Black Soldier Fly Diet Impacts Soil Greenhouse Gas Emissions From Frass Applied as Fertilizer

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Increased global production of animal-based protein results in high greenhouse gas (GHG) emissions and other adverse consequences for human and planetary health. Recently, commercial insect rearing has been claimed a more sustainable source of animal protein. However, this system also leaves residues called frass, which—depending on the insect diet—is rich in carbon (C) and nitrogen (N), and could thus be used as fertilizer in agriculture. The impact of this kind of fertilizer on soil GHG emissions is yet unknown. Therefore, we investigated the effect of black soldier fly (Hermetia illucens L.) frass derived from a carbohydrate (Carb-) or a protein (Prot-) based diet applied at two different application rates to an arable soil on C and N fluxes and microbial properties in a 40-day incubation experiment. CO2, N2O, NO, N2, CH4, water extractable organic C (WEOC), and inorganic N were continuously measured quantitatively. At the end of the incubation, microbial biomass (MB), stoichiometry, community composition, and abundance of functional genes were assessed. Along with a strong increase in WEOC and CO2, Carb-frass caused strong initial N2O emissions associated with high N and C availability. In contrast, Prot-frass showed lower CO2 emissions and N2O release, although soil nitrate levels were higher. At the end of incubation, MB was significantly increased, which was more pronounced following Carb-frass as compared to Prot-frass application, and at higher amendment rates. Fungal abundance increased most from both frass types with an even stronger response at higher application rates, whereas bacterial abundance rose following Carb-frass as compared to Prot-application. Abundance of functional genes related to ammonia-oxidizing bacteria and archaea were enhanced by high frass application but did not clearly differ between frass types. C use efficiency of microorganisms, as revealed by the metabolic quotient, was most strongly reduced in the high Prot-frass application rate. Overall, insect diet influenced available C and N in frass and thus affected mineralization dynamics, GHG emissions, and microbial growth. Overall, emissions were very high undermining the potential environmental benefit of insect based protein production and calling for more detailed analyses before frass is widely applied in agriculture.

Keywords: mineralization, nitrification, denitrification, N2O, CO2, insect frass, organic fertilizer
INTRODUCTION

Global meat consumption has quintupled during the last five decades due to increasing incomes and population growth (Godfray et al., 2018). To meet the demand of animal feed, insect protein is increasingly used as feedstuff for fish, poultry, pigs, crustaceans, and as pet food. Insects have a good conversion of protein, fast growth rates, and can be reared on a number of different materials including agricultural and food wastes (Berggren et al., 2019). After the larvae have been harvested, a waste product remains. This so-called “frass” is a by-product of insect production consisting of feed for the insects, their shed exoskeletons, and feces. It contains many essential plant nutrients and is considered a potential organic soil amendment or fertilizer to promote plant growth (Barragán-Fonseca et al., 2020; Poveda, 2021).

The chemical composition of frass and its nutrient content mostly depend on the insect species and its diet (Berggren et al., 2019; Poveda et al., 2019; Barragán-Fonseca et al., 2020; Quilliam et al., 2020) with the N concentration of frass being directly proportional to diet N (Fielding et al., 2013). Accordingly, C:N ratios of frass can vary largely from 3.3 (Kagata and Ohgushi, 2012) to 20.1 (Lovett and Ruesink, 1995) depending on insect species and feeding regime. A number of studies has assessed the potential use of frass as organic fertilizer and reported rapid mineralization of nitrogen (N) and carbon (C) from mealworm (Tenebrio molitor L.) (Houben et al., 2020) and grasshopper (Melanoplus borealis FIEBER and Chorthippus curtipennis HARRIS) frass (Fielding et al., 2013). When compared to mineral fertilizers, frass of black soldier fly (Hermetia illucens L.) has produced similar yields in maize (Zea mays L.) (Beesigamukama et al., 2020), cabbage (Brassica oleracea L.) (Choi et al., 2009), ryegrass (Lolium perenne L.) (Klammsteiner et al., 2020), bok choi (Brassica rapa subspec. chinensis (L.) HANELT), lettuce (Lactuca sativa L.), and potatoes (Solanum tuberosum L.) (Temple et al., 2013).

While fast mineralization of organic materials and the resulting release of plant nutrients is beneficial to plant growth, it also increases the risk of nutrient losses to the environment. Organic fertilizers such as slurry and biogas digestate, lead to increased CO₂ and N₂O emissions after application which has been attributed to the simultaneous application of high amounts of easily available C and N (Köster et al., 2011; Zhu et al., 2015). Application of manure with a larger content of easily degradable organic C substrates tended to generate higher N₂O emissions than those with more recalcitrant organic C, confirming that both C and N are major controls of N₂O emissions from agricultural soils (Zhou et al., 2017). Increased losses of greenhouse gases from soils after frass application are likely, due to its high share of easily available N and C. Accordingly, increased CO₂ and N₂O fluxes from forest soil amended with feces of the nun moth (Lymantra monacha L.) or feces and Scots pine needles (Pinus sylvestris L.) have been measured under laboratory conditions (Grüning et al., 2018). Information about CO₂ and N₂O emissions of frass from commercially reared insects applied to soils is lacking. However, for a complete life cycle assessment and environmental evaluation of protein production from insects, assessment of the CO₂ and N₂O release is essential.

The rapid mineralization of frass and release of greenhouse gases is facilitated by a growing microbial community after application of easily available N and C from frass to soil. Derived from larval exoskeletons, chitin is one main component of frass. It can serve as an N and C source for soil microorganisms and has been shown to be degraded especially by bacteria (Kielak et al., 2013), whereas fungal chitin degradation has also been observed (Saratchodząra et al., 1996). Recently, frass originating from different commercially reared insect species has been shown to promote fungal over bacterial growth (Watson et al., 2021). The same study also showed an increase in archaeal abundance relative to bacterial abundance for some frass types and application rates and at the same time observed accumulation of NO₃⁻, which was believed to derive from archaeal oxidation of ammonium (Watson et al., 2021). Consequently, frass application seems to alter the microbial community composition in soil, which has consequences for N and C fluxes. Thus, the impact of frass on the soil microbial community composition and microbial functions needs to be included in investigations to understand the underlying mechanisms of N and C cycling.

Therefore, this study aimed to assess the risk of greenhouse gas emissions (CO₂, N₂O, CH₄) after application of frass of different qualities and quantities to an agricultural soil. We expected that the chemical quality of frass controls C and N turnover processes in soil and hypothesized (I) that CO₂ emissions will increase with increasing availability of organic C (Corg) and (II) highest N₂O emissions will occur when both Corg and NO₃⁻ availability is high. Furthermore, we hypothesized (III) that fungal abundance increases more strongly with higher C availability and (IV) that bacteria in general and bacterial nitrifiers are more abundant when N availability is high.

