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Microbial Responses to Cu, Ni and Zn in Metal Enriched Sewage Sludge Treated Soil

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Abstract: Three experiments were conducted on a slightly acidic, grassland clay loam amended with sewage sludge contaminated with Cu, Ni and Zn in different combinations (Cu-Ni, Ni-Zn and Cu-Zn). Metals were added to a low metal sludge at two levels, above and below current limits. The effects of these metals on microbial indices were studied over a 7-week laboratory incubation. Zn inputs had few effects on microbial indices. In contrast, Cu and Ni decreased CO_2 evolution at one week of incubation and led to an increase later. Biomass C was lower in high Cu and Ni soils whereas biomass N was lower in all three high metal treatments and there was evidence of a shift from bacterial to fungal biomass. Greater CO_2 evolution rates in the high metal soils appeared to be a response to increased stress on microorganisms. Zn seemed to be less toxic than Cu and Ni when applied at rates close to EC limits for sludge treated soil (DoE, 1999).

Key words: Metals, Sewage sludge, CO2 evolution, Biomass C, Biomass N, C:N ratio

Introduction

Sewage sludge is a complex organic material, derived mainly from human waste and left after wastewater from domestic and industrial sources has been treated (RCEP, 1996). With the increase in population and rapid development in industralization, the amount of sewage sludge is considerably increased.

In many countries, the problem associated with the disposal of sewage sludge is a significant issue. Sewage sludge produced at municipal treatment plants is disposed of in several ways. In some countries, large amounts of sewage sludge for many years have been pumped out to sea, in other countries it has been landfilled, incinerated or applied onto the land. Being a rich source of plant nutrients, particularly N and P (Smith *et al*., 1998), a substantial amount of sludge produced in many countries has been applied to the land, such as in many developing countries (e.g. Pakistan) that do not have appropriate treatment facilities (Younas *et al*., 1998). However, depending on its sources, it often contains considerable amount of metals or potentially toxic elements (PTEs).

The addition of sewage sludge to soil is one of the main causes of soil pollution by PTEs (Alloway, 1995). Some PTEs such as Zn, Cu, Ni, Co and Cr are essential or beneficial micronutrients for plant, animals and microorganisms (Alloway, 1995). However, all metals may be toxic at higher concentrations.

Adverse effects of PTEs on the soil microorganisms, resulting from the use of contaminated sewage sludge in agriculture, are a threat to soil fertility. Marked reductions in microbial biomass C and biomass N have been found in metal contaminated compared w ith uncontaminated soil (Fliessbach *et al*., 1994). A change in soil microbial diversity or a shift from bacterial to fungal population has also been reported in metal contaminated soils (Frostegard *et al*., 1993; Fliessbach *et al*., 1994; Khan and Scullion, 2000). Results obtained from studies on the influence of metals on soil respiration are somewhat inconsistent. Some authors (Hattori, 1992; Doelman and Haanstra, 1984) found a significant decrease while others (Fliessbach *et al*., 1994; Bardgett and Saggar, 1994) observed higher CO_2 evolution in metal contaminated soil.

The aim of the present study was to estimate effects of concentrations of PTEs (Ni, Cu and Zn) close to current limits (DoE, 1999) on microbial biomass and community structure in the short-term. Responses to Cu, Ni and Zn in sludge treated soils were assessed on the basis of CO_2 evolution, microbial biomass C, biomass N and biomass C:N ratio.

Materials and Methods

Standard experimental design: Three experiments were carried out using Cu, Ni and Zn at different input levels. Each experiment (Cu-Ni, Ni-Zn or Cu-Zn) involved adding two metals in sludges each at three concentrations (low, medium or high). Individual experiments included all combinations of each metal (2) and input rate (3). A relatively uncontaminated sludge was used for the low metal treatment whilst metals were added to this sludge to provide inputs which achieved total soil metal concentrations slightly below (medium) and above (high) (Table 1) the EC limits for sludge treated soils (DoE, 1999). All data were expressed as the mean of 3 replicates.

Soil and amendments: The common soil used in this study is characterised in Table 2. The total metal concentrations of the soil prior to sludge treatment were well below (Khan, 1999) the E.C. Directive Limits for sludge treated soil (DoE, 1999). The sewage sludge (undigested) used was obtained from Aberystwyth sewage works UK. This sludge was chosen because of its low general metal content and represented the low metal treatment. All sludges were dewatered and as required, metal salts (copper sulphate, nickel chloride or zinc sulphate) were added in appropriate combinations and concentrations. All metal inputs were added to sludge subsamples 1 week before mixing with soil in order to allow metal inputs to equilibrate with the sludge. Sludge was applied to the soil at the rate of 40 g (oven dry) per kg of oven dry soil. Amended soil was placed in 600 cm^3 plastic containers (200 g) or 250 cm^3 conical flasks (25 g, for CO_2 evolution measurement) and were incubated in the dark at $22\pm0.5°C$.

