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Microbial biofilm communities on Reverse Osmosis membranes in whey water processing before and after cleaning

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A B S T R A C T

The need for recovering valuable compounds and water from side streams has increased the use of Reverse Osmosis (RO) membrane filtration in the food industry. RO membranes, however, are highly susceptible to biofilm formation, which may decrease performance and increase industrial costs. In order to identify and characterize the biofilm forming communities, industrial RO membranes from whey water recovery lines in a dairy industry were investigated before and after Cleaning-In-Place (CIP) treatments. Phase contrast and Confocal Laser Scanning Microscopy (CLSM) were used to visualize the biofilms. The Heterotrophic Plate Count (HPC) and yeast population were enumerated, and 16S, 26S, and ITS rRNA sequencing was employed to identify the dominant isolates. A dense biofilm of the filamentous yeast species *Saprochaete clavata* and *Magnusiomyces spicifer* was observed together with budding yeasts and Gram-negative bacteria. The filamentous yeasts had long hyphae, which spatially dominated the biofilm on the retentate and permeate surface and they were not in-activated by the standard CIP treatment. Since neither plate counts nor DNA-based methods reflect the wide membrane coverage of the filamentous yeasts, their role in biofouling may easily be underestimated. We suggest that filamentous yeasts are included in further research on fouling of water treatment membranes in the dairy industry when investigating the effect of different CIP treatments or new RO membrane properties.

1. Introduction

The dairy industry is pursuing ways to be water neutral by decreasing the fresh water consumption as well as the environmental discharge. Whey from cheese production is considered a highly nutritional by-product, from which protein and lactose fractions can be recovered and at the same time the liquid fraction can be reconditioned for further purposes in the production. A representative example of such a process line can be found in the study of Stoica et al. (2018) [1]. The whey solution is first up-concentrated through Ultrafiltration (UF) for whey protein collection (R1). The permeate solution (P1) from UF is then subjected to RO filtration for lactose collection (R2). An evaporation process for whey protein and lactose powder reclamation follows the retentate collection from UF and RO. At the specific production site the permeate solution from the RO filtration (P3) is further treated through a second RO polisher filtration step (ROP) and a two stage UV-C (Ultraviolet) light treatment. After UV disinfection, the liquid is reused in direct or indirect product contact industrial processes.

The membrane type commonly used for water reconditioning is Thin Film Composite (TFC) RO membranes in a spiral wound configuration [1]. These membranes are highly efficient, but biofouling and flux reduction are well-known challenges. Therefore, CIP treatment is essential and should be applied regularly to remove fouling, regenerate membrane efficiency and increase membrane performance and life span [1–6].

CIP is a cleaning and sanitizing procedure in which alkaline and acidic detergents, disinfectants and hot water circulate through the interior surface of closed process equipment without dismantling [7]. Regardless of how thorough a CIP program is, some fouling will always be left facilitating further microbial regrowth on membrane surfaces [2,3,5,8]. Microorganisms surviving CIP cycles are more potent biofilm formers and can develop more resistant biofilm structures [2,6,9]. This is a problem for filtration efficacy, but could in theory also pose a risk for the quality of the up-concentrated solutions as well as the permeate water. In fact, recent reports show microbial presence in RO permeate water from water and dairy plants [10,11]. Skou et al. [11] indicate that some microorganisms can grow even in the low nutrient ROP permeate water. The microbiological issues have to be evaluated when process water is reused in the food industry in direct or indirect product contact, since they may affect the quality or safety of the food products

Table 1
The composition of the cleaning solutions used in CIP A, B and C programmes, applied in the process lines, from which the RO membrane elements were sampled in this study.

CIP treatments	Cleaning solutions	Composition
CIP A	Acidic pH 1.8–2.0/50 °C/45min.	Nitric acid Citric acid
	Water flush	
	Alkaline pH 11.0–11.5/50 °C/35min.	Potassium hydroxide EDTA Sodium hydroxide
	Water flush	
CIP B	Acidic pH < 2.5/50 °C/45min.	Methanesulfonic acid Citric acid
	Water flush	
	Alkaline pH 11.5/50 °C/35min.	Sodium hydroxide
	Water flush	
CIP C	Alkaline pH 9.5–10.3/50 °C/1min. Enzymatic 50 °C/40min.	Sodium hydroxide Alkylamine oxides Sulfates Subtilisin (non-specific protease) Alkylamine
	Alkaline pH 11.5/50 °C/20min.	Sodium hydroxide
	Water flush	
	Acidic pH < 2.5/50 °C/40min.	Methanesulfonic acid Citric acid
	Water flush	

[12]. However, as indicated in a recent review about microbial diversity on RO membranes, the effect of CIP on microbial diversity of RO elements has been poorly studied [8].

Biofilms are microbiological structures attached to surfaces and encased in a hydrated polymeric matrix, containing polysaccharides, proteins and nucleic acids, named extracellular polymeric substances (EPS). The EPS matrix enables microorganisms to stick together and offers protection from various stresses and harsh environmental conditions. Multi-genera biofilms are often thicker and more resistant than biofilms consisting of one genus [2,5,6,13] and several reports indicate the existence of multi-genera biofilms on UF and RO filtration membrane surfaces in the dairy industry [5,6,14]. However, most studies have been focusing on methods targeting bacteria.

Many dairies are now introducing treatment and fit-for-purpose re-use of the process liquids. Therefore, there is a need to understand better the total microbial diversity of RO membrane's biofilms in an industrial scale and how it is affected by different CIP treatments and membrane microstructure. Previously [1], we conducted a screening of six industrial RO membranes from a whey water recovery line in a dairy industry, employing CLSM and HPC to analyze the biofouling potential. We found multi-genera biofilm communities on the retentate and the permeate side of the RO membranes after CIP cleaning, even after relatively short (six months) use. In the current multi-approach study, we will identify the bacterial and fungal inhabitants of these biofilms and we will assess the impact of CIP treatments on the dominance of different genera, by combining selective media and sequencing methods and different microscopy techniques on both the retentate and permeate surface of the RO elements. The aim of this work is to provide essential knowledge of RO biofouling, which may help to develop more efficient removal strategies.

2. Materials and methods

2.1. A. RO membranes' sampling overview

In total, 12 TFC RO elements of spiral wound configuration were sampled from a whey water recovery line in the dairy industry. The characteristics of the sampled RO elements A-E and the sampling procedure were described previously [1]. All the RO elements belonged to the first of two consecutive RO filtration steps, since most dairy plants using whey water reconditioning employ only one RO filtration step.

