UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Monika NOVAK BABIČ

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DOKTORSKA DISERTACIJA

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FUNGI IN SELECTED HOUSEHOLD APPLIANCES AND THEIR TRANSMISSION FROM NATURAL ENVIRONMENTS

DOCTORAL DISSERTATION

Ljubljana, 2016

Na podlagi Statuta Univerze v Ljubljani ter po sklepu Senata Biotehniške fakultete in sklepa 44. seje Komisije za doktorski študij z dne 13. 11. 2013 je bilo potrjeno, da kandidatka Monika Novak Babič izpolnjuje pogoje za opravljanje doktorata znanosti na Interdisciplinarnem doktorskem študijskem programu Biomedicina, znanstveno področje Mikrobiologija. Za mentorico je bila imenovana doc. dr. Polona Zalar.

Doktorska disertacija je zaključek podiplomskega študija Biomedicine, področje mikrobiologije. Opravljeno je bilo na Katedri za molekularno genetiko in biologijo mikroorganizmov Oddelka za biologijo Biotehniške fakultete Univerze v Ljubljani. Analize strojnega učenja so bile izvedene na Inštitutu Jožef Štefan v Ljubljani.

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Monika Novak Babič

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AV NOVAK BABIČ, Monika, univ. dipl. mikr.

SA ZALAR, Polona (mentorica)

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LI 2016

IN GLIVE V IZBRANIH GOSPODINJSKIH APARATIH IN NJIHOV PRENOS IZ NARAVNIH OKOLIJ

TD Doktorska disertacija

OP X, 102 str., 1 sl., 4 pril., 129 vir.

IJ sl

JI sl/en

AI Glive so ubikvitarni organizmi, prisotni v zraku, vodah, tleh in tudi znotraj človekovih bivališč. V doktorski nalogi smo z gojitvenimi in molekularno genetskimi tehnikami raziskali glivno bioto v pomivalnih in pralnih strojih, kjer jih kot potrošniki ne pričakujemo. Nadalje smo glive osamili tudi iz vodovodne in podzemne vode. Ugotovili smo, da pomivalne stroje, vzorčene globalno, naseljuje združba gliv, ki jih sicer redkeje najdemo v naravi. O selektivnosti pogojev v strojih priča podobnost mikobiote ne glede na lokacijo. Predstavljajo jo poliekstremofilni črni kvasovki vrst *Exophiala dermatitidis* in *E. phaeomuriformis*, ki preživijo pri temperaturah do 45 °C in pH vrednostih med 2,5 in 12,5. Tudi pralni stroji vsebujejo številne glive, večinoma iz rodov *Fusarium* in *Candida*. Z metodo strojnega učenja smo pokazali, da na glivno raznolikost v pralnih strojih zlasti vplivata uporaba mehčalca in temperatura pranja. V vzorčeni vodi so bile najpogosteje prisotne glive rodov *Aspergillus* in *Aureobasidium*. Potrdili smo domneve, da na pojav gliv v vodi vpliva lokacija vodonosnika. Glede na presek osamljenih vrst iz vseh treh habitatov smo ugotovili, da vodovodna voda predstavlja vektor prenosa gliv v stroje, znotraj katerih življenjski pogoji selektivno vplivajo na namnožitev oportuno patogenih gliv iz rodov *Candida, Exophiala, Fusarium, Rhodotorula* in *Saprochaete*. Rezultati so pokazali ponovljivost sestave glivnih združb znotraj gospodinjskih strojev ter omogočili prepoznavo tveganja okužb s porajajočimi oportunističnimi patogeni predvsem pri ljudeh z oslabljenim imunskim sistemom.

KEY WORDS DOCUMENTATION (KDW)

DN Dd

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CX Fungal diversity/*Exophiala*/*Candida*/*Fusarium*/*Aureobasidium*/natural habitats/dishwasher/washing machine/drinking water

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PB University of Ljubljana, Biotechnical Faculty, University Postgraduate Study Programme in Biomedicine, Field: Microbiology

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TI FUNGI IN SELECTED HOUSEHOLD APPLIANCES AND THEIR TRANSMISSION FROM NATURAL ENVIRONMENTS

DT Doctoral Dissertation

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AL sl/en

AB Fungi are cosmopolitan organisms, globally present in air, water, soil and within human dwellings. In the doctoral thesis we investigated mycobiota with the use of culture-dependent and culture-independent techniques on samples obtained from the interior of dishwashers and washing machines, where fungal growth is not expected. We additionally isolated fungi from drinking- and ground-water. We found that dishwashers sampled globally were inhabited by fungi, otherwise rarely found in nature. Due to selective conditions within the appliances, mycobiota was very similar, regardless of the geographic location. It was mainly represented by polyextremophilic black yeasts *Exophiala dermatitidis* and *E. phaeomuriformis*, which were able to survive at temperatures up to 45 °C and at pH between 2.5 and 12.5. We also isolated many different fungi from washing machines; the majority belong to the genera *Fusarium* and *Candida*. Statistical machine learning method showed that fungal diversity in washing machines is affected by the washing temperature and by the use of fabric softener. From tap water we mostly isolated fungi from the genera *Aspergillus* and *Aureobasidium*, and showed that the occurrence of fungi in water is depending on the location of the aquifers. According to the cross-section of isolated species from all three sampled habitats we confirmed tap water as the vector for fungal transmission into the appliances and estimated that the conditions within the appliances selectively affect propagation of opportunistic pathogens from the genera *Candida*, *Exophiala*, *Fusarium*, *Rhodotorula* and *Saprochaete*. The results revealed the repetitive diversity of fungi in household appliances and confirmed the tap water as the main transmission vector. These results enable risk assesment for infection of immunocompomised people with emerging opportunistic fungal pathogens.

KAZALO VSEBINE

KAZALO ZNANSTVENIH DEL

KAZALO SLIK

KAZALO PRILOG

OKRAJŠAVE IN SIMBOLI

°C	stopinja Celzija
CFU	enote, ki tvorijo kolonije (ang. **c**olony **f**orming **u**nits)
DGGE	denaturacijska gradientna gelska elektroforeza (ang. **d**enaturing **g**radient **g**el **e**lectrophoresis)
DNA	deoksiribonukleinska kislina (ang. **d**eoxiribo**n**ucleic **a**cid)
EPS	izvencelični polimeri (ang. **e**xtracellular **p**olymeric **s**ubstances)
EXF	zbirka glivnih kultur na Oddelku za biologijo, Biotehniška fakulteta (ang. Culture collection of **ex**tremophilic **f**ungi)
ITS	notranji distančniki (ang. **i**nternal **t**ranscribed **s**pacer)
NGS	sekvenciranje naslednje generacije (ang**. n**ext **g**eneration **s**equencing)
PCR	verižna reakcija s polimerazo (ang. **p**olymerase **c**hain **r**eaction)

1 PREDSTAVITEV PROBLEMATIKE IN HIPOTEZE

1.1 TAKSONOMSKA UVRSTITEV GLIV, VLOGA V OKOLJU IN PATOGENEZA

Glive so v samostojno kraljestvo prvič uvrstili leta 1784. Razlogi za uvedbo samostojnega kraljestva so bile očitne razlike med glivami in rastlinami, kamor so glive uvrščali prej. Glive se od rastlin razlikujejo po tem, da nimajo klorofila, ne tvorijo korenin, stebel in listov, kopičijo glikogen ter so heterotrofni organizmi. Na podlagi poznejših dognanj v evolucijski biologiji glive danes uvrščamo v domeno evkariontov ter v samostojno kraljestvo, ki se podrobneje deli na sedem debel. Razdelitev gliv v debla temelji na molekularnih analizah različnih genetskih markerjev; tako ločimo debla Chytridiomycota, Neocallimastigomycotina, Blastocladiomycota, Microsporidia, Glomeromycota, Ascomycota in Basidiomycota (Hibbett in sod., 2007). Zaradi polifiletske narave predvsem skupine Zygomycota je le-ta po omenjeni shemi ostala neuvrščena v rang debla, temveč je razpadla v poddebla Mucoromycotina, Kickxellomycotina, Zoopagomycotina in Entomophthoromycotina. Izmed naštetih skupin tvorijo glive iz debel Ascomycota in Basidiomycota septirane micelije ter jih obravnavamo kot višje glive. Razvojni krog pri glivah lahko sestoji iz dveh stadijev – spolnega (telomorfni) in nespolnega (anamorfni), vendar pri nekaterih glivah spolna oblika ni poznana (de Hoog in sod., 2014). Glive najdemo na vseh kontinentih, v oceanih, zraku ter v oblakih; kot prostoživeče saprofite, simbionte ali zajedalce. Glede na njihovo zmožnost kolonizacije okolij jih lahko razdelimo v tri skupine – mezofilne glive, generaliste in specialiste. Mezofilne glive običajno naseljujejo okolja kjer so dejavniki za njihovo rast bolj ali manj konstantni. Nasprotno generaliste najdemo v okoljih, kjer dejavniki za rast zelo variirajo, vendar nikoli pri skrajno ekstremnih pogojih. Glive poznane kot specialisti, so prilagojene na skrajnostna okolja in jih opisujemo kot ekstremotolerantne ali ekstremofilne organizme (Gostinčar in sod., 2010). V naravi imajo vlogo razkrojevalcev organskih snovi, z vidika bioremediacije okolja pa so pomembne tudi zaradi sposobnosti razgradnje dolgoverižnih ogljikovih spojin, med katerimi je veliko onesnaževal (Taylor in sod., 1993). Nekatere glive so užitne, ljudem neškodljive ter zaradi teh lastnosti široko uporabljane v živilski industriji in biotehnologiji. V bazični znanosti so različne vrste gliv v uporabi kot modelni organizmi za preučevanje osnovnih procesov v celicah, tvorbe biofilmov, fiziologije, biotehnologije, genetike in molekularne biologije ter kot proizvajalke antibiotikov. Z vidika medicinske znanosti ter ekologije so glive pomembne kot naseljevalke človeških bivališč saj so

povzročiteljice bolezni pri rastlinah, živalih in ljudeh. Glivne okužbe ljudi postajajo globalno prepoznan zdravstven problem, čeprav je med več kot 100.000 opisanimi vrstami le za okoli 100 vrst znano, da so pogosto povzročiteljice okužb pri ljudeh ali živalih (de Hoog in sod., 2014). Glivne okužbe so v porastu predvsem pri bolnikih z rakom, virusnimi okužbami ter avtoimunskimi in kroničnimi boleznimi (Anaissie in sod., 2002). Število ljudi, obolelih z invazivnimi glivnimi mikozami, kot so invazivna aspergiloza, kandidemija in kriptokokni meningitis, je približno 12 milijonov (Parkin in sod., 2002; Park in sod., 2009; Brown in sod., 2012). Okoli 4,8 milijonov ljudi trpi zaradi alergijske bronhopulmonarne aspergiloze (Denning in sod., 2013), 12 milijonov ima alergijski sinusitis (To in sod., 2012), 6 milijonov ljudi pa oboleva za glivnimi okužbami oči (Lam in sod., 2002). Najpogostejše so glivne okužbe kože, nohtov in las, s čimer se spopada milijarda ljudi po svetu (Vos in sod., 2012).

1.2 PRISOTNOST GLIV V BIVALNEM OKOLJU LJUDI

1.2.1 Življenjski pogoji in pojav gliv znotraj bivalnih prostorov

Glive so razširjene globalno in so prisotne tudi znotraj človeških bivališč. Na koncentracijo glivnih spor in delov micelija vpliva več dejavnikov, kot so: temperatura, vlažnost zraka, prisotnost rastlin in živali. Temperatura stanovanj v povprečju dosega med 19 °C in 21 °C, vendar v določenih prostorih, kot so kopalnice, savne, bazeni ter kuhinje z gospodinjskimi aparati, odstopa od povprečja. Tako je v kopalnicah temperatura med 19 °C in 28 °C, v savnah od 40 °C do 100 °C, med 40 °C in 75 °C v pomivalnem stroju, med 30 °C in 95 °C v pralnem stroju, 50 °C do 70 °C v sušilnikih za perilo in med 80 °C do 90 °C v strojih za pripravo čaja ali kave. V primeru zamrzovalnih skrinj, hladilnikov ter v klimatskih napravah so temperature podpovprečne in dosegajo med -20 °C in 7 °C (Elektro Energija, 2015). Nihanje temperature vpliva na hitrost rasti, fiziologijo celic, tvorbo spor in patogenost nekaterih gliv (Onofri in sod., 2007).

Poleg temperature je pomemben dejavnik za rast in razmnoževanje gliv tudi relativna zračna vlaga. Vlaga je odvisna od temperature, namembnosti prostora in naprav v prostoru, nanjo pa vplivajo tudi vsakodnevne dejavnosti ter način gradnje. Gradbene nepravilnosti lahko negativno vplivajo na ohranjanje zračne vlažnosti (Atrij, 2008). Idealna relativna zračna vlaga prostora je med 40 in 60 %. Običajno jo dosežemo pri temperaturi zraka med

22 °C in 24 °C. Če je zračna vlaga nižja od idealne, je zrak suh. Zaradi suhega zraka se v prostorih poveča nastajanje prahu, s čimer se prenašajo spore nekaterih gliv. Ko je vlaga v zraku višja od idealne ter dosega med 60 in 90 %, se lahko na površinah sten razvije glivni micelij. Če se plesen razprostira po večjih površinah, lahko neugodno vpliva na imunski sistem ter povzroča alergije, okužbe dihalnih poti in oči, glavobol, v najhujšem primeru tudi mikoze. V notranjih prostorih so prisotne predvsem glive, ki se razširjajo po zraku s sporami ter jih najdemo tudi v zunanjem okolju, v vodi in na odmrlem rastlinskem materialu (de Hoog in sod., 2014; Adan in Samson, 2011).

Za ohlajanje ali ogrevanje bivalnih prostorov so v razvitem svetu pogosto v uporabi klimatske naprave. Zgrajene so iz sistema zadrževalnih posod in cevi, kjer se voda skladišči, uparja in meša z zrakom. Na ta način se zrak vlaži in ohlaja / segreva ter na koncu prehaja v želen prostor. V ceveh ob dolgotrajni uporabi nastaja vodni kamen, ki vsebuje biofilme bakterij in gliv. V sistemu nihata tako temperatura kot tudi zračna vlažnost. Te naprave predstavljajo problem predvsem v bolnišnicah in drugih rehabilitacijskih ustanovah, saj je preko njih možno razširjanje mikroorganizmov z aerosoli in pojav alergij ter okužb dihalnega sistema pri imunsko oslabljenih ljudeh (Macher in Girman, 1990). Sisteme klimatskih naprav v stavbah in avtomobilih običajno kolonizirajo glive iz rodov *Alternaria, Aspergillus, Aureobasidium, Candida, Cladosporium, Fusarium, Paecilomyces, Penicillium* in *Rhizopus* (Simmons in sod., 1999; Kelkar in sod., 2004).

Podobno kot pri klimatskih napravah, tudi v kopalnicah, savnah in kuhinjah prevladuje višja relativna zračna vlaga in višje temperature, za vzdrževanje higiene v teh prostorih pa dodatno uporabljamo mila in detergente. Pod vplivom teh dejavnikov se na stenskih površinah ter ploščicah kopalnic najpogosteje pojavljajo glive iz rodov *Acremonium, Aureobasidium, Cladophialophora, Cladosporium, Cyphellophora, Paecilomyces, Phialophora, Phoma, Ramichloridium* in *Scolecobasidium*. V večji meri so prisotne tudi kvasovke iz rodov *Candida* in *Rhodotorula*, kar 80 % vseh izolatov pa predstavljajo glive iz rodu *Exophiala* (Hamada in Fujita, 2000; Hamada in Abe, 2010). Iz aerosolov, ki se sproščajo neposredno pri uporabi tušev, so izolirali glive iz rodov *Acremonium, Alternaria, Aspergillus, Bipolaris, Cladosporium, Curvularia, Epicoccum, Fusarium, Mucor, Paecilomyces, Penicillium* in *Ulocladium*, kar nakazuje na pitno vodo kot medij za prenos

teh gliv v prostore (Anaissie in sod., 2002). V turških savnah in kopelih so na stenah in tleh prostorov položene ploščice, zračna vlaga pa dosega 80 - 100 %. Pri takih pogojih so iz tal in natikačev uporabnikov izolirali glive *Aspergillus, Candida, Epidermophyton, Exophiala, Penicillium* in *Trichophyton* (Goksugur in sod., 2006). Matos s sodelavci je ugotovila tudi, da razlike v temperaturi vplivajo na pojav gliv rodu *Exophiala*. Iz vročih predelov turških savn so izolirali *E. dermatitidis*, iz hladnejših pa *E. phaeomuriformis, E. mesophila* in *E. janselmanii* (Matos in sod., 2002). Za razliko od turških savn so finske savne suhe, vroče ter sestavljene pretežno iz lesenih delov, ki jih kolonizirajo glive iz rodov *Arthrobotrys, Cephalosporium, Dactylaria, Gliodendron, Harposporium, Mucor, Nematoctonus, Phialophora, Rhinocladiella, Stemphylium* in *Trichoderma* (Salonen in Ruokola, 1969). Če so glivne združbe v prostorih z visokimi temperaturami in visoko zračno vlago relativno dobro dokumentirane, pa se prostorom s povprečnimi temperaturami in nižjo zračno vlago namenja manj pozornosti. Do nedavnega so glivno združbo v kuhinjskih prostorih povezovali predvsem s sporogenimi glivami v zraku. Šele študija Adamsove in sodelavcev (2013) se je osredotočila na pojav gliv na kuhinjskih pultih, v koritih in odtokih. Dokumentirali so pojav glivnih rodov, ki so običajno prisotni bodisi v zraku ali v pitni vodi, in sicer *Botryotinia, Candida, Cladosporium, Claviceps, Coniosporium, Cryptococcus, Debaryomyces, Exophiala, Fusarium, Kondoa, Malassezia, Lewia, Ochroconis, Penicillium, Phoma, Rhodotorula, Sclerococcum, Stemphylium, Trichosporon* in *Verrucocladosporium* (Adams in sod., 2013).

1.2.2 Pomivalni in pralni stroji kot življenjski prostor

Ne samo znotraj bivalnih prostorov, tudi v manjših vlažnih celicah, kot so gospodinjski aparati, so življenjski pogoji primerni za razširjanje gliv, čeprav je do nedavnega prevladovalo mnenje, da temu ni tako. Pomivalni in pralni stroji so v gospodinjstvih razvitega sveta pogosti. Po statističnih podatkih o opremljenosti gospodinjstev s trajnimi potrošnimi dobrinami je bilo leta 2012 v Sloveniji 97,6 % stanovanj opremljenih s pralnimi stroji, 52,8 % gospodinjstev pa s pomivalnimi stroji (Statistični urad Republike Slovenije, 2012). Oba gospodinjska aparata za delovanje uporabljata vodo iz vodovodnega omrežja, pred ciklom pranja pa uporabniki v stroje dodajajo detergente, ki izboljšajo učinek pranja. V zadnjih letih so le-ta vse bolj pogosto biološko razgradljiva (Poročilo komisije…, 2009). Zaradi povečane skrbi za čisto okolje in manjšo porabo energije, se vedno pogosteje

uporabljajo varčni programi pranja. Le-ti med ciklom dosegajo nižje temperature (med 30 in 60 °C) in porabijo manj vode (Elektro Energija, 2015).

O mikroorganizmih in njihovih medsebojnih interakcijah v pomivalnih strojih je malo znanega. Prve posredne študije so povezovale okužbe ljudi z vrstami gliv iz rodu *Candida* s pomivalnimi stroji (Bennett, 1998; Nedret Koc in sod., 2002). Neposrednih študij, ki bi podrobno predstavile problematiko pojava gliv v pomivalnih strojih ni bilo. Nasprotno pa je bilo o prisotnosti mikroorganizmih v pralnih strojih narejenih precej raziskav, ki pa so bile usmerjene predvsem v bolnišnično okolje zaradi vzdrževanja higiene in možnosti prenosa patogenih mikroorganizmov iz bolnikov na perilo, preko perila pa na druge bolnike. Iz različnega perila so takoj po pranju v pralnih strojih izolirali dermatofitne glive rodu *Fusarium, Microsporum canis, Mucor* sp., *Trichophyton mentagrophytes* ter kvasovke iz rodov *Candida* in *Rhodotorula* (Blaser in sod., 1984; Shah in sod., 1988; Neely in Orloff, 2001; Tanaka in sod., 2006; Stapleton in sod., 2013). Novejše raziskave so se zaradi vse pogostejšega pojava zatohlega vonja oblačil osredotočile tudi na prisotnost gliv na različnih delih pralnih strojev, od kovinskih (boben), plastičnih (predalčki za detergente) do gume (tesnilo ob bobnu). Iz gume so najpogosteje izolirali bele in rdeče kvasovke, kot so *Candida* sp., *Cryptococcus* sp., *Rhodotorula minuta, R. mucilaginosa* in *R. slooffiae* (Gattlen in sod., 2010; Stapleton in sod., 2013). Plastične dele strojev so običajno naseljevale filamentozne glive *Alternaria* sp., *Aspergillus ochraceus, A. versicolor, Cladosporium cladosporioides, C. sphaerospermum, Fusarium oxysporum* in *F. solani* (Hamada, 2002; Gattlen in sod., 2010; Stapleton in sod., 2013). Iz temnih biofilmov v predalčkih za detergente, kjer je koncentracija detergentov najvišja, so osamili črne kvasovke *Aureobasidium* sp., *Capronia coronata, Exophiala alcalophila, E. equina, E. lecanii-corni, E. mesophila, Ochroconis constricta, O. humicola* ter *Phialophora olivacea* (Gattlen in sod., 2010; Isola in sod., 2013). Pri primerjavi glivne diverzitete znotraj strojev in glivne flore ljudi so ugotovili, da večina izolatov iz pralnih strojev pripada vrstam gliv iz okolja, le majhen delež izolatov iz strojev so povezali z normalno floro človeka (Stapleton in sod., 2013).

1.3 PRISOTNOST GLIV V GLOBALNEM VODNEM KROGU

Glive so kot del mikrobnih združb prisotne tako v površinskih vodah, izvirski vodi kot tudi v podtalnici. V vodo vstopajo iz različnih okolij kot so tla in rastlinski materiali, voda pa deluje tudi kot vektor za njihov prenos med habitati. Zaradi prisotnosti anorganskih in organskih molekul iz okolja, voda glivam omogoča vir hranil in ionov. Vode iz različnih okolij se razlikujejo glede na pH in trdoto, zaradi česar se določene glivne vrste lahko selektivno pojavljajo na določenih področjih (Hageskal in sod., 2006). V razvitem svetu ima večina gospodinjstev dostop do vodovodne vode, ki jo uporabljamo v kopalnicah, kuhinjah in za delovanje gospodinjskih aparatov. Vodovodna voda se pridobiva v postopku čiščenja surove vode iz površinskih voda ali podtalnice. Tako vodo s postopki filtracije in flokulacije najprej očistijo večjih delcev, obsevajo z UV svetlobo ter po potrebi klorirajo, kar pa ne odstrani nujno vseh mikroorganizmov (Defra, 2011).

1.3.1 Glive v sladkih vodah

Sladke vode predstavljajo 2,5 % vseh vodnih zalog na Zemlji. Večina sladke vode se nahaja v obliki ledu na obeh polih, nekaj je predstavljajo površinske vode in podtalnica. Sladke vode, podobno kot morska voda, vsebujejo različne primesi; od naravnih organskih snovi, do polutantov, kot rezultat antropogenega delovanja, npr. celulozo, lignin, glikogen in alifatske ogljikovodike. Ionska sestava sladkih voda se razlikuje glede na površino po kateri tečejo, glede na letne čase in pretočnost. Zaradi teh dejavnikov se lahko razmere med posameznimi točkami v naravi zelo razlikujejo (Defra, 2011). Zaloge sladke vode na Zemlji predstavljajo ledeniki, ki so zaradi ekstremnih življenjskih pogojev in odmaknjenosti od človeške kolonizacije zanimivi za preučevanje. Iz površine in globine različnih ledenikov ter iz ledeniške vode so osamili glivne rodove *Acremonium, Aspergillus, Aureobasidium, Candida, Cladophialophora, Cladosporium, Clavispora, Cryptococcus, Debaryomyces, Dioszegia, Exophiala, Filobasidium, Fusarium, Geotrichum, Leucosporidium, Meyerozyma, Metschnikowia, Mrakiella, Penicillium, Phialophora, Phoma, Pichia, Rhodosporidium, Rhodotorula, Sporidiobolus, Sporobolomyces, Trichosporon* in *Udeniomyces* (Catranis in Starmer, 1991; Ozerskaya in sod., 2009; Branda in sod., 2010; Vaz in sod., 2011; Zalar in Gunde-Cimerman, 2014). V površinskih vodah izvirov, rek in jezer, ki se polnijo z raztapljanjem ledenikov ter s padavinsko vodo, so do sedaj odkrili filamentozne glive iz rodov *Acremonium, Alternaria,*

Aspergillus, *Cladosporium*, *Fusarium*, *Penicillium*, *Scopulariopsis*, *Trichoderma* ter kvasovke rodov *Candida*, *Cryptococcus* in *Rhodotorula* (Hageskal in sod., 2006; Pereira in sod., 2009). Podtalnica se od površinske vode razlikuje v vsebnosti kisika, temperaturi, pretočnosti ter pH. Iz podtalnice so osamili glive iz rodov *Aspergillus*, *Cladosporium*, *Fusarium*, *Stachybotrys*, *Trichoderma* ter kvasovke *Candida*, *Kloeckera* in *Rhodotorula* (Hageskal in sod.,2006; Pereira in sod., 2009; Pereira in sod., 2010).

1.3.2 Pogoji za tvorbo biofilma v vodovodnem sistemu in pitni vodi

Pri procesu pridobivanja pitne vode iz surovih vodnih virov države upoštevajo globalna in lokalna splošna priporočila, pravilnike in standarde, ki jih predpisujejo Svetovna zdravstvena organizacija (WHO), Ameriška agencija za zaščito okolja (US EPA) ter Evropska Unija (EU). Nastajanje biofilmov in posledično količino mikrobnih celic v planktonski obliki spremljajo z rednimi monitoringi pitne vode, primerno število mikroorganizmov pa vzdržujejo z dezinfekcijo. Dezinfekcijo vode lahko glede na mesto delovanja razdelimo na primarno in sekundarno. S primarno dezinfekcijo odstranimo mikroorganizme v surovi vodi v zbiralnikih. Sekundarna ali rezidualna dezinfekcija preprečuje razrast mikroorganizmov v vodovodnem omrežju. Običajno postopek primarne dezinfekcije zagotavlja tudi rezidualno delovanje (Stopar, 2007). Mikroorganizme iz vode odstranimo s filtracijo, kemijsko koagulacijo, inaktiviramo jih z UV sevanjem, ionizacijo s kovinami, kloriranjem, ozoniranjem ali ultrasonifikacijo, pri čemer je uporaba klora globalno najpogostejši način dezinfekcije (Gray, 2014). V Sloveniji od 928 oskrbovalnih območij s pitno vodo 48,5 % nima dezinfekcije, brez redne dezinfekcije vode pa je 56,0 % oskrbovalnih območij (Lapajne in Sovič, 2012). Kljub uporabi dezinfekcije pitna voda ni sterilna, pri transportu od črpališč do uporabnikov je podvržena različnim dejavnikom, ki vplivajo na število mikroorganizmov ter njeno končno kakovost. Taki dejavniki v vodovodnem sistemu so kovine, kisikove in klorove spojine, temperatura, spremembe pretoka vode, spremembe pH in posledično tvorba biofilmov (Defra, 2011). Biofilmi so skupek združb mikroorganizmov, ki vključujejo bakterije, glive in praživali, pritrjene na abiotsko (kamen, plastika, kovine,…) ali biotsko (sluznice, koža,…) površino. Mikroorganizmi predstavljajo približno 15 % biofilma, preostanek predstavljajo ekstracelularni polisaharidi (EPS) (Donlan, 2002). Biofilmi nastajajo na interfazi med tekočino in trdno snovjo ter so zgrajeni iz treh slojev: povezovalni, temeljni (bazalni) in

sekundarni. Prvi, povezovalni sloj vsebuje molekule, pritrjene na površino, ki olajšajo vezavo mikrobnih celic. Temeljni biofilm vključuje začetne mikrobne kolonizatorje, ki se pritrjajo na svežo površino, sekundarni biofilm pa sekundarne kolonizatoje, ki se pritrjajo na predhodne mikroorganizme. Znotraj biofilmov so mikrobne celice fiziološko običajno drugačne kot v planktonski obliki. Razlike se pojavijo zaradi spremenjenih gradientov oksigeniranih in anoksičnih področij in zaradi razlik v koncentraciji hranil. Biofilm predstavlja zaščito za celice v stresnem okolju, omogoča jim daljše preživetje brez hranil, večа možnost preživetja pri kemijskih, fizikalnih in bioloških vplivih iz okolja (Doggett, 2000; Paterson in Lima, 2005; Cooper, 2010). Na nastanek biofilmov v antropogenih vodnih okoljih imajo velik vpliv gradbeni materiali v vodovodnih sistemih. Glive in bakterije so v večji meri prisotne na površinah iz železa ali jekla, na površinah iz PVC materialov pa je količina mikroorganizmov manjša (Doggett, 2000; Grabinska-Loniewska in sod., 2007). Biofilm nastane neodvisno od hidrofobnosti ali hidrofilnosti materiala, čeprav naj bi biofilmi teoretično nastajali hitreje na hidrofobnih površinah (plastika) kot na hidrofilnih (kovine) (Donlan, 2002). Material cevi vpliva tudi na kakovost dezinfekcije. Kovinski materiali zaradi korozije reagirajo z ostanki klora in tako preprečijo vdor klora v biofilm (Le Chevallier, 1999). Pri primerjavi učinka kloriranja na bakterijske biofilme so Lehtola in sodelavci (2005) ugotovili, da je kloriranje učinkovitejše v ceveh iz polietilena (PE) kot v ceveh iz bakra. Na tvorbo biofilmov v ceveh vpliva tudi raven dostopnih hranil, zato v nekaterih državah preverjajo vsebnost organskih snovi pred in po dezinfekciji vodovodnih sistemov. Hranila se običajno koncentrirajo na medfazni površini med trdnimi in tekočimi deli sistema, njihova količina pa se spreminja glede na pretok vode. Če so v ceveh prisotna hranila, dezinfekcija ni dolgotrajno učinkovita, saj se biofilmi ob prisotnosti hranil hitreje obnovijo (US EPA, 2002). Za glive je sicer znano, da so oligotrofne in lahko preživijo ter se razmnožujejo tudi pri zelo nizki vsebnosti hranil (Kinsey in sod., 2003). Sestava mikrobne združbe biofilmov v vodah je v veliki meri odvisna tudi od temperature. Biofilmi v vodovodnih sistemih najhitreje nastajajo pri temperaturah med 15 °C in 25 °C (Donlan in sod., 1994; Lund in Ormerod, 1995). Biofilmi, ki nastanejo pri temperaturi vode okoli 20 °C, so pretežno sestavljeni iz bakterij rodu *Pseudomonas*. Le-te predstavljajo 96 % vseh mikroorganizmov, prisotne pa so tudi nekatere praživali. Pri temperaturah 40, 50 in 60 °C so v biofilmih najbolj zastopane vrste gliv iz rodu *Aspergillus*, prisotne so nekatere vrste bakterij, praživali ni več (Rogers in sod., 1994). Pomemben dejavnik za

kakovost pitne vode je tudi pretok vode. Odvisen je od postavitve, dolžine in širine cevi, vzdrževanja in delovanja črpalk (US EPA, 2002). Pretok vode vpliva na možnost začetne pritrditve celic na površino, dostopnost hranil, izgubo ekstracelularnih polisaharidov ter strukturo biofilmov. Biofilmi, ki nastajajo pri laminarnem toku vode imajo višje celokupno število celic kot biofilmi, ki nastajajo pri turbulentnem toku. Biofilmi, nastali pri turbulentnem toku imajo višje število celic na volumsko enoto, tak biofilm je zgoščen in bolj stabilen (Pereira in sod., 2002). Pri nižjih pretokih vode so biofilmi manj zgoščeni, na videz debelejši in puhasti (Percival in sod., 1999).

