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Acute Toxicity and Inhibitory Response of Marine Microalgae (*Skeletonema costatum* Cleve 1873) Exposed to Water-soluble Fractions of Crude Oil, Diesel, Spent Engine Oil and their Composite Mixture

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Abstract

Petroleum oils and their derivatives continue to devastate marine and coastal water ecosystems despite significant technological advancements in extraction and transportation systems. This is particularly true given their toxicity to sentinel organisms including Skeletonema costatum. This study assessed the potential of water-soluble fractions (WSFs) of crude oil, diesel, spent engine oil, and their composite mixture, to inhibit the growth of marine microalgae, Skeletonema costatum. At 72 hours after exposure, the sensitivity of test organisms to the WSF of various petroleum hydrocarbons were assessed using potentials inhibition of cellular growth. The inhibition concentrations (IC_{50}) were determined as diesel 1.08% (10.8 g/l) > spent engine oil 2.27% (22.7 g/l) > crude oil 4.57% (45.7 g/l) > composite mixture 5.54% (55.4 g/l). The control population revealed an initial cell density of $2x10^4$ cells/ml to an average of $33.92x10^4$ cells/ml. The WSF of crude oil and its derivative hydrocarbons caused increasing inhibition of cellular growth as exposure concentrations increased. It was observed that trace levels of the hydrocarbons caused underlying cellular response which later manifested in growth inhibition of S. costatum. Petroleum hydrocarbons, even in trace levels, may contaminate marine water systems and exert toxicity on microalgae, leading to disruptions in the aquatic food chain.

Keywords: Acute toxicity, Petroleum hydrocarbons, *Skeletonema costatum*, Growth inhibition

Introduction

Crude oil is a complex mixture of hydrocarbons and organic compounds. It consists primarily of aliphatic and aromatic (mono and polycyclic) hydrocarbons; nitrogen-, oxygen- and sulfur-containing constituents and trace amounts of heavy metals such as cadmium, iron, nickel, vanadium and lead (Varjani *et al*., 2015; Laws, 2017). Petroleum hydrocarbons are one of the most vital organic pollutants in terrestrial and aquatic ecosystems (Ukpaka *et al*., 2020). The major sources of oil pollution are pipeline ageing and vandalism, leaning of oil tankers on high seas, flow station blowouts and random disposal of used engine oils by automobile technicians (Nwilo and Badejo, 2005).

Typically, toxicity assessment of chemical contaminants can range from subtle physiological changes and sub-lethal responses to mortality in

exposed population. Contaminant uptake can occur via cellular diffusion, ingestion, inhalation or dermal contact (Lee *et al*., 2015). Previous studies have measured sub-lethal outcomes through cellular growth inhibition, immunological response, genotoxicity, reproductive impairment, deformity and developmental abnormalities or behavioural dysfunction (Kennedy 2014; Colvin *et al*., 2020; Hafez *et al*., 2021). Marine phytoplankton are a broad range of photosynthetic microscopic organisms, which play a major role in primary production in the aquatic food chain (Olaleye and Kadiri, 2021). Higher trophic level organisms directly or indirectly rely on phytoplankton for energy (Buskey *et al*., 2016). Therefore, disturbances in balance of phytoplankton population or alteration of primary production can impact on other aquatic organisms within the marine environment. In a previous study, Yakub and Ajijo (2016) attributed cellular growth inhibition in marine microalgae, *Isochrysis*, and *Chaetoceros sp*. to exposure to Water Soluble Fractions (WSF) of diesel. Consequently, disruptions in phytoplankton population can lead to considerable changes in optimal functioning of marine ecosystems, since they play major roles in influx of primary energy to coastal food webs (Hallare *et al*., 2011). Hence, toxicological investigations to assess the impacts of WSFs of petroleum hydrocarbons and their derivatives would provide information useful for addressing their ecological effects on microalgae populations. This study assessed the growth inhibition in *Skeletonema costatum*, a marine microalgae, exposed to WSFs of crude oil, diesel, spent engine oil, and their composite mixture (ratio 1:1:1).

Materials and Methods

Isolation and Acclimation of *Skeletonema costatum*

Pure isolates of *Skeletonema costatum* were cultured in the Algae Culture Laboratory of the Nigerian Institute for Oceanography and Marine Research (NIOMR), Victoria Island, Lagos, Nigeria. The microalgae strain, which was originally isolated from the coastal waters of Lagos State, was cultured and maintained with f/2 medium (half strength of 'f' growth medium), according to Guillard and Ryther (1962) and Singh *et al*. (2017), but with little modification at 25°C and 14:10 hrs light-dark regime. Three batches of the cultures were maintained at a salinity level of 27 ppt in a filtered and autoclaved enriched medium. Subsequently, the microalgae were cultured in 100 ml medium in 250 ml conical flasks.

