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**DEVELOPMENT OF LUMINESCENT BACTERIA AS TRACERS FOR
GEOLOGICAL RESERVOIR CHARACTERIZATION**

FINAL REPORT

By
Jeannette W. King

October 1991

Performed Under Contract No. DE-AC22-90BC14666

Fairleigh Dickinson Laboratory
Abilene, Texas



**National Petroleum Technology Office
U.S. DEPARTMENT OF ENERGY
Tulsa, Oklahoma**

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Work Performed Under Contract No. AC22-90BC14666

Prepared for
U.S. Department of Energy
Assistant Secretary for Fossil Energy

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CONTENTS

LIST OF TABLES & FIGURES	iv
Foreward	v
Acknowledgements	vi
Abstract	vii
Executive Summary	ix
TECHNICAL DISCUSSION	1
Introduction	1
Background	2
Project Objectives	3
Technical Approach	4
Conclusions	4
Recommendations	5
TECHNICAL PROCEDURES	5
Background	5
Collection, Isolation and Optimization of Luminescent Bacteria	7
<u>Techniques for Measuring Growth</u>	8
<u>Luminescent Measurement Procedures</u>	9
<u>Culture Studies</u>	10
<u>Cultures Collected from Marine Environments</u>	11
Acclimation to Formation Waters	15
Transport of Luminescent Bacteria through Reservoir Cores	19
REFERENCES	24
BIBLIOGRAPHY	30
APPENDICES	
APPENDIX A	32
Quantitative Determinations of Microbial Population Density	
APPENDIX B	34
Spectrophotometer Readings of Bioluminescent Intensity	
Standard Curve Data	
Spectrophotometer Readings of Concentration of <u>Vibrio fischeri</u>	
APPENDIX C	38
Growth Media	

LIST OF TABLES AND FIGURES

TABLES

TABLE I.	QUALITATIVE COMPARISON OF GROWTH AND LUMINESCENCE IN PHOTOBACTERIUM AND MARINE MEDIA	12
TABLE II.	CULTIVATION OF BIOLUMINESCING BACTERIA FROM GULF OF MEXICO	13
TABLE III.	LUMINESCING COLONIES FROM MF	14
TABLE IV.	CHEMICAL COMPOSITION OF PRODUCTION WATER	16
TABLE V.	PROTOCOL FOR FORMATION WATER TESTING	17
TABLE VI.	RESULTS FROM SERIAL DILUTION TESTS	18
TABLE VII.	CARBONATE CORE ANALYSIS RESULTS	21

FIGURES

Figure 1.	Bioluminescent Bacteria (400x) in Total Darkness - Time Exposure 5 minutes	6
Figure 2.	Bioluminescent Bacteria (400x) in Total Darkness - Time Exposure 20 minutes	7
Figure 3.	Effect of Aeration on Luminosity	9
Figure 4.	Bottle Apparatus for Testing Bacterial Transport Through Cores	20
Figure 5.	Double Syringe Apparatus for Testing Bacterial Transport Through Cores	22

Foreward

The need for oil independence in this country cannot be underestimated. Therefore, the U. S. Department of Energy's role in bringing cost effective, advanced technologies to the industry is most appropriate. Their far-sightedness in promoting innovative oil recovery research while supporting field projects is acknowledged and greatly appreciated. A biological tracer for reservoir characterization is a novel technique which could be adapted to the field and applied by small oil companies defining reservoir characteristics to improve oil production.

Recent projections of rising oil imports only confirm that our economy is not shifting fast enough to energy sources other than oil and gas, and we are increasing our dependence on unreliable sources when we fail to recover our own vast reserves.

The bioluminescent tracer investigated in this project could make a significant contribution to reservoir characterization and also environmental modeling.

The opinions, observations, conclusions and recommendations herein expressed are those of the author and not the Department of Energy.

Acknowledgements

The support of the Department of Energy for the development of the bioluminescent tracer has been acknowledged. We also appreciate Parul Patel, who conducted many of the laboratory tests; Kristi King, who was responsible for the final report; Luther Winans, who helped set up the photomicrographic equipment, and our colleagues at Fairleigh Dickinson Laboratories, Inc. who encouraged and assisted us. Special thanks is due to Ginny King and Ronda Hyatt, who observed bioluminescent bacteria while studying shifting sands at Padre Island and inspired me to study their applications for advanced technologies.

Abstract

This report describes work accomplished to satisfy a feasibility study for the Department of Energy under Contract No. De-AC22-BC14666.

Bioluminescent cultures were acquired and tested for use as biological tracers for reservoir characterization by small independent oil companies. Initially these bacterial cultures were fastidious to work with, but when we finally determined their critical growth parameters simple test variations were developed that could be routinely accomplished. The intensity of their luminescence is easily distinguished by the human eye and requires no sophisticated technical knowledge or instrumentation.

Cultures were received from culture banks and collected from marine environments. In our laboratory they were screened using the criteria of optimum growth and luminescence. Three stock cultures proved to grow profusely even when variations were made in nutrient additions, salts, and temperature. These three selected cultures were not inhibited when introduced to formations and formation waters and were not overgrown by other bacteria. Cultures isolated from the Gulf of Mexico were overgrown by indigenous bacteria and therefore, they were eliminated from further screening and adaptation.

Experiments were performed according to three major task descriptions:

1. Establish growth and luminescing limitations of selected bacteria in various media, varying salt concentration and temperature.
2. Adapt cultures to formation waters.
3. Determine transport limitations of bioluminescent bacteria through representative reservoir cores.

Experiments in formation waters proved that when bioluminescing cultures were introduced to formation water, their growth exhibited a slight lag but was not inhibited; luminescence was not affected.

Berea sandstone and carbonate core tests established that these bacteria could be easily transported through cores and did no lose their luminescing capability.

Our research concludes that bioluminescent bacteria have potential as geological tracers because:

1. they adapt easily to oil reservoirs, in that
 - a. they thrive in brine conditions,
 - b. they live and reproduce without oxygen and
 - c. they move freely through sandstone and carbonate cores;
2. their luminescence is easily seen and not disputable.

Bioluminescent tracers would make remarkable candidates for further energy development applications and environmental monitoring.

Executive Summary

Developing techniques for improving our domestic oil reserves will play a key role in maintaining energy independence for the United States. Stripper well operators who produce more than fifteen percent of our oil are most susceptible to price fluctuation and the least capable of applying advanced technologies. Their time and technological resources are limited to local availability.

The Department of Energy has focused on reducing oil well abandonments by establishing a better understanding of oil reservoirs and their recoverability. This will include the characterization of reservoir rock, fluid and fluid movement. Such information will then be transferred to oil producers who can use it cost-effectively to increase their oil production.

We have investigated bioluminescent tracers to be used to profile well bore formations and well-to-well communication. This tracer can be cost-effectively applied as a tool for reservoir formation evaluation. It grows well in reservoir conditions, moves through sandstone and carbonate cores and is easily detected.

Transferring this technology to independent operators fits into the DOE's goal of reducing well abandonment by improving reservoir characterization and identifying economical production options.

TECHNICAL DISCUSSION

Introduction

This project was undertaken as a feasibility study to determine the potential for applying bioluminescent bacteria as geological tracers to be used in oil exploration and production. The need for an improved understanding of the geological characteristics of stripper wells has encouraged the investigation of applying new techniques which could be used by small independent oil companies. Independent operators depend heavily on available geological information for exploration, drilling and applying improved oil recovery techniques. Critical technological and economic decisions demand the accuracy of such information. Isopach maps, which are relied on heavily, are based on logging information from individual wells while the communication between wells is frequently speculative. Tracers are used to indicate formation characteristics and also to track underground fluid movement. Chemical and radioactive tracers are expensive, and require sophisticated application and interpretation, and very often initiate environmental concerns. Biological tracers are available and used in medicine and environmental testing, but have not been proven as useful in geological reservoirs or for that matter in the oil industry at all.

