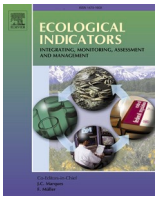
Ecological Indicators 121 (2021) 107032

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind



Integrated eDNA metabarcoding and morphological analyses assess spatio-temporal patterns of airborne fungal spores

Enrico Tordoni [a,*], Claudio G. Ametrano [a,b], Elisa Banchi [a,c], Silvia Ongaro [a], Alberto Pallavicini [a], Giovanni Bacaro [a], Lucia Muggia [a]

[a] Department of Life Sciences, University of Trieste, via Giorgieri 10, 34127 Trieste, Italy
[b] Grainger Bioinformatics Center, Field Museum of Natural History, Chicago, IL, USA
[c] National Institute of Oceanography and Applied Geophysics – OGS, via Piccard 54, 34151 Trieste, Italy

ARTICLE INFO

Keywords:
Aerobiology
Co-correspondence analysis
Detection efficiency
Next-generation sequencing
Sampling
Species diversity

ABSTRACT

Fungi represent relevant allergens and plant pathogens that can disperse on long ranges, potentially producing severe consequences on public health and agriculture. Up to 11% of the bioaerosol particles are fungal spores and mycelium fragments. Estimation of fungal species diversity in time and space is decisive but may be biased by abiotic conditions and sampling methods. Traditional morphological analyses of fungal spores have been widely applied in aerobiology in the past, while recently eDNA metabarcoding can complement these studies. Here, we used both morphological analysis (spore count and taxon identification) and high-throughput sequencing to disentangle spatio-temporal variation of fungi across Northern and Central Italy and to evaluate the detection efficiency of the two approaches. Our results showed that eDNA metabarcoding detects about three times more genera and has a higher detection efficiency than the morphological analyses. However, the efficiency is high in both spore count and eDNA metabarcoding methods when the most abundant or the rarest genera are considered but it can substantially vary between the two approaches when moderately abundant genera are analyzed. Furthermore, morphological spore determination resulted in higher variance explained by PERMANOVA analysis with respect to eDNA metabarcoding (26% and 13%, respectively), which leads to a better spatio-temporal characterization of the fungal genera. As both morphological analyses and eDNA metabarcoding methods capture significant interactions between seasons and sites, they could be preferably used as complementing approaches to reliably study airborne fungal diversity and variation.

1. Introduction

The atmosphere hosts high levels of biodiversity (Gandolfi et al., 2013; Mayol et al., 2017), and some microorganisms such as bacteria and fungi represent up to 11% of total airborne particles (Bauer et al., 2002; Burrows et al., 2009; Fröhlich-Nowoisky et al., 2009; Grinn-Gofroń et al., 2018). Fungi disperse their propagules in the atmosphere in form of spores or mycelium fragments; their size, shape, number, and range of dispersion depend both on the taxon characteristics and the abiotic conditions or climatic phenomena such as moisture, temperature, precipitations, winds, and availability of substrates (Grinn-Gofroń et al., 2018). These factors are relevant to make fungal aerial communities particularly variable, causing geographical and temporal heterogeneity even at finer scales (Bowers et al., 2013; Nacke et al., 2016). Furthermore, it is still questioned whether these airborne particles mainly originate from local sources (Skjøth et al., 2012) or travel across miles away from their origin under suitable weather conditions (e.g. Damialis et al., 2017; Mayol et al., 2017). New pieces of evidence emerged on how airborne samples change across time and space, or even across different land-use types (Banchi et al., 2018), while molecular data have provided also up to 10-fold higher resolution of the airborne biodiversity with respect to classical methods obtained by traditional microscopy (e.g. Kraaijeveld et al., 2015 for pollen; Banchi et al., 2018 for fungi). Indeed, conventional microscopy applied on aerobiological samples rarely accomplish species-level identification and is often biased by several issues, such as the low amount of sampling material,

Abbreviations: Co-CA, Co-Correspondence Analysis; FVG, Friuli Venezia Giulia; M, Marche; PERMANOVA, Permutational Analysis of Variance; U, Umbria; VA, Valle d'Aosta; VE, Veneto.

* Corresponding author.
E-mail address: etordoni@units.it (E. Tordoni).

https://doi.org/10.1016/j.ecolind.2020.107032
Received 3 April 2020; Received in revised form 3 September 2020; Accepted 30 September 2020
Available online 11 October 2020

the lack of distinctive morphological characters, and rigorous taxonomic classification along with a high intraspecific morphological variation (Núñez et al., 2016). Thus, resolving these drawbacks would be particularly important, both from an ecological and a clinical point of view, as some of these organisms are allergens or pathogenic agents with relevant implications for public health and agriculture.

Recent and increasing advances in molecular techniques such as High-Throughput Sequencing (hereafter HTS) and environmental DNA (eDNA, Taberlet et al., 2012) metabarcoding, brought useful insights into the diversity and composition of airborne assemblages (see Banchi et al., 2020c and references therein). Nowadays, eDNA metabarcoding analyses are widely applied in ecology-related fields such as species conservation and monitoring (Thomsen and Willerslev, 2015). These allow a better understanding of biodiversity thanks to a more exhaustive detection of species, especially the most elusive or the rarest ones (Deiner et al., 2017; Ruppert et al., 2019). Indeed, eDNA metabarcoding offers the possibility to assess and compare species diversity even without having specific training in taxonomy, and to detect those organisms which may not grow in culture, are dead or dormant (An et al., 2018).

While aerobiological studies implementing eDNA metabarcoding analyses have considered mainly pollen and bacterial diversity (Kraaijeveld et al., 2015; Korpelainen and Pietiläinen, 2017; Brennan et al., 2019), the fungal fraction has remained somewhat neglected (Banchi et al., 2020c). Even though significant progress has been made in fungal aerobiology in the last years, most of the studies were carried out using traditional morphological analyses in peculiar or spatially-constrained environments (e.g. cities, Núñez et al., 2017; Pyrri and Kapsanaki Gotsi, 2017; Antón et al., 2019), considering short period of time (Banchi et al., 2018) or focusing on specific medical interests (Chen et al., 2014). To the best of our knowledge, an assessment on the pattern of airborne fungal biodiversity coupled with a quantitative comparison between classical method (spore count based on morphological analysis) and modern eDNA metabarcoding has not been performed yet across seasons and a broad geographic region. Banchi et al. (2018, 2020c) strengthened the suitability of eDNA metabarcoding as a promising tool to improve data quality and sensitivity of aerobiological monitoring and developed a complete pipeline to analyze both the pollen and the fungal spore fractions in mixed aerobiological samples (Banchi et al., 2020b).

Here, we present a first, aerobiological study in which we investigated airborne fungal diversity and its variation across five areas distributed in North and Central Italy; the sampling was carried out for nine months using both morphological analysis and eDNA metabarcoding. By merging traditional and modern approaches we aimed at *i)* evaluating the detection efficiency (defined as the capability in capturing taxa diversity and variation across the sampling period) of eDNA metabarcoding with respect to standard morphological analyses based on spore count and *ii)* providing useful insights on the spatio-temporal patterns of aero-dispersed fungi.