Constitution of Water-soluble Fractions

Crude oil, diesel and spent engine oil were used as representative hydrocarbons. Crude oil was obtained from the Nigerian Upstream Petroleum Regulatory Commission, formerly the Department of Petroleum Resources (DPR), Lagos, Nigeria. Diesel was procured from a fuel retail outlet at Ilupeju, Lagos, Nigeria. Spent engine oil was obtained from an automobile dealership workshop at Victoria Island, Lagos, Nigeria. Water-soluble fractions were constituted using minor modifications of previously established protocols (Bhattacharjee and Fernando, 2008; Sadani *et al.*, 2011; Bamidele and Eshagberi, 2015). One part of oil was added to nine parts of filtered seawater at 27 ppt (1:9, v/v) in a one-litre volumetric glass flask. The flask was capped with a stopper and covered with aluminium foil to minimize the evaporation of volatile components. A magnetic stirrer was used to thoroughly and continuously mix the constituents at room temperature for 24 hrs at a speed of 250 rpm. Afterwards, the solution was transferred into a separating funnel and left to stand for 8 hrs. The aqueous phase was drained out, while the drawn-out fraction was designated 100% water-soluble fractions of the crude oil. This procedure was repeated for each of the oil samples. The resulting stock solution was kept in a refrigerator for 24 hrs and thereafter used to conduct bioassays.

Exposure of *Skeletonema costatum* to Water Soluble Fractions of Crude oil, Diesel, Spent engine oil and their Composite Mixture

Eight different concentrations (1%, 1.8%, 3.2%, 5.6%, 10%, 18%, 32% and 56%) of WSFs of crude oil, diesel, spent engine oil and their composite mixture were prepared according to the ISO 10253 recommended protocols, using filtered seawater and enriched with (f/2) growth media. Subsequently, these were inoculated with subcultures of microalgae (*S. costatum*) in an exponential growth phase, and at an initial density of $2x10^4$ cells/ml. The cultures were held in 250 ml polycarbonate flasks at 20°C and 27 ppt salinity on a 12:12 h light-dark cycle with cool-white fluorescent lights at approximately 25 µmol photons $m^{-2}s^{-1}$ irradiance. Controls were set up for microalgae, containing both growth media and algal inoculum without the test samples. For each concentration, samples were

constituted in triplicates, while the control was set up with six replicates. The growth of *Skeletonema costatum* was assessed, in each culture vessel, by counting cells in a microscope-coupled haemocytometer, every 24 hrs for a period of 72 hrs.

Data Analysis

Percentage inhibition was determined according to ISO 10253 (2006), and the average specific growth rate (μ) for each test culture was calculated using Eqn. 1.

$$\mu = \frac{ln\ N_L - lnN_0}{t_L - t_0} \quad \text{---------- (Eqn. 1)}$$

Where, t_0 = start time of test

t_L = time of test termination or the time of the last measurement within the exponential growth period in the control

N_0 = nominal initial cell density

N_L = measured cell density at time t_L.

The percentage growth inhibition was calculated using Eqn. 2:

$$I_{\mu i} = I_{\mu i} = \frac{\bar{\mu}_c - \mu_i}{\bar{\mu}_c} \times 100 \quad \text{---------- (Eqn. 2)}$$

Where: $I_{\mu i}$ = percentage inhibition (growth rate) for test flask *i*

μi = growth rate for test flask *i*

$\overline{\mu i}$ = mean growth rate for control.

The percentage growth inhibition values were used to perform Probit analysis on each test sample to estimate the inhibition concentrations (IC50) (concentration that inhibits cell growth by 50%).

Results

After exposure for 72 hrs, the cell density of *Skeletonema costatum* in control vessels increased from $2x10^4$ cells/ml (at t_0) to a maximum of $203.52x10^4$ cells/ml, on an average of $33.92x10^4$ cells/ml. In the test samples, cell density diminished significantly with increasing concentrations and duration of exposure for vessels containing WSFs of crude oil, diesel, spent engine oil, and their composite mixture (Figure 1). The pH ranged from 7.6 and 8.3 across test samples at 0 - 72 hrs (Table 1).

The calculated growth rate (μ) decreased with an increase in test concentrations, while the percentage inhibition increased. Toxicity of WSF of diesel peaked at 72 hrs with a corresponding IC_{50} of 1.08% (10.81 g/l), followed by spent engine oil at 2.27% (22.70 g/l) and crude oil at 4.57% (45.72 g/l). However, the WSF of composite mixture was observed to exert the least toxicity and had a corresponding IC_{50} of 5.54% (55.36 g/l) in the exposed population of *Skeletonema costatum*.

