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*Original Article*

# Determination of $N_2$ and $N_2O$ Fixation Rates in Leguminous Plants and Three Different Soil Types by Labeling Experiments with Stable Nitrogen Isotopes

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**Abstract:** Nitrogen (N) is an essential nutrient and plays an important role in plant growth and physiology. In addition, N is also the limiting nutrient in most terrestrial ecosystems. The increasing use of N fertilizers increases agricultural production, but also has negative impacts on biodiversity, water quality and increases emissions of greenhouse gases such as nitrogen oxides ($NO_x$) and nitrous oxide ($N_2O$) into the atmosphere.

$N_2O$ is a strong greenhouse gas and the product of microbial transformation processes of N introduced into soil and groundwater (nitrification and denitrification). The production of $N_2O$ in soils is highly dependent on oxygen and water content, soil temperature and texture and the available amount of reactive nitrogen ($NO_3^-$ or $NH_4^+$).

In agricultural soils, $N_2O$ emissions are also influenced by the type of fertilizer used, crops grown, soil pH and $NO_3^-$ concentration.

Refined forms of land management, such as the cultivation of legumes, can reduce the use of fertilizers and thus also the emission of $N_2O$. Legumes can use symbiotic nodule bacteria (rhizobia) to bind atmospheric $N_2$ and make it available to the plant. Non-symbiotic soil microorganisms such as cyanobacteria or other heterotrophic and autotrophic prokaryotes are also able to fix $N_2$.

Furthermore, the $N_2$-fixing enzyme nitrogenase, which is specific for most organisms, is not specific for other $N_2$ compounds, especially $N_2O$.

To study the $N_2$ and $N_2O$ fixation potential of soils and legumes, experiments with isotope-enriched N-gases (100 mol% $^{15}N_2$ and $^{15}N_2O$) were performed. Three different soil types (forest, meadow and wetland) as well as legume plants inoculated with rhizobia bacteria (Rhizobium *leucaneae*) (Leucaena *leucocephala*) were incubated in microcosms with different mixing ratios of $N_2$ and $N_2O$ over a period of one week. The measured $^{15}N$ enrichment was then used to determine the biological uptake rates.

Both in plants and in soils admixtures of up to 40% $N_2O$ had no influence on the $N_2$ fixation rate. $N_2$ uptake rates were 1.2 ± 0.4 ug N $gdw^{-1}$ $d^{-1}$in forest and grassland soils and 3.9 ± 1.2 ug N $gdw^{-1}$ $d^{-1}$ in wetland soils. In contrast, the $N_2$ fixation rates of leguminous plants were significantly higher (130 ± 40 ug N $gdw^{-1}$ $d^{-1}$), with the highest accumulation not in root nodules but in the roots. In both plants and soil, the $N_2O$ fixation of 0.2 ± 0.1 ug N $gdw^{-1}$ $d^{-1}$ could only be measured from a concentration of 4 vol%.

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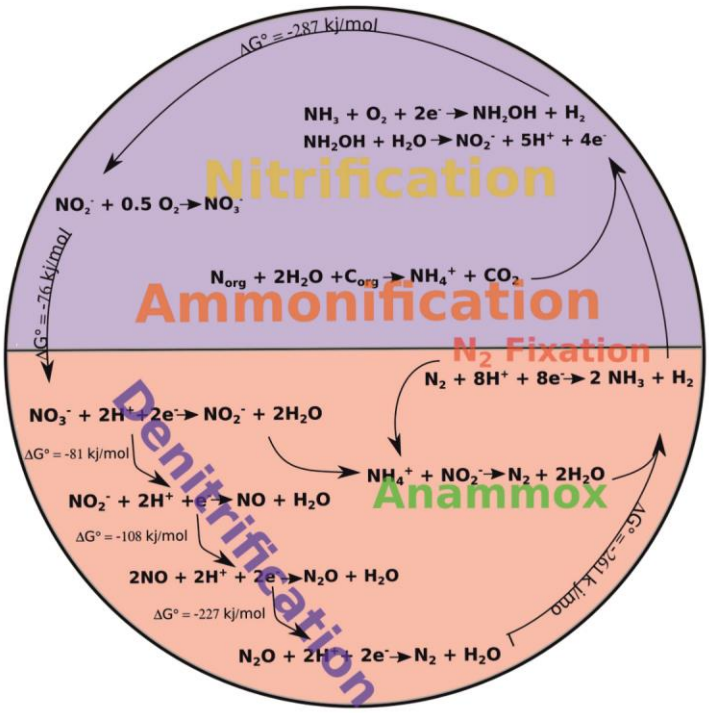
The results indicate that both soils and legumes have a high potential to bind atmospheric nitrogen in the form of both $N_2$ but not $N_2O$. The fate of nitrogen in soil needs to be further investigated. $N_2$ fixation seems to play an important role in wetland soils. Legumes in particular have the potential for $NH_4^+$ input and $N_2O$ reduction, as most of the solid nitrogen has been transferred to the roots, which can contribute significantly to soil nutrient input.

