IUFS J Biol 2011, 70(2):35-42

Biofilm formation on galvanized steel by SRB isolate obtained from cooling tower water

Derya Özüölmez [1,2]* and Ayşın Çotuk[2]

[1] *Wageningen University, Laboratory of Microbiology, Dreijenplein 10, 6703 HB, Wageningen, The Netherlands.*
[2] *Istanbul University, Faculty of Science, Department of Biology, Division of Fundamental and Industrial Microbiology, 34134, Vezneciler, Istanbul/Turkey*

Abstract

In this study, we investigated biofilm formation on galvanized steel coupons by anaerobic bacteria isolate including sulphate-reducing bacteria (SRB) isolated from cooling tower water in a lab-scaled experimental setup. The test coupons were exposed to the culture of anaerobic bacteria isolate during 744 hours. In the course of time, anaerobic bacteria isolate could form biofilm on galvanized steel coupons. According to the statistical analyses, there was no significant difference between sessile and planktonic SRB counts, while a positive correlation was found out between SRB counts ($P<0.01$). Extracellular carbohydrate appeared to be degraded by bacteria culture in the test system.

Keywords Sulphate-reducing bacteria (SRB), cooling tower water, galvanized steel, biofilm.
***Corresponding author:** Derya Özüölmez (e-mail: adaderya@gmail.com)
(Received :03.11.2010 Accepted: 25.01.2011)

Soğutma Kule Suyundan Elde Edilen SRB İzolatının Galvanizli Çelik Üzerinde Biyofilm Oluşturması

Özet

Çalışmada, laboratuvar ölçekli deney düzeneğinde, soğutma kule suyundan izole edilen sülfat indirgeyen bakteri (SRB) içeren anaerobik bakteri izolatının galvanizli çelik üzerinde biyofilm oluşturma yeteneği araştırılmıştır. Test kuponları 744 saat boyunca anaerobik bakteri izolatının içinde bırakılmıştır. SRB içeren anaerobik izolatının zaman içerisinde galvanizli çelik kuponlar üzerinde biyofilm oluşturabildiği saptanmıştır. İstatistiksel analizlere göre, sesil ve planktonik SRB sayılarının ortalamaları arasında anlamlı bir farkın olmadığı, bununla birlikte SRB sayıları arasında aynı yönde anlamlı bir ilişkinin olduğu tespit edilmiştir ($P<0.01$). Test sistemindeki bakteri izolatının hücredışı karbonhidratı kullandığı belirlenmiştir.

Anahtar kelimeler: Sülfat-indirgeyen bakteriler (SRB), soğutma kulesi suyu, galvanizli çelik, biyofilm.

Introduction

Open recirculating cooling tower water systems are used to dispose of residual heat generated in industrial processes. In industrial cooling systems, biofouling and corrosion are

the major causes of failure. Such systems offer ideal conditions for microorganisms to grow and proliferate (Choudhary 1998). Microorganisms present in cooling water form biofilm on all the parts of cooling systems that are in contact with process water, such as pipelines, heat exchangers and cooling towers. The development of anaerobic conditions at the bottom of the biofilm enhances the growth of anaerobic bacteria, especially sulphate-reducing bacteria (SRB) (Hamilton 1985).

A steel structure which is coated with a thin layer of zinc is called galvanized steel. Galvanized steel is commonly used as construction material in building water tanks and cooling towers because of its resistance to biofauling and biocorrosion, and its relatively cheap cost compared to stainless steel. In spite of its anticorrosive and antifouling properties, it was reported on several occasions that mixed species bacteria attached on galvanized steel and formed a biofilm layer which subsequently caused corrosion (Ilhan-Sungur et al. 2007; Ilhan-Sungur and Cotuk 2010).

The aims of our study were to isolate anaerobic bacteria including SRB from cooling tower water and to investigate its biofilm forming ability on galvanized steel coupons under laboratory culture conditions.

Materials and Methods

Experimental setup

Biofilm formation experiment was performed in 1 lt glass beaker containing 38 galvanized steel coupons and anaerobic bacteria culture ($2.5 \times 10^{7} \cdot ml^{-1}$) in Postage's medium C (800 ml). The beaker was placed into the anaerobic jar (2.5 L) and kept at 28 ^{0}C during the experiment. Anaerobic conditions were set up by using Anaerogen (Oxoid) sachets. The medium in the test system was magnetically stirred at 300 rpm with a Teflon bar throughout the experiment.

