Biol Fertil Soils (2007) 43:751–758
DOI 10.1007/s00374-006-0159-5

ORIGINAL PAPER

Effects of Cd, Zn, or both on soil microbial biomass and activity in a clay loam soil

Giancarlo Renella · Amar M. Chaudri · Céline M. Falloon · Loretta Landi · Paolo Nannipieri · Philip C. Brookes

Received: 5 February 2004 / Revised: 20 September 2006 / Accepted: 10 October 2006 / Published online: 2 December 2006

Abstract We investigated Cd, Zn, and Cd+Zn toxicity to soil microbial biomass and activity, and indigenous *Rhizobium leguminosarum* biovar *trifolii*, in two near neutral pH clay loam soils, under long-term arable and grassland management, in a 6-month laboratory incubation, with a view to determining the causative metal. Both soils were amended with Cd- or Zn-enriched sewage sludge, to produce soils with total Cd concentrations at four times (12 mg Cd g^{-1} soil), and total Zn concentrations (300 mg Zn kg^{-1} soil) at the EU upper permitted limit. The additive effects of Cd plus Zn at these soil concentrations were also investigated. There were no significant differences in microbial biomass C (B_C), biomass ninhydrin N (B_N), ATP, or microbial respiration between the different treatments. Microbial metabolic quotient (defined as qCO_2=units of CO_2–C evolved $unit^{-1}$ biomass C $unit^{-1}$ time) also did not differ significantly between treatments. However, the microbial maintenance energy (in this study defined as qCO_2-to-μ ratio value, where μ is the growth rate) indicated that more energy was required for microbial synthesis in metal-rich sludge-treated soils (especially Zn) than in control sludge-treated soils. Indigenous *R.leguminosarum* bv. *trifolii* numbers were not significantly different between untreated and sludge-treated grassland soils after 24 weeks regardless of metal or metal concentrations. However, rhizobial numbers in the arable soils treated with metal-contaminated sludges decreased significantly ($P<0.05$) compared to the untreated control and uncontaminated sludge-treated soils after 24 weeks. The order of decreasing toxicity to rhizobia in the arable soils was Zn>Cd>Cd+Zn.

Keywords Cadmium · Zinc · Toxicity · Microbial biomass · Activity · Rhizobia

G. Renella · L. Landi · P. Nannipieri
Department of Soil Science and Plant Nutrition,
University of Florence,
P.le delle Cascine,
28-50144 Florence, Italy

A. M. Chaudri (✉) · C. M. Falloon · P. C. Brookes
Agriculture and Environment Division, Rothamsted Research,
Harpenden,
Herts. AL5 2JQ, UK
e-mail: amar.chaudri@bbsrc.ac.uk

Introduction

There is a continuing debate whether Cd, Zn, or both can be responsible for the toxic effects on soil microbial biomass and activity and indigenous *Rhizobium leguminosarum* biovar *trifolii* in agricultural soils. This stems from previous work reported from the Woburn Market Garden Experiment in Southeast England (McGrath and Chaudri 1999; Smith and Giller 1992; Smith 2000), where field experiments were established for about 20 years to investigate the effectiveness of sewage sludges as soil conditioners and organic fertilizers for vegetable crops. The added sludges contained elevated concentrations of several metals, e.g., Cu, Ni, Cd, and Zn. Now, more than 30 years after sludge applications ceased, soils from the sludged plots still contain up to about four times more total Cd (about 12 mg Cd kg^{-1} soil) and maximum Zn concentrations at just above the upper EU limit of 300 mg Zn kg^{-1} soil (CEC 1986). The metal-contaminated soils also contained about half of the microbial biomass and had higher metabolic quotients than the non-contaminated soils (Brookes and McGrath 1984). Also, populations of indigenous *R. leguminosarum* bv. *trifolii* were ineffective at N_2-fixation (McGrath et al. 1988) in the previously sludged soils. They

attributed these adverse effects to the combined toxicity of the heavy metals in these soils, whereas other workers suggested that the observed toxic effects in sludged soil were primarily due to the high concentrations of Cd, which was the only metal significantly above the current EU limits (Smith 1996). However, from field experiments using different metal-enriched sludges, Chaudri et al. (1993, 2000) suggested that Zn at or below the current upper EU limit could decrease soil microbial biomass and activity.