**Keywords:** Nitrogen fixation; Nitrous oxide; Denitrification; Nitrogen Cycle; $^{15}N_2$- labelling; Stable nitrogen isotopes

## 1. Introduction

Nitrogen (N) is the most important limiting nutrient in all terrestrial ecosystems. N is critical for all life forms as it is needed for many vital substances such as nucleic acids, proteins and chlorophyll. Although about 78% of the Earth's atmosphere consists of diatomic nitrogen ($N_2$), this nitrogen is not available to most living organisms due to the strong triple bond between the N molecules. However, numerous prokaryotes, i.e. bacteria and archaea, developed the ability to convert $N_2$ into reactive nitrogen in the form of ammonia in a process called nitrogen fixation. Nitrogen fixation is the starting point of the global nitrogen cycle, which is a complex biogeochemical system of several N-transformations under different environmental conditions (Box. 1, Fig. 1).




**Box 1:**

Nitrogen cycle: The nitrogen cycle begins with the biological fixation of gaseous nitrogen ($N_2$) from the air, which is inaccessible to most plants. Microorganisms have the ability to

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bind $N_2$ through the enzyme nitrogenase as diazotrophic free life forms or in symbiotic relationship to plants.

The resulting reactive nitrogen can enter the biosphere in the form of ammonia. Further processing of the fixed nitrogen takes place in aerated soils exclusively by chemolithoautotrophic prokaryotes, which gain energy through ammonia oxidation. The first step is the oxidation of ammonia to nitrite via hydroxylamine, which is carried out by ammonia oxidizing prokaryotes. Important ammonia oxidizing groups are Nitrosomas, Nitrospira and Nitrosococcus among bacteria as well as numerous ammonia oxidizing archaea. Ammonia oxidation is followed by the transformation of nitrite into nitrate, mainly by the chemo-lithoautotrophic bacterial groups Nitrospira, Nitrobacter and Nitrococcus and Nitrospina. Both groups, ammonia-oxidizing prokaryotes and nitrite-oxidizing prokaryotes, obtain their energy from the respective nitrogen compounds. Since the energy yield from both compounds is comparably low, both groups are known to be slow growers and have to convert high amounts of ammonia to bind enough $CO_2$ to maintain cell growth. Nitrification occurs mainly in aerobic environments. However, since ammonia oxidation is important, e.g. for the removal of environmentally harmful ammonia in wastewater treatment plants, which can lead to eutrophication of the environment, the maintenance of nitrifying populations in wastewater treatment plants is an important task. A new, recently discovered metabolic pathway is anaerobic ammonia oxidation by the bacterium Planctomycetes (Anammox). An important feature of anammox is the production of gaseous $N_2$ without emission of $N_2O$, which is an advantage over aerobic nitrification.

The nitrogen cycle is closed by denitrification, i.e. the successive reduction of nitrate to gaseous nitrogen. Denitrification is an anaerobic process, and nitrate and gaseous intermediates of denitrification are an important source of energy for microorganisms living in anoxic environments.

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Without anthropogenic influences, biological $N_2$ fixation accounts for an input of about 98 Tg N $yr^{-1}$ into terrestrial ecosystems. Since the end of the 19th century, this input has decreased by about 20 Tg N $yr^{-1}$ due to land-use changes [1]. Marine biological $N_2$ fixation rates are about 121 Tg N $yr^{-1}$ and have remained constant over the last 150 years. The inputs of N by the Haber Bosch process introduce about 100 Tg N $yr^{-1}$, mainly into terrestrial ecosystems [1]. This contribution of the Haber Bosch process currently makes it possible to supply 27-40 % of the world's population due to increased productivity of agricultural soils [2].

