

## Laboratory Model for Formation of Heterogeneous Biofilms on Galvanized Iron and Survival of *A. hydrophila*

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doi: <http://dx.doi.org/10.13005/bbra/1551>

(Received: 06 August 2014; accepted: 31 October 2014)

Biofilms are composite highly hydrated structures of microbial cells that have implications in mass and energy transfer in aquatic ecosystems. Entry of potential pathogens and its concomitant shedding from the biofilm consortia formed on pipeline materials pose a grave threat to the quality of drinking water obtained by a community. A lab scale model biofilm consisting of heterotrophic micro flora from Mumbai water supply was developed on galvanized iron pipe pieces and entry of an emerging pathogen *A. hydrophila* isolate B8 studied using viable count techniques. Scanning Electron Microscopy and FT-IR spectroscopy was undertaken to assess the formation of rudimentary glycocalyx which enable the bacterial cells to adhere on to substratum while confocal laser scanning microscopy was used to observe the depth of the biofilm attained with progression of time. A stable heterotrophic biofilm from the autochthonous organisms present in the drinking waters supplied by Bhandup Municipal treatment plant to Mumbai city was attained within 16 days and *A. hydrophila* was able to enter the biofilm to become an integral part of the community within 2 days indicating that presence of pathogens in supplied drinking waters may not just be due to failure of water treatment plants but through preexisting biofilms on pipeline materials

**Key words:** Biofilms, *A. hydrophila*, Confocal Scanning Laser Microscopy, Scanning Electron Microscopy, Fourier Transform Infrared spectra .

Laid over the past 136 years Mumbai City has a network of water mains spread over a distance of 2400 km. With an average diameter varying from 80 mm to 1800 mm<sup>2</sup> it has progressively got crammed with various utility networks. Leakage through pipes and pipe joints contributes to an increase in the microbial load especially during the non-supply hours due to back flow particularly in areas with a high subsoil water table 2 . Thus bacterial quality of drinking waters

can deteriorate during distribution. This is aggravated by the ability of microorganisms to initiate corrosion through the formation of biofilms. Biofilms are functional consortia of sessile communities of microbial cells organized within extra cellular polymer matrices<sup>18,4,19</sup>. Entry of potential pathogens into such biofilms formed in the distribution pipelines thus pose a grave threat to the water quality obtained by the consumers, even when there is no known breach in the treatment procedures.

*Aeromonas* spp. are recognized as the etiological agent of gastroenteritis and various other extra intestinal diseases<sup>24,22,16</sup>. The World Health Organization has graded it as an emerging pathogen which is ubiquitous in tropical freshwater

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ecosystems (26). Though routine treatments are effective in decreasing the load of this pathogen, survivors do tend to exist<sup>3,16</sup> and their entry into autochthonous biofilms in the distribution pipelines can lead to serious health hazards.

Thus an attempt was made to study the formation of biofilm by the resident flora of drinking water on pipeline materials, and assess the ability of *A. hydrophila* B8 to enter into such preformed biofilm.

## MATERIALS AND METHODS

### Biofilm formation

Since examination of biofilms on the existing water distribution systems is difficult, a laboratory model for the development and observation of biofilms on pieces of pipeline material was developed using Bioreactor Culture Model assembly.

The model consisted of two sterile culture jars used in series and set up in triplicates as shown in figure. 1 The first vessel was used as a reservoir for tap water obtained from the Mumbai distribution systems and kept at a constant volume of 1750 ml. The effluent from this vessel entered into the second main bioreactor vessel containing 25 galvanized iron tiles of 1.3x0.8x0.3 cms. suspended by nichrome wire in 500 ml of tap water. The flow rate was adjusted using control valves such that for every drop of influent entering the second vessel one drop of effluent was collected in a sterile flask. Aeration through an air pump and use of magnetic stirrers enabled to mimic the shearing effects of liquid movement on the biofilm.

