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H_2O_2 modulates the energetic metabolism of the cloud microbiome

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Abstract. Chemical reactions in clouds lead to oxidation processes driven by radicals (mainly $HO^\bullet$, $NO_3^\bullet$, or $HO_2^\bullet$) or strong oxidants such as H_2O_2, O_3, nitrate, and nitrite. Among those species, hydrogen peroxide plays a central role in the cloud chemistry by driving its oxidant capacity. In cloud droplets, H_2O_2 is transformed by microorganisms which are metabolically active. Biological activity can therefore impact the cloud oxidant capacity. The present article aims at highlighting the interactions between H_2O_2 and microorganisms within the cloud system.

First, experiments were performed with selected strains studied as a reference isolated from clouds in microcosms designed to mimic the cloud chemical composition, including the presence of light and iron. Biotic and abiotic degradation rates of H_2O_2 were measured and results showed that biodegradation was the most efficient process together with the photo-Fenton process. H_2O_2 strongly impacted the microbial energetic state as shown by adenosine triphosphate (ATP) measurements in the presence and absence of H_2O_2. This ATP depletion was not due to the loss of cell viability. Secondly, correlation studies were performed based on real cloud measurements from 37 cloud samples collected at the PUY station (1465 m a.s.l., France). The results support a strong correlation between ATP and H_2O_2 concentrations and confirm that H_2O_2 modulates the energetic metabolism of the cloud microbiome. The modulation of microbial metabolism by H_2O_2 concentration could thus impact cloud chemistry, in particular the biotransformation rates of carbon compounds, and consequently can perturb the way the cloud system is modifying the global atmospheric chemistry.

1 Introduction

The atmosphere is an oxidizing medium where trace gases are transformed/removed by oxidation including methane and other organic compounds, carbon monoxide, nitrogen oxides, and sulfur gases. Evaluating the oxidizing power of the atmosphere is crucial since it controls pollutant formation and fate, aerosol production, and greenhouse radiative forcing (Thompson, 1992).

In this context, hydroperoxides (ROOH) contribute to the oxidizing power of the troposphere (Lee et al., 2000; Herrmann et al., 2015) by controlling the cycling of HO_x radicals ($HO^\bullet$, $HO_2^\bullet$). They can serve as temporary reservoirs of HO_x radicals since, for example, their photolysis and reactivity will regenerate $HO^\bullet$ radicals. Among hydroperoxide, hydrogen peroxide is a key gas-phase atmospheric chemical species (Vione et al., 2003), with concentrations in the gas phase at the ppbv level or less. The atmospheric concentration of H_2O_2 is impacted by a variety of meteorological parameters (e.g., actinic flux, temperature, and relative humidity) and is affected by the levels of chemical species such as VOCs, CO, O_3, and NO_x (Lee et al., 2000). One of the significant parameters controlling the evolution of H_2O_2 concentration is the actinic flux intensity. Diurnal and seasonal variations of hydrogen peroxide are shown by field measurements with higher concentrations during the day and in summer than during the night and in winter. This is linked to the atmospheric formation of H_2O_2 that results from a series of photochemical reactions creating free radicals followed by corresponding radical reactions with appropriate precursor substances.

In the presence of atmospheric liquid water (cloud, fog, rain), H_2O_2 is rapidly dissolved because of its high Henry

law constant (7.7×10^4 M atm^{-1} at 298 K; Sander, 2015). In this liquid phase, it is also produced by aqueous-phase reactivity (Möller, 2009). Several field campaigns have reported H_2O_2 concentrations in atmospheric water in the µM range (Gunz and Hoffmann, 1990; Marinoni et al., 2011; Deguillaume et al., 2014; Li et al., 2017). Hydrogen peroxide plays a central role in various important chemical processes in clouds. First, H_2O_2 is considered the most important oxidant for the conversion of sulfite to sulfate for pH lower than 5.5, thereby contributing significantly to the acidification of clouds and precipitations (Deguillaume et al., 2005; Shen et al., 2012). Second, the photolysis of H_2O_2 will lead to an efficient production of the hydroxyl radical $HO^\bullet$ (Arakaki et al., 2013), and recent studies have shown that this can be a dominant aqueous source (Bianco et al., 2015). They can also directly oxidize organic compounds in the aqueous phase (Schöne and Herrmann, 2015). Finally, H_2O_2 is involved in redox processes leading to the conversion of reactive free radicals and trace metals such as iron (Kieber et al., 2001; Deguillaume et al., 2005; Hems et al., 2017). Consequently, H_2O_2 is a key chemical compound controlling the aqueous-phase oxidant capacity and leading to the transformation of inorganic and organic compounds present in the atmospheric aqueous phase. The resulting inorganic and organic products can contribute to the aerosol phase when the cloud evaporates, leading to a climatic effect.

A few decades ago, living microorganisms were observed in cloud water (Sattler et al., 2001; Amato et al., 2005, 2007a, b; Wei et al., 2017). Particularly through measurements of adenosine triphosphate (ATP) and anabolic precursors or nutrient incorporation rates, it has been shown that cloud microorganisms are metabolically active and play an important role in cloud chemical reactivity (Sattler et al., 2001; Amato et al., 2007a; Hill et al., 2007; Vaïtilingom et al., 2012, 2013). Several studies performed on simplified or real microcosms have demonstrated that cloud microorganisms are able to degrade carbon compounds (Ariya et al., 2002; Amato et al., 2005, 2007c; Husarova et al., 2011; Vaïtilingom et al., 2010, 2011, 2013; Matulovà et al., 2014); recent studies have also shown that this could be the case in the air (Krumins et al., 2014).

