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ORIGINAL PAPER

Pollen monitoring: minimum requirements and reproducibility of analysis

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Abstract Training, quality assurance (QA) and quality control (QC) play an important role in building competence in monitoring and research in aerobiology. The main goals of this paper were to: (a) formulate an updated Minimum Requirements Report for pollen monitoring; (b) carry out a pilot QC exercise of staff involved in pollen counting from various national networks in order to examine between analysts reproducibility and develop a methodology that can be used in future QC exercises. A questionnaire survey was sent to coordinators of participating pollen monitoring networks. In addition, a total of 45 technicians from 15 European countries participated in the pilot QC exercise. All technicians were instructed to analyse two slides containing the following pollen types: (a) Poaceae and *Betula* pollen grains in the north of Europe; (b) Poaceae and *Olea* pollen grains in the south of Europe. Minimum Recommendations were produced based on the results of the questionnaire survey, published literature, and the outcomes of a workshop. In the QC exercise, it was noticed that technicians who followed the Minimum Recommendations and examined at least 10 % of the slide tended to have better indicators of precision and accuracy than those technicians who did not follow the Minimum Recommendations. The proposed Minimum Recommendations will help to improve the quality of scientific work, particularly for those who are considering the setting up of new monitoring sites. The results of the pilot QC exercise will help to develop a methodology that can be used again in the future, thereby ensuring data quality.

Keywords Aerobiology · Quality assurance · Quality control · Questionnaire

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1 Introduction

Ensuring the quality of aerobiological data is important when monitoring and reporting of airborne pollen counts. The *European Aeroallergen Network* (EAN) database was established in the late 1980s and provides a valuable service supplying pollen information to a variety of end users, including pollen allergy sufferers and health care professionals. The EAN database holds information from more than 600 pollen monitoring stations from all over Europe. The owners of the data held within EAN include charities, universities, government organisations and private individuals, and many operate within national networks. It is important to ensure data integrity, and so all data suppliers follow a standardised methodology based on the Minimum Requirements described by Jäger et al. (1995).

A number of papers have been published that have focused on the methodology and materials used in different sampling locations (e.g. Rantio-Lehtimäki et al. 1991; Galán et al. 1995; Tormo et al. 1996; Galán and Domínguez-Vilches 1997; Alcázar et al. 1999; Spieksma et al. 2000; Carvalho et al. 2008; Velasco-Jiménez et al. 2013), counting methods (e.g. Käpylä and Penttinen 1981; Leuschner 1999; Comtois et al. 1999; Cariñanos et al. 2000; Sikoparija et al. 2011) and data management (Jato et al. 2006). Such studies have attempted to evaluate and improve upon the standardised method.

The standardisation of pollen monitoring allows the comparison of data between sites, such as temporal and spatial variations in diurnal and daily concentrations, and seasonal characteristics (e.g. timing and intensity of pollen seasons), as well as trends over time (e.g. in relation to land use and climate change). The ability to produce comparable data also provides opportunities to construct models for predicting airborne pollen over large geographical areas (e.g. Siljamo et al. 2013; Sofiev et al. 2013; Vogel et al. 2008). Due to the interdisciplinary nature of aerobiology, this research is applicable to different subjects, such as agronomy, allergology, climatology, environmental health, forestry, meteorology and phenology.

Training, quality assurance (QA) and quality control (QC) play an important role in building competence in monitoring and research in aerobiology.

A biannual *European Basic Course on Aerobiology* for training technical staff and researchers has been running since 1993 under the aegis of the *International Association for Aerobiology* (IAA) and, more recently, the *European Aerobiology Society* (EAS). The course consists of theoretical and practical lessons that introduce students to, among other things, the concept of quality assurance for pollen monitoring.

At a European level, a Working Group examining QA and QC in aerobiology has been created in the framework of the EAS. Different tasks have been proposed to consolidate this Working Group: (a) organise training courses; (b) the use of adequate reference material for identification; (c) organize annual QC exercises; (d) produce an updated "Minimum Requirements" recommendations report (Galán 2009; EAS QC Working Group 2011).

