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Dr. KS Santhosh
Research Scientist (Non-Medical) Viral and Diagnostic Laboratory (VRDL), Shivamogga Institute of Medical Sciences, Sagar road, Shivamogga, India

Dr. DS Akhila
Assistant Professor, Centre for Science Education and Research, Nitte (Deemed-to-be University), Paneer campus, Deralakatte, Mangalore, India

Dr. MM Dechamma
Post-Doc Fellow, UCD-Centre for Food Safety, School of Public Health, Physiotherapy & Sports Science, University College Dublin, Belfield, Dublin D04 N2E5

Vittal Rajeshwari
Assistant Professor, Centre for Science Education and Research, Nitte (Deemed-to-be University), Paneer campus, Deralakatte, Mangalore, India

Dr. A Devivaraprasad Reddy
Scientist, Krishi Vigyan Kendra, Dr. Y.S.R. Horticultural University, West Godavari Dist., Venkataramannagudem, India

Correspondence
Dr. A Devivaraprasad Reddy
Scientist, Krishi Vigyan Kendra, Dr. Y.S.R. Horticultural University, West Godavari Dist., Venkataramannagudem, India

An integrative approach to understand the role of the nitrogen fixing microbial consortia in the environment

Dr. KS Santhosh, Dr. DS Akhila, Dr. MM Dechamma, Vittal Rajeshwari and Dr. A Devivaraprasad Reddy

Abstract
Mankind continue to transfigure the global nitrogen cycle at a record pace, reflecting an increased combustion of fossil fuels, growing demand for nitrogen in agriculture and industry, and pervasive inefficiencies in its use. This reactive nitrogen may be lost from ecosystems via leaching, as nitrate (NO_3^-), or as gaseous forms such as ammonia and nitrous oxide (N_2O). These nitrogen transformations are of significantly importance in both economic and environmental aspects. Regardless of more than 150 years of research on the nitrogen cycle there are still important questions to be answered with respect to nitrogen transformations and losses from terrestrial ecosystems. Nitrogen (N) is the seventh abundantly available element in the earth's atmosphere and is the key component of biomolecules (amino acids, nucleotides). The enzyme nitrogenase (nif) catalyzes the nitrogen fixing activity, which is performed by a wide range of microorganisms across various phylogenetic groups. However, high levels of inorganic nitrogen which is not assimilated to operate biological systems pose adverse effects on the nitrogen tolerant organisms. Most organisms integrate nitrogen either from inorganic nitrogen sources like ammonia (NH_3), nitrite (NO_2^-) and nitrate (NO_3^-) or from organic nitrogen compounds.
The link between biogeochemical nitrogen processes and microbial community dynamics can provide a more mechanistic understanding of the nitrogen cycle than the direct observation of nitrogen dynamics. Specific enzymes catalyze many of these reactions, and the molecular approach to study these enzymes are useful targets for understanding microbial processes such as assimilatory nitrate reduction, dissimilatory nitrate reduction, and N_2 fixation.

Keywords: global nitrogen cycle, enzyme nitrogenase, microbial community dynamics

1. Introduction
Nitrogen is the most abundant element in the atmosphere and is a major component of many biomolecule including amino acids, proteins, and nucleic acids. Consequently, nitrogen is one of the critically important nutrients required in relatively large quantities by all organisms. Animals receive their supply of nitrogen through the food they eat, but plants must integrate inorganic forms of this nutrient from the environment.
Nitrogen occurs in many different forms in the environment, which includes both inorganic (e.g., ammonia, nitrate) and organic (e.g., amino and nucleic acids). Nitrogen undergoes many different transformations in the ecosystem, changing from one form to another as organisms use it for growth and, in some cases for energy via nitrogen fixation, ammonification, nitrification, nitrate reduction and denitrification. The transformation of nitrogen into its many oxidation states is the key to productivity in the biosphere and is highly dependent on the activities of a diverse assemblage of microorganisms, such as bacteria, archaea, and fungi.
The availability of inorganic nitrogen from the natural environment is limited to meet the metabolic demands of plants. Because of this condition, the accessibility of inorganic forms of nitrogen is always a limiting factor for the productivity of plants particularly for plants growing in terrestrial and marine environments, and to a lesser degree in fresh waters where phosphate supply is usually the primary limiting nutrient, followed by nitrate. The dead biomass of plants, animals, and microorganisms contains large concentrations of organically bound nitrogen in various forms, such as proteins and amino acids. The process of decomposition is responsible for recycling the inorganic constituents of the dead biomass and preventing it from accumulating in large unusable quantities. Decomposition is carried out through the metabolic functions of a diverse array of bacteria, fungi, actinomycetes, microorganisms, and animals like moles, mice and rabbits. Since the mid-1900s, humans have been exerting an ever-increasing impact on the global nitrogen cycle. Human activities, such as making fertilizers and burning fossil fuels, have significantly altered the amount of fixed nitrogen in the Earth's ecosystems.

