

## The occurrence of potentially pathogenic filamentous fungi in recreational surface water as a public health risk

Katarzyna Góralska, Joanna Błaszowska and Magdalena Dzikowiec

### ABSTRACT

Microfungi occurring in surface water may represent an important health risk. Recreational water reservoirs are a potential reservoir of pathogenic fungi. The aim of the study was to assess the diversity of mycobiota in selected artificial bathing reservoirs with regard to its biosafety for the human population. The studies were conducted during the summer of 2016 in three research seasons (June (I), July and August (II), and September (III)), taking into account the various periods of recreational activities. Filamentous fungi were isolated from water samples collected at five different ponds utilized for recreation. From 162 water samples, 149 fungal taxa of filamentous fungi were identified: 140 were classified to species level and only nine to genus level. *Aspergillus fumigatus* was the dominant species. The highest species richness (S) was noted in June, with 93 fungal taxa (Menhinick's index from 2.65 to 4.49). Additionally, in season I, the highest diversity of fungal species was revealed (Simpson's diversity index from 0.83 to 0.99). The average number of CFU/1 mL sample ranged between 0.4 and 4.6 depending on the time of sampling and ponds. Of all the isolated species, 128 were clinically relevant (11 from RG-2 and 117 from RG-1), highlighting the need to introduce seasonal mycological monitoring of such reservoirs.

**Key words** | aquatic fungi, fungal occurrence, fungal water contamination, human pathogenic fungi, natural bathing areas

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### INTRODUCTION

Water reservoir contamination occurs when substances and chemical compounds, and allochthonous organisms, not present under natural conditions are discovered in reservoirs (Libudzisz *et al.* 2009). Microbiological contamination of the aquatic environment can come from natural sources – mainly soil in the immediate vicinity but also the transmission of microorganisms through air currents – or through anthropogenic sources, including wastewater, surface and ground runoff from industrial and agricultural areas, and landfills (Lucyga *et al.* 2011). The presence of potentially pathogenic fungi in water reservoirs, as well as

the lack of sanitary and epidemiological supervision over such objects, poses a risk of acquiring waterborne infections. Throughout the past 30 years in Europe, more than 400 different fungal species have been found in groundwater, surface water, and drinking water; among these species, 46 were classified as Biosafety Level-2 (Novak-Babič *et al.* 2018), meaning they cause various diseases, such as allergies and mycoses, in humans and animals.

In natural water environments, autochthonous species are most often represented by microscopic fungi from the following classes: *Chytridiomycetes*, *Oomycetes*, *Trichomyces*, and *Mucoromycetes* (which were once named *Zygomycetes*). Of the millions of estimated fungal species, only 3,000–4,000 are classified as aquatic fungi (Grossart & Rojas-Jimenez 2016) for which the water environment is

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a natural place of existence. The so-called water fungi belong to various taxonomic units and occur abundantly in water reservoirs in the form of vegetative mycelium, producing zoospores or other types of spores adapted to spread in water. The optimal temperature for the growth of most temperate aquatic fungi is 25 °C, but the fungi can also grow relatively well at temperatures as low as 10 °C. Genera such as *Alternaria*, *Aureobasidium*, *Cladosporium*, and *Penicillium* detected in aquatic environments are classified as secondary freshwater fungi since they originate from terrestrial habitats (Krauss et al. 2011).

Although it is very difficult to prove the existence of a causal relationship between the presence of microorganisms in recreational water areas and the acquisition of waterborne infection, several studies have confirmed the presence of waterborne fungal infections. Some of these studies have found a genetic relationship between waterborne fungal strains and strains of the same species isolated from clinical samples (Anaissie et al. 2001, 2003). Waterborne outbreaks of fungal infections, with *C. albicans*, *C. parapsilosis*, *Aspergillus* spp., *Mucor* spp., *Trichosporon asahii*, *Fusarium* spp., *Scedosporium* spp., and *Exophiala jeanselmei*, associated with hospital water containers have been observed among patients, particularly immunosuppressed individuals (Neblett Fanfair et al. 2013; Kanamori et al. 2016). A genetic study of 75 clinical and 156 environmental isolates of *Fusarium keratoplasticum* showed that strains isolated from clinical materials to be identical to those from plumbing biofilm samples (Short et al. 2014). Bandh et al. (2016) investigated the relationship between the presence of human pathogenic opportunistic fungi (*Aspergillus*, *Candida*, *Penicillium*, *Cryptococcus*, *Fusarium*, *Rhizopus*, and *Mucor*) in lake water and the incidence of fungal infections in the associated population of Kashmir, India: a higher incidence of fungal infection (9.84%) was found in people using lake water than people using only tap water (4.16%). Serious fungal infections (*Aspergillus* spp., *Scedosporium* spp., and *Rhizopus* spp.) of the lungs and brain resulting from the aspiration of contaminated water have been observed in people who have experienced near-drowning episodes (Leroy et al. 2006; Jenks & Preziosi 2015; Gerlach et al. 2016; Signore et al. 2017). Several reports of near-drowning accidents have clearly demonstrated that opportunistic moulds, including *Aspergillus* species, can

cause fungal pneumonia, and only a small amount of water (<150 mL) is needed to cause devastating infections (Ter Maaten et al. 1995). A relationship has been confirmed between natural disasters and subsequent fungal infections in people affected by disasters (Benedict & Park 2014). These reports show that pulmonary aspergillosis can be waterborne and it can be transferred either by air or water aerosol. Cutaneous mucormycosis caused by *Apophysomyces trapeziformis* occurred among 13 people who were severely injured after a tornado (22 May 2011) in Joplin, Missouri, USA. A report from Japan described disseminated aspergillosis caused by *Aspergillus fumigatus* to be associated with tsunami lung (Kawakami et al. 2012). Other fungal pathogens, such as *Rhizopus*, *Mucor*, *Fusarium*, *Scedosporium*, *Coccidioides immitis*, and *Apophysomyces elegans*, have also been implicated as agents of mycoses affecting the lungs, central nervous system and skin after a tsunami (Benedict & Park 2014). The results of these studies suggest that water should be considered as a potential transmission route for pathogenic fungi.

The results of research conducted in many countries revealed a high prevalence of potentially pathogenic fungi in the waters of natural bathing places, which poses a threat to human health (Dynowska et al. 2013; Biedunkiewicz & Góralska 2016). Although the presence of fungi in drinking water, swimming pools, and natural bathing places and its associated health risks have been documented, there are no international legal regulations for the mycological evaluation of drinking or recreational water (Novak Babič et al. 2017). The microbial parameters currently employed in testing (*Escherichia coli*, intestinal enterococci) have no indicative value regarding fungal contamination (Council Directive 2006/7/EC). The presence of specific species of filamentous fungi (*Aspergillus* spp. and *Rhizopus* spp.) in a water reservoir directly indicates a poor sanitary state and hence the epidemiological threat. This fact indicates the need for seasonal microbiological monitoring of the aquatic environment and thus supervision of the quality of bathing water in recreational aquatic areas. The taxonomic and phenological analysis of natural inland bathing sites indicate the highest species diversity of fungi during the summer corresponds with the period of time when inhabitants of countries with moderate climates use these types of recreational facilities with the highest frequency. The aim of

the study was to assess the diversity of the mycobiota in selected natural bathing places, with regard to biosafety implications for the human population. The qualitative composition of filamentous fungi before, during, and after the bathing season was compared.

