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Research Article

Synergistic Effect of Mixtures of Water Saturated Fractions of Benzene, Toluene and Hexane on the Biochemical Properties of Algal Culture

Denise, E. M.*, Anyadike, M. C. & Enang, A. E.

Department of Botany and Ecological Studies, University of Uyo, P.M.B. 1017, Uyo, Akwa Ibom State, Nigeria

***Correspondence to:** Dr. Denise, E. M., Department of Botany and Ecological Studies, University of Uyo, P.M.B. 1017, Uyo, Akwa Ibom State, Nigeria.

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Abstract

The synergistic effect of mixtures of Water Saturated Fraction (WSF) of benzene, toluene and n-hexane on the biochemical properties of algal culture was investigated in the laboratory in a four weeks' bioassay using the Winkler Technique. Synergistic analysis of 0%, 10%, 20% and 30% reveals that the mixtures of Water saturated fractions of benzene, toluene and n-hexane has a significant effect on the Dissolved Oxygen (DO) content of the algal culture with respect to change in concentrations ($P < 0.05$), but there was no significant effect on the Biochemical Oxygen Demand (BOD) content of the algal culture ($P > 0.05$). However, the lowest concentration of WSF recorded the highest amount of DO towards the terminal period of the experiment. During the synergistic analysis, there was consistent decrease in the values of DO as the culture aged. The Biochemical oxygen demand (BOD) followed the same pattern as the DO of the culture but there was an inconsistent decrease in the values of BOD as the culture aged. The result of the study could be used in determining the health or quality of water body polluted with crude oil hydrocarbons.

Introduction

Microscopic algae are typically found in freshwater and marine ecosystem living in both water column and sediment [1]. They could be unicellular species which exist individually, or in groups. Microalgae are aquatic organisms with chlorophyll and a thallus not differentiated into root, stem and leaf [2].

Algal studies in culture have led to the understanding of different biological pathways in many metabolic processes especially photosynthesis and products of algal metabolism. Some genera of unicellular algae are being used for the assay and detection of biological compounds like vitamins and growth-promoting or growth inhibiting substances [3]. Hydrocarbons are organic compounds having the empirical formulae (CH). They are of various forms and are abundantly found in nature. They are the major sources of crude oil. Benzene (C_6H_6), Hexane ($CH_2(CH_2)_4CH_3$) and Toluene ($C_6H_6CH_3$) are commonly found in Nigeria crude oil [4].

Benzene is a natural constituent of crude oil and is one of the most elementary petrochemical. It is a colourless and high flammable liquid with a sweet smell. Because of its high octane number, it is an important component of gasoline, comprising few percent of its mass. Toluene is also a colourless, insoluble liquid with the smell associated with paint thinners. It is a mono-substituted benzene derivative consisting of a methyl (CH_3) group attached to the phenyl group. Toluene is widely used as an industrial feed stock and as solvent. It is sometimes used as inhalant drugs for its intoxicate properties. Hexane is an Alkane of the six carbon atoms. Hexanes are significant constituent of gasoline. They are colourless at room temperature (27°C), odourless when pure, with boiling point between 50-70°C. Stephan (2015) recorded that hexane are widely used as cheap, relatively safe, largely uncreative and easily evaporated non-polar solvent [5].

Water saturated fraction is the solution of low molecular weight hydrocarbons naturally or artificially dissociated from petroleum hydrocarbon mixtures in contact with water. Although generally regarded as hydrophobic, many petroleum hydrocarbons are soluble in water to a limited extent [6]. This combination often contains less soluble, higher molecular mass components and more soluble products of chemical and biological degradation.

Dissolved oxygen is one of the most important factors in any aquatic eco system. The main sources of dissolved oxygen are atmosphere and photosynthetic process of producer organisms. The amount of dissolved oxygen in water depends on the surface area exposed, temperature etc. monitoring oxygen concentration also help to determine the health of that water body and it is one convenient way of feeling the pulse of an aquatic ecosystem [7]. The Biochemical Oxygen Demand is the amount of oxygen required by microorganisms to stabilize decomposable organic matter at a particular time and temperature. BOD test is widely used to determine the pollution strength of domestic and industrial wastes in terms of the oxygen that they require to deliver and produce CO_2 and H_2O [8]. BOD test is useful for determining the relative waste loading to treatment plants and the degree of oxygen demand removal provided by primary treatment, a high BOD therefore indicates the presence of large amount of organic pollution caused by microalgae in water [9] BOD have been used as an alternative measurement in determining the degree of organic pollution of water [10]. The aim and objective of this study is to access the synergistic effects of mixture of water saturated fractions of benzene, toluene and n-hexane on the biochemical properties of algal culture.

