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Cyanobacteria and algae in clouds and rain in the area of puy de Dôme, Central France

Running Title: Cyanobacteria and algae in clouds and rain

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ABSTRACT: The atmosphere contains diverse living microbes, of which the heterotrophic community has been the most studied. Microbes with other trophic modes, such as photoautotrophy, have received much less attention. Here, culture-independent and dependent methods were used to examine the presence and diversity of oxygenic photoautotrophic microbes in clouds and rain collected at or around puy de Dôme Mountain, central France. Cloud water was collected from the summit of puy de Dôme (1465 m above sea level (a.s.l.)) for cultivation and metagenomic analysis. Cyanobacteria, diatoms, green algae, and other oxygenic photoautotrophs were found to be recurrent members of clouds, while green algae affiliated with the Chlorellaceae were successfully cultured from three different clouds. Additionally, rain samples were collected below the mountain from Opme meteorological station (680 m, a.s.l.).

The abundance of chlorophyll $a$-containing cells and the diversity of cyanobacteria and green algae in rain were assessed by flow cytometry and amplicon sequencing. The corresponding downward flux of chlorophyll $a$-containing organisms to the ground, entering surface ecosystems with rain, varied with time and was estimated between $\sim 1$ and $>300$ cells cm$^{-2}$ day$^{-1}$ during the sampling period. Besides abundant pollen from Pinales and Rosales, cyanobacteria of the Chroococcidiopsidales and green algae of the Trebouxiaceae were dominant in rain samples. Certain members of these taxa are known to be ubiquitous and stress-tolerant and could use the atmosphere for dispersal. Overall, our results indicate that the atmosphere carries diverse, viable oxygenic photoautotrophic microbes and acts as a dispersal vector for this microbial guild.

IMPORTANCE: Information regarding the diversity and abundance of oxygenic photoautotrophs in the atmosphere is limited. More information from diverse locations is needed. These airborne organisms could have important impacts upon atmospheric processes and on the ecosystems they enter after deposition. Oxygenic photoautotrophic microbes are integral to
ecosystem functioning and some have the potential to affect human health. A better understanding of the diversity and the movements of these aeolian dispersed organisms is needed to understand their ecology, as well as how they could affect ecosystems and human health.

**INTRODUCTION**

The atmosphere harbors a diverse microbial assemblage that can be free-floating, adhered to particles, or suspended in water droplets (e.g. clouds, fog, and rain). Bacteria are typically present at concentrations between $10^4$ to $10^5$ cells m$^{-3}$ in air (1) and about $10^3$ to $10^4$ cells mL$^{-1}$ of water in clouds (2). Microbes aerosolized from the surface environment including bodies of water, soils, plant surfaces, and other sources can rise in the atmosphere, be incorporated into clouds, and return to the surface via dry or wet deposition (for a general review, see Frölich-Nowoisky et al. (3)). Some can help to form cloud droplets and ice crystals by acting as nuclei for the condensation and freezing of water (4, 5). Over mid-latitude continental areas, the microbial assemblage is often dominated by heterotrophic bacteria such as *Pseudomonas* and *Sphingomonas* both in terms of abundance and activity (2, 6). These heterotrophic microbes have been studied for their interaction with atmospheric processes potentially affecting cloud chemistry (7). Other microbial guilds, such as oxygenic photoautotrophic microbes, have received less attention regarding their abundance, diversity, and activity in the atmosphere.

Based upon previous studies, the main airborne oxygenic photoautotrophic microbes are green algae, cyanobacteria, and diatoms (8). These are found in/on and can be aerosolized from surface environments including lakes, trees, and building facades (9-12). Their abundance in the air was reported to be affected by meteorological parameters such as wind direction and speed (13). The aerosols containing cyanobacteria and algae are important to study due to their impacts upon human health (11, 14), ice nucleation (15), and surface ecosystems by deposition and
colonization (16). In general, the atmospheric dispersal and cycling of oxygenic photoautotrophs is not well documented (17). Cyanobacteria have been detected in clouds at variable abundances as part of a few studies (2, 18, 19). However, the diversity of oxygenic photoautotrophs in clouds remains understudied. Most studies have been conducted on atmospheric cyanobacteria and algae in near-surface air environments, snow, and rain (20-22). For in-depth reviews about algae and cyanobacteria in the atmosphere, see Tesson et al. and Wiśniewska et al. (8, 23).

This study aimed to investigate the diversity and abundance of cyanobacteria and algae in clouds and rain at and around the puy de Dôme mountain (1465 m, 680 m above sea level (a.s.l.), respectively), a mid-altitude rural area in central France. We investigated metagenomes of cloud water and cultured cloud water samples for oxygenic photoautotrophic microbes by enrichment.

Additionally, chlorophyll a-containing cells were enumerated in rain samples, the corresponding downward fluxes were estimated, and the diversity of cyanobacteria and algae was assessed by targeted amplicon sequencing.

RESULTS

Cyanobacteria and green algae in clouds

Cloud water from the summit of the puy de Dôme was collected and used directly to cultivate microbial oxygenic photoautotrophs. Cloud water was collected on 01/06/2010, 08/06/2010, and 29/06/2017 (dd/mm/yyyy) for enrichment cultures of oxygenic photoautotrophs (referred to as 01062010, 08062010, and 29062017 cultures, respectively). The physical and meteorological characteristics for each cloud during the cloud water collection process are presented in Table S1. Diversity metrics for the enrichment culture communities are presented in Table S2. The main eukaryotic oxygenic photoautotrophic microbes cultured were aeroterrestrial epiphytic green algae of the Chlorellaceae (Figure 1A). The most dominant Chlorellaceae
operational taxonomic unit (OTU) in the 08062010 and 29062017 cultures, and second most abundant in the 01062010 culture, was most closely associated with *Chloroidium saccharophila* SAG 211-9a. The 01062010 culture was dominated by an unclassified eukaryote (Figure 1A), whose taxonomy could not be resolved further. Surprisingly, no cyanobacteria were detected in the oxygenic photoautotrophic cloud water enrichments (Figure 1B). Sphingomonadaceae, Chitinophagaceae, and Rhizobiaceae bacterial families were dominant in the 01062010, 08062010, and 29062017 cultures, respectively (Figure 1B).