Microorganisms are also in direct interaction with oxidant species in clouds (iron, hydroxyl radical, hydrogen peroxide, etc.). Vaïtilingom et al. (2013) have demonstrated that microorganisms present in real cloud water are able to efficiently degrade hydrogen peroxide. This suggests that cloud microorganisms found strategies to survive and resist stresses encountered in this medium, and in particular oxidative stress. In this context, Joly et al. (2015) have conducted laboratory experiments to investigate the survival of selected strains (bacteria and yeasts) isolated from cloud waters, in the presence of various concentrations of hydrogen peroxide. The results showed that the survival rates of the studied strains were not affected by H_2O_2 exposure. In addition, the strains were exposed to artificial UV-visible light mimicking the natural solar irradiation inside clouds. No significant impact on the survival of the bacterial strains was observed.

These results have been confirmed in real cloud water, including the microbial community and chemical complexity (iron, H_2O_2, etc.), incubated in a photo-bioreactor designed to mimic cloud conditions (Vaïtilingom et al., 2013). Thanks to ADP/ATP ratio measurements, reflecting the energetic metabolism of microorganisms, exposed or not to solar radiation, it has been shown that microorganisms were not impacted by artificial light and consequently by the generation of radicals from H_2O_2 photo-reactivity. In addition, H_2O_2 is efficiently degraded by catalases and peroxidases involved in oxidative metabolism. Solar light did not modify the degradation rates of H_2O_2, demonstrating that the biological process was not inhibited by UV radiations and radicals.

Indeed, solar light can indirectly impact the viability of cells by the production of reactive oxygenated species (ROS), including $HO^\bullet$ and $O_2^{\bullet-}$ radicals. The main sources of these radicals are H_2O_2 photolysis or Fenton and photo-Fenton reactions involving iron (Fe) and H_2O_2. Most of these compounds can cross the cytoplasmic membrane by diffusion. Aerobic microorganisms can also produce similar ROS during respiration. These radicals can potentially damage major cellular components such as proteins, DNA, and lipids and lead to cellular death. Because microorganisms are usually protected against these ROS, they can specifically modify their metabolism to face oxidative stress taking place in clouds. Therefore, microorganisms utilize various mechanisms involved in oxidative stress metabolism such as (i) the production of pigments and antioxidant molecules (vitamins, glutathione, etc.) which can scavenge radicals or (ii) the production of specific enzymes such as superoxide dismutase which can transform $O_2^{\bullet-}$ into H_2O_2. H_2O_2 can be dismutated or reduced, respectively, by catalases and other peroxidases (Delort et al., 2017).

The studies from Vaïtilingom et al. (2013) and Joly et al. (2015) highlighted the interactions between biological activity and oxidants in clouds. It is crucial to consider all sinks and sources of H_2O_2, especially in atmospheric chemistry models, since H_2O_2 impacts many relevant processes in the atmosphere. In the present work, we artificially reproduced cloud conditions in microcosms to study the biotic and abiotic transformation of H_2O_2 and, conversely, the impact of hydrogen peroxide on the metabolism of cloud microorganisms.

For this purpose, we decided to study individually the effect of parameters interacting with H_2O_2: UV radiation, iron, and bacteria. Under various experimental conditions, the degradation rates of H_2O_2 were followed to highlight how individual parameters control its transformation. Moreover, the impact of H_2O_2 on the energetic state of the bacterial cells was evaluated by measuring the ATP concentration over time when the cells were exposed or not to H_2O_2. In order to confirm our laboratory results on the interaction between microorganisms and H_2O_2, we performed a corre-

lation analysis considering bio-physico-chemical parameters measured in real cloud samples collected at the PUY station. This work will lead to a better description of the mechanisms linking biological activity and cloud reactivity.

2 Material and methods

2.1 Bacterial strains and growth conditions

Pseudomonas graminis, 13b-3, DQ512786, *Pseudomonas syringae*, 13b-2, DQ512785, and *Sphingomonas* sp., 14b-5, DQ512789 were grown in 10 mL of R2A medium (Reasoner and Geldreich, 1985) under stirring (200 rpm) at 17 °C for approximately 17, 24, or 48 h, depending on the strain. 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.

Cells in the exponential growth phase were collected by centrifugation for 3 min at around 10 000 *g*. The supernatant was removed and the bacterial pellet was suspended and washed twice with an artificial cloud solution and incubated in microcosms to perform biodegradation experiments (see Sect. 2.2). The bacterial cell concentration was estimated by optical density at 575 nm to obtain a concentration close to 10^6 cell mL^{-1}. Finally, the concentration of cells was precisely determined by flow cytometry analysis (BD Facscalibur – Becton-Dickinson; $\lambda_{exc} = 488$ nm; $\lambda_{em} = 530$ nm) using a method based on the addition of a fluorochrome (SYBR Green) for their counting (Marie et al., 1999).

2.2 Incubations in 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 conditions and also in the presence of microorganisms. The experiments were performed under bulk conditions as cloud droplets cannot be reproduced in these bioreactors (Infors HT Multitron II).

