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# Nitrogen metabolism of wood decomposing basidiomycetes and their interaction with diazotrophs as revealed by IRMS

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ABSTRACT

Isotope ratio mass spectrometry (IRMS) is an advanced method to investigate carbon (C) and nitrogen (N) in organic samples. In particular, the N content, its isotope signature and the C/N ratio reveal important facts of nutrient cycling, niche separation and ecological food webs. In this study, the characteristics of N turnover of wood decomposing microorganisms were investigated.

The growth of the white rot causing basidiomycete *Trametes versicolor* is enhanced after addition of ammonia or urea, whereas the brown rot causing *Oligoporus placenta* is not accelerated. In addition, an interaction of each fungus with atmospheric $N_2$ assimilating (diazotrophic) bacteria was investigated. Cultivation experiments with a gas mixture of $^{15}N_2/O_2$ and subsequent IRMS analysis of dry biomass of the diazotrophs *Azotobacter croococcum*, *Beijerinckia acida* and *Novosphingobium nitrogenifigens* revealed that they assimilated up to 12% of their N from $N_2$. The experiments reflected N availability as a prerequisite for efficient growth of decomposing basidiomycetes and diazotrophs. Fungal–bacterial co-cultivation experiments showed that depending on the growth characteristics and bacterial $N_2$ assimilation activity N is transferred from certain bacteria into fungal biomass. Thus, the experiments gave a first indication of an interaction between wood decomposing basidiomycetes and diazotrophs, which is a novel pathway of fungal N acquisition.



## 1. Introduction

Interactions between bacteria and bacteria [1], bacteria and archaea [2–4] as well as between fungi and bacteria [5] have frequently been figured out to be a mutualistic partnership. The definition of a mutualistic interaction has been thoroughly discussed in the literature [6–8] and still undergoes fine tuning.

The principles of such interactions were studied employing elemental or molecular transfer between species. Nutrition turnover with a particular interest in N cycling was based on either the record of isotope abundances present in the environment [9–11] or on the fate of isotope labeled substrates (i.e., $^{13}C$, $^{15}N$) [12]. Both approaches applied isotope ratio mass spectrometry (IRMS).

The fractionation of isotopes is rather a result of catabolism than of anabolism, as catabolic reactions generally need lower activation energies than synthesis [9]. In most ecosystems, plants have the lowest $\delta^{15}N$ values, while saprophytes have higher $\delta^{15}N$ values [13], and ammonia species usually contain lower $\delta^{15}N$ values than nitrate species.

Beside IRMS, GC–MS [14,15], GC-C-IRMS [16,17] and HPLC-ESI-MS [18] were applied to recover $^{15}N$-enriched biomarkers, such as amino acids or chitin. In further studies on biomarkers of a high molecular weight, samples were pre-treated by electrophoresis for MS analysis [19] or by CsCl density-gradient supported ultracentrifugation for molecular biological analysis [20,21].

Fungal wood decomposition and N demand are already characterized in detail [22]. However, interactions of brown and white rot causing basidiomycetes and diazotrophs are postulated from acetylene reduction activity measurements, but have not been tested by isotope-enriched mass transfer [23,24]. Diazotrophs assimilate atmospheric $N_2$, reduce it to ammonia and ultimately transform it into organic N species [25]. Wood decomposing basidiomycetes are well adapted to access the main compounds of wood as C source [22]. A mutualistic interaction of brown and white rot causing basidiomycetes and diazotrophs is ought to intensify decomposition, as the lack of N in wood would be covered by bacterial $N_2$ assimilation, and, in turn, diazotrophs would participate in the fungal C pool (Fig. 1). Therefore, we assume that a mutualistic interaction is feasible, as the fungal decomposing activity would enhance nitrogenase catalyzed $N_2$ assimilation, which is triggered by ATP generation [26].

To prove that, we analyzed the environments and favored N substrates of both the white rot causing basidiomycete *Trametes versicolor* and the brown rot causing *Oligoporus placenta*. In

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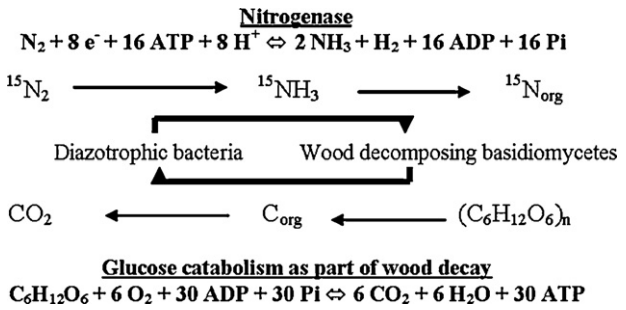


**Fig. 1.** Hypothetical interaction of diazotrophs and wood decomposing basidiomycetes.

addition, the $N_2$ assimilation rate and the interaction of each basidiomycete with the diazotrophs *Azotobacter croococcum* [27], *Novosphingobium nitrogenifigens* [28] and *Beijerinckia acida* [29] were investigated by tracing $^{15}N$-enriched atmospheric $N_2$ into fungal biomass by IRMS measurements.

## 2. Materials and methods

### 2.1. Sterilization procedure

All tools and media were sterilized following a standardized sterilization procedure (121 °C, $2 \times 10^5$ Pa, 20 min) in a laboratory autoclave (Varioklav Dampfsterilisator, H + P Labortechnik GmbH, Oberschleißheim, Germany).

### 2.2. Aqueous soil and wood extracts

Deionized water was added to 500 ml soil (collected from BAM Test Site Technical Safety, Baruth/Mark, Germany), 500 ml bark fragments from *Betula pendula* and 500 ml sawdust from *Pinus sylvestris*, respectively, to a final volume of 800 ml. The mixtures were sterilized (as mentioned in Section 2.1) and separated by filtration. The permeate was frozen in liquid $N_2$ and lyophilized (20 h at 10 Pa plus 4 h at 1 Pa, Lyophilisator Alpha 2–4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). Approximately 2–15 mg of each sample was analyzed by IRMS.

