Surface physical roughness correlating to biofilm attachment on galvanized aluminum surfaces by bacteria

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Abstract: Microbial biofilm formation on surfaces of materials is important information to better understand the adhesion mechanisms and to prevent bacterial colonization. Atomic force microscopy is a useful tool for examining bacterial biofilms formed on metal surfaces. The objectives of the present study were to evaluate the metal surface properties including roughness for attachment of the bacterium *Janthinobacterium lividum* isolated from drinking-water catridge and to establish the relationship between surface modification through galvanization and susceptibility to biofilm formation. Four metal coupons used in this study were Al Galvanic 0.3%, 5%, 55% and a pure zinc plate. The results showed that several roughness parameters including autocovariance, Z-range, mean roughness, and maximum height increased with bacterial attachment on the selective metal type surfaces. There was a strong positive correlation between different roughness parameters and the number of bacteria attached on the specific metal types. The highest population number of bacteria was observed on Al Galvanized 55% coupon, which was also the roughest surface among the test coupons with different galvanization treatments. Our data suggest that prevention of bacterial attachment on metal surfaces can be achieved by surface treatment to obtain better morphological characteristics.

Keywords: atomic force microscopy; biocorrosion; biofilm; surface characteristics; Zinc; galvanization; surface treatment

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1 Introduction

Microorganisms were first implicated in corrosion of metallic materials by von Volzogen Kuhr and van der Vlught (1934). Microbiological corrosion, also called the microbial influenced/induced corrosion (MIC), affects a wide range of industries, including oil fields, offshore industry, pipelines, pulp and paper, armaments, nuclear and fossil fuel power plants, and petroleum reservoirs (Kobrin, 1993; Gu et al., 2011a, b; Li et al., 2017), aviation and space (Gu, 2003; 2007; 2012). The cost due to failure and prevention was estimated to be 70% of corrosion in gas transmission. American refinery industries lose approximately $1.4 billion a year (see references in review by Dowling and Guezennec (1997). Microorganisms including the aerobic bacterial species and anaerobic sulfate-reducing bacteria (SRBs) as well as methanogens (Daniels et al., 1987), were implicated in the processes of corrosion (Gu, 2012; Gu et al., 2011a, b; Gu and Mitchell, 2013). In addition to SRBs, exopolymer (slime)-producing bacteria were also found to participate in the corrosion by a mechanism in which metal ions are complexed with functional groups of the exopolysaccharides, resulting in release of metallic species (Chen et al., 1995; 1997; Clayton et al., 1992). Similarly, fungi were involved in corroding aluminum and its alloys by a process of organic acids production (Videla et al., 1986), and they were causal microorganisms in materials including concrete (Gu et al., 1996; 1998), stone of cultural heritage (Hu et al., 2013; Meng et al., 2016, 2017), and polymers (Gu et al., 1996; 1998; Gu, 2003).

The biofilm formation on surfaces of materials is a prerequisite for the subsequently observed biofouling and biocorrison. Once the bacterial cells become attached on a submerged surface, they will proliferate and develop complex architecture of biofilm. In fact a number of factors affect bacterial attachment such as the physicochemical properties of substratum (Azeredo et al., 1999; Clint and Wicks, 2001; Liang et al., 2000) and the bacterial surface. Microbial adhesion and establishment of a complex community of microorganisms (microfouling) are prerequisites for substantial degradation of the underelying materials (Walch, 1992; Gu and Mitchell, 2013). Since all surfaces may act as substrata for formation of these biofilms (Costerton et al., 1995; Geesey and White, 1990; Marshall, 1980), subsequent attack of materials by microorganisms can take place either directly or indirectly, depending on a combination of factors (Gu et al., 1998; Gu et al., 2011a, b). In addition, other factors affecting the physical

environment also influence the extent of bacterial adhesion on surfaces, including ionic strength of the solution, type of cation, hydrodynamic force, and surface properties, e.g., hydrophobicity or hydrophilicity. Corrosion of metals is closely associated with the formation of complex microbial biofilms on surfaces. The microbial communities induce the formation of differential aeration cells under aerobic conditions because dissolved oxygen is consumed within microbial colonies (Gu and Mitchell, 2013). The decrease in oxygen levels provides an opportunity for anaerobic microorganisms to become established within biofilms, where oxygen concentration is low or not available.

