Assessing the N Cycling Ecosystem Function-Processes and the Involved Functional Guilds upon Plant Litter Amendment in Lower Himalaya

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Received: 10 April 2020
Accepted: 23 May 2020

Abstract

Nitrogen (N) cycling ecosystem function is crucial in primary productivity but also carry various ecological implications such as N losses to environment. In mountainous soil ecosystems, this important function beside soil characteristics is dependent on the litter produced through plant decay which may play a critical role in shaping the hosted soil microbial communities such as those involved in N cycling processes. This study aims at investigating the effects of plant litter amendment, believed to reduce the nitrogen leaching and improve soil health, on nitrogen cycling microbial communities and processes using litterbag approach at field station of COMSATS, Abbottabad. Plant litter collected from the stands of Pine (*Pinus wallichiana*) and understory *Indigo* shrub commonly known as *Indigo* Himalayan (*Indigofera heterentha* (*Fabaceae*) wall), near Abbottabad, native to the lower Himalaya, were applied to indigenous loamy soil in four treatments (i.e. Control, *Pine*, *Indigo* and *Pine + Indigo*). The N cycling processes (involved in nitrous oxide GHG emissions, potential nitrification activity – PNA and denitrification enzyme activity – DEA), through measuring enzymes activities and the abundances of nitrifying - *amoA* (ammonia oxidizing bacteria - AOB, ammonia oxidizing archaea - AOA) and denitrifying functional guilds (*nirS*, *nosZ*) were determined using quantitative PCRs by targeting their corresponding genes. The results revealed that the plant litter significantly influenced both nitrification and denitrification but also the size of microbial communities involved in these two

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processes. The number of archaeal and bacterial *amoA* gene copies increased with the application of *Indigofera* treatment with AOA size higher than the AOB with correlation matrix revealing that AOB were dominant in mediating the PNA rates. The DEA was found to increase upon *Indigofera* litterbag treatment where *nosZ* were less abundant but interestingly in contrast to *nirS* denitrifiers strongly correlated with DEA. Though the short term litter application enhanced the nitrification and denitrification rates, low abundance but dominant role of *nosZ* denitrifiers while AOB as main actors in nitrification across lower Himalayan soil are important to be studied in long perspectives since these functional guilds may differentially impact the N cycling function and thus carry strong implications for overall environment.

**Keywords**: ecosystem services, nitrification, *nosZ* gene, AOA, *Indigofera*, Himalaya

**Introduction**

Soil formed at the inter-section of lithosphere, biosphere, atmosphere, and hydrosphere consists of major components including mineral, organic, liquid and gas. Beside regulating the majority of ecosystem processes at landscapes, it also provides a range of ecosystem services thus plays an important role in sustaining humanity [1]. It supports plant growth and provides nutrients such as nitrogen [2], involves in flood mitigation, filtration of nutrients, recycling and detoxification of waste and gaseous emissions and enable humans to live in a healthy environment [3]. In mountainous regions, soil is important for agriculture productivity but also impacts overall environment through rich biodiversity and several functions such as N cycling.

These regions are among the highly prone areas to be affected by the global warming which may ultimately leads to variations in structure and diversity of microbial community and even extinction of few species [4] and thus the associated functions. In mountainous regions, the plant litter through decay of plant biomass contributes to maintaining the nutrient transformations and is critical in shaping the various forms of nutrients and substrates for hosted microbial communities [5]. This along with changing climate has a very significant impact on soil processes and therefore on the provision of ecosystem services [3]. Moreover, human perturbations including fires, fertilization and land-use changes has substantially altered the soil properties and subsequent nutrient fluxes hence contribute to climate change events. For example, the IPCC assessment reports have estimated a rise of up to 2°C in average global temperatures by the end of the century [6]. Such increased temperatures may also stimulate the nitrogen availability in soil contributing to increased amount of greenhouse gases [7] which may affect soil and overall environment [8]. Such impacts of climate change are expected to be severe particularly in arid and semiarid areas [9]. The altered nutrient cycling and the frequency, intensity and duration of the extreme climatic events have increased substantially especially during the 21st century hence may impact overall ecosystems [10].

