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Atmospheric Measurement Techniques Discussions

Interactive comment on "Autofluorescence of atmospheric bioaerosols – spectral fingerprints and taxonomic trends of native pollen" *by* C. Pöhlker et al.

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We thank referee #2 for her/his positive review and recommendation that "the study is most definitely worthy of publication in AMT". The comments by referee #2 have been very constructive to improve several sections and statements in our study. The comments and our answers are listed below. An updated version of Fig. 7 is shown here. Further, the new Figures S3, S5, and S7 have been prepared for the responses to referee #1 and #2 and are meant to be shown in the revised supplement file. In the following responses, the numbers of figures refer to the final numbers in the revised manuscript.

[2.1] Referee comment: I am unsure about the use of "native" in the context used here. Native implies pollen specific to a particular region however the pollen used here are observed throughout the world. I understand the authors wish to designate the pollen as chemically and physically unaltered which is an important issue. However another description maybe more appropriate.

Author response: We agree with the referee that the word "native" in its meaning of "indigenous origin or growth" may be misleading in the context of our study. Therefore, we replaced "native" by "natural" or "untreated" throughout the text to clarify that the pollen samples have not modified chemically and physically. In the title of the study, the word "native" has been omitted.

[2.2] Referee comment: p 5695 Line 6-9 Add "virus" to the list of PBAP mentioned. They are really the main PBAP types that are a few nanometers in size.

Author response: Viruses as typical PBAP in the nanometer size range have been added to the list:

"The major constituents of PBAP are microorganisms (e.g., bacteria and algae), reproductive units (e.g., pollen, fungal spores, bacterial spores and viruses), as well as fragments and excretions of various organisms (e.g., plant debris and bacterial vesicles) spanning a wide size range from a few nanometers to hundreds of micrometers (e.g., Kuehn and Kesty, 2005; Elbert et al., 2007; Burrows et al., 2009)."

[2.3] Referee comment: "eg". has been placed in front of some of the references throughout the text. Is there a reason for these selections?

Author response: The abbreviation "e.g." has been placed in front of some references to explicitly point out that in these cases only a selection of papers out of a larger number of relevant studies is cited.

[2.4] Referee comment: The author makes reference to the WIBS instrument however only the parameters of the WIBS-3 are discussed and shown in the diagrams. The

newer version WIBS-4 has important variations in the fluorescent wavelengths used for detection. Such differences should be referred to here.

Author response: All WIBS-related statements throughout the manuscript (text, figures, figure captions) have been changes to the newer and improved version WIBS-4.

[2.5] Referee comment: The author refers to the fact that some individual pollen grains showed far higher intensity compared to adjacent pollen grains. Was there any difference in the number of these high intensity pollen grains between the freshly collected and the purchased samples? Note should be made whether or not this so.

Author response: There was no obvious difference in abundance of the highly fluorescent grains between freshly collected and purchased samples. However, we observed a trend of increasing fluorescence intensity in all grains of a certain species with aging. It is not clear yet, how the abundance of highly fluorescent grains and the overall age-related intensity increase are linked. Please refer to the detailed discussion of this aspect in our response to comment [1.2] of referee #1.

[2.6] Referee comment: Reference is made to water uptake in altering the pollen grain morphology due to grain swelling. Do the authors think that the moist environment used in this study could cause extraction from the pollen? Roshchina has previously shown that fluorescent components are present in water extracts. Were there differences in the cytosol contributions between the moist and dry pollen for instance?

Author response: The interaction of water with pollen and its impact on pollen morphology and autofluorescence is complex and therefore requires a systematic discussion in future studies. We have observed extraction phenomena in water, however, they are not discussed in the current study on dry pollen properties. All data, shown here, are based on pollen in dry state, except several fluorescence microscopy images of pollen in moist state, which have been embedded in an aqueous mounting medium (for better perceptibility of pollen microstructure). These microscopy samples in moist state are clearly labeled in the figure captions. In these cases, we performed careful microscopy

comparisons between dry and moist samples, which confirm that the qualitative appearance of the fluorescence microstructure is not changed by extraction. The details of a comparison between dry and moist pollen are subject of ongoing experiments.

[2.7] Referee comment: Reference is also made to organelle fluorescence. Did the authors observe any chloroplast organelles under the microscope? Again note should be made whether or not this is so.

