

Microbiological survey of the aerial contamination in urban areas of the city of Murcia, Spain

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Resumen

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Estudio microbiológico de la contaminación aérea en áreas urbanas de la ciudad de Murcia, España.

La carga microbiana del aire de la ciudad de Murcia se analizó periódicamente a lo largo de un año en diez zonas diferentes de muestreo. La determinación de viables, tras la recogida de muestras por un método de impacto, indicó unos niveles globales de contaminación microbiana de dos órdenes de magnitud por m³, similar o inferior a la detectada en otras ciudades. Sin embargo, nuestros resultados permiten señalar que, como media, aproximadamente 5 x10³ microorganismos viables pueden ser inspirados al día por un ciudadano de Murcia al aire libre. La concentración de hongos en suspensión superó a la de las bacterias. Se detectaron algunas bacterias potencialmente patógenas, entre las que *Staphylococcus* fue el género más común. *Legionella* se detectó ocasionalmente en dos áreas muestreadas. Entre los hongos, el aislado con más frecuencia fue *Cladosporium*. La incidencia de bacterias y hongos mostró una distribución temporal diferente. Estos resultados son los primeros recogidos sobre la concentración y composición de la microflora aérea de esta ciudad.

Palabras clave: Contaminación aérea, Contaminación microbiana, Transporte aéreo de microorganismos

Abstract

Ten different urban areas of the city of Murcia were periodically sampled for aerial microbial content during one year period. Overall viable counting after air collection by an impaction method showed levels of microbial contamination of about two orders of magnitude per m³, which are either within or below the range reported for other population centers. However, it can be deduced from our results that about 5 x10³ viable microorganisms may be daily inhaled outdoor by Murcia citizens. The concentration of aerial fungal isolates surpassed that of bacterial contaminants. Several potentially pathogen bacteria were detected, among which *Staphylococcus* was the predominant genus. Also, *Legionella* was occasionally found in two environmental areas. Species pertaining to *Cladosporium* were among the most frequent isolated fungi. The seasonal distributions of bacteria and fungi followed a different temporal trend. The results of the present work are the first data available on the concentration and composition of the airborne microflora of this city.

Key words: Air contamination, Microbial pollution, Airborne microorganisms

Introduction

Atmospheric air is not merely a mixture of nitrogen, oxygen, carbon dioxide and other gases. This shaping agent of the biosphere also contains numerous small particles as well as alive elements which are associated to the suspended inert dust, including pollen, viruses, spores, and vegetative cells of both bacterial and fungal origin. In particular, microorganisms are natural components of most outdoor and indoor aerial environments (Madigan et al. 2008).

Microbial evaluation of the atmosphere has attracted some attention in the last decades because part of the airborne living population, collectively known as aeroplancton, may potentially be either allergenic or immunotoxic. However, the number of studies recently published dealing with fungal contamination or bacterial air sampling in urban areas is rather scarce (Mahdy & El-Sehrawi 1997; Lighthart 2000; Zhu et al. 2003; Stepalska & Wolek 2005). The low concentration of organic matter usually present in the air is unable to support stable heterotrophic growth. Although microorganisms survive thus poorly in this environment, more than 10.000 liters of air are being inhaled daily by an adult human (Cardona 2003). Therefore exposure to airborne microbial agents can often result in respiratory infections and other adverse health effects related to hypersensitivity disorders (Gorny et al. 2002).

The microbial load contained into the atmospheric air is mostly due to alloctonous microorganisms whose presence can be related to aerial transport from soil and other reservoirs as a function of the environmental conditions. The temporal persistence of air pollution is under seasonal variation because physical factors like

aerosol formation, temperature and humidity greatly influence microbial survival. Moreover, other mechanical factors, such as rain or wind, also affect the maintenance of the suspended transient contaminants by favoring either their sedimentation or their dispersal (Griffin 2007).

