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Long-term Evaluation of Culturable Fungi in a Natural Aging Biofilm on Galvanised Steel Surface

Fungi in aging biofilm on galvanised steel surfaces

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Fungi commonly found in municipal water can participate in natural biofilm formation on the surface of galvanised steel despite the toxic effect of zinc. Depending on the age of the biofilm, fungal diversity may vary. To examine this hypothesis, natural biofilm formation was allowed on galvanised steel surfaces over six months in a model recirculating water system. Fungal colonies with different morphologies were obtained monthly from biofilm and water samples and then identified by both morphological and molecular approaches. In addition, the biofilm layer was examined by electrochemical impedance spectroscopy (EIS) analysis and scanning electron microscopy (SEM). It was determined that fungi were included in the naturally aging biofilm formed on galvanised steel surfaces during the experiment. The diversity and the number of fungi in the biofilm and water changed over the experiment. All fungi isolated from the biofilm and water were found to be members of the Ascomycota phylum. *F. oxysporum* was the first fungus to be involved in the biofilm formation process and was one of the main inhabitants of the biofilm together with *Penicillium* spp. In addition, EIS data showed that the structure of the biofilm changed as it aged. The results of this study may lead to a better understanding of naturally aging biofilms involving fungi in municipal water systems, as well as the development of new strategies for effective disinfection of fungi based on biofilm age.

1. Introduction

Biofilm is one of the major problems encountered in municipal water systems. Besides being the most common threat to the performance and longevity of the water system, it also poses a significant risk to human health as it may contain opportunistic pathogenic microorganisms (1, 2). Biofilm studies in municipal water systems have generally focused on bacteria (1, 3, 4). However, under natural conditions, biofilms are usually complex communities including bacteria, fungi and other microorganisms (5, 6).

Although bacteria are known to be the pioneer colonisers, eukaryotes such as fungi or protozoa can be included in the biofilm as secondary colonisers. Fungi may contribute to the formation of the

extracellular polymeric substances (EPS) of the biofilm with their metabolic products humic acids and aliphatic compounds. Furthermore, fungal hyphae and yeast pseudohyphae formed during the maturation of the biofilm can form crosslinks, which makes the biofilm structure more stable and facilitates the attachment of bacteria (7, 8). Ruiz-Sorribas *et al.* reported that hyphae-rich biofilms on titanium coupon surfaces had greater biomass than hyphae-poor biofilms. Related to this, hyphae-rich biofilms showed higher bacterial EPS synthesis. When comparing the hyphae-poor and -rich models, no major differences in bacteria or fungi cell counts were observed, but there were differences in biomass (9). This result emphasises the significant role of fungi in microbial biofilms that can occur in artificial water systems. There are also studies showing that fungi in the biofilm cause corrosion as a result of their long-term presence on steel surfaces (10, 11). Members of the Ascomycota and Basidiomycota phyla were mainly reported in artificial water systems (10, 12, 13).

Similarly, the predominant Ascomycota phylum has been reported in municipal water systems. *Aspergillus*, *Penicillium*, *Candida cladosporium*, *Fusarium*, *Trichoderma* and *Paecilomyces* sp. have been noted in biofilms formed on steel surfaces. *A. niger*, *A. versicolor*, *P. chrysogenum* and *P. dipodomyicola* were reported as the most identified fungal species (2, 14). However, biofilm studies were generally carried out with pure or dual fungus-bacteria cultures, with plastic surfaces and in a short timescale (72 h) (15, 16). Also, studies on fungi were mainly focused on planktonic opportunistic pathogens (17–19). Studies evaluating planktonic and sessile fungal diversity and load simultaneously in municipal water systems are very limited (2, 20). These studies do not include detailed information on fungal diversity and load during the formation and maturation process of the biofilm.

It is essential to select materials for industrial systems that can prevent microbial biofilm formation. This is beneficial to reduce maintenance costs and create a long-lasting system. Galvanised steel is a commonly preferred metal with high antifouling and corrosion resistance (1). Although the antifouling property of galvanised steel is known, there are few studies showing that microbial biofilm can form on its surface (1, 21). Fungi commonly found in nature can also participate in the maturation and formation of a biofilm on the surface of galvanised steel (5). However, changes may occur in the amount of fungal colonisation and diversity depending on the age of the biofilm. To investigate this aspect, natural biofilm formation was allowed on galvanised steel surfaces for six months in a model water recirculation system and the diversity of culturable fungi in both the aging biofilm and water was investigated during the experiment. Also, the number of fungi and aerobic heterotrophic bacteria (AHB) in the biofilm and water samples was determined. In addition, EIS and SEM analyses were performed to obtain information about the structure of the naturally aging biofilm formed on the galvanised steel surfaces.

