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Multiple Substrate-Induced Respiration and Isothermal Calorimetry

Applicability in Risk Assessment of Contaminated Soil

Caroline Wright

ABSTRACT

Multiple substrate-induced respiration and isothermal calorimetry – applicability in risk assessment of contaminated soil

Caroline Wright

At present, soils face great threats. Consequences of human activities, such as climate change, acidification and contamination result in decreased soil health. This is a threat to human health and well-being, since our society is dependent on soil ecosystem services. The soil ecosystems provide resources, such as food and fresh water, regulate the climate and play key parts in important life supporting biological processes, e.g. cycling of carbon and nutrients. Due to increased awareness of the threats that soils face, and its importance to humans, soil quality monitoring has recently received increased attention.

Microorganisms run most biological processes in the soil, such as decomposition of organic material and nutrient cycling. Thus, microbial activity and diversity are considered useful biological indicators for soil quality monitoring. These biological properties can be examined using different methods.

The aim of the project was to evaluate the potential of multiple substrate-induced respiration (MSIR), using the MicroResp^{TM} system, and isothermal calorimetry for determining microbial activity and diversity in soils contaminated with copper (Cu) and polycyclic aromatic hydrocarbons (PAH). Thereby, the methods' applicability in risk assessment of contaminated soil could be decided. MSIR is considered appropriate for determining microbial activity and functional diversity, while isothermal calorimetry has not been tested as much in this area. The calorespirometric ratio (produced heat per unit CO_2) was calculated to evaluate potential relationships between heat and CO_2 at different contamination levels.

Although there was some variation between the methods, Cu had a clear effect on both microbial activity and functional diversity. Both methods were thus considered applicable in risk assessment of soil contaminated with Cu. The impact of PAH appeared to be more complex, the effects on microbial activity varied and PAH had little significant effect on functional diversity. Neither of the methods were therefore considered applicable for assessment of soil contaminated with PAH. The calorespirometric ratio did not provide useful results, and cannot be recommended for risk assessment purposes at present.

Keywords: Risk assessment, biological indicators, multiple substrate-induced respiration, MSIR, MicroResp^{TM}, isothermal calorimetry, calorespirometric ratio, contamination, Cu, PAH

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REFERAT

Multipla substrat-inducerad respiration och isotermisk kalorimetri – tillämplighet i riskbedömning av förorenad mark

Caroline Wright

I dagsläget utsätts marken för stora hot. Följder av mänsklig aktivitet, så som klimatförändringar, försurning och förorening försämrar markens kvalitet. Detta är ett hot mot människors hälsa och välmående, eftersom vårt samhälle är beroende av markens ekosystemtjänster. Markens ekosystem förser oss med exempelvis mat och rent vatten, reglerar klimatet, och har nyckelroller i viktiga biologiska processer, exempelvis cirkulering av kol och näringsämnen. På grund av ökad medvetenhet om hoten mot marken samt dess betydelse för människan, har kontroll av markens kvalitet börjat få ökad uppmärksamhet.

Mikroorganismer sköter de flesta biologiska processer som sker i marken, så som nedbrytning av organiskt material och cirkulering av näringsämnen. Därmed anses mikrobiell aktivitet och diversitet vara lämpliga biologiska indikatorer vid kontroll av markens kvalitet. Dessa biologiska egenskaper kan mätas med flera olika metoder.

Syftet med projektet var att utvärdera potentialen i att använda multipla substrat-inducerad respiration (MSIR), genom att använda systemet MicroResp^{TM}, samt isotermisk kalorimetri för att mäta mikrobiell aktivitet och funktionell diversitet i mark förorenad med koppar (Cu) och polycykliska aromatiska kolväten (PAH). Därmed kunde metodernas tillämplighet i riskbedömning av förorenad mark bestämmas. MSIR anses vara en lämplig metod i syfte att undersöka mikrobiell aktivitet och funktionell diversitet, medan isotermisk kalorimetri inte är lika beprövat. Kvoten mellan värmeproduktion och respirerad CO_2, *the calorespirometric ratio*, beräknades för att utvärdera eventuella samband mellan värmeproduktion och respiration vid olika föroreningskoncentrationer.

Trots att det förekom viss variation mellan metoderna, hade Cu en tydlig effekt på både mikrobiell aktivitet och funktionell diversitet. Båda metoder ansågs därför vara tillämpbara i riskbedömning av Cu-förorenad jord. PAH hade varierande effekt på mikrobiell aktivitet och liten signifikant effekt på funktionell diversitet. Ingen av metoderna ansågs därför tillämpbar i riskbedömning av jord förorenad med PAH. *The calorespirometric ratio* tillhandahöll ej användbara resultat, och kunde därmed inte rekommenderas i riskbedömningssyfte.

Nyckelord: Riskbedömning, biologiska indikatorer, multipla substrat-inducerad respiration, MSIR, MicroResp^{TM}, isotermisk kalorimetri, calorespirometric ratio, förorening, Cu, PAH

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PREFACE

This Master's thesis corresponds to 30 ETCS and is the final part of the M.Sc. in Environmental and Water Engineering at Uppsala University. My supervisor was Professor Dan Berggren Kleja and my subject reviewer was Associate Professor Anke Herrmann, both at the Department of Soil and Environment at the Swedish University of Agricultural Sciences.

The project was performed as a part of the science project APPLICERA, which is a collaboration between the Swedish University of Agricultural Sciences, the Swedish Geotechnical Institute, Chalmers University of Technology and Örebro University.

I would like to thank my subject reviewer Anke Herrmann, who with great engagement has provided valuable knowledge and assistance throughout the course of this project. You have patiently answered my questions which I am very grateful for. I would also like to thank my supervisor Dan Berggren Kleja for giving me the opportunity to carry out this project, for sharing his expertise and providing valuable feedback on the report.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Mänsklig aktivitet utgör ett stort hot mot markens funktion i dagsläget. Klimatförändringar och försurning till följd av bland annat förbränning av fossila bränslen, så som olja och kol, påverkar marken negativt. Även föroreningar från exempelvis gamla industriområden hotar markens hälsa. Försämrad markhälsa påverkar även människors hälsa och välmående eftersom vi på många sätt är beroende av marken. I marken finns ekosystem som förser vårt samhälle med många viktiga råvaror och tjänster. De förser oss till exempel med mat och rent vatten, och bidrar till reglering av klimatet genom att reglera koncentrationer av växthusgaser (exempelvis koldioxid, dikväveoxid och metan) i atmosfären. Markens ekosystem har också nyckelroller i många viktiga biologiska processer, exempelvis cirkulering av kol och näringsämnen. På grund av ökad medvetenhet om hoten mot marken samt dess betydelse för människan, har kontroll av markens kvalitet börjat få ökad uppmärksamhet.

Marklevande mikroorganismer, så som bakterier, har stor påverkan på markens funktion. De driver de flesta av de biologiska processer som äger rum i marken, exempelvis nedbrytning av organiskt material och cirkulering av näringsämnen. På grund av mikroorganismernas stora betydelse kan de också användas för att indikera markens kvalitet och funktion; de är lämpliga *biologiska indikatorer*. Två egenskaper som anses vara lämpliga biologiska indikatorer är mikroorganismernas aktivitet och mångfald. Det finns många olika metoder för att mäta dessa egenskaper.

När människors hälsa, miljön eller omkringliggande naturresurser hotas av förorenad mark, måste åtgärder vidtas. Som en del av processen att bestämma lämplig åtgärd bör en riskbedömning göras. Syftet med projektet var att utvärdera två metoders potential att mäta mikroorganismers aktivitet och mångfald, och därmed avgöra deras lämplighet i riskbedömning av förorenad mark. Metoderna mätte mikroorganismernas respiration (avgång av koldioxid, som är slutprodukten i många mikroorganismers ämnesomsättning) och värmeproduktion (värme bildas i markprocesser som frigör energi) till följd av tillsats av sju olika kolsubstrat, det vill säga organiskt kol som mikroorganismerna kan använda i sin ämnesomsättning. Respirationen och värmeproduktionen mättes i oförorenade jordprover samt jordprover förorenade med metallen koppar (Cu) och den organiska föroreningsgruppen polycykliska aromatiska kolväten (PAH) för att undersöka om föroreningarna hade någon påverkan på mikroorganismernas aktivitet och mångfald.

Cu är en essentiell, det vill säga livsnödvändig, metall som släpps ut i naturen i och med exempelvis användande av bekämpningsmedel i jordbruket och motorfordon. Trots att det inte är så giftigt för däggdjur, är det mycket giftigt för mikroorganismer. Därför var det föga förvånande att båda metoder visade att Cu generellt minskande mikroorganismernas aktivitet och mångfald. PAH är en föroreningsgrupp bestående av organiska ämnen. Exempel på källor för utsläpp av PAH är uppvärmning och ofullständig förbränning av organiskt material. De olika ämnena i gruppen PAH är olika giftiga, vissa är till exempel cancerframkallande. I mätningarna av mikroorganismernas respiration och produktion av värme, hade PAH en mer komplex effekt än Cu. Effekten på mikroorganismernas aktivitet varierade mellan jordar och metoder, och PAH hade liten signifikant effekt på den mikrobiella mångfalden.

Överlag visade mätning av både värmeproduktion och respiration potential för att korrekt kunna mäta mikroorganismernas aktivitet och mångfald i jord förorenad med Cu. Därför ansågs båda metoder vara tillämpliga i riskbedömning av mark som är förorenad av Cu. Det förekom viss variation mellan metoderna vilken bör redas ut i ytterligare försök. Effekten av PAH bedömdes vara alltför komplex för att metoderna skulle kunna tillhandahålla tillförlitliga resultat. Ingen av metoderna ansågs därför vara användbar i riskbedömning av PAH-förorenad mark.

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1 INTRODUCTION

1.1 BACKGROUND

1.1.1 Ecosystem services of soils

Soils are essential to human societies. Ecosystems which inhabit the soil provide a wide range of goods and services. These can be arranged as supporting, provisioning, regulating and cultural services (Haygarth and Ritz, 2009). The supporting services maintain life on Earth through processes such as primary production, nutrient cycling and soil formation. The provisioning ecosystem services supply food and fresh water as well as raw- and biomaterials and a platform for infrastructure. The regulating services regulate water supply and quality. They also regulate the climate through regulation of atmospheric concentrations of greenhouse gases, such as carbon dioxide (CO_2), nitrous oxide (N_2O) and methane (CH_4). Finally, the cultural ecosystem services provide spiritual, recreational, and cultural value (Haygarth and Ritz, 2009).

Soils are one of Earth's most complex systems (Ramsden and Kervalishvili, 2008). They are heterogeneous systems which make up a habitat to immense diversity (Haygarth and Ritz, 2009; Turbé et al., 2010), regarding abundance within species, species richness and function (Pulleman et al., 2012). The biodiversity in soil exceeds the biodiversity anywhere else in the biosphere. Without its diversity, the soil would not be able to deliver its important ecosystem services (Turbé et al., 2010).

As a result of increased awareness of the soils' essential ecosystem services, soil quality monitoring is receiving attention (Creamer et al., 2009; Pulleman et al., 2012). Soil quality, or soil health, has many definitions. What they all have in common, however, is that the soil should be able to function in a fashion that is consistent with its intended use, at present and in the future (Haygarth and Ritz, 2009). To ensure sustainable delivery of ecosystem services, assessment tools for soil quality monitoring are required (Turbé et al., 2010).

1.1.2 Biological indicators in soil quality monitoring

Although the soil biota runs most processes in soil, soil function has previously been associated with the soil's physical and chemical properties (Ritz et al., 2009). Due to the importance of biota to soil function, biological properties should be obvious indicators of soil quality (Nyberg et al., 2006; Black et al., 2008; Creamer et al., 2009; Ritz et al., 2009). Biological indicators have been used in specific situations for some time (Griffiths et al., 2016), but few have reached national success or the status as international standards (Ritz et al., 2009) and there is no obtainable standardized set (Pulleman et al., 2012).

Indicators are measurable substitutes to soil functions which might be too complex to assess (Turbé et al., 2010; Pulleman et al., 2012). Their purpose is to simplify information and measurements (Turbé et al., 2010). Various biological properties have been suggested as indicators. Ritz et al. (2009) developed a methodology in order to select appropriate indicators of soil quality. One of the candidates they considered to be applicable were abundance and diversity of nematodes, which was also suggested by Turbé et al. (2010). Nematodes show sensitivity regarding land use and soil type variations but the

measurement is, however, time consuming and requires specific knowledge (Turbé et al., 2010). Abundance and diversity of earthworms is also considered a useful indicator. These properties are easy to measure and sensitive to land use and soil type variations (Turbé et al., 2010). Most of the biological processes which take place in soil are run by microorganisms. They play key parts in fundamental processes, such as decomposition of soil organic material (SOM) and circulation of carbon and nutrients (Nyberg et al., 2006; Van Der Heijden et al., 2008; Ritz et al., 2009; Pulleman et al., 2012). Consequently, microbial activity and diversity are indicators of functions in which microorganisms participate (Turbé et al., 2010).

Diversity can be assessed using a variety of indexes (DeJong, 1975), e.g. The Shannon Index, established by Pielou (1975; Stevens et al., 2003) and the Simpson Index, established by Simpson (1949; DeJong, 1975). The Shannon Index puts much weight on the species richness, while the Simpson Index is more influenced by the species evenness (DeJong, 1975). However, biodiversity has often been ignored in monitoring of soil, since it is considered an ambiguous concept, and thus difficult to measure. Instead, methods which evaluate the soil's functional diversity have been established (Turbé et al., 2010). The functional diversity is the distribution of functions within the soil community (Stevens et al., 2003). The Shannon Index and the Simpson Index can also be applied for estimations of functional diversity.