Med organizme, ki jih najdemo v pitni vodi in predstavljajo komponente biofilmov spadajo tudi glive. Delci glivnih hif in spore se v biofilm običajno prilepijo naknadno, lahko pa so glive tudi primarni kolonizatorji. Število celic, ki tvorijo kolonije v mililitru (CFU/mL) je v biofilmih 1000 – 5000 krat večje kot v tekoči vodi (Grabinska-Loniewska in sod., 2007). Od tega je število kvasovk v biofilmih med 0 in 8,9 CFU/cm^2, število filamentoznih gliv pa med 4,0 in 25,2 CFU/cm^2 (Doggett, 2000). Glivni deli, predvsem hife, zamrežijo strukturo biofilmov, zaradi česar jih je težje odstraniti iz sistema. Tvorba biofilmov pri kvasovkah je raziskana pri vrstah iz rodov *Candida, Saccharomyces, Cryptococcus* in *Aureobasidium*. Biofilmi, ki jih tvorijo kvasovke so podobni bakterijskim biofilmom. Veliko glivnih vrst raste v dimorfni obliki kar pomeni, da lahko preklapljajo med kvasno in filamentozno morfologijo. Ta lastnost jim omogoča hitro prilagoditev na razmere in boljšo kolonizacijo površin. Nasprotno pa filamentozne glive v nespolni fazi življenjskega cikla nimajo rastne dinamike, ki jo omogočata cepitev in brstenje pri kvasovkah (Harding in sod., 2009). O biofilmih filamentoznih gliv so poročali Anaissie in sodelavci (2003), ki so le-te našli v vodovodnih sistemih bolnišnic. Tvorba biofilmov je raziskana pri rodovih *Aspergillus, Penicillium, Coriolus, Trichoderma* in nekaterih dermatofitnih glivah ter poteka v več fazah. Začetna faza vključuje adsorpcijo propagul (spor, fragmentov hif, sporangijev) na površino. Sledi faza aktivne vezave na površino, pri čemer kaleče spore izločajo adhezivne substance. V tretji fazi začnejo z apikalno rastjo in razvejanjem hif nastajati mikrokolonije gliv. Sprva je rast enoslojna, nato postane invazivna. Ta faza vključuje tudi nastanek polimernega, izvenceličnega matriksa, ki omogoči rastoči koloniji močnejše pritrjanje na površino. Sledi faza začetnega zorenja kolonije, ko se hife povežejo v micelij. Tvorijo se sloji in svežnji hif, med katerimi potekajo vodni kanali. V naslednji fazi biofilm dozori, pri

glivah nastajajo sporogene celice, sklerociji in preživetvene strukture. V zadnji fazi se iz dozorelega biofilma odcepljajo strukture v planktonski obliki, sledi razširjanje v vodo (Harding in sod., 2009). Iz pitne vodovodne vode so različni avtorji najpogosteje osamili glive iz rodov *Acremonium, Altenaria, Arthrinium, Aspergillus, Aureobasidium, Beauveria, Botrytis, Chaetomium, Cladosporium, Epicoccum, Exophiala, Fusarium, Geotrichum, Gliocladium, Mucor, Paecilomyces, Penicillium, Phialophora, Phoma, Phomopsis, Rhizopus, Sporothrix, Trichoderma* in *Verticillium.* Ugotovili so, da lahko prisotnost nekaterih gliv, npr. nekaterih vrst iz rodov *Penicillium, Cladosporium, Aspergillus, Phialophora, Acremonium, Candida, Fusarium* in *Exophiala* vpliva na pojav alergij, okužb kože in sistemskih okužb pri ljudeh (Defra, 2011). Raziskav, kjer bi povezali vrstno sestavo gliv v vodovodni vodi in v biofilmih pomivalnih ter pralnih strojev do sedaj ni bilo.

1.3.3 Glive v odpadnih vodah

Glive kot ubikvitarni organizmi, ki naseljujejo zrak, sladke vode in gospodinjska okolja, so v končni fazi prisotne tudi v odpadnih vodah. Odpadne vode iz gospodinjstev in industrije se stekajo v čistilne naprave, kjer se med postopkom mehanskega in biološkega čiščenja prečistijo ter vrnejo v vodni krog. Glivne vrste *Candida albicans, C. dubliniensis, C. glabrata, C. krusei, C. utilis* in *Trichosporon beigelii,* osamljene iz vode v čistilnih napravah, so izvirale iz komunalne odpadne vode in so klasificirane kot oportuno patogene. Nekatere vrste gliv so v odpadnih vodah prisotne konstantno, npr. *C. albicans, C. krusei, C. tropicalis, C. utilis, Meyerozyma guilliermondii* in *Saccharomyces cerevisiae*. Določene vrste se tekom procesa čiščenja iz sistema ne odstranijo in se z očiščeno vodo vrnejo v naravni vodni krog. Med njimi so kvasovke *C. krusei, Debaryomyces polymorphus, M. guilliermondi, Rhodotorula* spp. ter filamentozne glive *Aspergillus* spp., *Mucor* spp., *Penicillium* spp. in *Rhizopus* spp. (Biedunkiewicz in Ozimek, 2009).

1.4 RAZISKOVALNE HIPOTEZE IN CILJI

V okviru doktorske disertacije smo si zastavili dve hipotezi:

1: Notranjost pralnih in pomivalnih strojev kolonizirajo kvasovke in filamentozne glive, ki jih uvrščamo v različne rodove.
2: Glive se v pomivalne in pralne stroje prenesejo z vodo iz vodovodnega sistema, ki je povezan s površinsko vodo ali podtalnico.

V okviru hipotez smo si zadali cilje, da bi ugotovili, ali pralne in pomivalne stroje naseljujejo glive in ali se te glive glede na identifikacijo po veljavnih taksonomskih kriterijih uvrščajo med poliekstremotolerantne oportuno patogene mikroorganizme. Nadalje smo želeli ugotoviti, ali so v glivnih združbah vodovodne, površinske vode ali podtalnice prisotne glive, ki naseljujejo notranjost pralnih in pomivalnih strojev in ali voda deluje kot vektor za določene vrste gliv, ki nato kolonizirajo notranjost strojev in se v njih številčno obogatijo.

2 ZNANSTVENA DELA

2.1 OBJAVLJENA ZNANSTVENA DELA

2.1.1 Pomivalni stroji – umetno ustvarjeno okolje za naselitev oportunističnih patogenih gliv

Naslov v originalnem jeziku: Dishwashers - a man-made ecological niche accommodating human opportunistic fungal pathogens

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POVZETEK

Gospodinjstva lahko naseljujejo mikroorganizmi, ki sicer v naravnem okolju niso pogosti ter jih le redko najdemo. V 189 pomivalnih strojih iz privatnih gospodinjstev iz 101 mesta smo opazili obogatitev gliv, ki sicer za rast zahtevajo specifične okoljske pogoje. Sto dva vzorca sta bila odvzeta v različnih mestih po Sloveniji, 42 iz ostalih evropskih držav, 13 vzorcev iz Severne in 3 iz Južne Amerike. Nadalje je bilo 5 vzorcev odvzetih iz Izraela, 10 iz Južnoafriške Republike, 7 iz Azije in 7 iz Avstralije. Glive so bile izolirane pri temperaturi 37 °C. Vrste iz rodov *Aspergillus, Candida, Magnusiomyces, Fusarium, Penicillium* in *Rhodotorula* smo osamili občasno, medtem ko sta bili vrsti črnih kvasovk *Exophiala dermatitidis* in *Exophiala phaeomuriformis* (Chaetothyriales) najdeni najpogosteje. Iz 62 % odstotkov pomivalnih strojev smo osamili glive, od tega v 56 % glive iz rodu *Exophiala*. Obe najdeni vrsti iz rodu *Exophiala* sta znani kot povzročiteljici sistemskih okužb pri človeku, pogosto pa kolonizirata tudi pljuča bolnikov s cistično fibrozo. Ugotavljamo, da pogoji v strojih kot so visoke temperature, visoka vlaga ter bazičen pH zagotavljajo življenjsko okolje, ki selekcionira oportunistično patogene glive za človeka.

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Dishwashers – A man-made ecological niche accommodating human opportunistic fungal pathogens

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ABSTRACT

Habitats in human households may accommodate microorganisms outside the common spectrum of ubiquitous saprobes. Enrichment of fungi that may require specific environmental conditions was observed in dishwashers, 189 of which were sampled in private homes of 101 towns or communities. One-hundred-two were sampled from various localities in Slovenia; 42 from other European countries; 13 and 3 from North and South America, respectively; 5 from Israel; 10 from South Africa; 7 from Far East Asia; and 7 from Australia. Isolation was performed on samples incubated at 37 °C. Species belonging to genera *Aspergillus*, *Candida*, *Magnusiomyces*, *Fusarium*, *Penicillium* and *Rhodotorula* were found occasionally, while the black yeasts *Exophiala dermatitidis* and *Exophiala phaeomuriformis* (Chaetothyriales) were persistently and most frequently isolated. Sixty-two percent of the dishwashers were positive for fungi, and 56 % of these accommodated *Exophiala*. Both *Exophiala* species are known to be able to cause systemic disease in humans and frequently colonize the lungs of patients with cystic fibrosis. We conclude that high temperature, high moisture and alkaline pH values typically occurring in dishwashers can provide an alternative habitat for species also known to be pathogenic to humans.

Introduction

During the last decade a growing number of biodiversity studies have focussed on remote habitats, like tropical and arid regions, and on environments where conditions are considered to be unfavourable for microbial growth. Extreme habitats in general are characterized by low diversity of species that are present with high numbers of individuals. In this respect indoor environments may also be viewed as extreme habitats as only a few hundred fungal taxa have thus far been encountered (Samson *et al.* 2002). A vast amount of literature is available on the airborne fungal flora in our homes and especially on *Aspergillus fumigatus* because of its potential impact on human health. Surprisingly, the wet environments in our houses have only recently received attention. Wet cells like bathrooms, sinks, kitchens and saunas were described as novel niches for adaptation of human pathogens (Hamada & Abe 2010; Lian & de Hoog 2010; Nishimura *et al.* 1986, 1987). Modern

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ways of life involve a growing number of electric appliances and especially those that use water may provide alternative ecological niches suitable for enrichment of extremotolerant fungi. Habitats in these devices since recently tend to become less extreme and easier to occupy for microbes due to decreasing ecological constraints of energy-saving regimens, such as the application of lower temperatures for washing clothes and dishes, and the use of less aggressive detergents.

In this article we focus on the fungal flora inside dishwashers, or more precisely the rubber seals of the doors. Due to washing of dishes used for food, dishwashers are consistently rich in nutrients under alkaline conditions, while intermittent high temperatures are prevailing and large amounts of water are processed in each washing cycle.

Material and methods

Isolation of strains

Samples were collected from dishwashers in private homes on six continents, while more extensively sampling was performed in Slovenia (Table 1). Samples were taken by rubbing the surface and inside of invaginations of rubber sealing located on the folding doors of dishwashers using sterile cotton swabs, pre-moistened in sterile physiological solution. Cotton swabs were placed in sterile tubes in plastic bags. The swabs were processed either immediately or stored at 4 °C and used within up to 2 weeks. Swabs were rubbed over the surface of culture media with malt extract agar (MEA) supplemented with chloramphenicol, and incubated at 37 °C for up to 7 d. Pure cultures were isolated and deposited at the Culture Collection of Extremophilic Fungi (EXF, Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia).

DNA extraction

Strains were transferred to fresh MEA plates and incubated for 3–7 d. DNA from yeast-like colonies was extracted with the PrepMan Ultra reagent (Applied Biosystems) according to the manufacturer's instructions. In the case of filamentous growth the DNA was extracted after mechanical lysis of approx. 1 cm^2 of mycelium using the protocol of Gerrits van den Ende & de Hoog (1999).

Molecular characterization

For the identification of *Exophiala* strains and filamentous fungi, a fragment of rDNA including internal transcribed spacer region 1, 5.8S rDNA and ITS 2 (ITS) was amplified and sequenced using primers ITS5 and ITS4 (White *et al.* 1990). Yeasts were identified based on their large subunit ribosomal DNA (LSU) sequence (partial 28S rDNA, D1/D2 domains), amplified and sequenced with primers NL1 and NL4 (Boekhout & Kurtzman 1996); when species groups were involved, further characterization has not been performed. BigDye terminator cycle sequencing kits were used in sequence reactions (Applied Biosystems, Foster City, CA, U.S.A.). Sequences were obtained with an ABI Prism 3700 (Applied Biosystems) and assembled and edited using SeqMan 3.61 (DNAStar, Inc., MI, U.S.A.). Sequences were automatically aligned using ClustalX 1.81 (Jeannmougin *et al.* 1998). Alignments were adjusted manually using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. (Tamura *et al.* 2007). Genotypes of *Exophiala dermatitidis* were determined according to Matos *et al.* (2003).

Mating and M13 fingerprinting

One strain each of *Exophiala dermatitidis* genotypes **A** (EXF-5718), **B** (EXF-5653) and **C** (EXF-5586) were mixed (**A** × **B**, **A** × **C**, **B** × **C**) on the surface of synthetic nutrient-poor agar (SNA) (Gams *et al.* 1998) and incubated for 1 week at 37 °C, followed by two weeks incubation at 4 °C. Streak plates were performed and single colonies were isolated, followed by DNA isolation with PrepMan Ultra reagent (Applied Biosystems). Five selected colonies of each listed genotype and ten of each mating reaction were subjected to micro/mini satellite-primed PCR (MSP-PCR) fingerprinting with M13 microsatellite primer performed according to (Andrighetto *et al.* 2000) with minor modifications. Amplified DNA fragments were separated by electrophoresis in 1 % agarose gel in 0.5 × Tris-acetate-EDTA TAE buffer, and visualized by Invitrogen SYBR® Safe DNA gel staining. The GeneRuler 100 bp DNA Ladder Plus (Fermentas) was used as the standard to determine size of fragments. The DNA fingerprints of selected isolates were determined with the aid of the software package Gene Tools (Syngene).

Physiology

Inocula of selected *Exophiala dermatitidis* and *Exophiala phaeomuriformis* strains were prepared by suspending a loop-full of cells in 1 ml physiological saline. The testing of pH tolerance was performed in 96 well microtiter plates in 300 µl of liquid malt extract medium (Gams *et al.* 1998) with set pH values prior autoclaving from 2.0 to 14.0, with the addition of appropriate amounts of 0.1 M HCl or 0.1 M NaOH. In the range of pH values form 2.0 to 3.0 and 11.0–12.5, the tested pH values were in steps of 0.1, while between pH 3.0 and 11.0 only full units were tested. At pH above 12.5–13.7 media were not autoclaved. Temperature tests were performed on MEA plates, onto which 10 µl of cell suspension was applied and incubated at the following temperatures: 5, 10, 25, 30, 37, 40, 42, 45, 47 and 50 °C. Growth was checked once a week for two weeks. Halotolerance was tested on MEA media containing 5 %, 10 % and 17 % NaCl (w/v).

Scanning electron microscopy

For scanning electron microscopy, 0.25 cm^2 pieces of rubber seal were cut off from a used dishwasher. Samples were subjected to fixation in 2.5 % glutaraldehyde, 4 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at room temperature for 24 h. Samples were rinsed with buffer and exposed to postfixation in 1 % OsO_4 in distilled water for 24 h at 4 °C. After rinsing the material was dehydrated through a graded series of alcohols, twice for 15 min for each step (30 %, 50 %, 70 %, 80 %, 90 %, 100 %), followed by the mixture of absolute alcohol and acetone (1:1) and 100 % acetone. Dehydrated material was dried in the mixture of acetone and hexamethyldisilazane (HMDS), ratio 1:1, twice for 30 min,

Table 1 – Data on isolation of fungi from dishwashers, with detailed information on the most frequently isolated taxa.

Country	Sampled dishwashers	Positive dishwashers	*Exophiala* (*E. dermatitidis*/ *phaeomuriformis*)	*Candida parapsilosis*	*Magnusiomyces capitatus*	*Fusarium dimerum*	*Pichia guilliermondii*	*Rhodotorula mucilaginosa*	Other fungi[a]/algae[b]
Slovenia	102	72	40 (31/10[c])	10	7	3	4	4	4[a]/1[b]
Australia	7	3	2 (2/–)	–	–	–	–	–	–
Austria	5	4	2 (2/–)	1	1	–	–	–	–
Belgium	4	2	1 (1/–)	1	–	–	–	–	1[a]
Brazil	3	3	1 (1/–)	–	–	–	–	–	–
Canada	7	6	–	1	–	–	1	–	4[a]
China	2	–	–	–	–	–	–	–	–
Croatia	1	1	–	–	–	–	–	–	1[a]
Denmark	5	1	1 (–/1)	–	–	1	–	–	–
Germany	5	3	3 (2/1)	1	–	–	–	–	–
Great Britain	2	1	–	–	–	–	1	–	–
Israel	5	3	1 (1/–)	–	1	–	1	–	–
Italy	10	1	1 (1/–)	–	–	–	–	–	–
Japan	5	2	2 (2/–)	–	–	–	–	–	–
France	5	1	–	–	–	–	–	–	1[a]
South Africa	10	8	6 (6/–)	2	–	–	1	–	–
Spain	5	–	–	–	–	–	–	–	–
USA	6	6	5 (5/–)	–	–	1	–	–	–
Total (%)	189 (100 %)	117 (61.9 %)	66 (54/12) (34.9 %)	16 (8.5 %)	9 (4.8 %)	5 (2.6 %)	8 (4.2 %)	4 (2.1 %)	12 (6.3 %)

a For details see Table 4.
b For details see Table 4.
c Co-occurrence of *Exophiala dermatitidis* and *E. phaeomuriformis* in a single dishwasher.

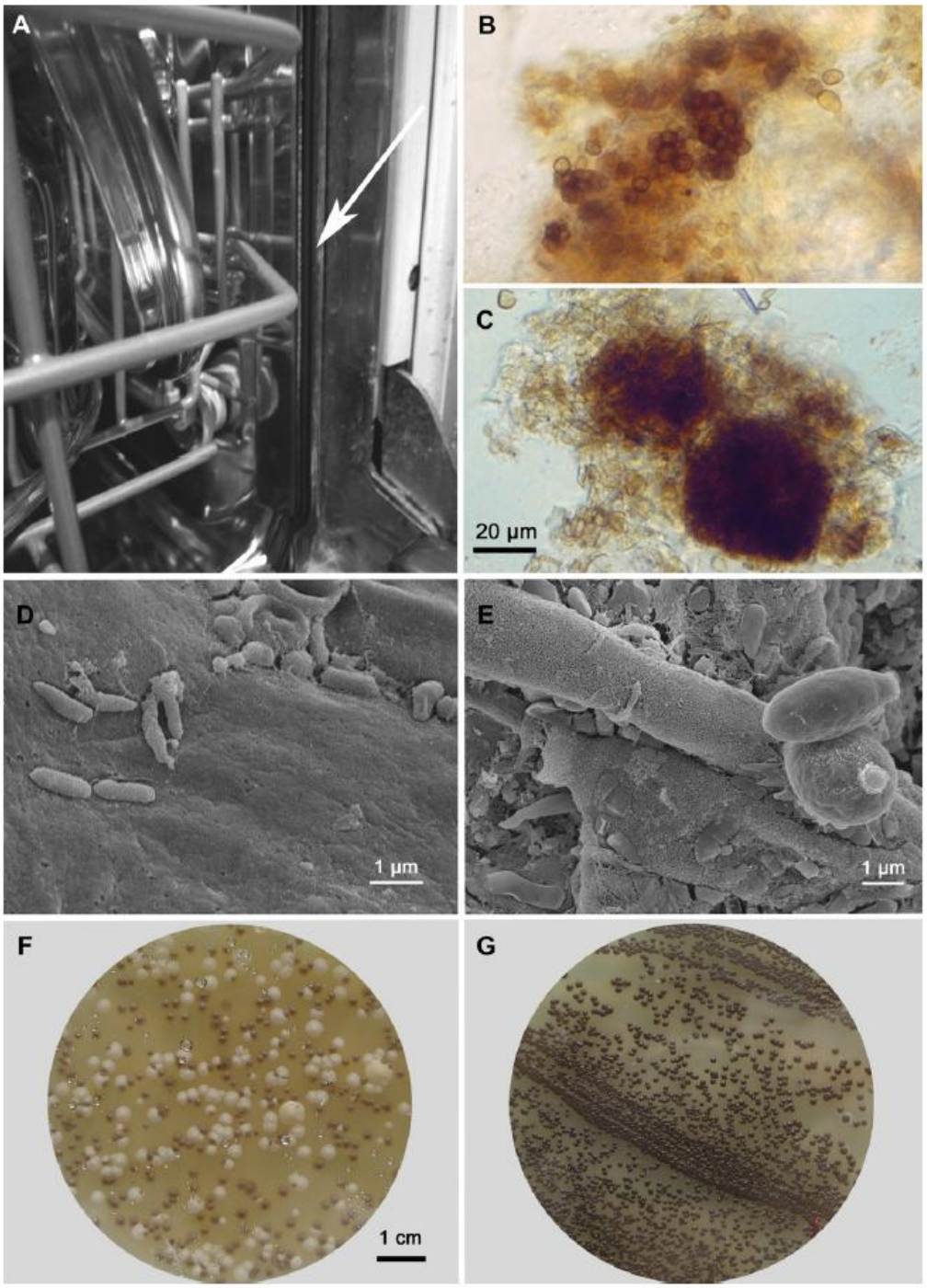

Fig 1 – (A) Sampling site in a dishwasher (indicated by an arrow); (B, C) *In vivo* morphology (separate muriform cells embedded in EPS) of a sample taken from the rubber seal of a dishwasher, of which *Exophiala dermatitidis*, genotypes A and C were isolated; (D) rubber seal surface with several bacterial cells; (E) rubber seal of a frequently used dishwasher over 6 y colonised with hyphae and yeast cells; (F, G) Primary isolation plates cultured from dishwasher swab samples, (F) *E. dermatitidis* (gen. A) and *Candida parapsilosis*; (G) *E. dermatitidis* (gen. A). Scale bar on picture C valid also for picture B. Scale bar on picture F valid also for picture G.

Table 2 – *Exophiala dermatitidis* strains from dishwasher's rubber, with location of sampling, hardness of water, genotypes, and GenBank accession numbers of ITS rDNA sequences.

Genotype	Country, city or town	Water hardness	No. of isolates	Representative strain – EXF no.	GenBank accession no. (ITS rDNA)
A	Slovenia, Goričko	S	3	EXF-5711	JF766637
A	Slovenia, Kostanjevica	S	2	EXF-5763	JF766638
A	Slovenia, Ljubljana and suburbs	MH	9	EXF-5580	JF766639
A	Slovenia, Ribnica	MH	1	EXF-5573	JF766640
A	Slovenia, Cerklje (Gorenjska)	MH	1	EXF-5578	JF766641
A	Slovenia, Ankaran	MH	1	EXF-5713	JF766642
A	Slovenia, Sežana	MH	2	EXF-6106	JF766643
A	Slovenia, Ormož	H	1	EXF-5649	JF766644
A	Slovenia, Bistrica ob Sotli	H	1	EXF-5721	JF766645
A	Slovenia, Mislinja	H	1	EXF-6096	JF766646
A	Slovenia, Jamnica	H	1	EXF-5771	JF766647
A	Australia, Traralgon	/	2	EXF-6100	JF766648
A	Austria, Graz	/	1	EXF-5767	JF766649
A	Belgium, Leuven	/	1	EXF-5727	JF766650
A	Brazil, São Paulo, Rio Claro	/	1	EXF-6276	JF766651
A	Germany, Bonn (Niederkassel)	/	2	EXF-5716	JF766652
A	Israel, Jerusalem	/	1	EXF-5827	JF766653
A	Japan, Kanagawa	/	2	EXF-5769	JF766654
A	South Africa, Cape Town	/	2	EXF-6110	JF766655
A	South Africa, Stellenbosch	/	2	EXF-6118	JF766656
A	USA, Salt Lake City	/	1	EXF-5652	JF766657
A	USA, Placerville	/	1	EXF-5656	JF766658
A2	Slovenia, Šentjernej	S	1	EXF-5761	JF766659
A2	Slovenia, Jamnica	H	1	EXF-5768	JF766660
A2	Australia, Traralgon	/	1	EXF-5758	JF766661
A2	Austria, Linz	/	1	EXF-5766	JF766662
A2	Italy, Viterbo	/	1	EXF-5770	JF766663
A3	Slovenia, Ljutomer	MH	1	EXF-5647	JF766664
A3	USA, Placerville	/	2	EXF-5654	JF766665
B	USA, Utah, Arches	/	2	EXF-5653	JF766666
B	Slovenia, Ljutomer	MH	1	EXF-5645	JF766667
B	Slovenia, Marijina vas	H	2	EXF-5760	JF766668
B	South Africa, Stellenbosch	/	3	EXF-6114	JF766669
B	Brazil, São Paulo, Rio Claro	/	1	EXF-6275	JF766670
C	Slovenia, Kamno	MH	1	EXF-5567	JF766671
C	Slovenia, Ljubljana	MH	1	EXF-5586	JF766672

In column of water hardness, the following abbreviations are used: S (soft: 4–8 dH), MH (moderately hard: 8–12 dH), H (hard: 12–18 dH), / (no data on water hardness).

followed by 15 min and 24 h incubation in HMDS, respectively. Dried material was mounted on specimens tub and coated with a 5 or 7 nm thick layers of platinum and gold. The samples were stored until required in a desiccator containing silica gel and viewed with a Jeol JSM-6500F scanning electron microscope.

Results

Direct inspection of some dishwashers's rubber seals showed structures interpreted to represent *Exophiala* muriform cells (Fig 1). Other fungal cells with yeast-like morphology were also found. Sampling and isolation of fungi from dishwashers of different age (6 m, up to 15 y), with different frequency of usage (once per month up to 3 times per day), at geographically different locations was performed (Table 1). Samples were collected from 102 dishwashers in Slovenia and 87 dishwashers from other countries on several continents. Thirty-eight percent of dishwashers were negative for fungi in the studied conditions, where incubation temperature was 37 °C. The most frequently encountered species were *Exophiala dermatitidis* and *Exophiala phaeomuriformis*, while *Candida parapsilosis*, *Pichia guilliermondii*, *Rhodotorula mucilaginosa*, *Magnusiomyces capitatus*, and *Fusarium dimerum* were less often but repeatedly isolated. On the primary isolation plates the growth was observed within 3–5 d after inoculation.

Data on *E. dermatitidis* and *E. phaeomuriformis* isolates, including their genotypes, are shown in Tables 2 and 3, respectively, while data on other filamentous fungi and other yeasts are given in Table 4. GenBank accession numbers of the sequences reported in this paper are also given in Tables 2–4. The prevalent genotype of *E. dermatitidis* in dishwashers was genotype **A** (Matos *et al.* 2003). Although black yeast colonies with different morphologies were often observed on primary isolation plates, generally only a single genotype was isolated per dishwasher. On three occasions, *viz.* in Slovenia (Ljubljana), Australia (Traralgon, South Victoria), and Brazil (Rio Claro) two genotypes were found in the same dishwasher: **A** plus **C**, **A** plus **A2**, and **A** plus **B**, respectively. The co-occurrence of two *Exophiala* species, *E. dermatitidis* plus *E. phaeomuriformis* was

Table 3 – *Exophiala phaeomuriformis* strains from dishwasher's rubber, with location of sampling, genotypes, and GenBank numbers of submitted ITS sequences.

Genotype	Country, city or town	Water hardness	No. of isolates	Representative strain – EXF no.	GenBank accession no. (ITS rDNA)
1	Slovenia, Ljubljana and suburbs	MH	5	EXF-5570	JF766611
1	Slovenia, Ljutomer	MH	2	EXF-5644	JF766612
1	Slovenia, Jesenice	MH	1	EXF-5569	JF766613
1	Slovenia, Lokev	MH	1	EXF-6108	JF766614
1	Slovenia, Gabrovka	H	1	EXF-5640	JF766615
1	Slovenia, Izlake	H	1	EXF-6121	JF766616
1	Slovenia, Maribor	H	1	EXF-6122	JF766617
1	Germany, Bonn	/	1	EXF-5826	JF766618
2	Denmark, Copenhagen	/	1	EXF-5715	JF766619

In column of water hardness, the following abbreviations are used: S (soft: 4–8 dH), MH (moderately hard: 8–12 dH), H (hard: 12–18 dH), / (no data on water hardness).

evident in a single case. *Exophiala* species were often accompanied by white yeasts (*P. guilliermondii*, *C. parapsilosis*, *M. capitatus*) or red yeasts (*R. mucilaginosa*). *Exophiala phaeomuriformis* was only isolated from European samples; the species exhibited two clearly different genotypes, indicated here as **1** and **2**. The majority of strains were genotype **1**, while one strain from Denmark represented genotype **2** (Table 3).

The ability of *Exophiala* species, the most commonly encountered fungal genus in dishwashers, to grow at different temperatures, pH and salinities is shown in Table 5. Strains of all genotypes of *E. dermatitidis* were able to grow between 10 °C and 45 °C, while genotypes **A**, **C** and the majority of strains of genotype **B** still showed growth at 47 °C. Strains of *E. phaeomuriformis* grew at 42 °C and mostly also at 45–47 °C. None of the isolates grew at 50 °C. Only genotype **C** of *E. dermatitidis* showed active growth at pH 2.5. All genotypes, with some variation within the groups, were extremely alkalotolerant being able to grow at pH 12.5. The growth spectrum of *E. phaeomuriformis* was even wider as all isolates could grow from pH 2.5–12.5. The morphology of *E. dermatitidis* strains subjected to different pH values was variable, growing as single cells and muriform clumps (Fig 2A) at low pH values, but transforming into filamentous growth with catenate conidial chains at pH 5.0 (Fig 2B) and maintaining this phenotype up to pH 12.5 (Fig 2C).

Table 4 – Remaining isolates (yeasts, filamentous fungi) from dishwasher's rubber, with location of sampling, GenBank numbers of submitted sequences, used for identification (ITS rDNA/LSU rDNA).

Identification	Country, city or town	No. of isolates	Representative strain – EXF no.	GenBank accession no. (ITS/LSU)
Acremonium strictum	Canada	1	EXF-6129	JF766680/–
Aspergillus niger group	Canada	1	EXF-6083	JF766673/–
Aureobasidium pullulans	Slovenia	1	EXF-5628	JF766676/–
Candida inconspicua	Slovenia	1	EXF-5648	–/JF766626
Candida parapsilosis	Slovenia	9	EXF-5540	–/JF766620
	Austria	1	EXF-6088	–/JF766621
	Belgium	1	EXF-5728	–/JF766622
	Canada	1	EXF-6126	–/JF766623
	Germany	1	EXF-5717	–/JF766624
	South Africa	2	EXF-6112	–/JF766625
Magnusiomyces capitatus	Slovenia	7	EXF-5633	JF766683/JF766627
	Israel, Jerusalem	1	EXF-6084	JF766681/–
	Austria, Graz	1	EXF-6087	JF766682/–
Fusarium dimerum	Slovenia	2	EXF-5636	JF766677/–
	USA	1	EXF-5658	JF766679/–
	Denmark	1	EXF-6079	JF766678/–
Paecilomyces variotii	Canada, Pointe Claire	1	EXF-6125	JF766675/–
Penicillium sp.	Croatia, Medžimurje	1	EXF-6098	JF766674/–
Pichia sp.	Slovenia, Ormož	1	EXF-5650	–/JF766632
Pichia guilliermondii	Slovenia	4	EXF-5541	–/JF766628
	Great Britain, Sutherland	1	EXF-6089	–/JF766629
	South Africa, Cape Town	1	EXF-6111	–/JF766630
	Canada, Pointe Claire	1	EXF-6127	–/JF766631
Rhodotorula calyptogenae	France, Martinique	1	EXF-6094	–/JF766634
Rhodotorula mucilaginosa	Slovenia	4	EXF-5543	–/JF766635
Sporopachydermia cereana	Belgium, Leuven	1	EXF-5729	–/JF766636
Prototheca aff. *wickerhamii*	Slovenia, Cerkno	1	EXF-5544	–/JF766633

Table 5 – Growth of *Exophiala dermatitidis* and *E. phaeomuriformis* at different temperatures, pH values and high NaCl concentration.

	Temperature tests [°C]				pH tests				Growth on NaCl [%]
	10	42	45	47	2.5	4	10	12.5	17
E. dermatitidis									
Genotype **A**	+	+	+	+	−/+	+	+	+	+
Genotype **A2**	+	+	+	−/+	−	+	+	+/−	+
Genotype **A3**	+	+	+	−	−/+	+	+	+	+(−[a])
Genotype **B**	+	+	+	+/−	+/−	+	+	+/−	+
Genotype **C**	+	+	+	+	+	+	+	+	+
E. phaeomuriformis									
Genotype **1**	+	+	+/−	+/−	+	+	+	+	+(−[a])
Genotype **2**	+	+	+/−	+/−	+	+	+	+	+

+: Growth; −: no growth; +/−: 50 % or more of tested strains are positive; −/+: 50 % or more of tested strains are negative.
Tested strains of *E. dermatitidis*, genotype **A** (n = 16): EXF-5573, EXF-5656, EXF-5767, EXF-5711, EXF-5718, EXF-5588, EXF-5576, EXF-5585, EXF-5649, EXF-5657, EXF-5713, EXF-5721, EXF-5724, EXF-5727, EXF-5827, EXF-5772, EXF-6110. Tested strains of *E. dermatitidis*, genotype **A2** (n = 5): EXF-5766, EXF-5761, EXF-5770, EXF-5768, EXF-5758. Tested strains of *E. dermatitidis*, genotype **A3** (n = 3): EXF-5647, EXF-5654, EXF-5655. Tested strains of *E. dermatitidis*, genotype **B** (n = 9): EXF-5760, EXF-6114, EXF-6115, EXF-6116, EXF-5653, EXF-5645, EXF-5646, EXF-5765, EXF-5712. Tested strains of *E. dermatitidis*, genotype **C** (n = 2): EXF-5586, EXF-5567. Tested strains of *E. phaeomuriformis*, genotype **1** (n = 10): EXF-5571, EXF-5644, EXF-6121, EXF-5575, EXF-5826, EXF-5569, EXF-5640, EXF-5642, EXF-6108, EXF-5570. Tested strains of *E. phaeomuriformis*, genotype **2** (n = 1): EXF-5715.
a Good growth on 5 % NaCl.

