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Fate of Microbial Carbon Derived From Biogas Residues Applied to Arable Soil

Dissertation
Submitted in fulfilment of the regulations to acquire the degree “Doktor der Agrarwissenschaften”
(Dr. sc. agr. in Agricultural Sciences)

to the

Faculty of Agricultural Sciences

presented by

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Tefenni
2015

This thesis was accepted as a doctoral thesis (Dissertation) in fulfilment of the regulations to acquire the degree “Doktor der Agrarwissenschaften” (Dr. sc. agr. in Agricultural Sciences) by the Faculty of Agricultural Sciences at the University of Hohenheim on: 26.06.2015

Date of oral examination: 15.07.2015

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1. Summary

Soil organic matter (SOM) is the major determinant of soil fertility as it has a number of positive impacts such as improving soil physical parameters, providing nutrients for crops, and supplying energy for the microbial biomass activity in soil. Loss of organic matter is a soil threat observed worldwide. Also, bioenergy crop cultivation may accelerate SOM loss due to higher biomass harvesting compared to food crops. It is necessary to supply adequate organic matter input to arable soils in order to maintain sustainable food and biofuel production. Biogas residues (BGRs), the side-products of biogas production, are rich in microbial and plant biomass; they thus can be used as a soil conditioner and contribute to replenishing the carbon (C) pool in soil. However, our knowledge on the contribution of BGRs particularly the microbial residues present in it to SOM formation is limited, even though scientific interest on SOM formation via microbial inputs is growing. Therefore, the objective of this thesis were i) developing an approach to label microbial biomass of biogas residues, ii) tracing the fate of labelled BGRs in arable soil, iii) determining the C flux within microbial food web, and iv) determining the impacts of other soil conditioners on the mineralization of BGRs.

In the first study a method was developed to label the autotrophic microorganisms in a biogas reactor using $KH^{13}CO_3$-amended cow manure as substrate. Analyses of phospholipid fatty acids (PLFA) and ether lipids confirmed the successful labelling of microorganisms, especially Gram-positive bacteria and methanogenic archaea. After removal of unused labelled carbonates by an acid fumigation approach, the labelled BGRs were incubated in soil for 378 days. The fate of ^{13}C was traced in CO_2 and in bulk soil with a mass balance having 93% mean recovery. Results showed that about 40% of the C derived from BGRs was rapidly mineralized within the first seven days, and mineralization reached 65% at the end of experiment. The data could be fitted to a two-pool exponential degradation model assuming two C pools each decaying exponentially. The proportions of readily degrading and stable C pools were determined to be 51% and 49%, respectively, with half-lives of 3 days and 1.9 years, respectively. The long half-life of the stable C pool in BGRs may indicate a mid-term contribution to SOM. In addition, the mineralization of SOM was enhanced by BGR-application, i.e. priming effects were detected, thus their extensive application should be avoided.

A differential fatty acid approach was used in the second study for the separation of C input from BGRs to living biomass and non-living SOM. Phospholipid fatty acids (PLFA) as

indicators of living biomass were compared with total fatty acids (t-FA), which are found also in necromass. Using PLFA as biomarkers of specific microbial groups, C redistribution within the microbial food web was determined. Results showed that BGRs increased the microbial biomass in soil. The sum of ^{13}C-labelled PLFA and t-FA decreased during incubation to 60% and to 33%, respectively. The level of enrichment was different for the individual PLFA and indicated that Gram-negative bacteria were predating on Gram-positive bacteria. A contribution of ether lipids was also detected indicating C flow from decaying methanogens. This study confirmed that microbial biomass in BGRs applied to arable soil significantly contributes to SOM formation.

After determining the fate of microbial C derived from BGRs in arable soil, the impacts of other soil conditioners on the mineralization of BGRs were tested in the third study. For this, labelled BGRs were incubated in soil both alone and together with compost, biochar and untreated manure. The amount of C mineralized to CO_2 and the degradation rate constant of stable C pool were not affected by any of the co-amendments. However, manure resulted in a higher mineralization rate constant of the readily degrading C pool. C flow within microbial food web was from Gram-positive bacteria and methanogenic archaea to mainly Gram-negative bacteria and slightly to fungi in all treatments. This study showed that co-amending BGRs with other soil conditioners brings neither benefits nor harms in terms of the formation or the mineralization of soil organic matter.

The proposed labelling approach using $KH^{13}CO_3$ may be useful for tracing the fate of BGRs. The enrichment in both bacteria and archaea were sufficient to be measured in an incubation experiment lasting for more than one year. However, there are disadvantages of the proposed approach such as presence of highly enriched residual carbonates. The fumigation method should be optimized for a complete removal of the highly labelled residual carbonates which will increase the precision of the overall approach.

In conclusion, this thesis provides valuable information on the fate of microbial C derived from BGRs in arable soil and the outcomes may be helpful to decision-making for sustainable soil management.

2. Zusammenfassung

Die organische Bodensubstanz (OBS) ist ein wichtiger Faktor für die Bodenfruchtbarkeit, da sie eine Reihe von positiven Auswirkungen auf den Boden haben, wie die Verbesserung der bodenphysikalischen Parameter, Bereitstellung von Nährstoffen für Kulturpflanzen und die Energieversorgung für die mikrobielle Aktivität im Boden. Der Verlust an organischer Substanz ist eine weltweit beobachtete Bedrohung für Böden. Auch kann der Anbau von Bioenergiepflanzen den OBS-Verlust aufgrund der höheren Biomasseentnahme im Vergleich zum Anbau von Nahrungsmitteln beschleunigen. Daher ist es notwendig, Ackerböden eine angemessene Menge organischen Materials zuzuführen, um eine nachhaltige Nahrungsmittel- und Biokraftstoffproduktion aufrecht zu erhalten zu können. Gärreste, ein Nebenprodukte der Biogaserzeugung, sind reich an mikrobieller und pflanzlicher Biomasse, können daher als Bodenverbesserungsmittel verwendet werden, und führen zur Rückführung von Kohlenstoff (C), der aus dem Boden entfernt wurde. Allerdings ist unser Wissen über den Beitrag der Gärreste, insbesondere der darin enthaltenen mikrobiellen Rückstände, auf die OBS-Bildung begrenzt, obwohl das wissenschaftliche Interesse an OBS-Bildung über mikrobielle C-Einträge wächst. Daher waren die Ziele dieser Arbeit: i) Entwicklung eines Ansatzes zur Markierung der mikrobiellen Biomasse von Gärresten, ii) die Verfolgung des Schicksals der markierten Gärreste in Ackerboden, iii) die Bestimmung der Kohlenstoffumverteilung im mikrobiellen Nahrungsnetz, und iv) die Bestimmung der Auswirkungen anderer Bodenverbesserungsmittel auf die Mineralisierung von Gärresten.

In der ersten Studie wurde eine Methode entwickelt, um die autotrophen Mikroorganismen in einem Biogasreaktor zu markieren, der mit $KH^{13}CO_3$-beaufschlagter Rindergülle als Substrat betrieben wird. Analysen von Phospholipid-Fettsäuren (PLFS) und Etherlipiden bestätigten die erfolgreiche Markierung von Mikroorganismen, insbesondere von Gram-positiven Bakterien und Archaeen. Nach dem Entfernen des unverbrauchten markierten Karbonats durch Säurefumigation wurden die markierten Gärresten 378 Tage lang im Boden inkubiert. Das Schicksal von ^{13}C im CO_2 und im Boden wurde bei einer mittleren Wiederfindung von 93% verfolgt und eine Massenbilanz aufgestellt. Die Ergebnisse zeigen, dass etwa 40% des C aus den Gärresten innerhalb der ersten sieben Tage mineralisiert werden, und die Mineralisierung am Ende des Versuchs 65% erreicht. Die Daten konnten an ein Modell angepasst werden, das zwei C-pools mit jeweils exponentiellem Abbau annimmt. Die Anteile des schnell und des langsam abbaubaren C Pools liegen bei 51% bzw. 49%, die Halbwertszeiten bei 3 Tagen und 1,9 Jahren. Die lange Halbwertszeit des langsam abbaubaren C-Pool in der Gärreste deutet darauf hin, dass Gärreste mittelfristig einen Beitrag zur Bildung

von OBS liefern können. Darüber hinaus wird jedoch die Mineralisierung der organischen Bodensubstanz durch die Applikation der Gärreste verstärkt, d.h. es kann ein Priming-Effekt nachgewiesen werden, deshalb sollte ihre extensive Ausbringung vermieden werden.

In der zweiten Studie wurde mittels differentieller Fettsäureanalyse der C-Eintrag aus den Gärresten in lebende Biomasse und in nicht-lebende OBS erfasst. Die Phospholipid-Fettsäuren (PLFS) als Indikatoren für die lebende Biomasse werden verglichen mit Gesamtfettsäuren (t-FS), die auch in der Nekromasse zu finden sind. Mit PLFS als Biomarker für spezifische mikrobielle Gruppen konnten die C-Flüsse im mikrobiellen Nahrungsnetz bestimmen werden. Die Ergebnisse zeigen, dass Gärreste die mikrobielle Biomasse im Boden erhöhten. Die Summe von ^{13}C-markierten PLFS und t-FS sank während der Inkubation auf 60% bzw. 33%. Die Isotopenanreicherung ist für jede Fettsäure spezifisch, und Gram-negative Bakterien ernähren sich von Gram-positiven Bakterien. Auch ein Beitrag der Etherlipide wurde nachgewiesen, und zeigt einen C-Fluss aus abgebauten Methanogenen. Diese Studie bestätigt, dass die mikrobielle Biomasse in auf Äckern ausgebrachten Gärresten wesentlich zur OBS-Bildung im Ackerboden beiträgt.

Nach der Bestimmung des Schicksals von mikrobiellem C aus Gärresten in Ackerboden, testeten wir in der dritten Studie die Auswirkungen von anderen Bodenverbesserungsmitteln auf die Mineralisierung von Gärresten. Hierzu wurden markierten Gärreste sowohl allein als auch zusammen mit Kompost, Biochar und Rindergülle im Boden inkubiert. Keines der anderen Bodenverbesserungsmittel bewirkte eine Veränderung der Menge an C, die zu CO_2 mineralisiert wurde und der Abbauraten des langsam abbaubaren C-Pools. Allerdings führte Gülle zu einer höheren Abbaurate des schnell abbaubaren C-Pools. Der Fluss des C durch das mikrobielle Nahrungsnetz ging bei allen Behandlungen von Gram-positiven Bakterien und Archaeen hauptsächlich zu Gram-negativen Bakterien und Pilzen. Diese Studie zeigt, dass die gleichzeitige Ausbringung von Gärresten mit anderen Bodenverbesserungsmittel weder Nutzen noch Schaden in Bezug auf die Bildung oder die Mineralisierung der organischen Bodensubstanz mit sich bringt.

Der vorgeschlagene Ansatz zur Markierung der Gärreste mit $KH^{13}CO_3$ kann für die Verfolgung des Schicksals der Gärreste in Boden nützlich sein. Die Anreicherung in Bakterien und Archaeen war ausreichend, um in einem Inkubationsversuch mit einer Dauer von über einem Jahr gemessen werden zu können. Der vorgeschlagene Ansatz hat jedoch auch Nachteile, wie etwa die Anwesenheit hochangereicherter Restkarbonate. Die

Fumigationsmethode sollte optimiert werden, damit die hoch angereicherten Restkarbonate quantitativ entfernt werden können, um die Genauigkeit des Ansatzes zu erhöhen.

Zusammenfassend stellt die vorliegende Arbeit wertvolle Informationen über das Schicksal des mikrobiellen C aus Gärresten in Ackerboden zur Verfügung, und die Ergebnisse können hilfreich für Entscheidungen über eine nachhaltige Bodennutzung sein.

3. General introduction

3.1. Carbon cycle and soil organic matter

Terrestrial systems are one of the three main carbon reservoirs together with marine systems and the atmosphere. The amount of C stored in soil was estimated to be as high as 1115-2200 Pg C (Batjes, 1992; Eswaran et al., 1993; Somebroek, 1993). Thus, soil organic matter (SOM) is one of the largest pools of terrestrial organic carbon in biosphere (Jobbágy and Jackson, 2000) and carbon flux from soils is average 68 Pg C a-1 (Raich and Schlesinger, 1992) which is higher than net primary productivity of 56 Pg C a^{-1} (Field et al., 1998).

On the other hand, SOM can act also as a sink for atmospheric CO_2 (Guo and Gifford, 2002; Leinweber et al., 2008). Since SOM enhances soil fertility (Krull et al., 2003), it also increases the net primary production and photosynthetic CO_2 fixation. The ability of SOM to regulate atmospheric CO_2 levels can contribute to mitigate the global climate change. The potential of carbon sequestration by soils is estimated to be between 0.4-0.6 Pg C a^{-1} (Sauerbeck, 2001) and 0.6-1.2 Pg C a^{-1} (Lal, 2003) and sustainable C sequestration in SOM is necessary in order to function as a long-term sink for CO_2 (Miltner et al., 2012).

Decomposition and mineralization processes provide the necessary nutrients to the plants (Campbell, 1978). Along with supplying nutrients for crops, SOM also improves physical properties of soil (Six et al., 2002) and provides energy for microbial biomass activity (Joergensen et al., 1990). Thus, a reasonable amount of SOM in arable soils is necessary in order to remain its fertility as sufficient nutrients and adequate soil physical properties must be present in soil on the long term. SOM-depleted soils usually have poor nutrient levels and may have unfavorable pH. Such soils cannot support economically feasible large-scale food production to meet the nutrition requirements of a growing human population (Campbell, 1978).

Loss of organic matter is a soil threat observed worldwide. Bellamy et al. (2005) reported a loss of 0.6% a^{-1} (relative to the existing carbon content) in soils of England and Wales between 1978 and 2003. This decrease was larger in SOM-rich soils and did not depend on land use, which may indicate the impact of climate change (Bellamy et al., 2005). Changes in the manure management practices due to legal restrictions and replacement of slurry-based systems by straw-based systems decreased the organic carbon content of Belgian cropland (Sleutel et al., 2003). The improvements in tractor power lead to deeper ploughing which increased the mineralization of organic carbon and diluted the surface SOM levels (Smith et al., 2007). Land use change from natural vegetation to cultivated land releases organic carbon

to the atmosphere (Schlesinger, 1990; Wilson, 1978). In addition, the harvest index has increased by about 1.45% a^{-1} which means an increase in the amount of biomass removed from the agricultural fields (Ewert et al., 2005). Changes in management and technology (e.g. combine harvesters) decreases SOM stocks and can have large impacts in future (Poulton, 1996; Smith et al., 2005).

Removal of aboveground biomass as a problem for SOM stocks may aggravate when growing energy crops. For example corn stover is one of the most important bioenergy feedstocks due to its high abundance and cellulose content (Pacala and Socolow, 2004). It is the non-grain part of corn normally left on soil surface after harvest and has not been utilized until recently. The annual production of corn stover is more than 216 million tons in the USA where there is an increasing interest in bioethanol production (Sokhansanj et al., 2002). Due to its availability, there is a potential of collecting those residues for conversion to bioethanol (Sokhansanj et al., 2002) which will directly decrease the organic matter input to soils. The possible increase in the corn residue removal may deplete SOM stocks and may have other negative impacts e.g. on soil water reserves and crop yields (Lal et al., 2004; Wilhelm et al., 2004). It was reported that removal of 25% of the corn stover can significantly decrease SOM stocks (Blanco-Canqui and Lal, 2007).

3.2. Sources and stabilization mechanisms of soil organic matter

The chemical composition of SOM is determined by its precursor material. The main precursor material for soil carbon is plant materials (Kögel-Knabner, 2002). On the other hand, microbial biomass plays an important role in the transformation of plant derived organic matter, but its contribution to SOM is difficult to be quantified. According to Dalal (1998), microbial biomass performs critical functions e.g. nutrient transformation, despite its low (<5%) contribution to SOM. Utilization of plant materials by microorganisms result in SOM formation after cell death (Kindler et al., 2006; Kindler et al., 2009; Miltner et al., 2009). The contribution after cell death i.e. stabilization of necromass into SOM is supported by the findings of Liang et al. (2011) who showed that the necromass pool was almost 40 times the size of the living biomass pool. This might mean that 80% of the soil organic carbon is in necromass, assuming 2% living biomass contribution (Liang and Balser, 2011). Simpson et al. (2007) reported the contribution of microbial biomass to be higher than 50% of the extractable SOM fractions, 45% of humin and 80% of soil nitrogen.

Recently, Miltner et al. (2012) proposed a conceptual model that demonstrates microbial cell envelopes to be a significant source of SOM (Fig. 3.1). According to this model, bacterial

growth is initially stimulated by the plant material input. However, substrate depletion will eventually result in starvation of the microbes and their death. Cell envelope fragments of the death bacteria are stabilized as non-living SOM after interaction with soil minerals and form cell envelope fragments observed in soils by scanning electron microscopy (Miltner et al., 2012). This is supported by the stabilization of 25% of the carbon derived from labelled *E. coli* cells in an incubation experiment in soil as metabolites or non-living SOM (Kindler et al., 2006). Also, scanning electron micrographs of samples from a glacier showed that small-sized SOM fragments presumably derived from cell envelope residues increased with soil age (Schurig et al., 2013).

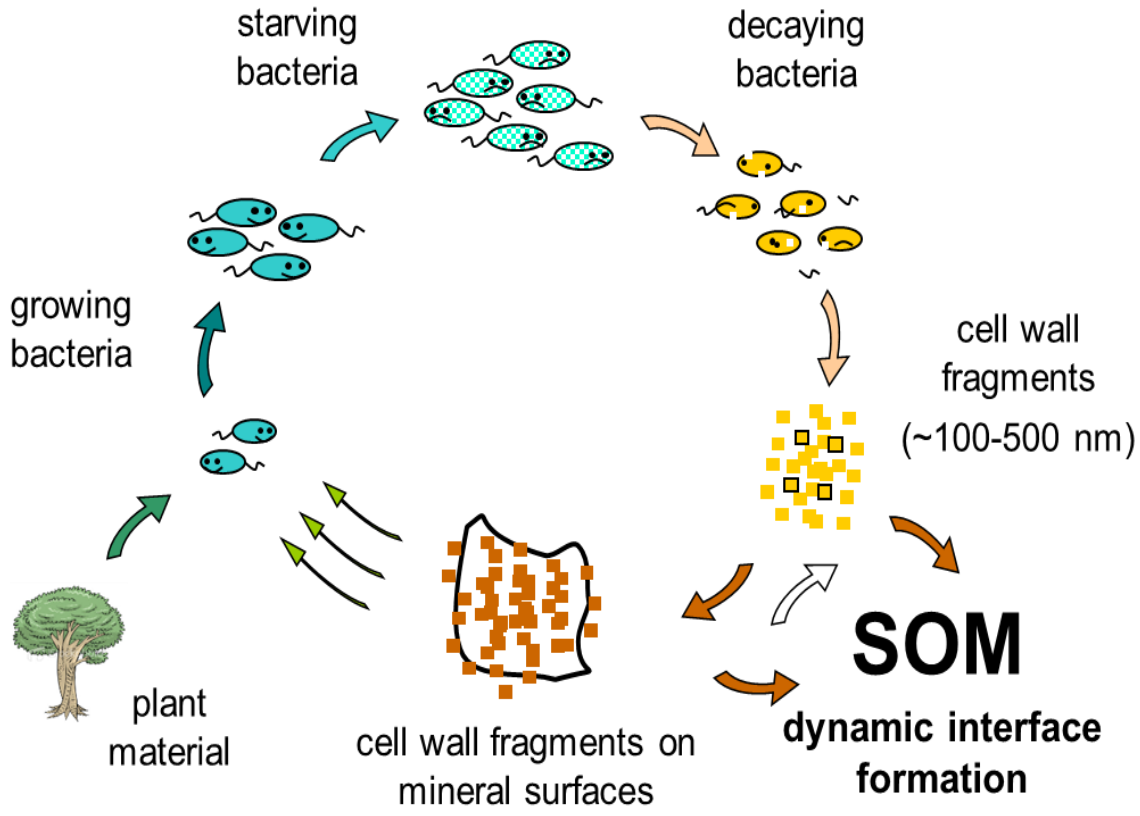

Fig. 3.1 Patchy fragment formation cycle as proposed by Miltner et al. (2012). It is shown that plant material is turned over by microorganisms which die after prolonged starvation. Their cell envelope fragments can be attached to soil minerals and are stabilized in non-living SOM.

Stable isotope based approaches can be used for the quantification of microbial input to SOM. As a general approach, the fate of ^{13}C labelled microbial biomass can be traced with a mass balance analyzing bulk soil and CO_2 using an elemental analyzer coupled to isotope ratio mass spectrometer and gas chromatography coupled to isotope ratio mass spectrometer, respectively (Kindler et al., 2006). However, in order to differentiate the contribution into living biomass and non-living soil organic matter, biomarkers need to be analyzed for their

concentrations and isotopic composition. Kindler et al. (2009) compared the label incorporations into phospholipid fatty acids and total fatty acids, thus quantified the contributions into living biomass and non-living SOM, respectively. Amino acid analysis can be also used in a similar way since proteins are a major component of microbial biomass (Miltner et al., 2009).

