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Yeasts and yeast-like fungi in tap water and groundwater, and their transmission to household appliances

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ABSTRACT

In the present study we describe the occurrence of fungi in 100 tap water and 16 groundwater samples from Slovenia. We used culture-dependent and culture-independent techniques. 28 fungal species belonging to 16 genera were isolated with selected culturing conditions, targeting human opportunistic yeasts and yeast-like fungi. Of special concern was the detection of *Aureobasidium melanogenum*, *Exophiala dermatitidis*, *Rhinocladiella similis*, *Candida parapsilosis* and *Rhodotorula mucilaginosa*. The DGGE analysis of ITS1 rDNA revealed from 6 to 16 bands hypothetically corresponding to different taxa, while pyrosequencing showed the presence of *Aspergillus* and *Exophiala*. According to the statistic machine learning methodology, the profile of fungi in water is determined by the concentration of calcium and magnesium ions and the presence of nitrate. *Exophiala* spp., *C. parapsilosis* and *R. mucilaginosa* are known as dominant contaminants of household appliances. It appears that they are transferred with water to dishwashers and washing machines, where they subsequently proliferate.

1. Introduction

Over the last 30 yr, fungi in indoor environments, in particular the clinical environments have increasingly been recognized as a health problem, linked to a growing immunocompromised population. Over the last 30 yr more than one billion people around the world have suffered from different fungal infections. These were also reported from more than 10% of autopsied patients (Lehrnbecher et al., 2010; Vos et al., 2010). Fungi can cause infections of skin, hair, nails, urinary and respiratory tract, catheter related and systemic infections. People not only come across pathogenic fungi in nature, but also in public places and households. Thus, fungi are present in indoor air, they can invade indoor damp walls (Adan and Samson, 2011), household wet cells, such as bathrooms and kitchens (Matos et al., 2002; Adams et al., 2013), and even extreme indoor habitats such as household appliances, for example dishwashers (Zalar et al., 2011) and washing machines (Gattlen et al., 2010; Novak Babič et al., 2015). Conditions inside household appliances used to be considered hostile to microbial growth. However, increased consumer awareness toward sustainable use of resources and hazardous chemicals, and novel technologies led to the development of household appliances operating at lower temperatures, with reduced amounts of water, and increased use of biodegradable detergents. These conditions are selective for thermotolerant, oxidative-stress resistant, and stress-tolerant fungi generally recognized as polyextremotolerant fungi, many of which are

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opportunistic human pathogens (Gostinčar et al., 2009; Zalar et al., 2011). Thus, dishwashers around the world are consistently colonized with polyextremotolerant yeasts, black-pigmented *Exophiala dermatitidis* and *Exophiala phaeomuriformis,* white *Candida parapsilosis*, red-pigmented *Rhodotorula mucilaginosa*, and filamentous *Fusarium dimerum*, *Fusarium oxysporum* and *Fusarium solani* species complexes (Zalar et al., 2011; Gümral et al., 2015). Surprisingly, mycobiota of washing machines showed an overlap with the mycobiota of dishwashers in the occurrence of *C. parapsilosis, R. mucilaginosa* and *E. phaeomuriformis*, whereas members of the *F. oxysporum* species complex, recovered from dishwashers with very low frequency, have been isolated with the highest frequency from washing machines (Novak Babič et al., 2015).

The majority of *Exophiala* species are opportunistic pathogens that can cause cutaneous and subcutaneous infections, lung and neurotropic infections, mainly of immunocompromised but also of immunocompetent individuals (de Hoog et al., 2009; Machouart et al., 2011). Both *R. mucilaginosa* and *C. parapsilosis* have been reported as newly emerging pathogens, causing primarily catheter-related infections and opportunistic nosocomial fungemias in immunocompromised patients (Neofytos et al., 2007; Pfaller et al., 2007; Van Asbeck et al., 2009; Miceli et al., 2011). Various *Fusarium* species are causative agents of approximately 80% of human fungal infections. They produce mycotoxins in water (Kelley et al., 2003), cause localised subcutaneous infections, sinusitis, and onychomycosis (O'Donnell et al., 2010; Sutton and Brandt, 2011; Garnica and Nucci, 2013).

Fungi might enter household appliances via air or water, food and waste, with influence of humans and their pets. Colonization of both dishwashers and washing machines with largely overlapping fungal species points at the water supply system as the main vector. Although several studies investigated the presence of fungi in water, their primary focus was on fungal genera that can be dispersed from water to air due to sporulation (Anaissie et al., 2002). Therefore, genera such as *Acremonium*, *Alternaria, Aspergillus*, *Aureobasidium, Cladosporium*, *Fusarium, Kloeckera, Paecilomyces, Penicillium, Phialophora, Phoma, Scopulariopsis, Stachybotrys* and *Trichoderma* were mostly isolated. Among yeasts, the presence of the genera *Candida, Cryptococcus* and *Rhodotorula* has been reported (Kinsey et al., 1999; Göttlich et al., 2002; Paterson and Lima, 2005; Hageskal et al., 2006; Grabinska-Loniewska et al., 2007; Pereira et al., 2010). No study has so far focused on the potential presence of human opportunistic pathogenic yeast and yeast-like fungi in public tap water systems as an entry point to household appliances, where selection and enrichment of selected species occurs.

In the present study we focused on the diversity of human opportunistic pathogenic yeasts and yeast-like fungi in groundwater and tap water, based on culturing techniques. In parallel, we analysed fungal communities in raw water sources (rivers, groundwater), selected tap water samples, as well as water after waste water cleaning treatment, by the analysis of ITS1 rDNA amplicons from total DNA by Denaturing Gradient Gel Electrophoresis (DGGE). Using next generation sequencing (NGS) technology, we have analysed a single tap water sample by pyrosequencing of ITS2 rDNA. We investigated the potential correlations between the appearance of yeasts and yeast-like fungi, detected by culture-dependent techniques, and water characteristics, using machine learning methodology. The overall aim was to determine whether tap water acts as the main vector for the inoculation of fungi in household appliances, where extreme abiotic conditions promote settlement and proliferation of selected human opportunistic fungal pathogens.

