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 #2.

In the manuscript by Mathonat et al., the authors describe the potential contribution of airborne microorganisms to nitrogen cycling by combining reanalysis of meta-G and meta-T datasets with laboratory exploration of bacterial isolates and rainwater incubation experiments. Overall, this provides valuable and interesting insights into the potential activity of these microbes under humid conditions. However, several concerns regarding the experimental design, data integration, and interpretation of results arise and should be addressed before the manuscript is accepted, as detailed below.

Thank you for this positive assessment of our work, and for your relevant comments. Please find our point-by-point responses below.

The study showed that cloud water samples did not demonstrate enhanced gene abundance (Fig. 1A) or expression levels (Fig. 1B) compared to clear atmosphere samples. The data also show that nitrogen processing genes are less abundant in clouds than in clear atmosphere, and RNA:DNA ratios are also lower in clouds. What does it means? This should be addressed in the discussion of why cloud environments were not enriched in the expected microbial activity.

Certain functions such as glutamate metabolism, nitrite reduction, and nitrate assimilation and reductive dissimilation, were equivalently represented in MG or MT regarless of atmospheric conditions (cloud or clear air). On the other hand, biological nitrogen fixation was more prevalent in clear atmosphere, both in MG and in MT.

The ratio between the relative abundance of transcripts in MTs respect to that of their corresponding genes in MGs (RNA:DNA ratio) is often considered as a proxy for microbial activity (e.g., Baldrian et al., 2012). We considered ratios statistically >1 as indicative of increased microbial activity for the related functions, and vice versa for ratios <1. To clarify, the revised text will include (Material and Methods, section 2.1):

The ratio between the relative abundance of transcripts in MTs respect to that of their corresponding genes in MGs (RNA:DNA ratio) is often considered as a proxy for microbial activity (e.g., Baldrian et al., 2012). The proportion of reads identified as involved in each N-cycling functions (i.e., nitrification, denitrification etc) in each MG and MT were summed, and the corresponding "RNA:DNA ratio" of a given function, i.e., the number of related reads transcripts in a MT with respect to that of genes in the corresponding MG, was calculated for each sample.

Based on this, most N-related functions were actively expressed by microbial cells in both clouds and clear air. Numerous functions had statistically higher RNA:DNA ratio in clear atmosphere than in clouds, in particular organic N processing functions, which is indeed surprising regarding the conditions. We will discuss the fact that higher RNA:DNA ratio in clear air than in clouds could be related with alternating drying-wetting conditions (Section 4.1 of the Discussion):

Active nitrogen-related functions are maintained by bacteria in the atmosphere, which might be critical for their physiology despite much shorter residence times (a few days) than in any other environment (Burrows et al., 2009). Surprisingly, higher gene expression levels (RNA:DNA ratio) of these functions were observed in clear air than in clouds, where the presence of condensed water is rather expected to promote biological activity. Liquid water can be retained by efflorescent aerosols at RH <100%, which could be sufficient to sustain biological activity in clear atmosphere (Cruz and Pandis, 2000). In addition, it is possible that multiple dry-wet cycles occurred in particular before collecting the clear air samples, which could have contributed to enhance biological activity as compared with cloud water, as observed in soil (Xiang et al., 2008).

