# Biofilm forming ability of *Sphingomonas paucimobilis* isolated from community drinking water systems on plumbing materials used in water distribution

Parul Gulati and Moushumi Ghosh

## ABSTRACT

*Sphingomonas paucimobilis,* an oligotroph, is well recognized for its potential for biofilm formation. The present study explored the biofilm forming ability of a strain isolated from municipal drinking water on plumbing materials. The intensity of biofilm formation of this strain on different plumbing materials was examined by using $1 \times 1$ cm$^2$ pieces of six different pipe materials, i.e. polyvinyl chloride (PVC), polypropylene (PP), polyethylene (PE), aluminium (Al), copper (Cu) and rubber (R) and observing by staining with the chemical chromophore, Calcofluor. To understand whether biofilm formation occurs under flow through conditions, a laboratory-scale simulated distribution system, comprised of the above materials was fabricated. Biofilm samples were collected from the designed system at different biofilm ages (10, 40 and 90 hours old) and enumerated. The results indicated that the biofilm formation occurred on all plumbing materials with Cu and R as exceptions. The intensity of biofilm formation was found to be maximum on PVC followed by PP and PE. We also demonstrated the chemical chromophore (Calcofluor) successfully for rapid and easy visual detection of biofilms, validated by scanning electron microscope (SEM) analysis of the plumbing materials. Chlorination has little effect in preventing biofilm development.

**Key words** | biofilm, chlorine resistance, drinking water, pipe materials, *Sphingomonas*

**Parul Gulati**
**Moushumi Ghosh** (corresponding author)
Department of Biotechnology,
Thapar Institute of Engineering and Technology,
Patiala 147004,
India
E-mail: *mghosh@thapar.edu*

## INTRODUCTION

Waterborne contamination in ambient water bodies and its related diseases continue to have a major impact on water quality issues across the globe, pathogen contamination being the most serious issue ascribed for almost all ambient water bodies. Several *Sphingomonas* sp. are ubiquitously distributed in nature and have been isolated from drinking water and drinking water distribution systems. These strains have been well studied for their effective degradation/metabolism of complex hydrocarbons. The ability of *S. paucimobilis* to develop biofilm on surfaces of water distribution systems, for example stainless steel, has been reported as a major concern regarding water quality.

Most microbes in water distribution systems exist in biofilm on the inner surfaces of the pipelines (Bachmann & Edyvean 2006). The formation of biofilm is influenced by several factors, such as concentration and quality of disinfectants and nutrients, water flow velocity, hydraulic conditions, temperature and/or pipe materials (Zacheus *et al.* 2001). Pipe materials can influence biofilm formation, especially in its early stages (Waines *et al.* 2011; Chowdhury 2012). In addition to that, Flemming *et al.* (2002) estimated that about 95% of bacteria in drinking water distribution systems are attached to the surface of pipe inner walls, while only 5% are in the water phase and detected by sampling as commonly used for quality control. The pipe material used in water distribution systems is an important factor that influences the proliferation of the biofilm (Lehtola *et al.* 2005). Few studies have attempted to characterize

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*Sphingomonas* and evaluated their importance in drinking water systems, moreover their ability to form biofilms on plumbing materials commonly used has not been reported. It may be argued that the outcomes of studies involved in characterization of *Sphingomonas* in community drinking water systems would be important in understanding their role in waterborne infections and enable adequate treatment procedures for assuring water quality. Therefore the objective of this study was to characterize *Sphingomonas* sp. in community water distribution systems and determine their capability for biofilm formation on the plumbing materials commonly used in distribution systems. Biofilm simulation studies with the selected isolate on six types of pipe materials commonly used were designed and a simple visual approach to detect biofilms is also reported.

## MATERIALS AND METHODS

### Bacterial strains and culture conditions

Tap water samples were collected from 20 different municipal sites and 140 isolates were screened from the water samples on the basis of the morphology of the colonies. *Sphingomonas* selective growth medium is composed of L9 minimal medium which is supplemented with 1% each of glucose and sodium glutamate as carbon and nitrogen source with mineral salts supplementation as described by Yim *et al.* (2010). Optimal concentration for streptomycin and piperacillin was determined by performing an antibiotic susceptibility test at 0, 50, 100, 150, 200, 250, 300, 350 and 400 μg/ml. The isolates were further screened on *Sphingomonas*-specific growth medium containing 100 and 50 μg/ml streptomycin and piperacillin, respectively. One isolate with demonstrable biofilm forming ability was selected for further studies and characterized by 16S sequencing. The 16S sequence of the isolate was submitted in the GenBank database as *Sphingomonas paucimobilis* strain MG6.

### Molecular characterization of isolates

DNA of the selected isolate was extracted following the method as described by Moore *et al.* (2004). DNA was amplified using a set of universal primers: 27F (AGAGTTTGATCMTGGCTCAG) and 1510R (TACGGYTACCTTGTTACGACTT). The reaction mixture consisted of 1X PCR (Polymerase Chain Reaction) buffer, 0.2 mM each dNTPs (Deoxyribonucleotide triphosphate), 0.5 μM of each primer, 1 unit Taq Polymerase and 2 μl DNA. PCR was performed at 94 °C for 5 minutes, 94 °C for 1 minute, followed by 30 amplification cycles of 54 °C for 1 minute, 72 °C for 1 minute with the final cycle of 73 °C for 3 minutes. The sample was sequenced by automated DNA Sequencer AB3500xL (Applied Biosystem). The phylogenetic tree was constructed by Mega 7.0 software using the Neighbor-Joining method. The evolutionary distances were computed using the Kimura 2-parameter method and the 16S sequence of the isolate was submitted in the GenBank database as *Sphingomonas paucimobilis* strain MG6.