N cycling in these soil ecosystems is important keeping in view the soil fertility perspectives but is also crucial from environmental quality perspectives such as N losses. For example, the nitrous oxide emissions through soils can contribute to global warming phenomenon having climate changes implications. In 21st century the mountainous regions are particularly expected to experience the extreme exposure to climate change affecting soils and residing communities. The projected increase in warming and intense precipitation [11] may also affect the soil productivity and biogeochemical cycling. For example, an increase in temperature could enhance the N2O emissions from ecosystems through impact on nitrification and denitrification processes and the ratio of N2O/NO3"− [12]. Nitrogen fertilization regimes have increased the N losses through N leaching, and volatilization thus affected the environment through global warming, and eutrophication etc. [13]. Since pre-industrial times atmospheric N2O concentrations have increased by 19% [14] while an increase in soil nitrates has also triggered the N leaching hence ground water pollution [15].

In face of such climate change events the uneven precipitation may lead to increased soil organic carbon storage particularly in Himalaya region [16]. Such severe impacts need crucial management measures through increasing the adaptive capacity of farmers [17] and various mitigation strategies such as application of organic amendment to soils may contribute in slow release of nutrients and improving the soil health. The practice is also known to enhance the soil nutrient (carbon) stocks in degraded soils [18]. Plant litter may also increase the nutrient storage in soils hence improving the soil health. Such as the organic carbon added through amendments is an important indicator of soil quality which controls the soil physicochemical properties [19] but also enhances soil productivity [20] through increased availability of carbon and nitrogen [21]. Plant litter is a source of organic matter used in microbial decomposition [2], provided organic matter plays an important role in ecosystems by retaining and supplying plant nutrients, reducing soil erosion, and enhancing water holding capacity [18]. For example, the farming practices such as application of plant litter and use of green manure, composts and rotation with
intercropping are believed to enhance soil nutrient sequestration [22].

These practices in N cycling perspectives may help to manage the N release to the environment. Impact of plant litter amendment to N cycling processes, nitrification and denitrification and the involved microbial communities (nitrifiers, denitrifiers) is therefore crucial to understand their long-term impact on N losses. The current work using litterbag adopted approach was carried out to assess the effects of plant litter *(Pinus wallichiana* and *Indigofera heterantha)* amendments on nitrification, denitrification, and involved microbial communities (ammonia oxidizing archaea and bacteria (AOA, AOB) and nirS, nosZ denitrifiers) in Abbottabad, lower Himalaya.

**Materials and Methods**

**Experimental Setup**

Soil incubation experiment was conducted with RCBD plotted at the field farm of COMSATS Abbottabad (34°11’57.13’’N latitude, 73°14’50.00’’E longitude) with litter bag treatments. Litterbag is a standard apparatus used in plant litter decomposition studies [23]. The experiment was performed in three replicates with two factors, three treatments and three sampling dates including control, hence we randomized each factor in each block. Polyester litter bags with dimensions of 20×20 cm (0.04 m²) were filled with dried and chopped litter with following treatments; *Indigofera heterantha* (I), *Pinus wallichiana* (P), *Pinus wallichiana*±*Indigofera heterantha* (PI), litter of these plants was selected since these species are native to the studied region – lower Himalaya. The fourth treatment was control and was without any application of plant litter. There were three sampling dates, 7, 15 and 28 days for taking samples for further analyses. Each treatment was provided with 3 replicates and the total of 27 bags were prepared. The field experiment was set in 304.8×243.84 cm plot using randomized complete block design where the plot was divided into three blocks (4 columns and 3 rows each).

