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1   **Interlaboratory proficiency test in aerobiology using virtual slides – feasibility study**

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Abstract

This study examines the use of Virtual Slide Images with the aim of assessing their efficacy and usability in comparison to traditional microscopy with glass slides for the Quality Control of aerobiological samples. Three glass microscopy slides containing samples of airborne pollen were digitised. Six counters from two laboratories examined the glass slides and their data were used to calculate assigned values and acceptable coefficients of variation (CV%) for 7 pollen types. A total of 24 analysts from 12 countries examined the virtual slides using specialist OlyVIA software. Data from traditional glass and virtual slides were entered into tests for repeatability and intralaboratory reproducibility following the norm EN 16868:2019. Participants also completed a questionnaire reflecting on the efficacy and usability of Virtual Slide Images for interlaboratory Quality Control. Data from traditional glass and virtual slides were comparable but coefficients of variation were generally larger for virtual slides than glass slides. Participants who examined <10% of the slide were more likely to produce results outside the limits of the study. The use of virtual slide technology is not for everyone and, in the current study, we found that opinion was polarised but it was interesting to note that there were no differences in response based on years of experience. There are advantages and disadvantages of the two methods, and we recommend virtual slides are used as an adjunct to glass slides for use in aerobiology Quality Control and other aspects of palynological training and assessment.

Keywords: Aerobiology; Quality Assurance; Quality Control; Questionnaire; Virtual Slide Images

1. Introduction

This study was organized by the European Aerobiology Society (EAS) Working Group on Quality Control which is responsible for ensuring representativeness and reproducibility of the methods used in routine aerobiological monitoring. In addition to repeatability and intralaboratory reproducibility the norm (EN 16868:2019 (CEN 2019)) requires regular assessment of interlaboratory reproducibility and accuracy.

The methodology for interlaboratory Quality Control (QC) has been proposed and implemented in previous large scale exercises organised under the auspices of the EAS (Galán et al. 2014; Šikoparija et al. 2017). However, a common feature of the former interlaboratory QC tests was the time required for completion. The same sample is analysed by several pollen monitoring laboratories, and so the slide needs to travel around Europe until all participants have received and analysed it. This takes a great deal of time and effort (Smith et al. 2019). For example, the QC exercise for *Ambrosia* pollen took a total of 531 days from when the exercise commenced until all 69 analysts reported their results (Šikoparija et al. 2017).

One method that could significantly reduce the time taken to conduct interlaboratory QC tests, is virtual microscopy (Rocha et al. 2009). Virtual Slide Images (.vsi) are microscope slides that have been scanned (digitalized) by taking high-resolution multi focus micrographs, which are stitched together using image-processing software (Weinstein et al. 2009; Pantanowitz et al. 2011). The virtual slides can be viewed on a computer screen using specialist software to examine selected areas at high magnification (Koch et al. 2009; Rocha et al. 2009; Weinstein et al. 2009). The technique is becoming increasingly common in research, consultation, teaching, and quality control in pathology (Rocha et al. 2009; Vyas et al. 2016) and could be translated to aerobiology.

58   With this in mind, we piloted the use of Virtual Slide Images with the aim of assessing their

59   efficacy and usability in comparison to traditional microscope slides.

60

61   **2.   Materials and Methods**

62

63   This project was approved by BioSense Institute Internal Review for its use of human subjects, and

64   all data have been anonymised.

65

66   *2.1.   Materials for analysis*

67   In this study, segments of three 24-hour samples collected in Serbia were digitized (i.e. 11 March

68   2018, 10 August 2014, and 24 April 2018). Detailed digitalization of a 14x48mm sample is very

69   time consuming (about 30 h) and produces very large files (about 200 GB) we therefore decided to

70   only do this for the central part of the sample, i.e. a 5x48 mm section situated at about 5mm from

71   the edges of the tape. The z-axis was limited to 28 microns and 21 cross sections at 1.4 micron

72   spacing, which reduced the file size to about 25 GB (scanning time around 6h). For this purpose,

73   Olympus BX51 microscope with UPLSAPO 40x / 0.90 objective lens (180 micron working distance)

74   and Olympus Soft Imaging Solutions with XC10 digital camera were used.

75   This exercise did not aim to test the knowledge of participants and their ability to identify

76   different pollen types, rather it was to determine whether a range of different pollen types with

77   different morphological characteristics could be counted with a degree of reproducibility on

78   Virtual Slide Images.

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80

81 *2.2.   Assigned values*

82 In order to determine the correct values in the slides, six counters from two laboratories were

83 asked to analyse the microscope slides using the normal methods used in their laboratories.

84 Assigned values for selected pollen types were determined (Galán et al. 2014; Šikoparija et al.

85 2017) as the robust average after outliers were removed using Hampel's test (Šikoparija et al.

86 2017). Only pollen types with an assigned value more than 10 pollen/m^3 were deemed suitable for

87 further analysis (CEN 2019).

88

89 *2.3.   Virtual slides between analysts comparison*

90 A call for participation in this QC exercise was sent by the European Aerobiology Society's QC

91 Working Group to active aerobiological monitoring stations in Europe. Virtual slides were analysed

92 using the Olympus OlyVIA ver.2.9.1 build 13771 software (freely available from

93 https://www.olympus-lifescience.com/en/support/downloads/). Participants were requested to

94 analyse a minimum of 10% of the slide surface using a magnification they felt comfortable with

95 (Galán et al. 2014). The analysed surface depends on the size of the display and so participants

96 were asked to submit a screenshot of the display, after choosing the magnification they wanted to

97 use, so that the area of slide examined in pixels could be verified. A list of pollen types likely to be

98 found on each slide (not exhaustive) was supplied to counters to aid identification (Appendix 1).

99

100 *2.4.   Questionnaires*

101 Participants were asked to fill in a questionnaire reflecting on the efficacy and usability of Virtual

102 Slide Images for interlaboratory Quality Control. The questionnaire included 7 questions on a

103 Likert Scale of 1 to 7 (ranging from strongly disagree to strongly agree) and based on similar

104 studies in literature (Blake et al. 2003; Burthem et al. 2005; Koch et al. 2009; Evered & Dudding

105 2011; Hanna et al. 2019):

106 1. The Virtual Slide Images and OlyVIA software were easy to install;

107 2. The guidance and supporting material provided were sufficient for helping me prepare for the

108   QC exercise;

109 3. The OlyVIA software was easy to use;

110 4. The manoeuvrable images studied with the OlyVIA software were of sufficient resolution to

111   allow identification of pollen;

112 5. The ability to conduct the laboratory exercise on my own schedule with the computer

113   technology was an advantage;

114 6. Navigating the images with the computer and OlyVIA software was easier than that of glass

115   slides with a microscope;

116 7. The computer technology saved me time compared to using light microscopy.

117   There were also questions about gender and the number of years of experience counting pollen

118 (< 5 years, 5-10 years and >10 years). In addition, the questionnaire included two open ended

119 questions where participants could say what they liked most about using Virtual Slide Images and

120 their suggestions for improving the system.

