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Evaluation of the Richness, Species Diversity, and Biosafety of Saprotrophic Fungal Communities in Constructed Wetlands and Biological Wastewater Ponds

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Abstract: The present study was focused on the characterization of the biocenotic diversity of saprotrophic fungi, taking into account the impact of various treatment systems and technological stages as well as the physicochemical properties of wastewater. The biodegradation potential, the hazard posed to humans, animals, and the environment, and the effectiveness of elimination of propagules were evaluated. The most effective elimination of fungal propagules was recorded in hybrid constructed wetland systems with horizontal (HF-CW) and vertical (VF-CW) wastewater flow, especially in the VF-HF objects. The fungal communities present in wastewater from small constructed wetland (CW) and wastewater stabilization ponds (WSP) were dominated by ubiquitous terrestrial molds accompanied by a minimal number of yeasts. The similarity of the species composition of the fungal communities between the treatment plants was generally low, whereas the species diversity together with the population size was very high at the various stages of wastewater treatment. Species with potential pathogenicity to humans and animals accounted for over 45%, i.e., were classified as BioSafety Level 1 and 2 (BSL-1 and BSL-2 groups), and potentially phytopathogenic fungi represented 31.5% of the mycobiota species composition. The dynamics of fungal growth were correlated with the content of organic pollutants and nutrients (nitrogen and phosphorus) and with oxygen deficiency. The accumulation of nitrates corresponded to the decline in the frequency of fungi in treated wastewater. The lowest efficiency of the removal of fungi was exhibited by the biological wastewater stabilization ponds.

Keywords: constructed wetlands (CW); biological wastewater stabilization ponds (WSP); saprotrophic fungi; biodiversity; hazards



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1. Introduction

The so-called small domestic wastewater treatment plants include collective treatment plants, i.e., wastewater stabilization ponds neutralizing wastewater supplied from a small area by a local sewer system or transported wastewater, and domestic wastewater treatment plants. Special interest has been aroused by wastewater treatment technologies referred to as “constructed wetlands” (CW), i.e., artificial systems reflecting natural processes occurring in wetland ecosystems based on the involvement of microorganisms and aquatic or hygrophilous plants [1–5]. In practice, mainly hybrid soil plant treatment plants with subsurface wastewater flow are used. The older types of these systems, referred to as one-stage treatment plants, were based on a one soil-plant bed (usually comprising common reed or willow plants) and a horizontal wastewater flow system (HF-CW “horizontal flow constructed wetland”), or sometimes a vertical flow system (VF-CW “vertical flow constructed wetland”) [3]. More recent technologies (multistage or hybrid wastewater

treatment plants) consist of two or three beds with a horizontal and vertical (HF-CW and VF-CW) wastewater flow. Multistage sewage treatment plants facilitate more effective biological treatment of wastewater in comparison with one-stage treatment plants [3]. CW systems are regarded as green technologies that are eco-friendly and effective in the treatment of domestic or municipal wastewater containing large amounts of organic matter, industrial wastewater contaminated by heavy metals, petroleum, hydrocarbons, or pharmaceuticals, and agricultural wastewater [6].

Investigations of the effectiveness of neutralization and removal of pollutants in wastewater treatment plants located in areas with no sewer systems are mainly focused on the removal of organic pollutants as well as nitrogen and phosphorus, i.e., factors responsible for water eutrophication and soil contamination [1,3,4,7–12]. Given the fecal load and contamination with intestinal bacteria in domestic wastewater, greater attention has been paid to the characterization and elimination of these microorganisms, including pathogenic bacteria [12–21]. In turn, microscopic fungi present in constructed wetland systems have been investigated less widely. As reported by Kharitonov et al. (2022) [21], most of these studies were focused on endomycorrhizal fungi (VAM fungi) associated with hydrophytes. Only a few reports [20–22] have described saprotrophic fungal communities. Many of these organisms (e.g., *Candida albicans* yeasts) or molds (e.g., dermatophytes and *Aspergillus fumigatus*) are dangerous opportunistic pathogens and causative agents of systemic and cutaneous mycoses in humans and animals. Domestic wastewater is also a source of plant pathogenic fungi, e.g., various *Fusarium* species that are detected most frequently [23]. On the other hand, filamentous fungi in consortia with other microorganisms are involved in the removal of organic compounds, C, N, and other nutrients [20] through incorporation thereof into their biomass [24] and via a co-metabolic pathway [25]. Fungi play a very important role in the biodegradation of complex and hardly biodegradable organic compounds, e.g., lignin and other aromatic compounds of plant origin or hair keratin. The biodegradation process is facilitated by the production of a wide range of degradation enzymes, i.e., polyphenol oxidases (peroxidases and laccase) or keratinolytic proteases by these microorganisms [25–28]. These properties allow fungi to remove not only environmental pollutants of natural origin, but also many xenobiotics, which is the main rationale behind the use of these microorganisms in the bioremediation process [25].

The research hypothesis formulated in the present study assumes that (1) the mycobiota of small domestic wastewater treatment plants, such as biological wastewater ponds and constructed wetlands, is dominated by microfungi of terrestrial origin, including potential human and animal pathogens and phytopathogens, and (2) the wastewater treatment plant type and the physicochemical properties of wastewater have an impact on the quantitative and qualitative composition of the mycobiota and the effectiveness of removal of these microorganisms from wastewater.

This study provides a comprehensive analysis of the species richness and diversity of saprotrophic fungal communities present in domestic wastewater treated in small treatment plants: biological wastewater pond systems (WSP) and several constructed wetland (CW) facilities, taking into account the different stages of wastewater treatment and seasonal dynamics.

2. Materials and Methods

2.1. Experimental Object

The study was carried out in six small domestic wastewater treatment plants located in dispersed development areas in the Lublin Province (south-eastern Poland). Five of them were one-stage wastewater treatment plants and hybrid constructed wetlands located in Leitnie (I), Jastków (III), Dąbrowica (IV), Janów (V), and Sobieszyn (VI). Additionally, the research was carried out in wastewater stabilization ponds (WSP) in Ludwin (II). The location of the analyzed wastewater treatment plants is shown in Figure 1, and their basic technological parameters are presented in Table 1.

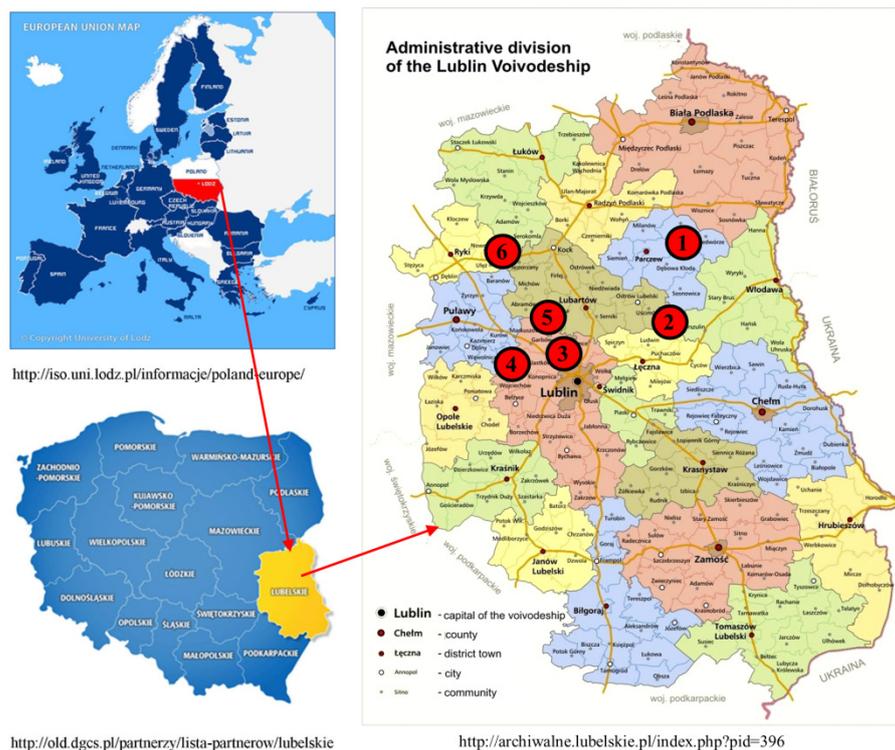


Figure 1. Location of the analyzed wastewater treatment plants; 1—Leitnie; 2—Ludwin; 3—Jastków; 4—Dąbrowica; 5—Janów; 6—Sobieszyn.

Table 1. Technological parameters of the studied small wastewater treatment plants in Lublin Province, Poland; VF—vertical flow, HF—horizontal flow.

Technological Parameters	Number and Name of the Object					
	(I) Leitnie	(II) Ludwin	(III) Jastków	(IV) Dąbrowica	(V) Janów	(VI) Sobieszyn
Capacity (m ³ /d)	150	137	1.2	0.6	0.45	38
Plants	reed <i>Phragmites australis</i> car. Trin. Ex Steud	-	willow <i>Salix viminalis</i> L.	reed <i>Phragmites australis</i> car. Trin. Ex Steud and willow <i>Salix viminalis</i> L.	reed <i>Phragmites australis</i> car. Trin. Ex Steud and willow <i>Salix viminalis</i> L.	reed <i>Phragmites australis</i> car. Trin. Ex Steud
Active capacity of septic tank [m ³]	-	-	13.7	4.6	8.5	75.0
Number of beds/ponds	2 beds	4 ponds	1 bed	4 beds	2 beds	4 beds
Area of beds/ponds [m ²]	A—800 B—4000	A—2030 B—666 C—666 D—2611	186	(A) VF-HF—48 (B) HF-VF—48	VF = 18 HF = 30	A—307 B—328 C—331 D—321
Depth of bed/pond [m]	0.8	A—3.0 B—3.0 C—3.0 D—1.8	1.1	VF = 0.8 HF = 1.0	VF = 0.8 HF = 1.0	0.8
Method of wastewater inflow	gravitational	pump	gravitational	pump	pump	pump

Table 1. Cont.

