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Four Swedish long-term field experiments with sewage sludge reveal a limited effect on soil microbes and on metal uptake by crops

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Abstract

Purpose*:* To study the effect of sewage sludge amendment on crop yield and on microbial biomass and community structure in Swedish agricultural soils.

Materials and methods: Topsoil samples (0-0.20 m depth) from four sites where sewage sludge had been repeatedly applied during 14-53 years were analysed for total C, total N, pH and PLFAs (phospholipid fatty acids). Heavy metals were analysed in both soil and plant samples, and crop yields were recorded.

Results and discussion: At all four sites, sewage sludge application increased crop yield and soil organic carbon. Sludge addition also resulted in elevated concentrations of some heavy metals (mainly Cu and Zn) in soils, but high concentrations of metals (Ni and Zn) in plant materials were almost exclusively found in the oldest experiment, started in 1956. PLFA analysis showed that the microbial community structure was strongly affected by changes in soil pH. At those sites where sewage sludge had caused low pH, Gram-positive bacteria were more abundant. However, differences in community structure were larger between sites than between the treatments.

Conclusions: At all four sites long-term sewage sludge application increased the soil organic carbon and nitrogen content, microbial biomass and crop yield. Long-term sewage sludge application led to a decrease in soil pH. Concentrations of some metals had increased significantly with sewage sludge application at all sites, but the amounts of metals added to soil with sewage sludge were found not to be toxic for microbes at any site.

Keywords Heavy metals • Long-term field experiments • Mycorrhiza • Phospholipid fatty acids • Microbial community structure

1 Introduction

Soil organic matter is a key attribute of soil fertility. The pool of soil organic C can be increased either indirectly by applying mineral fertilisers, through higher yields, or directly by adding organic amendments such as sewage sludge (Kätterer et al. 2013). Sewage sludge provides organic matter and essential plant nutrients, but may also contain unwanted metals, xenobiotic substances and pathogens (Singh and Agrawal 2008).

In Sweden, the political goal is that all kinds of waste are recycled and that "by 2015 at least 60 per cent of phosphorus compounds present in wastewater will be recovered for use on productive land" (Environmental Objectives Portal 2012). The production of sewage sludge from 402 wastewater treatment plants in Sweden was 203 500 Mg dw in 2010 and 25% of this was used in agriculture (Statistics Sweden 2012). This is less than in the Scandinavian neighbour countries Norway and Denmark (57% and 54% in 2009; Eurostat, 2012), but more than in Finland (3%; Lindfors 2012). The ReVAQ project in Sweden has led to a certification system for sludge which is run by the Swedish Water and Wastewater Association (Kärrman et al. 2007). The aim is to reduce heavy metals and other contaminants in wastewater, a process which allows for continuous improvement of the incoming wastewater, as a way of improving the quality of the sludge applied on agricultural land. Today there are over 37 wastewater treatment plants producing sludge certified by ReVAQ, corresponding to over 50% of Swedish sludge from wastewater (Revaq 2012), but the use of sewage sludge as a fertiliser in agriculture is still the subject of much debate, mostly concerning the possibilities for accumulation of heavy metals, especially cadmium (*e.g.* Linderholm et al. 2012).

The main objective of this study was to investigate the effect of continuous application of sewage sludge on soil microbial biomass and community structure. Long-term experiments are needed to be able to detect effects on the microbial biomass (Diacono and Montemurro 2010). Amendment with organic materials such as sewage sludge generally increases the microbial biomass, but high doses may cause negative effects on enzymatic activities in soil (Singh and Agrawal 2008), and inconclusive results presented in the literature makes it uncertain if sludge addition will affect microbial community structure, and whether these potential changes are negative for soil health and fertility (MacDonald et al. 2011).

Cadmium (Cd) was also of special interest due to its high toxicity, long body retention time and high mobility in the environment (Alloway and Jackson 1991). Its concentration has increased in soils as a result of industrial emissions with subsequent atmospheric deposition, but emissions have decreased since the mid-1970s, due to cleaning at point sources (Pacyna et al. 2009). The average concentrations of Cd in sewage sludge in Sweden are now below the Swedish threshold value of 2 mg kg^{-1} DM (Suppl. Table A). Due to less atmospheric deposition and use of P fertiliser with a low Cd content, the Cd content in Swedish wheat grains has decreased from the peak concentrations recorded during the 1970s (Kirchmann et al. 2009). Therefore, we hypothesized that the effect of heavy metals on soil microorganisms, as a result of repeated sewage sludge amendment, was not significant.

2 Methods

2.1 Sites

Soil samples were taken for analysis from four sites where sewage sludge has been repeatedly applied in long-term field experiments situated in different parts of Sweden; Ultuna, Lanna, Petersborg and Igelösa (Table 1). In these four experiments, at least one sewage sludge treatment is included in the experimental design. At Ultuna and Lanna, the sewage sludge treatments are being compared with other organic and mineral fertilisers, while at Igelösa and Petersborg comparisons are being made between different levels of sewage sludge and different levels of mineral fertiliser. All sites are designed as block experiments, with four full replicates per treatment.

In the Ultuna experiment, all organic fertilisers, including sewage sludge, are applied every second year, corresponding to 4 Mg C ha^{-1}. The Lanna experiment has a similar design, with 8 Mg ash-free dry matter ha^{-1} applied every second year. At Lanna, an additional sewage sludge treatment is included in which metal salts (Cd 0.098, Cu 3.036 and Ni 6.250 kg ha^{-1}) are added together with the sludge. The experiments at Petersborg and Igelösa have an identical design, with three levels of sewage sludge (0, 4 or 12 Mg dry matter ha^{-1} every fourth year) compared with three levels of NPK fertiliser (0 N, ½ normal N and normal N). The plots are 6 m × 20 m at Igelösa and Petersborg, 8 (or 6) m × 14 m at Lanna . These three sites have normal conventional management, including mould-board ploughing to a depth of 22-24 cm. At Ultuna, the plots are 2 m × 2 m and the soil is manually managed within frames, down to a depth that should be comparable to the other sites.

The sewage sludge applied to plots is obtained from nearby wastewater plants, Kungsängsverket in Uppsala (Ultuna), Ryaverket in Gothenburg (Lanna), Sjölunda in Malmö (Petersborg) and Källby in Lund (Igelösa). All of these in principle use the same method for P removal, which is precipitation with $FeCl_3$. Prior to 1976, $AlSO_4$ was used for precipitation in Uppsala. The concentration of heavy metals has decreased since the 1970's in all these wastewaters, exemplified by a ten-fold decrease of Cd and Pb in the Uppsala sludge (Börjesson et al. 2012). Recent mean values for sewage sludge in Sweden are given in Suppl. Table A.

In the Ultuna experiment, different spring-sown crops have been grown. At Lanna, only cereals, mainly oats and barley, have been used. Petersborg and Igelösa have a 4-year crop rotation, with a few exceptions consisting of winter wheat, sugar beet, spring barley and oilseed rape.

2.2 Soil sampling

Topsoil samples (0-20 cm depth) from the four sites were taken from plots that had received sewage sludge, and for comparison, from unfertilised and N-fertilised plots. Ultuna was sampled in September 2009 and Lanna in (autumn) 2010, just before the biennial application of sludge. Igelösa and Petersborg were sampled in June 2011, two years after sludge spreading. Samples from Igelösa were taken from all plots with different levels of sewage sludge, but without mineral N fertilisation, while at Petersborg sampling also included the highest level of N fertilisation combined with the highest level of sewage sludge and the control without sewage sludge.

2.3 Analyses

Grain, dried green mass, and soil samples were analysed after digestion in 2M HNO_3 with an inductively coupled plasma-mass spectrometer (Elan 6100 ICP-MS; Perkin Elmer, Waltham MA 02451, USA) for eight metals (Al, Cd, Cu, Fe, Mn, Ni, Pb and Zn) in topsoil and nine metals (Cd, Co, Cr, Cu, Mo, Ni, Pb, Se and Zn) in crop material. Concentrations of total soil C and total N were determined by dry combustion (LECO CNS Analyzer; LECO Corporation, St Joseph MI 49085, USA) and pH was measured in distilled water.

Phospholipid fatty acids (PLFA) were analysed according to the method described by Börjesson et al. (2012), whereby 1 g freeze-dried sample was fractionated with 5 ml chloroform, 20 ml acetic acid and 5 ml methanol. The methanol phase was used for PLFA analysis in all samples. The chloroform phase was also saved for determination of the neutral lipid fatty acid (NLFA) 16:1ω5 in samples from Petersborg and Igelösa. For principal component analysis (PCA), 25 PLFAs that were present at all sites were converted to percentage of total moles for evaluation of microbial community structure.

Statistical analyses were done with the use of JMP software ver. 6.0.3 (SAS Institute Inc., Cary NC 27513, USA); including analysis of variance, comparison of treatment effects (Tukey-Kramer HSD), and principal components obtained from covariance matrices of PLFA data.

3 Results

3.1 Yields and soil pH

At all four sites, sewage sludge had exerted a positive effect on crop yields (4% per year or more compared with unfertilised plots) and on soil organic matter levels (Fig. 1, Table 2 and 3). It should be noted that normal fertilisation differs between the sites. It is 80 kg N ha^{-1} year^{-1} at Ultuna and Lanna, while the dosage at Petersborg and Igelösa is according to fertiliser recommendations in the area, i.e. 140 kg N ha^{-1} for wheat and slightly lower for other grains.

