Plant Soil (2020) 449:249–266
https://doi.org/10.1007/s11104-020-04467-7

REGULAR ARTICLE

Carbon and nitrogen cycling in a lead polluted grassland evaluated using stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) and microbial, plant and soil parameters

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Received: 18 February 2019 / Accepted: 19 February 2020 / Published online: 14 March 2020

Abstract
Aims Carbon (C) and nitrogen (N) cycling are key ecosystem functions potentially altered by heavy metal pollution. We used an ecosystem approach to study the long-term effect of lead (Pb) on C and N cycles in a natural grassland in a former shooting range.
Methods Microbial activity was evaluated by substrate-induced respiration (SIR) in situ, adding isotopically labelled C_4-sugar to the soil. C and N contents and natural abundance of isotopes were measured in grass leaves, soil and microbial biomass together with root biomass.
Results A reduced microbial activity and microbial biomass per area, together with a higher soil C stock and C:N ratio suggested a lower microbial decomposition in high Pb compared to low Pb areas. A more closed N cycle in the high Pb area was indicated by 2–3‰ lower $\delta^{15}N$ in leaves and soil compared to low Pb areas. Higher $\delta^{13}C$ in leaves and higher root biomass but similar leaf nutrient contents indicated plant responses and adaptions to the high Pb.
Conclusions The applied ecosystem approach revealed that Pb slowed down the C and N cycles, possibly by indirect effects rather than by direct toxicity. The ecosystem seems to have adapted to altered conditions.

Keywords Ecological risk assessment · Field studies · Heavy metal contamination · Soil microbial respiration · Soil nutrient cycling · Stable isotopes

Responsible Editor: Xinhua He.

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Introduction

Many studies show that soil pollution by heavy metals can adversely affect the activity, species composition and abundance of soil microorganisms (Brookes 1995; Bååth 1989; Giller et al. 1998; Giller et al. 2009; Hinojosa et al. 2010). Soil microorganisms respond rapidly to perturbations and have therefore been viewed as providing an early sign for soil degradation (Hinojosa et al. 2010; Pankhurst et al. 1995). It has been proposed that microbial processes should be more included in risk assessments of polluted soil, with the aim to protect the soils' ecological function (ECHA 2016; EFSA 2016). Two key microbially mediated ecosystem processes potentially altered by heavy metal pollution are carbon (C) and nitrogen (N) cycling. Mineralization of C and N by soil microorganisms is tightly linked to plant production, controlling ecosystem processes and properties together (Bardgett et al. 2005; Wardle et al. 2004). However, processes at contaminated sites are rarely studied using a holistic ecosystem perspective or a community response which combine signals from soil, microorganisms and plants (Clements and Rohr 2009; Gómez-Sagasti et al. 2016; Krumins et al. 2015;

Rantalainen et al. 2006). Also, there is a need for more field studies to detect long-term changes of contaminated ecosystems (Gallagher et al. 2018; Giller et al. 2009; Krumins et al. 2015).

Soil microbial respiration is the most direct way to assess the activity of microorganisms in soil C cycling and its rate is directly related to the size of the active microbial biomass (Anderson and Domsch 1978; Anderson 1982; Babich and Stotzky 1985; Marstorp and Witter 1999). Respiration studies on polluted soils are mostly performed in the lab under controlled conditions, as natural fluctuations of environmental variables were regarded as problematic, hampering interpretation from the effect of pollutants (Brookes 1995). Indeed, responses of in situ basal respiration (BR) to metal contamination have not been consistent so far (Bian et al. 2015; Ramsey et al. 2005a; Ramsey et al. 2005b; Smorkalov and Vorobeichik 2011). However, soil sampling and processing disrupts the intimate relations between microorganisms, plant roots and soil particles. Such disturbances may release pollutants and previously protected labile substrates, which can trigger an immediate microbial response (Kapustka 1999). In fact, sub-optimal conditions in the field may produce the build-up of fractions of organic matter that can be decomposed rapidly under optimal conditions in the laboratory (Gregorich et al. 1994). For this reason, results on microbial respiration in the lab may not be directly transferrable to the field, favoring in situ studies in undisturbed soils. One major obstacle in the field is that soil respiration is a variable mix of respiration driven by photosynthates (so-called autotrophic respiration by roots and mycorrhizal fungi) and microbial respiration from heterotrophic decomposition of soil organic matter (SOM) (Comstedt et al. 2011; Ekblad and Högberg 2001; Hanson et al. 2000; Högberg et al. 2001). Another factor hampering the interpretation of the effect of metal pollution on soil respiration is that the effect of metal toxicity on microorganisms is difficult to distinguish from limitations of substrate availability (Hinojosa et al. 2010). To overcome these problems we applied an earlier developed method in which naturally isotopically labelled cane sugar is added to the soil (Ekblad and Högberg 2000; Ekblad and Högberg 2001; Högberg and Ekblad 1996). This enables microbial respiration of the added sugar to be distinguished from other respiration in the soil and overcomes substrate limitations. To our knowledge, this substrate-induced respiration (SIR) approach has never been applied to analyze microbial respiration of metal-polluted soil in situ.

Microbial biomass, often determined with the fumigation extraction method (Jenkinson and Powlson 1976), is an important measure to indicate the total size of the microbial community, including active, potentially active, and dormant fractions (Blagodatskaya and Kuzyakov 2013). Lower microbial biomass in polluted areas is generally reported (Bååth 1989; Giller et al. 2009), preferably expressed per total organic C or N (TOC or TON) (Bastida et al. 2008; Hinojosa et al. 2010). In this study we combine the measurement of microbial biomass and SIR to enable a comparison of the various microbial metabolic states. While the measurement of total microbial biomass includes all metabolic states, the measurement of SIR indicates the active part of the microbial biomass; thus, indicating the organisms that can readily utilize available substrates and grow (Blagodatskaya and Kuzyakov 2013).

Natural variations in stable isotopes in plants, soil and microbial biomass are ideal tools to increase our understanding of C and N cycles in ecosystems (Averill and Finzi 2011; Bowling et al. 2008; Buchmann and Ehleringer 1998; Dawson et al. 2002; Dijkstra et al. 2008; Högberg 1997; Kahmen et al. 2008). For instance, plant $\delta^{13}C$ can be used to assess intrinsic water use efficiency (Dawson et al. 2002). Plant $\delta^{15}N$ reflects the net effect of a range of processes such as N uptake, mineralization, nitrification, and losses through denitrification (Dawson et al. 2002; Hobbie and Högberg 2012; Högberg 1997). The $\delta^{15}N$ in plant leaves mirror the isotopic signature of the plant available N during the growing season, while soil C and N isotopes and contents integrate over longer time scales, e.g. decennia or centuries (Craine et al. 2015; Menichetti et al. 2013). Microbial $\delta^{15}N$ correlates with higher C and N availability and ecosystem N processing and has therefore been mentioned as a tool to study ecosystem function (Dijkstra et al. 2006; Dijkstra et al. 2008). To our knowledge, no study on polluted sites have combined measurements of $\delta^{13}C$ and $\delta^{15}N$ in plants, SOM, and microbial biomass to unravel effects from metal pollutants on C and N cycles.