Author response: Fluorescence microscopic evidence for chloroplast organelles in the pollen grains was not observed. This can be explained either by the absence of chloroplasts in pollen or by the fact that the optical filter for the red fluorescence channel (λex = 560/20 nm, λDichroic = 595 nm, λAbsorp = 630/30 nm) shows poor overlap with the emission range of chlorophyll pigments (λAbsorp = 680 nm) and thus potentially present chloroplasts are not recognized. The following statement has been added in p. 5714, l. 6:

“However, microscopic evidence for chloroplasts in pollen grains has not been observed in the current study, which can be explained either by the absence of chloroplasts or inappropriate filter settings in the fluorescence microscopy analysis.”

[2.8] Referee comment: Viability and fluorescence are very interesting aspects of PBAP fluorescence studies. While it may be true that pollen florescence increases with loss of viability, I am not convinced the same is true for fungal spores. While Wu and Warren show that fluorescence increases with decreasing viability other work such as Kanaani et al have shown the fluorescent intensity and percentage for fungal spores decrease with age. Given that fungal spores can be considered to lose viability once released, loss of viability could be linked to decreases in fluorescence.

Author response: The contradicting observations by Wu and Warren (1984a,b) and Kanaani et al. (2007) indicate that the link between viability and autofluorescence is not fully understood, yet. We follow the referee’s suggestion and add Kanaani et al. (2007) as a reference. Moreover we have modified our statement in p. 5709, l. 18 to

better represent the current state on discussion of this aspect:

"In contrast, other studies report decaying fluorescence levels with increasing age of airborne fungal spores (e.g., Kanaani et al., 2007). Ultimately, more research is needed to understand the quantitative relationship between metabolic state and autofluorescence properties of biological material."

[2.9] Referee comment: Was there much variation in the fluorescence intensities from one run to the next for the recorded EEMs for the pollen under investigation? State.

Author response: We did not observe strong variations in intensity between subsequent EEM runs for the same pollen sample. It can be stated that the measured EEMs of all pollen species represent a stable and reproducible result. The following statement was added to the manuscript (p. 5705, l. 16) to clarify this aspect:

"In subsequent EEM runs of the same pollen samples, no strong variations in the EEM appearance (i.e., intensity, spectral pattern) were observed. The obtained spectra can be regarded as a stable and reproducible result."

[2.10] Referee comment: The authors refer to the chlorophyll peak seen in some the samples originating from chloroplasts. However chloroplast paternal inheritance is very rare (although it can occur). Indeed, in the literature, pollen are generally not considered to possess chloroplasts. Might the chlorophyll signal originate from chlorophyll in a free state or bound to the cell wall or protein?

Author response: We thank the referee for this helpful comment. Our study is not intending to provide a final answer to the question, how and where chlorophyll fluorescence originates in pollen grains. In our study, we discuss paternal inheritance as one potential explanation, however, we agree with the referee that this is an rare phenomenon. Chlorophyll in free state or bound to the cell wall is another plausible explanation. We have implemented this explanation into the text (p. 5714, l. 6):

"Another explanation for the chlorophyll signal is the presence of chlorophyll molecules,

which may occur in free state or bound to the pollen cell wall."

[2.11] Referee comment: Could the small particles seen in Fig. 6 be aeroallergen/starch particles from a ruptured pollen grain?

Author response: We are confident that the small particles in Fig. 6 are not starch particles from ruptured grains for three reasons: (i) the observed particles in the C. betulus sample ($\sim$ 4-6 μm) are substantially larger than cytosolic starch vesicles ($\sim$ 1 μm); (ii) no ruptured pollen grains were found in C. betulus; (iii) the observed particles exhibit a clear cell wall, which suggests a more cellular rather than cytosolic character. To clarify this aspect, the following statement has been added to p. 5715, l. 21:

"Further, it also appears unlikely that the adhered particles are cytosolic starch granules from pollen grain rupture because of substantial differences in particle size and the absences of ruptured grains in the C. betulus sample (compare Fig. S5)."

Fig. S5, which illustrates the size and shape of the small particles in C. betulus in comparison with cytosolic starch granules, has been implemented into the supplemental material.

[2.12] Referee comment: The PCA analysis shows good separation between certain pollen species. Given the term "fingerprint" is used in the title how confident are the authors that, if given an unknown sample of the pollen species used here, that it could actually be identified? State level of confidence.

