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Molecular Modulation of Single and Multi-species Biofilm Formation by Orally-associated Bacteria

An honors thesis presented to the Department of Biological Sciences University at Albany State University of New York In partial fulfillment of the Honors Program Requirements

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Abstract

Biofilms are aggregates of bacterial cells attached to a surface. Oral biofilms ("plague") are major contributors to tooth decay (dental caries) and are a potential conduit for infection and disease. These biofilms have been shown to be resistant to removal by traditional oral hygiene practices. Novel prophylactic and in situ treatment methods are therefore needed to address this problem. While Oral biofilms have been shown to contain hundreds of species of bacteria we focus on three relevant organisms: *Streptococcus mutans*, *Streptococcus sanguinis*, and *Actinomyces naeslundii* (A. oris). Of the many bacteria involved in cavity formation and tooth decay, *S. mutans* is recognized as the principle causative agent and pioneer. *S. sanguinis* and *A. oris* are pioneer-colonizing commensal organisms that are involved in the initial establishment of oral biofilms and are associated with significant human diseases including endocarditis and actinomycosis. Our group previously identified small organic molecule inhibitors of biofilm formation in *Pseudomonas aeruginosa*. We hypothesize that these compounds directly affect cell-to-cell signaling ("quorum sensing") pathways that are involved in biofilm formation. Presently, members of our lab have tested the efficacy of similar compounds on the three individual aforementioned strains. In this work, we focus on how these organic compounds interact with communal biofilms consisting of two or more strains.
INTRODUCTION

A biofilm is an aggregate of bacterial cells that are attached to a surface and are typically encased in an extracellular polymeric matrix (EPS) consisting of proteins, polysaccharides, and nucleic acids. Biofilms have been associated with oral cavities, infections, and contamination of medical devices such as catheters and surgical implants (Ge X., et al, 2008; Grey, W. T., et al, 1997; Kuramitsu H. K., et al, 2007; Loo C. Y., et al, 2000; Zhang, K., et al, 2009). Biofilms are of particular interest as they are resistant to antibiotic treatments and/or physical removal (Suntharalingam, P. et al, 2005). It is thus important that novel biofilm treatment and removal methods be developed and elucidated. Previously our group identified small molecule inhibitors of biofilm formation for the bacterium *Pseudomonas aeruginosa* (Cady NC, et al, 2012). These molecules were also shown to inhibit cell signaling (aka: quorum sensing) in this organism. We believe that the inhibition of quorum sensing through the use of small molecule inhibitors is a prospective treatment method for the removal of bacterial biofilms.

Oral biofilm forming bacteria such as *Streptococcus mutans*, *Streptococcus sanguinis*, and *Actinomyces naeslundii* (oris) behave differently than *P. aeruginosa*. All three oral strains are Gram-positive whereas *P. aeruginosa* is a Gram-negative strain. Most notably, the conditions the strains prefer to grow in differ. Previous work focused on elucidating common growth conditions for all three Gram-positive strains. Following optimization of the growth assay, Steve Kasper and Samantha Testa conducted multi-well high throughput screening of cysteine sulfoxide derivatives inspired from naturally occurring compounds. These were provided by our collaborator Professor Rabi Musah (Cady NC, et al, 2012). The structures of these compounds are not included in this work as they are pending patent protection. This
work successfully isolated effective small molecule inhibitors of orally associated biofilm forming bacteria.

Biofilms are complex composites of hundreds of species of bacteria; essentially they are microscopic multi-species communities. Oral biofilms in particular have been shown to contain over 500 distinct bacteria (Blehert, D.S., et al, 2003; Kolenbrander, P. E., 2000; Kuramitsu H. K., et al, 2007). It has been shown that the interactions of species within biofilms can produce novel functions and morphologies otherwise not seen in the isolated strain by itself. “It is not merely the presence of a single organism in a complex community which determines the properties of a biofilm, but it is the interactions between the biofilm residents which is crucial” (Kuramitsu H. K., et al, 2007). It is important then, to study not only single species biofilms but also multi-species biofilms. The focus of this work is to compare the effects of five of the compounds: Compound 3, Compound 11, Compound 13, Compound 16 and compound 21, that were particularly active in individual strain experiments, to the effects they have on multispecies co-culture biofilms.
**Materials and Methods**

**Bacterial strains, media, and chemicals**

*Streptococcus mutans* Clarke was obtained from ATCC (ATCC® Number: 25175™).

*Streptococcus sanguinis* strain 10566 and *Actinomyces naeslundii* strain MG1, hereby referred to as *Actinomyces oris*, were obtained from Alexander Rickard, University of Michigan. All strains were propagated on Brain Heart Infusion (BHI) agar. Liquid cultures of all strains were prepared by growing single colonies in 5mL Brain Heart Infusion and incubating overnight at 37 °C with 5% CO₂.

Thermo Scientific 164590 optical bottom plates with #1.5 borosilicate coverglass base were used for biofilm formation experiments. Optical density and fluorescence measurements were performed in a Tecan M-200 plate reader. Images of SYTO 9 stained films were taken using Nikon 80i epifluorescence microscope or with a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). BHI and sucrose were obtained from Sigma Aldrich. SYTO 9 was obtained from Invitrogen. Organic Compounds were obtained from Rabi Musah, University at Albany.

90% Brain Heart Infusion + 2% sucrose media (90% BHIS) was prepared diluting 5mL 20% sucrose (w/v) in 45mL BHI. 10% BHIS, 25% BHIS, 50% BHIS, and 75% BHIS were prepared diluting with sterile H₂O. 90% Brain Heart Infusion (90% BHI) was prepared diluting BHI with sterile H₂O (4.5 mL BHI + 0.5 mL H₂O). 10% BHI, 25% BHI, 50% BHI, and 75% BHI were prepared in a similar fashion.
**Growth Optimization Assay**

Liquid cultures of desired strains were grown overnight as previously described. The next day the cultures were vortexed, 5 μL of the cell solution was removed and added to 495 μL of desired media (10% BHI/BHIS, 25% BHI/BHIS, 50% BHI/BHIS, 75% BHI/BHIS, 90% BHI/BHIS). This diluted culture was then vortexed and added to a flat glass bottom 96 well plate, 100 μL per well. The Optical Density (OD) was analyzed at 600 nm. Plates were taped and incubated for 48 hours at 37 °C with 5% CO₂. Biofilm growth was quantified using SYTO 9 fluorescence assay.

**Small Molecule Inhibitor Screening Assay**

Liquid cultures of desired strains were grown overnight as previously described. The next day the cultures were vortexed to resuspend all cells. In the case of single species screening, 5 μL of the cell solution was removed and added to 490 μL of 25% BHIS. In the case of multispecies co-culture screening, 5 μL of each strain’s cell solution was removed and added to 485 μL of 25% BHIS. In the case of tri-culture screening, 5 μL of each strain’s cell solution was removed and added to 480 μL of 25% BHIS. 5μL of desired compound (3, 11, 13, 16 or 21) at a 1 mM or 5 mM concentration was then added to this solution. For solvent controls 5 μL of NaOH or H₂O was added in place of the addition of compound. For normal growth, baseline control 5 μL of additional 25% BHIS was added in place of the addition of compound. The culture was then vortexed and added to a flat glass bottom 96 well plate, 100 μL per well. The Optical Density (OD) was analyzed at 600 nm. Plates were taped and incubated for either 48 hours or 48 hours with an addition media change at 24 hours, at 37 °C with 5% CO₂. Biofilm
growth was quantified using SYTO 9 fluorescence assay. Standard fluorescent microscopy and imaging was conducted using the Nikon 80i epifluorescence microscope and the Leica TCS SP5 confocal microscope. Three dimensional images were taken using a Leica TCS SP5 confocal microscope.