Biotechnological developments during the last few decades have established cost-effective processes in many industrial areas formerly dominated by chemical industries. Antibiotics, microbial enzymes and cancer treatments as well as the use of microorganisms to produce energy and chemical products have been demonstrated as more economical and environmentally safe industrial processes. Microorganisms can be inexpensive to produce and transport.

Bioluminescent reactions have been the source of rapid and routine detection and inumeration procedures in the laboratory. They have demonstrated sensitivity, rapidity, accuracy, and reproducibility (Chappelle, 1975). The ultimate objective of developing these bioluminescent reactions as biological tracers for field geoscience application is to reduce the time and expense included in geological characterization, and to promote oil recovery activities in regions where advanced technologies have been previously unaffordable.

Bioluminescent bacteria fulfill the requirements as tracers for oil field characterization. These bacteria have been established as candidates for geological tests because:

1. They have been adapted to oil reservoirs.
 - a. They thrive in the formation brines tested.
 - b. They can live and reproduce where no oxygen is available.
 - c. They do not lose their luminescent capabilities in reservoir conditions.
 - d. They move freely through reservoir cores.
2. Their luminescence is easily seen and not disputable requiring no instrumentation for detection.

Background

The Department of Energy in its policy statement of October 1988 stated that:

DOE's future EOR research will focus on developing a better understanding of the anatomy of a reservoir - its composition, fracture patterns and fluid movement. . . . And, new, novel recovery techniques, such as microbial EOR . . . will be explored for potential applications to stripper wells.

United States Department of Energy
October 1988

Current low oil prices discourage investment in oil and gas exploration and development. Therefore, our research efforts must be directed toward keeping those wells producing which have not been shut-in and increasing production where primary recovery production has been exhausted.

Stripper wells in the United States contribute more than fifteen percent of all U.S. production. Although this amount may seem low, small independent oil producers who operate these stripper wells represent the backbone of the American oil industry in the same way farmers are the backbone of American agriculture. Small independent oil companies are the most susceptible to price fluctuation and the least capable of applying advanced technologies. The loss of stripper production contributes to unemployment and increases our dependence on foreign oil. Cost-effective

improved oil recovery techniques that can be applied by independent operators in our mature oil fields will only help keep us more energy independent.

In order to produce additional oil, a better understanding of the reservoir, the geology and the fluids must be available to operators. They need to know where the most productive zones are and how best to recover that oil. Well logs (mud logs, open-hole and cased hole wireline logs, computer-generated logs, measurement-while-drilling-logs, etc.) can be used to identify producing zones, wet zones and highs and lows, but additional information about communication between wells would tell operators if channeling is occurring and where EOR could be most effectively applied.

A bioluminescent tracer could be applied which would profile well bore formation and well-to-well communication information. This cost-effective tracer could be easily implemented by stripper well operators to keep old wells producing and increase oil production in areas determined to have large reserves.

Project Objectives

This project was initiated as a feasibility study to determine the potential of bioluminescent bacteria for application as geological tracers that could be used by small independent oil companies.

The objectives of this project have been met and accomplished and include:

1. accumulation of bioluminescent bacteria and information relating to growth and luminescent investigations,
2. adaption of bioluminescent bacteria to formation waters, and
3. transportation of bioluminescent bacteria through representative cores.

Some variations to the originally proposed task descriptions were made in the best interest of the goal objectives and are appropriately indicated. The experimental data have been summarized and are reported in this document.

Technical Approach

An extensive amount of literature relating to oil recovery and bioluminescence was accumulated. This literature included bioluminescent phenomena such as inhibition, induction, adaptation and monitoring. The information directly used in this project is listed in the REFERENCE section. The BIBLIOGRAPHY includes additional informative articles.

Luminescent bacteria were collected from marine environments at night when it was overcast, so luminescence could be more easily observed. The literature had indicated that these bacteria should be removed from the gut of marine fish and tiny invertebrates, but so many luminescing bacteria were available that collecting larger animals proved unnecessary. They were also acquired from culture banks.

All bioluminescing bacteria were screened according to nutrients, salt tolerance, pH and temperature. Those selected for best growth and luminescence were subjected to experiments using formation waters and then for transport through Berea sandstone and carbonate cores.

Conclusions

Bioluminescent bacteria grow well in the formation brine where no oxygen is present - in other words in typical oil field reservoirs. The presence of oxygen was determined to be necessary for luminescence, but not for growth (Johnson, 1947). This phenomenon has been corroborated in the published literature (Shoup, 1929).

The reaction itself includes the mixed function oxidation of reduced flavin mononucleotide and long-chain aldehyde by molecular oxygen with the aid of the bacterial luciferase catalyst. The system can be repressed and induced (Nealson, 1970; Nealson, 1972; Watanabe, 1972; Coffey, 1967; McElroy, 1947). The details of regulation should be studied as a possible complement or alternative to other tracing and monitoring schemes. Applications in other areas of the oil industry might prove to be more accurate and inexpensive than currently used techniques.

Contrary to our original predictions, cultures acquired from culture banks adapted more readily and completely to formation waters than did those collected from marine environments. One particular culture, Vibrio fischeri, exhibited growth and dramatic luminescence even after many

transfers.

Transportation of bioluminescent bacteria through Berea sandstone and carbonate cores was almost immediate, even without applying external pressure.

Bioluminescent bacteria grow in geological reservoir conditions and maintain their capability to luminescence. They move freely through sandstone and carbonate formations. The data accumulated during this project establishes the laboratory parameters for using bioluminescent bacteria as geological tracers. In addition to enhanced oil recovery these natural bioluminescent tracers have great potential for application in the environmental monitoring of natural resource and fossil fuel development and clean-up.

Recommendations

The mechanisms of bioluminescence are well documented (Hastings, 1968; Nealson, 1979; Makemson, 1973). Now that the growth and luminescence of these bacteria in oil field reservoir conditions have been established, their extended application to energy and environmental developments is essential.

Adaptation to higher temperatures and the limitations of other parameters while maintaining visible luminescence will be a significant accomplishment.

The data accumulated in this project should be used to develop a commercial test product which oil producers could use as tracers. The scale-up and field applications of bioluminescent monitoring will determine its limitations and regulation. This system could provide an excellent source of information for completing geological reservoir characterizations and for monitoring oil degradation in marine environments.

TECHNICAL PROCEDURES

Background

Many researchers have demonstrated that luminescent reactions can be used for in vitro monitoring procedures in

the laboratory (Chappelle, 1968). The total amount of light emitted during the reaction is a function of the appropriate concentrations and reactions of luciferase, the enzyme which catalyzes the reaction; luciferin, the reduced compound which is oxidized in the appropriate environment to produce fluorescence; ATP (adenosine triphosphate) which is present as the principle carrier of chemical energy in the cells of all life forms, and oxygen. The glowing light is produced as the final product of the chemical conversion of the luciferin to oxyluciferin and light, catalyzed by luciferase and driven by the dephosphorylation of ATP (Eymers, 1947; McElroy, 1969). Because of this unique reaction, bioluminescent tests have been developed as automated testing methods in diagnostic medicine (Gauthier, 1979). The light generated can be measured and is directly proportional to the amount of ATP present. Intermediates in this reaction have been isolated and purified (Balny, 1975). Variations of this monitoring scheme have not been developed for industrial applications, but have great potential.

