Potential for phenol biodegradation in cloud waters

Audrey Lallement[1], Ludovic Besaury[1], Elise Tixier[1], Martine Sancelme[1], Pierre Amato[1], Virginie Vinatier[1], Isabelle Canet[1], Olga V. Polyakova[2], Viatcheslay B. Artaev[3], Albert T. Lebedev[2], Laurent Deguillaume[4], Gilles Mailhot[1] and Anne-Marie Delort[1*]

[1]Université Clermont Auvergne, CNRS, SIGMA Clermont, Institut de Chimie de Clermont-Ferrand, F-63000 Clermont-Ferrand, France
[2]Lomonosov Moscow State University, Chemistry Department, Leninskie Gory 1/3, Moscow, 119991, Russia
[3]LECO Corporation, 3000 Lakeview Avenue, St. Joseph, Michigan, 49085, USA
[4]Université Clermont Auvergne, CNRS, Laboratoire de Météorologie Physique, F-63000 Clermont-Ferrand, France

Correspondence to: Anne-Marie Delort (a-marie.Delort@uca.fr)

Abstract. Phenol is toxic and can be found in many environments, in particular in the atmosphere due to its high volatility. It can be emitted directly from manufacturing processes or natural sources, and it can also result from benzene oxidation. Although phenol biodegradation by microorganisms has been studied in many environments, the cloud medium has not been investigated yet as the discovery of active microorganisms in cloud is rather recent.

The main objective of this work was to evaluate the potential degradation of phenol by cloud microorganisms. Phenol concentrations were measured by GC-MS on two cloud samples collected at the PUY station (summit of puy de Dôme, 1465 m a.s.l., France): they ranged from 0.15 to 0.21 µg L^{-1}.

The strategy for investigating its potential biodegradation involved a metatranscriptomic analysis and metabolic screening of bacterial strains from cloud water collected at the PUY station for phenol degradation capabilities (from the 145 tested strains, 33 were isolated for this work).

Among prokaryotic messenger RNA enriched metatranscriptomes obtained from 3 cloud water samples, different from those used for phenol quantification, we detected transcripts of genes coding for enzymes involved in phenol degradation (phenol monooxygenases and phenol hydroxylases) and its main degradation product, catechol (catechol 1,2-dioxygenases). These enzymes were likely from Gamma-proteobacteria, a dominant class in clouds, more specifically the genera *Acinetobacter* and *Pseudomonas*.

Bacterial isolates from cloud water samples (*Pseudomonas* spp., *Rhodococcus* spp. and strains from the Moraxellaceae family) were screened for their ability to degrade phenol: 93% of the 145 strains tested were positive. These findings highlight the possibility of phenol degradation by microorganisms in clouds.

Main findings. Metatranscriptomic analysis suggested that phenol could be biodegraded in-clouds, while 93% of 145 bacterial strains isolated from clouds were able to degrade phenol.

Key words. Cloud water, phenol, biodegradation, metatranscriptomics, puy de Dôme.

1 Introduction

Due to its toxicity, phenol is one of the main pollutants listed by U.S Environmental Protection Agency (US EPA list) and its concentration in drinking water is inspected and regulated (Michalowicz and Duda, 2007). In France, phenol limit concentration in drinking water is 0.5 µg L^{-1}. Phenol is issued from natural sources such as

39 organic matter decomposition and biomass burning (Schauer et al., 2001), but it mainly results from industrial
40 processes. For instance phenol is involved in the production of oils, xylene, plastics, drugs, explosives, dyes,
41 pesticides; it is also present in oil refining and wood and leather preservatives (Gami et al., 2014; Schummer et
42 al., 2009). The annual phenol production exceeds 10.7 million tones worldwide in 2016 (Merchant Research &
43 Consulting Itd). Phenol has an environmental impact, particularly on the aquatic biota (microorganisms,
44 protozoa, invertebrates and vertebrates) (Babich and Davis, 1981; Duan et al., 2018). Phenol represents also a
45 risk for human beings because it can be rapidly absorbed through the skin and by inhalation through the lungs. In
46 particular it provokes cutaneous exfoliation and cardiac arrhythmias; it is also toxic to the liver and kidneys
47 (Babich and Davis, 1981; Lober, 1987) (National Library of Medicine HSDB Database:
48 https://toxnet.nlm.nih.gov/cgibin/sis/search/a?d-bs+hsdb :@term+@DOCNO+113).
49 Phenol can be found in all environmental compartments (soil, water), including the atmosphere (Atkinson et al.,
50 1992; Rubio et al., 2012). Even if its volatility is low (≤ 7% at 25 °C; The National Institute for Occupational
51 Safety and Health (NIOSH)), phenol is present in the gas phase, but this polar compound can also be transferred
52 to the aqueous phases of the atmosphere (rain, snow, clouds) thanks to its solubility described by the Henry's
53 law constant (H = 3.2 10^3 M atm^{-1} at 298 K and mass accommodation = 2.7 10^{-2} at 283 K; (Harrison et al., 2002;
54 Heal et al., 1995)). Phenol can also be formed by the oxidation of precursors such as benzene directly in the
55 atmosphere both in the gas and the aqueous phase (Grosjean, 1991; Harrison et al., 2005; Herrmann et al., 2015;
56 Vione et al., 2004). The production of phenol by the aqueous phase reactivity is expected to be less efficient than
57 in the gas phase. Indeed, benzene is precursor of phenol but it will not accumulate in the droplet in significant
58 amount due to its relatively low Henry's law constant (H = 1.8 10^{-1} M atm^{-1}). Phenol concentration ranges from
59 2.8 to 8.9 µg L^{-1} (0.03 to 0.09 µM) in cloud waters and it reaches up to 91.3 µg L^{-1} (0,97 µM) in rain (Harrison et
60 al., 2005; Schummer et al., 2009).
61 In the gas phase, phenol is transformed into nitrophenols either in the presence of $HO^•$ and $NO_2^•$ (during the day)
62 or in the presence of $NO_3^•$ and $NO_2^•$ (during the night) (Atkinson et al., 1992; Olariu, 2001; Olariu et al., 2002).
63 In the aqueous phase, phenol can undergo transformations that should be much faster than in the gas phase
64 leading to the formation of nitrophenols (Vione et al., 2004). Recent studies show that direct photolysis should
65 be competitive to the radical driven one for phenol (Rayne et al., 2009) and that phenol exposed to
66 atmospherically relevant photochemical conditions lead to the production of low-volatile compounds such as
67 light-absorbing molecules (HULIS). In-cloud processing of phenol can therefore be a source of Secondary
68 Organic Aerosol (SOA) (Gilardoni et al., 2016; Sun et al., 2010).
69 A great number of studies have been conducted to assess the biodegradation of phenol by microorganisms
70 including bacteria, fungi, yeast and algae in the context of environmental and water treatment chemistry
71 (Michalowicz and Duda 2007). Most of those microorganisms were isolated from soils (including the
72 rhizosphere) and waters (fresh and marine waters, waste waters and sediments) where contamination by phenol
73 has been studied (Basha et al., 2010; Kafilzadeh et al., 2010; Michalowicz and Duda, 2007; Mishra and Kumar,
74 2017; Sandhu et al., 2009; Sridevi et al., 2012; Tian et al., 2017). Only one team has focused on atmospheric
75 phenol uptake by microorganisms (Sandhu et al., 2007, 2009). They studied microbial community on leaves
76 directly in contact with phenol in the air and found that they were able to degrade it. Many studies are based on
77 direct measurement of the biodegradation activity of microbial isolates, in particular for biotechnological
78 application in industrial effluent decontamination (Basha et al., 2010; Michalowicz and Duda, 2007; Mishra and