**SYTO 9 Fluorescence Assay**

Biofilm growth was quantified using SYTO 9 nucleic acid stain. After incubation of 96-well plate Optical Density of overall growth was taken using the Tecan at 600 nm (“planktonic growth”). Supernatant was removed from each well. Wells were rinsed with 200 µL diH₂O (2x). Each well was stained with 100 µL SYTO 9 fluorescent dye (15 µL dye + 10 mL diH₂O). Plates were incubated 20-30 minutes at room temperature. SYTO 9 was removed and each well was rinsed 1x with 200 µL diH₂O. Then, 100 µL diH₂O was added to each well and fluorescent intensity was measured with the Tecan at 480 nm excitation/520 nm emission.
Results

A. Prior Work

Figure 1 shows results from our initial experiments in biofilm growth optimization. As shown, the presence of sucrose in the media leads to robust biofilm formation. Only S. sanguinis grew well without sucrose. S. mutans and S. sanguinis biofilm growth becomes increasingly robust in a linear fashion with increased concentration of BHI. A. oris biofilm growth, on the other hand, is maximal at 50% BHI and higher concentrations.

Shown in Figure 2 are results from Samantha Testa and Steve Kasper’s initial screening of 21 distinct small molecule inhibitors against individual model oral bacterial strains. The graph depicts the resulting percent change in biofilm biomass, with respect to normal biofilm formation (no compound added), as a result of the addition of a compound. The compounds had a variety of activity having an array of effects on biofilm formation.

Several of the 21 compounds inhibited all three strains; i.e. compounds 3, 5, 16, and 17. Addition of Compound 16 resulted in the greatest reduction in fluorescence with a percent change around -80% for A. oris and S. sanguinis and close to 100% reduction for S. mutans. Comparatively, the other compounds had reductions less than 50%, with compounds 3 and 5 having the most similar results across the three strains, having around 35% and 25% reduction for each strain, respectively.

Some compounds were shown to promote biofilm formation for all three strains; i.e. compounds 8, 9, 13, 14, 15, and 21. Compounds 13 and 14 both had percent changes for all three stains that were close to one another, around 35%. Compound 21 increased biofilm formation by 45% and 50% for S. mutans and A. oris, respectively. This was statistically the
largest increase in growth among the 6 compounds that promoted biofilm formation for all three strains. For *S. sanguinis*, Compound 21 caused a smaller increase in biofilm growth, with only about a 20% change.

The remaining eleven compounds had differential activity, promoting one strain while inhibiting another. For example, Compound 1 promoted growth in *S. mutans* and *A. oris* with increases of 45% and 60%. However Compound 1 slightly inhibited *S. sanguinis*, with a percent change of -5% in Figure 2. Compound 11 probably has the most interesting result of the remaining “differential activity” compounds. For *S. mutans* the addition of Compound 11 to growth media resulted in about a 45% change, increasing biofilm formation. However, for *A. oris* it had a statistically insignificant effect on biofilm formation, and in the case of *S. sanguinis* the compound resulted in a -25% change in biofilm growth.
Figure 1. Results from Growth Optimization Assay. Blue, red, and green bars represent strains grown in the presence of 2% sucrose (S. mutans, A. oris, S. sanguinis respectively). Light gray, gray, and black bars represent strains grown without sucrose (S. mutans, A. oris, S. sanguinis respectively). The plate was incubated for 48 hours at 37 °C with 5% CO₂. All data represents the average of 3 or more wells.
Figure 2. Percent change in biofilm growth (biomass) for individual bacterial strains due to addition of organic compounds, with respect to normal growth without compounds. All strains were grown in 25% BHIS. Plates were incubated for 24 hours, media was removed, fresh media added, and incubation continued for a total of 48 hours. Blue represents *S. mutans*, red represents *A. oris*, and green represents *S. sanguinis*. All data represents the average of 3 wells.
B. Effects of Compounds 11, 16, & 21 on Mono-cultures

Figure 3 demonstrates the quantified results of a compound screening of Compounds 11, 16, and 21 against mono-cultures of each model strain. As in Figure 2, the graph depicts the resulting percent change in biofilm biomass, with respect to normal biofilm formation (no compound added), as a result of the addition of a compound. As seen in Figure 3, compound 16 greatly inhibited biofilm growth for all three bacterial strains, with a percent change around -70% for *S. mutans* and *A. oris* and around -80% for *S. sanguinis*. Also demonstrated in this graph, compounds 11 and 21 promoted biofilm formation in all three stains. Compound 11 increased growth for *S. mutans* by 20%, for *A. oris* by 25%, and for *S. sanguinis* by 24%. Compound 21 increased growth for *S. mutans* by 15%, for *A. oris* by 22%, and for *S. sanguinis* by 10%. 
Figure 3. Percent change in biofilm growth (biomass) for individual bacterial strains (monocultures) due to addition of either compound 11, 16, or 21, with respect to normal growth without compounds. All strains were grown in 25% BHIS. Blue represents *S. mutans*, red represents *A. oris*, and green represents *S. sanguinis*. The plate was incubated for 48 hours at 37 °C with 5% CO₂. All data represents the average of 3 or more wells.
C. Effects of Compounds 11, 16, & 21 on Multi-Species Co-culture

The graph in Figure 4 shows data from a screening of compounds 11, 16, and 21 against multi-species co-cultures, two species per well. It illustrates the resulting percent change in biofilm biomass resultant from the addition of one of the compounds. According to Figure 4, compound 16 greatly inhibited the biofilm growth of all three combinations of bacterial strains. Specifically, a change in the biofilm formation of -24% was seen for the *S. sanguinis* & *S. mutans* co-culture, *S. sanguinis* & *A. oris* biofilm formation was reduced by 79%, and *A. oris* & *S. mutans* biofilm formation changed by -61%.

Compound 11 promoted biofilm growth for all three co-culture combinations. *S. sanguinis* & *S. mutans* increased the most with a change of 22%, *S. sanguinis* & *A. oris* the least with an increase in growth of 6%, and *A. oris* & *S. mutans* had a resultant percent change of 10%. Compound 21 promoted the *S. sanguinis* & *S. mutans* as well as the *A. oris* & *S. mutans* co-cultures, with seemingly no change in biofilm formation for the *S. sanguinis* & *A. oris* co-culture. Specifically, *S. sanguinis* & *S. mutans* biofilm formation was increased by 24%, the larger of the two, while *A. oris* & *S. mutans* had a resultant change of 7%.

Table 1 is a compilation of 2-dimensional fluorescent confocal images which correspond to the data shown in Figure 4. The images are only segments restrained by the field of view and are not demonstrative of the whole biofilm. However, images were taken of sections that were more or less representative of the whole biofilm. The 1st column (top to bottom) of the chart is representative of normal biofilm growth for the three co-culture combinations. Moving across the rows (left to right), the images depict films grown in the presence of compound 16, compound 11, and lastly compound 21. Biofilms grown in the presence of compound 16,
shown in column 2, appear barren compared to their corresponding normal biofilm growth, depicted in column 1. Also notable in Table 1 is the “speckled” morphology seen in any of the cultures grown with S. mutans (rows 1 and 3). By comparison, the images of S. sanguinis & A. oris biofilms (row 2) are quite unique. Each image across row two looks like a different film.

Figure 4. Percent change in biofilm growth(biomass) for multispecies co-cultures due to addition of compounds 11, 16, or 21, with respect to normal growth without compounds. All strains were grown in 25% BHIS. Blue represents S. sanguinis & S. mutans, red represents S. sanguinis & A. oris, and green represents A. oris & S. mutans. Plate was incubated for 48 hours at 37°C with 5% CO₂. All data represent the average of 3 or more wells.
Table I. Fluorescence microscopy corresponding to multispecies co-culture experiments (shown in Fig. 4).

