An efficient enrichment technique for isolation and quantification of indigenous diesel fuel-utilizing bacteria present in freshwater sediments

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The toxic effects of petroleum products on the environment, particularly diesel fuel, have been well studied. Equally well researched are the methods for efficient bioremediation of petroleum pollution. Many studies have demonstrated the ability of indigenous microbiota, found in petroleum-polluted environments, to biodegrade diesel fuel. In this study, we quantified the diesel fuel-utilizing bacteria in sediments from two freshwater lakes and then began an enrichment process using a regimen of constant aeration, addition of $(NH_4)_3HPO_4$ and diesel fuel. The enrichment process was performed in a tabletop bioremediation station based on an Eastern European ship-based bioremediation system. Over a three week period we observed significant increases in diesel fuel-utilizing bacteria, from 1.12 x 10^5 to 5.40 x 10^8 cfu/g dry weight in sediments from a lake that has historically been exposed to anthropogenic petroleum hydrocarbons, and from 1.63 x 10^5 to 5.10 x 10^9 cfu/g dry weight from another lake with no such exposure. In addition, we identified the predominant diesel fuel-utilizing microbiota using 16S rDNA sequence analysis. These results demonstrate that diesel fuel-utilizing bacteria can be recovered from lake sediments using this technique.

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Abbreviations: AOA: Atlas Oil Agar, TSA: tripticase soy agar, CFU: colony forming units, HDPE: high density polyethylene, PVC: poly-vinyl chloride.

Introduction

The pollution of aquatic ecosystems by petroleum hydrocarbons presents a significant risk to aquatic organisms [1-6). From catastrophic accidents to routine discharges, the mechanisms by which crude oil and/or its refined fractions are released into the environment have been well documented. While accidental tanker spills are high profile and infamous sources of petroleum contamination, the cumulative pollution caused by discharges from ships and factories, and from leaky storage tanks, poses a much greater environmental problem [7-9]. Among the common sources of hydrocarbon pollution are overflow spills that occur during fueling of recreational and commercial vessels, and discharges of bilge and ballast water [10, 11].

Numerous methods have been developed to remediate petroleum pollution. Physical and chemical methods of remediating petroleum spills are generally costly and do not completely eliminate the water-soluble fraction of petroleum [6, 12, 13]. On the other hand, biological remediation techniques are more cost-efficient and rely upon microorganisms to

metabolize pollutants, oxidizing, for example, petroleum hydrocarbons into carbon dioxide [8, 14]. Petroleum-degrading bacteria are reportedly ubiquitous in the aquatic environment [7, 12, 15]. However, the extent of the involvement of these indigenous organisms in the bioprocessing of petroleum contamination is unknown. Understanding their role in the natural bioremediation process would allow us to better evaluate the risk from petroleum products released to an aquatic environment, but efficient culturing techniques are lacking. Diesel fuel is particularly harmful to aquatic organisms and our long term goals focus on better understanding its fate when it enters an aquatic ecosystem. Our goal was to develop a technique that would allow for the rapid enrichment for diesel fuel-utilizing bacteria from freshwater lake sediments. This technique would be used for a variety of industrial and environment bioremediation applications such as starter cultures for diesel-fuel biodegradation biochemistry studies or in on-board bilge/ballast water treatment systems.

Materials and Methods

Sediment Collection

Sediments were obtained from two west Michigan lakes: Spring Lake, in northwestern Ottawa county, and Lake Wolverine (also known as Cleveland Lake), in northern Muskegon county. Spring Lake lies within a densely populated area, has a developed shoreline, is subject to high rates of motorized boat traffic, and contains sediments that have had a putatively high exposure to various hydrocarbons. Lake Wolverine is located in a rural area and lies within the Owassippe Scout Reservation, owned by the Boy Scouts of America, who prohibit motorized boating on the lake. Lake Wolverine's sediments can thus be assumed to have been minimally impacted by anthropogenic hydrocarbon exposure. Approximately three gallons of wet Spring Lake sediments were collected from several locations in the lake using a PONAR sampler and combined. We used combined sampling to increase the diversity of microbes. Approximately one gallon of wet Lake Wolverine sediments was collected with a bucket 2-3 m from shore. Sediments were kept in sealed plastic containers at 4^0C prior to analysis.