**Collection and Preparation of Plant Litter**

Litter from *Pinus wallichiana* and *Indigofera heterantha* was collected from Baghnotar near Abbottabad. After collection, the plant material was air dried to constant mass for 15 days. The stems and leaves were separated and cut into 2-4 cm length and the proportion of each organ in each plant was determined. Then each sample was weighed to have 20 grams, in which the proportion of each organ (leaves and stems) was respected. *Pine* stem proportion was 45% and leave proportion 55%, *Indigofera* stem proportion was 58% and leaves 42%, the mixture proportion was respected. The *Pine±Indigofera* sample was prepared on 1:1 ratio on weight basis, and again in the *Pine±Indigo*, the proportion of each organ for each plant was respected.

**Litter and Soil Sampling and Chemical Analysis**

Litter and soil samples were collected from the experimental site after 7 days of burying litterbags at 5 cm soil depth followed by 15- and 28-day intervals. Soil pH was determined in 1.5 (w/v) soil: water suspensions with a pH meter, after equilibration for 1 hour. Soil collected locally from the field farm of COMSATS Abbottabad was loamy in texture (sand 52%, silt 29%, 19 clay %), EC 0.81 d S m⁻¹ with 7.8 pH and average moisture contents of 28%. Soil moisture content was measured by weighing 5 g of fresh soil after sampling and dried in oven at 105°C for 24 hrs. Soil NO₃⁻N was measured after sampling. The moisture contents in soil samples were adjusted by oven drying of few grams of soil. Stock solution of nitrate was prepared after drying potassium nitrate (KNO₃) in an oven at 105°C for 24 hrs. 1 g KNO₃ was dissolved in water and diluted to 1000 ml. From the stock solution, NO₃⁻ standards were prepared as 0, 2, 4, 6, 8 and 10 ppm. 10 g soil was mixed with 100 ml distilled water and shaken for 1 h. Then the sample was filtered, 50 mL clear sample was added with 1 mL 1N HCl and mixed properly. The absorbance from the UV visible spectrophotometer was read at 220 nm. A regression plot was drawn to determine the concentration of nitrate in soil samples and data were statistically analyzed using Statview software (SAS 1999).

**Extraction and Preparation of Nucleic Acid (DNA)**

Three independent DNA extractions were performed for each frozen soil subsamples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories), according to the protocol of the manufacturer. The concentration of the soil DNA was determined by putting the known concentrations on the gel and the samples were diluted to run further analyses accordingly as detailed below.

**Gene Abundances of Nitrifiers and Denitrifiers Communities**

The abundance of nitrifiers and denitrifiers were estimated by quantitative PCR assays carried out as described previously [24]. The final reaction volume was 25 μl for nirS and nosZ with 1μM for nirS and nosZ of forward and reverse primers; 12.5 μL of QuantiTect SYBR® Green I PCR kit (Qiagen) for nirS and nosZ; 0.5 μg and 0.4 μg of T4 protein (Qbiogene, Carlsbad, CA USA) for nirS and nosZ respectively. Standard curves
for nirS and nosZ were obtained by performing qPCR assay on serial dilutions of known amount of plasmid DNA containing the targeted gene (from $10^7$ to $10^1$ copies). Microbial community quantification was carried out as described previously [24]. The genes encoding enzymes involved in ammonia-oxidation (amoA) for both bacteria and archaea were quantified to know the size of bacterial (AOB) and archaeal (AOA) ammonia-oxidizers in studied samples where the method and thermocycler conditions were as described by [25] and [15]. Independent qPCR assays were performed for each sample and the mean was calculated accordingly.

Potential Nitrification Activity

Fresh soil samples were used to quantify the potential nitrification activity (PNA) as described previously [26]. Briefly, 4 g fresh soil weighing about 2.5 g dry soil was mixed with 10 ml reagent solution and suspensions were shaken at 175 rpm in orbital shaker. After 1, 8, 24 and 48 hours, 2 ml liquid suspension was taken, and ammonium oxidation was blocked using 2 ml KCl (2 M) and afterward centrifugation at 3000 rpm for 2 minutes. Recovered supernatant solution was put in bottles and stored in freezer at 4ºC. Then at the end 0.8 ml supernatant was added with 1.5 ml NH₄Cl buffer and 0.4 ml Diazzo solution. After 30 minutes absorbance was measured against the standards at 530nm wavelength using UV/VIS Spectrophotometer afterwards potential nitrification activity was calculated as μg NO₂-N h⁻¹.

