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Growth of Microbial Cells on Modified Montmorillonites with Crude Oil Hydrocarbons as Carbon Source

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The effect of modified montmorillonites on microbial growth with crude oil as carbon source was investigated in aqueous clay/oil microcosm experiments with microorganism community that is predominantly *Alcanivorax spp* as the hydrocarbon degraders. The unmodified montmorillonite was treated with didecyldimethylammonium bromide, hydrochloric acid and the relevant metallic chloride to produce organomontmorillonite, acid activated montmorillonite and homoionic montmorillonites respectively which were used in this study. The parameters used for the assessment of the effect of the clay samples on microbial growth were maximum cell yield, specific growth rate and percentage utilization of hydrocarbons. The study estimated percentage utilization of the hydrocarbons based entirely on growth in the exponential phase. The study indicated that organomontmorillonite, acid activated montmorillonite and potassium-montmorillonite did not stimulate microbial growth whereas unmodified montmorillonite, calcium- and ferric(III)-montmorillonite were able to stimulate the growth of the microbial cells. The estimated hydrocarbon utilization indicated that appreciable percentage of hydrocarbons was utilized in Ca- and Fe(III)-montmorillonite-oil-microbe systems (63 and 57 % respectively) unlike in organo-, potassium- and acid activated montmorillonite-oil-microbe systems with low hydrocarbon utilization (25, 21 and 15 % respectively).

Keywords: Clay minerals, Crude oil, Microbial growth, Specific growth rate.

INTRODUCTION

Microbial degradation can take place both in the presence and absence of oxygen. If molecular oxygen is present as the terminal electron acceptor, the microbial degradation is aerobic or oxic, but if it proceeds in the complete absence of oxygen with other substances acting as electron acceptor (such as nitrates and sulphates), the microbial degradation is anaerobic or anoxic [1-3]. There could be a combination of both oxic and anoxic microbial degradation in some systems as have been reported to take place in near surface accumulations of tar sands [4,5]. For microbial growth to take place, in addition to the presence of electron acceptor and nutrients under favourable conditions, the microbes should be able to obtain carbon from a given substrate in order to build new cell materials and derive energy from transforming the substrate [6]. Crude oil hydrocarbons are known to meet the later condition and are therefore readily biodegraded under favourable conditions. Microbial growth with organic pollutants such as crude oil and its fractions in terrestrial and aquatic environments as carbon source in the presence of colloidal minerals such as clay minerals is either stimulated or inhibited [7-14]. Clay minerals have been reported to have the ability to adsorb not just organic compounds but microorganisms as well with varied implications on microbial degradation of target pollutants [15-18]. However, the effects of modified montmorillonites such as acid activated montmorillonite, organo-montmorillonite and homoionic interlayer montmorillonite clays on the growth kinetics of microorganisms using crude oil hydrocarbons as sole carbon and energy source is not well reported. Acid activated clay minerals have been the subject of much research, having been discovered to have good catalytic properties especially for many organic reactions as a result of their high surface area and acidity. Acid mine drainage interacting with clay minerals is one of the natural ways of having acid activated clay minerals [19,20]. During acid activation, the main goal is to obtain partially dissolved material that is of increased surface area, porosity and surface acidity [21,22]. For industrial or scientific research purposes, acid activated clay mineral is prepared by washing or treating the clay mineral with strong mineral acids such as sulphuric acid or hydrochloric acid. During the process of activation, the acid exchanges its protons for the interlayer exchangeable cations and partially dissolves the clay crystalline structure by leaching some of the cations such

as Mg^{2+}, Al^{3+} or Fe^{2+}. Consequently, acid activated clay mineral with increased surface acidity, specific surface area and porosity compared with the starting clay mineral is formed [21,23].

There are several applications of smectites on account of their high cation exchange capacity (CEC), one of which is the production of organoclays. Producing organoclay minerals in practice requires the replacement of the interlayer exchangeable inorganic cations with organic cations through ion-exchange reactions. The resultant organoclay modifies the surface of the original clay mineral from being hydrophilic to being hydrophobic [24,25].