2. Materials and methods

2.1. Isolation and cultivation of fungi from water

Slovenia can be divided into 5 geographical regions: Alpine and Subalpine, Littoral, Pannonian and Dinaric Karst regions, which differ in geology, and thus also in water characteristics. The study mainly focused on water sampling in the Ljubljana valley, which is partly Subalpine and Dinaric Karstic, while the remaining samples originated from 9 Slovenian cities, representing all geographical regions mentioned above. Samples of tap water were collected from regularly used water pipes (running tap water) of 100 private homes in different locations in Slovenia. Out of these, 50 samples were obtained in the capital city of Ljubljana, and originated from the 8 main water supply systems, while 50 samples originated from the following cities and sub-urban areas: Bohinj, Celje, Mislinja, Laško, Litija, Logatec, Ljutomer, Ormož, Portorož, Postojna, Ravne na Koroškem, Radomerje, Rodica, Ruše, Sečovlje, Sežana, Trebnje, Trebče and Velenje (Fig. 1). Additionally, 16 samples of groundwater used for tap water were obtained from Ljubljana. In the case of running tap water, 5 l of cold water were collected according to the standard SIST ISO 5667-5:2007. The groundwater samples were collected at main water supplies in sterile containers by employees of Waterworks and Sewage Company, according to the standard SIST ISO 5667-5:2007 (VO-KA, Ljubljana). An aliquot of 1 L of each sample was filtered twice using 0.45 μm membrane filters (Merck, Millipore), which were placed on Dichloran Rose Bengal Agar (DRBC; Oxoid Ltd., England) (Pereira et al., 2010), and were each incubated at 30 and 37 °C for 5–7 d. Pure cultures of fungi were transferred to malt extract agar (MEA) and deposited in the Ex Culture Collection of the Infrastructural Centre Mycosmo, MRIC UL, Slovenia: http://www.ex-genebank.com/, at the Department of Biology, Biotechnical Faculty, University of Ljubljana.

2.2. Genomic DNA extraction from pure cultures and from water samples

DNA from 3 d old yeast cultures grown on malt extract medium (MEA; Biolife, Italy) was extracted using PrepMan Ultra reagent (Applied Biosystems) following the manufacturer's instructions. DNA of filamentous fungi was extracted from 7 d old cultures grown on MEA using mechanical lysis of 1 cm^2 of mycelium, following instructions of Van den Ende and de Hoog (1999). Genomic DNA from water samples was obtained from 3 l of water, filtered through 0.45 μm membrane filters (Merck, Millipore) and extracted using PowerWater DNA Isolation Kit (MO BIO Laboratories Inc.) according to the manufacturer's instructions. DNA samples ready for downstream applications were stored at −20 °C.

2.3. Identification of pure cultures

Fungi were identified according to their morphological characters, but their identification was complemented with rDNA nucleotide sequence analyses of internal transcribed spacer region 1, 5.8S rDNA and ITS 2 (ITS). For amplification and sequencing primers ITS5 and ITS4 were used (White et al., 1990). Yeasts were identified by sequencing D1/D2 domains of 28S rDNA, (large subunit of ribosomal DNA; LSU) using primer set NL1 and NL4 (O'Donnell, 1993). All sequences were obtained at Microsynth AG, Switzerland using an ABI Prism 3700 Big Dye Sequencer (Applied Biosystems). Sequences were assembled by FinchTV 1.4 (Geospiza, PerkinElmer, Inc.). Fungi were identified with the BLAST algorithm at NCBI web page (Altschul et al., 1990) and by use of other taxonomically important databases (Centraalbureau voor Schimelcultures, Utrecht, the Netherlands CBS). Software Molecular Evolutionary

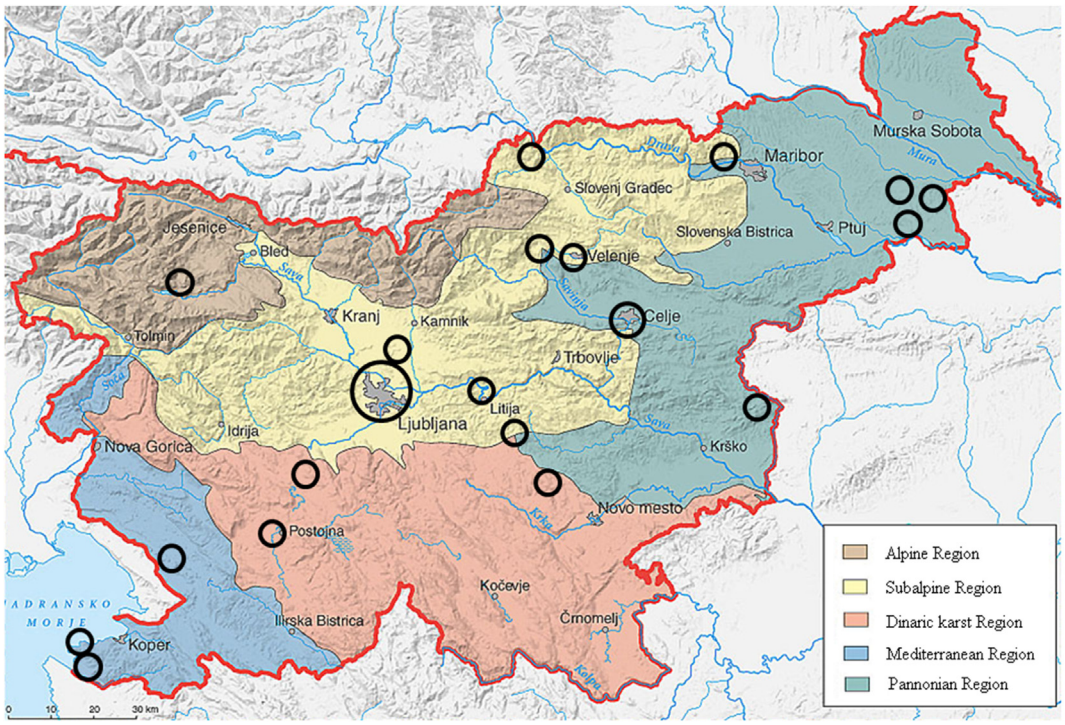

Fig. 1. Map of geographic regions of Slovenia (source: Anton Melik Geographical Institute, Ljubljana, Slovenia) with indicated sampling sites. Samples of tap water were collected from 100 private homes in different locations (indicated with black circles) positioned in 5 geographic regions of Slovenia. Fifty samples were obtained from the capital city of Ljubljana, while 50 samples originated from the following cities and sub-urban areas: Bohinj, Celje, Mislinja, Laško, Litija, Logatec, Ljutomer, Ormož, Portorož, Postojna, Ravne na Koroškem, Radomerje, Rodica, Ruše, Sečovlje, Sežana, Trebnje, Trebče and Velenje.