Here we present a case study at an old shooting range, a natural grassland polluted with lead (Pb) in central Sweden. Outside the maximum shot fall zone, less polluted areas exist within the same field, which makes this area excellent to study the impact of Pb under

the same ecological and environmental conditions. While our approach can be applied to other metal-polluted soils, Pb in firing range soils are of particular international concern because it is an important cause of contaminated sites worldwide (ITRC 2005; Sanderson et al. 2011; Sorvari et al. 2006). Also, potential impacts of Pb from shooting ranges on the environment and human health can be high (Fayiga and Saha 2016; Sanderson et al. 2011; Sanderson et al. 2018).

In this study, we aim to gain a deeper understanding on the effect of Pb on C and N cycles under field conditions by analyzing the natural abundance of $\delta^{13}C$ and $\delta^{15}N$ and C and N contents in plants, soils, and microorganisms, and perform in situ microbial respiration using an isotope labelling technique. In this way, we combine fast-responding microbial parameters with slower-responding soil and plant parameters to achieve a holistic ecosystem approach that may uncover long-term changes. Based on what is known from the literature, we hypothesized that high Pb contaminated areas would show indications of lower microbial activity and slower C and N cycling compared to low Pb contaminated areas.

Materials and methods

Survey area

The abandoned Munkatorp shooting range is located 4 km North East from the city center of Örebro, Sweden (59°18′08.8”N 15°16′32.0″E), and currently forms part of a nature reserve (Fig. 1). Before 1970, the area was used as a pasture. Clay target shooting activities (skeet and trap) and military training took place between 1970 and 2008, with about 50,000–100,000 firings annually (Eriksson 2011; Lin et al. 1995). Due to the directional nature of the shooting activities (ITRC 2005), the highest Pb concentrations (400–4000 mg Pb kg^{-1}) are found 120–180 m from the firing point in the middle of the field (Fig. 1) (Eriksson 2011). The west side has levels around 50 mg Pb kg^{-1} and is used as a low Pb polluted comparison. This low Pb side, however, contains polycyclic aromatic hydrocarbons (PAH) due to the presence of clay target remains (Table 1). The whole field has an even species composition and is dominated by the grass *Alopecurus pratensis* L., with sparse occurrences of other grasses such as *Deschampsia cespitosa* (L.) P. Beauv., *Dactylis glomerata* L., *Festuca* sp. and *Agrostis* sp. and some herbs (e.g. *Centaurea jacea* L.). The areas have a similar total above ground plant biomass with 982 ± 308 g m^{-2} (mean ± SD) in the low Pb area and 1193 ± 333 g m^{-2} in the low Pb area. The soil has an organic topsoil with the mineral component having a clay-loam texture.

The measurements and sampling were performed 12 and 14 October 2016. Weather conditions were stable, with a 100% cloudy cover and air temperatures of 8 °C and 5 °C respectively. Three groups of plots, consisting of three 243 mm diameter plots each, were laid out at a distance between groups of 10–15 m and 1–2 m between plots at the precise locations for the highest Pb concentrations based on the previous screening of the area with X-Ray Fluorescence (XRF). In a similar way, 3 × 3 plots were laid out in the low Pb polluted area at a distance of about 130 m from the high polluted area. Thus, there were in total 18 plots; 9 in the low Pb area and 9 in the high Pb area. All plots were placed at grass-dominated locations.

Tracer study with $\delta^{13}C$ for in situ microbial respiration

To get a relative measure of microbial activity in situ, we applied an approach involving C_4 sugar as an isotopic tracer (Ekblad and Högberg 2000; Högberg and Ekblad 1996). Cane sugar is a C_4 sugar that has a slightly higher abundance of ^{13}C ($\delta^{13}C \approx -10‰$) than the endogenous C in a C_3 ecosystem ($\delta^{13}C = -25$ to $-28‰$). Only microorganisms will respire the added sugar because roots exclusively respire C products that are assimilated by the plant. By analyses of the $\delta^{13}C$ of the CO_2 evolving from the soil with or without adding cane sugar, the fraction of C_3 (root and microbial) and C_4 (microbial respiration) can be calculated (see Menichetti et al. 2013).

Soil respiration measurements were performed on two of the plots in each group of three plots in the two areas applying methods described previously (Ekblad and Högberg 2000; Menichetti et al. 2013). About 1 h prior to the first soil respiration measurement, a 243 mm PVC collar was inserted 2 cm into the soil and standing vegetation was cut away from the plot. This was made to avoid measuring dark respiration of leaves. The collar was then removed until the start of the measurement, assuring no CO_2 would be build up above the soil surface prior to the measurement. The two plots were then treated with either 250 ml H_2O Milli RX distilled water as a control and BR measurement, or 250 ml of

