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INVESTIGATION OF BIOCIDE RESISTANCE OF BACTERIA FORMING UNWANTED BIOFILM IN WATER TREATMENT SYSTEM

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Abstract. Biofilm formation in water-related systems may cause bacteria to develop resistance to various chemical biocides. Resistance developed to biocides by bacteria is thought to be similar to the plasmid-mediated antibiotic resistance. In this present study, the resistance exhibited by 61 biofilm bacteria isolated from a wastewater treatment system to commercially widely used WET-TREAT 2002 and WET-TREAT 2008 biocides was researched. It was intended to put forward the relationship between the biocide resistance of bacteria whose resistance was detected and plasmids they carried. At the end of the study, bacteria treated with 10 different doses (1, 0.5, 0.2, 0.1, 0.01, 0.001, 0.0001, 0.00001, 0.000001, and 0.0000001%) of each of the two biocides for 24, 48 and 72 h were determined to develop resistance at different amounts depending on the exposure time and dose. This resistance to different biocides at different concentrations was determined to originate from a single or multiple plasmids the bacteria had.

Keywords: biofilm bacteria, biocide resistance, plasmid profile.

AIMS AND BACKGROUND

Biocides are chemical substances formed by one or more active substances, and can control or kill microorganisms containing bacteria, fungi, algae, mold, or yeast. They are inorganic or synthetic organic molecules. They are chemical agents used as antiseptics and disinfectants for disinfection, sterilisation of surfaces and protection of materials against microbial degradation[1,2]. Among the widespread biocide applications are the control of microbial growth in foodstuffs, textile products, construction materials, corrosion formation and petroleum products[3–6] . Today it is known that bacteria could develop resistance to biocides due to the extensive use of biocides in industrial environments[7,8]. Mechanisms involved in resistance to biocides are the prevention of permeability, biofilm formation, efflux systems, plasmids, target site mutations and overexpression of the target[9–11]. Biofilm formation on surfaces in aqueous systems starts to play a role of a reservoir which significantly increases the number of microorganisms. Biofilm sloughing leads to the spread of infections and contamination of remote areas of the water system and thus is important in terms of clinical and public health[12]. It is reported that bacteria are

10–100 times more resistant to antimicrobials, antiseptics and industrial biocides, due to metabolic changes they develop when they are in the biofilm structure[13–18]. It should be remembered that overdoses of biocides are not preferred because they adversely affect environmental cycles and have toxic effects[19]. Therefore, correct selection and application of the biocides ensure effective results in the fight against industrial problems. In order to achieve this result, sensitive tests should be used to show that biocides are effective against microorganisms existing in industrial environments, and application methods and concentrations should be determined accurately[12]. Whether it is used for industrial purposes or in clinical trials, selection of an appropriate biocide is very important. Therefore, within the scope of the development of the control program, data on the killing capacity of the biocide, the cost of the biocide and the system should be determined. This present study investigated the destruction of unwanted biofilms formed by bacteria in the water treatment system with two commercial biocides and the resistance developed by the biofilm bacteria against biocides.

EXPERIMENTAL

Bacteria. In this study, isolations from the biofilm structures having caused problems in the Wastewater Treatment Plant affiliated with Koycegiz Dalyan Environmental Protection Directorate (Mugla, Turkey) were performed, and 61 bacteria thought to be different according to the identification studies were used.

Biocide resistance testing of bacteria. In the present study, the following 2 biocides were used: WET-TREAT 2002 (W-2002) (2.2-dibromo-2-cyanoacetamide) and WET-TREAT 2008 (W-2008) (the isothiazolinone-based biocide). The chemical compositions and some chemical and physical properties of these biocides are shown in Table 1. By diluting with sterile distilled water, 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 of both biocides were prepared according to their prospectuses[20]. 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 3×10^8 CFU (Colony forming units)/ml of live bacteria[21]. To determine the bacteria 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 an appropriate amount of neutraliser (0.5% sodium thiosulphate, or 0.4% sodium dodecyl sulphate) were added to the sterilised Tryptic Soy Agar (TSA) in aseptic conditions. The media prepared this way were placed on empty sterile 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 completely. Five µl of 18–24 h fresh bacterial cultures adjusted to 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. The growth status of the bacteria inoculated on Petri dishes was recorded after 48-h incubation at 30°C (Ref. 22).