Maintenance of SOM level mechanisms need to be known for proper management. The stabilization of SOM depends on different mechanisms including chemical, physical and biochemical stabilization (Christensen, 1996; Lützow et al., 2006; Six et al., 2002; Sollins et al., 1996; Stevenson, 1994). First of all, SOM can be chemically stabilized due to interactions with mineral surfaces and metal ions. These inter-molecular interactions are described to occur within organic matter or between organic matter and inorganic soil components (Lützow et al., 2006). Thus, soil mineralogy is an important control of soil carbon stocks (Torn et al., 1997). The second mechanism, physical stabilization, occurs via aggregate formation, intercalation within phyllosilicates, encapsulation in organic molecules, and hydrophobicity which prevents organic matter to be in contact with water, soil microbes and enzymes, resulting in protection of soil organic matter against biodegradation (Elliott and Coleman, 1988; Lützow et al., 2006). The chemical composition of SOM is responsible for the last stabilization mechanism, biochemical stabilization, because it contains recalcitrant compounds, which can only be degraded very slowly (Six et al., 2002). For example, the condensed nature, hydrophobicity and non-specific structure of lignin make it difficult to be decomposed (Paul, 2006). This mechanism is a selective preservation process leading to an accumulation of recalcitrant molecules (Lützow et al., 2006). Those recalcitrant molecules can be categorized into compounds with primary (plant litter and rhizodeposits) and those with secondary recalcitrance (microbial products, humic polymers and production of charcoal) (Lützow et al., 2006), however, the chemical composition of SOM is very complex and difficult to be categorized (Kelleher and Simpson, 2006).

Proper management of arable soils is necessary to avoid carbon depletion. Lal (2004) reported various recommended management options instead of traditional agricultural methods for SOM accumulation via carbon sequestration. For example, no-till farming is an option, because tillage brings soil microorganisms in contact with previously inaccessible organic matter and thus increases SOM decomposition. SOM accumulation in the soil surface (0-10 cm) with no-till practice has been already reported even though no difference was observed in the whole soil profile (Blanco-Canqui and Lal, 2008). Increasing crop biodiversity in soils

instead of growing monocultures continuously, avoiding intensive use of chemical fertilizers, as well as using efficient irrigation techniques may contribute to soil organic carbon sequestration for a sustainable agriculture (Lal, 2004). Another important management option is avoiding residue removal so that large quantities of above-ground biomass is returned to the soil (Lal, 2004). The above-ground biomass includes shoot, leaves and other harvest residues of plants. This resource amounts to as much ast $4x10^9$ Mg a^{-1} in the world (Lal, 2005) and its application to soil provides energy sources and habitats to soil microbiota and closes element cycles (Lal, 2008). Nevertheless, some loss via harvest is unavoidable in agricultural soils and extra organic matter input is necessary for protecting SOM stocks. Recent increase in biogas sector results in generation of large amounts of nutrient-rich biogas residues as side-products which can be used as a soil amendment. However, careful investigation of amendments is necessary to estimate their possible negative impacts.

3.3. Biogas production

Biogas is a valuable energy source which can be produced theoretically from any type of biomass containing long-chain organic polymers (Deublein and Steinhauser, 2011; Weiland, 2010). The main substrates used for biogas generation are energy crops and livestock excrements which at the end are converted to a mixture of CO_2 and CH_4 along with other trace gases. The quality of biogas depends on its methane content which varies between 45-70% (Rasi, 2009).

The global interest in replacing fossil fuels by renewable fuels for decreasing greenhouse gases is increasing. For example Germany is targeting to supply up to 23% of its energy demand from bioenergy in 2050 (FNR, 2014). Among other renewable energy sources, bioenergy has the advantage to allow production according to demand. The number of biogas plants, thus, increased in Germany from 190 to 3552 between the years 2003 and 2012. Also, installed electrical capacity has already passed the level of 7500 MW (FNR, 2014). Together with biogas, bioethanol and biodiesel productions are also increasing which at the end causes land use change from arable or native lands to bioenergy crop fields.

Bioenergy crop production may have severe impacts on soil C stocks. This impact depends on the type of bioenergy crop planted and the former vegetation on the field (Don et al., 2012). While conversion of annual cropland to perennial crops increases the C stocks, conversion of perennial crops or grassland to annual crops results in a reduction of SOM (Anderson-Teixeira et al., 2009; Poeplau et al., 2011). The most common feedstock for bioenergy production is maize (Birch et al., 2003) which is an annual crop and SOM losses may occur in

the near future. As discussed in section 3.1, larger removal of biomass from arable land cropped with bioenergy plants than with crops is another disadvantage of bioenergy on soil systems, particularly with respect to SOM loss.

The formation of biogas from complex polymers is a stepwise process summarized in Fig. 3.2. These individual steps are carried out by specific microorganisms partly in syntrophic interrelationships (Angelidaki et al., 1993). Initially, complex polymers are converted to acetate, hydrogen and volatile fatty acids by hydrolysing and fermenting microorganisms. These microorganisms are generally *Firmicutes* such as *Clostridia* (Liu et al., 2009). Later, acetogenic bacteria convert the volatile fatty acids produced during the first step to acetate, hydrogen and CO_2 (Gujer and Zehnder, 1983). In the last step, methanogenic archaea produce CH_4 from either acetate or H_2 and CO_2. Methanogens detected in biogas digesters are mainly hydrogenotrophic, which means that they use H_2 and CO_2 as intermediates to be converted to CH_4 (Scherer et al., 2000), especially if high concentrations of ammonia are found in the substrate (Demirel and Scherer, 2008). This is due to inhibition of acetotrophic pathway and conversion of acetate to H_2 and CO_2 by syntrophic acetate oxidizing bacteria under increasing concentrations of NH_3 (Schnurer and Nordberg, 2008). At the end of the process, biogas residues are formed as nutrient-rich side products (Båth and Rämert, 1999) .

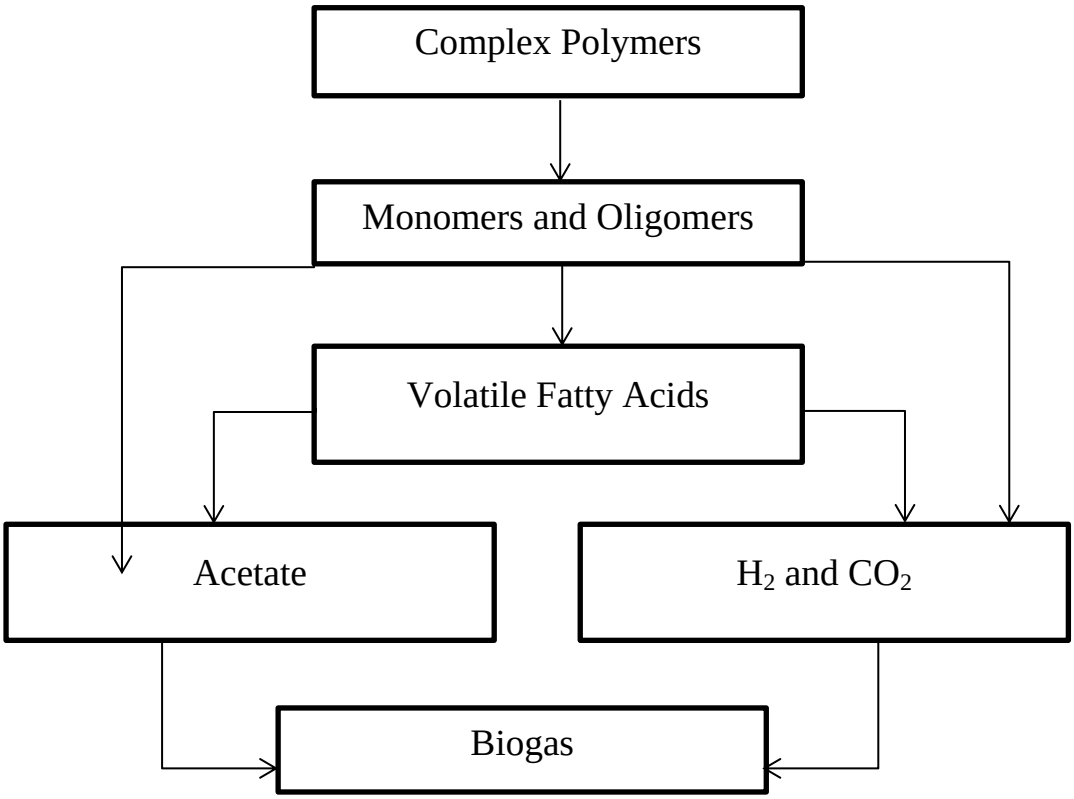

Fig. 3.2 Stages of anaerobic digestion for the production of biogas according to Gujer and Zehnder (1983).

3.4. Biogas residues as a soil conditioner

As discussed in Section 3.1. there is a global concern in increasing or at least preserving the level of SOM in arable soils. SOM level is result of input and degradation. It is difficult to control SOM degradation rates, but organic matter input can be easily manipulated in arable soils. This is especially necessary when growing bioenergy crops since more biomass is removed than food crops. Therefore, this additional export of organic matter needs to be compensated.

Applying organic amendments to arable soils can be an option for this compensation. In principle, organic matter amendment improves SOM (Grandy et al., 2002),, stimulates microbial activity (Debosz et al., 2002; Marinari et al., 2000), improves soil physicochemical properties (Tester, 1990), SOM soil structure, water holding capacity (Joshua et al., 1998), nutrients (Jakobsen, 1995), and enzymatic activities (Blagodatsky and Richter, 1998; Liang et al., 2003). In addition, biological control can be facilitated by organic matter amendments (Baker and Cook, 1974; Hodges and Scofield, 1983; Hoitink and Boehm, 1999; Lumsden et al., 1983). Organic amendments can be also used for remediating soils (Tejada et al., 2006).

However, fresh organic matter amendments can also have stimulatory impacts on the mineralization of SOM, the so called priming effects (Bingeman et al., 1953; Dalenberg and Jager, 1989). Large amount of carbon and nutrients can be released in short-term due to priming effects. Mechanisms of the priming effects were recently reviewed by Kuzyakov et al. (2000). Generally, priming effects are considered to occur immediately after the substance input (Dalenberg and Jager, 1981, 1989; Pascual et al., 1998); they depend on soil C and N content (Hart et al., 1986) and on the amount of the substance added (Asmar et al., 1994; Dumontet et al., 1985; Kawaguchi et al., 1986; Mary et al., 1993). However, these processes can only be precisely determined using stable or radioactive isotope labelling approaches (Kuzyakov et al., 2000).

An example of organic amendment is animal manure. Animal manure is rich in organic matter and nutrients (Barker and Zublena, 1995) and its application to cultivated soils has increased over the last decades due to its easy availability and ability to bring the nutrients back to the soil (Pagliai and Vignozzi, 1998). Another commonly used organic amendment is compost. Composts are the products of aerobic composting processes where raw organics are transformed into biologically stable substances (Cooperband, 2002; Epstein, 1996). Mineralization of C input from composts is lower than from untreated manure (Bernal and Kirchmann, 1992) or municipal waste (Busby et al., 2007). Biochar is another organic

amendment which reduces greenhouse gas emissions from soil (Singh et al., 2010; Woolf et al., 2010) and nutrient leaching (Singh et al., 2010), increases stability of labile organic matter amendments (Keith et al., 2011) and native soil (Liang et al., 2010). Recently, the nutrient-rich side products of biogas production systems, the biogas residues, are used as organic amendments to soil (Båth and Rämert, 1999), but little is known about priming effects of this material.

The application of BGRs to soil was suggested by Arthurson (2009) to close the element cycles. It also improves humus balance (Monnet, 2003; Odlare, 2005). However, the low C:N ratio can lead to decreased N-mineralization and bioavailability (Dahlberg et al., 1988; Demuynck et al., 1985). Generally crop yields after BGRs application are as high as with other fertilizers e.g. manure (Dahlberg et al., 1988). In addition, no phytotoxic effects of BGRs were observed unlike reported for bioethanol and biodiesel residues (Gell et al., 2011).

Biogas residues are known to increase crop yield and thus can replace or complement other organic fertilizers (Arthurson, 2009). The characteristics of BGRs determine the efficiency of fertilization. For example, BGRs have higher nutrient contents (Garg et al., 2005), lower C:N ratio, and a higher pH than untreated organic waste (Asmus et al., 1988). During biogas production process, C is mineralized to CO_2 or converted to CH_4, whereas N is retained (Tambone et al., 2009). In addition, organic N is transformed to ammonia (Tambone et al., 2010), which results in increased plant-available N in BGRs compared to the untreated organic waste; e.g. manure.

There are contradictory results found in the literature about the impacts of BGRs on soil parameters. Odlare et al. (2008) reported an increase in active microorganisms as well as substrate induced respiration due to BGRs. However, according to Ernst et al. (2008) stimulation of microbial biomass and basal respiration, as well as readily-degradable nutrients are lower in BGRs compared to conventional slurry. A bias in comparing untreated and treated organic fertilizers is that their C:N ratios are different, so that application according to N-input results in low C input by BGRs (Ernst et al., 2008). Fate of BGR-derived carbon in CO_2 and bulk soil, together with cycling in food-web should be traced. Co-amendments using other organic soil conditioners should be tested to modify possible negative effects caused by BGRs.

3.5. Use of stable isotopes to investigate soil carbon cycle

Basically, there are two stable isotope methods used in functional soil ecology: natural abundance and labelling (Staddon, 2004). The natural abundance approach is based on the different isotopic composition of different carbon pools which is derived from the discrimination against ^{13}C in different biochemical pathways (Staddon, 2004).

Alternatively, it is possible to track the fate of a compound by labelling approach i.e. adding ^{13}C-labelled substrate to the soil system. This approach has an advantage that many carbon pools of interest can be labelled and studied with high sensitivity (Staddon, 2004). For example Richnow et al. (2000) investigated the flux of polycyclic aromatic hydrocarbon in a contaminated soil. The same approach can be used for studying incorporation of organic substances to soil organic matter (Gunina et al., 2014; Liu et al., 2014; Torres et al., 2014). As an advanced approach, position specific labelling can be used to explain biochemical pathways (Apostel et al., 2013). Stable isotope labelling is especially important in soil carbon cycling studies since it can separate mineralization of a carbon amendment from the native SOM (Cheng, 2009; Nottingham et al., 2009), which is important to determine priming effects (see section 3.1 for priming effects). In most cases, it is necessary to determine the fate of a compound in specific microbial groups. For this reason, phospholipid fatty acid (PLFA) analysis is widely used (Cavigelli et al., 1995; McKinley et al., 2005; Sims, 2008). Since PLFAs are specific for microbial groups and found only in living cells (Zelles, 1999), they can be used as biomarkers. Combining PLFA with total fatty acid analysis allows us to distinguish the contribution of a substance to living biomass and non-living SOM, separately (Kindler et al., 2009). Mainly Gram-positive bacteria, Gram-negative bacteria and fungi can be distinguished (Cavigelli et al., 1995) using PLFA approach. It is also possible to relate PLFA to more specific groups like type I methanotrophs (Bowman et al., 1993) or actinomycetes (Ringelberg et al., 1997), however, for a species-specific determination nucleic acid based stable isotope labelling is necessary (Dumont and Murrell, 2005; Lueders et al., 2004; Manefield et al., 2002; Radajewski et al., 2000). DNA based isotope labelling gives the possibility of a functional gene analysis with a further metagenomics approach (Friedrich, 2006), however, it is less sensitive than PLFA or RNA based labelling approach due to the necessity of a cell division for DNA replication (Neufeld et al., 2007). PLFA based labelling is the most sensitive approach. Thus, a small amount of label and only partial incorporation of the labelled substance into PLFA is sufficient for an analysis.

In order to evaluate the use of BGRs as soil conditioners it is necessary to quantify their contribution and impacts to soil organic matter. Stable isotope labelling approaches can be used to precisely determine the fate of carbon derived from biogas residues in soil.

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4. Overview of the thesis

Biogas residues (BGRs) might be a sustainable organic amendment for soils due to their microbial biomass content, which may significantly contribute to the formation of soil organic matter (SOM). It was hypothesized that BGRs have influence on the composition and turnover of the SOM and thus the thesis aimed to determine the fate and effects of BGRs as a soil amendment. Therefore, the objectives of this study were i) developing a labelling approach for BGRs, ii) determining fate of microbial carbon derived from BGRs using a mass balance approach, iii) quantifying priming effects, iv) determining carbon (C) fluxes within microbial food web, and v) evaluating the impacts of co-amendment of BGRs with other soil conditioners.

The first study (section 5) includes the labelling of microbial portion of BGRs using $KHCO_3$. The aim was to label the hydrogenotrophic methanogens as well as anaerobic bacteria active in anaerobic digestion, which can use labelled $KH^{13}CO_3$ for production of CH_4, CO_2 as well as their metabolism. For this, we operated lab-scale fed-batch biogas reactors and used $KH^{13}CO_3$ amended cow manure as substrate. At the end of anaerobic digestion, the labelled BGRs were collected and further incubated in soil in a 378-day experiment in order to develop a mass balance of the label and quantify priming effects. Mineralized CO_2 data was fitted to a two pool model for the determination of readily-degradable and stable C pools in BGRs.

The fate of fatty acids derived from BGRs was studied in the second study (section 6) to analyse C flux in the microbial food web using a differential approach of phospholipid fatty acids (PLFA) and total fatty acids (t-FA). PLFA are only found in membranes of living cells unlike t-FA which occur also in death cells. Thus, it was aimed to separate the contribution of microbial C derived from BGRs into living biomass and non-living SOM for a better understanding of its stabilization and of C flow through the microbial food web. The label incorporation/loss in specific PLFA as biomarkers for microbial groups was analysed using compound specific gas chromatography - isotope ratio mass spectrometry (GC-IRMS), which allowed to quantify the C flow between Gram-positive bacteria, Gram-negative bacteria and fungi. Also, PLFA profiles of BGR-amended and non-amended soils were compared for the determination of impacts of BGR amendments on the soil community.

A shorter incubation experiment was performed to test the short-term influence of several organic amendments on the mineralization of BGRs in soil (section 7). Compost, biochar and manure were incubated in soil both alone and together with BGRs. It was hypothesized that

when more than one organic amendment is applied to soil, these labile organic materials may interact with each other, which potentially changes the mineralization trends. CO_2 was captured during the incubation experiment and its concentration as well as isotopic composition was measured. Changes in the proportions and degradation rate constants of C pools of BGRs due to co-amendment with other soils conditioners were estimated. In addition, microbial group specific incorporation of carbon was determined.

5. The contribution of biogas residues to soil organic matter and CO_2 emissions in an arable soil

Soil Biology & Biochemistry (86) 2015, 108-115

doi:10.1016/j.soilbio.2015.03.023

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Abstract

The biogas production process generates as side-products biogas residues containing microbial biomass which could contribute to soil organic matter formation or induce CO_2 emissions when applied to arable soil as fertilizer. Using an isotope labelling approach, we labelled the microbial biomass in biogas residues, mainly G+ bacteria and methanogenic archaea via $KH^{13}CO_3$, and traced the fate of microbial biomass carbon in soil with an incubation experiment lasting 378 days. Within the first seven days, 40% of the carbon was rapidly mineralized and after that point mineralization continued, reaching 65% by the end of the experiment. Carbon mineralization data with 93% recovery could be fitted to a two-pool degradation model which estimated proportions and degradation rate constants of readily and slowly degrading pools. About 49% of the carbon was in the slowly degrading pool with a half-life of 1.9 years, suggesting mid-term contribution to living and non-living soil organic matter formation. Biogas residues caused a priming effect at the beginning, thus their intensive application should be avoided.

Introduction

Bioenergy is considered a promising carbon (C) neutral option for reducing greenhouse gas emissions from fossil fuel burning and is currently being promoted in many countries. Germany, for example, is planning to produce 85% of its electricity via renewables by 2050, and is actively phasing out nuclear power and cutting fossil fuel use (Schiermeier, 2013). The number of biogas plants in Germany has increased from 139 in 1992 to 7850 in 2013 (German Biogas Association, 2014). Animal manure and slurries are valuable resources for biogas production and have the potential to sustain an important portion of annual renewable energy generation (FNR, 2008; Holm-Nielsen et al., 2009). Due to the development of the biogas sector (Weiland, 2010), a large amount of by-products, or biogas residues (BGRs), are produced which should be returned to soil to mitigate C loss.

BGRs are side-products of anaerobic digestion consisting mainly of microbial biomass and its residues as well as non-fermented feedstock leftovers (Gerardi, 2003). They improve crop growth and can replace other organic fertilizers (Arthurson, 2009) due to their high nutrient content and ability to improve the physical properties of soil (Garg et al., 2005). BGRs also have higher fertilization efficiency than untreated amendments such as manure, because during anaerobic fermentation the ratio of plant available ammonium (NH_4^+) to total Nitrogen (N) increases, C/N ratio decreases, and a higher pH is reached (Asmus et al., 1988). This is because N is retained while C is mineralized during biogas production (Tambone et al., 2009). Additionally, organic matter mineralization leads to the transformation of organic N into ammonia (Tambone et al., 2010). Unlike biodiesel and bioethanol residues, BGRs are reported to have no phytotoxic effects (Gell et al., 2011). In addition to supporting plant production, BGRs can provide organic matter input to arable soils (Odlare, 2005) because they consist of digested plant residues and microbial biomass residues (Gerardi, 2003), and thus contain recalcitrant organic materials.

Soil organic matter (SOM) is a sink for carbon dioxide (CO_2), affects soil respiration (Leinweber et al., 2008) and determines soil fertility (Krull et al., 2003). Therefore, there is global concern over the loss of SOM (Bellamy et al., 2005; Schulze and Freibauer, 2005). Although plant litter has been considered to constitute the major part of SOM (Beuch et al., 2000; Pelz et al., 2005), it is argued that the role of microbial biomass is underestimated given that after cell death, microbial biomass residues will contribute to non-living SOM (Miltner et al., 2012; Simpson et al., 2007). Also, microbial biomass is turned over much faster than plant litter in soil (Kästner, 2000; Schink, 1999; Van Kessel et al., 2000). This might mean that the

flux from microbial C to SOM is in the same order of magnitude as that from plant detritus C (Kindler et al., 2006). In an experiment using isotopically labelled *Escherichia coli*, it was shown that 44% of the applied Gram-negative (G^-) biomass was either incorporated into the indigenous soil microbiome or associated with non-living SOM (Kindler et al., 2006). BGRs are rich in microbial biomass and its residues and they are applied to soil as fertilizers. However, their contribution to SOM formation and/or CO_2 emissions is unknown. Thus, we aimed to determine their fate in soil in an incubation experiment using stable isotope tools.

