A surface modified microbial polymer inactivates quorum sensing molecules and incapacitates Sphingomonas paucimobilis biofilm formation in plumbing material

Parul Gulati¹ and Moushumi Ghosh ²

¹ Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, India
² Department of Biotechnology and affiliate faculty, Centre of Excellence Emerging Materials, Thapar Institute of Engineering and Technology, Patiala, India

E-mail: mghosh@thapar.edu

Abstract: We report the potential antibiofilm properties of a chemically modified biopolymer on the formation of biofilms by Sphingomonas paucimobilis MG6, an opportunist pathogen isolated from distributed drinking water systems. The biopolymer obtained from the producer bacteria K. terrigena was quarternized and examined for safety using RAW 264.7 cell lines. The modified biopolymer (N-methyl biopolymer: NMB) at 1mg/ml inactivated AHL homologs of S. paucimobilis at ambient temperature. GC-MS and C. violaceum biosensor assays were used to evaluate loss of AHL molecules. On NMB coated coupons loss of biofilm formation under static conditions, was visualized by a rapid calcofluor assays and scanning electron micrographs (SEM). Applicability was further investigated in a flow through apparatus afforded with different plumbing materials, coated with NMB. Leaching of NMB was not observed and a complete absence of biofilms was noted on coated materials, especially in PVC. SEM and viable counts corroborated these observations. Overall, results of this study suggest that the novel material offers as a potential sustainable approach for controlling biofilms in drinking water systems.

Keywords: Antibiofilm, Biopolymer, N-methylbiopolymer, plumbing, quorum sensing, S. paucimobilis

1. Introduction

Sphingomonas, considered as opportunistic pathogen [17],[25],[28] is endowed with the capability to survive in oligotrophic niches important for human life and sustenance-such as water supply, reverse osmosis systems[32]. Biofilm formation by Sphingomonas on a diverse range of surfaces [10], [11], [31] including plumbing materials and water distribution systems has been demonstrated [10]. Biofilm formation on these surfaces has been linked to bacteremia, catheter infections (intravascular), infections in the urinary and biliary tract, skin, ventilator associated pneumonia, myositis [27], diarrheal disease [7] in dental unit waterlines[26], tap water [12] and in hospital water system [27]. Sphingomonas paucimobilis has been frequently implicated in the above and in addition as a candidate for evoking metal corrosion and biofouling of distribution systems used for water [19].
Chlorine and biocide resistance of this strain exacerbates the risk to consumers. To prevent the detrimental effects of biofilm produced by Sphingomonas, it is indispensable to employ novel strategies for inhibition of biofilm. Since biofilm formation is governed by quorum sensing, interference and blocking quorum sensing signals responsible for biofilm formation can be an effective, non-disruptive strategy. Biopolymers with several novel functions have gained importance as surface applications in recent times. Their role in interference with quorum sensing signals (N-acyl-homo-serine lactones (AHLs)) in gram negative bacteria can be important in circumventing biofilms in water distribution systems without evoking residual after effects. Polymers originating from biological sources are used in a wide range of applications due to unique structural and functional attributes [20]. Moreover biopolymers have intrinsic safety, amenability of structural modification and have been used for water purification. The present study attempted to elucidate the role of a biopolymer from K. terrigena with quorum signal binding activity responsible for biofilm formation by S. paucimomilis. To simulate the applicability in distributed water systems different plumbing materials were coated with the surface modified biopolymer and analyzed for biofilm production in a flow through system previously reported.

