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Double Impact Model for Deciphering Substrate Uptake by Bacteria under Controlled Nutrient Release Conditions

T. Sampson[1*], C. J. Ogugbue[1], E. Ekaka-a[2] and G. C. Okpokwasili[1]

[1]Department of Microbiology, University of Port Harcourt, P.M.B 5323, Port Harcourt, Nigeria.
[2]Department of Mathematics and Computer Science, Rivers State University of Science and Technology, Port Harcourt, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author TS designed the study, performed the statistical analysis, wrote the protocol and first draft of the manuscript. Author CJO reviewed the first draft of the manuscript and managed the analyses and technical aspects of the study in collaboration with author GCO. Author EE aided in the mathematical modelling. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Bacterial growth kinetics under slow nutrient delivery conditions were studied and evaluated on a model basis in order to determine the impact of substrates on bacterial growth dynamics.
Study Design: The study was carried out in a controlled laboratory condition using an agar base slow-release fertilizer formulation, composed of NPK and Urea (in capsular and granular forms).
Place and Duration of Study: The study was carried out at the Environmental Microbiology Laboratory, University of Port Harcourt, Nigeria, for a 35 – day period.
Methodology: A 0.25% concentration of crude oil was used as a hydrocarbon substrate. Three bacterial isolates (*Pseudomonas* sp., *Bacillus* sp. and *Micrococcus* sp.) were employed in this study. The crude oil degradation rate was monitored using oil and grease method while nitrate nitrogen utilization rate was analyzed by the distillation method.
Results: Bacterial growth rate was observed to be limited by the substrate concentration [S] and the

**Corresponding author: E-mail: tonye4good62@yahoo.com;*

substrate saturation constant/affinity (K_s). The substrate saturation constant/affinity (K_s) was evaluated to be 0.25S, while a bacterial growth ratio ($\frac{\mu}{\mu_{max}}$) was determined to be 0.8.

Conclusion: These findings as well as the developed models could serve as important tools for monitoring the progress of a bioremediation process/strategy. They are therefore recommended for application in the field of environmental biotechnology.

Keywords: Double impact model; bacterial growth ratio; limiting conditions; crude oil; substrate.

1. INTRODUCTION

The petroleum industry, which is a major sector in Nigeria, accounts greatly for most of the environmental challenges in Nigeria. The negative impacts associated with this sector of the economy is mainly due to contamination of the environment following oil spills. These spills often occur as a result of pipeline leakages following vandalism [1] or inadvertently during transportation or spontaneous processes.

These spills affect the ecosystem to a large extent resulting in the loss of livelihood as well as loss in biodiversity [2]. In Nigeria, the Niger Delta region records the highest cases of crude oil pollution due to the increasing activities of petroleum industries in this area which presents attendant ecological problems that affect both terrestrial and aquatic ecosystems [3,4].

Different remediation methods are usually employed to cleanup affected sites. These methods range from physical to biological methods. However, bioremediation (a biological method) is a very useful alternative to physical and chemical methods of cleanup [5] as hydrocarbons are biodegradable in nature. Bioremediation is a process whereby microorganisms utilize hydrocarbon substrates as sole sources of carbon and energy for growth [6]. It relies on the ability of microorganisms to convert these organic and inorganic materials into simpler substances, making them to become non-toxic, thus restoring the environment to its pristine state [7]. The success of a bioremediation process is therefore dependent on the affinity of the microbial species for the substrate (contaminant). These substrates are necessary for the growth of the degrading species and the growth rate of the degrading bacteria among other factors, depends on the level of interaction between the bacterial species and the contaminant.

Models are important tools in monitoring and evaluation of a bioremediation process. Modeling involves the conversion of problems into formulations that offer precision as well as direction to the solution of such problems; it also helps in better comprehension of the system been modeled and results in better system design or control and application of modern computing ability.

In this paper, the interplay between bacterial species and their substrates during bioremediation was harnessed to develop a model. This novel model, based on the bacterial-substrate affinity, could be a veritable tool for growth rate monitoring and bioremediation process evaluation.

2. MATERIALS AND METHODS

2.1 Sources of Substrates

2.1.1 Hydrocarbon substrate

The hydrocarbon substrate (0.25%, v/v) used in this study was prepared by introducing 0.5 ml of sterile crude oil into 200 ml of mineral salt solution (MSS) contained in a 500 ml capacity Erlenmeyer flask.