Currently, there is considerable scientific interest in the impacts of not only BGRs but also other organic fertilizers. Ribeiro et al. (2010) reported that both hen manure and compost enhanced C mineralization rates by 200% - 500% compared to unamended soil, due to the significant amount of readily degradable organic C, which could be estimated by soluble organic C contents. To study C turnover precisely, the priming effect, which is defined as increased decomposition of native organic C after addition of readily decomposable organic substances to the soil, must be considered (Dalenberg and Jager, 1981; Kuzyakov et al., 2000). Bol et al. (2003) determined the priming effect after application of slurry as a fertilizer. We calculated from their data that 41-45% of CO_2 was generated due to the positive priming of the native soil depending on the nature and concentration of SOM. Although the impacts of several soil amendments such as slurry or plant residues have been studied extensively, so far the research on BGRs is limited. For example, Alburquerque et al. (2012) studied the biodegradability of BGRs from different co-digestion reactors and reported that BGRs from cattle slurry-glycerin mixtures are highly unstable and readily biodegradable, which can result in strong CO_2 emissions and loss of N-fertilizer value through N-immobilization in soils. Chen et al. (2012) reported mineralization of BGR-derived C as 6.4% of the organic C added to soil via BGRs from an agricultural maize-based biogas plant. However, their calculation was based on a difference method and the priming effect was not considered. Another attempt to investigate the mineralization of BGR-C focussed on natural abundances of stable isotopes (Chen et al., 2011). However, those results showed that BGRs induced the release of inorganic C as CO_2 and the authors concluded that this approach is not suitable for investigating the sources of short-term BGR-SOM interactions. The main problem encountered when using the natural abundance stable isotope approach is that inorganic C interferes with the two-end-member mixing equation. We presume that isotopic labelling of the BGR-C and removal of the inorganic C prior to incubation can overcome this problem and contribute significantly to the determination of the fate of the microorganism-derived BGR-C,

i.e. the contribution to CO_2 emissions (quantification of the priming effect) and SOM, and thereby provide recommendations for the use of BGRs as organic fertilizers.

However, labelling of BGRs is not straightforward and has not been applied so far. Our approach is based on the principle of labelling the microorganisms responsible for the anaerobic digestion of organic feedstock. We operated a biogas reactor fed by manure amended with ^{13}C-enriched carbonate in order to label the living biomass, and especially the hydrogenotrophic methanogens, which utilize CO_2 together with H_2 to produce biogas. The overall objectives of the present study were: i) to develop a labelling approach for BGRs, ii) to trace the fate of BGR-C in soil using a mass balance, and iii) to quantify the priming effect.

Materials and Methods

Chemicals

Isotopically labeled $KHCO_3$ with 98 atom% ^{13}C was obtained from Campro Scientific, Germany. All other chemicals were obtained from ThGeyer (Germany) at the highest grade available.

Production of BGRs

The BGRs were produced in lab-scale fed-batch reactors. The reactors had a working volume of 200 ml and were designed to allow daily manual feeding without contact to air. Inoculum was taken from a full-scale biogas plant (Gundorf, Sachsen, Germany). Cow manure was used as substrate with a loading rate of 2 g total volatile solids l^{-1} d^{-1}. Hydraulic retention time of the substrate was 30 days. After process stabilization, 150 mg $KH^{13}CO_3$ (98 atom% ^{13}C) per day was added together with the substrate for 28 days. Due to the expected high enrichment of CO_2 and CH_4, it was not possible to analyze the isotopic enrichment by isotope ratio monitoring mass spectrometry. Instead, the isotopic enrichments of the formed CH_4 and CO_2 were measured during the process using a 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to a 5975C inert quadrupole MSD with Triple Axis Detector (Agilent Technologies, Santa Clara, CA, USA). This approach is less sensitive to small deviations in the isotopic composition, and thus less precise at natural abundance level. For our purpose, i.e. confirmation of labelling of CH_4, the precision was by far sufficient. After 28 days, the BGRs were collected and freeze-dried for further processing. As a control to account for natural abundance of ^{13}C, unlabelled BGRs were also produced similarly using unlabelled $KHCO_3$. Prior to soil incubation, the BGRs were fumigated with 6 M HCl in a desiccator for 4

days to remove residual carbonates containing unused isotopic label (Harris et al., 2001), freeze-dried and stored at 4 °C.

Soil sampling and characteristics

Soil was sampled in October 2012 from Bad Lauchstädt static fertilization experiment site (51°24'N, 11°53'E) from a plot with mineral P and K treatment and a crop rotation of sugar beet, spring barley, potato and winter wheat since 1902 (Körschens, 1994). The soil is a Haplic Chernozem and has a loamy texture and 22% clay content. Soil characteristics are given in Table 5.1. After sampling, the soil material was stored at 4 °C until further use.

Table 5.1 Characteristics of soil, biogas residues (BGRs) and untreated manure used in the study.

	C (g kg^{-1})	**N (g kg^{-1})**	**C/N**	**pH**
Soil	17.9±1.4	1.6±0.1	11	6.2
Labelled BGRs	229.9±24.6	19.6±3.0	12	7.0
Unlabelled BGRs	225.3±13.5	19.3±0.1	12	7.0
Untreated manure	367.9±12.5	24.8±3.2	15	6.8

Soil incubation

Soil was sieved through a 2 mm sieve and homogenized prior to incubation. The water content was adjusted to 50% of water holding capacity for optimum microbial activity. Three sets of reactors containing only soil, soil with unlabelled BGRs, and soil with labelled BGRs, respectively, were set up. Characteristics of BGRs are given in Table 5.1. The amendment rate was 0.066 g N kg^{-1} dry soil, similar to other studies (Abubaker et al., 2012; Chen et al., 2012; Müller and von Fragstein und Niemsdorff, 2006), i.e. 8.61 µg ^{13}C g^{-1} soil (initial amount added). The addition of the labelled BGRs increased the amount of ^{13}C by approx. 5% of the natural background.

Incubations were performed at 20 °C in closed-reactor systems as previously described (Kindler et al., 2006). Briefly, glass reactors (5l volume, 10 cm diameter, 55 cm height) were filled with soil and aerated with humidified air at a flow rate of 30 ml/min. CO_2 produced in the reactors was captured in two traps, each of which contained 100 ml of 2 M sodium hydroxide (NaOH). As O_2 in the reactors was consumed, additional O_2 was supplied from flexible gas bags, which acted as self-dosing systems. Soil was sampled for bulk C analysis on

days 0, 7, 21, 42, 112, 259 and 378, whereas CO_2 was sampled on days 0, 7, 21, 42, and then every 35 days.

Analysis of concentration and isotope composition of CO_2, bulk SOM, fatty acids, and archaeal lipids

The amount of CO_2 trapped in NaOH was determined using a multi NC 2100S Total Organic Carbon Analyser (Analytik Jena, Jena, Germany). Isotopic composition of CO_2 was determined using a method modified from Miltner et al. (2004). Briefly, 2 ml aliquots of NaOH-trapped CO_2 were acidified with phosphoric acid in headspace vials and the headspace gas was analysed by isotope ratio gas chromatography - mass spectrometry (irm-GC/MS) using a HP 6890 Plus Gas Chromatograph (GC) (Hewlett-Packard, Wilmington, USA) coupled to a Finnigan MAT 252 IRMS with a GC Conflo combustion interface (Finnigan, Bremen, Germany). CO_2 was separated from other gases at 40 °C using a Poraplot Q-HT Plot FS column (25 m × 0.32 mm ×10 µm, Agilent Technologies, Santa Clara, CA, USA).

Carbon content and the isotopic composition of dried bulk soil samples was determined by elemental analysis – IRMS using a Flash 2000 Organic Elemental Analyser (Thermo Fisher Scientific, Waltham, MA, USA) coupled via a Conflo IV interface to a Delta V Advantage IRMS (Thermo Fisher Scientific, Waltham, MA, USA). Organic carbon content of the dried samples was determined after complete removal of inorganic carbon by washing with 6M HCl.

Fatty acids were extracted according to Bligh and Dyer (1959), and phospholipids were separated from glycolipids and neutral lipids by column chromatography over silica gel. Analysis of phospholipid fatty acids (PLFAs) was performed as described previously (Miltner et al., 2004). Monounsaturated and cyclopropyl lipids were evaluated as biomarkers of G^- bacteria (Fanin et al., 2013) and saturated branched fatty acids as biomarkers for Gram-positive (G^+) bacteria (O'leary and Wilkinson, 1988). PLFAs 18:2 cis 9, cis 12 and 18:1ω9 were evaluated as fungal biomarkers (Cavigelli et al., 1995). The PLFAs 14:0, 16:0 and 18:0 occur generally in all living cells (Tavi et al., 2013). The relative ^{13}C label distributions in the PLFAs were calculated by dividing the amount of ^{13}C in the respective microbial group to the sum of ^{13}C in all microbial groups.

Archaeal ether lipids were extracted using a modified four-step Bligh and Dyer extraction according to Sturt et al. (2004), two times using a mixture of methanol, dichloromethane and phosphate buffer (2:1:0.8, v:v:v) and two times with 5% trichloroacetic acid substituted for

the phosphate buffer. Phytanyl (C_{20} isoprenoid) and biphytanyl (C_{40} isoprenoid) side chains were released from archaeal diether and tetraether lipids of the phospholipid and glycolipid fractions using boron tribromide and subsequent reduction with lithium triethylborohydride as described in Bradley et al. (2009). Stable C isotope ratios of phytane and biphytane were determined on a Thermo Finnigan Trace GC Ultra coupled to a DELTA Plus XP isotope ratio mass spectrometer via a GC Combustion Interface III. Compounds were separated on a RTX-5MS fused silica capillary column (30 m × 320 µm × 0.25 µm, Restek, Bad Homburg, Germany) using a temperature programme of 60 °C (held 1 min) to 150 °C at 15 °C min^{-1} and then to 320 °C at 4 °C min^{-1} (held 22.5 min).

Calculation and statistical analyses

The ^{13}C data were fitted to a 2-pool exponential degradation model according to Qualls and Haines (1992).

$$CO_2(t) = 100 - \left[(a)e^{-k_1 t} + (100 - a)e^{-k_2 t}\right] \quad (5.1)$$

where $CO_2(t)$ is the percentage of C mineralized to CO_2 at time t; *a* is the percentage of the readily degrading C pool; k_1 and k_2 are the degradation rate constants of the readily and slowly degrading pools, respectively. The parameters *a*, k_1 and k_2 were estimated by a non-linear least squares Levenberg-Marquardt algorithm using Sigma Plot 12.0.

The decomposition of SOM in labelled BGR-amended reactors was calculated by the Eq. (5.2) suggested by Kuzyakov and Bol (2006).

$$C_{SOM} = \frac{C_t \times (\delta_t - \delta_{BGR})}{(\delta_S - \delta_{BGR})} \quad (5.2)$$

where C_t is the carbon of total CO_2; δ_t, δ_{BGR} and δ_S are the $\delta^{13}C$ values of CO_2, labelled BGRs and soil, respectively. The priming effects (PE) were calculated from the equation below

$$PE = C_{SOM}^{Soil+BGR} - C_{SOM}^{Soil} \quad (5.3)$$

where $C_{SOM}^{Soil+BGR}$ and C_{SOM}^{Soil} are the amounts of SOM-derived C–CO_2 from soils amended and not amended with BGRs, respectively (Kuzyakov and Bol, 2006).

All the analyses were performed in triplicate and significant differences were determined by ANOVA. Rates of CO2 production in control, unlabelled and labelled BGR amended reactors were compared by a Tukey test. All statistical analyses were performed using SPSS 21. The results are given as mean±standard deviation.

Results

Production of the labelled BGRs using $KHCO_3$

The enrichment of ^{13}C of both CH_4 and CO_2 increased with time in labelled reactors during the 28 days of anaerobic digestion in the presence of $KH^{13}CO^3$ (Fig. 5.1). At the end of incubation, the isotopic compositions of CH_4 and CO_2 were 10.07±0.20 atom% ^{13}C and 33.67±0.39 atom% ^{13}C, respectively. Both gases were thus highly enriched in ^{13}C.

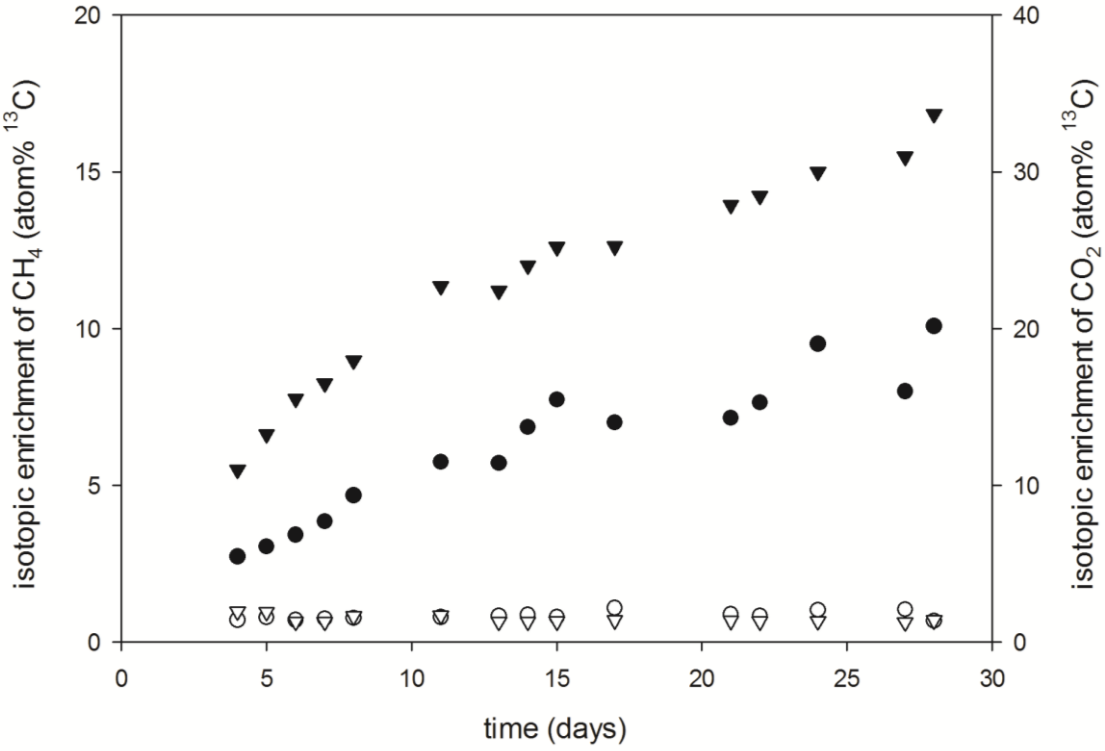

Fig. 5.1 Isotopic enrichment of ^{13}C in biogas after amendment of labelled (filled symbols) or unlabeled (empty symbols) $KHCO_3$. Circles and triangles represent isotopic enrichment in CH_4 and CO_2, respectively.

The isotopic compositions of individual PLFA in the labelled BGRs are given in Fig. 5.2. Straight-chain and branched saturated PLFAs were highly enriched in ^{13}C, whereas the enrichments in mono and polyunsaturated PLFAs were lower but significant (except 16:1ω7). The PLFA 16:1ω7 had the highest enrichment of 6.11±2.90 atom% ^{13}C. The relative ^{13}C label distributions in the PLFAs after accounting for the concentrations of the individual PLFAs are presented in Fig. 5.3. Around 58%, 13% and 5% of the ^{13}C was incorporated into G^+, G^- and fungal biomarkers, respectively. About a quarter of the label was detected in general biomarkers.

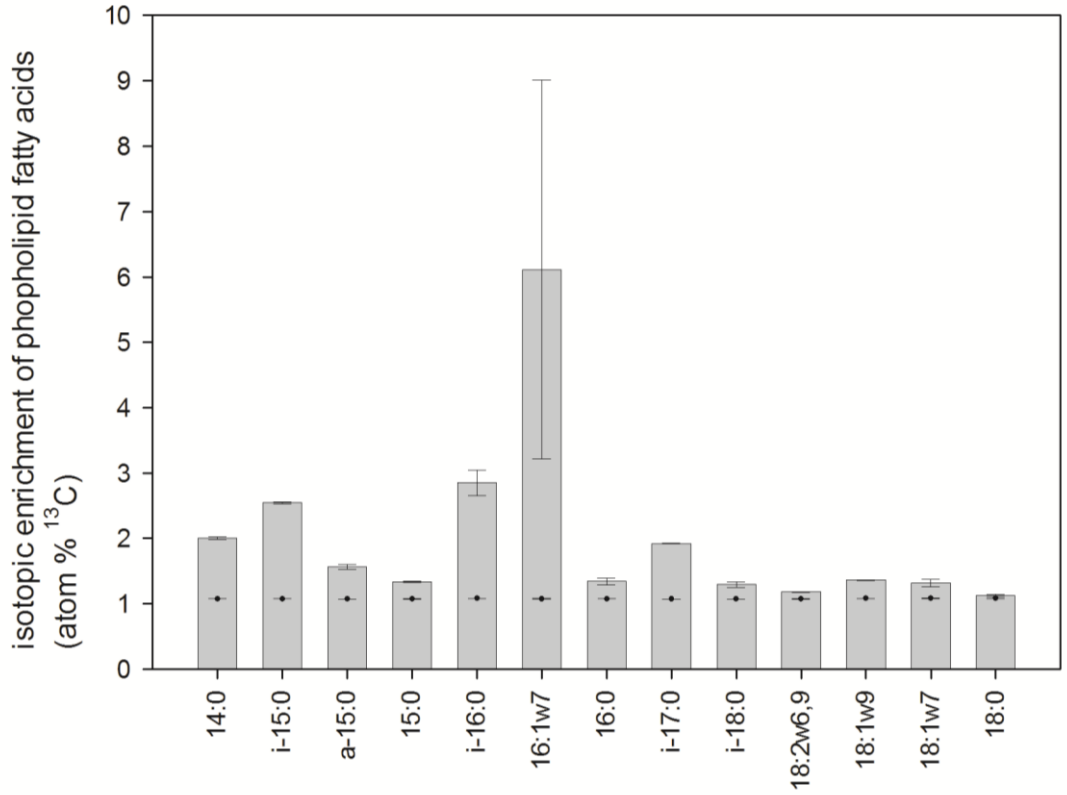

Fig. 5.2 ^{13}C enrichment in phospholipid fatty acid (PLFA) groups as a result of labelling with $KHCO_3$ represented by bars. Symbols indicate the natural isotopic composition of the individual PLFA groups in biogas residues which were produced using unlabelled $KHCO_3$. Error bars indicate standard deviation of replicate samples.

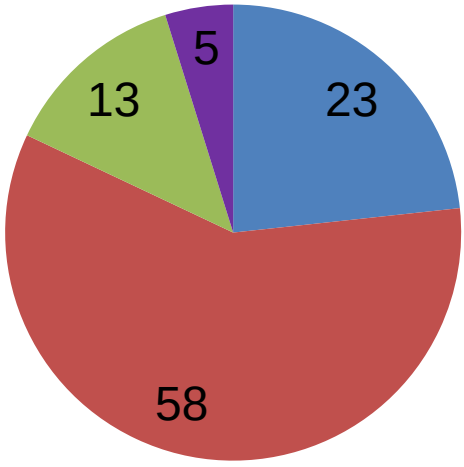

Fig. 5.3 Relative ^{13}C label distribution into general (■), Gram-positive (■), Gram-negative (■) and fungal (■) biomarkers in biogas residues prior to incubation with soil. The total ^{13}C in all microbial phospholipid fatty acids corresponds to 100%.

Similarly to PLFA, strong enrichment in ^{13}C of up to 4.25±0.12 atom% was detected in archaeal lipids (Fig. 5.4). Enrichment was higher in phytane compared to acyclic biphytane, which are derived from intact polar diether and tetraether lipids, respectively. Furthermore, isotopic enrichment was higher in the phospholipid fraction than in the glycolipid fraction.