The main aim of this work was to determine the microbiological content of the aerial ambient in the city of Murcia, Spain, with special focus on the bacterial and fungal contaminants. To this purpose the concentration and diversity of the predominant microbial populations was recorded during an annual period. Several relevant physicochemical parameters of the air in this city are being periodically obtained through automated analysis stations under surveillance of the City Council. However, to our knowledge, the results contained in this work are the first biological data reported on the concentration and composition of the airborne microflora of this city.

Materials and methods

Sample protocol

To analyze the bacterial and fungal microbiota suspended in the outdoor air of Murcia, samples were collected from ten representative sites selected according to differential characteristics. Areas for recreational use as well as zones with diverse population density and traffic intensity were included. General features of the sampling points are shown in table 1.

We used a portable MAS-100 air sampler device (Merck) based on the principle of air impaction. For each determination 100 liters of air were captured. Air flow was adjusted at 100 liters/min and directed over the surface of a petri dish

Place no.	Denomination	Main features
1.1	Salitre Garden- Lagoon	Closed recreational green area
1.2	Salitre Garden- Children park	Closed recreational green area
2	Ronda Norte	Intense road traffic, main street
3	Guadalupe	Green residential area
4	Gran Via	High population density, intense traffic
5	La Fama Avenue	Densely populated zone, old buildings
6	Circular Square	Green area with intense road traffic
7	Old Bridge	Road traffic, Segura river
8	Ronda de Levante	Intense road traffic
9	Cathedral Square	Pedestrian area

Tabla 1. Áreas de muestreo de aire seleccionadas en la ciudad de Murcia.

Table 1. Selected places for air sampling in the city of Murcia.

containing appropriate solid culture media (see below). The impact rate was equivalent to 11 m/sec to ensure efficient capture of particles above 1 μm and no loss of viability due to impact stress (Meier & Zingre 2000). Before each sampling session, the head of the air sampler was properly sterilized as a control. In all cases, the samples were obtained between 16:00 p.m. and 19:00 p.m. Duplicated samples were taken twice a month during an entire year (October 2007-September 2008) and the monthly results averaged. Microbial concentration for each temporal series was expressed as mean values of colony-forming units (CFU) per m^3 of air analyzed. In parallel, a correlation was established between the values found and the temperature and relative humidity recorded by the Meteorological Service of the University of Murcia.

Culture media

All media employed in this study were supplied by VWR International Eurolab S.L. Non-selective media were used for total bacteria counting (nutrient agar) and for bacterial growth examination (agar LB, agar-chocolate). Agar-MacConkey was employed as selective medium for isolation and identification of enterobacteria of the coliform group. Haemolytic activity was assayed on Columbia blood-agar medium. Isolated colonies of bacteria were also cultured on Hugh-Leifson differential medium to assess for fermentative or oxidative properties and motility. In addition, *Legionella* BCYE/GVPC-agar was used to evaluate the presence of the causative agent of legionnaires' disease. Estimation of airborne fungi was carried out by growth on Sabouraud-agar medium supplemented with chloramphenicol. The respective culture media were properly sterilized by autoclaving and spread into sterilized petri dishes which were adjusted to the air sampler when required. Sampled plates were incubated at 35-37°C for 48 h in the case of bacterial analysis and at 28-29°C for 72 h in the case of fungal sampling.

Other methods

After the incubation period, the plates were examined for CFU counting. Since in all cases 100 liters of air were sampled, the number of viable counts obtained in each plate was multiplied by 10 to reflect the microbial content in 1 m^3 of captured air. In addition to microscope observations,

other routine procedures for identification of bacteria included culture on Kligler iron-agar (slants), Gram staining, oxidase test with Kovac's reagent, and catalase reaction. In some cases the bacterial colonies that developed were subjected to microscope examination and to biochemical analysis with API Biomerieux strips. Fungal isolates were microscope examined in fresh or lactophenol blue-stained preparations. Bacterial and fungal identification was carried out according to general procedures contained in established manuals (Buchanan & Gibbons, 1975; Samson & Van Reenen-Hoekstra 1988; Germain & Summerbell 1996; Prescott et al. 2004).

