Heliyon 9 (2023) e13075

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Isolation and characterization of crude-oil-dependent bacteria from the coast of Ghana using oxford nanopore sequencing

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ARTICLE INFO

Keywords:
Jubilee oil
Coast of Ghana
Hydrocarbon
Bacteria
Sequencing

ABSTRACT

The utilization and improper use of crude oil can have irreparable damage on the environment and human populations. This study sought to isolate hydrocarbon utilizing bacteria from 1% v/v pristine seawater and 1% v/v crude oil using enrichment culture techniques. Whole genome sequencing of DNA using the Oxford Nanopore sequencing technique with Fastq WIMP as the workflow at 3% abundance was undertaken. The results showed that the most abundant isolates identified using this technique at specific sampling sites were, *Acinetobacter junii* (51.9%), *Alcanivarax pacificus* (15.8%), *Acinetobacter haemolyticus* (21.6%), *Pseudomonas aeruginosa* (23.4%), *Alcanivorax xenomutans* (24.7%), *Alcanivorax xenomutans* (23.0%) *Acinetobacter baumannii* (40.0%) and *Acinetobacter junii* (14.2%). Cumulatively, the most abundant isolates in the 8 sampling sites were *Acinetobacter junii* (17.91%), *Alcanivorax xenomutans* (11.68%), *Pseudomonas aeruginosa* (7.68%), *Escherichia coli* (7.67%), *Acinetobacter haemolyticus* (3.40%), *and Alkanivorax pacificus* (3.10%). Spearman's rank correlation analysis to examine the strength of relationship between the physicochemical parameters and type of bacteria isolated, revealed that salinity (0.8046) and pH (0.7252) were the highest. Isolated bacteria from pristine seawater, especially *Escherichia coli* have shown their capacity for bioremediating oil spill pollution in oceanic environments in Ghana.

1. Introduction

No amount of emphasis can be placed enough on how crucial it is for mankind to utilize the natural environment on a global scale. However, the misuse, exploitation, and poor use of these resources have permanently damaged the ecosystem, which has had an impact on human populations. Several human endeavors including petroleum hydrocarbon extraction have contributed significantly to the release of pollutants into the environment [1]. Specifically, there have been concerns with heavy metal contamination, organic and inorganic compounds absorption, ingestion and their introduction into the food chain [2]. Also, the crude oil value chain spanning

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https://doi.org/10.1016/j.heliyon.2023.e13075
Received 25 November 2022; Received in revised form 16 January 2023; Accepted 16 January 2023
Available online 25 January 2023

exploration, storage, transportation and final usage has the potential to exacerbate environmental pollution [3]. The crude oil is the original mixture of a variety of petroleum hydrocarbons including alkanes, cycloalkanes, and various aromatic hydrocarbons. Other organic compounds contain relatively higher amounts of nitrogen, oxygen, and sulfur, plus trace amounts of metals such as lead, iron, nickel, copper, and vanadium [4]. Crude oil is the most widespread contaminant within the marine environment [5]. There is ample evidence to support the devastating nature of these crude oil related issues, owing to spills and leakages from distribution channels and storage tanks as well as effluent discharge from production platforms into the immediate environment [3,6]. Pollution by hydrocarbons in marine environments may be the consequence of various natural (natural seepages), anthropogenic activities (discharge during tanks or ships transportation and pipeline failures) as well as other chronic pollution channels (ships, harbours, oil terminals, freshwater run-off, rivers and sewage systems) [7]. Spills from offshore drilling, pipelines and ships are equally significant contributions to the problem. Additionally, according to the Oil Spill Intelligence Report, spills in the size range of at least 34 tons have occurred in the waters of 112 nations since 1960 [5]. Commercial production of crude oil started in Ghana in late 2010 due to the discovery of the product in large quantities in 2007 at Cape Three Point in the Western Region. The first major spillage of 584 barrels in Ghana, made up of low-based mud drilling fluids occurred on December 26, 2009 in the drilling field of Cape Three Point. Another mud spill of 7 barrels took place on March 23, 2010 at the same location [8] (Ghanaian Daily Graphic, 2010). Additionally, a Ministerial Committee set up by the Ministry of Environment, Science and Technology recommended that Jubilee's partner, KOSMOS, was fined $35 million for negligent spilling of 699 barrels of oil mud which contains poisonous heavy metals (eg. arsenic, cadmium, iron, lead, mercury, nickel, zinc, etc.) on three occasions with the potential to affect Ghana's offshore ecosystem [9]. To mitigate these in an environmentally friendly and cost-effective manner, the use of oil eating microbes (bacteria) has been recommended [10,12].

Bacteria are the most predominant microbes in petroleum hydrocarbon biodegradation. They are the main degraders of spilt crude oil in several ecosystems [10]. Petroleum hydrocarbons can serve as the only carbon source for several bacteria for growth and respiration [11]. Bacterial species of genera *Achromobacter, Acinetobacter, Arthrobacter, Azoarcus, Brevibacterium, Cellulomonas, Corynebacterium, Flavobacterium, Marinobacter, Micrococcus, Nocardia, Ochrobactrum, Pseudomonas, Stenotrophomaonas* and some pieces of *Vibrio* are reported examples of hydrocarbon degraders. Their fungi counterpart also include genera *Aspergillus, Amorphoteca, Fusarium, Graphium, Neosartoria, Paecilomyces, Penicillium, Sporobolomyces, Talaromyces*. For yeast, the genera of *Candida, Pichia, Pseudozyma Rhodotorula* and *Yarrowia* also play vital roles in petroleum hydrocarbon pollutant degradation [12].

In Ghana, biodegradation of marine crude oil pollution using native bacterial consortium is yet to be fully exploited. The concept of microbial biodegradation has been successfully employed for soil pollutants remediation in mechanic workshops. This is also common for small scale refineries/processing units although this approach has rarely been employed on a large-scale basis for marine and oceanic pollution control.