A fundamental understanding of bacterial adhesion on morphologically different surfaces of materials, and their impact on the substratum materials requires integration of information from several disciplines, including materials science and engineering, microbial ecology, and physiology. Basic microbiological information is required for a better understanding of the role of microorganisms in corrosion and deterioration. Traditionally, microscopic techniques were used to evaluate the bacterial attachment on the substratum (Caldwell et al, 1997). Scanning electron microscopy (SEM) was also commonly used for this purpose. However, this technique is comparatively time-consuming and does not provide detailed information about the morphologies of the substratum nor *in situ* bacterial biofilms. In contrast, atomic force microscopy (AFM) has a unique advantage in offering information about the biofilm/substratum interactions *in situ*. In addition, it can obtain topographical details of the cells under fully hydrated and realistic state, and measure the roughness of the substratum quantitatively. The objectives of this study were to assess metal surfaces physical properties using AFM and elucidate the relationship between surface morphologies and bacterial attachment.

2 Materials and Methods

2.1 Preparation of Metal Coupons

Four types of metals with different surface treatments were used in the present study and they were Al Galvanized 0.3%, 5%, 55%, and pure Zinc plate (99.8% purity). The coupons (10 mm$\times$10 mm) were degreased by immersing in acetone over night, dried in fume hood and stored in a desiccator prior to AFM study.

2.2 Microorganism and Culturing Conditions

A bacterium, initially isolated from a drinking water filter, was described by Gu and Cheung (2001) and was then identified as *Janthinobacterium lividum* with 99.9% similarity using 16S rRNA sequences. At the starting of the experiment, each type of the coupons was introduced into an individual Erlenmeyer flask containing sterile 1% Nutrient Broth medium (Difco Lab., Detroit, Michigan, USA) and 1.0 ml of fresh culture of *J. lividum*. All flasks were incubated at 25°C in the darkness on a shaker. During the incubation period, one set of coupons was removed from each flask for examination using SEM and the sample preparation procedures are described below. At the same time, another set of coupons was removed for AFM.

2.3 Preparation for Scanning Electron Microscopy

The coupons after removal from the flasks were first treated with 3% glutaraldehyde buffer in 0.2 M Na cacodylate overnight in glass vials as described before (Gu et al., 1998). The samples were then washed with 0.2 M Na cacodylate three times, further fixed in 1% OsO_4 in 0.1 M Na cacodylate for at least 1 h, and rinsed with Na cacodylate and deionized water, respectively. Dehydration of the samples was achieved in an ethanol-distilled water series of 40 to 80% ethanol with a 10% increment and then 90 to 100% with a 5% increment. Samples were kept in 100% ethanol and sealed in glass vials until they were critical point dried in liquid CO_2. After drying, samples were coated immediately with Palladium-Gold and observed on a Leica Cambridge Scan 440 Scanning Electron Microcopy.

2.4 Preparation for Atomic Force Microscopy

The morphologies of the four types of metal coupons were analyzed before the experimental exposure in order to characterize surface physical properties including roughness. A Nanoscope IIIA AFM (Digital instruments, Santa Barbara, CA, USA) operating in the tapping mode was used to view the morphologies of the metal coupons. The AFM probe had a cantilever length 125 µm and the tip radius of the curvature was 5-10 nm. The Digital Nanoscope software (version 4.23) was used to process the data of the roughness of the metal surface. Similarly, the Nanoscope was also used in examination of biofilmed surfaces of the four types of materials.

3 Results

3.1 Surface Morphologies of Different Metal Coupons

The four different metal coupon surfaces containing variable Zn for surface treatment against corrosion could not be distinguished visually but the differences were clearly revealed using AFM. Information obtained by AFM was expressed in term of several quantitative parameters including Z-range, mean roughness, maximum height, raw mean, autocovariance, and the surface roughness. Representative AFM micrographs of the four metal coupons are shown in Figure 1. Variability of metal surface physical properties was due to galvanization process upon surface treatment to variable degrees. Using these several roughness parameters mentioned above, surface roughness could be ranked in the following order: Al Galvanized 55% > Al Galvanized 5% > Zn > Al Galvanized 0.3%. For example, the depth of the valley on surface of Al galvanized 55% was 2.72 µm while it was 399 nm for Al galvanized 3% by the surface treatment process (Table 1).