### 2.3. Cultivation of basidiomycetes

The reference basidiomycetes *Oligoporus placenta* (FPRL 280, Forest Products Research Laboratory, Watford, United Kingdom) and *Trametes versicolor* (CTB 863A, Centre Technique du Bois, Paris, France) were cultivated in 50 ml of liquid diazotrophic medium [30]. The medium was enriched by addition of 1 ml of 50% D-glucose solution and 250 μl of (i) 60 g $l^{-1}$ urea, (ii) 117 g $l^{-1}$ $NaNO_3$, (iii) 107 g $l^{-1}$ $NH_4Cl$ and (iv) deionized water as N-free control, respectively. The final concentration of (i)–(iii) was adjusted to 10 mM N. The fungal inocula were excisions of 5 mm × 5 mm of each basidiomycete grown on solid diazotrophic medium. The cultivation was carried out at 21 °C, 70% humidity and an incubation time of 7 to 35 days. After incubation, the mycelium was separated via filtration (Whatman No. 1, Schleicher & Schuell GmbH, Dassel, Germany), washed with distilled water, frozen in liquid $N_2$ and lyophilized (see above). Each mycelium was weighted and analyzed by IRMS. The N sources as well as potentially N-containing compounds for media preparation were analyzed as purchased.

Both basidiomycetes were also cultivated on a complex medium which contained 5% barley malt extract (Villa Natura Gesundprodukte GmbH, Kirn, Germany). After three weeks of incubation at 21 °C and a humidity of 70%, they were separated, rinsed and dried as mentioned above. These samples were analyzed by IRMS.

### 2.4. Cultivation of diazotrophs

*Azotobacter croococcum* (DSM 281, azotobacter Broth [30]), *Beijerinckia acida* (DSM 1714, beijerinckia agar [30]) and *Novosphingobium nitrogenifigens* (DSM 19370, nutrient agar pH 7: 5 g $l^{-1}$ peptone and 3 g $l^{-1}$ meat extract [30], German Collection of Microorganisms and Cell Cultures GmbH, Brunswick, Germany) were cultivated on 20 ml solid media according to respective recommendation and on solid diazotrophic medium. The experiments were carried out on Petri dishes (94/16, PS, w/vents, Paul Boettger OHG, Bodenmais, Germany) and in three replicate incubations. The incubation was carried out at 21 °C for three weeks in an exsiccator including a glass vial with 10 ml of sterile water to maintain humid conditions. At the beginning of the incubation time, the exsiccator was evacuated for 15 min with a vacuum pump (CVC 2000II, Vacuumbrand GmbH & Co. KG, Wertheim, Germany) to $4 \times 10^3$ Pa and then refilled with air under sterile conditions.

The experiment was repeated in an exsiccator, which was evacuated in the same manner, but refilled with a gas mixture of 75 vol% $^{15}N_2$ (98 atom% $^{15}N$) and 25 vol% $O_2$ (672793-SPEC, Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany). After incubation, the biomass was collected from the medium surface with a spatula. The biomass samples were frozen in liquid $N_2$, lyophilized, weighted and analyzed with IRMS.

### 2.5. Co-cultivation of basidiomycetes and diazotrophs

Both basidiomycetes were co-cultivated with each diazotroph in 20 ml liquid diazotrophic medium in Petri dishes (94/16, PS, w/vents, Paul Boettger OHG) for three weeks and in three replicate cultivations (i.e., two basidiomycetes × three diazotrophs × three replicates = 18 batches). The bacterial inocula consisted of approximately 20 μg cells harvested from pre-cultures on favored solid media as mentioned above. The fungal inocula was carried out as described above. The co-cultivation was carried out in an exsiccator filled with air and another one filled with a gas mixture of $^{15}N_2/O_2$ (Sigma–Aldrich Chemie GmbH) as described above. After incubation each mycelium was separately removed with a spatula and rinsed with sterile deionized water to separate attached bacteria. The mycelium was put into a reaction vial, while the medium suspension and the washing water were centrifuged at 8000 rpm for 10 min (Labofuge M, Heraeus Instruments GmbH, Berlin, Germany) to collect the bacterial biomass. The resulting bacterial biomass and the mycelia samples, respectively, were frozen, lyophilized as mentioned above, weighted and analyzed by IRMS.

### 2.6. IRMS measurements

The IRMS measurements were performed in an elemental analyzer (Vario EL III, Elementar Analysensysteme GmbH, Hanau, Germany) coupled to an isotope ratio mass spectrometer (IsoPrime, GV Instruments Ltd., Manchester, United Kingdom). The combustion proceeded in $O_2$ (purity: 99.9999%, AirLiquide Deutschland GmbH, Düsseldorf, Germany) for 90 s, and the carrier gas was He (purity: 99.9999%, AirLiquide). The reference gases $CO_2$ (purity: 99.998%, AirLiquide) and $N_2$ (purity: 99.9999%, AirLiquide) were used as gas standards. Caseine (Merck KGaA, Darmstadt, Germany) was measured 3–5 times before and after the measurements and after every sixth sample. If samples enriched in $^{15}N$ are analyzed, the $m/z = 29$ is difficult to determine, because the relevant faraday cup is adjusted to a low ion current. The problem was solved by low sample sizes. Reducing the amplifier resistance of the faraday cup or isotope dilution are further alternatives.

The elemental analyzer was calibrated with sulfanilic acid ($C_6H_7NO_2S$, MW: 173.18 g $mol^{-1}$, 41.6% C, 8.1% N, Merck KGaA) for C and N in the range of 0.1–20.0 mg.