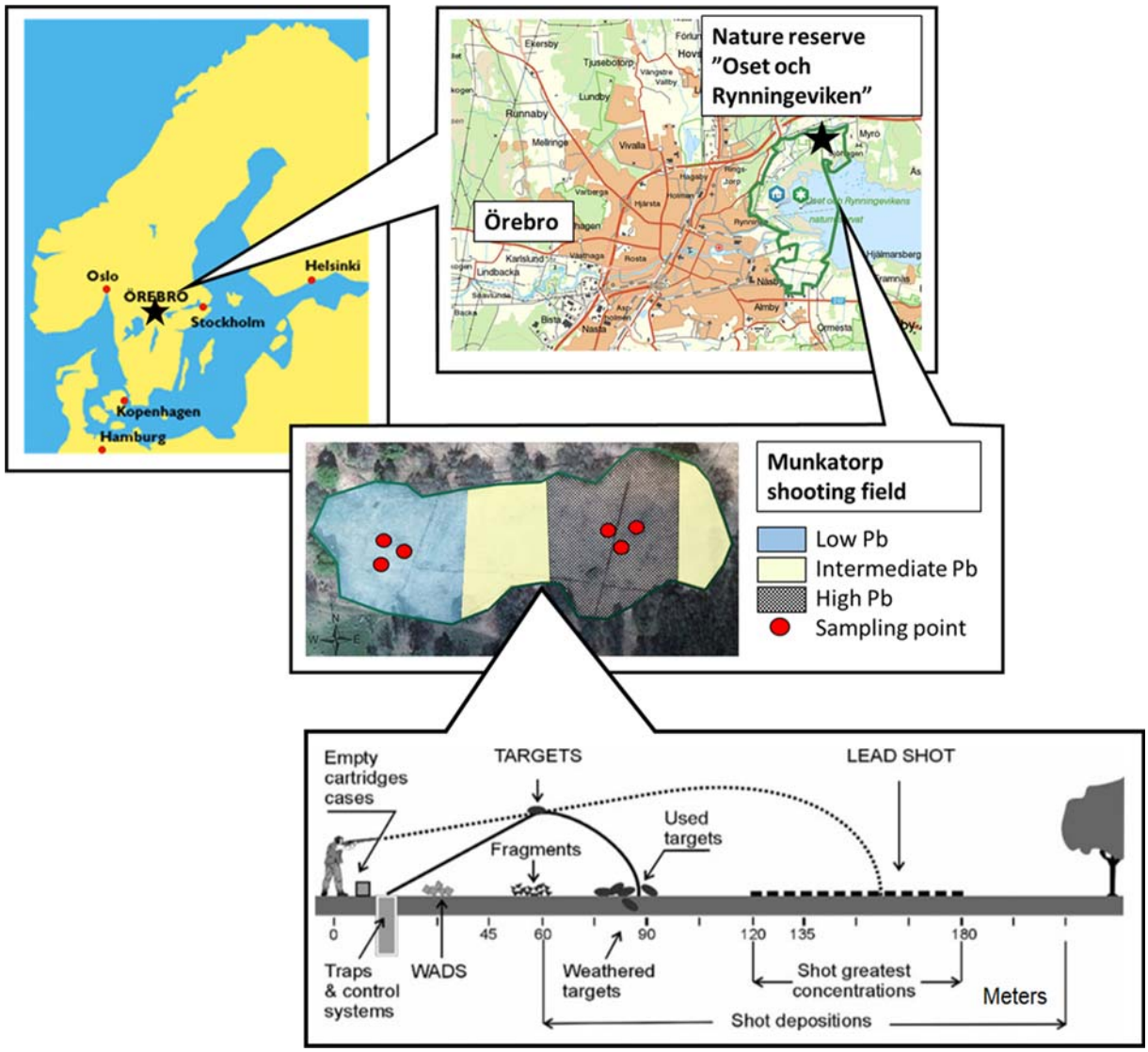

Fig. 1 Location of Örebro and the Pb-polluted site Munkatorp in central Sweden. The shooting field is approximately 220 m long and 50 m wide. Sampling points in low and high Pb polluted parts of the field are indicated. Lowest panel: Schematic representation of distances of fall out of shooting debris and Pb shot (modified from ITRC 2005)

cane sugar solution for a SIR measurement (66.7 g L^{-1} cane sugar, 2.67 g NH_4Cl L^{-1} and 1.95 g KH_2PO_4 L^{-1}). The N and P were included to assure that microbial growth would not be nutrient limited (Ekblad and Nordgren 2002). At 1 h after sugar or water additions, a headspace over the soil was created by pressing an air-tight opaque PVC cylinder, with a diameter of 243 mm and a height of 120 mm, 2 cm into the soil and placing a removable lid with a 12 kg weight on top (Ekblad and Högberg 2000). A plastic cover was used as a wind shield to minimize impact from possible pressure differences leading to air leakage (Menichetti et al. 2013). The air in the headspace was circulated in a closed loop through a portable spectroscopic instrument (Picarro G2201i; Picarro Inc., Santa Clara, California USA; calibrated at Örebro University isotope laboratory prior to sampling) enabling continuous measurements of the concentration and the $\delta^{13}C$ of the CO_2 in the headspace. Sampling was in each case made for at least 20 min. After this the headspace was removed and the position was marked with a low PVC ring of the same material as the cylinder. The first measurement, 1 h after

Table 1 Soil contamination of low and high lead (Pb) polluted areas at 0–5 cm and 5–15 cm depth of a grassland in a former shooting range. Only results for contaminants of potential concern at shooting ranges are shown. Arsenic (As), copper (Cu), nickel (Ni), and lead (Pb) in *aqua regia*, Pb and antimony (Sb) measured by X-Ray Fluorescence (XRF), and sum of 16 polycyclic aromatic hydrocarbons as listed by the US Environmental Protection Agency (∑16 EPA PAH) are shown. For analysis of metals in *aqua regia*, the values show a mean concentration (mg kg DM^{-1}) for 2 composite samples, while for XRF mean ± SD for n = 3 samples are shown

Depth	Pb	As - aqua regia (mg kg^{-1})	Cu - aqua regia (mg kg^{-1})	Ni - aqua regia (mg kg^{-1})	Pb - aqua regia (mg kg^{-1})	Pb - XRF (mg kg^{-1})	Sb - XRF (mg kg^{-1})	∑16 EPA PAH (mg kg^{-1})
0-5 cm	low	6,0	29	22	73	66 ± 19	< LOD	6.7
0-5 cm	high	7,0	22	17	2050	4668 ± 95	< LOD	< 1.3
5-15 cm	low	4,3	32	20	40	61 ± 24	< LOD	–
5-15 cm	high	7,3	30	20	1740	2583 ± 253	< LOD	–

addition of sugar, gives an indication of microbial activity; while a second sampling, 48 h later, provides an indication of microbial growth or changed activity (Dilly 2001; Nordgren 1992). The first two minutes of the CO_2 analyses were discarded because there was a two-minute lag period until the increase in CO_2 concentration was steady in the sampling loop (Menichetti et al. 2013). The proceeding two minutes were taken to determine the respiration rate from the slope, representing the most linear segment of the curve. This respiration rate (ppm/min) was converted to mg CO_2 C m^{-2} h^{-1} by multiplying with the molar volume of gas in headspace and tubes, corrected for air pressure and air temperature. All except the first two minutes of the data was used to calculate the $\delta^{13}C$ of the respired CO_2 using a Keeling plot approach (Keeling 1958; Pataki et al. 2003). Microbial respiration (SIR-C_4) was then calculated using a mass balance equation (Ekblad and Högberg 2000) with the formula:

$$pC4 = \frac{\delta^{13}C_{SIR} - \delta^{13}C_{BR}}{\delta^{13}C_{C4} - \delta^{13}C_{BR}} \quad (1)$$

Where *p*C4 is the proportion of C_4 respiration of total respiration, $\delta^{13}C_{SIR}$ is the estimated isotopic signature of respired CO_2 following cane sugar addition (SIR), $\delta^{13}C_{BR}$ is the estimated isotopic signature of respiration after water addition (BR), and $\delta^{13}C_{C4}$ is the isotopic signature of the added cane sugar solution (−10.80‰).

Sampling of soils and plant materials

Green grass leaves were collected from all three plots at all six groups and were pooled into one sample per group. Leaves were placed at −20 °C in the end of the sampling day. Soil samples were taken with a steel soil corer (7.2 cm diameter; height 5 cm) at two depths (0–5 cm and 5–15 cm). Three cores (subsamples) were taken from each plot and depth and the material from within each plot was pooled making it into two samples per plot, one sample per depth. This resulted in a total of thirty-six soil samples; two Pb levels x nine plots x two depths. The collected soil samples were stored at 4 °C for no longer than 10 days, then sieved through a 2 × 4 mm mesh while all roots were carefully separated before freezing to −20 °C for long-term storage.

Roots were thoroughly washed with cold tap water to clean out the soil from the roots and then sorted into fine roots (diameter < 1 mm), large roots (>1 mm), and dead roots. The roots were considered to be living if they showed tensile strength and had white vascular tissue (Boström et al. 2007). Dry matter (DM) content of the sieved soil fractions, grass leaves, and roots were determined after freeze-drying at −60 °C. The dry bulk density of each soil sample was calculated after subtracting the root weight from the soil cores. Microbial biomass in the upper 5 cm of plots was determined by fumigation extraction using 0.5 M K_2SO_4 (Jenkinson and Powlson 1976; Vance et al. 1987). Extracts were stored at −20 °C and then freeze-dried. Measurements on soil properties and root biomass were performed on each sample, but results were then averaged to give one value per plot for all parameters.

Total Pb concentration was measured three times per individual sample on the fresh sieved soil samples using a handheld XRF (Thermo Niton XL3 Analyzer, Thermo Fisher Scientific Inc., Waltham, MA, USA) and adjusted for soil water content. One composite soil sample per high/low Pb area and 0–5 cm/5–15 cm depth was used to measure the pH in a solution of 1:2.5 soil: deionized

water. Composite soil samples were also sent to ALS Scandinavia AB and analyzed according to ISO 18287:2008 for the sum of 16 polycyclic aromatic hydrocarbons as listed by the US Environmental Protection Agency (∑16 EPA PAH), and to Eurofins Environment Testing Sweden AB for total phosphorous (P) (ISO 11466/EN13346 mod./ICP-AES). Grass leave samples were sent to Eurofins Environment Testing Sweden AB for analysis of total P (NMKL No 1611998 mod./ICP-AES) and Pb (NMKL No 1611998 mod./ICP-MS.). In June 2019, the soil at the areas was sampled again; taking out at least 30 subsamples per area and depth, combined to 2 composite samples per area and depth, then sent to Eurofins Environment Testing Sweden AB for metal analysis in *aqua regia* by ICP-AES (ISO 11885:2009/SSa 028311 utg 1).