2. Materials and methods

2.1. Study area and data collection

Airborne biological particles were sampled as described in (Banchi et al., 2020a, 2020b) in five localities selected in Northern and Central Italy (Fig. S1 of Supplementary material). The sampling was performed by the collaborators of the Regional Agency for Environmental Protection (ARPA) units using a volumetric sampler (VPPS 2010, Lanzoni) with aspiration pump (10 l/min) mounted with sticky tape (Melinex®) and placed on the roof of a building at about 15–20 m from the ground. This sampling approach is established by the Italian national guidelines for aerobiological monitoring (www.pollnet.it). The sampler follows the suction slit impactor developed by Hirst (Levetin 2004) and can collect airborne particles in the range of 5–100 μm. The five sampling sites were selected to maximize the geographical and climatic diversity over Northern and Central Italy [i.e., two sites in North-Eastern Italy (FVG, Friuli Venezia Giulia and VE, Veneto), two in Central Italy (M, Marche and U, Umbria) and one in North-Western Italy (VA, Valle d'Aosta); Fig S1]. The sampling was performed for two weeks per month (during the 2nd and 4th week of the month) during nine sequential months (starting on 1st March and ending on 30th November 2017). In total, we collected 18 samples for each site that is two records per month; for instance, F1 and F2 represented the first and second half of March in site FVG. Due to operational constraints, the beginning and ending parts of the weekly tapes (two days) were used for morphological analyses while the central parts (five days) for the molecular ones.

According to Köppen climate classification, the climate conditions in FVG, Marche, Umbria and Veneto are classified as humid-subtropical (Cfa), while VA is classified as oceanic (Cfb) (Kottek et al., 2006).

2.2. Microscopy analysis

The tapes used for morphological inspection were placed on a glass slide, mounted in basic fuchsine and observed at a light microscope Olympus BH-2. Spore counting was performed following the guidelines published by the Italian national agencies for environmental protection (i.e. ISPRA - Istituto Superiore per la Protezione e la Ricerca Ambientale, ARPA - Agenzie Regionali per la Protezione dell'Ambiente and APPA - Agenzie Provinciali per la Protezione dell'Ambiente; available at http://www.pollnet.it/pubblicazioni.asp) in which at least 15% of the tape surface is inspected for pollen counting. For this study, we delimited a longitudinal area of 2 cm^2 (usually 2 × 1 cm), at the center of the mounted tape, to identify and count fungal spores at a magnification of 1000×. This area corresponds to 20% of the total area and covers almost the whole length (one sampling day) of the tape piece on which airborne particles stick. Fungal spore identification was based on the illustration manual of air samples of Smith (2000). Spores were counted for each individual genus that could be determined.

2.3. Molecular analyses: library preparation and sequence analyses

DNA extractions from the sampling tapes were performed with ZR Fungal/Bacterial DNA MicroPrep™ Kit (Zymo Research) following Banchi et al. (2018, 2020b). Firstly, DNA was extracted from the tape of each individual sampling day of the week; secondly, the DNA extractions were pooled in equal amount into week samples for DNA metabarcoding analysis. This sampling procedure resulted in a total of two records for each month and 18-week samples for each site; each week was treated further in the analyses as an individual "sample", resulting in a total of 90 samples. We used as controls, i.e. negative samples, six not-exposed tapes to assess possible contaminations due to samples processing and we included them in all PCR amplifications and sequencing steps. The fungal ITS2 fragment was amplified using one forward primer (ITS-u2_F, corresponding to the reverse complement of the primer ITS-u2 from Cheng et al., 2016) coupled with three reverse primers (ITS-p4, Cheng et al., 2016; ITS-f4, Banchi et al., 2020b; ITS4U_R, White et al., 1990) as described in detail in Banchi et al. (2020b).

Amplicons for HTS sequencing of the airborne samples were obtained with two sequentially PCR amplifications as in Banchi et al. (2018): the first, primary PCR, amplifies the target sequence; the second, outer PCR, is performed to attach the molecular identifiers (MID) for multiplex sequencing to the PCR products. For each sample, the two PCR amplifications were performed independently for either of the three primer pairs; the three reactions were pooled after the outer PCR, to ensure that the three PCR products were successfully amplified. Primary PCR reaction mix contained 2 μl DNA template (~10 ng), 8 μl SSOAdvanced™ SYBR® Green Supermix (Bio-Rad) and 200 nM of forward and reverse primers in a final volume of 15 μl. The PCR amplifications were performed in a CFX 96™ PCR System (Bio-Rad) with the following cycling profile: 98 °C for 30 s and 35 cycles at 95 °C for 10 s and 60 °C for 20 s. The outer PCR reaction mix contained 0.5 μl of the first PCR

product, 8 µl SSOAdvanced™ SYBR® Green Supermix (Bio-Rad) and 200 nM of each primer (10 µM) in a final volume of 15 µl. This cocktail was processed for 12 PCR cycles with the previous amplification conditions. The amplicons were run on a 2% agarose gel from which ~ 400 bp products were gel extracted, quantified with Qubit™ Fluorimeter (Thermo Fisher Scientific) and pooled in equal amount to prepare the libraries. The libraries were sequenced with an Ion Torrent Personal Genome Machine (PGM, Thermo Fisher Scientific) on two 316™ chips (Thermo Fisher Scientific). The sequence data are available at the NCBI short read repository under the accession number PRJNA576572.

Bokulich et al. (2013) suggested to perform a primary filtration, based on quality score and length, and a second filtration, based on the number of reads that represent the OTUs. Thus, the obtained sequences were de-multiplexed, trimmed (from primers and adapters), and quality filtered (minimum length 200 bp, minimum average quality score 20) with CLC Genomics Workbench v.12 (Qiagen). Reference-based Operational Taxonomic Units (OTUs) clustering and taxonomic assignment were performed with Microbial Genomics module in CLC Genomics Workbench v.12 (Qiagen), using as reference a fungal ITS2 database prepared following Banchi et al. (2020a). The parameters were set as follows: 97% similarity (70% for the new OTUs), minimum occurrence of two to remove singletons as suggested in Auer et al. (2017).

2.4. Statistical analyses

Due to some technical problems occurred to the samplers at the localities Marche and FVG, in order to have comparable data between eDNA and spore count, six samples had to be excluded from the dataset (F1, F4, F17, M8, M10, M15) while the remaining 84 have been considered for the following analyses. All the recovered OTUs were standardized to genus level.

To compare the pattern of genera accumulation in each site along the sampling period (i.e., the cumulative number of genera observed from the first to the last sampling week), we first computed genus accumulation curves for spores and eDNA independently using 'collector' method in 'vegan' R package (Oksanen et al., 2019, data not shown).

Once accumulation curves were calculated for each site and to make the observed richness comparable across sites, we calculated the ratio (spores/eDNA) for each curve. Furthermore, we assessed the detection efficiency of genera between the two analysis methods by displaying the pattern of the relative abundance of the two most abundant (*Cladosporium* and *Alternaria*), two relatively abundant (*Coprinus* and *Epicoccum*) and one of the rarest genera (*Oidium*).

To assess if assemblages characterized by the two methods shared common patterns, symmetric Co-Correspondence Analysis (Co-CA, see ter Braak and Schaffers, 2004; Schaffers et al., 2008) was performed by pooling all sites and seasons. Symmetric Co-CA aimed at finding gradients along which weighted covariances of site scores were maximized (ter Braak and Schaffers, 2004); none of the two abundance matrices plays any explanatory or predictive role. Co-CA was computed using R package 'cocorresp' (Simpson, 2009) on relative abundance data; Pearson product-moment correlation coefficient (r) for the site scores of ordination axes was also estimated.

