MICROBIAL METABOLISM OF DIESEL FUEL IN A TROPICAL ISLAND AQUIFER

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I certify that I have read this thesis and that, in my opinion, it is satisfactory in scope and quality as a thesis for the degree of Bachelor of Science in Global Environmental Science.

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For my mother, Laura Hong.

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ABSTRACT

Petroleum and petroleum-derived product contamination, also known as petroleum hydrocarbon (PH) contamination, has been a significant global environmental issue for decades. While PH degradation has been well studied in soils and surface environments, we still know little about the microbial ecology of PH degradation in subsurface aquatic ecosystems. In November of 2021, the Red Hill Bulk Fuel Storage Facility on O‘ahu spilled 19,000 gallons of diesel jet fuel (JP-5) into the Red Hill Shaft, potentially contaminating the groundwater aquifer the shaft draws from. However, due to limited knowledge of PH contamination in the context of O‘ahu’s aquifers, it is unknown how microbial communities in groundwater ecosystems can facilitate the degradation of these refined fuels. Therefore, we performed an experiment focused on quantifying the changes of microbial communities in the presence of JP-5 and their ability to transform or remove refined diesel hydrocarbons.

Groundwater was incubated with JP-5 for a period of 28 days. Throughout the experiment, samples for scanning excitation-emission fluorescence spectroscopy, flow cytometry, Total Organic Carbon, and microbial community profiling were collected. Fluorescence spectroscopy and dissolved organic carbon allowed for the analysis of dissolved organic matter compositional changes and JP-5 consumption while flow cytometry measurements and DNA allowed for the analysis of microbial growth and community compositional changes. My results demonstrated that diesel fuel addition induced a significant and rapid growth of microbes as well as the enrichment of a singular microbe, *Novosphingobium* (Class Alphaproteobacteria, Family Sphingomonadaceae), which came to numerically dominate the community. Throughout

the experiment, Total Organic Carbon decreased along with Total Nitrogen, indicating that the microbes were metabolizing JP-5. While ongoing research is further characterizing the chemical composition of petroleum hydrocarbons as the fuel biodegrades, from this experiment we can infer that our aquifers on O‘ahu can be a valuable source of bacterial bioremediation for future PH contamination events.

Keywords: jet fuel, microbes, groundwater.

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1.0 INTRODUCTION

Petroleum and petroleum-derived product contamination, also known as petroleum hydrocarbons (PH), has been a significant environmental issue around the world for decades, polluting terrestrial, marine, and underground ecosystems. These events are commonly divided into two main categories: surface and subsurface contamination which includes groundwater and soil environments. While the majority of accidental PH releases occur in surface marine environments, subsurface contamination events are particularly threatening as they can pollute groundwater resources which millions of people rely on for freshwater (Marigómez, 2014).

Petroleum hydrocarbon pollution is a common occurrence, leading to the development of a wide variety of remediation techniques–however, these are often developed for surface contamination events. Surface events allow for the usage of chemical and mechanical remediation techniques and are additionally aided by weathering for PH removal (Das & Chandran, 2010). Chemical dispersants are often less toxic than the oil itself, but in the context of subsurface environments, they may exacerbate the threat of aquifer contamination rather than remediate it (American Society for Microbiology, 2011; Das & Chandran, 2010).

Subsurface contamination is particularly concerning in the context of underground fuel storage, a widespread practice throughout developed nations. Within the U.S., the practice of storing fuel and refined petroleum hydrocarbons in underground storage tanks has been designated as one of the major sources of groundwater pollution (Borden & Kao, 1992). According to a national study conducted by Dietz et al., the U.S. currently has almost 800,000 underground storage tanks containing petroleum or other

hazardous substances (1986). Conservative estimates show that 10% or more of such tanks have leaked which directly translates to thousands of gallons of refined hydrocarbons having polluted groundwater resources (Borden & Kao, 1992).

Subsurface petroleum hydrocarbon contamination events are limited to treatments that either remove, reduce, or mitigate the toxicity of petroleum contamination; some techniques include air sparging, multiphase extraction, soil vapor extraction, and excavation (Miles et al., 2024). The most common method for subsurface treatment however is pump and treat. Pump and treat involves flushing out any contaminated water from the system and treating it above ground to remove pollutants; when properly designed this can remove the majority of pollutants from the system but may require several years of pumping (Borden & Kao, 1992). Studies have shown that pump and treat is particularly successful in the initial stages of contamination and containing contaminant plumes, but it is generally ineffective for the complete removal of contaminants in subsurface environments (Speight, 2020). In addition, this technique is limited by conditions where contaminant concentrations are high, the source of contamination has not been completely removed, the contaminant plume is large, and when groundwater flow is either slow or complex (Speight, 2020). Significant residual contamination is often left after pumping and treating, showing the constraints of this technique.

As an alternative remediation strategy, research has shifted toward the development of bioremediation strategies utilizing certain microbes to degrade petroleum within contaminated environments (Miles et al., 2024). This mechanism is particularly valuable in the face of groundwater contamination, where there are fewer remediation

choices as the previously mentioned techniques may be financially or technically unfeasible (Miles et al., 2024). The lack of development for remediation strategies in subsurface environments shows a significant need for applicable practices for groundwater PH contamination and a critical need for further research in bioremediation, especially with refined PH products. Bioremediation research has expanded greatly in recent years due to its promise as a remediation technique for petroleum hydrocarbon pollution. It has been widely recognized as the most environmentally friendly remediation technique for PH removal and is less costly than chemical or mechanical techniques commonly used (Truskewycz et al., 2019). In addition to its ecological and economic value, the technique can be applied to a diverse range of environments due to its flexibility. Bioremediation is particularly valuable in the context of limited access contamination events.

Aerobic biodegradation comprises the majority of research within the field of petroleum hydrocarbon degradation. Research on anaerobic degradation is significantly more limited (Laczi et al., 2020) because degradation occurs at slower rates under anaerobic conditions (Olajire & Essien, 2014). More than 79 genera of bacteria have been identified with the capability of degrading petroleum hydrocarbons and fuel components indicating the potential of indigenous bacteria to be able to degrade PH contaminants within their own environment (Truskewycz et al., 2019; Xu et al., 2018). Both common and rare species of bacteria have been isolated for the purpose of biodegradation in the context of petroleum hydrocarbons. Of these bacteria, different species have been shown to metabolize different chemical compounds within petroleum and petroleum-derived products, such as naphthalene and BTEX (benzene, toluene, ethylbenzene, xylene)

(Truskewycz et al., 2019). While a wide range of bacteria are able to metabolize many of the components of petroleum and petroleum-derived products, there are essentially no bacteria that can degrade all of its chemical compounds (Xu et al., 2018). Compounds with high molecular weights are often resistant to biodegradation and persist in the environment unless physically or chemically removed (Marigómez, 2014). However, high levels of contaminant removal can be achieved when utilizing a consortium of bacteria with diverse metabolisms to degrade the various components of petroleum and petroleum-derived products (Xu et al., 2018).

1.1 Culture-Independent Microbiology

Recent developments in technology have allowed for the culture-independent study of microorganisms (Rappé et al., 2003). Many microbial degradation and bioremediation technologies have been focused on pure microbial cultures or a simple consortium of bacterial species that have been isolated through classical isolation methods (Laczi et al., 2020). While a prominent approach, limiting biodegradation of petroleum hydrocarbons to lab-cultured bacterial species has produced many caveats in the field of bioremediation. It has been observed that lab-cultured bacteria produced varying results in experiments on the degradation of petroleum hydrocarbons in laboratory conditions versus field conditions, suggesting that degradation efficiency may be enhanced by the inclusion of other synergistic bacteria within the microbial community (Laczi et al., 2020). Combining omics alongside well-established techniques for culture-independent observation of intact microbial communities in degradation

studies such as flow cytometry can allow for the study of enrichable consortia within a system as either an individual or on a community level (Laczi et al., 2020).

1.2 Aquifer Contamination

Groundwater is a critical resource on which approximately half of the world's population depends as their main source of freshwater (Sampat, 2000). Remote areas such as islands are particularly dependent on groundwater as a freshwater resource and it has been argued that island aquifers are some of the world's most vulnerable aquifer systems (White & Falkland, 2009). Island aquifers tend to be shallow and highly permeable, occurring as a freshwater lens overlying seawater (White & Falkland, 2009). This type of aquifer is called a basal lens and is the most widely used aquifer for freshwater in tropical islands within the Pacific (Nichols et al., 1996). Due to their high permeability, they are particularly susceptible to contamination as precipitation drives the recharge of groundwater, further emphasizing the importance of maintaining integrity for island aquifers (Nichols et al., 1996).

