Assessing the response of degradative biofilms to groundwater pollutants
W-946

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Abstract

There is limited knowledge of interspecies interactions in biofilm communities. In this study, *Pseudomonas* sp. GJ1, a 2-chloroethanol (2-CE) degrading organism, and *Pseudomonas putida* DMP1, a *p*-cresol degrader, produced distinct biofilms in response to model mixed waste streams comprised of 2-CE and various *p*-cresol concentrations. The two organisms maintained a commensal relationship, with DMP1 mitigating the inhibitory effects of *p*-cresol on GJ1. A triple labeling technique compatible with confocal microscopy was used to investigate the influence of toxicant concentrations on biofilm morphology, species distribution, and exopolysaccharide production. Single species biofilms of GJ1 shifted from loosely associated cell clusters connected by exopolysaccharide to densely-packed structures as *p*-cresol concentrations increased, and biofilm formation was severely inhibited at high *p*-cresol concentrations. In contrast, GJ1 was abundant when associated with DMP1 in a dual species biofilm at all *p*-cresol concentrations, although at high *p*-cresol concentrations it was only present in regions of the biofilm where it was surrounded by DMP1. Evidence in support of a commensal relationship between DMP1 and GJ1 was obtained by comparing GJ1-DMP1 biofilms with dual species biofilms containing GJ1 and *Escherichia coli* 33456, an adhesive strain that does not mineralize *p*-cresol. Additionally, the data indicated that only tower-like cell structures in the GJ1-DMP1 biofilm produced exopolysaccharide, in contrast to the uniform distribution of EPS in the single-species GJ1 biofilm.

Introduction and Problem Statement

Biofilms of environmental and medical significance frequently consist of diverse populations of microorganisms (4, 9). Moreover, it has been recognized that the metabolism of complex organic pollutants often involves the concerted efforts of multi-species bacterial consortia (8, 19). In biofilms that detoxify mixed organic wastes, interspecies bacterial interactions may influence biofilm structure and development. For example, species organization may enhance biofilm activity or the resistance of a given species to toxicants. Toxicants may also provide a selective pressure that could shift the species distribution in a biofilm, ultimately influencing biofilm activity.

Exopolysaccharide (EPS) is an integral structural and functional component of biofilm systems. It can account for up to 90% of the organic matter in a biofilm (21). EPS influences biofilm architecture and helps protect organisms within a biofilm (1, 15). Numerous studies have been done to characterize the composition and quantity of EPS produced (13, 14) but few studies have explored the relationship between EPS, biofilm architecture, and environmental conditions.

Recently, scanning confocal laser microscopy (SCLM) has developed into a powerful tool for elucidating the three-dimensional architecture and species distribution of biofilm systems (5, 18) and may be useful for correlating biofilm structure with interspecies relationships, such as mutualism or commensalism. In this study, a triple labeling technique that was compatible with confocal microscopy was developed to fully characterize the response of a dual species biofilm to two organics that are commonly found in mixed organic waste. The biofilm consisted of *Pseudomonas putida* DMP1, a *p*-cresol degrading organism, and *Pseudomonas* sp. GJ1, a 2-chloroethanol (2-CE) degrading organism. The biofilm morphology, species distribution, and EPS production were examined using SCLM for biofilms grown with various toxicant concentrations. This information provided insight into the interspecies relationships present in the biofilm.

Objectives

The goal of this proposal is to determine the effects of changing toxicant concentrations on the structure and organization of multi-species degradative biofilms and how changes in community structure affects contaminant degradation. The specific aims are as follows:

1. To measure the kinetic parameters required to describe the growth of the members of two dual-species consortia;
2. To use the aforementioned kinetic parameters in a computer simulation of biofilm development;
3. To cultivate biofilms using the consortia under different concentration regimes;
4. To obtain three-dimensional structural information about the species distribution within the resultant biofilms using confocal scanning laser microscopy;
5. To compare the laboratory-acquired biofilm images with predictions from the computer simulation.