79 Kumar, 2017; Sridevi et al., 2012). Alternatively others used molecular based approaches and reported microbial
80 genes of phenol or catechol degrading enzymes (Brennerova et al., 2009; Fang et al., 2013; Sandhu et al., 2009;
81 Sharma et al., 2012; Silva et al., 2013; Suenaga et al., 2009) or gained knowledge from metatranscriptomic
82 analyses of microbial communities (Auffret et al., 2015). Ajaz et al. (2004) have identified thirty bacterial strains
83 resistant to phenol in garden soil and Padmanabhan et al. (2003) have done DNA-SIP with ^{13}C-labeled phenol to
84 identify 6 phenol-degrading populations in soil thanks to 16S rRNA gene analysis. Main bacterial genera able to
85 biodegrade phenol are *Pseudomonas*, *Rhodococcus*, *Acinetobacter* and *Bacillus*, other genera are also described
86 such as *Arthrobacter*, *Alcalinogenes, Burkholderia, Thauera*, *etc.* (Basha et al., 2010; Fang et al., 2013; Jadeja et
87 al., 2014; Michalowicz and Duda, 2007; Padmanabhan et al., 2003; Silva et al., 2013). Major biodegradation
88 pathways for aerobic bacteria have been established (Figure 1) : first phenol can be oxidized into catechol by
89 phenol hydroxylases or phenol monooxygenases, then the ring cleavage can be catalyzed by dioxygenases,
90 catechol 1,2-dioxygenase produces cis-cis-muconate ("ortho" pathway) while catechol 2,3-dioxygenase leads to
91 2-hydroxymuconate semialdehyde ("meta" pathway). Finally these products are integrated in the central
92 metabolism of the bacteria and end up in CO_2 production (Basha et al., 2010). Alternative pathways have been
93 described with anaerobic microorganisms. In these cases, phenol is carboxylated by a carboxylase in the para
94 position to produce 4-hydroxybenzoate and this metabolite is further metabolized in benzoyl-CoA *via* anaerobic
95 routes before its ring opening step (Basha et al., 2010).
96 Although phenol is present in clouds, to our knowledge its transformation by microorganisms in these specific
97 environments has never been assessed. Bacterial density usually ranges from 10^4 to 10^5 cells per mL of cloud
98 water (Vaïtilingom et al., 2012). In spite of the numerous atmospheric stresses, it has been shown that
99 microorganisms can survive in clouds, maintain metabolic activity and degrade organic compounds (Delort et
100 al., 2010, 2017; Hill et al., 2007; Sattler et al., 2001; Vaïtilingom et al., 2013). Among bacteria known for phenol
101 degradation, *Pseudomonas* (Gamma-proteobacteria) and *Rhodococcus* (Actinobacteria) notably are frequently
102 found viable and potentially active in clouds (Amato et al., 2017a, 2017b).
103 The aim of this work was to explore the potential for phenol biodegradation in clouds. First, phenol
104 concentration was quantified in atmospheric waters, and cloud water metatranscriptomes were checked for the
105 presence of transcripts of phenol-degrading genes; second, bacterial strains isolated from cloud water were
106 screened for phenol biodegradation ability.

107 **2 Materials and Methods**

108 **2.1 Chemical reagents**

109 Phenol (>99%) and hydrogen peroxide (30%) were obtained from Fluka, sodium chloride (>99%),
110 dichloromethane (>99.8%) and sulfuric acid (>95-97%) were from Sigma Aldrich, acetonitrile (>99.9%) was
111 from VWR Chemicals, NaOH (99%) from Merck, and $MgSO_4$ (>98%) from Carlo Erba reactifs – SDS.