The homoionic clays are usually generated by exchanging the interlayer cations of unmodified clays (usually clays with high cation exchange capacity such as montmorillonites) with excess metallic chloride of the required metallic ion to produce the desired homoionic interlayer clay *via* cation exchange reactions [26].

This study investigated the effects of acid activated montmorillonites, organo montmorillonites and homoionic montmorillonites on microbial growth so as to gain understanding of which form(s) of montmorillonite would be useful in further biodegradation and bioremediation studies. The main questions addressed in this study are:

• Do the modified montmorillonite clay minerals stimulate or inhibit microbial growth in comparison with the unmodified form?

• How may microbial growth kinetics be used for estimating hydrocarbon utilization by the microbes in the log phase?

EXPERIMENTAL

Bentonite Berkbent 163 supplied by Steetley Bentonite & Absorbent Ltd was the source of the montmorillonite used in preparing acid activated montmorillonite, organo montmorillonite and homoionic montmorillonite. Didecyldimethylammonium bromide and other chemicals used in this study were supplied by Sigma Aldrich. The microbial communities employed in this study were isolated from beach sediment sample that consists fine sand particles collected in a sterilized glass bottle (Duran) from a site at St Mary's lighthouse near Whitley Bay, Newcastle upon Tyne (N 55° 04' 18", W 01° 26' 59"), United Kingdom. The sediment samples were stored at 4 °C in cold room until commencement of the experiment. The Bushnel-Haas broth used as the nutrient source and nutrient agar were supplied by Sigma Aldrich. The crude oil was an undegraded North Sea crude oil originally supplied by British Petroleum.

Production of modified clays

Acid activated montmorillonite: Acid activated clay minerals were prepared following the procedure reported in Ugochukwu *et al.* [27]. The procedure involved the following clay mineral acid activation operating conditions: temperature of 70 °C, 3 M HCl, reaction time of 45 min and solid/liquid ratio of 1:3 (w/w) for acid activation of the montmorillonite clay mineral. The produced acid activated clay mineral was thoroughly washed to ensure that there was no free HCl in the clay samples. To confirm that the acid activated montmorillonite did not contain free HCl, the final supernatant separated from solids after washing was decanted and tested for chloride using ion-chromatography.

Organo-clay: The organo-montmorillonite was prepared by the reported method [27]. The procedure involved dispersing 50 g of montmorillonite in 1.5 L of de-ionized water and stirring for 24 h. Didecyldimethylammonium (DDDMA) bromide aqueous solution corresponding to 35 % cation exchange capacity of the clay was added to the clay suspension and stirred for 24 h. The resulting clay suspension was centrifuged and the supernatant separated from the solids. The resulting solids (organo-montmorillonite) were washed several times by adding de-ionized water and centrifuging. The organo-montmorillonite obtained was dried at a temperature of 48 °C and stored in a dessicator.

Homoionic montmorillonite: The procedure reported by Reddy *et al*. [29] was employed in the preparation of the homoionic montmorillonites as has been described in Ugochukwu *et al*. [28]. Samples were generated from exchanging the interlayer cations of montmorillonites with K^+, Ca^{2+} and Fe^{3+} to produce, K-montmorillonite, Ca-montmorillonite and Fe-montmorillonite.

Characterization of the clay materials: The procedure for X-ray diffraction (XRD), Fourier transform infrared (FTIR), surface area, cation exchange capacity (CEC), pH and the total organic carbon (TOC) of the clay samples are as reported in Ugochukwu *et al*. [27,28].