During the 1980s, it was proposed that standard testing methods for QC include within laboratory repeatability and between laboratory reproducibility (Spetz 1995). Different national aerobiological monitoring networks implement QC exercises, e.g. the *Spanish Aerobiology Network* (REA), in an attempt to determine reproducibility of analysis undertaken by technicians in the network (Oteros et al. 2013). QC measures from valid samples are crucial to offer quality results and essential for comparative studies among different geographical regions. However, only a few studies have been focused on proficiency testing to improve data quality in bio-monitoring networks (Berti et al. 2009; Oteros et al. 2013).

This paper signifies the next step in the consolidation of the EAS QC Working Group. The first goal was to formulate an updated *Minimum Requirements Report* for all members involved in the EAN that was based on the previous Minimum Requirements proposed by Jäger et al. (1995). The second goal was to carry out a pilot QC exercise of staff involved in pollen counting from various national networks in order to examine between analysts reproducibility and develop a methodology that can be used in future QC exercises, thereby ensuring data quality.

2 Materials and methods

2.1 QC questionnaire and Minimum Requirements Report

The first step towards formulating an updated *Minimum Requirements Report* for aerobiological

monitoring stations included in the EAN was to send a questionnaire to network coordinators. The QC questionnaire refers to different topics related to: (a) the pollen trap; (b) preparation and counting of the samples; (c) data management; (d) QC tests and courses; (e) additional questions and comments. The results of the questionnaire survey were then presented to a special EAS QC Working Group workshop organised at Perugia University (Italy) on the 27 November 2009 (Galán 2010). The first draft of the "*Minimum Requirements*" report was formulated during this meeting and then disseminated among network coordinators for ratification.

2.2 QC between analysts reproducibility

The EAS conducted a pilot QC exercise of staff involved in pollen counting from various national networks, in order to examine between analysts reproducibility and develop a methodology that can be used in future QC exercises. EAN members were contacted and offered the opportunity to participate in the QC exercise. A total of 45 technicians from 15 European countries participated (Appendix 1): Austria, Croatia, Germany, Poland, Finland, France, Italy, Macedonia, Portugal, Serbia, Spain, Switzerland, Turkey, Ukraine and UK.

All technicians were instructed to analyse two slides containing the following pollen types: (a) Poaceae and *Betula* pollen grains in the north of Europe; (b) Poaceae and *Olea* pollen grains in the south of Europe. The QC exercise was limited to these three pollen types (birch, grass and olive) in order to reduce the error in the analysis caused by counters examining unknown or unusual pollen types. Two slides with "moderate" levels of the mentioned pollen types were supplied to participating staff in the north and south Europe:

- Northern Europe: (a) One slide from Bad Tatzmannsdorf (Burgenland, Austria) for *Betula* pollen (slide dated 10/04/12; $n = 34$ technicians), termed VIE100412; (b) One slide from Vienna (Austria) for Poaceae pollen (slide dated 30/05/12; $n = 34$ technicians), termed VIE300512.
- Southern Europe: (a) One slide from Córdoba (Andalucía, Spain) for Poaceae and *Olea* pollen (slide dated 17/04/11; $n = 11$ technicians), termed COR140411; (b) One slide from Córdoba for Poaceae and *Olea* pollen (slide dated 07/05/11; $n = 10$ technicians), termed COR070511.

Moderate values have been considered because they represented the sort of levels often encountered on daily slides, but were not excessively high and so did not cause too much work for the participants. For this reason, no more than a daily average of 300 pollen grains/m^3 has been considered for these pollen types.

Three different counting methods were employed by participating sites: longitudinal (horizontal) transects, latitudinal/transversal (vertical) transects and random fields (Scheifinger et al. 2013). Daily average pollen concentrations were expressed as pollen grains per cubic metre of air (pollen grains/m^3). Members of staff at the Medical University of Vienna and the University of Córdoba examined the Northern and Southern European slides, respectively, before they were sent to the first participating site, and then checked the slides after the last participant had returned them. This was done to ensure that the slides had not been damaged during transit and that the same counters, using the same methods, were able to reproduce their original results (i.e. the pollen grains had not moved on the slide). No notable differences were found between pollen counts conducted before and after the slides had been sent to participating sites.

Between analysts reproducibility was determined following the method used by the *Spanish Aerobiology Network* (REA), as described by Oteros et al. (2013).

A first step was to study normality of the data distribution by using the Lilliefors test (Starink and Visser 2010). Outliers were identified following ISO confidence levels for statistical outliers (ISO 5725 1994), i.e. 95 % for outliers classed as "stranglers" and 99 % for those classed as "statistical outliers". A maximum of three counters (trained staff that analysed the slides) per country were selected aleatory for calculating outliers. This was because the inclusion of higher raw data from some labs could skew the final result. As a result, the number of elements (n) considered for outliers identification in samples VIE100412 and VIE300512 was reduced to 24.