Procedures

Strains, plasmids, and media. The strains and plasmids used are listed in Table 1. All strains were grown in MMV (10), a minimal medium supplemented with 50 μg Kanamycin mL^{-1}. During all biofilm colonization processes, 0.1% succinate was used as the sole carbon source because both GJ1 and DMP1 had approximately the same growth rate when grown on succinate. All strains were cultured at ambient temperature.

Growth and toxicity studies. *Pseudomonas* sp. GJ1 harboring pSMC21 was grown in MMV with the following concentrations of 2-chloroethanol (Aldrich, Milwaukee, WI): 5, 10, 15, 20, 25, and 30 mM. The specific growth rates of *Pseudomonas* sp. GJ1 harboring pSMC21 in suspended culture were determined. All growth experiments were performed at least twice and the average values are reported. *Pseudomonas putida* DMP1 harboring pVLT33 was grown in MMV with the following concentrations of *p*-cresol (Aldrich, Milwaukee, WI): 0, 0.0002, 0.0005, 0.0007, 0.0009, 0.0019 mM. The suspended culture growth rates of DMP1 were determined.

The effect of *p*-cresol on the specific growth rate of GJ1 was determined by growing GJ1 in MMV with 20 mM 2-CE and the following *p*-cresol concentrations: 0, 0.0002, 0.0005, 0.0007, 0.0009, 0.0019 mM. The effect of 2-CE on the specific growth rate of DMP1 was determined by growing DMP1 at the optimal *p*-cresol concentration (0.0007 mM) with various concentrations of 2-CE (0, 5, 10, 15, 20, 25, 30 mM).

Bench-scale flow cell. Biofilms were prepared, as described previously, in bench-scale parallel plate flow cells (reactor volume of 0.35 mL (5)). A cover glass was glued to a plastic frame using General Electric Silicone Rubber Adhesive Sealant RTV 102 (GE Silicones, Waterford, NY). Flow cells were operated in recirculating (start-up) or continuous modes at flow rates of 0.86 mL/min or 0.12 mL/min, respectively.

GJ1 and DMP1 dual species biofilms. In order to colonize the surface, medium containing equal concentrations of exponentially growing GJ1 (harboring pSMC21) and DMP1 (harboring pVLT33) was recirculated through a flow cell for 6 hours. Subsequently, sterile medium containing 20 mM 2-chloroethanol and either 0.0007 or 0.0019 mM *p*-cresol was continuously pumped through the flow cell for 45 hours. MMV (15 mL) was pumped through each flow cell to remove cells that were not attached to the cover glass. The biofilms in the flow cells were then stained and imaged as described below.

GJ1 and 33456 dual species biofilms. Exponentially growing *E. coli* 33456 harboring pVLT33 was recirculated through a flow cell for 6 hours to promote colonization. Exponentially growing *Pseudomonas* sp. GJ1 harboring pSMC21 was then recirculated through the same flow cell for 3 hours. After both colonization steps, sterile medium containing 20 mM 2-chloroethanol and either 0.0007 or 0.0019 mM *p*-cresol was continuously pumped through the flow cell for 45 hours. In order to maintain 33456,

which can not grow on either 2-chloroethanol or *p*-cresol, 0.002% glucose was added to the medium. The addition of glucose did not influence strain GJ1, which is unable to grow on glucose. The resulting biofilms were rinsed, stained, and imaged.

Single species biofilms. Flow cells were colonized as described above with medium containing either GJ1 harboring pSMC21, 33456 harboring pVLT33, or DMP1 harboring pVLT33. The colonized flow cells were then switched to the continuous flow conditions described above. Again, 33456 biofilms were switched to medium containing 0.002% glucose. The GJ1 biofilms were rinsed and stained with calcofluor white (but not stained with SYTO 59) prior to imaging. The 33456 and DMP1 biofilms were rinsed and stained with both SYTO 59 and calcofluor white before imaging.

Staining biofilms with SYTO 59. After rinsing, flow cells containing either *E. coli* 33456 or *Pseudomonas putida* DMP1 were stained with 20 μM SYTO 59 (Molecular Probes, Inc., Eugene, OR), a soluble nucleic acid dye that emits in the red region, for 10 minutes.