Sediment Enrichment

The sediment enrichment protocol was developed in our lab to provide a starter culture for a continuous flow system that was adapted for research purposes from an industrial bioremediation wastewater treatment system used in Ukraine and other Eastern European countries [16, 17]. For each sediment source, one L of wet sediment was transferred to each of two bioreactors, constructed from19-L HDPE buckets that had been disinfected with a 10% bleach solution and rinsed thoroughly with tap water prior to use. Water was added to achieve a total volume of 15 L. To aerate the sediments, aeration coils, constructed from ¾ inch diameter PVC pipe, were installed on the bottom of each bioreactor. The aerators used filtered lab air and provided saturation of the systems with oxygen as well as thorough mixing. The bioreactors were covered with Plexiglas lids, which were then covered with aluminum foil to preclude the passage of light into the bioreactors. Based on previous industrial experimental protocols and those developed in the lab [17], nitrogen and phosphorus were added to both bioreactors in the form of 1.0 g $(NH_4)_3HPO_4$ (0.44 mM) on alternate days. Once every 24 hours, 750 ml of

diesel fuel-enriched water were added to one bioreactor, resulting in a final concentration of approximately 100 parts per million (ppm). The diesel fuel enriched water was created by diluting 40 ml of No. 2 diesel fuel, obtained from a gas station (Exxon Mobile, Georgetown Twp., Michigan), to a final volume of 25 L with water and mixing constantly by compressed air by means of a PVC aeration coil, similar in construction to those installed in the bioreactors. The other bioreactor—the control, or non-enriched reactor—received 750 ml of tap water each day. Every three days, aeration of the bioreactors was suspended for 30 minutes, and the top 5 L were siphoned off of each bioreactor to remove toxins or by-products that may have accumulated. The volumes were replenished to 15 L with water, and aeration was resumed.

Enumeration of Bacteria

On the day the bioreactors were initiated, and then every three days afterwards for three weeks, a ~20 ml sample was removed from each bioreactor using a 25 ml sterile polypropylene tube. Three 1-ml aliquots of this sample were dried at 80^0C for 24 hours to determine the dry weight of the mixing sediments. A series of 10-fold dilutions was made from each sample, and spread plates were inoculated from these dilutions to determine the number of viable cells per gram of dry weight in the sediments. Two types of media were inoculated: 1X tripticase soy agar (TSA) (Difco Labs, Detroit, MI) for the purpose of enumerating general heterotrophic bacteria and Atlas Oil Agar [AOA, 18] that included 0.5% diesel fuel, rather than crude oil, in order to enumerate bacteria in the sediments that were capable of utilizing diesel fuel as a carbon source. The plates were incubated at room temperature (22^0C) for seven days, after which no new colonies ever appeared on either of the two media. After the seven days, colonies were counted, and these data were used to calculate colony forming units (CFU) per gram of dry weight for the diesel-enriched and the non-enriched sediments from each sediment source.

Sub-culturing and Identification of Bacteria

The first and last sets of AOA plates (i.e. those inoculated on the first and last days of the enrichment experiments) for each sediment source were examined, and colonies of each unique morphology (color, border, elevation), were streaked for isolation on new AOA and TSA plates. The selected bacteria were Gram stained, tested for their preferred growth temperature (4^0C, 22^0C, 30^0C, or 37^0C), and used in PCR amplifications and DNA sequence analysis in order to identify the genera of the diesel fuel-utilizing bacteria that were present in the sediments. Whole cells of each culture were added to the reaction as a suspension made by vortexing a loop-full of bacteria in 0.5 ml sterile dH_2O. The 16S rRNA gene of each bacterium was amplified by PCR, using the universal primers 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3'). The PCR reagents were from the PCR Core System I kit from Promega (Madison, WI). Final concentrations for the reactions were: 1X Mg-free reaction buffer; 1.5 mM Mg^{2+}; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 1 pmol/ul each of 27F and 1492R; and 0.24 mM of Tergitol Type NP-40. To each reaction, 2.5 units of *Taq* DNA polymerase were added after an initial incubation at 95^0C for five minutes. Total reaction volume was 50 ul. Amplifications were then carried out for 30 cycles of 30 seconds of denaturation at 95^0C, 30 seconds of annealing at 52^0C, and 60 seconds of extension at 72^0C. After a final extension at 72^0C for five minutes,