121

122 *2.5.   Data analysis*

123 The results from the analyses of Virtual Slide Images were examined in relation to coefficients of

124 variation (CV%) as described in EN 16868:2019 (CEN 2019) and z-scores as presented in previous

125 QC studies of aerobiological data (Galán et al. 2014; Šikoparija et al. 2017). Acceptable coefficients

126 of variation were calculated based on the assigned values determined from the analysis of

127 microscope slides (CEN 2019). Questionnaire data were analysed using the non-parametric

128 Kruskal–Wallis one-way analysis of variance to determine if there were significant differences

129 between responses based on the number of years of experience counting pollen. Results were

130 deemed significant with a *p*-value < 0.05. The analysis packages used were Microsoft® Excel for

131 Mac Version 16.32 and SPSS 26.

132

133 **3.   Results**

134

135 *3.1.   Assigned values and tests for repeatability and intralaboratory reproducibility*

136 Six counters from two laboratories: Laboratory for palynology University of Novi Sad Faculty of

137 Sciences, Serbia (Lab A) and Belgian Institute for Health, Sciensano (Lab B), examined microscope

138 Slide 1 (11-03-2018) and microscope Slide 2 (10-08-2019). Following analysis of the microscope

139 slides, assigned values were determined for *Alnus*, *Ambrosia*, *Artemisia*, *Corylus*,

140 Cupressaceae/Taxaceae, Poaceae and Urticaceae. The acceptable coefficients of variation (CV%)

141 were calculated for each pollen type. One daily average Urticaceae pollen concentration from Lab

142 A was deemed to be an outlier following the Hampel test and was removed from the analysis and

143 not used for calculating the assigned value (Table I).

144   Repeatability was tested using data from the glass slides for one counter from Lab A as

145 defined in the norm EN 16868:2019 (Section 8.4.2)(CEN 2019): one slide; same analyst; same

146 method; minimum three replicates per analyst. All results were within the acceptable coefficients

147 of variation for each pollen type (Table I).

148   Intralaboratory reproducibility was examined using data from the glass slides for Lab A, Lab

149 B and all laboratory staff together following EN 16868:2019 (Section 8.4.3), with the same

150   acceptable coefficients of variation used as those for repeatability (CEN 2019). The majority of

151   results were within the acceptable coefficients of variation for each pollen type. There was one

152   result for Cupressaceae/Taxaceae that was outside acceptable limits from Lab B (CV% of 34). As

153   previously mentioned, one participant from Lab A also reported an anomalously low Urticaceae

154   pollen concentration and this was removed as an outlier (before it was removed the CV% for Lab A

155   and all labs together was > 10) (Table I).

156   Following the norm (CEN 2019), only pollen types with an assigned value more than 10

157   pollen/m^3 were deemed suitable for further analysis. Poaceae had an assigned value of 9

158   pollen/m^3 and as a result should not have been examined further, but it is interesting to note that

159   the CV% was greater than 30 for all tests of repeatability and intralaboratory reproducibility for

160   this pollen type (Table I).

161

162   *3.2.   Virtual slides between analysts comparison*

163   A total of 24 analysts (Appendix 2) participated in the study from 12 countries (Belgium, France,

164   Germany, Italy, Lithuania, Netherlands, Portugal, Serbia, Slovakia, Spain, Switzerland, Turkey). We

165   initially gave participants 2 months to submit their results, but this deadline was extended for an

166   additional month because of a number of delays. The first information was received within one

167   month of commencing the exercise. Most data were delivered during the third month of the

168   exercise after the deadline was extended.

169   Data from Virtual Slide Images were compared to the assigned values and thresholds for

170   acceptable coefficients of variation (CV%) calculated from examining microscope slides. Results of

171   the analyses are shown in Figures 1−7, both for acceptable coefficients of variation (CV%) as used

172   in tests for repeatability and intralaboratory reproducibility following the norm EN 16868:2019

173   (CEN 2019) and z-scores as described in previous exercises carried out by the European

174   Aerobiology Society Working Group on Quality Control (Galán et al. 2014; Šikoparija et al. 2017).

175   Four of the counters who analysed the microscope slides also analysed virtual slides (QC1,

176   QC8, QC9, and QC10). The change to virtual slides did not noticeably affect their performance, as

177   all of their results were within the limits of CV% for pollen types with an assigned value >10

178   pollen/m^3. On the whole, however, coefficients of variation were generally larger for virtual slides

179   than glass slides.

180   A number of results for each pollen type exceeded the CV thresholds, and from the data it

181   was possible to identify several potential errors in identification. For example, participant QC15

182   appeared to have mis-identified a pollen type as being *Artemisia* (Fig. 3), participant QC16 seemed

183   to count the majority of *Corylus* as *Betula* (Fig. 4) and participants QC6 and QC18 counted

184   Urticaceae as Cannabaceae (Fig. 7).

185   The area of the virtual slides analysed by participants had a notable impact on the results.

186   Better results were achieved if a larger area of the slide was analysed. Four counters (QC1, QC2,

187   QC3 and QC4) analysed the whole of the virtual slide surface (100% of the virtual slide and ~36%

188   of the original glass slide) and generally recorded low CV%. A total of 8 out of the 24 participants

189   (33%) examined <10% of the surface of the slides (<10% of the whole slide, not just the virtual

190   slide). It was observed that 24 results exceeded the limits of the study and 12 of these (50%) were

191   from participants who examined <10% of the slide (Figs 1−7).

192

193   *3.3.   User opinion*

194   All 24 participants responded to the questionnaire survey. Seventeen respondents were female

195   and 7 were male. Seven respondents had less than 5 years' experience counting pollen, 5

196   respondents had between 5 and 10 years' experience and 11 had more than 10-years' experience

197 counting pollen (1 respondent did not answer this question). The results of questionnaire (Fig. 8)

198 show that the majority (>70%) of respondents agreed that the Virtual Slide Images and OlyVIA

199 software were easy to install, the guidance and supporting material provided were sufficient for

200 helping them prepare for the QC exercise, the OlyVIA software was easy to use and having the

201 ability to conduct the laboratory exercise on their own schedule with the computer technology

202 was an advantage. Responses were particularly positive about the guidance and supporting

203 material (87.5% agreed). Less people agreed that the manoeuvrable images studied with the

204 OlyVIA software were of sufficient resolution to allow identification of pollen (62.5%). There were

205 fewer positive responses (<30%) when people were asked about the ease of navigating the images

206 with the computer and OlyVIA software and whether the computer technology saved them time

207 compared to using light microscopy. The results of the Kruskal Wallis test showed there were no

208 significant differences in responses based on years of experience.

209 When participants were asked what they liked most about using Virtual Slide Images, over

210 a third mentioned advantages of using the computer rather than a microscope. These included the

211 possibility of more than one person being able view the same image at the same time, the large

212 field of view, ease of handling and that there was no time pressure and they were able to perform

213 the analysis anywhere at any time. In addition, several respondents recognised that virtual slides

214 could potentially save time compared to a traditional QC exercise and highlighted the fact that

215 virtual slides could not be damaged and can be permanently stored.

216 On the other hand, three respondents had nothing positive to say about the Virtual Slide

217 Images (they responded to both open ended questions, but all their responses were negative).

218 Suggestions for improving the use of Virtual Slide Images were primarily concerned with the focus

219 (z-axis) and the manoeuvrability of the slide image (x- and y-axis). Comments largely supported

220 the answers to the Likert scale questions, with several participants complaining the virtual slides

221   took longer to analyse (one respondent saying that one slide had taken all day) and that it was not

222   as ergonomically comfortable as sitting at a microscope.

223

224   3.4 Technical difficulties

225   Slide 3 (24-04-2019) was removed from the study because of reported problems in the way the

226   images were stitched together in the virtual slide and because some participants complained that

227   analysis was rather complicated due to the fact that not all pollen types on Slide 3 were

228   frequently encountered in all parts of Europe (Appendix 1). A common complaint was that the

229   slide images were too large and took too long to download. Approximately one third of

230   participants reported problems with focussing, including blurry images.