To clarify the situation, we studied the specific effects of Zn and Cd, both singly and in combination, on soil microbial biomass and respiration and on the survival of indigenous *R. leguminosarum* bv. *trifolii* (white clover rhizobia) at concentrations of these two metals similar to those found in the Woburn Market Garden Experiment soils. *R. leguminosarum* bv. *trifolii* was studied as it is an agronomically important soil microorganism and because it is sensitive to Cd (Smith 1996) and Zn (Chaudri et al. 1993, 2000) pollution. We used a different soil type under long-term arable and grassland management to test the toxicity of Cd and Zn to a wider soil base rather than focus exclusively on the Woburn soil. The experiment was carried out until the labile sludge C was mineralized and the effects of metals predominated, so that the effects of the metals on microbial parameters could not be confounded by microbial activity supported by freshly added and readily available C in the sludge.

Materials and methods

Sewage sludge characteristics and pretreatment

An aerobically digested sewage sludge cake, containing low heavy-metal concentrations, was obtained from the Banbury Water (Banbury, UK). The sludge contained 36% organic C, 6% total N, and total heavy-metal mean concentrations of (in milligram per kilogram oven dry weight): 2.62 Cd, 49 Cr, 588 Cu, 81 Pb, and 555 Zn.

For spiking the sludge with metal solutions, 500 g portions of the sludge cake were enriched with 100 ml sulfate solutions of either Cd, Zn, or Cd+Zn, thoroughly mixed, and incubated moist for 7 days at room temperature. Then the spiked sludges were slowly air-dried at room temperature in a fume cupboard to almost complete dryness before remoistening with deionized water and remixing and drying in the same way. The unspiked sludge was remoistened with the same volume of deionized water and was mixed and dried similarly. Finally, all sludge samples were slowly oven-dried at 20°C temperature increments up to 80°C and then completely dried at this temperature. Dried sludges were then milled to <1 mm diameter particles in an agate ball mill.

Soil preparation and incubation

Two silty clay loam soils (Aquic Paleudalf, USDA classification 1992), containing 27% clay and a pH in H_2O of 5.8, were sampled in May 1998 from the Highfield Long-Term Ley Arable Experiment at Rothamsted in Southeast England. These soils have been under continuous wheat or permanent grass ley for more than 50 years. The arable soil contained 1.58% organic C and 0.16% total N, and the grassland soil contained 3.39% C and 0.38% N. Both soils were sampled to depths of 0–23 and 0–10 cm, respectively, using a 5-cm-diameter Dutch auger. For each soil, the collected soil cores were bulked and mixed separately in the field before being sieved (<2 mm) in the laboratory, and all visible plant and root residues and soil fauna were discarded. After sieving, the soils were moistened to 50% water holding capacity and preincubated at 25°C for 10 days.

The total aqua regia extractable metal concentrations of the unamended arable soil were (in milligram per kilogram oven dry weight): 80 Zn, 25 Cu, 29 Ni, 40 Pb, 41 Cr, and 0.51 Cd, and those of the grassland soil were: 84 Zn, 17 Cu, 26 Ni, 44 Pb, 34 Cr, and 0.51 Cd.

The different sludges were carefully mixed into the soils by hand until it was not possible to distinguish the sludge from the soil particles, to give a final addition rate equivalent to 40 t sludge dry solids ha^{-1}, and added soil metal concentrations of (in milligram per kilogram oven dry weight): 12 Cd (4× EU limit), 300 Zn (EU limit), or 12 Cd+300 Zn. The unamended soils were mixed similarly.

All soils were incubated at 25°C in the dark in 1 l brown jars with separate vials containing deionized water and 20 ml 1 M NaOH for 7, 28, 70, 140, and 180 days in triplicate. The vials containing NaOH and water were replaced weekly. Initial biomass measurements (time 0) were made at the time the incubation commenced.

Chemical and biological analysis

At each incubation time, samples of each soil and treatment were divided into subsamples for the different analyses. Available Cd and Zn were estimated by extraction with 1 M NH_4NO_3 (Deutsch Institut für Normung 1997). Cumulative CO_2-evolution was determined by autotitration of aliquots of 1 M NaOH (Tinsley et al. 1951). Microbial biomass C was determined by the fumigation–extraction method (Vance et al. 1987; Wu et al. 1990) and microbial biomass ninhydrin N by the colorimetric ninhydrin-reactive N method (Amato and Ladd 1988; Joergensen and Brookes 1990). Soil ATP concentrations were estimated using the phosphoric acid method (Webster et al. 1984) as modified by Ciardi and Nannipieri (1990).

Throughout the text, the unamended soil will be referred to as the "control" soil, the soil receiving uncontaminated sludge as the "sludge-only" soil, the soil receiving both Cd and Zn as the "Cd+Zn" soil, and the soils receiving Cd and Zn separately as the "Cd-only" and "Zn-only" soil, respectively.

