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Research Article

Luminescence Inhibition of Water-soluble Fractions of Crude Oil, Diesel and Spent Engine Oil on Freshly Isolated *Vibrio campbellii*

Beatrice Omolola Bello[1, 3,*], Olayide Samuel Lawal[2], Gbenga Emmanuel Olumayede[2], Ogochukwu Angela Agwu[3], Stanley Ozoemena Agbo[1]

[1] *Department of Animal and Environmental Biology, Federal University Oye-Ekiti, Ekiti State, Nigeria*
[2] *Department of Chemistry, Federal University Oye-Ekiti, Ekiti State, Nigeria*
[3] *Nigerian Institute for Oceanography and Marine Research, Victoria Island, Lagos, Nigeria*

*Correspondence Email: belloob@niomr.gov.ng

Abstract

Inhibitory effects of water-soluble fraction (WSF) of crude oil, diesel, spent engine oil, and their composite mixture on luminescent *Vibrio species* isolated from a tropical marine fish were assessed to determine the acute toxicity and suitability of the bacteria. This was evaluated with a slight modification of the procedure for freshly prepared luminescent marine bacteria according to the International Organization for Standardization. Toxicity was assessed following bacterial exposure to different concentrations of the WSFs. Water quality parameters and total polycyclic aromatic hydrocarbons (PAHs) were determined using standard methods. Chemical analysis revealed cumulative loads of PAHs in the WSFs of crude oil (7.22 mg L^{-1}) > diesel (6.92 mg L^{-1}) > composite (6.23 mg L^{-1}) > spent engine oil (4.58 mg L^{-1}). The WSFs of crude oil and diesel showed rapid inhibition of bacterial light yield (11–81%), with maximum inhibition at 100% treatment level. However, luminescence inhibition was evident at 45 mins following exposure to spent engine oil (41–80%) for most of the treatment level tested, even though the 100% treatment level showed rapid (T_0) and elevated percent inhibition at 75%. Exposure to composite mixtures of the hydrocarbons (1:1:1) revealed a gradual rise in percent inhibition with time. The EC_{50} values noted for the entire test samples during the period of exposure (0–60 mins) were between 14.79 and 257.57 mg L^{-1}. Results obtained from this study revealed low toxic impacts of the WSFs and the observed sensitivity of the freshly isolated bioluminescent bacteria from our tropical waters. More so, it further demonstrates the potential for application of the bioluminescent *Vibrio campbellii* in standardized toxicity testing. *Vibrio campbellii* can be utilized to assess toxicity outcome in the event of bioremediation of oil residues but equally as a simple and generic indicator of oil spill and consequences on coastal waterways.

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Introduction

Operational accidents or pipeline sabotage can lead to the release of petroleum hydrocarbons into the ambient environment. Thus, spillage resulting from exploration, exploitation, and transportation of crude oil and its derivatives constitutes enormous global environmental concerns, mainly due to the recalcitrance of these pollutants when present in various environmental matrices, including soil, sediment, and water. Biota exposures to oil residues have been associated with toxic outcomes in various aquatic ecosystems [1–3]. In aquatic ecosystems, however, petroleum hydrocarbons are immiscible with water and often differentially partition between the water column and sediment. A

