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Characterization of the bacterial community structure and physicochemical properties of produced water from A4 well, north Hamada oilfield in Libya

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ABSTRACT

Produced water is contaminated water that is withdrawn from underground formations to the surface during oil and gas production. Samples from A4 Well - North Hamada Oilfield were collected and analysed to evaluate the quality of the produced water. Physico chemical analysis such as pH, salinity, electrical conductivity, total dissolved solids (TDS), sulphate, carbonate, bicarbonate, hardness, heavy metals, total petroleum hydrocarbons (TPHs) and Benzene, Toluene, Ethylbenzene and Xylene (BTEX) were analysed. The radioactive survey was conducted to measure the activity of alpha (α) and beta (β) particles and gamma (γ) rays. Culture-independent approach was carried out to investigate the bacterial diversity of produced water. 16S rRNA gene amplification technique using a universal bacterial oligonucleotide primer set was conducted. The results show wide variations in the different properties measured. The key contaminants of concern in the produced water that is more likely to affect the environment negatively are high concentrations of the following: salts content (109980 ± 129.12 m g/L of TDS), EC (169200 ± 105.36 µS/cm), salinity (84158 ± 50.48 mg/L), TPHs (58.5 ± 1.55 mg/L), BTEX (57.87 ± 2.65 mg/L) and some heavy metals (Ba 11.9 ± 1.1ppm, Fe 311 ppm ± 12.8 ppm, Se 3.61± 0.42 ppm and Sr 122.± 3.6 ppm). Two oil-degrading bacterial strains were isolated. The isolates are closely related to *Kocuria rosea* strain DSM 20447 with 99.81% gene sequence similarity and *Nocardia coubleae* strain OFN N12 with 98.57% gene sequence similarity. This study emphasises the importance of studying the microbial communities' structure that inhabit such oil by-products using genetic techniques.

Keywords: produced water; North Hamada oilfield; *K. rosea* DSM 20447; *N. coubleae* OFN N12; Libya.

1. Introduction

During oil and gas production, a considerable amount of water trapped in subsurface formations is generated and comes out to the surface, it is known as brine or formation water [1,2]. Produced water has characterized as very salty water with a level of salinity up to four times higher than the salinity of seawater. This is

because of the fact that produced water contains high concentrations of mineral salts, in addition to the organic matters represented in oil, grease, and volatile organic compounds such as (BTEX), polycyclic aromatic hydrocarbons (PAHs), organic acids and phenols [3]. The physicochemical and biological properties of produced water greatly vary, depending on the geological formation of reservoirs, the geographical location of the field and the type of product, whether it is gas or oil [4,5].

Petroleum reservoirs are extreme ecosystems for microbial life due to their high toxicity, hydrophobicity and low water activity, high temperature, salinity, and pressure. Therefore, they house a unique and complex ecosystem of microorganisms that can tolerate and adapt to these harsh conditions [6]. However, oil reservoirs offer a wide range of habitats for several species of microorganisms, such as bacteria and archaea. It attributes to the fact that oil reservoirs contain various phases such as crude oil, produced water and solid surfaces from rock and organic matter; thus, a broad spectrum of these organisms has been isolated from the produced water obtained during oil production[7,8].

Two bacterial identification approaches, culture-dependent and culture-independent are used to identify the bacterial species isolated from produced water that has been obtained from petroleum reservoirs worldwide. For instance, in the North Sea [9], Japan [7], California [10,11], Siberia [12], China [13,14] and Brazil [15]. This is including fermentative organisms, hydrocarbon-degrading bacteria (HDB), methanogens, nitrate-reducing bacteria NRB, manganese and iron reducers, sulphate reducers SRB, aerobic organisms and acetogens [16].

The microorganisms that were isolated and identified in these studies have enhanced not only our understanding of petroleum microbiology but also improved environmental applications and created new industries. They include but are not limited to the oil spill treatment [17] and play an active role in the microbial enhancement of petroleum recovery [18,19].

