THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF CHEMICAL ENGINEERING

AN OUNCE OF PREVENTION: EFFORTS TO DISPLACE BIOFILMS USING AN
ENGINEERED BIOFILM FOR REVERSE OSMOSIS APPLICATIONS

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ABSTRACT

The use of an engineered biofilm to prevent undesired biofouling in membrane systems offers a novel approach to addressing a fundamental problem faced in water purification. The goal of this project was to determine the efficacy of this beneficial biofilm model when used as a treatment for established biofilms rather than as a preventative measure. The ability of the engineered strain of *E. coli* to displace and replace an established biofilm was investigated via 96-well crystal violet assays, dead-end filtration tests, and SEM imaging. It was found that application of the beneficial strain after fouling had occurred only resulted in further fouling, and that the beneficial strain was unable to outperform the control strain when growing in a competitive environment. Future work may show that the model can be used to displace less-aggressive foulants. At present, however, the beneficial biofilm is only effective when formed in advance of fouling, and cannot be used to remove an established biofilm.

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Chapter 1

Problem Statement

The purpose of this project was to investigate the potential application of an engineered biofilm as a treatment for established biofilms of undesired microbes in membrane systems. Such undesired biofilms inhibit the processing of saltwater and wastewater sources to produce freshwater, which is necessary to support life and to generate power.[1,2] Growth of global populations and industry has led to an increased demand for reliable and affordable methods of water purification, as well increased reliance on lower-quality water sources.[3,4] Water treatment membranes have served as one of the most effective means of removing contaminants from low-quality sources.[4] Particulate matter and microbes can be removed by microfiltration and ultrafiltration membranes, while reverse osmosis (RO) membranes allow for the removal of salts and other dissolved contaminants.[5]

However, this technology does face some significant challenges. One of the most detrimental challenges to membrane systems is biofouling, when microbes attach to the membrane surface and establish a polymeric matrix.[6-8] Once formed, such biofilms are difficult to combat via traditional membrane-cleaning methods. They greatly reduce membrane flux and trap salt in the biofilm near the membrane surface, leading to a decline in rejection and increasing the osmotic-pressure barrier for filtration.[9] Their interference with performance also causes an increase in energy consumption.[10] While disinfectants and other common biofilm treatments may be initially effective, biofilms are often able to adapt to and overcome these methods.[11] In RO processes, two

of the most commonly occurring foulants are *P. aeruginosa* and *S. wittichii*.[12-15] In order to combat these resilient microbes, new solutions to membrane biofouling are of particular importance.

It was previously shown by Wood, et al. that an engineered biofilm could be used to significantly reduce undesired biofilm formation on membrane surfaces.[16] After being intentionally formed on a membrane surface, this beneficial biofilm, engineered in *E. coli*, generates nitric oxide (NO) bubbles. NO serves as a general biofilm dispersant, and was shown to be effective against *P. aeruginosa*, one of the most problematic biofoulants for membrane systems due to the aggressive nature of its growth.[17,18] The beneficial biofilm also limits its own growth via a quorum sensing (QS) circuit, the LasI/LasR system of *P. aeruginosa*, thereby ensuring that acceptable membrane flux is maintained despite its formation.

Having shown the effectiveness of this engineered biofilm as a preventative measure, its utility as a treatment after undesired biofouling had already occurred was of significant interest. Using the beneficial strain as an intermittent treatment rather than a prolonged protective measure would be more practical and reduce costs. Furthermore, Hong, et al. had shown that biofilm dispersal using a second cell type was possible in mircofluidic devices, supporting the notion that biofilm dispersal could be effective in membrane systems.[19] Considering these results, particularly the ability of the engineered biofilm to disperse potential foulant microbes, it was hypothesized that the beneficial biofilm microbes would be capable of displacing an established biofilm.

Chapter 2

Background and Hypothesis

The use of engineered microbes to combat biofouling in membrane systems is a novel idea, shown to hold promise as a preventative measure in both dead-end and cross-flow reverse osmosis applications by Wood, et al.[16] This project investigated the potential of this approach as a treatment to displace pre-existing biofilms. The efficacy of the engineered, NO-producing strain (*E. coli* TG1/pBdcAE50Q-*lasI-lasR*/pBNos) was tested for its ability to displace a consortial foulant biofilm of *P. aeruginosa* PAO1/pMRP9-1 and *E. coli* TG1/pBdcAE50Q-*lasR*/pBad; a strain identical to the beneficial strain except for its ability to produce NO (*E. coli* TG1/pBdcAE50Q-*lasI-lasR*/pBad) was used as a negative control. Analysis was performed by 96-well assay and dead-end filtration tests, as well as scanning electron microscopy (SEM).

2.1 The beneficial strain is able to limit its own growth via quorum sensing

The ability of the beneficial strain (*E. coli* TG1/pBdcAE50Q-*lasI-lasR*/pBNos) to control its own growth is crucial to its function as a biofilm control measure. While its growth on a membrane does reduce flux, the QS circuit that limits the extent of its growth ensures that performance is maintained at an acceptable level. This circuit, LasI/LasR QS, was taken from *P. aeruginosa*, and begins with the *lasI* promoter for LasI protein. Once produced, LasI in turn generates the 3*o*C12HSL QS signal, which accumulates until reaching a threshold value. The greater the cell density, the sooner this threshold is reached. At this point, the signal binds to the

LasR protein, creating a complex that signals the *lasI* promoter to begin production of a second protein: bdcAE50Q. This protein is responsible for biofilm dispersal, and prevents further growth of the beneficial biofilm once it has reached its threshold density by preventing more cells with the QS circuit from adding to the established biofilm.

