Supporting Information for:

Quorum sensing between *Pseudomonas aeruginosa* biofilms accelerates cell growth

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Materials. PDMS (Sylgard 184 silicone elastomer kit) was from Dow Corning (Midland, MI). Poly(ethylene glycol) diacrylate (M_n = 575), 2,2-dimethoxy-2-phenyl-acetophenone, *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C_{12}-HSL), carbenicillin, carbon tetrachloride, hexanes, and dimethyl sulfoxide were from Aldrich (Milwaukee, WI). Heptane, sodium chloride, potassium chloride, 10X phosphate buffered saline, and HEPES buffer were from Fisher Scientific (Pittsburg, PA). SU-8 2050 photoresist was from MicroChem (Newton, MA). Fluorescein disodium salt hydrate, $Na_2HPO_4 \cdot 7\ H_2O$, KH_2PO_4, $MgCl_2 \cdot 6\ H_2O$, and $MgSO_4$ were from Alfa Aesar (Ward Hill, MA). Ethanol was from Pharmco-Aaper (Brookfield, CT). (3-acryloxyproply) trichlorosilane was from Gelest Inc (Morrisville, PA). Bacto™ tryptone, Bacto™ yeast extract, Difco™ casamino acids, and Difco™ granulated agar were from BD Biosciences (Franklin Lakes, NJ). D-Glucose was from Mallinckrodt (Paris, KY). FM® 4-64 styryl membrane dye was from Molecular Probes/Invitrogen (Eugene, OR).

Preparation of glass cover slips. During pilot experiments we found that it was necessary to clean glass cover slips carefully with a piranha solution and silanize the resulting glass cover slips under dry, inert conditions. *WARNING:* piranha solutions are hazardous and should be handled with extreme care. Careful cleaning and silanization of the cover slips reduced the delamination of PEGDA hydrogels from the glass surface. We found that replacing the culture media 1–2 times per day during biofilm formation also reduced delamination. Delamination of hydrogels with microchambers (e.g. features <1-mm deep) was particularly problematic when

the surfaces were not carefully cleaned; delamination of hydrogels with mesostructures was typically not a problem. Despite optimizing the surface preparation of the glass slides, delamination of micro- or meso-structured hydrogels occasionally occurred during the growth of biofilms under static conditions and for long periods of time (e.g. >4 days).

Glass cover slips (Fisherbrand, 45 x 50 mm; thickness #1.5) were placed in a glass rack and submersed for 20 min in a glass tank containing a piranha solution. The cover slips were removed, rinsed thoroughly by dipping the rack in a second tank containing ddH_2O, and dried for 10 min at 110 °C or with a stream of nitrogen. Smaller glass cover slips (Corning, 22 x 22 mm; thickness # 2) used for ESEM imaging were rinsed with ethanol, dried under a stream of nitrogen, and incubated in a plasma cleaner for 10 min. All cover slips were silanized immediately after cleaning. We purged a glass chamber containing a 1 mM solution of (3-acryloxyproply) trichlorosilane in 4:1 heptane / CCl_4 with argon and silanized cover slips for 10 min. The silanization of smaller cover slips was performed in a glass Petri dish inside a glove bag. The cover slips were removed from the silanization solution, washed thoroughly with hexanes, ethanol, and ddH_2O. Cover slips were cured at 110 °C for 10 min and stored in the dark.[1]

Hydrogel Stability. We tested the stability of the hydrogels to degradation and delamination in a variety of liquids for 5 – 7 days at 37 °C (i.e. typical growth conditions), including: ddH_2O, M8 minimal media, 5% DMSO in M8. We also tested liquid cultures of *P. aeruginosa* PAO1 in M8 minimal media. Hydrogels were stable in ddH_2O and M8 minimal media for at least 14 days. Hydrogels were also stable in 5% aqueous solutions of DMSO, and are therefore compatible with solutions of synthetic nonpolar compounds, such as *N*-acyl homoserine lactones dissolved

in DMSO. The long-term stability of hydrogels was compromised in the presence of liquid cultures of bacteria. Gels with mesostructures were stable in liquid cultures of PAO1 up to ~ 48 h, and microstructured gels for 3 – 5 days before we observed the delamination of the hydrogels from the coverslip due to the swelling and buckling of PEGDA. Replacing the liquid nutrient media at least once a day can ameliorate the problem of delamination, but also has the disadvantage of possibly disrupting established metabolite gradients. Although not used in our current experiments, a possible solution for preventing delamination in the presence of liquid bacterial cultures is the addition of a crosslinking agent to the PEGDA pre-polymer solution. The addition of the crosslinker will allow the polymerized gel to form branched polymers instead of linear polymers, thus providing rigidity to the gel and preventing swelling. For example, Tsutsui and coworkers[2] use PETA (pentaerythritol tetraacrylate) to crosslink PEGDA hydrogels in their experiments in order to prevent delamination in the presence of mammalian cell cultures.

To keep the PEGDA gels hydrated we stored them in a Ziploc bag containing several wet paper towels. Alternatively, we placed a PDMS jig around the hydrogel to create a "moat" and filled it with a minimal amount of liquid nutrient media.