For irradiation conditions the bioreactor was equipped with lamps that emit UV radiation (Sylvania Reptistar; 15 W; 6500 K) to mimic solar light measured directly in clouds at the PUY station (Fig. S1 in the Supplement). 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 (see Vaïtilingom et al., 2013), while for dark conditions they were amber Erlenmeyer flasks.

All incubation flasks contained 100 mL of artificial cloud solution under agitation (130 rpm); its composition was first described in Vaïtilingom et al. (2011). 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. Stock solutions of this artificial cloud medium were prepared with the following concentrations: 200 µM for acetic acid (CH_3COOH; Acros organics), 145 µM for formic acid (HCOOH; Fluka), 30 µM for oxalic acid ($H_2C_2O_4$; Fluka), 15 µM for succinic acid ($H_6C_4O_4$; Fluka), 800 µM for ammonium nitrate ($H_4N_2O_3$; Fluka), 100 µM for magnesium chloride hexahydrate ($MgCl_2$, $6H_2O$; Sigma-Aldrich), 50 µM for potassium sulfate (K_2SO_4; Fluka), 400 µM for calcium chloride dihydrate ($CaCl_2$, $2H_2O$; Sigma-Aldrich), 2000 µM for sodium chloride (NaCl; Sigma-Aldrich), 1100 µM for sodium hydroxide (NaOH; Merck), and 315 µM for sulfuric acid (H_2SO_4; Sigma-Aldrich). Finally, the obtained solution was adjusted to pH 6 as necessary with a few drops of the solutions of NaOH or H_2SO_4 used for the preparation of the marine artificial cloud water solution and sterilized by filtration (polyethersulfone membrane, 0.20 µm; Fisher Scientific) before use.

For biotic conditions, the flasks were inoculated at 10^6 bacterial cells per mL. The artificial cloud water solution was 10 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 a 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 min and all manipulations were performed under sterile conditions.

Depending on the conditions, hydrogen peroxide and an 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 to 20.8 µM) for H_2O_2 and 0.5 µM (with a dispersion of mean values ranging from BDL – below detection limit – to 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), which are ligands characterized by high complexing constants ($K > 10^{20}$). 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 because its chemistry has been studied in detail by Li et al. (2010).

Hydrogen peroxide solution was prepared from a commercial solution (H_2O_2, 30 %, not stabilized; Fluka Analytical); 1 : 1 stoichiometry iron complex solution was prepared from iron (III) chloride hexahydrate ($FeCl_3$, $6H_2O$; 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 and 4 µM, respectively, in agreement with the real concentrations detected in samples collected at the PUY station multiplied by a factor of 10 when median values measured in marine cloud waters are considered (Deguillaume et al., 2014).

In addition, the working temperature was fixed at 17 °C, which is the average temperature of cloud samples in summer. For all the incubation conditions, samples were taken at regular intervals, and stored at −20 °C before analysis.

2.3 Analyses

Hydrogen peroxide was quantified with a miniaturized Lazrus fluorimetric assay (Lazrus et al., 1985; Vaïtilingom et al., 2013). This method is based on a reaction between hydrogen peroxide, horseradish peroxidase (HRP), and 4-hydroxyphenylacetic acid that produces a fluorescent dimeric compound. Fluorescence readings (Safire II TECAN©; $\lambda_{exc} = 320$ nm, $\lambda_{em} = 390$ nm) were made in a 96 well plate format.

Bioluminescence was used to analyze adenosine triphosphate (ATP) concentrations (Glomax® 20/20 single tube luminometer from Promega). This technique is based on an enzymatic reaction involving luciferin and luciferase. The protocol used was adapted from Biothema© commercial kit instructions (Koutny et al., 2006).

In order to determine the survival rate of microorganisms in the presence of hydrogen peroxide (20 µM), plate counts were performed on an R2A agar medium at the beginning of each experiment and after 3, 7, and 24 h of incubation. Plates were incubated for 3 days at 17 °C before CFU counts.

2.4 Determination of the initial degradation rates of hydrogen peroxide

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 h) normalized with the concentrations of cells. During these two hours no cell growth was observed.

2.5 Cloud sampling and statistical analysis

Cloud water sampling was performed at the summit of the PUY station (summit of the Puy de Dôme, 1465 m a.s.l., France), which is part of 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° and then with sterile water 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 (northwest, southwest, west, and northeast). They can also be 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 (H_2O_2, SO_4^{2-}, NO_3^-, Cl^-, acetate, formate, oxalate, Na^+, NH_4^+, Mg^{2+}, K^+, Ca^{2+}), temperature, and pH (see Supplement Table S1 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 in total) were selected after the constraints related to this statistical analysis (e.g., the cloud events with more than 10 % of missing values (parameters) were not considered) were applied.

In addition, a statistical significance test was evaluated using the PAST software (Hammer et al., 2001). The mean difference was considered to be statistically significant for a p-value less than 0.05.

3 Results

The interactions between H_2O_2, which is one of the major oxidants present in clouds, and microorganisms were investigated by performing experiments in artificial cloud microcosms, but also by considering chemical and biological parameters measured in real cloud samples over a long period at the PUY station.

