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Pollen and Flora as Bioindicators in Assessing the Status of Polluted Sites: The Case Study of the Mantua Lakes (SIN "Laghi di Mantova e Polo Chimico"; N Italy)

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Abstract: An integrated floristic and palynological approach was carried out at the site of national interest "Laghi di Mantova e Polo Chimico" to obtain an environmental assessment useful for monitoring polluted sites. The flora of highly contaminated sectors (area A and area B) was surveyed, and the floristic composition and ecological strategies of the species were compared with a control sector (area C). A total of 195 species were observed in the three sectors. Pollen preservation of six selected species was checked as a bioindicator of environmental health in the same sectors. Area A and area B likely share similar environmental pressures, including anthropogenic stressors such as pollution, geographical proximity and a similar set of habitats, leading to similarities in flora composition. Similarly, the incidence of pollen without cytoplasm is higher in area A (9.3%) and area B (7.6%) than in area C (2.5%). The floristic differences among the sectors and the quantity of empty or abnormal pollen, together with the CSR strategies adopted by the species, suggest that the effects of anthropogenic impact on local vegetation can be detected at both macroscopic and microscopic levels. The discovery of the protected species *Narcissus pseudonarcissus* in area C is noteworthy, which may be important in directing efforts towards the protection of plant communities in this sector.

Keywords: palynology; flora; bioindication; CSR strategy; environment conservation; environmental pollution; soil pollution

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1. Introduction

Human industrial activities are often a major source of pollution, introducing potentially hazardous compounds to organisms into atmospheric, terrestrial and aquatic ecosystems [1]. Therefore, to better understand how pollution affects the flora and vegetation of a certain area, appropriate plant bioindicators can be selected and studied (e.g., [2,3]). This is particularly relevant when dealing with sites of natural interest that may be endangered by the persistent presence of polluting activities.

The site of national interest (SIN) "Laghi di Mantova e Polo Chimico" ("Lakes of Mantua and Chemical Hub"), as defined by Italian law 179/2002, is an environmentally critical site located in the city of Mantua, in the region of Lombardy, Northern Italy [4]. The SIN includes the following: (i) an industrial area, largely responsible for local pollution; (ii) the Mantuan Lakes; and (iii) two special areas of conservation (SACs), which are included in the Natura 2000 network [5], namely the "Riserva Regionale della Vallazza" (Natura 2000 code: IT20B0010) and part of the "Ansa e Valli del Mincio" natural reserve (Natura 2000 code: IT20B0017) [4,6]. Accordingly, the Regional Agency for Environmental Protection (ARPA) of Lombardy identifies three main environmental compartments within the SIN: (i) "Polo Chimico" ("Chemical Hub"); (ii) "Lago di Mezzo e Inferiore" ("Middle and Lower lakes"); and (iii) "Vallazza, aree umide e Fiume Mincio" ("Vallazza, wetlands and Mincio River") [4]. The "Vallazza, aree umide e Fiume Mincio" are SIC areas (Sito di Interesse Comunitario = Site of Community Importance) and consist of wetlands of special interest in areas of conservation [6] (Figure 1).

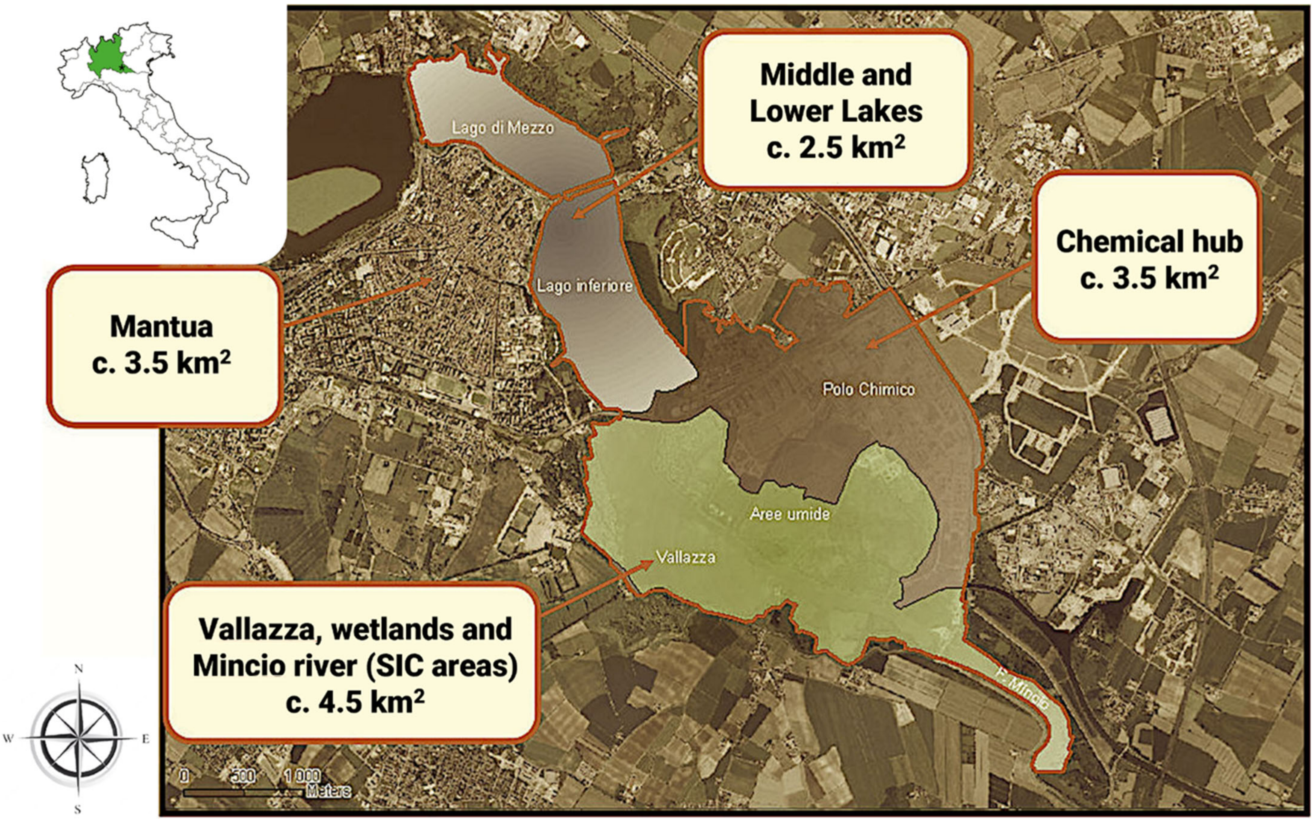

Figure 1. "Laghi di Mantova e Polo Chimico": environmental compartments within the SIN (modified from ARPA Lombardy). In the map of Italy (top-left), the province of Mantua is marked with a black asterisk, within the Lombardy region (green).

The flora, typical of marshlands and humid soils, includes reed beds and sedge meadows, enriched by *Iris pseudacorus* L., *Colchicum autumnale* L., *Anemone nemorosa* L. and *Acer campestre* L., among others [7]. The SIC areas host a rich hydro-hygrophyte flora, and many bird species of community importance as well. Noteworthy is the presence of *Narcissus pseudonarcissus* L., a plant species of community importance.