Microbial cell culture and growth conditions. *P. aeruginosa* PAO1 colonies were grown on Luria-Bertani (LB) agar (1.5%) infused with nutrients. Liquid cultures of *P. aeruginosa* PAO1 were grown in M8 media (241 mg/L $MgSO_4$, 4 mg/L glucose, 5 mg/L casamino acids, 12.8 g/L $Na_2HPO_4{\cdot}7H_20$, 3 g/L KH_2PO_4, and 0.5 g/L of NaCl). *P. aeruginosa* pTdK-GFP was grown in M8 liquid media or on LB agar containing 200 μg/mL carbenicillin. *P. aeruginosa* PAO-MW1 pUM15, *P. aeruginosa* PAO-MW1 p67T1, and *P. aeruginosa* PAO1 p67T1 were grown in LB media or on LB agar plates containing 300 μg/ml carbenicillin.

Construction of *P. aeruginosa* PAO-MW1 p67T1. Plasmid p67T1, expressing d-Tomato was transferred to *P. aeruginosa* PAO-MW1[3] via conjugal mating using *E. coli* HB101 (pRK2013, p67T1) as the donor strain.[4] Donor and recipient cell cultures containing appropriate antibiotics (described above) were grown to saturation overnight, harvested by centrifugation, and the cells were resuspended in fresh, antibiotic-free LB broth. The absorbance at λ=600 nm (OD_{600}) of the donor and recipient cultures was adjusted to OD_{600} = 1, and 3 mL of donor and recipient culture were mixed and filtered through a 0.45 μm pore membrane filter (47-mm diameter). The filter was incubated on the surface of an LB agar plate overnight at 37 °C. The cells from the filter were washed out into 6 mL of fresh LB broth and serial dilutions were plated onto LB agar containing 300 μg/mL carbenicillin and 50 μg/mL tetracycline to select for transconjugates.

Construction of *P. aeruginosa* PAO1 p67T1. Plasmid 67T1 was isolated from *E. coli* HB101 (pRK2013, p67T1)[4] using a QIAprep® Spin Miniprep Kit (Qiagen, Valencia, CA), and stored at -20 °C. Electrotransformation of p67T1 into *P. aeruginosa* PAO1 was performed as described elsewhere.[5] We selected transformants on LB agar plates containing 300 μg/mL carbenicillin.

Epifluorescence microscopy. We performed epifluorescence microscopy on an inverted Nikon Eclipse TE2000 equipped with an Andor iXon^{EM}+ DU-897 EMCCD (Andor Technology, South Windsor, CT, USA). Images were acquired using a 4X objective (Nikon PlanApo 4X/0.2 DM). Samples were illuminated with a 120 W mercury arc lamp (X-cite Series 120, EXFO, Mississauga, Ontario, Canada) using a 500/20x excitation filter, a 535/30m emission filter, and dichroic cube 86002 v2 (Chroma Technology, Rockingham, VT, USA). Data was collected on the EMCCD with the shutter off and a 31 ms exposure time, unless otherwise noted. Images of

cells were collected using Metamorph software (version 7.5.6.0, MDS Analytical Technologies, Downington, PA, USA). Image leveling was performed with Adobe Photoshop CS2.

Confocal laser scanning microscopy (CLSM). CLSM images for Figure 2 were obtained on a Zeiss 510 Meta. *P. aeruginosa* PAO1 and PAO-pTDK-GFP biofilms were grown in M8 media at 37 °C for 72 h in chambers embossed in microstructured 15% PEGDA hydrogels. The M8 media was exchanged out once daily in order to prevent delamination of the hydrogel. After 72 h, the media was removed and the biofilms were stained with FM® 4-64 styryl membrane dye before imaging. Image analysis was performed with Zeiss LSM 510 software (v. 4.2).

CLSM images for Figure 6 were obtained on a Nikon C1. Images were converted to TIFF stacks in ImageJ, and three-dimensional reconstructions and orthogonal views of image stacks were generated using Metamorph. Image analysis was done using COMSTAT[6] and a fixed threshold value for all stacks.

Environmental scanning electron microscopy (ESEM). ESEM images were obtained on a FEI Quanta. Samples were placed on a 4 °C stage, and imaged at 1 – 6 torr and 15 – 20 kV. *P. aeruginosa* PAO1 biofilms were grown in M8 media at 37 °C for 1, 3, or 5 days in chambers embossed in microstructured 15% PEGDA hydrogels. Biofilms were imaged directly without staining or fixation, or using the staining / fixation method described by Priester and coworkers.[7]

Determining the diffusion coefficient of fluorescein in a 15% PEGDA gel. The diffusion of small molecules through the hydrogel in one dimension follows Fick's laws of diffusion (Eq. 1),

where J is the particle flux, D is the diffusion coefficient (cm^2/s), C is concentration, x is distance, and t is time.[8]

$$\partial_t C = -\partial_x J = +D\partial_x^2 C \qquad \textit{Eq. (1)}$$

The solution to Eq. 1 is a sigmoidal curve that relaxes outward as a function of time and distance (see Berg 1993, Fig 2.5). The diffusion coefficient is constant for each hydrogel and small molecule pair at a particular temperature. In addition, small molecules of the same charge and approximate size will generally have similar diffusion coefficients in a given gel. We decided to approximate the range of D for various secondary metabolites by using the diffusion of fluorescein through the hydrogel as a model.

A 15 mm x 3 mm piece was cut out of a 4 mm thick PDMS slab and filled with a 15% PEGDA pre-gel solution. The PEGDA was polymerized by UV exposure for 150 s and the gel was soaked in ddH_2O. The gel was removed from the water and we cut out a rectangular well at one end of the hydrogel. We filled the well with 40 μL of an aqueous fluorescein solution (10 mM). The PDMS was left intact as a jig for the fluorescein solution. The hydrogel was placed under a 0.63x objective on a Zeiss Discovery V12 stereoscope, which made it possible to image the entire gel. We imaged the fluorescence intensity as a function of time and space by capturing images every 20 min for 400 min (Fig. S1).