Permutational Analysis of Variance (PERMANOVA; Anderson, 2001) was performed independently for the two assemblages treating Site (five levels: FVG; M, U, VA, VE) and Season (three levels: Spring, Summer, Autumn) as fixed effects along with their interaction to test whether pattern of variation across sites and among seasons could be detected. PERMANOVA tests were performed according to the following specifications: Bray-Curtis similarity, 4999 permutations of residuals under a reduced model, and type III sums of squares; when factors resulted significant, pairwise comparisons were performed using t statistic and 4999 permutations.

PERMANOVA were performed using PRIMER 6 software (Clarke and Warwick, 2005) and the add-on package PERMANOVA + (Anderson et al., 2008), all other statistical analyses were computed in R 3.6.2 (R Core Team, 2020).

3. Results

In the whole dataset, we observed 22.21 ± 8.66 (mean ± SD) genera based on spore count method, whereas 61.78 ± 47.81 genera were detected using eDNA metabarcoding. Furthermore, contrasting patterns in genus richness were observed across sites and seasons (Fig. S2 in Supplementary material). The most recurrent genera (i.e., those detected in more than 85% of the samples) using morphological analyses were *Cladosporium*, *Coprinus*, *Leptosphaeria*, *Alternaria*, *Epicoccum*, *Torula*, *Stemphylium*, *Exosporium*, and *Periconia*. In contrast, *Alternaria*, *Cladosporium*, *Calocybe*, *Epicoccum*, *Phoma*, and *Polyporus* were the most frequently detected in the eDNA metabarcoding dataset.

Interestingly, the ratio of the genus accumulation curves showed a similar trend (Fig. 1) during the sampling period in FVG, U, and VE, presenting a peak between the weeks 5 and 7 (Spring) and decreasing progressively towards the end of the sampling period (Autumn). In contrast, M displayed extremely low values of the ratio whereas VA showed a peak in the central part of the sampling period. Notably, the ratio of the species accumulation curves showed a similar trend across sites and sampling period.

The trends of the five representative taxa showed a strong taxon-dependency (Fig. 2). The detection efficiency was high in both spore count and eDNA metabarcoding methods when the most abundant (*Alternaria* and *Cladosporium*) and the rarest (*Oidium*) taxa are considered. Alternatively, when relatively abundant genera are analyzed, contrasting pattern can be detected. As an example, in the present analysis, the variation of *Coprinus* was better captured (i.e., higher abundance) by spore counting, while that of *Epicoccum* by eDNA metabarcoding.

Symmetric Co-CA (Fig. 3) displayed a rather concordant pattern between the two analytical methods showing both a strong highly significant correlation on the first two Co-CA axes ($r_1 = 0.84$, $r_2 = 0.61$; $P < 0.001$ for both correlation coefficients) as well as a high variance explained (≈ 90%). Interestingly, two samples (VA2, F2) emerged from the cloud of points both in spore count and eDNA, indicating consistency between the two datasets for these records.

The results of the PERMANOVA analysis (Table 1) displayed a significant variation between Season, Site and their interaction in both spore count and eDNA metabarcoding; in particular, spore count had twice of overall variation explained (26%) with respect to eDNA metabarcoding (13%). Post-hoc test performed on the Season-Site interaction showed large variability in the statistical significance of pairwise comparisons (see Table S1 of Supplementary material for further details).

4. Discussion

4.1. Detection efficiency of eDNA metabarcoding vs spore counting

Our results report that detection of taxonomic entities is partially discordant between the morphological and molecular approaches. However, the slightly different lists of the most abundant taxa reported by spore counting and eDNA metabarcoding are likely due to the type of spores (morphology and taxonomy) that are counted. We observed about three times more taxa recovered by eDNA metabarcoding than by spore counting. Interestingly, the ratio of the genus accumulation curves showed a similar trend across sites meaning that the accumulation of new taxa as a function of the time remains quite constant in either the spore counting or the molecular methods. In particular, these curves shared a similar pattern in FVG, U, VE, showing a peak around Spring, and a progressive decrease of taxa towards the Autumn, likely suggesting a substantial control of the climate on airborne fungal assemblages, as already reported in the literature (e.g. Favero-Longo et al., 2013; Grinn-Gofroń et al., 2018). Alternatively, in Marche (M) shallow values

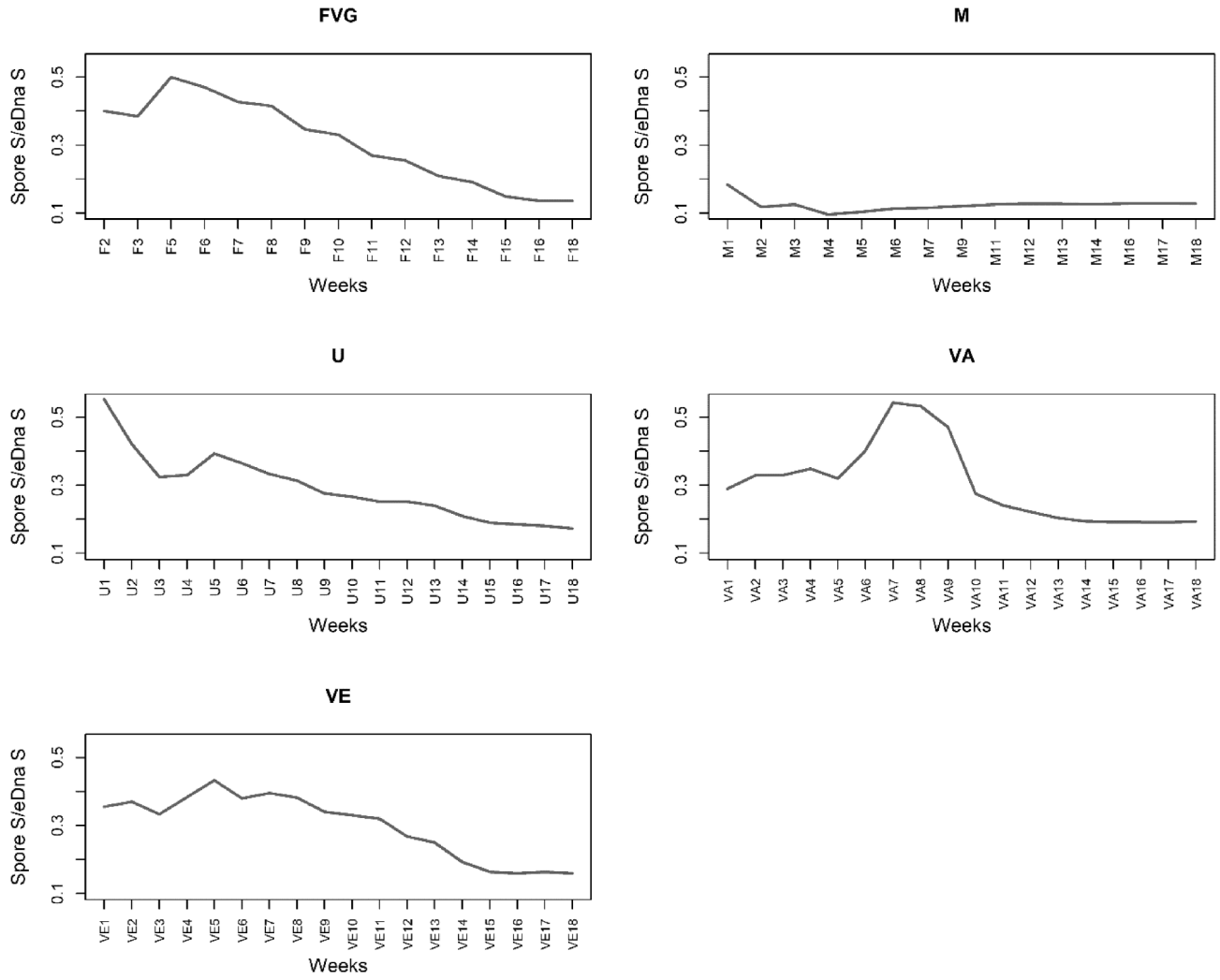


Fig. 1. Ratio between observed richness calculated using genus accumulation curve with spore count (Spore S) and eDNA (eDNA S) across the five sites (FVG: Friuli Venezia-Giulia, M: Marche, U: Umbria, VA: Valle d'Aosta, VE: Veneto) during nine months (March-November 2017). x-axis shows the sampling period (e.g. VA1 and VA2 refer to the first and second half of March, respectively).