2. Materials and Methods

2.1 Natural Biofilm Formation

The experiments were carried out in a model water recirculation system over six months. The system was made of polyethylene (PE100) of 70 cm height, 44 cm diameter and 100 l volume. The water in the system was constantly recirculated during the experiment. There was a recirculating water pump to provide water circulation (33 l min^{-1}) and heated to keep constant temperature at 27°C by a heater (100 W). The system was filled with 80 l of municipal water (pH: 7.11, Cl: 5.1 mg l^{-1}, dissolved oxygen: 8.5 mg l^{-1}). 2 l of water was discharged from the system regularly every day and the same amount of make-up water was added.

For natural biofilm formation, galvanised steel was used as test material. ASTM G1–03(2017)e1 (22) 50 × 25 × 1 mm size coupons were cut from 235 g m^{-2} zinc coated sheets (23). The coupons were washed with sterile distilled water, cleaned with acetone and then dried in a Pasteur oven. Both surfaces of the coupons were sterilised under ultraviolet lamp (UVC, 254 nm, 15 W) in a laminar flow device (Thermo Fisher Scientific, USA) for 30 min before the experiment. The coupons were placed into plexiglass coupon carriers and then the carriers were put into the model system under aseptic conditions, parallel to the flow of water. After placing the coupons into the system, the system was operated continuously for six months to allow formation of natural biofilms on the coupons' surfaces. During the experiment, microbiological analyses were performed both from the biofilm and water samples after 0.5 h and monthly. The biofilm layer on the coupon surfaces was also examined by SEM.

For EIS analysis, galvanised steel coupons were prepared in 25 × 25 × 1 mm dimensions. Only the

top coupon surfaces (1 cm^2) were in contact with the solution. All other surfaces were coated with silicon. The coupons were rinsed with ethanol for 3 min then air dried. The coupons were sterilised under ultraviolet lamp for 30 min (24). A carrier grid was prepared and placed on the model system to hang the electrochemical test coupons. The coupons were connected to the grid by copper wire. Biofilm formation was allowed on the surface of galvanised steel coupons for six months and monthly measurements were made by removing them from the system simultaneously with other coupons used for microbiological analysis.

2.2 Microbiological Analyses

2.2.1 Bacteriological Analysis

For enumeration of planktonic AHB, 2 l water sample was passed through a sterile nylon membrane filter (142 mm diameter and 0.22 µm pore diameter), then the filter was placed in a sterile plastic bag containing 20 ml sterile municipal water and kept in the stomacher (IUL Instruments) device for 2 min. From the concentrated sample in the plastic bag, dilution series were prepared from 10^{-1} to 10^{-4}. 0.1 ml of each dilution were spread on R2A plates in triplicate and then the plates were incubated at 27°C for 7 days (25). After the incubation period, the bacterial colonies were counted and colony-forming units per millilitre (CFU ml^{-1}) were calculated.

To count sessile AHB, biofilm samples were scraped from 25 cm^2 surfaces using sterile scalpel and cotton swab and were then suspended in 20 ml sterile municipal water by vortexing (26). Biofilm homogenates of the two coupons were serially diluted from 10^{-1} to 10^{-4}. 0.1 ml of each dilution were spread on R2A plates in triplicate and the plates were incubated at 27°C for 7 days (25). After incubation, the bacterial colonies were counted and CFU per square centimetre (CFU cm^{-2}) were calculated.

2.2.2 Mycological Analysis

To quantify planktonic fungi, 100 ml water samples were concentrated by filtration through 0.45 µm pore-sized nitrocellulose filters. These filters were placed on dichloran 18% glycerol agar (DG18) plates in triplicate and incubated at 27°C for 7 days (18, 27). After the incubation period, the fungal colonies were counted and CFU per 100 millilitre (CFU 100 ml^{-1}) were calculated.

To enumerate sessile fungi, 1 ml of each diluted biofilm homogenate were spread on DG18 agar plates in triplicate and incubated at 27°C for 7 days (27). After the incubation period, the fungal colonies were counted and CFU cm^{-2} were calculated.

The fungal colonies obtained from water and biofilm samples were examined macroscopically and microscopically and the pure colonies were subcultured on slanted potato dextrose agar (PDA) tubes and stored at 4°C. The pure fungal isolates were identified by using classical morphological and molecular methods.

2.3 Morphological and Molecular Identification of Fungal Isolates

For morphological characterisation, the fungal isolates were inoculated into malt extract agar (MEA) and PDA and then identified to genus level according to generally accepted standards (28). Afterwards, the isolates were inoculated into various media (Czapek yeast autolysate agar, Czapek-dox agar and Czapek yeast autolysate agar with 20% sucrose, 25% glycerol nitrate agar, MEA and PDA) and then identified to species level according to morphological and physiological characteristics (29–31).