1.1.2.1 Multiple substrate-induced respiration

During microbial decomposition of SOM, some of the organic carbon is assimilated into biomass while some is respired to the atmosphere as CO_2 (Schlesinger and Andrews, 2000; Harris et al., 2012; Bölscher et al., 2016). Multiple substrate-induced respiration (MSIR) measures the respiration in the soil. MSIR measurements reflect microbial activity, and can therefore be used to assess e.g. carbon cycling and decomposition of organic material (Ritz et al., 2009). It is an established and frequently used method in soil quality monitoring (Jensen and Mesman, 2006), and it is used on larger scales in some countries, such as the UK and Switzerland (Pulleman et al., 2012). Measurements of the microorganisms' respiration can also be used to assess microbial functional diversity (Turbé et al., 2010).

There is a variety of different methods available for measuring respiration (Black et al., 2008). The MicroResp™ system (Campbell et al., 2003), is ranked as one of the best (Black et al., 2008). There is, however, some debate regarding MicroResp™'s applicability on larger scales. Its high throughput is what makes it applicable for large scale soil monitoring (Black et al., 2008; Ritz et al., 2009). However, both Black et al. (2008) and Ritz et al. (2009) pointed out that there is still need for further testing across different land uses, and according to Creamer et al. (2009), MicroResp™ should be used carefully on large scales.

1.1.2.2 Isothermal calorimetry

As opposed to MSIR, which only takes into account the CO_2 produced in complete metabolic processes, isothermal calorimetry is an energetics approach which includes net heat production from all catabolic and anabolic processes in soil (Herrmann and Bölscher, 2015). It includes complete and incomplete aerobic metabolic processes, anaerobic metabolic processes and abiotic processes (Haglund et al., 2003). Heat production is

considered a suitable substitute for assessing microbial activity (Braissant et al., 2010), and isothermal calorimetry has been proven effective when it comes to accurately measuring heat production in soils (Harris et al., 2012; Herrmann et al., 2014).

While MSIR is considered suitable for monitoring soil quality (Jensen and Mesman, 2006), there is not much research about applying isothermal calorimetry for this purpose. However, it could potentially function as an alternative, or supply complementary information, to respiration measurements (Herrmann et al., 2014).

1.1.2.3 The calorespirometric ratio

Measurements of CO_2 and heat can be combined using the calorespirometric ratio (produced heat per unit CO_2; equation 1 on page 8). It has been used to assess microbial metabolism and metabolic efficiency, i.e. how efficiently soil microorganisms decompose soil organic carbon (SOC; Barros et al., 2010). For decomposition of organic material with identical composition, higher ratios indicate lower metabolic efficiency, since more waste heat is released per unit CO_2 (Herrmann and Bölscher, 2015). The calorespirometric ratio is not, however, established in risk assessment of contaminated soil.

1.1.3 Soil contamination and remediation

Contamination of soil is one of the main threats to soil quality (Howard, 1993; Creamer et al., 2010). Exposure to contaminants, such as metals and organic contaminants, can harm soil organisms which seriously impacts soil ecosystems (Creamer et al., 2010). This project focuses on the toxic effects of the metal copper (Cu) and the organic contaminant group polycyclic aromatic hydrocarbons (PAH) on soil microorganisms.

1.1.3.1 Copper

Although Cu is an essential metal (Berggren Kleja et al., 2006; Wang et al., 2010), it is very toxic to microorganisms (Sternbeck, 2000). The sensitivity varies a lot among species (Berggren Kleja et al., 2006), bacteria are particularly sensitive (Wang et al., 2010). Wang et al. (2009) showed that Cu reduces the soil microorganisms' ability to carry out their functions. Exposure to Cu has also been shown to affect microbial activity (Wang et al., 2007, 2010; Li et al., 2015) and diversity (Wang et al., 2007). According to the work of (Airoldi and Critter, 1996), exposure to Cu results in reduced microbial growth and ability to degrade glucose. The fraction of bioavailable Cu determines its toxicity. The two major factors which affect the bioavailability are pH and SOM content (Berggren Kleja et al., 2006).

1.1.3.2 Polycyclic aromatic hydrocarbons

PAH occur in mixtures containing different compounds of varying toxicity (CCME, 2008; Naturvårdsverket, 2009), which can obstruct the assessment of potential risks (CCME, 2008). The toxicity of PAH is also influenced by the bioavailability (El-Alawi et al., 2002). The presence of PAH in soil can both increase and decrease microbial activity and diversity. Some bacteria and fungi can utilize PAH as a carbon source (CCME, 2008; Turbé et al., 2010), which increases microbial activity. At high concentrations, however, the toxic effect leads to decreased activity (Dawson et al., 2007;

CCME, 2008). Sverdrup et al. (2002) observed no effects on bacterial diversity following PAH exposure.

Due to the threats of contamination, remediation of contaminated soil is important to improve soil function. Excavation of contaminated soil is usually required to reduce direct risks to human and environmental health. However, excavation practices greatly impact the soil environment, and result in e.g. soil erosion and disturbance of soil ecosystems (US EPA, 2008). Disturbed soil ecosystems is a big problem since it takes a long time for them to be restored once their function is lost or reduced (Haygarth and Ritz, 2009). It is therefore important to develop a methodology for monitoring the effects of contaminants on soil and ecosystem function without facing negative environmental consequences.

Even though microbial properties have been used and recommended as biological indicators of soil contamination (Brookes, 1995; Winding et al., 2002; Dawson et al., 2007) there is still need to investigate the potential of MSIR and isothermal calorimetry for this purpose.

1.2 AIM

The aim of the project was to evaluate the applicability of MSIR, using the MicroResp^{TM} system, and isothermal calorimetry in risk assessment of contaminated soil. This was done by assessing the effects of Cu and PAH on microbial activity and microbial functional diversity, using both methods. The applicability of each method was evaluated separately, but potential to combine the two, using the calorespirometric ratio, was also investigated.

Three specific questions were formulated:

- Is there a relationship between contamination level and microbial heat production in soil contaminated with Cu and soil contaminated with PAH?
- Is there a relationship between contamination level and microbial respiration in soil contaminated with Cu and soil contaminated with PAH?
- How do different concentrations of Cu and PAH influence the calorespirometric ratio?

1.3 HYPOTHESIS

According to previous research, MSIR is a suitable method for monitoring soil function. Therefore, similar results were expected in this project. Even though there has not been much testing of isothermal calorimetry's applicability per se, it has been proven to accurately measure heat flows from soil, which reflect microbial activity. Thus, isothermal calorimetry was assumed to be useful as well.

The hypotheses to the questions were:

- The heat production was expected to decrease with increasing Cu concentration, due to the well-known toxicity of Cu to microorganisms. The response of PAH on heat release was expected to be a bit more complex, since PAH can both increase and decrease microbial activity.

- For the same reasons as above, the microbial respiration was expected to decrease with increasing Cu concentration while the respiration response to PAH was expected to be more complex.
- The calorespirometric ratio was expected to increase with Cu concentration, since an increase indicates decreased metabolic efficiency. Due to the uncertainties regarding heat and respiration responses to PAH, it remained unclear how the ratio would change at different PAH concentrations.

2 METHODS AND MATERIAL

2.1 LYSIMETER FIELD EXPERIMENT

The soils which were analyzed in the project were prepared in a lysimeter field experiment at the Swedish University of Agricultural Sciences (SLU). Figure 1 shows a picture of the lysimeter park in the summer of 2016.

Figure 1 The lysimeter field experiment in the summer of 2016.

The experiment was initiated in October 2015 (a timeline describing when the different events of the lysimeter experiment took place is shown in Figure 2). The soil which was used in the experiment was sampled from two grasslands in the Uppsala area, Krusenberg (59.741117 °N, 17.682137 °E) and Nåntuna (59.796459 °N, 17.670756 °E). In October 2015, soil from both sites was mixed with 1 M $CuCl_2$ and 1 M CaO to achieve soil with high concentration of Cu (the Cu spike soil). The CaO was added to neutralize the protons released when Cu^{2+} was bound to soil material. The Cu spike soils were placed in lysimeters in the lysimeter park, and put to rest during the winter of 2015/2016 to allow leaching of excess Cu. The Cu spike soils had a target concentration of 3,000 mg kg^{-1}.

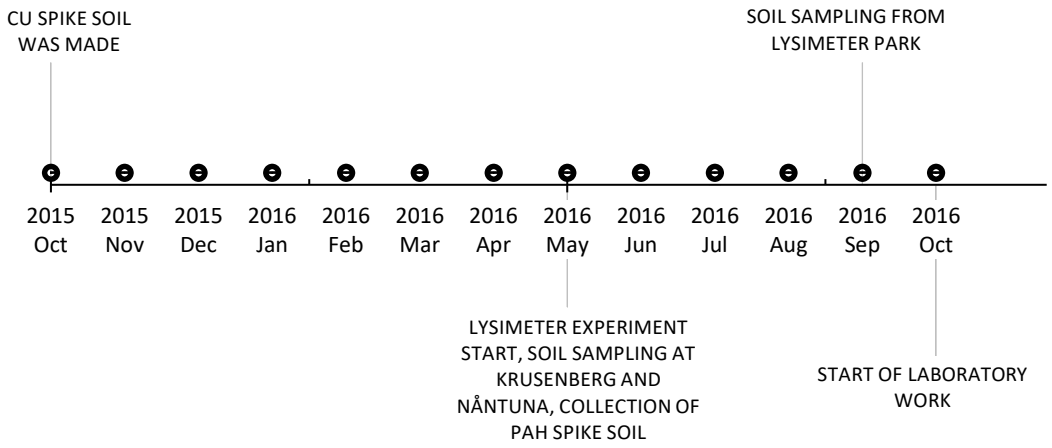

Figure 2 Timeline describing the implementation of the lysimeter field experiment.

In May 2016, soil was sampled from both locations, and soil with high PAH concentration (the PAH spike soil) was collected at the Nässjö impregnation work. The PAH spike soil contained PAH compounds with low (L), medium (M) and high (H) molecular weight at a total concentration of 14,000 mg kg^{-1}. Soils of different Cu and PAH concentration (Table 1) were achieved by mixing the spike soils with uncontaminated soil from Krusenberg and Nåntuna.

Table 1 Initial Cu and PAH-L (PAH with low molecular weight) concentration (mg kg^{-1}) for Cu and PAH treatment 1, 2 and 3. Treatment 1 had the lowest concentration and treatment 3 had the highest concentration

Treatment	Cu conc. (mg kg^{-1})	Treatment	PAH-L conc. (mg kg^{-1})
Cu1	200	PAH1	15
Cu2	430	PAH2	60
Cu3	1100	PAH3	170

The concentrations were chosen with consideration to the following guideline values: Treatment 1 (Cu1, PAH1) was based on the Swedish Environmental Protection Agency's guideline values for contaminated soil (Naturvårdsverket, 2009). They are values for less sensitive land use, such as industrial areas, and correspond to a 50 % protection of the soil organisms. The values for sensitive land use, such as residential areas, correspond to a protection of 75 % (Naturvårdsverket, 2010), but were excluded since such low concentrations were expected to have little effect on the soil. Treatments 2 and 3 (Cu2, PAH2 and Cu3, PAH3) were based on guideline values specified for larger cities. They correspond to a 25 % and 10 % protection of the soil organisms (SWECO, 2009). The PAH concentrations correspond to guideline values for PAH-L, since the concentrations of PAH-L were higher than PAH-M and PAH-H in the PAH spike soil.

Three replicates of uncontaminated samples and each treatment were prepared. A layer of gravel, 60 cm for the uncontaminated and Cu soils and 40 cm for the PAH soils, was placed in the bottom of each lysimeter (Figure 3a and b). To avoid spreading of PAH, a 20 cm layer of activated carbon was added to the PAH lysimeters. About 50 kg of soil was then put in the lysimeters in around 50 cm thick layers. By both weighing the soil and measuring the soil layer, a soil density similar to natural conditions was achieved. Geo textile was placed beneath the gravel and between the layers. The soil was sowed with a seed mix, and to avoid weather conditions preventing growth, the lysimeters were

watered with 1 L of water each per week between the 22nd of June and the 10th of August 2016.

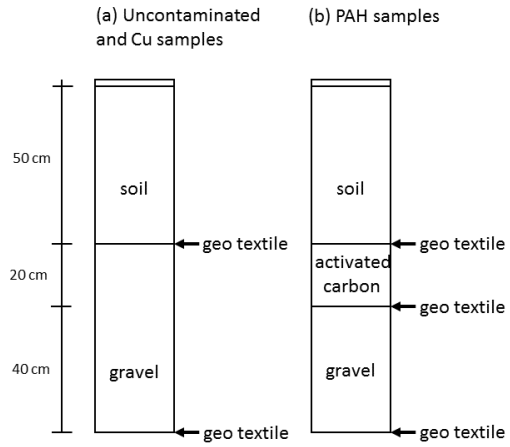

Figure 3 Schematic structure of the lysimeters. (a) The uncontaminated samples and Cu samples had a 60 cm layer of gravel beneath a 50 cm layer of soil. (b) The PAH samples had a layer of gravel of 40 cm in the bottom, which was followed by a 20 cm layer of activated carbon and a 50 cm layer of soil.

2.2 LABORATORY EXPERIMENT

2.2.1 Sampling and preparation of soil

Sampling from the lysimeter field experiment took place at the end of September 2016. The soil samples were sieved through a 2 mm sieve. Grass, roots and other remaining pieces of visible organic material were removed manually. The water content was adjusted to 45 % of the water holding capacity (Harris et al., 2012; Herrmann et al., 2014). The samples were incubated for 10-12 days at 25 °C to avoid effects of the preparations on the microbial community (Herrmann et al., 2014). Due to lack of space in the isothermal calorimeters, all samples could not be analyzed simultaneously. Therefore, the samples were stored at 8 °C during the period before incubation.