Despite the unambiguous importance of this naturalistic region, the chemical analyses performed by ARPA on local environmental compartments have shown the soil to be severely polluted by light and heavy hydrocarbons, VOCs (BTEXS), metals (especially mercury in the petrochemical area), PCBs, dioxins and furans [4]. The aquifer is heavily polluted by hydrocarbons, VOCs (BTEX), styrene and cumene in the petrochemical area, MTBE and ETBE in the ex-refinery area, and halogenated organic compounds and metals [4]. In addition, sediments at the bottom of the canals distributed throughout the region, the Mincio river, docks and wetlands are polluted with heavy hydrocarbons, metals (mercury in particular) and dioxins [4]. Supernatant, composed of viscous liquid substances derived from chemical activities, has been found in the aquifer in the ex-refinery area and in some sites inside the petrochemical area [4]. Precise quali-quantitative chemical data on aquifer pollution are publicly available and described in aquifer monitoring campaign reports (November 2019 [8], December 2022 [9]), while only qualitative data on soil pollution are currently available [4].

Considering these dramatic environmental conditions, the Mantua SIN stands out as an interesting case study for observations on flora and plant responses to stress induced by local chemical alterations in soil and water. Since pioneering work in evolutionary biology, it has been known that environmental conditions play a key role in the selection of plant species [10–14]. Selective pressure exerted by anthropogenic sources is no exception and has been shown to play a part in selecting plants in a certain environmental context, favouring some wild synanthropic species and/or disfavouring other plants [3,15–18]. The morpho-physiological status, flora composition and the geographic origin of the species living in a habitat are among the aspects that should be considered to assess environmental conditions through plant bioindicators. For example, significantly higher levels of secondary metabolites, such as flavonoids and phenolic compounds which are involved in plant–environment relationships, have been observed in the leaf extracts of plants growing in industrial areas compared to those growing in residential zones [19].

Concerning flora composition, the spread of stress-tolerant plants was found to be a very good indicator of decreasing environmental quality along the river Aniene (Latium, Italy) [20]. In Northern France, a turf of heavy-metal-tolerant plants were observed around one of the largest smelters in the world (Bois des Asturies in Auby), with soils polluted especially by zinc, cadmium and lead [21]. Both metal-accumulating plants, or metallophytes, and heavy-metal-sensitive species can adopt some strategies that allow them to cope with the negative consequences of heavy metal toxicity [22]; nonetheless, heavy metals can be disruptive to plant health. In roots, which are the first organ encountering heavy metals in soils, high levels of toxic elements hamper the overall growth performance of stressed plants, causing a decrease in mitotic activity and the suppression of root growth, consequently altering water balance and nutrient absorption [23]. Moreover, high levels of metals in bioaccumulating plants were found to inhibit chlorophyll production and increase oxidative stress [24].

In addition, the presence of alien species can be regarded as a further indicator of poor environmental quality, as the ability of aliens to colonise new habitats reflects their broad tolerance to environmental stress, defined as the maintenance of high values of fitness-related traits [25]. In the waters of the Mantuan lakes, physiological and ecological traits of the two introduced invasive aquatics, namely *Nelumbo nucifera* Gaertn. and *Ludwigia hexapetala* (Hook. et Arn.) Zardini, H.Y. Gu et P.H. Raven, demonstrated advanced photosynthetic efficiency. This characteristic results in faster growth rates and higher productivity than the three native macrophyte species *Nuphar lutea* (L.) Sm., *Nymphaea alba* L. and *Trapa natans* L. [26,27].

Thus, floristic analysis of a site, highlighting the occurrence of plant species and their ecological strategy, is a good botanical approach to assess the environmental quality of polluted contexts.

Less common in these types of studies is the observation of pollen, as well as its morphology and status, used as a bioindicator. Pollen is the most widespread plant part in terms of time and space all over the world [28]. It may be used as an ecological indicator in many environmental contexts and for a variety of purposes [29–32] based on its role as a microscopical witness of different plant species, or considering its state of preservation (with or without cytoplasm). In fact, mature pollen grains are filled with cytoplasm, but empty (lacking cytoplasm) pollen grains can often be observed in fresh pollen samples. The loss of cytoplasm can be physiological [33–35] or due to anomalies during pollen development [36], as well as an abnormal tapetum that cannot supply sufficient nutrients for pollen development [37]. Interspecific differences in empty pollen incidence have also been reported [38]. Nonetheless, pollen non-viability and cytological anomalies could be related to exposure to environmental pollution [39–41]. Although it is evident that air pollution has effects on pollen content and its molecular composition [42], in most cases, it is not possible to exclude the simultaneous influence of air and soil pollution on pollen development [40]. Low amounts of Cd, Ni and Pb and other trace elements were measured in the pollen of *Ambrosia artemisiifolia* L. growing in polluted soils [43]. This evidence demonstrates that heavy metals, and pollution in general, can enter anthers and interfere with pollen grain formation [44]. Therefore, the state of preservation of pollen in anthers may be influenced by soil conditions, and anomalous pollen can be formed under anomalous environmental conditions.

Aim of the Study

As said above, the ARPA agency, which continuously monitors environmental matrices, attested that all of "Laghi di Mantova e Polo Chimico" SIN is highly polluted. The 2022 aquifer analysis campaign report [9] is available, while the quantitative data on soils are not public. Based on the research on the possible relationships between flora and pollen content in contaminated soils, the present study introduces integrated floristic and palynological analysis to obtain a preliminary environmental assessment of the pollutant-stressed SIN. The aim of the research is to verify how the combination of flora and pollen analyses may be used as a proxy to check the environmental health in known polluted sites. In the floristic study, on one hand, the flora of highly contaminated sectors was surveyed, while on the other hand, the floristic composition and ecological strategies of the observed species were compared with a control sector. In addition, the pollen morphology of selected species was analysed, particularly considering the presence of the cytoplasm in pollen as a preliminary index of the general health of plant species growing in a polluted site.

We collected pollen samples and analysed them with a botanical–palynological approach. Chemical information on the pollutants directly extracted from plants is not among the aims of this paper. However, Massa et al. [45] demonstrated the ability of plants growing in polluted soils to tolerate or accumulate heavy metals in tissues and organs, thus possibly reflecting exposition to these substances.

2. Materials and Methods

The SIN "Laghi di Mantova e Polo Chimico", approximately 1027 hectares, includes a surface hydrology characterised by numerous basins that are affected by anthropogenic containment actions (e.g., canals, ditches) and the withdrawal–restitution of water used in industrial cooling processes. The study was conducted in three sectors that have a different location and different characteristics within the SIN area. Different impacts on flora and related pollen were therefore expected. Two sectors, named "area A" and "area B", were chosen close to the Lower Lake (Lago Inferiore) in order to study the pollution impact on the flora (Figure 2). These sectors were identified as being close to the industrial area and, based on the data reported in the 2019 aquifer analysis campaign, had similar pollution contamination [8]. The 2022 aquifer analysis campaign [9] showed high pollution and a similar pollution impact in sectors A and B; contamination with arsenic, manganese, ETBE and total hydrocarbons has worsened over the years in some spots, whereas benzene contamination has increased in one spot and decreased in another, and vinyl chloride contamination has decreased. Area A and area B are different in both extension and shape (Figure 2). They are commonly used as biking or walking routes and are thus subject to trampling [46] and show traces of human management, such as tree planting and swathing.