A generic characteristic of diffusive behavior is that the diffusing substance should spread as the square-root of time. For diffusion in d-dimensions:

$\langle r^2 \rangle \sim (2d)\, D\, t$ *Eq. (2)*

Thus, by plotting the position of the leading edge of the fluorescein band as a function of time, we can approximate D. We used multiple linescans to measure fluorescein fluorescence intensity. By thresholding at multiple values of fluorescence (150 – 350 AFUs), we determined the distance to the leading edge of the fluorescence band. All line scans produced very clean behavior where $r \sim \sqrt{(t)}$; that is, r^2 versus t has a clear linear relationship ($R^2 \sim 0.99$; an example is shown in **Figure S2**). Presumably, one of these line scans corresponds closely to x, and the slope of the line plotting $\langle r^2 \rangle$ over t will be $2D$. Fitting them made it possible for us to bracket the value of D in the range $1.3 – 2.2 \times 10^{-6}$ m^2/s (0.08 – 1.3 mm^2/min).

Figure S1. Diffusion of fluorescein through 15% PEGDA hydrogel. A solid slab of hydrogel was cast inside a PDMS jig, and a reservoir was cut at one end of the gel and filled with an aqueous solution of fluorescein (10 mM). We took images at 20 min intervals for 400 min to determine the distance that the leading edge of the diffusing fluorescent band had migrated.

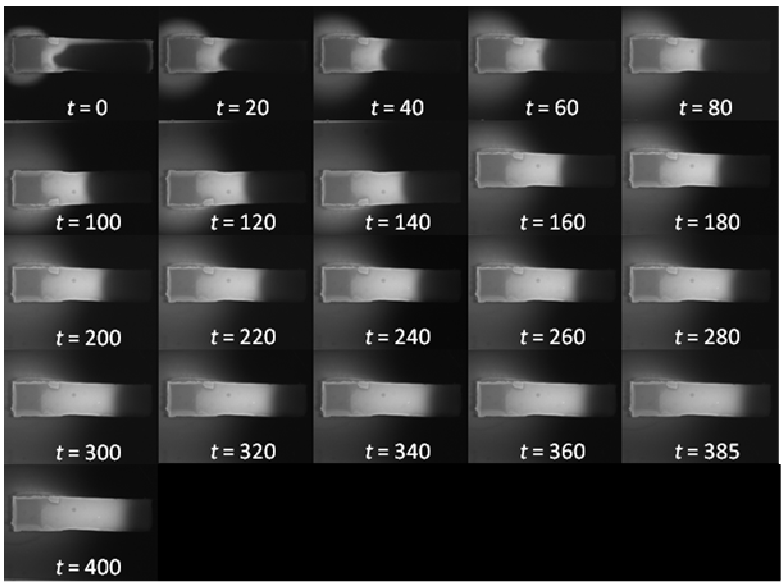


Figure S2. Plot of the position of the leading edge of the diffusing fluorescent band versus time. We determined an approximate range of potential values of D for metabolites diffusing between bacterial biofilms in embossed PEGDA hydrogels. We used multiple line scans to measure fluorescein fluorescence intensity and determine the distance to the leading edge of the fluorescence band. Various threshold values were applied to the fluorescent intensity as there was no sharp frontal boundary to the diffusing band. Fitting all line scans at the various threshold values produced a linear relationship and allowed us to bracket the value of *D* between 1.3 – 2.2 $\times 10^{-6}$ m^2/s (0.08 – 1.3 mm^2/min).