### Biofilm production

Biofilm formation was estimated by using a modified crystal violet assay (Merritt *et al.* 2005). A volume of 300 μl of a standardized bacterial suspension in mid-log phase was revived in biofilm-inducing media and dispensed in each well of the polystyrene plates and incubated at 30 °C. The unbound cells were removed by inverting the microtiter plate and vigorously tapping followed by rinsing the wells with phosphate buffer (pH 7.2). The adherent cells were stained with 200 μl of 1% (w/v) crystal violet solution for 5 minutes. The wells were washed with deionized water extensively and the plates were allowed to dry. Following washing, 200 μl of 30% (v/v) acetic acid was added to each well for 15 minutes; 100 μl aliquots were then transferred to a fresh microtiter plate and the absorbance measured at 585 nm.

To further evaluate the biofilm profile of *Sphingomonas*, the formazan-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed on biofilms as described by Kairo *et al.* (1999) with the modification of Walencka *et al.* (2005). Biofilm was grown in sterile flat-bottomed 96-well polystyrene microplates at 30 °C. After every 12 hours, bacterial suspension was removed till 72 hours, and 150 μl of PBS (phosphate buffered saline) and 50 μl of MTT at a concentration of 0.3% were added for 2 h at 37 °C. After incubation, the MTT solution was removed from the wells and 150 μl of dimethyl

sulfoxide (DMSO) and 25 μl of 0.1 M glycine buffer (pH 10.2) were immediately added to the wells to dissolve the formazan crystals formed in the wells. Incubation was carried out for 15 min at room temperature. The absorbance of the solution was measured at a wavelength of 570 nm using a Microplate Reader (Teccan, Austria).

## Determination of chlorine resistance

Overnight grown bacteria were harvested by centrifugation at 8,000 rpm for 5 minutes and washed twice in sterile phosphate buffered saline (pH 7.2). After the final centrifugation, the bacteria were suspended in buffer to an optical density of 1.05 (measured at 580 nm), and 10 μl and 20 μl of this suspension was transferred to each of the reaction tubes containing 10 ml of the PBS. The final cell concentration in each tube was approximately $4.0 \times 10^6$ per ml and $8.0 \times 10^6$ per ml, respectively. One tube served as an untreated control and received no chlorine but instead an equivalent volume of sample and sterile chlorine free distilled water was added. Active chlorine present in sodium hypochlorite is 46.15 g/L. The applied sodium hypochlorite concentrations in the tubes were varied from 2 to 8 mg/L. After time ($t$) = 0, 30, 60, 90 and 120 minutes at 30 °C, 50 μl of the sample was plated to rescue surviving bacteria. The plates were incubated at 30 °C for 48 hours, and colonies were enumerated (Ridgway & Olson 1982).

## Biofilm formation on individual plumbing materials under static conditions using Calcofluor assay

### Biofilm production on different materials

A 10× stock solution of Calcofluor fluorescent brightener (disodium-4,4′-bis((4,6-dianilino-1,3,5-triazin-yl)amino)stilbene-2,2′-disulphonate) (Sigma) was prepared at a final concentration of 80 mg/l. Whole plumbing materials ($1 \times 1$ cm$^2$) were removed using sterile forceps and rinsed with 50 ml of PBS to remove unattached cells and unbound dye. A hand-held UV-lamp long wave (Blak-Ray Lamp model UVL- 2 1, UV-366 nm) was used to detect the presence of biofilm on each surface (Lehtola *et al.* 2005). The images were captured using the CCD (Charge-Coupled Device) detector and stored as separate digital files (Gel Doc XR+ System, USA). Fluorescence intensity of Calcofluor stained coupons was observed to detect biofilm formation.

### Effect of chlorine treatment on biofilm formation ability

The effect of chlorine treatment was observed by inoculating a standardized suspension ($8 \times 10^6$ cells/ml) of *Sphingomonas* in two setups: one containing rich medium (Luria Broth) and the other containing filter sterilized water from a municipal distribution system. Sterilized PVC coupons were added. After every 12 hours, three coupons from each setup were taken out and incubated in 8 mg/L sodium hypochlorite solution for 1, 3 and 5 hours, respectively. After staining with Calcofluor, it was visualized under a UV lamp (Blak-Ray Lamp model UVL- 2 1, UV-366 nm). A duplicate of each coupon was placed in fresh medium/water and visualized by Calcofluor staining after 96 hours.

### Effect of chlorine treatment on combinations of *Sphingomonas* with waterborne pathogens

Biofilm production of *Sphingomonas* in combination with waterborne pathogens *Escherichia coli* O157:H7 ATCC 32150, *Shigella flexneri* 2a and *Salmonella typhimurium* ATCC 25315 was performed as described above by crystal violet assay. Chlorine resistance was observed by inoculating the standardized suspension of *Sphingomonas* in combination with pathogens in both medium (Luria Broth) and filter sterilized water. Sterilized PVC coupons were added in both setups and the rest of the experiment was carried out as described above.