Kocuria is a Gram-positive coccoid bacterium whose cells are arranged in pairs, short chains, mostly tetrads and irregular clusters[20]. Its species are members of the phylum of Actinobacteria, class Actinobacteria, order Actinomycetales and family Micrococcaceae [21]. Members of this genus are aerobic, non-encapsulated, oxidase-negative, non-spore-forming and catalase-positive. They were isolated from different environmental and ecological habitats with more than 18 identified *Kocuria* species based on the 16S rRNA phylogenetic studies [22].

K. rosea has smooth, creamy to pinkish colonies on nutrient agar and tolerates extreme conditions [23]. The recent study of Méndez *et al.*, [24] confirmed the isolation of the *K. rosea* strain DSM 20447 from a petroleum oil-contaminated soil in Central Chile, using 16S rRNA gene sequences analysis. They characterised the bacterium as a hydrocarbon-degrading bacterium. Akbari *et al.*, [25] also isolated *K. rosea* strain ABR6 from oil storage tanks of the Isfahan Oil Refining Company in Iran. The isolated bacterium has been recommended to be used as a crude oil recovery tool from petroleum sludge.

The genus *Nocardia* is a group of saprophytic and can be found worldwide. *Nocardia* members are gram-positive, actinomycetes, filamentous and branching with colonies that produce aerial hyphae [26]. There are more than 90 identified species of *Nocardia* based on the latest update to the taxonomy of this genus [27].

The strains of *N. coubleae* were previously isolated from oil products. Two novel strains of the *Nocardia* genus were isolated from the contaminated soil by crude oil in the Ahmadi oilfield in Kuwait during the Gulf war in 1997 [28]. They were proposed as *N. coubleae* OFN N11 and *N.coubleae* OFN N12T. However, there is no previous study that reports the isolation of *K. rosea* or *N. coubleae* from produced water.

Despite all the economic growth of the Libyan state, to which the oil industry has contributed mainly, the available knowledge about the microbial diversity of petroleum products and

especially the produced water is extremely limited. Therefore, a comprehensive characterization of the microbial structure of these by-products from the oil-rich region is much needed.

The objectives of this study were to provide a complete physicochemical characterization of produced water from the A4 Well, North Hamada Oilfield in Libya, and to introduce an approach for microbial identification of the unique bacterial isolates from this type of water using 16S rRNA gene sequencing analysis.

2. Material and methods

2.1. Site description and sample collection

The North Hamada oilfield was discovered in 1959, and the first oil well was drilled there on 18th November 1973. It is located 250 miles south of Tripoli, on the southern bank of the Ghadames basin, between 29’ 00’ to 29’ 40’ N and 12’ 35’ to 13’ 10’ E [29].

The trip to the oilfield started on 31/8/2021 and was lasted four days. During the field visit, it was found that the well has 4 tanks and one separator. Samples were collected from Tank 2, Tank 3, and the separator. Samples of two tanks were oil, while the separator sample contained produced water. Therefore, the oil-production water sample from the separator was collected in Sterile Pyrex bottles in triplicates (N = 3). Sample replicates were stored at (4 °C) in darkness until used. Field parameters were measured before the samples were transported to the laboratory of Libyan Petroleum Institute (LPI). Produced water samples for heavy metals analysis were filtered with 0.45 mm filters (Whatman™ membrane filters, Germany) and acidified with nitric acid (HNO_3 with the concentration of 15.6 M) to pH < 2 as described in [30].

2.2. Physicochemical properties of produced water

Physicochemical properties of produced water including pH, electrical conductivity (EC), dissolved oxygen (DO) and carbon dioxide CO_2 were measured in the field laboratory according to the standard methods Table1. The rest of properties such as Bicarbonate, Carbonate, Chloride, salinity, total Hardness, Calcium Hardness, Magnesium Hardness, Calcium, Magnesium, Sodium, Sulphate, TDS, TPHs, BTEX and heavy metals, were analyzed once the samples arrived to the LPI Laboratories according to the standard methods mentioned in table 1 [31].

