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# Biotransformation of methanol and formaldehyde by bacteria isolated from clouds. Comparison with radical chemistry

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## ABSTRACT

The kinetics of biodegradation of methanol and formaldehyde in phosphate buffer at pH 7 by 4 bacterial strains (*Pseudomonas* spp., *Bacillus* sp. and *Frigoribacterium* sp.) isolated from cloud water at the puy de Dôme mountain have been investigated using <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy. We showed that biodegradation occurred at 5 °C and 17 °C, respectively average and summertime temperature considered within the cloud system at this site. They ranged from 10<sup>-19</sup> to 10<sup>-21</sup> mol cell<sup>-1</sup> s<sup>-1</sup> both at 5 and 17 °C for formaldehyde, and from 10<sup>-21</sup> to 10<sup>-23</sup> mol cell<sup>-1</sup> s<sup>-1</sup> at 5 and 17 °C for methanol. Metabolic intermediates were identified, with notably production of C3 compounds (glycerol, 1,2- and 1,3-propanediol) from formaldehyde by the strain *Bacillus* sp. In order to evaluate to which extent microbiological oxidation of organic compounds has to be considered as an alternative route to radical chemistry in cloud water, the biodegradation rates measured were compared with rates related to the reactivity of organic species with free radicals <sup>•</sup>OH (daytime chemistry) and NO<sub>3</sub> (nighttime chemistry) under two cloud situations (urban and remote cases). Clearly, measured biological and chemical reaction rates were in the same range of magnitude and their relative contribution varies according to the scenarios we tested, including the temperature of the clouds (5 or 17 °C), the category of the clouds (urban and remote) and the diurnal cycle (day and nighttime). Except for the degradation of methanol at 5 °C in remote clouds, our results show that biotransformation processes could be the main sink for C1 compounds in liquid clouds ( $T \geq 5$  °C = "warm cloud") during the night and both in polluted and non polluted clouds.

## 1. Introduction

Large quantities of Volatile Organic Compounds (VOCs) are emitted into the atmosphere by natural and/or anthropogenic sources. Oxidation of VOCs leads to the formation of gaseous and particulate secondary products which, in turn, have significant impact on the atmospheric composition (Atkinson et al., 2006; Kawamura et al., 2005). In this context, secondary organic aerosols (SOA) are formed by gas-to-particle conversion of these low volatile organic products. These particles account for a significant fraction of ambient tropospheric aerosols and impact on

atmospheric processes, climate and human health (Finlayson-Pitts and Pitts, 2000; Hallquist et al., 2009).

Oxidation of VOCs occurs both in the atmospheric gas and liquid phases (cloud, rain). In the gas phase, VOCs are primarily transformed by photolysis and/or radical chemistry and reactions with other oxidants such as O<sub>3</sub>. In the presence of clouds and depending on their solubility, several VOCs (Van Pinxteren et al., 2005) are significantly transferred into the atmospheric liquid phase where they can be oxidized. Methanol (CH<sub>3</sub>OH) is a significant component of the volatile organic carbon in the atmosphere (Heikes et al., 2002) and is the predominant oxygenated compound in the mid to the upper troposphere (Singh et al., 2000, 2001); its life time in the atmosphere has been evaluated to 1–2 weeks (Dufour et al., 2007). The anthropogenic primary source and oxidation taking place in the atmosphere represent 11–20% of the methanol while the biogenic contribution mainly issued from plant metabolism is the most significant part (80–90%) (Galbally and Kirstine, 2002;

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Tie et al., 2003; Jacob et al., 2005; Brunner et al., 2007). Methanol plays an important role in atmospheric chemistry as it influences the oxidizing capacity of the atmosphere by reacting with hydroxyl radicals ( $\cdot\text{OH}$ ) or by producing peroxy radical ( $\text{RO}_2$ ) and formaldehyde (HCHO). Formaldehyde is a key atmospheric VOC since it is an important tracer of Non-Methane Volatile Organic Compound (NMVOC) emissions and photochemical activity. HCHO is a primary emission product from biomass burning (Carlier et al., 1986) and fossil fuel combustion (Anderson et al., 1996; Holzinger et al., 1999). However, its major source in the atmosphere is the photochemical oxidation of methane and non-methane hydrocarbons (Altshuller, 1993).

Methanol and formaldehyde are soluble and efficiently transferred into atmospheric waters (Henry's law constants respectively equal to  $2.2 \times 10^2 \text{ M atm}^{-1}$  and  $10^3 \text{ M atm}^{-1}$ ) (Sander, 1999). Very few papers report methanol concentrations in atmospheric waters; it was found at concentrations of  $0.7 \mu\text{M}$  in rain (Snider and Dawson, 1985). Formaldehyde concentration varies in a very wide range of values (see Table 1); from  $0.001 \mu\text{M}$  in rainwater in rural areas (Kieber et al., 1999) to  $710 \mu\text{M}$  in highly polluted fog (Jacob et al., 1984). The average concentration measured in cloud water at the puy de Dôme station is  $2.9 \mu\text{M}$  (Parazols, 2007) and depends on the air mass origins ( $6.5 \mu\text{M}$  for "polluted" category and  $2.0 \mu\text{M}$  for "remote" category).

It is generally admitted that the reactivity of organic acids in clouds is driven by the presence of free radicals ( $\cdot\text{OH}$ ,  $\text{NO}_3$ ) or oxidants such as  $\text{O}_3$  or  $\text{H}_2\text{O}_2$  (Herrmann et al., 2005; Tilgner and Herrmann, 2010). However, recent studies raised the idea that microbial activity can play a significant role in the liquid phase transformation of some organic compounds (Ariya and Amyot, 2004; Deguillaume et al., 2008; Delort et al., 2010). It is now well established that living microorganisms are present in clouds (Ahern et al., 2007; Amato et al., 2005, 2007a,c; Bauer et al., 2002; Fuzzi et al., 1997; Sattler et al., 2001). They are active in clouds as shown by ATP (Adenine Tri Phosphate) measurements (Amato et al., 2007c) and CTC (5-Cyano-2,3-ditotyl Tetrazolium Chloride) staining (Hill et al., 2007), these two techniques describe the

metabolic activity and the energetic state of the cells. This implies the uptake of nutrients from the liquid phase by cells and suggests that they can develop within cloud water. One key question for cloud chemistry now is to quantify biological oxidation as respect to chemical and photochemical processes. In the recent paper, from Vaitilingom et al. (2010), it was shown that cloudborne microorganisms could indeed contribute to the transformation of atmospheric organic compounds (carboxylic acids: acetate, succinate and formate), particularly during the night.

The aim of this study is to extend this work to the transformation processes of two important atmospheric organic compounds in clouds: methanol and formaldehyde, considering both biological activity and photochemical reactivity. It is known that the metabolic pathway involving C1 compounds results in very similar intermediates as radical chemistry (Fig. 1). However, contrarily to radical chemistry, only microorganisms can either reduce or oxidize compounds such as formaldehyde.

