© by PSP Volume 26 – No. 8/2017 pages 5021-5031 Fresenius Environmental Bulletin

EXAMINATION OF QUORUM-SENSING RESPONSES OF BIOCIDE-RESISTANT BIOFILM BACTERIA ISOLATED FROM A WASTEWATER TREATMENT SYSTEM IN INDUSTRIAL ENVIRONMENT

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ABSTRACT

Bacteria attaching surfaces contacting water reproduce in ~~it~~ the matrix they produce and form a slime layer called biofilm. Increased antimicrobial resistance of bacteria forming biofilm is thought to result from quorum sensing (QS) systems' becoming active. To remove unwanted biofilms from industrial environments, the most commonly used agents are antimicrobial agents called biocides. In the present study, how 61 different bacteria species out of 84 bacteria isolated from the waste water treatment system formed biofilm, and their resistance to Chloramine T trihydrate (Merck) and Penwater BC8120 (Hidrokim), widely used commercial biocides, were investigated. In addition, the relationship between QS systems and biocide resistance of the bacteria identified to be resistant was determined. At the end of the study, it was found that the bacteria treated with ten different concentrations of the biocides (%1, %0,5, %0,2, %0,1, %0,01, %0,001, %0,0001, %0,00001, %0,000001, %0,0000001) for 24 hours, 48 hours and 72 hours developed resistance at various levels depending on the dosage and duration of the application. However, it was also determined that the resistance of numbered 73 to 0,1%, 0,2%, 0,5% and 1% concentrations of Chloramine T trihydrate and the resistance of bacteria numbered 84 to 0,01%, 0,0001%, 0,00001%, 0,2% and 1% concentrations of Chloramine T trihydrate were based on the QS systems.

KEYWORDS:
Biofilm, biocide resistance, quorum sensing (QS) response, wastewater treatment system

INTRODUCTION

Biofilms are microbial communities developing in animate or inanimate surfaces. A polymeric extracellular slime layer (EPS = extracellular polysaccharide substances) forms the basis of this community [1]. EPS is released by biofilm bacteria and holds them together [2]. Many bacteria can initiate biofilm formation by attaching to surfaces such as live tissues, implants in the body, and wastewater, potable water or natural water systems [3, 4]. Bacteria prefer to cling to a surface rather than free-swimming in the aquatic environment because the surface to which they are attached is their supply of nutrients brought to that surface by the flowing water and is rich in oxygen due to water flow [5, 6]. Biofilm layers having been intensively investigated since the 1930s lead to substantial amounts of economic losses by causing unwanted residues and stratification called biofouling on industrial / domestic water systems, heat exchangers, water pipes, ships' hulls, and water treatment, storage and distribution facilities [7].

To remove unwanted biofilms from industrial environments, the most commonly used methods are antimicrobial agents called biocides and mechanical cleaning [8, 9]. Mechanical cleaning can be expensive, because it usually requires a significant amount of tool use and labor. In some cases, for instance, if the contaminated area is not reached, it cannot be used. The use of biocides and disinfectants may be ineffective if microorganisms in the biofilm build up resistance to antimicrobial agents [9, 10]. In industrial applications, biocides are used to control microbial growth in food, textiles, building materials or petroleum products [11]. Bacteria in the biofilm structure due to metabolic changes they undergo are reported to be 10-100 times more resistant to antimicrobials, and antiseptic and industrial biocides than are planktonic bacteria [12, 13, 14]. Compared to planktonic cells, they are more resistant to antibacterial agents, iodine, iodinepolyvinyl-pyrrolidone complex, chlorine, monochloramine, peroxygens and biocides such as glutaraldehyde, and to heat [8, 9, 11, 15]. It should be kept in mind that biocide doses exceeding the limits will not only lead to corrosion in the system and thus to economic losses but also will have negative effects on aquatic organisms in the environment where water is used or discharged. However, administration of high doses of antimicrobials is not preferred because they adversely affect environmental cycles and have toxic

effects on the environment [10].

Biofilm formation and resistance developing due to biofilm are thought to result from bacterial conjugation, plasmid, biofilm specific substances such as EPS and quorum sensing systems (QS) known as the perception of the environment [16]. Therefore, to clarify the behavior of biofilm bacteria and to reveal the molecular mechanisms of the development of resistance to antimicrobials will only be possible with QS studies. Such clinical trials are very few [17, 18]. Insufficiency of industrial and environmental studies on the issue is quite noteworthy. This study aimed to determine biofilm formation by bacteria isolated from a wastewater treatment system in the presence of various commercial biocides and to reveal the role of QS responses in biocide resistance. QS responses to different biocides and to different concentrations of those biocides obtained in the present study are also expected to contribute to the development of studies on biocides.