2.2.2 Microbial activity and functional diversity

2.2.2.1 Carbon substrate solutions

Seven different carbon substrates, α-ketoglutaric acid, citric acid, D-glucose, γ-aminobutyric acid, L-alanine, N-acetyl glucosamine and α-cyclodextrin, were used in the experiment (Herrmann et al., 2014). To avoid reduced microbial activity due to substrate deficiency, the substrate addition should lead to saturation of the microbial metabolism (Lerch et al., 2013). The work of Lerch et al. (2013) showed that, to saturate the microbial metabolism, the addition of carbon should correspond to at least 10 % of the SOC content. Since the soils had similar content of SOC (Table 2), the same substrate solutions were used for both soils. Nåntuna required a higher addition of carbon to satisfy the microbial metabolism. So, using the SOC content of Nåntuna, it was calculated that 1.4 mg C g^{-1} soil, corresponding to 11 and 10 % of the SOC in Krusenberg and Nåntuna respectively, should be added. The substrates were solved in Milli-Q (ultra-pure) water. The added weights of the carbon substrates varied since they had different carbon content.

2.2.2.2 Reference samples

Reference samples with similar heat capacity as the soil samples, were prepared before the main experiment start. To calculate the heat capacity of the soil samples, the soils' proportions of water (heat capacity: 4.18 J g^{-1} K^{-1}), organic material (heat capacity: 1.30 J g^{-1} K-1) and mineral (heat capacity: 0.83 J g^{-1} K^{-1}) were determined. The volume of Milli-Q water which had similar heat capacity compared to the soil samples was calculated. Milli-Q water was chosen for the reference samples because it is an inert medium, i.e. does not release heat during incubation at constant temperature. The calculated volumes for the Krusenberg and Nåntuna soils were similar. Therefore, an average value was calculated to 1.78 ml Milli-Q water. The same reference samples could thus be used during the whole experiment.

2.2.2.3 Substrate-induced respiration and heat production

Heat production and respiration following substrate addition were measured simultaneously, using a method developed by Herrmann and Bölscher (2015). Soil weights corresponding to 4 g of dry soil were placed in 20 ml reaction vessels, and 300 µl of the carbon substrate solutions were added dropwise to seven separate vessels. Milli-Q water was added to the soil in an eighth vessel as a control sample. To get a starting value, the absorbance of the CO_2 traps was measured using a spectrophotometer (SpectraMax Plus 384, Molecular Devices) at 572 nm after which they were placed in the vessels. The reaction vessels were then closed and placed in the isothermal calorimeters (TAM Air, TA Instruments) together with the reference samples. Heat production was measured continuously throughout the measurement. The duration of all measurements was initially planned to be 6 h. However, on the first day it was discovered that the respiration in the Nåntuna samples amended with α-ketoglutaric acid and citric acid was very high. A duration of 6 h would affect the accuracy of the results. Therefore, the duration of these measurements was reduced to 3.5 h. At the end of the experiment, the absorbance of the CO_2 traps was measured a second time. All measurements were performed at 25 °C.

In order to calculate the produced CO_2, the absorbance data were fitted to a calibration curve ($y = 5.95x^{-3.64}$, $R^2 = 0.97$; Figure A1), with absorbance on the x-axis and equilibrium CO_2 (µg) on the y-axis, which was made during the main experiment. To only account for the heat and CO_2 produced following substrate addition, the production in the Milli-Q water control samples were subtracted for all samples. The soil samples amended with α-ketoglutaric acid and citric acid were also corrected for abiotic processes (see below) to only take biotic processes into account. The basal respiration (see below) was found to be negligible and was thus not accounted for.

2.2.2.4 Abiotic processes

Herrmann et al. (2014) showed that addition of the carboxylic acids α-ketoglutaric acid and citric acid to sterile soil lead to significant heat production, indicating that the addition of these carbon substrates resulted in heat production from abiotic processes. Therefore, measurements of heat from abiotic processes were made for samples amended with α-ketoglutaric acid and citric acid. This was also done for samples amended with Milli-Q water to assess basal respiration. Similar to previous preparations, soil corresponding to 4 g of dry weight was placed in 20 ml glass reaction vessels. The soil samples were

sterilized by covering the vessels with aluminum foil and heating them to 120 °C for 20 minutes in an autoclave (3150 EL, Tuttnauer). After two days, the samples were put in the autoclave again, and the procedure was repeated. The carbon substrates and Milli-Q water were sterilized through a 0.2 µm filter to containers sterilized with ethanol before adding them to the soil. The production CO_2 and heat was determined as described above (Herrmann and Bölscher, 2015). The samples amended with Milli-Q water were stored in an incubator during the measurement due to lack of space in the calorimeters. The duration of the measurements was 6 h for the Krusenberg samples and 3.5 h for the Nåntuna samples, and the temperature was 25 °C.

2.3 THE CALORESPIROMETRIC RATIO

To determine if there were any relationships between heat production and respiration at different Cu and PAH concentrations, the calorespirometric ratio, γ, (mJ μg^{-1}) was calculated (equation 1).

$$\gamma = \frac{Q}{CO_2} \quad (1)$$

Where Q (mJ g^{-1} soil) is the produced heat and CO_2 (µg g^{-1} soil) is the respired CO_2.

2.4 THE SHANNON INDEX

To evaluate the microbial functional diversity, i.e. how well the microorganisms could utilize the carbon substrates at different contaminant concentrations, the Shannon Index was calculated (equation 2). Normalized heat and CO_2 data were used. Data were normalized by dividing the response from the addition of one substrate with the sum of the responses from all substrates (Table A2, A3, A4, and A5).

$$Shannon\ Index = -\sum_{i=1}^{s} p_i \cdot \ln p_i \quad (2)$$

Where p is the heat or CO_2 response to addition of one substrate as a proportion of the total response, and s is the number of substrates.

2.5 THE SIMPSON INDEX

To examine the catabolic evenness, i.e. how even the utilization of the substrates was in the two soils at different contamination levels, the Simpson Index was calculated (equation 3). For the Simpson Index calculation, normalized heat and CO_2 data were used.

$$Simpson\ Index = \frac{1}{\sum_{i=1}^{s} p_i^2} \quad (3)$$

2.6 STATISTICAL ANALYSIS

All statistical analyses were performed in R version 2.15.1 (R Development Core Team, 2008), using the "Vegan: Community Ecology Package" (Oksanen et al., 2011). PRIMER6 (Clarke and Gorley, 2005) was used to illustrate multi-variate statistics results. Effects of Cu and PAH on microbial functional diversity were examined with principal component analysis (PCA) using normalized heat and CO_2 data. Significant functional diversity differences between soil treatments along ordination axes were analyzed by posthoc one-way ANOVA, followed by Bartlett's test and Tukey multiple pair test comparison on principal component scores. The similarity of the heat and CO_2 data sets

was determined using the Mantel dissimilarity test based on the Pearson product-moment correlation coefficient (999 permutations).

3 RESULTS

3.1 SOIL CHARACTERISTICS

Krusenberg was a sand/loamy sand, while Nåntuna was a sandy loam/loam. The pH in Nåntuna was around 8.4, and was higher than the pH in Krusenberg, which was around 5.5 (Table 2). Cu had no effect on pH, i.e. the protons released when Cu^{2+} was bound to the soil were efficiently buffered by the added CaO. The Cu concentration in Krusenberg and Nåntuna had decreased to 60 and 80 % of the initial concentrations (cf. Tables 1 and 2). The analysis of the PAH concentrations had not been done within the timeline of this project, and were assumed to be equal to the initial concentrations (Table 1) in the analysis of the results. The soils had similar content of organic carbon and total nitrogen (Table 2).

Table 2 Soil pH and Cu concentrations in the uncontaminated samples and Cu samples. Content of organic carbon and total nitrogen in the uncontaminated samples. The values are mean ± standard deviation (n = 3). The soil samples for pH and Cu concentration were sampled in September 2016, and the samples for organic carbon and total nitrogen were sampled in June 2016

Soil and treatment	pH (water)	Cu conc. (mg g^{-1} soil)	Org. C (%)	Tot. N (%)
Krusenberg soil	5.5 ± 0.03	9 ± 1	1.3 ± 0.03	0.1 ± 0.003
Cu1	5.4 ± 0.03	121 ± 10	-	-
Cu2	5.4 ± 0.04	260 ± 15	-	-
Cu3	5.6 ± 0.02	652 ± 47	-	-
Nåntuna soil	8.4 ± 0.03	7 ± 1	1.4 ± 0.1	0.1 ± 0.01
Cu1	8.4 ± 0.03	163 ± 11	-	-
Cu2	8.4 ± 0.04	359 ± 92	-	-
Cu3	8.3 ± 0.05	900 ± 25	-	-

3.2 OVERALL MICROBIAL ACTIVITY

3.2.1 Effects on heat production

Cu had an apparent impact on heat production in both soils (Figure 4a and c, Table 3). The heat production decreased the most between the uncontaminated samples and the Cu1 samples, which had Cu concentrations around 150 mg kg^{-1}. Comparing the uncontaminated samples to the Cu3 samples, there was a decrease from about 200 to 90 mJ g^{-1} soil h^{-1} in the Krusenberg soil and 1,000 to 300 mJ g^{-1} soil h^{-1} in Nåntuna soil. The data were fitted to logarithmic curves (R^2 = 0.95 for Krusenberg and 0.98 for Nåntuna).

The impact of PAH on heat production varied between the soils (Figure 4b and d, Table 3). The heat production increased from approximately 200 mJ g^{-1} soil h^{-1} in the uncontaminated samples to 300 mJ g^{-1} soil h^{-1} in the PAH3 samples in the Krusenberg soil, while there was a decrease in Nåntuna soil from around 1,000 mJ g^{-1} soil h^{-1} to 800 mJ g^{-1} soil h^{-1}. However, both soils followed linear dose-response (R^2 = 0.82 for Krusenberg and 0.68 for Nåntuna).

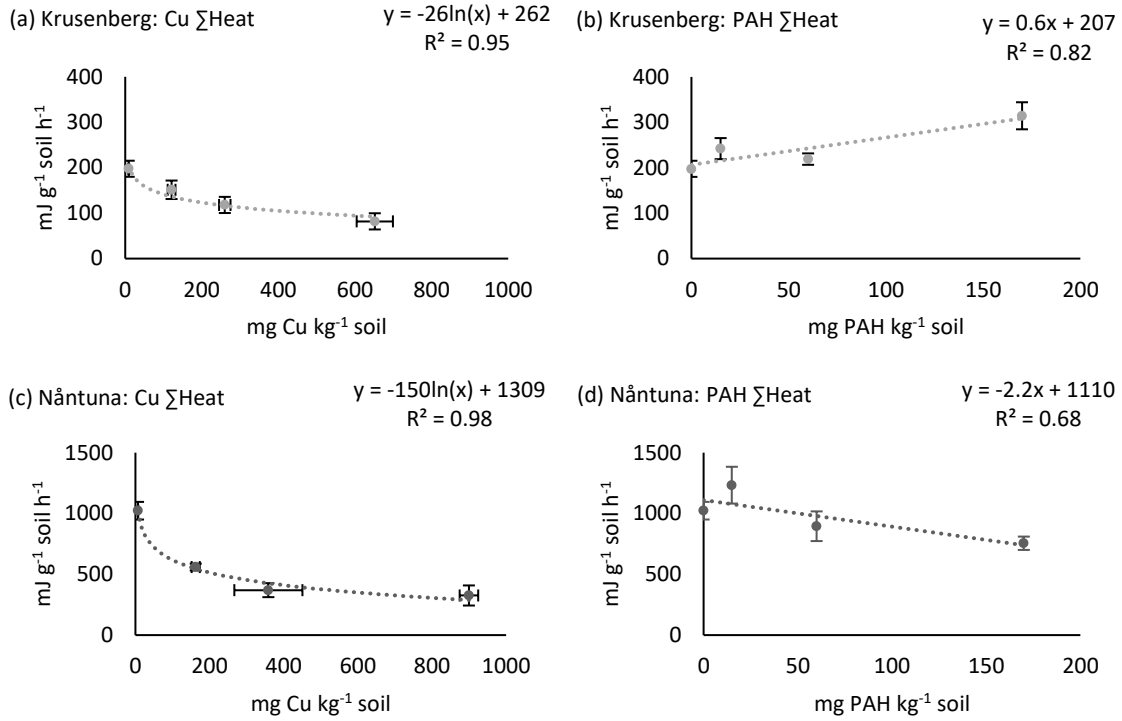

Figure 4 Effect on substrate-induced heat production of (a) Cu in Krusenberg soil (b) PAH in Krusenberg soil (c) Cu in Nåntuna soil (d) PAH in Nåntuna soil. The values represent mean values, vertical and horizontal error bars represent standard deviation (n = 3).

The addition of Cu lead to a greater heat production decrease in the Nåntuna samples than in Krusenberg (Table 3). At Cu3, the heat production in Krusenberg and Nåntuna were 41 and 32 % of the heat production in the uncontaminated samples. PAH1 lead to an increased heat production of approximately 20 % in both soils. At the highest concentration, however, the heat production in the Krusenberg samples had increased with around 60 %, while it had decreased to 74 % of the uncontaminated samples in Nåntuna.

Table 3 Percentage of the heat production in contaminated samples compared to uncontaminated samples. The values for Cu concentration represent mean ± standard deviation (n = 3)

Krusenberg			Nåntuna		
Treatment	Conc. (mg kg^{-1})	Heat (% of uncont.)	Treatment	Conc. (mg kg^{-1})	Heat (% of uncont.)
Cu1	121 ± 10	77	Cu1	163 ± 11	55
Cu2	260 ± 15	60	Cu2	359 ± 92	36
Cu3	652 ± 47	41	Cu3	900 ± 25	32
PAH1	15	122	PAH1	15	121
PAH2	60	111	PAH2	60	88
PAH3	170	159	PAH3	170	74

3.2.2 Effects on respiration

Cu had a decreasing effect on respiration in Krusenberg soil (Figure 5a, Table 4), which was similar to the heat production responses to Cu in both soils (Figure 4a and c). The respiration decreased from around 20 to 2 µg g^{-1} soil h^{-1} comparing the uncontaminated samples to Cu3. The respiration decreased logarithmically with increased Cu (R^2 = 0.98).