For molecular characterisation, the fungal isolates were inoculated into MEA and incubated at 25°C for 7 days. Then genomic DNA was extracted from the pure cultures using a microbial DNA isolation kit (MO BIO Laboratories, Inc, USA). Internal transcribed spacer (ITS) regions of the rDNA were used for molecular characterisation. These regions were amplified using the primer pairs V9G forward (5–TTACGTCCCTGCCCTTTGTA–3) and LS266 reverse (5–GCATTCCCAAACAACTCGACTC–3) (32, 33). Polymerase chain reactions (PCR) were conducted in 25 ml final reaction volume. Each tube contained genomic 1 µl of DNA, 2.5 µl of 2.5 µM forward and reverse primers, 2.5 µl of 10 × Taq buffer KCl-$MgCl_2$, 2.5 µl of 25 mM $MgCl_2$, 2 µl of 2.5 mM dNTP mix, 0.25 µl of 5U $µl^{-1}$ Taq DNA polymerase and 11.75 µl of RNase-free water. DNA amplification was performed in a thermocycler with an initial denaturation step for 5 min at 95°C, followed by 35 cycles of denaturation for 45 s at 95°C, annealing for 30 s at 56°C with an extension of 2 min at 72°C. A final extension at 72°C was performed for 6 min (32, 33). To confirm the amplification of solely the ITS, 5 µl of PCR products together with the marker was resolved by gel electrophoresis on 1% agarose gel containing 5 µg ml^{-1} GelRed in 1 × TAE buffer. ITS region was sequenced and then

the nucleotide sequences were compared with the deposited sequences in the National Centre for Biotechnology Information GenBank database.

2.4 EIS Measurement

The EIS method gives information about the events occurring between the metal and solution interphase and the kinetics of the surface. The results of EIS analysis are further analysed by fitting them with appropriate equivalent electrical circuits. The equivalent electrical circuits can provide significant information about the structure of the biofilm on the metal surface.

The EIS measurements were performed with a potentiostat/galvanostat (Gamry-G/750, USA). The electrochemical tests were carried out in a corrosion cell (1 l), with a carbon rod as a counter electrode, a saturated calomel electrode as a reference electrode and the galvanised steel coupons as the working electrode. The water of the model recirculating system was used as the working solution (800 ml). EIS measurements were obtained at instantaneous open circuit potential, in the frequency range from 10^{-5} Hz to 10^{-1} Hz, with 5 mV of amplitude. Gamry-Echem Analyst 6.33 software was used to analyse the data.

The Kramers–Kronig transformations were performed to validate EIS experimental data. The values of the goodness of fit were determined by chi-squared value. The values of the goodness of fit for galvanised steel were around 10^{-4}, falling in the middle of the determined range (34).

2.5 Surface Characterisation

The biofilm layer on the coupon surfaces was examined by SEM. The coupons were fixed with 2.5% glutaraldehyde, followed by dehydration in a graded series of ethanol (30%, 50%, 80% and 95%) and air-dryed (35). The dried samples were coated with gold and imaged with a FEI Quanta 450 FEG SEM.

3. Results and Discussion

3.1 Biofilm Formation and Microbial Growth

Due to the toxicity of zinc to many microorganisms, galvanised steel is assumed to exhibit strong antifouling properties (36). However, a heterogeneous, yellowish-greenish colored biofilm layer containing fungi and AHBs was observed to form on galvanised steel surfaces during the experiment (**Figures 1** and **2**). SEM images also confirmed the presence of microorganisms (**Figure 3**). Ilhan-Sungur and Çotuk and Minnoş *et al*. also reported the presence of aerobic bacteria in natural aging biofilms formed on the galvanised steel surfaces (1, 21). However, to the authors' knowledge, no other study has reported the presence, number and diversity of fungi at specified time intervals in natural aging biofilms on galvanised steel surfaces for a long time.

Unlike bacteria, fungi in biofilms were difficult to detect during the first three months of SEM examinations. As a matter of fact, it was determined that the number of fungi in the biofilm was low (0.8 CFU cm^{-2}, 1.2 CFU cm^{-2} and 3.6 CFU cm^{-2}, respectively) (**Figure 1**). Siqueira *et al.* were able to detect fungal colonisation in natural biofilms formed on polyethylene surfaces only after 3 months under fluorescent microscopy (37). Also, it was observed in SEM images that the fungal hyphae were not heterogeneously

