Carbon flow in *Agaricus bisporus'* compost: a stable isotope biomarker study

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Abstract

The white button mushroom, *Agaricus bisporus,* is commercially produced on compost. The interaction between *A. bisporus* and the microbiota in the compost was examined using stable isotope techniques and phospholipid derived fatty acids (PLFAs) . Microcosms were constructed with phase II-end compost and different fungal and bacterial densities: industry standard fungal density, low fungal density, low bacterial density (autoclaved compost), and compost without *A. bisporus* spawn. ^{13}C labelled glucose was added to track the transfer of carbon from the compost into the microorganisms and CO_2 over 24-day incubation. A parallel experiment with different fungal densities was run in which additional ^{13}C labelled glucose was added at day 14 to alleviate any substrate limitation. The results showed that *A. bisporus* suppresses bacteria in the compost, with only 33.1% ± 6.8% and 27.3% ± 4.8% of initial bacterial biomass present after 24 days for different fungal densities, whereas in absence of *A. bisporus* the bacterial biomass increased with 127% ± 4.6%. However, low bacterial density compost did not significantly reduce the growth of *A. bisporus* (17 ± 6.8 mg mycelium g^{-1} compost) when compared to non-autoclaved compost (12 ± 1 mg mycelium g^{-1} compost). The composition of the bacterial community changed when *A. bisporus* was present, with a relative increase in activity of Gram- bacteria compared to Gram+ bacteria over time. There was little indication that the growth of *A. bisporus* is restricted by carbon limitation.

Keywords: *Agaricus bisporus*; compost; PLFA; microbial interactions; ^{13}C labelling

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Introduction

Agaricus bisporus, also known as the white button mushroom, is cultivated as a commercial crop in the Netherlands. The market value of the country's total export of button mushroom amounted to €400 million in 2013, making the Netherlands the second largest producer of button mushrooms in Europe (Logatcheva et al., 2014). Furthermore, it is a high quality food rich in essential nutrients (Atila et al., 2017) and bioactive compounds such as anti-cancer polysaccharides (Zhang et al., 2014a).
Commercially produced button mushrooms are grown on straw-based, pasteurized compost. The compost goes through two distinct composting phases before the addition of *A. bisporus*. In phase I (PI) wheat straw, horse manure, gypsum and water are mixed and during a time span of 3-6 days. During this phase a microflora, consisting mainly of actinobacteria and fungi, is established (Zhang et al., 2014b). The temperature increases to 80 °C due to microbial activity and mesophilic organisms are replaced by thermophilic microbiota (Carrasco et al., 2018). The thermophilic fungi, such as *Scytalidium thermophilum*, are beneficial for mushroom production since they promote the hyphal growth rate of *A. bisporus* (Wiegant et al., 1992; Straatsma et al., 1994). Phase II (PII) starts with pasteurization of the PI compost, followed by conditioning at 50 °C to remove the ammonia, insects and unwanted organisms that could cause rotting or spoilage of the mushroom (Jurak, 2015). After pasteurisation and the establishment of the compost microflora, *A. bisporus* spawn is added to the PII-end compost. After the inoculation of the compost starts Phase III (Figure 1). The spawning of the compost is usually in the form of rye grains inoculated with the fungal mycelium. During phase III, the fungi fully colonizes the compost in 16-19 days, during which 50% of the lignin, 15% of xylan and 10% cellulose is removed from the compost compared to PII-end compost (Jurak, 2015). The remaining compost contains 27% total carbohydrates. Growers then add a peat casing layer on top of the compost, which is colonized by *A. bisporus.* On its surface pins will form after the temperature and humidity are lowered and ventilation is increased, also called induction. These pins, or primordia, will grow to become the fruiting body of the mushroom (Figure 1). This last step represents the final phase of the commercial cultivation of the fungus, known as phase four (PIV). After PIV and the harvesting of the mushrooms, 56% of the xylan and an estimated 46% of the cellulose has not been consumed by *A. bisporus,* and 44% of the lignin remains compared to PI compost (Jurak, 2015), although these estimates are likely too high as they include *A. bisporus* own cellulose as well. Xylan fragments are only partially available as a source of carbon to the fungus (Jurak et al., 2015), leaving a significant amount of carbohydrates unused in the production of button mushrooms as waste. This industrial production of *A. bisporus* results in a large amount of waste from the spent compost after cropping, as 1 kg of fresh mushroom results in 5 kg spent compost (Finney et al., 2009).
In an effort to reduce the waste products from compost, previous studies have examined the nutrient uptake from the compost to the mycelium, which is not yet fully understood (Durrant et al., 1991; Iiyama et al., 1994; Jurak et al., 2015; Vos, 2017). In particular, which part of the mycelium is responsible for and the role of other microorganisms in this nutrient transport. These bacterial-fungal interactions are complex but important for the compost ecosystem functioning (Deveau et al., 2018). For instance, the hyphae of the fungi can facilitate the transport of bacteria to be more efficient (Kohlmeier et al., 2005), due to the presence of an amphipathic film on the hyphae made by hydrophobines (Wessels, 1997). Bacteria in turn provide a suitable environment for *A. bisporus* by suppressing competitors, facilitating the degradation of complex carbohydrates and promoting hyphae elongation (Kertesz and Thai, 2018). Furthermore, bacteria are a nutrient source for *A. bisporus*, since the mushroom is able to degrade living and dead bacterial biomass as its sole nutrient source (Fermor and Wood, 1981; Fermor et al., 1991). Previous work by Vos et al. (2017) on microbial biomass in *A. bisporus'* compost showed that the presence of *A. bisporus* lowers the microbial biomass and affects the composition of the microbial community. The proposed mechanism for this is that enzymes secreted by *A. bisporus* degrade the lignocellulose in the compost. The degradation products are nutrients for bacteria, which are in turn consumed by *A. bisporus* as a potential way to obtain its vitamins (Vos et al., 2017). The contribution of the microbial biomass to the fungal biomass has been

estimated to be less than 10% (Sparling et al., 1982), although the contribution might be higher during the initial stages of the fungal development. It is clear that microbial organisms in the compost play a key role in the cultivation of the button mushroom, but their contribution in nutrient acquisition for the developing mushroom has not yet been quantified, neither have all the trophic interactions between the organisms in the compost bed been established.

For establishing these trophic interactions and reconstruction of the present food web, biomarkers such as phospholipid-derived fatty acids (PLFA) have been proven to be useful in identifying organisms and estimating the microbial biomass (Frostegard and Baath, 1996; Boschker and Middelburg, 2002; Klamer and Bååth, 2004; Vos et al., 2017). These phospholipid fatty acids are present in bacteria, fungi, and other eukaryotes as structural component of the cell membranes. Some are characteristic to particular organisms and can thus be used as a specific biomarker for these taxa or as an environmental biomarker (Ruess and Chamberlain, 2010; Frostegård et al., 2011). In addition, the quantification of the PLFAs can be used to assess the amount of total biomass of an organism.

Stable isotope techniques in combination with PLFA analysis can further elucidate the structure of food webs. Certain organisms can have a specific isotopic signature. These signatures can be used to identify them in their natural environment and asses their trophic interactions (Ruess and Chamberlain, 2010). By introducing an isotopically labelled substrate into the food web, the route of the substrate can be traced through the food web. The absolute uptake of the labelled substrate can be quantified by the amount of incorporated label in the PLFAs. As the ^{13}C enrichment due to label addition is usually orders of magnitude greater than due to metabolic fractionation, it removes the need to consider the isotopic fractionation in lipid metabolism (Ruess and Chamberlain, 2010). Furthermore, as the incorporation of the labelled substrate indicates active metabolism, it can be used to quantify the active biomass present in the system. These stable isotope labelling techniques in combination with PLFA biomarker are commonly used in biogeochemical and environmental studies but have not been used before to study *A. bisporus'* compost system.

This study aims at further elucidating the interaction between bacteria and fungi using stable isotope techniques combined with biomarkers analysis. The carbon flow between organisms inside the compost bed will be studied quantitatively by looking at biomass, as well as qualitatively using labelling techniques. The PLFAs of the different microbial groups and their isotopic composition will be used to study the interaction between the microbiota and assess their carbon uptake. In addition, the carbon budget will be evaluated using the isotopic carbon ratio in the bulk carbon measurements, CO_2 respiration data and PLFA data. The focus will be on the interaction of *A. bisporus* with the microbial community in the compost and how their biomass is connected to the carbon flow in time. This will add insights into the conditions for optimal mushroom growth and the nutrient uptake efficiency of the mycelium.

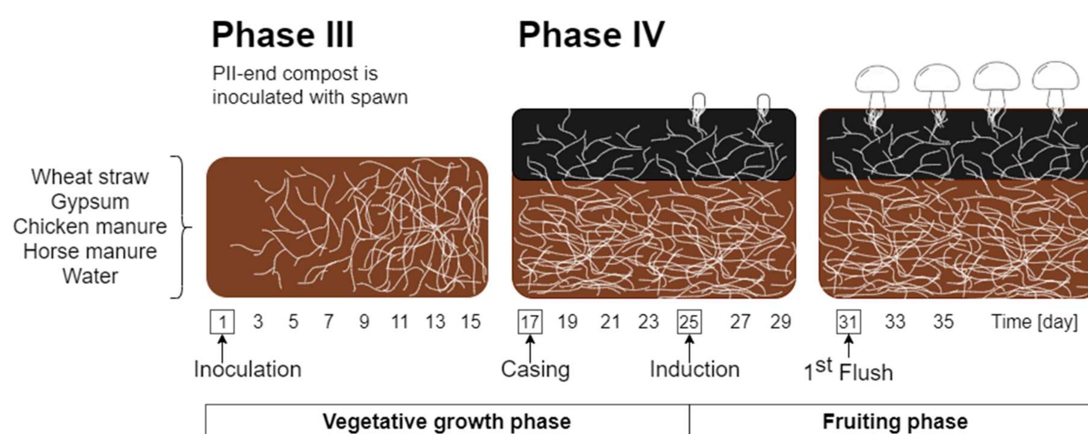


Figure 1 Schematic representation of the cultivation process of *A. bisporus.* Spawn is added to PII-end compost and in PIII the fungus colonizes the compost. The fruiting phase starts as pins start to form at the surface of the casing layer, which eventually develop into to the mushrooms' fruiting body.

Methods

Incubation experiment

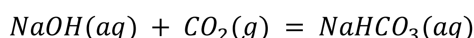
An incubation experiment with *Agaricus bisporus* strain A15 was carried out at Utrecht University for a total of 24 days. This incubation period is equal to the period that runs between inoculation of the compost to the mushroom pinning in the commercial production (Figure 1). Microcosms were constructed with 20 grams of PII-end compost (65.6% water content, CNC Grondstoffen©) in 300 mL glass bottles, loosely covered with aluminium foil to minimize any contamination but to allow air exchange with the atmosphere. Different conditions were tested in the microcosms by altering fungal and bacterial biomass. An additional parallel experiment was conducted to test substrate limitation, by adding additional glucose after two weeks of incubation.

Two conditions were set up to test the effect of the fungal density by adding 5 spawn grains per 20 grams of compost (1.5% spawn of total wet weight, industry standard) and 2 spawn grains supplemented by 3 sterile rye grains per 20 gram of compost (0.6% spawn of total wet weight compost). In another set of microcosms, the biomass of bacteria was reduced by autoclaving PII-end compost three times up to a temperature of 121 °C for 30 minutes before inoculating it with 5 spawn grains of *A. bisporus*. PII-end compost not inoculated with *A. bisporus* spawn served as a control. The four treatments are industry standard fungal density (1F), low fungal density (0.5F), low bacterial density (0.5B) and no *A. bisporus* spawn added (0F). To each treatment 1.1 mg ^{13}C-glucose and 0.05 mg ^{15}N-NH_4Cl per gram of compost was added before starting the incubation. Unlabelled ^{12}C-glucose and ^{14}N-NH_4Cl was added to the microcosms that served as a control for the isotopic background values for each treatment.

The parallel experiment was set up with PII-end compost inoculated with either 5 spawn grains or 2 spawn grains, in the same conditions as aforementioned. The normal fungal density with additional glucose is denoted as 1F*, the low fungal density as 0.5F*. To these parallel microcosms we added 1.1 mg ^{13}C-glucose and 11.4 mg additional unlabelled glucose with 0.05 mg ^{15}N-NH_4Cl per gram of compost. The extra addition occurred after 14 days of incubation to test substrate limitation. The microcosms consisted of biological triplicates which were incubated in the dark at 22°C and 80% relative humidity for 3, 7, 14, 24 days, with an additional timestep at 17 days for the parallel microcosms (see Appendix A for sampling diagram).

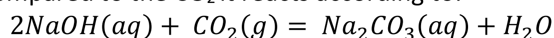
CO_2 production measurements

CO_2 sensors (GC-0006-W ExplorIR® 20%) were used to quantify the total respired CO_2 from the microcosms. Additionally, a CO_2 trap with 0.3M sodium hydroxide solution was used to distinguish the labelled fraction of this gas from the total. The microcosms were capped with an airtight lid for a two-hour period before ending the incubation. During this time, the NaOH trapped the CO_2 with the following reaction:



$$NaOH(aq) + CO_2(g) = NaHCO_3(aq)$$

If the NaOH is in excess compared to the CO_2 it reacts according to:



$$2NaOH(aq) + CO_2(g) = Na_2CO_3(aq) + H_2O$$

The isotopic values of the respired CO_2 in the NaOH solution were measured using a Gas Bench (Thermo Scientific™). The captured CO_2 was released by adding 80% phosphoric acid into helium filled glass tubes. Samples of the NaOH solution were used as blanks to correct for CO_2 uptake from the atmosphere into the NaOH traps during handling.

The CO_2 concentration in the bottles was measured with a CO_2 sensor for two replicates at each time point, until a stable value was measured for one minute using GasLab® software. The production rate was based on the amount of CO_2 accumulated in the microcosm after it was closed from the atmosphere for a one-hour period. The CO_2 production rate was calculated per gram of dry weight compost and corrected for the initial concentration of CO_2 present in the microcosm.

