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**Research Paper**

# Kinetics of Substrate Utilization and Bacterial Growth of Organophosphate Pesticide Degraded by Pseudomonas aeruginosa and Bacillus subtilis in Bauchi Metropolis.

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***Abstract***
*To demonstrate the potential use of bioremediation in polycyclic aromatic hydrocarbons contaminated soil using glyphosate as a model pollutant, a laboratory study with the objectives of investigating, evaluating and comparing the methods of natural attenuation, biostimulation, bioaugmentation, and combined biostimulation and bioaugmentation was performed. The study dealt with glyphosate biodegradation in soil using cattle dung fortified with $KH_2PO_4$ and pure culture of Pseudomonas aerugonisa and Bacillus subtilis as source of biostimulation and bioaugmentation, respectively. Each treatment strategy contained 5% (w/w) glyphosate in soil as a sole source of carbon and energy. After 6 weeks of remediation, the results revealed that natural attenuation, biostimulation, bioaugmentation, and combined biostimulation and bioaugmentation exhibited 42%, 59.4%, 67.2%, and 87% glyphosate degradation, respectively. Also, the total hydrocarbon-degrading bacteria (THDB) count in all the treatments increased throughout the remediation period. The highest bacterial growth was observed for combined biostimulation and bioaugmentation treatment strategy. A first-order kinetic model was fitted to the biodegradation data to evaluate the biodegradation rate and the corresponding half-life time was estimated. The model revealed that glyphosate contaminated-soil microcosms under combined biostimulation and bioaugmentation treatment strategy had highest biodegradation rate constants, k (0.06284 $day^{-1}$) as well as lowest half-life times, $t_{1/2}$ (11.03 days) than other remediation treatment methods. Therefore, the kinetic parameter values showed that the degree of effectiveness of these bioremediation strategies in the cleanup of glyphosate contaminated soil is in the following order: natural attenuation < biostimulation < bioaugmentation < combined biostimulation and bioaugmentation. Thus, the present work will contribute to the development of strategies for in situ treatment of polycyclic aromatic hydrocarbons contaminated soils.*
***Keywords:*** *bioremediation, biostimulation, bioaugmentation, first-order kinetics, glyphosate*

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## I. Introduction

Glyphosate (N-(phosphonomethyl)glycine), a synthetic phosphonate compound, is a broad-spectrum, post-emergent, and non-selective systemic herbicide used to eliminate grasses and herbaceous plants (Baylis, 2000; Manassero *et al.*, 2010; Zhang *et al.*, 2018). Glyphosate is one of the most widely used herbicides in the world against annual and perennial weeds in agriculture, urban areas, domestic gardens and silviculture (Aparico *et al.*, 2013; Shushkova *et al.*, 2016). Glyphosate acts on plants by inhibiting the activity of enolpyruvyl shikimate-3-phosphate synthase, an enzyme for aromatic amino acid biosynthesis in the shikimate pathway (Duke *et al.*, 2012). The massive use of this molecule has been reported to weaken plant defense systems (Johal and Huber, 2009; Feng *et al.*, 2020), disturb the metabolism (Cattani *et al.*, 2017) and cause DNA or liver damage (Munner and Boxall, 2008; Feng *et al.*, 2020) both for terrestrial and aquatic animals (Caglar and Kolankaya,2008; Seralini *et al.*,2014) In 2015, the International Agency for Research on Cancer of World Health Organization (WHO) classified glyphosate as "probably carcinogenic to humans" based on epidemiological, animal and in vitro studies. Glyphosate contaminates the aqueous environment from various

sources, such as industrial effluents and agricultural runoff (Munner and Boxall, 2008; Feng *et al.*, 2020). (Xing *et al*., 2017) reported that glyphosate concentration in waste stream could achieve up to 2560 mg $L^{-1}$. Thus, it is necessary to remove glyphosate from the Environment. There are several processes used for the removal of glyphosate, e.g. biodegradation, photodegradation, ozone oxidation, adsorption, membrane processes, flocculation and filtration (Jonsson *et al*., 2013). Among these technologies, biological treatment is considered as an easy, eco-friendly and cost effective process (Nourouzi *et al*., 2012). Many microorganisms were reported to be able to utilize glyphosate as carbon, nitrogen or phosphorus source (Lerbs *et al*., 1990; Klimek *et al*., 2001; Obojska *et al*.,2002; Sviridov *et al*., 2012; Firdous *et al*., 2017). The two main degradation pathways in glyphosate-degrading microorganisms are conversions to aminomethylphosphonic acid (AMPA) through the cleavage of C-N bond catalyzed by an oxidase, and to sarcosine through the direct cleavage of C–P bond, catalyzed by C–P lyase (Zhang *et al.*, 2018). Single AMPA or sarcosine pathway or both pathways have been frequently found in glyphosate degrading microorganisms using glyphosate as a phosphorus source. However, the systems biology approach to determine the kinetics of substrate utilization and bacterial growth still needs to be understood.

