Journal of Pollution
Special Issue
JOS 20-21687
Research Article Received Date: 04, Nov 2020

Incidence of the Genus *Aspergillus* and its Species in the Atmosphere of São Paulo, Brazil and its Relations with the Environment Atmospheric

Incidence of *Aspergillus* spp. in Brazil

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Abstract

Bioaerosols are biological materials suspended in the air, and they also include fungi. The fungal genus *Aspergillus* is relevant in the respiratory infection of critical patients, and *Aspergillus fumigatus* is the most frequent species in the Metropolitan Region of São Paulo (MRSP), Brazil.

Aims: Characterizing the frequency of anemophilous fungi of the genus *Aspergillus* in the atmosphere of São Paulo can provide relevant information regarding environmental monitoring.

Methods and Results: This study evaluated the atmosphere of the MRSP for the presence of fungi during one collection year, amounting to 420 air samples. The air samples were collected with the equipment MAS 100-ECO (Merck®, Fr.) and M air T (Millipore®). The atmospheric variables of temperature and humidity were measured. The highest humidity and air temperature were found in the summer season, along with the highest concentration of bacteria and the fungal genus *Aspergillus*. The highest concentration of PM_{10}, NO_2, and NO occurred in winter along with the highest concentration of CFU/m^3 of fungi. The *Aspergillus* genus ranked first with a 35.01% incidence in relation to the other fungal genera. The *Aspergillus fumigatus* showed a remarkable variation in its frequency in the studied areas ($p=0.021$).

Conclusions: The most common fungal genus in the atmosphere of the São Paulo Metropolitan Region was *Aspergillus*. Its frequency was maintained throughout the collection period as already reported in other studies.

Keywords: Aspergillus • Air • Pollution • Public health • Environment

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Received: 04, Nov 2020; **Accepted:** 23, August 2021; **Published:** 15, September 2021

Introduction

Bioaerosols are biological materials suspended in the air, including bacteria (0.25 μm to 8 μm), fungi (1mm to 30mm), pollen (17mm to 58mm), and viruses, and even smaller particles that can be easily transported into the atmosphere. Their role in the environment, as well as the technologies involved in their collection and analysis, are the main challenges in studies in this area [1,2]

Fungi that use the atmosphere as a way to disperse their spores are called anemophily. They also disperse fragments that can cause fungal infections. Among anemophilous fungi, 87% do not cause any type of disease, 10% act as opportunistic pathogens, and 3% are pathogenic in fact [3].

Exposure to one of the allergenic components should be considered a strong risk factor for the prevalence of respiratory diseases. The concentration of fungi in outdoor and indoor measurements correlates with seasonal patterns observed in atmosphere counts [2,4].

The most prevalent fungal genera in the atmosphere are *Cladophyalophora* spp., *Alternaria* spp., *Aspergillus* spp., and *Penicillium* spp. Several species belonging to these genera present fungal pathogens responsible for infection in immunocompromised patients [5,6].

The genus *Aspergillus* is the most incident in urban environments and inhabits mainly the soil, and it can be found in organic material. There are more than 200 species of *Aspergillus*, however, only a few of them are pathogenic to humans, and the most common is *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger,* and *Aspergillus terreus* [5,6].

This fungal genus is relevant for patients with critically respiratory infections, and *Aspergillus fumigatus* is the most frequent species (80%-90% of cases) which causes invasive lung disease; other species have been isolated less frequently, such as *Aspergillus flavus* and *Aspergillus terreus*.

Humans and animals continuously inhale fungal fragments, but in general, these particles are efficiently eliminated by the immune system. *Aspergillus* spp. isolation is more frequent when construction works occur in hospitals and animal recovery centres or outbreaks reported in intensive care units, and it can be found in the air conditioning system [7,8].

Aspergillosis is the disease caused by *Aspergillus* spp., whose clinical manifestations are determined by the patient's immune response and may be present in allergic, saprophytic, or invasive ways [8,9].