## Flow through simulation of biofilm formation on plumbing materials

A laboratory-scale simulated distribution system was fabricated comprising of six different sets of identical drinking water distribution pipes normally used. The pipe materials chosen were polyvinyl chloride (PVC), polypropylene (PP), polyethylene (PE), aluminium (Al), copper (C) and rubber (R). The length of the distribution pipes was one metre, with an internal diameter of 3 cm. Tap water was pumped from the contact tank (30 litres of tap water) with a flow

rate of 1.0 L/min into the simulated drinking water distribution pipe using a peristaltic pump. The water in the tank was inoculated with an overnight culture of *Sphingomonas paucimobilis* to a count of $10^5$ CFU/ml.

### Electron microscopy examination

Biofilm of *Sphingomonas* developed on different pipe materials after 4 days was examined by scanning electron microscopy (SEM).

### Energy dispersive X-ray spectroscopy

The scanning electron microscope (SEM) was equipped with an energy dispersive X-ray spectroscopy (EDX) analyzer. It provided the elemental analysis of biofilm developed on different pipe materials.

### Statistical analysis

Regression coefficient ($R^2$) was applied to obtain the relationship between log count of biofilm cells and the types of pipe materials during different biofilm ages.

## RESULTS

### Molecular identification of strain

The strain isolated from a municipal water distribution system was characterized by 16S rRNA sequencing using universal primers. The phylogenetic tree was constructed using sequences of representative bacteria. The isolated strain was identified as *Sphingomonas paucimobilis* strain MG6 (Figure 1); the sequence of this strain has been deposited in the GenBank database (Accession No. KX594380).

### Biofilm formation kinetics

The crystal violet and MTT assays have been suggested as methods for the estimation of biomass quantity and metabolic activity of the cells, respectively. Figure 2(a) shows that the biofilm formation commences after 24 hours of bacterial growth which is the stationary phase of cells. Maximum biofilm formation was observed after 40 hours. Exopolysaccharides are secreted when the cells reach the stationary phase that facilitates the attachment of cells to the surface (Dunne 2002). With progress of time, increase in exopolysaccharide production further accelerates the


**Figure 1** | Phylogenetic tree of *Sphingomonas paucimobilis* strain MG6.

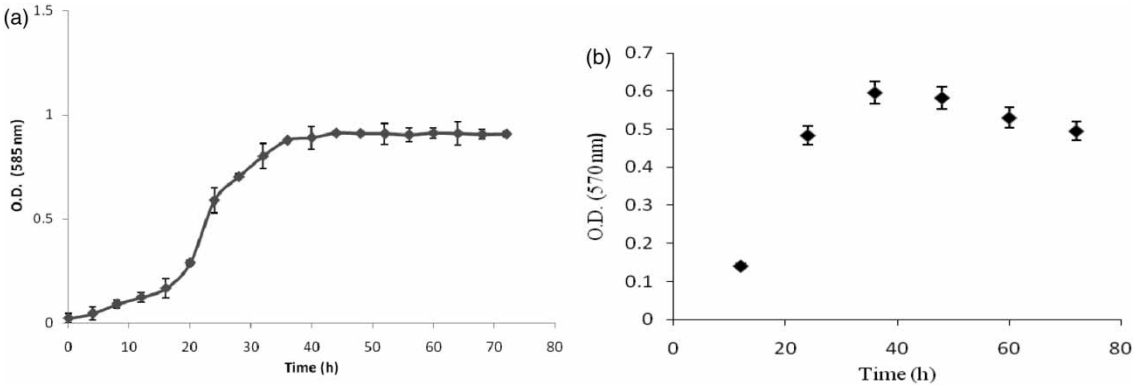


**Figure 2** | Biofilm profile of *S. paucimobilis* MG6 by (a) crystal violet assay and (b) MTT assay.

rate of biofilm formation. For the determination of viability of *Sphingomonas* cells in biofilm and number of adhering bacteria, the MTT assay was performed (Figure 2(b)) which further confirmed the biofilm forming ability of *Sphingomonas*.

## Determination of chlorine resistance

This study was carried out to estimate the effect of residual chlorine on the growth of *Sphingomonas.* It was found that the dosage of sodium hypochlorite had a profound effect on the reduction of log CFU/ml for *Sphingomonas*. Four different concentrations of sodium hypochlorite, i.e. 2, 4, 6 and 8 mg/L were used. *Sphingomonas* was found to be chlorine resistant at the concentration of 2 mg/L at a contact time of 90 minutes for different microbial loads (Figure 3).

## Calcofluor assay

### Biofilm production on different materials

Different plumbing materials like PVC, PP, PE, Al, R and Cu were used to develop biofilms. Results were recorded by visualizing the biofilm under a UV lamp after equal intervals of 12 hours till 72 hours with each material. The Calcofluor assay revealed the biofilm formation on PVC, PP, PE and Al pipe materials; however, variation was observed in intensity (Figure 4). Also, an increase in intensity was observed as time increased. The intensity was less in the case of copper and rubber suggesting the possibility of less biofilm formation on these materials.