C, N, and $\delta^{13}C$ and $\delta^{15}N$ natural abundance measurements

Freeze-dried microbial extracts, soil, and grass leaves were milled to powder with a ball-mill and their C and N content and isotopic signatures were determined using an elemental analyzer (EuroEA3024; Eurovector, Milan, Italy) coupled on line to a continuous flow Isoprime isotope ratio mass spectrometer (Isoprime Ltd., Manchester, UK). For analysis, 5 mg of freeze-dried soil, 5 mg of microbial extract, or 1.5 mg of grass leaves per sample was used. The results of $\delta^{13}C$ and $\delta^{15}N$ were expressed as parts per thousand (‰) deviations, in the ratio of the heavy to the light isotope of each element, from the international standards (Vienna Pee Dee Belemnite, V-PDB, for ^{13}C and atmospheric N_2 for ^{15}N). The working standard used was a wheat flour with a $\delta^{13}C$ of −26.231‰ and a $\delta^{15}N$ of 5.817‰. The working standard had been calibrated with the international standards IAEA-CH6 for ^{13}C and IAEA-N1 for ^{15}N. The precision of analyses of 10 standard samples was 0.056‰ for $\delta^{15}N$ and 0.016‰ for $\delta^{13}C$.

Microbial C and N were calculated using the formula:

$$Biomass\ C = EC/kEC \tag{2}$$

$$Biomass\ N = EN/kEN \tag{3}$$

where: EC or EN = (organic C or N extracted from fumigated soils) – (organic C or N extracted from non-fumigated soils); kEC and kEN reflect the extraction efficiency of the microbial biomass C and N and we applied a kEC = 0.4 (Vance et al. 1987) and a kEN = 0.54 (Brookes et al. 1985). Isotopic signatures of the microbial biomass (MB) were calculated using mass balance equations (Dijkstra et al. 2006):

$$\delta^{13}C_{MB} = \left[\delta^{13}C_F{*}C_F - \delta^{13}C_E{*}C_E\right]/C_{MB} \tag{4}$$

$$\delta^{13}N_{MB} = \left[\delta^{13}N_F{*}N_F - \delta^{13}N_E{*}N_E\right]/N_{MB} \tag{5}$$

Where F is fumigated, and E is the extractable non-fumigated fraction. The results were reported separately for water and sugar treated plots, since the addition of cane sugar and nutrients induces microbial growth and alters isotopic signatures.

Statistical analysis

The effect of pollution level, soil depth, and the interaction effect of pollution level and depth on soil parameters, microbial, and root biomass was performed using two-way ANOVA tests in JASP 0.9.2.0 (https://jasp-stats.org/). Tukey's post-hoc test and simple main effects analysis was used after ANOVA to identify significant different groups ($P \leq 0.05$). For the other variables (respiration and leaves), two sample – two tailed student's t-tests assuming equal variance were carried out to test whether differences were statistically significant ($P \leq 0.05$) between low and high Pb contaminated areas. Significance was validated with the Benjamini-Hochberg procedure as a multiple comparisons post-hoc test, allowing a false discovery rate of 10% (Benjamini and Hochberg 1995).

Results

Soil contamination and properties

The *aqua regia* Pb concentrations in the composite samples show a high level of Pb with an average contamination level of 2050 mg Pb kg^{-1} in the 0–5 cm soil and a concentration of 1740 mg kg^{-1} in the 5–15 cm layer of the high Pb area (Table 1). The lower Pb area had much lower levels of Pb, and are around the reference level of 50 mg kg^{-1} for sensitive land use as imposed for Sweden (Swedish-EPA 2016). Other metals of potential concern in shooting ranges, like arsenic (As), copper (Cu), and nickel (Ni), did not differ in

concentration levels between the high and low Pb area, while antimony (Sb) was below detection limits. The *aqua regia* Pb concentrations in the composite samples were similar to the XRF values in the low Pb area, while in the high Pb polluted area concentrations obtained by XRF were 2 times higher in the upper 5 cm. There were no significant differences in soil moisture content, bulk density, and pH between the two areas (Table 2).

Soil C and N

Integrated over both soil layers (0–15 cm), a significant increase in total C stock was observed from 6.9 kg m^{-2} in the low Pb area to 8.1 kg m^{-2} in the high Pb area, a rise of 17% (Table 3). Total N stock (0–15 cm) was not different between low and high Pb areas. The C:N ratio decreased with soil depth and was significantly higher in the high Pb polluted area compared to the low Pb polluted area at both soil depths, as well as integrated over the whole 0–15 cm (Table 3). The $\delta^{13}C$ signatures of the soil were similar in the two areas. In contrast, the $\delta^{15}N$ was significantly lower in the high Pb area compared to the low Pb area and the difference was 3‰ in the upper 5 cm and 1.9‰ at 5–15 cm depth. Both the $\delta^{13}C$ and the $\delta^{15}N$ increased with soil depth.

In situ microbial respiration

The basal respiration rate was similar in the two areas and total respiration was roughly doubled 1 h after addition of the sugar (SIR) compared to the watered plots (BR) and increased by a further 50% relative to the respiration from the watered plots 48 h later (Fig. 2). The isotopic signatures of basal respiration ($\delta^{13}C_{BR}$; only water added) was −27.3‰ in the low Pb plots and − 26.1‰ in the high Pb plots. One hour after sugar addition, the mean isotopic signature ($\delta^{13}C_{SIR}$) had increased to −21.7‰ in the low Pb area and to −23.5‰ in the high Pb area. After a further 48 h the mean $\delta^{13}C_{SIR}$ had increased to −18.1‰ in the low Pb plots and to −19.1‰ in the high Pb plots, suggesting an increasing contribution of the added C_4 sugar (−10.8‰) to microbial respiration. When these isotope values were used to calculate the microbial respiration of the added C (SIR-C_4) applying eq. (1) we found that the respiration of the added C (SIR-C_4) was 72 mg C m^{-2} h^{-1} in the low Pb area and only 39 mg C m^{-2} h^{-1} in high Pb area, a significant reduction of 44% (Fig. 2a). After 48 h there was at least a doubling of the SIR-C_4 compared to the value after 1 h and the low and high Pb polluted plots showed similar rates of SIR-C_4 (Fig. 2b). The C_3 respiration of SIR behaved in a similar way in the low and high Pb polluted areas; it increased by 30–40% 1 h after sugar addition but was 48 h later back to a similar rate as in the water treated plots (BR).

Microbial biomass

Total microbial biomass C and N per gram soil was not different between the topsoil of the low Pb area and the high Pb area (Table 4). Total microbial biomass C and N on an area basis in the 0–5 cm layer was 42% lower in the high Pb plots compared to the low Pb plots. Expressed as a % of soil total organic C or N (TOC or TON), microbial biomass C was 38% lower and N was 34% lower in the high Pb area compared to the low Pb area. No significant differences in the microbial C:N ratios and the $\delta^{13}C$ and the $\delta^{15}N$ of the microbial biomass were detected between the areas, this is due to high variability among the samples (Table 4, Fig. 3ab). Cane sugar treated plots had increased $\delta^{13}C$ values compared to the watered control by an average of 2.7‰ (Table 4), indicating that 17.5% of the microbial biomass was of C_4 origin.

