5%; $p < 0.01$). However, below 1.5 mg Cd kg^{-1}, little change in effect was seen with time (Fig. 6c). A positive effect of 6.0% ($CL_{95\%}$ = −12.7 to 28.7%; $p = 0.557$) was seen in 2001, although in this case, two of the $k = 3$ studies are from the silty clay loam soils in which the effect of the Cd sludge treatment was least (Fig. 2c).

Discussion

A statistical review of the LTSE experimental data has previously been commissioned by the UK Department for Environment, Food and Rural Affairs. Repeated measure ANOVA and multiple regression were used to combine experimental data for each of the LTSE field sites (with the exception of Shirburn) into a single analysis (Department for Environment, Food and Rural Affairs, 2008). In this case, a significant experimental confound was identified that affected data interpretation. The sludge used to produce the Zn experimental treatment also contained significantly higher concentrations of Cu and Cd than the uncontaminated control, whereas the Cd sludge treatment also contained significantly higher concentrations of Zn (Table 4; Department for Environment, Food and Rural Affairs, 2008).

Given that the Cu sludge treatment was least affected by confounding metal contamination, the effect of Cu on *Rhizobium* MPN was determined first and then used to "adjust" the data by adding $-m \times \ln([Cu])$ to the *Rhizobium* MPN observed in soils receiving the Zn and Cd sludge treatments, where m is the slope of the regression between ln([Cu]) and *Rhizobium* MPN and [Cu] is the total concentration of Cu in a given plot (Department for Environment, Food and Rural Affairs, 2008). For the Cu sludge treatment, the Department for Environment, Food and Rural Affairs (2008) observed a −13% decrease in *Rhizobium* MPN as total Cu concentrations increased from background levels to the UK statutory limit. This is in agreement with the results shown in Fig. 3b, where mean total Cu ranges from <170 to 200 mg kg^{-1}; however, the overall effect for the soils where total Cu was closest to the statutory limit were less than half this value. As part of investigations into the effects of Zn and Cu on *Rhizobium leguminosarum* biovar *trifolii* (sampled at an additional long-term sludge experiment at the ADAS Research Centre in Gleadthorpe, Nottinghamshire, UK), Chaudri et al. (2000) reported a lowest observed effect concentration (LOEC) for Cu (i.e., the concentration at which the observed difference between *Rhizobium* MPN in contaminated and uncontaminated soil is >1 $\log_{10}$ unit) of ≥250 mg kg^{-1}. Soils received an application (100 t ha^{-1}) of contaminated sludge in 1982, followed by an additional application in 1986, giving an overall metal loading of 2077 kg Cu ha^{-1}, and were sampled 10 yr after the final sludge application was made. In this case, *Rhizobium* MPN had decreased to approximately 3.5 $\log_{10}$ cells g^{-1} soil in soils where total Cu remained at 254 mg kg^{-1}, in comparison to soil receiving uncontaminated sludge where the *Rhizobium* MPN was approximately 4.5 $\log_{10}$ cells g^{-1} soil. Calculating the log response ratio for this difference ($\ln(R) = \ln(3.5) - \ln(4.5)$; Eq. [2]) corresponds to a difference of approximately −22% (Eq. [15]), which is twice as much as any of the effect sizes observed for the Cu sludge treatment during the current investigation. However, during the course of the LTSE, total Cu was found to be >250 mg kg^{-1} in only four cases: at Hartwood and Shirburn in 1999 (Dose–Response Levels 4–5) and at Auchincruive in 2003 (Level 4). The effect at Hartwood (−19.3%) agreed more with previous findings but occurred at a lower concentration of total Cu (206.5 mg kg^{-1}).

Chaudri et al. (2008) also reviewed the findings of the LTSE, reporting percentage reductions in *Rhizobium* MPN, determined