112 **2.2 Cloud water analysis**

113 **Cloud sampling:** Cloud waters have been sampled at the PUY station (summit of the puy de Dôme, 1 465 m
114 above the sea level, 45°46' North, 2°57' East, France) which is part of the atmospheric survey networks EMEP
115 (the European Monitoring and Evaluation Programme), GAW (Global Atmosphere Watch), and ACTRIS

116 (Aerosols, Clouds, and Trace gases Research Infrastructure). The sampling site is fully described in Deguillaume
117 et al. (2014). The global meteorological context was examined through 120 h back-trajectories of the air masses
118 sampled using the HYSPLIT model (HYbrid Single-Particle Lagrangian Inte-grated Trajectory). Two cloud
119 water samples collected in 2016 (October 21th and October 26th) were analyzed in this work for phenol
120 quantification by GC-MS. Three other samples were previously sampled and analyzed in 2013 (November 05th),
121 2014 (June 27th) and 2016 (February 16th) (Lebedev et al., 2018). Samples were collected using a sterilized cloud
122 droplet impactor and immediately filtered through Minisart® PES filter (0.22 µm porosity; Sartorius, Germany)
123 under sterilized conditions; these have been stored at -25 °C.
124 **GC-MS analysis:** Sample preparation was carried out according to US EPA 8270 method. Prior utilization, all
125 the glassware was cleaned with piranha reagent composed of 6 mL of sulfuric acid mixed with 2 mL of hydrogen
126 peroxide. The reagents were kept in the glassware for one night and after all the glasses were washed two times
127 with ultrapure water and two times with dichloromethane. With clean dishes, cloud waters kept frozen were
128 melted at room temperature and the pH adjusted to pH = 2 and 11. Organic compounds were extracted tree times
129 with dichloromethane (keeping the ratio 10 mL of water for 1 mL of CH_2Cl_2). All the dichloromethane fractions
130 were then dried with $MgSO_4$ and evaporated to 1 mL using a rotary evaporator under reduced pressure;
131 temperature of the water bath was 20 °C. Samples were kept at 4 °C until analysis
132 All analyses related to cloud samples collected the 21th and 26th of October 2016 were performed at Saint Joseph,
133 MI at LECO Corporation (USA). Accurate GC-MS measurements were performed with a high resolution time-
134 of-flight mass spectrometer Pegasus® GC-HRT in GC mode (software ChromaTOF-HRT). The obtained EI
135 mass spectra were used for phenol identification by utilizing high mass accuracy data and retention time
136 (Lebedev et al., 2013). Phenol concentrations were measured using naphthalene D8 as internal standard.
137 Response factor (0.7) was calculated using standard solution of phenol. Phenol concentrations measured in cloud
138 water samples collected the 5-11-2013, 27-06-2014 and 16-02-2016 are extracted from Lebedev et al. (2018).

139 **2.3 Analysis of metatranscriptomes**

140 **Transcriptomic analysis:** Cloud water samples were collected on November 17th, 2014, for three consecutive
141 periods of 5 h. The cloud air mass origin remained stable over the duration of sampling as attested by air mass
142 backward trajectories (Figure SM1). The clouds droplets collected by impaction were immediately transferred by
143 gravity into sterile collection bottles (Nalgene, Rochester, U.S.A) through sterile (autoclaved) silicone tubing.
144 Before cloud sampling operations started, control samples were made by pouring 200 mL of sterile water into
145 the collection device and through the tubing, and by processing it in parallel of the cloud water samples,
146 including sequencing and data treatment. These controls were clearly distinct from samples: based on their
147 contribution to identified ribosome sequences, these contained mostly Enterobacteriaceae (66%), Dikarya
148 (9.2%), Streptococcaceae (5.4%), Vibrionaceae (2.8%) and Micrococcaceae (1.2%), *i.e.* not the taxa of interest
149 here. Conservatively, the sequences present in controls were further removed from sample files (BWA-MEM; li
150 et al., 2013). Immediately after collection, water sample were filtered (MoBio 14880-50-WF) within an UV-
151 sterilized laminar flow hood installed at the sampling site. The filters were then put into ~5 mL of RNA Later
152 solution (Sigma, Steiheim, Germany) and stored at -80 °C until further processing. Briefly, total RNA were
153 extracted from filter halves using MoBio Power Water RNA kit and bacterial ribosomal RNA were depleted
154 using MICROB*Express* Bacterial mRNA Enrichment kit (Life Technologies). Metatranscriptomes of the

155 messenger RNAs were then obtained by multiple displacement amplification using REPLI-g WGA & WTA kit.;
156 Shotgun libraries were sequenced on Illumina MiSeq paired-end 2*300 bp. Sequencing reads were quality
157 checked (FastQC, Andrews, 2010) and trimmed (PRINSEQ-Lite, Schmieder and Edwards, 2011) before
158 assembling the mate pairs using PANDA-SEQ (Masella et al., 2012). Annotations were made against
159 UNIPROTKB database (Leinonen et al., 2006), including protein sequences for bacteria, archaea, and fungi
160 using BLASTX software (best hits with e-value < 10^{-4}). All steps were performed using custom scripts. The
161 sequence files have been deposited to European Nucleotide Archive (ENA) under the study accession number
162 PRJEB25802.
163 **Bioinformatics treatment:** Known enzymes involved in phenol degradation were found in KEGG Database
164 (see Figure 1). We only focused on aerobic metabolism as cloud environment is highly oxidative. Four
165 nucleotide sequences databases were created from NCBI corresponding to phenol hydroxylases (69 sequences),
166 phenol monooxygenases (29 sequences); catechol (regrouping catechol 1,2-dioxygenases and catechol 2,3-
167 dioxygenases) (145 sequences) and a fourth database including genes coding for putative phenol degradation
168 enzymes (38 sequences). The sequences from the cloud metatranscriptomes corresponding to the different
169 created databases were then extracted suing Bowtie2 (very-sensitive option; Langmead and Salzberg, 2012). The
170 affiliation of the extracted sequences was determined using blastn on a local server (e-value = 0.00001; Camacho
171 et al., 2009).