MATERIALS AND METHODS

Sampling and Isolation of Biofilm Bacteria producing EPS. Samples were collected from the slime biofilm layer which caused problems in the water system of Wastewater Treatment Plant (Mugla) run by Koycegiz Dalyan Environmental Protection Directorate. The samples were brought to the laboratory in sterile containers within two hours and analyzed. From the biofilm scrapings analyzed, two repetitive inoculations were performed. The media in which inoculations were performed were the casein hydrolysate of Glucose Yeast Agar (GYC agar) [19] and ESP medium (environment stimulating slime production) [20].

Identification of Isolates through Their Basic Cultural, Microscopic and Biochemical Properties. During inoculation, isolates forming a mucoid colony and producing potential biofilm were purified through stripe inoculation in petri dishes including an ESP medium. Basic microscopic, cultural and biochemical properties of the isolates were determined using conventional methods.

To achieve this purpose, the isolates' cell morphologies, gram reactions, pigmentation, oxygen requirements, growth conditions at + 4 ° C and + 42 ° C and reaction results obtained by common biochemical were analyzed. In addition, in order to obtain a single colony from the 24-hour active cultures of the isolates, stripe inoculation was carried out in the selective media such as Enterococcus agar, Eosin methylene-blue agar, Salmonella-Shigella agar, Mannitol Salt Phenol-Red agar, Pseudomonas agar, and Petri dishes were incubated for 24-72 hours in the appropriate media at appropriate temperatures. At the end of the incubation, each medium was checked to find out whether the samples grew, if there was growth, typical colony morphologies were recorded [21, 22].

Sixty-one bacteria thought to be different from each other after the diagnostic studies were selected for use in the study.

Detection of Biofilm Production Capacities of Bacteria. Biofilm formation capabilities of the isolates obtained as pure culture were qualitatively investigated using the modified standard tube method [23, 24]. Isolates were inoculated in tubes including 10-ml Tripticase Soy Broth (TSB) (Oxoid) at a density of McFarland No. 1 and incubated for 12 hours at 37°C. Then the contents were slowly emptied. Then, the tubes were washed with 0.01 M phosphate buffer solution (pH 7.2). After washing, 1% safranin solution was put in the tubes and the tubes were left at room temperature for 30 minutes. The dye solution was emptied, and then the tubes were washed twice with the phosphate buffer solution, turned upside down on filter paper and left there to dry up. The formation of a colored film on the tube wall the next day was considered positive.

Determining the Biocide Resistance of Bacteria. In the present study, monochloramine (NH2Cl) [Chloramine T trihydrate (c7h7clnano2s. 3h2o)], an oxidizing agent which is a potential alternative of chlorine, and Penwat BC 8120, a quaternary ammonium compound (QAC), were used. The chemical compositions and chemical and physical properties of these biocides are shown in Table 1.

Based on the product information of both biocides, 1000, 2000, 5000, 10000 mg / l (0.1-0.2-0.5-1%) and 0.001, 0.01, 0.1, 1, 10, 100 mg / l (0.0000001-0.000001-0.00001-0.0001-0.001-0.01%) concentrations were prepared by diluting with sterile distilled water [25].

During biocide resistance trials, suspensions turbidimetrically prepared from 24-h active cultures of the bacterial strains with sterile physiological serum in accordance with Mc Farland No. 1 standard were used. The suspensions included $3x10^8$ CFU (Colony Forming Units) / ml of live bacteria [26].

To determine the bacteria's resistance to biocides, different concentrations of biocides (0.0000001-0.000001-0.00001-0.0001-0.001-0.01-0.1-0. 2-0.5-1%) and appropriate amount of neutralizer (0.5% sodium thiosulfate, or 0.4% sodium dodecyl sulfate) were added to the sterilized Tryptic Soy Agar (TSA) in aseptic conditions. The media prepared this way were placed on sterile empty petri dishes, and frozen so that no water droplets would remain in the media. Then they were kept at the room temperature until they dried thoroughly. Five µl of 18-24h fresh bacterial cultures adjusted to

TABLE 1
According to the characteristics of biocides label and prescribing information

Trade Name	Chemical Composition	Application		Physical Features				pH	Solubility in water	Manufacturer
		Concentration-Density	Temperature	Method	Phase	Smell	Color			
Chloramine T trihydrate	C7H7ClNaNO2S.3H2O	1,5-2,5 mg/l	Cold water	Spray Wash	Solid	Weak Chlorine Odor	Yellowish white	8-10 (50 g/l-20 °C)	Good soluble	Merck
Penwater BC 8120	Quaternary ammonium compound	0,9-1,15 g/cm3	Cold water	Dip Wash	Clear Liquid	Odorless	Light blue	4,5-6 (20°C)	Good soluble	Hidrokim

match the 0.5 McFarland standard were taken with a micropipette and inoculated on the pre-numbered surfaces of the petri dishes for each bacterium through spotting. After 48-h incubation at 30 °C, growth status of the bacteria inoculated on petri dishes was recorded [21].