3.1 Experiments in artificial cloud water microcosms

Experiments were conducted in microcosms mimicking cloud conditions in which all important parameters, including H_2O_2, iron, light, and the presence of bacteria, could be studied individually or in complementarity.

3.1.1 Hydrogen peroxide degradation in artificial cloud water

H_2O_2 degradation was monitored periodically over a 8 h period. The kinetic profiles were similar for the three strains. Results obtained with *Pseudomonas graminis* (13b-3) are illustrated in Fig. 1, whereas the results obtained with the other strains are presented for information in Fig. S2.

Under abiotic conditions (Fig. 1, orange traces), the degradation of hydrogen peroxide is clearly effective in the presence of artificial solar light and an Fe-[EDDS] complex, due to the photo-Fenton reaction, with an initial degradation rate of 1.07×10^{-9} mol L^{-1} s^{-1} (Table 1a). After 150 min this degradation rate decreases in parallel with EDDS by oxidation (Li et al., 2010). In the presence of the Fe-[EDDS]

Table 1. Initial rates of abiotic degradation **(a)** and biotic degradation **(b)** of H_2O_2 measured in artificial cloud water. Values are expressed in 10^{-9} mol L^{-1} s^{-1}. Standard errors were calculated.

(a)	Light + Fe-[EDDS]	Fe-[EDDS]	Light	
	1.07 ± 0.18	0.22 ± 0.05	0.14 ± 0.08	
(b)	Light + Fe-[EDDS] + bacteria	Fe-[EDDS] + bacteria	Light + bacteria	Bacteria
Pseudomonas graminis 13b-3	1.55 ± 0.25	1.93 ± 0.18	2.15 ± 0.02	2.07 ± 0.01
Pseudomonas syringae 13b-2	1.75 ± 0.15	1.27 ± 0.04	1.72 ± 0.14	1.18 ± 0.08
Sphingomonas sp. 14b-5	1.97 ± 0.06	1.01 ± 0.21	0.87 ± 0.04	0.76 ± 0.11

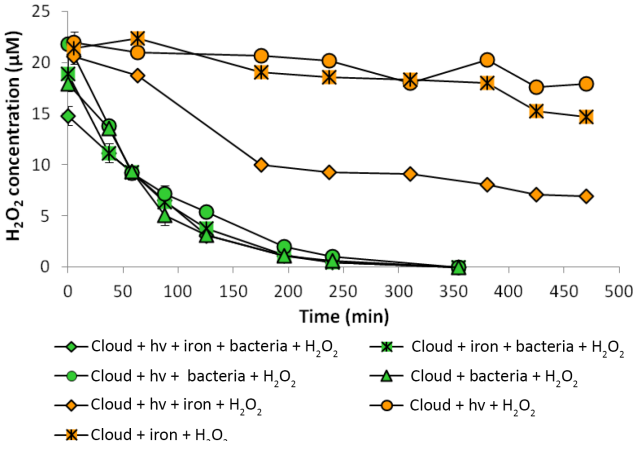


Figure 1. Evolution of H_2O_2 concentration as a function of time (min) under abiotic conditions: light + Fe-[EDDS] (orange diamond), light (orange circle), Fe-[EDDS] (orange square with black cross), and biotic conditions: light + Fe-[EDDS] + *Pseudomonas graminis* (13b-3) (green diamond), Fe-[EDDS] + *Pseudomonas graminis* (13b-3) (green square with black cross), light + *Pseudomonas graminis* (13b-3) (green circle), and *Pseudomonas graminis* (13b-3) (green triangle). Values shown are averages of triplicates plus/minus 1 standard deviation. Where error bars are not visible, they are smaller than the symbol.

complex alone and in the absence of light, hydrogen peroxide is almost not degraded. Indeed, the degradation rate of H_2O_2 due to the Fenton reaction is much lower (2.23×10^{-10} mol L^{-1} s^{-1}) than the value obtained with the photo-Fenton reaction. Exposing the microcosm only to our light conditions, the photolysis reaction of H_2O_2 is extremely slow (1.38×10^{-10} mol L^{-1} s^{-1}) due to the low absorption of H_2O_2 in the solar spectrum measured inside a cloud and that was reproduced by the lamps used for these experiments (Fig. S1).

For the biotic conditions (Fig. 1, green traces), the initial biodegradation rates are summarized in Table 1b. These results show that, under our experimental conditions, hydrogen peroxide was degraded more efficiently in the presence of bacteria even if the values obtained stay within the same order of magnitude compared to the abiotic conditions with artificial light and an Fe-[EDDS] complex. *Pseudomonas graminis* (13b-3) and *Pseudomonas syringae* (13b-2) are the most active strains, followed by *Sphingomonas* sp. (14b-5). For each strain, biodegradation rates are within the same order of magnitude without wide variations depending on the tested conditions, i.e., in the presence or absence of artificial light and of the Fe-[EDDS] complex.

The selected strains all degrade H_2O_2 within the same order of magnitude (average value for the three strains and for the condition with iron and light 1.76×10^{-9} mol L^{-1} s^{-1} and with iron without light 1.40×10^{-9} mol L^{-1} s^{-1}). In Vaïtilingom et al. (2013), the biodegradation rates of H_2O_2 were found within the same order of magnitude (average value for two distinct clouds with light 0.98×10^{-9} mol L^{-1} s^{-1} and without light 0.29×10^{-9} mol L^{-1} s^{-1}). The results obtained are within the same order of magnitude of values in a real cloud environment, thereby validating our microcosm conditions. This demonstrates that under our experimental conditions, the selected strains degrade H_2O_2 like the microbiome of real clouds. In addition, it validates our approach of separately analyzing the influence of each parameter (Fe, H_2O2, light, ...) on the microbial energetic state metabolism in an artificial marine cloud solution detailed in the next section.