The sampling was divided in three phases. Phase I included sampling of a RO element before CIP A (RO-A) and one after CIP A (RO-B), to assess the impact of CIP on biofouling level and diversity [1]. Phase II included the sampling of RO-BB, –C and -D, all collected after CIP B. For these elements, a different scenario was investigated: for RO-BB a different CIP program was employed, for RO-D the impact of a shorter membrane operating time (six months instead of three-four years) was looked at, and for RO-C an additional high heat treatment step after CIP B (78 °C/20min.) was tested for impact on biofouling level and diversity. All the RO elements were from parallel pressure vessels in the same whey water treatment line. RO-B, –BB, –C and -D were collected from the same pressure vessel. The membranes RO-BB, –C and -D were sampled both on the retentate and permeate side [1]. The CIP A and B differ in their formulation of the alkaline and acidic solutions used (Table 1). The RO elements A, B, BB, C, D, F1, F2, G1, G2, H1 and H2 belonged to Hypershell™ RO-8038-30 model, produced by Dow/Filmtec. The material was TFC, composed by a polyamide layer, on a polyethersulfone porous layer, on top of a non-woven fabric support material. A fourth element from a different production line (RO-E) was included and sampled on both the retentate and permeate side.

Table 2
Operating characteristics of the additional six RO elements sampled in Phase III of this study.

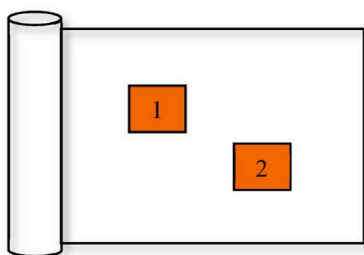| RO membrane element	F1	F2	G1	G2	H1	H2
Phase	III					
Use	Whey water treatment					
Cleaning formula	CIP B Elements collected after alkaline solution washing & water flushing					
Operating time	3–4 years					
RO side sampled	Permeate					

Fig. 1. Sampling outline for the RO membrane elements of Phase III; permeate side. Two spots were sampled from one sheet from each of the RO elements.

This element differed in both membrane composition and CIP programme used (Table 1) [1]. The RO-E element belonged to Hot Water Sanitizable RO Series model (HSRO-8038-30), produced by Alfa Laval Nakskov A/S. The material of this element was TFC with a top polyamide layer on a polyester support material.

Due to the initial observation of filamentous yeast biofilms on the permeate side of the elements in Phases I and II, the focus in Phase III was on the biofilm level and diversity on the permeate side. For this purpose, six more elements were sampled from the same whey water treatment line, after a washing step with alkaline solution for 30 min and water flushing. The elements encoded as RO-F1, -F2, -G1 were from the same pressure vessel and RO-G2, -H1 and -H2 from a parallel vessel (Table 2). The CIP B treatment was used on the elements examined in Phase III.

2.2. B. RO sampling procedure

The sampling procedure of the RO elements A-E was previously described [1]. Briefly, the RO elements were unfolded and the membrane sheets were divided into spots (10×10cm) evenly distributed across the membrane surface. In Phase I and II, four spots were sampled from two non-consecutive sheets on the retentate surface, in total eight spots from each element. In Phase II, one spot was additionally sampled from two non-consecutive sheets on the permeate surface, in total two spots from each element. In Phase III, two spots were sampled from one sheet on the permeate surface only, in total two spots from each element (Fig. 1).

Sampling for determination of the microbial population level and isolation of the different species was done by swabbing with sterile 10×10cm compress tissues pre-moisturized in 0.9% Saline Peptone Solution (SPS: 1 g/L peptone, 8.5 g/L sodium chloride, pH 7.2 ± 0.2). Tissues were placed in sterile stomacher bags with 50 mL of 0.9% SPS and mixed in Stomacher® 400 Circulator (Seward Limited, UK) for 1min. Ten-fold dilutions in 0.9% SPS followed. Non-selective Plate Count Agar (PCA, CM0325) was used for HPC enumeration and MYPG/antibiotics agar (10 g/L Glucose, 5 g/L Peptone, 3 g/L Yeast Extract, 3 g/L Malt Extract, 20 g/L Agar, pH 5.6 ± 0.2), supplemented with 0.1 g/L Chloramphenicol (Sigma C0378) and 0.05 g/L Chlorotetracycline (Sigma C4881) was used for yeast enumeration. In Phase I, HPC was enumerated on the retentate surface of RO-A and –B, while in Phase II HPC was enumerated on both the retentate and permeate surface of RO-BB, –C, -D and -E. In Phase III, HPC and yeast population were enumerated only on the permeate surface of RO-F1, -F2, -G1, -G2, -H1 and -H2 elements. Plates were incubated up to six days at 25 °C to allow for detection of the slow-growing strains. Total colony-forming-units (CFU) were determined and expressed as $\log_{10}(CFU/cm^2)$.

2.3. C. Microscopic and macroscopic photos

In order to visualize the biofilms on the retentate and permeate surface of the RO elements, CLSM microscopy was applied to RO membrane coupons, using the CLSM instrument Zeiss LSM 800 (Carl

Zeiss Inc.) equipped with an inverted microscope as described previously [1]. Phase contrast microscopy using the upright microscope Olympus BX43 (Olympus Scientific Solutions Americas Corp.) was applied on specimens scraped directly from the RO elements surface. Photos from the specimens from the upright microscope and from the RO membrane coupons surfaces were taken using a Samsung 16 MP, f/1.9, 28 mm (wide), 1/2.6″, 1.12 μm, OIS, AF camera.

2.4. D. Isolation of colonies from direct samples

Direct samples were collected by scraping using plastic sterile loops (Sigma-Aldrich) from the 12 RO elements, on the retentate and permeate surface. When no visual biofilm was observed, swabbing was applied instead, using sterile 10×10cm compress tissues. Direct samples were both streaked on non-selective PCA and yeast selective MYPG/antibiotics agar. Tissues were placed on PCA and MYPG/antibiotics agar and removed after one day. All plates were incubated at 25 °C up to six days. Material from individual colonies was purified prior to DNA extraction and sequencing.

2.5. E. Sampling from RO permeate streams

Complementary to RO membrane sampling, water permeate samples (P1–P6) were also collected from the same whey water process line after 10 h of production, following the last CIP B cycle. HPC and yeast population was determined in these samples [1]. However, the microbial population level and identification results cannot be directly compared with the RO-A to RO-D membranes' results, as the water samples were collected after the removal of these elements.

2.6. F. Determination of the dominant species from CFU plates

The individual colonies, grown at the highest dilutions on HPC and MYPG/antibiotics agar for CFU determination from Phases I, II, III and water sampling, were isolated and purified for sequencing and further identification.

DNA from all the isolates (direct and CFU) from Phase I, II, III and water sampling was extracted using BioRad™ Kit (BIO-RAD/20 ml/Cat. # 732–6030). For the PCR reactions the primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 907R (5′-CCG TCA ATT CCT TTR AGT TT-3′) were used for 16S rRNA sequencing, targeting bacteria. For yeast, the primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) were used for 26S rRNA and primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-GCATATCAATA AGCGGAGGA-3′) for ITS sequencing. PCR products were sequenced by Macrogen, (Netherlands). The results were analysed using CLC Genomics Workbench 10 and NCBI Database for identification.

