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Toxicity of Water-Soluble Fractions of Petrol on Some Freshwater Microalgae

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ABSTRACT: This study assessed the toxicity of water-soluble fractions of petrol on *Chlorella vulgaris* and *Scenedesmus obliquus*. Water soluble fractions of petrol were prepared into various concentrations as follows: 0%, 5%, 10%, 25%, 50%, 75% and 100%. These treatments were inoculated with 5 mL of the test microalgae and read at 750 nm at two-day intervals for 14 days using a UV/VIS Spectrophotometer. Both *Chlorella vulgaris* and *Scenedesmus obliquus* exhibited stimulatory growth response at lower concentrations (5%, 10%, 25% and 50%) while higher WSF concentrations (75% and 100%) were inhibitory to their growth. Comparatively, *Chlorella vulgaris* showed better growth response than *Scenedesmus obliquus*. The concentration of TPH was 5831.25 µg/L in the 25% petrol WSF before inoculation and it reduced to 3849.99 µg/L and 3879.50 µg/L for *C. vulgaris* and *S. obliquus,* respectively at the end of the experiment. Based on the results of total petroleum hydrocarbon, the test microalgae were shown to have bioremediation potentials. The oxidative biomarker activity for *C. vulgaris* and *S. obliquus* was not dependent on the concentrations of petrol WSF. Therefore, these species can be exploited as potential candidates in bioremediation of hydrocarbon contaminated aquatic environments when the level of contamination is low.

Keywords: Petroleum, Water-soluble fraction, *Chlorella, Scenedesmus,* TPH, Bioremediation

Introduction

Algae are non-vascular photosynthetic plants characterised by simple structures for reproduction. They are aquatic and are present in brackish, fresh and marine water bodies. They have also been found in extreme environments such as hot springs, deserts, ice and on tree barks where they form associations with-in lichen (Akoma and Chris-Iwuru, 2016). Their sizes range from single-celled microscopic forms to multi-cellular complex forms such as thallose, colonial and filamentous forms. They play important roles in the maintenance of ecological balance. In the aquatic environment, they are the primary producers and their productivity is a determinant of the status of organisms higher in the aquatic food chain, directly or indirectly (Akoma and Chris-Iwuru, 2016). Algae serve as indicators of the quality of water due their short life cycles and ability to grow fast, which enable them respond quickly to changes in environmental parameters. Therefore, alterations in the composition of algal species in water is an important bioindicator of pollution (Denise *et al.,* 2022).
Petroleum is a naturally-occurring complex mixture of organic compounds which is the product of the incomplete decomposition of biomass across a long geological time period. The most essential components of petroleum are hydrocarbons. Other elements, including nitrogen and sulphur, are present in crude oil at concentrations ranging from <0.1 - 0.9% and <0.1 to 6%, respectively, while oxygen makes up as much as 2% of the total contents (Akoma and Chris-Iwuru, 2016). There are three categories of petroleum components according to their densities; these are the light, medium and heavy weight fractions. Hydrocarbons are the principal components of the major fuels, and they are present in biofuels, solvents, oils, waxes, paraffin and plastics (Bhattacharjee and Fernando, 2008). The continual increase in the use of petroleum products has led to higher rates of pollution by these products and their by-products with attendant adverse environmental effects. Oil pollution is generally caused by oil spillage. The fraction of oil which is soluble in water is known as the water-soluble fraction (WSF). It is the part which is left in

water upon the completion of mechanical processes and weathering. The chemical composition of the oils determines its solubility. Lighter fractions generally exhibit higher solubility. The hydrophobicity and chemical structure of a hydrocarbon are key factors in its toxicity (Bamidele and Eshagberi, 2015). Some oil components are relatively water-soluble and may leak into aquatic ecosystems and affect the resident biota, especially planktonic microorganisms like microalgae, whose growth and development are completely dependent on water quality (Morales-Loo and Goutx 1990; Parab et al. 2008).