3.1.2 Impact of the H_2O_2 on the microbial energetic state in artificial marine cloud solution

The impact of the presence of H_2O_2 on the energetic state of the bacterial cells was evaluated by measuring the time evolution of ATP concentration for the three strains (Fig. 2). The ATP concentration was measured in the presence (Fig. 2a, b, c – black square) or absence (Fig. 2a, b, c – white square) of H_2O_2. In the absence of H_2O_2, a strong increase in ATP concentration was observed, reflecting an active metabolism of the bacteria. In contrast, in the presence of H_2O_2, the results were clearly different and can be described in two phases. In the first phase, ATP concentration was decreasing, while in a second phase it was progressively increasing (*Pseudomonas graminis*, 13b-3) or stabilizing (*Pseudomonas syringae*, 13b-2, *Sphingomonas* sp., 14b-5). The kinetics of ATP concentration evolution and H_2O_2 degradation are closely related. As discussed earlier (Fig. 1), the H_2O_2 initially present (20 µM) was entirely degraded in approximately 3 h (depending on the strain); this corresponds exactly to the end of the ATP

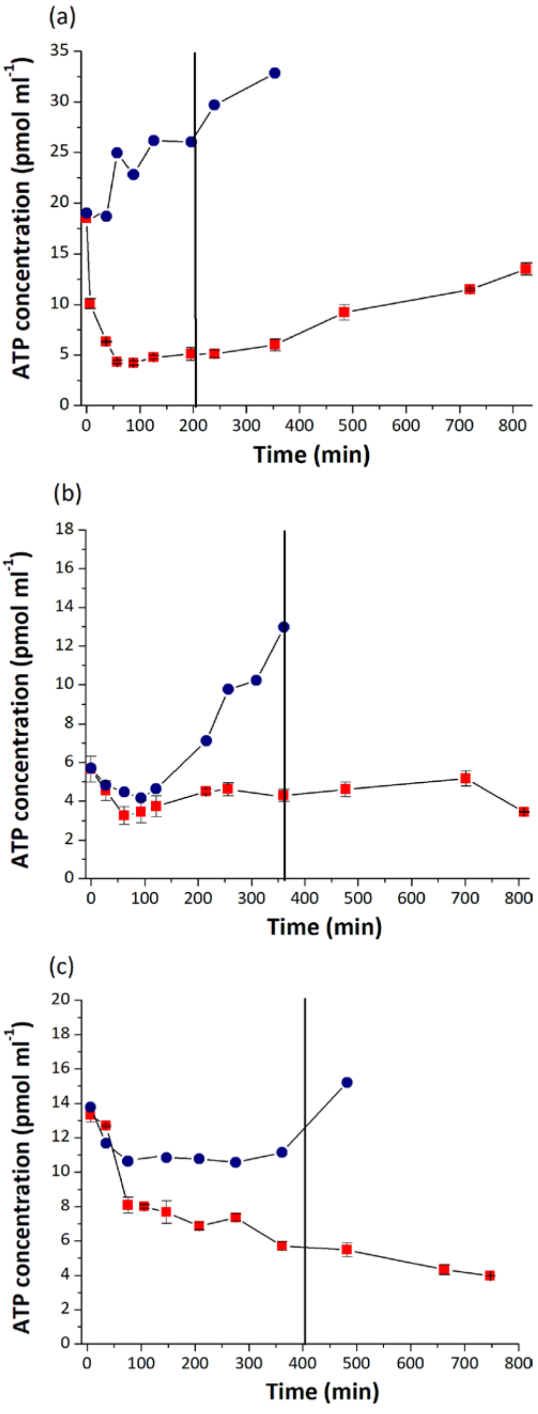


Figure 2. ATP concentration (µM) as a function of time (min) in the presence (red square) or absence (navy circle) of H_2O_2 for the three strains: **(a)** *Pseudomonas graminis* (13b-3), **(b)** *Pseudomonas syringae* (13b-2), and **(c)** *Sphingomonas* sp. (14b-5). The vertical bar illustrates the time corresponding to the total degradation of H_2O_2.

decrease. Complementary experiments were performed with incubations of the cells in the presence or absence of light and/or an iron complex (Fe-[EDDS]) under conditions similar to that described previously in the presence of H_2O_2 alone. The results obtained for the three strains are reported in Fig. SM3 (*Pseudomonas graminis*), Fig. S4 (*Pseudomonas syringae*), and Fig. S5 (*Sphingomonas* sp.).

The results show that light and iron complex have no impact on the ATP concentration decrease. The measured ATP concentration in the presence or absence of an artificial light and/or iron(III) complex is similar to that observed in the presence of H_2O_2 alone. The ATP concentration is thus only linked to the presence of H_2O_2.

