

Bioremediation of Crude and Refined Oil-Polluted Fresh Water Using *Chlorella vulgaris* Isolated from a Pond

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Abstract Crude and refined oil contamination of the aquatic environment is one of the major environmental problems that lead to unmanageable loss of biological life that impacts negatively on global economy particularly in the areas of petroleum production and transportation. Bioremediation potential of *Chlorella vulgaris* isolated from a pond in Uwani, Enugu State, Nigeria was studied using standard methods. The organism utilized crude oil heavily, kerosene moderately and petrol minimally as shown by the varying degree of turbidity produced during fourteen days of growth in mineral salts- oil medium. Biodegradation experiment was carried out for forty-two days and the results showed that there was a decrease in pH and an increase in the absorbance of the mineral salts - oil medium. The percentage of degradation of the crude oil, kerosene and petrol by the organism was 80%, 70% and 60% respectively. There was a reduction in the peak numbers and peak areas of the gas chromatograms of the total petroleum hydrocarbons and polyaromatic hydrocarbons of the residual crude oil as well as the residual kerosene and petrol. This work therefore indicated that the microalga *C. vulgaris* can be used for the bioremediation of crude and refined oil - polluted aquatic environments.

Keywords Crude Oil, Kerosene, Petrol, *Chlorella vulgaris*, Pond, Bioremediation

1. Introduction

Petroleum - based products are the major source of energy for the industry and daily life and petroleum is a major pollutant of the environment. Crude oil is a nationally - occurring complex mixture of hydrocarbons and non - hydrocarbon compounds which at appropriate concentration, possess a measurable toxicity towards living

organisms. The toxicity of crude oil and petroleum products varies widely depending on their concentration, composition, environmental factors and the biological state of organisms at the time of the contamination [2].

Nigeria is continually faced with the challenges of oil spillage during exploration processes and transportation. Some of these challenges include contamination of nearby water bodies, death of aquatic organisms and contamination of farmlands. The health hazards created by oil exploration are the major contributors to the disease burden in oil - bearing communities [3]. One promising treatment method is to exploit the ability of microorganisms to remove these organic pollutants from contaminated sites. This alternative treatment strategy that is effective, minimally hazardous, economical, versatile and environmentally - friendly, is known as bioremediation [4].

Some microorganisms have the astonishing, naturally - occurring catabolic diversity to degrade, transform or accumulate a huge range of products including hydrocarbons, polychlorinated biphenyls, radionuclides and metals [5]. Several microorganisms including fungi and bacteria are involved in biodegradation process. Biodegradation processes vary greatly but frequently, the final product is carbon IV oxide [6].

Most of the biological treatment technologies involve the use of bacteria but microalgae have already been applied for effluent treatment either as single species as is the case of *Chlorella*, *Scenedesmus* or *Arthrospira* [7,8] to treat and remove nitrogen, phosphorus and chemical demand in different types of effluents.

Algae play important roles in returning the environment altered by various contaminants to its original state. They are highly adaptive and can grow autotrophically, heterotrophically or mixotrophically in any environment resulting in higher concentration within themselves as compared to the surrounding water [9]. Microalgae are not unique in their bioremoval capabilities but they offer

advantages over other biological materials in some conceptual bioremoval process schemes. Absorption of heavy metals by algae can be an effective process for the removal and recovery of heavy metals ions from aqueous solution [10].

Pollutant - degrading mixotrophic algae are excellent agents for the remediation of carbon - polluted environments. [11]. Examples of algae widely used and studied in bioremediation include *Chlorella vulgaris*, *Anabaena inaequalis*, *Ascophyllum nodum* and *Nostoc spp.* *Chlorella vulgaris* and *Scenedesmus dimorphus* are highly efficient for ammonia and phosphorous removal during biotreatment of oil - contaminated water.

Monteiro et al. [12] observed that strains of the *Scenedesmus obliquus* tested have proven effective in removing a toxic heavy metal cadmium from aqueous solution, hence supporting their choice for bioremediation strategies of industrial effluents. Raposo et al. [13] analyzed the capacity of *Chlorella vulgaris* and the autochthonous flora of the effluents to remove some of the compounds present in the effluents.

Over the decades, oil spillage has adversely affected the rivers and streams especially in the oil - producing communities. Plants and animals at these contaminated sites are also adversely affected, hence the need to completely clean - up such sites, therefore in this work, the bioremediation of crude and refined oil - polluted fresh water using *Chlorella vulgaris* was carried out. It is hoped that this study will show that the micro alga *Chlorella vulgaris* isolated from a non-oil impacted environment (pond) possesses the ability to degrade crude oil and refined petroleum products and can be used to remediate oil spillage especially in fresh water environment and should not be regarded as a nuisance organism.

2. Materials and Methods

2.1. Samples Collection

The crude oil was obtained at Eleme Petrochemical Company Limited in Eleme Local Government Area of Rivers State, Nigeria. Samples of Kerosene (DPK) and Petrol (PMS) used were obtained from Anccor Filling Station, Uwani in Enugu South Local Government Area of Enugu State, Nigeria while water samples were obtained from a pond located at the premises of Commercial Agricultural Development Programme, Uwani, Nigeria, using sterile screw-capped bottles which were opened and inserted into the pond at a depth of 30cm below the water surface with their mouths downward. They were thereafter turned so that the water flowed into them. The bottles were thereafter aseptically closed and transported to the laboratory in an ice packed container for the isolation, characterization and identification of *Chlorella vulgaris*.

2.2. Isolation of *Chlorella Vulgaris*

This was carried out using the spread plate method described by Robert [14]. An aliquot (0.1ml) of the pond water was spread on the surface of Petri dishes containing sterile *Chlorella agar* using a sterile glass rod. Triplicate plates were prepared which were covered and incubated in an inverted position at 28°C for 7 days.