Test material

Galvanized steel was used as test material since it is frequently preferred as construction material in cooling towers. The coupons (20x20x0.5 mm) were prepared according to ASTM G1-72 standards (American Society for Testing Material, 1975). The cut edges of the coupons were coated with epoxy zinc phosphate primer (Moravia, Turkey) and then covered with epoxy finish coating (Moravia, Turkey) to prevent corrosion starting at these edges.

Microbiological analyses

Anaerobic bacteria isolates including SRB were isolated from several cooling towers. Postgate`s medium B (PB) was used for the isolation of anaerobic bacteria isolate including SRB (Postgate 1984). One of the rapidly and intensively growing anaerobic bacteria isolates was selected for further experiment and inoculated onto semi-solid Postgate's medium C (PC) in order to obtain the anaerobic bacteria isolate which was able to reduce sulphur compounds to H_2S, as well as SRB. Inoculated Petri dishes were placed into anaerobic jars in an atmosphere of less than 1% oxygen and 9-13% carbon dioxide, which was produced by using Anaerogen sachets (Oxoid, Thermo Fisher Scientific). The jars were incubated in the dark at 28°C for 21 days. Several black colonies, which have similar macroscopic traits, were aseptically picked up by using a sterile Pasteur pipet under a stereo microscope and transferred into the liquid PB medium. This process was performed twice (Feio et al. 1998; Hines et al. 2007). The bacteria isolate obtained was used for the biofilm formation experiment.

Gram staining was carried out to observe the morphology of the isolated anaerobic bacteria isolate (Holt et al. 1994). Endospore staining was used to monitor bacteria endospores (Cotuk 2003).

At sampling hours (2, 4, 6, 12, 24, 48, 72, 96, 168, 360 and 744) three coupons were taken out of the test system and also 50 ml of culture was replaced with 50 ml sterile PC medium. These three coupons were used for enumeration of SRB, EPS extraction and carbohydrate analysis. The medium of the test system was analyzed at the sampling hours for various

physico-chemical parameters such as pH, chloride (Cl^-), sulphate (SO_4^{2-}) and hydrogen sulfide (H_2S).

For enumeration of planktonic SRB, 5 ml of test system culture was serially diluted from 10^{-1} to 10^{-10}. One coupon was used to estimate the sessile bacteria counts at each sampling time. In order to remove unattached bacteria, the coupon was dipped into the sterile PC medium several times. The biofilm layer that formed on each surface of the coupon was collected by using sterile cotton swab and suspended in 10 ml PC medium by vortexing. The resulting suspension was serially diluted from 10^{-1} to 10^{-8}.

The sessile and planktonic SRB counts were determined by the most probable number (MPN) technique using PB medium. The MPN tubes were incubated in the dark at 28 ^{0}C for 21 days. The growth of SRB was evidenced by the presence of black FeS precipitate and turbidity.

One coupon was assigned for EPS extraction at each sampling time and the recovery of EPS was carried out according to the method of Zhang et al. (1999). The carbohydrate amount of the extracted EPS solution was determined by the phenol-sulphuric acid method. Standard glucose solutions (10-100 ppm) were prepared and used to draw standard curves (Dubois et al. 1956).

Statistical analyses of data

The bacteria counts were transformed to $\log_{10}$ and standard deviations of means were calculated. Statistical evaluation of the results was carried out by Pearson and Spearman`s product moment correlation coefficient test. The Mann-Whitney U test was employed to detect bacterial counts. All statistical analyses have been done using SPSS Statistics v17 software.

Results

The PB and PC media, which are specific for the isolation of SRB, were used to isolate anaerobic bacteria including SRB. The black precipitate and black colonies were indicative for iron sulfide (FeS) as a reduction of sulphate to sulfide. Elliptical and round shaped, black and brown colonies were aseptically transferred into PB medium to be kept for further experiments. Rod and curved rod bacteria morphologies were observed by phase contrast microscope (Fig. 1).