Grass leaves and root biomass

The total C, N and P contents of grass leaves, as well as the C:N and N:P ratio, were not altered by the Pb pollution (Table 5). The $\delta^{13}C$ signature of grass leaves in the high Pb area was enriched by 2‰ relative to the leaves in the low Pb area. In contrast, the $\delta^{15}N$ in the leaves from the high Pb area was depleted by 2‰ relative to the leaves from the low Pb area (Table 5, Fig. 3ab). The leaf Pb content ranged from <0.045–0.23 mg kg^{-1} in the low Pb area to <0.045–8.1 mg kg^{-1} in the high Pb area and was not statistically different between the areas. Total root biomass was three times higher in the top 5 cm soil in high Pb plots compared to low Pb plots (Table 6). This is mainly because of contribution of fine roots (<1 mm), while the biomass of roots with a diameter > 1 mm and dead roots did not differ between the areas. Between 5 and 15 cm depth, root biomass was much smaller and showed no difference between the areas.

Table 2 Soil properties of low and high lead (Pb) polluted soil at 0–5 cm and 5–15 cm depth of a grassland in a former shooting range. Dry bulk density, soil moisture, pH, and phosphorous (P) concentration are shown. Values show mean concentration (mg kg DM^{-1}) ± SD for n = 3 and *P*-values show results of ANOVA

Depth	Pb	Dry bulk density (g cm^{-3})	Soil moisture (% of DM)	pH	P (mg kg^{-1})
0-5 cm	low	0.43 ± 0.05	60.4 ± 7.4	5.52 ± 0.07	1.3
0-5 cm	high	0.27 ± 0.13	60.8 ± 13.4	5.62 ± 0.19	1.3
5-15 cm	low	0.83 ± 0.11	37.0 ± 3.5	5.47 ± 0.04	0.94
5-15 cm	high	0.89 ± 0.09	40.3 ± 6.7	5.40 ± 0.21	0.95
P-value[1]	Pb	0.41	0.67	0.91	na
	depth	***	***	0.34	na
	Pb x depth	0.09	0.59	0.56	na

[1] * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

Discussion

Negative impact on microbial biomass and activity

Negative effects on microbial biomass and activities were found in the high Pb area compared to the low Pb area. Firstly, the respiration of C_4 C 1 h after sugar addition was 44% lower in the high Pb compared to the low Pb area (Fig. 2a). Secondly, the total microbial biomass on an area basis and per gram C, as determined by fumigation extraction, show a similar reduction in response to high Pb (Table 4). Thirdly, the fact that BR was similar in the two areas (Fig. 2) together with a three times higher fine root biomass in the high Pb compared to the low Pb area also suggests that microbial respiration was reduced in the high Pb area. However, 48 h after sugar addition the respiration of C_4 C was increased 2–3 times, and was similar in low and high Pb areas. This suggests that the microorganisms were carbon limited and managed to increase their activity also in the high Pb area.

The isotopic signatures of the microbial biomass indicate that about 17.5% of the microbial C was of C_4 origin in the plots that received the C_4 sugar. Nevertheless, this contribution is similar to the increase in total SIR between the first and second sampling but markedly smaller than the recorded increase of the C_4 component of respiration over the same period (Fig. 2). This supports the view that microbial activity was highly C limited. The above microbial measurements taken

Table 3 Soil carbon (C) and nitrogen (N) concentration and stocks, C:N ratios and $\delta^{13}C$ and $\delta^{15}N$ of 0–5 cm, 5–15 cm and 0–15 cm depth of low and high lead (Pb) polluted soil of a grassland site in a former shooting range. Values show mean ± SD for *n* = 3 and *P*-values show results of ANOVA for 0–5 cm and 5–15 cm and t-tests for the integrated layer of 0–15 cm

Depth	Pb	C (%)	N (%)	C:N	C (kg m^{-2})	N (kg m^{-2})	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
0-5 cm	low	11.23 ± 0.45	0.90 ± 0.03	12.46 ± 0.42	2.42 ± 0.31	0.19 ± 0.03	−28.37 ± 0.22	5.23 ± 0.5
0-5 cm	high	17.89 ± 6.22	1.36 ± 0.47	13.16 ± 0.16	2.18 ± 0.66	0.17 ± 0.05	−28.41 ± 0.22	2.2 ± 0.85
5-15cm[1]	low	5.37 ± 0.13	0.50 ± 0.01	10.75 ± 0.08	4.47 ± 0.57	0.42 ± 0.05	−27.87 ± 0.20	6.37 ± 0.22
5-15cm[1]	high	6.64 ± 0.57	0.56 ± 0.05	11.82 ± 0.13	5.97 ± 1.04	0.51 ± 0.09	−28.04 ± 0.14	4.52 ± 0.22
P-value[2]	Pb	0.06	0.09	***	0.38	0.05	0.40	***
	depth	**	**	***	0.28	0.72	**	***
	Pb x depth	0.17	0.18	0.21	0.10	0.13	0.58	0.08
0–15 cm	low	7.32 ± 0.13	0.63 ± 0.00	11.32 ± 0.18	6.89 ± 0.6	0.61 ± 0.05	−28.04 ± 0.19	5.99 ± 0.31
0–15 cm	ligh	10.39 ± 2.23	0.83 ± 0.17	12.26 ± 0.14	8.14 ± 0.39	0.67 ± 0.04	−28.16 ± 0.03	3.74 ± 0.03
P-value[2]		0.08	0.12	**	*	0.17	0.33	***

[1] results of C and N stock (kg m^{-2}) for 5–15 cm were recalculated to similar volume as 0–5 cm to facilitate ANOVA testing