3.2 Bacterial Attachment to Metal Surfaces

Since these metals had different surface morphological characteristics, adhesion by the bacterium *J. lividum* was also variable among the 4 treatments. After 7 days of incubation,
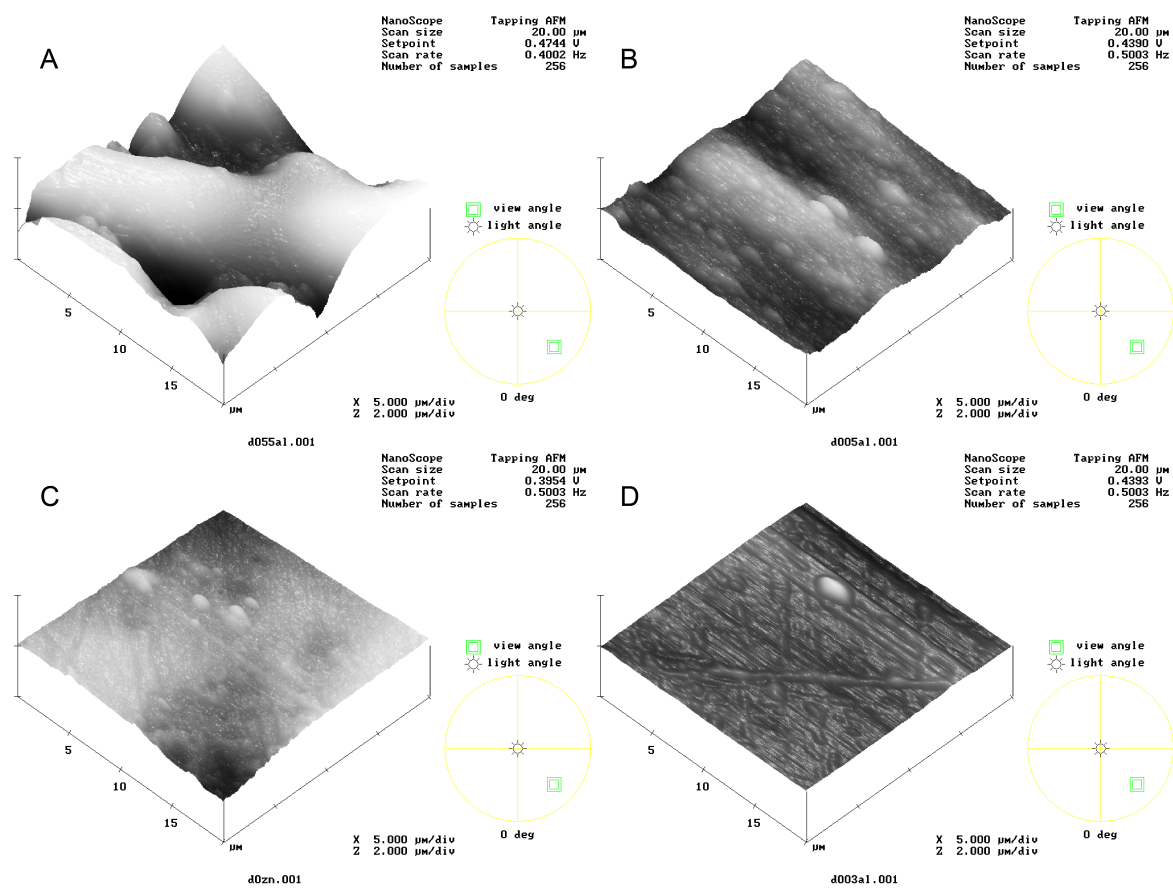
Figure 1. The AFM micrographs of four different metal coupons. (A) Galvanized 55% Al; (B) Galvanized 5% Al; (C) Zn; (D) Galvanized 0.3 % Al.

the metal coupons were removed from the incubation and the number of bacterial cells attached onto the metal coupons was quantified from SEM images. It was observed that the number of bacterial cells attached to each coupon surface was significantly different. Al Galvanized 55% coupons showed the highest density of the bacterial cells attached than all the other types of coupons. The order of bacterial attachment followed: Al Galvanized 55% > Al Galvanized (Galfan) 5% > Al Galvanized 0.3% > Zn (Figure 2) in agreement of the surface roughness observed (Table 1).