In an attempt to remedy these challenges, oil waste management companies in Ghana have tried to employ conventional methods

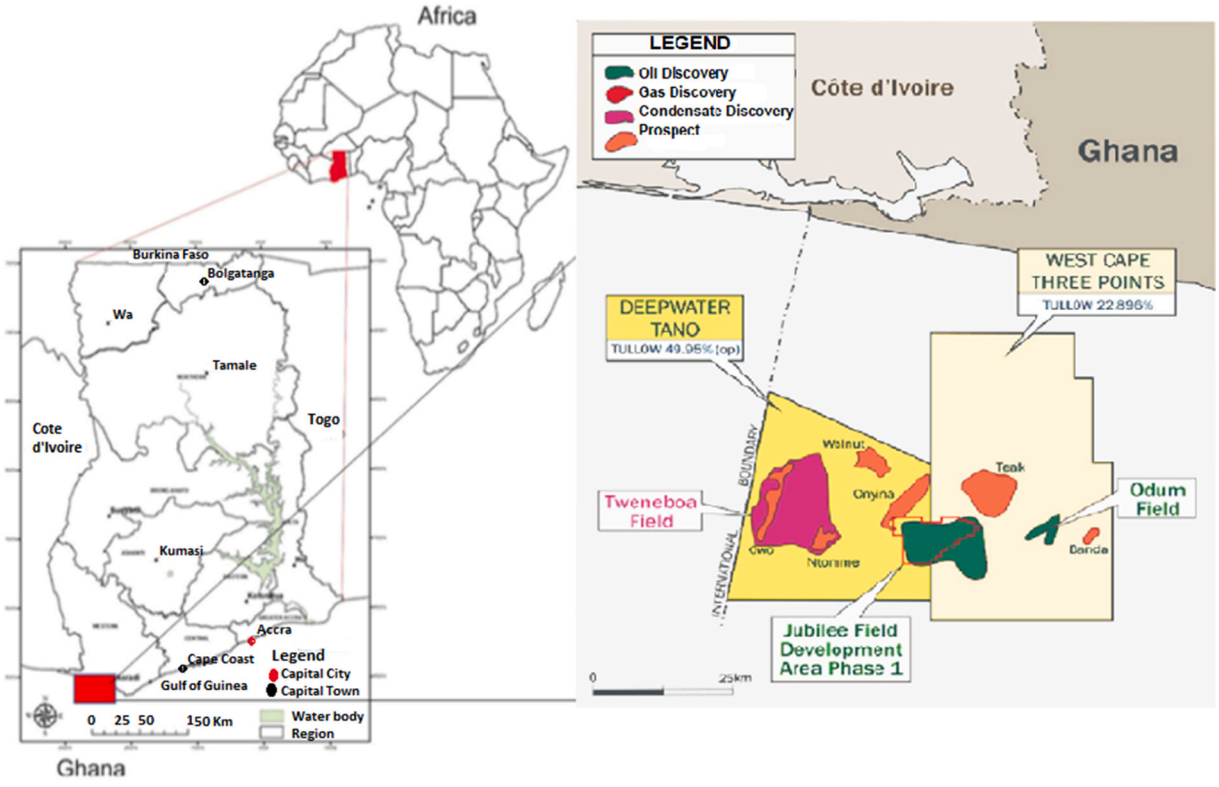

Fig. 1. Map of Ghana showing the Jubilee Oil Field where oil samples were collected [21].

such as chemical stabilization, thermal treatment, precipitation and solidification. However, these approaches have failed to yield the needed results due to its high cost and potentially detrimental consequences to the environment [13,14]. There is therefore the need for a paradigm shift in combating this challenge. Till date, published data in relation to the use of bacteria to bioremediate oil polluted fields are yet to be fully explored. Studies to elicit empirical data in this area is urgently warranted. The present study investigated the capacity of indigenous bacterial strains to remediate crude oil spills in marine environments along the coast of Ghana.

2. Methodology

2.1. Study design

A cross sectional study design was employed for this study from February 2021 to May 2022.

2.2. Study area

Ghana has a coastline that is about 550 km long (Fig. 1). It is generally low lying with contours not exceeding 30 m above sea level. Coastal communities are mainly permanent indigenous settlements which are exposed to the varying conditions of the sea [15,16]. Several shores within coastal communities continue to serve as natural harbors aiding trade among the coastal communities. The beaches along the coast also attract tourist, whiles some are wetlands and estuaries serving as habitat for aquatic lives [17].

Over the years, Ghana's climate has become drier and unpredictable with rainfall patterns showing variation of high and low distributions along the coast and in the country at large. There has generally been a reduction in the average annual rainfall and a transfer of the heaviest rains to the south -western corner of the country [18]. The location of Ghana within the tropical region has caused weather phenomena from the mesoscale, like thunderstorms to El Nino Southern Oscillation on the larger scale [19] to influence its weather systems to varying degrees. However, the Inter Tropical Convergence Zone (ITCZ) accounts for the June to September main rainy season. Also, the February to May rainy season which is the smaller of the two is as a result of the interaction between the eastward moving mid-latitude cold air and the tropical warm air, to create the appropriate moisture conditions that come into Ghana from the Atlantic Ocean to create rainfall [20].

2.3. Study approach

The study approach involved the *In vitro* bioaugmentation of microcosms, where simulated crude oil spills on the sea were