2. Materials and methods

2.1. Microorganism and culture conditions:

Overnight grown cultures of Sphingomonas paucimobilis MG 6, were used to produce biofilms on plumbing materials as described [9]. Extracellular biopolymer was obtained from K. terrigena (Accession number EU082029) and purified and quarternized [14]. Briefly, the biopolymer (500 mg; 10 mmole) was mixed with 1% (v/v) acetic acid (50 ml) and formaldehyde (0.5 ml; 0.22 mmole) was added. After stirring, the suspension at room temperature for 30 min, sodium borohydride (41.25 mg; 1.12 mmole) was added. The pH of the suspension was then adjusted to 10 by NaOH (1 M). White precipitates were filtered and washed until they attained a pH of 7. Azeotropic distillation was used to remove trapped moisture was removed by refluxing with dry benzene. Evaporation of benzene resulted in N-methylbiopolymer (NMB) (yield 86%; 430 mg). The cytotoxic activity and cell viability of the modified biopolymer was analyzed using RAW 264.7 macrophages and MTT assay [13]. RAW 264.7 cells (National Centre for Cell Sciences, Pune, India) were propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin solution, 1% L-glutamine and HEPES[4-(2-hydroxyethyl)-1-
piperazinethanesulfonic acid]. A 5% carbon dioxide concentration and incubation temperature of 37°C was used during the experiment. Plates with 96 wells were afforded with cells (50,000 cells/0.2 mL/well), incubated overnight for attachment; the medium in wells was replaced with NMB-containing medium the following day. A stock solution of 1 mg/mL was prepared in sterile saline, suitably diluted in culture medium to obtain working solutions (0.2, 0.4, 0.6, 0.8 and 1 mg/L) and 0.2 mL was added. For cell viability measurements, cells were incubated for 24 or 48 hours and the MTT assay as described earlier [33] was used.

2.2. Biofilm Inhibition assay:
Twenty microliters of inoculum (optical density, O.D.600 =0.02) was added along with 0.03 mg/ml NMB in each well of the polystyrene plates and incubated at 37°C for up to 72 hours. The plates were removed every 4 hours and biofilm formed was measured by crystal violet assay. The percentage of biofilm inhibition was calculated by:

\[ \text{Percentage of Biofilm inhibition} = \left( \frac{\text{Control} \ O.D. \ 585 \ nm - \text{Test} \ O.D. \ 585 \ nm}{\text{Control} \ O.D. \ 585 \ nm} \right) \times 100 \]

2.3. Simulation of biofilm inactivation in flow through system
A laboratory-scale simulated distribution system [9] was fabricated with minor modifications. Different sets of pipe material with the exception of Copper and were used. These included: polyvinyl chloride (PVC), Polypropylene (PP), polyethylene (PE), Aluminum (Al), and rubber (R). The distribution pipe has a length of 1 m and an internal diameter of 3 cm. Tap water was continuously pumped from the contact tank (30 liters of tap water) at a flow rate of 1.0 L/min into the simulated drinking water distribution pipe using a peristaltic pump. Overnight grown S. paucimobilis MG6 culture was used as inoculum, the concentration of viable cells was \(10^5\) CFU/ml of water pumped in the contact tank.

2.4. Biofilm inactivation on coupons with and without modified biopolymer
To check the inhibition of biofilm formed on plumbing materials under static conditions, NMB was added to sterile coupons and dried for 1 hour. S. paucimobilis MG6 was inoculated on all the coupons in water and Sphingomonas selective growth (SSG) medium and biofilm was allowed to form for 72 hours. These were then removed using sterile forceps, washed with 50 ml of 1xPBS to free unadhered cells. 80 mg/l calcofluor was added for staining and washed with PBS to remove unbound dye. The presence of biofilm on the surfaces was visualized by a hand-held UV-lamp long wave (Blak-Ray Lamp model UVL- 21, UV-366...
nm)[19].
To check for leaching of NMB, coupons as well as plumbing materials were pre coated with different concentrations of NMB ranging from 0.1 to 1 mg/ml by dip coating method. The coupons were allowed to dry for 1 hour and were placed in water and media. The coupons and materials were continuously analyzed for 72 hours for leaching of NMB using calcofluor assay.

2.5. Scanning Electron Microscopy (SEM) of PVC coated with modified biopolymer
Biofilm of S. paucimobilis MG6 developed on PVC coated with and without NMB was examined after 3 days by scanning electron microscopy (SEM) [1]. The samples of biofilms were fixed using glutaraldehyde solution (2.5%) overnight at 4°C. Excess glutaraldehyde was removed by washing was carried with 1x Phosphate Buffer Saline (PBS). Dehydration of biofilm samples was performed with 30% ethanol followed by 50%, 60%, 70%, 80%, 90%, 95% to 100% ethanol respectively. Gold coating of samples were done prior to analysis by SEM (JSM541-V, JEOL, Japan) [15].