Author response: We thank the referee for this critical question. We cannot provide a final answer to this question because a quantitative single particles analysis is needed to evaluate if our PCA results provide a reliable template for online pollen recognition. However, we reworked the PCA section in our study to discuss this aspect more adequately and to sketch directions for further research. The following changes have been implemented: (i) A proxy for pollen grain shape has been added as an additional PCA input parameter. We discuss the question if this increases the reliability of pollen

recognition. (ii) Four further pollen species (J. californica, A. retroflexus, P. alba and F. sylvatica) have been included into the analysis, which extent the statistical basis. (iii) In addition, the influence of age on autofluorescence has been analyzed for two species (P. alba and F. sylvatica). (iv) The PCA section in p. 5716-5717 has been reworked. The improved version with modified statements and references to the additional experiments is shown here:

"We utilized principal component analysis (PCA) to visualize general taxonomic trends in pollen fluorescence properties, as observed in Fig. 5. PCA reduces complex datasets to fewer dimensions and preserves most of the variability. It often provides insights into general underlying structures of the dataset in question. In other words, the PCA in this study is an attempt to display the diverse autofluorescence and morphology properties of pollen, based on few basic parameters. Figure 7 displays three PCA bi-plots which illustrate taxonomic trends based on fluorescence data only (Fig. 7A), based on fluorescence data in combination with pollen grain size (Fig. 7B), and based on fluorescence data in combination with pollen grain size and shape (Fig. 7C) (Sect. 2.4). In Fig. 7A two principal components (PC) span the fluorescence variability of all analyzed pollen species and the four eigenvectors shown (total intensity as well as relative intensities of the modes A, B, and C) represent the main distinctive features. It can be seen that: (i) the intensity eigenvector spreads out the pollen species according to their overall fluorescence intensity, with Poaceae being highest, followed by Asteraceae as well as Salicaceae, and Betulaceae being lowest. (ii) The diametric eigenvectors for mode A and mode C spread the pollen according to their fluorescence mode patterns. For example, the species J. nigra and B. fontinalis are characterized by a strongly fluorescent mode C and the absence of mode A (see Fig. 4D,G). In contrast, B. papyrifera and P. alba exhibit a dominant mode A and a rather weak intensity for mode C (see Fig. 4E and Fig. S4N). (iii) In addition, a certain clustering of species, which belong to the same family, is observed in the bi-plot, however pollen families are not clearly separated. In order to increase the taxonomic resolution in the PCA bi-plot, the autofluorescence-based input data is complemented by pollen grain size

and shape as morphology-related parameters in dry state. Size and shape can vary substantially across pollen species with diameters ranging from $\sim$10 μm to $\sim$ 50 μm and an axis aspect ratio (major/minor axis) ranging from $\sim$ 1 (spherical grains) to $\sim$ 1.8 (elongated grains) (Table 1, Fig. S7). About 90 % of the species in this study reveal rather spherical pollen grains (aspect ratio 1.1-1.2) and many species show grain diameters at about 25 μm (Fig. S7). Thus, the parameters size and shape alone do not provide a clear specificity and separation of pollen families. The combination of autofluorescence- and morphology-related parameters increases the separation and the clustering of pollen families as shown in Fig. 7B,C. Here, pollen grain size yields a clear improvement, whereas grain shape has only a minor effect. In Fig. 7B,C pollen families cluster in different sectors of the PCA bi-plot (e.g., clear separation of Poaceae, Fagaceae, and Juglandaceae), however, with some overlapping regions (e.g., Poaceae and Asteraceae). We can conclude that the PCA representation provides a good categorization of the characteristic autofluorescence and morphology parameters of pollen. Autofluorescence, size, and shape are the basic parameters for online LIF instrumentation. Thus, the trends and overall separation of species in Fig. 7 may be helpful to develop algorithms for pollen recognition in online applications. However, it has to be kept in mind that the situation is complicated by two further effects. First, environmental influences (i.e., water) and pollen aging (compare Sect. 2.1) can change the fluorescence and morphology properties. The examples P. alba and F. sylvatica clearly illustrate the influence of age, which impacts the positioning in the PCA bi-plot. For both species the relative intensity of mode C increases with age (compare Fig. S3) and therefore the markers in PCA bi-plot are shifted along the mode C eigenvector (illustrated by black arrows in Fig. 7C). Second, differences in fluorescence properties on single particle level cause further uncertainty in the PCA positioning (see Sect. 3.1). The defined positions in Fig. 7 represent the average properties of the pollen bulk samples, whereas the heterogeneity on single particle level may be more adequately represented by more diffuse spots, as qualitatively visualized for B. fontinalis and Q. robur in Fig. 7C. Ultimately, we suggest that the general trends and clustering in Fig.