However, intensive inputs of reactive nitrogen increasingly affect terrestrial and aquatic ecosystems, causing eutrophication and air pollution. The release of large quantities of greenhouse gases, mainly in the form of $N_2O$ and $NO_x$, accounts for more than 20 % of total greenhouse gas emissions. In addition, the Haber Bosch Process contributes more than 1.5 % to global energy consumption annually [2]. Both greenhouse gas emissions and energy

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consumption are expected to increase, as it is estimated that 50% more food will have to be produced by 2050 [3].

Several approaches are discussed to address the problems associated with increasing N inputs, such as dietary change, progress in agricultural engineering and waste reduction [4,5]. For this reason, new approaches are needed to quantify N inputs and transformations in order to track the whereabouts of N in different domains and scales.
Natural isotope ratios of nitrogen compounds can help to elucidate different paths of the nitrogen cycle and to identify the most important actors responsible for the assimilation and transformation of N from micro- to ecosystem levels [6,7].
Isotope techniques have been used for several decades to track the global nitrogen cycle [7]. Applications can be divided into studies with natural abundance isotope ratios and $^{15}N$ enrichment studies. Applications with natural $^{15}N/^{14}N$ ratios use the advantages of fractionation processes in microbial and inorganic N-transformations. The ratio $^{15}N/^{14}N$ of atmospheric nitrogen is 0.004 [8].

The nitrogen isotope composition of organic matter, dissolved components and gases is given in the delta notation:

$$\delta^{15}N = \frac{R_{Sample} - R_{Standard}}{R_{Standard}} \times 1000$$

Due to its homogeneous isotopic composition, air is commonly used as a reference material [8]. Microbial N-transformations, especially nitrification and denitrification, are in most cases associated with a strong isotope fractionation. The isotope fractionation, e.g. in denitrification, can be expressed as:

$$\alpha = \frac{1000 + \delta^{15}N_{Nitrite}}{1000 + \delta^{15}N_{Nitrate}}$$

$N_2$ fixation is characterized by only minor isotope fractionation with an average fractionation factor of about 1.0020 [6]. A higher fractionation occurs in nitrification, where $NO_3^-$ is depleted by a factor of 1.0285 compared to $NH_4^+$ [6]. Highest fractionation factors are observed during nitrification, whereby the $N_2O$ loss during incomplete nitrification shifts the isotope ratio of $NO_2^-$ to $N_2O$ by a value of 1.0600. The same applies to the $N_2O$ loss during denitrification with a fractionation factor of about 1.0305. Complete denitrification consumes $NO_2^-$ relative to $NO_3^-$ by a α value of 1.0060 [6].

Isotope fractionation also occurs during assimilation of $NH_4^+$, $NO_2^-$ and $NO_3^-$ with fractionation factors of 1.0158, 1.0210 and 1.0142, respectively. This means that the organic

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compounds produced are always enriched with $^{15}N$ compared to their starting material, which leads to the rule of thumb: "You are what you eat, +1 permille". Therefore, N-isotopes can be used to evaluate trophic interactions within ecosystem food webs [9].

Other approaches to the use of isotopes for the investigation of N fluxes and transformations are the use of enriched N compounds, i.e. compounds with a significantly different nitrogen isotope composition than the natural isotope ratio. Materials such as $^{15}N_2$, $^{15}N_2O$, $^{15}NO_3^-$ and $^{15}NH_4^+$ enable the tracking and quantification of the fate of reactive nitrogen in microorganisms and plants from microorganism- to ecosystem scale [7]. $^{15}N$ enrichment methods enable N fluxes and turnover to be determined directly in a system [7]. In addition, isotopic enrichment of compounds such as DNA, lipids or cellulose in combination with spectroscopic and molecular methods allows the isolation and visualization of key individuals and hotspots of the N cycle and new insights into biogeochemical systems.