Therefore, the specific objectives of this study are (1) To investigate the organophosphorus biodegradation efficiency in a freshly contaminated soil with Organophosphorous pesticide under Different Bioremediation Strategies (2) To investigate and evaluate the effects of bioattenuation, biostimulation, bioaugmentation, and combined biostimulation and bioaugmentation on glyphosate degradation through the application of a first-order kinetic model equation

## II. Materials and Methods

### 2.1 Materials

Fresh uncontaminated soil, with no prior history of oil contamination was excavated from Abubakar Tafawa Balewa University School Farm, Bauchi-Nigeria between 0-30cm from the soil surface. The soil was sieved on 2mm sieve to enhance proper mixing and extract consisting mainly of stones and dead plant debris discarded. The sieved soil was contaminated artificially organophosphate pesticide (Round up) to a pollutant level of 50 000 ppm. Cow dung (stimulant) was sun dried, grinded and homogenized; the stimulant was package in polythene bags prior to their application for bioremediation experiment. Physicochemical and microbiological analyses were performed on the soil and the organic stimulant (cow dung). Initial quantity of water in kilograms was applied to all the treatment options in the microcosm's base on the calculation of the experimental design. In the subsequent weeks, percentage moistures were determined and used to adjust the water lost and what need to be added. Moisture content level in each microcosm was increased from 3.5% to 12% using distilled water. The soil matrix was properly mixed at ambient temperature ($25^0$C- $30^0$C). Each of the contaminated soil (5 kg) were stacked into eleven wooden boxes lagged internally with formica material to prevent absorption of organophosphate pesticide and moisture reacting with the wooden surface. Each box had dimension of 15cm height × 40cm length × 40cm width with soil layer $0.024m^3$ deep. Various treatment options were prepared according to Table 1

### 2.2 Soil Sampling

A clean soil sample was obtained from a site where there was no previous history of contamination. Before loading the wooden boxes (Reactor), the soil sample was grinded and large particles were removed. Then, it was air-dried at room temperature for 48 h. The volume of clean soil sample for each process, varied according to the experimental design of the different treatment options. Reactor 1 (NA), Reactor 2 (BA), Reactor 3 (BS fortified with $KH_2PO_4$), Reactor 4 (BA+ BS fortified with $KH_2PO_4$).

### 2.3 Loading of the Microcosm

Clean soil samples were placed in the microcosm and mixed thoroughly by a PVC stirrer for homogenization. Afterwards, the soil samples were contaminated by adding 50000ppm of organophosphate pesticide (round- up herbicide) to each reactor. Distill water was added to the reactors to sustain minimum of 20% water content. Then, the soil samples in all reactors were mixed again for homogenization

### 2.4 Experimental Design for the Bioremediation of Glyphosate Spiked Soil

Four wooden boxes used as bioreactors were prepared for each treatment as shown in Figure1, designated as bioattenuation (treatment $A_1$), biostimulation (treatment $A_2$), bioaugumentation (treatment A3), and combined biostimulation and bioaugumentation (treatment A4). Each bioreactor contained 5 kg of soil contaminated and well mixed with 100 ml of 50 g of Organophosphate pesticide (5% w/v) dissolved in water. The bioreactor under treatment ($A_2$) was amended with 8.0 kg of organic manure (cow dung) fortified with 0.04088kg of $KH_2PO_4$ (biostimulation), and the bioreactor under treatment ($A_3$) were amended with 50 ml of inoculum $2.6\times10^5\ CFU/g$ (combine *Bacillus subtilis & Pseudomonas aeruogonisa)*) (bioaugumentation). The

bioreactor under treatment ($A_4$) was amended with both 50ml of inoculums (hybrid) and 8.0 kg of cow dung, fortified with 0.04088kg of $KH_2PO_4$ (combined biostimulation and bioaugumentation). The bioreactor under treatment $A_1$ was not amended with either Organic manure or inoculum (natural bioattenuation). The bioreactor or microcosm under organic manure treatment had a C: N: P ratio of 100:10:1(Beolchin *et al.*, 2010; Agarry *et al*., 2015). Soil in the bioreactor used for some experimental optional design was sterilized by autoclaving at $121^0$C for 15 min, all the bioreactors with its contents were incubated at a room temperature ($28^0C \pm 2^0C$) for Six weeks. The water (moisture) content of soil in each bioreactor was adjusted every week by addition of distilled water in order to make up for water losses used by microbes to enhance their activities. In order to avoid anaerobic conditions, the samples in the microcosms were tilled and aerated daily to enhance oxygenation and kept moist during the experimental period. Samples were taken weekly and analyzed for residual pesticide

