TÜBİTAK

Turk J Biol
36 (2012) 313-318

doi:10.3906/biy-1103-5

Biofilm formation comparison of the SANIPACKING® cooling tower fill material against standard polypropylene fill material in a recirculating model water system

İrfan TÜRETGEN[1], Nazmiye Özlem ŞANLI YÜRÜDÜ[1], Imke NORDEN[2]

[1]Department of Biology, Faculty of Science, İstanbul University, 34134 Vezneciler, İstanbul - TURKEY

[2]GEA 2H Water Technologies GmbH, Wettringen - GERMANY

Received: 04.03.2011

Abstract: Cooling towers are heat rejection systems that are used in some industrial applications, and they have the potential to develop infectious concentrations of *Legionella pneumophila*. SANIPACKING® cooling tower fill material and standard polypropylene fill material were compared in terms of biofilm formation potential and anti-*Legionella* activity within a 4-month period using a laboratory-scale recirculating water system. The recirculating water system was experimentally infected with a *L. pneumophila* standard strain (ATCC 33152) suspension and operated continuously until all experiments had been completed. Results showed that the *L. pneumophila* rapidly multiplied in a short time during the study within the model system bulk water. No *L. pneumophila* was isolated from the SANIPACKING® surface during the 4-month period, whereas intensive colonization occurred on the standard polypropylene material surface in the first month. Heterotrophic bacterial counts on the surfaces showed that a significantly low accumulation was recorded on the SANIPACKING® surface in comparison to the standard polypropylene material. Total bacterial counts on surfaces, determined by epifluorescence microscopy (using DAPI), revealed that significantly low counts of microorganisms colonized on the SANIPACKING® surface.

Key words: Biofilm, SANIPACKING®, *Legionella*, cooling tower, fill material

Model su sisteminde SANIPACKING® soğutma kulesi dolgu malzemesi ile standart polipropilen malzemenin biyofilm oluşturma potansiyellerinin karşılaştırılması

Özet: Soğutma kuleleri, endüstriyel uygulamalarda kullanılan ve sıcaklık düşürmeye yarayan ama aynı zamanda *Legionella pneumophila* bakterisinin yüksek sayılara ulaştığı cihazlardır. Bu araştırmada laboratuar ölçekli soğutma kulesi model sisteminde 4 ay süreyle SANIPACKING® marka soğutma kulesi dolgu malzemesi ve standart polipropilen dolgu malzemesi birbiriyle biyofilm oluşturma potansiyeli ve anti-*Legionella* özelliği bakımından karşılaştırılmıştır. Model sistem standart *L. pneumophila* suşu (ATCC 33152) ile deneysel olarak enfekte edilmiş ve tüm deneyler bitene kadar kesintisiz çalıştırılmıştır. Analiz sonuçları, model sistemin su fazında bu bakterinin hızla çoğaldığını göstermiştir. 4 aylık süreç boyunca SANIPACKING® yüzeyin üzerinden *L. pneumophila* bakterisi izole edilememişken, standart polipropilen yüzeyde ilk aydan itibaren yoğun kolonizasyon tespit edilmiştir. Heterotrofik bakteri sayısı itibariyle de SANIPACKING® yüzeyde, standart yüzeye göre anlamlı derecede düşük miktarda kolonizasyon saptanmıştır. DAPI boyası kullanılarak yapılan epifloresans mikroskopi analizleri de bu sonucu desteklemiştir.

