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The impact of metal pollution on soil faunal and microbial activity in two grassland ecosystems

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Capsule: Feeding activity and functional diversity influenced by metal pollution.

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Abstract

In this study the influence of metal pollution on soil functional activity was evaluated by means of Bait lamina and BIOLOG® EcoPlates™ assays. The *in situ* bait lamina assay investigates the feeding activity of macrofauna, mesofauna and microarthropods while the BIOLOG® EcoPlate™ assay measures the metabolic fingerprint of a selectively extracted microbial community. Both assays proofed sensitive enough to reveal changes in the soil community between the plots nearest to and further away from a metal pollution source. Feeding activity (FA) at the less polluted plots reached percentages of 90% while plots nearer to the source of pollution reached percentages as low as 10%. After 2 and 6 days of incubation average well colour development (AWCD) and functional richness (R') was significantly lower at the plots closest to the source of pollution. While the Shannon Wiener diversity index (H') decreased significantly at sites nearer to the source of pollution after 2 days but not after 6 days of incubation. Arsenic, Cu and Pb correlated significantly and negatively with feeding activity and functional indices while the role of changing environmental factors such as moisture percentage could not be ruled out completely. Compared to the Bait lamina method that is used *in situ* and which is therefore more affected by site specific variation, the BIOLOG assay, which excludes confounding factors such as low moisture percentage, may be a more reliable assay to measure soil functional activity.

***Key words*:** Bait lamina strips, BIOLOG® EcoPlates™, soil fauna, trace metals, soil physicochemical properties

Highlights:

► Feeding activity was lower at most metal polluted plots compared to less polluted plots.

► Indices AWCD, R and H' proved sensitive indicators of microbial functioning.

► Differences in moisture levels complicate the assessment of metal related effects.

1. Introduction

Amongst other factors the rate of organic matter decay in soils primarily depends on the biomass and metabolic activity of soil micro– and macrofauna present in the soil and litter layer (Eisenbeis, 2006; Hättenschwiler et al., 2005; Stefanowicz, 2006). In metal–polluted soils, sensitive species are replaced by more resistant ones, which often are not able to perform the same ecological functions (André et al., 2009; Davis et al., 2004; Van Beelen and Doelman, 1997). Disturbances of organic matter decomposition may lead to abnormalities in the turnover of elements, reduced soil porosity, restricted availability of nutrients to plants and reduced ecosystem productivity (Ruiz et al., 2008; Witkamp and Ausmus, 1976). Because of the central role soil fauna play in the fragmentation of organic matter, measurements of feeding activity can act effectively as an indicator of the integrity of the soil community (Filzek et al., 2004b; Kools et al., 2009).

One screening method to estimate soil faunal feeding activity is the bait lamina assay, which was developed by von Törne (1990). Bait lamina are made of rigid plastic strips each having sixteen holes, filled with bait which mimics dead plant material. When placed into the soil, this substrate will be utilized by soil fauna, resulting in a measure of the feeding activity of macro–, meso and micro–organisms in the soil (Kratz, 1998; Jensen et al., 2006; Weeks et al., 2004). This method has been proven to be successful in various ecological studies

investigating the effect of fluctuating soil moisture, temperature and climate change on feeding activity (Gongalsky et al., 2008; Larink, 1993; Meyer, 1996; Simpson et al., 2012). In addition the bait lamina assay has also been used in ecotoxicological studies where the impact of metal pollution on feeding activity was investigated (André et al., 2009; Filzek et al., 2004b). The first bait lamina studies acknowledged that it was difficult to disentangle the effects of micro– and macrofauna on feeding activity (Simpson et al., 2012). However, recent studies both in microcosms and in the field have shown that macrofauna (i.e. earthworms) (Förster et al., 2004; van Gestel et al., 2003), mesofauna (i.e. enchytraeids) and microarthropods (i.e. collembolan and ascari) (Helling et al., 1998; Römbke et al., 2006) are the main feeders on bait lamina. Moreover, Gongalsky et al. (2004) suggested that micro–organisms cannot contribute to perforation of bait lamina during the short duration (i.e. 14 days) typically used in such studies. Thus the feeding activity on bait lamina strips alone forms an incomplete picture of soil feeding activity.

Insam (1997) proposed a 96–well microplate with 31 carbon substrates plus control (BIOLOG® EcoPlates™) as a new set of substrates for community level physiological profiling (CLPP) in environmental samples. By applying this method we gain insight into the functioning of the soil microfauna involved in carbon cycling (Schutter and Dick, 2001). Loss in the ability of the microbial biomass to maintain its wide range of functions (e.g. changes in catabolic evenness or uniformity of substrate use) is considered as a warning of decreased soil health (Chapman et al., 2007). However, the principle criticism leveled at this method is that it relies upon the growth of a selectively extracted microbial population, which may not represent the true functioning of the whole soil, including macro fauna (Smalla et al., 1998). Consequently, *in situ* field assessment of soil activity instead of observations based on a cultured selection of micro fauna would be preferential.

In the field, the metal related stress response of soil organisms is difficult to pinpoint because of the large number of environmental and soil related factors that could potentially cause the same response (Stefanowicz et al., 2010). Soil fauna feeding activity and functional diversity are affected by factors such as, plant coverage (i.e. plant species richness; Birkhofer et al., 2011; and grassland versus forest; Stefanowicz et al., 2010, 2012), organic matter content (Bot and Bernites, 2005), clay mineralogy (Chen, 1998), acidity (Chapman et al., 2013; Eggleton et al., 2009), soil moisture and temperature (Gongalsky et al., 2008; Simpson et al., 2012). On the other hand metal bioavailability within the soil compartment is also affected by factors such as soil pH, clay and organic matter content. These factors are known to control metal adsorption/desorption in soils and in turn, may affect metal toxicity to soil fauna. In general metal mobility (Cd, Cu, Pb and Zn) in soils (i.e. soil solution concentrations and free ion activities) decreases with increasing pH and increasing clay and soil organic matter content (Oorts et al., 2006; Sauvé et al., 2000; Takáč et al., 2009). The metalloid As has intermediate properties between metals and non metals, tending to form anions instead of cations (Moreno–Jiménez et al., 2009). Inorganic forms of arsenite are considered more mobile and toxic than organic forms (Jedynak et al., 2009). At high pH values As adsorptions will be lower. This is because negatively charged arsenite species are repulsed by negatively charged surface sites which increase As bioaccessibility (Song et al., 2006; Yang et al., 2002). Moreover, As tends to bind to OH-groups of fulvic and humic acids forming ester-like bonds, while clay minerals with large surface areas such as Fe-,Al-, and Mn hydro(oxides) are an important sink for soluble As forms (Song et al., 2006).

Furthermore exposure cannot be expressed similarly for each organism in the soil ecosystem, e.g. soil microbes may be immersed in soil solution films surrounding soil particles, while invertebrates can be partially exposed after dermal (i.e. capable of adsorbing metals) contact with soil solution films (e.g. earthworms). For other invertebrates (e.g. some

arthropod species) metal uptake takes place through the ingestion of metal associated with particulate matter, the food or the soil solution (McGeer et al., 2004). Metal bioavailability and the reaction of soil fauna to changing environmental parameters are undeniably interlinked and the effect of one should thus be interpreted in combination with the other.

The general aim of the present work was to assess changes in the soil feeding activity and functional diversity at two sites with different levels of metal pollution, taking into account changing soil physicochemical properties. This was done firstly by applying the *in situ* bait lamina assay which primarily investigates the feeding activity of macrofauna, mesofauna and microarthropods and secondly by using the BIOLOG® EcoPlates™ assays to gain insight into the functioning of the soil microfauna related to carbon cycling.

2. Materials and Methods

2.1 Study sites

Sampling took place in the vicinity of the city of Antwerp (Northern Belgium) at two sites with different distance to an historically polluted site where a still active metal refinery is situated today; Fort 8 (F8) (0.3 km) and Hobokense Polder (HOP) (3 km) (Figure 1). Although both sites share similar grassland habitats, in comparison to HOP the grass sites at F8 nearest to the source of pollution show a lower diversity in grass species. Three independent grassland plots per site (~200 m apart) were randomly selected each with 2 subplots 30 cm apart (seen as replicates). Each subplot contained 20 bait lamina sticks (16 + 4 control strips). Sampling was conducted in the Spring of 2012. Previous studies on the same sites investigated metal and metalloid accumulation in soil, vegetation and a broad range of animal species (invertebrates, passerine birds, small mammals) (Janssens et al., 2001; Rogival et al., 2007; Vermeulen et al., 2009).

2.2 Soil sampling and characterisation

After removal of the visible organic layer (± 2 cm), soil was collected from 1 m x 1 m plots. At all 4 corners and in the middle of the plot, five subsamples in total, at a depth of 10 cm were collected. The subsamples were placed together in a plastic bag and thoroughly mixed (Rubio and Ure, 1993). Fresh soil samples were used for the BIOLOG® EcoPlates™ and pH measurements (i.e. conducted on the same day as sampling) while the rest of the soil was dried and used for measuring soil particle size, organic carbon content and metal content. Soil was sampled from the same plots where the bait lamina strips were placed.

Soil samples were air dried at room temperature for 24 h and sieved (2 mm mesh) after which 0.2–0.3 g was weighed into 50 ml polypropylene tubes. The soil was further oven dried (Pidomat, R2) for another 72 h at 60 °C. After drying, samples were placed into a desiccator to cool down before they were weighed. Pseudo total metal content was measured by adding a mixture of hydrochloric acid (HCl; 37 %) and nitric acid (HNO_3; 69 %) in a ratio 3:1 (v/v) (aqua regia). Samples were left to passively digest for 42 h and subsequently heat digested for 60 minutes in a HotBlock™ Pro (Environmental Express, 54 place SC154) at 125°C (Agemian et al., 1980; Rogival et al., 2007). All digested samples were diluted with ultrapure water up to 40 ml, (MilliQ®, Bedford, MA, USA) and stored until metal analyses. Soil metal(loid) concentrations (As, Cd, Cu, Ni, Pb and Zn) were measured with an inductively coupled plasma optic emission spectrometer (ICP–OES; Thermo scientific, ICAP 6300 Duo, Waltham, MA, USA). Analytical accuracy was verified using standard certified reference material from the Community Bureau of Reference, (BCR); light and sandy soil (BCR 142R) and sewage sludge (BCR 146R) (IRMM, Geel). Blanks and certified reference materials were prepared in the same manner as the samples and analyzed for quality assurance. All values were equal to 90–110 % of the certified values. Concentrations are reported on a dry–weight basis.