Table 1: Target total metal concentrations (mg kg^{-1}) (estimated from soil and sludge) of soil treated with contaminated sludge and EC limits

Metal	Cu	NI	Zn
Low	35	28	177
Medium	112	58	220
High	182	98	325
EC Limits (DoE,1989)*	135	75	300

*For soils of pH 6 to 7

Table 2: Some properties of unamended (prior to sludge treatment) soil

pH	CEC* (cmol kg^{-1})	L01**	Clay	Silt	Sand
		-----------------------%---------------------------------			
6.3	61.4	10.1	29.8	41.6	28.5

*Cation exchange capacity , **Loss on ignition

Table 3: CO_2-C evolution (mg h^{-1}) from sludge treated soil contaminated with varying levels of Cu, Ni and Zn

Treatment	1 week	3 weeks	7 weeks
a: Cu-Ni experiment			
Cu			
Low	9.88 a	3.14 c	1.25 b
Medium	9.62 a	3.52 b	1.36 b
High	8.99 b	3.87 a	1.51 a
Significance	*	***	**
Ni			
Low	9.92 a	3.30b	1,28b
Medium	9.55 ab	3.45 b	1.29 b
High	9.01 b	3.78 a	1.56 a
Significance	*	**	***
b: Ni-Zn experiment			
Ni			
Low	9.65 a	3.40	1.21 b
Medium	9.26 b	3.47	1.22 b
High	8.72 c	3.45	1.34 a
Significance	***	NS	**
Zn			
Low	9.12	3.42	1.25
Medium	9.16	3.46	1.25
High	9.35	3.45	1.27
Significance	NS	NS	NS
c: Cu-Zn experiment			
Cu			
Low	10.29 b	3.52 b	1.34 b
Medium	10.42 b	3.77 a	1.35 b
High	10.79 a	3.74 a	1.43 a
Significance	**	**	*
Zn			
Low	10.04 c	3.63 b	1,41
Medium	10.42 b	3,53 b	1.37
High	11.04 a	3.86 a	1.34
Significance	***	**	NS

*p<0.05, **p<0.01,***p<0.001, NS = non-significant Means with a common letter suffix in a column do not differ at a 5% level of probability (least significant difference test, LSD)

Soil moisture content (-50 kPa) was adjusted to slightly drier than its water holding capacity by centrifugation (Piper, 1950). This moisture condition was kept constant by addition of distilled water at the end of every second day.

Soil analysis: Soil pH was determined using a glass electrode in 1:2.5 w/v soil-water suspensions (MAFF, 1986). Organic matter content was measured as weight loss on ignition (L0I) of oven dry soil (Ball, 1964) at 400°C. Cation exchange capacity (CEC) was measured by the method described by Chapman (1965). Total IHNO_3 digestion) and extractable (0.5 M EDTA) metals (McGrath and Cegarra, 1992) were measured by atomic absorption spectroscopy.

Table 4: Biomass C (mg kg^{-1}) of sludge treated soil contaminated with varying levels of Cu, Ni and Zn

Treatment	--------3 weeks--------		-----------7 weeks----------	
	Cu	Ni	Cu	Ni
a: Cu-Ni experiment				
Low	1379 a	1344 a	578 a	566 a
Medium	1284 b	1261 b	469 b	505 a
High	1066 c	1124 c	458 b	461 b
	***	***	**	**
b: Ni-Zn experiment				
Treatment	--------3 weeks--------		-----------7 weeks----------	
	Ni	Zn	Ni	Zn
Low	1488 a	1403	608 a	579
Medium	1310 b	1247	549 b	562
High	1188 b	1336	505 b	520
	***	NS	**	NS
c: Cu-Zn experiment				
Treatment	--------3 weeks--------		-----------7 weeks----------	
	Cu	Zn	Cu	Zn
Low	1420 a	1301 b	595 a	584
Medium	1394 a	1330 b	580 a	564
High	1261 b	1445 a	520 b	548
Significance	***	***	**	NS

**p<0.01, ***p<0.001, NS = non-significant
Means with a common letter suffix in a column do not differ at a 5% level of probability (least significant difference test, LSD)