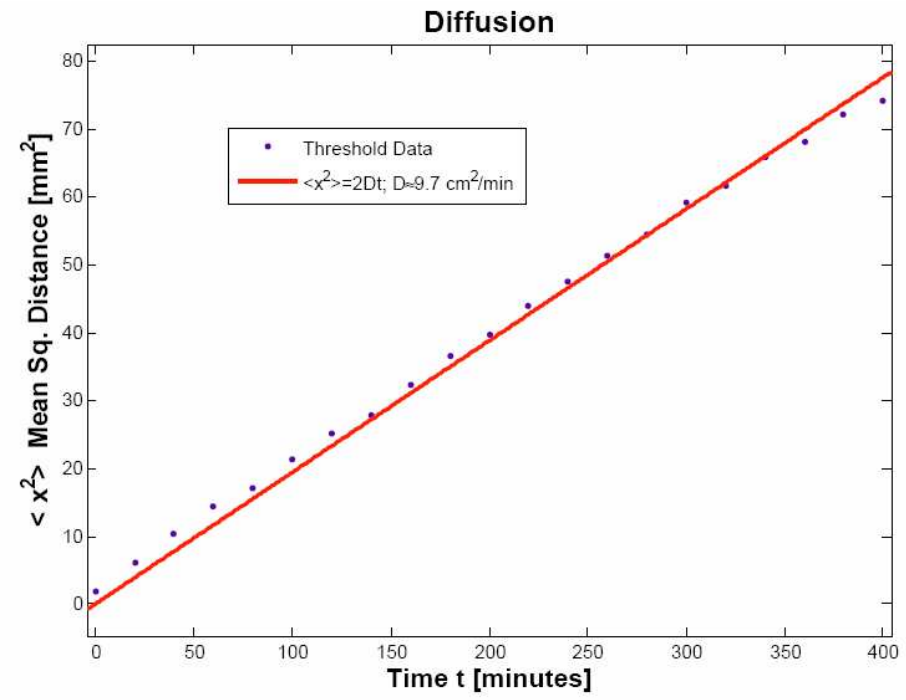


Figure S3. Effect of 3O-C_{12}-HSL on *P. aeruginosa* PAO1 cell growth rate in M8 minimal nutrient broth. 3O-C_{12}-HSL concentrations are indicated by different line patterns: 0 µm (solid), 0.1 µm (dotted), 1 µm (dashed), 10 µm (dash-dot). Growth curves for each concentration of HSL were performed in triplicate. The error (standard deviation of the mean) in the growth curves was very small; for clarity error bars are only shown for 0 µm and 10 µm 3O-C_{12}-HSL.

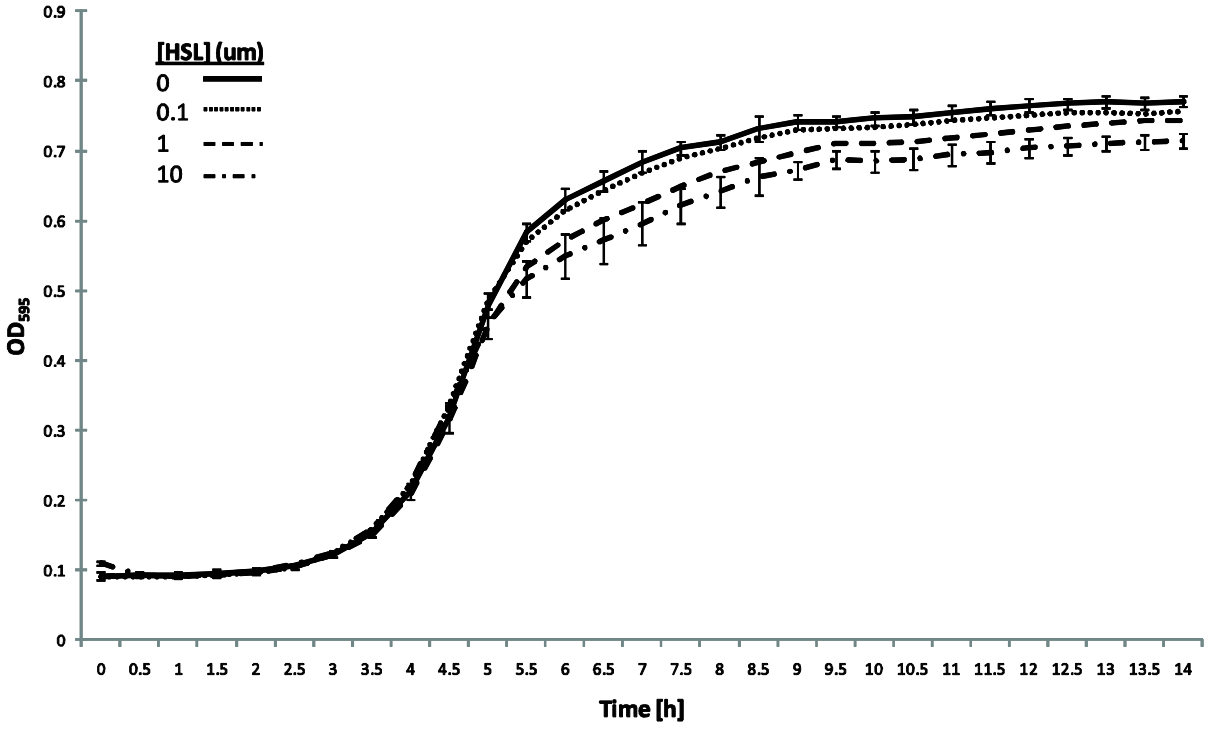


Figure S4. Biofilm position within the hydrogel array does not affect cell growth rate. **A**) Cartoon depiction of an 81-chamber array. Yellow squares represent chambers inoculated with PAO-MW1 or PAO-MW1 p67T1. Light blue squares are filled with M8 media. **B**) Graph showing cell growth rate of PAO-MW1, measured by OD_{595} , as a function of distance from the center chamber. **C**) Graph showing cell growth rate of PAO-MW1 p67T1, measured as d-Tomato fluorescence (labeled on the plot as 'RFP'), as a function of the distance from the center chamber.

