Atmospheric
Chemistry
and Physics
Discussions

Interactive comment on "H$_2$O$_2$ modulates the energetic metabolism of the cloud microbiome" *by* Nolwenn Wirgot et al.

Nolwenn Wirgot et al.

a-marie.delort@univ-bpclermont.fr

Received and published: 6 October 2017

Review Comment on "H2O2 modulates the energetic metabolism of the cloud micro-biome"

General Comments This manuscript describes experiments and statistical analysis of ïňfield data that indicate that cloud bacteria have a strong impact on the loss of H2O2 from cloud water and that the bacteria exhibit depleted ATP after exposure to H2O2. The work is important because it provides additional evidence that the presence of living microorganisms in cloud water strongly affects the chemistry of the cloud water with implications for cloud processing and downstream outcomes. This work is novel and of high quality. I have provided speciïňAc and technical comments below.

C1

Answer: First of all we would like to thank Referee #1 for his great interest in our work and for all the remarks he made to improve the manuscript, including corrections of the English language. Changes are highlighted in yellow in the revised manuscript (see supplementary file)

SpeciïňAc Comments:

Comment: Line 32: change to "formation and fate" to indicate formation and degrada-tion may be affected C1 Answer: Ok, done

Comment: Line 76, 235: Here and elsewhere, I would suggest eliminating the use of "microïňĆora" and use either "microbial community", "microorganisms", or "micro-biome" Answer: Ok, done

Comment: Line 114: 10481 g should be rounded to realistic signiïňAcant ïňAgures Answer: Replaced by "around 10000 g"

Comment: Line 116 – 118: Please add a citation for the technique Answer: Marie, D., Brussaard, C.P.D., Partensky, F., Vaulot, D. : Flow cytometric analysis of phytoplankton, bacteria and viruses, Robinson, J.P., Ed. Curr. Protoc. Cytom., John Wiley & Sons, 11.11, 1-15, 1999.

Comment: Line 120: use "... cloud water solution..." Answer: Ok, done

Comment: Line 127: Please brieïňĆy state how the pH was adjusted. Answer: This sentence was added lines 156-157 "Finally, the obtained solution was adjusted to pH 6 as necessary with a few drops of the solutions of NaOH or H2SO4 used for the preparation of the marine artificial cloud water solution".

Comment: Line 150-151: Please clarify that the H2O2 and iron complex were added at in situ cloud water concentrations, but all other constituents and bacteria were added at 10x the in situ concentration as stated in Lines 128-131. How does this concen-tration discrepancy affect the overall chemical reactivity of the cloud water medium as compared to in situ cloud water? How does this difference affect the activity of the

microbes? Is there any concern that the microorganisms would be less stressed or vulnerable under the artïïňĄcial conditions than actual cloud water conditions?

Answer: As stated in Lines 199-202 of the original manuscript: "Hydrogen peroxide and iron complex (Fe-[EDDS]) were added or not to the solution in the incubators. These two compounds are present in marine cloud water collected at the PUY station at average concentrations of 7.5 μM (with a dispersion of mean values ranging from 0.1 − 20.8 μM) for H2O2 and 0.5 μM (with a dispersion of mean values ranging from BDL. ïĂ■ 4.9) for Fe(III) (Deguillaume et al., 2014)". Therefore the concentrations used here for marine cloud water are thus compatible with real values at the PUY station when multiplied by a factor ten (20 μM for H2O2 and 4 μM for Fe(III) complex).

We have moved this paragraph to the Material and Method section lines 126-135. Of course any change in the concentrations can affect cloud metabolism, we show here that the major factor impacting ATP content is H2O2 while the presence of Fe(III)-EDDS does not modify this effect to a great extent. H2O2 concentration can indeed vary with atmospheric scenarios as stated in the introduction and discussion. This is what we have demonstrated from statistical analyses (Figure 4 and p values), ATP concentrations are correlated to H2O2 concentrations.

Comment: Line 164-166: This passage is not very clear with respect to language and technical aspects and needs to be re-written. What is "afïňĆine function"? Answer: It is actually an "affine "function (mathematical function)

Comment: Line 166: Clarify how the initial degradation rate was calculated. Via the ïňĄrst two time points? Or other?

