Referee 1

1. Page 2, line 54: The authors use Bowers (2011) to support a statement about bacteria playing a significant role in the composition and dyamics of bioaerosols amongst all the different airborne microorganisms. The cited paper, though, focuses on the effect of weather and land-use type over diversity and abundance of airborne bacteria in the Colorado Front Range, it doesn't make any statements about other airborne bioaerosols. The reviewer suggests to revise this reference.

The referee is right. We will change the reference with Gong et al., 2020
Gong, J., Qi, J., E, B., Yin, Y., Gao, D.: Concentration, viability and size distribution of bacteria in atmospheric bioaerosols under different types of pollution. Environmental Pollution 257, 113485, https://doi.org/10.1016/j.envpol.2019.113485, 2020.

2. Page 2, line 57: The authors use Bauer et al. (2002) as a citation to substantiate the atmospheric concentration over land of bacteria, but the cited paper is about the carbon content of fungal spores. The reviewer suggests to use a more fitting reference or explicitate the connection between the number of bacteria in the atmosphere and the cited paper.

The referee is right. We will change the reference by citing Burrows et al. , 2009.
Burrows, S. M., Butler, T., Jöckel, P., Tost, H., Kerkweg, A., Pöschl, U., Lawrence, M.G.: Bacteria in the global atmosphere – Part 2: Modeling of emissions and transport between different ecosystems, Atmospheric Chem. Phys. 9, 9281–9297. https://doi.org/10.5194/acp-9-9281-2009, 2009.

3. Page 3, line 65: rather than Lighthart (2006), the reviewer suggests using "Lighthart B. Shaffer B.T. Marthi B. Ganio L.M. (1993) Artificial wind gust liberation of microbial bioaerosols previously deposited on plants. Aerobiology9, 189–196." The latter is better suited to reference a statement about airborne bacterial agglomerates, as also cited by Lighthart (2006) itself.

The referee is right. In the revised manuscript the reference will be changed as suggested.

4. Page 3, line 67: Tong and Lighthart (1999) does not contain detailed information about particle size. The reviewer suggests to remove or change this reference.

We will remove the Tong and Lighthart (1999) reference in the revised manuscript.

5. Page 3, line 77: Bauer et al. (2003) it's not present in the reference section. The only Bauer paper in the reference section is from 2002 and is about carbon content of fungal spores. Said paper does not detail any interaction between bacteria and the atmosphere. The reviewer suggests adjustment to this reference.

In the revised manuscript, the reference Bauer et al. (2003) will be added.

6. Page 4, line 96-97: "where trandisciplinary studies gathering […] issues are possible". "Gathering issues" is an odd phrasing, the reviewer suggests substituting "gathering" with "addressing" or to better explicitate the meaning of the sentence.

In the revised manuscript we will substitute the word "gathering" with "addressing" as suggested.