172 **2.4 Biodegradation of phenol by bacterial strains from cloud waters**

173 **Bacterial strains**: Bacterial strains were isolated from cloud waters sampled at the PUY station and identified as
174 previously described in Vaïtilingom et al. (2012). From our lab strain collection, we choose all the potential
175 bacteria that could biodegrade phenol. From the 145 strains tested, 33 of the strains were isolated for this work,
176 the others were published earlier (see Table SM1).
177 119 *Pseudomonas*, 24 *Rhodococcus* strains and 2 strains form the Moraxella family were selected for the
178 screening of phenol degradation (see Table SM1). More precisely Pseudomonas strains included 4 *P.*
179 *fluorescens*, 10 *P. graminis,* 1 *P. grimontii*, 2 *P. poae*, 1 *P. reactans*, 1 *P. reinekii*, 3 *P. rhizosphaerae*, 35 *P.*
180 *syringae*, 2 *P. trivialis*, 2 *P. veronii*, 1 *P. viridiflava* and 57 *Pseudomonas sp. Rhodococcus* strains included 1 *R.*
181 *erythropolis*, 1 *R. enclensis* and 22 *Rhodococcus sp.* Moraxella family strains were 1 *Moraxella sp* and 1
182 *Psychrobacter sp*.
183 *Pseudomonas* and *Rhodococcus* strains represent 20.4% and 4.10% of the 584 strains of our cloud bacterial
184 collection. From our experience, at the genus level, *Pseudomonas* and *Rhodococcus* are among the most frequent
185 bacteria in clouds: *Pseudomonas* strains in particular have been frequently isolated by culture (Vaïtilingom et al.,
186 2012; Joly et al., 2013), and both targeted and untargeted molecular analyses (and metagenomes, respectively)
187 demonstrated high occurrence in the bacterial communities. These represented 0.1 to >2% of the prokaryotes
188 ribosome sequences in amplicon sequencing investigations (Amato et al., 2017a). Based on the biomass in
189 clouds (~10^4 bacteria cells mL^{-1}; Vaïtilingom et al., 2012), and assuming even ribosome amplification between
190 bacterial groups, we can infer the presence of ~10^3 *Pseudomonas* mL^{-1} and ~10^2 *Rhodococcus* mL^{-1} of cloud
191 water.
192 **Incubations**: All the strains were grown in 25 ml of R2A medium for 48 h at 17 °C, 130 rpm (Reasoner and
193 Geldreich, 1985). Then cultures were centrifuged at 4 000 rpm for 15 min. Bacteria pellets were rinsed first with

194 5 mL of NaCl 0.8% and after with Volvic® mineral water previously sterilized by filtration under sterile
195 conditions using a 0.22 µm PES filter. Cells were re-suspended in 5 mL of 0.1 mM phenol solution, prepared in
196 Volvic® mineral water, and incubated at 17 °C, 130 rpm agitation during 5 days in the dark. To know the
197 concentration, the OD for each strain was taken during the experiment. Strains concentration was around 10^9
198 cells mL^{-1}. The ratio number of bacterial cells / phenol concentration was kept to that measured in cloud waters,
199 in clear all the concentrations were multiplied by a factor 10^4. Indeed the mean bacteria concentration is around
200 10^5 cells mL^{-1} of cloud water while that of phenol can reach 0.008 µM (0.74 µg L^{-1}, see the result section) in
201 clouds collected at the PUY station. We showed in the past that when the cell / substrate ratios are kept constant
202 the rates of biodegradation are constant (Vaïtilingom et al., 2010). The temperature (17 °C) corresponds to the
203 average temperature at the PUY station in summer under cloud condition. It is well known that under culture
204 conditions in a laboratory, a lag time can be observed before bacteria starts to biodegrade phenol that
205 corresponds to the induction period of the gene expression (Al-Khalid and El-Naas, 2012).
206 Before sampling, evaporation of water has been compensated by adding Volvic® mineral water. A control
207 experiment was performed by incubating phenol without bacteria; phenol concentration remained stable with
208 time (0.1 mM of phenol was obtained at the end of the experiment). For phenol quantification over time in the
209 incubation experiments, 600 µL samples were centrifuged at 12 500 rpm for 3 min and the supernatants were
210 kept frozen until HPLC analysis.
211 **Phenol HPLC analysis:** Before analysis, all the samples were filtered on H-PTFE filter (pore size at 0.2 µm and
212 diameter of 13 mm from Macherey-nagel, Germany). Phenol detection was done on HPLC VWR Hitachi
213 Chromaster apparatus fitted with a DAD detector and driven by Chromaster software. Isocratic mode was used
214 with a reverse phase endcapped column (LiChrospher® RP-18, 150 mm x 4.6 mm, 5 µm, 100 Å). Mobile phase
215 was composed of acetonitrile and filtered water (Durapore® membrane filters, 0.45 µm HVLP type, Ireland) in
216 25/75 ratio with a flow rate at 1.2 mL min^{-1} (adapted from Zhai, 2012). Sample injection volume was 50 µL,
217 spectra were recorded at 272 nm and the run time was 10 min.
218 **Phenol degradation:** Percentage of phenol degradation was calculated by the following equation:
219
$$\text{Phenol degradation (\%)} = 100 - \frac{[Phenol]_{final} \times 100}{[Phenol]_{initial}} \qquad (1)$$
220 Limit of phenol quantification was 3.8 µM. Strains are not considered active below 5% of phenol degradation,
221 corresponding to 5 µM.
222 Comparison of strain phenol degradation abilities was done using a non-parametrical Kruskal-Wallis test (p.
223 value < 0.05) with Past software.

224 **3 Results**

225 **3.1 Phenol quantification in cloud waters**

226 The objective of this paper was to explore the ability of microorganisms isolated or present in cloud waters
227 collected at the PUY station to degrade phenol. From the 145 tested strains, 33 were isolated in this work, the
228 others were already published (Table SM1). We first checked its presence in cloud waters sampled at the PUY
229 station by performing GC-MS analysis. Figure SM2 presents the back trajectories of the air masses

230 corresponding to the 5 cloud events at the PUY station. The air mass origins of the 5 cloud samples determined
231 from these back trajectories were classified as described in Deguillaume et al. (2014) and are reported in Table 1.
232 The GC-MS analysis performed on cloud samples allowed reliable identification and quantification of phenol in
233 all samples (Table 1), the measured phenol concentrations ranged from 0.15 to 0.74 µg L^{-1}. Figure SM3 (A)
234 represents, as a typical example, the total ion current chromatogram of the cloud sample collected the 16th of
235 February 2016, the corresponding mass-chromatogram based on the ion 94 current (characteristic for phenol) is
236 represented in Figure SM3 (B). Quantification was done using similar mass chromatograms of all samples and
237 the identification was proven by the correct retention time and accurate mass measurements (calculated:
238 94.0413; experimental: 94.0414).