Photographs taken in our laboratory of the bioluminescent bacteria are shown as Figures 1 and 2. The pictures were taken at night in total darkness with a black velvet drape over the microscope and camera. We used a Nikon photomicrographic attachment, Microflex UFX, fitted to a Nikon Microscope. A small cut of Photobacterium Agar with luminescing colonies (24hr old) was placed on a glass slide on the microscope stage. Pictures were taken with 400X magnification using 3200 speed 35mm film. The only light was that given off by the bacteria. The picture in Figure 1 was a time exposure for 5 minutes; the picture in Figure 2 was exposed for 20 minutes.

Figure 1

Bioluminescent Bacteria (400x) in Total Darkness -
Time Exposure 5 minutes

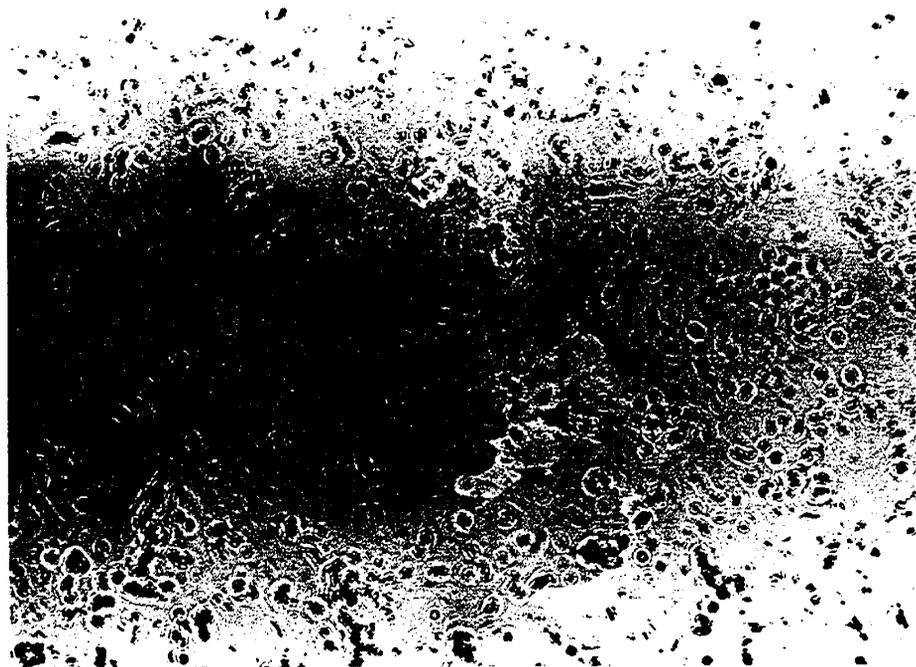
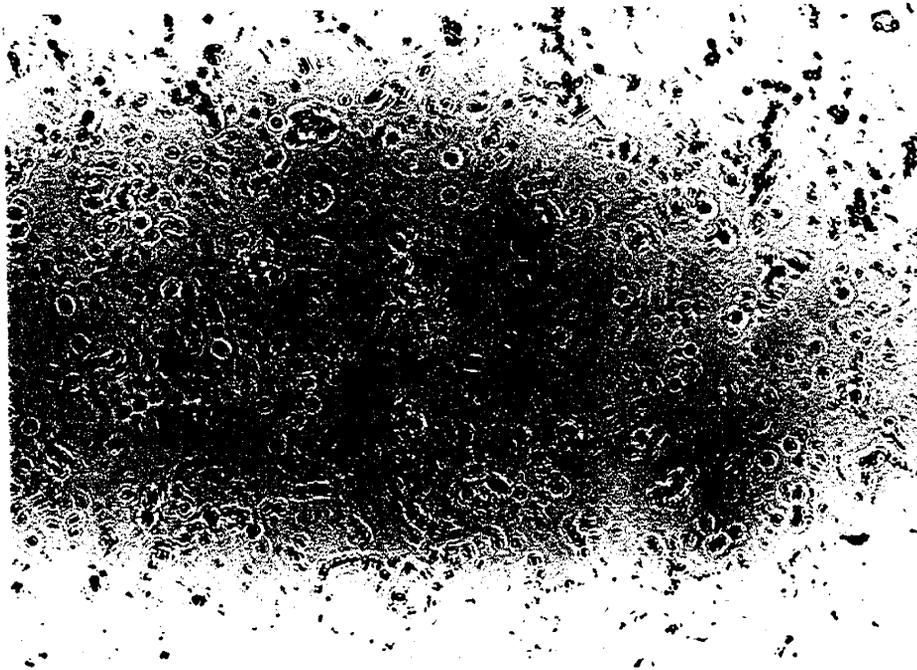


Figure 2

Bioluminescent Bacteria (400x) in Total Darkness -
Time Exposure 20 minutes



Collection, Isolation and Optimization of Luminescent Bacteria

Luminous bacteria are ubiquitous, but indicate characteristic geographic and seasonal distribution patterns (Shilo, 1979). Changes in temperature, nutrients, salt and oxygen concentration determine growth and luminescent variations. They are versatile heterotrophs and have been isolated from the seawater of tropical, temperate and polar regions and from the surface to several thousand meters deep (Ruby, 1980). Usually associated with living and nonliving sources of organic matter, they derive nitrogen from protein or its degradation products rather than inorganic sources (Harvey, 1952; Hendrie, 1971; Baumann, 1979). One species,

Vibrio fischeri, has been reported to grow using nitrate as the sole nitrogen source (Coffey, 1967). Many live in symbiotic associations with marine animals, which are their source of nitrogen.

Luminescing bacteria are facultative anaerobes (Hastings, 1975). Although all strains are reported to grow without oxygen (Hendrie, 1970), only one species grows in salt free broth and luminescence occurs only when oxygen is present. Free oxygen can be introduced into the medium (and very brilliant cultures are obtained) or aeration by agitation can be used (Barnard, 1902). Nonluminescent cultures of luminous bacteria grow in the absence of oxygen; however, they regain their luminescence when oxygen is reintroduced (McElroy, 1948). Most abundant growth occurs in sea water or when the medium contains 3% sodium chloride (Oginsky, 1959). The sodium chloride concentration may be varied from 2 to 5% without altering the kinetics of light production (Kempner, 1968). Light does not develop in appreciable amounts unless the concentration of salt approximates that required.

Most species grow optimally between 17°C and 25°C. Beijerinck (Harvey, 1948) studied luminescent bacteria as early as 1889 and continued reporting on them through the first quarter of this century. He confirmed that for some Photobacteria luminescence was most intense at 23-25°C, whereas growth peaked at 29-30°C. Farghaly reported growth ranges from 10-40°C (Farghaly, 1950). Earlier investigations which found thermal inactivation to be reversible (Brown, 1942) have been confirmed in our laboratory. Our tests confirm that luminescence ceased at 5°C, but reoccured at approximately 15°C and gained intensity as the temperature rose until 37°C when luminescence began to diminish.