Apart from the impact of Cu on respiration in Krusenberg, there were no other clear relationships (Figure 5b, c and d). In Nåntuna, Cu1 and 2 lead to decreased respiration (Figure 5c, Table 4). Comparing the uncontaminated samples to Cu3, there was a slight increase from about 130 to 140 µg g^{-1} soil h^{-1}. In Krusenberg, the respiration remained approximately unchanged at around 30 µg g^{-1} soil h^{-1} despite increasing PAH concentration (Figure 5b). In Nåntuna soil, the respiration increased from around 130 to 220 µg g^{-1} soil h^{-1} between the uncontaminated and PAH1 samples (Figure 5d). For PAH2 and PAH3, it decreased to approximately 70 µg g^{-1} soil h^{-1}.

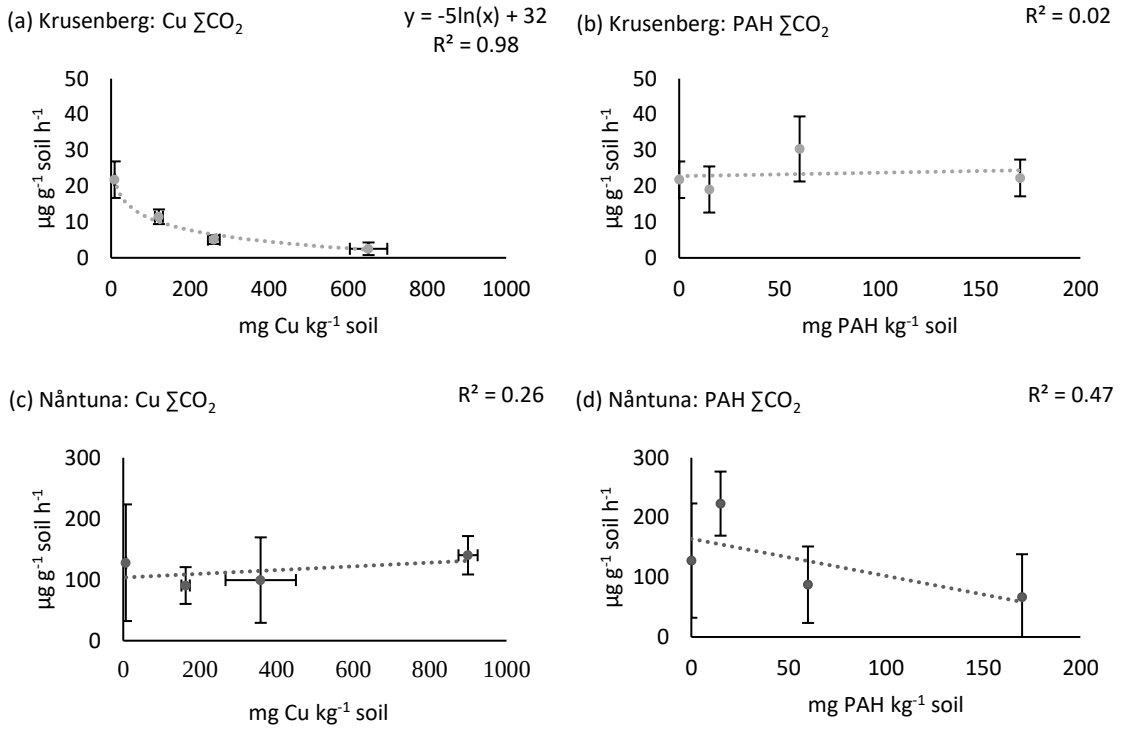

Figure 5 Effects on substrate-induced respiration of (a) Cu in Krusenberg soil (b) PAH in Krusenberg soil (c) Cu in Nåntuna soil (d) PAH in Nåntuna soil. The values represent mean values, vertical and horizontal error bars represent standard deviation (n = 3).

The respiration in the Krusenberg Cu1 samples was approximately 50 % of the respiration in the uncontaminated samples (Table 4). For Cu2 and Cu3, the respiration was 23 and 12 %. There was also an initial decrease to around 70-80 % of the uncontaminated samples following Cu treatment 1 and 2 in the Nåntuna samples. In the Cu3 samples, the respiration was 10 % higher than in the uncontaminated samples. The response to PAH1 in Krusenberg soil was a decrease to 87 %, which was followed by an increase to about 140 %. At PAH3, the respiration was approximately the same as in the uncontaminated samples. In Nåntuna soil there was a respiration increase of 75 % at PAH1, but at the two higher concentrations it declined. The respiration was around 50 % of the uncontaminated samples in the PAH3 samples.

Table 4 Percentage of the respiration in contaminated samples compared to uncontaminated samples. The values for Cu concentration represent mean ± standard deviation (n = 3)

Krusenberg			Nåntuna		
Treatment	Conc. (mg kg^{-1})	CO_2 (% of uncont.)	Treatment	Conc. (mg kg^{-1})	CO_2 (% of uncont.)
Cu1	121 ± 10	53	Cu1	163 ± 11	71
Cu2	260 ± 15	23	Cu2	359 ± 92	78
Cu3	652 ± 47	12	Cu3	900 ± 25	110
PAH1	15	87	PAH1	15	175
PAH2	60	139	PAH2	60	68
PAH3	170	102	PAH3	170	52

3.2.3 The calorespirometric ratio

The calorespirometric ratio decreased linearly (y = -0.009x + 9.6, R^2 = 0.82) with increasing Cu concentration in the Nåntuna soil samples (Figure 6a). There was no linear relationship between the calorespirometric ratio and Cu in Krusenberg soil. However, the ratio increased with increasing Cu concentration. There were no clear relationships between the calorespirometric ratio and PAH concentration (Figure 6b).

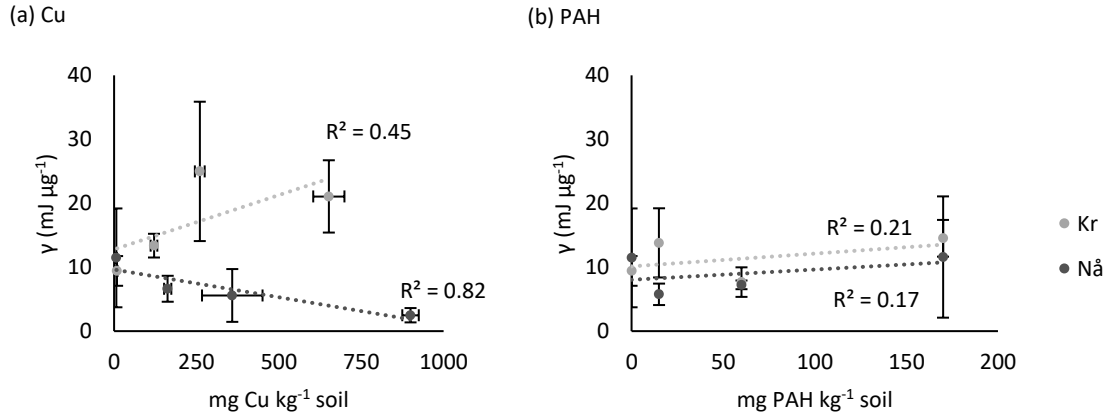

Figure 6 The calorespirometric ratio, γ, for (a) the uncontaminated samples and Cu treatments in Krusenberg (Kr) and Nåntuna (Nå) soil, and (b) the uncontaminated samples and PAH treatments in Krusenberg and Nåntuna soil. The values are mean values and the error bars are standard deviation (n = 3).

3.3 MICROBIAL FUNCTIONAL DIVERSITY

3.3.1 Heat production

The substrate-induced heat production in the Nåntuna soil was generally higher than in the Krusenberg soil (Figure 7a and b). PAH appeared to have a more complex effect on heat production than Cu in both soils. The substrates which generated the highest accumulated heat among the Krusenberg soil samples were α-ketoglutaric acid, citric acid and D-glucose (Figure 7a). The heat production appeared to decrease with increasing Cu concentration for all substrates. PAH appeared to either have no effect or an increasing effect on heat production.

The samples amended with α-ketoglutaric acid had the highest heat production in the uncontaminated samples and PAH samples in the Nåntuna soil (Figure 7b). The produced heat following the addition of α-ketoglutaric acid increased at PAH1, but decreased at PAH2 and PAH3. Cu appeared to have a decreasing effect on the heat production

following the addition of α-ketoglutaric acid. The heat production in the samples amended with the other substrates were not affected as much by Cu as the heat production in the samples amended with α-ketoglutaric acid.

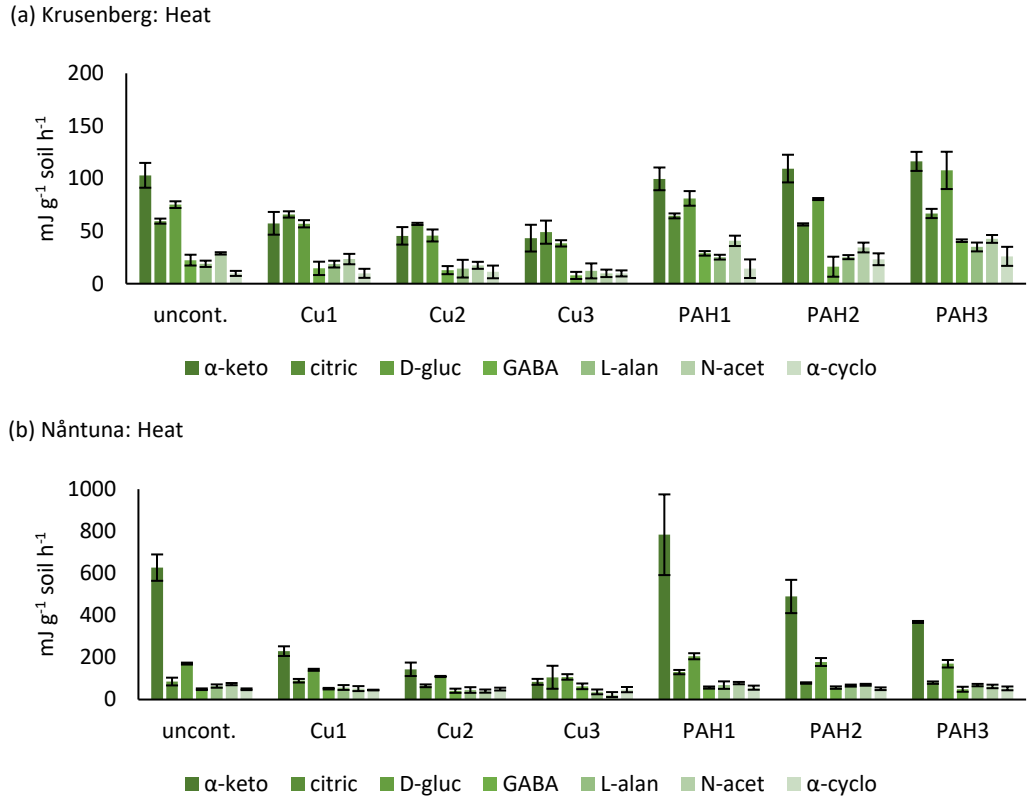

Figure 7 Substrate-induced heat production in uncontaminated (uncont.), Cu and PAH soil samples after addition of seven carbon substrates (α-keto = α-ketoglutaric acid, citric = citric acid, D-gluc = D-glucose, GABA = γ-aminobutyric acid, L-alan = L-alanine, N-acet = N-acetyl glucosamine, α-cyclo = α-cyclodextrin). Impact of Cu and PAH on heat production for each substrate. The error bars represent standard deviation (n = 3). (a) Soil samples from Krusenberg (b) Soil samples from Nåntuna.

Abiotic processes had an impact on the total produced heat in samples amended with the carboxylic acids (Table 5). Especially for the Krusenberg Cu treatments amended with α-ketoglutaric acid, in which the measured abiotic heat was larger than the total. For Cu treatments 1 and 2, the increase was within the standard deviation. For Cu3, however, it was not. Overall, the biotic heat production decreased in the Cu samples and increased in the PAH samples in Krusenberg soil.

The abiotic heat production was not as prominent in Nåntuna samples compared to the Krusenberg samples, except for Cu3 amended with α-ketoglutaric acid, in which the abiotic heat was almost as large as the total (Table 5). The Cu treatments had a decreasing effect on biotic heat production, except for Cu3 amended with citric acid. The PAH1 treatment had an increasing effect on biotic heat production for both substrates, which decreased for PAH2 and PAH3.