Wood, et al. confirmed the self-control ability of the QS circuit in *E. coli* by comparison to a strain that was identical except for the inclusion of LasI, which prevented it from controlling its biofilm density. The self-control strain exhibited less growth when analyzed by both a 96-well crystal violet biofilm assay and confocal imaging.[16] Furthermore, dead-end filtration tests showed that the self-control strain reduced flux decline by 50% and improved salt rejection by 11% when compared to the control strain.[16]

2.2 The beneficial biofilm is able to reduce the formation of undesired biofilms

While QS is responsible for the ability of the beneficial biofilm to limit its own growth, its dispersal of other microbes is via a different mechanism. The *bNOS* gene from *Bacillus anthracis* was included in the gene circuit for the beneficial strain. Arabinose is used to induce *bNos* and triggers the production of nitric oxide synthase (NOS), generating the NO that acts as a dispersant for other microbes. The effectiveness of NO production by the beneficial strain on reducing membrane fouling by other microbes was confirmed using 96-well assays, which indicated that the biofilm formation by *P. aeruginosa* was reduced by 80%.[16]

Use of the beneficial strain was also tested on nanofiltration membranes (DOW NF90) analyzed by confocal microscopy and both dead-end and cross-flow filtration tests. Imaging indicated that the beneficial biofilm reduced undesired biofouling by *P. aeruginosa* 40 fold, while

dead-end filtration showed considerably improved flux for the beneficial strain compared to the NO-negative control.[16] In cross-flow filtration, the beneficial strain reduced flux decline by approximately 40% when compared to the NO-negative control.[16]

2.3 Displacement of biofilms with engineered microbes is achievable

Once the ability of the beneficial biofilm to disperse other foulant microbes was confirmed, its potential for use as a treatment for the displacement of established biofilms was of significant interest. It had previously been shown by Hong, et al. that a second cell type could be used to disperse an existing biofilm in microfluidic devices.[19] In their experiment, however, both the cells in the biofilm to be removed and those used for dispersion utilized the same dispersal protein; both were signaled by bdcAE50Q.[19] While the same protein is generated in the QS circuit of the beneficial strain used in this project, the purpose was instead to test the ability of NO to disperse existing biofilms. While likely more difficult to achieve, this approach offered more promise as a treatment in membrane systems, as it would be effective against a variety of microbes and not only those susceptible to a specific dispersal protein.

2.4 Hypothesis

The goal of this experiment was to investigate the use of an engineered biofilm, previously shown to effectively reduce undesired biofouling in membrane systems, as a treatment to displace established biofilms. If possible, this beneficial biofilm could be used to remove foulant biofilms on membrane surfaces and replace them with its own, growth-limited biofilm capable of dispersing

other microbes. Such an ability would offer an alternative to traditional biofouling treatment methods used in membrane systems, which often have limited effectiveness.

The ability of this NO-producing, beneficial biofilm to disperse other potential foulants, including the particularly aggressive *P. aeruginosa*, was well established by Wood, et al.[16] Furthermore, displacement of established biofilms by a second cell type was shown to be possible by Hong, et al.[19] Therefore, it was hypothesized that, under the right conditions, the engineered-biofilm strain would be capable of displacing and replacing an established biofilm of deleterious microbes.

Chapter 3

Materials and Methods

The purpose of this project was to determine if the beneficial, NO-producing strain could displace an established biofilm in membrane systems. To more closely emulate real-world conditions, a consortial solution was used to form the foulant biofilm, resulting in more aggressive growth and thereby making it more difficult to remove. The effects of the beneficial strain were analyzed by 96-well crystal violet assay, dead-end filtration, and SEM imaging. For comparison, a negative control without the ability to produce NO was also tested.

3.1 Bacterial strains and cultures

Three strains of *E. coli* were utilized during experimentation. The foulant biofilm to be displaced was a consortial solution of *E. coli* TG1/pBdcAE50Q-rfp-lasR/pBad and *P. aeruginosa* PAO1/pMRP9-1, with the latter at a lower initial concentration. Biofilm displacement was attempted using the NO-producing *E. coli* TG1/pBdcAE50Q-*lasI-lasR*/pBNos (the beneficial strain) with *E. coli* TG1/pBdcAE50Q-*lasI-lasR*/pBad (the control strain) used as a negative control without the ability to produce NO. All strains were streaked on lysogeny broth (LB) plates with chloramphenicol (Cm) at 150 μg/mL and carbenicillin (Cb) at 200 μg/mL.

The foulant strains were inoculated and grown in LB, while the beneficial and control strains were grown in minimal medium with 0.4% glucose (M9G); the concentrations of Cm and Cb in these cultures were 300 and 250 μg/mL, respectively. Cultures were grown at 37 °C, 250

rpm shaking speed. For experimentation, the foulant cultures were centrifuged at 4 °C and 5,000 rpm for 10 minutes and then diluted to the desired optical densities (measured at 600 nm) in M9GCm300/Cb250 (µg/mL). Before being centrifuged and diluted in the same manner, cultures of the beneficial and control strains were diluted 25 times and allowed to grow for an additional 2-2.5 hours in order to produce cells in the exponential growth phase. The cultures were then induced with 15 mM L-Arginine and 1% Arabinose to begin NO production by the beneficial strain and allowed an additional 1.5-2 hours of growth. When diluted in M9GCm300/Cb250 (µg/mL), L-Arginine and Arabinose were also added at the above concentrations to allow continued production of NO.