Technological Parameters	Number and Name of the Object					
	(I) Leitnie	(II) Ludwin	(III) Jastków	(IV) Dąbrowica	(V) Janów	(VI) Sobieszyn
Capacity (m ³ /d)	150	137	1.2	0.6	0.45	38
Wastewater receiver	drainage ditch	drainage ditch	pond	soil	soil	drainage ditch
Average hydraulic load [m ³ /m ² /d]	0.031	-	0.007	0.012	0.037	0.014
Hydraulic retention time in the bed [day]	8	in summer—49 in winter—98	36	VF = 9.6 HF = 16	VF = 9.6 HF = 13.4	23

Three of the constructed wetlands (I, III, and VI) were one-stage wastewater treatment plants based on the use of one plant species. The other two (IV and V) represented a multistage soil-plant treatment plant using two plant species. The wastewater stabilization pond (II) consisted of a system of four biological aerobic and anaerobic ponds.

The one-stage sewage treatment plant in Leitnie (I) established in 1992 is characterized by a horizontal sewage flow (HF CW) and the use of *Phragmites australis* car. Trin. Ex Steud. It treats domestic wastewater delivered by septic tankers from the septic tanks of private farms, schools, recreation centers, small agricultural and food industry plants, and health care facilities. The maximum capacity of the treatment plant is 150 m³ · d⁻¹ (Table 1).

The wastewater stabilization pond (WSP) established in 1992 (object II in Ludwin) (Table 1) is a thorough treatment plant receiving wastewater from the sewer system (up to 90 m³ · d⁻¹) and wastewater transported from holiday resorts (approx. 150 m³ · d⁻¹).

Object III established in 1994 (Jastków) is based on the technological one-stage soil-plant system with a horizontal sewage flow (HF CW) and *Salix viminalis* L. It is intended for treatment of domestic wastewater from a 10-person household. Its maximum treatment capacity is 2 m³ · d⁻¹ (Table 1).

The multistage treatment plant IV (Dąbrowica) established in 2006 has two beds for a horizontal and vertical wastewater flow (VF-HF CW; HF-VF CW) and is based on the use of reed and willow. It is intended for treatment of wastewater from an individual 6-person household. The maximum capacity of the treatment plant is 0.6 m³ · d⁻¹ (Table 1).

The multistage soil-plant household wastewater treatment plant in Janów established in 2008 (object V) has one bed for a horizontal and vertical wastewater flow (VF-HF CW) and is based on the use of reed and willow. It operates in a 3-person household with a maximum capacity of 0.45 m³ · d⁻¹ (Table 1).

Object VI (Sobieszyn) established in 1995 is a one-stage wastewater treatment plant with a vertical flow (VF CW) and with the use of reed. It is intended for treatment of domestic wastewater discharged from a school complex. The maximum capacity of this treatment plant is 60 m³ · d⁻¹ (Table 1). A collective list of the technological parameters of all the small wastewater treatment plants is presented in Table 1.

2.2. Study Material

Raw wastewater samples collected at the individual treatment stages and treated wastewater samples were analyzed in the study. The types of samples collected from each treatment plant and their numbers are shown in Table 2. The wastewater samples were collected in accordance with Polish standards (PN-74/C-04620/00, PN-EN 25667-2: 1999) [29,30] in 4 terms: February, May, August, and November in 2008–2009. The samples were collected in sterile 100 cm³ glass bottles. Five 100-cm³ samples were collected from each type of sewage in each collection round. In the laboratory, the samples were combined into one aggregate sample (500 cm³) in sterile 1000 cm³ plastic bottles. Next, the

aggregate samples were shaken for 10–15 min to remove the microorganisms from the solid suspension and disperse the fungi evenly in the liquid suspension.

Table 2. Types of sewage samples taken for analyses from individual treatment plants and numbered; “-” not tested.

Treatment Plants (No.)	Type of Wastewater	Sample Number
Leitnie (I)	raw wastewater	1
	treated wastewater	2
Ludwin (II)	raw wastewater	3
	Wastewater—1 pond (anaerobic)	4
	wastewater post biological stage—post 2nd oxygen pond	5
	treated wastewater—sedimentation pond	6
Jastków (III)	raw wastewater	7
	wastewater post mechanical stage (settler)	8
	wastewater post biological stage (bed with willow)	9
	wastewater post chemical stage (carbonate silica rock—opoka)	10
	pond water inflow—inflow from a deposit with carbonate silica rock—opoka	11
	pond water outflow—outflow to the river	12
Dąbrowica (IV)	raw wastewater	13
	wastewater post mechanical stage (settler)	14
	wastewater post biological stage—bed A with willow (system I)	15
	wastewater post biological stage—bed B with reed (system I)	16
	wastewater post biological stage—bed C with reed (system II)	17
	wastewater post biological stage—bed D with willow (system II)	18
Janów (V)	wastewater post chemical stage (carbonate silica rock—opoka)	19
	raw wastewater	20
	wastewater post mechanical stage	-
	wastewater post biological stage—bed A with reed	22
	wastewater post biological stage—bed B with willow	23
Sobieszyn (VI)	wastewater post chemical stage—bed with (carbonate silica rock—opoka)	24
	raw wastewater	25
	treated wastewater	26

The research material prepared in this way was subjected to mycological and physico-chemical analyses. The selected physicochemical properties of the raw domestic wastewater flowing into the constructed wetlands and the wastewater stabilization pond, and the properties of treated wastewater are shown in Table 3. The physicochemical analyses were performed at the Water and Wastewater Analysis Laboratory of the University of Life Sciences in Lublin (Poland) in accordance with the methodology specified by PN-73/C-04537.01—“Water and Wastewater. Determination of the content of phosphorus compounds. General provisions and scope of the standard” [31], PN-73/C-04576.00—“Water and wastewater. Determination of the nitrogen content, General provisions and scope of the standard [32]”, and “Reference methodologies for analysis of wastewater samples” specified by the Regulation of the Minister of the Environment 2006, Annex 10 (Poland) [33].

Table 3. Characteristics of the physicochemical properties (mean values) of raw and treated domestic wastewater from small wastewater treatment plants (2008–2010).

Parameters	Number and Name of the Object											
	Leitnie (I), (n = 11)		Ludwin (II), (n = 11)		Jastków (III), (n = 11)		Dąbrowica (IV), (n = 11)		Janów (V), (n = 10)		Sobieszyn (VI), (n = 11)	
	A	B	A	B	A	B	A	B	A	B	A	B
temperature	17.0 ± 4.7	17.9 ± 4.3	17.3 ± 4.7	16.9 ± 5.4	17.0 ± 4.4	15.7 ± 4.6	16.8 ± 4.2	15.2 ± 4.5	17.4 ± 4.1	16.0 ± 4.8	17.1 ± 4.3	14.8 ± 3.3
pH	1 2 7.07–8.30 ± 0.39	1 2 7.15–8.04 ± 0.33	1 2 7.00–7.65 ± 0.20	1 2 7.79–8.26 ± 0.14	1 2 6.89–7.21 ± 0.12	1 2 7.17–8.33 ± 0.31	1 2 7.10–8.04 ± 0.30	1 2 7.17–9.65 ± 0.96	1 2 7.18–7.98 ± 0.25	1 2 7.08–9.45 ± 0.70	1 2 7.08–7.55 ± 0.14	1 2 7.18–7.83 ± 0.21
O ₂ (mg · dcm ⁻³)	0.22 ± 0.18	2.17 ± 2.63	0.41 ± 0.33	6.18 ± 3.02	0.27 ± 0.09	2.58 ± 1.41	0.29 ± 0.15	7.14 ± 2.84	0.30 ± 0.13	3.40 ± 1.55	0.29 ± 0.16	7.08 ± 1.52
general suspensions (mg · dcm ⁻³)	1302 ± 1777	155 ± 132	164 ± 50	28.6 ± 23.5	360 ± 337	21.9 ± 15.3	284 ± 127	17.3 ± 11.0	226 ± 212	12.5 ± 8.1	155 ± 90	32.9 ± 22.9
BOD5 (mgO ₂ · dcm ⁻³)	525 ± 200	116 ± 70	191 ± 79	19.5 ± 12.5	341 ± 185	14.1 ± 6.8	267 ± 69	6.8 ± 10.1	279 ± 79	7.2 ± 11.5	181 ± 57	22.6 ± 23.0
COD (mgO ₂ · dcm ⁻³)	1065 ± 422	300 ± 244	372 ± 80	90 ± 27	639 ± 289	46.4 ± 20.5	593 ± 214	31.6 ± 23.8	500 ± 100	30.7 ± 26.5	381 ± 145	63 ± 37
N og. (mg · dcm ⁻³)	179 ± 47	74 ± 38	98 ± 16	63 ± 18	97 ± 31	36.9 ± 16.8	171 ± 36	58 ± 23	81 ± 17	24 ± 7	73 ± 28	39.5 ± 22.0
N-NH ₄ (mg · dcm ⁻³)	127 ± 32.9	43 ± 33	80 ± 21.2	10.77 ± 11.85	66.4 ± 16.4	2.78 ± 6.3	129 ± 20.3	9.7 ± 13.5	63.3 ± 19.0	6.6 ± 7.6	55.4 ± 18.4	29.5 ± 17.9
N-NO ₃ (mg · dcm ⁻³)	1.29 ± 3.12	15.62 ± 25.48	0.38 ± 0.37	10.77 ± 11.85	1.10 ± 0.88	0.89 ± 1.26	0.91 ± 0.86	38.8 ± 21.9	1.34 ± 1.17	14.2 ± 8.6	0.21 ± 0.18	9.95 ± 14.10
P og.(mg · dcm ⁻³)	61.3 ± 17.7	33.8 ± 17.8	29.9 ± 3.9	10.2 ± 3.1	37.1 ± 11.0	7.6 ± 2.2	48.0 ± 6.9	4.4 ± 4.1	28.9 ± 5.7	1.0 ± 1.0	27.8 ± 18.9	8.7 ± 4.9

Notes: A—raw wastewater; B—treated wastewater; 1—minimum value; 2—maximum value.