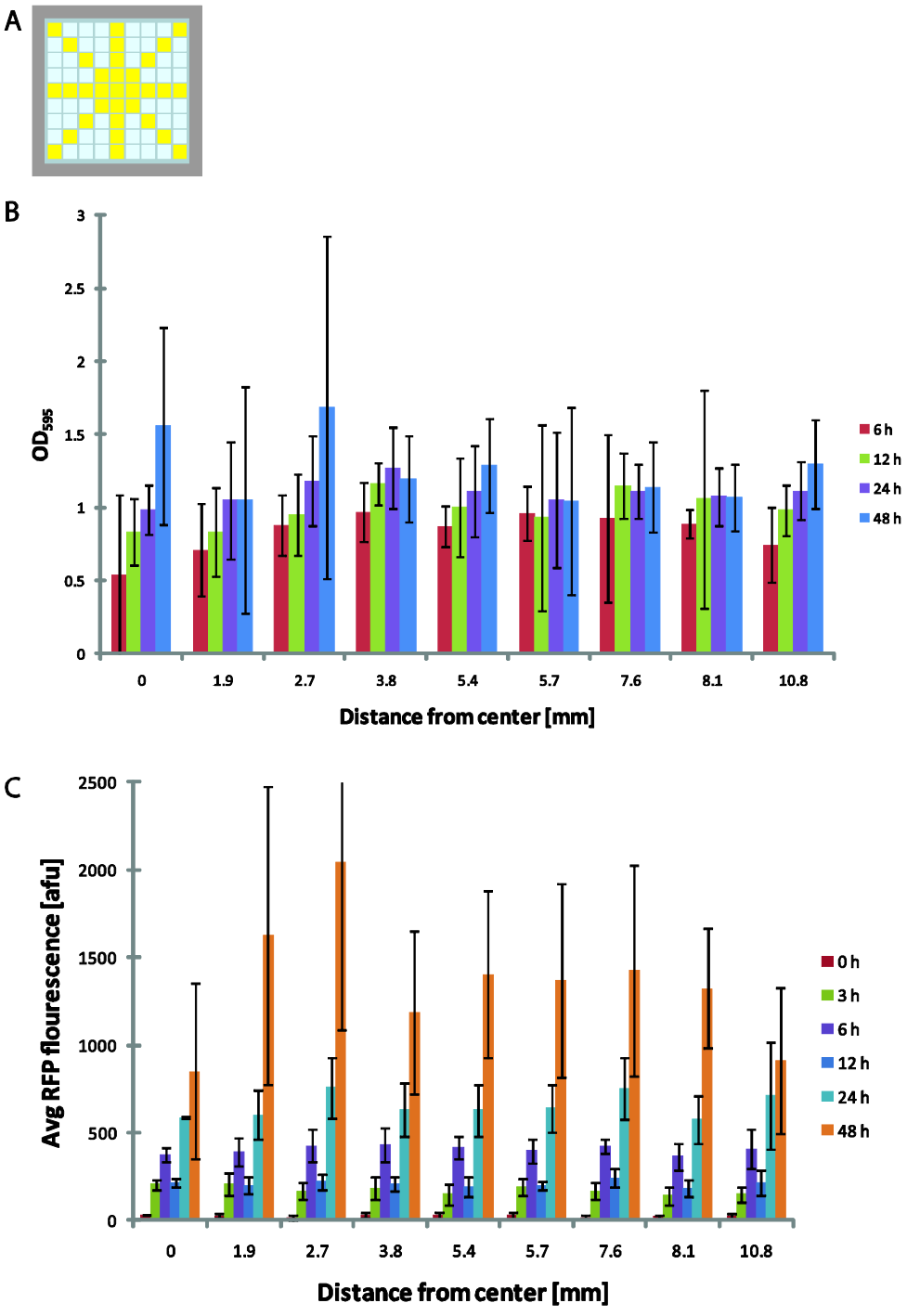

A
B
3
2.5
2
1.5
1
0.5
0
OD595
0
1.9
2.7
3.8
5.4
5.7
7.6
8.1
10.8
Distance from center [mm]
6 h
12 h
24 h
48 h
C
2500
2000
1500
1000
500
0
Avg RFP flourescence [afu]
0
1.9
2.7
3.8
5.4
5.7
7.6
8.1
10.8
Distance from center [mm]
0 h
3 h
6 h
12 h
24 h
48 h

Figure S4

Figure S5. Effects of HSLs on bacterial cell growth in hydrogel chambers. **A**) Cartoon depiction of an 81-chamber array. The blue square represents the center chamber inoculated with PAO1. Yellow squares represent chambers inoculated with PAO-MW1 *p*67T1. Light blue squares are filled with M8 media. **B**) Graph showing cell growth rate of PAO-MW1 *p*67T1, measured as d-Tomato fluorescence (labeled on the plot as 'RFP'), versus the distance from the center chamber when PAO1 and PAO-MW1 *p*67T1 were inoculated simultaneously. **C**) Graph showing cell growth rate of PAO-MW1 *p*67T1, measured as d-Tomato fluorescence (labeled on the plot as 'RFP'), versus the distance from the center chamber when PAO-MW1 *p*67T1 was inoculated into a hydrogel containing a nascent PAO1 biofilm in the center chamber.