Table 1. Strains and Plasmids

Strain or Plasmid	Description[a]	Reference or Source
Strains		
Pseudomonas sp. GJ1	2-chloroethanol degrader	(10)
Pseudomonas putida DMP1	*p*-cresol degrader	(20)
E. coli 33456	Wild-type *E. coli*	American Type Cultu Collection, (17)
Plasmids		
pVLT33	broad host range plasmid, Km^r	(7)
pSMC21[b]	pUCP-based plasmid containing *gfpmut2*, Ap^r, Cb^r, Km^r	(3)

[a] Ap^r, ampicillin resistance; Cb^r, carbenicillin resistance; Km^r, kanamycin resistance.
[b] pSMC21 is identical to pSMC2 but it has an additional Km^r gene

Staining biofilms with calcofluor white. After staining biofilms with SYTO 59, all flow cells were stained in the dark with 0.025% Calcofluor white M2R (Sigma Chemical Company, St. Louis, MO) for 1 minute. This dye binds to β-linked polysaccharides, such as cellulose and chitin (11). Calcofluor white can not penetrate intact cell membranes and does not stain viable cells (12). The biofilms were then rinsed with 2 mL of MMV to decrease background fluorescence. The stained flow cells were imaged using confocal microscopy.

Confocal microscopy. Confocal microscopy was performed using a Bio-Rad MRC-1024 laser scanning confocal imaging system (Bio-Rad Microsciences, Cambridge, MA) equipped with a Nikon Diaphot 200 inverted microscope (Mikon, Inc., Tokyo, Japan). All images were obtained with a 20X lens. The 488 line from a Kr/Ar laser was used to simultaneously excite both GFP and SYTO 59. GFP was detected using a standard fluorescein filter set (522/35 band pass filter) and SYTO 59 emissions were detected using a 605/32 band pass filter. The 363 nm line from an argon ion UV laser (Model # ENT 622, Innova Technology/Coherent Enterprises, Santa Clara, CA) was used to excite the calcofluor white, which was then detected using a 455/30 band pass filter. The GFP emissions were directed to the green channel, the SYTO 59 emissions to the red channel, and the calcofluor white emissions to the blue channel. The resulting images were processed using Adobe Photoshop 5.0 (Adobe Systems, Inc., San Jose, CA) software.

Results

Growth and toxicity studies. The growth and inhibition curves for GJ1 and DMP1 are presented in Fig. 1. DMP1 grew optimally at 0.0007 mM *p*-cresol and experienced substrate inhibition for *p*-cresol concentrations greater than 0.0007 mM (Fig.

1A). In contrast, the growth of GJ1 was unaffected by 2-CE at all concentrations tested and was an order of magnitude slower than that of DMP1 (Fig. 1B). DMP1 was unable to grow on 2-CE (Fig. 1C) and GJ1 was unable to grow on *p*-cresol (Fig. 1D). Thus, each member of the biofilm had an independent carbon source. For purposes of investigating interactions between GJ1 and DMP1 in a biofilm, 0.0007 or 0.0019 mM *p*-cresol concentrations were selected; 0.0007 mM was the optimal concentration for DMP1 growth, while DMP1 substrate inhibition occurred at 0.0019 mM *p*-cresol. A 2-CE concentration of 20 mM was chosen.

Both substrates inhibited the growth of the organism not able to utilize it as a carbon source. DMP1 growth was inhibited by the presence of 2-CE (Fig. 1C). At 20 mM 2-CE, the 2-CE concentration used for all biofilm studies, the planktonic specific growth rate of DMP1 was diminished to one-fourth of that with no 2-CE. GJ1 growth was also inhibited by the presence of *p*-cresol in the medium (Fig. 1D). At 0.0007 or 0.0019 mM *p*-cresol, the conditions investigated in this study, the specific growth rate decreased to two-thirds or one-third of that with no *p*-cresol, respectively.

