A high-throughput fluorescence-based assay for the detection of petroleum hydrocarbon degrading bacteria

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Abstract

Over the past 100 years, oil spills and long-term waste deposition from oil refineries have significantly polluted the environment. These contaminants include highly toxic petroleum hydrocarbons which have widespread negative effects on human health and ecosystem functioning. Natural attenuation of long chain and polyaromatic hydrocarbons is slow and often incomplete. Bioaugmentation of polluted soils with wild bacteria with enhanced capacity for petroleum hydrocarbon degradation could speed up this process. However, the isolation and characterization of bacterial crude oil degradation efficiency can present a substantial bottleneck in developing and implementing these bioremediation strategies. Here, we develop a low-cost, rapid, high-throughput fluorescence-based assay for detecting crude oil degradation by bacteria using the dye Nile Red, which fluoresces when in contact with hydrophobic substances. To determine whether Nile Red fluorescence could be used as a proxy for crude oil content, we incubated varying concentrations of Nile Red (1-10%) with crude oil in 96-well plates and found a strong correlation between fluorescence intensity (FI) and optical density (OD). We assessed whether cell culture media (Luria Broth and Minimal Salt Media) quenched or augmented the fluorescence of the Nile Red but found no statistical difference in the fluorescence of Nile Red in the presence of either media. To calculate crude oil degradation based on FI, we developed a robust linear model and propose that crude oil depletion efficiency can be easily obtained by subtracting the % decrease in fluorescence intensity from T_0 to T_{end}. To determine whether this assay could identify bacteria with enhanced metabolic capacities to break down crude oil, we screened three wild type bacteria isolated from a former oil refinery in Bay Point, CA and identified one strain (*Cupriavidus* sp. OPK) with superior crude oil depletion efficiencies (up to 83%) in only three days. To probe whether this assay could also be used to understand the functional traits used by bacteria to degrade crude oil, we combined the assay with fluorescence microscopy and discovered that bacteria degrade oil using different strategies (i.e. long-distance transport via biofilms, direct attachment through adherence, and sequestration in biosurfactant-based vesicles) which affected depletion rates. This Nile Red assay can be used to identify bacteria for removing pollutants from soil through bioaugmentation while also providing qualitative data which could be used to develop bio-inspired solutions or biomimetic materials to remove toxins from soil and water.

Keywords: bioaugmentation, bioremediation, petroleum hydrocarbons, fluorescence microscopy, environmental microbiology

Introduction

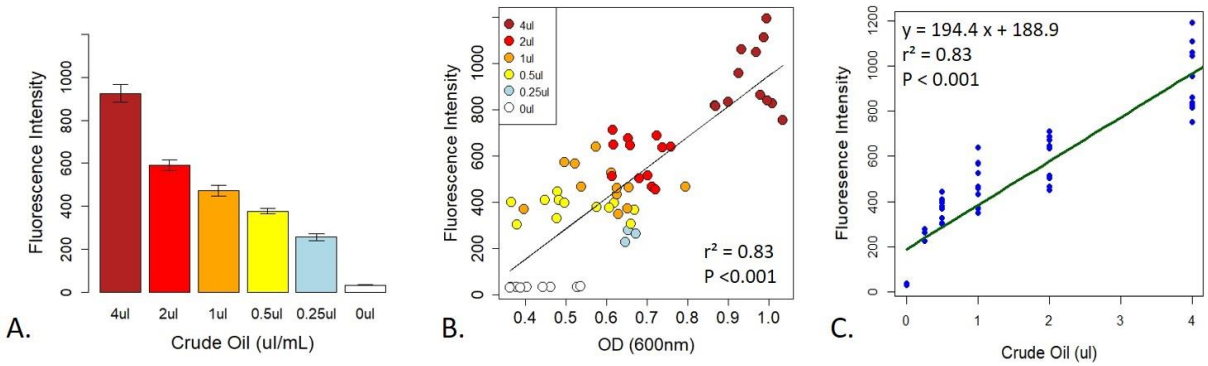
Over 10 million tons of crude oil have been spilled into the ocean and on land as a result of equipment failure, transportation accidents, and human tampering since the 1970s (Jernelöv 2010; ITOPF 2017). Such incidents negatively impact local ecosystems and decrease biodiversity in contaminated areas (Das and Chandran 2011). As many of these compounds are carcinogens (e.g. pyrene, benzene), oil spills also present an unprecedented threat to human health. High molecular weight polyaromatic hydrocarbons (PAHs) like benzo(a)anthracene, benzo(a)pyrene, and benzo(g,h,i)perylene can accumulate to toxic levels in the air (de Gouw et al. 2011; Srogi 2007). Petroleum hydrocarbons can also contaminate land and water reservoirs, making these areas unsuitable for agriculture or settlement (Maliszewska-Kordybach dand Smreczak 2000). Particularly in developing countries, these risks go unmitigated. For example, after a series of spills from Shell Oil pipes and refineries in the Niger Delta from the 1990s to early 2000s, drinking wells are currently contaminated with benzene levels that are 900x the safe level (Lindén and Pålsson 2013).