2.6. AHL Extraction
The OD600 of overnight grown S. paucimobilis MG6 was adjusted to O.D 600~ 1.80. The culture was centrifuged at 10,000 rpm for 15 minutes and cell pellet was discarded. The supernatant was filtered through 0.2 μm membranes. The filtrate was mixed with acidified ethyl acetate (0.01% Glacial Acetic Acid) and extracted for 15 minutes. Mixture was set aside for 10 minutes in a separating funnel for obtaining upper organic and lower aqueous layer. After extracting the aqueous layer twice the entire organic layer was pooled and dried (Rota-vapour) under vacuum at 40-50ºC. The dried residues were dissolved in1 ml of 20% acetonitrile.

2.7. Well diffusion assay for quorum sensing inhibitory activity:
A simple well diffusion assay was applied for detecting the presence of N-Acyl Homoserine Lactone (AHL) following incubation with NMB, in S. paucimobilis MG6. The biosensor strain have been used extensively; C. violaceum 026 is the mini Tn5 double mutant defective in the synthesis of violacein pigment, but retains the ability to respond to C4-C6 AHLs. Five milliliter of molten semi-solid Luria agar (0.3% w/v) was inoculated with 50 μl of an overnight grown culture of C. violaceum 026 and immediately poured over the surface of LB agar plates. When the overlaid agar solidified, wells were punched in the agar and filled with the cell-free supernatant and adjusted to a final solution of 50 μl by addition of sterile LB. Petri dishes were
incubated overnight (28°C), then analyzed the same for induction or inhibition of synthesis of violacein [23]. Purified AHL incubated with the NMB was analyzed in parallel for biosensor activity.

2.8. Gas Chromatography-Mass Spectrometry

Purified S. paucimobilis MG6 AHL (incubated) with and without NMB was examined by gas chromatography-mass spectrometry (GC-MS) (PerkinElmer Clarus 680 GC, India) [5]. Samples were injected into ELITE-5MS capillary column (30.0 m, 0.25 mm, 250 µm df). The operation parameters used were: Helium as carrier gas; injector temperature: 260°C; 1 ml/min flow rate. The temperature of the oven temperature was programmed as: 60 °C (2 min); followed by 300 °C at the rate of 10 °C /min and 300 °C, where it was held for 6 min. MS detector conditions were: transfer line temperature 240 °C; ion source temperature >240 °C; and ionization mode impact at 70 eV.

3. Statistical analysis: Data was converted to mean ± standard deviation [15] analyzed by one-way analysis of variance (ANOVA). In addition this was followed by Tukey's test or two-way ANOVA wherever applicable. The Graph Pad Prism was applied and results were considered significant for p<0.05.

4. Results

The survival of RAW 264.5 macrophages was not affected at 24 hours of incubation in the presence of NMB (modified biopolymer) significantly (p>0.05) (Table 1).

| Biopolymer dose (mg/L) | Cell Viability (%) |
|------------------------|--------------------|
| 0.00                   | 100% ± 3.27        |
| 0.02                   | 99.78% ± 4.11      |
| 0.40                   | 98.97% ± 7.10      |
| 0.60                   | 99.03% ± 5.32      |
| 0.80                   | 99.64% ± 7.31      |
| 1.00                   | 98.67% ± 6.76      |

The modified biopolymer treated macrophages upon microscopic observation, was visible
as a monolayer culture without any distinctive morphological changes at bactericidal doses over an incubation period of 48-72 hours. The modified biopolymer (NMB) was adjudged safe from these results.

Biofilm formation of S. paucimobilis MG6 (Accession No KX594380) was studied by crystal violet assay. We observed sloughing of biofilms usually by 96 hours therefore the 72 hours study was considered more relevant. Sloughing of biofilms has been reported in plumbing materials recently [7] in line with our observations.

Figure 1 depicts the influence of NMB on biofilm progression over time.

![Graph showing inhibition of biofilm formation by S. paucimobilis MG6 with NMB. Complete loss of biofilm was observed at NMB concentration of 1mg/ml. Data is the mean±SD of two experiments performed in triplicate.](image)

**Figure 1:** Inhibition of biofilm formation by S. paucimobilis MG 6 observed with NMB. Complete loss of biofilm was observed at NMB concentration of 1mg/ml. Data is the mean±SD of two experiments performed in triplicate.

