Paenibacillus Naphthalenovorans Biofilms Interact with and Degrade Naphthalene as Observed using Various Microscopy Techniques

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Abstract: The role of *Paenibacillus naphthalenovorans* biofilm formation in degrading a model polycyclic aromatic hydrocarbon (PAH) (naphthalene) was investigated *via* an array of microscopy techniques. The early stages of biofilm formation near a naphthalene crystal that was deposited on a glass coverslip were assayed qualitatively by growing the biofilms in batch bioreactors using either a rich carbon medium or a medium which contained naphthalene as a lone carbon source. Our results demonstrated that the biofilm selectively grew immediately adjacent to the edge of the PAH crystal, leading to a biofilm that facilitated the PAH degradation. Moreover, because PAH compounds are often found in capillary spaces in the subsurface, quantitative capillary-experiments were executed to evaluate the ability of *P. naphthalenovorans* biofilms in the degradation of a capillary-bound PAH contaminant. These capillary-experiments demonstrated that a biofilm forms at the pore's opening, and that, when compared to a diffusion process in a liquid medium, this biofilm substantially increased the rate at which the PAH is cleared from the pore. These results provide an enhanced understanding of the means of biofilm adhesion and development in a presence of the model PAH compound investigated. Moreover, the work presented here demonstrates approaches not used before for monitoring biofilm formation.

Keywords: AFM, Biodegradation, Biofilm, Fluorescence microscope, Naphthalene, Optical microscope, Polycyclic aromatic hydrocarbon (PAH), Scanning electron microscope (SEM), Transmission electron microscope (TEM).

1. INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs)

Organic molecules that contain two or more aromatic rings are referred to as PAHs. Examples include acenaphthene, phenanthrene, pyrene, fluorine, fluoranthene and naphthalene [1, 2]. PAH compounds can arise in the environment from both natural sources, such as volcanic flare-ups and range fires or from anthropogenic sources, such as oil and coal processing, fuel burning and industrial emissions [18, 19] As persistent environmental pollutants, these molecules have been shown to have carcinogenic and mutagenic impacts on human health [2, 23].

PAH Remediation Methods

Because of the stability associated with aromatic ring structures in the environment, several technologies have been used to treat soil and sediments contaminated with PAH compounds. Some of these technologies are based on incineration of physically removed contaminated soil or sediments [2, 26, 36]. Chemical and physical treatment methods are often expensive and reserved for large sites mainly due to their large capital costs [25]. Moreover, chemical treatments may necessitate the use of hazardous chemicals, which significantly limits the use of such methods. For smaller sites or treatment of materials that cannot be inexpensively extracted, chemical or biological treatments are generally employed [22].

Biological Treatment of PAH

Because of the limitations of chemical treatments, biological treatment, which may require longer treatment times, may be the preferred option for many sites [18, 20-24, 26]. Biological methods are based on the transformation of PAH compounds to nonhazardous forms through the use of biological materials (plants or microorganisms) [3, 13, 18-27, 33, 41-43]. Bioremediation offers a safe and lucrative option for treatment. The rate of the pollutants' bioremediation relies on environmental conditions, types and quantities of the microorganisms, and chemical structure and nature of compounds to be degraded [1].

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Many bacterial species are known for their abilities to degrade PAHs [18-26]. Most of these bacteria are isolated from contaminated soil or water. Previous studies have identified pure and mixed bacterial cultures which can transform PAHs, especially naphthalene and phenanthrene, to less hazardous chemical species [18]. Microbial strains with ability to metabolize PAH with different degradation rates have been also isolated [18]. For example, Kelley, *et al*. [24] showed that *Mycobacterium spp*. collected from oil polluted areas degraded 55% of naphthalene up to carbon dioxide in a week. They showed that naphthalene was predominantly transformed to cis- and trans-1,2-dihydroxy-1,2-dihydronaphthalene with the ratio 25:1 by dioxygenase and monooxygenase, correspondingly [24]. In another study [11], Dua and Meera used naphthalene oxygenase purified from *Corynebacterium renale* grown on naphthalene to review the degradation process. This isolated enzyme created cis-1,2-dihydronaphthalene as the predominate biodegradable metabolite. Several studies revealed the degradation of PAH to carbon and energy by different microorganisms that belong to different genera including *Marinobacter, Cycloclasticus*, *Pseudoalteromonas* and *Neptunomonas*, *Sphingomonas, Pseudomonas, Burkholderia a*nd *Mycobacterium* [12-21]. Others [6, 39] showed benzo(a)pyrene degradation by fungi species: *Saccharomyces cerevisiae, Neurospora crassa, Cunninghamella bainieri* with further production of trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene, 3-hydroxybenzo(a)pyrene and 9-hydroxybenzo(a)pyrene.

