Effect of different disinfection treatments on the adhesion and separation of biofilm on stainless steel surface

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ABSTRACT

Attachment and separation of sulfate-reducing bacteria (SRB) biofilm on stainless steel (SS) in simulated cooling water with and without different sterilization treatments was investigated by calculation of surface energy, theoretical work of adhesion and analysis of Scanning Electron Microscope/Energy Dispersive Spectrometer. Two types of biocides, glutaraldehyde and Polyhexamethylene guanidine (PHMG), and electromagnetic treatment were used in this paper. The results show that PHMG had the best bactericidal performance, followed by glutaraldehyde, and electromagnetic treatment was the lowest one. The theoretical work of adhesion was used to quantitatively evaluate the adhesion of biofilm on the surface of the metal. Theoretical work of adhesion between biofilm and SS in simulated cooling water increased with time. The theoretical adhesion work and adhesive capacity of biofilm to SS surface increased after treating with glutaraldehyde while decreasing after treating with PHMG and electromagnetic field. As the theoretical adhesion work decreased, the biofilm was gradually removed from the stainless steel surface. On the contrary, the biofilm adhered more firmly. The results of SEM were also consistent with the calculation results of theoretical adhesion work. The results obtained indicated that electromagnetic treatment had the lowest effect in sterilization but the best in biofilm separation.

Key words | adhesion, biocide, biofilm, contact angle, electromagnetic treatment, stainless steel

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HIGHLIGHTS

- The adhesion of biofilm on the stainless-steel (SS) surface was quantitatively analyzed.
- The effects of three sterilization treatments on the biofilm on the SS surface were compared.
- Electromagnetic treatment significantly reduced the adhesion work of biofilm on SS surface, although its sterilization performance was low.

INTRODUCTION

Microorganisms in natural water can have serious negative effects on various water treatment systems, such as plugging on the surface of filters and reverse osmosis membranes (Chen *et al.* 2017; Sánchez 2018), reducing the heat exchange performance of condensers and producing microbiologically influenced corrosion (MIC) (Chandra *et al.* 2019; Flemming 2020). MIC is a momentous source of corrosion in oil and gas pipelines, reservoir souring and a variety of surface structures (Sowards *et al.* 2014; Liu *et al.* 2018; Jia *et al.* 2019). MIC often induces a localized pitting corrosion (Zhou *et al.* 2020), and has been implicated as a possible

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mechanism in at least 20% of all serious corrosion phenomena (Hou *et al.* 2017). The most common corrosive microorganisms are sulfate-reducing bacteria (SRB), which are considered to be a serious problem for metals because they can obtain electrons from the metal matrix directly or indirectly and produce toxic HS^-, which can promote corrosion of metals (Li *et al.* 2018; Gu *et al.* 2019). Meanwhile, they can gather on the pipe surface and eventually form complex biofilms, which may affect heat exchange performance and make the corrosion of the pipe metals more serious (Gungor *et al.* 2015; Chen *et al.* 2018).

Biofilm, which is durable and resistant to physical removal, is formed by bacteria adhering to the contact surface, secreting polysaccharide matrix, fibrin, lipid protein, and so on, and encapsulating itself in the secretion (Seviour *et al.* 2019). In the metabolic process, the bacterial biofilm on the metal surface will cause changes of pH, dissolved oxygen, and redox potential in the local microenvironment, leading to the formation of active pitting corrosion (Chu *et al.* 2020). It's well known that the genetic and physiological behavior of bacteria in biofilms is different from that of planktonic bacteria. Biofilm could offer more nutrient supply and better resistance to environmental stress factors for sessile bacteria. Meanwhile, it could also enhance rates of horizontal gene transfer, thereby increasing gene transmission and genetic variation, improving survival rate under changing growth conditions or environmental stress (Bjarnsholt 2013). Furthermore, the sessile cells in biofilms are much more difficult to eradicate by conventional means than the planktonic cells due to strong adherence to surfaces and physical repulsion of antimicrobial substances (Gazula *et al.* 2019). Therefore, biofilm on metal surfaces becomes a culprit of pitting corrosion under biofilm and invalidates the metal materials.