Literature Review

In recent years, much research has been done on microalgae, majority of those work centers on distribution in their aquatic environment. Literatures on the effect of hydrocarbon on the growth of microalgae and synergistic effect of hydrocarbon compounds on biochemical properties of algal culture are scarce.

Bhattachargee *et al.* (2008) in an investigation on the effect of water soluble fraction of diesel on growth of responses of marine microalgae, *Chaetoceris calcitans* in the controlled laboratory conditions for a short-term period of 96 hours, observed differential growth responses at comparatively lower diesel concentrations (5, 10, 20 and 40%) though high concentration (80%) was found to suppress the growth effectively [11].

Megharaj *et al.* (2011) reported that benzene, toluene, ethyl benzene and xylene (BTEX) are of great environmental interest due it its availability to microorganisms (microalgae). This availability also influenced by vitality and reactivity, impacts on biodegradation and bioremediation in water and soil environments, with even dissolved components within the pore water considered bioavailable [12].

EL-Dib *et al.* (2001) investigate the fuel oil effect on the growth species diversity and chlorophyll a content of fresh water microalgae and observe that when subjected to different concentrations (0.03,0.07'0.012,0.25 and 0.5g/l) of aqueous extract of reference fuel oil (EPA, USA, API oil No2, 38% aromatic 1274). There was significant decrease in chlorophyll 'a' as the concentration of fuel oil was increased and that total algae counts and growth rate decrease in response to the studied fuel oil. The study also reveals high diversity value in all treated aqueous culture and high concentration of fuel oil significantly decrease carbohydrate and protein content of alga cells [13].

An experiment carried out by Phatarpekar and Ansari (2001) on toxic effect of water soluble fraction of four different fuel oils on microalga, *Tetraselmis gracilis*. On applying different concentrations of water soluble fraction, a decrease in cell population was observed, depending on different physicochemical properties, the crude oil showed different inhibitory effects on the growth of *Tetraselmis gracilis* petrol was the most toxic and produced inhibitory effect even at low concentrations while hydrocarbon, inhibited growth at higher concentrations. The increase in toxicity was attributed to the preserve of aromatic hydrocarbons of medium to higher molecular weight and their chemical modifications. Data from the experiment showed that different parental for environmental damage depending on the types and concentration of soluble and dispersed hydrocarbons present in the water soluble fraction (WSF) [14].

An assessment by Sushama *et al.* (2008), 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 the 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 and 40%) caused reduction in DNA and RNA 0f the diatom. At lower concentration, it caused reduction in protein and RNA content indicating increase metabolism. High concentration of oil and its fraction heal inhibitory effect on growth protein content and nucleic acid content. This indicate that biosynthesis of this molecules may be probably target for toxicity of oil [15].

Materials and Methods

Hydrocarbon and Source

The hydrocarbons used for this research were Benezene and Hexane. It was bought from Thomas Gold Chemical Ventures in Benin, Edo State.

Preparation of Water Saturated Fraction

The water saturated fraction was prepared according to the method used by Anderson *et al.* (1974). Samples of benzene, toluene and hexane (100ml each) were slowly mixed with distilled water respectively in the ratio 1:9 in a 4 litre screw-cap conical flask. These were placed on Gallen- Kamp table top magnetic stirrer and stirred with 71cm magnetic rod for 24hours for each sample at room temperature (27°C). After the stipulated time, the oil-water mixtures were allowed to stand for a long time in a separating funnel. The filtrates which are the water-saturated fraction were separated from the supernatant and this is referred to as stock or 100% water soluble fraction (WSF) [4].

Preparation of Growth Media

The microalgae species were grown in an artificial medium; Chu's modified No 10 mediums [16] the composition of the modified medium is shown in the table below;

Table 1: *Composition of the modified Chu No. 10 culture medium*

a. A stock solution was made by dissolving the salts listed in the amount indicated (in grams) each 100ml of distilled water.