Kinetics of Biofilm Formation

Kinetics of biofilm formations follows four main stages. These are the reversible or initial attachment stage, irreversible attachment stage, biofilm maturation and finally, the partial dispersal of a biofilm [32]. The rate of biofilm's kinetics depends on different factors including environmental factors such as the concentration and composition of pollutants in the environment, the pH, salinity and temperature of the environment [35]; the properties of the surface to which bacterial cells attach to such as roughness, stiffness, hydrophobicity, and porosity [40]; and the properties of bacteria forming the biofilms such as motility, surface charge and makeup of surface biopolymers [10]. With all the above factors, the kinetics of biofilm formation may vary significantly depending on the overall conditions under which the biofilms are forming. For example, Eriksson *et al*. showed that biofilms of contaminated Arctic soil formed on pyrene crystals in 3 months during incubation under 22°C. In comparison, *Pseudomonas spp*. formed biofilms on glass surfaces placed into medium that contains crude oil in 48 hours [9]. Therefore, the rate at which biofilms form or degrade is expected to largely depend on all factors involved in the biofilm formation process and should be quantified for each customized bioremediation experiment.

Biofilms in Bioremediation

Bacteria that accomplish bioremediation are often found to form biofilms. Consequently, studies of the role of biofilms in the degradation of PAH compounds are needed. Previous studies, which focused on suspension cultures lack data needed to quantify the relationship between PAH degradation and biofilm formation or the role that these biofilms play in the PAH degradation process.

Several previous studies have reported the role of bacterial biofilms in the degradation of PAH compounds [29, 31]. For example, Rodrigues, *et al*. [29] evaluated bacterial adhesion to PAH-crystals which served as lone carbon and energy sources for the biofilms. In their study, the colonization of the gfp-labeled derivative strain of *Pseudomonas putida* ATCC 17514 was monitored on fluorene and phenanthrene crystals *via* confocal laser scanning microscopy. As a result, it was shown that *P. putida* grew on phenanthrene, forming a biofilm on the accessible crystalline surfaces. In comparison, biofilm formation on the more soluble fluorine was insignificant. Their results highlighted the impact of substrate type on biofilm formation, showing the difficulties of biofilm formation on soluble substrates.

In another study, Wick, *et al*. [38] showed biofilm formation on anthracene crystals by the anthracene-degrading *Mycobacterium* strain LB501T. Their findings pointed to that bacterial cells favored growth on etched craters in the anthracene crystals due to the ease of consuming PAH-dissolved materials [38]. However, this study lacked microscopy analyses needed to demonstrate the interactions between the PAH crystal and the biofilm.

In the work reported here, we investigate both qualitatively and quantitatively the abilities of biofilms to degrade naphthalene. We hypothesized that initially the biofilm forms near the edge of the naphthalene crystal where the concentration of the PAH on the surface is the highest. As the biofilm grows near the crystal, it facilitates the biodegradation of naphthalene.

Moreover, we further hypothesized that when the naphthalene is contained in a pore, the biofilm selectively forms at the mouth of the pore, thus enhancing the degradation of the pore-located naphthalene. To test our hypotheses, we utilized an array of light, scanning, fluorescent, and atomic force microscopy techniques to assess the ability of bacteria to grow biofilms and to biodegrade naphthalene. The knowledge obtained from these results will be important to enhance the fundamental understanding of how to control naphthalene biodegradation for bioremediation of PAH in the environment.

2. MATERIAL AND METHODS

Bacterial Cultures

Paenibacillus naphthalenovorans are Gram-positive, rod-shaped, and motile bacteria by peritrichous flagella. The bacterial culture was bought from the American Type Culture Collection (ATCC BAA-206, Manassas, VA) [7]. These bacteria form white, glowing, mucoid colonies during growth on tryptic soy agar (TSA) and are strictly aerobic [7, 8]. For the study of the properties of bacterial colonies, the microorganisms were grown on tryptic soy agar (TSA) for 72 hours. Then, a bacterial inoculum was transferred into a 250 ml flask that contained one of two different growth media. To enable effective comparisons, the first medium used was a tryptic soy broth (TSB) as a carbon-rich media [7]. The second medium was a Mineral Salt Basal (MSB) solution, which contained per liter: 40 ml of Na_2HPO_4+KH_2PO_4 buffer (pH 6.8); 20 ml of Hutner's vitamin-free mineral base [7]; and 1.0 g of $(NH_4)_2SO_4$. To this MSB solution, 0.05g/l naphthalene (99% purity, Sigma-Aldrich) was added to serve as a lone source of carbon and energy [14]. For each media, growth curves over a period of two days were obtained for three different cultures grown at 28^{o}C and 150 rpm. During each growth period, the absorbance of the bacterial solution was determined as a function of time using UV/Vis. spectroscopy at 600nm. Our results indicated that the exponential phase of bacterial growth was reached after 17 hours when grown in TSB and after 28 hours when grown in the naphthalene enriched MSB growth medium, respectively. At the end of each growth experiment, five tubes that contain 1 ml bacteria each were frozen at –80^{o}C for future experiments. Prior to any new experiment, a new tube with frozen bacterial culture in it was allowed to thaw at room temperature. The bacterial cells were then allowed to grow and similarities between the growth curves of the culture collected and the original growth curves obtained for the purchased culture from ATCC confirmed that the bacterial cells were reserved.