There are currently some effective methods to inhibit biofilm growth and MIC in industrial pipelines, including physical and chemical methods. In practice, the most commonly used chemical biocides include various oxidants such as sodium hypochlorite, chlorine dioxide and ozone, and non-oxidants such as glutaraldehyde, isothiazolinone and benzalkonium chloride. Glutaraldehyde is a highly effective bactericide, which can kill various microorganisms. The bactericidal effect of glutaraldehyde mainly depends on the aldehyde group, which can alkylate the sulfhydryl, hydroxyl, carboxyl and amino groups of bacterial proteins, causing protein coagulation and leading to the death of bacteria. Polyhexamethylene guanidine (PHMG), is a cationic bactericide, its bactericidal effect mainly lying in the highly active guanidine group (Mattheis *et al.* 2013). Its advantage is that drug-resistant bacteria will not appear after long-term use. Physical methods are also used, such as ultraviolet radiation, electromagnetic treatment (Mattheis *et al.* 2013; Machuca *et al.* 2014). By modifying the physical properties of water, such as density, viscosity, permeability, surface tension, gas solubility, and electromagnetic oscillation effect, electromagnetic treatment technology could open a new way to control undesirable microbial proliferation. Meanwhile, modification of frequency and intensity of electromagnetic field will produce an oscillation effect on the cell membrane, which can relax the bonds between ions and protein molecules and eventually cause the membrane to rupture (Qian *et al.* 2016; Liu *et al.* 2017). However, the current researches on the above sterilization methods mainly lie in their bactericidal effect, and there are few studies on the removal effect of biofilms.

In general, the initial step of biofilm formation is to adhere microorganisms to the surfaces through non-specific physicochemical interactions (Jucker *et al.* 1996). Adhesion characteristics are a major factor affecting the balance between biofilm formation, growth and separation, and its quantification is essential for understanding, predicting and modeling biofilm development. Adhesion of microorganisms to various interfaces has been explained by the classical Derjaguin–Landau–Verwey–Overbeek (DLVO) theory of colloid stability (Hermansson 1999). Since then, the theory has been used not only as a qualitative model, but also in a quantitative way to calculate the changes of adhesion free energy involved in order to explain microbial adhesion (Hermansson 1999).

Thus, this paper investigated the adhesion properties of biofilm to the surface of stainless steel in simulated cooling water containing SRB, and the effects of attachment and detachment of biofilm on an SS surface with two chemical biocides and a physical treatment in a quantitative way.

EXPERIMENTAL

Strain and culture condition

The experimental SRB strain used in this study was isolated from the sludge of a pond and then purified. The analysis of the sample microbial population indicated that the strain was desulphovibrio, belonging to δ-proteobacteria.

SRB was cultured in a medium that contained 0.5 g·L^{-1} K_2HPO_4, 1.0 g·L^{-1} NH_4Cl, 0.5 g·L^{-1} Na_2SO_4, 0.1 g·L^{-1} $CaCl_2$, 2.0 g·L^{-1} $MgSO_4{\cdot}7H_2O$, 0.1 g·L^{-1} vitamin C, 3.5 g·L^{-1}sodium lactate, and 1.0 g·L^{-1} yeast extract. The pH value of the medium was maintained between 7.2 and 7.4 by adding diluted NaOH solution. The prepared liquid medium was autoclaved at 121 °C for 20 minutes. After

the autoclaved medium was cooled, SRB was added to the medium and cultured at 35 °C.

The number of SRB in different solutions during the experiment was determined by flat colony counting method.

Experimental medium

The SRB culture was inoculated into sterilized simulated cooling water at a ratio of 1:10 (V/V). Table 1 illustrates the composition of the simulated cooling water. Throughout the experiment, sterile liquid paraffin was used to seal the bacterial system to maintain an anaerobic environment.

Formation and treatment of biofilm

Type 304 (UNS S30408) stainless steel was used as experimental material. The coupons, with a working area of 1 cm^2, were polished with different grades of emery papers to obtain a smooth surface. Then the coupons were degreased with alcohol, rinsed with sterilized deionized water, and finally immersed in the test solution containing bacteria at 35 °C. After immersing for 7 days, different concentrations of biocides were added into the test solution or the experimental system treated in an electromagnetic field for different times. After the treatment, the coupons were taken out and allowed to dry in the air for contact angle measurement. The coupons were processed according to the method used in the literature for SEM and EDS measurements (Jia *et al.* 2018).