MATERIALS AND METHODS

Experimental Concept

We set up an automated incubation experiment under controlled laboratory conditions simulating organic fertilizer application in summer before sowing of the next winter crop. Frass from black soldier fly was applied to an agricultural soil. CO₂, N₂O, NO, N₂, and CH₄ fluxes from soil were measured continuously, and soil samples were collected to analyze soil mineral N and Corg dynamics. At the end of the incubation experiment, main soil microbial domains as well as functional genes related to N cycling were analyzed and the microbial biomass (MB) and its stoichiometry was quantitatively assessed.

Soil

The soil for the experiment was collected from a field site of the Höhere Landbauschule Rotthalmünster, Germany (latitude N48°21′, longitude E13°11′, elevation 360 m above sea level). It was sieved to 10 mm, air dried, and stored at 4°C until setup of the experiment. The soil was classified as a Haplic Luvisol with a silty loam texture (19% clay, 71% silt, 10% sand). Soil properties were: total C 1.23%, total N 0.14%, C:N 8.76, pH (CaCl₂) 6.74.
Prior to the experimental setup, soil was rewetted to 50% water-holding capacity (WHC, equivalent to 18.3% gravimetric water content) and preincubated for 4 weeks at 20°C.

Frass
For this experiment, we compared two types of frass from black soldier fly (H. illucens) differing in their feeding regime. In the carbohydrate-based feeding regime (Carb), larvae were reared on pre-consumer vegetable wastes; in the protein-based feeding regime (Prot), larvae were reared on brewer's spent grains and spent coffee grounds. Frass was frozen and dried at 70°C for 10 h as it is required for sterilization before application in agriculture. We applied both frass samples in two application rates: 0.13 g N kg⁻¹ equivalent to 170 kg N ha⁻¹ (−170), the maximum organic fertilization per year according to the German Fertiliser Ordinance (DüV, 2017), based on the EU’s Nitrates Directive (Council directive of 12 December 1991 concerning the protection of waters against pollution caused by nitrate from agricultural sources, 2008). The second dose was 0.39 g N kg⁻¹ (equivalent to 510 kg N ha⁻¹, −510), the maximum fertilization rate for compost for a three year period according to the German Fertiliser Ordinance (DüV, 2017). Even when lower amounts of N are used, spots with high fertilization are likely to occur when organic fertilizers are not evenly incorporated on a field site. An overview of treatments and added amounts of N and C is presented in Table 1. A more detailed analysis of nutrients in frass is included in the Supplementary Table 1.

### Table 1 | Chemical composition of frass types and added amounts of N and C.

| Feeding regime | Carb-170 | Carb-510 | Prot-170 | Prot-510 |
|----------------|----------|----------|----------|----------|
| Total nitrogen (% DM) | 2.65 | 3.86 | 4.46 | 51.3 |
| Total organic C (% DM) | 44.6 | 51.3 | 16.8 | 13.3 |
| C:N | 16.7 | 1.4 | 1.7 | 0.9 |
| Water extractable org. C (% DM) | 16.7 | 1.4 | 1.7 | 0.9 |
| Water extractable N (% DM) | 9.80 | 1.65 | 9.80 | 1.65 |
| N addition (g kg⁻¹) | 0.13 | 0.39 | 0.13 | 0.39 |
| N addition (kg ha⁻¹) | 170 | 510 | 170 | 510 |
| Corg addition (g kg⁻¹) | 2.20 | 6.60 | 2.20 | 6.60 |

Automatized Laboratory Incubation Experiment and Gas Analysis
The 40 days incubation experiment was carried out under fully controlled conditions using an automated soil incubation system with artificial atmosphere described in detail by Rummel et al. (2021a). Three kg pre-incubated soil (dry mass equivalent) was evenly mixed with frass. Then, soil moisture was adjusted to 70% water-filled pore space (WFPS, equivalent to 75% WHC or 27.4% gravimetric water content) by spraying H₂O dest. onto the soil-frass-mixture and thoroughly stirring it with a spoon. The equivalent of 2.5 kg dry soil was filled into acrylic glass pots (inner diameter 172 mm, total height 210 mm) with a porous ceramic plate at the bottom. Soil height in the pots was 8.5 cm, and bulk density was 1.3 g cm⁻³. The remaining soil-frass-mixture was filled into plastic boxes (bulk density 1.3 g cm⁻³) and closed with lids. All treatments were replicated four times.

Pots were tightly closed with transparent acrylic glass lids with rubber seals, and the outside of pots was covered with dark plastic sheets to prevent algal growth. To replace headspace atmosphere, pots were alternately evacuated using a rotary vacuum pump (Pfeiffer Vacuum GmbH, Asslar, Germany) and flushed with a gas mixture (82% He, 18% O₂) for 12 h. The gas mixture was prepared by using stainless steel capillaries of different length and inner diameter. The outlet of each pot was connected to flow-through multi-position valves (16 ports, Vici Valco Instruments, Houston, TX, USA) with multi-position actuator control modules (Vici Valco Instruments) controlled by Trilution Software (Gilson Inc., Middleton, WI, USA) via an interface module (506C System Interface, Gilson Inc., Middleton, WI, USA). The selected stream outlet tube of the multi-position valve was connected to a gas chromatograph (GC-450, Bruker, Billerica, USA) equipped with a thermal conductivity detector (TCD) for measurement of CO₂, O₂, and high concentrations of N₂O and a pulsed discharge detector (PDD, Vici AG International, Schenkon, Switzerland) for measurement of N₂O, N₂, and CH₄. The sample gas outlet of the GC was connected to a flow-through massflowmeter (Alicat Scientific, Tucson, AZ, USA), and a trace-level gas analyzer (CLD 88Yp, Eco Physics AG, Dürnten, Switzerland) equipped with a chemiluminescence detector (CLD) to analyze NO concentrations. During the incubation experiment, the headspace of the pots was flushed at a flow rate of ~40 ml min⁻¹. To add up to the required 300-mL-flow of the NO analyzer, samples were diluted with synthetic air. Processing of GC data was done using CompassCDS software (SCION Instruments, Livingston, UK). Data from the NO analyzer and flowmeter were read out every 10 sec via a serial port. The analytical precision of the GC was determined by repeated measurements of standard gases (CO₂, N₂O, N₂, CH₄) and was consistently <2%. Detection limits were 0.2 µg N₂O-N kg⁻¹ h⁻¹, 1.1 µg N₂-N kg⁻¹ h⁻¹, and 0.4 µg CH₄-C kg⁻¹ h⁻¹.