3. Results

3.1. A. Dominant isolates from RO membrane elements

3.1.1. i. Phase I

As previously reported [1], RO-A had macroscopically obvious biofouling on the retentate surface, while CLSM photos on RO-A coupons revealed a dense biofilm consisting of filamentous yeasts, budding yeasts and bacteria. By comparing representative macroscopic and CLSM photos of membrane coupons with phase contrast microscopy photos on direct scraping biofouling material from RO-A membrane, we show that filamentous yeasts dominated the biofilm due to the larger cell size compared to budding yeasts and bacteria (Table 3).

HPC on the retentate side of RO-A, collected before CIP A was 5.94 ± 0.12 $\log_{10}(CFU/cm^2)$, while after CIP A the microbial population had decreased to 4.20 ± 0.13 $\log_{10}(CFU/cm^2)$ [1]. According to rRNA sequencing (Table 4), on the retentate side of RO-A before CIP A, the dominant bacteria belonged to *Pseudomonas* sp. while strains

Table 3

Macroscopic photos of sheets from spiral RO elements, CLSM on coupons and phase contrast microscopy photos of direct scrapings from the same RO elements on the retentate and permeate side, before and after CIP. The elements had been in use for 3–4 years. CLSM photos: Orange stain: Con A-carbohydrate matrix, green stain: Syto 9-nucleic acids and red stain: Sypro Ruby-protein matrix of the biofilm.
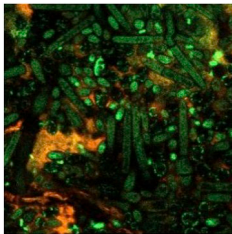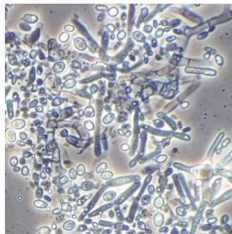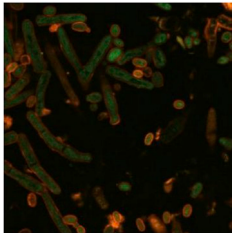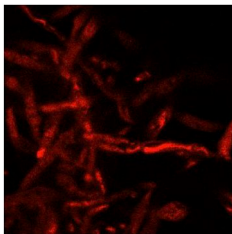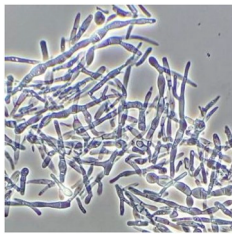
RO elements 3–4 years	Macroscopic image	CLSM	Phase contrast microscopy 400x magnification
RO-A Before CIP A Retentate side			No visual biofouling
RO-B After CIP A Retentate side			
RO-B After CIP A Permeate side			

Table 4

16S, 26S and ITS rRNA sequencing of direct isolates (direct scrapings from RO retentate and permeate surface) and dominant isolates (colonies isolated from the CFU plates of the highest serial dilutions) from RO-A and RO-B elements. E-value is 0.00 and (%) Identity is 96.5–100.

Membrane 3–4 years	RO Side	16S/26S/ITS rRNA Sequencing results	
RO-A before CIP A	Retentate	*Pseudomonas* sp.	Dominant
		Sporopachydermia lactativora	Direct isolate
RO-B after CIP A	Retentate	*Saprochaete clavata/ Magnusiomyces spicifer*	Dominant
		Sporopachydermia lactativora	Dominant
		Pichia norvegensis	Direct isolate
	Permeate	*Saprochaete clavata /Magnusiomyces spicifer*	Direct isolate

belonging to the budding yeast *Sporopachydermia lactativora* were isolated by direct sampling. Filamentous yeast strains were not detected on HPC, although they could be observed microscopically on the RO-A retentate surface (Table 3), since the non-selective HPC plates/25 °C were overgrown by bacteria after 18 h of incubation.

The CIP A treatment had visually removed biofouling from the retentate surface of RO-B (Table 3) and therefore direct scraping sample was not obtained. However, CLSM showed that only bacteria were removed, while much of the yeast network remained [1]. The strains isolated from RO-B retentate side samples on the non-selective (PCA) agar plates, belonged to the filamentous yeast species *Saprochaete clavata* and *Magnusiomyces spicifer* and the budding yeast *Sporopachydermia lactativora* (Table 4). The yeast colonies needed more than three days to be macroscopically visible on PCA plates/25 °C.

When the RO-B membrane was examined on the permeate surface,

we observed macroscopically obvious fouling which, according to phase contrast microscopy and CLSM, consisted of filamentous yeasts (Table 3). Isolates from scrapings, taken from the RO-B permeate side belonged to the filamentous yeast species *Saprochaete clavata and Magnusiomyces spicifer* (Table 4).
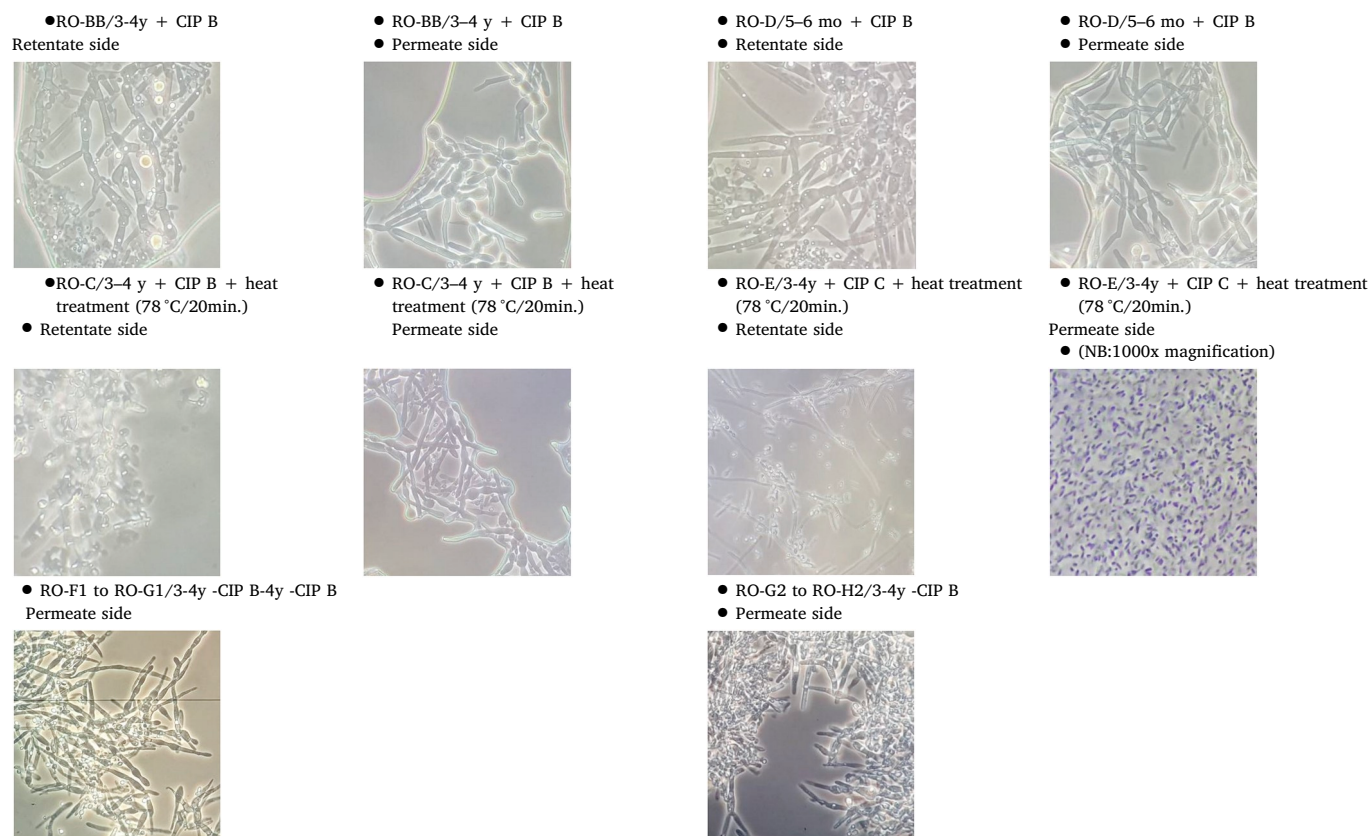
3.1.2. ii. Phase II