PLFA extraction

The compost samples were freeze-dried and homogenized by mechanical milling (Herzog HP-MA) into a fine powder. The lipids were extracted from the compost samples using a modified Bligh and Dyer method (Bligh and Dyer, 1959). The freeze-dried samples were sonicated for 10 minutes in a volumetric mixture 2:1:0.8 of MeOH:CH_2Cl_2:P-buffer, at the end of which the supernatant fluid was collected. This procedure was repeated a total of three times, after which phase separation of the supernatant fractions was induced by the addition of P-buffer and CH_2Cl_2 (DCM). The DCM layer was collected quantitively and dried under a N_2 stream. The total lipid fraction was separated into an apolar, neutral, and polar lipid fraction by eluting the sample in DCM, acetone, and methanol through a column of activated silica. The methanol fraction was collected and dried to obtain the polar lipid fraction. The sample was methylated with methanolic NaOH and toluene:MeOH (1:1, v:v) at 37 °C for 30 minutes together with a fatty acid methyl ester standard (C19:0). The reaction was stopped by adding acetic acid and water, after which it was washed three times with hexane. The collected hexane layer was dried and together with the C12:0 internal standard taken up in 220 µl hexane. To remove any water remnants or other substances that could interfere in the chromatography, the PLFAs samples were cleaned over a 1 cm column of a mix of aluminium oxide and sodium sulphate prior to analysis.

Bulk analysis

The bulk carbon and nitrogen concentrations, together with their isotopic distribution, were analysed with an Elemental Analyzer attached to an isotope-ratio mass spectrometry (EA-IRMS, Thermo Fisher Scientific). The obtained $\delta^{13}C$ and $\delta^{15}N$ values were used to calculate the amount of label incorporated into the bulk compost at each timestep. Background values were taken from the microcosms to which unlabelled glucose was added to calculate the total uptake of ^{13}C.

Isotope analysis

The PLFA samples were analysed using gas chromatography combustion isotope ratio mass spectrometry (GC-c-IRMS, Delta plus XP Thermo Finnigan). PLFAs were identified based on their retention times compared to the retention time of the fatty acid methyl esters (FAME) standards (Supelco 37 Component FAME Mix). Equivalent chain lengths (ECL) were calculated based of the retention times of C12:0, C16:0 and C19:0. For example:

$$ECL_{PLFA} = 12 + (16 - 12) \cdot \frac{RT_{PLFA} - RT_{C12:0}}{RT_{C16:0} - RT_{C12:0}}$$

Of the internal standards, C19:0 was also used to calculate the concentrations of all PLFAs. The $\delta^{13}C$ values of the PLFAs were measured using GC-c-IRMS with an apolar column (J&W CP-Sil 5 CB GC Column, 25 m, 0.32 mm, 0.12 µm) . The program started at an oven temperature of 70 °C and increased to 180°C at 2°C/min to reach 320°C at 20°C/min. The flow of the helium carrier gas was set to 1.5 ml/min.

The isotope ratios and PLFA concentrations were corrected and analysed using a custom R script, based on the Rlims package from NIOZ-DES (Soetaert et al.).

The $\delta^{13}C$ from the GC-c-IRMS were used to calculate the absolute ^{13}C uptake in each PLFA. The stable isotope signature of carbon or δ(delta) ^{13}C is defined as:

$$\delta^{13}C = \left({}^{13}C/{}^{12}C_{\ sample} \Big/ {}^{13}C/{}^{12}C_{\ standard} - 1 \right) * 1000$$

Where the international standard for carbon is the Vienna Pee Dee Belemnite with an isotope ratio (R_{VPDB}) of $^{13}C/^{12}C$=0.0112373. To calculate the absolute amount of label incorporated (^{13}C) by the organisms, the excess ^{13}C is multiplied by the concentration (C). The excess of ^{13}C is the increase of the fraction (F) ^{13}C after labelling relative to the control samples:

$$Total\ uptake: {}^{13}C = F_{sample} - F_{background} * C$$

Where the fraction is defined as:

$$F = R/(R + 1)$$

R is derived from the measured $\delta^{13}C$ values as:

$$R = (\delta^{13}C/1000 + 1) * R_{VPDB}$$

The total uptake was used to compare the incorporation of the label among PLFA biomarkers. The biomass of the bacteria and fungi was calculated from the PLFA concentration output of the GC-c-IRMS. The PLFAs used to identify and calculate bacterial biomass are given in Table 1. The fungal biomass was based on PLFA 18:2ω6, which is converted to total mycelial biomass based on literature values. To calculate the fungal biomass, it was assumed that 40 nmol of 18:2ω6 equals 1 mg mycelium (Vos et al., 2017) and that the fungal mycelium consists of approximative 50% C (Klamer and Bååth, 2004; Joergensen and Wichern, 2008). The bacterial biomass is calculated on the assumption that 50% of the bacterial biomass is carbon, and 363.6 nmol bacterial PLFA is 1 mg C (Bratbak and Dundas, 1984; Vos et al., 2017). For bulk compost, the concentration of total carbon present in each microcosm was quantified using the percentage of carbon in bulk compost.

Statistics

PLFA concentrations, CO_2 and bulk carbon were tested for significant difference ($p<0.05$) among the different treatments and sampling points using one-way ANOVA and Bonferroni post-hoc tests. The data was analysed in R (version 3.4.1). Data was tested for normality and equal variance prior to analyses. Results are reported as mean ± standard deviation, concentrations are always given per gram of dry weight compost unless specified otherwise.

Table 1 *PLFA biomarkers used for identification and biomass calculation of the microbial community*

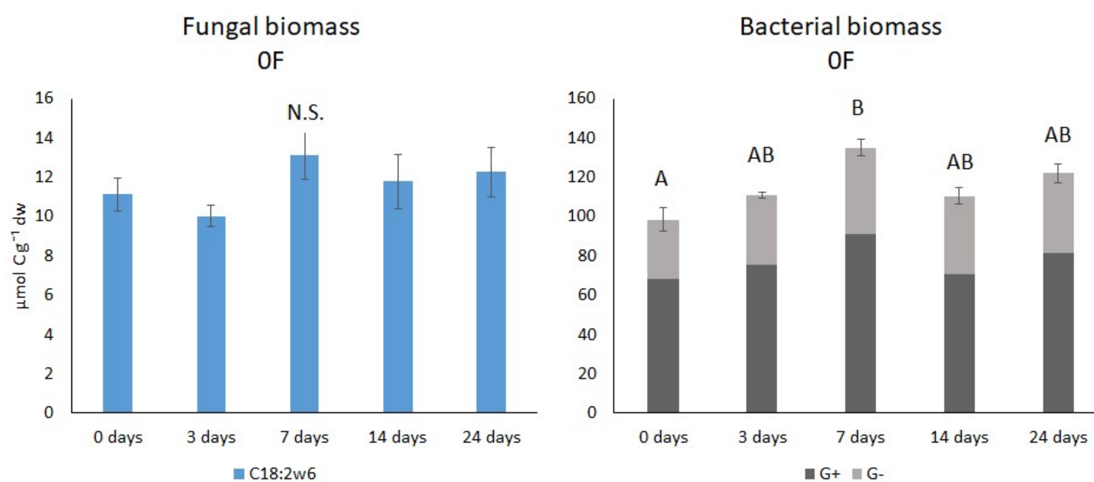
Organism	**PLFA**	**Reference**
Gram-positive bacteria	i14:0, i15:0, ai15:0, i17:0, ai17:0	(Zelles, 1997, 1999)
Gram-negative bacteria	cy17:0, cy19:0, C16:1w7	(Zelles, 1997, 1999)
Bacteria (general)	18:1ω7c	(Zelles, 1997, 1999)
Actinobacteria	10Me-C16:0	(Zelles, 1999)
Fungi	18:2ω6,9c	(Matcham et al., 1985; Frostegard and Baath, 1996)

Results

Effect of fungal density on total PLFA concentrations

One-way ANOVA tests were conducted to assess the statistical significance of the effect fungal spawn density had on the compost's endogenous bacterial and fungal biomass over time. The treatments tested were no spawn (0F), low spawn density (0.5F) and industry standard spawn density (1F). For the fungal biomass, there was a significant difference between the treatments measured at day 0 [$F(2,3)=25.57$, $p=0.013$] and day 7 [$F(2,3)=10.27$, $p=0.0455$]. Post-hoc analysis using the Bonferroni method showed that at day 0 the treatment without any spawn (11.1 ± 0.8 µmol C g^{-1} compost) was significantly different than the 0.5F treatment (16 ± 1.5 µmol C g^{-1}) and 1F treatment (9.7 ± 0.8 µmol C g^{-1}). For day 7, the mean of 0.5F treatment was significantly lower than that of the 1F treatment, but not different to the 0F treatment. At 14 days, conditions 1F and 0.5F decreased in concentration, though the difference between the two treatments was not significant [$F(2,3)=7.695$, $p=0.0659$]. For the timepoints at day 3, 14 and 24 no significant difference between the groups was found. These results show that the initial fungal biomass at the start of the incubation was significantly different in the microcosms without *A. bisporus*. Throughout the incubation the difference in fungal biomass became statistically insignificant between the three treatments, although remained variable (Figure 2). A complete overview of all concentrations is provided in Appendix B, Table B1.

The effect of the different spawn density treatment on the bacterial biomass was also examined. A significant difference was found between the 0F, 0.5F and 1F treatments for day 14 [$F(2,3)=92.89$, $p=0.002$] as well as for day 24 [$F(2,3)=156.8$, $p=0.0009$]. At day 14, the bacterial biomass was significantly higher in the microcosms without *A. bisporus* than in the 0.5F and 1F treatments (Appendix B, Table B2). At the end of the incubation, the 0F treatment had a total bacterial PLFA of 135 ± 5.0 µmol C g^{-1}, significantly higher than for low spawn (35.4 ± 4.4 µmol C g^{-1}) and normal spawn treatment (17 ± 1.2 µmol C g^{-1}). The significant decrease in bacterial biomass occurred at different timepoints for the different treatments, after 14 days in microcosm with five *A. bisporus* spawn ($p<0.001$) and after 17 days in 0.5F ($p<0.01$). These results show a significant decrease in bacterial biomass in the microcosms in presence of *A. bisporus*.



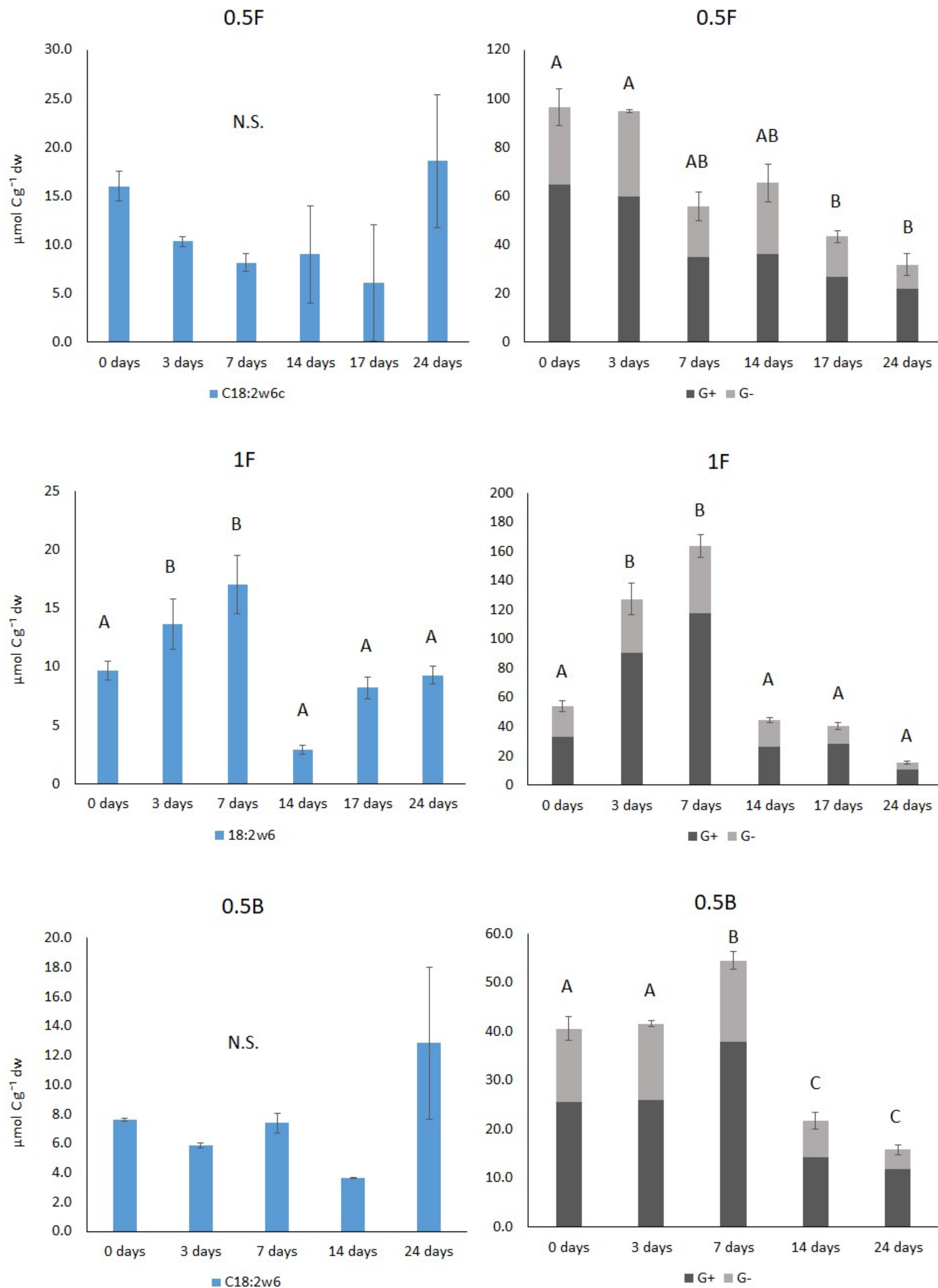


Figure 2 PLFA concentration after 0, 3, 7, 14, 17 and 24 days for 0F, 0.5B, 0.5F and 1F. Error bars represent SD, n=2. Significance ($p<0.05$) is indicated with letters, not sharing any letters means significantly different at $p<0.05$ level (ANOVA with Bonferroni post hoc analysis).