Toxicity Tests of Neutralizers. Eight ml of neutralizing agent, 1 ml of sterile water and 1 ml of 3x108 CFU / ml bacterial suspension were put in a sterile tube and kept in 20 ° C water bath for 5 min. After the desired contact time of the bacterial suspension and neutralizing agent was ended, the tube contents were vortexed and 1 ml of the mixture was taken and spread over Petri dishes containing ESP Agar twice. To check the counts, 0.1 ml of bacterial suspension was spread on the petri dishes twice. After the petri dishes were incubated at 37 ° C for 48h, the colonies were counted and whether neutralizers were toxic to bacteria was determined [27].

The test results demonstrated that 0.5% sodium thiosulfate and 0.4% sodium dodecyl sulfate used to neutralize Chloramine T trihydrate were not toxic to bacteria. However, when the mixture of Tween 80 (3%) and lecithin (0.3%) was used to neutralize the Penwater BC 8120, it was observed that Tween 80 was toxic. Thus, for the neutralization of Penwater BC 8120, Tween 80 was replaced with 0.4% sodium dodecyl sulfate determined to be nontoxic to the bacteria.

Statistical Analysis. In the present study, in order to determine the effects of biocides on biofilm formation and bacterial growth, the computer program GraphPad (Prism) 2.01 was used. For the statistical comparisons, one-way Analysis of Variance (ANOVA) was used. For the statistical analysis, P value of ≤0.05 was considered significant.

Preparation of the Test Bacteria to Determine the QS Responses of Biocide-Resistant Biofilm Bacteria. After the screening, QS responses of biocide-resistant biofilm bacteria were determined using the following reference strains: *Chromobacterium violaceum* ATCC 12472 (DSM 30191=NCIB 9131=NBRC 12614=CV026) and A*grobacterium tumefaciens* ATCC 19358 (DSM 30147=NCIB 9042=NBRC 13532=NT1. To achieve the goal, the replica plates which the bacteria resistant to different concentrations of biocides formed on the TSA medium were taken from inoculated colonies and then they were adjusted to 0.5 McFarland using the sterile physiological saline. Each of the standardized bacterial suspensions was prepared to examine their QS responses.

Production and Storage of *C. violaceum* and A. *tumefaciens* Reference Strains. All the *C. violaceum* strains used in the experiment were kept in a deep freezer at −80°C for long-term storage (3-6 months). For daily use, C. *violaceum* was inoculated on the Nutrient Agar (NA) and incubated for 24 hours at 30 ° C. A. *tumefaciens* was inoculated on the Rhizobium medium and incubated at 37 ° C for 24 hours. Then, for daily use, it was stored at + 4 ° C maximum for 7 days.

Detection of QS Responses. QS responses of the test bacteria prepared as mentioned above were investigated using the AHL (N-Acyl-homoserine lactones) method developed from several studies in the literature [28-31]. In the AHL method, equal amounts of Luria Bertani Agar (LBA) were distributed to each well of the microplates and dried at room temperature for 2 hours. 50µl of *C. violaceum* and *A. tumefaciens* reference strains incubated in the Luria Bertani Broth (LB) at 30 ° C for 18 hours and adjusted to match the 0.5 McFarland turbidity standard were distributed to each well. Likewise, 50µl of the test bacteria taken from a standardized solution were distributed to each well. Detection of AHL signal molecules was performed using *C. violaceum* and *A. tumefaciens* reference strains. These strains were grown in the LB medium solidified with 1.2% agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl). In addition, gentamicin (20 mg / ml) was added for the A. *tumefaciens* strain and kanamycin (20 mg / ml) was added for the *C. violeceum* strain. For the detection of AHL molecules with the acyl side chain of 4-8 carbons, the *C. violaceum* reference strain was used. AHL molecules present in the medium stimulate the production of violacein, a purple pigment in the *C. violaceum* reference strain [28, 32].

Thus, the purple pigment production during the incubation indicated the presence of the quorum-sensing signal molecule N-butanoyl-L-homoserine lactone (BHL) [28]. *A. tumefaciens* strain carrying the plasmid pZLR4 was used as another reference

strain. A. *tumefaciens* strain produced a blue-green pigment in the presence of X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopy ranoside) in the media through the stimulation by AHL molecules with the N-acyl side chain of 6-12 carbons (Bruhn et al., 2005; Ulusoy, 2007). The formation of blue-green pigment during incubation indicated the presence of the quorum-sensing signal molecule N- (3-okzodo-dekanoyl) -L-homoserine lactone (OdDHL) [29, 30].

TABLE 2
Basic cultural and microscopic characteristics of the isolates.