Table 1. Physicochemical analytical methods used to characterize the produced water

Property	Method	Instruments and manufacturers
EC	ASTM D-1125	HI99301Hanna EC Meter, Italy
pH – value @ 25 ºC	ASTM D-1293	Knick digital pH meter 646, Berlin-Germany
CO_2	ASTM D-513	AZ-0002 pSense High Accuracy CO2 Meter, Madrid-Spain
Bicarbonate	ASTM D-1067	Titration with standard acid
Carbonate	ASTM D-1067	Titration with standard acid
Chloride	ASTM D-516	Titration with silver nitrate
Total Hardness	ASTM D-1126	Titration with Na_2H_2 EDTA
Calcium Hardness	ASTM D-1126	Titration with Na_2H_2 EDTA
Magnesium Hardness	ASTM D-1126	Titration with Na_2H_2 EDTA
Calcium	ASTM D-511	Titration with standard EDTA
Magnesium	ASTM D-511	Titration with standard EDTA
Sodium	ASTM D-2791	Corning 400 Flame Photometer,UK
Potassium	ASTM D-2791	Corning 400 Flame Photometer,UK
Sulphate	DR 2500	Gravimetric method using barium chloride

TDS	ASTM D 5907	-
TPHs	USA EPA 413.2	Horiba OCMA-500 Oil Content Analyzer, HORIBA Jobin Yvon IBH Ltd, UK
Salinity	Calculated as NaCI (mg/l) (Cations + Anions)(	-
DO	Polarographic measurement method	HI9146-04 Hanna DO Meter, USA
BTEX	5021A	GC-FID, Thermo Fisher Scientific Inc, USA
Heavy Metals	EPA 200.7	AAS ICP-OES, Agilent Technology, USA

During the sampling, the survey for Naturally Occurring Radioactive Material (NORM), including α, β particles and ɣ rays, was conducted using Digilert 100 Handheld Radiation Detector (Keison Products, UK) and TracercoTM T202 (Johnson Matthey Public Limited Company, UK).

2.3. Bacterial isolation, purification and morphological characterization

Bacterial cells of the production water were collected and filtered (0.22 µm pore size) under aseptic conditions. The pure cultures of bacteria were obtained using a dilution plate technique. Bacterial cells on the filters were resuspended in sterile saline solution and tenfold serial dilutions were made to the dilution of 10^7. 0.1 mL of suspensions were plated on nutrient agar medium (Oxoid CM0003B nutrient agar, Thermo Fisher Scientific, UK) and then incubated at 37 °C for 48 h. Thereafter, colonies were picked and purified twice. Stock cultures of each isolate were prepared from an individual colony from the second round of purification. The isolated colonies were then morphological characterised based on their colour, size, shape, texture, aspect, and height. The gram stain technique was carried out to confirm the purity of the strained cells. Finally, the microscope examination (BRESSER Researcher Bino 40-1000x Microscope, Germany) was performed to observe the morphological features of the isolated bacteria.

2.4. Genetic identification of bacterial isolates

2.4.1. Genomic DNA extraction

The isolated and purified colonies were carefully collected and inculcated in 5 mL of liquid medium prepared using Accumix Nutrient Broth (Tulip Diagnostics (P) Ltd, India), then incubated over-night at 37°C under 250 rpm agitation. 1 mL of the liquid culture was used for DNA extraction using the EasyPure® Bacteria Genomic DNA Kit (TransGen Biotech, Beijing), and the steps followed the manufacturer's guidelines.

2.4.2. PCR amplification of 16S rRNA gene

The 16S rRNA gene was used to identify the isolated bacteria. The target gene was amplified by polymerase chain reaction (PCR) using universal primers set (16S-27F and 16S-1492R) is shown in table 2. The reaction mixture for PCR amplification contains a green master mix of 25 µL mixed with 2 µL of each forward and reverse primer (10 pmol/ µL) and 5 µL of template DNA and equal volume to 50 µL by added nuclease-free water. The PCR cycles were as follows: initial denaturation at 94°C for 2 min, denaturation, 35 cycles at 94°C for the 40s, annealing at 55°C for 30s, and 1 min of extension at 72°C. Then Cycling was completed by a final extension step at 72 °C for 10 min. Thereafter, PCR products were checked on an agarose gel.