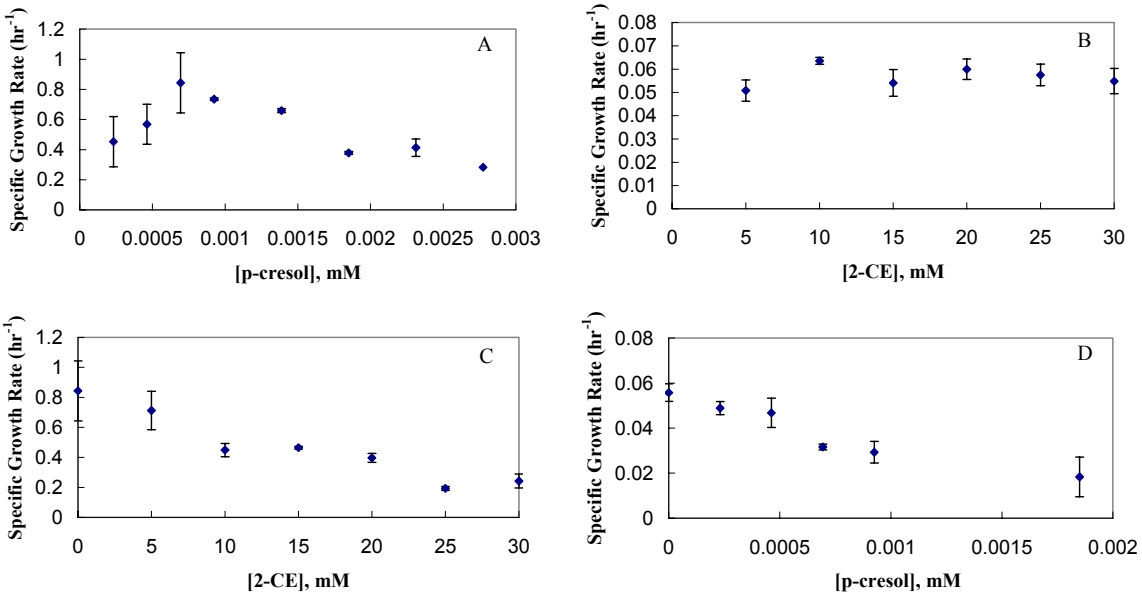

Figure 1. Growth and inhibition kinetics. Left column, *Pseudomonas putida* DMP1. Right column, *Pseudomonas* sp. GJ1. (A) *Pseudomonas putida* DMP1 growth on p-cresol. (B) *Pseudomonas* sp. GJ1 on 2-chloroethanol. (C) Inhibition of *Pseudomonas putida* DMP1 growth by 2-chloroethanol. (D) Inhibition of *Pseudomonas* sp. GJ1 growth by p-cresol.

A summary of the observed relationship between the pseudomonads, GJ1 and DMP1, and their respective carbon sources is shown in Fig. 2. DMP1 grows on *p*-cresol but was inhibited by 2-CE and GJ1 grows on 2-CE but was inhibited by *p*-cresol. Since DMP1 grew much faster than GJ1 under all planktonic conditions, the inhibition of DMP1 by 2-CE was not an important variable and was not explored in this study.

Figure 2

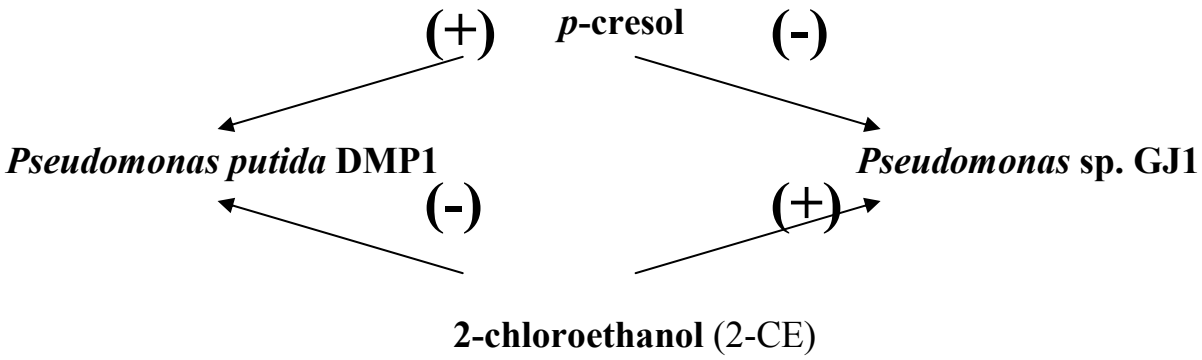

Figure 2. Relationship between strains DMP1 and GJ1 and their respective carbon sources.