the products were stored at 4^0C until analysis by agarose gel electrophoresis (within 24 hours). PCR products were separated by agarose gel electrophoresis (1.5% agarose in TAE buffer) and visualized by ethidium bromide staining. Successful amplifications manifested as bands at approximately 1.4 kbp in length. These bands were excised from the agarose gel and purified using the Geneclean Spin Kit (Q-Bio Gene, Irvine, CA). The sequences of the PCR DNA products were determined by Ohio State University sequencing core facility, and the results were used to identify the genera of the bacteria by comparison with known sequences in the Ribosomal Database Project [19].

Statistical Analysis

Statistical calculations were done using SPSS 12.0 software (SPSS Inc., Chicago, IL). The initial and final bacterial counts for the two sediment sources were compared using t-tests.

Results

Enrichment for diesel-utilizing bacteria

At the initiation of the experiment, when the sediments were placed in the bioreactors prior to any diesel or mineral supplementation, the sediments from the Spring Lake sample contained 1.12 x 10^5 CFU of diesel fuel-utilizing bacteria per gram (dry weight) (SD = 1.00 x 10^4) and 2.65 x 10^6 CFU/g general heterotrophic bacteria (SD = 6.95 x 10^5) (Table 1 and Figure 1). After 21 days of the enrichment protocol the sediments from the Spring Lake sample contained 5.40 x 10^8 CFU/g diesel fuel-utilizing bacteria (SD = 1.13 x 10^7) and 9.10 x 10^8 CFU/g general heterotrophic bacteria (SD = 2.14 x 10^8) (Table 1 and Figure 1). In the control bioreactor, where sediments were aerated and mineral-enriched, but not diesel fuel-enriched, the final number of diesel fuel-utilizing bacteria was 2.60 x 10^7 CFU/g (SD = 1.17 x 10^6) and the general heterotrophic bacteria numbered 6.40 x 10^7 CFU/g (SD = 2.98 x 10^7) (Table 1 and Figure 1).

Initial bacterial enumeration values for the sediments from the Lake Wolverine sample were 1.63 x 10^5 CFU/g diesel fuel-utilizing bacteria (SD = 1.92 x 10^4) and 3.28 x 10^7 CFU/g general heterotrophic bacteria (SD = 6.95 x 10^6) (Table 1 and Figure 2). Final, post-enrichment values for the sediments from the Lake Wolverine sample were 5.10 x 10^9 CFU/g diesel fuel-utilizing bacteria (SD = 2.77 x 10^9) and 1.90 x 10^9 CFU/g general heterotrophic bacteria (SD = 1.40 x 10^8) (Table 1 and Figure 2). In the control bioreactor, where sediments were aerated and mineral-enriched, but not diesel fuel-enriched, the final count of diesel fuel-utilizing bacteria was 4.47 x 10^6 CFU/g (SD = 1.21 x 10^6) and the general heterotrophic bacteria numbered 9.10 x 10^7 CFU/g (SD = 8.73 x 10^6) (Table 1 and Figure 2).

Microbial Plating and Identification

In order to evaluate the increase in diesel fuel-utilizing bacteria caused by our enrichment protocol, we developed a simple and straight forward method for enumeration. We successfully adapted the Atlas oil agar (AOA) media to use diesel fuel as the sole carbon source. This technical development allowed us to monitor the change (increase) in diesel fuel-utilizing bacteria over time. Additionally, the technique allowed us to perform simple microbiological analysis on any cultured bacteria. To determine if any of the cultured bacteria represented novel taxa we identified, by 16S rDNA, bacterial colonies that appeared to have unique colony morphology. Identifying the taxonomies of the organisms obtained from the bioreactors gives important insight into a consortium's ability to metabolize diesel fuel

Table 1. Numbers of diesel fuel-utilizing and general heterotrophic bacteria from Spring Lake and Lake Wolverine sediment samples preceding and following 21 days of constant aeration, daily diesel fuel enrichment (experimental bioreactors), and mineral enrichment on alternating days. All means and standard deviations are calculated from triplicate plate counts.