**Table 1**
IRMS analysis of fungal biomass and potential *in situ* substrates. The mean values of three replicate measurements and standard deviations are given.

| Organic sample | N in % | C in % | C/N | $\delta^{15}N_{AIR\ N_2}$ in ‰ | $\delta^{13}C_{VPDB}$ in ‰ |
|---|---|---|---|---|---|
| Caseine | 14.42 (±0.1) | 49.14 (±0.6) | 3.41 (±0.l) | 6.29 (±0.1) | −21.97 (±0.1) |
| *O. placenta* on malt extract medium | 3.43 (±0.5) | 42.89 (±0.2) | 12.50 (±2.0) | 4.80 (±0.4) | −27.54 (±0.1) |
| *T. versicolor* on malt extract medium | 2.99 (±0.3) | 43.63 (±0.6) | 14.59 (±1.6) | 4.47 (±0.5) | −27.28 (±0.1) |
| Saw dust extract from *Pinus sylvestris* | 0.37 (±0.1) | 49.48 (±0.5) | 133.73 (±10.5) | – | −25.74 (±0.1) |
| Bark extract from *Betula pendula* | 1.18 (±0.1) | 43.08 (±0.4) | 36.51 (±0.2) | 4.26 (±0.4) | −26.48 (±0.1) |
| Soil extract | 2.33 (±0.l) | 15.69 (±0.3) | 6.73 (±0.2) | −6.98 (±0.2) | −26.19 (±0.1) |
| Yeast extract | 11.11 (±0.1) | 40.41 (±0.l) | 3.64 (±01) | −0.71 (±0.1) | −25.24 (±0.1) |

The mass spectrometer was calibrated with an IAEA secondary standard. The standard compound for C and N calibration was L-glutamic acid with $\delta^{13}C_{VPDB} = 26.39 \pm 0.04$‰ and $\delta^{15}N_{AIR\ N_2} = -4.5 \pm 0.1$‰ (USGS 40, International Atomic Energy Agency, Vienna, Austria) [31].

### 2.7. Calculation

The difference of the ion current ratios between sample and reference was denoted as δ value. This enables highly precise measurements of C and N without isotope reference materials [32].

The $\delta^{15}N$ value in ‰ is determined according to Eq. (1) considering the international standard AIR $N_2$ as reference.

$$\delta^{15}N_{AIR\ N_2} = \left( \frac{R(^{15}N/^{14}N)_{Sample}}{R(^{15}N/^{14}N)_{AIR\ N_2}} - 1 \right) \times 1000 \quad (1)$$

N is measured as $N_2$ with $m/z = 28$ ($^{14}N_2$) and 29 ($^{14}N^{15}N$), respectively.

The $\delta^{13}C$ values in ‰ are calculated analogously to Eq. (1) with Vienna Pee Dee Belemnite (VPDB) as international standard. Carbon is detected as four natural isotopologues with the $m/z = 44$ ($^{12}C^{16}O^{16}O$), 45 ($^{13}C^{16}O^{16}O$ or $^{12}C^{17}O^{16}O$) and 46 ($^{12}C^{16}O^{18}O$).

In tracing experiments with $^{15}N_2$, the $^{15}N$ abundance of the biomass was determined in %, too. For the determination, the $\delta^{15}N$ value was used in Eq. (2).

$$^{15}N_{sample} = \frac{100}{\left\{1/\left[\left(\left(\delta^{15}N_{AIR\ N_2}/1000\right)+1\right) \times \left(\left(^{15}N/^{14}N\right)_{AIR\ N_2}\right)\right]\right\} + 1} \quad (2)$$

The absolute isotope ratio of N in AIR $N_2$ is $^{14}N/^{15}N = 272.0 \pm 0.3$ and corresponds to the natural $^{15}N$ abundance of 0.3663% [33,34].

## 3. Results and discussion

### 3.1. N contents in basidiomycetes, wood species and soil

The elemental composition of the biomass of *O. placenta* and *T. versicolor* as well as samples of their natural environments were investigated (Table 1). Both basidiomycetes contained 42–44% C and approximately 3% N, which is consistent with previous results [13]. The other half of the dry biomass is presumably oxygen and hydrogen of carbon hydrates or cell wall polymers like chitin and trace elements. Due to the fact that the fungal environmental compartments are known to have a low N content [13], the N contents of soil, bark and sap wood were extracted from a 500 ml matrix. Analysis of dried extracts revealed that sap wood was almost N-free and bark contained less than 1.0% N (Table 1). Presumably, the N content of wood compartments is not sufficient for the fungal growth characteristics. The N contents of soil extract were 2–3%, suggesting a potential fungal N source, as basidiomycetes are known for their efficient intramycelial transport systems [35]. As element contents and δ values of fungal substrates differ significantly [13], the results only represent a first glance on the *in situ* conditions and may elucidate multiple factors affecting microbial decomposition.

The components of the cultivation media were analyzed, and all were N-free except for diazotrophic medium containing yeast extract. Considering the total N content of 11.1%, yeast extract was a minor N source in the medium and was present with 5 mg $l^{-1}$ N and a $\delta^{15}N$ value of −0.7‰.

The standard deviations of IRMS measurements were minor, indicating homogeneous samples, and high instrumental reproducibility of the measurements (Table 1). However, a divergence was obtained between replicate biological measurements suggesting variances in fungal growth. Therefore, defined conditions and a distinct comparison of educts and products are needed for isotope analysis in order to obtain reliable data sets.

The IRMS measurements were evaluated by the work standard caseine which had a low variance (Table 1). At measurements of highly $^{15}N$-enriched biomass, an increase of the subsequent standard $\delta^{15}N$ value occurred to a maximum of 7.5‰. Non-labeled samples were saved from this effect, because they were analyzed in temporal distance.

### 3.2. Impact of N salts on the growth of basidiomycetes

*In vitro* experiments with *T. versicolor* and *O. placenta* cultivated in N-limited and C-enriched diazotrophic medium amended with (i) urea, (ii) ammonia, (iii) nitrate and (iv) deionized water as N-free control, respectively, revealed that distinct N substrates were preferred. Both basidiomycetes grew slowly on traces of N derived from yeast extract of diazotrophic medium (Fig. 2A and B). The N content of the mycelia of both fungi decreased significantly over time (Table 2), and was lower on diazotrophic medium compared to malt extract medium (Table 1). The $\delta^{15}N$ values of both basidiomycetes cultivated on diazotrophic medium without additional N substrates converged over incubation time to those of the yeast extract. These results suggest that both basidiomycetes adjusted their metabolism to N-limited conditions, which was also mirrored in high C/N ratios.