Surface analysis

The contact angle measurements were carried out using K100-MK2 automatic surface tension meter (KRUSS, Germany). SEM and EDS measurements were conducted using Hitachi Scanning Electron Microscope (SU-1500) combined with EMAX energy spectrometer.

RESULTS AND DISCUSSION

The growth of SRB in the simulated water

The growth curve of SRB measured in simulated cooling water is shown in Figure 1. Under certain conditions, the growth of microorganisms follows some rules. Generally, it can be divided into four stages: lag, logarithmic, stable and senescent stages. It can be seen from Figure 1 that the growth of SRB also obeys this rule. When SRB was added to fresh medium, the bacteria usually did not grow immediately. Then, the growth of bacteria entered the logarithmic stage. During this stage, SRB cells proliferated rapidly in the form of secondary division, and the number increased exponentially. The number of bacteria reached the maximum at the third day of propagation, which was about three orders of magnitude higher than the initial number. At this time, it was found that the medium became turbid. After logarithmic growth, the number of SRB dropped somewhat and then remained stable, and the number of newly reproduced cells was equal to the number of decayed cells. When the nutrient in the solution was insufficient, the mortality rate was greater than the growth rate, which led to a significant decrease in the number of bacteria and eventually tended to extinction.

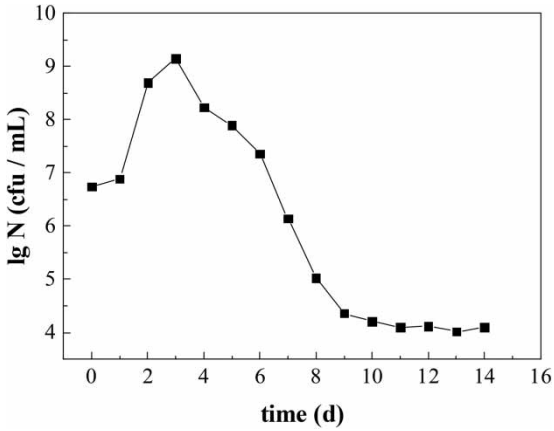

Figure 1 | Growth curve of SRB in simulated cooling water.

Table 1 | The composition of the simulated cooling water (mmol/L)

NaCl	$NaHCO_3$	Na_2SO_4	$MgSO_4$	$CaCl_2$
7.50	2.00	3.50	0.25	0.50

Calculation and analysis of theoretical adhesion

The adhesion of microorganisms on a solid surface is a thermodynamic process. The adhesion energy between the biofilm and sample surface in a water solution could be determined based on surface free energy and its components, which can be calculated from the surface contact angle. Young's equation describes the relationship between solid surface free energy γ_S, liquid surface tension γ_L, solid-liquid interfacial free energy γ_{SL}, and contact angle θ. The formula is as follows:

$$\gamma_S - \gamma_{SL} = \gamma_L \cos\theta \quad (1)$$

According to Lewis acid-base theory proposed by Van Oss *et al.* (1986, 1988), the solid surface free energy γ_S is composed of the non-polar component, namely the Lifshitz-van der Waals component (γ_S^{LW}), and the polar component, namely the acid-base component(γ_S^{AB}):

$$\gamma_S = \gamma_S^{LW} + \gamma_S^{AB} \tag{2}$$

The acid-base component of surface energy is the geometric mean of the electron-acceptor (Lewis acid) γ_S^+ and electron-donor (Lewis base) γ_S^-. So:

$$\gamma_S = \gamma_s^{LW} + 2\sqrt{\gamma_s^+ \gamma_s^-} \tag{3}$$

When a liquid and a solid are placed in intimate contact, the solid-liquid interfacial free energy γ_{SL} can be presented as follows:

$$\gamma_{SL} = \gamma_S + \gamma_L - W_a \tag{4}$$

where W_a is the work of adhesion (the work required to divide the two phases from contact). Combining the components of surface free energy,1 Wa can be expressed as follow (Bargir *et al.* 2009):

$$Wa = 2\left(\sqrt{\gamma_S^{LW}\gamma_L^{LW}} + \sqrt{\gamma_S^+\gamma_L^-} + \sqrt{\gamma_S^-\gamma_L^+}\right) \tag{5}$$