Soil pH had dropped in the sewage sludge treatment at the Ultuna site from 6.5 at the start of the experiment to 4.9 at the time of sampling in 2009, while pH had remained between 6.1 and 6.7 in the other treatments. At Lanna, the pH had also dropped in the plots treated with sewage sludge, although so far only from 6.6 in 1996 to 6.1 in 2010. The other two sites were limed in 1998, and in 2010 the pH values ranged between 6.9 and 7.3 at Petersborg and between 7.4 and 7.7 at Igelösa.

3.2 Metals in soil

The concentrations of metals were generally much higher in soil samples from Ultuna than soils from the other sites, not only in the sewage sludge-treated soil but also in the normally fertilised and unfertilised soils, which can be regarded as background (Table 4), due to the geological history in this region (Eriksson et al. 2010). At Ultuna, the concentrations of Cd, Ni, Pb and Zn in the sewage sludge-treated soil were higher than background levels, seemingly due to high inputs during the early years of the experiment. For example, the concentration of Cd in sewage sludge amounted to 9.1 mg kg^{-1} in 1972, but then decreased rapidly to around 2 mg kg^{-1}, and in 2009 it was down at 0.65 mg kg^{-1}. No increase in heavy metal concentrations has been observed since the 1970s, according to historical data (Table 4). An exception is Cu, soil concentrations of which have increased steadily because Cu levels in sludge have remained high. High concentrations of Fe in the Ultuna sludge-treated soil are an effect of addition of $FePO_4$, produced by P removal through precipitation with $FeCl_3$ during wastewater treatment.

At Lanna, metal concentrations in soils were generally low (Table 5), but those of Cu and Zn had increased in all sewage sludge-treated plots, while Cd and Ni concentrations were only significantly higher when these metals were added as soluble salts together with the sludge (Treatment G). For the other metals, including Pb, no significant differences were observed.

Data from the experiments at Igelösa and Petersborg are reported in detail by Andersson (2012). However, it should be noted that average Cu concentrations were also higher at these sites through sewage sludge addition (Table 6). For the other metals, there has been a trend for a slight increase, although barely significant.

Sewage sludge amendment decreased soil pH and the correlations between pH values and metal concentrations were strongly negative (Suppl. Table B), most obviously for Zn (r=-0.858).

3.3 Metals in plants

Analysis of metals in samples of silage maize harvested in 2009 in the Ultuna experiment showed that only Ni and Zn levels were significantly higher in plant material from the sewage sludge-treated plots, compared to the control plots (Table 7). Cadmium levels in crop samples from both the unfertilised and the sewage sludge treated-plots were considerably higher than in the corresponding treatments at the other sites. In order to compare silage maize with previous metal uptake by crops in this treatment, data from fodder rape, which was also harvested as green biomass, were used (Table 7). With the exception of Cu and Zn, which had similar uptake, metal concentrations were considerably lower in 2009 than in 1974.

Analyses of metals in wheat grain at Lanna (Table 8) showed that there was significantly more Ni in sewage sludge plots when spiked with Ni in salt solution. Similarly, there were elevated levels of Cd in the treatment with sludge spiked with metals, but for Cr, Co, Cu, Pb and Zn no significant differences were found. Molybdenum was found to be higher in crops fertilised with calcium nitrate ($CaNO_3$). However, Mo in grain was correlated with soil pH ($r=0.94; p=0.0061$), similarly to what was found in silage maize from Ultuna.

An interesting observation is the effect of N fertilisation on Cd uptake in wheat grain at Igelösa (Fig. 2). Accumulation of Cd was low in that soil and plant uptake was not affected by sewage sludge application, but concentrations increased in crops as an effect of NPK fertilisation, with N was applied as ammonium nitrate (N27).

3.4 PLFAs

The concentrations of total PLFAs were highest in the sewage sludge-treated soils at all sites (Tables 9-11). Correlations between soil organic matter and total PLFA content were highly positive at all sites, and PLFA:soil C ratios were similar independent of site or experimental treatment (Fig. 1).

Data from the Ultuna experiment showed that almost all the PLFAs identified had increased in the sewage sludge treatment. The variation within treatments was rather high, with CV (coefficient of variation = standard deviation divided by mean value) up to 25% in the sewage sludge treatment. There was a shift in the sludge plots towards more branched PLFAs (*e.g.* i16:0, i17:0, br18:0, 10Me17:0), indicating relatively more Gram-positive bacteria compared with other bacterial groups (Table 9). There was also a strong effect on cy17:0 and cy19:0.

In samples from Lanna, the highest total values were obtained from the two sewage sludge treatments, and the addition of extra heavy metals as soluble salts did not seem to affect the microbes at all (Table 10). The sludge-treated plots also had the highest concentrations of most individual PLFAs. In contrast to the Ultuna site, the effect of sludge treatments on mono-unsaturated PLFAs, *e.g.* 16:1ω7 and 18:1ω7, was not negative in Lanna.

In samples from Petersborg and Igelösa, sludge amendment caused a 31% increase in total PLFAs at Petersborg and a 33% increase at Igelösa, comparing the highest application rate with the unfertilised control (Table 11). Almost all individual PLFAs increased in equal proportions due to sludge amendment on these two sites, *i.e.* no inhibitory effect could be observed. If we calculate the PLFAs as concentrations (nmol PLFA g soil^{-1}), some of the PLFAs had more than doubled, *e.g.* i14:0 at Igelösa and 18:2 at Petersborg. At Petersborg, fertilisation with mineral N caused a further increase in microbial biomass (Table 11), although a lower proportion of carbon was incorporated into microbes according to the PLFA analysis (Fig. 1).

Analysis of microbial community structures with PCA of individual PLFAs showed that differences between sites were more pronounced than differences between treatments, with samples from the same sites clustered together (Fig. 3). However, some of the samples from Ultuna were extremely separated. Comparisons (Suppl. Table B) showed that PC (principal component) 1 was dominated by the presence of PLFA 16:0, which is ubiquitous in most organisms and thus of limited use for explaining differences. PC 2 had strong positive correlations with most of the mono-unsaturated PLFAs, while the eukaryotic biomarkers PLFAs 18:2+18:3 were negatively correlated with some branched PLFAs (i16:0, i17:0, br18:0

and 10me17:0), while other branched PLFAs (i14:0, a15:0, and 10Me18) had a positive correlation. The PLFA cy19:0, regarded as an indicator of stressed production of mono-unsaturated PLFAs (Kaur et al. 2005), was also negatively correlated with PC 2.

The PCA were also interpreted in relation to sites and treatments by comparing Tables 9-11. Some PLFAs appeared to be site-specific, *e.g.* at Ultuna i14:0, a15:0 and 16:1ω5 had low values, while 10me16:0 was more common that at the other sites. Petersborg had high values of 14:0, while Lanna had high values of i15:0 and 17:1, but low values of 18:2+3 and 18:1ω9. The treatments at Ultuna differed greatly, with values for the sewage sludge treatment being particularly extreme, with high ratios of the PLFAs 16:0, i17:0 and cy19:0, but low 18:1ω7, 18:1ω5 and 10me18:0.

A finding that can be regarded as more general was a positive correlation between pH and the mono-unsaturated PLFAs at all sites (*e.g.* 18:1ω7 with r=0.855 for N=60; Suppl. Table B). This was also obvious in the PCA (Fig. 4), where these PLFAs clustered together in the left part of the panel. There was also a negative correlation between the mono-unsaturated PLFAs and metals in the soil samples (Table 4; *cf.* Suppl. Table B).

3.5 Neutral lipid fatty acids (NLFAs)

NLFAs, among which 16:1ω5 is regarded as a biomarker for arbuscular mycorrhiza (*e.g.* Bååth 2003), were only found in samples from Igelösa and Petersborg (Fig. 5). Mineral fertilisation with NPK had a strong negative effect on the NLFA 16:1ω5 at Petersborg, which declined in concentration from 3.3 to 1.3 and 0.6 nmol NLFA g DM soil^{-1} in the fully fertilised treatments, while only a small but non-significant decrease could be observed in the plots with the highest sewage sludge dose. At Igelösa, no significant change was observed, but in contrast a trend for an increase in the NLFA 16:1ω5 at the lower dose of sewage sludge, as also seen at Petersborg.

4 Discussion

4.1 Metals in soil and crops

Amending soil with sewage sludge increased soil organic matter and microbial biomass at all four sites investigated. In addition, lower soil pH values in sludge-treated soil, for example at Ultuna (pH 4.9 after 53 years), did not cause lower microbial biomass. However, our results contradict earlier reports from the 1980s and 1990s about negative effects of sewage sludge fertilisation on microbial biomass and activity on soils in Britain (Brookes and McGrath

1984), but also in the Ultuna experiment (*e.g.* Witter et al. 1993). In the latter report, negative effects were attributed to metal toxicity. In order to test this hypothesis, we can first compare estimates of microbial biomass in the sludge-treated plots at Ultuna: Using the factor 5.85 mg ATP g^{-1} biomass C according to Tate and Jenkinson (1982) and 0.172 mmol PLFA g^{-1} biomass C according to Joergensen and Emmerling (2006), there has been an increase from 231 µg biomass C g^{-1} DM soil in 1990 (ATP data from Witter et al. 1993) to 517 µg biomass C g^{-1} DM soil in 2009. For the other treatments in the Ultuna experiment, the two methods have given similar biomass values (within 10% difference).