Enumeration of indigenous *R. leguminosarum* bv. *trifolii*

The most probable number (MPN) method was used to estimate the number of indigenous white clover rhizobia in the treated soils, using a tenfold dilution series (Vincent 1970), and *Trifolium repens*, cv. Menna, as the trap host. Briefly, moist soil (10 g oven dry basis) was added to 90 ml sterile deionized water and shaken on a rotary shaker for 30 min to initially give a 10^{-1} soil suspension. Seven further tenfold dilution steps in triplicate were carried out from the original suspension, 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 growth cabinet under conditions of a 14-h day at 20°C, 2.5×10^{3} lx, and 16°C nights. All tubes were assessed for the extent of nodulation after 3 weeks, and the MPN of rhizobia was calculated using the MPNES computer program (Woomer et al. 1990).

Microbial physiological indices

The metabolic quotient (qCO_2) was calculated as mg CO_2–C evolved g^{-1} biomass C day^{-1}. The efficiency of microbial biomass formation (E) in both soils during the first 7 days of sludge decomposition was calculated as $E\% = 100\times[(\text{Biomass C in sludged soild at 7 days}) - (\text{Biomass C in Control soils at 7 days})]/(\text{sludge C added})$. The qCO_2/μ ratio determined as the slope of the regression between the metabolic quotient (qCO_2) and the growth rate (μ) is an expression of the microbial maintenance energy (Pirt 1975). The μ value (expressed as nanomole ATP per day for all the sludge-amended soils) was calculated from the relationship: $[\ln(ATP_7) - \ln(ATP_0)]/(\Delta T)$, where ATP_0 and ATP_7 are the ATP concentrations of soils at day 0 and day 7, respectively, and ΔT is the time interval in days. The ATP concentration of the sludge itself was not significantly different from zero (data not shown).

Statistical analysis

All results are means of three replicates. The significance of differences was calculated from the Tukey–Kramer test ($P<0.05$ level). A significant decrease in rhizobial numbers was defined as one order of magnitude difference (i.e., one $\log_{10}$ unit) or more compared to control treatments and between treatments. A one-way analysis of variance (ANOVA) was also carried out for both the grassland and arable soils after $\log_{10}$ transformation of the rhizobial data.

Results and discussion

Cadmium and zinc availability

In soils mixed with metal-spiked sludges, NH_4NO_3-exchangeable Cd and Zn increased during incubation in both the arable and grassland soils, being greater in (Cd+Zn) soils (Fig. 1). Exchangeable Cd ranged from 0.6 to 1.2 mg kg^{-1} soil in the control arable soil and from 0.06 to 0.2 mg kg^{-1} soil in the control grassland soil, accounting for 5 to 10% and 0.5 to 2.5% of the total Cd, respectively (Fig. 1). In both the control soil and the sludge-only soil, extractable Cd and Zn concentrations were very small (0.02 and 0.8 mg kg^{-1}, respectively) throughout the incubation period (data not given). Soil exchangeable Zn ranged from 10 to 30 mg kg^{-1} soil in the arable soil and from 3 to 15 mg kg^{-1} soil in the grassland soil, representing 3 to 8% and 0.8 to 4% of the total Zn concentrations, respectively (Fig. 1). In (Cd+Zn) soils, Cd extractability increased in the presence of Zn in both the arable and grassland soils, whereas Zn concentrations in the presence of Cd were similar in both soils. The increasing Cd and Zn mobility during incubation was probably because of the release of these metals due to mineralization of the organic matter with which they were complexed.

Soil respiration and sludge mineralization

The basal respiration rate of the control grassland soil was about fivefold faster than that of the control arable soil (Table 1, Fig. 2). The mineralization of the sludges proceeded differently in the arable and grassland soils (Fig. 2). The phase dominated by the rapid mineralization of C derived from the sludge ended within 20 days in the grassland soil and 30 days in the arable soil. After this phase, the soil concentrations of mineralized sludge C ranged from 1% (Cd+Zn soils) to 4% (sludge-only soil) in the grassland soil and from 13% (Zn soil) to 16% (unamended sludge) in the arable soil (Table 1).

The metals added with the sludges did not cause any significant changes in the cumulative evolution of CO_2–C between unamended and metal-amended sludges after 180 days of incubation (Fig. 2). The cumulative respiration of the sludge-amended grassland soils was lower than that of the unamended soil. In addition, even if the grassland soil amended with no metal sludge had a higher cumulative respiration than soils receiving metal-spiked sludges, no significant differences were observed due to metal enrichment in this soil (Fig. 2).

