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Effect of Water Saturated Fractions of Mixtures of Benzene and Toluene on Growth of *Pediastrum duplex*

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ABSTRACT
The aim of this study was to investigate the effect of water saturated fraction of mixtures of benzene and toluene on the growth of *Pediastrum duplex* in the laboratory using dry weight technique. The result shows that algal biomass of *Pediastrum duplex* increased with increase in culture age and the concentration of water saturated fraction. There was fluctuation in biomass production at early age and mid age of the culture. The result of this study reveal a general growth stimulation trend of the WSF of the mixture of these hydrocarbons on biomass production of *Pediastrum duplex*, hence could be useful in setting standards for bioremediation and control of aquatic environmental contamination involving benzene and toluene.
Keywords: *Pediastrum duplex*, Benzene, Hydrocarbons, Toluene, Algal biomass.

INTRODUCTION

Algae are as chlorophyll-containing photosynthetic organisms without true roots, stems, leaves but have primitive reproductive structures (Opute and Kadiri, 2013). They are simple, composed of one cell, or grouped together in colonies, or as organisms with many cells, sometimes forming simple tissues. The majority of algae that are intentionally cultivated fall into the category of microalgae also referred to as phytoplankton, microphytes, or planktonic algae (Opute and Kadiri, 2013). Benzene is an organic chemical compound with the chemical formula C_6H_6 with melting point 5.53^oC and boiling point 80.1^oC. The benzene molecule is composed of 6 carbon atoms joined in a ring with 1 hydrogen atom attached to each (Lide, 2005). Benzene is a natural constituent of crude oil and is one of the elementary petrochemicals. Benzene is classed as an aromatic hydrocarbon because of the cyclic continuous pi bond between the carbon atoms (Arnold *et al*., 1958). Toluene also known as toluol is a colourless, water-insoluble liquid that has a smell associated with paint thinners with melting point -95^oC and boiling point of 111^oC. It is a mono-substituted benzene derivative, consisting of a CH_3 group attached to a phenyl group. It is an aromatic hydrocarbon that is widely used as an industrial feedstock and a solvent (Hogan *et al*., 2011).

Microscopic forms of algae (phytoplankton) play a variety of role. They are primary producers in aquatic environment, produce and supply oxygen to other biota in aquatic habitats, they form the base of the aquatic food chain, serves as indicators of water quality, mitigation of global warming. Seaweed and certain other algae have been reported to be importance source for food for man, especially for people of coastal countries like China, Japan, Korea (Behzadi, 2007). These algae form an important ingredient in soap and also spices for flavouring meat. They are rich in protein, organic mineral nutrient and inorganic calcium. Algae are used in waste water treatment facilities due to their abilities to take up minerals from the water and hence reduce the fertility of the water and hinder the proliferation of harmful bacteria. Algae bioreactors are used by some power plants to reduce carbon dioxide emission (John *et al*., 2002). Algae also have health consequences as some species produce harmful and dangerous substances known as algal toxins.

Pediastrum duplex

The word '*Pediastrum*' is derived from the Greek word 'pedion' meaning flat or plain and 'astron' meaning star (Brown, 1956 and Bold, 1985). This name implies its shape. The genus is a one-celled thick colonial form which consist of many cells as many as 2 (rarely), 4, 8, 16, 32, 64, 128 cells depending on the species. Most species are a star-shaped colony that is a flat, circular or sometimes irregularly subcircular configuration (Prescott, 1978).

Pediastrum duplex is a type species of fresh water green algae in the genus *Pediastrum*. They form nonmotile coenobia (colonies) with a fixed number of cells. These coenobia are flat and have circular shape. The cell bodies are polygonal, granulated & have horn-like projections.

This paper is aimed at assessing the effect of the water soluble fractions of mixtures of benzene and toluene on the growth of *Pediastrum duplex* and also to assess the toxicity of these hydrocarbons to the growth of *Pediastrum duplex*.

LITERATURE REVIEW

Hydrocarbons present in crude oil are toxic to many organisms especially those at early life stages (Lee *et* al., 2011). In recent years, research has centered on acute and chronic effect of hydrocarbons, many of these studies emphasizes the effect of the entire oil. Scanty literatures exist on the water saturated fractions of hydrocarbons on growth of microalge.

Santana de Almeida *et al*., (2007) investigated the effect of seawater contaminant with benzene, toluene and xylene and studied their degradation by ionizing radiation and observed that a major concern with leaking petroleum is the environmental contamination by toxic and low water-soluble components such as benzene, toluene and xylenes (BTX). These hydrocarbons have relatively high pollution potential because of their significant toxicity. The objective of their study was to evaluate the contamination of seawater by the main pollutants of the output and transport of petroleum such as benzene, toluene and xylenes and their removal by exposure to ionizing radiation. The minimal detected limits obtained by FIDGC were of 0.50g/L for benzene, 0.70µg/L for toluene and 1.54µg/L for xylenes and the obtained experimental variability was 15%.