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fraction of the oil residue, when dissolved in water, is assumed to become bioavailable for uptake by exposed organisms [4–5]. The water-soluble fraction of oil residue has been reported to contain a mixture of polycyclic aromatic hydrocarbons (PAHs), monoaromatic hydrocarbons, phenols, and heterocyclic compounds, containing nitrogen and sulfur, including heavy metals [6–8]. When natural water bodies are contaminated with petroleum hydrocarbons, there is a tendency for them to accumulate in organisms, particularly plankton, which is a critical component of the aquatic food chain. As aquatic organisms exhibit different tolerance limits when exposed to different pollutants, including petroleum hydrocarbons, it is important to assess the susceptibility and vulnerability of relevant bio-indicators. Thus, bioassays are necessary to assess the ecological risks of contamination due to crude oil and its derivatives. Typically, bacteria, algae, small mammals, fish, plants, and invertebrates constitute the critical indicator organisms for monitoring oil spillage in aquatic ecosystems [8–9]. Aquatic microorganisms, particularly heterotrophic bacteria, are major decomposers that can break down a wide range of organic compounds. As a result, they play major roles in the biogeochemical cycling of nutrients, which helps to sustain other aquatic life forms. To conserve this trophic level, therefore, it is necessary to monitor their response to various pollutants by developing sensitive and easily measurable toxicity indicators [10–11]. The bioluminescent bacteria are a relevant bioindicator, mainly because of their pre-valence and distribution in temperate and sub-tropical waters around the world [12–13]. Crude oil dissolves in water sparingly to produce the water-soluble fraction, which is the dissolved fraction suitable for ingestion by aquatic organisms. According to a prior study, crude oil contains a complex mixture of hazardous elements such as phenols, heterocyclic compounds, heavy metals, and PAHs such as benzene, toluene, ethylbenzene, and xylene (BTEX) [4]. Similarly, Perhar and Arhonditsis [14] reported that crude oil and its compounds are hazardous to aquatic species such as plankton and other microbial organisms, particularly during their early life stages. WSFs are complex combinations of refractory hydrocarbons that tend to stay in the environment during oil spills and can potentially be hazardous to aquatic organisms [15–16].

Exposures to toxic substances have been reported to inhibit the cellular action of light production and thus negatively affect the intensity of luminescence [8]. A relatively short generation time of about 30 mins, at room temperature presents the marine bacterium *Vibrio fischeri*, a relevant candidate and an accepted biosensor to assess the toxicity of effluents and wastewaters [17]. While *Vibrio fischeri* has proven to be a sensitive model in several experimental setups, research information following exposure to water-soluble fractions of crude oil and its derivatives is scarce. The purpose of this study, therefore, is to assess the suitability and susceptibility of the bioluminescent bacteria, *Vibrio campbellii*, to water-soluble fractions of crude oil, diesel, spent engine oil, and their composite mixture.

Materials and methods

1) Isolation of bioluminescent bacteria species

Fresh coastal fish species (*Mugil cephalus*) were obtained from fish landing sites on Victoria Island, Lagos State, Nigeria. Bioluminescent bacteria were isolated from the skin of the fish after initial enrichment in a 3% NaCl solution for about 4 hrs. Inoculums were picked from luminous spots on the fish and streaked on luminous medium (30 g of NaCl, 0.88 mL of glycerol, 10 g of bacteriological peptone, and 15 g of agar in 1 L distilled water) and incubated at 28±2 ^{o}C for 12 to 18 h. Bioluminescent colonies were subsequently selected, further purified on the same medium, and stored on slants for further studies and identification. The morphological, physiological, and molecular characteristics of the isolates were determined as described by Agwu and Aguta [13]. The 16S ribosomal unit was amplified by polymerase chain reaction (PCR) using universal primers for the bacteria: forward primer 5'–3' (27F AGAGTTTGATC MTGGCTCAG) and reverse primer 5'–3' (1492R AGA GTT TGATCMTGGCTCAG). The partial 16S rRNA was submitted to the GenBank with accession number, MT512030.

2) Preparation of water-soluble fraction

Water soluble fraction (WSF) was prepared using the procedures described by previous studies[18-20] as follows:

One part each of crude oil, automatic gas oil (diesel), and spent engine oil (each oil under the same procedure separately) was added to nine parts of filtered, autoclaved saline water of 3 ppt (1:9, v/v) in 1 L glass volumetric flasks. Each of the flasks was capped with a stopper and covered with aluminium foil to minimize the evaporation of volatile components of the oil. Mixing was done at room temperature on a magnetic stirrer, continuously for 24 h at a revolution rate of 250 per minute. At the end of the mixing, each of the mixtures was allowed to stand for 8 h in a separating funnel. The aqueous phase was drawn out, and the solution obtained was designated 100% WSF (stock solution), which was kept in a refrigerator for 6 h until required for use in the assay.