239 **3.2 Possibility of in-cloud phenol degradation by the cloud microbiome using a meta-transcriptomic**
240 **analysis**

241 The presence of transcripts involved in the biodegradation of phenol (Figure 1) was investigated from
242 prokaryotic messenger RNA enriched metatranscriptomes obtained from 3 cloud water samples. Sequence data
243 were looked for the presence of transcripts of genes involved in phenol biodegradation among the 281 sequences
244 included in our database (more details about the affiliation of the sequences are given in Table SM2).
245 Gene transcripts were detected for all the enzymes, except the catechol 2,3-dioxygenase, showing a possible
246 implication of the microorganisms in the degradation of phenol in cloud (Figure 2). However the number of hits
247 and the relative abundance of the transcripts coding for the different enzymes varied according the cloud
248 samples. Two hundred fifty-seven hits (sequence homology) could be counted for cloud 2, for only 70 in cloud 1
249 and 130 in cloud 3. Transcripts corresponding to the enzyme involved in the first step of oxidation of phenol
250 leading to catechols (phenol hydroxylases and phenol monooxygenases) were the most abundant in clouds 2 and
251 3 while those corresponding to the cleavage of the catechol ring (catechol 1,2-dioxygenase) were dominant in
252 cloud 1. For all the samples the transcripts corresponding to putative phenol degradation enzyme pathways (i.e.
253 none explicitly described enzymes) remained low. However the slight differences observed between the 3 cloud
254 samples are not significant when analyzed by a non-parametrical Kruskal-Wallis test.
255 Figure 3 presents the relative abundance of putative taxonomic affiliation of microorganisms involved in phenol
256 biodegradation, based on the information associated with sequences in the databases. All the sequences were
257 affiliated with Gamma-proteobacteria, from only two genera, namely *Pseudomonas* and *Acinetobacter*,
258 corresponding to only four species (*P. fluorescens, A. gyllenbergii, A. oleivorans* and *A. pitii*) matched with
259 cloud transcripts, among a total of 50 (Table SM2). This very low diversity was unexpected considering that
260 sequences from 50 bacterial genera including 109 species were used for our search in data bases. In addition the
261 relative abundance of sequences affiliated to a bacterial species varied a lot with the considered enzymes and
262 clouds (Figure 3).
263 Gamma-proteobacteria were found to contribute up to 21% of the ribosome sequences identified in bacteria in
264 targeted sequencing investigations. *Pseudomonas* in particular was highlighted as one of the most represented
265 genus (contributing alone up to 2% of the ribosome sequences) and most active genera based on its
266 representation in transcriptomes and consecutive high ribosomal cDNA:DNA ratio (Amato et al, 2017a; Figure
267 SM4). *Acinetobacter* and *Rhodococcus* were much less represented (<0.1% of the ribosome sequences) but also
268 accounted for groups of interest regarding potential metabolic activity.

269 *Rhodococcus* were previously isolated from clouds at the PUY station (Vaïtilingom et al., 2012) but genes for
270 phenol degradation affiliated with this genus were not detected here.

271 3.3 Screening of bacterial strains isolated from cloud waters for their ability to biodegrade phenol

272 From our strain collection of 826 culturable microorganisms isolated from clouds collected at the PUY station
273 between March 2003 and June 2016, we selected strains belonging to genera of interest concerning their
274 potential ability for phenol biodegradation. We choose to test specifically *Pseudomonas* and *Acinteobacter*
275 strains as they were detected in our metatranscriptomic analysis. As no *Acinetobacter* was available in our
276 bacterial collection we choose closely related genera namely two strains of *Moraxella* and *Psychrobacter*. In
277 addition *Rhodococcus* is well-known to biodegrade phenol in the literature (as well as *Pseudomonas* and
278 *Acinetobacter*). *Pseudomonas* and *Rhodococcus* are also the most frequently found genera in culturable bacteria
279 from clouds (Renard et al., 2016; Vaïtilingom et al., 2012). Altogether 145 bacterial strains were tested (Table
280 SM1). The percentage of phenol degradation measured by HPLC after 5 days of incubation at 17 °C is reported
281 in Figure 4 and in Table SM1. As the objective of the work was to perform a large screening with different types
282 of cells, incubation duration of 5 days was chosen to be sure that the induction period necessary for laboratory
283 experiments was long enough to be able to detect biodegradation ability for all the tested cells. This time is quite
284 long for a cloud but the objective here is not to evaluate a rate of biodegradation but to investigate the potential
285 of biodegradation of microorganisms present in cloud waters.
286 We found that 93.1% of the 145 tested strains were able to degrade phenol after 5 days of incubation. Globally,
287 in our experimental conditions, all the families tested were very good phenol degraders (see Figure 4A). No
288 significant difference was found in the capacity of phenol degradation between *Pseudomonas*, *Rhodococcus* and
289 *Moraxellaceae* strains. A focus on the *Pseudomonas* strains according to their species is presented in Figure 4B.
290 The mean capacity of phenol degradation varied between 31 and 67% (for *Pseudomonas rhizosphaerae* and
291 *Pseudomonas graminis* respectively), however no significant difference was observed between the species
292 according to the Kruskal-Wallis test. Considering specifically *Pseudomonas syringae* strains which are the most
293 abundant species among cultural strains present in cloud waters (Renard et al., 2016), only 2 of them out of 35
294 were not capable of degrading phenol (strains PDD-32b-31and PDD-69b-20, see Table SM1).