### Effect of chlorine treatment on biofilm formation ability

PVC coupons were removed from filter sterilized surface water/medium at different intervals of time and incubated

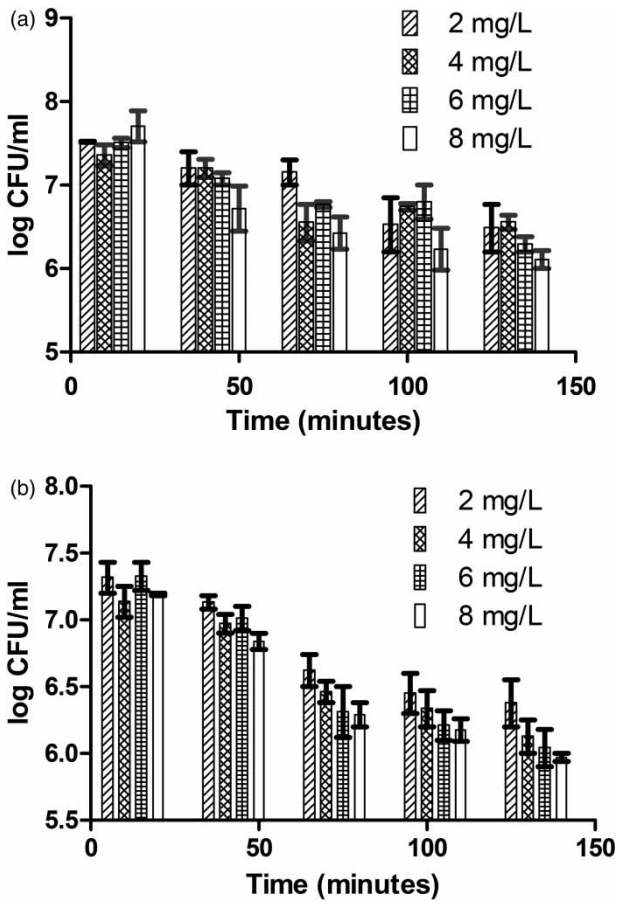


**Figure 3** | The log CFU/ml of *Sphingomonas paucimobilis* MG6 at different concentrations of sodium hypochlorite of (a) $4 \times 10^6$ cells/ml, (b) $8 \times 10^6$ cells/ml.

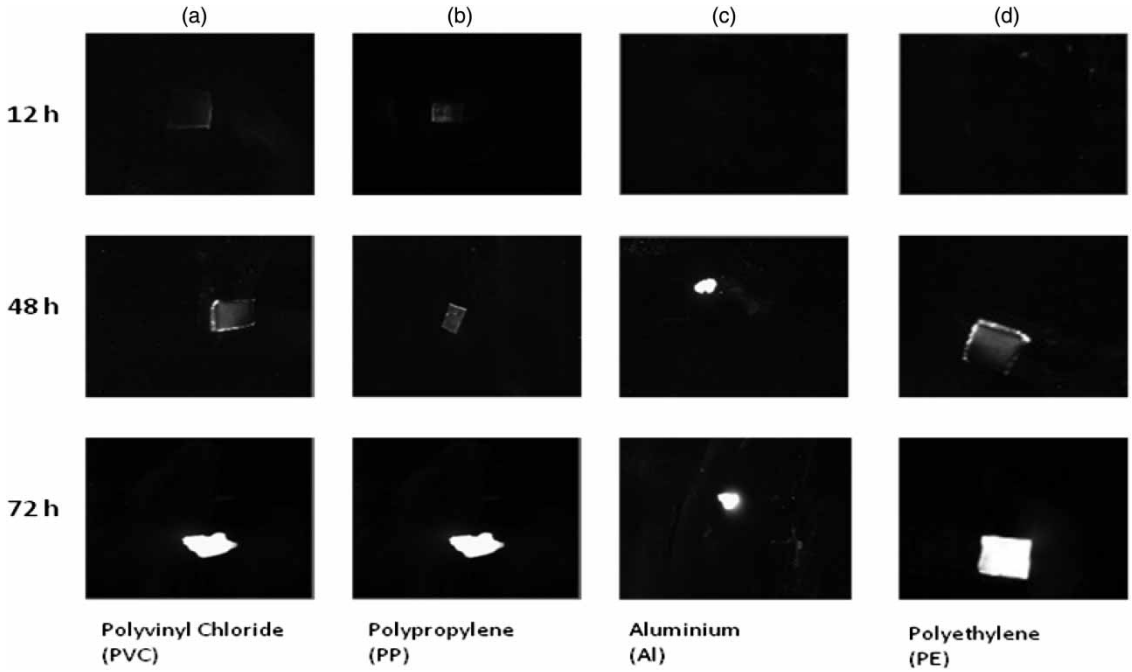


**Figure 4** | Calcofluor assay performed by *Sphingomonas paucimobilis* MG6 carried out on (a) polypropylene, (b) polyvinyl chloride, (c) aluminium, and (d) polyethylene coupons.

for 1, 3 and 5 hours, respectively. The intensity recorded varied with time; as depicted in Figure 5, the intensity increased as the time of biofilm development increased. With the increase of the time span of chlorine treatment, little or no decrease in intensity of biofilm development was observed either with medium or with water. Replicates of coupons were placed in fresh medium/water after each interval till 96 hours. After visualization, it was observed that a slight increase in intensity occurred, which marks the biofilm development following chlorine treatment, confirming the chlorine resistance of *Sphingomonas*.

### Effect of chlorine treatment on *Sphingomonas* in combination with waterborne pathogens

The detection of various waterborne pathogens from drinking water systems prompted us to study the biofilm production ability of *Sphingomonas* in the presence of three pathogens (Figure 6(a)). Pathogens were found to increase the biofilm production ability of *Sphingomonas; Shigella* being the highest followed by *Salmonella* and *E. coli*, making it possible to observe the effect of chlorination on *Sphingomonas* in combination with waterborne pathogens.