Along with the culture-dependent approach, we used metagenomics to explore cloud-borne oxygenic photoautotrophs. Clouds were collected on 11/10/2013, 14/10/2013, and 05/11/2013 (denoted as 11102013, 14102013, and 05112013, respectively; Table S1). Cloud-borne oxygenic photoautotrophs were identified based upon taxonomy in six cloud metagenomes—three obtained for this study (11102013, 14102013, 05112013), and three that were previously described (24) (17112014-1, 17112014-2, 17112014-3). Oxygenic photoautotrophs were detected in all six cloud metagenomes, indicating that these were persistent members of the microbial assemblage in clouds (Table 1). Bacillariophyceae (diatoms) and Chrysophyceae (golden algae) were >1% of the SSU rRNA gene sequences across all six clouds, while the cyanobacteria were <1% of the sequences (Table 1).

In addition to taxonomy, we investigated functional genes related to photosynthesis. Overall, based on the UniProtKB identifiers and their associated gene ontology identifiers (GO IDs), the functional genes related to photosynthesis by cyanobacteria were rare in the metagenomes, ranging from 2 to 103 sequences in all clouds (Figure 2). The main genes identified were related to *psb* genes, which encode for components of photosystem II (Data Set S1). The dominant bacterial genera associated with the detected UniProtKB identifiers of the

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photosynthesis-related genes were *Lyngbya*, *Synechococcus*, and *Pseudanabaena* (Figure 2). The 11102013, 17112014-2, and 17112014-3 clouds only had 2 functional gene sequences associated with photosynthesis, and each were 100% associated with *Oscillatoria*, *Microcystis*, and *Crocosphaera*, respectively (Figure 2). Functional genes associated with photosynthesis by eukaryotic photoautotrophs were not detected in any of the clouds.

Previously, Amato et al. (2) investigated the microbes that were potentially active in clouds. DNA and RNA were extracted from cloud water and amplicons of fragments of the 16S rRNA and 18S rRNA genes (for prokaryotes and eukaryotes, respectively) were generated. Based on the ratio of the relative abundance of a specific OTU in the 16S or 18S rRNA and 16S or 18S rRNA DNA of a sample, an OTU's activity can be gauged (25). This is not a perfect method of assessing activity, but it can be used as a qualitative metric (26). Here we re-examined the data specifically for different types of oxygenic photoautotrophic microbes. Eukaryotic algae had RNA/DNA average ratios ranging from 0.59 to 2.11 and likely were mainly inactive (Table 2). In contrast, the cyanobacteria appeared to be more active with RNA/DNA ratios averaging 41.69 and reaching values as high as 1100 (Table 2) Unfortunately, the cyanobacteria that were detected were associated with unclassified phylotypes, and could not be classified further with the sequence data available.

**Chlorophyll a-containing cells in rain**

We collected 16 rain samples over a 3-week period at our rural sampling site (Opme) and assessed the chlorophyll a-containing cell abundance by flow cytometry. The main meteorological parameters and chemical composition of the rain samples are shown in Table S3. There were chlorophyll a-containing cells present in every rain event over the sampling period. Their concentration ranged from 9 to 2750 cells mL$^{-1}$ of rainwater, corresponding to downward
fluxes of ~1 to 328 chlorophyll $a$-containing cells cm$^{-2}$ day$^{-1}$ being deposited with rain (Figure 3A). The chlorophyll $a$ content in cells vary throughout their lives and environmental conditions (27) and cell membranes could rupture during manipulation, allowing chloroplasts to be detected. These factors could affect the enumeration of chlorophyll $a$-containing cells. In addition to these measurements, the diversity of cyanobacteria and green algae in the rain samples was assessed by amplicon sequencing of the 16S and 18S rRNA genes, specific for each respective group. The diversity data from the sequencing is presented in Table S4. There was a significant positive correlation between the Chao1 index and biomass (the number of chlorophyll $a$-containing cells; $p = 0.04$, $\rho = 0.52$, Data Set S2). Additionally, there was a significant positive correlation between air masses coming from the west and the number of chlorophyll $a$-containing cells ($p = 0.02$; $\rho = 0.57$, Data Set S2). Despite the variable concentrations/fluxes, we enriched oxygenic photoautotrophic microbes from each rain event by incubation in MWC (28) or BG11 medium (ATCC medium 616), as indicated by observation of green-tinted cultures.

**Oxygenic photoautotrophs in rain**

The dominant cyanobacteria present varied across rain events. Unclassified members of the *Nostocales* order were the most prominent cyanobacteria that range from not detected to 100% of the OTUs in the rain samples (Figure 3B). The *Chroococcidiopsaceae* varied from not detected to 75%, being most represented by an OTU associated with an uncultured cyanobacterium. *Aliterellaceae* ranged from not detected to 50% of OTUs with the most dominant OTU being closely related to *Aliterella* CENA595 (29). The *Microcoleaceae* ranged from not detected to 8.9% of OTUs with the dominant OTU matched to *Tychonema* CCAP 1459-11B (30) (Figure 3B). The *Microcoleaceae* family’s relative abundance was significantly positively correlated with pH ($p = 0.036$, $\rho = 0.53$; Data Set S2) and with the concentration of...
chlorophyll $a$-containing cells ($p = 0.028$, $\rho = 0.55$; Data Set S2). These cyanobacteria might have been an important component of the group of chlorophyll $a$-containing cells that were detected. There were no cyanobacteria present on 25 May, 01 June, and 06 June (Figure 3B).