Answer: The text was changed as follows lines 187-190: The processing of data was done with the Origin 6.1 software. The graphs representing the hydrogen peroxide concentration decrease as a function of time were plotted. The degradation rates have been calculated from the initial slopes (the first five time points i.e. between 0 and 2 hours) normalized with the concentrations of cells. During these two hours no cell

growth was observed.

Comment: Line 174-175: What is the ïňĄxed part of the sampler? What alcohol was used? How does alcohol vapor affect cloud water chemistry as the samples are collected?

Answer: Only the metal sheet is disinfected by alcohol (70%) and washed with sterile water consequently alcohol has no impact on cloud water chemistry. The collector itself is not treated with alcohol and is autoclaved and kept sterile until use. The text was modified as follows lines 194-196: The detachable part of the impactor was sterilized beforehand by autoclave at 121°C for 20 min and the fixed part was rinsed with alcohol at 70° and then with sterile water just before sampling.

Comment: Line 180-206: This entire passage is redundant. This passage does not represent results. Please eliminate or work relevant parts into the Introduction, Methods or Discussion.

Answer: We fully agree with the reviewer, so we have moved and merged this section with the Material and Method section lines 109-174 as follows:

2 Material and methods 2.1. Description of the microcosms Microcosms were designed to simulate as much as possible the water phase of cloud waters. They provide the opportunity to work under artificial solar light condition and also in the presence of microorganisms. For irradiation condition the bioreactor was equipped with lamps that emit UV-radiation (Sylvania Reptistar; 15 W; 6500 K; UVA (up to 30%), UVB (up to 5%)) to mimic solar light measured directly in clouds at the PUY station (Fig. SM1). The incubation flasks were Pyrex crystallizers covered with a Pyrex filter and equipped with Teflon tubes of 8 mm Ø plugged with sterile cotton, letting air and light pass while for dark conditions they were amber Erlenmeyer flasks. All incubation flasks contained 100 mL of artificial cloud solution under agitation (130 rpm). This solution was mimicking cloud chemical composition from cloud samples classified as "marine" following the work from Deguillaume et al. (2014) at the PUY station. The major part of the collected

cloud samples were classified as marine (52%) supporting our choice for the artificial cloud composition. For biotic conditions, the flasks were inoculated at 106 bacterial cells per mL (Vaïtilingom et al., 2013). The three selected bacterial strains belonging to the Gamma-Proteobacteria (Pseudomonas) and Alpha- Proteobacteria classes (Sphingomonas) were isolated from cloud water and are representative of the genera most frequently found in cloud water samples (Vaïtilingom et al., 2012) collected at the PUY site. Depending on the conditions, hydrogen peroxide and iron complex (Fe-[EDDS]) were added or not to the solution in the incubators. These two compounds are present in marine cloud water collected at the PUY station at average concentrations of 7.5 μM (with a dispersion of mean values ranging from 0.1 – 20.8 μM) for H2O2 and 0.5 μM (with a dispersion of mean values ranging from BDL. – 4.9) for Fe(III) (Deguillaume et al., 2014). In the cloud aqueous phase, Fe(III) may be complexed by organic compounds. Recently, it has been hypothesized than iron can be chelated by other organic ligands of biological origin (Herckes et al., 2013; Herrmann et al., 2015), and in particular by siderophores (Vinatier et al., 2016) that are ligands characterized by high complexing constants (K>1020). Fe-[EDDS] was chosen as an iron(III) complex model because this ligand has a complexing constant for iron very close to the values for siderophores. Moreover, it is known to be stable at the working pH of 6.0 and because its chemistry has been studied in details by Li et al. (2010). In addition, the working temperature was fixed at 17°C which is the average temperature of cloud samples in summer.

2.2 Bacterial strains and growth conditions Pseudomonas graminis, 13b-3, DQ512786; Pseudomonas syringae, 13b-2, DQ512785, Sphingomonas sp., 14b-5, DQ512789 were grown in 10 mL of R2A medium (Reasoner and Geldreich, 1985) under stirring (200 r.p.m) at 17°C for approximately 17 h, 24 h or 48 h, depending on the strain. Cells in the exponential growth phase were collected by centrifugation for 3 min at around 10000 g. The supernatant was removed and the bacterial pellet was suspended and washed twice with an artificial cloud solution (2.2). The bacterial cell concentration was estimated by optical density at 575 nm to obtain a concentration close to 106 cell mL-1.