2.3. Characterization and Identification of the Isolates

The colonial and microscopic characteristics of the isolates were determined according to the scheme of Janse et al. [15]. The isolates were placed in a drop of sterile distilled water on microscopic slides and a drop of iodine was applied to them. The slides were thereafter examined under a compound microscope. The isolates were identified using a catalogue of algae as done by Robert [14].

2.4. Screening Test for Petroleum Hydrocarbons Utilization by *Chlorella vulgaris*

The test was carried out using mineral salts medium as described by Olukunle [16]. The medium was composed of the following (g/l): NaCl, 10.0; MgSO₄. 7H₂O, 0.42; KCl, 0.29; KH₂PO₄, 0.83; NaHPO₄, 1.25, Agar, 20.0 and distilled water, 1 litre. It was prepared and dispensed in 9ml amounts into test tubes. One millilitre of crude oil, kerosene and petrol was separately introduced into the test tubes which were thereafter capped and autoclaved at 121°C for fifteen minutes. Upon cooling, the tubes were inoculated with *Chlorella vulgaris* and incubated at 28°C for fourteen days in a rotary shaker operated at 75 revolutions per minutes after which they were observed for turbidity. Three uninoculated tubes each containing mineral salts –crude oil, mineral salts - kerosene and minerals oil –petrol served as controls

2.5. Time Course Test for Petroleum Hydrocarbons Utilization by *Chlorella vulgaris*

The mineral salts medium described by Olukunle [16] was used. It was prepared and dispensed in conical flasks in 99ml amounts. One millilitre (1%) of crude oil, kerosene and petrol was introduced separately into the flasks which were after that autoclaved at 121°C for fifteen minutes and allowed to cool. Upon cooling, the flasks were separately inoculated with the organism separately and incubated for forty - two days in a rotary shaker operated at 75 revolutions per minute. The pH and absorbance were determined at the beginning and end of the experiment. The pH was determined using a pH meter (JENWAY) while the absorbance was determined using a Spectrophotometer. Three uninoculated tubes each containing mineral salts – crude oil, mineral salts –kerosene and mineral salts –petrol were used as controls

2.6. Determination of the Residual Petroleum Hydrocarbons Content

This was determined as described by Amadi [17]. The content of each flask was sterilized at 121°C for fifteen minutes at the end of the forty- two days of incubation to destroy the *Chlorella vulgaris* and thereafter transferred into a separating funnel. The residual crude oil, kerosene and petrol were thereafter extracted with toluene. The funnel was corked tightly and tilted for five minutes. The flask was shaken and occasionally refluxed to let out air and left to stand for 30 minutes. The toluene-oil mixture was collected in a sample bottle and analyzed spectrophotometrically by diluting the mixture with a known volume of toluene and reading the absorbance at 420nm. A standard graph was prepared using known volume of the toluene.

The residual hydrocarbon content was determined from the formula.

$$GR \times WS$$

$$WT$$

Where GR = absorbance reading at 420nm X slope of the graph.

WS = initial volume of toluene used for the extraction.

WT = weight of the toluene-oil mixture.

$$\text{Percentage residual oil} = \frac{\text{oil applied} - \text{oil recovered}}{\text{oil applied}} \times 100$$

2.7. Gas Chromatographic Profiles of the Petroleum Hydrocarbons before and after Degradation by *Chlorella vulgaris*

The gas chromatographic profile of the petroleum hydrocarbons before and after degradation by *C. vulgaris* was determined as done by Onuorah et al. [18] using Agilent 6890 plus gas chromatograph equipped with ionizing detector, split injector and fused silica capillary column HP-1 of 30m length, 0.35mm internal diameter and 0.5m film thickness. The detector and injector temperatures were maintained at 300°C and 250°C respectively. The column temperature was programmed to rise from 80°C to 300°C with a rate of 3°C/min and final time of fifteen minutes. Nitrogen (O₂ free) was used as a carrier gas at a flow rate of 2ml per minute.

3. Results

The colonial and microscopic characteristics of the *Chlorella vulgaris* isolated from the pond water are shown in Table 1. The organism was small, green in colour, spherical, non – motile and non - flagellated with a single chloroplast with or without pyrenoid.

Table 1. Colonial and microscopic characteristics of the *Chlorella vulgaris* isolated from the pond

Colonial characteristics	Microscopic characteristics
Colonies were small, round and green in Petri dishes.	Colonies were small, non-flagellated, non-motile, spherical with a single, cup-shaped, thin chloroplast with or without pyrenoid

Table 2 showed the result of the screening test for petroleum hydrocarbons utilization by *Chlorella vulgaris*. The organism utilized crude oil heavily, kerosene moderately and petrol minimally.

Table 2. Screening test for petroleum hydrocarbons utilization by *Chlorella vulgaris*

Petroleum Hydrocarbons	Utilization by <i>Chlorella vulgaris</i>
Crude oil	+++
Kerosene	++
Petrol	+

+++ = heavy utilization

++ = moderate utilization

+ = minimal utilization

The changes in pH of the mineral salts -oil medium during forty-two days of degradation by *Chlorella vulgaris* are presented in Table 3. The medium containing crude oil had the lowest final pH of 4.0 while that containing petrol had the highest pH of 4.7.

Table 3. Changes in pH of the mineral salts - oil medium during forty two days of degradation by *Chlorella vulgaris*

Petroleum Hydrocarbons	Initial pH	Final pH
Crude oil	7.2	4.0
Kerosene	7.2	4.5
Petrol	7.2	4.7

Table 4 showed the changes in absorbance of the mineral salts - oil medium during forty-two days of degradation by *Chlorella vulgaris*. The organism had the highest absorbance in the medium containing crude oil while it had the lowest absorbance in the medium containing petrol.