3) Determination of PAHs and heavy metals

Concentrations of PAHs were determined as described by the United States Environmental Protection Agency [21–22], but with slight modifications. Briefly, 50 mL of water-soluble fractions of crude oil, diesel, spent engine oil, and their composites were measured into amber vials for cold extraction with 50 mL of dichloromethane for 30 min in a sonication bath [22–24]. The extract was dried to approximately 1 mL and applied to a silica gel column (4 mm i.d. × 90 mm) for clean-up. The eluates of the PAHs were evaporated under a gentle stream of nitrogen gas until 100 µL. The cleaned extract was reconstituted with 2 mL of 2, 2, 4-trimethylpentane and transferred for analysis into GC vials. Later, concentrated samples were assessed for 16 US EPA-priority PAHs in a gas chromatograph (GC-FID, Agilent 7890). Various concentrations of PAH standards were used to calibrate the GC-FID before analysis. The recovery of standards for the individual priority PAHs ranged from 75% to 96%. The GC-FID chromatograms of PAH congeners are shown in Figure 1.

4) Preparation of test samples

The physico-chemical parameters of the water-soluble fractions of crude oil, diesel, and spent engine oil and the diluent water (pH: 7.2, dissolved oxygen: 6.8 mg L^{-1} and salinity: 3.0 ppt) were determined and noted to be within acceptable limits for the experiment. A total of nine (9) test concentrations (6.25%, 8.3%, 12.5%, 16.7%, 25%, 33.3%, 50%, 66.7%, and 100% v/v dilution ratio) of the test samples were prepared using sterile seawater (30 g of NaCl dissolved in 1 L of distilled water).

5) Bacterial bioluminescence toxicity testing

Inhibitory effect of the test samples (water-soluble fractions of crude oil, diesel, spent engine oil and their composite samples (1:1:1 of WSF of crude oil, diesel, and spent oil, respectively)) on light emission of the isolated bioluminescent *Vibrio* sp. was evaluated following a slight modification of the procedure for freshly prepared luminescent marine bacteria according to the International Organization for Standardization (ISO) [25]. Various concentrations of the test samples were introduced into a fresh (≤ 24 h) bioluminescent *Vibrio* sp. culture of 10^6cfu at a ratio of 1:1. The intensity of light emission from the suspension immediately after the introduction of the test sample (T_0) and subsequently after 15, 30, 45, and 60 min after the addition of test samples, alongside a blank control (diluent water and bacteria culture, 1:1), was measured in a relative light unit (RLU) using a portable luminometer (MicroBioTests Inc.). Each test was done in three (3) replicates, and a temperature of 15 °C was maintained for the duration of the experiment by placing all cultures and test tubes in a regulated water bath. The toxicity data were analyzed to generate bioluminescence inhibitory percentages by comparing the response of the control to the corresponding response of the various treatments in the test samples. The percentage growth inhibition was calculated according to Nadri et al. [26]:

control OD − sample OD)/control OD × 100 (Eq. 1)

Where, control OD (optical density) is the absorbance of control.

Sample OD is the absorbance of a test sample in each of the various treatments.

Both the control OD and the test sample were subtracted from a blank before calculation.

The EC_{50} values and the correlation coefficient were calculated using a regression and correlation analysis of IBM SPSS Statistics 20 (Probit statistical analysis).

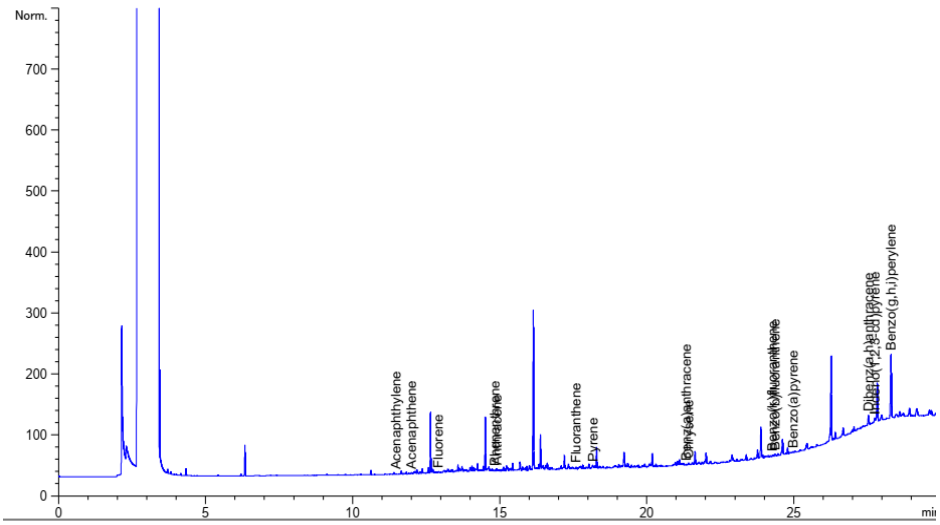

Figure 1 GC-FID thin layer chromatography of PAH congeners.