Genetics Analysis (MEGA) version 5.0 (Tamura et al., 2011) was used for alignments.

2.4. Pyrosequencing (454) of a tap water sample

Total DNA was extracted by PowerWater® DNA Isolation Kit (MO BIO Laboratories Inc.) from 3 l of a single tap water sample from Ljubljana, sampled according to standard SIST ISO 5667-5:2007. The DNA concentration was 3.55 ng μl^{-1}. The ITS2 of fungi was amplified using primer set ITS3 and ITS4 (White et al., 1990). Sequences and clusters were obtained with QIIME software package (Caporaso et al., 2010). Reads with less than 60% similarity were discarded, while sequences with 97% or more similarity were clustered into operational taxonomic units (OTU) and assigned to taxonomic identities from the sequence databases UNITE, NCBI, EMBL and DDBJ. Metagenome data was deposited in NCBI Short Read Archive (SRA) under following accession numbers: BioProject number: **SRP059666**, Sample number: **SRS965821**, Experiment number: **SRX1065967**, and Run number: **SRR2070801**.

2.5. DGGE analysis of fungal communities in water

DGGE was used for diversity visualisation within and between fungal communities in different water samples. Samples were derived from two aquifers: Ljubljana field (local name "Ljubljansko polje") at north, and Ljubljana moor (local name "Ljubljansko barje") at south of Ljubljana, Slovenia. Three samples of water from rivers were taken from river spring Ljubljanica in Vrhnika, river mouth Ljubljanica (Ljubljana), and from river Sava (Ljubljana). Additionally, 5 groundwaters, 5 tap waters and 2 water treatment plants from above mentioned aquifers were sampled. Total genomic DNA from water samples was extracted as explained in the section 'Genomic DNA extraction from pure cultures and from water samples', and amplified in two polymerase chain reactions (PCR): first with primer pair ITS4 (White et al., 1990) and EF4 (Smit et al., 1999), followed by second, nested PCR using primer set ITS1-gc (Gardes and Bruns, 1993) and ITS2 (White et al., 1990). Polyacrylamide gels were prepared according to Muyzer et al. (1993). The electrophoresis was run at 70 V for 16 h. The gel was stained with SYBR® Safe DNA Gel Stain (Life Technologies). Image of the gel was analysed using the GeneTools software (Syngene). DGGE patterns were analysed with software tool BioNumerics, version 7.1 (Applied Biomaths). Cluster analysis was performed using Pearson correlation with 1% optimization and presented as an UPGMA dendrogram.

2.6. Analysis of ionic concentrations in tap water samples

Cl^-, NO_3^-, NO_2^-, PO_4^{3-}, and SO_4^{2-} ions were measured with high performance ion chromatography (HPIC), implemented with Chromeleon equipment (Chromatography Management System) using eluent solutions 1.8 mM Na_2CO_3, 1.7 mM $NaHCO_3$ and the separation column HPIC-AS4A AC (Dionex). For the analytical procedure micro-membrane ASRS (Dionex) was used as suppressor and 12.5 mM H_2SO_4 as regenerator. The calibration curves were prepared and retention times determined. Based on the intensity of the signal, the concentration of each ion type was estimated according to Standard SIST EN ISO 10304-1:2009.

Na^+, Mg^{2+}, Ca^{2+} ions were measured with atomic emission spectrometry using Varian AA240 Atomic Absorption Spectrometra. Na-EDTA (Merck, Germany) was used as standard for the calibration curve. Ten-fold serial dilution of 10 ml of each water sample were analysed; the water sample flow through the machine was 5 ml min^{-1}. The mixture of ethylene and N_2O was used as the flame source. Final concentrations were determined following the method from Standard SIST EN ISO 11885:2009.

2.7. Statistical analysis of data using machine learning methods

To investigate the differences between samples in terms of the absence or presence of different fungal genera, a machine learning methodology that develops interpretable models, i.e. decision trees, was used. Decision trees typically do not require prior data transformation, and we did not perform data transformations. Decision trees (Breiman et al., 1984; Quinlan, 1993) are hierarchical models in which each internal node contains a test on a descriptive attribute of a water sample and each branch leaving this node corresponds to an outcome of this test. Each terminal node (leaf) of a tree represents a cluster of samples with similar values of the dependent variable. We used the decision tree learning algorithm from the CLUS data mining software (Blockeel and Struyf, 2002), which implements the paradigm of Predictive Clustering Trees (Blockeel et al., 1998). Default parameter settings for the learning algorithm were applied except that the minimum number of samples in tree leaves was set to 5 in order to get trees small enough to be easily interpreted. The independent variables (attributes) were the concentrations of Cl^-, NO_3^-, NO_2^-, SO_4^{2-}, Mg^{2+}, Ca^{2+} and Na^+ ions in mg l^{-1}. The dependent (class) variable in our analysis was the absence or presence of individual fungal genera: "no" if no fungi were present, and "yes" if any genus of the fungi studied was present in the sample.

3. Results

3.1. Chemical analyses of tap water

Ninety tap water samples from 5 geographic regions of Slovenia were analysed for ion content. Average concentrations of Cl^-, NO_3^-, NO_2^-, PO_4^{3-}, SO_4^{2-}, Mg^{2+}, Ca^{2+} and Na^+ ions are presented in Table 1. Main differences were observed for samples taken from different sites of Slovenia, especially when comparing concentrations of Cl^- and Ca^{2+}. Alpine and Subalpine sites were characterized by increased concentrations of Mg^{2+} and Ca^{2+}, whereas Cl^-, Ca^{2+} and Na^+ ions dominated in littoral sites. The water from the eastern, flat Pannonian part of the country had the highest concentrations of SO_4^{2-} and Mg^{2+}, while the water samples from Dinaric Karst had the highest concentrations of NO_3^- and Ca^{2+}.

3.2. Culturable yeasts and yeast-like fungi in tap water and groundwater samples

All 116 water samples were analysed for the presence of selected culturable fungi with emphasis on human opportunistic pathogenic species. Eighty percent of tap water samples were positive for at least one fungal species while samples of groundwater were positive in 69% (Table 2).