Although the number of spills has declined over the past ten years, clean-up of historic spills is non-existent or often incomplete (Jernelöv 2010). Ocean spills rely on burning or mopping-up crude oil and relying on native ocean bacteria to break down the remaining petroleum hydrocarbons (Kostka et al. 2011). Shoreline and inland spills are more difficult to clean up effectively. Environmental hazard teams often rely on chemical washing or removal of contaminated sand or soil, and where this is not possible, natural attenuation of petroleum hydrocarbons by native organisms (Das and Chandran 2011; Li et al. 2016). However, natural processes of degradation are often slow and only remove shorter-chain hydrocarbons. Long chain hydrocarbons and PAH often resist degradation because they are inaccessible

103 to organisms (due to mineralization, hydrophobicity, toxicity, and adsorption onto soil particles) and only
104 a small number of bacteria have the metabolic capacity to degrade these compounds (Bamforth and
105 Singleton 2005).
106
107 Bioremediation using bacteria isolated from polluted sites may enhance the speed and efficiency of
108 petroleum hydrocarbon removal (Das and Chandron 2011). Known bacterial petroleum hydrocarbon
109 degraders include *Alcanivorax borkumensis*, *Bacillus subtilis*, *Burkholderia cepacia*, *Pseudomonas*
110 *fluorescens*, *Pseudomonas marginalis*, and *Pseudomonas oleovorans* (Brooijmans et al. 2009; Rojo et al.
111 2009). These bacteria contain monooxygenases and dioxygenases which insert oxygen into petroleum
112 hydrocarbons to break down their carbon structure (Wang and Shao 2013). Some bacteria also produce
113 secondary compounds, namely biosurfactants, which make crude oil more accessible (Mohanty et al.
114 2013). Biosurfactants reduce the surface tension between the oil and water interface and allow
115 recalcitrant compounds, like PAHs, to precipitate into the aqueous phase, where they can then be
116 metabolized by bacteria.
117
118 Inocula could be made of selected petroleum degrading bacteria and then applied to polluted land as a
119 form of bioaugmentation. However, screening, identification, and characterization can be a time-
120 consuming process. To speed up this process, we sought to exploit the optical properties of the dye Nile
121 Red to develop a high-throughput fluorescence-based assay that uses 96-well microtiter plates and a plate
122 reader to detect bacteria that can degrade petroleum hydrocarbons. Nile Red is a colorless compound
123 which fluoresces red when in contact with hydrophobic substances (Greenspan and Fowler 1985). It is
124 often used as a stain for lipids and fatty acids (Greenspan et al. 1985; Rumin et al. 2015; Shrivastav et al.
125 2010), but has never been used, to our knowledge, as a way to measure crude oil content, as an assay for
126 crude oil degradation, or as a tool for understanding how bacteria degrade crude oil. Our research had