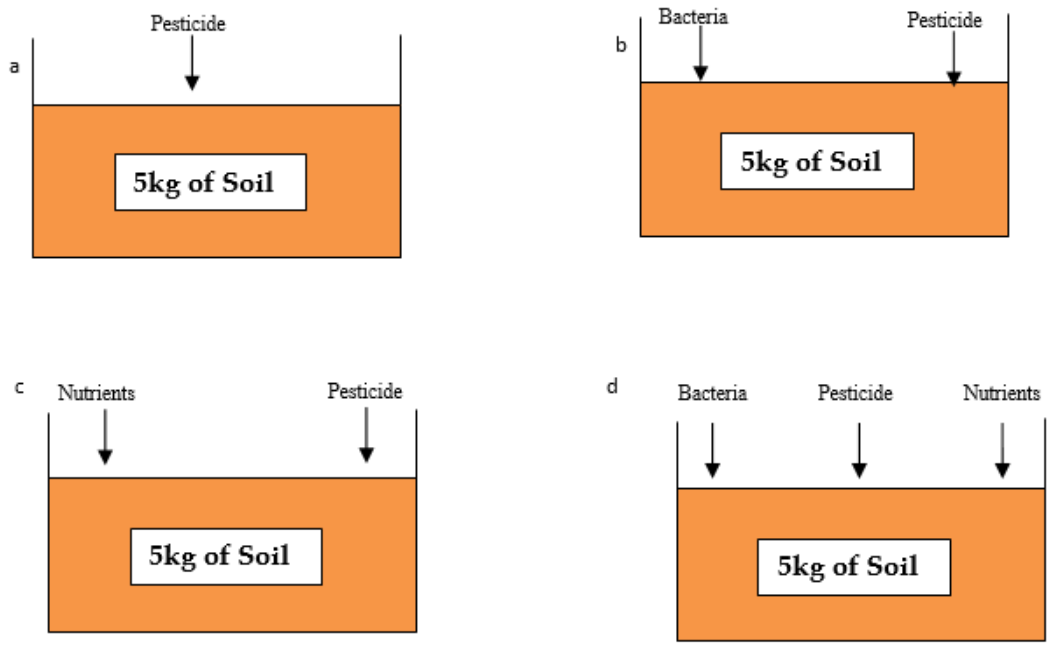


**Figure 1:** Schematics of Soil Treatment Reactors: (a) Natural Attenuations (NA), (b) Bioaugmentation (BA), (c) Biostimulation (BS) and (d) Biostimulation + Bioaugmentation (BS+BA).

Table 1: Configuration of the Treatment Microcosm

| Reactor | Microbial Inoculum Addition (Bioaugumentation) | Nutrient Addition (Biostimulation) | Pesticide Addition |
|---|---|---|---|
| A1(NA) | **NO** | **NO** | **YES** |
| A2 (BA) | **YES** | **NO** | **YES** |
| A3 (BS) | **NO** | **YES** | **YES** |
| A4 (BA) | **YES** | **YES** | **YES** |

**2.5 Microbial Analysis**

The pure isolate (*Bacillus subtlis and Pseudomonas aerugonisa*) in form of slants, was obtain from veterinary research institute in Vom, Jos, Plateau State. The slant was sub-culture using the streaking method on the nutrient agar in disposable petri dishes for 24 h in an incubator at 37 $^0$C to monitor its cell growth. A loopful of each stock culture of *Bacillus subtlis* and *Pseudomonas aerugonisa* were respectively inoculated into 3 different 250 ml Erlenmeyer flasks containing 100 ml of freshly prepared sterile nutrient broth medium (0.8 %) made up of yeast extract 2.0 g/L, peptone 5.0 g/L, NaCl 5.0 g/L, and agar 15.0 g/L, incubated at 37 $^0$C for 48 hrs to monitor a turbid suspension. 1 mls of the stock solution was serially diluted into 9 mls of sterile nutrient broth(same constituents above) in a serial dilution in triplicate for each stock solution to get a viable counts of 30-300 cfu/ml. 200 milliters ($2.3\times10^5\ CFU/g$ *Bacillus subtilis*, $2.5\times10^5\ CFU/g$ *Pseudomonas aeruogonisa* and $2.6\times10^5\ CFU/g$ Combine *Bacillus subtilis & Pseudomonas aeruogonisa)* of the inoculums was inoculated on the surface of the microcosm and was also used for the bioaugumentation experimental design option to study the biodegradation of glyphosate in soil. All the microcosms were mixed three times every week for aeration for the (6) weeks. After mixing, these microcosms were kept away from sunlight at room temperature in order to prevent rate of dehydration.