7 represent a stable separation of different pollen species. In the next step, this observation has to be reviewed by a systematic single particles analysis (online LIF or single particle fluorescence microscopy) to evaluate the level of confidence for pollen recognition."

References:

Kanaani, H., M. Hargreaves, Z. Ristovski, and L. Morawska (2007), Performance assessment of UVAPS: Influence of fungal spore age and air exposure, Journal of Aerosol Science, 38(1), 83-96.

Robinson, N. H., Allan, J. D., Huffman, J. A., Kaye, P. H., Foot, V. E. and Gallagher, M.: Cluster analysis of WIBS single-particle bioaerosol data, Atmos. Meas. Tech., 6, 337-347, 10.5194/amt-6-337-2013, 2013.

Figure Captions:

Fig.1 (Figure number in revised paper: Fig7.) Results of principal component analysis (PCA) illustrating taxonomic trends in pollen autofluorescence. (A) Bi-plot with scores of PCA based on pollen fluorescence properties only. Eigenvectors (red arrows) represent relative intensities of modes A, B, and C, as well as total intensity level, as most distinctive features. (B) Bi-plot with scores of PCA based on pollen fluorescence properties and grain size. Eigenvectors represent intensity of modes A-C total and grain size. (C) Bi-plot with scores of PCA based on pollen fluorescence properties, grain size, and grain shape. Eigenvectors represent intensity of modes A-C total, grain size, and grain shape (axis aspect ratio). In (C) age-related changes in fluorescence are shown for P. alba and F. sylvatica as black arrows. Heterogeneity in fluorescence properties on single particle level (Sect. X.X) is illustrated qualitatively for B. fontinalis and Q. robur by diffuse spots around marker, which represent average properties.

Fig.2 (Figure number in revised paper: FigS3.) EEMs showing influence of age on fluorescence properties by means of two pollen species: (A) Populus alba harvested

in March 2011 versus (B) Populus alba from March 2013; (C) Fagus sylvatica from April 2009 versus (D) Fagus sylvatica from May 2013. Spectra show that fluorescence intensity of pollen bulk samples tends to increase with age, however, spectral signature in EEMs is conserved. P. alba shows slight intensity increase with age (Fig. S3A versus Fig. S3B), corresponding with comparably slight intensity increase in fluorescence microscopy images (Fig. S2A versus Fig. S2B). In contrast, F. sylvatica shows strong intensity increase with age (Fig. S3C versus Fig. S3D), corresponding with similarly strong intensity increase in fluorescence microscopy images (Fig. S2C versus Fig. S2D).

Fig.3 (Figure number in revised paper: FigS5.) Microscopy images in bright field (left) and fluorescence mode (right) showing the “contaminating” small particles in the Carpinus betulus sample (in (B), right arrows) in comparison with cytosolic starch granules (in (B), left arrows). Ruptured pollen grains were not observed in the commercially obtained C. betulus sample. Pollen rupture as shown in (A) and (B) has been caused by mechanical stress. Comparison of the “contaminating” particles (4-6 μm) and cytosolic starch granules ($\sim$ 1 μm) shows substantial differences in particles size. Further, (A) illustrates the clear and blue cell wall-like fluorescence of the small particles (arrows). Scale bar = 30 μm.

Fig.4 (Figure number in revised paper: FigS7.) Overview of pollen grain size and shape (represented as aspect ratio) in dry state for 29 species analyzed in this study. Aspect ratio of $\sim$ 1 approximates spherical grains, whereas aspect ratio >1 represents elongated grains. Typical aspect ratio for most pollen species in this study is 1.1 – 1.2. Many pollen species show grain size $\sim$ 25 μm. No clear separation of families observed, based on size and shape. Error bars represent one standard deviation. Color code represents pollen families.