Both basidiomycetes developed similar amounts of biomass over time with and without nitrate as additional N substrate in diazotrophic medium (Fig. 2A and B). The growth rates were low compared to other N-enriched media, and therefore nitrate was a non-favourite N substrate. Moreover, the $\delta^{15}N$ values of the mycelia did not correspond to those of nitrate.

In the presence of urea or ammonia, *T. versicolor* produced more biomass compared to diazotrophic medium without N addition, whereas *O. placenta* had a similar biomass production over time (Fig. 2A and B). The $\delta^{15}N$ values of biomass from *T. versicolor* converged to the respective N substrates. The $\delta^{15}N$ values of the low biomass of *O. placenta* differed from the N substrate (Table 2), indicating that a mix of N sources from urea or ammonia and yeast extract from the diazotrophic medium were metabolized.

To test the effect of yeast extract, the previously mentioned set of experiments was repeated with diazotrophic medium excluding yeast extract. While *T. versicolor* did not grow after 10 weeks of

**Table 2**
IRMS analysis of biomass obtained from *O. placenta* and *T. versicolor* cultivated at different N substrates. Mean values and standard deviations of three replicate measurements are given.

| N substrate[a] | *Oligoporus placenta* | | | | | *Trametes versicolor* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | N in % | C in % | C/N | $\delta^{15}N_{AIR\ N_2}$ in ‰ | $\delta^{13}C_{VPDB}$ in ‰ | N in % | C in % | C/N | $\delta^{15}N_{AIR\ N_2}$ in ‰ | $\delta^{13}C_{VPDB}$ in ‰ |
| *Two weeks of incubation* | | | | | | | | | | |
| N traces $\delta^{15}N = -0.71 \pm 0.01$ | 1.66 (±0.1) | 35.70 (±0.9) | 21.51 (±3.1) | −0.05 (±0.1) | −16.14 (±0.2) | 1.75 (±0.4) | 41.17 (±0.9) | 23.53 (±5.3) | −0.40 (±0.4) | −16.02 (±0.3) |
| Urea $\delta^{15}N = 2.55 \pm 0.01$ | 2.98 (±0.1) | 36.81 (±0.7) | 12.35 (±0.4) | 1.29 (±2.0) | −15.53 (±0.1) | 3.26 (±0.9) | 41.18 (±1.4) | 12.63 (±3.2) | 1.60 (±1.1) | −13.93 (±0.5) |
| $NH_4Cl$ $\delta^{15}N = -0.59 \pm 0.1$ | 2.79 (±0.2) | 38.53 (±1.6) | 13.81 (±0.4) | −2.89 (±0.5) | −15.84 (±0.1) | 4.61 (±0.5) | 41.04 (±0.8) | 8.09 (±0.9) | −4.48 (±0.1) | −13.08 (±0.3) |
| $NaNO_3$ $\delta^{15}N = 1.39 \pm 0.4$ | 2.20 (±0.4) | 34.95 (±0.6) | 15.89 (±2.3) | −1.71 (±0.2) | −16.04 (±0.2) | 1.99 (±0.3) | 40.51 (±0.7) | 20.36 (±3.4) | −1.90 (±0.2) | −15.81 (±0.3) |
| *Four weeks of incubation* | | | | | | | | | | |
| N traces $\delta^{15}N = -0.71 \pm 0.001$ | 0.98 (±0.3) | 36.01 (±1.1) | 36.74 (±9.7) | −0.12 (±0.3) | −15.92 (±0.2) | 0.56 (±0.5) | 36.17 (±0.8) | 64.59 (±5.8) | 0.23 (±1.0) | −16.12 (±0.4) |
| Urea $\delta^{15}N = 2.55 \pm 0.01$ | 2.47 (±1.0) | 36.87 (±0.4) | 14.93 (±10.6) | 1.97 (±1.9) | −15.43 (±0.1) | 2.48 (±0.4) | 39.03 (±1.4) | 15.74 (±3.0) | 3.10 (±1.1) | −12.95 (±0.6) |
| $NH_4Cl$ $\delta^{15}N = -0.59 \pm 0.1$ | 2.18 (±0.2) | 36.63 (±0.4) | 16.80 (±1.7) | −0.88 (±1.0) | −15.62 (±0.1) | 2.82 (±0.3) | 39.75 (±0.5) | 14.10 (±1.2) | −0.22 (±0.9) | −13.39 (±0.2) |
| $NaNO_3$ $\delta^{15}N = 1.39 \pm 0.4$ | 1.82 (±0.4) | 34.73 (±0.5) | 19.08 (±4.2) | −2.22 (±0.6) | −16.10 (±0.2) | 1.79 (±0.3) | 37.03 (±1.5) | 20.69 (±3.6) | −2.23 (±0.1) | −15.24 (±0.2) |

[a] Amended N substrates in diazotrophic medium were adjusted to 10 mM N. The $\delta^{15}N$ values of the N substrates, the standard deviations and N traces are listed.