2.1.2 Nutrient substrate and concentration

Slow-release fertilizers (SRF) in capsular and granular formulations, prepared according to the methods of Sampson et al. [8] was used. Each SRF contained 2 g of respective fertilizer (NPK and Urea).

2.2 Bacterial Biomass

Three bacterial consortium was isolated and identified as *Pseudomonas* sp., *Bacillus* sp. and *Micrococcus* sp. Suspensions of a 24h old pure cultures of the isolates were used to inoculate the experimental setups below.

2.3 Experimental Setup

The setups depicted below were used in this study to determine the interaction between the

bacterial species and the substrate (crude oil) and its role in substrate removal:

Set up Aw = 200 ml MSS + 2 g NPK Capsular SRF + Biomass
Set up Bw = 200 ml MSS + 2 g NPK Granular SRF + Biomass
Set up Dw = 200 ml MSS + 2 g Urea Capsular SRF + Biomass
Set up Ew = 200 ml MSS + 2 g Urea Granular SRF + Biomass
Set up Gw = 200 ml MSS - No fertilizer + Biomass: Control 1
Set up Hw = 200 ml MSS - No fertilizer, no Biomass: Control 2

In order to avoid sampling error, destructive approach was employed in the determination of various parameters in water contained in each setup. Each setup consisted of six (6) disposable vials in triplicates simulating same conditions with each set of vials sacrificed after each sampling interval.

2.4 Study Duration

The experimental setup was monitored for a 35-day period during which changes in bacterial biomass, nutrient (nitrate) concentration and total hydrocarbon content (THC) were investigated at seven (7)-day intervals.

2.5 Measurement of Nutrient Utilization: Determination of Nitrate Nitrogen by Distillation Method

One milliliter water sample was weighed into a distillation flask. This was followed by the addition of 20 ml distilled water and 0.4 g magnesium oxide. The distillation flask was held on a retort stand on a distillation hot plate and then connected to a receiver flask via a Liebig condenser. To this receiver was added 10 ml of 2% boric acid with a few drops of double indicator. The flask was heated to distil out the ammonia as the distillate in the receiver. The boric acid indicator changed to greenish from purple and was titrated with 0.1N HCl to purple in a back titration.

$$\% NH_4^+ = \frac{\text{Titre value x inoculums volume of sample x } 10}{Weight\ of\ sample}$$

2.6 Estimation of Total Hydrocarbons Content (Oil and Grease Method)

The crude oil in the water sample was extracted using an organic solvent, xylene. After extraction, anhydrous sodium sulphate was added into the sample extract to remove any water collected alongside the extract. It was allowed to stabilize on a spectrophotometer for 15 minutes. Thereafter, the absorbance of the extract was read at 420 nm wavelength through a 1 cm glass cuvette.

2.7 Bacterial Growth Kinetics - Substrate Affinity Model

2.7.1 Assumptions

The mathematical model adopted was based on the following assumptions-

(a) The temperature is constant.
(b) Water in pores of the soil aggregate constitutes the liquid phase and the remaining part of the aggregate is considered as the solid phase. No gas phase exists because the aggregate is saturated with water.
(c) Only three components, substrate, oxygen and biomass, are involved in biodegradation;
(d) The transport resistances of substrate and oxygen to and through the micro colonies attached to the surface of soil particles are negligible.

2.7.2 Model formula

Differential models for single – substrate limited process [9] based on Monod's Kinetics were adopted.

$$\mu = \frac{\mu_{max} \cdot S}{K_s + S} \quad (1)$$

3. RESULTS AND DISCUSSION

The initial bacterial count of the water sample at the start of the experiment (day zero) was 4.9 x 10^4 cfu/ml. However, after 7 days, bacterial counts increased in all setups albeit at varying degrees: Setup Aw (2.23 x 10^7 cfu/ml); Setup Bw (1.80x 10^6 cfu/ml); Setup Dw (2.00 x 10^6 cfu/ml); Setup Ew (1.30 x 10^6cfu/ml) and control setup Gw (2.51 x 10^5cfu/ml). By the end of the experiment, rapid proliferation of the consortia was obtained in various setups as indicated below (Table 1): Setup Aw (2.29 x 10^{11} cfu/ml); Setup Bw (1.82 x 10^9 cfu/ml); Setup Dw (1.91 x 10^{10}cfu/ml); Setup Ew (1.29 x 10^8 cfu/ml) and control setup Gw (1.32 x 10^7cfu/ml).