295 4 Discussion and conclusion

296 Phenol was present in the cloud water samples at concentrations ranging from 0.15 to 0.74 µg L^{-1}; these values
297 are within the range usually measured in atmospheric waters at remote sampling sites (3.0 to 5.4 µg L^{-1}, Harrison
298 et al., 2005), but globally the concentrations measured at the PUY station are rather in the lower range of values.
299 Although the concentration of phenol remains within the same order of magnitude in the 5 cloud samples, it
300 seems that the origin of the air masses had an impact on this concentration; it was 3 times lower in non-polluted
301 air masses (West) than in polluted ones (North West/North).
302 The results reported combining molecular approach and biodegradation assays involving culturable bacteria
303 indicate that phenol-degrading microorganisms are present in clouds. Molecular approach allowed detection of
304 transcripts belonging to *Pseudomonas* and *Acinetobacter* strains but not for the sequences of the other strains
305 present in Table SM2. It was surprising not to find *Rhodococcus* sequences as this genus is well-known to
306 degrade phenol as reported in the literature. In parallel *Rhodococcus* strains isolated from clouds were very

307 active phenol degraders but no *Acinetobacter* have been isolated from clouds. This difference reflects the
308 complementarity but also the bias of each approach (molecular *vs* cultural). Meta-transcriptomic can be biased
309 by technical issues (extraction, sequencing, *etc.*) or by the creation of uncomplete database. In the future, the
310 database for phenol degradation could be improved by integrating more sequences, especially considering other
311 data banks than NCBI. For instance the catechol operon sequences of *Pseudomonas synringae* (Berge et al.,
312 2014) could be added to the data base. We recently published the genome sequence of *Pseudomonas syringae*
313 32b-74, *Pseudomonas graminis* 13b-3 and *Rhodococcus enclensis* 23b-28 which are degrading phenol (Table
314 SM1) (Besaury et al., 2017a, b; Lallement et al., 2017); they could be used to implement the database. Finally in
315 the future the genome of many phenol degraders (Table SM1) could be also sequenced and integrated.
316 On the other hand it is well known that culturable microorganisms only represent 1% or less than the total
317 community, notably in clouds (Amann et al., 1995, Vaïtilingom et al., 2012). Strains of *Acinetobacter*,
318 *Pseudomonas* and *Rhodococcus* genera are known to degrade phenol in other environments (Basha et al., 2010;
319 Gami et al., 2014; Michalowicz and Duda, 2007; Sandhu et al., 2007). The cloud microbiota as described from a
320 culturable approach harbors species usually affiliated with the phyllosphere (Amato et al., 2017b; Vaïtilingom et
321 al., 2012). Sandhu et al. (2007) explored the presence of phenol degraders among microbial communities on
322 plant leaves. They did not find *Pseudomonas* but they isolated *Acinetobacter* and *Rhodococcus* strains, and
323 noticed globally a low diversity of phenol degraders. Only the genes encoding for the ortho pathway for phenol
324 degradation that involves the catechol 1,2-dioxygenase activity were present in both Proteobacteria and
325 Actinobacteria. Similarly, we did not find transcripts of genes coding for catechol 2,3-dioxygenase but only
326 those coding for phenol hydroxylase, phenol monooxygenease and catechol 1,2-dioxygenase. In principle
327 bacteria can have either *ortho* or *meta* pathways, or both, but their expression is dependent on phenol
328 concentration. The enzyme catechol 1,2-dioxygenase is produced at low phenol concentration while catechol
329 2,3-dioxygenase enzymes become dominant at high phenol concentrations (3 mM) (Sandhu et al., 2009). This
330 might explain why bacteria from clouds and the phyllosphere only produce catechol 1,2-dioxygenase as the
331 phenol concentration in the atmosphere is much lower than in polluted surface water for instance (in the range of
332 a few µg L^{-1} versus 100 to 1000 µg L^{-1}) (Gami et al., 2014; Harrison et al., 2005; Schummer et al., 2009; Sturaro
333 et al., 2010).
334 In our study we focused on *Pseudomonas* strains as they are the most frequent culturable strains (Vaïtilingom et
335 al., 2012) and belong to the most active strains in cloud waters (Amato et al., 2017a). We observed that these
336 strains likely issued from the phyllosphere, *P. graminis P. syringae, P. fluorescence, P. poae* and *P. viriflava*
337 were able to degrade phenol. In the literature, *P. aeruginosa* and *P. putida* are the most popular phenol degraders
338 (Basha et al., 2010; Der Yang and Humphrey, 1975; Erhan et al., 2004; Gami et al., 2014; Kumar et al., 2005;
339 Molin and Nilsson, 1985). Interestingly Bartoli et al. (2015) showed that the genome of several *P. syringae*
340 pathogens of woody plants contained a catechol operon, while it was not the case for other *P. syringae* strains
341 pathogens of herbaceous plants. These results strongly suggested that the presence of enzymes present in the
342 catechol pathway could help the degradation of aromatics present in lignins. In addition Berge et al. (2014)
343 showed that some *P. syringae* strains from phylogroups 1 and 3 that were Ice Nuclei Active (INA^+) also
344 contained the catechol operon. In our case we also measured the ice nucleation activity of the 35 *Pseudomonas*
345 *syringae* strains as described in Joly et al. (2013). Figure SM5 presents the strains which were INA^+ (T>-8 °C)
346 *versus* their phenol degradation ability. Among the phenol degrader, 57.6% of the bacteria were INA^+.

Clouds can be considered as medium for microorganism transport and INA^+ bacteria are suspected to induce precipitations and thus participate to the water cycle (Morris et al., 2008). Consequently, the presence of *Pseudomonas syringae* in clouds combining ice nucleation and phenol degradation properties can be of major importance for the pathogenicity on woody plants, in terms of epidemiology, dispersion of pathogens and emergence of plant diseases.

We showed that microorganisms from clouds were able to degrade phenol. The question raises what is the potential impact of this biotransformation on the fate of phenol in real clouds? First the presence of transcripts of phenol-degrading enzymes measured directly *in situ* demonstrates a real in-cloud activity of microorganisms. However, these data do not give any exact quantitative contribution of the microbial activity to the phenol transformation in real clouds. On the other hand the large screening performed with selected cloud strains showed that they have the enzymatic equipment for phenol degradation. Future work should be conducted to evaluate this potential for phenol biodegradation in real clouds where a larger microbial diversity is present. In particular, precise biodegradation rates should be determined under "realistic cloud conditions" to evaluate its real impact. It will be also very important to compare the relative contribution of biological degradation *versus* radical chemistry, especially with photochemistry. It is well known that phenol can react with $^{\bullet}OH$, $NO_2^{\bullet}$, $NO_3^{\bullet}$ radicals alone or in combination to give rise to catechol, 2-nitrophenol, 4-nitrophenol and 2,4-diniropenol, these compounds can be further degraded in intermediates after the ring cleavage (Harrison et al., 2005).