[2] * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

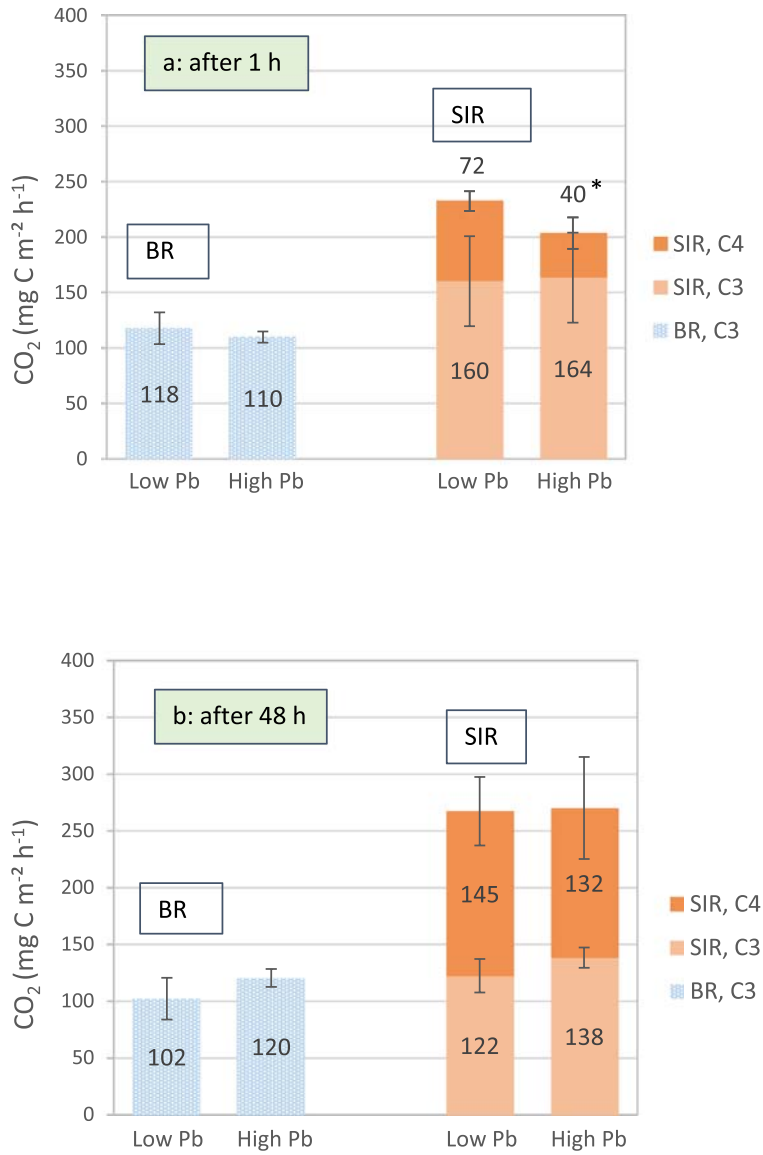

Fig. 2 In situ basal (BR) and substrate induced respiration (SIR) after addition of either deionized water or cane sugar solution (C_4-sugar) to plots in a low lead (Pb) polluted area and high Pb polluted area of a grassland in a former shooting range. (**a**) 1 h after addition (**b**) 48 h after addition. The contribution of endogenous C (C_3) and added C (C_4) to the respired CO_2 is shown. Error bars show SD for $n = 3$. Statistically significant results of t-tests between low and high Pb polluted area means are indicated with * = $P \leq 0.05$

together suggest that a larger fraction of the microbial biomass may be inactive/dormant (Blagodatskaya and Kuzyakov 2013) in the high Pb compared to the low Pb area and labile substrates were needed to activate it (see further discussions below).

Lower $\delta^{15}N$, higher $\delta^{13}C$, and higher root biomass in plants at high Pb areas

The 2–3‰ higher $\delta^{15}N$ of plant leaves and SOM in the low Pb compared to the high Pb area suggests an effect of Pb on the N cycle. Isotopic fractionation against ^{15}N during plant uptake can cause isotopic differences between the N source and the plant, but when N is limiting, plant uptake will reduce the soluble soil N and plants will have a similar $\delta^{15}N$ as the source (Högberg 1997). In our study, the $\delta^{15}N$ of plant leaves and of SOM in the upper 5 cm of the soil were similar, the horizon with much of the fine root biomass and probably highest N uptake. This suggests isotopic fractionation during uptake to be minor and a need for an alternative explanation to the observed $\delta^{15}N$ differences. Soil processes such as nitrification and denitrification discriminate against ^{15}N leading to losses that are ^{15}N depleted

Table 4 Microbial biomass of the upper soil layer (0–5 cm). Soils were sampled 48 h after in situ treatment with MilliQ water or cane sugar in low and high lead (Pb) polluted areas of a grassland in a former shooting range. Microbial biomass carbon (MBC) and nitrogen (MBN) are shown per gram soil, per m^2 and, as a % of total soil organic carbon or nitrogen (TOC or TON). Also provided are microbial C:N ratios and microbial isotopic signatures ($\delta^{13}C$ and $\delta^{15}N$). Values show means ± SD for $n = 3$ and results of ANOVA

Pb	Treat-ment	MBC (mg C g^{-1} soil)	MBN (mg N g^{-1} soil)	MBC (g C m^{-2})	MBN (g N m^{-2})	MBC (% of TOC)	MBN (% of TON)	microbial C:N	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Low	water	1.25 ± 0.19	0.17 ± 0.01	28.50 ± 6.38	4.00 ± 0.77	1.19 ± 0.10	2.04 ± 0.27	7.22 ± 1.48	−26.48 ± 1.10	8.21 ± 0.46
Low	sugar	1.39 ± 0.22	0.19 ± 0.01	27.64 ± 6.25	3.77 ± 0.57	1.27 ± 0.15	2.17 ± 0.08	7.30 ± 0.83	−22.97 ± 2.45	8.64 ± 3.88
High	water	1.18 ± 0.48	0.17 ± 0.05	14.40 ± 8.22	2.05 ± 0.98	0.73 ± 0.46	1.36 ± 0.68	6.67 ± 1.08	−25.78 ± 0.57	5.45 ± 3.58
High	sugar	1.23 ± 0.16	0.17 ± 0.02	17.94 ± 8.17	2.48 ± 1.14	0.79 ± 0.18	1.44 ± 0.37	7.27 ± 0.25	−23.87 ± 0.19	5.83 ± 3.09
P-value[1]	Pb	0.56	0.58	*	*	*	*	0.64	0.90	0.15
	treat-ment	0.55	0.69	0.76	0.86	0.65	0.67	0.58	**	0.83
	Pb x treat-ment	0.75	0.47	0.62	0.55	0.92	0.90	0.66	0.34	0.99

[1] * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

Table 5 Grass leaf properties at low and high lead (Pb) polluted plots in a grassland of a former shooting range. Carbon (C), nitrogen (N), phosphorous (P) and Pb contents are shown, along with C:N and N:P ratios and $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures. Values show means (%, ‰ or mg kg^{-1} DM) ± SD for $n = 3$; *P*-values show results of t-tests

Grass leaves	Low Pb	High Pb	*P*-value[1]
C (%)	46.33 ± 0.37	46.37 ± 0.20	0.88
N (%)	3.17 ± 0.22	3.01 ± 0.32	0.51
C:N	14.64 ± 0.93	15.54 ± 1.76	0.48
$\delta^{13}C$ (‰)	−31.72 ± 0.16	−29.72 ± 0.25	***
$\delta^{15}N$ (‰)	3.94 ± 0.22	1.98 ± 0.17	***
P (%)	0.25 ± 0.006	0.23 ± 0.025	0.15
N:P	12.5 ± 0.58	13.3 ± 0.28	0.11
Pb (mg kg^{-1})	0.10 ± 0.11	4.68 ± 4.17	0.13

[1] * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

relative to the source (Dawson et al. 2002; Högberg 1997). This result in a positive correlation between $\delta^{15}N$ and the total loss of N from a system (Craine et al. 2015; Högberg 1997). Thus, a plausible explanation to the higher $\delta^{15}N$ in the low Pb compared to the high Pb system, is that the N losses have been somewhat lower in the high Pb area, implying that high Pb resulted in a more closed N cycle. In support of this hypothesis, we found a higher SOM stock and C:N ratio in the high Pb area compared to the low Pb area (Table 3), suggesting that SOM decomposition (N mineralization) and N cycling has slowed down. Similarly, higher N mineralization rates and lower C:N ratios in soil correlated with an enrichment of $\delta^{15}N$ in plant leaves in an unpolluted forest soil (Garten 1993; Garten Jr and Miegroet 1994). The reduced N cycling has not significantly altered the plant-available N since we found around 3% N in grass leaves in both areas, which is well within the range of the 1–6% found in plants worldwide (Reich et al. 1998; Wright et al. 2004). A molecular approach, analyzing gene copies of different genes involved in N cycling, may reveal more details on which processes have been affected (Hallin et al. 2009; Wallenstein and Vilgalys 2005). Previous studies on trees in heavy metal polluted mine tailing soil and an industrial urban soil also found a depletion in ^{15}N (Parraga-Aguado et al. 2014; Radwanski et al. 2017), which is in agreement with our finding in this study. On the contrary, in a forest soil surrounding a copper smelter, various tree and herbaceous species classified according to their mycorrhizal association showed an increase in ^{15}N, which was linked to increased rooting depth and/or a decrease in mycorrhizal formations (Chashchina et al. 2018).