231

232   **4.   Discussion**

233

234   The digital capture of glass slide preparations to produce Virtual Slide Images is still a relatively

235   new technology (Evered & Dudding 2011). Although digital slides are increasingly being employed

236   in medicine for the teaching and assessment of histology and pathology (Vyas et al. 2016) and can

237   also be used for training, intralaboratory quality control, interlaboratory quality assurance and

238   image analysis (Rocha et al. 2009; Evered & Dudding 2011). Digital images are not expensive to

239   duplicate, they do not deteriorate, break or disappear, they are easy to store and are available to

240   multiple users simultaneously (Koch et al. 2009; Pantanowitz et al. 2011).

241   Light microscopy using traditional glass slides, on the other hand, is the established tool in

242   aerobiology (Oteros et al. 2015) and can be considered the gold standard for the analysis of

243   aerobiological samples. There are certain advantages to traditional microscopy, as analysts are

244 familiar with the equipment and have full control of XYZ stages, and glass slides are cheap to

245 prepare (Evered & Dudding 2011). However, glass slides are easily broken (Evered & Dudding

246 2011) which is a potential problem when conducting large scale interlaboratory QC exercises

247 where samples are sent to multiple sites (Galán et al. 2014; Šikoparija et al. 2017).

248

249 *4.1   Virtual slides between analysts comparison*

250 In this investigation, glass slide microscopy was considered to be the gold standard as described

251 for previous studies related to dermatitis and pathology (Koch et al. 2009; Vyas et al. 2016). The

252 results from the glass slides produced by one analyst were examined for repeatability and all data

253 from counters who used traditional microscopy were included in tests for intralaboratory

254 reproducibility following the norm EN 16868:2019 (CEN 2019). In addition, a total of 24 analysts

255 participated in between analyst comparisons using Virtual Slide Images. It was found that data

256 from traditional glass and virtual slides were comparable but coefficients of variation were

257 generally larger for virtual slides than glass slides. Some of this variation could be attributed to

258 reported problems with focusing the OlyVIA software and analysts coming into contact with pollen

259 types they were not normally accustomed to seeing. However, the results allowed identification of

260 several possible errors in identification, thereby highlighting the potential for the system to be

261 used in training and Quality Control.

262   It was noticeable that 33% of participants looked at less than 10% of the slide but made up

263 50% of results that were outside of the limits of the study. This is not particularly surprising as a

264 number of studies have now highlighted the fact that the area of the slide examined has a

265 significant impact on the quality of the data produced (Galán et al. 2014; Šikoparija et al. 2017;

266 Smith et al. 2019). This is further evidence that networks should follow the recommendations that

267 analysts should examine at least 10% of the slide surface (Mandrioli et al. 1998; Šikoparija et al.

268 2011; Galán et al. 2014). In addition, Poaceae had an assigned value of <10 pollen/m^3 and a CV%

269 greater than 30 for all tests of repeatability and intralaboratory reproducibility, thereby

270 highlighting the importance of selecting pollen types that are present on the slides in sufficient

271 numbers (Šikoparija et al. 2017; Smith et al. 2019).

272

273 *4.2 User opinion*

274 Participants were invited to give their opinion on the efficacy and usability of digital slides. On a

275 positive note, participants were satisfied with the amount of guidance and supporting material

276 provided, and generally agreed that the virtual slides and OlyVIA software were easy to install and

277 use. However, it is known that one disadvantage of digital microscopy is the large amount of

278 digital storage space needed for image data (Vyas et al. 2016). Indeed, several participants

279 commented that they experienced problems when downloading the slides because of their size.

280 Results of the questionnaire survey showed that many participants also liked the fact that

281 they could conduct the exercise in their own time. Moreover, participants mentioned the benefit

282 of being able to examine and discuss the slides with colleagues. This highlights the potential of

283 using Virtual Slide Images as a training tool.

284 The survey did, however, identify some issues with the usability of the system and

285 respondents were not impressed by the ability of the OlyVIA software to navigate around the

286 slides and did not think the virtual slides saved time compared to traditional glass slides. This is in

287 agreement with a previous study conducted by Vyas et al. (2016) who compared whole slide

288 digital images and traditional glass slides in the detection of common microscopic features seen in

289 dermatitis. The authors observed the efficiency of using glass slides was superior to digital slides,

290 and that glass slides were generally read faster (Vyas et al. 2016). Hanna et al. (2019) also

291 witnessed a 19% decrease in efficiency (increase in turnaround time) using digital pathology slides.

292   It should be remembered, however, that virtual slides are not meant to test all microscope

293   skills as field selection and focussing with virtual slides has been likened to operating a camera

294   (Burthem et al. 2005). With this in mind, it is important to note that more than 60% of participants

295   in the current study agreed there was sufficient resolution to allow identification of pollen.

296   Similarly, Blake et al. (2003) described the successful change from using traditional microscopes

297   and glass slides to using virtual slides. In their study the authors reported that, when asked, the

298   vast majority of medical students on the histology course they delivered rated digital images as

299   having excellent resolution (Blake et al. 2003).

300

301   *4.3.   Evaluation*

302   Rocha et al. (2009) defined digital slide quality by the following factors: (A) Quality - condition of

303   the original slide; (B) Completeness - the slide should be accessible in its entirety; (C) Image quality

304   - attributes of the digital slide (e.g. sharpness, contrast, colour) should be comparable to those of a

305   real microscope; (D) Usability – such as smooth scrolling and magnification options.

306   The quality of the scanned slide is important (Rocha et al. 2009) but so is the spectrum of

307   pollen types present and previous QC exercises have focused on only a few regionally important

308   allergenic pollen (Galán et al. 2014; Šikoparija et al. 2017). It was clear that a number of

309   participants struggled with the sample on Slide 3 collected in Serbia during the Spring of 2018,

310   which contained pollen types with similar morphological characteristics such as *Broussonetia,*

311   *Celtis*, *Morus*, and Urticaceae (Appendix 1). These pollen types are not commonly encountered in

312   large numbers in all parts of Europe, and this contributed to Slide 3 being omitted from the final

313   analysis. It should also be remembered that different aerobiological laboratories use different

314   methods, which include a variety of staining agents that result in pollen of different hues (or no

315   stain at all) and a range of adhesives that can make slides look different. Indeed, one participant

14

316 did mention that the colouration of the slides made it difficult to identify the pollen. Analysts

317 become accustomed to the techniques used in their own labs and this needs to be considered in

318 interlaboratory QC tests and compromises made. However, there should also be recognition that

319 you cannot please everyone all the time.

320   In an attempt to reduce the size of the virtual image, only part of the exposed portion of

321 the slide (5x48mm) was digitised. This is, however, the area typically examined during routine

322 monitoring using longitudinal transects (e.g. Galán et al. (2007)). Assigned values were calculated

323 using the data from the glass slides. This allowed us to compare counts made from traditional

324 glass slides with those from and Virtual Slide Images, although the results show there was more

325 variation in the data from virtual slides than traditional glass slides.

326   The quality of all images is extremely important when using virtual slides as a testing tool

327 (Koch et al. 2009). The high-resolution multi focus micrographs used in this study were generally of

328 sufficient resolution for the identification of pollen, but there were limitations and participants

329 often requested improvements in this regard. Problems related to the stitching together of the

330 images also caused Slide 3 to be omitted from the study.

331   The usability of the current system is also rather limited, and the ultimate goal would be

332 for technology that can rapidly upload images, proficiently focus, and effortlessly navigate across

333 virtual slides in the same way as operators do with glass slides (Koch et al. 2009). In order to make

334 the files used in this study acceptable for online transfer, the size of the files had to be reduced

335 and the image spacing of the z-stack restricted (i.e. 28 microns with 20 layers at 1.4 micron step).