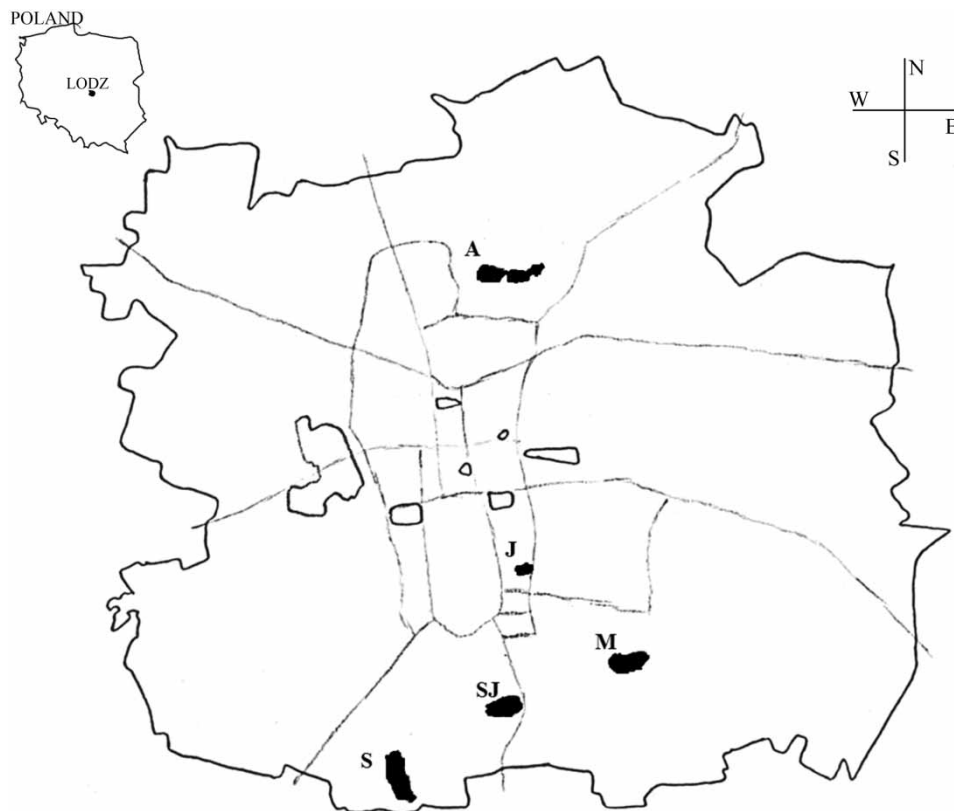
## MATERIALS AND METHODS

The research area included five artificial water reservoirs used as public bathing places, located in recreational areas in Lodz. The city is situated in Central Poland, in a temperate climate zone with the following characteristics: four distinct seasons, a mean annual air temperature of 7.5 °C, and a mean annual relative air humidity of 80%. The area of the city is 293.25 km<sup>2</sup>, making it the fourth largest city in Poland; it is inhabited by 690,422 people (2,354 people/km<sup>2</sup>). Within the city, there are 19 rivers and streams, partly covered with urban infrastructure.

The research covered five artificial water reservoirs: Jan's ponds (SJ), Mlynek (M), Stefanski's ponds (S), Jasien pond (J), and Arturowek complex (A) (Figure 1). The analysed reservoirs are used as recreational bathing places by residents during the summer. The Lodz Sanitary Inspectorate recognizes three (SJ, S and A) of the analysed water reservoirs as official bathing areas, while the other two (M and J) are not considered official bathing sites; therefore, they are not subject to sanitary control.

Jan's ponds (SJ) is surrounded by parkland comprising grassy areas and old stands, mainly linden and ash. The area of the pond is 4.3 ha and its depth is up to 2.53 m. SJ is empty in winter but is filled by the Olechowka River in spring. This urban bathing place is accompanied by a rich recreational infrastructure. Its most recent modernization was performed in 1995.

Mlynek (M) is a water reservoir on the course of the Olechowka River located close to the city limits and woods. It is 3.5 ha in surface area and up to 2.75 m deep.



**Figure 1** | Localization of examined ponds in Lodz city: SJ – Jan's pond; M – Mlynek; S – Stefanski's ponds; J – pond at Jasien; A – Arturowek complex.

The shore of the pond is high with a large number of reeds but no beaches. There is a varied recreational infrastructure.

Stefanski's ponds (S) consist of two water bodies located on the course of the Ner River. They cover an area of 11.4 ha and their maximum depth is about 4 m; their surroundings include rich stands such as black alder, white willow, birch, poplar, and chestnut. It is used as an urban bathing place and is accompanied by a rich recreational infrastructure. During the sampling period, the reservoirs were undergoing renovations.

The Jasien pond (J) is located in the city centre on the course of the Jasien River, within the park surrounding a historical palace. The area of the pond is 3.46 ha and the maximum depth is 4 m. The shore is covered with a gabion system; on one side with beach and large gang-board. There is a varied recreational infrastructure.

The Arturowek complex (A) is located at the source of the Bzura River and is a part of the Lodz's Hills Landscape Park. The Arturowek complex consists of three interconnected water bodies: upper (area 1.08 ha), middle (2.58 ha), and lower (3.05 ha), with a maximum depth of 2.1 m. Two ponds with a rich recreational infrastructure are used as bathing reservoirs and the third (smallest) is a retention reservoir. The entire Arturowek complex was recently modernized during a LIFE+ project entitled 'Eco-hydrological reclamation of recreation reservoirs'. Ponds were desludged and equipped with gabion systems.

Research was conducted during the summer of 2016 in three research seasons: I in June (before the start of the bathing season), II in July and August (peak bathing season during the holidays), and III in September (after the bathing season). The time of sampling was selected to align with the greatest use of these water reservoirs by people. Weather conditions in the first and second seasons of the research period were similar. Due to frequent rainfall, water levels were very high. However, before the third season, there was a period of drought, significantly lowering the water level; this was especially noticeable in the SJ reservoir, where the coastline moved by more than 2 m. Samples were always taken at the same time (between 6 and 8 AM) and weather conditions were similar (windless and rainless). The samples were collected in places most frequently used by people (gang-boards, beaches, designated bathing areas, and harbours).

Water samples were taken in triplicate 1.5–2 m from the shore of the water reservoirs at a layer 15 cm below the surface; the sampling was performed using Whirl-Pak<sup>®</sup> bags (Nasco, USA) with a volume of 500 mL. The number of sampling locations was dependent on the size of the water body: three sampling points on SJ, three on M, four on S, three on J, and five on A. In total, 162 samples were collected. During each collection, the air and water temperatures and water pH were measured (pHep Tester, Pocket pH Tester, HANNA instruments, Romania). The collected samples were delivered to the laboratory within an hour. The samples (500 mL) were concentrated by centrifugation to a volume of 10 mL, and then 1 mL of each sample was seeded on Sabouraud dextrose agar (SDA, Biomerieux, France) with chloramphenicol (repeated three times) and Czapek-Dox medium (Biomerieux, France) (repeated three times).

As the study focused on fungi that could pose a potential threat to human health, Sabouraud's dextrose agar was chosen for the growth of clinically relevant species, whereas Czapek-Dox medium was used to obtain sporulation of moulds (mostly of genera *Aspergillus* and *Penicillium*) to facilitate identification. Incubation was carried out for 5–7 days at 24 °C. After 7 days of incubation, the number of colonies was counted and expressed as the number of colony-forming units (CFU)/1 mL for water samples. For slow-growing fungi, incubation was prolonged for 14–21 days to achieve sporulation. From the obtained filamentous fungal isolates, microscopic preparations were made according to the Gerlach technique by pressing the adhesive tape to the mycelium and then transferring it to a microscope slide, using lactophenol and aniline blue staining (Gerlach 1972).