Salts/Nutrients	g/100ml
$CaCl_2.2H_2O$	3.67
$MgSO_4.7H_2O$	3.69
$NaHCO_3$	1.26
K_2HPO_4	0.87
$NaNO_3$	8.5
Na_2SiO_3	2.84

b. An iron solution was prepared by dissolving 3.35g citric acid ($C_6H_8O_7$). H_2O) in 100ml distilled water, then 3.35g ferric citrate ($FeC_6H_5O_3.5H_2O$) was added. This mixture was autoclaved and refrigerated (0°C) in darkness by wrapping in aluminum foils.

c. A trace element solution was made by dissolving the salts below in the amounts (mg) indicated in 1litre distilled water. The mixture was autoclaved and kept sterile.

Table 2: *Trace elements compositions of the modified Chu No. 10 culture medium*

Salts/Nutrients	g/100ml
$CuSO_4.5H_2O$	19.6
$ZnSO_47H_2O$	44.0
$CoCl_2.6H_2O$	20.0
$MnCl_2.4H_2O$	36.0
$NaMnO_4.2H_2O$	12.6
H_3BO_3	618.4

Table 3: *Vitamin Stock*

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

The final culture medium was prepared by adding aseptically, 1ml of each of the six stock solutions in step (1) above to 1ml of stock solution in step (2) above to 1ml of each of the stock solution in step (3) above and 1ml of vitamin stock. The resulting solution was then made up to 1 litre with distilled water in 1 litre volumetric flask.

Experimental Design

The experiment was carried out in completely randomized design (CRD). The treatments were assigned at random with control experiment. The water saturated fractions are analyzed in three different concentrations (10%, 20% and 30) with the control experiment (0%).

Culture Vessels

A five hundred mililitre (500ml) transparent bottle with the height of 16cm and diameter of 6cm was used to set up the experiment. The vessels were twelve (12) in total number. They were washed with detergent and ringed with a mixture nitric acid and sulphuric scid to remove any trace of spore present. The vessels were turn upside down to dried and for easy drainage of water.

Treatment Preparation

The various treatment concentration was obtained by serial dilution of WSF of the mixed hydrocarbons with the growth medium. 10% consist of 10ml of WSF and 90ml of growth medium, 20% consist of 20ml of WSF and 80ml of the growth media then 30% consist of 30ml of WSF and 70ml of the growth medium. The control experiment was made up of only the growth medium (0%).

Aerial Inoculation

The samples were naturally inoculated by being exposed to air for twenty-four (24) hours. This was done to determine the colonization pattern of microalgae on the artificially polluted samples. After the inoculation, the samples were kept in the green house to avoid contamination in an open place.

Preparation of Reagents

Preparation of Winkler a Solution (Manganous sulfate solution)

Dissolve 480g $MnSO_4.4H_2O$, 400g $MnSO_4.2H_2O$ or 364 g $MnSo_4.H_2O$ In distilled water, filter, and dilute to 1 Litre the $MnSO_4$ solution should not give a color with starch when added to an acidified potassium iodide (KI) solution.

Preparation of Winkler B Solution (Alkali-iodide-azide)

For saturated or less than saturated sample; Dissolve 500g NaOH (or 700g KOH) and 135g NaI (or 150.g Kl) in distilled water and dilute to I litre. Add 100g NaN_3 dissolved in 40mL distilled water. Potassium and sodium salts may be used interchangeably. This reagent should not give a colour with starch solution when diluted and acidified.

Starch

Use either an aqueous solution or soluble starch powder mixtures. To prepare an aqueous solution, dissolve 2g laboratory- grade soluble starch and 0.2g salicylic acid, as a preservative, in 100mL hot distilled water.

Sodium Thiosulphate ($Na_2S_2O_3.5H_2O$)

0.0250M of Sodium thiosulphate was prepared with the addition of 6.204g of sodium thiosulphate in 1000ml of distill water which dissolved with proper stirring and thoroughly shaking.

Determination of Dissolved Oxygen and Biochemical Oxygen Demand of the Algal Culture

After the D.O was determined for the first day of the practical set up (day 0), further test was carried out after every five (5) days. The value for BOD was determined from the differential value of DO between the 1st day and fift days later.

Procedure

- Oxygen bottle were fill with sample until it brims over.
- 1ml of winkler A and B solution each was added.
- The bottles were cork and mix by inverting it 5 to 6 times.
- The precipitate was allowed to settle.