Over the last 20 years, emissions of heavy metals in Europe have declined and for most metals, deposition in 2005 was only around 20% of that in 1980 (Pacyna et al. 2009). A similar decline has taken place in metal concentrations in sewage from Swedish wastewater plants (*e.g.* Börjesson et al. 2012). In the Ultuna experiment, most of the metal enrichment in sewage sludge-treated soil originated from applications made before 1974. Since then, application of sludge to soil has only mainly affected the Cu content in soil. Copper from water pipes has dissolved to a high extent into Uppsala’s drinking water, causing Cu enrichment in sewage sludge. However, it should be mentioned that since 2008 these levels have also decreased radically due to improved treatment techniques (Uppsala Vatten 2012). A conclusion regarding the sludge treatment at Ultuna is that lower inputs of heavy metals during recent decades have allowed the microbial biomass in the soil to increase and utilise a large proportion of the organic matter (*cf.* Börjesson et al. 2012). Chaperon and Sauvé (2007) investigated the toxicities of Ag, Cu, Hg and Zn to the enzymes dehydrogenase and urease in an agricultural soil, and calculated EC_{50}-values (concentrations giving 50% of the enzymatic activity in the control) to be 268 mg (4.221 mmol) Cu kg $soil^{-1}$ for dehydrogenase and 491 mg Cu kg $soil^{-1}$ for urease. For Zn, the corresponding values were 926 mg Zn kg $soil^{-1}$ for dehydrogenase and 1178 mg Zn kg $soil^{-1}$ for urease. These values are lower than what could be found in the sewage sludge-treated soil at Ultuna (Table 4), but other enzymes may be more sensitive, such as N_2-fixation: McGrath et al. (1995) estimated that free-living cyanobacteria were reduced by 50 % at metal concentrations 114 mg Zn, 33 mg Cu, 17 mg Ni, 2.9 mg Cd, 80 mg Cr and 40 Pb mg kg $soil^{-1}$. Since these values are lower than the Ultuna values, an inhibitory effect could be expected, but we must also take the effect of organic matter into account, and thus the threshold value, the exact mechanism and the identification of the most inhibitory metal need further investigation.

Crop yields have increased at all four sites through sewage sludge amendment and even the much higher metal concentrations at Ultuna than at the other sites have not caused any decrease in crop yields. Furthermore, metal concentrations in crops treated with sewage sludge have not been elevated. This was very obvious for Cu, for which high concentrations in the soil and an increase over recent years did not result in elevated levels in plants (for data on Igelösa and Petersborg; see Andersson 2012).

A metal of special interest is Cd, since high contents in food can cause human kidney damage (*e.g.* Åkesson et al. 2005). Strict regulations limiting the use of sewage sludge on agricultural soils are in place in Sweden and other countries. The sludge-treated soil at Ultuna has higher Cd concentrations (0.73 mg Cd kg^{-1}) than are currently permissible due to additions of metal-rich sewage sludge before 1975. At the other sites, concentrations are below the threshold limit of 0.4 mg Cd kg^{-1} soil, despite the fact that Cd has been added as a soluble salt together with sludge on eight occasions at Lanna, and large amounts of sewage sludge have been added during 30 years at Petersborg and Igelösa. In an earlier investigation of the Ultuna site, Bergkvist et al. (2003) analysed total Cd in HNO_3 extracts in archived soil samples from the sewage sludge treatment and found an increase from around 0.15 mg Cd kg^{-1} DM soil in original samples from 1956 up to 0.84 mg Cd kg^{-1} DM in 1997. However, they found no elevated concentrations below the topsoil (0.1-0.2 m). Bergkvist (2003, p. 33) found that the net transport of Cd through the soil profile was very low and suggested that there was crop-driven recirculation, with Cd leaching from the topsoil to the subsoil. Bergkvist et al. (2003) also found large variations in Cd concentrations in grain and straw fractions of a barley crop from the Ultuna site, measured in samples from five different years (1978-1997), but the concentration never exceeded 0.05 mg Cd kg^{-1} DM in grain samples from sewage sludge-amended plots. It can be added that the permissible limit for Cd in flour for some Swedish mills is 0.08 mg Cd kg^{-1}, while EU legislation cites 0.2 mg Cd kg^{-1}, limiting the use of the grain as food. All cereal samples from the sites in our study had Cd concentrations below both these limits (Table 8, Fig. 2). This means that they were also below what Kirkham (2006) regarded as the normal Cd concentration in plants, namely 0.1 mg Cd kg^{-1}. The general trend for Cd in Swedish wheat grain has been a decline from 0.08 to 0.04 mg Cd kg dw^{-1} since the peak concentrations in the 1970s (Kirchmann et al. 2009). The Cd contents in European topsoils are lowest (<0.1 ppm) in Scandinavia and in Portugal (Pan et al., 2010).

An increase in Cd uptake by crops fertilised with $CaNO_3$ was observed at Igelösa. This is a well-known phenomenon which has been observed in several studies in Sweden. For example in a study using two cultivars and three sites, Wångstrand et al. (2007) showed that Cd concentrations in wheat grain were significantly correlated with N fertilisation rate. The most likely cause is a decrease in available sorption sites due to sorption of Ca, which increases the activity of Cd in the soil solution and makes it available for plant uptake (Gray et al. 2002). Cadmium accumulation also varies between species and varieties of the same species, although it does not seem to be bound to a specific region of the chromosome (*Ci* et al. 2012). It may also be influenced by soil type, where high pH values and high contents of clay and organic matter can reduce the solubility of Cd (Giller et al. 1998; Mitchell et al. 1999; Gibbs et al. 2006; Eriksson 2009; Gao et al. 2010; Larsson Jönsson and Asp 2011). However, in the Igelösa samples in our study, the Cd uptake in plants was positively correlated with soil pH ($r=0.39, p=0.018$), which adds support to the hypothesis that an anion (in this case NH_4^+) from the fertiliser applied replaces Cd at active soil particle sites and makes Cd more plant-available, as suggested by Lorenz et al. (1994).

A dependence of plant uptake of Mo on soil pH was observed in samples from all sites, due to increased solubility of Mo with increasing pH (Tyler and Olsson 2001).

A review by Smith (2009) concluded that Zn was the most crucial metal for soil microbial processes due to the increase in labile forms, particularly under acidic conditions. This can also be manifested as higher crop uptake of Zn (Smith 2009), as found in maize silage in our study (Table 7). Villar and Garcia (2008) showed that bioleaching, *i.e.* solubilisation of metal ions with the aid of *Thiobacillus* spp., needed a pH of 2-3 to initiate release of Cr and Cu, while solubilisation of Ni and Zn started already at pH 6-6.5. At a site in New Zealand that had received over 1000 Mg ha^{-1} of wet sewage sludge, corresponding to >150 Mg dry weight, Speir et al. (2003) observed a strong relationship between plant uptake of Zn and Zn in soil solution, but not for other metals, and attributed this to lowered pH. This supports our findings of increased Ni and Zn in silage maize from sewage sludge-treated plots at Ultuna, where soil pH had decreased to 4.88 after more than 50 years of application.

4.2 Microbial composition (PLFAs and NLFAs)

The microbial community analysis showed that there was a larger difference between soil types than between treatments. This has also been reported by others, *e.g.* Bünemann et al. (2008) in a study with C and P additions. Among samples from the Ultuna experiment, there was a large variation between treatments. This effect is also evident in the PCA plot (Fig. 3), where the separation of Ultuna samples from the other samples is partly due to different lengths of experimental period. However, it is most likely that these differences were caused by the specific soil tillage applied at this site. The Ultuna soil is manually managed; all tillage is done by hand so that soil compaction through machinery is avoided. As a result, large differences in bulk density could develop between treatments due to different soil C contents (Kätterer et al. 2011).

As reported earlier (Börjesson et al. 2012), the microbial community structure, measured as PLFAs, was significantly affected by differences in soil pH in the Ultuna experiment. Soil pH values in the sewage sludge-treated plots at Ultuna and Lanna have decreased over the years, while acidification has been prevented through liming at Petersborg and Igelösa. At Ultuna, the decrease in mono-unsaturated PLFAs was inversely proportional to the increase in branched PLFAs, indicating a shift from Gram-negative to Gram-positive bacteria. High ratios of cy17:0 and cy19:0, indicating stress among Gram-negative bacteria, were most obvious in the sewage sludge treatment at Ultuna. The effect of pH on Gram-negative bacteria is well documented (*e.g.* Aciego Petri and Brookes 2009; Fernández-Calviño et al. 2010), but Gram-negative bacteria are also known to increase after nitrogen fertilisation, both through mineral fertilisers and manure (Peacock et al. 2001). Petersen et al. (2003) also found increased concentrations compared with the control of cy17:0 in Danish soils amended with high levels of sewage sludge, but the assumed stress effect on Gram-negative bacteria gradually decreased during the growing season.