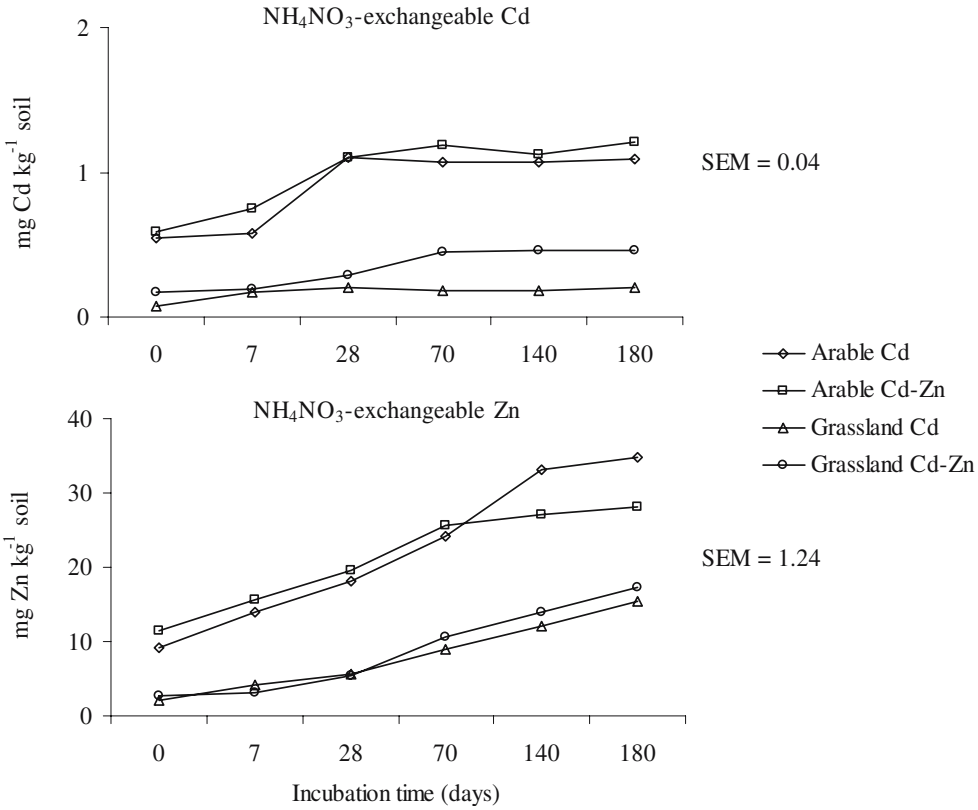

Fig. 1 Chemical extractability of Cd and Zn in the arable and grassland soils amended with Cd-, Zn-, and Cd+Zn-enriched sludge. *SEM*s indicate the pooled standard errors of the mean (n=3)

Soil microbial biomass

Sludge addition increased microbial biomass in both the grassland and arable soils whether estimated by ATP, biomass-C, or biomass-N concentrations (Fig. 3). In the grassland soil, the sludge addition increased the soil microbial biomass by about 20% over the control soil, whereas it increased by nearly a factor of two in the arable soil after 7 days of incubation (Fig. 3). No significant differences in biomass concentrations were observed between the metal treatments, although the availability of both Zn or Cd increased during incubation (Fig. 1). In both the arable and grassland soils, ATP and biomass-C concentrations were largest during 0 to 7 days of incubation, while biomass N reached a maximum concentration after 28 days of incubation (Fig. 3).

Significant decreases in soil microbial biomass after incorporation of Cd-contaminated sludges have been reported at soil concentrations of 50 (Dar 1996) and 100 mg Cd kg^{-1} (Zibilske and Wagner 1982), whereas small adverse effects of metal-enriched sludges on biomass-C concentrations were reported by Rost et al. (2001). The smaller biomass increase observed in the grassland than the arable soil might be due to the large initial microbial biomass concentration of the former soil, a value which could be near to maximum soil carrying capacity.

The high variability of biomass C and N observed in the first 28 days of incubation was probably caused by the extremely large concentrations of K_2SO_4-extractable organic C and total N in the sludge-treated soils. This caused high "control" values (data not shown) and increased analytical error, so that the early biomass measurements

Table 1 Net sludge carbon mineralization after 30 days incubation

Soil	Soil	Sludge	Cd	Zn	Cd+Zn
			mg CO_2–C kg^{-1} soil		
Arable	311 (42)	1,760 (88)	1,658 (107)	1,436 (106)	1,690 (99)
Grassland	1,725 (124)	2,093 (134)	1,917 (69)	1,818 (113)	1,810 (90)
			Mineralization of sludge organic C (%)		
Arable		16.5 (1.2)	15.3 (0.9)	12.8 (1.0)	15.7 (1.2)
Grassland		4.2 (0.2)	2.2 (0.1)	1.0 (0.1)	1.0 (0.2)

The "Soil" column shows the cumulative basal respiration values, whereas the other columns show cumulative respiration values induced by the addition of control or the different metal-spiked sludges. Values in parentheses are the standard errors of the means (n=3).