3.2 96-well crystal violet assays

For each trial, 96-well polystyrene plates were inoculated with the consortial foulant solution in M9GCm300/Cb250 (µg/mL) at the optical densities (at 600 nm) to be tested. Plates were incubated for 24-48 hours at 37 °C without shaking. Blank M9GCm300/Cb250 (µg/mL) was included in the outermost wells for analysis. The solution in 48 of the 96 wells was removed via pipette, leaving 12 wells with the fouling solution untreated. Half of the emptied wells were then filled with the beneficial strain and half with the control strain at the optical density (600 nm) to be tested. For analysis, half of the blank wells were also emptied and replaced with M9GCm300/Cb250 (µg/mL) containing L-Arginine and Arabinose at the same concentration as the treated wells. Plates were then incubated at 37 °C without shaking for an additional 24-48 hours. A variety of initial foulant and treatment concentrations, as well as incubation times, were tested, as shown in Table 1.

Table 1. 96-well crystal violet assay conditions

Trial No.	Initial Foulant Concentrations (OD$_{600nm}$)		Fouling Time (hr)	Initial Treatment Concentrations (OD$_{600nm}$)		Treatment Time (hr)
	E. coli TG1/pBdcAE50Q-rfp-lasR/pBad	*P. aeruginosa* PAO1/pMRP9-1		*E. coli* TG1/pBdcAE50Q-*lasI-lasR*/pBNos	*E. coli* TG1/pBdcAE50Q-*lasI-lasR*/pBad	
1	0.5	0.1	24	0.5	0.5	24
2	0.5	0.1	24	2.0	2.0	24
3	1.0	0.2	48	0.5	0.5	48

To investigate the effect of competition with foulant microbes on the growth of the treatment strains, an additional trial was performed in which the treatment strains were charged simultaneously. Conditions used in this trial are shown in Table 2.

Table 2. 96-well crystal violet assay conditions for simultaneous charging

Trial No.	Initial Foulant Concentrations (OD$_{600nm}$)		Initial Treatment Concentrations (OD$_{600nm}$)		Growth Time (hr)
	E. coli TG1/pBdcAE50Q-rfp-lasR/pBad	*P. aeruginosa* PAO1/pMRP9-1	*E. coli* TG1/pBdcAE50Q-*lasI-lasR*/pBNos	*E. coli* TG1/pBdcAE50Q-*lasI-lasR*/pBad	
4	0.5, 1.0	0.1	0.5	0.5	49

After the treatment period, a Tecan plate reader was used to measure total biofilm formation at 540 nm. Wells were then emptied and stained with 0.1% crystal violet; after rinsing, ethanol was used to dissolve the crystal violet and total cell growth was measured at 620 nm. Biofilm formation was normalized by cell growth to reduce any growth effect. Biofilm formation in the treated wells was compared to that in the untreated wells after the full fouling and treatment periods. It was also compared to biofilm formation of the fouling solution after the initial fouling period only, which was measured by analyzing a separate plate at this point in the experiment.

3.3 Dead-end filtration tests

Dead-end filtration tests were conducted in parallel with the 96-well assay experiments described above. One trial was conducted using only *P. aeruginosa* as the foulant; the remainder

used the same optical densities as those in the 96-well experiments. Conditions for all dead-end trials are listed in Table 3.

Trial No.	Initial Foulant Concentrations (OD$_{600nm}$)		Fouling Time (hr)	Initial Treatment Concentrations (OD$_{600nm}$)		Treatment Time (hr)
	E. coli TG1/pBdcAE50Q-rfp-lasR/pBad	*P. aeruginosa* PAO1/pMRP9-1		*E. coli* TG1/pBdcAE50Q-*lasI-lasR*/pBNos	*E. coli* TG1/pBdcAE50Q-*lasI-lasR*/pBad	
1	0.0	0.1	24	0.5	0.5	24
2	0.5	0.1	24	0.5	0.5	48
3	0.5	0.1	24	2.0	2.0	48
4	1.0	0.2	48	0.5	0.5	48

Prior to experimentation, DOW NF90 membranes were placed above the spacers and under the o-rings in Advantec MFS UHP-76 stirred cells (effective membrane area of 35.3 cm^2). Each 450-mL stirred cell was sterilized with 95% ethanol and rinsed with deionized (DI) water before being charged with up to 300 mL of the fouling solution to be tested. After the fouling period, the stirred cells were drained and charged with up to 300 mL of the treatment solutions.

After treatment, the stirred cells were drained and flux through the membranes was analyzed for 15 mM, 10 mM, 5 mM, and 0 mM (DI) sodium chloride solutions in series. Filtration was conducted at 50 psi and 400 rpm. As with the 96-well trials, a stirred cell was also analyzed after the fouling period to provide an untreated comparison. Flux through all fouled and treated membranes was compared to a baseline test of a membrane incubated with M9G only for 24 hours, allowing decline due to biofilm formation to be compared to that caused solely by the salt and sugar content of M9G.

3.4 SEM imaging

SEM imaging of membranes was performed for a single trial. Fouling and treatment were conducted in stirred cells as described above; conditions for this trial are shown in Table 4.