Denitrification Enzyme Activity

Denitrification enzyme activity (DEA) was quantified through incubation of fresh soil in the airtight plasma for 4 h at room temperature and N₂O was measured every half hour for a period of 2h with 4 readings in total, on a GC following the principle as detailed previously [27]. The soil in plasma contained the substrate and the conditions in plasma were anaerobic to block the last step of denitrification process to allow the N₂O accumulation [28].

Statistical Analysis

The obtained data for community abundances and enzyme activities were statistically analyzed. A linear model equation was used for calculation of microbial enzyme activities which were also analyzed through Statview to know the differences among treatments. The acquired data in three replicates for different variables were analyzed through Student’s t test (p<0.05) and an ANOVA test as required by using Statview software (SAS 1999).

Results

Soil Physicochemical Properties

Soil characteristics including soil moisture, pH and nitrate concentrations observed during the experiment for different treatments and sampling days are presented in the following table.

<table>
<thead>
<tr>
<th>Sampling Days</th>
<th>Treatments</th>
<th>Percent moisture</th>
<th>Soil pH</th>
<th>Soil NO₃-N (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Control</td>
<td>-</td>
<td>7.8</td>
<td>5.1±1.6</td>
</tr>
<tr>
<td></td>
<td>Pine</td>
<td>10±0.06</td>
<td>7.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Indigofera</td>
<td>10±0.05</td>
<td>7.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Pine + Indigofera</td>
<td>10±0.06</td>
<td>7.8</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
<td>-</td>
<td>7.8</td>
<td>9.0±1.3</td>
</tr>
<tr>
<td></td>
<td>Pine</td>
<td>99.89±18.5</td>
<td>7.2</td>
<td>4.7±0.06</td>
</tr>
<tr>
<td></td>
<td>Indigofera</td>
<td>117±8.2</td>
<td>7.2</td>
<td>5.4±0.63</td>
</tr>
<tr>
<td></td>
<td>Pine + Indigofera</td>
<td>115.1±16</td>
<td>7.1</td>
<td>4.2±1.29</td>
</tr>
<tr>
<td>15</td>
<td>Control</td>
<td>-</td>
<td>7.8</td>
<td>7.0±0.22</td>
</tr>
<tr>
<td></td>
<td>Pine</td>
<td>94±10.4</td>
<td>8.1</td>
<td>5.7±1.22</td>
</tr>
<tr>
<td></td>
<td>Indigofera</td>
<td>133.3±28.8</td>
<td>8.1</td>
<td>5.4±2.3</td>
</tr>
<tr>
<td></td>
<td>Pine + Indigofera</td>
<td>144.4±77</td>
<td>7.8</td>
<td>6.1±2.26</td>
</tr>
<tr>
<td>28</td>
<td>Control</td>
<td>-</td>
<td>7.8</td>
<td>11.5±0.75</td>
</tr>
<tr>
<td></td>
<td>Pine</td>
<td>154±5.4</td>
<td>7.6</td>
<td>10.7±1.50</td>
</tr>
<tr>
<td></td>
<td>Indigofera</td>
<td>131.6±2.4</td>
<td>7.8</td>
<td>9.0±1.18</td>
</tr>
<tr>
<td></td>
<td>Pine + Indigofera</td>
<td>135±4.8</td>
<td>7.9</td>
<td>10.2±0.86</td>
</tr>
</tbody>
</table>
are summarized in Table 1. Soil pH was 7.8 at day 0 and a slight drop of 0.6 unit observed after the plant litter treatment at 7 days, while there was an increase of 0.9 units observed on day 15 in Pine and *indigofera* treatments and Pine + *indigofera* gained 0.6 units. The pH at day 28 remained closer to day 0. Soil nitrates, depict the nitrification rates but also act as substrate for denitrification, were recorded differently for various treatments. For example, the nitrate concentrations at the start of experiment were 5.1 mg/kg and after addition of plant litter it remained around 4.7, 5.4 and 4.2 mg/kg for *pine*, *indigofera* and Pine + *indigofera* treatments respectively, where the NO$_3^-$ in control showed the highest concentration at day 28 after sampling and the lowest on day 15. The *pine* litterbag application showed the highest nitrate concentration on day 28 while the lowest on day 7. Interestingly, an increase in NO$_3^-$ concentration after application of *indigofera* litter was observed from day 0 to day 7 in comparison to initial concentration. In contrast, the application of *Pine* and *Pine + Indigo* litter resulted in a decrease in NO$_3^-$ concentration during the 1st week of experiment (Table 1). On basis of this observation the quantifications of the genes encoding the enzymes for nitrification and denitrification processes were carried out for the treatments applied with *Indigofera* litter.