Table 1. Changes in bacterial biomass in various setups during the study period

Sample	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
Aw	$4.9X10^4$	$2.23X10^7$	$1.70X10^8$	$1.70X10^9$	$2.80X10^{10}$	$2.29X10^{11}$
Bw	$4.9X10^4$	$1.80X10^6$	$1.40X10^7$	$1.49X10^8$	$1.49X10^8$	$1.82X10^9$
Dw	$4.9X10^4$	$2.00X10^6$	$1.35X10^8$	$1.58X10^8$	$1.60X10^9$	$1.91X10^{10}$
Ew	$4.9X10^4$	$1.30x10^6$	$1.62X10^6$	$1.23X10^7$	$1.23X10^8$	$1.29X10^8$
Gw	$4.9X10^4$	$2.23X10^5$	$2.51X10^5$	$2.30X10^6$	$1.32X10^7$	$2.30X10^7$

Table 2. Changes in nitrogen content (mg/L) in various setups during the study period

Sample	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
Aw	1.95	42.1	45.31	58.8	59.1	59.7
B w	1.95	25.38	30.21	40.4	45.2	46.2
Dw	1.95	20.6	22.4	25.3	36.1	38.3
Ew	1.95	8.82	9.3	10.7	15.1	20.41
Gw	1.95	1.91	1.82	1.5	1.47	0.99

The results in Table 1 also show that the control experimental setup, Gw witnessed a rather slow growth rate compared to the treated samples during the period of study. This implies that the slow release fertilizers influenced the observed changes in the bacterial biomass during the experimental period.

Changes in nutrient concentration of various setups with time were obtained. A different nutrient formulation was used in each setup and they included granular NPK SRF (Set up Aw), capsular NPK SRF (Set up Bw), granular urea SRF (Set up Dw), and capsular urea SRF (Set up Ew). The control setup Gw was not amended with any nutrient formulation. On zero day, the setups had an initial concentration of 1.95 ± 0.05 mg/L nitrate nitrogen. However, by day 35, there were marked changes in the nitrate nitrogen concentration of the water samples as thus: 59.7 ± 0.19, 46.2 ± 0.07, 8.3 ± 0.15, 20.41 ± 0.20 and 0.99 ± 0.0105 mg/L nitrate nitrogen for samples Aw, Bw, Dw, Ew, and Gw, respectively.

During the study period, the bacterial consortium was able to appreciably degrade the crude oil as indicated by the residual total hydrocarbon content of the setups (Table 3). The initial crude oil concentration of the setups was 11250mg/L on day 0 with a gradual concentration extinction of the petroleum hydrocarbons with time in all setups though at varying magnitudes. More than 50% of the petroleum hydrocarbons in the control was degraded by the microbes despite the non-addition of nutrients in that set up.

The results obtained from this investigation (Tables 1 – 3) have shown the impact of nutrient release rate on the pattern of substrate uptake by bacteria. Statistical analysis using the one way analysis of variance showed there was a significant difference in the mean values of the various treatment options, Aw – Ew, compared to the control Gw ($p > 0.05$, $f(5,36) = 34.378$, PV = 0.00). However, while there was a positive correlation between bacterial biomass and the nutrient source (nitrate nitrogen) ($r = 0.530$), there was a strong negative correlation between bacterial biomass and total hydrocarbons content ($r = -0.957$), using the two tailed Pearson's correlation model.