Table 1. Characteristics of biocides label and prescribing information

Trade name	Chemical composition	Application			Physical features			pH	Solubility in water	Manufacturer
		concentration-density	temperature	method	phase	odor	colour			
W-2002	2,2-dibromo-2-cyanoacetamide	1.10 g/cm^3	cold water	dip wash	solid	odourless	clear	(20°C, 1%) > 4.0	good soluble	PETEC Chemical
W-2008	Isothiazolin based	1.00 g/ cm^3	cold water	dip wash	solid	odourless	clear	6.0–9.5 (20°C)	good soluble	PETEC Chemical

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.

Plasmid DNA isolation from bacteria showing resistance to biocides. To perform the isolation, procedures described in the instructions of the geneJET™ Plasmid Miniprep Kit (Thermo Scientific, USA) were performed as instructed.

Plasmid electrophoresis and identification of plasmids. At the end of this procedure, 15–20 µl sample of the solution containing plasmid DNA obtained from each bacterium as described above was loaded onto 0.6% agarose gel containing 0.5 × TBE buffer and 0.5 µl ethidium bromide and the obtained content was electrophoresed at 100 V. Plasmid DNA bands in the ethidium bromide-stained gel were visualised with the Gel Imaging System and then their photographs were taken. After the photographs were loaded on to a computer, the sizes of the plasmids were determined by comparing them with the plasmids of two reference strains whose sizes were known. *Escherichia coli* V517 (carrying plasmid pVA517C) and *Salmonella typhimurium* isolate (carrying plasmid-90 kbps) were used as reference strains.

RESULTS AND DISCUSSION

Biocide resistance of biofilm bacteria. Of the 61 biofilm-derived isolates examined in the present study, 9 showed susceptibility or resistance to the W-2002 biocide at different levels, the remaining 52 bacteria did not develop resistance to any concentration of the biocide (in other words they reproduced in the medium). Hence, the experiments with biocide were continued with 9 bacteria. The other 52 bacteria were not given in Tables 2 and 3. Of the 9 bacteria resistant to the W-2002 biocide, 3–9 developed resistance in 24 h, 3–9 in 48 h and 3–9 in 72 h (Table 2). Because 24 of the 61 biofilm bacteria showed susceptibility or resistance to the W-2008 biocide at different rates, while the remaining 37 bacteria did not show resistance to any concentration of the biocide (in other words they reproduced in the medium), the trials with this biocide were continued with 24 bacteria. The other 37 bacteria were not given. Of the 24 bacteria resistant W-2008 biocide, 5–19 developed resistance in 24 h, 5-22 in 48 h and 5–22 in 72 h (Table 3). The results obtained regarding each of the two biocides used in this study revealed that biofilm-producing bacteria could develop quite high levels of resistance to many other commercially widely used biocides.

