Sampling bias and sampling errors in pollen counting in aerobiological monitoring in Italy†

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Information about airborne pollen concentration is of concern for health authorities across Europe. The reliability of data estimates depends on the accuracy and precision of pollen counts. In Italy, pollen counts are carried out on slides for microscopic evaluation and are regulated by the national Standard UNI 11108:2004. Our results showed that counts performed according to the Italian standard may result in a significant bias in the number of pollen grains counted and this will have an impact on final estimates of pollen concentration. For the same sample size, confidence intervals vary in relation to pollen abundance, either in terms of number of grains or of number of species. The sample size suggested by the standard (20% of the target surface) may result in errors in pollen counts ranging from 7–55% of the mean value, and in missing 22–54% of the taxa present on the slide.

Introduction

Aerobiological monitoring provides data about the atmospheric content of biological particulate matter—mostly represented by pollen and moulds—as well as its abundance and timing. This information is relevant in epidemiology, allergopathy prophylaxis, diagnosis and therapy.[1]

In Europe and Italy many aerobiological monitoring centres analyse the airborne pollen load and produce reports and forecasts for the public (http://www.polleninfo.org/). Monitoring centres within each national network usually work according to a reference protocol, for example the Spanish Aerobiology Network (REA)[2] and the monitoring network of the United Kingdom (http://www.pollenuk.co.uk/aero/ABPollenUK.html).

In Italy, airborne pollen collection and analysis are carried out according to a standard method published by the Italian Organization for Standardization[3] (in Italian: UNI, www.uni.com) In brief, the method consists in the identification and count (by optical microscope) of airborne pollen grains contained in a known air volume and drawn on a known surface by means of a vacuum pump. The main steps of the Standard UNI 11108:2004 are described in Table 1.

The Standard describes (i) the device to be used, (ii) its locational method, (iii) the procedures for preparing the sampling surface, (iv) the method for preparing the slides for microscopic examination, (v) the counting scheme for slide analysis, (vi) the method adopted to convert the pollen counts into atmospheric pollen concentration data. As shown in Table 1, the quality of airborne pollen concentration data depends on many factors, such as the suitability of the sampler location and its correct functioning, accuracy in sampling surface and microscopic slide preparation, and the slide sampling strategy. Another issue affecting data quality is the operator's ability in pollen identification.

However, even when assuming an unbiased location of the device, the correct functioning of the whole apparatus (device, preparation of the sampling surface and slides) and the

Table 1 Main issues defined in the Italian standard UNI 11108:2004

Steps	UNI 11108:2004 recommendations
(i) Device	Volumetric Hirst-type sampler; vacuum pump produces an airflow of 10 l/min (14.4 m^3/day); intake orifice size 2 × 14 mm.
(ii) Device installation	In free air circulation; on top of buildings or on roof terraces, at a height of 15–20 m.
(iii) Sampling surface preparation	Application of silicon fluid on a Melinex ® tape for particle adhesion; tape is mounted on a drum moving at 2 mm*h^{-1}, 1-week period.
(iv) Slide preparation	Sampled surface corresponding to 1-day time window is mounted on a slide and coloured with fuchsin glycerol gelatine.
(v) Slide analysis	Light microscopic identification and counting at 250× or 400× by means of horizontal homogeneously distributed sweeps, avoiding the upper and lower edges; sample surface = a tape portion of 14 × 48 mm; sampled portion ≥ 20% of the sample surface.
(vi) Concentration calculation	Data needed: A = sample area (14 × 48 mm); V = sampled air volume (14.4 m^3/day); a = sampled area; N = number of pollen grains counted per taxon; airborne pollen concentration = (A/a) × (1/V) × N.

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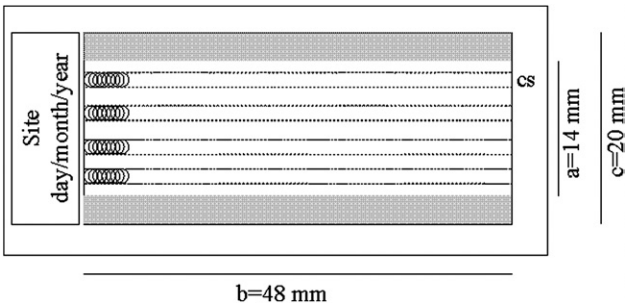

Fig. 1 Scheme of a daily slide: a × b = standard sample area after the standard UNI 11108:2004 (or central area of the tape); c × b = adhesive tape (or whole sampling surface); routine scheme adopted by the aerobiological centre of San Michele a/A for the counting: 4 continuous sweeps (cs) at 400×, diameter of microscope field = 0.5 mm.

employment of well-trained operators, there are several questions related to the adopted counting scheme (Fig. 1).