7. Page 6, line 167-168: what sensor does the PID use to measure concentration?

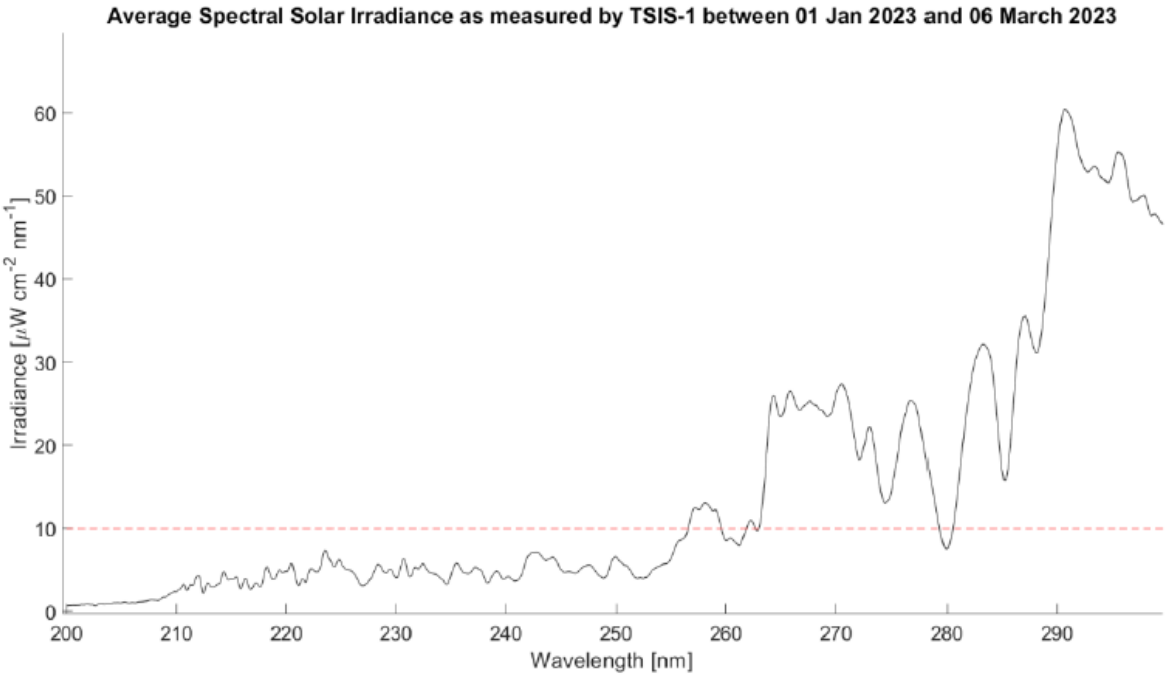
The PID (Proportional-Integral-Derivative) controller is a control loop mechanism employing feedback aimed at keeping the value of a certain parameter equal to a pre-defined setpoint value within a defined error. A PID controller is a mathematical construct that is then converted to an algorithm by the use of a software tool (developed by LabView in this case). It continuously calculates an error value as the difference between a desired setpoint (SP) and a measured process variable (PV) and applies correction based on proportional, integral, derivative terms in order to minimize the error on each loop cycle.
PV is the process variable read by a sensor and sampled by the hardware platform connected to the sensor (NI-cRIO, based on Labview program language).
In the first case the PVs are the CO2 and SO2 concentration values read as output of the corresponding gas analysers (sensors) connected to the cRIO platform. In the second case the PV is the ChAMBRe internal pressure as output of the pressure gauge (sensor) mentioned in the text.

For the reason above, In the revised manuscript, we will rephrase the sentence as follows:
"Two 5-lpm MFCs are designed for injection of CO_2 and other gases (i.e. SO_2, CO, NO and NO_2), respectively, whose concentration in the chamber can be selected by the operator (ppm or ppb units); a PID (Proportional–Integral-Derivative) controller, using the gas concentration values read from the corresponding gas analyzer, keeps the gas concentration in ChAMBRe constant during the experiment.
A 30-lpm MFC regulates the injection of dry air inside the chamber. In this case, the PID controller (using the ChAMBRe pressure values measured by pressure sensor mentioned above) allows to maintain a pre-defined pressure gap between inside and outside the chamber."

8. Page 8, Figure 2: The reviewer has some issues with the presented irradiance spectra. First of all, given that the data are presented over multiple wavelengths, shouldn't the correct unit for the y-axis be μW cm-2 nm-1 as it actually represents a spectral, rather than an integrated, irradiance? The irradiance in the UV-C

range seems unusually high also for the Sun measured on the terrace of the Genoa Physics Department. Even if it's not clear due to the coarse resolution of the y-axis scale, it seems that UV-C at 200 nm is well above 10 µW cm-2 nm-1. That value seems unusually high. As a reference, the reviewer reports here a plot of average spectral solar irradiance measured by the TSIS-1 instrument on the International Space Station between the 1st of January and 06 March 2023. The instrument measures the solar irradiance at the top of atmosphere and normalizes it to 1 AU (i.e.: Sun-Earth distance). This measurement happens before any interaction between solar radiation and ozone happens in the Earth's atmosphere and, therefore, before reduction of any UV radiation. Data were originally in W m-2 nm-1 but were converted to µW cm-2 nm-1 by multiplying the original data by 100 in order to have units consistent with those presented in the paper. The plot shows the spectrum between 200 and 300 nanometers for better visualization of the range of interest and it shows that even above the Earth's atmosphere the irradiance up to (roughly) 250 nm

is well below 10 µW cm-2 nm-1. These values are almost constant with time: the plot contains also a shaded area which details the temporal standard deviation (SD), but the shading is not visible as the SD values are close to zero (maximum SD = 0.1263 uW cm-2 nm-1).