of the ratio were recovered with a slight increase of taxa towards the Autumn period. We speculate that this is caused by the close presence of the sea to the collecting site in Marche, as here the sampler was only a few kilometers far from the coast. Only in VA the curve presents a very high peak in the distribution pattern, which can be attributed to the short vegetative period in this site (VA). Indeed, VA is the coldest, and northernmost site included in the survey, for which Spring and Summer seasons are shorter than in the other sites.

We observed also a higher variance explained by PERMANOVA analysis in spore count rather than eDNA metabarcoding. Even though this can appear as counterintuitive, the higher richness (α diversity) observed in the fungal assemblage often is not directly linked to its complementarity (i.e. β diversity). Accordingly, spore count is more prone to operator bias (as the operator cannot always discriminate all the fungal species based on spores, i.e. assess the true α diversity) and this enhances morphological clustering of two or more closely related taxa. On the other hand, eDNA can catch a much higher degree of taxa variation (β diversity) in the airborne sample through time, potentially leading to a taxonomic homogenization among the fungal assemblages. However, the detection efficiency of either method may be dependent on the morphological characteristics of the spores themselves. Indeed, *Alternaria* and *Epicoccum* conidiospores are multicellular and among the biggest aero-dispersed spores. They were more abundant in the spore count at the end of Summer and the beginning of Autumn, which is the known sporulation period of *Alternaria* in Europe (Vloutoglou et al., 1995). In contrast, according to the eDNA metabarcoding results, *Alternaria* seems to occur during almost the whole sampling period, presenting even cyclical peaks of abundance. This discrepancy between the two sets of results is likely due to the multicellularity of the *Alternaria* and *Epicoccum* conidiospores, which would let the DNA be easier amplifiable though the number of spores counted is relatively low in comparison to other taxa. *Epicoccum* is known to sporulate during relatively dry periods and spores are dispersed easily by wind turbulences (Ščevkova et al., 2016). Morphological inspections recovered *Epicoccum* spores during the Summer season, and higher numbers of counted spores did correspond with peaks of eDNA metabarcoding reads.

The opposite case is observed for *Oidium* and *Coprinus*. *Oidium* disperses by unicellular, relatively big conidiospores (on average $12 \times 22\,\mu m$), which are seldom recovered in the morphologically inspected samples. This is further corroborated by its low abundance in eDNA, which seems to be determined by the low amplifiability of its DNA, as the number of *Oidium* reads is extremely low, almost hardly detectable, during the whole surveyed period. There are few peaks of *Oidium* spores reported for Spring and early Summer, but these did not correspond with a high number of reads in the eDNA metabarcoding.

The high discrepancy in the detection of the taxon *Coprinus* between the spore counting and the eDNA metabarcoding might be caused by

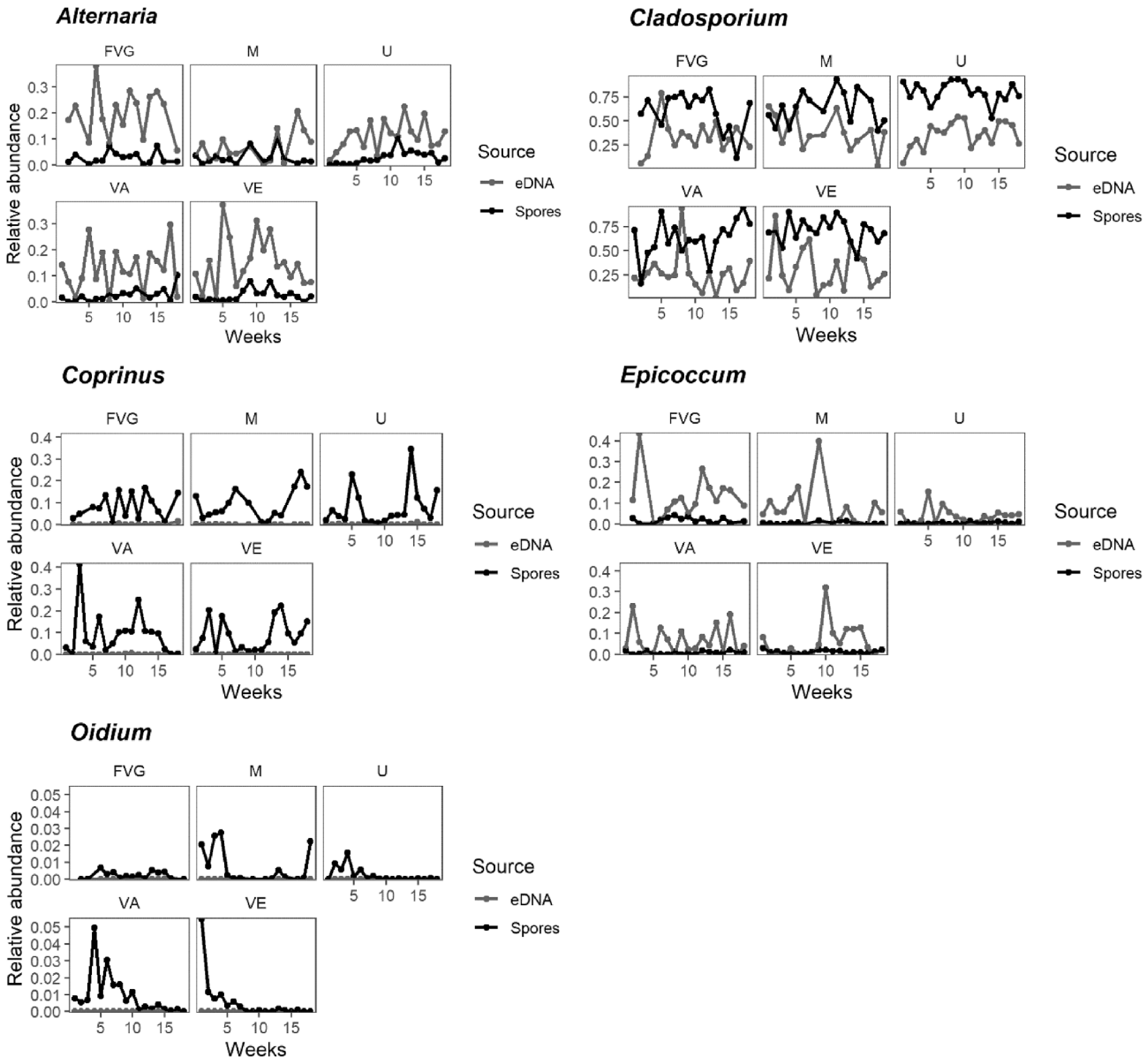


Fig. 2. Patterns of relative abundance analyzed for the two most common (*Cladosporium* and *Alternaria*), relatively abundant (*Coprinus* and *Epicoccum*) and of one of the rarest genera (*Oidium*) during the sampling period (nine months, March-November 2017) in each sampling site (FVG: Friuli Venezia-Giulia, M: Marche, U: Umbria, VA: Valle d' Aosta, VE: Veneto) comparing both spore count and eDNA metabarcoding data.

inefficient DNA extraction for its spores (being these highly melanized) or by primer bias during amplification and sequencing, as this latter resulted in an extremely low presence of reads corresponding to *Coprinus*. *Coprinus* basidiospores are rather small and released in high number with a very efficient and peculiar dispersing strategy (i.e. gills liquefy from the bottom up of the umbrella, as spores mature, the caps peel and mature spores are positioned at best to be caught by wind currents; Bender and Enderle, 1988), which is indeed observed in the aerobiological samples, as even up to 4000 spores per sample were counted for *Coprinus*.