Microbial growth experiments

Enrichment culture preparation for microbial cell proliferation: The initial enrichment culture was prepared by mixing 20 g of beach sand, 100 mL of Bushnel-Haas medium (which was prepared by dispersing 0.327 g of Bushnel-Haas broth in 100 mL de-ionized water) and 250 mg of crude oil in a 250 mL conical flask under a sterile atmosphere provided by bunsen burner flame. The flask was then closed with a cotton wool and continuously shaken. For the purpose of further enrichment and proliferation of the cells, several weights (0.25-0.5 g) of the crude oil were used as carbon source in several subcultures after the initial culture. Microbial growth was monitored in the subculture for each case by collecting 1 mL of cell suspension every two days and measuring absorbance at 600 nm using a UV-visible spectrophotometer. The growth curve obtained enabled determination of the exponential and stationary phase and hence an estimate of when there will be active cells in the system. Several subcultures were consequently prepared by sampling the cells when they were still very active. Cell growth was also monitored *via* standard cell plating. The plates were prepared as follows: 28 g of nutrient agar and 3.27 g of Bushnel-Haas broth were dispersed in 1 L of deionized water and then autoclaved. Crude oil (500 mg) was added and the mixture well shaken. The contents were then poured into several Petri-dishes and allowed to gel. They were then stored at 4 °C and subsequently used for cell plating as samples are withdrawn periodically from the test subculture. The procedure used in carrying out the cell plating was as follows: 1 mL of cell suspension from the subculture was used in preparing 10^1 to 10^7 fold dilution. 0.1 mL of each of the above stated dilutions was spotted on the plate and carefully spread. The plates were incubated for 24 h at 28 °C and enumerated.

Effect of clay samples on the microbial growth: A set of experiments (in triplicate) for determining the effect of clay mineral samples on microbial growth was carried out as follows: 250 mg of each of the clay samples (unmodified montmorillonite, acid activated montmorillonite, organomontmorillonite and homoionic montmorillonite) were each mixed with 50 mg of crude oil and 10 mL Bushnel-Haas medium and inoculated with the microbial cells from the final enrichment culture. Sacrificial samples were collected every four days until the 12th day for microbial growth tests.

Growth kinetics: Microbial growth kinetic equation can be fundamentally described as follows:

$$N = N_0 e^{\mu t} \quad (1)$$

where: N = the cell population at time t; N_0 = the initial cell population at time = 0; μ = the specific growth rate.

In order to evaluate the specific growth rate we generate the equation difference at two different points corresponding to two different time periods along the line describing the exponential phase of the microbial growth.

This leaves us with the equation:

$$\mu = (\ln N_2 - \ln N_1)/t_2 - t_1 \quad (2)$$

μ is the specific growth rate, N_1 and N_2 are the cell population at time t_1 and t_2, respectively.

If we assume logarithmic kinetics of substrate disappearance in the exponential phase applies to all biodegradation systems, then we apply the logarithmic kinetic equation:

$$ds/dt = \mu_{max} (S_0 + X_0 - S) \quad (3)$$

Integrating equation 3 would give:

$$S = S_0 + X_0 [1 - \exp (\mu_{max} t) \quad (4)$$

where: S = substrate concentration at the end of exponential phase; S_0 = Initial substrate concentration; μ_{max} = maximum specific growth rate; t = time to end of exponential phase; X_0 = amount of the substrate required to produce initial cell population [29].

Re-arranging equation 4 would give eqn. 5 as:

$$(S_0 - S)/S_0 = X_0 [\exp (\mu_{max} t) - 1]/S_0 \quad (5)$$

Given that the substrate concentration employed in the 9th subculture and the initial substrate concentration employed during microbial growth in the presence of clays are equal, it would be safe to assume the equivalence of S_0 and X_0.

Hence:

$$(S_0\text{-}S)/S_0 = \exp (\mu_{max} t) - 1 \quad (6)$$

Percentage of substrate utilized by cells =

$$[\exp (\mu_{max} t) - 1] \times 100 \quad (7)$$

Cells were enumerated *via* standard cell count as described above. Maximum cell yield and specific growth rate were consequently used to compare the kinetics of the cell growth among samples and hence the effects of the clay samples on microbial growth. Also, the logarithmic kinetics of substrate disappearance was used to estimate the percentage of the substrate utilized to the end of the exponential phase. Statistical analysis employed was a 2-sample t-test to analyze for significant statistical difference among the samples. All comparisons were with reference to the control experiment hence the validity of the 2-sample t-test.