Titration

- 2 (ml) concentration of H_2SO_4 added.
- 3 to 4 drops of starch were added.
- Titrate with 0.025M of sodium thiosulphate ($Na_2S_2O_3.5H_2O$)

Formular for Calculation

$$Dissolved\ Oxygen\ (mg/L) = \frac{ml\ x\ Normality\ of\ titrant\ x\ 8\ x\ 1000}{Volume\ of\ water\ sample\ (=\ 100\ ml)}$$

Statistical Analysis

Two factor analysis of variance without replication were used to test the significant effect of mixtures of WSF of benzene, toluene and hexane on the biochemical properties of algal culture ($F_{0.05}$).

Results

Results of Dissolved Oxygen (DO) using Winkler Test carried out at interval of every five (5) days on the various concentrations (0%, 10%, 20%, 30%) is shown on the Table 4 below.

Table 4: *Dissolve Oxygen Values of Culture (in mg/l)*

Concentrations	Day 0	Day 5	Day 10	Day 15	Day 20	Day 25
0%	55.5	45	39	27	26	25
10%	50.5	49	39.5	30	26	24
20%	50.5	44	30	23.5	22.5	17
30%	59	35	30	25.5	19.5	17

Table 4 above shows the values of the Dissolved oxygen of the culture across different concentrations of the mixture of WSF of benzene, toluene and n-hexane in a four weeks' bioassay. There was a consistent decrease in DO in all concentrations as the day increased.

Results of Biochemical Oxygen Demand (BOD) determined after the interval of 5 days using Winkler Test is recorded below in the table 5.

Table 5: *BOD Values of Culture (in mg/l)*

Concentrations	Day 5	Day 10	Day 15	Day 20	Day 25
0%	10.5	6	12	1	1
10%	1.5	9.5	9.5	4	2
20%	6.5	6.5	6.5	1	5.5
30%	24	4.5	4.5	6	2.5

Table 5 shows the Biochemical oxygen demand value of the culture medium across the different concentrations of mixture of WSF of benzene, toluene and n-hexane in a four-week bioassay. There was an inconsistent decrease the BOD value as the day progress.

Discussion

Biochemical properties such as dissolve oxygen and biochemical oxygen demand are major indicators of water quality. The survival of aquatic life depends on a sufficient level of dissolved oxygen in water. Primarily, low dissolve oxygen results from excessive growth of algae (Eutrophication) caused by Phosphate and Nitrate compounds [12]. Ecotoxicology of hydrocarbons are highly variable, depending on their type and concentration, exposure time, slate of environmental condition and the sensitivity of affected species [17,6].

In this study, there was consistent decrease in the values of DO as the culture aged. The Biochemical oxygen demand (BOD) followed the same pattern as the DO of the culture but there was an inconsistent decrease in the values of BOD as the culture aged. This could be due to the reduction in photosynthesis, Eutrophication and increased decomposition activities of dead algal cells [12]. However, at the end of the experiment there was significant effect of the mixtures of WSF of benzene, toluene and hexane on the Dissolved Oxygen (DO) content of the algal culture with respect to change in concentrations ($P < 0.05$), but not so on the Biochemical Oxygen Demand (BOD) content of the algal culture ($P > 0.05$).

The lowest concentration of WSF recorded the highest amount of DO towards the terminal period of the experiment. This was in aligned with the findings reported by Shushama *et al.* (2008) in a study on the effect of Bombay high crude oil (BHC) on growth and metabolism of the phytoplankton, *Thalassioria sp* reported an acute toxicity at high concentrations of crude oil while stimulatory was observed at lower concentration [15]. Also, the highest amount of DO in lower concentrations of the water saturated fractions of benzene, toluene and hexane as observed towards the end of the experiment could be due to high population of algal cells and increased photosynthesis. This finding corroborated with the work carried out by Bhattachargee *et al.* (2008) in an investigation on the effect of water soluble fraction of diesel on growth of responses of marine microalgae, *Chaetoceris calcitans* in the controlled laboratory conditions for a short-term period of 96 hours, observed differential growth responses at comparatively lower diesel concentrations [11].

Conclusion

In this study, the mixtures of WSF of benzene, toluene and hexane has significant effect on the DO content of algal culture, whereas no significant effect was recorded on the BOD of the culture.

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