# Top-down engineering of oil field microbiomes to limit souring and control oil composition during extraction operations

Ethan Hillman[1], Louis Caceres Martinez[1], Gozdem Kilaz[1], and Kevin Solomon[2]

[1]Affiliation not available
[2]University of Delaware

June 7, 2022

**Abstract**

Microbial processes sour oil, corrode equipment, and degrade hydrocarbons at an annual global cost to the oil and gas industry of nearly $2 billion. However, top-down control of these microbial processes can reduce their damage and enhance oil recovery. Here, we screened microbial communities from five oil wells in the Illinois basin and evaluated nutrient injection strategies to control metabolism and community composition. Molasses and molybdate supplementation stimulated significant gas and organic acid production while completely suppressing corrosive H2S formation in samples from two wells. These changes were accompanied with significant shaping of the microbiome community. Simulations of field operations via a lab-scale mini-coreflood validated that oil well microbiomes can be engineered to also shape oil hydrocarbon composition in situ. These pilot studies validate the potential of economical and sustainable top-down microbiome engineering to control microbes in oil extraction and enhance the economic viability of oil recovery.

Top-down engineering of oil field microbiomes to limit souring and control oil composition during extraction operations

Ethan T. Hillman[1,2¥], Louis Edwards Caceres-Martinez[3], Gozdem Kilaz[3], and Kevin V. Solomon[1,2,4++*]

[1] Department of Agricultural and Biological Engineering, Purdue University, West Lafayette, IN 47907

[2] Purdue University Interdisciplinary Life Sciences, Purdue University, West Lafayette, IN 47907

[3] School of Engineering Technology, Fuel Laboratory of Renewable Energy (FLORE), Purdue University, West Lafayette, IN 47907

[4] Department of Chemical & Biomolecular Engineering, University of Delaware, Newark, DE 19716

[¥=] Present Address: Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109, USA

[++] Present address: Department of Chemical & Biomolecular Engineering, University of Delaware, Newark, DE 19716

***** Correspondence: kvs@udel.edu

**Abstract**

Microbial processes sour oil, corrode equipment, and degrade hydrocarbons at an annual global cost to the oil and gas industry of nearly $2 billion. However, top-down control of these microbial processes can reduce their damage and enhance oil recovery. Here, we screened microbial communities from five oil wells in the Illinois basin and evaluated nutrient injection strategies to control metabolism and community composition. Molasses and molybdate supplementation stimulated significant gas and organic acid production while completely suppressing corrosive $H_2S$ formation in samples from two wells. These changes were accompanied

with significant shaping of the microbiome community. Simulations of field operations via a lab-scale mini-coreflood validated that oil well microbiomes can be engineered to also shape oil hydrocarbon composition *in situ* . These pilot studies validate the potential of economical and sustainable top-down microbiome engineering to control microbes in oil extraction and enhance the economic viability of oil recovery.

*Topical Heading:* Biomolecular Engineering, Bioengineering, Biochemicals, Biofuels, and Food

*Keywords:* Microbial enhanced oil recovery, top-down microbiome engineering, miniature coreflood, cultivation screening, comprehensive two-dimensional gas chromatography

**Background:**

Fossil fuels are likely to remain a significant energy source for at least the next 30 years as economies transition globally towards more renewable energy sources.[1–3] Extracting trapped oil from aging wells can not only increase the overall production of existing wells but also minimize the need to drill new wells and, ultimately, decrease the cost of extracting oil from existing reservoirs. Expensive polymer and/or surfactant/polymer formulations that require large amounts of funding and research effort are used to extract oil not recovered by primary extraction techniques.[4–6] Additionally, native and exogenous microbes have been harnessed as a more cost-effective alternative for secondary and tertiary oil recovery – an approach coined microbial enhanced oil recovery (MEOR).[7,8] Microbial activity in oil wells, however, can either be harnessed to benefit the oil recovery process or be disruptive and hinder the process. Microbial metabolic products can liberate trapped oil: produced gases displace immobile oil, organic acids dissolve carbonaceous deposits and increase well permeability, solvents dissolve and mobilize large hydrocarbons from the pores, and biosurfactants act as emulsifiers.[7,9] On the other hand, microbes can also degrade and sour oil, and produce metabolites that corrode the well casing, flowlines, and pipelines. Globally, pipeline corrosion alone can result in nearly $2 billion dollars (USD) of damage and loss each year.[7,10] This corrosion is due primarily to oxidation of microbially-produced hydrogen sulfide ($H_2S$) to sulfuric acid.[10,11]Engineering the composition and metabolism of oil well microbial communities promises to enhance the productivity and economic viability of oil extraction operations.

Engineering microbiomes has emerged as a sustainable approach to develop processes for a number of industries from health and nutrition to agriculture and fuels.[12–14] To engineer microbial communities, there are two common general approaches: bottom-up and top-down microbiome engineering.[15] Generally, bottom-up microbiome engineering pertains to constructing communities of microbial species and strains with desired attributes and synergies to carry out a task or set of tasks. In MEOR, bottom-up approaches often focus on biofilm or surfactant production via communities centered around natural and/or engineered strains of *Pseudomonas* ,*Bacillus* , *and Enterobacter* which are able to extract up to 26% of the additional trapped oil.[7,16–19]However, the ecology of engineering bottom-up communities is very complex and developing stable communities that colonize a natural, fluid ecosystem, like that of the oil well microbiome, is exceedingly difficult.[20] Strains may fail to colonize because they do not fill a particular ecological niche in the community and can change the native microbiome composition in unpredicted and uncontrollable ways.[21] Emerging bottom-up strategies to overcome this challenge such as artificial syntrophy where microbes exchange metabolites for mutual survival are difficult to develop and can fail catastrophically if a single species is lost due to unanticipated competition with native microbes.[22,23]Lastly, strains used in bottom-up approaches are often genetically engineered, which raises both ecological concerns and creates regulatory burden related to the introduction of genetically modified organisms in the environment.[14,24]

In contrast, top-down microbiome engineering manipulates environmental factors such as nutrients, pH, temperature, and ionic strength to tailor a native community for a desired outcome or task.[15]This strategy does not require any bacterial species to be introduced into the community or colonize a new environment, but instead leverages the present microbes.[20,25] Tuning environmental factors, or synthetic ecology, is typically more cost-effective and is easily testable in controlled parallel experiments.[26]Similarly, top-down MEOR is often much more economical than the synthetic polymers used for standard secondary and tertiary oil recovery.[27,28] However, not all microbiomes will respond to changes in these environmental factors and they

are likely to have different responses depending on the composition of microbes and environmental factors surrounding them.[29] Up to 89% of MEOR trials have successfully produced additional oil but to varying degrees.[29,30] Therefore, top-down microbiome engineering conditions must be rapidly screened *in vitro* in a high-throughput, cost-effective manner to identify both candidate oil well communities and optimal MEOR intervention conditions. Taken together, the facts that top-down engineering approaches are cost-effective, can be rapidly screened, and do not require scientific ecological barriers suggest that it is an attractive strategy for developing MEOR formulations for field trials.

In this study, we use top-down engineering strategies to screen wells and optimize oil extraction operations for the Illinois basin via MEOR. Through *in vitro* cultivation, we identified candidate wells that would be responsive to MEOR intervention and characterized the response of their microbial community as a function of specific nutrient supplementation. We established that simple molasses injection coupled with inorganic salt solutions could be used to stimulate microbial activity that reduced $H_2S$ production and stimulated desired gas and organic acid production. Simulation of field recovery operations via a miniature coreflood experiment confirmed the ability of these interventions to reduce oil souring and modify the specific hydrocarbon composition of produced oil. Ultimately, this work demonstrates that top-down microbiome engineering strategies can significantly benefit oil recovery operations while improving economic and environmental sustainability.

**Results and Discussion** :

Wells in the same basin have unique properties and microbial communities

Wells at mesophilic temperatures and moderate depths [1500-3000 ft, 457-914 m] are promising sites for successful MEOR applications[29–31] within the Illinois basin. We selected five such wells from this basin that were all approximately 27 @C (80 @F) in order to hold the temperature constant between trials and simplify cross-well comparisons (Table 1). The wells had depths of 1336-2258 ft (407-688 m) while the pH remained fairly neutral (pH 6 – 7). DNA was extracted from the produced water (effluent) of each well to assess the native microbial community composition via 16S rRNA gene profiling (**Supplemental Figure 1** ). We found that each well had a distinct microbial composition with various sulfur-reducing ($H_2S$-producing) and MEOR-relevant[9,19,31] archaea and bacteria. Common bacteria phyla include Proteobacteria, Bacteroidota, Firmicutes, Synergistota, Spirochaetota, Verrucomicrobiota, Desulfobacterota and Campilobacterota, of which the latter three are prominent sulfur-metabolizing bacteria.[32–35] These populations were distinct from others in the literature,[36,37] a finding that is not unexpected given that each community is driven by different environmental conditions such as temperature, salinity, pH, nutrient availability, and oil gravity among other factors.[19] However, there is no known correlation between native microbial community and MEOR success. Therefore, we pursued *in vitro* functional screening to evaluate candidate wells for MEOR top-down microbiome engineering.