Table 4. SEM trial conditions

Trial No.	Initial Foulant Concentrations (OD$_{600nm}$)		Fouling Time (hr)	Initial Treatment Concentrations (OD$_{600nm}$)		Treatment Time (hr)
	E. coli TG1/pBdcAE50Q-rfp-lasR/pBad	*P. aeruginosa* PAO1/pMRP9-1		*E. coli* TG1/pBdcAE50Q-*lasI-lasR*/pBNos	*E. coli* TG1/pBdcAE50Q-*lasI-lasR*/pBad	
1	0.5	0.1	24	0.5	0.5	24

At the end of the treatment period, membranes were removed from the stirred cells and allowed to dry. Samples cut from these membranes were sputter-coated with iridium and imaged under an FEI Nova NanoSEM 630 FESEM at magnifications providing a 10-μm scale.

Chapter 4

Results and Discussion

4.1 Attempted displacement in 96-well assays increased total biofilm formation

The first 96-well assay trial used treatment and control strains at an initial concentration comparable to the initial overall density of the consortial foulant solution. Results (shown in Figure 1) indicate significantly greater biofilm formation resulted in wells after treatment.
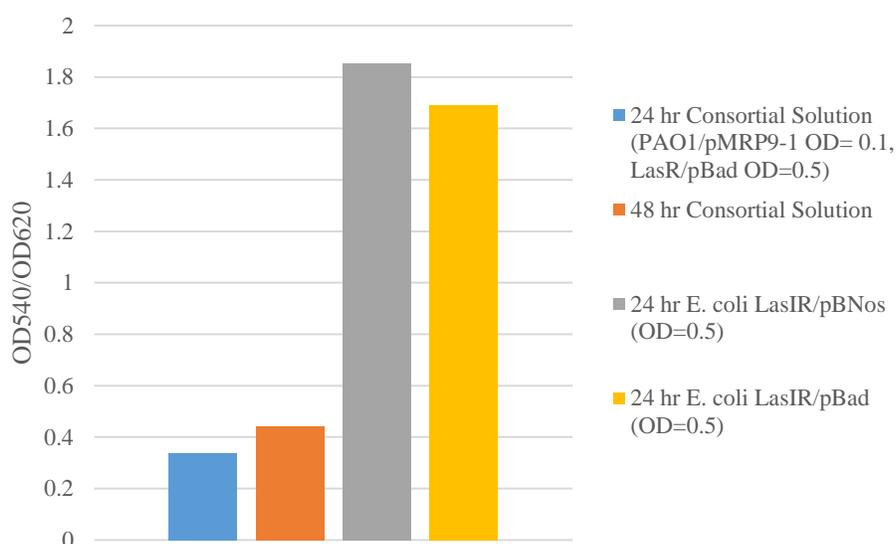

Figure 1. Results of 96-well assay trial no. 1

Following this trial, the effect of increasing the treatment strain concentrations by a factor of four was investigated. Figure 2 illustrates that this increase did not improve the performance of the beneficial strain as a treatment measure.
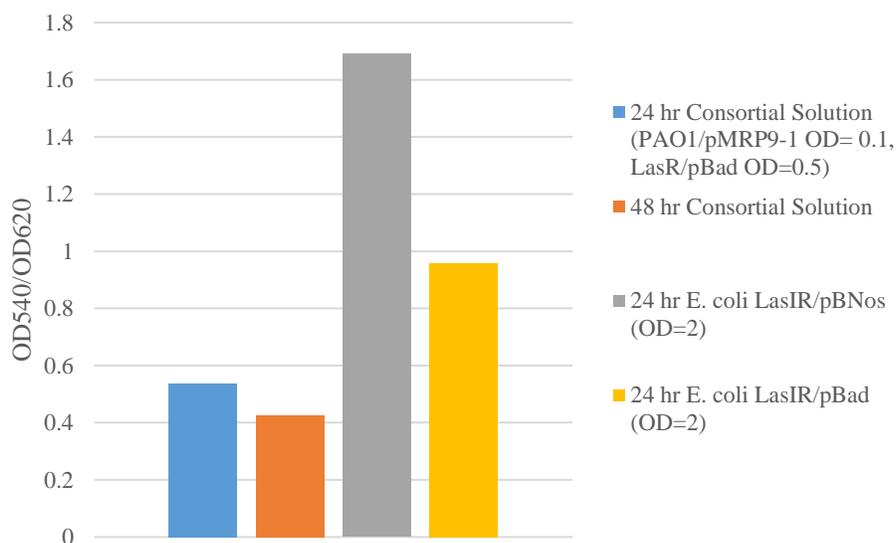
Figure 2. Results of 96-well assay trial no. 2

Finally, in trial no. 3, the treatment strain initial concentrations were returned to their original levels and those of the consortial solution components were increased. For this trial, both fouling and treatment times were also extended. Results (shown in Figure 3) once again showed that treatment with the beneficial strain was unsuccessful.
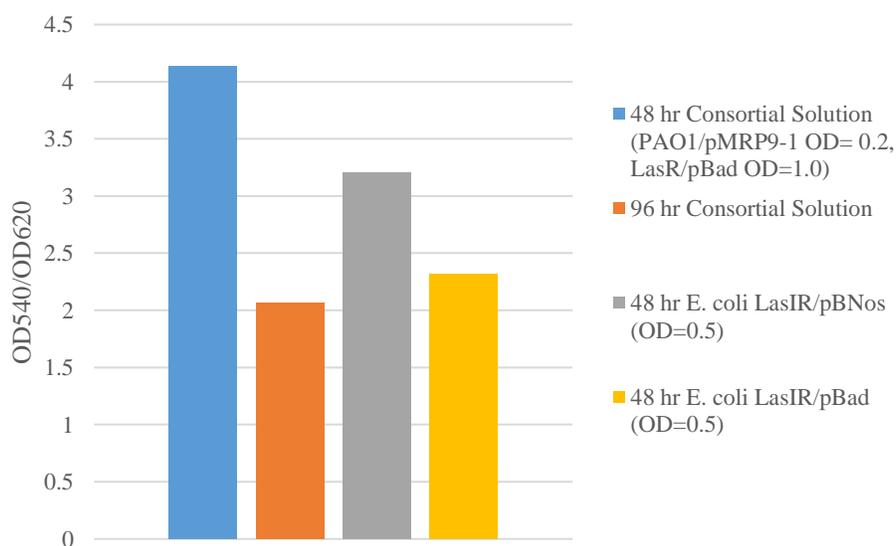