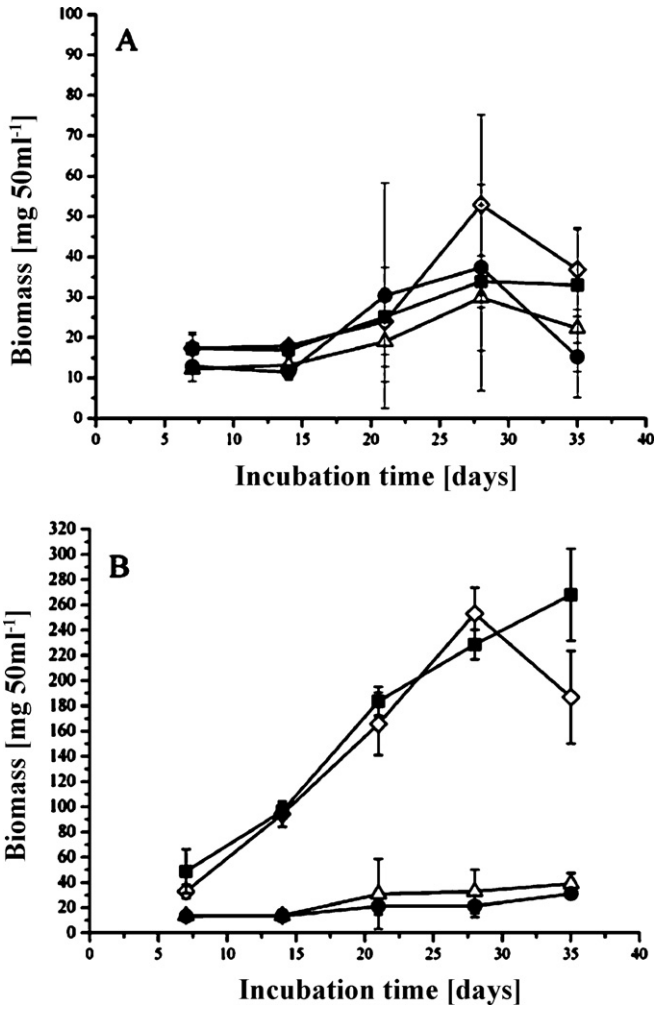


**Fig. 2.** Biomass formation versus time of *O. placenta* (A) and *T. versicolor* (B) grown in diazotrophic medium with addition 10 mM N of (◊) urea, (■) $NH_4Cl$, (△) $NaNO_3$ and (●) deionized water. Mean values and standard deviations of three replicate measurements are given.

incubation, *O. placenta* developed weak on all added N substrates but also in the N-free control (data not shown).

Growth experiments with other white rot causing basidiomycetes, such as *Hypholoma fasciculare* and *Pleurotus ostreatus*, revealed a similar affiliation to reduced N species like *T. versicolor* (data not shown), leading to the assumption that the addition of adequate amounts of reduced N species generally accelerates the growth of basidiomycetes.

### 3.3. $N_2$ assimilation rates in diazotrophs

Diazotrophs were selected according to N substrates, atmospheric $N_2$ fixation activity and to phylogenetic genera [27–29]. *A. croococcum*, an ubiquitous bacterium originally isolated from soil, was cultivated on solid diazotrophic as well as on specific azotobacter medium [30] under air and under a gas mixture of $^{15}N_2/O_2$ (Fig. 3A). The N content of the bacterial biomass was in both media and both atmospheres approximately 10% with a C/N ratio of 4–5. The $^{15}N$ abundance was in the range of the natural isotope abundance under air but was 12–13% when cultivated under a gas mixture of $^{15}N_2/O_2$, indicating a very efficient fixation and assimilation of $N_2$. However, traces of N in the medium or in the inoculum may diluted the $^{15}N$ abundance due to impurities in $CaCO_3$, which was added in high amounts to the azotobacter medium [30].