Table 3. Changes in total hydrocarbon content (mg/L) of crude oil contaminated water in various setups during the study period

	Total hydrocarbon content (mg/L)					
Sample	**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 35**
Aw	11250	8750	5500	4000	3870	2925
Bw	11250	9000	5500	4350	4129	3440
Dw	11250	9500	6250	4500	4230	3645
Ew	11250	9000	7000	5300	5000	3791
Gw	11250	10000	7650	6700	5600	5085

The positive correlation between bacterial biomass and the nutrient source (nitrate nitrogen) was as a result of the slow nutrient delivery behavior of the fertilizers used. The slow-release fertilizers showed an early stage nutrient deficiency phenomenon, which later released the active nutrients gradually with concomitant increase in bacterial biomass. The strong negative correlation between bacterial biomass and total hydrocarbons content on the other hand shows that the increase in bacterial biomass was inversely related to the residual total hydrocarbon content. This therefore shows a direct proportionality between the rate of increase in bacterial biomass and that of uptake of substrate (hydrocarbon) or removal rate by the degrading species. This, therefore, implies that crude oil degradation is greatly influenced by bacterial growth and nutrient availability.

Mathematical Modelling

From the results, bacterial count changed with respect to time. The specific rate of change/growth rate (μ) is calculated as;

$$\text{Specific growth rate } (\mu) = \left(\frac{1}{X0}\right) * \left(\frac{dx}{dt}\right)$$

The results from this study revealed that the rate of hydrocarbon removal correlates with the rate of increase in biomass. Also, changes in the bacterial growth rate resulted in a concomitant reduction in the total hydrocarbons content, with respect to time. However, the growth rate is limited by the substrate concentration [S] and the substrate saturation constant/affinity (K_s).

➢ Kinetics parameters

From the experimental results obtained, the kinetics parameters (μ_{max} and K_s) were calculated (as shown in Table 4) based on Monod's Kinetics:

$$\mu = \frac{\mu_{max} * S}{K_s + S}$$

Where;

μ = Specific growth rate
μ_{max} = Maximum Specific growth rate
S = concentration of substrate in aqueous phase
K_s = affinity constant for substrate.

Table 4. Bacterial Kinetic parameters per week

Substrate	μ_{max}	K_s
Hydrocarbon (SRF)	0.69	1155.05
Nitrate (SRF)	0.69	8.60

➢ Relationship between specific growth rate and substrate concentration

A hyperbolic function/relationship was seen to exist between specific growth rate and substrate concentration with respect to time (Figs. 1 and 2). If the concentration of S is reduced, the population growth rate will decrease. If concentration of S increases to a specific limit where growth rate is at maximum, then S is no longer regarded as a limiting factor. When $K_s = S$, the term $\left(\frac{S}{(Ks + S)}\right)$ becomes half $\left(\frac{1}{2}\right)$ and the growth rate becomes equal to $\frac{1}{2}$ maximum rate.

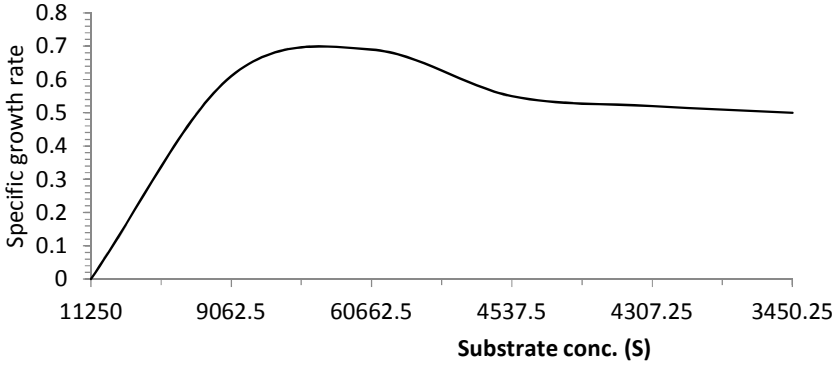

Fig. 1. Relationship between specific growth rate (μ) and Hydrocarbon Conc. [s_h]

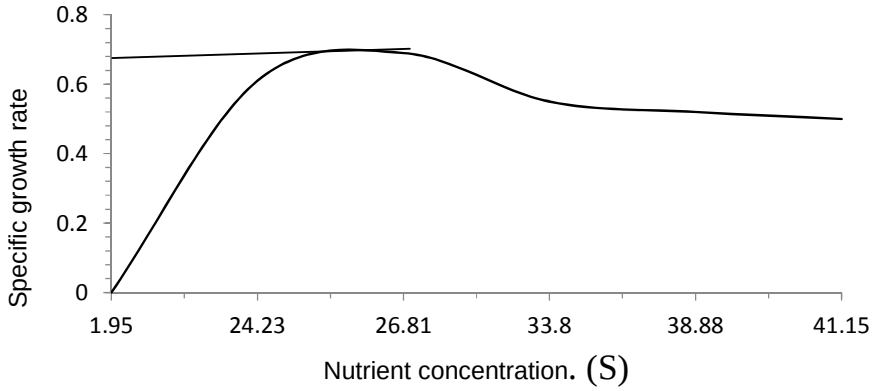

Fig. 2. Relationship between specific growth rate (μ) and nutrient Conc. [S_n]