### Formation of biofilms in the model

A stable heterotrophic biofilm (as detected by absence of significant variations in the load of organisms) was obtained within 16 days. Entry of *Aeromonas hydrophila* B8 in the water influent tank at a density of  $1 \times 10^3$  to  $3.5 \times 10^3$  cfu/ml was then initiated and maintained throughout the study period of 37 days. *A. hydrophila* B8 was isolated from Mumbai municipal waters and was selected based on its survival ability of 51 days in tap and distilled water, and its high BATH hydrophobic index (7,23) of 0.67 which facilitates good adherence.

### Analysis of the biofilm

Analysis of the tile sample was performed

by removing it aseptically from each set, gently washing them thrice in sterile tap water, scraping with a sterile scalpel to detach the biofilm, and suspending in 5 ml of sterile saline. The samples were vigorously vortexed for 10 min, serially diluted and plated in duplicates on R2A agar (Hi-media) at RT for 48 hrs for heterotrophic counts and on Ampicillin Dextrin Agar (Hi-media) at RT (30-32°C) for 24 hrs for *A. hydrophila* counts. At the same time influent and effluent waters samples were collected and used for analysis of their heterotrophic and *A. hydrophila* populations

The biofilms developed after 20 days were also studied nondestructively under

1. Scanning electron microscopy<sup>21</sup> wherein the biofilm tiles were air dried and given an argon gas induced gold shadow coating under vacuum using Hummer V technics machine. The tile were loaded onto SEM Cameca microscope and observed at various magnifications ranging from 543x to 2459x
2. Specular reflection technique of Fourier Transform Infrared Spectroscopy<sup>17</sup> was studied on a specially developed tile of dimensions 1.5x4x0.3 cms. Air dried biofilm tiles collected after 20 days were placed in Bomem-Hartmann and Braun FTIR-MB series and specular reflection spectra analyzed using Win Bomem Easy software<sup>17</sup>. A tile piece wiped clean with carbon tetrachloride to remove all traces of organic matter was used as the blank.

### Biofilm Depth Analysis

The biofilms formed after 3, 5, 18 and 20 days of immersion were scanned under Confocal Laser Scanning Microscope to determine the depth of the biofilm<sup>15</sup>. The saline rinsed tile samples were stained by 0.1 mg/ml ethidium bromide for 2 mins and observed under CLSM BioRad MRC 1024 controlled by an internal operating software Laser Sharp Processing OS/2. The images were viewed using Argon ion laser through a Plan-Apo oil objective lens.

The 50 fields in the samples were scanned in the xy and z plane to note areas of depth variation. Differences due to depth variation per tile were analyzed using unpaired t test and areas with mean depth variation were chosen for photography and depth computation. False coloring using ThermoLUT coloring software to

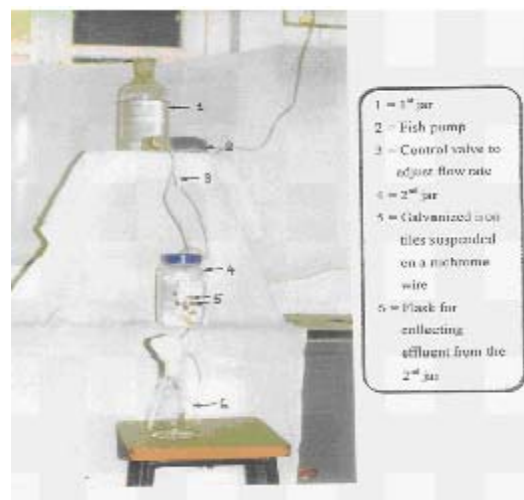
indicate intensity of fluorescence at each point of the biofilm was done, such that black/blue indicates minimum intensity while red denotes the maximum and thus highlight the metabolic status of the biofilm cells