<table>
<thead>
<tr>
<th></th>
<th>BHIS</th>
<th>Compound 16</th>
<th>Compound 11</th>
<th>Compound 21</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. sanguinis</em> &amp; <em>S. mutans</em></td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td><em>S. sanguinis</em> &amp; <em>A. oris</em></td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td><em>A. oris</em> &amp; <em>S. mutans</em></td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>
D. 3-d Imaging Highlighting Compound 16

Table II is a compilation of results from 3-dimensional microscopy of mono-cultures of our three model strains of oral bacteria. The table focuses on normal biofilm growth (left column) and biofilms grown in the presence of compound 16 (right column). As qualitative data, the key points of these figures are the morphologies of each species. The biofilms formed by each individual strain has a unique appearance characteristic of that strain.

As seen in Table II, *S. mutans* has a “dotted” or “speckled” appearance. The biofilm covers the field of view uniformly with a series of small aggregates of bacteria. Within the biofilm there are spaces where no bacteria seem to be present. *A. oris* and *S. sanguinis*, on the other hand, each cover the field of view completely with growth, leaving no empty space. These strains have more of a “lawn”-like appearance, particularly *S. sanguinis*. They cover the field of view in a sea of green fluorescence. Though they share the “lawn” appearance, unlike *S. sanguinis*, *A. oris* biofilms also form large elevated “plateau” areas within the biofilm. Thus *A. oris* biofilms have more topography than either *S. sanguinis* or *S. mutans*. In summation, each strain has a distinct morphology; *S. mutans* biofilms have a “speckled” appearance, *S. sanguinis* forms more of a “lawn,” while *A. oris* is a combination of “lawn” and “plateaus.”

The addition of Compound 16 to the growth conditions results in vastly different morphologies for the biofilms (Table II). When compared to the large, robust, uniform “green sea” appearance of the normal biofilms, biofilms grown in the presence of compound 16 look barren. Instead of uniformity, the biofilms tend to aggregate into small clusters (at best).

Explicitly, *S. mutans* biofilms grown in the presence of compound 16 have an even more “speckled” appearance, with larger amounts of empty space and smaller aggregates (Table II).
A. oris biofilms grown in the presence of compound 16 lose their uniformity and “lawn” appearance. Instead they are almost void of any growth. The biofilm does form some small “plateau” aggregates. However, they are not nearly as large, nor as expansive as in normal A. oris biofilm growth. The S. sanguinis biofilm has also lost its uniformity, however has retained both small and large areas of aggregation. As such the biofilm no longer resembles a “lawn,” but is more of a series of small and large “islands.” Interestingly, the biofilm formed in the presence of compound 16 has more numerous and larger topographical features, “plateaus,” than seen in the normal S. sanguinis biofilm.

Table II. Three-dimensional confocal microscopy of mono-cultures of our three model strains of oral bacteria.

<table>
<thead>
<tr>
<th></th>
<th>BHIS</th>
<th>Compound 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. mutans</td>
<td><img src="image1" alt="S. mutans BHIS" /></td>
<td><img src="image2" alt="S. mutans Compound 16" /></td>
</tr>
<tr>
<td>A. oris</td>
<td><img src="image3" alt="A. oris BHIS" /></td>
<td><img src="image4" alt="A. oris Compound 16" /></td>
</tr>
<tr>
<td>S. sanguinis</td>
<td><img src="image5" alt="S. sanguinis BHIS" /></td>
<td><img src="image6" alt="S. sanguinis Compound 16" /></td>
</tr>
</tbody>
</table>
Table III depicts images from 3-dimensional microscopy of multi-species co-culture biofilms. The left column depicts normal biofilm growth while the right column illustrates biofilms grown in the presence of compound 16. The data in this table is qualitative, focusing on morphology and appearance. Among the images of normal biofilm growth the *S. sanguinis* & *S. mutans* and the *A. oris* & *S. mutans* biofilms more closely resemble each other than they do the third co-culture of *S. sanguinis* & *A. oris*. The biofilms that contain *S. mutans* as one of the two bacterial strains have a mostly flat, uniform, “lawn”-like appearance. In contrast, the *S. sanguinis* & *A. oris* biofilm resembles the *A. oris* biofilm seen in Table II, a “lawn” with “plateaus.”

The addition of compound 16 to the growth media resulted in some interesting changes in morphology. The *S. sanguinis* & *S. mutans* and the *A. oris* & *S. mutans* biofilms both lost their “lawn” appearance. While there still is growth that is fairly uniform, it has been interrupted with areas of no growth. As a result the *A. oris* & *S. mutans* biofilm has a “speckled” appearance, characteristic of *S. mutans* mono-cultures. Compared to this, the *S. sanguinis* & *S. mutans* biofilm has much larger aggregates. Finally, the *S. sanguinis* & *A. oris* biofilm lost most of its topography when grown with compound 16, though it retained its uniformity. As a result it has a plain “lawn” appearance, and resembles the *S. sanguinis* & *S. mutans* and the *A. oris* & *S. mutans* biofilms formed under normal growth conditions.
Table III. Three-dimensional confocal microscopy of co-cultures of our three model strains of oral bacteria.

Figure 5 demonstrates the quantified results of screening compound 13 at different concentrations (1 mM and 5 mM) against co-cultures. NaOH was also tested to control for any effect the solvent the compound is dissolved in might have. The graph depicts the percent change in SYTO-9 fluorescence as a result of the addition of the solvent or compound. Fluorescence is an indicator of biofilm biomass.

Addition of NaOH had a significant effect on *S. sanguinis* & *S. mutans* co-culture growth with a 28.7% reduction in growth. However, it also significantly promoted *A. oris* & *S. mutans* and tri-culture biomass with 40.5% and 37.2% increases in fluorescence respectively. For *S. sanguinis* & *A. oris* a 7.4% increase was witnessed.
Both 1 mM and 5 mM of compound 13 dissolved in NaOH promoted biofilm formation across the board. Compound 13 at 1 mM had the most significant effect on S. sanguinis & A. oris with an 86.7% increase in fluorescence. At a 1 mM concentration compound 13 increased growth for S. sanguinis & S. mutans, A. oris & S. mutans, and for tri-culture by 43.0%, 41.3%, and 36.7% respectively. Compound 13 at 5 mM also had the most significant effect on S. sanguinis & A. oris with a 66% increase in fluorescence. S. sanguinis & S. mutans, A. oris & S. mutans, and tri-culture biofilm biomass increased by 44.8%, 40.0%, and 30.9% respectively.

Table IV is a compilation of 2-dimensional fluorescent images taken with the Nikon 80i epifluorescence microscope corresponding to the data in Figure 5. The images are only segments restrained by the field of view and are not demonstrative of the whole biofilm. However, images were taken of sections that were more or less representative of the whole biofilm. These data are qualitative and do not give much information toward elucidating the effect of the compounds on biomass. However, we can note that the “speckled” morphology noted in Table I is less distinguishable in the images of Table IV. Furthermore, the addition of NaOH, column 2, seems to cause changes in morphology for all combinations tested. In general it seems that NaOH leads to more robust, uniform, healthier biofilms.

The addition of compound 13, columns 3 and 4 of Table IV, also seems to result in considerable changes in morphology, except for the case of the S. sanguinis & S. mutans co-culture, row 1. The S. sanguinis & S. mutans combination seems to increase in fluorescence without any drastic changes in the general appearance of the biofilm. In general, for the other co-cultures the addition of compound 13 seems to lead to an appearance consisting of a great
deal more of areas of congregation and very high fluorescence. This is most dramatic in the case of the *S. sanguinis* & *A. oris* co-culture. Perhaps, these are “plateaus” as were seen in the 3-d images of Table II and Table III. Finally, as was seen in Table I, *S. sanguinis* & *A. oris* co-cultures, row 2, seem to have the most unique appearance going across the row.