**Table 1 Characteristics of candidate wells from the Illinois basin**

| Well ID | State | Depth (ft) | Temp (@C) | pH |
|---|---|---|---|---|
| B | IL | 1597-1626 | 27 | 7 |
| C | IL | 1585-1590 | 27 | 6.5 |
| D | IL | 1336-1343 | 27 | 7 |
| G | IL | 1620-1626 | 27 | 6 |
| S | IN | 2226-2258 | 27 | 6.5 |

In vitro screening identifies candidate wells and nutrient profiles for MEOR

In order to evaluate which candidate wells would respond to nutrient supplementation, *in vitro* microcosms were created in an anaerobic environment using a mixture of nine-parts oil well effluent and one-part crude nutrient source stock [40% corn syrup or molasses]. In addition to culture optical density, we monitored the microbial production of gases and organic acids that may enhance oil recovery by tracking microcosm

headspace pressure and pH, respectively, over the course of 1 week (**Figure 1** ). While two of the wells (Wells B, C,) did not respond to nutrient supplementation, microcosms from Well D and G responded to both molasses and corn syrup supplementation. One replicate from Well S demonstrated a moderate gas production on molasses while all replicates showed a drop in pH. The responses of Wells D and G were more reproducible, generating up to 13 PSI of pressure as well as a 2 pH-unit-drop in the culture pH. These findings are consistent with other biostimulation[36] results and further supports the notion that not all wells respond to nutrient supplementation and may respond in different ways (e.g., more acids than gases or vice versa). Overall, cultures grown on molasses, a commonly used nutrient source in MEOR,[9] produced more pressure and had larger pH drops suggesting that it had stronger microbe-activating potential for MEOR. Therefore, molasses was chosen as the crude nutrient source for our future screening efforts. Bacteria from the orders Lactobacillales, Enterobacterales, Bacteroidales, and Campylobacterales dominated the molasses microcosms from Well D and G comprising [?] 96% of the total reads (**Supplemental Figure 2** ). These bacteria are collectively known to produce $CO_2$ and $H_2$gases as well as acetic, lactic, butyric, and propionic acids that have roles in MEOR[9,19,31]. The increasing pressure and drop in pH imply that the production of some or all of these was stimulated by nutrient supplementation. However, production of $H_2S$ by Campylobacteria, such as *Sulfospirillium* and*Malaciobacter,* [33,38] may have also been stimulated. While our results indicated at least two responsive wells for MEOR intervention, successful MEOR biostimulation requires limited production of corrosive $H_2S$ while also increasing the gas, acid, and/or solvent production for oil recovery.

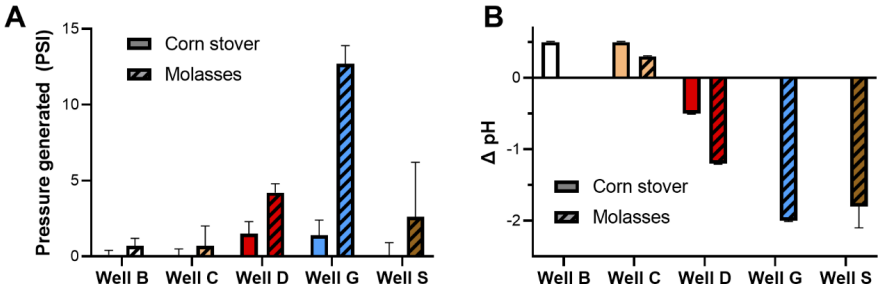


**Figure 1 – Carbon source screening of well candidates.** A) Accumulated pressure generated from microcosms over the 7-day span. B) Change in pH in microcosms over the 7-day experiment. Carbon sources screens: corn syrup (solid bars) and molasses (stripped bars). Error bars represent standard deviation, n=3.

Micronutrient supplements control metabolism and shape microbiome composition in vitro

To investigate if micronutrients can modulate the microbiome composition of the microcosms for top-down engineering in MEOR, our *in vitro*screening system was used to track metabolism and community composition under various micronutrient supplements. *In vitro* screening focused on Well D because it was one of the two most responsive wells. Moreover, Well G was heavily dominated by one genus (*Malaciobacter* , class Campylobacteria) (**Supplemental Figure 1** ), with less microbial diversity to adapt to the micronutrient conditions. Again, Well D was stimulated with molasses and supplemented with a variety of micronutrients including chloride (NaCl and KCl), phosphate ($K_2HPO_3$ and $KH_2PO_3$), nitrate ($KNO_3$), molybdate ($Na_2MoO_4$), and a combination of both nitrate and molybdate. Chloride salts were used as a negative control while phosphate salts were tested as a phosphate source, which have been added in other MEOR studies.[9,31,36] To reduce sulfate reduction, nitrate and molybdate salts were tested. Nitrate is a competing electron acceptor in place of sulfate in several sulfur-reducing bacteria,[11,39–41] while molybdate has been shown to inhibit $H_2S$ generation in different aqueous and marine contexts.[40–43]

Gas and organic acid production varied strongly as a function of micronutrient supplementation (**Figure 2A, Supplemental Figure 3** ). Nitrate- and molybdate-supplemented communities from Well D produced significant amounts of pressure. The cumulative effects of combination treatments were additive, resulting in the most observed pressure generation. Similarly, nitrate dropped the pH of the culture the most (to a pH ˜4) where the molybdate treatment had a slightly smaller effect (Supplemental Figure 3B). These

results suggest that chloride and phosphorous do not alter microbial metabolism in the microcosms and that nitrate best stimulated the production of gases and acids for MEOR. There was some additional benefit to adding molybdate in combination with nitrate as it generated the most gas and one of the larger pH changes. Because only select microbes can convert nitrate into $N_2$ and/or ammonia, or use it as a nitrogen source for synthesis of DNA and amino acids, supplemented nitrate will confer a competitive advantage to specific microbes in an otherwise nitrogen-limited environment such as an oil well. In turn, we expect that the differences in the metabolic outputs (**Figure 2A** ) are reflective of the differences in the microbial community composition as a result of the added compounds.

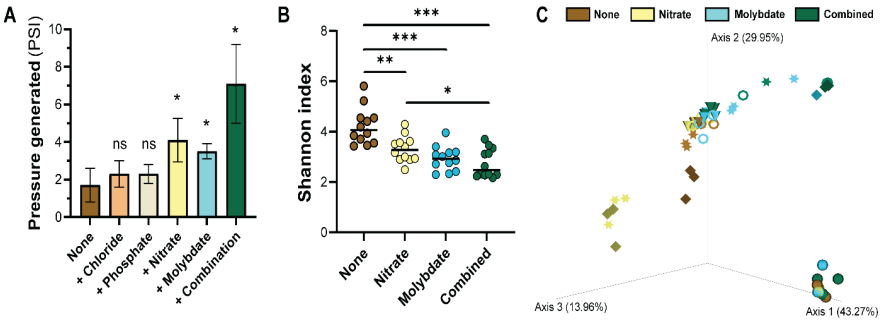


**Figure 2 – Non-essential nutrients stimulate metabolism and manipulate the microbiome composition.** A) Pressure generated in microcosms from Well D & G when treated with various nutrient supplements; T-tests performed with respect to None. B) Diversity score (Pielou's evenness) of microcosms after treatment with micronutrients (days 3, 4, 5 and 7). C) Unweighted Unifrac PCoA plot of Well D microcosms after micronutrient supplementation; shapes represent days, colors indicate treatment group: spheres = day 1, diamonds = day 3, star = day 4, rings = day 5, cones = day 7. Error bars represent standard deviation, n=3; * = $p < 0.05$, ** = $p < 0.005$, and *** = $p < 0.001$.

While the fate of nitrate is ultimately determined by the microbial community, we found that nitrate and molybdate supplements shifted the microbial community composition in different ways. Nitrate, molybdate, and the nitrate-molybdate combination treatments provide a selective pressure that selects for or enriches specific species reducing the alpha diversity (Shannon index) of the cultures from Well D (**Figure 2B** ). However, the treatments do not enrich for the same species - the beta diversity of the communities as estimated by principal coordinate analysis (PCoA) analysis of the 16S rRNA gene composition (**Figure 2C** ) reveal distinct groups of data points. Moreover, the time evolution of these communities diverge over the first three days (spheres to diamonds) as a result of their different selective pressures. However, the combined molybdate and nitrate treatment converges on that of molybdate alone (**Figure 3C** ) suggesting that the selection pressure applied by molybdate is stronger than that of nitrate and, ultimately, determines microbial metabolism and community structure. Hydrogen sulfide inhibition in the literature is more sensitive to molybdate than nitrate[42,43], adding further support for the strong selective pressure of molybdate.