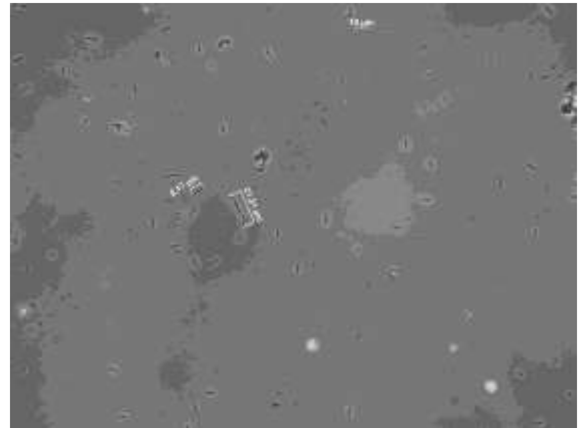

Figure 1. Phase-contrast photomicrograph of bacteria culture including SRB.

As a result of Gram staining, both Gram positive and Gram negative bacteria were observed (Fig. 2) and terminal and subterminal positioned endospores were monitored by endospore staining.

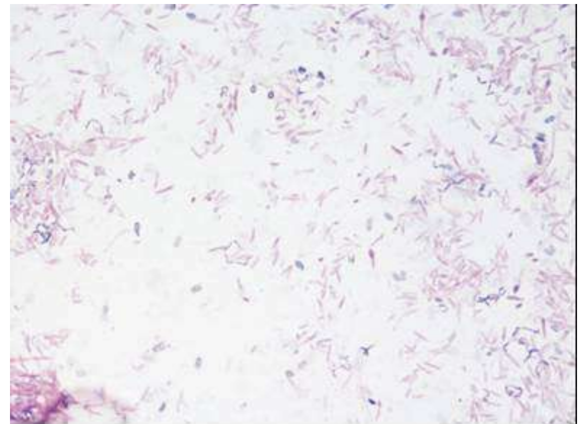

Figure 2. Photomicrograph of Gram staining preparation of bacteria culture.

Various physico-chemical parameters of the test system medium were determined at each sampling hour (Table 1). There was no

significant change in chloride ion concentration in the test system during the experiment and established to be around 1400-1700 ppm. The increases in sulphate ion concentration in the test system were constantly followed by decreases. The maximum value was detected at 24h as 2129 ± 43.51 ppm, while the minimum value was 1583 ppm at 4h. Sulfide concentration in the test system was determined to be around 1 mmol/l during the course of the study. Changes in pH were in parallel with the changes in sulphate concentrations and were between 6.35 and 7.09 throughout the study.

Table 1. SO_4^{2-}, Cl^-, H_2S and pH values of the test system culture for each sampling hour.

Hours	SO_4^{2-} ppm	Cl^- (ppm)	H_2S (mmol/l)	pH
0	1699 ± 0.00	1600	1.0866 ± 0.0027	6.35
2	1975 ± 130.54	1600	0.0559 ± 0.0261	6.62
4	1583 ± 76.15	1500	1.1463 ± 0.0109	6.51
6	1891 ± 54.39	1500	1.0677 ± 0.2220	6.52
12	1729 ± 65.27	1600	1.0808 ± 0.0272	6.52
24	2129 ± 43.51	1500	1.0752 ± 0.0102	6.61
48	2045 ± 32.64	1400	1.0577 ± 0.0139	6.50
72	1737 ± 32.64	1400	1.0577 ± 0.0054	6.40
96	1614 ± 54.40	1400	1.0675 ± 0.0052	6.61
168	1622 ± 21.76	1600	1.0651 ± 0.0016	6.72
360	2029 ± 76.15	1700	1.0534 ± 0.0039	6.89
744	2029 ± 141.42	1400	1.0651 ± 0.0016	7.09

±, Standard deviation

While the total carbohydrate concentration was 1304.512 ± 90.77 µg/cm^2 at 2h, which was the maximum value, it dropped to 675.336 ± 55.46 µg/cm^2 at the 6h, which was the minimum value during the course of the experiment. After 6h, increasing total carbohydrate concentration on the coupons reached to 923.657 ± 21.91 µg/cm^2 at 24h and decreased again to 720.059 ± 10.68 µg/cm^2 at 48h. After 48h, total carbohydrate concentration on the coupons progressively increased and was determined as 988.412 ± 59.39 µg/cm^2 at 744h. (Fig. 3). The quantites of carbohydrate detected in biofilm seemed not to be correlated with the SRB counts.