Table 4. Changes in absorbance of the mineral salts - oil medium during forty-two days of degradation by *Chlorella vulgaris*

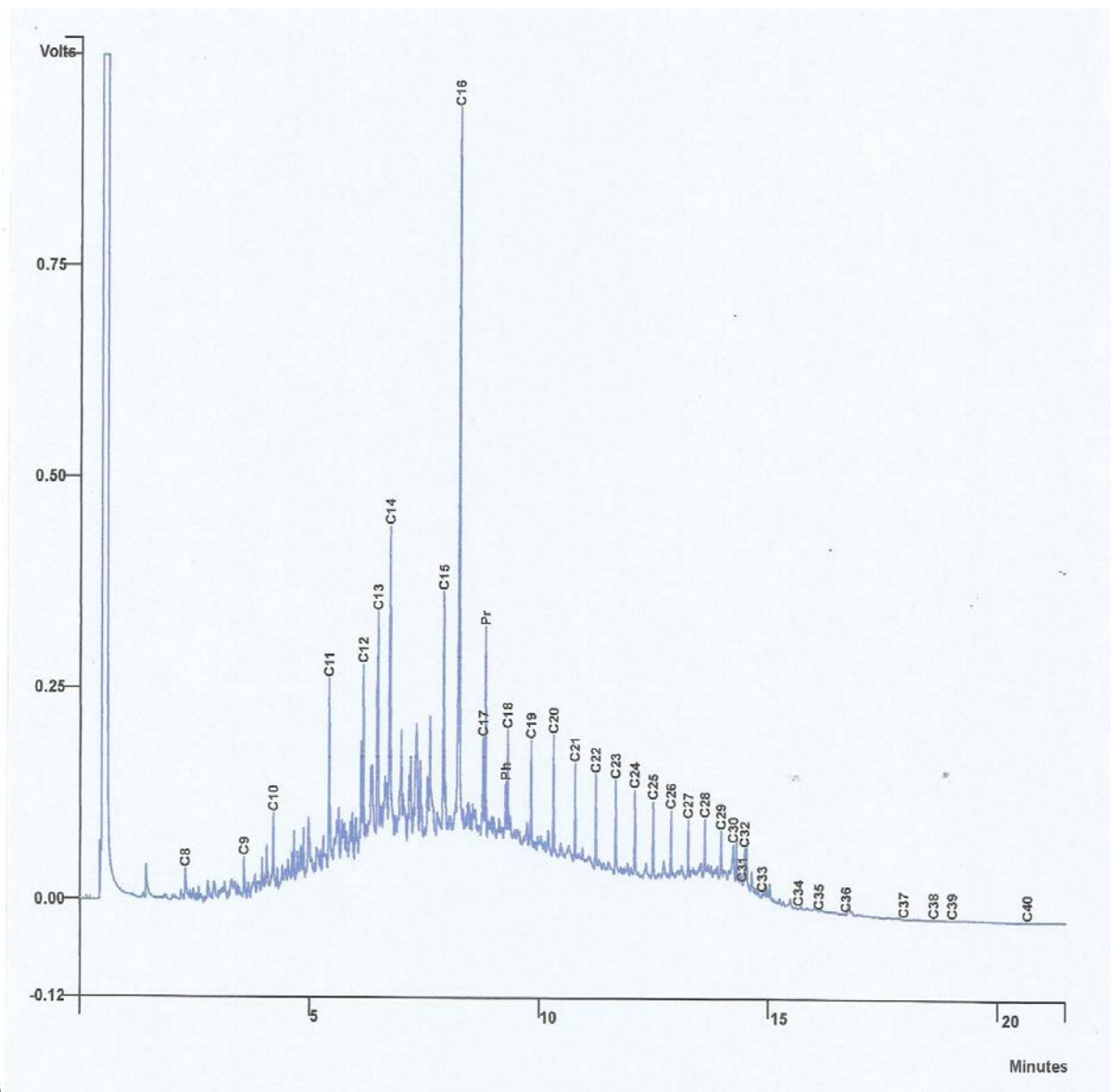
Petroleum Hydrocarbons	Initial absorbance	Final absorbance
Crude oil	0.460	0.928
Kerosene	0.416	0.852
Petrol	0.464	0.875

The residual oil content of the mineral salts -oil medium at forty-two days of degradation by *Chlorella vulgaris* is shown in Table 5. The residual oil content of the medium containing crude oil had the lowest volume while that containing petrol had the highest volume of oil recovered.

Table 5. Residual oil content of the mineral salts - oil medium at forty two days of degradation by *Chlorella vulgaris*

Petroleum hydrocarbons	Initial oil content	Final oil content	Degradation %
Crude oil	1.0	0.2	80
Kerosene	1.0	0.3	70
Petrol	1.0	0.4	60
controls	1.0	1.0	0

Figures 1 – 4 showed the gas chromatographic profiles of the unused crude oil (total petroleum hydrocarbons and polyaromatic hydrocarbons), kerosene and petrol respectively. The peak areas were 13,434, 722; 2,919, 034; 13,955,470 and 5,558, 402 for the total petroleum hydrocarbons and polyaromatic hydrocarbons of the unused crude oil, kerosene and petrol respectively.



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Figure 1. Gas chromatographic profile of the total petroleum hydrocarbons content of the unused crude oil

