# *IN-VITRO* RETENTION OF DIFFERENT $^{15}$N SOURCES AT BIOCHAR

Klaus-P. Götz[1]*, Klaus D. Wutzke[2]

[1]Agricultural Climatology, Faculty of Life Sciences, Humboldt-University of Berlin, Albrecht-Thaer-Weg 5, 14195 Berlin, Germany

[2]Research Laboratory, University of Rostock, Ernst-Heydemann-Str. 8, 18057 Rostock, Germany

## Abstract

*Biochar, which can be used for improving soil fertility, is a carbon rich, heterogeneous, and chemically complex material. It is known that biochar ab/adsorbs nutrients, but there is still a lack of knowledge regarding these effects. To investigate the ability of biochar to adhere nitrogen an in-vitro experiment was conducted. Different inorganic (ammonium, nitrate, and ammonium nitrate) and organic $^{15}$N sources (urea; valine, phenylalanine, isoleucine, and glutamic acid; also yeast protein as a high molecular weight compound) were added to Pyro-biochar and to HTC-biochar to estimate retention after 15 and 30 days, and also to examine the effect of washing on $^{15}$N. The $^{15}$N in-vitro retention (%) of $^{15}$N-inorganic and $^{15}$N-organic substances at Pyro-BC and HTC-BC, at days 15 and 30, was less than 10% for both biochar's. This amount could substance specific clearly reduced by washing. The high molecular weight $^{15}$N yeast protein adhered to 30% for HTC-biochar, which was 3-fold higher than for Pyro-biochar. Therefore, the manufacturing process of the two biochar's had a significant impact on in-vitro retention. The functional groups, and therefore Van-der-Waals-interactions, chemical reaction, the affiliation of amino acids and the molecular size of compounds, such as yeast protein, can affect the $^{15}$N retention of these substances.*

**Key words:** *$^{15}$N technique, Pyro-biochar, HTC-biochar, $^{15}$N retention*

## 1. INTRODUCTION

The importance of N as an essential nutrient, the rates of its anthropogenic input in production systems, the unavoidable losses to the environment, and the complexity of the biological, physical, and chemical factors that regulate N cycling processes all contribute to the necessity of understanding the soil N cycle (van Groenigen et al. 2015). Plant's use of both inorganic N (ammonium ($NH_4^+$) and nitrate ($NO_3^-$), and also organic N (e.g. free amino acids), is dependent on the availability and production of the different N moieties (Andresen et al. 2015 and references therein). Soil carbon compounds also occur in different forms. The two pools of carbon--inorganic and organic—may co-exist. Organic forms can be subdivided into "recalcitrant carbon," or carbon resistant to decay, such as humus, and also "labile carbon," in which the carbon is bioavailable in the form of easily degraded compounds such as oils, sugars, and alcohols (Wilson 2014). Biochar, which can be used for improving soil fertility, is a carbon- rich, heterogeneous, and chemically complex material, and its various actions in soil are difficult to tease apart and explain mechanistically. Biochar results from heating biomass in a no- or low-oxygen environment. Technically this can be accomplished by a variety of methods. A very good, comparative overview of biochar in terms of production, physico-chemical properties, and applications is provided by Kambo and Dutta (2015). The resulting charcoal product resembles a blackened, shrunken version of the original biomass. The product now has been converted from the lignin-cellulose-hemicellulose complex to a broad spectrum of carbon allotropes. The result is a collection of disjointed graphite crystals, having hexagonally-shaped carbon rings, with some leftover hydrogen and oxygen attached, along with minerals in the original biomass (Wilson 2014). Depending upon the type of biochar, precursor feedstock, production process and conditions, application rate, surface area, porosity, type of soil, and climate, the crop yield response can be either productive or counterproductive in response to the application of biochar to soil (Kambo and Dutta 2015, include other references). From the moment biochar is removed from the kiln, its surfaces begin to oxidize, and form a variety of new compounds. These changes result in different molecules being attached to the surface, and composed primarily of oxygen, hydrogen, and carbon. These functional groups are able to bond with various nutrients and