The presence of water-soluble fraction of petroleum hydrocarbons affects algae in varying ways. These effects are dependent on the species involved, its type and the concentrations of petroleum hydrocarbons present (Poh *et al.*, 2020). Some of the effects which have been identified are induction of oxidative stress, interruption of synthesis of proteins, adverse effects on cell membranes, and reduction in the concentrations of chlorophyll-a in the cells. Also, the content of bioactive compounds in the organism may be altered in response to exposure to these chemicals (Parab *et al.*, 2008; Kadiri and Eboigbodin, 2012; Salinas-Whittaker *et al.*, 2020).

The impacts which petroleum hydrocarbons have on microalgal species have been assessed in a number of studies including Ezenwaeani and Kadiri (2023); Akoma and Chris-Iwuru, (2016); Kadiri and Eboigbodin, (2012). However, the bulk of available literature focuses on crude oil and other particular hydrocarbons without paying attention to the fractions which are soluble (Denise *et al.*, 2019). Furthermore, Nigerian literature assessing the impacts of the petrol fractions present in water on microalgae particularly, are scanty. This study therefore aim to ascertain the impacts of varying concentrations of water-soluble fractions of petrol on selected microalgae to better understand their response to hydrocarbon pollution.

Materials and methods

Study area: This study was conducted in the University of Benin, Benin City, Nigeria. The experiments were carried out in the Limnology and Phycology Laboratory of the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin.

Test microalgae: Two species of microalgae (*Chlorella vulgaris* and *Scenedesmus obliquus*) were selected for the study. The algal samples were obtained from fishponds around Benin City and were inoculated into a growth medium for the isolation of pure cultures. These cultures were then subjected to microscopic examination to determine the algal species present in the samples. After this, isolation of desired species was done and was followed by series of subculturing to obtain monocultures of *C. vulgaris* and *S. obliquus.*

Culture media: Chu's modified No. 10 medium was employed for the cultivation of microalgae (Chu, 1942). Under aseptic conditions, 10 mL each of the macronutrient stock solution, micronutrient stock solution, iron solution, trace stock solutions, and vitamin stock were added together and made up to 1 litre of using distilled water to make 10 litres of Chu 10 medium.

Petrol source and WSF preparation: The petrol used in this study was purchased at All Season Petroleum Filling Station along the Ugbowo-Lagos Road in Benin City, Edo State. A 1000mL conical flask with a screw cap was used for the combination of petrol and distilled water mixture which was done in a ratio of 1:9 (v/v) as outlined in Kadiri and Eboigbodin (2012). The mixture was stirred at constant speed (150 rpm) for 24 hours using a magnetic stirrer. The mixture was then transferred to a separating funnel and allowed to separate into two distinct phases before decanting. The aqueous phase separated from the petrol was regarded as 100% WSF stock solution.

Experimental setup: Six treatments which were in replicates were used during the experiments in addition to the control (5%, 10%, 25%, 50%, 75% and 100% WSF), with each placed in its own appropriately labelled culture bottle. These treatments were obtained via serial dilution of the WSF with the Chu 10 growth medium. Five millilitres (5 mL) of the algal culture was inoculated with the aid of a syringe into the culture vessels containing the different treatments. After inoculation, they were partially capped to allow for effective respiration and to prevent contamination. The vessels were placed close to a north-facing window to ensure maximum exposure to sunlight and their positions were rotated every day to ensure uniform exposure to light.

Measurement of algal growth and percentage inhibition: Algal growth was determined turbidimetrically at 48 hour intervals using a 72I UV/Visible Spectrophotometer set to a wavelength of 750nm.

Percentage Inhibition: The percentage inhibition of *C. vulgaris* and *S. obliquus* were determined using the formula:

$$\textit{Percentage inhibition}\ (\%) = 100 - \left(\frac{\textit{measured biomass}}{\textit{theoretical biomass}} * 100\right)$$

Where: measured biomass = absorbance of test algae in treatments and
theoretical biomass = absorbance of test algae in control.