Outliers were calculated by transforming the raw data to z scores and eliminating the outer 5 % of the data. z scores have been calculated with Formula 1:

$$Z = \frac{\chi_i - \bar{X}}{S}$$

χ_i: daily average pollen concentration by individual participant (raw data), S: standard deviation of the sample, $\bar{X}$: mean of the sample.

To define outliers, the following conditions have been considered: (1) Only results with a z score outside the range [−1.96, 1.96] were considered outliers; (2) To be considered an outlier, $\chi_i - \bar{X}$ must also be more than 10, where χ_i is a raw score and $\bar{X}$ is the mean of the sample. This was because z score values are strongly influenced by the sample mean: if the sample mean is very low, the z score cannot identify true outliers.

Daily average pollen concentrations produced by individual participants were compared with the assigned value. The assigned value (X) is an approximation of the unknown real value of the slide. It was calculated as the average, taking into account the central 95 % of data, omitting outliers, calculated by z scores. The assigned value (X) depends directly on the sample mean ($\bar{X}$), but with several modifications to make it closer to the real value of the slide, the population mean. The standard deviation of this 95 % sub-sample is called the standard deviation of proficiency (S').

The confidence limits (CL) of the assigned value were also defined, i.e. the values that should be considered as true, taking into account that an acceptable error must be assumed. In this occasion, it has been assumed that the true value lies between the upper limit (UL) and the lower limit (LL) with 95 % probability (Abraira 2002a, b), using Formula 2:

$$\mathrm{CL} = X \pm \frac{1.96 \times S'}{\sqrt{n}}$$

X assigned value, S' standard deviation for proficiency, n size of the sample

Variation coefficient (VC) has been calculated taking into account the assigned value and the standard variation using the following selection criteria:

1. Only pollen types whose assigned value (X) was over 10 were taken into account, because the VC is strongly influenced by low means
2. VC ≥30 was deemed unacceptably high when referring to pollen types with an X value between 10 and 25.
3. VC ≥20 was deemed unacceptably high when referring to pollen types with an X value between 25 and 100.
4. VC ≥15 was deemed unacceptably high when referring to pollen types with an X value between 100 and 300.

Calculation of the VC for each pollen type was essential, since a high VC would mean high variability in the sample. In such a case, the dataset could not be used for the QC exercise because there is no guarantee that the assigned value would be a true representation of the real value.

Absolute error (AE) has been considered as a value recorded by each technician and assigned a value, Formula 3:

$$\mathrm{AE} = \chi_i - X$$

χ_i value recorded by each technician, X assigned value

Relative errors (RE) have been obtained taking into account Formula 4:

$$\mathrm{RE} = \begin{cases} \frac{\chi_i - \mathrm{UL}}{X} \times 100, & \chi_i > \mathrm{UL} \\ 0, & \mathrm{UL} > \chi_i > \mathrm{LL} \\ \frac{\chi_i - \mathrm{LL}}{X} \times 100, & \chi_i > \mathrm{LL} \end{cases}$$

χ_i value recorded by each technician (raw data), X assigned value, UL (upper limit) or LL (lower limit) the confidence limit value nearest to χ_i.

For considering significant error, both conditions must be met: RE >20 % and AE >*10*.

- Number of erroneous elements (NEE) is defined as the number of staff members that have committed significant errors.
- Percentage of erroneous elements (PEE) is the percentage of NEE with respect to the total of participants in the analysis of the slide.
- Average of relative error (ARE) has been defined as the average of the RE committed by every participant.
- Average of absolute error (AAE) has been defined as the average of the AE committed by every participant.

The QC exercise examining between analysts reproducibility has been performed in two steps: (a) In an attempt to find out the general reproducibility, results from all counters together were examined by analysing the precision indicator (VC) and accuracy (PEE and ARE); (b) Two groups of pollen counters

were created and the different quality indicators (VC, PEE, ARE and AAE). Both groups were compared by Student's *t* test: Group 1—counters that do not follow the updated Minimum Recommendations on pollen counting and examine less than 10 % of the slide by longitudinal (horizontal) and latitudinal (vertical) by random fields; Group 2—counters that follow the updated Minimum Recommendations and examine more than 10 % of the slide by longitudinal (horizontal) and latitudinal (vertical) transects. For comparing these groups, samples 5 and 6 have been used.