In addition of examining the presence of phenol degrading pathways, we also looked for biological pathways leading to the potential formation of phenol from benzene (Choi et al., 2013; Tao et al., 2004), involving toluene monooxygenases in 8 species of Actinobacteria, Alpha- and Beta-proteobacteria (Table SM3). None of these sequences were found in the cloud prokaryote metatranscriptomes. This result should be confirmed by incubating cloud microorganisms directly with benzene to assess the real potential of cloud microorganism to produce phenol under these conditions. Consequently the origin of phenol in cloud waters could only result of the mass transfer from the gas phase to the aqueous phase or of the production *via* radical processes in the atmosphere. For instance the production of phenol in the gas phase can result from the reactivity of $^{\bullet}OH$ radical with benzene (Grosjean, 1991; Volkamer et al., 2002). Considering that benzene has a very low solubility in water, it is likely that the production of phenol mainly occurs in the gas phase and is transferred to the water phase. The contributions of biotic or abiotic transformation of benzene into phenol in the water phase should remain minor processes.

In conclusion, this is the first report showing that cloud water is inhabited by microorganisms that have phenol degradation ability. The study was centered on bacteria present in cloud waters collected at the PUY station where phenol concentrations were measured by GC-MS and found in the range of 0.15 to 0.74 $\mu g\ L^{-1}$. Metatranscriptomic analysis suggested that phenol could be biodegraded in-clouds, while a large screening of isolated strains showed that the enzymatic equipment to degrade phenol was not rare. These two combined approaches suggested that *Pseudomonas*, *Acinetobacter* and *Rhodococcus* strains were the major genera potentially involved in phenol biodegradation. Further work is needed to evaluate the relative contribution of this biological activity and radical chemistry (particularly photochemistry) to phenol transformation. For that, experiments will be set up to measure phenol biodegradation rates under realistic cloud conditions and compare them with abiotic degradation rates. This will bring valuable information to better describe the fate of this pollutant in the atmosphere. Since phenol is highly toxic and is one of the main pollutants listed by U.S

387 Environmental Protection Agency (US EPA, 1981), this work will help to better assess its impact on health and
388 air quality. The most probably, microorganisms could participate to a natural remediation process of the
389 atmosphere.

390 **Acknowledgments**

391 This work was mainly funded by the French ANR program BIOCAP (ANR-13-BS06-0004), the ANR-DFG
392 program CHLOROFILTER (ANR-DFG-14-CE35-005-02) and CNRS EC2CO program FONCOMIC.
393 The authors also acknowledge the financial support from the Regional Council of Auvergne, from the
394 Observatoire de Physique du Globe de Clermont-Ferrand (OPGC), from the Fédération de Recherche en
395 Environnement through the CPER Environnement founded by Région Auvergne-Rhône-Alpes, the French
396 ministry, and FEDER from the European community

397 **Ethics statements**

398 This work does not involve human or animal subject. There is no ethical problem.

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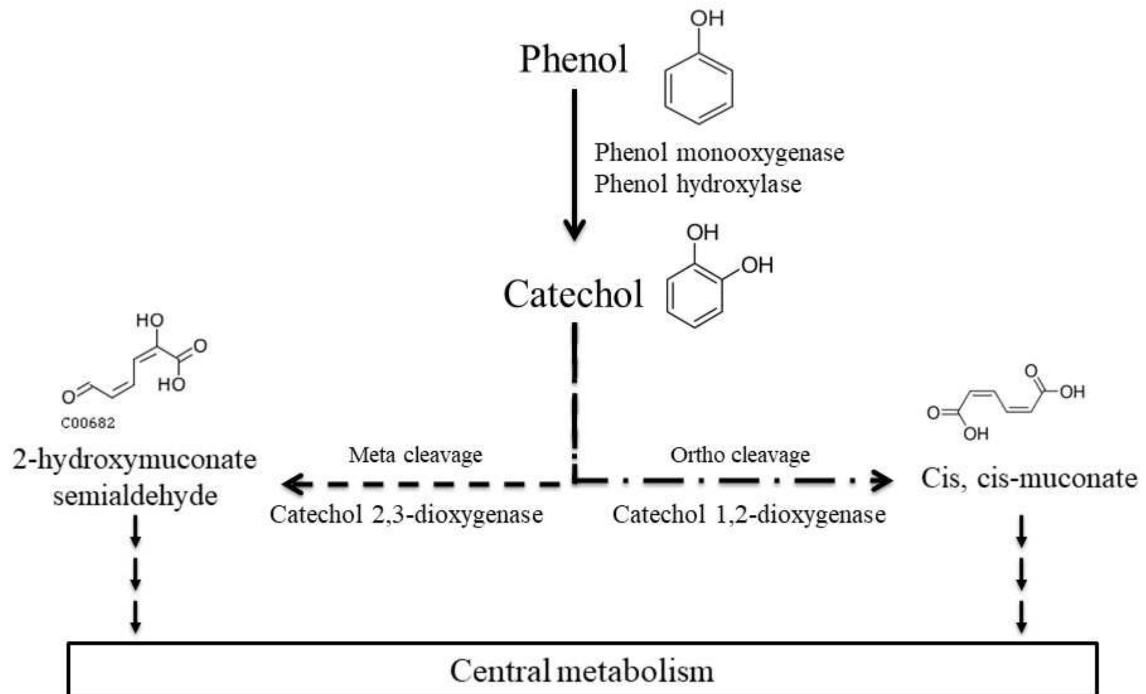

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606 **Figure 1: Main phenol biodegradation pathways described for aerobic microorganisms as referred in KEGG**
607 **database.**

