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A long-term atmospheric baseline for intercontinental exchange of airborne pathogens

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ABSTRACT

The atmosphere is a potential pathway for global-scale and long-range dispersal of viable microorganisms, promoting biological interconnections among the total environment. We aimed to provide relevant baseline information for long-range long-term intercontinental exchange of potentially infectious airborne microorganisms of major interest in environmental and health-related disciplines. We used an interannual survey (7-y) with wet depositions fortnightly collected above the boundary layer (free troposphere) at a remote high-elevation LTER (Long-Term-Ecological-Research) site, analyzed by 16S and 18S rRNA genes, and compared to a database of 475 well-known pathogens. We applied a conservative approach on close relatives of pathogenic species (>98% identity) standing their theoretical upper limit for atmospheric baseline relative abundances. We identified c. 2–3% of the total airborne microbiota as potential pathogens. Their most frequent environmental origins were soil, aquatic, and anthropogenic sources. Phytopathogens (mostly fungi) were the potential infectious agents most widely present. We uncovered consistent interannual dynamics with taxa foreseeable over time (i.e., predictable seasonal behavior) and under recurrent environmental scenarios (e.g., Saharan dust intrusions), respectively, being highly valuable microbial forensic environmental indicators. Up to 8 bacterial and 21 fungal genera consistently showed temporal abundances and recurrences unevenly distributed. Incidence of allergenic fungi was lower in summer, and significantly higher in spring. Close relatives to *Coccidioides posadasii* consistently showed higher signals (i.e., high specificity and high fidelity) in winter, whereas *Cryptococcus neoformans* had a significant signal in spring. Along Saharan dust intrusions, the bacterial phytopathogens *Acidovorax avenae* and *Agrobacterium tumefaciens* and the fungal phytopathogens *Pseudozyma hubeiensis* and *Peniophora* sp. consistently showed higher signals. Potential human pathogens showed low proportion, being mostly fungal allergens. Microorganisms related to obligated human, amphibian and fish pathogens were commonly found in winter. More studies in remote field sites above the boundary layer will unveil whether or not a similar trend is found globally.

1. Introduction

The atmosphere is a potential pathway for global-scale and long-range dispersal of viable microorganisms (Griffin, 2007; Hervàs et al., 2009; Polymenakou, 2012; Prospero et al., 2005) promoting interconnections among the total environment (atmosphere – hydrosphere – biosphere – lithosphere – anthroposphere) (Santl-Temkiv et al. 2021). Airborne particles are vectors for intercontinental disease transmission and spreading of allergens, and the need of monitoring at the global scale the microorganisms present in aerosols has been recently stated (Smith, 2013, Cáliz et al. 2018). Scientific knowledge is still mostly missing to estimate background abundances and distribution of airborne pathogens and to properly determine whether a pathogen has been intentionally (or accidentally) released and where and how a biothreat agent emerges. This information is fundamental for accurate decision-making policies (NAS Consensus Report, 2014).

Recent studies on common pathogens have explored environmental effects related to agronomy (e.g., Gonzalez-Martin et al., 2014), climate change (Hellberg and Chu, 2016), air pollution (Liu et al., 2018), and the implications for human health of microorganisms present in the atmosphere (Polymenakou, 2012) and transported in desert dust plumes (Griffin, 2007). The need to predict the risk and outbreaks dynamics of

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airborne diseases, and the development of strategies to minimize the risk of severe pandemics has been emphasized (West et al., 2008). The topic is therefore of major interest although it lacks comprehensive long-term studies yet to establish basic environmental baseline information on the natural abundance and distribution of pathogens in the atmosphere and the interconnections among the different Earth compartments. The combination of air sampling and molecular tools has been highlighted as a new opportunity to predict biological threats (West et al., 2008), and the potential of metagenomics for biosurveillance and monitoring of airborne biothreats in public health has been shown for urban areas (Be et al., 2014). Health disciplines (human and veterinary medicine, and public health), agriculture and microbial forensics share many practices, aims and motivations that would benefit for more detailed ecological studies combining molecular ecology, environmental microbiology, and atmospheric dynamics. More specifically, microbial forensics has specific needs because of the requirements for "evidence" and "proof" in the context of law enforcement and international policy (NAS Consensus Report, 2014). High throughput sequencing and bioinformatics may help to support environmental microbial forensics approaches, since they represent powerful techniques to identify and characterize pathogens (Skowronski and Lipkin, 2011) after some limitations and methodological aspects are carefully considered (Schmedes et al., 2016).