To determine which taxa were specifically selected against and enriched in these microcosms, we evaluated the 16S rRNA gene composition of the microbial community. Interestingly, we found that after Day 1 of cultivation, there was little effect across the treatments with only a slight increase in the *Enterobacteriaceae* in the molybdate and combined treatments (**Supplemental Figure 4** ). After Day 3, however, the treatments varied noticeably from the untreated microcosms (**Figure 3A** ). For example, nitrate microcosms saw a bloom of *Sulfospirillium* in days 3 and 4, while the molybdate and combined treatments each saw a bloom in *Enterobacteriaceae sp.* and *Lachnoclostridiumsp.,* which are MEOR-relevant bacteria. Many *Sulfospirillium* species contain both nitrate- and sulfur-reducing pathways, so it is reasonable to expect that the nitrate substrate allowed these species to outgrow organisms that could not use this nutrient. Similarly, a few *Enterobacteriaceae* have been found to reduce molybdate[44,45] which may have provided a similar advantage to these microbes. At the same time, molybdate is structurally similar to sulfate and has been shown to

inhibit the growth of $H_2S$ producers[43] which may account for the absence of *Desulfovibrio* , *Dethiosulfovibrio* ,*Sulfospirillium* , and other sulfate reducing bacteria in the molybdate and combined treatment microcosms. After 7 days, the cultures primarily consisted of members of *Bacteroides* and*Lachnoclostridium* regardless of treatment suggesting that the community may have shifted to scavenge or use other nutrient sources to survive. Similar progressions of microcosms toward primarily Bacteroidetes and Clostridia have been seen in previous studies[36] suggesting these groups of bacteria have the advantage over other organisms in anaerobic consortia when the initial nutrients have been depleted at the end of the cultivation. Additionally, no known $H_2S$ reducing bacteria were detected with molybdate and combination treatments suggesting that the effects of the molybdate were effective over this whole time-course of the cultivation. In contrast, *Sulfurospirillium* species were present at several timepoints in the nitrate microcosms. In a previous $H_2S$ inhibition study, molybdate was also found to be the strongest inhibitor of $H_2S$ reducing bacteria[43] from marine enrichment cultures. Where previous findings suggested that the addition of these inhibitors had no effect on the composition of the microbial community,[42] we find that they can drastically shape the community composition and metabolism. However, our findings that Well D and G (**Supplemental Figure 4)** respond differently to the nitrate and molybdate inhibitors further support previous assertations that the efficiency of $H_2S$ inhibitors depends on the composition of the microbiome.[42,43] While changes in microbial community composition indicate a response to our top-down engineering, they are not definitive of metabolic output, which must be characterized directly.

Hydrogen sulfide production reduced or suppressed by nitrate and molybdate treatments

To assess if these supplements resulted in metabolic changes that reduce or inhibit $H_2S$ production, we measured $H_2S$ in the headspace of the *in vitro* microcosms from Well D. Untreated microcosms from Well D generated, on average, approximately 220 ppm (+/- 35) of headspace $H_2S$ after 10 days of cultivation (**Figure 3B** ). Nitrate-treated microcosms reduced the amount of $H_2S$ to ˜150 ppm on average, while the molybdate and combined treatments limited the $H_2S$ to approximately 2 ppm or less. Taken together with the sequencing data, this suggest that the nitrate treatment here likely allowed for competitive metabolism of nitrate over sulfate as has been reported to decrease $H_2S$ production.[11,41,43] Biostimulation with nitrate alone, however, was not able to completely inhibit $H_2S$ production and enriched for specific organisms that can utilize both nitrate and sulfate,[35] like*Sulfurospirillium,* [11,46]. We hypothesize that these taxa may have either simultaneously utilized both electron acceptors or switched to sulfate metabolism once the nitrate was consumed, resulting in modest decreases in $H_2S$ production. Additionally, nitrate supplementation generally allows other species an advantage over the sulfate-reducing bacteria like*Desulfovibrio* and *Dethiosulfovibrio* species, which were present only in our controls, but does not directly inhibit these organisms that contribute to the production of $H_2S$. On the other hand, molybdate proved very effective at inhibiting $H_2S$ generation both on its own and in combination with nitrate. Molybdate has been proposed to inhibit $H_2S$ generation by binding to the sulfate adenylyltransferase (Sat, ATP sulfurylase) complex and blocking the generation of ammonium persulfate precursor to sulfide while also depleting the ATP pools of the cell.[43] We believe that this form of selection may explain why the molybdate effectively inhibits $H_2S$ formation while also limiting the number of $H_2S$ generating organisms in the community, which is also consistent with our decreased alpha diversity finding (**Figure 2** ). Based on this understanding, molybdate strongly inhibits the sulfate-reducers, which allows other microbes to ferment the molasses to organic acids and gases for MEOR, whereas nitrate only limits $H_2S$ production through competition until it is all consumed, after which the presence of microbes that can consume both nitrate and sulfate may dominate.

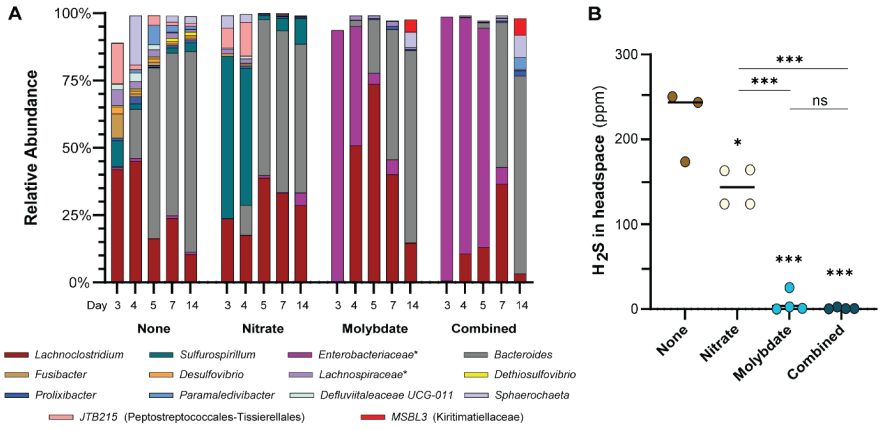


**Figure 3** – Top-down nutrient treatments inhibit and enrich specific microbes and their metabolism. A) Microbiome composition of microcosms with and without treatment across days 3 – 14. B) $H_2S$ headspace measurements of microcosms after 14 days of treatment, T-tests performed with respect to None except where indicated. Error bars represent standard deviation, n=4 (except for "None", n=3); * = $p < 0.05$, ** = $p < 0.005$, and *** = $p < 0.001$. *Enterobacteriaceae* * and*Lachnospiraceae* * indicate unspecified genera within these two Families.

To evaluate how persistent the impacts of these treatments are, we passaged the communities into media that contained the same, different, or no supplements. After three days of growth, microcosms were passaged three separate times into fresh media with and without supplements. Subcultures of untreated communities with either no supplement or only nitrate produced moderate amounts of $H_2S$ although lower than the untreated parent culture, while molybdate treatment of untreated subcultures matched that of the initial molybdate-treated cultures with little to no $H_2S$ formation after the passages ([?]7 ppm $H_2S$) (**Figure 4, Supplemental Figure 5A** ). When cultures were passaged from nitrate treatments and selection pressure was removed or kept the same (**Figure 4B** ; no supplement or $NO_3^{-1}$ only, respectively), the $H_2S$ production resumed and remained a little lower than the level of the parent culture (˜100 ppm). This suggests that the nitrate treatment may have enriched for $H_2S$-reducing bacteria or at best suppressed their metabolism initially. However, if nitrate selection is applied to molybdate cultures, $H_2S$ levels remained below that of nitrate treatment of the native microbiome. Therefore, we suspect that the reduced alpha diversity of molybdate treatment (**Figure 2** ) reflects a direct inhibition or loss of many of the sulfur-reducing species, which are either temporarily limited or possibly enriched by the nitrate treatment. As before, molybdate was able to limit the $H_2S$ production of the sulfate-reducing communities to very low levels (**Figure 4B** ), further demonstrating that it was effective even against actively $H_2S$-generating communities like those from the nitrate-treated group that otherwise produce $H_2S$ (**Figure 4C** ). Molybdate-treated subcultures, regardless of parent culture, generated less $H_2S$ (**Supplemental Figures 5A and D** ). Interestingly, when molybdate microcosms were subcultured and had their selection pressure removed (**Figure 4C** , **Supplemental Figure 5B and C** ), $H_2S$ was produced although at low levels (<25 ppm) with each sequential passage producing more $H_2S$ demonstrating that continuous application of this selection pressure is needed to permanently suppress $H_2S$. This suggest that $H_2S$-generation is inhibited by molybdate, but the associated microbes may still be present at low levels. Lastly, subcultures of microcosms generated from combination treatment (**Figure 4D** ) behaved like molybdate subcultures for combined supplement and molybdate passages. However, after three passages, $H_2S$ generation of these communities treated with nitrate began to return to higher levels. This suggests that the supplemented nitrate substrate in the combination treatment may have continued enriching for nitrate consumers, specifically some that harbor both nitrate and sulfate reducing pathways, so that sulfate reduction resumed once the nitrate was consumed. Throughout the subculture cultivations, the microbial community compositions were similar to their respective nutrient screening microcosms which were dominated by *Enterobacteriaceae, Bacteroides, and Lachnoclostridium*

(**Figure 3, Supplemental Figure 6** ). Ultimately, the combination of molybdate and nitrate best limits $H_2S$ (**Supplemental Figure 5E** ) and provides some redundancy of inhibitors in the case one of the selection pressures is resisted or lost; however, molybdate is the most important for effectively limiting $H_2S$ production from these oil well communities (**Supplemental Figure 5D** ). From this *in vitro* screening system, we showed that oil well microbial communities can be engineered and controlled from the top down using nitrate and molybdate, structural analogs of sulfate, to inhibit sulfate-reducing bacteria and $H_2S$ generation.