Biofilm Formation in Batch Bioreactors

In this study, naphthalene was chosen as the model PAH to investigate because, of all PAH's, it has the simplest chemical structure. Naphthalene, bicyclo(4.4.0)deca-1,3,5,7,9-pentene, is composed of only two fused benzene rings [18, 20]. At room temperature and under normal atmospheric conditions, this C10H8 hydrocarbon is a crystalline, aromatic, white, and solid [20]. The ability of *P. naphthalenovorans* to form biofilms nearby and far away from naphthalene were tested in batch bioreactors. 0.0144±0.001 mg of naphthalene was melted onto glass coverslips. These coverslips, together with the defined quantity of naphthalene, were then placed in sterilized 6-flat well tissue culture plates (Falcon, Fisher Scientific Co.). Each well was filled with 10 ml of MSB medium that contained 2% of *P. naphthalenovorans* grown till the late exponential phase of growth in MSB as described above. After a predetermined amount of time, which varied from two to seven days, the glass coverslips with biofilms formed on them were washed each with 3-5 drops of sterile MSB medium to remove loosely bound biofilms and was sacrificed and prepared for imaging using optical light microscopy, fluorescence microscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM), and atomic force microscopy (AFM). For each condition tested, at least three glass coverslips were investigated.

Optical Light Microscopy

The structure and the location of biofilms grown in batch bioreactors as described above were investigated *via* 20X magnification using an optical light microscope (Nikon Eclipse Ti-S, camera - DCM130). Samples imaged were the glass coverslips with naphthalene and biofilm growth on them at 3 and 7 days of biofilm growth.

Fluorescence Microscopy

P. naphthalenovorans biofilms were investigated using fluorescence microscopy equipped with micromanipulators (Leica Fluorescence Microscope, Leica DMFSA) [5]. To enhance the quality of the images, a SYTO® 9 green fluorescent nucleic acid stain was applied to the formed biofilm. 5 ml of SYTO® 9 was diluted (1:1,000) with water, vortexed to

homogenize the dye, then incubated to 30 minutes. The adherent biofilm was stained *in situ* on coverslips and then washed by sterile water directly. Several dye concentrations were investigated in order to establish the 0.001 concentration used which did not bleach the biofilm. After that, samples were washed with water, dried and examined by Fluorescent microscopy.

Transmission Electron Microscopy (TEM)

TEM (JEOL TEM, JEM 1200 EX, Tokyo, Japan) was used to investigate the following properties of the bacteria that grew within the biofilm: size, shape and capsule formation (particularly after storage of bacteria in refrigerator and after growth with naphthalene), motility - flagellum availability, and deformation of biofilm after grown in MSB with naphthalene. By examining the various properties of the bacteria as observed by TEM, we were also able to ensure that the medium was not contaminated with other microorganisms. To investigate the aforementioned bacterial properties, bacterial cultures were grown for one day in TSB and in MSB with 0.05 g/L of naphthalene. The electron microscopy grid was held with forceps and the bacterial suspension was applied onto the grid and left for 1 minute, and then drawn off from the edge of the grid with a filter paper to prepare a sample for TEM examination. A drop of negative stain (1% formaldehyde and 0.5% glutaraldehyde) was immediately applied to the slide with the use of a pipette. The extra liquid was drawn off from the edge of the grid with a filter paper again. After that, all samples were examined using TEM. The images were captured by means of a Digital Camera set on a JEOL MegaView III.