Isolate No	Cell morphology	Gram reaction	Pigment	O_2 demand	Growth at +4°C	Growth at 42°C
1	Bacillus	+	Dark yellow	Facultative	+	++
2	Bacillus	+	Ab	Facultative	+	++
3	Bacillus	-	Ab	Facultative	+	++
5	Bacillus	-	Ab	Facultative	++	+
7	Bacillus	-	Yellow	Aerobic	+	+
8	Bacillus	-	Ab	Facultative	+	-
9	Bacillus	+	Ab	Facultative	++	+
10	Coccobacillus	-	Ab	Facultative	++	++
11	Bacillus	-	Ab	Aerobic	+	++
12	Coccus	+	Ab	Facultative	++	+
13	Coccobacillus	-	Ab	Facultative	-	+
14	Bacillus	+	Ab	Facultative	+	+
15	Bacillus	+	Ab	Facultative	+	+
17	Bacillus	+	Ab	Facultative	-	++
18	Bacillus	-	Ab	Facultative	++	++
19	Bacillus	+	Ab	Facultative	-	++
20	Bacillus	-	Ab	Facultative	+	-
21	Bacillus	+	Ab	Facultative	+	-
23	Coccobacillus	-	Ab	Facultative	+	+
27	Bacillus	-	Ab	Facultative	+	-
28	Bacillus	+	Ab	Facultative	+++	-
30	Coccobacillus	-	Ab	Facultative	++	+
31	Bacillus	-	Ab	Facultative	+++	-
32	Bacillus	+	Ab	Facultative	+	++
33	Bacillus	+	Ab	Aerobic	+++	++
34	Bacillus	+	Ab	Facultative	++++	+
35	Bacillus	-	Ab	Facultative	+++	-
36	Bacillus	-	Ab	Facultative	+++	++
37	Bacillus	+	Ab	Facultative	+++	+
38	Coccus	+	Ab	Aerobic	+++	-
39	Bacillus	-	Ab	Facultative	+++	-
40	Bacillus	-	Ab	Facultative	+++	+
41	Bacillus	+	Ab	Facultative	+++	++
43	Bacillus	-	Pale pink	Facultative	-	+
44	Bacillus	-	Dark yellow	Facultative	++	++
45	Bacillus	-	Ab	Facultative	++++	++
46	Bacillus	+	Ab	Aerobic	+++	++
47	Bacillus	-	Ab	Facultative	+++	-
48	Bacillus	-	Ab	Facultative	+++	+
49	Bacillus	-	Ab	Facultative	+++	-
50	Bacillus	-	Ab	Facultative	+++	++
52	Bacillus	-	Ab	Facultative	+++	+
53	Bacillus	+	Ab	Facultative	++	+
54	Bacillus	-	Ab	Facultative	-	-
57	Diplo-coccobacillus	-	Ab	Facultative	++	++
60	Bacillus	-	Ab	Aerobic	-	-
61	Bacillus	-	Ab	Facultative	++	++
62	Bacillus	-	Ab	Facultative	++	++
63	Bacillus	+	Ab	Facultative	++	+
64	Coccus	+	Ab	Facultative	+++	+
65	Bacillus	-	Ab	Aerobic	++	+
66	Bacillus	+	Ab	Facultative	++	+
67	Bacillus	+	Ab	Facultative	-	-
68	Bacillus	+	Ab	Facultative	+++	+
69	Bacillus	+	Ab	Facultative	+++	+
70	Bacillus	+	Ab	Aerobic	++	+
73	Coccobacil	+	Brown	Facultative	-	+
74	Bacillus	+	Ab	Facultative	+++	+
82	Bacillus	+	Ab	Aerobic	-	-
84	Streptococcus	-	Ab	Facultative	++	++

(-): No growth, (+): Very weak growth, (++): Weak growth, (+++): Strong growth, Ab: Absent

RESULTS AND DISCUSSION

Eighty-four potential biofilm producing and mucoid-colony forming isolates isolated from biofilm samples were identified based on the data given in table 2 and table 3. Then 61 bacteria species considered to be different from each other were isolated from these 84 bacteria.

Having considered their growth characteristics in the selective media, these isolated 61 bacteria species were determined to belong to the following genera: *Enterobacter, Salmonella, Bacillus, Pseudomonas, Escherichia, Acinetobacter, Staphylococcus, Proteus, Achromobacter, Rautella, Providencia, Klebsiella, Nitrosomonas, Flavobacterium* and *Myxococcus*. Some of the isolates were identified at the species level. However, because molecular diagnostics methods were not used, all the isolates were used by using bacteria numbers given by the researchers in the present study. Based on the standard tube method performed, all of these bacteria were identified as biofilm producing bacteria.

After the biocide resistance tests, it was demonstrated that increasing concentrations of commercial biocides used in the present study decreased biofilm formation capability of some bacteria. However, biofilm formation capability of some bacteria was not affected, and even these bacteria developed resistance to the administered doses of biocides.

TABLE 3
The results of biochemical tests of isolates.