**Table IV.** Two-dimensional images taken using the Nikon 80i epifluorescence microscope with the 10x objective, corresponding to Figure 5.
Figure 5. Percent change in biofilm growth (biomass) for multispecies co-cultures due to addition of compound 3, with respect to normal growth without compounds. All strains were grown in 25% BHIS. Blue represents the solvent control, addition of NaOH. Red represents addition of 1mM compound 13 dissolved in NaOH. Green represents addition of 5mM Compound 13 dissolved in NaOH. The plate was incubated for 48 hours with a 24 hour media change at 37°C with 5% CO₂. All data represents the average of 3 wells.

F. Effects of Compound 3 (1mM vs. 5mM) on Co-cultures

Figure 6 depicts the quantified results of screening compound 3 at different concentrations (1 mM and 5 mM) against co-cultures. Water was also tested to control for any effect the solvent the compound is dissolved in might have. The graph depicts the percent
change in SYTO-9 fluorescence as a result of the addition of the solvent or compound.

Fluorescence is an indicator of biofilm biomass.

In general the addition of the solvent, H₂O, had little effect on biofilm formation. The only significant value shown is for the *S. sanguinis* & *S. mutans* co-culture which had a 17.9% reduction in biomass. The other three combinations had negligible changes, especially within the error for each. *S. sanguinis* & *A. oris* growth was promoted by 2.6%, *A. oris* & *S. mutans* growth was reduced by 3.8%, and tri-culture growth was reduced by 4.5%.

Figure 6 shows that 1 mM compound 3 modestly reduces biofilm formation for all combinations tested. Compound 3 at 1 mM reduced fluorescence by 37.6% for *S. sanguinis* & *S. mutans*, 33.5% for *A. oris* & *S. mutans*, and 32.5% for tri-culture. *S. sanguinis* & *A. oris* was affected the least with only an average percent change of -0.8%. 5mM Compound 3 greatly reduced biofilm formation for all combinations of bacteria tested. The *S. sanguinis* & *S. mutans* co-culture’s biomass was reduced by 91.1%, the *S. sanguinis* & *A. oris* biomass was reduced by 91.3%, the *A. oris* & *S. mutans* biomass was reduced by 94.9%, and the tri-culture biomass was reduced by 94.0%.
Table V is a compilation of 2-dimensional fluorescent images taken with the Nikon 80i epifluorescence microscope corresponding to the data in Figure 6. The images are only segments restrained by the field of view and are not demonstrative of the whole biofilm.

**Figure 6.** Percent change in biofilm growth (biomass) for multispecies co-cultures due to addition of Compound 3, with respect to normal growth without compounds. All strains were grown in 25% BHIS. Blue represents the solvent control, addition of H$_2$O. Red represents addition of 1mM Compound 3 dissolved in H$_2$O. Green represents addition of 5mM Compound 3 dissolved in H$_2$O. Plate was incubated for 48 hours with a 24 hour media change at 37°C with 5% CO$_2$. All data represents the average of 3 wells.
However, images were taken of sections that were more or less representative of the whole biofilm.

This data are qualitative and do not provide much information toward elucidating the effect of the compounds on biomass. Again, we can note (like in Table IV) that the “speckled” morphology noted in Table I is less distinguishable in the images of Table V. Images where H₂O was added, column 2, tend to have more empty space and a generally more disrupted appearance. The biofilms seem less uniform, except perhaps in the case of S. sanguinis & A. oris, row 2. The addition of 1mM compound 3, column 3, seems to change the morphology of each combination, especially those with A. oris, rows 2-4. These biofilms have less “fluffy” morphologies. Finally, the 5 mM Compound 3, column 4, images have tremendously reduced uniformity and barely resemble a biofilm at all. These biofilms look like they are small groups of cells or potentially even single cells.
Table V. Two-dimensional images taken using the Nikon 80i epifluorescence microscope with the 10x objective, corresponds to Figure 6.

![Table VI](image)

**G. 2-d (20x) and 3-d Imaging Highlighting 5mM Compound 3**

Table VI is a compilation of two-dimensional fluorescent images taken with the Nikon 80i epifluorescence microscope using the 20x objective. The images correspond to the data in Figure 6 and highlight the effect of 5mM Compound 3 on biofilm formation. The images are
only segments restrained by the field of view and are not demonstrative of the whole biofilm. However, images were taken of sections that were more or less representative of the whole biofilm.

Based on these images we can further qualitatively analyze the effect of compound 3 at a 5 mM concentration. It is very clear that the biofilm has been drastically disturbed. There are large areas void of growth and little to no uniformity or complexity. The *A. oris* & *S. mutans* co-culture appears to have a different morphology than the other three combinations. The biofilm consists of small cells and groups of cells. In comparison all of the other three combinations, which contain *S. sanguinis*, have long string-like formations.

**Table VI.** Two-dimensional images taken using the Nikon 80i epifluorescence microscope with the 20x objective, corresponds to Figure 6.

![Images](image1.png)

Table VII is a compilation of 3-dimensional fluorescent images taken with the Leica TCS SP5 confocal laser scanning microscope. The images correspond to the data in Figure 6 and highlight the effect of 5 mM compound 3 on biofilm formation. The images are much larger segments and are fairly demonstrative of the whole biofilm. Regardless, images were taken of
sections that were more or less representative of the whole biofilm. In comparison to the normal biofilm growth the biofilms treated with 5 mM compound 3 appear less uniform, less thick, and contain many spots void of growth. These images clearly illustrate a reduction in biomass. Interestingly, the images still strongly resemble a biofilm and seem to indicate a decent amount of growth. This is particularly interesting when compared to previous two dimensional images that appear almost to be single cells and practically absent of biomass.

Table VII. Three-dimensional images taken with the Leica TCS SP5 confocal laser scanning microscope highlighting the effect of 5mM Compound 3 on biofilm formation.
Discussion

A. Prior Work

Prior experiments elucidated 48 hour incubation at 37°C with 5% CO₂ as optimal incubation conditions for our three model strains. We also had found A. oris difficult to grow on polystyrene thus leading to the use of glass bottom plates for our experiments. These plates allowed for the use of fluorescent analysis and imaging, previously we had used crystal violet and absorbance measurements to quantify our data and to image our biofilms. Crystal violet has the tendency to stick to the walls of the well, even after multiple rinses, and misrepresent the abundance of bacteria present. Thus switching to glass bottomed plates, SYTO 9 dye, and fluorescent analysis simultaneously increased the stability of our biofilms while also decreasing statistical error.

Figure 1 summarizes the end result of our attempts to optimize our growth assay. S. mutans has been proven to thrive off the presence of sucrose in the oral cavity. We thus hypothesized that sucrose would be a beneficial addition to our BHI growth media. Other work has shown that simple sugars are indeed beneficial to the growth of some strains of oral bacteria, with sucrose being particularly effective in increasing biofilm growth. As shown in Figure 1 all of our model bacterial strains grew better in the presence of 2% sucrose, thus proving our hypothesis correct. Of the three strains only S. sanguinis was able to grow proficiently without sucrose.