Bioreactor	Bacterial Type	Mean Initial Count and Standard Deviation (cfu/g dry weight)	Mean Final Count and Standard Deviation (cfu/g dry weight)
Spring Lake, Diesel Fuel-Enriched	Diesel Fuel-Utilizing	1.12×10^5 ($SD = 1.00 \times 10^4$)	5.40×10^8 ($SD = 1.13 \times 10^7$)
Spring Lake, Control	Diesel Fuel-Utilizing	1.12×10^5 ($SD = 1.00 \times 10^4$)	2.60×10^7 ($SD = 1.17 \times 10^6$)
Lake Wolverine, Diesel Fuel-Enriched	Diesel Fuel-Utilizing	1.63×10^5 ($SD = 1.92 \times 10^4$)	5.10×10^9 ($SD = 2.77 \times 10^9$)
Lake Wolverine, Control	Diesel Fuel-Utilizing	1.63×10^5 ($SD = 1.92 \times 10^4$)	4.47×10^6 ($SD = 1.21 \times 10^6$)
Spring Lake, Diesel Fuel-Enriched	General Heterotrophic	2.65×10^6 ($SD = 6.95 \times 10^5$)	9.10×10^8 ($SD = 2.14 \times 10^8$)
Spring Lake, Control	General Heterotrophic	2.65×10^6 ($SD = 6.95 \times 10^5$)	6.40×10^7 $SD = 2.98 \times 10^7$)
Lake Wolverine, Diesel Fuel-Enriched	General Heterotrophic	3.28×10^7 $SD = 6.95 \times 10^6$)	1.90×10^9 ($SD = 1.40 \times 10^8$)
Lake Wolverine, Control	General Heterotrophic	3.28×10^7 $SD = 6.95 \times 10^6$)	9.10×10^7 ($SD = 8.73 \times 10^6$)

and may help to address the limiting factors to growth in a more targeted fashion.

We were able to identify bacterial representatives of four different genera from the sediments of the two lakes: *Acidovorax, Acinetobacter, Janthinobacterium,* and *Pseudomonas* (Table 2). We did not identify any new taxa through our identification process. Overall, the most frequently observed genus of diesel fuel-utilizing bacteria was *Acinetobacter*. This predominance becomes more pronounced following enrichment with diesel fuel: two out of three subcultured organisms from the initial set of Spring Lake AOA plates were *Acinetobacter*, while three out of four were of this genus on the final set of plates; no *Acinetobacter* were recovered from the initial set of Lake Wolverine AOA plates, but seven out of eight organisms cultured from the final set were *Acinetobacter*.

Discussion

Our study indicates that diesel-fuel utilizing microorganisms are present in relatively high abundance in aquatic sediments, independent of previous diesel fuel exposure, and these microorganisms responded to enrichment with diesel fuel. A novel component of our work included enriching for diesel-fuel utilizing bacteria from freshwater sediments that lacked pre-exposure to diesel fuel or other aliphatic hydrocarbons (Lake Wolverine). These results demonstrate that the enrichment protocol rapidly and significantly increased the number of indigenous diesel fuel-utilizing bacteria in the sediments in as little as 14 days. The enrichment system is very simple and inexpensive to manufacture and could be easily scaled for desired applications. The “settling” step and removal of excess liquid allowed us to create a semi-continuous flow system that

Table 2. Characterization of lake sediment bacteria grown on Atlas Oil Agar (AOA). Isolate designations are acronyms of brief physical descriptions of the colonies that were selected from the AOA plates. SL=Spring Lake; LW=Lake Wolverine. Gram Stain: GNR = Gram Negative Rod; GNC = Gram Negative Coccus. Temperatures tested for preferred growth were 4^0C, 22^0C, 30^0C, and 37^0C.