Effect of bacterial density on total PLFA concentrations

To test the effect of bacterial density on fungal biomass, non-autoclaved compost and autoclaved compost with equal spawn density was compared. In this comparison between 1F and 0.5B, the concentrations of fungal PLFA were analysed using t-test at 95% confidence interval. The PLFA concentration of the fungal biomarker was not significantly different at day 0 ($t(2)=-3.4942$, $p=0.07304$). For day 7, the 1F treatment had significantly higher fungal biomass ($t(2)=-5.2647$, $p=0.03424$) compared to the low bacteria treatment. At 14 days, the fungal biomass decreased in both treatments, both 1F and 0.5B had similar levels of fungal biomass (Appendix B, Table B1). No statistical difference was found between the two conditions at day 24 ($t(2)=0.96941$, $p=0.4346$). At the end of the incubation, the fungal biomarker increased in both treatments, to 9.3 ± 0.7 µmol C g^{-1} in the normal bacteria treatment and 13 ± 5.2 µmol C g^{-1} in the low bacteria treatment. These results show that for the initial 7 days of the incubation, the autoclaved compost had significantly lower fungal biomass than non-autoclaved compost. However, after 14 days of the incubation the two treatments became more similar due to a sharp decrease in fungal biomass in the 1F treatment.

In addition to the fungal biomass, the effect of autoclaving the compost on the bacterial biomass was analysed between the industry standard (1F) and low bacteria (0.5B) treatments. The bacterial biomass was not statistically different at day 0 ($t(2)=-2.1877$, $p=0.1602$) for 1F and 0.5B. Between day 3 and 14, the bacterial biomass was significantly lower in 0.5B by 2-fold, compared to non-autoclaved compost ($t(5.7102)=-3.0641$, $p=0.02355$). At day 24, the 1F treatment decreased in bacterial PLFA, resulting in a similar bacterial biomass for autoclaved and non-autoclaved ($t(1.2753)=-0.0227$, $p=0.9849$). The results indicate that the starting bacterial biomass was similar at day 0 between autoclaved and non-autoclaved compost. The bacterial biomass was significantly lower for 0.5B at day 3-14 of the incubation but was at similar levels at the end of the incubation (Figure 2).

The bacterial composition changed over time when *A. bisporus* was present. The proportion of Gram-bacteria in compost without *A. bisporus* was 30% ± 2.2% average over the entire incubation. For the 0.5F treatment the proportion of Gram- bacteria increased at day 14 to an average of 39.9% ± 0.5%, an increase of 9.6% ± 3% from day 0. After 24 days, the proportion of Gram- bacteria decreased again to 27% ± 2.5% of total bacterial biomass, indicating a shift in bacterial composition (Appendix B, Table B7).

The total bacterial biomass decreased in the microcosms in which *A. bisporus* was present (Table 2). In the microcosms where *A. bisporus* was absent there was an increase in the bacterial biomass by 127% ± 4.6 %. In the autoclaved compost the bacterial biomass decreased by 62% ± 2.6%. For 1F treatment the bacterial biomass changed with a 73% ± 4.8% decrease. The bacterial biomass decreased by 67% ± 6.8% for the 0.5F treatment. Overall, bacterial biomass decreased in all three conditions that *A. bisporus* was present.

Table 2 *Fungal biomass (in mg mycelium g^{-1} dry weight compost) and bacterial biomass (mg g^{-1} dry weight compost) (mean ± SD)*

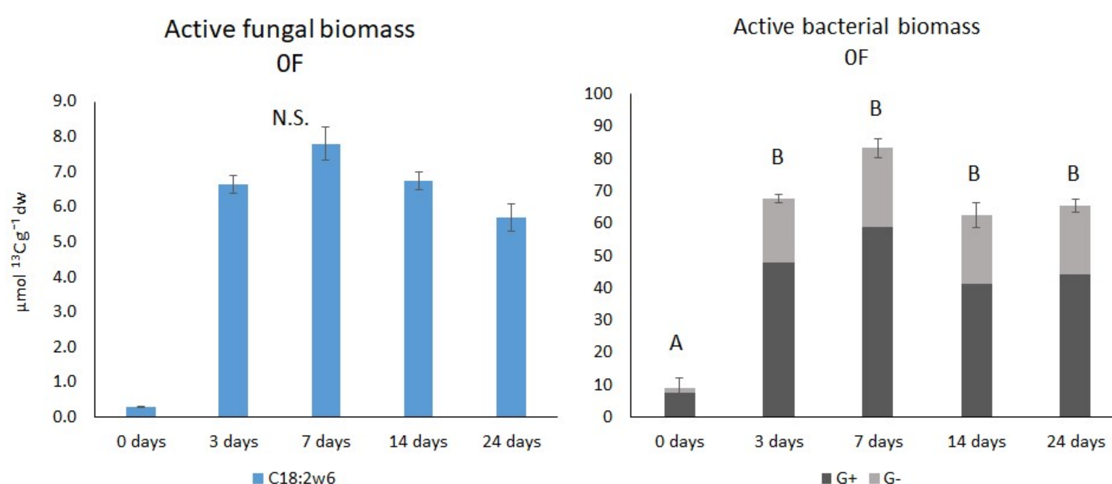
	Day 0		**Day 14**		**Day 24**	
	Fungi	**Bacteria**	**Fungi**	**Bacteria**	**Fungi**	**Bacteria**
0F	14.6 ± 1.1	68 ± 2.3	15.5 ± 1.8	78 ± 2.2	16.1 ± 1.7	86.3 ± 0.2
0.5F	21.0 ± 2.03	67 ± 2.3	11.8 ± 6.6	45 ± 2.2	24.4 ± 8.9	22 ± 3.8
1F	12.7 ± 1.1	39 ± 3.2	3.8 ± 0.5	30.9 ± 0.4	12.2 ± 1.0	10.5 ± 1.0
0.5B	10.0 ± 0.1	28.1 ± 0.9	4.76 ± 0.03	14.8 ± 0.8	16.9 ± 6.8	10.6 ± 0.4

Incorporation of the label in PLFA

The absolute uptake of the label into the PLFAs is a measure for the active part of the total biomass. The active biomass was analysed similarly as the total fungal and bacterial biomass. A significant effect of the difference in spawn density was found at day 0 [F(2,3)=25.57, p=0.01]. The labelled fungal biomarker was significantly higher in the microcosms without *A. bisporus* than in 0.5F and in 1F treatments (Appendix B Table B2). These results indicate that at the start of the incubation the active fungal community was very variable in the compost (Figure 3).

At day 3, 7, 14 and 24 no significant difference in active fungal biomass was found using one-way ANOVA between 0F, 1F and 0.5F. However, the proportion of active fungal biomass of the total fungal biomass did shift in time between the three treatments. Notably, only 5% of the total fungal biomass was active in autoclaved compost at day 3. This increased slowly over time to 32% at day 24. In contrast, 66% of the total fungal biomass was active in 0F at day 3, decreasing over time to 46% active biomass at day 24. Both 1F and 0.5F had a more stable trend, with 58% and 56% active fungal biomass at day 3, decreased slightly to 37% and 38% of active biomass at day 14 and remained at that level at day 24 with 41% and 36% active fungal biomass.

The active bacterial biomass was also compared among the different spawn density treatments. For the first three days, the difference of ^{13}C incorporation of bacterial PLFAs in all three conditions was insignificant [F(2,3)=2.83, p=0.204]. After three days, 68% ± 2.3 % of the bacterial biomass was active in 0F. Moreover, for 0.5F, 73% ± 2.8% bacterial biomass was active and 74% ± 0.6% in 1F at day three. At day 14, both 1F and 0.5F treatments had significantly lower active bacterial biomass than 0F [F(2,3)=91.04, p=0.002]. At day 24 also, the bacterial biomass was significantly lower in microcosms with *A. bisporus* spawn present compared to 0F [F(2,3)=181.8, p<0.001]. At the end of the incubation, 1F had a mean of 6.2 ± 0.6 μmol $^{13}C\ g^{-1}$, which is 37% active biomass of the total bacterial biomass. In the 0.5F microcosms 41% of the total biomass was active biomass. In contrast, the 0F microcosms without spawn remained relatively constant with a higher activity of 53% of the total bacterial biomass at the end of the incubation. These results indicate that also the active bacterial biomass decreased in the microcosms to which *A. bisporus* spawn was added.



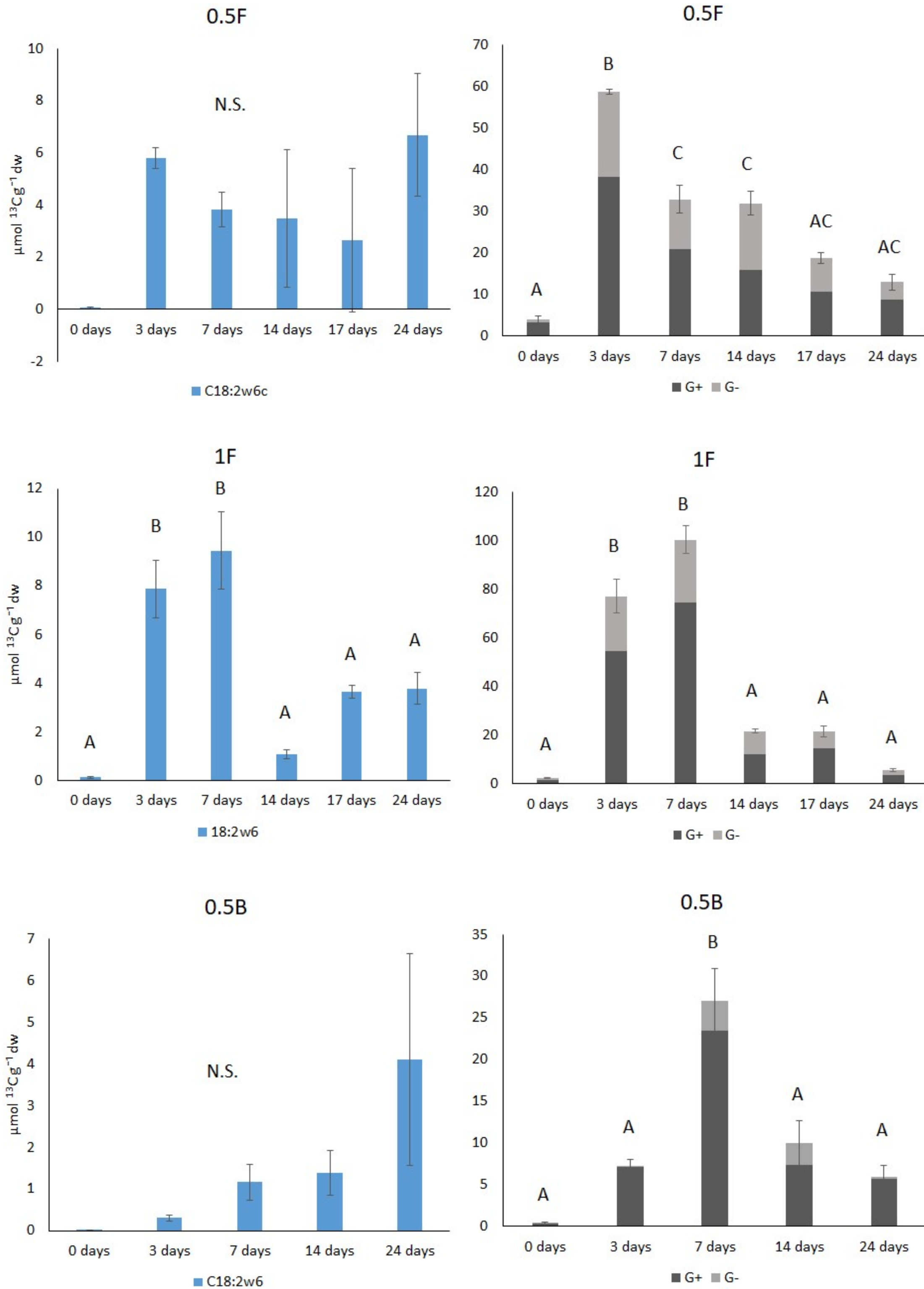
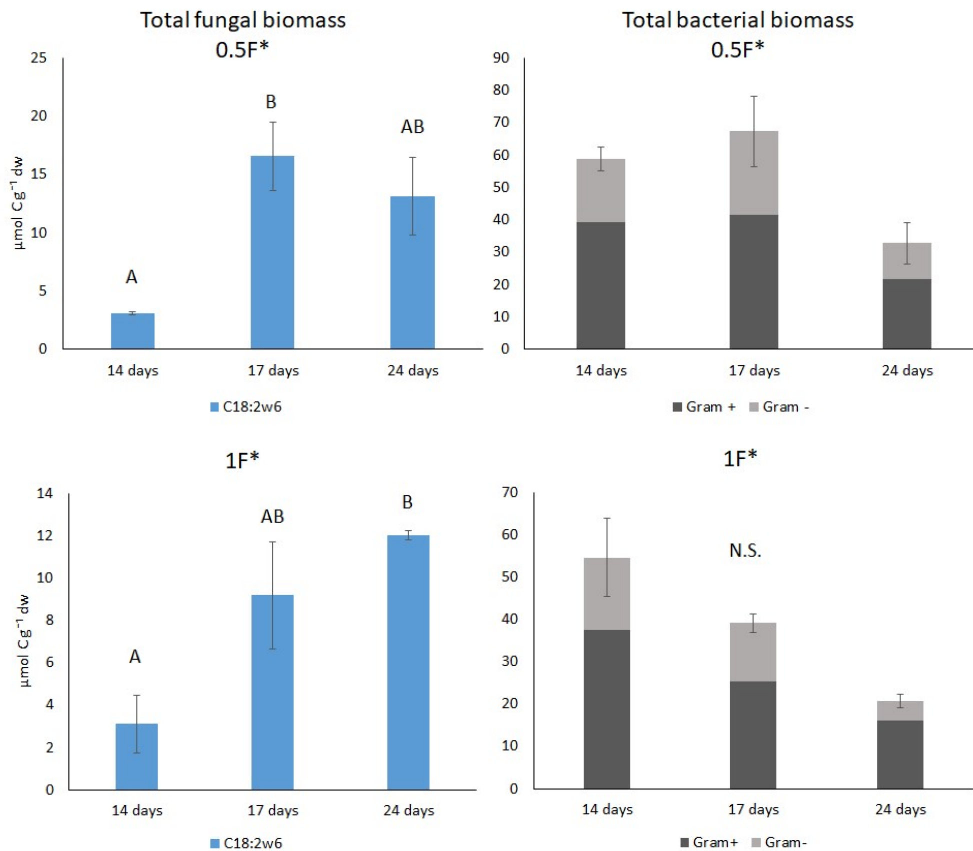


Figure 3 PLFA ^{13}C absolute uptake for fungal and bacterial biomarkers. Error bars represent SD, n=2. Significance ($p<0.05$) is indicated with letters, not sharing any letters means significantly different at $p<0.05$ level (ANOVA with Bonferroni post hoc analysis).