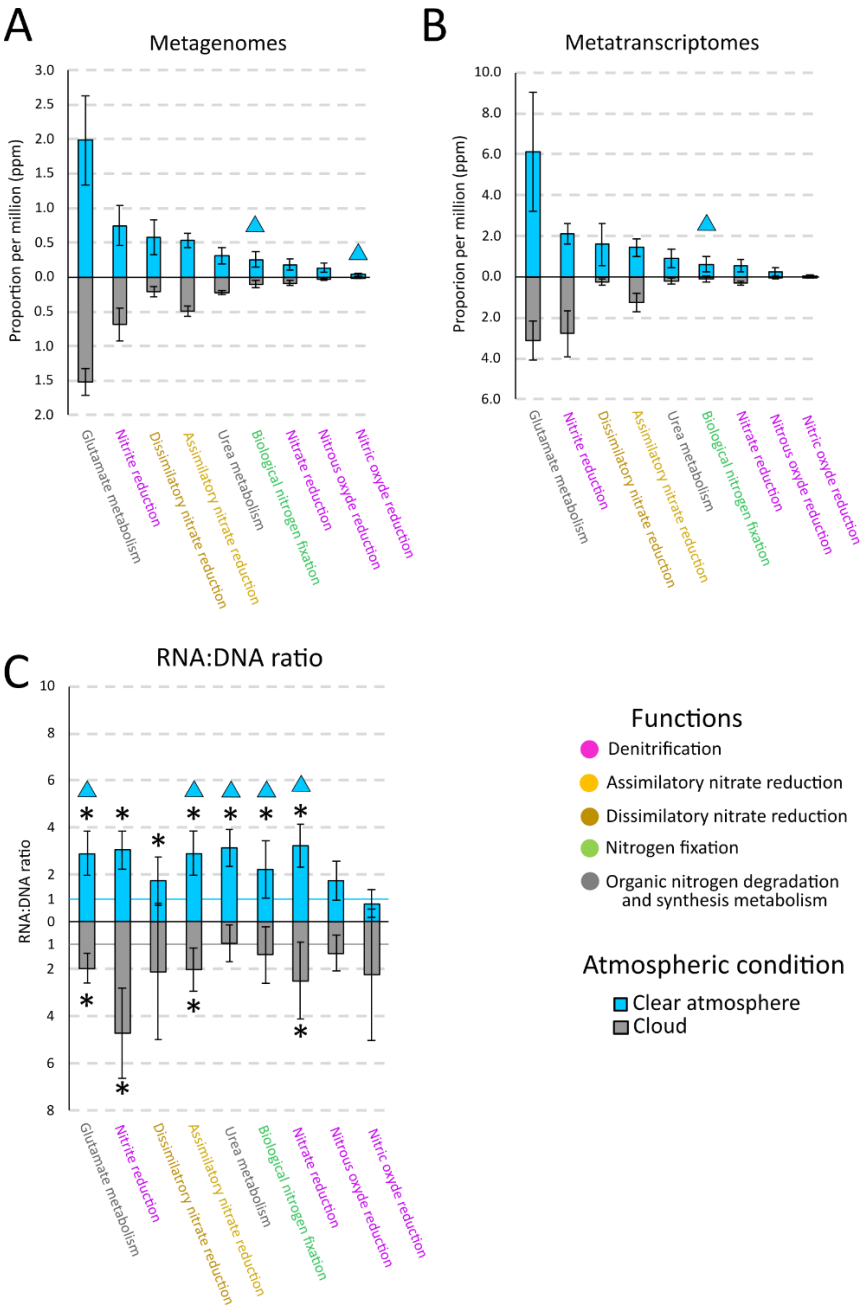
In line 422 the author argue that it is due to higher N2 abundance in open atmosphere. But other factors may also lead to these results, and should be acknowledged/discussed, such as methodological difference in sampling approach, the volume of air sampled for open air and for possible scavenged ones due to rain washdown.

Thank you for raising this concern. The sampling methodology for the production of MG and MT is fully presented in Péguilhan et al., 2025. Briefly, in total, 9 cloud and 6 clear air conditions were sampled in 2019 and 2020, each during daytime periods of 2 to 6 consecutive hours, using between two and four high-flow-rate impingers (HFRIs; DS6 model, Kärcher SAS, Bonneuil-sur-Marne, France), running at an air flow rate of 2 m^3 min^{-1}. Samples were collected directly into a nucleic acid preservation buffer, at a concentration of 0.5X for clear atmosphere and 1X concentration for clouds, in order to compensate for concentration/dilution due to evaporation/water accumulation, so as to reduce potential methodological biases. A detailed description of these devices and their suitability for the collection of biological material can be found in Šantl-Temkiv et al., 2017.
Regarding rain washdown, this is not relevant here are as the rainfall events described in the manuscript article are distinct from the aerosol and cloud samples that based gene expression analyses.