minerals (Wilson 2014). Given its pores and its electrical charges, biochar is capable of both absorption and adsorption. Absorption (AB-sorption) is a function of pore volume. The larger pores absorb water, air, and soluble nutrients, similar to a normal sponge. Adsorption (AD-sorption) depends on surface area and charge. The surfaces of biochar, both internal and external, adsorb materials by electro-chemical bonds, and function as would an electric sponge. Quantification of the respective involvement of nitrogen sources at various transformation processes (e.g. N uptake, mineralization, immobilization, and gaseous losses) is not possible, as a result of the same chemical identity of the nitrogen. Although it is known that biochar ab/adsorbs nutrients, the potential for reversibility of these effects is poorly understood. A charge of biochar having organic nutrients (compost/digestate) before application would only be useful if the nitrogen is later released from the biochar and can become plan-available. Natural nitrogen isotope values for synthetic fertilizers and for fertilizers that may be permitted by EU Regulation 2092/91 fall within a relatively narrow range, close to 0 %, with 80% of the samples lying between −2% and 2%, and 98.5% of the data having $\delta^{15}N$ values less than 4% (Batemann and Kelly 2007). The fertilizers that may be permitted in organic agriculture have a higher mean $\delta^{15}N$ value of 8.6%, and exhibit a broader range in $\delta^{15}N$ values, from 0.6–36.7%. Therefore, the enriched stable isotope $^{15}N$ (enrichment of the tracer > 0.366 atom% $^{15}N$) is more effective when investigating these processes in the plant-soil system, especially at levels relating to both controlled-condition and agricultural ecosystem-focused experiments (Bedard-Haughn et al. 2003). To investigate the ability of two biochar's to adhere/retain nitrogen, an *in-vitro* experiment was conducted, as part of research reported by Reibe (2015). Using the $^{15}N$-dilution technique, different inorganic and organic $^{15}N$ substances were added to both Pyro-biochar (Pyro-BC) and HTC-biochar (HTC-BC), to estimate time-dependent retention after 15 and 30 days, and also the effect of washing on $^{15}N$ levels.

## 2. MATERIAL AND METHODS

### *2.1 Biochar's*

Pyro-BC was manufactured from maize silage by pyrolysis at 600 °C for 30 minutes, and HTC-BC from maize silage using hydrothermal carbonization at 230 °C for 4 h (Reibe 2015), and both were used in the conduct of this experiment.

### *2.2 In-vitro experiment*

Biochar's were dried at 60 °C to constant weight and sieved to < 2 mm. To 150 mg biochar (in 2 ml reaction tubes; Eppendorf, Germany) 1.5 ml solution of the respective $^{15}N$ source (Table 1) was added and gently shaken. Following experimental setup, the tubes remained (for the respective appropriate time durations) at a constant temperature of 15 °C in an incubator (Memmert GmbH & Co. KG, Germany). Sampling was performed after 15 and 30 days, respectively. Following the respective incubation times, the reaction tubes were first gently shaken, and the biochar $^{15}N$-solution mixture was quantitatively transferred into a paper filter (MN 616, diameter: 11 cm, thickness: 0.2 mm, Macherey-Nagel GmbH & Co. KG, Germany). For the 'washing' treatment, 2 x 1.5 ml of water was added to the Pyro-BC and HTC-BC. After the passage of the aqueous phase, the paper filters with the biochar inside were dried at 60 °C to constant weight. After drying, 4 mg of each sample was weighed on tin slices (28 mm, IVA Analysentechnik e. K., Meerbusch, Germany) for analysis. The $^{15}N$ enrichment and corresponding N amounts were measured with an isotope ratio mass spectrometer (Sercon 20-20, UK). This experimental approach involves the factor's biochar (Pyro-BC, HTC-BC), nine different $^{15}N$ sources (Table 1), sampling date (15, 30 days), and washing (without and with). These factor levels/treatment combinations were combined, and repeated three times.

