Bioremediation Of Condensate Polluted Fresh Water Ecosystem

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Abstract: Biostimulation with N.P.K fertilizer (option C) bioaugmentation with indigenous hydrocarbon utilizing bacteria (HUB) (option B) and a combination of both biostimulation and bioaugmantation (option A) as well as option without any remedial treatments, positive and negative controls (options D and E) were evaluated *ex-situ* in the remediation of artificially condensate polluted fresh water sample collected from African Regional Aquaculture centre (ARAC), Aluu in Rivers state. The monitoring period was 56 days. There was an increase in the total heterotrophic bacterial (THB) and hydrocarbon utilizing bacterial (HUB) counts in all the period except in the negative control option E, which was added sodium azide. Results of physicochemical parameters using ANOVA showed that pH, alkalinity and chemical oxygen demand (COD) were significantly different at 5 percent levels ($P<0.05$) in the treatment options, while there were no significant difference ($P>0.05$) in the following parameters, salinity, biochemical oxygen demand BOD) and total hydrocarbon content (THC) in the treatment options. Using least significant difference (LSD), treatment D and E were fond to be different from treatment A,B and C. the percentage total petroleum hydrocarbon (TPH) losses from Gas Chromatograph (GC) results, showed the following % TPH losses; option A 99.4%, option B, 99.0%, option C 88%, option D 65%, and option E 23% respectively Characterization an identification tests of HUB reveal that the following genera *Bacillus, Klebsiella, Citrobacter, Alcaligen, Arthrobacter, Proteus, and Enterobacter* were implicated in the biodegradation process. The results suggest that the combination of biostimulation and bioaugmentation or the use of bioaugmentation with indigenous HUB would be more effective in the bioremediation of condensate polluted fresh water ecosystem.
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1. Introduction

Condensate is a low density mixture of hydrocarbons which are in gaseous state under reservoir conditions and which becomes liquid when the pressure or temperature is reduced. It is similar to light stabilized crude oil, used as feedstock in refining and petrochemical industries (Egiazarov et al, 1995, Givens and Michael, 2003).

The release of condensate into the environment as a result of activities of oil producing companies, equipment failure, pipeline corrosion and sabotage introduces toxic substances into the environment. Such as aromatic hydrocarbon (benzene, toluene, xylene, ethylebenzene). Thiols (Mercaptans) cyclo hexane, hydrogen sulphide and high THC (Egiazarov *et al*; 1975; Ayotamuno *et al*; 2007). These substances impact the environment negatively. Of all natural resources, the aquatic environment is the most severely threatened by oil pollution. The resultant effect leads to loss of aesthetic values of natural beaches, damage to marine, wild life, modification of the ecosystem through species elimination delay in flora and fauna succession, loss of mangrove for biodiversity, decline in fishery/agricultural resources production among other socio economic effects in the oil producing communities of the Niger Delta, Nigeria (Ayatamuno *et al.,* 2006; Ayotamuno, *et al*., 2007; Zaki *et al.,* 2014).

The applications of conventional oil spill clean up techniques (mechanical removal, sediment relocation and chemical treatment) are inadequate and expose personnel to health hazards. Oil spills of coastal regions and open sea are poorly containable, much of the oil can, however, be eliminated by the hydrocarbon-degrading activities of microbial communities, in particular the hydrocarbonoclastic bacteria (HUB) or hydrocarbon utilizing bacteria (HUB). These organisms can help remedy the ecological damage caused by oil pollution of aquatic habitats. (Macaulay, 2015).

In recent past, several literatures have shown that bioremediation has high potential for elimination of pollutants from the environment with least negative impact at relatively low cost. Bioremediation is the use of microorganisms to accelerate the natural decomposition of hydrocarbon pollutants into nontoxic residues. Many researchers have demonstrated high bioremediation effectively for oil polluted environment (terrestrial and aquatic) by adopting various strategies to enhance bioremediation (Vadali, 2001; Odokuma and Dickson, 2003; Obire and Akinde, 2004; Egbuehi *et al.,* 2005. Adenipekun