Table 2. Resistance of bacteria against W-2002 biocide by using spotting method

Concentration (%)	Sensitive *n* (%)			Resistant *n* (%)			Sensitive + resistant (*n* = 9) Toplam		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
1	9(100)	9(100)	6(66.6)	0	0	3(33.3)	9(100)	9(100)	9(100)
0.5	3(33.3)	3(33.3)	3(33.3)	6(66.6)	6(66.6)	6(66.6)			
0.2	6(66.6)	6(66.6)	6(66.6)	3(33.3)	3(33.3)	3(33.3)			
0.1	5(55.5)	4(44.5)	4(44.5)	4(44.5)	5(55.5)	5(55.5)			
0.01	3(33.3)	2(22.2)	2(22.2)	6(66.6)	7(77.8)	7(77.8)			
0.001	0	0	0	9(100)	9(100)	9(100)			
0.0001	3(33.3)	3(33.3)	3(33.3)	6(66.6)	6(66.6)	6(66.6)			
0.00001	3(33.3)	4(44.5)	4(44.5)	6(66.6)	5(55.5)	5(55.5)			
0.000001	3(33.3)	3(33.3)	3(33.3)	6(66.6)	6(66.6)	6(66.6)			
0.0000001	3(33.3)	3(33.3)	3(33.3)	6(66.6)	6(66.6)	6(66.6)			

Sensitive – no growth; resistant – growth; $P \leq 0.05$ statistically significant.

Table 3. Resistance of bacteria against W-2008 biocide by using spotting method

Concentration (%)	Sensitive *n* (%)			Resistant *n* (%)			Sensitive + resistant (*n* = 24) Toplam		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
1	11(45.8)	9(37.5)	8(33.3)	13(54.2)	15(62.5)	16(66.7)	24(100)	24(100)	24(100)
0.5	9(37.5)	6(25)	3(12.5)	15(62.5)	18(75.0)	21(87.5)			
0.2	9(37.5)	5(20.8)	2(8.3)	15(62.5)	19(79.2)	22(91.7)			
0.1	10(41.6)	5(20.8)	3(12.5)	14(58.4)	19(79.2)	21(87.5)			
0.01	5(20.8)	2(8.3)	2(8.3)	19(79.2)	22(91.7)	22(91.7)			
0.001	11(45.8)	4(16.6)	4(16.6)	13(54.2)	20(83.4)	20(83.4)			
0.0001	5(20.8)	4(16.6)	4(16.6)	19(79.2)	20(83.4)	20(83.4)			
0.00001	19(79.1)	19(79.1)	19(79.1)	5(20.9)	5(20.9)	5(20.9)			
0.000001	11(45.8)	4(16.6)	4(16.6)	13(54.2)	20(83.4)	20(83.4)			
0.0000001	6(25)	4(16.6)	4(16.6)	18(75.0)	20(83.4)	20(83.4)			

Sensitive – no growth; resistant – growth; $P \leq 0.05$ statistically significant.

Plasmid profile. Plasmid screening was performed in the colonies growing in the 0.001% concentration in which 8 of the 9 bacteria resistant to the W-2002 biocide reproduced. Analysis of the plasmid profiles of the bacteria resistant to the W-2002 biocide demonstrated that 6 (75.0%) of the 8 bacteria resistant to the W-2002 biocide carried plasmids of various sizes, and that bacteria numbered 17 and 62 did not carry plasmids (Fig. 1). Five (83.3%) of the 6 bacteria carrying plasmids carried 7.0 kbp plasmids. Two (40%) of the 5 bacteria (Nos 45 and 47) carried only that plasmid whereas the other three (60%) bacteria (Nos 31, 35 and 40) carried it with the other plasmids (Fig. 2). Plasmid screening was also performed in the colonies growing in the 0.001% concentration in which 11 of the 24 bacteria resistant to the W-2008 biocide reproduced. Analysis of the plasmid profiles of the bacteria resistant to the W-2008 biocide demonstrated that 10 (90.9%) of the 11 bacteria resistant to the W-2008 biocide carried plasmids of various sizes, and that bacterium numbered 16 did not carry a plasmid (Fig. 3). The number of the plasmids carried by the 10 bacteria varied from 1 to 4. While 3 bacteria (Nos 17, 45 and 62) carried 1 plasmid, 2 bacteria (Nos 39 and 40) carried 4 plasmids. The 90-kbp plasmid is the most common plasmid profile carried alone (25%) or with the other plasmids (75%) (Nos 17 and 62) (Fig. 4). The most common plasmids among the plasmid profiles of the bacteria showing resistance to the two different biocides in the study were 57-, 90-, 0.5-, 7.0 and 5.8-kbp plasmids (Table 4). This resistance determined in this study was considered to be plasmid mediated, because 6 (75%) of the 8 bacteria resistant to W-2002 and 10 (90.9%) of the 11 bacteria resistant to W-2008 were determined to carry plasmids. In a study, resistance to quaternary ammonium compounds (QACs) in the different *Staphylococcus* species was encoded by the *qacJ* gene carried by the 2.65 kbp plasmid which plays