Colony forming unit (CFU) was determined by using the formula; CFU/g = $\frac{number\ of\ colonies \times dilution\ factor}{volume\ of\ culture\ plate}$

**2.7 Kinetic Model Analysis**

A mass balance in the experimental microcosm was used to find a kinetic model for degradation of total hydrocarbon. The kinetic model can be defined as shown in Equation (1) (Komilis *et al*., 2009; Yaman, 2020)

$$-r = -\frac{dc}{dt} = kC^n \cdots\cdots\cdots (1)$$

Where; r: reaction rate, k: biodegradation rate, C: concentration, t: time, n: reaction order.

Eq. (1) is a typical first-order model. The use of first order kinetics in the description of biodegradation rates in environmental fate models is common because mathematically the expression can be easily incorporated into the model (Coulon *et al*., 2012). The following relation of substrate concentration to time can be obtained as given in Eq. (2):

$$\ln C = a + k_1 t \cdots\cdots\cdots (2)$$

The constants, k and n, are found by plotting concentration vs. time and determining the best suitable line. The half time ($t_{1/2}$) can be calculated as follows (Equation (3)) (Tellez *et al*., 1995; Chemlal *et al*., 2012)

$$t_{\frac{1}{2}} = \frac{\ln 2}{k} \cdots\cdots\cdots (3)$$

## III. Results and Discussion

**3.1 Microbial Analysis**

The results obtained from this study showed that microorganisms can successfully biodegrade TPH, particularly when combined BS and BA processes are used. Results of this study also showed that BA has a higher effect on biodegradation efficiency than BS process. Adding nitrogen and phosphorus, along with microbial inoculation and aeration can create an optimum condition for microorganisms to degrade TPH. Analysis of the contaminated soil indicated that the C: N: P ratio was 100:10:1, which corresponds to the nutrients needed for microorganisms (Alexander *et al*., 1999; Li *et al*., 2007; Agarry and Oghenejoboh, 2015; Yaman, 2020). In addition, the initial soil contained low numbers of TPH biodegraders. Therefore, adding optimum amount of nutrients and introducing TPH degraders (Addition of consortium of *Pseudomonas aerugonisa* and *Bacillus subtilis)* on pesticide polluted soil, apparently increasing the microbial populations in the contaminated soil an offers interesting possibility of enhancing more efficient TPH biodegradation of the soil after few weeks. Counts at the end of the investigation revealed that sample A4 in the organophosphate pesticide contaminated soil treated with combined biostimulation and bioaugumentation strategies supported more bacteria growth. Figure 1 shows the variation of total heterotrophic bacteria counts (THBC) against bioremediation time, the counts are in order of A4>A2>A3>A1 with maximum count (A4) obtained at week 9. This indicated that in the soil, the addition of *Pseudomonas aerugonisa* and *Bacillus subtilis* (bioaugumentation) and cow dung fortified with $KH_2PO_4$ (biostimulation) resulted in the highest number of bacteria at the beginning of remediation period as compared to natural attenuation (or bioattenuation). The lower bacteria population in natural attenuation treatment may be due to the presence of low nutrients (limiting nutrient) and the distribution of nutrient ratio especially of C:N:P that is needed in the correct ratio for bacteria growth

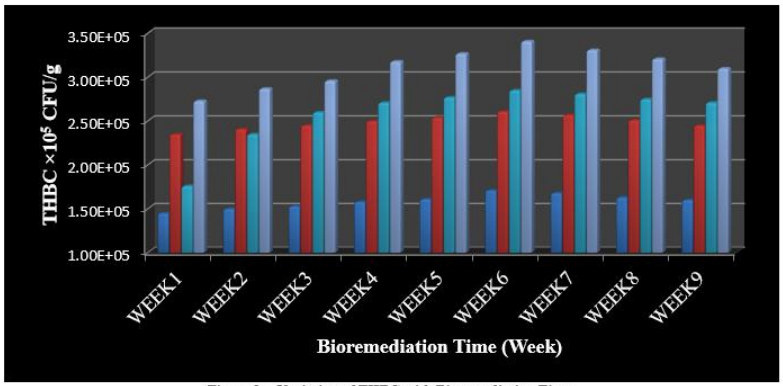


**Figure 1: Variation of THBC with Bioremediation Time**

Natural Attenuation (A1)
Bioaugumentation (A2)
Biostimulation (A3)
Combined Bioaugmention and Biostimulation (A4)