3 Results

3.1 QC questionnaire and Minimum Requirements Report

The questionnaire was completed by coordinators of 26 different regional/national networks involved in EAN, representing 23 different countries (Fig. 1). The results of the questionnaire survey were taken into account when preparing the updated *Minimum Requirements.* For example, the majority of networks examined slides using longitudinal (horizontal) (60 %) or latitudinal (vertical) (30 %) transects, and less than 10 % said that they used random fields.

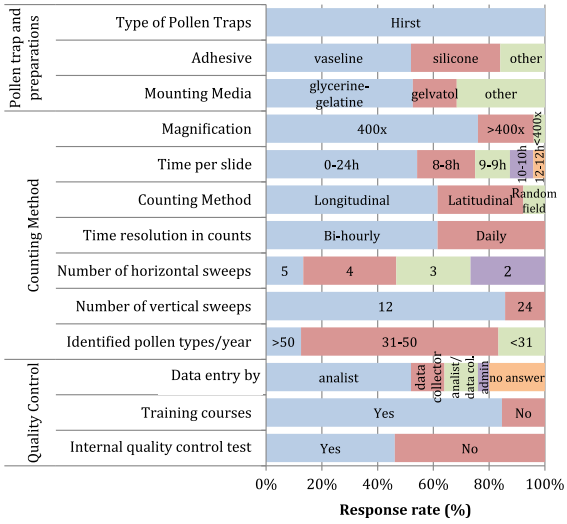

Fig. 1 Results of quality control questionnaire of pollen monitoring networks involved in EAN. *Y* axis shows the formulated question

The *Minimum Requirements* for pollen monitoring networks are:

1. *Sampler position* the sampler must be placed on a readily accessible, flat, horizontal surface. It should be on the roof of a building, increased from the ground, and away from the edge of the building in order to reduce the effect of turbulence.
2. *Flow rate* 10 l/min.
3. *Control of flow rate* Check weekly.
4. *Adhesive* Silicon (polydimethilsiloxane) or vaseline (can include vaseline and paraffin wax mixture).
5. *Mounting media* glycerine gelatine or polyvinyl alcohol (e.g. Gelvatol or Mowiol).
6. *Staining* no staining is allowable but the use of basic fuchsine or safranin is recommended.
7. *Minimum surface examined* 10 % of whole deposition area.
8. *Counting methods* Longitudinal (horizontal) or latitudinal (vertical) transects.
9. *Monitoring period* Whole year.
10. *Training* e.g. attend national or international courses or probationary/training periods with a particular emphasis on identifying main pollen types, operating the pollen trap, preparation of the samples for analysis.
11. *Internal validation of counted samples* within analysts QC among different staff members.
12. *External validation of counted samples* QC between laboratories in the frame of a national network, among different networks at international level, and among different project partners.
13. *Reporting* written report produced annually.

Note that in the case of counting method, these Minimum Requirements recommended that operators examine a minimum of 10 % of the slide surface by longitudinal (horizontal) or latitudinal (vertical) transects (No. 7).

3.2 Quality control between analysts reproducibility

Lilliefors test showed that the data were normally distributed. Outliers were identified following ISO confidence levels as described in the Material and Methods chapter and were taken into account when

Table 1 Summary parameters of pollen counters involved in the QC exercise (including those who follow and do not follow the Minimum Recommendations)

Sample	Slide	Pollen type	*N*	*X*	UL	LL	*S'*	VC	NEE	PEE (%)	ARE
1	COR170411	Poaceae	11	15	18	13	4	29.0	0	0.0	9.4
2	COR170411	*Olea*	11	220	238	202	29	13.0	0	0.0	5.2
3	COR070511	Poaceae	10	50	54	46	6	12.2	0	0.0	2.6
4	COR070511	*Olea*	10	74	82	67	11	15.0	0	0.0	3.9
5	VIE100412	*Betula*	34	93	101	85	14	15.5	6	17.6	9.5
6	VIE300512	Poaceae	34	51	55	47	7	14.2	6	17.6	9.6
Average								16.5		5.9	6.7

