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Original article

Biofilm formation on copper and its control by inhibitor/biocide in cooling water environment

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ABSTRACT

The present study has successfully identified the nitrate reducing bacteria present in the cooling water system and also investigated the performance of industrially applied biocide and inhibitor on the bacterial inhibition. In order to carry out the objective of this study, facilities and methods such as 16S rRNA gene sequencing, Lowry assay, SEM, EIS, ICP-MS and weight loss analysis were being utilized. In this study, two out of the five morphologically dis- similar colonies identified through 16S rRNA gene sequencing, namely the *Massilia timonae* and the *Pseudomonas*, were being utilized in the biocorrosion study on copper metal. From the surface analysis using SEM demonstrated the phenomenon of biofilm formation on the copper surface. 2-methylbenzimidazole has the addition of methyl group in the diazole ring position of benzimidazole it has create basicity environment and inhibit the metal deterioration. Meanwhile, it is also deducible from the EIS and protein analysis that com- bination of biocide with either of the inhibitors gives rise to better biocorrosion suppression (0.00178 mpy and 0.00171mpy) as compared to the sole effect of either biocide or inhibitor (0.00219 mpy, 0.00162 and 0.00143). Biocorrosion system biocide with MBM was found to exhibit 65% corrosion inhibition efficiency. Moreover, adoption of 2-Methylbenzimidazole seems to display better performance as compared to Multionic 8151, which is adopted in cooling water system.

1. Introduction

Microbial corrosion is a predominant worldwide challenge in the oil, processing industry and gas industry. Generally, the problem of corrosion can give rise to two basic issue that eventually result in economic losses industrially (Narenkumar et al., 2018a, b; Ituen et al. 2020; Kokilaramani et al. 2020). The first problem is the failure of equipment which subsequently incurred a cost of replacement and shutdown of plant. While the second problem is the reduction of plant efficiency in view of loss in heat transfers area due to fouling and accumulation of corrosion products (Heggs, 1992). For instance, Ecsom who provides almost 90% of power requirement for the natives of South Africa reported detection on MIC of copper steel in virtually all of the cooling water systems in their power plant (Beech et al. 1999; Lide 2004). And the costs incurred due to the maintenance and down time of the plant can be accounted to millions of dollars every year (Vuet al. 2009; Narenkumar et al., 2016). It is important to note that the phenomenon of metallic corrosion not solely leads to substantial economic losses in power plant but the oil and gas industry as well.

Copper which is well-known for its durability, excellent thermal conductivity and mechanical workability has been widely adopted in systems like cooling tower (Martinez et al. 2004). However, the performance of this metal can be adversely affected particularly in aggressive environment particularly the cooling water system (Li et al. 2018; Idora et al 2015). This is in view that the presence of bacteria in the cooling water environment has not only initiated

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the formation of biofilm on metal surface, it also induced changes to the condition in the metal/electrolyte interface (Satoh et al. 2009; Eliades et al. 2009; Palaniappan and Toleti 2016). The biofilm formation which results from the exopolymeric substance excretion and cell adhesion will then give rise to reduction in the thermal efficiency of copper as well as localised corrosion attack (Busalmen et al. 2002; Jin et al. 2014). The corrosion resistance nature of this metal due to its natural oxide eventually become functionless in view that the cuprous ion is now moving outward rather than the inward movement of oxygen (Rao et al. 1998). Microorganisms implicated in biocorrosion of different metals are physiologically diverse. And the main group of bacteria conjoint with the corrosion of copper and their alloys are the nitrate-reducing bacteria, which has received little attention from the researchers typically on their role in biocorrosion (Al-Nabulsi et al. 2015).