Bacterial adhesion on surfaces was positively correlated to the surface roughness parameters. The correlation coefficient was 0.807, 0.891, 0.894 and 0.900 for autocovariance, Z-range, mean roughness and maximum height, respectively (Table 2).

Figure 2. Number of attached bacterial cells on the four different types of coupons with different galvanization.

4 Discussion

Microbial involvement in the corrosion of metals has been known for a long time (Gu and Mitchell, 2013; Gu et al., 2011a, b). Large amount of information based on research using *Pseudomonas aeruginosa* is also readily available. In this study, surface morphological characteristics of roughness of several systematic treated metal types were investigated
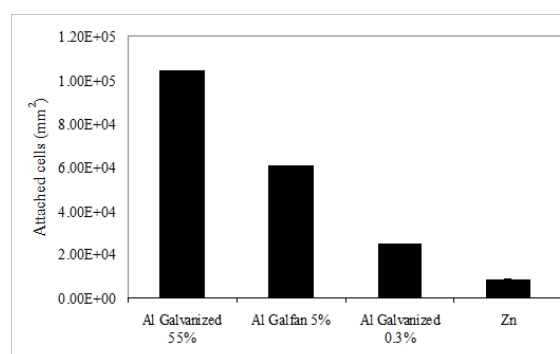
to determine the relationship between surface roughness and bacterial attachment using AFM and SEM. A previous study showed that as the roughness of the stainless steel surface decreased, fewer bacterial cells attached to the surface using a mixed bacterial culture derived from poultry processing plant (Arnold and Bailey, 2000). Our results are in good agreement with theirs despite of the different materials types and the galvanization of Al used. The difference is that their

Table 1. Different metal surface roughness parameters as shown by atomic force microscopy

Measurement	Metal			
	Galvalume 55% Al	Galfan 5% Al	Galvanized 0.3% Al	Zn
Autocovariance[1]	246542	25540	1131	16406
Z-range[2]	2770	960	403	744
Mean Roughness[3]	405	122	22.26	98.18
Raw mean[4]	3.2	4.45	5.34	5.26
Maximum height[5]	2.72 μm	990 nm	399 nm	775 nm

[1] Autocovariance (nm^2) indicates the degree of surface structural or morphological regularity. The regularity is inversely related to the magnitude of the number.
[2] The Z-range (nm) controls the vertical range of the image. This is the difference between the highest and lowest points within the given range.
[3] The mean roughness (Ra, nm) is the mean value of the surface relative to the center plane.
[4] The raw mean (nm) is the SD of the Z-values within the given three-dimensional area.
[5] The maximun height (nm) is the difference in height between the highest and lowest points on the surface relative to the mean plane.
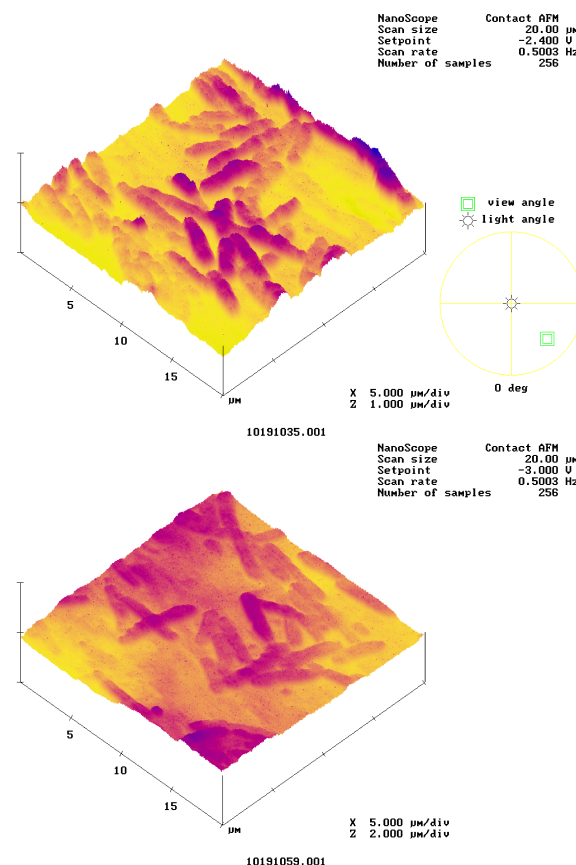
Table 2. Correlation between surface roughness of the test materials and the bacteria attached