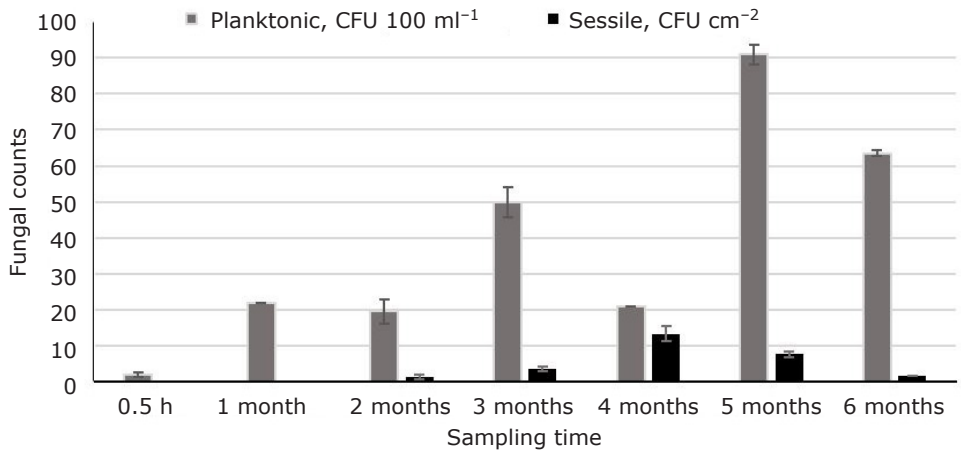

Fig. 1. The total number of planktonic and sessile fungi over six months

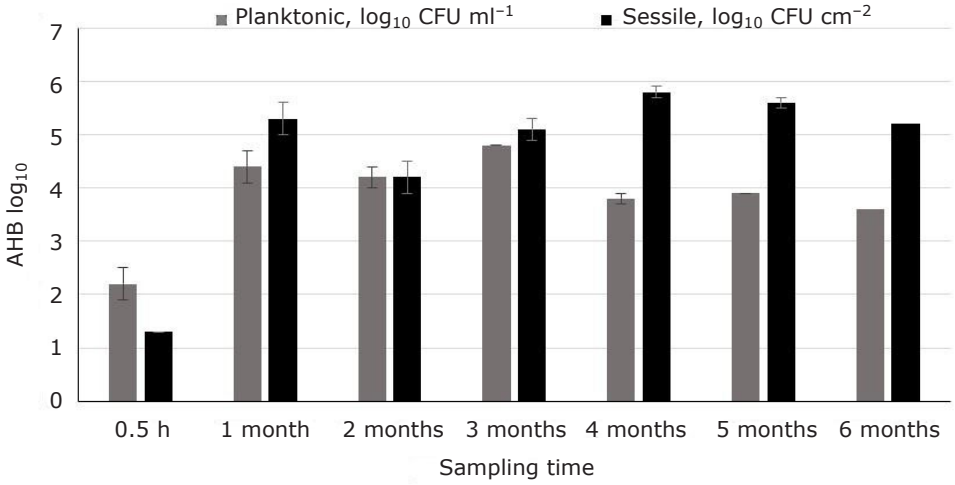

Fig. 2. The total number of planktonic and sessile aerobic heteretrophic bacteria over six months

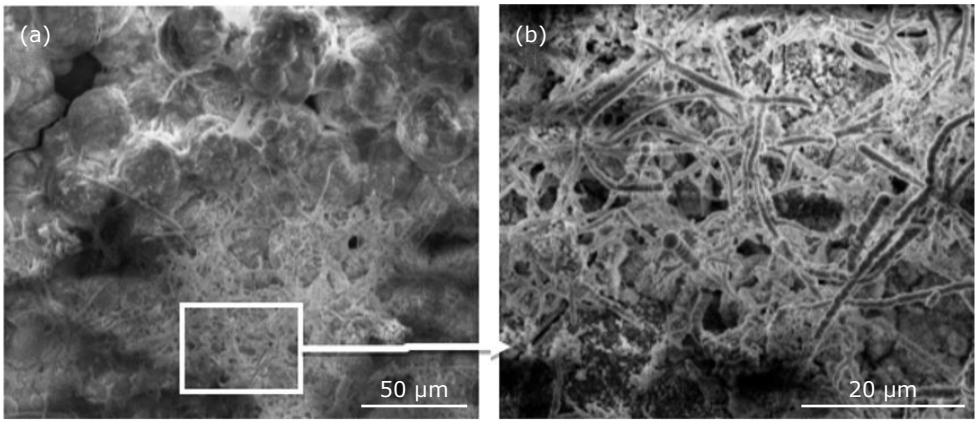

Fig. 3. SEM micrographs of galvanised steel coupon surfaces after four months: (a) 50 µm; (b) 20 µm

distributed on the surface and formed regional settlements (**Figure 3**) as reported by Elvers *et al*. (8). Furthermore, fungal hyphae were present as a separate layer on top of the biofilm layer and not embedded in EPS (**Figure 3**). Electrical equivalent circuits generated from the EIS data also showed that fungi existed as a discrete layer in the biofilm (**Figure 4**), as reported by Juzeliunas *et al*. (38).