Oxidative stress response measurement: The oxidative stress response of *C. vulgaris* and *S. obliquus* were determined by determining the activities of catalase, superoxide dismutase, glutathione peroxidase and the concentration of total protein. All assays were done using standard methods as outlined in Ezenweani and Kadiri (2023).

Total petroleum hydrocarbon (TPH) determination: Total petroleum hydrocarbon was determined using the WSFs of the petroleum fuel before and after the experiment. Analysis of TPH followed the USEPA 8015 method for the Gas Chromatography analysis of Diesel Range Organics (DRO) (USEPA, 1996; Weisman, 1998)
Statistical analyses: Microsoft Excel spreadsheets and SPSS were used for statistical analyses. The means and standard errors were calculated, while analysis of variance was used to assess statistically significant differences ($p<0.05$) in the growth of microalgae across the treatments.

Results

Growth response of S. obliquus in petrol WSF: The growth response of *Scenedesmus obliquus* in petrol water-soluble fractions is represented in Figure 1. The least growth response was that of the 100% fraction, and the best was that recorded from the 5% fraction. Generally, there was an increasing trend in the growth of *Scenedesmus obliquus* at 0%, 5%, 10%, 25% and 50%. For the first 4 days, growth rates were low for all concentrations. However, exponential growth was recorded for 5% and 10% from day 4 to day 14. There was a gradual increase in the growth of *Scenedesmus obliquus* in 25% and 0% WSF until day 14. The growth of the organism in the 75% fractions increased until day 10 after which it decreased. At 75% and 100% WSF, there was minimal growth of the species. Using ANOVA, the differences in growth response between the different treatments was found to be statistically significant ($p < 0.001$)

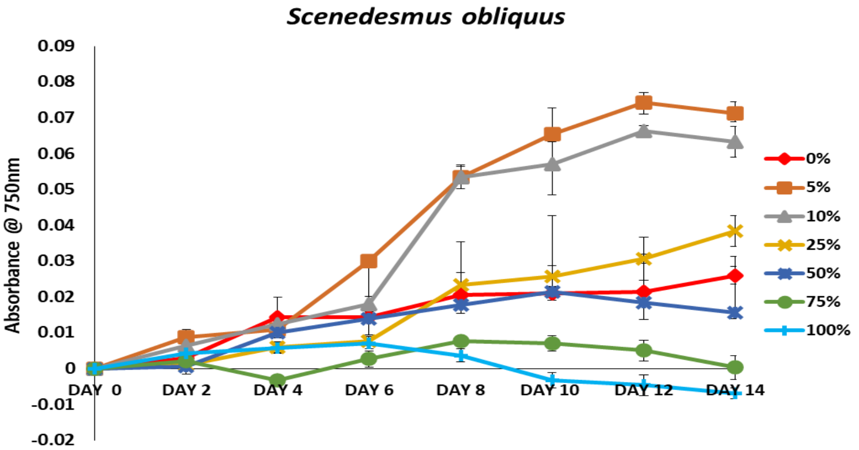

Figure 1: Growth response of *S. obliquus* to petrol WSF

Growth response of C. vulgaris to petrol WSF: Figure 2 shows the growth response of *Chlorella vulgaris* in petrol water-soluble fractions. The results show that the highest growth response was recorded in the 5% WSF fraction, and the least was recorded in the 100% WSF fraction. The growth response of *Chlorella vulgaris* was exponential from day 0 through day 14. The trend for the 10% fraction increased till day 6, followed by a drastic reduction at day 8, and another peak at day 14. The 25% fraction featured a lag phase till day 8 followed by increased growth till day 12 and another lag till day 14. For the 50% fraction, growth increased from day 2 till day 14 with a lag phased from day 8 to 12. The other concentrations (0%, 75% and 100%) increased gradually till day 6, with a lag phase till day 14. Furthermore, the growth response of *C. vulgaris* at different concentrations of petrol WSF were significantly different ($p < 0.001$).