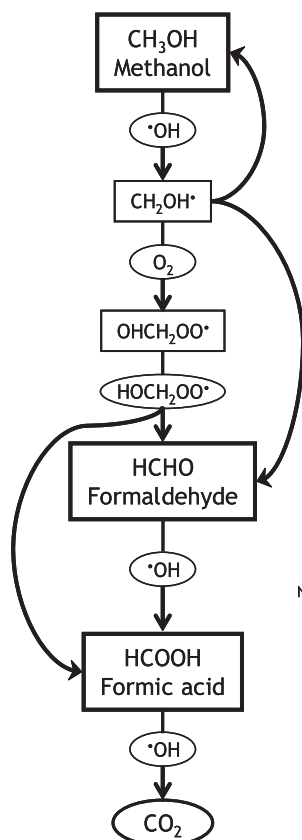
14 cloud samples have been collected between March 2003 and October 2004 at the puy de Dôme station (1465 m.a.s.l,  $45^\circ 46'$  North,  $2^\circ 57'$  West, France, see for more information: <http://www.obs.univ-bpclermont.fr/SO/beam>) (Marinoni et al., 2004; Amato et al., 2005, 2007a). From these samples, we isolated by cultivation more than one hundred strains of bacteria, fungi and yeasts. These included a large proportion of non sporing bacteria able to grow at low temperature. Amato et al. (2005, 2007b) demonstrated their abilities to degrade methanol and formaldehyde at  $27^\circ\text{C}$ . Here, we examined the effect of temperature on biological activity by determining the rates of biodegradation at  $5^\circ\text{C}$  and  $17^\circ\text{C}$ ; these temperatures correspond to the mean annual temperature and to the maximal temperature observed at the puy de Dôme summit when clouds form, respectively (see the database at: <http://www.obs.univ-bpclermont.fr/opgc/index.php>).

The results obtained with 4 bacterial strains belonging to the genera *Pseudomonas*, *Bacillus* and *Frigoribacterium*, the most current microorganisms isolated from clouds, are presented. The metabolism of these strains was investigated using  $^1\text{H}$  and/or  $^{13}\text{C}$  NMR spectroscopy. This provided indication about the pathways

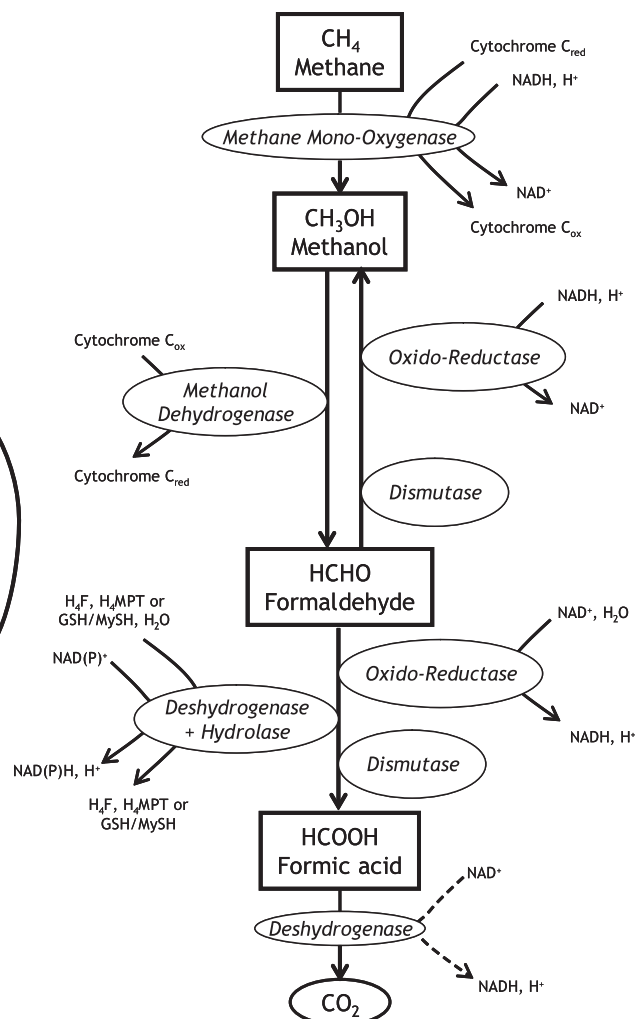
**Table 1**  
Methanol and formaldehyde concentrations measured in atmospheric waters (rain, fog and cloud) at different sampling sites under various influence (continental, marine, urban, polluted).

Compound	Sites	Samples	Concentrations ( $\mu\text{M}$ ) Min–Max or (average)	References
HCHO	Wilmington, Rural with maritime influence (NC, US)	Rain	<0.001–13	Kieber et al., 1999
	San Rita, Rural (Arizona, US)	Rain	(7.33)	Snider and Dawson, 1985
	Florence, Urban (Italy)	Rain	0.16–14.8	Largiuni et al., 2002
	Heraklion, Coastal Urban (Crete)	Rain	0.4–11.1	Economou and Mihalopoulos, 2002
	Gdansk Wrzeszcz, Urban (Poland)	Rain	1.7–44	Polkowska et al., 2006
	Los Angeles, Urban (California, US)	Rain	0.85–45	Kawamura et al., 2001
	Los Angeles, Urban (California, US)	Rain	0–20	Grosjean and Wright, 1983
	Galicía, rural with anthropogenic influence from a thermal power plant (Northwest Spain)	Rain	0.2–2.1	Peña et al., 2002
	Sierra Nevada, Rural (US)	Cloud	8–14	Collett, Jr. et al., 1990
	5 sites, Virginia, Rural (US)	Cloud	0.8–228	Munger et al., 1995
	Los Angeles, Urban (US)	Cloud	6–43	Richards, 1995
	Henninger flat, Rural (California, US)	Cloud	0.3–36	Grosjean and Wright, 1983
	San Pedro Hill, Coastal (California, US)	Cloud	13.6–37.3	Igawa et al., 1989
	Henninger flat, Inland Mountainous site (California, US)	Cloud	45.9–61.5	Igawa et al., 1989
	puy de Dôme mountain (air masses from various origins) (Fr)	Cloud	0.1–14.3 (2.0–remote) (6.5–polluted)	Parazols, 2007
	San Joaquin, Polluted (California, US)	Fog	53–710	Jacob et al., 1984
	San Joaquin, Polluted (California, US)	Fog	2.3–410.2	Collett, Jr. et al., 1999
	5 sites, Urban/remote/marine (California, US)	Fog	0.3–76.7	Grosjean and Wright, 1983
	Fairbanks, Urban (Alaska, US)	Ice fog	16.7–38.7	Grosjean and Wright, 1983
	Riverside campus, Polluted (California, US)	Fog	4.1–228	Igawa et al., 1989
$\text{CH}_3\text{OH}$	San Rita, Rural (US)	Rain	0.7	Snider and Dawson, 1985