Scanning Electron Microscopy (SEM)

SEM (S-570 Hitachi, Ltd., Tokyo, Japan) was used to image the biofilms which formed on the glass coverslips in the batch bioreactors described above. The glass coverslips with biofilms and naphthalene were chemically fixed by glutaraldehyde. Each specimen was then freeze-dried in a Lyphilizer (Lyophilizer - The Virtis Co.) for 24 hours after which it was plunged directly into liquid nitrogen. After that, each specimen was attached to a metallic stub and coated with a thin layer (approximately 20-30 nm) of gold using a sputter coater for approximately 20 minutes (Technics Hummer V (Anatech) [5]. Each coated specimen was then observed using SEM. A Digital Camera (Quartz Imaging Corporation) placed on the Hitachi SEM was used to capture the images.

Atomic Force Microscopy (AFM)

Topographic images of *P. naphthalenovorans* biofilms were acquired in air at room temperature using a PicoForce™ scanning probe microscope with Nanoscope IIIa controller and extender module (Bruker Inc., Santa Barbara, CA) [28]. All images were collected in contact mode using silicon nitride cantilevers with a manufacturer reported spring constant of 0.06 N/m (DNP-S, Veeco Inc., Santa Barbara, CA). Height, deflection, and friction images were simultaneously acquired at a scan rate of 1 Hz, a resolution of 512 pixels per line and 512 lines per image. The acquired AFM images did not undergo any specific image processing except for first order flattening. The dimensions of the bacterial cells in the biofilms imaged were determined *via* image analysis using the cross-sectional feature of offline AFM. For each biofilm imaged, the dimensions of at least 15 cells were quantified. In addition to the dimensions, surface roughness values for the biofilms were calculated from the high-resolution AFM height mode images using the roughness feature in the Nanoscope software [30]. For each biofilm, the average squared mean roughness (R_{rms}) value was calculated by averaging the roughness values obtained for twenty 200 nm^2 areas on approximately 10-20 cells located in each biofilm.

Biodegradation of Naphthalene via Biofilm Formation in Capillaries

To check how biofilms of *P. naphthalenovorans* facilitate the biodegradation of naphthalene, capillaries filled with known masses of naphthalene were placed in MSB solutions with 1% of *P. naphthalenovorans* cultures grown as described above. Prior to filling the capillary with naphthalene, the capillary was sterilized, dried, and weighed. The capillaries (Fisherbrand* Pasteur Pipettes - 9 in. Borosilicate Glass) were filled by melting naphthalene into the capillary until it reached a pre-marked reference on the capillary. The melted naphthalene was allowed to air-dry prior to placing it in the MSB bacterial solution. Control experiments to assess the dissolution of naphthalene in MSB were run in parallel by placing naphthalene-filled capillaries in MSB solutions with or without bacteria. All capillaries and bacterial solution vessels were sealed to prevent naphthalene's evaporation. Experiments were conducted in triplicates. After 10 days, the capillaries were removed from the vessels, washed extensively with 100 µl of distilled water to remove any loose biofilms, dried in air, and weighed. To compare all experiments, the naphthalene weight loss due to biodegradation or due to dissolution was normalized by the weight of naphthalene measured at the beginning of the experiment.

3. RESULTS AND DISCUSSION

Formation of External Polymeric Substances (EPS) Capsule

TEM images of P. naphthalenovorans are shown in Figure **1**.

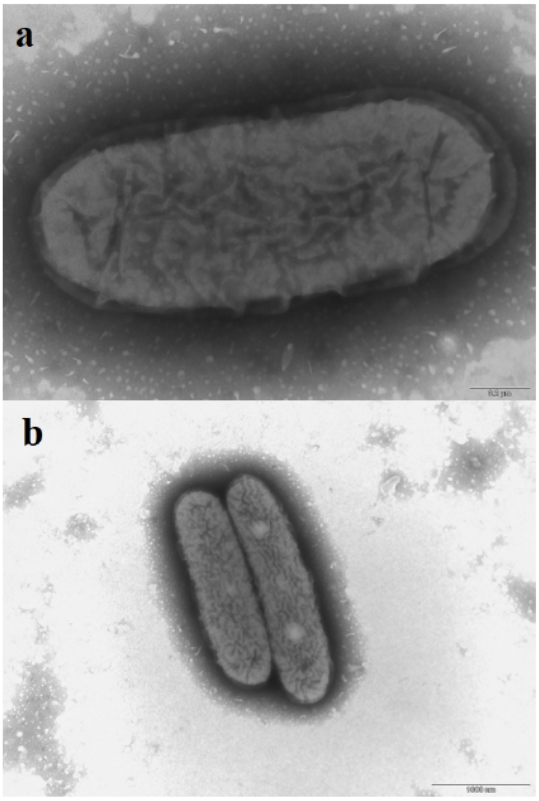


Figure 1: Representative transmission electron micrographs of (**a**) *P. naphthalenovorans* cells (size increased 4.25 thousand times), scale bar 0.2 µm and (**b**) *P. naphthalenovorans* cells in capsule (size increased 2.75 K thousand times), scale bar 1µm. Figures **1** (**a**) and (**b**) were depicted after first cultivation of suspension in TSB to simply study cells' morphologies.