Since all the elements in Phase II were collected after CIP B treatment, low numbers of microbial population were expected. However, visual biofouling was detected across the retentate and - surprisingly-the permeate surface of elements RO-BB, –C, -D and –E [1]. Mainly filamentous yeasts were observed on the direct scraping samples taken during membrane autopsy, using the phase contrast microscope (Table 5), as well as in the CLSM observations [1].

Whether the membranes had been in use for three to four years (RO-BB) or only six months (RO-D) the HPC on the retentate side were at similar levels, 5.69 ± 0.63 and 5.56 ± 0.58 $\log_{10}(CFU/cm^2)$, respectively [1]. Although both RO-BB and -D were collected after CIP treatment (CIP B), they had similar microbial population levels with RO-A collected before CIP treatment (CIP A) in Phase I. RO-BB and -D had relatively high microbial population levels on the permeate side as well; 4.51 ± 0.60 $\log_{10}(CFU/cm^2)$ and 3.88 ± 0.50 $\log_{10}(CFU/cm^2)$, respectively.

According to isolation and sequencing results, the biofilm on RO-BB retentate side consisted of bacteria belonging to *Raoultella* sp. and on the permeate side to *Raoultella* sp. and *Escherichia* sp. (Table 6). On RO-D, the dominant isolates on the retentate side belonged to *Raoultella* sp., *Enterobacter* sp., *Escherichia* sp., *Enterococcus* sp. and on the permeate side to *Enterobacter* sp., *Lelliottia* sp. and *Acinetobacter* sp. However, the application of an additional high heat treatment to RO-C decreased HPC below the LOD (1.3 $\log_{10}(CFU/cm^2)$ [1]. In RO-C, strains belonging to

Table 5

Phase contrast microscopy photos from direct scrapings from RO-BB to RO-G2 retentate and permeate side. Magnification 400x.

● RO-BB/3-4y + CIP B Retentate side	● RO-BB/3-4 y + CIP B ● Permeate side	● RO-D/5-6 mo + CIP B ● Retentate side	● RO-D/5-6 mo + CIP B ● Permeate side
● RO-C/3-4 y + CIP B + heat treatment (78 °C/20min.) ● Retentate side	● RO-C/3-4 y + CIP B + heat treatment (78 °C/20min.) Permeate side	● RO-E/3-4y + CIP C + heat treatment (78 °C/20min.) ● Retentate side	● RO-E/3-4y + CIP C + heat treatment (78 °C/20min.) Permeate side ● (NB:1000x magnification)
● RO-F1 to RO-G1/3-4y -CIP B-4y -CIP B Permeate side		● RO-G2 to RO-H2/3-4y -CIP B ● Permeate side	

Bacillus sp. and *Acinetobacter* sp. were isolated from scrapings from both the retentate and permeate side.

RO-E had lower HPC on the retentate and permeate side as well; $3.07 \pm 0.38 \log_{10}(CFU/cm^2)$ and $1.79 \pm 0.00 \log_{10}(CFU/cm^2)$, respectively [1]. In RO-E, strains belonging to *Pseudomonas* sp. and *Acinetobacter* sp. were dominant on the retentate side, while *Pseudomonas* sp. were directly isolated on the permeate side (Table 5).

Filamentous yeasts belonging to the sister genera *Saprochaete clavata* and *Magnusiomyces spicifer* were repeatedly isolated through direct sampling on both the retentate and permeate side of all the elements on Phase II, apart from the permeate side of RO-E (Tables 5 and 6). On the retentate side of RO-E the following isolates belonging to closely related filamentous yeast genera were identified after direct sampling: *Trichosporon jirovecii, Geotrichum klebahnii, Saprochaete clavata, Magnusiomyces spicifer*. Filamentous yeasts were also observed on the retentate and permeate side of RO-C, but the high heat treatment had probably inactivated the yeasts, since no growth was observed on the yeast selective agar.

3.1.3. iii. Phase III

The microbial population on the permeate surface of the six RO elements in Phase III was between 2.87 ± 0.49 and $4.77 \pm 0.46 \log_{10}$ (CFU/cm^2) (Fig. 2). The isolated strains from the higher dilutions of HPC and MYPG/antibiotics agar plates belonged to *Saprochaete clavata* and *Magnusiomyces spicifer* (Identity 99–100%, E-value 0.00). Strains belonging to *Enterobacter* sp. were directly isolated on the permeate side of these six elements. Filamentous yeasts dominated the biofilms of both retentate and permeate side of all the elements of Phase III, according to phase contrast microscopy photos from the direct scraping samples (Table 5).