We will provide more information about sample acquisition in the revised manuscript as (Material and Methods Section 2.1):
Briefly, 6 aerosol (clear atmosphere) and 9 cloud samples were originally collected in 2019-2020, from the instrumented atmospheric station of puy de Dôme Mountain summit (1,465m asl, France) (Baray et al., 2020). For both atmospheric conditions, multiple high-flow rate impingers were deployed in parallel, each running at an air flow rate of 2 m3.h-1 (Šantl-Temkiv et al., 2017), and the samples were accumulated for 2 to 6 h, directly into a nucleic acid preservation buffer using high-flow rate impingers,. at 0.5X concentration for clear atmosphere and 1X for clouds in order to compensate for concentration/dilution due to evaporation/water accumulation, so as to reduce potential methodological biases. Clear atmosphere samples were collected at relative humidity (RH) ranging from 41% to 78% (55% on average) while clouds were characterized by RH = 100% and liquid water content >0.

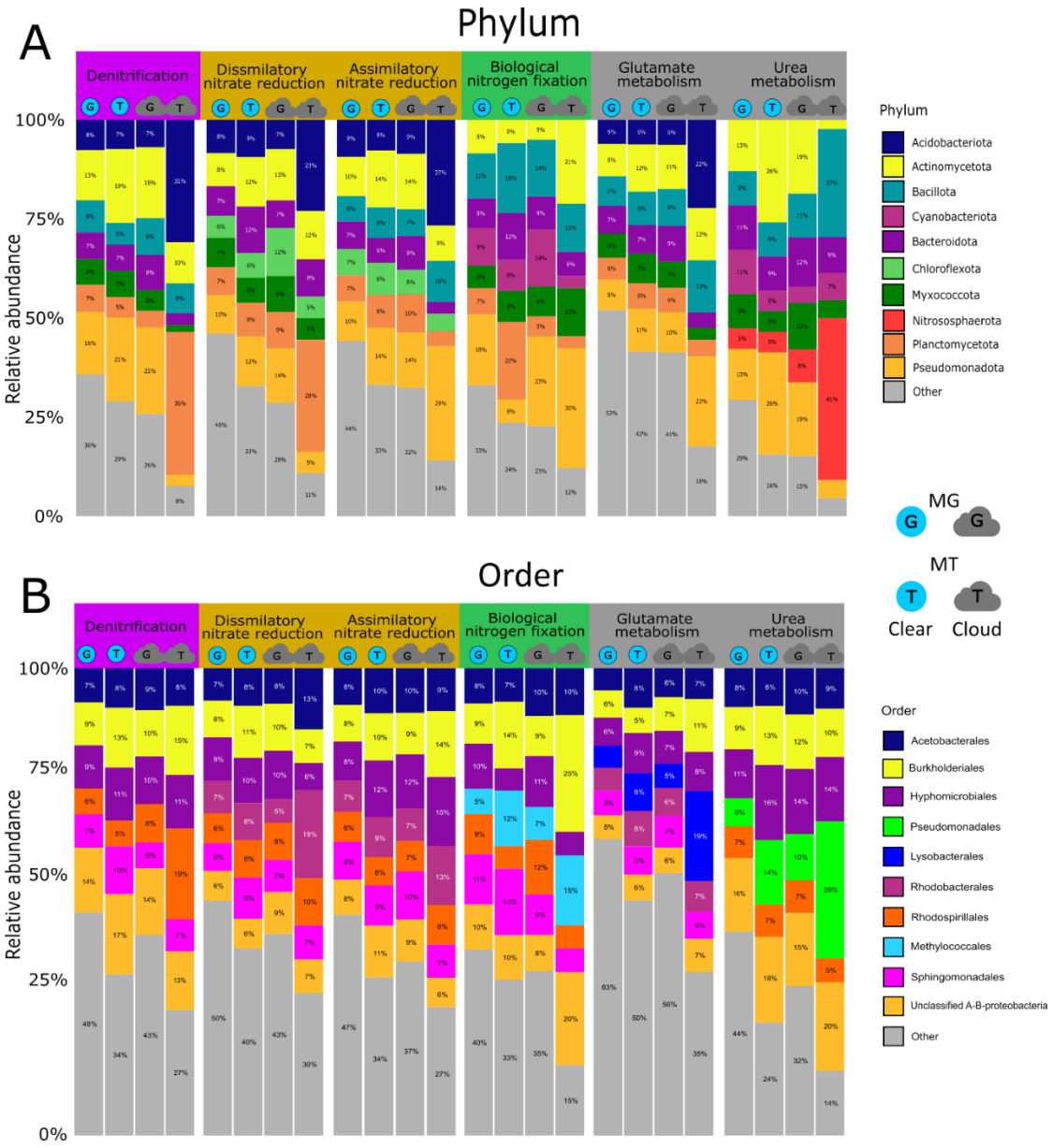
We also include, in the Results section 3.6:
Under clear-sky conditions, the atmospheric samples used to generate the data exhibited relative humidity (RH) values ranging from 41% to 78%, with a mean of 55% ; no relationship between the expression of biological functions and RH could be detected (see (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.