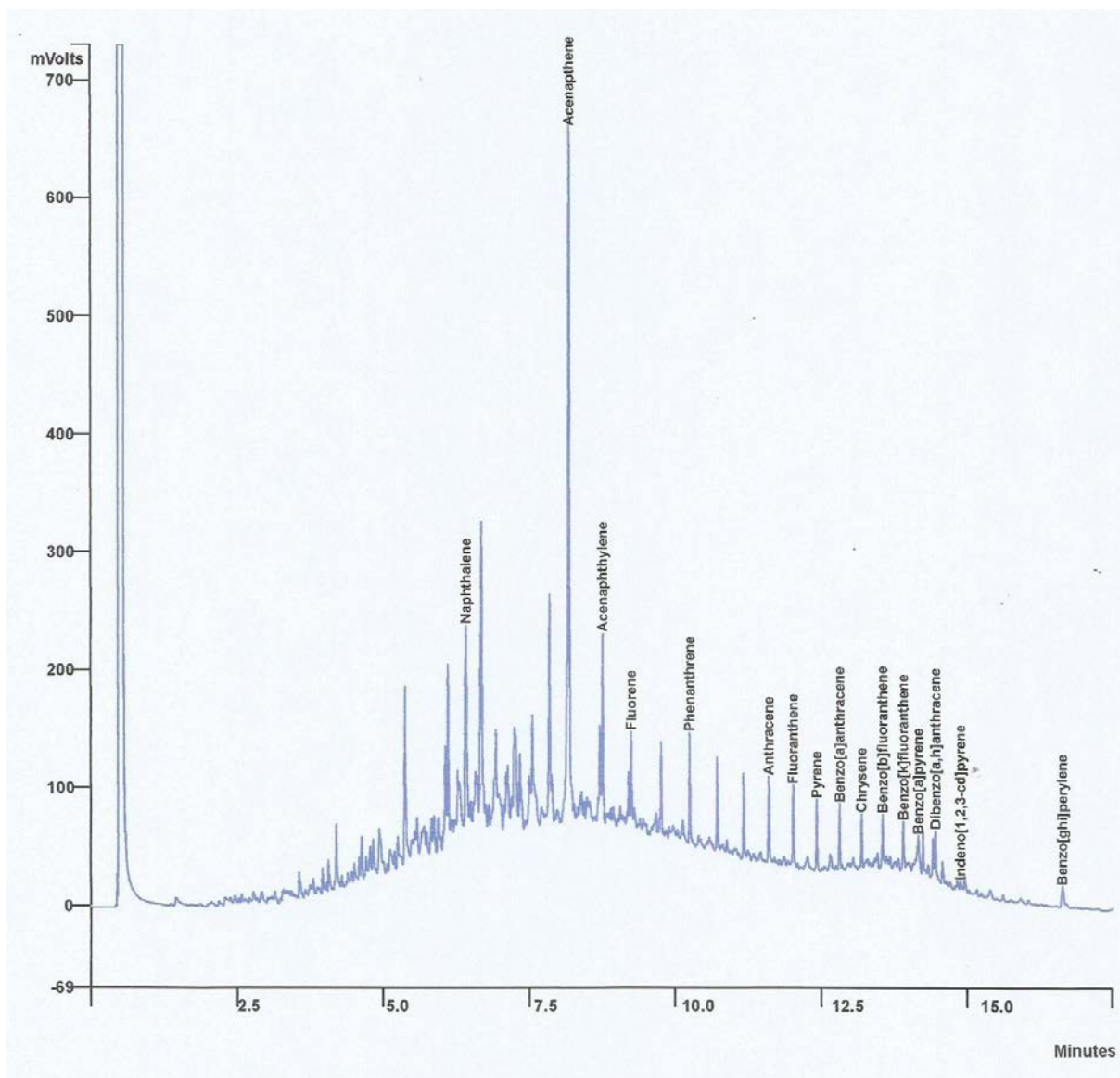


Figure 2. Gas chromatographic profile of the polyaromatic hydrocarbons of the unused crude oil