In this study we used the $^{15}N$ isotope label to determine the N-uptake rates of soils by diazotrophic microbes. In addition, we incubated legume plants vaccinated with rhizobia. We select three different soil types, grassland, forest and wetland soils, to compare the $N_2$ fixation in these soils, which differ in their microbial community structure. In addition, we measured $N_2$ in pulses (Leucaena *leucocephala*) inoculated with rhizobia (Rhizobium *leucaneae*).
The aim of the study was to compare the uptake rates of bare soil types and to compare them with the uptake of legumes. The aim is to determine the potential to increase the N input of leguminous plants compared to unmodified soils. This could provide insight into the potential of N-input by biological N-fixation by leguminous plants compared to diazotrophic N-inputs.
In addition, we have investigated the ability of the enzyme nitrogenase to bind $N_2O$. Since the enzyme nitrogenase is not specific for $N_2$, but also has the ability to bind $N_2O$, legume plantations could be a significant sink for the greenhouse gas $N_2O$.

## 2. Materials and Methods

In order to determine the uptake rates of soils and legumes, microcosmic incubations were performed in which soils and plant samples were incubated under a $N_2/N_2O$ mixed atmosphere.
Soils from grassland and forest were taken from three different soil types in the Hainich National Park, Thuringia, Germany [10], while soil samples from wetlands were taken from the Hartusov-Mofette field observatory [11-12]. Soil samples were taken with a 2.6 inch snail at three different locations in each soil type, thoroughly mixed, sieved at 2 mm and then air-dried at 40°C. Soil samples were then collected from the field observatory at a temperature of 40°C. Soil samples were subsequently placed in 120 ml vials where soil moisture was adjusted to 60% water holding capacity (WHC) with sterilized deionized water. The sample vessels were sealed gas-tight with butyl rubber plugs fastened with crimp caps. The mixing ratios within the headspace were adjusted for different amounts of labeled and

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unlabeled $^{15}N_2$ and $^{15}N_2O$. In order to determine the $^{15}N_2$ fixation and the influence of different amounts of $N_2O$ on the $N_2$ fixation rates, 40% $^{15}N_2$ were mixed with different percentages of unlabeled $^{14}N_2O$. The mixing ratios were in the range of:

40% $^{15}N_2$ : 40% $^{14}N_2$ : 20% $O_2$ (treatment 1)
40% $^{15}N_2$ : 40% $^{14}N_2O$ : 20% $O_2$ (treatment 2)
40% $^{15}N_2$ : 36% $^{14}N_2$ : 4% $^{14}N_2O$ : 20% $O_2$ (treatment 3)
40% $^{15}N_2$ : 39.6% $^{14}N_2$ : 0.4 $^{14}N_2O$ :20% $O_2$ (treatment 4)

Additional experiments were conducted with different mixing ratios of labeled $^{15}N_2O$ within an oxic $^{14}N_2$ matrix. Concentrations ranged at:

40% $^{14}N_2$ : 40 % $^{15}N_2O$ : 20% $O_2$ (treatment 5)
76% $^{14}N_2$ : 4% $^{14}N_2O$ :20% $O_2$ (treatment 6)
79.6% $^{14}N_2$ : 0.4% $^{15}N_2O$ : 20% $O_2$ (treatment 7)
79.996% $^{14}N_2$ : 0.004% $^{15}N_2O$ : 20% $O_2$ (treatment 8)

Each of the eight treatments was performed in triplicate to determine the biological variability of the N-fixation. Incubation was performed under light conditions and lasted 72 hours. In order to take leakages and gas consumption, especially oxygen, into account, the mixing ratios in the headspace of each bottle were readjusted after 36 hours. After opening the flask, the gas was released.
Soils were air-dried, ground and measured for their $^{15}N/^{14}N$ ratio with a Delta V Advantage IRMS (Thermo Fisher Scientific, Bremen, Germany) coupled to an elemental analyzer.
Plant samples were taken from the flasks and separated into root, nodule and leaf components. The samples were air-dried at 40°C and then ground, weighed in tin capsules and measured in the same way as soil samples.
The mean uptake rate was determined by using the mean of three biological replicas with the same treatment.