The reported RNA:DNA ratios for genes with abundances close to zero in the Meta-G and Meta-T (Fig. 1C) are problematic and may represent mathematical artifacts rather than biologically meaningful expression levels. When the DNA abundance approaches zero, even minimal RNA detection yields inflated ratios that are statistically unstable and probably lack biological relevance. For example, genes with <0.1 ppm bp in the metagenome could show high RNA:DNA ratios purely due to noise in the measurements, not genuine transcriptional activity. therefore, it is recommended to establish a minimum abundance threshold below which RNA:DNA ratios should not be calculated or interpreted.
Thank you for this comment, which helps improving data's representation. We agree that such low proportion may generate baises, and the revised manuscript will include a new Fig.1 (see below) showing only functions with representation greater than 0.1 ppmbp in MG.



On the same line- I have doubts regarding the nitrification taxa presented in Fig. 2. These genes show extremely low values in both MG and MT in Fig. 1 (<1% of nitrogen-related reads), yet Fig. 2 presents detailed phylum and order-level taxonomic breakdowns for these barely detectable genes. And it is most noted for the cloud water MT samples, presenting 100% abundance of Acidobacteriota at the phylum level, and 100% Cytophagales at the order level (which belongs to the Bacteroidota phylum). Can the author explain this discrepancy? My suspected idea is that it results from the low gene abundance and thus outliers may dominant your data.

Thank you for raising this point. An error regarding the affilitions shown in Fig.1B/denitrification was indeed detected, and this will be corrected in the revision. In addition, as for Fig.1, the taxa with less than 0.1 ppmbp representation have been removed for better readibility. The new figure will be as follows :



The detection of anammox-related genes and transcripts is surprising, as anammox bacteria are obligate anaerobes. Could the authors discuss potential explanations for this observation? Clarification on whether the RNA:DNA ratios for anammox genes suggest active expression or baseline transcription would help interpret whether these organisms are potentially active in atmospheric conditions or simply represent transported but inactive populations.

As indicated in the text (Sections 3.1 and 4.1), very few transcripts and genes were detected for Anammox, regardless of atmospheric conditions, which is consistent with the strictly anaerobic nature of anammox bacteria. With regards to its low representation, Anammox will be removed from the new Figure 1 (see response to previous comment).

The bacterial strains screened for the nifH gene were pre-selected to include 34 known nitrogen-fixing taxa (e.g., Pseudomonadales, Sphingomonadales). This targeted selection can introduce positive bias, making the finding that 15% of tested strains were nifH-positive potentially unrepresentative of the broader rainwater bacterial population. What is the total number of rain-borne isolated bacteria? The authors should clarify whether this 15% reflects the prevalence among all culturable cloud bacteria or only among pre-selected candidate taxa, and be careful about extrapolating this percentage to the entire atmospheric microbial community without appropriate caveats (see e.g., line 434 in the discussion).

It is correct that the strains screened for nifH were selected for belonging to taxa known to include nitrogen-fixers, such as Pseudomonadales, Sphingomonadales, Burkholderiales, Rhizobiales, Mycobacteriales, Rhodospirillales, Hyphomicrobiales, and Rhodobacterales. These are among the most frequent viable bacteria in clouds (Vaïtilingom et al., 2012).