Table 5 Total heat production and contribution from abiotic and biotic processes in soil samples amended with α-ketoglutaric acid and citric acid. The values are mean ± standard deviation (n = 3)

Treatm.	Heat prod. (mJ g^{-1} soil h^{-1})					
	α-ketoglutaric acid			citric acid		
	total	abiotic	biotic	total	abiotic	biotic
Kr soil	103 ± 12	70 ± 10	33 ± 8	59 ± 2	29 ± 13	30 ± 15
Cu1	57 ± 11	69 ± 13	0	66 ± 3	33 ± 7	33 ± 9
Cu2	45 ± 8	66 ± 23	0	57 ± 1	36 ± 12	21 ± 11
Cu3	43 ± 13	79 ± 9	0	49 ± 11	41 ± 5	8 ± 6
PAH1	100 ± 11	62 ± 6	38 ± 13	64 ± 2	32 ± 3	32 ± 2
PAH2	109 ± 13	70 ± 2	40 ± 14	56 ± 1	30 ± 3	26 ± 2
PAH3	116 ± 9	73 ± 2	44 ± 7	67 ± 4	33 ± 2	34 ± 6
Nå soil	627 ± 63	51 ± 6	576 ± 68	86 ± 19	21 ± 18	65 ± 8
Cu1	230 ± 23	58 ± 6	172 ± 22	89 ± 9	33 ± 7	56 ± 12
Cu2	144 ± 32	69 ± 11	74 ± 23	65 ± 7	37 ± 9	28 ± 11
Cu3	84 ± 14	63 ± 28	21 ± 15	106 ± 55	39 ± 25	66 ± 80
PAH1	783 ± 192	75 ± 14	708 ± 192	130 ± 10	44 ± 11	87 ± 8
PAH2	490 ± 79	40 ± 20	449 ± 98	79 ± 3	31 ± 20	48 ± 22
PAH3	369 ± 5	50 ± 10	318 ± 6	80 ± 6	28 ± 15	52 ± 10

3.3.2 CO_2 production

The CO_2 production in Nåntuna samples was higher than in Krusenberg (Figure 8a and b). The Krusenberg samples with the highest CO_2 production were samples amended with α-ketoglutaric acid and D-glucose, except for the Cu3 treatment where all substrate amendments lead to low CO_2 production (Figure 8a). The addition of Cu appeared to have a decreasing effect on CO_2 for all substrates, and PAH had no apparent effects.

The CO_2 production in the Nåntuna samples amended with α-ketoglutaric acid and citric acid was higher than the other substrates (Figure 8b). There were no apparent impacts of the Cu treatments. The CO_2 production in the PAH samples amended with α-ketoglutaric acid and citric acid increased at the PAH1 treatment, but returned to a level similar to the uncontaminated samples at PAH2 and PAH3.

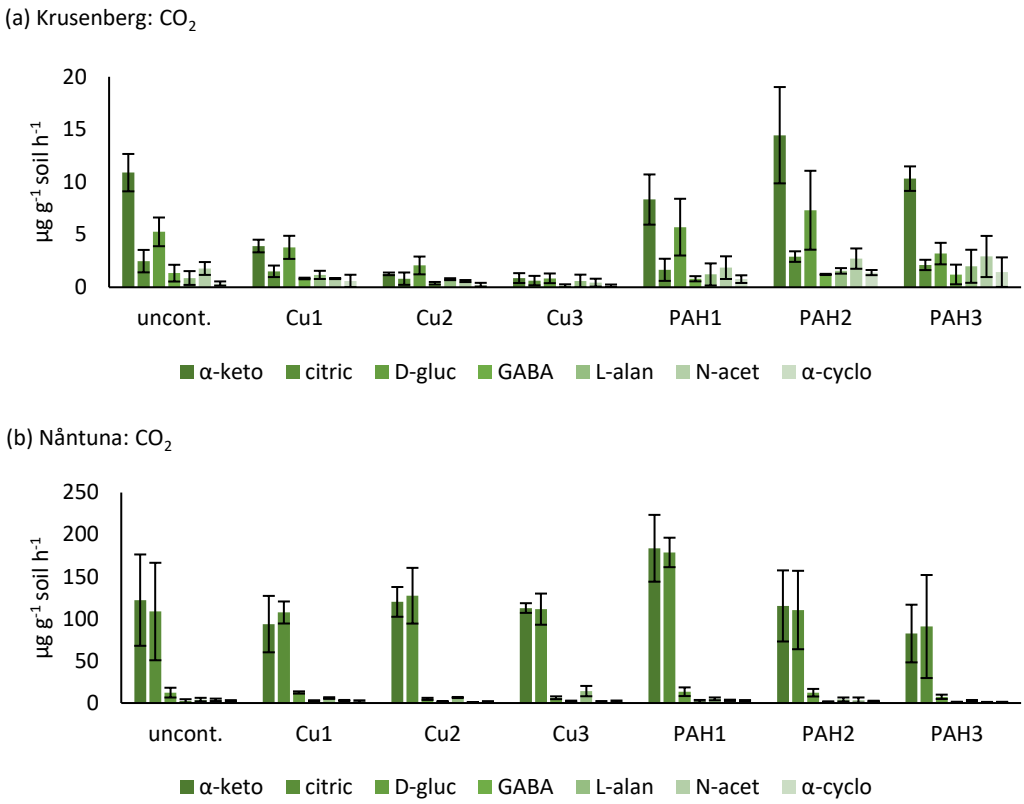

Figure 8 Substrate-induced CO_2 production in uncontaminated (uncont.), Cu and PAH soil samples after addition of seven carbon substrates (α-keto = α-ketoglutaric acid, citric = citric acid, D-gluc = D-glucose, GABA = γ-aminobutyric acid, L-alan = L-alanine, N-acet = N-acetyl glucosamine, α-cyclo = α-cyclodextrin). Impact of Cu and PAH on CO_2 production for each substrate. The error bars represent standard deviation (n = 3). (a) Soil samples from Krusenberg (b) Soil samples from Nåntuna.

There was almost no CO_2 production in the abiotic Krusenberg soil samples (Table 6). There was, however, abiotic CO_2 production in the Nåntuna samples. Cu reduced the biotic CO_2, for both substrates in the Krusenberg soil, while the biotic CO_2 increased in Nåntuna, comparing the uncontaminated samples to the Cu3 samples. PAH appeared to lead to diverse responses in both soils and for both substrates.

Table 6 Total CO_2 production and contribution from abiotic and biotic processes in soil samples amended with α-ketoglutaric acid and citric acid. The values are mean ± standard deviation (n = 3)

	CO_2 prod. (µg g^{-1} soil h^{-1})					
Treatm.	α-ketoglutaric acid			citric acid		
	total	abiotic	biotic	total	abiotic	biotic
Kr soil	11 ± 1.8	0.1 ± 0.2	11 ± 2	2 ± 1	0.09 ± 0.2	2 ± 1
Cu1	4 ± 0.6	0.03 ± 0.02	4 ± 0.6	2 ± 0.6	0.1 ± 0.2	1 ± 0.7
Cu2	1 ± 0.1	0.1 ± 0.1	1 ± 0.1	0.8 ± 0.6	0.1 ± 0.1	0.7 ± 0.5
Cu3	0.9 ± 0.5	0.2 ± 0.1	0.7 ± 0.3	0.6 ± 0.5	0.1 ± 0.09	0.5 ± 0.5
PAH1	8 ± 2.4	0.2 ± 0.3	8 ± 3	1.7 ± 1	0.02 ± 0.03	2 ± 1
PAH2	14 ± 4.6	0	14 ± 5	3 ± 0.5	0.02 ± 0.03	3 ± 0.5
PAH3	10 ± 1.2	0	10 ± 1	2 ± 0.5	0	2 ± 0.5
Nå soil	122 ± 54	54 ± 13	69 ± 49	108 ± 58	72 ± 27	36 ± 35
Cu1	94 ± 34	47 ± 9	46 ± 27	107 ± 13	86 ± 9	22 ± 7
Cu2	120 ± 18	74 ± 8	47 ± 24	127 ± 33	96 ± 43	38 ± 66
Cu3	112 ± 6	41 ± 20	71 ± 17	111 ± 19	67 ± 5	45 ± 17
PAH1	183 ± 40	71 ± 15	113 ± 55	178 ± 18	92 ± 17	87 ± 35
PAH2	115 ± 42	82 ± 3	38 ± 35	110 ± 47	95 ± 12	29 ± 32
PAH3	83 ± 34	62 ± 11	26 ± 28	91 ± 61	76 ± 8	29 ± 43

3.3.3 Principal component analysis

3.3.3.1 Comparison of Krusenberg and Nåntuna soil

Regarding heat production data (Figure 9a), the separation was primarily along principal component 1 (PC 1; $p < 0.001$). PC 1 explained 88 % of the total variance, and was mostly influenced by α-ketoglutaric acid. The uncontaminated Krusenberg samples were separated from the uncontaminated Nåntuna samples ($p < 0.001$), indicating different functional diversities of the microbial communities in the two soils. Principal component 2 (PC 2) accounted for 5 % of the separation, but there was no significant separation along PC 2. The substrate with the highest influence on PC 2 was citric acid.

For the respiration data (Figure 9b), there was a separation along PC 1 ($p < 0.001$), which explained 64 % of the total separation. However, there was no significant separation between the uncontaminated Krusenberg and Nåntuna soils. The total variation was explained to 26 % by PC 2, but there was no significant separation along PC 2 either. The substrates with the highest influence on PC 1 and 2 were α-ketoglutaric acid and citric acid. The Mantel dissimilarity test indicated that the heat production and respiration data sets were similar ($p < 0.01$).

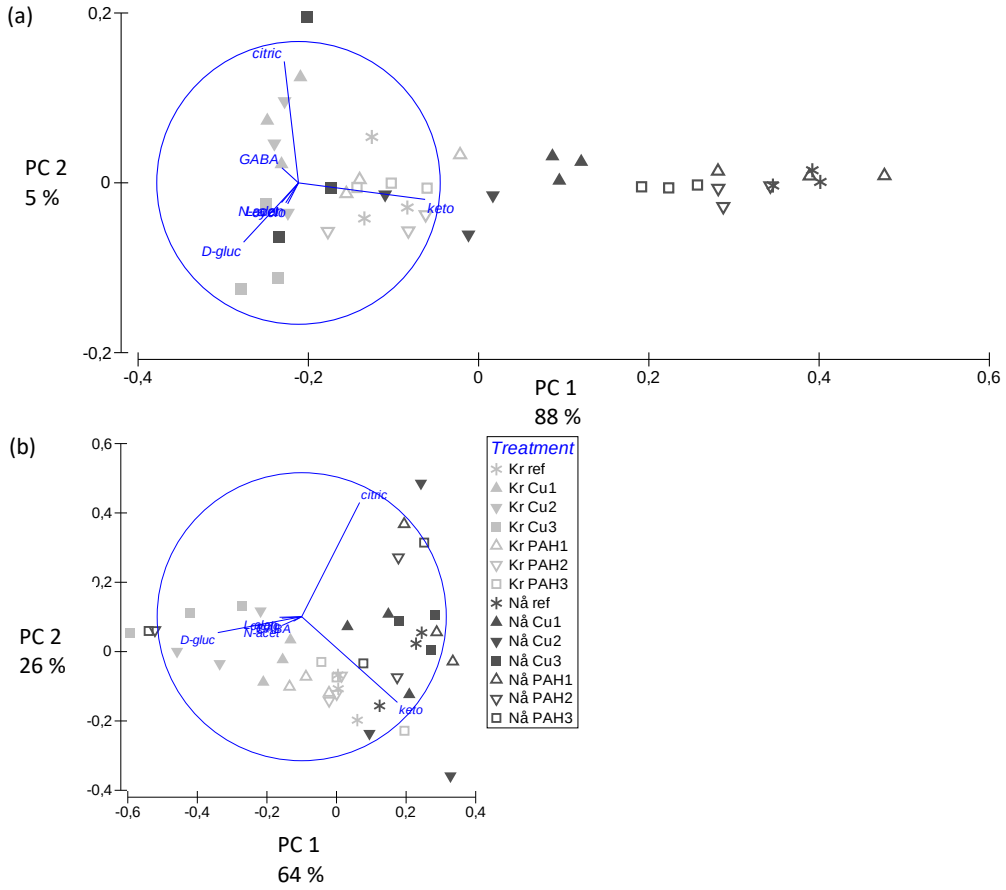

Figure 9 Microbial functional diversity, Cu and PAH treatments included, in Krusenberg and Nåntuna soil samples (a) using heat data (b) using respiration data.

3.3.3.2 Impact of Cu and PAH in Krusenberg soil

Analyzing heat data, there was a separation along PC 1 ($p < 0.001$), which explained 46 % of the separation (Figure 10a). The substrates with the biggest influence on PC 1 were α-ketoglutaric acid and D-glucose. There was no significant separation between the uncontaminated samples and the lowest or middle Cu concentration. However, there was a separation ($p < 0.05$) comparing the uncontaminated samples to the Cu3 samples, indicating a significant impact on microbial functional diversity. There was also a separation along PC 2 ($p < 0.01$). The separation along PC 2, which was mostly impacted by citric acid, accounted for 29 % of the variation. There was significant separation between the Cu1 and Cu3 treatment ($p < 0.01$) and the Cu2 and Cu3 treatment ($p < 0.05$). There were no significant separations between the uncontaminated samples and any of the PAH samples.

The separation of the CO_2 data along PC 1 explained 69 % of the total separation ($p < 0.001$; Figure 10b). There was a significant separation between the uncontaminated samples and the Cu2 samples ($p < 0.01$), as well as the uncontaminated samples and the Cu3 samples ($p < 0.001$). There was also significant separation between Cu treatment 1 and 3 ($p < 0.05$). There were no significant separations between the uncontaminated samples and the PAH samples. PC 2 explained 19 % of the separation, but there was no significant separation along PC 2. PC 2 was mostly impacted by D-glucose. Comparing the heat and CO_2 data sets, the Mantel test showed similarity ($p < 0.01$).