PVC coupons were removed, incubated for 1, 3 and 5 hours and the effect of chlorine treatment was visualized by Calcofluor staining. It was observed that *Sphingomonas* with *Shigella flexneri* 2a showed maximum resistance to chlorine as the biofilm formation is maximum in this case followed by *Salmonella typhimurium* and *Escherichia coli* O157:H7. The results can be visualized by observing the variation in the intensity of stained Calcofluor (Figure 6).

### Flow through simulation of biofilm formation on plumbing materials

These results were corroborated by observations from the flow through simulation experiment. Viable cells of *Sphingomonas* were recorded from different scrapings of plumbing materials at different ages (Table 1). Variation was observed between different plumbing materials with log count of biofilm cells on polyvinyl chloride being the highest followed by polypropylene and polyethylene.

### Scanning electron microscopy micrographs

Biofilm formation of *Sphingomonas* on different pipe materials is explained using scanning electron micrographs.

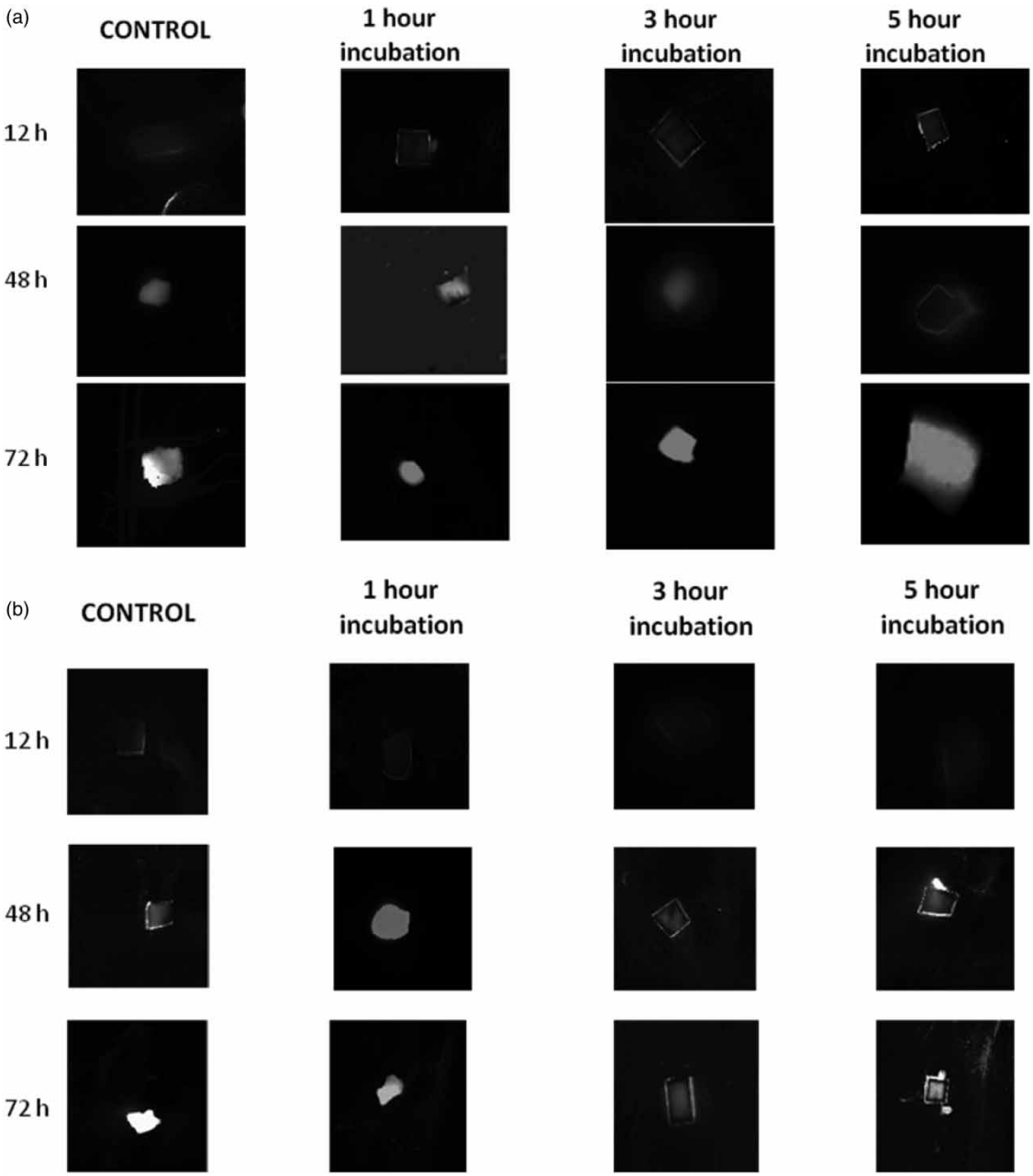


**Figure 5** | Effect of chlorine treatment on biofilm development on PVC using (a) water and (b) medium.

Figure 7 shows that a fully matured biofilm was formed at 72 hours. A clear indication of the cells embedded in a polymer matrix and exopolysaccharides could be obtained. *Sphingomonas paucimobilis* growing on copper and rubber pipes produced a smaller amount of exopolysaccharides than on the other types of pipes, hence the low biofilm formation.

