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

Fungi in the aging biofilm on the galvanized steel surfaces

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<ABSTRACT>

Fungi commonly found in municipal water can participate in the natural biofilm formation on the surfaces of galvanized steel despite the toxic effect of Zn, and also depending on the age of the biofilm, fungal diversity may vary. To examine this hypothesis, natural biofilm formation was allowed on galvanized steel surfaces over 6 months in a model recirculating water system. Fungal colonies with different

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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 electrochemistry impedance spectroscopy (EIS) analyses and scanning electron microscopy. It was determined that the fungi were included in the natural aging biofilm formed on the galvanized steel surfaces over the experiment. The diversity and the number of fungi in the biofilm and water changed over the experiment. All of the 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 also it is 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 ages. The results of this study may lead to a better understanding of natural 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.

Keywords: Fungi, Galvanized steel, Natural biofilm, Aging biofilm, Recirculating water system.

1.Introduction

Biofilm is one of the major encountered problems in mun water systems. Besides being the most common threat to the performance and longevityof 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 considered as complex communities including bacteria, fungi etc. (5, 6).

Although bacteria are mainly known to be the pioneer colonizers, eukaryotes such as fungi or protozoa can be included in the biofilm as secondary colonizer. Fungi

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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). Also, Ruiz-Sorribas *et al.* (9) reported that hypha-rich biofilms on Ti coupon surfaces had greater biomass than hypha-poor biofilms, and releated to this, hypha-rich biofilms showed higher bacterial EPS synthesis. When comparing the hyphae-poor and -rich models, no major differences in (bacteri, fungi) cell counts were observed, but were in biomass. This result emphasizes the significant role of fungi in microbial biofilms that can occur in the man-made water systems. As a matter of fact, there are also studies showing that fungi in the biofilm cause corrosion as a result of their long-term presence in the biofilm on steel surfaces (10, 11). The members of Ascomycota and Basidiomycota phylum were mainly reported in man-made water systems (10, 12, 13).

Similarly, the predominantly Ascomycota phylum has been reported in the municipal water systems. *Aspergillus, Penicillium, Candida Cladosporium, Fusarium, Trichoderma* and *Paecilomyces* sp. were notified in the biofilms formed on the steel surfaces. *A. niger, A. versicolor, P. chrysogenum* and *P. dipodomyicola* were reported as the most identified fungal species (2, 14). However, it was realized that biofilm studies were generally carried out with pure or dual (fungus-bacteria) cultures, with plastic surfaces and in a short-term (72 h.) (15, 16). Also, it was noticed that the studies on fungi were mainly focused on planktonic opportunistic pathogens (17-19). In addition, the studies evaluating planktonic and sessile fungal diversity and load simultaneously in the municipal water systems are very limited (2, 20). In addition,

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these studies do not include detailed information on the fungal diversity and load during the formation and maturation process of the biofilm.

It is essential to select materials for industrial systems for preventing microbial biofilm formation, which is beneficial for reducing maintenance costs and creating a long-lasting system. For this reason, galvanized steel is a commonly preferred metal with its high antifouling and corrosion resistance in man-made water systems (1). Although the antifouling property of galvanized 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 galvanized steel (5).

However, changes may occur in the amount of fungal colonization and diversity depending on the age of the biofilm. To investigate this aspect, natural biofilm formation was allowed on galvanized steel surfaces for 6 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 also determined. In addition, Electrochemistry impedance spectroscopy (EIS) and scanning electron microscope (SEM) analyzes were performed to obtain information about the structure of the natural aging biofilm formed on the galvanized steel surfaces.

2. Materials and Methods

2.1 Natural Biofilm Formation

The experiments were carried out in the model water recirculation system over 6 month. The system was made of PE100 (Polyethylene) with 70 cm height, 44 cm diameter, 100 L volume. The water in the system was constantly recirculated during

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the experiment. There was a recirculating water pump to provide water circulation (33 L/min) and heated to keep constant temperature at 27◦C by a heater (100W). The system was filled with 80 L of municipal water (pH: 7.11, Cl: 5.1 mg/L, dissolved oxygen: 8.5 mg/L). 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, galvanized steel was used as test material. ASTM G1–72 (22) 50×25×1 mm size of 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. The both surfaces of the coupons were sterilized under ultraviolet lamp (UVC, 254 nm, 15 W) in a laminar flow device (Thermo Fisher Scientific, United States) for 30 min before the experiment. The coupons were placed into the 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 over 6 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. Also, the biofilm layer on the coupon surfaces was examined by SEM.