and Fasidi, 2005; Atagana, 2006; Ayatamuno *et al*., 2006; Abid *et al*; 2007, Abu and Atu, 2007; Liu *et al.,* 2011; Malik and Ahmed, 2002; Agary *et al*., 2012). However, intrinsic bioremediation has been observed to be a very slow process, which could take years to yield the desired results (Mitchell *et al*, 2000, Vadali, 2001). Biostimulation and Bioaugmentation are methods of bioremediation geared towards enhancing and speeding the process. Biostimulation involves the addition of appropriate microbial nutrients to oil polluted environment to stimulate the indigenous microbial flora of the waste to bring about its degradation (Obire and Akinde, 2004). Bioaugmentation is the addition of exogenous or indigenous petroleum degraders into the polluted environment. Sometimes they are genetically engineered. A mixture of both biostimulation and bioaugmentation could also be used in bioremediation process (Odokuma and Dickson, 2003; Mukred et al., 2008). Biodegradation is the primary mechanism of bioremediation (elimination of pollutants by lose of microbes) however, physical and chemical processes such as evaporation, dissolution, sedimentation, emulsification, oxidation, aggregation, volatilization, sorption, are also important (Vidali, 2001).

This study was armed at evaluating Biostimulation bioaugmentation, combination of biostimulation and bioaugmentation and intrinsic bioremediation (natural attenuation) in the remediation of condensate polluted fresh water ecosystem.

2. Materials And Methods

2.1. Sample

Fresh water sample was obtained from African Regional aquaculture centre, (ARAC), Aluu Rivers State. Nigeria (4^o 54.42' $N6^o$ 54.48' E) Water sample was collected in 4 liter plastic container and transported in ice-park to the laboratory and refrigerated at 4^oc. The petroleum condensate was abstained from the facility of Obete Gas Plant located in Ogba-Egbema community of Rivers State. Owned by Total Exploration and Production Nigeria Limited (Total E&P). All reagents employed were of analytical grade and were obtained from BPH chemical Ltd, Poole, England. Nutrient agar (NA) and potato dextrose agar (PDA) were obtained from International Diagnostic Groups, English. Fitter paper (Whatman No.1) was obtained from WER Bauston Ltd.

2.2. Enumeration of microbial populations

The total heterotrophic bacterial (THB) count of water sample and that of bioremediation test set up were performed on NA (oxoid) using spread plate method (APHA, 1998). Plates were properly labeled and incubated at 37^oc for 24 h. The HUB count of water sample and the bioremediation test set up were carried out in duplicates on mineral salt agar (MSA) of mills *et al.,* (1978) as modified by Okpokwasili and Odokuma (1990), the total fungal (TF) count of water sample was estimated by plating 1ml of serial dilution on PDA plates in duplicates. Approximately 10ml of 10% lactic acid was added. Incubation was at 30^oC for 5-7 days. The same techniques were employed for hydrocarbon utilizing fungi (HUF) counts. Sterile filter papers (whatman No.1) saturated with crude oil were aseptically placed on the inside lid of each plate and kept in an inverted position for both HUB and HUF counts. Isolation and identification of HUB was accomplished on basis of their cultural morphological characteristics and by use of Gram's staining. The pure stock culture isolates were further subjected to series of biochemical tests for identification and characterization using the determination scheme of Holt *et al.,* 1994). Physicochemical parameters of water sample and bioremediation monitoring set up analysed included pH, alkalinity, salinity, BOD, COD, and THC. They were determined using methods adopted from Stewart *et al*, (1974). Physicochemical properties of condensate were determined using methods adopted from ASTM,(2003). For TPH, Gas chromatogram Flame Ionization Detector (FID) was employed. (USEPA, 3630 C (1996) HP5890 series 11GC).

2.3. Experimental Design Set Up

Bioremediation tests were carried out in Five 2L Erlemeyer flasks. The flasks were labeled A,B,C,D and E. to each flask was dispensed 100ml mineral salt broth (MSB) and sterilized by autoclaving at 121oc for 15mins. To each flask was added 300ml of fresh water sample. To each set up was added 20ml of the condensate. Into flasks A and C were added 5ml of 10% NPK fertilizer. Isolates were subcultured into nutrient broth and allowed to stand for 6h. 5ml of the mixed culture were inoculated into Flask A and B. by use of sterile syringe. To flask E was added 4 grams of sodium azide (negative control) there was no addition of fertilizer and bacteria culture. The bioremediation set up were allowed to stand for 56 days physicochemical and bacteriological analyses were carried out in two weeks /interval.

Table 1: Bioremediation Test Set up

Option	A	B	C	D	E
	FW+CD+BT+FT	FW+CD+BT	FW+CD+FT	FW+CD	FW+CD+SA

Key: FW=fresh water, CD= condensate, BT= bacteria FT= fertilizer, SA= sodium azide

2.4. Statistical Analysis

Analysis of variance (ANOVA) and least significant difference (LSD) tests at 95% confidence level were employed for a data analysis.