Finally, the concentration of cells was precisely determined by flow cytometry analysis (BD Facscalibur Becton-Dickinson; λexc= 488 nm; λem = 530 nm) using a method based on the addition of a fluorochrome (SYBR-green) for their counting (Marie et al., 1999).

2.3 Biodegradation assays Biodegradation assays were performed in marine artificial cloud water solution that mimics real cloud conditions as described in Vaïtilingom et al. (2011). Stock solutions were prepared with the following concentrations: 200 μM for acetic acid (CH3COOH; Acros organics), 145 μM for formic acid (HCOOH; Fluka), 30 μM for oxalic acid (H2C2O4;Fluka), 15 μM for succinic acid (H6C4O4; Fluka), 800 μM for ammonium nitrate (H4N2O3; Fluka), 100 μM for magnesium chloride hexahydrate (MgCl2, 6H2O; Sigma-Aldrich), 50 μM for potassium sulfate (K2SO4; Fluka), 400 μM for calcium chloride dihydrate (CaCl2, 2H2O; Sigma-Aldrich), 2000 μM for sodium chloride (NaCl; Sigma-Aldrich), 1100 μM for sodium hydroxide (NaOH; Merck), 315 μM for sulfuric acid (H2SO4; Sigma-Aldrich). Finally, the obtained solution was adjusted to pH 6 as necessary with a few drops of the solutions of NaOH or H2SO4 used for the preparation of the marine artificial cloud water solution and sterilized by filtration (Polyethersulfone membrane, 0.20 μm; Fisher Scientific) before use. The artificial cloud water solution was ten times more concentrated than a real cloud water solution in order to stabilize the pH. This was also the case for bacteria concentration because the bacteria/substrate ratio should be kept identical to that of real cloud. Indeed, it has been demonstrated that if this ratio is maintained, the degradation rate remains constant (Vaïtilingom et al., 2010). The equipment was sterilized by autoclaving at 121°C for 20 minutes and all manipulations were performed under sterile conditions. Biodegradation assays were performed in marine artificial cloud solutions inoculated with bacterial cells and incubated in a bioreactor (Infors HT Multitron II) at 17°C in the presence or absence of hydrogen peroxide solution, of iron complex solution and under irradiation or obscurity condition. At regular intervals, samples were taken and stored at -20 °C. Hydrogen peroxide solution was prepared from a commercial solution (H2O2, 30%; not stabilized Fluka Analytical). 1:1 stoichiometry iron complex solution

was prepared from iron (III) chloride hexahydrate (FeCl3, 6H2O; Sigma-Aldrich) and from (S,S)- ethylenediamine-N,N'-disuccinic acid trisodium salt (EDDS, 35% in water). The hydrogen peroxide solution and the iron complex solution were freshly prepared before each experiment and the final working concentrations were fixed at 20 μM and 4 μM respectively, in agreement with the real concentrations detected in samples collected at the PUY station multiplied by a factor ten when median values measured in marine cloud waters are considered (Deguillaume et al., 2014).

Comment: Line 220-221: Redundant. Answer: We agree with the referee, it was changed line 231 by "For the biotic conditions, the initial biodegradation rates are summarized in Table 1(b)."

Comment: Line 222-225: Is there any signiïňĄcance to the fact that the Sphingomonas isolate is less active on H2O2 or that Sphingomonas and Pseudomonas 13b-2 seem not to recover with respect to the ATP concentration as well as Pseudomonas 13b-3? Could the authors discuss further?

Answer: Of course each individual strain can behave slightly differently, the tested strains here are model strains. In principle as Sphingomonas are well represented in the cloud microbiome this could impact the whole system. However we have shown that the H2O2 biodegradation rates measured here are within the same order of magnitude as those measured with real cloud water (Vaitilingom et al 2013), so it proves that this impact is not so high. In addition, concerning the ATP concentrations, our in-lab experiments are validated by the statistical analyses performed with the 37 cloud events (figure 4). Also the growth rate of Sphingomonas is not changed in the presence of H2O2 (Figure 3). In conclusion the differences between Pseudomonas and Sphingomonas have no major consequence on the global response of the system.