The image in Figure **1a** clearly shows the nuclear materials of the bacterial cells while the image in Figure **1b** clearly shows the external polymeric substances' (EPS) capsule. A previous study by Gutierrez, *et al* [19] showed that bacteria that form biofilms containing significant quantities of EPS have enhanced degradation rates of PAH. According to the best of our knowledge, the TEM micrograph shown in Figure **1b** is the first report that *P. naphthalenovorans* cells have a capsule of EPS (combines both LPS, proteins and eDNA). The presence of a capsule around *P. naphthalenovorans* implies that these cells utilize such capsule to stabilize themselves in various environmental conditions [4]. Such ability to survive in the environment infers that this bacterium can be an ideal candidate to be used in PAH clearing in different environments [32]. The presence of EPS on the bacterial surface can facilitate the bacterial ability to degrade the PAH including the model naphthalene.

Optimal Growth Conditions

To determine the appropriate naphthalene concentration needed to be dissolved in the MSB media to grow *P. naphthalenovorans,* the concentration of naphthalene in the MSB was varied and bacterial growth was monitored. The ability of the bacteria to grow in MSB solutions with five different naphthalene concentrations (0.001, 0.005, 0.006, 0.0065, 0.0075 and 0.01 g/L) was examined by measuring the absorbance of the bacterial solution at 600 nm (Figure **2**).

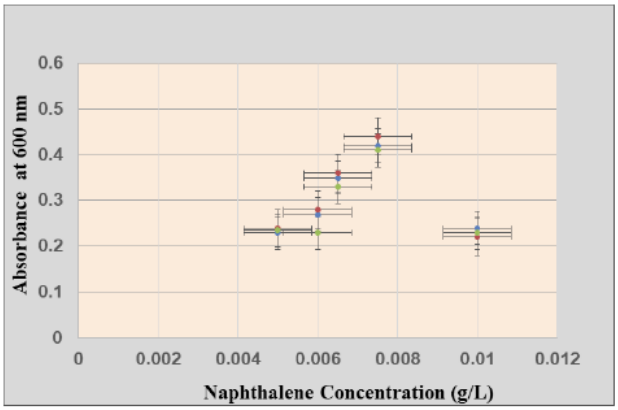


Figure 2: A scatter plot that shows the bacterial concentration in terms of light absorbances measured at 600 nm versus naphthalene concentration in the solution. The different symbols indicate the triplicates performed. Error bars indicate the standard deviation calculated for the triplicate experiment.

Our results indicated that, of the conditions tested, the bacterial growth was the highest when the naphthalene concentration was 0.0075 g/L. Using concentrations higher than 0.0075 g/L was toxic for bacterial growth as indicated by bacterial growth reduction while using lower concentrations was not sufficient to grow bacteria as indicated by reduction of growth or absence of it. Therefore, all the biofilm experiments performed in batch bioreactors were carried out using MSB media with a naphthalene concentration of 0.0075 g/L.

Biodegradation of Naphthalene via Biofilm Formation in Batch Bioreactors

Our first hypothesis states that bacterial cells will form biofilms preferentially near by the PAH contaminate source (naphthalene) compared to far away from the PAH source due to minimized mass transfer diffusion limitations. To test our hypothesis, the

growth of biofilms of *P. naphthalenovorans* on glass coverslips that have a certain weight of a naphthalene on their surface and immersed in MSB media in a batch bioreactor was investigated using an array of microscopy techniques. To check the biofilm formation, our first step was using SEM. Although SEM images indicated the presence of biofilms (Figure **3**), the location of the biofilm with respect to the contaminate source could not be quantified using this technique.

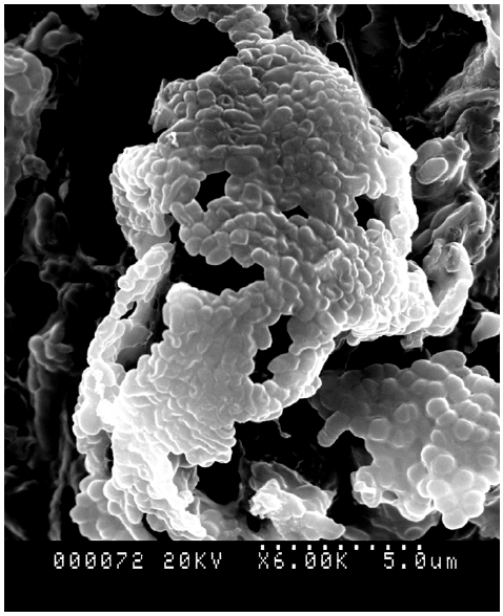


Figure 3: A scanning electron micrograph of the 7 days' old cells of *P. naphthalenovorans* on a glass coverslip with melted naphthalene on it (size increased 6.0 thousand times), scale bar 5 µm.