In the present study, we surveyed potential pathogens present in a long term ecological research (LTER) study that fortnightly collected samples in wet deposition (both rain and snow) from a high-altitude mountain in the Central Pyrenees (LTER site Aigüestortes, NE Spain) over seven years (Cáliz et al. 2018). We applied a conservative approach as a preliminary view about close relatives of pathogenic species, covering the most widely recognized pathogens by means of rRNA genes amplicon metagenomics. We analyzed the temporal dynamics, and we showed occurrence, dynamics, and the baseline oscillation range for a wide repertoire of potential environmental pathogens. Since samples were collected at a remote high-elevation site above the boundary layer —free troposphere— (Ontiveros et al. 2021), we addressed intercontinental transport of potential pathogens that were deposited on the Mediterranean region (DeLeon-Rodriguez et al., 2013; Triadó-Margarit et al., 2019). Overall, we provide relevant baseline information and expected environmental ranges of variation for long-range intercontinental exchange of potentially infectious airborne microorganisms, covering the most characteristic bacteria and fungi representatives.

2. Methods

Samples of wet atmospheric precipitations (rain and snow, n = 137) were fortnightly collected above the boundary layer (Ontiveros et al. 2021) along a 7-y survey (May 15, 2007 to October 15, 2013) at the High Mountain LTER-AT site, a Long Term Ecological Research field station within the protected area of the Aigüestortes i Estany de Sant Maurici National Park in the Pyrenees, NE Spain (42°33′N 0°53′E, c.a. 1700 m a.s.l.). Samples were collected using a passive MTX ARS 1010 automatic sampler (MTX, Bologna, Italy) equipped with two plastic containers (labelled dry and wet, respectively) installed on a metallic structure with 1.1 m legs. This collector discriminated between dry and wet atmospheric deposition using a hygroscopic sensor that activated an aluminum lid to cover/uncover the containers, respectively. The wet container remained fully covered and in the dark preventing atmospheric inputs until the hygroscopic sensor was activated. Water samples from the wet container were filtered using pre-combusted (450 °C, 4 h) Whatman GF/F filters and then dried in a laboratory oven for 4 h and aseptically kept in a dark and dry place. The containers were carefully cleaned and repeatedly washed with sterile MilliQ water after each use. We had previously tested that wet depositions collected above the boundary layer can substantially reduce near-surface aerosols contamination, and can be a cost-effective useful proxy for monitoring intercontinental exchange of microorganisms from the high-atmosphere (Triadó-Margarit et al. 2019, Ontiveros et al. 2021).

Each precipitation event was tracked with the Vertical Velocity NOAA HYSPLIT Model and GDAS meteorological data (direction and wind speed at 3000 MASL) as reported earlier (Cáliz et al., 2018, Ontiveros et al. 2021). Additional meteorological data can be found elsewhere (http://loopweb.org/loopweb2018/index.php/lter-research/meteorological-data).

For the molecular and bioinformatic analyses, after DNA extraction and purification (PowerSoil Isolation Kit, Mobio Laboratories), multiplexing PCR amplification and Illumina MiSeq sequencing were carried out for 16S and 18S rRNA genes (V4 and V9 hypervariable regions, respectively) (Cáliz et al., 2018) following the methods of the Research Technology Support Facility at the Michigan State University (https://rtsf.natsci.msu.edu/), and raw sequence datasets were processed using the UPARSE pipeline (Edgar, 2013). Briefly, after merging of paired reads, filtering by an expected error of 0.25 and read length of 253 bp, ca. 42% of the original reads were retained (i.e., 5,622,407 and 6,417,502 reads for 16S and 18S rRNA genes, respectively). The reads were dereplicated and clustered into operational taxonomic units (OTUs) at cut-off 3% identity after chimera removal (UCHIME) and excluding singletons. More than 92% of the globally trimmed, quality filtered sequence pool was mapped back into OTUs. A total of 4164 prokaryote and 8248 eukaryote OTUs were obtained and taxonomically assigned with SILVA_119 (Quast et al., 2013). Chloroplast, mitochondria, Metazoa, Embryophyta (mostly pollen), and unclassified reads were excluded for downstream analyses. A total of 3630 prokaryote and 5814 eukaryote OTUs were finally compiled. In order to minimize biased effects for differences in sampling effort, the original OTU table was average rarefied (100 random subsamplings) and set to a depth of 12,500 prokaryotes and 10,000 eukaryotes sequences per sample. Environmental descriptive terms —ENVO terms— (Buttigieg et al., 2013) were extracted from the closest GenBank matches (99% identity) using the SEQenv pipeline (Sinclair et al., 2016). For studying the effect of Saharan intrusion episodes (n = 96), two categories were defined, i.e., (i) the "dust group", for samples collected <48 h since the last reported Saharan dust episode, and (ii) the "dust-free group", for rain/snow samples collected after a period ≥20 days free of any reported Saharan dust intrusion event. Temporal monitoring of Saharan dust plumes was obtained from www.calima.ws.