Results

1) Physicochemical parameters

The physicochemical parameters of the test samples and diluent water with an average of (pH: 7.2, dissolved oxygen: 6.8 mgL^{-1}, and salinity: 3.0 ppt) are presented in Table 1.

Table 1 Physicochemical parameters of the test samples and diluent water

Parameters	Water soluble fractions		
	pH	Dissolved oxygen ($mg\ L^{-1}$)	Salinity (ppt)
Crude oil	7.18±0.01	6.39±0.01	3.02±0.02
Diesel	7.17±0.01	6.88±0.05	3.03±0.02
Spent engine oil	7.16±0.01	6.41±0.01	3.01±0.02
Composite	7.18±0.02	6.31±0.02	3.03±0.01
	Diluent water		
Diluent water	7.12±0.00	7.02±0.01	3.02±0.03

2) Isolation and characterization of bioluminescent bacteria

A pure culture of bioluminescent, short-rod, and gram-negative bacteria was obtained after a repeated purification protocol. The bacterial isolate was capable of fermenting glucose, fructose, lactose, and sucrose but was unable to utilize citrate. Also, the culture showed a negative response tothe Voges-Proskauer test but was positive for a methyl red test. A sequenced fragment of the 16S rRNA was used for molecular identification of the isolate in a Megablast, which revealed 99.90% similarity to *Vibrio campbellii*.

Toxicity exposures at different treatment levels of water-soluble fractions of petroleum hydrocarbons

Total polycyclic aromatic hydrocarbon levels were determined in WSFs of crude oil, diesel, spent engine oil and their composite mixture.

Cumulative levels for different PAHs determined in WSFs of crude oil, diesel, spent engine oil, and their composites are shown in Figure 2.

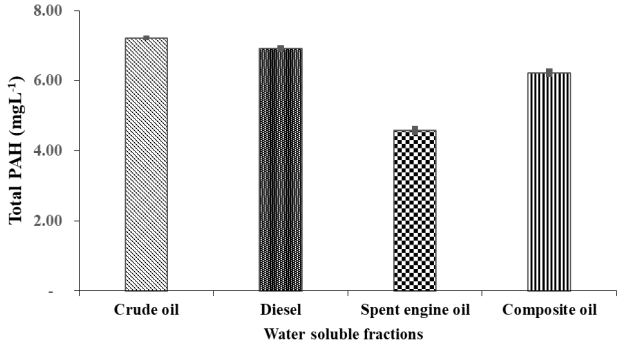

Figure 2 Total PAH concentrations (mgL^{-1}) in WSF of crude oil, diesel, spent engine oil, and their composites. Error bars represent the standard deviation of the mean (mgL^{-1}) ($p< 0.05$).

3) Inhibition of Vibrio campbellii luminescence in a water-soluble fraction of crude oil

The Inhibition of *Vibrio campbellii* luminescence due to exposure to various levels of water-soluble fractions of crude oil was compared with the experimental control and expressed as percentage inhibition (PI). As depicted in Figure 3, the PI was noted to be highest at T0, which is the time immediately after the introduction of the toxicant to cell cultures for most of the treatment levels, except at 6.25 and 33.30%. However, empirical values of the PI declined slightly with increasing exposure time. Toxicity increased with increasing treatment concentrations until 100% to inhibit 79% luminescence, while the least treatment level of 6.25% recorded 11.41% inhibition. In contrast, control exposures did not result in luminescence inhibition.

