Biofilm Formation on Copper and Galvanized Steel Surfaces in a Cooling-Water System

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Abstract

Cooling water systems provide ideal aquatic environment for the microorganism multipication. Copper and galvanized steel are frequently used in the construction of cooling towers because of their well-known antifouling property. The current study would examine the changes in total aerobic mesophilic heterotrophic bacteria (TAMHB) counts, the presence of *Legionella* and free-living amoeba on copper and galvanized steel surfaces and the presence of biocide in a cooling water system. In this study biofilm was confirmed by SEM on copper and galvanized coupons in experiments. Statistical analysis demostrated that heterotrophic bacteria counts were significantly ($P<0.01$) higher on the surface of galvanized steel coupons than on the surface of copper coupons. Our results showed that the isolates were characterized as members of genus *Pseudomonas* or were closely related to this genus. Free-living amoeba were detected on the surface of copper coupons and galvanized steel coupons whereas *Legionella pneumophila* was not established by culture method.

Keywords: Biofilm, Cooling water systems, Copper, Galvanized steel, *Legionella*
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Introduction

A recirculating cooling system reuses the same water by it passing through heat exchangers, cooling towers to remove the heat. So the heat has been transferred into the cooling system from equipment or industrial processes (Stoitchkov and Dimitrov 1998; Flemming 2002).

Cooling water systems provide an ideal aquatic environment (nutrient supply, pH, temperature, etc.) for the microorganism multiplication (Choundhary 1998; Ludensky 2003). Bacterial population in these systems exceed a million of cfu ml^{-1}. High levels of bacteria increase the risk of microbiologically influenced corrosion (MIC) development favoring biofilm formation on wet surfaces (Licina et al. 1996; Martinez et al. 2004) and a decrease in efficiency of a heat exchanger (Characklis 1990).

Copper (especially heat exchanger) and galvanized steel (especially cooling tower) are commonly used for the structure of cooling water systems because of their anticorrosive and antifouling properties (Wagner and Little 1993).

Biofilm in cooling water systems is a fairly common problem. When the biofilm layer could not be cleaned, pathogen bacteria living there can lead to fatal diseases. *Legionella,* among these bacteria, also survive as intracellular parasites of free-living amoeba (Wadowsky et al. 1988). According to the Center for Disease Control, when bacterial populations reached or exceed 500,000 colonies the risk of *Legionella* growth will enhance per ml (Young 2000).

The aim of this study was to examine the changes in total aerobic mesophilic heterotrophic bacteria (TAMHB) counts, the presence of *Legionella* and free-living amoeba

on copper and galvanized steel surfaces and the presence of biocide in a cooling water system.

Material and Methods

Cooling water system

This study was performed by using an open recirculating flow system of a hotel cooling water system. For this purpose a modified Pedersen device was put in parallel to the heat exchanger system with copper coupons (Pedersen 1990). Furthermore galvanized steel coupons were located into water basins in a cooling tower (Pedersen 1990). The capacity of this cooling water system was 15 tonnes. Water loss by evaporation and blow-down was counterbalanced with potable water as the make-up water. The cooling water system was treated with biocide (izothiazolone, concentration of 30 ppm) and corrosion inhibitors (phosphate based, concentration of 60 ppm). Biofilms were permitted to develop for ten months on copper and galvanized coupons within the aqueous phase of the system.

Test materials

Copper and galvanized steel coupons (50 x 25 x 1 mm) were prepared according to guidelines in ASTM G1-72 (ASTM 1975).

Copper coupons: Surface of copper coupons (99.9% purity) were sanded. The total surface area of each coupon was determined.

Galvanized steel coupons: The thickness of the zinc coating covering the stainless steel was 5μm. The cut areas of all the coupons were coated with epoxy zinc phosphate primer (Moravia Turkey) and then covered with epoxy finish coating (Moravia, Turkey) to avoid the initiation of corrosion at these disturbed areas. The total surface area of each coupon was determined.

Microbiological analysis

Three coupons of each material were removed monthly from the cooling water system during ten months. Biofilms on their surfaces were scraped by sterile swab, suspended in sterile tap water and vortexed for 60 s (Gagnon and Slawson 1999). The resulting suspensions were serially diluted to 10^{-1}-10^{-7}.