In fact, some scientists and statisticians predict that by 2030, the amount of nitrogen fixed by human activities will exceed that fixed by microbial processes. Increase in available nitrogen can alter ecosystems by increasing primary productivity and impacting carbon storage. Because of the importance of nitrogen in all ecosystems and the significant impact from human activities, nitrogen and its transformations have received a great deal of attention from ecologists.
The nitrogen cycle describes the series of processes by which the element nitrogen, which makes up about 78% of the Earth's atmosphere, cycles between the atmosphere and the biosphere. Plants, bacteria, animals, and manmade and natural phenomena all play a role in the nitrogen cycle. The fixation of nitrogen (conversion of gaseous nitrogen (N_2) into forms usable by living organisms) occurs because of atmospheric processes such as lightning, but most fixations are carried out by free-living and symbiotic bacteria. Plants and bacteria participate in symbiosis such as the one between legumes and rhizobia or contribute through decomposition and other soil reactions. Bacteria like *Rhizobium*, or the actinomycete *Frankia* which modulates members of the plant families Rosaceae and Betulaceae, utilize atmospheric nitrogen and convert it to an inorganic form (usually ammonium, NH_4^+) that plants can use. The plants then use the fixed nitrogen to produce vital cellular products such as proteins. The plants are then eaten by animals, which also need nitrogen to make amino acids and proteins. Decomposers acting on plant and animal materials and waste, return nitrogen back to the soil. Human-produced fertilizers are another source of nitrogen in the soil along with pollution and volcanic emissions, which release nitrogen into the air in the form of ammonium and nitrate gases. The gases react with the water in the atmosphere and are absorbed by the soil with rain water. Other bacteria in the soil are key components in this cycle converting nitrogen containing compounds to ammonia, NH_3, nitrates, NO_3^-, and nitrites, NO_2^-. Nitrogen is returned to the atmosphere by denitrifying bacteria, which convert nitrates to dinitrogen gas.

2. Nitrogen

2.1 Background

Nitrogen (N) was discovered by Daniel Rutherford, a Scottish physician in 1771. The name nitrogène was suggested by a French chemist Jean-Antoine-Claude-Chaptal in 1790. This chemical element can appear as a colourless gas, liquid or solid depending on the standard pressure and temperature conditions. Earth's atmosphere constitutes of about 78% of nitrogen which is the richest and essential chemical component of majority of the key biomolecules (e.g., amino acids, nucleotides). Yet atmospheric nitrogen (N_2) is inert which means that it cannot react with other chemicals to form a new compound. Rise in the accessibility of natural inorganic N at times boosts the primary production. However, high levels of inorganic N that cannot be assimilated to operate ecological systems (i.e., N saturated ecosystems) will cause adverse effects on the smallest amount tolerant organisms. Apart from these, there are reactive forms of nitrogen which readily react with other compounds present in the environment causing harmful effects on both the environment and human life. This reactive nitrogen comprises of nitrous oxide (N_2O), nitrogen oxides (NO_x), ammonia (NH_3), nitrate (NO_3), nitrite (NO_2) (Galloway *et al*. 2008) [23], ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) and form the foremost common ionic (reactive) types of dissolved inorganic nitrogen in aquatic ecosystems (Wetzel, 2001; Rabalais, 2002) [75, 49]. These ions occur naturally because of atmospheric deposition, surface and groundwater run-off, dissolution of nitrogen-rich geologic deposits, N2 fixation by nitrogen-fixing prokaryotes and biological degradation of organic matter (Wetzel, 2001; Rabalais, 2002) [75, 49].

3. Nitrogen mineralization and immobilization: mechanisms; microorganisms/ enzymes involved; C: N ratio

In the ecosystem the biochemical cycling of N can be divided into external and internal N cycle. In the external cycle, the addition or removal of N from the ecosystem takes place in the process of dinitrogen (N_2) fixation, dry and wet N deposition, N fertilization, N leaching, run-off erosion, denitrification and ammonia volatilization. The internal N cycle consists of those process which convert N from one chemical form to another or transfer N between various ecosystem pools. The N cycle includes the process like plant assimilation of N, return of N to soil in plant litterfall and root turn over, N mineralization (the conversion of organic N to inorganic N), microbial immobilsation of N (the uptake of inorganic N by microorganisms), and nitrification (the production of nitrite (NO_2^-)) from ammonium (NH_4^+) or organic N.
Paul and Clark (1989) estimated that the sum of all output fluxes of the external N cycle globally is about $0.25X10^{15}$g-Nyr^{-1}, while net N mineralization in soils is more than 14 times this amount (about $3.5X10^{-15}$ g-Nyr^{-1}). However, because net N mineralization is the difference between actual N mineralization and microbial immobilization of N, gross N mineralization rates may be over two orders of magnitude greater than all output fluxes of N combined.