2.1. Identification of potential pathogens

The presence of potential pathogens was tested against a *de-novo* 16S and 18S rRNA gene database holding 283 bacterial and 192 eukaryotic well-known pathogens (Ecker et al., 2005). Only OTUs > 98% identical and with the highest BLAST alignment coverage (>90%) to the inventoried pathogens were considered for downstream analysis. Several fungal OTUs matched human and animal pathogenic fungi (http://www.mycologylab.org) and phytopathogens under quarantine regulations in Europe (http://www.q-bank.eu). We however acknowledge that additional confirmation tests are needed (e.g. by studying specific virulence markers) to fully support pathogenic capacities, even for rRNA gene sequence identities of 100% and full alignment coverage. Our study should therefore be taken as a conservative approach that shows the potential of the samples to contain pathogens at the highest end.

2.2. Data sharing

The genetic dataset is available in the Sequence Read Archive (SRA) National Center for Biotechnology Information (NCBI) under accession PRJEB14358.

2.3. Statistical analyses

All statistical analyses were run in the R environment (http://www.r-project.org/) and the graphs were performed with ggplot2 package

(Wickham, 2009). The IndVal index (labdsv package) was used to identify microbial taxa as "indicator species" (Roberts, 2016). The index combines relative abundance (tied to the concept of specificity) and relative frequency of occurrence (i.e., fidelity) (Dufrêne and Legendre, 1997); *p-values* were adjusted for multiple testing using the Bonferroni correction. A significance cut-off of 0.01 was established. Correlation analyses based on Spearman's rank order coefficient were used to inspect the association degree between the OTU abundance distributions and chemical composition of wet depositions.

3. Results

3.1. Long-term atmospheric baseline for potential pathogens and potential sources

In the whole temporal dataset we identified as potential pathogens c. 2–3% of the total airborne microbiota, i.e. 77 bacterial OTUs (2.2 %) and 191 eukaryal OTUs (3.3%, >97% of them fungi). These potential pathogens reached mean relative abundances close to 20% in micro-eukaryotes and to 10% in bacteria. We noticed substantial differences in relative abundances for the different potential hosts (supplemental Fig. S1). As a whole, the presence of potential pathogens for plants, humans, and other animals showed higher mean abundances in eukarya than in bacteria. Higher relative abundances of opportunistic pathogens were observed for bacteria (mean value 3.4%) than for eukarya (mean value 0.7%), and *Pseudomonas fluorescens* was the opportunistic pathogen most commonly retrieved (Fig. 1).

Plant pathogens were frequently found in the eukaryal (12% mean relative abundance) and bacterial datasets (4%). All in all, fungi affecting plants were the most commonly found airborne pathogens (Fig. 2) with *Taphrina carnea*, *Daedaleopsis confragosa*, *Pseudocercospora fijensis*, *Peniophora nuda*, *Peniophora* sp., *Boeremia exigua*, *Roestelia* sp., and *Sporobolomyces* reaching the highest abundances. Additionally, *Pseudozyma hubeiensis*, *Phoma destructiva*, *Leptosphaeria biglobosa* and *Ophiosphaerella herpotricha* showed relative abundances >0.3% and frequencies of appearance >0.65. Plant pathogenic bacteria were also noticeable (mean proportion 4.4%), mostly *Acidovorax avenae*, *Ralstonia solanacearum,* and *Agrobacterium tumefaciens*.

We found that for humans and other mammals, the most abundant potential bacterial pathogens were *Stenotrophomonas maltophilia*, *Yersinia pseudotuberculosis* and *Arcobacter cryaerophilus* with baseline values ranging between c. 0.5 and 0.4% (Fig. 1). A close match with *Bacillus anthracis* was observed, although this result should be carefully considered because of the high conservation in the 16S rRNA gene sequence within the *Bacillus* group. For fishes and amphibians, we detected *Renibacterium* and *Flavobacterium branchiophilum* with mean relative abundances <0.5%.

The fungi *Cryptococcus neoformans* var. *neoformans* (obligated pathogen of humans and other mammals) was detected at relative abundances up to 2.5% (Fig. 2), and the allergen *Cladosporium cladosporioides* was also recurrently found. We also detected the presence of *Coccidioides*, a severe fungal pathogen endemic from some arid and semi-arid regions. Members of black yeasts showed high occurrence too, with *Exophiala* spp. (Chaetothyriales, potentially affecting fish and amphibians) as a main component (Fig. 2). The opportunistic *Aureobasidium,* mainly comprising saprobic fungi (organic matter decomposers), was recurrently found and notably abundant (c.a. 0.5%).