We will revised the corresponding Results Section 3.4 to recall this preselection, as :

Thirty-four (34) strains of Alphaproteobacteria and Gammaproteobacteria isolated from clouds in earlier work were tested by PCR for carrying the N_2 fixation biomarker gene (nifH). These strains were selected to belong to taxa that include nitrogen-fixers, and they are among the most frequent viable bacteria in clouds (Vaïtilingom et al., 2012). In total, 5 strains (~15%) were positive, of which 4 Sphingomonadales and 1 Rhizobiales (Supplement Table S1).

In the Section 4.2.4 of the Discussion about N_2 fixation, we will replace « *the screening of isolates indicates that ~15% of Pseudomonadota strains isolated from the atmosphere carry this function* » by « *the screening of isolates indicates that this function is not rare in Pseudomonadata viable in the atmosphere* »

Moreover, our estimates for N_2 fixation (Section 4.2.4) are indeed based on the assumptions that « *half of the airborne bacteria (5×10^{19} cells) are Pseudomonadota, and that 15% are diazotroph* », which clearly is an upper estimate considering that the 15% are for preselected strains. From this, we still get to the conclusion that biological nitrogen fixation in clouds « *likely represents an unsignificant fraction of global biological nitrogen fixation* ».

It will be clearly specified in the revised text that :

« *Although a more precise quantification would be needed, this upper estimation indicates that biological nitrogen fixation in clouds likely represents an unsignificant fraction of global biological nitrogen fixation compared to…* »

In Table 3 it is shown that the amoA gene remained below the detection limit even after incubation, including in samples showing the highest ammonium reduction in Fig 4 (20230922-RAIN-TF). If ammonium oxidation (nitrification) were responsible for the observed ammonium loss, it would be expected that amoA gene abundance would increase during incubation as ammonia-oxidizing bacteria proliferated. This absence of amoA suggests that ammonium depletion may have occurred through alternative pathways rather than nitrification? A direct examination of the expressed genes through RNA analysis (either RT-PCR to measure selected expressed genes, or RNA-seq to directly explore the activity of nitrification genes during ammonium removal) would dramatically enhance your findings. The presented results provide weak support for the proposed nitrification activity.

In the nitrogen cycle mediated by microorganisms, ammonium can follow two pathways: either nitrification (leading to nitrate production), or incorporation into the biomass, through glutamate metabolism (assimilation). However, there was no increase in nitrate concentration during rainwater incubations and the amoA gene (biomarker for nitrification) was not detected. Consequently, nitrification does not appears as the most likely process for explaining ammonium depletion. Rather, the bioassimilation of ammonium through glutamate is probable. Furthermore, the genes and transcripts of this function are, by far, the most abundant of N-related functions in both MGs and MTs in clouds and aerosols.

While the authors rightly use broader terminology such as "atmospheric microorganisms" and "airborne microbial communities" in the discussion and acknowledge that nitrogen transformations can occur in clear atmosphere conditions, they do not adequately address this fundamental distinction throughout the manuscript. They extrapolate results "to cloud environments based on rainwater data" and make claims about processes occurring "in clouds" without clarifying whether they are studying indigenous cloud-resident microbes or rain-scavenged airborne populations. The terminology and interpretations throughout the manuscript should be revised to consistently refer to "airborne scavenged microbes," "rain-deposited atmospheric microbes," or "atmospherically transported microbes" rather than implying a distinct "cloud-borne community," as the experimental design does not adequately distinguish between these fundamentally different microbial sources.

The manuscript is structured around two distinct and independent experimental approaches. The first consists of a reanalysis of atmospheric metagenomic (MG) and metatranscriptomic (MT) datasets obtained under dry and cloudy atmospheric conditions from the study by Péguilhan (2025), while the second is based on laboratory incubations of fresh rainwater samples.

Particular attention was paid to the terminology used throughout the manuscript to designate « *atmospheric microorganisms* » in general, as these are not taxonomically distinguishable between clouds, clear air and precipitation (Péguilhan et al., 2021, 2023).

The rainwater incubation conditions are acknowledged as a limitation (particularly the relatively high temperature compared to actual cloud conditions). This also affects the implications, which are at present understated. Incubation at 17°C would likely accelerate microbial growth and metabolism above natural rates, and continuous shaking, and 5-day duration do not reflect the transient, episodic nature of cloud events (range of hours). These conditions may fundamentally alter community dynamics, succession, and biogeochemical rates, making the measured bioassimilation rates weak proxies for actual atmospheric processes.
See our responses below.