Eighty eight percent of groundwater samples and 48% of tap water samples contained filamentous fungi from the genus *Aspergillus*, which were not identified to the species level. More than a half of the groundwater samples were positive for *A. melanogenum* (56%), followed by *E. dermatitidis* (12%), *C. parapsilosis* (12%) and *F. dimerum* (6%), while other fungal species were detected only sporadically. Colonies of *Exophiala* species usually appeared after 3 d of cultivation at 37 °C. If they were not observed after 7 d at this temperature, plates were moved to 4 °C for 2 additional weeks. *A. melanogenum* was present in 25% of tap water samples together with the following black yeasts, all assigned to Biosafety Level-2 (BSL-2): *E. dermatitidis* (6%), *E. phaeomuriformis* (5%), *Exophiala lecanii-corni* (3%), *Exophiala oligosperma* (1%) and *Rhinocladiella similis* (8%). Additionally, tap water hosted ubiquitous opportunistic pathogenic yeasts *R. mucilaginosa, C. parapsilosis* and non-pathogenic *Meyerozyma guillermondii,* detected in 13%, 11%, and 10% of samples, respectively. Filamentous fungus *F. dimerum* was isolated from 6% of the samples, while other fungal species appeared sporadically. In Fig. 2 the comparison of mycobiota between groundwater and tap water samples with the focus on taxa detected in household appliances (Zalar et al., 2011; Novak Babič et al., 2015) is presented. Black yeast-like *A. melanogenum* and *E. dermatitidis* were isolated from both water types with lower presence in tap water than in groundwater. The percentages of *C. parapsilosis* and *F. dimerum* were in the same range in both water types, while yeasts like *R. mucilaginosa, M. guilliermondii* and black fungi *R. similis*, and *E. phaeomuriformis* were isolated in larger amounts from tap water.

Table 1
Average of ion concentrations in tap water samples according to geographic locations in Slovenia.

Geographic site	Concentration of ions (mg l^{-1})							
	Cl^-	NO_3^-	NO_2^-	PO_4^{3-}	SO_4^{2-}	Mg^{2+}	Ca^{2+}	Na^+
Alpine and Subalpine	8.70	6.13	0.00	0.00	11.50	13.17	65.81	4.05
Littoral	42.73	3.00	0.00	0.00	12.70	9.10	76.00	22.30
Pannonian	12.10	6.50	0.00	0.00	19.40	14.10	31.30	9.19
Dinaric Karst	7.50	9.30	0.00	0.00	7.10	10.20	86.90	3.40

3.3. Mycobiota in water detected with cultivation independent methods

3.3.1. DGGE analysis

DGGE profiles of fungal communities from different water samples were obtained to characterize mycobiota in water from 2 rivers, 5 groundwaters, 5 household tap waters and water from 2 waste water treatment plants, all located in the area of two Ljubljana aquifers (Ljubljansko polje and Ljubljansko barje). DGGE profiles showed two separated groups, one including water samples from north aquifer Ljubljansko polje and another group with samples collected from south aquifer Ljubljansko barje. High similarity (96–98%) was observed inside each group, regardless of whether it was an environmental water sample (river, groundwater) or tap water sample (Fig. 3). For instance, mycobiota from river-1 (Sava) was related to the samples of groundwater located in the basin of the same river (groundwater-1a, 1b). Similar results were observed for samples of river-2 (Ljubljanica mouth at north) and river-3 (Ljubljanica spring at south), which also included comparison to samples of tap water and water derived from waste water treatment plants. Since profiles of samples derived from the same aquifer are closely related, DGGE profiles indicate that preceding water treatments had no significant effect on the composition of fungal species in the water.

3.4. Next generation sequencing

Analysis of the fungal community in a single tap water sample (W3-Hrastje) performed with 454 pyrosequencing revealed only two genera and three species: out of 3259 reads, *Aspergillus* sp. represented 95% of all detected OTUs, *Aspergillus conicus* (3% of OTUs) and *E. dermatitidis* (0.1% of OTUs). Other detected OTUs (1.5%) were only identified at the level of families (Polyporaceae, Trichocomaceae, Mycosphaerellaceae), order (Pleosporales), classes (Sordariomycetes, Eurotiomycetes) or phylum (Ascomycota). The majority of detected fungal OTUs belonged to the Ascomycota. Only 0.4% of 3259 OTUs was classified as Basidiomycota (family Polyporaceae) (Table 3).

Table 2
The list of species isolated from 16 groundwater and 100 tap water samples from Slovenia, their frequency of isolation, EXF- and GenBank accession numbers.