Soil pH was determined using a glass electrode (744 Metrohm, Switzerland) on fresh soil samples in a 1:5 (v/v) suspension of soil in KCl (1 M) (Vanhoof et al., 2007). The organic matter content of the soil samples was determined by loss on ignition (LOI) (Schumacher, 2002). For this purpose, dry soil was incinerated at 550 °C for 4 h (Heiri et al., 2001). The weight loss should then be proportional to the amount of organic carbon contained in the sample. A conversion factor of 1.724 has been used to convert organic matter to organic carbon based on the assumption that organic matter contains 58 % organic C (i.e., g organic matter/l.724 = g organic C) (Nelson and Sommers, 1996).

Furthermore particle size distribution was analyzed using laser diffraction (Malvern Mastersizer S., Worcestershire, UK) (Queralt, et al., 1999). Soil texture was based on the results of the particle size distribution which was calculated and described according to the GRADISTAT program (Blot and Pye, 2001).

2.3 Bait lamina assay

Bait–lamina strips were purchased from *Terra Protecta*®, GmbH. Each stick (16 cm long and 0.5 cm wide) contained 16 holes (0.5 cm apart from each other) filled with the bait substance, which comprises a wet mixture of cellulose, bran flakes and activated coal (70: 27:3 w/w) (Kratz, 1998). Bait strips were placed in the soil at three randomly selected grassland plots. Each group consisted of two (1 m x 1 m) plots that were 30 cm apart (Figure 1). A thin stainless steel blade was used to make a slit in the soil, into which the bait lamina strips were vertically placed. Control strips were checked weekly as an indication on how fast the feeding activity (FA) is progressing without disturbing the real experiment. After three weeks, when FA on the control strips was between 40–60%, the experimental strips could be carefully removed for counting. The number of empty (FED), partially empty (PFED) or

intact (UNFED) holes were recorded and expressed as a percentage. Feeding activity per site was calculated as the sum of FED and PFED holes.

Each week the soil temperature and moisture percentage was measured in situ. Soil temperature (°C) was measured with a propagation thermometer (Gardman, 16055) that penetrated 9 cm into the soil surface layer and was left in the soil for 10 minutes before it was removed and read. Soil moisture percentage (M%) was measured by inserting two metal probes (10 cm) into the soil surface. Moisture % is calculated inside the TRIME device and read as an output on the screen (TRIME–TDR, IMKO).

2.4 Biolog Ecoplates

The BIOLOG® EcoPlates™ (Biolog, Hayward) measure the capacity of the microbial community to metabolize a wide range of different carbon sources. This is a surrogate for microbial diversity and activity of soil microbial communities (Gagliardi et al., 2001). The plates contain 31 different organic substrates such as sugars or amino acids, as well as a dried mineral salts medium and a tetrazolium redox dye (Garland and Mills, 1991; Insam, 1997). Every individual well contains only one specified carbon source. Formation of a purple colour occurs when the microorganisms, which can utilize the carbon source, begin to respire and grow. The respiration of the cells oxidizes the carbon source while reducing the dye yielding a blue colour (Garland, 1997). Soil samples from each plot (i.e. at the four corners and in the middle of each 1 m x 1 m plot) were placed into a plastic bag and mixed by shaking. A 10 g sample was taken from each bag and stirred for 30 minutes on a rotary shaker (3017, GFL) in 100 ml physiological water (9 g NaCl + 1 L of sterile, MilliQ). Subsequently the supernatant was decanted and filtered through 'Whatman No. 40' filter paper with a pore size of 8 µm. The resulting filtrate was diluted 10^5 times with sterile physiological water (9 g NaCl + 1L of water (MilliQ®, USA)) and a 120 µl aliquot of the resulting suspension was added to each

well of the Ecoplates, which were incubated at 23 °C for 1, 2, 3 and 6 days. Measurements of the dye reduction were made with a micro–plate reader (EL x 808 ultra, Bio–tek instruments inc.) at 600 nm.

Microbial response in each microplate that expressed AWCD was determined according to (Frąc et al., 2012; Gomez et al., 2004).

$$AWCD = \Sigma OD_i / 31$$

where OD_i is optical density value from each well, corrected by subtracting the blank well (inoculated, but without a carbon source) values from each plate well (Garland and Mills, 1991; Weber et al., 2007). Functional richness (R) values were calculated as the total number of oxidized carbon substrates. The H' value gives an indication of the functional diversity of each soil (Stefanowicz et al., 2010) and is calculated as follows:

$$H' = -\Sigma p_i (\ln p_i)$$

where p_i is the proportional activity on each substrate (OD_i) to the sum of activities on all substrates ΣOD_i. Both R and H' were calculated using a standard OD of 0.25 as threshold for positive response (Garland, 1997). Community reaction patterns are typically analyzed at defined time intervals over 2 to 5 days (BIOLOG, Inc., 2007). Therefore the functional indices AWCD, R and H' of day 2 and 6 will be presented. The following five groups of carbon substrates were used: (1) carbohydrates; (2) carboxylic and acetic acids; (3) amino acids; (4) polymers; and (5) amines and amides according to Weber and Legge, 2009 (Supplementary information: Table S1). For each series the corrected absorbance values of the substrates were summarized and expressed as a percentage of total absorbance value of the plate corresponding to a particular time interval (Weber and Legge, 2009; Chodak and Niklinska, 2010).

2.5 Statistics

Normality of the data was checked with the Shapiro–Wilk test. Data was $\log_{10}(x+1)$ transformed to obtain a normal distribution. To compare measured characteristics of F8 with that of HOP an unpaired t–test was conducted between soil physicochemical properties (pH, OC %, clay %, M %, ° C) and pseudo total metal content (As, Cd, Cu, Ni, Pb, Zn). Hierarchical cluster analyses was performed with Ward's method and Euclidian distance to group plots; according to soil physicochemical properties and pseudo total soil metal concentrations and in a separate analyses according to carbon source utilization for which the AWCD was used. Pearson's correlation analyses was performed to investigate which soil physicochemical properties and soil metal concentrations correlate significantly with FA and functional indices. Similarly differential carbon source utilization was based on AWCD and expressed as a percentage of total carbon utilization per plot. The outcome of the AWCD, R and H' indices per plot were analyzed by ANOVA and mean comparisons between treatments were performed using Tukey's post–hoc test at $p < 0.05$. Since our study takes place at two sites each with three plots and two replicate subplots a nested ANOVA was performed to investigate the effect of "site" and "plots within sites" on FA and functional indices. A principle component analysis (PCA) was conducted to visually explore the association between soil physicochemical properties, metal concentrations, FA and functional indices. The gradient length is the amount of variation explained by the combination of environmental variables and is expressed as standard deviation (SD). Since a short gradient length (SD < 1) was determined using a preliminary Correspondence Analyses (CA), a linear response model was used. Scores were divided by SD and log transformed. Scaling was focused on inter species distance. The relationship between soil metal concentrations and FA and functional indices were further investigated with linear regression analysis and analysis of covariance (ANCOVA) to better understand the effect of metal pollution taking into account sampling

site. All assumptions needed to perform ANCOVA (i.e. normal distribution of data and homogeneity of variance) were met.

The data were analyzed with the statistical packages SPSS (IBM SPSS Software, Inc.) and GraphPad Prism 6 (GraphPad Software, Inc.), while the nested ANOVA was performed in Minitab® 17 (Minitab, Inc.) and ordination models were calculated using CANOCO version 4.5. (ter Braak and Smilauer, 1998).

3. Results

3.1 Difference in soil physicochemical properties and pseudo total metal content between sites and plots

Data recorded for the soil physicochemical properties and metal content per subplot are presented in Table 1. Unpaired t–test's showed significant differences between the three plots at F8 and HOP for: M % ($t = 3.11$; $p = 0.011$), °C ($t = 4.09$; $p = 0.002$), As ($t = 4.57$; $p = 0.001$), Cu ($t = 2.34$; $p = 0.042$), Pb ($t = 3.64$; $p = 0.005$) (i.e. in all cases $n = 3$). All other variables; pH, organic carbon %, clay %, Cd, Ni and Zn did not differ significantly between the plots at F8 and HOP. At both sites a patchy distribution of metal contamination is observed with much higher As, Cu, Pb concentrations at F8/Gr 2.1, 2.2, 3.1, 3.2 than at F8/Gr 1.1–1.2. Also at HOP we see that HOP/Gr 1.1–1.2 has higher Cd, Ni and Zn concentrations than the rest of the subplots.

3.2 Feeding activity and functional indices

The percentage of FED, PFED and UNFED holes per plot is presented in Figure 2. In general the FA at HOP was higher than at F8. The lowest FA (i.e. FED + PFED) was observed at F8/Gr 3 with FA of ± 10% (almost no FED recorded at the subplots), while the

most active feeding was observed at the subplots HOP/Gr 2.2 and 3.2 (overall the highest FA was observed at HOP/Gr 3) with FA of ± 90%.

Figure 3 demonstrates the results of the calculated indices after 2 and 6 days of incubation; AWCD (i.e. average carbon source utilization), R' (i.e. number of utilized substrates) and H' (i.e. variety of utilized substrates, gives an indication of functional diversity). After 2 days the plots further away from the pollution source indicated significantly ($p < 0.05$) higher AWCD, R' and H' values compared to the plots nearer to the pollution source (i.e. with the exceptions of F8/Gr 1). After 6 days both AWCD and R' have increased and follow the same trend as seen after 2 days (i.e. significantly lower at F8/Gr 2 and 3). In contrast, the H' index is similar at all plots after 6 days of incubation (i.e. polluted and less polluted) (Figure 3).

The nested ANOVA indicated significant variability among plots for all functional indices measured at day 2 and 6 but this was not always the case for sites at day 2 and 6 (Table 3). In other words this is an indication that the differences between plots were of more importance than the variability among sites. Such an observation is to be expected since soil is such a variable entity.