Table 2. Primer sequences used for PCR amplification and sequencing

Primer	Primer sequence

1492R	5'-CGGCTACCTTGTTACGACTT-3'
27F	5-'AGAGTTTGATCCTGGCTCAG-3'

2.4.3. Sequence of PCR products

PCR products were purified using the ExoSap-It kit (Applied Biosystems, USA). Sequencing analyses were carried out on the purified PCR products using the Big Dye Terminator V3.1 mix (Applied Biosystems, USA). Sequencing reaction products were purified using the BigDyeXteminator kit (Applied BiosystemsUSA). Traces were then produced by capillary electrophoresis using the 3730xl DNA Analyzer (Applied Biosystems, USA). The obtained 16S rDNA sequence data was then aligned with known 16S rDNA sequences in the Gen bank to identify the isolated bacteria. The basic local alignment search tool (BLAST), at the National Center for Biotechnology Information (NCBI) was used for alignments https://blast.ncbi.nlm.nih.gov/Blast.cgi [32]. The percentage of homology scores was used to identify the bacterial isolates.

3. Results and Discussion

3.1. Physicochemical and radioactivity characteristics of produced water

The results of physicochemical properties (Table 3) indicated that most properties have higher values than the recommended values of World Health Organization (WHO) regulations [33] and the United States Environmental Protection Agency guidelines (USEPA) [34]. The high value of EC (169200 ± 105.36 µS/cm) may be explained by the fact that it is a reflection of the concentration of the TDS (109980 ± 129.12 mg/L) in the sample. A higher concentration of TDS increases the EC. This finding is consistent with that of Joel *et al.*, [35] who suggest that the high value of TDS could be due to suspended and dissolved solids inherent in the formation of the water samples. An observation worth mentioning from the values in table 3 is that the pH (5.51 ± 0.01) value is considered to be lower than the accepted limits 6-9. It may be due to the high concentration of CO_2 (20240 ± 8.54 mg/L), which in turn led to the formation of carbonic acid.

The radioactivity survey was performed during the sampling. The outcomes of screening levels of the total radioactivity presented in the form of α, β particles and ɣ radiation revealed that the concentrations were lower than the recommended activity concentrations of WHO [33]. Therefore, no further radioisotope-specific analysis is required.

Table 3. Physicochemical and radioactivity properties of produced water (N = 3)

Property	Results (Mean ± SD)
EC	169200 ± 105.36 µS/cm@25ºC
pH – value @ 25 ºC	5.51 ± 0.01
CO_2	20240 ± 8.54 mg/L
Bicarbonate	189 ± 8.718 mg/L
Carbonate	0 mg/L
Chloride	94515 ± 63.41mg/L
Total Hardness	66000 ± 86.9 mg/L
Calcium Hardness	32100 ± 67.27 mg/L
Magnesium Hardness	33900 ± 57.61 mg/L
Calcium	12840 ± 32.08 mg/L
Magnesium	8238 ± 38.57 mg/L
Sodium	18400 ±152.97 mg/L
Potassium	7800 ±40.36 mg/L
Sulphate	200 ± 13.45 mg/L
TDS	109980 ± 129.12 mg/L
TPHs	58.5 ± 1.55 mg/L
Salinity	84158 ± 50.48 mg/L
DO	0.09 ± 0.02 mg/L

α, β Particles	± 0.03 µSv/hr
ɣ rays	0 Bq/cm^2
Total radiation	0.179 ± 0.018 µSv/hr

Results of the volatile aromatic hydrocarbons of BTEX concentrations have shown in the table 4. Four volatile organic compounds, namely ethylbenzenes, p,m-Xylene and o-Xylene exceeded the Permissible limits of WHO [33]. Gas Chromatography (GC) analyses of the volatiles show that benzene and toluene concentrations were less than the detection limits. Although benzene is slightly soluble in water, it has a concentration of less than ethylbenzene (11.95 ± 0.15 ppm) in the water sample. It may stand to reason that biodegradation is relatively slower for some volatile aromatic hydrocarbons than others. In General, BETX usually cannot be detected far from the discharge point because of the volatile nature of such aromatic hydrocarbons.