3.1.3 Impact of H_2O_2 on the survival of the microbial strains

We also checked that the decrease in ATP in the presence of H_2O_2 was not due to cell mortality. Results of the number of culturable bacteria in the presence or absence of H_2O_2 are shown in Fig. 3. The evolution of the cell concentration was not significantly different when cells were incubated in the presence or absence of hydrogen peroxide. The decrease in ATP is therefore not linked to a lower concentration of cells, but to a modification of metabolic pathways due to H_2O_2 presence. The total number of cells increased by a factor of 5 to 10 after 24 h, showing that bacteria were also able to divide and grow.

3.2 Impact of H_2O_2 on the microbial energetic metabolism in real cloud environment

In the previous section, we showed that H_2O_2 had a strong impact on the energetic metabolism of cells under our microcosm conditions. To go further, we looked at the potential impact of H_2O_2 on microbial energetic states in real cloud samples by carrying out statistical analyses based on data measured on real cloud water collected at the PUY station.

For this, principal component analysis (PCA) was used. In order to perform this multivariate statistical analysis, Table S1 was built.

The result of the PCA analysis is presented in Fig. 4. The first two dimensions contain practically 50 % of the total inertia (total variance of the data table), reflecting the validity and reliability of the result. The PCA shows that if we consider all important parameters in the collected cloud samples, a strong correlation appears between ATP and H_2O_2 concentrations (longer vectors and very close on the PCA). There is no correlation between ATP concentration and the number of bacteria (vectors practically orthogonal); this shows that H_2O_2 is linked to the energetic state of the cells and not to their concentration. Also, there is no correlation between ATP and markers of pollution such as the pH values, the NO_3^-, SO_4^{2-}, and NH_4^+ concentrations, or even the temperature that could impact microbial metabolism.

In addition, a Spearman rank correlation test (non-parametric test) was performed based on the 37 cloud samples to confirm the correlation between H_2O_2 and ATP. The

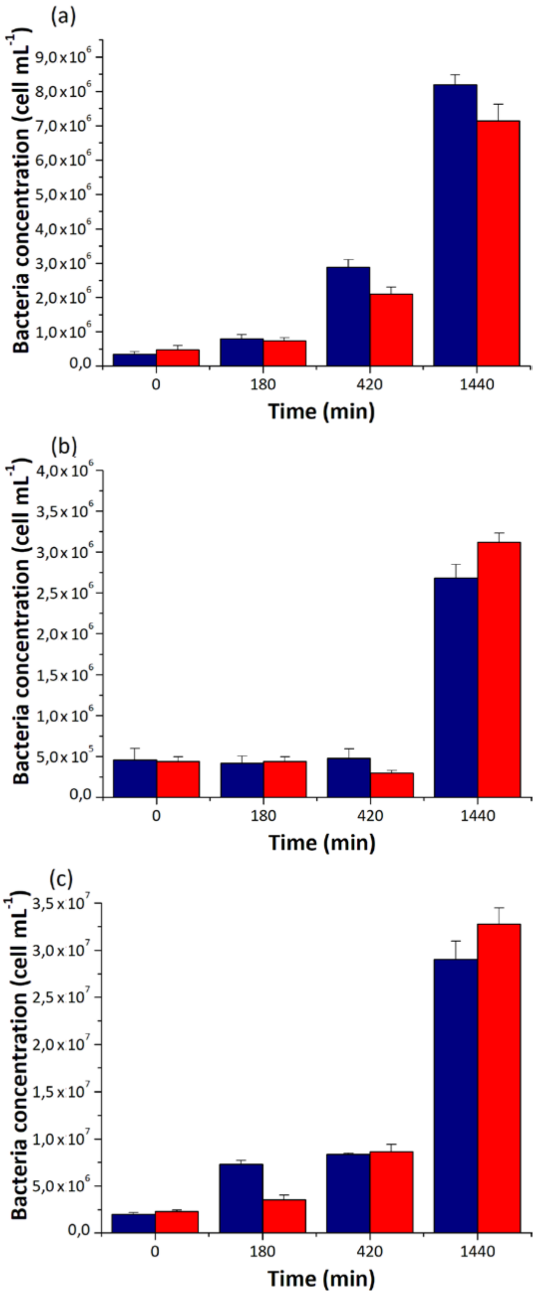


Figure 3. Bacterial cell numbers measured by plate-counting in the presence (red) and absence (navy) of H_2O_2 at 20 µM for the three strains: **(a)** *Pseudomonas graminis* (13b-3), **(b)** *Pseudomonas syringae* (13b-2), and **(c)** *Sphingomonas* sp. (14b5). Error bars represent the standard deviation from the means ($n = 3$).

values used for this test are presented in Table S1. A p-value of 0.0047 was obtained with a Spearman coefficient of 0.45 (Zar, 1972). This shows an extremely strong correlation between H_2O_2 and ATP, as theoretically the Spearman coefficient must be greater than 0.27 for 37 observations and the p-value less than 0.05 (significance threshold). To confirm that ATP depletion due to H_2O_2 impact was not linked with the