Identification of the strains	Frequency of isolation	Representative strain – EXF[a] no.	GenBank accession no.	BSL[d]
Groundwater				
Aspergillus spp.	14	/	/	1–2
Aureobasidium melanogenum	9	EXF-8476	**KP034983** (ITS[b])	1
Candida parapsilosis	2	EXF-8460	**KP034964** (LSU[c])	1
Exophiala dermatitidis genotype A	2	EXF-8493	**KP034991** (ITS)	2
Fusarium dimerum	1	EXF-8478	**KP034999** (ITS)	1
Rhinocladiella similis	1	EXF-8262	**KP034994** (ITS)	2
Rhodotorula mucilaginosa	1	EXF-8464	**KP034970** (LSU)	1
Sporidiobolus salmonicolor	1	EXF-8680	**KP034975** (LSU)	1
Trichosporon coremiiforme	1	EXF-8679	**KP034976** (LSU)	1
Tap water				
Aspergillus spp.*	48	/	/	1–2
Aureobasidium melanogenum	25	EXF-8432	**KP034984** (ITS)	1
Candida pseudointermedia	2	EXF-9894	**KP034967** (LSU)	1
Candida orthopsilosis	1	EXF-8409	**KP034968** (LSU)	1
Candida parapsilosis	11	EXF-8411	**KP034965** (LSU)	1
Candida pararugosa	1	EXF-10051	**KP034966** (LSU)	1
Candida saitoana	1	EXF-10054	**KP034969** (LSU)	1
Clavispora lusitaniae	1	EXF-8458	**KP034982** (LSU)	2
Debaryomyces hansenii	4	EXF-8402	**KP034981** (LSU)	1
Exophiala alcalophila	2	EXF-9876	**KP034990** (ITS)	1
Exophiala dermatitidis genotype A*	5	EXF-8470	**KP034992** (ITS)	2
Exophiala dermatitidis genotype B	1	EXF-8435	**KP034993** (ITS)	2
Exophiala lecanii-corni	3	EXF-9878	**KP034985** (ITS)	2
Exophiala mesophila	1	EXF-8424	**KP034986** (ITS)	1
Exophiala oligosperma	1	EXF-8434	**KP034988** (ITS)	2
Exophiala phaeomuriformis genotype 1	5	EXF-8441	**KP034987** (ITS)	2
Exophiala xenobiotica	2	EXF-8261	**KP034989** (ITS)	1
*Fusarium dimerum**	6	EXF-8427	**KP035000** (ITS)	1
Galactomyces candidum	1	EXF-10052	**KP035008** (ITS)	1
Meyerozyma caribbica	1	EXF-9902	**KP034974** (LSU)	1
*Meyerozyma guilliermondii**	10	EXF-8455	**KP034973** (LSU)	1
Pichia fermentans	3	EXF-8414	**KP034980** (LSU)	1
Pseudozyma crassa	3	EXF-9893	**KP034979** (LSU)	1
Rhinocladiella similis	8	EXF-8433	**KP034995** (ITS)	2
Rhodotorula mucilaginosa	13	EXF-8417	**KP034971** (LSU)	1
Rhodotorula slooffiae	4	EXF-8420	**KP034972** (LSU)	1
Trichosporon montevideense	1	EXF-10056	**KP034977** (LSU)	1
Yarrowia lipolytica	2	EXF-8418	**KP034978** (LSU)	1

*Species isolated from the sample of tap water, which was also subjected to NGS analysis.
[a] EXF, strain accession number in Ex Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Infrastructural Centre Mycosmo, MRIC UL, Slovenia).
[b] ITS, internal transcribed spacer region of ribosomal DNA.
[c] LSU, large subunit of ribosomal DNA.
[d] BSL, Biosafety level (de Hoog et al. 2009).

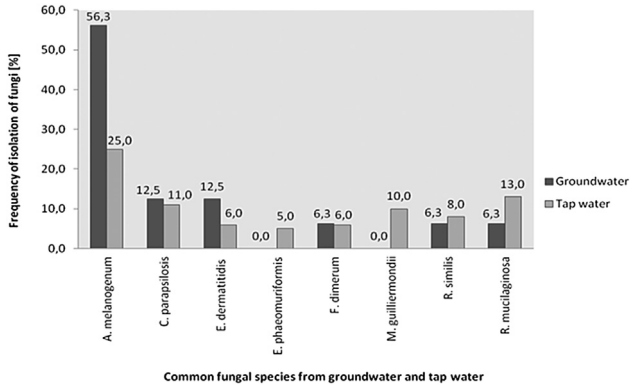

Fig. 2. Comparison of the occurrence of fungal species in ground- and tap water samples from Slovenia, with the focus on taxa detected in dishwashers (Zalar et al., 2011) and/or washing machines (Novak Babič et al., 2015). *Aureobasidium melanogenum* was predominant in both water types. The frequency of isolation was higher from ground water (56%) and lower from tap water (25%). *Exophiala dermatitidis* was frequent in groundwater samples (12%) upon tap water (6%), while the percentage of occurrence of *Candida parapsilosis* and *Fusarium dimerum* was the same in both water sources. *Rhodotorula mucilaginosa, Meyerozyma guilliermondii* and *Exophiala phaeomuriformis* were only found in tap water.

3.5. Statistical analyses of the occurrence of fungi in water in relation to water characteristics

To determine the correlation between ion composition of tap water and the occurrence of culturable fungi, the results of the analyses of the 90 tap water samples were processed with machine learning methods. In the resulting decision tree (Fig. 4), we observed two separate groups of ions determining fungal presence in the tap water. In the first group, the presence of fungi was positively correlated with high concentrations of Ca^{2+} (more than 52.9 mg l^{-1}), high concentrations of Mg^{2+} and SO_4^{2-}. In the second group, the presence was primarily correlated with concentrations of Ca^{2+} lower than 52.9 mg l^{-1}. In this group only the concentration of NO_3^- had an important positive influence on the presence of fungi in tap water. Surprisingly, the decision tree model did not include Cl^- as a factor influencing the presence of fungi in tap water systems. To verify the apparent low importance of Cl^- for the absence/presence of fungal species, we calculated the mutual information between the latter and each of the ion concentrations: this was the lowest for the Cl^- ion concentration.