The end goal of our optimization experiments was to elucidate a common set of conditions that promotes stable, robust biofilms for all three strains. To this end, our data points to the optimal media for simultaneous growth of all three strains to be 25%-50% BHI
with 2% sucrose (Figure 1). If we were interested in studying single strains 90% BHIS would be the best media choice for *S. mutans* and *S. sanguinis* biofilm growth. This is because, for these strains, as BHI concentration increases biofilm biomass increases linearly. This differs from *A. oris* whose biofilm formation shares a parabolic relationship to BHI concentration, reaching a maximum around 25-50% BHI. However, we are interested in studying multispecies biofilm formation. Therefore, the optimum media choice would be in this *A. oris* climax range of 25-50% BHIS, at a concentration where all three strains grow well.

Figure 2 shows previous work done by Samantha Testa and Steve Kasper. They screened 21 organically inspired compounds against mono-cultures of our model oral bacterial strains. Previously in our lab Jason Behnke screened a library of similar organic compounds (compounds A-Z) against *Pseudomonas Aeruginosa* (Cady NC, et al, 2012). These compounds had a variety of activity, and many were shown to be involved in quorum sensing pathways. The compounds in Figure 2 also had a variety of activity having an array of effects on biofilm formation. We used these data to select compounds for more detailed analysis, imaging, and use in our multi-species experiments.

Specifically we chose compounds 11, 16, and 21 for our initial work with multi-species co-culture screening. We chose compound 16 as it demonstrated the greatest reduction in biofilm formation of all compounds screened, with a percent change around -80% for *A. oris* and *S. sanguinis* and close to 100% reduction for *S. mutans*. Compound 21 was selected because it was one of a few compounds that promoted all of the three strains. It also demonstrated the largest statistical increase in growth (45% for *S. mutans* and 50% for *A. oris*) among the six compounds that promoted biofilm formation for all three strains. Finally we
selected compound 11 because it had interesting differential activity. Specifically compound 11 promoted *S. mutans* (45% change) and inhibited *S. sanguinis* (-25% change) with seemingly no effect on *A. oris.*

In our future work we will examine other compounds from this screening pool, selecting based on similar criteria (Figure 2). That is, we will focus on which compounds promote all three strains, which compounds inhibit all three strains, and which compounds show differential activity that might yield interesting results, elucidating further upon how these species interact with one another in a communal biofilm. Compounds 3 and 5 would be interesting as reduction compounds. They had the most similar results across the three strains, having around 35% and 25% reduction for each strain, respectively. Two good promoting compounds we would like to study further are Compounds 13 and 14. Both had percent changes for all three stains that were close to one another, around 35%. We also will examine compounds that have been particularly effective in blocking cell-to-cell communication in quorum sensing models. Compounds 3, 5, 8, 13, 16, 17, and 20 are good choices for study, based on quorum sensing experiments conducted by Sam Testa and Steve Kasper. We chose to focus on compounds 3 and 13 later in this paper for these reasons.

B. Compounds 11, 16, & 21 on Individual Strains

Figure 3 is shows the percent change in SYTO 9 stained biofilm from a screening of compounds 11, 16, and 21 against mono-cultures of our three model organisms. Based on prior work done in our lab (Figure 2) it was expected to see inhibition of growth for all three strains when compound 16 was added to the culture. This hypothesis was upheld, as shown in Figure
3 compound 16 resulted in a minimum of 70% reduction in biofilm formation for the three strains tested.

It was further hypothesized from prior work that compound 11 would have differential activity. Previously we had seen an increase in biofilm formation for \textit{S. mutans}, little effect for \textit{A. oris}, and a decrease in biofilm formation for \textit{S. sanguinis}. However, the results in Figure 3 do not coincide with previous results. Instead of differential activity, a promotion of biofilm formation was seen for all three strains. It is possible that this is a result of contamination due to poor experimental technique. Another possibility is that the compound has degraded over time, taken on a new structure and therefore changed in activity.

Comparatively, for compound 11 where previously a 45\% increase had been seen, in this experiment we saw a 20\% increase in the biofilm formation of \textit{S. mutans}. For \textit{A. oris} the previously insignificant change of 1.5\% increased in this experiment to a 25\% intensification of biofilm growth. Finally and most peculiar of all, instead of an inhibition of biofilm formation resulting in a -25\% change, Figure 3 shows an increase in \textit{S. sanguinis} biofilm formation of 24\%.

As for compound 21, it was theorized that an increase in biofilm growth would be seen across all three strains, but most especially for \textit{S. mutans} and \textit{A. oris}. This trend was seen again, however in prior work \textit{S. mutans} and \textit{A. oris} had percent changes of 45\% and 50\% whereas in Figure 3 they have increases above normal growth of only 15\% and 22\%. Thus they had about 3 fold and 2 fold decreases in their previous potency. Similarly, \textit{S. sanguinis} biofilm formation was promoted as expected but by half as much as seen in initial experiments (20\% change vs. 10\% change).
Compound 16 seems to be undoubtedly able to inhibit biofilm formation. However, the results in Figure 3 for compound 11 and compound 21 demand the repetition of this experiment. Perhaps there was some contamination within the growth media, or perhaps the compounds have degraded over time and therefore lost some activity. If results are still aberrant from previous work then it will be necessary to obtain a fresh supply of our small molecule inhibitors.

C. Compounds 11, 16, & 21 on Multi-Species Co-culture

In-vivo bacterial biofilms do not consist of merely one species but several, if not hundreds (Blehert, D.S., et al, 2003; Kolenbrander, P. E., 2000; Kuramitsu H. K., et al, 2007). Therefore, it is important to study not only single species biofilms in-vitro but multi-species biofilms as well. The data presented in Figure 4 is from one such experiment. Our three compounds of interest were tested against co-culture biofilms of S. sanguinis & S. mutans, S. sanguinis & A. oris, or A. oris & S. mutans.

Based on our mono-culture experimental results, we hypothesized that compound 16 would inhibit all three co-cultures having inhibited each individual strains biofilm formation. However, we also thought it might be possible that some complimentary degradation by the co-cultures might be able to render the compound ineffective. Since compound 21 had enhanced biofilm formation for each strain, it was postulated that growth would increase for all three combinations of co-culture. However, we were unsure how the multi-species biofilms might behave.
For compound 11 we expected to see some interesting effects through microscopy. Because it had promoted biofilm formation for *S. mutans* and inhibited biofilm formation for *S. sanguinis*. If this result carried over to the co-cultures, we expected to see marked differences between the morphologies of control co-culture biofilms and those with compound 11.

Because compound 11 enhances biofilms of *S. mutans*, inhibited biofilms of *S. sanguinis* and had no effect on biofilms of *A. oris* we expected to see for *S. sanguinis* & *S. mutans* a biofilm almost entirely composed of *S. mutans*, for *S. sanguinis* & *A. oris* a predominately *A. oris* biofilm, and for *S. mutans* & *A. oris* a strongly *S. mutans* biofilm.

Figure 4 supports the hypothesis that compound 16 would inhibit all of the combinations. However, it is interesting to note that for the *S. sanguinis* & *S. mutans* co-culture biofilm formation was only reduced by 24%. For individual strains the smallest reduction was the -68% change seen in Figure 3 for *S. mutans*. In the other two co-cultures the reduction was two-to-three fold greater at -61% change and -79% change. Perhaps there is some sort of resistance involved with this combination. It would be interesting to see if this kind of result persists in future experiments.

In Figure 4 compound 11 modestly promoted growth for all three strains. This was not entirely unexpected. The Compound had been seen to promote biofilm formation for *S. mutans* and inhibit biofilm formation for *S. sanguinis*. Perhaps in the co-cultures where *S. mutans* was present the species was able to thrive. Furthermore, for the combinations with *S. sanguinis*, the species would be inhibited allowing for its partner species to thrive off of nutrients that might not have been previously available due to competition. Indeed the greatest increase in growth was seen for the *S. sanguinis* & *S. mutans* combination, where *S.
sanguinis would have been inhibited and S. mutans promoted. Furthermore, the least increase in biofilm growth was seen for S. sanguinis and A. oris where S. sanguinis would likely have been inhibited, while A. oris would have been unaffected by the compound but still slightly promoted due to the reduced competition for resources.