Area A is 3925.65 m^2 wide, bordering the Lower Lake to the north, a small bog to the south, a trail connecting it to the road to the east and the site of the ex-refinery to the west.

Area B is 19,971.90 m^2 wide and has a convoluted shape; it borders area A to the south-east, follows Lago Inferiore and then grassland from the east to the north-east, borders a small road to the North and follows the ex-refinery's border.

A third sector, named area C, relatively distant from the industrial site but still within the SIN and used as a walking route, served as the control sector. It is a 16,995 m^2 wide, rectangular-shaped trail bordering an ex-farm's abandoned fields on its western and eastern sides, as well as a road and a humid zone of the regional reserve (Riserva Regionale della Vallazza) on its northern and southern sides, respectively. Area C was chosen because the Environmental Agency (ARPA) has not yet recorded soil pollution locally [4], and no pollutants analysed in the 2019 and 2022 aquifer analysis campaigns exceeded law limits in groundwater, with the exception of arsenic [8,9]. Therefore, area C can be regarded as a sector subject to the same anthropic impacts as area A and area B (trampling [46] and vegetation management), excluding soil pollution (or at least to a lesser extent), both due to its distance from the A and B sectors and to the data reported by ARPA.

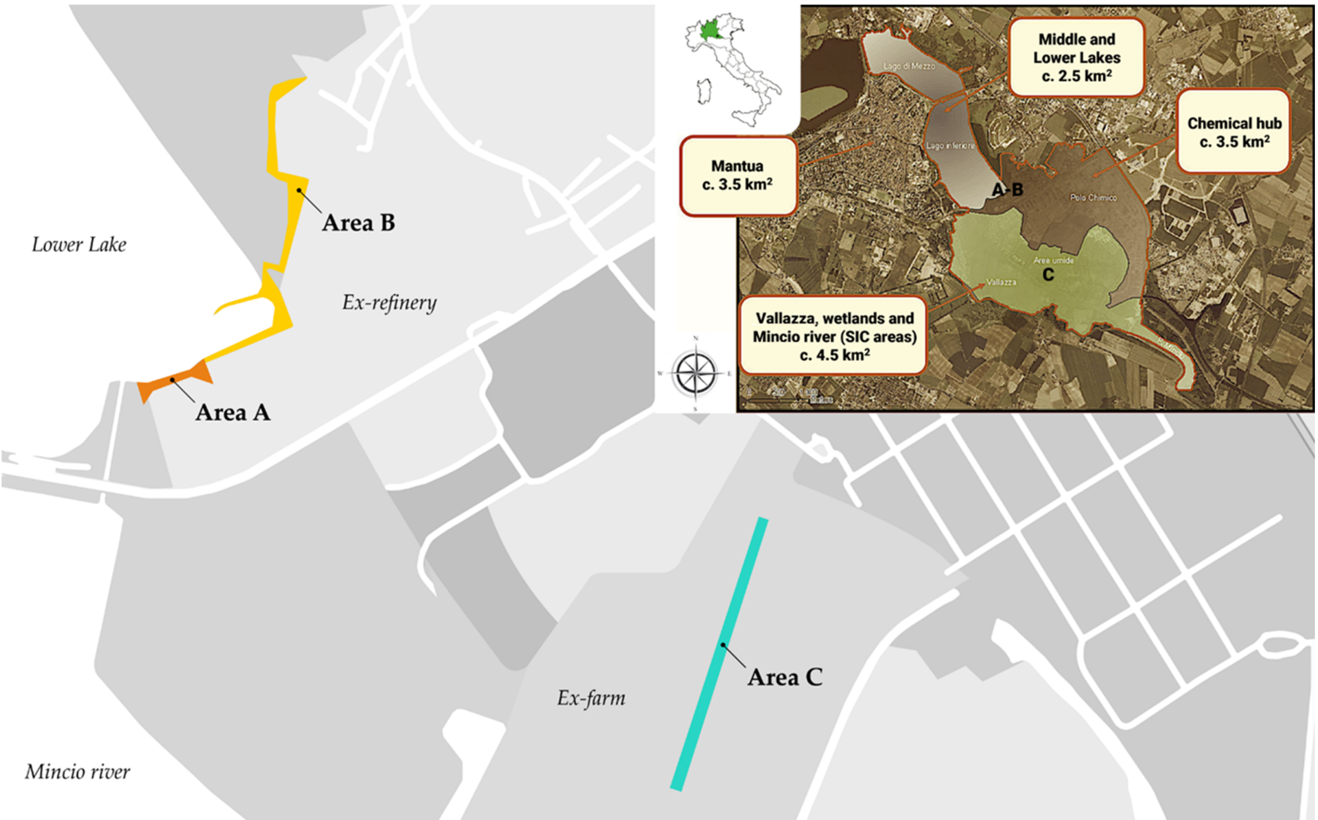

Figure 2. SIN "Laghi di Mantova e Polo Chimico": overview of the studied site with location of the sectors of interest (area A, area B, area C). In the top-right section, the province of Mantua is marked with a black asterisk and the Lombardy region is coloured in green in the map of Italy; while the location of the studied sectors is marked with their respective letters in the map of the SIN.

2.1. Flora

The vascular flora checklist was carried out during eight site inspections, which took place in 2021 (April–June) and 2022 (February–May). Species identification was performed according to Rothmaler [47] and Pignatti et al. [48], and their nomenclature follows Pignatti et al. [48]. Species occurrence was described according to *Acta Plantarum* [49] IPFI [50] (a project initially based on Conti et al.'s Italian flora checklist [51] and continuously updated [52]), in accordance with the definitions by Pyšek et al. [53], except for the term "cryptogenic", which was used in accordance with the definitions by Salvai and Dose [54].

A presence/absence matrix of the species found in the three sectors was used to calculate the Bray–Curtis similarity index for each possible pairing of sectors. Bray–Curtis similarity indices obtained were then used for hierarchical clustering analysis (UPGMA algorithm).

The competitor, stress-tolerant and ruderal (CSR) strategy of species was calculated using StrateFy [55] and then plotted in a ternary plot. The plant traits used to calculate CSR strategies via StrateFy [55] were leaf area, leaf dry matter content and specific leaf area (Table 1). Values input into StrateFy were calculated using data obtained from the TRY Plant Trait Database [56]; for each species, each trait value input into StrateFy was the arithmetic mean of all the values of that trait (when two or more values were available) obtained from the TRY Plant Trait Database [56]. Data obtained from the TRY Plant Trait Database were used to calculate the CSR strategy of 55 out of 106 herbaceous species and

24 out of 27 woody species, while the necessary trait data to calculate it using StrateFy [55] were not available for the other species.