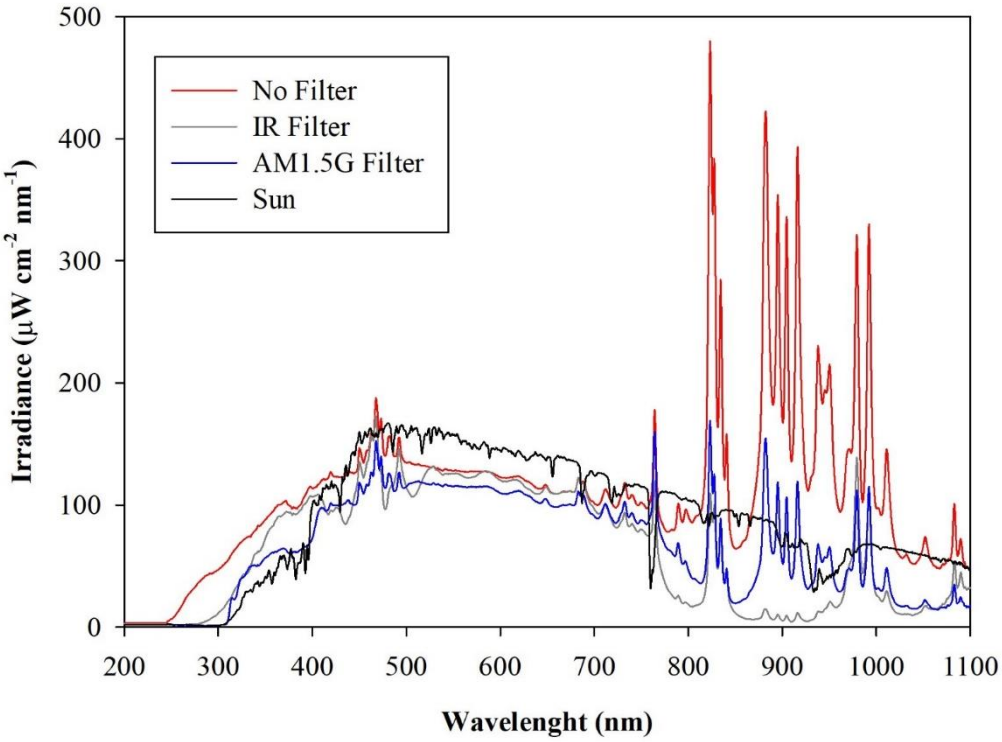


Data used in this plot are accessible through the following API link: https://lasp.colorado.edu/lisird/latis/dap/tsis_ssi_24hr.csv?time,wavelength,irradiance&time>=2023-01-01T00:00:00Z&time<=2023-03-07T23:59:59Z&formatTime(yyyy-MM-dd'T'HH:mm:ss) and their description can be found at: https://lasp.colorado.edu/lisird/data/tsis_ssi_24hr

Again the presented data are top of atmosphere, so at the surface of the Earth these UV values should be even lower.

Given that UV-C are the most harmful for biological particles, any excess in this spectral range in the Solar Simulator could severely impact bioaerosols' viability. Due to this reasons, the reviewer kindly asks the author to better explain the Solar Simulator spectra and the measured sun irradiance.

The referee is right. We will change the Y axis unit using μW cm^{-2} nm^{-1} in the revised manuscript. Thanks to the detailed comment of the Referee, we found an error in figure 2 reported in the manuscript: not only in the original manuscript the "No filter" irradiance in the UV range is lower than IR filter, AM1.5G filter and sun irradiance (and this is not possible) but also the absolute values are wrong. We revised data from spectrometer, and we found an error in the subtraction of background noise collected in "dark condition" in the UV range (with the optic fiber covered by a black hood). Below the corrected figure will be replaced in the revised manuscript. The irradiance MDL of the spectrometer varies between 3 and 5 μW cm^{-2} nm^{-1} and, in the case of measure spectra with Solar Simulator and AM 1.5G filter and sun, it has met below 300 nm.
In conclusion, with the AM1.5G filer and Sun, we don't observe any sizeable irradiance below 300 nm.



9. Page 12, figure 5: What does the error bars represent? Standard deviation? Standard error? Where does this uncertainty comes from? Repeated WIBS measurements at the same time?

The bar error is the uncertainty of bacteria concentrations calculated following the Poisson statistics i.e. the standard deviation. We will add this sentence in the revised manuscript.

10. Page 13, line 319: the acronym MISG is explained here, but was already used at page 7, line 173. Please move the acronym explanation at the first occurrance of it in the text.

We will move the acronym explanation at the first occurrence of MISG in the revised manuscript.

11. Page 16, line 420: the acronym OD (which I assume stands for "Optical Depth") is used but never explained. Please explain it.

We will add the acronym of OD (Optical Density) to the revised manuscript.

12. Pages 17, lines 437-438: it is stated "Data, obtained by CFU counting on agar plates, are averaged and used to figure out the uncertainty". What's the uncertainty metric? Standard deviation?