## Radical chemistry



## C<sub>1</sub> metabolism



**Fig. 1.** Non-exhaustive schemes of the ways of degradation of C<sub>1</sub> compounds by radical chemistry and by metabolism. The scheme corresponding to radical chemistry has been drawn from reactions given in Monod et al. (2000) and references therein. C<sub>1</sub> metabolism scheme was reconstructed from Hanson and Hanson (1996), and Delort (2006) and references therein. Known enzymes are described for each reaction in which they can be involved, as well as cofactors when required (the dot line in the case of Formate, dehydrogenase signifies the existence of several types of this enzyme, with and without a cofactor). NAD: Nicotinamide adenine dinucleotide; H<sub>4</sub>F: Tetrahydrofolate; H<sub>4</sub>MPT: Dephospho-tetrahydromethanopterin; GSH: Glutathione; MySH: Mycothiol.

involved and allowed determining the rates of biotransformation under experimentally controlled conditions. Extrapolating to the conditions existing in the liquid phase of clouds issued from urban and remote air masses during daytime (high concentration of radicals, mainly ·OH) and during nighttime (lower level of radicals, mainly ·NO<sub>3</sub>), the contribution of microbial activity is compared to the degradation of methanol and formaldehyde with that from radical chemistry. The objective is to evaluate the extent to which microbiological processes of organic compounds can be considered as an “alternative” route to radical chemistry in cloud water.

## 2. Materials and methods

### 2.1. Conditions of incubation for the biodegradation tests

For each isolated strain, liquid pure pre-culture was incubated at 17 °C or 27 °C in either TSA (Trypcase Soy broth, Bioré, Marcy l’Etoile, France) or R2A broth culture media (Reasoner and Geldreich, 1985). A large volume (about 400 mL) of culture was

then incubated under similar conditions and cells were harvested by centrifugation (4000 g, 15 min, 4 °C) after 24 or 48 h of growth and rinsed twice in NaCl 0.8%. The cell pellet was then resuspended in the test medium composed of 25 mL of 0.1 M phosphate buffer at pH 7.0, supplemented with 20 mM <sup>13</sup>C-formaldehyde (H<sup>13</sup>CHO) (Eurisotop, solution at 20% v/v in water) or methanol (CH<sub>3</sub>OH) (Aldrich). The isotopic form of formaldehyde was used because the unlabeled commercial product contains methanol for stabilizing the solution. In addition, <sup>1</sup>H NMR could not detect the hydrated form of formaldehyde (see for more details Amato et al., 2007b) and <sup>13</sup>C NMR allowed to monitor the fate of <sup>13</sup>C and to identify metabolites. Incubations were carried out for 24–64 h at 5 °C or 17 °C in the dark under aerobic conditions (200 rpm). Samples for analysis were taken at regular intervals of time. One millilitre of the incubation medium was centrifuged (12,000 g, 3 min) to pellet cells; and supernatants were kept frozen (–40 °C) until measurement. The experimental blank was made in parallel for each strain and consisted in cells suspended in phosphate buffer in the absence of carbon source.

In the case of *Bacillus* sp 3B6, incubations with  $^{13}\text{C}$ -formaldehyde were performed using a perfusion system (see section 2.4) instead of agitation in flasks.  $^{13}\text{C}$  NMR spectra were recorded *in vivo* on the sample without separation of the cells.

Cell concentration in the test media was adjusted based on the optical density ( $\text{OD}_{575\text{nm}}$ ) to match the cell/substrate ratio existing in cloud water. In order to express the rates of degradation as  $[\text{mol}][\text{cell}]^{-1}[\text{s}]^{-1}$ , the exact cell concentration was determined by standard dilution plating of the incubation medium on R2A or TSA and CFU (Colony Forming Units) counts after 2 days incubation at  $17^\circ\text{C}$  or  $27^\circ\text{C}$ .

## 2.2. Measurements by $^1\text{H}$ NMR

Supernatants from biodegradation test media were prepared for  $^1\text{H}$  NMR by mixing a volume of  $450\ \mu\text{L}$  of the sample with  $50\ \mu\text{L}$  of sodium tetra deuterated trimethylsilyl propionate (TSPd<sub>4</sub>, Eurisotop) in solution in  $^2\text{H}_2\text{O}$ .  $^2\text{H}_2\text{O}$  was used for locking and shimming, while TSPd<sub>4</sub> constituted a reference for chemical shifts (0 ppm) and quantification. Final volumes of  $500\ \mu\text{L}$  of prepared samples were put in 5 mm-diameter NMR tubes. Acquisition of spectra was made at 400.13 MHz,  $21^\circ\text{C}$ , on a Bruker Avance 400 spectrometer, by collection of 32 scans ( $45^\circ$  pulse, 4789.27 Hz SW, 65.536 data points, 6.84 min total acquisition time). Water signal was eliminated by presaturation, and no filter was applied before Fourier transformation. Using Bruker software (X-Win NMR), baseline was corrected before integration for quantification. The concentration of metabolites was calculated as following:

$$[m] = \frac{(9 \times A_0 \times [\text{TSPd}_4])}{(b \times A_{\text{ref}})} \quad (\text{E1})$$

where  $[m]$  is the concentration of the compound to quantify,  $A_0$  is the area of  $m$  resonance;  $A_{\text{ref}}$  is the area of TSPd<sub>4</sub> resonance; 9 and  $b$  respectively are the numbers of protons of TSPd<sub>4</sub>, resonating at 0 ppm, and of metabolite  $m$ .

## 2.3. Formaldehyde assay

Formaldehyde resonates at about 4.5 ppm in  $^1\text{H}$  NMR, and so it was masked by the residual signal of water, making it undetectable by this technique at  $21^\circ\text{C}$ . Because of this, formaldehyde was assayed by a sensitive automatic analyser (Aerolaser AL4021). The principle is based on the reaction of formaldehyde with acetylacetone and ammonia, resulting in the formation of the detected fluorochrome complex ( $\lambda_{\text{excitation}} = 400\ \text{nm}$ ;  $\lambda_{\text{emission}} = 510\ \text{nm}$ ), for which light emission is directly proportional to formaldehyde concentration.

Alternatively  $^{13}\text{C}$  labeled formaldehyde was analyzed by  $^{13}\text{C}$  NMR as described previously (Amato et al., 2007b) or by *in vivo*  $^{13}\text{C}$  NMR (see section 2.4).  $^{13}\text{C}$  NMR analysis allows following specifically the  $^{13}\text{C}$  labeled metabolites issued from the transformation of  $^{13}\text{C}$ -formaldehyde by the bacterium.