In Hawai‘i, roughly 99% of water used by households is sourced from groundwater resources (Gingerich & Oki, 2000). On O‘ahu, a singular basal lens aquifer provides 77% of the island's total freshwater (Jedra, 2021). This high reliance on O‘ahu's aquifer has warranted a "sole source aquifer" designation from the EPA: When reliance on an aquifer for drinking water meets or exceeds 50%, the aquifer is designated as a sole source aquifer (SSA) for which, if contaminated, there would be no reasonable alternative resource for freshwater (EPA, 2023b).

1.3 Red Hill 2021 Fuel Release

In November of 2021, the U.S. Navy accidentally released 19,000 gallons of diesel fuel (jet propellant-5; JP-5) into the Red Hill drinking water shaft, potentially contaminating the groundwater aquifer it draws water from (EPA, 2023a). Earlier that year, in May of 2021, a pressure surge event caused a pipeline joint failure, spilling JP-5 directly onto the tunnel floor of the Red Hill Bulk Fuel Storage Facility. The fuel was collected into a fire suppression system where it remained until November. In November, the stored fuel was accidentally released directly into the Red Hill Shaft, impacting 93,000 people at the Joint Base Pearl Harbor-Hickam and Red Hill Housing at Pu‘uloa. The fuel release caused the closure of the Halawa Shaft, located adjacent to the Red Hill Shaft, due to potential contamination spread.

In response to the contamination event, the Navy conducted a pump and treat remediation tactic (EPA, 2023a). According to the EPA, concentrations of fuel have been decreasing over time, however, assessment of the aquifer and evaluation of the effectiveness of the technique continue to be monitored by the EPA and Hawaii State Department of Health (DOH) (2023a). Threats to drinking water resources are still unknown as stakeholders work to identify how the Red Hill Shaft contamination has spread in terms of direction, flow rate, and whether the contamination is capable of spreading to other aquifers on the island.

The Red Hill Bulk Fuel Storage Facility holds approximately 180 million gallons of fuel and consists of 20 underground tanks (Jedra, 2021). These tanks sit a mere 100 feet above the SSA that supplies 77% of O‘ahu’s freshwater and since 1943, the facility has recorded a minimum of 73 fuel leaks, amounting to at least 180,000 gallons of fuel

(Jedra, 2021). Due to the limited knowledge of PH contamination in the context of O‘ahu’s aquifers, it is imperative to understand how bioremediation can assist or hinder the degradation of petroleum hydrocarbons in groundwater.

1.4 Significance

It is evident that the importance of understanding and researching petroleum and petroleum-derived contamination events in tropical island aquifers is substantial. Due to the Red Hill fuel spill of 2021 as well as ongoing efforts beginning in 2023 to de-fuel the tanks, this work is particularly relevant, timely, and engaging. There are numerous gaps within the field of PH contamination of groundwater resources, particularly in the context of place-based application and evaluation, but also in terms of bioremediation strategies.

To potentially fill such holes within the field of PH contamination remediation, we developed and experimentally tested three core hypotheses, utilizing modern techniques to better understand the complexity and nuances of groundwater microbial growth upon and degradation of PH. We hypothesized that the addition of diesel fuel to groundwater would induce the rapid growth of microbes. Additionally, we hypothesized that the fuel addition would particularly enrich a subset of taxa from the groundwater community that would remove a significant fraction of the carbon and nitrogen from the system as the fuel was metabolized. To test these hypotheses groundwater was incubated for 1 month with added JP-5 to study the degradation of petroleum hydrocarbons in groundwater as well as its resulting impacts on groundwater microbial communities. Throughout the experiment, samples for scanning excitation-emission fluorescence spectroscopy, flow cytometry, dissolved organic carbon, and microbial community

profiling were collected from biological replicates and two control incubations (where either microbes or fuel was excluded). Fluorescence spectroscopy and dissolved organic carbon allowed for the analysis of fluorescent dissolved organic matter compositional changes and JP-5 consumption while flow cytometry measurements and DNA allowed for the analysis of microbial growth and community compositional changes. Results from our experiment showed that groundwater microbial communities grew rapidly on the JP-5 amendment, reaching and maintaining densities ten times higher than the controls over the 28 days. Community compositions shifted in response to the domination of a single bacteria, *Novosphingobium*, and a significant fraction of the added JP-5 carbon and ambient nitrogen was removed over the experiment, suggesting rapid and sustained biodegradation by natural groundwater microbes.

2.0 METHODS

2.1 Water Collection

On 26 April 2023 a total of 20 liters of groundwater was collected from Kamehameha Schools Well B (21.341667, -157.8525, 265m deep, 66.7m above sea level, established in 1997); access was granted by the Hawai‘i Department of Health (Dr. Robert Whittier). Before groundwater was collected, water was discharged steadily at high volume from the main pump for 6 minutes, followed by 15 minutes of purging from the sampling spigot itself. Groundwater was collected in a sterilized polycarbonate carboy triple rinsed with sample water prior to final collection.

2.2 Experimental Set-Up

The experiment was conducted at C-MORE Hale on the campus of the University of Hawai‘i at Mānoa and ran for a total of 28 days from April 27, 2023, to May 25, 2023. Three different treatments (treatment names are italicized) were used for the experiment: replicated *JP-5 treated live* incubations of groundwater amended with JP-5, one *JP-5 treated sterile control* (filter-sterilized groundwater amended with JP-5 to control for the temporal dynamics of JP-5 in the absence of microbial degradation), and one *live groundwater control* (unfiltered and unamended groundwater to control for the temporal dynamics of the natural microbial community). JP-5 treated live replicates were conducted in triplicate, with three parallel 1 L capacity glass conical separatory funnel incubation vessels containing 1100 mL groundwater amended separately with 2 mL of JP-5 and mixed by shaking the separatory funnel for 30 seconds three times. For the JP-5

treated sterile control, 1100 mL of groundwater was filtered through a 0.2 μm sample-flushed polyethersulfone filter (Millipore Sterivex) before 2 mL of JP-5 was added and mixed. For the live groundwater control, 1100 mL of groundwater was added and mixed for 30 seconds with no addition of JP-5. See Table 1 for a summary of each treatment and their parameters.

After assembling the treatments, each flask was then wrapped with aluminum foil to block out light and kept at room temperature for the duration of the experiment (4 weeks). Each flask was allowed to sit for 10 minutes prior to the initial sampling to allow undissolved JP-5 to form a layer at the top of the groundwater. After 7 days, 500 mL of groundwater was transferred from the bottom of the separatory funnels into 1 L amber glass bottles to remove the continuous source of JP-5 from the system. By isolating the groundwater from the layer of JP-5, we were able to study the microbial degradation of dissolved JP-5 within treatments from 7d to 28d. The leftover groundwater within the separatory funnels was discarded and the bottles were sealed and stored in the dark at room temperature for the remainder of the experiment.

Table 1: Treatment summary.

Treatment Name	Number of Replicates	Microbial Presence	Fuel Addition
JP-5 treated live	3	Yes	Yes
JP-5 treated sterile control	1	No	Yes
Live groundwater control	1	Yes	No

Four types of samples were collected throughout the experiment: total organic carbon (TOC), microbial communities (DNA), 3-D scanning fluorescence spectroscopy

(fDOM), and flow cytometry (FCM). TOC samples were collected in 20 mL scintillation vials with a septum cap. The vial was rinsed with 4 mL of sample water prior to collection and then acidified with a drop of concentrated HCl before being stored at room temperature in the dark. Microbial community cells were collected by peristaltic pump filtration onto 0.2 μm Sterivex filters and stored at -80˚C. Samples for fDOM were collected in 4 mL amber vials and stored at 4˚C. 1 mL of sample water was collected for FCM in 2 mL cryovials, then fixed with 17 μL of 32% paraformaldehyde for a final concentration of 0.5% before being stored at -80˚C.

On day 0, 2, 7, 14, and 28 all sample types were collected (deemed *low frequency time points*). Additional samples for fDOM and FCM were collected every 6 hours for the first 48 hours of the experiment, then daily until groundwater from the separatory funnels was transferred to the amber bottles at 7d. After the transfer, samples were collected from the amber bottles every 6 hours for the next 48 hours before switching to daily until day 14; these were deemed *high frequency time points*. See Table 2 for the sampling schedule.

Table 2: Sampling schedule. Sampling began on April 27th, 2023, and finished on May 13, 2023. High frequency samples were only fluorescence spectroscopy (fDOM) and flow cytometry (FCM). Low frequency samples include both fDOM, FCM, adding dissolved organic carbon (TOC) and DNA.