The identification was made based on macroscopic and microscopic characteristics using keys: de Hoog et al. (2019), Fassatova (1983), and Watanabe (2002). Current species names were checked according to Index Fungorum Website.

Clinically relevant species were classified to risk groups (RGs) and biosafety levels (BSLs) according to the online version of the Atlas of Clinical Fungi (de Hoog et al. 2019). These two parameters are commonly used to characterize the occupational health risks and intrinsic virulence associated with the pathogenicity of fungal species.

Microorganisms used for laboratory work are classified into four RGs (WHO 2004), but RG-4 does not apply to fungi. General criteria for attribution of fungi in different

categories were explained by de Hoog (1996), but the application to individual species is still under debate. According to the Atlas of Clinical Fungi (de Hoog et al. 2019), the categories of RGs are defined as follows:

RG-1: Saprobies or plant pathogens occupying non-vertebrate ecological niches or commensals. Infections are coincidental, superficial, and non-invasive or mild.

RG-2: Species principally occupying non-vertebrate ecological niches, but with a relatively pronounced ability to survive in vertebrate tissue. In severely immunocompromised patients, they may cause deep, opportunistic mycoses. The category also includes pathogens causing superficial infections.

RG-3: Pathogens potentially able to cause severe, deep mycoses in otherwise healthy patients.

The levels of laboratory biosafety (BSL) are based on intrinsic virulence (Risk Groups 1–4) and routes of infection (WHO 2004). Each BS level describes the microbiological practices, safety equipment and facility safeguards for the corresponding level of risk associated with handling a particular agent; no special precautions for category BSL-1, while specific procedures are required for BSL-2, BSL-3, and BSL-4 (BSL-4 does not apply to fungi).

Qualitative and quantitative assessments of biodiversity were applied. The species richness (S) was expressed as the number of species found in the water of the examined reservoirs. Additionally, Menhinick's index (species richness index) and Simpson's diversity index were also calculated (Magurran 2004). Menhinick's richness index is calculated as the ratio of the number of species (S) and the square root of the total number of individuals (N):

$$D_{Me} = \frac{S}{\sqrt{N}}$$

Simpson's diversity index is a measure of diversity which takes into account the number of species present (n), as well as the relative abundance of each species (N):

$$D = 1 - \frac{\sum n(n-1)}{N(N-1)}$$

The obtained data were analysed using the  $\chi^2$  test and the Spearman rank correlation. Additionally, for

comparison of fungal counts obtained from media, the non-parametric Kruskal–Wallis ANOVA was used. All calculations were performed using the STATISTICA 13.2 software. For all test, the significance level was assumed to be  $\alpha \leq 0.05$ .

## RESULTS

In total, 149 different taxa of filamentous fungi were identified: 140 classified to the species and only nine to genus level (Supplementary Table S1). In water reservoirs (SJ, M, and A), fungi belonging to *Oomycota*, *Zygomycota*, *Ascomycota*, and *Basidiomycota* were identified. No representatives of the *Zygomycota* were found in S, while no fungi from the *Basidiomycota* were found in J (Table 1 and Figure 2). The greatest species richness (S) was found in SJ (79 species), while the smallest number of species was found in M (47) and A (42) (Figure 3). Significant differences in species richness (S) were observed between research seasons and examined ponds ( $\chi^2 = 31.20$ ;  $df = 8$ ;  $p = 0.0001$ ). Statistically significant differences between species richness and reservoirs were noted for season I ( $\chi^2 = 14.61$ ;  $df = 4$ ;  $p = 0.0056$ ) and for season III ( $\chi^2 = 22.5$ ;  $df = 4$ ;  $p = 0.0002$ ); no significant difference was observed for season II ( $\chi^2 = 9.06$ ;  $df = 4$ ;  $p = 0.0595$ ).

The highest number of species (93) and the number of colonies (308) was noted in water samples in research season I; only 68 species and 175 colonies were found in season II; and 70 species and 289 colonies during season III. The Kruskal–Wallis test showed a statistically significant difference in the number of fungal colonies obtained on media depending on the research season only for two ponds: S ( $p = 0.013$ ) and A ( $p = 0.021$ ). The average number of CFU/1 mL ranged between 0.4 and 4.6. For pond A, the highest differences between CFU/1 mL were noted in relation to seasons; in season III, the value of CFU (2.9/1 mL) was over 3.5 and 7 times higher than in season I and II, respectively. More detailed data regarding CFU/1 mL are given in Table 2.

Based on the Menhinick's index, the greatest species richness was observed in SJ (season I and III), S (season I), and A (season I), and the least in S (season III). Simpson's diversity index was the highest for SJ and A for all

**Table 1** | Genera of fungi isolated from the examined water reservoirs in three research seasons

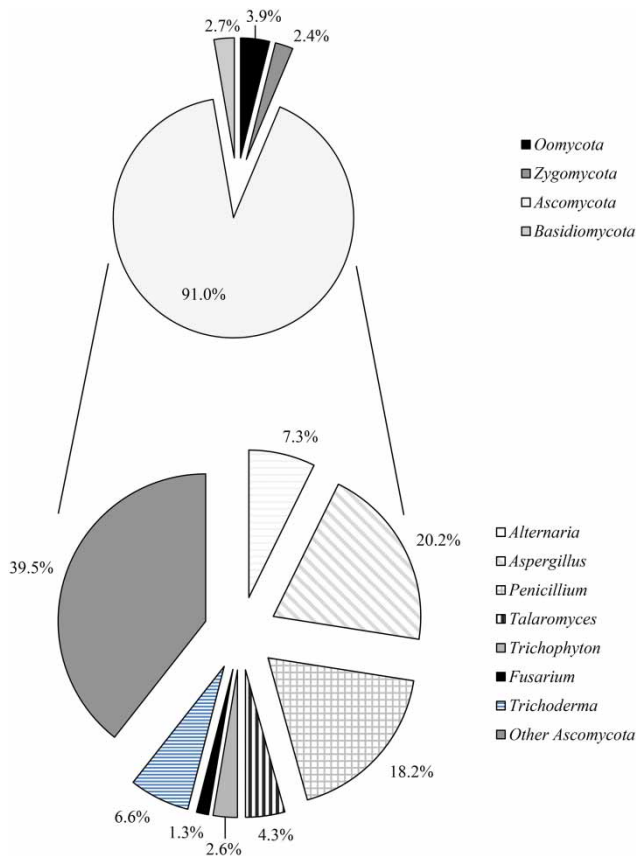
No.	Phylum	Classis	Genus	Pond [X/A] <sup>a</sup>															Total number of species	Number of clinically relevant species
				SJ			M			S			J			A				
				I	II	III	I	II	III	I	II	III	I	II	III	I	II	III		
1	<i>Oomycota</i>	<i>Oomycetes</i>	<i>Globisporangium</i>		X/1													1	–	
2			<i>Phytophthium</i>					X/1				X/1						2	–	
3			<i>Pythium</i>		X/1					X/1			X/1	X/2				3	3	
4			<i>Achlya</i>			X/1			X/1					X/1		X/1		1	1	
5			<i>Aphanomyces</i>	X/1														1	–	
6	<i>Zygomycota</i>	<i>Mucoromycetes</i>	<i>Lichtheimia</i>															X/1	1	1
7			<i>Mucor</i>			X/1		X/1				X/1						1	1	
8			<i>Rhizopus</i>	X/1	X/2													2	2	
9			<i>Syncephalastrum</i>											X/1				1	1	
10	<i>Ascomycota</i>	<i>Dothideomycetes</i>	<i>Neofusicoccum</i>		X/1	X/1			X/1			X/1	X/1					1	–	
11			<i>Neoscytalidium</i>											X/1				1	1	
12			<i>Cladosporium</i>	X/1					X/1									1	1	
13			<i>Hortaea</i>		X/1													1	1	
14			<i>Tripospermum</i>			X/1												1	1	
15			<i>Sydozia</i>	X/1		X/1								X/1				1	1	
16			<i>Arthrographis</i>						X/1					X/1				1	1	
17			<i>Taeniolella</i>	X/1														X/1	1	1
18			<i>Alternaria</i>	X/4	X/2	X/2	X/1	X/1	X/1	X/2	X/1	X/1	X/3	X/1	X/1			X/2	7	7
19			<i>Coniothyrium</i>							X/1								1	1	
20			<i>Didymella</i>	X/1														1	–	
21			<i>Medicopsis</i>	X/1		X/1												1	1	
22			<i>Phoma</i>				X/1	X/1		X/1								2	2	
23			<i>Stagonosporopsis</i>							X/1				X/1				1	1	
24		<i>Eurotiomycetes</i>	<i>Onychocola</i>						X/1					X/1			X/1	1	1	
25			<i>Cladophialophora</i>								X/1							1	1	
26			<i>Cyphellophora</i>	X/1					X/1									2	2	
27			<i>Exophiala</i>	X/1														1	1	
28			<i>Aspergillus</i>	X/2	X/3	X/7	X/5	X/6	X/2	X/4	X/3	X/1	X/4	X/6	X/7	X/5	X/2	X/4	23	21
29			<i>Neosartorya</i>			X/1								X/1				1	1	
30			<i>Paecilomyces</i>	X/1														X/1	1	1
31			<i>Penicillium</i>	X/7	X/3	X/4	X/3	X/3		X/8	X/2		X/8	X/3	X/8	X/2	X/1	X/3	18	14
32			<i>Talaromyces</i>		X/1		X/1		X/1		X/2	X/2		X/1	X/2	X/3			8	8
33			<i>Ajellomyces</i>		X/1	X/1		X/1		X/1				X/1	X/1				1	1