## 2.4. *In vivo* $^{13}\text{C}$ NMR study

*In vivo* NMR experiments were performed at 500 MHz on Bruker Avance NMR spectrometer using a perfusion system described in Choroa et al. (2009). *Bacillus* sp. 3B6 cells were perfused with phosphate buffer containing 10% of  $^2\text{H}_2\text{O}$  and saturated with air. The perfusion rate was controlled by peristaltic pump (flow rate  $20\ \text{mL min}^{-1}$ ). Bacterial suspension circulated continuously in the system. Benzene was used as an internal reference to calibrate chemical shifts (128.39 ppm relative to TMS (0 ppm)) in a sealed

capillary placed in 10 mm sample tube. Spectra were acquired with power-gated  $^1\text{H}$  decoupling WALTZ16 sequence. Acquisition parameters were as followed: 1000 scans (corresponding to 15 min acquisition for each spectrum);  $30^\circ$  carbon pulse (7  $\mu\text{s}$ ); relaxation delay 1 s; acquisition time 0.14 s; spectral window 30.303 Hz; data points 8000 zero filled to 16000.

## 2.5. Calculation of degradation rates in cloud water

The biodegradation rates of the compound C ( $k_c$ ) have been determined by linear regression fits as follows:

$$k_c = (k \times [C]_0) / N_{\text{cells}} \left[ \text{mol cells}^{-1} \text{s}^{-1} \right] \quad (\text{E2})$$

with  $[C]_0$  the initial concentrations of selected compound C ( $\text{mol L}^{-1}$ ),  $k$  the pseudo-first order decays ( $\text{s}^{-1}$ ),  $N_{\text{cells}}$  the concentration of cells participating to the biodegradation and determined by CFU counts ( $\text{cells L}^{-1}$ ).

The calculations of the estimated degradation rates in cloud water have been determined by:

- for bacteria:

$$R_c = k_c \times N_{\text{cells}(\text{cloud})} \left[ \text{mol L}^{-1} \text{s}^{-1} \right] \quad (\text{E3})$$

- for free radicals:

$$R_c^* = k_{c,\text{radical}} \times [\text{Radical}] \times [C]_{\text{cloud}} \left[ \text{mol L}^{-1} \text{s}^{-1} \right] \quad (\text{E4})$$

with  $[C]_{\text{cloud}}$  the initial concentrations of selected compound C ( $\text{mol L}^{-1}$ ) in the cloud water,  $k_c$  the biodegradation rate of compound C ( $\text{mol cell}^{-1} \text{s}^{-1}$ ),  $N_{\text{cells}(\text{cloud})}$  the bacterial concentration in cloud water ( $\text{cells L}^{-1}$ ),  $[\text{Radical}]$  ( $\text{mol L}^{-1}$ ) the concentration of free radical  $\cdot\text{OH}$  or  $\text{NO}_3$  from literature and  $k_{c,\text{radical}}$  ( $\text{L mol}^{-1} \text{s}^{-1}$ ) is the 2nd order chemical rate constant between compound C and  $\cdot\text{OH}$  or  $\text{NO}_3$  from the literature.

## 3. Results and discussion

Four strains of bacteria, *Pseudomonas graminis* (DQ512786), *Sphingomonas* sp. (DQ512776), *Pseudomonas syringae* (DQ512783), *Frigoribacterium* sp (DQ512796) and *Bacillus* sp 3B6 (DQ512741), isolated from cloud water samples collected at the puy de Dôme summit, were studied. Their isolations and identifications were described elsewhere (see Amato et al., 2007a); briefly pure strains were isolated from individual colonies on Petri dishes and were identified by DNA sequencing and comparison with known sequences in genomic data banks (<http://www.ncbi.nlm.nih.gov/BLAST/>). These strains were selected as they belong to genera frequently encountered in atmospheric waters (Amato et al., 2005, 2007a; Fuzzi et al., 1997; Ahern et al., 2007). *Pseudomonas* strains are also usually found on vegetation, *Frigoribacterium* in cold environments and *Bacillus* are more ubiquitous (Amato et al., 2007a).

### 3.1. Biotransformation of formaldehyde and methanol at $5^\circ\text{C}$ and $17^\circ\text{C}$

Biotransformation of formaldehyde and methanol by the 4 selected strains was investigated using mainly  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy as previously described in Amato et al. (2007b) (see Table S1 in the supplementary data). Our experiments involved formaldehyde and methanol at concentrations higher than those in



cloud water (20 mM instead of a few  $\mu\text{M}$ ) to allow analysis by NMR. However the substrate to cell ratios used in the incubation media were similar to those found in natural clouds. In a previous work (Väitilingom et al., 2010) we showed that, at the concentrations used, the biodegradation rates did not depend on cell or substrate concentrations at given substrate to cell ratio.

### 3.1.1. Biotransformation of methanol

The biodegradation rates of methanol measured from  $^1\text{H}$  NMR experiments at both  $5^\circ\text{C}$  and  $17^\circ\text{C}$  by the four bacterial strains are reported in Table 2. They are expressed as mole of compound degraded per cell and per second. At  $17^\circ\text{C}$ , all strains degraded methanol, at rates ranging from  $10^{-21}$  to  $10^{-23}$  mol cell $^{-1}$  s $^{-1}$ . At  $5^\circ\text{C}$ , the rates of methanol transformation were lower and could be only measured for two strains, *P. syringae* and *Frigoribacterium* sp.; these were  $5.8 \times 10^{-22}$  and  $2.5 \times 10^{-23}$  mol cell $^{-1}$  s $^{-1}$ , respectively.

Considering the metabolic pathways involved in the transformation of C1 compounds, no intermediate was detected, suggesting that methanol was directly oxidized into formaldehyde, then formate and  $\text{CO}_2$  as described in Fig. 1.

### 3.1.2. Biotransformation of formaldehyde

The biotransformation of  $^{13}\text{C}$ -formaldehyde at  $5^\circ\text{C}$  and  $17^\circ\text{C}$  was studied by  $^1\text{H}$  NMR or  $^{13}\text{C}$  NMR spectroscopy. The degradation rates measured are reported in Table 1 for the four bacterial strains. Formaldehyde was efficiently degraded by all four studied bacterial strains, with values ranging between  $10^{-19}$ – $10^{-21}$  mol cell $^{-1}$  s $^{-1}$  at both temperatures  $17^\circ\text{C}$  and  $5^\circ\text{C}$ . Thus, contrarily to methanol, low temperature was not a limiting factor for formaldehyde bacterial degradation.

In addition metabolites detected on NMR spectra allowed to confirm the metabolic routes described in Fig. 1. First, the 4 strains were able to produce methanol by reduction of formaldehyde; in all cases methanol remained in the incubation medium and accumulated since its degradation was much slower (see above). Second, the 4 strains were also oxidizing formaldehyde into formate. In the case of *Bacillus* sp, *P. syringae* and *P. graminis*, formate concentration decreased with time as it was further transformed into  $\text{CO}_2$ . This result is consistent with our previous observations showing that *P. syringae* and *P. graminis* were good formate degraders (Väitilingom et al., 2010). In the case of *Bacillus* sp, the formation of  $^{13}\text{CO}_2$  was clearly detected (see later Fig. 2). In the case of *Frigoribacterium* sp. formate originating from the oxidation of formaldehyde accumulated in the incubation medium. This later point was verified by incubating this strain in the presence of formate: its degradation was extremely slow and the measured degradation rates were  $0.2 \times 10^{-22}$  and  $1.2 \times 10^{-21}$  mol cell $^{-1}$  s $^{-1}$  at  $5^\circ\text{C}$  and  $17^\circ\text{C}$ , respectively.