Metal contamination in soils has previously been reported by Abaye et al. (2005), who investigated agricultural soils in England fertilised with sewage sludge from 1942 to 1961. They found that Gram-negative bacteria were much more common than Gram-positive. Likewise, Sandaa et al. (2001) found higher amounts of bacteria belonging to the α-subdivision in soils amended with contaminated or heavy metal-spiked sewage sludge in Germany, and Piotrowska-Seget et al. (2005) found that metal-tolerant bacteria isolated from soils were mainly Gram-negative, some of them with plasmid-associated tolerance to Zn and Cd. This may also point towards the profound effect of pH caused by sewage sludge at Ultuna, since Gram-negative bacteria were much less abundant there, and therefore the effect of metals can be ruled out. Gram-positive bacteria are also assumed to consume older organic material (*e.g.* Waldrop and Firestone 2004), which fits with our observation of a decline in soil organic carbon during the last 15 years in the sewage sludge plots at Ultuna (Börjesson et al. 2012). Furthermore, in a long-term experiment in Germany, where metal-amended sewage sludge had been applied on five occasions between 1980 and 1989, the PLFA profile of the most metal-contaminated soils was very similar to that in the Ultuna sludge samples, with a decrease in 16:1ω5, 18:1ω7, 10Me18:0 and an increase in 14:0, i15:0, 16:0 and cy19:0 relative to the mineral fertilised treatment (Witter et al. 2000). Those authors also performed a test for metal tolerance and found that the study soils had developed a tolerance for Zn. A tolerance to Zn has presumably also developed at Ultuna, since Zn values have been at constantly high levels for a long time.

Some PLFAs seem to be site-specific: At Lanna, very low ratios of both the PLFAs 18:2 and 18:1ω9 (Table 10) indicate that fungi are less abundant there compared with the other three sites. Some PLFAs were obviously treatment-specific, but the effects were not consistent between the different sites, thus providing further proof that the sites, and their chemical and physical soil properties, were more important than the treatments for determining the microbial community structure.

The lack of significant change in the amounts of the PLFA 18:2 indicates that there was no obvious effect on fungi in the sludge-amended plots in our study, which is in accordance with some previous reports (*e.g.* Marschner et al. 2003, Suhadolc et al. 2010). However it contradicts some others in which metal inputs decreased bacterial-fungal ratios of biomarker PLFAs (Khan and Scullion 2000), but negative effects on fungal gene fragments in Cu-containing sewage sludge have also been reported (Macdonald et al. 2011).

The lack of response in NLFA content in the soil samples from sewage sludge-treated plots at Petersborg and Igelösa contradicts previously reported negative effects of sewage sludge on the mycorrhizal fungus *Glomus mossae* (Jacquot-Plumey et al. 2003), but their result were obtained with metal-spiked sludge mixed with quartz. This further exemplifies the necessity to perform studies in long-term field experiments and the significance of adsorption or covalent binding of metals with organic matter (Alloway and Jackson 1991). At normal doses, P added with sewage sludge, mainly as insoluble $FePO_4$, obviously did not affect the mycorrhiza in this study, although this could have been expected (Jansa et al. 2006).

5 Conclusions

- At all four sites investigated, long-term sewage sludge application increased soil fertility in terms of soil organic matter (total C and N), microbial biomass and crop yield.
- Long-term sewage sludge application led to a decrease in soil pH values.
- Concentrations of some metals, mainly Cu and Zn, had increased significantly with sewage sludge application at all sites.

- The amounts of metals added to soil with sewage sludge were found not to be toxic for microbes at any site. Earlier observations of lower microbial biomass at the Ultuna site were no longer detectable.

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Figure legends

Fig. 1 Correlation between Total C (% of dry weight soil) and Total PLFAs, given as mean values for topsoil samples from the four sites. Numbers correspond to the following treatments: **Petersborg**: 1 = A0 (no sludge, no NPK), 2 = B0 (1 ton sludge, no NPK), 3 = C0 (4 ton sludge, no NPK), 4 = A2 (no sludge, normal NPK), 5 = C2 (4 ton sludge, normal NPK); **Igelösa**: 6 = A0 (no sludge, no NPK), 7 = B0 (1 ton sludge, no NPK), 8 = C0 (4 ton sludge, no NPK); **Lanna**: 9 = Cropped, unfertilised, 10 = $CaNO_3$, 11 = Sewage sludge, 12 = Sewage sludge + metals; **Ultuna**: 13 = Cropped, unfertilised, 14 = $CaNO_3$, 15 = sewage sludge.

The fitted line for correlation has the equation: Total PLFAs = 33.01 * Total C (%) – 0.900; with r=0.958 and $p<0.0001$ ($n=15$). Error bars show standard deviation for each treatment ($n=4$)

Fig. 2 Cadmium in soil samples and in wheat grain from Igelösa 2011. Bars = standard deviation, $n=4$. A= No sewage sludge; B= 4 ton sludge every 4th year; and C=12 ton sludge every 4th year. 0=No NPK; 1=half of normal NPK; and 2=normal NPK. Different letters above error bars indicate significant difference (Tukey-Kramer HSD, $\alpha=0.05$)

Fig. 3 Principal components of 25 PLFAs in all four experiments (N=60). Data points are mean values for each treatment, with standard error for $n=4$

Fig. 4 Principal component analysis based on correlation matrix of individual PLFAs (mol-%) and certain variables in topsoil samples from all four sites ($n=15$)

Fig. 5 Effects of sewage sludge and mineral N on the mycorrhizal biomarker NLFA 16:1ω5 in samples from Petersborg and Igelösa. A = No sewage sludge; B = 4 ton sludge every 4th year; and C = 12 ton sludge every 4th year. 0 = No NPK; and 2 = normal NPK. Error bars are standard deviation for $n=4$. Different letters above error bars indicate significant difference (Tukey-Kramer HSD, $\alpha=0.05$)

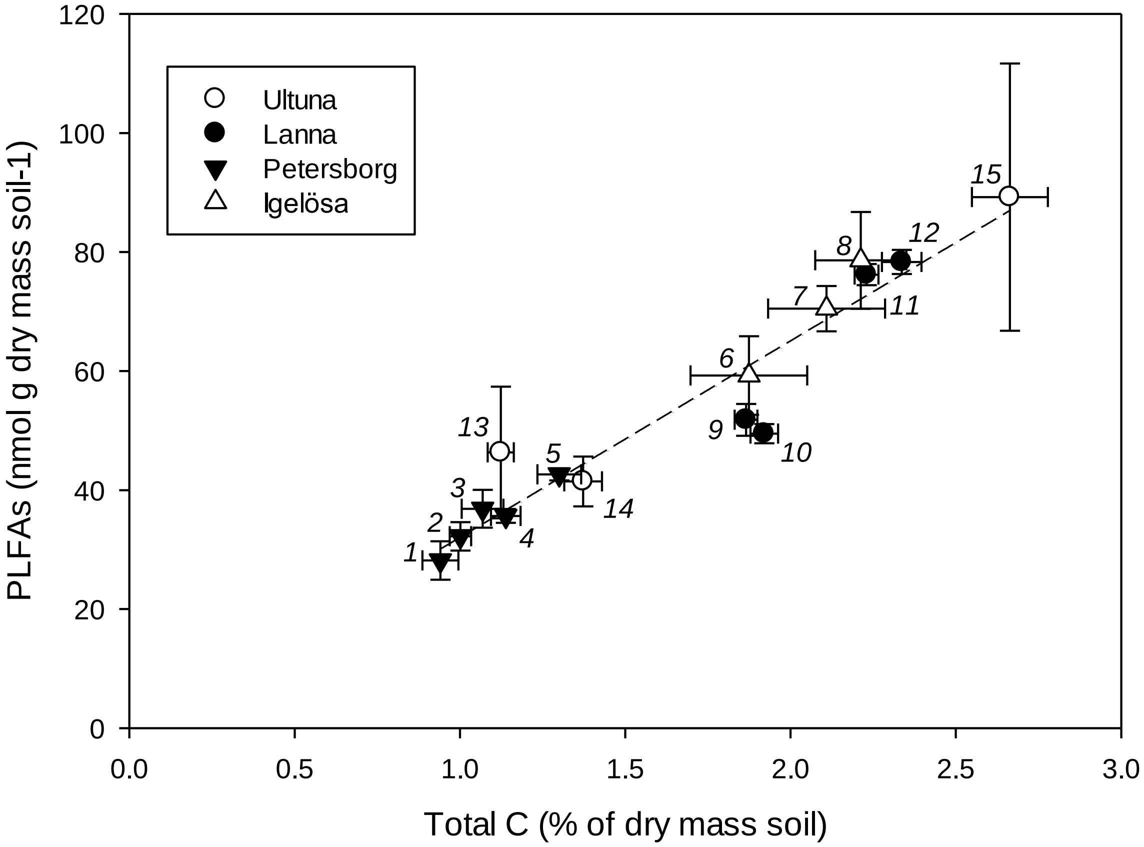

120
100
80
60
40
20
0
PLFAs (nmol g dry mass soil-1)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Total C (% of dry mass soil)
Ultuna
Lanna
Petersborg
Igelösa
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Fig. 1.