Cladosporium is known for its high sporulation rate (Bensch et al., 2012) and its conidiospores are ever-present in aerobiological samples: they are counted up to few thousand (5000) and estimated even up to several thousand (10000–15000) in some samples. Results obtained by spore counting and eDNA metabarcoding for this genus are concordant, and in general, the peaks of spore counting correspond to high numbers of reads in the molecular results.

Although eDNA metabarcoding allows the detection of rare or unculturable taxa in many studies, from our results it emerges that spore counting is still essential to evidence the presence of the rarest taxa among airborne fungi. This holds true also for the genera *Bipolaris*, *Exosporium*, *Glyomastix*, *Sporormiella*, *Spegazzinia*, *Tilletia*, *Torula*, or *Beltrania*, which have a very characteristic shape and are well identifiable by morphological inspection but their infrequent presence on the collecting tapes does not make them be amplified and sequenced. This fact is likely due to PCR primers and amplification biases. On the other hand, eDNA metabarcoding helps to discriminate between genera with very similar spores which may be included under "bigger" categories, i.e. morphological clusters in the spore count analyses. This is the case of the genera *Leptosphaeria*, *Lophistoma*, *Phaeosphaeria*, *Paraphaeosphaeria* and *Keissleriella*, which cannot be properly segregated if the atlas of Smith (2000) is used for identification. On the contrary, their ambiguous morphological determination was resolved by the eDNA metabarcoding. These genera were particularly abundant in the Summer period in all the sites and their spores represented in few cases more than half of the whole spore counted for the samples.

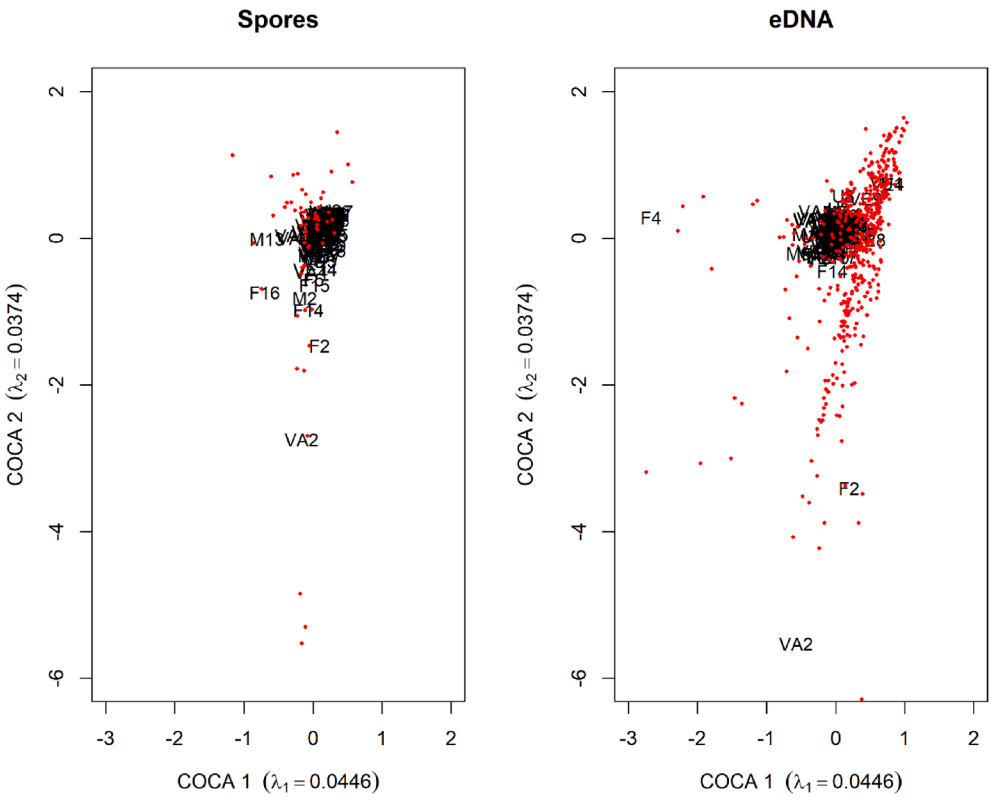


Fig. 3. Symmetric Co-CA between weighted average scores of two assemblages derived from morphological analyses (Spores, left panel) and DNA metabarcoding (eDNA, right panel). Red dots represent taxa, while text is site label as described in method section (e.g. VA2 represents the site VA sampled in the second half of March). Even though the two panels show different ordinations in the multivariate space of the two assemblages (spores vs eDNA), the two axes represent the same ordination aiming at maximizing weighted covariance between the weighted averaged species scores of the two assemblages.

Table 1
PERMANOVA output computed on Bray–Curtis similarity matrices for two assemblages deriving from HTS (eDNA) and morphological analyses (Spores), respectively. df: degrees of freedom, MS: Mean squares.

Source of variation	df	MS	Pseudo-F	Variance explained (%)
eDNA				
Site	4	3527	2.11**	5.95
Season	2	3421	2.05**	3.36
Site × Season	8	2057	1.23*	3.67
Residual	69	1669	–	87.02
Spores				
Site	2	1370	3.06***	9.35
Season	8	1408	31.38**	5.83
Site × Season	69	806	1.80**	10.74
Residual	83	449	–	74.08

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

The high correlation between the first two axes observed in the symmetric Co-CAs also corroborates a substantial consistency of the patterns detected by the two methods. Notwithstanding this, our results might be argued by the fact that samples used for either molecular and morphological analyses are not exactly the same. This is due to operational constrains that are intrinsic to the sampling procedure. Indeed, although there were two samplers available, one was used exclusively by the ARPA units for delivering the weekly pollen bulletins; these tapes were treated with fuchsine and could not be used for any further analyses. The second sampler was available for our investigation. We therefore opted to use the same tape for both morphological and molecular analyses to not introduce further biases. In the case we had exactly comparable samples, we should have cut the tapes longitudinally, but this would have severely affected the amount of pollen and spores available for the eDNA metabarcoding analyses, that was already very poor in certain weeks. Because the approach followed by Banchi et al. (2018) proved reliable, we decided to apply it also in the present study.