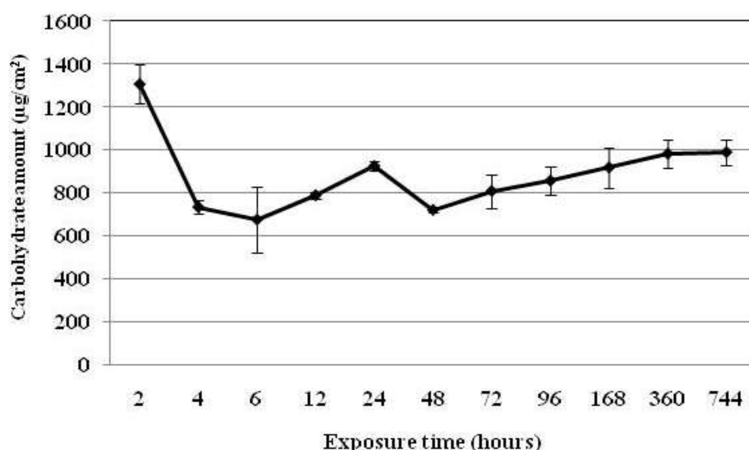

Figure 3. Quantities of carbohydrate detected in biofilm formed on galvanized steel coupons. Error bars represent the standard deviation.

The planktonic and sessile SRB counts were shown in Fig 4. The maximum SRB count in biofilm was detected as 9.75 cells/cm^2 at 12h, whereas the minimum sessile SRB count was 6.39 cells/cm^2 at 360h. The cell concentrations of planktonic SRB in the culture increased to a maximum of 10.88 cells/ml at 2h, the minimum value was detected as 8.96 cells/ml at168h. It was determined that planktonic and sessile SRB counts decreased with time ($P<0.01$ and $P<0.01$, respectively). According to Spearman's rank correlation coefficient test, it was established that there was a positive correlation between planktonic and sessile SRB counts ($P<0.01$). T-test analysis revealed that there was no significant difference between the planktonic and sessile SRB counts.

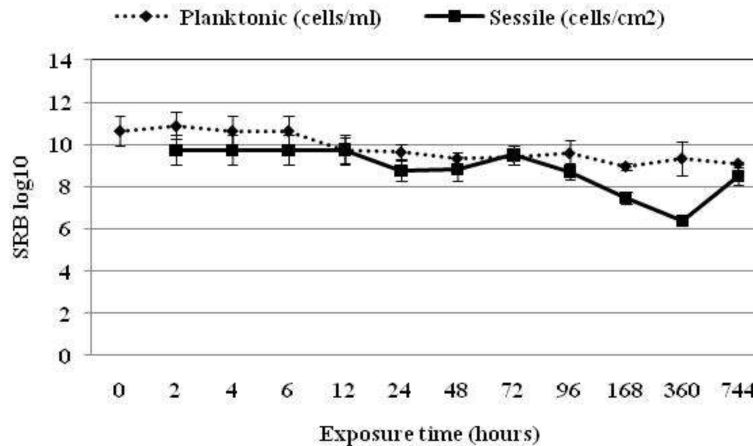

Figure 4. Planktonic and sessile SRB counts during the course of experiment. Error bars represent the standard deviation.

Discussion

The increases in sulphate ion concentration in the test system were constantly followed by decreases. This might be attributed to the addition of make-up medium to the test system at sampling hours. The sulfide concentrations in the test system were determined to be around 1 mmol/l during the course of the study. According to the statistical analyses, no correlation was found between sulfide concentrations and sessile and planktonic SRB counts. O'Flaherty et al. (1998) reported that inhibition of SRB was influenced by H_2S concentration in media between 6.8 and 7.2 pH values. The pH values in the test system were between 6.35 and 7.09, however the H_2S concentration was low. According to our results, it can be considered that H_2S could not affect SRB growth negatively.

According to the Mann-Whitney U test, there was no significant difference between the planktonic and sessile SRB counts in the test system. Additionally, no marked difference in SRB counts was observed during the experiment. This might be due to the discharge and make-up of the test system culture at each sampling hour. By this means, planktonic SRB counts didn't change significantly and SRB counts remained at a constant level. According to the Spearman's rank correlation coefficient test, there was positive correlation between sessile and planktonic SRB counts ($P<0.01$). Similarly, in a study of Ilhan-Sungur and Cotuk (2010), SRB counts in water and biofilm were reported to be positively correlated. Beech et al. (1994) and Ellwood et al. (1982) also reported similar results. This showed that SRB in biofilm were continously pouring into the planktonic phase.