For enumeration of TAMHB, 100 μl diluted biofilm suspensions were spread plated in triplicate onto R2A Agar plates and incubated at 27°C for 7 days (Reasoner and Geldrich, 1985). After the incubation, the number of colonies was enumerated under a colony counter (WTW, model BZG 30) and recorded as CFU/ml. Predominant bacteria in biofilm developed on coupons were identified by API 20 NE systems and API 20 E (Biomeriux) and also using additional biochemical tests.

For the enumeration of *L. pneumophila*, 5 ml of biofilm homogenate was treated with KCl–HCl for 15 min (pH 2.2). Also 2ml of biofilm homegenate was incubated at 50°C. Pretreated samples were inoculated (0.1 ml) buffered charcoal-yeast extract (BCYE, Oxoid, UK) agar containing glycine, vancomycin, polymyxin, natamysin and incubated at 37°C for 10-14 days. Analyses were carried out in triplicate. Colonies consistent with *L. pneumophila* morphology were subcultured to tryptone soy agar. Definitive serogroup identification was performed by latex agglutination (Dennis 1988; British Standard 1998)

In order to determine free living amoebae, biofilm suspensions were inoculated on nonnutrient agar plate seeded with *E. coli* suspension. The plates were incubated at 30°C and examined daily for up to 7 days using an inverted light microscope for the presence of amoebae (Aksözek 2001).

Scanning electron microscopy (SEM)

Copper and galvanized steel coupons were examined for biofilm by scanning electron microscopy (SEM) at the end of the experimental duration of exposure of 1 and 10 months. Coupons were fixed with 2.5 % glutaraldehyde, followed by dehydration in a graded series of ethanol and air-drying (Campanac et al. 2002). The dried samples were coated with a palladium layer (15 nm) and imaged with a Jeol JSM-6335 F electron microscope.

Statistical analysis of data

Bacterial counts were $\log_{10}$ transformed and standard errors of the means were calculated. Mann-Whitney U test was employed to detect statistically significant changes in the bacteria counts. Spearman's correlation coefficients test was used to examine the relationship between bacterial counts measured on copper and galvanized steel surfaces. The results were analysed by Spearman's correlation coefficients test and Mann-Whitney U test using SPSS for Windows Version 11.5. Statistical significance for all analysis was accepted at a $P<0.01$.

Results

Biofilm was confirmed by SEM on copper and galvanized coupons in experiments (Fig. 1).

During ten months the result of TAMHB on copper and galvanized steel surfaces are shown in Figure 2. Statistical analysis demostrated that TAMHB counts were significantly ($P<0.01$) higher on the surface of galvanized steel coupons than on the surface of copper coupons. Predominant viable microorganisms isolated on copper and galvanized steel biofilms were shown in Table 1. All isolates were Gram-negative and non-spore forming bacteria.