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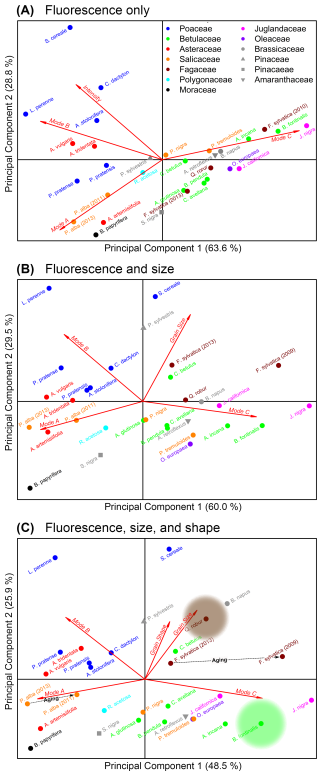
(A) Fluorescence only
Poaceae
Betulaceae
Asteraceae
Salicaceae
Fagaceae
Polygonaceae
Moraceae
Juglandaceae
Oleaceae
Brassicaceae
Pinaceae
Pinaceae
Amaranthaceae
Principal Component 2 (28.8 %)
Principal Component 1 (63.6 %)
(B) Fluorescence and size
Principal Component 2 (29.5 %)
Principal Component 1 (60.0 %)
(C) Fluorescence, size, and shape
Principal Component 2 (25.9 %)
Principal Component 1 (48.5 %)

Fig. 1.

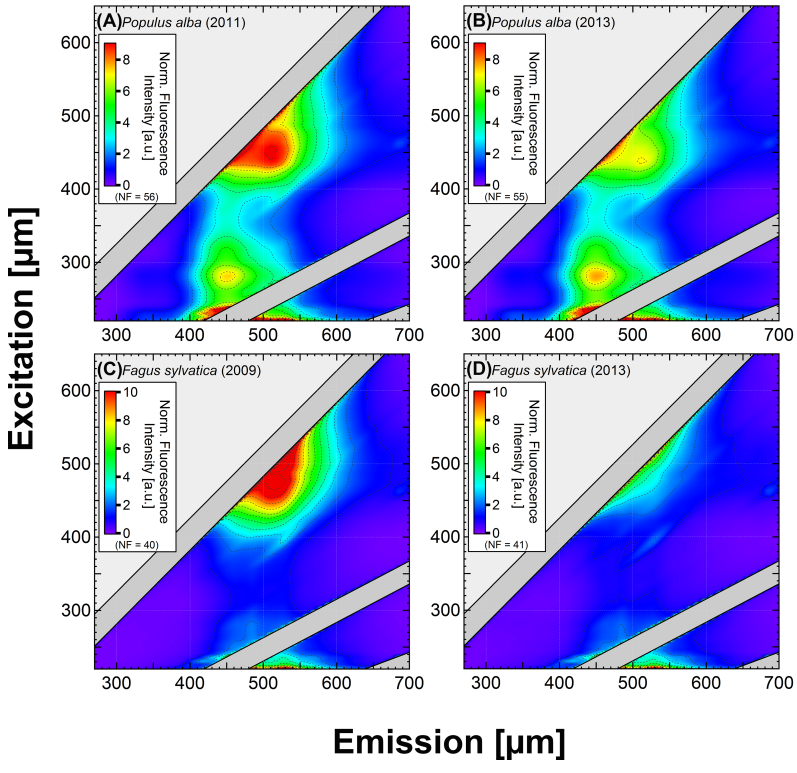
(A) Populus alba (2011)
(B) Populus alba (2013)
(C) Fagus sylvatica (2009)
(D) Fagus sylvatica (2013)
Norm. Fluorescence Intensity [a.u.]
(NF = 56)
(NF = 55)
(NF = 40)
(NF = 41)
Excitation [µm]
Emission [µm]

Fig. 2.