Net N mineralization = gross N mineralization – microbial immobilization of inorganic N.

Net nitrification = gross nitrification – microbial immobilization of NO_3^-

Net rate can be estimated by measuring the changes in inorganic- N pool sizes, gross rates can also be estimated by techniques using two N isotopes (eg: ^{14}N, and ^{15}N)
Immobilization is the reverse of mineralization. All living things require N. Therefore, microorganisms in the soil compete with crops for N. Immobilization refers to the process in which nitrate and ammonium are taken up by soil organisms, thereby rendering nitrogen unavailable to crops.

$$\underset{\text{Ammonium}}{NH_4^+} \rightarrow \underset{\text{Nitrite}}{NO_2^-} \rightarrow \underset{\text{Nitrate}}{NO_3^-}$$

Incorporation of materials with a high carbon to nitrogen ratio (e.g. sawdust, straw, etc.), will increase biological activity and cause a greater demand for N, and thus result in N immobilization. Immobilization only temporarily locks up N. When the microorganisms die, the organic N contained in their cells is converted by mineralization and nitrification to plant available nitrate.
The conversion of organic nitrogen (N) in soil organic matter (SOM) to ammonium (NH_4^+) is by a process called ammonification. Often, the ammonium is rapidly converted to nitrate (NO_3^-) by the microbial process of nitrification.
The amount of inorganic N ($NH_4^+$and NO_3^-) originating from SOM is termed N mineralization (NM). Nitrogen

mineralization is a product of the amount of organic N in the soil and the N mineralization rate (NMR). Soil organic matter originates from crop residues, manures, and other organic amendments applied to soils. As these organic materials decompose, a portion becomes part of SOM. Trumbore (2000) showed that the SOM mean age can vary from a few years to several centuries in temperate soils. The more the mean age, the less decomposable the SOM fraction and lower the NMR. Mean age of a few years are characteristic of SOM that originates from recent additions of crop residues, roots, manure, and other organic amendments.

4. Nitrogen transformation

Nitrogen (N) is one of the most commonly applied elements to increase the yield. Each year approximately, about 67.84 million tons of nitrogen is applied to agricultural lands all over the world, and the total cost is up to $44.2 billion (Liu *et al*., 2009) [38]. After application of nitrogen as manure, depending on its chemical composition, nitrogen undergoes various transformations in atmosphere, water, soil via microbial processes.

Nitrogen has nine different chemical forms in soil depending on the oxidative states including nitrogen dioxide(NO_2), nitrate(NO_3), nitrite(NO_2^-), nitric oxide(NO), nitrous oxide(N_2O), dinitrogen trioxide (N_2O_3), ammonia(NH_3), ammonium(NH_4^+), and organic nitrogen(N).

4.1 Nitrogen fixation

Nitrogen fixation has a profound agronomic, economic, and ecological impact because the availability of fixed nitrogen defines agricultural production throughout the world (Smil, 2004) [55].

In nature, the availability of nitrogen is in in fixed or combined form as nitric oxide by lightning and ultraviolet rays but most of the nitrogen are immobilized as ammonia, nitrites, and nitrates by soil microorganism. These microorganism plays significant role in nitrogen fixation. Microorganisms assimilate nitrogen either from inorganic sources, like NH_3 and nitrate (NO_3^-), or from organic nitrogen compounds (Cabello *et al*., 2004) [13]. The atmospheric nitrogen enters the food chain primarily via biogeochemical method known as nitrogen fixation, that consists of reducing N_2 to NH_3.

4.1.1 Types of nitrogen fixation

The two types of N fixation are physical and biological Nitrogen Fixation. Majority of nitrogen fixation take place by biological methods (90%) and less by physicochemical methods (10%).