Isolate designation	Source / Plating	Gram stain	Preferred Growth Temperature (^{0}C)	Genus (% sequence match from Ribosomal Database Project [Cole et al. 2005])
BWOI	SL / Day 0	GNR	22	*Acinetobacter junii* (99.8)
WRO	SL / Day 0	GNR	22	*Acinetobacter calcoaceticus* (94.2)
YFBC	SL / Day 0	GNR	22	*Pseudomonas borealis, P. brassicacearum,* or *P. aurantiaca* (all 98.6)
BWM	SL / Day 21	GNR	22	*Acinetobacter calcoaceticus* (98.9)
FWG	SL / Day 21	GNR	22	*Acinetobacter calcoaceticus* (98.9)
WTB	SL / Day 21	GNC	22	*Acinetobacter calcoaceticus* (98.7)
YB	SL / Day 21	GNR	30	*Acidovorax facilis* (97.1)
BU	LW / Day 0	GNR	22	*Pseudomonas fluorescens* (97.9)
LYB	LW / Day 0	GNC	22	*Pseudomonas fluorescens* (98.7)
WOR	LW / Day 0	GNR	22	*Janthinobacterium agaricidamnosum* (96.4)
WSWD	LW / Day 0	GNR	4	*Janthinobacterium lividum* (98.5)
BWS	LW / Day 21	GNR	22	*Acinetobacter calcoaceticus* (99.6)
CCBBD	LW / Day 21	GNR	22	*Pseudomonas fluorescens* (97.8)
CCBBL	LW / Day 21	GNR	22	*Acinetobacter calcoaceticus* (99.6)
OI	LW / Day 21	GNR	22	*Acinetobacter haemolyticus* (96.3)
OWI	LW / Day 21	GNC	22	*Acinetobacter calcoaceticus* (99.6)
WOB	LW / Day 21	GNC	22	*Acinetobacter calcoaceticus* (99.2)
WS	LW / Day 21	GNC	22	*Acinetobacter junii* (99.4)
WTH	LW / Day 21	GNC	22	*Acinetobacter calcoaceticus* (99.2)

maintained active cultures by adding a continuous carbon source (diesel fuel) and removal of any potential toxic end-products.

The initial increase in heterotrophic bacteria in both the control as well as experimental bioreactors is probably a function of growth from the availability of latent nutrients caused by the aeration and mixing of the sediments. After day four however, only the bioreactor supplemented with diesel fuel resulted in a significant increase in both heterotrophic and diesel fuel-utilizing bacteria in sediments from both sources (Figures 1 & 2). The increasing biomass for the diesel-fuel bioreactors compared to the control bioreactors after day four would indicate that carbon from the diesel fuel is being assimilated into the system (Figures 1 & 2).

Our enrichment results are consistent with similar studies in which petroleum-degrading bacteria were obtained from the environment and enriched by exposure to a particular petroleum compound [20-24]. These studies, however, sought petroleum-degrading bacteria in polluted soils or sediments; the authors did not consider ecosystems with minimal pre-exposure to petroleum products. Other studies did the same with soil and water samples