Isolate No	Nitrit test	Gas production	H_2S	Jelatinase	Motility	Oksidase	Katalase	Lysine decarboxylase	Urea	O/F	İndol	Methyl red	VP	Citrate
1	-	-	-	-	-	-	-	-	+	-	-	-	-	-
2	Red	-	-	++++	-	+	+	-	+	OF	-	-	-	-
3	Red	+	-	-	+	-	-	+	-	-	+	+	-	-
5	-	-	-	+	+	-	+	-	-	O	-	-	-	-
7	-	-	-	-	+	-	+	-	-	-	-	-	-	-
8	Red	-	-	-	+	-	-	-	-	-	-	-	-	-
9	-	-	-	-	+	-	-	-	-	-	-	-	-	-
10	Red	+	-	-	+	-	+	+++	-	OF	+	-	+	+
11	Red	-	-	+++	+	-	-	-	-	F	-	-	+	-
12	-	-	-	-	+	-	+	-	-	-	-	-	-	-
13	Red	+	-	-	+	-	+	++	-	OF	-	-	+	+
14	-	-	-	-	+	+	+	-	-	-	-	-	-	-
15	-	-	-	-	+	-	-	-	-	OF	-	+	+	-
17	-	-	-	-	+	-	-	-	-	OF	-	-	+	-
18	Red	-	-	++	+	-	+	+	-	OF	-	-	-	-
19	-	-	-	-	+	-	-	-	-	-	-	-	-	-
20	-	-	-	+	+	-	+	++	-	O	-	-	-	-
21	-	-	-	-	+	-	-	-	-	-	-	-	-	-
23	-	-	-	-	+	-	-	+++	-	OF	-	+	-	+
27	Red	-	-	-	+	-	+	-	-	-	-	-	-	-
28	-	-	-	-	+	-	-	-	-	-	-	-	-	-
30	Red	+	-	-	+	-	+	+	-	OF	-	-	+	+
31	Red	-	-	-	-	+	-	-	-	-	-	-	-	-
32	-	-	-	++	+	+	+	-	-	OF	-	-	+	-
33	Red	-	-	+	+	-	+	-	-	OF	-	-	+	-
34	-	-	-	-	-	-	+	-	-	OF	-	-	-	-
35	Red	-	-	-	+	-	+	+	-	OF	-	-	+	+
36	Red	+	-	+++	+	-	-	++	-	OF	-	-	-	-
37	Red	-	-	-	+	-	+	-	-	-	-	-	-	-
38	-	-	-	-	+	-	-	-	-	-	-	-	-	-
39	-	-	-	-	+	+	+	-	-	-	-	-	-	-
40	Red	-	-	-	+	-	+	++	-	OF	-	+	+	+
41	-	-	-	-	+	-	-	+++	-	OF	-	+	+	-
43	-	+	-	-	+	-	-	+++	-	-	+	+	-	-
44	Red	-	-	-	+	-	-	++	-	-	-	-	-	-
45	Red	-	-	-	+	-	-	+	-	OF	+	+	-	-
46	Red	-	+	++	+	-	-	-	-	-	-	-	+	+
47	Red	-	-	-	+	-	+	++	-	-	-	-	-	-
48	Red	+	-	+	+	-	+	++	-	OF	-	+	+	-
49	Red	-	-	++++	+	-	-	++	-	OF	+	+	+	+
50	-	-	-	-	+	-	-	-	+	OF	-	-	-	+
52	-	-	-	-	+	+	+	-	-	OF	-	-	-	-
53	-	-	-	-	+	-	-	-	-	-	-	-	-	-
54	-	-	-	-	+	-	-	-	-	-	-	-	-	-
57	Red	+	-	++	+	-	-	++	-	OF	-	-	-	-
60	Red	+	-	-	+	-	-	+	+	OF	+	+	+	+
61	Red	-	-	-	+	-	-	+	-	OF	+	+	-	-
62	Red	-	-	-	+	-	-	+	-	-	+	+	-	-
63	-	-	-	-	+	-	-	-	-	-	-	-	+	-
64	-	-	-	-	+	-	-	-	-	-	-	-	+	-
65	-	-	-	++	+	-	-	-	-	-	-	-	-	-
66	-	-	-	-	+	-	+	-	-	-	-	-	-	-
67	-	-	-	-	-	-	+	-	-	-	-	-	-	-
68	-	-	-	-	+	+	+	-	-	-	-	-	+	-
69	-	-	-	-	+	+	+	-	-	-	-	-	-	-
70	-	-	-	-	+	+	+	-	-	-	-	-	+	-
73	-	-	-	++++	+	+	-	-	-	-	-	-	+	+
74	-	-	-	+++	+	-	+	-	-	-	-	-	+	-
82	-	+	-	+++	+	+	-	-	-	-	-	-	+	+
84	-	+	-	-	+	-	-	+++	-	-	-	+	+	-

(-): No growth, (+): Very weak growth, (++): Weak growth, (+++): Strong growth, O: Oxidative, F: Fermentative, OF: Oxidative / Fermentative

This result was considered statistically significant (P≤0.05) (Table 4 and 5). Because Chloramine T trihydrate caused the distortion of the broth at 1% and 0.5% concentrations when 0.4% SDS (sodium dodecyl sulfate) was used as a neutralizer, SDS was replaced with 0.5% sodium thiosulphate.