Double Impact Model for Growth Rate Determination

The growth or increase in bacterial biomass is a function of crude oil concentration and availability of nutrients as both factors/substrates impact simultaneously on bacterial populations. McGee et al. [10] has proposed a model to describe the above condition and this is thus evaluated under these growth conditions.

$$\mu/\mu_{max} = \{[A/(A+B)]*C\} + \{[B/(A+B)]*D\} \quad (2)$$

$$\mu = \mu_{max}\{[A/(A+B)]*C\} + \mu_{max}\{[B/(A+B)]*D\} \quad (3)$$

Where;

$$A = \frac{K_1}{S_1}$$
$$B = \frac{K_2}{S_2}$$
$$C = \frac{S_1}{K_1 + S_1}$$
$$D = \frac{S_2}{K_2 + S_2}$$

1 = Crude oil hydrocarbon substrate under SRF condition
2 = Nutrient substrate under SRF condition

From the Experimental Results and Calculations

K_1 = 1155.05
S_1=4537.5
K_2= 8.60
S_2 = 33.8
μ_{max} = 0.69

Substituting

A = 0.25
B = 0.25
C = 0.80
D = 0.80

Hence

$\mu = \mu_{max}\{[A/(A+B)]*C\} + \mu_{max}\{[B/(A+B)]*D\} = 0.55 week^{-1} = 0.5x10^{12}$ cfu/ml/day

Model Evaluation

From the experimental results above, A = B and C = D.

The model becomes:

$$\mu = [\mu_{max}\{(\tfrac{1}{2})*C\}]*2 = \mu_{max}* C$$

But $C = \frac{S_1}{K_1 + S_1}$

Therefore:

$\mu = \frac{\mu_{max} \cdot S_1}{K_1 + S_1}$; recall equation 1, above

Also:

$$\frac{\mu}{\mu_{max}} = \frac{S_1}{K_1 + S_1}$$

Proof:

$$\frac{0.55}{0.69} = \frac{4537.5}{1155.05 + 4537.5} = 0.80 = C$$

Hence:

$$\frac{S}{K_s + S} = 0.80$$

$$S = (K_s + S)*0.8$$

$$K_s = (S - 0.8S)/0.8 = S(1 - 0.8)/0.8 = 0.25S$$

Based on these kinetics parameters, K_s is determined by multiplying the substrate concentration by 0.25.

$$K_s = 0.25S.$$

However, when;

$$\mu = 0.5\mu_{max},$$

$$\frac{0.345}{0.69} = \frac{S}{K_s + S}$$

$$0.5 = \frac{S}{K_s + S}$$

$$(K_s + S)*0.5 = S$$

$$K_s = \frac{S - 0.5S}{0.5} = \frac{S(1 - 0.5)}{0.5} = S$$

Deductions

1. The growth ratio, $\frac{\mu}{\mu_{max}} = 0.8$; in a substrate limiting condition.
2. When $\frac{\mu}{\mu_{max}} = 1$, $K_s = 0$
3. When $\frac{\mu}{\mu_{max}} > 0.8$, K_s becomes lower, indicating higher rate of substrate uptake (reduction in total hydrocarbon content).
4. When $\frac{\mu}{\mu_{max}} < 0.8$, K_s, becomes higher, indicating lower rate of substrate uptake.
5. When $\frac{\mu}{\mu_{max}} = 0.5$, $K_s = S$; growth rate is at $\frac{1}{2}\mu_{max}$ (half maximum rate)
6. The growth ratio, $\frac{\mu}{\mu_{max}} = \frac{S}{Ks+S} = 0.8$; $\frac{S}{0.25S + S} = 0.8$: This implies the growth rate can be analytically determined at any point by measuring the substrate concentration and calculated using the formula, $\frac{\mu}{\mu_{max}} = \frac{S}{0.25S + S}$.