The referee is right, the sentence is not clear. We will modify the sentence in the revised manuscript as:
"Data, obtained by CFU counting on agar plates, are weighted averaged and used to figure out the uncertainty (standard error of the weighted mean) of the bacterial concentration in the solution. The weights are the relative uncertainties of CFU number on agar plates following the Poisson statistics."

13. Page 18, figure 6: What are the error bars? Standard deviation? Standard Error? Also, while for CFU ml-1 the uncertainty should come from the fact that bacteria are spread in duplicates (page 17, line 434), where does the uncertainty of the QTx TOT come from? Was it also measured in duplicates?

The referee is right. We never explained how calculate the uncertainty of QTx TOT. We will add this sentence in the revised manuscript at the Quantom TX section: "To evaluate the uncertainty on the bacteria count (QTx TOT), we repeated the measurement on the same sample 10 times, and we found an instrument repeatability of 5%. This uncertainty is much higher of the statistical error of total counting (assuming the Poisson statistic), and, for this reason, we adopted a 5% uncertainty to all Quantom Tx counts."
In addition, we will add a sentence under the figure: "Error bars have, in most cases, the same size of the data points and they are calculated as previously described."

14. Page 18, line 463: "OD600nm and QTx TOT have the same value of the b parameter". Looking at Table 1 that's not the case: for OD600nm the value is (3.3+-0.1)x10^-2, while for QTx TOT the value is (3.4+-0.5)x10^-2. If the meaning of the sentence was that the two values are not significantly different, please add a statistical test confirming it.

We will change the phrase in: The b values of OD600nm and QTx TOT are compatible within their uncertainties, and this result is expected since the OD600nm is an indirect measurement of the total concentration of cells in suspension.

15. Page 19, lines 476-477: "the number of the counted colonies are averaged, to retrieve the bacterial concentration in the solution and its statistical uncertainty". Again, what's the metric for this uncertainty?

We will revise the part from line 469 to 481 to explain better the preparation and the metric used to calculate the uncertainty of counted colonies. In the revised manuscript this part will be replaced by:
"To prepare the inoculum for the chamber experiments, the E. coli is grown in 30 ml of fresh TSB nonselective medium, in a shaking incubator at 37 °C and 200 rpm and its growth is followed by checking the OD600nm value until the mid-exponential phase. When OD600nm ~ 0.5, 20 ml of this liquid preparation is centrifuged at 3000 rpm for 10 min. Afterward, the bacteria pellet, separated by surnatant, is resuspended in 20 ml of sterile physiological solution (NaCl 0.9 % w/v) to prepare a suspension of approximately 10^8 CFU mL^{-1}, as verified by standard dilution plating. To retrieve the bacterial concentration, the average of CFU counting on agar plates and the uncertainty are calculated following the same metric described in 2.3.1 paragraph.
For the experiments performed at ChAMBRe, the typical bacterial concentration in the inoculum is 10^7 CFU ml-1: to reach this concentration, a further dilution step is needed (i.e., typically 1:10 or 1:5) before the injection (see Massabò et al, 2018 for details)."

16. Page 20, line 499-501: The coefficient of variation of the experiments should be moved to the results section and then possibly discussed in terms of reproducibility of ChAMBRe experiments (see also comment #24).

We will move the coefficient of variation to the results section. See the answer of comment 24 about the discussion in terms of reproducibility.

17. Page 20: "Dark" conditions are never explained. Intutively, it just means with the Solar Simulator turned off, but please explicitate that.

We will clarify the dark condition in the revised manuscript as follows: "solar simulator off" and "solar simulator on" to dark and light condition, respectively.

18. Page 22, Figure 7: in the caption the colours are swapped and wrong: red is indicated for total bacteria and green for viable, while it should be red for viable and blue for total.

The Referee is right: In the caption of figure 7, we will indicate as blue the total bacteria and red viable bacteria trend in the revised manuscript.

19. Page 22, lines 563-564: "particles, in the size range of 1-2 μm (τ=2-3 hours), the same of E.Coli". Commas make the sentence awkward to read. The reviewer suggests rephrasing to "particles in the same size range of E.Coli (1-2 μm) and τ of 2-3 hours".

In the revised manuscript, we will rephrase the sentence as suggested by the referee.

20. Page 23, line 573: The usage of "Then" at the beginning of the sentence is confusing, it seems to imply that dead bacteria after ozone treatment were exposed to NOx. The reviewer suggests to rephrase it starting with "In another experiment …".

We will replace "Then" with "In another experiments" in the revised manuscript.

21. Page 24, Paragraph "Experiments with E.Coli and the Solar Simulator": all the results reported here must be discussed in view of the UV-C intensity in the Solar Simulator (see comment #8).