The relationship between the surface energy of solid and liquid and the contact angle can be obtained by combining Equations (1), (4) and (5).

$$(1+\cos\theta)\gamma_L = 2\left(\sqrt{\gamma_S^{LW}\gamma_L^{LW}} + \sqrt{\gamma_S^+\gamma_L^-} + \sqrt{\gamma_S^-\gamma_L^+}\right) \tag{6}$$

In order to calculate the parameters γ_S, γ_S^+ and γ_S^-, it is necessary to measure the contact angle between the solid and three probe liquids with known γ_L^{LW}, γ_L^+ and γ_L^- values. Distilled water, ethylene glycol and formamide were used in this paper. The γ_S^{LW}, γ_L^+ and γ_L^- values are quoted from the literature (Ge *et al.* 2011).

In a certain system where stainless steel (subscript '1') and biofilm (subscript '2') are in water solution (subscript '3'), the theoretical work of adhesion W_{123} could be obtained by resolving the various works of adhesion (Bargir *et al.* 2009):

$$W_{132} = W_{12} + W_{33} - W_{13} - W_{23} \tag{7}$$

where W_{12}, W_{33}, W_{13}, W_{23} can be calculated by Equations (5). W_{12}, W_{23} represents the work of adhesion between biofilm and coupon, and between biofilm and solution medium respectively. Based on the above equations, the theoretical adhesion work W_{123} of microorganism on SS surface in water solution can be calculated and analyzed. The larger the W_{123}, the easier it is for the biofilm to adhere to the SS surface in water.

Theoretical adhesion of SRB on the SS surface

First of all, the contact angles of the coupons with biofilm in the three kinds of probe liquids were measured, as shown in Table 2. The surface free energy and its components of biofilm and coupons were calculated by Eps. (6), as shown in Table 3. According to Table 3, the γ_S^{LW} values were much bigger than γ_S^{AB} values, so it dominated in the surface free energy, which played a major role in biofilm adhesion. As the bacteria in the solution grow over time, more and more microorganisms and their metabolites attached to the surface of the SS, thereby continuously reducing the hydrophobicity of the SS surface. When bacteria in the solution died massively, the value of γ_S decreased correspondingly.

After substituting data listed in Table 4 into Equations (5) and (7), the theoretical work of adhesion of SRB on a coupons surface in simulated cooling water (W_{132}) at different time can be obtained. The values of W_{132} depended on the D-value of W_{12} and W_{23}. Although both W_{12} and W_{23} values decreased at day 12, the difference in value was the

Table 2 | Advancing contact angles of the coupons with biofilm in three probe liquids (°)

immersion time(d)	0	1	2	4	8	12
Water	84.35	58.55	53.70	58.39	57.40	60.62
Ethylene glycol	56.74	49.40	36.94	40.42	35.64	37.04
Formamide	66.26	57.64	46.85	42.19	42.08	45.69

Table 3 | Surface free energy and its components of biofilm on SS

	Surface free energy and its components (mJ·m^{-2})				
Immersion time(d)	γ_s	γ_s^{LW}	γ_s^{AB}	γ_s^+	γ_s^-
0	27.47	21.31	6.17	1.76	5.41
1	33.42	27.48	5.92	0.28	31.26
2	41.55	36.85	4.73	0.19	29.48
4	76.78	66.10	10.66	1.33	21.38
8	55.96	52.99	2.96	0.10	21.90
12	45.46	43.53	1.98	0.05	19.59

Table 4 | Theoretical work of adhesion of biofilm on SS

	Components of theoretical work of adhesion ($mJ \cdot m^{-2}$)			theoretical work of adhesion ($mJ \cdot m^{-2}$)	
Immersion Time (d)	W_{12}	W_{13}	W_{23}	W_{33}	W_{132}
1	65.69	79.80	110.76	145.60	20.73
2	72.48	79.80	115.92	145.60	22.36
4	92.70	79.80	134.27	145.60	24.23
8	81.10	79.80	118.43	145.60	28.46
12	73.70	79.80	108.57	145.60	30.93

largest. This was due to the Van der Waals force strengthening the interaction between the SS and biofilm in the water (Choi *et al.* 2017). It can be seen from Table 4 that the value of W_{132} increased gradually with time from 20.73 $mJ \cdot m^{-2}$ in 1d to 30.94 $mJ \cdot m^{-2}$ in 12d. The adhesion behavior of biofilm on the SS surface was enhanced with time in the simulated cooling water.