Techniques for Measuring Growth

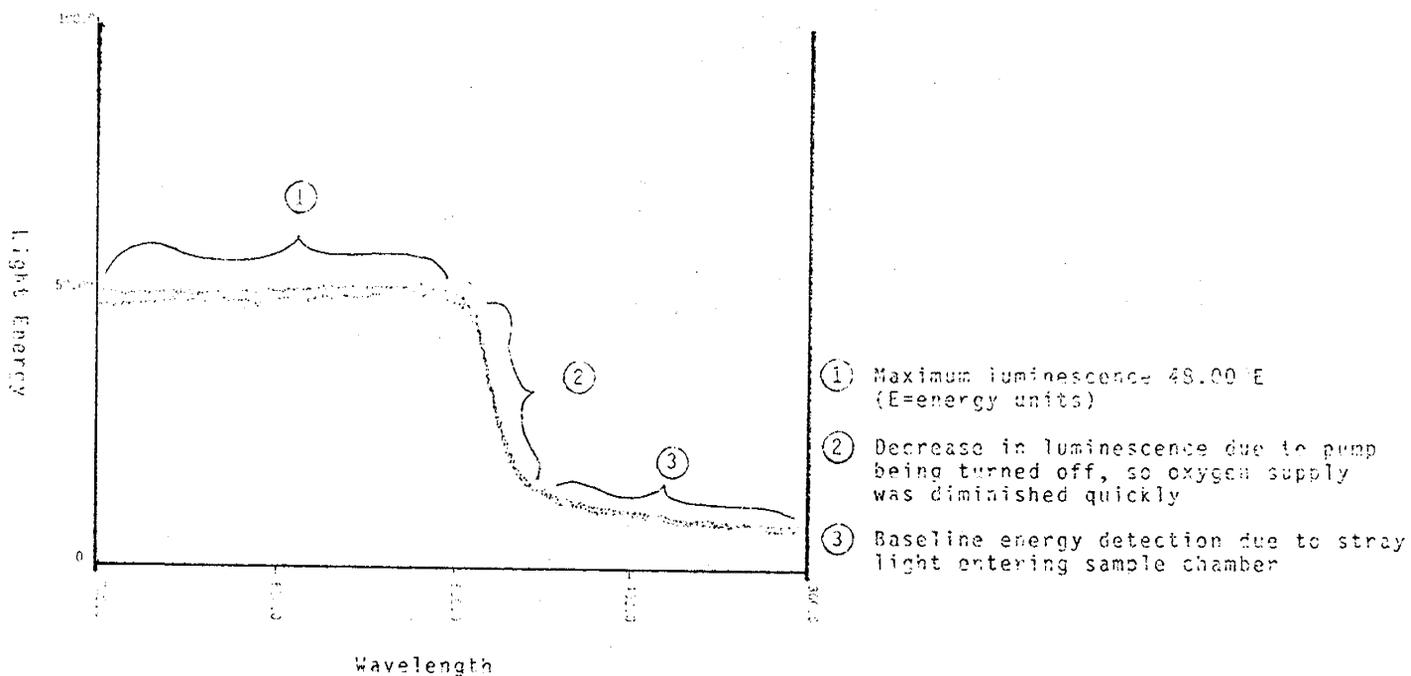
Many different approaches to measuring the growth and luminescence are available. Initially growth population density changes were measured using the Millipore Filter Techniques and Standard Plate Count Method (see Appendix A). Later Optical Density Measurements and the Soft Agar Method were used (see Appendix A) because of their simplicity. Luminescence was monitored qualitatively. During culture screening the qualitative determinations, either negative or positive, were observed by the naked eye. In the adaption to formation water studies quantitative measurements were made by using the Millipore Filter technique.

Luminescent Measurement Procedure

Various kinds of photomultiplier photometers have been used for measuring light intensity of bioluminescence (Mitchell, 1970; Hasting, 1965; Hastings 1971). Quantitative determinations of luminescent intensity were made using the Shimadzu Spectrophotometer (see Appendix B).

Sterile Pb broth and M broth (see Appendix C) were used as blanks depending on selected growth medium. When bioluminescence was being detected, a black strip of tape was placed over the deuterium lamp source in the instrument, so that only bacterial luminescence was detected. Time did not permit us to test sample variations. Aerating the sample Vibrio fischeri 7744 in Pb broth gave 50 Energy units at 700nm; whereas when aeration stopped the luminosity dropped to 5 Energy units and 300nm (see Figure 3).

Figure 3
Effect of Aeration on Luminosity



Culture Studies

Three standard cultures were selected from the literature and ordered from the catalogue of the American Type Culture Collection in Rockville, Maryland because of their reported growth and luminescence adaption to a wide range of temperatures and nutrient concentrations. Photobacterium phosphoreum has been the subject of bioluminescent investigation since 1878 (Breed, 1957). It is identified as being coccobaccili, Gram negative, isolated from such variable conditions as the Baltic Sea and the West Indies. Its bluish green luminescence is easily distinguished and has been reported to give off enough light to read the dial of a watch in a totally dark room (Forsyth, 1910). In an extensive discussion by Harvey visibility by the human eye varies depending on wave lengths. Luminous bacteria emit light only in the visible range (Harvey, 1952). Phosphorescence (the bluish-green bioluminescence) and good growth require salt solutions equivalent to a 3 % sodium chloride solution. Therefore, this culture was also considered as a reasonable candidate for adaption to formation water.

Photobacterium fischeri was named for Professor Bernhard Fischer, one of the earliest students of luminescent bacteria (Breed, 1957). It is now classified as Vibrio fischeri because the short cells are motile, grow optimally between 25° and 32°C and retain luminescence ten days to two weeks with optimal temperature for luminescence 20°-40°C.

Achromobacter fischeri peculiarly means without color, grows optimally at 25-28°C, and is mobile by means of peritrichous flagella. Recently it has been reclassified as Vibrio fischeri.

Several months of testing various media (see Appendix) formulations and temperatures were required for luminescence to develop and be retained for any period of time. The critical condition was finally determined to be simply boiling the liquid media for at least two minutes before autoclaving and/or dispensing. Photobacterium medium was the only agar out of all the broths and agars tested which maintained intense luminescence for more than five days.

Although published papers reported that Photobacterium phosphoreum has been the culture of choice for laboratory experiments, the culture received by us from ATCC gave poor luminescence and optimum growth population of only 1.1×10^5 cfu/ml in Photobacterium broth and no luminescence in

Marine broth. Population densities were monitored by MF (see Appendix A). Both incubations were at 26°C with shaking for aeration. Vibrio fischeri, ATCC #7744, which had previously been named Achromabacter fischeri and Vibrio fischeri, ATCC #25918, were acquired and tested in standard media, Photobacterium broth and Marine broth (see Appendix C and TABLE I).

Cultures received in the laboratory had been freeze-dried and stored as pellets in double glass vials. Aseptically each vial was opened, the inner vial was removed and the pellet dropped into 0.5ml sterile broth and gently mixed with sterile pipette with which 0.1ml was removed on to agar slant. Incubation was stationary at room temperature (app. 26°C). After 18 hours, growth and luminescence were checked. At 24 hours, transfers were aseptically made into 10ml of broth in a 50ml shake flask and incubation was continued (with shaking for aeration in shake flasks). Growth and luminescence continued to be monitored (see TABLE I).

Refrigerating broth cultures inhibited luminescence, which was redeveloped after returning to 17°C or above. Broth cultures were stored at room temperature for more than one year during which time luminescence diminished, but was reactivated after transferring a small aliquot (usually 1%) into fresh medium and incubating 8-12 hours.

Cultures Collected from Marine Environments

Four collection locations were selected on the Gulf of Mexico where bioluminescent bacteria had been observed:

1. Port Aransas
2. Malaquite Beach
3. Rockport Bay, and
4. Bob Hall Pier.

Water samples were collected in one liter polyethylene containers and stored at 30°C until they reached the laboratory, because that was the temperature of the water where sampling was done. A loopful of Gulf water was streaked on to Petri dishes of Marine Agar and Photobacterium Agar. A 0.2ml sample was placed on each of two agar plates and swirled for even distribution. These inoculated Petri plates were incubated at 30°C.