N, size of sample (number of counters); *X*, assigned value; UL, upper limit; LL, lower limit; *S'*, standard deviation for proficiency; VC, variation coefficient; NEE, number of erroneous elements; PEE, percentage of erroneous elements; ARE, average of relative error. Outliers were identified following ISO confidence levels

Table 2 Comparison of summary parameters between Group 1 (do not follow the Minimum recommendations) and Group 2 (do follow the Minimum recommendations)

S	Slide	Pollen type	Group 1								Group 2							
			N	*X*	*S'*	VC	NEE	PEE (%)	ARE	AAE	*N*	*X*	*S'*	VC	NEE	PEE (%)	ARE	AAE
5	VIE100412	*Betula*	18	94	21	22	4	22.2	9.3	14.8	16	90	21	23.7	2	12.5	9.7	15.2
6	VIE300512	Poaceae	18	52	11	21.5	5	27.8	14.8	11.3	16	48	6	12.5	1	6.3	3.8	4.5
Average						21.9		25	12.1	13.1				18.1		9.4	6.8	9.8

N, size of simple; *X*, assigned value; *S'*, standard deviation for proficiency; VC, variation coefficient; NEE, number of erroneous elements; PEE, percentage of erroneous elements; ARE, average of relative error; AAE, average of absolute error

calculating the Summary Parameters shown in Table 1. The average VC was 16.5 %. All slides were suitable for proficiency testing (all VC were deemed acceptable).

The average PEE was 5.9 %. The highest PEE was observed for Poaceae (sample 6; assigned value = 51) and *Betula* (sample 5; assigned value = 93).

The mean average relative error (ARE) in this study was 6.7 %, the largest ARE was observed in sample 6 (9.6 %) and the lowest average ARE in sample 3 (2.6 %).

Two groups of counters were also compared: Group 1—counters that do not follow the Minimum Recommendations when counting pollen and read less than 10 % of the slide by longitudinal (horizontal) or latitudinal (vertical) transects or by random fields; Group 2—counters that do follow the Minimum Recommendations when counting pollen and read more than 10 % of the slide by longitudinal or latitudinal transects. For comparing groups, samples 5 and 6 were used due to the fact that all participants who examined the other slides (1–4) followed the Minimum Recommendations. The results (Table 2) show that Group 1 had poorer Summary Parameters than those obtained by Group 2. Differences in VC were small (21.9 vs. 18.1), but differences in error were larger: PEE (25.0 vs. 9.4), ARE (12.1 vs. 6.8) and AAE (13.1 vs. 9.8). The results of Student's *t* test show that there were no significant differences between the two groups; $p = 0.86$ for *Betula* in VIE100412 and $p = 0.12$ for Poaceae in VIE300512. More of the counters who did not follow the Minimum Recommendations, and examined <10 % of the slide, were outside of the thresholds of Relative errors than those who did follow the Minimum Recommendations (Fig. 2).