Figure 4 demonstrates the percentage of carbon source utilization (i.e. carbohydrates, carboxylic and acetic acids, amino acids, polymers and amines and amides) per plot after 6 days of incubation. The similarity of carbon source utilization between plots is supported by the lack of significance in the diversity index H' measured after 6 days of incubation (Figure 3).

3.3 Relation between soil physicochemical properties, pseudo total metal and bioassays (feeding and functional diversity)

Ordination of FA and functional indices after 2 and 6 days (i.e. AWCD, R' and H') in relation to each other and the soil physicochemical properties is visualized in the PCA (Figure 5). The outcome of the cluster analysis (Supporting information Figure S1 and S2) is

indicated on Figure 5. The oval shapes separates sites into three clusters based on carbon source utilization while the grey rectangles separate plots into two clusters based on soil physicochemical properties and pseudo total metal content. The PCA further separates plots into three clusters the polluted plots (F8/Gr 3 and 2) with low FA and carbon source utilization, the intermediate plots (F8/Gr 1 and HOP/Gr 1) and lastly the less polluted plots (HOP/Gr 2 and 3) with active feeding and carbon source utilization. Feeding activity and functional indices cluster together opposite to the pseudo total metal concentrations indicating a negative association between pseudo total metal concentrations and soil functioning. The correlation between the measured indices and metal concentrations ($R \geq 0.7$) to PC 1 and PC 2 is summarized underneath the ordination diagram (Figure 5). Arsenic, Cu and Pb showed the strongest positive correlation to PC 1 and Ni to PC 2 while FA and the functional indices of day 2 and 6 correlates negatively to PC 1. The short arrow length of M%, °C, pH, clay% and OC% indicate that they are less important in separating plots than the longer arrowed pseudo metal concentrations (i.e. longer arrow length).

The outcome of the Pearson's correlation analysis (presented in Table 2) is in accordance to ordination of variables in the two dimensional space of the PCA observed in Fig 5. Soil M% and soil temperature correlated positively and significantly to functional indices and FA of both 2 and 6 days while As, Cu and Pb concentrations correlated negatively and significantly to functional indices and FA.

Linear regression analysis shown in Figure 6 further depicts the significant correlation between FA, functional indices and the strongest correlating metals As and Pb.

The results of the ANCOVA showed that there was no significant effect of As, Cu and Pb on FA. However, there was a significant main effect of site (F8 vs HOP), suggesting that higher levels of FA are associated with the site where it is measured (Table 4 and in the nested ANOVA Table 3 (A)).

The ANCOVA further showed a significant effect of As concentrations on AWCD, R', H'. While no significant effect on functional indices were observed for Cu and Pb. In contrast to FA there was no significant main effect of site on the functional indices: AWCD, R' and H' (Table 4).

4. Discussion

4.1 The role of soil physicochemical properties on feeding activity and functional indices

Field experiments took place early spring when soils were moist and prevailing temperatures were low and fluctuated between 6–12 °C. Feeding activity of the macro and meso soil fauna is known to occur down to temperatures of 5 °C (Gongalsky et al., 2008) which is lower than the mean minimum soil temperature that was observed at F8/Gr 3.1–3.2 (5.75 °C). Although soil temperatures were significantly higher at HOP compared to F8 the average soil temperature differed by only one degree Celsius. This could be explained by the time of day when the readings were taken (i.e. F8 early morning and HOP approximately one hour later–always in the same order). However, soil texture (i.e. clay content), grass species, height, density and shading at both sites were comparable and therefore the effective soil temperature taken at the same time of day could not have differed significantly between plots from both sites.

Even though the size of the microbial biomass in surface and subsurface soils stay rather constant throughout the year, seasonal changes in soil temperature affect their activity especially in temperate regions (Blume et al., 2002; Sistla et al., 2013). Palmisano et al. (1991) established that seasonal effects such as cold temperature that suppress activity could be reversed by incubating environmental samples collected in the winter at a summer temperature of 22 °C. Similarly in our study the role of low soil temperature on microbial

functional diversity tested with the BIOLOG® assay was reversed after incubation at 23 °C. Therefore in our study the effect of soil temperature on the soil microbial community is negligible.

The plots at HOP had significantly higher moisture levels than at F8 which could in turn increase soil activity (Willis and Raney, 1971). Furthermore, soil respiration and population proliferation is best in the moisture range of 20% to 60% (Katznelson and Stevenson, 1956). All our plots fell within this moisture range. Soil faunal proliferation favor optimal soil moisture levels (Eggleton et al., 2009; Larink and Sommer, 2002; Spehn et al., 2000). However, studies using bait lamina strips that specifically investigate the effect of moisture levels on FA are scarce. In the study of Simpson et al. (2012) moisture levels ranged from 22–46% between deciduous woodland plots. In our study the average soil moisture differed in a narrow range from 29% at F8 to 38% at HOP. Similar to our study a TDR probe was used to measure soil moisture percentages. Plots with highest moisture levels showed increased FA but this was not the case for all used bait strips (Simpson et al., 2012). Furthermore Gongalsky et al. (2008) established that the effect of soil moisture on FA in forest soils was less evident than the role of soil temperatures (i.e. –4°C to 24°C). However Gongalsky et al. (2008) did not measure the *in situ* soil moisture levels but instead added distilled water 220%, 260%, 300% while the experimental temperature range were broader than in our study. Different study aims and methodologies complicate the comparisons between outcomes of our study with that of others.

Bioavailability is considered to consist of three dynamic processes: physicochemical desorption, physiological uptake, and toxicodynamic redistribution inside the organism (Ardestani and van Gestel, 2013; Hamelink et al. 1994). Several factors can influence the solubility and mobility of metal ions and therefore the bioavailability to organisms. These factors include pH, OC and clay content which was measured in our study. However, other

factors such as the chemical form of metals in soil (i.e. metal speciation), ions competing for binding sites and the time the metal has been in the soil can have an effect on metal bioavailability (Lofts et al., 2004). The study of Smolders et al. (2009), which investigated influences of soil properties and aging on trace metal toxicity in soils, found that increased organic matter and clay content in soils increases the sorption capacity of metal ions while decreased pH increases free metal ion activity($Cu^{2+,}$ Pb^{2+}) and a increase in pH increases As^{3-} availability. However, the effect of pH on free metal ion activity counteracts the effect of metal adsorption due to an increase in ion competition to bind to active sites of toxicity and the overall effect of soil pH on total metal-based toxicity is thus small (Oorts et al., 2006).

Measured soil properties in our study did not differ significantly between F8 and HOP while As, Cu and Pb pseudo total metal concentrations did. Overall pseudo total metal concentrations more than the measured soil physicochemical properties in this study proved important in explaining changes in soil functioning. It could be argued that pore water metal concentrations and 0.01 M $CaCl_2$ extracted metals are a better indication of toxicity than total metal concentrations (van Gestel et al., 2009; Hobbelen et al., 2006). However others studies have reported pseudo total metal concentrations to be closer related to soil fauna functional parameters than pore water metal concentrations (André et al., 2009; Davis et al., 2004; Filzek et al., 2004b; Niemeyer et al., 2012; Van Beelen et al., 2004; Stefanowicz et al., 2010). Within the scientific community there is no generally accepted method for predicting metal toxicity in soils; possibly no chemical assessment allows prediction of metal toxicity across a wide range of species because of the different routes of toxic exposure in soil (Smolders et al., 2009).

4.2 The effect of pseudo total metal concentrations on feeding activity and functional indices

According to the Flemish soil quality standards (VLAREM, 2012) all the plots at both sites can be classified as polluted. Fort 8 showed a significantly higher pseudo total metal content than HOP for As, Cu and Pb but not for Cd, Ni and Zn. These observations confirm earlier results of metal concentrations measured at these sites (Janssens et al., 2001; Rogival et al., 2007; Vermeulen et al., 2009). In our study FA proved higher at HOP than at the more polluted plots of F8. In line with this, HOP demonstrated a greater rate of substrate utilization (AWCD) and an increase in the number of utilized substrates (R') compared to the more polluted plots of F8. Arsenic, Cu and Pb correlated negatively and significantly to FA, AWCD, R' and H' while concentrations of Cd, Ni and Zn did not.

At low concentrations metals play an integral role in the life processes of soil fauna. Some metals, such as Cu, Ni and Zn, serve as micronutrients and are used for redox–processes; to stabilize molecules through electrostatic interactions; as components of various enzymes; and for regulation of osmotic pressure. Many other metals (e.g., As, Cd and Pb), have no biological role and are non–essential and potentially toxic already at low concentrations (Bruins et al., 2000; Vijver et al., 2000). However, at high concentrations both essential and non–essential metals can be toxic to soil fauna (Rathnayake et al., 2010; Utgikar et al., 2004).

Our study is not the first to observe a strong impairment of primarily macro– and mesofauna feeding activity at sites associated with high metal concentrations in the same range as those measured in our study (As: 16.01–189.70 (mg/kg dw), Cd: 4.02–14.05, Cu: 27.67–515.90, Pb:147.10–1373, Zn: 77.73–619.40). Niemeyer et al. (2012) observed a strong negative correlation between total metal concentrations (i.e. Cd: < 0.2–62 (mg/kg dw), Cu: 44–108, Pb: 23–2200 and Zn: 80–3300), low organic matter content, low vegetation coverage and FA measured at sites situated within the boundaries of a abandoned lead smelter in Santo Amaro, Brazil. In the same study the significant decrease in total species richness of

arthropods and community composition of surface dwelling invertebrates correlated with the bait lamina results. Also André et al. (2009) measured concentrations of Cd: 0.1–4.3 (mg/kg dw), Pb: 9.6–34.1, Zn: 33.8–1109 (i.e. Cunha Baixa uranium mine, Portugal) and Filzek et al. (2004a) concentrations of Cd: <0.3–227 (mg/kg dw), Cu: 2.55–682, Pb: 113–5680, Zn: 462–9650 (i.e. Avonmouth Cd/Pb/Zn–smelter, South–West England) which demonstrated that FA differed among sites with different levels of contamination and that lower FA levels can be contributed to a decrease in abundance and biodiversity of key decomposer groups.