3. Results

The microbial growth profile (THB and HUB) during the monitoring period of the various bioremediation options are illustrated in Figures 1-2 while the physicochemical parameters during the same period are represented in Figures 3-8.

Table 2 shows the physicochemical characteristics of the fresh water sample. The result showed that the fresh water body had very low THC of < 0.5 mg/l and pH of 7.01 indicating neutrality. The physicochemical characteristics of condensate sample used in the study is shown in Table 3. It reveals a high values of TPH of 15,342. 4 mg/l and a high octane number of 56.

The HUF and TF counts as well as HUB and HUF of the fresh water source, are presented in Table 4. It indicates that HUF were not detected in the fresh water sample. The HUB load was $6.0x10^3$cfu/ml. The THB and HUB counts of (growth profile) during the bioremediation monitoring period are illustrated in Figures 1-2. They followed the same pattern. They increased exponentially from initial day to day 14 and gradually increased to day 28 and declines from day 42 to day 56. That of option E (negative control) declined sharply to zero from day 28 to day 42 and 56.

Changes in physicochemical parameters during the bioremediation monitoring are illustrated in Figures 3-8.

TPH% loss in various treatment options including positive and negative controls at day 56 are shown in Table 5. The highest % in TPH was recorded in option A with 99.4%. While option E was the lowest negative control with 23.0%.

Statistical analysis results of growth profile of THB and HUB shows that thee was significant difference in treatment options. At 5% levels ($P<0.05$). The LSD further reveals that treatment E was different from other treatments at 5% levels. For physicochemical parameters. Statistical analysis showed that there was significant difference at 5% levels ($P<0.05$) for pH, alkalinity and COD, whereas there were no significant difference ($P> 0.05$) for alkalinity, BOD and THC. LSD showed that treatments D and E were different from treatments A B and C for THC.

Table 2: physicochemical characteristics of habitat fresh water sample

Parameter	Value
PH	7.01
Alkalinity	7.2
Salinity(mg/l)	17.34
BOD(mg/l)	5.76
COD (mg/l)	18.0
THC (mg/l)	<.0.5

Table 3: Physicochemical characteristics of condensate sample

Parameter	Method	Value
Specific gravity	ASTM D1298	0.747
Reid Vapour Pressure (Kgf/Cm3)	ASTM D323	0.45
Octan Number	ASTM D2699	56 oN
Sulphur content (% wt)	ASTM D4294	0.0169
TPH (Mg/l)	15,342.4	

Table 4: Bacterial and fungi counts of habitat fresh water sample

TYP of Count	value (cfu/ml)
THB	$1.3x10^5$
HUB	$6.0x10^3$
TFC	$1.0x10^3$
HUF	ND

ND= Not detected.

Table 5: percentage (%) loss in TPH of the various bioremediation options at day 56 monitoring period in condensate polluted fresh water sample

Option	TPH loss (%)
A	99.40
B	99.00
C	88.79
D	65.80
E	23.00

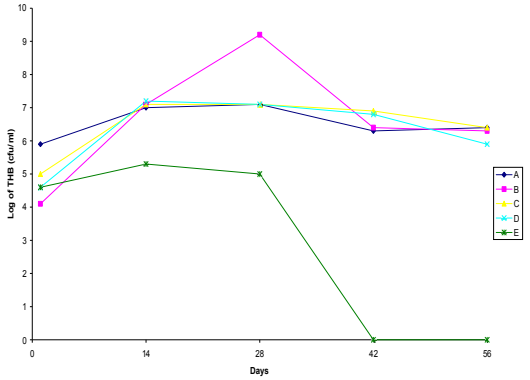

Fig 1: Growth Profile of the THB in Condensate polluted fresh water sample during the monitoring of the various bioremediation options

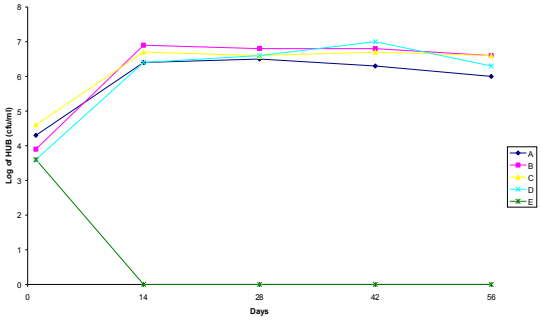

Fig. 2: Growth profile of HUB in condensate polluted fresh water sample during the monitoring of the various bioremediation options