One pot was not airtight leading to drying out of soil and non-evaluable measurements. Therefore, treatment Prot-170 only had three replicates for gas measurements and final soil sampling.
Soil Analyses
Samples of pre-incubated soil were taken prior to experimental setup. After opening pots at the end of the experiment, soil from each pot was homogenized and a sample was taken for analyses. During the incubation experiment, soil samples were taken from the plastic boxes every 2–5 days. Subsamples were analyzed for soil mineral N, water-extractable C (WEOC), and soil water content. For analysis of mineral N (NO$_3^-$, NO$_2^-$, and NH$_4^+$), fresh soil was extracted with 2 M KCl solution (1:4 w/v) for 60 min. The pH of the extraction solution was adjusted to pH = 7 by addition of 0.1% 2 M KOH. Samples were filtered with 615 µm filter paper (Macherey–Nagel GMBH and Co. KG, Düren, Germany) and extracts were analyzed colorimetrically using the San$^{\text{TM}}$-Continuous-Flow Analyzer (Skalar Analytical B.V., Breda, The Netherlands). To determine WEOC, fresh soil was homogenized with H$_2$O$_{\text{bidest}}$ (1:5 w/v) (Burford and Bremner, 1975). Samples were centrifuged, filtered with 0.45 µm polyether sulfone filters (Labsolute, Renningen, Germany), and stored at −20°C. The extracts were analyzed for organic C and total N content using a multi N/C 2100 S Analyzer (Analytik Jena, Jena, Germany). Soil water content was determined by oven drying at 105°C. Soil pH was measured in 0.0125 M CaCl$_2$ solution.

Soil Microbial Biomass
C and N in microbial biomass (MBC and MBN respectively) was estimated using the chloroform fumigation extraction (CFE) method from a subsample of fresh 2 mm-sieved soil (Brookes et al., 1985; Vance et al., 1987; Joergensen and Brookes, 2005). Subsamples of 10 g of fresh soil were used as non-fumigated sample (nF), a second was fumigated (FUM), and a third was oven dried at 105°C for dry matter analysis. nF and FUM samples were extracted with 0.5 M K$_2$SO$_4$ (1:4 w/v) and filtered. Extracts were analyzed for total organic C (TOC) and total N (tN) using a multi N/C® 2100 S Analyzer (Analytik Jena, Jena, Germany). Soil water content was determined by oven drying at 105°C. Soil pH was measured in 0.0125 M CaCl$_2$ solution. MBC was estimated using the chloroform fumigation extraction (CFE) method from a subsample of fresh 2 mm-sieved soil (Brookes et al., 1985; Vance et al., 1987; Joergensen and Brookes, 2005). Subsamples of 10 g of fresh soil were used as non-fumigated sample (nF), a second was fumigated (FUM), and a third was oven dried at 105°C for dry matter analysis. nF and FUM samples were extracted with 0.5 M K$_2$SO$_4$ (1:4 w/v) and filtered. Extracts were analyzed for total organic C (TOC) and total N (tN) using a multi N/C® 2100 S Analyzer (Analytik Jena, Jena, Germany). Soil water content was determined by oven drying at 105°C. Soil pH was measured in 0.0125 M CaCl$_2$ solution.

Molecular Analyses
Soil DNA was extracted from fresh, 2 mm-sieved soil with the FastDNA® Spin Kit for Soil (MP Biomedicals, Santa Ana, USA) following the manufacturer's instructions but with modifications according to Hemkemeyer et al. (2014). Briefly, 0.5 g fresh soil was bead beaten twice at 6.5 m s$^{-1}$ for 45 s in a FastPrep® Instrument (MP Biomedicals) and subsequently centrifuged at 14,000 × g. After protein precipitation of the supernatant, the solution was mixed with binding matrix, which, in addition, was washed twice with 1 ml 5.5 M guanidine thiocyanate (Carl Roth, Karlsruhe, Germany) in order to remove soil contaminants. After transfer to spin filters and washing twice with kit-derived “SWS-M,” DNA was eluted with 100 µl PCR-grade water (kit’s “DES”). Subsequently the eluate was added back to the binding matrix and centrifuged again to increase DNA elution yield. Soil DNA extracts were tested for PCR inhibitors using a DNA amplification inhibition test as described previously by Guerra et al. (2020) and diluted fifty-fold prior to quantitative real-time PCR (qPCR).

Quantification of microbial domains/kingdoms was done targeting the Internal Transcribed Spacer 1 region (ITS1) for fungal quantification and the small ribosomal subunit (16S rRNA) for quantification of bacteria and archaea. Cloning fragments for bacterial and archaeal qPCR standards originated from the following species Bacillus subtilis (bacteria) and Methanobacterium oryzae (archaea) and were produced from DNA via conventional PCR with the primers F27 and 1391R (bacteria; Lane, 1991; Blackwood et al., 2005, respectively) and A109f and Ar1384r (archaea; Großkopf et al., 1998; Lueders et al., 2004, respectively). Plasmids containing the cloning fragment were produced by molecular cloning with the pGEM®-T Vector System II Kit (Promega Corporation, Madison, USA) utilizing Escherichia coli JM109 High Efficiency Competent Cells. Plasmid purification from transformed cells was done with the HiYield® Plasmid Mini DNA Kit (Süd-Laborbedarf GmbH). Standard serial dilutions were made as follows: 1 × 10$^8$–1 × 10$^2$ copies µl$^{-1}$ (bacteria), and 1 × 10$^5$–1 × 10$^4$ copies µl$^{-1}$ (archaea). Fungal qPCR standards were produced via conventional PCR with the primers NSA3 and NLC2 (Martin and Rygiewicz, 2005) using the fungal species Metarhizium brunneum, and directly used in the qPCR omitting the molecular cloning step. The serial dilution for the fungal standards was 1 × 10$^8$–1 × 10$^4$ copies µl$^{-1}$. Detection of microbial domains/kingdoms was performed in duplicates with a sample dilution of 1:50 in a Light Cycler® 480 Instrument II (Roche Diagnostics, Mannheim, Germany) with a total reaction volume of 20 µl containing 2 µl of the 1:50 diluted sample volume. Fungal ITS1 region amplification was performed using the primers NS11 and 58A2R (0.4 µM) (Martin and Rygiewicz, 2005) in a SYBR Green I HRM dye system (1 × concentrated, LightCycler® 480 SYBR Green I Master.
Cumulative emissions were calculated for CO2 and N2O losses:

\[ \text{EF} (\%) = \frac{E_{\text{Treatment}} - E_{\text{Control}}}{\text{Input}} \times 100 \]

Where \( E_{\text{Treatment}} \) are the mean cumulative CO2 or N2O emissions (mg kg\(^{-1}\)) from the respective treatment, \( E_{\text{Control}} \) are the mean cumulative CO2 or N2O emissions from the Control (mg kg\(^{-1}\)), and Input is the amount of N or C that was added with frass (mg kg\(^{-1}\)).