After returning to the laboratory the plates were observed for luminescent bacteria. Luminescent colonies

TABLE I

QUALITATIVE COMPARISON OF GROWTH AND LUMINESCENCE
IN PHOTOBACTERIUM AND MARINE MEDIA

Growth Observations

Incubation Time	<u>Vibrio fischeri</u> 7744				<u>Vibrio fischeri</u> 25918				
	M		Pb		M		Pb		
	broth	agar	broth	agar		broth	agar	broth	agar
18 hrs	+	+	+	+		++	+	++	-
36 hrs	++	++	++	++		++	++	++	++
5 da	++++	++++	+++	+++		+++	++	+++	+++
1% transferred to fresh media									
24hrs	++	++	+	++		+	-	++	-
48hrs	+++	+++	+++	+++		+++	+++	+++	+++
Observations of Luminescence									
18 hrs	-	+	-	-		-	-	-	-
36 hrs	+	+	++	++		-	-	-	-
5 da	+	sl	++++	++++		-	-	-	-
10% transferred to fresh media									
24 hrs	+++	+++	-	++		-	-	-	-
48 hrs	-	sl	+	+++		-	-	-	-

- = no growth or luminescence
 + = some growth or luminescence
 ++ = moderate amount or growth or luminescence
 +++ = abundant growth or luminescence
 ++++ = maximum amount of growth or luminescence

were picked and transferred to the appropriate agar and incubated at 25°C using sterile techniques. This process was continued daily until all luminescent colonies were isolated. Isolated colonies were transferred to appropriate agar slants for storage and later testing (see TABLE II).

TABLE II
CULTIVATION OF BIOLUMINESCING BACTERIA
FROM THE GULF OF MEXICO

Sampling Location	Incubation Time			
	24 hrs		48 hrs	
	Pb agar	M agar	Pb agar	M agar
Port Aransas	++++	++++	++++	++++
Malachite Beach	++++ l	++++ l	++++ l	++++ l
Rockport Bay	++++	++++	++++	++++
Bob Hall Pier	++++	++++	++++	++++

- = no growth
+ = some growth
++ = moderate growth
+++ = abundant growth
++++ = maximum growth
l = luminescence

Nonluminescent bacterial growth greatly exceeded the growth of luminescent microorganisms. Because luminescing bacteria were observed only in the water samples from Malachite Beach, additional M agar plates were streaked, incubated at 26°C for 24, 48 and 72 hours, and luminescing colonies were picked for isolation. Luminescence lost intensity after 24 hours of incubation.

Observations from Gulf water collections indicated that these luminescent bacteria were inhibited by the growth of nonluminescent bacteria. The luminescent colonies that grew on the Pb and M agar were only observed on the outer edges of the agar plates. No single luminescent colonies grew in the midst of nonluminescing bacterial growth, only when two or more luminescing colonies were together and those had very limited growth time. However, when these luminescing colonies were singly transferred, they showed better growth although luminescence diminished quickly. Additionally, we observed that the luminescing bacteria moved away from the nonluminescing colonies, confirming indeed that they are mobile and microaerophilic or anaerobic.

Gulf water samples were subjected to the Millipore Filtration Technique (MF), as follows:

Each container of gulf water was thoroughly mixed before sampling. The sterilized Millipore filter apparatus was assembled. A 10ml aliquot of each gulf water sample was poured through the Millipore apparatus with a 0.2(μ) filter in place. Following rinsing the filter was removed to a 60mm Petri dish containing M agar. Incubation was at room temperature (26°C) using damp towels to maintain humidity.

Results are shown in TABLE III.

TABLE III

LUMINESCING COLONIES FROM MF

Sampling Location	24 hr Incubation cfu/sample	48 hr Incubation cfu/sample
Port Aransas 1	50	50
Port Aransas 2	0	0
Port Aransas 3	5	0
Port Aransas 4	5	5
Malachite Beach	10	10
Rockport Bay	0	0
Bob Hall Pier 1	0	0
Bob Hall Pier 2	2	0

Luminescent colonies looked like tiny points of light sprinkled around the agar plate. Luminescence was limited to 24 hours (unlike standard cultures which retained luminescence until agar dried and cultures died). Color pigment of luminescent colonies was easily distinguished from nonluminescent colonies even in the light. Attempts to isolate "wild type cultures" from Millipore filters were unsuccessful; colonies appeared to be growing under the filter or inside the filter and could not be picked for isolation transfer.

Luminescence in the Gulf isolated microorganisms diminished after 24 hours. Color pigment could not be differentiated between luminescent and nonluminescent colonies, whereas the color of colonies acquired from the culture bank

could be easily distinguished in the daylight. Photobacteria in a nonluminescing phase are white and opaque, and luminescing colonies are shiny yellow.

Acclimation to Formation Waters

That biological activity has been present in geological formations as much as physical and chemical activity has long been established. The negative aspects are well known to miners and oil field developers. Biogenic activity is a constant reminder as we continue to look for fossil energy resources. Zobell (Kutznetson, 1963) determined that when pressure is increased, the temperature optimum and maximum for development of some microbial species is increased, but luminescent limitations have not yet been determined.

For those reasons it is entirely appropriate to assume that bacteria, particularly those from marine environments, might thrive in geological reservoirs. The challenge, then, was to test the adaption of marine, bioluminescing bacteria to waters collected from geological formations.

The first suggestions regarding using microorganisms to increase oil production were made in 1926 (Kutzentson, 1963). Since that time research in this country and abroad, particularly in eastern Europe, has continued with particular emphasis on microbiological prospecting, microbial enhanced oil recovery and bioremediation of oil wastes. Water containing dissolved salts is a basic criterion for culturing appropriate luminescing microorganisms.

Variations in chemical compositions differ sharply in various underground waters and have a strong influence on the indigenous microflora. However, the predominant hydrogeological elements closely resemble the defined trace elements in marine environments. Therefore, acclimating marine, and specifically luminescent bacteria from marine environments, was attempted with relative ease.

Water associated with oil production has different compositions depending on the source. Some different types of water have been categorized (Collins, 1985) as meteoric, interstitial, connate, diagenetic, formation, juvenile, condensate and seawater. For the purposes of this projection, we collected water which was referred to as formation water by the operator, but might be more accurately defined as production water since it was recovered after drilling.

Several formation water samples were available in our laboratory for this project. Three sources are described as follows:

- 1-Jensen Lease, Jones County, Texas, Lueders West Field, Upper Hope Lime formation,
- 2-Wessels Lease, Runnels County, Texas, Palo Pinto formation,
- 3-Washington County, Oklahoma, Bartlesville Sand formation.

TABLE IV shows the chemical composition of one water sample analyzed in May 1991. This one is from Washington County, Oklahoma and was the most recently collected. (Salinity is measured by the amount of chloride in the water and is usually about 1.8 times the chloride content. For reference, seawater contains about 20,000 mg/l chloride.)

TABLE IV

CHEMICAL COMPOSITION OF THE PRODUCTION WATER

Parameter	mg/l
pH	6.5
P-alkalinity	0
T-alkalinity	100
hardness (CaCO ₃)	18,900
Chlorides	72,550
Sulfates	90
Chlorine Residual Free	0
Chlorine Residual Total	0
Hydroxides	0
Carbonates	0
Bicarbonates	100
Total Dissolved Solids	91,650
Conductivity (um/cm)	137,475

Vibrio fischeri 7744 was selected for formation water testing because it had been established as the most adaptable and intensely luminescent culture. Ten serial dilutions of formation water and standard Pb were prepared from 1%-100% formation water. Quadruplicate tests were made for each.

TABLE V

PROTOCOL FOR FORMATION WATER TESTING

Sample Designation	Photobacterium broth(ml)	Formation Water(ml)
a 1-4	10	0
b 1-4	9	1
c 1-4	8	2
d 1-4	7	3
e 1-4	6	4
f 1-4	5	5
g 1-4	4	6
h 1-4	3	7
i 1-4	2	8
j 1-4	1	9
k 1-4	0	10

Results of the 24 and 48 hour Serial Dilution Test can be seen in TABLE VI.