The M13 fingerprint of strains of *E. dermatitidis* resulted in 7 fragments (2290, 1830, 1400, 1120, 900, 700, 590 bp) in genotype **A**, in 7 fragments of slightly different sizes (2300, 1380, 1180, 860, 760, 580 and 340 bp) in genotype **B**, and in 8 fragments (2100, 1600, 1450, 1300, 1100, 850, 800, 660 bp) in genotype **C** (Fig 3). The fingerprints were consistently present in all 5 strains of each genotype. Mating events mostly resulted in fingerprints of either one genotype. Only in a single case (mating of genotypes **A** × **B**) a mixed profile was observed: seven fragments were identical to genotype **A** (2290, 1830, 1400, 1120, 900, 700, 590 bp), while one fragment (760 bp) was characteristic for genotype **B** (Fig 3, lane 24).

Discussion

Wet rooms, such as bathrooms, kitchens and steam baths in our households tend to harbour fungal biota. Recurrent groups of species are those with mucous propagules found in white, red and black yeasts and yeast-like fungi, coelomycetes, and species of the genera *Fusarium* and *Acremonium* (Anaissie *et al.* 2001; Göttlich *et al.* 2002). *Cladosporium* species are mostly those of the *C. sphaerospermum* complex (Zalar *et al.* 2007), comprising species that are not easily dispersed in air (B. Andersen, pers. comm.). Black yeast-like organisms grow very slowly and may therefore be overlooked, but their consistent occurrence has been noted in the classical studies by Nishimura *et al.* (1986, 1987) and confirmed in several recent papers (Göttlich *et al.* 2002; Hamada & Abe 2010; Lian & de Hoog 2010; Matos *et al.* 2002). Black yeast-like fungi discovered in bathrooms and kitchens belong to different species of the genera *Exophiala* (Nishimura *et al.* 1987) and *Cladophialophora* (Lian & de Hoog 2010).

Dishwashers as habitats for fungi share a number of characteristics with certain wet indoor environments including continuous moisture, high pH due to the regular use of detergents, and temporarily increased temperatures. In some niches in wet rooms, such as sinks, the concentration of organic matter may be high. Dishwashers also intermittently experience temperatures as high as 60–80 °C, high organic loads, high concentrations of salt to prevent calcareous accumulation and aggressive alkaline detergents. The seals of dishwashers are less rich in nutrients and might be even used as nutrition source for microorganisms (Rose & Steinbuchel 2005). Our study focused on these rubber seals of household dishwashers that can provide support and extent protection to microorganisms.

Fungal biota isolated from dishwasher rubber seals were similar to those reported from wet rooms, particularly in the occurrence of red, white and black yeasts, and *Fusarium* species. Among the white yeasts *Candida parapsilosis* was prevailing. This species is known from hospital settings where it forms extracellular slimes as a component of biofilms (Levin *et al.* 1998) in catheters, tubing systems and on prosthetic materials. These provide portals of entry to the human body. *Candida parapsilosis* has frequently been reported as an agent of opportunistic fungemia in severely compromised patients (de Hoog *et al.* 2009). Also members of *Rhodotorula* are known from catheter-related infections (Neofytos *et al.* 2007).

Fusarium dimerum was repeatedly encountered (Table 4). This species is apparently a water-transmitted fungus (Hageskal *et al.* 2006) that has been identified as an opportunistic human pathogen causing disseminated or localized infections in immunocompromised patients (Austen *et al.* 2001; Bigley *et al.* 2004; Krcmery *et al.* 1997; Letscher-Bru *et al.* 2002). Schroers *et al.* (2009) mentioned an investigation at the Loyola University Medical Center (S. Johnson *et al.*, unpublished data) where plumbing systems were supposed to be a source for *F. dimerum*. Zhang *et al.* (2006) concluded for opportunistic fusaria in general that they might have nosocomial origin.

The isolation of *Magnusiomyces capitatus* from dishwashers was unexpected. This species has first been found as an opportunistic invader of debilitated patients causing pulmonary infections (Gemeinhardt 1976), and has been repeatedly detected in disseminated infections in leukemic patients

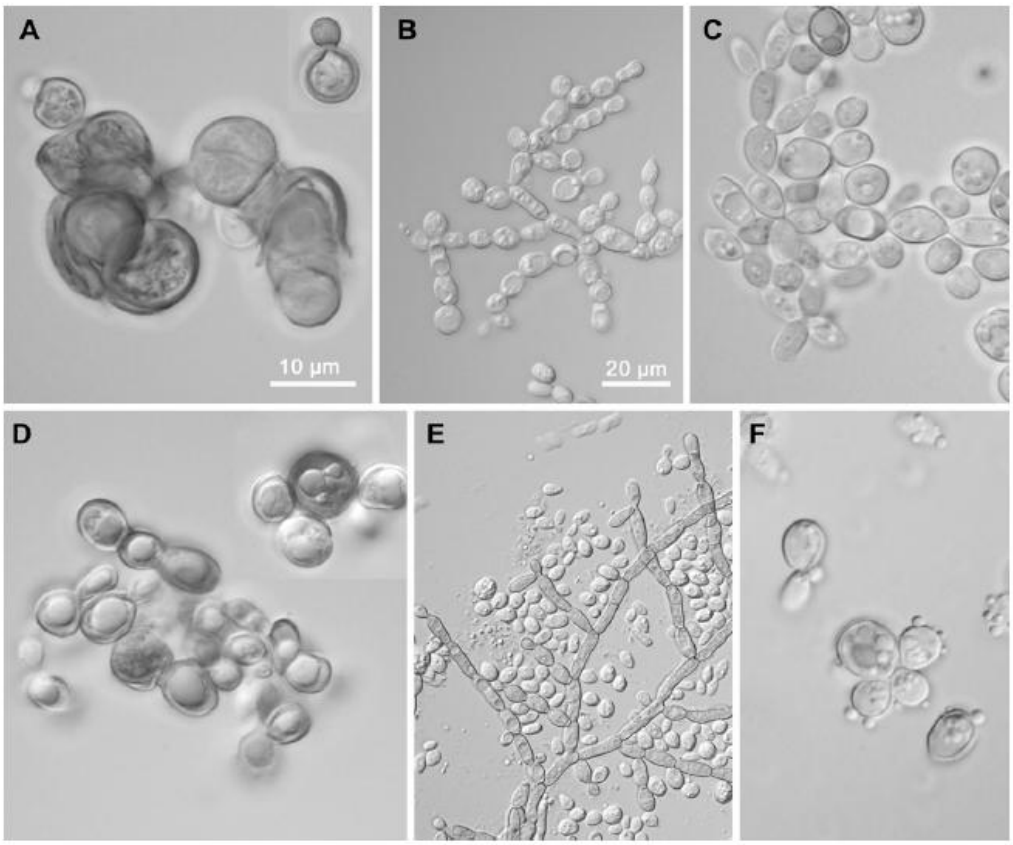

Fig 2 – Morphology of two *Exophiala* species growing in liquid malt extract medium with different pH values. (A–C) *Exophiala dermatitidis*, (A) pH 2.5, muriform cluster of cells (strain EXF-5766); (B) pH 6.0, catenate conidial chains (strain EXF-5647); (C) pH 12.0, catenate conidial chains (strain EXF-5766). (D–F) *Exophiala phaeomuriformis* (strain EXF-5642), (D) pH 2.5, budding cells; (E) pH 6.0, catenate chains of conidia on conidiophore; (F) pH 12.5, budding cells. Scale bars on pictures C, D, F as indicated on picture A (10 µm). Scale bars on pictures E as indicated on picture B (20 µm).

(reviewed by de Hoog *et al.* 2009). The natural habitat of this fungus is not known as only two strains derived from heated woodpulp have been found in the environment (Smith & Poot 2003). Our recurrent isolation of this fungus from dishwashers suggests that it has found a niche in the human-made environment of household machinery.

The composition of black yeast biota of wet indoor environments is determined by temperature relations. The study

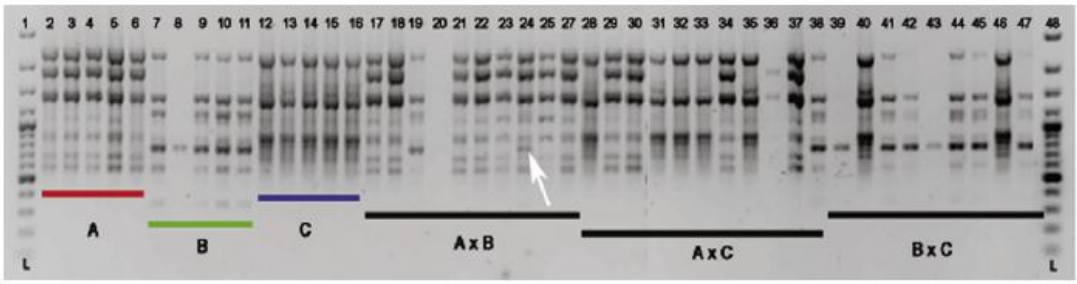

Fig 3 – MSP-PCR fingerprints of *E. dermatitidis* single strains and of strains subjected to mating obtained with the M13 primer. L, molecular size marker GeneRuler 100 bp DNA Ladder Plus, Fermentas (lanes 1, 48). Lanes 2–6: genotype A (EXF-5718); lanes 7–11: genotype B (EXF-5653); lanes 12–16: genotype C (EXF-5586); lanes 17–27: genotypes A × B; lanes 28–38: genotypes A × C; lanes 39–47: genotypes B × C. The white arrow indicates the unique fragment, present in the fingerprint after mating.

of fungi in Norwegian tap water revealed 94 different species belonging to 30 genera (Hageskal *et al.* 2006), including different species of black yeast genus *Phialophora*. Similar fungi were noted in Finnish tap water, where also species of the genus *Exophiala* were found (Kärkkäinen *et al.* 2009). Göttlich *et al.* (2002) noticed an abundance of *Phialophora*-like species (now known as *Cadophora*; G. S. de Hoog *et al.*, unpublished data) in cold tap water. *Cadophora* species are clinically insignificant and have never been reported to cause human or animal infections. At high water temperatures, *Exophiala* species are prevalent. Matos *et al.* (2002, 2003) and Sudhadham *et al.* (2008) noted the very regular presence of *Exophiala dermatitidis* and *Exophiala phaeomuriformis* in steam baths. Matos *et al.* (2002) noticed small differences in ecological preferences of the two species: *E. dermatitidis* occurred in steam rooms, whereas *E. phaeomuriformis* was mostly encountered in the warm but less extreme environments of adjacent halls and bathrooms. This matched with physiological differences: *E. dermatitidis* tolerates higher temperatures, while the production of extracellular capsules (Yurlova & de Hoog 2002) also enhances its adhesion to smooth plastic surfaces and tiles of steam rooms. These properties are less pronounced in *E. phaeomuriformis*. The results of our worldwide screening revealed that domestic dishwashers represent another important extreme habitat for these species that was overlooked until recently. Roughly one-third of sampled dishwashers was infested mostly with one of the two *Exophiala* species. The water hardness (Tables 2, 3) seems to have an important role in the persistence of *E. dermatitidis* and *E. phaeomuriformis* in dishwashers. The majority of isolates of both species were found in medium hard (*E. dermatitidis*: 59 %, n = 19; *E. phaeomuriformis*: 75 %, n = 9) and hard water areas (*E. dermatitidis*: 22 %, n = 7; *E. phaeomuriformis*: 25 %, n = 3). In areas with soft water only *E. dermatitidis* was found, representing 20 % (n = 6) of all isolates of this species. Karuppayil & Szaniszlo (1997) found that the concentration and availability of Ca^{+}-ions can influence the growth form in *E. dermatitidis* and supposedly also its virulence. Lower Ca^{+} concentrations favoured the non-polarized, muriform growth type, matching the invasive form of black fungi in human chromoblastomycosis, whereas higher concentrations promoted polarized growth such as the development of hyphae or budding cells.

The high prevalence of the two *Exophiala* species can be explained by their remarkable thermotolerance, halotolerance and pH tolerance (Table 5), the combination of which has previously not been observed in fungi. They could therefore be classified as polyextremotolerant microorganisms, in accordance with Bowers *et al.* (2009). These authors use the term "polyextremophiles" for anaerobic Bacteria and aerobic Archaea from African alkaline and hypersaline lakes that are able to grow optimally at elevated salt concentration (between 3.7 and 4.3 M Na^{+}), alkaline pH (above pH 9.5), elevated temperatures (between 46 and 66 °C) (Bowers *et al.* 2009). Active growth for *Exophiala* was recorded with up to 47 °C, in a pH range between 2.5 and 12.5, and with up to 17 % NaCl salinity. The latter is important for survival of osmotic stress during the dry periods in the dishwasher, as well as during washing when a high concentration of salt is added to prevent accumulation of calcareous deposits in the machine. The black yeasts under adverse conditions are able to produce an extremotolerant ecotype, expressed as meristematic, heavily melanised muriform cells. This was also the prevailing morphology exhibited on the rubber sealing of the examined dishwashers (Fig 1). It seems that the muriform phenotype in dishwashers is a result of multiple stress factors, such as changing pH (from 6.5 to 9.5 in a washing cycle), alternation of dry and wet periods, low and high salinity and increase of temperature from 10 to 80 °C, all experienced cyclically, according to the frequency of usage.

Thermophilic members of *Exophiala* and related genera are frequently encountered as agents of human disease, both in compromised and in otherwise healthy hosts. Both species identified, but particularly *E. dermatitidis*, have a significant opportunistic potential. In our study *E. dermatitidis* and *E. phaeomuriformis* were consistently found in dishwashers indicating a preferred habitat. Special attention to this habitat has to be paid particularly in hospital wards with immunocompromised patients. Wet rooms are also a likely source of contamination of the lungs of patients with cystic fibrosis, where *E. dermatitidis*, and to a lesser extent *E. phaeomuriformis* are regular colonizers (Haase *et al.* 1990). Although there were so far no reports on the infection of healthy humans in households using dishwashers, the potential hazard they represent should not be overlooked. Based on a screening of 2300 samples of faeces from humans with and without underlying disease revealed that *E. dermatitidis* is present in 0.5 % of the samples (de Hoog *et al.* 2005).

The genotype **A** of *E. dermatitidis* (Matos *et al.* 2003, matching genotype I of Uijthof *et al.* 1998) was prevalent in our study, comprising 67 % of strains of this species isolated. This is the most common genotype, with an apparent higher infective ability than genotype **B**, judging from the number of isolates from systemic disease as well as intestinal prevalence (Hiruma *et al.* 1993; de Hoog *et al.* 2005). Several genotypes may be found in a single dishwasher. MSP-PCR fingerprint data suggest that occasional recombination takes place (Fig 3), although teleomorphs are not known in this species.

The dishwasher habitat enriches a limited biota of polyextremotolerant fungi. Mixed biofilms formed on the rubber seals of dishwashers are rich in excreted polymeric substances (EPS). These EPS have multiple roles in the protection of the microbes. They provide a hydrated matrix that regulates the passage of ions from the neighbouring environment, prevents mechanical damage and increases heat resistance of microorganisms, whether the embedded microbe is in its hydrated state or in its dry form (Sterflinger 1998). The presence of such fungi in dishwashers increases the risk of infection through tableware or otherwise.

The ability of opportunistic fungi to survive near-boiling temperatures needs special attention. Amongst them black yeast represent a potential threat, due to their ability to convert into extremotolerant meristematic forms (Karuppayil & Szaniszlo 1997; Sterflinger 1998). The black yeast genus *Exophiala*, particularly *E. dermatitidis* and *E. phaeomuriformis* show a surprising combination of meristematic morphology, thermotolerance and opportunistic pathogenic behaviour. Their human association has been documented in colonisation of the lungs of patients with cystic fibrosis and occurrence as causative agents of neurotropic disseminated infections (Hiruma *et al.* 1993; Machouart *et al.* 2011), particularly in East Asia (Sudhadham *et al.* 2008).

In conclusion we have shown that dishwashers are among household machineries that provide a specific habitat for polyextremotolerant, potentially human pathogenic fungi. The co-existence of different genotypes of the same species possibly enables genetic recombination resulting into new genotypes with unknown pathogenic potential. Knowing that these fungi coinhabit our homes, further research is imperative as only this could reveal, whether the presence of *E. dermatitidis* inside our households poses any threat to human health.

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2.1.2 Pomivalni stroji zagotavljajo selektivno ekstremno okolje za oportuno patogene, kvasovkam podobne glive

Naslov v originalnem jeziku: Dishwashers provide a selective extreme environment for human-opportunistic yeast-like fungi

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POVZETEK

Urbano življenje je proizvedlo umetna okolja, ki iz mikrobiološkega stališča zagotavljajo ekstremne življenjske pogoje. Mnogi mikroorganizmi kot so npr. termofilni predstavniki črnih kvasovk iz rodu *Exophiala*, se v takih okoljih lahko razmnožijo. V pomivalnih strojih se na tesnilu vrat ter v stoječi vodi v notranjosti stroja neprestano kopičijo številne črne, bele in rdeče kvasovke. Mnoge od njih so predvsem znane kot povzročiteljice oportunističnih okužb pri človeku. V preglednem članku so podatki iz literature podprti s študijo, ki vključuje 937 gospodinjstev iz 15 mest v Turčiji. Glive so bile najdene v 265 vzorcih (28,3 %). Z uporabo sekvenciranja rDNA regije so bile kot najpogostejše identificirane vrste *Exophiala dermatitidis* (n = 116), *Candida parapsilosis* (n = 44), *E. phaeomuriformis* (n = 35), *Magnusiomyces capitatus* (n = 22), *Rhodotorula mucilaginosa* (n = 15) in *Clavispora lusitaniae* (n = 14). V članku je povzeta tudi vloga pomivalnih strojev pri prenosu gliv na ljudi.

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REVIEW

Dishwashers provide a selective extreme environment for human-opportunistic yeast-like fungi

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Abstract Urban life has led to the creation of human-made environments that, from a microbiological perspective, provide extreme life conditions. Certain non-ubiquitous microorganisms such as thermophilic members of the black yeast genus *Exophiala* are enriched within these habitats for which no counterpart is known in nature. Dishwashers consistently accumulate a number of specific black, white and red yeasts on the rubber seals of doors and in stagnant water at the interior. Several of these yeasts are primarily known as agents of human opportunistic infections. In this review, the literature data are supported by a screening study involving 937 households in 15 cities in Turkey. Fungi were detected in 230 samples (24.5 %). Using rDNA sequencing, the prevalent species were identified as *Exophiala dermatitidis* (*n*=116), *Candida parapsilosis* (*n*=44), *E. phaeomuriformis* (*n*=35), *Magnusiomyces capitatus* (*n*=22), *Rhodotorula mucilaginosa* (*n*=15), and *C. lusitaniae* (*n*=14). The possible role of dishwashers in transmitting disease is discussed.

Keywords *Candida parapsilosis* · *Exophiala* · Extremophile · Indoor environment · *Magnusiomyces capitatus* · Opportunistic pathogen

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Introduction

Daily life in urban settings takes place in human-made environments, which often have no identifiable counterpart in nature. Many of these environments, which can be considered as microbiologically extreme, provide artificial habitats to a limited number of fungi whose natural niche is often unknown. The airborne fraction of this fungal flora is associated with a wide range of adverse health effects, including infectious respiratory disease, toxicity, and allergy (Méheust et al. 2014). Less is known about health risks of fungi in wet cells such as bathing facilities and kitchen equipment, which are characterized by (i) high and low temperatures, (ii) elevated salt concentrations, (iii) acidic and basic conditions, and (iv) toxicity, along with (v) low nutrient availability. Fungi that are

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particularly selected by such complexes of stress factors have been referred to as 'polyextremotolerant' (Gostinčar et al. 2011).

Zalar et al. (2011) and Dogen et al. (2013a) observed that the thermophilic black yeasts *Exophiala dermatitidis* and *E. phaeomuriformis* show widespread colonization in the rubber seals of household dishwashers. The dishwasher environment is particularly unique in providing conditions of high temperature and alkalinity. Black yeasts have long been known as colonizers of wet human-made environments such as humidifiers (Nishimura and Miyaji 1982), private (Dogen et al. 2013a; Lian and de Hoog 2010) and public (Nishimura et al. 1987) bathing facilities, Turkish steam baths and Finnish saunas (Matos et al. 2002; 2003). These and other members of *Exophiala* are also known from other habitats, which are all human-made, such as creosoted railway sleepers (Dogen et al. 2013b; Gumral et al. 2014; Sudhadham et al. 2008), and are otherwise known as opportunistic pathogens on healthy or compromised humans (de Hoog et al. 2000; Matsumoto et al. 1993; Woo et al. 2013; Zeng et al. 2007). The latter behavior is underlined by the regular finding of opportunistic *Candida* species and *Magnusiomyces capitatus*, for which no natural habitat is known either.

In the present paper, we review recent findings demonstrating that dishwashers provide an extreme and unique environment for selected opportunistic fungi, particularly for thermophilic black and white yeasts (Dogen et al. 2013a; Zalar et al. 2011). The conclusions were verified by a screening study involving 937 households in 15 cities in Turkey. We also verified the results of previous reports demonstrating differences in fungal prevalence with hardness of municipal waters across the households studied.

Materials and methods

Sample area

We collected samples from 15 cities in Turkey (Adana, Ankara, Antalya, Burdur, Erzurum, Eskişehir, Gaziantep, Isparta, Istanbul, Izmir, Kahramanmaraş, Kars, Osmaniye, Sivas, and Şanlıurfa) during the study period of June to August 2013. The types and characteristics of water in these cities, including the elements naturally found in water, are presented in Table 1. All water samples were free from nitrite and ammonia, allowing the water to be drinkable. The study protocol was reviewed and approved by the Ethic's Committee of Gülhane Military Medical Academy, Ankara, Turkey.

Sample collection

A total of 937 samples were collected from dishwashers in different urban private dwellings. The samples were obtained using sterile cotton swabs moistened with sterile physiological saline. The insides of the invaginations of the rubber seals located on the folding doors of each dishwasher were swabbed, as described by Zalar et al. (2011). Samples were transported in sterile tubes and inoculated onto malt extract agar (MEA; Oxoid; Basingstoke, U.K.) culture plates containing 100 μg/ml chloramphenicol (Sigma; Steinheim, Germany). The plates were incubated at 37 °C and monitored daily for up to 21 days for evidence of fungal growth. Strains with slow, yeast-like growth, either white, black or red, were selected for further study.

DNA extraction, PCR, and molecular analysis

DNA extraction and PCR amplification were performed as described previously (Turin et al. 2000). The ribosomal DNA (rDNA) sequences spanning the internal transcribed spacer (ITS) region were amplified with the universal fungal primers ITS1 and ITS4 using an Applied Biosystems PRISM 3130XL Genetic Analyzer (Refgen Biotechnology; Ankara, Turkey). CAP contig assembly software included in the BioEdit Sequence Alignment Editor software version 7.0.9.0 was used to edit the sequences (Hall 1999). The assembled DNA sequences were examined using the BLAST (nucleotide-nucleotide) software program from the National Center for Biotechnology Information (National Institutes of Health; Bethesda, MD, U.S.A.).

Genotyping *Exophiala* isolates

Sequences were aligned with ClustalW (Tamura et al. 2011) and compared through neighbor-joining phylogenetic tree analyses using the Molecular Evolutionary Genetic Analysis (MEGA) version 5.05 software package (www.megasoftware.net). Genotype indications of *E. dermatitidis* (A, B, C) and *E. phaeomuriformis* (1, 2) were made according to Zalar et al. (2011). The genotypes were compared using the local CBS research database (www.cbs.knaw.nl/collections) containing approximately 10.000 black yeast sequences.

Physiology

All yeast isolates were tested for several growth characteristics. First, thermotolerance tests were performed in 96-well microtiter plates containing 300-μl malt extract broth (MEB) inoculated with 10 μl of a cell suspension in each well. The plates were then incubated at 5, 10, 40, 42, 45, and 47 °C. The growth in the media samples was assessed daily for 2 weeks. Visual and spectrophotometric readings were performed at 450 nm using a Thermo Scientific Multiskan™-GO Microplate Spectrophotometer (Vantaa, Finland). The tolerance at pH 2.5, 4, 10, and 12.5 was assessed in the MEB using appropriate amounts of 0.1 M HCl and 0.1 M NaOH.

Table 1 Water characteristics of the study areas in Turkey

City	Water type	pH	Total hardness (°F)	Ca (mg/l)	Mg (mg/l)	NO3 (mg/l)	Cl (mg/l)	Na (mg/l)	K (mg/l)
Adana	Hard	8.1	22.38	51.75	22.94	0.5	0.05	21.63	0.15
Ankara	Soft	7.63	9.41	26.88	6.53	0.3	0.01	9.09	0.06
Antalya	Very hard	7.68	37.48	121.85	17.04	3.1	0.04	34.14	0.18
Burdur	Medium hard	7.52	10.31	36.96	2.59	3.3	0.06	12.04	0.16
Erzurum	Medium hard	7.68	18.82	52.94	13.57	8.3	0.05	17.95	0.15
Eskişehir	Very hard	7.9	31.2	48.05	46.62	1	0.04	30.16	0.13
Gaziantep	Medium hard	7.6	13.06	48.56	2.23	0.6	0.04	12.62	0.04
Isparata	Medium hard	8.54	15.09	31.49	17.53	0.8	0.01	14.52	0.09
Istanbul	Medium hard	7.8	13.69	47.92	4.15	0.5	0.02	13.23	0.12
Izmir	Medium hard	7.94	17.18	46.32	13.6	1.7	0.05	16.24	0.12
Kahramanmaraş	Medium hard	8.33	19.24	48.37	17.36	0.7	0.04	18.59	0.1
Kars	Medium hard	7.71	18.02	53.3	11.4	1.1	0.05	17.41	0.11
Osmaniye	Medium hard	8.32	14.67	37.42	12.91	0.4	0.05	14.81	0.07
Sivas	Medium hard	8.02	18.56	60.24	8.5	0.3	0.05	17.94	0.11
Şanlıurfa	Medium hard	8.14	16.6	43.08	14.16	1.3	0.06	15.41	0.13

Halotolerance was assessed in MEB supplemented with 5, 10, or 17 % NaCl (*w/v*). Tolerance to 100 μg/ml cycloheximide (Sigma) was also tested on MEA (Dogen et al. 2013a; Gumral et al. 2014; Zalar et al. 2011).

Results

Of the 937 dishwasher samples, 230 (24.5 %) were positive for fungi, including 116 black yeasts, 79 non-black yeasts, and 35 polyfungal populations of black and non-black yeasts, at a selective temperature of 37 °C. From a total of 265 yeasts, *Exophiala dermatitidis* was the most common species (43.8 %) identified, followed by *Candida parapsilosis* (16.6 %), *E. phaeomuriformis* (13.2 %), *M. capitatus* (8.3 %), *Rhodotorula mucilaginosa* (5.6 %), *C. lusitaniae* (5.3 %) (Fig. 1), other *Candida* species (6.4 %), *Trichosporon asahii* (0.4 %), and *Yarrowia lipolytica* (0.4 %). *Exophiala dermatitidis* isolates were identified according genotypes A (n=86), B (n=20), and C (n=10). Isolates of 19 *E. phaeomuriformis* were assigned to genotype 1, and 16 isolates were assigned to genotype 2 (Table 2). The *Exophiala* isolates grew on MEA within 3–7 days, and the remaining yeast isolates grew within 1–3 days. The presence of black yeasts in dishwashers was not correlated with (i) the type or age of the dishwasher, (ii) its cleanness, (iii) or the type of detergent used.

Black yeasts were detected in isolates from all 15 cities, while non-black yeasts were identified in isolates from 7 out of 15 cities. Prevalence of black yeasts according to water hardness was 30 % in soft, 0–36.7 % in medium hard, 7 % in hard, and 9–28 % in very hard water. Genera of non-black yeasts were identified in 19 % in soft, 4.4–62.5 % in medium hard, and 20 % of very hard water, while there were no data for fungi from areas with hard water. We also compared the prevalence of black and non-black yeasts among the 7 cities where both types of yeasts were detected. The black yeast prevalence was found to be higher (15.6–30 %) in 4 cities (Ankara, Eskişehir, Isparta, and Istanbul) compared to that of non-black yeasts (4–20 %). In contrast, the prevalence of non-black yeasts was higher (43.3–62.5 %) in the remaining 3 cities (Erzurum, Sivas, and Kars) than that of black yeasts (28–36.7 %). *Candida parapsilosis* was detected in all 7 cities, while *M. capitatus* and *R. mucilaginosa* were identified in 5 out of 7 cities (Table 2).

Exophiala dermatitidis genotype A and *E. phaeomuriformis* genotype 1 strains grew at temperatures ranging from 5 to 47 °C, at pHs ranging from 2.5 to 12.5, and tolerated cycloheximide and 5–17 % salinity (Table 3).

A selection of the study isolates were deposited at the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands. In addition, the ITS rDNA sequences of these isolates were deposited in GenBank (Table 4).

Discussion

Dishwashers represent a selective environment favoring the growth of a small number of stress-tolerant fungi. Zalar et al. (2011) conducted the first global screening of dishwasher fungal communities and identified 120 strains representing 14 genera. A high prevalence of fungi (61.9 %), particularly black yeasts (28 %), was noted. Later studies yielded fungi in 17.7 % of the samples (Dogen et al. 2013a), while in the present study, 24.5 % of dishwashers were positive for the presence of yeast-like species. Previous data also revealed the consistent presence of a fungus that is not commonly encountered in the environment, *M. capitatus* (Dogen et al. 2013a; Zalar et al. 2011), with rates of prevalence similar to

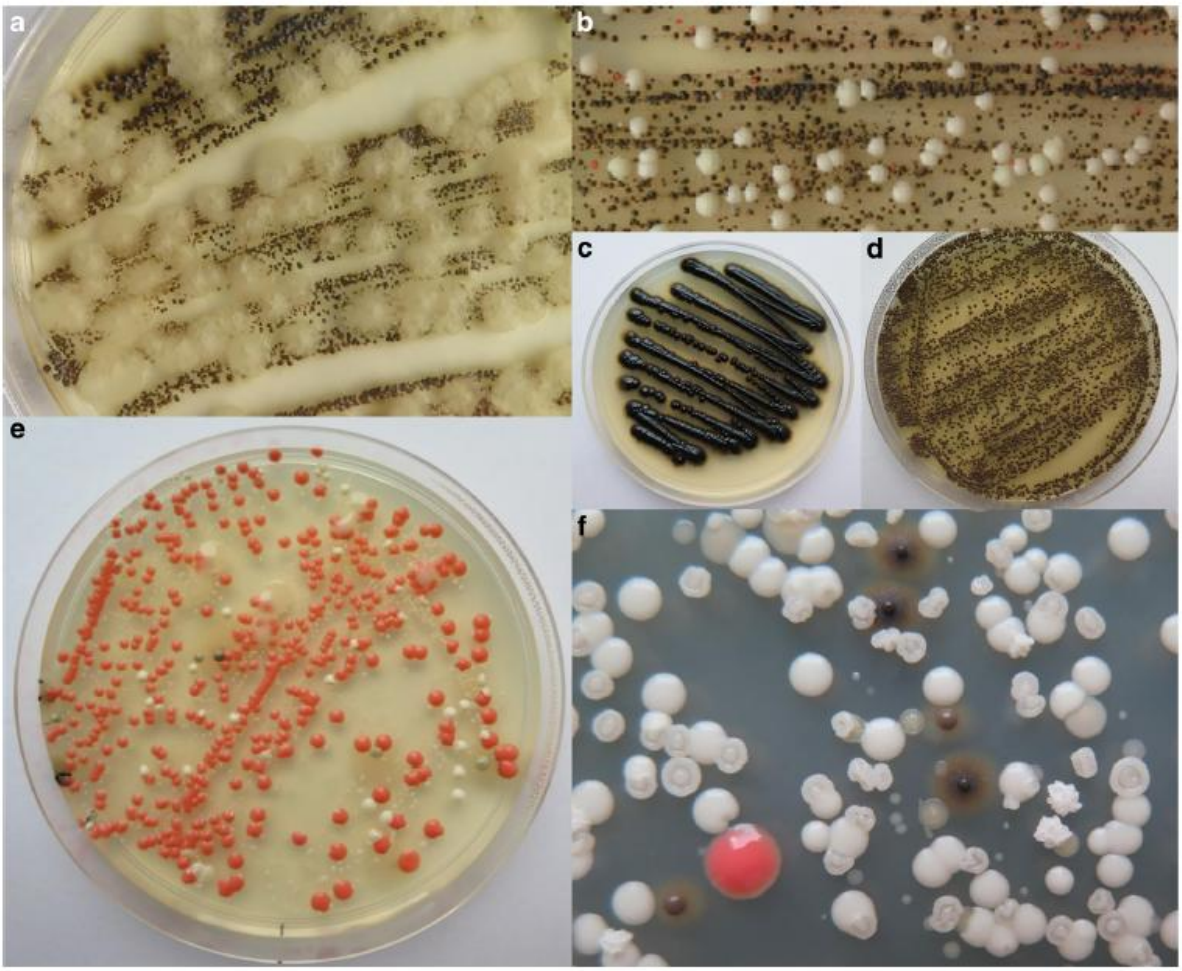

Fig. 1 **a** swab sampling – *Exophiala dermatitidis* and *Magnusiomyces capitatus*; **b** *E. dermatitidis*, *E. phaeomuriformis*, *Candida parapsilosis*, and *Rhodotorula mucilaginosa*; **c** pure culture – *E. dermatitidis*; **d** swab sampling – *E. dermatitidis*; **e** swab sampling – *C. parapsilosis*, *R. mucilaginosa*, and *C. lusitaniae*; **f** *E. dermatitidis*, *E. phaeomuriformis*, *C. parapsilosis*, *R. mucilaginosa*, and *C. lusitaniae* (16×magnification)

those observed in the present study (16.6 and 8.3 %, respectively). The prevalence of black yeasts in the study of Dogen et al. (2013a) conducted in Turkey was 15.2 %, while in the present analysis, conducted across a wider geographic range, it was 16.1 %. Zalar et al. (2011) noted that the frequency of black yeasts in dishwashers varied among countries. Indeed, in the present study, we recovered the highest rate of black yeasts (36.7 %) in Sivas, whereas we did not identify any black yeast in Gaziantep (Table 2). At a regional scale, however, the presence of fungi, and the relatively large proportion of chaetothyrialean black yeasts, appears to be relatively consistent.