### *2.3 $^{15}N$ sources and $^{15}N$ calculation*

The applied $^{15}N$ sources (Table 1) were divided into inorganic compounds (ammonium, nitrate, and ammonium nitrate) and organic compounds (urea; amino acids: valine (Val), phenylalanine (Phe), isoleucine (Iso), and glutamic acid (Glu)). Lyophilized yeast protein (YP) (*Wutzke et al., 1983) was used as a relevant high-molecular compound. All sources were highly enriched with $^{15}N$ between 87 and

98 atom% $^{15}N$. Ammonium chloride served as the ammonium source, and potassium nitrate as the nitrate source.

To 150 mg biochar 1.5 ml of a 30 mM $^{15}N$ solution of the respective nitrogen source was added, which corresponds to 0.675 mg $^{15}N$ (=100%) per reaction tube. $^{15}N$ was expressed on an excess basis. The measured $^{15}N$-enrichment (atom-% $^{15}N$), the $^{15}N$-enrichment (atom-% $^{15}N$ excess above natural abundance of 0,3663 atom-%) of Pyro-and HTC-BC treated only by water for 15 and 30 days, without and with washing (data not shown), was subtracted, and the corresponding $^{15}N$ amount (Faust et al., 1981) and the $^{15}N$ retention (%) were calculated.

**Table 1.** $^{15}N$ sources, their $^{15}N$ enrichment (Atom-%$^{15}N$), $^{15}N$ content (mg $^{15}N$ $g^{-1}$) and N content (%).

| N Source | Atom-% $^{15}N$ | mg $^{15}N$ $g^{-1}$ | N Content (%) |
|---|---|---|---|
| Ammonium[a] | 98.0 | 274.8 | 26.17 |
| Nitrate[b] | 98.0 | 145.4 | 13.85 |
| Ammonium nitrate[c] | 98.0 | 367.3 | 34.98 |
| Urea | 95.0 | 474.5 | 46.62 |
| Valine (Val) | 94.2 | 120.6 | 11.95 |
| Phenylalanine (Phe) | 96.4 | 87.5 | 8.47 |
| Isoleucine (Ile) | 94.2 | 107.7 | 10.67 |
| Glutamic acid (Glu) | 93.0 | 94.8 | 9.52 |
| Yeast protein* (YP) | 87.9 | 48.6 | 5.49 |

[a)] Ammonium chloride; [b)] Potassium nitrate; [c)] $^{15}NH_4{}^{15}NO_3$

*2.4 Statistical analysis*

Data were expressed as means and the $^{15}N$ retention tested by one-way ANOVA followed by Tukey´s HSD test ($P \leq 0.05$) using SPSS Statistics Desktop 25.0 for Windows. Student's *t*-test was used for comparison of treatment without (-) and washing, and sampling date, 15 and 30 days, respectively.

## 3. RESULTS AND DISCUSSION

Biochar has receiving considerable research attention due to its potential importance, and value concerning both agronomic and environmental applications. It has a high specific surface area, a high density of negative surface charges, and characteristic pores and surface functional groups, all potentially quite valuable, both agronomically and environmentally. Biochar has been reported to be able to improve soil fertility by sequestrating C, enhancing retention of nutrients, and also suppressing greenhouse gas emissions to the air (Gai et al. 2014).

The retention of the $^{15}N$ sources at Pyro-BC (Table 2), generated from maize silage, and heated for 30 minutes at 600 °C, after 15 days for the *in-vitro* experiment was low and similar for ammonium, nitrate, Val, and Iso by a mean value of ~ 3.8%. For the $^{15}N$ compounds-- ammonium nitrate, urea, Phe and Glu--the $^{15}N$ retention was 1.6-fold higher, and with a mean ~ 6.2%. The high molecular $^{15}N$-YP showed the highest $^{15}N$ retention 11.37% at Pyro-BC, which was significant compared to the other $^{15}N$ sources. Application of high molecule weight compounds, such as animal-based (non-manure) fertilizers, is a common practice. The natural nitrogen isotope composition of animal protein materials including bone, hair, and muscle protein are principally determined by diet, and are generally enriched relative to the $\delta^{15}N$ composition of the dietary protein. For example, fertilizer products from animals that are herbivores would be expected to be isotopically lighter than products made from animals that are carnivores. This

could explain a relatively large range in fishmeal fertilizers if the different products are derived from fish that are at different trophic levels. Alternatively, it could be due to a difference in how the products are processed (Bateman and Kelly 2007).