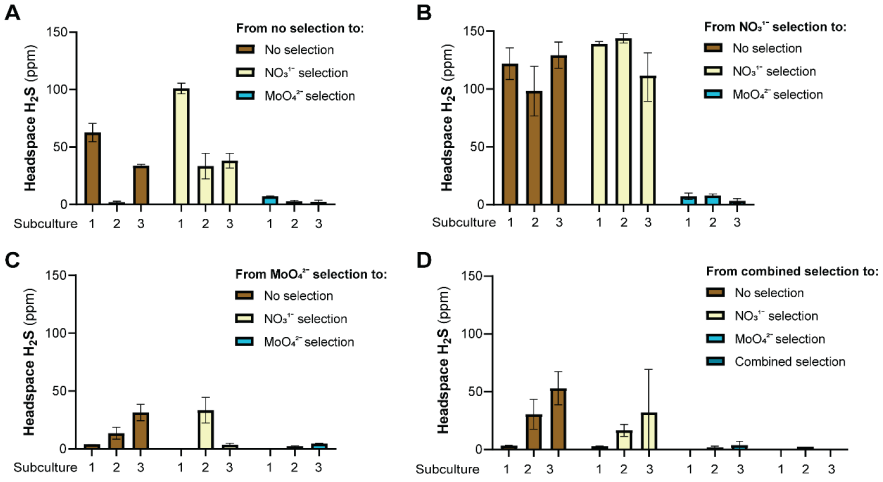


**Figure 4–Continued application of $H_2S$ inhibitors effective limits $H_2S$ production in microcosms cultures.** Headspace $H_2S$ from microcosms 7 days after subculture from parent cultures with A) no, B) nitrate, C) molybdate, or D) combined supplements and subsequent treatment with no (brown), nitrate (yellow), molybdate (aqua), or combined (teal) supplementation. Error bars represent standard deviation, n=3. Additional comparisons and statistical analysis are shown in Supplemental Figure 5.

Mini-coreflood experiments validate in vitro community control

To evaluate the effect of our top-down engineering approach on oil recovery, we applied the same treatments to a miniature coreflood system. The mini-coreflood (MCF) design (**Figure 5A-C** ) was used to simulate microbial enhanced secondary oil recovery from representative core material to evaluate the effects of the carbon and micronutrient supplements in parallel. In addition to pressure generated by the microbes in the MCF, we evaluated the composition of the microbial community and the produced oil. After initial oil recovery from brine flooding was completed, 9:1 mixtures of effluent and molasses-nutrient supplements were injected into the MCFs and allowed to sit for 10 days in an anaerobic environment to seed the core and incubate the growing microbes. Similar to our *in vitro*microcosms, we found that molasses was able to induce microbial growth and activity, generating up to 26 PSI. Nitrate-treated floods generated the most pressure while the molybdate and combination treatment generated less pressure (**Figure 5D** ). We suspect that the suppression of $H_2S$ and the limitation of sulfate-reducing bacteria account for the differences in pressure generation between the molybdate-treated cultures and the untreated or nitrate-treated cultures. Because increased pressure applies more force on the trapped oil deposits, the amounts of oil recovered trended with the pressure produced (**Figure 5E/F** ). We also found that the microbial community in the MCF was distinct from our *in vitro*screening, reflecting the contribution of the high petroleum hydrocarbon concentrations in these samples. MCF microbial communities drastically shifted toward *Bacteroides* under the molybdate and combination treatment whereas the untreated and nitrate-treated MCFs were dominated by *Lachnoclostridium* (**Figure 5G** ). As at the end of the*in vitro* microcosms, both *Bacteroides* and*Lachnoclostridium* were prominent members of the final MCF microbiome (**Figure 4, Figure 5G** ), but these MCF trials contain significant amounts of the oil hydrocarbons that can be used as an addi-

tional carbon source. While the V4-V5 16S rRNA gene region amplified here is not specific enough to identify the species of these genera, microbes belonging to the *Lachnoclostridium* group have been found to degrade some hydrocarbons and some reduce sulfate to $H_2S$.[47–49] Similarly, we observed increases in the abundance of organisms belonging to the genus*Pseudomonas* which is not a prominent producer of gases and has been linked to naphthalene,[50] normal paraffin,[51] and polycyclic hydrocarbon degradation.[52,53] As shown in (**Figure 5E/G** ) the proportion of n-paraffin dropped from ˜35 %w/w to ˜25 %w/w of the relative abundance while*Pseudomonas* sp. increased in abundance in the molybdate and combination MCFs. This condition suggests that *Pseudomonas sp.*degraded around 30% of the long chain n-paraffins (C14 – C29) in a period of 14 days (**Supplemental Figure 7A, Supplemental Table 1** ). Although isolates were not identified here, *Pseudomonas* sp., such as *P. proteolytica, P. xanthomarina, and P. aeruginosa* , are expected to induce n-paraffin degradation starting from n-tetradecane (C14) by alkane hydroxylases systems.[53–55] Certainly, biodegradation of long chain n-paraffins is recognized to have a positive influence in oil recovery processes by means of reducing the viscosity of the crude oil while increasing its fluidity.[56] The relative abundance of monocyclo-paraffins increased from ˜30 %w/w to ˜40 %w/w in total corresponding to the increment of the C9 – C17 fraction for all treatments (**Supplemental Figure 7B, Supplemental Table 1** ), likely due to degradation of the n-paraffins. However, no specific trend was observed for the abundance of iso-paraffins. Aromatics such as naphthalene also decreased in abundance with treatment and the presence of *Pseudomonas* sp. (**Figure 5F/G** ). Species such as *P. mendocina, P. putida, P. fluorescens, P. paucimobilis, P. vesicularis, P. cepacia, P. testosteronei, P. aeruginosa,* and *P. stutzeri* have been reported to induce naphthalene biodegradation.[52,57,58] In contrast, the triaromatic fraction (C17) increased as a response of the supplements here applied. The relative abundance of dicyclo- and monocyclo-paraffins increased when these treatments were applied, likely because of n-paraffin degradation, and thus, changed the overall composition of the produced oil (**Figure 5E/G, Supplemental Table 1** ). While the results presented in Figure 5 represent one trial of these treatments, an additional replicate treatment at a different time (**Supplemental Figure 8A-C** ) showed n-paraffin degradation for all treatments. However, the same trend for the aromatic polycyclic hydrocarbon fraction is not seen in the replicate likely because the microbiomes of the produced water used to seed these wells were different (**Supplemental Figure 8D** ). Regardless of these specific differences, we observed reproducible control of the oil well microbiome as a function of nutrient supplementation to alter produced oil paraffin profiles while limiting $H_2S$ production and pressurizing the reserve.

Our results demonstrate a clear correlation between pressure generation and oil recovery in MEOR processes. While the $H_2S$-limiting treatments did not substantially enhance pressure generation in the presence of hydrocarbons and confined geometry, we were able to validate the role that molybdate plays in controlling souring and corrosion by oil well microbes. However, field trials and economic analysis are needed to determine the viability of our process relative to current surfactant-based oil recovery processes. The modest improvements in oil recovery may be offset by the increased operating costs needed for corrosion maintenance, surfactant production, and microbial control via more expensive ammonium quaternary disinfectants currently used.[19] Moreover, we demonstrated that the specific oil compositions were a strong function of microbial activity, which could be controlled via top-down engineering. Further optimization of the nutrient formulation, such as increased amounts of molasses or other nutrients, may enhance pressure generation or alter the microbial dynamics so that more oil can be recovered and specific hydrocarbon compounds can be enriched or depleted. In agreement with the *in vitro* findings, these results suggested that we can control the microbial population in small scale oil recovery settings and can use that to concurrently modulate oil composition.