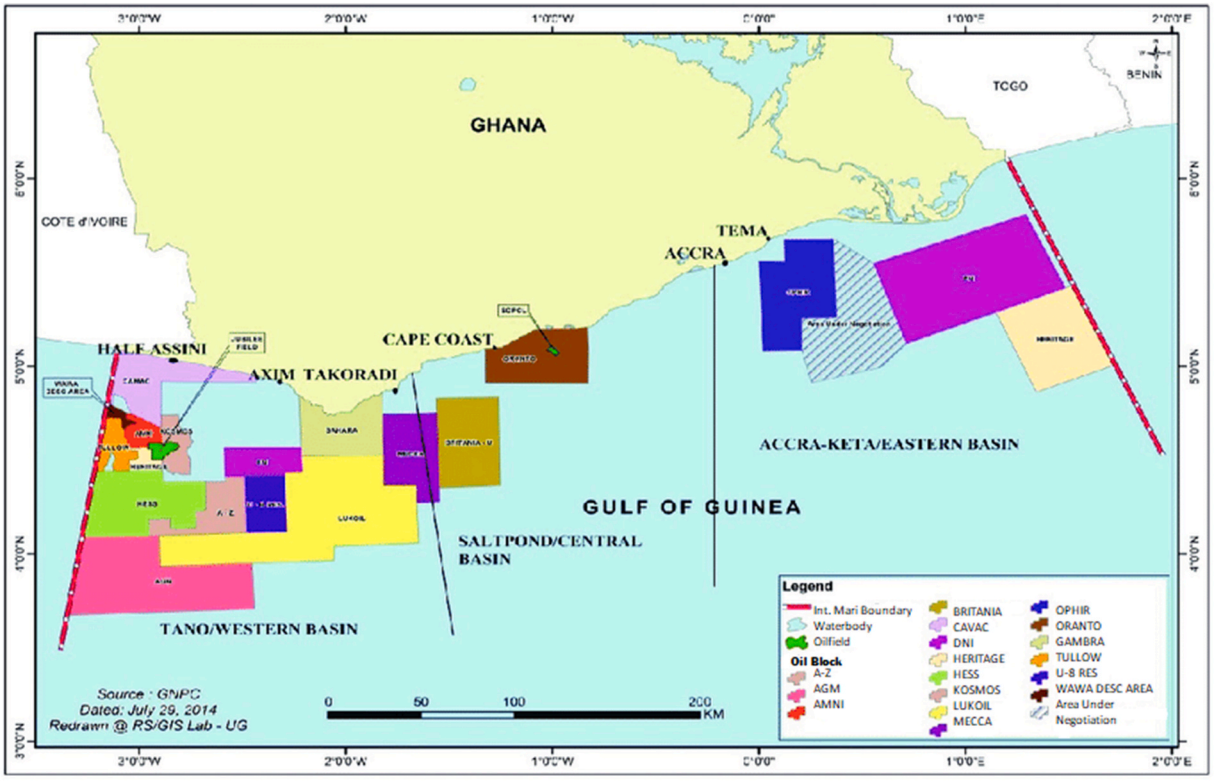

Fig. 2. Map Showing Three Distinct Oil Basins in Ghana where water samples were collected.

remediated using indigenous cultured bacteria within specific time frames.

2.3.1. Crude oil sampling

Samples of Jubilee crude oil were obtained from the Radiation Protection Institute (RPI) of the Ghana Atomic Energy Commission (GAEC). The samples comprise oils from different wells within a specific geographical location (60 km offshore between the Deepwater Tano and West Cape Three Points blocks), pumped in sessions at different times (Fig. 1). The first batch of oils were pumped in December 2010 and the batch used for this analysis was the 31st composite obtained in February 2016 [22]. About 1 L of the crude oil sample was collected in a plastic bottle, clearly labeled and kept at room temperature in the laboratory without any treatments prior to the analysis.

2.3.2. Sea water sampling

Sea water samples were collected from the shores of Keta, Accra, Takoradi, Axim, Tema, Winneba, Saltpond and Cape Coast representing the 3 geological basins of the coast of Ghana (Fig. 2).

Sample collection was done on 3 consecutive occasions between the months of April and July 2021. The samples were collected using sterile plastic scoops into sterile 250 ml polyethylene bottles (Kane-EM Industries, Ghana) and immediately stored on ice. Prior to collection, the plastic bottles were autoclaved after washing and rinsing them with dilute nitric acid followed by distilled water. Sea water was collected onshore, at 10 M and 20 M offshore at each sampling location and homogenized to ensure a representative sample. Samples were kept on ice in an ice chest, transported to the laboratory and stored in a refrigerator at 4 °C [23]. Selected physico-chemical parameters analyzed included; electrical conductivity, pH, temperature, salinity and Total dissolved solids (TDS). These were measured on site using a low range combo HANNA HI98129 m (Slovenia) following the manufacturer's instructions.

2.3.3. Laboratory analysis

Microbiological analyses were carried out within 24 h after sample collection according to International Standards organization (ISO) and Organization for Economic Cooperation and Development (OECD) standards [24,25].

2.3.3.1. Preparation of Mineral Salt Medium (MSM) for bacterial growth. Mineral salts medium (MSM) and Agar (without carbon content) used in this study was prepared using 10 g NaCl; 0.42 g $MgSO_4{\cdot}7H_2O$; 0.29 g KCI; 0.83 g KH_2PO_4; 1.25 g K_2HPO_4; 0.42 g $NaNO_3$ and dissolved in 1 L of distilled water. The mineral salt agar was prepared by dissolving 15 g of bacteriological Agar in 1 L MSM [26]. The media was autoclaved at a pressure of 15 pounds per square inch (psi) using a BST-PA20L portable autoclave (Bionics Scientific, India) for 30 min and readied for inoculation.

2.3.3.2. Isolation and selection of crude-oil-degrading bacteria. Enrichment culture techniques were employed for the isolation of bacteria [27]. A set of eight (8) 250 ml Erlenmeyer flasks containing 100 ml of sterile mineral salts medium (MSM) (autoclaved and allowed to cool) were inoculated with 1 ml of crude oil and 1 ml of sea water each. An IKA vortex GENIUS 3 (USA) was used to shake the mixture and stored at 28 °C for 7 days in an Avantor INCU-Line incubator, USA. The control set up did not include crude oil to test for contamination. A 1 ml aliquot of the set up was sub-cultured onto a fresh 100 ml MSM with 1 ml crude oil every 7 days for 4 consecutive times and incubated under the same conditions. After 4 cycles, 1 ml of the culture was pipetted into nutrient broth for storage and further analysis.

2.4. Identification of crude-oil -degrading bacteria

2.4.1. Gram staining

Preliminary bacterial identification was done using gram staining to determine the success of the bacteria culture and enrichment processes.

The Gram staining method by Coico [28] was adopted for this work.

The slides were then observed under a microscope of magnification: 100× 1.25 oil (Richter Optica).

2.5. DNA extraction and whole genome sequencing

2.5.1. Bacterial DNA extraction

Extraction of the genomic DNA from bacterial cultures was done using a Zymo DNA extraction kit (Zymo Research, USA) following the manufacturer's protocol. This was carried out at the Biotechnology Center of the Biotechnology and Nuclear Agricultural Research Institute of the Ghana Atomic Energy Commission. The eluted DNA was stored at -20^{O}C until further analysis. The quality of the extracted DNA was determined using 1% agarose gel and viewed under a high performance ultraviolet transilluminator (UVP, Cambridge, UK).

2.5.2. Whole genome sequencing of extracted DNA using the oxford nanopore sequencing method

The DNA extracted from the bacterial cultures were sequenced using an Oxford nanopore sequencer from the Noguchi Memorial Institute, Accra Ghana following the manufacturer's instructions.

2.5.2.1. Purification of DNA using AMPure XP beads. The AMPure XP beads were re-suspended in solution by vortexing. Molecular grade water was used to prepare fresh 80% ethanol from stock. About 10 mM of Tris-HCL (2-Amino-2-hydroxymethyl-propane-1,3-diol/C4H11NO3) buffer at pH 7.5–8.0 was prepared with 50 mM NaCl. About 25 μl of resuspended AMPure XP beads was added to each DNA sample (45 μl–50 μl), and mixed by flicking the tube. The mixture was incubated on a HulaMixer (Thermo Fisher Scientific-USA) for 5 min at room temperature. The sample was briefly spun down using a GCM series mini centrifuge (Thomas Scientific – USA) and pelletized on a magnet. The tube was kept on the magnet (DynaMag™-96 Side Skirted Magnet, Thermo Fisher Scientific) and the supernatant pipetted off. The tube was kept on the magnet (DynaMag™-96 Side Skirted Magnet, Thermo Fisher Scientific) and 200 μl of freshly prepared 80% ethanol used to wash the beads without disturbing the pellets. The ethanol was subsequently removed using a pipette and discarded. The preceding step was repeated once. The washed tube with beads was spun down (GCM Series Mini Centrifuge Thomas Scientific) and placed back on the magnet and residual ethanol pipetted off and briefly allowed to dry. The tube was removed from the magnetic rack and pellets were re-suspend in 22 μl of 10 mM Tris-HCI (pH 7.5–8.0) and incubated for 2 min at room temperature. The magnetic rack was used to pellet the beads from the eluate to obtain a clear and colourless liquid. About 20 μl of the eluate was pipetted into a clean 1.5 ml Eppendorf DNA LoBind tube.