Its transmission occurs by inhalation of the spores that are deposited in the airways, causing infections of the lower respiratory tract and sinus of the face, while saprophytic manifestation includes otomycosis and pulmonary aspergilloma. In allergic processes, this fungus may be associated with allergic sinusitis and bronchopulmonary aspergillosis. In invasive diseases, it acts in the central nervous system, the cardiovascular system, and also other tissues that may be infected as a result of hematogenous diseases. The clinical manifestations resulting from the infection depend on the virulence of the fungus, the intensity of exposure, the patient's immune status, and the presence of previous lung disease. In healthy individuals, inhalation usually does not cause disease [9-11].

Exposure of fungal spores to polluted urban air can affect their allergenicity. However, individuals may be exposed to more potent allergens, especially during the first 12 hours of exposure to allergens and urban air pollution [12].

The Metropolitan Region of São Paulo (MRSP) is a megalopolis with a population of 21 million, corresponding to more than 11% of the total population of Brazil, and this carries one of the saddest marks of urban development in the country: pollution. In the MRSP the air is infested with chemical material generated by human activities, mainly from the intense circulation of vehicles that affect the quality of life of the population, since air pollution can cause serious health problems.

The Brazilian National Health Surveillance Agency (ANVISA) defines the current legislation for indoor air control in the country and proposes the maintenance of air conditioning systems for environmental control in buildings with these systems. The plan complies with the parameters regulated by ANVISA Resolution 9/2003.

Additionally, studies conducted in the RMSP to identify polluting sources have revealed that vehicular emissions and soil resuspensions are the main sources identified of suspended Particulate Matter (PM_{10}) and Organic Carbon (OC). The estimated contribution of fungal biomass is 2% and 8% of the total PM_{10} and OC, respectively, quite representative for urban areas in Brazil [13].

Research studies related to indoor air quality analyze contamination by microorganisms not considering the external air circulating the analyzed sites. These studies refer to buildings and their occupants, raising individual information such as construction and maintenance of the building, current and historical use of the building, ventilation, moisture control, surface materials, activities, and lifestyle of the occupants. However, the production and even dispersion of these microorganisms influence this context, increasing or reducing their levels, modifying their emissions in the atmosphere under the influence of the speed and direction of winds, precipitation, temperature and air instability [2,14].

Another line of research using anemophilous fungi is from bioindicators, which require expansion of knowledge. This direction has led researchers to study anemophilous fungi because of the growing interest in allergenic microorganisms and the search for new environmental indicators of pollution, since their frequency and diversity may be associated with environmental factors [15].

The medical importance of fungal spores in the air has been emphasized in connection with allergic reactions with a wide spectrum of clinical forms. As these spores are components of the bioaerosol and are also considered as indicators of the level of air pollution, a better understanding of these phenomena requires a detailed survey of the particles transported by air [16]. Characterizing the frequency of anemophilous fungi of the genus *Aspergillus* in the atmosphere of São Paulo can bring relevant information regarding environmental monitoring. This air quality monitoring can assist public policies and control agencies.

Methods

Collection and analysis of fungi

This study evaluated the atmosphere of the RMSP for the presence of fungi during one collection year between September 2017 and August 2018. Two locations were analyzed: the city of São Paulo in the neighbourhood Cerqueira Cesar and the city of Ibiúna, in the district of Votorantim, the two cities being 60.6 km^2 apart (Figure 1).

Figure 1: Identification of collection points.

A total of 420 air samples were collected as follows: 118 in summer, 99 in winter, 141 in spring, and 125 in autumn. Of the total, 136 are from the rural area and 284 from the urban area. The air samples were collected with the equipment MAS 100-ECO (Merck®, Fr.) and M air T (Millipore®), which have the equivalent capacity [18]

The collections took place once a week, with three samples in each location and one-hour intervals between them. Each collection had a final volume of 250 L (0.25 m^3), which allowed the isolation of colony-forming units and identification of the isolated genera.