By considering the answer to comment n. 8, we believe that no additional discussion is needed.

22. Page 24, lines 593-594: This sentence ending with "and data are here gathered" is not very readable. Please clarify its meaning.

The referee is right; in the revised manuscript, we will revise the sentence to: "No significant differences in results appeared changing the intensity of the Solar Simulator operated with the AM1.5G filter and the data with a Solar Simulator intensity of 100% are here reported."

23. Page 24, lines 598-599: "V:T (…) reaches zero after an hour". In figure 9, though, V/T appears to be 0 already at the 30 minute mark. What's the correct result? Also, please keep consistent the format of V:T in both text and figures (either V:T or V/T everywhere).

The referee is right; in the revised manuscript we will modify the sentence to: "The viable concentration collapses quickly, reaching zero after 30 minutes. The comparison between V:T ratio obtained for dark and light baseline is shown in Figure 9."
We will correct in the revised manuscript the Y axis of figure 8 and 9 reporting V:T instead of V/T.

24. Page 25, lines 615-617: The reviewer thinks that reproducibility would need a more in depth discussion. First of all, in the text it is never explicitated the amount of performed replicates, with the exception of the caption of figure 7 where it is stated that eight repetitions were done for the baseline experiments. This point is, instead, extremely important as the reproducibility of the baseline experiments is crucial for the application of ChAMBRe itself. The reviewer encourages the authors to clearly state how the baseline experiments are performed in terms of replicates and how uncertainty and error propagation (if any) is estimated. Furthermore, the sentence here is not very clear, it suggests that the reproducibility (20% in terms of what? How was this reproducibility calculated?) is in line with the experimental sensitivity. What does this mean? That the

experimental uncertainty is as big as the reproducibility? If so that would imply that any variation of viability could be inputtable to an error in reproducibility. The reviewer strongly suggest to clarify and clearly quantify all the parameters tied to the reproducibility and experimental uncertainty so that the reader can have a clear idea of the uncertainties tied to the ChAMBRe experimental protocol presented in this paper.

The Referee is right: some points are missing (for example the number of experiments as suggested) and the discussion in terms of reproducibility and uncertainties is not always clear and not well described. In the first version of manuscript, we have discussed the results in terms of average and std error of C_0 and τ and these values have been retrieved starting from the averaged results on number of experiments. Thanks to the Referee comment, we have thought that a revised following approach should be a better choice. For this reason, in the revised manuscript, we will add a column to table 3 reporting the number of experiments and we will review the "Results" and "Discussion, conclusion and perspectives" sections describing how we have calculated the uncertainties of C_0 and τ for total and viable *E.coli* concentration and V:T ratio. All data have been reviewed following this procedure:

- For each experiment, C_0 and τ have been retrieved fitting the data with the function $C(t) = C_0 e^{-\frac{t}{\tau}}$;
- In the tables 2 and 3 average and standard deviation of C_0 and τ of all experiments are reported.

The results have been discussed in terms of τ of V:T ratio and its coefficient of variability of baseline experiments compared to the values obtained with gases experiments.

Here the sections we would insert in the revised manuscript:

3.1 Baseline experiments with E. coli in dark conditions.

E. coli behaviour in a set of eight replicated experiments, led from separate cultures, was first determined in dark conditions. The average total concentration and standard deviation of *E. coli* inside the chamber at t = 0 (three minutes after the conclusion of the injection to allow proper mixing/homogenization inside the ChAMBRe volume) was (0.34 ± 0.08) cells cm^{-3}, as measured by the WIBS; the average viable concentration and standard deviation, determined by the Andersen impactor sampling at t = 0, was (0.04 ± 0.02) cells cm^{-3}. The viable concentration at t = 0 was obtained by measuring the CFUs on three petri consecutively sampled; the coefficient of variation on the CFUs collected on the three petri, resulted equal to 12%.

The average ratio and standard deviation of viable:total (V:T in the following) bacteria concentration inside ChAMBRe, at t = 0 turned out to be V:T = (0.13 ± 0.07). The total and viable bacteria concentration values, measured inside ChAMBRe, depended the V:T ratio in the inoculum to be injected (biological effects between each bacteria culture) and on the aerosolization process affecting the bacteria viability. The bacteria viable concentration in the inoculum was determined via standard dilution plating while the bacteria total concentration was calculated by the Quantom Tx. During baseline experiments, the V:T ratio of the inoculum ranged between 0.25 ± 0.03 and 0.50 ± 0.06. Time-trends of the averaged total and viable concentration of the bacteria, nebulized inside ChAMBRe, are shown in Figure 7. Bacteria lifetime in ChAMBRe can be calculated by fitting the data of each experiment with an exponential function as:

$$C(t) = C_0 e^{-\frac{t}{\tau}} \qquad (2)$$

where C_0 is the total or viable concentration of *E. coli* just after the injection (t = 0) and τ is the total or viable bacteria lifetime, respectively. In table 2, the average and standard deviation of C_0 and τ for the *E. coli* total and viable concentration of eight experiments are reported.