The most frequent environmental origins (EnvO) for airborne pathogens matched mainly soil, aquatic, and anthropogenic sources (Supplemental Figs. S2 and S3). Interestingly, we noticed different predominant sources according to the potential targeted hosts and the obligated vs. opportunistic categories. Thus, soil sources were predominant for airborne plant pathogens, aquatic sources for fish-amphibian, and bodily fluids and waste for human obligated pathogens, respectively. Opportunistic airborne pathogens showed a larger repertory of environmental sources.

3.2. Recurrent seasonal dynamics in airborne pathogens

The relative abundances of potential pathogens showed significant seasonal temporality in the long-term dataset (Supplementary Fig. S4, and Tables S1 and S2). Indicator values (IndVal) unveiled consistent seasonal patterns with several foreseeable microbial genera (8 bacteria and 21 fungi), showing temporal abundances and recurrences unevenly distributed (Fig. 3).

The plant pathogenic bacteria *Acidivorax avenae* and *Agrobacterium tumefaciens* were preferentially and recurrently found in summer (Fig. 3, left panel). Conversely, *Pseudomonas syringae* was mainly present in autumn and secondarily in summer. Genera which contain obligated human pathogens, as *Corynebacterium*, *Stenotrophomonas* and *Yersinia,* and amphibian and fish pathogens like *Renibacterium,* were commonly found in winter. The predicted environmental sources for these bacterial genera were mostly soils and aquatic environments (Fig. S5).

Fungal phytopathogens were mostly found in winter and autumn, with presence of only a few animal pathogens (Fig. 3, right panel). The black yeast *Exophiala,* and *Coccidioides posadasii* consistently showed higher signals in winter whereas *Cryptococcus neoformans* var. *neoformans* had a significant signal in spring. Indicator species descriptions were further refined by multi-level pattern analysis (indicspecies R package), which considered different seasons combinations. *Exophiala, Coccidioides* and *Cryptococcus*, were classified as non-summer occurring taxa. Within the most abundant plant pathogens, *Roestelia* sp. and *Peniophora* sp. recurrently showed higher signal during autumn whereas *Leptosphaeria biglobosa* appeared to be prevailing in winter. Conversely, *Pseudozyma hubeiensis* was negligible in winter. Again, the predicted environmental sources were mostly soils (Fig. S6). More details on the full catalogue of bacterial and eukaryotic pathogens surveyed (Table S3), and on sample characteristics and temporal meteorological data (Table S4) can be found in Supplementary Information.

3.3. Pathogens relative abundances and dynamics under Saharan dust and non-dust episodes

Initially, as a whole we did not observe significant differences in the relative abundance of potential pathogens, related to presence or absence of Saharan dust in aerosols along the seven-years temporal survey (Fig. S7). However, a deeper analysis unveiled that potential bacterial phytopathogens showed higher relative abundance during Saharan dust intrusion episodes (Mann-Whitney Test p-value < 0.01). Conversely, fungal pathogens of animals and opportunistic eukaryal pathogens showed a significantly lower prevalence under Saharan influence (Mann- Whitney Test p-value < 0.01). No differences were observed for human pathogens or allergens (Fig. S7).

We explored which of these potential pathogens showed higher specificity and fidelity in Saharan dust intrusion events using the IndVal values (Fig. 4). Interestingly, the bacterial plant pathogens *Acidovorax avenae* and *Agrobacterium tumefaciens*, consistently showed higher signals under Saharan influence (Fig. 4, left panel). Conversely, *Janthinobacterium agaricidamnosum* was characteristic of non-dust situations. The fungal plant pathogens *Pseudozyma hubeiensis* and *Peniophora* sp. (Fig. 4, right panel) consistently showed high IndVal signals under Saharan influence. The remaining pathogenic fungi showed higher IndVal indices under non-dust conditions.

4. Discussion

We have surveyed seven consecutive years of atmospheric samples collected by passive natural deposition in a high-elevated mountain above boundary layer, after a high frequency sampling effort. Our pioneering study is a first step for generating basic background knowledge on the natural levels and dynamics of globally dispersed microorganisms and on the expected ranges of variation for long-range intercontinental exchange of potentially infectious airborne microorganisms, covering

Fig. 1. Long-term atmospheric baseline for relative abundances of potential bacterial pathogens. Note x-axis squared scale. Only taxa with relative abundances >0.1% are shown. Host preferences and percentage of 16S rRNA gene sequence identity to known species are highlighted.