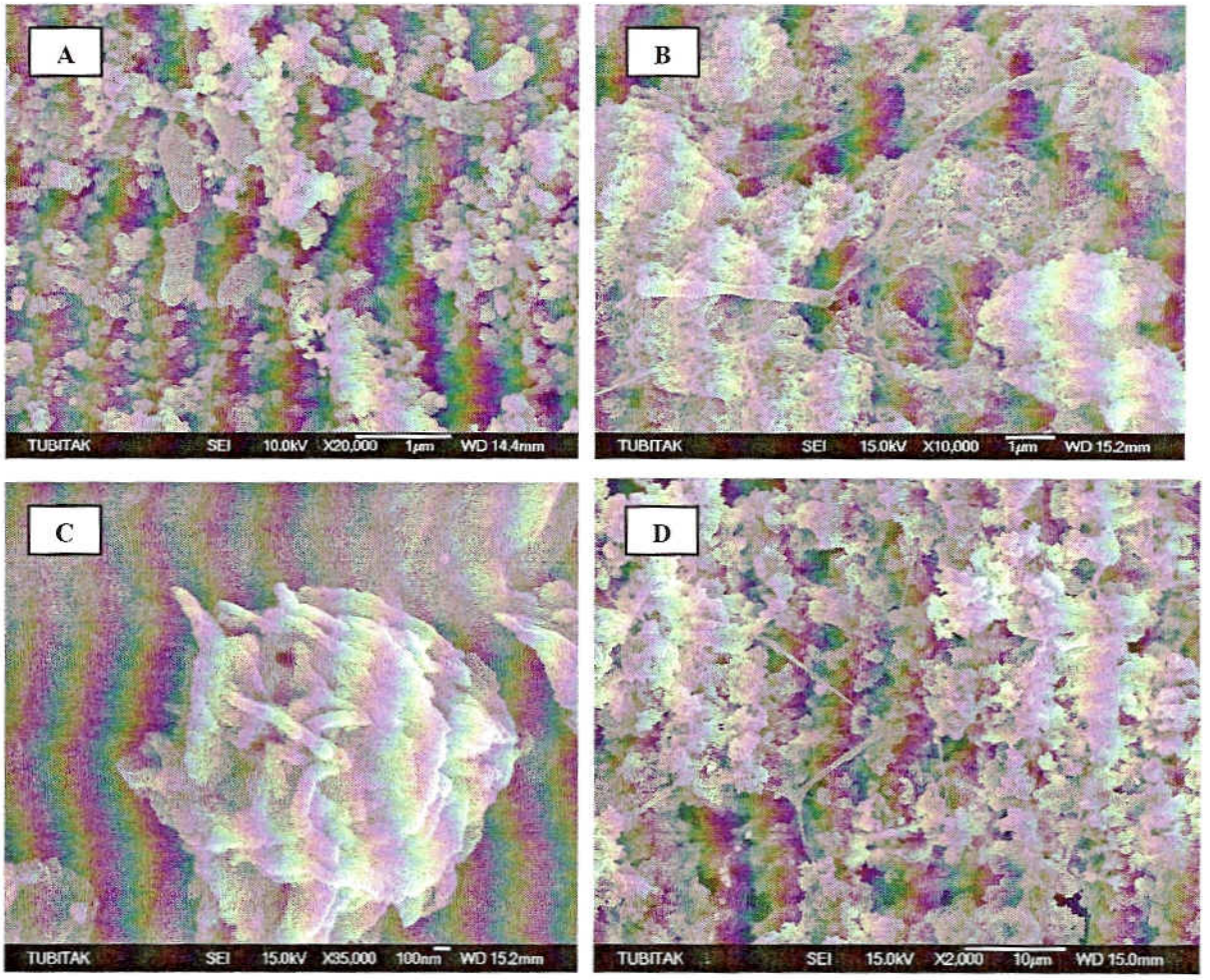

Figure 1. SEM micrograph of the biofilm formed on copper (A,B) and galvanized steel (C,D) surfaces, 1th month (A,C), 10th month (B,D)

Table 1. Predominant viable microorganisms in the biofilm on copper and galvanized steel surfaces

Names of isolated bacteria
Burkholderia cepacia (syn *Pseudomonas cepacia*)
Pseudomonas spp.
Brevundimonas vesicularis (syn *Pseudomonas vesicularis*)
Moraxella spp.
Stenotrophomonas maltophila
Comamonas testosteroni (syn *Pseudomonas testosteroni*)
Pseudomonas stutzeri
Pseudomonas fluorescens
Pseudomonas spp.
Sphingomonas paucimobilis (syn *Pseudomonas paucimobilis*)
Pasteurella spp.
Ochrobactrum anthropi

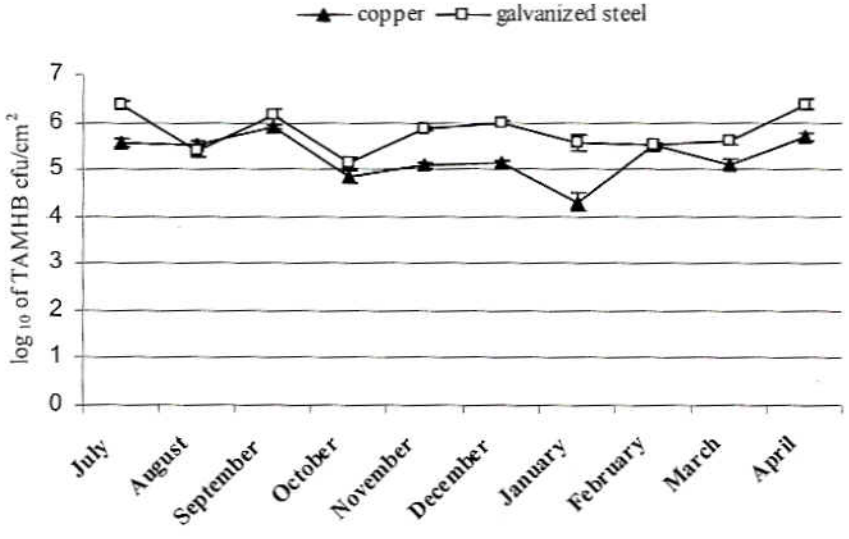

Figure 2. TAMHB in the biofilm on copper and galvanized steel surfaces. Error bars represent the standard deviation. CFU:Colony forming unit.