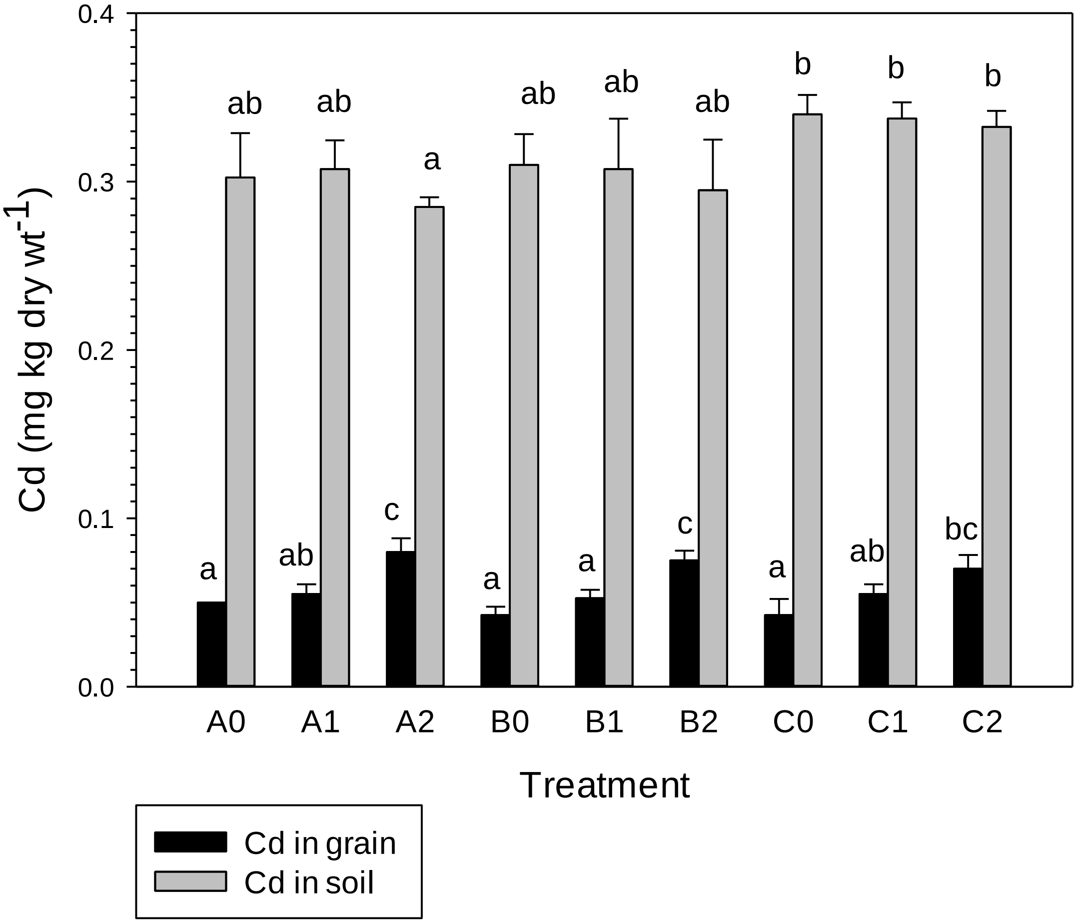

Cd (mg kg dry wt^{-1})
0.0
0.1
0.2
0.3
0.4
a
ab
ab
ab
c
a
a
ab
a
ab
c
ab
a
b
ab
b
bc
b
A0
A1
A2
B0
B1
B2
C0
C1
C2
Treatment
Cd in grain
Cd in soil

Fig. 2.

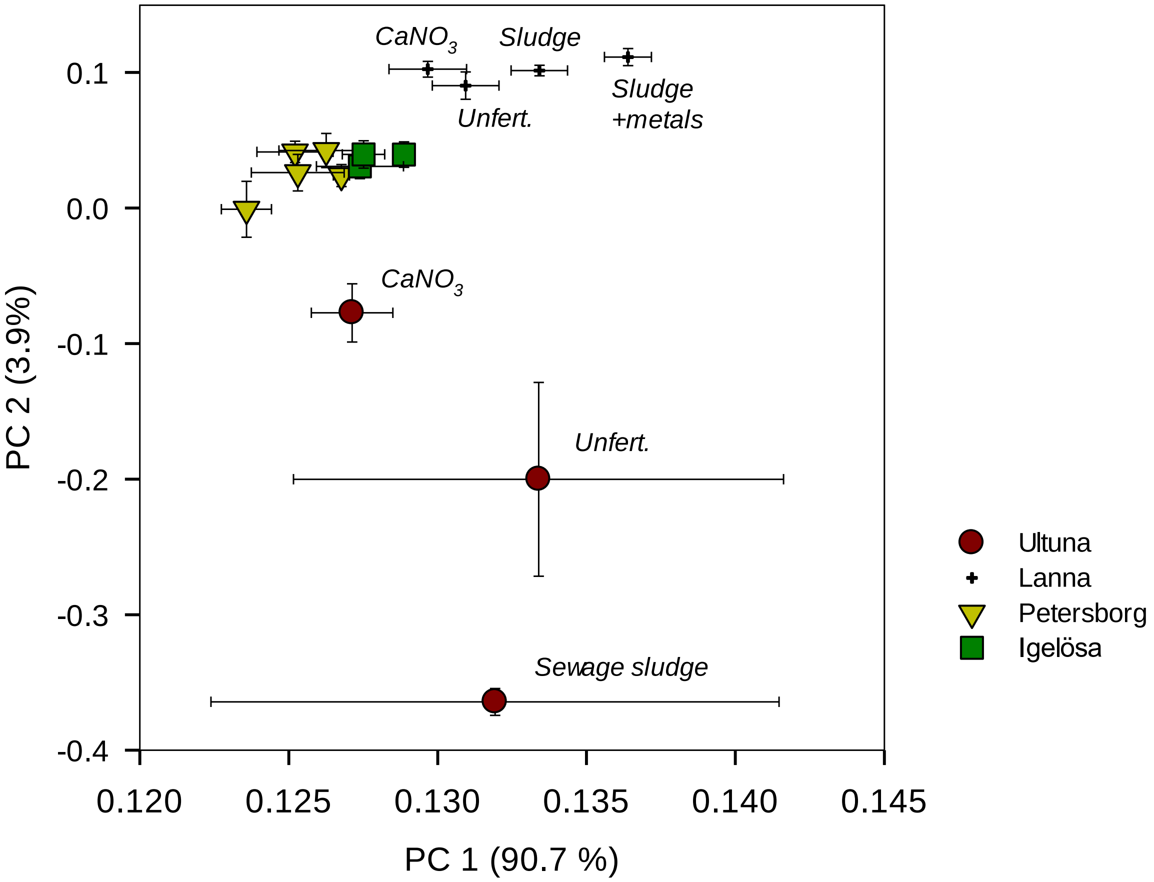
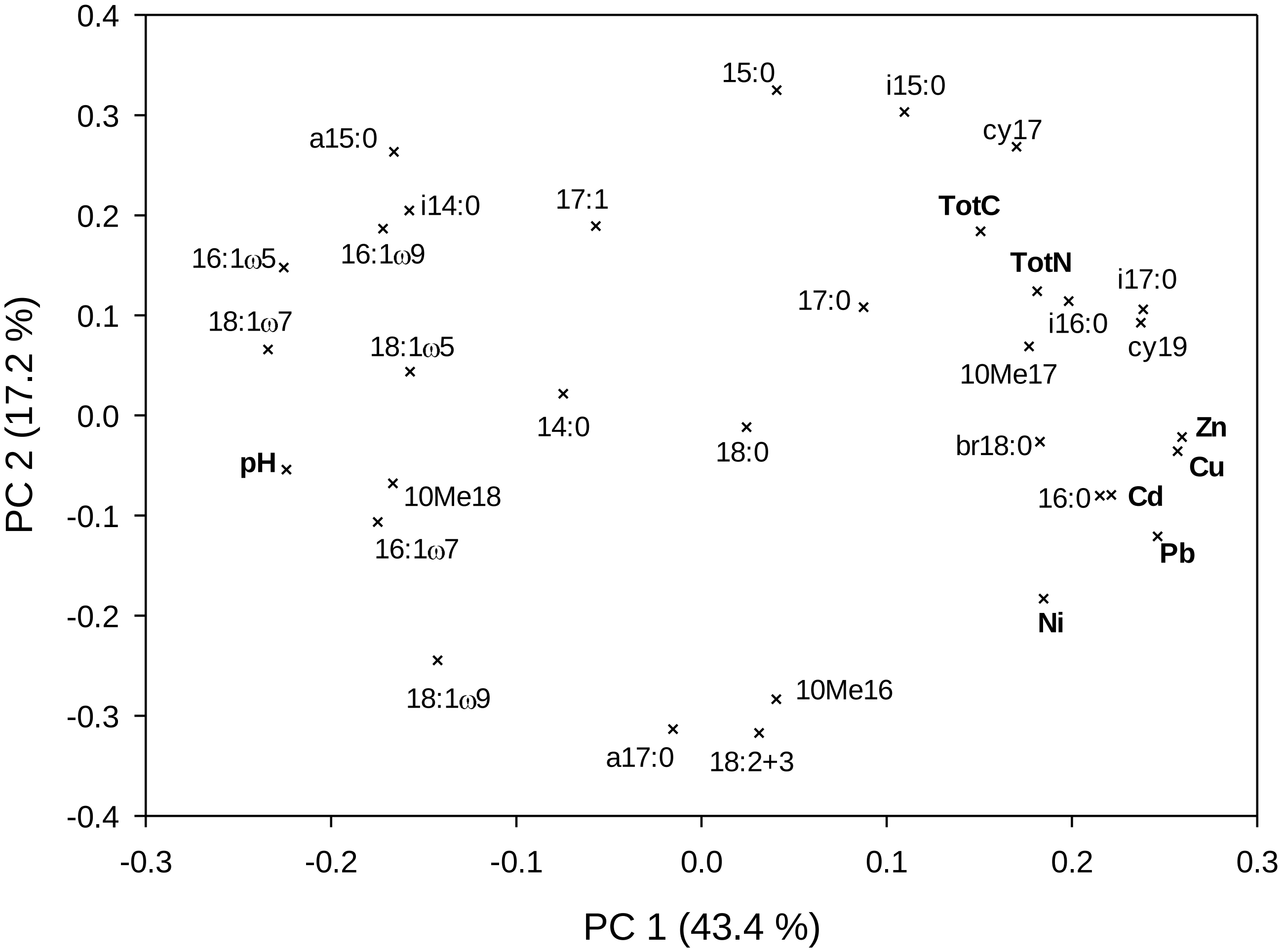

CaNO$_3$
Sludge
Unfert.
Sludge
+metals
CaNO$_3$
Unfert.
Sewage sludge
0.1
0.0
-0.1
-0.2
-0.3
-0.4
PC 2 (3.9%)
0.120
0.125
0.130
0.135
0.140
0.145
PC 1 (90.7 %)
Ultuna
Lanna
Petersborg
Igelösa

Fig. 3.