Special attention was paid to the metabolism of *Bacillus* sp 3B6 for different reasons: first, although representatives of this genus are very frequently found in the atmosphere, few studies have

linked their metabolism to atmospheric processes; second, this particular strain was found to degrade herbicides (Durand et al., 2006), to synthesize oligosaccharides (Matulová et al., 2011) and was thought to be a good candidate having specific properties toward C1 compounds. Its metabolism was thus studied in details using a more sophisticated approach, namely *in vivo*  $^{13}\text{C}$ -NMR. The monitoring of  $^{13}\text{C}$ -formaldehyde biotransformation at  $17^\circ\text{C}$  by *Bacillus* sp 3B6 is presented in Fig. 2A. Methanol ( $\delta = 49.17$  ppm), formate ( $\delta = 170.24$  ppm) and  $\text{HCO}_3^-$  ( $\delta = 160.5$ ) are the metabolites of formaldehyde expected from the C1 metabolic pathway described in Fig. 1. Fig. 3 shows that the transformation of  $^{13}\text{C}$ -formaldehyde was completed after 200 min, that of formate after 300 min while methanol decreased very slowly after 200 min over time after its formation (this is consistent with the very low degradation rate of methanol by this strain, see Table 2). When *in vivo*  $^{13}\text{C}$  NMR is performed,  $^{13}\text{CO}_2$  is distributed between the aqueous phase ( $\text{H}^{13}\text{CO}_3^-$ ) and the gas phase ( $^{13}\text{CO}_{2\text{gas}}$ ).  $\text{H}^{13}\text{CO}_3^-$  is in the measurement area of the spectrometer and can thus be detected by  $^{13}\text{C}$  NMR;  $^{13}\text{CO}_{2\text{gas}}$  is out of the measurement area and doesn't give any  $^{13}\text{C}$  NMR signal. As soon as  $\text{H}^{13}\text{CO}_3^-$  is formed, part of it goes to the gas phase ( $^{13}\text{CO}_{2\text{gas}}$ ), as a result the concentration of  $\text{H}^{13}\text{CO}_3^-$  seems to remain constant with time. Besides these main metabolites, other  $^{13}\text{C}$  NMR signals (Fig. 2B) indicated the presence of glycerol ( $\delta = 72.37$ ;  $63.05$  ppm), propane-1,2-diol ( $\delta = 68.28$ ,  $67.75$ ,  $18.78$  ppm) and propane-1,3-diol ( $\delta = 61.76$ ,  $34.13$  ppm). The biotransformation of formaldehyde into propane-1,2- and 1,3-diol has been already described in *Escherichia coli* (Hunter et al., 1984). These 3 metabolites were accumulating with time in the medium (see Fig. 2A). In addition transient signals of low intensity corresponding to the carbons of serine could be identified ( $\delta = 60.65$ ,  $62.45$  and  $174$ ,  $0$  ppm). The presence of these metabolites demonstrated that formaldehyde can enter serine metabolism, which is connected to the metabolism of glycerophospholipid and then to that of glycerolipid involving glycerol. Glycerol can then be converted into propane-1,3-diol and propane-1,2-diol. (<http://www.genome.jp/kegg/pathway.html>, see Figs. S1–S4 in the supplementary data). This complex metabolism, involving a large number of enzymes, shows that functionalization and condensation to larger molecules (C3 compounds) from formaldehyde (C1 compound) is also possible.

### 3.2. Comparison with radical chemistry

Oxidation processes in the atmosphere are largely catalyzed by free radicals, of which the principals are  $\cdot\text{OH}$  and  $\text{NO}_3$ . Radical hydroxyls are generated by photochemical pathways and are implicated in the oxidation processes existing in the atmosphere during the day, while nitrate radicals are the most efficient radical oxidants during the night (Finlayson-Pitts and Pitts, 1997). Hence, in order to compare microbiological with radical chemistry

**Table 2**

Biodegradation rates (mol cell $^{-1}$  s $^{-1}$ ) of methanol and formaldehyde at  $5^\circ\text{C}$  and  $17^\circ\text{C}$  by microbial strains isolated from cloud samples. Given values are results of first approximation calculations, in which degradation rates were assumed to be linear over time.

Compound	Methanol		Formaldehyde	
	Biodegradation rate (mol cell $^{-1}$ s $^{-1}$ )			
Strain and temperature	$5^\circ\text{C}$	$17^\circ\text{C}$	$5^\circ\text{C}$	$17^\circ\text{C}$
<i>Pseudomonas graminis</i>	0	$5.6 \times 10^{-22}$	$8.1 \times 10^{-21}$	$1.9 \times 10^{-20}$
<i>Pseudomonas syringae</i>	$5.8 \times 10^{-22}$	$5.7 \times 10^{-21}$	$8.6 \times 10^{-20}$	$1.4 \times 10^{-19}$
<i>Frigoribacterium</i> sp.	$2.5 \times 10^{-23}$	$3.5 \times 10^{-23}$	$6.4 \times 10^{-21}$	$6.4 \times 10^{-21}$
<i>Bacillus</i> sp.	0	$2.9 \times 10^{-21}$	$3.1 \times 10^{-21}$	$2.0 \times 10^{-20}$
Average ( $\pm$ Standard deviation)	$1.5 (\pm 2.9) \times 10^{-22}$	$2.3 (\pm 2.6) \times 10^{-21}$	$2.6 (\pm 4.0) \times 10^{-20}$	$4.6 (\pm 6.3) \times 10^{-20}$