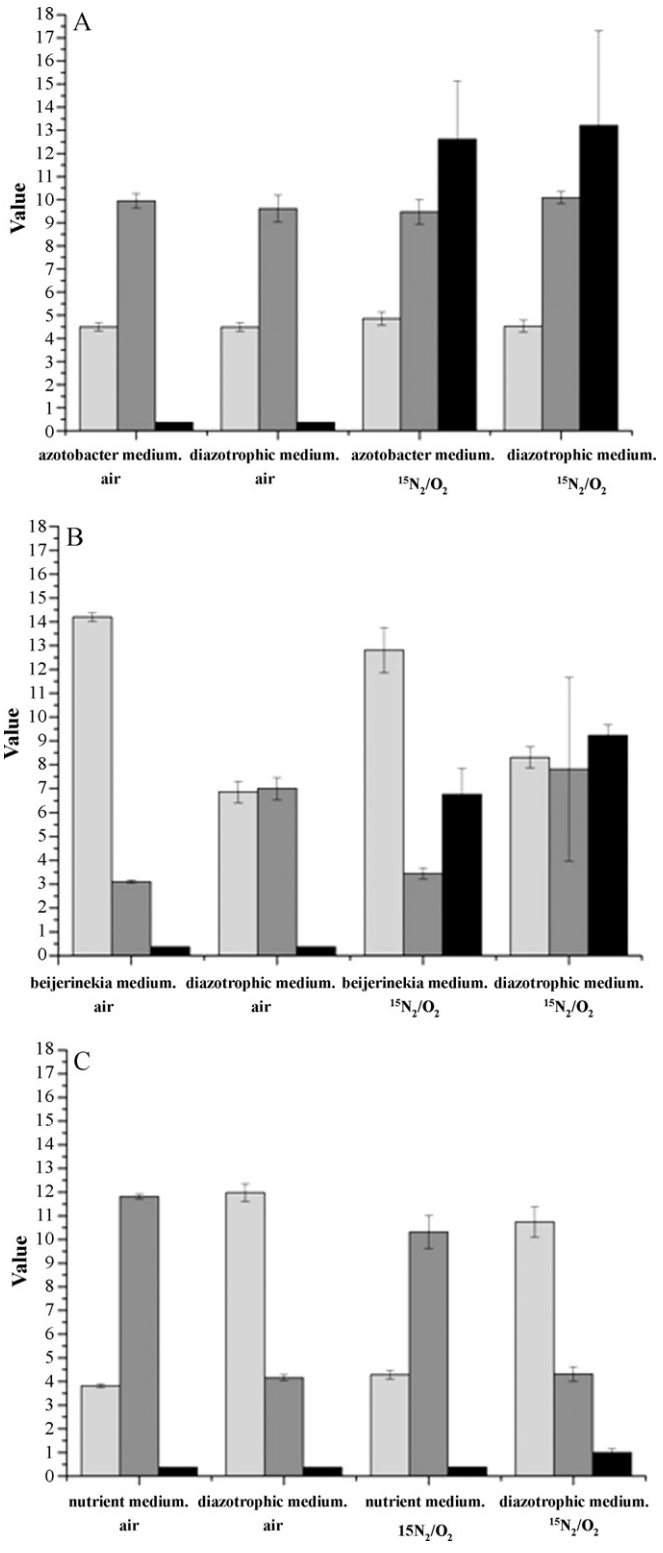


**Fig. 3.** C/N ratio (grey), N content in % (m/m) (dark grey) and $^{15}N$ abundance in % (black) after two weeks of incubation of *A. croococcum* (A), *B. acida* (B) and *N. nitrogenifigens* (C). The corresponding media and atmospheric composition are denoted Mean values and standard deviations of nine replicate measurements are given.

*B. acida* was able to assimilate even low amounts of N of the diazotrophic medium, resulting in N contents of 7–8% instead of 3–4% on beijerinckia medium (Fig. 3B). Beijerinckia medium is N-free, does neither contain $CaCO_3$ nor several trace elements used in diazotrophic medium, and may therefore explain the reduced N content in the biomass of *B. acida*. The $^{15}N$ abundance of its biomass in the gas mixture of $^{15}N_2/O_2$ was 7–9% indicating an efficient assimilation of atmospheric $N_2$. In addition, *B. acida* covered the whole medium surface rapidly and enabled the efficient discovery of nutrients such as trace concentrations of N. Such an approach is similar to mycelia-forming basidiomycetes, as their network of fine hyphae allows a nutrient uptake and transport. Unlike basidiomycetes, *B. acida* developed a sort of mucilage that covers cells and substrate surfaces.

*N. nitrogenifigens* was recently isolated as a strictly aerobic diazotroph from pulp and paper waste water and was characterized with nitrate reductase and urease enzyme activity [28]. The recommended nutrient medium for *N. nitrogenifigens* had approximately 1.1 g $l^{-1}$ N, which is a high amount of organic N compared to diazotrophic medium. In cultivation experiments, *N. nitrogenifigens* grown on nutrient medium had a significant higher N content than cultivated on diazotrophic medium (Fig. 3C). Biomass of *N. nitrogenifigens* cultivated on diazotrophic medium and under a gas mixture of $^{15}N_2/O_2$ revealed that $N_2$ assimilation is feasible but not preferred, as the $^{15}N$ abundance was less than 2%. If nutrient medium was applied, no enrichment in $^{15}N$ abundance occurred and therefore no $N_2$ assimilation can be assumed.

IRMS studies confirmed that all tested diazotrophs fixed atmospheric $N_2$, although their assimilation characteristics differed significantly depending on species and media. In addition, each diazotroph assimilated beside atmospheric $N_2$, N sources originated from the medium in trace amounts. Therefore, further environmental conditions such as availability of N sources determined whether and to which extent bacterial $N_2$ assimilation takes place *in situ*. Trace elements like molybdenum, vanadium and iron are essential for diazotrophic key proteins and are more abundant in soil than in wood [36]. Therefore, considerable $N_2$ assimilation without soil contact is assumed as improbable although harsh environments select towards a broad diversity of diazotrophs [37].

### 3.4. Tracing N in co-cultivated diazotrophes and basidiomycetes

Interactions in N exchange of diazotrophs and basidiomycetes were determined in co-cultivation experiments on diazotropic medium under a gas mixture of $^{15}N_2/O_2$ and air (Tables 3 and 4). As control *T. versicolor* (Table 4A) and *O. placenta* (Table 3A) were also cultivated separately at these conditions. The total biomass of co-cultivations revealed high standard deviations, resulting from a significant biological variation at low concentrations and non-avoidable residues after the separation procedure. However, a negative impact of the $^{15}N_2/O_2$ atmosphere on fungal development was not observed, as the N contents in both basidiomycetes were similar under both atmospheres. The $^{15}N$ abundance of fungal biomass was within the range of the natural abundance and also similar under both atmospheres underlining that both basidiomycetes do not assimilate atmospheric $N_2$ (Tables 3A and 4A).

Co-cultivation experiments of *O. placenta* with diazotrophs revealed that *A. croococcum* marginally affected fungal growth (Table 3B), *B. acida* reduced fungal growth (Table 3C) whereas *N. nitrogenifigens* was overgrown by *O. placenta* (Table 3D).

*A. croococcum* enhanced the N content in *O. placenta*, although the bacterial N content was lower than in previous experiments (compare Table 3B with Fig. 3A). However, the $^{15}N$ abundance was almost similar under a gas mixture of $^{15}N_2/O_2$ and air indicating that *O. placenta* rather metabolized *A. croococcum* than hosted it as a $N_2$ assimilating partner. The colonies from initially inoculated *A. croococcum* were recovered visually after incubation, but they were hard to separate from the mycelium. Thus, the IRMS results may be biased and may explain why increased N contents were observed in both organisms.