Results

Microbial concentration as a function of the sampling site

The results obtained for bacteria and fungi in the various areas analyzed during our study are summarized in table 2. No extreme variations were recorded when the different sampling sites were compared. In all cases the presence of airborne fungi outnumbered the bacteria, and the total viable microorganisms (bacteria + fungi) accounted for a maximum of $7,0 \times 10^2$ and a minimum of $3,3 \times 10^2$, which is within the usual range of airborne microbial contamination (Atlas & Bartha 2002). The data also reveal that, on the average, bacteria represent only 27% of the suspended microflora in Murcia as compared to fungi (73%).

Consistently, the site no. 1.1 (Salitre Garden-Lagoon) was the place showing the higher number of total CFU/ m^3 . This recreational place shows a large inflow of people together with a wide variety of plants, birds (mostly pigeons) and pets (dogs). On the contrary, the site no. 9 (Cathedral Square), which is a restricted pedestrian area, registered the lowest counts for both bacteria and fungi. The highest bacterial concentration in the air was detected in place no. 5 (La Fama Avenue), coincident with an urban zone of low sanitary general conditions (Table 2).

Bacterial diversity

The most frequent types of colony morphologies were investigated further in each sampled plate for microbial identification. By staining procedures, 77% of the bacterial colonies examined

Area no.	Bacteria (CFU/m ³) x10 ²	Fungi (CFU/m ³) x10 ²	Total (CFU/m ³) x10 ²
1.1	1,37	5,67	7,04
1.2	1,02	4,3	5,32
2	1,64	3,63	5,27
3	1,94	3,91	5,85
4	1,25	3,15	4,4
5	2,45	3,5	5,95
6	1	4,32	5,32
7	1,35	2,71	4,06
8	1,77	5	6,77
9	0,6	2,63	3,23
Total average:	1,44	3,88	5,32

Tabla 2. Concentración media de microorganismos en el aire en las diferentes zonas estudiadas (los valores máximos y mínimos de cada serie se indican en negrita)

Table 2. Average aerial microbial concentration in the different zones studied (maximal and minimal values in each series are in bold).

contained Gram-positive cells while 23% were composed of Gram-negative cells. When cell morphology was examined, the incidence of coccoid cells (65%) predominated over those with bacilar shape (35%). Likely, these frequencies are due to the differential cell wall structure (with a wider layer of peptidoglycan in Gram-positive cells) and to the lower surface/volumen ratio showed by cocci as compared to bacilli.

Biochemical characterization of the isolates was also performed to identify the examined bacteria till the genus level. The results are summarized in table 3. Species of *Staphylococcus* represented almost one half of the aerial bacterial population, followed far away by species of *Acinetobacter*, spore-forming *Bacillus*, *Streptococcus*, *Corynebacterium* and *Pseudomonas*. Enterobacteria were occasionally present, as well as *Listeria*, *Lactobacillus* and *Haemophilus*.

By using the selective and specific culture medium *Legionella* BCYE/GVPC-agar we were also able to detect *Legionella* in the sampling areas no. 4 (Gran Vía) and no. 8 (Ronda de Levante), although only during July 2008. No serotyping of the isolated strains was additionally performed.

Fungal diversity

In our study, almost all airborne fungi isolated grew in filamentous forms and gave rise to characteristic colonies on Sabouraud plus chloramphenicol solid medium. Colony morphology, coupled to microscope analyses of both hyphae and spores, allowed to establish the relative abundance of the more common genera detected. Four genera were essentially predominant among fungi, be-

sides a number of forms less frequently isolated that were uncharacterized. *Cladosporium* (69%) was the air contaminant most usually detected and this result is coincident with other reported studies on the fungal airborne microflora in open air environments (Oliveira et al. 2005; Flores-Tena et al. 2007). Also present in the sampled air were found species of *Alternaria* (14%), *Aspergillus* (11%) and *Penicillium* (6%).