Table 5: Microbial biomass N (mg kg^{-1}) of sludge treated soil contaminated with varying levels of Cu, Ni and Zn

a: Cu-Ni experiment				
Treatment	--------3 weeks----------		-----------7 weeks----------	
	Cu	Ni	Cu	Ni
Low	317 a	295 a	124 a	128 a
Medium	306 a	288 a	123 a	118 a
High	217 b	259 b	103 b	104 b
Significance	***	***	**	**
b: Ni-Zn experiment				
Treatment	--------3 weeks--------		-----------7 weeks----------	
	Ni	Zn	Ni	Zn
Low	289 a	288 a	120 a	119 a
Medium	269 b	273 a	114 b	116 a
High	250 c	248 b	110 b	108 b
Significance	***	***	**	**
c: Cu-Zn experiment				
Treatment	--------3 weeks--------		-----------7 weeks----------	
	Cu	Zn	Cu	Zn
Low	270 a	269 a	111a	105 a
Medium	247 b	239 b	101a	96 ab
High	236 c	245 b	74b	87 b
Significance	***	***	***	****

*p<0.01, ***p<0.001, NS = non-significant
Means with a common letter suffix in a column do not differ at a 5% level of probability (least significant difference test, LSD)

Soil respiration was estimated by measuring the CO_2 concentration change in flasks sealed for 6 hours (Sparling, 1981) and data expressed in terms of C output. Respiration measurements started after one week and continued at frequent intervals until the end of the experiment (7 weeks). Gas chromatography (Pye-Unicam Series 104 Chromatographl was used to analyse CO_2 in the headspace of the flasks.

A fumigation-extraction method was used for estimating microbial biomass C (Vance *et al*., 1987) and biomass N (Brookes *et al*., 1985). Standard factors for converting extractable C (2.64) and N (1.85) to microbial C and N respectively were based on the above studies. Soil was fumigated with chloroform in a dessicator for 24 hours. The fumigated and non-fumigated soils were then extracted with

Table 6: Microbial biomass C to N ratio of sludge treated soil contaminated with varying levels of Cu, Ni and Zn

Treatment	--------3 weeks--------		-----------7 weeks----------	
a: Cu-Ni experiment				
	Cu	Ni	Cu	Ni
Low	4.35 b	4.67	4.75	4.45
Medium	4.19 b	4.39	4.04	4.28
High	4.93 a	4.42	4.47	4.53
Significance	***	NS	NS	NS
Treatment	--------3 weeks--------		-----------7 weeks----------	
	Ni	Zn	Ni	Zn
b: Ni-Zn experiment				
Low	5.17	4.87 b	5.06	4.85
Medium	4.89	4.55 b	4.83	4.83
High	4.76	5.40 a	4.61	4.82
Significance	NS	*	NS	NS
Treatment	--------3 weeks--------		-----------7 weeks----------	
	Cu	Zn	Cu	Zn
Low	5.28 b	4.84 c	5.39 b	5.78
Medium	5.70a	5.62b	5.84b	6.12
High	5.36 b	5.89 a	7.03 a	6.38
Significance	***	***	***	NS

*$p<0.05$, ***$p<0.001$, NS = non-significant

Means with a common letter suffix in a column do not differ at a 5% Level of probability (least significant difference test, LSD)

1 M K_2SO_4. The organic C concentrations of the filtrates were analysed using a Shimadzu Total Organic Carbon Analyser (TOC-5050). For microbial biomass N, 25 ml of the above filtrates was dried and digested with concentrated H_2SO_4. Steam distillation followed by titration with dilute HCl was carried out for nitrogen measurements (Bremner and Mulvaney, 1982).

Biomass C and biomass N were used to calculate microbial biomass C:N ratios so as to provide an indication of shifts in population structure between bacterial and fungal biomass (Paul and Clark, 1996).

Statistical analysis: For data analysis, the statistical package "STATGRAPHICS Version 6.0" (Manugistics, 1992) was used. Data were analysed by two-way analysis of variance and mean differences were compared using the least significant difference test (LSD).

Results and Discussion

Microbial biomass C, biomass N and microbial C:N ratio were calculated at 3 and 7 weeks of incubation. At 7 weeks, soil microbial responses were broadly similar to those measured at 3 weeks. Therefore, in most cases results on these two occasions are described together. Also due to a consistency of microbial response to metal treatments, data for individual measurements will be considered together.

CO_2-C evolution patterns at 1, 3 and 7 weeks are shown in Table 3. With increasing Ni inputs, there was a decrease in CO_2-C evolution at 1 week followed by a later increase. The pattern of response to Cu inputs was variable. In the Cu-Ni experiment, an initial decrease (at 1 week) in response to Cu inputs was followed by an increase. However, in the Cu-Zn experiment, CO_2-C evolution was increased at all three occasions by Cu inputs. Higher Zn inputs did not affect CO_2-C evolution when combined with Ni, but caused a significant increase (except at week 7) when combined with Cu.