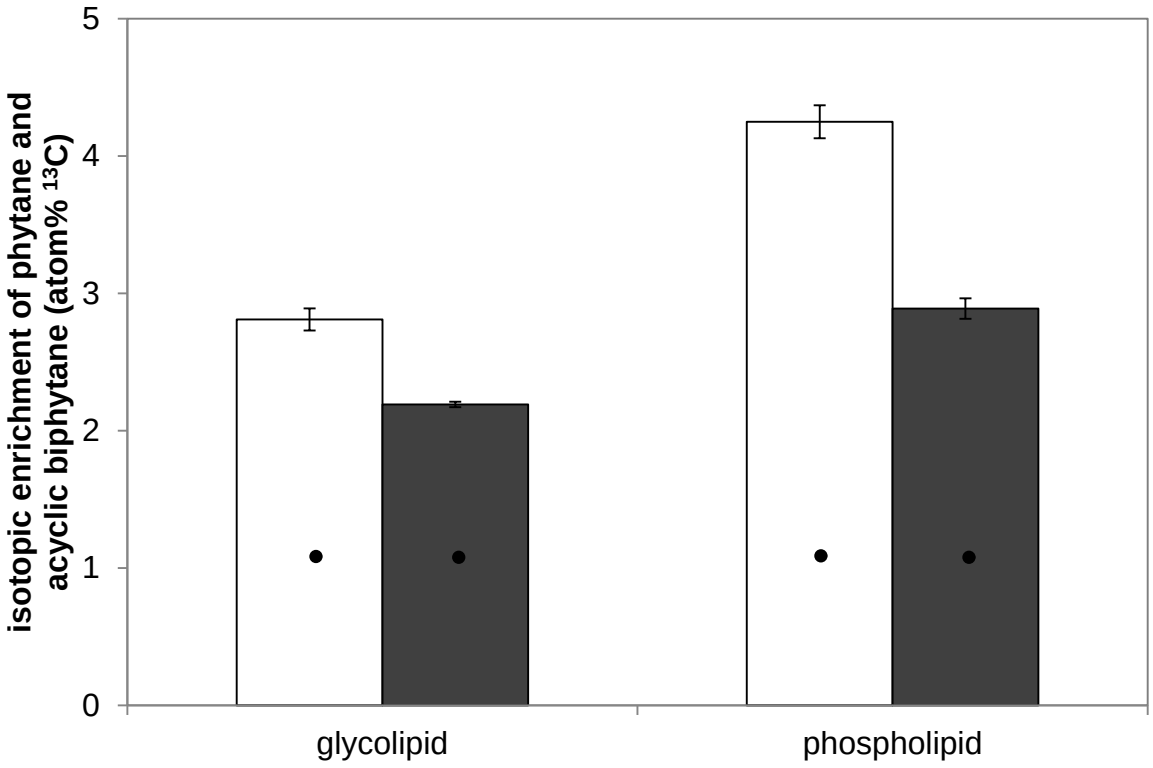

Fig. 5.4 ^{13}C enrichment in archaeal lipids as a result of labelling with $KH^{13}CO_3$. Enrichment in phytane and acyclic biphytane was represented by open and filled bars, respectively. The natural isotopic composition of the archaeal lipids in biogas residues produced in the reactors amended with unlabelled $KHCO_3$ was represented by (●) symbol. Error bars indicate standard deviation of replicate samples.

The BGRs were fumigated in a desiccator with 6M HCl in order to remove residual carbonates as recommended for correct interpretation (Harris et al., 2001). The contents and their isotopic compositions of total C and organic C in labelled BGRs are given in Table 5.2. There was no significant difference between the amounts of total and organic C of BGRs ($p>0.05$). However, the pronounced difference in isotopic compositions between total and organic C indicates that very little but highly enriched inorganic C is left after fumigation. Assuming an isotopic composition of the inorganic C in the labelled BGR similar to the one of the CO_2 in the biogas (33 at% ^{13}C), we estimate the inorganic C to be in the range of 2 mg g^{-1}, which is within the range of variation of the analysis of total and organic C.

Table 5.2 Carbon (C) content of labelled biogas residues after fumigation to remove carbonates.

carbon content (g kg^{-1})		isotopic composition of ^{13}C (atom%)	
total C	**organic C**	**total C**	**organic C**
229.9±24.6	249.8±6.5	1.64±0.08	1.38±0.01

Soil incubation experiment with BGRs

The CO_2 production rates are given in Fig. 5.5. All reactors showed a peak in respiration at the beginning of the experiment due to mixing and this peak lasted for about 70 days. After that time, about 0.13 mmol CO_2 kg^{-1} soil d^{-1} was produced by each reactor irrespective of the BGR amendment. BGRs significantly stimulated CO_2 production rates (i.e. they caused a priming effect) within the first week of the experiment compared to the control reactor without BGR additions ($p<0.001$). During this period, about 4.8 times more CO_2 was generated in the BGR-amended reactors than in control reactors. CO_2 production rates of soils amended with labelled and unlabelled BGRs were not statistically different ($p>0.05$).The impact of BGR decreased after seven days and no significant difference was observed between labelled, unlabelled and non-amended treatments from day 21 ($p>0.05$).

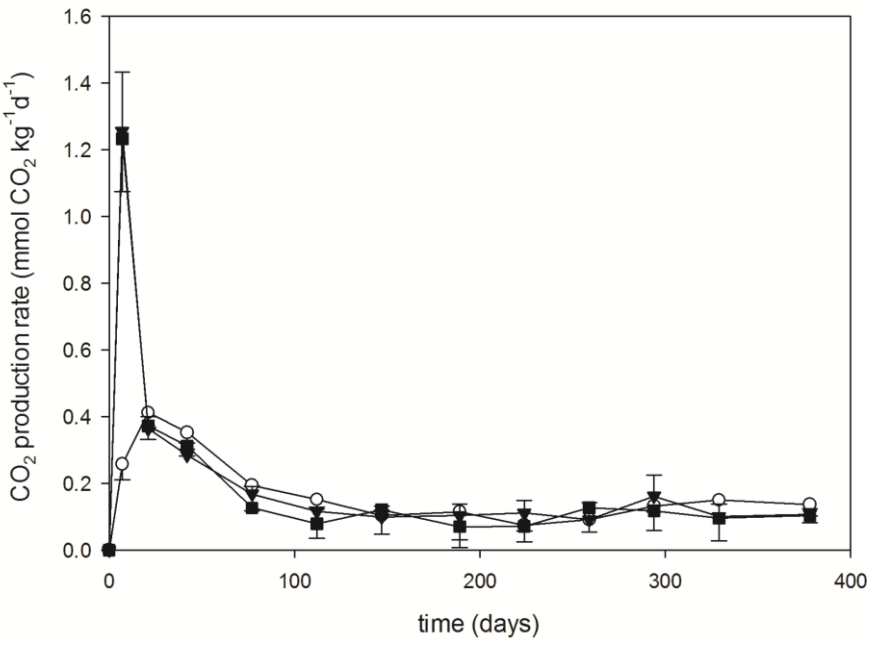

Fig. 5.5 CO_2 production rates of unamended soil (-○-); soil amended with unlabelled biogas residues (-■-) and soil amended with labelled biogas residues (-▼-). Error bars indicate standard deviation.

In order to track the fate of organic C, residual inorganic C was subtracted from CO_2 prior to the mass balance calculation. Inorganic C was released as CO_2 until the first sampling time point, as no inorganic C was found in soil after 7 days (Table 5.3). Therefore measurements of inorganic C were discontinued after day 42.

Table 5.3 Total and organic carbon (C) in the soil amended with biogas residues (BGRs).

Days	Total C derived from BGRs (mmol ^{13}C kg^{-1})	Organic C derived from BGRs (mmol ^{13}C kg^{-1})	Difference
0	0.447±0.030	0.316±0.043	Significant ($p<0.05$)
7	0.163±0.073	0.136±0.085	N.S.[a]
21	0.112±0.071	0.107±0.083	N.S.[a]
42	0.135±0.071	0.117±0.065	N.S.[a]

[a] not statistically significant ($p>0.05$)

The fate of microbial BGR-C (corrected for inorganic C contribution) is presented in Fig. 5.6. The amount of ^{13}C in soil decreased to 43% of the initial value after seven days. There were no statistically significant changes in the ^{13}C remaining in soil after that time ($p>0.05$). A similar trend was observed in terms of mineralization to CO_2. 40% of the initial C was captured in CO_2 traps after seven days. Mineralization continued until the end of the experiment to a final value of up to around 65%. The mean recovery was calculated as 93% of the initial value, taking the total detected ^{13}C in CO_2 and remaining in soil into consideration. The recovery was constant during the experiment ($p>0.05$).

Mineralization data was fitted to the two-pool model ($R^2 = 0.99$) based on Eq. (5.1) resulting in Eq. (5.4)

$$CO_2(t) = 100 - (51.43e^{-0.2005t} + 48.57e^{-0.0010t}) \quad (5.4)$$

The model thus estimates the fractions of the readily and slowly degrading C pools to be 51.43% and 48.57%, respectively. The degradation rate of the readily degrading pool was 0.2005 d^{-1}, which corresponds to a half-life of about three days. At the second stage (after about 15 days), the stable pool was dominant and the degradation rate was 0.0010 d^{-1}, which corresponds to a half-life of 1.9 years.

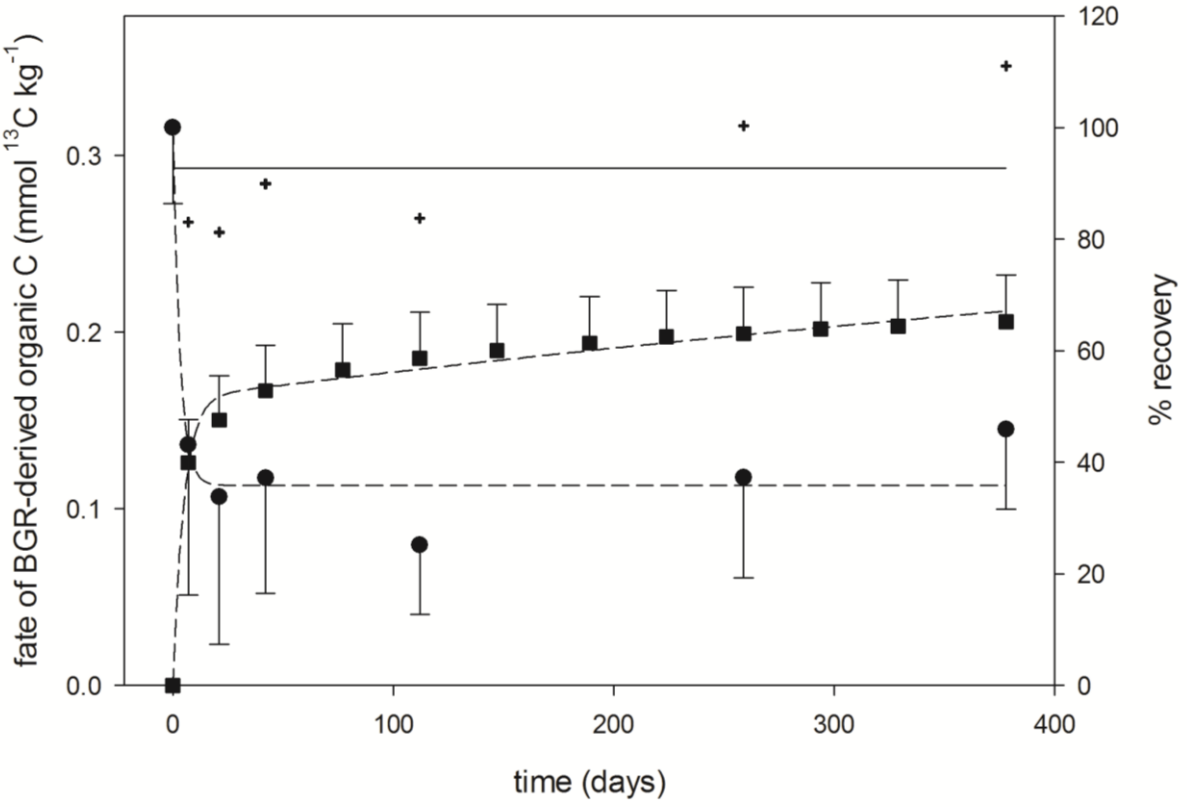

Fig. 5.6 ^{13}C in CO_2 (■) and remaining in soil (○). Symbols represent measured concentrations; the dashed line represents the fitted model. The horizontal solid line shows the average of calculated recoveries for each sampling point (+). Error bars indicating standard deviation of replicate samples were plotted in one direction only in order to avoid overlapping.

Stimulation of SOM mineralization with the BGR amendment / Priming effect

The priming effect was calculated for the first 7 days, when the CO_2 production was significantly stimulated by BGR-amendment. During this time 8.79±1.26 mmol CO_2 kg^{-1} was produced in the labelled BGR-amended reactors, from which 6.65±0.93 mmol CO_2 kg^{-1} was produced via SOM mineralization as calculated by Eq. (5.2). At the same point, unamended soil reactors produced only 1.80±0.32 mmol CO_2 kg^{-1}, showing that 73% of the SOM-derived CO_2 produced in BGR-amended reactors occurred due to the priming amounting to 4.85±0.98 mmol C kg^{-1}.

Discussion

In the present study, we labelled microorganisms and their residues in the BGRs by adding $KH^{13}CO_3$ to the biogas reactor. By labelling BGRs, we were able to trace their fate in soil for a period of 378 days using a mass balance approach. This allowed us to estimate the priming

effect, contribution to SOM and CO_2, and also to determine the degradation rates of readily and slowly degrading C pools in the BGRs.

Labelling of BGRs using isotopically labelled $KH^{13}CO_3$

The effectiveness of the labelling approach could be estimated from the enrichment in ^{13}C of the produced biogas. Microorganisms utilized $KHCO_3$ together with the manure C and produced labelled CH_4. We can presume that both methanogenic archaea and bacteria consumed the carbonate efficiently based on the ^{13}C enrichment in CH_4 and CO_2. However, CO_2 could potentially be labelled due to abiotic release directly from residual labelled carbonate. Labelled CH_4, however, may only be produced by biological activity of methanogens (Ferry, 1993). Autotrophic, likely methanogenic, archaeal activity is additionally indicated by the strong label incorporation into phytane and acyclic biphytane derived from archaeal diether- and tetraether lipids, which are typical for methanogenic archaea (Koga et al., 1993). This confirms that successful labelling of the archaeal biomass and its residues in BGRs was accomplished by the approach applied here.

In order to test for potential ^{13}C enrichment of bacteria in BGRs, we analysed ^{13}C-enrichment in phospholipid fatty acids (PLFAs). PLFAs are considered to originate only from living cells and are not found in dead cells or storage products (Zelles, 1999). Thus, they may serve as indicators of living biomass. The enrichment in PLFA groups in the BGRs showed that mainly branched saturated and monounsaturated PLFAs were enriched in ^{13}C (Fig. 5.2). Branched saturated fatty acids are indicators of G^+ bacteria and these bacteria contained the highest amount of label in our study (Fig. 5.3). High enrichment in PLFA 16:1ω7 might be an indicator of anaerobic oxidation of methane because it was found that CH_4 was utilized as C source by microorganisms harboring this PLFA (Bannert et al., 2012). However, the relative contribution of this PLFA was much lower than that of the G^+ PLFA biomarkers (Fig. 5.3). The fungal biomarker, polyunsaturated fatty acid, were slightly but significantly enriched. The role of fungi is probably not critical in the anaerobic digestion of manure (Toerien and Hattingh, 1969). Anaerobic digestion is commonly described as a process driven by *Bacteria* hydrolysing organic C into acetate, syntrophic acetate oxidizers producing CO_2 and H_2, and methanogenic *Archaea* producing methane from these intermediate products (Ferry, 1993; Zinder and Koch, 1984). Instead, enrichment of fungal fatty acids might be related to cross-feeding on labelled microbial biomass. In sum, enrichment of ^{13}C in archaeal, bacterial and fungal biomarkers showed that labelling of microbial biomass was successful.

Fate of BGR carbon derived from anaerobic microorganisms

BGRs induced CO_2 release within seven days of application to soil. The BGRs may have stimulated the soil microbes by supplying labile organic C and N. Similarly, Chen et al. (2012) found that BGR treatment increased CO_2 emissions significantly during the first two days of the treatment. Biodegradability tests also showed that maximum respirometric activity of various BGRs occurred within the first 24 hours (Alburquerque et al., 2012). It is therefore likely that biodegradation of BGRs and inorganic C release to CO_2 emissions is a short-term process.

We determined the mass balance of the ^{13}C derived from BGRs and fitted the data to a two-pool model suggesting that BGRs consisted of a readily degrading fraction and a slowly degrading fraction (Qualls and Haines, 1992). The readily degrading fraction comprised about 51% of the BGRs with a half-life of 3 days indicating the decay of readily degradable compounds. It is likely that an important fraction of BGRs is comprised of archaeal methanogens and anaerobic bacterial biomass. The high enrichment of methane via carbonate labelling indicates that methanogens, especially hydrogenotrophic methanogens, were abundant in the BGRs (Borja et al., 1996; Zinder and Koch, 1984). Concurrent label incorporation into diether- and tetraether-derived phytane and acyclic biphytane further supports the occurrence of autotrophic methanogens in the BGRs. Cell lysis due to the aerobic conditions in the soil (Loesche, 1969) might explain the very short half-life of the BGR-derived C. Similarly, G^+ bacterial biomass may have also contributed to the readily degradable C pool as 58% of the label in PLFAs was detected in G^+ bacterial biomarkers. In contrast, biomarkers for G^- bacteria contained only 13% of the label and the calculated half-lives of the BGRs were considerably shorter than the half-life of G^- bacterial biomass of 7 days (Kindler et al., 2006).

The fraction of slowly degradable C is around 49% with a half-life of 1.9 years according to the two-pool model. The stable components of the BGRs could be composed of stabilized cell debris or C which was assimilated by other microorganisms in the microbial food web as suggested by Kindler et al. (2006). However, plant residues in BGRs cannot explain the stable C, because these residues are not labelled and thus do not contribute to $^{13}CO_2$ production. The significant contribution of the slowly degrading pool with a relatively long half-life shows that BGRs could contribute to mid-term C storage in soil. Interestingly, the half-life of the slowly degrading C pool in BGRs was similar to one of the G^- C which has been reported to be 1.3 years (Kindler et al., 2006). Sewage sludge-cotton waste as a soil

amendment has a stable C half-life of around 127 days ($k = 0.0055\ d^{-1}$) which could be prolonged by composting (Bernal et al., 1998). To obtain the highest benefits from BGR recycling in soil, stable organic matter is required (Alburquerque et al., 2012). Highly unstable fertilizers do not contribute to C storage in soils and have leaching potential due to nutrient release. They can cause undesired immediate CO_2 emissions (Marstorp, 1996; Paustian et al., 1997). Thus, a balance between readily degradable and stable C source in soil amendments is advantageous. As they consist of almost equal proportions of readily and slowly degradable fractions, BGRs meet this criterion. Given their capacity to replenish organic matter removed from arable soils by extensive plant production, BGRs could play an important role in sustainable agriculture and mitigate the impacts of increased mineralization associated with global climate change (Holm-Nielsen et al., 2009; Lal, 2004).

Hitherto reported C mineralization after manure, compost and maize BGR amendments was 31%, 11% and 6.4% of the initial amount, respectively (Chen et al., 2012; Hartz et al., 2000). We report much higher mineralization of C since we tracked the fate of microorganism-derived C. As microbial biomass could contribute as much as 50% to SOM depending on soil type (Simpson et al., 2007), further stabilization of microbial-derived BGRs would be beneficial. In order to improve stability and storage of C in soil, co-application of BGRs together with more stable soil additives such as manure and compost should be investigated.

The priming of C, i.e. the intensive mineralization of soil-derived organic matter with the application of fresh matter inputs, is of both scientific and practical importance (Broadbent, 1948; Kuzyakov et al., 2000). High priming effect equals high additional CO_2 production from the native soil in addition to the respiration of the amendment as well as SOM loss and may lead to soil degradation (Bingeman et al., 1953). Mineral N fertilizers and readily decomposable organic substances were reported to result in priming via acceleration of SOM mineralization and/or N immobilization (Kuzyakov et al., 2000). For example, 41-45% of CO_2 (more than 70% of the soil-derived C) was produced due to the increased mineralization of the native soil C after slurry amendment (Bol et al., 2003). Fresh organic matter input was found to stimulate microbial decomposition of SOM that was stabilized for as long as 2500 years (Fontaine et al., 2007). Priming is commonly considered to be a short-term process that occurs immediately after addition of the amendment to soil (Dalenberg and Jager, 1981; Kuzyakov et al., 2000; Pascual et al., 1998). It is a very important concern as it may cause a decline in SOM stocks. Therefore, we calculated the priming for the first sampling point after seven days and found that 73% of the SOM-derived CO_2 release after BGR-amendment was

due to the priming effect. However, the mineralization of unlabelled components of BGRs e.g. undigested plant residues was ignored. Instead, our estimate assumes that the label is representative for the bulk C of the BGR. With our approach, however, we were able to specifically label the microbial residues in the BGR, which can be assumed to be mineralized more rapidly than the plant residues remaining in the BGR. If such an unlabelled, slowly degrading fraction were present in the BGR, our calculations would slightly overestimate the contribution of BGR-derived C to the total CO_2 production and thus underestimate the contribution of SOM-derived CO_2. The priming effect therefore may be slightly larger than we estimated. Our results thus indicate that extensive and continuous application of BGRs should be avoided.

This study provides information on the mineralization of organic C derived from BGRs as well as the methodology for tracing the fate of microbial C rather than inorganic C. The applied method may be useful for studying the fate of organic C associated with microbial biomass. The fate of inorganic C and total organic C (including stable undigested plant residues) in BGRs have been studied recently (Chen et al., 2012; Chen et al., 2011), and this paper complements these studies by unravelling the fate of microbial C in the BGRs after BGR addition to soil.

Conclusions

The proposed method of labelling biogas residues via $KH^{13}CO_3$ may prove useful for studying the contribution of biogas residues to soil organic matter, notwithstanding its disadvantages such as low enrichment of organic carbon and highly enriched residual carbonates. To increase the precision of the approach, the fumigation method should be optimized or another method applied to remove all enriched carbonates prior to amendment. Our model shows that around half of the biogas residues are readily degradable while the other half are relatively stable. The stable portion could contribute to living and non-living SOM which may be vital for mid-term carbon storage in soils. Since priming effects were detected, intensive and continuous application of biogas residues should be avoided. This will most probably not result in the desired increase in SOM contents of the amended soils. Some benefits could be achieved by applying biogas residues in combination with other stability-enhancing substances such as compost or biochar. Further field studies are needed to examine the impact of biogas residues on soil under natural climatic conditions.