Comment: Line 248: Which previous conditions are referred to here?

Answer: We refer to the experiments in the presence of H2O2 alone. The sentence has been modified lines 257-259 as follows: Complementary experiments were performed

with incubations of the cells in the presence or absence of light and/or iron complex (Fe-[EDDS]) under conditions similar to that described previously in the presence of H2O2 alone.

Comment: Line 263-275: This passage is either restating the Methods, or should be moved to the Methods. The Methods should include how data were collected and how statistical analyses were performed. Here it might be better to discuss the ïňĄnal set of data that resulted – i.e. Line 268 – 269 where it is explained how many events were selected for use. Then followed by the presentation of the PCA results. Also, here and in the Methods it would be good to state how many sampling events were available. Then it could be stated that 37 events (of xx total) were selected after the constraints (e.g. no more than 10 percent of missing values) were applied. Line 268: It is not entirely clear exactly what the 10 percent refers to. Does this mean that no more than 10 percent of data for any speciïňĄc sample or any speciïňĄc parameter was missing?

Answer: We took into account the referee's remark and moved this paragraph to the methods section lines 192-210 as follows:

"2.6 Cloud sampling and statistical analysis Cloud water sampling was performed on the summit of the PUY station (summit of the puy de Dôme, 1465 m a.s.l., France) which is part of the atmospheric survey networks EMEP, GAW, and ACTRIS. The detachable part of the impactor was sterilized beforehand by autoclave at 121°C for 20 min and the fixed part was rinsed with alcohol at 70° just before sampling. Between 2004 and 2013, 89 cloud events were collected at the PUY station. The origin of these clouds can be analyzed according to their back trajectories in four sectors (North/West, South/West, West and North/East). They can be also considered in four different categories considering their chemical composition (marine, continental, highly marine and polluted) as described in Deguillaume et al. (2014). Various parameters were measured including ATP, bacteria and fungi concentration, inorganic and organic species concentration (H2O2, SO42-, NO3-, Cl-, acetate, formate, oxalate, Na+, NH4+, Mg2+, K+, Ca2+), temperature and pH (see Table SM1 for details). More information about

the cloud sample collection is given in Deguillaume et al. (2014). These data were used in this study to achieve statistical analyses. R software 3.1.2 was used to carry out principal component analysis (PCA). The data of 37 cloud events (of 89 total) were selected after the constraints related to this statistical analysis (e.g. the cloud events with more than 10 percent of missing values (parameters) were not considered) were applied. In addition, statistical significance test was evaluated using PAST software (Hammer et al., 2001). Mean difference was considered to be statistically significant for a p-value less than 0.05."

Comment: Line 310: Since the speciïňĄc transcriptomic /metabolomic response of the microorganisms was not determined, the authors should indicate that the organisms "likely" or "probably" responded to the conditions using the mechanisms stated.

Answer: We agree with the referee, this is only a hypothetical mechanism. The text has been changed as follows lines 312-315:

This reveals that microorganisms are able to manage the stress induced by H_2O_2 through their metabolism. It is likely that they could respond using enzymes involved in H_2O_2 degradation (e.g. catalases, peroxidases, etc.) and other typical antioxidant molecules (glutathione, etc.).

Comment: Line 324: avoid "very" and other qualitative wording Answer: OK changed to "high"

Comment: Line 327-332: This passage is not clear. Do you mean that formate metabolism could be inhibited by presence of H_2O_2? Please expand this discussion a little more to make the intended points.

Answer: We agree it was not clear enough, so we have added this sentence Lines 336-338:

"Indeed formate contributes to the anti-oxidant strategy of this bacterium to supply NADH which is known to be decreased under oxidative conditions, formate helps thus

to control the cellular redox potential (see Fig. 5)."

Comment:Table 1: What is the rationale for the number of signiïňĄcant ïňĄgures shown in each case. Should they be different for different data sets? Answer: Sorry but I do not understand this question.

Comment: Line 333-334 and Figure 6 legend: Please edit to indicate that this is a hypothesized mechanism. Since the actual response of cells was not measured, these mechanisms cannot be known with certainty.