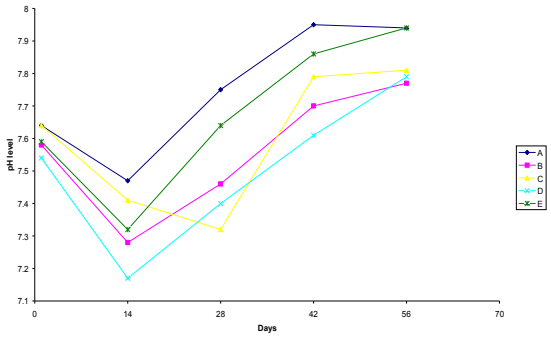

Fig 3: Changes in pH level in condensate polluted fresh water sample during monitoring of the various bioremediation options

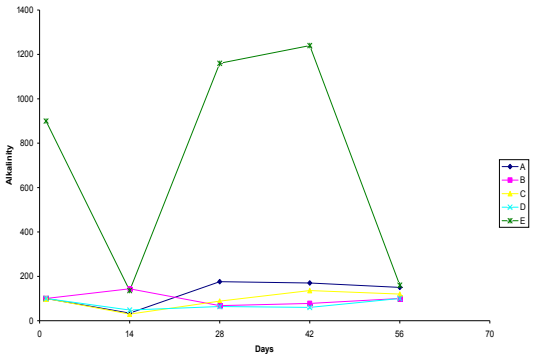

Fig 4: Changes in alkalinity level in condensate polluted fresh water sample during monitoring of the various bioremediation options

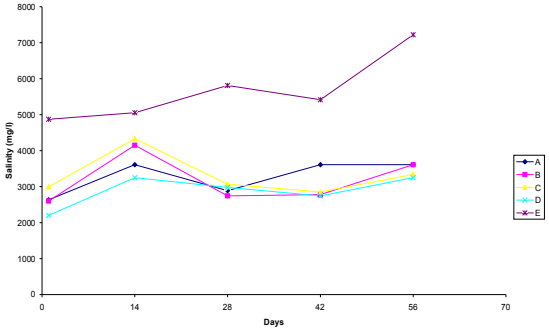

Fig 5: Changes in salinity level in condensate polluted fresh water sample during monitoring of the various bioremediation options

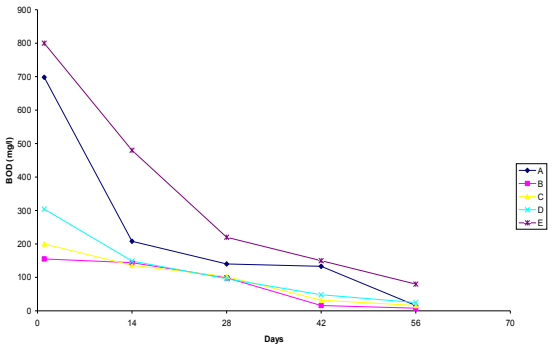

Fig. 6: Changes in BOD level in condensate polluted fresh water sample during monitoring of the various bioremediation options

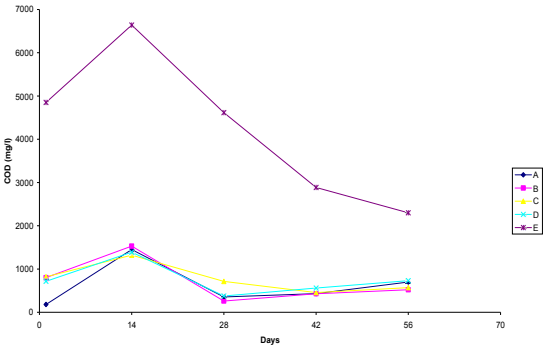

Fig. 7: Changes in COD level in condensate polluted fresh water sample during monitoring of the various bioremediation options

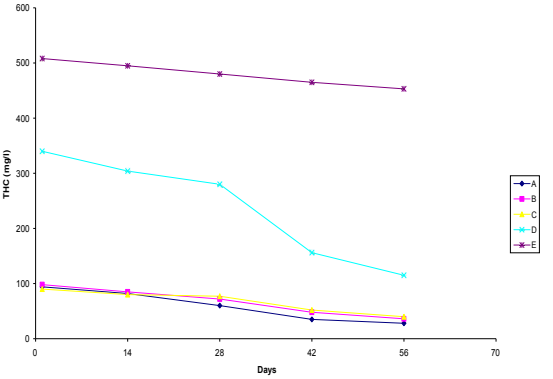

Fig 8: Changes in THC level in condensate polluted fresh water sample during monitoring of the various bioremediation options