Acknowledgements

The present study was funded by the Helmholtz Association of German Research Centres within the initiative and networking fund for the Department of Bioenergy (UFZ) and supported by the HIGRADE graduate school. The authors thank Ellen Kandeler (University of Hohenheim), Juergen Proeter (German Biomass Reseach Centre - DBFZ), Sabine Kleinsteuber (UFZ) and Heike Streuber (UFZ) for fruitful discussions, Harald Wedwitschka (DBFZ) for inoculum and manure supply, J. Wendt and X. Prieto (MARUM) for their assistance with the ether lipid analyses, Ursula Guenther (UFZ) for help with the isotope analyses, and Marc Pages Gallego (Rovira i Virgili University) and Neeraja Venkataraman (University of Bayreuth) for assistance in the laboratory. We are also grateful to two anonymous reviewers for the constructive suggestions which improved the quality of the manuscript.

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6. Fate of fatty acids derived from biogas residues in arable soil

Submitted to Soil Biology & Biochemistry

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Abstract

Biogas residues are rich in microbial biomass and contribute to organic matter formation when applied to soils. Here we present a detailed analysis of the fatty acids derived from biogas residues in arable soil. We applied a differential approach using phospholipid fatty acids and total fatty acids to evaluate the carbon dynamics in living biomass and non-living soil organic matter. Biogas residue addition increased the microbial biomass in soil. The sum of ^{13}C-labelled phospholipid fatty acids decreased to ~ 60% during incubation whereas the decrease of t-FA was higher (to 33%). Compound-specific fatty acid analysis showed fatty acid specific incorporation or loss of ^{13}C. Overall, microbial biomass in biogas residues may be a significant contributor to soil organic matter formation.

Introduction

Soil organic matter (SOM) is the largest active carbon (C) pool in the terrestrial environment (Jobbágy and Jackson, 2000). Carbon stored in soil has been estimated at 1115-2200 Pg C (Batjes, 1992) and soil respiration was calculated to average 68±4 Pg C a^{-1} based on extrapolations from terrestrial biome areas (Raich and Schlesinger, 1992). This flux contributes to the recycling of C from gross primary productivity of 100-120 Pg C a^{-1} and is similar to the net primary productivity of 50-60 Pg C a^{-1} (Houghton and Woodwell, 1989). SOM also determines soil fertility (Krull et al., 2003) by contributing to microbial activity (Joergensen et al., 1990), crop development, and soil physical properties (Six et al., 2002).

However, loss of SOM has recently been reported to occur in several regions of the world due to changes in land use and soil management (Sleutel et al., 2003; Wang et al., 2003) and climate change (Bellamy et al., 2005), and there is a concern that it might be a global phenomenon (Schulze and Freibauer, 2005). In addition, complete removal of above-ground biomass in agricultural soils has long been known to decrease the level of SOM (Barber, 1979). This means that growing bioenergy crops may deplete SOM stocks due to higher biomass removal, especially for maize cropping (Blanco-Canqui and Lal, 2007). One possible way of mitigating the loss of SOM is by applying fresh organic matter amendments that contribute to the formation of SOM. Application of biogas residues to arable soils, especially those used for growing bioenergy crops, can potentially compensate carbon loss.

Biogas residues (BGRs) may serve as a new type of organic fertilizer resulting from the development of the biogas sector. They are residual products of anaerobic digestion, and their application to soil could close the global element cycling and indirectly reduce greenhouse gas emissions (Arthurson, 2009). BGRs are potentially sustainable alternatives to mineral fertilizers, which contribute to the nutrient cycle and require energy input for production and distribution on the field.

Plant litter is the major source of SOM formation (Kögel-Knabner, 2002) but microbial assimilation of easily degradable plant litter occurs prior to SOM formation (Fließbach et al., 2000; Pelz et al., 2005) so that microbial biomass contributes significantly to SOM formation (Miltner et al., 2012). The contribution of microbial biomass can be as high as 50% of the extractable soil organic matter fractions (Simpson et al., 2007). Other authors even report that up to 80 % of the SOM are of microbial origin (Liang and Balser, 2011). Given that, BGRs along with their microbial biomass residues from the fermentation process (necromass) could

make a significant contribution to SOM formation, it is essential that the potential for SOM formation or loss due to BGR-amendment be investigated in detail. Recently, Coban et al. (2015) quantified the mineralization of ^{13}C-labelled microbial biomass from BGRs to be about 65% after one year of incubation in soil and the remaining amount was found to contribute to SOM formation. However, the contribution of BGR-biomass to the soil microbial biomass and non-living SOM was not evaluated. Tracing isotopically labelled carbon in phospholipid fatty acids (PLFAs) and total fatty acids (t-FAs) can explain the fate of carbon derived from bacteria (Kindler et al., 2009). PLFAs are only found in membranes of living cells (Pinkart et al., 2002) and do not exist in storage products (Zelles, 1999); t-FAs, in contrast, also occur in dead cells. PLFAs can also be used for the estimation of biomass (Frostegård and Bååth, 1996). Additionally, microbial groups have specific PLFA compositions thus PLFAs are used as useful biomarkers in ecological studies (Boschker and Middelburg, 2002). In order to determine which organisms use a substance as a carbon source, PLFA analysis with stable isotope labelling is combined (Boschker et al., 1998).

In the present study we evaluated the contribution of microbial biomass derived from BGRs to the soil microbial biomass and non-living SOM. The fate of ^{13}C-labelled fatty acids was determined and the partitioning into PLFAs and t-FAs was applied.

Materials and Methods

Chemicals

All chemicals were obtained from ThGeyer (Germany) at the highest purity grade available, unless stated otherwise. Isotopically labelled $KH^{13}CO_3$ (98 atom%) was obtained from Campro Scientific (Germany).

Labelling microbial biomass in BGRs

The method for labelling the BGRs and the setup of the incubation experiment have been described previously (Coban et al., 2015). Briefly, 98 atom% ^{13}C labelled $KH^{13}CO_3$ amended manure was used as the substrate in a biogas reactor in order to label the microbial biomass in BGRs. The isotopic compositions of methane and CO_2 produced in biogas reactors were 10.07 ± 0.20 atom% ^{13}C and 33.67 ± 0.39 atom% ^{13}C, respectively. In addition, both bacterial PLFAs and archaeal phospholipid ether lipids (phytane) were found to be labelled; they showed an average enrichment of 1.69 atom% ^{13}C and 4.25 atom% ^{13}C, respectively, after 28 days of anaerobic digestion. These results indicated that autotrophic methanogenic bacteria

and archaea were labelled by this approach. Approx. 58%, 23%, 13% and 5% of the ^{13}C was found to be incorporated into Gram-positive, general, Gram-negative and fungal biomarkers, respectively (Coban et al., 2015). Unlabelled BGRs were also produced similarly using unlabelled $KHCO_3$. The BGRs contained approx. 1980±890 µmol PLFAs kg^{-1}.The BGRs were fumigated in a desiccator with 6M HCl for four days in order to remove the highly labelled $KHCO_3$ which might have been left over. Characteristics of the BGRs are given in Table 6.1.

Soil

A sample Haplic Chernozem with a loamy texture was taken from the long-term agricultural "Static Fertilization Experiment" site in Bad Lauchstädt (51°24'N, 11°53'E, Germany). The soil has been cultivated with a crop rotation of sugar beet, spring barley, potato and winter wheat and fertilized with 60 kg P ha^{-1} and 230 kg K ha^{-1} since 1902 (Körschens et al., 1990). The characteristics of the soil are given in Table 6.1.

Table 6.1 Characteristics of soil and biogas residues (BGRs) used in the study (Coban et al., 2015).

	C (g kg^{-1})	**N (g kg^{-1})**	**C/N**	**pH**
Soil	17.9±1.4	1.6±0.1	11	6.2
Labelled BGRs	229.9±24.6	19.6±3.0	12	7.0
Unlabelled BGRs	225.3±13.5	19.3±0.1	12	7.0

The labelled BGRs were applied to the soil at a level of 0.066 g N kg^{-1} soil, which corresponds to 8.61 µg ^{13}C g^{-1} soil or 5% of the ^{13}C naturally present in the soil. Approx. 5100±1460 pmol PLFAs g^{-1} soil was added to soil via BGRs in this application rate. Soil incubation was performed at 20 °C in closed reactor systems as described previously (Kindler et al., 2006). Briefly, 1.2 kg of sieved (2 mm) and homogenized soil was filled into glass reactors (4.3l volume, 10 cm diameter, 55 cm height) after mixing with either unlabelled or labelled BGRs. In addition, control reactors with unamended soil were included. The water content of the soil was adjusted to 50% of its water holding capacity. During incubation for 378 days, the soil was aerated with humidified air at a flow rate of 30 ml/min in a closed circuit. The system included flexible gas bags providing O_2 in dependence of CO_2 production. Soil samples for PLFA and t-FA analyses were taken on days 0, 7, 21, 42, 112, 259 and 378.

Samples were frozen immediately after sampling without drying and stored at -20°C until analysis. The first soil sampling for FA extraction was carried out shortly (<20 min.) after mixing the soil with biogas residues.

Extraction and analysis of PLFAs and t-FAs

PLFAs were extracted from the soil using a phosphate buffer/methanol/chloroform mixture according to Bligh and Dyer (1959) and separated from other lipids in the extracts by column chromatography over silica gel (Unisil, Clarkson Chromatography Products, South Williamsport, PA). The extracted PLFAs were methylated with methanol and trimethylchlorosilane (9:1, v:v) according to Thiel et al. (2001). t-FAs were methylated with the same reagent directly in the soil sample, extracted from the soil matrix with diethyl ether and purified by passage over silica gel (Mallinckrodt Baker Germany, Griesheim, Germany). Analysis of methylated PLFAs and t-FAs was performed as described previously (Miltner et al., 2004). Concentrations of PLFAs and t-FAs were determined using a 7890A gas chromatograph (GC; Agilent Technologies, Santa Clara, USA) equipped with 5975C inert XL mass spectrometer (MS) triple axis detector (Agilent Technologies, Santa Clara, USA) and a HP-5MS column (30 m × 0.25 mm × 0.25 µm; Agilent, Santa Clara, USA). One µl aliquots were injected in splitless mode with Helium tracer gas flowing at 2.5 ml min^{-1}. The temperature program was as follows: initial temperature 45 °C (hold for 2.25 min) then heat to 300 °C (3 min) at 40 °C min^{-1}. Isotopic enrichment in the fatty acid methyl esters was determined using Finnigan MAT 252 isotope ratio mass spectrometer (IRMS) coupled to a HP 6890 Plus gas chromatograph (GC) (Hewlett-Packard, Wilmington, USA) via a GC combustion interface (Finnigan, Bremen, Germany). A BPX-5 column (50 m x 0.32 mm x 0.5 µm) was used to separate the fatty acids (SGE, Darmstadt, Germany). The temperature program was as follows: initial temperature 70 °C (hold for 1 min), heat to 130 °C (0 min) at 20 °C min^{-1}, heat to 150 °C (5 min) at 2 °C min^{-1}, heat to 165 °C (5 min) at 2 °C min^{-1}, heat to 230 (0 min) at 2 °C min^{-1}, heat to 300 °C (5 min) at 20 °C min^{-1}. Isotope values were corrected for C introduced by methylation. Saturated branched PLFA and 10me 18:0 in particular for actinomycetes were regarded as biomarkers for Gram-positive bacteria, while monounsaturated PLFA and polyunsaturated PLFA were regarded as biomarkers for Gram-negative bacteria and fungi, respectively.

Calculations and statistics

The amount of background ^{13}C at natural abundance bound to fatty acids in the unamended soil was subtracted from the amount in soil amended with labelled BGRs to calculate the excess value derived from BGRs.

All the analyses were performed in triplicate and the results were presented as mean ± standard deviation. Significant differences between BGR-amended and unamended soils were determined with independent samples t-test.

Results

Impact of BGRs on the soil microbial community

The concentration and distribution of PLFAs in unamended soil and labelled BGR-amended soil at the beginning of the experiment is presented in Fig 6.1a. The addition of BGRs roughly doubled the concentrations of almost all of the PLFAs from 8,020±840 to 16,700±500 pmol/g soil. This increase in PLFAs ($p<0.05$) due to the amendment of BGRs was statistically significant. Since there is a direct correlation between microbial biomass and PLFAs, we can estimate a doubling of the microbial biomass, as well (Frostegård and Bååth, 1996). The significantly different PLFA concentrations between unamended soil and labelled BGR-amended soil was observed in saturated methyl branched PLFA (except 10 Me 18:0), some of monounsaturated PLFAs (18:1 cis and 18:1 trans), cy-17:0, 15:0 and 16:0 ($p<0.05$). However, the concentration of the PLFA 18:2 was similar in unamended and BGR-amended soils ($p>0.05$).

At the end of the experiment after 378 d, the PLFA concentrations were 14,650±1300 pmol/g soil (~85 % of the initial value) in BGR-amended soil and 10,300±615 pmol/g soil in the unamended soil. The difference between labelled BGR amended and unamended soils decreased until the end of experiment, compared to the beginning of the experiment, however was still significant for most of the individual PLFAs (Fig 6.1b): monounsaturated PLFAs, polyunsaturated PLFAs, and some of unsaturated straight-chain PLFAs ($p<0.05$). Interestingly, most of the saturated methyl branched PLFA concentrations in BGR-amended and unamended soil was not significantly different ($p>0.05$).

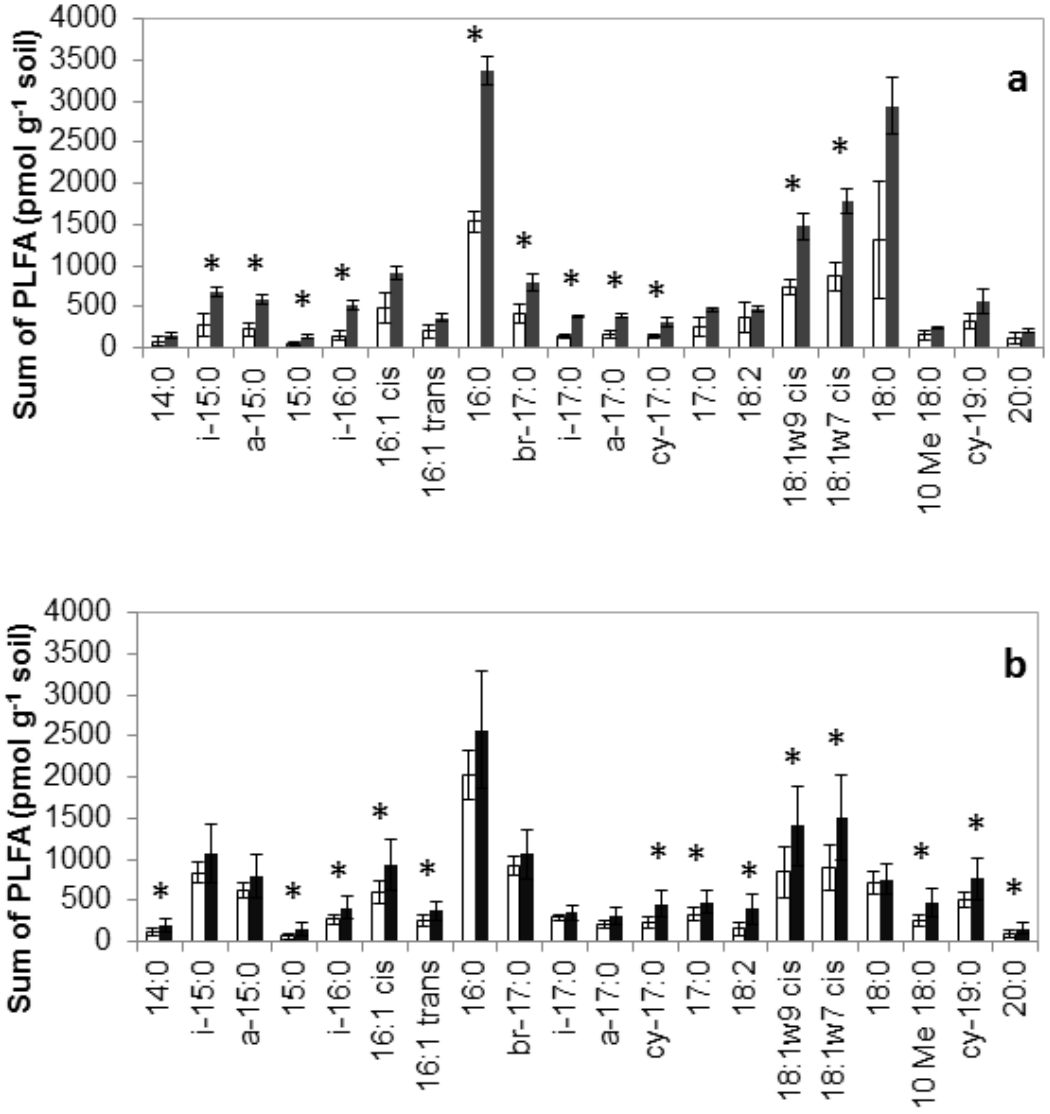

Fig. 6.1 Distribution of phospholipid fatty acids (PLFAs) at the beginning (a) and at the end (b) of the 378 days incubation. ■ = soil amended with biogas residues (BGRs), □ = unamended soil. Error bars indicate standard deviation of replicate samples and statistically significant differences between BGR-amended and unamended soil were represented by (*) sign.

Fate of labelled carbon in PLFAs

The amount of ^{13}C derived from labelled BGRs (excess) in the PLFAs tended to decrease from 1,800± 200 pmol ^{13}C g^{-1} soil during the experiment, to values of around 60% of the initial values (Fig. 6.2), which indicates metabolization of roughly 40 % of the label found in PLFAs initially, although we also have to consider that the standard deviations were very high. Due to this high variability there was no statistically difference between the PLFA concentrations at the beginning and end of the experiment, even though some of the time points differed from each other. In addition, the BGRs contained labelled archaeal biomass,

but the amount of archaeal lipids was below the detection limit in the soil-BGRs mixture and thus could not be quantified. Archaeal biomass is about half of the bacterial biomass in the BGRs (Tay et al., 2001) and has a three-fold average label level: archaeal lipids were found to be highly labelled with values of about 4.3 at% in comparison to 1.7 at% for bacterial PLFAs (Coban et al., 2015). With this assumption, we can estimate the amount of excess ^{13}C in archaeal biomass of the BGRs to be 150% (50% concentration × 300% label level = 150%) of that in bacterial biomass.

The shifts of the ^{13}C label within the PLFAs can be shown by the changes from the beginning to the end of the experiment (Fig. 6.3). Mainly, ^{13}C in saturated branched PLFAs e.g. i:15:0, a-15:0, i-16:0, i-17:0 and some of saturated straight-chain PLFAs e.g. 14:0. 15:0, 17:0, and 20:0 was mineralized and/or metabolized. On the other hand, incorporation was detected in some of the mono- and polyunsaturated PLFAs as well as in 10 Me 18:0 and to a smaller extent in 18:0. The label in cyclopropyl PLFAs 17:0 and 19:0 increased and decreased, respectively. No significant incorporation or loss was detected in PLFA 16:0.

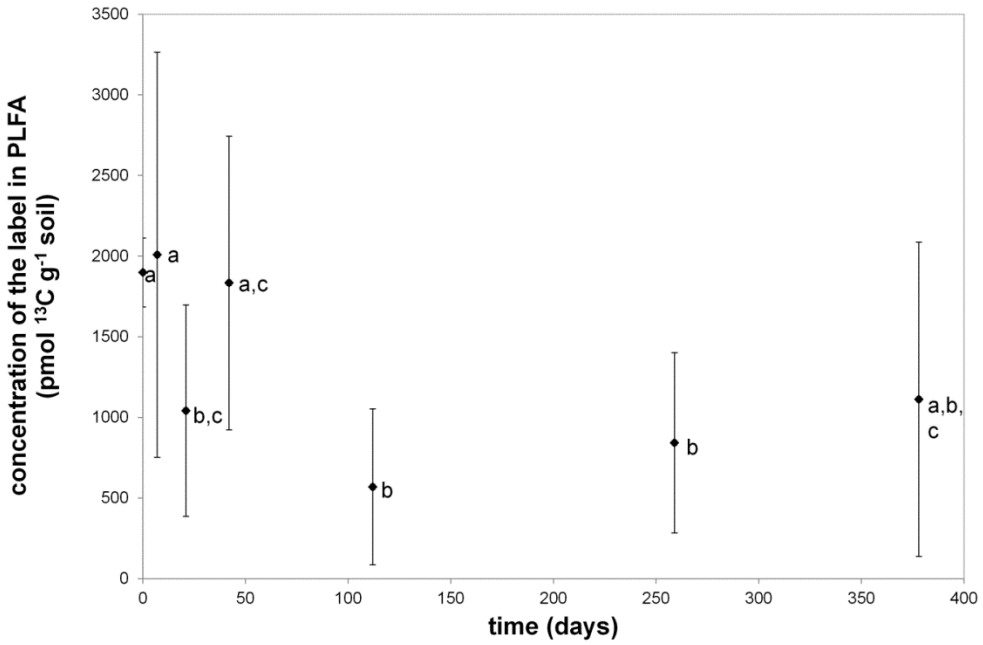

Fig. 6.2 Fate of ^{13}C derived from biogas residues in phospholipid fatty acids (PLFA) during 378 days of incubation. Error bars indicate standard deviation of replicate samples. Significant differences were indicated by different lowercase letters.