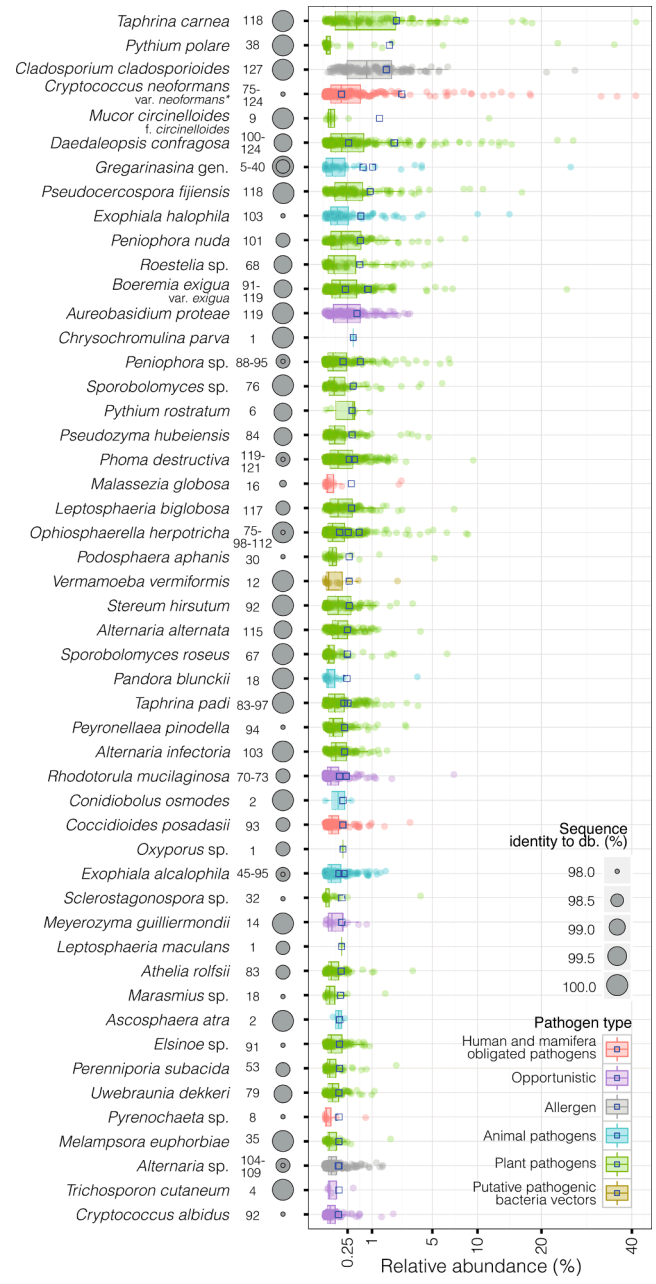

Fig. 2. Long-term atmospheric baseline for relative abundances of potential eukaryal pathogens (mostly fungi). Note x-axis squared scale. Only taxa with relative abundances >0.1% are shown. Host preferences and percentage of 18S rRNA gene sequence identity to known species are highlighted.

the most representative bacteria and fungi pathogens. A global disease monitoring and surveillance platform for humans, animals and plants pathogens has been recently emphasized as a fundamental Global Health aim (Lemon et al. 2007) and surveillance and modelling is a common practice for many plant diseases (Brown and Hovmøll, 2002). It is challenging however to properly split a natural event of pathogens from a deliberate (or accidental) release to feed decision-making and early warning systems (Skowronski and Lipkin, 2011). The lack of baseline information for natural abundances and distributions of pathogens in nature has been identified as a major limitation in medicine, public health and microbial forensics to facilitate rapid responses and better disease control policies (NAS Consensus Report, 2014). Temporal survey programs focused on the composition and structure of the atmospheric microbiota are still lacking because of the logistic challenges and methodological limitations (Barberán et al., 2014; Bowers et al., 2013; Tipton et al., 2019; Woo et al., 2013, Šantl-Temkiv et al. 2021). Interestingly, rain and snow samples collected at high-altitude mountains are a proxy for the microbiota composition in the free troposphere submitted to long-range transport (DeLeon-Rodriguez et al., 2013; Triadó-Margarit et al., 2019), minimizing local surface contaminations.

We uncovered consistent interannual dynamics with potential pathogenic representatives foreseeable over time (i.e., predictable seasonal behavior) or under recurrent environmental scenarios (e.g., Saharan dust intrusion events), respectively. Therefore, indicator taxa could be consistently identified as highly valuable microbial forensic signals. One of the best examples of temporal indicators is *Coccidioides* spp., a potentially severe and understudied fungal pathogen endemic from arid and semiarid regions of North and Central America (Kirkland and Fierer, 2018; Kollath et al., 2019), that we identified as an indicator taxon for winter. From the local endemic regions aerosolized soil particles would have been injected into high atmospheric altitudes and airborne transported across the Atlantic Ocean. This consistent seasonal pattern is probably linked with predominant western air mass provenance and the general atmospheric circulation (Cáliz et al., 2018), given that winter is the season with the highest precipitation of Atlantic origin in the LTER-AT site (Table S4 and http://loopweb.org/loopweb2018/index.php/lter-research/meteorological-data). *Coccidioides* spp. poses a risk to human health, since there is evidence of coccidiomycosis outbreaks following dust storm exposure (Griffin, 2007; Tong et al., 2017), although still some experimental limitations related to the low aerial concentrations prevent air surveillance of this pathogen (Kollath et al., 2019). Collecting concentrated bioaerosols directly washed from the atmosphere by rain and snow may circumvent the problem of under detection biomass levels, which technically limits many aerosols studies (Dommergue et al., 2019, Triadó-Margarit et al., 2019).