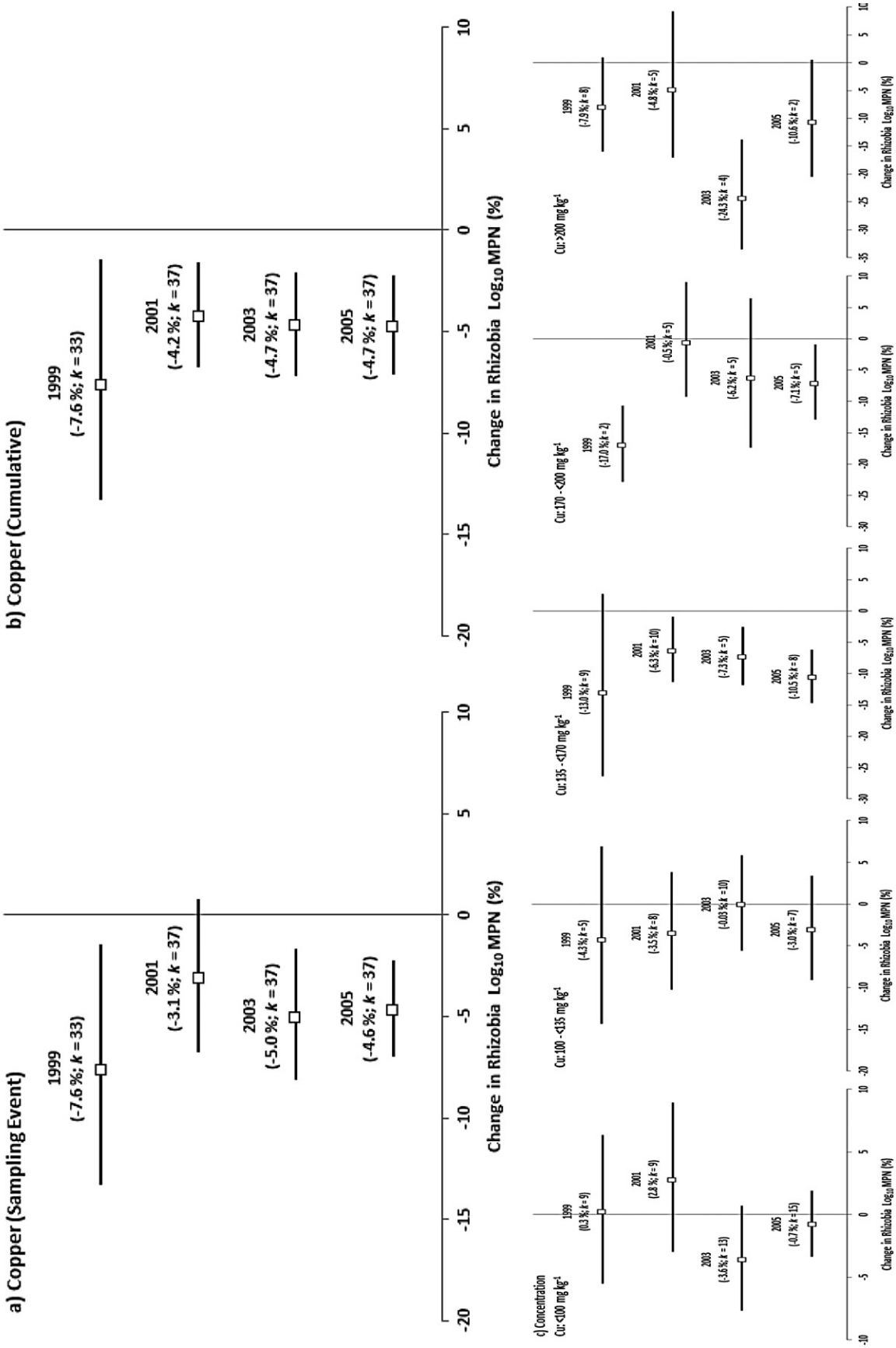

Fig. 5. Forest plots showing the change in effect of the Cu sludge treatment on *Rhizobium* most probable number of cells (MPN) with time: (a) after each sampling event, (b) cumulative effect with time, and (c) the change in effect with time and increasing concentrations of total Cu. Each point (□) represents the summary effect size for specified sampling events, with the exception of the cumulative effect, which represents the mean effect over consecutive years. The number of primary studies (*k*) used to calculate each effect size is indicated. Horizontal lines represent 95% confidence limits. Effects are not statistically significant if 95% confidence limit crosses the zero line.