To examine the effect of bacterial density on fungal activity, 1F (non-autoclaved) and 0.5B (autoclaved) were compared. For day 0 there was no significant difference in fungal active biomass between the two conditions ($t(2)=-3.6072$, $p=0.067$). For day 3 to 14 the active fungal biomass in 1F was significantly higher ($t(10)=-3.1267$, $p=0.01039$) with a 6-fold higher concentration than 0.5B (Appendix B, Table B2). At the end of the incubation, the active fungal biomass was similar for both 1F and 0.5B ($t(2)=0.07107$, $p=0.9498$). These results are in line with the total fungal biomass and show that the active fungal biomass was significantly lower in the autoclaved compost for the first half of the incubation.

The relative abundances of active biomass of Gram+ and Gram- bacteria showed a non-significant trend over time. The total biomass of Gram+ bacteria at day 14 in both the 1F and 0.5F microcosms was 10% lower than 0F. As the Gram+ bacteria active biomass decreased, the active biomass of Gram-bacteria simultaneously increased to 43% and 39% for 1F and 0.5F, respectively. After 24 days the active biomass was similar again in relative abundance to the start of the incubation for Gram+ and Gram-, both in active and total biomass (Appendix B, Table 6B).

Effect of additional glucose during incubation



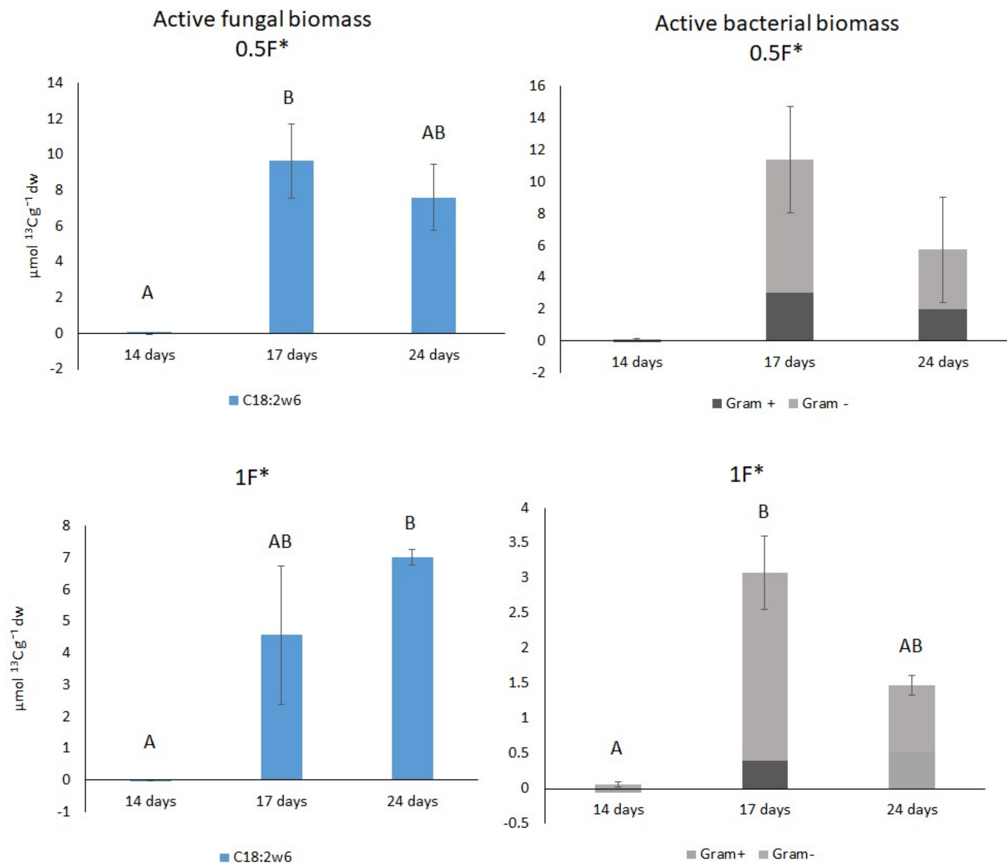


Figure 4 PLFA total concentrations and ^{13}C absolute uptake for fungal and bacterial biomarkers for the parallel experiment, glucose was added at day 14. Error bars represent SD, n=2. Significance (p<0.05) is indicated with letters, not sharing any letters means significantly different at p<0.05 level (ANOVA with Bonferroni post hoc analysis).

The total biomass of the microcosms with added substrate at day 14 (1F*,0.5F*) was compared to the total biomass in the parallel microcosms without added glucose (1F, 0.5F). Both conditions had similar concentrations of fungal and bacterial biomass at day 14. After 24 days, the fungal biomass was significantly higher in the microcosm with added glucose than without added glucose ($t(2)=-4.9923$, $p=0.03786$) in the 1F microcosms, but not in 0.5F treatment. The mean fungal PLFA was 12.0 ± 0.23 µmol C g^{-1} in 1F*, compared to 9.3 ± 0.7 µmol C g^{-1} in 1F. For both 0.5F and 1F treatments, the total bacterial biomass was not significantly different. Overall, the addition of glucose did not influence the bacterial biomass but had some effect on the fungal biomass. The effect of additional glucose on fungal biomass was only significant for industry standard spawn treatments and only after 24 days.

For the parallel microcosms, the active bacterial biomass measured by label incorporation showed a non-significant compositional change compared to the total biomass (Figure 4). A relatively large proportion of the active biomass was made up of Gram- bacteria: at day 24 the Gram- bacteria comprised 51% and 41% of the total active bacteria biomass for 1F* and 0.5F*, respectively. Of the total bacterial biomass, 21% and 23% consisted of Gram- bacteria at day 24 in 1F* and 0.5F*, respectively. The Gram- bacteria thus were relatively more active if additional glucose was added, though they were not more dominant in total biomass.

Actinobacteria

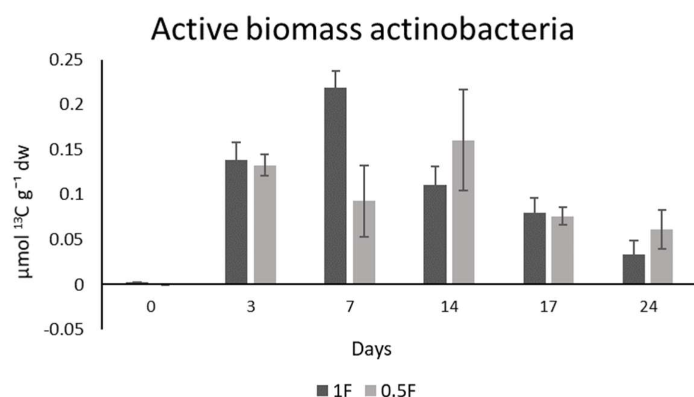


Figure 5 ^{13}C incorporation into 10-MeC16:0, biomarker Actinobacteria. Error bars represent SD, n=2

Actinobacteria were analysed separately from total bacterial biomass as they influence growth of *A. bisporus* (McGee, 2018). The data for actinobacteria was only based on the biomarker 10Me-C16:0 that was present in all chromatograms from the industry standard (1F) and low (0.5F) fungal density treatments (Figure 5). The active actinobacterial biomass followed the same general trend as the total bacterial biomass, with higher activity in the first half of the incubation than the last half. After 24 days, 0.4 ± 0.1 µmol $^{13}C\ g^{-1}$ for 0.5F was present, which is a 2.5-fold decrease compared to day 14. Compared to day 7, the active biomass decreased by 8-fold by day 24 to 0.23 ± 0.08 µmol $^{13}C\ g^{-1}$ for 1F. The concentration of 10Me-C16:0 showed a similar pattern as the ^{13}C incorporation (Appendix C).The concentrations of biomarkers for actinobacteria were relatively low, but roughly follow similar trend as total bacterial biomass.

Fungal/Bacterial ratio

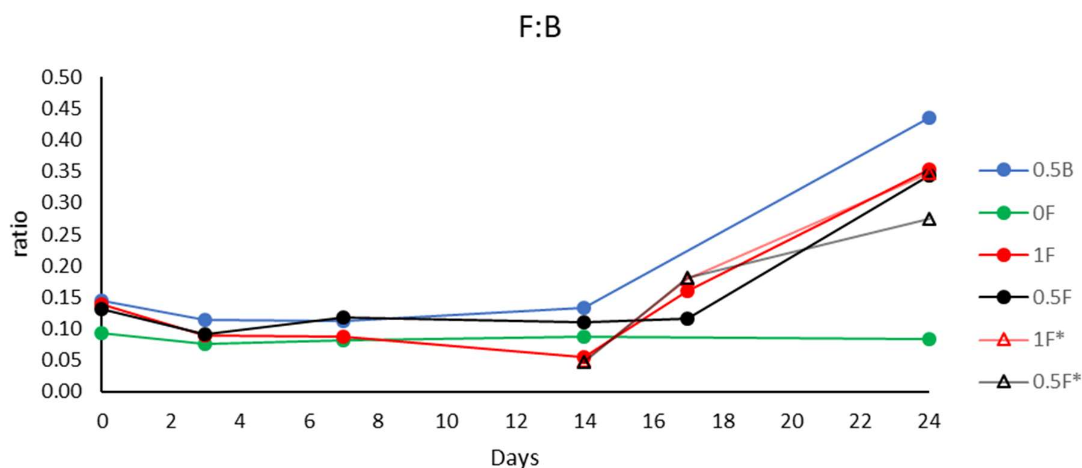


Figure 6 Fungal/bacterial ratio. The ratio is calculated from the PLFA concentrations of the biomarkers in Table 1, the fungal biomarker divided by all bacterial and fungal PLFAs.

The Fungal/Bacterial ratio is an expression of the amount of fungal biomass compared to the microbial biomass (Figure 6). The ratio showed that the bacterial to fungal PLFA ratio stayed relatively the same during the first 0-14 days. The high concentration of the fungal biomarker at the start of incubation coincided with a high microbial biomass in the PII-end compost in all treatments, as the total concentrations of all the PLFAs were much higher in the first 0-14 days. After 14 days, the proportion

of fungal PLFA increased relative to the bacterial PLFAs in the microcosms with *A. bisporus,* despite a decrease in total absolute amount of fungal PLFA. For the microcosms that started with low spawn density, the increase in fungal PLFA relative to bacterial PLFAs was seemingly later, as the shift is seen at day 17. The negative control without *A. bisporus* remained around a ratio of 0.1 throughout the incubation, indicating no shift in the fungal to bacterial biomass ratio. Overall, the presence of *A. bisporus* affected the F:B ratio only after two weeks, when fungi became more dominant.

Specific uptake fungal biomarker

The different treatments of the microcosms affected the specific uptake of the fungal biomarker 18:2ω6 (Figure 6). The PLFA 18:2ω6 is a general fungal biomarker, encompassing both the pre-existing fungal community as well as *A. bisporus*. In the control treatment without *A. bisporus,* showed rapid enrichment above natural $\delta^{13}C$ abundance during the first 3 days (1834‰ ± 81‰) and 18:2ω6 had a higher $\delta^{13}C$ value compared to the other treatments. The fungal biomarker in the autoclaved compost remained low the initial 3 days (53‰ ± 11‰) but increased slowly until day 14. The 0.5F and 1F conditions had similar specific uptakes at day 3, 1187‰ ± 51‰ and 1284‰ ± 25‰, respectively. The decrease in uptake seen in the other conditions indicate that most label was taken up in the first three days by the pre-existing microbial community. At 14 days, the autoclaved compost reached highest uptake (632‰ ± 378‰), whereas both 1F and 0.5F reached their lowest values. The parallel experiment showed a similar uptake rate of the label at day 14-17 as the other microcosms at day 0-3. In contrast to other treatments, in both the 1F* and 0.5F, the fungal biomarker did not decrease in $\delta^{13}C$ after the first three days of label addition but remained high until the end of incubation. The specific uptake decreased at day 14 for 1F, 0.5F, 0.5B and 0F, although the latter remained higher than the other three treatments. At the end of the incubation, the specific uptake for 0.5B was 423‰ ± 147‰, slightly lower than 0.5F (535‰ ± 13‰) and 1F (650‰ ± 103‰). Overall, the specific uptake of the fungal biomarker remained high for 0F with a steady decline over time, whereas 0.5B slowly increased in $\delta^{13}C$ until day 14. Both 1F and 0.5F were following the trend of 0F until day 14, where there was a sharp decrease in $\delta^{13}C$ for both treatments.