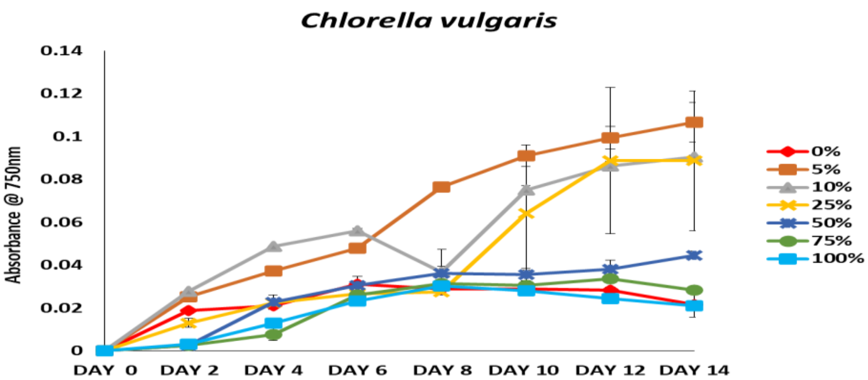

Figure 2: Growth response of *C. vulgaris* to petrol WSF

Percentage inhibition: The percentage inhibition of the microalgae used in this study upon exposure to varying concentrations of petrol WSF is presented in Figure 3. The results show that both *S. obliquus* and *C. vulgaris* experienced stimulatory response at 5%, 10%, 25% of petrol WSF, indicating that the response of both organisms was not inhibited. Negative percentage values were also obtained for 10% and 25% petrol WSF. The response of *S. obliquus* was inhibited in 50% WSF, while the response of *C. vulgaris* was stimulated at this same concentration. Percentage inhibition for higher concentrations (75% and 100%) of *C. vulgaris* and *S. obliquus* in petrol WSF were positive, which indicated an inhibitory response of the microalgae to the treatments. As concentration increased, there was a corresponding increase in the percentage inhibition of petrol WSF.

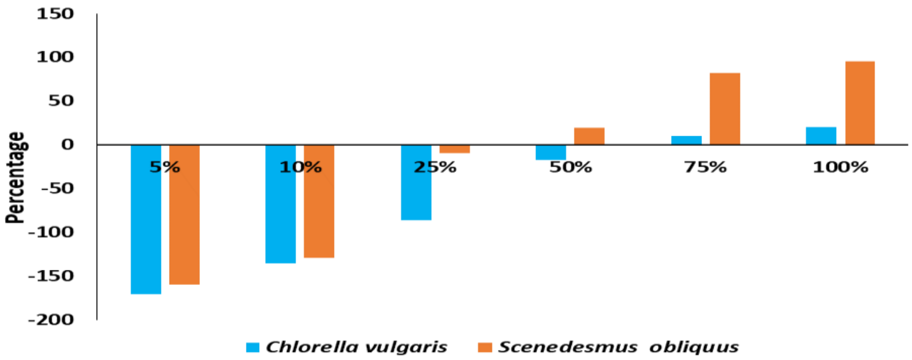

Figure 3: Percentage inhibition of *Chlorella vulgaris* and *Scenedesmus obliquus* in WSF of petrol

Total petroleum hydrocarbon concentration: Figures 4 and 5 show the total petroleum hydrocarbon concentration in the 25% petrol WSF treatment before and after the experiment. The concentration of TPH in the stock solution containing 25% WSF of petrol was 5831.25 µg/L at the start of the experiment (day 0). In the setup inoculated with *C. vulgaris*, the concentration of TPH at the end of the experiment (day 14) dropped to 3849.99 µg/L, while the setup containing *S. obliquus* recorded a final TPH concentration of 3879.50 µg/L on termination of the experiment. This represents a 33.47% and 33.97% reduction in TPH concentrations by *S. obliquus* and *C. vulgaris*, respectively.