Among green algae, the Trebouxiiales order dominated the rain samples accounting for 0.1 to 6% of OTUs across the rain events with a peak in abundance on 25 May (Figure 3C). An uncultured *Trebouxia* alga was the most dominant sequence associated with OTUs matched to this order. Other Trebouxiophyceae varied in abundance from not detected to 0.76%. The other green algae detected were lower in abundance. Members of the order Chlorellales were only detected in some rain events in relative abundances up to about 0.3% (13 May; Figure 3C). The Chlamydomonadales detected in some of the rain samples peaked in relative abundance on 25 May at about 0.11% (Figure 3C). Members of the Pedinomonadales were only detected on 3 May at about 0.01% relative abundance (Figure 3C).

Other than cyanobacteria and algae, pollen was also detected in rain (Figure 3D). Notably, pollen from the order Pinales dominated the eukaryotic sequences at early time points, and then decreased in relative abundance across the sampling time frame (Figure 3D). The order Pinales was dominated by phylotypes associated with the genus *Pinus*, pine trees, which are abundant in the sampling region. During the final days of the sampling period the relative abundance of Pinales decreased and the relative abundances of the Rosales and Poales increased. Therefore, there were significant negative correlations between the relative abundances of Pinales and Rosales ($p = 1.7\times10^{-9}$, $\rho = -0.96$; Data Set S2) as well as Pinales and Poales ($p = 2.5\times10^{-6}$, $\rho = -0.90$). There were no significant correlations between pollen sequences’ relative abundances and chemical/meteorological parameters or geographic origins of the air masses (Data Set S2).

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Finally, it should be mentioned that taxa other than oxygenic photoautotrophic microbes were abundant in the amplicons from rain samples, although the primers used to generate 16S rRNA and 18S rRNA gene amplicons were designed for excluding them (Cya 359f/Cya 781r and Euk528f/CHLO02r, respectively (31-33)). The use of these primers might need to be reexamined for future investigations.

DISCUSSION

Biodiversity and sources of cyanobacteria and algae in clouds and rain

Various genera of microalgae and cyanobacteria associated with the terrestrial and phyllosphere environments have previously been found in atmospheric samples (8). Microbes of the phyllosphere experience many of the same stressors as airborne microbes (34), so they might be better suited to living and surviving in the atmosphere than those from other sources. Overall, our data indicated that many of the microalgae and cyanobacteria detected in clouds and rain were of terrestrial origin, but some were likely of marine origin.

The BG11 media used for enrichment of cloud water selected for freshwater cyanobacteria and algae. The predominant photoautotrophic microbes enriched from clouds under these conditions were green algae of the Chlorellaceae (Figure 1B). The main OTU was associated with Chloroidium saccharophila SAG 211-9a. This organism was originally isolated from tree sap (35, 36), which suggests again that the phyllosphere could be an important source of atmospheric microalgae. Furthermore, Chloroidium is known to have a wide variety of habitats that range from freshwater to being lichen-associated (36). Chloroidium isolated from atmospheric samples were able to grow in a simulated freshwater environment (15).

We focused on enrichment of freshwater oxygenic photoautotrophs because these organisms might be able to colonize the local environment after atmospheric transport. The puy
de Dôme region is inland, far from oceanic sources, but it has been noted that the microbial communities in clouds at puy de Dôme often retain a marine signature (2, 24, 37, 38). Most of the clouds that were characterized for this study were of marine origin. However, the single cloud of continental origin (11102013) had similar relative abundances of SSU sequences of oxygenic photoautotrophic microbes compared to the marine clouds (Table 1).

Cyanobacteria were present in low relative abundances in the metagenomes across three clouds sampled for this study (11102013, 14102013, and 05112013) and the three clouds that were characterized previously (17112014-1, 17112014-2, 17112014-3) (24) (Table 1). Based upon the UniProtKB annotation of functional genes detected in the metagenomes, however, there were diverse cyanobacteria present. Different cyanobacterial genera associated with the marine environment were detected including *Lyngbya*, *Crocosphaera*, and *Synechococcus*. *Lyngbya* is a common cyanobacterium found in microbial mats in intertidal areas and *Crocosphaera* and *Synechococcus* are common in the ocean (39, 40). Additionally, *Synechococcus* and *Lyngbya* have both been found in air samples (10, 41). Besides marine cyanobacteria, there were also terrestrial cyanobacteria such as *Gloeocapsa* and *Gloeobacter*, which were all present in the 14102013 metagenome (Table 1). *Gloeocapsa* has been found in the atmosphere previously (42, 43) and *Gloeobacter* has been found in terrestrial environments including caves (44).

Cyanobacteria such as *Lyngbya* and *Gloeocapsa* were found to have a wide distribution amongst various atmospheric locations in Asia, Antarctica, Polynesia, Nearctic, and the Palearctic (11), in addition to some cloud samples from this study. Their heterogeneous presence in this study could be due to various reasons relating to meteorological conditions of the different locations over which the air mass traveled.
Despite detecting a clear marine signature in the cloud metagenomes, many of the algae and cyanobacteria in rain at the sampling site/time could be from other environments. The major cyanobacterial phylotypes detected in rain were of the order *Chroococcidiopsidales*. This contains two families: *Chroococcidiopsaceae* and *Aliterellaceae*. A major OTU detected was related to the genus *Aliterella* whose type strain was isolated from the Atlantic Ocean off the coast of Brazil (29). This genus has also been found on rocks in the Atacama Desert (45) and in rain collected in Hawaii (43). It has a cosmopolitan distribution and might be stress tolerant (as per its presence in a desert and in the atmosphere). Uncultured members of the *Chroococcidiopsaceae* and unclassified members of the *Nostocales* were also detected in the samples. Members of these families have been previously detected in atmospheric samples in Hawaii (43). A member of the *Microcoleaceae*, matching with *Tychonema* CCAP 1459-11B, a freshwater cyanobacterium (30), was dominant in some of the rain samples.