The analyses consisted in the following measurements: wastewater temperature (mercury thermometer), pH—potentiometric method, dissolved oxygen concentration—WTW multi-parameter Multi 340i meter (WTW, Weilheim, Germany), total suspended solids—direct weight method with the use of medium quantitative filters, 5-day biochemical oxygen demand (BOD₅)—dilution method with the use of the WTW Oxi 538 oxygen meter (WTW, Weilheim, Germany) to determine the oxygen content, chemical oxygen demand (COD_{Cr})—dichromate method with the use of the MPI 2010 photometer from WTW (WTW, Weilheim, Germany) after oxidization of samples in a thermoreactor at 148 °C, total nitrogen—AQUALYTIC PC spectrophotometer (AQUALYTIC, Dortmund, Germany) after oxidization of samples in a thermoreactor at 100 °C, phosphate content—WTW MPI 2010 photometer (WTW, Weilheim, Germany), ammonia—AQUALYTIC PC spectrophotometer (AQUALYTIC, Dortmund, Germany), and nitrates—Slandi LF photometer (Slandi, Michałowice, Poland).

2.3. Methods for Isolation and Identification of Fungi

Filamentous fungi (molds) and yeasts were isolated with the plate dilution method. The well-established plate count method is useful and widely applied in studies of culturable bacteria and fungi in wastewater treatment plants [7,18]. The fungal isolation was carried out using the Martin medium [34] for saprotrophic fungi and the Sabouraud medium [35] for potential human and animal fungal pathogens. A volume of 10 cm³ was collected from each pooled sample and a series of 10th dilutions were prepared. In the case of treated sewage, the starting (undiluted) material was analyzed as well. For isolation of fungi, 1 cm³ of the dilution (and/or undiluted sample) was collected and supplemented with an appropriate selective medium with uniform mixing. Saprotrophic fungi were grown for 5–7 days at 25 °C, and potentially pathogenic fungi were cultured for 3–7 days at 30 °C. In all cases, three parallel plate replicates were used. Colonies of mold fungi growing on the Martin medium were transferred (10⁻²–10⁻³ dilutions) onto slants with glucose–potato (PDA) medium composed of (g · dcm⁻³) potatoes—250, glucose—2, agar—20, and distilled H₂O—1 dcm³. Colonies of mold fungi from the Sabouraud medium were transferred (10⁻²–10⁻³ dilutions) onto slants with the same medium composition but without antimicrobial antibiotics. Yeast colonies were transferred onto wort–agar slants composed of (g · dcm⁻³) wort 5%—1 dm³ and agar—20 g. The fungal genus and species composition was determined by collecting all colonies from one randomly selected plate (3 replicates per series) when there were at least 20 colonies on the plate. When there were several colonies on the plates, all colonies from 3 replicates were collected.

Filamentous fungi were identified based on macromorphological features on the slants and/or plates as well as micromorphological features in the microcultures. Yeasts and yeast-like fungi were identified using Fungichrom I biochemical assays (EliTech France SAS) and observations of morphological features in intravital preparations and microcultures. The final genus and species classification was conducted based on various systematic studies of microscopic fungi [36–39]. The names of fungal species were verified in the Index Fungorum (<http://www.indexfungorum.org> accessed on 25 August 2021) using both names in the species list. The older but still accepted nomenclature of species was used in the analysis of the fungal collection.

2.4. Analysis of Results

To determine the dominant fungal genera and species, the coefficients of genus and species dominance (D) were calculated using the formula [40]:

$$D = 100 (Sa:S)$$

where Sa—sum of isolates of genus or species a; S—sum of isolates of the studied group of fungi (molds, yeasts). The following scale was adopted to assess the frequency of the genera and species of filamentous fungi and yeasts: < 1%—sporadic, 1–5%—rare, 6–10%—frequent, 11–25%—very frequent, 26–50%—abundant, and >50%—mass occurrence [41].

The Marczewski-Steinhaus formula was used to determine the species similarity between the fungal communities from the analyzed treatment plants [42]:

$$S(1,2) = w / (a + b - w)$$

where $S(1,2)$ —similarity of species from two communities, a —number of species in community 1, b —number of species in community 2, w —number of species common to both communities. If both sets have all elements in common, the similarity is $S = 1$, i.e., 100%; in turn, $S = 0$ when there are no common elements for both sets.

The analysis of the species diversity of the fungal communities, taking into account the number of isolates (strains) of individual species present in raw wastewater, detected at the subsequent stages of treatment, and present in the entire wastewater treatment plant, was carried out by calculation of the Simpson coefficient (based on the probability theory) according to the following formula [43]:

$$D = \sum_{i=1}^s \left(p_i^2 \right)$$

where D —species diversity coefficient, p_i —isolates (strains) of species “ i ” in the fungal community, with $p_i = n_i/N$ (n_i —number of strains of species “ i ”, N —total number of species). The values of the Simpson coefficient are in the range from 0 (low diversity) to $1-1/S$, and S is the number of species in the community. A greater diversity is reflected by a greater Simpson coefficient value.

2.5. Statistical Analysis

Significant differences between the total frequency of fungi (total values for the genera) and the specific stage of wastewater treatment were demonstrated with the use of one-way ANOVA followed by a post-hoc test and Fisher’s test at the significance level of $\alpha = 0.05$

The Pearson correlation coefficient (r) was calculated at the significance level $\alpha = 0.05$ to establish the correlation between the total fungal frequency and the physicochemical parameters of the raw and treated wastewater.

The Principal Component Analysis (PCA) was carried out to determine the correlation between fungal populations that were most frequently recorded in the wastewater (dominant species) and the wastewater treatment system (treatment plant type). In this experiment model, two graphs should be read together: the score plot and the loading plot. The score plot is a map of observations and their location with respect to each other based on the variables in the loading plot [44]. The closer distance of variable p (type of treatment plant) in the loading plot to observation n (fungal species) in the score plot or the location of the data in the same part of the graph indicates a greater impact of the variable on the observation. The loading plot shows the impact of the variable (type of treatment plant) on the individual principal component (PC) and indicates correlations between the variables.

The STATISTICA v.12.0 software (StatSoft Poland) was used in the statistical analysis of the research results.

3. Results

3.1. General Characteristics of Mycobiota in Constructed Wetlands and Wastewater Stabilization Ponds

In the two-year study (2008–2009), 10 499 fungi were identified on the two isolation media (Martin medium—2008–2009, Sabouraud medium—2008) in four terms each year (February, May, August, November). In this group, there were 10 044 (95.7%) molds and 455 (4.3%) yeasts. The collected strains represented 41 genera and 102 species of terrestrial (non-aquatic) filamentous fungi as well as 4 and 9 yeast taxa (Table S1—Supplementary Material). The total number of mold genera in the analyzed treatment plants was similar and ranged from 18 (one-stage treatment plants with reed—I, VI) to 24 (treatment plants with willow—III, IV, V). Yeasts were represented by 3 genera and 4–5 species, except for the

treatment plants I and VI, where from 2 to 4 species were isolated (Table S1). The dominance coefficients (Table S2—Supplementary Material, Figure 2) indicated that seven genera, i.e., *Acremonium*, *Aspergillus*, *Fusarium*, *Geotrichum*, *Penicillium*, *Phoma*, and *Trichoderma*, accounted for 82% of the total number of the isolated strains of filamentous fungi. The most frequently isolated strains represented the genus *Penicillium* and *Trichoderma*, which were classified as abundant (29% of molds) and very frequent, respectively. Six other genera accounting for 12%, i.e., *Alternaria*, *Gliocladium*, *Mucor*, *Paecilomyces*, *Phialophora*, and *Verticillium*, were classified as rare (1–5%). The other 28 genera representing 68% of the total genus composition of the filamentous fungal biota were classified as sporadic (<1%). Their total frequency corresponded to 5.12% of the total number of isolated molds (Table S2, Figure 2). The yeast populations were characterized by the highest proportion of *Rhodotorula* (40%), including *Rh. rubra* (97% of the genus).