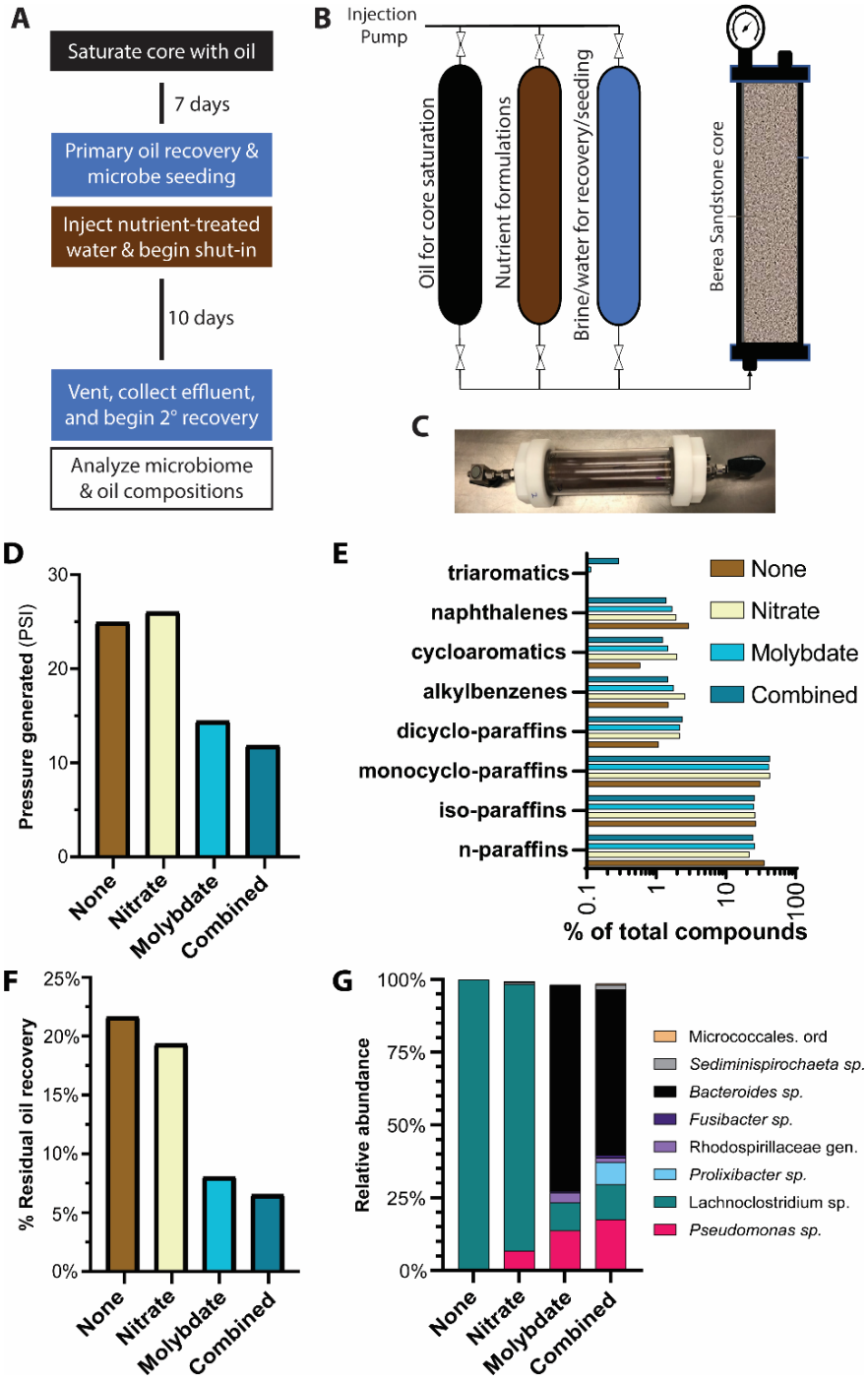


**Figure 5** – Top-down nutrient formulations control microbial metabolism in ways the affect both oil yield and composition. A) Overview of mini-coreflood (MCF) experimental design where cores are saturated with oil, accessible oil (primary recovery) is extracted with brine and microbes are seeded with produced water, nutrients are subsequently injected, and reactors are shut-in for ten days before they are vented and flooded with brine to recover any of the oil liberated by the microbial processes (i.e. secondary recovery). B) MCF experimental setup showing the pump which injects either oil, sterile brine, well water for seeding,

or nutrient supplements with the MCF canisters encasing the core material and C) an actual image of assembled MCF apparatus. D) Pressure generated during 10 day mini-coreflood by various supplements. E) Chemical composition of produced oil after microbial mini-coreflood. F) Fraction of residual oil in place that was recovered after 10-day microbial shut-in period. G) Composition of microbes in effluent from the microbial mini-coreflood after 10-day shut in period. This data represents one trial which is the most data complete, a replicate performed at a different time from different produced water is presented in the supplement (Supplemental Figure 8).

## Conclusions

Microbial activity of oil wells has a significant impact on oil recovery efficiencies, the souring of produced oil or $H_2S$ generation, and final oil composition. Top-down engineering approaches via direct nutrient injection and supplementation can be used to shape community structure and control metabolism. We validated the ability of molasses supplemented with nitrate and/or molybdate to apply different selective pressures which were either competitive or inhibitory of $H_2S$ production, respectively. Molybdate supplementation in particular provided strong selective pressure whose effects persisted over multiple generations or passages once removed, demonstrating a strong capacity to shape community composition and function. While these did not enhance oil recovery in our coreflood experiments under the conditions tested, top-down engineering had demonstrable impact on the composition of oil recovered and inhibiting microbial souring processes. Our work highlights the power of microbiome engineering for the improvement of oil recovery operations and develops a workflow for the rapid screening and evaluation of candidate oil wells.

## Methods

*Cultivation*

Effluent oil well water was collected from several wells around the Illinois oil basin and transported to the laboratory (West Lafayette, IN, USA) the same day. For cultivation of the oil well microbiome, 9 ml of effluent water was mixed in Hungate tubes with either 1 ml of sterile 40% (w/v) corn syrup or 1 ml of sterile 40% (w/v) molasses in an anaerobic chamber (PLOS, Grand Rapids, MI, USA) with an atmosphere of 85% $N_2$, 10% $CO_2$, & 5% $H_2$. Cultures that were treated with additional non-essential micronutrient supplements [final concentration of 0.5 g/L potassium nitrate, 0.5 g/L sodium molybdate, 0.25 g/L potassium monophosphate and 0.25 g/L potassium diphosphate, or 0.25 g/L sodium chloride and 0.25 g/L potassium chloride] – solutions were sterile filtered and equilibrated overnight in the anaerobic chamber. After nutrient solutions were added, cultures were thoroughly mixed and incubated at 27 @C. Pressure measurements were taken every 24 hours with a pressure gauge (APG, Logan, UT, USA),[59] 1 ml of culture was removed for microbiome analysis and pH monitoring by pH test strips (pH2-8, MilliporeSigma, St. Louis, MO, USA). After sampling, the cultures were then vented to a gauge pressure of 0 and replaced in the incubator; cultures were monitored and sampled for 7-10 days.

*DNA extraction, PCR, Sequencing & analysis.*

To evaluate the composition of the native oil well, ˜1L of effluent water was prefiltered with Glass Fiber Filters (1.2 µm pore, MilliporeSigma) and then biomass was concentrated on Express PLUS Membrane Filters (0.22 µm pore, MilliporeSigma) similar to previous work.[60] DNA was then extracted from the microbial biomass on the second filter using the DNeasy PowerFecal Kit (QIAGEN, Germantown, MD, USA) along with the FastPrep 24 5G homogenizer (MP Biomedicals, Santa Ana, CA, USA) as per instructed by the manufacturers. All DNA was stored at - 20 °C until further analysis. DNA concentration and quality were assessed on a NanoPhotometer (Implen NP80, Los Angeles, CA, USA). The microbial community composition of oil well microcosms was assessed by DNA extracted from a 1-ml aliquot of the cultures as mentioned above. The 1 ml aliquots were centrifuged at 10,000g for 7 min to pellet the cells. After removing the supernatant, the microbial DNA was extracted with the same DNeasy PowerFecal kit as noted above.