**Table 3**
Biomass, N-content and IRMS results of *O. placenta* (A) and of co-cultivation of *O. placenta* with *A. croococcum* (B), *B. acida* (C) and *N. nitrogenifigens* (D) on diazotrophic medium under air and a gas mixture of $^{15}N_2/O_2$, respectively. Mean values and standard deviations of six replicate measurements of separated fungal and bacterial biomass are given.

| | Organism | Atmosphere | Biomass in mg 20 ml$^{-1}$ | N-content in % | $\delta^{15}N_{AIR\ N_2}$ in ‰ | $^{15}N$ abundance in % |
|---|---|---|---|---|---|---|
| A | *O. placenta* | Air | 7.43 (±0.5) | 0.16 (±0.1) | 3.21 (±2.3) | 0.37 (±3 10$^{-3}$) |
| | *O. placenta* | $^{15}N_2/O_2$ | 13.10 (±2.4) | 0.06 (±0.1) | 8.66 (±6.0) | 0.37 (±2 10$^{-3}$) |
| B | *O. placenta* | Air | 8.70 (±0.7) | 0.48 (±0.3) | 5.54 (±4.8) | 0.37 (±2 10$^{-3}$) |
| | *A. croococcum* | | 8.50 (±0.3) | 2.05 (±0.12) | 4.76 (±2.1) | 0.37 (±1 10$^{-3}$) |
| | *O. placenta* | $^{15}N_2/O_2$ | 9.10 (±0.l) | 1.03 (±0.71) | 362.75 (±3 10$^2$) | 0.50 (±1 10$^{-1}$) |
| | *A. croococcum* | | 1.40[a] | 2.83[a] | 56.79[a] | 0.36[a] |
| C | *O. placenta* | Air | 7.17 (±0.3) | 0.29 (±0.1) | 6.09 (±2.1) | 0.37 (±1 10$^{-1}$) |
| | *B. acida* | | 13.47 (±1.4) | 7.04 (±0.6) | −0.19 (±2.1) | 0.37 (±3 10$^{-5}$) |
| | *O. placenta* | $^{15}N_2/O_2$ | 6.3 (±0.5) | 0.27 (±0.1) | 26335.50 (±9 10$^3$) | 9.13 (±2.9) |
| | *B. acida* | | 12.67 (±1.4) | 9.14 (±1.2) | 40989.26 (±7 10$^3$) | 13.37 (±2.1) |
| D | *O. placenta* | Air | 8.75 (±0.1) | 0.46 (±0.2) | 14.86 (±1.6) | 0.37 (±1 10$^{-3}$) |
| | *N. nitrogenifigens* | | 2.55 (±0.1) | 1.71 (±0.1) | 13.24 (±4.0) | 0.37 (±1 10$^{-3}$) |
| | *O. placenta* | $^{15}N_2/O_2$ | 15.83 (±1.6) | 2.34 (±0.4) | 241.6 (±1 10$^2$) | 0.45 (±1 10$^{-1}$) |
| | *N. nitrogenifigens* | Not sufficient biomass for IRMS measurement | | | | |

[a] The biomass from three replicate cultivations was pooled for a single IRMS measurement.

*B. acida* also enhanced the N content in *O. placenta* (Table 3C), but co-cultivations were dominated by bacterial biomass. Under the $^{15}N_2/O_2$ atmosphere, *B. acida* assimilated more $N_2$ than without *O. placenta*, and the bacterial biomass had a $^{15}N$ abundance of approximately 12–13% (compare Table 3C with Fig. 3B). The presence of the fungus may forced *B. acida* to fix more $N_2$ from the gas phase. However, high $^{15}N$ abundance was also determined in the biomass of *O. placenta*. Although the growth of *O. placenta* was negatively affected in presence of *B. acida*, we conclude that bacterial fixed $N_2$ was transferred to the fungus.

However, the standard deviation of $^{15}N$ abundance and the $\delta^{15}N$ values of biomass retrieved from experiments at a gas mixture of $^{15}N_2/O_2$ were considerably higher than those at air. Nevertheless, the shift in $^{15}N$ abundances between both types of experiments was significant and reproducible and revealed that *B. acida* assimilated and transferred $^{15}N_2$ (Table 3C). In case of the other diazotrophs a non significant enrichment in $^{15}N$ was detected due to the high standard deviations.

Biomass of *N. nitrogenifigens* in co-cultivation was recovered in both low amounts and low N contents compared to incubations in the absence of *O. placenta* (compare Table 3D and Fig. 3C). However, the bacterial biomass collected after incubation under a gas mixture of $^{15}N_2/O_2$ was to low for subsequent IRMS measurements. In contrast, *O. placenta* developed well in co-culture and produced a similar amount of biomass compared to pure culture incubations under both atmospheres. However, the fungal N content was higher in the presence of *N. nitrogenifigens*, indicating that the N of the bacterial biomass was at least partly metabolized. A shift in the fungal $^{15}N$ abundance was not observed, and therefore no active bacterial–fungal interaction occurred. In addition, *N. nitrogenifigens* may suffer from non-optimal conditions in the acidic mycosphere of *O. placenta* caused by acidic excretion [22].

The aforementioned diazotrophs were also co-cultivated with *T. versicolor*, which was regarded as a fungus with a high affinity to reduced N substrates (Table 2) and a less acidic mycosphere than *O. placenta*.

When *T. versicolor* and *A. croococcum* were co-cultivated, the separation difficulties occurred in the same manner as for *O. placenta* and *A. croococcum*. Mycelium and bacterial colonies were strongly connected to each other, while the biomass of *T. versicolor* dominated, and the amount of bacterial biomass was probably overestimated due to fungal contamination. The N content was approximately 2% in both bacterial and fungal biomass suggesting that *A. croococcum* was rather metabolized than being a mutualistic partner. Tracing experiments under a gas mixture of $^{15}N_2/O_2$ did not result in an increase of $^{15}N$ abundance of any of both organisms (Table 4B).