2.5.2.2. Determination of concentration of DNA using qubit dsDNA Assay Fluorometer (thermofisher). To determine of the concentration of DNA, the required number of Qubit assay tubes (0.5 ml) for standards and samples was obtained using the required standard tubes (2). The Qubit assay tube lids were labeled appropriately to prevent interference with the sample/standard reads. The Qubit working solution was prepared in a clean plastic tube by diluting the Qubit dsDNA (double stranded) HS reagent (Fluorophores) in the Qubit dsDNA HS (High sensitivity) buffer in the ratio 1:200. A 190 μl aliquot Qubit working solution was pipetted into the tubes to be used for standards 1 and 2. About 10 μl of Qubit working standards (1 and 2) was then added to the appropriate tubes and mixed by vortexing for 2–3 s. A 197 μl volume of Qubit working solution was pipetted into each sample assay tube. A 200 μl final volume for the sample was achieved by adding 3 μl of each sample to the sample assay tubes already containing 197 μl of Qubit working solution and mixed by vertexing for 2–3 s. All the tubes (Samples and standards) were allowed to incubate at room temperature for 2 min. DNA concentration in the samples were measured on the Qubit Fluorometer after calibration of the device with the standards.

2.5.2.3. Normalization of quantified DNA samples. Normalization is required to reduce the concentration of the DNA in each sample that was higher than 100 ng/μl for accurate sequencing reads. The normalization step was not required since all the Qbit values for the samples were all less than 100 ng/μl (Table 4). A 7.5 μl aliquot of DNA was pipetted for the next steps.

2.5.2.4. Rapid barcoding. The 7.5 μl of DNA amplicons from the previous step were obtained. A 2.5 μl aliquot of fragmentation mix (Rb 01–12) was added to the sample. The content was mixed gently by flicking the tube and spun down using a portable bench-top centrifuge (GCM Series Mini Centrifuge, Thomas Scientific). The barcoded sample was incubated at 30 ^{O}C for 1 min and then at 80 ^{O}C for 1 min using Veriti™ Dx 96-well thermal cycler (Thermofisher Scientific-USA) and briefly cooled on ice. All the barcoded samples were pooled into a 1.5 ml Eppendorf tube (super pool) and the total volume noted.

2.5.2.5. Concentration of the super pooled library and ligation of the rapid adapter (RAP). A 1 μl aliquot of Rapid Sequencing Adapter (RAP) was added to 10 μl of barcoded DNA totalling 11 μl pool. The combination was gently mixed by flicking the tube and content spun down with a mini centrifuge. The mixture was incubated for 5 min at room temperature for complete reaction (ligation) between the RAP and barcoded DNA. The prepared library was stored on ice briefly before loading to the MinION (SpotOn) flow cell.

2.5.2.6. Priming and loading the SpotON flow cell. The MinION flow cell was obtained and MinKNOW GUI software opened on a computer making sure the device was connected to a reliable internet source. The flow cell was carefully inserted into the MinION. The number of active pores available for sequencing was checked (800 nanopores or above) to ensure a successful run. The sequencing buffer (SQB), loading beads (LB), flush tether (FLT) and one tube of flush buffer (FB) were thawed and shook thoroughly. The flow cell priming mix was prepared by adding 30 μl Flush tether (FLT) directly into one tube of flush buffer (FB) and mixed by pipetting up and down. The flow cell priming port cover was opened by turning the cover clockwise 90°. Air bubbles were eliminated using a P1000 pipette set at 200 μl. A continuous butter was ensured from the priming port across the sensor array. An 800 μl aliquot of the priming mix was loaded into the flow cell through the priming port. A mandatory 5-min pause was observed. The content of the loading beads (LB) was thoroughly mixed by pipetting up and down. The library (11 μl of barcoded DNA with RAP) was prepared for analysis by adding 34 μl of sequencing buffer (SQB), 25.5 μl of Loading Beads (LB) and 4.5 μl of Molecular grade water. The SpotON flow cell sample port was opened to make the SpotON port accessible. Additional 200 μl of the priming mix was loaded into the flow cell via priming port. The prepared library was mixed by pipetting up and down prior to loading. A 75 μl aliquot of the sample was added into the flow cell via SpotON sample port, in dropwise manner. The SpotON sample and priming ports were closed and MinION covered with lid. The sequencing run was started immediately using the MinKNOW software.

3. Results

3.1. Baseline characterization of sea water and crude oil samples

Table 1 below shows the results for physicochemical analysis of sea water samples from along the coast of Ghana. The parameters

measured for the sea water included temperature, pH, conductivity, TDS and Salinity. There were no significant variations in the parameters measured for the sampling sites. The highest and lowest temperatures were 27.2 and 26.0 corresponding to Cape Coast and Keta respectively. The mean temperature recorded for all the sampling sites was 26.8. The highest electrical conductivity value was 53.12 (Cape Coast) while the lowest (51.28) was recorded at Tema. The mean EC value for all sampling sites obtained was 52.15. Salinity values ranged between 34.02 (Saltpond) and 31.94 (Tema) with the average salinity value for all sampling sites being 32.84.

Spearman's Rank Correlation analyses were performed to examine the strength of relationship between the parameters (predictors) and the type of bacteria cultured at the various sites. The results are presented in Table 2. Salinity had the highest correlation ($\rho = 0.8046$) with type of bacteria. This suggests a strong positive correlation between the two variables. Similarly, pH had a strong positive correlation coefficient of 0.7252. Both relationships were found to be statistically significant at significance level of 0.05. All the independent factors were found to have a positive correlation with the type of Bacteria with the exception of Electrical Conductivity which showed a negative but insignificant correlation with the type of bacteria.

Table 3 presents the result of a Dominance analysis performed to compare the relative importance of the predictors. The result clearly ranks salinity as the most important predictor of the type of bacteria followed by pH with electrical conductivity being the least important predictor.