## RESULTS AND DISCUSSION

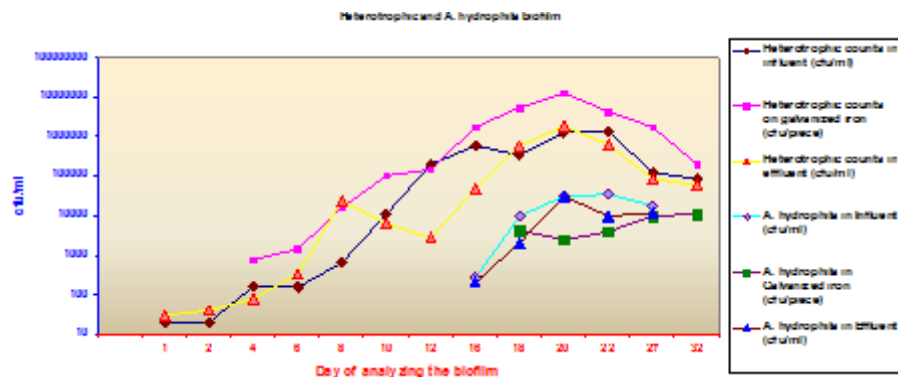
Since the material used in distribution pipeline of Mumbai city is galvanized iron the biofilm formation was studied on this material in a lab simulated model, the results of which are shown in Fig. 2. Initially by the first and second day, though heterotrophic counts ranging between  $1.5 \times 10^3 \pm 0.7$  to  $3.8 \times 10^3 \pm 0.9$  cfu/ml were detected in influent and effluent waters, no biofilm development was observed. But by the 4<sup>th</sup> day, organisms at a low count of  $5 \times 10^2$  cfu/tile were detected on the tile indicating adherence the initial step in the formation of biofilm. The counts continued to increase up to the 16<sup>th</sup> day of set up to a density of  $2 \times 10^6$  cfu/tile, except for a small drop observed on the 10<sup>th</sup> day, which could be due to temporary sloughing of cells. Since steady state autochthonous biofilm was attained by the 16<sup>th</sup> day, entry of *A. hydrophila* B8 was initiated. By the 18<sup>th</sup> day, presence of *A. hydrophila* B8 was detected at a density of  $420 \pm 26$  cfu/tile which jumped to  $5 \pm 0.8 \times 10^3$  cfu/ml within 2 days. The counts of *A. hydrophila* B8 continued to rise attaining a density of  $1.3 \times 10^5$  cfu/tile at the end of the 37 day study period.

Yu and McFeters<sup>27</sup> studied the response of a drinking water isolate *Klebsiella Kp1* to disinfectants by growing it as biofilms on stainless steel in a reactor made of wide mouth pint jars. In

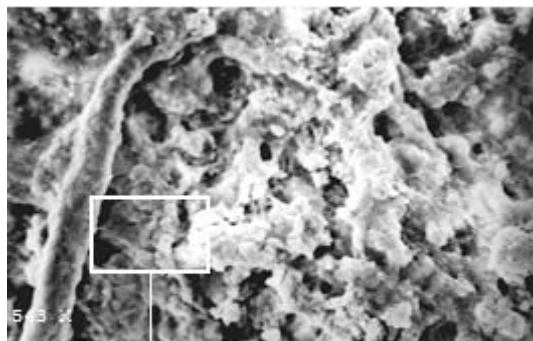
our study also similar jars were used but biofilms were cultivated on galvanized iron tiles. Mackerness *et al.*,<sup>18</sup> studied the entry of *E. coli* into a lab model biofilm of heterogeneous organisms from drinking water supplies of London on glass and bitumen painted mild steel tiles and found that it could enter into the biofilm in low numbers. Rate of bacterial adhesion is responsive to physical characteristics like hydrophobicity and presence of divalent ions<sup>9,11</sup>. Thus surface topography and chemical structure may influence the way cells interact to form biofilms. Presence of divalent ions like magnesium and calcium were routinely found within the range of 25 to 65 ppm during the months of high incidence of planktonic *A. hydrophila* counts in the distributed waters of Mumbai city (data not shown) and this could be one of the