**Table 4**
Biomass, N-content and IRMS results of *T. versicolor* (A) and of co-cultivation of *O. placenta* with *A. croococcum* (B), *B. acida* (C) and *N. nitrogenifigens* (D) on diazotrophic medium under air and a gas mixture of $^{15}N_2/O_2$, respectively. Mean values and standard deviations of six replicate measurements of separated fungal and bacterial biomass are given.

| | Organism | Atmosphere | Biomass in mg 20 ml$^{-1}$ | N content in % | $\delta^{15}N_{AIR\ N_2}$ in ‰ | $^{15}N$ abundance in % |
|---|---|---|---|---|---|---|
| A | *T. versicolor* | Air | 16.8 (±0.8) | 0.09 (0.1) | 4.37 (±4.4) | 0.37 (±1 10$^{-3}$) |
| | *T. versicolor* | $^{15}N_2/O_2$ | 15.5 (±3.0) | 0.16 (0.1) | 1.57 (±0.8) | 0.37 (±1 10$^{-4}$) |
| B | *T. versicolor* | Air | 16.23 (±1.4) | 1.72 (±0.1) | −0.15 (±0.6) | 0.37 (±1 10$^{-4}$) |
| | *A croococcum* | | 3.73 (±1.0) | 2.51 (±0.3) | 7.39 (±4.5) | 0.39 (±2 10$^{-3}$) |
| | *T. versicolor* | $^{15}N_2/O_2$ | 7.77 (±0.3) | 1.62 (±0.6) | 101.40 (±92.8) | 0.40 (±4 10$^{-2}$) |
| | *A croococcum* | | 6.30 (±0.9) | 2.54 (±0.1) | 22.66 (±6.1) | 0.37 (±2 10$^{-3}$) |
| C | *T. versicolor* | Air | 6.93 (±1.6) | 1.43 (±0.5) | 6.20 (±2.5) | 0.37 (±9 10$^{-4}$) |
| | *B. acida* | | 13.80 (±1.2) | 6.41 (±0.7) | −0.17 (±2.3) | 0.37 (±6 10$^{-5}$) |
| | *T. versicolor* | $^{15}N_2/O_2$ | 8.07 (±0 4) | 0.83 (±0.5) | 40291.08 (±6 10$^3$) | 13.18 (±1.6) |
| | *B. acida* | | 11.13 (±0.4) | 8.91 (±1.2) | 37922.17 (±1 10$^4$) | 12.52 (±2.8) |
| D | *T. versicolor* | Air | 15.23 (±0.6) | 0.78 (±0.2) | 6.19 (±1.1) | 0.37 (±4 10$^{-4}$) |
| | *N. nitrogenifigens* | | 0.50[a] | 0.78[a] | 41.78[a] | 0.36[a] |
| | *T. versicolor* | $^{15}N_2/O_2$ | 16.13 (±1.1) | 1.15 (±0.3) | 14.80 (±6.4) | 0.37 (±4 10$^{-4}$) |
| | *N. nitrogenifigens* | | 3.03 (±0.9) | 2.27 (±1.3) | 103.40 (±50.0) | 0.40 (±4 10$^{-4}$) |

[a] The biomass from three replicate cultivations was pooled for a single IRMS measurement.

In contrast, co-cultivation of *B. acida* and *T. versicolor* produced more bacterial than fungal biomass under both atmospheres. The N content of *B. acida* was similar to that in pure culture, while N content of *T. versicolor* was higher compared to IRMS results obtained from pure culture incubations (compare Table 4A and C). The $^{15}N$ abundances were 12–13% in both bacterial and fungal biomass (Table 4C). Therefore, we conclude that N was actively transferred from bacterial into fungal biomass. However, for a mutualistic interaction an increase in fungal biomass compared to exclusive fungal incubations was expected. The $\delta^{15}N$ values were similar to those obtained in the co-cultivation experiments with *O.placenta* (compare Tables 3C and 4C). Although, these values are characterized by high standard deviations they underline the $^{15}N$ assimilation in case of *B. acida* but not for *A. croococcum* and *N. nitrogenifigens*.

If *N. nitrogenifigens* was co-cultivated with *T. versicolor*, more bacterial biomass was recovered than in the corresponding co-cultivation experiments with *O. placenta* (compare Tables 4D and 3D). This was probably caused by the strong acidic mycosphere of *O. placenta*. However, in both co-cultivations the bacterial biomass is very low, and the fungal biomass prevailed. The bacterial N content was lower in co-cultivation than in pure culture incubation (compare Table 4D and Fig. 3C), whereas the N content of *T. versicolor* after co-cultivation with *N. nitrogenifigens* increased compared to the corresponding pure culture incubation (Table 4A and D). Tracing experiments under a gas mixture of $^{15}N_2/O_2$ did not result in an increase of the $^{15}N$ abundance of any of both organisms. The results suggest that *N. nitrogenifigens* did not significantly fix $N_2$ and that the bacterial inoculum was metabolized by the fungus.

Considering the low growth rates of diazotrophs in the presence of any fungus, a mutualistic interaction is difficult to detect. Bacterial inocula with higher cell numbers or longer incubation time could overcome such limitations, but enriched bacterial inocula may also serve as an additional N substrate and longer incubation time may coincide with other nutrient depletion.

## 4. Conclusions

N substrates of basidiomycetes and experiments under N-limiting conditions confirmed that these fungi are highly adapted to low environmental N concentrations. Co-cultivations revealed that both fungi may also receive N from diazotrophs, but such interactions are very strain specific and generally not mutualistic. To conclude from our laboratory based studies to applied wood protection, wood preservatives containing reduced N species as active agents or stabilizers could enhance the activity of white rot causing basidiomycetes if ammonia or urea is released over time. Interactions between diazotrophs and white rot causing fungi have the potential to accelerate wood decomposition, as ammonia is a favored fungal N substrate and the first intermediate of nitrogenase catalyzed $N_2$ assimilation. However, ideal conditions for bacterial $N_2$ fixation and assimilation such as availability of molybdenum, vanadium or iron are presumably not present in wood only, and therefore interactions between diazotrophs and white rot causing fungi are more likely in soil-wood environments. On N-depleted timber constructions N-efficient brown rot prevails [22]. Interaction between brown rot causing fungi and diazotrophs is not described nor can now be assumed.

IRMS has been proven to be an appropriate instrumental method to trace and quantify fungal and bacterial N turnover and fungal–bacterial interactions, and reflects a link between C and N mass cycle. Future studies will focus on further bacterial–fungal interactions or mass spectrometric measurements to trace molybdenum and vanadium as essential elements of diazotrophic proteins.

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