The bacteria's susceptibility to Chloramine T trihydrate biocide (neutralizer = 0.5% sodium thiosulphate) was statistically significant (P≤0.05) (Table 4). The bacteria's susceptibility to Penwater BC8120 biocide was statistically significant (P≤0.05) (Table 5). When ten different concentrations of the biocides were considered together, it was determined that the number of bacteria resistant to Chloramine T trihydrate was 20-34 in 24h, 33-49 in 48h, 35-51 in 72h, whereas the number of the bacteria resistant to Penwater BC8120 was 10-45 in 24h, 12-45 in 48h, 12-5 in 72h. These results regarding the two biocides used in the present study suggested that biofilm-producing bacteria could also develop high resistance to many other commercially used biocides.

To break the resistance of the biofilm layer, disinfection of industrial systems should be regularly performed with the appropriate dose of the biocide [31, 32]. Appropriate dose and appropriate treatment time play an important role in the selection of the biocides to be used in industrial facilities for disinfection [33]. In the present study, the number of bacteria developing resistance was 40 when the highest dose (1%) of Chloramine T trihydrate was used, and 1 of them produced biofilm most in 24h, 25 in 48h and14 in 72h. When they were treated with 0.1% biocide, the number of bacteria developing resistance was 45 and while 3 of them produced biofilm most in 48h, 42 produced biofilm most in 72h. When they were treated with the lowest dose (0.0000001%) of the same biocide, the number of bacteria developing resistance was 51, and 2 of them produced biofilm most in 24h, 17 in 48h and 32 in 72h. A little different from the case Chloramine T trihydrate was used, when Penwater BC 8120 was used, biofilm production was high in all the three periods (24h, 48h and 72h) depending on the dose of the biocide. For example, the number of bacteria developing resistance was 14 when the highest dose (1%) of Penwater BC8120 was used, and 6 of them produced biofilm most in 24h, 1 in 48h and 7 in 72h. When they were treated with the 0.01% concentration of the same biocide, the number of bacteria developing resistance was 45 and 4 of them produced biofilm most in 48h whereas 1 produced biofilm most in 72h. When they were treated with the lowest dose (0.0000001%) of the same biocide, the number of bacteria developing resistance was 10 and 3 of them produced biofilm most in 48h while 4 of them produced biofilm most in 72h.

TABLE 4
Resistance of bacteria against Chloramine T trihydrate (Nötralizer= %0,5 Sodyum Tiyosülfat) biocide

Concentration	Sensitive n (%)			Resistant n (%)			Sensitive+ Resistant (n=61) Toplam		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
%1	41 (67.2)	28 (45.9)	24 (39.3)	20 (32.8)	33 (54.1)	37 (60.7)			
%0.5	35 (57.4)	22 (36.1)	15 (24.6)	26 (42.6)	39 (63.9)	46 (75.4)			
%0.2	31 (50.8)	18 (29.5)	13 (21.3)	30 (49.2)	43 (70.5)	48 (78.7)			
%0.1	30 (49.2)	20 (32.8)	15 (24.6)	31 (50.8)	41 (67.2)	46 (75.4)			
%0.01	31 (50.8)	14 (22.9)	11 (18.0)	30 (49.2)	47 (77.1)	50 (82.0)			
%0.001	31 (50.8)	12 (19.7)	11 (18.0)	30 (49.2)	49 (80.3)	50 (82.0)	61	61	61
%0.0001	27 (44.3)	14 (22.9)	11 (18.0)	34 (55.7)	47 (77.1)	50 (82.0)	(100.0)	(100.0)	(100.0)
%0.00001	35 (57.4)	26 (42.6)	26 (42.6)	26 (42.6)	35 (57.4)	35 (57.4)			
%0.000001	32 (52.5)	14 (22.9)	10 (16.4)	29 (47.5)	47 (77.1)	51 (83.6)			
%0.0000001	27 (44.3)	14 (22.9)	10 (16.4)	34 (55.7)	47 (77.1)	51 (83.6)			

Sensitive: No growth, Resistant: Growth. (P≤0.05 Statistically significant)