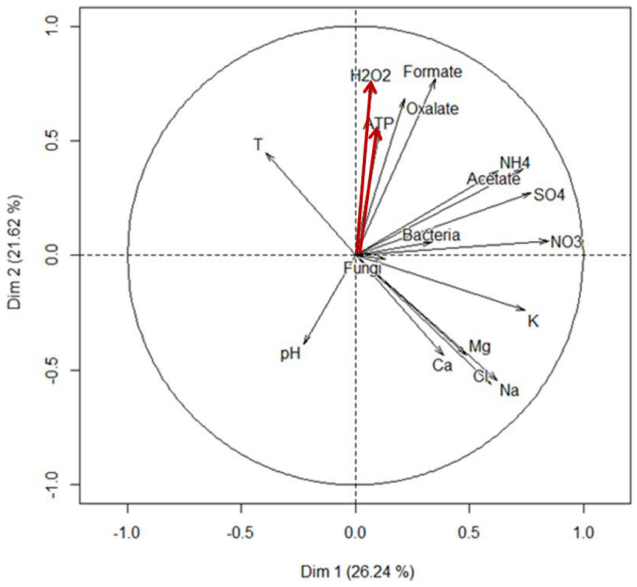


Figure 4. Variables' factor map (PCA) of the 37 cloud events on the PC1-PC2 plane based on 17 variables.

mortality of cells, a Spearman rank correlation test was also performed to evaluate the correlation between ATP and total microorganism concentrations (sum of bacteria and fungi concentrations in Table S1) (p-value superior to 0.37).

Figure 4 suggested that ATP or H_2O_2 could also be correlated with formate and oxalate since the vectors were relatively close. A Spearman rank correlation test (non-parametric test) was thus performed based on data extracted from the 37 cloud samples (Table S1). A strong correlation was obtained between ATP and formate (p-value = 0.0043, Spearman's coefficient = 0.46), and between H_2O_2 and formate (p-value = 0.00015, Spearman's coefficient = 0.58). The ATP–oxalate correlation is rather weak (p-value = 0.030, Spearman's coefficient = 0.36) and much lower than the ATP–H_2O_2 correlation; similar values were obtained for oxalate and H_2O_2 (p-value = 0.035, Spearman's coefficient = 0.35).

4 Discussion

Our objective was to study in detail the interactions between cloud microorganisms and H_2O_2.

First, we looked at the mechanisms involved in H_2O_2 transformations under laboratory conditions by isolating each parameter to determine its impact on H_2O_2 (artificial light, Fe-[EDDS] complex and bacteria). Degradation rates of hydrogen peroxide were precisely determined for different microbial strains frequently found in cloud water samples collected at the PUY site. The results show that all bacterial strains studied under these conditions degrade H_2O_2 within the same order of magnitude as abiotic conditions.

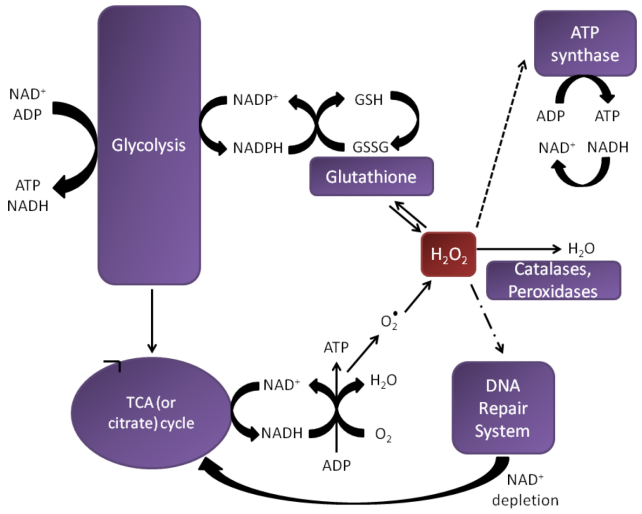


Figure 5. Hypothetical mechanism that could explain the impact of H_2O_2 on cell metabolism and ATP concentration. Interconnection between ATP synthesis and cellular redox potential (NAD^+ / NADH, $NADP^+$ / NADPH ratios). NAD^+ depletion related to the DNA repair system. Adapted from Oka et al. (2012). - - - arrows: inhibition of ATP synthase; –.–.– arrows: NAD^+ depletion related to the DNA repair system.

The degradation rates of H_2O_2 by bacteria are not impacted by the presence of light and Fe-[EDDS] and consequently by the generation of $HO^\bullet$ radicals. On the opposite end, in these laboratory experiments mimicking real cloud conditions, we have shown that H_2O_2 has a strong impact on the microbial energetic state of the cells. This strong decrease in ATP concentration is not linked to the number of cells as bacteria are able to divide and grow in the presence of H_2O_2. 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) and other typical antioxidant molecules (e.g., glutathione).

A few studies reported the decrease in ATP concentration in microorganisms (Perricone et al., 2003), plants (Tiwari et al., 2002), or mammalian cells (Spragg et al., 1985; Josephson et al., 1991; Sporn and Peters-Goldenwhen, 1988; Hyslop et al., 1988; Oka et al., 2012) exposed to H_2O_2. Figure 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 synthesizing ATP from ADP (Tamarit et al., 1998). Second, H_2O_2 could impact different metabolic pathways which are interconnected, including glutathione metabolism, glycolysis, the TCA cycle, and the DNA repair system. The functioning of the enzymes in these pathways and also the activity of the ATP synthase are dependent on the redox potential of the cells (NAD^+/NADH, $NADP^+$/NADPH ratios), and as a consequence the ATP concentration is regulated by this redox potential (Haddock and Jones, 1977; Singh et al., 2007; Oka et al., 2012). If for instance NAD^+ is depleted when the repair system is activated to avoid potential DNA damages induced by H_2O_2, then ATP is depleted, and finally all the metabolic pathways involving these compounds are impacted and a complete change in the metabolome can be expected.