The summer and spring temporal signals were however mostly overlapped with the Saharan dust events that in the Mediterranean region specially occur in warm seasons. Saharan aerosolized dust is mostly transported towards the Caribbean region (Prospero et al., 2005) and, under specific meteorological conditions, dust is also transported towards continental Europe and the Mediterranean region, mostly in late spring and summer (Moulin et al. 1997). Interestingly, our approach allowed us to identify a few indicator phytopathogenic taxa simultaneously connected to both Saharan dust intrusions and temporal dynamics most of them bacteria (*Acidovorax avenae* and *Agrobacterium tumefaciens*) and only a few fungi (*Pseudozyma hubeiensis*). Conversely, most of the potential pathogens had a temporal spring-summer signal uncoupled to Saharan dust events (e.g., *Cryptococcus neoformans* and the phytopathogens *Ophiosphaerella herpotricha, Alternaria infectoria* and *Tilletia caries*). This point deserves further research and more detailed temporal surveys and model simulations (Ontiveros et al. 2021) because the frequency of dust storms, temperature and humidity are currently changing in some sensitive places of the world in response to changes in land use and anthropogenic forcing climate changes (Goudie, 2014, Ginoux et al., 2012) increasing the global dust emissions (Tegen et al., 2002). The signature of different pathogens has commonly been reported in airborne dust (e.g., Hervàs et al., 2009; Meola et al., 2015; Polymenakou et al., 2008) and their effects reviewed and discussed earlier (e.g., Griffin, 2007; Kellogg and Griffin, 2006; Prospero et al., 2005). The decline of entire biomes as coral reefs has been related to the presence of pathogens in recurrent dust events (e.g., Garrison et al., 2003). Higher levels of dust (due to desertification) and increased wind speed (with extreme events) expected for the new climatic scenarios may exacerbate the dispersal and persistence of some pathogens, especially those better adapted to the harsh conditions prevailing in the high atmosphere (Hellberg and Chu, 2016).

Phytopathogens were the potential infectious agents most widely present in the long-term dataset collected in our LTER-AT site. Long-range aerial dispersal of crop plants pathogens had been previously identified as one of those most prominent and severe consequences of