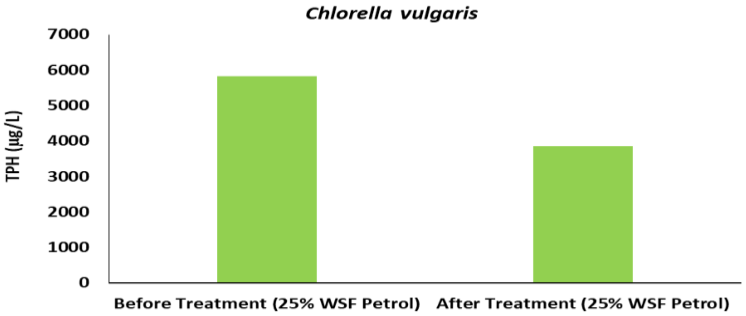

Figure 4: Total petroleum hydrocarbon of concentration of 25% WSF treatment with *C. vulgaris*

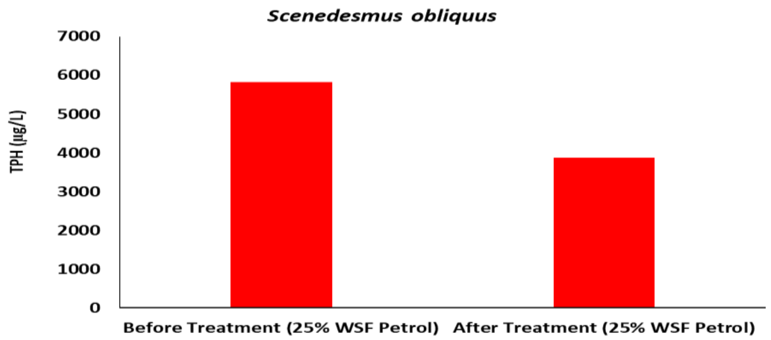

Figure 5: Total petroleum hydrocarbon of concentration of 25% WSF treatment with *S. obliquus*

Oxidative stress response: The values obtained for enzyme activity and the concentrations of total proteins for *C. vulgaris* and *S. obliquus* are presented in Tables 1 and 2, respectively. The activity of SOD for *C. vulgaris* ranged from 0.1003 in the 100% petrol WSF to 5.7644 in the 75% petrol WSF. GPX activity ranged from -0.0018 to 0.0018. The minimum catalase activity recorded was -0.2526 in the 100% petrol WSF, while the maximum was 0.8947 in the 25% petrol WSF. The concentrations of total proteins ranged from 0.19 in 75% petrol WSF, to 1.14 in the 0% petrol WSF. For *S. obliquus* in petrol WSF, the minimum activity of SOD, GPX, catalase and total protein concentrations were 0.613, -0.007, -1.421 and 0.19, respectively, and the maximum values were 8.521, 0.0018, 0.211 and 1.71, respectively.

Table 1: Enzyme activity for *Chlorella vulgaris* in petrol WSF (Mean ± S.E.)

Concentrations	SOD	GPX	Catalase	Total proteins
0%	^{b}0.9190±0.4393	^{a}-0.0053±0.0000	^{a}0.0000±0.0000	^{a} 1.14±0.01
5%	^{b}1.4202±0.0355	^{a}-0.0053±0.0001	^{a}0.7895±0.0059	^{a} 0.57±0.23
10%	^{b}3.7594±0.0401	^{a}-0.0070±0.0001	^{a}0.4737±0.0115	^{a} 0.57±0.12
25%	^{b}2.2556±0.0372	^{a}-0.0070±0.0004	^{a}0.8947±0.0036	^{a} 0.57±0.00
50%	^{b}1.0025±0.0003	^{a}-0.0018±0.0000	^{a}0.1184±0.0008	^{a} 0.76±0.01
75%	^{b}5.7644±0.0494	^{a}0.0000±0.0000	^{a}-0.1579±0.0012	^{a} 0.19±0.01
100%	^{b}0.1003±0.0000	^{a}0.0018±0.0000	^{a}-0.2526±0.0013	^{a} 0.95±0.02

Means preceded by superscript "b" are significantly different from means preceded by superscript "a" ($p < 0.05$)

Table 2: Enzyme activity for *Scenedesmus obliquus* in petrol WSF (Mean ± S.E.)