Potential Nitrification Activity (PNA) Across Various Litter Bag Treatments

Potential nitrification activity (PNA) was found to be increasing with the experiment time of plant litter application with high values for the treatments with litter as compared to control (Fig. 1) Overall the rates of the PNA were higher after 28 days in comparison to initial and ranged from 0.025 to 0.034 µg NO$_3^-$-N per g dry soil for *Indigofera* litter treatment in contrast to control which remained 0.08 µg NO$_3^-$-N per g dry soil. The addition of *indigofera* plant litter resulted in PNA as 0.1, 0.21, 0.21 µg NO$_3^-$-N per g dry soil at day 7, 15 and 28, respectively. It was also observed that the increase in PNA continued for overall duration, but this increase was stronger at initial days. For example, the change in PNA was significant at day 7 and 15 in comparison to control whereas with respect to day 15, a non-significant difference at day 28 (p<0.05) was observed (Fig. 2, Fig. 3).

Denitrification Enzyme Activity (DEA) Across Various Litter Bag Treatments

Similar to the observations for PNA, the denitrification enzyme activity was found to be significantly higher for the litter bag treatments as compared to the control. However, this increase was prominent with experiment time where the highest values for DEA were observed after 28 days of litter application. As shown in the Fig. 3, the *indigofera* plant litter caused an increased DEA with values recorded as 0.022, 0.27, 0.038 µgN-N$_2$O/g dry soil at day 7, 15 and 28, respectively. Although the increase in PNA was sharp just after the day 7, the trend for the DEA was relatively different with slight increase at earlier days but the highest values for the DEA at day 28 denote that the application of the litter has caused a significant increase in DEA where it overall ranged from 0.022 to 0.038 µg N$_2$O-N per g dry soil for the *Indigofera* treatment while in control it was 0.021 µg N$_2$O-N per g dry soil.

Fig. 1. The community gene abundance of nitrifiers (archaeal and bacterial *amo*A (AOA and AOB)) and denitrifiers (*nir*S and *nos*Z) in number of gene copies per ng DNA.
Community Gene Abundances of Nitrifiers (amoA Gene)

The size of the ammonia oxidizers controlling the nitrification process are described by the number of gene copies of the amoA genes in ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) per ng of DNA which we have assessed by quantifying the archaeal and bacterial ammonia monoxygenase gene through qPCR as shown in (Fig. 1). The abundances of AOA ranged from 1.13E + 07 to 2.63E + 07 while the abundance of AOB ranged from 2.77E + 06 to 1.51E + 07 per ng DNA. The abundances of these nitrifying functional guilds were recorded higher at all days-intervals as compared to control. Overall, the size of AOA community was significantly higher in indigofera treatment as compared to AOB community. The gene abundance of the AOA ranged from 2.20E + 07 to 2.63E + 07 gene copies ng⁻¹ DNA for indigofera treatment which was higher than control while the size of AOB community ranged from 5.63E + 06 to 1.51E±07 gene copies ng⁻¹ DNA, for indigofera treatment, in contrast to control which was 2.77E + 06 gene copies ng⁻¹ DNA. Overall, the abundance of the AOA was observed higher than the AOB but for both the control was always less than the treatments showing that the application of plant litter has significantly increased the community enrichment both for AOA and AOB.