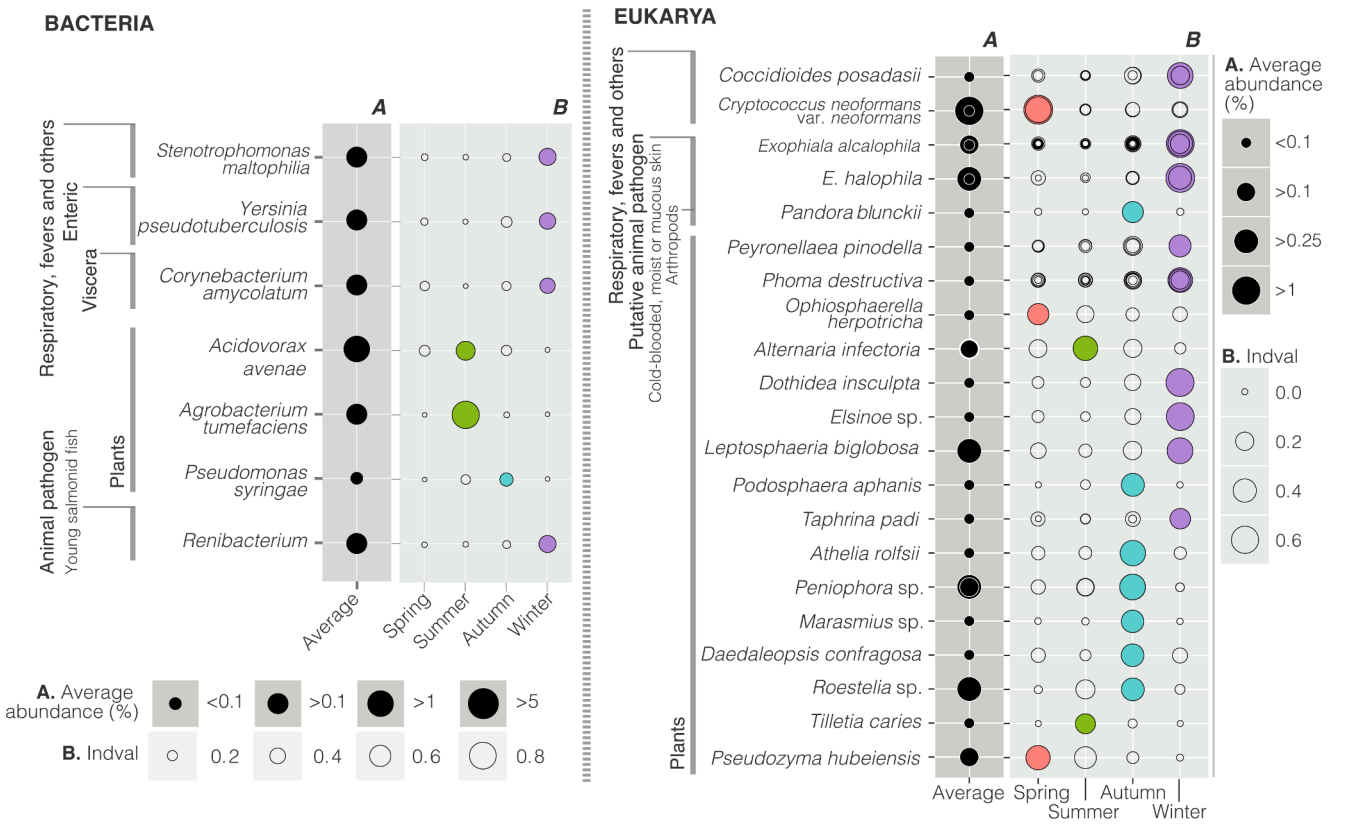

Fig. 3. Indicator airborne bacteria and eukarya taxa (IndVal) for the different seasons along the interannual long-term study. Panel A shows averaged relative abundances of the different potential pathogens; panel B shows the IndVal index for each season. Only taxa with enough statistical support are listed (IndVal index values > 0.3 and p-value < 0.05 for bacteria; IndVal index values > 0.2 and p-value < 0.01 for eukarya). Colored spots shows the season where the taxa were significantly over represented (i.e., both high specificity and high fidelity).