Concentrations	SOD	GPX	Catalase	Total proteins
0%	^{b}3.0911±0.0511	^{a}-0.0053±0.0001	^{a}-0.0526±0.0014	^{a}0.57±0.03
5%	^{b}0.6126±0.0006	^{a}-0.0053±0.0000	^{a}-0.1228±0.0005	^{a}1.71±0.40
10%	^{b}0.6683±0.0060	^{a}-0.0035±0.0002	^{a}-0.0175±0.0007	^{a}1.71±0.02
25%	^{b}1.3367±0.0046	^{a}-0.0070±0.0004	^{a}0.2105±0.0002	^{a}0.57±0.01
50%	^{b}4.5113±0.0065	^{a}-0.0035±0.0001	^{a}-1.4211±0.0109	^{a}0.19±0.01
75%	^{b}8.5213±0.0116	^{a}0.0018±0.0000	^{a}0.0000±0.0000	^{a}0.19±0.00
100%	^{b}6.5163±0.0579	^{a}0.0018±0.0001	^{a}-0.0526±0.0013	^{a}0.19±0.01

Means preceded by superscript "b" are significantly different from means preceded by superscript "a" ($p < 0.05$)

Discussion

This study was carried out to assess the effects of petrol water-soluble fractions on *Scenedesmus obliquus* and *Chlorella vulgaris.* The results observed showed that there was growth stimulation for *Scenedesmus obliquus* from 5% to 75% petrol WSF. The ability of the species to carry out microbial degradation, accumulation, and the use of petrol as a carbon source has been documented. Akoma and Chris-Iwuru (2016) reports that the stimulation of algal growth by low concentrations of petroleum hydrocarbons can be attributed to the species' abilities to incorporate carbon and hydrogen atoms from these hydrocarbons into the synthesis of carbohydrates and proteins within their cells, causing an increase in algal biomass. This agrees with the results obtained by Denise *et al.* (2022) who assessed growth response of three microalgae species to hexane, benzene, toluene and xylene and reported that certain concentrations of petroleum products allow some algae to grow and produce biomass. Growth stimulation in microalgae by petrol WSF has been noted in Bamidele and Eshagberi (2015). However, at 0% and 100% petrol WSF, the growth of *S. obliquus* was retarded. At 0%, growth retardation observed was possibly due to the absence of carbon for the microalgae to use in energy generation, while at 100% WSF, the growth retardation of the species can be attributed to the toxicity of petrol at this concentration exceeding the tolerance threshold of the species. This agrees with Fabregas *et al.* (2021) where the growth of *Tetraselmis suecica* was inhibited at high concentrations of petroleum oils. This further points to the fact that when the concentrations of hydrocarbons in water are above a certain limit, even the microorganisms which are able to thrive in the presence of lower concentrations will be killed off (Salinas-Whittaker *et al.*, 2020). Growth stimulation in *Chlorella vulgaris* was recorded from 5% to 50% petrol WSF. This is attributed to its ability of *C. vulgaris* to use petroleum as carbon sources (Bhattacharjee and Fernando, 2008). However, at higher concentrations (75% and 100%) the toxicity tolerance limit of *C. vulgaris* was exceeded resulting to growth retardation particularly from Day 4 onwards. This could be attributed to the increased presence of toxic components of the hydrocarbons which are able to interfere with cellular processes including respiration and photosynthesis, thereby affecting the metabolic functions of the algae (Edema *et al.,* 2007). Similar results have been recorded in previous studies. Parab *et al.* (2008) recorded growth retardation of *Thalassiosira* sp. at 100% petrol WSF. Similarly, concentration of 75% WSF of petrol retarded the growth of *Microcystis aeruginosa* in the reports of Akoma and Chris-Iwuru (2016). The growth response of *C. vulgaris* was more than that of *S. obliquus* at the same WSF concentrations indicating that *C. vulgaris* was more efficient at converting petrol into a carbon source for energy and food production. Denise *et al.* (2022) recorded similar differences in growth stimulation of microalgae in WSF of all petroleum fuels where the growth of *C. vulgaris* was more inhibited than those of *Anabaena flosaquae* and *Nitzschia palea*.