Table 1: pH values of the water soluble fractions of crude oil, diesel, spent engine oil and composite mixture tested on *Skeletonema costatum* at 0 and 72 hours duration of exposure

Conc (%)	Crude oil		Diesel		Spent engine oil		Composite mixture	
	t_0	t_{72}	t_0	t_{72}	t_0	t_{72}	t_0	t_{72}
0.00	8.30	8.20	8.30	8.20	8.30	8.20	8.30	8.20
1.00	8.12	8.19	8.22	8.19	8.09	8.17	8.14	8.09
1.80	8.11	8.21	8.16	8.00	8.07	8.16	8.13	8.08
3.20	8.11	8.18	8.18	8.00	8.12	8.11	8.11	8.00
5.60	8.07	8.12	8.13	8.10	8.08	8.10	8.11	7.96
10.00	8.10	8.00	7.90	7.80	8.06	8.11	8.03	8.01
18.00	8.09	8.10	8.00	7.90	8.06	8.09	8.00	7.80
32.00	8.03	7.98	7.80	7.70	8.04	8.06	8.01	7.70
56.00	8.02	8.00	7.90	7.80	8.02	8.04	7.80	7.70

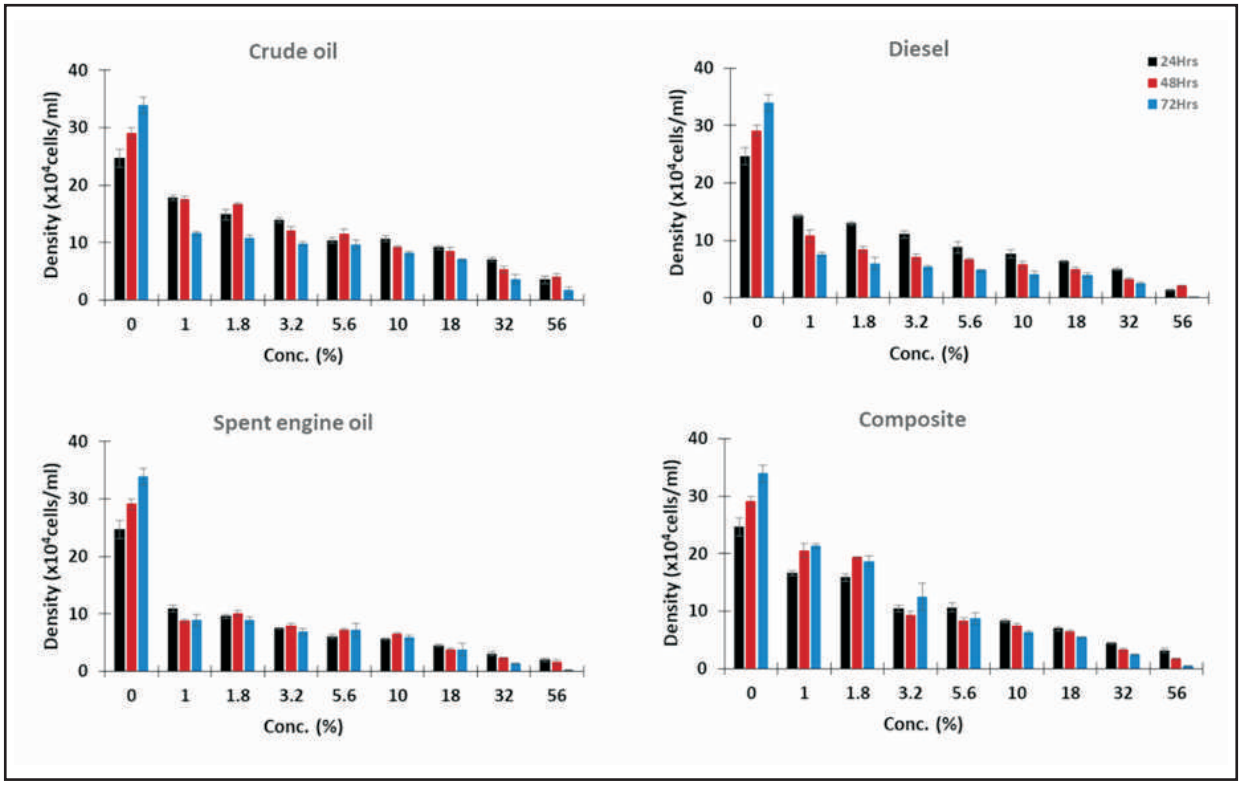

Figure 1: Cellular growth inhibition of *Skeletonema costatum* exposed to the water soluble fractions of crude oil, diesel, spent engine oil, and their composite mixture. Bars represent the mean, while error bars represent the standard deviation of mean.

Table 2: Specific growth rate, percentage inhibition of cellular growth, and inhibition concentration (IC_{50}) values for *Skeletonema costatum* exposed to water soluble fractions of crude oil (A), diesel (B), spent engine oil (C) and composite mixture (D).