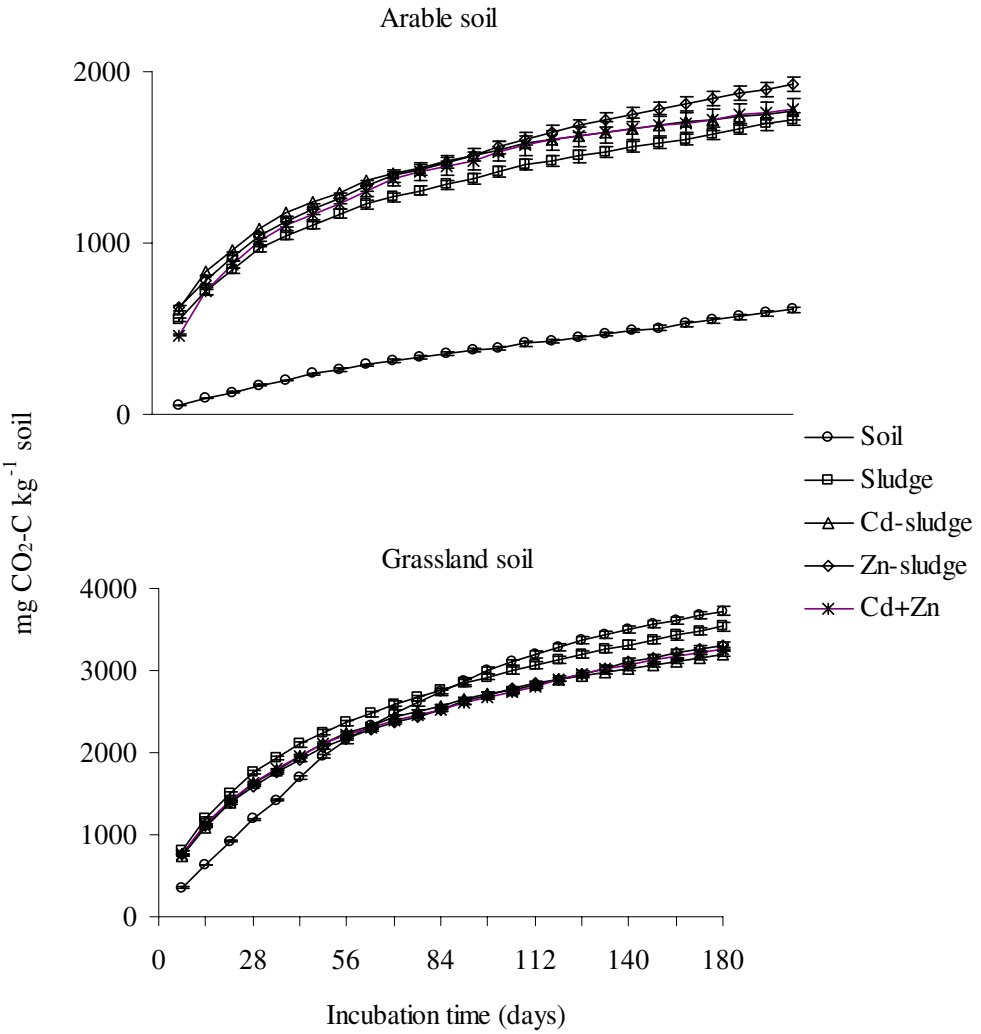

Fig. 2 Cumulative respiration rates of the arable and grassland soils. *Error bars* are standard errors of the mean (SEM) (n=3)

are unreliable. However, the effect largely disappeared after this initial period, and thereafter biomass C and N trends more closely resembled those of ATP.

Microbial physiological indices

The metabolic quotient (qCO_2) of the sludge-amended arable soils increased during the early mineralization period, reaching values up to three times higher than those of the grassland soils treated with the same sludge types (Fig. 4). However, at the end of the early decomposition phase, the qCO_2 values of the sludge-amended grassland and arable soils were similar (Fig. 4).

The efficiency of microbial biomass formation (E) during the first 7 days of sludge decomposition in the arable soil was significantly lower (P<0.05) after an amendment with Cd+Zn sludge compared to the uncontaminated sludge-treated soil but not with the Zn- or Cd-contaminated soils (Table 2). In contrast, compared to the uncontaminated sludged soil, the E values in the grassland soils were significantly smaller (P<0.05) in the Zn contaminated soil but not in the Cd or Cd+Zn soils.