Table 1. Criteria used for data collection from TRY Plant Trait Database [56]. The 'Trait ID' is the code for a macro-group of traits; the 'Data ID' indicates which of the traits in the macro-group was used. Mean values of the traits obtained were thus input into StrateFy [55]. References to datasets consulted are reported in Supplementary Materials (File S1).

TRY Trait ID	TRY Data ID	TRY Plant Trait Name	Measure Unit
3110	6577	Leaf area: in case of compound leaves, leaf, petiole and rachis included	mm^2
47	258	Leaf dry matter content per leaf water-saturated mass (LDMC)	%
3116	6583	SLA: petiole included	$mm^2\ mg^{-1}$

2.2. Pollen

Pollen was sampled during four sampling sessions, which took place in 2022 (February–May). A total of 21 samples, including flowers and anthers, were hand-collected and placed in paper bags from random individuals of 6 species selected among those found in all sectors, namely *Alopecurus myosuroides* Huds., *Amorpha fruticosa* L., *Lamium purpureum* L., *Potentilla reptans* L., *Prunus spinosa* L. and *Veronica persica* Poir. This resulted in three samples, one per sector, for each species, except *L. purpureum*, which was collected twice per sector during separate sampling sessions. Each sample was georeferenced. Under a stereomicroscope at 20× magnification, anthers were opened with a mounted needle and tweezers, and pollen was directly put on a slide. Then, pollen was coloured using basic fuchsin jelly and covered with a coverslip. The slide was sealed with paraffin, thus becoming a permanent preparation. Pollen was counted at 400× magnification with a light microscope, and its identification was checked with the help of the reference collection of fresh pollen of the Laboratory of Palynology and Palaeobotany of Modena. The fraction of empty pollen grains versus total pollen grains was counted in each sample. One sample (*A. myosuroides*, collected in area B) was excluded from the dataset due to the low number of pollen grains observed (two pollen grains), likely due to the involuntary collection of immature flowers or low pollen production of the plant (see below, par. 4.3).

Differences in the mean empty/total pollen fraction between the three sectors and between the species were assessed with a one-way ANOVA test, followed by pairwise Tukey's test if significant. p-values < 0.05 were considered statistically significant. All statistical analyses were performed with Past 4.11 version [57].

3. Results

The results of flora and pollen analyses are reported below, especially focusing on species composition and the incidence of empty pollen in the three analysed sectors.

3.1. The Flora of the Sectors A, B, C

A total of 195 species were observed in the 3 sectors. The complete floristic list with the occurrence and CSR strategy of each species is reported in the Supplementary Materials (Table S1).

Area A was the richest in species (87) despite being the smallest one, while area B and area C hosted the same number of species (55). The flora of each sector had a mostly herbaceous habitus that mostly consists of native and non-invasive species, including both herbaceous and woody species (Figure 3, Table 2). However, the percentage of non-native invasive species among herbaceous and woody species substantially differed: invasive herbaceous species made up approximately 6–7% of the total herbaceous species, while the amount of invasive woody species was much higher, from approximately 14 to 25% in the three sectors (Table 2).

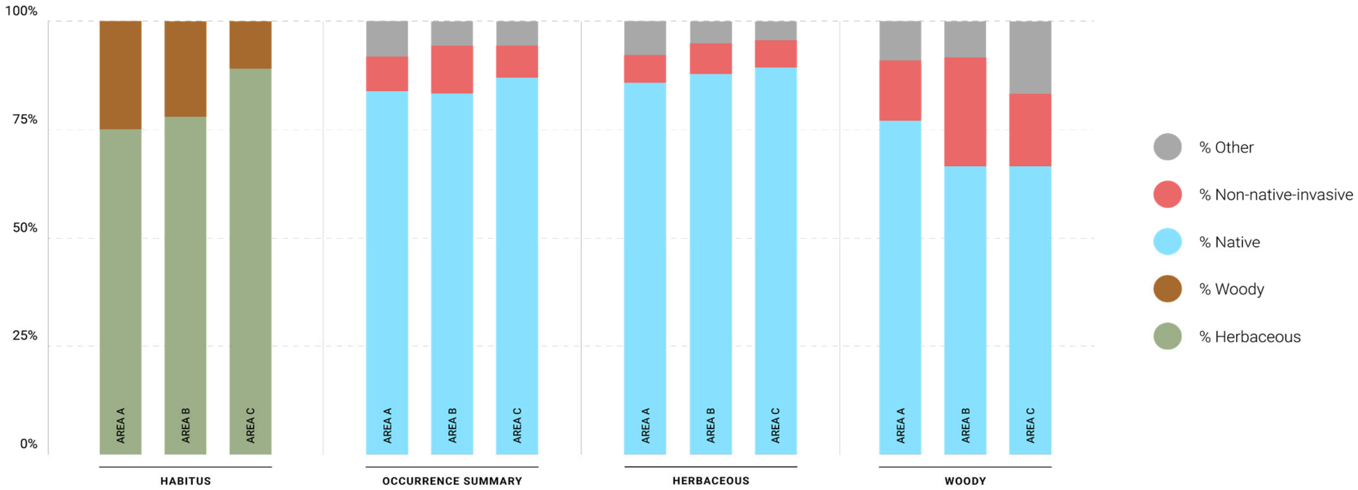

Figure 3. SIN "Laghi di Mantova e Polo Chimico": The herbaceous/woody species (habitus) and their occurrence in the three studied sectors (area A, area B, area C).

Table 2. SIN "Laghi di Mantova e Polo Chimico": Number, incidence of habitus and occurrence (number and, in brackets, percentage) of the species observed in the three sectors (area A, area B, area C).

Species		Area A	Area B	Area C
Habitus	Herbaceous	65 (74.7%)	43 (78.2%)	49 (89.1%)
	Woody	22 (25.3%)	12 (21.8%)	6 (10.9%)
	Total	87	55	55
Occurrence	Native	73 (84.0%)	46 (83.6%)	48 (87.3%)
	Non-Native Invasive [1]	7 (8.0%)	6 (10.9%)	4 (7.3%)
	Other [1]	7 (8.0%)	3 (5.5%)	3 (5.4%)
	Total	87	55	55
Occurrence (Herbaceous)	Native	56 (86.2%)	38 (88.4%)	44 (89.8%)
	Non-Native Invasive [1]	4 (6.1%)	3 (6.9%)	3 (6.1%)
	Other [1]	5 (7.7%)	2 (4.7%)	2 (4.1%)
Occurrence (Woody)	Native	17 (77.3%)	8 (66.7%)	4 (66.6%)
	Non-Native Invasive [1]	3 (13.7%)	3 (25.0%)	1 (16.7%)
	Other [1]	2 (9.0%)	1 (8.3%)	1 (16.7%)

[1] "Invasive" is the total of "Invasive alien", "Invasive archaeophyte" and "Invasive neophyte" species; "Other" is the total of non-"Native" and non-"Invasive" species.