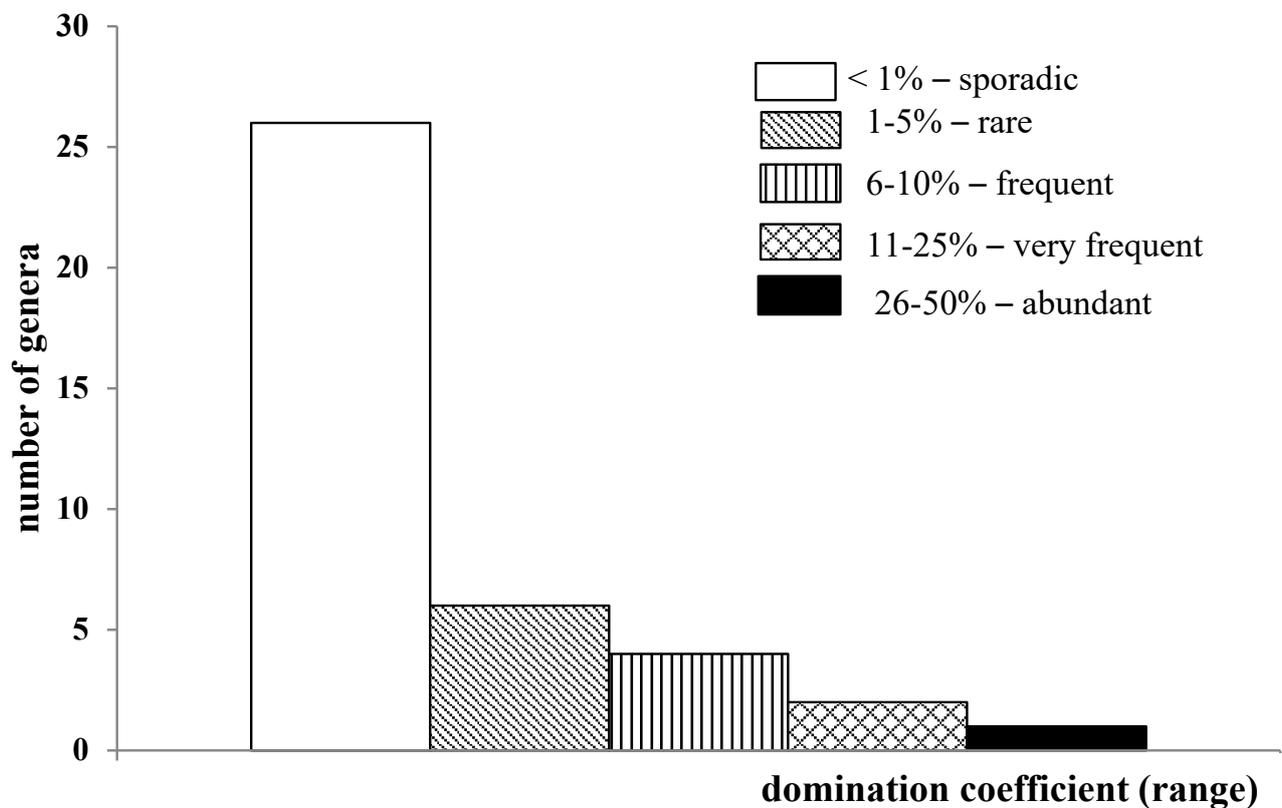


Figure 2. Frequency of genera of filamentous fungi recorded in small sewage treatment plants; *Cladosporium*, *Beauveria*, *Bjerkandera*, *Botrytis*, *Broomella*, *Chaetomium*, *Cladosporium Colletotrichum*, *Chrysosporium*, *Cylindrocarpon*, *Dactylaria*, *Doratomyces*, *Epicaccum*, *Isaria*, *Mariannea*, *Mortierella*, *Pithomyces*, *Pseudeurotium*, *Pythium*, *Rhizoctonia*, *Scedosporium*, *Sclerotinia*, *Scopulariopsis*, *Sporothrix*, *Talaromyces*, *Thielavia*, *Tilachlidium*, *Torula*, *Trichocladium*; *Alternaria*, *Gliocladium*, *Mucor*, *Paecilomyces*, *Phialophora*, *Verticillium*; *Acremonium*, *Aspergillus*, *Fusarium*, *Geotrichum*; *Phoma*, *Trichoderma*; *Penicillium*.

The genus *Candida* (approx. 34% of yeast records) was the second largest population with the dominant *C. guilliermondii* species (49% of the genus). The other two genera, *Saccharomyces* and *Trichosporon*, were less numerous (Table S3, Figure 3). Taxonomically, the genus *Geotrichum* is classified as yeast-like fungi (Ascomycota-Sacharomycetales). However, due to the absence of budding forms and the presence of typical hyphae, this genus was morphologically assigned to filamentous fungi in this study. The analysis of the species structure of the investigated wastewater mycobiota indicated the presence of single mold species with a low frequency among the dominant genera (Table S1, Table 4). In the case of the genus *Penicillium*, three populations, i.e., *P. chrysogenum*, *P. expansum*, and *P. verrucosum*,

accounted for over 60% of the total number of these fungi. The same proportion was recorded in the case of three of the five identified *Acremonium* species (*A. kiliense*, *A. murorum*, and *A. strictum*). One population was selected within the other five dominant genera, i.e., *G. candidum* (84.4% of the genus population), *Ph. herbarum* (87.7%), *T. viride* (99.3%), *A. fumigatus* (70.1%), and *F. solani* (approx. 50%). In total, the populations of the 11 aforementioned species represented approx. 60% of the total number of micromycetes present in the wastewater treatment plants (Table 4).

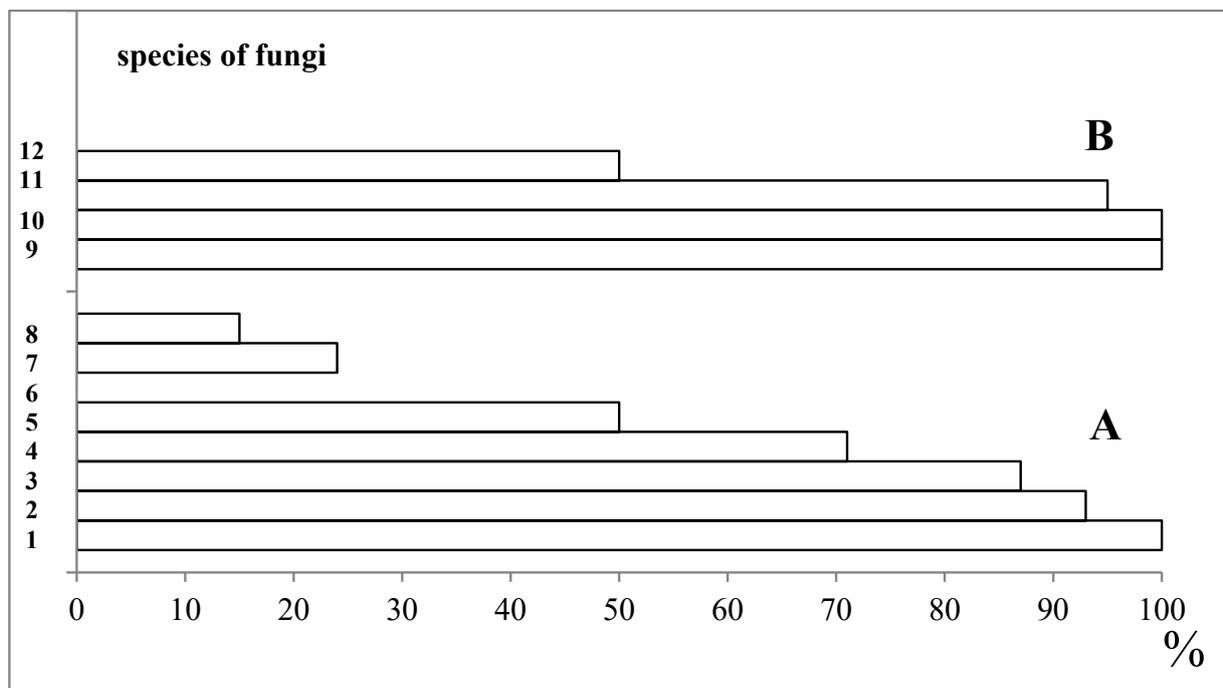


Figure 3. Share (w%) of dominant species in the genus structure; (A)—filamentous fungi, (B)—yeasts; (1) *Trichoderma viride*, (2) *Phoma herbarum*, (3) *Geotrichum candidum*, (4) *Aspergillus fumigatus*, (5) *Fusarium solani*, (6) *Penicillium expansum*, (7) *P. verrucosum*, (8) *P. chrysogenum*, (9) *Saccharomyces cerevisiae*, (10) *Trichosporon beigelii*, (11) *Rhodotorula rubra*, (12) *Candida guilliermondii*.

In terms of systematics, the fungi isolated in the domestic raw and treated wastewater were classified into 20 orders from 4 divisions: Ascomycota, Zygomycota, Basidiomycota, and Oomycota. The majority of the fungi (14 orders) represented Ascomycota; the other orders belonged to 3 Basidiomycota, 2 Zygomycota, and 1 Oomycota divisions (Table S1). Assuming that the taxonomic structure of fungal communities is reflected in the proportional share of the number of species from a given taxonomic group (order) in the total number of species expressed in % [41], it was calculated that 90% of the mycocenosis of the six small wastewater treatment plants represented Ascomycota. The most numerous were Hypocreales (26.17% of all micromycetes) and Eurotiales (22.43%). Pleosporales and Saccharomycetales (8.42% each) as well as Microascales and Glomerellales (5.61% each) were classified as frequent orders (Table S1). In the spatial structure [41] referred to as the proportional share of the number of isolates (= number of records) per representatives of the same taxonomic group (order) compared to the total number of isolates expressed in %, the dominance and co-dominance hierarchy was similar to that in the taxonomic structure. The highest number of propagules was found in the case of Eurotiales and Hypocreales (34.08% and 30.66% in the total micromycete community, respectively). Saccharomycetales (10.9%) and Pleosporales (9.9%) were subdominant orders (Table S1).

Table 4. Frequency and share (in %) of the dominant species of filamentous fungi (at least 3% of total molds); I—Leitnie, II—Ludwin, III—Jastków, IV—Dąbrowica, V—Jastków, VI—Sobieszyn; 1—number of strains isolated on Martin medium in 2008, (2) number of strains isolated on Martin medium in 2009, (3) number of strains isolated on Sabouraud medium in 2008.