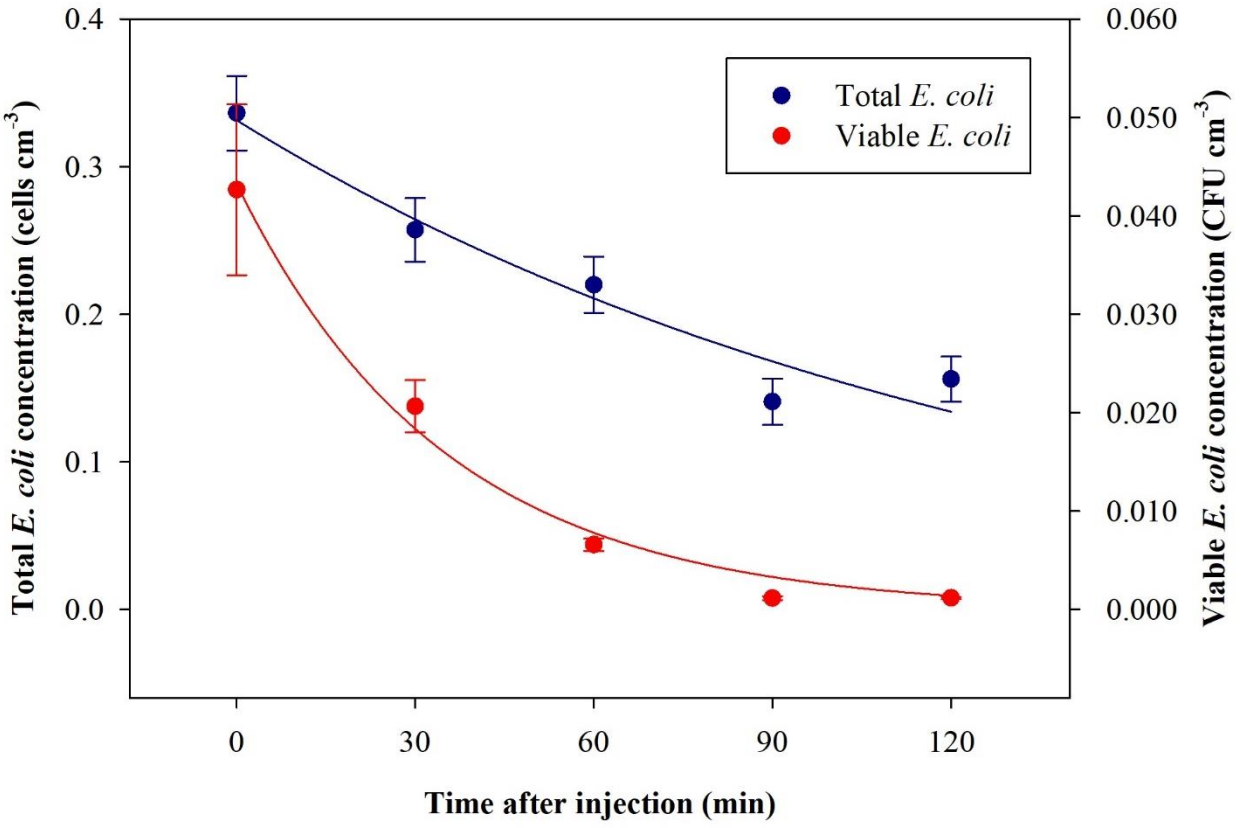


Figure 7: Time-trend of *E. coli* average bacteria total (blue) and viable (red) concentration inside ChAMBRe obtained by eight repetitions of baseline experiments.

Table 2: C_0 and τ (average ± std deviation) of the exponential fit for total and viable concentration of *E. coli*.

Exponential function	Total *E. coli*	Viable *E. coli*
C_0	(0.33 ± 0.08) cells cm^{-3}	(0.04 ± 0.02) CFU cm^{-3}
τ (min)	153 ± 22	32 ± 5

The total *E. coli* averaged lifetime is about 150 minutes; this value agrees with data reported in (Massabò et al., 2018) for generic aerosols: particles in the same size range of *E. coli* (1-2 μm) and τ = 2-3 hours. The viable *E. coli* averaged lifetime is about 32 minutes, lower than the aerodynamic lifetime, this indicating the difficulty of this microorganism to survive in the atmospheric medium.