Generally, the character of good corrosion resistance in copper has made it a common candidate in the heat transfer application, typically in the cooling water. However, the condition in cooling water system which encourages the growth and multiplication of microorganisms has gives rise to the issue of metallic biocorrosion and less effective heat transfer system. As reported by Kear et al., (2004) the proposed mechanism of copper electro-dissolution in chloride media made up of:

Type 1

$$\mathrm{Cu} + 2Cl^- \leftrightarrow \mathrm{Cu}Cl_2^- + e^- \quad (1)$$

Type 2

$$\mathrm{Cu} \leftrightarrow Cu^+ e^- \quad (2)$$

$$Cu^+ 2Cl^- \leftrightarrow \mathrm{Cu}Cl_2^-$$

Type 3

$$\mathrm{Cu} + Cl^- \leftrightarrow \mathrm{CuCl} + e^- \quad (3)$$

$$\mathrm{CuCl} + Cl^- \leftrightarrow \mathrm{Cu}Cl_2^-$$

Here, the direct formation of cuprous chloride species from copper is represented by type (1) and (3) while case (2) demonstrated the dissolution to cuprous ion, which is believed to be the controlling kinetic of anodic copper dissolution in system with no inhibitor adoption (Antonijevic et al. 2009).

The biocide or corrosion inhibitor into the cooling water as a mean to control the biofouling problem; we are therefore concerned on the inhibition efficiency of the currently adopted inhibition material particularly to the biocorrosion on copper. Although it is a common practice to add the biocide or inhibitor at every other time interval, the extent of inhibition and interference between the two materials were not known (Narenkumar et al., 2017a,b). It is thus important for us to study on the degree of efficiency by the biocide and inhibitor in controlling and preventing the biocorrosion issue. Additionally, the effectiveness of the currently adopted inhibitor was also compared with another industrially adopted inhibitor, 2-methylbenzimidazole (Ramesh and Rajeswari, 2005).

The susceptibility of copper to corrosion when chloride ions are present, which is the cooling water system with chloride detected in this case, it is thus motivate us to investigate on the role of bacteria on metallic corrosion. Nitrate reducing bacteria (NRB), such as Pseudomonas, can be considered as the most common/ dominant bacteria found in cooling water environment which is responsible for the reduction of nitrate to ammonia via nitrite (Jia et al.2017). Meanwhile, the ammonia ion will then alter the condition of the cooling water and give rise to the biocorrosion phenomena. Ideally, this study can also serve as a benchmark to future biocorrosion study in view of the lack in current study on copper corrosion particularly those induced by the nitrate-reducing bacteria.

2. Materials and methods

2.1. Sample collection and enumeration and identification of bacteria

Sample of cooling water was collected from the cooling tower located root top level of E1 building in National University of Singapore, Engineering Drive, Singapore. Water sample was serially diluted (10 folds) by standard serial dilution method and pour plate technique was employed for the isolation of aerobic bacteria. The enriching medium (modified Winogradsky's) was presented in Table1.Medium was being utilised to enumerate the nitrate reducing bacteria present in the cooling water system. Five morphologically dissimilar colonies were detected and isolated by 16S rRNA gene sequencing analysis. Two of the colonies, namely the *Massilia timonae* and *Pseudomonas*, were chosen and further enriched in freshly prepared enriching medium by streak plate method. Well-growth bacteria were then enriched in liquid enriching medium before stored under refrigeration condition for further usage. These isolates were used for the biocorrosion studies.

2.2. Biocide and inhibitors

The biocide bronopol or 2-bromo-2-nitropropane-1, 3-diol (CAS: 52–51-7) was used as microbicide or micro biostat to control the slime-forming bacteria, fungi and algae (Narenkumar et al., 2018a,b) Inhibitors namely multionic 8151 (comprised of low molecular weight polymeric silt dispersant) and 2-Methylbenzimidazole (which is the addition of methyl group in the diazole ring position of benzimidazole) was used.