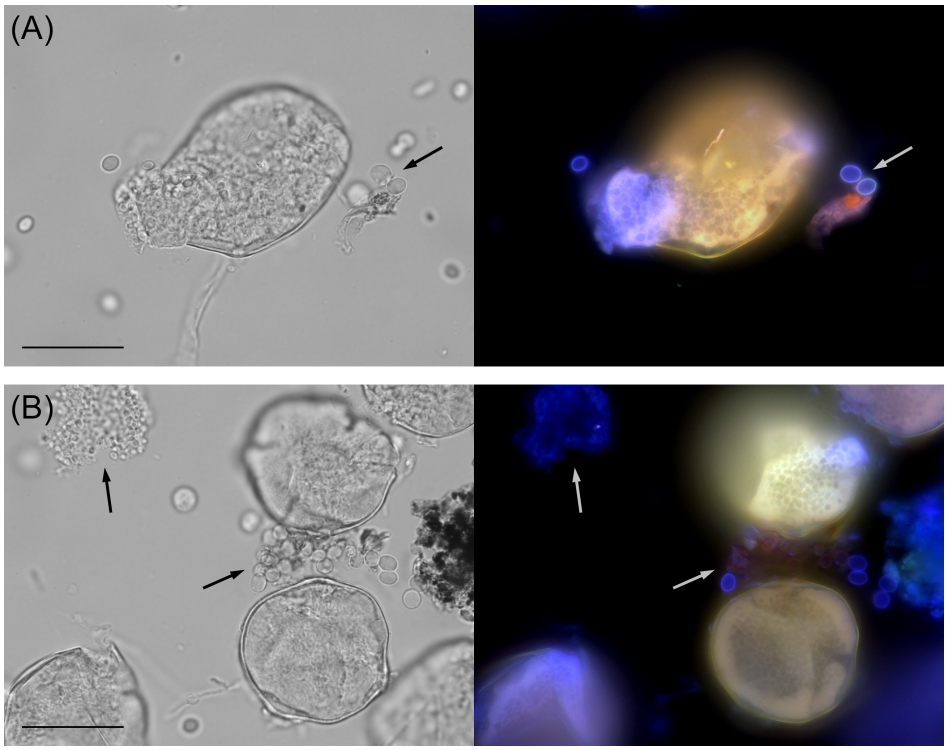
(A)
(B)

Fig. 3.

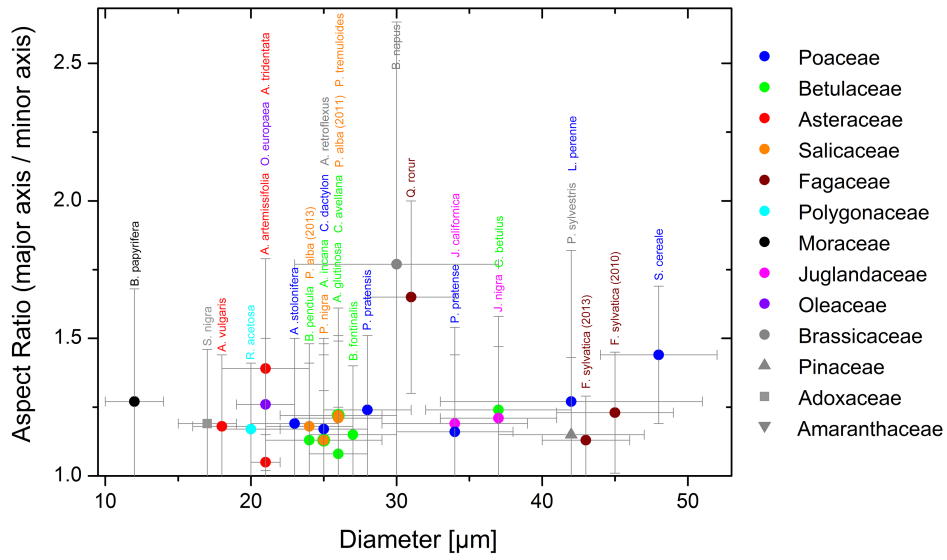
Aspect Ratio (major axis / minor axis)
Diameter [µm]
1.0
1.5
2.0
2.5
10
20
30
40
50
B. papyrifera
S. nigra
A. vulgaris
R. acetosa
A. artemissifolia
O. europaea
A. tridentata
A. stolonifera
B. pendula
P. alba (2013)
P. nigra
A. incana
C. dactylon
A. retroflexus
A. glutinosa
C. avellana
P. alba (2011)
P. tremuloides
B. fontinalis
P. pratensis
B. nepusi
Q. rorur
P. pratense
J. californica
J. nigra
C. betulus
P. sylvestris
L. perenne
F. sylvatica (2013)
F. sylvatica (2010)
S. cereale
Poaceae
Betulaceae
Asteraceae
Salicaceae
Fagaceae
Polygonaceae
Moraceae
Juglandaceae
Oleaceae
Brassicaceae
Pinaceae
Adoxaceae
Amaranthaceae

Fig. 4.