## 3. Results

Table 1 shows results for labeling experiments conducted with bare soils. Uptake rates were normalized for uptake [ug] per gram dry weight of soil per day.

| | | Uptake rate [ug N $gdw^{-1}$ $d^{-1}$] | |
|---|---|---|---|
| **Treatment** | **Grassland soil** | **Forest soil** | **Wetland soil** |
| 1 | 0.28 ± 0.19 | 0.67 ± 0.45 | 3.6 ± 1.85 |
| 2 | 0.74 ± 0.24 | 0.95 ± 0.61 | 3.71 ± 1.97 |
| 3 | 1.08 ± 0.45 | 0.81 ± 0.51 | 3.81 ± 2.12 |
| 4 | 1.08 ± 0.64 | 1.01 ± 0.64 | 3.63 ± 2.02 |
| 5 | 0.43 ± 0.10 | 0.33 ± 0.28 | 1.41 ± 0.82 |

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| | | | |
|---|---|---|---|
| 6 | 0.10 ± 0.05 | 0.03 ± 0.03 | 0.21 ± 0.11 |
| 7 | - | - | - |
| 8 | - | - | - |

**Table 1.** Results for uptake rates in experiments conducted with bare soils.

For forest soils, uptake rates did not vary significantly from those of grassland soils (p=0.56). The mean uptake rates were between 0.80 ± 0.38 and 0.88 ± 0.18 ug $gdw^{-1}$ $d^{-1}$. The uptake rates of wetland soils were significantly higher than in both grassland soils and forest soils (p<0.01), with constant values between about 3.6 and 3.8 ug $gdw^{-1}$ $d^{-1}$.

Admixtures of different amounts of $^{14}N_2O$ (up to 40 Vol%) did not influence the apparent uptake rate. No significant changes in $^{15}N_2$ uptake were observed for all treatments and all soil types.

The uptake rates for treatments with $^{15}N_2O$ (treatment 5 - 8) were slightly lower than in treatments 1 to 4 and in experiments with $^{15}N_2O$ concentrations below 4 ppm no uptake could be observed.

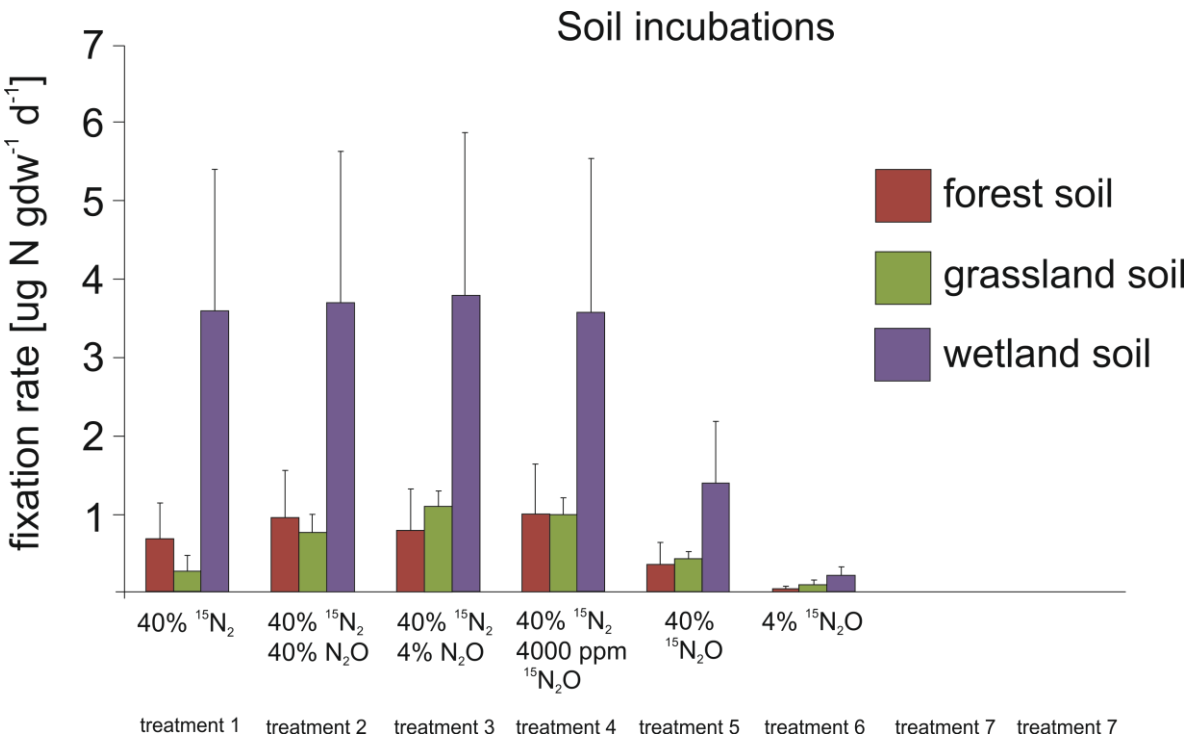


**Figure 2.** Uptake rates in bare soils determined by $^{15}N_2$ and $^{15}N_2O$ labelling. Error bars represent the 2σ standard deviation of all three replicas.