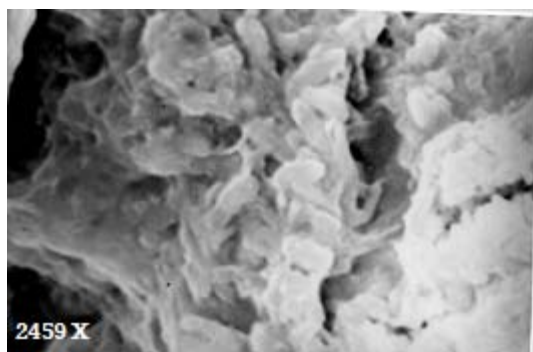
**Fig. 1.** Culture model assembly for development of biofilm on galvanized iron tile



**Fig. 2.** Heterotrophic Biofilm formation and entry of *A. hydrophila*



**Fig. 3.** SEM Images of 18 day old biofilm

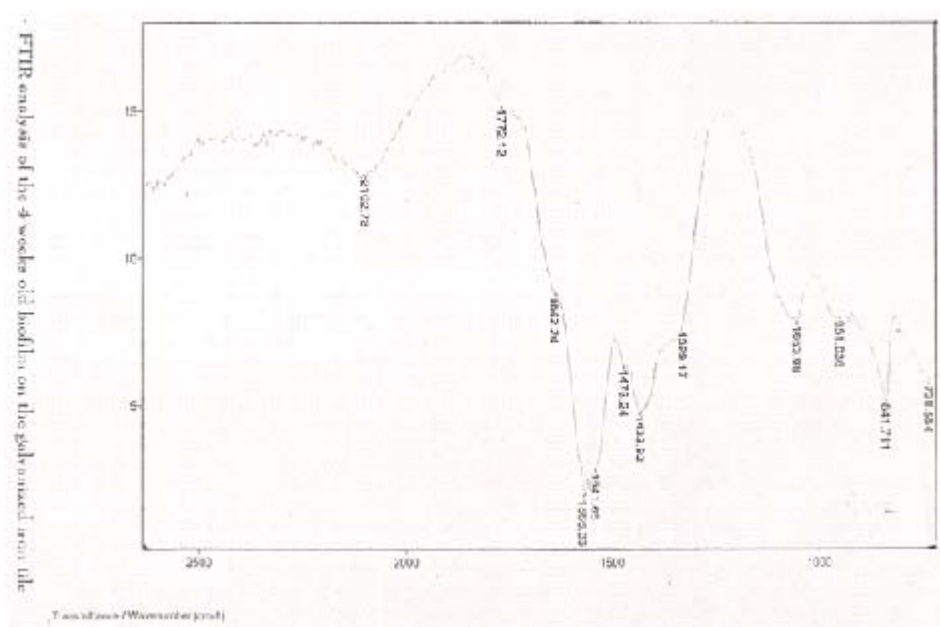


**Fig. 4.** Magnified portion of the above image showing biofilm architecture

reasons that can facilitate nonspecific attachment and support the entry of this species into the biofilm consortia.

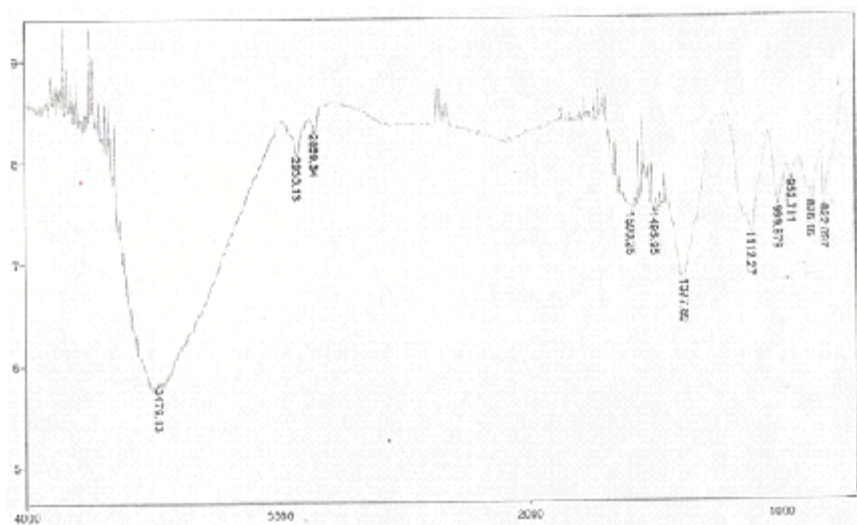
SEM was performed to obtain a photographic evidence of the formation of biofilm and observe the surface topography of the adhering bacteria without undue introduction of artifacts. Fig 3 shows dense clumps of bacterial colonies with many water channels going into the biofilm at magnification of 543X while a magnified (2459x) portion of the same image shows a number of bacterial cells in an exopolymer matrix (Fig4).