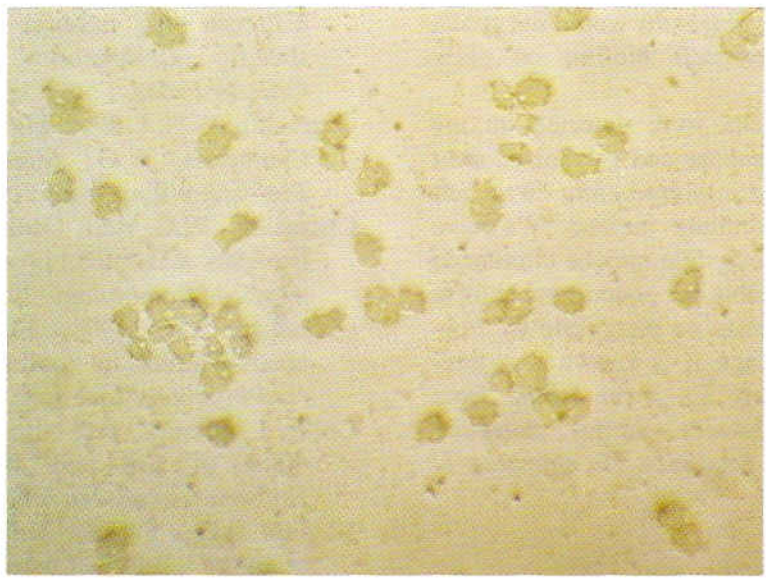

Figure 3. Free living amoeba culture isolated from biofilms

Free-living amoeba was grown in samples taken from both copper and galvanized steel coupons during ten months (Fig. 3). However, *L. pneumophila* could not be grown samples taken from the same materials.

Discussion

It is known that copper and Zn has a toxic effect on many microorganisms (Thurman and Gerba 1989; Kim et al. 2002, Gadd and Griffiths 1978; Cohen et al. 1991). Izothiazolone (biocide) that is used in our system is also toxic to these microorganisms. However our findings show the presence of biofilm formed on copper and galvanized steel surfaces. Similarly, Turetgen and Cotuk (2007) reported biofilm formation on copper and galvanized steel surfaces in a model cooling tower system. In addition the formation of biofilm on copper and galvanized steel surfaces are referred to in various researches in different experiments (Lethola et al. 2004; Dogruoz et al. 2009; İlhan-Sungur et al. 2007; Chang et al. 2003).

We found that TAMHB counts on the surface of galvanized steel coupons were significantly higher than those of copper coupons. In fact, in a model cooling tower system, Turetgen and Cotuk (2007) reported a similar result which was also in the absence of biocide in a model cooling tower system

Pseudomonas genus are acknowledged to be the pioneer colonizers in the process of biofilm formation and often found in the primary stage of biofilm formation in aquatic environments (Characklis and Cooksey 1983). In our study, strains that are identified as *Burkholderia, Brevundimonas, Comamonas, Pseudomonas* and *Sphingomonas* belong as members of *Pseudomonas* genus or are closely related to this genus. In our previously study, we pointed out *Pseudomonas* spp. attached on galvanized steel after 8 hours and it disappeared in the biofilm layer on galvanized steel after 456 hours (Dogruoz et al. 2009). However, Kimiran et al. (2008) reported that *Pseudomonas* genus were not isolated in the biofilm on copper and galvanized steel surfaces during six months in a model cooling tower system. These findings can be explain with quorum sensing, bacterial antogonism and bacterial enzymes (Kjelleberg and Molin 2002; Zeybek et al. 2005; Boyd and Chackrabarty 1994). Cell- to-cell signaling has recently been demonstrated to play a role in cell attachment and detachment from biofilms (Davies et al. 1998). Xie et al. (2000) showed

that *Porphyromonas gingivalis* would not attach to *Streptococcus cristatis* biofilm on glass slides.

Free-living amoeba were detected on the surface of copper coupons and galvanized steel coupons whereas *L. pneumophila* was not established by the culture method. We have thought that *L. pneumophila* may be eliminated with biocides and viable but nonculturable form or *L. pneumophila* may be phagosyte by free-living amoeba. In fact, it is known that free-living amoeba keep *L. pneumophila* from harmful effect of bioside. Further molecular techniques will be necessary for detecting *L. pneumophila*.

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