Firstly, the Standard considers as sample area only the central region of the slide, 14 mm wide—the same size as the inlet orifice—and 48 mm long. However, particles contained in the sampled air volume may impact all over the adhesive tape, whose dimensions are 20 × 48 mm, due to a drift effect. Considering only the central portion of the slide may create the basis for a bias in the estimates arising from the pollen counts.

Secondly, the Standard assumes that pollen counts performed in microscopic fields lying on the same vertical profiles of the slide statistically represent the same number of particles (UNI 11108:2004, p. 4). By definition, it implies that the mean and the total number of pollen grains counted on individual sweeps are statistically the same. Although this assumption has a clear impact on the reliability of the final result, no information is given about the extent of its validity.

Thirdly, the Standard recommends sampling at least 20% of the sample area. Nevertheless, a survey of centres belonging to the Italian Aerobiology Association network revealed a mean examined surface of 13.1%.[4] Therefore, it is crucial to gather information on the effect of the sample size on the final estimates of pollen load on the slide.

The effects of sample size, counting scheme and the abundance of taxa on the estimates have already been studied by Comtois *et al.*,[5] Kapyla and Pettinen,[6] and Cariñanos *et al.*[7] Instead, we intend to evaluate certain specific aspects related to the Italian Standard UNI 11108:2004 in order to provide useful information for its future revision. Since estimates of airborne pollen concentrations are based on pollen counts on the slides, we will concentrate on the sampling bias and sampling error related to the location and density of countings within the slide. In particular, this study addresses the following questions:

1. What is the bias regarding the number of pollen grains and the number of species if only the central portion of the slide is taken into account?

2. To what extent can estimates originating from different sweeps of the same slide be considered as statistically representing the same number of particles?

3. What is the sampling error in relation to the sample size (expressed as a proportion of the target area)?

Experimental procedures

Data set

A set of 365 daily aerobiological samples (slides) collected from October 2006 to September 2007 was considered in the present study. The slides were obtained using a Hirst-type volumetric spore trap (Lanzoni VPPS 2000) located at San Michele a/A (Trento, Northern Italy, Lat N: 46°11′ Long. E: 11°08′ elevation: 227m a.s.l.), where aerobiological monitoring has been performed since 1990. The 365 slides had been previously examined following a counting scheme of 4 continuous longitudinal sweeps (see Fig. 1). To investigate the effect of pollen density on estimates of the number of grains and species, the slides were organized according to the number of pollen grains counted and the number of taxa identified. Subsequently, three groups of n = 34 slides each were identified to represent different degrees of pollen abundance (Table 2):

(a) slides below the 10th percentile of counted pollen grains, considered as "low pollen concentration and low number of taxa";

(b) slides around the 50th percentile ±17 slides, considered as "median pollen concentration and median number of taxa";

(c) slides above the 90th percentile, considered as "high pollen concentration and high number of taxa".

To make the counting work affordable (one single slide may have up to 3,900 pollen grains, see Table 2), we selected a random sample of n = 4 slides from each group, resulting in a total of n = 12 (Table 2). Taxa identification and total of pollen grain counts were carried out using a light microscope (Leitz Diaplan) at 400×, scanning the whole adhesive tape (20 × 48 mm) with 40 horizontal continuous sweeps (0.5 × 48 mm each). Pollen counts (taxa and respective grain numbers) were kept separate for each sweep. To avoid observer error, the 12 slides were examined by

Table 2 Descriptive statistics of the available data set. Total: the set of daily aerobiological samples (slides) considered for this study. Group: the different degrees of pollen abundance identified within the Total set in relation to pollen density, low, median and high. Sample: the slides randomly selected within each Group

		Total (n = 365)	Group "low" (n = 34)	Group "median" (n = 34)	Group "high" (n = 34)	Sample "low" (n = 4)	Sample "median" (n = 4)	Sample "high" (n = 4)
pollen grains	min	1	1	112	765	1.0	150.0	3271.0
	median	142	3	142	1506	2.0	152.0	3279.5
	max	3969	4	184	3969	4.0	157.0	3969.0
pollen taxa	min	1	1	10	24	1.0	10.0	25.0
	median	10	3	10	26	3.0	10.0	29.5
	max	33	4	10	33	4.0	10.0	32.0

the same operator. All together, a total of n = 89,320 pollen grains were counted.