Anahtar sözcükler: Biyofilm, SANIPACKING®, *Legionella*, soğutma kulesi, dolgu malzemesi

Introduction

Legionella pneumophila bacterium, the agent responsible for Legionnaires' disease and Pontiac fever (1), was first discovered in a 1976 outbreak in Philadelphia, USA. This bacterium has been found in various natural aquatic environments, such as rivers, ponds, and lakes (2), as well as in soil. This bacterium is also found in man-made environments (cooling towers, hot water distribution networks, pools, and whirl spas) where the temperature of the water is kept between 28 and 50 °C (2-4). *Legionella* counts increase where biofilm and warm water temperatures are present. Therefore, cooling towers have the potential to develop infectious concentrations of *L. pneumophila*. A cooling tower is a heat rejection device that extracts waste heat to the atmosphere through the cooling of a stream of water to a lower temperature. To achieve better cooling performance, a medium called fill is used to increase the surface area between the air and water flows. Film fill is composed of thin sheets of plastic material to create an increased surface area upon which the water flows.

A biofilm is a community of cells embedded in a thick mucilaginous matrix of extracellular polymeric substances (EPS), which may consist of 90% or more polysaccharides (5). This EPS layer provides important functions for the protection, survival, and dispersion of biofilm-associated microorganisms (6). Biofilm formation in cooling tower systems is undesirable for operational and public health reasons. Bacterial biofilms in cooling towers develop most frequently on heat transfer surfaces, as temperatures there favor the rapid growth of *L. pneumophila* (7). If the cooling water is not treated with biocides, these problems may cause reduced heat transfer efficiency and therefore greater energy losses with possible production cutbacks or shutdowns (8). Most nonoxidizing biocides will not kill all of the different types of bacteria that are found in water cooling systems. The application of only one such nonoxidizing bactericide will result in the selection of unaffected bacteria. On the other hand, oxidizing biocides are nonselective in their action (9). Biocides attack targets of cell function, placing the bacterium under stress. Communities under stress have lower species diversity and select for fitter species. Where a bactericide is the stress factor, fitter species would be those resistant to or more tolerant of the specific bactericide. As diversity is inversely proportional to productivity, it would influence the corrosive nature of the biofilm. In this respect, it is clear that this kind of contamination cannot be completely avoided by proper water treatment (10). Therefore, modification of the solid surface onto which the biofilm attaches has been used to reduce biofouling (11,12).

The aim of this study was to compare the SANIPACKING® film fill material and standard polypropylene (PP) film fill material in terms of biofilm formation potential and anti-*Legionella* activity during a 4-month period using a model system. The experimental study was performed using a lab-scale recirculating model system under constant hydraulic conditions, which corresponds with the situation in cooling tower installations.

Materials and methods

Model system and experiment setup

The study was performed using a 100-L polypropylene lab-scale recirculating model system under constant hydraulic conditions. It is equipped with a recirculating pump in the basin and a heat source to facilitate evaporation (Figure 1). The cover has openings to ensure fresh air and daylight entry. A

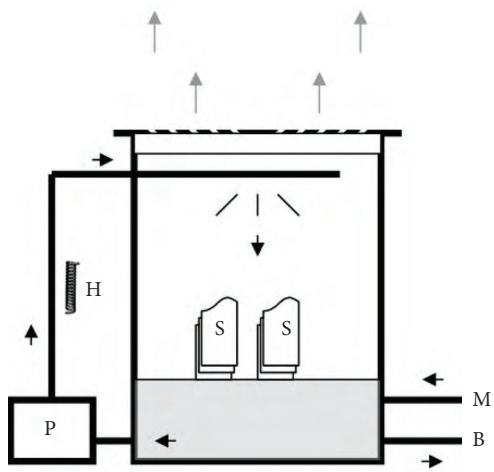

Figure 1. Schematic diagram of the model recirculating water system; arrows indicate the flow direction. P: pump, S: surfaces, H: heater, M: make-up water inlet, B: blowdown outlet.

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 37 °C. Both test materials were fixed with hangers over the bulk water surfaces without any contact with each other. Test materials were not submerged into the bulk water; the surfaces were only in contact with sprayed water through the nozzles. At the beginning of the experiment, the recirculating system was experimentally infected with a *L. pneumophila* standard strain (ATCC 33152) suspension (1 mL of *L. pneumophila* inoculum at 10^5 cells/mL) and operated continuously until all experiments had been completed. A rather low final inoculum was provided to the model system to mimic the natural entry of *L. pneumophila* from supply water. No chemicals (disinfectants, pH regulators, or antiscaling agents) were added to the system, to exclude their negative effects on microorganisms and biofilm formation.