Furthermore, the species composition of the dishwasher fungal biota also appears to be rather consistent. In order to verify this consistency, we investigated the presence of fungi in household dishwashers from 937 samples of private dwellings across 15 cities in Turkey. As in preceding studies, the thermophilic black yeasts *E. dermatitidis* and *E. phaeomuriformis* were found at high frequency, *C. parapsilosis* and *M. capitatus* were also repeatedly recovered. Most notably, *R. mucilaginosa* and *C. parapsilosis* were isolated from water sources, also some species from genus *Exophiala* are known to be waterborne such as *E. oligosperma*, *E. lecani-cornii*, and *E. mesophila* (de Hoog et al. 2011; Heinrichs et al. 2013; Shaker and Sharif 2012). The rest of the identified white yeasts are neither members of the common environmental saprobic biota nor the airborne fraction of indoor biota (Samson et al. 2010). For example, *M. capitatus* was found only once in heated wood pulp, with an unconfirmed report from a hollow tree trunk (Randhawa

Table 2 Distribution of thermophilic black yeasts and non-black yeasts in dishwashers sampled from 15 city centers throughout Turkey and several published studies

Study groups	Sample number	*E.dermatitidis*	Genotype A	Genotype A2	Genotype A3	Genotype B	Genotype C	*E.phaeomuriformis*	Genotype 1	Genotype 2
In the present study (Turkey, City)	937	116	69	9	8	20	10	35	19	16
Adana	106		2	1	1	–	2		1	–
Ankara	100		18	–	–	3	3		3	3
Antalya	100		6	1	–	1	–		1	–
Burdur	50		–	–	–	1	–		–	–
Erzurum	45		6	–	1	–	1		5	–
Eskişehir	50		2	–	–	4	1		3	4
Gaziantep	57		–	–	–	–	–		–	–
Isparta	90		8	1	–	1	–		1	3
Istanbul	90		15	1	–	5	–		1	2
Izmir	50		1	1	3	3	–		–	–
Kahramanmaraş	38		1	1	–	–	–		–	2
Kars	50		6	3	1	–	–		3	1
Osmaniye	50		1	–	–	–	–		–	–
Sivas	30		3	–	2	1	3		1	1
Sanlıurfa	31		–	–	–	1	–		–	–
Turkey, Mersin (Dogen et al. 2013a, b)	153	21	13	–	–	7	1	3	2	1
18 countries (Zalar et al. 2011)	189	58	39	5	3	9	2	12	11	1

Study groups	*C.guilliermondii*	*C.kefyr*	*C.lusitaniae*	*C.parapsilosis*	*C.tropicalis*	*M.capitatus*	*F.dimerum*	Other yeasts[a]	Total
In the present study (Turkey, City)	8	3	14	44	1	22	–	22	265
Adana	–	–	–	–	–	–	–	–	7
Ankara	1	1	–	6	–	7	–	4	49
Antalya	–	–	–	–	–	–	–	–	9
Burdur	–	–	–	–	–	–	–	–	1
Erzurum	3	2	6	3	–	8	–	6	41
Eskişehir	–	–	1	5	1	2	–	1	24
Gaziantep	–	–	–	–	–	–	–	–	–
Isparta	–	–	–	4	–	–	–	–	18
Istanbul	–	–	–	6	–	3	–	3	36
Izmir	–	–	–	–	–	–	–	–	8
Kahramanmaraş	–	–	–	–	–	–	–	–	4
Kars	3	–	4	17	–	2	–	2	42
Osmaniye	–	–	–	–	–	–	–	–	1
Sivas	1	–	3	3	–	–	–	6	24
Sanlıurfa	–	–	–	–	–	–	–	–	1
Turkey, Mersin (Dogen et al. 2013a, b)	–	–	–	2	–	2	–	–	28
18 countries (Zalar et al. 2011)	8	–	–	16	–	9	5	16[b]	119

[a] *T. asahii* ($n=1$), *Y. lipolytica* ($n=1$), *C. inconspicua* ($n=2$), *C. intermedia* ($n=1$), *C. krusei* ($n=2$), and *R. mucilaginosa* ($n=15$)

[b] Please check Zalar et al. 2011, for detailed information

Table 3 Growth characteristics of *Exophiala* species, *Candida parapsilosis*, and *Magnusiomyces capitatus* isolates at various temperatures, pH values, and salinity concentrations

Strains (number)	Temperatures						pH values				Growth on NaCl			Cycloheximide
	5 °C	10 °C	15 °C	42 °C	45 °C	47 °C	2.5	4	10	12.5	5 %	10 %	17 %	
E. dermatitidis (n=116)														
Genotype A (n=69)	65/69	+	+	+	+	64/69	+	+	+	+	65/69	65/69	65/69	+
Genotype A2 (n=9)	8/9	+	+	+	+	+	+	+	+	+	+	+	+	+
Genotype A3 (n=8)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Genotype B (n=20)	17/20	+	+	+	+	19/20	+	+	+	+	19/20	19/20	19/20	+
Genotype C (n=10)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E. phaeomuriformis (n=35)														
Genotype 1 (n=19)	17/19	17/19	+	+	18/19	16/19	+	+	+	+	+	+	17/19	+
Genotype 2 (n=16)	15/16	+	+	+	15/16	14/16	+	+	+	+	+	+	15/16	+

et al. 2001), while all remaining known strains of this species originated from human patients (de Hoog 2014b; de Hoog and Smith 2004).

There is some resemblance of dishwasher biota to that of bathing facilities, particularly with respect to the frequent isolation of black yeasts (Lian and de Hoog 2010). *Exophiala dermatitidis* has also been recovered from hot springs (Sudhadham et al. 2008), however Sudhadham et al. (2008) proposed a natural life cycle for *E. dermatitidis* that included intestinal passage in frugivorous tropical animals.

Rhodotorula species are a known part of indoor wet cells, which are characterized by intermittent humidity and high temperature, with nutrient depletion as a constant factor. The natural habitat of *C. lusitaniae* and *C. parapsilosis* is enigmatic; they have occasionally been found in sugary environments such as fruits, but are otherwise associated with mammals, either as opportunists or as intestinal colonizers (Kurtzman et al. 2011).

The relative enrichment of the above black and white yeasts in the dishwasher environment provides clues into

Table 4 CBS and GenBank accession numbers (ITS rDNA sequences) for the selected study isolates

Isolate	Sampling area	CBS no.	GenBank accession No. (ITS rDNA)
E. dermatitidis genotype A	Osmaniye	139110	KP658832
E. dermatitidis genotype A2	Kahramanmaraş	139113	KP658835
E. dermatitidis genotype A3	Izmir	139114	KP658836
E. dermatitidis genotype B	Burdur	139117	KP658839
E. dermatitidis genotype C	Adana	139120	KP658842
E. phaeomuriformis genotype 1	Antalya	139124	KP658846
E. phaeomuriformis genotype 2	Isparta	139127	KP658849
C. guilliermondii	Kars	139.39	KP658851
C. inconspicua	Sivas	139.38	KP658852
C. intermedia	Sivas	139.37	KP658853
C. kefyr	Erzurum	139.36	KP658854
C. krusei	Istanbul	139.35	KP658855
C. lusitaniae	Erzurum	139.34	KP658856
C. parapsilosis	Kars	139.47	KP658857
C. tropicalis	Eskişehir	139.33	KP658858
M. capitatus	Ankara	139.32	KP658859
R. mucilaginosa	Istanbul	139.31	KP658860
T. asahii	Ankara	139.48	KP658861
Y. lipolytica	Erzurum	139.30	KP658862

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some of the factors that are essential for their colonization. Chaetothyrialean black yeasts show (i) thermotolerance (≥42 °C), (ii) acidotolerance (pH<3), (iii) alkalitolerance (pH>9), (iv) halotolerance (NaCl>10 %), and (v) tolerance of oxidative stress and harsh detergents (Dogen et al. 2013a, 2013b; Zalar et al. 2011). Fungi that colonize harsh environments mostly have low competitive ability against ubiquitous saprobes. For example, the predominant fungus in dishwashers, the black yeast *E. dermatitidis*, has been found in other hostile habitats such as on creosoted railway sleepers (Dogen et al. 2013b; Gumral et al. 2014; Sudhadham et al. 2008).

The yeast-like fungi isolated from dishwashers rubber seals were shown to be tolerant to a wide range of temperatures, pH levels, and salt concentrations, indicating a degree of 'polyextremotolerance' (Gostinčar et al. 2011), which is rarely observed in other classes of fungi (Table 3). In agreement with previous reports (Dogen et al. 2013a; Zalar et al. 2011) we observed active growth of *E. dermatitidis* and *E. phaeomuriformis* at 5–47 °C, across a wide pH range (2.5–12.5) and at up to 17 % NaCl, with tolerance to cycloheximide (Table 3). We recovered genotype A of *E. dermatitidis* more frequently than genotypes B and C, with a ratio of 8.6:2:1. Genotype A of *E. dermatitidis* is known to be more successful at entering urban environments (Dogen et al. 2013a; Sudhadham et al. 2008; Zalar et al. 2011) and in infecting patients. This genotype shows the highest pulmonary colonization rate in human patients with cystic fibrosis (Kondori et al. 2011; Packeu et al. 2012), and caused fatal cerebritis in immunocompetent adults in East Asia (de Hoog et al. 2000; Matsumoto et al. 1993). A recent review noted that in nearly 40 % of patients with *E. dermatitidis* systemic infections the underlying risk factors remain unknown (Patel et al. 2013). *E. phaeomuriformis* has been observed in deep infections (Zeng et al. 2007), and long-term *E. phaeomuriformis* colonization in the lungs of cystic fibrosis patients has been reported (Packeu et al. 2012).

The prevalent white yeasts in dishwashers, such as *C. parapsilosis*, *M. capitatus*, and *C. lusitaniae*, are almost exclusively known from clinical settings. Members of the *C. parapsilosis* species complex, i.e., *C. parapsilosis sensu stricto*, *C. metapsilosis*, and *C. orthopsilosis*, are important nosocomial opportunists, representing the second most common cause, after *C. albicans*, of fungemia in immunocompromised patients. They also have a marked ability to produce biofilms (Bonfietti et al. 2012; Ruiz et al. 2013), entering the host via cathethers and prosthetic materials. The predominance of *C. parapsilosis s. str.* in dishwashers is consistent with its prevalence in human infections. This species has also been reported in fruit juices (Maciel et al. 2013) and in the fecal samples of synantropic wild birds (Lord et al. 2010). It was regularly isolated from water sources (Pires-Gonçalves et al. 2008) and from residential surfaces (Adams et al. 2013). Latest research revealed that *C. parapsilosis s. str.* is not only consistently present on dishwasher rubber seals but also inhabits the interior of washing machines, presenting a possible route of infection (Novak-Babič et al. 2015). *Candida lusitaniae* was also recovered from the soil of parks, schools, and kindergarten recreational areas (Wójcik et al. 2013), and from decaying wood in tree trunk hollows (Randhawa et al. 2001). It is a very rare (~1 %) cause of candidemia in patients with hematological malignancies (Atkinson et al. 2008). In the present study, we recovered *C. lusitaniae* (5.2 %) as the sixth most common fungus dwelling in dishwashers, while Zalar et al. (2011) have not recorded it at all.

Magnusiomyces capitatus is primarily associated with pulmonary infections in leukemic patients (García-Ruiz et al. 2013; Gimenia et al. 2005), and has been occasionally isolated from patients with oral infections (Hattori et al. 2007). *Magnusiomyces capitatus* is phylogenetically and phenotypically closely related to *Saprochaete clavata*. These two species are often misidentified (Desnos-Ollivier et al. 2014). All strains but one, which was confirmed to be *M. capitatus*, from Zalar et al. study (2011) were later identified as *S. clavata* (Vaux et al. 2014). In 2012 in France an outbreak of rapidly fatal cases of invasive fungal infections due to *S. clavata* was reported and no common infection source has been confirmed (Vaux et al. 2014). Although it is known that this species can be transmitted via contaminated medical devices (Ersoz et al. 2004) or can be associated with dairy products (Gurgui et al. 2011). It appears that this newly recognized emerging pathogen can survive dishwasher conditions. A detailed taxonomic study is necessary to ascertain inasmuch the teleomorph species *Magnusiomyces capitata* and the anamorph *Saprochaete clavata* truly belong to different species or whether a single, variable complex is concerned.

The fact that uncommon opportunists, some of which are hardly known outside humans, are enriched by the dishwasher environment provides insight into the ecological preferences of these organisms. Given their wide range of resistance to divergent stressors, combined with their consistent occurrence in human hosts, it may be hypothesized that dishwashers, and other human-made environments provide enrichment conditions for these fungi. The fact that only a very small fraction of all known opportunists (de Hoog et al. 2014a) is found in the dishwashers indicates that 'polyextremotolerance' is not a general prerequisite for opportunism. Rather, a common colonization history from an as-yet-unrevealed source seems likely. After enrichment in the dishwasher, with up to 10^6 CFU/cm^2 of dishwasher rubber seal (N. Gunde-Cimerman, personal communication) human hosts might be contaminated via fungus-loaded aerosols that are liberated when opening the dishwasher, via traumatic inoculation or repeated contact with contaminated surfaces. To date, there is no hard evidence supporting these transmission routes.

Gostinčar et al. (2011) speculated that the highly variable and stressful environment of the dishwasher may generate evolutionary adaptation. The possibility is not excluded that such increased stress-tolerance would consequentially enhance the fungus' opportunistic ability in the human host. However, since this evolution takes place outside the human hosts, it does not necessarily increase fungal virulence. Whether or not change takes place, data should be compared of the entire life cycle with habitat jumps. Currently, environmental data on the dishwasher biota mainly concern artificial, i.e., human-dominated habitats, while their behavior in natural settings remains unknown. Although previous studies of black yeast-like fungi in groundwater (Göttlich et al. 2002; Heinrichs et al. 2013) did not list *E. dermatitidis* amongst isolates, *E. dermatitidis* has recently been found in groundwater-derived municipal drinking water networks in Slovenia (Novak Babič et al. submitted), which suggests a closed route from nature to dishwasher. At this point in time health risks for human patients remain difficult to estimate. Intuitively, for severely immunocompromised patients, dishwashers may convey a risk that is comparable or due to higher infection doses even higher to that of bathing facilities (Anaissie et al. 2001).

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2.1.3 Oportuno patogene vrste iz rodov *Candida* in *Fusarium* izolirane iz uporabnikom dosegljivih delov pralnih strojev

Naslov v originalnem jeziku: *Candida* and *Fusarium* species known as opportunistic human pathogens from customer accessible parts of residential washing machines

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POVZETEK

Omejitve pri porabi energije so spremenile navade potrošnikov pri uporabi gospodinjskih pralnih strojev. Razviti so bili stroji, ki pri svojem delovanju uporabljajo nižje temperature pranja, manjše količine vode in biološko razgradljive detergente. Taki pogoji v pralnih strojih selektivno vplivajo na obogatitev oportunistično patogenih gliv za človeka. Osredotočili smo se na izolacijo gliv iz dveh uporabniku dostopnih delov pralnih strojev, ki pogosto vsebujejo mikrobne biofilme: predali za detergente in tesnila na vratih. Od 70 gospodinjskih pralnih strojev, vzorčenih v Sloveniji, smo iz 79 % osamili glive. Skupno smo izolirali 72 sevov, ki pripadajo 12 rodovom in 26 vrstam. Med njimi so glive iz kompleksov *Fusarium oxysporum* in *Fusarium solani*, *Candida parapsilosis* ter *Exophiala phaeomuriformis* skupno predstavljale 44 % vseh izoliranih gliv. Te vrste so sicer poznane kot oportunistični patogeni človeka, vendar so pogosto povzročiteljice okužb kože, nohtov ter oči tudi pri zdravih posameznikih. Analiza strojnega učenja je pokazala, da so prisotnost detergentov, mehčalcev ter temperatura pranja ključni dejavniki za kolonizacijo pralnih strojev z glivami. Tri pralne stroje s prevladujočim neprijetnim vonjem smo dodatno analizirali na prisotnost gliv in bakterij. V teh primerih so bile glive izolirane v manjšem številu (7,5 %), prevladovale pa so bakterije vrst *Micrococcus luteus*, *Pseudomonas aeruginosa* in *Sphingomonas* sp.

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Candida and *Fusarium* species known as opportunistic human pathogens from customer-accessible parts of residential washing machines

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ABSTRACT

Energy constraints have altered consumer practice regarding the use of household washing machines. Washing machines were developed that use lower washing temperatures, smaller amounts of water and biodegradable detergents. These conditions may favour the enrichment of opportunistic human pathogenic fungi. We focused on the isolation of fungi from two user-accessible parts of washing machines that often contain microbial biofilms: drawers for detergents and rubber door seals. Out of 70 residential washing machines sampled in Slovenia, 79% were positive for fungi. In total, 72 strains belonging to 12 genera and 26 species were isolated. Among these, members of the *Fusarium oxysporum* and *Fusarium solani* species complexes, *Candida parapsilosis* and *Exophiala phaeomuriformis* represented 44% of fungi detected. These species are known as opportunistic human pathogens and can cause skin, nail or eye infections also in healthy humans. A machine learning analysis revealed that presence of detergents and softeners followed by washing temperature, represent most critical factors for fungal colonization. Three washing machines with persisting malodour that resulted in bad smelling laundry were analysed for the presence of fungi and bacteria. In these cases, fungi were isolated in low numbers (7.5 %), while bacteria *Micrococcus luteus*, *Pseudomonas aeruginosa*, and *Sphingomonas* species prevailed.

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Introduction

Infections by opportunistic human pathogenic fungi are becoming an increasing health concern all over the world. The number of patients who are at risk of invasive fungal mycoses (invasive aspergillosis, candidaemia, cryptococcal meningitis) is around 12 million (Parkin et al. 2002; Park et al. 2009; Brown et al. 2012). About 4.8 million patients suffer from allergic bronchopulmonary aspergillosis (Denning et al. 2013), 12 million have allergic fungal sinusitis (To et al. 2012), and 6 million have fungal eye infections (Lam et al. 2002). About 1 billion people around the world suffer from skin, nail, and hair infections (Vos et al. 2012). At the same time, fungal infections are increasing as the number of patients suffering from cancer, AIDS, and autoimmune or chronic diseases increase (Anaissie et al. 2001).

Water environments in nature represent reservoirs for a large spectrum of microorganisms. Some of these invade our homes *via* the tap-water system (Pereira et al. 2010), and these can represent a potential health risk, particularly to immunocompromised people. Recent studies have shown that they can also invade water-connected household appliances, such as washing machines, and dishwashers. Consumer awareness toward a sustainable use of resources and hazardous chemicals, led also to the development of machines operating at lowered washing temperatures and with reduced amounts of water and biodegradable detergents. The selection of these conditions can promote thermotolerant, oxidative-stress resistant, and generally stress-tolerant microbes and may lead to an accumulation of opportunistic human pathogenic species in equipments and possibly also in other indoor-environments (Gostincar et al. 2009).

The discovery that dishwashers from residential households can be colonized with the polyextremotolerant and opportunistic human pathogenic black yeast *Exophiala dermatitidis* and other potentially pathogenic fungi (Zalar et al. 2011) received considerable public attention. Although it had been known already earlier that washing machines accommodate bacteria and fungi in visible and non-visible biofilms that can often result in malodour of clothes inside washing machines or laundries in healthcare facilities and residential homes. Different species of the bacterial genera *Acinetobacter*, *Bacillus*, *Clostridium*, *Corynebacterium*, *Escherichia*, *Micrococcus*, *Pseudomonas*, and *Staphylococcus* have been the most frequently isolated (Robinton et al. 1968; Blaser et al. 1984; Smith et al. 1987; Perry et al. 2001; Panagea et al. 2005). In comparison, fungi have been reported less frequently and belonged to genera such as *Alternaria*, *Aspergillus*, *Candida*, *Capronia*, *Cladosporium*, *Cryptococcus*, *Fusarium*, *Penicillium*, *Rhodotorula*, and *Trichosporum* (Munk et al. 2001; Hamada 2002; Gattlen et al. 2010; Kubota et al. 2012; Stapleton et al. 2013).

Further studies have indicated that malodour usually is associated with the bacterial degradation of various substances present in detergents (Munk et al. 2001). As washing machine colonizing microorganisms can also cross-contaminate clothes during washing cycles, they also present a threat for humans as they may case cutaneous and other infections and may lead to the development of nosocomial infections in hospital environments (Munk et al. 2001; Gattlen et al. 2010). Such nosocomial infections have been reported for the bacteria *Clostridium difficile*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Rozman et al. 2013), and for dermatophytic fungi and yeasts belonging to the genera *Microsporum*, *Trichophyton*, and *Candida* (Shah et al. 1988; Tanaka et al. 2006).

In the present study, we focused on the presence and diversity of fungi that inhabited 70 residential washing machines in Slovenia. With emphasis on potentially pathogenic species that have not been studied to date, we sampled easily accessible plastic drawers for washing powder and softener, and the rubber door seals. These parts are often covered visibly with persistent, even stained microbial biofilms. As they are also frequently manipulated by consumers, they require special attention. Additionally, three of the sampled washing machines producing strong malodour persistently, were sampled to identify involved fungi and bacteria. Isolated fungal strains were tested for their ability to grow at 37 °C, commercial softener and produce esterases and proteases. Eventually, we used a machine learning approach for the identification of the most important factor that supports fungal growth in washing machines.

Materials and methods

Washing machines sampled

Seventy three residential washing machines were sampled for fungi, three of these also for bacteria. The mean time of use of machines was 3.5 y. These washing machines were also used from once per week to once per day, at 30 °C–95 °C, and were from different producers and located in different geographical sites in Slovenia. Samples were taken from the washing powder and fabric softener drawers, and from the rubber door seals around the washing machine doors (Table 1).

Isolation of fungi and bacteria

Sterile cotton swabs pre-moistened in saline solution (0.9 % w/v NaCl) were used for taking samples by rubbing the surface of the drawers and the rubber door seals. These swabs were kept in sterile tubes and were processed either immediately or stored at 4 °C for up to 7 d. Swabs were rubbed over the surface of malt extract agar (MEA) supplemented with 0.05 g L^{-1} chloramphenicol, and incubated at 25 °C and 30 °C for up to 7 d. Visibly developing fungi were then transferred to fresh MEA plates. All pure cultured fungal isolates were tested for their ability to grow at 37 °C on MEA.

Bacteria and fungi were isolated from three washing machines selected due to their intense malodour, and from three washing machines without malodour (Tables 2 and 3), using the same sampling technique as described above. Additional samples were taken from the inner parts of the washing machine drum, the water-supply connector, and the wastewater connector. These swabs were rubbed over the surface of R2A culture medium (Conda, Spain) and nutrient agar (NA; Biolife, Italy), and incubated at both 25 °C and 37 °C for up to 7 d.

Table 1 – Frequency of occurrence of strains, their sampling sites and GenBank accession numbers of recruited DNA barcodes from the 70 residential washing machines from Slovenia.

Identification	Frequency of isolation	Representative strain – EXF no.	GenBank Accession no.
Washing powder drawer			
Aureobasidium pullulans	1	EXF-6298	KJ481250 (ITS)
Candida parapsilosis	3	EXF-8293	KJ481229 (LSU)
Cladosporium sphaerospermum	2	EXF-8279	KJ500007 (*act*)
Exophiala lecanii-corni	1	EXF-6140	KJ481253 (ITS)
Exophiala mesophila	1	EXF-6138	KJ481254 (ITS)
Exophiala phaeomuriformis genotype 1	3	EXF-8235	KJ481255 (ITS)
Fusarium oxysporum species complex (FOSC)	9	EXF-5661	KJ481241 (*tef*)
Fusarium proliferatum	1	EXF-5664	KJ481246 (*tef*)
Fusarium solani species complex (FSSC)	2	EXF-5665	KJ481247 (*tef*)
Fusarium verticillioides	2	EXF-5553	KJ481244 (*tef*)
Meyerozyma guilliermondii	1	EXF-8240	KJ481231 (LSU)
Mucor circinelloides	1	EXF-6296	KJ481257 (ITS)
Ochroconis sp.	1	EXF-5565	KJ481236 (*act*)
Penicillium crustosum	2	EXF-8272	KJ481238 (*benA*)
Phoma radicina	1	EXF-6297	KJ481248 (ITS)
Phoma fimeti	1	EXF-5551	KJ481249 (ITS)
Fabric softener drawer			
Aureobasidium melanogenum	1	EXF-8259	KJ481251 (ITS)
Candida parapsilosis	9	EXF-8289	KJ481227 (LSU)
Cladosporium pseudocladosporioides	1	EXF-5563	KJ500008 (*act*)
Cladosporium halotolerans	1	EXF-5564	KJ500009 (*act*)
Exophiala equina	1	EXF-5566	KJ481252 (ITS)
Fusarium oxysporum species complex (FOSC)	7	EXF-8264	KJ481243 (*tef*)
Mucor racemosus	1	EXF-5556	KJ481258 (ITS)
Penicillium brevicompactum	1	EXF-5558	KJ481240 (*benA*)
Penicillium crustosum	3	EXF-8276	KJ481239 (*benA*)
Rhodotorula slooffiae	1	EXF-5557	KJ481234 (LSU)
Rubber door seal			
Candida parapsilosis	2	EXF-8290	KJ481228 (LSU)
Cladosporium bruhnei	1	EXF-5660	KJ500006 (*act*)
Cryptococcus diffluens	1	EXF-6329	KJ481230 (LSU)
Exophiala phaeomuriformis genotype 1	1	EXF-6326	KJ481256 (ITS)
Fusarium oxysporum species complex (FOSC)	2	EXF-6333	KJ481242 (*tef*)
Fusarium proliferatum	1	EXF-6330	KJ481245 (*tef*)
Meyerozyma guilliermondii	1	EXF-6331	KJ481232 (LSU)
Rhodotorula mucilaginosa	3	EXF-6325	KJ481233 (LSU)
Rhodotorula slooffiae	1	EXF-6328	KJ481235 (LSU)
Ochroconis sp.	1	EXF-6327	KJ481237 (*act*)

EXF, strain accession number in Ex Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Infrastructural Centre Mycosmo, MRIC UL, Slovenia); *act*, partial sequence of the gene encoding for actin; *benA*, partial sequence of the gene encoding beta tubulin; ITS, internal transcribed spacer region of ribosomal DNA; LSU, large subunit of ribosomal DNA; *tef*, translation elongation factor 1 alpha partial sequence.

For the six washing machines selected for the malodour comparison, samples of waste water (50 mL) and incoming tap water (500 mL) were also collected. For the isolation of fungi, the water samples were filtered through 0.45-μm membrane filters (Merck, Millipore). The filters were then placed on dichloran rose bengal chloramphenicol agar (DRBC; Oxoid, England), and MEA (Biolife, Italy) with addition of chloramphenicol. The culture media were incubated at both 25 °C and 30 °C for up to 7 d. For determination of the bacteria in the incoming and waste water, 10 mL of each were filtered through 0.22-μm membrane filters (Merck, Millipore). These filters were placed on NA and R2A culture media and incubated at both 25 °C and 37 °C for up to 7 d. All of the pure microbial cultures obtained are deposited in the Ex Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Infrastructural Centre Mycosmo, MRIC UL, Slovenia).

DNA extraction

DNA was extracted from freshly growing yeast and yeast-like colonies on MEA, and from bacterial colonies on NA, using PrepMan Ultra reagent (Applied Biosystems) according to the manufacturer instructions. DNA from filamentous fungi was extracted after mechanical lysis of 1 cm^2 of mycelium using the protocol of Gerrits van den Ende & de Hoog (1999).

Molecular characterization and identification of strains

Filamentous fungi were first identified on the basis of morphological characters that allowed the recognition of genera. Molecular barcodes were then selected for further characterizations and for species typing. For all filamentous fungi and for genotyping *Exophiala* strains, the internal

Table 2 – Bacteria and fungi isolated from three washing machines characterized with intense malodour.

Bacteria	Frequency of isolation	Representative strain– EXB no.	Fungi	Frequency of isolation	Representative strain – EXF no.
Washing powder drawer					
Agrobacterium tumefaciens	1	EXB L-612	*Sporobolomyces ruberrimus*	1	EXF-8989
Pseudomonas putida	1	EXB L-602			
Roseomonas genomospecies	1	EXB L-618			
Sphingobium yanoikuyae	1	EXB L-1236			
Stenotrophomonas maltophila	1	EXB L-601			
Fabric softener drawer					
Bacillus horneckiae	1	EXB L-598	*Sistotrema brinkmannii*	1	EXF-8992
Microbacterium sp.	1	EXB L-599			
Micrococcus luteus	3	EXB L-610			
Micrococcus yunnanensis	2	EXB L-617			
Ochrobactrum anthropi	1	EXB L-600			
Drum interior					
Brevibacterium casei	1	EXB L-604	*Penicillium chrysogenum*	1	EXF-8995
Micrococcus luteus	3	EXB L-619			
Micrococcus yunnanensis	1	EXB L-603			
Paracoccus marcusii	1	EXB L-1232			
RUBBER DOOR SEAL					
Achromobacter xylosoxidans	1	EXB L-613	*Penicillium sanquifluum*	1	EXF-8994
Brevundimonas diminuta	1	EXB L-605			
Kocuria rhizophila	1	EXB L-622			
Micrococcus luteus	2	EXB L-623			
Pseudomonas aeruginosa	2	EXB L-1228			
Interior of water supply connector tube					
Blastomonas natatoria	1	EXB L-1238	*Phialophora europaea*	1	EXF-8990
Chryseobacterium daecheongense	1	EXB L-625			
Methyloversatilis sp.	1	EXB L-1234			
Sphingomonas koreensis	1	EXB L-606			
Sphingomonas sp.	1	EXB L-1233			
Sphingopyxis chilensis	1	EXB L-1237			
Interior of waste water connector tube					
Massilia timonae	1	EXB L-629	*Penicillium chrysogenum*	1	EXF-8996
Micrococcus luteus	3	EXB L-626			
Pseudomonas aeruginosa	1	EXB L-1230			
Pseudomonas nitroreducens	1	EXB L-607			
Water from water supply system					
Acinetobacter sp.	1	EXB L-614	*Alternaria alternata*	1	EXF-8991
Micrococcus sp.	1	EXB L-608	*Cladosporium cladosporioides*	2	EXF-8997
Sphingomonas yabuuchiae	1	EXB L-1239	*Cladosporium pseudocladosporioides*	1	EXF-8998
Sphingomonas sp.	1	EXB L-1235	*Neosartorya fischeri*	1	EXF-8993
			Penicillium chrysogenum	1	EXF-9011
			Sporobolomyces ruberrimus	1	EXF-9005
Waste water from washing machines					
Klebsiella variicola	1	EXB L-1241	*Penicillium chrysogenum*	1	EXF-9012
Pseudomonas aeruginosa	1	EXB L-1231	*Simplicillium chinense*	1	EXF-9013
Pseudomonas putida	1	EXB L-609			
Shewanella putrefaciens	1	EXB L-615			

EXB, accession number for Bacteria and EXF- for Fungi in Ex Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Infrastructural Centre Mycosmo, MRIC UL, Slovenia).

transcribed spacer regions 1 and 2 and the 5.8S rDNA was amplified and sequenced using primers ITS5 and ITS4 (White et al. 1990). For *Penicillium* strains, partial beta tubulin gene exons and introns (*benA*) were amplified and sequenced with primers Bt2a and Bt2b (Glass & Donaldson 1995); for *Cladosporium* strains the partial actin gene (*act*) with primers ACT-512F and ACT-783R (Carbone & Cohn 1999); for *Fusarium* strains the nuclear translation elongation factor 1-alpha (*tef*) with primers EF1 and EF2 (O'Donnell et al. 1998). Yeast were identified based on their large subunit ribosomal DNA (LSU) sequence (partial 28S rDNA, D1/D2 domains), which were amplified and sequenced with primers NL1 and NL4 (Boekhout & Kurtzman 1996). BigDye terminator cycle sequencing kits were used in the sequence reactions (Applied Biosystems, Foster City, CA, U.S.A.). Sequences were obtained with an ABI Prism 3700 Big Dye Sequencer (Applied Biosystems) at Microsynth AG, Switzerland. The sequences were assembled with the software FinchTV 1.4 (Geospiza, PerkinElmer, Inc.) and

Table 3 – Bacteria and fungi isolated from three washing machines not associated with malodour.