At the time of the collection, a disposable Petri dish containing 20 mL of culture medium was used, with modified Dichloran Rose Bengal Chloramphenicol (DRBC m) for fungal isolation and Tryptic Soy Broth (TSB) for the cultivation of bacteria [16]. The dishes containing DRBCm were incubated in a bacteriological incubator set at 30ºC + 2ºC for up to 7 days for isolation and phenotypic sex identification following a specific method.

After incubation, the concentrations of fungi and bacteria were analyzed. Bacteria were not cultivated. The fungal isolates were identified by genus, and fungi belonging to the genus *Aspergillus* were identified by species. The species *A. flavus*, *A. fumigatus*, *A. orizeae*, *A. ochraceus*, *A. glaucus*, *A. nidulans,* and *A. terreus* were identified by phenotypic methods and confirmed by the MALDI-TOF MS system [14,25]

The identification of species belonging to the Section *Nigri* was not broken down with the basic taxonomy or with the MALDI-TOF MS system.

Collection and analysis of the environmental variables

The atmospheric variables of temperature and humidity were measured during collections using a hygrograph placed in both locations. The analysis of atmospheric conditions and concentration of pollutants was carried out according to the seasons.

Data on pollutants, in turn, were obtained from the *Companhia Ambiental do Estado de São Paulo*–Environmental Company of the State of São Paulo (CETESB) in daily online reports. The system presents daily values of pollutants and meteorological parameters for the selected station.

In this study, the Cerqueira Cesar station was used for points located in the city of São Paulo and data from the Sorocaba station was used to monitor the collections in Ibiúna. The O_3 data for the São Paulo points were obtained at the Pinheiros station, as Cerqueira Cesar does not take these measurements. The following pollutants were analyzed, all in daily averages: nitrogen oxides (NOx), carbon monoxide (CO), sulfur dioxide (SO_2), inhalable particles (PM_{10}), and nitrogen dioxide (NO_2).

Results

Analysis of environmental variables

The highest humidity and air temperature were found in the summer season, along with the highest concentration of bacteria and the fungal genus *Aspergillus*.

The summer season also showed more bacteria in the air both in the urban and rural areas. The highest concentration of CFU/fungi was present in the rural area during the winter season (Table 2).

Table 2. Average and standard deviation (SD) of temperature, humidity, CFU of fungi, and CFU of bacteria according to the seasons of the year in the urban and rural areas of São Paulo, Brazil (2017-2018).

Location	Season of the year	Humidity Average SD	Max-Min	Temperature Average SD	Max-Min	CFU/FUNG Average SD	CFU/BAC Average SD	***Aspergillus* spp. n(%)**

Urban Area	Spring	24.5	15-80	23	16-30	23.1	29.2	22
		± 19.8		± 6.0		± 44.9	± 75.2	13.90%
	Summer	54.5	22-74	27.4	20-37	43.2	44	54
		± 14.2		± 4.1		± 73.2	± 47.4	32.00%
	Autumn	47.2	15-75	26.1	15-40	57.7	28.1	51
		± 14.9		± 5.4		± 78.7	± 51.2	30.20%
	Winter	49.3	14-83	16.5	13-31	91.3	55.3	42
		± 16.2		± 5.2		± 87.5	± 58.1	24.90%
Rural Area	Spring	30.3	46-86	21	18-32	49	36.3	10
		± 18.6		± 6.2		± 49.21	± 76	17.90%
	Summer	75.8	62-88	24.5	18-28	132	78.2	17
		± 15.42		± 4.7		± 76.4	50.8	30.40%
	Autumn	41.7	40-84	14.8	16-31	117.8	53.5	16
		± 15.0		± 5.5		± 78.6	± 52	28.60%
	Winter	30.6	31-88	9.6	16-32	155.4	62.6	13
		± 16.4		± 5.2		± 88.8	± 57.6	23.20%

In the two studied regions, the highest concentrations of PM_{10}, NO_2, and NO occurred in winter along with the highest concentration of CFU/m^3 of fungi.

The concentrations of SO_2, CO, bacteria, and the genus *Aspergillus* showed different characteristics during the study period, presenting low concentrations in the rural area when compared to the measurements in the urban area (Table 3).

