Quantitative Microbiological Analysis of Biofilm Communities from the Surfaces of Different Cooling Tower Materials

Ayten Kimiran-Erdem*, Nazmiye Ozlem Sanli-Yürüdü, Elif Özlem Arslan-Aydoğdu, Nihal Dogruoz, Zuhal Zeybek, Irfan Türetgen, Aysin Cotuk

Istanbul University, Faculty of Science, Department of Biology, 34134, Vezneciler, Istanbul-Turkey

Abstract

Biofilms are complex communities of microorganisms attached to surfaces or associated with interfaces. Since biofilm formation is influenced by the type of surface materials, in the current study it was aimed to compare copper, stainless steel, galvanized stainless steel, polyvinyl chloride, polyethylene, polypropylene, ceramic and glass surfaces for biofilm formation rate. In this study, both monthly collected water and biofilm samples were analyzed in terms of total coliforms, faecal coliforms, *Pseudomonas*, aerobic mesophilic heterotrophic bacteria (at 22 and 37°C) and amoebas. We found that plastic polymers, especially polyethylene and polypropylene, supported the lowest total aerobic mesophilic heterotrophic bacterial numbers. Although the protozoa (amoeba) could found on to all of the surfaces, *Pseudomonas* species could harbour none of them. It can be concluded that selection of the suitable pipe material could reduce waterborne disease and minimize the possibility of biofilm development associated with the operation of cooling tower systems.

Keywords: Biofilm, cooling tower, surface material, bacteria, amoeba.
***Corresponding author:** Ayten Kimiran-Erdem (E-mail: kimiran@istanbul.edu.tr)

Introduction

Biofouling occurs in nearly every industrial water-based process, including water treatment and distribution, pulp-paper manufacturing, the operation of cooling towers and also in medical devices such as dental unit water lines, catheters or ventilators (Percival et al. 1998; Gagnon and Slawson 1999; Momba and Binda 2002). It is generally accepted that bacteria have a tendency to attach to surfaces and initiate biofilm formation (Xu et al. 1998). Biofilms are defined as functional consortia of microorganisms organized within their extracellular polymeric substances (EPS), which facilitate irreversible attachment of cells to the surface, inorganic precipitates derived from the bulk aqueous phase and/or corrosion products of the metal substratum (Beech and Gaylarde 1999). Biofilm layer provides mechanical stability (Stoodley et al. 2002), ideal growth conditions for microorganisms and also protects its inhabitants from physico-chemical alterations occurring in the bulk water phase (Costerton 1999). In particular, protozoa may play an important role as predators of biofilm bacteria, however they can also act as protection for bacteria against exogenous influences i.e. disinfection. Biofilms lead to many undesired conditions in industry, such as decreased heat transfer in cooling towers, deterioration/corrosion of materials, increased resistance to antimicrobial compounds and growth in drinking water distribution systems (Lechevallier et al. 1988, Rogers et al. 1994b; Xu et al. 1998; Momba and Binda 2002; Schwartz et al. 2003; Sanlı-Yurudu et al. 2007). Furthermore, since biofilm can harbour infective microorganisms, detachment of cells from biofilms in water systems may result in the potential transmission of pathogens

via contaminated food, drinking water, or aerosols and have potential to increase the risk of pathogen exposure to patients (Stoodley et al. 2002).

The accumulation of microorganisms on the surfaces of pipe materials and the formation of biofilms depend on many factors prevailing in the water system, e.g. types of surface materials, pH, microbial occurrence in water, concentration and quality of nutrients, the microbial quality of intake water, and disinfectants, the presence of a disinfectant residual, water temperature, and hydraulics of the system (Niquette et al. 2000; Zacheus et al. 2000; Momba and Binda 2002).

The characteristics of the surface material composing pipes may greatly influence the densities of bacteria fixed in a distribution system (Niquette et al. 2000; Momba and Makala 2004; Türetgen and Cotuk 2007). A wide range of cooling tower construction materials is used. During the study period, bulk water was used as the water source; materials such as copper (Cu), stainless steel (SS), galvanized stainless steel (GSS), polyvinyl chloride (PVC), polyethylene (PE), polypropylene (PP), ceramic (C) and glass (G) were preferred as test pipes for the study of biofilms.