As in previous microcosm studies,[61] the microbial community was then assessed by amplifying and sequencing the V4-V5 region of the 16S rRNA gene from the extracted DNA (primers: 515f - forward, 5' GT-

GYCAGCMGCCGCGGTAA 3'; and 926r - reverse 5' CCGYCAATTYMTTTRAGTTT 3').[62] 16S rRNA gene PCRs were set up using Phusion Polymerase MasterMix (Thermo Fisher Scientific, Waltham, MA, USA) as follows PCR-grade water (22 µl), Phusion mastermix (25 µl), forward primer (10 µM, 1.0 µl), reverse primer (10 µM, 1.0 µl), and template DNA (1.0 µl) in a total reaction volume of 50 µl; for samples that had very low template concentration up to 5 µl of DNA template was used and the water volume reduced to 17 µl. PCR amplification conditions were set to 98 °C for 0.5 min; 35 cycles of 98 °C for 15 s, 50 °C for 30 s, 72 °C for 60 s; and 72 °C for 10 min, and then a 4 °C hold. The resulting amplicons were confirmed by gel electrophoresis, purified using the Clean and Concentrator kit (Zymo Research Irvine, CA, USA), and then labeled using unique 8-bp tagged i5 (i509-i516) index forward primers and 8-bp tagged i7 (i713-i724) index reverse primers as suggested by the manufacturer (Illumina San Diego, CA, USA). Pooled amplicons were then multiplexed and sequenced via 2 x 250-bp paired reads on an Illumina MiSeq at the Purdue Genomics facilities. Sequences were analyzed using the QIIME2 pipeline[63] which quality-filtered, joined paired reads, checked for chimeras, and denoised the data (via DADA2[64]). The operational taxonomic units (OTUs) were assigned using the SILVA SSU database (SILVA 138)[65]for assigning taxonomy to the representative OTUs. All alpha and beta diversity metrics were calculated using QIIME's built in analysis tools.

*Micro GC H2S quantification*

An Agilent 490 Micro GC (Agilent Technologies, Palo Alto, CA, USA) equipped with parallel Molsieve 5A and PoraPLOT U columns and a thermal conductivity detector was used for H2S analysis with Helium carrier gas (15PSI). The column and injector temperatures were isothermally set to 60 degC for the duration of the 120 s run. The injector was set to split mode with an injection time of 40 ms and a backflush time of 10 s. $H_2S$ standards were prepared as previously described[66] where, briefly, $H_2S$ was generated by reacting $Na_2S$ (Sigma-Aldrich, Saint Louis, MO, USA) with excess HCl (Fisher) in a 20 ml Hungate bottle with a butyl rubber stopped in the anaerobic chamber. Six working calibration standards at concentrations corresponding to 25, 50, 100, 250, 750, and 1,500 ppm of $H_2S$ were prepared by diluting from a 5 µmol/ml stock. Either a 2 ml headspace of standard or the microcosm samples was withdrawn and injected into the instrument with a gas syringe. Standards were run in triplicate to create a calibration curve while the biological samples were each run once.

*Mini-coreflood set up*

These studies used high-permeability (>400 mD), 1" diameter by 6" long Berea Sanstone™ cores (Cleveland Quarries, Vermilion, OH, USA) that were dried to completeness in an oven at 110 °C overnight. Once dried, the cores were saturated with a synthetic brine comprising a salinity (9400 mg/L and a hardness of 250 mg/L) closely resembling fluids from the representative wells to determine pore volumes (PV) by the mass of brine that was retained in the core. Following brine saturation, mini-coreflood reactors were assembled in the anaerobic chamber and they consisted of a PVC sleeve (1" inner diameter, McMaster-Carr, Cleveland, OH, USA) that surrounded the core and a plastic canister to hold the core in place (built by Purdue University's Chemistry Precision Machine Shop, West Lafayette, IN, USA). The plastic canisters were then filled with DI water that was pressurized to 80 PSI to apply pressure on the sleeve and keep it tightly fit to the core. Once mini-coreflood reactors were assembled, dead oil from the representative wells was processed and injected into the core at a rate of 0.5 ml/min until the core was saturated with oil; the amount of brine displaced represented the volume of original oil in place (OOIP) before the primary water flood. The primary flood was carried out by injecting of 2 PV effluent oil well water at rate of a 1 ml/min to displace the oil for primary recovery and simultaneously seed microbes into the core. The secondary flood was carried out with 1 PV of a mixture of nine parts well water to one part of the molasses solution with supplements as indicated in the "Cultivation" methods section above. The flow rate of the secondary flood was 1 ml/min, and no additional oil was recovered during this nutrient injection step. Mini-corefloods were shut-in for 10 days inside the anaerobic chamber (27 °C) replicating ground conditions. Pressure generated over the 10-day period was measure with an in-line pressure gauge. Oil generated by the microbial pressure was collected and then an additional 2 PV of brine was used as a final water flood to recover oil displaced by the microbial activity. The collected brine flood volumes were centrifuged, and DNA was extracted from the pellet; V4-V5 16S rRNA

gene regions were amplified and sequenced as described above (see "*DNA extraction, PCR, Sequencing & analysis* ").

*Chemical quantitation via GCxGC-FID*

The quantitative chemical characterization of the oil samples was carried out using a comprehensive two-dimensional gas chromatography system Agilent 7890B GC (GCxGC) coupled to a flame ionization detector (FID). A thermal modulator cooled with liquid nitrogen (LECO Corporation, Saint Joseph, MI), an Agilent 7683B series injector, and a HP 7683 series autosampler were also used. A reversed phase column configuration was selected having a primary mid-polar column DB-17ms (30 m x 0.25 mm x 0.25 μm) and a secondary nonpolar column DB-1ms (0.8 m x 0.25 mm x 0.25 μm). Both columns were provided by Agilent (Santa Clara, CA). Ultrahigh purity helium (99.9999 %) was used as the carrier gas at a constant flow rate of 1.5 mL/min, and a front inlet septum purge flow of 3 mL/min and a front inlet gas saver flow of 20 ml/min were set in the GC x GC system. A split inlet liner with glass wool suitable for low pressure drop provided by Agilent (Santa Clara, CA) was used to protect the system due to the nature of the samples under study. The temperature of the front inlet, initial oven temperature, and the target oven temperature were set at 280 °C, 40 °C (hold time of 0.2 min), and 260 °C (hold time of 5 min), respectively. An oven temperature ramp rate of 3 °C/min was considered for a total GC method time of analysis of 4,712 seconds (78.5 min) per sample. Secondary oven and modulator temperature offsets were set at 50 °C and 15 °C, respectively. A modulation period of 2.5 s was used to avoid wraparound. Samples of 0.5 μL were injected to the system using a manual dilution factor of 100 (10 μL of sample in 1 ml of pentane) and automatic split ratio of 20:1. The development and refinement of the GCxGC-FID classification map (**Supplemental Figure 9** ) was made based on the methodology described in a previous study.[67] Thus, the chemical characterization encompassed nine hydrocarbon groups and carbon numbers as follows: n-paraffins (C6-C32), iso-paraffins (C6-C32), monocyclo- (C6-C30), dicyclo- (C8-C20), and tricyclo-paraffins (C10-C16), alkylbenzenes (C6-C20), cycloaromatics (C9-C18), naphthalenes (C10-C19), and triaromatics (C14-C19). Data were processed in ChromaTOF software version 4.71.0.0 optimized for GC x GC – FID (LECO Corporation, Saint Joseph, MI) with a signal-to-noise ratio of 50. Weight percentage (wt. %) relative to each hydrocarbon class and carbon number was calculated via normalizing the peak area by integration of the GC x GC chromatograms extracting solvent and column bleed peaks using Microsoft Excel - 365.

*Statistical Methods*

T-tests were performed using GraphPad Prism's in-software analysis tools (https://www.graphpad.com/guides/prism/latest/statistics/stat_key_concepts_ttests.htm). Relative abundance, alpha diversity, and beta diversity metrics were calculated in QIIME2[63] and the results (such as PCoA) were visualized using QIIME2 view (https://view.qiime2.org/).

**Abbreviations:**

FID: flame ionization detector, GCxGC: comprehensive two-dimensional gas chromatography, $H_2S$: hydrogen sulfide, MEOR: microbial enhanced oil recovery, MCF: mini-coreflood, OTU: operational taxonomic unit, PCoA: principal coordinate analysis, PCR: polymerase chain reaction

**Declarations:**

*Ethics approval and consent to participate:* Not applicable

*Consent for publication:* Not applicable

*Availability of data and materials* : The sequencing datasets supporting the conclusions of this article are available under the NCBI SRA: SUB11545621. Additional Data including GCxGC data can be found in Supplemental Materials file.

*Competing interests* : The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

*Funding:* This project was financially supported by the Pioneer Oil Company (Vincennes, IN) and the Purdue Enhanced Oil Recovery Lab.

*Author Contributions:* ETH: Conceptualization, Formal analysis, Data curation, Investigation, Methodology, Project administration, Supervision, Validation, Visualization; Roles/Writing - original draft. LECM: Formal analysis, Methodology, Data curation, Investigation, Writing - review & editing. GK: Funding acquisition, Project administration, Resources, Writing - review & editing. KVS: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing. All authors have read and agreed to the published version of the manuscript.