Table 3. Average and Standard Deviation (SD) of the concentration of gases, CFU of fungi, and CFU of bacteria according to the seasons of the year in the urban and rural areas of São Paulo, Brazil (2017- 2018).

Location	**Season of the**	**PM_{10}**	**SO_2**	**NO_2**	**CO**	**NO**	**CFU/ FUNG**	**CFU/ BAC**	***Aspergillus* spp. N**

	year	Average	Average	Average	Average	Average	Average	Average	
Urban Area	Spring	12.4	0.7	28.5	0.35	16.1	23.1	29.2	22
		± 16.2	± 6.9	± 20.4	± 9.5	± 13.3	± 44.9	± 75.1	13.90%
	Summer	23	1.7	42.6	4.9	25.7	43.2	44	54
		± 11.6	± 1.5	± 21.5	± 10.6	± 18.5	± 73.2	± 47.4	32.00%
	Autumn	1.6	31.6	52.4	23.6	27.2	57.7	28.1	51
		± 5	± 27	± 32.5	± 20.4	± 11.1	± 78.7	± 51.2	30.20%
	Winter	25.1	39.1	73.9	40.8	49.6	91.3	55.3	42
		± 17.2	± 30.1	± 31.1	± 30.9	± 19.9	± 87.5	± 58.1	24.90%
Rural Area	Spring	6.4	3.1	4.6	0.6	2.1	48.9	36.3	10
		± 16.5	± 7.9	± 19.5	± 9.9	± 13.3	± 49.2	± 76.0	17.90%
	Summer	11.1	7	8.9	3	2.3	132	78.2	17
		± 12.5	± 25.2	± 29.4	± 19.0	± 16.1	± 76.3	± 50.8	30.40%
	Autumn	11	6.3	1.7	9	0.7	117.8	53.5	16
		± 5.3	± 27.2	± 33.6	± 20.8	± 11.6	± 78.6	± 51.9	28.60%
	Winter	16.5	5.1	9.8	6	4.5	155.4	62.6	13
		± 14.7	± 30.6	± 31.6	± 31.3	± 18.3	± 88.8	± 57.6	23.20%

Fungal analysis results

Fungal isolates were also classified by the pigmentation of the hyphae. Hyaline spores were more frequently present in the urban area (66.5% N=182) and demacious in the rural area (72.4% N=181). The highest incidence of sex was in the fall, with 2 or 3 different sexes being more frequent in each air sample.

During this study, 542 fungal isolates were obtained, being 319 (59%) in rural areas and 223 (41%) in urban areas. They were classified into 14 different genera. The number of rural samples was 136 (32.2%), and the total number of urban samples was 286 (67.7%) (Figure 1; Table 4).

Figure 1. Distribution of isolated fungal genera in air sampling carried out in rural and urban areas of São Paulo, Brazil during one year of collection (2017-2018).