Based on the Bray–Curtis similarity index calculated on the presence/absence matrix, hierarchical clustering analysis and the similarity index for each individual sector pairing showed that area A and area B clustered together at 0.458 similarity, while area C clustered on its own between 0.350 and 0.360 similarity with the area A–area B cluster (Figure 4). The percentages of plant strategies calculated for each sector are reported in Figure 5.

Sector	Area A	Area B	Area C
Area A	1	0.469	0.366
Area B	0.469	1	0.360
Area C	0.366	0.360	1

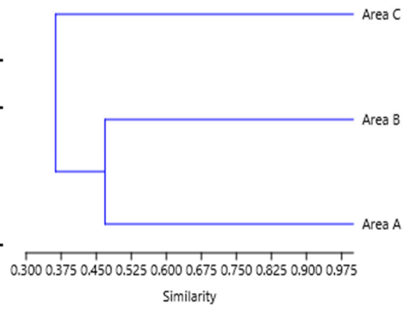

Figure 4. SIN "Laghi di Mantova e Polo Chimico": Bray–Curtis similarity (index matrix to the left, dendrogram to the right) of the flora of the three investigated sectors (area A, area B, area C) based on a presence/absence matrix of the species. The dendrogram (**right**) is based on the Bray–Curtis index matrix (**left**).

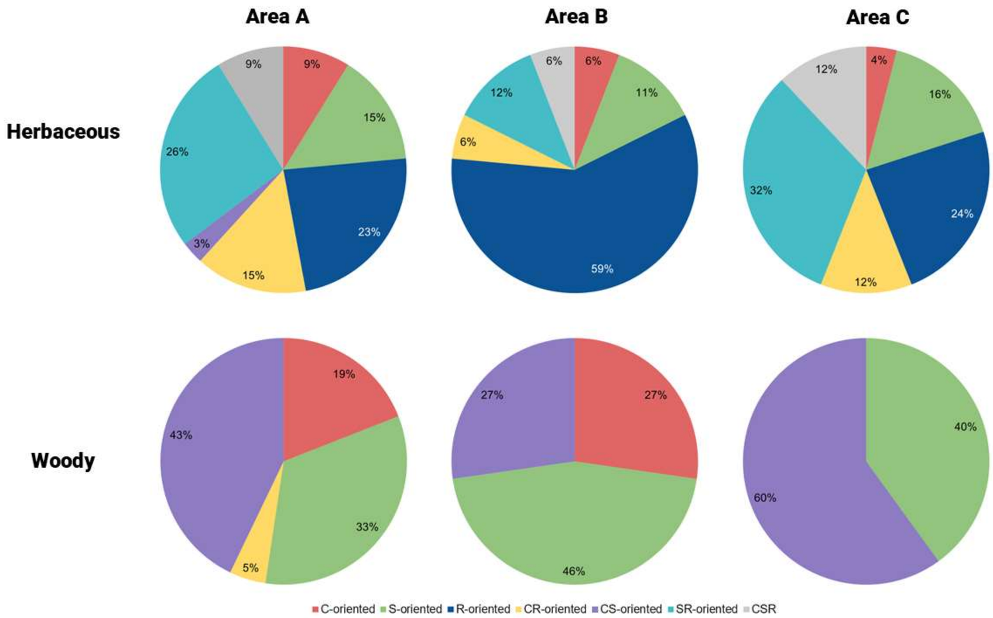

Figure 5. SIN "Laghi di Mantova e Polo Chimico": CSR ecological strategies of species found in the three sectors. C: competitor (red); S: stress-tolerant (green); R: ruderal (blue); CS: mixed competitor/stress-tolerant; CR: mixed competitor/ruderal; SR: mixed stress-tolerant/ruderal; CSR: mixed competitor/stress-tolerant/ruderal. The CSR strategy was calculated for 55 herbaceous species and 23 woody species observed in the surveys in the 3 sectors (area A, area B, area C).

3.2. Pollen Analysis

As reported above, six species found in the three sectors were selected for pollen analyses. The total number of pollen grains observed ranges from 46 (*Lamium purpureum*, sample from area C) to 5696 (*Potentilla reptans*, sample from area C), depending on the different pollen production and interspecific variability, as observed in morphological studies (e.g., [58,59]).

Empty and full pollen grains were observed in all the samples (Figure 6; Table S2), ranging from 1 to 15% of empty grains in the different species (Table 3). Differences were found in 'empty pollen grains' versus 'total pollen grains' (one-way ANOVA test, F = 11.72, $p = 0.001$) in the three sectors. More specifically, the area C average value was different from that of both area A (pairwise Tukey's test, Q = 6.607, $p < 0.001$) and area B (pairwise Tukey's test, Q = 4.728, $p = 0.013$), whereas the difference between the area A and area B average values was not statistically significant (pairwise Tukey's test, Q = 1.571, $p = 0.523$). No significant differences were found when testing the average incidence of empty pollen in each species (one-way ANOVA test, F = 0.4194, $p = 0.8533$) (Table 3).