The variation in concentrations of pesticide residual content (mm/kg and %) against bioremediation time is shown in Figures 2 and 3 respectively. Biodegradation of the organophosphate pesticide started very fast during the first week of remediation in all the treatments and slowly continued up to the 9th week (day 63). The concentration of glyphosate was reduced from the initial concentration of 50 000 mg/kg to 29 000 mg/kg, 20 300 mg/kg, 16 400 mg/kg, and 6 500 mg/kg in six(6) weeks of remediation and corresponding to 42%, 59.4%, 67.2%, and 87% glyphosate reduction was achieved under A1, A3, A2 and A4 respectively. This observation revealed that during the glyphosate biodegradation in soil, addition of organic manure (Cow Dung fortified with $KH_2PO_4$) and bacterial inoculums individually resulted in a more effective bioremediation response than the natural attenuation. Similar observations have been reported for naphthalene (Agarry and Oghenejoboh, 2015), kerosene (Shabir *et al.*, 2008; Agarry *et al.*, 2010 and Agarry and Oghenejoboh, 2015) and spent engine oil (Abdulsalam and Omale; 2009 and Abdulsalam *et al*., 2011). Nevertheless, bioaugmentation strategy elicited a higher biodegradation of glyphosate than the biostimulation treatment. This is in agreement with the observation of Bento *et al*., (2003) and Agarry and Oghenejoboh, 2015) who reported that among the individual methods of natural attenuation, biostimulation, and bioaugmentation that were used for the remediation of a soil contaminated by diesel oil, bioaugmentation method elicited higher diesel oil degradation than others. In contrast, other workers have shown that biostimulation strategy enhanced the bioremediation of kerosene contaminated soil (Shabir *et al.*, 2008; Agarry *et al.*, 2013; Agarry and Oghenejoboh, 2015), crude oil contaminated soil (Lu *et al.*, 2010), spent engine oil contaminated soil (Abdulsalam *et al.*,2011) and lubricating oil contaminated soil (Agarry *et al.*, 2013) than bioaugmentation. Generally, in this work, the combination of biostimulation (Cow Dung fortified with $KH_2PO_4$) and bioaugmentation treatment strategy (which has not been reported for glyphosate removal) showed relatively greater glyphosate reduction than other treatments during the whole period of remediation.