Effects of different treatments on the adhesion of SRB biofilm on the SS surface

The simulated cooling water containing SRB was sterilized with different concentrations of glutaraldehyde, PHMG and different times in electromagnetic field. The results presented in Table 5 shows that PHMG had the best sterilization performance for SRB, and the sterilization rate was 100% at a concentration of 10 mg/L. The sterilization performance of glutaraldehyde was the second, and the sterilization rate could be 98.00% at a concentration of 80 mg/L. The reason why PHMG had better bactericidal properties than glutaraldehyde is that PHMG is a guanidine polymer. Its effective bactericidal effect may be related to the following aspects. First of all, the polymer is positively charged and therefore tend to attract negatively charged bacteria. At the same time, the membrane formed by the polymer blocks the respiration channel of microorganism, which makes the microorganism suffocate rapidly (Choi *et al.* 2017). In addition, PHMG forms a complex with the phospholipid bilayer, which disrupts the osmotic balance and destroys plasma membrane, leading to cell leakage. And it can diffuse through the cell membrane and react with nucleic acid, thereby inhibiting the division of bacteria and making bacteria lose their reproductive capacity (Brzezinska *et al.* 2018). Compared with chemical fungicides, electromagnetic treatment was less effective. The sterilization effect after 90 minutes of electromagnetic treatment was 56.75%.

After the SS samples was immersed in the bacterial system for 7 days, a biofilm was formed on the SS surface. Then the samples were treated with the above three sterilization treatment methods to observe the change of adhesion and detachment of the biofilm on the SS surface. The various work of adhesion after different treatments are listed in Table 6. It revealed that after PHMG and electromagnetic treatment, the value of W_{132} decreased significantly, while increased after glutaraldehyde treatment.

Table 5 | Sterilizing rate of SRB with different treatments

Glutaradehyde	Concentration (mg/L)	0	5	10	20	80
	Sterilizing rate (%)	–	23.03	46.58	83.12	98.00
PHMG	Concentration (mg/L)	0	10	50	100	200
	Sterilizing rate (%)	–	100.00	100.00	100.00	100.00
Electromagnetic Treatment	Treatment time (min)	0	5	20	60	90
	Sterilizing rate (%)	–	29.07	36.01	47.36	56.75

Table 6 | Theoretical work of adhesion (W_{132}) of biofilm on SS surface with different treatments

Glutaradehyde	Concentration (mg/L)	0	5	10	20	40	80
	W_{132} ($mJ \cdot m^{-2}$)	25.94	22.81	28.22	31.77	34.59	38.63
PHMG	Concentration (mg/L)	0	5	10	50	100	200
	W_{132} ($mJ \cdot m^{-2}$)	25.94	25.24	19.79	9.97	10.56	11.88
Electromagnetic treatment	Time (min)	0	5	10	20	30	60
	W_{132} ($mJ \cdot m^{-2}$)	25.94	19.41	14.39	9.57	5.28	1.58