No.	Strains		Wastewater Treatment Plants (No.)						Total	%
			I	II	III	IV	V	VI		
1.	<i>Aspergillus fumigatus</i>	1	0	5	15	58	31	0	109	3.61
		2	17	42	41	7	33	40	180	
		3	0	6	13	9	26	20	74	
								363		
2.	<i>Fusarium solani</i>	1	15	29	9	0	0	15	68	4.370
		2	4	2	11	0	0	18	35	
		3	4	40	63	146	49	63	365	
								468		
3.	<i>Geotrichum candidum</i>	1	38	25	99	189	0	44	395	8.19
		2	34	37	13	113	44	21	262	
		3	22	11	63	38	11	21	166	
								823		
4.	<i>Penicillium chrysogenum</i>	1	7	19	39	71	1	6	143	4.62
		2	0	0	15	14	10	0	39	
		3	0	31	121	44	64	23	283	
								465		
5.	<i>P. expansum</i>	1	9	27	30	52	1	11	130	6.64
		2	6	68	18	36	21	0	149	
		3	0	74	216	55	27	16	388	
								667		
6.	<i>P. verrucosum</i>	1	8	28	54	35	0	19	144	6.32
		2	28	49	114	47	89	8	335	
		3	0	13	101	37	0	5	156	
								635		
7.	<i>Phoma herbarum</i>	1	0	0	0	0	0	5	5	7.23
		23	15	49	211	83	126	148	722	
			0	0	0	0	0	0	0	
								727		
8.	<i>Trichoderma viride</i>	1	53	109	254	66	133	56	671	15.01
		2	27	194	29	16	90	5	361	
		2	93	72	139	61	90	21	476	
								1508		
9.	<i>Verticillium lecanii</i>	1	9	101	38	90	14	5	257	3.51
		23	10	0	0	0	4	0	14	
			0	65	0	17	0	0	82	
								353		
TOTAL			489	1096	1706	1284	864	570	6009	59.83

3.2. Species Similarity, Diversity, and Determinants of the Growth of Fungal Communities in the Wastewater Treatment Plants

It was shown (Table 5) that the total coefficients of the species similarity in the fungal communities inhabiting the different types of small wastewater treatment plants were relatively low, as they exceeded 50% in only one case (treatment plants III and IV). Greater similarities in the species composition were recorded in the spring–summer period (May,

August) than in the autumn–winter period (November, February) (Table 5). The wastewater treatment plants II and V as well as I and II were similar in terms of the presence of dominant species. This was indicated by the location of the variables in the loading plot (PCA analysis). The analysis also showed that three of the 11 dominant species, i.e., *T. viride*, *Ph. herbarum*, and *P. verrucosum*, were closely associated with a specific treatment plant. Their occurrence was correlated with treatment plants I and II (Leitnie and Ludwin), VI (Sobieszyn), and IV (Dąbrowica), respectively, as evidenced by the compatibility of the position of the variables in the loading plot with the data in the score plot (Figure 4). Very high values were obtained for all treatment plants and the treatment stages in the species diversity analysis based on the number and frequency of species and calculation of Simpson coefficients (Table 6). A clear decline in species diversity was only detected in the case of wastewater treated in one-stage treatment plants I and III and in multistage object IV. High coefficients of species diversity in the microbiota of the raw and treated wastewater were noted in each study term, regardless of the season of the year (data available on request from the authors).

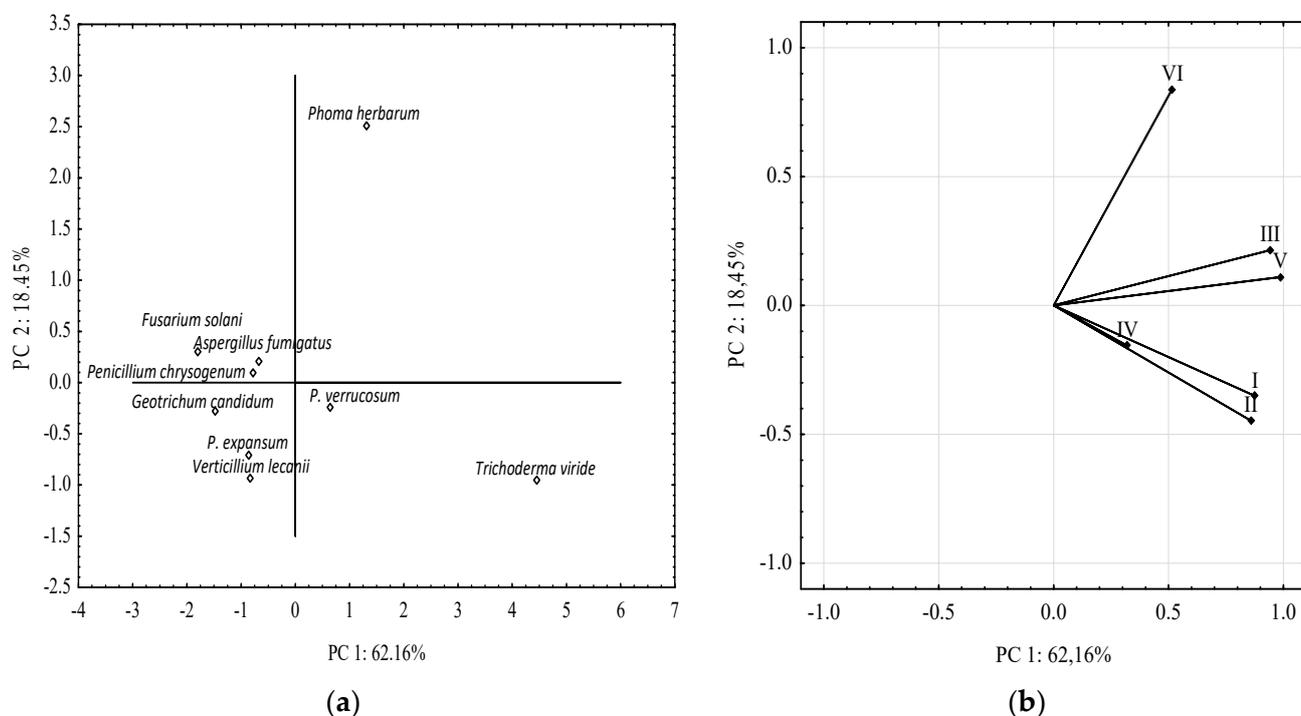


Figure 4. Principal component analysis PCA of the distribution of dominant populations of fungi (a) in relation to the type of treatment plant (b); Explanations: the model explained 80.61% of the variability by two principal components (PC1 and PC2). Wastewater treatment plants I (92%), II (96%), and V (77%) were significantly correlated with the PC1 variable. Wastewater treatment plant IV was significantly correlated with the PC2 variable.

Table 5. Species similarity coefficients (S—expressed in %) of fungal communities in the analyzed small wastewater treatment plants; *—detection of species on Martin medium (2008/2009) and Sabouraud medium (2008), total; I—Leitnie, II—Ludwin, III—Jastków, IV—Dąbrowica, V—Janów, VI—Sobieszyn.

Wastewater Treatment Plant	Analysis (Month)				Means	Total for Analysis
	February	May	August	November		
I–II	28.6 *	36.4	43.8	21.7	32.6	34.4
I–III	35.5	50.0	26.7	26.1	36.4	45.0

Table 5. Cont.

Wastewater Treatment Plant	Analysis (Month)				Means	Total for Analysis
	February	May	August	November		
I–IV	20.6	33.4	21.1	18.9	23.5	34.3
I–V	13.0	32.1	16.7	22.2	21.0	37.5
I–VI	47.8	32.0	22.2	35.0	34.3	44.7
II–III	34.2	34.2	38.7	30.0	34.3	43.4
II–IV	35.1	25.6	32.4	28.6	30.4	43.2
II–V	20.7	18.0	32.0	26.5	24.3	35.1
II–VI	30.3	27.3	42.1	21.6	30.3	38.5
III–IV	34.3	43.7	42.9	41.0	40.5	56.0
III–V	20.0	26.5	26.3	22.2	23.8	47.0
III–VI	48.1	30.0	31.3	35.6	36.3	46.8
IV–V	19.2	24.2	31.2	28.3	25.8	40.8
IV–VI	30.0	19.4	29.4	32.5	27.8	36.1
V–VI	33.4	21.4	18.5	35.5	27.2	44.6

Table 6. Simpson species diversity coefficients for fungal communities in raw wastewater and at each wastewater treatment stage.

Wastewater Treatment Plants/Type of Wastewater	Saprotrophic Fungi—Martin Medium				Potentially Pathogenic Fungi—Sabouraud Medium	
	2008—Means for:		2009—Means for:		2008—Means for:	
	Analysis	Wastewater Treatment Plants	Analysis	Wastewater Treatment Plants	Analysis	Wastewater Treatment Plants
Leitnie (I)						
1	0.993	0.989	0.992	0.977	0.998	0.998
2	0.499		0.996		0.250	
Ludwin (II)						
3	0.998		0.997		0.990	
4	0.998	0.975	0.998	0.968	0.998	0.980
5	0.998		0.998		0.998	
6	0.998		0.998		0.998	
Jastków (III)						
7	0.996		0.998		0.997	
8	0.997		0.997		0.998	
9	1.000	0.937	0.999	0.961	0.998	0.870
10	0.999		0.999		0.993	
11	0.998		0.499		0.995	
12	0.997		0.498		0.991	

Table 6. Cont.

Wastewater Treatment Plants/Type of Wastewater	Saprotrophic Fungi—Martin Medium				Potentially Pathogenic Fungi—Sabouraud Medium	
	2008—Means for:		2009—Means for:		2008—Means for:	
	Analysis	Wastewater Treatment Plants	Analysis	Wastewater Treatment Plants	Analysis	Wastewater Treatment Plants
Dąbrowica (IV)						
13	0.996		0.997		0.996	
14	0.997		0.998		1.000	
15	0.998		0.999		0.999	
16	0.999	0.909	0.999	0.948	1.000	0.932
17	0.998		0.998		0.996	
18	0.996		0.930		0.999	
19	0.500		0.750		0.500	
Janów (V)						
20	0.998		0.998		0.996	
22	0.998	0.733	0.998	0.972	0.998	0.976
23	0.999		0.999		1.000	
24	1.000		1.000		1.000	
Sobieszyn (VI)						
25	0.996	0.990	0.992	0.987	0.997	0.993
26	0.998		0.992		0.997	

Notes: (I): raw wastewater, 2—treated wastewater; (II):—raw wastewater, 4—anaerobic pond I, 5—aerobic pond II, 6—treated wastewater (sedimentation pond); (III): 7—raw wastewater, 8—post mechanical stage, 9—post biological stage (bed with willow), 10—post chemical stage (opoka), 11—pond water inflow, 12—pond water outflow into the river; (IV): 13—raw wastewater, 14—post mechanical stage, 15—post biological stage—bed A with willow (system I), 16—post biological stage—bed B with reed (system I), 17—post biological stage—bed C with reed (system II), 18—post biological stage—bed D with willow (system II), 19—post chemical stage (opoka); (V): 20—raw wastewater, 22—post biological stage—bed A with reed, 23—post biological stage—bed B with willow, 24—post chemical stage—bed with opoka; (VI): 25—raw wastewater, 26—treated wastewater.