Since microbial biofilms cause problems both in medicine and industry (Xu et al. 1998), it would be beneficial to control biofilms. The main purpose of the current study was to compare the effect various pipe materials on biofilm formation and selection of the appropriate manufacturing material for water systems.

Material and Methods

Model system and test coupons

The experimental study was performed using a 100-liter polypropylene lab-scale cooling tower water system under constant hydraulic conditions, which correspond more with the situation in cooling tower installations. It is equipped with a recirculating pump in the basin and a heat source to facilitate evaporation. Cover lid has openings to ensure fresh air and daylight entry (Fig. 1). A supply of potable water was used to replenish water lost by evaporation and blowdown (partial draining). Throughout the experiment, the water temperature was kept constant at 29°C.

Materials that are commonly used in construction of cooling tower systems were selected (Cu, SS, GSS, PVC, PE, PP, C, and G, as control due to its chemically inert characteristic). All the materials are in rigid form, certified and commercially available. The coupons (20 x 50 x 1 mm) were washed with detergent, rinsed with distilled water, immersed in 70% ethanol for 5 min and air dried before use (Bloomfield et al. 1993). Biofilms were allowed to develop for 5 months on coupons within the aqueous phase of the system. Coupons were inserted vertically into water basins. No chemicals (disinfectant, pH regulators or anti-scaling agents) were added to the system, to exclude their negative effects on microorganisms and biofilm formation.

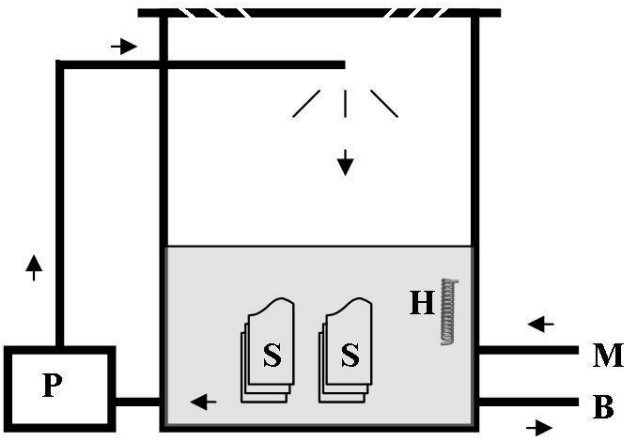

Figure 1. Schematic diagram of model recirculating water system, arrows indicate the flow direction. P: pump, S: surfaces, H: heater, M: make-up water inlet, B: blowdown outlet

Microbiological Assessments of Biofilm and Water Samples

Both water and biofilm samples were collected from the model system monthly. Samples were analyzed in terms of total coliforms (TC), faecal coliforms (FC), *Pseudomonas*, aerobic mesophilic heterotrophic bacteria (AMHB) (at 22 and 37°C) and amoeba.

Analysis of Water Samples

Total and faecal coliforms were detected by membrane filtration on selective media, Endo-NKS (Sartorious) for total coliforms and mFC agar (Sartorious) for faecal coliforms, according to the Standard Methods (APHA 1998). 100 ml water samples were filtered through membrane filter (0.45 µm pore-size) (Sartorius), and were then placed on the selective media. The mFC and Endo-NKS plates were incubated at 44^{o} and 37°C, respectively, for 18–24 h. Then the characteristic blue colonies were counted as FC. TCs were detected by production of typical red colonies with a metallic surface sheen or atypical dark red colonies without sheen.

For isolation of *Pseudomonas* species each water samples (100 ml) were filtered by cellulose membrane filter (0.45 µm) and these filters were placed on Pseudomonas CFC selective supplement added Pseudomonas base agar (Oxoid). All plates were incubated at 27 ºC for 48 hours (Donnell et al. 2005).

Plate count agar (PCA) (Oxoid) plates were inoculated with 0.1 ml bulk water samples for AMHB counts. After inoculation plates were incubated at 37 ºC and at 22 ºC for 24-48 hours.

In the amoeba analysis, each of water samples was examined to wet-mount preparation under light microscope (40x) directly. Also, each of water samples (100 ml) were filtered by cellulose membrane filter (0.45 µm) and these filters were placed on non-nutrient agar (NNA) seeded with heat-killed *Escherichia coli*. All plates were incubated at 30ºC and examined microscopically for amoeba (trophozoite and cyst) everyday for 10 days (Jeong and Yu 2005).