Measurement	R^2
Autocovariance	0.8068
Z-range	0.891
Mean Roughness	0.8937
Maximum height	0.8998

test materials were stainless steel with several surface polishing treatments while ours are a collection of industrially manufactured materials with galvanization to variable extent. In addition, we used a pure culture of *J. lividum* whileas they used a mixed culture of bacteria. However, the results showed that as the roughness parameters of metal coupons increased, the bacterial attachment to the metal coupons also showed a trend of increase. Such information has great potential for applications involving materials of Al with feasible galvanization for surface modification and treatments to improve the materials against biofilm formation.

Videla et al. (1987) stated that the nature of the metal surface plays an important role in biofilm formation no matter it was facilitated or hindered microbial attachment. In order to elucidate the effect of surface roughness of the coupons on bacterial attachment, bacteria on surfaces of the coupons were assessed from SEM micrographs and correlation analysis was then carried out. Results showed that high correlation was observed between various roughness parameters and the

number of bacterial cells attached on the metal coupons as shown in the results derived from enumeration of images of SEM (Figure 3). Fewer bacteria were observed on the least rugged surfaces of the Al Galvanized 0.3%. Similarly, Arnold and Bailey (2000) reported that decrease in the measurements of roughness parameters by AFM for the treated surfaces corresponded to a reduction of bacterial attachment. Nevertheless, our results showed that there was a positive correlation (r^2, 0.89) between surface roughness parameters measured by AFM and bacterial attachment.

Figure 3. Atomic force microscopy images of bacteria deposited on surfaces of metals treated with galvanization with preference for rough surfaces.

In our study, galvanized Al 55% coupons had the roughest surface and the most abundant number of attached *J. lividum*. This suggests that Al Galvanized 55% was favorable for bacterial attachment. However, the susceptibility to biofouling and biocorrosion needs to be further investigated in the future study. It would be interesting to know the settlement of invertebrate larvae on Al Galvanized 55% coupons because bacterial biofilm is thought to play a role in the induction and inhibition of larvae settlement (Holmstrrmöm and Kjelleberg, 1994). Moreover, Xu et al. (1999) also showed that bacteria may not participate in the corrosion process directly but they can change the nature of the substratum so as to facilitate the corrosion process.

5 Conclusions

Surface treatment of materials can be effective means to prevent microbial deposition and colonization of materials surfaces. The surface roughness of the galvanized coupons increased with an increase of the attached bacteria when incubated under laboratory conditions. The high correlation between surface morphological characteristics of metal coupons and the number of bacteria attachment can offer effective material engineering against bacteria in applications.

Acknowledgments

Conflict of Interest

All authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

References

Arnold, J.W. and Bailey, G.W., 2000. Surface finishes on stainless steel reduce bacterial attachment and early biofilm formation: scanning electron and atomic force microscopy study. Poultry Science, 79(12), 1839-1845.
https://doi.org/10.1093/ps/79.12.1839

Azeredo, J., Visser, J. and Oliveira, R., 1999. Exopolymers in bacterial adhesion: interpretation in terms of DLVO and XDLVO theories. Colloids and Surfaces B: Biointerfaces, 14(1-4), 141-148.
https://doi.org/10.1016/S0927-7765(99)00031-4

Caldwell, D.E. and Lawrence, J.R., 1986. Growth kinetics of *Pseudomonas fluorescens* microcolonies within the hydrodynamic boundary layers of surface microenvironments. Microbial Ecology, 12(3), 299-312.
https://doi.org/10.1007/BF02011173

Caldwell, D.E., Wolfaardt, G.M., Korber, D.R. and Lawrence, J.R., 1997. Do bacterial communities transcend Darwinism? pp. 105-191. *In*: Jones JG (ed), Advances in Microbial Ecology. Springer, Boston.