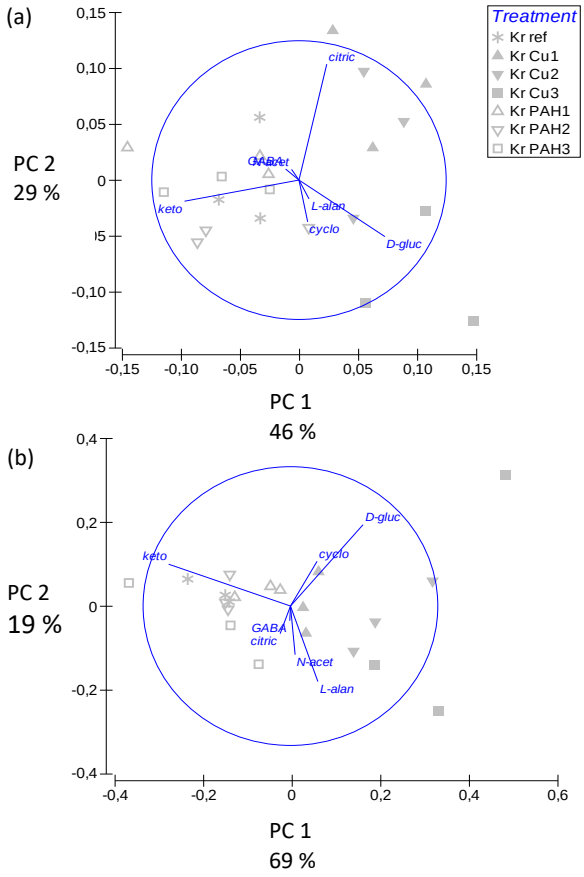

Figure 10 Effects of Cu and PAH on microbial functional diversity in Krusenberg soil samples (a) using heat production data (b) using respiration data.

3.3.3.3 Impact of Cu and PAH in Nåntuna soil

There was a separation, primarily along PC 1 ($p < 0.001$), accounting for 93 % of the total variation of the heat production data (Figure 11a). The substrate with the most impact on PC 1 was α-ketoglutaric acid. There was separation between the uncontaminated samples and all Cu treatments ($p < 0.001$). The Cu1 treatment was separated from the Cu3 treatment ($p < 0.001$), and the Cu2 treatment was separated from Cu3 ($p < 0.05$). Additionally, the uncontaminated and PAH1 samples were significantly separated from the PAH3 samples ($p < 0.05$). PC 2 explained only 5 % of the total separation with no significant separations along PC 2.

For the CO_2 data (Figure 11b), PC 1 and PC 2 explained 58 and 41 % of the total variance. However, there were no significant separations between the uncontaminated samples and the Cu and PAH samples. The substrates with the biggest impact on PC 1 were α-ketoglutaric acid and D-glucose and the substrates which had the biggest influence on PC 2 were α-ketoglutaric acid and citric acid. The outcome of the Mantel test was not significant, indicating that the data sets for heat production and respiration were not similar.

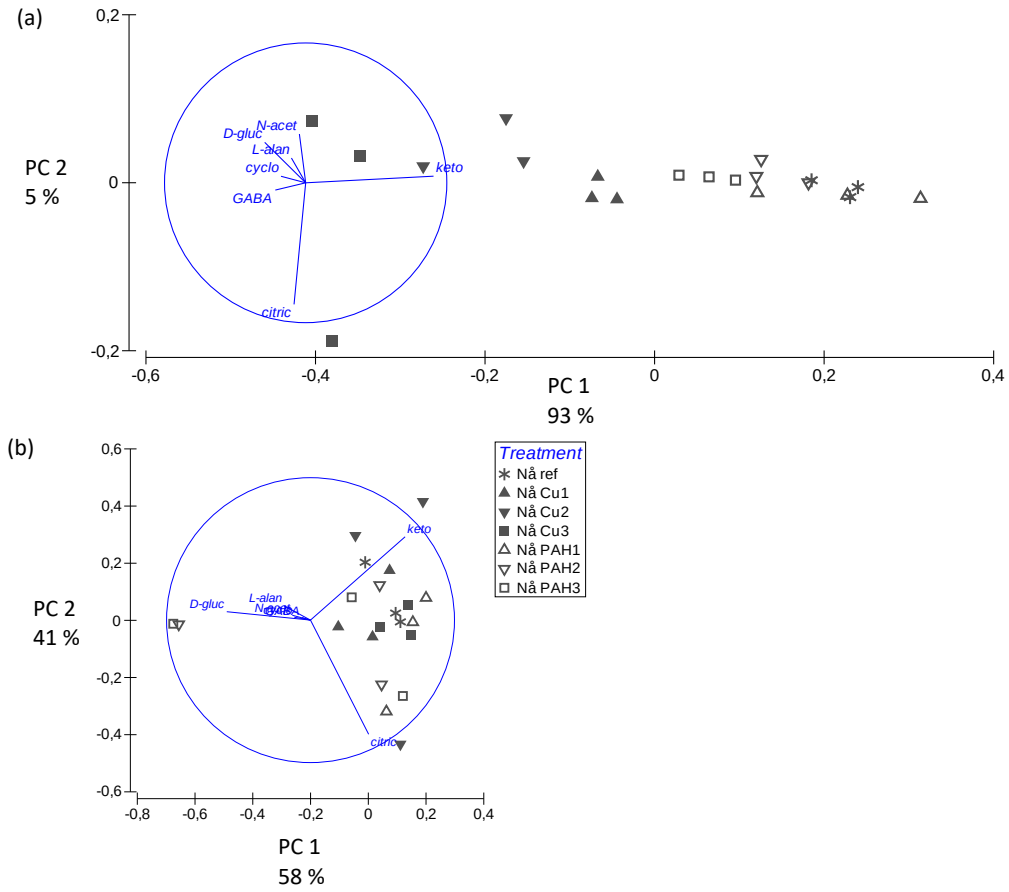

Figure 11 Effects of Cu and PAH on microbial functional diversity in Nåntuna soil samples (a) using heat production data (b) using respiration data.

3.3.4 The Shannon Index

The Shannon Index, calculated using both heat and respiration data, showed that the microbial functional diversity was higher in the uncontaminated Krusenberg samples than Nåntuna (Table 7). The heat data for Krusenberg indicated that Cu had a decreasing effect on functional diversity. There was an indication that PAH had an effect on functional diversity in Krusenberg, but there was no apparent pattern. According to the respiration data, Cu affected the microbial functional diversity in Krusenberg samples, but there was no clear pattern. There was an increased functional diversity at PAH1 compared to the uncontaminated samples, which remained constant at PAH2 and PAH3. In Nåntuna soil samples, the microbial functional diversity increased in the samples treated with Cu and PAH, according to the heat production data. There were no apparent patterns, when calculating the Shannon Index using respiration data.

Table 7 The Shannon Index in uncontaminated soil samples and at different Cu and PAH concentrations using heat production and respiration data. The values are mean ± standard deviation (n = 3). (*Cu concentration (mean ± standard deviation, n = 3); PAH concentration)

Treatment	Conc. (mg kg^{-1} soil)	Shannon Index (heat data)	Shannon Index (resp. data)
Krusenberg soil	*9 ± 1; 0	1.7 ± 0.03	1.4 ± 0.09
Cu1	121 ± 10	1.6 ± 0.1	1.7 ± 0.1
Cu2	260 ± 15	1.6 ± 0.03	1.6 ± 0.23
Cu3	652 ± 47	1.5 ± 0.09	1.3 ± 0.56
PAH1	15	1.8 ± 0.07	1.5 ± 0.04
PAH2	60	1.7 ± 0.04	1.5 ± 0.11
PAH3	170	1.8 ± 0.03	1.5 ± 0.32
Nåntuna soil	*7 ± 1; 0	1.4 ± 0.05	1.3 ± 0.09
Cu1	163 ± 11	1.8 ± 0.02	1.4 ± 0.15
Cu2	359 ± 92	1.8 ± 0.02	1.0 ± 0.26
Cu3	900 ± 25	1.6 ± 0.1	1.2 ± 0.12
PAH1	15	1.4 ± 0.18	1.1 ± 0.11
PAH2	60	1.5 ± 0.05	1.3 ± 0.06
PAH3	170	1.6 ± 0.04	1.3 ± 0.18

3.3.5 The Simpson Index

The Simpson Index showed that the Krusenberg microbial community had a more even substrate utilization than Nåntuna (Table 8). This was apparent using both heat and respiration data. The Simpson Index calculated with Krusenberg heat data declined with increasing Cu, pointing to less even utilization of the substrates. The respiration data showed increased evenness at Cu1 and Cu2, which decreased at Cu3 to a similar evenness as the uncontaminated samples. Both heat and respiration data indicated increased evenness with increasing PAH concentration for Krusenberg. Cu had an increasing effect on evenness according to the Simpson Index, calculated with Nåntuna heat production data. This was not as apparent when analyzing CO_2 data. There was an increase at the Cu1, which then decreased. At Cu3, the evenness was similar to the uncontaminated samples. The PAH treatments resulted in increased evenness using both heat and respiration data. However, the evenness was higher using heat production data.

Table 8 Simpson Index in uncontaminated soil samples and at different Cu and PAH concentrations using heat production and respiration data. The values are mean ± standard deviation (n = 3). (*Cu concentration (mean ± standard deviation, n = 3); PAH concentration)

Treatment	Conc. (mg kg^{-1} soil)	Simpson Index (heat data)	Simpson Index (resp. data)
Krusenberg soil	*9 ± 1; 0	4.6 ± 0.2	3.3 ± 0.5
Cu1	121 ± 10	4.3 ± 0.7	4.4 ± 0.6
Cu2	260 ± 15	4.3 ± 0.3	4.2 ± 1.5
Cu3	652 ± 47	3.4 ± 0.5	3.5 ± 1.6
PAH1	15	5.1 ± 0.7	3.7 ± 0.2
PAH2	60	4.7 ± 0.3	3.6 ± 0.4
PAH3	170	5.2 ± 0.3	3.8 ± 1.5
Nåntuna soil	*7 ± 1; 0	2.9 ± 0.2	2.7 ± 0.1
Cu1	163 ± 11	5.2 ± 0.2	3.3 ± 0.8
Cu2	359 ± 92	5.4 ± 0.3	2.0 ± 0.6
Cu3	900 ± 25	4.5 ± 0.5	2.7 ± 0.4
PAH1	15	2.9 ± 0.7	2.3 ± 0.2
PAH2	60	3.4 ± 0.3	2.9 ± 0.2
PAH3	170	4.1 ± 0.3	2.8 ± 0.4

4 DISCUSSION

4.1 OVERALL MICROBIAL ACTIVITY

4.1.1 Effects of Cu

According to the heat data, Cu had a decreasing effect on microbial activity in both soils. This was not surprising due to the well-known toxicity of Cu to microorganisms (Wang et al., 2007, 2010; Li et al., 2015). There were large initial decreases, indicating effects of Cu on microbial activity at relatively low concentrations, even lower than the guideline value for less sensitive land use (Naturvårdsverket, 2009). Although the heat decrease followed a similar pattern in both soils, the percental decrease compared to uncontaminated samples was greater in Nåntuna. The reason for this could be the higher fractions of available Cu, i.e. higher Cu concentrations, in the Nåntuna soil, which were probably due to more leaching in the Krusenberg spike soil. A possible reason for the higher losses due to leaching could be the diverse properties of the soils. Physical and chemical properties, primarily pH and SOM, affect the proportion of adsorbed Cu (Berggren Kleja et al., 2006). In a soil with lower pH, such as Krusenberg, a larger proportion of Cu is leached. Variation due to pH differences could have been avoided by adjusting the pH in the soils, as was done by Griffiths et al. (2008). However, for the sake of the objective of this project, comparing two soils with different properties provides more useful information. In addition, soil texture may have had an influence on the results. The Krusenberg soil was a sand/loamy sand which probably supported the leaching of Cu.

There was an apparent relationship between Cu concentration and respiration for Krusenberg soil samples, which followed the same pattern as for the heat data of both soils. Similar to the Krusenberg heat data, the initial decrease was the most prominent. As was mentioned above, this indicates effects on microbial activity at a concentration corresponding to less sensitive land use, maybe even sensitive land use. There was no relationship between Cu concentration and respiration in the Nåntuna samples. There was high variation between the replicates, which probably had a large impact on the results, and the results should be interpreted with this in mind.

4.1.2 Effects of PAH

The responses to PAH were complex, as expected (CCME, 2008). There is not as much knowledge of the impacts of PAH on soil microorganisms as Cu, and further investigation is needed in this area. Exposure to PAH lead to a linear heat production increase in Krusenberg samples, and a linear decrease in the Nåntuna samples. In both soils, however, there was an initial increase at the lowest PAH concentration, corresponding to the guideline value for less sensitive land use (Naturvårdsverket, 2009). This could possibly be due to microorganisms utilizing PAH as a carbon source (CCME, 2008; Turbé et al., 2010). It is not known why the responses vary between the soils. It could be due to different microbial community composition, with different abilities to degrade PAH. Another possible reason is different concentrations in Krusenberg and Nåntuna samples. If the PAH concentrations were higher in the Nåntuna samples, as they were for Cu, the decrease could be due to a greater toxic effect (Dawson et al., 2007; CCME, 2008). There were no relationships between PAH concentration and respiration, possibly due to the

complex effects of PAH to soil organisms (CCME, 2008). The respiration results should, however, be analyzed with respect to the big differences between replicates. For both heat and respiration data, there was no knowledge about actual PAH concentrations, which prevents proper interpretation of PAH impacts.

4.1.3 The calorespirometric ratio

The calorespirometric ratio has been found to be applicable for evaluation of metabolic efficiency in soils (Barros et al., 2010). The results of this project, however, do not agree. In the Cu soil samples, the calorespirometric ratio was inconsistent between the soils. The ratio for Krusenberg increased, indicating decreased metabolic efficiency (Herrmann et al., 2014; Herrmann and Bölscher, 2015). This is in line with the initial hypothesis that Cu contamination decreases metabolic efficiency. However, there was no apparent relationship between the ratio and Cu concentration in Krusenberg samples. The ratio in Nåntuna samples decreased in a linear fashion, indicating an increased metabolic efficiency (Herrmann et al., 2014; Herrmann and Bölscher, 2015).

PAH had no apparent impact on the calorespirometric ratio. This was hardly surprising considering the complex responses to PAH in the measurements of both heat production and respiration. The fact that there was no knowledge of actual PAH concentrations inhibits a proper analysis of the results, as was also mentioned above. It is also important to point out that the results could have been affected by variation between replicates.