Plant $\delta^{13}C$ mirrors intrinsic water use efficiency, the ratio of net photosynthesis to transpiration, during the growing season (Dawson et al. 2002). Plants with lower stomatal conductance will save water but at the same time reduce the CO_2 concentration in the leaves and therefore reduce the isotopic fractionation during photosynthesis (Farquhar et al. 1989). Leaf CO_2 concentration can also be reduced by high rates of photosynthesis (Marshall et al. 2007). Thus, the 2.0‰ higher $\delta^{13}C$ in leaves from the high Pb compared to the low Pb plots (Fig. 3a) could theoretically be a result of more closed stomata or higher photosynthetic activity, or a combination of both (Dawson et al. 2002; Marshall et al. 2007; Scheidegger et al. 2000). High Pb in plant cells has been found to negatively affect normal plant-water relations through impairments of stomatal regulation and through effects on water uptake by roots (Rucińska-Sobkowiak 2016; Seregin and Ivanov 2001). Photosynthesis can be impaired by Pb, caused by direct inhibition of the

Table 6 Root biomass at 0–5 cm and 5–15 cm depth of low and high lead (Pb) polluted grassland soil in a former shooting range. Values show mean root content (g kg^{-1} DM) ± SD for n = 3; *P*-values show results of ANOVA

Depth	Pb	Total root (g kg^{-1} soil)	Root < 1 mm (g kg^{-1} soil)	Root > 1 mm (g kg^{-1} soil)	Dead root (g kg^{-1} soil)
0-5 cm	Low	30.3 ± 13.7	17.7 ± 9.8	11.3 ± 5.1	1.4 ± 1.0
0-5 cm	High	100 ± 64.3	78.4 ± 43.5	18.8 ± 19.6	2.8 ± 2.8
5-15 cm	Low	2.9 ± 1.1	2.1 ± 1.1	0.69 ± 0.53	0.11 ± 0.09
5-15 cm	High	4.7 ± 5.6	2.9 ± 2.7	1.6 ± 2.7	0.14 ± 0.21
P-value[1]	Pb	0.10	*	0.49	0.42
	Depth	*	**	*	0.05
	Pb x depth	0.11	*	0.59	0.43

[1] * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

synthesis of several important enzymes in the chloroplasts and indirectly by metal-induced water stress, Mg and Fe deficiency, and stomatal closure (Seregin and Ivanov 2001). The four times higher root biomass in the high Pb compared to the low Pb area speaks against a negative effect of Pb on photosynthesis and rather points towards effects on the water balance or other unknown effects. At present, we cannot decide which mechanism caused the difference in leaf $\delta^{13}C$ between high and low Pb areas. Possibly, a dual isotope approach measuring both $\delta^{13}C$ and $\delta^{18}O$ in grass leaves may differentiate between biochemical and stomatal limitations and may reveal whether water use efficiency and/or photosynthesis was affected (Scheidegger et al. 2000).

How can we explain increased biomass allocation to roots? Reduced root growth and development is reported as a phytotoxic effect of Pb in some vascular plant species in short-term pot experiments (de Vries et al. 2013; Nagajyoti et al. 2010; Påhlsson 1989; Zhang et al. 2014), but plants exposed to low concentrations of Pb have been found to promote growth of root systems (Seregin and Ivanov 2001). However, it is not clear to what extent results from such studies can be transferred to field conditions. A meta-analysis of plant biomass allocation in response to environmental variables showed that allocation to roots was most strongly associated with lower nutrient availability and to a lesser extent with drought stress (Poorter et al. 2012). With regard to nutrient availability, we found normal N and P concentrations and N:P ratios in grass leaves (Hejcman et al. 2016) and no differences between high and low Pb areas (Table 5). We cannot exclude that the similar leaf nutrient concentrations is the result of higher root biomass successfully compensating for a difference in N or P availability. Water stress, on the other hand, would also potentially explain the difference in leaf $\delta^{13}C$ between the two areas. However, soil water content did not differ at sampling. Also, it is unlikely that water availability differs between the two areas since the site is flat and has the same soil parent matter. Therefore, the main trigger for the increase in root biomass remains unknown.

Pb and plant-soil-microbe interactions affecting C and N cycling

Plants, soil and microorganisms interact in multiple ways (Bardgett 2017; Pregitzer et al. 2010; Schweitzer et al. 2008; Wardle et al. 2004) and their response to Pb contamination will be intertwined (Giller et al. 1998). A slower C and N cycling may be caused by direct toxic effects on the microbial community but may also be caused indirectly by changes in the soil environment or by changes driven by plants. It is known that some metals such as Pb may decrease the amount of substrate available for respiration through the formation of complexes, making it difficult to distinguish a metal toxicity effect from an effect on substrate availability (Giller et al. 1998). Indeed, our results after adding labile substrates suggest that a limited access to labile C may have caused lower respiration rates and accumulation of C in the high Pb area. A higher C sequestration may also be related to a higher microbial turnover time or microbial carbon use efficiency (Spohn et al. 2016).

Alternatively, the increased input of root-derived C from the three times larger root biomass in the high Pb area has resulted in the larger C storage. It has been reported that root-derived litter contributes more to the build-up of SOM than aboveground litter (Clemmensen et al. 2013; Kätterer et al. 2011). In addition to a higher input of root-derived C, root traits themselves can influence C cycling, nutrient cycling and soil formation and structural stability (Bardgett et al. 2014). For instance, dense root systems with higher specific root lengths can reduce nitrous oxide emissions and N leaching from soil (Abalos et al. 2014), which is in line with our finding of a relative $\delta^{15}N$ depletion and an increase in root biomass in the high Pb area. Generally, plants with conservative traits, e.g. more allocation of C to roots, are associated with slow rates of N cycling and high soil C sequestration (Wardle et al. 2004).

Methodological considerations

The in situ stable isotope technique applied by us allow studies of microbial respiration in an environment relatively undisturbed by the sampling. The removal of plant shoots, insertion of the soil collar 2 cm into the soil, and the creation of a headspace above the soil may have caused some disturbance to respiration (Heinemeyer et al. 2011; Mills et al. 2011) and CO_2 diffusion (Davidson et al. 2002; Rochette and Hutchinson 2005). Thorough tests of the method have shown such problems to be minor (Ekblad and Högberg 2000; Ekblad et al., 2002) and should nonetheless be similar on all plots. One assumption when applying this method is that root respiration of the added sugar did not occur, and thus respiration of C_4-C was entirely microbial. Indeed, experiments on grasses and trees suggest that plants or mycorrhizal fungi in symbiosis do not take up and respire exogenously fed sugars (Bingham and