2.3 Preparation of systems for biocorrosion and inhibition study

Biocorrosion studies of copper was performed following a procedure as described in our earlier study (Narenkumar et al., 2017a,b). Metal coupons composed of greater than 99.9% of copper and other substances such as O, Pb, Bi and IMP were utilised in this study. Before each experiment, the coupons were polished with various grade of emery paper (400, 800, 1200 & 2500) for mirror surface. The coupons were then washed with distilled water and degreased with acetone before dried at room temperature.

Effect of identified NRB on biocorrosion of copper coupons was investigated using the following system. The systems prepared, with and without NRB, were incubated for a period of 10 days before the coupons were removed for analysis and the detailed systems was presented in the Table 2. For each system, 200 ml of sterilised cooling water sample were used. For those system comprised of bacteria, 10 ml enriching medium was added as source of nutrient for growth of bacteria. Bronopol and inhibitors were added to the system after the 3 days after the growth of bacteria and biofilm formation on metal surface.

2.4 Characterization of biocorrosion and its inhibition

Table 1
Components of enriching medium used.

Components	Chemical Structure	Usage gm/l
Potassium Phosphate Dibasic Anhydrous	K_2HPO_4	1.000
Magnesium Sulfate	$MgSO_4 \cdot 7H_2O$	0.200
Ferrous Sulfate	$FeSO_4 \cdot 7H_2O$	0.050
Calcium Chloride Dihydrate	$CaCl_2 \cdot 2H_2O$	0.100
Sodium Molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.001
D-glucose Anhydrous	$C_6H_{12}O_6$	10.00
Potassium Phosphate Monobasic	KH_2PO_4	1.080
Ammonium Chloride	NH_4Cl	0.540
Sodium Nitrate	$NaNO_3$	1.270

Table 2
Allocation of system for the study of biocorrosion

System	Composition	Average Weight Loss (g)	IE (%)
a	Control: Sterilised cooling water	0.0015	–
b	Control + Massilia	0.0025	–
c	Control + Pseudomonas	0.0056	–
d	Control + Pseudomonas + 5 ppm Bronopol	0.0051	18.93
e	Control + Pseudomonas + 2 ppm MBI	0.0042	45.0
f	Control + Pseudomonas + 25 ppm M8151	0.0046	27.9
g	Control + Pseudomonas + 5 ppm Bronopol + 2 ppm MBI	0.0032	62.9
h	Control + Pseudomonas + 5 ppm Bronopol + 25 ppm M8151	0.0059	45.36

After 10 days of incubation at temperature of 25 °C, copper coupons were taken out from the system for biocorrosion study through the following experiments:

2.4.1. Scanning electron microscopy (SEM) analysis

The surface morphological characteristics of the copper coupons were examined using the SEM (Jeol JSM 5600L) at magnification ranging of 1000X and 2000X with accelerating voltage of 15 kV. In order to visualise the biofilm formation on metal surface, coupons of diameter 1.1 cm and thickness of 0.1 cm were used. After 10 days of incubation, coupons removed from system will be fixed with 3% glutaraldehyde in a phosphate buffer solution (PBS, pH 7.3–7.4) for four-hours before they were dehydrated with an acetone gradient (at 0%, 25%, 50%, 75%, 95% and 100%). Subsequently, the coupons were air-dried and coated with platinum (30 mA for 30sec) using Ion sputter Jeol model JFC 1100 before studied with SEM.

2.4.2. Weight loss study

Weight of each polished coupons were recorded before incubate in cooling water sample. Ten days later, metal coupons removed from each system were pickled with pickling solution (1 L of water containing 500 ml of 178 hydrochloric acid, sp.gr. 1.19) at 37 °C for three min and rinsed with distilled water. Each coupon was weighed again to determine the amount of copper loss. The coupons for weight loss study were duplicated such that an average weight loss can be determined.