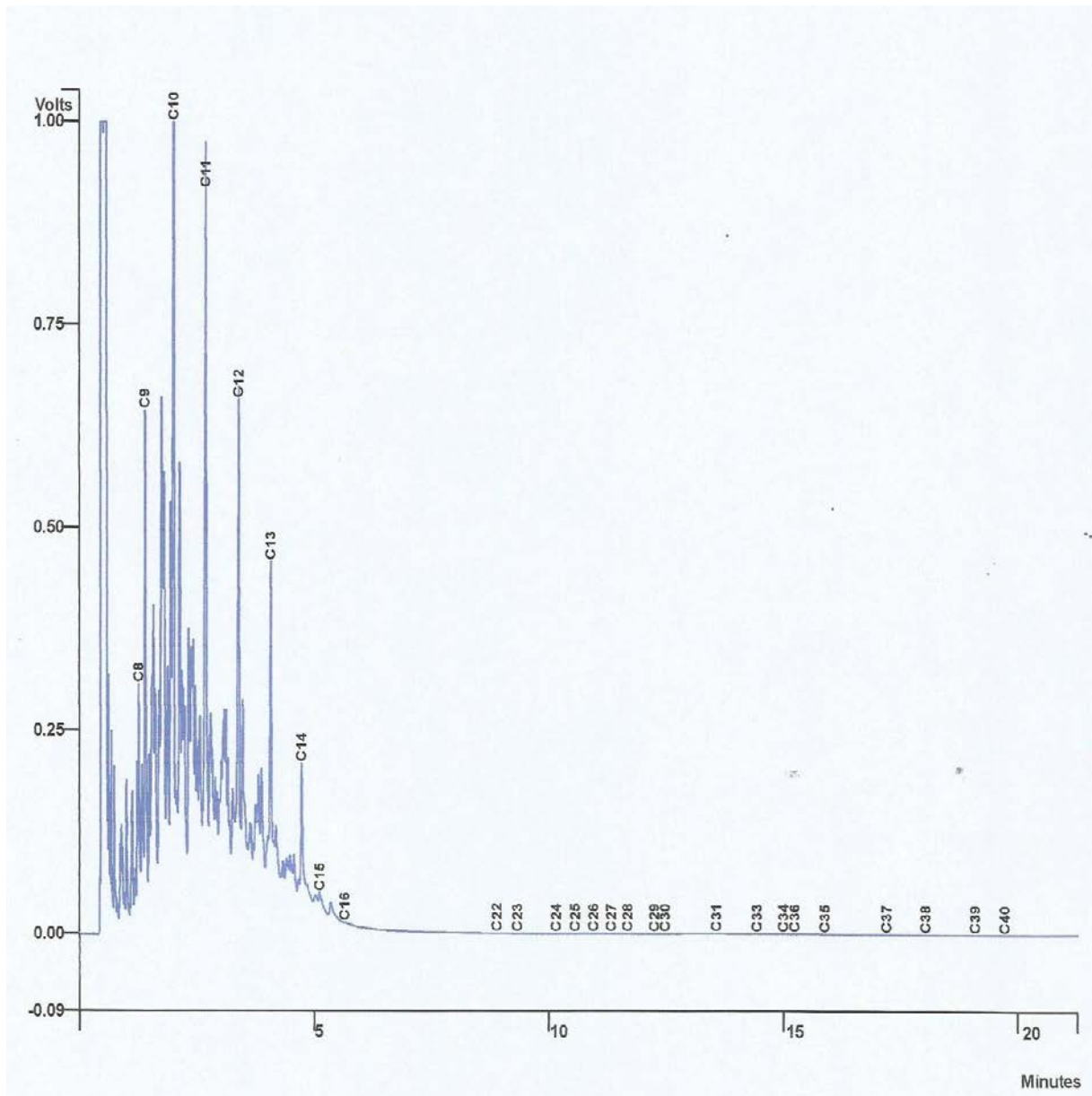


Figure 3. Gas chromatographic profile of the unused kerosene

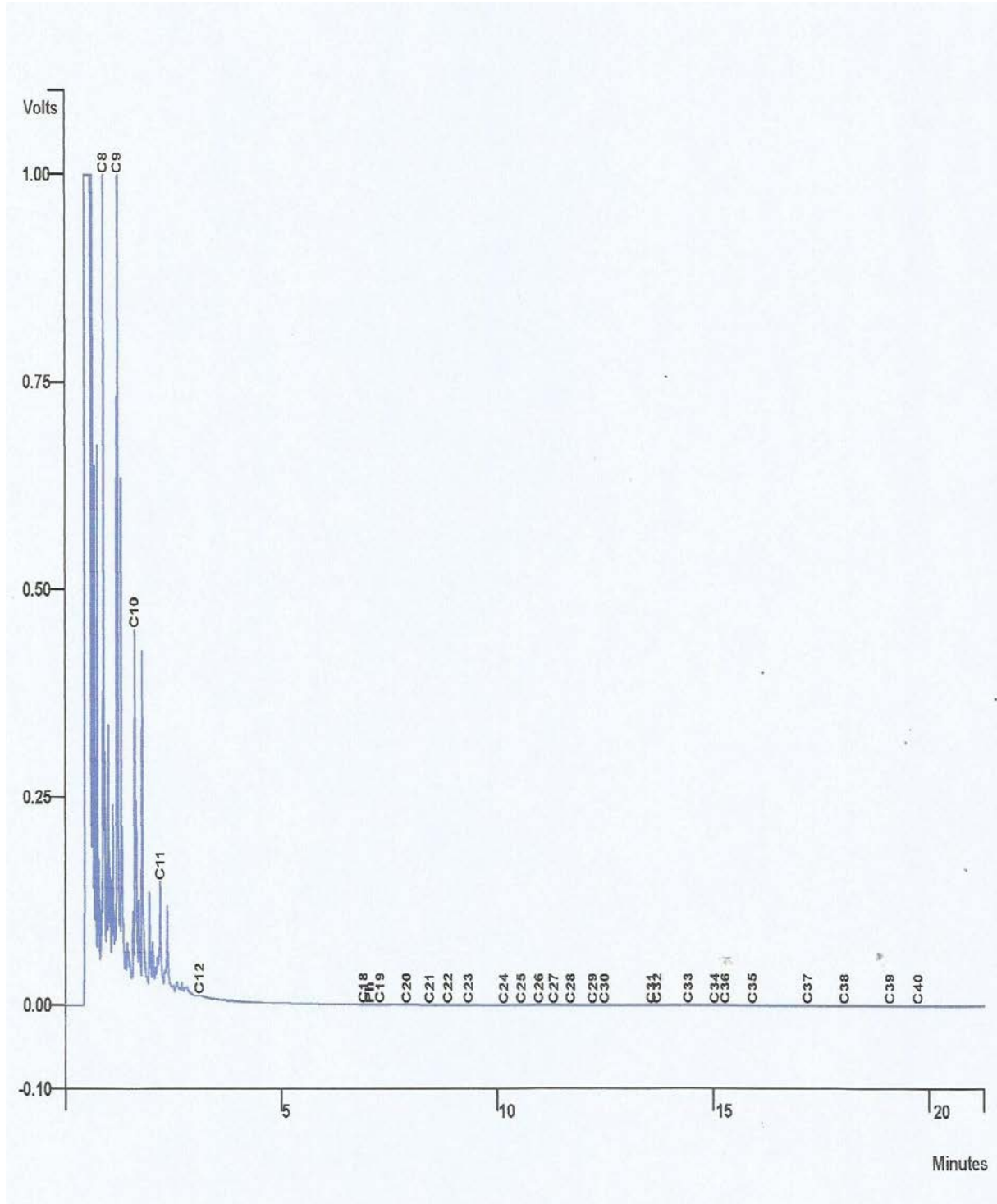


Figure 4. Gas chromatographic profile of the unused petrol

The gas chromatographic profiles of the residual crude oil, kerosene and petrol respectively are presented in Figures 5-8. The peak areas of the total petroleum hydrocarbons and polyaromatic hydrocarbons of the residual crude oil, kerosene and petrol were 246,285; 109,387; 22,668 and 12,283 respectively.