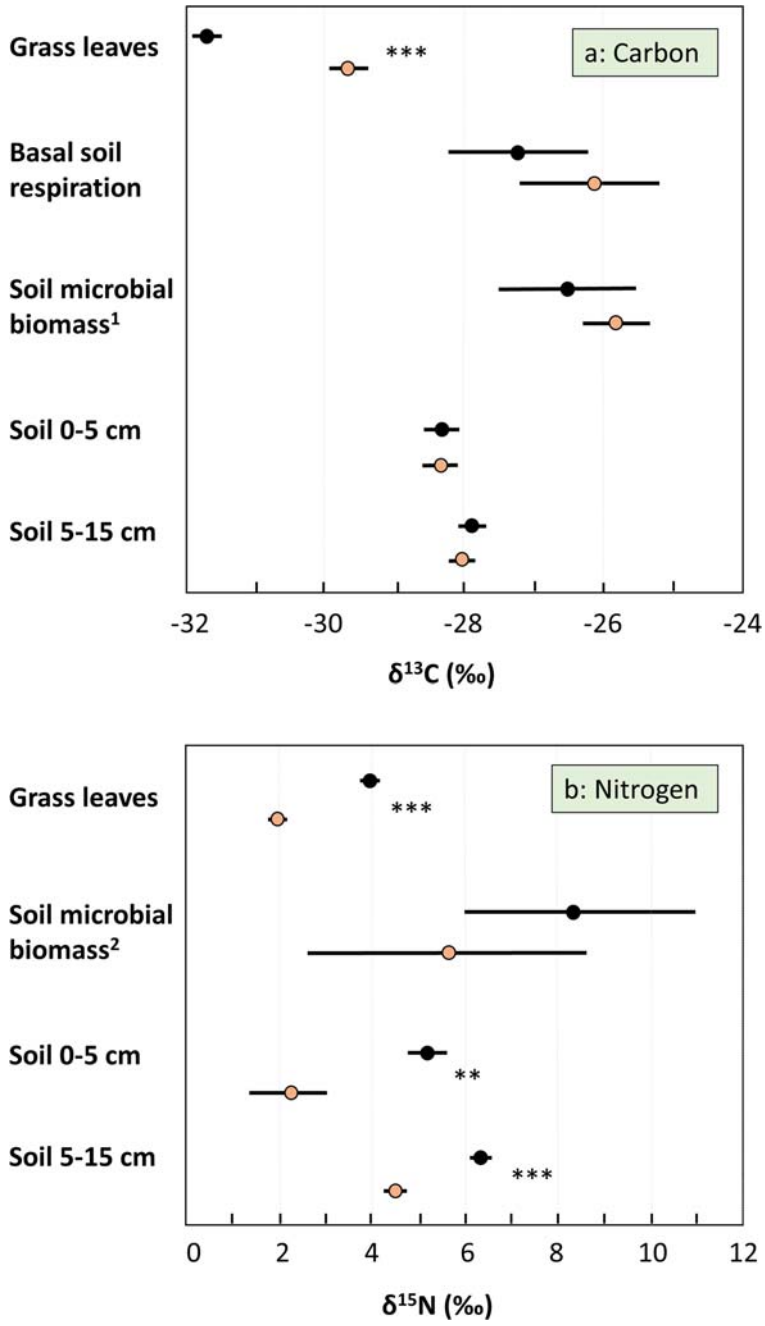

Fig. 3 Patterns of natural abundance of isotopic signatures **(a)** Carbon (C) isotopes ($\delta^{13}C$) **(b)** Nitrogen (N) isotopes ($\delta^{15}N$) of grass leaves, microbial biomass and soil of low lead (Pb) (closed circles; ●) and high Pb polluted plots (orange circles; ○) in a grassland in a former shooting range. Error bars show SD when larger than the symbols. Statistically significant differences from ANOVA (soil, microbial biomass) and t-test (basal soil respiration and grass leaves) between areas with low Pb and high Pb polluted plots are marked with * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$. [1]Soil microbial biomass $\delta^{13}C$ show water-treated plots only as the cane sugar changed microbial biomass $\delta^{13}C$. [2]Soil microbial biomass $\delta^{15}N$ show an average of water- and cane sugar treated plots

Stevenson, 1993, Cheng et al., 1993; Ekblad and Högberg 2000). A more recent review of the uptake of organic C from the soil by plants (Schmidt et al. 2013) show this to be rather exceptional or marginal for green terrestrial plants (Johansson et al. 2015).

Despite that the studied grass covered field is very flat and the low and high Pb areas have a very similar parent matter, soil, and vegetation, we cannot be completely sure that the detected differences between the two areas were present already before the shooting activities were initiated. Further studies on grasslands and other ecosystems are therefore needed to test the generality of our results and the applicability of our methods.

The lower $\delta^{15}N$ of plants and soil was not reflected in a significantly lower $\delta^{15}N$ value of microbial biomass in the high Pb soil. However, the error in the ^{15}N analyses

of the microbial biomass was rather large due to small available biomass. An increased number of samples and possibly larger analytical sample size would be desirable in follow-up studies.

Ecological risks of a long-term disturbed ecosystem in perspective

Although this long-term polluted grassland, like many other polluted ecosystems, can hold a natural vegetation cover, our research shows that Pb pollution has caused hidden changes in ecosystem functions. Some of these changes may restrict further spreading of Pb from the soil, reducing the ecological risks off-site. For instance, although high root biomass and the increased C contents in the highly polluted soil are signs of ecosystem disturbance, this also naturally creates a situation of "phytostabilization" (Chaney et al. 1997; Salt et al. 1995). Increased soil C is limiting Pb mobility in soil and bioavailability to organisms as SOM has been recognized as a critical component for heavy metal retention in soils by contributing to adsorption and complexation (Sanderson et al. 2011). In addition, metals can be immobilized in the root zone in several ways by adsorption onto roots, accumulation and absorption by roots, and precipitated within the root zone (Chaney et al. 1997; Kucharski et al. 2005; Salt et al. 1995). Also, a high root density and well-established vegetation cover prevent erosion caused by wind and surface runoff. Furthermore, Pb is a metal which generally accumulates in roots, with very little translocation to shoots (Siedlecka 1995). Thus, despite detecting various negative effects of Pb on nutrient cycling, the slowly adapted ecosystem as a whole seems to be able to bear strong resistance to contamination, as previously reported (Radwanski et al. 2017; Rantalainen et al. 2006; Selonen and Setälä 2015).

Conclusion

In this study, we studied a natural grassland that was polluted by Pb from shooting activities. We found important differences in plant, soil and microbial properties, which indicated C and N cycles have slowed down resulting in a higher soil C:N ratio and accumulation of SOM. The Pb pollution caused reduced microbial biomass and activity, possibly by indirect effects via substrate limitation rather than via direct toxicity. Yet, the ecosystem seems to have adapted to the new conditions, rather than to have deteriorated. We show in this study that an ecosystem approach with stable isotope techniques, fast-responding microbial parameters and slower-responding plant and soil parameters can be used to evaluate nutrient cycling in situ. We studied one contaminated site and further studies on grasslands and other ecosystems are needed to test the general applicability of our findings. In general, very little is known on in situ functional ecosystem responses in long-term polluted areas and more research is needed to unravel patterns. Such an improved understanding is needed to feed discussions on what parameters, directions and rate of change can indicate if nutrient cycles are impaired.

Acknowledgements This research was funded to AE from Structor Miljöteknik AB and the Faculty Board of Business, Science and Engineering of Örebro University. The authors would like to thank Structor Miljöteknik AB for XRF data and technical support during sampling, and three anonymous reviewers for constructive comments. Siamak Sobhanei is acknowledged for his language support.

Funding Information Open access funding provided by Örebro University.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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