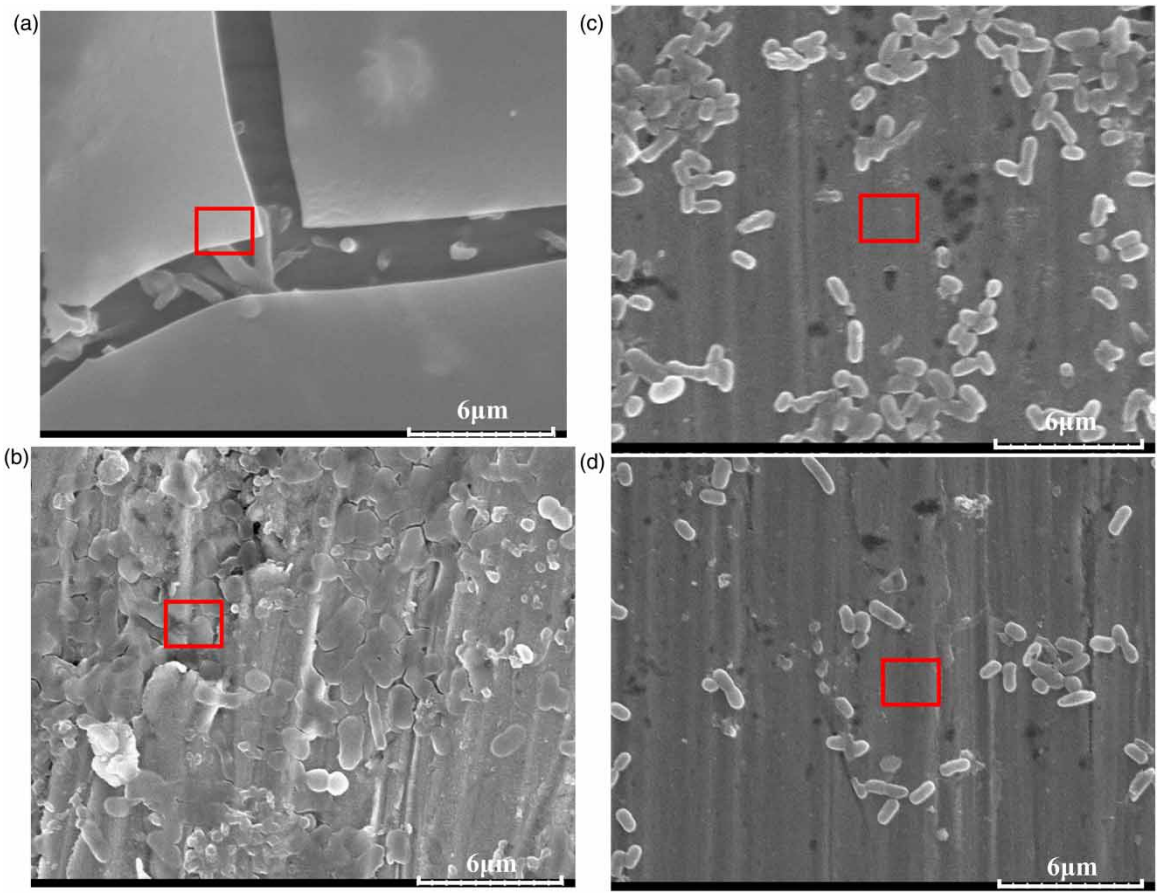

Figure 2 | SEM image of SS surface exposed to SRB for 7d with and without treating. (a) Without treating; (b) 80 mg/L glutaraldehyde; (c) 50 mg/LPHMG; (d) 60 min electromagnetic treatment.

Table 7 | Results of EDS analysis of SS surface after different treatments (atomic percent %)

	Sterilizing treatment			
Element	**Without treating**	**80 mg/L glutaraldehyde**	**50 mg/L PHMG**	**60 min electromagnetic treatment**
C	63.32	62.21	/	/
O	11.82	21.83	6.32	6.57
Cr	5.83	3.03	17.51	18
Fe	17.29	10.11	68.61	67.02
Ni	1.73	0.91	8.56	8.18

With the increase of glutaraldehyde concentration, the value of W_{132} increased gradually, indicating that the adhesion of biofilm on the SS surface increased. The value of W_{132} reached 38.63 $mJ{\cdot}m^{-2}$ at a concentration of 80 mg/L, which is 50% higher than the value before treatment. It demonstrated that glutaraldehyde has good bactericidal properties but cannot separate the biofilm from the surface of stainless steel. This is because the two aldehyde groups contained in glutaraldehyde can react with amino groups and have an irreversible crosslinking effect (Dassanayake *et al.* 2020). Glutaraldehyde can pass through the plasma membrane and solidify the macromolecular substances in the membrane, such as proteins or lipids containing amino groups and imino groups, without changing the original membrane structure, thereby making the adhesion of the biofilm on the SS surface stronger (Dassanayake *et al.* 2020).

The value of W_{132} decreased with the increase of PHMG concentration. The W_{132} value reached the minimum (9.97 $mJ{\cdot}m^{-2}$) at a concentration of 50 mg/L, which was at least 60% lower than that of the untreated samples, indicating that the presence of PHMG reduced the ability of biofilm to adhere to SS surfaces. PHMG not only has remarkable bactericidal performance, but also can effectively remove biofilm from the SS surface by hanging

microorganisms through the long molecular chains of PHMG (Brzezinska *et al.* 2018).