The sessile SRB counts detected in our study were greater than the sessile SRB counts reported by Ilhan-Sungur et al. (2007), a study

in which a pure SRB strain (*Desulfovibrio* sp.) was tested in a similar test system. This difference was probably due to the bacteria species used in both studies. Biofilm formation abilities of bacteria can be different when they are pure or in dual- or mixed-species biofilm. Andersson et al. (2008) investigated biofilm formation and adherence properties of thirteen bacterial species in pure and mixed cultures and reported that strains unable to form single-strain biofilm were able to form strong biofilm when cocultured with other species, which indicates the synergistic effects of bacteria on metabolism and biosynthesis. Dual-species biofilm formation may be much faster than single-species biofilm formation, because two strains may affect each other by quorum sensing and induce growth, which leads fast biofilm formation (Andersson et al., 2008). James et al. (1995) showed pure cultures of two different bacterial species' biofilms in a laboratory reactor were thinner, whereas a biofilm containing both species was thicker. A large number of factors, such as cell surface hydrophobicity, nutrient supply, hydrodynamic forces, surface changes, production of EPS, LPS and presence of other adhesion proteins such as fimbria and flagella all play an important role on the rate and extent of attachment of microbial cells (Denkhaus et al. 2007).

Extracellular carbohydrate appeared to be degraded by bacteria culture in the test system. Likewise, it was reported by Zhang and Bishop (2003) that bacteria in mixed cultures degrade their own EPS material when they pass into the starvation phase. Carbohydrate amount in EPS detected in our study was much higher than the carbohydrate amount reported in the study of Ilhan-Sungur et al. (2007). This may be attributed to the bacterial species present in the test system. The composition and production ability of EPS vary depending on bacterial species and also the amount of EPS secreted in a biofilm depends on carbon substrates available both inside and outside of the cell (Sutherland 2001). In order to obtain the anaerobic bacteria isolate, black and brown colonies formed in the semi-solid PC medium were picked up and observed under the phase contrast microscope, and several rod, curved rod and spiral shaped cells in different sizes were seen. Gram staining examination showed that both Gram positive and negative bacteria were present in the culture. These might be an indication of any change in bacteria morphology as well as the presence of more than one bacteria species in the culture. Besides, endospore staining preparations were observed under light microscope and bacteria seemed to contain terminal and subterminal positioned, oval shaped endospores. Some SRB species such as *Desulfotomaculum* (Campbell and Postgate 1965), *Desulfosporosinus* (Stackebrandt et al. 1997), *Desulfosporomusa* (Sass et al. 2004), *Desulfovirgula* (Kaksonen et al. 2007) contain endospore which enables them to survive for long periods of time in unfavorable conditions. Further molecular analysis will be performed to determine the bacteria species present in this bacteria culture.

Acknowledgements

We are grateful to Prof. Dr. Nurhan Cansever and Asst. Prof. Dr. Esra İlhan-Sungur for their helpful discussions and to Dr. Nihal Doğruöz for her skilled technical support. This study was supported by the Research fund of Istanbul University (Project no: T-18/15122006)

References

American Society and Testing and Material (ASTM), (1975) Standard recommend practice for preparing, cleaning and evaluating corrosion test specimens, In: *Annual book of ASTM standards*, Designation: G1-72, American Society for Testing Materials, Philadelphia, 0300137510, pp.626-629.

Beech, I.B., Sunny Cheung, C.W., Patrıck Chan, C.S., Hill, M.A., Franco, R., Lino, A.R. (1994) Study of parameters implicated in the biodeterioration of mild steel in the presence of different species of sulphate-

reducing bacteria. *International Biodeterioration and Biodegradation,* 34: 289–303.

Campbell, L.L, Postgate, J.R. (1965) Classification of the spore-forming sulfate-reducing bacteria. *Bacteriological Reviews*, 29(3): 359–363.

Choudhary, S.G. (1998) Emerging microbial control issues in cooling water systems. *Hydrocarbon Processing*, 77(5): 91-102.