RESULTS AND DISCUSSION

Characterization of clay mineral samples

XRD and FTIR: The d-spacing of air dried acid activated montmorillonite (BA) which is 14.8 Å indicates that the protons located at the interlayers of the clay may have been highly hydrated by moisture (Table-1). This could be due to the high charge/size ratio of protons. The organomontmorillonite (BO) on heat treatment at 300 °C showed the least layer collapse at 13.2 Å in comparison with unmodified montmorillonite (BU) at 10.6 Å and acid activated montmorillonite at 10.1 Å (Table-1). This is due to the intercalation of didecyldimethylammonium (DDDMA) in the interlayer of this clay sample. The d-spacing of air dried Ca-montmorillonite and Fe(III)-montmorillonite at 14.6 Å and 14.8 Å respectively are higher than that of unmodified montmorillonite due to relatively higher charge/size ratio leading to attraction of more water layer under air-dry condition. The IR absorption band at 3623 cm^{-1} in the spectra for treated and untreated montmorillonite is assigned to OH-stretching of AlAlOH which is typical of dioctahedral smectites such as montmorillonites (Table-1). The absorption bands at 2861 and 2935 cm^{-1} (Table-1) observed with organomontmorillonite are assigned to symmetrical and assymetrical vibration stretch of CH_2 respectively from the hydrocarbon moiety of DDDMA of the organoclay sample. The absorption band at 1430 cm^{-1} is due to calcite (carbonate) and does not appear in the spectra of BA and B-Fe as it was digested during acid activation and ionic exchange, respectively (Table-1).

TABLE-1
BASAL SPACING OF 001 REFLECTIONS AND SELECTED FTIR ABSORPTION BANDS OF THE CLAY SAMPLES

Sample	XRD			FTIR	
	d-spacing (Å)			Absorption band (cm^{-1})	
	Ethylene glycolated	Air dried	Heat treated	OH-stretch	Carbonate
BU	17.1	12.5	10.6	3623	1430
BO	16.8	14.2	13.2	3623, 2935, 2861	1430
BA	16.8	14.8	10.1	3623	–
B-K	17.0	12.6	10.0	3623	1430
B-Ca	17.0	14.6	10.2	3623	1430
B-Fe	17.0	14.8	10.3	3623	–

BU = unmodified-; BA = acid activated-; BO = organo-; B-K = potassium-, B-Ca = calcium- and B-Fe = ferric-montmorillonite.

Hydrocarbon degrading bacteria enrichment culture: Fig. 1 shows that day 6 is the most probable period of having active cells in the culture hence the point of sampling the cells in order to maintain active cells. Consequently, the microbial enrichment culture was grown in a mineral salts growth medium (Bushnell-Haas) with crude oil as sole carbon and energy source. Different amounts of the substrate (0.25-0.5 g) were used and sampling done at the sixth day for fresh subculture until the 9th subculture which was the final enrichment culture.

The absorbance – time curve for the 9th subculture (final enrichment subculture) using crude oil as sole carbon and energy source is presented in Fig. 2.

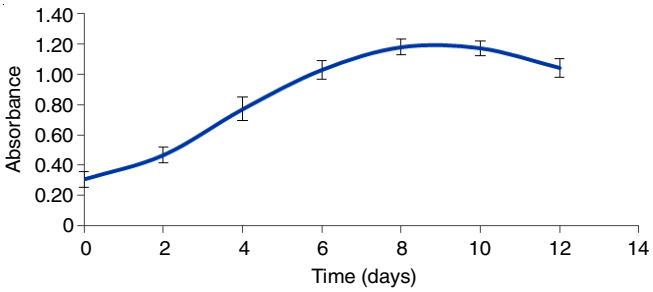

Fig. 1. Microbial growth curve for initial culture with crude oil (500 mg) as sole carbon and energy source. Values are presented as mean ± one standard deviation