Figure 3

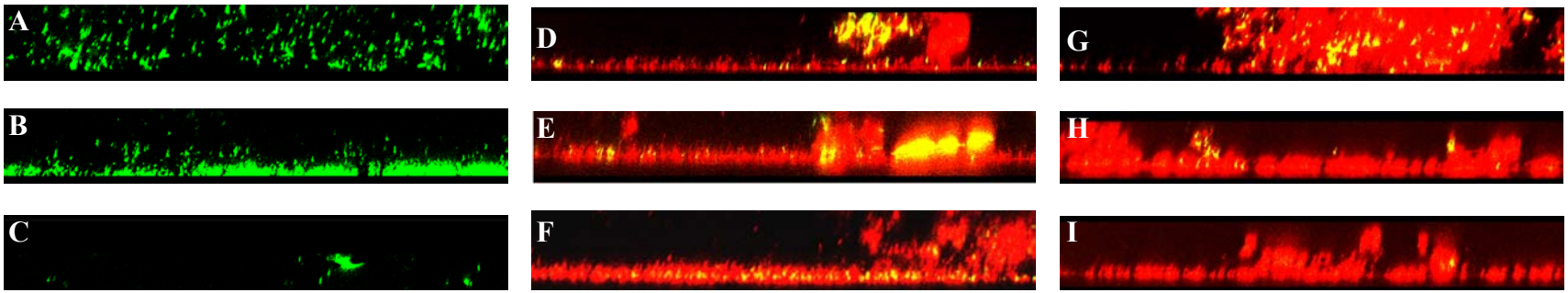

Figure 3. Vertical sections of biofilms. Left column, *Pseudomonas* sp. GJ1 (green) monoculture biofilms. Middle column, *Pseudomonas* sp. GJ1 (yellow) and *Pseudomonas putida* DMP1 (red) coculture biofilms. Left column, *Pseudomonas* sp. GJ1 (yellow) and *E. coli* 33456 (red) coculture biofilms. A, D, and G were grown on succinate. B ,E, and H were grown on 20 mM 2-CE and 0.0007 mM *p*-cresol. C ,F, and I were grown on 20 mM 2-CE and 0.0019 mM *p*-cresol. 200X magnification.

Biofilm morphology, species distribution, and EPS production. Vertical sections of GJ1 single species biofilms (Fig. 3A, B, and C), GJ1-DMP1 dual species biofilms (Fig. 3D, E, and F), and GJ1-33456 dual species biofilms (Fig. 3G, H, and I) were obtained using confocal microscopy. These biofilms were cultured in medium containing succinate (Fig. 3A, D, G), 20 mM 2-CE and 0.0007 mM *p*-cresol (Fig. 3B, E, H), or 20 mM 2-CE and 0.0019 mM *p*-cresol (Fig. 3C, F, I). In all images, green or yellow cells correspond to GJ1 and red cells correspond to either DMP1 (Fig. 3D, E, and F) or 33456 (Fig. 3G, H, and I). Multiple images were collected for each set of experimental conditions; a representative image is presented in all cases.

A distinct change was observed in the GJ1 single species biofilm morphology as the *p*-cresol concentration increased (Fig. 3, top to bottom). When GJ1 biofilms were cultured in medium with succinate (in the absence of *p*-cresol), a biofilm consisting of loosely associated cell clusters formed (Fig. 3A). These cell clusters were connected by exopolysaccharide (data not shown). When the GJ1 biofilm was developed in medium containing 20 mM 2-CE and 0.0007 mM *p*-cresol, a morphological change resulted (Fig. 3B). The biofilm was dense and attached to the substratum. A more dramatic change in morphology resulted when the GJ1 biofilm was developed in medium containing 20 mM 2-CE and 0.0019 mM *p*-cresol (Fig. 3C). Under these conditions, only a few clusters of GJ1 were able to survive. In contrast, DMP1 single species biofilms were dense and closely attached to the substratum under all *p*-cresol concentrations (data not shown).