a role in multidrug resistance[23]. Another literature review revealed a gap related to studies investigating resistance regarding 2.2-dibromo-2-cyanoacetami- and isothiazolinone-based biocides which are widely used in industry[24].

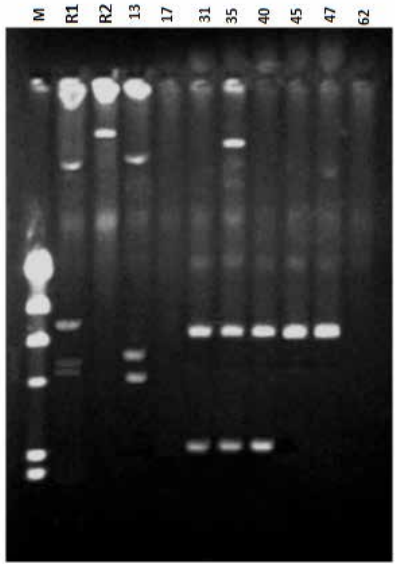

Fig. 1. Plasmid electrophoresis photograph of W-2002 resistant bacteria (M – marker; R1 – reference; 1 – *E. coli* V 517; R2 – reference 2: *S. typhimurium* isolate; 13–62 – bacteria no)

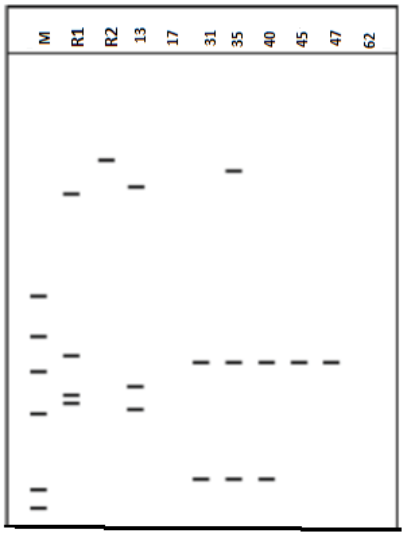

Fig. 2. **S**chematic view of plasmid electrophoresis photograph (see Fig. 1) of W-2002 resistant bacteria. R1 – 53.7; 7.2; 5.6; 5.1 kbp, R2 – 90 kbp, 13: 57; 5.8; 4.8 kbp, 17:-, 31: 7,0; 2,5 kbp, 35: 70; 7,0; 2,5 kbp, 40: 7,0; 2,5 kbp, 45: 7,0 kbp, 47: 7,0 kbp, 62: –

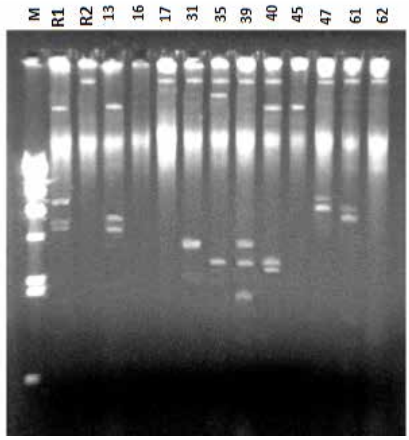

Fig. 3. Plasmid electrophoresis photograph of W-2008 resistant bacteria (M – marker; R1 – reference; 1 – *E. coli* V 517; R2 – reference 2: *S. typhimurium* isolate; 13-62 – bacteria no)