Answer: We agree with the reviewer. The text and the Fig. 5 legend have been changed as follows:

Lines 318-322. "Fig. 5 illustrates how H_2O_2 could affect the concentration of ATP in the cells. First H_2O_2 could directly inhibit the ATP synthase, a membrane protein synthetizing ATP from ADP (Tamarit et al 1998). Second H_2O_2 could impact different metabolic pathways which are interconnected including glutathione metabolism, glycolysis, TCA cycle and DNA repair system."

Legend: Figure 5: Hypothetical mechanism that could explain the impact of H_2O_2 on cell metabolism and ATP concentration. Interconnection between ATP synthesis and cellular redox potential (NAD+/NADH, NADP+/NADPH ratios).

Comment: Line 342: It would be good to examine the response of the organisms on a transcriptomic basis as well to conïňĄrm what genes are expressed in response to the H_2O_2 stress.

Answer: This is a good suggestion; we have changed the text line 346 as follows: To go further in the understanding of the modulation of the metabolic pathways (including carbon, nitrogen, amino-acids or sugars) induced by H_2O_2, a combined metabolomic and transcriptomic approach could be used.

Comment :Technical Corrections: Answer: We thank the reviewer for these valuable corrections. Changes have been made in the revised manuscript.

Line 40: use "parameters" Line 60: use "...A few decades ago, living microorganisms were observed in cloud water..." Line 62: use "nutrient" Line 64: change "Few" to "Several" Line 69: "...to efﬁciently degrade..." Line 70: eliminate "to" and "to the" Line 70: eliminate "have" Line 79: use "radiation" Line 81: eliminate the ﬁrst occurrence of "the" Line 87: eliminate "the" Line 88: instead of "Thanks to the fact that..." use "Because..." Line 90: eliminate "the" Line 91: eliminate the ﬁrst occurrence of "of" Line 104-106: This sentence should be re-written. Something like "It is crucial to considerall sinks and sources of H2O2, especially in atmospheric chemistry models, since H2O2 impacts many relevant processes in the atmosphere." Line 114: "g" should be italicized Line 121: eliminate the space after "concentrations" Line 129-130: use "...the bacterial cell concentration..." Line 134: replace "consisted" with "were performed" Line 139: add a space between the number value and the unit Line 142 and elsewhere: use "rpm" Line 164-166: This passage is not very clear with respect to language and technical aspects and needs to be re-written. What is "afine function"? Answer: "affine function" Line 187-190: The processing of data was done with the Origin 6.1 software. The graphs representing the hydrogen peroxide concentration decrease as a function of time were plotted. The degradation rates have been calculated from the initial slopes (the first five time points i.e. between 0 and 2 hours) normalized with the concentrations of cells

Line 168: Eliminate "The". Add the company for R. Line 170: use "less than" instead of "inferior" Line 174: use "sterilized beforehand"; replace "during" with "for" Line 232 and elsewhere: use "within the same order of magnitude" Line 233: replace "than" with "of" Line 234: use "...separately analyze..." Line 76, 235: Here and elsewhere, I would suggest eliminating the use of "microﬂora" and use either "microbial community", "microorganisms", or "microbiome" Line 235: use "clouds" Line 236: eliminate both "the"s Line 245: use "strain" Line 254-257: Redundant and restates methods. Eliminate the ﬁrst two sentences and replace the next two with something like "Results for the number of culturable bacteria in the presence or absence of H2O2 are shown in Figure 3. " Line 260: replace "was multiplied" with "increased" Line 287:

comma after "ATP" Line 288: use "less than" instead of "inferior" Line 292: replace "as" with "since" Line 304: replace "to" with "at" Line 312: use "reported" Line 330: eliminate the second occurrence of "the" Figure and Tables For ﬁgures and tables, I would suggest using the following wording: "Values shown are averages of triplicates plus/minus one standard deviation" "Symbols are averages of triplicates and error bars represent the standard error. Where error bars do not appear they are smaller than the symbol"

Please also note the supplement to this comment:
https://www.atmos-chem-phys-discuss.net/acp-2017-581/acp-2017-581-AC1-supplement.pdf