336 The results of our study indicate that, for more precise identification of pollen where fine

337 morphological features need to be seen, a thicker z-stack with finer step must be used. This is

338 particularly important in melissopalynology. As a result, much larger files would need to be

339 produced for use in quality control following the norm DIN 10760: 2002 (DIN 2002) for the

15

340   determination of the relative frequency of pollen in the analysis of honey. For smooth online views

341   of virtual slide images the application of a client-server-based data management system such as

342   the Net Image Server SQL would be needed ([https://www.olympus-](https://www.olympus-lifescience.com/en/microscopes/virtual/vs120/net-image-server-sql/)

343   [lifescience.com/en/microscopes/virtual/vs120/net-image-server-sql/](https://www.olympus-lifescience.com/en/microscopes/virtual/vs120/net-image-server-sql/)).

344   The use of virtual slides as a tool for quality assurance programmes has certain advantages,

345   not least the ability to distribute identical images from a single original slide to multiple users at

346   different sites thereby avoiding the problems and costs related to sending slides between

347   laboratories by post (Burthem et al. 2005; Rocha et al. 2009). Such exercises can customarily take

348   months to complete, as shown in aerobiology (Šikoparija et al. 2017) and other disciplines such as

349   pathology (Rocha et al. 2009). Whereas, in this study, all data were returned within three months.

350   It is important that participants in proficiency tests examine the same material, and there is

351   potential for the use of digital slides in quality control programmes and for measuring accuracy

352   (Rocha et al. 2009). For instance, in a pilot study assessing the use of virtual slides in

353   haematological quality assessment, Burthem et al. (2005) reported comparable results from both

354   glass and digital slides. As a result, the authors recommended that digital virtual slides could be

355   used as a supplementary resource to glass slides in educational aspects of haematological

356   morphology and external quality assessment (Burthem et al. 2005). However, the use of virtual

357   slide technology is not for everyone and, in the current study, we have found that opinion was

358   polarised but it was interesting to note that there were no differences in response based on years

359   of experience. Both traditional glass and virtual slides test common skills such as identification,

360   and it is recognised there are advantages and disadvantages of the two (Burthem et al. 2005; Koch

361   et al. 2009). We therefore recommend that, as with the study by Burthem et al. (2005), virtual

362   slides are used as an adjunct to glass slides for use in aerobiology quality control and other aspects

363   of palynological training and assessment.

371

Figure Legends

373

Figure 1. Results between analyst comparison using virtual slides for *Alnus*: (A) Acceptable

coefficients of variation(CV%) as used for repeatability and intralaboratory reproducibility

following the norm EN 16868:2019; (B) z-scores as described in previous exercises carried out by

the European Aerobiology Society Working Group on Quality Control (Galán et al. 2014; Sikoparija

et al. 2017). Results from participants who examined <10% of the digital slide marked in bold.

379

Figure 2. Results between analyst comparison using virtual slides for *Ambrosia*: (A) Acceptable

coefficients of variation(CV%) as used for repeatability and intralaboratory reproducibility

following the norm EN 16868:2019; (B) z-scores as described in previous exercises carried out by

the European Aerobiology Society Working Group on Quality Control (Galán et al. 2014; Sikoparija

et al. 2017). Results from participants who examined <10% of the digital slide marked in bold.

385

Figure 3. Results between analyst comparison using virtual slides for *Artemisia*: (A) Acceptable

coefficients of variation(CV%) as used for repeatability and intralaboratory reproducibility

following the norm EN 16868:2019; (B) z-scores as described in previous exercises carried out by

17

389   the European Aerobiology Society Working Group on Quality Control (Galán et al. 2014; Sikoparija

390   et al. 2017). Results from participants who examined <10% of the digital slide marked in bold.

391

392   Figure 4. Results between analyst comparison using virtual slides for *Corylus*: (A) Acceptable

393   coefficients of variation(CV%) as used for repeatability and intralaboratory reproducibility

394   following the norm EN 16868:2019; (B) z-scores as described in previous exercises carried out by

395   the European Aerobiology Society Working Group on Quality Control (Galán et al. 2014; Sikoparija

396   et al. 2017). Results from participants who examined <10% of the digital slide marked in bold.

397

398   Figure 5. Results between analyst comparison using virtual slides for Cupressaceae/Taxaceae: (A)

399   Acceptable coefficients of variation (CV%) as used for repeatability and intralaboratory

400   reproducibility following the norm EN 16868:2019; (B) z-scores as described in previous exercises

401   carried out by the European Aerobiology Society Working Group on Quality Control (Galán et al.

402   2014; Sikoparija et al. 2017). Results from participants who examined <10% of the digital slide

403   marked in bold.

404

405   Figure 6. Results between analyst comparison using virtual slides for Poaceae: (A) Acceptable

406   coefficients of variation (CV%) as used for repeatability and intralaboratory reproducibility

407   following the norm EN 16868:2019; (B) z-scores as described in previous exercises carried out by

408   the European Aerobiology Society Working Group on Quality Control (Galán et al. 2014; Sikoparija

409   et al. 2017). Results from participants who examined <10% of the digital slide marked in bold.

410

411   Figure 7. Results between analyst comparison using virtual slides for Urticaceae: (A) Acceptable

412   coefficients of variation (CV%) as used for repeatability and intralaboratory reproducibility

413   following the norm EN 16868:2019; (B) z-scores as described in previous exercises carried out by

414   the European Aerobiology Society Working Group on Quality Control (Galán et al. 2014; Sikoparija

415   et al. 2017). Results from participants who examined <10% of the digital slide marked in bold.

416

417   Figure 8. Results of the questionnaire study to participants involved in the interlaboratory

418   proficiency test using virtual slides (% responses)

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420

421

422   **References**

423

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479

Appendices

Appendix 1

The list of pollen types likely to be found on each slide (not exhaustive), which was supplied to counters to aid identification:

- Slide 1 (11-03-2018): *Alnus, Corylus, Fraxinus, Populus,* Taxaceae/Cupressaceae*, Ulmus*.

- Slide 2 (10-08-2014): *Ambrosia*, Apiaceae, *Artemisia*, Cannabaceae, Chenopodiaceae, *Plantago* Poaceae, Urticaceae, *Xanthium*.

- Slide 3 (24-04-2018): *Alnus*, Apiaceae, *Betula*, Brassicaceae, *Broussonetia, Carpinus, Celtis*, Cyperaceae, *Fagus, Fraxinus, Juglans, Morus*, Pinaceae, *Platanus,* Poaceae, *Quercus, Rumex, Salix,* Taxaceae/Cupressaceae, Urticaceae.

Appendix 2

The following counters participated in this QC exercise: Arandjelovic, A.; Bekil, S.; Bruffaerts, N.; Bucher, E.; Cislaghi, G.; de Weger, L.; Dovydaityte, D.; Kolek, F.; Graber, M-J.: Hoebeke, L.; Iannotta, M.P.; Leier-Wirtz, V.; Martínez-Bracero, M.; Navarro, D.; Oliver, G.; Pereira, C.; Pereira, J.; Plaza, M.; Radisic, P.; Ribeiro, H.; Sallin, C; Ščevková, J.; Šikoparija, B.; Trajkovska, G.; Tosunoglu, A.; Verstraeten, C.