(continued)

Table 1 | continued

No.	Phylum	Classis	Genus	Pond [X/A] <sup>a</sup>															Total number of species	Number of clinically relevant species
				SJ			M			S			J			A				
				I	II	III	I	II	III	I	II	III	I	II	III	I	II	III		
34			<i>Arachniotus</i>			X/1				X/1								1	-	
35			<i>Chrysosporium</i>			X/1		X/1		X/1		X/1	X/2		X/1		X/1	2	2	
36			<i>Trichophyton</i>	X/1		X/1	X/1	X/1		X/1		X/1		X/1				3	3	
37		<i>Incertae sedis</i>	<i>Dissitimurus</i>	X/1									X/1					1	1	
38			<i>Scolecobasidium</i>	X/1			X/1											2	2	
39			<i>Staphylotrichum</i>												X/1			1	1	
40		<i>Leotiomycetes</i>	<i>Scytalidium</i>	X/1	X/1	X/2		X/1		X/3		X/1			X/1	X/1		3	3	
41		<i>Orbiliomycetes</i>	<i>Arthrotrichum</i>									X/1						1	1	
42		<i>Sordariomycetes</i>	<i>Coniochaeta</i>			X/1				X/1							X/1	2	2	
43			<i>Phaeoacremonium</i>					X/1										1	1	
44			<i>Acremonium</i>	X/2	X/1			X/1		X/2								4	4	
45			<i>Bisfusarium</i>							X/1								1	1	
46			<i>Fusarium</i>	X/1													X/1	2	1	
47			<i>Neocosmospora</i>					X/1	X/1								X/1	1	3	
48			<i>Hypomyces</i>		X/1				X/1									1	1	
49			<i>Ilyonectria</i>	X/1														1	1	
50			<i>Cordyceps</i>						X/1			X/1						2	2	
51			<i>Metarhizium</i>				X/1								X/1			1	1	
52			<i>Sarocladium</i>	X/2		X/1	X/1		X/1									2	2	
53			<i>Trichoderma</i>		X/1	X/5	X/1	X/4	X/2	X/2		X/2		X/1		X/1	X/1	5	5	
54			<i>Papulaspora</i>	X/1			X/1		X/1	X/1	X/1							3	3	
55			<i>Plectosphaerella</i>	X/1					X/1						X/1			1	1	
56			<i>Verticillium</i>	X/1														1	-	
57			<i>Pseudallescheria</i>													X/1		1	1	
58			<i>Scopulariopsis</i>		X/1			X/1										2	2	
59			<i>Cladorrhinum</i>												X/1			1	1	
60			<i>Humicola</i>		X/2	X/1		X/2				X/1			X/2	X/1	X/1	4	4	
61			<i>Thermothelomyces</i>						X/1									1	1	
62			<i>Arthrimum</i>		X/1													1	1	
63	<i>Basidiomycota</i>	<i>Agaricomycetes</i>	<i>Bjerkandera</i>	X/1			X/1		X/1						X/1	X/1		1	1	
64			<i>Riopa</i>						X/1								X/1	1	1	
65			<i>Phanerodontia</i>			X/1												1	1	
66			<i>Sporotrichum</i> sp.					X/1										1	1	
<b>Total</b>																		<b>149</b>	<b>128</b>	

<sup>a</sup>X is the occurrence of any species of the genus. A is the number of species isolated within the genus.



**Figure 2** | The frequencies of phyla and genera found in water samples of examined ponds.

research seasons, and the lowest in the S pond in season III (Table 2). The lowest species richness (0.680) and species diversity indexes (0.144) were obtained for pond S in season III, with the mean number of fungi being 2.1 CFU/1 mL of water sample (Table 2).

In the examined ponds, depending on the sampling date, differences for water pH and water and air temperature were noted. The physicochemical parameters of the analysed tanks are presented in Table 3.

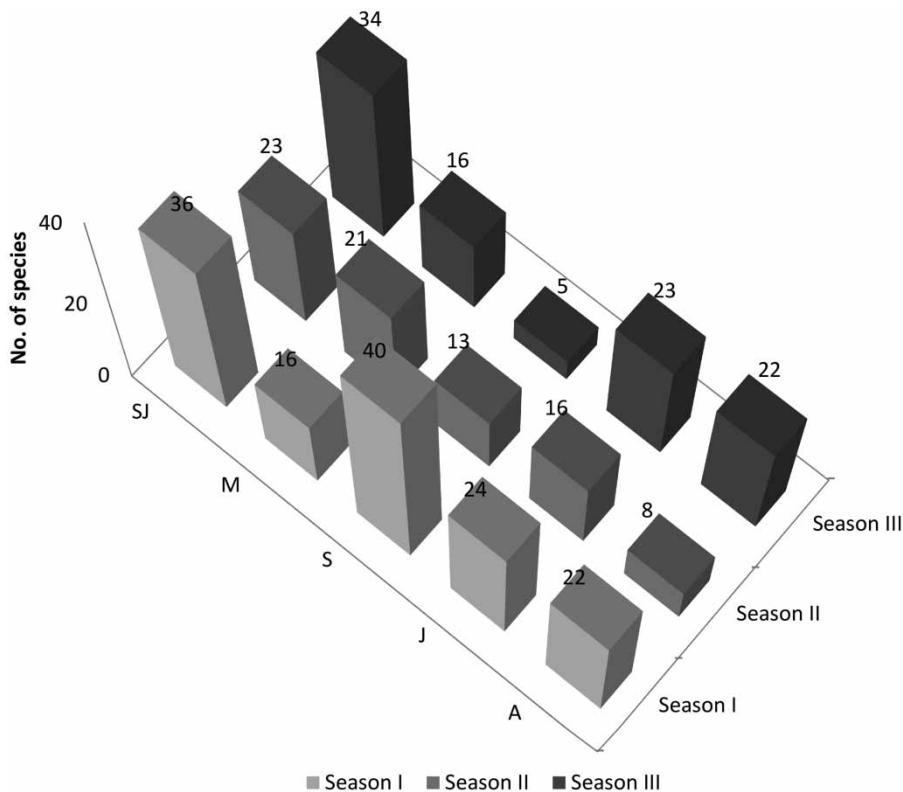
A statistically significant negative correlation (Spearman rank correlation) was found between the number of species from the SJ reservoir and the pH of its water ( $r = -0.737$ ) (Figures 4 and 5). There was no correlation between the number of species in the M, J, and A reservoirs and pH and water temperature, while positive correlations were found between the number of species in the S pond and water pH ( $r = 0.59$ ) and water temperature ( $r = 0.75$ ) (Figure 4). No significant correlation was observed between

the number of colonies and air temperature in any pond (Figure 5).