Each experiment was done in triplicate. At the end of the incubation periods, colonies on all plates were counted by a Colony Counter Device (åCOLyte Super Colony Counter, Synbiosis) and expressed as colony-forming units per ml (log cfu ml^{-1}).

Analysis of Biofilm Samples

Three coupons of each material were removed monthly from the basin, dip-rinsed in sterile phosphate buffer to remove unattached cells. Biofilms on surfaces were scraped by sterile scalpel, suspended in phosphate buffer

and vortexed for 60 s (Gagnon and Slawson 1999).

Homogenated biofilm samples were diluted in 1/200 ratio, from diluted biofilm homogenates, 0.1 ml liquid was drawn and transferred to PCA, mFC, Endo-NKS and Pseudomonas CFC selective supplement added Pseudomonas base agar (Oxoid) for the isolation of AMHB (at 22°C and 37°C), FCs, TCs and *Pseudomonas* spp., respectively. All plates were incubated suitable temperatures and periods. At the end of the incubation periods, bacterial colonies on the plates were counted by a Colony Counter Device (åCOLyte Super Colony Counter, Synbiosis).

Analyses were carried out in triplicate. At the end of incubation period, attached bacterial counts were expressed as log cfu cm^{-2}, and calculated using the following equation:

Attached viable count (cfu cm^{-2}) = N x D /surface area of slides where:

N = average number of colonies and

D = dilution factor.

For the analysis of amoeba each biofilm suspensions were examined to wet-mount preparation under light microscope (40x) directly. Also, 10 µl of biofilm suspensions were taken and inoculated on non-nutrient agar (NNA) seeded with heat-killed *Escherichia coli*. All plates were incubated at 30ºC and examined microscopically for amoeba (trophozoite and cyst) everyday for 10 days (Jeong and Yu 2005).

Statistical Analysis

Results were analyzed statistically by Student's *t*-test. Differences were considered significant when $p<0.05$.

Results

The planktonic populations of bacteria varied between 2.3 and 5.9 log cfu 100 ml^{-1}. It has been observed that planktonic bacteria counts in water samples were generally higher than their sessile counterparts on the surfaces of tested materials (Cu, SS, GSS, PVC, PE, PP, C and G (Table 1, 2, 3).

Table 1. Comparison of the numbers of total coliform colonies occurring in the biofilm and planktonic phase of the model system.

Age of biofilms (in months)	Means no. of Total Coliforms								
	On surfaces (log cfu cm^{-2})								In planktonic phase (log cfu 100 ml^{-1})
	PP	PE	C	GSS	Cu	G	SS	PVC	
1	3.3	4.0	4.2	4.8	4.1	3.9	3.9	3.8	5.0
2	5.3	5.3	5.2	5.7	5.2	5.2	5.1	5.4	5.6
3	4.3	4.2	4.6	4.6	4.8	3.8	4.0	4.0	5.4
4	3.7	4.0	4.1	3.9	3.8	3.9	3.6	4.0	2.3
5	4.2	3.9	4.1	4.1	3.8	4.2	3.8	5.6	4.9

PP: polypropylene, PE: polyethylene, C: ceramic, GSS: galvanized stainless steel, Cu: copper, G: glass, SS: stainless steel, PVC: polyvinyl chloride

Table 2. Comparison of the numbers of AMHB colonies at 37°C occurring in the biofilm and planktonic phase of the model system.

Age of biofilms (in months)	Means no. of Aerobic Mesophilic Heterotrophic Bacteria (AMHB) (at 37°C)								
	On surfaces (log cfu cm^{-2})								In planktonik phase (log cfu 100 ml^{-1})
	PP	PE	C	GSS	Cu	G	SS	PVC	
1	1.7	1.7	2.8	3.0	2.9	2.1	2.6	2.4	3.5
2	5.1	5.2	5.3	5.6	5.1	5.2	5.2	5.3	5.9
3	4.1	4.4	4.7	4.8	4.8	4.2	4.4	4.4	5.1
4	2.9	2.9	2.9	3.3	3.2	3.2	3.9	3.0	2.3
5	3.0	3.2	3.2	3.6	3.1	3.1	3.4	3.3	3.5

Table 3. Comparison of the numbers of AMHB colonies at 22°C occurring in the biofilm and planktonic phase of the model system.