The retention of $^{15}N$ after washing was reduced, and was both low and similar for ammonium, nitrate, Val, and Iso (mean ~ 2.1%) which corresponds to a reduction of ~ 45%, when compared to 'without washing'. Further, for ammonium nitrate, urea, Phe, and Glu, the $^{15}N$ retention was 1.8-fold higher, and with a mean ~ 3.9%, and also low. The retention of the $^{15}N$-YP at Pyro-BC was only minimally lowered by washing, nevertheless was 7.59% markedly higher than for the other inorganic and organic $^{15}N$ sources. Washing with 2 x 1.5 ml of water led immediately to lowering the retention of the examined $^{15}N$ sources. A significant reduction (data in bold) was in the range between 27% (Urea) and 60% (Phe), indicating different retention capacities of the $^{15}N$ sources at Pyro-BC, with the exception of Val, Glu, and YP.

For example, for Val the isopropyl group results in it being a non-polar aliphatic amino acid. Iso is also classified as a non-polar, uncharged, branched-chain, aliphatic amino acid. Phe is classified as neutral, and also non-polar because of the inert and hydrophobic nature of the benzyl side chain. However, The Glu molecule, in mildly acid water solutions, assumes an electrically neutral zwitterion structure.

**Table 2.** Retention of inorganic and organic of $^{15}N$ sources at Pyro-Biochar (manufactured from maize silage by pyrolysis at 600 °C for 30 minutes) after 15 and 30 days of *in-vitro* incubation at 15 °C. (Different letters within a column indicate significant differences between $^{15}N$ sources; bold numbers for reduction/changes indicate significance between treatment without (-), washing, respectively; bold numbers for retention/changes indicate significance between day 15 and day 30, without (-), with washing, respectively.)

| **Time** | **Day 15** | **Day 15** | **Day 15** | **Day 30** | **Day 30** | **Day 30** |
|---|---|---|---|---|---|---|
| **Biochar** | **Pyro-BC** | **Pyro-BC** | **Pyro-BC** | **Pyro-BC** | **Pyro-BC** | **Pyro-BC** |
| **Treatment** | - | **Washing** | | - | **Washing** | |
| **$^{15}N$ source** | **Retention** (%) | **Retention** (%) | Reduction (%) | **Retention** (%) | **Retention** (%) | Changes (%) |
| Ammonium | 3.83$^{c}$ | 2.70$^{bc}$ | **30** | 2.61$^{b}$ | 3.24$^{abc}$ | +24 |
| Nitrate | 3.94$^{c}$ | 2.07$^{bc}$ | **47** | **3.16**$^{b}$ | 1.99$^{c}$ | **37** |
| Ammonium nitrate | 6.71$^{b}$ | 4.05$^{b}$ | **40** | **4.54**$^{b}$ | **5.20**$^{ab}$ | +14 |
| Urea | 6.63$^{b}$ | 4.85$^{ab}$ | **27** | **2.89**$^{b}$ | **3.46**$^{abc}$ | +20 |
| Valine | 3.67$^{c}$ | 1.83$^{bc}$ | 50 | 2.79$^{b}$ | 2.90$^{bc}$ | +4 |
| Phenylalanine | 6.10$^{bc}$ | 2.42$^{bc}$ | **60** | 4.25$^{b}$ | 3.20$^{abc}$ | 25 |
| Isoleucine | 3.70$^{c}$ | 1.72$^{bc}$ | **53** | 4.21$^{b}$ | **2.85**$^{bc}$ | 32 |
| Glutamic acid | 5.31$^{bc}$ | 4.30$^{b}$ | 19 | 3.13$^{b}$ | 3.08$^{abc}$ | 1 |
| Yeast protein | 11.37$^{a}$ | 7.59$^{a}$ | 33 | 7.73$^{a}$ | 5.45$^{a}$ | 29 |

The retention at Pyro-BC of the $^{15}N$-YP was after 30 days with 7.59% markedly higher as the other inorganic and organic $^{15}N$ sources with ~ 3.5% retention. For nitrate, ammonium nitrate and urea the $^{15}N$ retention at day 30 (without washing) was significant lower 3.16, 4.54, 2.89 (data in bold), 3.94, 6.71, 6.63, respectively, (day 15), indicating reversible retention of $^{15}N$ of the biochar-nitrogen solution.