Authors may explore the option of the cloud transition zone: the between cloud and open atmosphere region, which studies imply is at near saturated humidity, and with a wide atmospheric coverage than previously estimated. The present study may indicate this region might be optimal to bacterial activity, as it should be with longer atmospheric duration, and higher temperatures than in the clouds.
Thank you for this suggestion. The following text will be inserted in the revised manuscript (Perspectives Section).
Atmospheric regions such as the cloud transition zone (i.e., the interface between clouds and the free atmosphere) exhibit humidity levels close to saturation and a broader atmospheric coverage than previously estimated (Calbó et al., 2024; Ruiz de Morales et al., 2024). This region may be particularly favorable for bacterial activity, as it is expected to experience longer atmospheric residence times and higher temperatures than those prevailing within clouds.

In line 393, the global estimates for ammonium processing (5.5×10^{7} kg year^{-1}) and in line 412, nitrate transformation (2×10^{7} kg year^{-1}) are derived by extrapolating the average bioassimilation rates measured in the 17 °C rainwater incubations to the total estimated airborne biomass and assuming a 15% cloud fraction. This calculation chain seems to contain multiple uncertainties: (a) laboratory rates that likely overestimate natural rates (difference in incubation and ambient temp, and thus growth rate, incubation time, etc.), (b) extrapolation from a single location to the entire atmosphere, (c) uncertain estimates of global airborne biomass, and (d) the assumption that all cloud-associated bacteria exhibit similar metabolic activity regardless of cloud type (or open atmosphere), altitude, RH, temp, or geographic location. A supported/convincing estimate should include sensitivity analyses to provide uncertainty ranges for these global estimates. In addition, a comparison with other independent estimates (from e.g., atmospheric chemistry models) would strengthen yours.
Thank you for raising this major point.
Quantitative data derived from rainwater incubation experiments were used to estimate the extent of possible N-biotransformation processes carried out by microorganisms in the global atmosphere. Assumptions include the fact that biodiversity in rain water samples is representative of « atmospheric microbes » on their whole, and that the biological transformation rates observed in rainwater can be transposed to clouds. It is already clearly stated in the current manuscript (Perspectives section) that :
« *However, the accuracy of these transformation estimates at global scale could be improved. For instance, the measured bioassimilation rates were obtained from rainwater incubations at 17°C, which do not fully represent atmospheric aerosol conditions, as the liquid bulk phase does not account for the particulate nature of aerosols, and the temperature is relatively high compared to actual atmospheric conditions.* »

Estimates are provided on the global scale. We are aware of the high spatial and temporal variability of microbial biomass and biodiversity existing in the atmospheric environmnent. To fully emphasize the associated likely high variability of nitrogen biological processing in the atmosphere, and the uncertainties linked with our estimates, we will revise the manuscript, as :
« *From our data, assuming that our samples are representative of the global atmosphere, we estimate a global ammonium processing by bacteria in clouds of ~5.5 × 10^{7} kg year^{-1}, considering the average bioassimilation rate measured in rainwater incubations (4.61×10^{-8} µmol cell^{-1} h^{-1}), a total airborne biomass of 5 × 10^{19} bacteria (Whitman et al., 1998), and a cloud fraction of 15% (Lelieveld and Crutzen, 1990).* » (Section 4.2.2).
And, in the Perspective section :

« Moreover, while these estimates are scaled to the global level, atmospheric conditions, nitrogen speciation and concentrations and microbial diversity and abundance are subject to strong spatial and temporal variability. Hence, nitrogen-related biological processes are expected to be highly variable in space and time as well. Our estimates, which are based on samples collected on a single location, for limited periods of time, and derived from laboratory incubations under defined fixed conditions are therefore associated with high uncertainties. Addressing variations and heterogeneity of these processes will require deeper investigations. One could envision enhanced microbial nitrogen cycling activity following major nitrogen release events (e.g., fertilizer application in agriculture).».

The statistical description in the methodology section is lacking. Authors should expand and describe all tests selected in the analysis of the data.