Bacteria	Frequency of isolation	Representative strain– EXB no.	Fungi	Frequency of isolation	Representative strain – EXF no.
Washing powder drawer					
Halomonas hamiltonii	2	EXB L-1347	*Fusarium oxysporum*	1	EXF-9794
Micrococcus luteus	1	EXB L-1340			
Fabric softener drawer					
Brevibacterium casei	1	EXB L-1355	*Candida parapsilosis*	2	EXF-9781
Micrococcus luteus	1	EXB L-1339	*Meyerozyma guilliermondii*	1	EXF-9786
Drum interior					
Bacillus amyloliquefaciens	1	EXB L-1349	*Cladosporium cladosporioides*	1	EXF-9795
Bacillus pumilus	1	EXB L-1350	*Cladosporium langeronii*	1	EXF-9796
Micrococcus luteus	3	EXB L-1357	*Penicillium viridicatum*	1	EXF-9797
Rubber door seal					
Acinetobacter sp.	1	EXB L-1358	*Aspergillus fumigatus*	1	EXF-9799
Bacillus sp.	1	EXB L-1343	*Penicillium bialowiezense*	1	EXF-9800
Bacillus subtilis	1	EXB L-1354	*Penicillium expansum*	1	EXF-9798
Micrococcus luteus	1	EXB L-1342	*Penicillium glabrum*	1	EXF-9792
Pseudomonas pseudoalcaligenes	1	EXB L-1353			
Interior of water supply connector tube					
Blastomonas natatoria	1	EXB L-1344	*Aureobasidium pullulans*	1	EXF-9785
Brevundimonas aurantiaca	1	EXB L-1359			
Sphingobacterium spiritivorum	1	EXB L-1360			
Interior of waste water connector tube					
Acinetobacter sp.	1	EXB L-1361	*Debaryomyces hansenii*	1	EXF-9780
Pseudoxanthomonas sp.	1	EXB L-1345	*Exophiala phaeomuriformis*	1	EXF-9788
			Ochroconis constricta	1	EXF-9793
Water from water supply system					
Micrococcus luteus	1	EXB L-1351	*Aspergillus versicolor*	1	EXF-8692
Pseudomonas pseudoalcaligenes	1	EXB L-1352	*Aureobasidium melanogenum*	1	EXF-8428
			Trichoderma citrinoviride	1	EXF-6299
Waste water from washing machines					
Pseudomonas aeruginosa	1	EXB L-1229	*Penicillium oxalicum*	1	EXF-8693

EXB, accession number for Bacteria and EXF- for Fungi in Ex Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Infrastructural Centre Mycosmo, MRIC UL, Slovenia).

automatically aligned. The alignments were manually adjusted using Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 (Tamura *et al.* 2011). Taxa were identified through BLAST searches (Altschul *et al.* 1990) by recruiting the sequence database at http://www.ncbi.nlm.nih.gov/ in June 2014.

Machine-learning analysis

For the investigation of differences between samples in terms of absence or presence of different fungal genera, machine-learning methods were used. The J48 algorithm for the induction of decision trees in the WEKA data-mining package (Witten *et al.* 2011) was used, which is a reimplementation of the well-known C4.5 algorithm (Quinlan 1993). Default parameter settings for J48 were applied, but reduced error pruning of the tree was used instead of the standard C4.5 pruning. The dependent (class) variable in our analysis was the absence or presence of fungi: 'no'if no fungi were present, and 'yes' if any fungus was present in the sample. The independent variables (attributes) were age of washing machine (years), frequency of washing machine use (times per week), use of detergents (yes/no), use of fabric softeners (yes/no), temperature programmes used for washing (one binary attribute for 30 °C, 40 °C, 60 °C and 90 °C as yes/no and two numeric attributes for minimum and maximum temperatures) and temperature of isolation of fungi (one binary attribute for 25 °C, one for 30 °C as yes/no and one for the actual temperature value).

Growth of fungal strains from washing machines on selected softener

Solid culture media containing a selected commercial fabric softener were prepared with distilled water, with the fabric softener as the only source of nutrients. The fabric softener was diluted to concentrations of 50 %, 25 %, 10 %, 5 %, and 1 % (v/v). Medium prepared only with distilled water and agar was used as a negative control. The diameters of fungi on media with different concentrations of softener were measured after 3 weeks of incubation at 25 °C and 30 °C and compared to negative controls. When the diameter on media with softener was larger than in the negative controls, the growth was assigned as positive. As a replacement for softener, consumers often use acetic acid, and thus, fungal growth was also tested on liquid yeast nitrogen base medium supplemented with 1 % (v/v) acetic acid. Fungal growth observed on MEA was used for comparisons. The media were inoculated with 10 μL fungal suspensions, prepared with saline and incubated at 25 °C and 37 °C for 2 weeks.

Production of different extracellular enzymes

For testing whether the fungal strains can break down the substrates containing fatty acids and proteins, one representative isolate of each species from the washing machines was tested for the production of extracellular proteases and esterases. Proteolytic activity was tested using agar supplemented with milk, while for testing of the esterase activity, agar with the addition of Tween 80 was used (Paterson & Bridge 1994).

Results

Different communities of fungi known also as opportunistic human pathogens inhabiting drawers for washing powder, fabric softener, and rubber door seals

No fungi were isolated in 21 % ($n = 15$) of the 70 sampled washing machines. The mean age of these washing machines was 6 y and the temperature most often used for washing within this group was 60 °C. In the 79 % of washing machines that were positive for fungi, the most often used temperature for washing was 40 °C. Drawers for fabric softeners were fungus positive in 80.6 % cases; drawers for powder in 74.4 % and rubber door seals in 52.6 %. This sampling resulted in a total of 72 fungal isolates (Table 1), of which 43 (60 %) had earlier been reported as an opportunistic human pathogen (Table 4). The most frequently isolated fungi were members of the *Fusarium oxysporum* species complex (FOSC; 19 strains) and *Candida parapsilosis* (14 strains), followed by different species of black yeasts from genus *Exophiala* (7 strains).

Most often, there were three different types of fungal communities for each single sampling site. Filamentous fungi prevailed over yeasts in drawers for washing powder. This filamentous fungal community was composed of members of the FOSC and *Fusarium solani* species complex (FSSC) that occurred together with *Cladosporium sphaerospermum*, *Exophiala phaeomuriformis* genotype 1, *Exophiala mesophila*, *Exophiala lecanii-corni*, *Meyerozyma guilliermondii*, *Candida parapsilosis*, and *Penicillium crustosum*. The most frequently isolated species were from the FOSC, followed by *Exophiala* species and *C. parapsilosis* (Figs 1 and 2). The second most common community was observed most frequently in the drawers for fabric softener, and it was represented by a predominance of *C. parapsilosis*, followed by members of the FOSC and *P. crustosum*. The third most common community was dominated by yeasts, and was mainly observed on the washing machine rubber door seals. This was represented by the red yeast *Rhodotorula mucilaginosa*, the white yeast *C. parapsilosis*, the black yeast-like *E. phaeomuriformis*, and *Ochroconis* species. In this community *R. mucilaginosa* and *C. parapsilosis* prevailed.

Increased occurrence of fungi in washing machines primarily correlates with use of fabric softener

The occurrence of fungi in drawers for washing powder and softener and on rubber door seals were statistically analysed with the machine-learning model (Fig 3). Correlations of fungal diversity and key variables such as the age and frequency of use of washing machines, the temperatures used in washing cycles, and the use of detergents were tested. The accuracy of the model when used for predicting the presence or absence of fungi was 82.5 % when evaluated on the training data and 73.2 % when evaluated with the 10-fold stratified cross-validation procedure. The 10-fold stratified cross-validation gives a more realistic estimate if the model is used for predictions of unknown samples. The decision tree suggests that the type of washing machine, its time of use, and its frequency of use are not important parameters for the presence of fungi (i.e., they do not appear in the tree). The key variables that most influenced the presence of fungi at these sampled sites were the use of fabric softener, the washing temperature, and the temperature used for the isolation of the fungi. When both detergents were used (washing powder and fabric softener; first leaf in the decision tree in Fig 3), the diversity of fungi was higher than in cases where one or both of these detergent types were not used. In four cases when neither of these detergent types was used, no fungi were isolated from the chosen sample sites. The temperature of cultivation also appeared to be important - incubation at 25 °C resulted mainly in the isolation of filamentous fungi, while at 30 °C, filamentous fungi and white and black yeasts were isolated.

Characterization of the fungal isolates for selected virulence factors

The isolated fungal strains were characterized in terms of their growth at 25 °C and 37 °C, their proteolytic and esterase activities, and their use of softener as sole source of carbon (Table 5). When examined for their thermotolerance, all fungi grew well at 25 °C on MEA because they formed solid colonies within 2 weeks incubation. As the ability to grow at 37 °C is an important factor for fungal pathogenesis in human (Anaissie et al. 2001), the isolated fungi were also tested for their ability to grow at this temperature. *Aureobasidium pullulans*, *Mucor racemosus*, *Ochroconis* spp. and the species of *Cladosporium*, *Penicillium*, and *Phoma* did not grow at 37 °C, while all other isolated taxa grew well at 37 °C (Table 5). Another important fungal virulent factor is the production of esterases and proteases (Ishida et al. 2012). All of the tested fungi showed esterase activities at 25 °C, while *A. melanogenum*, *E. phaeomuriformis*, FOSC and FSSC members, *Fusarium proliferatum*, *Fusarium verticillioides*, *M. guilliermondii* produced esterases at 37 °C. The tested strains of *A. melanogenum*, *Cladosporium bruhnei*, *C. sphaerospermum*, *F. solani*, *F. verticillioides*, *Mucor circinelloides*, *P. crustosum*, and *Penicillium brevicompactum* produced proteases at 25 °C. At 37 °C, protease activity was measured only for *A. melanogenum*, *F. solani*, *F. verticillioides*, and *M. circinelloides*.

All tested strains, listed in Table 5, except *Phoma fimeti* grew well on the medium with 1 % fabric softener (Fig 4), while *F. verticillioides* and *P. crustosum* even grew on the medium containing 5 % fabric softener. No growth was observed at higher concentrations of fabric softener. The growth of 26 of the isolated strains was tested on medium with 1 % acetic acid but none of the tested fungi developed colonies.

Table 4 – Fungal species isolated in this and other studies from different parts of washing machines, along with their natural habitats and opportunistic pathogenic potential.

Species	Washing machine site	Habitats	BSL	Human pathogenicity	Vector of transmission	References for fungi isolated from washing machine
Alternaria sp.	Plastic parts	Air, water, soil, plants, buildings	1	Allergic reactions, sinusitis, toxin production	Air, water	Gattlen et al. 2010.
Aspergillus ochraceus	Plastic parts	Air, water, soil, plants, buildings	1	Allergic reactions, sinusitis, pulmonary infections, antromycosis, toxin production	Air, water	Gattlen et al. 2010.
Aspergillus versicolor	Plastic parts	Air, water, soil, plants, buildings	1	Allergic reactions, sinusitis, pulmonary infections, toxin production	Air, water	Gattlen et al. 2010.
Aureobasidium pullulans var. *pullulans*	Drawer for washing powder	Air, water, soil, limestone, plants	1	Extrinsic allergic alveolitis, phaeohyphomycosis	Air, water	This study
Aureobasidium pullulans var. *melanogenum*	Drawer for fabric softener	Air, watery habitats, soil, plants	1	Extrinsic allergic alveolitis, phaeohyphomycosis	Air, water	This study
Aureobasidium sp.	Different parts	Air, water, soil, limestone, plants	1	Extrinsic allergic alveolitis, phaeohyphomycosis	Air, water	Hamada 2005
Candida albicans	Laundered towels	Soil, water, human skin	2	Invasive candidiasis, catheter infections, urinary tract infections, vulvovaginitis, endocarditis, peritonitis, joint infections, meningitis	Water, hands	Blaser et al. 1984.
Candida parapsilosis	Drawer for washing powder and softener, rubber	Soil, water, marine environment, plants, insects, human skin	1	Invasive candidiasis, catheter infections, urinary tract infections, vulvovaginitis, endocarditis, peritonitis, joint infections, meningitis	Water, skin, insects	This study
Candida sp.	Different parts, laundered towels, lab coats, clothes	Soil, water, marine environment, plants, insects, human skin	1	Candidiasis, catheter infections, urinary tract infections, vulvovaginitis, meningitis	Water, skin, insects	Stapleton et al. 2013, Neely & Orloff 2001
Capronia coronata	Metal parts	Water, wood, plants	1	Unknown	Water	Gattlen et al. 2010.
Cladosporium bruhnei	rubber	Air, water, bathrooms	1	Sinusitis, keratitis, skin and lung infections	Air, water	This study
Cladosporium halotolerans	Drawer for fabric softener	Air, water, bathrooms, salterns	1	Sinusitis, keratitis, skin and lung infections	Air, water	This study
Cladosporium cladosporioides	Different parts	Air, water, soil, bathrooms	1	Sinusitis, keratitis, skin and lung infections	Air, water	Hamada 2005
Cladosporium pseudocladosporioides	Drawer for fabric softener	Air, water, soil	1	Sinusitis, keratitis, skin and lung infections	Air, water	This study

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Table 4 – (continued)

Species	Washing machine site	Habitats	BSL	Human pathogenicity	Vector of transmission	References for fungi isolated from washing machine
Cladosporium sphaerospermum	Drawer for powder, rubber	Air, water, bathrooms, salterns	1	Sinusitis, keratitis, skin and lung infections	Air, water	This study, Gattlen *et al.* 2010.
Cladosporium sp.	Plastic parts, water from WM	Air, water, bathrooms	1	Sinusitis, keratitis, skin and lung infections	Air, water	Gattlen *et al.* 2010, Hamada 2002.
Cryptococcus diffluens	rubber	Air, flowers, trees, sea water	1	Subcutaneous cryptococcosis, skin, nail infections	Air, water	This study
Cryptococcus sp.	Metal parts	Air, flowers, trees, sea water	1	Subcutaneous cryptococcosis, skin, nail infections	Air, water	Gattlen *et al.* 2010.
Exophiala alcalophila	Different parts	Soil, water, bathrooms	1	Unknown	Water	Hamada 2005
Exophiala equina	Drawer for fabric softener, soap dispenser	Water, cold blooded animals	1	Subcutaneous phaeohyphomycosis	Water	This study, Isola *et al.* 2013.
Exophiala lecanii-corni	Drawer for washing powder, soap dispenser	Water, biofilms from water supply	2	Cutaneous, subcutaneous phaeohyphomycosis, systemic infections	Water	This study, Isola *et al.* 2013.
Exophiala mesophila	Drawer for washing powder, soap dispenser	Water, steam bath, bathrooms	1	Cutaneous, subcutaneous phaeohyphomycosis	Water	This study, Isola *et al.* 2013.
Exophiala phaeomuriformis genotype 1	Drawer for washing powder, rubber	Water, natural hot springs, steam bath, bathrooms, dishwashers	2	Cutaneous, subcutaneous and systemic infections	Water	This study
Fusarium oxysporum species complex	Drawer for washing powder and softener, rubber, other parts, towels	Air, water, soil, plant material, animals	2	Sinusitis, keratitis, onychomycosis, peritonitis, pneumonia, thrombophlebitis, osteomyelitis, subcutaneous infections	Air, water, plants	This study, Stapleton *et al.* 2013.
Fusarium proliferatum	Drawer for washing powder, rubber	Air, water, soil, plant material	1	Sinusitis, endophthalmitis, onychomycosis, pneumonia, subcutaneous infections	Air, water, plants, soil	This study
Fusarium solani species complex	Drawer for washing powder, different parts, towels	Air, water, soil, plant material, animals	2	Sinusitis, keratitis, onychomycosis, peritonitis, pneumonia, thrombophlebitis, osteomyelitis, subcutaneous infections	Air, water, plants, soil	This study, Stapleton *et al.* 2013.
Fusarium sp.	Washed laundry	Air, water, soil, plant material	1	Sinusitis, endophthalmitis, onychomycosis, pneumonia, subcutaneous infections	Air, water, plants, soil	Neely & Orloff 2001

Fusarium verticillioides	Drawer for washing powder	Air, water, soil, plant material	2	Sinusitis, keratitis, onychomycosis, peritonitis, pneumonia, thrombophlebitis, osteomyelitis, subcutaneous infections	Air, water, plants, soil	This study
Meyerozyma guilliermondii	Drawer for washing powder, rubber	Soil, sea water, dishwashers, skin	1	Blood stream, pulmonary, cutaneous infections	Air, water	This study
Microsporum canis	Washed laundry	Soil, animals	2	Hair, skin infections	Water, clothes, animals	Shah et al. 1988, Blaser et al. 1984.
Mucor circinelloides	Drawer for washing powder	Soil, plant material, air	1	Allergic reactions	Air, plants, soil	This study
Mucor racemosus	Drawer for washing softener	Soil, plant material, air	1	Allergic reactions	Air, plants, soil	This study
Mucor sp.	Washed laundry	Soil, plant material, air	1	Allergic reactions	Air, plants, soil	Neely & Orloff 2001
Ochroconis humicola	Soap dispenser	Water, soil, air, animals	1	Pulmonary infections	Air, water	Isola et al. 2013
Ochroconis sp.	Drawer for washing powder, rubber	Water, soil, air, animals	1	Pulmonary infections	Air, water	This study
Penicillium brevicompactum	Drawer for washing powder	Water, soil, air, plant material	1	Allergic reactions, pulmonary infections, sinusitis	Air, water, soil	This study
Penicillium crustosum	Drawer for washing powder and softener, rubber	Water, soil, air, plant material	1	Allergic reactions, pulmonary infections, sinusitis	Air, water, soil	This study
Penicillium sp.	Rubber, plastic parts of	Water, soil, air, plant material	1	Allergic reactions, pulmonary infections, sinusitis	Air, water	Gattlen et al. 2010.
Phialophora olivacea	Soap dispenser	Water, fruits, air	1	Unknown	Water, air	Isola et al. 2013.
Phoma fimeti	Drawer for washing powder	Plant material, marine environment, water, cement	1	Unknown	Air, water	This study
Phoma radicina	Drawer for washing powder	Plant material, marine environment, water, cement	1	Unknown	Air, water	This study
Rhodotorula minuta	rubber	Soil, water, sea water, air, fruits, bathrooms	1	Fungemia, endocarditis, meningitis	Air, water	Gattlen et al. 2010.
Rhodotorula mucilaginosa	rubber, plastic, metal	Soil, water, air, fruits, bathrooms, dishwashers	1	Fungemia, endocarditis, meningitis	Air, water	This study, Gattlen et al. 2010.
Rhodotorula slooffiae	Drawer for washing softener, rubber, metal parts	Soil, water, air, fruits, bathrooms	1	Fungemia, endocarditis, meningitis	Air, water	This study, Gattlen et al. 2010.

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Table 4 – (continued)

Species	Washing machine site	Habitats	BSL	Human pathogenicity	Vector of transmission	References for fungi isolated from washing machine
Rhodotorula sp.	Different parts, towels	Soil, water, air, fruits, bathrooms	1	Fungemia, endocarditis, meningitis	Air, water	Stapleton *et al.* 2013.
Scolecobasidium constrictum	Different parts	Water, soil, air, bathrooms	1	Pulmonary infections	Air, water	Hamada 2005
Trichosporon domesticus	Plastic parts	Soil, water, human skin	2	Superficial, subcutaneous, systemic infections, pneumonitis	Water	Gattlen *et al.* 2010.
Trichophyton mentagrophytes	Laundered socks	Soil, human skin, animals	2	Onychomycosis, hair, skin infections, Tinea pedis	Clothes, animals	Tanaka *et al.* 2006.

BSL, Biosafety level (de Hoog *et al.* 2009).

Fungi are not the causative agents of malodour in washing machines

The six residential washing machines tested for bacteria and fungi had been used from 1 month up to 10 y with different frequencies of use. In one of these six cases, fabric softener had never been used. In all six cases, the most frequently used temperature program ran at 40 °C.

While fungi prevailed over bacteria in the sampled tap water, very low numbers of fungal colony forming units (CFU) were recovered from washing machines with malodour, and in waste water samples (Fig 5). Although the microbiota isolated from the different parts inside the washing machines was very diverse, it was dominated by *Micrococcus luteus*, followed by different *Pseudomonas* and *Sphingomonas* species in washing machines with malodour. Only a few species from the genera *Pseudomonas*, *Klebsiella*, and *Shewanella* were isolated from the waste water. Fungi of the genera *Penicillium* and *Cladosporium* occurred rarely, if at all (Table 2). In comparison, the three washing machines that did not have intensive malodour were mainly colonized with *M. luteus*, different *Bacillus* species, and *Pseudomonas pseudoalcaligenes*, with only *P. aeruginosa* isolated from waste water. Fungi were detected more often in these machines, compared with those with malodour. Here, different *Penicillium* species were isolated, followed by the fungal genera *Aspergillus*, *Cladosporium*, and *Aureobasidium*. Also white and black yeasts from the genera *Candida*, *Meyerozyma*, *Debaryomyces*, and *Exophiala* were detected, which were not isolated from the machines with malodour (Table 3).

Discussion

Infections with opportunistic human fungi can occur in many ways (de Hoog *et al.* 2009). Presence of fungi that are known also as opportunistic human pathogens in household appliances might represent a so-far largely overlooked risk factor for fungal infections (de Hoog *et al.* 2009). Dishwashers and washing machines may have become more microbe-friendly environments than they used to be in the past because they use now lower temperatures and less aggressive detergents without bleach (Beadle & Verran 1999) promoting the selection of species that are stress-resistant and have become known as opportunistic human pathogens (Gostincar *et al.* 2011). This ecological trend is confirmed also in the present study, as 40 °C was the washing temperature of choice for most users. Gram-negative bacteria in planctonic form can survive washing temperatures up to 50 °C, while Gram-positive bacteria can survive up to 60 °C (Munk *et al.* 2001). Although there is little data on the highest temperatures that fungi can survive during the washing cycle, it has been reported that *Candida albicans* strains can be successfully recovered at lower temperatures, while 60 °C is needed for the inactivation of *Trichophyton rubrum* (Hammer *et al.* 2011). It is also known that certain filamentous fungi and yeasts can survive temperatures above 60 °C, or even near to 100 °C (Rogers *et al.* 1994; Sterflinger 1998).

The main way for fungi to enter washing machines is *via* the water supply system and/or dirty laundry. This study did

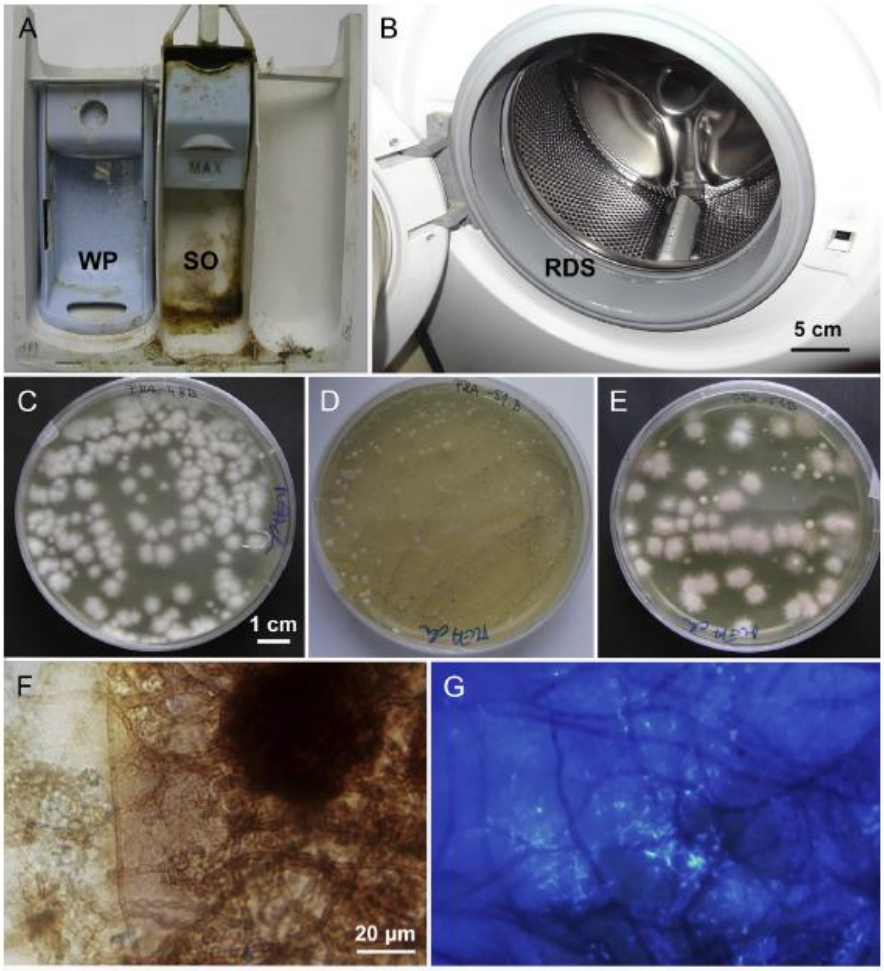

Fig 1 – Fungi in biofilm formation from selected parts of washing machines. (A) Drawers for washing powder (WP) and fabric softener (SO) covered with visible dark brown blemishes. (B) Rubber door seal (RDS). (C–E) Isolation culture media (MEA with chloramphenicol): (C) With members of the *Fusarium oxysporum* species complex. (D) With dense colonies of *Exophiala phaeomuriformis* genotype 1 among white yeast colonies of *Candida parapsilosis*. (E) With pink colonies of *Fusarium oxysporum* accompanied with colonies of *Candida parapsilosis*. (F, G) Fungal/bacterial biofilms viewed with light microscopy (F) and fluorescent microscopy (G): Autoflourescence of fungi and bacteria. Scale bar in (B) (5 cm) applies also for (A). Scale bar in (C) (1 cm) applies also for (D, E). Scale bar in (F) applies also for (G).

not focus on dermatophytes, which are transferred mainly *via* the laundry, but primarily on water-borne fungi. Fungi entering household appliances *via* the tap-water system, might present a health risk, since they are enriched within the devices such as washing machines. Thus, many investigations focused on the presence of microbes in groundwater and in domestic water systems and pipes. Spores of filamentous fungi from the genera *Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Trichoderma*, black yeasts from the genera *Aureobasidium*, *Cladophialophora*, *Exophiala*, and *Phialophora*, white yeasts from the genera *Candida*, *Meyerozyma*, *Pichia*, and *Saccharomyces*, and red yeasts from the genera *Rhodotorula* and *Sporobolomyces* have all been retrieved from tap water (Anaissie *et al.* 2001; Göttlich *et al.* 2002; Gonçalves *et al.* 2006; Hageskal *et al.* 2007, 2009; Sammon *et al.* 2010). Previous studies of fungi in different sites of washing machines revealed contamination with filamentous species from the genera *Alternaria*, *Aspergillus*, *Capronia*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Trichosporon*, and of yeasts from the genera *Candida*, *Cryptococcus*, and *Rhodotorula* (Gattlen *et al.* 2010; Stapleton *et al.* 2013). Hamada (2002) analysed rinsing and washing water from washing machines for fungal contamination, and reported the presence of *Exophiala*, *Phoma*, *Cladosporium*, *Scolecobasidium*, *Penicillium*, and *Phialophora*.

During the washing cycle, water-borne fungi entering a washing machine may become completely inactivated, retain their viability without colonizing surfaces, or become

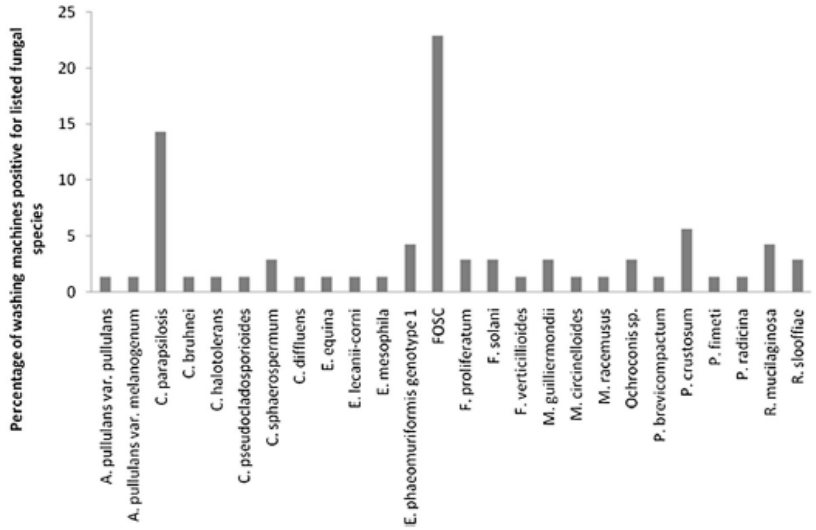

Fig 2 – Occurrence of different fungal species in washing machines. Members of *Fusarium oxysporum* species complex (22.9 %) were detected most often, followed by *Candida parapsilosis* (14.3 %), *Penicillium crustosum* (5.7 %), *Exophiala phaeomuriformis* (4.3 %) and *Rhodotorula mucilaginosa* (4.3 %).

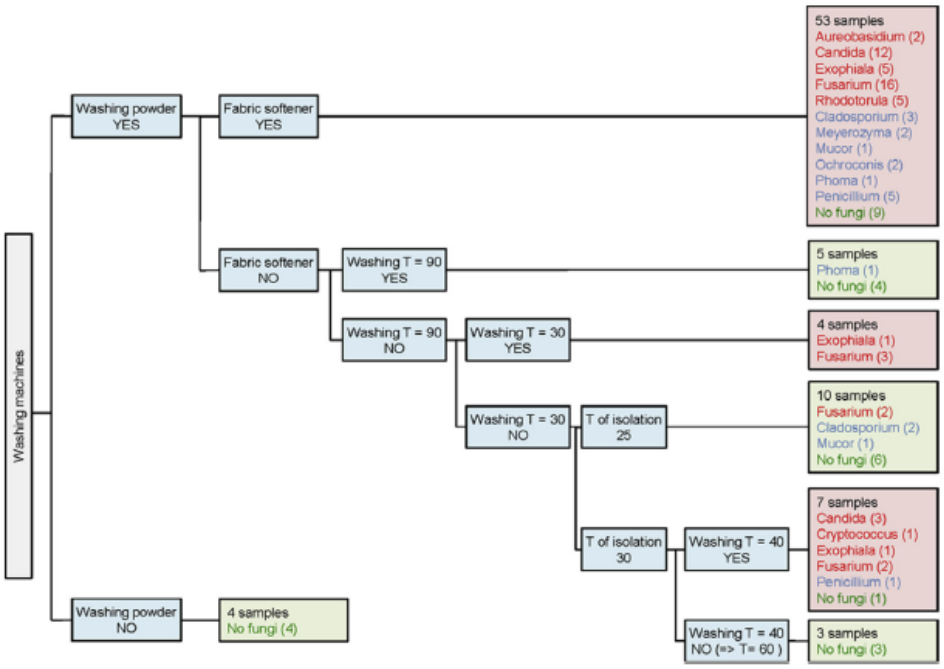

Fig 3 – Decision tree for samples from 70 washing machines not showing malodour, generated with J48 machine-learning method. Internal nodes (blue boxes) represent conditions for values (or presence) of different factors including use of washing powder and fabric softener, washing temperature, and temperature used during taxon isolation. 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 provides information of 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 with possible pathogenic potential, while blue colour indicates fungi without pathogenic potential. The red colour of the leaf means that the majority of samples contained at least one fungal genus, while the green colour of the leaf means that in the majority of samples no fungi were found.

Table 5 – Selected growth characteristics of fungi isolated from washing machines.