Also microbial parameters can act as sensitive indicators of soil functioning. Ghosh et al. (2004) observed significant negative correlations between As concentrations (ranging from 11–30 mg/kg dw) and microbial biomass, soil respiration and enzyme activity. In our study As concentrations at almost all sites exceeded the concentration range found by Ghosh et al. (2004). In a long term Cu exposure study by Brandt et al. (2010) initial total concentrations of 150 mg/kg dw affected bacterial community structure and functioning. In our study Cu concentration exceeded those of Brandt et al. (2010) at sites nearest to the source of pollution. Furthermore the study of Stefanowicz et al. (2010) conducted on polluted grassland soils (Cd: 2–506 mg/kg dw, Pb: 0.1–33.2 mg/kg dw and Zn: 0.1–72.1 mg/kg dw) demonstrated that total metal concentrations (i.e. perchloric acid extracted) correlated negatively with microbial biomass, activity and functional diversity. In other case studies with much lower total metal concentrations correlations between metal contamination and microbial functional structure were less evident. Niklińska et al. (2006) indicated that microbial communities at polluted sites Cu: 0.6–10.8 and Zn: 2.4–44.8 mg/kg dw were still able to perform the same ecological functions as the communities extracted from the reference site. Also Bååth et al. (1998) found no significant differences in microbial parameters between the polluted Pb: 0.09–4.31 mg/kg dw (Diethylenetriamine pentaacetic acid (DTPA) extraction) and reference sites, while,

corresponding to the result of the present study, moisture content complicated the interpretation of results.

Incubation time of 2 and 6 days played an important role in well colour development and was significantly affected by site and plots within sites. Even if particular microorganisms have a low capacity to utilize the carbon source in a particular well, colour change will occur with an increase in incubation time. Whether this colour change is due to the absence of certain species or due to the growth rate of the species cannot be derived from our Biolog results alone. In this study the microbial diversity index differed significantly between plots after 2 days but not after 6 days of incubation. Functional diversity (H') can be defined as the number of distinct functions that are carried out by a community (i.e. the percentages of carbon sources utilized per plots is similar on day 6), but does not measure the different species within the community (Gaston, 1996). Diversity is a useful indicator of the well–being of an ecological system; however there is considerable debate on the role diversity plays in ecosystem functioning (Franklin et al., 2001). One of the hypothesis being that many species are 'redundant' since they play equivalent roles in an ecosystem (Bell et al., 2005; Nannipieri et al., 2003; Torsvik et al., 1998). There is some evidence that support this hypothesis in the review of Nielsen et al. (2011) who concluded that species diversity, on average, led to improved functioning (i.e. greater biomass, decomposition rates, respiration), especially in very species poor communities. However the relationship was non–linear and indicated that certain key species (i.e. earthworms or rhizobia bacteria) have a disproportional large effect on soil functioning compared to species number and diversity. In our study pollution tolerant species present at the more polluted plots may have the ability to metabolize the same carbon sources as the more sensitive key species in the less polluted plots. However, the tolerant species do not share the same capacity to colonize and grow at the speed of the key metal sensitive species present at the less polluted plots. This might explain the fact that after 2 days

incubation we do see a difference in functional diversity however at 6 days the number of slower growing species with the same function has increased and the capacity to distinguish between functional diversity is less evident. The observed changes in growth rate caused by a change in the functional structure of the microbial community may be an indication of the resilience of the soil community to adapt. The latter can be physiologically within an organism (e.g. point mutations or horizontal gene transfer) or on a community level (e.g. extinction of metal-sensitive species or the replacement/shifts in the community structure towards metal tolerant species) under prevailing metal concentrations (Brandt et al., 2010; Mertens et al., 2010).

There is also evidence that refutes the redundancy hypothesis; e.g. Hol et al. (2010) proved that the loss of a few rare key microbe species has been shown to impact plant–herbivore above ground relationships. Also Brussaard et al. (1997) highlights the importance of species biodiversity in protecting environments against soil borne disease; and that diversity ensures nutrient and water use efficiency. This suggest that the loss of seemingly redundant species can have unexpected consequences.

Finally, soil faunal community structure and activities are difficult to elucidate using a single monitoring approach. Combining the BIOLOG and Bait lamina assays allowed us to simultaneously investigate the response of a wide range of soil fauna (i.e. macro and meso invertebrates and soil microbes) that confirmed their suitability for monitoring studies focusing on metal polluted sites. However, the ANCOVA indicated that feeding activity measured by the bait lamina assay was significantly affected by sampling site and since this method is measured *in situ*, field related factors such as moisture percentage could potentially overshadow the effect of metal pollution. In contrast the same analysis showed that functional indices measured with the BIOLOG assay proved more sensitive to metal pollution (i.e. As concentrations) while the effect of site was of less importance. Therefore the BIOLOG assay

which excludes confounding factors such as low soil temperature and moisture percentage may therefore be a more reliable assay to measure soil functional activity.

5. Conclusion

The present study demonstrated correlation between decreased feeding activity, metabolic capacity and functional richness and increased pseudo total metal concentrations of As, Cu and Pb. While environmental impact studies often focus on the response of micro or macro soil fauna separately this study aimed to combine both. Moisture percentage correlated positively with FA and functional indices and the effect of significantly lower moisture levels at plots nearer to the source of pollution could not be disentangled from metal related effects. Furthermore a reduction in the microbial functional diversity (H') was observed after 2 days but not after 6 days of exposure which may indicate that ecological functioning is more robust to stressors than the community structure (i.e. specific species present), a phenomenon called functional redundancy. The added value of the combined use of the bait lamina and BIOLOG assay is that results of both an *in situ* field test that measures the feeding activity of primarily macrofauna, mesofauna and microarthropods are combined with an assay that measures the metabolic fingerprint of a selectively extracted microbial community. Therefore the metal related response of a wide range of soil fauna can be simultaneously investigated by using both methods. However, the BIOLOG assay which excludes confounding factors such as low soil temperature and moisture percentage may be a more reliable assay to measure soil functional activity.

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Figure legends:

Figure 1: Schematic presentation of sampling sites (i.e. Fort 8 and Hobokense Polder) and plots in relation to the pollution source. Each one of the subplots contained sixteen plus four control strips (i.e. 16 + 4) while plots were approximately 200 m apart.

Figure 2: Bait lamina feeding activity percentages per plot; FED (hole completely empty), PFED (hole partially empty), UNFED (hole completely filled). Feeding activity is calculated as FED + PFED.

Figure 3: A) Average well–color development (AWCD) (i.e. average carbon source utilization), **B)** Richness (i.e. number of utilized substrates), **C)** Shannon–Wiener index (H') (i.e. variety of utilized substrates, gives an indication of functional diversity) per plot at the sites; Fort 8 (F8) and Hobokense Polder (HOP). Vertical bars represent the mean with standard deviation. Uppercase letters indicate significant differences between plots after 2 days of incubation while lowercase letters indicate differences between plots after 6 days (ANOVA, Tukey's mean separation test, $p < 0.05$).

Figure 4: Percentage total carbon source utilization measured per plot (i.e. results after 6 days of incubation).

Figure 5: PCA of soil physicochemical properties, feeding activity (FA) and functional indexes; average well color development (AWCD), functional richness (R'), Shannon Wiener diversity index ('H) according to the first and second principle components (see Table 2) that together explained 94.6% of the total variation. The oval shapes separates sites into three clusters based on carbon source utilization while the grey rectangles separate plots into two clusters based on soil physicochemical properties and pseudo total metal content. The triangle

surrounds the measured bioassays for example FA and functional indices which cluster together in close proximity.

Figure 6: Linear relationship between arsenic (As), lead (Pb) and feeding activity (FA), average well color development (AWCD), richness (R') and the Shannon Wiener diversity index ('H) (i.e. of day 6), linear relationships are indicated with r^2 and p values.

Figure 1:

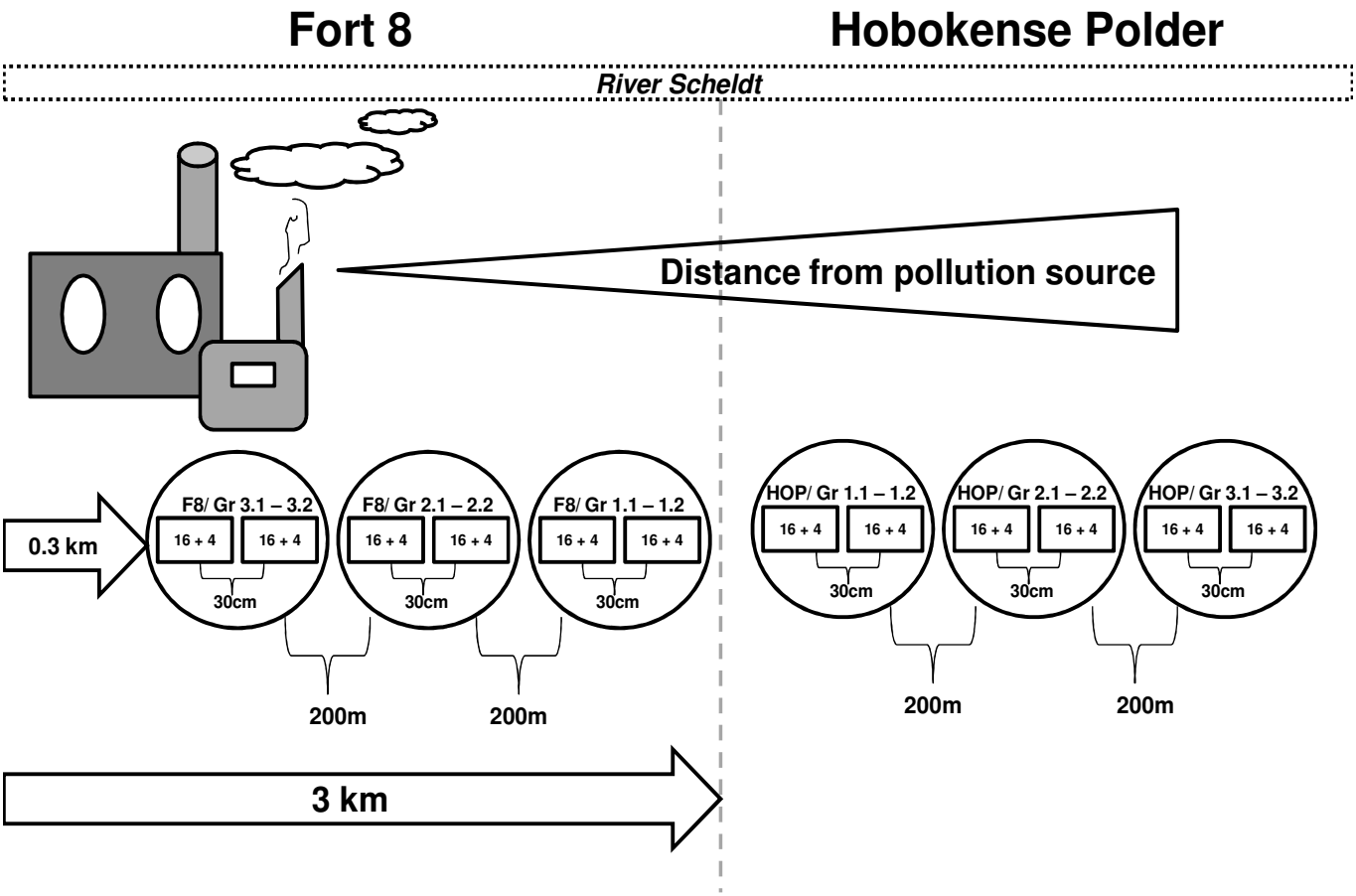


Figure 2:

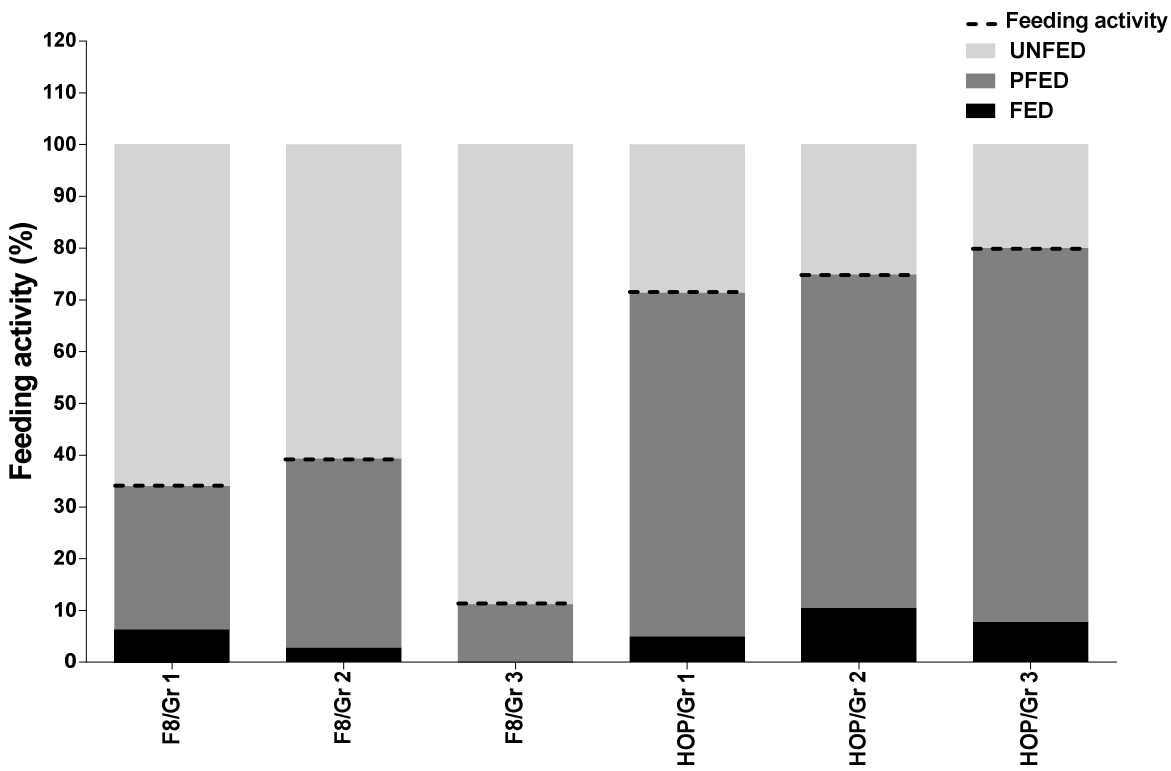

Feeding activity
UNFED
PFED
FED
Feeding activity (%)
120
110
100
90
80
70
60
50
40
30
20
10
0
F8/Gr 1
F8/Gr 2
F8/Gr 3
HOP/Gr 1
HOP/Gr 2
HOP/Gr 3

Figure 3:

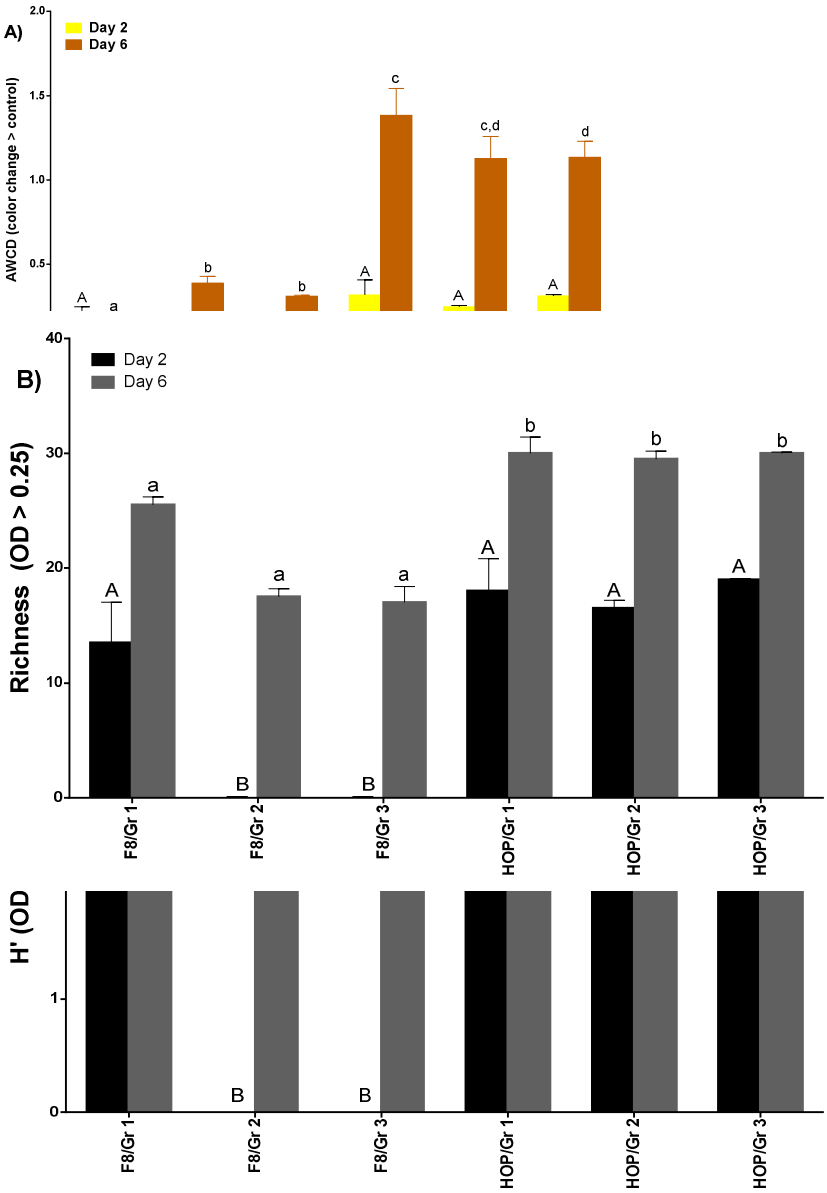

A)
Day 2
Day 6
AWCD (color change > control)
2.0
1.5
1.0
0.5
A
a
b
b
A
c
A
c,d
A
d
B)
Day 2
Day 6
Richness (OD > 0.25)
40
30
20
10
0
A
a
B
a
B
a
A
b
A
b
A
b
F8/Gr 1
F8/Gr 2
F8/Gr 3
HOP/Gr 1
HOP/Gr 2
HOP/Gr 3
H' (OD
1
0
B
B
F8/Gr 1
F8/Gr 2
F8/Gr 3
HOP/Gr 1
HOP/Gr 2
HOP/Gr 3

Figure 4:

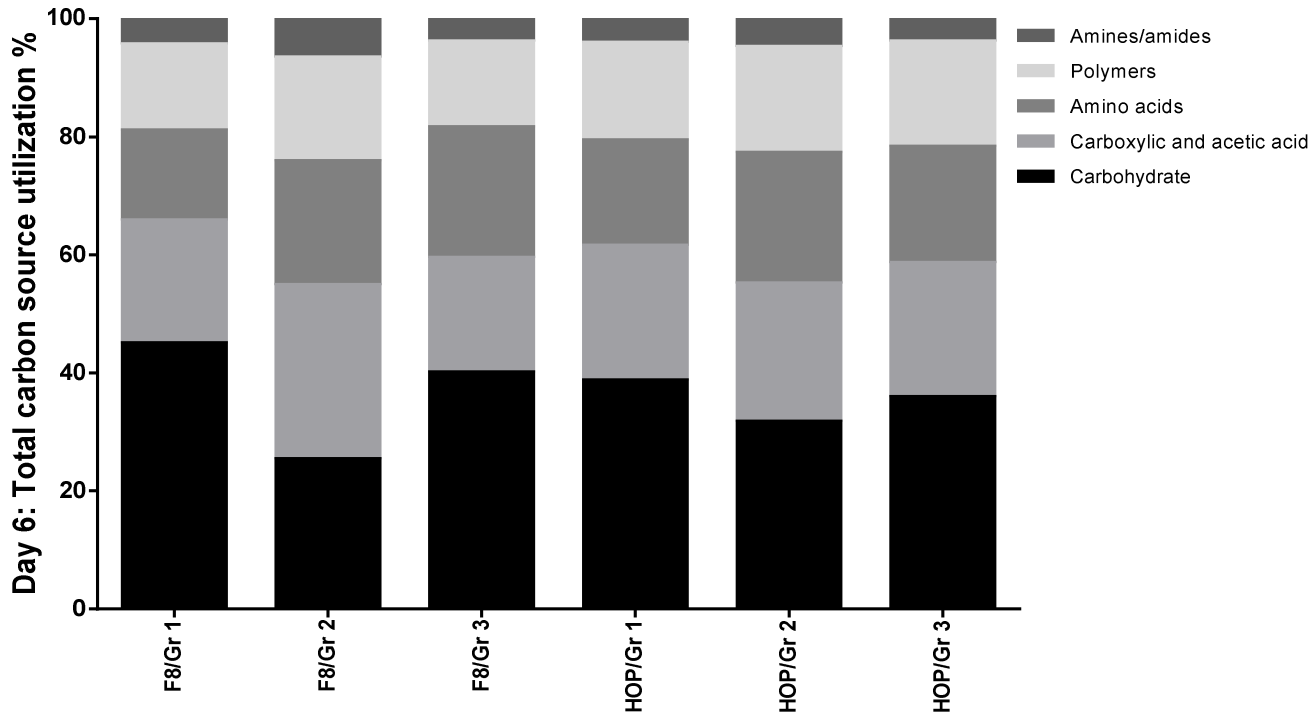
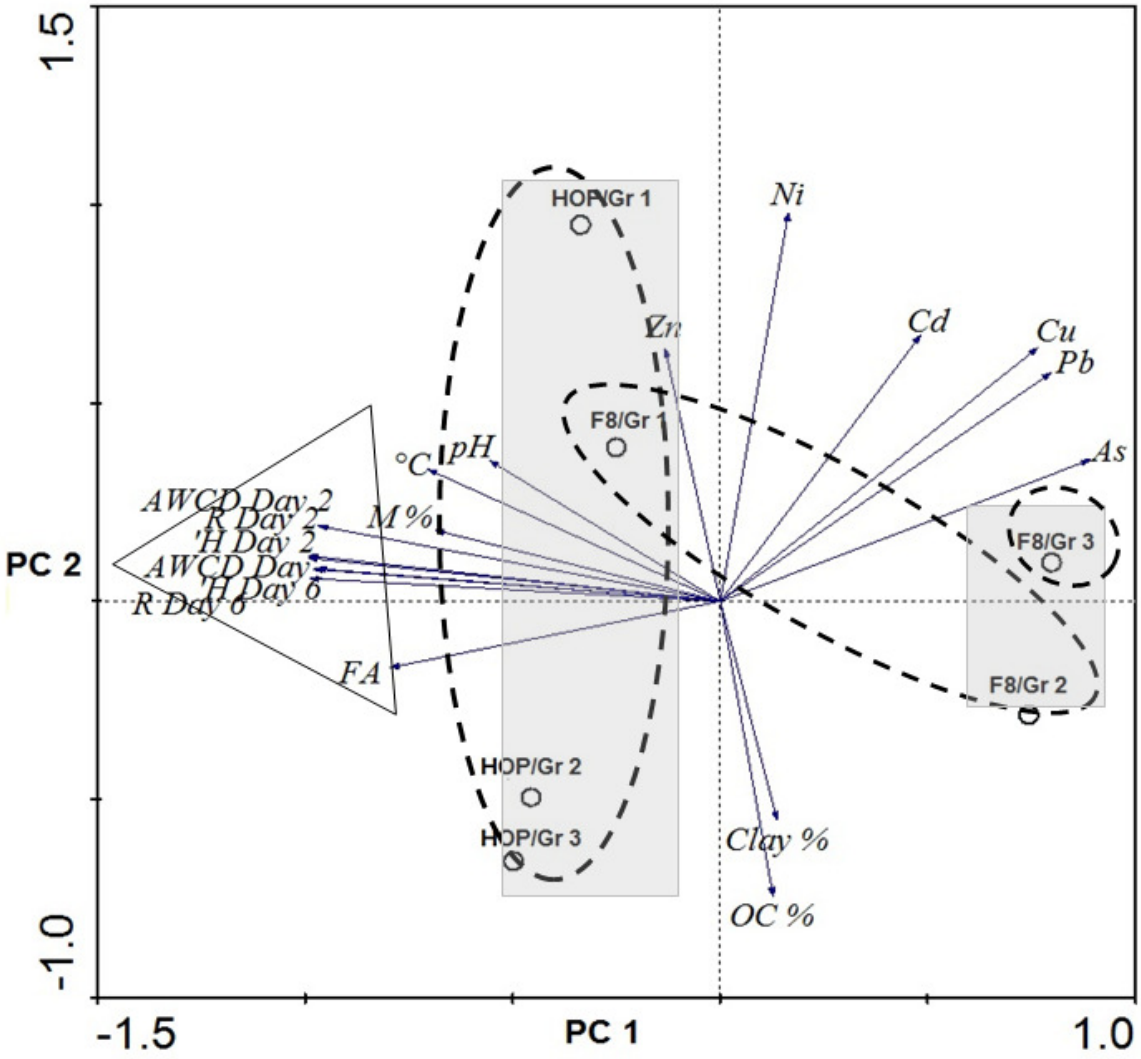


Figure 5:



	PC 1 (93.2 %)		PC 2 (1.4 %)
As	0.88	Ni	0.87
Cu	0.74	clay %	–0.73
Pb	0.78	OC %	–0.96
Feeding activity	–0.78		
AWCD (2)	–0.96		
R (2)	–0.98		
'H (2)	–0.97		
AWCD (6)	–0.96		
R (6)	–0.99		
'H (6)	–0.99		

Figure 6:

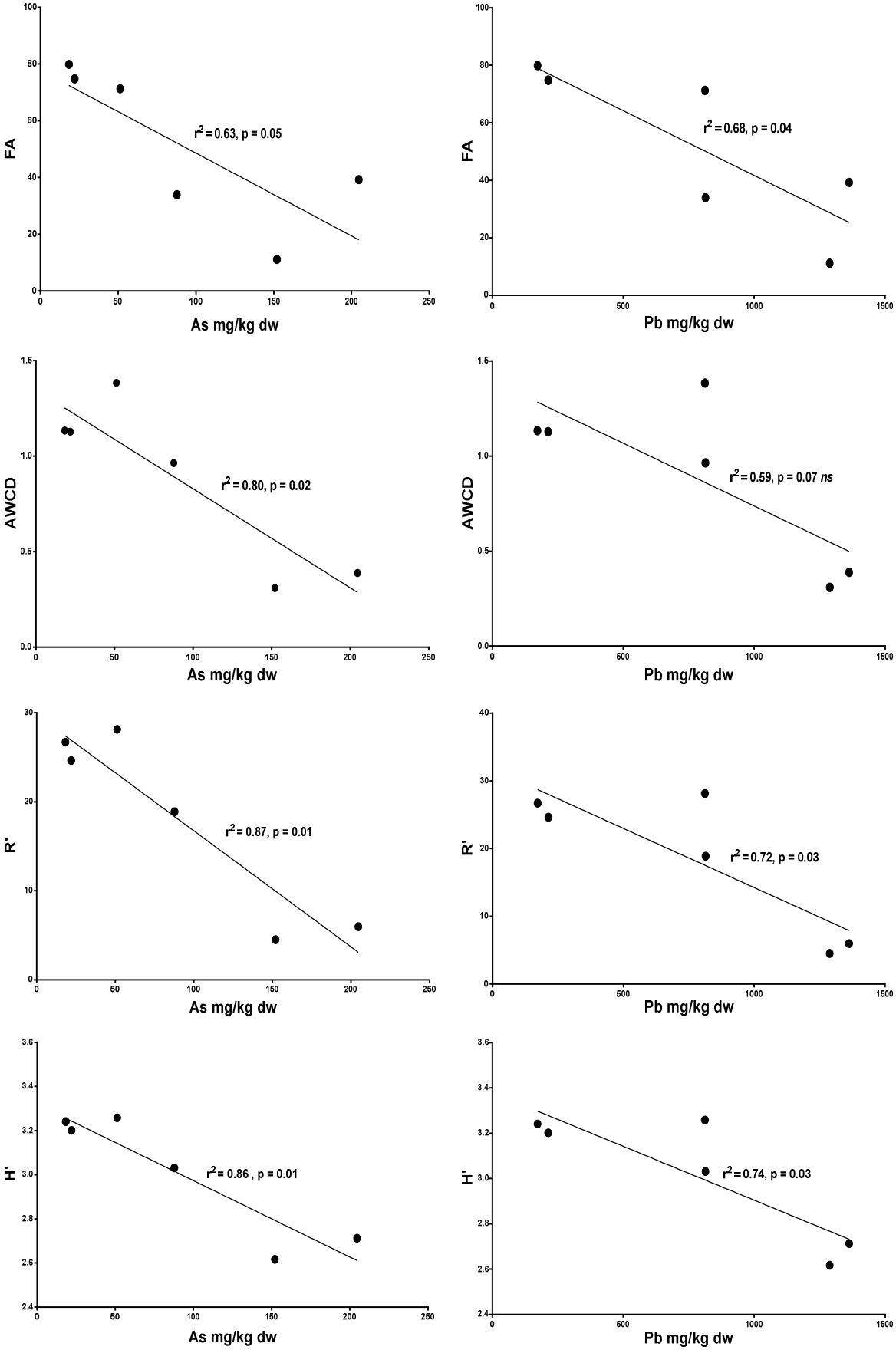

FA
As mg/kg dw
r² = 0.63, p = 0.05
FA
Pb mg/kg dw
r² = 0.68, p = 0.04
AWCD
As mg/kg dw
r² = 0.80, p = 0.02
AWCD
Pb mg/kg dw
r² = 0.59, p = 0.07 ns
R'
As mg/kg dw
r² = 0.87, p = 0.01
R'
Pb mg/kg dw
r² = 0.72, p = 0.03
H'
As mg/kg dw
r² = 0.86 , p = 0.01
H'
Pb mg/kg dw
r² = 0.74, p = 0.03