In addition, for EIS analysis, galvanized steel coupons werep repared in 25×25×1mm 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 sterilized under ultraviolet lampfor 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

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wire.Biofilm formation was allowed on the surface of galvanized steel coupons for 6 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 each of the dilutionswere spread on R2A plates in triplicate and then the plates were incubated at 27◦C for 7 days (25). After incubation period, the bacterial colonies were counted and a colony-forming unit per milliliter (CFU/mL) were calculated.

For counting of sessileAHB,biofilm samples were scraped from the surfaces of 25 cm^2 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 each of the dilutionswere 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 centimeter (CFU/cm^2) were calculated.

2.2.2 Mycological analysis

In order 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, incubated at 27◦C for 7

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days (27, 28). After incubation period, the fungal colonies were counted and CFU per 100 milliliter (CFU/100 mL) was calculated.

For enumeration of sessile fungi, 1 mL each of the diluted biofilm homogenates were spread on DG18 agar plates in triplicate, incubated at 27◦C for 7 days (28). After incubation period, the fungal colonies were counted and CFU per square centimeter (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 characterization, the fungal isolates were inoculated into malt extract Agar (MEA) and PDA and then identified to genus level according to the generally accepted standards (29). 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 levels according to the morphological and physiological characters (30-32).

For molecular characterization, 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 microbial DNA isolation kit (MO BIO Laboratories, Inc., USA). Internal transcribed spacer (ITS) regions of the rDNA were used for molecular characterization. These regions were amplified using the primer pairs V9G forward (5-TTACGTCCCTGCCCTTTGTA-3) and LS266 reverse (5-GCATTCCCAAACAACTCGACTC-3)

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(33, 34). Polymerase chain reaction (PCR) reactions were conducted in a 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 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 (33, 34). 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 GelRed in 1×TAE buffer. ITS region was sequenced andthen the nucleotide sequences were compared with the deposited sequences in the NCBI 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 analyzed 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 (SCE) as a reference electrode, and the galvanized steel coupons as the working electrode.The waterof the model recirculating system was used as the working solution (800 mL). EIS measurements were obtained at instantaneous open circuit potential (OCP), in the

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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 (KK) 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 galvanized steel were around 10^{-4}, falling in the middle of the determined range (35).

2.5 Surface characterization

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 (36). The dried samples were coated with gold and imaged with a FEI Quanta 450 FEG scanning electron microscope.

3.Results and Discussion

3.1 Biofilm Formation and Microbial Growth

Due to the toxicity of zinc to many microorganisms, galvanized steel is assumed to exhibit strong antifouling properties (37). However, a heterogeneous, yellowish-greenish colored biofilm layer containing fungi and AHBs was observed to form on galvanized steel surfaces during the experiment (Figure 1, 2). SEM images also confirmed the presence of microorganisms (Figure 3). Ilhan-Sungur and Çotuk (1), and Minnoş *et al*. (21) also reported the presence of aerobic bacteria in natural aging biofilms formed on the galvanized steel surfaces. 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 galvanized 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

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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.* (38) were able to detect fungal colonization in natural biofilms formed on polyethylene surfaces only after 3 months under fluorescent microscopy. Also, it was observed in SEM images that the fungal hyphae were not heterogeneously 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*. (39).

The highest number of sessile fungi was determined as 13.3 CFU/cm^2 at fourth months of biofilm. As a matter of fact, SEM images showed that fungi were densely present in the 4-month-old biofilm (Figure 3). Doggett (40) 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

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lowest and highest numbers of planktonic fungi were detected as 2 CFU/100 mL at 0.5 h and 91 CFU/100 mL at fifth month. When the 6-months results were analyzed, 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 (40) 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*. (15) 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 andit could be due to nutrient competition.