Data analysis

To quantify the bias caused by drift on the tape, total pollen grain counts and number of taxa from the 20 × 48 mm area were compared with total counts from the 14 × 48 standard sample surface centred on the inner part of the adhesive tape. Differences were tested by the Wilcoxon signed-rank test.[8]

Confidence intervals (CI, P = 95%) of different sweeps were calculated in order to estimate at what level of confidence the assumption about the homogeneous distribution of pollen grains along individual vertical profiles of the tape can be considered valid

The error associated with the sample size in pollen counts was estimated with the standard error (SE) for different sample sizes.

The effect of sample size on the number of taxa identified was evaluated by calculating the percentage of taxa identified in relation to the total sampling effort. This was done by rarefaction analysis[9] carried out on the basis of 1,000 iterations of random sweep selections.

Results and discussion

Sampling bias

Fig. 2 reports the mean number of pollen grains for each sweep (all slides). There is a distinct drift when particles are sucked through the orifice and impact on the collection surface (adhesive tape).

Overall, the percentage of pollen grains falling on lateral areas ranged between 1.5% and 31.3% of the total pollen count and was higher for slides with a low pollen concentration. This is because percentages can vary widely when the pollen concentration is low. Note that slides with a low concentration have a maximum total number of grains of n = 4 (see Table 2). On average, the difference between the number of pollen grains captured in the central area of the tape (14 × 48 mm) and those counted on the entire surface (20 × 48 mm) was statistically significant ($p < 0.01$).

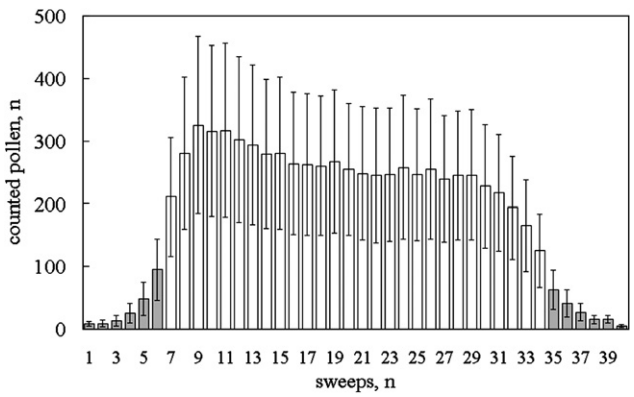

Fig. 2 Pollen distribution per sweep (n = 40) on the entire adhesive surface (20 × 48 mm, see Fig. 1). Each sweep is 0.5 × 48 mm; x axis represents the whole adhesive breadth (20 mm). Medium data counts on 12 slides. Bars represent the standard error. White: central area of the slide (14 × 48 mm) suggested by the standard UNI 11108:2004 grey: lateral portion (3 × 48 mm each).

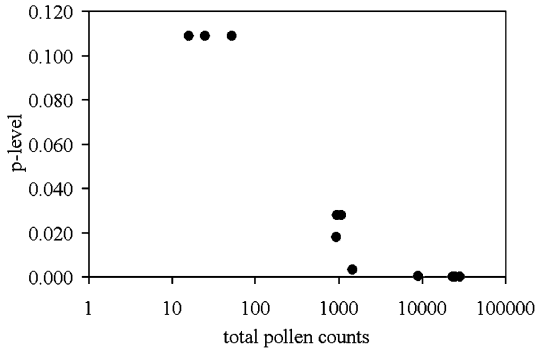

Fig. 3 Statistical significance of differences between pollen counts on total sampled area and standard sample surface.

Considering data sets for each taxon and collection date, differences between the two counts were significant only for samples with medium and high pollen density (Fig. 3), *i.e.* those with a higher pollen load and of particular interest to allergopathy prophylaxis.

As far as the number of taxa are concerned, no significant difference was found between the number of species identified on the central area and those on the whole surface. The maximum difference amounted to one species per slide.

Confidence of estimates

As revealed by Fig. 2, there are differences among the mean values of the various sweeps. When considering a defined P level (P = 95%), the confidence interval (CI) varies between 5.9% and 57.3% of the mean (Table 3), with the widest CI (>30% of the mean) occurring for low values of pollen density. This suggests that the assumption of homogeneity inherent in the Italian standard remains quite ambiguous and cannot be generalised, unless a desired CI width is identified. It is worth noting that the CIs reported were calculated with reference to the central portion of the tape and that they are likely to increase if the entire surface is considered. These results suggest the importance of defining a target precision level of daily pollen counts and—on this basis—identifying an adequate sampling technique adaptable to pollen density.