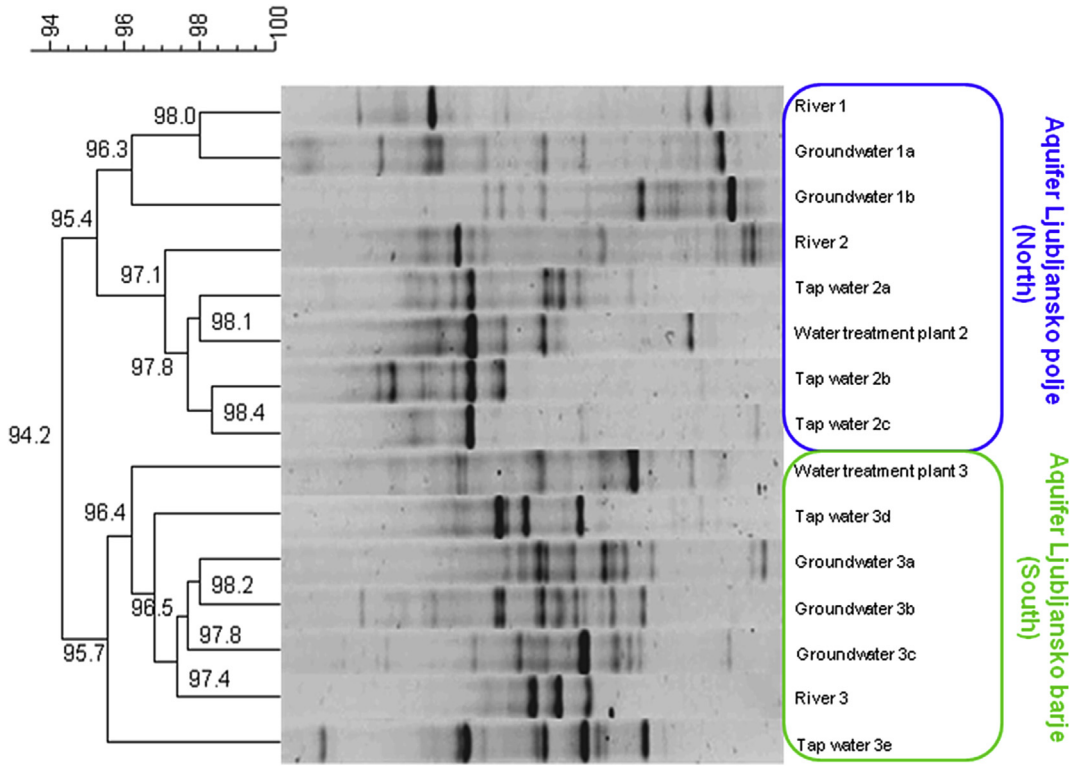

Fig. 3. DGGE profiles of fungal communities from different water samples. Rivers, groundwater, tap water and waste water samples from 2 different aquifers of Ljubljana valley were compared. The blue box shows cluster of samples from north aquifer Ljubljana field (Slovenian name "Ljubljansko polje"), and the green box indicates cluster of samples from south aquifer Ljubljana moor (Slovenian name "Ljubljansko barje"). Numbers 1, 2, and 3 indicate the samples from rivers and surrounding areas. Similarities between communities derived from the same aquifer are apparent (96–98%). Fungal communities inside clusters are closely related regardless of the type of water.

4. Discussion

Seventy one percent of the Earth is covered with water. Only 0.6% of this water, originating in glaciers, rivers, lakes and groundwater, is fresh water (Wurzbacher et al. 2011). The World Health Organisation (WHO), the US Environmental Protection Agency (US EPA) and the European Union (EU) issue directives, determine legislations, set recommendations and require testing of drinking water quality. In EU, standards for water sources are defined in Directive 2000/60/EC and Council Directive 80/778/EC. Tap water in Slovenia is regularly checked for bacteria and chemicals, as determined in the European Council Directive 80/778/EC for drinking water (European Union, 1980). Mainly groundwater and to a lesser extent recycled surface water derived from rivers is used as tap water in Slovenia. Among 928 water supply facilities 56% are regularly disinfected with chlorine, while 44% of water supply sources are not disinfected at all (Lapajne and Sovič, 2012). After disinfection, the total count of mesophilic bacteria, coliforms, *Escherichia coli* and spores of *Clostridium perfringens* are determined (European Union, 1980). Water quality legislations, with the exception of some white papers and recommendations (US EPA, 2002; US EPA, 2006), do not mention fungi or set limits for fungi in drinking water (European Union, 1980; US EPA, 2002; Defra, 2011). As a consequence the presence of fungi in tap water is not measured, and its potential influence on human health is rarely investigated (Defra, 2011).

Table 3
Analysis of tap water sample performed with 454 Pyrosequencing, where 3259 reads were obtained. Highest taxonomic unit is presented on the top of the table.

Taxonomic unit	OTU-s	Percentage of fungal community (%)
Fungi	8	0.24
Ascomycota	12	0.37
Eurotiomycetes	2	0.06
Sordariomycetes	2	0.06
Pleosporales	2	0.06
Mycosphaerellaceae	2	0.06
Trichocomaceae	9	0.28
Polyporaceae	12	0.37
Aspergillus sp.	3100	95.12
Aspergillus conicus	106	3.25
Exophiala dermatitidis	4	0.12

Filamentous fungal genera reported in tap water worldwide in previous studies included *Acremonium, Alternaria, Arthrinium, Aspergillus, Beauveria, Botrytis, Chaetomium, Cladosporium, Epicoccum, Fusarium, Geotrichum, Gliocladium, Mucor, Paecilomyces, Penicillium, Phoma, Phomopsis, Rhizopus, Sporothrix, Trichoderma* and *Verticillium* (Kinsey et al., 1999; Gonçalves et al., 2006; Kanzler et al., 2008; Sammon et al., 2010; Defra, 2011; Heinrichs et al., 2013a, 2013b). Selected species of *Aspergillus, Fusarium,* and *Penicillium*, some of which are also isolated from tap water, are known producers of mycotoxins (Kelley et al., 2003). Yeasts isolated from tap water were black yeast-like genera *Aureobasidium, Exophiala, Phialophora,* white yeasts of the genera *Candida, Cryptococcus* and red yeasts from the genus *Rhodotorula* (Kinsey et al., 1999; Göttlich et al., 2002; Hageskal et al., 2006; Grabinska-Loniewska et al., 2007; Pereira et al., 2010).

Amongst the most important opportunistic human pathogens that have been detected so far in tap water are *Cryptococcus neoformans, Stachybotrys chartarum,* and representatives of the genera *Acremonium*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Candida*, *Chaetomium*, *Cladosporium*, *Exophiala*, *Fusarium*, *Mucor*, *Nectria*,