PC 2 (17.2 %)
PC 1 (43.4 %)
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.3
-0.2
-0.1
0.0
0.1
0.2
0.3
15:0
i15:0
a15:0
cy17
i14:0
17:1
TotC
16:1ω5
16:1ω9
TotN
i17:0
17:0
18:1ω7
i16:0
18:1ω5
cy19
10Me17
14:0
Zn
18:0
br18:0
pH
Cu
10Me18
16:0
Cd
16:1ω7
Pb
Ni
10Me16
18:1ω9
a17:0
18:2+3

Fig. 4.

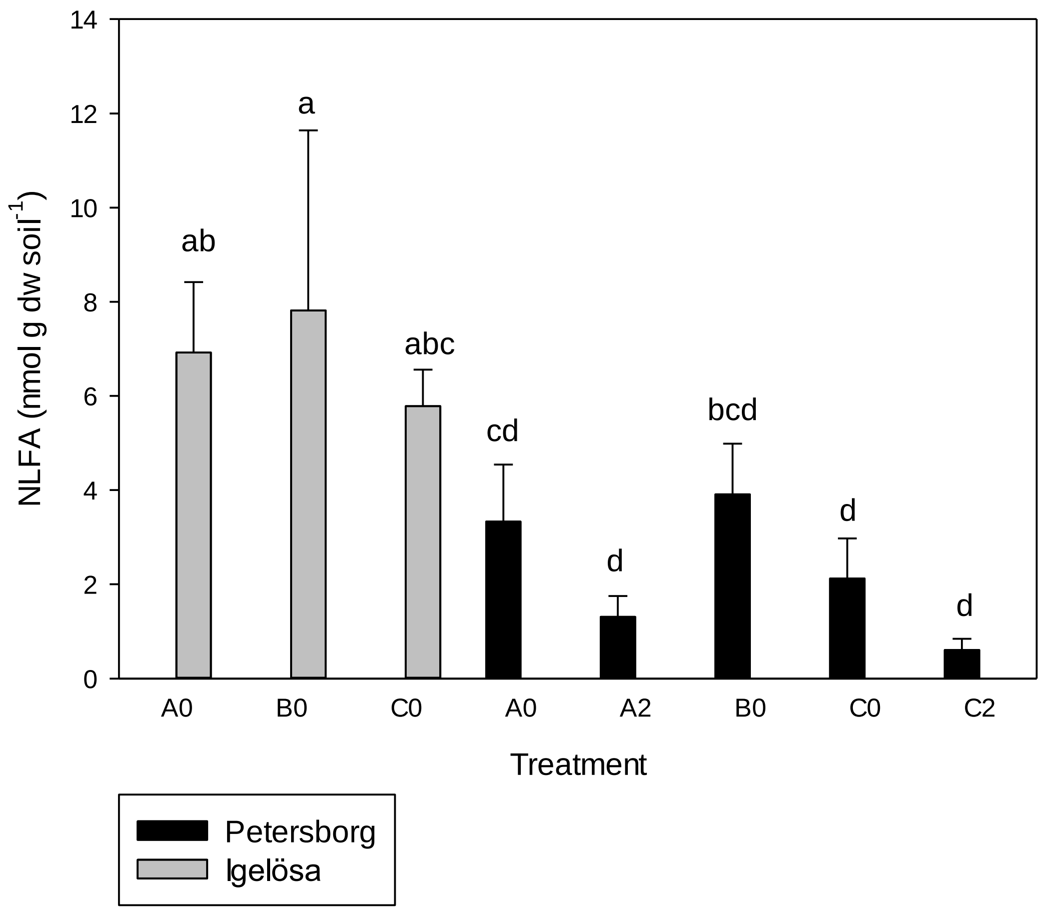

NLFA (nmol g dw soil^{-1})
14
12
10
8
6
4
2
0
ab
a
abc
cd
d
bcd
d
d
A0
B0
C0
A0
A2
B0
C0
C2
Treatment
Petersborg
Igelösa

Fig. 5.

Table 1

Characteristics of the four study sites

Site	Coordinates	Start (Year)	Soil type/Clay content	Amount of sludge applied
Ultuna	59°49’N, 17°39’E	1956	Clay loam (36.5% clay)	4 Mg C ha^{-1} every 2nd yr
Lanna	58°21’N, 13°06’E	1997	Silty clay (42% clay)	8 Mg dry matter ha^{-1} every 2nd yr
Petersborg	55°32’N, 13°00’E	1981	Sandy loam (14% clay)	4 or 12 Mg dry matter ha^{-1} every 4th yr
Igelösa	55°45’N, 13°18’E	1981	Loam (26% clay)	4 or 12 Mg dry matter ha^{-1} every 4th yr

Table 2
Properties of topsoil at the four study sites. Ult=Ultuna (measured 2009), Lan=Lanna (2010), Pet=Petersborg (2011), Ige=Igelösa (2011).
Original = Start years: Ultuna 1956, Lanna 1997, Petersborg and Igelösa 1981

Treatment	Total C (%)				pH			
	Ult	Lan	Pet	Ige	Ult	Lan	Pet[b]	Ige[b]
Original at start	*1.50*	*1.98*	*1.2*	*1.9*	*6.5*	*6.65*	*6.8*	*7.0*
Unfert. cropped	1.12	1.87	0.94	1.88	6.23	6.45	6.80	7.11
Normal fertilised	1.37	1.92	1.14	(2.0)[a]	6.68	6.55	6.62	n.m.
Sewage sludge	2.66	2.23	1.30	2.21	4.88	6.08	6.51	7.05

[a] = measured 2006

[b] It should be noted that all experiments at Petersborg and Igelösa were limed in 1998

n.m. = not measured

Table 3
Crop yields in the long-term field experiments, given as mean values (± standard deviation) relative to the fully NPK fertilised treatment as a reference at each site, *n=4.*
For Petersborg and Igelösa, treatments were: A = No sewage sludge, B = 4 Mg sludge every 4th year, C = 12 Mg sludge every 4th year; 0 = No NPK, 1 = half of normal NPK, 2 = normal NPK. Treatments with yields in italics are set to 100

Ultuna 1956-2009		Lanna 1997-2010			Petersborg 1982-2011	Igelösa 1982-2011
B. Cropped, unfertilised	56.5 (30.7)	I. Cropped, unfertilised	36.6 (7.7)	A0.	49.7 (17.5)	56.1 (19.5)
C. Calcium nitrate	*100*	B. Calcium nitrate	*100.0*	A1.	85.6 (8.7)	80.4 (10.6)
O. Sewage sludge	139.3 (67.8)	F. Sewage sludge	105.7 (25.6)	A2.	*100.0*	*100.0*
		G. Sewage sludge+metals	102.9 (26.3)	B0.	58.0 (23.6)	62.0 (20.4)
				B1.	90.1 (14.5)	87.2 (10.3)
				B2.	103.7 (11.0)	104.2 (6.0)
				C0.	64.6 (20.6)	70.8 (18.5)
				C1.	91.3 (12.1)	90.9 (10.7)
				C2.	106.7 (6.8)	104.7 (11.6)

Table 4

Mean metal concentrations (mg kg^{-1} DM) in soil samples from the Ultuna experiment (± standard deviation) on earlier analysis occasions and in those taken in 2009. Different letters within rows indicate significant difference (Tukey-Kramer HSD, α=0.05)

	Sewage sludge			Calcium nitrate 2009	Unfertilised 2009
	1968*	1974*	2009		
Al (g kg^{-1})	n.m.	n.m.	20.7 (0.6) ^{a}	20.2 (0.2) ^{a}	20.0 (0.39) ^{a}
Cd	0.74	0.72 (0.04)	0.73 (0.02) ^{a}	0.24 (0.02) ^{b}	0.23 (0.01) ^{b}
Co	11.6	11.6 (0.3)	*n.m.*	*n.m.*	*n.m.*
Cu	69	84 (4)	196 (4.6) ^{a}	27.8 (1.95) ^{b}	26.7 (0.57) ^{b}
Fe (g kg^{-1})	n.m.	n.m.	34.7 (0.7) ^{b}	27.6 (0.4) ^{a}	27.4 (0.3) ^{a}
Mn	413	420 (7)	478 (16) ^{a}	464 (6) ^{a}	457 (25) ^{a}
Ni	40.8	34.5 (0.4)	27.2 (0.92) ^{a}	22.9 (0.77) ^{b}	22.7 (0.79) ^{b}
Pb	38.5	40.4 (0.7)	41.0 (3.06) ^{a}	21.6 (0.70) ^{b}	21.3 (0.67) ^{b}
Zn	272	268 (9)	271 (9.18) ^{a}	87.6 (4.22) ^{b}	85.9 (3.00) ^{b}