2.4.3. Electrochemical impedance spectroscopy (EIS) analysis

EIS was utilised in the present study for electrochemical properties of the corroded copper surface incubated in cooling water system for 10 days. All the EIS analysis was conducted in three-electrode system with platinum electrode as the counter-electrode, Ag/AgCl electrode as the reference electrode and coupons embedded in a sample holder of corrosion cell as working electrode. The working electrode featured an exposed surface area of 0.785 cm^2 in the electrolyte which is made up by the aliquot from each individual system transferred in a beaker. A frequency response analyser of Autolab /PGSTAT 20 was used to perform the EIS scanning while the analysis was done using the Autolab Version 4.9 (Metrohm) software. The range of frequency used was 5 MHz to 100 kHz. All electrochemical measurements were performed after the open-circuit potential was stabilized, which is taken to be 100 s for the present study. Results obtained were then modelled and simulated using the EQUVRT software and two equivalent circuit models were used for fitting the data such as R(QR) and R(Q(R(QR))) respectively. Tafel plots and potentiodynamic scanning curves were measured with a scan rate of 0.5 mV/s (Parthipan et al. 2017a)

2.4.4. Accelerated-Leaching study by inductively-coupled argon plasma – Mass spectroscopy (ICP-MS)

In this study, the concentration of copper metal leached off and dissolved in the electrolyte were analysed using the Agilent 7500a inductively-coupled argon plasma – mass spectroscopy (ICP-MS) as shown below.

2.4.5. Protein analysis

The protein analysis with Lowry method is one of the most commonly utilised techniques in estimation of protein concentration in a biological sample (Dubois 1956). In this method, the protein present in sample will first be treated with the copper ion in an alkaline solution. Subsequently, the aromatic amino acids formed will reduce the phosphomolybdate phosphotungstic acid in the Folin Reagent and give rise to blue colour of the sample. The protein concentration can then be estimated with absorbency reading at 750 nm coupling with the standard curve of Bovine Serum Albumin (BSA) solution plotted.

3. Results

3.1. Identification of bacteria isolated from cooling water system

Preliminary identifications of the isolated bacteria by biochemical test confirmed the Gram-negative nature, Rod shape, Catalase and oxidation positive of the bacterium. A comparison using the 16S rRNA gene sequences from databases revealed that the sequences of the five species successfully isolated from the cooling water system displayed highest levels of similarity with genus *Massilia* and *Pseudomonas* (*Massilia timonae* 1544 *and Pseudomonas* sp. 1413). Two species were chosen for further study of biocorrosion on copper metal in cooling water environment in view of their distinct morphological structure in terms of colonies size since all the colonies identified displayed pale white to yellow on the nutrient agar plate. Sequence alignment with 16S rRNA revealed that more than 98% of the species isolated displayed similarity with *Massilia timonae* and *Pseudomonas*. Furthermore, the nucleotide sequence of the isolated species has been deposited in the GenBank database under the accession number of FJ755909 and FJ755915. The optimal antibacterial activity of bronopal, MBM and M8151were found to be 5, 2 and 25 ppm respectively.

3.2. Protein analysis and growth curve

In order to determine the extent of inhibition for the optimum amount of biocide/inhibitor found, protein concentration of the respective systems was investigated with the Lowry assay via Folin Reaction. The total protein present in the *Massilia timonae* and *Pseudomonas* system were found to be 65 mg/L and 90 mg/L after 10 days incubation.

3.3. Weight loss

The weight loss analysis calculated for copper coupons in the presence /absence of bacteria/ biocide/inhibitor are presented in Table 2. In presence of bacteria higher weight loss (0.0025 and 0.0056 g) was observed compared to control (0.0015). whereas presence of biocide and inhibitor (system d to h) lower values of 0.0051, 0.0042, 0.0046, 0.0032 and 0.0059 g respectively. Notably, weight loss was highly reduced in the combination of bronopol and MBM (System g), and thus, inhibition efficiency was 65%.