Fungal species	EXF- no.	Growth/activity under different temperature conditions											
		Malt extract medium		Esterase activity		Proteolytic activity		1 % Acetic acid		Water agar with 1 % fabric softener		Water agar with 5 % fabric softener	
		25 °C	30 °C	25 °C	30 °C	25 °C	30 °C	25 °C	30 °C	25 °C	30 °C	25 °C	30 °C
Aureobasidium melanogenum	**8259**	+	+	+	+	+	+	–	–	+	–	–	–
Aureobasidium pullulans	6298	+	–	+	–	+	–	–	–	+	–	–	–
Candida parapsilosis	8293	+	+	+	–	–	–	–	–	+	–	–	–
Cladosporium bruhnei	5660	+	–	+	–	+	–	–	–	+	–	–	–
Cladosporium halotolerans	5564	+	–	+	–	–	–	–	–	+	–	–	–
Cladosporium pseudocladosporioides	5563	+	–	+	–	–	–	–	–	+	–	–	–
Cladosporium sphaerospermum	8279	+	–	+	–	+	–	–	–	+	–	–	–
Cryptococcus diffluens	6329	+	+	+	–	–	–	–	–	+	+	–	–
Exophiala equina	5566	+	+	+	–	–	–	–	–	+	–	–	–
Exophiala lecanii-corni	6140	+	+	+	–	–	–	–	–	+	–	–	–
Exophiala mesophila	6138	+	+	+	–	–	–	–	–	+	–	–	–
Exophiala phaeomuriformis genotype 1	8235	+	+	+	+	–	–	–	–	+	+	–	–
Fusarium oxysporum	5661	+	+	+	+	–	–	–	–	+	+	–	–
Fusarium proliferatum	5664	+	+	+	+	–	–	–	–	+	+	–	–
Fusarium solani	**5665**	+	+	+	+	+	+	–	–	+	+	–	–
Fusarium verticillioides	**5553**	+	+	+	+	+	+	–	–	+	+	+	+
Meyerozyma guilliermondii	8240	+	+	+	+	–	–	–	–	+	+	–	–
Mucor circinelloides	**6296**	+	+	+	–	+	+	–	–	+	–	–	–
Mucor racemosus	5556	+	–	+	–	–	–	–	–	+	–	–	–
Ochroconis sp.	5565	+	–	+	–	–	–	–	–	+	–	–	–
Penicillium brevicompactum	5558	+	–	+	–	+	–	–	–	+	–	–	–
Penicillium crustosum	8272	+	–	+	–	+	–	–	–	+	–	+	–
Phoma fimeti	5551	+	–	+	–	–	–	–	–	–	–	–	–
Phoma radicina	6297	+	–	+	–	–	–	–	–	+	–	–	–
Rhodotorula mucilaginosa	6325	+	+	+	–	–	–	–	–	+	–	–	–
Rhodotorula slooffiae	5557	+	+	+	–	–	–	–	–	+	+	–	–

Isolates indicated in bold grew at 37 °C and had both proteolytic and esterase activities.

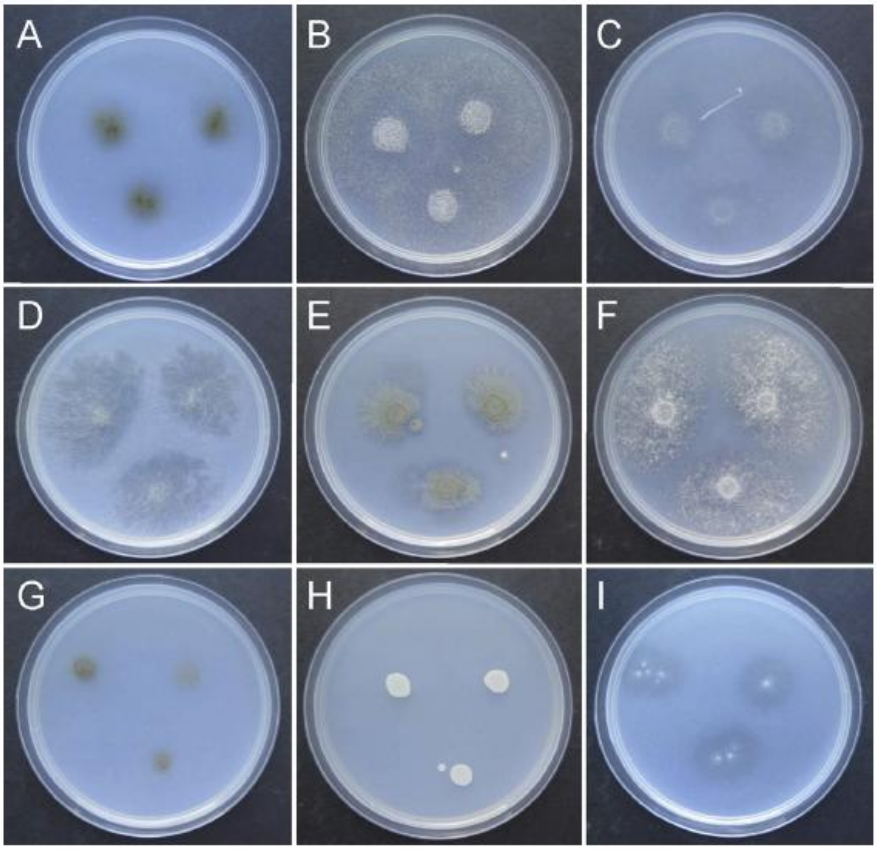

Fig 4 – Growth of pure cultured fungi after 2 weeks at 25 °C on water agar containing 1 % commercial fabric softener. (A) *Cladosporium bruhnei*. (B) *Fusarium verticillioides*. (C) *Fusarium oxysporum*. (D) *Mucor circinelloides*. (E) *Penicillium crustosum*. (F) *Fusarium solani*. (G) *Exophiala phaeomuriformis* genotype 1. (H) *Meyerozyma guilliermondii*. (I) *Aureobasidium pullulans*.

selectively enriched. Compared to their planktonic form, microorganisms in biofilms can survive higher temperatures and are more resistant to the 'cleaning' effects of detergents (Gattlen *et al.* 2010), and thus the development of biofilms is favoured inside washing machines. Biofilm formation is further influenced by the presence of the appropriate nutrients and conditions, such as moisture and the type of material (Lund & Ormerod 1995; Hageskal *et al.* 2007), production of extracellular polysaccharides, and diversity of the microorganisms present (Doggett 2000; Steenbergen *et al.* 2001; Pereira *et al.* 2002; Kinsey *et al.* 2003).

The potential build-up of biofilm formation in washing machines and on laundry can result in persistent malodour (Munk *et al.* 2001). Past studies have identified the main cause of malodour as a result of bacterial degradation of different substances including detergents and dirt on clothes (Munk *et al.* 2001), which results in the production of volatile organic compounds, and in particular dimethyl disulphide (Stapleton *et al.* 2013). The fungal species *R. mucilaginosa*, *F. oxysporum*, and *F. solani* have also been investigated for the production of volatile organic compounds; however, they were not classified as producers of such (Gattlen *et al.* 2010; Stapleton *et al.* 2013). In different studies, bacteria from the genera *Brevundimonas*, *Micrococcus*, *Moraxella*, *Ochrobactrum*, *Pseudomonas*, *Roseomonas*, *Shewanella*, *Sphingobacterium*, *Sphingomonas*, and *Stenotrophomonas* have been connected with washing machine malodour (Legnani & Leoni 1997; Labows *et al.* 1999; Gattlen *et al.* 2010; Kubota *et al.* 2012; Stapleton *et al.* 2013). Munk *et al.* (2001) observed adhesion and survival of *Staphylococcus epidermis*, *Escherichia coli*, and *P. aeruginosa* to textiles at low temperatures of washing (less than 60 °C) and with use of detergents without bleach. Almost all of the isolated bacterial species were previously reported from freshwater or from household surfaces such as shower curtains, kitchen sponges, and dish racks (Munk *et al.* 2001).

The 73 washing machines included three with persistent malodour. When the fungi were isolated from the inner parts of these machines, only six fungal isolates (*Penicillium chrysogenum*, *Penicillium sanquifluum*, *Phialophora europaea*, *Sistotrema birkmannii*, and *Sporobolomyces ruberrimus*) were retrieved and bacterial communities consisting of *Micrococcus*, *Pseudomonas*, and *Sphingomonas* clearly dominated. By means of contrast, the three washing machines without malodour accommodated 15 fungal strains from ten different genera, mainly

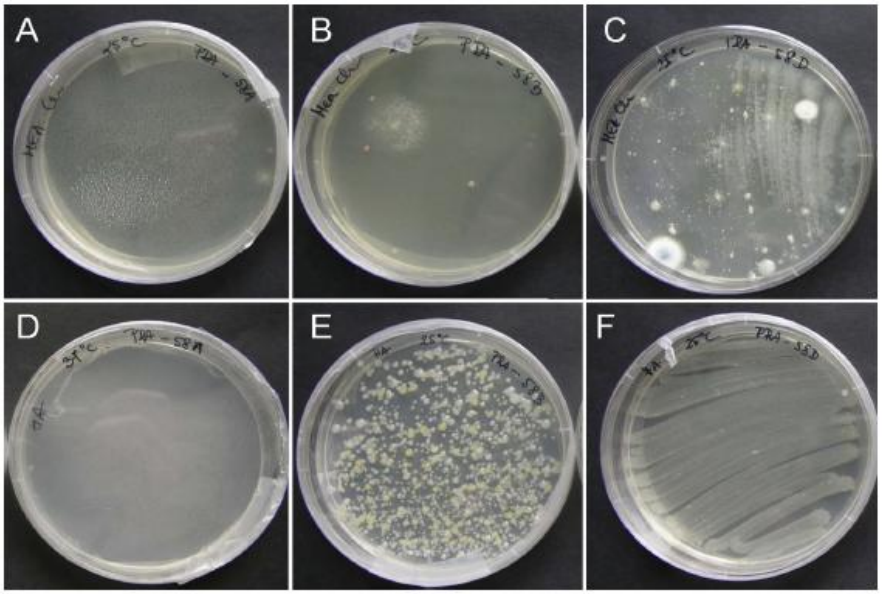

Fig 5 – Growth of fungi and bacteria from different sites of washing machines with malodour. The different samples were smeared either on MEA supplemented with chloramphenicol for the isolation of fungi (A–C), or on nutrient agar for bacteria (D–F). (A, D) Sample from washing powder drawer, showing no fungi (A) and no bacteria (D). (B, E) Sample from fabric softener drawer showing several yeast colonies (B) and numerous bacteria (E). (C, F) Sample from rubber door seal, showing several yeast (C) and numerous bacterial colonies (F).

different *Penicillium* species, followed by *Cladosporium* species and *Candida parapsilosis*. *Micrococcus* and *Bacillus* species were the most frequently isolated bacteria, while other bacterial genera were only sporadically identified. These findings indicate that the malodour is associated with the presence of bacteria, especially from the genera *Pseudomonas*, *Shewanella*, and *Sphingomonas*. The presence of fungi does not have any effects on malodour formation. *Pseudomonas* species are typical waterborne opportunistic bacteria that have been previously reported from washing machines (Legnani & Laoni 1997), and in particular as biofilms on elastomeric and polyethylene surfaces, and less so on metal surfaces (Moritz *et al.* 2010). These are known producers of dimethyl polysulphides and ammonium, which can cause a 'swampy' odour. In our study, *P. aeruginosa* and *P. putida* prevailed, and both of these bacteria originate from water and soil. *Pseudomonas aeruginosa* is known as a human pathogen that can cause wound infections (Kelly *et al.* 2004; Feazel *et al.* 2009; Gattlen *et al.* 2010), while *P. putida* can break down aliphatic and aromatic hydrocarbons and organic toxins of the herbicide atrazine, and it is not classified as a human pathogen (Palleroni 1992). Bacteria from genus *Sphingomonas*, which mainly originate from water, can cause wound and respiratory infections in immunocompromised people, and the corrosion of metals (White *et al.* 1996). *Micrococcus luteus*, which was the most frequently detected bacteria in the present study, are commensals and are only rarely pathogenic. They can be isolated from water, dust, and human skin. On the surface of skin, they break down fatty acids to produce volatile organic compounds that result in malodour (James *et al.* 2004).

Microbial degradation of detergents might be a reason for malodour and biofilm persistence. Detergents are mixtures of different chemical components that include aromatic hydrocarbons (polyvinylpyrollidone), alcohols (terpineol, sorbytol), surfactants (anionic, non-ionic, cationic, zwitterionic), fragrances (citral, lymonene), enzymes (amylase, protease, lipase), and bleaches (sodium percarbonate) (ZPS 2009; Isola *et al.* 2013). Not only bacteria, but also fungi have been described as having the ability to degrade washing detergents. Hamada & Abe (2009) tested the growth of different bathroom-colonizing fungi on media containing different components of detergents like fatty acids and anionic and non-ionic surfactants. Most of these fungi grew on fatty acids and anionic surfactants, while the growth on non-ionic surfactants varied from species to species. Detergents containing bleach successfully prevent both bacterial and fungal growth (Beadle & Verran 1999; Hamada & Abe 2009).

The machine-learning analysis used in our study indicates that in washing machines where both washing powder and fabric softener are used, the diversity of fungi is significantly higher than in washing machines where only one or none of these are used. The use of fabric softener presents a key parameter that influences fungal colonization of washing machines. To the best of our knowledge, there have been no reports on the growth of fungi on commercial fabric softeners. In contrast to washing powder, softeners do not include bleach. When we tested the growth of 26 of the most representative fungal isolates from the washing machines on media that contained a commercial fabric softener, all of the tested fungi, except *P. fimeti*, assimilate the softener at least to

a concentration of 1 %. Acetic acid at 1 %, which is in some cases used as an alternative for commercial fabric softeners, completely inhibited the growth of these same fungal strains.

Amongst these fungi isolated from washing machines, filamentous fungi prevailed over yeast, in contrast to the mycoflora detected in dishwashers (Zalar et al. 2011). Surprisingly, around 30 % of the washing machines in the present study were colonized with species from the genus *Fusarium*: FOSC, FSSC, *F. proliferatum*, and *F. verticillioides*. The FOSC and FSSC fungi are causative agents of approximately 80 % of human fungal infections (O'Donnell et al. 2010; Sutton & Brandt 2011; Garnica & Nucci 2013). Members of FOSC and FSSC are also known for their ability to form biofilms on surfaces of contact lenses and polyvinyl chloride pipes (Short et al. 2011), and thus these are often involved in eye (mycotic keratitis) or catheter-related (Wey & Colombo 1997; Mukherjee et al. 2012) infections. In nature, representatives of FOSC and FSSC have been isolated from plants, plant materials, soil, air, and water, and have primarily been seen as plant pathogens and soil inhabitants (O'Donnell et al. 2004; Zhang et al. 2006; Smith 2007).

Cladosporium pseudocladosporioides and *C. sphaerospermum* at least occasionally colonize washing machines. Both are examples of stress-resistant cosmopolitan fungi disseminated through air and colonizing water and bathrooms and habitats with lowered water activities such as salterns (de Hoog et al. 2000; Zalar et al. 2007; Pereira et al. 2010). They were also isolated from the water distribution system of hospitals (Hayette et al. 2010). Representatives of the cosmopolitan genus *Penicillium* were also isolated. *Penicillium crustosum* and *P. chrysogenum*, which dominated among the fungi in the washing machines with malodour, are not recognized as opportunistic human pathogens (de Hoog et al. 2009); instead, they are primarily known as food spoilage organisms.

Species of the genus *Exophiala* were isolated in 8.5 % of cases. Different species of the genus *Exophiala* are oligotrophic and can be commonly found on rocks and in water (Sterflinger 1998), and also in water-related human-made environments, such as bathrooms, water pipes for taps, and saunas (Matos et al. 2002; Biedunkiewicz & Schulz 2012). The majority of *Exophiala* species are classified as opportunistic pathogens that can cause cutaneous and subcutaneous infections, and lung infections (de Hoog et al. 2009) and known from biofilms (Hamada & Abe 2009; Isola et al. 2013; Heinrichs et al. 2013). The present study resulted in the isolation of *E. phaeomuriformis*, *E. mesophila*, *E. equine*, and *E. lecanii-corni*, which have all been reported as human pathogens. All of these species are able to cause infections in humans (Woo et al. 2013; Najafzadeh et al. 2013). They decompose aromatic hydrocarbons (Isola et al. 2013), assimilate different detergents (Hamada &Abe 2009) and survive high temperature and high pH (Zalar et al. 2011).

Candida parapsilosis has been reported as an emerging pathogen (van Asbeck et al. 2009; Miceli et al. 2011). It was the second-most frequently detected species (14.3 %) in washing machines investigated here and in dishwashers (Zalar et al. 2011). *Candida parapsilosis* is a ubiquitous microorganism that can be isolated from soil, water, and plants (Deresinski et al. 1995) and occurs on catheters and other prosthetic materials (Levin et al. 1998). It is a causative agent of opportunistic fungemia in immunocompromised patients (Barone & Branchini 1998; de Hoog et al. 2009). *Rhodotorula mucilaginosa* prevailed amongst the red-pigmented yeasts in dishwashers (Zalar et al. 2011) and in the present study of washing machines. Members of the genus *Rhodotorula* are known to form biofilms (Gattlen et al. 2010) and have been involved in catheter-related infections (Neofytos et al. 2007) and fungemia in cancer and AIDS patients (Pfaller et al. 2007). It is capable of behaving in a vigorous and highly competitive manner and therefore dominates various habitats (Cray et al. 2013). These red yeasts are very common in the environment and have been isolated from air, soil, food, and saline water (Wirth & Goldani 2012).

We were able to show that the majority of the analysed washing machines were colonized with various fungal species of which several are known also as opportunistic human pathogens. Fungi and bacteria commonly occur in water and water supply systems as single propagules, however, typically in low numbers. Within washing machines, they can become established as colonies and in biofilms that may release cells or conidia during washing cycles. Accordingly, washing machines may present a reservoir for these fungi from where they are further disseminated to clothes and wastewater. It appears that cloth washing at temperatures below 60 °C, mild detergents and commonly used fabric softeners can lead to an increased presence of microbial diversity in washing machines. The processes during washing may allow the selective enrichment of thermotolerant species and are not capable of eliminating non-thermotolerant species. Washing regimes recruiting reduced amounts of water, lowered water temperatures and biodegradable detergents may increase the diversity and quantity of microbes in households and could present a health risk specifically for immunocompromised people. The regular cleaning of washing powder drawers with bleach or bleach containing cleaners helps to restrict or remove microbial biofilms. Performances of such cleaning procedures are recommended by washing machine manufacturers.

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2.1.4 Kvasovke in kvasovkam podobne glive v pitni vodi in podtalnici ter njihov prenos v gospodinjske stroje

Naslov v originalnem jeziku: Yeasts and yeast-like fungi in tap water and groundwater, and their transmission to household appliances

Avtorji: Monika Novak Babič, Polona Zalar, Bernard Ženko, Sašo Džeroski, Nina Gunde-Cimerman

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POVZETEK

V raziskavi smo opisali pojav gliv v 100 vzorcih pitne vode ter 16 vzorcih podtalnic iz Slovenije. Pri tem smo uporabili tako gojitvene tehnike kot tudi molekularno-genetski pristop. Z gojitvenimi metodami smo iz vzorcev vode osamili 28 glivnih vrst iz 16 različnih rodov. Osredotočili smo se na kvasovke in kvasovkam podobne oportuno patogene glive. Med njimi posebno skrb vzbuja odkritje gliv *Aureobasidium melanogenum*, *Exophiala dermatitidis*, *Rhinocladiella similis*, *Candida parapsilosis* in *Rhodotorula mucilaginosa*. DGGE analiza, narejena na podlagi ITS1 regije rDNA iz vzorcev celokupnih DNA je razkrila med 6 do 16 hipotetično različnih glivnih enot, medtem ko smo z metodo pirosekvenciranja potrdili prisotnost rodov *Aspergillus* in *Exophiala*. S statistično metodo strojnega učenja smo ugotovili, da na prisotnost gliv v vodi vplivajo kalcijevi in magnezijevi ioni ter prisotnost nitrata. Vrste *Exophiala* spp., *C. parapsilosis* in *R. mucilaginosa* osamljene iz vode, so znane kot naseljevalke gospodinjskih strojev. Predvidevamo, da se preko vode prenesejo v pomivalne in pralne stroje, kjer se namnožijo.

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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

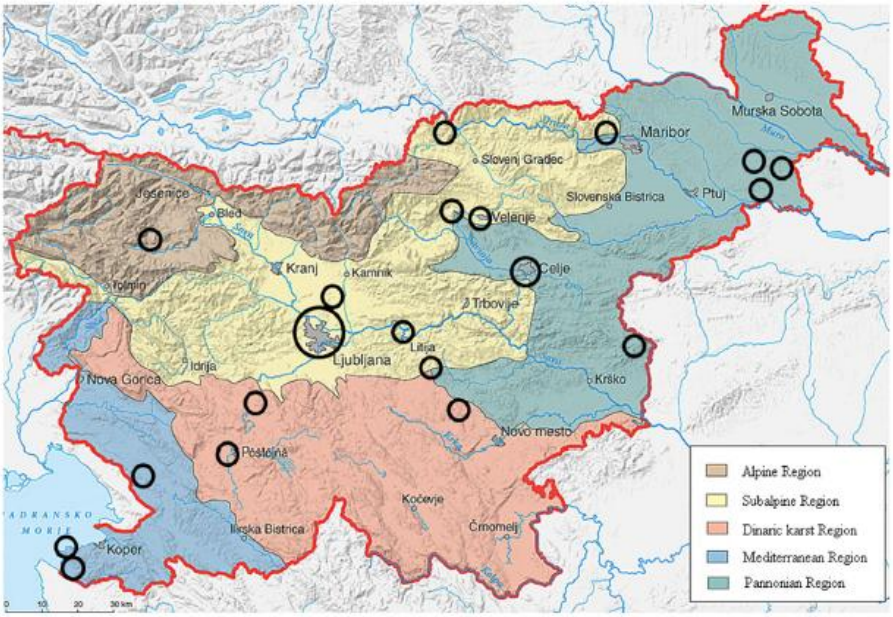

Fig. 1. Map of geographic regions of Slovenia (source: Anton Melik Geographical Institute, Ljubljana, Slovenia) with indicated sampling sites. Samples of tap water were collected from 100 private homes in different locations (indicated with black circles) positioned in 5 geographic regions of Slovenia. 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).

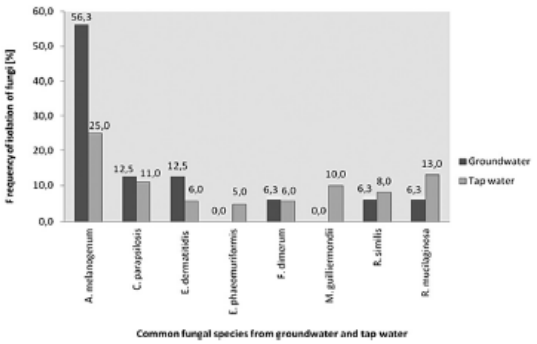

Fig. 2. Comparison of the occurrence of fungal species in ground- and tap water samples from Slovenia, with the focus on taxa detected in dishwashers (Zalar et al., 2011) and/or washing machines (Novak Babič et al., 2015). *Aureobasidium melanogenum* was predominant in both water types. The frequency 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.

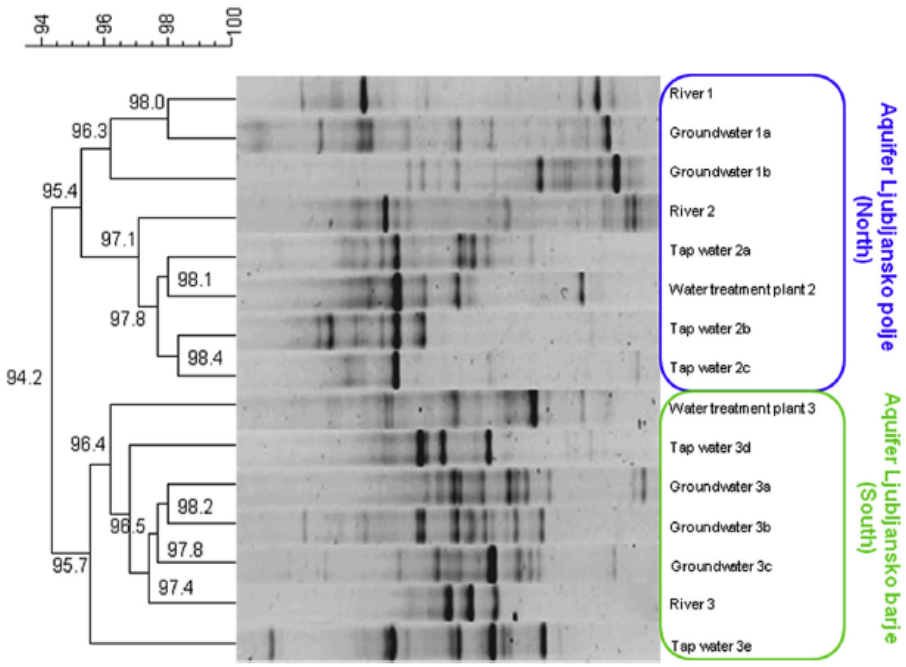

Fig. 3. DGGE profiles of fungal communities from different water samples. Rivers, groundwater, tap water and waste water samples from 2 different aquifers of Ljubljana valley were compared. The blue box shows cluster of samples from north aquifer Ljubljana field (Slovenian name "Ljubljansko polje"), 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,*

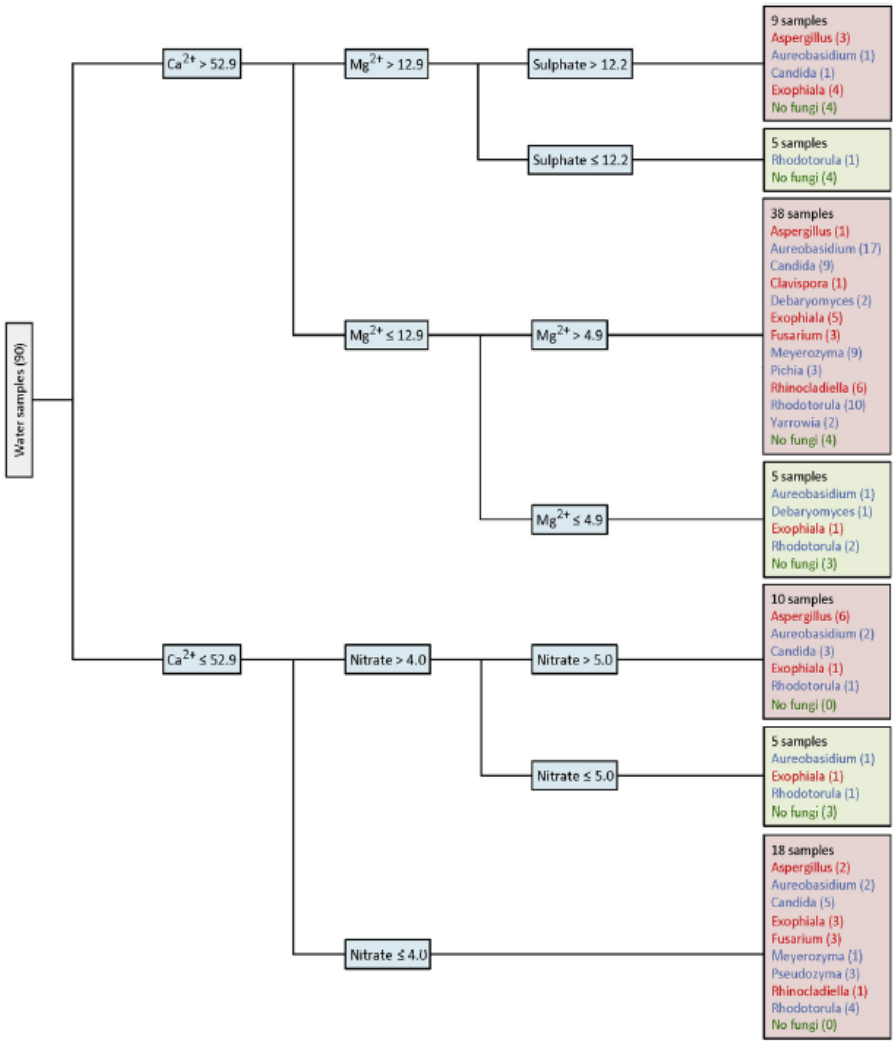

Fig. 4. Decision tree for the 90 water samples generated with the Predictive Clustering Trees machine learning method. All studied fungal genera are considered. Internal nodes (grey boxes) represent concentrations of different ions. Tree leaves (red and green boxes) contain the samples that satisfy all 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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3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

Zaradi naraščanja števila prebivalstva, daljšanja življenjske dobe v razvitem svetu in naraščanja števila ljudi s kroničnimi ali avtoimunskimi boleznimi postajajo oportuno patogene glive globalno prepoznan zdravstven problem, s katerim se spopada nekaj več kot milijarda ljudi po svetu (Vos in sod., 2012). Ker so glive razširjene globalno, so prisotne tudi znotraj človeških bivališč, kjer na njihov pojav in pogostost vplivajo temperatura, vlaga, število oseb v prostoru ter prisotnost rastlin in živali (de Hoog in sod., 2014). Najprimernejši pogoji za pojav plesni v prostorih so pri temperaturah višjih od 22 °C in pri relativni zračni vlažnosti nad 60 % (Elektro Energija, 2015). Pojav plesni v prostorih neugodno vpliva na imunski sistem, pojav alergij, okužb dihalnih poti, v najhujšem primeru pa na razvoj mikoz (de Hoog in sod., 2014). V različnih študijah so glive osamili iz vlažnih prostorov, savn, kopalnic in kuhinj. Le-te so najpogosteje pripadale rodovom *Acremonium, Alternaria, Aspergillus, Aureobasidium, Candida, Cladophialophora, Cladosporium, Cyphellophora, Exophiala, Fusarium, Meyerozyma, Mucor, Ochoconis, Paecilomyces, Penicillium, Phialophora, Phoma, Ramichloridium, Rhizopus, Rhodotorula* in *Scolecobasidium* (Hamada in Fujita, 2000; Matos in sod., 2002; Adams in sod., 2013). Izum gospodinjskih strojev je moderni družbi omogočil skrajšanje časa, ki ga porabimo za opravljanje gospodinjskih del. Če so bili pralni in pomivalni stroji v preteklosti redkost v gospodinjstvih, je imelo v svoji posesti po podatkih Statističnega urada Republike Slovenije v letu 2012 97,6 % gospodinjstev pralni ter 52,8 % pomivalni stroj (Statistični urad Republike Slovenije, 2012). Ekološke smernice v modernem času stremijo k uporabi t.i. varčnih strojev, ki med ciklom pranja dosegajo nizke maksimalne temperature, pri tem pa porabijo čim manj električne energije in vode. Da bi kljub temu vzdrževali kakovostno pranje posode / perila kot pralna sredstva uporabljamo detergente z različnimi dodatki, ki izboljšajo učinek pranja (npr. encimi, belila) (Isola in sod., 2013). Prav tako s standardi, kot je npr. NSF / ANSI Standard 184 zagotavljajo, da gospodinjski stroji dobro očistijo posodo ali perilo. Proizvodi certificirani po tem standardu naj bi po uporabi programa pranja zmanjšali prisotnost bakterij za 99,9 % (NSF International, 2008). Pralni in pomivalni stroji so po splošnem prepričanju ljudi dolgo časa veljali za prostor, ki ni primeren za naselitev mikroorganizmov. Pojav gliv v gospodinjskih aparatih, njihova vloga ter morebitni vplivi na zdravje ljudi so slabo raziskani. Prisotnost gliv v povezavi s

pralnimi stroji se v literaturi omenja predvsem zaradi neprijetnega, zatohlega vonja prostorov kjer so stroji nameščeni ter zaradi možnega prenosa nekaterih glivnih vrst med okuženo osebo ter ostalimi lastniki perila, ki se istočasno pere v stroju (Shah in sod., 1988; Munk in sod., 2001). Pomivalni stroj, kjer so bile oprane stekleničke za mleko so omenjali kot možen vir okužbe novorojenčkov z vrsto *Candida glabrata* (Nedret Koc in sod., 2002).