4.1.1.1 Physical Nitrogen Fixation

Physical Nitrogen Fixation is further classified into natural and industrial nitrogen fixation.

a) Natural Nitrogen fixation

During process of thunder and lightning, N_2 and O_2 of the air react to form nitric oxide (NO). Further these nitric oxides are oxidized with oxygen to form nitrogen peroxide (NO_2).

$$N_2 + O_2 \rightarrow 2N0$$
$$2NO + O_2 \xrightarrow{\text{Oxidation}} 2NO_2$$

Formed nitrogen peroxide dissolved in rain water to form nitrous acid (HNO_2) and nitric acid (HNO_3). Later it gets reacted with alkaline radicals to form nitrates (NO_3^-) and nitrites ($N0_2^-$).

$2NO_2 + H_2O \rightarrow HNO_2 + HNO_3$; HNO_3 + Ca or K salts → Ca or K nitrates

Soluble nature of nitrates makes them to easily get dissolved in water and it can be directly absorbed by the roots of the plants.

b) Industrial Nitrogen Fixation

Till 20th century, plants and animals depended on microbes to scavenge nitrogen from the atmosphere. Currently around 99% of the inorganic inputs of nitrogen fertilizers are produced by the Haber-Bosch synthesis. In the Haber-Bosch process, nitrogen from air is combined with hydrogen from natural gas in the presence of catalyst under high pressure and temperature, to produce ammonia. Synthesised ammonia can be utilised to produce a plant fertilizer. The production of ammonia is an exothermic and reversible process.

$$N_{2\,(g)} + 3H_{2\,(g)} \leftrightarrow 2NH_{3\,(g)}\ \Delta H = -92\ KJ\ mol\text{-}1$$

The Haber-Bosch process uses catalysts like iron or ruthenium at temperature ranging from 400 -650°C (750-1000°F) and a pressure at 200-400 atmospheres to force nitrogen and hydrogen together. The elements then move out of the catalyst and into industrial reactors to convert elemental nitrogen into fluid ammonia. Then the fluid ammonia is used to produce various types of fertilizers.

4.1.1.2 Biological Nitrogen Fixation

Prior to the industrial revolution, biological nitrogen fixation (BNF) which is carried out by a group of prokaryotes (termed as diazotrophs) were the dominant source of N to the atmosphere. Diazotrophs converts inert N_2 into useful ammonia catalysed by the enzyme nitrogenase.

Biological N fixation is performed by a phylogenetically diverse list of bacteria and archaea that may live in close symbiotic association with plants or are “free living” or non-symbiotic.

4.1.2 Nitrogen Fixers

Annually 95% of the total global nitrogen is fixed by diazotrophs. There are two types of nitrogen fixing micro-organisms: asymbiotic and symbiotic.

a) Asymbiotic nitrogen fixation

The organisms which fix nitrogen without direct interaction with other organisms are called as asymbiotic organisms. In 1981, Winogradsky observed that a bacterium (*Clostridium pasteurianum*) is responsible for enhancing the nitrogen content in the soil when it is exposed to atmosphere. The rate of nitrogen fixation varies depending on the type of bacteria. Free living organisms which fix the atmospheric nitrogen include aerobic bacteria (*Azotobacter*), anaerobes (*Clostridium*) and cyanobacteria (*Anabaena, Nostoc, Aulosira, Cylindrospermum Trichodesmium*). The use of consortia of nitrogen fixers with various agricultural crops like cereals and grasses found to be profitable and it also contributed to the N economy of the plants. The inoculation of *Azospirillium* to crop plants enhances the plant growth, nitrogen content, nutrient assimilation, root size and function (Okon and Kapulnik, 1986) [44].

Azotobacter is a Gram-negative, non-symbiotic, aerobic diazotroph which belongs to the γ-subclass of Proteobacteria. Genus *Azotobacter* comprises of *A. chroococcum, A. vinelandü, A. beijerinckii, A. paspali, A. armeniacus, A. nigricans* and *A. salinestri.* They are heterotrophic aerobic bacteria that fix nitrogen non-symbiotically with G+C content of 63–67.5 % (Becking, 2006) [5] and are widely distributed in soils, water and sediments (Tejera, 2005) [65]. They have the capability of synthesizing antibiotics, plant growth promoting substances, vitamins, exopolysaccharides, pigments and antagonistic substances against a variety of pathogens. Cyanobacteria are aerobic photoautotrophs which requires water, carbon dioxide, inorganic substances and light. Photosynthesis is their principal mode of energy metabolism. Many free-living blue-green algae fix atmospheric nitrogen and do not compete with crop plants or heterotrophic soil microflora for carbon and energy because they are phototrophic in nature. Nitrogen-fixing ability has been shown by various heterocystous cyanobacteria (*Nostoc, Anabaena, Aulosira*) non-heterocystous unicellular (*Gloeocapsa, Aphanothece, Gloeothece*) and filamentous (*Oscillatoria, Plectonema*) cyanobacteria. In the non-heterocystous forms, the photosynthesis was found to be disconnected from nitrogen fixation. The fixation predominantly occurs during the dark period and therefore engages non-photosynthetic cells. The species which possess the potential of developing effective bio fertilizers include heterocystous and filamentous forms belonging to the order *Nostocales* and *Stigonematales* in which the nitrogenase activity and oxygenic photosynthesis are separated. Species of *Nostoc*, *Anabaena*, *Tolypothrix*, *Aulosira*, *Cylindrospermum* and *Scytonema* contribute significantly to soil fertility.