The biofilm samples were thus imaged using various optical light and fluorescence microscopy techniques (Figure **4**).

All images in Figure **4** clearly show that the *P. naphthalenovorans* biofilm preferably grew near the contaminate source. Figures **4B**, **4C**, and **4D** show a break sandwiched between the PAH source and the edge of the biofilm. The edge of the gap indicates the original position for the naphthalene melted drop on the glass coverslip. We speculate that the biofilm started growing directly near the PAH drop. With time, however, the size of the PAH drop was diminished due to both vaporization and bacterial consumption. The distance between 3 days biofilm and naphthalene on the Figure **4B** is 20 µm. The distance between 7 days biofilm and naphthalene on the Figure **4C** is 150 µm. All the distances were measured by the ImageJ Program (Version 1.43, April 2010, National Institutes of Health). Our optical and fluorescence microscopy results clearly indicate that *P. naphthalenovorans* biofilms grow near the PAH contaminate source. These microscopy results suggest that the bacterial biofilms are possibly degrading the PAH as was seen by the gaps that formed in all parts of Figure **4**.

The Effect of Naphthalene on the Structure of P. Naphthalenovorans Biofilms

AFM was used to image the *P. naphthalenovorans* biofilms in contact mode in air (Figure **5**).

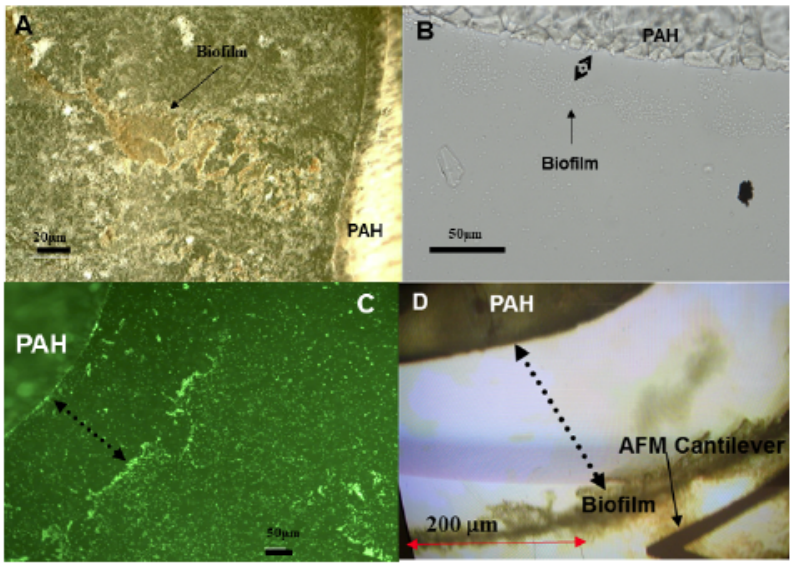


Figure 4: Images of *P. naphthalenovorans* biofilms grown on glass coverslips nearby naphthalene drops using: A. Light microscopy with 20X magnification after 7 days, scale bar is 20 µm. B. fluorescent microscopy without staining after 3 days, scale bar is 50µm. C. fluorescent microscopy with SYTO Dye staining after 7 days, scale bar is 50µm. D. optical microscopy of the AFM screen for 7 days biofilm, scale bar is 200 µm.