The metabolic quotient (qCO2) was calculated from the average daily basal respiration of the last seven days of incubation, as the quotient of mg CO2-C d\(^{-1}\) kg\(^{-1}\) soil dry matter and the microbial biomass carbon (g MBC kg\(^{-1}\) soil dry matter) at the end of incubation, and is expressed as mg CO2-C g\(^{-1}\) MBC.

Microbial biomass elements, metabolic quotient, and molecular data were checked for homoscedasticity and normal distribution using residual-vs.-fitted- and q-q-plots, respectively. If necessary, data were Box-Cox transformed using R package MASS (Venables and Ripley, 2002) prior to calculating one-way ANOVA using car (Fox and Weisberg, 2011). If requirements were still not met, the Kruskal-Wallis test was performed. As post hoc tests either estimated marginal means using emmeans (Lenth, 2019) or Dunn test using FSA (Ogle et al., 2018) were employed and supported by cld command from multcomp (Hothorn et al., 2008) and cldList command from rcompanion (Mangiafico, 2018), respectively.

RESULTS

Soil Mineral N and Water-Extractable Organic C

Chemical composition of frass types controlled C and N mineralization dynamics. Water extractable organic C (WEOC) content increased directly after addition of frass to soil (Figure 1). WEOC content was highest in Carb-170 and Carb-510, reaching maximum values of 120 and 301 mg C kg\(^{-1}\) on 2 DAO, respectively, then rapidly decreased. WEOC dynamics then followed a similar pattern in all treatments with smaller increases between 16 and 24 DAO and after 33 DAO.

Soil mineral N content was 42.68 mg NO\(_3\)\(^-\)N kg\(^{-1}\) and 0.58 mg NH\(_4\)\(^+\)N kg\(^{-1}\) at onset of the incubation experiment (Figure 2). In the unamended Control treatment, soil NO\(_3\)\(^-\) content stayed on a similar level throughout the experiment. Both frass types had contrasting effects on soil mineral N dynamics. In treatments amended with Prot-170 and Prot-510, soil NO\(_3\)\(^-\) content increased after frass application, reaching maximum values in Prot-510 on 8 DAO. In Carb-170 and Carb-510, soil NO\(_3\)\(^-\) content decreased after frass application and was below detection on 2 and 5 DAO in Carb-170. Toward the end of...
the incubation experiment, soil NO$_3^-$ content increased in Carb-
treatments.

Soil NH$_4^+$ content increased in all frass treatments directly 
after onset of incubation (Figure 2B). Highest NH$_4^+$ content was 
measured in the treatments with triple frass addition (Carb-510, 
Prot-510) on 2 and 5 DAO, after which soil NH$_4^+$ decreased 
below detection limits (<0.16 mg N kg$^{-1}$). Toward the end of 
the incubation experiment, NH$_4^+$ content slightly increased in all 
treatments reaching values around 1 mg N kg$^{-1}$. NO$_2^-$ content 
was below detection (<1.16 mg N kg$^{-1}$) in all treatments at all 
sampling dates.

In Control, Carb-170, and Prot-170, there was slight net N 
immobilization (−6 to −3 mg kg$^{-1}$), while addition of Carb-510 
and Prot-510 led to net N mineralization (41 and 10 mg N kg$^{-1}$, 
respectively, Table 2).

**Soil C Fluxes and Cumulative Emissions**

CO$_2$ fluxes from soil were higher in all frass treatments 
compared to the unamended Control treatment (Figure 3). In 
Control, CO$_2$ fluxes of around 0.1 mg CO$_2$-C kg$^{-1}$ h$^{-1}$ were 
constant throughout the incubation experiment. The highest 
CO$_2$ fluxes (11 mg CO$_2$-C kg$^{-1}$ h$^{-1}$) were measured in Carb-
510 during the first days after onset of incubation. After the 
initial peak, CO$_2$ fluxes decreased throughout the incubation 
experiment in all treatments. CO$_2$ fluxes from Carb-510 and 
Carb-170 were higher than from Prot-510 and Prot-170,
Figure 2 | (A) NO$_3^-$ and (B) NH$_4^+$ content in soil during the incubation experiment (mean ± standard deviation for n = 4, for Prot-170 n = 3).

Table 2 | Microbial biomass and net N mineralization (mean ± standard deviation for n = 4, net mineralization for Prot-170 n = 3).

|             | Net N mineralization (mg N kg$^{-1}$) | Microbial biomass N (mg kg$^{-1}$) | Microbial biomass C (mg kg$^{-1}$) | Microbial biomass C:N (mol mol$^{-1}$) |
|-------------|---------------------------------------|-----------------------------------|-----------------------------------|--------------------------------------|
| Initial     | –                                     | 37.0                              |                                   | 113.8                                |
| Control     | –4.0 ± 2.8                            | b c                               | 17.2 ± 6.2                        | c                                    | 82.7 ± 8.3                           | e 6.0 ± 1.5 ab                        |
| Carb-170    | –2.8 ± 9.0                            | b c                               | 60.9 ± 4.6                        | a                                    | 250.0 ± 11.3                         | c 4.8 ± 0.3 b                         |
| Carb-510    | 41.2 ± 7.2                            | a                                 | 70.2 ± 2.3                        | a                                    | 434.0 ± 31.4                         | a 7.2 ± 0.5 a                         |
| Prot-170    | –5.7 ± 15.1                           | c                                 | 45.6 ± 1.1                        | b                                    | 167.2 ± 21.5                         | d 4.3 ± 0.5 b                         |
| Prot-510    | 10.4 ± 12.4                           | b                                 | 66.9 ± 7.2                        | a                                    | 333.0 ± 21.3                         | b 5.8 ± 0.6 ab                        |

Different letters in one column indicate a significant difference between treatments.
respectively. CO₂ fluxes were positively correlated with soil WEOC content over the total incubation period ($R^2 = 0.8$, $p < 0.001$).

Cumulative CO₂ emissions were higher in frass-amended treatments compared to the unamended Control treatment and higher in the treatments with higher frass input (Table 3). When triple amounts of C were added, cumulative CO₂ emissions increased by a factor of 2.8–2.9 for both frass types. Accordingly, emission factors (EF) for C lost as CO₂ were stable within one frass type but about three times higher in Carb than in Prot (EF: 13–13.5% and 4.5–5%, respectively). Cumulative CO₂ emissions were positively correlated with MBC at the end of the experiment (Supplementary Figure 4A).

In one replicate of Carb-510, CH₄ fluxes were measured from 27 to 30 DAO reaching maximum fluxes of 4.2 µg CH₄–C kg⁻¹ h⁻¹ and 0.83 mg CH₄–C kg⁻¹ in total (Supplementary Figure 1).