Electromagnetic treatment significantly reduced the adhesion of biofilm on the SS surface. As the time of electromagnetic treatment increased, the value of W_{132} decreased gradually. The value decreased from 25.94 mJ·m^{-2} before treatment to 9.57 mJ·m^{-2} after 20 min of treatment, which was almost the same as the PHMG treatment with a concentration of 50 mg/L. The W_{132} value continued to drop to 1.58 mJ·m^{-2} after 60 min of electromagnetic treatment, which was 94% less than that of the untreated coupon. The results in Tables 5 and 6 show that the electromagnetic treatment had the lowest sterilization performance compared with the two bactericides, but had the largest reduction in the theoretical adhesion work of the biofilm on the SS surface, showing the best biofilm separation effect on the SS surface.

SEM/EDS analysis of biofilm

SEM/EDS were used to observe the influence of different treatment methods on the mature biofilm on the stainless steel surface, as shown in Figure 2. According to Table 6, the sterilization treatment methods with the largest or smallest theoretical adhesion work were selected for SEM/EDS analysis. Table 7 describes the results of EDS analysis of the boxed areas in the figures. In the process of biofilm formation, extracellular polysaccharides (EPS) are produced by SRB. These EPS are easy to adhere to the metal surface and form biofilm that can be seen in SEM images (Figure 2(a)), which is one of the important factors leading to MIC (Li & Ning 2019). The analysis and detection of EDS showed the sum of the atomic percentages of C and O was as high as 75.14%, which were the major elements of the organism. The biofilm on the surface of SS became denser and no detachment of the biofilm was found (seen from Figure 2(b)) after treating with glutaradehyde for 6 h, and the EDS results shows that the SS surface also contained high content of C and O, implying the surface was mainly covered by biofilm. This might be due to the cross-linking effect of glutaraldehyde (Brzezinska *et al.* 2018). Both the results of Figure 2(c) and Table 7 demonstrated that most of the biofilm on the SS surface was removed after treating with PHMG for 6 h at the concentration of 50 mg/L, which is consistent with the above calculation and analysis results. Only the elements Fe, Cr, Ni, O were detected in the test area, indicating that most of the biofilm had been removed from the surface. Figure 2(d) displayed that there was no obvious biofilm and only a few SRB on the SS surface after treating with electromagnetic field for 60 min. This might be due to the fact that the frequency and intensity of electromagnetic field will change greatly in a very short period of time, resulting in an oscillation effect. Electromagnetic treatment may also change the physical and chemical properties of the biofilm on the SS surface, thereby reducing the adhesion ability of biofilm to stainless steel. The results of EDS were similar to that treated with PHMG; that is, only the elements O, Cr, Fe, Ni were detected on the surface, and most of the biofilm was removed from the SS surface. Compared with the sample treated with PHGM, there was less SRB adhesion on the surface after electromagnetic treatment. The above surface analysis results are consistent with the results from W_{132} analysis.

CONCLUSIONS

(1) The theoretical work of adhesion W_{132} of SRB on the SS surface in simulated cooling water increased with time, and the adhesion ability of biofilm on SS surface was enhanced.
(2) PHMG has the best bactericidal effect on SRB, followed by glutaraldehyde, and electromagnetic treatment had the lowest bactericidal effect.
(3) The value of W_{132} increased with the glutaraldehyde concentration, indicating that the adhesion of the biofilm on the SS surface was enhanced, while the value of W_{132} decreased with PHGM concentration and the time of electromagnetic treatment, indicating that the adhesion of the biofilm on SS surface was weakened.
(4) The SEM/EDS results verified the analysis results of theoretical adhesion work W_{132}. The biofilm on the SS surface couldn't be removed after treating with glutaraldehyde, but it could be removed basically by PHMG or electromagnetic treatment.

Among these three treatment methods, although electromagnetic treatment is not very effective in sterilization, it has a good stripping effect on the biofilm on the SS surface. Therefore, from the perspective of controlling the adhesion of biofilms on solid surfaces, electromagnetic treatment is an ideal treatment method.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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