3.2. B. Whey water permeate sampling

The CFU/mL of the water permeate samples after each of the water treatment steps has been presented in our previous work [1]. The identification results of the dominant isolates from these samples are presented in Table 7. Both selective (MYPG/antibiotics) and non-selective (PCA) for yeast media were used during sampling. In the water permeate sample after UF (P1), the dominant isolates were identified as *Pseudomonas* sp. and the yeasts *Sporopachydermia lactativora, Candida pseudoglobosa* and *Saprochaete clavata/Magnusiomyces capitatus*. In the water permeate sample after the first RO (P2), *Saprochaete clavata* and *Sporopachydermia lactativora* were detected. In the water permeate sample after the second RO (P3), *Stenotrophomonas* sp./*Pseudomonas* sp. were detected. After UF, the cfu levels were in the order of 1–3 $\log_{10}$ (CFU/mL), but after P2 the cfu number fell to the order of 1 $\log_{10}$(CFU/mL) or below the LOD (1 cfu/mL). The values were thus far below those set for drinking water quality.

4. Discussion

4.1. A. CIP persistent multispecies biofilms on RO membrane surfaces

In this multi-approach study, we investigated industrial RO membranes used for different time spans and exposed to different CIP treatments from a whey water recovery line to assess the level and diversity of the microbial population in the biofilm communities. By combining selective media and sequencing methods for yeasts, and different microscopic techniques, we showed that multi-genera biofilm communities resistant to CIP were established on both the retentate and permeate surface of the RO elements, even after only six months of use. Persistent biofilms on the retentate side of RO membranes for whey

Table 6

16S, 26S and ITS rRNA sequencing of direct isolates (direct scrapings from RO retentate and permeate surface) and dominant isolates (colonies isolated from the CFU plates of the highest serial dilutions) from RO-BB, –C, -D and -E elements. E-value is 0.00 and (%) Identity is 96.5–100.

Membrane	RO side	16S/26S/ITS rRNA Sequencing results	
RO-BB	Retentate	*Raoultella* sp.	Dominant
3–4 years		*Pichia norvegensis*	Direct isolate
+ CIP B	Permeate	*Raoultella* sp.	Dominant
		Escherichia sp.	Dominant
		Saprochaete clavata /Magnusiomyces spicifer	Direct isolate
RO-D	Retentate	*Raoultella* sp.	Dominant
5–6 months		*Escherichia* sp.	Dominant
+ CIP B		*Enterobacter* sp.	Dominant
		Enterococcus sp.	Dominant
	Permeate	*Lelliottia* sp.	Dominant
		Enterobacter sp.	Dominant
		Acinetobacter sp.	Dominant
		Saprochaete clavata /Magnusiomyces spicifer	Direct isolate
		Pichia norvegensis	Direct isolate
		Sporopachydermia lactativora	Direct isolate
RO-C	Retentate	*Bacillus* sp.	Direct isolate
3–4 years	Permeate	*Acinetobacter* sp.	Direct isolate
+ CIP B			
+ heat treatment			
(78 °C/20min.)			
RO-E	Retentate	*Pseudomonas* sp.	Dominant
+ CIP C		*Acinetobacter* sp.	Dominant
+ heat treatment		*Trichosporon jirovecii*	Direct isolate
(78 °C/20min.)		*Geotrichum klebahnii*	Direct isolate
		Saprochaete clavata /Magnusiomyces spicifer	Direct isolate
	Permeate	*Pseudomonas* sp.	Direct isolate

Table 7

Identification of the water permeate samples' isolates. 16S rRNA and 26S rRNA sequencing was used for bacteria and yeasts isolates, respectively. E-value is 0.00 and (%) Identity is 98–100%.

Water permeate sample	Media	Sequencing results
P1	PCA	Pseudomonas sp.
	MYPG	*Sporopachydermia* lactativora
		Saprochaete *clavata*/Magnusiomyces *capitatus*
		Candida pseudoglobosa
P2	MYPG	Saprochaete *clavata*
		Sporopachydermia lactativora
P3	PCA	Stenotrophomonas sp. /Pseudomonas sp.
P4	*CFU/mL < LOD*	
P5	*CFU/mL < LOD*	
P6	*CFU/mL < LOD*	

4.2. B. Effect of CIP and membrane microstructure to biofilm communities

Our results indicate that the different CIP recipes had different cleaning efficiencies depending on their composition. CIP A consisted of an acidic solution, containing the strong inorganic nitric acid and the organic citric acid followed by an alkaline solution, containing potassium hydroxide, sodium hydroxide and the chelating agent EDTA. This formulation removed efficiently the bacterial population in RO-B, but the yeast biofilm remained attached on membrane surface. However, CIP B, which consisted of an acidic solution of two organic acids (methanosulfonic and citric acid) and an alkaline solution of sodium hydroxide, was neither efficient in removing the bacterial nor the yeast population from RO-BB and -D. In fact, the microbial level of RO-BB and -D on the retentate side was similar to RO-A *before* CIP application. Also the permeate side of RO-BB and RO-D had high bacterial numbers.

RO membranes have extremely small pores (0.0001–0.001μm) and bacteria are theoretically incapable to pass to the permeate surface. That the same species were found on both the retentate and permeate side of different membrane elements in the same or parallel vessels could indicate potential leakage in RO membranes or in the RO
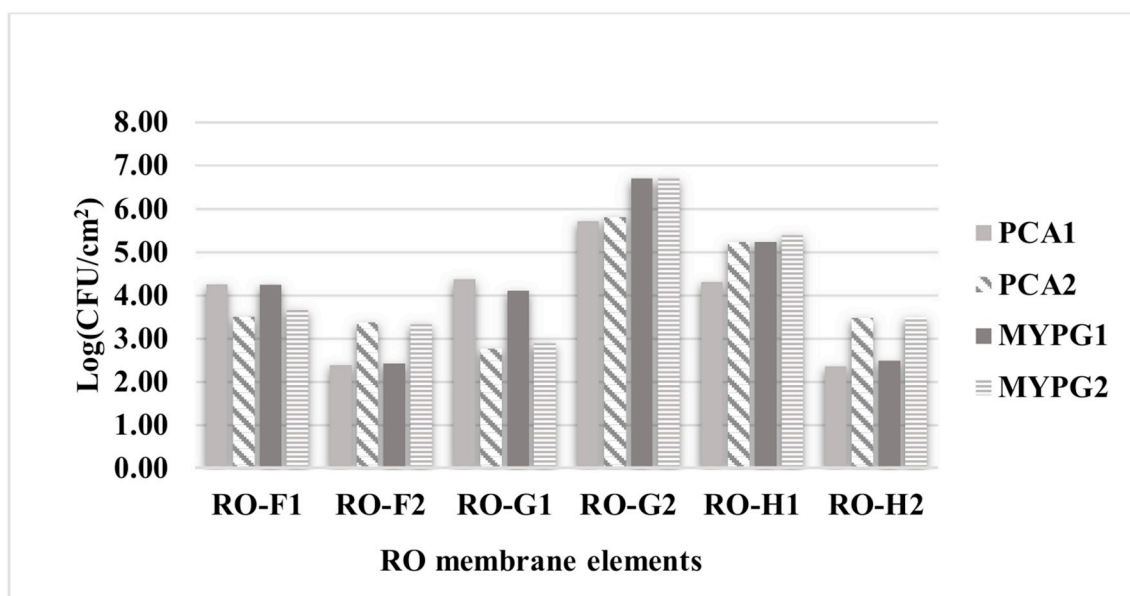
concentration after CIP have been previously reported by Anand et al. [14]. Looking at bacterial levels and diversity, they found microbial populations at similar levels, namely between 3.5 and 5.5 $\log_{10}$(CFU/cm^2) already after two, four, and six months of use.