Orlu and Ogbalu (2013) considered the evaluation of the effect of water soluble fraction of Bonny Light Crude Oil and the aqueous extract of *Lepadagathis alopecuroides* on reproduction (fertility & hatchability) in *Clarias gariepinus* and the result showed that there was concentration dependent reduction in hatchability of eggs exposed to *L. alopecuroides*. A highly significant ($p<0.01$) but negative linear correlation ($r= -0.9327$) was observed between percent hatchability and the concentration of crude oil water soluble fraction. The result confirmed that both *L. alopecuroides* and crude oil water soluble fraction are capable of inhibiting hatchability of *Clarias gariepinus* and reducing the reproductive capacity of this species in the wild.

Katayama *et al*., (2003) examined the effect of spilled oil on microbial communities in a tidal flat and reported that the proportion of oil susceptible bacteria in the isolates decreases with addition of oil. It was also observed that oil susceptible bacteria were unable to assimilate petroleum compounds coupled with inhibition of growth.

Kadiri and Emmanuel (2006) in their studies with the water soluble fraction (WSF) of Bonny Light Crude Oil using microalgae reported that algae grown in 10%, 25%, 50%, 75% and 100% concentrations of water soluble fraction and growth response shows that there was stimulation of growth at lower (10%, 25% and 50%) and suppression at higher concentrations (75% and 100%) and also maximum growth occurred in 50% concentration of water soluble fraction (WSF) of crude oil in all algae.

Praepilas and Pakawadee (2001) investigated the potential of microalgae (*Scenedesmus quandricauda* and *Chlorella sp*.) utilizing industrial waste water as a cheap nutrient for their growth. The culture gave the highest lipid content at 18.58% and 42.86% in cases of *S. quandricauda* and *S. obliquss* in addition, under salt stress (1.0M NaCl) *S. obliquss* demonstrate the highest lipid content at 50% which was more than the case of no NaCl adding. However, the concentration of NaCl does not affect lipid accumulation in case of *S. quandricanda*. Many microalgae can accumulate lipids due to excess photosynthesis and some species

can accumulate amount of lipids under heterotropy or environmental stress such as nutrient deficiency or salt stress (Takagi *et al*., 2006).

Stepaniyan (2008) considered the effect of crude oil on basic functional characteristics (growing speed, photosynthesis and death) of microalgae of the Barent sea using specific species (*Laminaria saccharina*, *Fucus vesticulosus*, *Ascophyllum nodosum*, *Porphyra umbilicalis*, *Palmaria palmaria*, and *Enteromorpha prolifera*) and concluded that Kelp are more resistant to the influence of oil, red and green algae are less resistant under short term, influence of oil toxin depressed photosynthesis and increases respiration while in a long term, the rate of growth was reportedly reduced.

An assessment by Sushama *et al*., (2007) on the effect of Bombay High Crude Oil (BHC) and its water soluble fraction (WSF) on growth and metabolism of the phytoplankton, *Thalassiosira sp*, the study revealed signs of acute toxicity at higher concentration of crude oil (0.5%) and water soluble fraction (40%) while stimulating effect was observed at lower concentration (0.01 and 0.1%) of BHC and 5%, 10% of WSF. WSF at higher concentration (20 & 40%) caused reduction in DNA and RNA of the diatom. At lower concentration, it caused reduction in protein and RNA content indicating increased metabolism. High concentration of oil and its fraction reveal inhibitory effect on growth protein content and nucleic acid content. This indicated that biosynthesis of this molecules may be probably target for toxicity of oil.

MATERIALS AND METHOD

Hydrocarbon and Source

The hydrocarbons used for this research were Benzene and Toluene. They were bought from Thomas Gold Ventures in Benin, Edo State.

Sample Collection

Unialgal culture of the microalgae (*Pediastrum duplex*) used for this study was isolated from water samples collected from Domita Farms situated at Latitude N 05^{o} 01.164’ and Longitude E 007^{o} 59.833’ with 71m elevation above ground level in Uyo, Akwa Ibom State.

Isolation of Pure Culture of Microalgae

The pure culture was obtained by series of sub-culturing in modified Chu. No 10 artificial growths medium. An aliquot of the water sample collected was taken and used as inoculums to inoculate modified Chu. No 10 artificial growths medium. This was done repeatedly over time in 10 different sets of sub culturing experiment spanning a period of three months until a purer culture of the test microalga was obtained. These were stored in enriched medium and kept on a south window in the Green House of the Botany & Ecological Studies department, University of Uyo, Uyo. A sub-sample was then taken for microscopic examination after three months of sub culturing and identification of alga species made using relevant texts (Prescott, 1975 and Kadiri and Azomani, 1999).