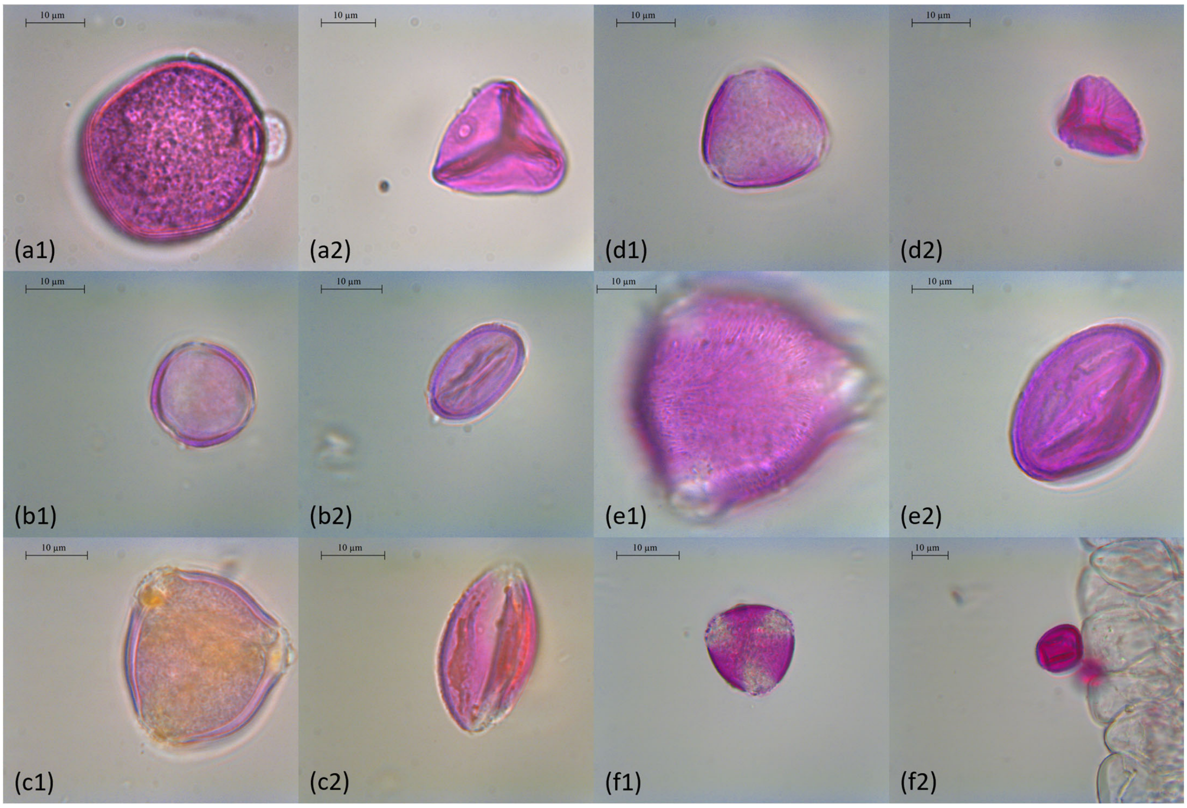

Figure 6. SIN "Laghi di Mantova e Polo Chimico": full (1) and empty (2) pollen grains observed in the samples. (**a**) *Alopecurus myosuroides*; (**b**) *Amorpha fruticosa*; (**c**) *Lamium purpureum*; (**d**) *Potentilla reptans*; (**e**) *Prunus spinosa*; (**f**) *Veronica persica*.

Table 3. SIN "Laghi di Mantova e Polo Chimico": 'empty' versus 'total' pollen grains. Mean values are grouped by sector and by species. Statistical assessment of results was performed by means of one-way ANOVA test, followed by, if significant, pairwise Tukey's test. Statistically significant differences between means are highlighted with different letters ([a] and [b] mark comparison between areas; [c] marks comparison between species).

Species	Total Pollen Grains	Area A	Area B	Area C	Mean
Alopecurus myosuroides Huds.	248	0.096 9.6%	N/A [1]	0.018 1.8%	0.057 [c] 5.7%
Amorpha fruticosa L.	4302	0.072 7.2%	0.079 7.9%	0.015 1.5%	0.056 [c] 5.6%
Lamium purpureum L. [2]	826	0.057 5.7%	0.057 5.7%	0.030 3.0%	0.048 [c] 4.8%
Potentilla reptans L.	7515	0.117 11.7%	0.114 11.4%	0.008 0.8%	0.080 [c] 8.0%
Prunus spinosa L.	1054	0.067 6.7%	0.062 6.2%	0.034 3.4%	0.054 [c] 5.4%
Veronica persica Poir.	760	0.150 15.0%	0.068 6.8%	0.041 4.1%	0.086 [c] 8.6%
Mean	2450.8	0.093 [a] 9.3%	0.076 [a] 7.6%	0.025 [b] 2.5%	

[1] Sample excluded from the dataset due to few pollen grains observed. [2] The species was sampled two times in two different sessions, resulting in two samples for each sector. The average value for each sector is reported in this table and was used in data analysis.

4. Discussion

Stress-tolerant species, heavy-metal-tolerant species and non-native invasive species were found, supporting the presence of polluted soils in the studied area. The floristic surveys showed that the presence/absence of species in area A and area B is more similar to each other than they are to area C. Similarly, the presence/absence of cytoplasm in pollen shows similarities between area A and area B, and the incidence of empty pollen is higher in these two sectors than in area C (Table 3). Interestingly, each species responded the same way in the three sectors, resulting in no significant differences in average empty pollen between species. Therefore, statistically significant differences in empty pollen means were observed between areas and not between species. The floristic differences and quantity of empty or abnormal pollen, together with the ecological strategies adopted by the species inhabiting the sectors, suggest that the effects of pollution and anthropogenic impact on local vegetation can be detected at macroscopic and microscopic levels.

4.1. Flora Composition of Sectors A, B, and C: The Role of Non-Native Invasive Species

The surveyed sectors host predominantly native flora (85% on average), and all of them include many wild synanthropic plants. Native synanthropic species reported in one or more sectors include *Humulus lupulus* L. [60], *Populus nigra* L. [60], *Quercus robur* L. [60], *Rosa canina* L. [60], *Salix alba* L. [60], *Salix caprea* L. [61] and *Sambucus nigra* L. [60], among woody species that testify some remnants of floodplain hardwood forest. The native synanthropic grasses and other herbs include a large number of species living in floodplain communities [61,62], such as *Achillea* gr. *millefolium* L., *Cirsium arvense* (L.) Scop., *Artemisia vulgaris* L. [60,61], *Carex hirta* L. [60], *Dactylis glomerata* L. [60,61,63], *Schedonorus pratensis* (Huds.) P. Beauv. [60], *Galium mollugo* L. [61], *Glechoma hederacea* L., *Hypericum perforatum* L., *Phragmites australis* (Cav.) Trin. ex Steud. [60], *Lamium purpureum* L. [61], *Lolium perenne* L. [61,64], *Medicago lupulina* L. [60,61], *Plantago lanceolata* L. [60,61,63,64], *Plantago major* L. [60,63], *Poa annua* L. [61,64], *P. pratensis* L., *P. trivialis* L. [60,61], *Senecio vulgaris* L., *Sonchus oleraceus* L. [61,64], *Stellaria media* (L.) Vill. [60,61,64], *Trifolium repens* L. [60,61] and *Veronica persica* Poir. [61]. Other native synanthropic herbaceous species found include *Alliaria petiolata* (M. Bieb.) Cavara et Grande [60], *Anisantha sterilis* (L.) Nevski [64], *Calystegia sepium* (L.) R. Br. [60], *Capsella bursa-pastoris* (L.) Medik. [60], *C. rubella* Reut. [64], *Chelidonium majus* L. [60], *Chenopodium album* L. [60,64], *Convolvulus arvensis* L. [60,64], *Daucus carota* L. [60,64], *Elymus repens* (L.) Gould [65], *Euphorbia helioscopia* L. [60], *Galium aparine* L. [60,64], *Geranium molle* L. [64], *G. pusillum* L. [60], *G. robertianum* L. [60], *Lamium purpureum* L. [60], *Malva sylvestris* L. [65], *Melilotus albus* Medik. [60], *Silene latifolia* L. [60], *Urtica dioica* L. [60,63] and *Vicia sativa* L. [64].

Non-native synanthropic species include *Acer negundo* L. [60,65], *Datura stramonium* L. [60], *Erigeron annuus* (L.) Desf. [60], *Erigeron canadensis* L. [65] and *Papaver rhoeas* L. [61,64].

Interestingly, several metallophytes or metal-tolerant species [66] were detected in the sectors: *Achillea* gr. *millefolium* [66], *Capsella bursa-pastoris* [67], *Echium vulgare* L. [67], *Elymus repens* [66], *Medicago lupulina*, *Plantago lanceolata* [66], *Phragmites australis* [66] and *Vicia cracca* L. [67] (Table S1).