In the plant experiments, uptake rates were three orders of magnitude higher than in soil experiments. Most markers were found in root samples where enrichment was highest, and uptake rates of up to 133 $ug^{-1}$ $gdw^{-1}$ $d^{-1}$ were measured.

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| | Uptake rate [ug N gdw$^{-1}$ d$^{-1}$] | | |
|---|---|---|---|
| **Treatment** | **Nodules** | **Roots** | **leaves** |
| 1 | 65.36 ± 20.36 | 132,50 ± 41.43 | 40.00 ± 35.01 |
| 2 | 38.22 ± 18.94 | 110.72 ± 49.64 | 14.65 ± 6,44 |
| 3 | 0.2 ± 0.1 | 2.4 ± 0.06 | 2.53 ± 0.36 |
| 4 | - | - | - |
| 5 | - | - | - |

**Table 2.** Uptake rates in legume plants determined by $^{15}N$ and $^{15}N_2O$ labelling. Error bars represent the 2σ standard deviation of all three replicas.

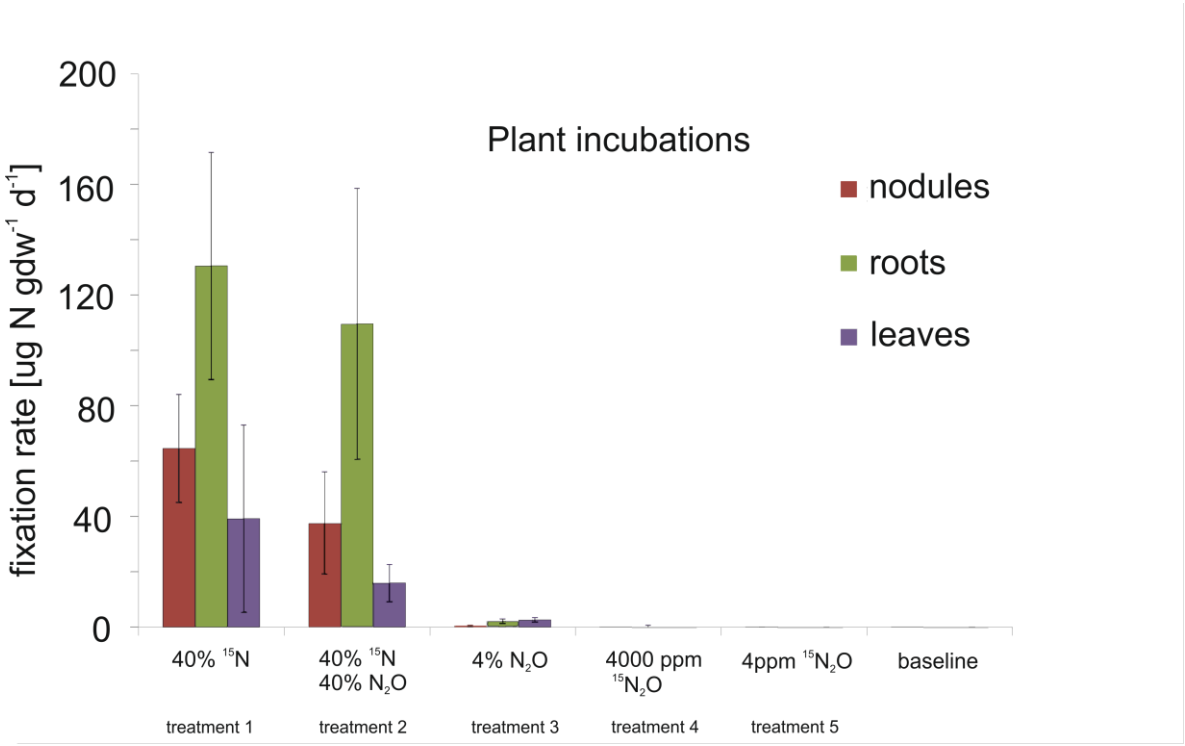


**Figure 3.** Uptake rates of plants determined by $^{15}N_2$ and $^{15}N_2O$ labelling. Error bars represent the 2σ standard deviation of all three replicas.