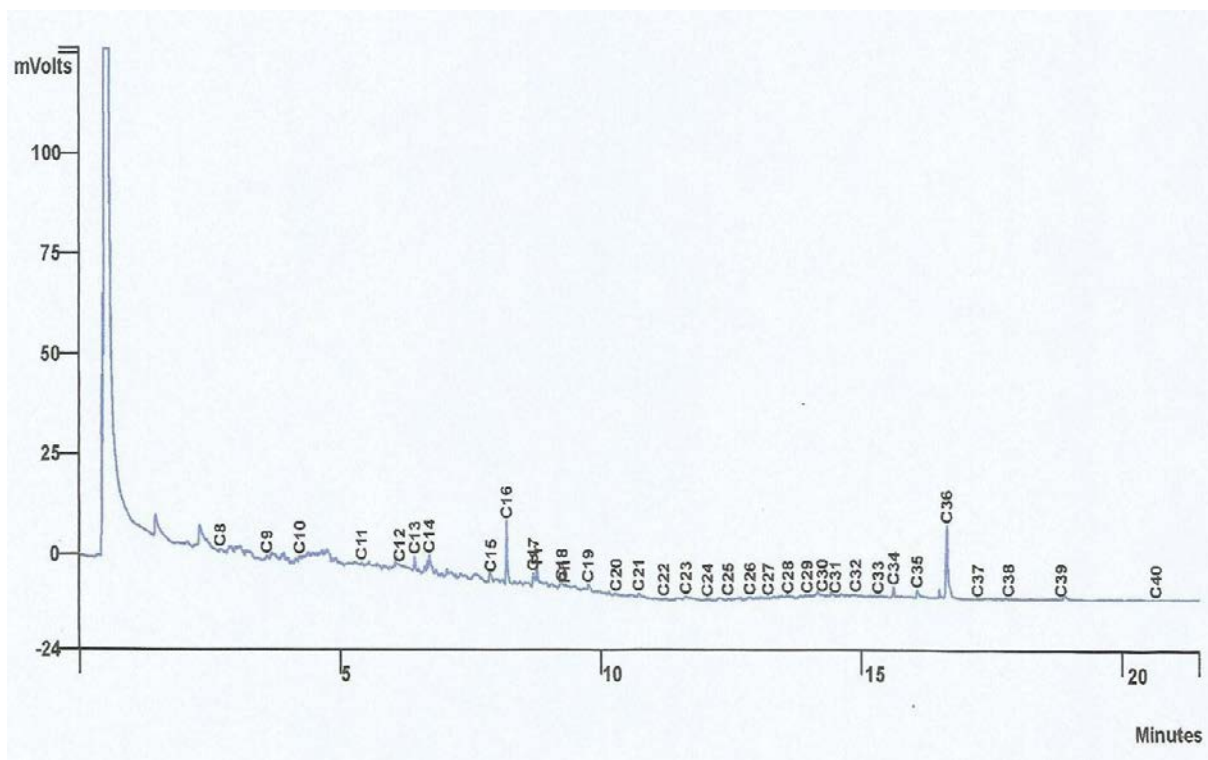


Figure 5. Gas chromatographic profile of the total petroleum hydrocarbons of the residual crude oil

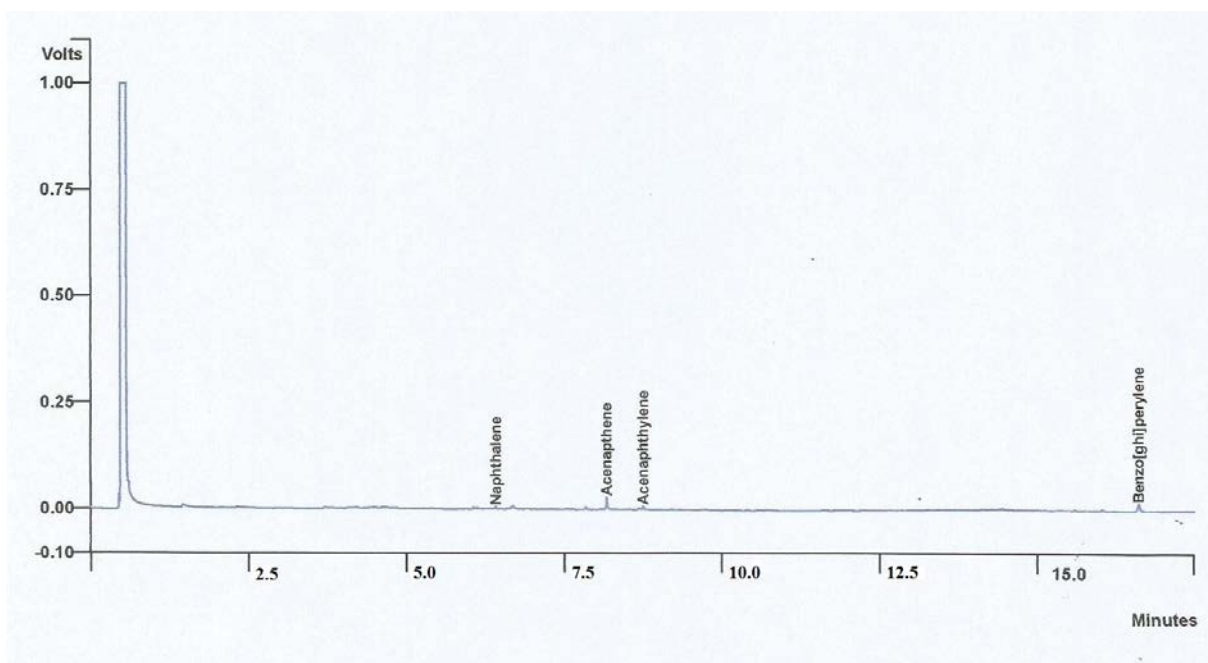


Figure 6. Gas chromatographic profile of the polyaromatic hydrocarbons of the residual crude oil