Figure 3. Results of 96-well assay trial no. 3

While biofilm formation in wells treated with the beneficial strain was below that in the fouled wells analyzed at the start of the treatment period, it was higher than biofilm formation in fouled wells at the end of the treatment period. Furthermore, it remained higher than those wells treated with the NO-negative control strain.

It should be noted that biofilm formation in wells treated with the beneficial strain was also greater than that in wells treated with the negative control in all of these trials. This suggested that the beneficial strain could not function effectively when competing with other microbes. Therefore, investigation into competitive growth of the treatment strains by simultaneously charging them with foulant microbes was carried out. This did show that the beneficial strain was able to prevent excessive fouling by the other microbes (Figure 4).
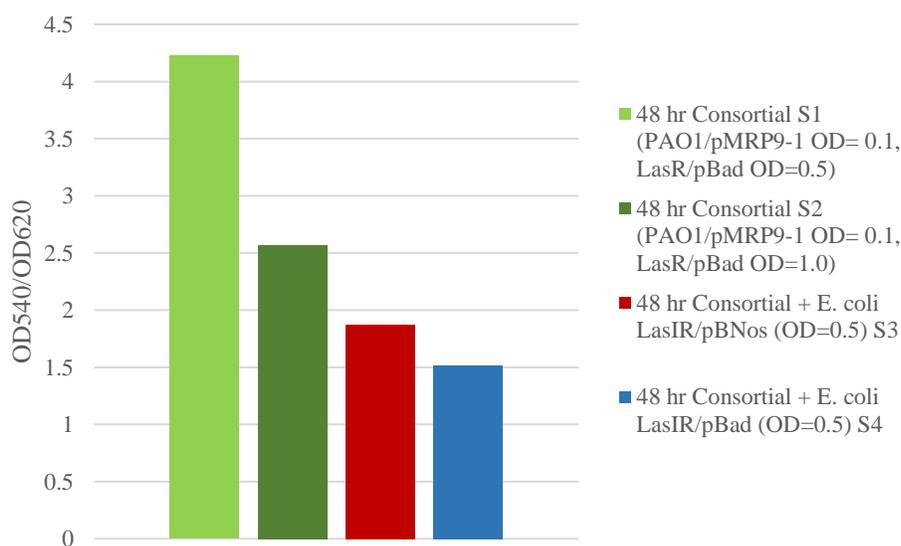

Figure 4. Results of 96-well assay trial no. 4 (simultaneous charging of foulant and treatment strains)

However, no advantage was shown over the negative control strain in this trial, and the results of the 96-well assay tests therefore indicate that the beneficial biofilm model cannot effectively be implemented as a treatment method for displacement of established biofilms.

Tabulated results for these trials are shown in Appendix A.

4.2 Membrane flux declined following treatment with the beneficial strain

Attempts to displace foulant biofilms from membrane surfaces in dead-end flux trials largely mirrored the results of the 96-well assay trials. In trial no. 1, treatment greatly increased biofouling over that caused by *P. aeruginosa* alone (Figure 5).
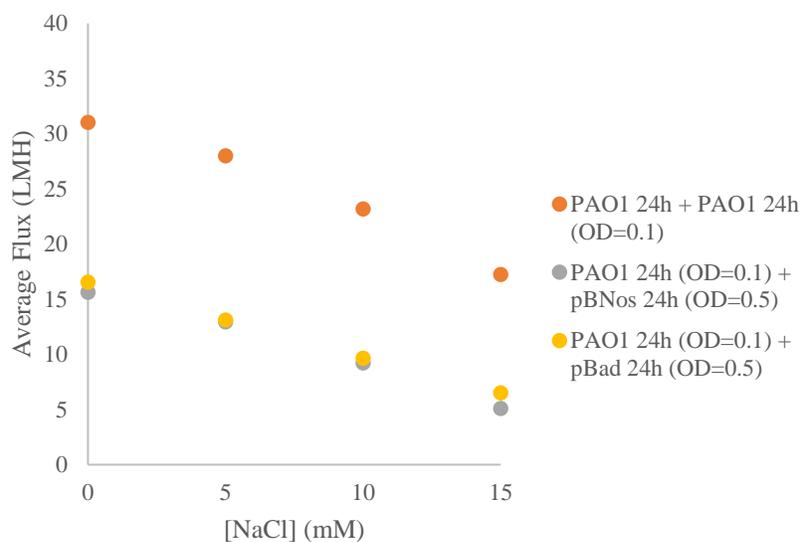