Genera	Relative Percentage
<i>Staphylococcus</i>	47
<i>Acinetobacter</i>	10
<i>Bacillus</i>	9
<i>Streptococcus</i>	8
<i>Corynebacterium</i>	8
<i>Pseudomonas</i>	7
<i>Yersinia</i>	3
<i>Proteus</i>	3
<i>Listeria</i>	2
<i>Lactobacillus</i>	2
<i>Haemophilus</i>	1

Table 3.- Frequencies of most common bacterial isolates (October 2007-September 2008)

Tabla 3.- Frecuencia relativa de bacterias aisladas (Octubre 2007-Septiembre 2008)

Seasonal variation of microbial contaminants

The mean values of seasonal isolations obtained throughout an entire year in each of the 10 areas sampled were computed to establish the data shown in figures 1 and 2. The microbial load due to bacteria was higher during October and decreased in a gradual fashion until January, when a small increase was detected, to decrease again thereafter. The lowest level of bacterial contami-

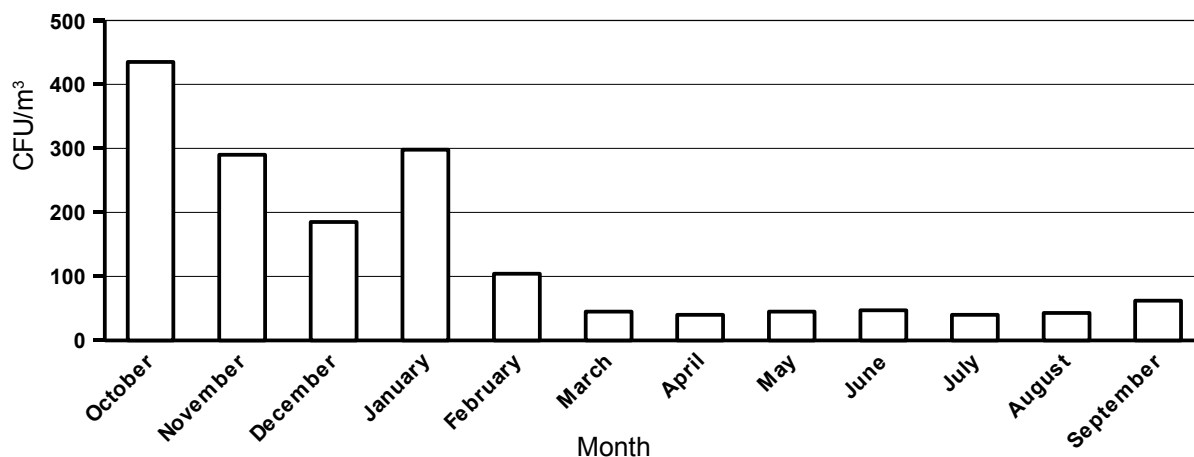


Figura 1. Variación estacional de la concentración de bacterias en el aire de las zonas muestreadas durante el periodo de un año.
 Figure 1. Seasonal distribution of bacterial concentration into the air of the sampled areas during one year period.