3.1.1 Pomivalni stroj kot življenjski prostor gliv

V predhodnih raziskavah higiene v gospodinjstvih so se v glavnem osredotočali na pojav enterobakterij na kuhinjskih površinah ter v pomivalnih strojih. Ugotovili so, da ima višja temperatura pranja v pomivalnem stroju večji vpliv na odstranjevanje mikroorganizmov kot izbira detergentov (Johanson in sod., 2004). Prav tako so temperature pranja znotraj pomivalnih strojev višje kot pri pranju na roke, zaradi česar naj bi z uporabo pomivalnih strojev odstranili več bakterij s posode kot pri klasičnem pranju. Pri pranju posode na roke se namreč kot pripomoček uporabljajo gobice za pomivanje, ki vsebujejo ~ $8{,}5*10^7$ CFU/ml mikroorganizmov, med njimi tudi glive rodu *Aspergillus*, po pranju pa jih je težko temeljito očistiti (Hilton in Austin, 2000; Alwakeel, 2007). V globalni raziskavi gliv v pomivalnih strojih smo za mesto vzorčenja izbrali tesnilo iz gume, ki omogoča tesnenje vrat stroja med ciklom pranja. Tesnilo je zaradi svoje lokacije v stroju v primerjavi z notranjostjo stroja izpostavljeno nižjim temperaturam ter manjšemu pretoku vode. Poleg tega ima hrapavo površino, zaradi česar se na njem lažje zaustavijo delci hranil in mikroorganizmi, kar omogoči tvorbo biofilma. Izmed 189 vzorčenih pomivalnih strojev smo prisotnost gliv potrdili pri 62 % strojev. Iz velike večine strojev smo osamili vrste iz rodov *Aspergillus, Candida, Magnusiomyces, Fusarium, Penicillium, Pichia* in *Rhodotorula* ter najpogosteje vrsti črnih kvasovk *Exophiala dermatitidis* in *E. phaeomuriformis*. Le-te smo potrdili pri 55 % na glive pozitivnih strojih, sopojav obeh vrst v stroju pa je bil zabeležen samo v enem primeru. V poznejši študiji glivne raznolikosti v pomivalnih strojih, kjer smo le-to preučevali v sodelovanju s sodelavci iz Turčije na vzorcu 937 pomivalnih strojev, se je izkazalo, da je glivna raznolikost v strojih ne glede na lokacijo presenetljivo podobna. Na glive pozitivnih strojev je bilo 2,5-krat manj kot v prvi študiji (24,5 %), kljub temu pa smo v primeru pozitivnih strojev v kar 43,8 % detektirali *E. dermatitidis*. Sicer so glivno bioto, tako kot v globalni študiji, poleg črnih kvasovk predstavljale še *Candida parapsilosis*, *Meyerozyma* (prej rod *Pichia*) *guilliermondii*,

Rhodotorula mucilaginosa ter v okolju redko najdena dimorfna gliva *Magnusiomyces capitatus*. Prisotnost vseh omenjenih gliv je bila potrjena tudi v študiji, kjer so podrobno preučili glivno združbo na različnih mestih znotraj pomivalnih strojev kot tudi njihov prenos iz strojev na kuhinjske površine. Pri tem ugotavljajo, da črne kvasovke na tesnilu pomivalnih strojev dosegajo do 10^6 CFU/cm^2, ostale glive pa se pojavljajo v številu od 10^2 do 10^5 CFU/cm^2 ter, da so kuhinje s pomivalnimi stroji pogosteje kolonizirane s črnimi kvasovkami kot tiste brez strojev (Zupančič in sod., 2016).

Pri obeh vrstah črnih kvasovk smo v obeh izvedenih študijah določili tudi genotipe (Matos in sod., 2002). Pri vrsti *E. dermatitidis* je v pomivalnih strojih v obeh študijah prevladoval genotip A, ki je v primerjavi z genotipom B tudi pogosteje najden v kliničnem materialu (de Hoog in sod., 2005). Rezultati eksperimenta MSP-PCR prstnega odtisa kažejo na možnost rekombinacije v stresnih pogojih med genotipoma A in B vrste *E. dermatitidis.* Teleomorfna oblika pri rodu *Exophiala* sicer ni opisana kljub temu, da so v genomu te glive potrdili obstoj genov za parjenje in mejozo (Chen in sod., 2014). Pri vrsti *E. phaeomuriformis* smo prvič opisali pojav dveh genotipov, poimenovanih 1 in 2. Večino izolatov te vrste je v globalni študiji pripadalo genotipu 1, medtem ko sta bila v primeru turške študije oba genotipa zastopana enakovredno.

Zaradi pogostosti *E. dermatitidis* in *E. phaeomuriformis* v strojih smo predvidevali, da so sevi teh vrst prilagojeni na življenjske pogoje znotraj strojev. Na izbranih posameznih sevih smo zato v obeh študijah testirali rast pri različnih temperaturah, pH in pri različnih koncentracijah soli (NaCl). Večina sevov je bila sposobni rasti pri temperaturi do 47 °C; da sta obe vrsti sposobni rasti pri visokih temperaturah so sicer opazili že Matos in sodelavci (2002), ki so prisotnost *E. dermatitidis* potrdili v vročih savnah, *E. phaeomuriformis* pa v nekoliko hladnejšem okolju kopalnic. Zmožnost preživetja pri visokih temperaturah pri vrsti *E. dermatitidis* je verjetno pogojena s tvorbo melaniziranih meristematskih skupkov in kapsule, ki celice ščiti pred izsušitvijo (Yurlova in de Hoog, 2002, Zupančič in sod., 2016). Takšen ekotip črnih kvasovk kot del biofilma na tesnilu pomivalnih strojev smo opazili tako pod svetlobnim kot tudi vrstičnim mikroskopom in je najverjetneje posledica kombinacije stresnih dejavnikov, ki so jim celice izpostavljene tekom ciklov pranja v pomivalnem stroju. Obe vrsti sta rasli tako pri kislem kot bazičnem pH vse do pH 12,5.

Pojav sicer šibke rasti pri tako visokem začetnem pH gojišča je najverjetneje posledica naknadnega znižanja le-tega zaradi metabolne aktivnosti preživelih glivnih celic v gojišču. Pri opazovanju mikromorfoloških sprememb smo ugotovili, da sevi *E. dermatitidis* v gojiščih z nizkim pH rastejo v obliki kvasnih celic in muriformnih skupkov, od pH 5,0 ter v bazičnem območju pa tvorijo psevdohife. *E. phaeomuriformis* je za razliko od *E. dermatitidis* ne glede na pH gojišča rastla v psevdohifni obliki. Pri testiranju rasti na slanih gojiščih smo ugotovili, da obe glivi dobro rasteta do 10 % soli, pri 17 % NaCl pa je bila njuna rast šibkejša, še zlasti pri *E. phaeomuriformis*. Prilagoditev na slanost je sicer dobro poznana pri mnogih psihrotolerantnih vrstah iz rodu *Exophiala* (de Hoog in sod., 2011), pri vrsti *E. dermatitidis* pa bi lahko predstavljala pomemben virulentni dejavnik pri bolnikih s cistično fibrozo (Haase in sod., 1991). Glede na rast pri teh pogojih smo obe glivni vrsti uvrstili med poliekstremotolerantne mikroorganizme, ki so sposobni rasti pri kombinaciji več različnih ekstremnih pogojev kot so visoke koncentracije soli (med 3,7 in 4,3 M Na^+), bazičen pH (nad 9,5) in visoke temperature (med 46 in 66 °C) (Bowers in sod., 2009).

Najpogostejši naseljevalki pomivalnih strojev *E. dermatitidis* in *E. phaeomuriformis* spadata v rod *Exophiala* (Ascomycota, Chaetothyriales), ki združuje dimorfne črne kvasovke. Za 17 od 38 opisanih vrst znotraj tega rodu različne študije potrjujejo, da so oportuno patogene za živali ali ljudi (Anaissie in sod., 2009). Povzročajo kromoblastomikozo, okužbe centralnega živčnega sistema ter okužbe dihal pri imunsko oslabljenih ljudeh. Slednje so pogoste predvsem pri bolnikih s cistično fibrozo (Chang in sod., 2000; de Hoog in sod., 2014; Taj-Aldeen in sod., 2006; Kondori in sod., 2011). V naravnem okolju sta bili vrsti občasno najdeni v tropskih gozdovih, na površini sadja ter v iztrebkih ptic in netopirjev (Sudhadham in sod., 2008). Pogosteje so ju osamili iz antropogenih okolij, kot so savne in kopalnice ter iz okolij, onesnaženih z ogljikovodiki (bencinske črpalke, železniški tiri) (Matos in sod., 2002; Gümral in sod., 2014). Dokazano so sposobne asimilacije toksičnih monoaromatskih spojin kot edinega vira ogljika, kar bi lahko bil eden od virulentnih dejavnikov teh gliv. Alkilbenzeni, ki jih te glive razgrajujejo v naravi, so namreč strukturno podobni nevrotransmiterjem v živčnem sistemu (de Hoog in sod., 2014; Matos in sod., 2002; Prenafeta-Boldú in sod., 2006).

Nepričakovan rezultat je bila osamitev gliv *Magnusiomyces capitatus* iz pomivalnih strojev. Iz novejših taksonomskih študij je sicer razvidno, da je za pravilno taksonomsko uvrstitev teh gliv potrebna ionizacija v matriksu z desorpcijo z laserjem in masnim analizatorjem časa preleta ionov (MALDI-TOF) in ne le zaporedje ITS regije v genomu (Desnos-Ollivier in sod., 2014). Z naknadno identifikacijo smo ugotovili, da večina pridobljenih izolatov iz pomivalnih strojev spada v vrsto *Saprochaeta clavata* (deblo Ascomycota). O ekologiji in naravnem rezervoarju vrst iz rodov *Magnusiomyces* / *Saprochaeta* je zelo malo znanega (de Hoog in Smith, 2011). Za vrsto *S. clavata* so malo poročali o patogenosti za ljudi, čeprav je taksonomsko ozko povezana s patogeno *M. capitatus*. O glivi *M. capitatus* so poročali kot o povzročiteljici invazivnih okužb, zlasti pri pacientih s hematološkimi obolenji (Pimentel in sod., 2005; Garcia-Ruiz in sod., 2013). Okužbe imunsko oslabljenih ljudi s to glivo so povezali s kontaminiranimi mlečnimi produkti (Ersoz in sod., 2004). Izgleda, da sta ti vrsti, sicer redko najdeni v naravi, v pomivalnih strojih našli novo ekološko nišo.

3.1.2 Glive kolonizirajo uporabnikom dostopna mesta v pralnih strojih

Glive v pralnih strojih so predhodne študije omenjale predvsem v kontekstu prenosa perilo-uporabnik in verižne kontaminacije različnega perila s potencialnimi patogeni ljudi in živali. Pri tem je bilo največ pozornosti namenjene vrstam iz rodu *Candida, Microsporum* in *Trichophyton*, ki so preživele na opranem perilu (Shah in sod., 1988; Tanaka in sod., 2006). Le malo pozornosti se je namenjalo ostalim nišam znotraj pralnih strojev, kjer je preživetje pogojeno s prilagojenostjo na visoke koncentracije detergentov in belil. V raziskavi pojava gliv v pralnih strojih smo želeli ugotoviti, katere glive naseljujejo predalčke za pralni prašek in mehčalec ter tesnilo vrat strojev. Pri 79 % vzorčenih strojev smo potrdili prisotnost gliv, kar smo v nadaljevanju povezali tudi z najpogosteje uporabljeno temperaturo pranja. V strojih, pozitivnih na glive, je bila le-ta 40 °C, medtem ko so uporabniki strojev, kjer gliv nismo osamili, uporabljali programe pranja s temperaturami med 60 in 95 °C. Podobno kot pri pomivalnih strojih, smo tudi pri pralnih strojih opazili konzistentnost glivne biote le, da so jo predstavljale druge vrste. V primerjavi s pomivalnimi stroji smo iz pralnih strojev izolirali veliko več filamentoznih glivnih vrst. Največ pralnih strojev (23 %) je bilo kontaminiranih z glivami iz *Fusarium oxysporum* kompleksa vrst, na drugem mestu je bila kvasovka *Candida parapsilosis* (14

%), ki spada med porajajoče se patogene (Miceli in sod., 2011). Pogoste so bile tudi filamentozne glive iz rodov *Penicillium, Cladosporium* ter ubikvitarna kvasovka *Rhodotorula mucilaginosa*. Čeprav smo iz 9 % strojev osamili glive iz rodu *Exophiala* pa presenetljivo iz nobenega od vzorčenih pralnih strojev nismo osamili vrste *E. dermatitidis*, ki je bila najpogosteje prisotna v pomivalnih strojih. *E. phaeomuriformis* smo potrdili le v 4 % vseh vzorčenih strojev. Dobljeni rezultati se ujemajo z rezultati predhodnih raziskav, kjer so glive iz pralnih strojev osamili iz bobnov, različnih plastičnih delov in tesnila. Najpogosteje so poročali o pojavu kvasovk iz rodov *Candida* in *Cryptococcus* ter vrst *Rhodotorula minuta, R. mucilaginosa* in *R. slooffiae* (Gattlen in sod., 2010; Stapleton in sod., 2013). Plastične dele so naseljevale vrste *Alternaria* sp., *Aspergillus ochraceus, A. versicolor, Cladosporium cladosporioides, C. sphaerospermum, Fusarium oxysporum* in *F. solani* (Hamada, 2002; Gattlen in sod., 2010; Stapleton in sod., 2013). Vrste *Aureobasidium* sp., *Capronia coronata, Exophiala alcalophila, E. equina, E. lecanii-corni, E. mesophila, Ochroconis constricta, O. humicola* in *Phialophora olivacea* pa so osamili iz temnih biofilmov v predalčkih za detergente (Gattlen in sod., 2010; Isola in sod., 2013).

Razlog za razlike med glivno bioto pomivalnih in pralnih strojev je najverjetneje temperatura pranja, zaradi katere v strojih pride do selekcije vrst. Ugotovili smo, da velika večina uporabnikov pomivalnih strojev pri ciklu pranja izbere višje temperature (~ 60 °C), medtem ko pri pranju perila večina uporablja programe pranja z nižjimi temperaturami (~ 40 °C). To smo v nadaljevanju potrdili tudi s testom rasti izbranih gliv pri temperaturah 25 in 37 °C, kjer so vse testirane glive bile sposobne rasti pri 25 °C, medtem ko pri 37 °C za večinoma filamentozne vrste *Aureobasidium pullulans*, *Mucor racemosus*, *Ochroconis* sp. ter vrste iz rodov *Cladosporium, Penicillium* in *Phoma* nismo zabeležili rasti. Za določitev ostalih faktorjev, ki vplivajo na pojav gliv v pralnih strojih smo uporabili statistično analizo strojnega učenja in ugotovili, da ima na pojav gliv v pralnih strojih najvišji vpliv uporaba detergentov, še posebej mehčalca. Razen vrste *Phoma fimeti*, so ostale rasle na gojišču z 1 % mehčalcem, vrsti *Fusarium verticillioides* in *Penicillium crustosum* pa tudi na gojišču s 5 % koncentracijo mehčalca. Ker smo med raziskavo ugotovili, da veliko ljudi namesto mehčalca pri pranju uporablja alkoholni kis, smo izbrane seve izpostavili 1 % ocetni kislini; v tem primeru pri nobenem od sevov nismo potrdili rasti. Iz literature je razvidno, da lahko nekatere vrste gliv iz rodov *Cladosporium, Exophiala* in *Scedosporium*

rastejo na gojiščih, ki so jim dodane le maščobne kisline ter anionski in neionski surfaktanti (Hamada, 2002; Hamada in Abe, 2010, Isola in sod., 2013). Detergenti vsebujejo različne snovi kot so aromatski ogljikovodiki, alkoholi, surfaktanti, dišave, encimi in belila, ki so jih glive sposobne razgraditi (Isola in sod., 2013). Pri izbranih sevih smo zato preverjali esterazno in proteazno aktivnost, ki mikroorganizmom omogoča razgradnjo maščobnih kislin in beljakovin. Pri vseh glivah smo opazili esterazno aktivnost pri 25 °C, medtem ko so vrste iz rodu *Fusarium* ter vrste *Aureobasidium melanogenum, Meyerozyma guilliermondii* in *Exophiala phaeomuriformis* izražale esterazno aktivnost tudi pri 37 °C. Manj gliv je pri danih pogojih izražalo proteazno aktivnost, vse razen *A. melanogenum*, ki spada med polimorfne glive, pa so spadale med filamentozne vrste (*F. solani, F. verticillioides, Mucor circinelloides*).

Pri razgradnji detergentov in pri nizkih temperaturah pranja lahko pride do namnožitve mikroorganizmov, zaradi katerih imajo obleke ali stroj po pranju neprijeten vonj (Munk in sod., 2001). V študiji smo izbrali 3 pralne stroje, pri katerih so uporabniki poročali o neprijetnem vonju ter 3 stroje kot kontrolno skupino, iz vseh smo osamili tako glive kot tudi bakterije. Izkazalo se je, da so stroji z neprijetnim vonjem vsebovali številčno manj gliv, z manjšo vrstno raznolikostjo kot stroji brez neprijetnega vonja, kar nakazuje na to, da glive niso glavne povzročiteljice neprijetnega vonja. V večini vzorcev smo iz strojev z neprijetnim vonjem osamili glive iz rodu *Penicillium* ter bakterije iz rodov *Micrococcus, Pseudomonas, Shewanella* ter *Sphingomonas*, kar se ujema z ugotovitvami predhodnikov (Stapleton in sod., 2013). Za navedene bakterije je značilno, da proizvajajo metabolne produkte z neprijetnim vonjem kot so dimetil disulfid in dimetil trisulfid (Palleroni in sod., 1992; James in sod., 2004; Stapleton in sod., 2013).

Pri vzorčenju pomivalnih strojev nismo zasledili večjega števila gliv iz rodu *Fusarium* (Ascomycota, Hypocreales), le-te pa so bile najpogosteje prisotne v pralnih strojih. Kolonije teh gliv so bele, oranžne ali roza barve in tvorijo zračni micelij. Rastni optimum dosežejo pri temperaturah med 25 °C in 35 °C in pri pH med 5 in 7 (de Hoog in sod., 2014). Vrste iz kompleksov vrst *F. solani* in *F. oxysporum* so vpletene v približno 80 % glivnih okužb pri ljudeh (O'Donnell in sod., 2010; Sutton in Brandt, 2011; Garnica in Nucci, 2013). Identificirane so kot oportunistični patogeni, ki povzročajo okužbe kože, oči,

okužbe preko katetrov, sintetizirajo mikotoksine ter so sposobne tvoriti biofilme na umetnih materialih kot so npr. kontaktne leče (Austen in sod., 2001; Bigley in sod., 2004; Krcmery in sod., 1997; Letscher-Bru in sod., 2002; Wey in Colombo, 1997; Mukherjee in sod., 2012). Oportunistični patogeni rodu *Fusarium* naj bi v splošnem povzročali bolnišnične okužbe pri bolnikih, ki prejemajo kemoterapijo ali imajo sladkorno bolezen (Zhang in sod., 2006). Tovrstne okužbe je težko odpraviti, saj so nekatere vrste rodu *Fusarium* odporne na večino antimikotikov, smrtnost pacientov je 37 % (Arikan in sod., 1999). Vodovodni sistemi, vlažne niše v prostorih in sistemi za hlajenje so se izkazali kot možen vir teh gliv (Schroers in sod., 2009). V naravi so sicer to glive prisotne na rastlinskem materialu, v tleh in zraku. Prvotno so bile okarakterizirane kot rastlinski patogeni (O'Donnell in sod., 2004; Zhang in sod., 2006; Smith, 2007).

Poleg gliv rodu *Fusarium* smo iz pralnih strojev v večjem številu osamili tudi filamentozne glive iz rodov *Cladosporium* in *Penicillium*. Oba rodova sta globalno gledano med najbolj razširjenimi glivami. Vrste rodu *Cladosporium* (Ascomycota, Capnodiales) vsebujejo melanin, zaradi česar tvorijo olivno zelene ali rjavkaste kolonije. Njihov rastni optimum je pri 25 °C, pri temperaturah višjih od 35 °C pa večina vrst ne raste. Znotraj rodu ločimo 3 komplekse vrst: *C. sphaerospermum, C. herbarum* in *C. cladosporioides* kompleks. Najbolj razširjene so vrste *C. herbarum, C. cladosporioides* in *C. oxysporum* (Bensch in sod., 2012). V naravi te glive najdemo v zraku, tleh, na rastlinskem materialu, v tropih, izolirali pa so jih tudi iz izjemno slanih okolij ter kopalnic (Zalar in sod., 2007; Hamada in Abe, 2010; de Hoog in sod., 2014). Glive iz rodu *Cladosporium* so redko povzročiteljice okužb pri ljudeh. Zaradi tvorbe spor in njihove številčne prisotnosti v zraku, so večinoma vpletene v oportunistične okužbe dihalnega trakta, kot so sinusitis ali pljučne okužbe, lahko pa povzročajo tudi alergije, okužbe kože in keratitis (de Hoog in sod., 2014). Čopičaste plesni iz rodu *Penicillium* (Ascomycota, Eurotiales) so pogosto izolirali iz vode, zraka ter tal (de Hoog in sod., 2014). Večina vrst dobro raste pri temperaturi 25 °C, oportuno patogeni vrsti *P. verrucosum* in *P. marneffei* pa sta sposobni rasti tudi pri 37 °C (Skoulidis in sod., 2004). Konidiji so lahko različno pigmentirani, zaradi česar so kolonije teh plesni bele, sive, rumene ali zelene barve. Mnoge vrste tega rodu so znane kot kvarljivci hrane, tvorijo pa tudi zdravju škodljive mikotoksine, lahko povzročajo okužbe

dihalnega trakta, keratitis ter alergije pri ljudeh z oslabljenim imunskim sistemom (de Hoog in sod., 2014).

Kvasovka *Candida parapsilosis* (Ascomycota, Saccharomycetales) je bila druga najpogostejša glivna vrsta, izolirana tako iz pralnih, kot tudi pomivalnih strojev. Predhodno je bila osamljena tudi iz kuhinjskih površin in vode (Adams in sod., 2013). Znano je, da proizvaja veliko ekstracelularnih polisaharidov, tvori lepljive biofilme in jo zaradi te sposobnosti najdemo na površini umetnih materialov. Zaradi vse pogostejše izolacije iz kliničnega materiala velja za porajajočega se patogena (van Asbeck in sod., 2009; Miceli in sod., 2011). Pogosto je povzročiteljica okužb preko katetrov ali drugega prostetičnega materiala, v hujših primerih lahko pri imunsko oslabljenih ljudeh povzroči tudi sepso (Levin in sod., 1995). V naravi je sicer prisotna v tleh, vodi in na rastlinskem materialu (Bodey in sod., 1992; Deresinski in sod., 1995).

3.1.3 Voda kot vektor prenosa gliv v gospodinjske aparate

Pomivalni in pralni stroj za svoje delovanje potrebujeta vodo, ki jo v veliki večini primerov dobimo preko priključitve na lokalno vodovodno omrežje. Čeprav je vnos gliv v stroje mogoč tudi preko perila, posode ter ostankov umazanije, pa je obema napravama skupna prav uporaba vode. Zaradi tega nas je zanimalo ali bi voda iz lokalnega omrežja lahko predstavljala vir gliv, ki naselijo stroje. Voda iz vodovodnega omrežja pri vzorčenih strojih po Sloveniji je pitna, kar pomeni, da zadošča kriterijem Evropske direktive za kakovost pitne vode 80/778/EC (Evropska Unija, 1980). Trenutna direktiva sicer ne vsebuje omejitev ali posebnega monitoringa za prisotnost gliv, čeprav je poročanj o glivah v vodi veliko. Najpogosteje omenjene so vrste iz rodov *Aspergillus, Cladosporium, Paecilomyces, Penicillium, Phialophora, Trichoderma* in *Verticillium*. Gre za glive, ki tvorijo spore, se razširjajo z aerosoli in predstavljajo najpogostejše tveganje za razvoj respiratornih infekcij pri ljudeh z oslabljenim imunskih sistemom (Defra, 2011). O prisotnosti kvasovk in kvasovkam podobnih gliv v pitni vodi ni veliko podatkov. Predhodniki so iz vode ali biofilmov na ceveh osamili vrste iz rodov *Aureobasidium, Candida, Cryptococcus, Exophiala, Geotrichum, Rhodotorula* in *Sporotrix* (Doggett, 2000; Göttlich in sod., 2002; Kinsey in sod., 2003; Hageskal in sod., 2006). Da bi potrdili prisotnost najpogosteje najdenih gliv v pomivalnih in pralnih strojih tudi v pitni vodi, smo se v glavnem

osredotočili na kvasovke in kvasovkam podobne glive. Glive smo osamili iz 80 % vzorcev vodovodne in 69 % vzorcev podzemne vode. V obeh primerih smo najpogosteje osamili filamentozne glive iz rodu *Aspergillus*, ki so jim sledile črne kvasovke in kvasovkam podobne glive iz rodov *Aureobasidium, Exophiala* in *Rhinocladiella*. *E. dermatitidis* in *E. phaeomuriformis* sta bili osamljeni iz 6 oz. 5 % vzorcev vodovodne vode, pri čemer je to prvi zabeleženi primer izolacije *E. dermatitidis* neposredno iz pitne vode. Izmed ostalih kvasovk smo tako kot v primeru pralnih in pomivalnih strojev najpogosteje izolirali vrsti *Candida parapsilosis* in *Rhodotorula mucilaginosa* (11 in 13 %). Prisotnost gliv rodov *Aspergillus* in *Exophiala* smo nato naknadno potrdili še z molekularno-genetsko metodo sekvenciranja naslednje generacije (NGS).

Glive rodu *Aspergillus* (Ascomycota, Eurotiales) spadajo med najbolj pogosto izolirane glive iz vode (Pereira in sod., 2010; Defra, 2011). Zaradi tega jih najdemo tudi v prostorih, ki so bili poplavljeni ali so nenehno vlažni (npr. kopalnice). Običajno so v takih prostorih najpogostejše vrste *Aspergillus flavus, A. fumigatus, A. niger, A. ochraceus* in *A. versicolor* (Klich, 2009). Proizvajajo mikotoksine, zaradi njihove sposobnosti tvorbe spor in razširjanja po zraku pa pogosto povzročajo okužbe dihalnega trakta, alergije, astmo, okužbe ušes, v redkih primerih pa tudi kronične micetome in invazivno aspergilozo (de Hoog in sod., 2014; Rementeria in sod., 2005).

Druga najpogosteje osamljena gliva iz vode je bila črna kvasovka *Aureobasidium melanogenum* (Ascomycota, Dothideales). *A. melanogenum* je bila kot samostojna vrsta na podlagi primerjave genomov opisana šele leta 2014 (Gostinčar in sod., 2014), pred tem je spadala v eno od varietet znotraj vrste *A. pullulans* (Zalar in sod., 2008). Kolonije teh gliv so rožnate in gladke, zaradi vsebnosti melanina pa s starostjo potemnijo. Sposobne so tvorbe velikih količin izvenceličnih polisaharidov, najpogosteje pululana. *A. melanogenum* je edina izmed vrst v rodu *Aureobasidium*, ki raste tudi pri 37 °C (Gostinčar in sod., 2014). Glive iz rodu *Aureobasidium* sicer najdemo v tleh, na rastlinskem materialu, osamili pa so jih tudi iz ledenikov, vode, kuhinj in kopalnic. Pogosto so povezane tudi z oligotrofnimi okolji (Hageskal in sod., 2006, Kanzler in sod., 2008; Gostinčar in sod., 2014). Med opisanimi vrstami je trenutno le za *A. melanogenum* potrjeno, da lahko povzroča lokalne okužbe kože, alergije, meningitis in pljučne mikoze (Collier in sod., 1998; Hawkes in sod.,

2005). Čeprav je bila ta gliva v naši raziskavi osamljena iz 56 % vzorcev podzemnih voda ter iz 25 % vzorcev pitne vode pa smo jo redko našli v pomivalnih (1 %) in pralnih strojih (2 %), kar nakazuje njeno občutljivost na pogoje znotraj strojev.

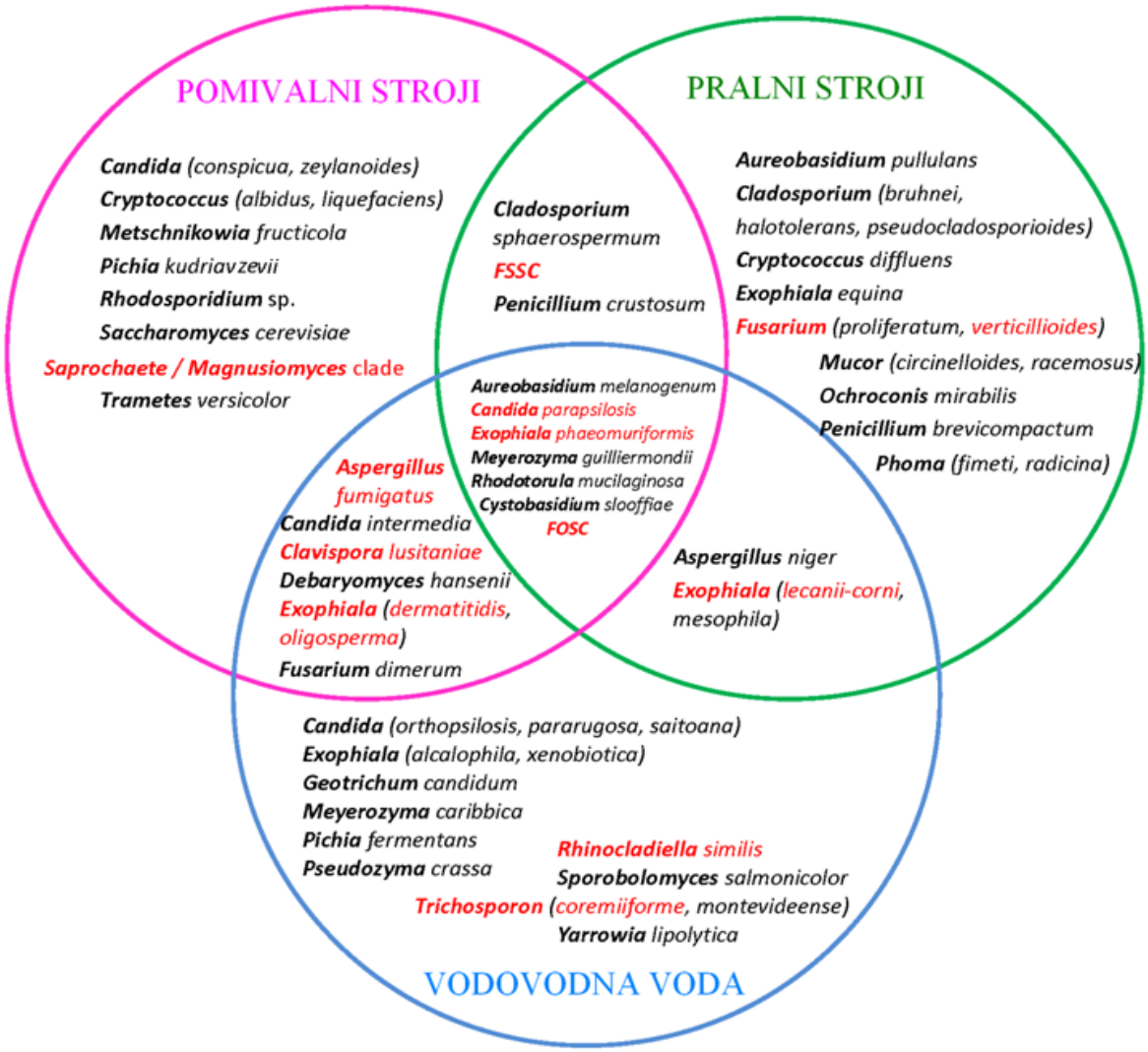
Izmed ubikvitarnih gliv smo poleg gliv iz rodu *Candida* v večjem številu osamili tudi rdeče pigmentirano kvasovko *Rhodotorula mucilaginosa* (Basidiomycota, Sporidiobolales). V naravi je razširjena v zraku, sladki in slani vodi, tleh, rastlinskem materialu, mlečnih izdelkih, na kuhinjskih površinah in znotraj gospodinjskih aparatov (Rose in Kurup, 1977; de Hoog in sod., 2014; Kanzler in sod., 2008; Adams in sod., 2013). Okužbe ljudi s to kvasovko so največkrat povezane z uporabo katetrov v bolnišnicah (Neofytos in sod., 2007). Pri imunsko oslabljenih ljudeh lahko povzroča tudi okužbe oči, meningitis in sepso (Pfaller in sod., 2007; de Hoog in sod., 2014).

Že v primeru študije gliv v pomivalnih strojih smo predpostavili, da ima trdota vode vpliv na pojav določenih glivnih vrst v strojih. Da bi to preučili, smo v primeru vzorcev vodovodne vode izvedli tudi kemijsko analizo ionov, dobljene rezultate pa nato s statistično metodo strojnega učenja primerjali s prisotnostjo glivnih vrst v taistih vzorcih. Izkazalo se je, da smo glive najpogosteje osamili iz vzorcev vode z višjo vsebnostjo kalcijevih ionov (Ca^{2+} > 53 mg/l) ter ob zmerni prisotnosti magnezija (5 mg/l < Mg^{2+} > 13 mg/l). Pri vzorcih, kjer je bila koncentracija kalcijevih ionov nižja, se je za prisotnost gliv kot pomemben dejavnik izkazala prisotnost nitrata (NO_3^-). Nepričakovano pa statistična analiza kot dejavnika, pomembnega za pojav gliv v vodi, ni prikazala kloridnih ionov (Cl^-). Znano je, da prisotnost kalcijevih ionov v okolju regulira obliko rasti pri *E. dermatitidis* (Karuppayil in Szaniszlo, 1997) in bi zato lahko pozitivno vplivala na pogostejše pojavljanje te glive v vodi. Poleg tega so za melanizirane glive na splošno v predhodnih raziskavah poročali o višji stopnji odpornosti na dezinfekcijska sredstva. Na slabšo učinkovitost dezinfekcije, ki temelji na kloriranju prav tako vpliva agregiranje gliv v skupke (Langfelder in sod., 2003; Mamane-Gravetz in Linden, 2005). Iz rezultatov analize strojnega učenja smo nadalje sklepali, da je za prisotnost gliv v vodi in posledično v gospodinjskih strojih pomembna tudi lokacija. Z metodo denaturacijske gradientne gelske elektroforeze (DGGE) smo zato primerjali glivne združbe iz različnih vzorcev vode (rečna, vodovodna, podzemna, odpadna voda) na področju Ljubljanske kotline. Pričakovali smo,

da bodo glivne združbe enake znotraj podobnih vzorcev (npr., med vzorci vodovodne vode). Presenetljivo pa so rezultati DGGE analize pokazali podobnost glivnih združb v vzorcih, ki so bili odvzeti bodisi znotraj vodonosnika Ljubljansko polje na severu, ali vodonosnika Ljubljansko barje na jugu. Pojav določenih glivnih rodov v različnih vzorcih vode se je tako izkazal vezan na lokacijo primarnega vodnega vira (zajetja). Da lokacija vpliva na pojav določenih glivnih rodov v vodi in posledično na njihovo prisotnost v biofilmih, je bilo pozneje potrjeno v primeru britanske študije biofilmov iz glav tušev (Moat in sod., 2016).