Despite some studies criticize the use of relative abundances (e.g. Gloor et al., 2017), these are routinely used when studying microorganisms such as bacteria and fungi (Bowers et al., 2013; Aguayo et al., 2018). Additionally, evidences from other taxonomic groups reported an overall good agreement between relative abundances detected through metabarcoding and true abundances of taxa (Leray and Knowlton, 2015; Serrana et al., 2019). Definitely, future researches should consider this aspect, likely proposing a method which firstly analyze morphologically the sampled tapes, and consecutively use them for DNA analyses.

4.2. *Spatio-temporal variability of fungal assemblages*

Particles that build the biological fraction of the aerosol are represented by pollen and other plant material, fungal spores and mycelia fragments, bacteria, viruses, unicellular algae or even microscopic arthropods (Burrows et al., 2009). The composition of aerobiological samples has been studied for many years and served to understand how biological assemblages may change in relation to abiotic factors, such as meteorological events (Grinn-Gofroń et al., 2018).

It is generally accepted that most of the sampled airborne particles come from local sources (Skjøth et al., 2012); however, recent studies suggested that they can be transported miles away from their origin under suitable weather conditions (Damialis et al., 2017; Mayol et al., 2017). In accordance to the local sources hypothesis, our findings showed that the interaction effect between Season and Site (see Table 1 and Table S1 of Supplementary material) explains part of the observed variability in the diversity of airborne fungi, therefore suggesting that taxonomic composition of airborne fungal spores changes substantially across the sampling period. This lets us speculate that airborne fungi detected in our survey majorly originate from local sources because there is a high diversity observed among Sites and Seasons. Both the morphological and molecular methods succeeded in catching the actual variability in the composition of some air-dispersed fungi (see Fig. 3) suggesting a spatio-temporal variability in the fungal assemblages. As in the present study, a strong effect of season and site was also observed in

previous studies (Favero-Longo et al., 2013; Fröhlich-Nowoisky et al., 2016; Grinn-Gofroń et al., 2018), which highlighted the strong control of climate and local weather conditions on the sporulation dynamics of fungi. We recovered the highest frequency and abundance of spores in the late Spring/early Summer period, while toward the Autumn months, the decrease of spore frequency is likely due to the lower temperature causing the death of the fungi (Antón et al., 2019).

The spatial context surrounding the sampling stations, considering the type of vegetation, pedology and land use may also influence the spatial patterns of fungal assemblages (Banchi et al., 2018). Indeed, the range of dispersion of some spores can be strongly influenced by the landscape characteristics and its alteration (i.e. natural vs anthropogenic land cover; Kauserud et al., 2005). Furthermore, the presence of hosts is determining for the completion of the reproductive cycle, i.e. the sporulation, for the majority of the plant pathogenic fungi, which justify the detection of *Alternaria*, for example, in the late Summer, when its plant host (i.e., corn, *Zea mays*) is at its full growth state.

Similar results were obtained by Abrego et al. (2018), who showed how a combination of aerial sampling coupled with probabilistic molecular species identification offers a highly effective method to assess fungal diversity over large areas.

4.3. Integrating morphological and molecular approaches to study the aeromycota

Studying the diversity and the dispersion patterns of aerobiological particles is becoming central in aerobiology, as the interest in aero-dispersed pathogens and allergens is increasing in multiple fields of research, spanning from medicine to agriculture. While the diversity and the seasonal variation of pollen have been in particular well documented across several regions and using different methodologies individually or combined (Damialis et al., 2017; Brennan et al., 2019), the diversity of airborne fungi (the aeromycota) is still limited known (Grinn-Gofroń and Bosiacka, 2015). Moreover, the majority of the published results are the outcome of either morphological or DNA analyses performed individually, as taxon determinations were performed on spores trapped on sticky tapes, filters or on those fungi grown out from spores trapped on culture growth media (Burge, 2002; Fröhlich-Nowoisky et al., 2009).

While pollen monitoring is carried out weekly worldwide (Buters et al., 2018) with a taxon accuracy up to the genus or even species level, spore monitoring is infrequent and spore calendars exist since recently only for Spain (Fuentes Anton et al., 2019). This is due to the difficulties of studying fungi in airborne samples because of the vast number of spores, the high variation of spore morphology (even intraspecific), and the contemporaneous presence in the air of spores from both ubiquitous and seasonal fungi. In most of the cases, only a pair of fungal genera (spore types) are included in the pollen calendars, and these usually correspond to the two allergenic and most frequent genera *Alternaria* and *Cladosporium* (Buters et al., 2018). Recently, Fuentes Anton et al. (2019) stressed the need to increase the knowledge of airborne fungal diversity and its seasonal variation proposing a preliminary spore calendar for the western part of the Iberian Peninsula. The monitoring of Fuentes Anton et al. (2019), though extended for two years, was still based on morphological inspections.

Since 2012, a growing number of studies based on metabarcoding analyses of airborne fungi have been published showing a steadily increase in the last three years (as reviewed by Banchi et al., 2020c). Banchi et al. (2018) were among the first suggesting the use of the molecular approach as supplement to the traditional morphological determination, stressing the evidence that eDNA metabarcoding would act as a promising approach to increases quality and sensitivity in aerobiological monitoring. The eDNA metabarcoding is independent of the degree of expertise and specialization of the operator and detects a higher taxonomic diversity, increasing up to ten-fold the accuracy of taxon recognition (Banchi et al., 2018, 2020c). Still, the eDNA metabarcoding approach does not offer a quantitative but a semi-quantitative approach (based on relative abundances) in the estimation of taxon diversity. Future studies should consider performing a more reliable quantification of the most abundant or interesting taxa by real-time quantitative PCR. On the other hand, molecular results might be biased by preferential amplification of specific sequences (i.e., preferential annealing of the primers to the DNA), especially if the mixed sample is composed by a high number of different taxa, or by the lack of representative, reference sequences in public databases. However, since 2018 the use of eDNA metabarcoding analyses have progressed impressively also because of the availability of reference databases for fungal sequences (Nilsson et al., 2018; Banchi et al., 2020b,c). Therefore, a microscopical inspection of the samples would still be preferable to assure that any of the rarely occurring taxa is not missed during DNA amplification and sequencing (Banchi et al., 2018), and to enable a quantification of the taxa by spore count.

Abrego et al. (2019) have highlighted the importance of combining morphological and molecular approaches to capture airborne fungal diversity in regional-level surveys, especially in the frame of large-scale biodiversity assessments or long-term monitoring programs. Likewise, our results strengthen this perception and support the combination of aerial sampling with the use of HTS to provide a highly effective tool for generating a reliable and exhaustive list of aero-dispersed fungi. This integrative approach should be considered in forthcoming aerobiology monitoring and for the development of spore calendars.

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.

Acknowledgments

The research was funded by the project “Finanziamenti di Ateneo per progetti di Ricerca scientifica (FRA2016)” assigned to LM by the University of Trieste.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2020.107032.

References

Abrego, N., Norros, V., Halme, P., Somervuo, P., Ali-Kovero, H., Ovaskainen, O., 2018. Give me a sample of air and I will tell which species are found from your region: Molecular identification of fungi from airborne spore samples. Mol. Ecol. Resour. 18, 511–524. https://doi.org/10.1111/1755-0998.12755.

Aguayo, J., Fourrier-Jeandel, C., Husson, C., Ioos, R., 2018. Assessment of passive traps combined with high-throughput sequencing to study airborne fungal communities. Appl. Environ. Microbiol. 84, e02637–e2717. https://doi.org/10.1128/AEM.02637-17.

An, C., Woo, C., Yamamoto, N., 2018. Introducing DNA based methods to compare fungal microbiota and concentrations in indoor, outdoor, and personal air. Aerobiologia 34, 1–12. https://doi.org/10.1007/s10453-017-9490-6.