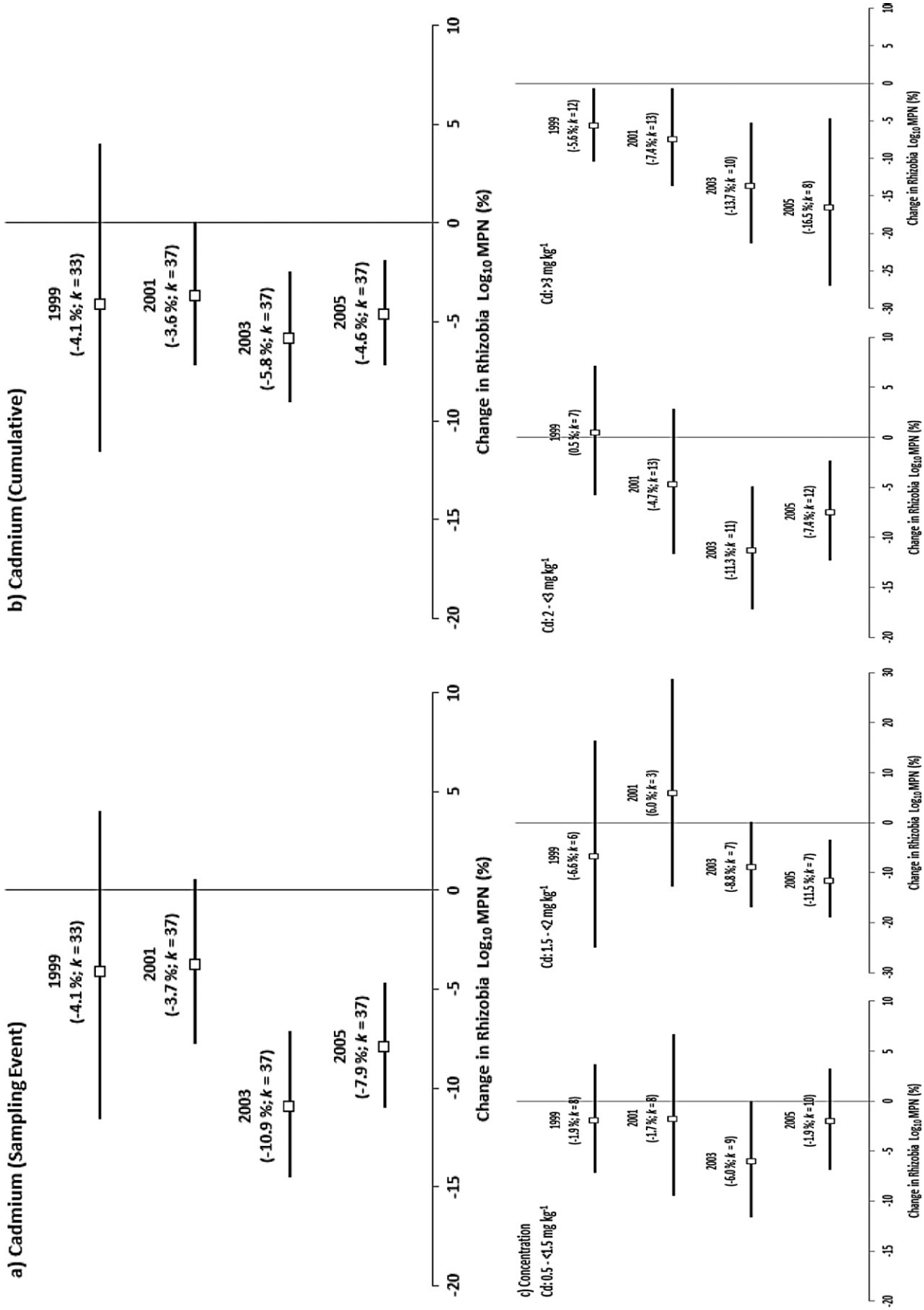

Fig. 6. Forest plots showing the change in effect of the Cd sludge treatment on *Rhizobium* most probable number of cells (MPN) with time: (a) after each sampling event, (b) cumulative effect with time, and (c) the change in effect with time and increasing concentrations of total Cd. Each point (□) represents the summary effect size for specified sampling events, with the exception of the cumulative effect, which represents the mean effect over consecutive years. The number of primary studies (*k*) used to calculate each effect size is indicated. Horizontal lines represent 95% confidence limits. Effects are not statistically significant if 95% confidence limit crosses the zero line.

by multiple regression, for soils where the total concentrations of Zn, Cu, and Cd were at the respective UK statutory limits. In soils receiving the Cu sludge treatment, reductions in *Rhizobium* MPN ranged from −5% (Hartwood and Rosemaund in 1999; Bridgets in 2005) to −31% (Gleadthorpe in 1999), although no consistent trends were seen with time. Combining the data using meta-analysis also revealed no significant change in *Rhizobium* MPN with time. Several significant relationships between *Rhizobium* MPN and total Cu have been found previously, particularly in 1999 (Table 6), although in the present study significant effects were not observed until total Cu increased above 135 mg kg^{-1}. Hence with regard to protecting *Rhizobium* populations, the current UK statutory limit for Cu appears to be sufficient.