Leaching of NMB from the coated coupons or plumbing materials used was not observed during the tenure of the study (96 hours). Biofilm production was reduced sharply and with lower (0.03mg/ml) doses of NMB and 45% biofilm reduction was noted. At a concentration of 1mg/ml, complete loss of biofilm was observed; therefore this concentration was used for coating the plumbing materials. Although evaluation of the physical effects during biofilm formation was not our objective, it was important to understand whether the different plumbing materials coated with NMB, actually supported biofilm formation by S. paucimobilis MG6 in the same fashion as observed under static conditions. The observations
of the flow through simulation experiment corroborated the fact that NMB coated plumbing materials failed to support biofilm development, albeit with different efficacies. There was a complete loss of biofilm formation on PVC; in PP, PE and Al notable decrease in biofilm formation was observed. Viable cells of S. paucimobilis MG6 were observed from different scrapings of plumbing materials obtained at various ages (Table 2).

**Table 2:** Comparison of biofilm cells log counts which developed on different plumbing materials with and without NMB treatment. PVC: polyvinyl chloride; PP: polypropylene; PE: polyethylene; Al: Aluminum. *The values represented are log CFU /ml of S. paucimobilis MG6.

| Tested plumbing materials: | Biofilm age | 24 hours | 48 hours | 72 hours | 96 hours |
|---------------------------|-------------|----------|----------|----------|----------|
| PVC                       | Untreated   | 90.2     | Not detected | 93.6     | Not detected | 94       | Not detected | 95.3     | Not detected |
|                           | NMB treated |          |          |          |          |          |          |          |          |
| PP                        | Untreated   | 87.4     | Not detected | 88.0     | Not detected | 89.0     | Not detected | 90.4     | 3.6       |
|                           | NMB treated |          |          |          |          |          |          |          |          |
| PE                        | Untreated   | 82.5     | Not detected | 83.3     | Not detected | 86.7     | 4.3       | 79.4     | 3.3       |
|                           | NMB treated |          |          |          |          |          |          |          |          |
We observed bacterial growth at 72 and 96 hours on PP, PE and Al. The surface composition of the surface is pertinent in sustaining biofilms [26]. Therefore the surface characteristics of these plumbing materials, following 48 hours of exposure to S. paucimobilis (in the flow through system) should provide an explanation. PVC is extensively used as a plumbing material for distribution of water, loss of biofilm production in NMB coated PVC thus assumes relevance. Since intensity of fluorescence reciprocates biofilm formation, the effect of NMB on biofilm inhibition could be clearly visualized (Figure 2).
Figure 2: Inhibition of S. paucimobilis MG6 biofilm on PVC coupons in water and medium. Presence of biofilms was analyzed using the calcofluor assay.

Distinct reduction in biofilm formation in the NMB coated coupons was also observed from scanning electron micrographs confirming the biofilm inhibition efficacy (Figure 3).

Figure 3: Scanning electron Micrographs of S. paucimobilis MG6 (control, Left) with 1 mg/ml NMB coated on PVC and used in the flow through system.

It has been suggested that for biofilm formation, AHL signaling is important. However, there is little evidence for AHL regulation of biofilm development in Sphingomonas. Therefore an attempt was made to detect AHL in the isolate. For this, the CV026 inhibition test was carried out since these molecules are expected to inhibit the induced CV026 and produce colorless colonies in purple background. Inhibition of AHL activity was observed from the diminished violacein production in C. violaceum CV026 in a concentration dependent manner.

Moreover, this particular assay is invaluable for ascertaining the acyl chains ranging from C10 to C14 [22]. The A. tumefaciens A136, a broad range biosensor, was also employed to confirm the presence of the C8 to C12-HSLs including oxo-C6-HSL; the good sensitivity to even low level of longer acyl side chain AHLS [8] is notable, for this biosensor assay. To
demonstrate whether NMB can degrade AHL signaling molecule of S. paucimobilis MG6, AHL incubated with NMB was used for evaluating its functionality by the biosensor. Chemical analysis for the presence of AHL upon treatment with NMB was carried out by GCMS. After incubation of NMB with dDHL, it was observed that [M-H] ions which are characteristic of this signaling molecule are absent, while the presence of specific ions at m/z 69, 86 and 128 confirmed the loss of dDHLs (Figure 4).