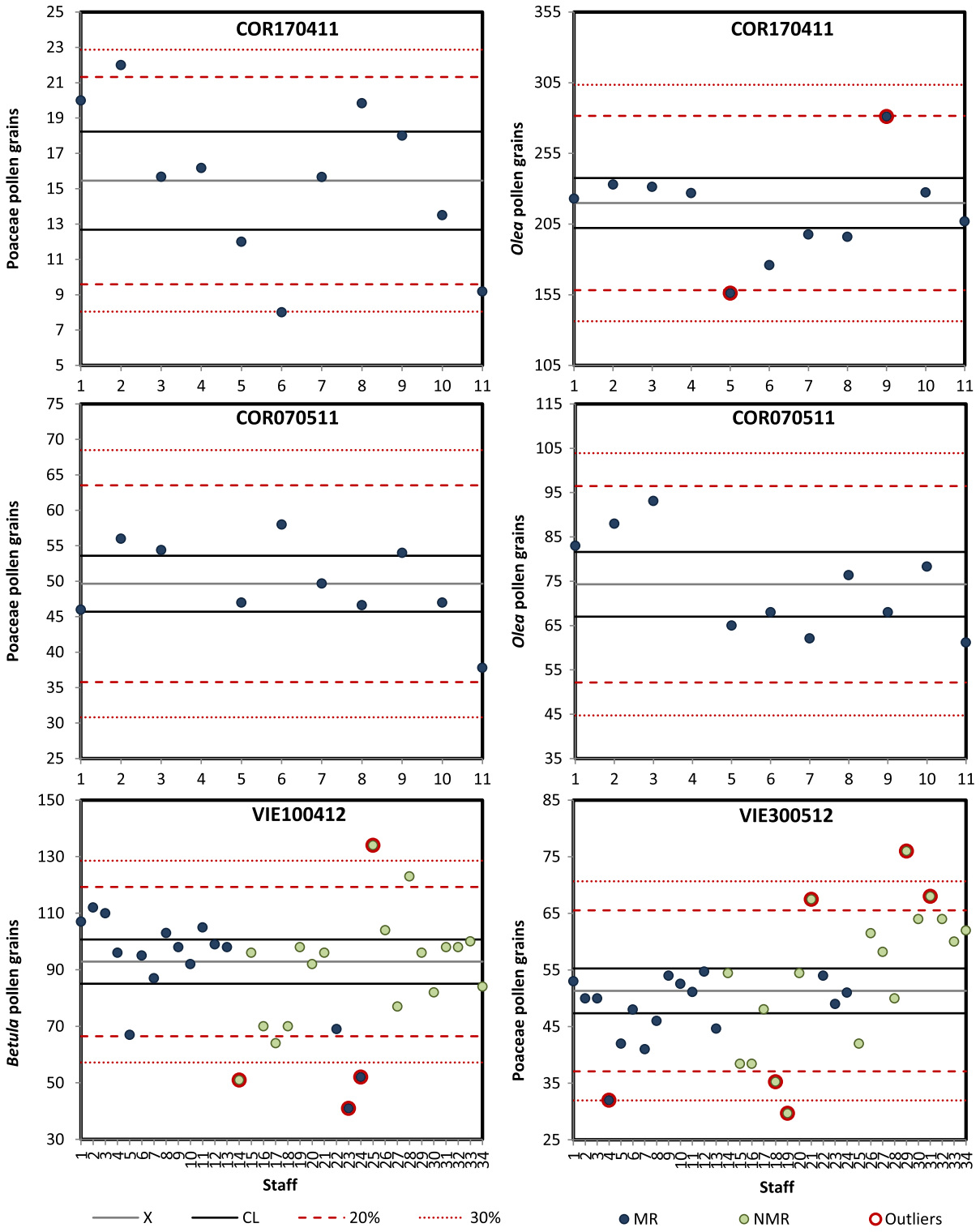

Fig. 2 Summary graphics of pollen counts. X = assigned value; CL = confidence limits; 20 and 30 % thresholds of relative errors; MR = group of pollen counters that follow Minimum Recommendations; NMR = group of pollen counters that do not follow the Minimum Recommendations

4 Discussion and conclusions

Jäger et al. (1995) published the Minimum Requirements in the methodology for *Routinely Performed Monitoring of Airborne Pollen Recommendations* for all members involved in the EAN. In the EAN, all monitoring stations use the Hirst type volumetric spore trap (Hirst 1952), and airborne pollen is expressed as a daily average of pollen grains per cubic metre of air (pollen grains/m^3). However, the results of the questionnaire study carried out by the EAS Working Group on Quality Control have highlighted some notable differences in sample preparation and analysis.

Sampling efficiency is a product of impaction efficiency and retention efficiency (Ogden et al. 1976); it is therefore important to use an efficient adhesive medium that takes into account the size of particles being sampled and remains stable in different environmental conditions (Comtois and Mandrioli 1997; Galán and Domínguez-Vilches 1997). The mounting medium must be water soluble and compatible with the adhesive in use, allowing long-term storage of the material and the option of staining (Käpylä 1989).

When examining slides, a method of sub-sampling is usually used. This is designed to reduce the amount of time required to produce the daily pollen counts. However, it also means that some precision will be lost. Such imprecision is always likely to be linked with the airborne pollen count unless aerobiologists examine the whole slide (Comtois et al. 1999; Sikoparija et al. 2011). There are three different sub-sampling methods commonly in use: longitudinal transects method (Galán et al. 2007), latitudinal transects method (Käpylä and Penttinen 1981; BAF 1995) and random field method (Mäkinen 1981). The most commonly used of these sub-sampling methods are the longitudinal (horizontal) and latitudinal (vertical) transect methods. This is because the random fields method, although probably the least time-consuming, does not allow for the estimation of valuable short-term (hourly or bi-hourly) concentrations (Käpylä and Penttinen 1981; Comtois et al. 1999).