In the study, dominated *A. fumigatus* was detected in all reservoirs. *Alternaria alternata*, *Chrysosporium inops*, and *Penicillium aurantiogriseum* were also frequently isolated (Supplementary Table S1). In research season I, *A. fumigatus* and *P. chrysogenum* predominated, but *Bjerkandera adusta* was also frequent. In research season II, *A. fumigatus*, followed by *A. alternata* and *C. inops*, were most commonly isolated. In the third season, *Aspergillus fisheri* and *A. fumigatus* were most often identified. In the SJ pond, the most frequently recorded species was *A. fumigatus*, but *P. waksmanii*, *P. citrinum*, and *P. aurantiogriseum* were also very often found. In the M, dominated *A. fumigatus*, however, *Trichoderma harzianum* was also frequently identified. In the S reservoir, *A. fumigatus* and *A. alternata* were most frequently recorded. In pond J, the most frequently identified species was *A. niger*, but *A. fumigatus*, *P. waksmanii*, and *C. inops* were often noted as well. The most common species in reservoir A was *A. fumigatus*, but *A. niger* and *P. chrysogenum* were also very common (Supplementary Table S1).

Almost 86% of all identified fungal species are known to be associated with human infections. Table 2 shows the occurrence of clinically relevant species according to individual research season and ponds. Among the isolated species, 11 belong to RG-2 and 117 belong to RG-1; in addition, 24 species were classified as BSL-2 and 104 as BSL-1. More detailed data on the number of species detected in RG-1 and RG-2 in the examined ponds in the three research seasons are presented in Supplementary Table S2. Statistically significant differences in the numbers of clinically relevant species were observed between ponds and research seasons ( $\chi^2 = 24.905$ ,  $df = 8$ ;  $p = 0.0016$ ). The highest prevalence of RG-1 and RG-2 organisms was noted in SJ (81); statistically significant differences were found between the occurrence of clinically relevant species in the examined reservoirs ( $\chi^2 = 14.24$ ,  $df = 4$ ,  $p = 0.0065$ ). Species classified as BSL-1 and BSL-2 were most common in SJ (65 and 16, respectively), but similar frequencies were observed in other ponds; however, statistically significant differences were found between water reservoirs with regard to the number of species classified as BSL-1 ( $\chi^2 = 11.91$ ,  $df = 4$ ,  $p = 0.0180$ ). A significantly higher total





**Figure 3** | Species richness (S) in individual water reservoirs in three research seasons.

number of BSL-1 and BSL-2 fungi was found in season I (119) compared to other seasons ( $\chi^2 = 12.28$ ,  $df = 2$ ,  $p = 0.0022$ ).

## DISCUSSION

Worldwide studies have indicated that potentially pathogenic fungi are widespread in the waters of natural reservoirs, and even in water intended for drinking (tap water and bottled water), which poses a threat to human health (Siqueira et al. 2011; Oliveira et al. 2013; Ashbolt 2015; Fisher et al. 2015; Biedunkiewicz & Górska 2016; Novak-Babič et al. 2016). According to the literature data, the filamentous fungi from different genera (*Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, *Trichoderma*, *Mucor*, and *Rhizopus*) have often been detected in surface-, ground-, and tap water (Siqueira et al. 2011; Bandh et al. 2016; Novak-Babič et al. 2016). In our study, filamentous fungi from the aforementioned genera

predominate, especially *Aspergillus* spp. and *Penicillium* spp., which have also been observed by other authors in various world regions (Siqueira et al. 2011; Bandh et al. 2016; Baumgardner 2017). It is worth mentioning that in our study, *A. fumigatus* was the most common species isolated from all analysed ponds. Similar results from samples of water from the Augustow Canal (Poland) were obtained by Cudowski et al. (2015). The species that most frequently appeared in the samples from lakes (Poland) was *Aspergillus heteromorphus* (Biedunkiewicz & Górska 2016). On the other hand, *Aspergillus niger*, which is frequently isolated from river water samples in India (Parveen et al. 2011), dominated one of the five examined bathing areas of this study.

Numerous studies on the presence of typical aquatic fungi, such as zoosporic fungi from the *Oomycetes* and *Chytridiomycetes*, can be found in the literature (Czeczuga et al. 2003; Kiziewicz et al. 2004; Hu et al. 2013; Cudowski et al. 2015; Godlewska et al. 2016; Valderrama et al. 2016); much fewer studies concern fungi that secondarily colonize

**Table 2** | Sampling stations characteristics with species richness (S) and species diversity

	SJ			M			S			J			A		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Number of species	36	23	34	16	21	16	40	13	5	24	16	23	22	8	22
Number of colonies (S)	68	40	69	35	60	21	99	38	54	82	25	57	24	12	88
Average number of colonies (CFU/1 mL) $\bar{x} \pm SD$	3.8 ± 4.965	2.2 ± 2.901	3.8 ± 1.618	1.9 ± 1.862	3.3 ± 4.935	1.2 ± 1.200	4.1 ± 4.267	1.6 ± 2.570	2.2 ± 1.180	4.6 ± 5.227	3.2 ± 2.333	1.4 ± 0.916	0.8 ± 1.215	0.4 ± 0.674	2.9 ± 5.413
Menhinick's species richness index <sup>a</sup>	4.37	3.64	4.09	2.70	2.71	3.49	4.02	2.11	0.68	2.65	3.20	3.05	4.49	2.31	2.35
Simpson's diversity index <sup>a</sup>	0.95	0.92	0.96	0.83	0.71	0.98	0.94	0.60	0.14	0.88	0.96	0.95	0.99	0.91	0.92
Number of clinically relevant species <sup>b</sup>	31	20	30	16	19	14	33	12	5	18	14	18	21	7	21
Number of species from															
<i>Zygomycota</i>	1	2	1	0	1	0	0	0	0	1	0	1	0	0	1
<i>Alternaria</i>	4	2	2	1	1	1	2	1	1	3	1	1	0	0	2
<i>Aspergillus</i>	2	3	7	5	6	2	3	2	1	3	6	6	4	2	4
<i>Cladosporium</i>	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0
<i>Penicillium</i>	7	2	3	3	2	0	6	2	0	6	3	6	2	1	3
Dermatophytes	1	0	1	1	1	0	1	0	1	0	1	0	0	0	1

<sup>a</sup>Based on all identified species.<sup>b</sup>Number of clinically relevant species with defined risk group (RG) and biosafety level (BSL).