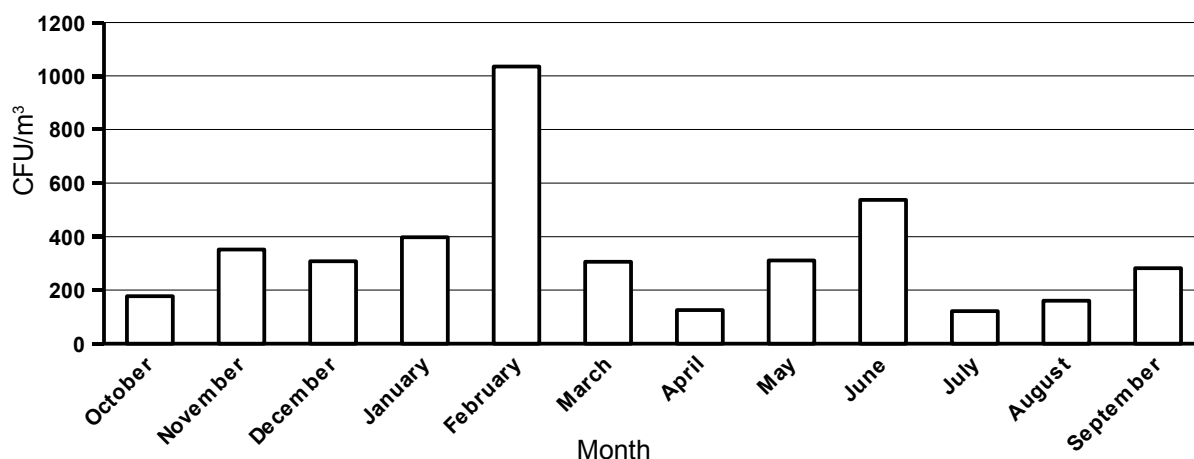


Figura 2. Variación estacional de la concentración de hongos en el aire de las zonas muestreadas durante el periodo de un año.
 Figure 2. Seasonal distribution of fungal concentration into the air of the sampled areas during one year period.

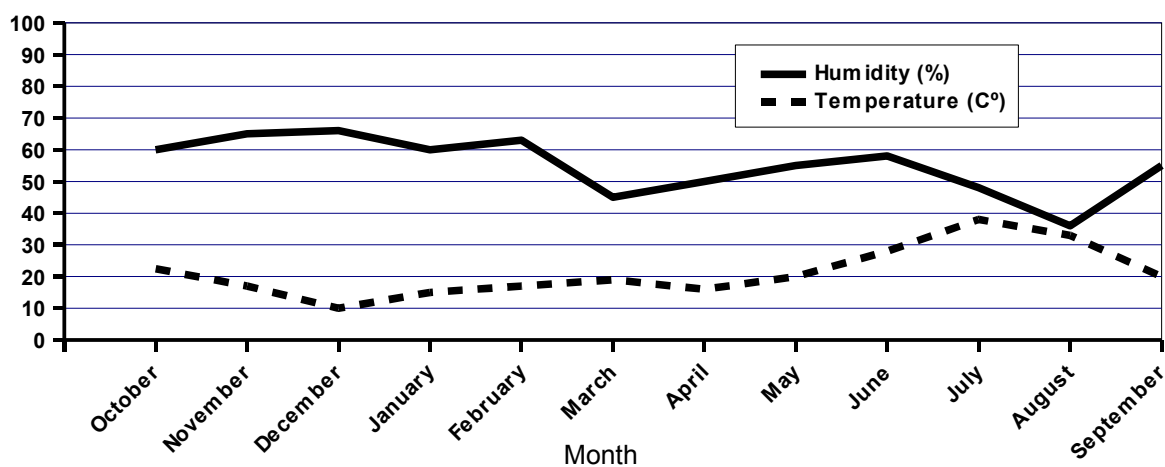


Figura 3. Temperatura y humedad relativa del aire registrada durante el periodo de muestreo.
 Figure 3. Air temperature and relative humidity recorded during the sampling period.

nation was found in the period between March and September (Fig. 1). However, the temporal distribution of the fungal microbiota appears to follow a different pattern (Fig. 2). In close relation to humidity and temperature values (Fig. 3) fungal concentration increased rather steadily during autumn, peaked during February and less dramatically again in June, while the rest of the year showed a level below 4×10^2 CFU/m³. The lowest value for fungi was recorded in July (coincident with the minimal value for bacteria). Notably, this month was unusually warm, with daytime temperatures often surpassing 40°C (Fig. 3).