Elemental composition of biofilm formed on different pipe materials as determined by EDX is shown in Table 2. It represented the weight percentage of different elements found at 12, 48 and 72 hours on different pipe materials. EDX is more useful when our objective is to compare the composition of biofilm formed on different materials. It was observed that carbon and oxygen are the dominant

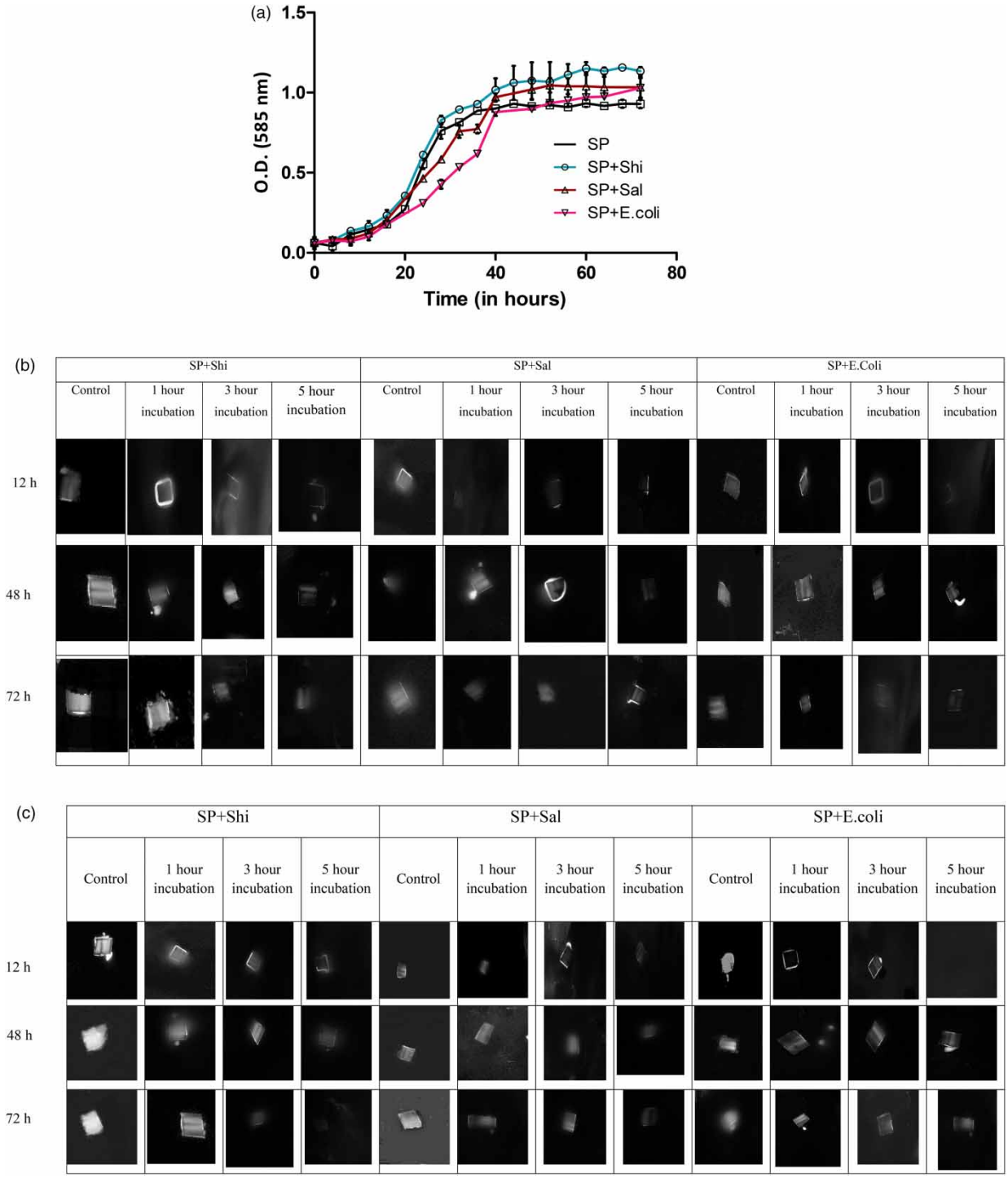


**Figure 6** | (a) Biofilm production of *Sphingomonas* in combination with waterborne pathogens, (b) effect of chlorine treatment on PVC in medium, and (c) effect of chlorine treatment on PVC in water.

elements in biofilm formed on all the plumbing materials followed by calcium and chloride. Carbon and oxygen are interdependent and inversely related to each other as increase in carbon content is followed by consumption of oxygen by cells in PVC as cells are more metabolically active in this case. The inverse is the case with PE. Little

**Table 1** | The relationship between log counts of biofilm cells which formed on different pipe materials at different ages

| | $R^2$ | | |
|---|---|---|---|
| | Biofilm ages (hours) | | |
| **Tested pipe materials** | **24** | **48** | **96** |
| PVC | 92.7 | 92.6 | 94.9 |
| PP | 88.1 | 84.2 | 91.7 |
| PE | 84.7 | 84.6 | 73.3 |
| Al | 74.5 | 79.0 | 85.9 |
| Cu | 75.8 | 74.4 | 84.1 |
| R | 74.1 | 73.2 | 72.3 |

variation is observed in PP and Al. Weaker signals from magnesium, potassium, chromium and iron were also obtained. EDX allowed better knowledge of observed structures.