Table I. Assigned values and results of repeatability and intralaboratory reproducibility as defined in the norm (EN 16868:2019). **SPT** (standard deviation for proficiency testing = Robust standard deviation); **Assigned value** (Assigned value (X) = robust average); **n =** Datasets after the removal of outliers (Hampel test); **1 counter CV%** = Repeatability; **Lab A CV%** = Laboratory A Intralaboratory Reproducibility; **Lab B CV%** = Laboratory B Intralaboratory Reproducibility; **All CV%** = All counter Intralaboratory Reproducibility; **Allowed CV%** = The acceptable coefficients of variation (CV)

Pollen type	SPT	Assigned value	n =	1 counter CV%	Lab A CV%	Lab B CV%	All CV%	Allowed CV%
Alnus	6.12	42	8	19	19	5	15	20
Ambrosia	9.01	83	8	12	12	3	11	20
Artemisia	2.58	23	8	3	5	4	11	30
Corylus	4.61	34	8	15	12	18	14	20
†Cup/Tax	6.26	25	8	14	22	34	25	30
Poaceae	2.81	9	8	33	33	31	32	NA
Urticaceae	18.61	349	7	3	3*	4	5*	10

†Cupressaceae/Taxaceae

*Outliers removed (Hampel test)

Repeatability - EN 16868:2019 CV% (Section 8.4.2)

Intralaboratory Reproducibility - EN 16868:2019 CV% (Section 8.4.3)

The acceptable coefficients of variation (CV), calculated only for taxa with an assigned value > 10 EN 16868:2019 CV% (Section 8.4.2).
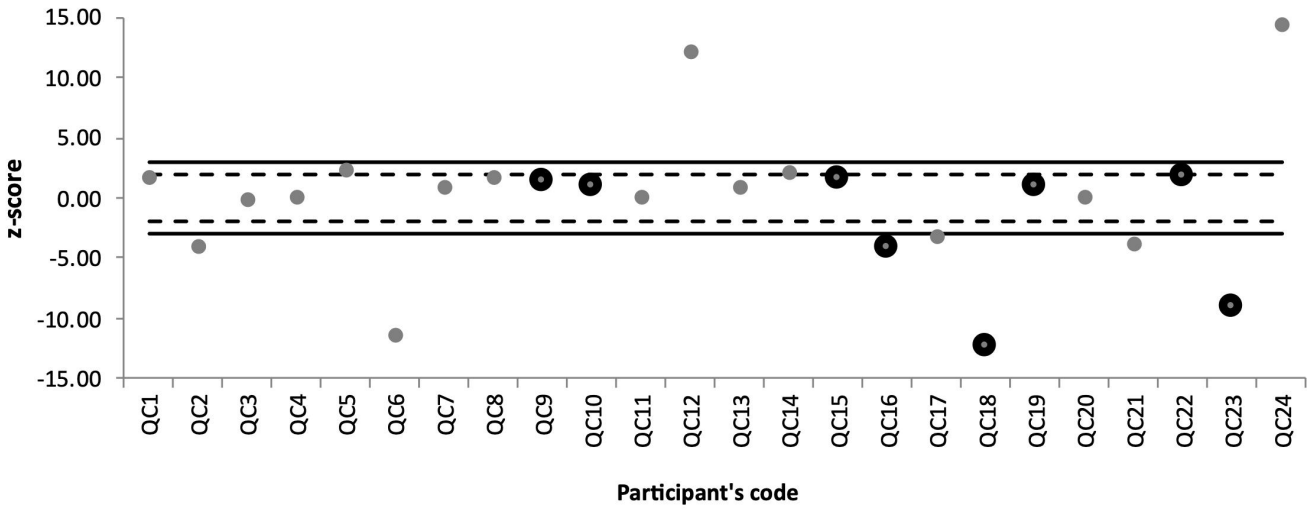
Poaceae