127 five key objectives: (1) to determine whether there was a correlation between fluorescence intensity (FI)
128 and crude oil content, (2) to determine whether cell culture media (Luria Broth vs. Minimal Salt Media) or
129 the addition of bacteria caused variations in the fluorescence intensity of the Nile-red stained oil, (3) to
130 develop a new model to calculate crude oil content based on FI, (4) to identify whether a Nile-red based
131 assay could detect bacterial strains isolated from the natural environment with enhanced capacity to
132 degrade crude oil, and (5) to determine whether fluorescence microscopy of aliquots taken from the assay
133 could be used to deepen our understanding of the functional traits bacteria use to degrade crude oil.
134 Based upon statistical analysis of assay data, we show that this assay can measure and calculate crude oil
135 degradation by bacteria down to the level of 1.25 nl/µl in as little as three days. Furthermore, it can
136 provide new information on the mechanisms bacteria use to break down crude oil when combined with
137 fluorescence microscopy. Potential applications of this assay include the rapid detection of novel bacteria
138 for bioremediation and the development of new bio-inspired solutions and materials for the removal of
139 petroleum hydrocarbons from the environment.
140
141 **Results & Discussion**
142
143 To determine whether the fluorescence intensity of Nile-Red stained crude oil was correlated with crude
144 oil content, we set up 96-well plates with two serial dilutions of crude oil (1 µl - 16 µl, and 250 nl – 4 µl,
145 equivalent to 1-8% and 0.125%-2% crude oil respectively) as described in the method section. Each plate
146 received four treatments: LB without bacteria, LB with bacteria, MSM without bacteria and MSM with
147 bacteria. For these initial proof-of-concept assays, we randomly selected a bacterial strain (*Cupriavidus*
148 sp. strain OPK) from our freezer stocks as a model bacterium. At day 0 (T_0), there was a strong correlation
149 between fluorescence intensity (FI) and optical density (OD) for all treatments (cells vs. no-cells with LB
150 or MSM) (**Fig. 1A-B**). This indicates FI is a strong proxy for crude oil content. After T_0, for the wells

151 containing bacteria the correlation between FI and OD varied as cells proliferated and the amount of crude
152 oil declined. To determine the strength of the relationship between FI and crude oil content, we created
153 several linear models from the T_0 data for each of the treatments (described fully in the method section).
154 The final model used for calibration was based on the MSM with bacteria treatment (**Fig. 1C**). The model
155 had an R^2 of 0.83 with a y-intercept of 188.9 ± 22.37 and a concentration coefficient of 194.4 ± 11.11 with
156 a residual standard error of 124 on 61 degrees of freedom ($F_{1,61}$= 305.9, p <0.0001). The standard errors
157 and their fitted values were randomly distributed, the residual errors were normally distributed in the Q-
158 Q plot, and Cooks' Distances among all points was less than 1 (**SI Fig. 3**). The final calibration equation is
159 y = 194.4x + 188.9. To calculate crude oil depletion efficiency, we developed an additional approach based
160 on the following equation: DE = FIT_0-FIT_3/FIT_0 , where DE is the 'depletion efficiency' and FI_x is the
161 fluorescence intensity on day x. The calibration curve and depletion efficiency equation thus provide two
162 approaches to detect and quantify bacterial crude oil degradation.



163

164 **Figure 1: Correlation between fluorescence intensity and crude oil content**. There was a strong relationship
165 between fluorescence intensity and crude oil content when crude oil was incubated with Nile Red (A). This
166 relationship was confirmed by the strong correlation between crude oil fluorescence intensity and optical density
167 on D_0 (B). Figure 1.C represents the linear model calibrating fluorescence intensity with crude oil content. All
168 fluorescence intensity data is based on excitation at 535nm and emission at 650nm.
169

170 To optimize assay parameters, we conducted a series of experiments to determine whether cell culture
171 media or the presence of bacteria could alter the fluorescence of Nile Red-stained crude oil, leading to an

172 over or under estimation of crude oil content. We found media type did not interfere, in terms of
173 fluorescence quenching or overlapping in excitation/emission spectra, with the fluorescence of the Nile-
174 Red stained crude oil. T-tests showed that there was no difference in fluorescence intensity in wells filled
175 with bacteria and LB or MSM (t = 1.27, df = 30.25, p = 0.21) or in wells without bacteria in LB or MSM (t =
176 0.88, df = 33.26, p = 0.38). This indicates that cell autofluorescence is minimal and does not over-lap with
177 the crude oil fluorescence. However, during the course of these experiments we found that the microbial
178 rate of crude oil degradation was almost 10% greater in the presence of MSM. *Cupriavidus* sp. strain OPK
179 grown in LB degraded on average 74% of the crude oil while *Cupriavidus* sp. strain OPK in MSM reduced
180 crude oil by 83% (t = -2.8, df = 3.96, p = 0.04); however, there was no difference in the final biomass
181 between the two treatments (t = -1.78, df = 2.77, p = 0.18). (**SI Fig. 1**). This is likely due to the fact that in
182 MSM crude oil is the only source of nutrients while in LB there are other nutrients (e.g. amino acids from
183 yeast extract) which could support bacterial metabolism. These results suggest using Minimal Salt Media
184 in the assays may provide a more accurate measure of bacterial crude oil degradation.
185
186 To further test whether this assay could be used to distinguish differences in the efficiency of bacterial
187 degradation of crude oil, we tested three strains of bacteria previously isolated from Shell Pond, a
188 petroleum contaminated site in Bay Point, CA (**Fig. 2**). The site was a former Shell Refinery, where
189 petroleum byproducts and other chemicals were desposited from the 1950s until the 1970s. Of the three
190 bacterial species, two species (*Cupriavidus* sp. strain OPK and *Rhodococcus erythropolis* strain OSPS1)
191 performed the best, depleting 69 ± 0.03% and 62 ± 0.03% of crude oil, respectively. The *Pseudomonas* sp.
192 strain BSS only depleted 49 ± 0.03% of the crude oil.