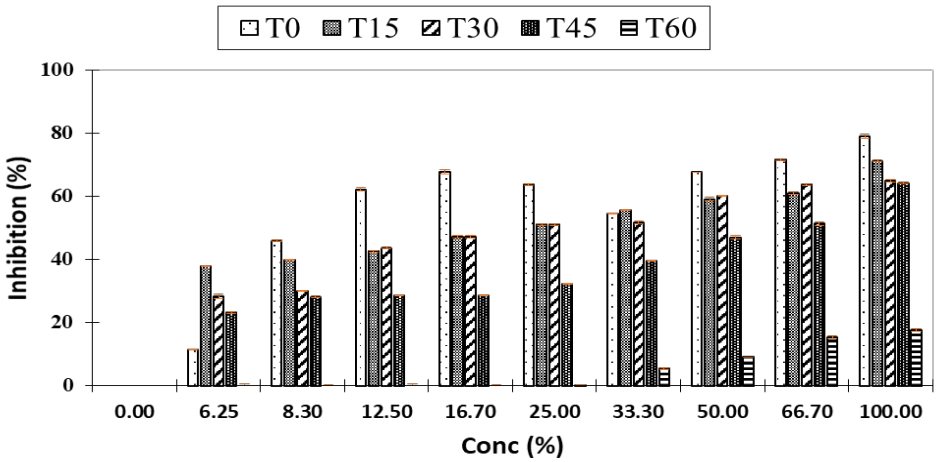

Figure 3 Inhibition of *Vibrio campbellii* luminescence upon treatment in various concentrations of water-soluble fractions of crude oil at different exposure times. The data represents the mean of the triplicate samples and the standard deviation.

4) Inhibition of Vibrio campbellii luminescence in a water-soluble fraction of diesel

There was no measurable inhibition of luminescence in the control setup. However, various concentrations of the water-soluble fraction of diesel showed considerable inhibition of bacterial luminescence that ranged from 0 to 80%. Inhibition was particularly high at T0, T15, and T30 (Figure 4), across the various concentrations, even though it was more remarkable at 100% treatment level.

5) Luminescence inhibition of *Vibrio campbellii* in a water-soluble fraction of spent engine oil

When compared with bacterial luminescence in the control test, the toxicity of the water-soluble fraction of spent engine oil was particularly remarkable at 45 min, though high across treatment levels, as shown in elevated PI (Figure 5). Percent inhibition increased with the severity of the treatment and ranged between 41 and 100% at concentrations of 8.30 and 81%, respectively.

6) Inhibition of *Vibrio campbellii* luminescence in a water-soluble fraction of a composite of crude oil, diesel, and spent engine oil

Percent inhibition of *Vibrio campbellii* luminescence determined at different concentrations of the composite of crude oil, diesel, and spent engine oil (1:1:1) revealed that toxicity increased correspondingly with the severity of the chemical contaminant (Figure 6). This is in contrast with the observed sustained luminescence in the control experiment and at lower treatment concentrations (6.25 and 8.30%) at T45 and T60.

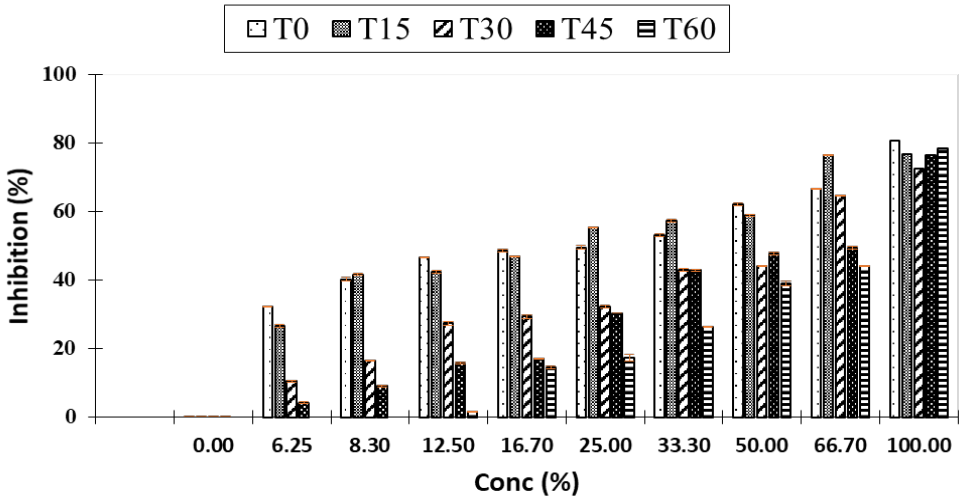

Figure 4 Inhibition of *Vibrio campbellii* luminescence upon treatment in various concentrations of water-soluble fraction of diesel at different exposure times. The data represent the mean of the triplicate samples and the standard deviation.