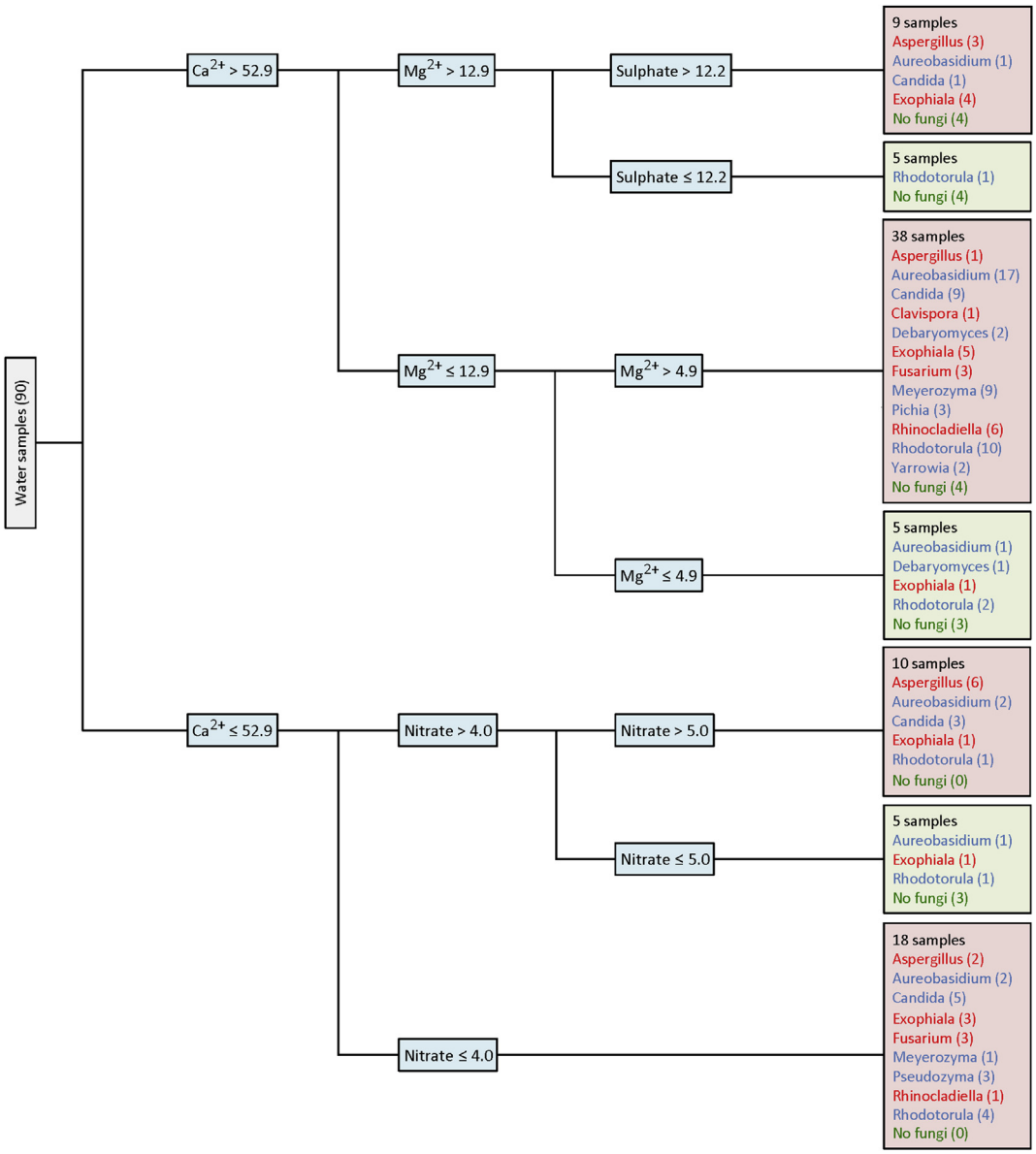

Fig. 4. Decision tree for the 90 water samples generated with the Predictive Clustering Trees machine learning method. All studied fungal genera are considered. Internal nodes (grey boxes) represent concentrations of different ions. Tree leaves (red and green boxes) contain the samples that satisfy all of the conditions on the path from the tree root to the given leaf. Each leaf gives: total number of samples in a leaf (in black), fungal genera in these samples with numbers of samples for each genus (in red and blue), and number of samples where no fungi were found (in green). Red colour indicates genera belonging to BSL-2 level, while blue colour indicates fungi classified in BSL-1. The red colour of the leaf means that the majority of samples contained at least one genus of fungi, while the green colour of the leaf means that in the majority of samples no fungi were found.

Paecilomyces, *Penicillium*, *Phialophora*, *Phoma*, *Rhizopus*, *Rhodotorula*, *Scopulariopsis*, and *Sporothrix* (Defra, 2011).

In our study, 80% of tap water samples harboured fungi. This is in accordance with Niemi et al. (1982) who reported that 50%–100% of water samples were positive for fungi. We have focused on yeasts and yeast-like fungi with pathogenic potential, thus *Aspergillus* isolates were not identified to the species level. Among yeasts and yeast-like fungi we mostly isolated black pigmented fungi *A. melanogenum*, *R. similis,* and *Exophiala* spp., white yeasts *Candida* spp., *M. guilliermondii* and red yeasts from the genus *Rhodotorula*. In addition to these, representatives of the genera *Clavispora, Debaryomyces, Galactomyces, Meyerozyma, Pichia, Pseudozyma, Trichosporon,* and *Yarrowia* were detected. Among them *E. dermatitidis, Pseudozyma crassa* and *Yarrowia lipolytica*, which are rarely isolated from the environment, were identified from 6%, 3% and 2% of the tap water samples, respectively. To our knowledge, this is the first report of the isolation and cultivation of *E. dermatitidis,* the dominant opportunistic human pathogen in dishwashers, from tap water. Our results obtained with pyrosequencing from a single tap water sample also confirmed the results of culture dependent techniques. *Aspergillus* spp. and *E. dermatitidis* were detected, but other taxa (*F. dimerum, M. guillermondii*), which have been isolated and cultured from a single tap water sample, have not been identifiable by ITS2 rDNA to the species level.

Black yeast-like *Aureobasidum pullulans* has previously been reported mainly from oligotrophic environments (Gostinčar et al., 2014), but also in approx. 5% of groundwater samples (Kanzler et al., 2008). Four varieties of the genus *Aureobasidium* (Zalar et al., 2008) were in 2014 elevated to the species level (Gostinčar et al. 2014). Among them only *A. melanogenum* was

reported to be involved in human infections, causing phaeohyphomycosis and other localized infections (Hawkes et al., 2005). Surprisingly, *A. melanogenum* was the only *Aureobasidium* species detected in the water samples. It was present in 56% of groundwater samples and in 25% of tap water samples with CFU even up to 160 l^{-1}. However, *A. melanogenum* was isolated in low amounts from dishwashers (1%) and from washing machines (less than 2% of the samples) (Zalar et al., 2011; Novak Babič et al., 2015), indicating its sensitivity to the extreme conditions within household appliances.