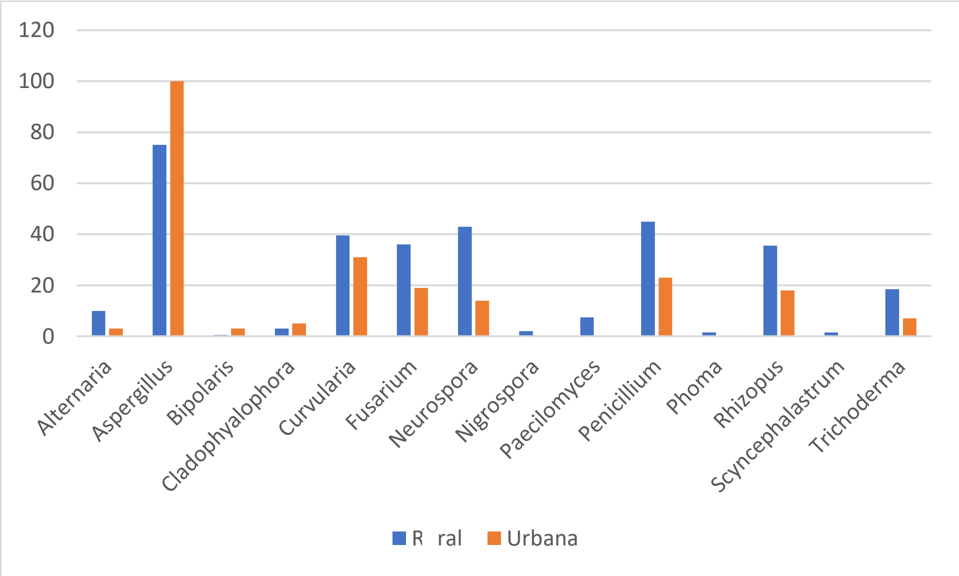

Table 4. Frequency of isolated fungal genera in atmospheric air in urban areas in one year of collection (2017/2018).

Fungal genera	Urban Area	Rural Area	p-value
Alternaria	16 (94.1%)	1 (5.9%)	0.017
Aspergillus	169 (75.1%)	56 (24.9%)	0.001
Bipolaris	4 (80.0%)	1 (20.0%)	0.495
Cladophylophora	8 (88.9%)	1 (11.1%)	0.283
Curvularia	65 (70.7%)	27 (29.3%)	0.598
Fusarium	49 (77.8%)	14 (22.2%)	0.081
Neurospora	49 (62.8%)	29 (37.2%)	0.237
Nigrospora	1 (33.3%)	2 (66.7%)	0.236
Paecilomyces	6 (60.0%)	4 (40.0%)	0.511
Penicillium	48 (57.1%)	36 (42.9%)	0.013
Phoma	------	2 (100%)	0.099
Rhizopus	45 (65.2%)	24 (34.8%)	0.531
Scyncephalastrum	------	2 (100%)	0.099
Trichoderma	25 (83.3%)	5 (16.7%)	0.1

The genus *Aspergillus* ranked first with a 35.01% incidence in relation to the other genera, followed by 12.68% for *Curvularia,* 12.10% for *Penicillium*, 10.37% for *Neurospora*, 9.22% for *Fusarium*, 9.51% for *Rhizopus*, and others with a lower incidence.

The frequency of the genus *Aspergillus* showed a different behaviour from the other genera, with a higher incidence in the urban area (57.2%) of the total isolates. All other genera had a higher incidence in rural areas. The genera *Nigrospora*, *Paecilomyces*, *Phoma,* and *Syncephalastrum* were not isolated in the urban area and this may show that they develop better in rural regions. In the rural region, there is greater fungal diversity than in the urban region, with 14 species identified against 10.

The fungi belonging to Section *Nigri* appear in 38.0% (123/323) of the identified species, followed by *A. flavus* with 31% (100/323) and *A. fumigatus* with 21.0% (68/323). A relevant factor is that these species have a higher concentration in the urban area. *Aspergillus fumigatus* showed a remarkable variation in its frequency in the studied areas (p=0.021) (Figure 2; Table 5).

Figure 02. Distribution of isolated species of the genus *Aspergillus* in air samples in urban and rural areas of São Paulo, Brazil in one collection year (2017-2018).