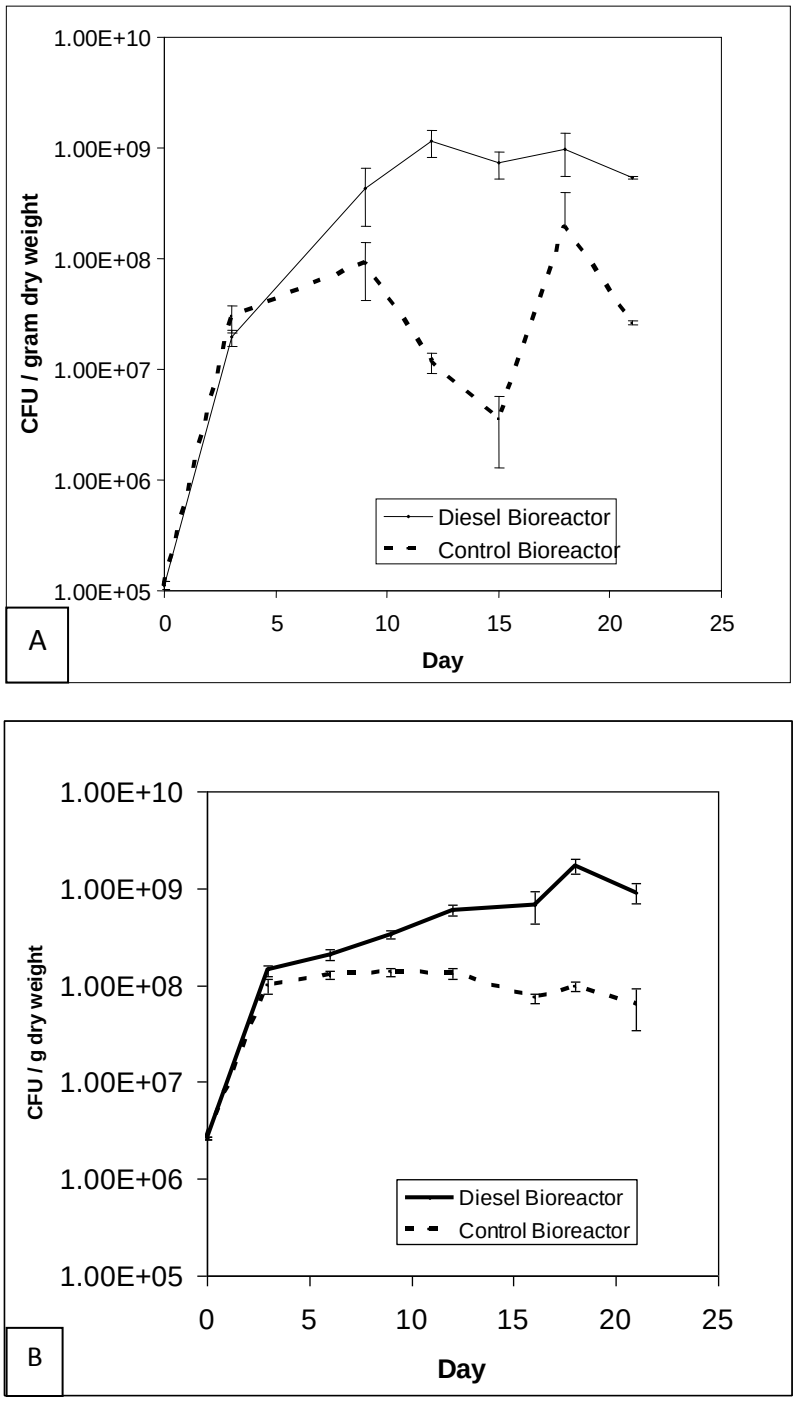

Figure 1. Bacteria from Spring Lake sediments. (A) Diesel fuel-utilizing bacteria from the diesel fuel enriched bioreactor and control bioreactor enumerated using diesel AOA plating. (B) General heterotrophic bacteria from the diesel fuel enriched bioreactor and control bioreactor enumerated using TSA plates.

(polluted only), but neither carried out experiments to maximize the numbers of such organisms [25, 26]. No studies have examined the effect of diesel fuel on the microbial composition of fresh water sediments that have not experienced pre-exposure to any type aliphatic hydrocarbons. Furthermore, the abovementioned studies showed the natural