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*. (41) stated that the number of bacteria in 3-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. Also Lutterbach and França (42) 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. Although the surface

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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.* (43) studied fungal colonization on PVC surfaces for 95 weeks, and stated that CFU count can be a problem in fungi measurement. Because, they found out that for the same amount of biomass, a budding yeast colony or a filamentous fungus spore can produce significantly more CFU number than a hyphal mycelium. Therefore, it is debatable that bacteria occupy how much area 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*. (41) investigated the numerical and biodiversity changes of fungi in biofilms formed on various surfaces (Ti6Al4V, 254SMO, Intershield Inerta160) for 3 months. 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 a synergistic and an antagonistic interactions between bacteria and fungi involved in biofilm at different stages of the maturation process (44, 45).

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 at 0.5 h and 4.8 $\log_{10}$ CFU/mL at third month. During the experiment, the number of planktonic AHB was found to be lower than the sessileones. This situation can be interpreted as bacteria preferthe biofilm over the water phase due to its advantages such as meeting their nutritional needs and protection from disinfection. Ilhan-Sungur and Çotuk (1), and Minnoş *et al.* (21)

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reported similar findings in their natural biofilm studies with galvanized steel exposed to municipal water.

Based on the numbers of bacteria and fungi, it can be said that the biofilm matured in the fourth month and the shedding from the biofilm occurred in the fifth month (Figure 1, 2). Besides, the electrical equivalent circuits generated from the EIS data also confirmed the structural changes of the biofilm (Figure 4) (Table 1). 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*. (46). 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, and their isolation could be done. The fungal isolates were identified by morphological and molecular methods and the results are given in Table 2. Significant changes were observed in fungus diversity in the biofilm over the experiment. Similar findingswere reported for biofilms formed on 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 galvanized steel

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surfaces during the experiment. Only *Candida metapsilosis* was detected as a yeast species. Doggett (40), 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%) and *Cladosporium cladosporioides* (99.79%) 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 also *C. metapsilosis* participated in the biofilm again. In the fifth month, *P. citrinum* (100% similarity) accompanied to *P. chrysogenum and F. oxysporum* in the biofilm. It was determined that while *F. oxysporum*, which was first and frequently also detectedin the biofilm during the experiment, was not present in the 6-months old biofilm, *P. chrysogenum*, detected in the third month, was exist (Table 2). The results indicated that the natural biofilms formed on galvanized steel surfaces has a dynamic structure in terms of fungal diversity.

F. oxysporum being one of the fungi that firstly appeared in the biofilm and generally remained in the biofilm throughout the experiment is a piece of evidence that this fungus is one of the biofilm's main inhabitants. Fish and Boxall (6) and Preciado et al. (20) reported that *Fusarium* replaced other fungal genera such as *Aspergillus* and *Cladosporium*, which were dominant in the biofilms on HDPE and PVC

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surfaces in the first days and then dominated the fungal community by the time. The *Fusarium oxysporum* species complex was also reported to be among the fungal species predominantly found in the biofilm that forms on the plastic sealing surfaces of machinery directly connected to municipal water (47). It is proposed that the ability of the *Fusarium* genus to produce EPS can promote biofilm formation (48).

It was determined that *Penicillium* species were included in the biofilm from the second month and the different species (*P. polonicum*, *P. chrysogenum* and *P. citrinum)* were found in the biofilms of different ages (Table 2). According to these findings, it can be said that the biofilm may reach a certain maturity in order for *Penicillium* species to settle on the biofilm, or *Penicillium* species can hardly adhere to the surface. Siqueira and Lima (49), in their study in which was examined the biofilm formation of *Aspergillus, Alternaria, Botrytis, Penicillium* spp. for 24 h, reported that the spores of *Penicillium* species adhered to the surface later than the other fungal isolates. In addition, the hydrophilic or hydrophobic properties of the fungal spore had 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.* (12) stated that especially *Penicillium chrysogenum* type fungus only attached to carbon steel 1045 surfaces. In addition, in this study, the presence and survival of *P. chrysogenum* in the biofilm since the third month and even after the shedding of the biofilm may suggest that this fungus is one of the 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 2). However, fungi of the genus *Aspergillus* were found in the majority of biofilm studies

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conducted on municipal water distribution systems (40, 45). This may indicate that fungi of the genus *Aspergillus* did not preferentially attach to the galvanized 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 (50). 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 2). *F. oxysporum*, which was identified in the first month, was found to remain in the water phase over 6 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). As a matter of fact, *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, 45).