assigned value = 9 Pollen/m^3
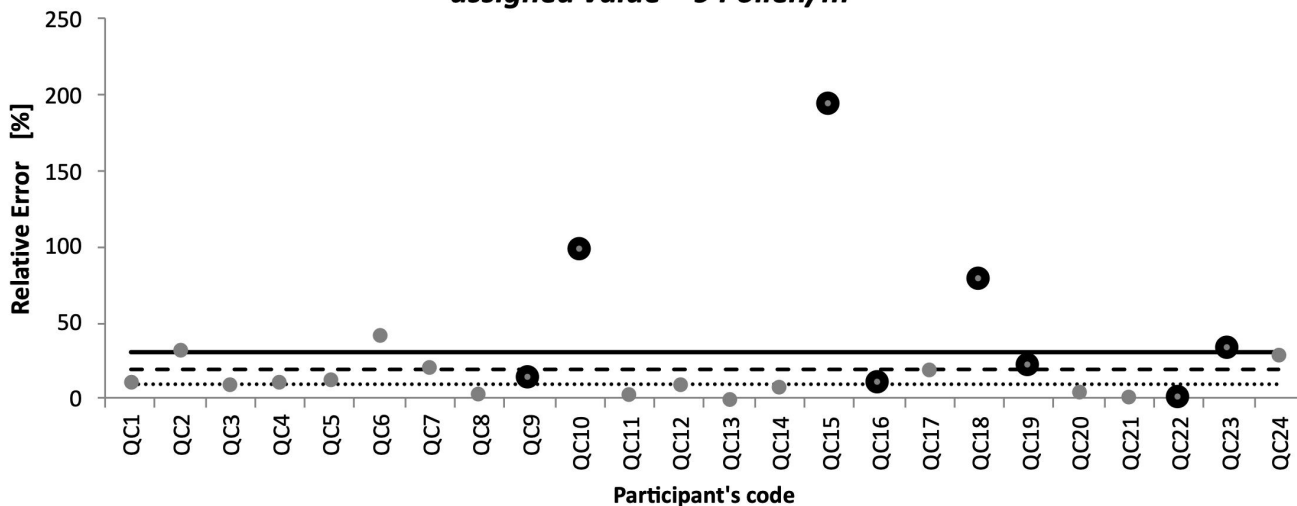
Poaceae

assigned value = 9 Pollen/m^3
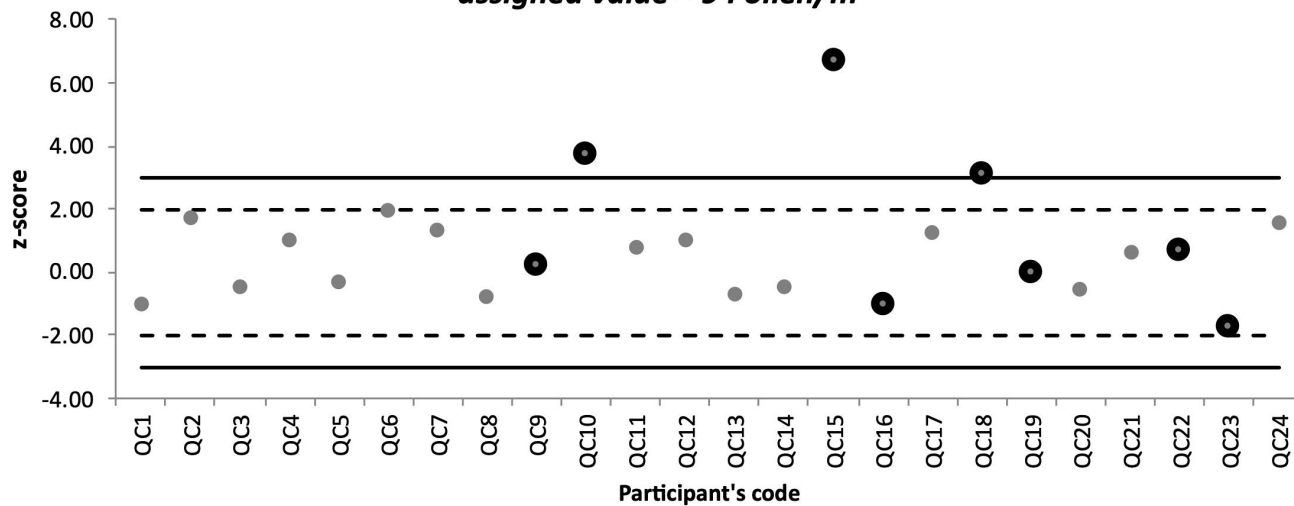
Urticaceae
assigned value = 349 Pollen/m^3
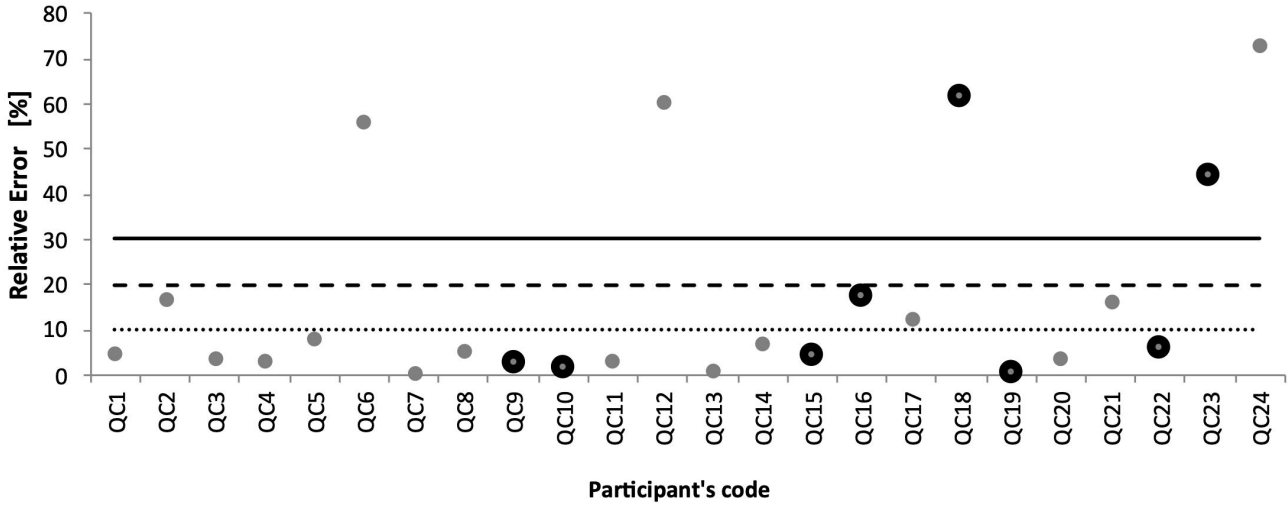
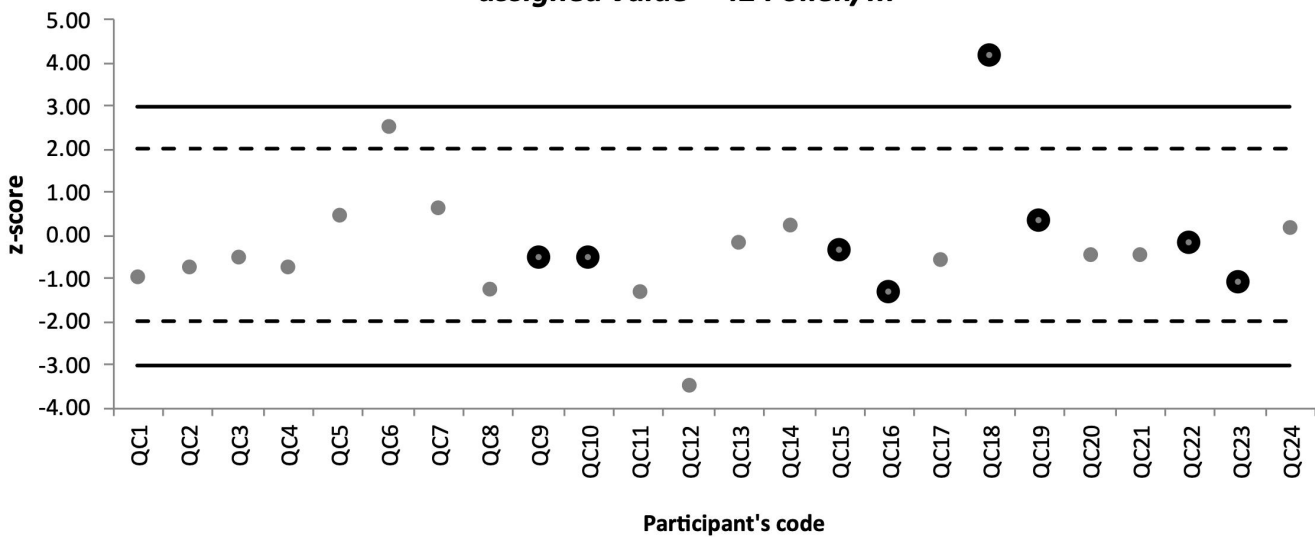