Finally compound 21 promoted the S. sanguinis & S. mutans as well as the A. oris & S. mutans co-cultures, with no noticeable effect for the S. sanguinis & A. oris combination. Previously, compound 21 had promoted biofilm growth for all three individual strains. We therefore hypothesized that the percent change for all three co-cultures would be positive as well. This was accepted for two out of the three combinations where we saw promotion of biofilm growth. However, it did not agree with the results of the S. sanguinis & A. oris co-culture. Perhaps some interaction or metabolic product reduces effects of the compound. However, it might be more likely that some sort of error was involved. Perhaps the compound has degraded over time into other molecular structures that do have novel activity. Regardless, further testing must be conducted to clarify if this is a normal occurrence. It might be that quantified data might not illuminate the answers we seek but rather be up to inventive imaging techniques to elucidate what is occurring.

Fluorescence measurements tell us a great deal about our model organisms, how they grow, and how they are affected by our organically inspired compounds. However, fluorescence alone fails to clarify exactly what is occurring. Table I is a compilation of two-dimensional images taken with the confocal microscope of our co-cultures. Through imaging we are able to see the effects of our compounds, see the changes in morphological structure,
see the reduction or promotion of fluorescence and growth, and learn a little more about the 
behaviors of oral biofilm forming microbes.

Our hypotheses for this experiment are consistent with those for Figure 4, stated above.
Having seen reduction in biofilm formation for all of the individual strains with the addition of 
compound 16, both in graphs and charts as well as in images, we expected to see this for the 
three combinations as well. This hypothesis was largely supported by the data shown in Table I. 
Column 2 corresponds to biofilms formed in the presence of compound 16. When compared to 
their normal growth counterparts in column 1, they are barren. Where in column 1 the field of 
view is covered in robust, uniform biofilms and therefore filled with green fluorescence, the 
images in column 2 are void of life and are largely black and empty. It should be noted that 
there still is growth for the S. sanguinis & S. mutans combination. However, if we consult 
Figure 4 we see that for this combination only a -24% change resulted. Further experiments 
may clarify whether this is a consistent trend, or perhaps some sort of error. Regardless, for 
the other two combinations over 60% reduction was quantified in Figure 4 and further 
represented here through microscopy.

It is difficult to gather a great deal of information from the images in columns 3 and 4 of 
Table I besides that they appear to be healthy, robust, stable, uniform biofilms. Differences in 
brightness, i.e. fluorescent intensity, can be noted. However, it is difficult to draw such a 
conclusion lacking the quantified data, especially because these images are only of segments of 
the biofilm and while are hopefully representative images; they are not pictures of the whole. 
That said, in Figure 4 a promotion of biofilm growth was noted for both compounds. Ideally we 
would like to be able to differentially stain the two different species comprising this biofilm, to
see their relative contributions to the overall film. This way if any interesting occurrences such as one strain dominating the biofilm or localization to certain areas of the biofilm, we would be able to differentiate and clarify as such. At this time, however, we do not have the appropriate labeled antibodies to perform this differential staining.

Something of note that can be readily identified from this table is a general characterization of the biofilm morphologies developed by each strain. As will be further articulated later in this paper, each strain has been seen to have a specific visual pattern (Table II and Table III). Specifically, *S. mutans* biofilms have a “speckled” morphology; *S. sanguinis* forms more of a “lawn,” while *A. oris* forms a combination of “lawn” and “plateau” features. This has been seen in prior work as well as presented here in the tables of confocal images. Information to this end has yet to be elucidated for our co-culture biofilms. However, seen in the confocal images of Table I are some interesting phenomena, important to note and study further.

Firstly, each co-culture biofilm that contains *S. mutans*, save *S. sanguinis* & *S. mutans* grown with compound 21, has a similar appearance. This “dotted” or “speckled” morphology is very like that seen for *S. mutans* mono-cultures in previous work and demonstrated here in Table II. Perhaps *S. mutans* is dominating these biofilms, out-competing the other strain in the culture. It is also possible that the location of the image is only a segment of localized growth, and that this trend is merely related to consistently poor imaging. Acquisition of differential staining techniques would likely help elucidate exactly what is occurring.

Another trend to note is that each of the images of *S. sanguinis* & *A. oris* is quite unique. This is especially true when compared to the trend stated above with the *S. mutans*
morphology. By contrast, these images each have a distinct morphology of their own. Beyond this however, not much can be said without some way of distinguishing *A. oris* from *S. sanguinis*.

D. 3-d Imaging Highlighting Compound 16

We hoped to use confocal microscopy to elucidate a better understanding of the morphologies of our biofilms. This would prove particularly useful for our co-cultures where distinctions between strains might be distinguishable. Furthermore, said distinctions would surely be revealing into the interactions and behaviors of multi-species biofilm formation and growth.

Table II is a compilation of results from confocal microscopy of mono-cultures of our three model strains of oral bacteria. We hypothesized distinct morphologies might be seen for each individual strain, as had been observed throughout previous experimentation and imaging. Furthermore, Table II focuses specifically on compound 16, which was hypothesized to yield greatly reduced biofilm growth based on fluorescence data and prior observation (Figure 2, Figure 3).

Both of these hypotheses were confirmed. Firstly, in Table II each strain has a distinct morphology; *S. mutans* biofilms have a “speckled” appearance, *S. sanguinis* forms more of a “lawn,” while *A. oris* is a combination of “lawn” and “plateaus.” These findings fit with observations in earlier experimentation. In further experiments these results should be verified through further microscopy. They could be key to our understanding of multi-species biofilms.
Our second hypothesis, that images of compound 16 would show decreased biofilm formation was confirmed. Compared to the images of normal biofilm growth those of biofilms grown in the presence of compound 16 have reduced growth, with much less fluorescence and bacteria visible and a great deal more black and empty space. This is consistent with quantified data in Figure 2 and Figure 3 where biofilm formation was inhibited by a minimum of 70% across the three strains. In addition to reduction of growth Table II shows notable changes in the morphologies of our model biofilms, resultant from the addition of compound 16. The biofilms seem to tend to form small aggregates (at best), as opposed to the typical robust, uniform biofilm.

*S. mutans* biofilms grown in the presence of compound 16 have an even more “speckled” appearance, with larger amounts of empty space and smaller aggregates. *A. oris* biofilms grown in the presence of compound 16 lose their uniformity and “lawn” appearance retaining some small “plateau” aggregates. However, these are not nearly as large, nor as expansive as in normal *A. oris* biofilm growth. The *S. sanguinis* biofilm has also lost its uniformity, however has retained both small and large areas of aggregation. As such the biofilm no longer resembles a “lawn,” but is more of a series of small and large “islands.”

Interestingly, the *S. sanguinis* biofilm formed in the presence of compound 16 has more numerous and larger topographical features, “plateaus,” than seen in the normal *S. sanguinis* biofilm. Whether this is a consistent occurrence or some form of error has yet to be proven. It is possible in all of these cases that these are not fully representative images. The confocal only is able to image a section of the well in which the biofilm has been grown. While we do our best to take representative images, it is possible that these images are the exception under
these conditions for what is happening. Further experimentation and imaging under the same conditions could help verify these results.