Anderson, M.J.A., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26, 32–46. https://doi.org/10.1111/j.1442-9993.2001.01070. pp. x.

Anderson, M.J., Gorley, R.N., Clarke, K.R., 2008. PERMANOVA + for PRIMER: guide to software and statistical methods. PRIMER-E, Plymouth.

Antón, S.F., de la Cruz, D.R., Sánchez, J.S., Reyes, E.S., 2019. Analysis of the airborne fungal spores present in the atmosphere of Salamanca (MW Spain): a preliminary survey. Aerobiologia 35, 447–462. https://doi.org/10.1007/s10453-019-09569-z.

Auer, L., Mariadassou, M., O'Donohue, M., Klopp, C., Hernandez-Raquet, G., 2017. Analysis of large 16S rRNA Illumina data sets: impact of singleton read filtering on microbial community description. Mol. Ecol. Res. 17, e122–e132. https://doi.org/10.1111/1755-0998.12700.

Banchi, E., Ametrano, C.G., Greco, S., Stankovich, D., Muggia, L., Pallavicini, A., 2020a. PLANiTS: a curated sequence reference dataset for plant ITS DNA metabarcoding. DATABASE baz155. https://doi.org/10.1093/database/baz155.

Banchi, E., Ametrano, C.G., Tordoni, E., Stanković, D., Ongaro, S., ARPA working group, Tretiach, M., Pallavicini, A., Muggia, L., 2020b. Environmental DNA assessment of