*Acknowledgments:* We would like to thank the members of Purdue's Enhance Oil Recovery Lab, specifically Dr. Nate Schultheiss, Dr. Thomas Everett, Dr. Jeremy Holtsclaw, Dr. Katherine Wehde, and Timothy Henderson, who made this work possible by coordinating well samplings, provided experimental feedback, and supplying technical supported and insight for our work. We would also like to thank Dr. Stephen Lindemann and his lab at Purdue Food Science for providing access to their Micro GC for $H_2S$ analyses. We are also grateful to the Purdue University Genomics Core for their role in sequencing the 16S rRNA gene amplicons and the Purdue Institute of Inflammation, Immunology, and Infectious Disease for access to the Bell computing cluster which allowed us to analyze the sequencing reads. Additionally, we are thankful to both Bindley Bioscience Center and the Fuel Laboratory of Renewable Energy at Purdue University for the research space and support staff where this research was carried out.

**References**

1. U.S. Energy Information Administration. International Energy Outlook 2021. https://www.eia.gov/outlooks/ieo/pdf/IEO2021_Narrative.pdf. Published 2021. Accessed December 31, 2021.

2. Raffa P. Where is research on fossil fuels going in times of climate change? A perspective on chemical enhanced oil recovery. *MRS Commun* . 2021;11(6):716-725. doi:10.1557/s43579-021-00131-y

3. Energy Transition Outlook 2021 Executive Summary. A global and regional forecast to 2050. 2021:40. https://eto.dnv.com/2021.

4. Mohsenatabar Firozjaii A, Saghafi HR. Review on chemical enhanced oil recovery using polymer flooding: Fundamentals, experimental and numerical simulation. *Petroleum* . 2020;6(2):115-122. doi:10.1016/j.petlm.2019.09.003

5. Farajzadeh R, Kahrobaei S, Eftekhari AA, Mjeni RA, Boersma D, Bruining J. Chemical enhanced oil recovery and the dilemma of more and cleaner energy. *Sci Rep* . 2021;11(1):1-14. doi:10.1038/s41598-020-80369-z

6. Sheng JJ. *Modern Chemical Enhanced Oil Recovery: Theory and Practice* . Gulf Professional Publishing; 2010.

7. Nikolova C, Gutierrez T. Use of Microorganisms in the Recovery of Oil From Recalcitrant Oil Reservoirs: Current State of Knowledge, Technological Advances and Future Perspectives. *Front Microbiol* . 2020;10(January). doi:10.3389/fmicb.2019.02996

8. She H, Kong D, Li Y, Hu Z, Guo H. Recent Advance of Microbial Enhanced Oil Recovery (MEOR) in China. *Geofluids* . 2019;2019. doi:10.1155/2019/1871392

9. Lazar I, Petrisor IG, Yen TF. Microbial Enhanced Oil Recovery.*Pet Sci Technol* . 2007;25(September):1353-1366. doi:10.1016/S0376-7361(09)70098-6

10. Bermont-Bouis D, Janvier M, Grimont PAD, Dupont I, Vallaeys T. Both sulfate-reducing bacteria and Enterobacteriaceae take part in marine biocorrosion of carbon steel. *J Appl Microbiol* . 2007;102(1):161-168. doi:10.1111/j.1365-2672.2006.03053.x

11. Hubert C, Voordouw G. Oil field souring control by nitrate-reducing Sulfurospirillum spp. that outcompete sulfate-reducing bacteria for organic electron donors. *Appl Environ Microbiol* . 2007;73(8):2644-2652. doi:10.1128/AEM.02332-06

12. Foo JL, Ling H, Lee YS, Chang MW. Microbiome engineering: Current applications and its future. *Biotechnol J* . 2017:1600099. doi:10.1002/biot.201600099

13. Lee ED, Aurand ER, Friedman DC, Group EBRCMRW. Engineering Microbiomes—Looking Ahead. *ACS Synth Biol* . 2020;9(12):3181-3183.

14. Ke J, Wang B, Yoshikuni Y. Microbiome Engineering: Synthetic Biology of Plant-Associated Microbiomes in Sustainable Agriculture. *Trends Biotechnol* . 2021;39(3):244-261. doi:10.1016/j.tibtech.2020.07.008

15. Lawson CE, Harcombe WR, Hatzenpichler R, et al. Common principles and best practices for engineering microbiomes. *Nat Rev Microbiol* . 2019;17(12):725-741. doi:10.1038/s41579-019-0255-9

16. Youssef N, Simpson DR, Duncan KE, et al. In situ biosurfactant production by Bacillus strains injected into a limestone petroleum reservoir. *Appl Environ Microbiol* . 2007;73(4):1239-1247. doi:10.1128/AEM.02264-06

17. Yue M, Zhu W, Song Z, Long Y, Song H. Study on distribution of reservoir endogenous microbe and oil displacement mechanism. *Saudi J Biol Sci* . 2017;24(2):263-267. doi:10.1016/j.sjbs.2016.09.014

18. Lin X, Zheng X, Liu R, et al. Extracellular Polymeric Substances Production by ZL-02 for Microbial Enhanced Oil Recovery. *Ind Eng Chem Res* . 2021;60(2):842-850. doi:10.1021/acs.iecr.0c05130

19. Quraishi M, Bhatia SK, Pandit S, et al. Exploiting Microbes in the Petroleum Field : Analyzing the Credibility of Microbial Enhanced Oil Recovery (MEOR). *Energies* . 2021;14(4684):1-30.

20. Diaz-Colunga J, Lu N, Sanchez-Gorostiaga A, et al. Top-down and bottom-up cohesiveness in microbial community coalescence. *Proc Natl Acad Sci U S A* . 2022;119(6):1-11. doi:10.1073/pnas.2111261119

21. Walter J, Maldonado-Gómez MX, Martínez I. To engraft or not to engraft: an ecological framework for gut microbiome modulation with live microbes. *Curr Opin Biotechnol* . 2018;49:129-139. doi:https://doi.org/10.1016/j.copbio.2017.08.008

22. Lindemann SR, Bernstein HC, Song HS, et al. Engineering microbial consortia for controllable outputs. *ISME J* . 2016;10(9):2077-2084. doi:10.1038/ismej.2016.26

23. Amor DR, Bello MD. Bottom-up approaches to synthetic cooperation in microbial communities. *Life* . 2019;9(1). doi:10.3390/life9010022

24. Rottinghaus AG, Ferreiro A, Fishbein SRS, Dantas G, Moon TS. Genetically stable CRISPR-based kill switches for engineered microbes.*Nat Commun* . 2022;13(1):1-17. doi:10.1038/s41467-022-28163-5

25. Gilmore SP, Lankiewicz TS, Wilken SE, et al. Top-Down Enrichment Guides in Formation of Synthetic Microbial Consortia for Biomass Degradation. *ACS Synth Biol* . 2019;8(9):2174-2185. doi:10.1021/acssynbio.9b00271

26. Pacheco AR, Osborne ML, Segrè D. Non-additive microbial community responses to environmental complexity. *Nat Commun* . 2021;12(1):1-11. doi:10.1038/s41467-021-22426-3

27. Portwood JT. A Commercial Microbial Enhanced Oil Recovery Technology: Evaluation of 322 Projects. In: Vol All Days. SPE Oklahoma City Oil and Gas Symposium / Production and Operations Symposium. ; 1995.

28. Nikolova C, Gutierrez T. *Marine Hydrocarbon-Degrading Bacteria: Their Role and Application in Oil-Spill Response and Enhanced Oil Recovery* . INC; 2022. doi:10.1016/b978-0-323-85455-9.00005-9

29. Zahner RLL, Tapper SJJ, Marcotte BWGWG, Govreau BRR. Lessons Learned From Applications of a New Organic-Oil-Recovery Method That Activates Resident Microbes. *SPE Reserv Eval Eng* . 2012;15(06):688-694.