Date	Hours	Day	Sampling Type	Sampled
4/27/23	0	0	low frequency	fDOM/FCM/TOC/DNA
4/27/23	6, 12, 18	0.25, 0.50, 0.75	high frequency	fDOM/FCM
4/28/23	24, 30, 36, 42	1, 1.25, 1.50, 1.75	high frequency	fDOM/FCM
4/29/23	48	2	low frequency	fDOM/FCM/TOC/DNA
4/30/23	72	3	high frequency	fDOM/FCM
5/1/23	96	4	high frequency	fDOM/FCM
5/2/23	120	5	high frequency	fDOM/FCM
5/3/23	144	6	high frequency	fDOM/FCM
5/4/23	168	7	low frequency	fDOM/FCM/TOC/DNA
5/4/23	174, 180, 186	7.25, 7.50, 7.75	high frequency	fDOM/FCM
5/5/23	192, 198, 204, 210	8, 8.25, 8.50, 8.75	high frequency	fDOM/FCM
5/6/23	216	9	high frequency	fDOM/FCM
5/7/23	240	10	high frequency	fDOM/FCM
5/8/23	264	11	high frequency	fDOM/FCM
5/9/23	288	12	high frequency	fDOM/FCM
5/10/23	312	13	high frequency	fDOM/FCM
5/11/23	336	14	low frequency	fDOM/FCM/TOC/DNA
5/12/23	504	21	high frequency	fDOM/FCM
5/13/23	672	28	low frequency	fDOM/FCM/TOC/DNA

2.3 Fluorescence Spectroscopy of Jet Propellent-5

In this experiment, fluorescence spectroscopy was utilized to track the presence of JP-5 at high frequency throughout the incubation period. Jet propellent-5 (JP-5) is a kerosene-based jet fuel refined through a straight distillation of crude or shale oil or the distillation of such in the presence of a catalyst (Agency for Toxic Substances and Disease Registry [ATSDR], 2017). Like all petroleum products, a key component of JP-5's composition is hydrocarbons–compounds made up of organic carbon and hydrogen. JP-5 contains more than 200 aliphatic and aromatic hydrocarbons (i.e. benzene, toluene, ethylbenzene, naphthalene, and xylene) as well as a wide variety of additional additives not found in plain kerosene such as antioxidants, inhibitors (static, corrosion, and fuel

system icing), biocides, lubrication improvers, and thermal stability improvers (ATSDR, 2017). JP-5 is composed of both soluble and insoluble components, many of which are fluorescent (See Table 3 for major components of JP-5, their qualitative fluorescence and solubility). Previous work has demonstrated the fluorescence characteristics of similar refined diesel fuels. Some components of JP-5 diesel fuel lend the fuel a specific spectral signature which allows us to track that signature as a proxy for fuel concentration (Wasswa et al., 2019). To track the fluorescence of JP-5 in this experiment we identified a characteristic fluorescence signature in our groundwater background (Figure 6) and constructed a standard curve to evaluate the consistency and linearity of the relationship between concentration and fluorescence (Figure 1). We performed fluorescence spectroscopy on a series of dilutions created by mixing fresh and aged JP-5 with Mili-Q water, with concentrations ranging from 0 ppb of JP-5 to 5,000 ppb. Scanning fluorescence spectroscopy was conducted on respective samples utilizing a HORIBA Aqualog fluorometer following the methods outlined by Nelson et al. (2015).

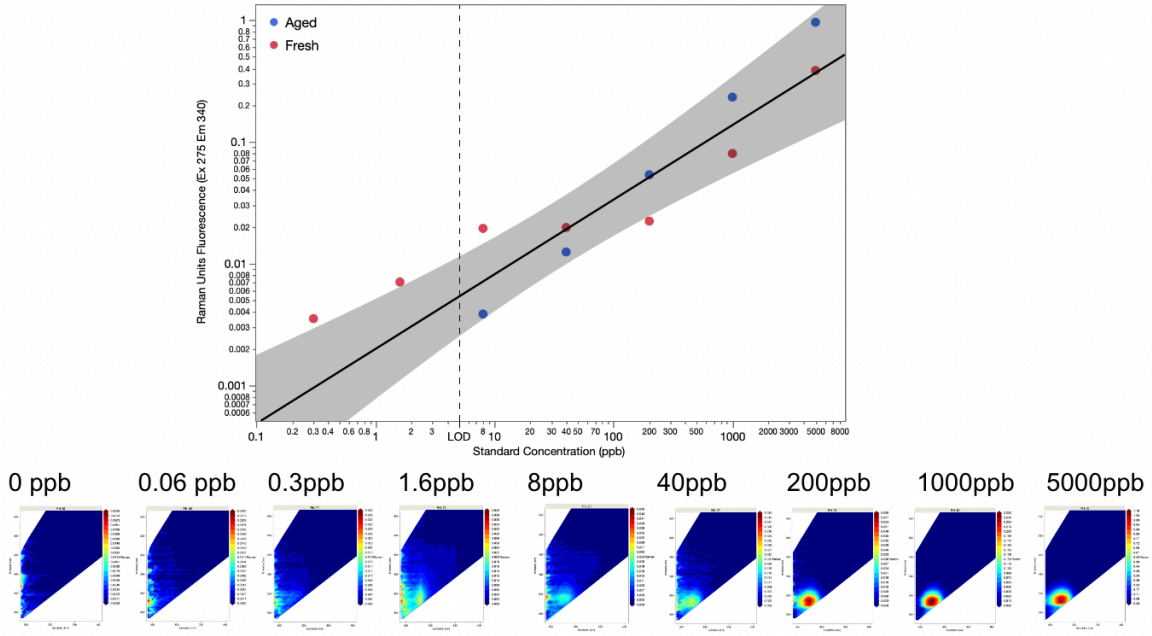

Figure 1: Standard curve and excitation-emission fluorescence spectroscopy scans of JP-5 dilution series. The dotted line indicates the approximate empirical limit of detection (LOD) at roughly 5 ppb. The samples were measured first immediately after mixing and equilabration (same day; "Fresh" in red) re-measured after 1 week of incubation in the dark at room temperature ("Aged" in blue).

Table 3: Fluorescence and solubility of dominant JP-5 components in water.

Compounds	Solubility	Fluorescence
Alkenes	Insoluble	Non-fluorescent
Alkyl aromatic	Varies	Fluorescent
Aliphatic hydrocarbons	Insoluble	Non-fluorescent
1,2,3,4-Tetramethylbenzene	Insoluble	Fluorescent
Branched paraffins	Insoluble	Non-fluorescent
BTEX (benzene, toluene, ethylbenzene, xylene)	Soluble	Fluorescent
Biphenyl	Insoluble	Fluorescent
n-Paraffins	Insoluble	Non-fluorescent
Naphthalene	Insoluble	Fluorescent

2.4 Laboratory Analysis of Bulk Total Carbon and Nitrogen

Total organic carbon samples were submitted to and processed by the SOEST Laboratory for Analytical Biogeochemistry. Samples were analyzed using the Shimadzu TOC-L combustion analyzer for both Total Nitrogen (TN) and Non-Purgeable Organic Carbon (NPOC) which uses a 680˚C combustion platinum catalytic oxidation method (Shimadzu Corporation, 2010). The instrument combusts the sample before measuring carbon content as CO_2 by non-dispersive infrared gas analysis to measure NPOC while simultaneously measuring TN (Shimadzu Corporation, 2010).

2.5 Microbial Abundances and Community Profiling

To track microbial abundances, flow cytometry samples were processed following Nelson et al. (2015) on a Cytoflex Flow Cytometer. Samples were stained with 7 µL of 40 µL mL^{-1} of Hoechst 34580 prior to processing for a final concentration of 1 µg mL^{-1}. Microbial community profiling was done using amplicon sequencing of the 16S ribosomal subunit gene. Sterivex filter housings were gently removed and the filter was excised with a sterilized blade. The filters were then placed into 2 mL tubes containing Lysing Matrix D (MP Biomedicals) and homogenized using a FastPrep 96 (MP Biomedicals) at 1800 RPM for 3 minutes. DNA was extracted from this homogenate using a NucleoMag DNA Microbiome extraction kit (Macherey-Nagel). The extracted DNA was then submitted to the Microbial Genomics and Analytical Laboratory at the University of Hawai‘i at Mānoa for 16S amplicon-based metagenomic library preparation. The V4 region of the small subunit (16S) ribosomal RNA gene was

amplified from each genomic DNA template using forward-indexed primers 515F-Parada: GTGYCAGCMGCCGCGGTAA and 806R-Apprill: GGACTACNVGGGTWTCTAAT (Walters et al., 2016) following the protocols of the Earth Microbiome Project (Caporaso et al., 2018), with the following minor changes: we did not pool triplicate reactions, and we used 1uL of genomic template. Amplicons were cleaned and normalized to equimolar concentrations using the SequelPrep kit (Thermo Fisher Scientific) and then pooled and sequenced on the Illumina MiSeq platform (600 cycle V3 chemistry). Sequences were demultiplexed using a custom probabilistic script and microbiome profiling was implemented in the MetaFlow|mics pipeline (Arisdakessian et al., 2020) following the specific settings presented by Jani et al. (2021): sequences were assembled, denoised and quality trimmed using DADA2 (Callahan et al., 2016), globally aligned with mothur (Schloss et al., 2009) to the Silva (v132) global SSU rRNA alignment database (Yilmaz et al., 2014), bayesian consensus classified (70%) to the Genus level (Wang et al., 2007), and clustered into 99% sequence identity operational taxonomic units using vsearch (Rognes et al., 2016) refined to reduce intragenomic taxonomic splitting errors using LULU (Frøslev et al., 2017). Sequences that could not be classified at the Domain level or were classified as chloroplast or mitochondrial 16S were discarded and sequencing depth was subsequently standardized to 10,000 random reads per sample. All bioinformatics were deployed on the C-MAIKI gateway (Cleveland et al., 2022) at the University of Hawai‘i at Mānoa by the Center for Microbial Oceanography: Research and Education.