Both the morphology and species distribution of the dual species GJ1-DMP1 biofilms were influenced by the *p*-cresol concentration. When the GJ1-DMP1 biofilms were developed on succinate, the two species were intermingled in the biofilm (Fig. 3D). Under these conditions, yellow GJ1 cells were found near the biofilm-bulk fluid interface. In contrast to the case where a GJ1 biofilm was developed on succinate alone and formed loosely associated cell clusters, the biofilm containing both GJ1 and DMP1 formed a relatively tightly-packed biofilm, even in the tall structures, resembling that of a DMP1 single species biofilm (data not shown). When the biofilm was developed in medium containing 20 mM 2-CE and 0.0007 mM *p*-cresol, the yellow GJ1 cells were present only between layers of the red DMP1 cells (Fig. 3E). Similarly, GJ1 was only found deep within the biofilm when the biofilm was developed in 20 mM 2-CE and 0.0019 mM *p*-cresol (Fig. 3F). There was also a decline in the GJ1 population in the biofilm when the *p*-cresol concentration was increased to 0.0019 mM. When the medium contained either 0.0007 mM or 0.0019 mM *p*-cresol, GJ1 was no longer evident near the biofilm-bulk fluid interface.

To determine if the apparent protection of GJ1 from *p*-cresol by DMP1 was due to the specific ability of DMP1 to degrade *p*-cresol or if any organism could offer the same protection, GJ1 was grown in dual species biofilms with *E. coli* 33456, which can form biofilms but was unable to degrade *p*-cresol. When cultured on succinate, the GJ1-33456 biofilm had both species intermingled (Fig. 3G), which was similar to the GJ1-DMP1 biofilm grown under the same conditions (Fig. 3D). When the GJ1-33456 biofilm was developed in 20 mM 2-CE and either 0.0007 mM or 0.0019 mM *p*-cresol, only a few yellow clusters of GJ1 cells were present (Fig. 3H and 3I), significantly fewer than in the GJ1-DMP1 biofilm.

When cultured on succinate, GJ1 and DMP1 were homogeneously distributed in horizontal sections of the biofilm at both 15 and 30 μm above the substratum (Fig. 4A, B, and C). When exposed to 0.0007 mM *p*-cresol, large clusters of GJ1 cells were concentrated beneath DMP1 in the cell towers and in the lower layers of the biofilm (Fig. 4D, E and F). When exposed to 0.0019 mM *p*-cresol, GJ1 was only present in small clusters in the lower layers of the biofilm (Fig. 4G, H, and I). At 15 μm above the substratum, there were distinct clusters of yellow GJ1 cells homogeneously interspersed with clusters of red DMP1 cells (Fig. 4G).