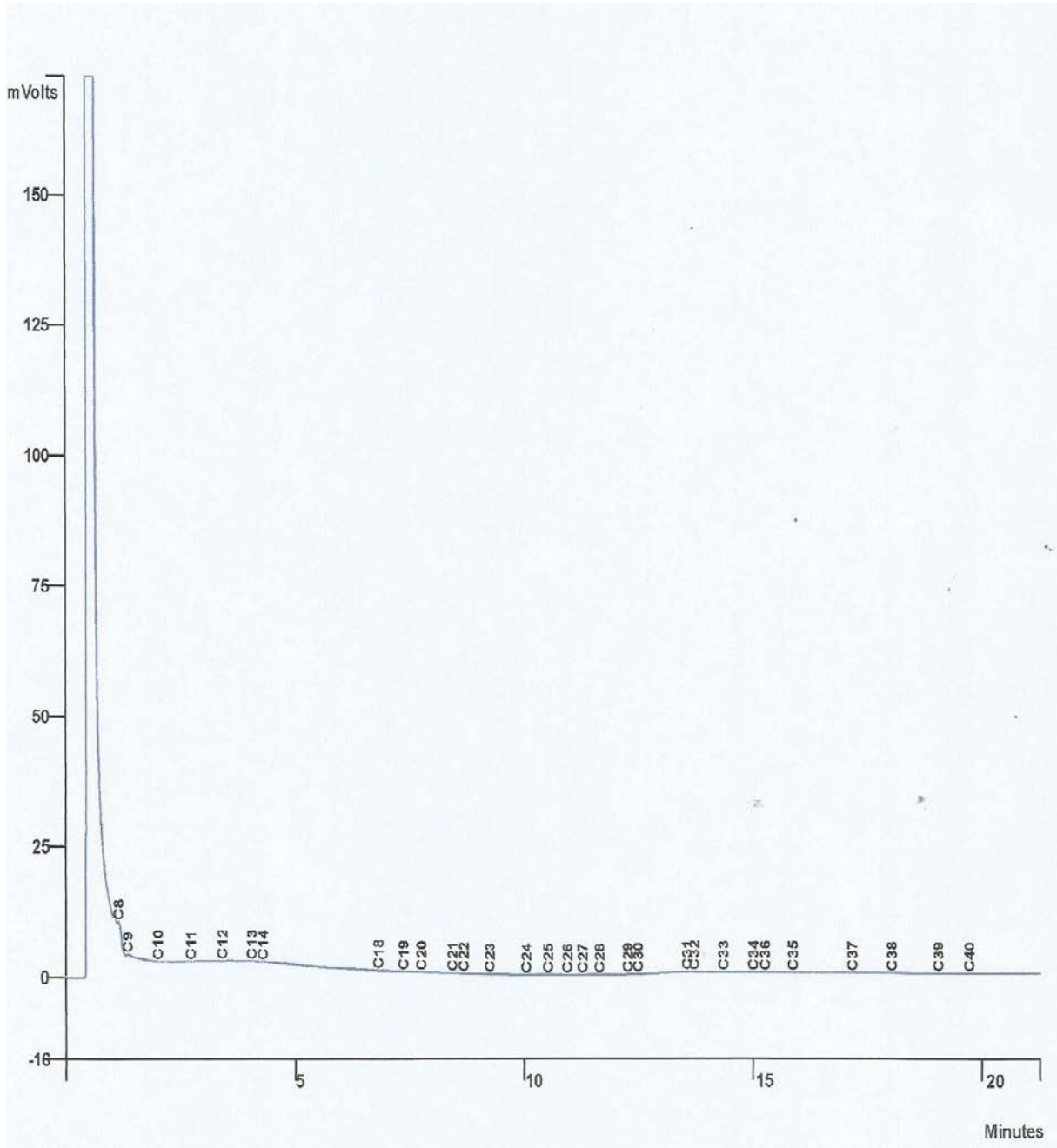


Figure 7. Gas chromatographic profile of the residual kerosene

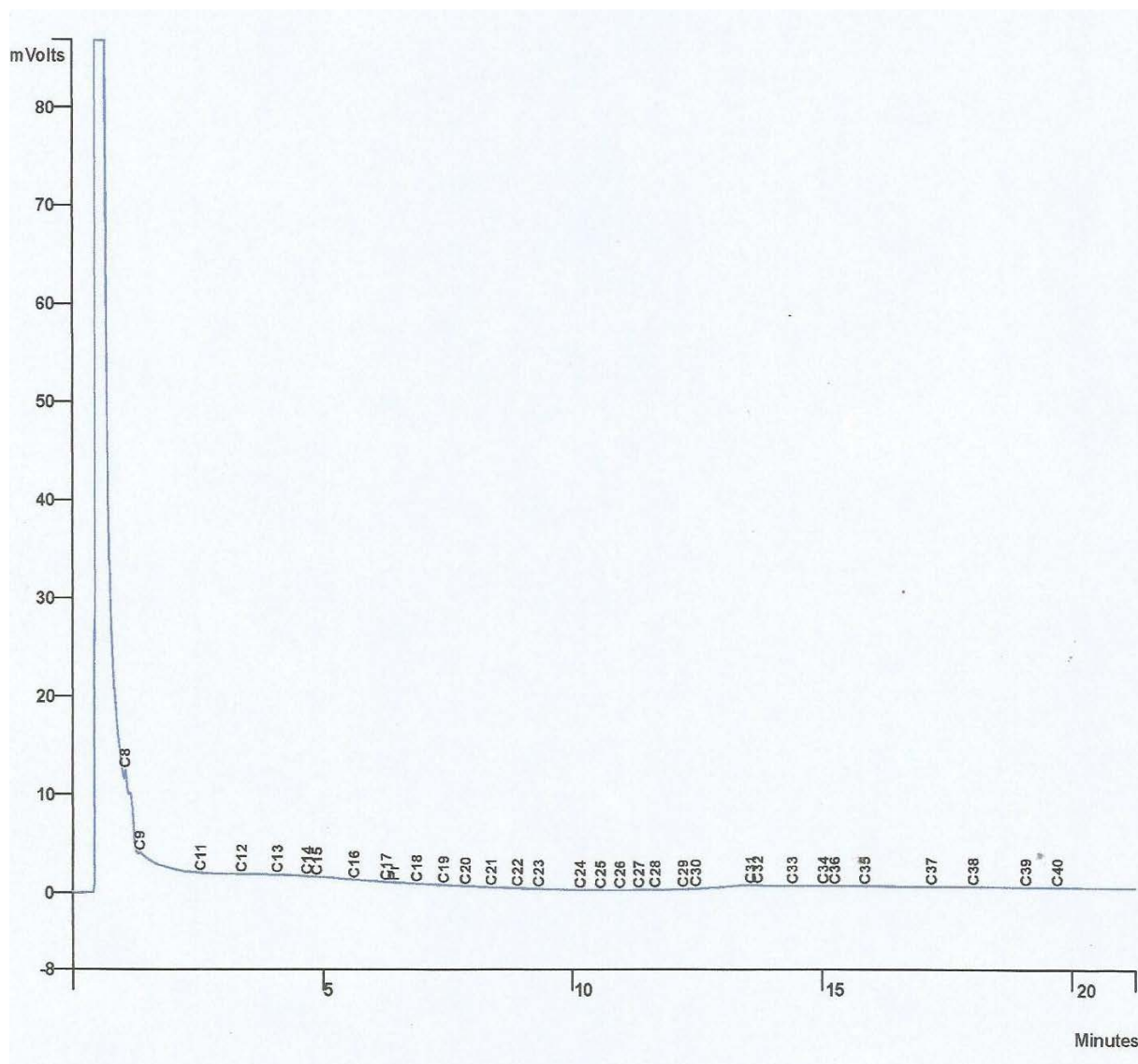


Figure 8. Gas chromatographic profile of the residual petrol

4. Discussion

The algae isolated from the pond water were identified as *Chlorella vulgaris* (Table 1). This result agreed with Janse et al. [15] who also identified *Chlorella vulgaris* from fresh water.

The result of the screening test for Petroleum hydrocarbons utilization by *Chlorella vulgaris* showed heavy crude oil utilization, moderate and minimal utilization of kerosene and petrol respectively as shown by the varying degree of turbidity produced in the mineral salts -oil medium (Table 2)

There was a progressive decrease in the pH of the mineral salts - oil medium at forty-two days of petroleum hydrocarbons degradation by *Chlorella vulgaris* from the neutral to the acidic level. The highest final pH (4.7) was obtained in the mineral salts - petrol medium inoculated with *Chlorella vulgaris*, final pH (4.5) was obtained in the mineral salts - kerosene medium inoculated with *C.*

vulgaris while the lowest pH (4.0) was obtained in the mineral salts - crude oil medium inoculated with *C. vulgaris* (Table 3).

The reduction in pH may be attributed to the production of acidic metabolites by *C. vulgaris* while growing in the mineral salts - oil medium. This result agreed with Rahman et al. [19] that reported that the utilization of crude oil as sole source of carbon and energy by microorganisms resulted in their growth and the concomitant production of acids.

There was an increase in the absorbance of the mineral salts -oil medium during the forty-two days of the degradation by *Chlorella vulgaris* (Table 4). The absorbance of the mineral salts - crude oil medium containing *C. vulgaris* was higher than that of the kerosene and petrol. The increase in absorbance indicated growth as a result of the utilization of oil as carbon and energy source by the *C. vulgaris*.

The residual oil content of the mineral salts - crude oil