There were diverse algae present in clouds. Chlorellales members were cultivated from clouds (Figure 1A) and detected in rain (Figure 3C). Previously, green algae such as *Chlorella* and *Chlorococcum* were cultured from rain (20). Overall, the most abundant group of green algae in our rain samples were of the Trebouxiales order (Figure 3C), with phylotypes associated with uncultured *Trebouxia* being the most dominant. Certain members of the *Trebouxia* are prominent phycobionts of lichen (46) and have been detected in air and rain samples in South Korea (47) and air samples in Spain (48). The Trebouxiophyceae, which includes Chlorellales and Trebouxiales, have also been found in the marine environment (49), suggesting a cosmopolitan distribution of this taxonomic class.

Other than microbial oxygenic photoautotrophs, pollen was detected in the rain samples. The pollen did not correlate with any of the physicochemical parameters we measured. This fact,
along with their large sizes, suggests the pollen grains were most likely derived from local sources, emphasizing the importance of local landscapes on the atmospheric biological content (50). The types of pollen that were detected varied over the three-week sampling period. In anemophilous species, pollen is obviously largely dispersed aerially with well-known seasonality (47). The beginning of our sampling (May) likely corresponded with pine pollen season and the end could have corresponded with the end of the pine pollen season (June).

Because there were conflicting correlations with a western origin of rain air masses with air temperature and the number of chlorophyll a-containing cells, respectively, it is not possible to discriminate between a geographical source and an actual environmental temperature effect with this data. In a recent study, which included our sampling area, local landscapes and meteorological conditions were significant factors affecting the composition of the airborne microbial community (50).

Based upon the taxonomic affiliations, the cyanobacteria and algae detected in rain were likely derived from a mixture of locations including marine, freshwater, and terrestrial habitats. Many of the marine species were probably transported by the clouds and some of the freshwater and terrestrial phylotypes were transported by a combination of the clouds and the locally generated bioaerosols washed-out of the air column by the rain.

**Survival and potential activity**

Since many of the oxygenic photoautotrophs in our rain samples have a cosmopolitan distribution, atmospheric transport could be a substantial dispersal mechanism for these organisms (51). However, for successful dispersal the organisms must survive atmospheric stressors, including osmotic shocks, light, oxidants, and freezing. Many Trebouxiophyceae tolerate desiccation and other stressors that they would experience in the atmosphere (52).
Osmotic shocks and dehydration stress occur in both terrestrial and phyllosphere habitats. It was speculated that airborne cyanobacteria were more tolerant to different relative humidities than algae (53). The cellular biochemical properties of cyanobacteria and algal cells may be key to their survival in the atmosphere. Algae of the genus Chloridium, such as Chloridium sp. UTEX 3007 (54), can potentially survive a variety of atmospheric stressors because of intracellular desiccation resistance-promoting sugars (36, 54). Some green algae isolated from the atmosphere have carotenoids, thick cells walls, and intracellular antioxidants which help them to survive atmospheric stressors (55). Many members of the Trebouxiophyceae have mycosporine-like amino acids in the cytoplasm which help to protect the algae from UV radiation and oxidants (56). Mycosporine-like amino acids are also found in cyanobacteria such as Lyngbya purpurem (57). Other cyanobacteria such as Synechococcus, in particular, are known to survive various osmotic, oxidative, and temperature stressors (40). The Chroococcidiopsaceae family was prominent in our rain samples and a member of this family, Chroococcidiopsis, is well-known to be highly tolerant to desiccation and radiation (58). The relative abundances of this family were negatively correlated with increasing air temperature (ranging from 14°C to 22°C; Table S3), a trend that was previously reported for the abundance of various cultivated algae (59).

Amongst atmospheric stressors, freeze-thaw stress has been found to affect bacteria the most (60). The ability of microbial cells to tolerate freezing can be an important factor for survival during atmospheric transport. Algae isolated from atmospheric samples were able to survive freezing to a greater extent than aquatic algae (15). The 01062010 and 08062010 cloud water enrichments were revived after being stored in glycerol at -20°C for several years. This method for storing microorganisms could have an impact upon the mortality of cells and thus...
potential composition of the microbial community (61). Green algae and cyanobacteria, the most abundant oxygenic photoautotrophs in the atmosphere (41), are more amenable to cryopreservation than other oxygenic photoautotrophs (62). The dominance of the Chlorellaceae in the cloud enrichments could be due to a larger number of organisms of this taxon present in the culture when it was frozen. This would increase the likelihood of survival of this taxon as opposed to other lower abundant taxa. However, when comparing 08062010 (stored at -20°C for several years) and 29062017 (which was never stored at -20°C) cultures, they shared a dominant OTU in the family Chlorellaceae that matched with Chloroidium saccharophila SAG 211-9a. The fact that this alga was the major phylotype in two cloud enrichments and survived cryopreservation in the 01062010 and 08062010 cultures supports its tolerance to atmospheric stressors.

In addition to their vegetative states, many cyanobacteria and algae go through different stages of development. Many of the resting states that the cells manifest, which are also part of their reproductive cycle, impart stress-tolerance and allow the microbe to survive harsh conditions (for a review see Ellegaard and Ribeiro (63)). For example, unequal autospores are used for reproduction in Chloroidium and small autospores could be amenable to aeolian transport (36). Small resting states would be more aerodynamically favorable for long-distance transport as compared to larger vegetative cells.