Slika 1 povzema rezultate izolacije in identifikacije gliv, osamljenih iz pomivalnih in pralnih strojev ter vodovodne vode. Na podlagi dobljenih rezultatov je razvidno, da smo iz vseh treh okolij osamili glive *Aureobasidium melanogenum, Candida parapsilosis, Cystobasidium slooffiae, Exophiala phaeomuriformis,* glive iz *Fusarium oxysporum* kompleksa vrst (FOSC) ter ubikvitarni kvasovki vrst *Meyerozyma guilliermondii* in *Rhodotorula mucilaginosa*. Za te glive lahko sklepamo, da so se po prenosu z vodo sposobne prilagoditi različnim življenjskim pogojem znotraj obeh gospodinjskih aparatov. Iz Slike 1 je prav tako razvidno, da so nekatere glive, osamljene iz vode, uspešnejše pri kolonizaciji bodisi pralnih strojev (*Aspergillus niger, Exophiala lecanii-corni* ter *E. mesophila*) ali pomivalnih strojev (*A. fumigatus, C. intermedia, Clavispora lusitaniae, Debaryomyces hansenii, E. dermatitidis, E. oligosperma* in *Fusarium dimerum*). Razlike se najverjetneje pojavijo zaradi temperature pranja, ki je bila pri vzorčenih pomivalnih strojih običajno višja kot pri pralnih strojih. To je razvidno na primeru vrst črnih kvasovk iz rodu *Exophiala,* kjer so že v študijah Matos in sodelavcev (2002) ter de Hoog-a in sodelavcev (2011) ugotovili, da se nekatere vrste pogosteje pojavljajo v okoljih z višjo temperaturo (npr. *E. dermatitidis*), druge pa bolje rastejo pri mezofilnih pogojih (npr. *E. mesophila*). Nekatere glive, ki smo jih sicer osamili iz vode, se v strojih niso pojavile, zaradi česar lahko sklepamo, da pogoji v strojih inhibirajo njihovo rast (nekatere vrste iz rodov *Candida, Exophiala, Pseudozyma, Rhinocladiella, Trichosporon* in *Yarrowia*). Na to, da voda kljub vsemu ni edini vir vnosa gliv v stroje, pa nakazujejo preostale glive iz ločenih združb v pomivalnih oz. pralnih strojih. V pomivalnih strojih so to predvsem kvasovke ali kvasovkam podobne glive, ki so povezane s hrano (rodovi *Candida, Cryptococcus, Metschnikovia, Pichia, Saccharomyces* in *Saprochaete*). V pralnih strojih pa gre večinoma

za filamentozne glive, prisotne v zraku, tleh ter na rastlinskem materialu. Le-te se v pralne stroje najverjetneje prenesejo preko perila (rodovi *Aureobasidium*, *Cladosporium*, *Fusarium*, *Mucor*, *Ochroconis*, *Penicillium* in *Phoma*) (Slika 1).



Slika 1: Glivne vrste, osamljene iz vodovodne vode (modra barva), pralnih strojev (zelena barva) in pomivalnih strojev (roza barva).

Legenda: Barvni krogi vključujejo glive, osamljene iz pomivalnih in pralnih strojev ter vodovodne vode, s preseki združb s skupnimi predstavniki. Rodovi gliv so označeni s krepkim tiskom; glive, ki spadajo v 2 stopnjo biološke varnosti pa z rdečo barvo.

3.1.4 Vpliv selekcije v gospodinjskih aparatih na pojav gliv v odpadnih vodah

Gospodinjski stroji so priključeni na vodovodno omrežje in zaradi odpadnih voda po končanem postopku pranja tudi v globalni vodni krog. Pri DGGE analizi, ki smo jo opravili tekom študije o glivah v vodah smo med drugim ugotovili, da je glivna sestava v odpadnih vodah podobna glivni sestavi podzemne in vodovodne vode. Takšen rezultat lahko nakazuje, da se glive po namnožitvi v strojih preko odplak vrnejo v naravno vodno okolje. Za kvasovke *Candida krusei, Debaryomyces polymorphus, M. guilliermondi, Rhodotorula* spp. ter filamentozne glive *Aspergillus* spp., *Mucor* spp., *Penicillium* spp. in *Rhizopus* spp. je znano, da so tudi po procesu čiščenja odpadne vode prisotne v vodi, ki se vrne v okolje (Biedunkiewicz in Ozimek, 2009).

Glive so globalno prisotne v naravi, od zraka, sladke in slane vode, ledenikov, tal ter rastlinskega materiala. Prav tako so prisotne v zraku človekovih bivališč, kot tudi na stenah, v kopalnicah in kuhinjah. Tekom doktorske naloge smo potrdili hipotezi, da pomivalne in pralne stroje naseljujejo različne glive ter pokazali, da življenjski pogoji znotraj izbranih gospodinjskih aparatov selektivno vplivajo na namnožitev oportuno patogenih gliv ter gliv, ki spadajo med porajajoče patogene. Potrdili smo tudi domneve, da lahko vodovodna voda predstavlja vektor prenosa gliv v stroje ter, da ionska sestava vode selektivno vpliva na pojav gliv v vodovodni vodi. Rezultati DGGE analize so pokazali konstantnost glivne diverzitete tako v pitni kot v odpadni vodi, kar priča o prenosu oportuno patogenih gliv iz strojev nazaj v okolje. Dobljeni rezultati doprinašajo k boljšemu poznavanju pojava in sestave glivnih združb v vodovodni vodi, pralnih in pomivalnih strojih ter omogočajo poznavanje tveganja okužb z glivnimi patogeni za rizične skupine ljudi.

3.2 SKLEPI

1. Glivna diverziteta v pomivalnih strojih

V dveh ločenih študijah pomivalnih strojev (N=189, 937) smo skupno osamili 23 vrst gliv iz 14 rodov; najpogosteje črne kvasovke *Exophiala dermatitidis* in *E. phaeomuriformis* so bile prisotne v 19 % vseh strojev. Stalno glivno bioto so v obeh študijah predstavljale še vrste *Candida parapsilosis, Magnusiomyces capitatus, Meyerozyma guilliermondii, Rhodotorula mucilaginosa,* ki so sposobne tvoriti biofilme na umetnih podlagah kot je guma.

1a. Fiziološke lastnosti črnih kvasovk omogočajo njihovo naselitev v pomivalnih strojih

Črne kvasovke *E. dermatitidis* in *E. phaeomuriformis* so prilagojene na specifične življenjske pogoje v pomivalnih strojih. Rastejo pri temperaturah do 45 °C, izbrani genotipi tudi v pH območju od 2,5 do 12,5 ter do 17 % NaCl v gojišču. Vsi genotipi *E. dermatitidis* lahko kot edini vir ogljika izkoriščajo tesnila iz gume.

2. Glivna diverziteta v pralnih strojih

Iz 70 pralnih strojev, vzorčenih v Sloveniji, smo osamili 26 vrst gliv iz 12 rodov. Najpogostejši so bili izolati gliv kompleksa vrst *Fusarium oxysporum* (23 % pralnih strojev) ter kvasovka *Candida parapsilosis* (14 %), pogosto prisotni pa predstavniki rodov *Aureobasidium, Cryptococcus, Cladosporium, Exophiala, Meyerozima, Mucor, Penicillium* in *Rhodotorula*.

2a. Temperatura pranja in prisotnost mehčalca vplivata na naselitev gliv v pralnih strojih

Vsi testirani glivni izolati iz pralnih strojev so rasli pri 25 °C, le nekateri pri 37 °C. 96 % izoliranih vrst gliv je raslo na komercialnem mehčalcu kot edinem viru hranil. S statistično analizo strojnega učenja smo ugotovili, da imata predvsem prisotnost mehčalca in temperature pranja od 30 do 60 °C pomemben vpliv na pojav gliv v strojih. Esterazno in proteazno aktivnost, kot virulentne faktorje gliv, je pri 25 °C izražala večina sevov, pri 37 °C pa le vrste iz rodu *Fusarium* ter vrste *A. melanogenum, Exophiala phaeomuriformis, Meyerozyma guilliermondii* in *Mucor circinelloides.*

3. Glivna diverziteta v vodovodni in podzemni vodi

Iz 100 vzorcev vodovodne vode in 16 vzorcev podzemne vode iz področja Slovenije smo izolirali 28 glivnih vrst iz 16 rodov. Najpogostejše so bile glive rodu *Aspergillus* in črne kvasovke vrste *Aureobasidium melanogenum*. Pogosto prisotne so bile še vrste iz rodov *Candida, Rhodotorula, Exophiala, Meyerozyma* in *Rhinocladiella.* Črno kvasovko *Exophiala dermatitidis* smo prvič izolirali iz vodovodne in iz podzemne vode. Prisotnost gliv rodov *Aspergillus* in *Exophiala* v vodovodni vodi smo potrdili tudi z negojitveno metodo sekvenciranja naslednje generacije (NGS).

3a. Trdota vode, vsebnost nitratov in lokacija vplivajo na pojav gliv v vodi

S statistično analizo strojnega učenja smo pojav gliv v vodovodni vodi povezali z višjo vsebnostjo kalcijevih (Ca^{2+} > 53 mg/l) in magnezijevih ionov (5 mg/l < Mg^{2+} > 13 mg/l) ter s prisotnostjo nitrata. DGGE analiza primerjave glivnih združb iz različnih vzorcev vode na področju Ljubljanske kotline je pokazala povezavo med pojavom gliv v vzorcih vode in lokacijo primarnega vodnega vira, oz. vodonosnika.

4. Skupne glive iz vseh treh okolij, njihov prenos z vodo in selekcija v strojih

Iz pomivalnih in pralnih strojev ter vodovodne vode smo osamili glive *Aureobasidium melanogenum, Candida parapsilosis, Cystobasidium slooffiae, Exophiala phaeomuriformis,* glive iz *Fusarium oxysporum* kompleksa vrst (FOSC) ter ubikvitarni kvasovki vrst *Meyerozyma guilliermondii* in *Rhodotorula mucilaginosa*. Sklepamo, da te glive vstopijo z vodo, se naselijo in razmnožujejo znotraj obeh vrst gospodinjskih aparatov.

Iz pomivalnih strojev smo izmed gliv, ki se prenašajo z vodo, osamili vrste *A. fumigatus, C. intermedia, Clavispora lusitaniae, Debaryomyces hansenii, E. dermatitidis, E. oligosperma* in *Fusarium dimerum,* ki so odporne na višje temperature. Nasprotno so mezofilne vrste iz vodovodne vode *Aspergillus niger, Exophiala lecanii-corni* in *E. mesophila,* naseljevale le pralne stroje. Razlike med glivno diverziteto v obeh strojih so posledica predvsem temperature pranja, ki je pri pranju v pomivalnih strojih običajno višja kot pri pranju v pralnih strojih.

Rezultati doprinašajo k boljšemu poznavanju pojava in sestave glivnih združb v vodovodni vodi, znotraj pralnih in pomivalnih strojev ter omogočajo poznavanje tveganja okužb z glivnimi patogeni pri rizičnih skupinah ljudi.

4 POVZETEK (SUMMARY)

4.1 POVZETEK

V zadnjih letih so zaradi ekološke ozaveščenosti proizvajalci gospodinjskih strojev znižali temperature pranja in količino porabljene vode, uveljavilo pa se je tudi pranje z biorazgradljivimi detergenti. V pralnih strojih so iz različnih delov osamili glive *Fusarium* sp., *Microsporum canis*, *Mucor* sp., *Trichophyton mentagrophytes* ter kvasovke rodov *Candida* in *Rhodotorula*, pomivalne stroje pa so posredno povezovali z okužbami gliv iz rodu *Candida*. Oba gospodinjska aparata za svoje delovanje potrebujeta vodo, ki zaradi tega predstavlja eno od poti za vnos mikroorganizmov v stroje. Čeprav je mikrobiološka kakovost vodovodne vode nadzorovana tudi z dezinfekcijskimi postopki, pa se vode iz različnih področij razlikujejo glede na pH, trdoto, vsebnost hranil in prisotnost biofilmov v cevnih sistemih. Za glive je znano, da lahko preživijo tudi v oligotrofnih razmerah, z malo hranili. Iz pitne vodovodne vode so različni avtorji najpogosteje osamili večinoma filamentozne glive rodov *Acremonium, Altenaria, Arthrinium, Aspergillus, Aureobasidium, Beauveria, Botrytis, Chaetomium, Cladosporium, Epicoccum, Exophiala, Fusarium, Geotrichum, Gliocladium, Mucor, Paecilomyces, Penicillium, Phialophora, Phoma, Phomopsis, Rhizopus, Sporothrix, Trichoderma in Verticillium.* Raziskav, kjer bi povezali vrstno sestavo gliv v vodovodni vodi in v biofilmih pomivalnih ter pralnih strojev do sedaj ni bilo mogoče zaslediti. Tekom doktorske naloge smo preučevali prisotnost gliv v pomivalnih in pralnih strojih ter v podzemni in vodovodni vodi kot vektorju za prenos gliv v stroje. V globalni študiji pomivalnih strojev iz 18 držav smo iz tesnil na vratih strojev izolirali 23 vrst gliv iz 15 rodov, v študiji 937 pomivalnih strojev iz področja Turčije pa 14 vrst gliv, ki so pripadale 6 rodovom. Glivna sestava je bila v obeh primerih presenetljivo podobna, saj smo obakrat najpogosteje osamili oportuno patogene črne kvasovke *Exophiala dermatitidis* in *E. phaeomuriformis* ter kvasovke *Candida parapsilosis, Magnusiomyces capitatus*, *Meyerozyma guilliermondii* in *Rhodotorula mucilaginosa*. Ugotovili smo, da so sevi vrst *E. dermatitidis* in *E. phaeomuriformis* poliekstremotolerantni, saj so rasli pri temperaturah od 10 °C do 45 °C, brez ali do 17 % NaCl v gojišču in v pH območju od 2,5 do 12,5. Za seve teh vrst iz Turčije smo potrdili odpornost na cikloheksimid pri koncentraciji 100 µg/ml. Z metodo parjenja in prstnega odtisa smo ugotovili, da je med genotipi znotraj vrste *E. dermatitidis* mogoča rekombinacija, čeprav spolna oblika te glive ni poznana. Pri raziskavi pralnih strojev smo

se osredotočili na uporabnikom dosegljive dele kot so predalčki za detergente in tesnilo ob vratih strojev. Iz 70 v Sloveniji vzorčenih pralnih strojev smo iz omenjenih okolij osamili 26 vrst iz 12 rodov. Glivno bioto so v glavnem predstavljale glive rodov *Cladosporium, Exophiala, Penicillium* in *Rhodotorula,* najpogostejše pa so bile glive iz *Fusarium oxysporum* kompleksa ter *Candida parapsilosis*. Za razliko od pomivalnih strojev iz pralnih strojev nismo osamili *E. dermatitidis* pač pa mezofilne vrste iz rodu *Exophiala* kot sta npr. *E. mesophila* in *E. equina*. Ugotovili smo tudi, da lahko vsi testirani glivni izolati rastejo pri 25 °C, pri 37 °C pa za *Aureobasidium pullulans, Mucor racemosus, Ochroconis* sp. ter vrste rodov *Cladosporium, Penicillium* in *Phoma* nismo zabeležili rasti. Pri testiranju esterazne aktivnosti smo rast pri 25 °C prav tako zabeležili pri vseh sevih, pri 37 °C pa le pri vrstah iz rodu *Fusarium* ter vrstah *A. melanogenum, Meyerozyma guilliermondii* in *Exophiala phaeomuriformis.* Proteazno aktivnost so pri danih pogojih izražale vrste *A. melanogenum, Fusarium solani, F. verticillioides* in *Mucor circinelloides*. Ugotovili smo, da so so glive iz pralnih strojev, razen *Phoma fimeti* sposobne rasti na vodnem agarju z dodatkom 1 % mehčalca. Pri vrstah *Fusarium verticillioides* in *Penicillium crustosum* smo rast zabeležili tudi na gojišču s 5 % koncentracijo mehčalca. V raziskavi je veliko ljudi poročalo o uporabi alkoholnega kisa namesto mehčalca, zato smo seve iz pralnih strojev testirali še na rast v gojišču z 1 % koncentracijo ocetne kisline. Pri nobeni od testiranih gliv nismo zabeležili rasti. Statistična analiza strojnega učenja je potrdila ugotovitve, da ima prisotnost detergentov, še posebej mehčalca, pomemben vpliv na pojav gliv v strojih. Največ gliv in najvišjo raznolikost smo opazili pri strojih, kjer se pri pranju uporabljata tako mehčalec kot pralni prašek. Statistična analiza je prav tako izpostavila tudi pozitiven vpliv nižjih temperatur pranja (30 - 60 °C) na glivno raznolikost in število. Pri preučevanju vpliva mikroorganizmov na neprijeten vonj v pralnih strojih smo ugotovili, da je le-ta povezan s prisotnostjo bakterij rodov *Micrococcus, Pseudomonas, Shewanella* in *Sphingomonas.* Iz strojev z neprijetnim vonjem smo namreč osamili veliko več bakterijskih vrst in zelo malo gliv v primerjavi s stroji, ki neprijetnega vonja niso imeli. Pri obeh tipih gospodinjskih strojev je voda ključnega pomena, zato smo sklepali, da bi lahko služila kot vektor prenosa gliv v stroje. Z metodo filtracije smo preučili 100 vzorcev vodovodne vode in 16 vzorcev podzemnih voda iz področja Slovenije in izolirali 28 glivnih vrst iz 16 rodov. V obeh tipih vode smo najpogosteje opazili glive iz rodu *Aspergillus* (48 in 14 % vzorcev) ter črne kvasovke vrste *Aureobasidium*

melanogenum (25 in 9 %). Pogosto smo izolirali še vrste rodov *Candida, Rhodotorula, Exophiala, Meyerozyma* in *Rhinocladiella.* Število glivnih kolonij v 1 litru vzorcev vode se je gibalo od 0 do neštevnega (>300). *E. dermatitidis* je bila prvič neposredno izolirana tako iz vodovodne kot tudi podzemne vode, število kolonij pa v nobenem od vzorcev ni preseglo števila 10. V izbranem vzorcu vodovodne vode smo nato na podlagi ITS2 regije prisotnost gliv rodov *Aspergillus* in *Exophiala* potrdili tudi z molekularno-genetsko metodo sekvenciranja naslednje generacije (NGS). Statistična analiza strojnega učenja je pojav gliv v vodovodni vodi povezala z višjo vsebnostjo kalcijevih (Ca^{2+} > 53 mg/l) in magnezijevih ionov (5 mg/l < Mg2+ > 13 mg/l), ob nižjih koncentracijah kalcija pa se je za prisotnost gliv v vodi kot pomemben dejavnik pojavil nitrat. Kloridni ioni se, presenetljivo, niso izkazali kot eden izmed dejavnikov, ki bi vplivali na pojav in raznolikost gliv v vodah. DGGE analiza, s katero smo primerjali glivne združbe iz vzorcev rek, vodnjakov, vodovodnih voda ter vzorcev vode iz čistilnih naprav na področju Ljubljanske kotline je pokazala, da je pojav gliv v različnih vzorcih vode neodvisen od tipa vode, ampak je vezan na lokacijo primarnega vodnega vira in vodonosnika. Da je pojav nekaterih glivnih vrst v strojih povezan z vstopno vodo je razvidno iz podatka, da smo iz vseh treh okolij osamili glive *Aureobasidium melanogenum, Candida parapsilosis, Cystobasidium slooffiae, Exophiala phaeomuriformis,* glive iz *Fusarium oxysporum* kompleksa (FOSC) ter ubikvitarni kvasovki vrst *Meyerozyma guilliermondii* in *Rhodotorula mucilaginosa*. Te glive so prisotne v vodi, po prenosu v stroje pa so se sposobne prilagoditi življenjskim pogojem znotraj obeh gospodinjskih aparatov. Kljub vsemu pa so nekatere glive iz vode uspešnejše pri kolonizaciji bodisi pralnih strojev (*Aspergillus niger, Exophiala lecanii-corni* ter *E. mesophila*) ali pomivalnih strojev (*A. fumigatus, C. intermedia, Clavispora lusitaniae, Debaryomyces hansenii, E. dermatitidis, E. oligosperma* in *Fusarium dimerum*). Nastale razlike bi lahko bile posledica temperature pranja, ki je bila pri vzorčenih pomivalnih strojih višja kot pri pralnih strojih. Primer so vrste iz rodu *Exophiala,* pri katerih je znano, da se vrsti *E. dermatitidis* in *E. phaeomuriformis* pojavljata v okoljih z višjo temperaturo, *E. equina, E. mesophila i*n *E. oligosperma* pa so bile osamljene iz okolij z mezofilnimi pogoji. Za nekatere vrste rodov *Candida, Exophiala, Pseudozyma, Rhinocladiella, Trichosporon* in *Yarrowia* iz vode, ki se v strojih niso pojavile smo sklepali, da pogoji v strojih negativno vplivajo na njihovo rast. Da voda ni edini vir vnosa gliv v stroje, nakazujejo preostale glive iz ločenih združb v pomivalnih oz.

pralnih strojih. V pomivalnih strojih so to predvsem kvasovke in kvasovkam podobne glive, povezane s hrano (*Candida, Cryptococcus, Metschnikovia, Pichia, Saccharomyces* in *Saprochaete*). V pralnih strojih pa filamentozne glive, prisotne v zraku, prsti in na rastlinskem materialu, ki se v pralne stroje lahko prenesejo preko perila (*Aureobasidium, Cladosporium, Fusarium, Mucor, Ochroconis, Penicillium* in *Phoma*). Odpadne vode iz gospodinjstev se vračajo v reke, jezera in morja. Končni rezultati, predstavljeni v doktorski nalogi, doprinašajo k poznavanju ekologije glivnih združb v pralnih in pomivalnih strojih ter njihovega pojava in prenosa iz vodovodne vode. S tem omogočamo poznavanje tveganja okužb s porajajočimi in oportunističnimi glivnimi patogeni med ljudmi z oslabljenim imunskim sistemom.

4.2 SUMMARY

In the last years, increasing ecological awareness as well as the need for low energy consumption among producers of household appliances lead to lowered temperatures of washing and reduced amount of water, as well as to the use of biodegradable detergents. Recent studies report the presence of fungal species *Fusarium* sp., *Microsporum canis*, *Mucor* sp., *Trichophyton mentagrophytes*, as well as yeasts of the genera *Candida* and *Rhodotorula* from different parts of washing machines. Fungal contamination of dishwashers was indirectly associated with infections of fungi from genus *Candida*. Both types of household appliances use water, which might serve as the entry route for microorganisms. Although the microbiological quality of the drinking water is regularly controlled with disinfecting procedures, water from different areas may vary depending on the pH, water hardness, nutrient content and the presence of biofilms in piping systems. Fungi can survive in the oligotrophic conditions present in water. Most frequently reported filamentous fungi in drinking water belong to the genera *Acremonium, Altenaria, Arthrinium, Aspergillus, Aureobasidium, Beauveria, Botrytis, Chaetomium, Cladosporium, Epicoccum, Exophiala, Fusarium, Geotrichum, Gliocladium, Mucor, Paecilomyces, Penicillium, Phialophora, Phoma, Phomopsis, Rhizopus, Sporothrix, Trichoderma* and *Verticillium*. To the best of our knowledge there were no reports, which would link the diversity of fungi in drinking water to the diversity and presence of fungi detected in dishwashers and washing machines. During the doctoral thesis we studied the presence of fungi in dishwashers and washing machines using culturable and unculturable techniques. We also wanted to test the assumption that groundwater and drinking water can act as a possible vector for the transmission of fungi into the appliances. In a global study of dishwasher's rubber seals sampled in 18 countries, we isolated 23 fungal species from 15 genera, while in the study of 937 dishwashers from a single country (Turkey) 14 fungal species that belong to 6 genera were isolated. Fungal diversity was in both cases very similar. Amongst the most numerous isolates were opportunistic pathogenic black yeasts *Exophiala dermatitidis* and *E. phaeomuriformis*, followed by yeasts and yeast-like fungi *Candida parapsilosis, Magnusiomyces capitatus, Meyerozyma guilliermondii* and *Rhodotorula mucilaginosa*. Black fungi *E. dermatitidis* and *E. phaeomuriformis* are polyextremotolerant microorganisms, since they were able to grow at temperatures from 10 °C to 45 °C, up to 17 % NaCl, and in the pH range from 2.5 to 12.5. Strains of these

species obtained from Turkey, which were also tested for cycloheximide resistance at concentration of 100 mcg/ml all showed resistance. After mating of different strains and subsequent fingerprint, we proved the recombination ability among the genotypes of the species *E. dermatitidis*, although sexual form of the fungus is not yet known. In the washing machine study we focused on the fungal isolation from user-accessible parts, such as compartments for detergents and rubber seal on the door. From 70 washing machines sampled in Slovenia, we isolated 26 fungal species from 12 genera. Fungal biota is mainly composed of species belonging to the genera *Cladosporium, Exophiala, Penicillium* and *Rhodotorula*, while the most common were fungi from *Fusarium oxysporum* species complex and the yeast *Candida parapsilosis*. Unlike in dishwashers, *E. dermatitidis* was not isolated from washing machines, but only the mesophilic species *E. mesophila* and *E. equina*. All the tested fungal isolates were able to grow at 25 °C, while the species *Aureobasidium pullulans, Mucor racemosus, Ochroconis* sp. and species of the genera *Cladosporium, Penicillium* and *Phoma* did not grow at 37 °C. At 25 °C the esterase activity was evident in all tested fungal strains, while some activity at 37 °C was observed only for *Fusarium* species, *A. melanogenum, Meyerozyma guilliermondii* and *Exophiala phaeomuriformis*. Protease activity was observed at 37 °C for *A. melanogenum, Fusarium solani, F. verticillioides* and *Mucor circinelloides*. We also tested the growth of isolates on the medium with added 1 and 5 % fabric softener as a sole nutrient source. Except *Phoma fimeti*, all tested strains were able to grow on the water medium with 1 % fabric softener, while the growth of *Fusarium verticillioides* and *Penicillium crustosum* was recorded also with the addition of 5% fabric softener. During our research many people reported the use of alcohol vinegar instead of fabric softener, thus we tested also the growth of isolated strains in the medium containing 1 % acetic acid. We did not observe growth in any of the tested fungi. Statistical analysis based on machine learning confirmed that the presence of detergent, and specifically fabric softener, has a significant impact on the occurrence of fungi in washing machines. The highest fungal diversity was observed in machines where both, fabric softener and washing powder were used. Statistical analysis also highlighted the positive impact of low washing temperatures (30-60 °C) on the fungal diversity. When examining the influence of microorganisms on the smelly odor in the washing machines, we found that it is mainly associated with the presence of bacteria belonging to the genera *Micrococcus, Pseudomonas, Shewanella* and *Sphingomonas*. From washing machines with

an unpleasant odor we isolated more bacterial species and very few fungi in comparison to the non-smelly machines. Because both types of household appliances use water we assumed that water can serve as a vector of transmission of fungi into the machines. We examined 100 samples of drinking water and 16 groundwater samples from different areas of Slovenia by filtration and subsequent cultivation and isolated 28 fungal species belonging to 16 genera. In both kinds of water we frequently observed fungi of the genus *Aspergillus* (48 and 14 % of the samples), and the black yeasts-like species *Aureobasidium melanogenum* (25 and 9 %), followed by the species of the genera *Candida, Rhodotorula, Exophiala, Meyerozyma* and *Rhinocladiella*. The number of fungal colonies in 1 liter of water samples ranged from 0 to uncountable (> 300). To the best of our knowledge we reported for the first time cultivation of *E. dermatitidis* directly from drinking water as well as groundwater, although the number of colonies did not exceed the number of 10 per liter. The presence of fungi of the genera *Aspergillus* and *Exophiala* was also confirmed in the selected drinking water sample with the next-generation sequencing (NGS) method based on the ITS2 rDNA sequence. Statistical analysis based on machine learning approach connected the emergence of fungi in tap water with a higher content of calcium (Ca^{2+} > 53 mg/l) and magnesium ions (5 mg/l < Mg^{2+} > 13 mg/l). At lower concentrations of calcium, however, the presence of fungi in water was dependent on nitrate. Surprisingly, chloride ions did not have any significant effect on the occurrence and diversity of fungi in the water. DGGE analysis, in which we compared the fungal communities from the rivers, groundwater, drinking water and water from water treatment plants in the area of the Ljubljana basin, showed that the occurrence of fungi is independent of the water type, but is linked to the aquifer location. Occurrence of certain fungal species in household appliances is connected to the water inlet, evident from the data of isolation of the following fungi from dishwashers, washing machines and water: *Aureobasidium melanogenum, Candida parapsilosis, Cystobasidium slooffiae, Exophiala phaeomuriformis*, fungi from *Fusarium oxysporum* species complex (FOSC) and the ubiquitous yeasts *Meyerozyma guilliermondii* and *Rhodotorula mucilaginosa*. These fungi were able to adapt to the living conditions within both household appliances. Nevertheless, some waterborne fungi were able to successfully colonize only washing machines (e.g. *Aspergillus niger, Exophiala lecanii-corni* and *E. mesophila*), or only dishwashers (e.g. *A. fumigatus, C. intermedia, Clavispora lusitaniae, Debaryomyces hansenii, E. dermatitidis,*

E . oligosperma and *Fusarium dimerum*). The observed differences may be the resulty of different temperatures inside the appliances. Sampled dishwashers were usually operating on higher temperatures but maximally at 68 °C, in comparison to sampled washing machines, where the possible 95 °C is rarely used. As an example we can list different species from genus *Exophiala*, where temperature plays the key role in habitat occupation, since *E. dermatitidis* and *E. phaeomuriformis* occur in environments with high temperatures, while the majority of species, represented by *E. equina, E. oligosperma* and *E. mesophila*, were isolated only from environments with mesophilic conditions. Some species of the genera *Candida, Exophiala, Pseudozyma, Rhinocladiella, Trichosporon* and *Yarrowia* were isolated from water samples, but were detected as well inside the appliances. Thus, we conclude that the conditions inside the appliances may have negative impact on their growth. Remaining fungi from separated groups in dishwashers and washing machines indicated that the water is not the only source for fungal contamination of the household appliances. Fungi belonging to such dishwasher group were mostly yeasts and yeast-like fungi associated with food (*Candida, Cryptococcus, Metschnikovia, Pichia, Saccharomyces* and *Saprochaete*), while the washing machine group was represented mainly by filamentous fungi associated with air, soil and plant material. These could be introduced to the washing machines with laundry (*Aureobasidium, Cladosporium, Fusarium, Mucor, Ochroconis, Penicillium* and *Phoma*). The results presented in the doctoral thesis, contribute to the knowledge of fungal communities within washing machines and dishwashers, and reveal the possible transmission route via tap water to the appliances. These findings enable risk assesment of infections potentially caused by emerging and oportunistic fungal pathogens particularly among immunocompromised people.

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ZAHVALA

Za sofinanciranje doktorskega študija preko Inovativne sheme (pogodba št. 1244) se zahvaljujem Ministrstvu za izobraževanje, znanost in šport ter Univerzi v Ljubljani: »*Doktorski študij je delno sofinancirala Evropska unija, in sicer iz Evropskega socialnega sklada. Sofinanciranje se izvaja v okviru Operativnega programa razvoja človeških virov za obdobje 2007-2013, 1. Razvojne prioritete Spodbujanje podjetništva in prilagodljivosti; prednostne usmeritve 1. 3: Štipendijske sheme*.«

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Dovoljenje založnika za objavo članka »Dishwashers - a man-made ecological niche accommodating human opportunistic fungal pathogens« v tiskani in elektronski verziji

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Dovoljenje založnika za objavo članka »*Candida* and *Fusarium* species known as opportunistic human pathogens from customer-accessible parts of residential washing machines« v tiskani in elektronski verziji

Article title: *Candida* and *Fusarium* species known as opportunistic human pathogens from customer-accessible parts of residential washing machines
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