b) Symbiotic nitrogen fixation

This type of biological nitrogen fixation takes place in plants harbouring diazotrophs within their tissues. Plants acquire atmospheric nitrogen with the help of diazotrophs to which they are associated. Based on the degree of association and interdependency between plants and microbes, either they have loose associations with free-living nitrogen fixers or intercellular endophytic associations and endosymbioses.

Association of rhizobacteria with plants display the simplest form of nitrogen-fixing symbiosis. These Rhizobacterium colonises the plants and respond to root exudates via chemotaxis which helps the plant in acquisition of nutrients. These soil bacteria have the ability to induce nodules to the roots of legume plants and within these nodules they fix nitrogen. This association is mutually beneficial and allow in obtaining a niche for the bacteria and nitrogen for the growth of plants. Nitrogen fixers associated with plants are divided in two main groups: root-nodule bacteria and plant growth-promoting rhizobacteria (PGPR).

Rhizobium and *Frankia* form root-nodules in association with legumes and actinorhizal plants. Some plants show endosymbiotic interactions with nitrogen-fixing cyanobacteria like *Nostoc(Nostocazollae)*. Various *Proteobacteria*, *Actinobacteria*, *Bacilli*, and *Cyanobacteria* exhibit plant growth-promotion and develop associative or endophytic associations with cereals (van Egeraat, 1975; Vanderlinde *et al*., 2014) [69, 70]. *Azoarcus, Herbaspirillum,* and *Gluconacetobacter* colonize on the surface of plant, then spread and multiply within tissues without causing damage. They show obligatory or facultative association between them and exhibit complex interactions with their host hosts that varies from mutualism to parasitism. They enter the plant tissues through stomata or root hairs which are confined within the infection thread and released into the cytoplasm of cells in the cortex. Later, the bacteria are differentiated into bacteroid forms which can reduce dinitrogen into ammonia which can be assimilated by the host plants (Liu *et al*., 2009, Eskin *et al*., 2014) [38, 20].

4.1.3 Mechanism of nitrogen fixation

Leguminous plants belonging to the family Papilionacae like bean, pea, gram and soybean develops root nodules. They serve as a major site for N_2 fixation which contains enzymes like nitrogenase and leghaemoglobin (leguminous haemoglobin). The nitrogenase has two components: Mo-Fe protein (molybdoferredoxin) called as dinitrogenase and Fe-protein (azoferredoxin) called as dinitrogenase reductase. The nitrogenase enzyme catalyses the conversion of atmosphere dinitrogen (N_2) to NH_3. Free di-nitrogen in the atmosphere is bound to MoFe protein till complete reduction occurs. Ferredoxin donates an electron to Fe-protein which hydrolyses ATP to ADP and reduces the MoFe protein, thereby leading to the reduction of the substrate N_2. The electrons and ATP are supplied from the photosynthesis and respiration processes of the host cells. *In vitro,* for the reduction of one molecule of dinitrogen, 16 ATP molecules are required, whereas *in situ*, 20-30 are needed. Thus, ammonia is the stable product of nitrogen fixation which is immediately protonated to form ammonium ions (NH_4^+). Because of the toxicity, protonated form of ammonia is immediately used by plants for amino acid synthesis. The activity of nitrogenase is influenced by oxygen. Leghaemoglobin (Lb) present in root nodules act as an oxygen scavenger and is involved in protecting the enzyme (nitrogenase) activity.

5. Conclusion

Nitrogen transformation process is vital for life and a key role for productivity in the environment during the course of transformation, some of the organisms use for their metabolic activity either for energy or growth. Thus, nitrification and denitrification process are mediated by microbes i.e. archaea, bacteria and fungi and the process may occur in days or it may take years. Culture dependent and culture independent techniques has revealed many microbes in the pathway and understanding the role of microbes in different hopes for better utilization of efficient strains, which can be used for various applications.

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