4.2 MICROBIAL FUNCTIONAL DIVERSITY

4.2.1 Influence of carbon substrates

The substrates which were primarily responsible for the functional diversity variations between the soils were α-ketoglutaric acid and citric acid. Soil samples amended with these substrates were also greatly affected by Cu. This is consistent with Kong et al. (2006), who showed that exposure to Cu reduced the microorganisms' ability to utilize carboxylic acids as carbon substrates. α-ketoglutaric acid and citric acid are both intermediates in the tricarboxylic acid (TCA) cycle, which is a part of the metabolism of aerobic organisms (Yin et al., 2012). Addition of these acids might therefore contribute to increased microbial metabolism. It is not completely clear why some carbon substrates lead to more production of heat and CO_2 than others.

4.2.2 Abiotic processes

There was abiotic heat production in the sterile soil samples amended with carboxylic acids, as was also shown by Herrmann et al. (2014). At present, further investigation is needed to determine the source of abiotic heat production in soil (Herrmann et al., 2014). A possible reason could be adsorption of carboxylic functional groups of organic matter to iron oxide, which has been proven to be a reaction that releases heat (Gu et al., 1994). The abiotic heat production in the Krusenberg Cu samples amended with α-ketoglutaric acid exceeded the total. This indicates that Cu had such toxic effect in the Krusenberg soil that there was no microbial heat production in these samples. It is important to point out, however, that the analyses of total and abiotic heat production were done in different soil samples, and that some variation could be due to soil heterogeneity.

Herrmann et al. (2014) showed that there was no significant CO_2 production in sterilized soil samples amended with α-ketoglutaric acid and citric acid. The results of this project, however, showed CO_2 production from sterilized Nåntuna soil samples. The reason for this is not completely clear. One reason could be that the sterilization was unsuccessful. This is not likely, however, since the abiotic CO_2 production in the Krusenberg soil samples was insignificant. Taking the different pH values of the soils into account, a more probable reason is that the soil carbonate has reacted with the carboxylic acids, which leads to a release of CO_2.

4.2.3 Effects on microbial functional diversity

The Shannon Index, calculated using heat data, indicated that the microbial functional diversity in uncontaminated Krusenberg samples exceeded the microbial functional diversity in Nåntuna samples. This was a bit surprising since the Nåntuna soil samples had higher microbial activity, i.e. higher heat production and respiration, than Krusenberg. The difference was not as apparent using respiration data, the Shannon Index was a bit higher for Krusenberg, but according to the PCA the difference was not significant. The Simpson Index, calculated with both heat and CO_2 data, was also higher for Krusenberg, indicating a more even use of the substrates. Much of the activity in Nåntuna samples came from the addition of α-ketoglutaric acid and citric acid, which explains the lower Shannon and Simpson Index scores for the Nåntuna samples.

Comparing the Nåntuna heat and respiration data with PCA, gave an indication that the methods did not provide the same information. This was verified by the Mantel dissimilarity test. The heat data showed an apparent impact of Cu on microbial functional diversity, according to both the Shannon Index and the PCA, while the respiration data showed no significant effects of Cu. The heat data were consistent with Kong et al. (2006) and Wang et al. (2007) who showed that Cu affect microbial diversity. The Cu treatments also lead to an increased Simpson index, i.e. more even substrate utilization, according to the heat production data. This was due to the microorganisms' decreased ability to utilize carboxylic acids, i.e. α-ketoglutaric acid and citric acid, in Cu contaminated soil (Kong et al., 2006). The fact that the methods provided different information could be explained by the presence of incomplete metabolic processes in the soil, which are only included by calorimetry (Herrmann and Bölscher, 2015). If these processes were heavily impacted by Cu, it would not be shown using MSIR. MSIR is considered an appropriate method for soil monitoring. However, it underestimates the impacts of Cu on microbial functional diversity. The differences between the results using MSIR and isothermal calorimetry support the idea to use both methods.

The data for Krusenberg soil samples were similar, indicating that measurements of heat and CO_2 provided the same information. This indicates that the metabolic processes in the Krusenberg samples produce both heat and CO_2. The Shannon Index and PCA for Krusenberg heat and CO_2 data showed that Cu had an impact on microbial functional diversity. This is consistent with Wang et al. (2007), who showed an effect of Cu on microbial diversity. The heat data showed that both functional diversity and evenness were reduced by Cu, which was also shown by Kong et al. (2006). The PAH treatments had little significant effect on microbial functional diversity and substrate use evenness in both soils.

4.3 APPLICABILITY IN RISK ASSESSMENT

Diverse results regarding the methods' applicability in risk assessment have been shown. MSIR is generally considered an appropriate method for monitoring soil function (e.g. Jensen and Mesman, 2006). The method reflects carbon cycling, which is a fundamental function of soils, and has high throughput (Ritz et al., 2009). However, there was a lot of variation between replicates using MSIR, compared to isothermal calorimetry. While isothermal calorimetry may be more work- and time consuming than the MicroResp^{TM} system, it has been proven to accurately measure heat production in soil (Harris et al., 2012; Herrmann et al., 2014). This agrees with the results of this project. Isothermal calorimetry also has the advantage of including other microbial processes (Herrmann and Bölscher, 2015), which are not taken into account by MSIR. Due to its lower capacity, however, isothermal calorimetry might not be applicable in routine risk assessment investigations.

The heat and respiration data did not provide the same information for the Nåntuna samples regarding effects on microbial functional diversity. To only use MSIR could thus lead to an underestimation of the contamination situation. The abiotic CO_2 production in the Nåntuna samples amended with the carboxylic acids could also give a misleading picture of the microbial respiration. MSIR does not take abiotic processes into consideration, and thus the CO_2 production could be misinterpreted as respiration.

However, both methods are considered applicable in soil contaminated with Cu. The calorimetry data decreased similarly for both soils with little variation, as did the MSIR data for Krusenberg. The variation among the replicates could have contributed to the absence of a pattern in the Nåntuna samples. Variation should be considered, but it seems unnecessary to discard the method completely because of the variation among the Nåntuna MSIR data. Instead, further investigation is needed. PAH appears to have complex effects on microbial activity, and has little significant effect on microbial functional diversity. Therefore, neither of the methods can be considered applicable in risk assessment of soils contaminated with PAH. The calorespirometric ratio is not considered applicable in risk assessment due to the diverse results. Further testing is needed to determine why the ratio increased with increasing Cu concentration in the Krusenberg soil samples and decreased in Nåntuna samples.

The objective of the project was not to evaluate the differences between the two soils. However, including two soils with different properties has increased the robustness of the results. The diverse results also emphasize how complex soil systems are, and that there is need for further research and testing. When risk assessment is performed, site-specific properties (e.g. soil properties and contaminant type) must always be considered. These properties determine the effectiveness of the chosen remediation technique (Leitgib et al., 2008).

4.4 UNCERTAINTIES

Generally, which has been mentioned above, there were larger variations between replicates using MSIR than isothermal calorimetry. Variation does not necessarily mean that the methods are not stable. The replicates used in the experiments were field replicates. Thus, the variation could be due to variation between the soil samples, and not

instability of the methods. Both methods required only a small amount of soil. This gives soil heterogeneity a big influence on each soil sample (Creamer et al., 2009). To reduce variation that is not related to the stability of the methods, lab replicates should have been included in the experiment.

The actual PAH concentrations had not been determined within the timeline of this project, as has also been mentioned above. Therefore, it is to be expected that the PAH concentrations have been overestimated. This is a major uncertainty for the PAH results. Because of the diverse soil properties and Cu concentrations, it is likely that the PAH concentrations also vary between the soils.

5 CONCLUSIONS

The answers to the formulated questions were:

- There was a relationship between Cu concentration and substrate-induced heat production in both soils. The heat production decreased logarithmically with increased Cu concentration. There were linear relationships between PAH concentration and heat production. However, the relationships varied between the soils. The heat production increased with PAH concentration in the Krusenberg soil samples, and decreased in the Nåntuna soil samples.
- There was a relationship between substrate-induced respiration and Cu concentration in the Krusenberg soil. The respired CO_2 declined logarithmically with increased concentration of Cu. There were no relationships between respiration and Cu in Nåntuna soil samples or respiration and PAH in both soils.
- The calorespirometric ratio increased with increased Cu in Krusenberg soil samples, and decreased in the Nåntuna samples. The calorespirometric ratio was not influenced by PAH concentration.

There is potential to use both methods in risk assessment of soils contaminated with Cu. MSIR is already frequently used in some countries. Therefore, I would especially like to promote isothermal calorimetry as a new candidate for evaluating microbial activity and functional diversity. Although the throughput of isothermal calorimetry is not as high as for MSIR, it measures microbial activity with high precision. Combining the methods by calculating the calorespirometric ratio did not give consistent results for soil contaminated with Cu in this project, and should therefore be used with caution. The microbial responses to exposure of PAH are at present regarded as too complex to be accurately evaluated with MSIR or isothermal calorimetry.

As a final remark, the importance of site-specific properties must be emphasized. A method can show one thing at one site but something else at another. Although this project evaluated the impacts of Cu and PAH in two diverse soils, there is still need for further investigation, including other soil types and contaminants, to verify the methods' applicability in risk assessment of contaminated soil.

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APPENDIX

A1 CALIBRATION CURVE

Figure A1 shows the calibration curve, and the equation which was used to transform absorption data to CO_2 production data (µg).

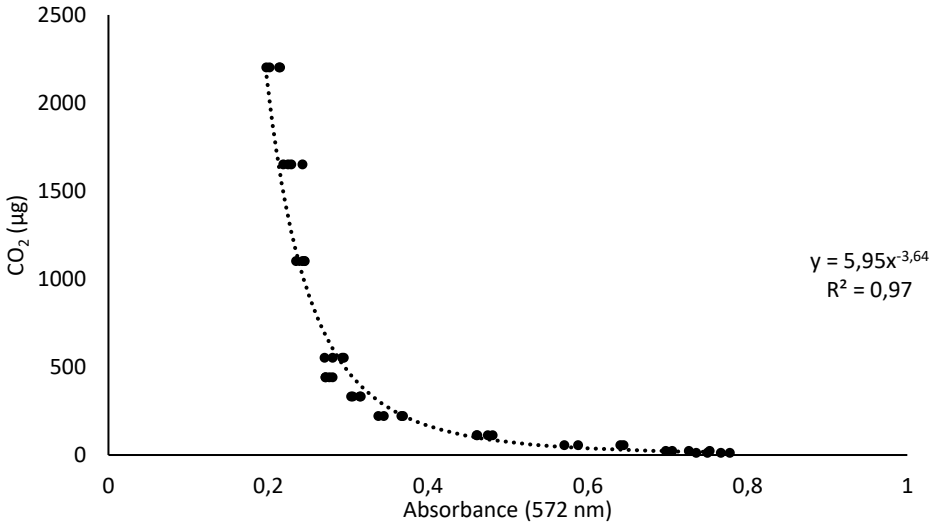

Figure A1 Calibration curve for CO_2 data.

A2 HEAT DATA

Table A2 Heat production (mJ g^{-1} h^{-1}) in all replicates from addition of each substrate, and sum of heat production

Sample	α-keto	citric	D-gluc	GABA	L-alan	N-acet	α-cyclo	Sum
Kr ref1	20.87	32.53	78.67	26.63	20.08	28.18	10.93	217.89
Kr ref2	28.00	15.22	72.47	16.67	15.50	30.09	7.00	184.96
Kr ref3	18.94	12.36	74.26	23.89	21.14	28.17	11.37	190.12
KrCu1A	0	26.91	57.97	7.47	14.91	19.04	4.88	131.17
KrCu1B	0	36.92	53.10	19.72	20.77	28.77	12.51	171.80
KrCu1C	0	20.34	59.73	16.47	20.15	22.39	12.16	151.24
KrCu2A	0	18.19	45.29	8.74	12.06	15.50	7.15	106.94
KrCu2B	0	10.27	51.82	13.46	23.56	21.30	18.13	138.54
KrCu2C	0	20.83	40.42	16.40	7.16	15.53	8.28	108.62
KrCu3A	0	9.35	39.44	6.97	12.88	8.36	7.84	84.84
KrCu3B	0	0	40.63	11.57	18.89	13.88	13.08	98.05
KrCu3C	0	2.55	34.95	4.98	4.69	7.24	8.37	62.78
KrPAH1A	18.01	19.34	88.21	31.18	27.46	46.32	11.42	241.94
KrPAH1B	19.41	21.75	80.70	28.36	22.91	38.75	7.14	219.02
KrPAH1C	49.06	28.51	74.33	26.95	25.30	37.11	24.14	265.40
KrPAH2A	36.50	17.81	80.46	5.95	23.92	29.96	29.12	223.73
KrPAH2B	12.04	12.17	81.42	17.65	24.34	39.26	17.90	204.78
KrPAH2C	31.47	8.73	79.49	24.82	27.46	33.96	22.65	228.58
KrPAH3A	35.96	28.20	106.90	40.80	32.85	41.80	17.28	303.79
KrPAH3B	44.18	20.52	90.40	39.69	32.12	39.24	25.31	291.46
KrPAH3C	28.49	29.98	125.82	42.13	39.73	46.58	35.35	348.08
Nå ref1	628.60	57.30	173.10	45.48	70.81	75.91	50.52	1101.72
Nå ref2	566.44	55.83	165.62	53.13	55.43	67.21	44.19	1007.85
Nå ref3	496.02	46.43	173.94	48.39	65.87	76.70	51.68	959.01
NåCu1A	148.36	52.66	135.84	47.76	50.42	60.87	43.14	539.05
NåCu1B	159.45	38.78	143.60	52.61	50.68	56.51	46.38	547.99
NåCu1C	183.90	53.40	144.50	54.73	70.51	38.59	45.79	591.41
NåCu2A	89.51	20.41	111.91	53.23	54.93	39.96	56.33	426.27
NåCu2B	71.58	9.07	106.65	34.95	50.99	47.72	49.81	370.77
NåCu2C	32.94	25.47	110.24	37.33	29.93	32.65	42.94	311.49
NåCu3A	11.18	17.81	98.32	63.12	47.46	33.11	36.77	307.77
NåCu3B	0	108.62	122.57	76.15	37.10	10.56	60.98	415.98
NåCu3C	0	7.41	101.51	49.18	24.70	27.28	43.74	253.83
NåPAH1A	492.06	78.79	212.52	62.80	79.38	82.44	53.45	1061.44
NåPAH1B	869.49	67.22	189.26	54.10	48.00	71.90	48.30	1348.27
NåPAH1C	721.33	71.71	215.61	53.81	78.31	80.11	67.35	1288.23
NåPAH2A	535.80	46.40	194.35	60.77	61.58	70.96	53.56	1023.43
NåPAH2B	414.60	23.16	183.05	59.24	71.14	74.10	56.31	881.60
NåPAH2C	360.79	36.23	157.62	50.10	66.13	65.33	44.14	780.35
NåPAH3A	311.60	45.96	188.54	62.56	75.24	70.97	63.13	818.00
NåPAH3B	302.71	42.13	169.27	42.98	65.79	61.55	45.98	730.41
NåPAH3C	312.73	41.11	152.74	41.09	65.15	54.25	49.99	717.05

