Bacterial contribution to nitrogen processing in the atmosphere

by F. Mathonat et al.

Author response to comments by Referees

All referee comments are shown in black, our author responses in **blue**; suggested new manuscript text is indicated in *red*; text citations from the original manuscript are *in italic*.

REFEREE #1.

The manuscript explores nitrogen cycling by natural airborne microbial communities using a combination of genetic and biogeochemical tools. Based on their data, the authors conclude that a significant fraction of the airborne microbial community has the potential to process organic and inorganic nitrogen compounds while airborne. Through rainwater incubations, they estimate the contribution of the community in processing these compounds, particularly for bio-assimilatory purposes. They conclude that while the contribution of the airborne microbial community to nitrogen cycling is insignificant on a global scale, it may be relevant for the survival of microbial cells while airborne.

Overall, I find the manuscript innovative and relevant to the field and recommend it for publication. However, I kindly ask the authors to address the following issues prior to publication:

Thank you for your positive assessment of our work and for your constructive comments, which helped elaborating an improved version of the manuscript. Please find our point-by-point responses below.

Equation 4: I had difficulty understanding this equation and was unable to reconcile the units. I would appreciate it if the authors could explain the equation in more detail and provide an example in the text showing how they performed the calculations.

The equation : « $Gene\ abundance = [DNA] \times NA \times 10^{-9} / (n \times mw)$ » is used for converting a concentration of DNA into a concentration of corresponding gene copy numbers. This was proposed by Whelan et al., 2003, and is commonly applied to qPCR to prepare standard curves. In the equation, *[DNA]* is the concentration of the recombinant plasmid in ng μL^{-1}, *NA* is the Avogadro's constant (6.023×10^{23} mol^{-1}), *n* is the length of the gene sequence in base pairs (bp) and *mw* is the average molecular mass of a base pair (660 g $mole^{-1}$ bp^{-1}).

As an example, consider the 16S rRNA gene of *Pseudomonas syringae* PDD-32b-74 isolated from clouds (GenBank A.N. HQ256872). The plasmid pEX-A128 including the target region of the 16S rRNA gene sequence (amplified by the universal primers EUBf/EUBr, see Materials and Methods of the manuscript) was provided by a subcontracted company (Eurofins Genomics), at a concentration of 19.68 ng μL^{-1}. The plasmid (vector plus gene insert) has a total length of 2799 bp (2450 and 349 bp, respectively). To calculate the concentration in number of gene copies per µL in this solution, the DNA concentration is multiplied by Avogadro's number to convert to molecules·mol^{-1}. The factor 10^{-9} is applied to convert nanograms to grams. On the other side of the equation, the number of base pairs (2799 bp) is multiplied by the average molecular weight of a base pair (660 g·mol^{-1}).

This gives : $19.68 \times 6.023 \times 10^{23} \times 10^{-9} / (2799 \times 660) = 6.42 \times 10^{9}$ copies.μL^{-1}

From this, a standard curve in copies.μL^{-1} was produced.

To clarify the methods used for gene quantification, reference to Whelan et al., 2003 will be included and the text revised as follows, so as to include additional important information (DNA concentration in the plasmid stock solutions and length) (Material and Methods Section 2.3.6):

Plasmids including the target gene regions were provided by a subcontracted company (Eurofins Genomics, Lille, France), at a concentration of 19.68 ng μL^{-1} for 16S rRNA and 11.38 ng μL^{-1} for amoA. The plasmid (vector + gene insert) for 16S rRNA and amoA genes have a total length of 2799 bp (2450 + 349 bp, respectively) and 2982 bp (2450 bp + 532 bp, respectively). From equation (4), the plasmid stock solutions for 16S rRNA and amoA genes were therefore at concentrations of 6.42×10^9 copies.μL^{-1}, and 3.48×10^9 copies.μL^{-1}, respectively. Standards for quantification were obtained by decimal dilutions of the stock solutions, …

It is fascinating that the authors were able to determine transcripts in the clear sky samples. What was the relative humidity and how does that fit with what has been reported in the literature for microbical acitivity in relation to RH?

Thank you for raising this aspect, more information will be included in the revised manuscript (Section 3.2 dedicated to MG/MT reanalysis results), as:

Under clear-sky conditions, the atmospheric samples used to generate the data exhibited relative humidity (RH) values ranging from 41% to 78%, with a mean of 55% ; no relationship between the expression of biological functions and RH could be detected (see (Péguilhan et al., 2025) for further details). Nevertheless, RH is known to impact the viability of model airborne bacteria, with often higher survival at extreme low or high RH levels (Cox and Goldberg, 1972; Wright et al., 1969), and to influence their gene expression patterns (Barnes and Wu, 2022). Larger datasets remain necessary to examine such relationships in the natural environment.

I wonder if these transcripts could have been produced prior to aerosolization and preserved in the airborne state due to cell inactivity, which would include the turnover of the transcripts. Could the authors exclude this possibility and discuss the consequences for their interpretation?