The highest number of sessile fungi was determined as 13.3 CFU cm^{-2} at four months of biofilm. As a matter of fact, SEM images showed that fungi were densely present in the four-month-old biofilm (**Figure 3**). Doggett (39) reported 4–25 CFU cm^{-2} fungi in biofilms on the surfaces of 20–73 year-old iron and PVC water pipes. Göksay Kadaifciler and Demirel (2) reported that the average number of fungi in biofilm samples on steel surfaces with an average age of 12 years was 4 CFU cm^{-2}. They also stated that older steel surfaces had a higher number of fungi in their biofilm than younger ones (2). Although the materials and media used in isolation in the aforementioned studies are different from this study, the average number of fungi detected is similar. It can be concluded from the findings and from the limited number of reported fungal-focused biofilm studies that it takes a long time for fungi to take place in natural biofilms and their numbers increase as the biofilm ages.

It was determined that the planktonic fungi in the water increased their numbers until the third month and then there were fluctuations in their numbers. The lowest and highest numbers of planktonic fungi were detected as 2 CFU 100 ml^{-1} at 0.5 h and 91 CFU 100 ml^{-1} at fifth month. When the six-months results were analysed, the number of planktonic fungi in the water was found to be greater than the number of sessile ones in the biofilm (**Figure 1**). A similar finding was also reported in the study conducted by Göksay Kadaifciler and Demirel (2). This could be due to the spores formed in the biofilm being unable to adhere to the surface or germinate due to nutrient competition with bacteria. Doggett

(39) reported that fungal spores were found loosely on the surface of the biofilm on the iron surfaces of the municipal water distribution system and fungal hyphae development could not be detected. Barros Afonso *et al*. also reported from the biofilm study they conducted with two different fungi and bacteria species that bacteria with a high growth rate and metabolic activity inhibited fungal spore germination and it could be due to nutrient competition (15).

Unlike fungi in the biofilm, the presence of sessile AHB was detected at 0.5 h and their number was determined as 1.3 $\log_{10}$ CFU cm^{-2} (**Figure 2**). This result indicates that bacteria may be primary hosts in the biofilm. The number of AHB was found to be quite high from the first month, with the highest value being 5.8 $\log_{10}$ CFU cm^{-2} at the fourth month. Comparing the counts of sessile AHB and fungi in the biofilm at the fourth month, it was noticed that the number of AHB in the biofilm was 10^5 times the number of fungi. Rajala *et al*. stated that the number of bacteria in three-months old biofilms formed on the surfaces of titanium alloy (Ti6Al4V), super austenitic stainless steel (254SMO) and epoxy-coated carbon steel (Intershield Inerta160) coupons were 10^3–10^4 times greater than the fungal ones (40). Also Lutterbach and França reported that the average number of AHB in one-month old biofilms on AISI-304 stainless steel surfaces was 18.5 times higher than the number of fungi (41). Although the surface materials and biofilm ages studied differ, the results show that the number of bacteria in the biofilm is significantly higher than the fungal ones. However, Webb *et al.* studied fungal colonisation on PVC surfaces for 95 weeks and stated that CFU count can be a problem in fungi measurement (42). They found that for the same amount of biomass, a budding yeast colony or a filamentous fungus spore can produce significantly more CFU than a hyphal mycelium. Therefore, it is debatable how much area bacteria occupy in the biofilm as biomass compared to fungal hyphae. In addition, although the numbers of fungi in the biofilm are less than bacteria, they may play an essential role in the formation and maturation of the biofilm. Rajala *et al*. investigated the numerical and biodiversity changes of fungi in biofilms formed on various surfaces (Ti6Al4V, 254SMO, Intershield Inerta160) for three months (40). They suggested that despite their low numbers, fungi can provide an anoxic microhabitat by consuming the oxygen in the environment for anaerobic sulfate reducing bacteria or methanogenic archaea and thus play a role in the alteration of the biofilm structure. In addition, there may be synergistic and antagonistic interactions between bacteria and fungi involved in biofilm at different stages of the maturation process (43, 44).

It was determined that similar to fungi, the number of planktonic bacteria in the water increased until the third month.The lowest and highest planktonic AHB counts were 2.2 $\log_{10}$ CFU ml^{-1} at 0.5 h and 4.8 $\log_{10}$ CFU ml^{-1} at the third month. During the experiment, the number of planktonic AHB was found to be lower than the sessile ones. This situation can be interpreted as bacteria prefer the biofilm over the water phase due to its advantages such as meeting their nutritional needs and