Monitoring the process and progress of bioremediation is very crucial. Most monitoring involves analyzing for changes in physicochemical parameters as well microbiological parameters such as changes in cell biomass as well as community diversity. Several researchers have evaluated bioremediation reports on model basis, including the use of Monod's kinetics model to numerically simulate the fate of hydrocarbon pollutants [11]. However, there is a paucity of information on kinetics models of crude oil degradation [12] under slow nutrient delivery condition.

The results from this study conform to the fact that the rate of hydrocarbon removal correlates with the rate of increase in biomass [13]. It suggests that a change in bacterial growth rate gives a concomitant reduction in the total hydrocarbon content with respect to time. The same was observed for the hydrocarbon removal rate as well as the substrate affinity constant (K_s). This implies that growth rate is influenced by the concentration of nutrient. Table 4 as well as Figs. 1 and 2 respectively showed the kinetics parameters and the relationship between specific growth rate (μ) and substrate concentration, which indicate that the higher the growth rate the higher the substrate removal rate and the lesser the substrate affinity (K_S). Hence, low K_s indicates high substrate uptake (i.e high substrate removal rate). The K_s value is therefore an important parameter in monitoring the efficacy of a particular treatment option.

Model Based Comparative Analysis for NPK and Urea Slow-Release Fertilizers

Based on the model parameters above, the K_s values for NPK and urea slow-release fertilizers are evaluated and used as a comparison index.

Average Substrate Values

S_h = 4175mg/l……NPK SRF
S_h = 4900mg/l ……..urea SRF
S_n = 49.6mg/l ……NPK SRF
S_n = 18.0mg/l ……urea SRF

Where,

h = hydrocarbon substrate
n = nutrient concentration

Recall:

$K_s = 0.25S$

1. $K_{sh} = 0.25*4175 = 1043.75$…NPK SRF
2. $K_{sh} = 0.25*4900 = 1225$…….Urea SRF
3. $K_{sn} = 0.25*49.6 = 12.4$…… .NPK SRF
4. $K_{sn} = 0.25*18.0 = 4.5$……Urea SRF

This comparative analysis for NPK and urea slow-release fertilizers, based on the model evaluation results, showed that NPK SRF has lower K_s value for hydrocarbon substrate than urea SRF. Urea SRF has lower K_s value for nutrient concentration than NPK SRF. This implies NPK fertilizer induced higher bacterial affinity for hydrocarbon substrate than urea slow release fertilizer, under both conditions. Also, urea recorded higher activity (lower K_s) for nutrient than NPK fertilizer. This means higher affinity indicates the substrate is in short supply or limiting.

4. CONCLUSION

This study has revealed the growth dynamics of bacteria during crude oil degradation, under slow nutrient delivery conditions. From the kinetics studies, it was observed that nutrient greatly stimulated bacterial uptake of crude oil hydrocarbon while using it as a source of carbon and energy. This means the bacterial growth rate is affected by nutrient concentration and hydrocarbon content. This gave rise to the double impact model: μ/μ_{max}= {[A/(A+B)]*C}+ {[B/(A+B)]*D}, from which the model was modified and K_s was calculated based on the substrate concentration [s]; K_s = 0.25S. This model is therefore a simple one for determining bacterial affinity for a particular substrate at any given time without undue permutations and hence, could be a useful tool in optimizing bioremediation processes.

Also, bacterial growth ratio ($\frac{\mu}{\mu_{max}}$) was determined to be 0.8. This finding is very critical in crude oil degradation studies. This is because it can serve as a preliminary index in bioremediation monitoring, where a value (ratio) lower than 0.8 indicates a lower rate of substrate uptake (reduction in total hydrocarbon content) and a ratio higher than 0.8 indicates a higher rate of substrate uptake.

Hence, in this paper, a simple model for determining the affinity of bacteria for a particular substrate at any given time without going through a long series of calculation is presented. These models are useful tools that can be employed in the optimization of a bioremediation process and are therefore recommended for application in the field of environmental biotechnology for the cleanup of polluted aquatic ecosystems.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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