Urticaceae
assigned value = 349 Pollen/m^3
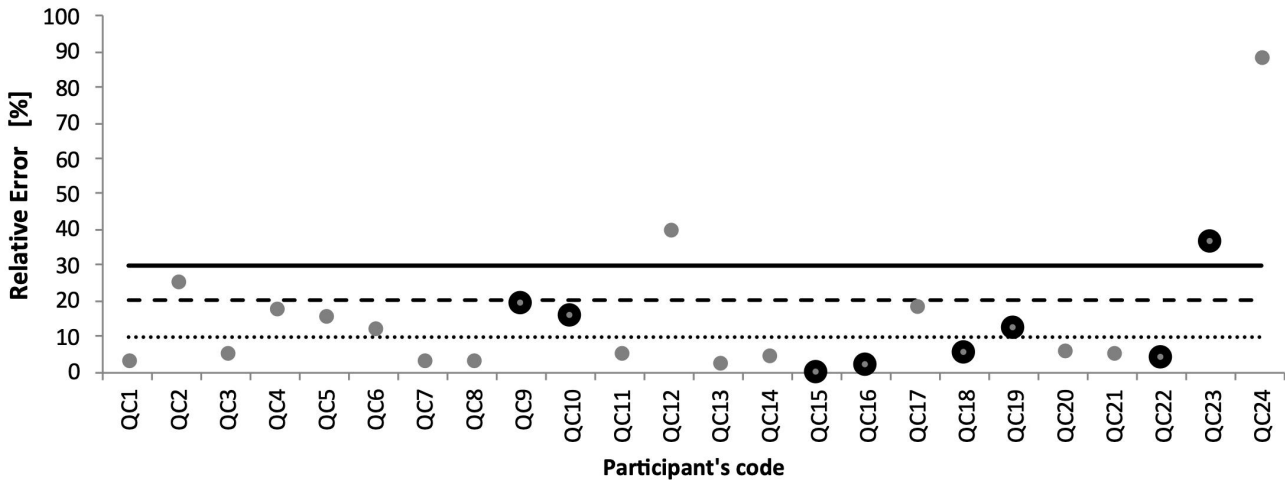
Alnus
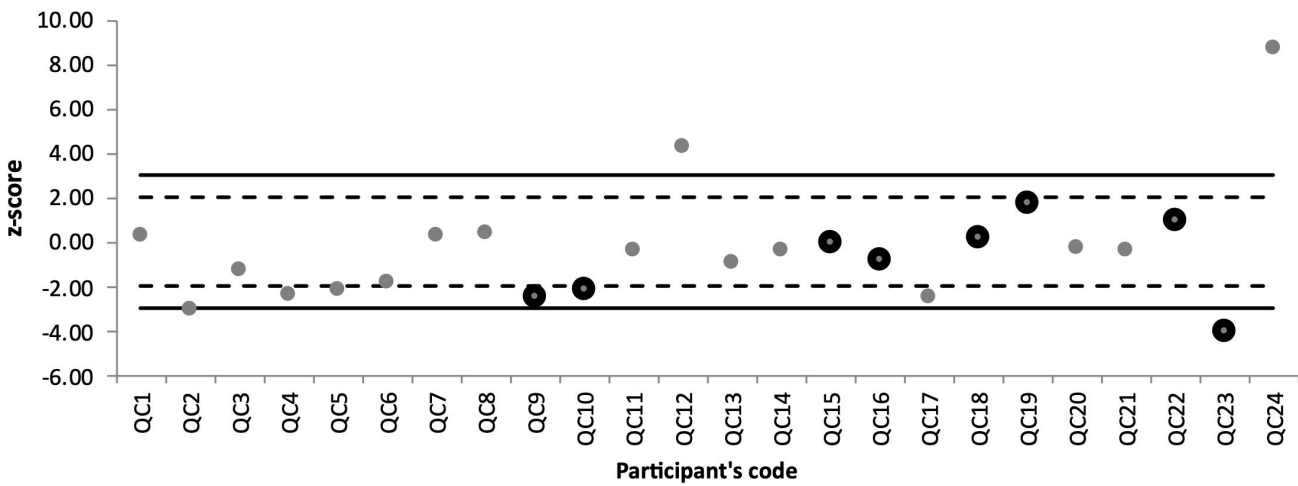**assigned value = 42 Pollen/m³**
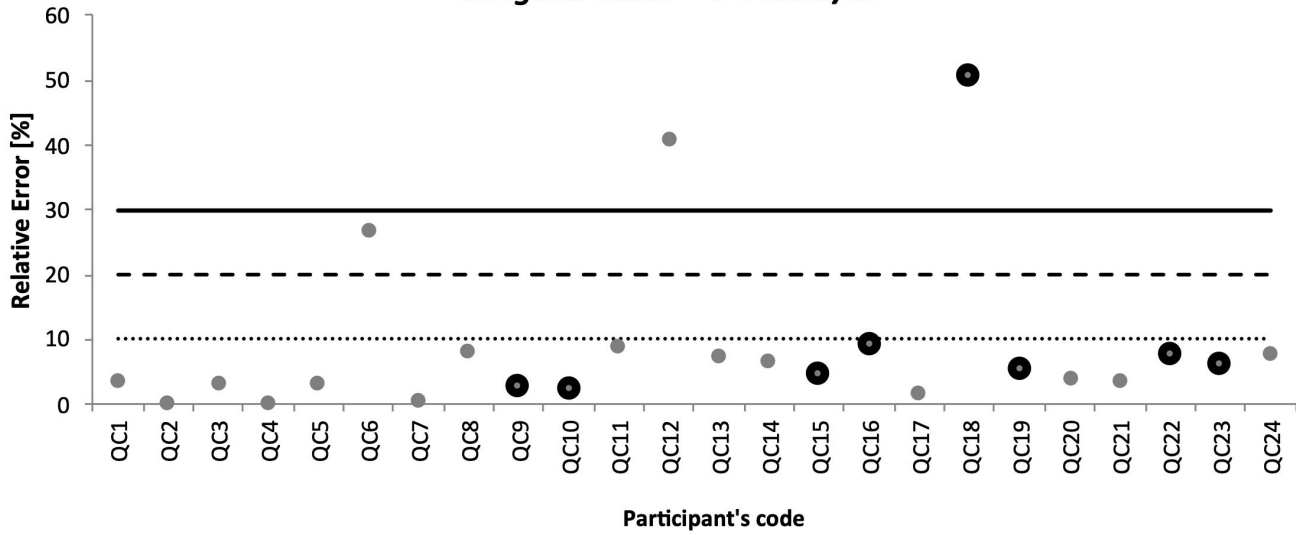
Alnus
assigned value = 42 Pollen/m³
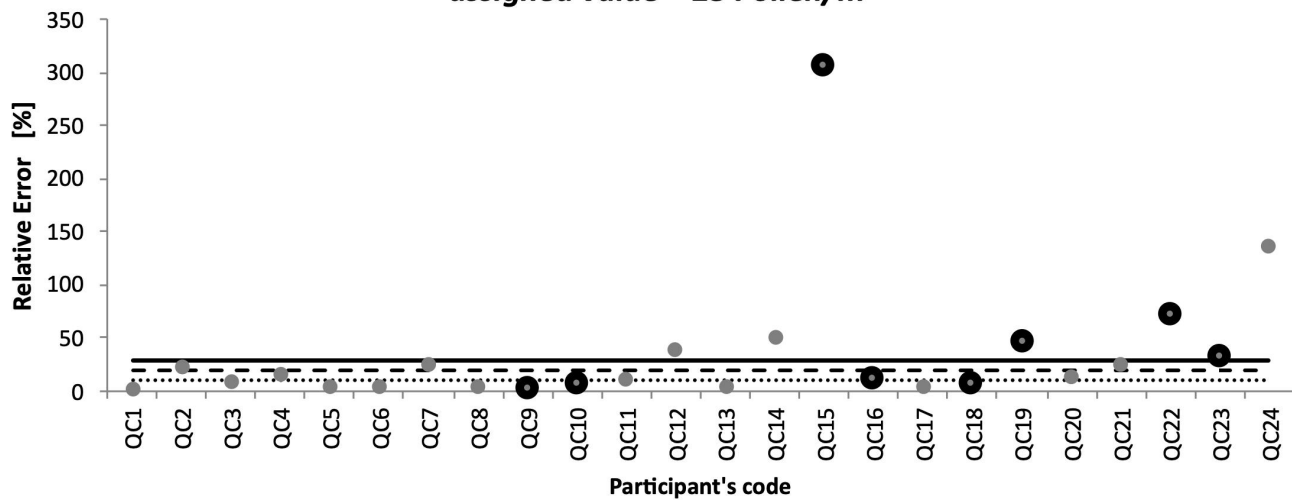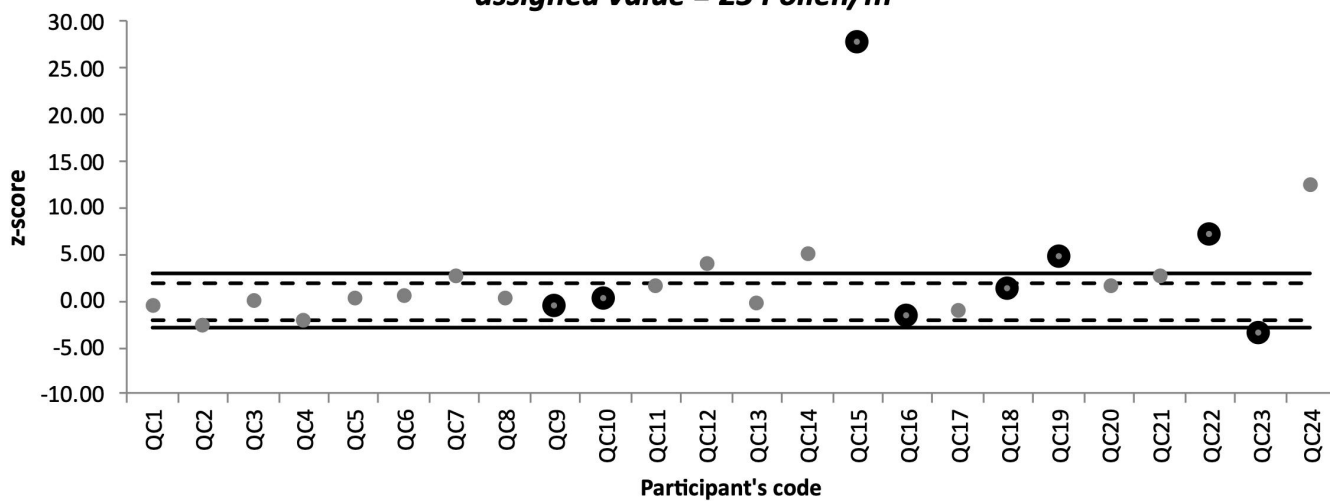
Ambrosia

assigned value = 83 Pollen/m³

Ambrosia

assigned value = 83 Pollen/m³

Artemisia
assigned value = 23 Pollen/m³

Relative Error [%]