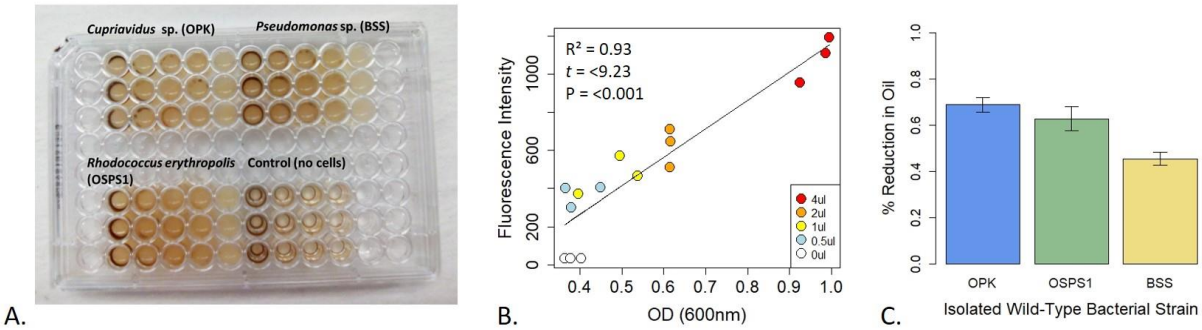


Fig. 2. Design and performance of a fluorescence-based assay to detect bacteria that degrade crude oil. A. Assay of three bacteria isolated from Shell Pond after 3 days of exposure to crude oil (concentrations ranging from 0.125% - 2%) in Minimal Salt Media (MSM). Consumption of crude oil can be seen in wells with bacteria. B. Correlation between fluorescence intensity (FI) and optical density (OD). Each point in the plot represents the treatments from 2A (decreasing from 4 µl to 0.5 µl per well, for a concentration of 2 to 0.125 % crude oil per 200 µl well volume) at T_0. Correlation between FI and OD for each treatment was >0.9 at T_0, demonstrating the replicability of this assay. C. Amount of crude oil consumed by each bacterial strain when grown in 2% crude oil.

To determine whether this assay could also be used to elucidate the microbial mechanisms used to degrade crude oil, for the above experiments we extracted 1 µl of each bacterial strain from the 96-well plate at T_3, T_{10}, and T_{20} for analysis using fluorescence microscopy. The bacteria with the highest rates of degradation, *Cupriavidus* sp. strain OPK, formed biofilms connecting the bottom of the oil layer with the bottom of the microtiter well. We observed red fluorescent 'networks' showing that the bacteria transported the crude oil through the biofilm (**SI Fig 5**). Transport of crude oil through a network may allow for the efficient diffusion of petroleum hydrocarbons across highly specialized membranes embedded with monooxygenases and dioxygenases. Enzymes, like alkB and almA, are known to reside in bacterial outer membranes and two enzymes (alkB and p450cam) also seem to be secreted and embedded in biofilms (Gkorezis et al. 2016; French et al. in prep). In contrast, the *Rhodococcus erythropolis* strain OSPS1 attached directly to crude oil droplets (**Fig. 3B**). Other studies have shown that bacteria, such as *Alcanivorax borkumensi*, can attach to crude oil using exopolysaccharides and pili (Broojimans et al. 2009). Strain OSPS1 may be using similar methods for cell adherence to crude oil. The least efficient strain, *Pseudomonas* sp. strain BSS, produced biosurfactant-based vesicles 4.17 -12.34 µm