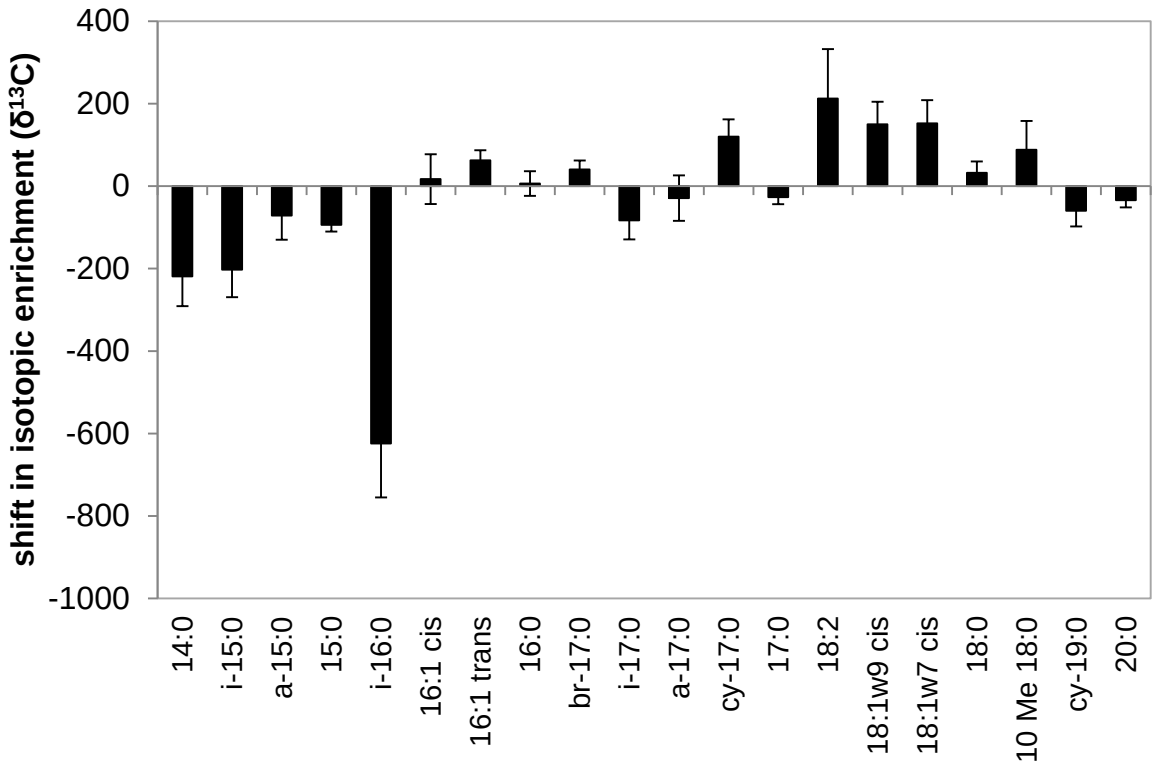

Fig. 6.3 Shift of the isotopic composition of PLFA until the end of incubation. Positive values indicate incorporation whereas negative values indicate loss. Error bars indicate standard deviation of replicate samples.

Fate of labelled carbon in t-FAs

Similar to PLFAs, the amount of ^{13}C (excess) derived from labelled BGRs in t-FAs decreased from 5,100±2,100 pmol ^{13}C g^{-1} soil during the experiment to around 33% of the initial amount (Fig. 6.4). However, this decrease was not statistically significant, and no statistically significant differences between any time points were observed ($p>0.05$). We can estimate that archaea may have contributed up to 8,000 pmol ^{13}C g^{-1} soil to t-FAs, if we make similar assumptions as for PLFAs.

Unlike in PLFAs, the isotopic enrichment of ^{13}C in t-FAs derived from labelled BGRs decreased in almost all fatty acids until the end of experiment. Only the FA 18:2 and 18:1w9 showed significant label acquisition (Fig. 6.5). Neither incorporation nor loss of t-FA was detected in a-15:0, 15:0, br-17:0, 18:1w7 cis and 18:0.

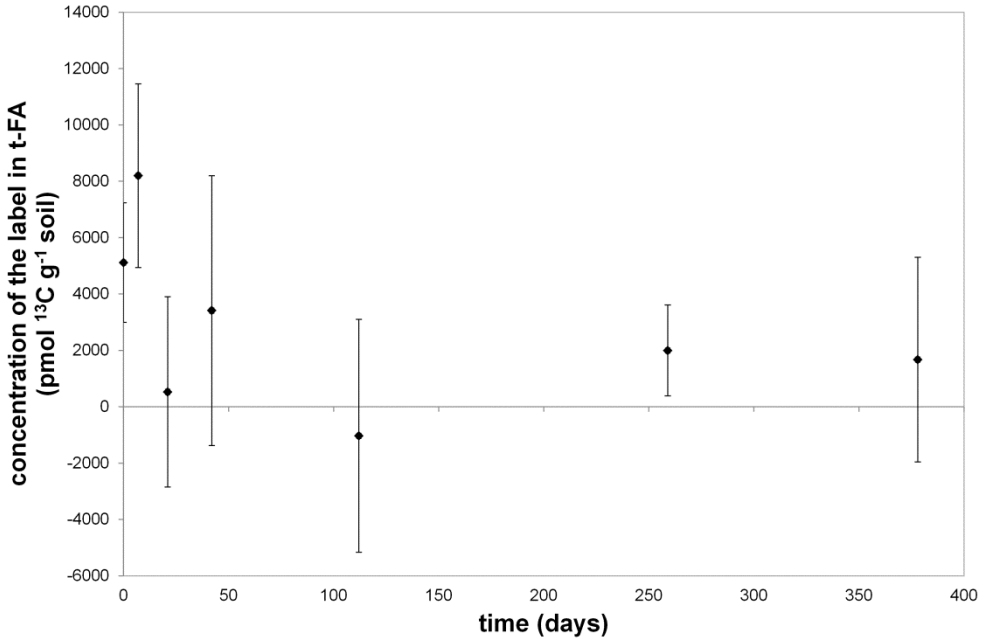

Fig. 6.4 Fate of ^{13}C derived from BGRs in t-FA during the experiment. Error bars indicate standard deviation of replicate samples. No significant differences were detected.

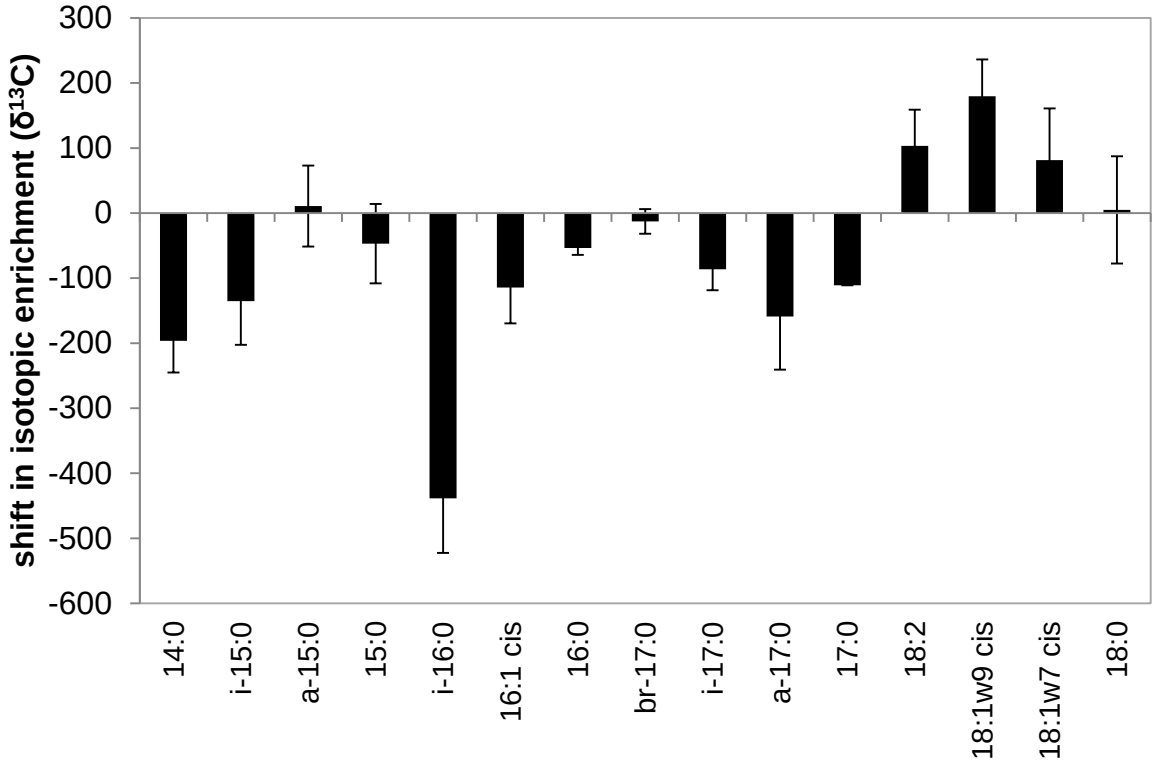

Fig. 6.5 Shift in the isotopic enrichment of t-FA until the end of incubation. Positive values indicate incorporation whereas negative values indicate loss. Error bars indicate standard deviation.

Discussion

In the present study, the fate of fatty acids derived from ^{13}C-labelled BGRs during incubation in soil was investigated in order to evaluate the contribution of BGR-biomass to the living soil microbial biomass and non-living SOM. Details of the overall mass balance are presented elsewhere (Coban et al., 2015). The fatty acids were assigned to living microbial biomass or non-living soil organic matter by comparison of the label within PLFAs, representing living cells, and within t-FAs, which include also the fatty acids derived from necromass and SOM. The label amount was higher in the t-FAs but also a stronger decrease of the label in the t-FAs than in the PLFAs occurred. Shifts of the label incorporation in the individual t-FAs and PLFAs were detected indicating fatty acid specific incorporation/loss during the experiment.

Impact of BGRs on soil community

The increase in the PLFAs immediately after BGR-amendment to the soil shows that BGRs contributed to microbial biomass in the soil environment. Since biogas residues were fumigated with a strong acid before mixing with the soil, it is likely that cell death of some of the microorganisms present in the biogas residues occurred. Thus, microbial biomass input to the soil through application of biogas residues refers to the input of microorganisms survived the fumigation process or the next generation of native soil microbes that may have rapidly grown on the residuals of biogas residue microorganisms. Later on this biomass could be used as substrate by other bacteria during the incubation, as well. Living soil microbial biomass makes up only a small part (<5%) of SOM (Dalal, 1998), however, has the ability to produce recalcitrant molecules after cell death (Liang et al., 2011; Miltner et al., 2012). Thus, an average doubling of living microbial biomass in soil due to the amendment with the BGRs may indicate a potential for SOM formation. Since the concentrations of saturated methyl branched PLFAs increased with the BGR amendment, it is likely that Gram-positive bacterial biomass was added to soil with or supported by the amendment with BGRs. It is difficult to estimate whether Gram-negative bacterial biomass in soil was also increased, since only some of monounsaturated methyl branched PLFA concentrations were higher than that of in the BGR-amended soil. In addition, fungal biomarker PLFAs did not increase with the BGR-amendment. This effect of the BGRs on the microbial community composition of the soil is consistent with the microbial composition of the BGRs, which was dominated by Gram-positive bacteria (Coban et al., 2015).

Microbial community composition of the BGRs-soil mixture may presumably be affected by this biomass input. In biogas plants, methanogenic archaea produces methane from the intermediates produced by bacteria (Zinder et al., 1984) and thus, BGRs contain both bacterial and archaeal biomass. In addition, there will be heterotrophic secondary consumer bacteria feeding on the decaying primary biomass produced during the biogas fermentation process. This can explain the rapid increase in bacterial PLFAs in soil due to BGRs amendment. Unfortunately, archaeal ether lipids cannot simply be hydrolysed using the standard PLFA approach (Gattinger et al., 2003) and they were too low in concentration for analysis within the BGR-soil mixture; thus we could not quantify archaeal biomass input to soil via BGRs. Assuming that archaeal biomass is about half of the bacterial biomass in biogas reactors (Tay et al., 2001), we can consider a significant archaeal biomass input to soil together with anaerobic bacteria. Fungi do not contribute to biogas production and were thus not found in BGRs indicated by the low abundance of fungal biomarker PLFA in the BGRs and the lack of increase of fungal biomarkers in the BGR-amended soil.

The impact of BGRs in the present experiment decreased with time and the difference between concentrations of PLFAs in unamended and BGR-amended soil was reduced. Interestingly, concentrations of monounsaturated PLFAs and polyunsaturated PLFAs, the biomarkers for Gram-negative bacteria and fungi, respectively, were significantly higher than that of unamended soil at the end of the experiment. This showed that the instant and longer-term effects of the BGRs on the composition of microbial community in soils were different. In addition, Gram-positive bacterial biomarkers had generally similar concentration in unamended and BGR-amended soils at the end of the experiment.

Fate of PFLAs derived from BGRs

During the experiment there was a decreasing trend for the label remaining in PLFAs (Fig. 6.4), even though no significant changes were detected due to high variations in the data ($p>0.05$). The decrease in sum of PLFAs was less pronounced than the decrease of the label in the bulk soil carbon to 35% of its initial value (Coban et al., 2015) indicating differences between mineralization or metabolization of PLFAs and other biomass components. Other biomass components like proteins are considered to turn over more slowly (Fan et al., 2004; Kelleher and Simpson, 2006; Knicker et al., 1993; Miltner et al., 2012; Miltner et al., 2009). Carbohydrates are considered to be readily-degradable, but amino sugars bound to the microbial cell envelopes were found to be rather stable and dominantly associated to necromass (Glaser et al., 2004). What should also be taken into account is that BGRs

contained labelled C not only in bacteria but also in archaea, which is not determined by the classical PLFA approach (Sturt et al., 2004). The turnover of C derived from archaea may be different than that from bacteria and affected mineralization or metabolization of bulk C. In addition, it has to be taken into account that ^{13}C-labelled PLFA may be synthesized during the experiment if soil bacteria grow on labelled components of BGR other than PLFA, e.g. amino acids. The observed concentration is always the net result of PLFA synthesis and degradation

The shift in isotopic enrichment of individual PLFAs interestingly showed specific trends for the different PLFAs, indicating that the incorporation/loss of labelled C depended on the individual PLFAs and might depend on the microbial group. The ^{13}C label in saturated branched PLFAs, indicative of Gram-positive bacteria (McKinley et al., 2005), decreased during the experiment, as did the label in some saturated straight-chain PLFAs found in almost all organisms (Cavigelli et al., 1995). While the label in saturated branched and saturated straight-chain PLFAs was decreasing, the label in mono- and polyunsaturated PLFAs as well as 10 Me 18:0 was increasing. Monounsaturated PLFAs are indicators of mostly Gram-negative bacteria (McKinley et al., 2005). Thus, our results may indicate a carbon flow from Gram-positive bacteria to Gram-negative bacteria i.e. some Gram-negative bacteria are micropredators or they feed on necromass. Several studies showed that *Firmicutes*, which are mostly Gram-positive, dominate the community in biogas digesters (Klocke et al., 2007; Kuo et al., 1997; Schlüter et al., 2008), which can indicate a Gram-positive bacterial biomass input via BGRs. In addition, 58% of the label in the BGRs were detected in Gram-positive biomarker PLFAs which is considerably higher than that in Gram-negative biomarker PLFAs (13%) and fungal biomarker PLFA (5%) as reported previously (Coban et al., 2015). Lueders et al. (2006) identified the bacterial predators in a soil microbial food web as *Lysobacter* spp., *Myxococcales*, and *Bacteroidetes*, all of which belong to Gram-negative bacteria, and this matches perfectly with the results of the present study. Those gliding micropredators are specialized in the degradation of biomacromolecules and can attack both dead organic matter or bacteria and living organisms (Reichenbach, 1999). Gliding ability of bacteria, e.g. *Myxobacteria* was suggested to be beneficial in soil environments to access non-diffusing substrates (Reichenbach, 1999). The reason that those bacteria presumably attacked Gram-positive bacteria might depend on cell membrane structure, because the outer membrane in Gram-negative cells might prevent access of hydrolytic enzymes of the predator bacteria to the peptidoglycan layer (Begunova et al., 2004; Jurkevitch and Davidov, 2007) thus making Gram-positive bacteria more prone to lysis by

these micropredators. A higher abundance of Gram-positive bacteria might also explain why they are a favourable target for bacterial predators.

However, a ^{13}C loss or gain in fatty acid biomarkers does not necessarily indicate a mass flow between organisms. Without an analysis of other microbial components like amino sugars, peptides or nucleic acids, it is difficult to link the gain of ^{13}C in Gram-negative biomarker PLFAs to the loss of ^{13}C in Gram-positive biomarker PLFAs. Nevertheless, several microbial groups uptook/lost the fatty acid C derived from biogas residues. Biogas residues contain a complex microbial community different than that of arable soils. Some of the microbial groups may have not survived in the new habitat and eventually die. This was previously supported by introducing an isotopically labelled bacteria to soil (Kindler et al., 2006). Independent of the assimilation of microbial necromass by other soil microbes, their cell wall fragments will contribute to the formation of SOM (Miltner et al., 2012). Fungal biomarkers also gained the isotopic label during the experiment, and the uptake of the label into fungal biomass may indicate a significant role of fungi in labile C turnover in soil.

Fate of t-FAs derived from BGRs

Similar to the results of the PLFA analysis, the portion of t-FAs remaining in soil at the end of experiment (33%) was lower than the initial amounts, even though no statistically significant changes were detected ($p>0.05$). This decrease was accompanied by significant shifts in the isotopic composition for several FA ($p<0.05$).

We could not fit the sum of fatty acid data from the present study to a two-pool biomass turnover model (Kindler et al., 2006; Kindler et al., 2009) because the data were highly variable and thus did not allow the identification of a clear pattern over time. This was caused mainly by the variability in each group of fatty acids which resulted in a high variability of sum of t-FA as the sum of these groups. The isotopic enrichment or loss in t-FA groups was different than that in PLFA groups. While most of the fatty acids lost the label during the experiment, t-FAs 18:2, 18:1w9 cis and 18:1w7cis gained the label. The label incorporation into those t-FAs was consistent with the label incorporation in PLFAs. A shift in individual t-FAs was previously explained by loops of microbial metabolization and cross-feeding between microorganisms (Kindler et al., 2009).

Conclusions

Tracing the fate of fatty acids in soils can contribute to our understanding of the soil carbon cycle and soil organic matter formation. During the incubation of BGRs in soil, isotopically labelled carbon in phospholipid fatty acids and total fatty acids was mineralized or metabolized to a lower extent than the bulk carbon. Isotope analyses of fatty acid groups showed specific trends for different fatty acid groups regarding label incorporation or loss. Overall, we have shown that biogas residues provide microbial carbon which is stabilized in soil and can contribute to the formation of soil organic matter.

Acknowledgements

We are thankful to the funding of Helmholtz Association of German Research Centres within the framework of the program `Energy and Land Use´ of the Department of Bioenergy (UFZ) and graduate school HIGRADE. Also, we would like to thank Ellen Kandeler (University of Hohenheim) and Daniela Thrän (DBFZ-UFZ) for fruitful discussions, Harald Wedwitschka (DBFZ) for inoculum and manure supply, Ursula Guenther (UFZ) for help with the isotope analyses, Marc Pages Gallego (Rovira i Virgili University) for assistance with the fatty acid extractions, and Anne Carney for the English proofreading.

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7. Impacts of compost, biochar and manure on carbon mineralization of biogas residues applied to soil

Submitted to European Journal of Soil Science

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Summary

Biogas residues contain microbial biomass which contributes to the formation of soil organic matter. However, whether the potential of biogas residues to increase soil organic matter can be enhanced by co-application with compost, biochar or manure is unknown. The aim of this paper is to evaluate the impacts of co-amendment on the mineralization of biogas residues, carbon dioxide emissions, and the carbon flow to the microbial food web. The fate of ^{13}C labelled microbial biomass present in biogas residues co-applied with compost, biochar and manure to soil was determined by analysing CO_2 and biomarker phospholipid fatty acids. The total amount of mineralized carbon derived from biogas residues was not affected by co-amendment. However, while the mineralization rate constant of the slowly degrading carbon pool was not affected by co-amendments, manure co-amendment resulted in a higher mineralization rate constant of the readily degrading carbon pool of biogas residues. The incorporation of carbon was mainly to Gram-negative biomass and was the lowest in manure co-amendment indicating differences in bioavailability of the carbon source. Overall, co-amendment of biogas residues with compost, biochar or manure brings neither benefits nor harms in terms of the contribution to CO_2 emissions or formation of soil organic matter.

Introduction

Soil organic matter (SOM) is a major factor determining the fertility of soil. It supplies energy for microbial biomass activity (Joergensen *et al.*, 1990), provides nutrients for crops and improves the physical properties of the soil (Six *et al.*, 2002). Recently, a loss of SOM over the last decades has been observed (Bellamy *et al.*, 2005). One possible reason for such a loss may be an increased biomass export on agricultural areas. Biomass export is enhanced due to an increased use of agricultural areas for bioenergy crops. To maintain soil fertility and functioning, this loss has to be compensated for, for example by amending soils with organic matter. Side-products of the biogas production process, biogas residues (BGRs), can be used as a soil conditioner. A recent study investigated the mass balance of carbon (C) derived from biogas residues applied to arable soil, indicating mid-term contribution to living biomass and/or non-living SOM (Coban *et al.*, 2015). However, degradation of SOM was also stimulated by BGR-amendments indicating potential lack of its sustainability (Coban *et al.*, 2015). We hypothesize that the co-amendment of BGRs with other soil conditioners can minimize negative impacts of BGRs on SOM stability and can increase the contribution of BGRs to SOM formation.

BGRs are rich in nutrients , including appreciable amounts of plant-available inorganic nitrogen, making them a valuable fertilizer (Arthurson, 2009). In addition, the application of BGRs to soils has recently been suggested to promote the recycling of C on bioenergy croplands (Arthurson, 2009). All this makes them promising soil conditioners. Formation of SOM via organic amendments is a complex process and strongly depends on the chemical composition of the amendment. BGRs are composed of microbial biomass together with undigested plant residues which cannot be utilized by microorganisms during anaerobic digestion within biogas plants. An increase in microbial biomass during BGR-application to soil was also reported (Chen *et al.*, 2012). Microbial biomass residues (necromass) account for the majority of organic matter in soils and microbial cell wall envelopes were proposed to be a significant source of SOM (Miltner *et al.*, 2012), which makes the microbial biomass present in BGRs a significant contributor to the formation of SOM, although they also have been shown to stimulate the degradation of SOM (Coban *et al.*, 2015).