Age of biofilms (in months)	Means no. of Aerobic Mesophilic Heterotrophic Bacteria (AMHB) (at 22°C)								
	On surfaces (log cfu cm^{-2})								In planktonic phase (log cfu 100 ml^{-1})
	PP	PE	C	GSS	Cu	G	SS	PVC	
1	0	0	2.5	2.6	2.7	1.6	2.0	2.2	3.3
2	2.4	2.1	2.3	3.2	3.4	2.2	2.9	2.9	4.1
3	2.9	2.3	2.5	3.9	4.1	2.9	3.7	3.6	5.0
4	3.7	3.7	3.8	3.7	3.2	3.2	3.2	3.6	2.3
5	0	2.8	2.6	0	2.9	2.9	0	3.4	0

The results showed that all of the materials were rapidly colonized by microorganisms. In a month, the number of all culturable bacteria on the surfaces had reached the level of 3-4.8 log cfu cm^{-2}, except *Pseudomonas* (Table 4). After 3 months, it has been observed that biofilms on nearly all surfaces reached steady state, except PVC.

Table 4. Comparison of the numbers of *Pseudomonas* spp. colonies occurring in the biofilm and planktonic phase of the model system.

Age of biofilms (in month)	*Means no of Pseudomonas* spp.								
	On surfaces (log cfu cm^{-2})								In planktonic phase (log cfu 100 ml^{-1})
	PP	PE	C	GSS	Cu	G	SS	PVC	
1	0	0	0	0	0	0	0	0	1.6
2	0	0	0	0	0	0	0	0	1.6
3	0	0	0	0	0	0	0	0	1.7
4	0	0	0	0	0	0	0	0	1.5
5	0	0	0	0	0	0	0	0	0.2

In the first two-months period, TC counts were increased gradually and have reached maximum level at the second month, on GSS surfaces (Table 1). The most colonized surface by coliforms was PVC, at the end of the examination period.

It was found that AMHB counts which incubated at 37°C have reached maximum level

on all surfaces at the second month, after that this counts were gradually decreased. The results of AMHB counts which incubated at different temperatures (22 and 37°C) were not statistically different ($p<0.05$). AMHB counts at 22°C on Cu have reached maximum level at the third month, decreased at the fifth month.

It has revealed that biofilm formation on GSS generally increased. On the other hand, plastic polymers, especially PP were supported the lowest bacterial numbers (Table 1, 2 and 3). Neither FC nor *Pseudomonas* was detected in any of the materials examined during the 5 months-periods. In bulk water samples, *Pseudomonas* number was increased dramatically at first three month whereas it was decreased last two month (Table 4). FC was not found from bulk water samples in the system. On the other hand, in bulk water samples, TC and AMHB (22 and 37°C) counts have increased gradually 2nd and 3rd month, have decreased in 4th and 5th month.

Although direct microscopic examinations of all water samples and biofilm samples for amoeba have been found as negative, culture results can be seen in Table 5. These organisms have grown from water samples during five months. Amoeba were isolated from Cu, SS, GSS, G, C, PE surfaces during five months, except on PVC and PP materials at 4th and 5th months (Table 5).

Table 5. Presence of free living amoeba on different materials in the biofilm and planktonic phase of the model system.

Age of biofilms (in month)	Amoeba								
	On surfaces (log cfu cm^{-2})								In planktonic phase (log cfu 100 ml^{-1})
	PP	PE	C	GSS	Cu	G	SS	PVC	
1	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+
4	-	+	+	+	+	+	+	-	+
5	-	+	+	+	+	+	+	-	+

Discusson

In the current study, it has been determined the number of bacteria in biofilm which were formed on different materials. Studies have reported that biofilm layer reached to stationary phase after 100-150 days in model systems (Boe-Hansen et al. 2002). In this study, the population of microorganisms increased after two and three months. The culturable bacterial numbers on the surfaces were quite variable when examining the formation of biofilm according to months. One reason of the fluctuations in the bacterial numbers on surfaces may be heterogeneity of biofilm. It is known that the biofilm consisted of several groups of microbes with nutrient requirements, and the presence of microbial groups with different physiology can vary in the biofilm with time (Zacheus et al. 2000).