216 in diameter which contained small oil droplets ranging from 0.11 to 2.98 µm in diameter (**Fig. 3C; SI Fig.**
217 **3A**) and/or bacteria (**SI Fig. 3B**). We have observed a similar phenomenon in other wild-type strains of
218 bacteria, which produce biosurfactant-based vesicles when exposed to crude oil (**SI Fig. 4**). Previous
219 studies have shown that *Pseudomonas* species produce biosurfactants, namely rhamnolipids, during the
220 degradation of PAHs (Hua and Wang 2014; Mulligan and Gibbs 2004). Our follow-up experimental
221 research has also shown that biosurfactants can spontaneously form vesicles 5-150 µm in diameter in the
222 presence of water and crude oil (**SI Fig. 5**). This selective degradation of PAHs may explain why the strain
223 degraded less crude oil than the other two strains tested. These results suggest that bacteria employ
224 different strategies for breaking down crude oil which may impact their overall efficiency in removing
225 toxic compounds from the environment.

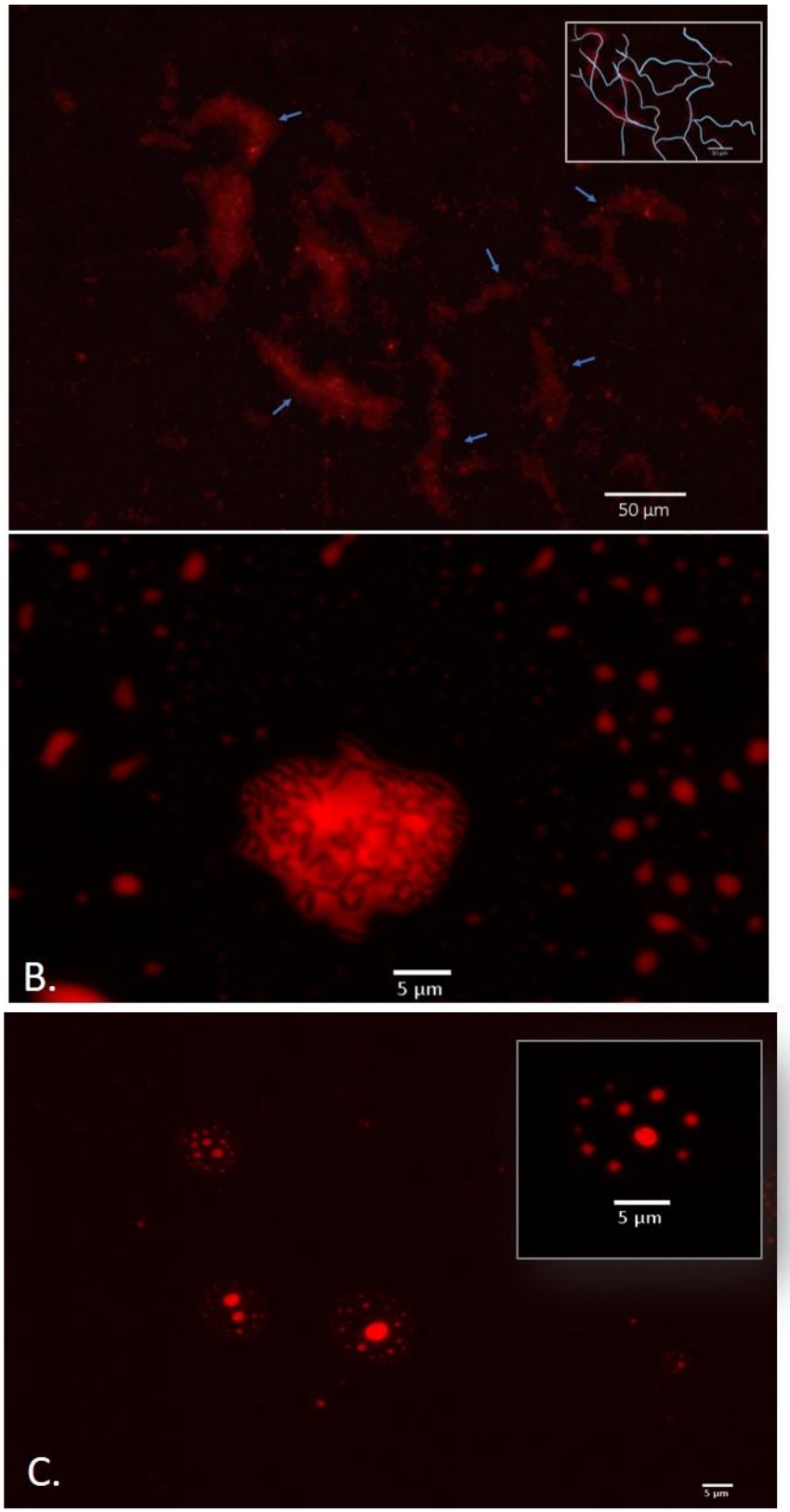


Figure 3 Bacterial strategies for crude oil degradation. A. *Cupriavidus* sp. OPK transported crude oil through biofilm networks after exposure to 1-12.5% crude oil for 3 days. Blue arrows point to channels within the network; the inset

box shows a rough tracing of the network for clarity. B. *Rhodococcus erythropolis* strain OSPS1 (black rods) adhered to crude oil droplets. The oil droplet depicted is 21.03 um in diameter. C. *Pseudomonas* sp. strain BSS produced biosurfactants when exposed to crude oil which spontaneously formed vesicles. The vesicle depicted inset is 11.31 µm in diameter and contains small oil droplets ranging in size from 790 nm - 1.33 µm in diameter. All images are false-colored red; detailed description of image acquisition can be found in the Methods section.

In addition to quantitative data on microbial crude oil depletion efficiency, qualitative data on how bacteria degrade petroleum could be used to select specific strains for different applications. For example, strains like *Cupriavidus* sp. strain OPK and *Rhodococcus erythropolis* strain OSPS1 might be more suitable for direct application to polluted soils as inoculum. They could also be applied in large quantities to bioreactors containing polluted soil. In contrast, strains like *Pseudomonas* sp. BSS might be more suitable for clean up of soils and water dominated by recalcitrant PAHs. In addition, further genetic, molecular, and ecological analysis of bacteria screened using the Nile Red assay could lead to new ways to augment these capabilities or to wholly new approaches to bioremediation (**Fig. 4**). For example, research on the relationship between biofilm formation and petroleum degradation could lead to bioinspired filters which could be used for water purification. Similarly, understanding the structure, chemical composition and function of biosurfactant-based vesicles could be used to create synthetic vesicles for enhanced removal of recalcitrant PAHs from highly polluted environments where living inoculum might not survive. These are just two examples of how our group is currently incorporating insights from this assay to develop new bioremediation strategies. Consequently, we anticipate that the high-throughput Nile Red assay presented here could be used by microbiologists, ecologists and engineers for a variety of applications within the fundamental and applied sciences.