3.4. Surface analysis

Copper specimens were immersed in various cooling water system for a period of 10 days before they were taken out from the system. Surface morphology of the layer formed on surface of copper was analysed using the SEM. It is observable from the SEM micrographs that the control (Fig. 2a), *Massilia timonae* and *Pseudomonas* isolated were capable of forming biofilm on the copper surface (Fig. 2b & c). Whereas, addition of biocide no great difference was detected but the amount of bacteria attachment was reduced (Fig. 2d). As shown in Fig. 2e & f, it is interesting to detect of the distinct morphology formed in the *Pseudomonas* system when MBM (Fig. 2f) was adopted. Although there is no reduction of bacteria attached on the copper surface, it is believed that MBM reacted chemically and alter the biofilm layer rather than detaching the bacteria from the copper surface as observed from system with biocide adoption. Undoubtedly, the combination of bronopol with MBM (Fig. 2g) demonstrated better inhibition as compared to the combination with M8151 (Fig. 2h) as revealed by the EIS results too.

3.5. Electrochemical study

The corrosion performance of bacteria on copper metal was evaluated using the electrochemical impedance carried out with open circuit potential in cooling water systemsare shown in Fig. 3 and Table 3 respectively. The control system i_{coor} is observed as 0.454 μA/cm2, while system b to h showed 0.347, 0.202, 0.186, 0.138, 0.036, 0.151, and 0.146 respectively. It is observable from the plots and tables that the presence of bacteria in the cooling water environment results in higher surface resistance and this is justifiable with the formation of intact biofilm layer at the metal surface (Fig. 3 b & c). It is also noteworthy that the corrosion potential was slightly shifted as compared to the control system (Fig. 3a).

4. Discussion

As reported by Gallego et al, *Massilia timonae* is one of the five species under the genus *Massilia* belonging to the family of *Oxalobacteraceae* (Betaproteobacteria). This species was first isolated from the blood of an immunocompromised patient in 1998. Subsequently, other species of genus *Massilia* were also successfully isolated from environmental samples particular in soil (Gallego et al. 2006). As published by La Scola et al, (1998) the species of *Massilia timonae*, which is gram-negative and motile, featured as medium straight rod morphologically. This species which digested gelatine at 25 °C best survived at the temperature range of 25to 30 °C on MacConkey agar as well as nutrient broth with no NaCl component. And one of the many characteristics of this species that agrees well with our study is its nitrate reducing capability.

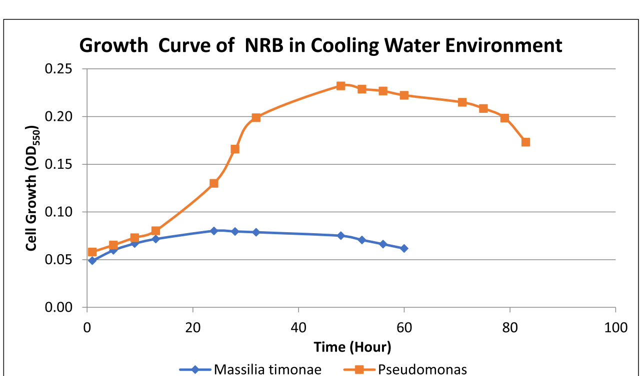

Fig. 1. Growth curve of NRB in cooling water environment.

The genus of *Pseudomonas*, belonging to the family of *Pseudomonads*, is one of the most abundant microbes found from water due to its predilection for growth in moist environment. In general, the genus of *Pseudomonas* can be described as Gram-negative, non-spore forming and motile in movement. Morphologically, they are straight rods measuring 0.5 to 1.5 μm. Although their metabolism is fermentative-less, they can grow without the presence of oxygen as long NO_3 is available as a respiratory electron acceptor (Dhandapani et al. 2020). As shown in the growth curve, it is observable that the growth of *Pseudomonas* is more active as compared to the *Massilia timonae*. This is justifiable with the fact that *Pseudomonas*, which belongs to the slime forming bacteria, is readily grow in the rich inorganic medium. Hence, this species was chosen for biocide and inhibitor study of biocorrosion on copper metal in cooling water environment. Bacterial production of catalase enhances the microbial corrosion of metal surface due to increasing the oxygen reduction current and oxidizes ferric into ferric oxide which might to the formation of red color in the growth system (Busalmen et al. 2002). The strain *Pseudomonas* was previously reported to enhance the corrosion (Parthipan et al. 2017b). Therefore, isolated this strain can accelerate corrosion.