The percentage inhibition calculated for *Chlorella vulgaris* in this study showed that there was inhibition of the species only at 75% and 100% petrol WSF, while that of *S. obliquus* showed inhibition of the species at 50%, 75% and 100%. Higher concentrations of the petrol WSF corresponded with higher inhibition of the microalgae species. This is in line with several studies (Bamidele and Eshagberi, 2015; Salinas-Whittaker *et al.,* 2020).

Chlorella vulgaris and *Scenedesmus obliquus* are known for their ability to grow in nutrient-rich environments. Studies have shown that in environmental media contaminated with petroleum hydrocarbons, their ability to degrade hydrocarbons and utilise the mineralised nutrients in the generation of energy makes them good candidates for bioremediation of petroleum contaminated sites (Pereira *et al.,* 2012). The ability of the microalgae used in this study to effect bioremediation of total petroleum hydrocarbons as shown in Figures 4 and 5, revealed that the concentrations of TPH were reduced by 34% by both test microalgae (*C. vulgaris and S. obliquus*). This indicated that both species are capable of carrying out microbial bioremediation of petrol in contaminated sites. These results corroborate those obtained in previous studies which assessed the ability of these organisms to effect bioremediation of petroleum hydrocarbons. El-Sheekh *et al.* (2013) reported success in the use of *C. vulgaris* and *S. obliquus* in the biodegradation of crude oil at low concentrations. Similarly, both species in addition to *Spirulina maxima* were employed for the remediation of phenols by Radziff *et al*. (2021) and found to be effective.

The profiles recorded for oxidative stress biomarkers and the concentrations of proteins for both microalgae species upon exposure to petrol WSF showed that there were variabilities in the values for *C vulgaris* and *S. obliquus*. Antioxidant enzymes including SOD, GPX and catalase are produced by cells upon exposure to reactive oxygen species (Chia and Kwaghe, 2015). The trends of the values obtained for SOD, GPX, Catalase and total proteins showed that the values were not completely dependent on the concentrations of petrol WSF which the organisms were exposed to. However, GPX activity was higher at higher petrol WSF concentrations for both species. Similar results are reported in Chia *et al.* (2016) upon exposure of *S. quadricauda* the oxidative stress factors. Proteins contained in growth media are a reflection of the nitrogen concentrations in these media and a number of metabolic factors including down-regulation of protein synthesis by algae and the degree to which cross-linking of proteins and lipids occurs in the media (Pereira *et al.*, 2007). For both *C vulgaris* and *S. obliquus,* the concentrations of total proteins were higher at lower concentrations of petrol WSF. These results agree with those of Salinas-Whittaker *et al.* (2020) where protein content decreased on exposure of *Dunaliella tertiolecta* to WSF of a fuel oil/diesel mix.

Conclusion

This study assessed the growth response, inhibition, and total petroleum hydrocarbon removal potential of *Chlorella vulgaris* and *Scenedesmus obliquus* in varying concentrations of petrol WSF. The growth response of the species showed that there was slower growth of both species at higher concentrations of petrol WSF. The growth of both species was inhibited at 75% and 100%. Both species were found to be effective in the removal of total petroleum hydrocarbons from the media. Therefore, it is concluded that at low concentrations of petroleum hydrocarbons, *C. vulgaris* and *S. obliquus* are potential candidates for bioremediation.

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