3.0 RESULTS

3.1 Groundwater Bacterial Growth on Diesel Fuel

Flow cytometry data revealed a notable difference in bacterial growth patterns between the three treatments (JP-5 treated live, JP-5 treated sterile control, and live groundwater control). Over the initial 48-hour sampling period, there was minimal change among the treatments, with JP-5 treated live sample bacterial counts ranging from 4.9 to 130 cells/µL and live groundwater and JP-5 treated sterile controls ranging from 13 to 460 cells/µL and 5.2 to 140 cells/µL respectively (Figure 2, Table 4). After 72 hours of incubation, growth patterns began to diverge with a significant increase in bacterial counts within the JP-5 treated live samples (Figure 2), which showed consistent growth up until they reached a carrying capacity of $1.2 \times 10^4 \pm 2.1 \times 10^3$ cells/µL at 6 days, maintaining that concentration until the transfer at 7 days. In contrast, the JP-5 treated sterile and live groundwater controls remained relatively low in concentration at 7d (8.9 cells/µL and 8.8×10^2 cells/µL, respectively). Once transferred into bottles, bacterial counts remained roughly steady in all treatments through 28d (Figure 2). By 6 days and until the end of the experiment, the JP-5 treated live samples had a bacterial count roughly 100 times higher than the JP-5 treated sterile control and roughly 15 times higher than the live groundwater control (Table 4).

Table 4: Bacterial counts (cells) at selected time points across bottle incubations.

		Bacterial Concentrations (cells per microliter)				
Bottle	**Treatment**	**0 days**	**2 days**	**6 days**	**7 days**	**28 days**
Sample 1	JP-5 treated live	140	50	$1.3x10^4$	$1.2x10^4$	$1.2x10^4$
Sample 2	JP-5 treated live	99	44	$9.5x10^3$	$9.9x10^3$	$1.0x10^4$
Sample 3	JP-5 treated live	110	20	$1.3x10^4$	$1.1x10^4$	$1.2x10^4$
Sterile Control	JP-5 treated sterile control	140	29	34	8.9	120
Live Control	Live groundwater control	17	17	300	880	760

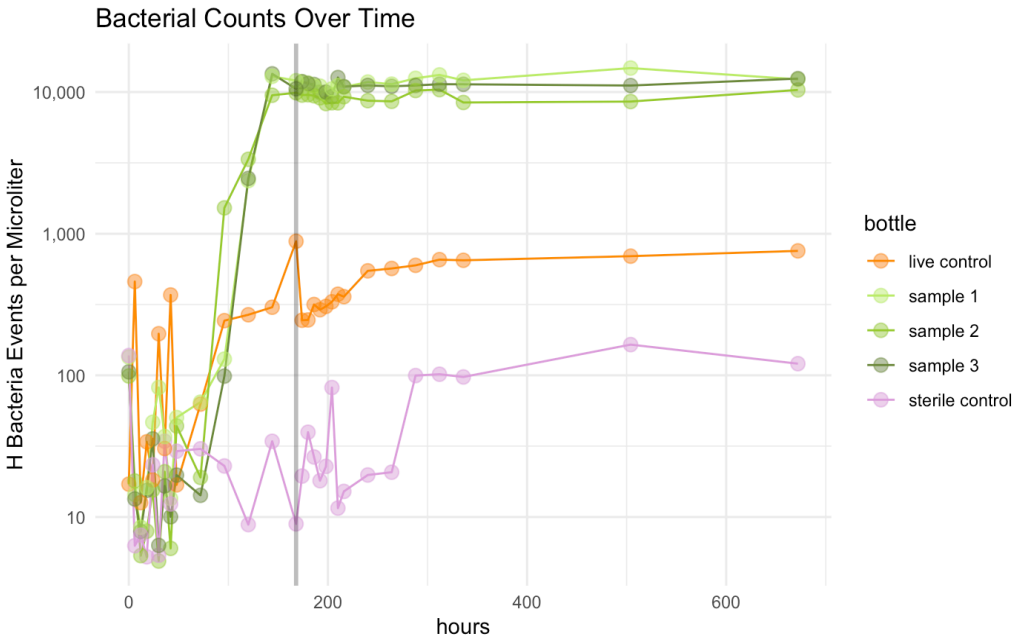

Figure 2: Groundwater bacterial growth increase in response to fuel addition.

Within 3 days bacterial counts within the JP-5 treated live samples were enriched relative to the live groundwater and JP-5 treated sterile controls, reaching carrying capacity by 6 days. Once transferred to bottles (denoted by the gray vertical line), bacterial counts in the JP-5 treated live samples remained high throughout the 28 day experiment.

3.2 Total Nitrogen and Non-Purgeable Organic Carbon Consumption

Total Nitrogen and non-purgeable organic carbon (NPOC) analyses revealed distinct patterns between the JP-5 treated live samples and controls. At the beginning of the experiment, Total Nitrogen (TN) concentrations within all treatments began at 40 µM, typical for groundwaters in Hawai‘i (Nelson et al., 2015; Richardson et al., 2015; Watson et al., 2023). However, between 2 and 7d, the nitrogen concentration within the JP-5 treated live samples decreased dramatically to under 10 µM (Figure 3), while the controls stayed near 40 µM, suggesting drawdown of TN coincident with microbial growth responses. After the transfer, all treatment concentrations of TN remained relatively constant.

Non-purgeable organic carbon analyses showed an initial increase in concentration for both the JP-5 treated live samples and JP-5 treated sterile control: Within the first 48 hours, beginning at approximately 100µM, concentrations increased by approximately 65 µM in the JP-5 treated sterile control and an average of 55 µM in the JP-5 treated live samples, suggesting ongoing solubilization of the diesel fuel. However, after the initial 48-hour period, concentration patterns diverged significantly between the JP-5 treated live samples and JP-5 treated sterile control: The JP-5 treated live samples continued to increase in concentration to approximately 300 µM while the JP-5 treated sterile control remained constant, such that at 7 days TOC was twice as high when microbial communities were active (Figure 4). The live groundwater control on the other hand showed low concentrations and minimal changes throughout the experiment: starting at 9.61 µM, peaking at 48 hours at 26.24 µM before decreasing to 12.51 µM at 28 days. After the transfer to bottles, consistent with removal of the surface layer diesel

source, concentrations decreased in both the JP-5 treated live samples and the sterile control by an average of 98.4 ± 27.1 µM and 49.78 µM respectively. Taken together these results indicate an initial 2-7 day period of solubilization of JP-5 into dissolved phase (TOC); once isolated from the JP-5 source TOC was utilized steadily at a rate of roughly 4.7 ± 1.3 µmol/L/day in the JP-5 treated live groundwater, roughly double the rate of removal in the JP-5 treated sterile control (2.4 µmol/L/day).