As agreed with the trend in EIS, the combination of biocide and inhibitor (system g & h) has significantly reduced the protein concentration for both the bacteria system (53.983 mg/L/ 52.700 mg/L). It is noteworthy that the *Pseudomonas* demonstrated faster growth rate as compared with the *Massilia timonae*, in which agrees well with the results from optical density (Fig. 1). The effect of biocide on inhibition of *Pseudomonas* was studied at different concentration levels (5 ppm Bronopol, 2 ppm MBI, 25 ppm M8151) based on the inhibition efficiency. In Fig. 3d observable that the corrosion potential slightly shifted to the active direction as compared to the system with biocide absent (Fig. 3a). The decrement of overall current densities also indicates that the suppression effect from biocide which is dominated in the anodic region. Bronopol concluded that the mechanism of its antibacterial action results from its interaction with essential thiols within the cell. Julia et al. (1998) reported that bronopol act as a catalyst for the oxidation of thiol groups to disulfides with the consumption of oxygen. Such catalysis reaction not only results in the alteration of redox, it also leads to the generation of free radicals. Consequently, the functions and growth of bacterial was inhibited. However, the traditional practice to adopt the biocide in killing microorganism is now recognized to be less effective, particularly when the bacteria are incorporated into biofilm (Zulkifli et al., 2017; Elumalai et al., 2021). The exo polymerix matrix formed can act as a diffusion barrier for penetration of biocide. Thus, the adoption of corrosion inhibitor has then gained recognition in effective control of biocorrosion (Ramesh and Rajeswari, 2005).

Results from the electrochemical test revealed that addition of either MBM and M8151 helps in corrosion inhibition for *Pseudomonas* (Fig. 3 e & f). In addition, it is observable that the effect of MBM (0.00162mpy) is better as compared to the M8151 (0.00143mpy), which is being used in NUS cooling tower. The higher inhibition efficiency may be attributed to the increased electron density thus leading to electron transfer mechanism from functional group to metal surface, producing coordinate bonding with a greater adsorption and inhibition efficiency (Beech 1998; Chauhan et al., 2020). This also agrees well with the finding that molybdate demonstrated little inhibition efficiency in view that the protective film formation is not favoured in electrolyte containing aggressive anions such as bromides (Antonijevic et al. 2009). One of the features that give rise to the importance of azole, an