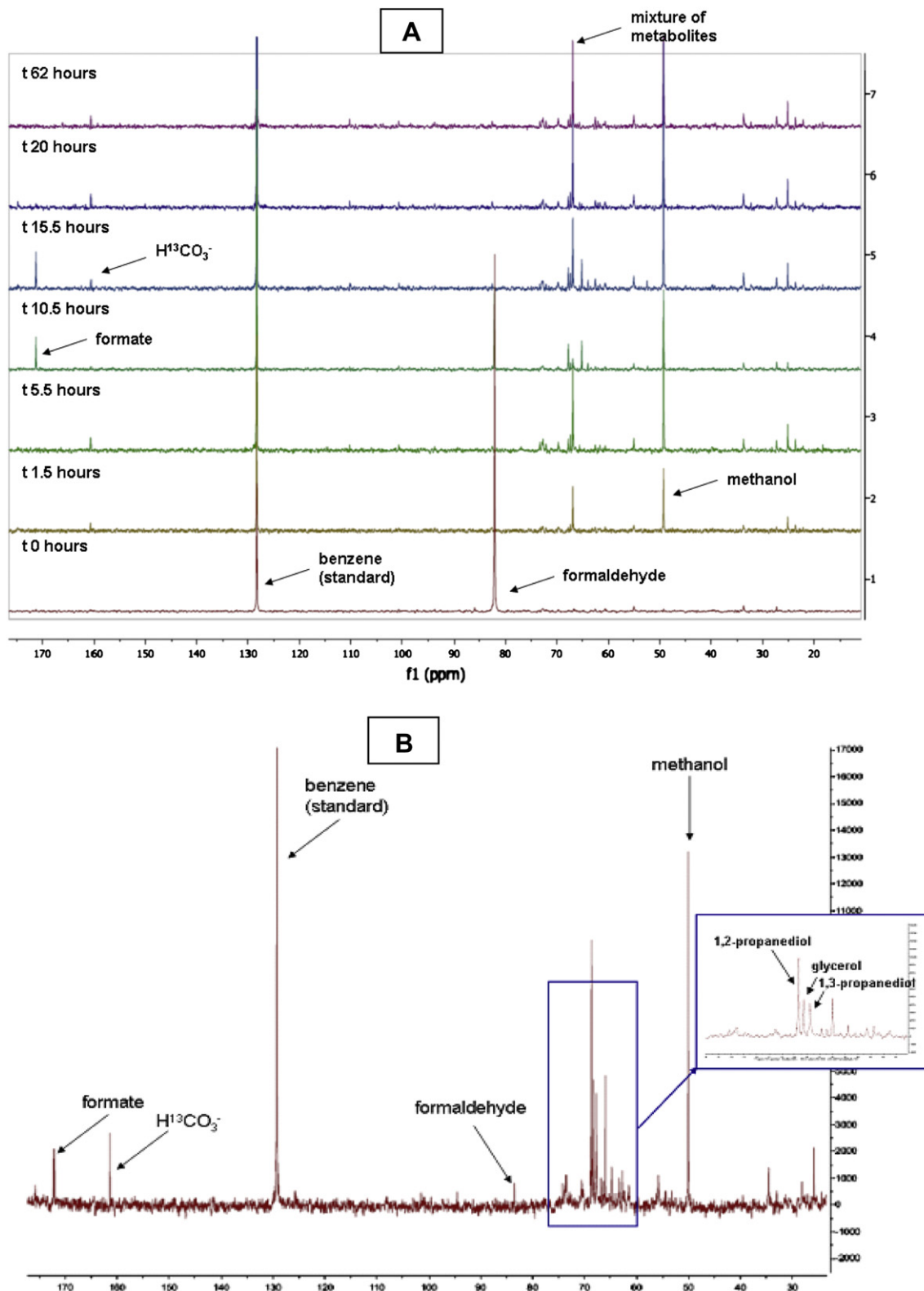
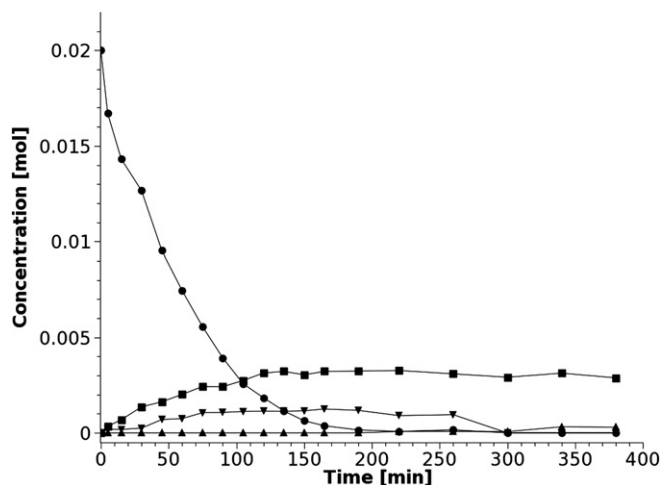


Fig. 2. A) *in vivo*  $^{13}\text{C}$  NMR spectra collected during the incubation of *Bacillus* sp 3B6 with  $^{13}\text{C}$  formaldehyde; B) Example of *in vivo*  $^{13}\text{C}$  NMR spectrum collected after 4 h. Cell concentration was  $10^9$  cells  $\text{mL}^{-1}$  and substrate concentration was 20 mM.

processes on the transformation of methanol and formaldehyde, we have considered their reactivity with  $\cdot\text{OH}$ ,  $\text{NO}_3^-$  (Table 3). The rate constants of  $\cdot\text{OH}$ ,  $\text{NO}_3^-$  measured at 25 °C have been extrapolated at 5 °C and 17 °C by using the temperature dependency of the

kinetic constants. For microbial activity the average rate between the four strains was used (Table 2).

We extrapolated our results to typical chemical and microbial concentrations measured in cloud waters at puy de Dôme: i)



**Fig. 3.** Time course of the concentrations of  $^{13}\text{C}$ -formaldehyde (●),  $^{13}\text{C}$ -methanol (■),  $^{13}\text{C}$ -formate (▼) and  $\text{H}^{13}\text{CO}_3$  (▲) measured by *in vivo*  $^{13}\text{C}$  NMR during the incubation by *Bacillus* sp 3B6 sp at  $17^\circ\text{C}$ .

concentrations of  $6.5\ \mu\text{M}$  and  $2.0\ \mu\text{M}$  for formaldehyde as mean values for urban and remote clouds respectively (Parazols, 2007); ii) active biomass of  $8.1 \times 10^4$  bacteria  $\text{mL}^{-1}$  (Amato et al., 2007c). As no methanol was measured at the puy de Dôme station, we used the value of  $0.7\ \mu\text{M}$  given by Snider and Dawson (1985).

For free radical concentrations, we have used the latest values published recently by Tilgner and Herrmann (2010) who considered clouds from urban origin ( $[\cdot\text{OH}] = 1 \times 10^{-14}$   $\text{mol L}^{-1}$  and  $[\text{NO}_3] = 2 \times 10^{-13}$   $\text{mol L}^{-1}$ ); and clouds from remote origin ( $[\cdot\text{OH}] = 5 \times 10^{-14}$   $\text{mol L}^{-1}$ ;  $[\text{NO}_3] = 5 \times 10^{-15}$   $\text{mol L}^{-1}$ ). These values are simulated by a cloud chemistry model that considers explicit multiphase chemistry. TMI (iron, manganese, copper),  $\text{H}_x\text{O}_y$ , nitrogen, sulphur and organic chemistry is taken into account and calculations are performed to simulate concentrations of radicals as

a function of chemical scenarios (remote and polluted environments) and photochemical conditions (night vs. day).

These conditions were used to quantify the relative participation of bacterial activity and free radicals reactivity to the oxidation of methanol and formaldehyde during the day and during the night (Fig. 4).