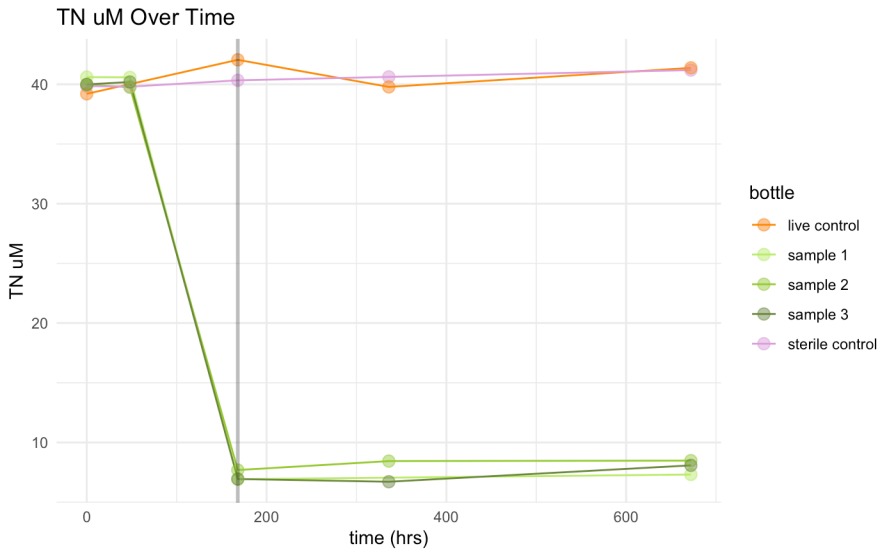

Figure 3: Removal of Total Nitrogen during bacterial growth. Total Nitrogen concentration patterns diverged after 48 hours between the JP-5 treated live samples and controls. After the transfer to bottles (denoted by the gray vertical line), TN concentrations remained relatively constant.

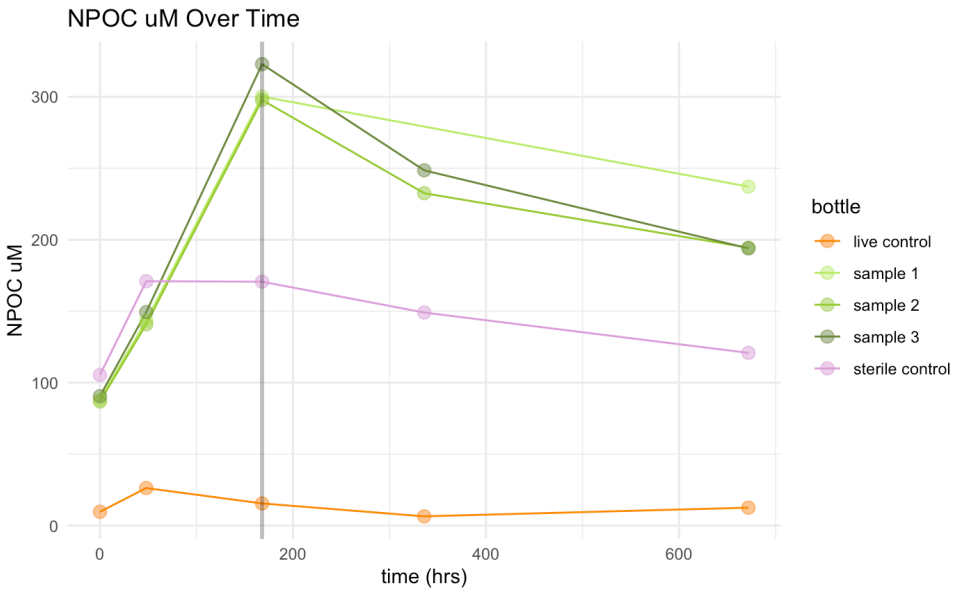

Figure 4: Accumulation and removal of Non-Purgeable Organic Carbon during bacterial growth. Treatments amended with JP-5 began the experiment with non-purgeable organic carbon (NPOC) concentrations at 100 µM, increasing in concentration until 48 hours. After 48 hours, the JP-5 treated live samples continued to increase in concentration until after the transfer (denoted by the gray vertical line), in which both the JP-5 treated sterile control and JP-5 treated live samples decreased in NPOC.

3.3 Compositional Changes in Fluorescence of Dissolved Organic Matter

Fluorescence spectroscopy was used to analyze and monitor the composition of organic matter throughout the experiment. Both the live groundwater and JP-5 treated sterile controls showed consistent levels of JP-5 throughout the experiment with the sterile control at full saturation and the live groundwater control at background levels (Figure 5). JP-5 signature patterns within the JP-5 treated live samples varied dramatically throughout the experiment: At the beginning of the experiment, the JP-5 signature showed fluorescence of 10 Raman Units of Water within JP-5 treated live

samples (Figure 5). For the first 4 days of the experiment, JP-5 treated live concentrations remained between 10 and 20 Raman Units before increasing through 6d. After 6d, concentrations stabilize, remaining between 35-60 Raman units throughout the rest of the experimental period.

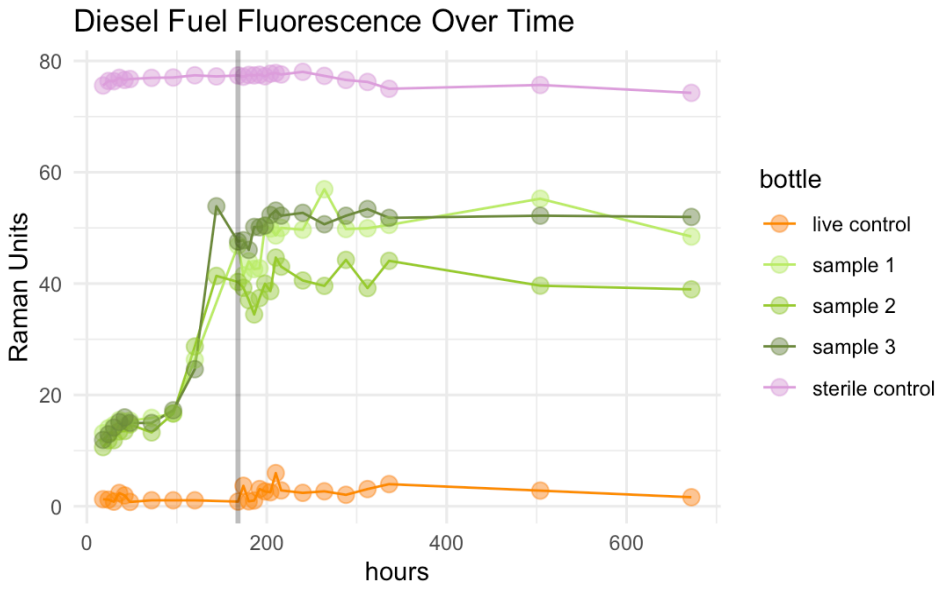

Figure 5: Increase in diesel fuel fluorescence in treatments amended with JP-5. JP-5 treated live samples began the experiment with a lower fluorescence than the JP-5 treated sterile control despite being amended with equal amounts of JP-5. The sterile control maintained a steady fluorescent signature throughout the experiment, while JP-5 treated live samples gradually grew until the transfer and remained steady thereafter.

The findings in Figure 5 are supported by the minimal change between the initial timepoint and final timepoint for both the live groundwater and JP-5 treated sterile control in the excitation-emission matrices (Figure 6). However, the JP-5 treated live

samples saw a change in their excitation-emission matrices, showing fluorescence in ranges outside of the JP-5 proxy (Figure 6).