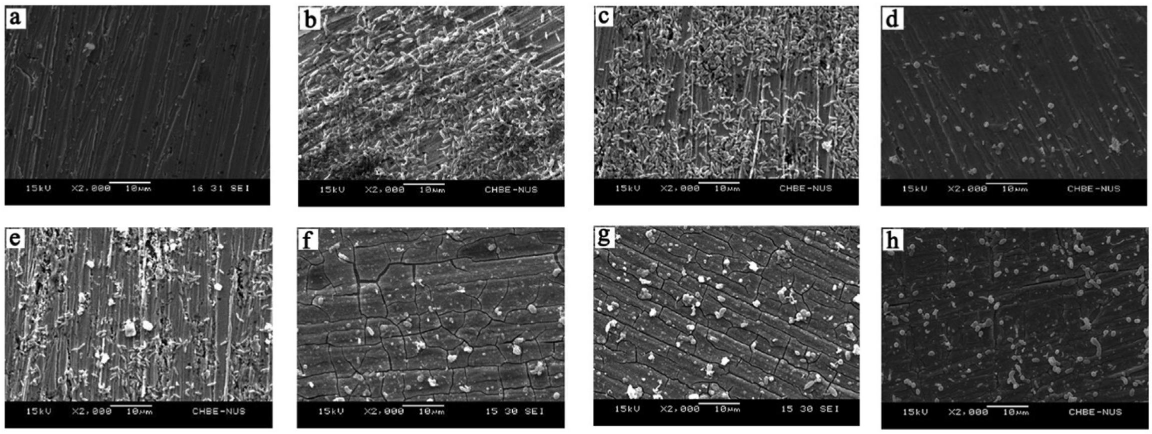

Fig. 2. Scanning electron microscopy of the copper coupon in presents and absence of bacteria and biocide/inhibitor a) Sterilised cooling water b) Massilia timonaec) Pseudomonas d) Pseudomonas + 5ppmBronopol e) Pseudomonas + 2 ppm MBM f) Pseudomonas + 25 ppm M815 g) Pseudomonas + Bronopol + MBM h) Pseudomonas + Bronopol + M815.