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. In fact, it has been reported that the majority of the fungi identified in the studies conducted in municipal water systems belong to the

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Ascomycota phylum (2, 6, 40). The member 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 as a possible situation. 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 recognized as mycotoxin producers (51). Despite being the least virulent member of the *C. parapsilosis* complex, *C. metapsilosis* has been linked to infant and child fatalities. Therefore, any genera or species of fungi proliferation may cause various allergies and/or irritant effects in immunocompetent individuals (52).

In addition 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 small percent of the total microorganisms present (53). 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 galvanized steel surfaces.

4. Conclusion

The following conclusions can be drawn from this study:

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- Fungi involved in natural aging biofilms formed on galvanized steel surfaces, and the number and diversity of fungi changed according to the biofilm ages.
- Only fungi of the phylum Ascomycota were found in the natural biofilm on the galvanized steel surfaces and, *F. oxysporum* and *Penicillium* spp. were the primary inhabitants of it.
- It is difficult to detect fungi in natural young biofilms by SEM analysis.
- The results might have important consequences for a better insight into natural aging biofilm containing fungi and might eventually lead to a strategy by which effective disinfection of municipal water systems can be done according to the age of biofilm.

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<Tables>

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Table I. Electrochemical parameters of the electrical equivalent circuits of the coupons.

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

μF cm^2: Microfarad per square centimeter

kΩ cm^2: Kiloohm per square centimeter

Table II. The identified culturable fungi in the water and biofilm samples over 6 months.

Time	WATER (Closest relatives of accession no /Similarity percentage)	BIOFILM (Closest relatives of accession no/Similaritypercentage)
0.5 h	*Penicillium chrysogenum* (MT601877.1/100%) *Aspergillus versicolor* (MF475945.1/98.83%) *Penicillium polonicum* (MT529240.1/100%)	-
1stmonth	*Penicillium spinulosum* (MH864670.1/99.78%) *Fusarium oxysporum* (JN232163.1/99.35%)	*Fusarium oxysporum* (JN232163.1/99.35%) *Cladosporium cladosporioides* (AF177736.1/99.79%)
2ndmonth	*Cladosporium cladosporioides* (LN482431.1/99.79%) *Fusarium oxysporum* (JN232163.1/99.35%)	*Penicillium polonicum* (MT529240.1/100%) *Candida metapsilosis* (MF797776.1/99.03%)
3rdmonth	*Aspergillus* sp. (MN905834.1/96.62%) *Cladosporium cladosporioides* (LN482431.1/99.79%) *Penicillium* sp. (MN905858.1/99.79%) *Fusarium oxysporum*(JN232163.1/99.35%)	*Fusarium oxysporum*(JN232163.1/99.35%) *Aspergillus versicolor* (KU318417.1/98.79%) *Penicillium chrysogenum* (MT601877.1/100%) *Aspergillus* sp. (MN905834.1/96.62%) *Penicillium polonicum* (MT529240.1/100%)
4thmonth	*Aspergillus* sp. (MN905834.1/96.62%) F*usarium oxysporum* (JN232163.1/99.35%) *Candida metapsilosis* (MF797776.1/99.03%)	*Candida metapsilosis* (MF797776.1/99.03%) *Fusarium oxysporum*(JN232163.1/99.35%) *Penicillium chrysogenum* (MT601877.1/100%)
5thmonth	*Aspergillus* sp. (MN905834.1/96.62%) *Penicilliumo xalicum* (MT529124.1/100%) *Fusarium oxysporum* (JN232163.1/99.35%) *Candida metapsilosis* (MF797776.1/99.03%) *Penicillium brevicompactum* (MT558924.1/99.79%)	*Fusarium oxysporum* (JN232163.1/99.35%) *Penicillium citrinum* (MN046972.1/100%) *Penicillium chrysogenum* (MT601877.1/100%)
6thmonth	*Penicillium chrysogenum* (MT601877.1/100%) *Fusarium oxysporum* (JN232163.1/99.35%)	*Penicillium chrysogenum* (MT601877.1/100%)