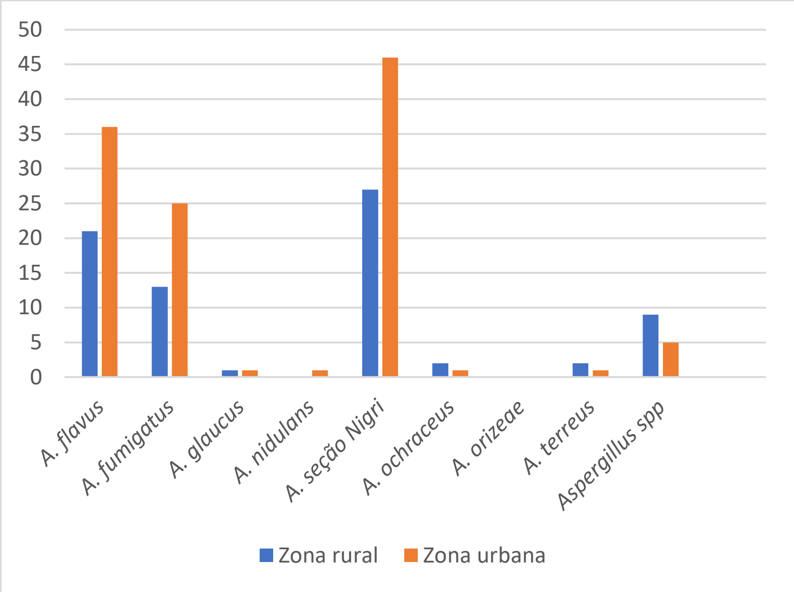

Table 5. Variation in the frequency of isolated species of the genus *Aspergillus* in atmospheric air samples of urban and rural areas in one year of collection (2017/2018).

Genus Aspergillus	Urban Area	Rural Area	p-value
A. flavus	69 (78.4%)	19 (21.6%)	0.356
A. fumigatus	47 (87.0%)	7 (13.0%)	0.021
A. glaucus	1 (50.0%)	1 (50.0%)	0.438
A. nidulans	2 (100%)	-----	0.562
A. section *Nigri*	97 (77.6%)	28 (22.4%)	0.336

Discussion

The methodology used to identify the isolated fungi

The use of the MALDI-TOF System in the identification of fungal species of the genus *Aspergillus* brought up some difficulties that must be addressed, such as defining the reproduction phase of each genus that favours the production of different proteins to obtain a better reading on the equipment. In this study, the characterization of species of the genus *Aspergillus* using the MALDI system was of great importance, as it confirmed the phenotypic evidence [4,15]

The non-dismemberment of the Section *Nigri* did not alter the results, as these section groups' microorganisms with similar environmental behaviours. Section *Nigri* appears in other studies with "*A. Niger*" more often with 40%, followed by *A. flavus* with 28.3% and *A. fumigatus* with 17.2%.

As reported in several studies, the diversity of anemophilous fungi is extensive, showing variations according to environmental factors that may or may not favour their development.

In this study temperature and humidity influence the concentration of fungi in the atmosphere and winter, they favour the increase of spores in the air. A study carried out in the city of Porto in Portugal confirms the environmental influence but presents different data. The authors report that during the two years of study, the highest concentrations of spores in the atmosphere were observed during the summer and autumn months, while the lowest concentrations were recorded in the winter and early spring months [20,26]

Together with more PM_{10} particles and NO_2 and NO gases, there was also the highest concentration of fungal spores, while the levels of SO_2 and CO gas concentrations were relative to the incidence of the genus *Aspergillus*, that is being higher in the urban area and lower in the rural area, both during the winter season.

The fungal genera varied according to the total concentration of fungi, with their highest frequency in the rural area. The environmental conditions of the autumn season favoured fungal diversity, while those offered by spring reduced this number, a fact already observed when characterizing the size, shape, and density of spores that are subject to fluctuations, depending directly on the humidity of the air, which when increased facilitates air transport; in contrast, the disintegration of airborne particles can occur, decreasing the binding forces and increasing the tensions [13,28]

According to this study, the highest concentration of CFU/m^3 in the urban area was due to the high concentration of isolates of the genus *Aspergillus*.

The higher concentration of the genus *Aspergillus*, in the urban region may demonstrate an adaptation to the most polluted areas, issues that still need to be studied. On the other hand, demacious fungi presented low concentrations in urban collections.

Importance of monitoring the genus *Aspergillus* in urban regions

Aspergillosis is the most common disease among human mycoses. It is caused by fungi of the genus *Aspergillus*. It is important to remember that fungi of the genus *Aspergillus* are ubiquitous in their distribution and are a serious threat to public health in indoor environments.

Aspergillosis is a multifaceted disease whose clinical manifestations are determined by the host's immune response and may be present in an allergic, saprophytic, or invasive manner.

Aspergillus is a fungus of universal distribution in nature, whose most common source of infection is the airway, and it has emerged as a cause of serious life-threatening infection in immunosuppressed patients.