While transiting through the atmosphere, if oxygenic photoautotrophs can survive atmospheric stressors, they have the potential to be metabolically active. After re-assessing a previous study on RNA/DNA ratios using rRNA amplicons, the eukaryotic algae were found to be relatively inactive with low ratios of RNA/DNA (Table 2). This could be consistent with their presence as resting states (e.g. spores) (63). Alternatively, this could indicate that cellular
functions of the eukaryotic algae were greatly affected by atmospheric stressors. In contrast the
cyanobacteria appeared to be more active with RNA/DNA ratios of not detected to 1100 (Table
2). Thus, they could affect cloud water droplet chemistry via carbon fixation, carbon and
nitrogen metabolism, or oxidant detoxification, similar to how heterotrophic bacteria were
hypothesized to be affecting cloud droplets (24). Consistent with other data, different genes
associated with oxygenic photosynthesis were persistently present in cloud metagenomes.
Nevertheless, not all genes associated with oxygenic photosynthesis and carbon fixation were
detected: for instance, no genes encoding for ribulose-1,5-bisphosphate carboxylase/oxygenase
(RuBisCO) were annotated (Data Set S1).

Potential impacts on ecosystems

The oxygenic photoautotrophs that are suspended in the air will eventually return to
different surface environments through dry or wet deposition (e.g. rain). Some eukaryotic algae
can promote ice formation, and thus could promote their deposition to new surface environments
by affecting cloud physics and precipitation patterns (15). All the rain samples analyzed
contained viable, culturable, aerially dispersed oxygenic photoautotrophs. As a result, rain
undoubtedly acts as a dissemination vector for oxygenic photoautotrophs to colonize new
environments. Near the rain sampling site of Opme is Aydat Lake, a freshwater lake of
approximately 65 hectares (6.5 × 10⁹ cm²). The cyanobacteria and green algae that were
transported through the atmosphere in this area were thus likely to be deposited in this lake
ecosystem, as well as other locations. Extrapolating the observed flux of chlorophyll a-
containing cells with precipitation to the surface area of the lake indicates that 10⁹-10¹²
chlorophyll a-containing cells enter the lake per each rainy day. These values are specific for this
region and sampling time and would likely differ compared to other areas in the world and/or
other periods of the year. Whether the rain-borne microbes will be successful in their new environment will vary depending upon the microbe’s viability and physiology, as well as atmospheric conditions (64). These new organisms could impact the local water quality and ecology, but the exact effects will depend upon their functional capabilities.

The ability to reproduce under different conditions is very important to dispersal success (15). *Trebouxia*, the most abundant green algae detected in our rain sequences, can be considered a “generalist” due to its ability to grow in freshwater and brackish water conditions (15). If these green algae can reproduce after atmospheric transport and deposition, their colonization could impact their new environments, just as aerially dispersed bacteria and fungi can affect the microbial composition and functioning of the environments they enter (65). Previously, the primary production rate of autotrophic communities in water was reported to be negatively impacted by aeolian-transported microbes (66). Notably, green algae were suggested to be better colonizers of water than aerially dispersed diatoms or cyanobacteria (67).

The transported microbes could also affect human health. It has been speculated that the allergic alga *Gonyostomium semen* could be aerially dispersed, thus leading to its expansion into new ecosystems (8). Here, phylotypes associated with toxin-producing oxygenic photoautotrophs including *Microcystis, Scytonema, Tychonema*, and *Anabaena*, whose toxins can have varying effects upon humans and other microbes (68-70), were detected in clouds (Figure 2) and rain (Figure 3). This illustrates the aerial spread of microbes of health-related and ecological interests and the possibility that they enter ecosystems with precipitation. Once they enter a new environment such as Aydat Lake, they could produce toxins which could affect human health or even the health of other algae, which are important to primary production. Even if a microbe does not establish a lasting presence in a system, it still could have long-term
impacts upon the resident microbial community (71). Through these methods, aeolian
transported oxygenic photoautotrophs could impact the health and biogeochemical functioning of
a lake ecosystem.

Conclusions and Perspectives

Our study indicates that there are diverse oxygenic photoautotrophs among the microbial
assemblages carried by the atmosphere. We detected cyanobacteria, diatoms, as well as red,
green, and golden algae as persistent members of cloud microbial assemblages. Many of the
organisms detected were associated with stress tolerant phylotypes that have widespread
distributions, and some were toxin producers with potential ecological and health-related
impacts. We were able to culture some of them, mostly the aeroterrestrial epiphytic green algae
of the Chlorellaceae. Cyanobacteria were not detected in cloud water enrichments under the
conditions utilized, but molecular approaches indicated that these are present and probably active
in clouds. Genes encoding for part of photosystem II of various cyanobacteria were present in
metagenomes. Rain acted to mediate the dispersal of many of these diverse oxygenic
photoautotrophs, with members of Chroococcidiopsidales and Trebouxiales being notable, into
new ecosystems. Despite small sample sizes and short time periods, our study provides key
information on the aeolian transport of cyanobacteria and green algae that is currently missing.
Microbiologists, ecologists, and meteorologists should work together to conduct long-term
studies to identify the sources of the oxygenic photoautotrophic microbes in rain and determine
their emission/depositional fluxes. Aeolian dispersal is more conducive to certain microbes and
understanding the physicochemical effects on their dispersal patterns and the exact biological
and ecological implications of their dispersal is an understudied aspect of aerobiology.
MATERIALS AND METHODS

Cloud and rain sampling

Clouds and rain were sampled from meteorological stations operated by the Observatoire de Physique du Globe de Clermont-Ferrand (OPGC) and parts of the national COPDD atmospheric survey network.

Clouds were sampled at the puy de Dôme (PDD) station (1465 m a.s.l., 45.7720° N, 2.9655° E) on 01/06/2010, 08/06/2010, and 29/06/2017 (dd/mm/yyyy) as described previously (38) (see Table S1 for sampling details). Clouds were sampled again at the same site on 11/10/2013, 14/10/2013, and 05/11/2013 (sample names 11102013, 14102013, 05112013, respectively), as described previously (2, 24). The cloud water samples were filtered through a 47 mm, 0.22 µm porosity membrane (MoBio 14880-50-WF) in a laminar flow hood, cut in half, and half-filters were stored at -80°C prior to use for metagenomic analyses following previous protocols (24). Backward trajectories of the air masses were generated with HYSPLIT (Figure S1) (72).