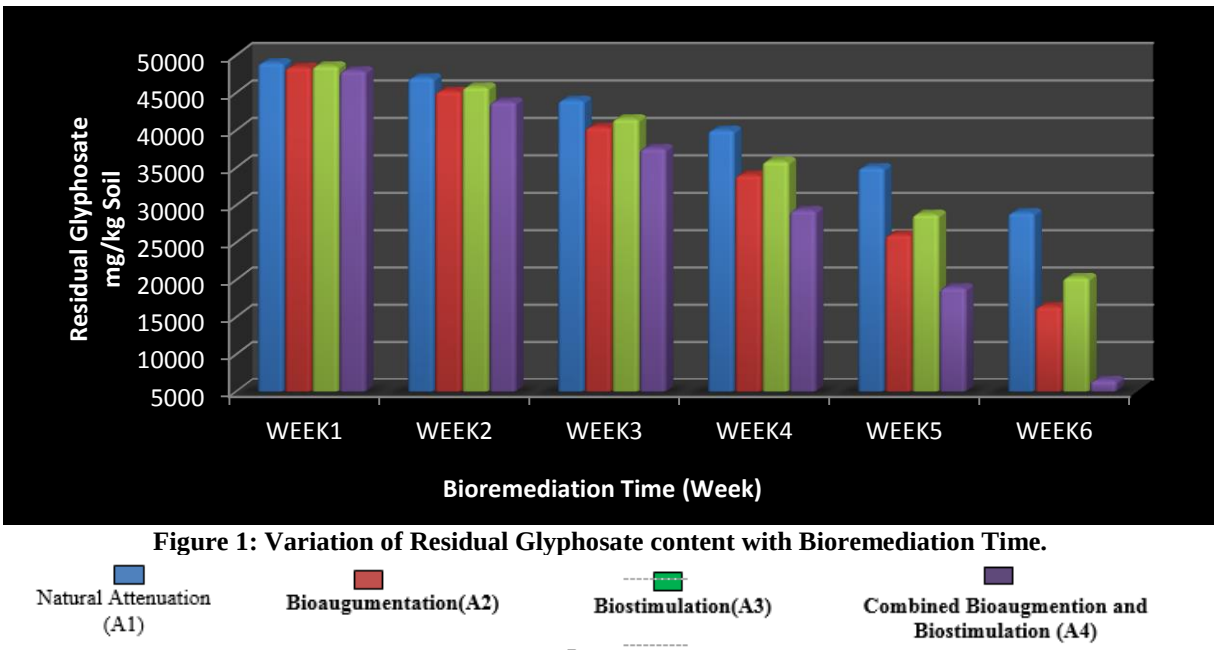


**Figure 1: Variation of Residual Glyphosate content with Bioremediation Time.**

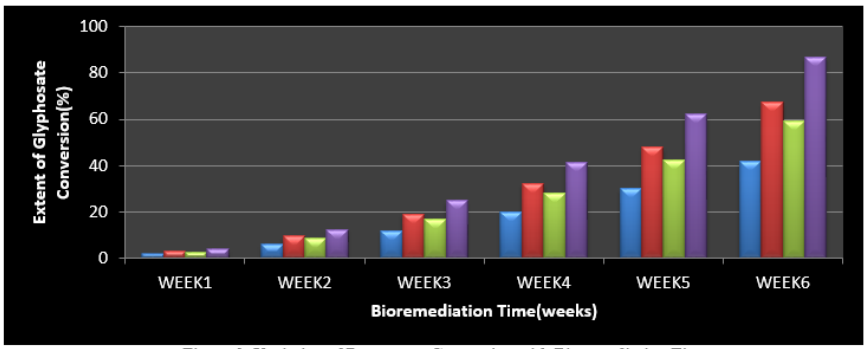


**Figure 3: Variation of Percentage Conversion with Bioremediation Time**

**Combined Bioaugmention and Biostimulation (A4)**

**Biostimulation (A3)**

**Bioaugumentation (A2)**

**Natural Attenuation (A1)**

### 3.2 Bioremediation Kinetics

The data obtained from the treatment tanks were applied to the first-order rate model. Several investigators have reported that first-order kinetics and Michaelis-Menten kinetic can be used for petroleum-hydrocarbon degradation (Brook *et al*., 2001; Shewfelt, 2005; Chemial *et al*., 2012). Figure 5-8 shows fits of the first-order models to data from the NA, BS + BA, BS, and BA treatment processes, which is validated by the high correlation determination of the $R^2$ values indicates that the combination of Bs and Ba A4 (0.89) has the highest regression fitting of the first order kinetic model in the observe trend of regression, A2 (0.83), A3 (0.81) and A1 (0.76) as shown in Table 2. The biodegradation rate constants (k) and half-life times ($t_{1/2}$) for the different remediation treatments are presented in Table 2. It is to be noted that the higher is the biodegradation rate constants, the higher or faster is the rate of biodegradation and consequently the lower is the half-life time. Table 2 shows that the biodegradation of glyphosate in soil under combined biostimulation and bioaugmentation treatment strategy had a highest (k=0.06284$d^{-1}$ ) and lowest ($t_{1/2}$) =11.03d) than that under bioaugmentation (k=0.03016$d^{-1}$, ($t_{1/2}$) =22.9823d), biostimulation ((k=0.0274$d^{-1}$, ($t_{1/2}$) =25.2973d)), and natural attenuation k=0.0238$d^{-1}$, ($t_{1/2}$) =29.07d), respectively. Therefore, value of the kinetic parameter showed that the degree of effectiveness of these bioremediation strategies in the cleanup of soil contaminated with naphthalene is in the following order: bioattenuation < biostimulation < bioaugmentation < combined biostimulation and bioaugmentation