airborne plant and fungal seasonal diversity. Sci. Total Environ. 738, 140249. https://doi.org/10.1016/j.scitotenv.2020.140249.
Banchi, E., Pallavicini, A., Muggia, L., 2020c. Relevance of plant and fungal DNA metabarcoding in aerobiology. Aerobiologia 36, 9–23. https://doi.org/10.1007/s10453-019-09574-2.
Banchi, E., Ametrano, C.G., Stanković, D., Verardo, P., Moretti, O., Gabrielli, F., et al., 2018. DNA metabarcoding uncovers fungal diversity of mixed airborne samples in Italy. PLoS ONE 13, e0194489. https://doi.org/10.1371/journal.pone.0194489.
Bauer, H., Kasper-Giebl, A., Loflund, M., Giebl, H., Zibuschka, R., Puxbaum, H., 2002. The contribution of bacteria and fungal spores to the organic carbon content of cloud water, precipitation and aerosols. Atmos. Res. 64, 109–119. https://doi.org/10.1016/S0169-8095(02)00084-4.
Bender, H., Enderle, M., 1988. Studien zur Gattung Coprinus (Pers.: Fr.) S.F. Gray in der Bundesrepublik Deutschland. IV. In Z. Mykol. 54, 45–68.
Bensch, K., Braun, U., Groenewald, J.Z., Crous, P.W., 2012. The genus *Cladosporium*. Stud. Mycol. 72, 1–401.
Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., Caporaso, J.G., 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat. Methods 10, 57–59. https://doi.org/10.1038/nmeth.2276.
Bowers, R.M., Clements, N., Emerson, J.B., Wiedinmyer, C., Hannigan, M.P., Fierer, N., 2013. Seasonal variability in bacterial and fungal diversity of the near-surface atmosphere. Environ. Sci. Tech. 47, 12097–12106. https://doi.org/10.1021/es402970s.
Brennan, G.L., Potter, C., de Vere, N., Griffith, G.W., Skjoth, C.A., Osborne, N.J., Petch, G. M., 2019. Temperate grass allergy season defined by spatio-temporal shifts in airborne pollen communities. Nat. Ecol. Evol. 3, 750–754. https://doi.org/10.1038/s41559-019-0849-7.
Burge, A.H., 2002. An update on pollen and fungal spore aerobiology. Current Rev. Allergens Clinical Immunol. 110, 544–552. https://doi.org/10.1067/mai.2002.128674.
Burrows, S.M., Elbert, W., Lawrence, M.G., Pöschl, U., 2009. Bacteria in the global atmosphere–part 1: review and synthesis of literature data for different ecosystems. Atmos. Chem. Phys. 9, 9263–9280. https://doi.org/10.5194/acp-9-9263-2009.
Buters, J., Schmidt-Weber, C., Oteros, J., 2018. Next-generation pollen monitoring and dissemination. Allergy 73, 1944–1945. https://doi.org/10.1111/all.13585.
Chen, B., Chao, H.J., Wu, C., Kim, H., Honda, Y., Guo, Y.L., 2014. High ambient *Cladosporium* spores were associated with reduced lung function in schoolchildren in a longitudinally study. Sci. Total Environ. 481, 370–376. https://doi.org/10.1016/j.scitotenv.2014.01.078.
Cheng, T., Xu, C., Lei, L., Li, C., Zhang, Y., Zhou, S., 2016. Barcoding the kingdom Plantae: new PCR primers for ITS regions of plants with improved universality and specificity. Mol. Ecol. Res. 16, 138–149. https://doi.org/10.1111/1755-0998.12438.
Clarke, K.R., Warwick, R.M., 2005. Primer-6 Computer Program. Natural Environment Research Council, Plymouth.
Damialis, A., Kaimakamis, E., Konoglou, M., Akritidis, I., Traidl-Hoffmann, C., Gioulekas, D., 2017. Estimating the abundance of airborne pollen and fungal spores at variable elevations using an aircraft: how high can they fly? Sci. Rep. 7, 1–11. https://doi.org/10.1038/srep44535.
Deiner, K., Bik, H.M., Mächler, E., Seymour, M., Lacoursière- Roussel, A., Altermatt, F., et al., 2017. Environmental DNA metabarcoding: transforming how we survey animal and plant communities. Mol. Ecol. 26, 5872–5895. https://doi.org/10.1111/mec.14350.
Favero-Longo, S.E., Sandrone, S., Matteucci, E., Appolonia, L., Piervittori, R., 2013. Spores of lichen-forming fungi in the mycoaerosol and their relationships with climate factors. Sci. Total Environ. 467, 26–33. https://doi.org/10.1016/j.scitotenv.2013.06.057.
Fröhlich-Nowoisky, J., Kampf, C.J., Weber, B., Huffman, J.A., PoÈhlker, C., Andreae, M. O., et al., 2016. Bioaerosols in the earth system: climate, health, and ecosystem interactions. Atmospheric Res. 182, 346–376. https://doi.org/10.1016/j.atmosres.2016.07.018.
Fröhlich-Nowoisky, J., Pickersgill, D.A., Després, V.R., Pöschl, U., 2009. High diversity of fungi in air particulate matter. PNAS 106, 12814–12819. https://doi.org/10.1073/pnas.0811003106.
Fuentes Anton, S., Rodríguez de la Cruz, D., Sánchez Sánchez, J., Sánchez Reyes, E., 2019. Analysis of the airborne fungal spores present in the atmosphere of Salamanca (MW Spain): a preliminary survey. Aerobiologia 35, 447–462. https://doi.org/10.1007/s10453-019-09569-z.
Gandolfi, I., Bertolini, V., Ambrosini, R., Bestetti, G., Franzetti, A., 2013. Unravelling the bacterial diversity in the atmosphere. App. Microbiol. Biotech. 97, 4727–4736. https://doi.org/10.1007/s00253-013-4901-2.
Gloor, G.B., Macklaim, J.M., Pawlowsky-Glahn, V., Egozcue, J.J., 2017. Microbiome datasets are compositional: and this is not optional. Front. Microbiol. 8, 2224. https://doi.org/10.3389/fmicb.2017.02224.
Grinn-Gofroń, A., Bosiacka, B., Bednarz, A., Wolski, T., 2018. A comparative study of hourly and daily relationships between selected meteorological parameters and airborne fungal spore composition. Aerobiologia 34, 45–54. https://doi.org/10.1007/s10453-017-9493-3.
Grinn-Gofroń, A., Bosiacka, B., 2015. Effects of meteorological factors on the composition of selected fungal spores in the air. Aerobiologia 31, 63–72. https://doi.org/10.1007/s10453-014-9347-1.
Kauserud, H., Lie, M., Stensrud, Ø., Ohlson, M., 2005. Molecular characterization of airborne fungal spores in boreal forests of contrasting human disturbance. Mycologia 97, 1215–1224. https://doi.org/10.1080/15572536.2006.11832731.
Korpelainen, H., Pietiläinen, M., 2017. Biodiversity of pollen in indoor air samples as revealed by DNA metabarcoding. Nordic J. Bot. 35, 602–608. https://doi.org/10.1111/njb.01623.
Kottek, M., Grieser, J., Beck, C., Rudolf, B., Rubel, F., 2006. World map of the Köppen-Geiger climate classification updated. Metereol. Zeit. 15, 259–263. https://doi.org/10.1127/0941-2948/2006/0130.
Kraaijeveld, K., de Weger, L.A.L.A., Ventayol García, M., Buermans, H., Frank, J., Hiemstra, P.S.P.S., et al., 2015. Efficient and sensitive identification and quantification of airborne pollen using next-generation DNA sequencing. Mol. Ecol Res. 15, 8–16. https://doi.org/10.1111/1755-0998.12288.
Leray, M., Knowlton, N., 2015. DNA barcoding and metabarcoding of standardized samples reveal patterns of marine benthic diversity. PNAS 112, 2076–2081. https://doi.org/10.1073/pnas.1424997112.
Levetin, E., 2004. Methods for aeroallergen sampling. Current Allergy and Asthma Reports 4, 376–383. https://doi.org/10.1007/s11882-004-0088-z.
Mayol, E., Arrieta, J.M., Jiménez, M.A., Martínez-Asensio, A., Garcias-Bonet, N., Dachs, J., et al., 2017. Long-range transport of airborne microbes over the global tropical and subtropical ocean. Nat. Comm. 8, 201. https://doi.org/10.1038/s41467-017-00110-9.
Nacke, H., Goldmann, K., Schöning, I., Pfeiffer, B., Kaiser, K., Castillo-Villamizar, G.A., et al., 2016. Fine spatial scale variation of soil microbial communities under european beech and Norway spruce. Front. Microbiol. 7, 2067. https://doi.org/10.3389/fmicb.2016.02067.
Nilsson, R.E., Larsson, K.H., Taylor, A.F.S., Bengtsson-Palme, J., Jeppesen, T.S., Schigel, D., Kennedy, P., Picard, K., Glöckner, F.O., Tedersoo, L., Saar, I., Kõljalg, U., Abarenkov, K., 2018. The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. Nuc. Ac. Res. 47, D259–D264. https://doi.org/10.1093/nar/gky1022.
Núñez, A., Amo de Paz, G., Ferencova, Z., Rastrojo, A., Guantes, R., García, A.M., Alcamí, A., Gutiérrez-Bustillo, A.M., Moreno, D.A., 2017. Validation of the hirst-type spore trap for simultaneous monitoring of prokaryotic and eukaryotic biodiversities in urban air samples by next-generation sequencing. Appl. Environm. Microbiol. 83 (13), e00472–e517. https://doi.org/10.1128/AEM.00472-17.
Núñez, A., Amo de Paz, G., Rastrojo, A., García Ruiz, A.M., Alcamí, A., Gutiérrez-Bustillo, A.M., Moreno Gómez, D.A., 2016. Monitoring of airborne biological particles in outdoor atmosphere. Part 1: Importance, variability and ratios. Internat. Microbiol. 19, 69–80. https://doi.org/10.2436/20.1501.01.
Oksanen, J., Blanchet, G.F., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2019. Vegan: community ecology package. R package version 2.5-6.
Pyrri, I., Kapsanaki Gotsi, E., 2017. Functional relations of airborne fungi to meteorological and pollution factor in a Mediterranean urban environment. Fungal Ecol. 30, 48–54. https://doi.org/10.1016/j.funeco.2017.08.007.
R Core Team, 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
Ruppert, K.M., Kline, R.J., Rahman, M.S., 2019. Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: a systematic review in methods, monitoring, and applications of global eDNA. Global Ecol. Cons. e00547 https://doi.org/10.1016/j.gecco.2019.e00547.
Schaffers, A.P., Raemakers, I.P., Sýkora, K.V., ter Braak, C.J., 2008. Arthropod assemblages are best predicted by plant species composition. Ecology 89, 782–794. https://doi.org/10.1890/07-0361.1.
Serrana, J.M., Miyake, Y., Gamboa, M., Watanabe, K., 2019. Comparison of DNA metabarcoding and morphological identification for stream macroinvertebrate biodiversity assessment and monitoring. Ecol. Ind. 101, 963–972. https://doi.org/10.1016/j.ecolind.2019.02.008.
Skjøth, C.A., Sommer, J., Frederiksen, L., Gosewinkel Karlson, U., 2012. Crop harvest in Denmark and Central Europe contributes to the local load of airborne *Alternaria* spore concentrations in Copenhagen. Atmos. Chem. Phys. 12, 11107–11123. https://doi.org/10.5194/acp-12-11107-2012.
Ščevkova, J., Dušička, J., Mičieta, K., Somorčik, J., 2016. The effects of recent changes in air temperature on trends in airborne *Alternaria*, *Epicoccum* and *Stemphylium* spore seasons in Bratislava (Slovakia). Aerobiologia 32, 69–81. https://doi.org/10.1007/s10453-015-9412-4.
Simpson, G.L., 2009. cocorresp: Co-correspondence analysis ordination methods. R package version 0.4-0.
Smith, E.G., 2000. Sampling and identifying allergenic pollen and molds. An illustrated identification manual for air samplers. Blewston Press, San Antonio, Texas.
Taberlet, P., Coissac, E., Hajibabaei, M., Rieseberg, L.H., 2012. Environmental DNA. Mol. Ecol. 21, 1789–1793. https://doi.org/10.1111/j.1365-294X.2012.05542.x.
Ter Braak, C.J.F., Schaffers, A.P., 2004. Co-correspondence analysis; a new ordination method to relate two species compositions. Ecology 85, 834–846. https://doi.org/10.1890/03-0021.
Thomsen, P.F., Willerslev, E., 2015. Environmental DNA–An emerging tool in conservation for monitoring past and present biodiversity. Biol. Con. 183, 4–18. https://doi.org/10.1016/j.biocon.2014.11.019.
Vloutoglou, I., Fitt, B.D.L., Lucas, J.A., 1995. Periodicity and gradients in dispersal of *Alternaria linicola* in linseed crops. Europ. J. Plant Pathol. 101, 639–653. https://doi.org/10.1007/BF01874868.
White, T., Bruns, T., Lee, S.J.W.T., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR protocols: a guide to methods and applications. Academic Press, New York, pp. 315–322.