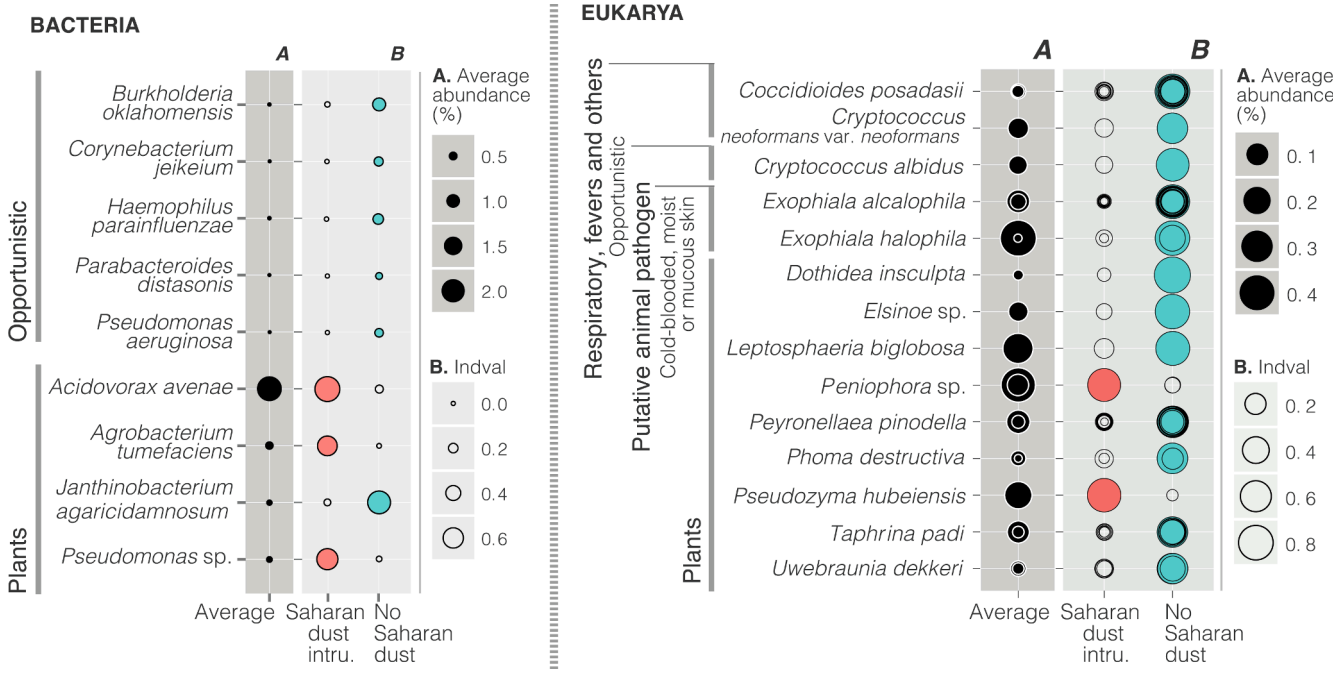

Fig. 4. Indicator airborne bacteria and eukarya taxa (IndVal) for Saharan dust intrusion episodes and dust-free periods. Panel A shows averaged relative abundances of the different potential pathogens; panel B shows the IndVal index for each escenario. Only taxa with enough statistical support are listed (see Fig. 3). Colored spots shows the conditions where the taxa were significantly over represented (i.e., both high specificity and high fidelity).

global airborne dispersal of microorganisms (Brown and Hovmøll, 2002) but interannual dynamics were poorly known. Our long-term study showed *Acidovorax avenae* and *Agrobacterium tumefaciens* – causing bacterial leaf blight and Crown gall diseases respectively to a large variety of plant crops – consistently linked to both summer atmospheric depositions and Saharan dust intrusion events. Conversely, the fungal pathogen *Leptosphaeria biglobosa* (causal agent of stem canker in oilseed rape) was characteristic from winter. Other bacteria and fungi for which the potential economic, environmental or social impacts are considered to be severe in EU (EU Regulation 2016/2031 Article 6–2) and included within the priority pests list, such as Candidatus *Liberibacter* spp., *Xylella fastidiosa,* and the fungi *Phyllosticta citricarpa* were never detected along the interannual survey. The chytrid fungus infecting amphibians (*Batrachochytrium dendrobatidis*) were not detected in the dataset either. Thus, our results support an unlikely aerial long-range intercontinental transport of *Batrachochytrium dendrobatidis* although short-distance airborne colonization cannot be ruled out (Kolby et al., 2015).

We acknowledge the limitations of our study to properly identify pathogens based on rRNA genes sequences identity alone. We carried out a conservative approach and although it is not sufficient to definitely state whether or not a given airborne microorganisms have pathogenic abilities, it stands the theoretical upper limit for their atmospheric baseline relative abundances. We only used for downstream analyses the OTUs having partial sequence identity >98% to inventoried pathogens, and not all microorganisms within these cluster are generally pathogenic. Even for partial sequence identities of 100% and full alignment coverage, additional confirmation steps are needed to fully corroborate the pathogenic capacity of the targeted OTUs (Ruppitsch et al. 2007). More experiments, such as the animal assay using some isolates or the comparison of epidemiological investigation data, are further needed to substantiate our findings. In the most recent literature, several investigations have used powerful molecular approaches to study airborne pathogens occurrence, abundance and distribution outdoor (Skowronski and Lipkin, 2011, West et al., 2008, Be et al., 2014), most of them looking for specific pathogens (e.g., Chow et al., 2016; Kolby et al., 2015, Dietzel et al., 2019) after some limitations and methodological aspects are also recognized (Schmedes et al., 2016). Although in our DNA-based study we did not specifically checked cell viability, there is consistent evidence of the presence of metabolically active cells (including pathogens) in the atmosphere (e.g., DeLeon-Rodriguez et al., 2013; Meola et al., 2015; Prospero et al., 2005, Polymenakou, 2012; West et al., 2008; Womack et al., 2010). The amplicon metagenomics satisfactorily covered cell identity but more specific assessment on pathogenicity needs of further molecular studies using specific qPCR tests on specific virulence markers (Kaushik and Balasubramanian, 2012).

Overall, our study supplies a wide-spectrum catalog and an atmospheric conservative baseline for long-range long-term air-dispersed potential pathogenic microorganisms. We unveiled predictable dynamics for some of them and the seasonal differences could be explained by the origin of air masses and the source of aerosols (Cáliz et al. 2018). More studies above the boundary layer in remote field sites will confirm whether or not a similar pattern is found globally or whether it is restricted to the general and regional air mass regimes of the Mediterranean area. More accurate molecular and experimental approaches will be needed to tell apart pathogens in massive datasets. Air mass circulation patterns may change in the new climate scenarios and the long-range transport of microorganisms is particularly responsive to climate variability (Prospero et al., 2005). Thus, unforeseeable consequences of airborne pathogens on new downwind sites is a research topic that should be carefully addressed in the future.

CRediT authorship contribution statement

Xavier Triadó-Margarit: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. **Joan Cáliz:** Data curation, Formal analysis. **Emilio O. Casamayor:** C onceptualization, Funding acquisition, Investigation, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106916.

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