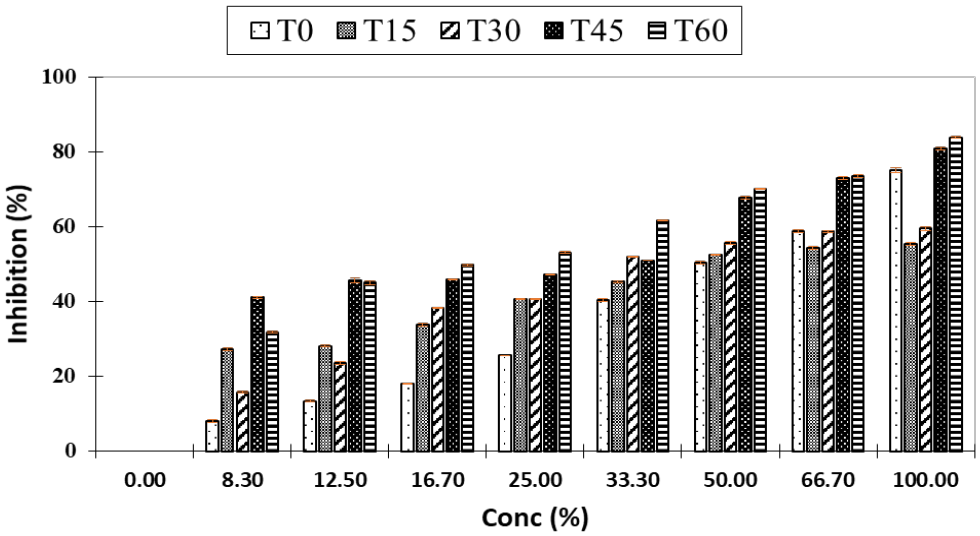

Figure 5 Inhibition of *Vibrio campbellii* luminescence upon treatment in various concentrations of water-soluble fraction of spent engine oil at different exposure times. The data represents the mean of the triplicate samples and the standard deviation.

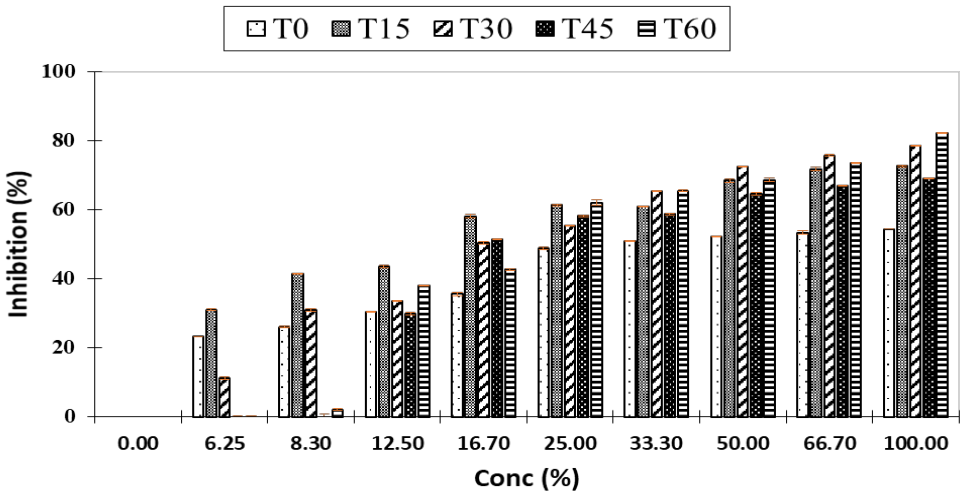

Figure 6 Inhibition of *Vibrio campbellii* luminescence upon treatment in various concentrations of water-soluble fraction of the composite mixture (1:1:1) at different exposure times. The data represents the mean of the triplicate samples and the standard deviation.