Classification of Experimental Microalga

Classification of *Pediastrum duplex* Meyen (1829)

Kingdom: Plantae
Phylum: Chlorophyta
Class: Chlorophyceae
Order: Chlorococcales
Family: Hydrodictyaceae
Genus: *Pediastrum*
Species: *Pediastrum duplex*

Plate 1: ***Pediastrum duplex***

Culture Medium

The microalga species was grown in an artificial medium; Modified Chu No. 10 Medium solution (Chu, 1942). The preparation of the medium is shown below:

- A stock solution was made by dissolving the salts listed below in the amounts indicated each in 100ml of distilled water or deionized water, autoclaved and kept sterile.

Table 1: A Stock Solution of Modified Chu No. 10 Culture Medium

Salt	Amount (grams/ml)
$CaCl_2.2H_2O$	3.67/100
$MgSO_4.7H_20$	3.69/100
$NaHCO_3$	1.26/100
K_2HPO_4	0.87/100
$Na_2SiO_3.9H_20$	2.84/100
$NaNO_3$	8.5/100

- An iron solution was prepared by dissolving 3.35g citric acid ($C_6H_8O_7.H_20$) in 100ml distilled water, then 3.35g ferric citrate $FeC_6H_5O_3.5H_20$. The mixture was autoclaved and refrigerated in darkness by wrapping in aluminium foils.
- Trace elements composition of the modified Chu No. 10 culture medium was prepared by dissolving the amounts in 1 litre of distilled water. The mixture was autoclaved and kept sterile. The salts include:

Table 2: Trace Elements Composition of the Modified Chu No. 10 Culture Medium

Salt	Amount (milligrams)
$CuSO_4.H_2O$	19.6
$ZnSO_4.7H_2O$	44.0
$CaCl_2.6H_2O$	20.0
$MnCl_2.4H_2O$	36.0
$NaMO_4.2H_2O$	12.6
H_3BO_3	618.4

Table 3: Vitamin Stock

Vitamins	g/ml
Cyanocobalamin (B_{12})	0.004/100
Thiamin	0.004/100
Biotin	0.004/100

Preparation of Water Saturated Fraction (WSF) of Benzene and Toluene

Water saturated (WSF) was prepared according to the method of (Anderson, *et al.*, 1974). A sample of hydrocarbon (Benzene and Toluene) was mixed in a volume of distilled water in the ratio 1:9 in a glass jar. This was placed on Gallen-kamp table top magnetic stirrer and stirred with 7cm magnetic rod for 24hours at room temperature.

After mixing, the oil-water is allowed to stand overnight in a separating funnel. The filtrate which is the water saturated fraction was separated from the supernatant (stock or 100% WSF). The stock was diluted with the culture medium serially to 5%, 10%, 15% WSF respectively.

Serial Dilution

Various concentrations of the WSF were prepared by mixing the stock WSF with a given volume of growth medium. The table below explains the process.

Culture Vessels

Twelve transparent round bottom bottle (700mls each) 17cm height by 6cm diameter). They were washed with detergent and ringed with water acidify to remove any trace of alga spore present. The vessels were turned upside down for easy drainage of water and allowed to dry.

Experimental Set Up

Five hundred (500) mls of the various concentrations of mixtures of water soluble fraction and growth medium were measured separately into the experimental vessels in replicate. Three (3) mls of the experimental microalga (*Pediastrum duplex*) was added to each replicate across all treatments as inoculum. Each was then plugged with sizeable cotton wool to limit evaporation and deposition of external spores. Each replicate along with its alga content were then arranged on a south window for photosynthesis.

Statistical Analysis

All data obtained were subjected to ANOVA and the means and standard deviation calculated using standard biometry.

RESULT

Table 4: Total Algal Biomass Production (Mg/L)

Concentration	Day 4	Day 8	Day 12	Day 14	Day 16
Control (0%)	0.062	0.041	0.031	0.053	0.073
5% (low)	0.064	0.044	0.032	0.107	0.218
10% (mild)	0.059	0.041	0.035	0.063	0.192
15% (high)	0.052	0.037	0.028	0.048	0.127
Mean	0.059	0.040	0.031	0.067	0.122
Standard Deviation	0.000	0.044	0.160	0.026	0.056

RESULTS AND DISCUSSION

Algal bioassay is one of the various methods of investigating pollution in aquatic environment. It is often used to determine the ability of the water system (natural or waste water) to support, accelerate or inhibit algal growth. It is applied to assess the sensitivity of a recovery water body to nutrient changes, effect of secondary and tertiary waste water effluents; nutrient limitations in water bodies of various geographical regions. Such alteration in water quality can be simulated in laboratory using algal cultures to measure their growth response to such changes (Birk and Hering, 2009).