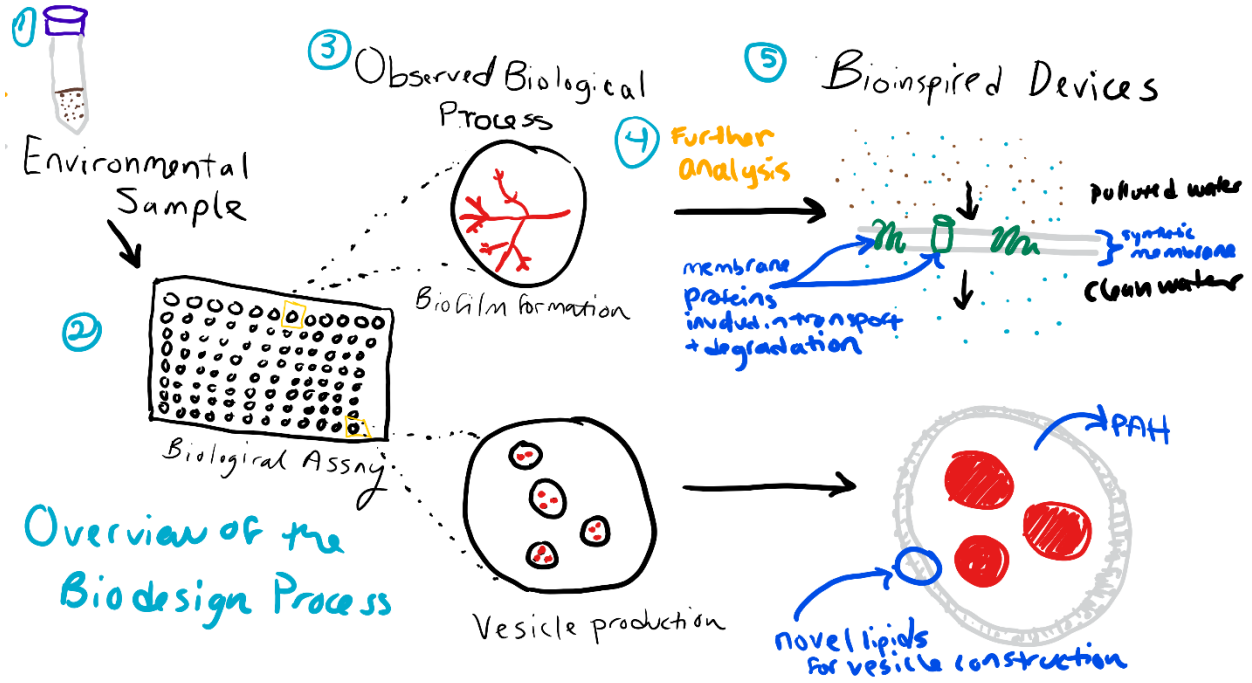


Figure 4: Incorporation of crude oil degradation assay into biodesign processes. Understanding how bacteria degrade crude oil could lead to the development of novel materials and devices for bioremediation. Examples include the creation of biofilters for water filtration (embedded with enzymes involved in hydrocarbon transport and degradation) and synthetic vesicles composed of bacterial-derived lipids for the removal of PAHs from complex hydrocarbon mixtures. The assay is compatible with a number of downstream analyses, including DNA extraction (e.g. to identify bacterial species, genes involved in hydrocarbon degradation) and mass spectrometry (e.g. LC/MS and MALDI to identify membrane proteins and lipids).

Conclusion

Toxic compounds released during crude oil spills and waste from crude oil refineries threaten ecosystem functioning, local biodiversity, and human health. Bioremediation using bacteria that efficiently degrade petroleum hydrocarbons can remove these contaminants from the environment. However, isolating, characterizing and identifying these bacteria can be a time-consuming process. We have developed a robust fluorescence-based assay for detection and quantification of crude oil and petroleum hydrocarbon degradation by native soil bacteria isolated from petroleum contaminated soils down to 1.25 nl/µl. We have further demonstrated that the Nile Red assay can be used to understand and exploit bacterial functional traits employed in petroleum hydrocarbon degradation. Future research could develop

270 variations on this fluorescence-based assay to rapidly identify bacteria that can degrade single
271 hydrocarbon substrates (e.g. pyrene) or other classes of toxic environmental compounds (e.g.
272 polychlorinated biphenyls, PCBs) (Hendricksen et al. in prep). Further multi-disciplinary research to
273 identify the specific genes, proteins and compounds underlying bacterial breakdown of contaminants like
274 crude oil could lead to the development of more efficient inoculum and may even lead to new bio-inspired
275 solutions for bioremediation.
276
277 **Methods**
278
279 *Growth of bacterial strains*
280 Bacteria were isolated from native soil taken from Shell Pond in 2016. These strains were kept at 80°C in
281 glycerol stocks. To revive each strain, a small amount of stock culture was incubated in 10 mL of LB at 37°C
282 for three days. Each strain was plated on LB plates to create single colonies. From here, single colonies
283 were selected and grown in 5 mL of LB for 16 hours.
284
285 *Nile Red stock solutions*
286 1 mM and 0.1 mM stock solutions of Nile Red were made in DMSO. These stock solutions were encased
287 in tin foil and kept in a -20°C freezer until use.
288
289 *Addition of Nile Red to crude oil*
290 To determine whether concentration of Nile red had an effect on fluorescence intensity, we conducted
291 several assays with three concentrations of Nile Red (0.1%, 0.5%, and 1%) for the 1 mM and 0.1 mM stock
292 solutions. First, we sterilized the crude oil by autoclaving it three times and filtering it twice with 0.22 µm
293 filters. Next, each concentration of Nile Red was incubated with 1 mL of crude oil for 20 minutes in 2 mL