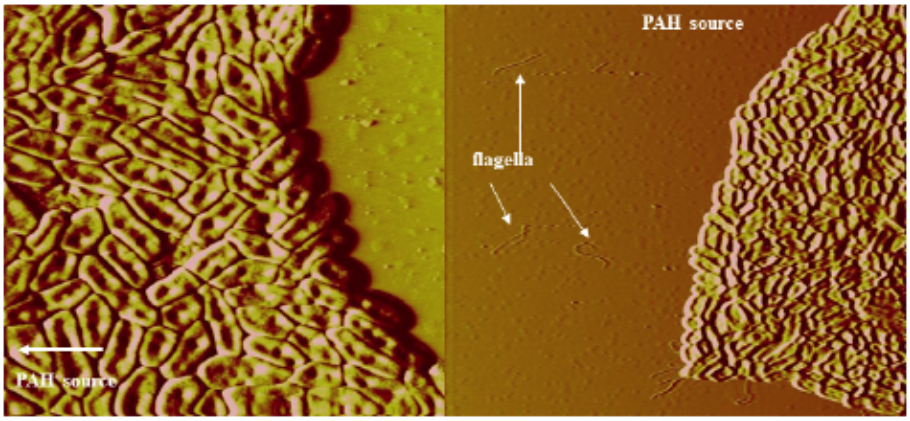


Figure 5: AFM deflection images of: (**A**) Biofilm nearby naphthalene grown in MSB with naphthalene only and (**B**) biofilm grown in TSB with 0.0075 g/L naphthalene.

Biofilms were allowed to grow for 3-7 days on glass coverslips immersed in MSB solutions that contained either 0.0075 g/L naphthalene alone or contained 0.0075 g/L naphthalene and TSB drop (0.05 g). AFM images of both biofilms were analyzed to quantify the roughness values of cells within the biofilms. Our results indicated that cells in both biofilms were heterogeneous in their roughness values. On average, the roughness of the cells in the biofilm that did grow using naphthalene as the lone carbon source (Figure **5A**) was found to be 54.7 nm compared to 28.5 nm average roughness value determined for cells in biofilms grown using both TSB and naphthalene (Figure **5B**).

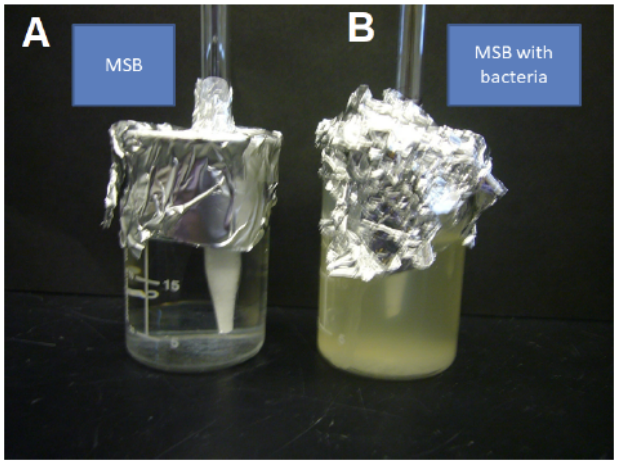
Our AFM images interestingly indicated that *P. naphthalenovorans* lose their flagella upon biofilm formation (Figure **5B**). Such observation requires further genetic analysis to determine if *P. naphthalenovorans* change their expression of the genes that control flagella formation upon exposure to naphthalene. Other studies in the literature indicated the loss of flagella of *Listeria monocytogenes* results upon biofilm formation. Therefore, bacterial motility supported by the presence of flagella is not required for biofilm formation [34].

In addition to roughness, the size of bacterial cells grown in TSB versus the size of those grown in MSB with naphthalene was compared at the nanoscale from AFM measurements (Figure **5**). AFM images of 15 bacterial cells grown in MSB media with PAH alone or in MSB media with PAH and TSB in air were analyzed using the cross-sectional offline feature of AFM for their length, width, and height. Our results showed that the length, width and height of bacterial cells grown in MSB with PAH alone were 1.21 ± 0.17 nm, 0.56 ± 0.10 nm and 41.1 ± 7.6 nm, respectively. In comparison, the length, width and height of bacterial cells grown in MSB with PAH and TSB were 2.13 ± 0.33 nm, 1.08 ± 0.18 nm and 218.5 ± 38.4 nm, respectively. Our results indicated that bacterial cells grown in MSB with PAH alone were collapsed in all three dimensions compared to those grown in MSB with TSB and PAH (results are significantly different, Mann Whitney Rank Sum Test, $P<0.001$). This is due to the degradation of the hard source of carbon and energy such as naphthalene. During the degradation of naphthalene, bacterial cells spend most of their energy not building cells, but for the utilization of hard structured carbon source to transfer it to more easily degradable components to get energy and carbon.

Biodegradation of Naphthalene in Capillaries

To confirm that *P. naphthalenovorans* biofilms facilitate the biodegradation of PAH compounds modeled by naphthalene, we carried out a controlled experiment that accounts for naphthalene dissolution and evaporation effects. The weights of the capillaries (Figure **6A**) that were placed in MSB alone were approximately the same as the initial weight of the capillaries with naphthalene in them (Figure **6A**).