The result of the effect of water saturated fractions of Benzene and Toluene on growth of *Pediastrum duplex* is shown in Table 4. Growth was assessed and evaluated as numerical increase and decrease in total algal biomass within a given time. In this study, there was an overall increased in algal biomass of *Pediastrum duplex* with increase in culture age and the concentration of water saturated fraction across all concentrations. This increase however was only observed after an initial fluctuation in biomass production at the early age and mid age of the culture between day 4 and 8 respectively. The increase was gradual and consistent throughout the duration of the investigation in all concentrations. This increase could have been occasioned by algal growth stimulation by the WSF of the mixture of these hydrocarbons hence the increasing biomass production observed across all treatments. This trend could be due to increase in photosynthetic activities arising from hydrocarbon influence or stimulation. Different authors have reported growth stimulation in lower concentration of hydrocarbons. Sushama *et al*., (2007) investigating the effect of Bombay High Crude Oil (BHC) and its water soluble fraction (WSF) on growth and metabolism of the phytoplankton (*Thalassiosira sp*). They reported acute toxicity at higher concentration of crude oil (0.5%) and water soluble fraction (40%) and stimulating effect at lower concentration (0.01 and 0.1%) of BHC and 5%, 10% of WSF. WSF at higher concentration (20 & 40%) caused reduction in DNA and RNA of the diatom. At lower concentration, it caused reduction in protein and RNA content indicating increased metabolism. High concentration of oil and its fraction heal inhibitory effect on growth protein content and nucleic acid content. This indicated that biosynthesis of this molecules may be probably target for toxicity of oil. Stepaniyan (2008) investigated the effect of crude oil on basic functional characteristics of algae (growing speed, photosynthesis and death) of the Barent sea using specific species (*Laminaria saccharina*, *Fucus vesticulosus*, *Ascophyllum nodosum*, *Porphyra umbilicalis, Palmaria palmaria*, and *Enteromorpha prolifera*). Their study shows that Kelp are more resistant to the influence of oil, red and green algae are less resistant under short term this was evidence as depressed photosynthesis and increases respiration while in a long term, the rate of growth was reportedly reduced. Also, statistical evaluation of results ($p<0.5$) shows that there was no significant difference in algal biomass produced in lower concentrations and that of high concentrations. This result is different from previous results reported for other microalgae investigated in similar conditions. This finding however further buttresses the fact that different microalgae respond different to different stressor under the same environmental condition. This again point to the different tolerant range and hence different toxicity pattern for various algae. The temporal drop in total biomass produced in day 12 could be due to nutrient depletion and exhaustion in limiting nutrient component of the growth medium. Terasa, *et al*. (2013) in their study on the influence of macro and micronutrients on the growth, biomass and lipid productivities of *Dunaliella tertiolecta* showing that with the exception of Nitrogen (N) and Iron (Fe) other nutrients added to the culture medium - Magnesium (Mg), Potassium (K), Manganese (Mn) and Zinc (Zn) - did not influence the studied parameters (algal biomass production). The best maximum and average dry weight of algal biomass produced at the 5th day were 141.8 mg/L/d and 63.1 mg/L/d by increasing the N concentration 10 times in comparison to the standard culture medium "artificial seawater medium with vitamins". In these conditions, the lipid content and maximum lipid productivity were respectively 33.5 % and 47.4 mg/L/d, corresponding to more than three times the value in the standard conditions (13.3 mg/L/d). Also, by increasing the Fe concentration 10 times in the culture medium, the maximum lipid productivity increased to almost double, i.e. from 14.6 mg/L/d to 28.0 mg/L/d, obtained in the 28th day of the test. The authors concluded that N and Fe addition in culture medium resulted in a significant increase in lipid productivity, suggesting that residual wastewaters rich in N or Fe could be

used to cultivate *D. tertiolecta* for lipid production. In this study , the resulting increase between day 12 and the end of the study after the temporal drop in biomass at day 12 could be due to growth stimulating influence arising from other nutrient factors present in the growth medium after the depletion of limiting nutrient component. This claim cannot be held tenaciously in this study since the nutrient status of the growth medium was not monitored during the various phases of this investigation. However, various literatures laid credence to this claim.

CONCLUSION

The investigation into the effect of water saturated fractions of mixtures of benzene and toluene on the growth of *Pediastrum duplex* reveal no adverse effect on the growth and biomass production at the investigated concentrations. The investigation reveal that growth and biomass production in *P. duplex* were enhance in all treatments.

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