Fig. 2. HPC and yeast counts on non-selective PCA/25 °C and selective for yeast MYPG/25 °C agar, respectively. Sampling was conducted on the permeate side of RO-F1, -F2, -G1, -G2, -H1 and -H2. Two spots were sampled from RO membrane permeate surface. Bars represent the microbial population in $\log_{10}$ (CFU/cm^2) from the two spots plated in PCA (PCA$_1$ and PCA$_2$) and MYPG (MYPG$_1$ and MYPG$_2$) agar media. LOD: 5 (cells/cm^2) or 0.7 $\log_{10}$ (CFU/cm^2).

installation fittings [3,4,10]. It has been reported that the use of caustic solutions on filtration membranes increases the pore size and decreases the pore density, while the application of the acidic solution afterwards tends to restore this balance [15]. Also high temperatures have been found to increase pore size [16]. At the sampling site we investigated, the acidic solution was applied first and the alkaline solution afterwards in RO-A,-B,-BB, –C, -D and RO-F1 to RO-H2 membranes. In all of these elements, filamentous yeasts were detected on both the retentate and permeate surface. However, in the RO-E membrane, where the yeasts were only detected on the retentate surface, the CIP C formulation included the application of the alkaline solution at the beginning, while the acidic solution was applied at the end of the programme.

Additionally, hydrophobicity and roughness of membrane material in relation with surface cell characteristics of the microbial cells could affect microbial adhesion [5,6,8,13]. Therefore, the hyphal cell [17], which is rich in chitin, a hydrophobic material, may attach more easily to the non-woven fabric support material of the permeate membrane surface.

In the future, it would be interesting to investigate if and to which extent induced pore size changes or fitting defects are involved in the transmission of microbial cells to the permeate side of the RO elements.

4.3. C. Filamentous yeast dominates the biofilm communities

To our surprise filamentous yeasts dominated the biofilms on both the retentate and the permeate surface, even after CIP application, according to microscopy results of this study and of Stoica et al. [1]. Furthermore, on the permeate side of RO-B and RO-F1 to RO-H2, only filamentous yeasts were isolated from the CFU plates of the higher dilutions. The filamentous yeast isolates were identified as *Saprochaete clavata* and *Magnusiomyces spicifer*. A filamentous yeast isolate belonging to *Blastoschizomyces capitatus* (later renamed *Magnusiomyces capitatus* [18]) was identified by Tang et al. [6] together with *Pseudomonas* sp., *Klebsiella* sp. *and Bacillus* sp. from scraping samples of a RO membrane used for whey filtration in a New Zealand dairy plant. This shows that filamentous yeasts can be found on RO membranes used in this type of production in different geographical settings.

Several recent studies of dishwashers report the isolation of *Saprochaete/Magnusiomyces* spp [19–22]. This indicates a certain tolerance to cleaning agents. The existence of filamentous fungi in water and their ability to form resistant biofilms in water distribution systems [23–25] could suggest water as a possible transmission route.

The yeasts *Saprochaete clavata* and *Magnusiomyces spicifer* are not considered pathogens. However, they have been associated with nosocomial outbreaks in severely immunocompromised persons [26–34].

The biofilm communities in our study included also budding yeast species (*Sporopachydermia lactativora*) and Gram-negative bacteria (*Raoultella* sp., *Pseudomonas* sp., *Escherichia* sp. *and Enterobacter* sp.*)* which seemed to be in much higher numbers according to colony forming units on non-selective media. The filamentous yeasts seemed to be dominant in terms of biomass as documented by CLSM and phase contrast microscopy. Interestingly, the filamentous yeasts were found to constitute a larger part of the countable population on the permeate surface in Phase III, when selective substrate was used.

We observed that although filamentous yeast cells can spread by filamentous growth on membrane surface covering a great area compared to bacteria, they grow considerably slower than bacteria and may be outgrown on non-selective media. A further complication of using cfu alone for the estimation of the microbial population level is that the difference in the cells' biomass could lead to underestimation. One filamentous cell spreading along a large area on membrane surface would only give rise to one count on an agar plate.

4.4. D. Dominant gram-negative bacteria in biofilm communities

Different bacteria species such as *Pseudomonas* sp., *Escherichia* sp.,

Enterobacter sp. and *Raoultella* sp.were isolated from the retentate and the permeate surface after CIP in high numbers. These bacteria are Gram-negative slime producing bacteria with high biofilm formation potential [35–37]. Al Ashhab et al. [9] showed, in a lab scale experiment using TFC RO membranes filtrating an artificial Tertiary Wastewater solution, that repeating cleaning cycles could select for the microbial groups that attach to the TFC material of RO membrane surface and favour those producing rigid and adhesive EPS. They found that γ-Proteobacteria and specifically *Pseudomonadaceae* were dominant. The *Enterobacter, Klebsiella, Raoultella, Pseudomonas* and *Escherichia* genera isolated in the current study, belong to the class γ-proteobacteria. The same study [9] found that consecutive cleaning cycles led to proliferation of Ascomycota phylum. The *Saprochaete* and *Magnusiomyces* genera belong to this phylum. This could be an indication that these filamentous species are wide spread in different water streams and repeated CIP cycles favour their dominance.

According to the Drinking Water Quality Guidelines [38,39], *E. coli* or thermotolerant coliforms and enterococci must not be detectable in 100 mL of water sample, if this is to be considered water of drinking quality. Some of the genera found in this study belong to coliforms (*Enterobacter* sp., *Escherichia* sp.), indicators of the microbiological water quality and safety. However, no *E. coli*, coliforms or enterococci were detected in the permeate water samples collected further down the same process line. Moreover, HPC and the total yeast population was below the LOD (1 cfu/mL) after the last UV-C treatment step (P6).

5. Conclusion

Twelve RO elements from a whey water filtration unit were sampled to study the biofilm communities before and after CIP. We detected high numbers of microbial population on the retentate and permeate surface of the elements, established already after six months on use. The biofilms consisted of the filamentous yeasts *Magnusiomyces spicifer* and *Saprochaete clavata*, the budding yeasts *Sporopachydermia lactativora* and Gram-negative bacteria such as *Pseudomonas* sp., *Raoultella* sp., *Escherichia* sp., *and Enterobacter* sp.. The results indicate that CIP treatments were inefficient at removing the biofilm structures, leading to fast microbial regrowth.