3.2 Experiments with E. coli and NOx in dark conditions.

A preliminary check was performed exposing the *E. coli* to O_3, which is recognized to be a strong antimicrobial agent (Kim et al., 1999; Giuliani et al., 2018; Thanomsub et al., 2022), hence the expected result was a complete viability loss. The exposure of bacteria to O_3 (concentration > 1000 ppb) resulted in a complete cell mortality, as expected. The initial condition immediately after the injection was V:T = (0.03 ± 0.01) and no CFUs were collected in any of the following samplings (starting 30 minutes after the injection).

In another experiments, bacteria were exposed to NO_2 and NO concentrations, 900 and 1200 ppb for both the pollutants. The exposure of bacteria to such pollutants showed a V:T reduction. The average results, obtained in a set of total eight experiments, are shown in Figure 8.

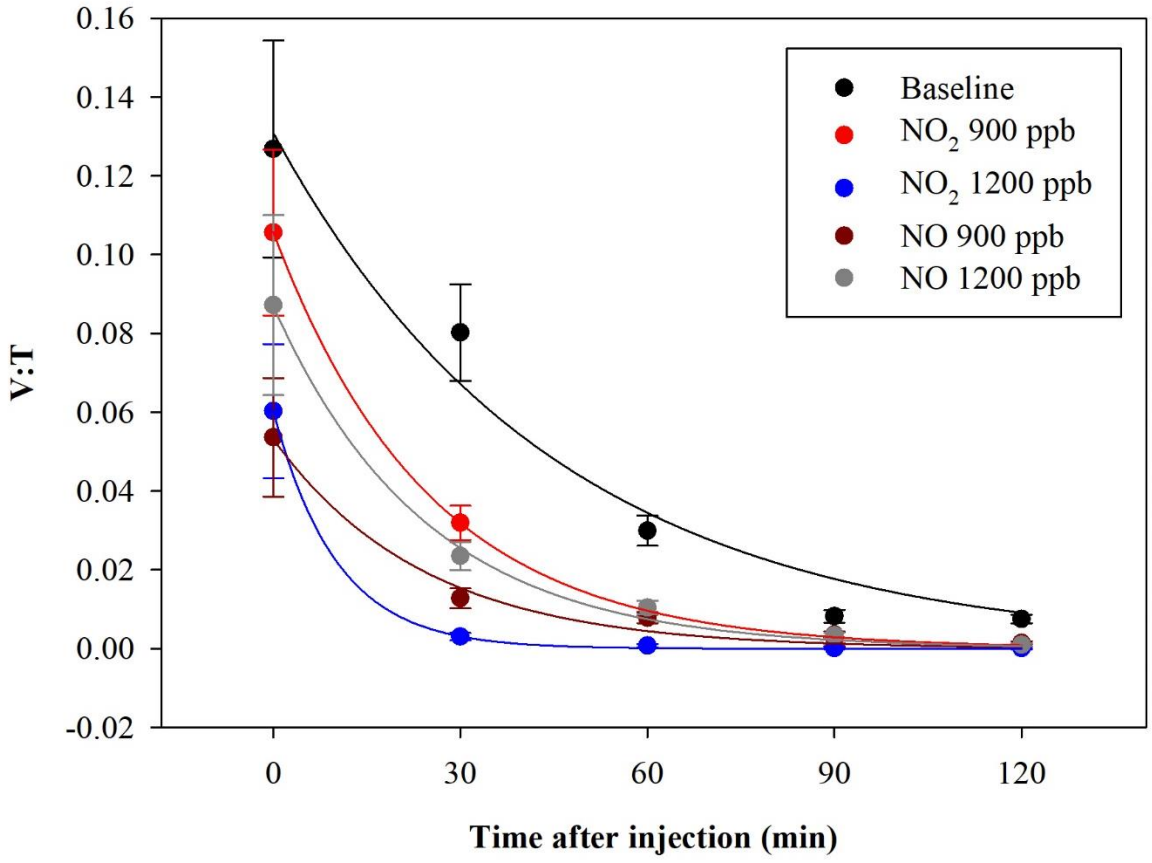


Figure 8: Time-trend of the V:T ratio for E. coli in baseline (black) and in the experiments with ChAMBRe maintained at a constant concentration of: NO_2 (900 ppb red and 1200 ppb blue) and NO (900 ppb dark red and 1200 ppb gray).