An overnight culture (vegetative seed) was used as the initial inoculum into each of the four 1% dilutions. After incubating 24 hours at 26°C with shaking a 5%, transfer was made into each flask of the next dilution (four flasks). Two 24 hour flasks were removed. One flask was used for MF plate count, the other was tested on a Pb agar plate. After 48 hours, tests were repeated. This process continued through seven dilutions after which time growth ceased (see TABLE V for description). The inoculum for this experiment were Vibrio fisheri 7744 in samples 1 and 3, and fresh Gulf water in samples 2 and 4.

Serial dilutions of formation waters were made and transfers were done when growth in the lesser dilution was in log phase. Doudoroff had reported that an adaption of the culture involving the selection of strains possessing special capabilities was necessary for the development of cultures upon transfer to a medium of a different composition from that to which the bacteria was accustomed (Doudoroff, 1942). Our results (Table VI) indicated adaptation of luminescent cultures only until forty percent of the new medium was formation water. This experiment was repeated several times with different formation water samples and similar results. However, transfer of cultures from synthetic medium (Photobacterium or Marine broth) directly to formation water showed immediate growth and no need for adaption by serial transfers. Luminescence was

TABLE VI

RESULTS FROM SERIAL DILUTION TEST

24 hr sample

Sample	Broth		24 Inc		48 hr Inc	
designation	growth	lum	growth	lum	growth	lum
a1	+++	+++	++++	++++	++++	++
a2	+	-	+	-	++	-
b1	++	++++	++++	++++	++++	+
b2	+	-	++++	-	++++	-
c1	++	++++	++++	++++	++++	+
c2	+	-	++++	-	++++	-
d1	++	+++	++++	++++	++++	++++
d2	+	-	++++	-	++++	-
e1	+	-	+	-	+	-
e2	+	-	+	-	+	-
f1	-	-	-	-	-	-
f2	-	-	-	-	-	-

48 hr sample

a1	++++	+	++++	++++	++++	++++
a2	-	-	-	-	+++	-
b3	+++	+	++++	++++	++++	++++
b4	+++	-	+++	-	+++	-
c3	++++	+	++++	++++	++++	++++
c4	++++	-	++++	-	++++	-
d3	-	-	-	-	-	-
d4	++++	-	++++	-	++++	-
e3	-	-	-	-	-	-
e4	-	-	-	-	-	-

Note: When no growth or luminescence occurred, the next transfer was not made.

apparent immediately and population densities showed a lag, but then resumed the same population density as in the synthetic medium. When serial dilutions were made, a precipitate became apparent as the concentrations of formation water were increased. Apparently this increased amount of precipitate restricted the growth of the bioluminescent bacteria, whereas, when a small inoculum (10% of the mature culture) was added directly to the formation water, the amount of precipitate was so insignificant it did not inhibit the growth of the luminescent bacteria.

From these tests we concluded that serial dilution adaption did not develop acclimation to formation waters. However, the immediate transfer of a 10% inoculum (10^8 col/ml) into formation water did not prohibit growth or luminescence. It was not within the context of this project to chemically analyze the precipitate which formed during serial dilution transfers, but the increase of that precipitate caused bacterial growth to diminish, whereas when the transfer was immediately made into formation water no precipitate formed and the bacteria thrived.

Transport of Luminescent Bacteria through Reservoir Cores

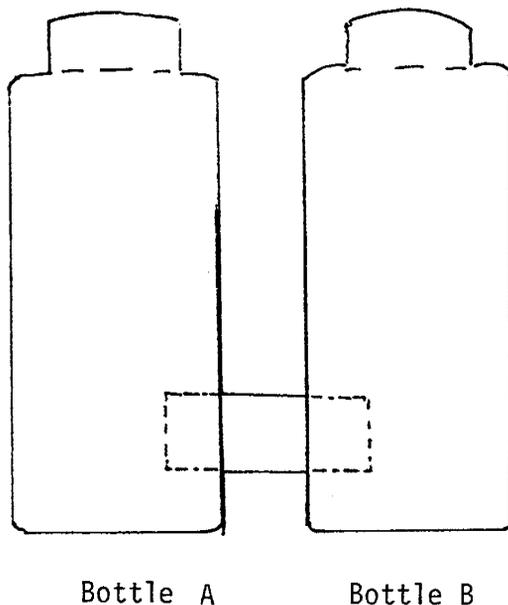
Different objectives exist between those researchers promoting microbial stimulation of oil bearing reservoirs (Yen, 1986) and those using microorganisms for plugging (Updegraff, 1987 and Knapp, 1984). The critical issue is the characterization of each formation and the role of the microbes in recovering oil. Where microbial transport is the objective, injection and penetration have been possible due to the appropriate selection of microbial cultures matched with the assessment of the reservoir rock and fluids (Moses, 1982). It has been reported that bacteria can move through reservoirs at a prodigious rate - 100m per day in a straight line or possibly 100 m per hour (Jack, 1990). Our studies were limited to cores considered appropriate candidates for microbial transport.

Included in the transport of bacteria through geological porous media are the considerations of the interaction between the core, the transport phenomena of the bacteria, and facilitating the bacterial transport under naturally occurring reservoir conditions. A reasonable baseline must be established before examining any variations in the reservoir parameters. Therefore, a reasonable starting place was the design of an apparatus in which tests could be repeated using variable conditions. The selected apparatus consisted of two straight sided containers (bottles) connected by a

core through which the transport of media and culture containing media could be observed. (see Figure 4)

Figure 4

Bottle Apparatus for Testing Bacterial Transport Through Cores



Berea sandstone cores from Cleveland Quarries Company were cut by Core Laboratories in Midland, Texas into several column sizes from vertical and horizontal directions. The drilled plugs selected for these tests had a permeability of 300mD and were 2 inches long and 1 inch in diameter from horizontal cuts. Each core (drilled plug) was wrapped tightly in Teflon and fitted snugly into the adjacent sides of the bottles. The bottles were autoclaved for 20 minutes with 1ml distilled/deionized water on the bottom of each bottle. Two hundred milliliters of sterile Photobacterium broth were added to each bottle. After one bottle was inoculated, the apparatus was incubated at 26°C without shaking. Periodic 1ml samples were aseptically removed for monitoring. Microbiological monitoring was done on Photobacterium agar plates to assess growth and luminescence.

An initial control experiment using phenol red was per-

formed to establish appropriate transport through the core and to detect any leakage. Equilibrium between the two bottles was almost immediate and was determined to be established within 15 minutes. Additional tests using bioluminescent bacteria and standard medium were followed by tests using formation waters. An established bioluminescing culture of Vibrio fischeri 7744 was used as the inoculum.

Bottle B was one-third filled with formation water. Bottle A was one-third filled with a mature bacterial culture of Vibrio fischeri in Pb broth. Within fifteen minutes luminescence was apparent in Bottle B, which had previously contained only formation water. Within eight hours culture equilibrium had been established in both bottles as indicated by the same amount of luminescence in each bottle.

This test was repeated several times using different formation waters and achieving the same results.

The carbonate cores that were used to test bacterial transport came from Coleman County Texas. Core Laboratories/Midland, Texas reported the core analysis shown in Table VII.