Heterotrophic bacterial analysis

A sheet of fill material was removed monthly from the basin and dip-rinsed in sterile phosphate buffer to remove unattached cells. Randomly chosen biofilms from the surface (10 cm^2 area) were scraped using a sterile scalpel, suspended in 5 mL of sterile phosphate buffer, and vortexed (Clifton Cyclone, UK) for 60 s (13). To get a heterotrophic plate count (HPC), 10-fold diluted biofilm homogenates and bulk water were spread-plated (0.1 mL) onto R2A agar plates (Oxoid, UK) and incubated at 28 °C for 10 days. After the incubation time, the number of colonies was enumerated under a colony counter (BZG 30, WTW, Germany) and recorded as CFU/mL. HPC determinations were performed by triplicate analyses.

***Legionella pneumophila* analysis**

A sheet of fill material was removed monthly from the basin and dip-rinsed in sterile phosphate buffer to remove unattached cells. Randomly chosen biofilms from the surface (10 cm^2 area) were scraped using a sterile scalpel, suspended in a 5 mL sterile phosphate buffer, and vortexed (Clifton Cyclone, England) for 60 s (13). The suspension was divided into 2 parts; the first was pretreated with heat (50 °C, 30 min) and the second was pretreated with acid (HCl-KCl solution, pH 2.2) after centrifugation (6000 rpm, 15 min) (14). The acid and heat pretreatments were used as decontamination methods. Pretreated samples were inoculated (0.1 mL) onto alpha-ketoglutarate-supplemented buffered charcoal-yeast extract (BCYE) agar containing glycine, vancomycin, polymyxin, and cycloheximide, and were incubated at 37 °C for 10 days (15). Colonies consistent with *Legionella* morphology were subcultured to tryptone soy agar and BCYE agar plates. Definitive identification was performed by latex agglutination (16).

DAPI counting

Biofilm homogenates were stained with 1.0 µg/mL DAPI for epifluorescence microscopy in a glass tube. After 30 min, incubations were terminated by vacuum filtration onto black polycarbonate filters with a pore size of 0.2 µm (Millipore, US) after mixing with filter-sterilized double-distilled water (17,18). The air-dried polycarbonate filters were mounted on a glass microscope slide with paraffin below the filter and coverslipped. A Nikon Eclipse 80i microscope equipped with a 100-W mercury lamp was used with nonfluorescing immersion oil. The number of bacteria was estimated from counts of 20 randomly chosen fields. An eyepiece with a calibrated graticule was used for all bacterial counting.

Statistical analysis

Plate counts and microscopic signal counts were $\log_{10}$ transformed and standard errors of the means were calculated. Differences between variables were tested for significance using a t-test; differences were considered significantly different at $P < 0.05$. Statistical analyses were performed using SPSS 11.0.

Results and discussion

Based on the *L. pneumophila* culture results on BCYE agar, no *L. pneumophila* was isolated from the SANIPACKING® surface within the 4-month period, whereas intensive colonization occurred on standard PP material surface in the first month (Figure 2). Heterotrophic bacterial counts on surfaces showed that a significantly lower accumulation was recorded on SANIPACKING® surfaces in comparison to standard PP material (Figure 3).