## DISCUSSION

The World Health Organization (WHO) estimated that about 1.2 billion ($10^9$) people lack access to safe drinking water and 3.4 million people die of water-related diseases every year (WHO 2002). One important factor responsible

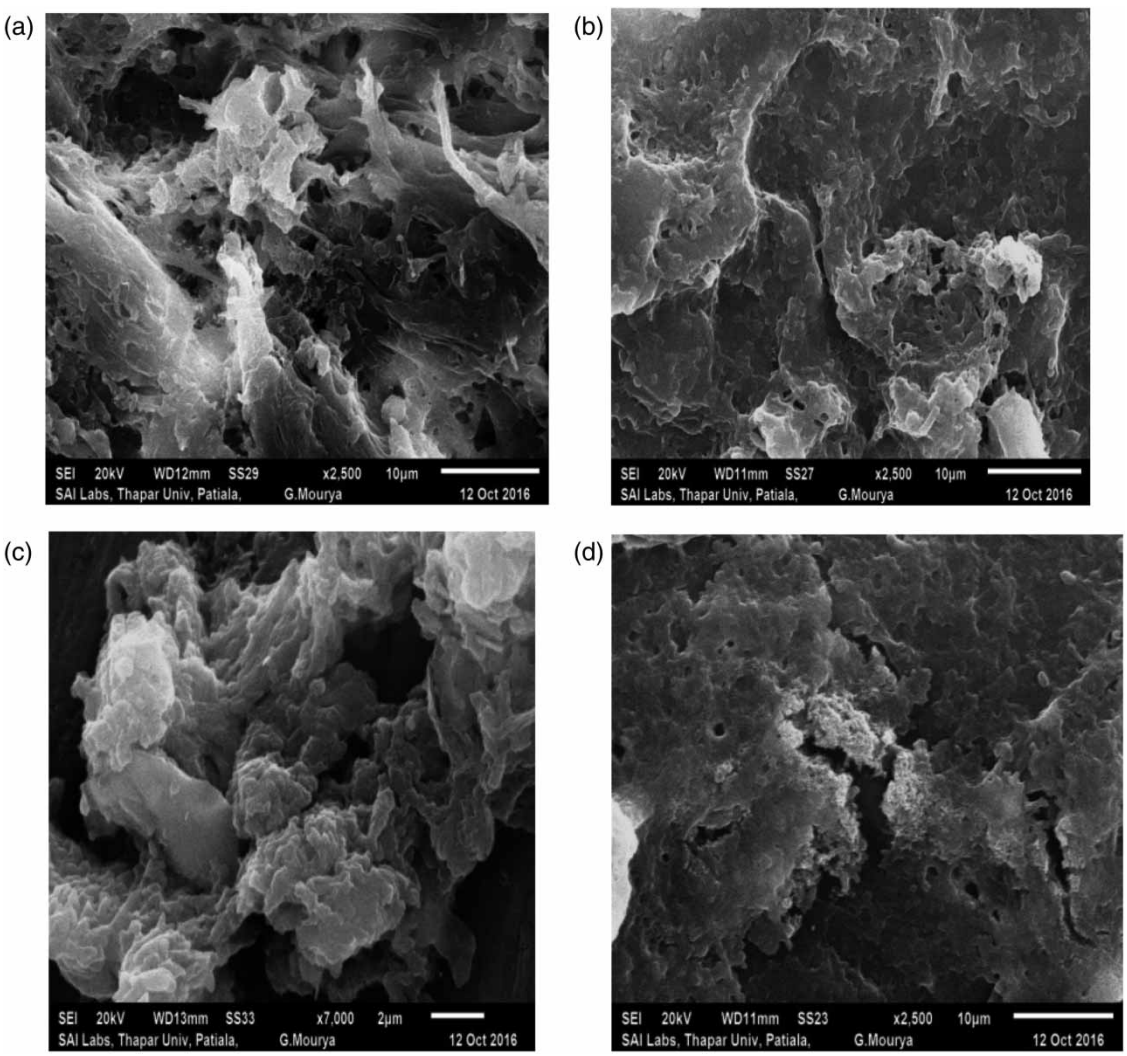


**Figure 7** | Scanning electron micrographs at different time intervals on (a) PVC, (b) PP, (c) Al, and (d) PE.

**Table 2** | EDX analysis of biofilm elemental composition (weight percentage) at different time intervals

| | PVC | | PP | | Al | | PE | |
|---|---|---|---|---|---|---|---|---|
| **Elements** | **12 hours** | **72 hours** | **12 hours** | **72 hours** | **12 hours** | **72 hours** | **12 hours** | **72 hours** |
| Carbon | 24.86 | 68.12 | 90.87 | 82.11 | 90.26 | 89.26 | 77.56 | 58.69 |
| Oxygen | 38.07 | 5.17 | 8.91 | 16.82 | 7.00 | 9.91 | 6.95 | 32.34 |
| Magnesium | 0.04 | 0.20 | 0.11 | – | 0.49 | 0.19 | 0.10 | 0.19 |
| Chloride | 12.46 | 22.62 | – | 0.62 | – | – | 14.71 | 1.61 |
| Potassium | 0.01 | – | – | 0.07 | – | 0.03 | – | 0.37 |
| Calcium | 24.51 | 3.53 | 0.03 | 0.13 | 0.31 | – | 0.57 | 4.20 |
| Chromium | 0.05 | 0.29 | – | – | – | 0.07 | – | 0.26 |
| Iron | – | – | 0.09 | 0.10 | – | 0.17 | – | – |
| Sodium | – | 0.08 | – | 0.15 | – | 0.07 | 0.11 | 0.57 |
| Silicon | – | – | – | – | – | – | – | – |
| Phosphorus | – | – | – | – | 1.94 | 0.30 | – | 1.78 |
| Total | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