* Data from Andersson and Nilsson (1975); extraction with 2M HNO_3 at 100°C (same as for samples from 2009)
n.m. not measured

Table 5

Mean metal concentrations (mg kg^{-1} DM) in soil samples from Lanna 2010 (± standard deviation). Different letters within rows indicate significant difference (Tukey-Kramer HSD, α=0.05)

	I. Cropped, unfertilised	B. Calcium nitrate	F. Sewage sludge	G. Sewage sludge + metals
Al (g kg^{-1})	21.0 (1.8) ^{a}	21.8 (2.1) ^{a}	21.8 (1.3) ^{a}	21.7 (0.7) ^{a}
Cd	0.121 (0.009) ^{b}	0.120 (0.005) ^{b}	0.140 (0.015) ^{b}	0.287 (0.017) ^{a}
Cu	9.09 (0.88) ^{b}	8.90 (0.79) ^{b}	20.8 (1.07) ^{a}	25.0 (1.32) ^{a}
Fe (g kg^{-1})	25.4 (2.6) ^{a}	26.4 (2.4) ^{a}	27.7 (1.8) ^{a}	26.4 (1.0) ^{a}
Mn	518 (175) ^{a}	483 (159 ^{a}	624 (215) ^{a}	461 (120) ^{a}
Ni	10.2 (0.95) ^{b}	10.2 (0.44) ^{b}	10.4 (0.63) ^{b}	17.9 (1.09) ^{a}
Pb	13.7 (0.81) ^{a}	14.1 (0.65) ^{a}	14.4 (0.86) ^{a}	14.4 (0.45) ^{a}
Zn	63.2 (6.1) ^{b}	65.6 (6.4) ^{b}	83.1 (5.6) ^{a}	79.3 (4.4) ^{a}

Table 6

Mean metal concentrations (mg kg^{-1} DM) in soil samples from Igelösa and Petersborg 2011 (± standard deviation for $n = 4$). A = No sewage sludge, B = 4 Mg sludge every 4th year, C = 12 Mg sludge every 4th year; 0 = No NPK, 1 = half of normal NPK, 2 = normal NPK. For each site, different letters within columns indicate significant difference (Tukey-Kramer HSD, $\alpha=0.05$)

	Cu		Zn		Cd		Pb		Ni	
Igelösa										
A0	15.3	(6.6) ^{a}	47.5	(1.9) ^{a}	0.30	(0.03) ^{a}	16.3	(0.50) ^{a}	12.0	(0.82) ^{a}
B0	22.3	(9.2) ^{a}	53.8	(1.0) ^{b}	0.31	(0.02) ^{ab}	16.8	(0.50) ^{a}	12.0	(0) ^{a}
C0	25.8	(1.7) ^{a}	58.0	(2.9) ^{b}	0.34	(0.019) ^{b}	17.3	(0.50) ^{a}	12.3	(0.50) ^{a}
Petersborg										
A0	9.4	(0.2) ^{c}	38.0	(2.9) ^{a}	0.24	(0.01) ^{a}	13.5	(0.58) ^{ab}	7.9	(0.39) ^{a}
A2	15.3	(12.5)^{abc}	38.3	(3.3) ^{a}	0.24	(0) ^{a}	12.8	(0.50) ^{a}	7.7	(0.66) ^{a}
B0	14.8	(1.5) ^{abc}	42.3	(2.8) ^{ab}	0.27	(0.01) ^{a}	14.0	(1.41) ^{ab}	8.3	(0.92) ^{a}
C0	21.0	(1.8) ^{a}	45.0	(1.6) ^{b}	0.26	(0.01) ^{a}	14.5	(0.58) ^{b}	8.2	(0.47) ^{a}
C2	20.3	(2.6) ^{ab}	45.3	(2.2) ^{b}	0.27	(0.01) ^{a}	14.0	(0.82) ^{ab}	8.3	(0.36) ^{a}

Table 7

Mean metal concentrations (mg kg^{-1} DM) in maize silage from the Ultuna experiment 2009 (± standard deviation, *n* = *4*). Different letters within rows indicate significant difference between treatments (Tukey-Kramer HSD, α=0.05). Data in italics from analysis of fodder rape, harvested as green mass at Ultuna 1974 (Andersson and Nilsson 1975)

	B. Cropped, unfertilised	C. Calcium nitrate	O. Sewage sludge		
				in fodder rape 1974	
Cd	0.109 (0.017) ^{a}	0.059 (0.014) ^{b}	0.130 (0.026) ^{a}	*0.303*	*(0.020)*
Co	0.032 (0.010) ^{a}	0.019 (0.009) ^{a}	0.017 (0.003) ^{a}	*0.094*	*(0.006)*
Cr	0.371 (0.414) ^{a}	0.137 (0.083) ^{a}	0.146 (0.060) ^{a}	*0.62*	*(0.18)*
Cu	3.56 (0.18) ^{a}	3.52 (0.69) ^{a}	4.11 (0.36) ^{a}	5.8	(0.2)
Mo	0.263 (0.083) ^{a}	0.392 (0.013) ^{a}	0.210 (0.012) ^{a}	*n.m.*	
Ni	0.358 (0.094) ^{b}	0.237 (0.117) ^{b}	0.606 (0.155) ^{a}	*4.43*	*(1.16)*
Pb	0.441 (0.153) ^{a}	0.366 (0.134) ^{a}	0.451 (0.314) ^{a}	*1.95*	*(0.50)*
Se	0.157 (0.065) ^{a}	0.130 (0.040) ^{a}	0.139 (0.037) ^{a}	*n.m.*	
Zn	13.2 (4.83) ^{b}	9.70 (0.78) ^{b}	91.0 (18.8) ^{a}	*98.3*	*(19.4)*

n.m. not measured

Table 8

Mean metal concentrations (mg kg^{-1} DM) in wheat grain from Lanna 2010 (± standard deviation). Different letters within rows indicate significant difference (Tukey-Kramer HSD, α=0.05)

	I. Cropped, Unfertilised	B. Calcium nitrate	F. Sewage sludge	G. Sewage sludge + extra metals
Cd	0.026 (0.003) ^{a}	0.032 (0.004) ^{a}	0.026 (0.004) ^{a}	0.059 (0.008) ^{a}
Co	0.0055 (0.0006)^{a}	0.0023 (0.0005)^{a}	0.0035 (0.0006)^{a}	0.0033 (0.0010) ^{a}
Cr	n.d.	n.d.	n.d.	n.d.
Cu	3.59 (0.16) ^{a}	3.91 (0.11) ^{a}	3.97 (0.12) ^{a}	3.93 (0.13) ^{a}
Mo	0.57 (0.17) ^{b}	1.07 (0.38) ^{a}	0.30 (0.03) ^{b}	0.33 (0.02) ^{b}
Ni	0.21 (0.06) ^{b}	0.12 (0.03) ^{b}	0.24 (0.05) ^{b}	0.63 (0.11) ^{a}
Pb	0.0055 (0.0013)^{a}	0.0090 (0.0028)^{a}	0.0063 (0.0010)^{a}	0.0075 (0.0072) ^{a}
Se	0.032 (0.0319 ^{a}	0.072 (0.032) ^{a}	0.024 (0.024 ^{a}	0.029 (0.039 ^{a}
Zn	26.8 (1.1) ^{a}	21.5 (1.7) ^{a}	32.6 (2.7) ^{a}	31.6 (2.3) ^{a}

n.d. = below detection limit

Table 9
PLFA levels (% of total moles) in topsoil samples from Ultuna 2009, given as mean values for $n = 4$ plots of each treatment. Different letters within rows (between treatments) indicate significant difference (Tukey-Kramer HSD, α=0.05)

	Cropped, unfertilised	Calcium nitrate	Sewage sludge
i14:0	0.29^{a}	0.20^{a}	0.11^{a}
14:0	1.61^{a}	1.34^{a}	1.50^{a}
i15:0	8.16^{a}	7.10^{a}	9.70^{b}
a15:0	5.23^{ab}	5.61^{b}	4.30^{a}
15:0	0.52^{a}	0.37^{a}	0.94^{a}
i16:0	2.60^{a}	3.13^{ab}	3.86^{b}
16:1ω9	1.06^{a}	1.61^{a}	0.69^{a}
16:1ω7	8.67^{b}	9.31^{b}	5.02^{a}
16:1ω5	3.01^{b}	3.12^{b}	1.32^{a}
16:0	12.88^{a}	11.62^{a}	17.43^{b}
17:1	4.60^{b}	4.02^{ab}	3.19^{a}
10me16	5.47^{a}	6.10^{a}	5.21^{a}
i17:0	1.88^{a}	2.14^{a}	4.77^{b}
a17:0	3.88^{a}	3.67^{a}	2.71^{a}
cy17:0	2.96^{a}	3.37^{a}	5.53^{b}
17:0	0.27^{a}	0.16^{a}	0.80^{b}
br18:0	0.98^{a}	2.27^{ab}	4.19^{c}
10Me17:0	1.83^{a}	1.61^{a}	2.67^{a}
18:2+18:3	9.54^{a}	6.00^{a}	5.16^{a}
18:1ω9	6.62^{ab}	7.89^{b}	5.36^{a}
18:1ω7	6.76^{b}	7.24^{b}	3.36^{a}
18:1ω5	1.29^{a}	1.53^{a}	0.65^{a}
18:0	3.09^{a}	2.87^{a}	4.02^{a}
10me18:0	3.57^{b}	4.40^{b}	1.76^{a}
cy19:0	3.25^{a}	3.36^{a}	5.79^{b}
Total PLFAs (nmol g soil^{-1})	46.3^{a}	41.5^{a}	89.2^{b}