The measurements preformed in microcosms do not reproduce what is really occurring in cloud droplets. First, incubations were performed with artificial cloud water and model strains; nevertheless, the obtained results were consistent with those obtained with real cloud water samples. Second, the potential growth of microorganisms during a cloud event could also modify transformation rates; this is only realistic for long cloud lifetimes (> 24 h). Finally, experiments were performed under bulk conditions and not with individual cloud droplets; only models can take into account the complexity of cloud conditions, in particular the multiphase aspect of cloud chemistry. To go further and integrate biodegradation rates into atmospheric chemistry models, complementary experiments should be performed and biodegradation rates should be expressed as $mol^{-1}\ cell^{-1}\ h^{-1}$.

However, the most important result of this work was to show the correlation between H_2O_2 concentrations and ATP concentrations. This result obtained under our microcosm conditions was confirmed using data measured in real cloud samples that experienced multiphase and real cloud conditions. Indeed, we have shown, thanks to statistical analyses, that there was also a high correlation between H_2O_2 and ATP concentrations in real cloud samples collected under various environmental conditions. We suggest thus that hydrogen peroxide modulates the global metabolism of cloud microorganisms.

Another interesting correlation was obtained between H_2O_2 and formate as well as ATP with formate. This could result from different concomitant processes. First, formate is the most oxidized carbon molecule before CO_2 generated from successive oxidations of the organic matter by radicals issued from H_2O_2. Second, it could reveal the impact of H_2O_2 on the C1 metabolism; it is known that C1 compounds can be transformed by cloud microorganisms (Husàrovà et al., 2011; Vaitilingom et al., 2010, 2011, 2013). In addition, Thomas et al. (2016) report the overproduction of formate in a strain of *Pseudomonas fluorescens* exposed to H_2O_2. Indeed, formate contributes to the anti-oxidant strategy of this bacterium to supply NADH, which is known to be decreased under oxidative conditions. Formate thus helps to control the cellular redox potential (see Fig. 5).

Finally, this work brings new insights into the interactions between H_2O_2 and the cloud microbiome and its potential consequences for cloud chemistry (see Fig. 6).

First, it confirms that cloud microorganisms are able to efficiently degrade hydrogen peroxide and potentially impact the global carbon budget and the oxidant capacity of clouds as already shown in Vaïtilingom et al. (2013). By decreasing H_2O_2 concentration, radical chemistry is less efficient

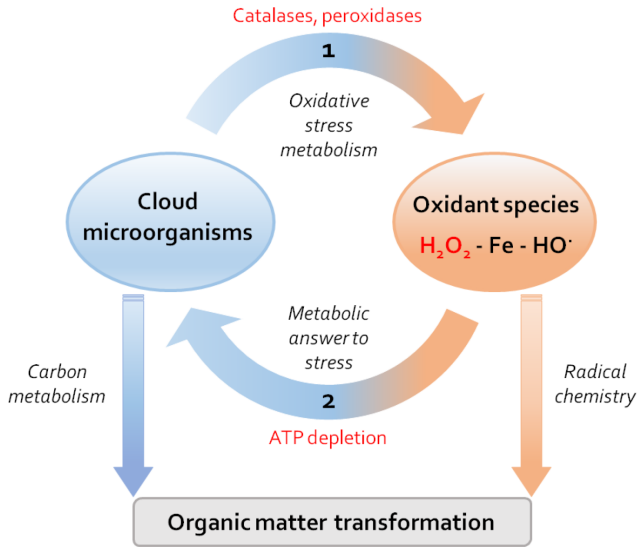


Figure 6. Interaction between H_2O_2 and cloud microorganisms and its potential consequences for atmospheric chemistry. (1) Cloud microorganisms degrade H_2O_2 thanks to their catalases and peroxidases (oxidative stress metabolism); as a result, this impacts the oxidant capacity of clouds. The concentration of radicals issued from H_2O_2 is decreased and radical chemistry is less efficient at transforming the organic matter. (2) H_2O_2 impacts the energetic metabolism of microorganisms that react to this stress. The depletion of ATP modulates the global carbon metabolism of the microorganisms, and consequently the transformation of the organic matter. These processes are modulated by the H_2O_2 concentration that varies depending on the atmospheric scenario.

at degrading the organic matter. Second, we show here for the first time that H_2O_2 impacts the energetic metabolism of the cloud microbiome and thus potentially modulates its carbon metabolism. As a consequence it can modify the final transformation of the organic matter in clouds. This reciprocal interaction between H_2O_2 and microorganisms and its subsequent impact on cloud chemistry is clearly dependent on H_2O_2 concentration.

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.

The next step could be to integrate biological data into numerical atmospheric models to better quantify the consequence of this modulation for atmospheric chemistry.

Data availability. The authors are very grateful to the OPGC/LaMP staff for collecting the cloud samples at the PUY station; see the database of cloud biological and chemical composition, publicly accessible at http://www.obs.univ-bpclermont.fr/SO/beam/data.php.

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Competing interests. The authors declare that they have no conflict of interest.

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