Area A had 32 more species than the other two sectors, despite its surface being 5 times smaller than that of area B and four times smaller than that of area C. The biodiversity richness observed in this disturbed sector may be due to the relatively wide variety of plant communities living in a small space. In fact, area A includes fragmented patchy grasslands, small shrublands and floodplain woods. Non-native invasive species (7: 8%), besides the already mentioned *E. canadensis* and *A. negundo*, included *E. annuus*, *Senecio inaequidens* DC., *V. persica*, and the woody species *Amorpha fruticosa* L. and *Parthenocissus quinquefolia* (L.) Planch.

Area B had the highest presence/percentage of non-native invasive species (6: 11%), which, besides *V. persica* and *A. fruticosa*, included *D. stramonium*, *Phytolacca americana* L., *Lonicera japonica* Thunb. and *Platanus hispanica* Mill. ex Münchh. Accordingly, area B is the one with the most surface bordering water bodies. Riparian habitats are known to be among the most subject to plant invasions [68–70] as they are assumed to be subject to high nutrient input and high disturbances caused by floods [68,71–73]; moreover, they are known to function as very effective ecological corridors for propagules of invasive species [74]. Therefore, the surface bordering with water bodies might be the reason for the higher number of invasive species in area A and area B. To this, it can can be added that non-native invasive plant species were well represented and prevalent in area B, where they are able to thrive in disturbed or resource-rich conditions [74], as well as settle in heavy-metal polluted environments [75–78] and highly tolerate pollution contexts [78,79].

Areas A and B likely share similar environmental pressures, including anthropogenic stressors such as pollution, geographical proximity (Figure 2) and a more similar set of habitats, which cause the similarity in flora composition.

Area C is slightly smaller than area B but hosts the same number of species and is the sector with the lowest presence/percentage of non-native invasive species (4: 7%). They include *Arundo donax* L., besides *E. annuus*, *V. persica* and *A. fruticosa*. Of special interest, area C hosts a population of *Narcissus pseudonarcissus* L. (Table S1), which is listed in Annex II of Council Directive 92/43/EEC ("Habitats Directive") under the synonym *N. pseudonarcissus* subsp. *nobilis* (Haw.) A. Fernández [80]. This nationally protected plant is native to Western Europe. Given the *N. pseudonarcissus* population's proximity to polluted zones, efforts should be directed toward the protection of this sector and the whole area from anthropogenic pollution that could alter plant communities, both directly by inducing toxic effects in organisms or eutrophication and indirectly by making the community more vulnerable to damaging factors. such as pathogens or invasive species (e.g., [81–84]).

4.2. Ecological Strategies: The Importance of Stress-Tolerant and Ruderal Strategies

The competitor, stress-tolerant and ruderal (CSR) theory aims to determine the ecological strategy of plant species, reflecting trade-offs in its functional traits in order to strive under conditions of competition, growth limitation mediated by abiotic factors or periodic biomass destruction [55]. This results in a tendency to invest in propagules in order to sustain the population in a disturbed environment (R-selection, "ruderals"); in resource retaining, metabolic performance optimisation and protection to survive in resource-poor or unstable environments (S-selection, "stress tolerators"); or in fast vegetative growth (C-selection, "competitors") [55]. The CSR theory has been used in the past as a proxy for observing vegetation response to environmental pollution [85], evaluating phytoremediation measures [86], describing the history of biological invasions [87] and predicting the restoration of plant communities [88]. Therefore, keeping track of community CSR strategies could serve as a bioindicator for ongoing changes or to predict community changes if environmental conditions change. The calculated CSR strategies are shown in ternary plots, suggesting a different strategy prevailing in herbaceous and woody species (Figure 7).

Among herbaceous species, S-selection combined with R-selection is the most common strategy in the study area, whereas species adopting a C-oriented strategy are present but less common in all the investigated sectors (4–9%). Moreover, C-selection is almost always mixed with S- and R-selection.

In area A, herbaceous flora mostly adopted SR-oriented ecological strategies (26%), with a consistent number of species leaning toward R-oriented (23%), S-oriented (15%) and CR-oriented strategies (15%). In area B, the majority of species (59%) adopted an R-oriented strategy, with other strategies being far less represented. In area C, the flora showed an ecological strategy selection similar to area A, with most species adopting SR-oriented (32%) and R-oriented (24%) strategies, with a similar percentage of species adopting S-oriented (16%) and CR-oriented strategies (12%).

These results could mean that area B could be the sector most subject to disturbance and of poor environmental quality due to the high percentage of strictly R-selected species. This is in agreement with the evidence that R-selected strategies are typical of disturbed areas [55,89] and that ruderal plant species are good indicators of decreasing environmental quality [20]. In contrast, area A and area C hosted a more diverse range of ecological strategies among herbaceous species.

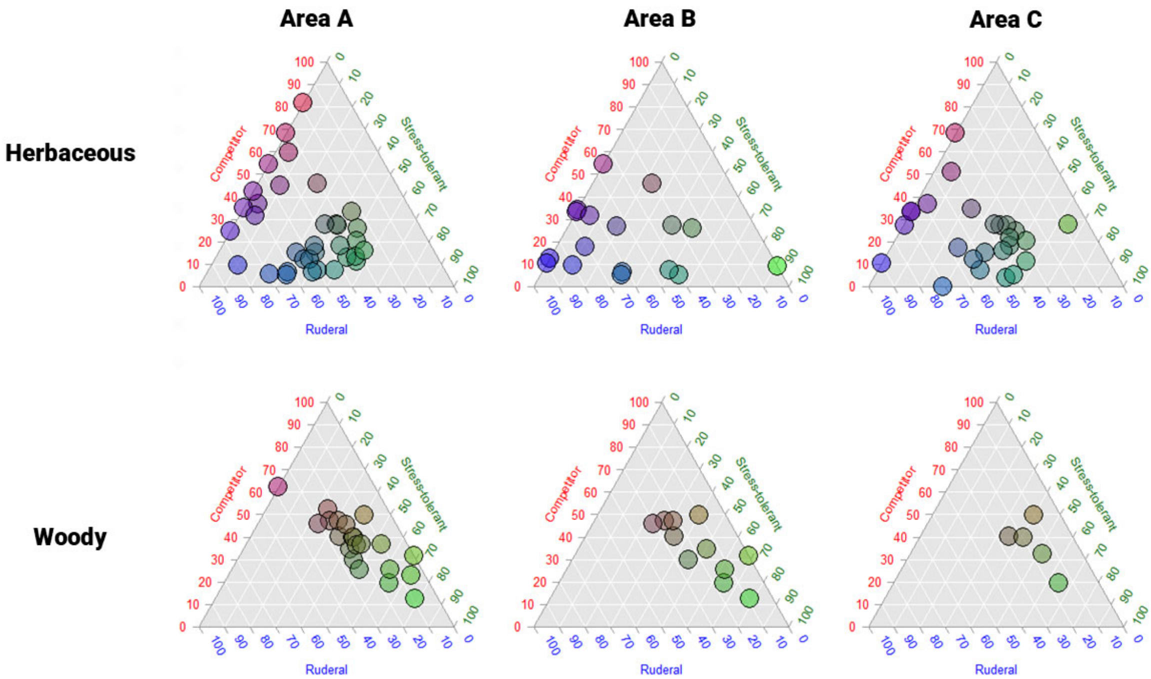

Figure 7. SIN "Laghi di Mantova e Polo Chimico": CSR ecological strategies of species found in the three sectors. The CSR strategy was calculated for 55 herbaceous species and 23 woody species observed in the surveys in the three sectors (area A, area B, area C). Main colours: competitor = red; stress-tolerant = green; ruderal = blue.