Table 1: Soil physicochemical properties (bulk soil pH (KCl), clay percentage (clay %), organic carbon percentage (OC %), soil moisture content (M %), soil temperature (°C)) and pseudo total metal concentrations (mg/kg dw) measured per subplot. Repeated measures (week 0-4) were done for M% and °C therefore the mean and standard deviation (stdev) is included.

	pH (KCl)	Clay %	OC %	M %	M% stdev	°C	°C stdev	As	Cd	Cu	Ni	Pb	Zn
F8/Gr 1.1	4.92	4.00	5.10	29.13	2.73	6.25	2.06	**78.05**	**6.95**	**104.50**	**23.16**	**659**	**98.81**
F8/Gr 1.2	4.87	3.97	3.74	29.45	3.68	6.25	2.06	**53.19**	**4.15**	**95.87**	**11.89**	**544.80**	**77.73**
F8/Gr 2.1	3.16	4.20	12.51	31.63	9.68	6.50	1.91	**189.70**	**8.80**	**239.90**	11.72	**1246**	**135.20**
F8/Gr 2.2	3.30	5.51	11.56	31.68	2.83	6.00	1.63	**149.90**	**10.11**	**193**	**14.45**	**1011**	**127.50**
F8/Gr 3.1	3.91	4.83	7.04	27.55	2.96	5.75	1.26	**139.70**	**11.11**	**515.90**	**20.98**	**1373**	**189.10**
F8/Gr 3.2	3.92	4.58	4.37	24.53	5.98	5.75	1.26	**121.40**	**5.14**	**228.20**	**13.98**	**844.90**	**108.50**
HOP/Gr 1.1	4.22	4.83	6.33	42.80	18.00	7.75	1.26	**33.17**	**10.50**	**211.90**	**21.18**	**721.20**	**600.30**
HOP/Gr 1.2	3.76	4.33	5.07	44.08	15.00	7.75	1.26	**51.26**	**14.05**	**238.90**	**25.45**	**593.90**	**619.40**
HOP/Gr 2.1	4.41	5.19	6.44	34.80	7.12	6.75	1.50	**15.30**	**4.02**	**27.67**	**8.06**	**146.40**	**81.54**
HOP/Gr 2.2	4.14	5.08	9.23	36.75	6.67	6.75	1.50	**20.86**	**6.38**	**44.79**	**14.76**	**197.30**	**171.30**
HOP/Gr 3.1	3.98	4.40	10.25	35.33	7.61	6.75	1.89	16.01	**5.69**	**36.02**	11.88	**147.10**	**174.50**
HOP/Gr 3.2	3.52	5.04	11.78	36.33	6.03	6.75	1.89	16.12	**4.20**	**28.93**	9.22	**147.30**	86.89

Soil concentrations were compared to the Flemish soil quality standards (VLAREM addendum 2.4.2 (Table 2), 2012). The standards include correction for organic matter and clay content, allowing a comparison between different soil types. Flemish soil remediation standards were exceeded per metal at the plots indicated in bold.

Table 2: Pearson's correlation analyses between soil physicochemical properties (i.e. pH(KCl), moisture percentage (M%), clay percentage (clay %), organic carbon percentage (OC%) and soil temperature (°C)), pseudo soil metal concentrations and feeding activity (FA) and functional indices; average well color development (AWCD), functional richness (R') and Shannon diversity index (H') measured after 2 and 6 days of incubation. n = 6, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

	pH		Clay %		OC %		M %		°C		As		Cd		Cu		Ni		Pb		Zn		FA		AWCD (6)		R' (6)		H' (6)		AWCD (2)		R' (2)		H' (2)	
	R	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*
FA	-0.04	*ns*	0.19	*ns*	0.30	*ns*	**0.72**	**	**0.76**	**	**-0.74**	**	-0.16	*ns*	**-0.67**	*	-0.28	*ns*	**-0.69**	*	0.21	*ns*			**0.78**	**	**0.82**	**	**0.75**	**	**0.79**	**	**0.74**	**	**0.73**	**
AWCD (6)	0.43	*ns*	-0.11	*ns*	-0.20	*ns*	**0.80**	**	**0.80**	**	**-0.82**	**	-0.15	*ns*	-0.58	*ns*	0.01	*ns*	**-0.67**	*	0.33	*ns*	**0.78**	**			**0.98**	***	**0.94**	***	**0.98**	***	**0.97**	***	**0.97**	***
R' (6)	0.44	*ns*	-0.07	*ns*	-0.11	*ns*	**0.79**	**	**0.74**	**	**-0.87**	***	-0.23	*ns*	**-0.67**	*	-0.05	*ns*	**-0.74**	**	0.24	*ns*	**0.82**	**	**0.98**	***			**0.94**	***	**0.97**	***	**0.98**	***	**0.98**	***
H' (6)	0.34	*ns*	-0.04	*ns*	0.01	*ns*	**0.81**	**	**0.78**	**	**-0.82**	**	-0.06	*ns*	-0.56	*ns*	0.01	*ns*	**-0.66**	*	0.36	*ns*	**0.75**	**	**0.94**	***	**0.94**	***			**0.93**	***	**0.93**	***	**0.92**	***
AWCD (2)	0.32	*ns*	-0.10	*ns*	-0.11	*ns*	**0.77**	**	**0.76**	**	**-0.85**	***	-0.18	*ns*	**-0.62**	*	-0.05	*ns*	**-0.73**	**	0.30	*ns*	**0.79**	**	**0.98**	***	**0.97**	***	**0.93**	***			**0.96**	***	**0.95**	***
R' (2)	0.53	*ns*	-0.18	*ns*	-0.20	*ns*	**0.66**	*	**0.68**	*	**-0.87**	***	-0.31	*ns*	**-0.68**	*	-0.07	*ns*	**-0.74**	**	0.17	*ns*	**0.74**	**	**0.97**	***	**0.98**	***	**0.93**	***	**0.96**	***			**0.99**	***
H' (2)	0.55	*ns*	-0.18	*ns*	-0.21	*ns*	**0.65**	*	**0.67**	*	**-0.85**	***	-0.32	*ns*	**-0.68**	*	-0.05	*ns*	**-0.73**	**	0.15	*ns*	**0.73**	**	**0.97**	***	**0.98**	***	**0.92**	***	**0.95**	***	**0.99**	***		

Table 3: Nested ANOVA's were conducted to investigate the effect of "site" and "plots within sites" on: FA **(A)** (i.e. measured only once at end of exposure period) and functional indices measured after 2 **(B)** and 6 **(C)** days of incubation, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

				FA		
	Source	**DF**	**SS**	**MS**	**F**	**P**
A	***Site***	1	6687.77	6687.7	27.70	**0.01****

Plot	4	965.61	241.40	1.73	0.26
Subplot	6	838.87	139.81		
Total	11	8492.24			

		AWCD Day 2					R' Day 2				H' Day 2			
	Source	**DF**	**SS**	**MS**	**F**	**P**	**SS**	**MS**	**F**	**P**	**SS**	**MS**	**F**	**P**
B	***Site***	1	0.02	0.02	9.91	**0.04***	2.37	2.37	5.32	0.08 ***ns***	0.42	0.42	4.60	**0.01****
	Plot	4	0.01	0.00	9.34	**0.01****	1.78	0.45	167.44	**0.00******	0.37	0.09	310.63	**0.00******
	Subplot	6	0.00	0.00			0.02	0.00			0.00	0.00		
	Total	11	0.03				4.17				0.79			

		AWCD Day 6					R' Day 6				H' Day 6			
	Source	**DF**	**SS**	**MS**	**F**	**P**	**SS**	**MS**	**F**	**P**	**SS**	**MS**	**F**	**P**
C	***Site***	1	0.08	0.08	7.86	0.05 ***ns***	0.69	0.69	7.71	0.05 ***ns***	0.01	0.01	11.64	**0.03***
	Plot	4	0.04	0.01	22.99	**0.001*****	0.36	0.09	42.47	**0.00******	0.00	0.00	5.48	**0.03***
	Subplot	6	0.00	0.00			0.01	0.00			0.00	0.00		
	Total	11	0.12				1.06				0.01			

Table 4: Analysis of covariance (ANCOVA) was performed to understand the effect of metal pollution taking into account sampling site (F8 versus HOP), not significant = *ns*, *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

		FA				AWCD				R'				H'			
Source	***df***	***SS***	***MS***	***F***	***p***	***SS***	***MS***	***F***	***p***	***SS***	***MS***	***F***	***p***	***SS***	***MS***	***F***	***p***
rrected model	4	7340.98	1835.24	11.16	0.00	1.67	0.42	9.50	0.01	1035.71	258.93	24.15	0.00	0.72	0.18	10.72	0.0
ercept	1	3730.24	3730.24	22.68	0.00	1.46	1.46	33.25	0.00	708.56	708.56	66.08	0.00	12.74	12.74	761.24	0.0
	1	0.24	0.24	0.00	0.97 *ns*	0.25	0.25	5.68	**0.04***	118.97	118.97	11.10	**0.01***	0.10	0.10	5.75	**0.0**
	1	282.13	282.13	1.72	0.23 *ns*	0.07	0.07	1.49	0.26 *ns*	34.15	34.15	3.19	0.12 *ns*	0.02	0.02	1.41	0.27
	1	40.72	40.72	0.25	0.63 *ns*	0.09	0.09	2.01	0.20 *ns*	36.68	36.68	3.42	0.11 *ns*	0.03	0.03	2.05	0.19
e	1	2055.68	2055.68	12.50	**0.01***	0.10	0.10	2.21	0.18 *ns*	58.78	58.78	5.48	0.05 *ns*	0.05	0.05	3.09	0.12
or	7	1151.27	164.47			0.31	0.04			75.06	10.72			0.12	0.02		
al	12	40592.78				11.36				5059.85				109.56			
rrected Total	11	8492.25				1.98				1110.77				0.84			