294 Eppendorf tubes. The Nile Red-crude oil solution was shaken periodically to assure the mixing of the two
295 substances. We found that more consistent results were found with the 1% concentration of the 1 mM
296 stock solution of Nile Red. This may be due to the low concentration of DMSO in the 1 mM stock solution,
297 which allows for a more even complexing of the dye and the crude oil. We observed that the DMSO in the
298 more diluted stock solution (0.1 mM) tended to pool at the bottom of the Eppendorf after complexing
299 with the oil for 20 minutes. To determine the stability of the Nile Red-crude oil solutions, we left these
300 solutions standing at room temperature in racks for 24 hours in full light. We observed a slight decrease
301 in FI (roughly 20%), likely due to the fact that Nile Red is photo-sensitive and some bleaching may have
302 occurred. As such, it is ideal to create new stock solutions of dyed crude oil before the start of each
303 experiment.
304
305 *Assay set up*
306 To set up each assay, 96 well plates were filled with crude oil which had been complexed with Nile Red as
307 described above (ranging from 16 µl - 250 nl). For the control wells, LB or MSM was added to the wells
308 until a total of 200 µl was reached. For wells containing bacteria, 100 µl of bacteria in LB or MSM at an OD
309 of 0.6 were added to each well; LB or MSM was then added to bring the total volume of each well to 200
310 µl. Each treatment was replicated three times. Assays were sealed with parafilm and placed on a shaking
311 incubator (120 rpm) in a dark room. To measure FI and OD, we used a Tecan plate reader. OD readings
312 were taken at 600 nm in a circular pattern with 4x4 readings. FI readings were taken at excitation 535nm
313 and emission 650 nm in a circular pattern with 6x6 readings. We measured FI and OD every day from T_0-
314 T_5 and then again at T_{10} and T_{20}.
315
316 *Staining of biosurfactant-based vesicles*

317 Vesicles were stained with a 1 µg/mL stock solution of FM™ 4-64 Dye (*N*-(3-Triethylammoniumpropyl)-4-
318 (6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide) which was made with a potassium
319 phosphate buffer (pH 7). Briefly, 20 µl of culture was incubated with 20 µl of dye for 30 minutes. 1 µl of
320 this solution was placed on a glass microscope slide and imaged with a fluorescent microscope.
321
322 *Microscopy*
323 1ul aliquots of media from 96 well plates were placed on glass microscope slides and imaged using a
324 Zeiss AxioImager M1 with a Hamamatsu Orca 03 12-bit grayscale digital color camera. Images were taken
325 at 40x and 100x. Nile Red was detected using the Texas Red filter. To image intact biofilms within each
326 well in glass-bottom 96-well microtiter plates, we used a Zeiss AxioObserver Z1 Live-Cell system with a
327 QImaging Retiga SRV and a QImaging 5MPix Micropublisher camera. Images were taken with a 0.01-0.05
328 second exposure time (depending on magnification) and analyzed using the Zeiss Axio Vision software.
329
330 *Statistical analysis*
331 We used t-tests to determine whether media type (LB or MSM) had a significant effect on crude oil
332 fluorescence. We used one-way analysis of variance (ANOVA) to determine whether Nile Red
333 concentration had a significant effect on crude oil fluorescence intensity. Differences among treatments
334 were assessed by reference to the standard F tests. We also used ANOVA to determine whether microbial
335 strain had an effect on degradation of crude oil. Crude oil depletion efficiency (%) was calculated by taking
336 the FI at D_0 and subtracting the FI at D_3, divided by the FI at Day_0. We used Pearson Correlation analysis
337 to determine whether there was a correlation between FI and OD. To create a calibration curve to relate
338 FI to amount of crude oil, we created linear models and compared these to quadratic and polynomial
339 models of the data, followed by visual examination of the residuals of each model to evaluate the
340 suitability of each model for explaining the data. We further tested the validity of the slope and y-intercept

values for the model using t-tests. The confidence interval for each is $\beta_o = b_o \pm tsb_o$ and $\beta_1 = b_1 \pm tsb_1$ where sb_o and sb_1 are the standard errors for the intercept and slope, respectively. To determine if there is a significant difference between the expected (β) and calculated (b) values we calculated t and compared it to its standard value for the correct number of degrees of freedom. We selected the model with the best 'fit' for the data based on R squared value and statistical significance. General statistics, ANOVA, and t-tests were conducted in R (v. 3.2.2, "Fire Safety") using packages stats (v. 3.4, R core team) and psych (v. 1.6.4).

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