Adjusting for the concentration of Cu in soils receiving the Zn sludge treatment, the Department for Environment, Food and Rural Affairs (2008) observed a decrease in *Rhizobium* MPN of −24% as total Zn increased from background concentrations to the UK statutory limit, in agreement with the overall effect determined for the Zn sludge treatment. Taking the confounding Cu contamination into account, no significant interaction was found between the effects of Zn and Cu, suggesting that there was no additive effect between the two metals in soils receiving the Zn sludge treatment (Department for Environment, Food and Rural Affairs, 2008). Similarly, Chaudri et al. (2000) did not observe an additive effect of Zn and Cu in soils receiving a combined sludge treatment contaminated with both Zn and Cu. In this case, significant reductions in *Rhizobium* MPN (>1 $\log_{10}$ unit), comparable to soils with high concentrations of total Zn (291–441 mg kg^{-1}), were seen in soils where Zn and Cu were present at concentrations of 230 to 328 and 159 to 223 mg kg^{-1}, respectively. However, the observed effect on *Rhizobium* MPN was greatly reduced in soils receiving a Cu sludge treatment where only Cu was present at concentrations of 160 to 254 mg kg^{-1} (Chaudri et al., 2000). Therefore, in the current situation, if it is assumed that Zn and Cu do not have an additive effect, then clearly Zn is having a significant effect on *Rhizobium* MPN at concentrations below both the UK statutory and advisory limits.

The temporal change in the effect of the Zn sludge treatment was also discussed by Chaudri et al. (2008). *Rhizobium* MPN were ≥1 $\log_{10}$ unit lower in soils receiving the Zn sludge treatment at Dose–Response Level 3 at eight of the nine LTSE field sites in 2005, with the exception of the Shirburn site, where no significant decreases were observed (Table 6). With the exception of 2005, the mean percentage reductions (1999 = −24.5% [n = 2]; 2001 = 33.7% [n = 6]; 2003 = 37.2% [n = 6]; 2005 = 44.3% [n = 8]) calculated by Chaudri et al. (2008), where total Zn was set equal to 300 mg kg^{-1}, were in agreement with the overall effects calculated here, for each sampling event, where total Zn ranged from 300 to <350 mg kg^{-1} (Fig. 4c). The range of calculated percentage decreases also increased with time, corresponding to the observed increases in confidence limits, indicating that the change in the effect of the Zn sludge treatment was not uniform at all sites. Chaudri et al. (2008) concluded that no clear threshold for the effect of Zn could be determined and that the observed decrease in *Rhizobium* is mostly proportional to the total concentration of Zn present in the soil. The results presented here support this conclusion, as the effect of the Zn sludge treatment was negative at all levels of the dose–response curve. In addition, Chaudri et al. (2000) and Broos et al. (2005) reported LOEC values for Zn in sludge-amended soils of 90 and 233 mg kg^{-1}, respectively, which are also consistent with the results presented here. Evidently the current UK statutory limit is not sufficient to prevent a decline in *Rhizobium* populations in sludge-amended soils. However, the results presented here indicate that the proposed advisory limit may also not be sufficient. Hence, it would be more practical to adopt a statutory limit for Zn based on a "critical" percentage reduction of *Rhizobium*, suggested by Chaudri et al. (2008), below which N_2 fixation is known to become impaired.