The formation of biofilm is affected with various factors. One of those factors is the surface materials, which are used in water systems. Especially, colonization of bacteria shows affinity on materials which are not inert chemically. In laboratory conditions, microorganisms have a tendency to attach on hydrophobic, apolar materials such as Teflon and other plastics (such as PP, PVC, PE). When the relevant literature was examined, it was found that hydrophobic material support the formation of biofilm (Rogers et al. 1994b). However, in contrast to results of other investigators (Rogers et al. 1994a; Zacheus et al. 2000), we found that plastic polymers, especially PE and PP, supported the lowest aerobic mesophilic heterotrophic bacterial

numbers. Our findings are coherent with Türetgen and Cotuk's results (2007).

Also, it has been found that galvanized steel surfaces supported significantly higher microorganisms than other surfaces of materials did. It can be concluded that due to the rough and rugged form of this layer, GSS may provide suitable niche for microorganisms for rapid biofilm formation (Pasmore et al. 2002). Obtained results confirm previous results which showed that GSS surfaces support more microorganisms than do other materials (Türetgen and Cotuk 2007). The number of bacteria on glass which was used as control material due to its inert chemical properties.

One possible survival strategy of microorganisms is the colonization of solid surfaces by the formation of biofilms. Possible advantages of the bacterial adherence are higher availability of nutrients attached to the surfaces (Schwartz et al. 1998). It has been indicated in related studies that the pioneers on the surfaces were a diverse mixture of Gram negative microorganisms, and also a large percentage of Gram negative bacteria consisted of pseudomonads. In present study, although *Pseudomonas* was found from bulk water samples in the model system, it is suprising that no *Pseudomonas* was detected in any of the materials. This is in agreement with the results of Stepanovic et al. (2004) who showed that biofilm formation by bacteria was significantly affected by the growth medium composition. However, concerning its nutrient supply *Pseudomonas* depends on a symbiotic life together with other bacteria and protozoa such as amoeba. Therefore, the life in community with other microorganisms in biofilms seems to be an advantage or disadvantage for the growth of *Pseudomonas*. In fact, our studies about interaction of microorganisms have been reported (Cotuk et al. 2005; Zeybek et al. 2005)

In the present study, it has been understood that direct microscopic examination method of free living amoeba, both water samples and biofilm samples, is not important when compared with the cultivation method. These microorganisms can highly be overlooked by direct microscopic examination. According to the cultivation method, it was found that amoeba could not hang on to PVC and PP materials. So, we assumed that amoeba were unable to graze because of absence of other bacteria on these materials. Therefore, these protozoa may prefer other surfaces which include more bacteria. This situation can be clarified with further studies so as to investigate the relationship of bacteria and amoeba.

The study was planned to quantify the distribution of bacteria introduced between bulk water and biofilm formed on different materials in model system. Selection of the suitable pipe material could reduce waterborne disease and minimize the possibility of biofilm development associated with the operation of cooling tower systems.

Acknowledgements

The model system was donated by Dizayn Teknik Plastic Pipes & Fittings Co.

References

APHA. (1998) *Standard methods for the examination of water and wastewater*. 20th edn. Washington, D.C.: American Public Health Association, American Water Work Association, Water Environment Federation.

Beech I.B. and Gaylarde C.C. (1999) Recent advances in the study of biocorrosion-an overview. *Revista de Microbiologia*, 30 (3): 177-190.

Bloomfield S.F., Arthur M., Begun K. and Patel H. (1993) Comparative testing of disinfectants using proposed European surface test methods. *Letters in Applied Microbiology*, 10: 287-294.

Boe-Hansen R., Albrechtsen H.J., Arvin E., Jorgensen C. (2002) Bulk water phase and biofilm growth in drinking water at low nutrient conditions, *Water Research*, 36: 4477-4486.

Costerton J.W. (1999) Introduction to biofilm. *International Journal of Antimicrobial Agents*, 11: 217-221.

Cotuk A., Dogruoz N., Zeybek Z., Kimiran-Erdem A., Ilhan-Sungur E. (2005) The effect of *Pseudomonas* and *Aeromonas* strains on *Legionella pneumophila* growth, *Annals of Microbiology*, 55 (3), 219-224.