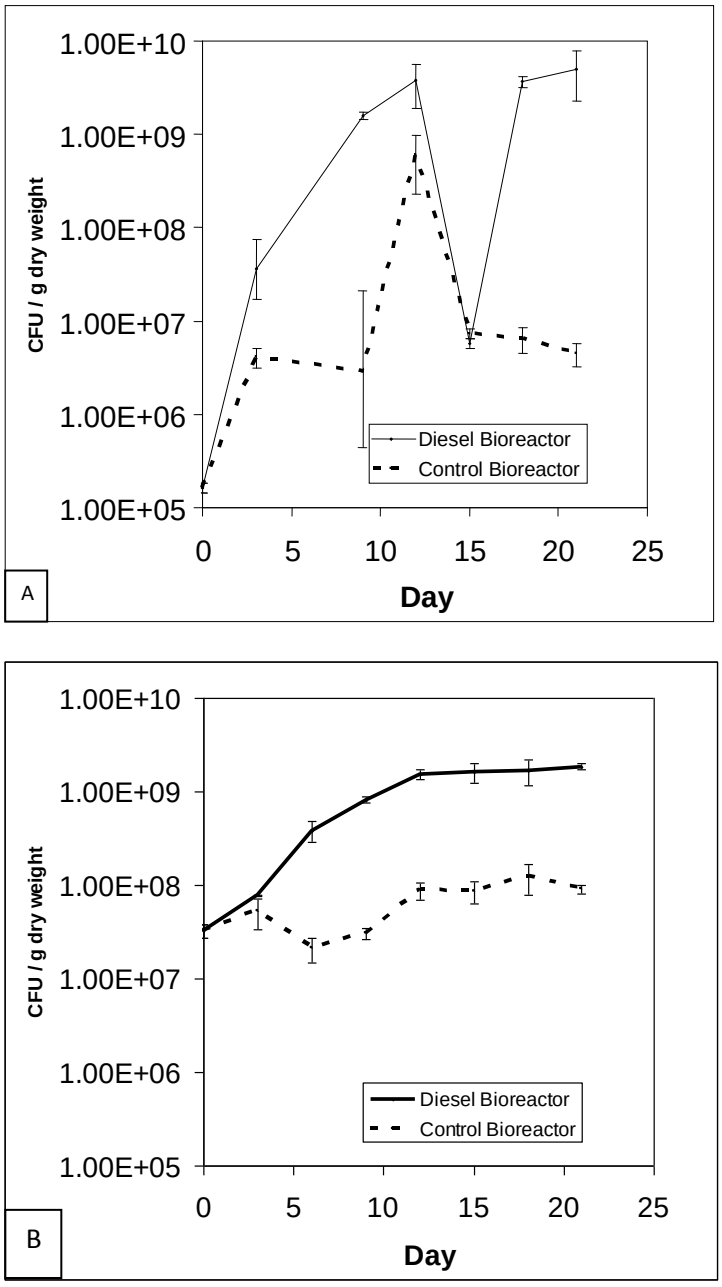

Figure 2. Bacteria from Lake Wolverine sediments. (A) Diesel fuel-utilizing bacteria from the diesel fuel enriched bioreactor and control bioreactor enumerated using diesel AOA plates. (B) General heterotrophic bacteria from the diesel fuel enriched bioreactor and control bioreactor enumerated using TSA plates.

responses of bacterial populations to exposure to such compounds, while ours aimed to determine the yield of cells under controlled, optimum conditions.

There are many experimental approaches that have been used to enrich for microorganisms from aquatic sediments that are able to metabolize environmental contaminants. Our technique possesses many advantages over these historical enrichment protocols. Many techniques involve adding sediments to closed systems (usually in a sealed flask) and rely on the headspace as the source of oxygen [27, 28].