The filamentous yeasts dominated the biofilm in all the RO elements developing long hyphae covering large areas of the membrane sheets. However, these relatively slow growing yeasts were not detected on the non-selective agar media when bacteria were also present in high numbers due to overgrowth by the latter. This, together with the fact that one yeast cell will be interpreted as one colony on agar plate even though it has much larger coverage and biomass, could lead to the yeasts being overlooked if selective methods for yeast isolation and identification are not used.

Increasing number of studies indicate the existence of multi-bacterial biofilm communities on RO membranes used for water treatment. Our findings suggest that it can be highly relevant to focus also on fungal contribution to the formation of resistant biofilms in RO membranes. A better understanding of the microbial development in relation to different CIP treatments and their interactions with membranes as well as potential routes of permeate contamination will help to design procedures for efficient food process water treatment for reuse.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.memsci.2019.117174.

References

[1] I.M. Stoica, E. Vitzilaiou, H. Lyng Røder, M. Burmølle, D. Thaysen, S. Knøchel, F. van den Berg, Biofouling on RO-membranes used for water recovery in the dairy industry, J. Water Process Eng. 24 (2018) 1–10 https://doi.org/10.1016/j.jwpe.2018.05.004.

[2] S. Anand, D. Singh, M. Avadhanula, S. Marka, Development and control of bacterial biofilms on dairy processing membranes, Compr. Rev. Food Sci. Food Saf. 13 (2013) 18–33, https://doi.org/10.1111/1541-4337.12048.

[3] H.F. Ridgway, A. Kelly, C. Justice, B.H. Olson, Microbial fouling of reverse-osmosis membranes used in advanced wastewater treatment technology: chemical, bacteriological, and ultrastructural analyses, Appl. Environ. Microbiol. 45 (1983) 1066–1084 https://www.ncbi.nlm.nih.gov/pubmed/6847180.

[4] P. Kumar, N. Sharma, R. Ranjan, S. Kumar, Z.F. Bhat, D.K. Jeong, Perspective of membrane technology in dairy industry: a review, Asian-Australas. J. Anim. Sci. 26 (2013) 1347–1358, https://doi.org/10.5713/ajas.2013.13082.

[5] A.N. Hassan, S. Anand, M. Avadhanula, Microscopic observation of multispecies biofilm of various structures on whey concentration membranes, J. Dairy Sci. 93 (2010) 2321–2329, https://doi.org/10.3168/jds.2009-2800.

[6] X. Tang, S.H. Flint, J.D. Brooks, R.J. Bennett, Factors affecting the attachment of micro-organisms isolated from ultrafiltration and reverse osmosis membranes in dairy processing plants, J. Appl. Microbiol. 107 (2009) 443–451, https://doi.org/10.1111/j.1365-2672.2009.04214.x.

[7] A. Thomas, C T Sathian, Cleaning-In-Place (CIP) System in Dairy Plant- Review, (2014), https://doi.org/10.9790/2402-08634144.

[8] O. Sánchez, Microbial diversity in biofilms from reverse osmosis membranes , A short review 545 (2018) 240–249, https://doi.org/10.1016/j.memsci.2017.09.082.

[9] A. Al Ashhab, A. Sweity, B. Bayramoglu, M. Herzberg, O. Gillor, Biofouling of reverse osmosis membranes: effects of cleaning on biofilm microbial communities, membrane performance, and adherence of extracellular polymeric substances, Biofouling 33 (2017) 397–409, https://doi.org/10.1080/08927014.2017.1318382.

[10] B. Buysschaert, L. Vermijs, A. Naka, N. Boon, B. De Gusseme, Online flow cytometric monitoring of microbial water quality in a full-scale water treatment plant, Npj Clean Water 1 (2018) 16, https://doi.org/10.1038/s41545-018-0017-7.

[11] P.B. Skou, B. Khakimov, T.H. Hansen, S.D. Aunsbjerg, S. Knøchel, D. Thaysen, F. van den Berg, Chemical characterization by gas chromatography-mass spectrometry and inductively coupled plasma-optical emission spectroscopy of membrane permeates from an industrial dairy ingredient production used as process water, J. Dairy Sci. 101 (2018) 135–146, https://doi.org/10.3168/jds.2017-12950.

[12] S. Casani, M. Rouhany, S. Knøchel, A discussion paper on challenges and limitations to water reuse and hygiene in the food industry, Water Res. 39 (2005) 1134–1146 https://doi.org/10.1016/j.watres.2004.12.015.

[13] C.G. Kumar, S. Anand, Significance of microbial biofilms in food industry: a review, Int. J. Food Microbiol. 42 (1998) 9–27 https://doi.org/10.1016/S0168-1605(98)00060-9.

[14] S. Anand, A. Hassan, M. Avadhanula, The Effects of Biofilms Formed on Whey Reverse Osmosis Membranes on the Microbial Quality of the Concentrated Product, (2012), https://doi.org/10.1111/j.1471-0307.2012.00848.x.

[15] A. Simon, W.E. Price, L.D. Nghiem, Influence of formulated chemical cleaning reagents on the surface properties and separation efficiency of nanofiltrationmembranes, J. Membr. Sci. 432 (2013) 73–82 https://doi.org/10.1016/j.memsci.2012.12.029.

[16] R.R. Sharma, S. Chellam, Temperature effects on the morphology of porous Thin Film composite nanofiltration membranes, Environ. Sci. Technol. 39 (2005) 5022–5030, https://doi.org/10.1021/es0501363.

[17] E. Paramonova, B.P. Krom, H.C. van der Mei, H.J. Busscher, P.K. Sharma, Hyphal content determines the compression strength of Candida albicans biofilms, Microbiology 155 (2009) 1997–2003 http://mic.microbiologyresearch.org/content/journal/micro/10.1099/mic.0.021568-0.

[18] G.S. de Hoog, M.T. Smith, C.P. Kurtzman, J.W. Fell, T.B.T.-T.Y (Eds.), Chapter 45 - Magnusiomyces Zender (1977), Fifth E. Boekhout, Elsevier, London, 2011, pp. 565–574 https://doi.org/10.1016/B978-0-444-52149-1.00045-8.

[19] J. Zupančič, M. Novak Babič, P. Zalar, N. Gunde-Cimerman, The black yeast exophiala dermatitidis and other selected opportunistic human fungal pathogens spread from dishwashers to kitchens, PLoS One 11 (2016) e0148166 https://doi.org/10.1371/journal.pone.0148166.

[20] A. Döğen, E. Kaplan, Z. Öksüz, M.S. Serin, M. Ilkit, G.S. de Hoog, Dishwashers are a major source of human opportunistic yeast-like fungi in indoor environments in Mersin, Turkey, Med. Mycol. 51 (2013) 493–498 https://doi.org/10.3109/13693786.2012.738313.