**Soil N Fluxes and Cumulative Emissions**

N₂O fluxes increased in all treatments immediately after onset of incubation reaching maximum fluxes within 48 h after frass addition and then decreased rapidly (Figure 4A). In Carb-510, N₂O fluxes ranged between 1 and 2 mg N₂O-N kg⁻¹ h⁻¹ for a short period of less than 24 h and in Carb-170, maximum N₂O fluxes were 0.8 mg N₂O-N kg⁻¹ h⁻¹. In contrast, in Prot-170 and Prot-510, maximum N₂O fluxes were 0.3 mg N₂O-N kg⁻¹ h⁻¹.

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**FIGURE 3** | CO₂ fluxes from soil during the incubation experiment (mean ± standard deviation for $n = 4$, for Prot-170 $n = 3$).

**TABLE 3** | CO₂, CH₄ emissions, emission factors for N₂O and CO₂, and CO₂ emissions per unit of microbial biomass ($q_{CO₂}$) (mean ± standard deviation for $n = 4$, net mineralization for Prot-170 $n = 3$).

|        | CO₂ (mg CO₂-C kg⁻¹) | CH₄ (mg CH₄-C kg⁻¹) | CO₂ emission factor (%) | N₂O emission factor (%) | $q_{CO₂}$ (mg CO₂-C d⁻¹ g⁻¹ MBC) |
|--------|---------------------|---------------------|-------------------------|-------------------------|----------------------------------|
| Control | 82.6 ± 7.6          | d                   | 13.0 ± 1.1              | 9.29 ± 2.57             | 20.0 ± 1.4 b                     |
| Carb-170 | 943.8 ± 71.6       | b                   | 13.5 ± 0.7              | 5.52 ± 0.99             | 26.8 ± 8.6 ab                    |
| Carb-510 | 2763.3 ± 144.8    | a                   | 4.5 ± 1.3               | 3.41 ± 0.50             | 44.5 ± 5.9 a                     |
| Prot-170 | 315.4 ± 65.2       | c                   | 5.1 ± 0.8               | 3.11 ± 0.13             | 26.8 ± 16.2 ab                   |
| Prot-510 | 874.6 ± 121.5      | b                   | 4.5 ± 1.3               | 3.31 ± 0.13             | 32.9 ± 2.3 ab                    |

*only one replicate.

Different letters in one column indicate a significant difference between treatments.
FIGURE 4 | (A,B) N₂O fluxes, (C) N₂ fluxes, and (D) NO fluxes from soil during the incubation experiment (mean ± standard deviation for \( n = 4 \), for Prot-170 \( n = 3 \)).

TABLE 4 | Cumulative N gas emissions (mean ± standard deviation for \( n = 4 \), for Prot-170 \( n = 3 \)).

| Treatment | N₂O (mg N₂O-N kg⁻¹) | NO (µg NO-N kg⁻¹) | N₂ (mg N₂-N kg⁻¹) | N₂O/(N₂O+N₂) | N₂O (only first peak) (mg N₂O-N kg⁻¹) | N₂O (after day 5) (mg N₂O-N kg⁻¹) |
|-----------|----------------------|-------------------|-------------------|--------------|----------------------------------------|-----------------------------------|
| Control   | 1.14 ± 0.14          | 1.61 ± 0.21       | 7.02 ± 3.52       | n.s.         | 0.16 ± 0.07                            | 0.25 ± 0.04                       |
| Carb-170  | 13.29 ± 5.48         | 14.42 ± 4.15      | 8.52 ± 9.66       | n.s.         | 0.69 ± 0.24                            | 0.66 ± 0.35                       |
| Carb-510  | 22.79 ± 1.35         | 46.99 ± 8.41      | 8.90 ± 4.19       | n.s.         | 0.72 ± 0.12                            | 2.98 ± 0.88                       |
| Prot-170  | 5.48 ± 3.39          | 32.94 ± 8.01      | 9.94 ± 6.59       | n.s.         | 0.39 ± 0.14                            | 1.78 ± 0.71                       |
| Prot-510  | 14.50 ± 1.98         | 145.55 ± 32.12    | 15.47 ± 13.40     | n.s.         | 0.58 ± 0.25                            | 7.24 ± 2.94                       |

Different letters in one column indicate a significant difference between treatments.

In the unamended Control treatment, N₂O fluxes decreased after its initial peak until 10 DAO after which they were close to zero (Figure 4B). In the frass-amended treatments, N₂O fluxes stayed on a similar level in Carb-170 and Prot-170 after 5 DAO. In Prot-510, N₂O fluxes increased again to ∼12 µg N₂O-N kg⁻¹ h⁻¹ until 22 DAO after which they decreased until the end of the incubation experiment. In Carb-510, N₂O fluxes increased after 10 DAO with small peaks around 17-20 DAO and 33 DAO.

NO fluxes followed similar patterns as N₂O fluxes, but on a much lower scale (Figure 4C). NO fluxes showed a first peak directly after onset of incubation with a subsequent decrease to values <0.1 µg NO-N kg⁻¹ h⁻¹ in most treatments. In Prot-510, NO fluxes increased after 10 DAO reaching highest values of ∼0.45 µg NO-N kg⁻¹ h⁻¹ around 20 DAO. After 5 DAO, NO fluxes showed a strong positive relationship with N₂O fluxes \((R^2 = 0.45, p < 0.001)\). Measurements of N₂ fluxes were started 48 h after onset of incubation to avoid contamination with N₂ of the soil air. In Carb-170, Prot-170, and Control, N₂ fluxes decreased throughout the incubation experiment (Figure 4D). N₂ fluxes increased in Prot-510, where they peaked around 5 DAO. In Carb-510, N₂ fluxes were lowest from 4 to 10 DAO, after which they increased to a similar level as Prot-510.
Cumulative N$_2$O emissions were higher in all frass-amended treatments compared to the Control (Table 4). During the first 5 days, cumulative N$_2$O losses were highest in Carb-510 and Carb-170, while cumulative N$_2$O losses were highest in Prot-510 and Prot-170 from 5 DAO until the end of the incubation experiment. Over the whole incubation period, cumulative N$_2$O losses were highest in Carb-510. Losses from −510 treatments were always higher compared to the −170 treatments. The N$_2$O EF was highest for Carb-170 and Carb-510 (EF = 9.3% and 5.5%, respectively). For Prot-170 and Prot-510, EF did not change with added amounts and was 3.3–3.4%.

Cumulative NO emissions were higher in frass-amended treatments than in the Control with highest NO losses in Prot-510. NO losses from −510 treatments were always higher compared to −170 treatments. Differences between cumulative N$_2$O losses were small and not statistically significant.

### Microbial Community

#### Soil Microbial Biomass, Structural, and Functional Community Composition

At the end of the incubation, microbial biomass C and N (MBC, MBN) were higher in frass-amended soils compared to the Control (Table 2, Supplementary Figures 2A,B). Treatments that received 510 kg N ha$^{-1}$ of frass showed four to five-fold higher MBC, those with 170 kg N were two to three-fold higher (Table 2). Carb-treatments had higher MBC, than Prot-treatments. MBN was about 3 to 4 times higher compared to the Control, but differences between frass amendments were less pronounced (Table 2). Interestingly, within frass amendments higher application rates led to higher molar microbial biomass C:N though this was not always significant (Table 2, Supplementary Figure 2C).