TABLE VII

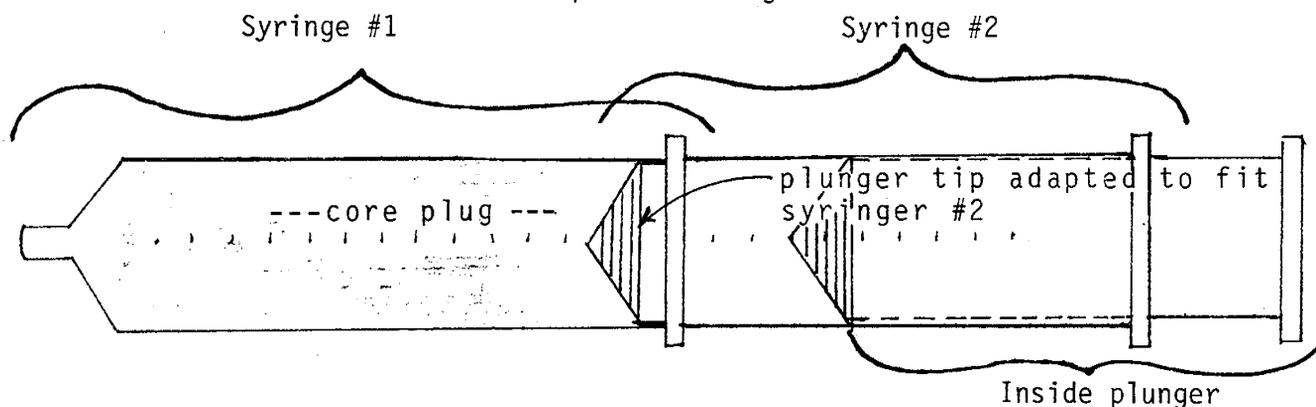
CARBONATE CORE ANALYSIS RESULTS

Permeability		Porosity Helium	Grain Density
(maximum)	(90 deg)		
mD	mD	%	gm/cc
0.47	0.47	19.8	2.69

A 2.5" long by 0.75" diameter core plug was fitted tightly into a polypropylene syringe, 3.5" long with a 1/16" in thickness; it has a 25ml total capacity. The plunger tip from inside the barrel of this syringe was fitted to a second outside syringe, in order that these could be attached in a series, and the second syringe used to inject the syringe containing the core plug. The second syringe could then be attached to a pump for introducing media with or without bacteria under pressure. (see Figure 5)

Figure 5

Double Syringe Apparatus for Testing Bacterial
Transport Through Cores



The core was flushed several times with 70% ethanol and rinsed with one volume of distilled/deionized water for every volume of ethanol. This apparatus dried overnight in a 37° incubator. The following day the apparatus was placed vertically over a dilution glass bottle held firmly in place with clamps to a ring stand. A 10ml aliquot of filtered (to remove excess salts) media was poured into syringe #2.

Pressure was manually applied to the barrel until media began to drip from syringe #1 (approximately 1 minute). A 25ml aliquot of luminescing culture, grown overnight, was added to syringe #2. Pressure was again applied until the uninoculated media had been recovered in the collection bottle. The apparatus was allowed to continue with only gravity flow. The "flow" was timed at one drop every seven seconds. After 24 hours, the flow had decreased to one drop every eleven seconds. The collection bottle glowed intensely when shaken indicating that enough luminescing bacteria had moved through the core to maintain luminescence in the recovered media. This experiment was repeated four times with the same results -- luminescing bacteria can be transported by gravity flow through a tight carbonate core without losing their luminescing capability. It had occurred to us that the bacteria might have travelled inside the wall of the syringe between the core and the syringe wall. However, it was too tight. The wall of the core was very smooth, after cutting and had to be firmly forced into the syringe so that the amount of liquid that could have travelled outside the core was negligible compared to that which moved through the core.

No field sites were available to test this procedure between communicating oil wells. Additional core tests, using cores selected from EOR field site candidates should

be performed to confirm the use of bioluminescent tracers in various kinds of cores. These should be followed by documented field tests on a scale small enough to be reasonable, but large enough to prove field application potential.

It is very unlikely that the photobacteria used in this study would be inhibited by bacteria indigeous to the reservoir formation. Such indigenous bacteria have been found by us to be extremely fastidious unlike the photobacteria which proved to be prolific in synthetic and formation water. Only the luminescing bacteria collected from the marine environments were overgrown by nonluminescing bacteria and these cultures were all eliminated after the first screening test.

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APPENDIX A

Quantitative Determination of Microbial Population Density

Standard Plate Count Method

The colony-counting technique referred to as the Standard Plate Count Method is the method most routinely used at Fairleigh Dickinson Research Center. A 1 ml aliquot of sample is aseptically pipetted into 99 mls of sterile phosphate dilution water in a milk dilution bottle. Serial dilutions are continued through $1:10^{-8}$. Using a sterile, special pipette, 0.1 ml and 1.0 ml are delivered from each dilution bottle to two respective Petri plates appropriately labelled. Duplicate plates are made so that one can be incubated using microaerophilic anaerobic conditions. Ten to fifteen ml warm, sterile agar are aseptically added to each plate and gently swirled to adequately distribute the sample. When the agar has solidified, the plates are inverted and incubated at the appropriate temperature for 2 days or until growth is complete. Colonies are then counted. This method is based on the assumption that each cell develops into one colony and that each colony is derived from one cell. Because not all cells are capable of reproduction, each cell might not yield a colony, as assumed. Therefore, the number of colonies counted is almost always lower than the true value. However, this is a "favorable error" because we can assume that there are more viable cells than actually counted.

Membrane Filter Technique

Predetermined dilutions of samples to be tested are made in sterile blanks of dilution water using aseptic techniques.

Assemble the sterilized Millipore filter holder components. With smooth sterile forceps aseptically center a sterile 47mm diameter Millipore filter on the screen with

gridded side up. Lock and seal the funnel to the base and seal firmly in the neck of the filtering flask. Connect this flask to a second side-arm connected to the vacuum source.

Filtration is accomplished by passing the sample through the filter under partial vacuum. Pour each sample dilution through filter set up, beginning with greatest dilution. Rinse the funnel with two or three portions of sterile, blank dilution water between samples. Unlock and remove funnel, immediately removing the filter with sterile blunt forceps and place on Petri dishes prepared with sterile pad or agar. Use rolling motion to avoid entrapment of air.

Incubate using humid conditions at temperature appropriate for each culture for 24 hours. (Incubate an additional 24 hours to confirm initial count).

Counting is most accurate if done with the aid of a low-power (10 to 15) magnification.

Optical Density Procedure

A quantitative measurement of the luminescent bacteria present in any given liquid culture was determined by Optical Density readings in the Shimadzu Spectrophotometer 6V-240. A standard curve was first prepared using McFarland nephelometer barium sulfate standards. These are numbered tubes in increasing opacity and the opacity of each tube is equated with a certain number of organism/ml. Each tube's density was read at 660nm and the absorbance values obtained were plotted against the number of organisms/ml for that tube. This standard curve is described and illustrated in Appendix B.

The procedure that was followed included: McFarland standards (see Appendix B) were prepared and the absorbance of each concentration was measured and recorded. A 2.0 ml aliquot of a 24 hour and 48 hour cultures of Vibrio fischeri 7744 in M broth was added to separate cuvettes. Absorbancy was measured and recorded and concentrations were calculated.

Sterile Pb and M broths were used as blanks depending on selected growth medium. Bioluminescent bacteria cultures in broth were diluted to 10^2 using the serial dilution method. Before reading the absorbance of each dilution of bacteria the absorbance was calibrated to 0- for each dilution using the sterile media dilution so that the uninocu-

lated media is subtracted out. For instance, before reading dilution 10¹ of bacteria the absorbance was zeroed at 10¹ of sterile media. The absorbance value of each dilution was then equated with the number of organisms/ml determined by the standard curve.

This method does not account for those cells that are dead or are not viable. Thus the method was only used in comparison with the soft agar method and was not solely relied on.