FTIR analysis of the biofilm samples through ATR technique are commonly used for the study of biofilms. Since in our study the sample tile material used neither contained zinc selenide nor germanium crystals specular reflection technique instead of ATR wherein radiation reflecting off the samples front surface was studied. Its spectra revealed the presence of peaks at an average wavelengths of 1642.24 /cm (amide I), 1566.33/cm and 1541.65/cm (amide II) and 1053.98 (C-O-C group of polysaccharide) after 64 scans (Fig 5). Such peaks are normally attributed to presence of nitrogen contained in organic matters of biological origin which were absent in that obtained of the blank tile (Fig 6) suggesting that it is the presence of the organisms and its glycocalyx

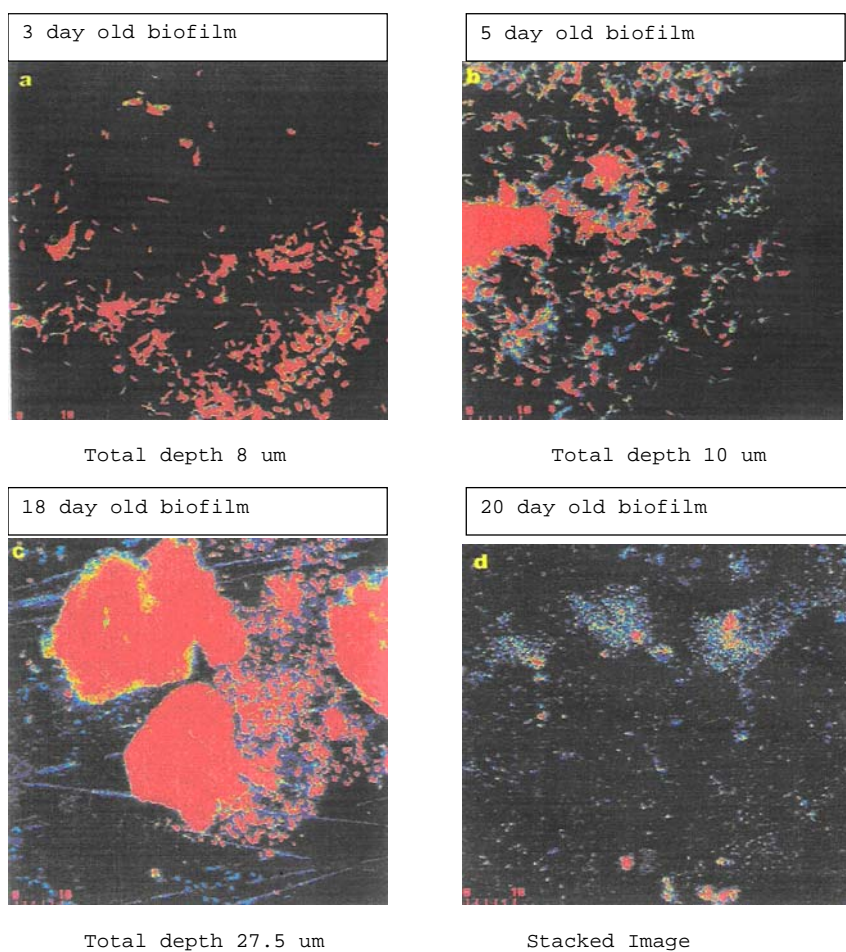


**Fig. 5.** Specular reflection FTIR analysis of a 4 week old biofilm formed on galvanized tile





**Fig. 6.** Specular reflection FTIR analysis of a blank tile without biofilm formation



**Fig. 7.** Confocal Laser Scanning microscopy Images of biofilm

and not the tile material that is responsible for such a FTIR spectra.