Compared to the Control, frass addition with 170 kg N ha$^{-1}$ increased the abundance of bacteria and fungi 1.5- and seven-fold, respectively (Supplementary Figure 3; Supplementary Table 2). Frass addition to supply 510 kg ha$^{-1}$ of frass-N, led also to a three-fold higher fungal abundance compared to 170 kg N ha$^{-1}$, while in the case of bacteria this was just 1.5-fold. Accordingly, the fungi-to-bacteria ratio increased following frass application and was highest in treatments with 510 kg N ha$^{-1}$ (Figure 5A). In contrast, archaea did not respond to frass addition at all (Supplementary Figure 3B) and, thus, the archaea-to-bacteria ratio decreased. This decline was stronger in carbohydrate-based treatments (Figure 5B). The same patterns were reflected by bacterial and archaeal amoA gene copy numbers though less pronounced (Figure 5C).

AOA amoA were not affected by the addition of frass, whereas gene copy numbers of AOB amoA were promoted by the Carb-510 treatment as compared to the Control (Figures 6A,B; Supplementary Table 2). The copies of nirK genes doubled in both treatments that added 510 kg N ha$^{-1}$ compared to Control (Figure 6C). The copies of nirS genes were only promoted by frass obtained from the carbohydrate-based feeding regime at both concentrations, where their abundance increased about five-fold (Figure 6D). Though the higher application rate of protein-based frass led to two-fold higher abundances, this was not reflected in the Dunn test. All four treatments increased nosZ clade I gene copies. The treatments with 510 kg N ha$^{-1}$ showed 2.6-fold higher nosZ clade I gene abundance, while in the treatments with 170 kg N ha$^{-1}$ this was 2.1-fold (Figure 6E). Gene copies of nosZ clade II were promoted in the Carb-510
treatments as compared to the Control without frass addition (Figure 6F).

Cumulative soil CO$_2$ and N$_2$O emissions were positively correlated with measurements of soil microbial population size (i.e., MBC, MBN, bacterial 16S rRNA, and fungal ITS gene abundance) (Supplementary Figures 4, 5). Furthermore, increased cumulative soil N$_2$O emissions were associated with increased denitrifier gene abundance (i.e., nir and nos genes) ($r = 0.73–0.79$, $p \leq 0.0005$).

The Prot-510 treatment had the highest mean CO$_2$-C emissions per unit of microbial biomass carbon ($q_{CO_2}$) and the control treatment had the lowest, with the other treatments being intermediate between the two, but not being statistically separable from the control and Prot-510 treatments by the estimated marginal means post-hoc test (Table 3; Supplementary Table 2).

**Microbial Ionome at the End of Incubation**

Further chloroform-labile elements derived from the microbial biomass were P, S, K, Na, Mg, Fe, Mn, and Cu, which broadly followed CaCl$_2$-extractable elements (Supplementary Tables 3 and 3.1). Accordingly, MBP followed the pattern of available

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**FIGURE 6** (A,B) Abundance of functional genes involved in ammonia oxidation, (C,D) nitrite reduction, and (E,F) nitrous oxide reduction. Means ± standard deviation ($n = 4$) and letters indicating significant differences (for statistical data see Supplementary Table 2) are shown.
Effect of Frass Quality on C and N Mineralization

The chemical composition of black soldier fly frass is largely dependent on the insects’ feeding regime and diet N content (Fielding et al., 2013; Barragán-Fonseca et al., 2020; Quilliam et al., 2020). The two frass types compared in this incubation study were obtained from different feeding systems, a carbohydrate-based diet (Carb-) and a protein-based diet (Prot-). Prot-samples had a higher total N content and lower C:N ratio compared to the carbohydrate-based feeding system (Carb-) leading to strong differences in N mineralization dynamics between both frass types. In the Prot-treatment, low C:N ratio of frass led to mineralization and nitrification of organic N and increasing soil NH$_4^+$ and NO$_3^-$ content. The effect was stronger with higher N application rate in Prot-510 than in Prot-170. In the Carb-treatments, soil NH$_4^+$ content increased immediately after incorporation of frass to soil, while NO$_3^-$ content decreased leading to net N immobilization. C:N ratio of organic material is known to be a primary control whether N is mineralized or immobilized during decomposition (Robertson and Groffman, 2015). Accordingly, both rapid N mineralization (Temple et al., 2013; Houben et al., 2020; Klammsteiner et al., 2020) and N immobilization (Lovett and Ruesink, 1995; Kagata and Ohgushi, 2013) have been reported after frass application depending on frass C:N ratio. However, we measured net N immobilization in Control, Carb-170, and Prot-170 and net N mineralization in Carb-510 and Prot-510 over the whole incubation period of 40 days irrespective of frass C:N ratio.

C mineralization dynamics depended on both amount of C input and C quality. Carb-frass was characterized by a high share of water-soluble C$_{org}$ leading to increases in water-extractable organic C (WEOC) content in soil directly after application with higher values in Carb-510 than Carb-170. A similar effect was visible in Prot-treatments, but on a much lower level, which is consistent with the very low content of water-soluble C$_{org}$ in the Prot-frass. Water-soluble C compounds are the first to be leached from decomposing organic material and are directly available for soil microorganisms (Kögel-Knabner, 2002). High shares of soluble and labile C$_{org}$ and high C mineralization rates have been reported for mealworm, gypsy moth, and grasshopper frass (Christenson et al., 2002; Fielding et al., 2013; Houben et al., 2020). Accordingly, soil CO$_2$ fluxes increased strongly in frass amended treatments and showed similar dynamics as WEOC. The very good correlation between CO$_2$ fluxes and WEOC content confirms that C quality was the main driver of C turnover. C addition was slightly higher in Carb treatments than in Prot-treatments, as frass addition was based on providing equal amounts of N. To compare the amount of C lost as CO$_2$ irrespective of C input, CO$_2$ emission factors can be used. In our study, CO$_2$ emission factors were similar within one frass treatment and roughly 2.5 times higher for Carb- than Prot-frass, confirming that C quality was most important in controlling C mineralization and turnover in our study. Thus, our results confirm that diet is a crucial factor controlling chemical composition of frass and its mineralization dynamics.

DISCUSSION

Effect of Frass Quality and Mineralization on N Gas Losses

N$_2$O fluxes were highly dynamic and followed different patterns between the first 5 days after onset (DAO) of incubation and the incubation period after 5 DAO. During the first days of incubation, N$_2$O emissions were mostly driven by high availability of easily degradable C and N leading to highest N$_2$O fluxes in Carb-510 and Carb-170. During peak N$_2$O emissions, the highest WEOC and high NH$_4^+$ content in soil were measured in Carb-510 and Carb-170, while NO$_3^-$ content was very low. In Prot-170 and Prot-510, N$_2$O fluxes also increased strongly, but on a lower magnitude, which can be attributed to the lower availability of C$_{org}$.