The quantitative reduction in the *E. coli* lifetime, due to the exposure to pollutants, can be evaluated considering the V:T ratio and fitting the data with an exponential curve, as previously described; the results are shown in Table 3.

Table 3: Initial values and τ (average and std deviation) of the exponential fit for V:T ratio of *E. coli* at different pollutants concentrations.

Exponential function	(V:T t = 0)	τ (min)	experiments #

Baseline	0.13 ± 0.07	40 ± 5	8
NO_2 900 ppb	0.11 ± 0.02	25 ± 2	2
NO_2 1200 ppb	0.06 ± 0.02	11 ± 2	2
NO 900 ppb	0.05 ± 0.01	26 ± 3	2
NO 1200 ppb	0.10 ± 0.02	25 ± 4	2

E. coli averaged lifetime in baseline experiments, calculated on the V:T ratio, turned out to be about 40 min. The exposure of *E. coli* to NO_2 reduced the lifetime to about 25 and 11 min with a concentration of 900 ppb and 1200 ppb respectively. The exposure to 900 ppb and 1200 ppb of NO decreased bacteria lifetime to 26 and 25 min, respectively and the values are similar to the value obtained with the lowest NO_2 concentration. The increase of the NO concentration did not correspond to a decrease in the *E. coli* viability, as observed with NO_2: these results suggest a greater toxic effect of NO_2 than of NO on *E. coli*. The literature of a comparison of the toxic effects of NO and NO_2 on *E coli* is poor. Some research articles have demonstrated negative effects of these two gases on bacterial strains: Kosaka et al. 1986 found a decrease in *E.coli* viability with increasing NO_2 concentration. Janvier et al, 2020 highlighted a significant adverse effect of NO_2 on some commensal skin bacterial strains. Mancinelli and McKay, 1983 found that a low concentration of NO is bacteriostatic for some organisms but not for others. It is worth noting that NO has a strong antimicrobial property, being an endogenously produced molecule that is critical for critical infection defence (Fang, 1997), although some bacteria are able to escape this NO action (Privett et al., 2012).

4 Discussion, conclusion and perspectives

The main result presented in this work is the assessment of a multi-step and well controlled protocol to perform experiments on the impact of air quality on bacteria viability by an atmospheric simulation chamber, ChAMBRe in this case. Even if the chamber configuration is still in progress and several new equipment will be deployed at ChAMBRe in the near future, the present set-up opens the possibility of systematic studies. The average τ of the V:T ratio of eight baseline experiments was 40 min with a standard deviation of 5 min; the coefficient of variation of 13% corresponds to the experimental sensitivity to changes in *E. coli* viability due to exposure to pollutants and/or other relevant parameters. The baseline reference must be experimentally determined for each bacteria strain and efforts are planned for repeating the observation with *Bacillus subtilis, Bacillus spizizenii* and *Pseudomonas fluorescens* in the near future. It is worthy to note that the experimental protocol returns the lifetime of total and viable bacteria injected in the chamber. The figure for total bacteria corresponds to the aerodynamic behavior of aerosol of diameter around 1 μm, already reported in (Massabò et al., 2008) while the lifetime of viable bacteria is much shorter (about half an hour) due to the difficulty of this microorganism to survive in the atmospheric medium. Such shorter lifetime posed clear constraints on the first experiments with exposure of *E. coli* to NO_x inside ChAMBRe. A time window of two hours after the bacteria injection was considered to observe the behaviour of *E. coli* viability and it was possible to quantify a lifetime reduction, in dark conditions, clearly related to NO and NO_2 concentration inside ChAMBRe. These findings

pave the road to systematic studies including other bacteria strains and pollutant species. In the near future With the *E. coli* exposed to the light produced by the Solar Simulator operated with the AM1.5 filter, the viability resulted very short even in the baseline conditions and therefore no further experiment with pollutants was performed. With other bacterial strains, the impact of light on viability will have to be reinvestigated. It is well known in the literature that the viable but non-culturable condition (VBNC) is a survival strategy of many bacteria in the environment in response to adverse environmental conditions (e.g., solar radiation). There is a growing scientific interest in studying VBNC cells, including to understand novel public health implications of VBNC cells. In our simulated experiments, we are investigating alternative methods to detect bacterial viability and VBNC state, such as "live and dead staining" by fluorescence microscopy. This assay can be used to monitor the viability of bacterial populations as a function of cell membrane integrity using different fluorescent dyes.

Further experiments with "flow cytometry" could certainly be more beneficial not only to enumerate live and dead bacteria, but also to evaluate the health and viability of bacterial cells by determining the activity of bacterial oxidases and reductases.

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