Davies et al (5) studied the FTIR spectra of the glycocalyx associated with the biofilm cells collecting a spectra over the range of 700 to 2000  $\text{cm}^{-1}$  and observed that the absorbance peak centered around 1050  $\text{cm}^{-1}$  for complex ring sugars and 1534  $\text{cm}^{-1}$  for amide II. ATR was also used by Bremer and Geesey (1) to study the protein components of the biofilm in which peaks of amide I (1638/ $\text{cm}$ ), amide II (1578 and 1536 / $\text{cm}$ ) were obtained. Geesey et al (10) during his deterioration studies of copper wire caused by bacterial cells found appearance of biofilms to be associated with changes in the absorption bands at 1640 / $\text{cm}$

Formation of such rudimentary glycocalyx as envisaged by SEM and FTIR spectra would enable bacterial cells to attach areas of pipe and still withstand moderate shear forces resulting from hydrodynamic turbulence within the system. The exopolymer matrix may also act in various degrees as a diffusion barrier, microbial sieve and absorbent (15) protecting the microbes from predation and desiccation<sup>12,13,20</sup>.

CLSM images of biofilms of different ages were obtained to study its metabolic status and the depth attained with increase in age. Fig 7(a) shows a 3 day old biofilm formed by the autochthonous flora of water. The cells have just adhered onto the tile. This biofilm had a depth of 8  $\mu\text{m}$  at its maximum aggregation loci. Fig 7(b) reveals a 5 day old biofilm at a depth of 10  $\mu\text{m}$ , wherein the organisms have started to form micro colonies and the cell size has slightly reduced. The small increase in size at this stage might be due to compaction of the biofilm structure. Fig 7(c) represents a 18 day old biofilm of 27  $\mu\text{m}$  depth. The figure shows three actively growing micro colonies (high intensity of red color) representing a well matured and metabolically active community. Since this was the time when *A. hydrophila* counts could also be detected, its metabolic status indicated that *Aeromonas* was well integrated and adapted to the phenotypic variations required to get adjusted to the sessile form of growth required for survival in nutrient limiting environments. Fig 7(d) represents a 20 day old well compacted biofilm attained at a depth of 70  $\mu\text{m}$ , where the individual cell size is highly reduced.

De Beer et al.,<sup>6</sup> found that CLSM revealed

their biofilm to have a heterogeneous structure consisting of cell clusters separated by channels which Stoodley et al.,<sup>25</sup> hypothesized might be liquid flow inside the biofilm. Lawrence et al.,<sup>14</sup> observed *Pseudomonas* biofilms to be more tightly packed at their attachment surfaces with 9 to 27% cellular material at this site and become increasingly diffuse near their outer regions, whereas *Vibrio* biofilms exhibited the opposite trend. In our study, biofilm micro colonies were found to be at maximum intensity in the center similar to that observed with *Vibrio* biofilms with the depth increasing by the end of the two week study period to 70  $\mu\text{m}$ .

WHO and US Environmental Protection Agency are considering imposing limits on the occurrence of *Aeromonas* detected in drinking waters<sup>8</sup>. Limits for the number of *Aeromonas* spp in drinking waters of India thus needs to be evaluated based on the inherent incidence of these organisms in raw waters and their survival strategies which allow them not just to enter but establish themselves in sessile states in preexisting heterotrophic biofilms of the distribution pipelines.

## ACKNOWLEDGEMENTS

The authors thanks Dr N. Naik of ACTREC, Mumbai for performing confocal laser scanning microscopy of the biofilms. Our thanks are also due to Dr V. Charu of HUL, Mumbai for performing and analyzing Specular reflection technique of Fourier Transform Infrared Spectroscopy.

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