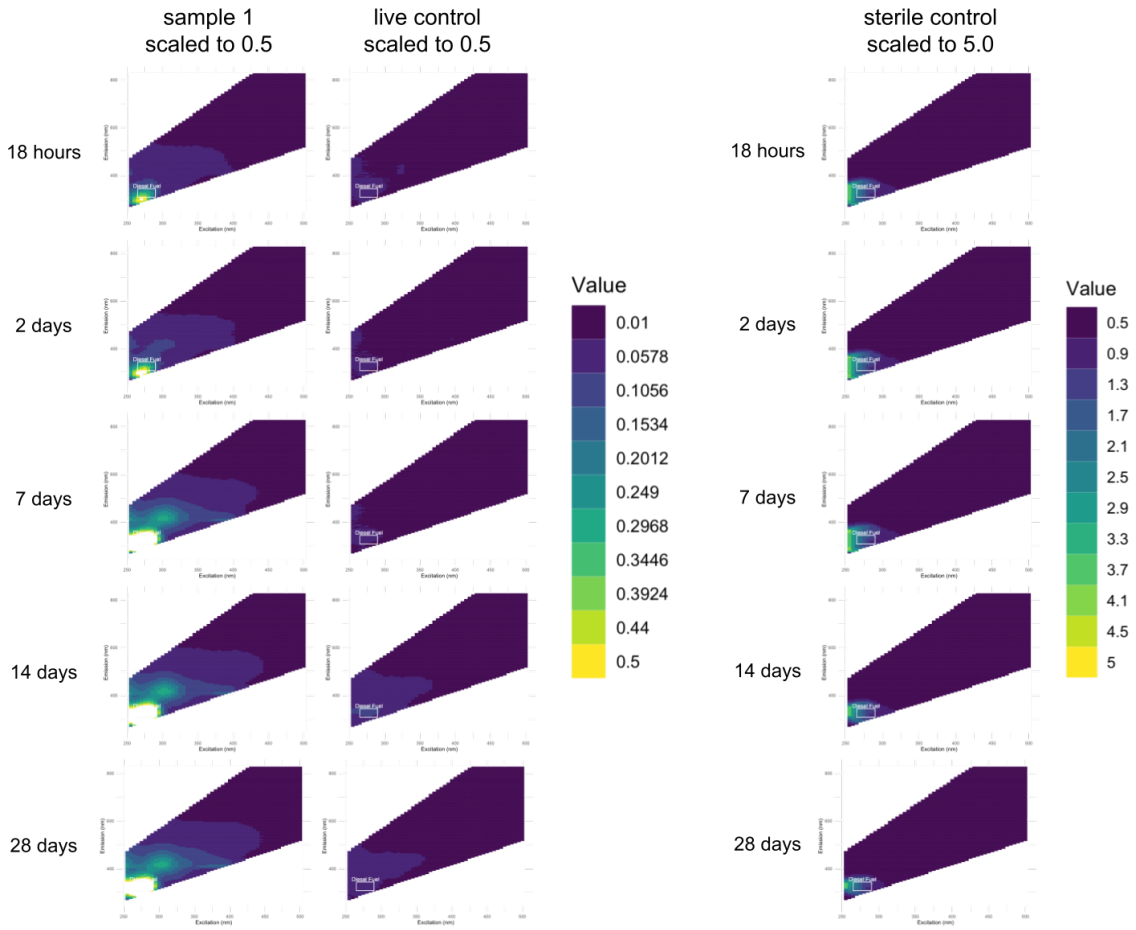

Figure 6: Excitation-emission fluorescence spectroscopy scans over time. Note the different scales used (see top of each column and respective contour legends). Scans show low initial fluorescence in JP-5 treated live sample 1 relative to the JP-5 treated sterile control, with both the sterile control and JP-5 treated live demonstrating a minimal change from 7 to 28 days, and sterile controls having a single lower emissions peak than treatments. There was minimal diesel fluorescence in the live groundwater control.

3.4 Microbial Community Compositional Shifts in Fuel-Amended Treatments

16S sequencing allowed for the tracking and monitoring of microbial community compositions throughout the experiment. The compositions of the microbial communities showed consistent differences between the JP-5 treated live and the live groundwater control between 2 and 7d (Figure 7). At the beginning of the experiment, the live groundwater control microbial community clustered with samples collected from the groundwater source. At the 2-day mark, JP-5 treated live samples clustered with that of the live groundwater control, but by the 7-day time point, the microbial compositions diverged due to treatment conditions: Samples collected from JP-5 treated live samples after the 7-day mark and through the full 28d clustered independently from those of the live groundwater control.

Figure 7: Multidimensional scaling plot showing the differentiation of microbial communities between bottle treatments. JP-5 treated live samples clustered closely together and separately from live groundwater samples after the 2d timepoint,

emphasizing differences between the two treatments' microbial communities. At 28 days, duplicate samples were taken from the JP-5 treated live samples.

3.5 *Novosphingobium* Growth in Fuel-Amended Treatments

The most abundant microbe within the experiment was *Novosphingobium* (Class Alphaproteobacteria, Family Sphingomonadaceae). In the presence of JP-5, by the 7-day transfer point, each of the three JP-5 treated live replicates were dominated by *Novosphingobium*, comprising over 80% of each microbial community (Figure 8). Comparatively, *Novosphingobium*'s relative abundance within the live groundwater control remained low throughout the experiment (0.4 to 16.3%). While there were few microbes within the JP-5 treated sterile control (Figure 2), of that small population, nearly 75% of the community was also composed of *Novosphingobium* at the final timepoint.

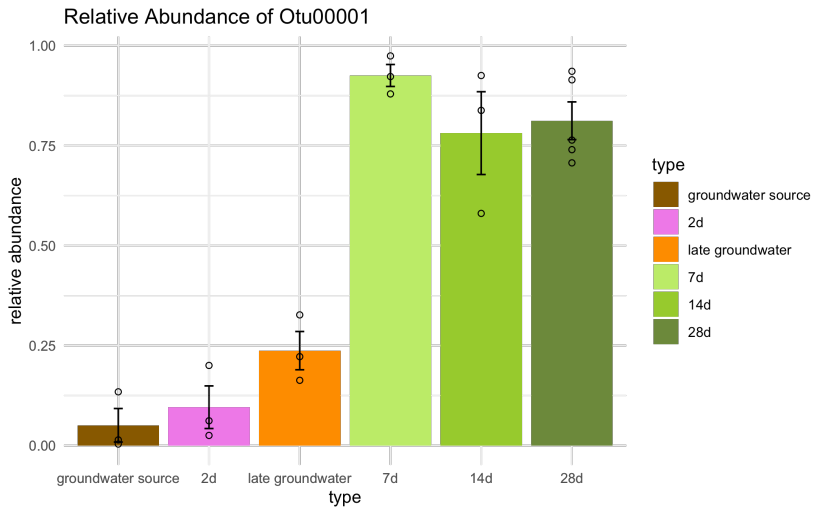

Figure 8: *Novosphingobium* mean relative abundance in each community type. OTU00001 represents *Novosphingobium* (Class Alphaproteobacteria, Family

Sphingomonadaceae) which showed the highest relative abundance amongst all microbes present within the community in the amended groundwater samples.

3.6 Additional Microbial Taxon Growth Patterns

While *Novosphingobium* was identified as the microbe with the highest relative abundance within fuel-amended treatments, an additional microbe that showed increased relative abundance in the presence of JP-5 was *Sulfuritalea* (Class Gammaproteobacteria, Family Rhodocyclaceae) (Figure 9). A suite of microbial taxa increased in relative abundance in later time points primarily within the live groundwater control, including an unidentified microbial species (Class Alphaproteobacteria, Clade NRL2), Burkholderiaceae, and *Rhodobacter* (Class Alphaproteobacteria, Family Rhodobacteraceae) (Figure 10a-c). Additional microbial taxa saw increases in relative abundances at the 2d time point including *Porphyrobacter* (Class Alphaproteobacteria, Family Sphingomonadaceae), *Dechloromonas* (Class Gammaproteobacteria, Family Rhodocyclaceae), and *Sphingopyxis* (Class Alphaproteobacteria, Family Sphingomonadaceae) (Figure 10d-f). However, after the 7d time point, the relative abundances of these microbes decreased significantly through the end of the experiment.

Other notable growth patterns and microbes within the experiment are shown in Figure 11. Figure 11 shows the growth patterns of Archaea which were present at the beginning of the experiment. The highest relative abundance of Archaea was in the live groundwater control. However, by the end of the experiment, Archaea were absent in all treatments except for the JP-5 treated sterile control.

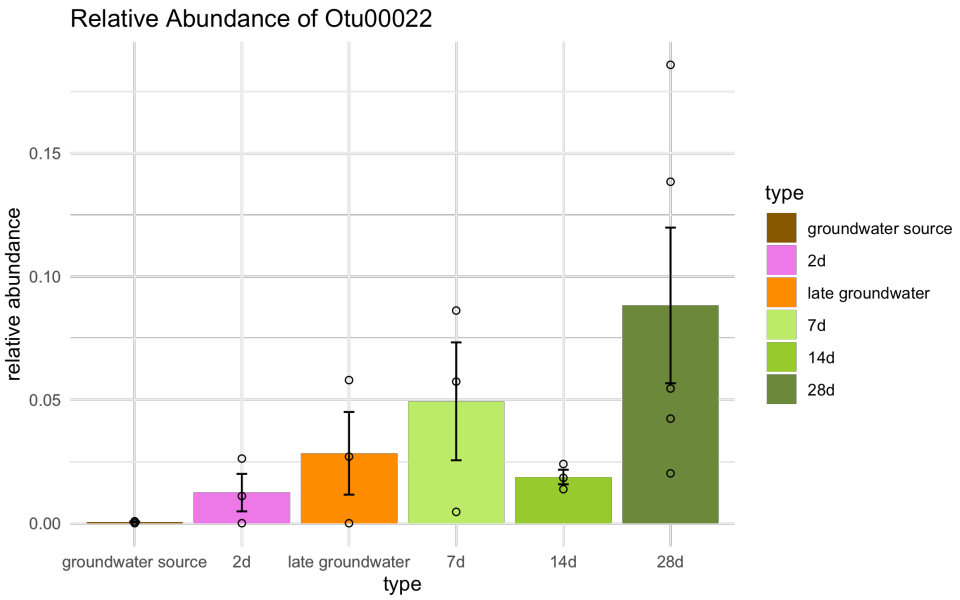

Figure 9: Increased OTU00022 relative abundances in 28d JP-5 treated live samples. Otu00022 represents *Sulfuritalea* (Class Gammaproteobacteria, Family Rhodocyclaceae) which was identified as an additional microbe that increased in relative abundance in late JP-5 treated live samples.