for sustenance of water-related disease is the treatment process in distribution systems which fail to comply with the appropriate treatment and disinfection specifications. If resistant pathogens survive conventional disinfection methods, these disinfection methods do not comply with appropriate specifications. Bacteria isolated from different environments with resistant phenotypes to various disinfection methods have been reported (Wojcicka *et al.* 2007). *Sphingomonadaceae* are common inhabitants of drinking water worldwide (Momba *et al.* 2000; Flemming *et al.* 2002; Stopforth *et al.* 2002) and have a remarkable capacity to cope with stress conditions and to adapt to new habitats (Besner *et al.* 2002; Flemming *et al.* 2002; Bachmann & Edyvean 2006; WHO 2011). A body of literature has explicitly indicated the risks of *Sphingomonas* contaminated drinking water systems as persistent sources of infection through biofilm formation. Health risks associated with *S. paucimobilis* include primary bacteremia, intravascular catheter infections, urinary tract infections, biliary tract infection, cutaneous infection (Peel *et al.* 1979), ventilator-associated pneumonia (Ensminger *et al.* 2006), myositis (Pegoraro *et al.* 2008), diarrheal disease (Cheong *et al.* 2008), nosocomial urinary tract infection from contaminated water bottles (Crane *et al.* 1981), bacteremia in dental unit water lines and tap water (Kanamori *et al.* 2016), bacteremia in hospital water systems (Perola *et al.* 2002), etc. Also, bacteremia, endophthalmitis, osteomyelitis and arthritis were associated with hospital drinking water distribution system in Hungary (Felföldi *et al.* 2010). Both staining using chemical probes and SEM indicated that the biofilm cells growing in different pipe materials were embedded in a polymer matrix and exopolysaccharides. Biofilm formation has been shown to be affected by several factors, including disinfectant effectiveness, the nature and concentration of biodegradable compounds in the water, pipe materials used for distribution system construction and water temperature. Proper management of these factors through adequate source water treatment, appropriate materials selection, maintenance of a clean distribution system, and minimization of water age are all important for biofilm control. However, few studies have emphasized the biofilm forming ability of *Sphingomonas* isolates on commonly used water distribution surfaces. We used the fluorescent probe, Calcofluor, to effectively visualize biofilm in the static cultures. The amount of fluorescence is a function of the affinity of Calcofluor for capsular polysaccharides (CPS) and the quantity of adherent CPS. Thus, a negative or weak result denotes minimal biofilm formation, which is exactly what the probe is intended to detect (Singla *et al.* 2014). The variation in biofilm growth was consistent and could be correlated to that obtained in the flow through simulation system. Piping material plays a very important

role in the maintenance of high water quality because it can supply the bacteria in water with nutrients through the leaching of organic compounds. Generally, maintaining a disinfectant residual is thought to limit the growth of organisms within the distribution system and, depending on the residual concentration, contact time, and the pathogens present, a disinfectant residual may afford some protection against contamination from intrusion (Besner *et al.* 2002). Chlorination is the primary recognized method of disinfection of water distribution systems, however, there may be other resistance populations of bacteria (Ridgway & Olson 1982; Goel & Bouwer 2004). The survival of *Sphingomonas paucimobilis* in water distribution systems might be due to its capability to resist chlorine. Drinking water supplies are not expected to be sterile.

Generally accepted specifications for drinking water make provision for heterotrophic plate counts of 100/ml, and in some cases 500/ml and even more, because most of the organisms concerned do not constitute a meaningful health risk. According to the WHO drinking water standard, 2–3 mg/L chlorine should be added to water in order to attain a satisfactory disinfection and maintain residual concentration (WHO 2008). *Sphingomonas* was found to be chlorine resistant at a concentration of 2 mg/L and a contact time of 90 minutes for different microbial loads. LeChevallier *et al.* (1988) suggested that low concentrations of chlorine are not effective in biofilms which prompted us to use a higher concentration of chlorine (8 mg/L) for studying its effect on biofilm development and *Sphingomonas* was found to be chlorine resistant.

Our observations of lower biofilm production on copper plumbing material, may be attributed to the release of copper residuals that have antibacterial properties, expressed by damage to cell membranes and nucleic acid structure (Bruins *et al.* 2000). Copper is the most popular modern plumbing material (Moritz *et al.* 2010) because of its low cost and the ease with which it can be machined into a wide variety of pipes and fittings. Moreover, Lehtola *et al.* (2004) reported that the formation of biofilm was slower in copper pipes than in PE pipes, and that copper ions led to lower microbial numbers in water; while plastic pipes such as PE, which have recently been used as cost-effective replacements of traditional metal plumbing, may release biodegradable organic compounds and phosphorus, which can promote microbial regrowth and biofilm formation.

Overall, our study provides valuable insights on biofilm formation by *Sphingomonas* in commonly used plumbing materials; the view that chlorine alone can be used as an effective disinfectant needs to be seriously reconsidered due to reasons stated. Alternative methods of disinfection such as application of quorum quenchers or ozone or combinational treatment may prove useful for containing *Sphingomonas* growth and biofilm formation.

## CONCLUSION

The present study demonstrated the ability of a *Sphingomonas* isolate from a municipal water distribution system to form biofilms on six different plumbing materials commonly used. Biofilm formation occurred both under static as well as circulatory conditions and could be visualized using the fluorescent chemical probe, Calcofluor, and validated by electron photomicrographs. Viable counts concurrently obtained from pipe materials agreed with these observations. Chlorine resistance of the strain raises concern and suggests an urgent need to develop effective disinfection strategies.

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## CONFLICT OF INTEREST

None.

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