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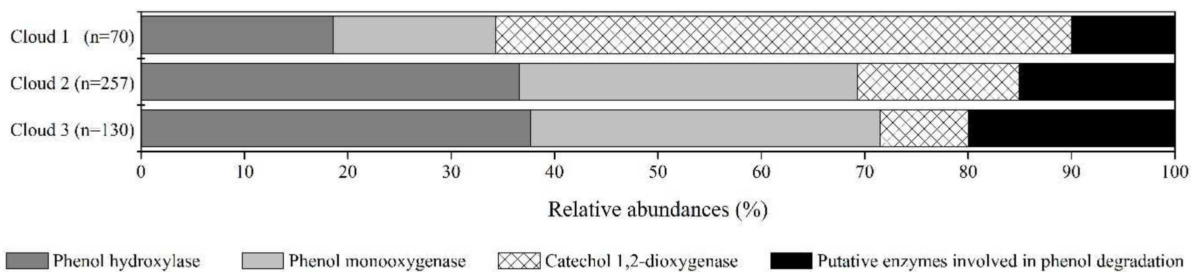
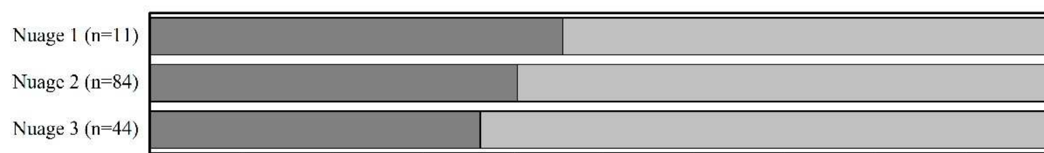
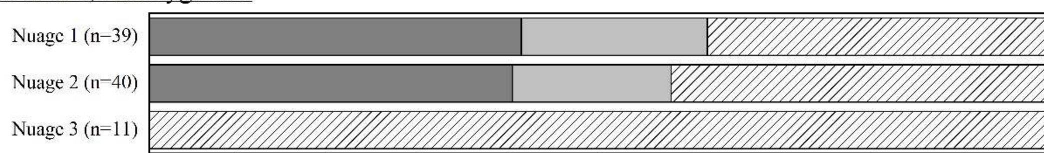


609 **Figure 2: Relative abundance of transcripts in cloud waters encoding for enzymes involved in phenol degradation**
610 **pathways. The absolute total number of hits for each sample is indicated (n).**





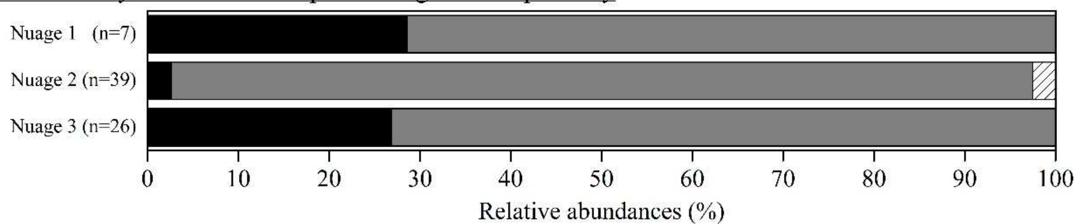


Figure 3: Relative abundance of the putative taxonomic affiliation of the microorganisms involved in phenol degradation. For the 5 databases, microorganisms associated to a matching sequence with cloud transcripts are plotted here; the absolute total number of hits for each sample is indicated (n).

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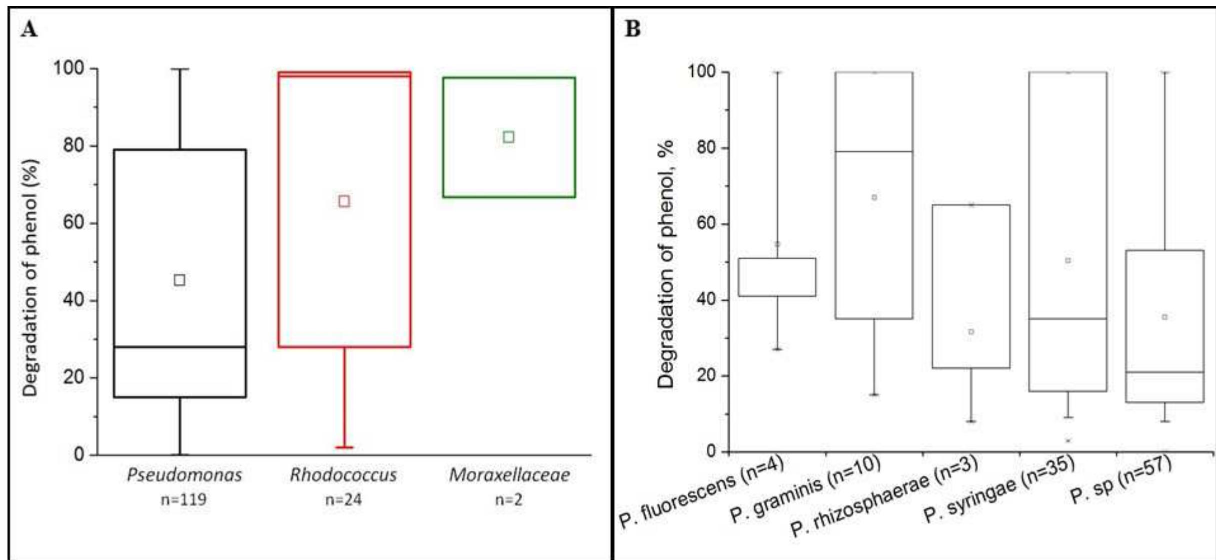

616 **Figure 4: Biodegradation of phenol by bacterial strains isolated from cloud waters. Results are expressed as the % of**
617 **phenol biodegradation measured by HPLC after 5 days of incubation at 17 °C. A) Results obtained for the 119**
618 ***Pseudomonas* strains, the 24 *Rhodococcus* strains, and the 2 strains from the Moraxellaceae family. B) Focus on the**
619 **Pseudomonas species. Only species groups with a minimum of 3 strains are plotted here.**

620

621 **Table 1: Phenol concentrations measured by GC-MS in the five cloud water sampled at the PUY station.**

Cloud water sampling date	Air mass origin	Phenol concentration (µg L^{-1})
05/11/2013[a]	West	0.52
27/06/2014[a]	West	0.73
16/02/216[a]	North East	0.74
21/10/2016b	North West/North	0.21
26/10/2016[b]	North West/North	0.15

622 [a] From Lebedev et al. (2018), [b] This work.