Thank you for this relevant comment. The revised text will include (Section 3.2) a brief discussion about RNA stability, and the likelihood that the transcripts were indeed likely produced by airborne cells, as:

Functional gene expression was evaluated considering their relative representation of transcripts (mRNA) in MT (Fig. 1B) respect to their corresponding genes in MG (RNA:DNA ratio, with higher values indicating higher expression levels). While ribosomal RNAs can persist up to several days at low temperatures (Schostag et al., 2020), the average half-life of an mRNA in a bacteria cell (Mycobacterium tuberculosis) at 37°C is between 2 and 5 minutes, which is much shorter than the average duration of atmospheric transport (~3–4 days; Burrows et al., 2009). Some studies have shown that under "stressful" conditions or during dormancy/inactive states, such as caused a shift in temperature, the half-life may increase by a factor of 2–3, but it still remains on the order of only a few tens of minutes (Rustad et al., 2013). Most of the transcripts identified in cloud and aerosol samples were therefore likely produced by the cells while airborne.

Denitrification is a process that occurs under oxygen-limited or anoxic conditions, where it replaces aerobic respiration. Do the authors have any indications that oxygen is limited for the cells while they are airborne? If so, why would the cells denitrify instead of using oxygen?

It is indeed an important consideration regarding the atmosphere that this an aerobic environment, and that this may be limiting for certain pathways such as denitrification.

Nevertheless, aerobic denitrification (AD) can occur, notably in *Pseudomonas* species that are frequent in the atmosphere. This was described in other taxa as well including Actinomycetes and yeasts. In AD, O_2 and NO_3^- compete for electrons, with O_2 being thermodynamically favored. The O_2 concentration regulates enzymatic activity and therefore the efficiency of denitrification, with three possible patterns observed across different microorganisms: efficiency decreases with increasing dissolved oxygen (DO) until a threshold is reached (Wilson and Bouwer, 1997), efficiency is optimal only within a specific DO concentration range (Chen and Ni, 2012) and rare tolerance to high DO concentrations (Ji et al., 2014; Zhang et al., 2011).

At present, there is no consensus on the mechanisms underlying AD, but several theories have been proposed (Hao et al., 2022): the microenvironmental theory, where oxygen diffusion is limited within cell aggregates ; the enzyme theory, where two type of nitrate reductases coexist and allow cell to reduce both oxygen and nitrate simultaneously (Kumar and Lin, 2010; Yang et al., 2020) ; the electron transfer theory, where a bottleneck in the respiratory chain prevents all electrons from being transferred to O_2, and these are redirected to denitrification enzymes (Chen et al., 2006; Kong et al., 2006; Robertson and Kuenen, 1984). The efficiency of this process depends on energy demand, O_2 concentration, and the presence of specific enzymes such as Nap, NAR, and NIR.

This will be developped in the revised manuscript (section 4.2.3 of the Discussion), as :

Denitrification is a process that generally occurs under oxygen-limited or anoxic conditions. However, some microorganisms are capable of performing aerobic denitrification (AD). Aerobic denitrifying bacteria are predominantly Gram-negative bacteria affiliated with Pseudomonadota, with nearly 50% of them belonging to Pseudomonas (Ji et al., 2015), a taxon frequent in the atmosphere (Vaïtilingom et al., 2012). In addition, yeasts with AD capacity were reported from surface sediments (Fang et al., 2021; Zeng et al., 2020), as well as Actinomycetes from aquatic ecosystems (Ma et al., 2022). In AD, O_2 and NO_3^- compete for electrons, with O_2 being thermodynamically favored. The O_2 concentration regulates enzymatic activity and therefore the efficiency of denitrification, with three possible patterns observed across different microorganisms: efficiency decreases with increasing dissolved oxygen (DO) until a threshold is reached (Wilson and Bouwer, 1997), efficiency is optimal only within a specific DO concentration range (Chen and Ni, 2012) and rare tolerance to high DO concentrations (Ji et al., 2014; Zhang et al., 2011). At present, there is no consensus on the mechanisms underlying AD, but

several theories have been proposed (Hao et al., 2022). The first is the microenvironmental theory, where oxygen diffusion is limited in cell aggregates. The second is the enzyme theory, which attributes aerobic denitrification to the activity of specific enzymes. For example, in Thiosphaera pantotropha, two nitrate reductases coexist: M-NAR (active only in the absence of O_2) and P-NAR (active even in the presence of O_2). This dual capacity allows the cell to reduce both oxygen and nitrate simultaneously, making denitrification possible under aerobic conditions (Kumar and Lin, 2010; Yang et al., 2020). The third theory, which is not mutually exclusive with the enzyme theory, is the electron transfer theory. It explains AD as the result of a bottleneck in the respiratory chain that prevents all electrons from being transferred to O_2. Instead, some electrons are redirected to denitrification enzymes, enabling the simultaneous use of O_2 and NO_3^- as electron acceptors (Chen et al., 2006; Kong et al., 2006; Robertson and Kuenen, 1984). The efficiency of this process depends on energy demand, O_2 concentration, and the presence of specific enzymes such as Nap, NAR, and NIR.