**Table 3** | Physicochemical parameters in analysed ponds during sampling

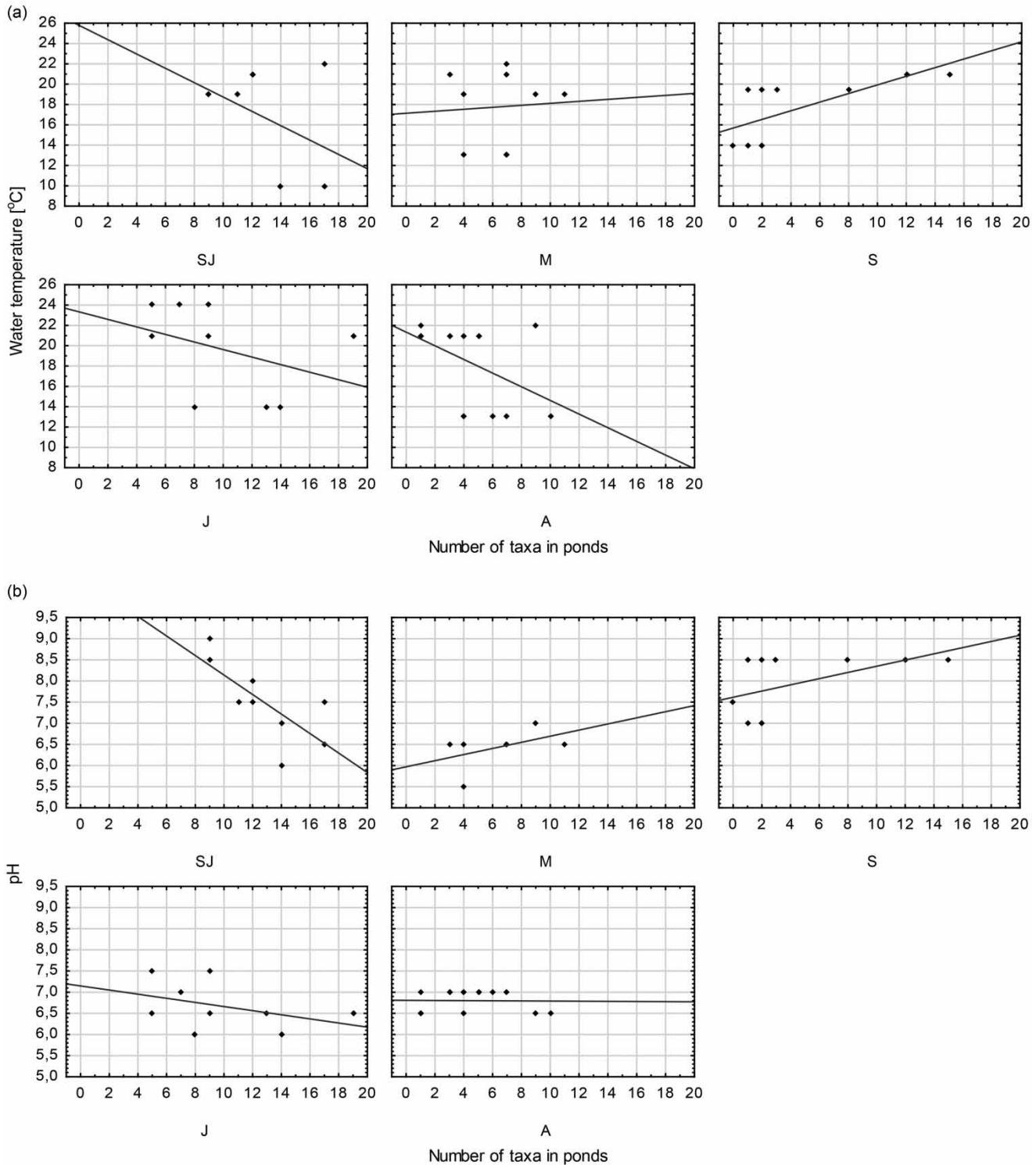
	Research season <sup>a</sup>														
	June (I)			July and August (II)						September (III)					
	18.8			17.8						15.8					
Average temperature (°C)	74.8			73.7						70.1					
Average humidity	Pond														
	SJ			M			S			J			A		
Season	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Air temperature (°C)	21	16	6.5	21	17.5	7.5	16	17	5.5	19	24	12	19	15	10
Water temperature (°C)	21.3	19	10	21.3	19	13	21	19.5	14	21	24	14	21.4	21	13
Water pH	7.67	8.3	6.5	6.5	6.67	6.67	8.5	8.5	7.13	6.5	7.3	6.17	6.8	6.8	6.8

<sup>a</sup>Water samples were collected from different ponds for 1 week in each research season.

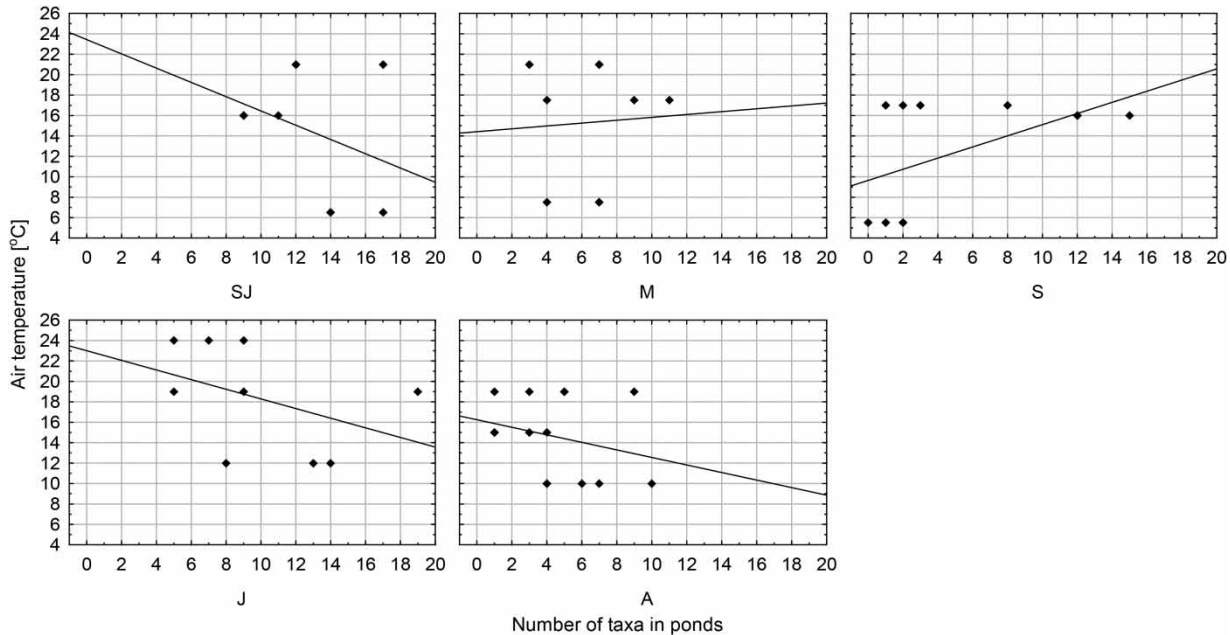
water (*Aspergillus*, *Penicillium*, *Fusarium*, and *Trichoderma*), many of which are potentially pathogenic and can cause mycosis in humans and animals (Arvanitidou et al. 2002; Parveen et al. 2011; Bandh et al. 2016; Di Piazza et al. 2017). In our study, only 13 of the isolated 149 taxa were aquatic. Similarly, species of moulds originally associated with terrestrial environments have been found to dominate in bathing lakes in northern Poland (Biedunkiewicz & Górska 2016), and Parveen et al. (2011) report the presence of 31 species of secondary aquatic fungi in water samples from a river in Raipur city (India). In contrast, Czczuga et al. (2003) report that as many as 108 out of 200 species in the Biebrza River, a natural water body in Poland, belonged to aquatic fungi; in addition, of 26 species isolated in the Horodnianska River, Poland, 20 were originally aquatic fungi (Kiziewicz et al. 2011). These data indicate that primary terrestrial fungi occur more frequently in anthropogenically modified water reservoirs than in natural waters, where autochthonous species dominate.