Soft Agar Method

A quantitative measurement of viable cells was determined using the soft agar method. Five ml aliquots of .7% agar were dispensed in the tube and held in a water bath at 45°C. Plates were prepared of 1.5% agar and kept at 10°C. Dilutions of the bacteria were prepared using the serial dilution method and immediately afterwards plates were poured. The contents of each dilution were vortexed and then poured into a tube from the water bath. The tube was vortexed and poured onto a cool agar plate. As soon as the media solidified the plate was inverted. Colonies were counted after 48 hours.

Time is very essential using this method. Even though the cool media plate enables the soft agar overlay to solidify quickly, the amount of time the cells remain in the tube after mixing should be as little as possible.

APPENDIX B

Spectrophotometer Readings of Bioluminescent Intensity

The intensity of the light produced by the bacteria was measured by the Shimadzu UV-240 Spectrophotometer which contained a photomultiplier. Since aeration of the broth culture is necessary for luminescence, the culture was moved through a flow cell by a peristaltic pump. To block out all light entering the sample chamber, black electric tape was placed over the openings of the deuterium-2 lamps. Before

each intensity reading, the flow cell was flushed with 70% ethanol followed by sterile distilled water and sterile media. Light intensity was measured in the Energy mode on a scale from 0 to 100 Energy units at a wavelength of 490nm (Hasting, 1977), and a slit width of 0.13nm.

A scan from 300 to 700nm was attempted to determine the optimum wavelength at which maximum intensity could be detected. However, there was no significant variation in intensity at different wavelengths except when the pump was turned off and the culture was left stationary. During this time, and within seconds, the bacteria use up all the oxygen in the broth and luminescence fades.

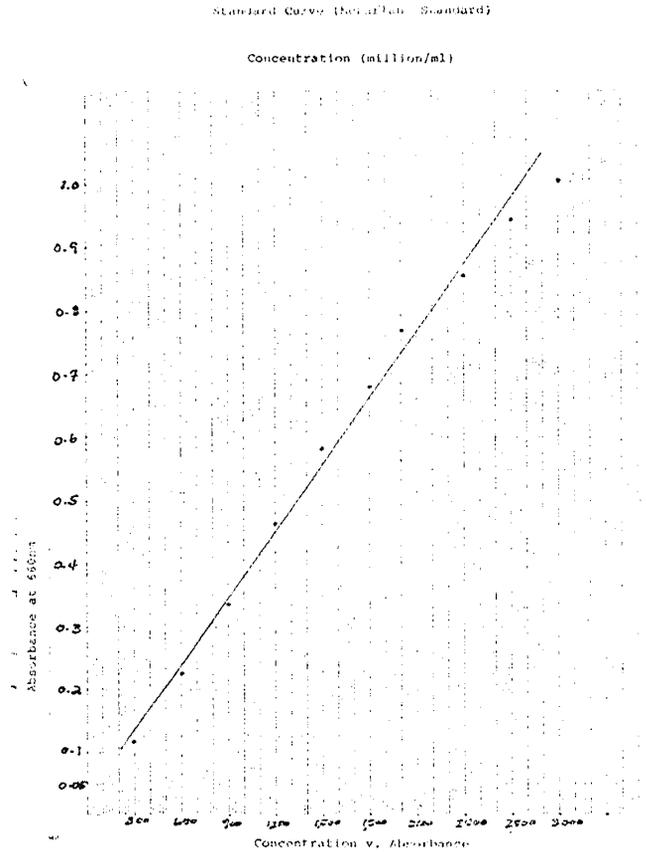
Although a particular wavelength at which maximum intensity could be observed was not able to be determined comparisons of intensity could be produced at a fixed wavelength, 490nm. It was hoped that personal communication with the manufacturer would shed light on techniques that might help to obtain better results for the specificity of wavelength. However, the manufacturer's technical representative told us that the detection of bioluminescence using the spectrophotometer was a unique application. Therefore, considerable time was spent adapting the spectrophotometer for measuring the intensity of maximum bioluminescence at a specific wavelength, because the instrument is designed to use a lamp outside the cuvette for measuring light intensity.

Standard Curve Data

McFarland Standard

Tube	Concentration	Absorbance at 660nm
1	300	.118
2	600	.227
3	900	.366
4	1200	.464
5	1500	.584
6	1800	.680
7	2100	.770
8	2400	.856
9	2700	.945
10	3000	1.046

Standard Curve (McFarland Standard)



Spectrophotometer Readings of Concentration of Vibrio fischeri

- I. Procedure: McFarland standards were prepared and the absorbancy at each concentration of $BaSO_4$ was recorded. Two mls of a 24 and 48 hour culture of Vibrio fischeri in Pb broth and a 24 hour culture in M broth were placed in cuvettes and their absorbancy was recorded, and their concentrative was calculated. Sterile Pb broth and M broth were used as the blanks.

II. Results:

Preparation of McFarland nephelometer barium sulfate standard

Tube	Barium chloride 1%(ml)	Sulfuric acid 1%(ml)	Corresponding app. density of bacteria (mil/ml)
1	0.1	9.9	300
2	0.2	9.8	600
3	0.3	9.7	900
4	0.4	9.6	1,200
5	0.5	9.5	1,500
6	0.6	9.4	1,800
7	0.7	9.3	2,100
8	0.8	9.2	2,400
9	0.9	9.1	2,700
10	1.0	9.0	3,000

III. Result Explanation:

- 1) Sample 1 is a 48 hr culture of Vibrio fischeri and sample 2 is a 24 hr culture of Vibrio fischeri. A concentration of = 4,000 million/ml in the 48 hr to = 3,000 million/ml in the 24 hr seem to indicate growth of = 1,000 million/ml in a 24 hr period.
- 2) Sample 3 is a 24 hr culture of Vibrio fischeri in Marine broth. Concentration seems to be = 5,000 mil/ml.
- 3) A number of factors could account for inaccurate readings:
 - a. Living bacteria cannot be distinguished from dead cells.
 - b. Luminescence of the organisms could have interfered with the readings even though I waited for the luminescence to die out before reading the absorbance.
 - c. 660 nm may not have the proper wavelength for reading that reduced the number of interference in the broth.
- 4) These results are merely a trial. They cannot be confidently used as accurate concentration readings.

APPENDIX C

Growth Media

Photobacterium broth (Pb)	
Tryptone	5.0g
Yeast Extract	2.5g
Ammonium Chloride	3.0g
Magnesium Sulfate	0.3g
Calcium Carbonate	1.0g
Monopotassium Phosphate	3.0g
Sodium Glycerol Phosphate	23.5g
Sodium Chloride	30.0g
Distilled/deionized water	1000ml
Marine broth (M)	
Peptone	5g
Yeast Extract	1g
Ferric Citrate	0.1g
Sodium Chloride	19.45g
Magnesium Chloride Dried	5.9g
Sodium Sulfate	3.24g
Calcium Chloride	1.8g
Potassium Chloride	0.55g
Sodium Bicarbonate	0.16g
Potassium Bromide	0.08g
Strontium Chloride	0.034g
Boric Acid	0.022g
Sodium Silicate	0.004g
Sodium Fluoride	0.0024g
Ammonium Nitrate	0.0016g
Disodium Phosphate	0.008g
Distilled/deionized water	1000ml
Minimal Medium	
Sodium Chloride	30g
Monopotassium Phosphate	3.7g
Ammonium Chloride	.5g
Magnesium Sulfate	.2g
Glycerol	2ml
Peptone	5g
Yeast Extract	3g
Distilled/deionized water	1000ml

Sea Water Complete (SWC)	
Sea Water	750ml
Distilled/deionized water	250ml
Glycerol	3ml
Peptone	5.0g
Yeast Extract	0.5g