## 4. Discussion

*Applicability of the $^{15}N_2$ method*

Isotopically enriched $^{15}N_2$ has been used for several decades to determine rates and fate of biologically fixed $N_2$ [7]. Direct $^{15}N_2$ marking has several advantages over other indirect methods, such as $C_2H_2$ reduction assays or gas measurements in the headspace [13,14,7]. Compared to other methods, direct $^{15}N_2$ marking experiments have the following advantages:

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- Direct determinations of $^{15}N$ fixation, without assumptions or calibration, which may be distorted due to experimental conditions.
- No control samples are required, which reduces the complexity and cost of the experiments.
- Even small amounts of $^{15}N_2$ are detectable.
- Fixed $^{15}N$ can be directly traced in metabolites such as amino acids or DNA, which makes it possible to track the fate of embedded $N_2$ in plants and ecosystems [6].

Due to the high cost of $^{15}N$ labelled gases, experiments with labelled $^{15}N_2$ are mainly limited to incubations in mesocosms or microcosms, but not to field applications [7]. This was also the case in our study. For our experiments we used 1728 ml $^{15}N_2$ and 480 ml $^{15}N_2O$, which can lead to costs of up to several thousands of Euros. Considering the relatively low enrichment of incubated soils and plants in our microcosm experiments, it is obvious that the costs for experiments in vessels larger than 5 liters exceed reasonable costs. But also the rates obtained from microcosm experiments can be very useful for the following reasons:

- Most microbial processes in microcosm experiments work on the nano- and micro-scale, which can also be represented in microcosms [6].
- Microcosm experiments can be performed in remote locations and with a variety of soils, plants or lichens and provide new data on unknown soil types [add number here, Atto manuscript].
- These data can be incorporated into biogeochemical models and combined with remote sensing techniques to provide information for higher spatial scales.

To the best of our knowledge, our data provide the first determinations of biological nitrogen fixation in forest and grassland soils determined by direct $^{15}N$ labelling [7,15]. In temperate forests, both symbiotic and asymbiotic $N_2$ fixation are considered important for N input [1]. $N_2$ fixation in soils of temperate hardwood forests is estimated to be about 0.369 g N $m^{-2}$ $yr^{-1}$ for purely non-symbiotic fixation and 1.604 g N $m^{-2}$ $yr^{-1}$ including symbiotic $N_2$ fixation [15]. Our estimate of non-symbiotic $N_2$ fixation in forest soils is 1.71 ± 0.35 g N $m^{-2}$ $yr^{-1}$, which is an order of magnitude higher than the fluxes reported by [15]. However, [16] reported increasing total nitrogen stocks between 2004 and 2009 in soils from the old deciduous forest from which the samples originate, suggesting enhanced nitrogen cycling in this region. Therefore, the higher uptake rates might be a regional pattern and not comparable with global rates of $N_2$ fixation.

$N_2$ fixation values from the grassland soil were similar to those from forest soils and ranged at 1.55 ± 0.72 g N $m^{-2}$ $yr^{-1}$. Compared to ecosystem data from [15], the rates determined with the direct $^{15}N_2$ method in our study are again higher than those obtained with the $C_2H_2$ method, with average $N_2$ fixation rates of up to 0.251 ± 0.393 g N $m^{-2}$ $yr^{-1}$.

The uptake rates in wetland soils showed a significantly higher uptake rate, which was 7.16 ± 0.1752 g N $m^{-2}$ $yr^{-1}$. Ecosystem-wide $N_2$ uptake rates in wetland soils are rare, but since wetlands are oligotrophic ecosystems, increased diazotrophic uptake is plausible [1]. In Spanish rice fields the fixation rates of $N_2$ ranged between 0.023 and 7.55 g N $m^{-1}$ $y^{-1}$. Similar to grasslands, cyanobacteria are extremely important drivers of N input in wetland soils.

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They contain specialized heterocyst cells that maintain nitrogen activity by protecting the enzyme from oxygen. Cyanobacteria are also known to maintain nutrient inputs in rice paddy soils and also provide the majority of N in natural wetland systems [7,15].