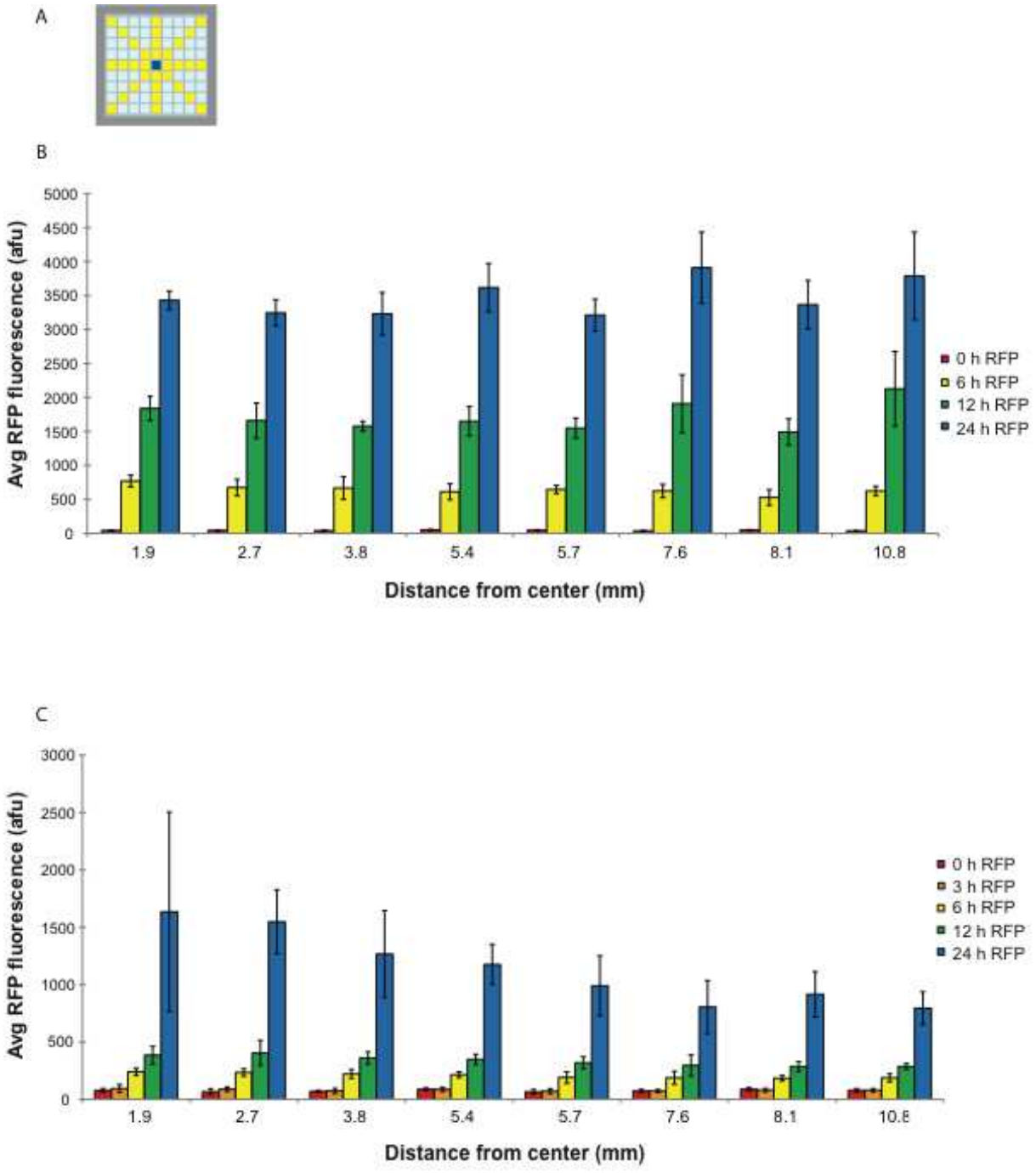

A
B
Avg RFP fluorescence (afu)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
1.9
2.7
3.8
5.4
5.7
7.6
8.1
10.8
Distance from center (mm)
0 h RFP
6 h RFP
12 h RFP
24 h RFP
C
Avg RFP fluorescence (afu)
3000
2500
2000
1500
1000
500
0
1.9
2.7
3.8
5.4
5.7
7.6
8.1
10.8
Distance from center (mm)
0 h RFP
3 h RFP
6 h RFP
12 h RFP
24 h RFP

Figure S5

Figure S6. RFP expression in *P. aeruginosa* PAO-MW1 p67T1 is not affected by homoserine lactone concentration. Fluorescence of d-Tomato was measured during the course of a growth curve experiment in a 96 well microtiter plate. The amount of expressed d-Tomato steadily increases over time; however there was no difference in the relative amounts of RFP produced in the presence (1 μM, dark grey bars) or absence (light grey bars) of 3O-C_{12}-HSL.