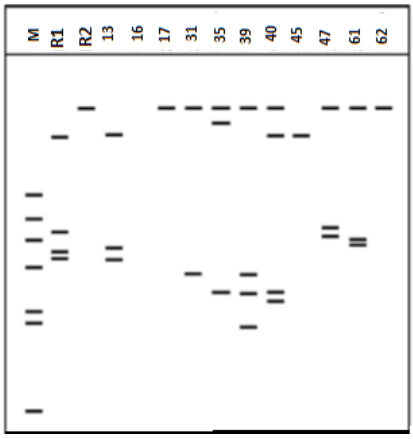

Fig. 4. **S**chematic view of plasmid electrophoresis photograph (see Fig. 3) of W-2008 resistant bacteria. R1 – 53,7; 7,2; 5,6; 5,1 kbp; R2 – 90 kbp, 13: 57; 5,8; 4,8 kbp, 16: –, 17: 90 kbp, 31: 90; 4.0 kbp, 35: 90; 70; 3,0 kbp, 39: 90; 4,0; 3,0; 2,0 kbp, 40: 90; 57; 3.0; 2.7 kbp, 45: 57 kbp, 47: 90; 7.5; 7.0 kbp, 61: 90; 6.5; 6.0 kbp, 62: 90 kbp

Table 4. Plasmids contained in the plasmid profiles of the bacteria showing resistance to the two different biocides in the study and the number of bacteria containing these plasmids

Plasmid profiles (kbp)	Number of bacteria containing plasmids	
	applied biocides	
	W-2002	W-2008
90	–	8
70	1	1
57	1	3
7.5	–	1
7.0	5	1
6.5	–	1
6.0	–	1
5.8	1	1
5.0	–	–
4.8	1	1
4.0	–	2
3.0	–	3
2.7	–	1
2.5	3	–
2.0	–	1
Number of bacteria containing plasmid	6	10
Number of bacteria without plasmid	2	1
Number of total bacteria	8	11

(–): no plasmid.

CONCLUSIONS

The Biocidal Product Directive defines biocide as active substances and preparations which contain one or more active ingredients, are introduced to the market in the ready to use form, exert a controlling effect on harmful organisms, prevent their action, or destroy them by chemical or biological means. Biocides ensure the active performance of chemical products and manufacturing processes in industry. They reduce maintenance and repair costs of products, and protect human and animal health against microorganisms, have the protection effect against the reduction of wood, water and fossil fuels, play an active role in the development of water-based systems and protect the atmosphere by preventing the emission of volatile organic compounds into the atmosphere. Therefore, the use of biocides is very important in industrial facilities. However, unwanted biofilm bacteria developing in the systems or equipment exhibit resistance to biocide applications. Because biocidal products are biocides, they by nature may have harmful effects

on humans, animals and the environment. The correct use of biocidal products in appropriate doses has gained great importance in recent years. In parallel, it was determined that as the concentration of the two commercial biocides used in this study increased, biofilm formation decreased in some bacteria but was not affected in some bacteria, and even resistance was developed to the administered doses of the biocide. These results were statistically significant ($P \leq 0.05$). However, the study also confirmed that biocides caused biofilm-producing bacteria to develop resistance at different levels depending on the dose and the duration of the applications. In addition, it was determined that the biocide resistance was caused by plasmids and that similar plasmid profiles (2.0 to 90 kbp plasmids) were effective in this resistance, which is thought to result from the fact that the transfer of plasmids between microorganisms was very common due to gene transfers in dynamic communities such as biofilm. In the literature, although considerable research has been devoted to antibiotic resistance, rather less attention has been paid to biocide resistance. In the control of harmful biofilm, there is a need for further research to enlighten the basis of bacterial resistance and to determine the appropriate dose and duration. The present study was aimed at contributing to biocide control programs in the fight against harmful biofilms in water treatment systems.

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