After 5 DAO, N$_2$O fluxes stayed on a stable level in Carb-170 and Prot-170 with higher emissions in Prot-170 reflecting the higher soil NO$_3^-$ availability. In Carb-510 and Prot-510, N$_2$O fluxes increased again. The interaction of C and N availability on controlling N$_2$O fluxes was validated by analysis of regression, highlighting a strong positive interaction between soil NO$_3^-$ and WEOC concentrations after 5 DAO. It is known, that denitrification in soil is largely controlled by the supply of readily decomposable organic matter (Azam et al., 2002; Chantigny et al., 2007; Loecke and Robertson, 2009). Thus, high N$_2$O losses have been explained by combined availability of C and N after incorporation of organic fertilizers (Köster et al., 2011; Zhu et al., 2015, Zhou et al., 2017) or plant litter (Rummel et al., 2013; Houben et al., 2020; Quilliam et al., 2020).
This rapid mineralization makes nitrification and coupled nitrification-denitrification potential pathways of N₂O production, while nitrifier denitrification is unlikely due to high C availability (Wrage et al., 2001; Wrage-Mönning et al., 2018). Furthermore, contribution of fungal denitrification is likely as increasing contribution of fungal denitrification has been reported under conditions of high C and N availability after litter application (Laughlin and Stevens, 2002; Wu et al., 2017; Senbayram et al., 2018; Zhong et al., 2018; Rummel et al., 2021a). This is in accordance with the promotion of fungi in our study and other studies with frass application (Lovett and Ruesink, 1995; Temple et al., 2013). To reliably distinguish between N₂O emission pathways, analysis of the N₂O isotopic signature is recommended in future studies (Yu et al., 2020).

In contrast to many other studies (Azam et al., 2002; Huang et al., 2004; Millar and Baggs, 2004, 2005; Frimpong and Baggs, 2010; Rummel et al., 2020), we did not find a correlation between CO₂ and N₂O fluxes in our study. Nevertheless, we did measure both high CO₂ and N₂O fluxes indicating that oxic and anoxic decomposition processes took place at the same time. Our results show that the N₂O emission dynamics are highly variable and N₂O fluxes may change rapidly within few hours. Therefore, peak emissions may easily be missed when only sampling once a day and continuous measurements are important to estimate the real gas losses to the environment and to allow for a reliable assessment of the environmental effects of frass.

NO fluxes followed a similar pattern as N₂O fluxes after 5 DAO and were positively correlated. The NO/N₂O emission ratio was always < 0.2 (Supplementary Figure 6A) indicating that NO was mostly produced from bacterial denitrification as the NO/N₂O emission ratio of bacterial denitrification is mostly around 0.01, while emission ratios of NO/N₂O from nitrification are often higher than 1 (Skiba et al., 1997; Cheng et al., 2004).

However, contribution of fungal denitrification to NO production has also been reported (Senbayram et al., 2018). N₂O fluxes decreased throughout the incubation experiment, with the exception of Prot-510, where the highest N₂O fluxes were measured on 5 DAO when N₂O fluxes were low. The product ratio of denitrification N₂O/(N₂O+N₂) was mostly < 0.5 (Supplementary Figure 6B) as more N₂ than N₂O was produced. Similar denitrification product ratios have been measured in studies using the same soil and similar soil moisture conditions (Rohe et al., 2021; Rummel et al., 2021b).

Cumulative N₂O emissions where higher in Carb-treatments than in Prot-treatments and higher in treatments receiving higher amounts of N. In Carb-treatments, the first 5 days contributed the largest part to the cumulative emissions, while in Prot-treatments, the second peak also largely contributed to cumulative emissions. The N₂O emission factors differed between frass types and application rates. In Prot treatments, EF were lower compared to Carb and did not change with application rate. This indicates that the amount of N lost as N₂O depended on the amount of available N and increased with higher N availability. In the Carb treatments, EF decreased with increasing frass input.

Compared to N₂O emissions from similar incubation studies with application rates of 150-160 kg N ha⁻¹, N₂O losses can be ranked as following: Prot-170 (3.4 mg N₂O-N kg⁻¹) < biogas digestate (12.6 mg N₂O-N kg⁻¹, Köster et al., 2015) < Carb-170 (13.3 mg N₂O-N kg⁻¹) < ammonium sulfate (14.1 mg N₂O-N kg⁻¹, Senbayram et al., 2012) < Prot-510 (14.5, mg N₂O-N kg⁻¹) < biogas residue (16.3 mg N₂O-N kg⁻¹, Senbayram et al., 2012) < Carb-510 (22.8 mg N₂O-N kg⁻¹) < cattle slurry (34.1 mg N₂O-N kg⁻¹, Köster et al., 2015). As soil type, incubation conditions, and especially soil moisture strongly affect N₂O emissions and dynamics, standardized values such as EF are better to compare emissions between studies. EF in our study were much higher than the emission factor of 1% of applied N estimated by the IPCC (IPCC, 2006). Actual emission factors in our study ranged between 3 and 9% of applied N, more similar to digestates (0-5.1%, Walling and Vaneckhauta, 2020) and manures (0.05-13.9%, Walling and Vaneckhauta, 2020). Thus, black soldier fly frass cannot be considered a ‘compost-like’ organic fertilizer or soil amendment, but should be treated similar to highly available organic fertilizers such as fresh manure or biogas digestates.

Effect of Frass Quality on Microbial Biomass, Community, and Activity

Both frass types increased MBC and MBN with Carb-frass and higher amounts both showing stronger increases. These results show the potential of frass for sequestering C as MBC is eventually converted into soil organic matter, which consists to a large part of microbial necromass (Khan et al., 2016). The high correlation between cumulative CO₂ and MBC was expected, as a larger microbial community releases more CO₂. When considering the metabolic quotient (qCO₂), which indicates the respiration per unit of microbial biomass and can be used as a first proxy of C use efficiency, it can be seen that the Prot-510 treatment, had a higher respiration per unit of MBC. Consequently, high Prot-frass application leads to less efficient conversion into microbial C and thus may less well contribute to SOC sequestration, as microbial physiology and efficient C use is key (Kallenbach et al., 2015). Carbon and nitrogen are well correlated in the soil microbial biomass (Xu et al., 2013), as both are required proportionally, explaining the higher N immobilization in the Carb-treatments by a community utilizing a resource with high C availability and a higher loss of N by mineralization in the Prot-treatments by a C-limited microbial community. Losses of C or N could be reduced by more balanced C to nutrient ratios, closer to microbial demand, of the frass applied. This should be tested in future studies by a simultaneous application of other nutrients in combination with frass, complementing the nutrient availability of the frass.