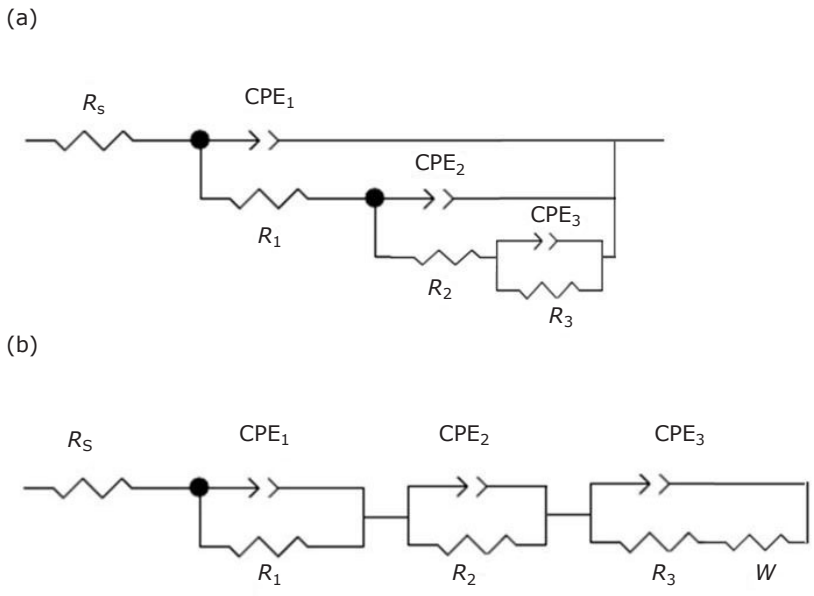

Fig. 4. Electrochemical impedance spectra of galvanised steel coupons with biofilm after: (a) 4 months; (b) 5 months. Circuit elements: W = Warburg element; R_s = resistance of the solution; R_1 = resistance of charge transfer; R_2 = resistance of the biofilm formed by microorganisms that primary adhere to the metal surface; R_3 = resistance created by fungi and the metabolic products they produce; CPE = constant phase element; CPE$_1$ = double layer capacitance; CPE$_2$ = biofilm layer; CPE$_3$ = porous diffuse layer containing fungi and metabolites

Table I Electrochemical Parameters of the Electrical Equivalent Circuits of the Coupons

	μF cm^2			kΩ cm^2			
Time	**C_1**	**C_2**	**C_3**	***R_1***	***R_2***	***R_3***	**W**
4th month	15.5 (0.86)	62.2 (0.54)	5.2 (0.77)	3.03	5.11	2.47	–
5th month	11.3 (0.94)	71.8 (0.92)	0.6 (0.83)	2.36	3.67	1.03	1.47

protection from disinfection. Ilhan-Sungur and Çotuk and Minnoş *et al.* reported similar findings in their natural biofilm studies with galvanised steel exposed to municipal water (1, 21).

Based on the numbers of bacteria and fungi, it can be said that the biofilm matured in the fourth month and shedding from the biofilm occurred in the fifth month (**Figures 1** and **2**). The electrical equivalent circuits generated from the EIS data also confirmed the structural changes of the biofilm (**Figure 4**) (**Table I**). In contrast to the fourth month, the Warburg element, which expresses diffusion, in the fifth month's electrical equivalent circuit may indicate that the diffusion process in the biofilm increased due to shedding (**Figure 4**), as reported by Unsal *et al*. (45). The fourth month's electrical equivalent circuit showed that the biofilm layer was parallel to the other layers formed on the metal surface. This phenomenon demonstrates that the dynamic system formed by microorganisms in the biofilm has a connection with the other layers formed. This connection makes the biofilm more resistant and compact. However, by the fifth month, the biofilm layer was serially connected to the other layers. This indicates that each layer advances in its own system, the biofilm's compact structure deteriorates and the stratification changes.

3.2 Identified Fungal Isolates

The fungi were present in the water phase from 0.5 h and in the biofilm from the first month when they could be isolated. The fungal isolates were identified by morphological and molecular methods and the results are given in **Table II**. Significant changes were observed in fungus diversity in the biofilm over the experiment. Similar findings were reported for biofilms formed on high density polyethylene (HDPE) and PVC surfaces exposed to municipal water (6, 20). In addition, it was determined that mainly filamentous fungal species were present in the natural biofilms on the galvanised steel surfaces during the experiment. Only *Candida metapsilosis* was detected as a yeast species. Doggett (39) and Fish and Boxall (6) also reported that filamentous fungi were predominant in natural biofilms in water distribution systems compared to yeasts.