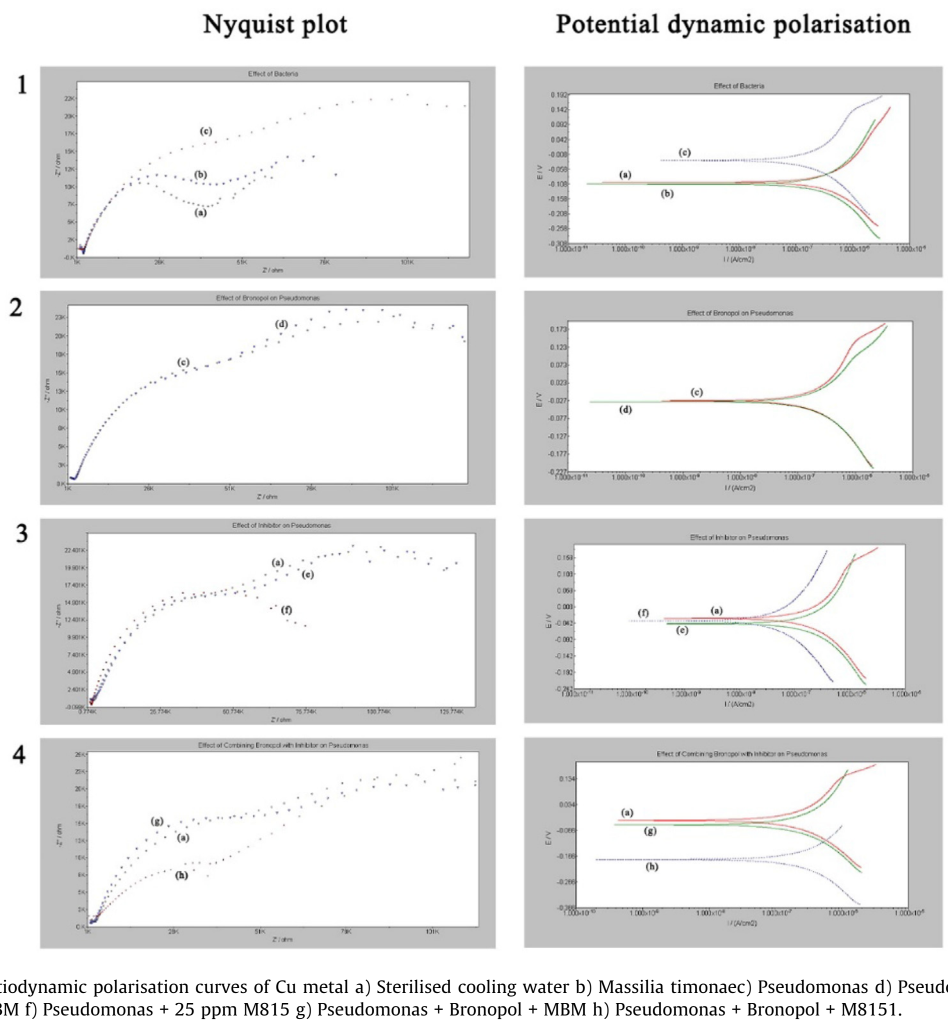

Fig. 3. Nyquist and Potentiodynamic polarisation curves of Cu metal a) Sterilised cooling water b) Massilia timonaec) Pseudomonas d) Pseudomonas + 5ppmBronopol e) Pseudomonas + 2 ppm MBM f) Pseudomonas + 25 ppm M815 g) Pseudomonas + Bronopol + MBM h) Pseudomonas + Bronopol + M8151.

organic compound, in corrosion inhibition is the presence of free electron pair in its structure. The free electron attributed by the nitrogen atom in the structure has give rise to the potential bonding site for copper atoms and its corrosion inhibition character (Parthipan et al., 2017a). And in this case, 2-methylbenzimidazole which is the addition of methyl group in the diazole ring position of benzimidazole has give rise to the increased basicity over that of benzimidazole. Therefore, one can then expect higher $\pi_{M \rightarrow L}$ acceptor strength and enhanced ability of ligand to bind with the transition metal ions and metal surfaces. However, it is worth noted that only two Cu(II) complexes can bind with each ligand due to steric hindrance (Sigma-Aldrich).

Table 3
Potentiodynamic polarisation parameters of Cu: Effect of bacteria

Systems	R_p(kΩ)	E_{corr}(V)	i_{corr}(μA/cm^2)	Corr Rate (mpy)
(a) Sterilised cooling water – Control	37.9	−0.110	0.454	0.00533
(b) Massilia	47.3	−0.109	0.347	0.00407
(c) Pseudomonas	64.7	−0.028	0.202	0.00195
(d) Pseudomonas with 5 ppm Bronopol	57.6	−0.031	0.186	0.00219
(e) Pseudomonas with 2 ppm MBM	47.6	−0.043	0.138	0.00162
(f) Pseudomonas with 25 ppm M8151	217	−0.041	0.036	0.00143
(g) Pseudomonas with Bronopol + MBM	51.0	−0.047	0.151	0.00178
(h) Pseudomonas with Bronopol + M8151	37.8	−0.177	0.146	0.00171

The effect of combining the biocide with inhibitor, in optimized concentration found, suggested that the combination of bronopol with MBM (Fig. 3 g) give rise to better inhibition efficiency as compared to that with M8151 (Fig. 3 h) due to forming the protective layer over the metal surface and also inhibit the bacterial biofilm on metal surface which is due to the antimicrobial activity. It is deducible from the EIS results that combination of biocide with inhibitor is more effective in suppressing the corrosion induced by *Pseudomonas.* It is noteworthy that the corrosion rate for cooling water with inhibitor adoption is lower as compared to the one with no inhibitor used. It can be concluded that 2 ppm of MBM displayed better inhibition efficiency (65%) for biocorrosion on copper in the cooling water environment. The inhibition mechanism of 2-methylbenzimidazole was found to be predominantly cathodic while M8151 appeared to be predominantly anodic. The inhibitor is adsorbed on copper metal and acts as a cathodic inhibitor by retarding the transfer of hydrogen and chloride from the bulk solution to the copper metal /solution interface.

5. Conclusions

In the present study illustrated that *Massilia timonae* and *Pseudomonas* is identified as an effective bacterium to aid the inhibition of biofilm formation by biocide and inhibitor. EIS confirmed that, that the combination of MBM inhibitor and biocide bronopol is suitable to be adopted in the cooling water system in view of lower dosage level when compared to the existing commercial M8152 inhibitor. From this result, it can be concluded that the combination of inhibitor and biocide significantly reduced the corrosion rate of the copper metal due to antibacterial action in cooling tower.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Further Reading

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