The highest concentration of Cd measured during the LTSE was 4.7 mg kg^{-1} at the Hartwood field site in 2001, where the soil received the Cd sludge treatment at Dose–Response Level 4 (Supplemental material). Hence all the concentrations of Cd considered here fall within the range of 0.6 to 17.6 mg kg^{-1}, across which Broos et al. (2005) saw no effect of Cd on *Rhizobium* in sludge-amended soils; nor was any significant effect observed in soils spiked with Cd salts, with total Cd concentrations of 31.5 and 35.4 mg kg^{-1}. Similarly, Renella et al. (2007) observed no effect of Cd in a grassland soil amended with spiked sewage sludge where total Cd was four times (12 mg kg^{-1}) the UK limit. A significant ($p < 0.05$) decrease in *Rhizobium* was observed in an arable soil receiving the same sludge treatment; however, the reduction was lower than in a soil amended with Zn-spiked sludge at the UK limit. Therefore if Cd does not appear to have an effect on *Rhizobium* at concentrations below the UK limit, then it is possible that the effect of the Cd sludge treatment observed during the LTSE is due to the confounding Zn contamination. Chaudri et al. (2008) observed no consistent trends with time in LTSE soils receiving the Cd sludge treatment, although the number of significant linear relationships between *Rhizobium* MPN and total Cd increased from $n = 0$ in 1999 to $n = 4$ in 2005, suggesting an increase in the effect with time (Table 6). However, the relationships seen at Gleadthorpe (in 2001), Rosemaund (in 2003), Shirburn (in 2003), and Pwllpeiran (in 2005) were also significant when regressed against total Zn (Chaudri et al., 2008). In addition, total Zn and total Cd were found to be significantly collinear at a number of sites (e.g., R^2 = 0.61–0.98 at Gleadthorpe, Hartwood, and Pwllpeiran in 2005). Therefore, it was not possible to fully determine which metal was having an effect, although it was presumed that the observed decreases in *Rhizobium* MPN were due to the presence of Zn (Chaudri et al., 2008).

The apparently anomalous change in the cumulative effect that was observed when the data for soils with a concentration of Cd exceeding the UK limit were removed from the meta-analysis could also be explained by the presence of Zn because little change in the range of mean Zn concentrations was observed (85.0–213.0 to 85.0–173.4 mg kg^{-1}) when the data were excluded. A comparable effect was also observed for soils where the mean total Cd ranged from 1.5 to <2 mg kg^{-1}, in which the mean total Zn also ranged from 100 to 173.4 mg kg^{-1} (Fig. 3c). Hence, during the course of the LTSE, Zn has been present in soils receiving the Cd sludge treatment at concentrations capable of producing an observable effect (Fig. 3a). Adjusting for the concentration of Cu in the Cd sludge treatment, the Department for Environment, Food and Rural Affairs (2008) observed a 15% decrease in *Rhizobium* MPN as concentrations of total Cd increased from background levels to the UK statutory limit. This is in agreement with the overall effect determined in 2005

for soils where total Cd was >3 mg kg^{-1}, ranging from 3.1 to 4.2 mg kg^{-1} (Supplemental Table S1). However, in this case total Zn also ranged from 113.3 to 192.1 mg kg^{-1} (with one outlier above 200 mg kg^{-1} at Shirburn; Supplemental Table S1). Hence these soils, predominantly receiving the Cd sludge treatment at Dose–Response Level 4, are largely comparable to soils receiving the Zn sludge treatment at Dose–Response Level 1. This level of Zn concentration is also capable of producing a 15% decrease in *Rhizobium* MPN. Below the UK statutory limits, the overall cumulative effect size observed for the Zn sludge treatment was approximately 3.5 times greater than that of the Cd treatment and approximately 20 times greater than that of the Cu sludge treatment (see above). In agreement with both Broos et al. (2005) and Renella et al. (2007), this would suggest that the order of metal toxicity is Zn > Cd > Cu. Given that Cd does not appear to have a significant effect on *Rhizobium* below 4 mg kg^{-1}, then the observed decreases in *Rhizobium* MPN in soils receiving the Cd sludge treatment could be caused by Zn. If this is so, then it gives further indication that an advisory limit of 200 mg Zn kg^{-1} may not be sufficient. Of the three heavy metals applied during the LTSE, clearly Zn has had the greatest impact on the *Rhizobium* populations of the receiving soils.