DNA sequences of fungal isolates obtained from the one-month-old biofilm were found to be similar to *Fusarium oxysporum* (99.35% similarity) and *Cladosporium cladosporioides* (99.79% similarity) species. While these species were not found in the biofilm in the second month, *Penicillium polonicum* (100% similarity) and *C. metapsilosis* (99.03% similarity) species were detected in the biofilm. The highest biodiversity in the biofilm was detected in the third month, with *Aspergillus versicolor* (98.83% similarity), *Aspergillus* sp. (96.62% similarity), *P. chrysogenum* (100% similarity), *P. polonicum* and *F. oxysporum*. It was determined that *P. chrysogenum* and *F. oxysporum* continued to exist in the biofilm in the fourth month and *C. metapsilosis* also participated in the biofilm. In the fifth month, *P. citrinum* (100% similarity) accompanied *P. chrysogenum* and *F. oxysporum* in the biofilm. It was determined that *F. oxysporum*, which was detected in the biofilm during the early part of the experiment, was not present in the six-month-old biofilm, while *P. chrysogenum*, first detected in the third month, existed (**Table II**). The results indicate that natural biofilms formed on galvanised steel surfaces have a dynamic structure in terms of fungal diversity.

F. oxysporum was one of the first fungi that appeared in the biofilm and generally remained in the biofilm throughout the experiment. It suggests that this fungus is one of the biofilm's main inhabitants. Fish and Boxall (6) and Preciado *et al*. reported that *Fusarium* replaced other fungal genera such as *Aspergillus* and *Cladosporium* which were initially dominant in biofilms on HDPE and PVC surfaces (20). The *F. oxysporum* species complex was also reported among the fungal species predominantly found in the biofilm formed on the plastic sealing surfaces of machinery directly connected to municipal water (46). It is proposed that the ability of the *Fusarium* genus to produce EPS can promote biofilm formation (47).

It was determined that *Penicillium* species were included in the biofilm from the second month.

Table II The Identified Culturable Fungi in the Water and Biofilm Samples Over Six Months

Time	Water (closest relatives of accession no/similarity percentage)	Biofilm (closest relatives of accession no/similarity percentage)
0.5 h	*Penicillium chrysogenum* (MT601877.1/100%)	nd[a]
	Aspergillus versicolor (MF475945.1/98.83%)	
	Penicillium polonicum (MT529240.1/100%)	
1st month	*Penicillium spinulosum* (MH864670.1/99.78%)	*Fusarium oxysporum* (JN232163.1/99.35%)
	Fusarium oxysporum (JN232163.1/99.35%)	*Cladosporium cladosporioides* (AF177736.1/99.79%)
2nd month	*Cladosporium cladosporioides* (LN482431.1/99.79%)	*Penicillium polonicum* (MT529240.1/100%)
	Fusarium oxysporum (JN232163.1/99.35%)	*Candida metapsilosis* (MF797776.1/99.03%)
3rd month	*Aspergillus* sp. (MN905834.1/96.62%)	*Fusarium oxysporum* (JN232163.1/99.35%)
	Cladosporium cladosporioides (LN482431.1/99.79%)	*Aspergillus versicolor* (KU318417.1/98.79%)
	Penicillium sp. (MN905858.1/99.79%)	*Penicillium chrysogenum* (MT601877.1/100%)
	Fusarium oxysporum (JN232163.1/99.35%)	*Aspergillus* sp. (MN905834.1/96.62%)
	–	*Penicillium polonicum* (MT529240.1/100%)
4th month	*Aspergillus* sp. (MN905834.1/96.62%)	*Candida metapsilosis* (MF797776.1/99.03%)
	Fusarium oxysporum (JN232163.1/99.35%)	*Fusarium oxysporum* (JN232163.1/99.35%)
	Candida metapsilosis (MF797776.1/99.03%)	*Penicillium chrysogenum* (MT601877.1/100%)
5th month	*Aspergillus* sp. (MN905834.1/96.62%)	*Fusarium oxysporum* (JN232163.1/99.35%)
	Penicillium oxalicum (MT529124.1/100%)	*Penicillium citrinum* (MN046972.1/100%)
	Fusarium oxysporum (JN232163.1/99.35%)	*Penicillium chrysogenum* (MT601877.1/100%)
	Candida metapsilosis (MF797776.1/99.03%)	–
	Penicillium brevicompactum (MT558924.1/99.79%)	–
6th month	*Penicillium chrysogenum* (MT601877.1/100%)	*Penicillium chrysogenum* (MT601877.1/100%)
	Fusarium oxysporum (JN232163.1/99.35%)	–

[a]nd = nondetected

Different species (*P. polonicum*, *P. chrysogenum* and *P. citrinum*) were found in biofilms of different ages (**Table II**). This suggests that the biofilm must reach a certain maturity in order for *Penicillium* species to settle on the biofilm, or that *Penicillium* species have difficulty adhering to the surface. Siqueira and Lima examined the biofilm formation of *Aspergillus*, *Alternaria*, *Botrytis* and *Penicillium* spp. for 24 h. They reported that the spores of *Penicillium* species adhered to the surface later than the other fungal isolates (48). In addition, the hydrophilic or hydrophobic properties of the fungal spore have been reported to play an essential role in the biofilm kinetics of fungi (15). Another possibility is that *Penicillium* may exhibit selectivity in attachment with respect to the surface. Marangoni *et al*. stated that especially *Penicillium chrysogenum* type fungus only attached to carbon steel 1045 surfaces (12). In this study, the presence and survival of *P. chrysogenum* in the biofilm after the third month and even after the shedding of the biofilm may suggest that this fungus is a long-term inhabitant of the biofilm.