Reduced CO_2 evolution rates with high metal inputs during the first week in the Cu-Ni and Ni-Zn experiments are consistent with the findings of Hattori (1989, 1992) and Doelman and Haanstra (1984). On the other hand, findings of high CO_2-C evolution rates during the later stages of the experiments in these same soils are in close accordance with results reported by Bardgett and Saggar (1994), They found increased respiration rates in moderately Cu contaminated soil compared with less and highly Cu contaminated soils over 4 weeks of incubation.

Zn and Ni applied at a rate of 200 mg kg^{-1} soil slightly increased, the CO_2 evolution over the following 2 days (Wilke, 1991), an effect attributed to the surviving microbial population mineralizing freshly killed microbial cells. In the study reported here, there was a similar increase for Zn but a decrease for Ni in CO_2-C evolution at one week. Thereafter, higher metal inputs consistently increased respiration. Between weeks 3 and 7, the overall reduction in microbial biomass C was large compared to any effects of metal inputs. Indeed, reductions in biomass C over this period tended to be less in the high metal soils. Therefore, it is unlikely that the mechanism suggested by Wilke (1991) would have had a marked effect on respiration in our experiments.

In all experiments, there was a very marked decrease in microbial biomass C and biomass N between weeks 3 and 7. These changes were broadly in line with reductions in CO_2 evolution. In most cases, lower values of microbial biomass C and biomass N were observed in high compared with low and medium nroal soils (Table 4, 5). However, an increase in biomass C was observed in high Zn soils at three weeks in one experiment (Table 4). In the other experiment and at 7 weeks, Zn inputs did not significantly affect biomass C, however, this was not the case for biomass N. In most experiments, effect of moderate metal inputs were also non-significant. Compared with the low metal soils the largest reduction in biomass C (20%) and N (33%) were observed in the high Cu soils in Cu-Ni and Cu-Zn experiments respectively.

Several studies (Chander and Brookes, 1993; Bogomolov *et al.*, 1996) have reported similar reductions in biomass C and biomass N with similar metal inputs to those used in the present study. However, in some studies where sludge metal contents were low (Banerjee *et al.*, 1997) these indices were either not affected or were increased by a sludge application. But this increase in biomass C and biomass N would be expected unless inhibited by toxic metal contents. There was a general increase in biomass C:N ratio with higher metal inputs (except Ni) especially after 3 weeks in all experiments (Table 6). This increase in biomass C:N ratio indicated a large increase in fungal relative to bacterial biomass in the high metal soils (Paul and Clark, 1996). These results are in accord with Fliessbach *et al.* (1994) who reported that fungal contribution to substrate induced respiration was more than 70% and increased up to 97% in soils with high metal content associated with a decrease in bacterial contribution. Hiroki *et al.* (1985) also noticed an increase in the number of fungal colonies with increase in Cu concentrations in the soil. Similarly, Khan and Scullion(1999) reported increased levels of ergosterol contents in metal contaminated than control soils. Other authors have also reported an increase in fungal counts (Hattori, 1992) and fungal phospholipid fatty acid (Frostegard *et al.*, 1993, Khan and Scullion, 1999) in metal contaminated compared with the non-contaminated soil.

Greater CO_2-C evolution was associated with lower biomass C in the high metal soils and may be due to the shift in substrate utilisation from biomass synthesis to maintenance

(Chander and Brookes, 1991; Bardgett and Saggar, 1994; Khan and Scullion, 1999). There were often indications of a shift in the balance of microbial populations, from bacteria to fungi, in response to treatments and this may have affected the above indices.

Microorganisms are dynamically involved in many basic ecologic processes, for example the mineralisation of C and N and the fixation of atmospheric N needed to maintain the fertility of soils. A shift from bacteria to fungi in the metal contaminated soil may greatly affect some processes like nitrification for example, where few species are involved. Giller *et al.* (1998) reported that the reduction in the competitiveness of the clover in mixed sward with ryegrass due to the absence of N_2-fixation if heavy metals were allowed to accumulate to toxic concentrations, would result in the rapid loss of clover from newly-established pastures.

In this study existing guidelines (DoE, 1989) were used to determine treatment levels. Close to these upper limits and in a moderately acidic soil, each of the three metals tested affected microorganisms in the short-term, but responses to Zn differed in many respects to those for Ni and Cu. There has been recent concern regarding the concentration of Zn in the soil (RCEP, 1996). However, in the current study Zn seemed to be less toxic to soil microorganisms than Cu and Ni, at the rates used.

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