Figure 4: Spectra of extracted AHL (N-dodecanoyl-L-Homoserine lactone) degradation by NMB in full scanning positive mode (a) Total Ion Chromatogram (b)-(d) Ion-fragments at different m/z carried out by GC-MS. These results indicated that NMB possibly binds to AHL molecules and subsequently degrades them.

5. Discussion

Earlier studies in this laboratory have explicitly demonstrated the capability of S. paucimobilis MG 6, an isolate from community drinking water systems, to form biofilms on a wide variety of plumbing materials. The strain is chlorine tolerant and supports multigeneric aggregation with S. flexneri 2a and E. coli 0157:H7. Furthermore a systematic study on mechanisms of biofilm formation using biofilm negative mutants conclusively
highlighted the involvement of quorum signaling molecules in Sphingomonas for biofilm formation. A complete loss of motility, aggregation along with exopolysaccharide production was noted in biofilm negative mutants. The latter properties are revoked upon supplementation with homolog of N-dodecanoyl-L- Homoserine lactone purified and characterized from S. paucimobilis MG6. Incubation with purified AHL with the modified biopolymer led to a complete loss of swarming motility and quorum sensing activity detectable on A. tumefaciences plate assays. It been suggested that Interference of the bacterial communication system by suitable antagonists can offer a benign yet effective strategy for biofilm prevention. To this end, microbial exopolymers are attractable classes of molecules and present an interesting proposition for sustainable application. The involvement of AHLs in biofilm formation by Sphingomonas assumes importance, in view of the safety of drinking water. Thus, it was the intent of this study to investigate the role of bacterial exopolymers modified chemically. The biopolymer from Klebsiella terrigena has been well characterized in our prior studies, besides the native biopolymer is able to inactivate to purified AHL but with very low efficiency. This biopolymer was therefore selected for chemical modification and designated as N-methyl biopolymer (NMB). Parallel results (not shown) suggest that NMB modulated the quorum sensing genes luxI and luxR activities leading to a loss of biofilm formation without affecting the cell integrity presumably by lactonolysis of lactone moiety of signal molecule. This results in the ring opened product and complete inactivation of the signal molecule crucial for biofilm formation.

Thus far, several studies have reported synthetic, semi-synthetic and natural molecules with antiquorum sensing activities. However, to date, no studies have evaluated antiquorum sensing molecules especially in materials used for plumbing or water distribution. The conditions of the environment define the growth of biofilms in biotechnological systems, in the human body, and in nature. Growth of microbes is strongly mediated by the nutrient availability, physical transport, and structure of biofilm [4], [16], [35]. For instance, it has been observed that biofilms develop different morphologies under different flow conditions [3], [6], [29], [11], [34] and are strongly affected by hydrodynamic shear forces in wastewater treatment plants [21], and internal transport limitations are among many factors that cause biofilms to be highly resistant to chemical stresses[16],[18]. It is clear that the flow distributions, hydrodynamic shear on surfaces in addition to important chemical constituents govern habitat conditions for microbial growth in biofilm [2], [15], [18], [30]. Several flow through systems has been experimented in an effort to
unravel interactions between the biofilm growth and their spatial patterns evoke by key environmental conditions [8]. In the present study it was important, first, to understand that whether the modified biopolymer could actually support biofilm formation by S. paucimobilis MG6 under static conditions. These observations were then correlated with the flow through simulation experiment. Viable cells of S. paucimobilis MG6 were recorded from different scrapings of plumbing materials at different ages (Table 2), direct staining using calcofluor and SEM images clearly indicated the functionality of the modified biopolymer in restricting biofilm formation. Violacein production is a simple assay for the detection of AHLs. Since, violacein is inducible by all the AHL (with varying N-acyl side chains from C4 to C8 in length) In CV026 [24]. Therefore this strain was deemed suitable for use as bioreporter for directly observing the effect of AHL treated with and without NMB in the present study. The bio-reporter studies and analytical results of direct incubation of AHL with NMB clearly substantiate the observations on inactivation of AHL which is translated to an impaired biofilm formation by S. paucimobilis.