At day 30, washing is only influencing the retention of $^{15}N$ nitrate, which was by 37% reduced from 3.16 to 1.99%.

Depending on the incubation time (between 15- and 30-days), washing influenced the $^{15}N$ retention of ammonium nitrate and Iso, both of whose retention increased markedly from 4.05 to 5.20 and from 1.72 to 2.85%, respectively, but for urea decreased from 4.85 to 3.46% within this time period. The increase of $^{15}N$ retention for these two (Pyro-BC-ammonium nitrate; Pyro-BC-amino acid) combinations may be due to a stronger detachment of unlabelled $^{14}N$ of the Pyro in the aqueous phase, which then increases the $^{15}N$ proportion of the biochar. The urea molecule has unshared pairs of electrons on both nitrogen atoms as well as on the oxygen atom of the carbonyl group (House and House 2017). As a result, there are multiple possible sites by which urea can form hydrogen bonds to other urea molecules or to solvent molecules. Therefore, association with urea itself is possible, with the greatest extent of association expected to occur in solvents that consist of larger, less polar molecules, or in those that do not form strong hydrogen bonds.

Feedstock types and pyrolysis temperature greatly influenced the biochar chemical and physical characteristics, which further influenced N adsorption ability of the biochar. The results from Gai et al. (2014) showed that biochar yield and contents of N, hydrogen, and oxygen decreased as pyrolysis temperature increased from 400 ˚C to 700 ˚C, whereas contents of ash, pH, and carbon increased with greater pyrolysis temperatures. Biochar from wheat straw, corn straw, and peanut shells at pyrolysis temperatures of 400, 500, 600, and 700˚C adhere substantial amounts of $NH_4^+$-N, and the sorption characteristics were well-fitted to the Freundlich isotherm model. The ability of biochar to sorb $NH_4^+$-N was the highest when it had the largest cation exchange capacity. Compared with $NH_4^+$-N, none of $NO_3^-$-N was adsorbed to biochar's at different $NO_3^-$ concentrations. Instead, some $NO_3^-$ -N was even released from the biochar materials. Gai et al. (2014) concluded that biochar can be used under conditions where $NH4^+$-N (or $NH_3$) pollution is a concern, but further research is needed to establish the environmental value of applying biochar to reduce $NO_3^-$-N pollution.

By hydrothermal carbonization at 230 °C for 4 h, the result was the HTC-BC. HTC-biochar has a larger surface area (8.27 $m^2$ $g^{-1}$) than does the Pyro-biochar (1.69 $m^2$ $g^{-1}$) (Reibe 2015). The retention of the $^{15}N$ sources at HTC-BC (Table 3) was after 15 days of the *in-vitro* experiment, with the exception of $^{15}N$-YP, which was similar for the $^{15}N$ compounds and with a mean ~6.9%, independent of the assignment to inorganic or organic compounds. The high molecular $^{15}N$-YP showed the highest $^{15}N$ retention, with 31.48% at HTC-BC, which additionally was a full 20% higher than Pyro-BC. Washing after 15 days led to a marked decrease, to a low $^{15}N$ retention of about ~3.2%. The significant reduction (data in bold) was in the range between 39% (Urea) and 63% (Ammonium), and statistically not significant for Glu and YP.