The qCO_2/μ ratio values, used in this study as an expression of microbial maintenance energy of the growing microbial biomass during sludge decomposition, were significantly higher (P<0.05) in the grassland soil mixed with Zn-spiked sludge and in the arable soil mixed with Zn-spiked sludge and Cd+Zn-spiked sludge (Table 2) compared to soils mixed with uncontaminated sludge.

The metabolic quotient qCO_2, generally considered as an indicator of microbial stress, was unaffected by the presence of heavy metals. High qCO_2 values observed immediately after sludge incorporation into soils were due to the increased metabolic rate preceding the onset of microbial growth (Fig. 4). Similar results were reported by Filcheva et al. (1996). In contrast, increased qCO_2-to-μ ratios indicated that more energy was required for microbial biomass synthesis in Zn and Cd+Zn soils than sludge-only soils (Table 2). This suggests that qCO_2 is more suitable for describing the metabolic status of the microbial biomass

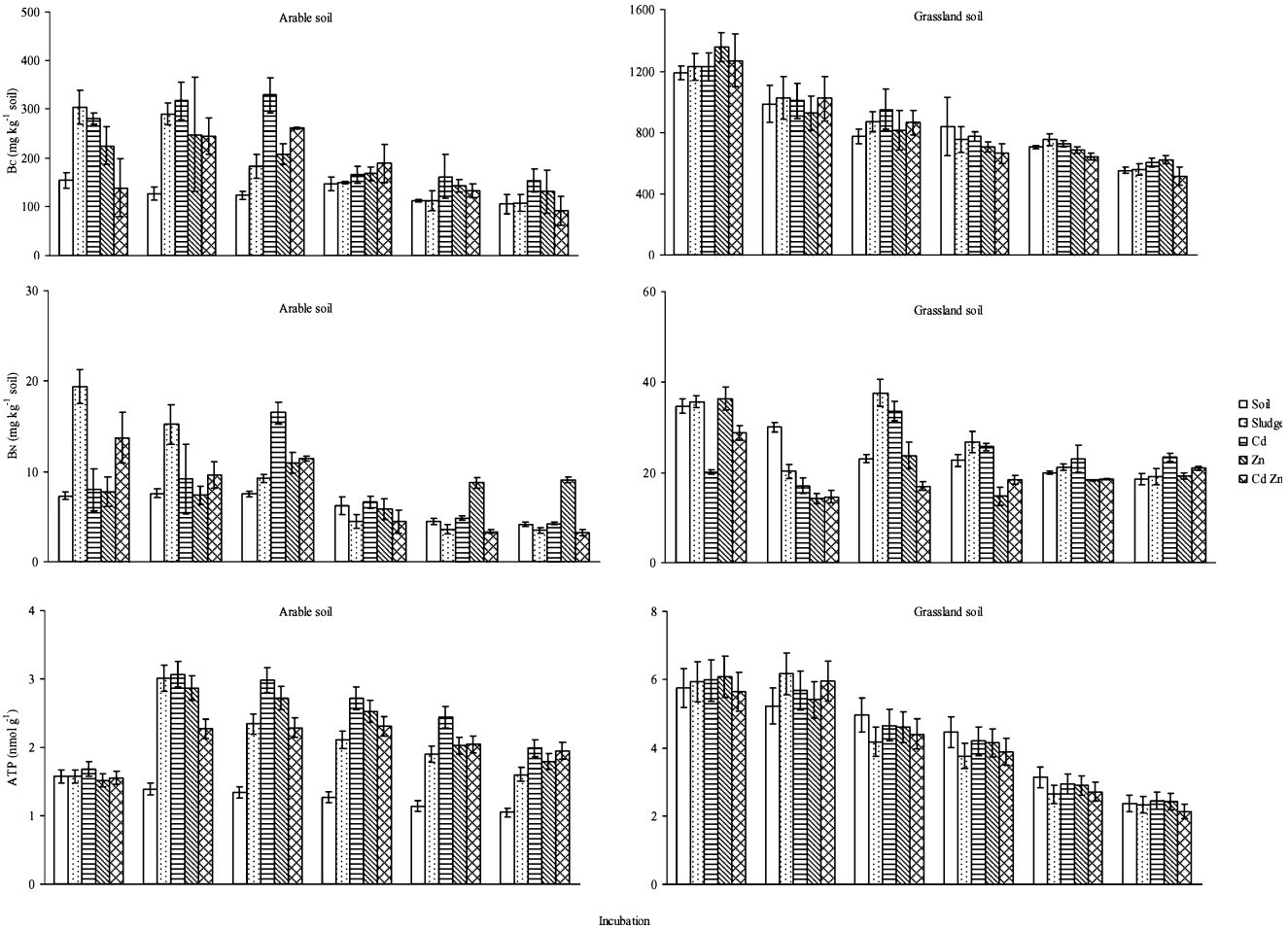

Fig. 3 Microbial biomass C and N content and ATP contents of arable and grassland soils amended with no metal- and metal-enriched sludges. *Error bars* are standard errors of the mean (n=3)

under steady-state or equilibrium conditions (i.e., no substrate recently added), whereas the qCO_2/μ ratio may better take into account the organic C respired and incorporated into the biomass during active microbial growth. Similar conclusions were drawn also by Renella et al. (2005), describing microbial growth in long-term Cd-