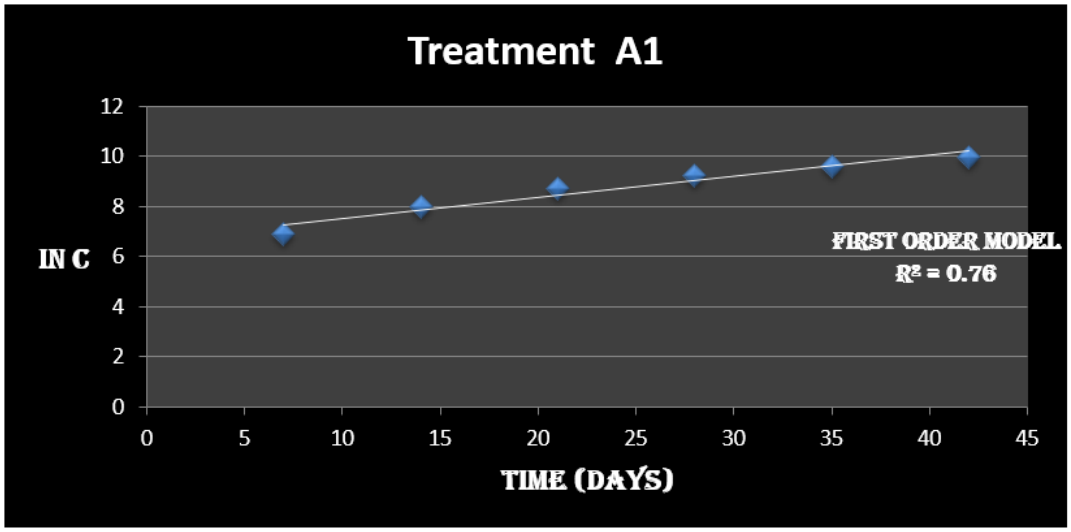


**Figure 2: Reaction rate data of First-Order Model for Bioattenuation.**

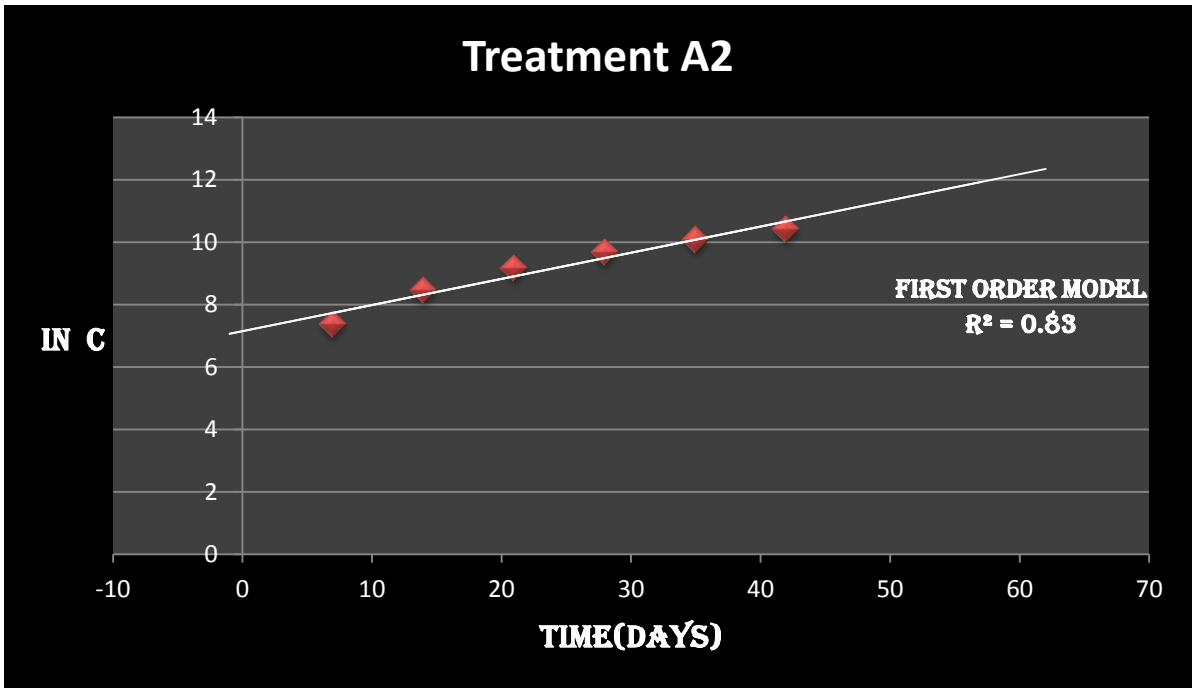


**Figure 3: Reaction rate data of First-Order Model for Bioaugumentation.**

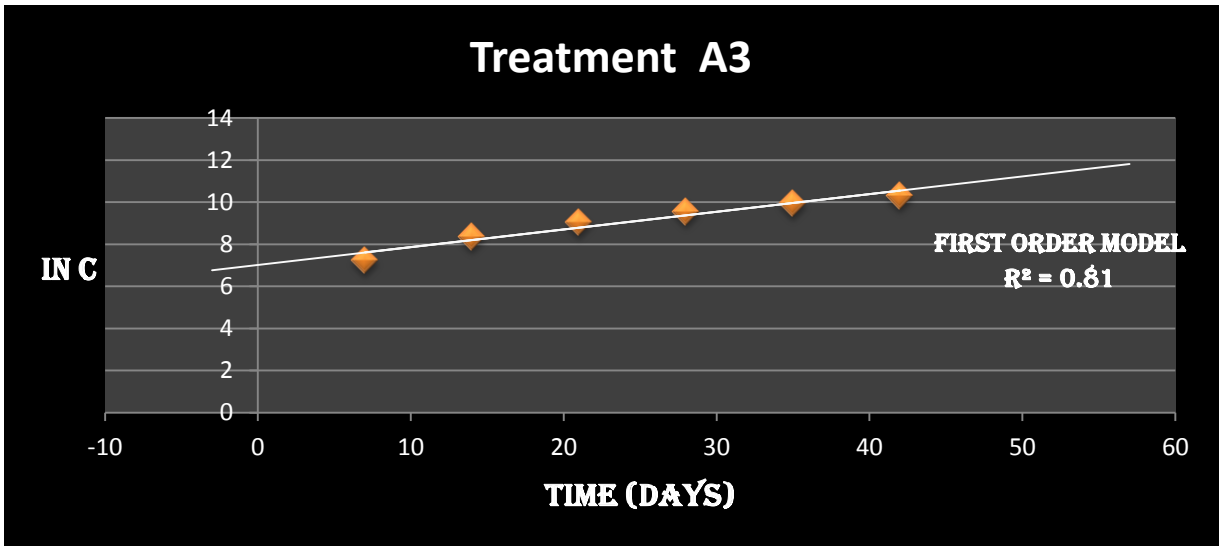


**Figure 4: Reaction rate data of First-Order Model for Bioaugumentation**

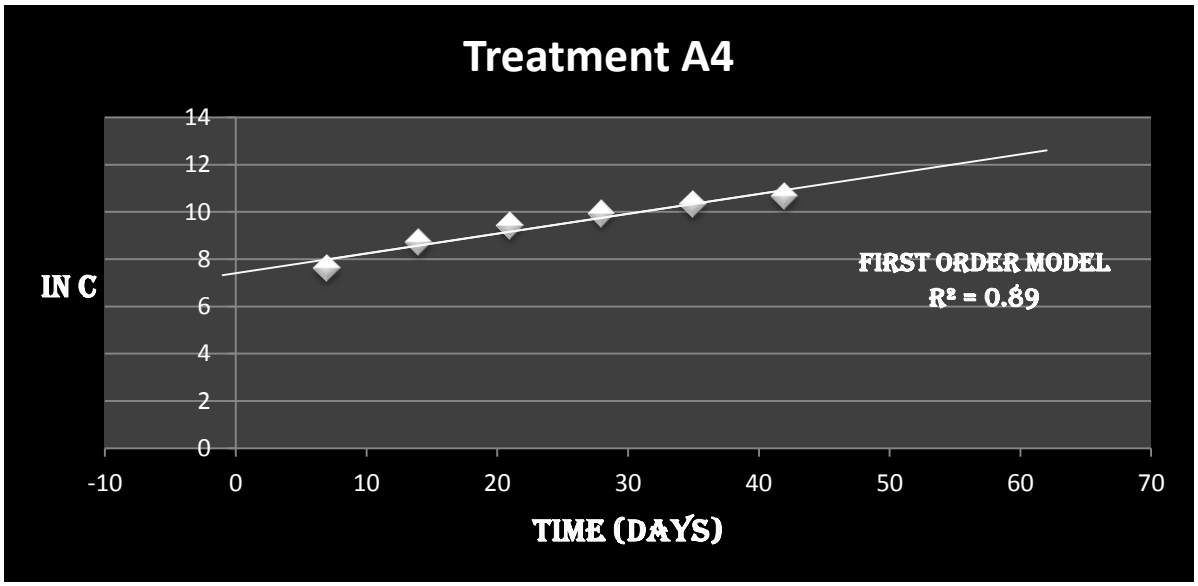


**Figure 5: Reaction rate data of First-Order Model for Combination of BA and BS**

**Table 2: Specific Degradation Rate Constant (k). Half life time ($t_{1/2}$) and Correlation Coefficient ($R^2$)**

| Bioremediation Methods | K(day)$^{-1}$ | $R^2$ | $t_{1/2(days)}$ |
|---|---|---|---|
| Bioattenuation (A1) | 0.02384 | 0.76 | 29.07 |
| Biostimulation (A3) | 0.0274 | 0.81 | 25.29 |
| Bioaugmentation (A2) | 0.03016 | 0.83 | 22.98 |
| Combined Biostimulation and Bioaugmentation (A4) | 0.06284 | 0.89 | 11.03 |

## IV. Conclusion

From this present study, it can be concluded that the biodegradation of glyphosate in the contaminated soil indicates the presence of PAH-degrading microbial communities; and that the rate of glyphosate reduction in soil could be enhanced by the addition of organic nutrients and inoculums, respectively. The soil treatment under combined biostimulation and bioaugmentation exhibited the highest degree of biodegradation with the highest biodegradation rate constant (k 0.06284 day$^{-1}$) and lowest half-life time ($t_{1/2}$=11.03days)) and the soil treatment under natural attenuation the least degradation with the lowest biodegradation rate constant ( k 0.02384day$^{-1}$ ) and highest half-life time ($t_{1/2}$= 29.07days). Thus, the use of biostimulation and bioaugmentation to enhance glyphosate biodegradation in the soil could be one of the severally sought bioremediation strategies of remediating natural ecosystem (environment) contaminated with Aromatic hydrocarbons

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