Chen, G., Clayton, C.R., Sadowski, R.A., Kearns, J.R., Gillow, J.B. and Francis, A.J., 1995. Influence of sulfate-reducing bacteria on the passive film formed on austenitic stainless steel AISI 304 (No. CONF-950304-). NACE International, Houston, Texas, United States.

Chen, G., Kagwade, S.V., French, G.E., Ford, T.E., Mitchell, R. and Clayton, C.R., 1996. Metal ion and exopolymer interaction: a surface analytical study. Corrosion, 52(12), 891-899.
https://doi.org/10.5006/1.3292082

Clayton, C.R., Halada, G.P. , Kearns, J.R., Gillow, J.B. and Francis, A.J., 1994. Spectroscopic study of sulfate reducing bacteria-metal ion interactions related to microbiologically influenced corrosion (MIC). pp.141-152. *In*: Kearns JR and Little BJ (eds), Microbiologically Influenced Corrosion Testing, ASTM STP 1232. American Society for Testing and Materials, Philadelphia, Pennsylvania.

Clint, J.H. and Wicks, A.C., 2001. Adhesion under water: surface energy considerations. International Journal of Adhesion and Adhesives, 21(4), 267-273.
https://doi.org/10.1016/S0143-7496(00)00029-4

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M., 1995. Microbial biofilms. Annual Reviews in Microbiology, 49(1), 711-745.
https://doi.org/10.1146/annurev.mi.49.100195.003431

Daniels, L., Belay, N., Rajagopal, B.S. and Weimer, P.J., 1987. Bacterial methanogenesis and growth from CO_2 with elemental iron as the sole source of electrons. Science, 237(4814), 509-511.
https://doi.org/10.1126/science.237.4814.509

Dowling, N.J.E. and Guezennec, J., 1997. Microbiologically influenced corrosion. pp. 842-855. *In*: Hurst CJ, Crawford RL, Garland JL and Lipson DA (eds), 2007. Manual of environmental microbiology. American Society for Microbiology Press, Washington DC.

Geesey, G.G. and White, D.C., 1990. Determination of bacterial growth and activity at solid-liquid interfaces. Annual Reviews in Microbiology, 44(1), 579-602.
https://doi.org/10.1146/annurev.mi.44.100190.003051

Gehrke, T., Telegdi, J., Thierry, D. and Sand, W., 1998. Importance of extracellular polymeric substances from *Thiobacillus ferrooxidans* for bioleaching. Applied and Environmental Microbiology, 64(7), 2743-2747.

Gu, J.-D., 2003. Microbiological deterioration and degradation of synthetic polymeric materials: recent research advances. International Biodeterioration & Biodegradation, 52(2), 69-91.
https://doi.org/10.1016/S0964-8305(02)00177-4

Gu, J.-D., 2007. Microbial colonization of polymeric materials for space applications and mechanisms of biodeterioration: a review. International Biodeterioration & Biodegradation, 59(3), 170-179.
https://doi.org/10.1016/j.ibiod.2006.08.010

Gu, J.-D. 2012. Corrosion, Microbial. pp. 613-623. *In*: Schmidt TM and Schaechter M (ed), Topics in Ecological and Environmental Microbiology, Elsevier, U.K..

Gu, J.-D. and Mitchell, R.(2013) Biodeterioration, pp. 309-341. *In*: Rosenberg E, DeLong EF, Lory S, Stackebrandt E and Thompson F (eds), The Prokaryotes. Springer-Verlag, New York.
https://doi.org/10.1007/978-3-642-31331-8_31

Gu, J-D. and Cheung, K.H., 2001. Phenotypic expression of Vogesella indigofura upon exposure to hexavalent chromium, Cr^{6+}. World Journal of Microbiology & Biotechnology. 17(5), 475-480.
https://doi.org/10.1023/A:1011917409139

Gu, J.-D., Ford, T.E. and Mitchell, R., 1996. Susceptibility of electronic insulating polyimides to microbial degradation.