As shown in the Table below, most of the produced water content of hydrocarbons (TPHs 58.5 ± 1.55 mg/L) was volatile hydrocarbons represented by BTEX (57.87 ± 2.65 ppm). A possible explanation for this might be that the efficiency of water separation and treatment processes was low. Therefore, to prevent the negative environmental impacts of such organic pollutants, further polishing such as physical, biological, thermal and or chemical treatments are required to be carried out.

Table 4. Concentrations of volatile hydrocarbons (BTEX) in produced water, N = 3, data expressed as a mean ± SD

Volatile hydrocarbon	Conc (ppm)	Volatile hydrocarbon	Conc (ppm)
Benzene	<0.001	p,m-Xylene	7.20 ± 0.38
Toluene	<0.001	o-Xylene	38.71 ± 3.27
Ethylbenzenes	11.95 ± 0.15	Total BTEX	57.87 ±2.65

Several heavy metals have been detected in the produced water sample; the results are depicted in Table 5. Analytical characterization of heavy metals in the produced water showed a variation in heavy metals. The results revealed a high concentration of Ba, Ni, Fe, Se, Cd and Sr (11.9 ± 1.1, 0.08 ± 0.003, 311 ± 12.8, 3.61± 0.42, 0.01 ± 0.002 and 122.± 3.6 ppm respectively), which exceeded the permissible limits under the provisions of the Libyan specifications No. 10 for the year 2008 [36]. The metals concentration of Cr, Cu, Zn and Hg (0.01 ± 0.004, 0.01± 0.003, 2.81± 0.3 and < $5x10^{-6}$ ppm respectively) were within the permissible limits of the Libyan specifications No. 10 for the year 2008 [36]. The rest of the heavy metals concentrations, Pb and As were lower than the detention limits. This variation in the heavy metals in the produced water can be explained by the geological formation and the age of the oil well [37].

Table 5. Heavy metals concentration in produced water (ppm) N = 3, data expressed as a mean ± SD

Element	Conc (ppm)	Element	Conc (ppm)
Ba	11.9 ± 1.1	Pb	< 0.03
Ni	0.08±0.003	Cd	0.01 ± 0.002
Cr	0.01±0.004	Se	3.61± 0.42
Cu	0.01±0.003	Sr	122.± 3.6
As	< 0.02	Zn	2.81± 0.3
Fe	311± 12.8	Hg	< $5x10^{-6}$

3.2. Isolation and genetic identification of bacterial community of produced water

3.2.1. Morphological characteristics of isolates

Two pure bacterial strains were isolated from produced water Figure1 using the dilution plate technique. The colonies grew on nutrient agar plates after 24-48 h of incubation under aerobic conditions. The first strain of the isolated bacterium was Gram-positive, cocci in shaped formed pairs or clusters under the microscope, 1–2 mm in size, small, smooth texture, and pale-cream to pale-pink Figure1, A. The colonies of the second isolate were Gram-positive bacillus bacterium, dry, plaster, star-shaped, chalking wrinkled and white Figure1, B.

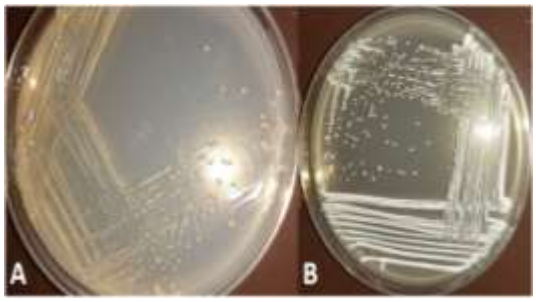

Fig. 1. Isolated colonies from produced water on solidified nutrient agar medium. The first and second bacterial isolates were labelled as A and B respectively

3.2.2. Genetic characterisation of bacterial isolates

Despite the phenotypic characterisation tools and biochemical assays to identify the bacterial species still being in practice applied, the genetic approach has become imperative and widely used for bacterial identification.