All three counting methods have previously been shown to produce comparable results (Mäkinen 1981; Cariñanos et al. 2000). Although the area of the slide examined is likely to make a noticeable difference between counts (Comtois et al. 1999). Some studies have focused on the minimum number of transects required for reading slides (Comtois et al. 1999; Sikoparija et al. 2011). However, the size of the microscope's field of view and amount of magnification should also be considered because they affect the area of the slide examined. For this reason, it is better to define a minimum area of the slide required for counting rather than the number of transects used. Mandrioli et al. (1998) gave a general recommendation that at least 10 % of the slide should be read. This was supported by the study of Sikoparija et al. (2011) and included in the updated Minimum Requirements proposed by the EAS QC Working Group.

These updated Minimum Requirements are based on the results of a questionnaire survey of network coordinators involved in the EAN, published literature that includes protocols prepared by different national or regional pollen monitoring networks (i.e. Mandrioli and Puppi 1978; Mandrioli 1994; BAF 1995; PAACB 2003; Galán et al. 2007; Albertini et al. 2009), and the results of a Workshop (Galán 2009; EAS QC Working Group 2011). This fulfils the first goal of this paper.

When defining the sampling method, any inherent human error should be taken into consideration, namely error in pollen identification, counting and data management. Some studies have focused on proficiency for pollen identification and counting (Pedersen and Moseholm 1993; Gottardini et al. 2009; Berti et al. 2009; Oteros et al. 2013). Several national or regional aerobiological monitoring networks have introduced a quality control system to guarantee the accuracy of information (Berti et al. 2009; Oteros et al. 2013). The second goal of this paper was to carry out a pilot QC exercise of staff involved in pollen counting from various national networks. For this reason, slides containing pollen types representative of northern Europe (*Betula* and Poaceae) and Southern Europe (*Olea* and Poaceae) were sent to staff from participating networks in order to examine between analysts reproducibility.

The QC exercise was limited to three pollen types (birch, grass and olive) in order to reduce the error in the analysis caused by counters examining unknown or unusual pollen types. However, it is recognised that the pollen spectrum varies between sites in different biogeographical areas, which could still cause identification to be problematical. For instance, the spring slides donated for the QC exercise from Austria contained low amounts of *Carpinus* and *Ostrya* pollen

from the Betulaceae family that could confuse people who are not used to seeing these pollen types (e.g. they might count *Ostrya* as *Betula*). Different slide preparations can also make identification difficult for people not used to a certain technique (e.g. some sites use stain, whilst others do not). As a result, it is recommended that future QC exercises should use slides from different sites, so that participants are not continually disadvantaged.

When carrying out such external validation of counting methods, it is important to analyse both precision and accuracy indicators. Oteros et al. (2013) adapted international standardised methodologies used in other disciplines, i.e. Analytical Chemistry, when carrying out quality control of the Spanish Aerobiological Network. The present paper has included new parameters (PEE and AAE) that are considered to be better suited to explaining aerobiological data. Precision and accuracy indicators are highly dependent on sample mean, as confirmed in previous studies about accuracy (Comtois et al. 1999; Oteros et al. 2013). For this reason, error was only considered significant when RE >20 % and AE >10. Taking into account these parameters, it was noticed that technicians who followed the Minimum Recommendations and examined at least 10 % of the slide (Group 2) tended to have better indicators of precision (VC) and accuracy (PEE, ARE and AAE) than those technicians who did not follow the Minimum Recommendations.

The present paper is the result of many years of work undertaken by the EAS Working Group on Quality Control, and the efforts of the aerobiological research community in general. The proposed update of the Minimum Requirements will help to improve the quality of scientific work, particularly for those who are considering the setting up of new monitoring sites. The results of the pilot QC exercise will help to develop a methodology that can be used in future QC exercises, thereby ensuring data quality. In addition, in order to improve the accuracy of the data and to promote the practice of quality control, it is important for future quality control exercises to include other pollen types and to involve a greater number of technicians from different networks.

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Appendix

EAS QC Working Group (http://eas.polleninfo.org/): G. Frenguelli, Italy; C. Galán, Spain; R. Gehrig, Switzerland; A.M. Pessi, Finland; C. Rogers, USA; M. Saar, Estonia; M. Smith, UK/Austria; D. Zühlke, Germany.

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