Besides repeating this experiment to validate the accuracy of our findings there a few directions we can take this in the future. Firstly, confocal microscopy only allows for a specific portion of the plate to be imaged (without compiling multiple image stacks from across a larger area). Thus there was not a complete set of images for compound 11 or compound 21. In addition to verifying our data we should run experiments to target these compounds and get 3D images of their resulting biofilms. It is especially important to understand how our mono-cultures are affected by the compounds so that we might better interpret the results with our co- and tri-cultures. We also will be moving on to examining other interesting compounds whose effects are shown in Figure 2. It will be interesting to see if different compounds cause different morphologies. Or perhaps all biofilm reducing compounds will result in the same appearance.

Table III presents image results from confocal microscopy of multi-species co-culture biofilms. Again we hypothesized that 3D imaging would help elucidate further upon cell-to-cell interactions and the intricacies of multi-species biofilms. We hypothesized based on 2D imaging results (Table I) that we would see reduction of growth resultant from compound 16, and distinct changes in morphologies.

We had expected each combination to be distinct in appearance. However, the normal biofilms of S. sanguinis & S. mutans and the A. oris & S. mutans biofilms more closely resemble each other than they do the third co-culture of S. sanguinis & A. oris. Both of these biofilms have S. mutans and as stated earlier with regard to Table I it seems that the strain might be
dominating. These *S. mutans* co-culture biofilms have a mostly flat, uniform, “lawn”-like appearance. The *S. sanguinis* & *A. oris* biofilm however, is a “lawn” with “plateaus.”

With the addition of compound 16 the two co-cultures containing *S. mutans* both lost their “lawn” appearance. They still had a good amount of uniform growth, however instead of a “lawn” of growth, the bacteria clustered into aggregates, leaving areas where no growth is present. This gives the *A. oris* & *S. mutans* biofilm a “speckled” appearance very similar to the morphology of *S. mutans* mono-cultures. Perhaps, *S. mutans* was able to resist the inhibitory effect of the compound, thus explaining the resulting morphology. A staining technique that specifically targeted *S. mutans*, or even targeted *Streptococcus* bacteria, might clarify if this indeed explains the change in appearance.

In comparison, the *S. sanguinis* & *S. mutans* biofilm has much larger aggregates. Perhaps this is some combination of the normal *S. mutans* “speckled” biofilm and the *S. sanguinis* morphology witnessed after the addition of compound 16 (Table II). This distinction would require more sophisticated differential staining techniques, given that both strains are *Streptococci*. Perhaps a strain genetically modified to express a fluorescent tag or antibodies that would label specific strains would be useful here.

The addition of compound 16 to the *S. sanguinis* & *A. oris* co-culture appears to have reduced the topography, leaving a “lawn” without “plateaus.” The biofilm still is uniform and has almost no empty space. It is quite similar to the *S. sanguinis* & *S. mutans* and the *A. oris* & *S. mutans* biofilms formed under normal growth conditions.

In the 2D images of Table I the addition of Compound 16 appears to obliterate the biofilms, leaving the images baren and void of life. Here however (Table III) the biofilms all
retained growth, just had altered morphologies. This difference is undoubtedly a difference in imaging technique. One explanation could be that our interest in witnessing morphology change directed imaging toward growth as opposed to images representative of the biofilm as a whole. It is very possible that large sections of the well were empty/void of life. Future experiments should attempt to capture a more representative image to give a better idea of the biofilm as a whole and the effects of the compounds.

E. Effects of Compound 13 (1mM vs. 5mM) on Co-cultures

Work by Stephen Kasper and Samantha Testa in our lab has shown compound 13 promotes biofilm growth for all three of our model strains (Figure 2). Additional work has shown that the concentration of the compound has an influence on its activity. Furthermore, in preliminary testing of compounds in Quorum Sensing models compound 13 stood out as having an interesting activity. Specifically, the addition of compound 13 seemed to increase cell-to-cell communication.

Figure 5 shows the percent change in fluorescence results for a screening of compound 13 against our three co-culture combinations and against tri-culture. In addition to testing a new compound at two different concentrations, this experiment was incubated for 48 hours, as before, but with the addition of a media change after 24 hours. Finally, the solvent in which compound 13 is dissolved (NaOH) was also screened against our combinations as a control.

Based on prior work we hypothesized compound 13 would increase biofilm biomass for all of combinations having previously promoted biofilm formation for individual model strains in mono-culture. Figure 5 confirms this hypothesis where we see that both 1 mM and 5 mM
compound 13 promoted biofilm formation across the board and had the greatest increase in biomass for the *S. sanguinis* & *A. oris* co-culture. Specifically, 1 mM compound 13 increased the *S. sanguinis* & *A. oris* co-culture growth by 86.7% and 5 mM compound 13 increased growth by 66.4%. This is interesting because the lower concentration resulted in a greater promotion of growth. One might assume that a higher concentration would result in greater activity, as is the case for compound 3 as seen in Figure 6.

This increased growth for the lower concentration is only for this one combination however. In the cases of *S. sanguinis* & *S. mutans*, *A. oris* & *S. mutans*, and for tri-culture the two concentrations have very comparable activity. Compound 13 at 1 mM induced increased biofilm formation of 43.0%, 41.3%, and 36.7%, respectively. However, 5 mM compound 13 resulted in changes of 44.8%, 40.0%, and 30.9%, respectively (Figure 5).

Another interesting observation one can note in Figure 5 is that the solvent, NaOH, is having an effect on biofilm formation. We had hoped that NaOH would have negligible activity, as having activity might suggest that our results for compound 13 might be related to the presence of NaOH. Indeed, the results for addition of NaOH to the *A. oris* & *S. mutans* combination as well as the tri-culture combination could be used to argue that exact point. Both of those combinations had percent changes of 40.5% and 37.2%, respectively, which is almost exactly the same values obtained for 1 mM compound 13 and very similar to those obtained for 5 mM compound 13 (40% and 31% respectively).

However, this might not be exactly the case. While the 5 mM compound 13 values are comparable, there is a lower concentration of NaOH due to an increased concentration of the solute. This suggests that perhaps if NaOH is playing some role, it is not the driving force behind
the observed increased growth. Furthermore, NaOH did not increase growth for the other two combinations. In fact an average reduction of growth by 28.7% is seen for *S. sanguinis* & *S. mutans* co-culture and there was a negligible change in growth for the *S. sanguinis* and *A. oris* co-culture (7.4%). For these two co-culture combinations compound 13 at both concentrations was seen to cause increased biofilm biomass. This caveat is most especially true in the case of *S. sanguinis* & *A. oris* as this combination had the largest promotion of growth following the addition of compound 13 at either concentration.

This quantitative data suggest a need for further exploration into the effects of NaOH on biofilm formation. The results in Figure 5 do not clearly elucidate whether it always promotes growth, or whether it is dependent on the species of bacteria present. It might be best to run some tests on mono-cultures to see if it is the presence of a particular strain that gave the results seen. For said troubleshooting experiment one might expect to see NaOH to have a strong effect on either *A. oris* or on *S. mutans*. Regardless, it would seem that compound 13 at either a 1 mM or a 5 mM concentration results in an increase in biofilm growth of all four of these combinations of multi-species biofilms. Whether or not the solvent plays a role can neither be strongly confirmed nor strongly denied.

Fluorescence data give the most accurate understanding of what is occurring as a result of the addition of one of the compounds. That said, imaging gives a qualitative understanding of what might be occurring and helps to elucidate a further understanding of what the compounds are exactly doing. Table IV is a compilation of 2D fluorescent images taken with the Nikon 80i epifluorescence microscope and corresponds to the data in Figure 5. The images are only segments restrained by the field of view and are not demonstrative of the whole biofilm.
However, images were taken of sections that were more or less representative of the whole biofilm.