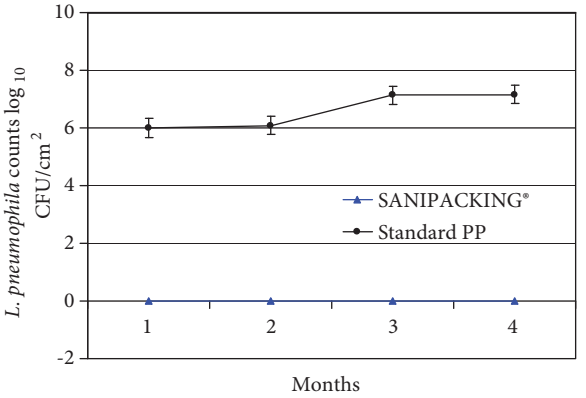

Figure 2. *L. pneumophila* counts on surfaces. Error bars represent standard deviation (SD). CFU: colony forming unit.

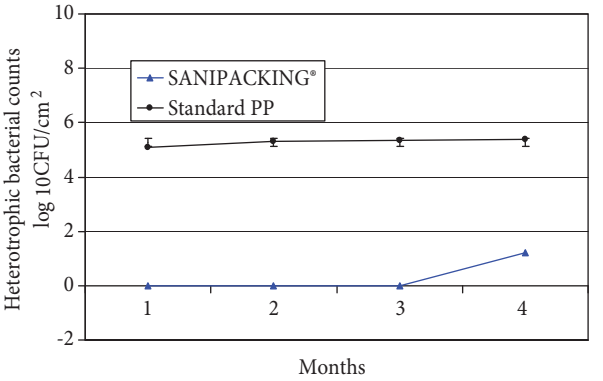

Figure 3. Heterotrophic bacterial counts on surfaces. Error bars represent SD. The value for the fourth month on the SANIPACKING® surface was 41 CFU/cm^2, which corresponds to 1.61 on the $\log_{10}$ base.

Total bacterial counts on the surfaces, determined by epifluorescence microscopy, revealed that significantly fewer microorganisms (more than 2 log) colonized on the SANIPACKING® surface (Figure 4). Heterotrophic bacterial counts in bulk water increased from 810 CFU/mL to 7810 CFU/mL within the 4-month period.

Culturable heterotrophic bacteria first appeared in the fourth month on the SANIPACKING® surface. The count on the SANIPACKING® surface was 41 CFU/cm^2, whereas the same parameter was 262,400 CFU/cm^2 on standard PP materials. This result indicates the antimicrobial effect of the SANIPACKING® surface, which reduces the biofilm formation on fill materials. Total bacterial counts, taken using epifluorescence microscopy, showed that bacteria could attach to the SANIPACKING® surface but were not viable or remained viable but not culturable. On the other hand, Figure 3 shows that the SANIPACKING® surface has a repellent or bactericidal effect on heterotrophic bacteria, which leads to reduced biofilm formation. This leads to less clogging of the SANIPACKING® fill during regular operation. It is known that cooling towers have been responsible for major Legionnaires' disease outbreaks. The anti-*Legionella* activity of the SANIPACKING® material could help to reduce the dissemination of *L. pneumophila* to the environment.

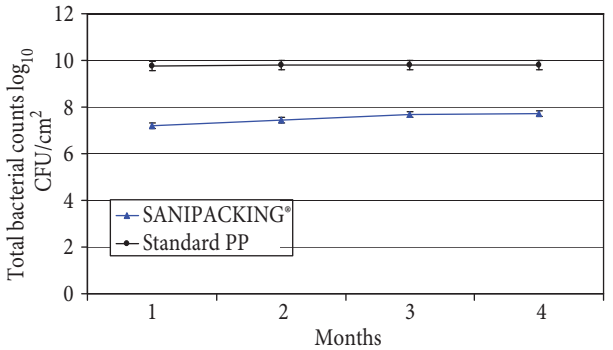

Figure 4. Total bacterial counts on surfaces. Error bars represent SD.

The objective of biocide treatment in a cooling water system is usually to keep heat exchangers relatively clean from any biofouling. Usually, this is achieved by continuous or periodic slug dosage of oxidizing biocides. The monitoring parameter is usually the number of colony forming units per milliliter of cooling water. If this number is 10^4 or below, the control program is considered to be successful (19).