Table 3 Selected slides; mean number of pollen counts on all sweeps of the standard sample area (central portion) and confidence intervals

Date of slide	Mean pollen grains, no.	Coefficient of variation	CI (P = 95%)	CI (P = 95%), % of mean
18/11/2006	1.64	81.52	0.52	31.55
08/12/2006	0.39	126.59	0.19	49.00
16/12/2006	0.43	147.96	0.25	57.27
28/12/2006	0.68	120.68	0.32	46.71
13/04/2007	799.89	22.95	71.07	8.88
15/04/2007	928.36	19.60	70.42	7.58
16/04/2007	856.04	15.19	50.33	5.88
29/04/2007	294.39	19.39	22.09	7.50
28/06/2007	49.36	39.25	7.50	15.19
05/07/2007	32.04	36.36	4.51	14.07
01/08/2007	32.75	34.37	4.36	13.30
22/09/2007	37.68	35.47	5.17	13.73

Sampling errors

Previous papers (see Maher[10] and references therein) have addressed the question of uncertainty in estimating pollen proportions. Here, error estimates in relation to the total number of grains and the number of taxa are briefly addressed. Fig. 4 reports the SE values in relation to sample size expressed as percentages of the target sample surface, considering the whole sampling surface and the central area. As far as the number of pollen grains is concerned, examination of 20% of the slide - the minimum area suggested by the Italian Standard - reveals an error rate ranging from 7% to 52% if only the central portion is considered; the SE increases to 20% and 55% respectively if the whole area is taken into account. The size of the SE depends on pollen density, with slides having a median-to-high pollen concentration showing a much lower SE than slides having a low concentration. However, in both cases, with the current counting scheme of continuous sweeps, it will be very difficult to achieve reasonable SE values (*e.g.* 5% of the mean estimate) especially if the entire sampling area is considered. The data reported in Fig. 4 suggest that it is necessary to identify a target precision level of mean estimates and to look for more effective counting (sampling) schemes to be used to carry out counts on the slides.

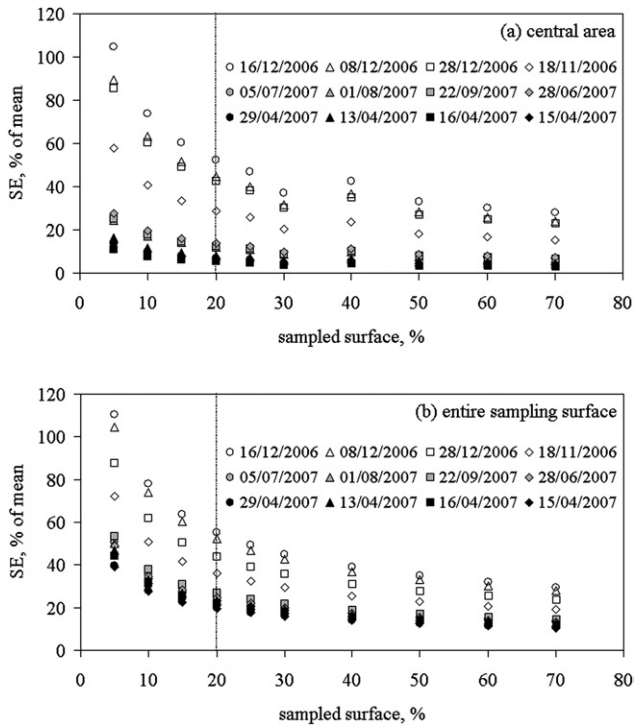

Fig. 4 Standard Error values in relation to the sampled surface percentage, considering (a) the central area and (b) the whole sampling surface. The dashed lines indicate the 20% sampling size suggested by the standard UNI 11108:2004.

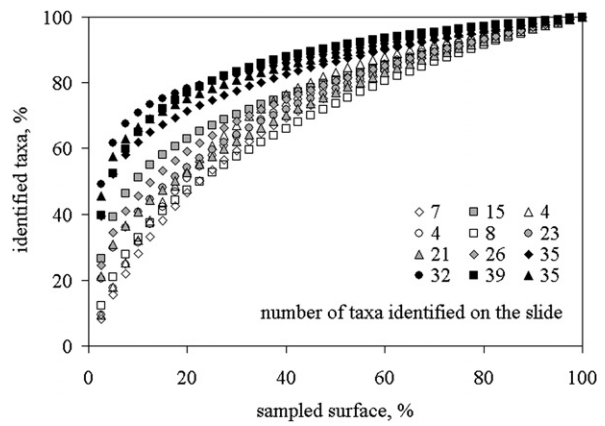

Fig. 5 Percent of species identified in relation to the percent of the area sampled. Results are the mean of 1000 iterations of random selection of the sampling sweeps. Numbers in the graph are the total number of taxa identified for each slide.