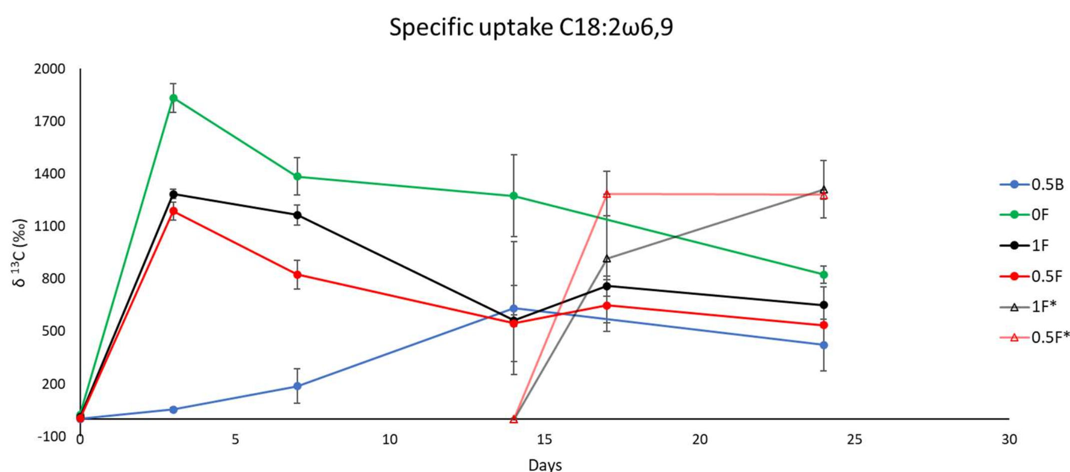


Figure 7 Specific uptake ($\delta^{13}C$) of 18:2ω6,9 over time for the different treatments. Error bars represent SD, n=2. Specific uptake: $\delta^{13}C = \delta^{13}C_{sample} - \delta^{13}C_{background}$

Bulk compost

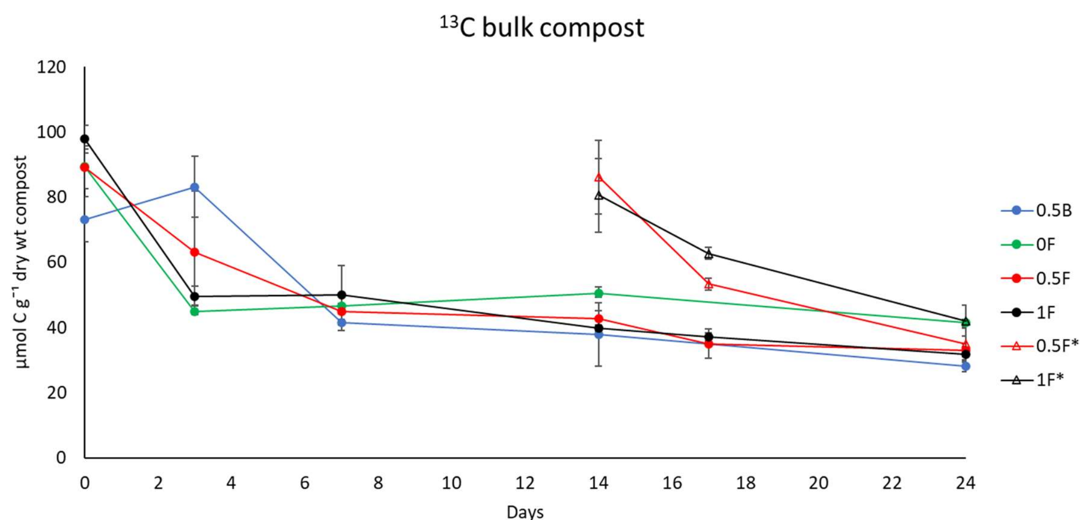


Figure 8 Amount of ^{13}C in bulk compost over time. Error bars represent SD, n=2.

The bulk compost was analysed for total amount of carbon as well as ^{13}C%. In the first three days of the incubation, the added label was quickly respired for 1F, 0.5F and 0F treatments (Figure 8). The respiration rate was 14.8 µmol ^{13}C g^{-1} per day for 0F in the first three days, whereas respiration averaged 2 µmol ^{13}C g^{-1} per day over the entire incubation. 0.5B showed a different trend, with higher amounts of ^{13}C in compost at day 3. The parallel experiment followed the same trend at day 14-24 as 1F and 0.5F at the beginning of the incubation. No significant differences in the organic ^{13}C-carbon in the compost between the treatments was found at the first four timepoints, except at 24 days (one-way ANOVA, $F(3.4)=8.794$, $p=0.031$). At the end of the incubation, the microcosms without *A. bisporus* (0F) contained significantly more ^{13}C in the compost than the other microcosms with a mean of 44 ± 1.1 µmol ^{13}C g^{-1} compared to 26 ± 1.7 µmol ^{13}C g^{-1} in 0.5B. Notably, this significant difference was only in ^{13}C-compost, the total concentration of carbon was not significantly different among the treatments at this time [$F(3,4)=0.963$, $p=0.492$]. Both 1F and 0.5F showed similar values to 0.5B at the end of the incubation (Appendix D, Table D2).

After 24 days, the ^{13}C% of the total bulk carbon was 0.33% ^{13}C for 0F. The proportion of ^{13}C in the bulk carbon was slightly lower for the other three treatments, with 0.24% ^{13}C for 0.5B, 0.27% ^{13}C for both 1F and 0.5F. For the two treatments with added glucose at day 14, the proportion was only slightly higher for 1F* with 0.36% ^{13}C and 0.28% ^{13}C for 0.5F* (Table D1 and D2). Overall, these results indicate that the presence of *A. bisporus* resulted in lower ^{13}C present in the bulk compost.

CO_2 respiration

The rate of total CO_2 respiration in the microcosms was compared among the different treatments using one-way ANOVA for the different timepoints. For day 0, no CO_2 data was measured as the samples were immediately processed for PLFA analysis. The total CO_2 respiration was significantly lower for 0.5B than the other treatments at day 3 [$F(3,4)=18.03$, $p=0.00868$]. The total CO_2 respiration in 0.5B was 1.6 ± 0.7 µmol C h^{-1} g^{-1}, whereas the respiration rate without *A. bisporus* was 4-fold higher. Even higher CO_2 respiration was measured in 0.5F with 7.7 ± 1.4 µmol C h^{-1} g^{-1}. At day 7 and during the rest of the incubation, the CO_2 respiration was not significantly different among the treatments. Total CO_2 respiration was lowest for 0.5B at day 3, but the different treatments did not affect respiration for longer time period.

The effect of the different spawn and bacterial treatments on respiration of the added label, i.e. $^{13}CO_2$ respiration rate, was analysed. The $^{13}CO_2$ respiration the rate was significantly different at day 3 [$F(3,4)=23.49$, $p=0.00531$] among the different treatments. The 0.5B microcosms' values were significantly lower with a mean of 0.019 ± 0.009 µmol ^{13}C h^{-1} g^{-1}, whereas the 0F and 0.5F treatments had a respiration rate 5-fold and 6-fold higher, respectively. At day 14, the $^{13}CO_2$ respiration rate was still significantly lower for the autoclaved compost [$F(3,7)=5.696$, $p=0.0271$]. The 0.5B treatment had a mean $^{13}CO_2$ respiration rate 6-fold lower than the 1F treatment. At day 7, 17 and 24 no difference between 0F, 1F, 0.5F and 0.5B was found (Appendix D Table D3).

The results for the amount of label that was respired in the parallel microcosms was compared with the same timepoints and treatment as the original incubation. Thus, the values measured on day 3 from the 1F and 0.5F microcosms were compared with the values from day 17, as the ^{13}C label and additional ^{12}C-glucose were added at day 14 instead of day 0. The low spawn treatments (0.5F) showed that the $^{13}CO_2$ respiration was significantly higher in the microcosms where glucose was added later ($t(3)=3.5329$, $p=0.03856$). The $^{13}CO_2$ respiration was 3 times higher at day 17 for 0.5F* than its counterpart 0.5F at day 3. The higher $^{13}CO_2$ respiration rate for the parallel microcosms indicate that the presence of *A. bisporus*, which has fully colonized the compost at day 17, caused a higher respiration rate than the compost endogenous microbiota.

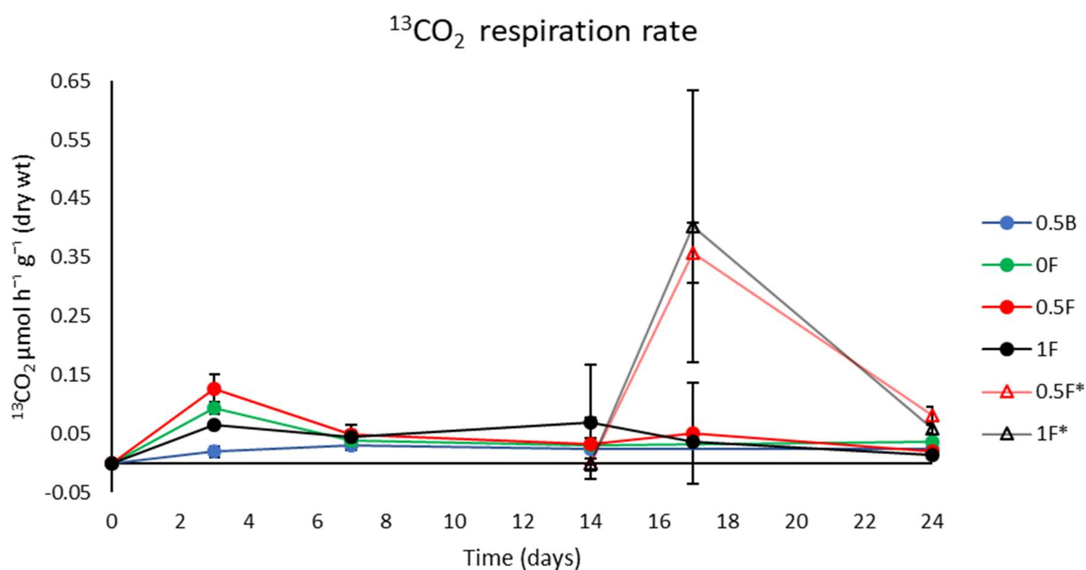


Figure 9 The rate of respiration of the $^{13}CO_2$ as measured from the CO_2 sensor. The ^{13}C% values were obtained from the NaOH traps. Error bars represent SD, n=2

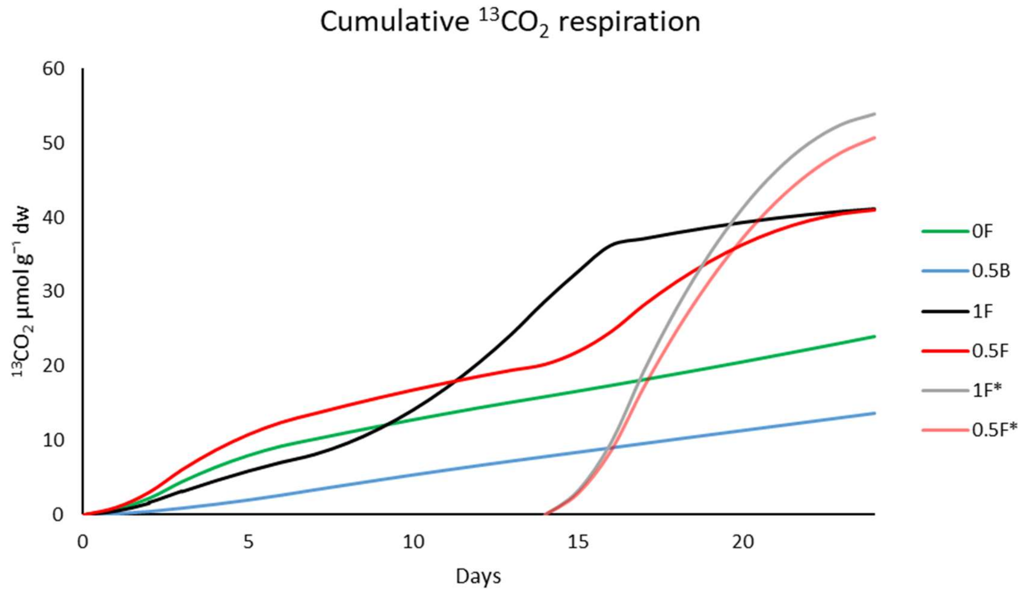


Figure 10 The cumulative $^{13}CO_2$ production over time, calculated interpolating the rate of respiration for each day from the measured rates. For the parallel treatments 1F* and 0.5F* the label was added at day 14.

As the CO_2 rate was measured at discrete timepoints, the total amount of CO_2 production was calculated by interpolating the rate of respiration for each day from the measured hourly rates. This rate was then multiplied by the number of incubation days in order a to obtain the total CO_2 respired at the different sampling points (Figure 10). The total cumulative $^{13}CO_2$ respiration interpolated over the entire incubation amounted to 0.91% ^{13}C of the total respired CO_2 for 0F (Appendix D, Table D3). This is comparable to the $^{13}CO_2$ respiration of 1F and 0.5F with 1.11% and 0.95% of the total respired CO_2 over the whole incubation period. 0.5B had an overall low $^{13}CO_2$ respiration of almost half of the other treatments, with 0.55% of $^{13}CO_2$ of the total CO_2 respiration. For the parallel experiment, the measurements were done from day 14 to day 24, not including the first two weeks of the incubation. Over this shorter period, the ^{13}C% was much higher with 8.17% and 4.94% of ^{13}C respiration for 1F* and 0.5F*. Consistently, these cumulative percentages showed that the activity over the entire incubation period was low for 0.5B compared to the other treatments. Furthermore, it highlights that the high respiration rate in the parallel microcosm resulted in high total CO_2 production.