In clouds from urban origin (Fig. 4A), at  $5^\circ\text{C}$ , in the presence of sunlight,  $\cdot\text{OH}$  radicals are present and bacterial activity would account for 0% and 5% of the degradation of methanol and formaldehyde respectively; during the night, biological activity would only compete with  $\text{NO}_3$  radicals and its participation to the oxidation of methanol and formaldehyde would reach 32% to 71%. At  $17^\circ\text{C}$ , the contribution of bacterial activity to the oxidation of methanol and formaldehyde would slightly increase (3% and 7%, respectively) during daytime and bacteria would become the major actors at night (79% and 90% respectively). In conclusion, in urban clouds, the contribution of bacteria is not negligible during the day under typical summer time conditions ( $17^\circ\text{C}$ ); this is especially true for formaldehyde degradation. At night, bacteria are much more active than  $\text{NO}_3$  at both temperatures for formaldehyde degradation, and for methanol at high temperature. However the activity of  $\text{NO}_3$  is the most important factor for methanol degradation at low temperature, and remains important at  $17^\circ\text{C}$ .

In clouds from remote origin (Fig. 4B), during day time, the influence of bacterial activity is less important than that observed in urban case, its contribution varies from 0% to 1% for methanol degradation (at  $5$  and  $17^\circ\text{C}$  respectively) and from 3% to 5% for formaldehyde degradation (at  $5$  and  $17^\circ\text{C}$  respectively) and is thus very low compared to  $\cdot\text{OH}$  activity. However, the reverse situation is observed during night time, bacterial activity becomes almost the only actor compared to  $\text{NO}_3$  it accounts for 95–99% of methanol degradation at  $5$  and  $17^\circ\text{C}$  respectively and for 100% of formaldehyde degradation at both temperatures. Clearly, these results show that  $\text{NO}_3$  have a very little influence on methanol and formaldehyde degradation in clouds from remote origin compared to enzymatic activity; as a consequence microbial activity would be driving the

**Table 3**

Degradation rates of methanol and formaldehyde extrapolated to cloud conditions (urban and remote) at  $5^\circ\text{C}$  and  $17^\circ\text{C}$ .

Cloud condition	Organism or compound (temp [ $^\circ\text{C}$ ])	Methanol		Formaldehyde	
		Reaction rate or reaction rate constant <sup>a</sup>	Rate in cloud water ( $\times 10^{-14}$ $\text{mol L}^{-1} \text{s}^{-1}$ )	Reaction rate or reaction rate constant <sup>a</sup>	Rate in cloud water ( $\times 10^{-14}$ $\text{mol L}^{-1} \text{s}^{-1}$ )
Urban and remote	Bacteria ( $5^\circ\text{C}$ )	$1.5 \times 10^{-22}$	1.2	$2.6 \times 10^{-20}$	209.5
	Bacteria ( $17^\circ\text{C}$ )	$2.3 \times 10^{-21}$	18.6	$4.6 \times 10^{-20}$	376.0
Urban	$\cdot\text{OH}$ ( $5^\circ\text{C}$ )	$8.7 \times 10^{8b}$	607.3	$6.1 \times 10^{8c}$	3974.5
	$\cdot\text{OH}$ ( $17^\circ\text{C}$ )	$9.5 \times 10^{8b}$	662.9	$7.1 \times 10^{8c}$	4618.1
	$\text{NO}_3$ ( $5^\circ\text{C}$ )	$1.9 \times 10^{5d}$	2.7	$3.4 \times 10^{5e}$	85.7
	$\text{NO}_3$ ( $17^\circ\text{C}$ )	$3.6 \times 10^{5d}$	5.1	$6.6 \times 10^{5e}$	43.9
Remote	$\cdot\text{OH}$ ( $5^\circ\text{C}$ )	$8.7 \times 10^{8b}$	3036.6	$6.1 \times 10^{8c}$	6114.6
	$\cdot\text{OH}$ ( $17^\circ\text{C}$ )	$9.5 \times 10^{8b}$	3314.5	$7.1 \times 10^{8c}$	7104.8
	$\text{NO}_3$ ( $5^\circ\text{C}$ )	$1.9 \times 10^{5d}$	0.1	$3.4 \times 10^{5e}$	0.3
	$\text{NO}_3$ ( $17^\circ\text{C}$ )	$3.6 \times 10^{5d}$	0.1	$6.6 \times 10^{5e}$	0.7

The following values of concentrations have been used for calculations of rate in cloud water:

- [bacteria] =  $8.1 \times 10^4$  cell  $\text{mL}^{-1}$  (Amato et al., 2007a–c);

- for urban case  $[\cdot\text{OH}] = 1 \times 10^{-14}$   $\text{mol L}^{-1}$  and  $[\text{NO}_3] = 2 \times 10^{-13}$   $\text{mol L}^{-1}$  (Tilgner and Herrmann, 2010); for remote case  $[\cdot\text{OH}] = 5 \times 10^{-14}$   $\text{mol L}^{-1}$ ;  $[\text{NO}_3] = 5 \times 10^{-15}$   $\text{mol L}^{-1}$  (Tilgner and Herrmann, 2010);

-  $[\text{CH}_3\text{OH}] = 7 \times 10^{-7}$   $\text{mol L}^{-1}$  (for urban and remote case);

- for urban case  $[\text{HCHO}] = 6.5 \times 10^{-6}$   $\text{mol L}^{-1}$  and for remote case  $[\text{HCHO}] = 2 \times 10^{-6}$   $\text{mol L}^{-1}$ .

<sup>a</sup> Reaction rate values ( $\text{mol s}^{-1} \text{cell}^{-1}$ ) are given for bacteria, and reaction rate constant values ( $\text{L mol}^{-1} \text{s}^{-1}$ ) are given for free radicals.