3.2. Assessment of enrichment culture technique with crude oil as the sole carbon source using gram staining

Gram staining was the technique employed to ascertain the success of the bacteria culture and enrichment processes. Crude oil was the sole carbon source used for the isolation process. On the whole, the results (Fig. 3A–H) showed the presence of a mixture of gram positive (violet to blue) and gram negative (pink to red) bacteria present in most the samples at varying degrees. It also showed that some the cells were rod-like (bacillus) and others spherical (cocci). Some were joined in chains (streptobacillus and streptococcus) and others were clumped together (Staphylococcus).

Specifically, results from Accra (Fig. 3A) showed that there were more spherical shaped (single and clumped together) bacteria compared to other features. There also appeared to show higher proportions of gram-positive bacteria compared to gram negative bacteria. Results from Tema (Fig. 3B) however had higher portions of streptococci compared to those from Accra. Another notable result showed that the bacteria from Winneba (Fig. 3C) had higher proportions of streptobacilli compared to the rest of the sampling sites. The rest had mixtures of different bacteria in varying degrees.

Assessment of Quality of Extracted DNA: Results from the Agarose gel electrophoresis of the extracted DNA of the samples are shown in Fig. 4. DNA bands were visualized for all the samples analyzed. All the 8 samples viewed in a high performance ultraviolet transilluminator showed tight bands with no detectable smearing. The negative control did not have any observable band.

3.3. Assessment of DNA concentration and normalization

The DNA concentrations were measured using Qubit dsDNA Assay Fluorometer (Thermofisher) and results shown in Table 4. The Standard solutions used to calibrate the equipment (S1 and S2) gave concentrations of 31.00 ng/ml and 12192.65 ng/m respectively on the fluorometer. A high proportion of extracted DNA had concentrations lower than 10.00 ng/ml. The highest and lowest concentration recorded were 10.40 and 1.96 ng/ml corresponding to Keta and Takoradi respectively. None of the readings exceeded the 100 ng/ml mark, indicating that there was no need for further dilution before proceeding to the next step.

3.4. Analysis of bacterial Community Composition -profiling of crude oil dependent bacteria

Bacterial diversity along the coast of Ghana was analyzed based on culture dependent methods and capacity to degrade crude oil. Bacterial isolates were obtained from 8 communities spanning the 3 geological basins of the coast of Ghana (Fig. 2). The types of bacteria isolated from each community in this study using the Oxford Nanopore Sequencing technique are presented in Table 5. The Oxford Nanopore sequencing technique using Fastq WIMP as the workflow at 3% abundance was used to analyse the DNA samples extracted from bacteria cultured on Mineral Salt Medium (MSM) with crude oil as the sole carbon source. A total of 1,468,046 reads were done and analyzed. Of this number, 1, 164, 690 reads were classified whiles 303,356 reads were unclassified. Out of the 1,164,690 bacteria classified using the Oxford Nanopore technique, the 6 most common bacteria species isolated at 3% abundance

Table 1
Physicochemical Parameters of Sea water.

Community	Temperature (^{o}C)	Conductivity (μS cm^{-1})	pH	TDS (mg L^{-1})	Salinity (mg L^{-1})
Accra	26.8	51.42	8.04	26.74	32.51
Tema	27.2	51.28	8.26	26.95	31.94
Keta	26.0	52.83	7.91	25.90	32.01
Winneba	26.4	51.48	8.12	26.41	33.35
Saltpond	27.1	52.18	8.70	26.82	34.02
Cape Coast	27.2	53.12	8.51	27.53	33.29
Takoradi	26.9	52.73	8.06	26.15	32.91
Axim	26.5	52.19	8.11	25.28	32.7 5

Footnote: Data on physicochemical parameters are presented as means of three replicate measurements TDS (Total dissolved solids).

Table 2
Correlation Analyses to examine the strength of relationship between the parameters.

Parameter	(1)	(2)	(3)	(4)	(5)	(6)
Temperature (1)	1.000					
Electrical Conductivity (2)	−0.0846	1.000				
PH (3)	0.7245*	0.1158	1.000			
TDS/g/L (4)	0.7269*	−0.0456	0.6273*	1.000		
Salinity (5)	0.3155	0.1801	0.7079*	0.2608	1.000	
Type of Bacteria	0.3510	−0.1059	0.7252*	0.5396*	0.8046*	1.000

Footnote: Numbers attached to parameters correspond with type of bacteria on the last row.

Table 3
Dominance Analysis on Relative Importance of factors for Bacterial Determination.

Parameters/Units	Dominance Stat.	Standardized Dominance Stat.	Ranking
Salinity (mg L^{-1})	0.3808	0.4286	1
TDS (mg L^{-1})	0.1471	0.1656	3
pH	0.2434	0.2740	2
Electrical Conductivity (μS cm^{-1})	0.0442	0.0497	5
Temperature (^{o}C)	0.0729	0.0820	4

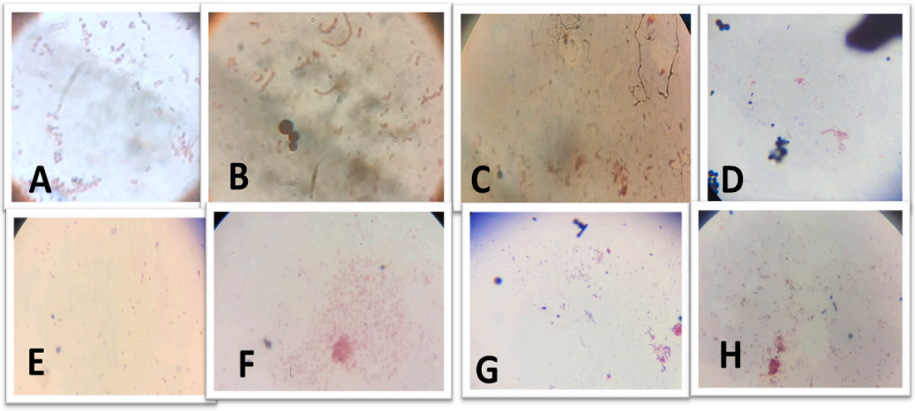

Fig. 3. Microscopic images of Gram-stained Bacteria Isolates. A: Accra isolate, B: Tema isolate, C: Weneba Isolate, D: Cape Coast isolate, E: Takoradi isolate, F: Axim isolate, G: Keta isolate, H: Saltpond isolate.