Table 10

PLFA levels (% of total moles) in topsoil samples from Lanna 2010, given as mean values for *n = 4* plots of each treatment. Different letters within rows (between treatments) indicate significant difference (Tukey-Kramer HSD, α=0.05)

	Cropped, unfertilised	Calcium nitrate	Sewage sludge	Sewage sludge + metals
i14:0	0.97^{ab}	1.06^{b}	0.89^{ab}	0.84^{a}
14:0	1.54^{a}	1.85^{a}	1.72^{a}	1.46^{a}
i15:0	9.64^{ab}	9.41^{a}	9.97^{bc}	10.36^{c}
a15:0	8.04^{a}	8.17^{a}	8.25^{a}	8.22^{a}
15:0	0.95^{ab}	1.01^{b}	0.88^{ab}	0.82^{a}
i16:0	3.18^{a}	3.17^{a}	3.08^{a}	2.98^{a}
16:1ω9	1.73^{a}	1.78^{a}	1.50^{a}	1.62^{a}
16:1ω7	7.76^{a}	7.64^{a}	7.84^{a}	8.33^{a}
16:1ω5	4.25^{ab}	4.44^{b}	3.55^{a}	3.87^{ab}
16:0	11.79^{a}	11.68^{a}	11.80^{a}	11.67^{a}
17:1	4.88^{a}	4.63^{a}	5.33^{a}	5.43^{a}
10me16:0	4.68^{a}	4.19^{a}	4.18^{a}	4.28^{a}
i17:0	2.48^{a}	2.53^{a}	2.66^{a}	2.54^{a}
a17:0	2.54^{a}	2.63^{a}	2.71^{a}	2.57^{a}
cy17	4.75^{a}	4.77^{a}	4.51^{a}	4.66^{a}
17:0	0.42^{a}	0.15^{a}	0.55^{a}	0.54^{a}
br18:0	1.50^{a}	1.50^{a}	1.48^{a}	1.33^{a}
10me17:0	1.89^{a}	1.80^{a}	1.92^{a}	1.80^{a}
18:2	1.72^{a}	1.63^{a}	1.22^{a}	1.40^{a}
18:1ω9	5.78^{a}	5.96^{a}	6.31^{a}	6.05^{a}
18:1ω7	7.63^{a}	7.80^{a}	7.73^{a}	7.90^{a}
18:1ω5	1.32^{a}	1.34^{a}	1.41^{a}	1.38^{a}
18:0	3.30^{a}	3.39^{a}	3.14^{a}	3.07^{a}
10me18:0	3.71^{ab}	3.85^{b}	3.43^{ab}	3.11^{a}
cy19:0	3.53^{a}	3.63^{a}	3.95^{a}	3.77^{a}
Total PLFAs (nmol g soil^{-1})	49.2^{ab}	46.8^{ab}	72.7^{de}	74.8^{e}

Table 11

PLFA levels (% of total moles) in topsoil samples from Petersborg and Igelösa 2011, given as mean values for *n = 4* plots of each treatment. A = No sewage sludge, B = 4 Mg sludge every 4th year, C = 12 Mg sludge every 4th year; 0 = No NPK, 1 = half of normal NPK, 2 = normal NPK. Different letters within rows (between treatments) indicate significant difference (Tukey-Kramer HSD, α=0.05)

PLFA	Petersborg					Igelösa		
	A0	A2	B0	C0	C2	A0	B0	C0
i14:0	1.05 ^{b}	1.20 ^{bc}	1.19 ^{bc}	1.27 ^{c}	1.21 ^{bc}	0.70 ^{a}	1.28 ^{c}	1.25 ^{c}
14:0	2.21 ^{a}	2.17 ^{a}	2.21 ^{a}	1.91 ^{a}	2.21 ^{a}	2.00 ^{a}	1.68 ^{a}	1.51 ^{a}
i15:0	8.11 ^{bc}	7.66 ^{a}	8.09 ^{abc}	8.25 ^{c}	7.81 ^{ab}	7.82 ^{abc}	7.84 ^{abc}	8.01 ^{abc}
a15:0	7.25 ^{ab}	6.93 ^{a}	7.53 ^{abc}	7.84 ^{bc}	7.67 ^{abc}	7.87 ^{bc}	7.70 ^{abc}	8.25 ^{c}
15:0	0.77 ^{a}	0.78 ^{ab}	0.81 ^{abc}	0.86 ^{c}	0.85 ^{bc}	0.77 ^{a}	0.77 ^{a}	0.75 ^{a}
i16:0	2.93 ^{ab}	3.04 ^{bc}	2.88 ^{a}	2.93 ^{ab}	3.04 ^{bc}	3.07 ^{c}	2.96 ^{abc}	3.02 ^{bc}
16:1ω9	1.73 ^{c}	1.49 ^{ab}	1.54 ^{abc}	1.43 ^{ab}	1.36 ^{a}	1.56 ^{bc}	1.40 ^{ab}	1.41 ^{ab}
16:1ω7	7.36 ^{a}	7.47 ^{ab}	7.79 ^{abc}	7.53 ^{abc}	7.61 ^{abc}	8.15 ^{abc}	8.33 ^{c}	8.31 ^{bc}
16:1ω5	4.14 ^{bc}	3.96 ^{ab}	4.03 ^{abc}	4.01 ^{abc}	3.69 ^{a}	4.30 ^{bc}	4.37 ^{c}	4.26 ^{bc}
16:0	11.96 ^{d}	11.79 ^{cd}	11.65 ^{cd}	11.68 ^{cd}	11.59 ^{cd}	11.24 ^{bc}	10.91 ^{ab}	10.66 ^{a}
17:1	3.89 ^{a}	3.81 ^{a}	3.88 ^{a}	3.79 ^{a}	3.59 ^{a}	3.76 ^{a}	3.75 ^{a}	3.67 ^{a}
10me16:0	5.19 ^{a}	4.38 ^{a}	4.74 ^{a}	4.72 ^{a}	4.96 ^{a}	5.15 ^{a}	4.73 ^{a}	4.82 ^{a}
i17:0	2.18 ^{c}	1.96 ^{ab}	2.12 ^{bc}	2.06 ^{abc}	1.94 ^{ab}	1.94 ^{ab}	1.87 ^{a}	1.95 ^{ab}
a17:0	2.72 ^{a}	2.85 ^{ab}	2.86 ^{ab}	2.94 ^{ab}	3.15 ^{b}	2.80 ^{ab}	3.02 ^{ab}	3.15 ^{b}
cy17:0	3.71 ^{b}	3.56 ^{ab}	3.54 ^{ab}	3.52 ^{ab}	3.56 ^{ab}	3.43 ^{ab}	3.38 ^{a}	3.41 ^{ab}
17:0	0.55^{abcd}	0.56 ^{bcd}	0.60 ^{d}	0.58 ^{cd}	0.60 ^{d}	0.52 ^{abc}	0.50 ^{ab}	0.48 ^{a}
br18:0	1.96 ^{a}	2.05 ^{a}	1.80 ^{a}	1.77 ^{a}	1.88 ^{a}	2.40 ^{b}	2.44 ^{b}	2.43 ^{b}
10me17:0	1.57 ^{a}	1.80 ^{b}	1.76 ^{ab}	1.66 ^{ab}	1.77 ^{ab}	1.59 ^{a}	1.65 ^{ab}	1.65 ^{ab}
18:2+18:3	2.61 ^{a}	4.56 ^{ab}	3.51 ^{ab}	4.14 ^{ab}	3.84 ^{ab}	4.48 ^{ab}	5.32 ^{b}	5.25 ^{b}
18:1ω9	6.47 ^{a}	7.19 ^{bc}	6.58 ^{ab}	6.74 ^{abc}	7.37 ^{c}	6.71 ^{ab}	6.96 ^{abc}	7.00 ^{abc}
18:1ω7	7.95 ^{a}	8.09 ^{a}	8.33 ^{ab}	7.90 ^{a}	7.90 ^{a}	8.98 ^{c}	9.07 ^{c}	8.84 ^{bc}
18:1ω5	1.48 ^{cd}	1.55 ^{d}	1.42 ^{cd}	1.36 ^{bc}	1.47 ^{cd}	1.27 ^{ab}	1.19 ^{a}	1.16 ^{a}
18:0	4.96 ^{d}	4.03 ^{bc}	4.33 ^{c}	4.01 ^{bc}	3.78 ^{b}	3.25 ^{a}	2.95 ^{a}	2.89 ^{a}
10me18:0	3.48 ^{b}	3.74 ^{b}	3.34 ^{b}	3.66 ^{b}	3.83 ^{b}	2.72 ^{a}	2.79 ^{a}	2.69 ^{a}
cy19:0	3.80 ^{b}	3.40 ^{a}	3.49 ^{ab}	3.48 ^{ab}	3.37 ^{a}	3.56 ^{ab}	3.21 ^{a}	3.25 ^{a}
Total PLFAs (nmol g soil^{-1})	28.2 ^{a}	35.7 ^{ab}	32.2 ^{a}	36.9 ^{ab}	42.6 ^{b}	59.3 ^{c}	70.5 ^{d}	78.6 ^{d}