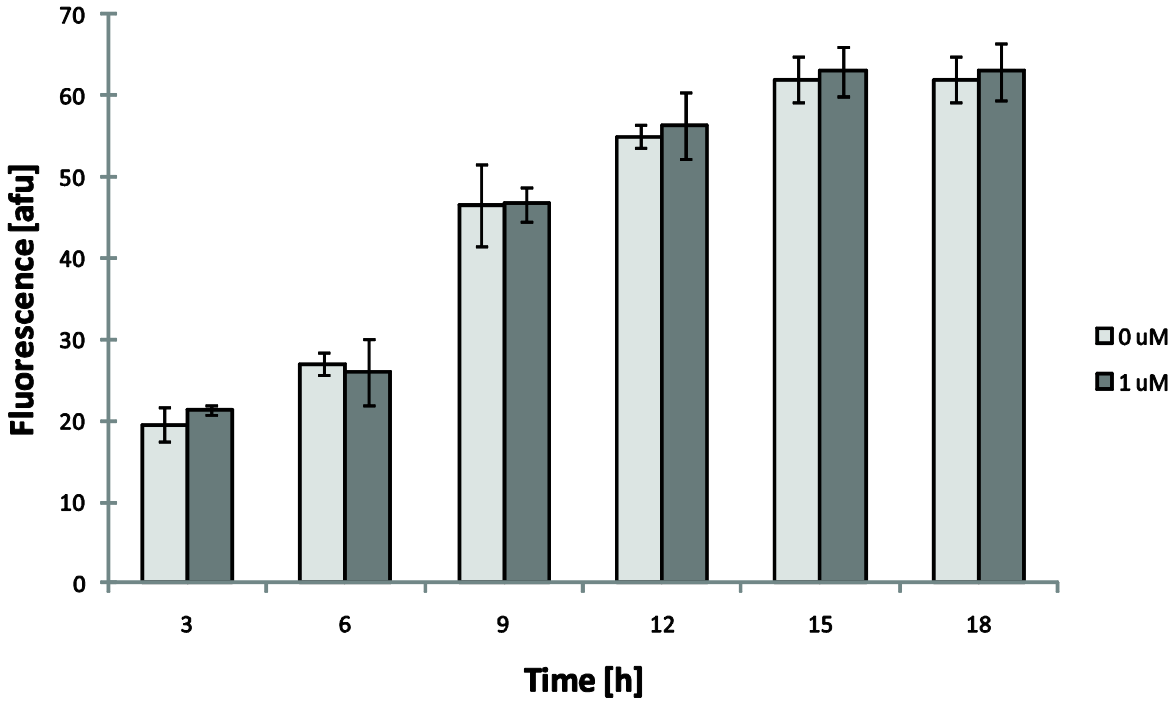


Figure S6

Figure S7. Expression of YFP is nearly undetectable from concurrent inoculation of PAO1 and PAO-MW1 pUM15. A) A plot of YFP fluorescent intensity in chambers containing PAO-MW1 pUM15 versus their distance (center-to-center) from the center chamber containing PAO1 at different time intervals. The two strains were inoculated concurrently. B) A plot of YFP fluorescent intensity in chambers containing PAO-MW1 pUM15 after 24 h incubation. The plot compares the large response at 24 h with a nascent biofilm compared to the response after 24 h with concurrent inoculation.

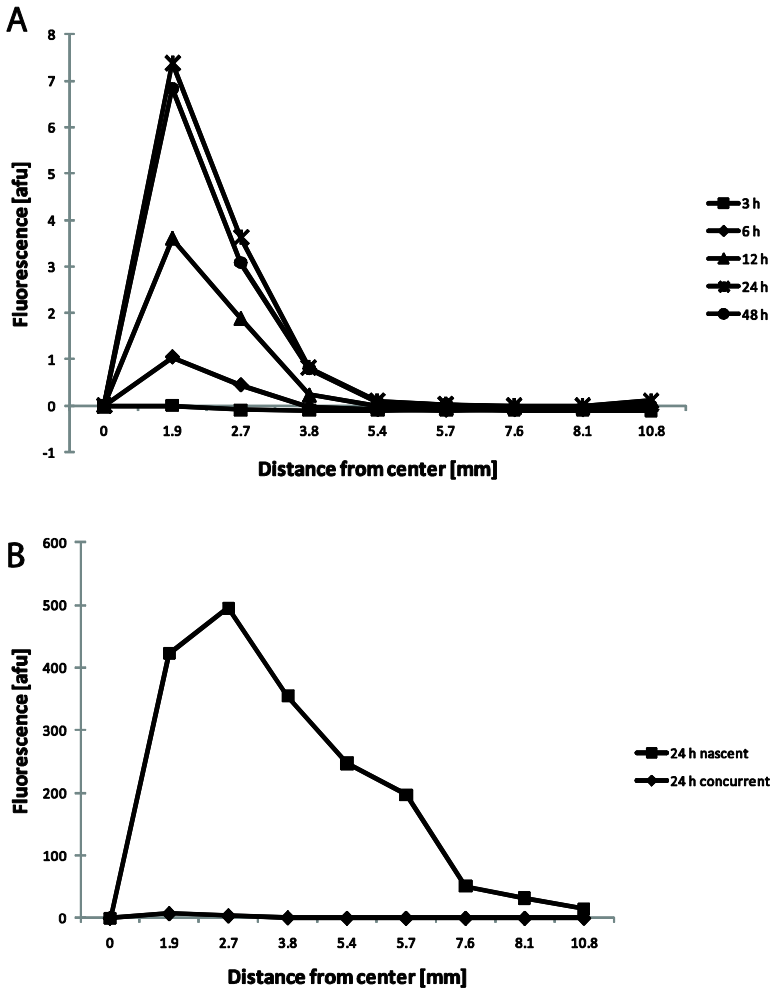


Figure S8. The observed HSL gradient visualized by YFP fluorescence in the presence of a nascent PAO1 biofilm closely matches the increase in growth rate of PAO-MW1 p67T1 cells in the presence of a nascent PAO1 biofilm as visualized by measuring d-Tomato fluorescence (labeled as 'RFP fluorescence'). Bars correspond to RFP fluorescence (left y-axis) and lines correspond to YFP fluorescence (right y-axis). Time points are in matching colors. The correspondence between the HSL gradient and increase in growth rate is easiest to see at 24 h (blue bars and line).