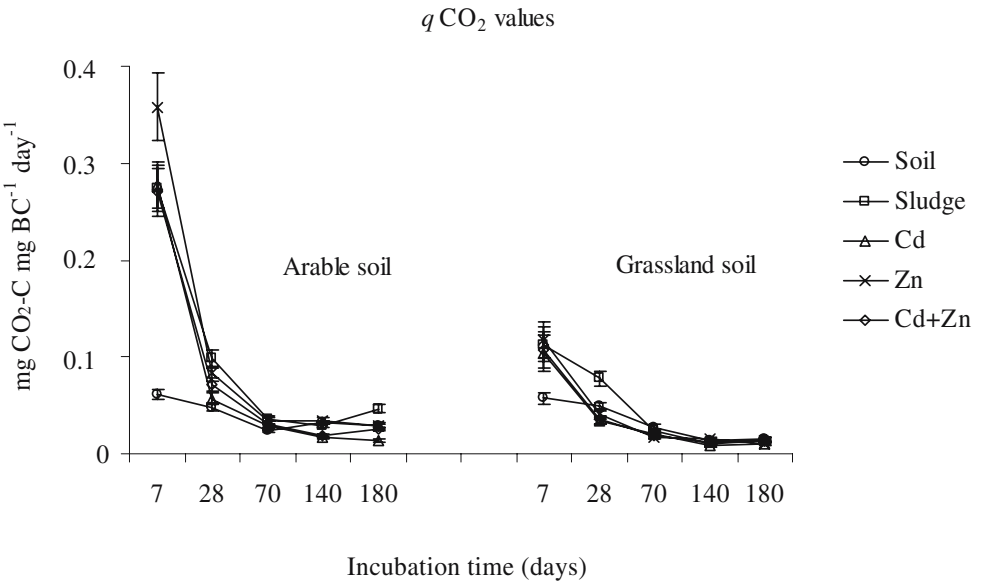

Fig. 4 Metabolic quotient of the arable and grassland soils amended with no metal- and metal-enriched sludges. *Error bars* are standard errors of the mean (n=3)

Table 2 Efficiency of the microbial biomass formation (E%) and the microbial maintenance energy (qCO_2/μ ratios) 7 days after sludge addition

	Soil			
	Arable		Grassland	
Treatment	E%	qCO_2/μ	E%	qCO_2/μ
Sludge	1.8[a]	2.8[a]	1.6[a]	9.4[a]
Cd	2.2[a]	7.4[a]	1.3[a]	21.5[a]
Zn	1.4[a]	9.8[b]	0.4[b]	56.9[b]
Cd+Zn	1.3[b]	13.0[b]	1.3[a]	14.0[a]

See text for calculations of E%, qCO_2, and growth rates (μ). Different superscript letters indicate significant differences ($P<0.05$) within the same column.

contaminated soils during plant residue decomposition. Lower efficiency of biosynthesis in heavy-metal-contaminated soils than in uncontaminated soils has also been reported by Giller et al. (1998), Chander and Brookes (1991), and Chander and Joergensen (2001). Microbial stress was not observed after the substrate-C-dominated phase ended, probably due to the start of the dormant phase as the readily mineralizable C added with sludge had been decomposed (Anderson and Domsch 1990). Nevertheless, lower respiration rates in the sludge-amended soils might be considered as an index of microbial stress if associated with changes in microbial biomass composition. For example, metal-resistant soil microorganisms generally show decreased catabolic versatility compared to non-resistant ones (Wenderoth et al. 2001).

Enumeration of indigenous *R. leguminosarum* bv. *trifolii*

Initial indigenous white clover rhizobial numbers in the grassland and arable soils were 8.0×10^4 and 2.4×10^5 cells g^{-1} oven dry soil, respectively. Rhizobial numbers did not change significantly in either control grassland or arable soils or in sludged soils over the 180-day incubation period (data not shown) (Fig. 5). Rhizobial numbers in the arable Zn, Cd, or Cd+Zn soils were significantly lower (one-way ANOVA $P<0.05$ and one $\log_{10}$ difference criteria) compared to both control and sludged soils by 180 days of incubation. The order of decreasing toxicity in the arable soils after 180 days was Zn>Cd>Cd+Zn. Differences between rhizobial numbers of the Zn-, Cd-, or Cd+Zn-treated soils were not statistically significant (Fig. 5).