-: Nondetected h: Hour

<Figures captions>

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Figure 1. The total number of planktonic and sessile fungi over 6 months. (CFU/100 mL: Colony forming unit per 100 milliliter, CFU/cm^2: Colony forming unit per square centimeter, h: Hour)

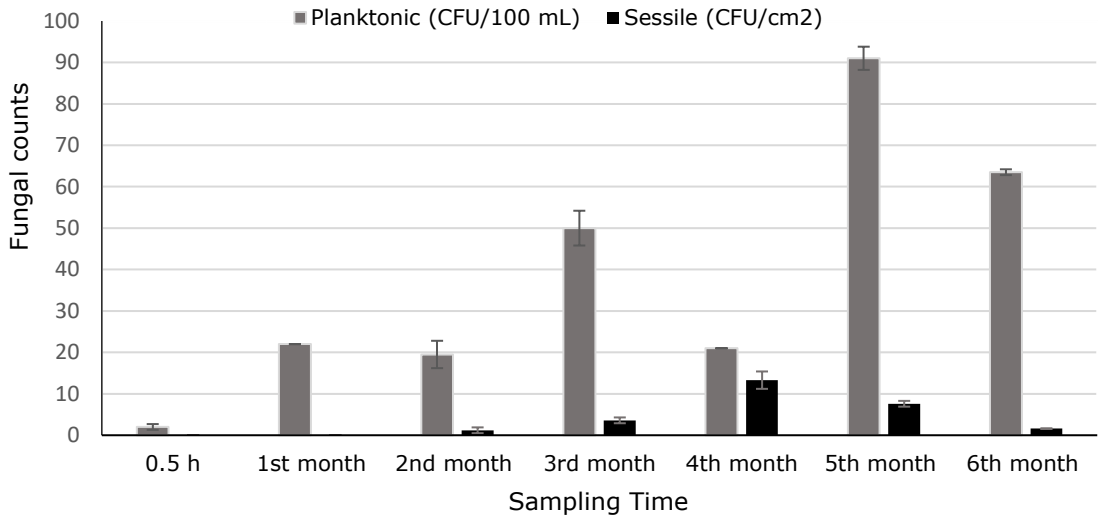

Figure 2. The total number of planktonic and sessile aerobic heteretrophic bacteria over 6 months. (log_{10} CFU/mL: log_{10} Colony forming unit per milliliter, CFU/cm^2: Colony forming unit per square centimeter, h: Hour)

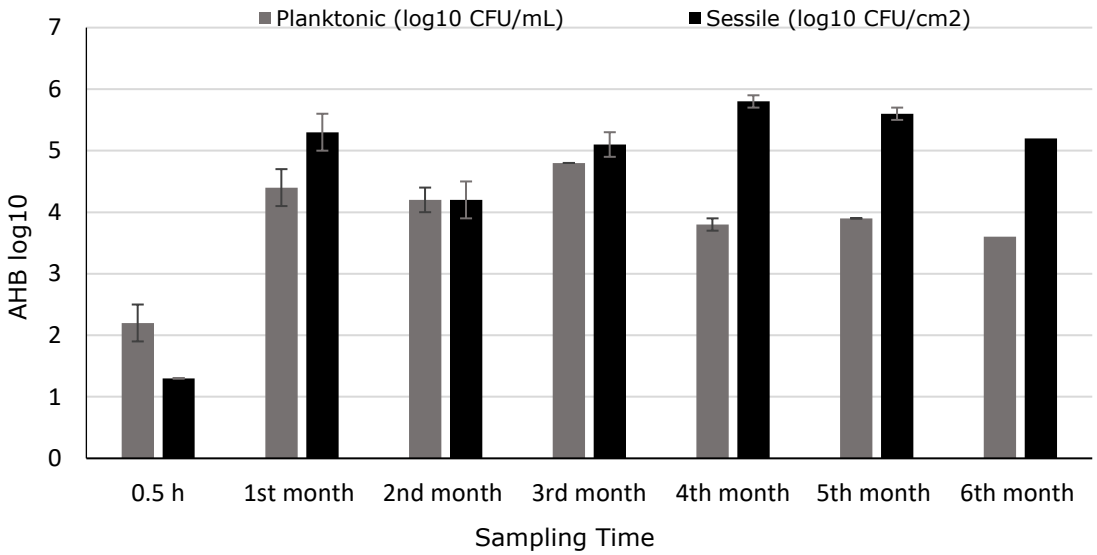

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Figure 3. SEM micrographs of the galvanized steel coupons surface after 4 months. Bars: a) 50 μm, b) 20 μm.