Figure 5. Results of dead-end filtration trial no. 1

In the trials conducted with a consortial fouling solution, far more fouling occurred than when fouling with a single microbe (Figure 6).
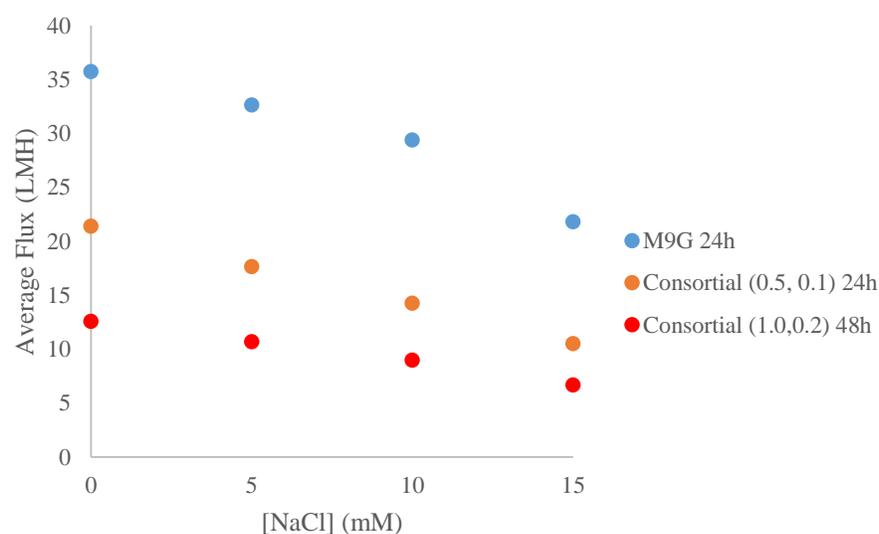
Figure 6. Results of consortial fouling in dead-end filtration trials

Nevertheless, treatment of consortial-fouled membranes only resulted in further flux decline. Furthermore, the beneficial strain either showed no improvement over the negative control strain or caused a higher degree of fouling, as in the 96-well trials. The results of trial no. 4, shown in Figure 7, illustrate these trends.
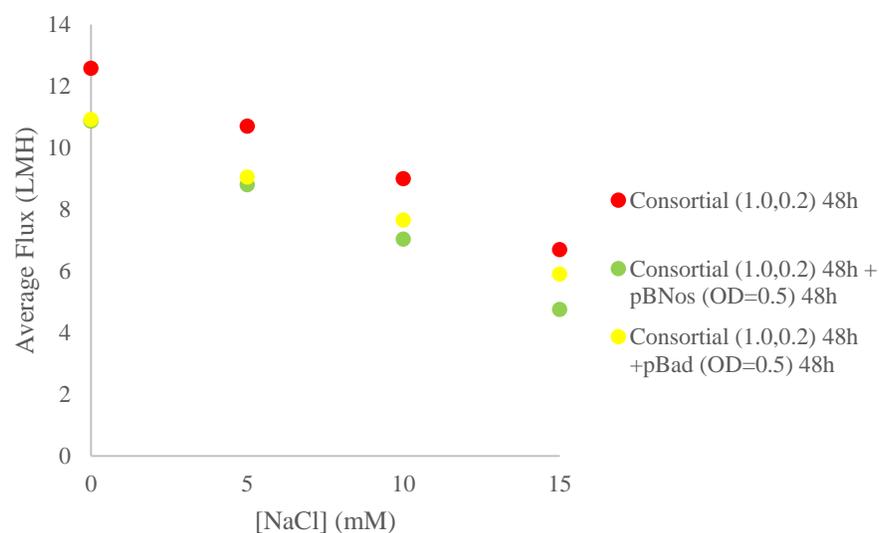

Figure 7. Results of dead-end filtration trial no. 4

Results of trials nos. 2 and 3 are shown in Appendix B.

4.3 SEM imaging indicates that membrane surfaces were completely fouled

All of the microbes used in these experiments are rod-shaped, meaning that SEM imaging cannot reliably distinguish between the individual strains. However, results of SEM imaging did show that both the samples treated with the beneficial and negative control strains (Figures 8 and 9, respectively) were similarly fouled, with no areas of unfouled membrane (Figure 10) visible.
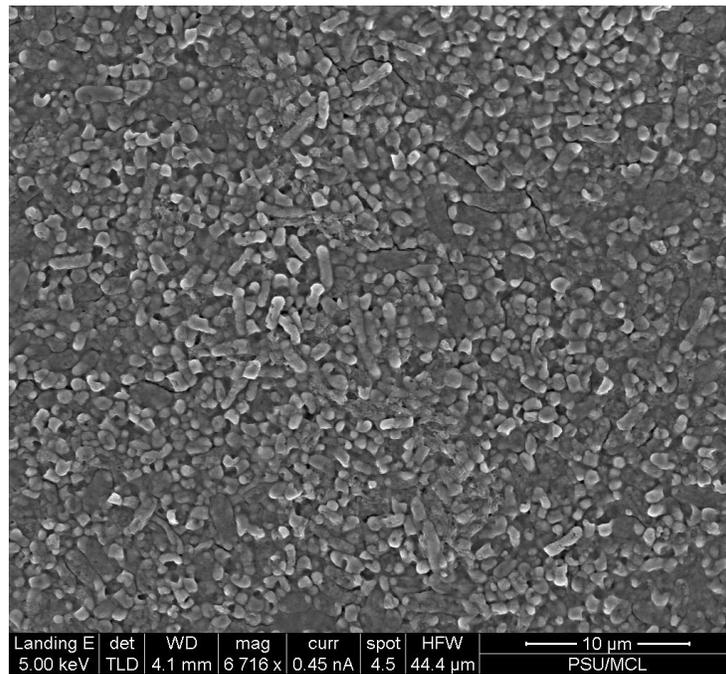
Figure 8. Consortial-fouled membrane treated with beneficial strain