The biofilm reproducibility of the model system was compared with a full-scale cooling tower system from a previous work to reveal how representative the model system is (20). No significant difference was found between biofilm, the HPC counts, and reproducibility from tested coupons between the full-scale and model systems, which was validated by a t-test of the biofilm densities on coupons of the 2 systems ($P < 0.05$). Similarly, a correlation was found between the model system and the full-scale towers in respect to pH and dissolved oxygen levels.

Biofilm is essential for the survival and multiplication of *L. pneumophila*. Interestingly, the growth of other environmental organisms stimulates the growth of *L. pneumophila* in aquatic environments (21,22). An infectious dose level for *L. pneumophila* has not been established; however, even low numbers of this bacterium constitute a risk for public health. Since cooling towers are ideal incubators, *L. pneumophila* could multiply in a very short time (4). The prolonged persistence of pathogenic bacteria in aquatic biofilms serves as a reservoir, and detachment through shear forces or other mechanisms permits continuous pathogen intrusion into bulk water (23). Material selection will not guarantee the absence of *L. pneumophila* or any other particular pathogen, nor will these measures prevent illnesses. Choosing the appropriate material will reduce populations of pathogens, thereby reducing the risk of associated illnesses. However, mechanical cleaning and chemical disinfection are strongly recommended from the beginning of tower operation (7).

To control biofilm in industrial systems, usage of mechanical techniques is not usually practical. The most widely practiced approach to controlling biofouling at minimal levels in industrial water systems is chemical treatment, either by the reduction of microbial numbers using biocides or removal using dispersants/enzymes (9,11,19,24-26). The effectiveness of a biocidal control program depends on several factors, such as transportation, adsorption, diffusion, penetration, and interaction at the target site. The differences in biocide efficacy also especially depend on the increased resistance of biofilm bacteria (19,24). Due to the problem of resistance and its potential environmental impact, alternative strategies for biofouling control need to be investigated and put into practice (27,28).

In response to ever-increasing environmental concerns, SANIPACKING® film fill material may be considered an innovative technology designed to decrease waterborne pathogens like *Legionella* and biofilm formation.

It could be concluded that the SANIPACKING® surface has effective anti-*Legionella* and antibiofilm activity as a fill material. Due to its chemical properties, *L. pneumophila* is unable to grow on it. The model system water temperature was set at 37 °C to provide the optimum growth temperature for experimentally seeded *L. pneumophila*. The results showed that *L. pneumophila* multiplied in a short time during the study within the model system. Regular biocidal treatment programs should be practiced before biofilm development. SANIPACKING® seems to facilitate of the meeting of effective control program criteria.

Acknowledgments

This work was supported by the Research Fund of İstanbul University (Project Number UDP-4160).

Corresponding author:

İrfan TÜRETGEN

Department of Biology,

Faculty of Science,

İstanbul University,

İstanbul - TURKEY

E-mail: turetgen@istanbul.edu.tr

References

1. Yu VL. Could aspiration be the major mode of transmission for *Legionella*? Am J Med 95: 13-15, 1993.
2. Yamamoto H, Sugiura M, Kusunoki E et al. Factors stimulating propagation of legionellae in cooling tower water. Appl Environ Microbiol 58: 1394-1397, 1992.
3. Bentham RH. Routine sampling and the control of *Legionella* spp. in cooling tower water systems. Curr Microbiol 41: 271-275, 2000.
4. Türetgen I, Sungur EI, Cotuk A. Enumeration of *Legionella pneumophila* in cooling tower water systems. Environ Monit Assess 100: 53-58, 2005.
5. Costerton JW. Introduction to biofilm. Int J Antimic Agents 11: 217-221, 1999.
6. Tsuneda S, Jung J, Hayashi H et al. Influence of extracellular polymers on electrokinetic properties of heterotrophic bacterial cells examined by soft particle electrophoresis theory. Coll Surf 29: 181-188, 2003.