The addition of frass clearly stimulated fungi and the higher the application rate the greater their abundance. This was not just indicated by an increased fungi-to-bacteria ratio, but also by higher microbial biomass C:N in 510 kg N treatments compared to 170 kg N reflecting higher fungal compared to bacterial C:N as often observed (Mouginot et al., 2014). Promotion of fungi by frass application have also been reported elsewhere (Lovett and Ruesink, 1995; Temple et al., 2013; Watson et al., 2021).
Higher MBC to microbial element ratios were also reflected in the MBC:MBP and MBC:MBS ratios. Higher MBC:MBP ratios may be associated with fungal biomass (Mouginot et al., 2014). Although investigations into soil MBC:MBS ratios and concurrent changes in fungal to bacterial ratios are few, a reduced MBC:MBS ratio with increased fungal contribution to soil microbial biomass has been observed (Heinze et al., 2010), which contradicts the findings of the current study where an increased MBC:MBS ratio is associated with a higher fungal to bacterial ratio. However, recently, it has been shown, that fungi have quite flexible C:N and C:P ratios, which may well be true for other elements (Camenzind et al., 2021). A reduced MBC:MBMn ratio in the Carb-510 treatment may be due to involvement of Mn in glycolysis and lignin degradation (Jakubovics and Jenkinson, 2001; Datta et al., 2017; Hemkemeyer et al., 2021) which may be of higher importance in the Carb frass. Homeostatic behavior of MBC,K, MBC:Na and MBC:Mg ratios could be observed in this study, indicating relatively stable requirements of K, Na and Mg, per microbial C, across a changing soil microbial community composition, supplied with substrates differing in availability of K and Na, but not in the availability of Mg.

Interestingly, archaeal 16S rRNA and amoA genes did not respond at all to amount and type of frass. This contrasted findings of a study by Watson et al. (2021) in which application of frass derived from other insect species (mealworm, buffalo worm) stimulated archaeal abundance, which was linked with nitrite accumulation (high ammonium oxidation rates). In our study, despite increases in bacterial 16S rRNA and amoA gene abundances, frass addition only led to small differences in ratios of archael and bacterial amoA gene copy numbers at the end of incubation. Future studies need to consider the potentially relevant role of archaea in N cycling to improve our understanding of N fluxes in frass amended soils.

With few exceptions, the abundance of denitrification genes showed a positive response to both frass types, revealing the promotion of the overall denitrifier community size. Furthermore, if the community size was increased, the increment depended on the amount of frass added, especially for nirK and nosZ clade I denitrifiers, indicating that these groups strongly respond to frass addition, regardless of the type of frass. In contrast to their nirK counterpart, nirS-carrying microorganisms did not respond to addition of frass obtained from the protein-based feeding regime, indicating that the quality of the added organic material is important for nirS denitrifiers. Different responses of nirK and nirS denitrifiers to fertilization have frequently been reported (e.g., Yang et al., 2017), supporting the idea of niche differentiation of nir-type denitrifiers (Jones and Hallin, 2010). Similarly to nirS denitrifiers, nosZ clade II denitrifiers did not respond to frass from the protein feeding regime, while clade I types were strongly promoted, again pointing to different ecological niches (Hallin et al., 2018).

**Suitability of Frass as Organic Fertilizer**

To focus on frass turnover processes and C and N fluxes between soil and atmosphere, no plants were included in our study. However, as insect frass has been portrayed as an organic fertilizer to be applied similarly to compost or manure, it is not unlikely that frass would be applied during the fallow period before sowing/planting. In accordance with previous studies (Fielding et al., 2013; Houben et al., 2020), we found rapid mineralization of C and N from frass. While fast release of plant nutrients may be beneficial to promote plant growth, our results show clearly that there is a high risk of greenhouse gas losses including CO$_2$, NO, N$_2$O, and N$_2$, we measured substantial CH$_4$ emissions from one replicate of Carb-510. Thus, CH$_4$ emissions after frass application are possible—most likely when high amounts of C$_{org}$ are added, mineral N content is low, and soil moisture is very high such as after heavy rainfall. To better understand greenhouse gas emission dynamics and potential CH$_4$ emissions after frass application, we recommend further laboratory and field studies with different soils and moisture conditions and varying levels of frass addition. High NH$_4^{+}$ content of soil further increases the risk of N losses through NH$_3$ volatilization when soil pH is high. Thus, we do not recommend this type of frass to be used as a “compost-like” fertilizer before planting/sowing, as, besides C lost as CO$_2$ from mineralization N losses can cause distinctly negative environmental effects to the detriment of the proposed environmentally friendly protein production using insects. Application of frass as a nutrient source to growing plants may be one strategy to reduce N losses, as NO$_3^{-}$ will be quickly taken up minimizing the potential of N$_2$O emissions. However, effects of frass on plant growth also need more thorough evaluation, as high amounts of frass added can inhibit growth of plants (Newton et al., 2005; Temple et al., 2013; Alattar et al., 2016). As an alternative, frass may be mixed with C-rich material to facilitate microbial N immobilization and N conservation. This at the same time can shed further light on the potential of frass to sequester C in the soil, which is required for a full life cycle assessment of frass and the insect industry as a whole. Nonetheless, these strategies also require further investigation.

As our study is the first to measure greenhouse gas losses after frass application to agricultural soil, it is self-evident that more studies with different types of frass (insect species, diet, frass treatments such as drying), soil types, and incubation conditions are needed to reliably assess the potential of greenhouse gas losses. It is important to consider the stoichiometric needs of microorganisms when applying frass to reduce emissions and nutrient losses, and whether additional nutrients could be applied to frass to compensate for stoichiometric imbalances. Furthermore, trace elements should be considered as they play key roles in nutrient cycling processes.

**CONCLUSIONS**

Insect frass is a source of C and nutrients for soil microorganisms. Our study showed that, depending on C, N, and nutrient content of the frass, N will be immobilized or mineralized, and C will be used more or less efficiently by soil microorganisms resulting in microbial, in particular fungal growth. Further, high nutrient availability after frass addition to soil can result in substantial rapid losses of C (as CO$_2$, partly CH$_4$) and N gases, especially N$_2$O. This reduces the fertilizer (N) and organic amendment
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AUTHOR CONTRIBUTIONS

PSR and FW designed the experiment and PSR carried it out. SAS extracted microbial biomass elements and DNA. LB tested DNA quality for inhibitory effects. SAS and LB employed qPCR of structural and functional genes, respectively. MH coordinated molecular measurements and processed data. MH and SAS statistically analyzed molecular and microbial biomass elements data. PSR prepared the manuscript with contributions from all co-authors. All authors contributed to interpretation of results.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.709993/full#supplementary-material

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