A section dedicated to statistics will be included in the Material and Methods Section:

2.3.8 Statistical analyses

All statistical tests were carried out using Past software (v.4.03) (Hammer et al., 2001). To compare the mean proportions of the different functional categories in metagenomic (MG) and metatranscriptomic (MT) datasets, a parametric Student's t-test or a non-parametric Mann–Whitney test was performed. RNA:DNA ratios were compared to a value of 1 using a one-sample Wilcoxon test, and the mean RNA:DNA ratios of the different functional categories between cloudy and clear-air conditions were assessed using the parametric Student's t-test or the non-parametric Mann–Whitney test.

For the rainwater incubation experiments, correlations among the different factors were evaluated using Pearson's rank correlation test. Mean values derived from the incubations (e.g., ion concentrations, bacterial abundances, etc.) were compared using the non-parametric Wilcoxon test.

In Figure 5, authors should clarify the significance of the impacting vectors. Significant ones can be marked with asterisks next to them, and please note the statistical tests used in the caption (as well as in the methodology...)

In the revision, the tex twill include of short description of PCA's results (Section 3.6 of the Results) :

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 ($p < 0.05$).

Table of PCA Eigen values (Fig. 5) and percentage of variance for each component:

	Eigen value	Percentage of variance	Cumulative percentage of variance
comp 1	7.7555819	33.719921	33.71992
comp 2	4.2131195	18.317911	52.03783
comp 3	3.8427927	16.707794	68.74563
comp 4	2.4968841	10.856018	79.60164
comp 5	2.3414263	10.180114	89.78176
comp 6	1.0902079	4.740034	94.52179
comp 7	0.7891131	3.430926	97.95272
comp 8	0.4708746	2.047281	100.00000

Did the author conduct rRNA depletion prior to meta-T sequencing? I couldn't find this information in the methodology, nor in the referenced paper by Péguilhan et al. (2025). Since ribosomal RNA typically comprises the majority of total RNA in microbial samples, information on rRNA removal would be helpful for understanding the meta-T data processing workflow.

Thank you for this relevant comment.
No rRNA depletion method was employed. As mentioned in the Supplemental Material and Tables S2-S3 of Péguilhan 2025, the datasets from sequencing contained significant and consistent proportions of rRNA in MGs and MTs, ~1-2% and ~90% of the total reads, respectively. These were removed numerically. We recall here the relevant text from the Supplemental Material of Péguilhan et al. 2025 :
« *The trimmed reads were screened with SortMeRNA (v 2.1b.6) (Kopylova et al., 2012) to filter and recover the rRNA gene reads in separated files, with the default parameters, "paired-out" option, and all the available databases. Non-rRNA gene reads were processed for functional analyses. The proportions of rRNA gene reads in MG and MT datasets were generally between 1-2 % and 80-94%, respectively. Sample 20201124AIR's MT consisted of only 12% rRNA gene reads and was therefore excluded from further analysis (Tables S2-S3). Human reads were filtered from the non-rRNA gene reads using Bowtie2 (v 2.4.2) (Langmead and Salzberg, 2012), against the NCBI Homo sapiens genome "hg38_2021-5-18" with default parameters (Tables S2-S3). Human reads were excluded from further analyses.* »

The manuscript discusses Fig. 1A and 1C but doesn't mention Fig. 1B. Please check and correct.
This will be corrected in the revised manuscript

In Figure 1 the nitrogen processing genes are not presented in the same order across panels A, B, and C. This can confuse and possibly mislead readers, making it harder to compare panels and understand the relationships between gene abundance, expression, and activity.
Thank you, the order of the nitrogen gene functions will be changed so that they appear in the same order on each panel (see the new Figure 1 in our response to comment above).

In addition, the presentation of panel C as bar charts can be improved, as the ratios are not cumulative based on 0, but rather based on 1. Consider changing to a box plot or dots, and keeping open atmosphere and cloud samples in the same direction (upwards) to make comparison easier.
We chose to keep the representation of RNA:DNA ratios as a mirrored bar chart (see the new Figure 1 in our response to comment above). Ratios significantly >1 are indicated by «* », and the comparison between clear atmosphere and clouds is provided by statistical analyses (triangles).

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