7. Harris A. Problems associated with biofilms in cooling tower systems. In: Keevil CV, Godfree A, Holt D, Dow C. eds. Biofilms in the Aquatic Environment. Springer-Verlag; 2000: pp. 139-144.

8. Flemming HC. Role and levels of real-time monitoring for successful anti-fouling strategies - an overview. Wat Sci Technol 47: 1-8, 2003.

9. Cloete TE, Jacobs L, Brözel VS. The chemical control of biofouling in industrial water systems. Biodegredation 9: 23-37, 1998.

10. Lück PC. Ecology and control of *Legionella* and *Pseudomonas* bacteria in drinking water systems. Tenth International Symposium on District Heating and Cooling, 2006.

11. Melo LF, Bott TR. Biofouling in water systems. Exp Ther Flu Sci 14: 375-381, 1997.

12. Rusin P, Bright K, Gerba C. Rapid reduction of *Legionella pneumophila* on stainless steel with zeolite coatings containing silver and zinc ions. Lett Appl Microbiol 36: 69-72, 2003.

13. Gagnon GA, Slawson RM. An efficient biofilm removal method for bacterial cells exposed to drinking water. J Microbiol Meth 34: 203-214, 1999.

14. Bopp CA, Sumner JW, Morris GK et al. Isolation of *Legionella* spp. from environmental water samples by low-pH treatment and use of a selective medium. J Clin Bacteriol 13: 714-719, 1981.

15. Dennis PJL. Isolation of legionellae from environmental specimens. In: Harrison TG, Taylor AG. eds. A Laboratory Manual for *Legionella*. Wiley; 1988: pp. 31-44.

16. Sedgwick AK, Tilton RC. Identification of *Legionella pneumophila* by latex agglutination. J Clin Bacteriol 2: 365-369, 1982.

17. Rodriguez GG, Phipps D, Ishiguro K et al. Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. Appl Environ Microbiol 58: 1801-1808, 1992.

18. Søndergaard M, Danielsen M. Active bacteria (CTC+) in temperate lakes: temporal and cross-system variations. J Plankton Res 23: 1195-1206, 2001.

19. Ludensky M. Control and monitoring of biofilms in industrial applications. Int Biodet Biodeg 51: 255-263, 2003.

20. Türetgen I. Comparison of the efficacy of free residual chlorine and monochloramine against biofilms in model and full scale cooling towers. Biofouling 20:81-85, 2004.

21. Armon R, Starosvetzky J, Arbel T et al. Survival of *Legionella pneumophila* and *Salmonella typhimurium* in biofilm systems. Wat Sci Tech 35: 293-300, 1997.

22. Storey MV, Ashbolt NJ, Stenström TA. Biofilms, thermophilic amoebae and *Legionella pneumophila* - a quantitative risk assessment for distributed water. Wat Sci Tech 50: 77-82, 2004.

23. Banning N, Toze S, Mee BJ. Persistence of biofilm-associated *Escherichia coli* and *Pseudomonas aeruginosa* in groundwater and treated effluent in a laboratory model system. Microbiology 149: 47-55, 2003.

24. Cloete TE. Biofouling control in industrial water systems: what we know and what we need to know. Materials Corr 54: 520-526, 2003.

25. Smith I, Fricker EJ, Eccles J et al. Laboratory observations of biocide efficacy in model cooling tower systems. ASHRAE Transactions: Research 4723 (RP-954), 314-324, 2004.

26. Bott TR. Biofouling control in cooling water. Int J Chem Eng 73: 1-4, 2009.

27. De Carvalho CCR. Biofilms: recent developments on an old battle. Recent Pat Biotechnol 1: 49-57, 2007.

28. İlhan Sungur E, Türetgen İ, Javaherdashti R et al. Monitoring and disinfection of biofilm-associated sulfate reducing bacteria on different substrata in a simulated recirculating cooling tower system. Turk J Biol 34: 389-397, 2010.