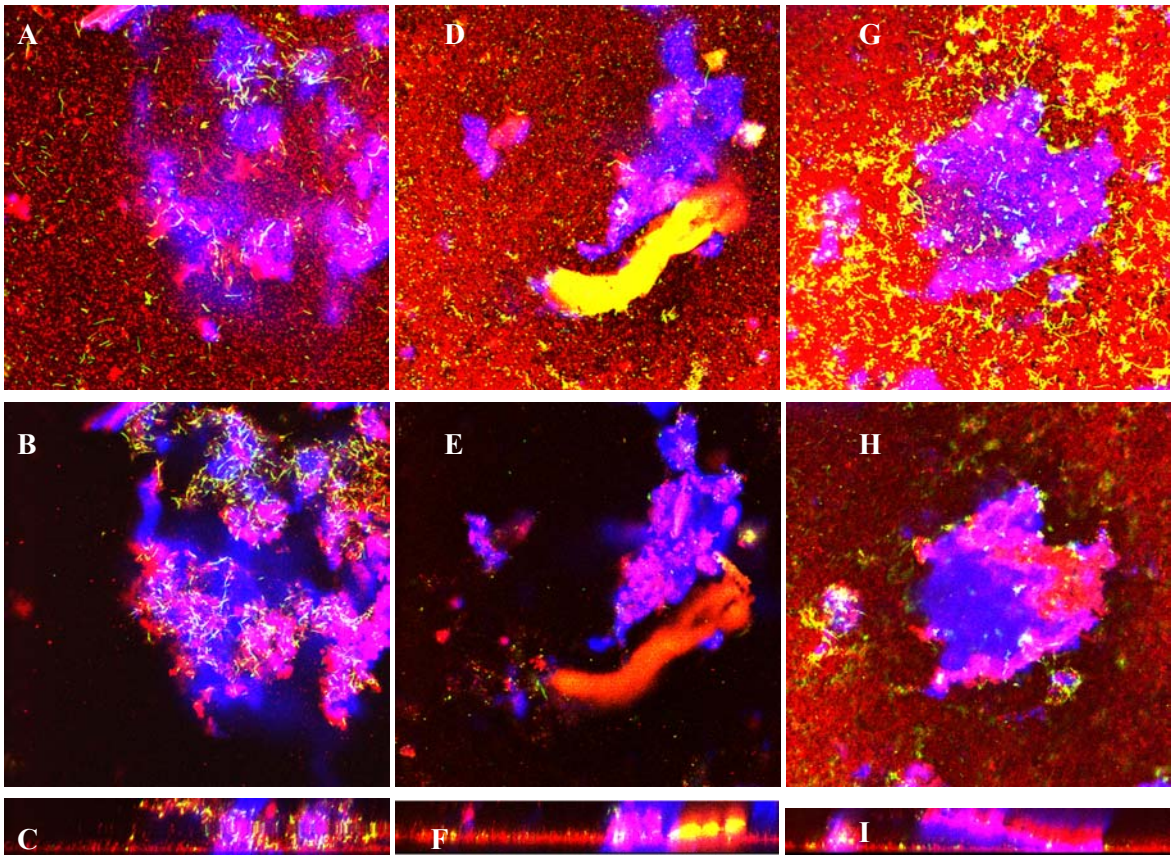

Figure 4: Horizontal and vertical sections of *Pseudomonas* sp. GJ1 (yellow) and *Pseudomonas putida* DMP1 (red) coculture biofilms with exopolysaccharides (blue). Left column, grown on succinate. Middle column, grown on 20 mM 2-CE and 0.0007 mM *p*-cresol. Right column, grown on 20 mM 2-CE and 0.0019 mM *p*-cresol. 200X magnification.

To examine the effect of the organics on EPS formation, GJ1-DMP1 dual species biofilms were labeled with calcofluor white (Figure 4). In these images, the color red corresponds to DMP1 cells, yellow corresponds to GJ1 cells, and blue corresponds to exopolysaccharides (EPS). Magenta regions, resulting from the overlap of red and blue pixels, represent DMP1 cells embedded in EPS. Similarly, white regions, resulting from the overlap of yellow and blue pixels, are GJ1 cells covered in EPS.

The EPS, which was produced by both GJ1 and DMP1 in single species biofilms (data not shown), was formed in similar quantities in the dual species biofilms under all three sets of environmental conditions. The EPS was formed only by cells located in tower- or mushroom-shaped clusters, where there was a high cell density (Fig. 4C, F, and I).

Discussion

The complementary metabolic activities of *Pseudomonas* sp. GJ1 and *Pseudomonas putida* DMP1 were combined in a biofilm to produce a single functional system in which both strains were stably maintained. The planktonic growth and toxicity data (Fig. 1) obtained for the two pseudomonads suggests that GJ1 had a limited chance of survival in the presence of *p*-cresol. It was also evident that a single strain could not effectively remediate a mixed waste stream containing 2-CE and *p*-cresol.

Figures 3 and 4 demonstrate that GJ1 benefited from the close interactions with DMP1 in a dual species biofilm. In the presence of *p*-cresol, GJ1 was present in the greatest proportion when it was paired with DMP1 in a biofilm. The fact that GJ1 was almost eliminated from single species (Fig. 3C) or GJ1-33456 dual species (Fig. 3I) biofilms at *p*-cresol concentrations of 0.0019 mM implies that GJ1 benefited from being coupled with DMP1, in addition to the non-specific resistance to toxicants that is associated with biofilms (1). GJ1 depended on DMP1 to mineralize *p*-cresol and thus detoxify the medium in its vicinity.