Label distribution

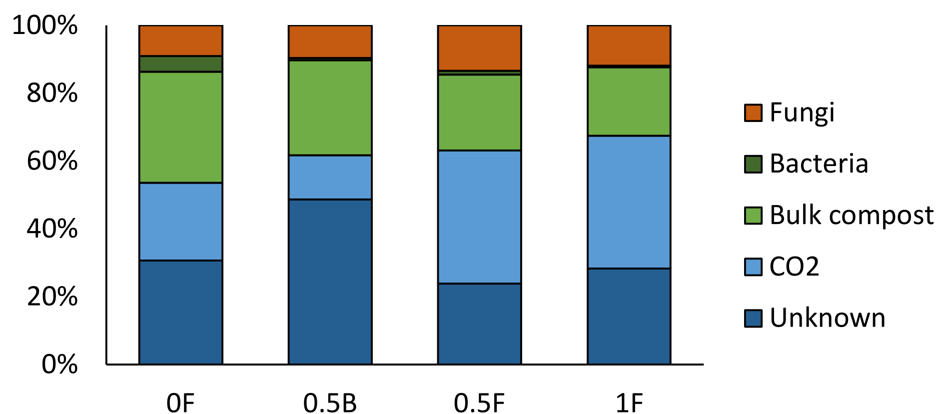


Figure 11 Label distribution at the end of the incubation in different compartment relative to day 0 (C%)

The total amount of ^{13}C-label added to each microcosm was 733 µmol, of which most was found in the bulk in day 0 samples (93%-70%). As the parallel microcosms 1F* and 0.5F* contained no ^{13}C-glucose at day 0, these microcosms were not evaluated in this comparison. The distribution of the label into the different compartments for the four conditions of 0F, 0.5F, 1F, and 0.5B was calculated using the CO_2 measurements and bulk carbon and the PLFA data at day 24 (Figure 11). The bulk carbon was separated in a fungal and bacterial compartment and the remainder as bulk compost. The amount of label at day 24 was expressed relative to the amount of label at day 0, as not all label added did make it into the microcosms. Of the label added in the 0F microcosms, 38% remained in bulk compost and 30% of the label was respired as CO_2 (Figure 11). In 0F condition, the bacterial compartment took up 4.6% of the label, whereas the other three conditions had a much lower bacterial label uptake of <1.2%. In 0.5B, only 13% of the ^{13}C-label was respired as CO_2. This condition also had the highest proportion of ^{13}C that was not in any of the measured compartments (47%). Of the label added to the 0.5F and 1F microcosms, most was respired as CO_2, 39% for both treatments. For both these treatments, 22-20% of the label remained in the bulk compost, which is approximately 10% lower than in 0F. The fungal compartment was comparable for all treatments, 9.0% in 0F to 13.3% for 0.5F. Overall, the results showed that the majority of the label was respired as CO_2, particularly for the 1F and 0.5F treatments. A large part was not accounted for or was in other unspecified compartments, particularly for 0.5B. The fungal and bacterial biomass was generally only a small part of the bulk compost, though the bacterial biomass in 0F was clearly larger than for the other three conditions (Table 2).

Discussion

The fungal-bacterial relationships and the carbon flow within the compost bed were examined by combining stable isotopes and biomarker analysis. This approach quantifies both biomass and activity of bacteria and fungi inside the compost bed. These biogeochemical techniques have successfully been applied to *Agaricus bisporus'* compost system. The different treatments of each microcosm allowed for close examination of all factors influencing the carbon flow.

Fungal dynamics

Fungal biomass and activity varied among the different treatments. Autoclaving of the compost effectively decreased the activity as well as biomass of the pre-existing microbial community, especially visible during the first 7 days of the incubation. At the start of the incubation, the incorporation of the ^{13}C label in the fungal and bacterial biomarkers was much less compared to the non-autoclaved compost. Moreover, the autoclaved compost was characterized by a lower respiration rate. This decrease in bacterial biomass and activity did not result in a lower fungal biomass at the end of the incubation when compared to the other treatments. These results are partly in line with previous studies, as Durrant et al. (1991) also successfully cultivated *A. bisporus* on axenic mushroom compost that was autoclaved three times. Here, also sterile air was used together with sterile casing in order to onset the formation of fruiting mushroom bodies. In contrast, Noble et al. (2009) found that significant formation of primordia only occurred in microcosms with non-axenic casing. This could indicate that even though the mycelial biomass is similar, the of primordia forming on autoclaved compost could be less than on non-autoclaved compost. It should also be noted there was high variability in results of the microcosms with autoclaved compost. This is partly due to the highly heterogeneous nature of the compost itself, partly due to the number of replicate samples. The number of replicates in the experiment was three biological replicates per treatment, however only two replicates of each were processed and analysed due to time limitation. Even with a small sample size, the high fungal biomass reached in autoclaved compost indicate that the microbial community is not of vital importance for *Agaricus bisporus,* for at least the first 1-2 weeks of its vegetative growth phase. In addition, Sparling et al. (1982) showed that only 10% of the mushroom biomass was from microbial biomass, suggesting that the bulk of the mushrooms carbon-nutrition is from straw carbon (Sparling et al., 1982). This does not consider the possible bacterial multiplication during PIII, which would increase the bacterial biomass in the compost. Bacterial consumption of *A. bisporus* could therefore be higher. Another explanation is that the microbial community was able to recover after the initial days of low activity. It is also possible that autoclaving the compost did not affect all pre-existing microbiota equally and that essential, growth promoting (thermophilic) microbiota remained in the compost. This is also seen in the results, as autoclaving the compost impacted Gram- bacteria more than Gram+ bacteria. Thus, if *A. bisporus* is more dependent on Gram+ bacteria, the effect of autoclaving on the fungal growth is minimal, as Gram+ bacteria were less affected by the autoclaving. However, this is somewhat in contradiction with previous literature, as there is some indication that Gram- bacteria are more suppressed by *A. bisporus* than Gram+ (Vos et al., 2017). The contradictive findings could be explained by a highly dynamic bacterial community with large fluctuations throughout the mushroom cropping process (Mcgee et al., 2017b; McGee, 2018).

The decrease in fungal biomass at day 14 is a remarkable and unexpected trend. It was mostly visible in the microcosms with *A. bisporus* in 1F microcosms as a sharp decrease in concentration and ^{13}C incorporation of the fungal PLFA and coincided with the decrease in bacterial PLFAs. At the same time, the specific uptake of ^{13}C in the fungal biomarker indicates that the fungal biomass was still active at this time. There are multiple possible explanations for this decrease in fungal biomass at day 14: 1) a limitation of space or nutrient transport, inhibiting further growth of *A. bisporus*, 2) a suppression of

the compost endogenous fungal community 3) limitations related to the use of PLFAs as a proxy for biomass. These three hypotheses will be expanded on in the following paragraphs. The possibility of the trend being an artefact of the method has also been explored. However, as the four treatments, the additional parallel experiments with the added glucose at day 14, and the background samples also showed this low 18:2ω6 concentration at day 14, it is likely not due to handling during extraction. Furthermore, the 0F samples were consistent in fungal biomass throughout and were processed at the same time in the same manner as all other samples, making it unlikely that the trend is due to an extraction or analytical method errors. These 0F background samples without *A. bisporus* indicate a stable endogenous fungal community in adequate growth conditions, whereas incubations under similar conditions found decreasing concentrations of 18:2ω6 in microcosms without *A. bisporus* (Vos et al., 2017). This indicates that the conditions of the incubation did not negatively impact the growth of the fungal community.

The first hypothesis that the decrease in biomass after 14 days is caused by a limitation of nutrients or space is supported by the observation that the sharp decrease was most pronounced in high, industrial standard density spawn condition, whereas the results for the low spawn density condition were more variable overall. As the mycelium had already colonized the compost at day 14, further growth could have been inhibited. This is not reflected in the results as there was continuous growth of fungal biomass up to 24 days (Figure 2 and Appendix E). Additionally, the specific ^{13}C uptake still shows active label uptake after 14 days. As the parallel microcosm had additional glucose at day 14, which resulted in only slightly higher fungal biomass for the normal spawn condition, the nutrient limitation would likely not be carbon, but possibly water limitation or phosphorus. However, (Bååth, 2001) found that in agricultural as well as natural soils, carbon is the most common limiting factor for fungal growth.

The second hypothesis is that *A. bisporus* heavily supressed the pre-existing fungal community at day 14. This is mostly based on the specific uptake of 18:2ω6, which differs among the different treatments (Figure 7). The specific uptake indicates the incorporation of ^{13}C label into 18:2ω6. The biomass decreased at day 14 coincides with a sharp drop in ^{13}C uptake of the fungal biomarker in the 1F and 0.5F conditions. As the fungal biomarker groups all fungi together (*A. bisporus* and the existing fungal community), the isotopic signature can be used to distinguish these two groups. The basis of this is the assumption that the 0F condition only consisted of the compost own pre-existing fungal community and that the autoclaved compost contained very little active fungal biomass at the start, so the isotopic signature solely indicates the growth of *A. bisporus*. The higher $\delta^{13}C$ signature seen in the 0F condition could possibly be due to a more rapid uptake of the label as compost endogenous fungi grew faster than *A. bisporus* (Straatsma et al., 1994). The $\delta^{13}C$ of *Agaricus* and other fungi could furthermore be distinguished by different PLFA signatures, as *Agaricus* species have a relatively high mol% of 18:2ω6 compared to other fungal species such as Ascomycetes, Zygomycetes and most other Basidiomycetes (Dembitsky et al., 1992; Klamer and Bååth, 2004; Barros et al., 2008). Thus, the pre-existing community has a high $\delta^{13}C$ signature with a lower mol% 18:2ω6 and faster growth, whereas *A. bisporus* itself has an overall lower $\delta^{13}C$ signature with high mol% 18:2ω6 and slower growth. The $\delta^{13}C$ values of 18:2ω6 for 1F and 0.5F could then be viewed as a combination of the two isotopic signatures. From day 14 onwards, both 1F and 0.5F 18:2ω6 specific uptake decreased, deviating from 0F and consequently following the same trend as for the autoclaved microcosms. These findings, together with the 14 day decrease in total fungal biomass, could indicate that at day 14 the existing fungal community was suppressed and possibly outcompeted by *A. bisporus*. Previous studies have found that *A. bisporus* can use other fungi as sole nutrient source (Fermor and Grant, 1985). Moreover, it was found that the mushroom produced enzymes that degrade fungal and actinomycete cell walls to obtain carbon, nitrogen, and phosphorus from the mycelium of the thermophilic fungi *Scytalidium thermophilum* in liquid culture. Thus, *A. bisporus* is capable to degrade fungal cell walls, yet it is unknown whether it

does so preferentially. DNA evidence could further prove the suppression of the compost fungal community by identifying the species present at the start of the incubation and after 14 days. The moment in time when the change in $\delta^{13}C$ of 18:2ω6 occurs, should be interpreted with caution as it is subject to the growth conditions of the experiment. Different initial incubation conditions, the experimental set-up, environmental controls, or the moment of measuring could possibly change the timing of the perceived 'tipping point'. A lower spawn density also seems to influence the timing, as low spawn density seems to have a later tipping point (Figure 6).

The third hypothesis is that the decrease in fungal biomass could be partly due to the conversion from PLFA to biomass. The biomass conversion based on PLFAs used here was taken from Vos et al. (2017), which is based on a slightly different extraction method. To estimate the biomass more accurately, a conversion factor calculated from pure mycelium to PLFA would have been preferred over literature values. However, due to COVID19-lab restrictions, this was unfortunately not possible. The use of PLFAs as a proxy for biomass can be questioned, as the fungal community changes its composition and different species have different proportions of PLFAs. At the start of the incubation, the fungal biomarker mostly relates to the pre-existing fungal community present in the PII-compost. As *A. bisporus* grew and became more dominant, the PLFA 18:2ω6 concentration decreased. As the total ^{13}C in the bulk remained constant and the fungi remained active, the decrease in fungal PLFAs could be explained by a different cell membrane composition of *A. bisporus.* This means that PLFAs are not a perfect proxy for biomass, specifically if a community changes. As the conversion to biomass is based on *A. bisporus*' PLFA composition, this might not be representative for the fungal community in the compost. The different proportion of total lipids can vary from 18 to 80 mg g^{-1} dry wt even within the class Basidiomycetes to which *Agaricus* belongs. The cell walls of *Agaricus bisporus* hyphae have been reported to consist 9.9-10.1% of lipids. The yeast cells present in the compost , e.g. *Trichosporon sp* or *Candida subhasi* (Mcgee et al., 2017a), can have over 20% of lipids in dry cell weight (Kolouchová et al., 2016). Thus, this difference in lipid content between *A. bisporus* and the pre-existing fungal community could have caused an overestimation of the pre-existing fungal community. In addition, Klamer and Bååth (2004) found a correlation between ergosterol, 18:2ω6, content and mean hyphal diameter. This suggests that the PLFA 18:2ω6 is not correlated to the biomass per se, but more so to the hyphal surface area. Thus, if conversion factors are calculated for fungi grown under optimum conditions with higher hyphal diameters, whereas the in vivo fungi have thinner hyphae, this will result in an overestimation of the fungal biomass. In this case, the hyphal thickness is unknown, but is a potential cause for discrepancy in the fungal biomass calculations.

Bacterial dynamics

The decrease of the total and active bacterial biomass at 14 days was detected only in *A. bisporus* presence. This trend was also visible in the fungal/bacterial ratio (Figure 6), which showed that the point where fungi became 'dominant' over bacteria in the compost was after 14 days. The stability of the bacterial biomass in the absence of *A. bisporus*, suggests that the fungus consumed these microorganisms. This is in line with previous findings that showed the fungus is capable of degrading bacteria and decrease the bacterial biomass in compost (Fermor et al., 1991; Vos et al., 2017). The suppression of bacterial biomass was more pronounced in the higher spawn density treatment than in the low spawn density treatment. The lower density of hyphae could have resulted in less enzymes released that degrade the pre-existing fungal and bacterial community (Fermor and Wood, 1981).