For the day-30 treatment, the retention at HTC-BC (without washing, with washing) was, with the exception of YP, 6.3% and 3.5%, and comparable with the values at day 15. Also, at this date $^{15}N$-YP showed the highest $^{15}N$ retention, with 33.56% and 18.76%, respectively, in comparison to the other $^{15}N$ sources. A significant reduction by washing at day 30 (data in bold) was in the range between 40% (Glu) and 58% (Phe), indicating different retention capacities for the $^{15}N$ sources at HTC-BC, with the exception in this case of ammonium, ammonium nitrate, and urea. The $^{15}N$ retention of urea and Iso decreased markedly between day 15 and day 30 (without washing), from 8.18% to 6.03%, and from 6.24% to 5.36%, respectively, and increased only for ammonium nitrate (with washing), from 2.84% to 3.68%. The increase of the $^{15}N$ retention, also shown for Pyro-BC, may be for HTC-BC also due to a detachment of unlabelled $^{14}N$ in the aqueous phase, which then increases the $^{15}N$ proportion of the biochar.

**Table 3.** Retention of inorganic and organic of $^{15}N$ sources at HTC-Biochar (manufactured by hydrothermal carbonization at 230 °C for 4 h) after 15 and 30 days of *in-vitro* incubation at 15 °C. (Different letters within a column indicate significant differences between $^{15}N$ sources; bold numbers for reduction/changes indicate significance between treatment without (-), washing, respectively; bold numbers for retention/changes indicate significance between day 15 and day 30, without (-), with washing, respectively.)

| | **Day 15** | **Day 15** | **Day 15** | **Day 30** | **Day 30** | **Day 30** |
|---|---|---|---|---|---|---|
| **Biochar** | **HTC-BC** | **HTC-BC** | **HTC-BC** | **HTC-BC** | **HTC-BC** | **HTC-BC** |
| **Treatment** | - | **Washing** | | - | **Washing** | |
| **$^{15}N$ source** | **Retention** (%) | **Retention** (%) | Reduction (%) | **Retention** (%) | **Retention** (%) | Reduction (%) |
| Ammonium | 4.33$^{b}$ | 1.60$^{b}$ | **63** | 4.67$^{b}$ | 2.90$^{b}$ | 38 |
| Nitrate | 7.91$^{b}$ | 3.06$^{b}$ | **61** | 8.33$^{b}$ | 3.73$^{b}$ | **55** |
| Ammonium nitrate | 6.80$^{b}$ | 2.84$^{b}$ | **58** | 5.62$^{b}$ | **3.68**$^{b}$ | 34 |
| Urea | 8.18$^{b}$ | 4.96$^{b}$ | **39** | **6.03**$^{b}$ | 4.81$^{b}$ | 20 |
| Valine | 6.48$^{b}$ | 2.64$^{b}$ | **59** | 6.16$^{b}$ | 2.93$^{b}$ | **53** |
| Phenylalanine | 7.99$^{b}$ | 3.69$^{b}$ | **54** | 7.46$^{b}$ | 3.16$^{b}$ | **58** |
| Isoleucine | 6.24$^{b}$ | 3.16$^{b}$ | **49** | **5.36**$^{b}$ | 3.18$^{b}$ | **41** |
| Glutamic acid | 7.20$^{b}$ | 3.93$^{b}$ | 45 | 6.60$^{b}$ | 3.98$^{b}$ | **40** |
| Yeast protein | 31.48$^{a}$ | 28.21$^{a}$ | 10 | 33.56$^{a}$ | 18.76$^{a}$ | **44** |

## 4. CONCLUSIONS

The *in-vitro* retention of $^{15}N$-inorganic and $^{15}N$-organic substances at Pyro-BC and HTC-BC, at days 15 and 30, was for both biochar's below 10%. This amount could substance specific clearly reduced by washing. The retention of $^{15}N$ substances at Pyro-BC, like urea, the amino acids Val, Phe, Iso, and Glu, with molecular masses of 60, 117, 165, 131, and 147 g/mol, respectively, was (without, with washing) assigned to different homogeneous groups. HTC-BC adhere the high molecular $^{15}N$ yeast protein to 30%, which was 3-fold higher as at Pyro-BC. Therefore, the manufacturing process of the two biochar's had a significant impact on *in-vitro* retention. The functional groups, and therefore Van-der-Waals-interactions, the chemical reaction (acidic, neutral, and alkaline) and affiliation (e.g. to the aromatic amino acids, like Phe; aliphatic, like Val, Iso or acidic amino acid, Glu), and also the molecular size of compounds, such as yeast protein, can affect the retention of these substances.

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