Generally, grassland soils contained larger numbers of rhizobia than arable soils in all treatments, although the differences in the control soil and sludged soil were not significant (i.e., when analyzed either by one-way ANOVA or by more than one $\log_{10}$ difference). The larger soil-organic-matter concentrations in the grassland soil than in the arable soil may have provided greater protection to the free-living rhizobia by decreasing metal bioavailability and hence toxicity (Fig. 1).

In the arable soils, negative effects of Zn and Cd on rhizobial numbers occurred at soil metal concentrations similar to those previously reported by Chaudri et al. (1992) in soils spiked with Zn and Cd sulfate, which provided soil metal concentrations similar to those of the Woburn Market Garden Experiment and soils used in this study.

Conclusion

Our laboratory study suggests that both Zn and Cd at 300 and 12 mg kg^{-1} soil, respectively, had adverse effects on soil microbial activity and indigenous clover rhizobial numbers, particularly in the arable soil. Effects in the grassland soil were less pronounced, presumably due to the protective effect of the soil organic matter. However, while incorporation of Cd- and Zn-contaminated sludge into soils

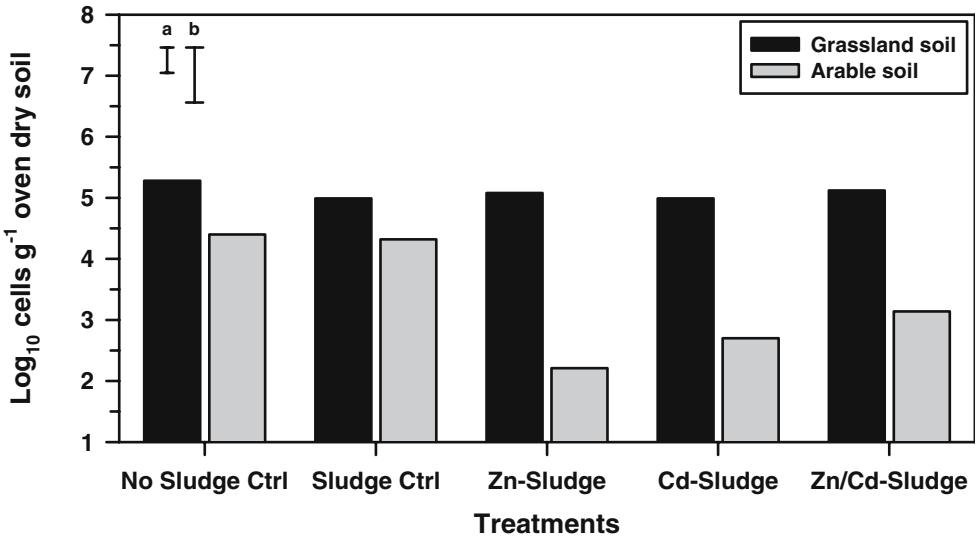

Fig. 5 Indigenous *Rhizobium leguminosarum* bv. *trifolii* in grassland and arable soils receiving no sludge (*Soil*), no metal sludge (*Sludge*), Cd, Zn, or Cd+Zn sludge after 180 days incubation. *Bars with different letters* indicate significant differences at $P<0.05$ (*a*=grassland soils and *b*=arable soils)

significantly decreased indigenous clover rhizobial numbers in some cases, it did not affect the total soil microbial biomass. The qCO_2-to-μ ratio values, expressing the maintenance energy and not the qCO_2 per se, were sensitive to the presence of metals, indicating that more energy was required for microbial biomass synthesis in soils amended with metal-contaminated sludge than in soils which received uncontaminated sludge.

However, in comparing these results with the data obtained from the Woburn Market Garden soils, it must be recognized that microbial responses measured in this study were measured after "fresh" additions of sludges and over a 6-month period, thus reflecting acute toxicity, rather than chronic toxic effects of Cd and Zn on soil microbial biomass and indigenous clover rhizobia over a 20-year period.

The current EU limit for Cd in soil is 3 mg kg^{-1}. However, sewage sludges now being applied to agricultural soils in the UK contain low Cd concentrations and therefore it is unlikely that higher soil Cd concentration, as found in soils from the Market Garden Experiment and this study, would develop elsewhere. Hence, the main metal of concern regarding its effects on soil microorganisms and their activities is currently Zn.

Acknowledgments Rothamsted Research receives grant-aided support from the Biological and Biotechnological Sciences Research Council.

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