To identify factors that may have had an impact on the dynamics of fungal growth in the wastewater, correlation coefficients were calculated between some physicochemical wastewater parameters and fungal abundance (total frequencies of genera). It was found that the number of fungi in the raw wastewater was significantly negatively correlated with the oxygen content ($r = -0.5386$) and significantly positively correlated with the level of organic matter based on BOD₅ and COD ($r = 0.8933$ and 0.9370 , respectively), total nitrogen and N-NH⁴⁺ ($r = 0.7199$ and 0.6202 , respectively), and phosphorus content ($r = 0.8635$). A weak statistically significantly positive correlation with the level of COD and phosphorus ($r = 0.5428$ and 0.4977 , respectively) and a highly significant negative correlation with the level of N-NO₃ ($r = -0.8174$) were found in the case of the treated wastewater.

3.3. Changes in the Composition and Frequency of Genera and Species during the Wastewater Treatment Process

The analysis of the species richness in the collected fungal material revealed the highest value of this parameter in the raw wastewater and the lowest value in the treated wastewater (Table 7). The highest efficiency in the reduction of the number of species (Martin medium) was found in the one-stage treatment plant with reed (I—59%) and in the one-stage object with willow (III—44%). A lower decline in the number of species (28–33%) was recorded in the multistage treatment plants with reed and willow (IV and V) and in the wastewater stabilization pond (II)—Table 7. The changes in the fungal population density

(total values) during the wastewater treatment in the same system (isolation on Martin medium) were reflected in a decline in the number of these microorganisms. However, the multistage treatment plants IV and V removed the propagules more efficiently (80% and 51%, respectively) than the one-stage objects, especially III and VI (26%). The lowest efficiency in reducing the total number of fungi was noted in the wastewater stabilization pond (II) (Table 7). This effect was confirmed by the statistical analysis (analysis of variance and Fisher's test). The numbers of potentially pathogenic filamentous fungi (1-year study with no statistical analysis) in the wastewater treated in some objects (II and III) were even higher than in the raw wastewater (Table 7). The highest efficiency of the multistage treatment plant IV (Dąbrowica) was also evidenced by the substantial reduction in the fungal frequency already at the mechanical treatment stage. In both multistage treatment plants (IV and V), the biological treatment caused a further significant decrease in the size of the micromycete population, and this effect increased considerably after the chemical stage (opoka bed) (Table 7).

The study indicated that the changes in the total frequency of fungal genera induced by the wastewater treatment process were primarily associated with fluctuations in the frequency of the dominant genera (approx. 82% of all fungi present in the wastewater) and, in particular, the species selected within these genera (Tables S4 and 8). The lowest rate of the removal of the propagules of the dominant genera was observed in the wastewater stabilization pond (II), which was reflected by the complete elimination of only two (*Aspergillus* and *Geotrichum*) of the seven dominant genera. The most efficient process was recorded in the multistage constructed wetland systems IV and V, where the population density of six and seven dominant genera, respectively, was reduced. Species of the genera *Aspergillus* (100%), *Geotrichum* (90–100%), and *Trichoderma* (82–100%) were removed at the highest rate. In the group of one-stage treatment plants, which were less effective in reducing the frequency of the dominant genera, the reed treatment plants (I and VI) were more efficient. In both these objects, five of the seven genera (*Acremonium*, *Aspergillus*, *Geotrichum*, *Fusarium*, and *Trichoderma*) exhibited reduced abundance or were eliminated (50–100%). An increased frequency of some genera in the treated wastewater was mainly noted in the one-stage treatment plants (I, III, VI) and in the wastewater stabilization pond (II) (Table S4). In the case of the dominant species (Table 8), the propagules of *Geotrichum candidum* were most effectively removed in all the wastewater treatment systems. The dynamics of changes in the *Trichoderma viride* abundance was much less evident. The greatest reduction of the number of propagules of this species in the treated wastewater was achieved in the multistage treatment plant V (from 86.5% to 90.4%). In contrast, an increase in the frequency of *T. viride* in the treated wastewater was recorded in the wastewater stabilization pond (II) and in the one-stage treatment plant III. The largest increase in the microbial abundance in the treated wastewater was noted in the case of *Phoma herbarum*. Its abundance in the treated wastewater was only limited in the multistage treatment plant IV, although the species was only present at the biological treatment stage (Table 8).

Table 7. Changes in the number of species and the total frequency of filamentous fungi at each wastewater treatment stage (cfu · 10³ · cm⁻³).

Mycological Parameters/Year	Wastewater Treatment Plant (No.)/Type of Wastewater																								
	I		II		III					IV					V			VI							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
	Number of species—Martin medium																								
2008	22	4	16	16	7	9	14	10	11	8	6	9	9	13	8	10	10	12	4	5	12	10	9	13	13
2009	14	13	12	12	10	7	12	13	9	6	7	6	11	7	10	10	12	12	9	12	6	9	5	14	11
Total	34	14 (59) *	24	20	16	16 (34) *	23	19	17	11	13	13 (44) *	15	15	15	17	18	19	10 (33) *	18	13	18	13 (28) *	20	19 (5) *
	Number of species—Sabouraud medium																								
2009	9	5 (44) *	13	13	13	9 (31) *	13	12	13	14	12	10 (23) *	10	12	4	11	12	13	4 (60) *	12	12	9	8 (33) *	13	11 (15) *
	Number of fungi—Martin medium																								
2008	262	101	125	134	142	142	210	178	84	91	123	161	212	178	146	97	102	162	9	166	129	61	32	212	127
2009	258	177	164	141	158	132	155	162	104	88	70	109	150	123	91	110	142	103	64	139	140	98	118	122	119
Total	260	139	145	138	150	137	183	170	94	90	97	135	181	151	119	104	122	133	37	153	135	80	75	167	123
Mean from 2 years		(46) *				(5) *						(26) *							(80) *			(51) *		(26) *	
***	n	hijkl	ijk	hijkl	jkl	ghijk	m	m	cd	bcd	d	ghij	m	kl	fg	de	fg	fghi	a	lł	be	b	b	łm	fgh
	Number of fungi—Sabouraud medium																								
2009	153	28	106	127	162	127	196	148	149	277	208	290	205	162	135	70	212	134	9	217	151	79	34	186	112
		(82) *				(20) **						(48) **							(96) *			(84) *		(40) *	

Notes: I—Leitnie: 1- raw wastewater, 2—treated wastewater; II—Ludwin: 3—raw wastewater, 4—post mechanical stage, 5—post biological stage, 6—treated wastewater; III-Jastków: 7- raw wastewater, 8—post mechanical stage, 9—post biological stage, 10—post opoka stage, 11—pond water inflow, 12—pond water outflow; IV- Dąbrowica: 13—raw wastewater, 14—post mechanical stage, 15—post bed H (system I), 16—post bed B (system I), 17—post bed C (system II), 18—post bed D (system II), 19—post opoka stage; V—Janów: 20—raw wastewater, 22—post bed A (willow), 23—post bed B (willow), 24—post opoka stage; VI- Sobieszyn: 25—raw wastewater, 26—treated wastewater; () *—% reduction vs. raw wastewater; () **—% stimulation vs. raw wastewater; *** the same letters indicate no significant differences between the means of fungal frequency; different letters indicate significant differences between the means.

Table 8. Total numbers of the most frequent species of filamentous fungi at subsequent wastewater treatment stages; () *—% reduction vs. raw wastewater; Explanation as for Table 6.

Fungal Species	Wastewater Treatment Plant (No.)/Treatment Stage																								
	I		II				III						IV						V				VI		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	22	23	24	25	26
<i>Geotrichum Candidum</i>	34	2 (94%) *	22	7	3	0	34	21	2	0	0	0	46	56	7	16	24	0	3	8	1	0	0	36	0
-Martin medium (means from 2 years) -Sabouraud medium	22	0	11	0	0	0	25	5	27	0	6	0	18	20	0	0	0	0	0	11	1	0	0	21	0
<i>Phoma herbarum</i> Martin medium 2009	42	63	18	5	9	17	17	20	25	36	40	73	0	0	11	8	44	20	0	16	33	47	30	69	79
<i>Penicillium expansum</i>	5	3	7	7	5	29	4	10	1	0	4	6	17	15	1	0	12	10	0	1	0	0	2	1	8
-Martin medium (means from 2 years) -Sabouraud medium	0	0	11	1	29	33	33	2	38	10	2	131	39	15	0	1	0	0	0	22	0	5	0	2	14
<i>Trichoderma viride</i>	29	11 (62%)	28	40	53	31	11	20	12	31	44	26	3	16	7	5	10	0	2	52	43	9	7	23 (86.5%)	10 (56.5%)
-Martin medium (means from 2 years) -Sabouraud medium	89	4 (95.5%)	10	19	29	14	21	27	9	6	71	5 (76.2%)	0	50	0	2	9	0	0	52	23	10	5	21	0

3.4. Analysis of the Biosafety of Wastewater-Colonizing Fungi

The analysis of the species composition showed the presence of 10% (16 species) of potential human and animal fungal pathogens (Table S1) representing causative agents of opportunistic infections (biosafety levels 2-BSL 2) in accordance with the biosafety classification [45]. *A. fumigatus*, *F. solani*, and *Acremonium kiliense* were the most abundant species. *A. flavus*, *Candida albicans*, *C. krusei*, *C. tropicalis*, *Scopulariopsis brevicanlis*, *Sporothrix schenckii*, *Trichosporon beigellii*, and several other opportunistic pathogen species were less numerous. In the BSL-1 group, which comprises fungi that cause mild and superficial infections, there were 35 species from de Hoog's list (1996) [45], including *G. candidum* and *T. viride*, which were often isolated from the wastewater treatment plants analyzed in this study. It was also found that over 30% of species (35) in the mycocoenosis structure in the small wastewater treatment plants were represented by phytopathogenic fungi, most often from the genera *Fusarium*, *Phoma*, *Alternaria*, and *Verticillium* (Table S1).