4. Discussion

The TPH of the condensate sample of 15, 342.4 mg/L used in this study indicates a very high hydrocarbon content capable of contaminating/polluting a given water body when spilled into the environment. This implies that condensate like crude oil is not safe to be discharged on land or into water body. The value of THC of the habitat water source which was negligible (< 0.5mg/l) and the BOD values of 5.76mg/l indicated that the water sources was not previously polluted by hydrocarbon (DPR, 2002). On pollution of the water samples artificially, there were initial increase in the values of (BOD and THC) in the different treatment options. There were marked decrease in the THC and BOD values during the bioremediation in all the treatment options. Except option E (negative control) from day 1 to day 56 during the period (Figures 6 and 8), leading to the various reduction in THC and in BOD values. Addition of indigenous bacterial consortium enhanced the degradation of petroleum hydrocarbon (TPH) leading to % losses in TPH. This observation is in general agreement with literature regarding the use of bioaugmentation (Vidali, 2001), Odokuma and Dickson, 2003; Mukred, *et al*., 2008; Liu, et al, 2011).

The results of the microbial counts (THB and HUB) showed that the condensate was utilizable source of carbon and energy for the bacterial cells. Moreover, the exponential growth pattern of bacteria from day one to day 14 (Figs. 1-2) indicates that the pollutant (condensate) was being metabolized as sole sources of carbon and energy within the period. The decline in THB and HUB counts from day 42 to 56 may be due to nutrient exhaustion with possible accumulation to toxic metabolites in the media which marked the on set of stationary and death phases (Nester *et al.,* 2009). The relative few or no growth observed in the negative control option during the bioremediation period was to the application of sodium azide (biocide) which eliminated micro organisms in the water sample. (Odokuma and Akubuenyi, 2008). This led to low percentage loss (23%) in TPH since the microorganisms were eliminated that would have metabolize the hydrocarbon (pollutant).

The observed loss in TPH in the negative control set up could be attributed to natural attenuations process (auto-oxidation, evaporation, volatilization and emulsification) other than biodegradation since micro organisms were eliminated. On the other hand the % loss in TPH of 65.8% in option D. (positive control) is attributed to the presence of the existing indigenous HUB in the water sample which had a higher % TPH loss than the negative control. This suggests that bacteria played important/ greater role in the degradation of the condensate (pollutant). The addition of 10% fertilizer and bacteria culture in option A, addition of indigenous bacterial culture alone in option B and addition of 10% fertilizer alone in option C enhanced the process of loss of TPH, hence higher % losses of TPH in these options (A, B, and C).

The decline in BOD during the period of bioremediation indicate that the amounts of degradable organic material present in the polluted water sample were being degraded (utilized by the microbes). BOD represents the amount of oxygen required for the microbial decomposition of organic matter in waste water sample; it is roughly proportional to the amount of degradable organic material present in the water sample (Nester *et al.,* 2009).

Changes in pH (Fig. 3) during the biodegradation period showed pH near neutrality. This favours most heterotrophic bacterial activity (Atlas, 1984). The pH levels throughout the period could be a function of the chemical composition of the pollutant (condensate) and the microbial activities.

Changes in salinity level during the period of remediation was observed to be relatively high in all the treatment options compared to the habit water sources before pollution with condensate (Fig. 4). This could be the effect of the condensate and the composition of the media (MSB). The changes in COD may have been supported by the microbial growth activities, since the highest THB during the monitoring of the bioremediation were recorded on day 14 (Fig.1) the highest values of COD in the various options were also recorded on day 14 (Fig.7). COD provides a measure of the oxygen equivalent of that portion of the organic matter in a water sample that is susceptible to oxidation (Stewart *et al,* 1974). The high values of COD recoded in the negative control through out the period may be due to chemical reactions in the system. The changes in alkalinity decrease and increase in the different options through the period of bioremediation may be due to the production of acidic metabolites (Delyan, *et al*., 1990).

In conclusion, the present study revealed that condensate has high hydrocarbon pollution potential and the combination of biostimulation with use of fertilizer plus bioaugmentation with introduction of indigenous HUB, or the use of biostimulation and bioaugmentation alone would be effective to enhance the bioremediation of condensate polluted water body from the results of the level of hydrocarbon losses. It also suggests that since HUB are present in the water body and had TPH loss of 65% without addition of fertilizer or introduction of indigenous HUB, all that could be needed is the application to the polluted

water, the right quantity and type of fertilizer for microbial growth. It is obvious from the present study that bacteria have the capacity to degrade hydrocarbon (condensate) in aquatic environment.

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