Rain was collected at the Opme meteorological station (680 m a.s.l., 45.712500° N, 3.090278° E) with an automated wet-deposition sampler (Eigenbrodt NSA 181/KHS), as previously described (73). Briefly, eight autoclaved 1-L glass bottles were kept at 4°C in the chamber with a new bottle designated for collection every 24 hours. Before putting new bottles in the carousel, the funnel was disinfected with 70% ethanol and rinsed with sterile water. The funnel was covered by a polytetrafluoroethylene (PTFE) lid that automatically opened when precipitation occurred, and which prevented dry deposition between rain sampling events. All rain water samples were collected and transported to the laboratory within 24 hours of the precipitation event. Sixteen rain samples were collected between 23 May, 2018 and 13 June,
2018. The rain water was filtered through 0.22 µm porosity membranes (MoBio 14880-50-WF) and membranes were stored at -20°C for later DNA extraction. The pH of filtered rain was determined and ion concentrations were measured by ion chromatography (Dionex, Sunnyvale, CA, USA) as previously described (2). The backward trajectories of the air masses (Figures S2) were generated with HYSPLIT (72).

**Cultivation of photosynthetic microbes**

Cloud water samples collected on 01/06/2010, 08/06/2010, and 29/06/2017 (dd/mm/yyyy) were incubated in BG11 medium (ATCC 616) under constant light (75 µE·m²·s⁻¹) at 20°C in the INFORS HT Multitron II equipped with Sylvania Gro-Lux F15W/Gro lights while shaking at 80 rpm to produce cultures named 01062010, 08062010, and 29062017, respectively. We used BG11 medium, which is designed to favor the growth of cyanobacteria and chlorophytes from freshwater environments, not diatoms. The study site is far from the ocean, thus we focused on cultivating organisms that could successfully survive if deposited in the local environment. This excluded marine organisms. The 01062010 and 08062010 cultures were preserved in 15% glycerol and stored at -20°C for about seven years. Once the 29062017 culture was established, the others were revived, and all were incubated in BG11 media at 15°C under constant illumination (75 µE·m²·s⁻¹) at 80 rpm. After incubating for 7 days, 5 mL of each culture was centrifuged at 8,000 × g for 3 minutes and the cell pellet was stored at -20°C until DNA extraction.

All rain samples were cultivated in MWC (28) or BG11 media. Visual observation of green tinted cultures indicated successful enrichment of oxygenic photoautotrophs.

**DNA Extractions**
DNA from the cloud enrichment cultures was extracted with a modified phenol-chloroform based protocol (74). A 50 mM glucose/10 mM EDTA/25 mM Tris-HCl solution was added to the cell pellet, then five freeze-thaws were performed by freezing at -80°C in ethanol (200 proof) and heating to 55°C. Lysozyme (0.4 mg L\(^{-1}\)) and EDTA (0.5 M) were added, and samples were shaken at 100 rpm at room temperature for 5 min. Ten percent SDS was added and followed quickly by extraction with phenol: chloroform: isoamyl alcohol (25:24:1). The emulsion was separated by centrifuging at 16,000 \(\times\) g for 3 min. The resulting aqueous phase was extracted with phenol: chloroform: isoamyl alcohol and centrifuged again. The aqueous phase from the second extraction was precipitated with 200 proof ethanol (1 mL) and 2 µg glycogen.

DNA was extracted from half of the cloud filters and from the whole rain filter using a MoBio PowerWater isolation kit (now, Qiagen, Hilden, Germany) following the manufacturer’s protocol.

Cloud Enrichment PCR and Sequencing

DNA extracts from cloud enrichments were sent for PCR amplification and sequencing by MR DNA (www.mrdnalab.com, Shallowater, TX, USA). The 16S rRNA amplicons were generated using the primer set 515f/806r (75) with a barcode on the forward primer (Table S2). HotStarTaq Plus Master Mix Kit (Qiagen, USA) was used for amplification with the following conditions: 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 53°C for 40 sec and 72°C for 1 min, and ending with a final elongation at 72°C for 5 min. To generate the 18S rRNA gene amplicon, PCR using 1391f (76) and Eukbr (77), with a barcode on the forward primer (Table S2) was performed with HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 53°C for 40 sec and 72°C
for 1 min, and ending with a final elongation at 72°C for 5 min. The products were confirmed on a 2% agarose gel. The 16S rRNA gene amplicons were sequenced with an Ion S5 XL instrument (Ion 530 chip) following the manufacturer’s guidelines. The 18S rRNA gene amplicons were combined in equal proportions and purified using calibrated Ampure XP beads (Beckman Coulter). These were used to prepare the DNA library for Illumina MiSeq 2×300 bp paired end sequencing following the manufacturer’s protocols. The corresponding sequence files were deposited at the EMBL-EBI European Nucleotide Archive under the study accession number PRJEB35708.