Participant's code

Artemisia
assigned value = 23 Pollen/m³

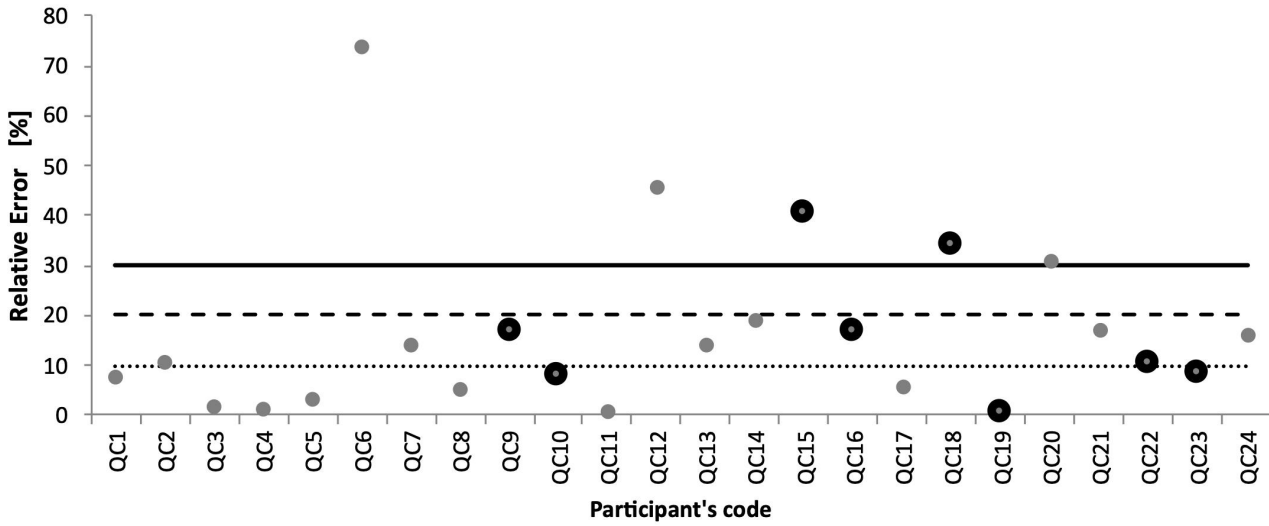z-score

Participant's code

Corylus
assigned value = 34 Pollen/m³
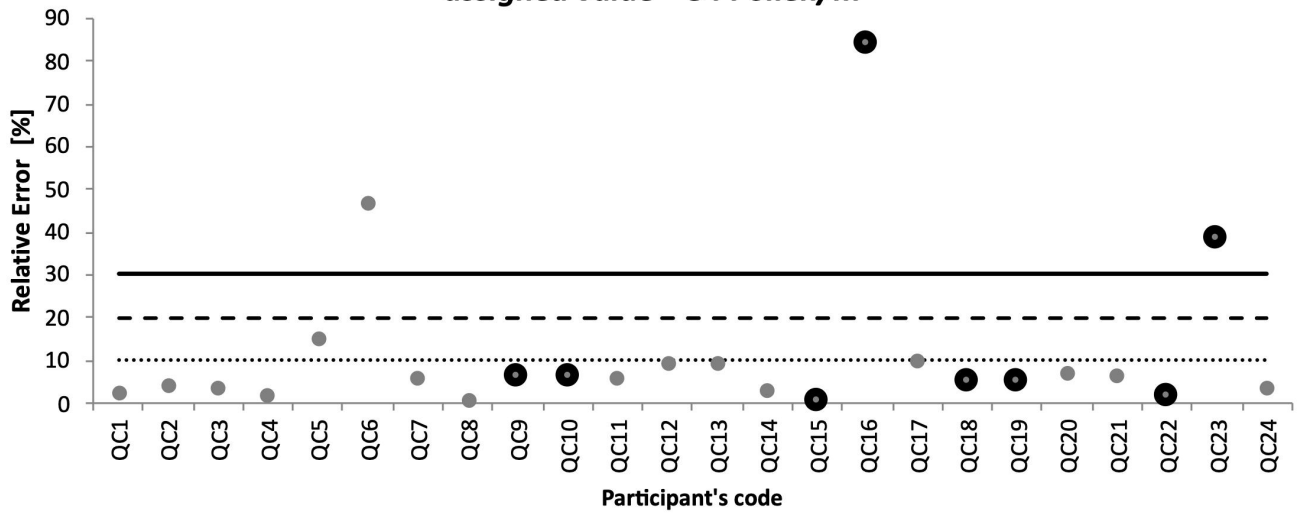
Corylus
assigned value = 34 Pollen/m³
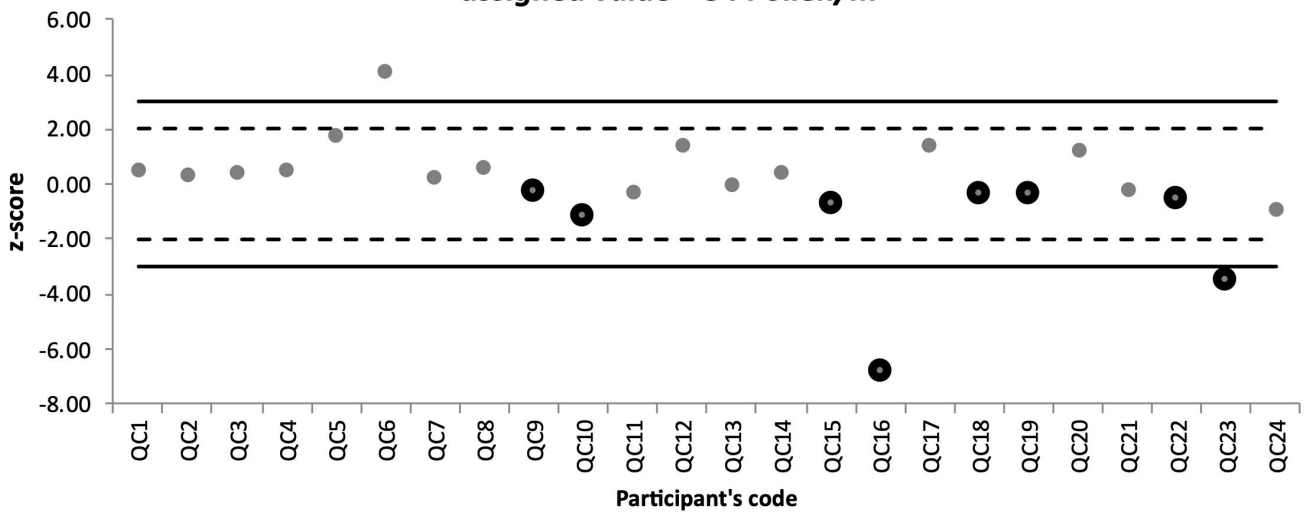
Cupressaceae/Taxaceae
assigned value = 25 Pollen/m^3

Cupressaceae/Taxaceae
assigned value = 25 Pollen/m^3