Waterborne fungal infections may occur in different ways, such as exposure during sports and recreation, drinking contaminated liquids, and from personal and home hygiene activities, including the inhalation of aerosols while showering. Fungal infections resulting from exposure to water of natural and anthropogenic water reservoirs (e.g. swimming pools, water parks, interactive fountains, and jacuzzis), primarily affects immunocompromised

people or those with trauma, including damaged skin (Hageskal et al. 2009; Baumgardner 2017). These infections commonly manifest as allergies and dermatomycoses (mycoses of the skin or nails) but sometimes manifest as *keratitis* and acute *otitis media* caused mainly by fungi from the following genera: *Candida*, *Fusarium*, *Aspergillus*, and *Paecilomyces* (Kamihama et al. 1997; Hageskal et al. 2009; Baumgardner 2017). Among filamentous fungi, species from the genera *Fusarium* and *Aspergillus* are the most common causes of fungal *keratitis* in the USA and other parts of the world. A study conducted from 2004 to 2008 in Philadelphia revealed an increase in the number of fungal *keratitis* cases caused by *Fusarium*, especially among contact lens users; four of the 28 patients with *keratitis* reported specific water exposure: two from lakes and one each from well water exposure and swimming while wearing contact lenses (Baumgardner 2017). Fungi from the genus *Fusarium* were sporadically isolated in the present study; however, they are commonly detected in surface water, groundwater, and even tap drinking water in Europe (Novak-Babič et al. 2017). Additionally, surface water can be a possible reservoir of dermatophytes, which are mainly zoophilic (*Microsporum canis*, *Trichophyton verrucosum*, and *T. mentagrophytes*) and geophilic species (*Microsporum gypseum*) that are highly transmissible between animal hosts or soil environment and people. On the contrary, anthropophilic dermatophyte species



**Figure 4** | The number of fungal taxa from water samples depending on (a) water temperature and (b) pH. (a) The significant positive correlation was found only in pond S. Spearman correlation coefficients were  $r = -0.406$  (SJ),  $r = 0.067$  (M),  $r = 0.745$  (S),  $r = -0.385$  (J), and  $r = -0.475$  (A). (b) The significant negative correlation was found only in pond SJ and significant positive correlation in pond S. Spearman correlation coefficients were  $r = -0.737$  (SJ),  $r = 0.472$  (M),  $r = 0.587$  (S),  $r = -0.403$  (J), and  $r = -0.020$  (A).



**Figure 5** | The number of fungal taxa water samples depending on air temperature. There was no significant correlation. Spearman correlation coefficients were  $r = -0.328$  (SJ),  $r = 0.059$  (M),  $r = -0.555$  (S),  $r = -0.418$  (J), and  $r = -0.275$  (A).

(*Trichophyton schoenleinii*, *T. tonsurans*, and *T. violaceum*) are most commonly isolated from water used for recreational facilities, such as swimming pools, jacuzzis, baths, and saunas (Novak-Babič et al. 2018). It is worth noting that *Trichophyton* spp. and *Epidermophyton floccosum*, which cause superficial fungal infections of the hair, fingernails, or skin, are the only fungal species considered as potential microbial hazards in the WHO guidelines for safe recreational water (WHO 2006). In these studies, three species of dermatophytes (*Trichophyton verrucosum*, *T. schoenleinii*, and *T. violaceum*) were detected in recreational waters. Contamination of bathing areas with these dermatophytes probably occurred as a result of water coming into contact with bathing people and their animals such as dogs. During sample collection, we observed that pet owners often walked there with dogs, which also entered the water.

Although the categories of RGs and BSLs were introduced to protect laboratory workers from occupational health risks, the classification of species to risk group organisms can also help to estimate the clinical relevance of isolates detected from sources outside the laboratory and determine the risk of environmental exposure to fungi.

The classification of a species to a specific RG indicates its pathogenicity. It needs to be highlighted that the potentially pathogenic fungi for humans are usually associated with soil, air, water, the human home, and hospital environments. It is estimated that of all the fungi described in the world, approximately 600 species are opportunistic pathogens (de Hoog et al. 2015), which are etiological agents of mycoses, mainly in immunocompromised persons. Over the last two decades, the increase in the number of immunocompromised patients may promote the spread of mycoses caused by environmental RG-1 and RG-2 fungi. In our study, almost 86% of the identified taxa of filamentous fungi were clinically relevant species: 11 belonged to RG-2 and 117 to RG-1, while the same species were, respectively, classified as BSL-2 (24) and BSL-1 (104). Another study performed in three lakes utilized for recreation in north-eastern Poland found a high percentage (73%) of clinically relevant fungal species – eight species out of 45 belonged to BSL-2 and 25 belonged to BSL-1 (Biedunkiewicz & Górska 2016). Additionally, 42 of the 79 identified species isolated from various sites in a swimming pool facility by Ekowati et al. (2017) were classified as BSL-1 and RG-1. The presence of such fungi in recreational waters represents a potential

threat of fungal infections being spread through water, especially in persons with immunodeficiency.

The RG and BSL classifications do not always coincide for a particular organism: for example, *Aspergillus flavus* is classified as both RG-1 and BSL-2. In this case in laboratories, BSL-2 should be maintained due to the easy spread of conidia and their small size (3.5 µm diameter). Although this species occurs in the environment as a saprobe, numerous reports indicate its pathogenicity towards humans: the Atlas of Clinical Fungi (de Hoog et al. 2019) notes about 40 publications on the pathogenicity of *A. flavus* based on cases of mycoses in humans. In our study, *A. flavus* was detected less frequently than other *Aspergillus* species, with *A. fumigatus* being most frequently isolated from water samples. De Hoog et al. (2019) list over 120 publications on the pathogenicity of *A. fumigatus* (RG-2 and BSL-2). This species is the main agent of pulmonary aspergillosis in patients with impaired immunity (de Hoog et al. 2019). Exposition to fungal propagules via water aerosols is believed to represent the most potential route of infection for bathers (Novak Babič et al. 2017).

It has been suggested that human skin might be made more susceptible to fungal infection, especially opportunistic black fungi from genera *Exophiala* and *Cladophialophora* by softening due to bathing (Lian & de Hoog 2010; Wang et al. 2018). Two such species (*Exophiala jeanselmei* and *Cladophialophora carrionii*) were occasionally identified in the water samples in the present study. *Exophiala* species are often isolated from indoor water sources, such as sinks, swimming pools, and bathing facilities, and also occur in surface water and municipal drinking water enabling biofilm formation (Oliveira et al. 2013; Novak Babič et al. 2017; Novak Babič et al. 2018). The clinical manifestations of *E. jeanselmei* (RG-2/BSL-2) mainly concern subcutaneous phaeohyphomycosis and black-grain mycetoma. The propagules are mostly hydrophilic and infections mostly being of traumatic origin (de Hoog et al. 2019).