The decrease in bacterial activity and biomass in the presence of *A. bisporus* indicate that the fungi suppressed other microbiota, but it does not conclusively prove that they predated on bacteria or consumed them for nutrients. The addition of extra glucose plus ^{13}C labelled glucose added at day 14 in the parallel experiment did not influence the amount of bacterial biomass in the microcosms. This

could indicate, as previously suggested, that *A. bisporus* does not consume bacteria as a source of carbon (Sparling et al., 1982), but possibly as a source of nitrogen (Fermor et al., 1991). According to Vos et al. (2017), bacteria could provide a source of vitamins, in addition to carbon. Indeed, the active vitamin B_{12} has been detected in the *A. bisporus*' mushroom's peel (Koyyalamudi et al., 2009) and is likely to be bacteria-derived. However, it still is undetermined how the mushroom exactly obtains this vitamin. Furthermore, in compost with low microbial activity, i.e. autoclaved compost, *A. bisporus* is still able to grow to a similar mycelial biomass. Thus, the results from this incubation do not conclusively indicate that *A. bisporus* consumes bacteria as an essential source for its nutrients.

The presence of *A. bisporus* also affected the bacterial composition. The Gram+ bacteria seem to have decreased more in comparison to Gram- bacteria. Furthermore, the Gram- bacteria showed a relatively higher activity than the Gram+, specifically in the parallel experiments. These results could indicate that Gram+ bacteria are suppressed more than Gram- bacteria by *A. bisporus*. This contradicts the finding from Vos et al. (2017), where a decrease in Gram- biomarker PLFAs relative to Gram+ biomarkers was found. This could be explained by a highly dynamic bacterial population in the compost throughout the cropping process (Mcgee et al., 2017b). Furthermore, others have found that the bacterial community is mostly dominated by Gram- bacteria after PIII (Zhang et al., 2014b). An indication for bacterial preference could be that *A. bisporus* produces antimicrobial metabolites against Gram+ bacteria more than against Gram- bacteria (Barros et al., 2008; Öztürk et al., 2011). However, there are also contradictory findings that *A. bisporus* does produce antibacterial metabolites against some Gram- bacteria (Tambekar et al., 2006; Ozen et al., 2011). These contradictory results might be due to different methods used (Alves et al., 2012). The preferential consumption of *A. bisporus* of Gram+ bacteria seems to be dependent on the study, implying that this could either be compost specific or opportunistic rather than preferential. If the consumption of Gram+ bacteria is preferential, this would be to *A. bisporus* own advantage, since Gram- bacteria, specifically the *Pseudomonas* genus, regulate the primordia formation of the mushroom (Rainey et al., 1990; Noble et al., 2009).

Effect of addition glucose

The fungal biomass was slightly higher at the high spawn density treatment when extra glucose was added than without additional glucose, possibly alleviating substrate limitation or any nutrient depletion. One drawback of the addition of a carbon source is that it could have affected the C/N ratio of the compost. This effect is likely to be minor, as only 0.25 mg of glucose was added to 20 grams of compost, an increase of approximately 4% in carbon.

The low spawn condition is lower in mycelial density and possibly did not experience any limitation yet. The $^{13}CO_2$ respiration in the parallel was very high, indicating that *A. bisporus* respired the labelled glucose faster than the pre-existing microbial community of the compost. The $^{13}CO_2$ respiration is much higher for the parallel experiment, at a moment where seemingly the bacterial and fungal PLFA was low. This could indicate that *A. bisporus* uses the glucose for energy rather than for biosynthesis, or the addition of easily degradable carbon effectively increased the activity of the microbiota.

The relative increase in active biomass is more pronounced in Gram- bacteria over Gram+ bacteria and is mostly observed in the microcosms with additional glucose. The addition of easily degradable carbon increased the microbial activity overall, as seen by the high $^{13}CO_2$ efflux, but specifically increased the microbial activity of Gram- bacteria. The added simple sugars can have a different effect than more complex carbohydrates on microorganisms (Lonardo et al., 2017). It has been proposed that Gram- bacteria are more associated with simple, labile carbon compounds, whereas Gram+ bacteria are more strongly associated with more complex, recalcitrant carbon (Fanin et al., 2019). This would explain why the extra glucose additional could be more beneficial for Gram- bacteria than for Gram+, indicating that they could use different strategies or use the substrate with different efficiencies.

Label distribution

Not all label added was recovered, as the ^{13}C in bulk compost and respired CO_2 account for approx. 77% at most. The large unaccounted-for compartment could in part be due to measurement inaccuracies or underestimation of the CO_2 produced. *A. bisporus* is known to have high CO_2 respiration for short moments of time, so called respiratory bursts (Vos, 2017). These burst take place every 13-20 hours, during which CO_2 production increases ≤ 3.5-fold for a 3-6 h duration. When taking the respiratory bursts into account, the CO_2 compartment for 1F increases with a maximum of 13.5 µmol $^{13}CO_2$ g^{-1} over the 24 days, an approximate increase of 13% of label that is accounted for. This would increase the total CO_2 compartment to 52% of the label, decreasing the unknown compartment by half to 15%. The actual amount of label respired as CO_2 is likely somewhere in between this maximum of 52% and the measured 41%. The underestimation of CO_2 is likely only partly responsible for the unaccounted part of labelled ^{13}C. Another possible explanation is that a part of the label was released as other molecules undetectable in our analytical window, i.e. volatiles, which are not detected with the methods used in this experiment. *A. bisporus* releases C8 compounds such as 1-octen-3-ol (Grove, 1981), which inhibits primordia formation (Noble et al., 2009). The total amount of volatiles released is relatively small compared to CO_2 respiration, but as these volatiles are molecules that on average consist of eight carbon atoms, the effect on the carbon budget could be substantial. Furthermore, the microbial community release volatiles of themselves, with 2-ethyl-1-hexanol in particular, in large amounts (Noble et al., 2009). The release of this compound alone in the presence of *A. bisporus* can amount to 1.47 µmol C per gram of casing layer. Due to the production of volatiles from fatty acids, it is unlikely that these volatiles release the labelled ^{13}C at the beginning of the incubation, but as the incubation lasts the effect on the carbon budget increases.

The autoclaved compost contained a higher amount of label in the bulk compost at the end of the incubation than its non-autoclaved counterpart, indicating that the substrate was less efficiently used. This is in line with previous work that showed that more cellulose was degraded in non-axenic than in axenic compost (Wood and Leatham, 1983). This indicates that the compost microbiome does increase the resource uptake efficiency during *A. bisporus* cultivation.

Implications

This study provides some indications that *A. bisporus* consumes bacteria for its nutrients, with Gram+ bacteria being affected more than Gram-. Further research with ^{13}C labelled bacteria, possibly separated in Gram- and Gram+ can further elucidate whether *A. bisporus* actively predates on these microorganisms and whether the bacterial cell wall structure contains a preference. The large variation in fungal biomass from week 1 to week 2 of incubation could be further studied using additional DNA SIP techniques. By identifying the species present at the start of the incubation and after 14 days, the suppression of the compost endogenous fungal community by *A. bisporus* could be confirmed. Furthermore, this study shows that both the compost endogenous bacteria and fungi are consumed by *A. bisporus* for its growth, although this study found that autoclaving the compost did not inhibit the mycelial growth significantly. These results indicate that not the microbiome in its entirety, but perhaps only a few select species are of vital importance for the successful cultivation of *A. bisporus.* Further research into which species in the compost are of essential importance for *A. bisporus* growth could give further knowledge on how to improve its growth.

Conclusion

The carbon flow within *A. bisporus* compost was traced and its microbiota quantitatively studied. The transfer of ^{13}C from bacteria to fungi through consumption is implied but not conclusively proven. The different fungal spawn densities did not result in different bacterial or fungal biomass at the end of the 24-day incubation, although the suppression of bacteria seem more efficient with higher fungal density. The microcosms with low fungal density had large variability and a slower growth of *A. bisporus*, as can be seen from the fungal/bacterial ratio. The results show that *A. bisporus* suppresses bacteria in the compost significantly after two weeks of incubation, coinciding with an increase in the fungal/bacterial ratio. This ratio more clearly shows the growth of *A. bisporus*, as the total fungal biomass seemingly decreases significantly at day 14. This decrease in fungal biomass could be caused by *A. bisporus* suppression of the pre-existent fungal community. However, low bacterial compost does not significantly reduce the growth of *A. bisporus*, indicating that the efficiency of its growth is dependent on particular microorganisms. The absence of compost endogenous microbiota decreased carbon cycling, as demonstrated by low respiration of the label. The composition of the bacterial community changed if *A. bisporus* was present, with a relative increase in activity of Gram- bacteria than Gram+ bacteria. There was little indication that *A. bisporus* growth is carbon limited , as both bacterial and fungal PLFAs were similar with and without additional glucose. The additional glucose further increased activity of the microbial biomass of specifically Gram- bacteria. Further research into the composition of the microbiota of the PIII compost using stable isotope labelling could further elucidate the relationship between *A. bisporus* and the microbial community. This study has improved the knowledge on the interaction of *A. bisporus* with the compost microbiota and their role in the carbon cycle within the compost bed.

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Appendices

Appendix A

Schematic diagram incubation

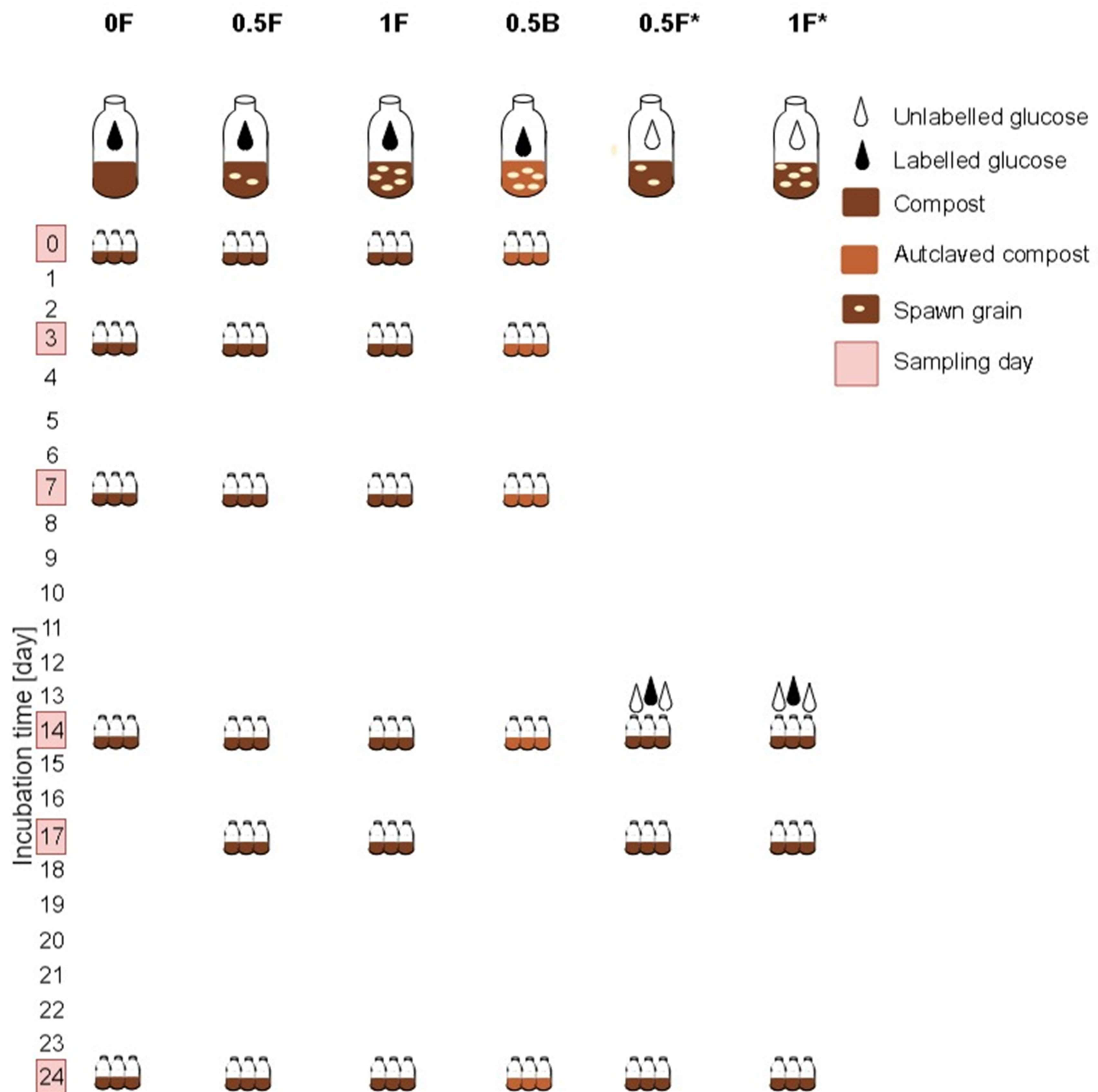

Figure A Schematic sampling diagram of the different conditions and sampling moments. Additional ^{13}C background samples at day 0 and day 14 not included in diagram.