4. Discussion

4.1. Evaluation of the Frequency in the Species Composition and the Taxonomic and Spatial Structure of Total Fungal Communities

The present study showed that the communities of microscopic fungi in raw domestic wastewater as well as wastewater treated in the constructed wetland facilities and in the biological wastewater stabilization pond (so-called small wastewater treatment plants located in rural areas) were dominated by terrestrial filamentous fungi. Similar observations have recently been reported by Kharitonov et al. (2022) [21] in their study performed with the use of the plate count method in a vertical constructed wetland. Our findings differ from the results of investigations of fungal communities present in analogous wastewater treatment systems reported by Kacprzak et al. (2003) [22]. They also differ from reports on the mycological condition of municipal wastewater [23]. Using the same mycological methods as in this study (isolation of fungi on Martin medium and microscopic identification of species), Kacprzak et al. (2003, 2005) [22,23] found that the abundance of yeasts was several times higher than the mold count. Since yeasts assimilate primarily easily available sources of C and energy, we believe that their low frequency in the examined wastewater (4% of all micromycetes) was a result of an insufficient level of an easily available fraction in the organic pollutants. This suggestion is supported by the equally low proportion (2.5%) of Mucorales representatives, i.e., sugar fungi assimilating simple nutrient substrates, in the analyzed fungal communities. It is also possible that the differences in the proportions of filamentous fungi and yeasts reported in this study and by Kacprzak et al. (2003, 2005) [22,23] were associated with differences in the level of the soluble fraction contained in organic wastewater pollutants. However, this issue cannot be elucidated fully, as no such data are available.

The present study showed that the fungal communities in the domestic wastewater were dominated by ubiquitous ascomycete molds (Ascomycetes) adapted to the utilization of a wide range of organic substrates, also in oligotrophic environments (treated wastewater). This is supported by data on the occurrence of many species of fungi from the genera *Penicillium*, *Aspergillus*, *Cladosporium*, *Fusarium*, and *Verticillium* in drinking water distribution systems [46]. The comprehensive analysis of the taxonomic structure of the collected micromycete strains revealed that *Ascomycota* accounted for 90% of the mycocoenosis of the examined wastewater, whereas *Zygomycota*, *Basidiomycota*, and *Oomycota* represented the other 10%. Similar proportions in the structure of systematic groups of fungi in constructed wetland wastewater treatment systems were shown by Usharani (2019) [20] and Kharitonov et al. (2022) [21]. The latter authors identified 39 genera and classified 32 as *Ascomycota*, 4 as *Basidiomycota*, 2 as *Zygomycota*, and 1 as *Zoopagomycota*. As shown in the present study, *Hypocreales* and *Eurotiales-Ascomycota* had the largest share in the taxonomic and spatial structure of the fungal communities. The high abundance of representatives of both orders (especially *Eurotiales*) in constructed wetland and biological wastewater treatment plants was indicated by Kacprzak et al. (2003) [22], Kharitonov et al.

(2022) [21], and Usharani (2019) [20]. It was also noted that, in terms of the dominance of taxonomic groups, the fungal communities of the rural wastewater treatment plants exhibited a great similarity to fungal communities inhabiting hydrogenic soils [41], with the prevalence of Hypocreales and Eurotiales-Ascomycota.

Over 80% of the mycobiota of the analyzed wastewater treatment plants were represented by populations of 7 of 46 genera (*Acremonium*, *Aspergillus*, *Fusarium*, *Geotrichum*, *Penicillium*, *Phoma*, and *Trichoderma*). In terms of the genus richness, however, this accounted for less than 20% of the total micromycete population. The greatest proportion, i.e., 70% of the genus composition, was constituted by sporadically occurring genera with a dominance coefficient of <1%. The quantitative dominance of only a few genera in the fungal community was associated with the greater abundance of single populations, i.e., *Aspergillus fumigatus*, *Fusarium solani*, *Geotrichum candidum*, and *Trichoderma viride*, and the lower frequency of 2–3 populations, as in the case of *Acremonium* and *Penicillium*. In total, these species accounted for less than 10% of the mycobiota species composition in the analyzed wastewater treatment plants. The other 100 species were classified as rare or sporadic in the frequency scale used in the study. Many genera and species identified in the present study were also reported by Kacprzak et al. (2003) [22], Fakhru'l-Razi et al. (2006) [47], Usharani (2019) [20], and Kharitonov et al. (2022) [21], who performed mycological analyses of constructed wetlands and biological wastewater treatment plants. They isolated, i.e., *Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Clonostachys (Gliocladium)*, *Trichoderma*, *Fusarium*, *Geotrichum (Dipoascus)*, *Penicillium*, *Paecilomyces*, and *Phoma*.

Key importance for the growth and development of terrestrial (geophilic) fungi in wastewater is ascribed to the presence of organic matter and the involvement of these organotrophs in the wastewater treatment process via organic matter decomposition. The present results suggest that at least populations that reached a considerable level of abundance were metabolically active and thus participated efficiently in the removal of organic pollutants from the analyzed wastewater. This statement is supported by the fact that the fungal community present in wastewater is regarded as one of the indicators of organic matter degradation [20]. In the case of terrestrial filamentous fungi (which predominated in the analyzed wastewater of the treatment facilities), this function requires adaptation to life in the aquatic environment. One of these factors is the production of wettable spores, as in the case of the *Acremonium*, *Fusarium*, *Geotrichum*, *Phoma*, and *Trichoderma* genera isolated in the wastewater. In the case of terrestrial species with non-wettable spores, such as *Penicillium* and *Aspergillus*, an important role is played by the ability to grow and sporulate inside colonized substrates (detritus particles or plankton) and spread in water through clustering. It has been shown [48] that many *Aspergillus* and *Penicillium* species present in wastewater reduce its salinity and alkalinity. They also remove chlorides, nitrite, and phosphate, the latter two with an efficiency of 87–97% and 22.8–32.1%, respectively.

The present study demonstrated that the physicochemical properties of wastewater are important determinants of intensive growth of some fungal species of fungi of terrestrial origin in this environment. In addition to the content and availability of various carbon and energy sources (organic pollutants), as evidenced by the positive correlation between the fungal frequency and the level of BOD₅ and COD, the abundance of fungi in the raw wastewater was correlated with the content of total nitrogen, ammonium nitrogen, and phosphorus. In the treated wastewater, a stimulating effect on fungal growth was exerted by the level of phosphorus and organic matter (COD values), whereas nitrates produced in the nitrification process during the wastewater treatment had an inhibitory effect. Noteworthy, nitrates are a good source of nitrogen for *Penicillium* and *Aspergillus* fungi. The high negative correlation between the species frequency and the oxygen content in raw wastewater indicated a stimulating effect of oxygen deficiency on the growth of many fungi. Since filamentous fungi are aerobic organisms, this effect may be surprising. However, as emphasized earlier, the wastewater was quantitatively dominated by a small number of species versus the total number of identified fungi. The selected populations, i.e., *A. fumigatus*, *F. solani*, *G. candidum*, *P. chrysogenum*, *P. expansum*, *P. verrucosum*, *Ph. herbarum*,

and *T. viride*, are able to grow in limited oxygen access conditions and some fungi can grow even in the temporary absence of oxygen [36]. Therefore, the relationship observed in the study indicates selection caused by oxygen deficiency and, consequently, disturbances in the biological balance in the fungal communities present in the analyzed wastewater.

4.2. Evaluation of Changes in the Species Composition and Frequency of Populations in the Fungal Communities Depending on the System and Wastewater Treatment Stage

As shown in the present study, the micromycete communities in the one- and multi-stage soil-plant wastewater treatment plants and the wastewater stabilization pond in the rural areas exhibited a 50–70% variation in the species composition. This was indicated by the relatively low Marczewski-Steinhaus species similarity coefficients ($S = 34\text{--}56\%$). Noteworthy, this similarity of the species composition was clearly higher than in the case of hydrogenic soils characterized by a maximum value of 30–32% [41,49]. An even greater diversity of fungal communities was noticeable in the analysis of the frequency of species, as evidenced by the high total values of Simpson species diversity coefficient ($D = 0.770\text{--}0.990$) for each treatment plant. In contrast, lower values of this coefficient for mycocoenoses in hydrogenic soils, i.e., $D = 0.501\text{--}0.875$, have been reported [41]. The Simpson coefficient reflects the rank of abundant species in communities [43]; therefore, its high values indicate great importance of the selected fungal populations in the functioning of constructed wetland systems and biological ponds.