Landing E | det | WD | mag | curr | spot | HFW | 10 µm
5.00 keV | TLD | 4.0 mm | 6 159 x | 0.45 nA | 4.5 | 48.4 µm | PSU/MCL
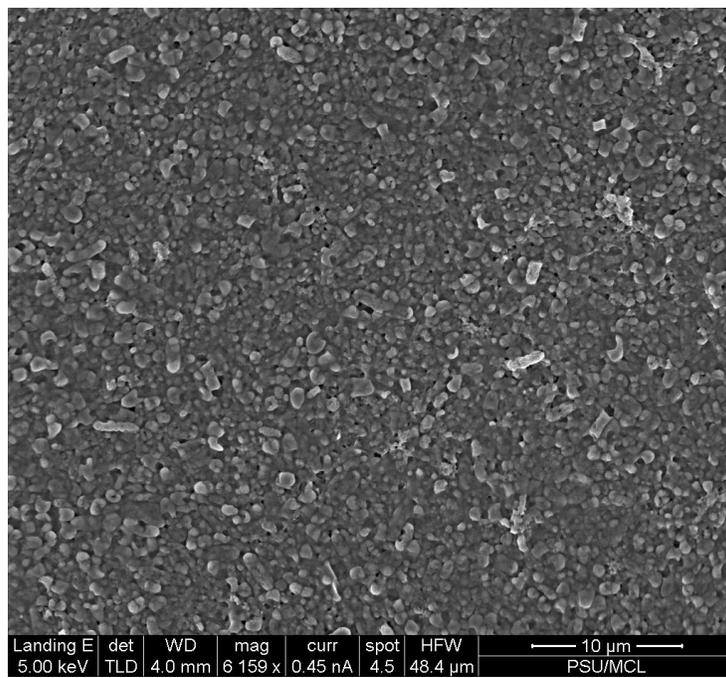
Figure 9. Consortial-fouled membrane treated with negative control strain

Landing E | det | WD | mag | curr | spot | HFW | 10 µm
5.00 keV | TLD | 3.9 mm | 7 342 x | 0.11 nA | 3.5 | 40.6 µm | PSU/MCL
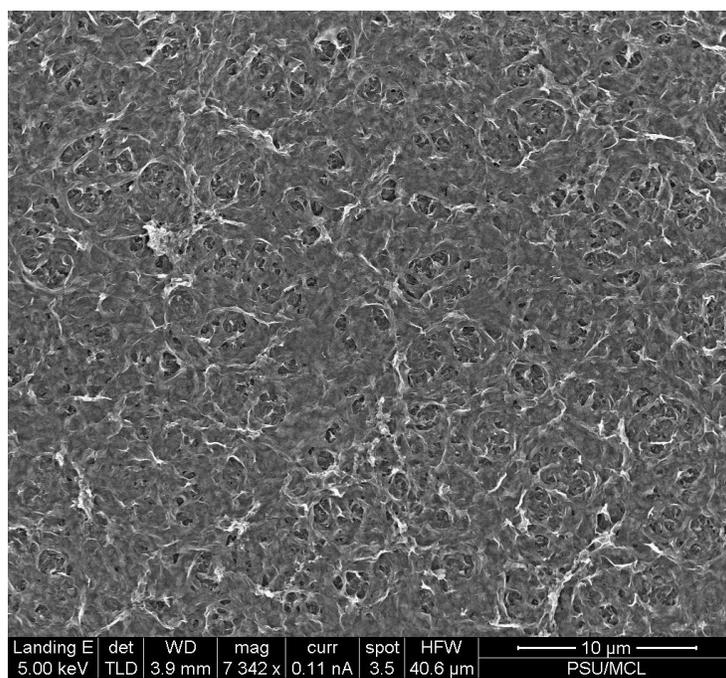
Figure 10. Unfouled DOW NF90 membrane surface

4.4 The beneficial strain does not offer a viable treatment option at this time

  While it cannot be asserted that no displacement of the original, foulant biofilms was achieved after treatment with the beneficial strain, these efforts show that treatment only causes greater overall fouling. Furthermore, when growing in a competitive environment, the beneficial strain offers no benefit over the negative control strain. While the self-control mechanism appears to reduce biofilm formation, NO-production appears to be ineffective unless the gas is released prior to the introduction of foulant microbes.

Chapter 5

Future Work and Conclusion

The objective of this project was to determine if the protective biofilm previously used to disperse potential foulants could be used to displace an established foulant biofilm. Results of 96-well assay, dead-end filtration, and SEM imaging all indicate that this is not possible. Treatment of an established biofilm using the beneficial strain only results in further fouling and greater flux decline. Furthermore, no advantage over the control strain was observed under the conditions tested.

Further investigation could take advantage of the microbes' fluorescent capabilities and utilize confocal microscopy to determine the degree to which each strain is present in the final biofilm. Knowing the extent of each strain's growth could allow for a better understanding of how the beneficial strain behaves in a competitive environment, and perhaps lead to discovery of conditions under which its use as a treatment measure would be possible. These experiments could also be repeated using less-aggressive foulants. While the beneficial strain was not effective against *P. aeruginosa* or the consortial biofilm, it may be a viable option for treating other microbes common in membrane systems.