The addition of energy-rich fresh organic matter may stimulate or inhibit mineralization of native SOM (Kuzyakov *et al.*, 2000). This so called priming effect may be induced by amendment of soils with organic substances or inorganic fertilizers, input of plant residues, and exudation of organic substances by roots (Kuzyakov *et al.*, 2000). The priming effect

mainly occurs immediately after the addition of the material to the soil (Kuzyakov *et al.*, 2000) but can be delayed for days or weeks (Kuzyakov, 2010). A priming effect due to amendment of soil with BGRs has been reported (Coban *et al.*, 2015).

In order to fully exploit the SOM forming potential of BGRs, it is necessary to mitigate adverse priming effects and reduce the mineralization of BGRs. Supplying additional organic matter with different properties to BGR-amended soils can have stimulatory or inhibitory impacts on the mineralization of BGRs and SOM. For example, Keith *et al.* (2011) applied biochar and labile organic matter (sugar cane residue) to a smectite-rich soil and found that mineralization of labile organic matter was reduced by biochar addition. This points to potential interactions between organic soil amendments, which might influence the mineralization in BGR-amended soils if other organic materials such as compost, biochar and manure are co-amended with BGRs. Such interactions have not been investigated so far.

Organic matter is stabilized by composting, an aerobic treatment. Compost is commonly applied to arable soils as a soil conditioner and increases soil microbial C and SOM (Baldi *et al.*, 2010). Spaccini *et al.* (2009) reported changes in the molecular characteristics of SOM after compost amendment indicating significant incorporation of compost-derived organic molecules into SOM. Mineralization of compost in soil is much lower than that of BGRs and untreated manure (Bernal & Kirchmann, 1992).

Another soil conditioner, biochar (also known as pyrogenic organic matter), increases soil C content and influences the chemical composition of SOM (Qayyum *et al.*, 2014). There are a number of studies reporting positive, negative, or no priming effects of biochar. A recent meta-analysis showed that biochar induces positive priming of native SOM and negative priming of fresh organic matter (Maestrini *et al.*, 2014). This may apply also to biochar addition to BGR-amended soils as biochar and BGRs provide C which is varying in its availability for microbial consumption and further mineralization. The extent to which readily-degradable organic matter has decomposed is one of the important characteristics defining the amendment quality (Lasaridi & Stentiford, 1998). We here use the term "stability" to characterize an amendment's tendency to produce CO_2 when applied to soil, with high stability referring to low CO_2 production. For our study, we selected compost, biochar and cow manure as co-amendments, because they have different levels of stability in this sense.

Untreated agricultural waste, for example manure, is also often used as a soil conditioner. SOM content and quality can be improved by application of manure (Xie *et al.*, 2014), and cattle slurry is known to increase soil microbial biomass (Kandeler & Eder, 1993). In addition, continuous application of farmyard manure was found to increase the organic C content and to improve the physical properties of soil (Prasad & Singh, 1980). Even though impacts of the organic amendments tested in this study (compost, biochar and manure) were reported previously, there is a lack of knowledge on how those organic amendments will affect the mineralization of SOM or BGR-derived C as a result of co-amendment due to interactions of the different C pools.

In the present study we investigate the impacts of co-application of three different soil amendments (compost, biochar, manure) on the mineralization of ^{13}C labelled microbial biomass C derived from BGRs. No data are available yet to identify how the mineralization/accumulation of C changes when the BGRs are co-applied with other soil amendments. Thus, the objectives of this study are: i) to evaluate the mineralization of ^{13}C-labelled BGR-C in soil co-amended with compost, biochar or untreated manure, ii) to analyse the effects of the co-amendments on the label incorporation into and redistribution in the living soil microbial biomass, and iii) to determine priming effects of the supplied organic materials with different stability and their interactions with BGRs to affect SOM mineralization.

Materials and Methods

Soil and soil amendments

A material from a Haplic Chernozem with loamy texture was used for the experiments and was sampled from the Bad Lauchstädt static fertilization experiment site (Germany) from a plot which received mineral phosphorus and potassium fertilization of 60 kg ha^{-1} and 230 kg ha^{-1}, respectively. A crop rotation of sugar beet, spring barley, potato and winter wheat has been applied since 1902. BGRs were produced in lab-scale biogas reactors as described previously (Coban *et al.*, 2015). Briefly, cow manure receiving reactors operating in steady-state were amended with isotopically labelled potassium carbonate (98 atom% ^{13}C $KHCO_3$, 150 mg d^{-1}) for 28 days for the labelling of microbial biomass. At the end of labelling, average isotopic enrichment in phospholipid fatty acids and phytane were 1.69 at.% and 4.25 at.%, respectively, indicating significant label incorporation to both bacterial and archaeal biomass (Coban *et al.*, 2015). As a control, unlabelled BGRs were produced in the same way

using unlabelled $KHCO_3$. Remaining inorganic C was removed by acid (6M HCl) fumigation in a desiccator lasting four days prior to amendment to the soil. A commercially supplied charcoal produced from pyrolysis of beach wood was used as biochar. Compost material was produced via composting of farmyard manure. Cow manure was used as received from the farm. Characteristics of the soil and amendments are given in Table 7.1.

Table 7.1 Characteristics of soil and soil amendments used in the study.

	C (g kg^{-1})	**N (g kg^{-1})**	**C/N**	**Isotopic composition (atom% ^{13}C)**	**pH**	**Amendment application (g kg^{-1} soil)**	**C added with amendment (g kg^{-1} soil)**
Soil	18±1	2±0	11	1.08±0.00	6.2		
Labelled BGRs	230±25	20±3	12	1.38±0.01	7.0	3.3±0.5	0.76±0.08
Unlabelled BGRs	225±14	19±0	12	1.08±0.00	7.0	3.3±0.5	0.74±0.05
Compost	339±05	23±0	15	1.08±0.00	8.2	2.9±0.0	0.97±0.01
Biochar	709±30	9±0	78	1.08±0.00	8.0	7.3±0.0	5.20±0.22
Manure	368±13	25±03	15	1.08±0.00	6.8	2.64±0.3	0.97±0.03

Soil incubation

Soil material was sieved through a 2 mm screen prior to incubation. In analogy to earlier studies (Müller & von Fragstein und Niemsdorff, 2006; Chen *et al.*, 2012) and in accordance to agricultural practice where amendments are mainly regarded as N fertilizers, we adjusted the amount of labelled and unlabelled BGRs, compost, biochar and manure amended to the soil to correspond to 0.066 g N kg^{-1} dry soil each in the range of similar studies. Samples with co-amendments (BGRs + another additive) thus received 0.132 g N kg^{-1}, with a 1:1 ratio of BGRs and the other additive. As the C/N ratio of the amendments differed, this resulted in different levels of C added to the soil (Table 7.1). The amount of ^{13}C amended with the labelled BGRs corresponded to about 5% of the bulk ^{13}C naturally present in the soil. The soil water content was adjusted to 50% of its water holding capacity. Incubations were performed in triplicate at 20 °C in 50 ml bottles each containing a test tube filled with 1 ml of 2 M NaOH for trapping CO_2. 200 µL NaOH was sampled after adequate mixing at days 0, 4, 10, 17, 35 and, 61 to determine the concentration and isotopic composition of the respired C. After each

sampling, tubes were replaced by freshly prepared tubes for capturing the respired C produced during the next time interval. Captured C from a set of three bottles excluding soil or amendment was also sampled to avoid CO_2 contamination from ambient air during the preparation of NaOH solutions. At the beginning and end of incubation, 2 g of soil samples from each bottle were taken for phospholipid fatty acid (PLFA) analysis after adequate mixing.

Analyses of C in respired CO_2 and living microbial biomass

The concentration of CO_2 in the NaOH traps was determined using a multi NC 2100S Total Organic Carbon Analyser (Analytic Jena, Jena, Germany). For analysis of the isotopic composition of CO_2 according to Miltner *et al.* (2004), 0.2 ml aliquots of NaOH were acidified with phosphoric acid in headspace vials and the headspace was analysed using an HP 6890 Plus gas chromatograph (GC) (Hewlett-Packard, Wilmington, USA) coupled to a Finnigan MAT 252 isotope ratio mass spectrometer (IRMS) via a GC combustion interface (Finnigan, Bremen, Germany). A Poraplot Q-HT Plot FS column (25 m x 0.32 mm x 10 µm) at 40 °C was used to separate CO_2 from other gases.

Phospholipid fatty acids are reported to be indicators of living biomass (Zelles, 1999). We therefore extracted PLFA according to Bligh and Dyer (1959), fractionated them by column chromatography over silica gel and converted them to fatty acid methyl esters with methanol and trimethylchlorosilane (9:1, v:v) at 70 °C for two hours as described previously (Thiel *et al.*, 2001; Miltner *et al.*, 2004). Phospholipid fatty acid concentrations were determined using an Agilent Technologies 7890A GC coupled to a 5975C inert XL mass spectrometer (MS) triple axis detector (Agilent Technologies, Santa Clara, USA). A BPX-5 column (30 m x 0.32 mm x 0.25 µm; SGE, Darmstadt, Germany) was used to separate fatty acid methyl esters. The temperature program for GC MS was as follows: initial temperature 45 °C (hold for 2.25 min) then heat to 300 °C (3 min) at 40 °C min^{-1}. The isotopic compositions of the PLFAs were determined using the IRMS mentioned above. A BPX-5 column (50 m x 0.32 mm x 0.5 µm) column was used for the separation (SGE, Darmstadt, Germany) and the temperature program was: initial temperature 70 °C (hold for 1 min), heat to 130 °C (0 min) at 20 °C min^{-1}, heat to 150 °C (5 min) at 2 °C min^{-1}, heat to 165 °C (5 min) at 2 °C min^{-1}, heat to 230 (0 min) at 2 °C min^{-1}, heat to 300 °C (5 min) at 20 °C min^{-1}. Saturated branched PLFA were regarded as biomarkers for Gram-positive bacteria, and 10me 18:0 as a biomarker for actinomycetes , monounsaturated PLFA as biomarkers of Gram-negative bacteria and polyunsaturated PLFA

as biomarker of fungi. The use of PFLA as biomarkers of various microbial groups is common practice in soil microbiology (Cavigelli *et al.*, 1995).

Calculations and statistical analysis

^{13}C mineralization was fitted to a two-pool model as suggested by Qualls and Haines (1992). This model assumes that the BGR-C is distributed to two pools, one readily degrading and one slowly degrading pool, and each decaying according to first order kinetics. Eq. (7.1) is used to estimate degradation rates:

$$CO_2(t) = 100 - \left[(a)e^{-k_1 t} + (100 - a)e^{-k_2 t}\right] \quad (7.1)$$

where $CO_2(t)$ is the C mineralized to CO_2 (% of intial ^{13}C) at time t (d); *a* is the fraction of the readily degrading C pool (%); k_1 and k_2 (d^{-1}) are the degradation rate constants of the readily degrading and slowly degrading pools, respectively. The parameters were estimated by non-linear least squares Levenberg-Marquardt algorithm using Sigma Plot 12.0.

For the determination of priming effects caused by co-amendment, we compared the amount of CO_2 produced in co-amended soils (BGRs+compost, biochar or manure) with the predicted CO_2 production using the data from separate single amendments in the equation below, assuming that total CO_2 production in co-amendments is simply an additive combination of CO_2 productions of respective single amendments:

$$C = C_1 + C_2 - C_{SOM} \quad (7.2)$$

where C_1 is the C-CO_2 in BGR-amended soil; C_2 is the C-CO_2 in soil amended with an organic matter other than BGRs (compost, biochar or manure); C_{SOM} is the C-CO_2 in unamended soil. This equation calculates the expected amount of C-CO_2 produced if no interactions occur due to co-amendment. A priming effect is assumed if the calculated values differ from the measured ones. This might indicate interactions between different C sources, resulting in a stimulation/inhibition of C decomposition.

All analyses were performed in triplicate and the results are given as mean ± standard deviation. The amount of excess $^{13}CO_2$ produced under different treatments (soil only, soil + BGR, and soil + BGR + compost/biochar/manure) was compared with one-way ANOVA separately for each time interval (e.g., 0-4, 4-10, etc.). Effect of co-amendment on total carbon mineralization was also tested using one-way ANOVA. Where appropriate, the Tukey post-hoc test was applied following ANOVA analysis. In labelling experiments, control

experiments using unlabelled BGRs were performed in parallel. The mean value of measured $^{13}CO_2$ concentrations in these control experiments were subtracted from measured $^{13}CO_2$ concentrations in labelled experiments to correct for naturally present ^{13}C. Measured and predicted values for total carbon mineralization for each treatment were compared with independent-samples t-test. All calculations were performed in SPSS 21 (IMB, New York, USA).

Results

Mineralization of BGR-derived C and its modelling

The release of ^{13}C-CO_2 derived from BGRs is presented in Fig. 7.1. The mineralization of C was initially rapid, and was decreasing with time approaching a stable state. At the end of the experiment, 37.4% ± 2.8% of the BGR-derived ^{13}C was mineralized to CO_2, whereas the mineralization of ^{13}C in the soils co-amended with compost, biochar, and manure was 34.9% ± 7.2%, 35.7% ± 2.2%, and 39.6% ± 8.9%, respectively. According to the results of ANOVA, mineralization of ^{13}C was not dependent on the treatment for all the time intervals ($p>0.05$).

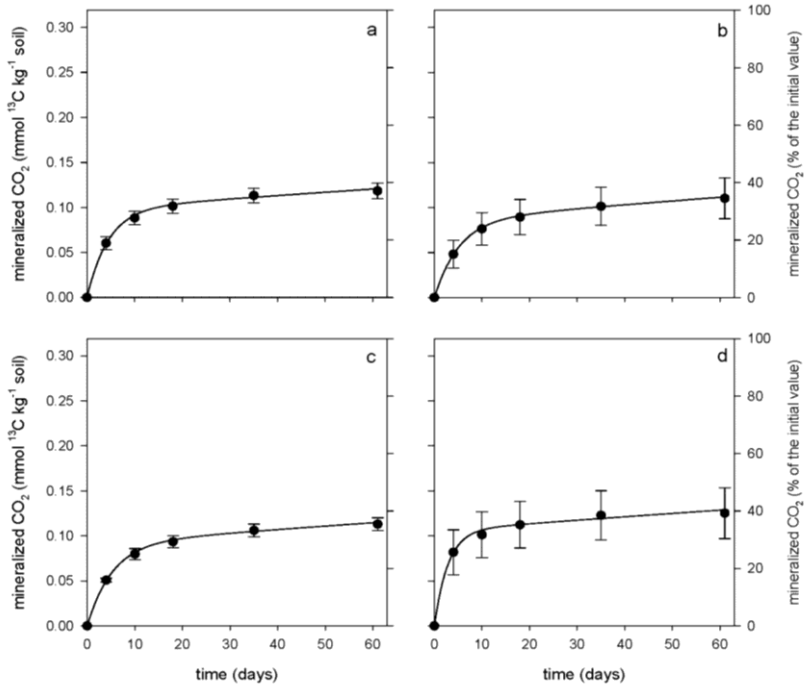

Fig. 7.1 Mineralization of ^{13}C in the soils amended with biogas residues when applied alone (a), with compost (b), with biochar (c) and with manure (d), respectively. Mean of measured values are depicted by (●) with error bars indicating standard deviation, and fitted model results are drawn as a solid line.

The parameters for the pool sizes and the fast and slow degradation rate constants k_1 and k_2 estimated by the model using Eq. (7.1) fitted to the measured mean values are presented in Table 7.2. About 31% of the BGR-C was present in the readily degrading C pool when applied alone and the remaining 69% constituted the slowly-degrading C pool. Co-amendment with compost, biochar and manure resulted in only slight changes in the fraction of the two C pools. The degradation rate constant of the readily degrading C pool derived from BGRs was 0.2236±0.0219 d^{-1} and did not change significantly with compost and biochar amendments. However, it increased to 0.3528±0.0539 d^{-1} with manure amendment. The degradation rate constant of the slowly degrading C pool was 0.0018-0.0020 d^{-1} for all treatments and thus was not influenced by co-amendment.

Table 7.2 Parameters of equation (7.1) estimated for the carbon derived from biogas residues (BGRs) in soil with different amendments. Results are presented in mean±standard error.

Amendment	R^2	Readily degrading pool		Slowly degrading pool	
		Fraction (% of initial)	Degradation rate constant (d^{-1})	Fraction (% of initial)	Degradation rate constant (d^{-1})
BGRs only	0.99	30.68±1.23	0.2236±0.0219	69.32±1.23	0.0018±0.0004
BGRs + compost	0.99	26.76±0.63	0.1944±0.0101	73.24±0.63	0.0020±0.0002
BGRs + biochar	0.99	28.51±0.99	0.1904±0.0145	71.49±0.99	0.0018±0.0003
BGRs + manure	0.99	33.05±1.51	0.3528±0.0539	66.95±1.51	0.0019±0.0006

Carbon flow into and within the microbial food web

In order to determine the C flow from BGRs to living soil biomass, PLFA analyses were conducted at the beginning and at the end of the incubation. Abundances of the isotopic label in biomarker PLFA were calculated for Gram-negative, Gram-positive and fungal biomarkers. In all treatments, label in the biomarkers for Gram-positive bacteria was lost during the incubation (Fig. 7.2). The ^{13}C label was incorporated into biomarkers for Gram-negative bacterial and fungal biomass in all the treatments. Loss of labelled ^{13}C from Gram-positive bacteria was higher in manure and compost co-amendments than in biochar and no co-amendment (BGRs only). Incorporation into Gram-negative biomarkers was the highest (115±22 µmol ^{13}C g^{-1} soil) with biochar amendment and lowest (19±4 22 µmol ^{13}C g^{-1} soil) with manure amendment. The incorporation into fungi was in the range 2-9 µmol ^{13}C g^{-1} and lower than that into Gram-negative bacteria soil in all treatments.

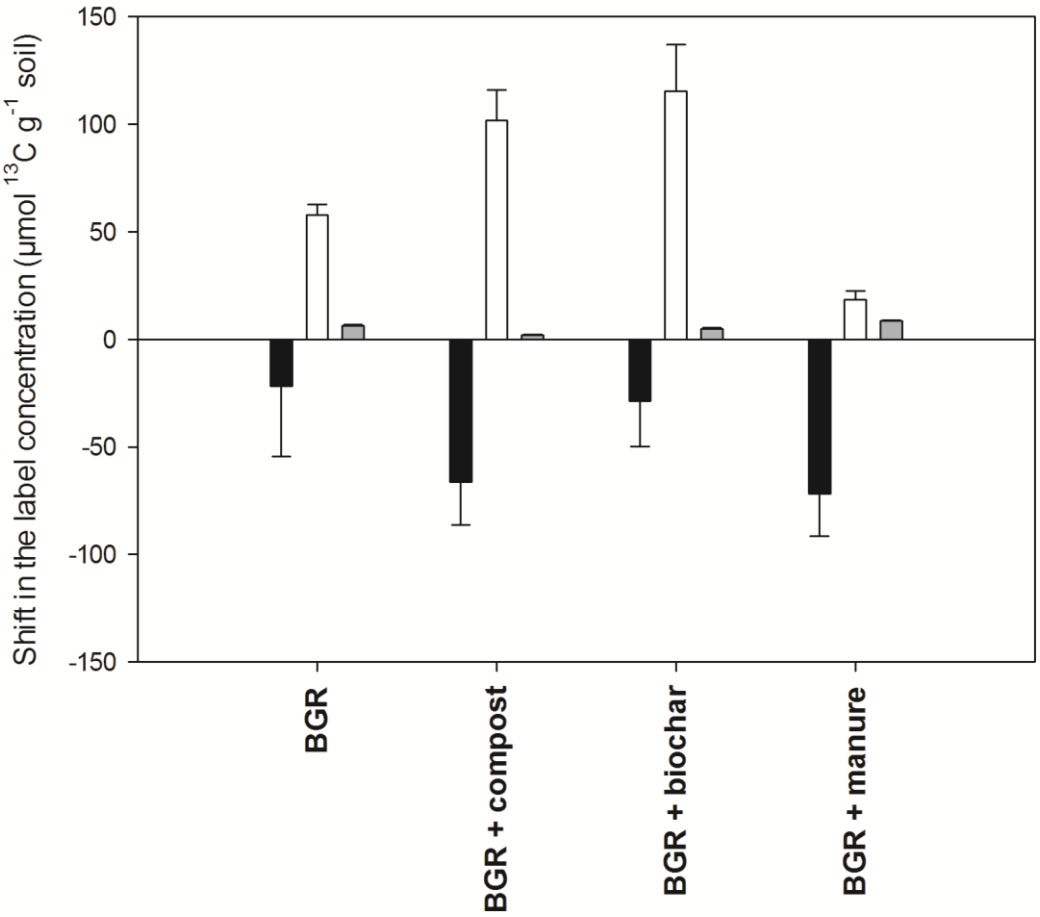

Fig. 7.2 Shift in the label concentration during the incubation. Positive values indicate ^{13}C incorporation to Gram-positive (■), Gram-negative (□), or Fungi (■) whereas negative values indicate loss of ^{13}C.