The PCA analysis of the mean population size on Martin medium revealed the greatest similarities in the frequency of the dominant species between the following wastewater treatment plants: the wastewater stabilization pond (II in Ludwin) and the one-stage treatment plant with reed (I in Leitnie) and between the one-stage treatment plant with willow (III in Jastków) and the multistage reed-willow system (IV in Dąbrowica). This was proved by the presence of different populations: *T. viride* in wastewater treatment plants I-II and *Penicillium* (including *P. verrucosum*) in objects III-IV. Elucidation of these relationships based on the available data on the operation of these systems and the physicochemical parameters of wastewater poses considerable difficulties. We believe that the differences in the amount of inflowing wastewater, its oxygenation degree, and the pollutant load may be one of the causes of this phenomenon. Objects I-II, i.e., the collective treatment plants, received large amounts of wastewater with a high load of organic pollutants as well as nitrogen and phosphorus, especially in the case of treatment plant I (Table 2). In wastewater treatment plants III-IV intended for single-family households, the amounts of inflowing wastewater and pollutants were significantly lower or at a similar level, as reported by Józwiakowski et al. (2019) [3]. These conditions may have been more beneficial for the growth of *Penicillium* than *T. viride*. The former fungi are “satisfied” with a relatively low concentration of organic C, which is associated with the slow growth of their vegetative mycelium and the poor increase in biomass. In turn, *T. viride* dominated in the wastewater of treatment plants I-II due to its rapid and high increase in biomass, high hydrophilicity, and high tolerance to oxygen deficiency and high concentrations of various organic and mineral pollutants [36].

The results of the present study (analysis of variance and Fisher’s test) proved that the frequency of fungi in the treated wastewater differed significantly from the frequency of fungi in the raw material. In our previous studies [13,50,51], we demonstrated that the so-called total number of saprotrophic and potentially pathogenic fungi was reduced by 93%–95% and 99% in the wastewater treatment process in the small pond and soil-plant wastewater treatment plants, respectively (multistage treatment plants). With regard to the species diversity assessed by calculation of the Simpson coefficient value in the present study, this effect was visible in only three of the six treatment plants: (one-stage with reed—I, one-stage with willow—III, and multistage with reed and willow—IV). The hybrid treatment plant (IV in Dąbrowica) was characterized by the highest efficiency in reducing the species diversity. The high Simpson coefficients for the wastewater treated in the other treatment plants was most probably related to the increase in the abundance of some

dominant species, in particular *Ph. Herbarum* and *Penicillium*, and less frequently *T. viride*, *Fusarium*, and *Verticillium*, during the mechanical and/or biological treatment stages. The intensification of the growth of many fungal populations during the treatment process indicates their involvement in the biodegradation of organic pollutants. However, the “shift” of this effect to the final treatment stage (chemical treatment with limestone and treated wastewater) noted in many treatment plants suggests an insufficient treatment outcome. In turn, secondary and passive enrichment of the “mycoplankton” of treated wastewater with fungi originating from the environment, e.g., from the macrophyte phyllosphere, is possible as well.

The present study indicates that the biological pond system (II in Ludwin), which was based exclusively on the microbiological treatment process, was the least effective in the elimination of fungal propagules. The enhancement of the wastewater treatment function with the use of a plant component increased the effectiveness of the elimination of mycological pollutants. This was most evident in the multistage treatment plants (IV in Dąbrowica and V in Janów) equipped with one (V) or two (IV) beds for the horizontal (HF-CW) and vertical (VF-CW) wastewater flow. The increased efficiency of removal of most of the dominant species in these treatment plants, often amounting to 100% especially in the double-bed biological treatment plant, was mainly a result of the use of the vertical wastewater flow and reed as a phytoremediation factor. As reported by Obarska-Pępkowiak et al. [52], VF-CW beds are better oxygenated due to the pulsed supply of wastewater and formation of oxygen microzones around submerged parts of the plant. The stem tissue in plants accumulates and supplies atmospheric oxygen (aerenchyma) to the underground parts. An increased oxygen supply stimulates the process of nitrification and accumulation of nitrates in wastewater, which does not support the growth of most fungi. The inhibition of fungal growth through accumulation of nitrates was evidenced by the high negative correlation ($r = -0.8174$) between the fungal frequency and the content of these ions in the treated wastewater. In the case of the one-stage wastewater treatment plants, the reduction of the micromycete abundance was weaker. The one-stage wastewater treatment plants with vertical sewage flow and reed (I in Leitnie and VI in Sobieszyn) were characterized by higher efficiency than the one-stage treatment plant with willow and the horizontal sewage flow (HF-CW) (III in Jastków). This was probably associated with the higher degree of wastewater oxygenation.

4.3. Evaluation of the Biodegradation and Bioremediation Potential of Fungal Communities in Wastewater Treatment Systems

The fungal communities identified in the sewage of the analyzed wastewater treatment plants are of the presence of populations with known capabilities of biodegradation of complex organic compounds of plant and animal origin. This group comprises the genera *Acremonium*, *Aspergillus*, *Clonostachys* (*Gliocladium*), *Geotrichum*, *Fusarium*, *Paecilomyces*, *Penicillium*, and *Trichoderma*, which are ubiquitous fungi with cellulolytic, proteolytic, lipolytic, chitinolytic, and even ligninolytic properties (*Trichoderma*) [36]. *Trichoderma*, *Penicillium*, *Paecilomyces*, *Fusarium*, and *Clonostachys* can also metabolize many different organic pollutants of anthropogenic origin, including polycyclic aromatic hydrocarbons, phenols, polyphenols, dyes, drugs with an aromatic structure, detergents, and other pollutants [28,52–57]. Microfungal genera identified in wastewater, e.g., *Alternaria*, *Aspergillus*, *Paecilomyces*, *Penicillium*, and *Phoma*, are able to immobilize heavy metals, often with high yields [20]. Additionally, saprotrophic fungi present in wastewater oxidize ammonium salts (heterotrophic nitrification), reduce nitrates (denitrification), and remove phosphates. Such metabolic activity has been exhibited by, e.g., *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium*, and *Trichosporon* [20,36]. Given these data, it seems reasonable to claim that microfungal communities present in small constructed wetlands and biological sewage ponds are suitable for the removal and neutralization of various natural and foreign organic pollutants (xenobiotics) as well as the transformation and accumulation of inorganic pollutants.

4.4. Evaluation of Mycological Hazards in Biological and Constructed Wetland Wastewater Treatment Plants

The present study showed that over 46% of the fungal species colonizing the analyzed wastewater treatment plants were capable of causing opportunistic infections (14.4%) or mild and rare infections (31.5%). *A. fumigatus* is one of the most dangerous opportunistic pathogens (group BSL-2) causing systemic mycoses and less frequently, superficial diseases. This fungus often causes human and animal infections, especially in wild wetland birds and poultry. It mainly causes pulmonary mycosis in immunocompromised humans and animals [58]. The greatest concerns among BSL-1 fungi are raised by *G. candidum* due to its high abundance. The fungus can cause oral, bronchial, and pulmonary geotrichosis and skin infections in immunocompromised subjects [36]. The opportunistic pathogens mentioned above were successfully eliminated from the wastewater treatment systems analyzed in this study. As shown by studies of municipal wastewater treatment plants [59], fungi detected in wastewater are commonly and abundantly present in the air of wastewater treatment plants (spores). The presence of a fungal aerosol comprising numerous genera of Ascomycota (e.g., *Alternaria*, *Fusarium*, *Cladosporium*, *Penicillium*, *Aspergillus*, *Sarocladium*) in reed constructed wetlands was reported by [60]. In addition to potentially pathogenic forms (infectious aerosol), the air contains typically saprotrophic fungi, e.g., the majority of *Penicillium* species. Inhalation of spores of these fungi can also be harmful due to their allergenic effects. Non-pathogenic bioaerosols may contain spores of toxicogenic fungi producing mycotoxins and causing immunotoxic diseases [61]. In addition to allergies and mycotoxicoses, inhaled spores may finally cause such mycoses as pulmonary aspergillosis [58]. Birds, in which *A. fumigatus* is the causative agent of 95% of aspergillosis cases, are especially susceptible to the disease [62]. Given these data, we believe that there is a high risk of formation of bioaerosols containing allergenic, toxinogenic, and pathogenic fungi, e.g., *Aspergillus*, *Penicillium*, or *Alternaria*, in both biological and constructed wetland wastewater treatment plants. Moreover, the incomplete elimination of such phytopathogenic fungi as *Fusarium*, *Verticillium*, and *Phoma* from wastewater also carries the risk of contamination of soil with these organisms.

5. Conclusions

The investigations were focused on the impact of the wastewater treatment system and physicochemical parameters on the dynamics of the growth of micromycete populations. Based on the research data, an analysis of the possibility to remove contaminants and fungal biosafety was carried out. The optimal technological system for elimination of these microorganisms from domestic wastewater was selected.

The fungal communities of small wetland constructed wastewater treatment plants and biological ponds are dominated (over 95% of the total number of fungi) by geophilic filamentous fungi, mainly Hypocreales and Eurotiales-Ascomycota. They are characterized by the presence of a small number of populations adapted to living in the aquatic environment (15% of the composition and 80% of the frequency of the identified genera). The *Trichoderma viride* populations dominated in objects with a higher wastewater treatment capacity. The *Penicillium* populations often showed preference for wastewater treatment plants with a low capacity. Potentially pathogenic species from the BSL-1 and BSL-2 groups posing a threat to humans and animals and phytopathogenic species accounted for 46.0% and 31.5% of the total micromycetes, respectively. The dynamics of fungal growth in the raw wastewater was influenced by the content of organic matter, nitrogen, phosphorus, and oxygen. The reduction in the frequency of fungi in the treated wastewater was correlated with the increase in the concentration of nitrates. The most efficient process of removal of fungal propagules was noted in the hybrid constructed wetland treatment plants with the vertical (VF-CA) and horizontal (CF-CW) wastewater flow, whereas the biological treatment plant exhibited the lowest efficiency.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w15010031/s1>, Table S1. List of species of filamentous fungi, yeasts, and the number of their records found in the studied small wastewater treatment plants: Table S2. Dominance coefficient (D) for the genera of filamentous fungi; I–VI—numbers of wastewater treatment plants; I—Leitnie, II—Ludwin, III—Jastków, IV—Dąbrowica, V—Jastków, VI—Sobieszyn: Table S3. Dominance coefficient (D) for the genera of yeasts; explanations as in Table S2: Table S4. Changes in the frequency of the populations of the dominant genera of filamentous fungi at each wastewater treatment stage.

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