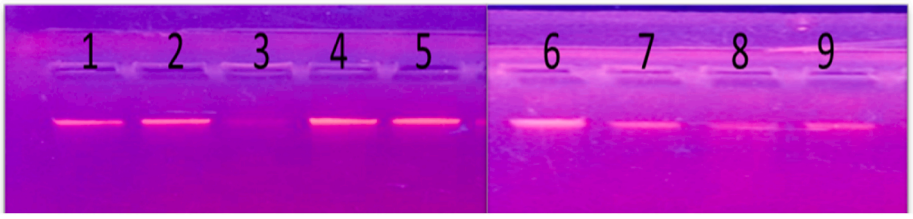

Fig. 4. Agarose gel electrophoresis of bacterial genomic DNA. 1: Accra isolate, 2: Tema isolate, 3: Negative control: 4: Weneba Isolate, 5: Cape Coast isolate, 6: Takoradi isolate, 7: Axim isolate, 8: Keta isolate, 9: Saltpond isolate.

were *Acinetobacter junii Alcanivorax xenomutans, Pseudomonas aeruginosa, Escherichia coli. Acinetobacter haemolyticus, and Alkanivorax pacificus* (Fig. 6). The phylogenetic tree showing the relationships between the identified species of bacteria is shown in Fig. 5.

At 3% species level, the total number of reads classified was 1,164,690. Majority of the Taxa classified were *Acinetobacter junii* with a percentage of 17.91, followed by *Alcanivorax xenomutans* representing 11.68% of the total number of taxa classified. The number of *Pseudomonas aeruginosa* and *Escherichia coli* bacteria classified were nearly the same with a percentage of 7.68 and 7.67% respectively, followed by *Acinetobacter haemolyticus* (3.40%) and *Alcanivorax pacificus* (3.10%), shown in Fig. 7.

In Fig. 8, each bar represents the percentage of the bacteria with the most counts for the site. More than half (51.9%) of the bacteria found in Takoradi (92) were *Acinetobacter Junii*. Similarly, *Acinetobacter junii* topped as the bacteria with the most counts in Tema (66)

Table 4
Qbit results showing concentration of DNA in samples.

Sample ID	Qubit Conc. (ng/ml)
S1	31.00
S2	12192.65
Accra	4.99
Tema	6.04
Keta	10.40
Winneba	3.75
Saltpond	9.13
Cape Coast	3.80
Takoradi	1.96
Axim	2.47
Negative Control	too low

Footnote: S1 (Standard 1), S2 (Standard 2).

Table 5
Bacteria Species identified in each community using the Oxford Nanopore Sequencing technique at 3% Abundance.

Bar Code	Community	Isolated Bacteria
66	Tema	*Escherichia coli, Alcanivorax xenomutans, Acinetobacter junii, Pseudomonas mendocina, Vibrio fluvialis,*
67	Keta	*Escherichia coli, Acinetobacter baumannii, Pseudomonas aeruginosa*
72	Accra	*Escherichia coli, Alcanivorax xenomutans, Pseudomonas aeruginosa, Stenotrophomonas acidaminiphila, Achromobacter xylosoxidans,*
73	Winneba	*Escherichia coli, Alcanivorax xenomutans, Pseudomonas aeruginosa, Stenotrophomonas acidaminiphila,*
76	Saltpond	*Escherichia coli, Pseudomonas aeruginosa, Alcanivorax xenomutans,*
83	Axim	*Escherichia coli, Alcanivorax pacificus, Acinetobacter haemolyticus,*
84	Cape Coast	*Escherichia coli, Alcanivorax pacificus, Acinetobacter haemolyticus, Pseudomonas mendocina, Thalassospira marina*
92	Takoradi	*Escherichia coli, Acinetobacter junii, Alcanivorax xenomutans,*

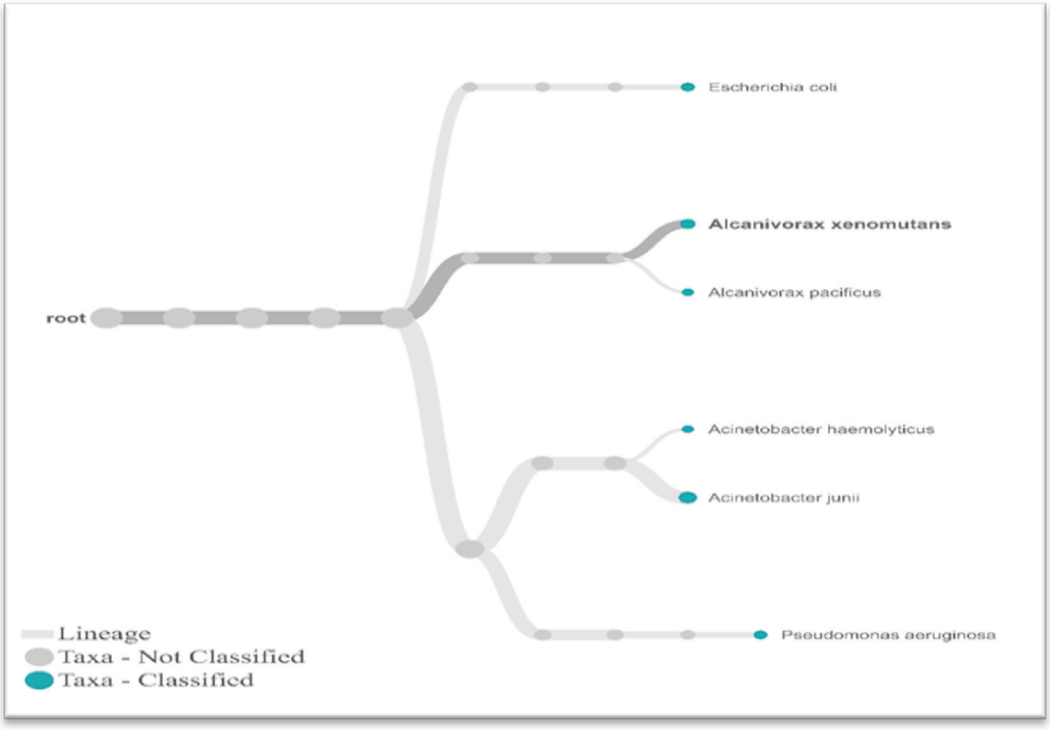

Fig. 5. Phylogenetic Representation of the Most Dominant Bacterial Species Isolated from Eight Communities at 3% abundance Using Fastq WIMP as the Workflow.