Journal of Applied Polymer Science, 62(7), 1029-1034. https://doi.org/10.1002/(SICI)1097-4628(19961114)62:7

Gu, J.-D., Roman, M., Esselman, T. and Mitchell, R., 1998. The role of microbial biofilms in deterioration of space station candidate materials. International Biodeterioration & Biodegradation, 41(1), 25-33. https://doi.org/10.1016/S0964-8305(98)80005-X

Gu, J.-D., Ford, T.E. and Mitchell, R. (2011a) Chapter 26: Microbial degradation of materials: general processes. pp. 351363. *In*: Revie W (ed), The Uhlig Corrosion Handbook (3rd ed), John Wiley & Sons, New York.

Gu, J.-D., Ford, T.E. and Mitchell, R. (2011b) Chapter 39: Microbial corrosion of metals. Pages 549557. *In*: Revie W, (ed), The Uhlig Corrosion Handbook (3rd ed.), John Wiley & Sons, New York .

Holmstrrmöm, C. and Kjelleberg, S., 1994. The effect of external biological factors on settlement of marine invertebrate and new antifouling technology. Biofouling, 8(2), 147-160. https://doi.org/10.1080/08927019409378269

Hu, H., Ding, S., Katayama, Y., Kusumi, A., Li, S.X., de Vries, R.P., Wang, J., Yu, X.Z. and Gu, J.-D., 2013. Occurrence of Aspergillus allahabadii on sandstone at Bayon temple, Angkor Thom, Cambodia. International Biodeterioration & Biodegradation, 76, 112-117. https://doi.org/10.1016/j.ibiod.2012.06.022

Kobrin, G., 1993. A Practical Manual on Microbiological Influenced Corrosion. NACE International, Houston, Texas.

Li, X.X., Yang, T., Mbadinga, S.M., Liu, J.F., Yang, S.Z., Gu, J.-D. and Mu, B.Z., 2017. Responses of microbial community composition to temperature gradient and carbon steel corrosion in production water of petroleum reservoir. Frontiers in Microbiology 8, 2379. https://doi.org/10.3389/fmicb.2017.02379

Liang, M.N., Smith, S.P., Metallo, S.J., Choi, I.S., Prentiss, M.

and Whitesides, G.M., 2000. Measuring the forces involved in polyvalent adhesion of uropathogenic Escherichia coli to mannose-presenting surfaces. Proceedings of the National Academy of Sciences, 97(24), 13092-13096. https://doi.org/10.1073/pnas.230451697

Marshall, K.C., 1980. Adsorption of microorganisms to soils and sediments. pp. 317-329. *In*: Bitton G and Marshall KC (eds), Adsorption of Microorganisms to Surfaces. John Wiley & Sons, Inc., New York, USA.

Meng, H., Luo, L., Chan, H.W., Katayama, Y. and Gu, J.-D., 2016. Higher diversity and abundance of ammonia-oxidizing archaea than bacteria detected at the Bayon temple of Angkor Thom in Cambodia. International Biodeterioration & Biodegradation, 115, 234-243. https://doi.org/10.1016/j.ibiod.2016.08.021

Meng, H., Katayama, Y. and Gu, J.-D., 2017. More wide occurrence and dominance of ammonia-oxidizing archaea than bacteria at three Angkor sandstone temples of Bayon, Phnom Krom and Wat Athvea in Cambodia. International Biodeterioration & Biodegradation, 117, 78-88. https://doi.org/10.1016/j.ibiod.2016.11.01

von Wolzogen Kuhr, C.A.H. and van der Vlugt, L.S., 1934. Graphitization of cast iron as an electrochemical process in anaerobic soils. Water, 18, 147-165.

Videla, H.A., De Mele, M.F.L., and Brankevich, G.J., 1987. Microfouling of several metal surfaces in polluted seawater and its relation with corrosion. Paper No. 365, Corrosion/87, NACE International, Houston, TX.

Walch, M., 1992. Corrosion, microbial. Encyclopedia of Microbiology, 1, 585-591.

Xu, L.C., Fang, H.H. and Chan, K.Y., 1999. Atomic force microscopy study of microbiologically influenced corrosion of mild steel. Journal of the Electrochemical Society, 146(12), 4455-4460. https://doi.org/10.1149/1.1392658