Correlation coefficient

The correlation coefficient for crude oil showed a significant percentage inhibition ($r = 0.683^*$, $p<0.05$) on *Vibrio campbellii* luminescence at TO exposure time. Furthermore, the composite oil sample showed a significant percentage inhibition ($r = 0.751^*$, $p<0.05$) on *Vibrio campbellii* luminescence at T15 exposure time. Also, for crude oil exposure, the percentage inhibition on *Vibrio campbellii* luminescence was observed between T15 and T60 ($r = 0.679^*$, $p<0.05$) and T30 and T60 ($r = 0.690^*$, $p<0.05$).

EC_{50} values upon toxicity assessment Vibrio campbellii in the water-soluble fraction of petroleum hydrocarbons

Effective concentration (EC_{50}) values determined from inhibition of bacterial luminescence upon treatment with water-soluble fractions of crude oil and its derivatives are shown in Table 2. EC_{50} values (at T0) for crude oil and diesel were 14.79 and 19.32 mg L^{-1} but increased gradually at T60 until 257 and 60.69 mg L^{-1}, respectively. On the other hand, spent engine oil and the composite mixture demonstrated higher T0 EC_{50} values of 48.25 and 46.68 mg L^{-1}, respectively, while lower values of 18.45 and 24.71 mg L^{-1} at T60.

Table 2 EC_{50} (mg L^{-1}) values obtained from bacteria luminescence test in water-soluble fractions of petroleum hydrocarbons

Time (mins)	Crude Oil	Diesel	Spent Engine oil	Composite
T0	14.79	19.32	48.25	46.68
T15	20.84	19.43	52.07	15.51
T30	26.3	43.7	42.4	21.72
T45	58.13	49.98	19.2	22.14
T60	257.57	60.69	18.45	24.71

Discussion

The physico-chemical parameters of the test samples and the diluent water (pH: 7.2, dissolved oxygen: 6.8 mg L^{-1} and salinity: 3.0ppt) were within the limits stipulated according to Nadri et al. [26]. Mirjani et al. [8] reported that *A. fischeri* grows maximally at pH 7. Earlier studies by Hashmi et al. [27] also stated that the growth of *A. fischeri* was inhibited at acidic and alkaline pH, as this increased the lag phase of the bioluminescent bacteria. In this study, the isolation of *Vibrio campbellii* further buttresses the presence of the luminescent bacteria in the environment, as earlier reported by Agwu and Aguta [13] following their isolation from the skin of fish species in Nigeria's coastal waters. These luminescent marine bacteria are ubiquitous, and as heterotrophs, they can survive on a broad range of living and non-living sources of organic matter [27, 11]. Considering that bioluminescence is a relevant microbial process to evaluate the potential toxicity of substances on microbial populations [28-30], this study exposed freshly isolated luminescent *Vibrio campbellii* to different concentrations of water-soluble fractions of crude oil, diesel, spent engine oil, or their composite mixtures. Similarly, Mohseni et al. [31] had previously isolated luminous Vibrio species from the Caspian Sea, which were highly sensitive to low concentrations of heavy metals and thus served as a bioindicator for the assessment of metal pollution in natural and wastewaters. Oil spillage constitutes a global as well as local environmental menace with both short- and long-term consequences. Therefore, the assessment of potential deleterious effects due to biota exposures to crude oil and its petroleum derivatives in the event of release and contamination of natural waters is crucial [33, 5].