A 16S rRNA gene sequence analysis was used in this study to overcome the drawbacks of using traditional methods. The bacterial isolates were identified and characterised to the strain level. The 16S rRNA gene sequences quality shown in the figure 2. The BLASTn search tool revealed that isolate (A) has a high degree of gene sequence identity (99.81%) with *K. rosea* strain DSM 20447 (GenBank accession number NR_044871.1). The isolate (B) has 98.57 % gene sequence identity to *N. coubleae* strain OFN N12 (GenBank accession number NR_104567.1) (Table 6). Therefore, based on 16S rRNA gene sequence analyses, bacterial isolates are classified into the genera of *Kocuria* and *Nocardia*. 27F cleaned sequences for both isolates are depicted in the appendix.

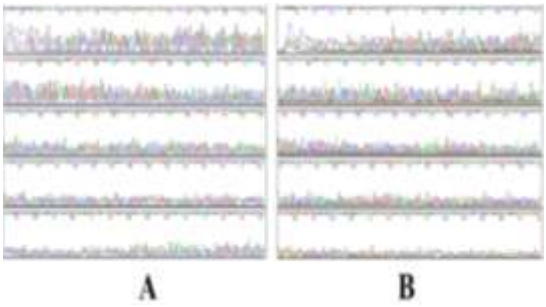

Fig. 2. Chromatograms depicting 16S rRNA gene sequence quality; A) Bacterial isolated of *K. rosea;* B) Bacterial isolate of *N. coubleae*

Table 6. BLAST results of the 16 S rRNA gene sequences of the isolated bacteria

No	Scientific Name	Max Score	Query Cover %	E value	Identity %	Length bp	Accession number
A	Kocuria rosea strain DSM 20447	1884	100	0	99.81	1481	NR_044871.1
B	Nocardia coubleae strain OFN N12	1755	100	0	98.57	1322	NR_104567.1

Ecosystems such as polluted water, which is known to demonstrate an alkaline or acidic nature, tend to be extreme and complex environments. It is due to the high concentration of various toxic organic and inorganic contaminants that can harm the environment [38]. The harsh physicochemical characteristics of these environments make them act as a home for bacterial strains that have been shown to have efficient levels of pollutant degradation under laboratory conditions [39].

In this study, the bacterial strains of *K. rosea* and *N. coubleae* have been isolated from oil-contaminated environments and described as an oil-degrading bacterium several times in the literature [24, 25, 40-42]. However, the current study is the first study that isolated *K. rosea* and *N. coubleae* from the produced water.

This study shows that the bacterial isolates tolerated the high content of TPHs 58.5 ± 1.55

mg/L and a total BTEX of 57.87 ± 2.65 ppm. The results provide a further support to the hypothesis that the isolates of *K. rosea* strain DSM 20447 and *N. coubleae* strain OFN N12 are more likely to be oil-degrading bacteria that inhabit such an extreme environment. It is encouraging to compare these results with that found in Wu *et al.*, [40], who isolated and identified another strain of the *Kocuria* genus, which is *Kocuria sp*. strain TIBETAN4, from soils around Qinghai Lake in China. The isolate was able to tolerate up to 12.5 mM phenol and enable to degrade 50 mM phenol within less than four days. Therefore, it has been described as an effective phenol-depredating bacterium.

According to the genotypic and phenotypic results described above, it is clear that the utility of the genetic approach (16S rRNA sequencing) is a promising tool in microbial identification that inhabits the oil reservoirs.

4. Conclusion

To conclude, the constituents present in the produced water that pose an environmental concern are the high levels of salt content, total dissolved solids and electrical conductivity. In addition, chemicals that cause hardness include calcium, magnesium, and sulphates, toxic elements such as heavy metals and the presence of petroleum hydrocarbon families. These results also show that the reservoir has a microbial population associated with oil degradation and has the potential for microbial-enhanced oil recovery.

Therefore, this work strengthens the idea that we must follow the environmental guidelines and regulations to safely dispose of the produced water. In addition, reinjection of the water back to the formation well or dilution with uncontaminated fresh water may be the best way to avoid its negative impacts on the ecosystem. More genetic analyses, such as a quantitative polymerase chain reaction (qPCR) are highly required for a better understanding of genetic characteristics that stand behind the ability of *Kocuria* and *Nocardia* bacteria to tolerate the crude oil.

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