In Italy, 27 hospitals had their demand analyzed for fungal infections. A total of 384 cases were found over a two-year period. Aspergillus infection was diagnosed in 32 clinical patients and 25 surgical patients. Aspergillosis was shown to be more severe than candidiasis, as the crude mortality rate was significantly higher, 63% *vs*. 46%.

Research related to the presence of *Aspergillus* in the atmosphere in urban regions accompanies works and/or constructions in hospital environments, representing one of the main risk factors, thus requiring protective measures. With changes in the hospital routine, there is often an increase in the incidence of pulmonary aspergillosis in immunocompromised or simply hospitalized patients during the reform performed [2,5,17,21]

Among the isolated species of the genus *Aspergillus*, the species *A. fumigatus* was found more frequently and is related to public health issues, causing several diseases in the respiratory system.

With the data obtained in this research, it was verified that the species *A. fumigatus* is widely distributed in the air of the urban area of the city of São Paulo, which shows the importance of the monitoring and surveillance of this fungus in external areas.

Environmental monitoring studies should be broader and consider other variables, such as the presence of fungi of the genus *Aspergillus*. With this analysis, the system may have better parameters for health care [19]

Regarding the methodology used to identify fungi, the taxonomic identification of fungi has always been quite complex, requiring observation of the formation of development and reproduction structures that are often not easy in culture media. The possibility of using the MALDI-TOF system as a support in the identification of species of the genus *Aspergillus* was of great value for this research.

Regarding environmental conditions, the highest concentrations of fungal spores were obtained in the winter season. Temperature and humidity directly influence the concentration of spores. The concentrations of SO_2 and CO and other pollutants can influence the concentration of fungi in the atmospheric air of the city of São Paulo; however, this study proved to be inconclusive and a longer collection time is needed to prove this correlation.

Regarding the importance of monitoring the genus *Aspergillus* in urban regions, the higher concentration of hyaline fungi and especially of the genus Aspergillus in the urban region may demonstrate an adaptation to the most polluted areas, issues that still need to be studied. On the other hand, demacious fungi presented low concentrations in urban collections.

The most common fungal genus in the atmosphere of the São Paulo Metropolitan Region was *Aspergillus*. Its frequency was maintained throughout the collection period, as already reported in other studies. Therefore, understanding the interactions between pollutants and the genus *Aspergillus*, which is the most common in urban environments, can help to decipher the general increase in allergy susceptibility observed in recent decades.

The frequency of *A. fumigatus* species could be related to several respiratory system issues caused by fungi.

This study can collaborate with public health investigations regarding the possibility of acquiring a fungal infection caused by fungi of the genus *Aspergillus*.

Competing interests

The authors declare that there is no conflict of interest.

Acknowledgements

We would like to acknowledge the support of Dr. Ana Paula Guarnieri Christ in the execution of the methodology of this study and to B.Sc. Valter Batista Duo Filho, Biomedical Scientist and Microbiology Specialist, for his contribution to the major review of the study.

Ethical considerations

Ethical issues have been completely observed by the authors.

DESCRIPTION OF AUTHORS CONTRIBUTION

The authors listed below participated effectively in the preparation of the manuscript: Incidence of the genus *Aspergillus* and its species in the atmosphere of São Paulo – Brazil and its relations with the environment.

The activities in each stage are described below.

1. Experiment organization and data collection:

Castro e Silva, DM; Gonçalves, FLT;

2. Tabulation, statistical analysis of data

Castro e Silva, DM; Marcusso, NMR

3. Identification of fungal isolates

Castro e Silva, DM; Marcusso, NMR; Dalmuth, AC; Moreno, AM; Moreno, LZ

4. Writing the text and standardizing the rules according to the jornal

Castro e Sillva, DM; Gonçalves, FLT; Cardoso, MRA

5. Revision of the text:

Castro e Silva, DM; Gonçalves, FLT; Cardoso, MRA

As this is true, I sign this declaration.

[Unreadable Signature]

Dulcilena de Matos Castro e Silva

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