30. Sen R. Biotechnology in petroleum recovery: The microbial EOR.*Prog Energy Combust Sci* . 2008;34(6):714-724. doi:10.1016/j.pecs.2008.05.001

31. Safdel M, Anbaz MA, Daryasafar A, Jamialahmadi M. Microbial enhanced oil recovery, a critical review on worldwide implemented field trials in different countries. *Renew Sustain Energy Rev* . 2017;74(January):159-172. doi:10.1016/j.rser.2017.02.045

32. Anantharaman K, Hausmann B, Jungbluth SP, et al. Expanded diversity of microbial groups that shape the dissimilatory sulfur cycle.*ISME J* . 2018;12(7):1715-1728. doi:10.1038/s41396-018-0078-0

33. Rojas CA, De Santiago Torio A, Park S, Bosak T, Klepac-Ceraj V. Organic Electron Donors and Terminal Electron Acceptors Structure Anaerobic Microbial Communities and Interactions in a Permanently Stratified Sulfidic Lake. *Front Microbiol* . 2021;12:0-19. doi:10.3389/fmicb.2021.620424

34. Hahn CR, Farag IF, Murphy CL, Podar M, Elshahed MS, Youssef NH. Microbial Diversity and Sulfur Cycling in an Early Earth Analogue: From Ancient Novelty to Modern Commonality. *MBio* . 2022. doi:10.1128/mbio.00016-22

35. Begmatov S, Savvichev AS, Kadnikov V V., et al. Microbial communities involved in methane, sulfur, and nitrogen cycling in the sediments of the barents sea. *Microorganisms* . 2021;9(11). doi:10.3390/microorganisms9112362

36. Zhan Y, Wang Q, Chen C, et al. Potential of wheat bran to promote indigenous microbial enhanced oil recovery. *J Ind Microbiol Biotechnol* . 2017;44(6):845-855. doi:10.1007/s10295-017-1909-0

37. Nazina T, Sokolova D, Grouzdev D, et al. The potential application of microorganisms for sustainable petroleum recovery from heavy oil reservoirs. *Sustain* . 2020;12(1). doi:10.3390/SU12010015

38. Iwanowicz DD, Jonas RB, Schill WB, Marano-Briggs K. Novel microbiome dominated by Arcobacter during anoxic excurrent flow from an ocean blue hole in Andros Island, the Bahamas. *PLoS One* . 2021;16(8 August):1-16. doi:10.1371/journal.pone.0256305

39. Varjani SJ, Gnansounou E. Microbial dynamics in petroleum oilfields and their relationship with physiological properties of petroleum oil reservoirs. *Bioresour Technol* . 2017;245(August):1258-1265. doi:10.1016/j.biortech.2017.08.028

40. Kögler F, Hartmann FSF, Schulze-Makuch D, Herold A, Alkan H, Dopffel N. Inhibition of microbial souring with molybdate and its application under reservoir conditions. *Int Biodeterior Biodegrad* . 2021;157(June 2020). doi:10.1016/j.ibiod.2020.105158

41. de Jesus EB, de Andrade Lima LRP, Bernardez LA, Almeida PF. Inhibition of Microbial Sulfate Reduction By Molybdate. *Brazilian J Pet Gas* . 2015;9(3):95-106. doi:10.5419/bjpg2015-0010

42. Nemati M, Mazutinec TJ, Jenneman GE, Voordouw G. Control of biogenic h2s production with nitrite and molybdate. *J Ind Microbiol Biotechnol* . 2001;26(6):350-355. doi:10.1038/sj.jim.7000142

43. Stoeva MK, Coates JD. Specific inhibitors of respiratory sulfate reduction: Towards a mechanistic understanding. *Microbiol (United Kingdom)* . 2019;165(3):254-269. doi:10.1099/mic.0.000750

44. Saeed AM, El Shatoury E, Hadid R. Production of molybdenum blue by two novel molybdate-reducing bacteria belonging to the genus Raoultella isolated from Egypt and Iraq. *J Appl Microbiol* . 2019;126(6):1722-1728. doi:10.1111/jam.14254

45. Rahman MF, Rusnam M, Gusmanizar N, et al. Molybdate-reducing and SDS-degrading Enterobacter sp. Strain Neni-13. *Nov Biotechnol Chim* . 2016;15(2):166-181. doi:10.1515/nbec-2016-0017

46. Lohmayer R, Kappler A, Lösekann-Behrens T, Planer-Friedrich B. Sulfur species as redox partners and electron shuttles for ferrihydrite reduction by Sulfurospirillum deleyianum. *Appl Environ Microbiol* . 2014;80(10):3141-3149. doi:10.1128/AEM.04220-13

47. Yutin N, Galperin MY. A genomic update on clostridial phylogeny: Gram-negative spore formers and other misplaced clostridia.*Environ Microbiol* . 2013;15(10):2631-2641. doi:10.1111/1462-2920.12173

48. El-Sayed WS, Al-Senani SR, Elbahloul Y. Diversity of dehalorespiring bacteria and selective enrichment of aryl halides-dechlorinating consortium from sedimentary environment near an oil refinery. *J Taibah Univ Sci* . 2018;12(6):711-722. doi:10.1080/16583655.2018.1495869

49. Sallam A, Steinbüchel A. Clostridium sulfidigenes sp. nov., a mesophilic, proteolytic, thiosulfate- and sulfur-reducing bacterium isolated from pond sediment. *Int J Syst Evol Microbiol* . 2009;59(7):1661-1665. doi:10.1099/ijs.0.004986-0

50. Dennis JJ, Zylstra GJ. Complete sequence and genetic organization of pDTG1, the 83 kilobase naphthalene degradation plasmid from Pseudomonas putida strain NCIB 9816-4. *J Mol Biol* . 2004;341(3):753-768. doi:10.1016/j.jmb.2004.06.034

51. Okoye AU, Chikere CB, Okpokwasili GC. Characterization of Potential Paraffin Wax Removing Bacteria for Sustainable Biotechnological Application. In: Vol Day 3 Wed,. SPE Nigeria Annual International Conference and Exhibition. ; 2019. doi:10.2118/198799-MS

52. Speight JG, El-Gendy NS. Chapter 9 - Chemistry of Biotransformation. In: *Introduction to Petroleum Biotechnology* . Gulf Professional Publishing; 2018:287-359.

53. Alsebri H, Hamad AA, Hassam MM. Biodegradation of petroleum hydrocarbons using indigenious bacterial and actinomycetes cultures.*Pakistan J Biol Sci* . 2020;23(6).

54. Das K, Mukherjee AK. Crude petroleum-oil biodegradation efficiency of Bacillus subtilis and Pseudomonas aeruginosa strains isolated from a petroleum-oil contaminated soil from North-East India. *Bioresour Technol* . 2007;98(7):1339-1345. doi:10.1016/j.biortech.2006.05.032

55. Li YP, Pan JC, Ma YL. Elucidation of multiple alkane hydroxylase systems in biodegradation of crude oil n-alkane pollution by Pseudomonas aeruginosa DN1. *J Appl Microbiol* . 2020;128(1):151-160. doi:10.1111/jam.14470

56. Lan G, Fan Q, Liu Y, et al. Effects of the addition of waste cooking oil on heavy crude oil biodegradation and microbial enhanced oil recovery using Pseudomonas sp. SWP- 4. *Biochem Eng J* . 2015;103:219-226. doi:10.1016/j.bej.2015.08.004

57. Barman SR, Banerjee P, Mukhopadhayay A, Das P. Biodegradation of acenapthene and naphthalene by Pseudomonas mendocina: Process optimization, and toxicity evaluation. *J Environ Chem Eng* . 2017;5(5):4803-4812. doi:10.1016/j.jece.2017.09.012

58. Samanta SK, Singh O V, Jain RK. Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. *Trends Biotechnol* . 2002;20(6):243-248. doi:10.1016/s0167-7799(02)01943-1

59. Hillman ET, Li M, Hooker CA, Englaender JA, Wheeldon I, Solomon K V. Hydrolysis of lignocellulose by anaerobic fungi produces free sugars and organic acids for two-stage fine chemical production with Kluyveromyces marxianus. *Biotechnol Prog* . 2021;e3172. doi:https://doi.org/10.1002/btpr.3172

60. Daly RA, Borton MA, Wilkins MJ, et al. Microbial metabolisms in a 2.5-km-deep ecosystem created by hydraulic fracturing in shales.*Nat Microbiol* . 2016;1(September):1-9. doi:10.1038/nmicrobiol.2016.146

61. RedCorn RM, Hillman ET, Solomon K V, Engelberth AS. Xanthobacter-dominated biofilm as a novel source for high-value rhamnose. *Appl Microbiol Biotechnol* . 2019;103(11):4525-4538.

62. Walters W, Hyde ER, Berg-lyons D, et al. Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Analysis. *mSystems* . 2016;1(1):e0009-15. doi:10.1128/mSystems.00009-15.Editor

63. Bolyen E, Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* . 2019;37(8):852-857. doi:10.1038/s41587-019-0209-9

64. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data.*Nat Methods* . 2016;13(7):581-583. doi:10.1038/nmeth.3869

65. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools.*Nucleic Acids Res* . 2013;41(D1):590-596. doi:10.1093/nar/gks1219

66. Varlet V, Giuliani N, Palmiere C, Maujean G, Augsburger M. Hydrogen sulfide measurement by headspace-gas chromatography-mass spectrometry (HS-GC-MS): Application to gaseous samples and gas dissolved in muscle.*J Anal Toxicol* . 2015;39(1):52-57. doi:10.1093/jat/bku114

67. Vozka P, Kilaz G. How to obtain a detailed chemical composition for middle distillates via GC x GC-FID without the need of GC x GC-TOF/MS. *Fuel* . 2019;247(January):368-377. doi:10.1016/j.fuel.2019.03.009