To sum up, our findings suggest that the modified biopolymer can effectively inhibit biofilm formation by S. paucimobilis especially on the surfaces indicated. The flow through system enabled a simulation of real time conditions. Although stricter validations will be necessary for enabling suitable modifications prior to commercial applications, the current results provide important insights for developing an effective and alternate strategy using this biopolymer to prevent biofilms in distributed water systems.

6. Conclusion

Overall, the present study demonstrated the applicability of NMB, a quarternized extracellular polymer produced by K. terrigena in inactivating AHL of S. paucimobilis. In absence of AHL molecules, S. paucimobilis fails to produce biofilm. The anti-biofilm effect could be replicated directly on plumbing materials under simulated conditions using a simple flow though setup. The results provide encouraging insights for further studies to exploit the applicability of NMB as a novel green material for controlling Sphingomonas biofilms in distributed water systems.

References:

[1] Asahi Y, Miura J, Tsuda T, Kuwabata S, Tsunashima K, Noiri Y, Sakata T, Ebisu S, Hayashi M 2015 Simple observation of Streptococcus mutans biofilm by scanning electron microscopy using ionic liquids AMB Exp.5 6-8.
[2] Battin TJ, Wille A, Satller B, Psenner R 2001 Phylogenetic and functional heterogeneity of sediment biofilms along environmental gradients in a glacial stream Appl Env.Microbiol.67
799-807.

[3] Besemer K, Singer G, Limberger R, Chlup A-K, Hochedlinger G, Hödl I, Baranyi C, Battin TJ 2007 Biophysical controls on community succession in stream biofilms. *Appl. Env. Microbiol* 73 4966-4974.

[4] Boessmann M, Neu T, Horn H, Hempel D 2004 Growth, structure and oxygen penetration in particle supported autotrophic biofilms *Wat. Sc. Tech.* 49 371-377.

[5] Burton EO, Read HW, Pellitteri MC, Hickey WJ 2005 Identification of Acyl-Homoserine Lactone Signal Molecules Produced by Nitrosomonas europaea Strain Schmidt *Appl. Env. Microbiol.* 71 4906-4909.

[6] Chang YC, Le Puil M, Biggerstaff J, Randall AA, Schulte A, Taylor JS 2003 Direct estimation of biofilm density on different pipe material coupons using a specific DNA-probe. *Mol. Cellu.Prob.* 17 237-243.

[7] Chan, S., Pullerits, K., Keucken, A. et al. 2019 Bacterial release from pipe biofilm in a full-scale drinking water distribution system. *npj Biofilms Microbiom.*, 5, 1-8.

[8] Farrand SK, Qin Y, Oger P 2002 Quorum-sensing system of Agrobacterium plasmids: analysis and utility. In: Methods in enzymology. Elsevier. p. 452-484.

[9] Gulati P, Ghosh M 2017 Biofilm forming ability of Sphingomonas paucimobilis isolated from community drinking water systems on plumbing materials used in water distribution. *J. Wat. Health*, 15 942-954.

[10] Gusman V, Medić D, Jelesić Z, Mihajlović-Ukropina M 2012 Sphingomonas paucimobilis as a biofilm producer *Arch. Biol.Sc.* 641 327-1331.

[11] Jang H-J, Choi Y-J, Ka JO 2011 Effects of diverse water pipe materials on bacterial communities and water quality in the annular reactor *J. Microbiol. Biotechnol.* 21 115-123.

[12] Kanamori H, Weber DJ, Rutala WA 2016 Healthcare outbreaks associated with a water reservoir and infection prevention strategies *Clin. Infect. Dis.* 62 1423-1435.

[13] Kaur T, Ghosh M 2017 Characterization and toxicity of a phosphate-binding exobiopolymer produced by Acinetobacter haemolyticus MG606 *J. Wat. Health* 15(1) 103-111.

[14] Khaira G, Ganguli A, Ghosh M 2014 Synthesis and evaluation of antibacterial activity of quaternized biopolymer from Klebsiella terrigena *J Appl. Microbiol.* 116 511-518.

[15] Kreft JU, Picoreanu C, Wimpenny JW, van Loosdrecht MC 2001 Individual-based modelling of biofilms *Microbiol.* 147 2897-2912.

[16] Kuehn M, Mehl M, Hausner M, Bungartz HJ, Wurzel S 2001 Time-resolved study of biofilm architecture and transport processes using experimental and simulation techniques: the role of EPS *Wat. Sci. Tech.* 43 143-151.