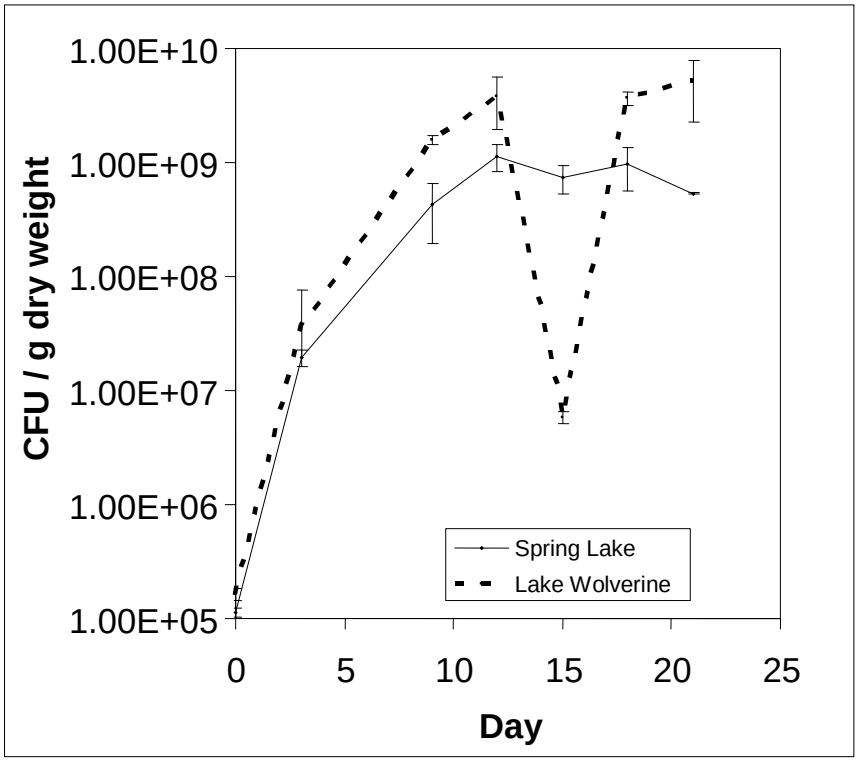

Figure 3. Comparison of the number of diesel fuel-utilizing bacteria between Spring Lake and Lake Wolverine sediments. The samples were enumerated using diesel AOA plates.

A closed system can result in a decrease of available oxygen as it is used by the growing culture; our system maintains constant saturation of oxygen in the liquid making it readily available to the growing cultures. Another common enrichment approach is to supplement the enrichment culture with a variety of minimal salts or trace metals solutions, which has the potential to change the natural composition of the medium provided by the sediments themselves [29-31]. Our system simply relies on the addition of ammonium phosphate to provide a source of nitrogen and phosphorous to the growing cultures. Lastly, the incubation time can vary widely, lasting from as short as a few weeks to as long as several months [29, 32, 33, 34]. Associated with incubation time is the subculturing approach, were some techniques seek to isolate pure cultures or to simply enhance the abundance of a microbial consortium [27, 28, 32, 35]. Our approach utilizes a very short incubation time with the goal of enhancing the concentration of naturally occurring microbiota, whether they are part of a consortium or a single species involved in the bioprocessing of the target compound.

While we did not find any novel taxa during our enrichment process, representatives of well-described bacteria found to be associated with petroleum metabolism were abundant in the inoculates. Based on the diverse metabolic properties of the four genera we did identify, it is not surprising that they were represented in our enrichment cultures. There have been many previous studies identifying these genera as participating in the biodegradation of hydrocarbons from soil, marine, and aquatic environments [21, 23, 27, 28, 29]. Such as, a

study that identified 130 isolates from a gasoline and diesel fuel station soil samples to the genus level, showed 16 of those to be of the Pseudomonas genera [23]. VanHamme et al. [24] frequently recovered *Pseudomonas sp.* and infrequently recovered *Acinetobacter sp.* from their soil samples, but never observed *Acidovorax sp.* nor *Janthinobacterium sp.* In our study, all of the organisms that were identified by PCR amplification and DNA sequence analysis were originally subcultured from AOA plates, and were capable of growth on AOA and TSA. None grew on AOA plates that lacked diesel fuel, and none grew on AOA only. To perform a more thorough ecological evaluation of the taxa present in these lake sediments, as the culturable bacteria represent only a small fraction of the total microbial population in the bioreactors, a more inclusive method, such as DGGE-PCR or PCR amplification and 454 sequence analysis of 16S rDNA could be used.

In conclusion, the results of the present study support the hypothesis that diesel fuel- utilizing bacteria are ubiquitous in freshwater sediments regardless of the level of prior exposure to anthropogenic petroleum products. Furthermore, these sediments can be easily and quickly enriched for petroleum-utilizing microbiota by aeration and addition of ammonium phosphate as the mineral source and diesel fuel as the hydrocarbon source. The development of this bench-top technique will aid in both the search for novel organisms capable of bio-processing petroleum hydrocarbons as well as provide the foundation to studies that wish to better understand the natural bioprocessing ability of indigenous microorganisms. Applications for this enrichment technique extend beyond the use of petroleum hydrocarbons as the enrichment additive and could be applied to studies that involve any environmental contaminate that can be used as a carbon source. We are currently in the process of applying this technique to the study of other anthropogenic sources of carbon in aquatic environments. The initial results of an enrichment assay using caffeine as the sole source of carbon are consistent with the findings presented above (A. Gibson et. al., unpublished).

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