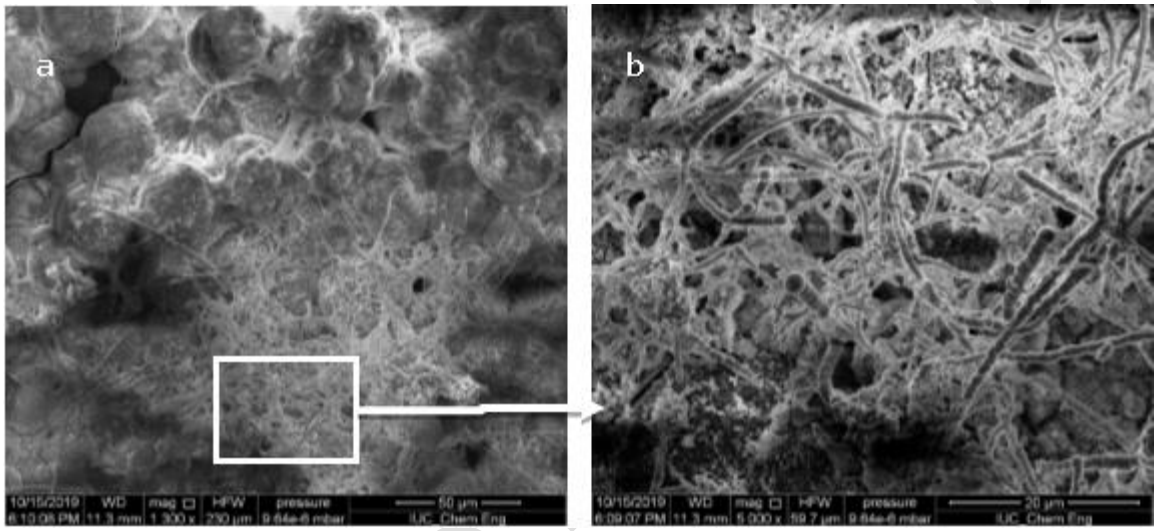

Figure 4. Electrochemical impedance spectra of the galvanized steel coupons with biofilm after 4 (a) and 5 (b) months. Circuit elements (*W:* Warburg element; R_s: resistance of the solution; R_1: resistance of charge transfer; R_2: resistance of the biofilmformed by microorganisms that primary adhere 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.

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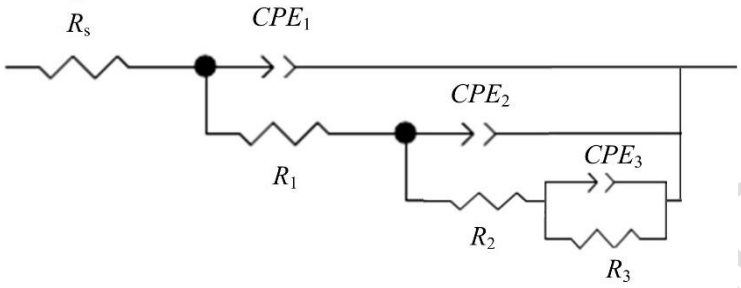
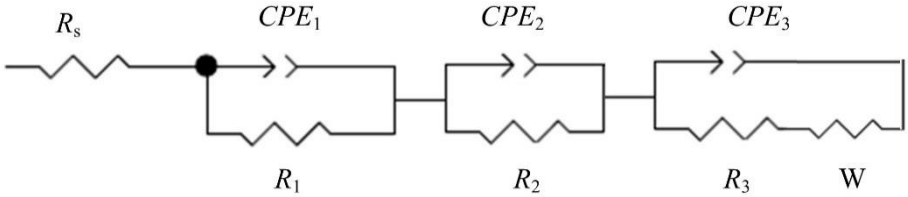
(a) 4.month



(b) 5.month



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