A3 NORMALIZED HEAT DATA

Table A3 Normalized heat data, i.e. impact on heat production of each substrate

Sample	α-keto	citric	D-gluc	GABA	L-alan	N-acet	α-cyclo	Sum
Kr ref1	0.10	0.15	0.36	0.12	0.09	0.13	0.05	1
Kr ref2	0.15	0.08	0.39	0.09	0.08	0.16	0.04	1
Kr ref3	0.10	0.06	0.39	0.13	0.11	0.15	0.06	1
KrCu1A	0	0.21	0.44	0.06	0.11	0.15	0.04	1
KrCu1B	0	0.21	0.31	0.11	0.12	0.17	0.07	1
KrCu1C	0	0.13	0.39	0.11	0.13	0.15	0.08	1
KrCu2A	0	0.17	0.42	0.08	0.11	0.14	0.07	1
KrCu2B	0	0.07	0.37	0.10	0.17	0.15	0.13	1
KrCu2C	0	0.19	0.37	0.15	0.07	0.14	0.08	1
KrCu3A	0	0.11	0.46	0.08	0.15	0.10	0.09	1
KrCu3B	0	0	0.41	0.12	0.19	0.14	0.13	1
KrCu3C	0	0.04	0.56	0.08	0.07	0.12	0.13	1
KrPAH1A	0.07	0.08	0.36	0.13	0.11	0.19	0.05	1
KrPAH1B	0.09	0.10	0.37	0.13	0.10	0.18	0.03	1
KrPAH1C	0.18	0.11	0.28	0.10	0.10	0.14	0.09	1
KrPAH2A	0.16	0.08	0.36	0.03	0.11	0.13	0.13	1
KrPAH2B	0.06	0.06	0.40	0.09	0.12	0.19	0.09	1
KrPAH2C	0.14	0.04	0.35	0.11	0.12	0.15	0.10	1
KrPAH3A	0.12	0.09	0.35	0.13	0.11	0.14	0.06	1
KrPAH3B	0.15	0.07	0.31	0.14	0.11	0.13	0.09	1
KrPAH3C	0.08	0.09	0.36	0.12	0.11	0.13	0.10	1
Nå ref1	0.57	0.05	0.16	0.04	0.06	0.07	0.05	1
Nå ref2	0.56	0.06	0.16	0.05	0.05	0.07	0.04	1
Nå ref3	0.52	0.05	0.18	0.05	0.07	0.08	0.05	1
NåCu1A	0.28	0.10	0.25	0.09	0.09	0.11	0.08	1
NåCu1B	0.29	0.07	0.26	0.10	0.09	0.10	0.08	1
NåCu1C	0.31	0.09	0.24	0.09	0.12	0.07	0.08	1
NåCu2A	0.21	0.05	0.26	0.12	0.13	0.09	0.13	1
NåCu2B	0.19	0.02	0.29	0.09	0.14	0.13	0.13	1
NåCu2C	0.11	0.08	0.35	0.12	0.10	0.10	0.14	1
NåCu3A	0.04	0.06	0.32	0.21	0.15	0.11	0.12	1
NåCu3B	0	0.26	0.29	0.18	0.09	0.03	0.15	1
NåCu3C	0	0.03	0.40	0.19	0.10	0.11	0.17	1
NåPAH1A	0.46	0.07	0.20	0.06	0.07	0.08	0.05	1
NåPAH1B	0.64	0.05	0.14	0.04	0.04	0.05	0.04	1
NåPAH1C	0.56	0.06	0.17	0.04	0.06	0.06	0.05	1
NåPAH2A	0.52	0.05	0.19	0.06	0.06	0.07	0.05	1
NåPAH2B	0.47	0.03	0.21	0.07	0.08	0.08	0.06	1
NåPAH2C	0.46	0.05	0.20	0.06	0.08	0.08	0.06	1
NåPAH3A	0.38	0.06	0.23	0.08	0.09	0.09	0.08	1
NåPAH3B	0.41	0.06	0.23	0.06	0.09	0.08	0.06	1
NåPAH3C	0.44	0.06	0.21	0.06	0.09	0.08	0.07	1

A4 RESPIRATION DATA

Table A4 Respiration (μg g^{-1} h^{-1}) in all replicates from addition of each substrate, and sum of respiration

Sample	α-keto	citric	D-gluc	GABA	L-alan	N-acet	α-cyclo	Sum
Kr ref1	9.74	2.63	5.42	2.24	0.36	2.18	0.14	22.71
Kr ref2	12.20	2.36	6.55	1.05	1.62	2.09	0.53	26.40
Kr ref3	8.93	0.68	3.83	0.74	0.67	1.08	0.40	16.33
KrCu1A	2.78	0.85	3.13	0.81	0.80	0.86	0.12	9.34
KrCu1B	3.14	1.51	3.19	0.92	1.57	0.86	0.42	11.62
KrCu1C	4.16	0.24	5.06	0.78	1.16	0.77	1.26	13.42
KrCu2A	0.57	0.02	3.05	0.30	0.79	0.63	0.31	5.67
KrCu2B	0.66	0	1.52	0.32	0.67	0.48	0.04	3.69
KrCu2C	0.91	0.90	1.65	0.52	0.85	0.66	0.38	5.87
KrCu3A	0	0.13	1.05	0.10	1.21	0.65	0.25	3.39
KrCu3B	0	0	0.32	0	0	0	0.17	0.49
KrCu3C	0.39	0.57	1.18	0.30	0.56	0.66	0.02	3.68
KrPAH1A	10.16	2.43	8.57	0.79	2.36	1.32	0.43	26.07
KrPAH1B	6.30	0.47	5.36	1.06	0.30	3.11	1.16	17.77
KrPAH1C	5.87	0.86	3.21	0.57	0.99	1.17	0.73	13.41
KrPAH2A	10.93	1.55	4.94	1.28	1.74	2.09	1.56	24.09
KrPAH2B	19.19	2.57	11.64	1.19	1.25	3.84	1.11	40.80
KrPAH2C	11.37	2.92	5.38	1.19	1.68	2.25	1.52	26.30
KrPAH3A	10.20	2.15	4.36	2.22	1.60	3.21	1.02	24.75
KrPAH3B	10.77	1.07	2.41	0.38	0.66	0.84	0.29	16.42
KrPAH3C	8.46	1.95	2.84	1.05	3.72	4.72	3.00	25.74
Nå ref1	122.65	73.51	18.94	4.85	6.42	4.94	3.55	234.87
Nå ref2	28.23	4.66	8.06	1.29	2.44	4.29	1.73	50.70
Nå ref3	51.90	27.44	10.06	1.17	3.34	2.16	1.83	97.90
NåCu1A	22.54	14.46	12.70	2.85	5.39	2.08	0.89	60.90
NåCu1B	37.55	27.63	11.10	2.85	5.30	3.47	1.29	89.19
NåCu1C	73.13	18.02	13.74	2.79	7.02	3.13	3.38	121.21
NåCu2A	69.50	0	3.51	2.46	7.28	1.06	1.73	85.54
NåCu2B	21.40	0	5.59	1.51	6.25	1.18	1.25	37.19
NåCu2C	45.45	113.53	5.72	1.51	6.13	0.82	2.15	175.30
NåCu3A	58.27	38.83	8.13	2.94	21.19	2.45	0.54	132.35
NåCu3B	63.90	30.46	4.95	1.89	9.77	0.34	1.81	113.13
NåCu3C	89.34	62.77	5.66	1.55	11.56	0.88	2.95	174.72
NåPAH1A	68.77	122.70	19.24	3.85	5.67	3.92	3.16	227.31
NåPAH1B	172.94	77.61	11.55	1.24	6.09	3.26	1.97	274.67
NåPAH1C	92.26	55.92	9.46	1.31	2.97	2.42	3.23	167.57
NåPAH2A	46.93	61.41	15.16	2.11	2.93	1.46	2.60	132.61
NåPAH2B	0	0	7.06	0.90	2.74	1.91	1.65	14.25
NåPAH2C	63.19	20.65	14.53	0.72	6.96	7.07	2.41	115.53
NåPAH3A	21.13	7.92	9.54	0.94	2.62	1.17	1.40	44.72
NåPAH3B	0	0	4.53	0.49	1.68	0.81	0.86	8.38
NåPAH3C	54.62	76.62	8.04	1.52	3.60	1.25	1.27	146.91

A5 NORMALIZED RESPIRATION DATA

Table A5 Normalized respiration data, i.e. impact on respiration of each substrate

Sample	α-keto	citric	D-gluc	GABA	L-alan	N-acet	α-cyclo	Sum
Kr ref1	0.43	0.12	0.24	0.10	0.02	0.10	0.01	1
Kr ref2	0.46	0.09	0.25	0.04	0.06	0.08	0.02	1
Kr ref3	0.55	0.04	0.23	0.05	0.04	0.07	0.02	1
KrCu1A	0.30	0.09	0.33	0.09	0.09	0.09	0.01	1
KrCu1B	0.27	0.13	0.27	0.08	0.14	0.07	0.04	1
KrCu1C	0.31	0.02	0.38	0.06	0.09	0.06	0.09	1
KrCu2A	0.10	0	0.54	0.05	0.14	0.11	0.05	1
KrCu2B	0.18	0	0.41	0.09	0.18	0.13	0.01	1
KrCu2C	0.15	0.15	0.28	0.09	0.14	0.11	0.06	1
KrCu3A	0	0.04	0.31	0.03	0.36	0.19	0.07	1
KrCu3B	0	0	0.66	0	0	0	0.34	1
KrCu3C	0.11	0.15	0.32	0.08	0.15	0.18	0.01	1
KrPAH1A	0.39	0.09	0.33	0.03	0.09	0.05	0.02	1
KrPAH1B	0.35	0.03	0.30	0.06	0.02	0.17	0.07	1
KrPAH1C	0.44	0.06	0.24	0.04	0.07	0.09	0.05	1
KrPAH2A	0.45	0.06	0.21	0.05	0.07	0.09	0.06	1
KrPAH2B	0.47	0.06	0.29	0.03	0.03	0.09	0.03	1
KrPAH2C	0.43	0.11	0.20	0.05	0.06	0.09	0.06	1
KrPAH3A	0.41	0.09	0.18	0.09	0.06	0.13	0.04	1
KrPAH3B	0.66	0.07	0.15	0.02	0.04	0.05	0.02	1
KrPAH3C	0.33	0.08	0.11	0.04	0.14	0.18	0.12	1
Nå ref1	0.52	0.31	0.08	0.02	0.03	0.02	0.02	1
Nå ref2	0.56	0.09	0.16	0.03	0.05	0.08	0.03	1
Nå ref3	0.53	0.28	0.10	0.01	0.03	0.02	0.02	1
NåCu1A	0.37	0.24	0.21	0.05	0.09	0.03	0.01	1
NåCu1B	0.42	0.31	0.12	0.03	0.06	0.04	0.01	1
NåCu1C	0.60	0.15	0.11	0.02	0.06	0.03	0.03	1
NåCu2A	0.81	0	0.04	0.03	0.09	0.01	0.02	1
NåCu2B	0.58	0	0.15	0.04	0.17	0.03	0.03	1
NåCu2C	0.26	0.65	0.03	0.01	0.03	0	0.01	1
NåCu3A	0.44	0.29	0.06	0.02	0.16	0.02	0	1
NåCu3B	0.56	0.27	0.04	0.02	0.09	0	0.02	1
NåCu3C	0.51	0.36	0.03	0.01	0.07	0.01	0.02	1
NåPAH1A	0.30	0.54	0.08	0.02	0.02	0.02	0.01	1
NåPAH1B	0.63	0.28	0.04	0	0.02	0.01	0.01	1
NåPAH1C	0.55	0.33	0.06	0.01	0.02	0.01	0.02	1
NåPAH2A	0.35	0.46	0.11	0.02	0.02	0.01	0.02	1
NåPAH2B	0	0	0.50	0.06	0.19	0.13	0.12	1
NåPAH2C	0.55	0.18	0.13	0.01	0.06	0.06	0.02	1
NåPAH3A	0.47	0.18	0.21	0.02	0.06	0.03	0.03	1
NåPAH3B	0	0	0.54	0.06	0.20	0.10	0.10	1
NåPAH3C	0.37	0.52	0.05	0.01	0.02	0.01	0.01	1