(A) Nominal concentration (%)	Growth rate (0 – 72h) Mean μ	Percentage inhibition (%)	IC_{50} (%)	(B) Nominal concentration (%)	Growth rate (0 – 72h) Mean μ	Percentage inhibition (%)	IC_{50} (%)
0.00	0.04	0.00		0.0.	0.04	0.00	
1.00	0.02	40.59		1.00	0.02	52.92	
1.80	0.02	37.95		1.80	0.02	61.19	
3.20	0.02	43.74		3.20	0.01	65.35	
5.60	0.02	44.34	**4.57**	5.60	0.01	69.44	**1.08**
10.00	0.02	50.30		10.00	0.01	74.79	
18.00	0.02	55.74		18.00	0.01	76.26	
32.00	0.01	78.59		32.00	0.00	92.12	
56.00	0.00	100.00		56.00	-0.04	100.00	

(C) Nominal concentration (%)	Growth rate (0 – 72h) Mean μ	Percentage inhibition (%)	IC_{50} (%)	(D) Nominal concentration (%)	Growth rate (0 – 72h) Mean μ	Percentage inhibition (%)	IC_{50} (%)
0.00	0.04	0.00		0.00	0.04	0.00	
1.00	0.02	47.20		1.00	0.03	35.74	
1.80	0.02	47.20		1.80	0.03	15.83	
3.20	0.02	56.17		3.20	0.03	21.41	
5.60	0.02	54.91	**2.27**	5.60	0.02	48.20	**5.54**
10.00	0.02	61.68		10.00	0.02	59.75	
18.00	0.01	77.33		18.00	0.01	64.80	
32.00	-0.01	100.00		32.00	0.00	94.55	
56.00	-0.03	100.00		56.00	-0.02	100.00	

Discussion

The pH is a parameter that contributes to variation in toxicity of certain contaminants when in contact with exposed populations. However, the pH values of cell culture and test media were within the recommended limits for optimum growth of *Skeletonema costatum* based on ISO 10253 (2006). This was evident in the quality of cellular growth of the control population as previously reported by Yakub and Ajijo (2016).

A decline in cell density with increasing concentration of WSFs indicated moderate toxicity of the hydrocarbons when in contact with *Skeletonema costatum*. The toxicity was high in the WSF of diesel, spent engine oil, and crude oil, but least in the composite mixture (Olaleye and Kadiri, 2021). Nevertheless, the petroleum hydrocarbons did not exert any synergistic effect because of the low toxicity outcome of the composite mixture. The large-scale transportation of hydrocarbon products via marine waterways, where leakages and accidents are inevitable, creates a major danger to marine microalgae, especially when spillage and contamination of marine waters occur. Previous findings have reported inhibition and eventual mortality of microalgae, when exposed to hydrocarbons and their derivatives (Ansari *et al*., 1997; Bhattacharjee and Fernando, 2008; Parab *et al*., 2008; Yakub and Ajijo, 2016). The mode of extraction of the soluble fraction of the hydrocarbons ensure that the relevant bioavailable portion is tested in the exposed microalgae population. Moreover, it ensures that constituent polycyclic aromatic compounds and metals in their soluble forms contribute to toxicity in microalgae (Paixa˜o *et al*., 2007).

Pereira et al. (2012) reported a trend in species sensitivity to biodiesel and diesel blends (*S. costatum* > *Nannochloropsis oculata* > *Tetraselmis chuii* > *Pseudokirchneriella subcapitata*) and observed that S. costatum was the most sensitive to WSFs, with comparatively stronger growth inhibition. The sensitivity of S. costatum was further confirmed with growth rate inhibition, when exposed to WSFs of diesel oil (10.8 g/L). The 72-hour IC_{50} of 5.54% (55.36 g/L) recorded for the WSF of the composite mixture suggests that the composite mixture was less toxic than the WSF of crude oil, diesel and spent engine oil. This could be due to stronger interaction among the mixture (potentiation effect), which might have diminished bioavailability and uptake in exposed cells. Interactions (including antagonism, potentiation, and synergies) are typically observed at medium or high dose levels (in comparison to the low doses). At low exposure levels, they are either unlikely to occur or have no toxicological significance (SCENIHR, 2012).

These substances may function separately, have various endpoints or different target organs, act independently or elicit similar reactions through shared or similar method of action. The growth rate of microalgae communities is closely related to energy production within any aquatic environment, and as such, any impairment or stress induction from the WSF of contaminating petroleum hydrocarbons may result in a significant decline and shift in the base of the food chain, along the trophic level.

Conclusion

In this study, an increase in concentration and exposure period of WSF of crude oil, diesel, spent engine oil and their composite sample caused growth inhibition for *Skeletonema costatum*. Hence, the hydrocarbons had significant toxic effects on *Skeletonema costatum* within the marine environment.

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