TABLE 5
Resistance of bacteria against Penwater BC8120 biocide

Concentration	Sensitive n (%)			Resistant n (%)			Sensitive+ Resistant (n=61) Toplam		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
%1	43 (70.5)	32 (52.5)	15 (24.6)	18 (29.5)	29 (47.5)	46 (75.4)			
%0.5	23 (37.7)	16 (26.2)	22 (36.1)	38 (62.3)	45 (73.8)	59 (63.9)			
%0.2	51 (83.6)	49 (80.3)	49 (80.3)	10 (16.4)	12 (19.7)	12 (19.7)			
%0.1	42 (68.9)	36 (59.0)	36 (59.0)	19 (31.1)	25 (41.0)	25 (41.0)			
%0.01	29 (47.5)	27 (44.3)	27 (44.3)	32 (52.5)	34 (55.7)	34 (55.7)			
%0.001	32 (52.5)	31 (50.8)	31 (50.8)	29 (47.5)	30 (49.2)	30 (49.2)	61	61	61
%0.0001	27 (44.3)	27 (44.3)	27 (44.3)	34 (55.7)	34 (55.7)	34 (55.7)	(100.0)	(100.0)	(100.0)
%0.00001	23 (37.7)	23 (37.7)	23 (37.7)	38 (62.3)	38 (62.3)	38 (62.3)			
%0.000001	16 (26.2)	16 (26.2)	16 (26.2)	45 (73.8)	45 (73.8)	45 (73.8)			
%0.0000001	23 (37.7)	23 (37.7)	23 (37.7)	38 (62.3)	38 (62.3)	38 (62.3)			

Sensitive: No growth, Resistant: Growth. (P≤0.05 Statistically significant)

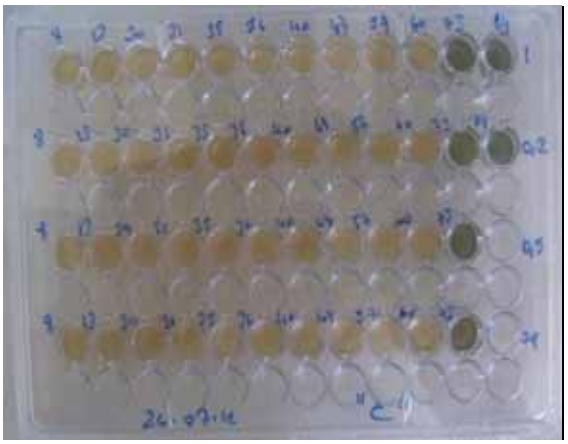

FIGURE 1
Biocide resistance of bacteria numbered 73 and 84 to Chloramine T trihydrate (When incubated with the *C. violaceum* reference strain)

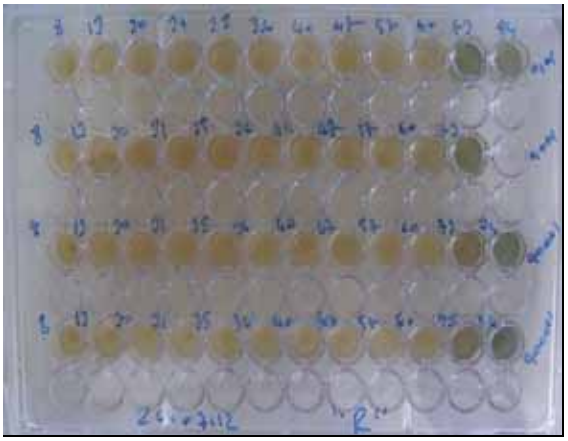

FIGURE 2
Biocide resistance of bacteria numbered 84 to Chloramine T trihydrate (When incubated with the *A. tumefaciens* reference strain)

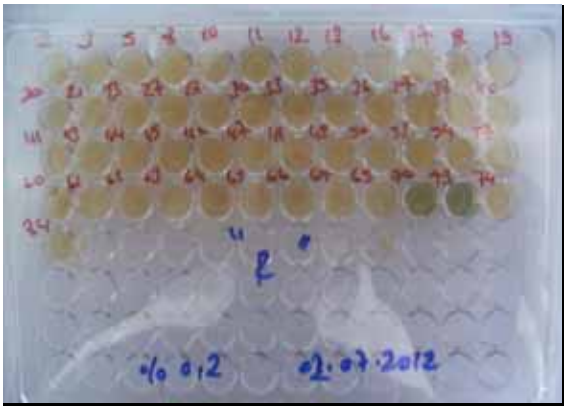

FIGURE 3
Biocide resistance of bacteria numbered 70 and 73 to Penwater BC8120 (When incubated with the *A. tumefaciens* reference strain).

Figures (Figures1, 2 and 3) show the formation of purple-blue-green pigments indicating that the biocide resistance of bacteria is based on the QS system.

In addition, 61 bacteria developed resistance to Chloramine T trihydrate more than they did to Penwater BC8120. For example, the numbers of bacteria developing resistance to 0.2% and 0.001% concentrations of Chloramine T trihydrate were 48 and 42 respectively whereas the numbers of bacteria developing resistance to the same concentrations of Penwater BC8120 were 12 and 11 respectively.