Earlier studies have demonstrated the varying effects of different hydrocarbons on bacterial cells and communities. Toxicity indicators ranged from inhibition of microbial biomass, reduction in species richness, evenness, and phylogenetic diversity to specific bacterial cell growth inhibition. However, when exposed to hydrocarbons, some bacteria that can potentially degrade hydrocarbons will grow and multiply, while the growth of susceptible individuals is greatly hindered [3]. The toxicity of WSFs of hydrocarbons tested in this study revealed elevated inhibition of luminescence immediately after microbial contact with crude oil or diesel (Figures 3 and 4) which coincided with increased concentrations of total PAHs (Figure 2). This observation contradicts the report of Mirjani et al. [8], which showed that different concentrations of total petroleum hydrocarbon (TPH) did not elicit toxicity in luminous *Aliivibrio fischeri* after 15 mins of incubation. The results of this study are also in contrast with the findings of Palamae et al. [33], who reported that the presence of crude oil at all concentrations had negative effects on the log bioluminescence per log number of viable cells after 15- to 105-min exposure.

Despite this, the subsequent decline in toxicity noted in this present study suggests that the bacterial cells may have recruited various adaptive mechanisms to survive in the surrounding substrates. Thus, certain microbes demonstrate exceptional adaptability that may include modification of membranes to tolerate toxic organic solvents, including aliphatic alcohols [34–35].

On the other hand, toxicity appeared to have been delayed upon treatment with spent engine oil, essentially because of the higher percent inhibition (PI) noted at T45 (Figure 5), while the composite showed a gradual elevation in toxicity (Figure 6). The difference in compositions of WSFs of the various petroleum hydrocarbons may have contributed to their differential toxicity. For spent engine oil, however, its constituents are greatly altered by various metals, including magnesium, copper, zinc, lead, and cadmium, aside from other aliphatic and aromatic hydrocarbons such as phenol, naphthalene, benzo(a) anthracene, benzo(a) pyrene, and fluoranthene, due to contamination and breakdown of additives [36]. Rodrigues et al. [4] reported that WSF of diesel and gasoline showed elevated concentrations of BTEX with low concentrations of PAHs. Meanwhile, the reverse was present in the WSF of petroleum, with an elevated concentration of PAHs, particularly naphthalene, and a low concentration of BTEX. In the course of this study, toxicity to exposed microbial cells was observed to be highest at 100% treatment concentration. Our observations are in agreement with a previous report that certain microbial processes such as cell viability, multiplication, growth, bioluminescence, life span, and reproduction are enhanced by moderate amounts of organic and inorganic chemicals, while elevated levels can result in toxicity [27].

On the whole, WSFs of crude oil, diesel, spent engine oil, and their composite utilized in this exposure study presented toxicity threshold (EC_{50}) values that ranged from 14.79 to 257.57mg L^{-1} during the exposure period of 0-60 min (Table 2). This indicates relatively low toxicity, according to the classifications of the Joint Group of Experts on the Scientific Aspect of Marine Environmental Protection [37] and the Offshore Chemical Selection Guidelines for Drilling and Production Activities on Frontier Lands [38]. Also, in an earlier study, *Vibrio fischeri* exposure to normal and branched alkanes yielded EC_{50} values >100 mgL^{-1}[39–40], thus placing them within the least toxic category rating of the Revised Notification Scheme for Offshore Chemicals [38]. In a related study, Mirjani et al. [8] reported that concentrations of TPHs from 30 to 220 mg L^{-1} did not show any short-term (15 min) or long-term (16 h) toxicity on a luminous *Aliivibrio fischeri*. Likewise, the bioluminescent *Vibrio campbellii* showed EC_{50} values of 0.97 mg L^{-1} and 14.54 mg L^{-1} following exposure to zinc and cadmium, respectively [31]. Taken together, petroleum hydrocarbons can elicit toxic responses in *Vibrio campbellii* and this holds some promise for application in field monitoring.

Conclusion

The intensity of light yield from a bacterial strain, *Vibrio campbellii*, isolated from the skin of a Nigerian coastal fish, *Mugil cephalus*, is a useful toxicity indicator, demonstrating sensitivity to various levels of WSFs of crude oil, diesel, wasted motor oil, or their composite combinations. Toxic responses to the various treatments were either rapid, delayed, or gradually increased over time, indicating variable sensitivity to the different components of the WSFs. When compared to the usage of imported freeze-dried bacterial strains, bioluminescent bacteria can be freshly isolated from tropical waters and evaluated for standardized toxicity testing due to their convenience of use and applicability in oil pollution monitoring.

Conflict of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article. All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript. The authors have no financial or proprietary interests in any material discussed in this article.

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