While qualitative and therefore limited in what information can be gathered we cannot some interesting changes in morphology following the addition of different substances. In Table IV column 2 on can see that NaOH changes the morphology of all of the combinations tested. What generally is seen is a more robust, more uniform biofilm. This is interesting when compared to the quantitative data as even in the cases where biomass was reduced or negligibly changed, row 1 and 2, the community that is there has less empty space within it. 3D imaging like that done for compound 16 in Table II and Table III and for compound 3 in Table VII would likely illustrate these changes most interestingly. Furthermore, if it were possible to differentially stain the strains we might see the greater complexity involved.

In columns 3 and 4 of Table IV we can note how compound 13 at 1 mM and at 5 mM concentrations affects the morphology of the biofilms. One can see that there are considerable changes in morphology for all combinations except for the *S. sanguinis* & *S. mutans* co-culture in row 1. This combination does not seem to be as drastically effected as the others. In addition, the 1 mM concentration seems very similar to the normal growth in BHIS. The other three combinations however, seem to have increased formation of congregations where large blobs of very high fluorescence can be noticed. This is most dramatic in the case of the *S. sanguinis* & *A. oris* co-culture. For this combination unlike the others, the biofilm goes from having a few internal spaces absent of growth to being a completely uniform sprawl of growth.

These congregations and areas of “blob” fluorescence may be nothing more than an issue with the dye sticking in the extra-polymeric matrix. However, perhaps these are actually
“plateaus” as were seen in the 3D images of Table II and Table III. Again, a more complete collection of 3D images along with differential staining techniques would greatly enhance our qualitative understanding of these bacterial communities and the complexity of their morphologies.

F. Effects of Compound 3 (1 mM vs. 5 mM) on Co-cultures

Work by Stephen Kasper and Samantha Testa in our lab has shown compound 3 reduces biofilm growth for all three of our model strains (Figure 2). Additional work has shown that the concentration of the compound has an influence on its activity. Furthermore, in preliminary testing of compounds in Quorum Sensing models compound 3 stood out as having an interesting activity. Specifically, the addition of compound 3 seemed to decrease cell-to-cell communication.

Figure 6 shows the percent change in fluorescence results for a screening of compound 3 against our three co-culture combinations and against tri-culture. In addition to testing a new compound at two different concentrations, this experiment was incubated for 48 hours, as before, but with the addition of a media change after 24 hours. Finally, the solvent in which compound 3 is dissolved (water) was also screened against our combinations as a control.

Based on prior work we hypothesized compound 3 would decrease biofilm biomass for all of combinations having previously reduced biofilm formation for individual model strains in mono-culture. Furthermore, for compound 3 in particular we expected to see a concentration dependence based on work done in our lab. Figure 6 confirms this hypothesis where one can see that 1 mM compound 3 modestly inhibited biofilm formation for all combinations (~35%)
save for the *S. sanguinis* and *A. oris* combination where no activity was really seen (0.8%). Also, the addition of 5 mM compound 3 reduced biofilm formation across the board. Furthermore while 1 mM compound 3 reduced a decent amount, 5 mM compound 3 reduced all four combinations by more than 90%.

The solvent control in this experiment was addition of water. We had hoped to see little to no effect as to confirm that any activity witnessed was indeed the influence of the compound. In general the addition of water had little effect on the fluorescence. The only significant value shown was for the *S. sanguinis* & *S. mutans* co-culture which had a 17.9% reduction in biomass. The other three combinations had negligible changes. That said, the results from imaging the biofilms showed some interesting changes in morphology.

Table V is a compilation of 2D fluorescent images taken with the Nikon 80i epifluorescence microscope corresponding to the data in Figure 6. The images are only segments restrained by the field of view and are not demonstrative of the whole biofilm. However, images were taken of sections that were more or less representative of the whole biofilm. Qualitatively we can assess that while H₂O did not greatly reduce the overall biomass of the biofilms the kind of film formed does seem to be more disrupted with more open and empty space within the biofilm (column 2 of Table V). The biofilms seem less uniform, except perhaps in the case of *S. sanguinis* & *A. oris*, row 2. Regardless, when compared to the biofilms formed after the addition of compound 3 at either concentration these biofilms seem healthy and look much more like their normal growth counterparts in column 1.
The addition of 1 mM compound 3, column 3, seems to change the morphology of each combination, especially those with *A. oris*, rows 2-4. These biofilms have less “fluffy” morphologies. The combinations that have *S. mutans* present also seem to obtain more of the “speckled” appearance previously noted in Table I. The most interesting information one can elucidate from this table however, is the effect caused by the addition of 5 mM compound 3. In column 4 of Table V, for every combination one sees a drastic reduction in the ability of the cells to come together and form their typical biofilms. This correlates well with Figure 6 where over 90% reduction of biomass was witnessed. These biofilms almost looks like they are single planktonic cells, though it is more likely that they are small groups of cells.

**G. 2-d (20x) and 3-d Imaging Highlighting 5mM Compound 3**

Table VI is a compilation of 2D fluorescent images taken with the Nikon 80i epifluorescence microscope using the 20x objective. With these images we can take a closer look at the biofilms and examine any differences they may have in morphology. To this end we see that the *A. oris* & *S. mutans* co-culture lacks the long string like formations that are present in the other three combinations. It is possible that these formations are specifically related to the presence of *S. sanguinis* as it is present in all three of the other combinations. Furthermore, instead of having these strings, the *A. oris* & *S. mutans* biofilm seems to consist of small groupings, giving the biofilm a single cell like appearance. This begs the question of whether this is a biofilm at all.

Based on the images in Table V and Table VI we get the impression that we are looking at planktonic growth rather than biofilm growth. However, if there was no biofilm like qualities
and these truly were single planktonic cells it would be expected that the rinsing step of our procedure would remove them from the well. Table VII is a compilation of 3D fluorescent images taken with the Leica TCS SP5 confocal laser scanning microscope. The first thing one can note is that there is definitely a reduction in biomass when 5 mM compound 3 is added to the well. Furthermore, we see that there is more empty space within the well whereas the normal growth is uniform and healthy. However, the most interesting thing we can take away from these images is the stark difference between them and the 2D images of Table V and Table VI. Whereas before we might have thought we were looking at planktonic growth, in Table VII we can clearly see that there are qualities characteristic of biofilms. While the 5 mM compound 3 biofilms are certainly less thick and heavily disrupted, they do form congregations and do have a greater presence than one might have thought based on the 2D images.
Conclusions

This study focused on the effects of small molecule inhibitors on single and multi-species biofilms, formed by orally-associated bacteria. Optimization assays elucidated a set of conditions common for all three strains that produced the formation of robust, uniform biofilms. These conditions are not necessarily best for the individual strains but have been shown to give consistent results for all three model organisms. Using these growth conditions we were able to successfully grow stable multi-species biofilms and test the activity of inhibitory compounds against these biofilms. Results from this screening were somewhat aberrant from the prior work. This may have been an issue of contamination, or could have been related to degradation of the compounds.

We saw that the activity of a compound on a monoculture biofilm can, but does not always, correlate to the activity of the compound when used on a multi-species biofilm. Also, certain compounds showed concentration dependent activity, where higher concentrations produced a greater effect. We also saw that the solvent in which a compound is dissolved could play a role in the changes observed. This seems particularly true in the case of morphological changes.

Using microscopic analysis, we elucidated that the compounds and solvents often affect the structure and morphology of the biofilm. Even in cases where the compound or solvent, showed negligible activity in terms of effecting biomass and growth, a dramatic change in appearance can still result. Microscopy also showed that each strain (S. mutans, S. sanguinis, and A. oris) forms a biofilm with a distinct appearance. It seems that the appearance of the multi-species biofilms might be in some part determined by which of these strains are present.
In some cases it seems that one species may be dominating the other(s). In the future, we hope to obtain antibodies that will differentially stain the strains, illustrating more clearly any specific inter-species interactions.
References


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