Appendix B

PLFA concentrations

Table B1 *Total Fungal PLFA (µmol C g⁻¹ dry wt compost)*

	0d	3d	7d	14d	17d	24d
0F	11.1 ± 0.8	10.0 ± 0.5	13 ± 1.2	12 ± 1.4		12 ± 1.3
0.5F	16 ± 1.5	10.3 ± 0.5	8 ± 1.0	9 ± 5.0	6 ± 5.9	19 ± 6.8
1F	9.7 ± 0.8	14 ± 2.2	17 ± 2.5	2.9 ± 0.4	8.2 ± 0.9	9.3 ± 0.7
0.5B	7.6 ± 0.1	5.8 ± 0.2	7.4 ± 0.7	3.6 ± 0.02		13 ± 5.2
0.5F*				3.1 ± 0.11	17 ± 2.9	13 ± 3.3
1F*				3 ± 1.4	9 ± 2.5	12.0 ± 0.2

Table B2 *Absolute uptake ^{13}C uptake Fungal PLFA (µmol ^{13}C g⁻¹ dry wt compost)*

	0d	3d	7d	14d	17d	24d
0F	0.29 ± 0.02	6.6 ± 0.3	7.8 ± 0.5	6.7 ± 0.24		5.7 ± 0.4
0.5F	0.047 ± 0.04	5.8 ± 0.4	3.8 ± 0.7	4 ± 2.6	3 ± 2.7	6.7 ± 2.3
1F	0.11 ± 0.03	8 ± 1.2	9 ± 1.6	1.1 ± 0.2	3.6 ± 0.3	3.8 ± 0.7
0.5B	0.009 ± 0.005	0.3 ± 0.07	1.16 ± 0.4	1.4 ± 0.5		4 ± 2.5
0.5F*				0.0004 ± 0.0007	10 ± 2.1	8 ± 1.8
1F*				-0.006 ± 0.003	5 ± 2.2	7.0 ± 0.2

Table B3 *Total bacterial PLFA (µmol C g⁻¹ dry wt compost)*

	0d	3d	7d	14d	17d	24d
0F	107 ± 6.1	120 ± 1.5	156 ± 4.2	122 ± 4.2		135 ± 5.0
0.5F	104 ± 7.5	102.9 ± 0.7	61 ± 5.9	73 ± 7.6	47 ± 2.5	35 ± 4.4
1F	60 ± 3.8	138 ± 10.8	176 ± 7.8	49 ± 1.7	43 ± 2.42	17 ± 1.2
0.5B	45 ± 2.4	45.5 ± 0.6	59 ± 1.8	24 ± 1.7		16.7 ± 1.0
0.5F*				64 ± 3.6	75 ± 10.9	35 ± 6.4
1F*				60 ± 9.2	42 ± 2.3	23 ± 1.6

Table B4 *Absolute uptake ^{13}C uptake bacterial PLFA (µmol ^{13}C g⁻¹ dry wt compost)*

	0d	3d	7d	14d	17d	24d
0F	11 ± 3.0	74 ± 1.2	90 ± 2.8	68 ± 3.7		72 ± 2.1
0.5F	4.5 ± 0.8	64.9 ± 0.6	36 ± 3.3	36 ± 2.8	21 ± 1.3	14 ± 2.0
1F	2.8 ± 0.4	85 ± 6.9	108 ± 5.7	24.0 ± 0.7	23 ± 2.1	6.2 ± 0.6
0.5B	0.3 ± 0.1	7.2 ± 0.8	29 ± 3.9	11 ± 2.7		6 ± 1.5
0.5F*				0.001 ± 0.0005	0.37 ± 0.09	0.10 ± 0.01
1F*				-0.00028 ± 0.00078	0.09 ± 0.01	0.05 ± 0.003

Table B5 *Total Gram+ bacterial PLFA (µmol C g⁻¹ dry wt compost)*

	0d	3d	7d	14d	17d	24d
0F	68 ± 3.6	75 ± 1.1	91 ± 2.8	70.4 ± 3.4		81 ± 3.6
0.5F	65 ± 6.4	60.0 ± 0.4	35 ± 4.1	36 ± 7.3	27 ± 1.7	22 ± 3.3
1F	33 ± 3.1	90 ± 10.4	118 ± 3.8	26 ± 1.2	28 ± 2.3	11 ± 1.1
0.5B	25.4 ± 0.6	25.9 ± 0.4	38 ± 1.5	14 ± 1.4		11.7 ± 0.9
0.5F*				39.4 ± 0.6	42 ± 7.0	22 ± 3.8
1F*				37 ± 7.6	25 ± 1.4	16 ± 1.5

Table B6 *Absolute uptake ^{13}C uptake Gram+ PLFA (µmol ^{13}C g⁻¹ dry wt compost)*

	0d	3d	7d	14d	17d	24d
0F	7.7 ± 3.0	47.9 ± 0.8	59 ± 2	41 ± 3.3		44 ± 1.8
0.5F	3.3 ± 0.8	38 ± 0.4	21 ± 2.4	16 ± 2.7	10.5 ± 0.7	9 ± 1.5
1F	1.6 ± 0.3	54 ± 6.9	74 ± 3.7	12.0 ± 0.3	15 ± 2.0	3.7 ± 0.5
0.5B	0.2 ± 0.01	7.1 ± 0.8	23.4 ± 1.2	7.3 ± 1.6		6 ± 1.4
0.5F*				0.0006 ± 0.0005	0.12 ± 0.03	0.026 ± 0.007
1F*				-1.8E-04 ± 7.8E-05	0.01 ± 0.0009	0.01 ± 0.0004

Table B7 *Total Gram- bacterial PLFA (µmol C g⁻¹ dry wt compost)*

	0d	3d	7d	14d	17d	24d
0F	30 ± 4.8	35.8 ± 0.7	44 ± 2.8	40 ± 2.5		40 ± 1.3
0.5F	31.7 ± 4.0	34.7 ± 0.5	20 ± 1.0	29 ± 2.0	17 ± 1.8	10 ± 2.4
1F	22 ± 2.2	37 ± 2.1	46 ± 5.6	18 ± 1.2	12.3 ± 0.8	4.7 ± 0.4
0.5B	15 ± 2.4	15.6 ± 0.5	16.5 ± 1.2	4.7 ± 1.0		4.0 ± 0.3
0.5F*				19 ± 3.5	26 ± 7.1	11 ± 5.1
1F*				17 ± 4.6	14 ± 1.7	4.6 ± 0.5

Table B8 *Absolute ^{13}C uptake Gram- bacterial PLFA biomass (µmol ^{13}C g⁻¹ dry wt compost)*

	0d	3d	7d	14d	17d	24d
0F	1.5 ± 0.5	19.6 ± 0.7	24 ± 1.9	21 ± 1.7		21 ± 1.0
0.5F	0.7 ± 0.19	20.3 ± 0.46	12 ± 2.1	15.8 ± 0.18	8 ± 1.1	4.3 ± 1.1
1F	0.6 ± 0.23	22.5 ± 0.45	15.7 ± 4.3	9.5 ± 0.70	6.8 ± 0.6	1.9 ± 0.3
0.5B	0.06 ± 0.01	0.1 ± 0.1	4 ± 2.2	3 ± 2.0		0.2 ± 0.5
0.5F*				0.00063 ± 8.3E-05	0.14 ± 0.04	0.046 ± 0.001
1F*				0.0014 ± 0.0006	0.069 ± 0.008	0.024 ± 0.0021

Appendix C

Total PLFA concentration actinobacteria

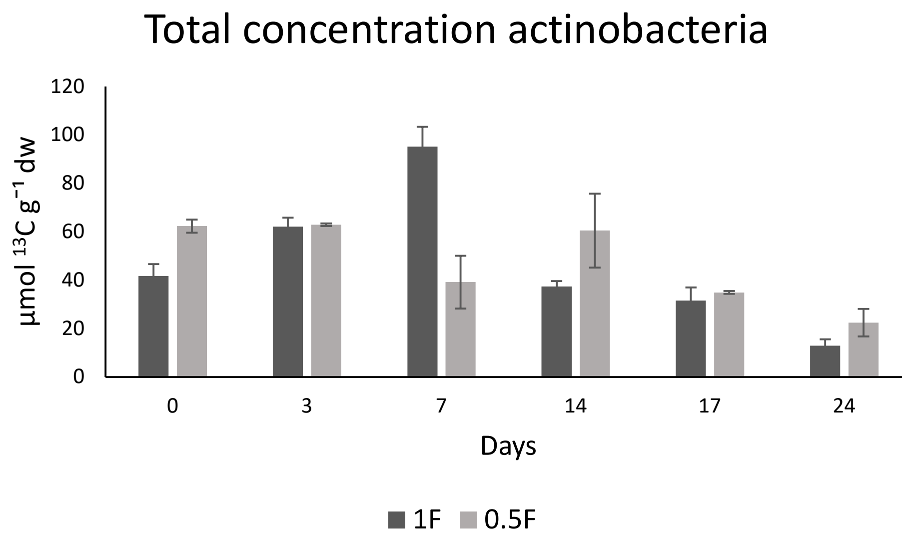

Figure C Total concentration 10-MeC16:0, biomarker Actinobacteria. Error bars represent SD, n=2

Appendix D

Bulk and CO_2 concentrations

Table D1 *Total C bulk compost (mmol C g^{-1} dry wt compost)*

	0d	3d	7d	14d	17d	24d
0F	24.3 ± 0.3	25.0 ± 0.9	26.5 ± 0.8	26.4 ± 0.6		26.0 ± 0.9
0.5F	26.6 ± 0.2	32 ± 7.1	25.4 ± 0.4	26 ± 1.28	24 ± 2.8	24.7 ± 0.4
1F	27 ± 1.1	25 ± 1.9	26 ± 1.4	26.3 ± 0.4	26.4 ± 0.7	25.1 ± 1.7
0.5B	25.7 ± 0.1	26 ± 1.6	27.4 ± 0.7	26.8 ± 0.3		26.1 ± 0.3
0.5F*				28± 4.3	26.8 ± 0.24	24.5 ± 0.8
1F*				25.1 ± 0.3	26 ± 2.0	24.8 ± 1.4

Table D2 *^{13}C absolute uptake bulk compost (µmol ^{13}C g^{-1} dry wt compost)*

	0d	3d	7d	14d	17d	24d
0F	89 ± 5.5	45 ± 1.9	47 ± 3.6	50 ± 2.0		41.4 ± 1.1
0.5F	89 ± 6.6	63.1 ± 7.1	44.9 ± 0.4	43 ± 1.3	35 ± 2.8	32.9 ± 2.0
1F	98 ± 4.2	50 ± 3.0	50 ± 9.1	40 ± 1.0	37.2± 1.2	31.9 ± 2.5
0.5B	73 ± 7.0	83 ± 9.4	42 ± 2.6	38 ± 9.7		28.0 ± 1.7
0.5F*				0.00063 ± 8.3E-05	0.14 ± 0.04	35.0 ± 4.8
1F*				0.0014 ± 0.0006	0.069 ± 0.008	42.1 ± 2.0

Table D3 *CO_2 ^{13}C incorporation (µmol ^{13}C h^{-1} g^{-1} compost)*

	Incorporation label CO2					
	3d	**7d**	**14d**	**17d**	**24d**	Cumulative*
0F	0.09 ± 0.01	0.04 ± 0.007	0.030 ± 0.002		0.04 ± 0.029	24 ± 5.8
0.5F	0.13 ± 0.02	0.05 ± 0.02	0.03 ± 0.05	0.05 ± 0.09	0.02 ± 0.003	41 ± 16.3
1F	0.07 ± 0.003	0.04 ± 0.02	0.07 ± 0.097	0.04 ± 0.005	0.01 ± 0.0002	41 ± 7.3
0.5B	0.02 ± 0.009	0.03 ± 0. 009	0.02 ± 0.018		0.02 ± 0.0008	13 ± 4
0.5F*			-6.5E-06 ± 1.7E-07	0.4 ± 0.05	0.08 ± 0.01	51 ± 1.3
1F*			-5.9E-05 ± 1.22E-06	0.04 ± 0.02	0.06 ± 0.008	54 ± 3.6

*in (µmol ^{13}C g^{-1})

Appendix E

Growth of *A. bisporus* during incubation

During incubation pictures were taken to track *A. bisporus* mycelial growth. To give some indication of its colonization rate, these pictures show the most important timepoints; initial growth (7 days), halfway point (day 14), and full colonization at the end of the incubation (24 days).

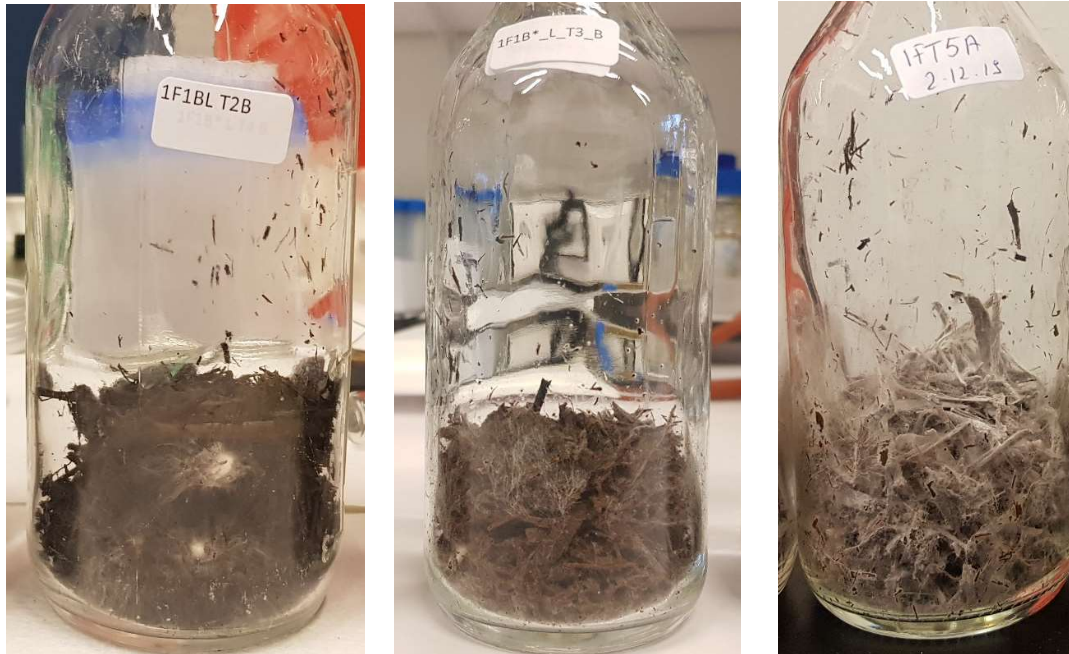

Figure E Photographs during incubation of *A. bisporus* colonization of the compost after 7 days, 14 days, and 24 days.