Mineralization of co-amendments and estimation of priming effects caused by co-amendment

Soil amended with BGRs produced 14.2±0.6 mmol CO_2 kg^{-1} and co-amendment with compost, biochar and manure resulted in a total CO_2 production of BGR-amended soil of 16.1±4.3, 14.1±0.5 and 24.1±2.22 mmol CO_2 kg^{-1}, respectively (Fig. 7.3). ANOVA analysis followed by the Tukey post-hoc test indicated that manure was the only amendment which increased total mineralization significantly (Table 7.3). In order to estimate priming effects caused by interactions of the co-amendments, the amount of CO_2 produced in co-amended (BGRs+compost, BGRs+biochar or BGRs+manure) soils was compared with the CO_2 production calculated from separate incubations (soil only, soil+BGRs, soil+compost, soil+biochar, soil+manure). Eq. (7.2) resulted in calculated CO_2 production of 16.7±0.8, 14.6±0.7, and 25.5±2.3 in compost, biochar and manure co-amended soils. These values are

within the range of measured CO_2 production (Fig. 7.3). No statistically difference was detected using independent-samples t-test ($p>0.05$).

Table 7.3 Significance values (*p*) calculated by Tukey test for determining the statistical differences of total CO_2 production between biogas residues (BGR) and co-amendments.

Amendment	BGR only	BGR + compost	BGR + biochar	BGR + manure
BGR only		0.85	1.00	0.02
BGR + compost			0.83	0.05
BGR + biochar				0.02

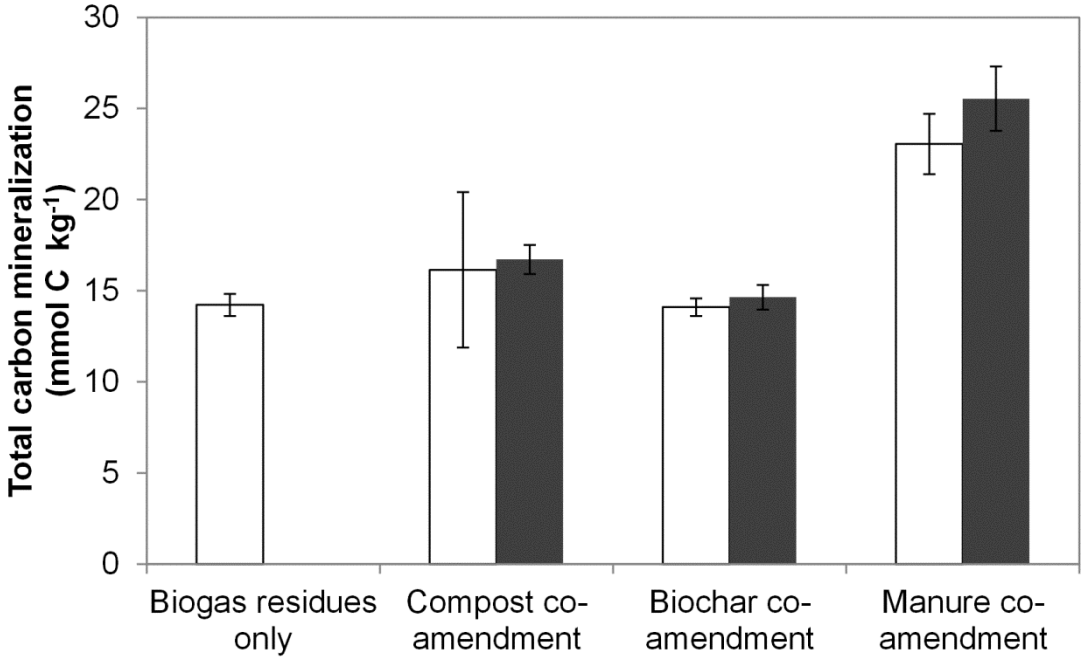

Fig. 7.3 Total carbon mineralization in soils amended with biogas residues when applied alone and co-amended with compost, biochar and manure. Empty bars indicate measured values whereas filled bars indicate additive predictions based on single-amendment experiments, assuming no interactions between different C-pools.

Discussion

In the present study, ^{13}C-labelled BGRs were applied to soil together with compost, biochar and manure and the fate of ^{13}C was traced. This allowed us to estimate degradation rate constants of readily degrading and slowly degrading C pools present in BGRs, and the impacts of co-amendment on them. The isotope tracing approach also allowed us to investigate the C flow within the microbial food web. Priming effects caused by possible interactions occurred within C from different amendments was estimated by CO_2 productions of separate and co-amendments.

Mineralization of BGR-derived C and its modelling

The change in daily mineralization with time showed that BGRs consist of a number of C pools degrading with different rates. Theoretically, an indefinite number of C pools could defined for BGRs similar to those of SOM or other amendments (Zimmerman *et al.*, 2011). However, we assumed two major C pools one readily degrading and the other one slowly degrading, and fitted the data to a two-pool model.

Fast decomposition of the readily degrading C pool can be estimated by the k_1 values in Table 7.2 which indicates the immediate decay of the microorganisms present in the BGRs. These microorganisms are presumably *Firmicutes* and *Archaea* which were active in biogas generation process (Liu *et al.*, 2009). The degradation rate constant (k_1) of the C derived from *E. coli* biomass was reported to be 0.10 d^{-1} which is approx. 2-3 fold lower than that of the BGR-derived C (Kindler *et al.*, 2006). Aerobic conditions in the soil incubation might be responsible for the rapid decay of the anaerobic microorganisms present in BGRs. Interestingly, manure amendment increased the degradation rate constant of readily degrading C pool, which may occur due to a supply of labile organic matter or easily available nutrients. The main difference between manure and other amendments used in the study was that manure has neither been composted nor fermented. Composting impacts the stability of organic matter amendment, for example increases the portion of aromatic structures (Chefetz *et al.*, 1996). Similarly, pyrolysis produces biochars with a highly recalcitrant nature. On the other hand, manure is known to be easily degradable even during storage (Møller *et al.*, 2004). We presume that manure amendment added biologically available organic matter and nutrients to the soils which increased the microbial activity. Increased microbial activity, then, caused faster mineralization of the readily degrading C pool present in BGRs, which can explain the increased k_1 value in manure co-amendment and can be attributed to a priming effect of manure on this pool. Since the readily-degradable C present in BGRs was limited, the cumulative mineralization of BGR-C in manure-co-amendment was not significantly higher than that of other treatments ($p>0.05$). The impact of manure co-amendment was an increase in k_1 value and earlier stabilization of mineralization rate (day 10) than of no or compost co-amendment (day 18) and biochar co-amendment (day 35). The decomposition of the slowly degrading C pool present in BGRs was very low with k_2 values of 0.0018-0.0020 d^{-1}, corresponding to half-lives of approx. one year. These half-lives are in the same range as the half-life of *E.coli* derived C, which was reported to be 1.3 year (Kindler et al. 2006). This indicates that the slowly degrading C pool derived from microorganisms present in BGRs

could be similar to that of *E.coli* which was represented by biomass metabolites, stabilized cell debris or C assimilated by other organisms in microbial food web (Kindler *et al.*, 2006). The C flow within the microbial food web can be demonstrated by the fate of ^{13}C in biomarker PLFA.

Carbon flow into and within the microbial food web

The main bacterial group in the anaerobic digesters fermenting manure are generally Gram-positive *Firmicutes* (Liu *et al.*, 2009), including anaerobic *Clostridia*. A decline of *Clostridia* could have occurred in the soil where the conditions are aerobic, which can also explain the high k_1 values. Interestingly, the incorporation of the label into biomass was higher than the loss of C from biomass (except for manure co-amendment), clearly indicating ^{13}C sources other than bacteria and fungi. This can be due to labelled methanogenic *Archaea* found in our BGRs which could have been assimilated by soil bacteria and fungi. The C flow was in the same direction in all treatments and these findings support the notion that microbial biomass input to soil is a C source for soil microorganisms (Kindler *et al.*, 2006) and might contribute to formation of SOM in long-term applications (Simpson *et al.*, 2007).

Incorporation of the label to the Gram-negative biomass increased with the stability of the amendment, reaching to a maximum with biochar amendment. This may be a hint that BGR-derived C was assimilated to a larger extend by soil microorganisms in case the additional C source is biologically less available. This is expected because soil microorganisms will preferentially degrade easily available and readily degradable substrates. However, even though incorporation into living Gram-negative biomass was lowest in manure co-amendment, the mineralization of BGR-C did not differ between treatments (Fig. 7.1). In manure co-amended soil, the living biomass might have been stabilized as necromass to a larger extent than in the other treatments, which may explain the lowest incorporation into living biomass. The biological availability of amendments with different levels of stability may also result in shifts of C to be mineralized/metabolized by the soil microorganisms, thus we also investigated the mineralization of co-amendments, not only BRG-C.

Mineralization of co-amendments and estimation of priming effects caused by co-amendment

As expected, biochar was a very stable co-amendment presumably due to its highly recalcitrant nature offering a strategy for decreasing CO_2 emissions (Lorenz & Lal, 2014). Compost co-amendment resulted an increase in CO_2 production, however it was not significant. Only manure co-amendment increased CO_2 significantly, indicating that the

organic matter amendments were mineralized according to their biological stabilities which were not affected by BGR-amendment.

Determining priming effects allows the quantification of changes in SOM mineralization due to BGR-amendment. For this determination, it is necessary to precisely characterise the isotopic composition of the fresh matter applied to the soil (Kuzyakov *et al.*, 2000). In the case of our BGRs, we labelled only autotrophic microbial biomass which was active during anaerobic fermentation. As a consequence, the label was not homogenously distributed within the C pool and was different in the biomarkers of Gram-positive bacteria, Gram-negative bacteria, fungi and archaea (Coban *et al.*, 2015). In addition, the undigested plant derived C was unlabelled, and has a similar isotopic composition as SOM because both were at natural abundance. Moreover, there were additional C sources such as compost, biochar and manure, which also have a similar isotopic composition as SOM. This prevents the separation of C-release from co-amendments and SOM. As there were three C sources in each of the co-amended treatments (SOM, BGRs, and the co-amendment), separation of these sources based on labelling with one isotope (^{13}C) only would have required much higher experimental effort. Thus, we estimated priming effects and interactions between BGRs and additional organic matter amendment by comparing the CO_2 production in co-amended soils with the sum of CO_2 produced in separate single amendments (See Materials and Methods). This comparison of predicted and measured values for the determination of priming effects is modified from another study (Mukherjee *et al.*, 2014). As presented in Fig 7.3, co-amendment with compost, biochar or manure did not further influence the mineralization of SOM. Co-amendment resulted in a CO_2 release which was not significantly different from the sum of separate amendments. This does not explain whether or not any of the amendments caused a priming effect. However, it means that co-amendment does not cause interactions which can decrease/stimulate priming effects caused by BGRs. It was reported that BGRs causes priming effects when applied alone (Bernal & Kirchmann, 1992; Coban *et al.*, 2015), which may result in a decrease in SOM stocks in case of extensive application. The extent of the priming effect might even increase with higher application rates of the amendment (Kuzyakov *et al.*, 2000). Co-amendment with other organic matter neither mitigates nor induces this effect, it is thus not a promising management option for BGR-applied soils.

This study investigated the impacts of common organic matter amendments on the mineralization of BGRs in arable soil. Even though the degradation rate constant of readily degrading C pool was increased by manure co-amendment, overall mineralization extent was

not affected by co-amendment. However, characteristics of the organic matter amendments can play a vital role. We only could test one type of biogas residues in this study, which were produced by anaerobic degradation of cow manure. Biogas residues of other feedstock should also be tested.

Conclusions

The mineralization of biogas residues amended to soil together with compost, biochar or untreated manure and their contribution to microbial biomass was investigated. The total amount of mineralized carbon derived from biogas residues after 61 days was not affected by co-amendment. Manure amendment resulted in a higher mineralization rate constant of the readily degrading carbon pool of biogas residues. However, it did not influence the mineralization rate constant of the slowly degrading carbon pool. With the aid of phospholipid fatty acids analysis, we could also show that part of the carbon was redistributed within the microbial food web. Carbon was lost from Gram-positive biomass present in biogas residues and incorporation to Gram-negative soil microorganisms increased during the incubation in all treatments. The three co-amendments differed, however, in the extent of their influence on the carbon flow into and within the microbial food web. Additional carbon input was observed indicating carbon sources other than bacteria, which are presumable methanogenic archaea. The carbon flow to Gram-negative biomass was highest with biochar co-amendment and lowest with manure co-amendment indicating an impact of bioavailable carbon sources. The co-amendments had not pronounced on net mineralization at the end of the experiment, and interactions with biogas residues in the effect on soil organic matter mineralization could not be detected, indicating neither benefits nor harms in terms of the contribution to or mineralization of soil organic matter.

Acknowledgements

This research was funded by the Helmholtz Association of German Research Centres within the initiative and networking fund for the Department of Bioenergy (UFZ) and supported by HIGRADE graduate school. The authors thank Ellen Kandeler (University of Hohenheim), Daniela Thraen (UFZ-DBFZ), Juergen Proeter (DBFZ), Sabine Kleinsteuber (UFZ) and Heike Streuber (UFZ) for productive discussions, Harald Wedwitschka (DBFZ) for inoculum and manure supply and Ursula Guenther (UFZ) for help with the isotope analyses.

Conflict of Interest

The authors declare that they have no conflict of interest.

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8. General conclusions and final remarks

Organic matter loss in soils is a critical problem particularly in arable soils due to its necessity for food and energy security. Global warming may also have negative effects on the carbon storage of soils so that the problem may rise in the near future. At the same time, management practices are changing with growing bioenergy crops, e.g. more plant biomass is removed from the field and carbon input from those sources are prevented. Manipulating the organic matter input can be a solution to increase or maintain soil carbon stocks. Bringing back the organic carbon removed from soil is possible by applying the residues of the biogas generation process, biogas residues, to the field as a soil conditioner Because, those side-products are rich in microbial biomass and microbial biomass was proposed to contribute significantly to soil organic matter formation. However, it is unclear whether biogas residues have significant impacts on the soil organic matter formation and/or CO_2 emissions from soils in particular whether these impacts are positive or negative. Therefore it was aimed to trace the fate of biogas residues and determine their impacts when applied to arable soil. Stable isotope tools combined with biomarkers were applied to reach the objectives.

Results showed that up to a sum of 65% of biogas residues was mineralized to CO_2 at the end of 378 days. The mineralization data could be fitted to a two-pool model assuming biogas residues to be consisted of two distinct carbon pools (a readily degrading and a stable) which degrade according to first order kinetics. The portion of stable carbon pool was about 49% and with its long half-life of about 1.9 years it can contribute to the formation of soil organic matter. However, biogas residues also stimulated the mineralization of soil organic matter at the beginning. Their intensive application should be avoided in order to prevent organic matter loss.

A differential fatty acid approach was used in the second study by comparing phospholipid fatty acid analysis with total fatty acid analysis. This study was another support that the differential fatty acid approach is a powerful tool to explain the dynamics of living biomass and non-living soil organic matter. In addition, carbon fluxes within the microbial food web could be estimated using fatty acids as biomarkers for microbial groups. During the incubation of biogas residues in soil, isotopically labelled carbon in phospholipid fatty acids and total fatty acids was mineralized or metabolized to 60% and 33%, respectively. This mineralization or metabolization of carbon was in a lower extent than that of the bulk carbon indicating carbon flow between groups of microorganisms. According to the changes in the isotopic composition of individual fatty acids, the carbon flow was estimated to occur from

Gram-positive bacteria to Gram-negative bacteria and fungi. Therefore, this is another indication that biogas residues may be one of the major contributors to soil organic matter formation when used as a fertilizer.

In order to test the impacts of co-amendment of biogas residues with other soil conditioners, we have conducted the third study. Mineralization patterns of biogas residues applied together with compost, biochar and manure were compared. The amount of mineralized carbon derived from labelled biogas residues was not affected by co-amendment. The degradation rate constant of the readily degrading carbon pool was increased by manure co-amendment, however, none of the co-amendments had an impact on the degradation rate constant of stable carbon pool. The direction of carbon flow within the microbial food web was also not affected by co-amendment and was from Gram-positive bacteria present in biogas residues to Gram-negative and fungal soil microorganisms in all treatments. The extent of co-amendment influence on the carbon flow into and within the microbial food web was the highest with biochar co-amendment and the lowest with manure co-amendment. This can be an indication that bioavailable carbon in the co-amendment may have influence on the extent of carbon flow within the microbial food web.

Microbial predators found in soil can rapidly transform living bacteria or remains of them into their metabolism. The resulting cycle of carbon within the microbial web can cause an accumulation of organic matter via the previously proposed patchy fragments formation cycle, after binding to mineral surfaces. The finding of this study on the fate of microbial biogas residues which are mainly Gram-positive bacteria and archaea, is providing valuable support on the microbial formation of soil organic matter. Previously model Gram-negative organism was already studied but it is still an open gap in literature how the fungal residues contribute to the soil organic matter, and the further studies can focus on this subject. The findings of this study should give valuable information on the management of arable soils as well, however, soil type, climate, type of biogas residues (substrate) may all have influence and careful consideration should be done prior to deciding a management option.

Advances in isotope methods are very beneficial for the precise detection of carbon fluxes which requires labelling of the material of interest in case the natural abundance is not different from the one of the background. Several attempts exist in literature for the determination of carbon input from biogas residues using natural isotope approach. None of them, however, was completely effective due to other factors, e.g. inorganic carbon with a different isotopic composition. To the best of my knowledge, this study presents results of

labelled microbial biomass found in the biogas residues for the first time. The efficiency of the labelling was sufficient for the determination of carbon mineralization and stabilization with the labile organic matter input as well as for identifying the carbon fluxes within the microbial food web. This approach can be used in other studies e.g. tracing the fate of methanogens in rice fields or in marine sediments where such kind of community is fundamental, as well.

It is shown that the microbial portion, which is known to have high turnover rates, can be found in biogas residues and the carbon input could be quantified using a labelling approach with $KHCO_3$. However, biogas residues also contain carbon derived from sources other than microbial biomass. Further research may focus on labelling other components of biogas residues which is biased due to its complex chemical structure. Since there are a number of studies on the decomposition of biogas residues without using labelling approach, thus including all parts of biogas residues, this study complements them by unravelling the mineralization and stabilization of microbial carbon found in biogas residues. The proposed labelling approach has also disadvantages, especially that highly labelled carbonates can be not completely removed with the acid fumigation approach and can be still present in the biogas residues. This should be further optimized in order to quantify carbon fluxes more precisely.

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Publications and presentations

Parts of this thesis were published in / submitted to scientific journals for publication or presented in conferences.

Publications

- **Coban, H.**, Miltner, A., Elling, F. J., Hinrichs, K.-U., Kästner, M., The contribution of biogas residues to soil organic matter and CO_2 emissions in an arable soil, Soil Biology & Biochemistry (86) 2015, 108-115.
- **Coban, H.**, Miltner, A., Kästner, M., Fate of fatty acids derived from biogas residues in arable soil, submitted to Soil Biology & Biochemistry.
- **Coban, H.**, Miltner, A., Centler, F., Kästner, M., Impacts of compost, biochar and manure on carbon mineralization of biogas residues applied to soil, submitted to European Journal of Soil Science.

Presentations

- **Coban, H.**, Miltner, A., Elling, F. J., Hinrichs, K.-U., Kästner, M., Contribution of biogas residues to soil carbon and CO_2 emissions when applied to arable soil, invited speaker at Biomass for energy – lessons from the Bioenergy Boom, November 2014, Leipzig, Germany.
- **Coban, H.**, Miltner, A., Kästner, M., Are biogas residues good alternative organic fertilizers, oral presentation at HIGRADE Spring Conference, March 2014, Leipzig, Germany.
- **Coban, H.**, Miltner, A., Kästner, M., Microbial biomass of biogas residues as a contributor to soil carbon cycle, poster presentation at American Geophysical Union (AGU) Fall Meeting, December 2013, San Francisco, CA, USA.
- **Coban, H.**, Miltner, A., Kästner, M., Fate and effect of biogas residues (BGR) on Soil Organic Matter (SOM) Carbon after application to arable soil, oral presentation at Biochars, Composts, Digestates (BCD), October 2013, Bari, Italy.
- **Coban, H.**, Miltner, A., Kästner, M., Effect of fermentation residues on the soil microbial communities and soil organic matter carbon after application on arable soils, poster presentation at Annual Conference of Association for General and Applied Microbiology (VAAM), March 2013, Bremen, Germany.

Acknowledgements

First of all I would like to thank Prof. Dr. Ellen Kandeler for her valuable supervision of the thesis and for being always helpful. Her guidance provided me the knowledge on soil science which was a new field for me. She was giving me very essential ideas which helped me to organize this study successfully.

I would like to thank Prof. Dr. Matthias Kästner and Dr. Anja Miltner for giving me the opportunity to make my PhD in the Department of Environmental Biotechnology. Prof. Kästner was always helping me to solve any problem in a fast and efficient way. Special thanks are going to Dr. Miltner for being there every time I ask for help.

Many thanks to Prof. Dr. Daniela Thrän for her support at the Department of Bioenergy. Thanks also to Dr. Sandro Pütz for involving me to the Group Bioenergy System Analysis.

I am thankful to Prof. Dr. Kai-Uwe Hinrichs and Felix Elling from the Centre for Marine Environmental Sciences for the cooperation regarding the ether lipid analysis. I would also like to thank Marc Pages Gallego, Neeraja Venkataraman, Dr. Karolina Nowak, Ursula Günther, Kerstin Ethner, Ines Mäusezahl, Dr. Uwe Kappelmeyer, Claudia Pietsch, and Kerstin Puschendorf for help in the laboratory.

I am very happy that I shared an office with Oksana Coban, Rania Saad, and Nancy Hachicho. Very thanks for the friendly working atmosphere.

Last but not least, I deeply thank to my wife, parents, and my sister for their understanding, encouragement and support. I am especially thankful to Oksana Coban not only for being a supportive wife but also a helpful colleague.