The results showed that the average weight change of the capillaries with bacteria was 0.103±0.079 g and change of the weight of control capillaries was 0.001±0.002 g. The average percentage change of capillaries with bacteria was 18% and change of control capillaries was 0.16% (Figure **6B**). The standard deviation of percent changes was 15% for capillaries with bacteria and 0.2% for control capillaries. The differences quantified in the PAH weight due to biofilm formation in comparison to that due to dissolution of the PAH compounds were statistically significant (Mann-Whitney Rank Sum Test, $P<0.01$).



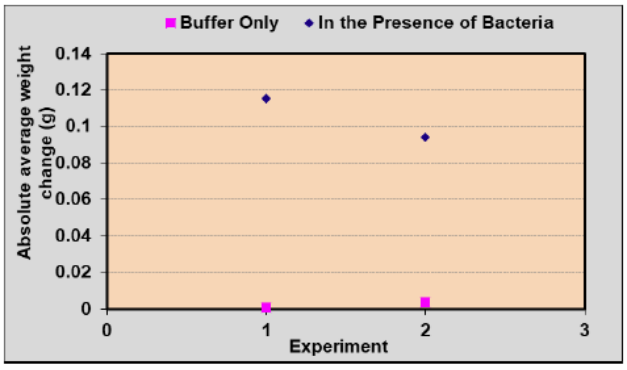


Figure 6: (**A**) Pictures that demonstrate the capillary experiment: 1 – the capillary was placed in a solution containing only the MSB media; and 2- the naphthalene was added to a capillary that was immersed in a MSB media that had been inoculated with the bacteria. (**B**) The average absolute change in naphthalene weight in 10 days for both experiments depicted in Figure 6A.

The fact that when capillaries of naphthalene were placed in MSB alone had a negligible change in their weight indicates that the dissolution of naphthalene is not a significant contributor to the weight loss of naphthalene during the experiment. The solubility of naphthalene in water has been reported to range between 30,000 µg/l and 40,000 µg/l at 25°C [37] and it has to be noted that the solubility of naphthalene in MSB can be considered to be similar to that in water [37]. Therefore, the MSB solution will quickly reach equilibrium with the naphthalene in the capillary. When the capillaries were placed in the MSB solution with bacteria in it, the naphthalene weight was reduced by 18% (Figure **6A**). This direct measurement indicates that *P. naphthalenovorans* biofilms facilitated the degradation of naphthalene.

CONCLUSIONS AND FUTURE RECOMMENDATIONS

This study is a step towards better understanding of how *P. naphthalenovorans* biofilms degrade naphthalene. Conducted experiments proved that *P. naphthalenovorans* prefer to form their biofilms near by the edge of the naphthalene crystal compared to far away from it and biofilm formation facilitates the biodegradation of naphthalene as a lone source of carbon. Achieved results will contribute to the creation of biopreparation methods for practical applications of bacterial remediation for the cleaning of naphthalene from the environment. Specifically, our results indicate that *P. naphthalenovorans* growth was the highest when the naphthalene concentration was 0.0075 g/L among concentrations tested. The distances between 3- and 7-days biofilm and naphthalene on coverslip were 20 µm and 150 µm. Acquired optical and fluorescence microscopy images suggested that the bacterial biofilms are possibly degrading naphthalene. Model experiment of naphthalene biodegradation showed that the weight was reduced by approximately 25%. The results of AFM quantified the length, width and height of cells imbedded within bacterial biofilms grown with PAH and these data might help to estimate the biofilm formation in environment.

Further research studies will be required to forecast the bioremediation routes of PAH in the environment. Running bioremediation tests in soil contaminated with PAH in soil columns will represent actual environments much better than capillaries. Such mimicking environment will enable us to accurately predict how bacterial transport and biofilm formation in soil columns can effectively treat contaminated soil with PAHs. Testing also the effect of biofilm formation under flow conditions and not in batch assays is important to predicting the abilities of biofilms to be retained in contaminated soil for effective bioremediation. Running similar experiments to the proposed above with other PAH compounds and synergetic mixture of PAH compounds will be necessary for modeling the soil complexity of PAH compounds. Assaying the structures of the PAH compounds after degradation will help us indicate how much PAH transformation to simple compounds occurred due to bacterial degradation and will help us decide if these compounds are simple enough to be consumed by the soil microflora for the treatment to be deemed sufficient and efficient. The impacts of different physical, chemical and biological factors on bioremediation and the abilities of bacteria to form biofilms that can be used to degrade PAH are important to investigate as well. These factors for example include pH, ionic strength, temperature, bacterial type and type of soil. The findings from this study along with findings from suggested studies will be

essential to predicting how biofilms can be used to degrade PAH compounds in variable soil environments.

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