*Comparison of N2 fixation rates between soils*

The insignificant difference between grassland and forest soil is consistent with the relative gene frequencies from the study areas [10]. Similar relative frequencies of *nifH* genes encoding for nitrogenase were determined for both the forest soil and the grassland soil, with the frequencies in the forest soil being slightly increased [10], suggesting a similar genetic potential for $N_2$ fixation in both soils. [10] suggested a higher importance of $N_2$-fixation by free-living nitrogen fixing bacteria in forest soils than in grassland soils, which cannot be confirmed by the present experiment, because of similar uptake rates in both soils. Grassland soils can store high amounts of carbon and their primary production is highly dependent on biological nitrogen fixation [17]. Legumes are not a dominant component of vegetation in most grasslands and account for only 1 to 5 % of grassland ecosystems [15]. In contrast, [17] found that especially autotrophic soil crusts of cyanobacteria consortia as well as free-living diazotrophic proteobacteria make up the largest part of N input into natural grassland ecosystems and thus underline the high importance of biological crusts not only for the carbon cycle, but also for N cycling [18].

However, both soil types show a high number of bacteria of the genus Rhizobium [10], which indicates that there is an even higher potential for symbiotic N fixation in both soil types, which could not be demonstrated with our experiment.

As already mentioned, the higher N uptake rates of the wetland soil might be related to the high number of cyanobacteria, which can amount to up to 5% of the microbial community in the sampled soils [19]. An increased input of N by cyanobacteria seems plausible, since this group of organisms finds good living conditions in wetlands due to often occurring anoxic conditions in these ecosystems, which are favorable for $N_2$ fixation and nitrogenase activity [2]. Elevated $N_2$-fixation rates in oligotrophic ecosystems compared to more nutrient rich ecosystems might also be related to a downregulation of $N_2$ fixation in the presence of other nitrogen compounds, especially of free-living diazotrophs [20].

Measured symbiotic N-fixation of legumes is three orders of magnitude higher than the diazotrophic uptake measured in soils, with most of the nitrogen being stored in the nodules and roots. The results thus illustrate the potential of legumes to act as a natural way of reactive N in soils [21]. Currently legume crops contribute >30% of human nutritional nitrogen [21]. Since most of fixed $^{15}N_2$ was measured within the roots and nodules, our results demonstrate the ability of biological $N_2$ fixation for direct belowground N input into soils.

The $^{15}N_2O$ fixation could only be measured at unrealistically high $N_2O$ concentrations. Thus, the nitrogenase does not appear to be an active $N_2O$ sink. The measured uptake rates at 4 % $^{15}N_2O$ may be due to the uptake of $^{15}N_2O$ reduced to $^{15}N_2$. Although $N_2O$ is not actively bound either in the soil or in legumes, $N_2$ fixation provides a good opportunity to naturally

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introduce N into the soil, especially through legumes and cyanobacteria, which reduces fertilization and production of $N_2O$.

## 5. Conclusions:

Microcosm experiments with $^{15}N_2$ and $^{15}N_2O$ have been conducted in order to determine $N_2$ and $N_2O$ uptake rates. The method was suitable to trace $^{15}N_2$ and $^{15}N_2O$ into bare soil samples and legume plants. Significant differences were measured in wetland soils compared to grassland and forest soils, whereas higher uptake rates in wetland resulted from the presence of cyanobacteria as well as the oligotrophic conditions and consequently the lack of other bioavailable nitrogen compounds like $NH_4^+$ or $NO_3^-$. Nitrogenase seems not to be a relevant source for $N_2O$, because $N_2O$ uptake could only be measured at a concentration of 4 ppm, which is too high for natural abundance occurrences. The presences of high amounts of $N_2O$ have also no influence on the fixation rate of $N_2$, since $N_2$ uptake rates did not vary up to $N_2O$ concentration as high as 40%. Uptake rates obtained by direct $^{15}N_2$ labelling were three orders of magnitude higher than in for non-symbiotic $N_2$ fixation in soils, showing the high potential to use legumes for increasing soil N input. Since most of fixed $N_2$ was transferred to roots, compared to shots and nodules, demonstrates that there is a high potential of belowground $N_2$ input, which might be an advantage compared surface input by litter and manure, because of lower $N_2O$ emissions.

**Author Contributions:**

**Funding:** Please add: "This research received no external funding".

**Conflicts of Interest:** Declare conflicts of interest or state "The authors declare no conflict of interest."

**Acknowledgements:**

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