Generally, woody species exhibit C- and S-oriented strategies. According to Pierce et al. [55,89], woody species apparently tend to be competitors or stress-tolerant species, while ruderal trees are not common among the natives in Europe. The woody flora of the studied sectors is no exception: most of the woody species in every sector adopted a CS-oriented strategy, followed by strategies oriented toward C- and S-selection.

4.3. Empty Pollen as Evidence of Environmental Stress

Abnormal pollen was found in the species analysed from the three sectors in the SIN area studied (Table 3). The 'empty' versus 'total' pollen grains was significantly lower in samples gathered in area C (2.5% on average), whereas both area A and area B had relatively higher empty pollen (9.3% and 7.6% on average, respectively). The average percentage of empty pollen did not significantly differ between areas A and B. In our study, the plants living in the more polluted sectors produced a higher amount of non-fertile empty pollen grains. This suggests that they may suffer from environmental stresses with the production of some anomalous pollen in anthers, a phenomenon described in many different plant species/families.

Many reasons can be at the basis of anomalous pollen production in certain species, including plant age [90], pollen hydration status and environmental temperatures in *Corylus avellana* L. [91]. The mortality of pollen grains may result from errors in meiosis producing pollen tetrads with two dead grains in *Typha latifolia* L. [92]. Variations in the cytoplasmic content of pollen in anthers have been often described as being linked to some asynchronous division and delayed degradation of tapetal cells, resulting in the formation of abnormal and empty pollen in *Jasminum sambac* Aiton [93] and in *Dyckia*-Bromeliaceae [94]. A high incidence of deformed or anomalous pollen in wild plants of *Quercus*, *Betula* and *Crataegus* interspecific hybrids [91,95,96], and pollen grains with little or no cytoplasm in *Cornus mas* L. [97], *Actinidia* interspecific hybrids [98], *Prunus persica* (L.) Batsch [99], *Cucumis melo* L. [100], *Triticum aestivum* L. [101] and *Castanea sativa* Mill. [102] were also observed.

Regarding sampled species, in anthers of *Alopecurus myosuroides*, the exclusive presence of empty pollen grains was especially observed at the beginning of the flowering stage in plants that remained sterile. Pollen lethality varies greatly, with the proportion of empty/anomalous pollen ranging from 73% up to almost 0% [103]. In *Lamium purpureum*, pollen fertility is very high: average proportions of good pollen grains vary between 96.2% and 99.8%, with the percentage of empty grains being more than 5% in a single plant only in exceptional cases [104,105]. The percentage of pollen fertility estimated by glycerine jelly staining and observation in *Veronica persica* is 73.3% [106]. Although no systematic data of morphological pollen observations were found on *Amorpha fruticosa*, *Potentilla reptans* and *Prunus spinosa*, observations of reference slides show some presence of empty or abnormal pollen among the fresh pollen.

Nonetheless, reproductive processes in plants have been shown to be very sensitive to pollution and anthropic alterations of soil [3,67,107]. Anomalies and increases in pollen abortivity and non-viability have been linked to many pollution contexts. Some examples include *Viola tricolor* L., growing in Zn- and Pb-contaminated sites [67]; the synanthropic species *Artemisia vulgaris*, *Barbarea vulgaris* W. T. Aiton, *Chelidonium majus*, *Cichorium intybus* L., *Daucus carota*, *Lamium maculatum* L., *Lactuca serriola* L., *Melilotus albus*, and *Pastinaca sativa* L., growing near sites subject to road-traffic emissions [108]; and *Ballota nigra* L., *Berteroa incana* (L.) DC., *Calluna vulgaris* (L.) Hull., *Calystegia sepium*, *Carduus acanthoides* L., *Chelidonium majus*, *Echium italicum* L., *Linaria vulgaris* Mill., *Matricaria chamomilla* L., *Picris hieracioides* L., *Raphanus raphanistrum* L., *Sinapis arvensis* L., *Trifolium pratense* L., *Typha latifolia*, *Pinus sylvestris* L., *Pinus nigra* J. F. Arnold, and *Robinia pseudoacacia* L., growing in multi-metal-polluted soils near industrial sites [109].

Although the presence of empty pollen cannot be univocally correlated with a response to pollution, the interesting results obtained from our analyses show a positive relation between anomalous pollen and soil pollution. This supports the notion that the development of empty pollen grains is favoured by polluted soils.

5. Conclusions

Data analysis highlights interesting similarities between the polluted sectors (areas A and B), while the control sector (area C) has a quite different flora and lower empty/total pollen ratio. All the sectors host mostly native flora, with area B having the highest percentage of non-native invasive species and area C having the lowest. Most species in area C adopt competitor-oriented (C, CS) and stress-tolerant-oriented (S, CS) strategies, similarly to area A, whereas the majority of species in area B adopted an R-oriented (R, CR, SR) strategy. The flora analysis highlights some differences in species present in the three sectors and the ecological strategies they adopt. As area B has a heavily ruderal flora, it is suggested that this sector is more subject to disturbance than the other two.

Although it is not possible to consider the mere presence of empty pollen in some species an indicator of pollution in an area, the general incidence of empty pollen in the studied species was higher in polluted sectors than in the control sector. Differences were recorded between areas rather than between species. Therefore, in this study, given the statistically significant difference in the average incidence of empty pollen grains between area C and the other two areas, as well as the absence of a statistically significant difference between area A and area B, there is first evidence that local pollution might be the reason why the studied species show increased pollen abortivity when collected in the polluted areas. Our data are in agreement with evidence of the relationships between pollen viability and morphology in air pollution conditions [110–112].

A botanical approach, intended as the floristic analysis of a site, highlighting the species occurrence status of plant species and their ecological strategy, is known to be a good way to assess environmental quality, making the whole flora of a site a bioindicator itself. The integrated floristic and palynological study presented in this paper, coupling flora analyses with rapid pollen screenings, may be a way to quickly and cheaply identify sites under pollutant stress, potentially addressing the need for further environmental assessment analyses. This approach might also be used as a low-cost and fast method to continuously check plant health in known polluted sites, possibly also using flora and pollen as proxies to assess the severity of pollution affecting the site or the effectiveness of any remediation measure taken over time.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15129414/s1, File S1: TRY Plant Trait Database contributing datasets; Table S1: Flora, occurrence and CSR strategies; Table S2: Pollen analysis data.

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