As for the number of taxa, Fig. 5 shows the relationship between the sample size (in % of the entire sample surface) and the number of taxa identified, which varies with the species richness of the sample (in % of the total number of species identified). Considering the standard sample size of 20%, the taxa recorded range between 46% and 78% of identified species, with the lowest percentage for the less rich samples.

According to our results, the reduced sample size reported from Italian centres (13.1% on average) may result in a considerable increase in error size. Questions arise about the need for more explicit precision requirements and more effective sampling schemes.

Conclusions

Reports and information on pollen concentrations in the atmosphere are based on monitoring data which are subject to errors from various sources. The reliability of this information is dependent on the precision and accuracy of the pollen counts. It was demonstrated that particles deviating from the trajectory of the inlet caused pollen grains to be deposited onto the lateral areas of the sampling tape. Thus, taking into account only the central portion of the tape results in a sampling bias, mainly regarding the number of pollen grains. The Standard UNI recommendation to avoid the edges of the particle deposition area seems therefore not to be justified and not appropriate to ensuring unbiased estimates of pollen load.

Restricting the sampled area to the central part of the tape was based on the assumption that pollen counts performed in microscopic fields lying on the same vertical profiles of the slide statistically represent the same number of particles. It implies that the mean and the total number of pollen grains counted on individual sweeps are statistically the same. However, the reported data showed high variability in the counts within each sweep, thus affecting the precision of the resulting estimates. We believe that it is necessary to have a clearer definition of expectations in terms of data variability before making unjustified assumptions. If the whole adhesive surface of the slide is considered to be the sample area, a new sampling strategy is necessary to account for differences in pollen distribution across the slide.

A common objection when a new methodology is proposed is that it will disrupt the time series comparability. Such an objection is relevant and changes in methodology need always to be considered with care. However, if a flaw in a method exists, the above objection should not lead to the maintenance of the *status*

quo, *i.e.* the manteinance of an error or a bias. Rather, steps should be undertaken to ensure that past and future data series can be linked: for example old and new methods could be run in parallel for some time in order to derive "bridging functions" between the two data series.

As Comtois[5] showed, errors in counts are related to the percentage of surface examined and on the sample abundance. Maintaining the same sampled surface, the number of identified taxa depends on the total number of species contained in the sample.[9] A slide sampling design should take into account all these factors by setting *a priori* a desired standard error for the data, taking into account the aim of the study and the expected use of the data. This will also help in understanding the actual meaning of possible differences over time and space.[10]

In this paper, we have considered only part of the entire process necessary "to produce" pollen data and we have identified some problems to be resolved in order to improve the reliability of the data. Considering the use of aerobiological data in the human health field, their reliability and comparability in time and space is crucial. Although the use of common Standard Operating Procedures (SOPs) represented by documents such as the UNI 11108:2004 is important, we believe that the overall design of aereobiological monitoring needs to be quality assured, from site selection to data reporting.

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References

1 E. Levetin and P. Van de Water, *Immunol Allergy Clin North Am*, 2003, **23**, 423–442; I. Chuine and J. Belmonte, *Grana*, 2004, **43**, 65–80.
2 C. Galán Soldevilla, P. Cariñanos Gonzáles, P. Alcázar Purificatión and E. Domínguez Vilches, in *Spanish Aerobiology Network (REA): Management and Quality Manual*, ed. Servicio de publicaciones de la Universidad de Córdoba, Córdoba, Spain, 2007, pp. 36.
3 UNI 11108:2004, *Air quality. Method for sampling and counting of airborne pollen grains and fungal spores*, ed. UNI Ente Nazionale Italiano di Unificazione, Milano, Italy, 2004, pp. 8.
4 E. Zieger, presented in part at the Workshop of the Italian Aerobiology Association (AIA)"*Sistema di qualità in aerobiologia: stato dell'arte e prospettive*", Perugia (I), February, 2007.
5 P. Comtois, P. Alcazar and D. Néron, *Aerobiologia*, 1999, **15**, 19–28.
6 M. Kapyla and A. Pettinen, *Grana*, 1981, **20**, 131–141.
7 P. Cariñanos, J. Emberlin, C. Galán and E. Dominguez-Vilches, *Aerobiologia*, 2000, **16**, 339–346.
8 F. Wilcoxon and R. A. Wilcox, in *Some rapid approximate statistical procedures*, ed. Lederle Laboratories, Pearl River, New York, 1964, pp. 59.
9 H. J. B. Birks and L. M. Line, *The Holocene*, 1992, **2**(1), 1–10.
10 L. J. Maher, *Review of Palaeobotany and Palynology*, 1972, **13**(2), 85–93.