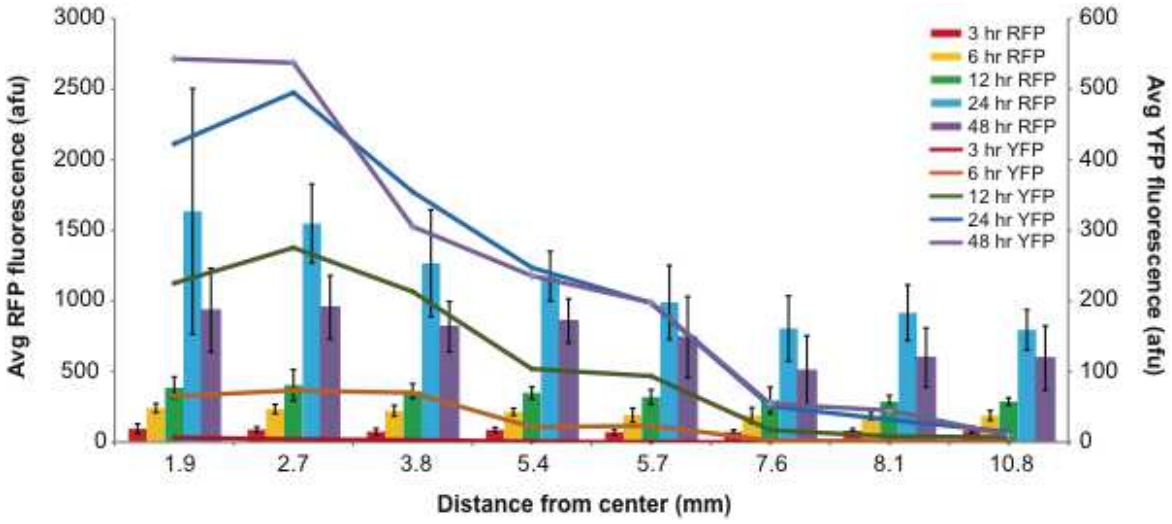


Figure S9. Positive and negative controls for pilot experiment to test whether exogenous 3O-C_{12}-HSL activates the QS circuit of PAO-MW1 pUM15. All 36 chambers of a 6 x 6 array were inoculated with PAO-MW1 pUM15. Chambers in the positive control contained 10 μm 3O-C_{12}-HSL; chambers in the negative control did not contain 3O-C_{12}-HSL. Gels were incubated at 37 °C for 10 h and YFP expression was quantified by epifluorescence microscopy. Images: Phase images of positive (A) and negative (B) control chambers, and fluorescent images of positive (C) and negative (D) control chambers. The dashed white line in panel D indicates the position of the chamber wall. The fluorescence gain for panels C & D was identical. Scale bar = 0.5 mm.

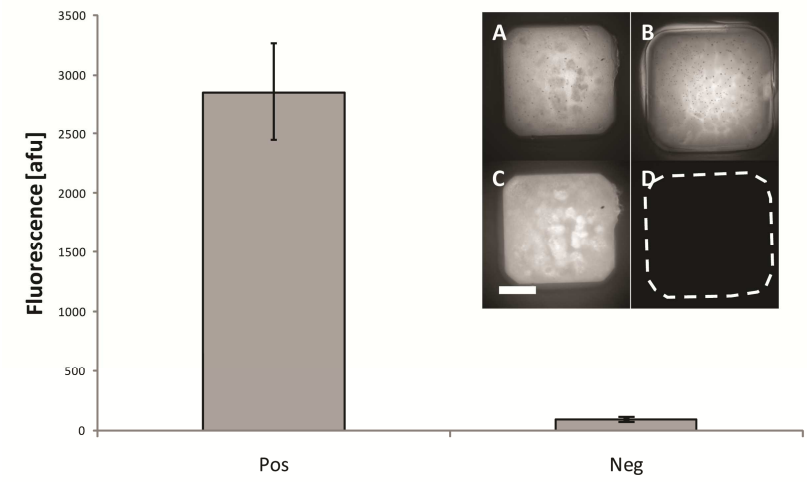
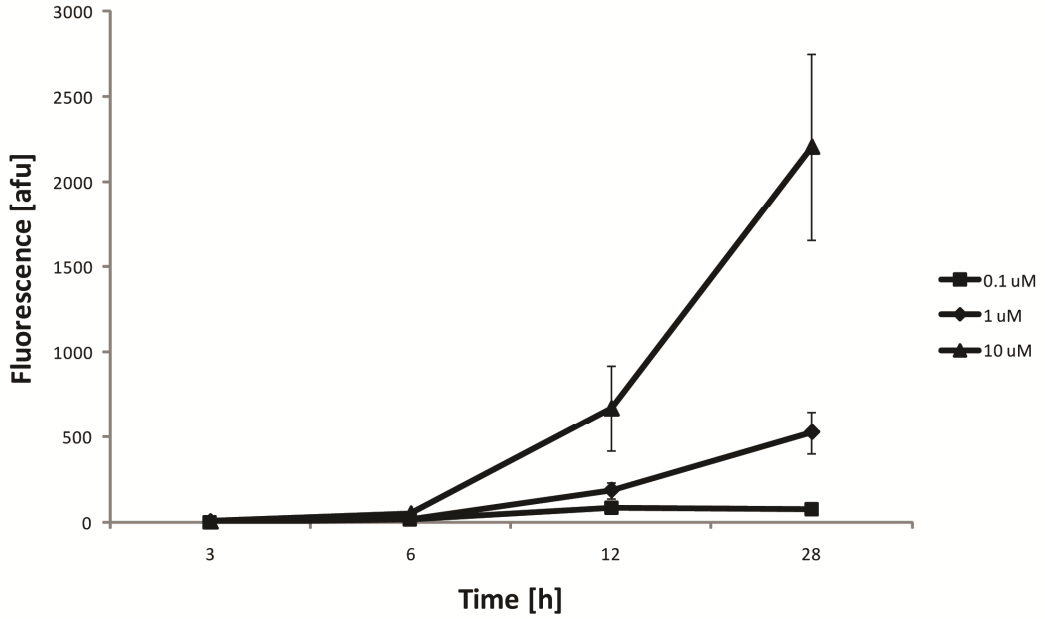


Figure S10. Dose-response curve relating the concentration of 3O-C_{12}-HSL to the fluorescence intensity of expressed YFP at different times in chambers embossed in a layer of 15% PEGDA.



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