### Rain Samples PCR and Sequencing

For the DNA extracts from rain samples, portions of the 16S rRNA genes targeting cyanobacteria (31) and of the 18S rRNA genes targeting green algae (78) were PCR-amplified. The 16S rRNA gene PCR reaction was composed of 34.3 µL sterile water, 5 µL 10X Green PCR buffer without Mg (ThermoFisher Scientific), 0.2 µL Platinum™ Taq DNA Polymerase (10 U µL⁻¹; ThermoFisher Scientific), 3 µL 25 mM MgCl₂, 1 µL 10 mM dNTPs, 2 µL 10 µM forward primer Cya359 F (31), 2 µL 10 µM reverse primer Cya 781 R modified from Nübel et al. (31), and 1 µL of template DNA (5-10 ng). The primer sequence of the modified reverse primer from Nübel et al. (31) was 5’ GACTACWGGGTATCTAATCCWTT 3’. The conditions were as followed: 94°C for 2 min, then 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and a final extension of 72°C for 5 min. For the 18S rRNA gene PCR 33.7 µL sterile water, 5 µL 10X Green PCR buffer, 0.3 µL Taq DNA polymerase (5 U µL⁻¹; Fischer BioReagents™, ThermoFisher Scientific), 3 µL 50 mM MgCl₂, 1 µL 10 mM dNTPs, 2 µL 10 µM EUK528f (32), 2 µL 10 µM CHLO02r (33), and 3 µL of template DNA (5-10 ng) were mixed. The conditions (78) were as follows: 94°C for 5 min., then 35 cycles of 94°C for 30 s., 55°C for 30 s., 72°C for 1
min., and a final extension of 72°C for 5 min. Both sets of amplicons were purified with MinElute PCR purification kit (Qiagen). Negative controls and positive controls were processed alongside the samples. The positive control for the 16S rRNA and 18S rRNA gene amplifications were DNA extracts from cultures of *Microcystis aeruginosa* PCC 9443 and an enrichment culture of rain water collected on 27/05/2018 in MWC media (28) containing algae (27052018), respectively. Purified amplicons from the rain sample DNA extracts were sent to GenoScreen (Lille, France) and library preparation followed the Metabiote®v2.0 protocol (GenoScreen, Lille, France) using 5 ng amplicon DNA. Library preparation quality was confirmed by capillary gel electrophoresis. The samples were sequenced on one line with a 2×250-bp paired-end MiSeq Illumina instrument. The sequence files were deposited at the EMBL-EBI European Nucleotide Archive under the study accession numbers PRJEB35600, samples ERS4058723 to ERS4058754.

**Bioinformatic analysis of PCR amplicons**

The 18S rRNA amplicons from photosynthetic cloud enrichment cultures were demultiplexed with sabre ([https://github.com/najoshi/sabre](https://github.com/najoshi/sabre)) and paired ends were assembled with the default parameters of the DADA2 (79) plugin in QIIME2 (v2020.2) (80). The 16S rRNA amplicons from oxygenic photoautotrophic cloud enrichment cultures were demultiplexed with software from MR DNA LLC ([http://www.mrdnalab.com/mrdnafreesoftware/](http://www.mrdnalab.com/mrdnafreesoftware/)). Both the 16S and 18S rRNA gene rain amplicons were demultiplexed with the CASAVA v1.0 software (Illumina) and the paired-ends were assembled with the FLASH tool (81) with trimming of poor-quality reads (phred score <30) and a 97% similarity requirement.

All fastq files were imported into QIIME2 (v2020.2) (80), trimmed and denoised with DADA2 (79) and grouped into amplicon sequence variants (ASVs). The ASVs were then
clustered into operational taxonomic units (OTUs) (97%) with the vsearch (82) plugin. A naive Bayes scikit-learn classifier (83, 84) for each primer set was trained using extracted reference full-length operon reads of the Silva v132 database (85) and used for classification. Taxa associated with negative controls and prokaryotic reads in eukaryotic-targeted amplicons (and vice versa) were filtered out from the samples. OTUs, that were of interest and lacked taxonomic resolution from the identification, were queried manually against NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the BLASTn algorithm in May 2020. Samples were rarefied and analyzed for Shannon’s diversity, Chao1, and observed OTUs in QIIME2 (v2020.2).

Cloud metagenomes

DNA extracts from cloud water samples 11102013, 14102013, and 05112013 were amplified by MDA using GenomiPHI (GE Healthcare) to aid in generating sequencing libraries. The libraries were prepared, sequenced, and analyzed as previously described (24) using an Illumina MiSeq 2 × 300 bp (Genoscreen, Lille, France). The sequence files were deposited at the EMBL-EBI European Nucleotide Archive under the study accession numbers PRJEB35574, samples ERS4058712 to ERS4058714. Methods for the taxonomic and functional annotation of the sequences have been described previously (24). In brief, taxonomic annotations were assigned based upon the SILVA database (85). The oxygenic photoautotrophs were identified based upon the taxonomy. The functional annotations were assigned based upon the UniprotKB protein database (86). With the UniprotKB identifiers, associated genera and gene ontologies identifiers (GO IDs) (87) were assigned. GO IDs associated with photosynthesis were selected. Additional cloud metagenomes obtained from Amato et al. (24) were reanalyzed to investigate
the presence of oxygenic photoautotrophic microbes (sequences files ENA accession numbers ERS2351639 to ERS2351641).

**Re-analysis of cloud RNA/DNA ratios**

Previously generated prokaryotic and eukaryotic ribosomal DNA and cDNA amplicons were re-assessed (2). Sequence files accession number in NCBI BioProject PRJNA380262 were reanalyzed to examine the potential activity of photosynthetic microbes in clouds. Known oxygenic photoautotrophs were manually selected from the OTUs based upon their taxonomic identification. The number of reads for each oxygenic photoautotrophic OTU were expressed as a percentage of the total community for the cDNA (representing rRNA) and DNA sequences. The RNA/DNA ratio for each OTU was calculated using the relative percentages. The OTUs were grouped at the phylum or class level and the RNA/DNA ratios were averaged for each taxonomic group. A taxon was considered to be active if the ratio was greater than 1, meaning that the relative abundance of the taxon was greater in the rRNA amplicons rather than in the rRNA gene amplicons. Phantom taxa, phylotypes present in the rRNA but not the rRNA genes (25), were excluded in this analysis.