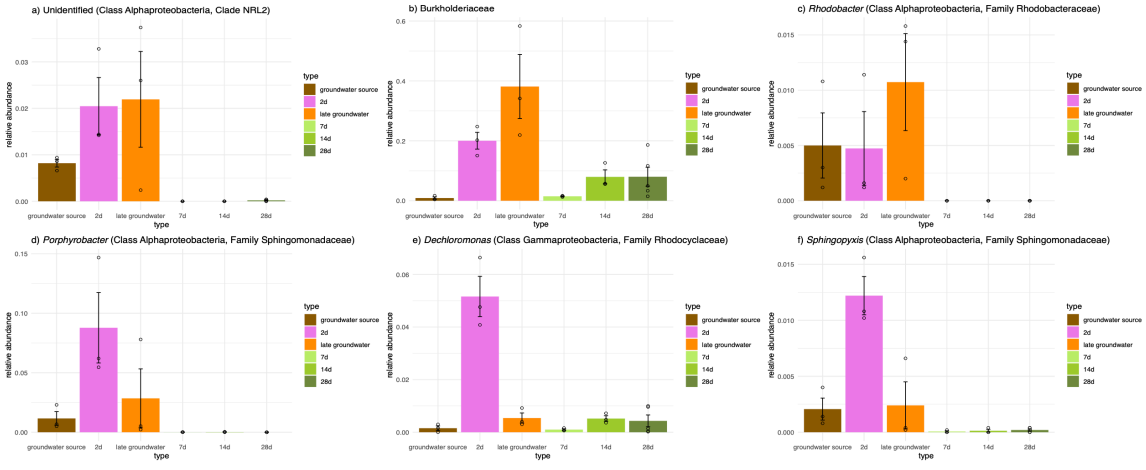

Figure 10: Growth patterns of abundant OTUs over time. Each row showcases Otus that shared similar growth patterns throughout the experiment and were selected if they exhibit mean relative abundances greater than 1%. Graphs a-c showcase Otus which saw

an increase in relative abundance in later time points within the live groundwater control. Graphs d-f saw increases in relative abundance by the 2d timepoint, however, their relative abundances decreased by the end of the experiment.

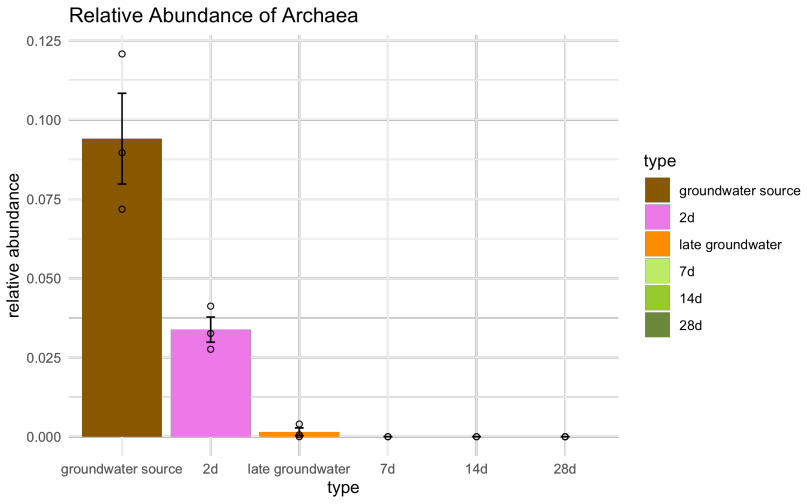

Figure 11: Summed Archaea relative abundances. Archaea relative abundances were highest in live groundwater controls and decreased throughout the experiment.

3.7 Microbial Contamination Event

Four days into the experiment, a contamination event occurred where sample 2 of the JP-5 treated live samples deviated biologically from the other two samples. Chemically, sample 2 was consistent with the patterns observed in samples 1 and 3, however, biologically, after the transfer at 7d, sample 2's microbial community saw an increase in *Pseudomonas* (Class Gammaproteobacteria, Family Pseudomonadaceae), climbing to over 30% of the microbial community by the end of the experiment (Figure 12).

The growth of *Pseudomonas* is consistent with the findings in the fluorescent patterns of sample 2. Sample 2's fluorescent signal of JP-5 was similar to the other JP-5 treated live samples, however, a new fluorescent signal emerged 4 days into the experiment, showing high fluorescence in the range of 509 (emission) and 390 Raman units (excitation) (Figure 13). These wavelengths may be associated with metabolic by-products of bacterial growth, supporting the patterns shown in Figure 12. Despite the initial detection of microbial by-products at day 4, increases in *Pseudomonas* relative abundance were not observed until day 28d.

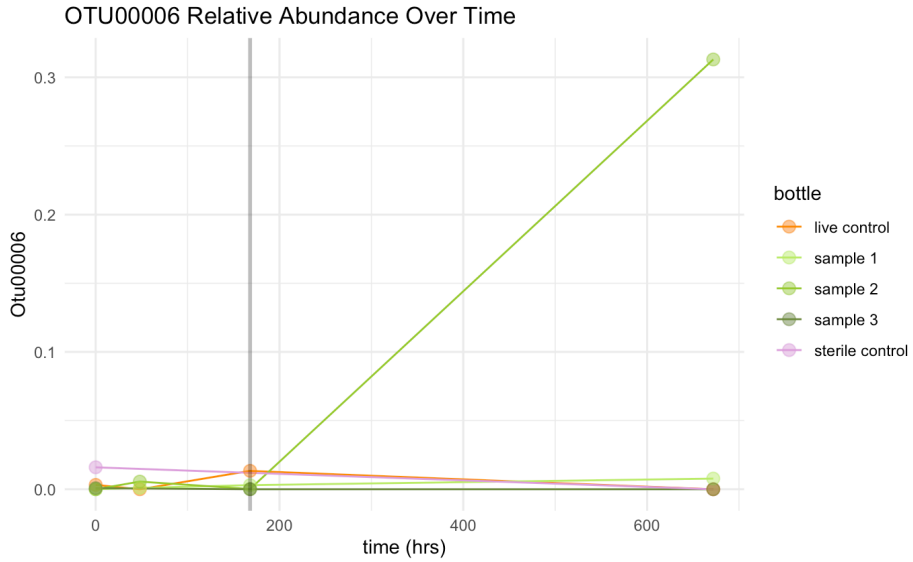

Figure 12: OTU00006 growth in relative abundance over time. OTU00006 represents *Pseudomonas* (Class Gammaproteobacteria, Family Pseudomonadaceae) which appeared in sample 2 after the transfer indicating a contamination event. OTU00006 does not appear in other samples.

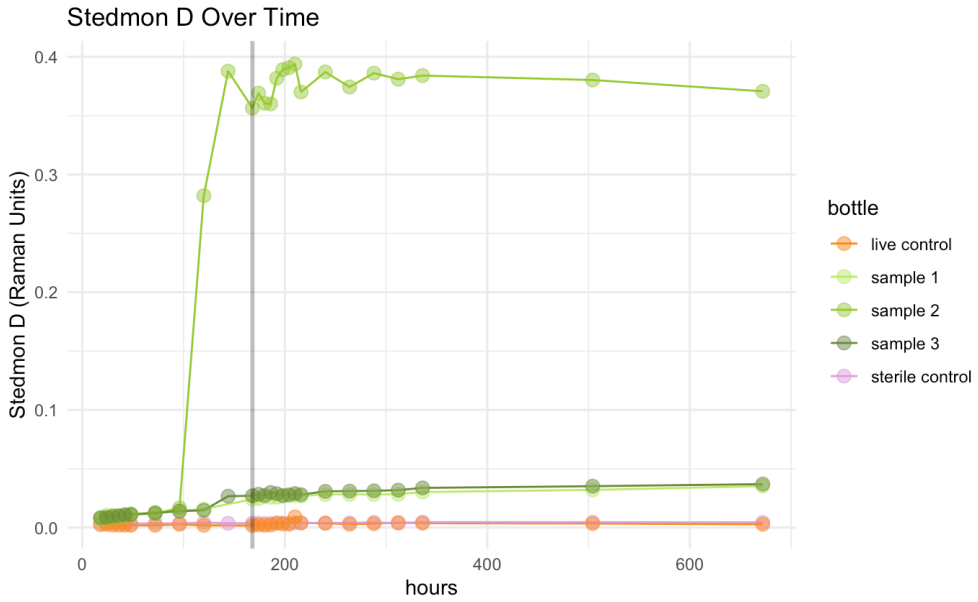

Figure 13: Increase of Stedmon D fluorescent signature over time. Stedmon D fluorescence is generally associated with Pseudomonas by-products and is primarily present in only sample 2 of the JP-5 treated live samples. While there is fluorescence shown in samples 1 and 3, these signals are significantly lower than what is seen in sample 2.