At this point, however, it appears that the beneficial strain has little chance of serving as an effective method of displacing undesired biofilms in membrane systems. If this approach is to be utilized, it is clear that "an ounce of prevention is worth a pound of cure."

Appendix A

Additional 96-Well Assay Results

Table 5. Results of 96-well assay trial no. 1

Solution	OD540 nm	OD620 nm	OD540 nm/OD620 nm
Consortial (24 hrs)	0.3666	1.0941	0.3369
Consortial (48 hrs)	0.6541	1.4911	0.4415
E. coli LasIR/pBNos (24 hrs)	1.4181	0.7848	1.8526
E. coli LasIR/pBad (24 hrs)	1.6813	1.0122	1.6893

Table 6. Results of 96-well assay trial no. 2

Solution	OD540 nm	OD620 nm	OD540 nm/OD620 nm
Consortial (24 hrs)	0.5423	1.0098	0.5379
Consortial (48 hrs)	0.6139	1.4588	0.4255
E. coli LasIR/pBNos (24 hrs)	1.6267	0.9963	1.6907
E. coli LasIR/pBad (24 hrs)	1.2002	1.2547	0.9594

Table 7. Results of 96-well assay trial no. 3

Solution	OD540 nm	OD620 nm	OD540 nm/OD620 nm
Consortial (24 hrs)	1.9240	0.4660	4.1322
Consortial (48 hrs)	1.2134	0.5893	2.0700
E. coli LasIR/pBNos (24 hrs)	1.9089	0.6081	3.2026
E. coli LasIR/pBad (24 hrs)	1.4074	0.6105	2.3188

Table 8. Results of 96-well assay trial no. 4 (simultaneous charging of foulant and treatment strains)

Solution	OD540 nm	OD620 nm	OD540 nm/OD620 nm
Consortial (24 hrs)	1.9816	0.4711	4.2275
Consortial (48 hrs)	1.2859	0.5034	2.5713
E. coli LasIR/pBNos (24 hrs)	1.8403	0.9850	1.8724
E. coli LasIR/pBad (24 hrs)	1.1656	0.7715	1.5144

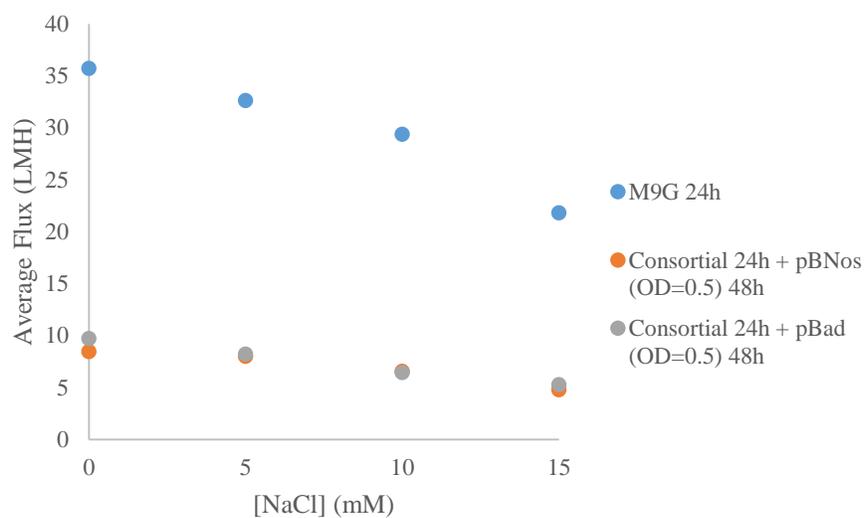# Appendix B

Additional Dead-End Test Results

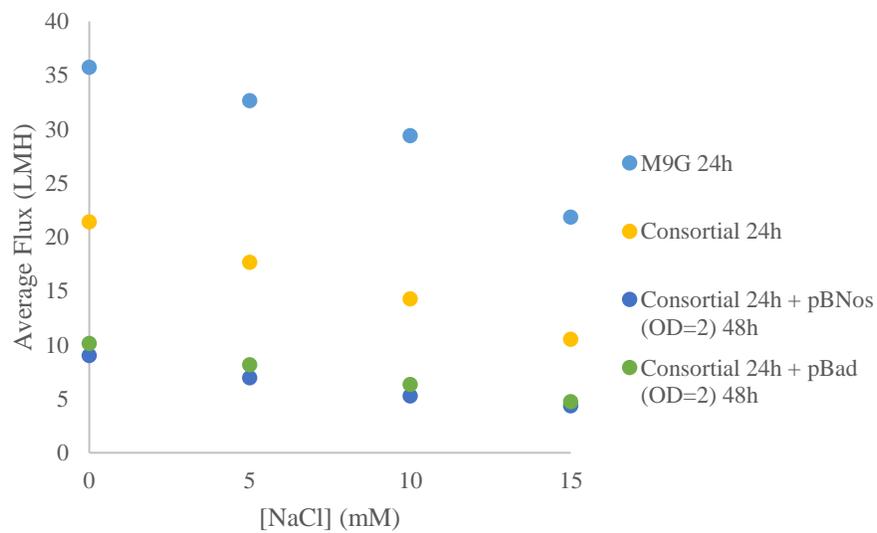Figure 11. Results of dead-end filtration trial no. 2

Figure 12. Results of dead-end filtration trial no. 3

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