The morphological changes in the GJ1-DMP1 biofilms, which resulted from exposure to *p*-cresol, caused GJ1 and DMP1 to become increasingly intermingled. Nielsen et al. recently found that a consortium of *Burkholderia* sp. LB400 and *Pseudomonas* sp. B13 (FR1), which interact metabolically to degrade chlorobiphenyl, experience morphological changes due to environmental conditions (16). A *p*-cresol concentration of 0.0007 mM resulted in GJ1 surrounding itself with strain DMP1 in the z-direction (Figs. 3E, 4D, 4E, and 4F). At higher *p*-cresol concentrations, such as 0.0019 mM, architectural changes were seen in the x-, y-, and z-directions (Figs. 3F, 4G, 4H, 4I). GJ1 enhanced its contact with DMP1, dispersing itself throughout the biofilm in the xy-plane (Fig. 4G); this appears to be the only location in the biofilm where GJ1 was able to survive. By surrounding itself with DMP1, GJ1 effectively reduced the local concentration of *p*-cresol to which it was exposed.

EPS production by the GJ1-DMP1 coculture biofilms appears to be unaffected by toxicant concentrations. Rather, EPS production appeared to be influenced by biofilm morphology; EPS was only found in tall vertical structures in the biofilm, such as tower- or mushroom-shaped clusters (Fig. 4C, 4F, and 4I). This suggests that there was a phenotypical difference between the bacteria in these tall vertical structures and the bacteria in other regions of the biofilm. Since EPS was only found in tall structures within the biofilm, this implies that the genes responsible for EPS production in strains GJ1 and DMP1 may be controlled by quorum sensing signals. Davies and coworkers have shown that quorum sensing plays an important role in *Pseudomonas aeruginosa* biofilm structure and development (6). Cross-strain cell-cell signaling may be occurring because GJ1 and DMP1 were both present in clusters producing EPS. Cross-strain quorum sensing has been demonstrated for a variety of organisms (2).

In summary, it was demonstrated that a triple labeling technique compatible with SCLM can be used to elucidate interspecies interactions in a dual species biofilm. The images obtained in this study indicate that there was a commensal interaction between

strains GJ1 and DMP1. As the concentration of *p*-cresol, an inhibitor of GJ1 growth and substrate for DMP1 growth, increased, GJ1 was found only surrounded by DMP1 in the biofilm. This technique is not limited to the study of commensal relationships; there is a need to gain an understanding of other bacterial interactions in biofilms, such as mutualism or ammensalism (9). An understanding of the mutual or commensal relationships in mixed culture biofilms will aid in the design of multi-species biofilms for the biosynthesis of specialty chemicals or biodegradation of xenobiotics that can not be metabolized by a single organism.

Conclusions

- We have developed a triple labeling technique compatible with confocal microscopy was used to investigate the influence of toxicant concentrations on biofilm morphology, species distribution, and exopolysaccharide production.
- We have used the technique to observe single species biofilms of GJ1, which shifted from loosely associated cell clusters connected by exopolysaccharide to densely-packed structures as *p*-cresol concentrations increased, and biofilm formation was severely inhibited at high *p*-cresol concentrations.
- GJ1 was abundant when associated with DMP1 in a dual species biofilm at all *p*-cresol concentrations, although at high *p*-cresol concentrations it was only present in regions of the biofilm where it was surrounded by DMP1.
- Evidence in support of a commensal relationship between DMP1 and GJ1 was obtained by comparing GJ1-DMP1 biofilms with dual species biofilms containing GJ1 and *Escherichia coli* 33456, an adhesive strain that does not mineralize *p*-cresol.
- The data indicated that only tower-like cell structures in the GJ1-DMP1 biofilm produced exopolysaccharide, in contrast to the uniform distribution of EPS in the single-species GJ1 biofilm.

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TRAINING ACCOMPLISHMENTS

- Eric S. Gilbert, Post-doc
- Stacie Cowan, Graduate student
- Ivan Chang, Undergraduate student
- Maya Lim, Undergraduate student
- Niki Rambhia, Undergraduate student
- Donald Chua, Undergraduate student
- Richard Tsai, Undergraduate student

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