medium was higher than those of the media containing kerosene and petrol (Table 5). The mineral salts - crude oil medium inoculated with *C. vulgaris* had the highest degradation percentage (80%) while the mineral salts - petrol medium inoculated with *C. vulgaris* had the lowest degradation percentage (60%). However, there was no degradation in the uninoculated tubes. Crude oil was degraded more than the kerosene and petrol. This may be attributed to the fact that crude oil is a complex mixture of hundreds of hydrocarbons and other substances such as nitrogen, phosphorus, sulphur hydrogen and oxygen. These constituents of crude oil must have favoured the increased growth and proliferation of the *C. vulgaris*, hence their greater utilization of crude oil more than kerosene and petrol which are processed petroleum products. The kerosene and petrol may have lost many of these constituents during refining.

The gas chromatographic profiles of the unused crude oil (total petroleum hydrocarbons and polyaromatic hydrocarbons) and unused kerosene and petrol showed high peak numbers and peak areas (figures 1-4). A decrease in the peak numbers and peak areas (figures 5-8) of the residual crude oil, kerosene and petrol indicated a reduction in the gas chromatographic detectable hydrocarbons after forty-two days of inoculation with *C. vulgaris*, hence the utilization of such oil by the organism. This result is in agreement with Onuorah et al. [18] who reported a decrease in the peak numbers and peak areas of residual oil after forty-two days of utilization by bacterial consortia

El-Sheekh et al. [20] evaluated the potential of two green algae *Scenedesmus obliquus* and *Chlorella vulgaris* to degrade crude oil. Experiments were performed by incubating algal cultures with 0.5, 1.0, 1.5 and 2.0% crude oil for fifteen days under heterotrophic conditions. The highest growth of *S. obliquus* occurred at 0.5% crude oil concentration while *C. vulgaris* had its highest growth at 2.0% crude oil concentration. They also reported that both algae can grow and degrade oil effectively when incubated with low concentration of oil.

5. Conclusions

This study showed that the remediation of crude oil (total petroleum hydrocarbons and polyaromatic hydrocarbons), kerosene and petrol - polluted sites by *Chlorella vulgaris* is attainable, as it is cheap and leads to environmentally - friendly products. The application of the alga in biomonitoring and restoration of aquatic systems favours the biodegradation and remediation of many organic pollutants. *Chlorella vulgaris* can therefore be considered as a key component in the clean - up strategy for petroleum hydrocarbons remediation.

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