Community Gene Abundances of Denitrifiers (nirS, nosZ Gene)

The results of gene abundances showed that nirS ranged from 6.15E + 07 to 7.12E + 07 gene copies ng⁻¹ DNA for indigofera treatment and 3.62E + 07 gene copies ng⁻¹ DNA for control while, the abundance of nosZ ranged from 6.27E + 04 to 1.12E + 05 gene copies...
Correlation Analyses of N Cycling Processes and Litter Bag Treatments

To understand the relation between the community gene abundances and the nitrification and denitrification enzyme activities, we carried out the correlation analyses between the PNA and DEA and sizes of the nitrifiers (AOA, AOB) and the denitrifiers (nirS, nosZ). The correlation analyses showed that PNA was positively correlated to the size of both bacterial and archaeal nitrifiers however this correlation was stronger for the AOB (r = 0.59, p<0.05) as compared to AOA (r = 0.43, p<0.05) representing that both type of nitrifiers were involved in nitrification process. The correlation analyses of the denitrification enzyme activity and the abundances of denitrifiers showed a positive correlation between the studied denitrifying communities and the DEA. However, this correlation was relatively stronger for the nosZ (r = 0.79, p<0.05) denitrifiers and compared to nirS (r = 0.46, p<0.05). Although the size of the nosZ denitrifiers was less as shown by the gene abundance data but the correlation analyses revealed a stronger relation of the DEA with the nosZ denitrifiers.

Discussion

Ecosystem services provided by soil and the residing microbial communities are crucial on earth [29]. N cycling in soil is mainly driven through nitrification and denitrification process thus control the N losses [30] where these processes upon fertilization and organic amendments are important from ecological perspectives for example in mountainous regions with various types of plant litter added to the ecosystems [31]. Here we investigated the N cycling processes upon plant litter addition to soil in lowe Himalayas.

Results showed an increase in soil NO:\textsuperscript{-}\textsubscript{3} concentration over the experiment time as compared to initial value at day 0 and up till day 7 upon addition of indigofera litter. However, a decrease in NO:\textsuperscript{-}\textsubscript{3} concentration was observed for pine and pine + indigo treatments during the first week. This could also be attributed to the linear N mineralization in control which might be produced due to the decomposition of native soil organic matter while the increase in NO:\textsuperscript{-}\textsubscript{3} concentration in indigofera treatment may be associated to its high C:N ratio since the indigofera belongs to leguminous group which is rich in nitrogen as observed previously [32]. Similarly, the decrease in nitrate concentration could be attributed to humid conditions with high plant litter C:N ratio as submerged conditions may support the denitrification activity [33], meanwhile plant litter usually high in C:N ratio may acts as substrates for microbes [34] which may also support NO:\textsuperscript{-}\textsubscript{3} utilization resulting in increased denitrification [35]. The significant decrease in NO:\textsuperscript{-}\textsubscript{3} for control treatment at day 15 may also be attributed to leaching phenomenon. It is pertinent to mention here that the precipitation occurred at day 8 and 9 might have triggered the leaching of the nitrate ultimately resulting in decreased soil nitrates. Overall, addition of plant litter like indigofera being leguminous family may cause high mineralization rates and this high plant nitrogen may contribute to the differences in N transformation processes [36]. Plant litter may also contribute favorably to microbial community abundance and activity and accelerates the N mineralization since the litter addition can promote the reduced NO:\textsuperscript{-}\textsubscript{3}-N losses than that of un-amended conditions [5]. In contrast, plant biomass with low N contents may negatively influence the plant available N through accelerated nitrogen immobilization [37]. Nitrates deficiency can also be referred to low denitrification activity whereas high nitrates and denitrification occurring in a system directly linked to the fact that the denitrification may serve as a sink for the net nitrification [36]. We observed that the plant litter significantly influenced both the PNA and DEA and corresponding gene with PNA followed a trend of sharp increase from start to day 28, where DEA was also increasing with time. The gene abundance of nosZ and nirS were increased since the application of plant litter with days (i.e. Control, I-7, I-15, I-28) where the minimum number of gene copies (2.57E + 04 per ng DNA) were observed in control and maximum after litter addition (1.12E + 05) at day 28 (Fig. 1, Fig. 2). The increase in nosZ gene abundance upon litter application may also hint the reduction of N\textsubscript{2}O to N\textsubscript{2} as nosZ is involved in this step [38]. Overall, nirS dominated the denitrification gene pool implying that the final product of denitrification in this case might be the nitrous oxide thus completing the cycle [39].