The approach to mitigating heavy metal accumulation in sludge-amended soils varies among different countries and largely depends on soil usage priorities and the extent of environmental impact tolerated (McGrath et al., 1994). The USEPA has performed an environmental risk assessment and analysis of exposure pathways whereby metal toxicity may occur in plants, animals, and humans. A number of potential exposure routes were considered by the USEPA, resulting in some of the highest metal loadings permitted with regard to the application of sewage sludge to agricultural land (Table 8); notably the only consideration of soil biota was the potential bioaccumulation of heavy metals in earthworms (McBride, 1995; McGrath et al., 1994). The majority of EU member states do permit a certain degree of metal accumulation, with the statutory limits set by the UK Sludge (Use In Agriculture) Regulations being among if not the highest in Europe (Table 8). In this case, the approach is to adopt statutory limits below the minimum concentrations of heavy metals known to have an adverse effect on soil microorganisms (McGrath et al., 1994). The most cautious method is the "metal-balance" approach adopted by countries such as Sweden, Denmark, and the Netherlands. This approach recognizes the extreme persistence of heavy metals and tries to match the rate of sludge application with that of loss processes to prevent a net accumulation of heavy metals. As a result of this, the statutory limits significantly restrict the recycling of sewage sludge to agricultural land in those countries (Table 8). Although there is little evidence to support the need for such conservative limits, Witter (1996) suggested that this approach provides more of an incentive to reduce metal emissions to wastewater, thus improving the overall quality of sewage sludge. This is clearly a desirable situation and an obvious solution to the problem of heavy metals accumulating in agricultural land due to the application of sewage sludge. The contaminated sludge treatments applied during the LTSE are clearly exceptional cases and, outside of the UK, their use in agriculture would not be permitted within any of the EU countries mentioned in Table 8, although application of the Zn sludge treatment would be permitted in the United States.

Conclusions

The total metal loadings applied during the course of the LTSE represent a case of acute exposure to heavy metal contamination, rather than a chronic accumulation, which may influence both the short-term and long-term response of soil microorganisms. However, the results presented here clearly show a decline in *Rhizobium* MPN due to the historical application of sewage sludge contaminated with Zn. Results further indicate that sludge-borne Zn has an observable effect on *Rhizobium* at concentrations below both the UK statutory limit and the UK advisory limit. Therefore a reduction in the UK statutory limit may be necessary to prevent the loss of *Rhizobium* from agricultural soils. No consistent trend

Table 8. Maximum permissible concentrations for heavy metals in sewage sludge and sludge-amended soils adopted by EU member states and the United States (adapted from Ministry of Agriculture, Fisheries and Food, 1993b; McGrath et al., 1994; Smith, 1996).

Metal	Total metal concentration										
	France	Germany	Italy	Spain	the Netherlands	UK†	Denmark	Finland	Norway	Sweden	USA
	mg kg^{-1}										
	Sludge										
Cd	20	10	20	20	1.25	–	1.2	1.5	4	2	5
Cu	1000	800	1000	1000	75	–	1000	600	1000	600	4300
Pb	800	900	750	750	100	–	120	100	100	100	840
Hg	10	8	10	16	0.75	–	1.2	1.0	5	2.5	57
Ni	200	200	300	300	30	–	45	100	80	50	420
Zn	3000	2500	2500	2500	300	–	4000	1500	1500	800	7500
	Soil‡										
Cd	2	1.5	3	1	0.8	3	0.5	0.5	1	0.5	20
Cu	100	60	100	50	36	135	40	100	50	40	750
Pb	100	100	100	50	85	300	40	60	50	40	150
Hg	1	1	1	1	0.3	1	0.5	0.2	1	0.5	8
Ni	50	50	50	30	35	75	15	60	30	15	210
Zn	300	200	300	150	140	300	100	150	150	100	1400

† The UK Sludge (Use in Agriculture) Regulations do not set maximum limits for heavy metals in sewage sludge. Instead, metal loadings (kg ha^{-1} yr^{-1}) are to be kept below a specified annual average over a 10-yr period (Table 1).

‡ Concentration in soils with pH 6 to 7.

in the effects of Cu and Cd were observed in the present study, nor have they been observed previously when considering each of the nine field sites separately. Hence, with regard to protecting *Rhizobium*, there does not appear to be anything present within the LTSE data set that would give cause for changing the current UK limit for Cu or Cd. The implication of this work is that continued evaluation of the long-term impact of sludge-borne heavy metals is necessary to prevent the occurrence of adverse effects that may have previously been overlooked.

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Long-term Impact of sewage sludge application on rhizobium leguminosarum biovar trifolii: an evaluation using meta-analysis

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