Additionally, allergenic fungi were also identified in the present study. Apart from numerous *Aspergillus* and *Penicillium* species, *Alternaria* and *Cladosporium* species were also detected. *Alternaria* and *Cladosporium* are cosmopolitan genera of saprobe commonly found on the dead plant material and are known to be ubiquitous laboratory

contaminants. *Alternaria* was represented in the present study by seven species, which were detected in all examined ponds. *Alternaria alternata* (RG-1/BSL-1) may cause skin lesions after trauma, often in immunocompromised patients or with chronic underlying metabolic disease, as well as in cases of keratitis, cutaneous infections and otitis, onychomycoses (de Hoog et al. 2019). Unlike *Alternaria*, only one species of *Cladosporium* (*C. sphaerospermum* RG-1/BSL-1) was identified in the present study.

Potentially pathogenic species show the ability to grow at body temperature (37 °C) and even within the fever range (38–42 °C) of the human host, which is an important requirement for systemic infection. Additionally, small spore sizes aid fungal entry and penetration through the host's barriers. Oliveira et al. (2013) reported that 66% of the species isolated from different drinking water sources were able to grow at 30 °C and they had spore sizes below 5 µm, while *A. fumigatus*, *A. viridinutans*, and *Cunninghamella bertholletiae* were able to grow at the higher temperature tested (42 °C). It is worth emphasizing that the majority of clinically relevant species isolated from water samples of examined ponds produce spores with a diameter below 5 µm (e.g. *Aspergillus* spp., *Penicillium* spp., *Acremonium* spp., and *Trichodetma* spp.). Of the species belonging to RG-1, only a few, including *Alternaria* spp., have spores larger than 5 µm.

Various indicators with different sensitivities are used to assess species richness and biodiversity (Goncalves et al. 2012; Mirzaie et al. 2013; Kanieski et al. 2018) and their application is related to the type of population analysed and the properties of the studied ecosystem. They are used to compare both the richness of the species and the diversity of the studied environments, changes in the structure of the ecosystem over time, and to assess the impact of environmental and anthropogenic factors on the studied populations. The indexes commonly used for assessing the structure of the water fungal community are Menhinick's species richness index and Simpson's diversity index. Menhinick's index is recognized as a species richness indicator for both high- and low-abundance samples (Mirzaie et al. 2013; Kanieski et al. 2018). Simpson's diversity index represents the probability that two individuals randomly selected from a sample will belong to different species. These indicators were used in the present study to identify

qualitative and quantitative differences in the fungal community in the studied recreational ponds at different sampling periods, i.e. June, July and August, and September. Simpson's diversity index indicated a high species diversity for most analysed ponds: for 10 out of the 15 analysed samples (i.e. various ponds and research seasons), Simpson's diversity index was above 0.9. It should be emphasized that particularly large discrepancies in the two indices were recorded in pond S (reservoir formed on the Ner River), where the indicators fell from 4.02 (Menhinick's index) and 0.942 (Simpson's diversity index) in June to 2.109 and 0.603 during period II and then to 0.680 and 0.144 during period III. In the period July–September, no significant changes in pH or water temperature were observed in comparison to other reservoirs; however, in September, work began to modernize water pond S to remove sludge from its bottom which could have disturbed the existing aquatic ecosystem. According to the literature data, these indicators display wide variation regardless of climate zone: considerable differences in Simpson's diversity index values have been found in Antarctic lakes (0.13–0.72) (Goncalves *et al.* 2012) and in urban lakes in China (0.39–0.98) (Zhang *et al.* 2018).

The abundance and species diversity of fungi depends on the properties of water reservoirs, such as standing water, flowing water, and reservoir volume, as well as the prevailing physicochemical conditions, such as access to light, the amount of oxygen dissolved in water, water pressure, and the presence of organic compounds (Pejman *et al.* 2009; Krauss *et al.* 2011; Pietryczuk *et al.* 2018). Environmental stress factors, such as high concentrations of heavy metals, sulphates, and nitrates, as well as low concentrations of oxygen, have been found to significantly reduce the diversity and biomass of hyphomycetes (Sole *et al.* 2008). Redundancy analysis (RDA) indicates that variations in water chemistry cause a significant proportion of the change in fungal community structure (86.2%), with fungi being negatively correlated with high metal and nutrient concentrations. Nitrates and phosphates stimulate fungal growth but at higher concentrations, this positive correlation between nutrients and fungal diversity may become reversed (Krauss *et al.* 2011). Sridhar *et al.* (2009) report low fungal diversity at higher concentrations of biogens. The recreational water bodies

examined in the present study are artificial reservoirs formed on small rivers flowing through the city of Lodz. These rivers are supplied with water from storm canals collecting pollution from the centre of the city, recreational plots and industrial plants, thus water is rich in organic matter and chemical pollutions. Additionally, they are subject to periodic increases in pollution due to illegal discharge of industrial wastewater that may affect the fluctuations in the number of microfungi in the ponds.

Most aquatic hyphomycete species tolerate pH within a range between 5 and 7: the number of species in water reservoirs declines below and above these values (Krauss *et al.* 2011). Anthropogenic acidification of water strongly slows the decomposition of leaves and other organic matter by inhibiting the activity of such enzymes as pectin lyase. Low pH values can also raise aluminium concentrations in stream water, which severely depresses fungal richness and activity (Baudoin *et al.* 2008). Additionally, greater increases in temperature resulting from sunlight irradiation, found in slow-flowing surface water, and the DNA-damaging effect of UV-radiation may lead to reduce the abundance of fungi and other microorganisms in the surface layer (Dand *et al.* 2009; Novak Babič *et al.* 2017). Although the physicochemical conditions in examined ponds varied widely depending on the time of sampling (temperature: 10–24 °C, pH: 6.5–8.5), it seems that these fluctuations did not appear to have a significant impact on the abundance of the fungal population. It should be emphasized that high air temperatures associated with strong solar radiation were recorded (mean 18 °C; maximum 33 °C) during the sampling period, both in June and July, that could modulate the abundance fungal communities. The abundance of fungi was found to be low in the samples, varying from 0.4 to 4.6 CFU/1 mL according to the time of sampling. Comparable CFU values were obtained for three lakes used for recreational purposes located in Olsztyn, northern Poland (Biedunkiewicz & Górska 2016), where the amounts of fungal propagules ranged between 190 and 550 CFU/L before swimming season, 375–7,000 CFU/L during the season, and 90–1,800 CFU/L after the season. Much lower abundance was observed in water samples taken from five Antarctic lakes (from 6.5 to 62.0 CFU/1 L) (Goncalves *et al.* 2012), and much higher levels in water samples taken in the Araçá Bay mangrove swamp, São Sebastião, Brazil

(Doi et al. 2018), where filamentous fungus colony density ranged from  $0.1 \times 10^4$  to  $4.6 \times 10^4$  CFU/100 mL.

## SUMMARY

Most human mycoses are caused by opportunistic fungi with a widespread occurrence in the environment (genera *Aspergillus*, *Fusarium*, and *Rhizopus*). Our study suggests that natural bathing areas can serve as reservoirs of potentially pathogenic fungi. Among the 149 taxa of filamentous fungi isolated, 128 (over 85%) were clinically relevant species belonging to risk group 1 and 2 (RG-1 or RG-2). Such a significant percentage of species associated with human fungal infections highlights the potential health risk for people bathing in natural bathing areas. Potentially pathogenic species of *Aspergillus*, *Cladosporidium*, *Alternaria*, *Fusarium*, *Penicillium*, and *Phialophora* have also been detected in other studies on surface waters used for recreation in other parts of the world. Hence, it is recommended that the degree of fungal contamination of natural and anthropogenic water reservoirs should be monitored, especially in the bathing season.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this paper is available online at <https://dx.doi.org/10.2166/wh.2020.096>.

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