[17] Laskin A, White D 1999 Preface to special issue on Sphingomonas *J. Ind. Microbiol.Biotech.* 23 231-235.

[18] Laspidou CS, Rittmann BE 2004 Modeling the development of biofilm density including active bacteria, inert biomass, and extracellular polymeric substances *Wat. Res.* 38 3349-3361.

[19] Lehtola MJ, Miettinen IT, Lampola T, Hirvonen A, Vartiainen T, Martikainen PJ 2005 Pipeline materials modify the effectiveness of disinfectants in drinking water distribution systems *Wat. Res.* 39 1962-1971.

[20] Lehtovaara BC, Verma MS, Gu FX 2012 Multi-phase ionotropic liquid crystalline gels with controlled architecture by self-assembly of biopolymers *Carb. Poly.* 87 1881-1885.

[21] Liu Y, Tay JH 2002 The essential role of hydrodynamic shear force in the formation of biofilm and granular sludge *Wat.Res.* 361653-1665.

[22] McClean KH, Winson MK, Fish I, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW 1997 Quorum sensing and Chromobacterium violaeceum: exploitation of
violacein production and inhibition for the detection of N-acylhomoserine lactones Microbiol.143 3703-3711.

[23] McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW et al 1997 Quorum sensing and Chromobacterium violaceum: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones Microbiol.143 3703-3711.

[24] Morohoshi T, Kato M, Fukamachi K, Kato N, Ikeda T 2008 N-acylhomoserine lactone regulates violacein production in Chromobacterium violaceum type strain ATCC 12472 FEMS Microbio. Lett.279 124-130.

[25] Oie S, Oomaki M, Yorioka K, Tatsumi T, Asamaki M, Fukuda T, Hakuno H, Nagano K, Matsuda M, Hirata N 1998 Microbial contamination of ʻsterile waterʻused in Japanese hospitals J.Hosp. Infect.38 61-65.

[26] Ono M, Niikaido T, Ikeda M, Imai S, Hanada N, Tagami J, Matin K 2007 Surface properties of resin composite materials relative to biofilm formation Den. Mat. J. 26 613-622.

[27] Pegoraro E, Borsato C, Dal Bello F, Stramare R, Fanin M, Palu G, Angelini C 2008 Sphingomonas paucimobilis associated with localised calf myositis J. Neurol, Neurosurg. Psychiat. 79 1194-1195.

[28] Perola O, Nousiainen T, Suomalainen S, Aukée S, Kärkkäinen U-M, Kauppinen J, Ojanen T, Katila M 2002 Recurrent Sphingomonas paucimobilis-bacteraemia associated with a multi- bacterial water-borne epidemic among neutropenic patients J. Hosp. Infect.50 196-201.

[29] Purevdorj B, Costerton JW, Stoodley P 2002 Influence of hydrodynamics and cell signaling on the structure and behavior of Pseudomonas aeruginosa biofilms App.Env.Microbiol.68 4457-4464.

[30] Rittmann B 1982 Comparative performance of biofilm reactor types Biotech.Bioengg. 24 1341-1370.

[31] Simões M, Simões LC, Vieira MJ 2010 A review of current and emergent biofilm control strategies LWT-Food Sc. Tech.43 573-583.

[32] Singer G, Besemer K, Schmitt-Kopplin P, Hödl I, Battin TJ 2010 Physical heterogeneity increases biofilm resource use and its molecular diversity in stream mesocosms PloS one 5(4) 1-11.

[33] Singh RP, Jain S, Ramarao P 2013 Surfactant-assisted dispersion of carbon nanotubes: mechanism of stabilization and biocompatibility of the surfactant J. Nanopart.Res. 15 1985-1991.

[34] Teodósio J, Simões M, Melo L, Mergulhão F 2011 Flow cell hydrodynamics and their effects on E. coli biofilm formation under different nutrient conditions and turbulent flow Biofoul.27 1-11.

[35] Venugopalan V, Kuehn M, Hausner M, Springael D, Wilderer P, Wuertz S 2005 Architecture of a nascent Sphingomonas sp. biofilm under varied hydrodynamic conditions App. Env. Microbiol.71 2677-2686.