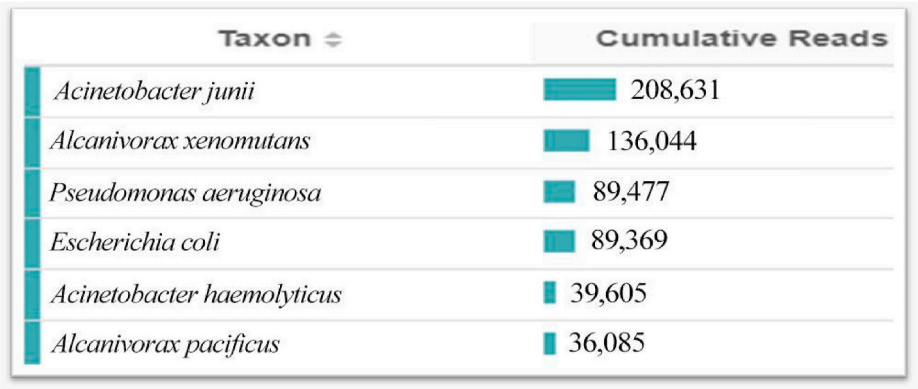

Fig. 6. Combined total abundance of species taxon cumulative reads from sampling.

but with a percentage of 14.2%. In Keta (67), *Acinetobacter baumannii* had the most counts of bacteria with a percentage of 40. Whereas in Winneba (73), *Alcanivorax xenomutans* was the bacteria with the most counts, having a percentage of 24.7%.

4. Discussion

The isolation and use of microorganisms for the cleaning of petroleum pollutants has been of major interest to several researchers in recent times. This substantial interest has been due to the proliferation of environmental pollution from petroleum hydrocarbon pollutants and the environmental sustainability in addition to cost effectiveness associated with bioremediation, compared to other conventional (chemical and physical) methods [29]. In this study, microcosm tests were employed for the isolation and characterization of bacteria along the coast of Ghana capable of degrading crude oil. Sea water samples were collected from 8 coastal communities (Accra, Tema, Keta, Winneba, Saltpond, Cape Coast, Takoradi and Axim) where there has not been any crude oil spillage for isolation of bacteria using enrichment culture techniques with mineral salts medium (MSM) and crude oil as the sole carbon source.

The 6 most common bacteria species successfully isolated at 3% abundance using oxford Nanopore Sequencing were *Acinetobacter junii Alcanivorax xenomutans, Pseudomonas aeruginosa, Escherichia coli. Acinetobacter haemolyticus, and Alkanivorax pacificus*. The specially prepared Mineral Salts Medium (MSM) [26] containing essential nutrients and minerals coupled with the crude oil and enrichment techniques ensured that bacteria which utilized crude oil were isolated. Hossain et al. [30] utilized enrichment culture techniques using petroleum oil (diesel and burned engine oil) as the sole carbon source. Their research was successful in the isolation of three (3) bacteria species although they did not use pure crude oil. Sudiana et al. [31] also employed a two-step enrichment culture technique using crude oil as the sole carbon source to isolate some bacteria taxa including α-subclass Proteobacteria, β-subclass Proteobacteria and γ subclass Proteobacteria. Furthermore, Erdogan et al. [32] employed enrichment culture techniques using crude oil as the sole carbon source to isolate 33 strains of bacteria in his work on contaminated soils.

The capacity to isolate crude oil degrading bacteria from an uncontaminated area was initially confirmed using the gram staining method. The technique made it possible to characterize the isolated bacteria into gram-positive and gram-negative strains as well as other morphological characteristics such as the shapes of the isolated bacteria. The results showed a mixture of these traits throughout the sampling sites. Samuel et al. [33] employed this technique for the initial confirmation of some bacteria species including *Escherichia coli*, *Micrococcus luteus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*. Also, Bhagobaty [34] employed gram staining in his work to gain a deeper insight into the microbial communities present in crude oil seeps located at Digboi oilfield in Assam, India. In their study, Zhuang et al. [35] demonstrated this technique by identifying 3 g-positive bacteria using the gram staining technique on naphthalene-degrading bacterial strains from oil-contaminated tropical marine sediments.

The Dominance Analysis performed in this work to compare the relative importance of the predictors (TDS, salinity, pH, conductivity, temperature, etc.) on type of bacteria, showed that salinity was the most important predictor of the type of bacteria to be isolated followed by pH at each sampling site. A similar result was obtained by Rath et al. [36], where salinity and pH were key in predicting the type of bacteria isolated. Additionally, Zhang et al. [37] ascertained the dependence of bacteria type on salinity. The reliability of this assertion was confirmed by the Spearman's Rank Correlation analyses, where the strength of relationship between the parameters measured and the type of bacteria cultured at the various sites was examined. The results clearly showed that Salinity had the highest correlation ($\rho = 0.8046$) with type of bacteria followed by pH with a positive correlation coefficient of 0.7252 with a statistical significance level of 0.05. Apart from the isolation and initial identification processes to confirm the success of the culturing process, there was the need for further characterization work using DNA sequencing to adequately place the bacteria in their proper genera and species levels.

The extraction was confirmed by gel electrophoreses where 7 out of the eight sampling sites gave tight bands when visualized in the high performance Ultraviolet transilluminator. The site that showed the faintest band was Takoradi which was corroborated by the gram staining results showing sparsely spaced bacterial isolates. Agarose gel Electrophoresis (2%) was utilized by Godini et al. [38] after the amplification of DNA fragments in their work. The Qubit fluorometric results for the concentration of DNA also showed the

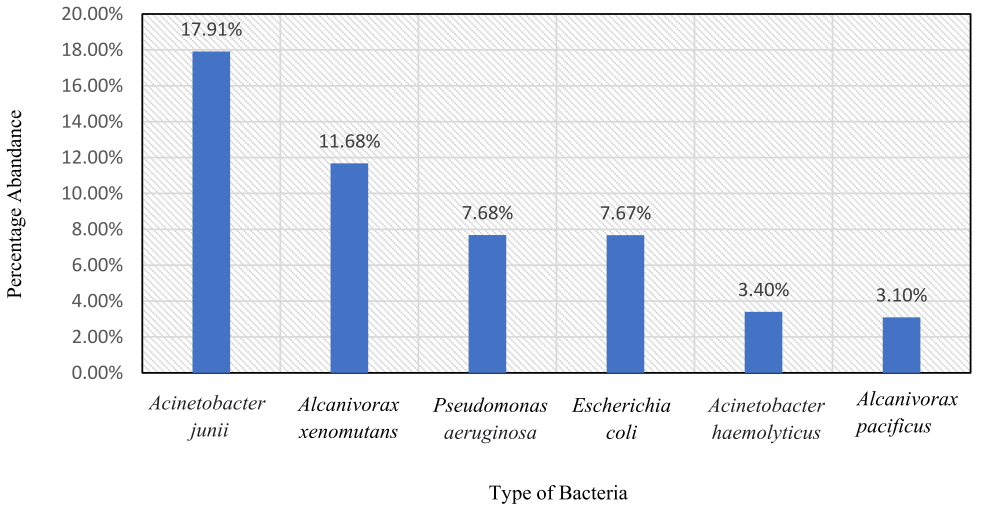

Fig. 7. The six most abundant bacteria identified as aggregate from the eight sampling sites using Oxford Nanopore Sequencing.