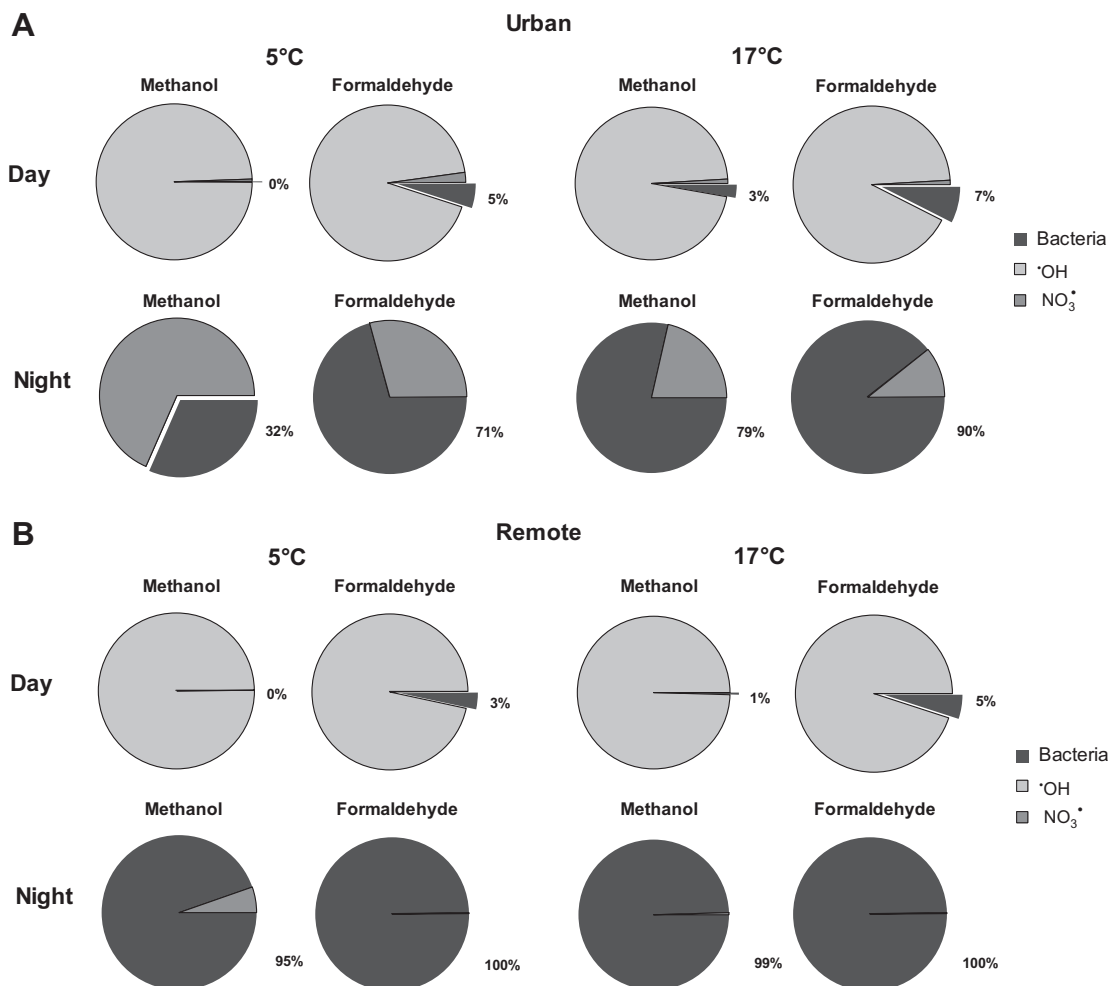
<sup>b</sup> Elliot and McCracken, 1989.

<sup>c</sup> Chin and Wine, 1994.

<sup>d</sup> Herrmann and Zellner, 1998.

<sup>e</sup> Exner et al., 1993.





**Fig. 4.** Estimated relative influence of bacterial activity and free radicals ( $\cdot\text{OH}$  and  $\text{NO}_3^{\cdot}$ ) on the degradation of methanol and formaldehyde in cloud water at 5 °C and 17 °C during the day and the night for both cloud air mass types: urban (A) and remote (B) cases. During nighttime (i.e. in the absence of photochemical reactivity),  $\cdot\text{OH}$  radicals are considered not present. The rates of degradation for cloud conditions are those reported in Table 2.

chemical reactivity of C1 compounds in warm clouds ( $\geq 5$  °C) during the night.

#### 4. Conclusions

The biodegradation of methanol and formaldehyde by strains from cloud water collected at puy de Dôme were already demonstrated to occur under optimal conditions of temperature (Amato et al., 2005, 2007b). Here we brought new evidences that biodegradation is also possible at *in situ* temperatures: 5 °C and 17 °C, corresponding to the annual average and maximum temperatures of clouds at puy de Dôme. This is consistent with their ability to grow at 5 °C (Amato et al., 2007a), and attests of the presence of cold tolerant enzymes. Also this study allowed the comparison between microbial transformations of methanol and formaldehyde with radical pathways.

The metabolic pathways of photo- and bio-degradation of methanol and formaldehyde presented some similarities. As an example, photochemistry, through the production of radicals, is involved in the progressive oxidation of organic compounds to  $\text{CO}_2$  in cloud water. The final step is constituted by the oxidation of formate (Monod et al., 2000; Herrmann et al., 2005), and this reaction can also be catalyzed by three out of the four microbial strains studied here (*P. graminis*, *P. syringae* and *Bacillus* sp). Methanol can be oxidized into formaldehyde by the four strains

tested or by reactivity with free radicals (see Fig. 1). The main difference between radical chemistry and biological activity is the ability of some microbes to catalyze the reduction of formaldehyde into methanol even under very oxidative conditions. In addition, we showed the ability of *Bacillus* sp strain to produce complex compounds such as propane-1,2-diol, propane-1,3-diol and glycerol. This last point is of importance concerning atmospheric chemistry as it suggests that not only successive oxidations that lead to shorten molecules can occur in cloud water but also reactions that can result in compounds of larger molecular size. In our study, C3 molecules have been built from a C1 compound. It is of major importance to be aware that such functionalization processes in the atmosphere, identified as determining for atmospheric sciences (Hallquist et al., 2009; Blando and Turpin, 2000), can be catalyzed by microorganisms.

Second, the relative contributions of microbial and radical activities ( $\cdot\text{OH}$  and  $\text{NO}_3^{\cdot}$ ) have been quantitatively evaluated. Clearly, measured biological and chemical reaction rates are in the same range of magnitude, and their relative contribution varies according to the scenarios we have tested, including the temperature of the clouds (5 or 17 °C), the origin of the clouds (urban and remote) and the diurnal cycle (day and nighttime). The general conclusions are the following: i) bacteria activity is always more favored at higher temperatures, therefore it will be limited to warm clouds; ii) during daytime,  $\cdot\text{OH}$  are obviously the preponderant actors.

However biotransformation processes could be the main sink for C1 compounds during nighttime where the radical chemistry is less efficient than enzymatic reactions. This is explained by the kinetic constant of NO<sub>3</sub> with these organic compounds that are lower than those with ·OH and by the lower NO<sub>3</sub> concentration during the night compared to those of ·OH during the day. The same conclusions were reached in our previous study where we had considered organic acids such as formate, succinate and acetate (Väitilingom et al., 2010); iii) the origin of air masses modulates the extent of the previous conclusion. Indeed NO<sub>3</sub> reactivity which is negligible at night compared to bacteria in remote clouds becomes of some importance in urban clouds, this is due to the higher concentration of NO<sub>3</sub> in polluted clouds compared to non polluted ones. However, we are aware that our experimental approach leads to some simplifications where some stressful factors for microorganisms are not considered (very acidic medium, presence of highly toxic chemical species that can characterize polluted clouds).

In conclusion, our results reinforce the hypothesis of the existence of a microbial activity in cloud droplets. It is therefore timely to consider biodegradation in parallel to free radicals oxidation among the processes catalyzing atmospheric transformation of organic matter in clouds and thus to complete cloud chemistry models integrating both chemical and biological components.

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## Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.atmosenv.2011.06.035.

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