Nitrogen fixation is an energetically costly process that microbes typically use only when other nitrogen sources are unavailable. This does not seem to be the case in the samples analyzed by the authors. Why would the cells rely on N2 fixation when other nitrogen sources are plentiful?

The statement that N2 fixation may occur in airborne cells is supported by metatranscriptomic data, with higher expression of the *nifH* gene under clear atmosphere condition compared to cloudy conditions, and by the screening of isolates, where this function is not rare. In some rainwater incubations, atmospheric nitrogen fixation is hypothesized to be carried out by the microorganisms detected in these experiments, as there is no decreased in ammonium and nitrate concentrations. The use of organic nitrogen sources by microorganisms is also a plausible explanation, but their actual bioavailability for airborne cells is likely limited.

In the revised text (Section 4.2.4), it will be added that :

Nitrogen fixation is an energy demanding process, but it enables microorganisms capable of performing it to assimilate atmospheric nitrogen for biomass production. Microbes only activate N_2 fixation when they lack access to more readily assimilable nitrogen sources, such as ammonium. This situation can occur in the atmosphere, where bacteria outside clouds or in the microenvironment of a droplet have limited access to easily bioavailable nitrogen (Khaled et al., 2021). This is supported by indications of amino-acid starvation in metatranscriptomes (Péguilhan et al., 2025). One could envision that the atmospheric environments serve as niches for nitrogen fixers: while certain bacteria grow rapidly by using available compounds such as ammonium, diazotrophs typically slower-growing can subsequently, or in parallel, develop in nitrogen-limited environments.

The authors suggest anoxygenic phototrophs as possible candidates for N2 fixation. What would these microbes use as electron donors for N2 fixation while airborne? Many of them depend on reduced sulfur compounds or hydrogen. Are these valid sources in this context?

Thank you for this relevant comment. The revised text will be added with the following information (Section 4.2.4) :

Anoxygenic phototrophs do not use water as the electron donor but instead exploit a variety of reduced organic (e.g., organic acids) or inorganic compounds such as Fe^{2+}, H_2, HS^-, $S_2O_3^{2-}$, NO_2^-, and AsO_3^{3-} (Trüper and Pfennig, 1981). While potential electron donors such as H_2S and thiosulfate are relatively scarce (<0.1–1 ppbv), H_2, whose concentration is around 500 ppbv, along with organic compounds such as formate and acetate, could support anoxygenic phototrophy, and nitrogen fixation from them.

The rainwater incubations lasted for several days. However, in the atmosphere, the retention time of microbes in rain droplets is much shorter. I would appreciate it if the authors could discuss the relevance of their estimates based on these long-term incubations.

Thank you for this relevant comment.

Rainwater incubations were conducted over 5 days. The average residence time of a bacterium in the atmosphere has been modeled at 3 to 4 days (Burrows et al., 2009), and this is expected much shorter in atmospheric droplets. It will be acknowledged in the text (Section 3.6) that we actually considered the rates to remain constant during the incubation time, which undoubtedly is an approximation:

This is a plausible duration for bacteria's residence in the atmosphere, estimated around 3 to 4 days (Burrows et al., 2009), but the actual time spent by cells within atmospheric droplets is expected much shorter. The data were

interpolated over the 5-day periods in order to determine rates, assuming, as a first order approximation, that these remained constant throughout the incubation time.

Lastly, I would appreciate a detailed discussion of Figure 5 (PCA plot) that summarizes the results.
The results from PCA will be developped briefly in the revised manuscript (Section 3.6), as:
PCA (Fig. 5) illustrates the variability of rain water samples composition, and its evolution during incubations. The 2 first components explain 52% of the variance and allow discriminating in particular marine from continental air masses. Samples from air masses originating from marine areas (Atlantic Ocean) were enriched in Na^+ and Cl^- ions, whereas samples from continental air masses contained higher levels of NH_4^+, NO_3^-, and SO_4^{2-} ($p < 0.05$, Spearman's rank correlation). Continental air masses were also characterized by higher ambient temperatures at the sampling site, smaller water volumes less acidic pH, and higher cell concentrations respect to marine air masses ($p < 0.05$). This is consistent with previous observations at this site (Péguilhan et al., 2021). Certain bacterial taxa could be also associated with air mass origin as well : the relative abundance of Sphingomonadales was significantly higher in samples from marine air masses, whereas Burkholderiales dominated in samples from continental air masses ($p < 0.05$). Finally, the bioassimilation rates of ammonium and nitrate were positively correlated with the relative abundance of Sphingomonadales and negatively correlated with that of Burkholderiales ($p < 0.05$), but they were independent from the initial concentrations of these ions, bacteria and amoA gene copies ($p > 0.05$).

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