The genus *Aspergillus*, which was frequently detected in the water phase during the experiment, was not found to be a permanent component of the biofilm (**Table II**). However, fungi of the genus *Aspergillus* were found in the majority of biofilm studies conducted on municipal water distribution systems (39, 44). This may indicate that fungi of the genus *Aspergillus* do not preferentially attach to the galvanised steel surface. The reason for

this may be that zinc has a toxic effect on many microorganisms. However, fungi of the genus *Aspergillus* are known to be among the most resistant fungal species against heavy metals (49). Another possibility is that antagonistic relationships between microorganisms in the biofilm may have prevented the long-term presence of *Aspergillus* in the biofilm.

After 0.5 h, DNA sequences of fungal isolates obtained from the water phase were found to be similar to *A. versicolor* (98.83% similarity), *P. chrysogenum* (100% similarity) and *P. polonicum* (100% similarity) species (**Table II**). *F. oxysporum*, which was identified in the first month, was found to remain in the water phase over six months. However, the diversity of fungi in the water phase changed during the experiment. As a result, the following fungi genus/species were identified in the water phase: *Aspergillus* sp. (96.62% similarity), *Penicillium* sp. (99.79% similarity), *P. brevicompactum* (99.79% similarity), *P. oxalicum* (100% similarity), *P. spinulosum* (99.78% similarity), *Candida metapsilosis* (99.03% similarity), *C. cladosporioides* (99.79.% similarity) and *F.oxysporum* (99.35% similarity). *Aspergillus*, *Penicillium*, *Fusarium* and *Cladosporium* have been reported as the dominant fungal genera detected in water samples in studies conducted with municipal water systems thus far (2, 17, 18, 44).

In line with the findings, it was determined that all of the fungal genera defined in the water phase could also be found in the biofilm and all fungi in both phases were included in the Ascomycota phylum. It has been reported that the majority of the fungi identified in the studies conducted in municipal water systems belong to the Ascomycota phylum (2, 6, 39). The members of Ascomycota phylum are known to spread in air, soil and aquatic ecosystems. For this reason, their predominance in municipal water systems associated with these ecosystems is considered possible. When compared to other phyla, Ascomycota phylum has a high species diversity including opportunistic pathogens of the respiratory tract. The spores of *Aspergillus*, *Penicillium* and *Cladosporium* are known to be potential allergens. *Fusarium* spp. are also opportunistic in pulmonary and bronchial infections. *Aspergillus*, *Penicillium* and *Fusarium* spp. are additionally recognised as mycotoxin producers (50). Despite being the least virulent member of the *C. parapsilosis* complex, *C. metapsilosis* has been linked to infant and child fatalities. Proliferation of any genera or species of fungi may cause allergies or irritant effects in immunocompetent individuals (51).

Traditional culture methods, considered the gold standard, allow the physiology of viable and culturable fungi to be studied. However, it is generally accepted that culture-dependent methods cover only a small percentage of the total microorganisms present (52). Culture-independent methods, on the other hand, are a powerful technique that can be used to identify not only culturable fungi, but also fungi with unknown culture conditions. However, there are difficulties in its use due to the cost and technical expertise required for this method. In this context, combined use of culture-dependent methods with culture-independent ones may be helpful in obtaining more comprehensive data on the fungal community in processes of biofilm formation and maturation on galvanised steel surfaces.

4. Conclusion

The following conclusions can be drawn from this study. Fungi are involved in naturally aging biofilms formed on galvanised steel surfaces and the number and diversity of fungi change according to the biofilm age. Only fungi of the phylum Ascomycota were found in biofilm on galvanised steel surfaces. Of these, *F. oxysporum* and *Penicillium* spp. were the primary inhabitants. It is difficult to detect fungi in natural young biofilms by SEM analysis. The results might have important consequences for a better insight into naturally aging biofilms containing fungi and might eventually lead to a strategy by which effective disinfection of municipal water systems can be carried out according to the age of the biofilm.

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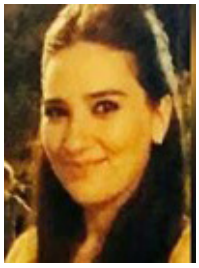

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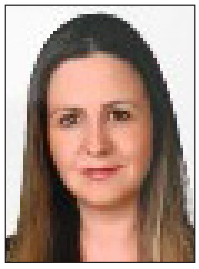

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