Donnell M.J.O, Tuttlebee C.M., Falkiner F.R., Coleman D.C. (2004) Bacterial contamination of dental chair units in a modern dental hospital caused by leakage from suction system hoses containing extensive biofilm. *Journal of Hospital Infection*, 59 (4): 348-360

Gagnon G.A. and Slawson R.M. (1999) An efficient biofilm removal method for bacterial cells exposed to drinking water. *Journal of Microbiological Methods*, 34: 203-214.

Jeong J. and Yu H.S. (2005) The role of domestic tap water in *Acanthamoeba* contamination in contact lens storage cases in Korea. *The Korean Journal of Parasitology*, 43: 47-50.

Lechevallier M.W., Cawthon C.D., Lee R.G. (1988) Factors promoting survival of bacteria in chlorinated water supplies. *Applied and Environmental Microbiology*, 54 (3): 649-654.

Momba M.N.B. and Binda M.A. (2002) Combining chlorination and chloramination processes for the inhibition of biofilm formation in drinking surface water system models. *Jornal of Applied Microbiology*, 92: 641-648.

Momba M.N.B and Makala N. (2004) Comparing the effect of various pipe materials on biofilm formation in chlorinated and combined chlorine-chloraminated water systems. *Water SA*, 30 (2): 175-182.

Pasmore M., Todd P., Pfeifer B., Rhodes M. and Bowman C.N. (2002) Effect of polymer surface properties on the reversibility of attachment of *Pseudomonas aeruginosa* in the early stages of biofilm development. *Biofouling*, 18, 65-71.

Percival S.L., Knapp J.S., Edyvean R.G.J. and Wales D.S. (1998). Biofilm, mains water and stainless steel. *Water Research*, 32 (7): 2187-2201.

Rogers J., Dowsett A.B., Dennis P.J., Lee J.V. and Keevil C.W. (1994a) Influence of temperature and plumbing material selection on biofilm formation and growth of *Legionella pneumophila* in a model potable water system containing complex microbial flora. *Applied and Environmental Microbiology*, 60: 1585-1592.

Rogers J., Dowsett A.B., Dennis P.J., Lee J.V. and Keevil C.W. (1994b) Influence plumbing materials on biofilm formation and growth of *Legionella pneumophila* in potable water system. *Applied and Environmental Microbiology*, 60: 1842-1851.

Sanli-Yurudu N.O, Kimiran-Erdem A. and Cotuk A. (2007) Studies on the efficacy of Chloramine T trihydrate (N-chloro-p-toluene sulfonamide) against planktonic and sessile populations of different *Legionella pneumophila* strains. *International Journal of Hygiene Environmental Health*, 210 (2):147-53.

Schwartz T., Hoffmann S., Obst U. (1998) Formation and bacterial composition of young, natural biofilms obtained from public bank-filtered drinking water systems. *Water Research*, 32 (9): 2787-2797.

Schwartz T., Kohnen W., Jansen B. and Obst U. (2003) Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiology Ecology*, 43: 325-335.

Stepanovic´ S., C´irkovic´ I., Ranin L. and Svabic´-Vlahovic M. (2004) Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Letters in Applied Microbiologyl*, 38: 428–432.

Stoodley P., Cargo R., Rupp C.J., Wilson S. and Klapper I. (2002) Biofilm material properties as related to shear-induced deformation and detachment phenomena. *Journal of Industrial Microbiology and Biotechnology*, 29: 361 – 367.

Türetgen I. and Cotuk A. (2007) Monitoring of biofilm-associated *Legionella pneumophila* on different substrata in model cooling tower system. *Environmental Monitoring and Assessment*, 125: 271-279.

Xu K. D., Stewart P.S., Xia F., Huang C.T. and McFeters G.A. (1998) Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Applied and Environmental Microbiology*, 64 (10): 4035-4039.

Zacheus O.M., Iivanainen E.K., Nissinen T.K., Lehtola M.J. and Martikainen P.J. (2000) Bacterial biofilm formation on polyvinyl chloride, polyethylene and stainless steel exposed to ozonated water. *Water Research*, 34 (1): 63-70.

Zeybek Z., Dogruoz N., Cotuk A. (2005) *In vitro* anti-*Legionella* activity of the *Bacillus* strains. *6th Congress of the International Federation of Infection Control*, October 13-16, İstanbul.