**Enumeration of chlorophyll a-containing cells**

Triplicates of fresh rain water samples of 0.45 mL were fixed with a final concentration of 0.5% glutaraldehyde and stored at 4°C in the dark until flow cytometry analysis. Just before analysis, fixed samples were amended with 1 vol of Tris-EDTA buffer (40 mM Tris; 1 mM EDTA; pH 8.0) and analyzed by a BD FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) to enumerate chlorophyll a-containing cells. Detection was on FL3 (excitation 488 nm; emission 670 nm) with detection thresholds set at 500 mV for FL3 and 380 mV for SSC. Acquisition time was 315-400 s. at a flow rate between 66 – 72 µL min\(^{-1}\) (high
setting). The flow rate was determined precisely for each sample through the acquisition time and the gravimetric loss from the water sample. All data was analyzed in the software BD CellQuest Pro™ (Becton Dickinson, Franklin Lakes, NJ). Chlorophyll a-containing cells were expressed both as rainwater concentration (cells mL⁻¹) and as a surficial flux (cells cm⁻² day⁻¹). The flux was calculated from the total number of cells in a rain sample for a given day divided by the area of the collection surface.

**Statistical Analyses**

Statistical analyses were conducted in OriginPro Version 2019 9.6 (OriginLab Corporation, Northampton, MA, USA). To correlate the presence of phylotypes with geographic origin, the two types of air masses were defined and statistically analyzed as described in Pouzet et al. (73).

**Data Availability**

The sequence files were deposited to the European Nucleotide Archive with project accession numbers PRJEB35574 (cloud metagenomes; ERS2351639 to ERS2351641), PRJEB35708 (cloud enrichments), and PRJEB35600 (rain samples; ERS4058723 to ERS4058754).

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Competing Interests: The authors declare no competing interests.

REFERENCES


Cyanobacteria and algae in clouds and rain


Table 1: Relative abundance of oxygenic photoautotrophic microbial small subunit (SSU) ribosomal sequences in cloud metagenomes. All clouds were of marine origin, based on back trajectories from NOAA HYSPLIT (Figure S1), except for sample 11102013, which was continental in origin.

<table>
<thead>
<tr>
<th>Sample</th>
<th>11102013</th>
<th>14102013</th>
<th>05112013</th>
<th>Amato et al. 2019</th>
<th>Amato et al. 2019</th>
<th>Amato et al. 2019</th>
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<td>This study</td>
<td>This study</td>
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<td>Amato et al. 2019</td>
<td>Amato et al. 2019</td>
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<td>0.27</td>
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<td></td>
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<tr>
<td>Chlorophyta</td>
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<td>Bacillariophyceae</td>
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<td>1.5</td>
<td>1.4</td>
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<td>Golden algae</td>
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<td>3.1</td>
<td>1.8</td>
<td>2.3</td>
<td>2.6</td>
</tr>
</tbody>
</table>
Table 2: Calculated RNA:DNA ratios of taxa including photosynthetic microbes in clouds

(Original data from Amato et al. (2)). The relative abundances of the taxa in the RNA and DNA reads were used to calculate ratios. The range is the minimum and maximum ratio observed for the corresponding taxon in the sample.

<table>
<thead>
<tr>
<th>Group</th>
<th>Polluted Cloud</th>
<th></th>
<th>Continental Cloud</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average ± Std</td>
<td>Range</td>
<td>Average ± Std</td>
<td>Range</td>
</tr>
<tr>
<td></td>
<td>Dev</td>
<td></td>
<td>Dev</td>
<td></td>
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<tr>
<td><strong>Prokaryotes</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>41 ± 119</td>
<td>nd* - 1100</td>
<td>11 ± 23</td>
<td>nd - 120</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyta</td>
<td>0.85 ± 0.94</td>
<td>nd - 2.9</td>
<td>1.2 ± 1.7</td>
<td>nd - 7.1</td>
</tr>
<tr>
<td>Cryptophyta</td>
<td>0.59 ± 0.82</td>
<td>nd - 2.9</td>
<td>0.67 ± 0.85</td>
<td>nd - 2.8</td>
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<td>Bacillariophyta</td>
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<td>nd - 101</td>
<td>0.95 ± 1.6</td>
<td>nd - 10</td>
</tr>
<tr>
<td>Dinophyceae</td>
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<td>nd - 25</td>
<td>2.2 ± 3.0</td>
<td>nd - 11</td>
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<td>1.3 ± 1.5</td>
<td>nd - 8.7</td>
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<td>Phaeophyceae</td>
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<td>nd - 8.7</td>
<td>0.74 ± 0.57</td>
<td>nd - 2.3</td>
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</tbody>
</table>

*nd: not detected
Figure 1: Microbial composition of oxygenic photoautotrophic enrichments. Cloud water was incubated with BG11 medium at 15°C under constant illumination. (A) The eukaryotic population of the three cloud enrichments at the family level and their relative abundances in 18S rRNA gene reads. (B) The taxonomic classification and relative abundance of bacteria in 16S rRNA gene reads in all three cloud enrichments at the family level.
Figure 2: Relative abundance of bacterial genera associated with detected photosynthetic genes in cloud water. The total number of reads of functional genes related to photosynthesis (n) in each cloud metagenome and their associated taxonomy at the genus level were based upon the UniProtKB database. All clouds were marine in origin except 11102013 which was continental in origin.
Figure 3: Abundance of chlorophyll a-containing cells in rain and the composition of cyanobacteria and green algae in rain from 23 May, 2018 to 13 June, 2018. (A) The concentration of chlorophyll a-containing cells in rain samples and the amount of rain collected. The downward flux is the amount of chlorophyll a-containing cells entering the surface environment per square centimeter of area, as inferred from concentration measurements. There were no rain events on 24 and 26 May or 02, 04, 08, and 12 June. (B) The composition of 16S rRNA partial amplicons with primers targeting cyanobacteria from fresh rain samples collected. The nd designation is for “not detected,” which were days when none of the phylotypes were detected in the microbial populations. On 01 June, only sequences associated with chloroplasts were detected. (C) The algal composition of 18S rRNA partial amplicons with primers targeting green algae from fresh rain samples collected. (D) The pollen composition of 18S rRNA partial amplicons with primers targeting green algae from fresh rain samples collected. The 48 hour back-trajectories of the air masses from NOAA HYSPLIT for all sampled rain is presented in Figure S2.