4.0 DISCUSSION

4.1 *Novosphingobium* is a Dominant Degrader of JP-5

During the experiment, JP-5 treated live samples saw significant microbial growth, producing cell counts that were magnitudes higher than both the JP-5 treated sterile and live groundwater controls. This growth primarily occurred between day 3 and day 6 of the experiment while bottles were incubated with a continuous layer of JP-5 (Figure 2). High bacterial concentrations were maintained for 3 weeks after the JP-5 source was removed, while Total Organic Carbon was steadily removed at rates more than twice that of the sterile control, indicating consistent metabolism of JP-5 hydrocarbons.

DNA analysis showed that the dominant bacteria within the microbial communities was *Novosphingobium*. *Novosphingobium* was consistently found at high relative abundances in the JP-5 treated live samples, comprising over 80% of the microbial communities at its peak abundance, supporting the majority of observed bacterial growth (Figure 8). The dominance of *Novosphingobium* in an aerobic fuel-amended environment is conducive to other findings that have identified Sphingomonadaceae as degraders of a wide range of hydrocarbons (Kertesz et al., 2019). While most of the community composition consisted of *Novosphingobium*, an additional microbe that saw an increase in relative abundance in the presence of JP-5 was *Sulfuritalea* (Figure 9). *Sulfuritalea* relative abundances failed to reach over 1%, however, *Sulfuritalea* has additionally been identified as a PH degrader, primarily degrading benzene (Kim et al., 2020).

4.2 Consumption, Dissolution, and Volatilization of JP-5

Non-purgeable Organic Carbon (NPOC) analysis revealed that microbes were able to consume the JP-5 at an approximate rate of 4.7 ± 1.3 μmol/L/day once the JP-5 source was removed. The rate of NPOC removal in the sterile control was 2.4 μmol/L/day, less than half that of the JP-5 treated live samples, indicating degradation rates attributable to microbial metabolism of 2.3 ± 1.3 μmol/L/day. The rate of NPOC removal in the sterile amended control could be attributed to losses from volatilization, from abiotic decomposition, or from degradation by the relatively low (but growing) biomass of bacteria accumulated over the course of the experiment (roughly 100-fold below that of the JP-5 treated live samples by the end of the experiment, but still dominated by *Novosphingobium*).

With the presence of a continuous source of JP-5 during the initial part of the experiment, NPOC concentrations increased from approximately 100 μM to 300 μM by the 7-day timepoint (Figure 4). This increase in NPOC concentrations can be attributed to the solubilization of light aromatic and polycyclic aromatic compounds of JP-5 such as BTEX and naphthalene into the groundwater (Truskewycz et al., 2019). The difference in the TOC of JP-5 in JP-5 treated live samples compared to the JP-5 treated sterile control by 7d (Figure 4) may be due to the higher solubility of microbial degraded products of previously insoluble compounds and/or the ability of microbes to increase the solubility of larger compounds of fuel in water. Certain microbes have been identified to be able to increase the dissolution of fuel into water through the formation of biofilm around fuel droplets, increasing the surface area of the fuel and therefore the rate of consumption and

dissolution of it into the water (Prasad et al., 2023). While these observations have occurred in marine environments, bacteria within other ecosystems such as aquifers may be capable of possessing these traits.

At the 2-day timepoint, the JP-5 treated live samples continued to see an increase of NPOC while the JP-5 treated sterile control concentrations of NPOC began to decline, potentially suggesting losses due to volatilization of organics. An alternative explanation for the difference observed in NPOC concentrations between the JP-5 treated sterile and JP-5 treated live samples could be attributed to the analysis process of NPOC. The Shimadzu TOC-L combustion analyzer sparges samples with air to drive off inorganic carbon before NPOC is measured (Shimadzu Corporation, 2010). Many compounds within JP-5 are volatile, meaning that during analysis, lighter fractions of JP-5 such as BTEX and saturated hydrocarbons with 1-5 ring structures could be volatilized (Truskewycz et al., 2019). This could account for the differences between JP-5 treated live samples and JP-5 treated sterile control between the 2-7 day timeframe. JP-5 treated live samples likely saw less volatilization due to microbial processes that converted volatile compounds of JP-5 into soluble organic carbon. There is potential for microbial activities such as chemolithotrophic CO_2 reduction to organic carbon via ammonia oxidation to occur within the experiment. These processes therefore could have resulted in higher NPOC concentrations than the sterile control. We did not see any evidence of high relative abundances of Ammonia Oxidizing Archaea or Ammonia Oxidizing Bacteria, but this may be an artifact of the massive growth of *Novosphingobium* limiting our analytical window for rarer taxa.

4.3 Microbial Impacts on Fluorescence Spectroscopy Analysis

Results generated by fluorescence spectroscopy are particularly puzzling due to the initial reduced diesel fuel fluorescence in JP-5 treated live samples. In early time points, diesel fuel fluorescence in JP-5 treated live samples was significantly lower than the JP-5 treated sterile control despite the equal amendment of 2 mL of JP-5 to 1.1 L of groundwater. Both treatments were fully saturated with JP-5, however, JP-5 treated live samples failed to reach the same Raman counts as the sterile control at the beginning of the experiment. Additionally perplexing is the gradual increase in diesel fuel fluorescence during the first 7 days. It would be expected that diesel fuel fluorescence would gradually decline as these compounds are metabolized, however, the opposite was observed (Figure 5).

We hypothesize that as the JP-5 dissolved into the groundwater, smaller fluorescent compounds were rapidly and preferentially consumed by the microbial community, resulting in a low fluorescence at initial time points. At the beginning of the experiment, there were likely no limiting factors that could have hindered microbial growth in JP-5 treated live samples as conditions were oxic and nitrogen levels were consistent with groundwater concentrations, enabling the microbes to consume dissolving components of JP-5 (American Society for Microbiology, 2011). However, as TN concentrations decreased between 2-7 days (Figure 3), this coincided with a gradual increase in diesel fuel fluorescence, potentially reducing the rate at which microbes were able to consume fluorescent components of the fuel and allowing diesel fuel within the groundwater to fluoresce more brightly. Incidentally, this points to a potential for

nitrogen limitation in our experiment, pointing the way for potential bio-remediation nitrogen amendment considerations in the future.

Excitation-emission scans support the observed findings in fluorescence spectroscopy, showing diesel fuel fluorescence at only later time points within JP-5 treated live samples. The excitation-emission scans of JP-5 observed within the experiment match the diesel scans produced by Wasswa et al. (2019). However, alongside the appearance of JP-5 fluorescence in late JP-5 treated live samples is the development of a secondary zone of fluorescence (Figure 6). In JP-5 treated sterile control excitation-emission scans, the majority of JP-5 fluorescence falls outside of the diesel fuel zone and into a secondary zone that more closely resembles excitation-emission scans of oil than diesel fuel (Wasswa et al., 2019). The impact of microbial activity is apparent between the JP-5 treated live and sterile treatments, however, the exact nuances of such are unknown.

4.4 *Novosphingobium* as a Candidate for In-situ Bioremediation

Novosphingobium proves to be a prime candidate for in-situ bioremediation. In-situ bioremediation techniques for groundwater contamination generally consist of two main practices: biosparging and bio-augmentation. Biosparging refers to the oxidization of the contaminated aquifer to increase oxygen concentrations and enhance the rate of naturally existing reactions performed by the groundwater's microbial community (Tripathi & Ram, 2018). While biosparging relies on the aquifer's existing microbial communities, bio-augmentation involves the introduction and usage of a research-based consortium of microbial cultures in contaminated groundwater sites. As a naturally

existing microbial species in O‘ahu’s aquifer, *Novosphingobium* is a significant potential candidate for biosparging, however, as numerous studies have shown success in culturing the genus, it can additionally be utilized in bio-augmentation efforts.

5.0 CONCLUSION

Findings from our experiment concluded that, in the presence of petroleum hydrocarbons, naturally occurring microbial genera within O‘ahu’s aquifer, *Novosphingobium*, saw significant growth and, within a week, dominated the groundwater microbial community. Capable of degrading and consuming PH NPOC, *Novosphingobium* is a viable candidate for bioremediation efforts for future PH contamination events in O‘ahu’s aquifer ecosystem. Future research would be necessary to resolve perplexing findings in organic matter solubilization and volatilization as microbial transformation and degradation progresses. Furthermore, a deeper analysis into the chemical degradation of JP-5 would aid in determining exactly which compounds of JP-5 were consumed by microbial species throughout the experiment.

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