Discussion

No analyses on the microbiological load of the urban air in the city of Murcia have been published prior to this work. In the present study we present a series of preliminary data related to such topic. The methods used to collect microorganisms in the atmosphere may include impaction, impingement, filtration or gravity deposition (Griffin 2007). Gravity deposition involves exposure of nutrient agar plates to open-air environments for some periods of time. However, this simple and inexpensive protocol has been shown unacceptable in comparison to volumetric assays (Gregory 1973). Impingement is also used in aeromicrobiology but requires the use of liquid media that often makes resulting counts less reliable (Terzieva et al. 1996). On the other hand, filtration methods, based on the trapping of microorganisms larger than the pore size on a filter that is later cultured, show the serious drawback of microbial desiccation due to the high flow rate needed and the time required prior to culture. Consequently, we selected an impaction method which enables air to move directly over the surface of petri dishes containing selective and non-selective agar media. In the procedure used, air flow can be controlled for volume and evenly distributed over the surface of culture media. Our detection system is easy to use due to its portability, shows relative low cost, and more importantly, allows the assessment of cultivable populations of bacteria and fungi per a known volume of air.

Air can be somehow compared to an ocean in many physical and biological aspects because of the existence of heterogeneous turbulences, mixture of components and irregular distribution of

the elements involved. Hence, data on airborne contamination may be influenced by multiple variables that are continuously changing and, consequently, they offer an informative flash rather than to describe a steady situation. However, in spite of the restrictions of this type of studies, our results clearly make possible to draw a contamination profile with general quantitative and qualitative conclusions.

Common levels of cultivable microorganisms from air vary from 10 to 10^4 CFU/m³ (Atlas & Bartha 2002). Our data indicate the existence of a significant level of microbial contamination in the urban air of Murcia which is, nevertheless, below or within the normal range of pollution found in similar studies performed on the outdoor air of other cities (Di Giorgio et al. 1996, Shaffer & Lighthart 1997, Mahdy & El-Sehrawi 1997, Lighthart 2000, Zhu et al. 2003, Nava et al. 2004, Oliveira et al. 2005). The results pointing that viable fungal counts generally exceeded the bacterial airborne concentration are in agreement with the fact that fungal spores are generally well adapted to survival in the absence of available water and nutrients in the atmosphere (Ingold 1971). Likewise, the relative abundance of Gram-positive bacterial cells with cocoid appearance versus bacilar shape might be explained on the bases of their structural cell wall composition, which results in more global resistance to hostile conditions of the aerial environment, such as desiccation or sun radiation. Taking into account our daily intake of air (more than 10.000 liters) and that a global mean value of 532 microorganisms per m³ was obtained as the average value from the overall data, this study reveals that one citizen of Murcia may inhale a total estimate of no less than about 5×10^3 microorganisms each day from the outdoor air.

The highest total microbial density corresponded to a garden area with relatively intense human and animal activities while the minor microbial pollution was found near the Cathedral building. Several potential pathogens, like *Staphylococcus* and other common Gram-positive bacteria, were detected in several areas of the Murcia air. The occasional finding of the potential pathogen *Legionella* in two sampled areas during July is of particular interest considering that the world's largest outbreak of Legionnaires' disease happened in Murcia during summer in the very re-

cent past (García-Fulgueiras et al. 2003). Epidemiological investigation implicated then the production of aerosols by infected cooling towers of air conditioning systems, that were presumably in close proximity to the two sampled areas that resulted positive in our study.

Viable bacterial contamination appears to reach a maximum peak during autumn in the air analyzed and a minimal level during spring and summer, which is in agreement with the deleterious effects of high temperature and sunstroke on most bacteria. On the other hand, our results are indicative that the fungal temporal distributions follows a different trend with highest incidence in February.

Air serves as a transmission vehicle for potential pathogens and public health requires constant investigation on the aeromicrobiological contamination of the urban air. It should be highlighted that, taking into account that usually less than 1% of the bacterial community in many environments is cultivable under the most favorable conditions (Amann et al. 1995), our approach based on data of viable counts may represent an underestimate with respect to the real microbial composition. This consideration, justify enough our idea that a continued program for surveillance, prevention and control of microbial pollution should be established as part of public efforts for an advanced quality of life.

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