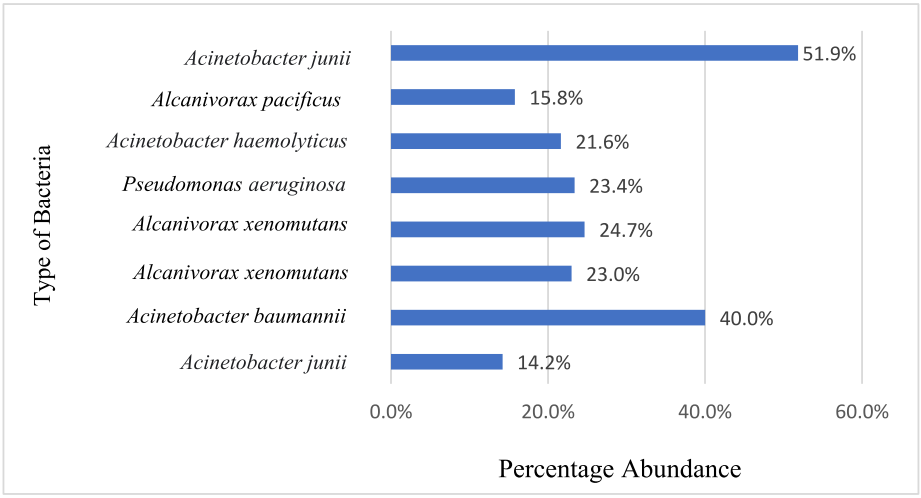

Fig. 8. Most abundant bacteria at each sampling site as identified by the Oxford Nanopore Sequencing.

least concentration of 1.95 ng/ml corresponding to Takoradi. Successful isolation of bacteria and subsequent extraction of DNA suggests the capacity of the isolated bacteria to utilize crude oil for growth and energy requirements. These life processes lead to the mineralization of the crude oil into water and carbon dioxide (CO_2). Furthermore, the sampling of sea water near the surface (less than 10 cm), coupled with the culture and isolation procedures being undertaken in oxygenated environments suggests that the bacteria isolated were either aerobic bacteria or facultative anaerobes. Grishchenkov et al. [39] in their work isolated bacterial strains (Pseudomonas sp. BS2201, BS2203 and Brevibacillus sp. BS2202) from petroleum-contaminated soil capable of degrading petroleum hydrocarbons under aerobic and anaerobic conditions.

In this study, the Oxford Nanopore Sequencing technique was used to analyse bacterial isolates from the 8 sampling sites. This technique has previously been used to analyse crude oil degrading and other bacteria elsewhere [40–43]. The technique identified several species of bacteria with the 6 most abundant from highest to lowest being; *Acinetobacter junii, Alcanivorax xenomutans, Pseudomonas aeruginosa, Escherichia coli, Acinetobacter haemolyticus*, and *Alkanivorax pacificus,* occurring in all 8 sampling sites in varying degrees. Similar work by Ohadi et al. [44], using mineral salts medium (MSM) and Iranian light crude oil (ILCO, 1%) as the sole carbon source also identified *Acinetobacter junii* as the most dominant bacterial isolate using 16S rDNA sequencing for identification.

Another study by Wang et al. [45] on Xiamen Island in China, identified *Alcanivorax* sp, *Acinetobacter* sp, and *Pseudomonas* sp as the most important oil degraders in the area. Ma et al. [46], whose work focused on isolating bacteria using culture-dependent enrichment methods from deep sea hydrothermal vents of the South Mid-Atlantic Ridge, among other isolates found *Acinetobacter* sp as the dominant bacteria isolate. The survival of these species on crude oil, despite the fact that there has not been any major oil spills persistent along the coast of Ghana implies their capacity to degrade crude oil. Other works have reported the degradation of crude oil

by the following species; *Acinetobacter junii* (46), *Alcanivorax xenomutans* [47], *Pseudomonas aeruginosa* [48], *Acinetobacter haemolyticus* [49] and *Alkanivorax pacificus* [50]. Interestingly, *Escherichia coli* was found to have survived on the crude oil as the sole carbon source in all 8 sampling sites during the experiment. This indicates its potential for environmental remediation of crude oil spills. At the time of this work, the degradation of crude oil by *Escherichia coli* has yet to be reported. However, transformant *Escherichia coli* has been implicated in crude oil (phenanthrene) degradation [51], and was also isolated in a used engine-oil contaminated soil by Musa [52].

5. Conclusion

Oil spillage and petroleum pollution are now a major cause of environmental vulnerability and Ghana in particular, stands the risk of petroleum pollution. In this study, *Acinetobacter junii, Alcanivorax xenomutans, Pseudomonas aeruginosa, Escherichia coli, Acinetobacter haemolyticus*, and *Alkanivorax pacificus* isolated from pristine sea water conditions (no crude oil contamination), using enrichment culture techniques with crude oil as the sole carbon source have demonstrated the capacity for crude oil degradation. The findings of this research present a good opportunity for the formulation of environmentally friendly approaches for oil spill remediation in Ghana., *Escherichia coli,* although being reported for the first time, presents a unique opportunity for crude oil degradation due to its ability to survive on crude oil in all 8 samples in appreciable amounts. Thus, the use of *E. coli* may require additional study to further establish their involvement and efficiency in crude oil degradation. Although this study looked at the diversity of crude oil degrading bacteria at the coast of Ghana, it did not compare the efficiency of crude oil degradation by the individual isolated bacteria. This study is necessary to identify the best performing bacteria to be used for the management of crude oil spill. Furthermore, the genetic identity of the various bacteria isolated from the coast of Ghana should be assessed for possible existence of unique strains with potentially enhanced ability to degrade crude oil.

Author contribution statement

Mark Kwasi Sarfo, Ph.D.: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Samuel Fosu Gyasi, Ph.D.: Conceived and designed the experiments; Analyzed and interpreted the data. Amos Tiereyangn Kabo-Bah, Ph.D.: Conceived and designed the experiments. Bright Adu, Ph.D.; Andrew Sarkodie Appiah: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data. Quaneeta Mohktar, BSc.: Performed the experiments; Contributed reagents, materials, analysis tools or data. Yaw Serfor-Armah: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgements

Special thanks go to the Biotechnology and Nuclear Agricultural Research Institute of the Ghana Atomic Energy Commission, the Regional Center for Energy and Environmental Sustainability (RCEES) of the University of Energy and Natural Resources (UENR) as well as the Noguchi Memorial Institute for their support during the research. Other thanks also go to the communities and fisherfolk for their support during the sampling campaigns.

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