[21] R. Gümral, B. Özhak-Baysan, A. Tümgör, M.A. Saraçlı, Ş.T. Yıldıran, M. Ilkit, J. Zupančič, M. Novak-Babič, N. Gunde-Cimerman, P. Zalar, G.S. Hoog, Dishwashers provide a selective extreme environment for human-opportunistic yeast-like fungi, Fungal Divers. 76 (2016) 1, https://doi.org/10.1007/s13225-015-0327-8.

[22] P. Zalar, M. Novak, G.S. de Hoog, N. Gunde-Cimerman, Dishwashers – a man-made ecological niche accommodating human opportunistic fungal pathogens, Fungal Biol 115 (2011) 997–1007 https://doi.org/10.1016/j.funbio.2011.04.007.

[23] G. Hageskal, A.K. Knutsen, P. Gaustad, G.S. de Hoog, I. Skaar, Diversity and significance of mold species in Norwegian drinking water, Appl. Environ. Microbiol. 72 (2006) 7586–7593, https://doi.org/10.1128/AEM.01628-06.

[24] G. Hageskal, N. Lima, I. Skaar, The study of fungi in drinking water, Mycol. Res. 113 (2009) 165–172 https://doi.org/10.1016/j.mycres.2008.10.002.

[25] M.S. Doggett, Characterization of fungal biofilms within a municipal water distribution system, Appl. Environ. Microbiol. 66 (2000) 1249 LP – 1251.

[26] M. Gurgui, F. Sanchez, F. March, J. Lopez-Contreras, R. Martino, A. Cotura, M.L. Galvez, C. Roig, P. Coll, Nosocomial outbreak of < em > Blastoschizomyces capitatus < /em > associated with contaminated milk in a haematological unit, J. Hosp. Infect. 78 (2011) 274–278, https://doi.org/10.1016/j.jhin.2011.01.027.

[27] S. Vaux, A. Criscuolo, M. Desnos-Ollivier, L. Diancourt, C. Tarnaud, M. Vandenbogaert, S. Brisse, B. Coignard, F. Dromer, Multicenter outbreak of infections by Saprochaete clavata, an unrecognized opportunistic fungal pathogen, mBio 5 (2014) 1–10, https://doi.org/10.1128/mBio.02309-14.

[28] T. Birrenbach, S. Bertschy, F. Aebersold, N.J. Mueller, Y. Achermann, K. Muehlethaler, S. Zimmerli, Emergence of Blastoschizomyces capitatus yeast infections, central europe, emerg, Inf. Disp. 18 (2012) 98–101, https://doi.org/10.3201/eid1801.111192.

[29] M. Picard, S. Cassaing, P. Letocart, X. Verdeil, C. Protin, P. Chauvin, X. Iriart, L. Cavalie, A. Valentin, B. Marchou, J. Ruiz, B. Riu-Poulenc, F. Huguet, C. Recher, Concomitant cases of disseminated Geotrichum clavatum infections in patients with acute myeloid leukemia, Leuk. Lymphoma 55 (2014) 1186–1188, https://doi.org/10.3109/10428194.2013.820290.

[30] M.I. Del Principe, L. Sarmati, M. Cefalo, C. Fontana, G. De Santis, F. Buccisano, L. Maurillo, E. De Bellis, M. Postorino, G. Sconocchia, G. Del Poeta, M. Sanguinetti, S. Amadori, L. Pagano, A. Venditti, A cluster of Geotrichum clavatum (Saprochaete clavata) infection in haematological patients: a first Italian report and review of literature, Mycoses 59 (2016) 594–601, https://doi.org/10.1111/myc.12508.

[31] A. Ulu-Kilic, M.A. Atalay, G. Metan, F. Cevahir, N. Koç, B. Eser, M. Çetin, L. Kaynar, E. Alp, Saprochaete capitata as an emerging fungus among patients with haematological malignancies, Mycoses 58 (2015) 491–497, https://doi.org/10.1111/myc.12347.

[32] V. Camus, M.-L. Thibault, M. David, G. Gargala, P. Compagnon, F. Lamoureux, C. Girault, J.-B. Michot, A. Stamatoullas, H. Lanic, F. Jardin, P. Lenain, H. Tilly, S. Leprêtre, Invasive Geotrichum clavatum fungal infection in an acute myeloid leukaemia patient: a case report and review, Mycopathologia 177 (2014) 319–324, https://doi.org/10.1007/s11046-014-9746-4.

[33] S. Favre, A. Rougeron, L. Levoir, B. Pérard, N. Milpied, I. Accoceberry, F. Gabriel, S. Vigouroux, Saprochaete clavata invasive infection in a patient with severe aplastic anemia: efficacy of voriconazole and liposomal amphotericin B with adjuvant granulocyte transfusions before neutrophil recovery following allogeneic bone marrow transplantation, Med. Mycol. Case Rep. 11 (2016) 21–23 https://doi.org/10.1016/j.mmcr.2016.03.001.

[34] L. Durán Graeff, D. Seidel, M.J.G.T Vehreschild, A. Hamprecht, A. Kindo, Z. Racil, J. Demeter, S. De Hoog, U. Aurbach, M. Ziegler, H. Wisplinghoff, O.A. Cornely, Invasive infections due to Saprochaete and Geotrichum species: report of 23 cases from the FungiScope registry, Mycoses 60 (2017) 273–279, https://doi.org/10.1111/myc.12595.

[35] B.S. Scales, R.P. Dickson, J.J. LiPuma, G.B. Huffnagle, Microbiology, Genomics, and clinical significance of the Pseudomonas fluorescens species complex, an unappreciated colonizer of humans, Clin. Microbiol. Rev. 27 (2014) 927–948, https://doi.org/10.1128/CMR.00044-14.

[36] M. Drancourt, C. Bollet, A. Carta, P. Rousselier, Phylogenetic analyses of Klebsiella species delineate Klebsiella and Raoultella gen. nov., with description of Raoultella ornithinolytica comb. nov., Raoultella terrigena comb. nov. and Raoultella planticola comb. nov. Int. J. Syst. Evol. Microbiol. 51 (2001) 925–932, https://doi.org/10.1099/00207713-51-3-925.

[37] M. Wu, X. Li, Chapter 87 - Klebsiella pneumoniae and Pseudomonas aeruginosa A2, in: Tang Yi-Wei, M. Sussman, D. Liu, I. Poxton, J.D. Schwartzman (Eds.), M.M.M, Second E. Schwartzman, Academic Press, Boston, 2015, pp. 1547–1564 https://doi.org/10.1016/B978-0-12-397169-2.00087-1.

[38] WHO, Guidelines for Drinking-Water Quality, fourth ed., (2011).

[39] ILSI Research Foundation, Water Recovery and Reuse: Guideline for Safe Application of Water Conservation Methods in Beverage Production and Food Processing, (2013).