Table S1: BIOLOG® EcoPlates™ carbon source guild groupings

Well No.	ID	C-source	Guild
1	*A1*	*Water (blank)*	
2	*A2*	*β-Methyl-D-Glucoside*	Carbohydrate
3	*A3*	*D-Galactonic Acid y-Lactone*	Carboxylic & acetic acid
4	*A4*	*L-Arginine*	Amino acids
5	*B1*	*Pyruvic Acid Methyl Ester*	Carbohydrate
6	*B2*	*D-Xylose*	Carbohydrate
7	*B3*	*D-Galacturonic Acid*	Carboxylic & acetic acid
8	*B4*	*L-Asparagine*	Amino acids
9	*C1*	*Tween 40*	Polymers
10	*C2*	*i-Erythritol*	Carbohydrate
11	*C3*	*2-Hydroxy benzoic Acid*	Carboxylic & acetic acid
12	*C4*	*L-Phenylalanine*	Amino acids
13	*D1*	*Tween 80*	Polymers
14	*D2*	*D-Mannitol*	Carbohydrate
15	*D3*	*4-Hydroxy Benzoic Acid*	Carboxylic & acetic acid
16	*D4*	*L-Serine*	Amino acids
17	*E1*	*α-Cyclodextrin*	Polymers
18	*E2*	*N-Acetyl-D-Glucosamine*	Carbohydrate
19	*E3*	*y-Hydroxybutyric Acid*	Carboxylic & acetic acid
20	*E4*	*L-Threonine*	Amino acids
21	*F1*	*Glycogen*	Polymers
22	*F2*	*D-Glucosaminic Acid*	Carboxylic & acetic acid
23	*F3*	*Itaconic Acid*	Carboxylic & acetic acid
24	*F4*	*Glycyl-L-Glutamic Acid*	Amino acids
25	*G1*	*D-Cellobiose*	Carbohydrate
26	*G2*	*Glucose-1-Phosphate*	Carbohydrate
27	*G3*	*α-Ketobutyric Acid*	Carboxylic & acetic acid
28	*G4*	*Phenylethyl-amine*	Amines/amides
29	*H1*	*α-D-Lactose*	Carbohydrate
30	*H2*	*D,L-α-Glycerol Phosphate*	Carbohydrate
31	*H3*	*D-Malic Acid*	Carboxylic & acetic acid
32	*H4*	*Putrescine*	Amines/amides

Figure S1: Clustering of study plots according to soil physicochemical properties (i.e. °C, M %, pH, clay % OC %) and pseudo total metal concentrations (i.e. As, Cd, Cu, Ni, Pb, Zn).

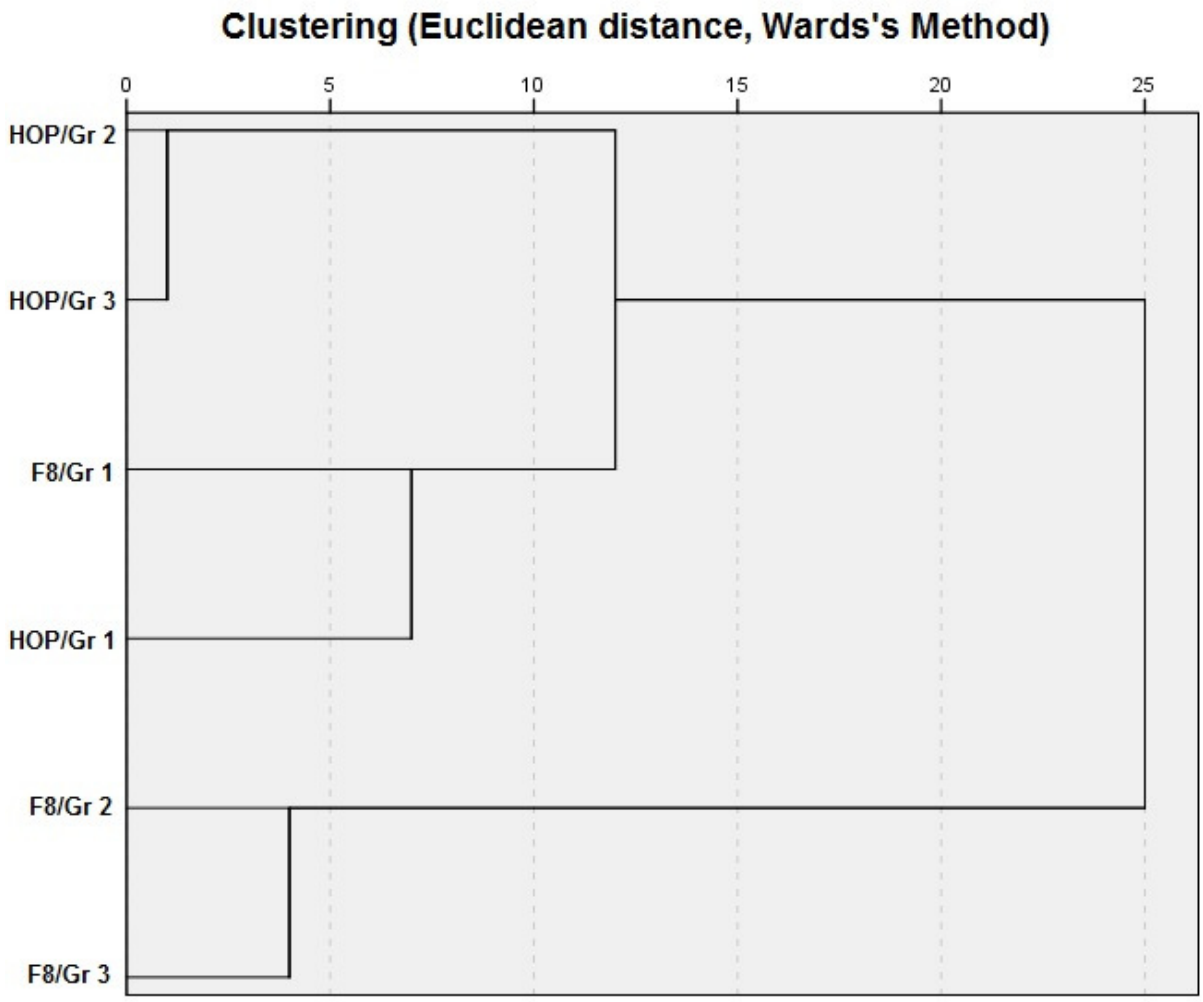
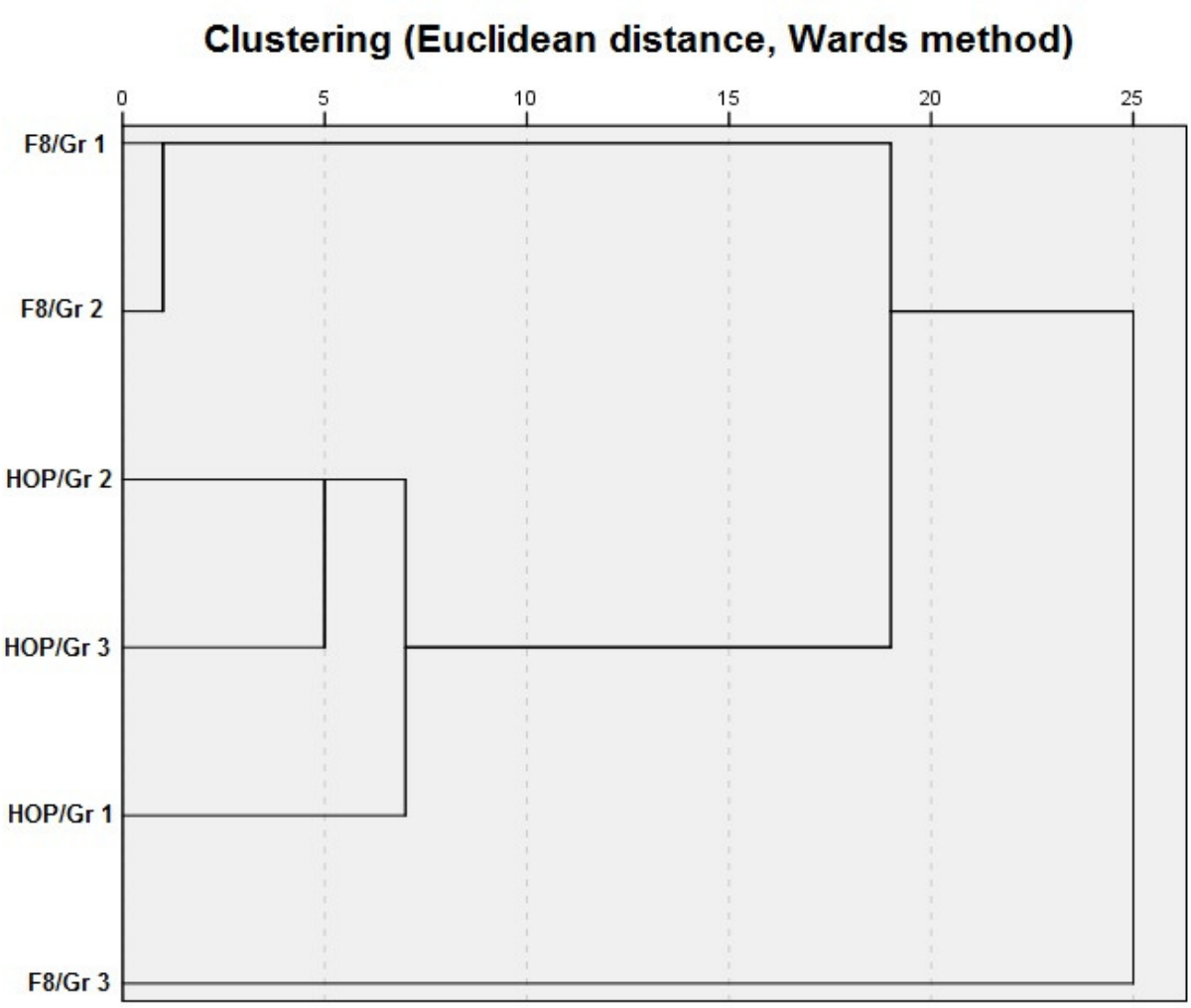


Figure S2: Clustering analysis of soil average well color development (AWCD) sampled along a grassland pollution gradient. The AWCD is an indication of the community level physiological profile (CLPP) or carbon source utilization.


Clustering (Euclidean distance, Wards method)
0
5
10
15
20
25
F8/Gr 1
F8/Gr 2
HOP/Gr 2
HOP/Gr 3
HOP/Gr 1
F8/Gr 3

48
49
50