The indigofera litter addition induced an increase in the size of bacterial amoA throughout the experiment at various sampling days and such observations about the stimulatory effects of plant litter addition to soil have previously been reported [40]. In the current study, AOA dominated in community size as compared to AOB however the nitrification might have been
controlled mainly by AOB as found through correlation analyses. In contrast, AOA are shown to be dominant nitrifying actors in stress or unusual environments like high temperature or constructed soils like Technosols [24, 41]. Similarly, this has been reported that a range microbial communities are involved in different function due to functional redundancy but their role differentially impact the overall process [42]. Moreover, the microbial communities present in an ecosystem may also subject to the prevailing environmental conditions thus are likely to evolve in the context of micro-environment or changing environment [43]. The studied soil system might have been under changing climate events as reported for Himalaya and meanwhile the dominance of AOA is observed to be high in harsh environment as compared to AOB [44]. Previous studies have also shown that AOA are more abundant than bacterial amoA in a variety of environments including terrestrial ecosystems [45, 46]. Overall, the trend for nitrifiers and denitrifiers community abundances was found to be positively increasing with experiment time. Such community enrichment may have happened owing to the greater number of carbon sources in indigofera treatment than control. Earlier, [47] reported that the organic amendment affected the microbial gene pool in the studied environment. In accordance to the gene abundance data, the correlation analyses showed a positive correlation between the PNA and both AOA and AOB where this relationship was found stronger for AOB ($r = 0.59$) than AOA ($r = 0.43$) (Fig. 4) as observed for AOB previously [24]. Similarly, a strong positive correlation between DEA and the abundance of nosZ denitrifiers was in line with earlier reports which proposed that the biological processes are positively correlated to the abundance of corresponding genes [48]. Moreover, the microbial communities often share similar ecological attributes which usually carry strong correlation to the ecosystem functioning [42]. It is pertinent to mention here that the addition of plant litter might have triggered the community enrichment between the AOA and AOB, with AOB being the dominant actors for nitrification crucial in is estimating the overall nitrification rates [49]. In DEA nosZ were less abundant, but due to their dominant role as shown by correlation analyses and involvement in reduction of N$_2$O to N$_2$, these are critical in deciding the overall nitrous oxide budget from soils [38, 50].

**Conclusions**

To conclude, the increased nitrification and denitrification enzyme activities and the abundances of the corresponding community genes gave us an insight...
into the N cycling processes but also the driving actors, upon plant litter – *indigofera* application, in studied soil in lower Himalaya. Positive correlation of nitrification with both AOA and AOB was found where AOB seems to be dominantly involved in nitrification in the studied soil. The nosZ despite of being low in size are major actors in DEA and nitrous oxide reduction crucial in overall greenhouse gas budget. Long term studies, to investigate the shifts in community enrichment and associated processes in the studied region are needed to ultimately assess N cycling processes under changing climate scenarios.

**Acknowledgements**

This work was financially assisted by research grants provided by International Foundation for Science (IFS - C/5429-1) and Higher Education Commission (HEC - No: PD-IPFP/HRD/HEC/2013/3003) of Pakistan. This work was also funded by Researchers Supporting Project number (RSP 2019/121), King Saud University, Riyadh, Saudi Arabia.

**Conflict of Interest**

The authors declare no conflict of interest.

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