Amongst other black yeast-like fungi we isolated representatives of the genera *Rhinocladiella* and closely related *Exophiala* spp. Species *R. similis, Exophiala alcalophila, E. lecanii-corni, Exophiala mesophila, E. oligosperma, E. phaeomuriformis* and *Exophiala xenobiotica* were isolated from 22% of water samples. In previous studies they have been isolated from groundwater, biofilms in tap water systems (Göttlich et al., 2002; Heinrichs et al., 2013a, 2013b), and from glaciers (Branda et al., 2010; Blasi et al., 2015). These species are all classified as human opportunistic pathogens, causing cutaneous or subcutaneous infections (de Hoog et al., 2009). An important finding is our discovery of the *E. dermatitidis* in tap water (6%) and groundwater samples (12.5%). This species is the dominant fungus in dishwashers worldwide (Zalar et al., 2011; Döğen et al., 2013; Gümral et al., 2015); however, its presence was not confirmed in other household appliances, like washing machines (Novak Babič et al., 2015). *E. dermatitidis* is a causative agent of phaeohyphomycosis, mycetoma, brain infections and colonizer of respiratory tract. Patients with cystic fibrosis represent a particular risk group (de Hoog et al., 2009; Kondori et al., 2011). Previous studies reported isolation of *E. dermatitidis* from glaciers (Branda et al., 2010; Blasi et al., 2015), bathrooms (Döğen et al., 2013), saunas (Matos et al., 2002; Blasi et al., 2015), railway sleepers (Gümral et al., 2014) and also beach sand (Efstratiou and Velegraki, 2009), but never directly from tap water.

Red yeasts of the genus *Rhodotorula,* known to form biofilms, are common in the environment and have been previously reported also from 10% of groundwater samples (Kanzler et al., 2008; Wirth and Goldani, 2012). We isolated *R. mucilaginosa* from 13% of tap water samples with up to 36 CFU l^{-1}. *R. mucilaginosa* can colonise kitchen surfaces (Adams et al., 2013), and was recently also isolated from household appliances such as dishwashers (2%) (Zalar et al., 2011), and washing machines (7%) (Novak Babič et al., 2015). It can cause catheter-related infections (Neofytos et al., 2007), eye infections, meningitis and fungemia in immunosuppressed individuals (Pfaller et al., 2007; de Hoog et al., 2009).

Yeasts from the genus *Candida* were isolated from both groundwater (12%) and tap water (16%) with up to 42 CFU l^{-1}. *C. parapsilosis* dominated among other *Candida* species (*Candida pseudointermedia, Candida orthopsilosis, Candida pararugosa, Candida saitoana*), while *Candida albicans* was not detected in any water sample. *C. parapsilosis* was previously reported from soil, plant material and tap water samples (Deresinski et al., 1995; Pires-Gonçalves et al., 2008; Lord et al., 2010). It can invade human indoor environments, in particular kitchens (Adams et al., 2013), and also household appliances (Gattlen et al., 2010). It was isolated from 15% of sampled washing machines (Novak Babič et al., 2015) and from approx. 5% of dishwashers (Zalar et al., 2011; Gümral et al., 2015). *C. parapsilosis* is an emerging pathogen, causing opportunistic fungemias (de Hoog et al., 2009), colonizing catheters and other prosthetic materials (Levin et al., 1998).

Other fungi, frequently present in washing machines and/or dishwashers, like *F. dimerum, F. oxysporum*, and *F. solani* species complexes were only detected in low amounts or were absent in tap water. However, *F. dimerum,* also previously reported as groundwater inhabiting species (Hageskal et al., 2006), present in 2% of dishwashers (Zalar et al., 2011), was isolated from 6% of tap water samples.

Machine learning analysis correlated measured water characteristics and the presence of fungi in water. This presence most strongly correlated with the concentrations of Ca^{2+}, Mg^{2+}, and NO_3^-. Thus, fungi were primarily isolated from samples with a combination of high concentrations of Ca^{2+} and Mg^{2+}, or low concentration of Ca^{2+} and presence of NO_3^-. It is noteworthy that the growth morphology of certain fungi can depend on the presence of some ions, such as Ca^{2+}, and also that some fungi can affect aqueous geochemistry in karst, i.e., rocks made of limestone (Wang and Szaniszlo, 2009; Hou et al., 2013). A remarkable conclusion, based on machine learning, was the very low importance of Cl^- in determining the fungal presence in tap water, indicating that naturally occurring chloride or additional chlorination for water treatment does not affect fungi. Similar results have been observed in a previous study of melanised fungal species (Defra, 2011).

The lack of influence of chlorination on water mycobiota and the resemblance between fungal communities in the raw water source to the communities detected in tap water was illustrated with DGGE analysis. Fungal DGGE profiles in selected rivers, groundwater, tap water, and waste water showed closely related profiles of fungal species between samples derived from the same aquifer and that these profiles were only little changed throughout the water cycle – also after chlorination of tap water, although we have to stress that this could not be quantified with the methodology used.

Many of the fungal species detected in tap water and groundwater can affect human health, causing allergies, eye, hair, skin and nail infections (Defra, 2011) as well as colonising the respiratory tract (e.g. in cystic fibrosis, and even without) (Kondori et al., 2011; Mukaino et al., 2006). Aging human populations, the high numbers of immunocompromised individuals and the lifestyles that are increasingly confined to indoor environments all contribute to an increased risk of allergies, severe infections with fungi, or systemic mycoses (de Hoog et al., 2009).

Our study revealed high occurrence of several human opportunistic fungi, in particular black-pigmented yeasts *Exophiala* spp., *A. melanogenum* and white yeast *C. parapsilosis*. These and other opportunistic pathogenic species present in low counts in tap water, enter household appliances via water. Here they undergo a strong selection process exposed to intermittent dry and wet conditions, changes in pH, together with the presence of man-made substrates and chemicals, such as rubber and detergents, and occasionally relatively high temperatures for fungi (40 °C and above). As a result, the enrichment of species, adapted to multiple abiotic stress factors via melanisation, meristematic growth, and production of extracellular polysaccharides, biofilm formation and phenotypic plasticity, occurs. This enrichment can increase the number of individual species, and considerably increases the risk of humans as a vector for fungal dispersal. It is thus a neglected health risk, particularly in hospitals (Anaissie et al., 2002; Warris et al., 2010), as well as in our households.

We conclude that a variety of fungal species, including opportunistic human pathogens, can be isolated from groundwater as well as tap water. The results of data mining show that the presence of fungal species in water is related to the concentration of certain inorganic ions. We do not yet know in which natural environments they are established before they are transferred – by the water system – to the extreme environments of household appliances. However, it is certain that some of these fungal species can establish themselves in dishwashers and washing machines, where they subsequently proliferate and can constitute a threat to human health.

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