In the present study, the biocide resistance of bacteria numbered 73 to 0.1%, 0.2%, 0.5% and 1% concentrations of Chloramine T trihydrate (Figure 1) and the biocide resistance of bacteria numbered 84 to 1%, 0.2% (Figure 1) and 0.01%, 0.0001%, 0.00001% (Figure 2) concentrations of the same biocide and the biocide resistance of bacteria numbered 70 and 73 to the 0.2% concentration of Penwater BC8120 (Figure 3) were based on the QS systems.

According to the obtained results, the biocide resistance of bacteria numbered 73 to 0.1%, 0.2%, 0.5% and 1% concentrations of Chloramine T trihydrate and the biocide resistance of bacteria numbered 84 to 1%, 0.2% and 0.01%, 0.0001%, 0.00001% concentrations of the same biocide were based on the QS systems. Although there are many different AHL molecules in different gram (-) bacteria, in the present study only BHL and OdDHL producing bacteria have been identified because of the reference strains used. In the bacteria numbered 73 and 84, biofilm production in aforementioned biocide concentrations was determined to depend on these two signal molecules. In another study, of the 20 isolates isolated from cystic fibrosis patients determined to have different biofilm formation ability, 45% did not produce the OdDHL signal molecule and 80% did not produce the BHL signal molecule [34]. In Ulusoy's study (2007), of the isolates investigated, 20 produced the OdDHL signal molecule, but none produced the BHL signal molecule [35]. As is seen in Figure a, when the Chloramine T trihydrate biocide resistance of bacteria numbered 73 and 84 inoculated with the *C. violaceum* reference strain was examined, it was determined that both bacteria exhibited QS response by producing the AHL molecule. In another study in which the tests conducted by using the *C. violaceum* CV026 strain, *Aeromonas hydrophila* and *Yersinia ruckeri* isolates produced the BHL signal molecule, but *Vibrio anguillarum, Vibrio alginolyticus, Pseudomonas fluorescens* isolates did not produce the BHL signal molecule (Ulusoy, 2007).

It might be possible to establish an association between the QS systems and biofilm formation and biocide resistance in bacteria which have not been determined to exhibit the QS response through the scanning of different AHL molecules, autoinducers, peptides and reference strains through the scanning of different AHL molecules, autoinducers, peptides and reference strains, it might be possible to establish an association between the QS systems and biofilm formation and biocide resistance in bacteria which

have been determined not to exhibit the QS response.

CONCLUSION

Biofilm-forming organisms are often isolated from manmade water systems such as all kinds of water-related equipment, storage and distribution systems, evaporative condensers and cooling towers in industrial facilities. Legionnaire's disease, Pontiac fever, cholera, dysentery, septic shock, cystic fibrosis, and mastitis outbreaks are directly related to leakage and contamination by, and circulation systems of these industrial systems. In order to control potentially pathogenic organisms and to keep biofouling (biological pollution) to a minimum in such systems and in treatment plants, biocides with antimicrobial properties are used primarily. Biocide usage assures the effective working of a system by preventing negative conditions such as the decrease in heat transfer resulting from biological development and stratification, increases in pumping costs, occurrence of structural damage in the system due to corrosion caused by microorganisms, and regression of other water treatment chemicals such as corrosion inhibitors and deposit formation inhibitors. Therefore, the use of biocides in wastewater treatment plants is very important. However, biofilm bacteria resist to the effects of biocides in various ways. Of these ways, the most noticeable ones are the limited diffusion of biocides into the biofilm, different growth rates of biocides in the biofilm and the adverse effects of changes in the microenvironment on bacteria.

Indeed, the increasing concentrations of the two commercial biocides used in the present study led to decreases in biofilm formation in some bacteria, but had no effects on some bacteria, and these unaffected bacteria even developed resistance to the certain doses of biocides. These results were statistically significant ($P \leq 0.05$). However, the present study also confirmed that biocides caused biofilm-producing bacteria to develop resistance at various levels depending on the dose and duration of the applications. Therefore, it is quite important to apply biocides in an appropriate dose and duration to combat bacteria developing biofilm and biofouling which lead to energy and economic losses in industrial facilities by affecting the performance of the facility and pose a risk for the public health and environment. Therefore, to develop biofilm fighting methods based on the use of biocides, it is necessary to develop new strategies in which resistance development does not occur.

In the present study, acquisition of QS responses in certain bacteria exhibiting biocide resistance is of great importance in terms of solving the problem of harmful biofilm production and resistance to biocides. The results obtained in the study are considered to contribute to the understanding and prevention of biocide resistance of biofilm-producing microorganisms causing problems in water purification systems.

ACKNOWLEDGEMENT

This study was supported by Scientific Research Project Office (SRPO) of Muğla Sıtkı Koçman University, Muğla (Project number SRP-10-32).

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Received: **05.01.2017**
Accepted: **25.05.2017**

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