Cord-Ruwisch, R. (1985) A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. *Journal of Microbiological Methods*, 4: 33-36.

Cotuk, A. (2003) *Genel Mikrobiyoloji Laboratuvar Yöntemleri*, Nobel Tıp Kitabevleri, İstanbul.

Denkhaus, E., Meisen, S., Telgheder, U., Wingender, J. (2007) Chemical and physical methods for characterization of biofilms. *Microchimica Acta,* 158: 1–27.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28: 350-356.

Ellwood, D.C., Keevil, C.W., Marsh, P.D., Brown, C.M., Wardell, J.N. (1982) Surface-associated growth. *Philosophical Transactions of the Royal Society of London, Series B*, 297: 517–532.

Feio, M.J., Beech, I.B., Carepo, M., Lopes, J.M., Cheung, C.W.S., Franco, R., Guezennec, J., Smith, J.R., Mitchell, J.I., Moura, J.J.G., Lino, A.R. (1998) Isolation and characterization of a novel sulphate reducing bacterium of the *Desulfovibrio* genus. *Anaerobe*, 4: 117-130.

Hamilton, W.A. (1985) Sulphate-reducing bacteria and anaerobic corrosion. *Annual Review of Microbiology*, 39: 195-217.

Hines, M.E., Visscher, P.T., Teske, A., Devereux, R. (2007) Sulfur Cycling, In: Hurst C.J. (ed), *Manual of Environmental Microbiology*, ASM Press, Washington, D.C., 9781555813796, pp. 497-510.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., Williams, S.T., (1994) *Bergey's Manual of Determinative Bacteriology*, 9th ed., Williams & Wilkins, Baltimore, USA, 0683006037.

Ilhan-Sungur, E., Cotuk, A. (2010) Microbial corrosion of galvanized steel in a simulated recirculating cooling tower system. Corrosion Science, 52(1): 161-171.

Ilhan-Sungur, E., Cansever, N., Cotuk, A. (2007) Microbial corrosion of galvanized steel by a freshwater strain of sulphate reducing bacteria (*Desulfovibrio* sp.). *Corrosion Science*, 49: 1097-1109.

James, G.A., Beaudette, L., Costerton, J.W. (1995) Interspecies bacterial interactions in biofilms. *Journal of Industrial Microbiology*, 15: 257–62.

Kaksonen, A.H., Spring, S., Schumann, P., Kroppenstedt, R.M., Puhakka, J.A. (2007) *Desulfurispora thermophila* gen. nov., sp. nov., a thermophilic, spore-forming sulfate-reducer isolated from a sulfidogenic fluidized-bed reactor. *International Journal of Systematic and Evolutionary Microbiology*, 57: 1089-1094.

O'Flaherty, V., Mahony, T., O'Kennedy, R., Colleran, E. (1998) Effect of pH on growth kinetics and sulphide toxicity thresholds of a range of methanogenic, syntrophic and sulphate-reducing bacteria. *Process Biochemistry*, 33(5): 555-569.

Postgate, J.R. (1984) *The sulphate reducing bacteria*, 2nd ed., Cambridge University Press, Cambridge, 0521257913.

Sass, H., Overmann, J., Rütters, H., Babenzien, H.-D., Cypionka, H. (2004) *Desulfosporomusa polytropa* gen. nov. sp. nov., a novel sulfate-reducing bacterium from sediments of an oligotrophic lake. *Archives of Microbiology*, 182: 204–211.

Stackebrandt, E., Sproer, C., Rainey, F.A., Burghardt, J., Pauker, O., Hippe, H. (1997)

Phylogenetic Analysis of the Genus *Desulfotomaculum*: Evidence for the Misclassification of *Desulfotomaculum guttoideum* and Description of *Desulfotomaculum orientis* as *Desulfosporosinus orientis* gen. nov., comb.nov. *International Journal of Systematic Bacteriology*, 47(4): 1134-1139.

Zhang, X., Bishop, P.L., Kinkle, B.K. (1999) Comparison of extraction methods for quantifying extracellular polymers in biofilms. *Water Science and Technology*, 39 (7): 211-218.

Zhang, X., Bishop, P.L. (2003) Biodegradability of biofilm extracellular polymeric substances. *Chemosphere,* 50: 63–69.