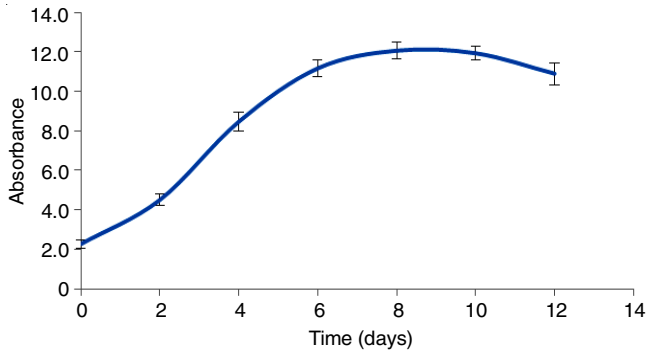

Fig. 2. Microbial growth curve for final enrichment culture (9th subculture) with crude oil (0.5 g) as sole carbon and energy source. Values are presented as mean ± one standard deviation. The absorbance data was obtained after 10 fold dilution of the cell suspension

The final enrichment culture (9th subculture) has a growth rate (specific growth rate of about 0.008 h^{-1}) that is consistent with other subcultures and produced maximum absorbance (Fig. 2) as a result of relatively high cell density. This 9th subculture was the source of the microbial cells used for subsequent experiments in studying the effect of clay minerals on microbial growth with crude oil as sole carbon and energy source (Fig. 3).

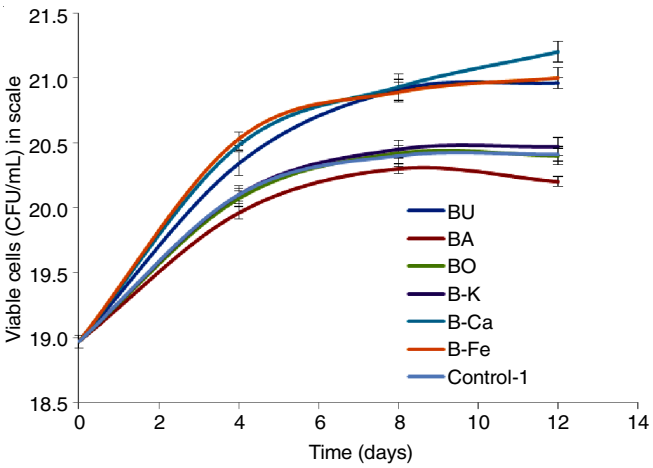

Fig. 3. Effect of modified montmorillonites on growth of hydrocarbon degrading bacteria. BU, BA, BO, B-K, B-Ca, B-Fe, represent unmodified-, acid activated -, organo-, potassium- calcium- and ferric-montmorillonite and Control-1 is (control without clay). Control-2 (control with neither clay nor cells) and clay controls all have zero value for viable cell count and have not been plotted above. The exponential phase is not very well defined with respect to its termination point but here we apply 160 h. Hence, the cells were in the exponential phase for 192 h

The maximum cell yield, specific growth rate and estimated percentage hydrocarbon utilization are presented in Table-3.

The acid activated montmorillonite appears to depress microbial growth as the maximum cell yield and specific growth rate constant for cells in unmodified montmorillonite (BU) and Control-1 are higher than those in the acid activated clay samples (Table-3 and Fig. 3). The 2-sample t-test at 95 % confidence interval with respect to maximum cell yield and specific growth rate indicates that generally, Control-1 and BU are statistically significantly different from BA (P-values < 0.05). The mean percentage utilization of hydrocarbon to the end of the exponential phase by the microbes in the experiment containing BA (15 %; Table-3) is quite low and statistically significantly different from those of Control-1 and BU (28 and 42 %; Table-3). The lowered pH of the acid activated clay samples (Table-1) could be responsible for this depression of microbial activity. This result is consistent with the report of Alexander [30] that showed that at extremes of acidity or alkalinity, activity of microbes declines.

Organo montmorillonite (BO) appears not to stimulate microbial growth as the maximum cell yield and specific growth rate in this sample are both less than that in either Control 1 or BU. The mean percentage utilization of hydrocarbon to the end of the exponential phase by the microbes in the experiment containing BO (21 %; Table-3) is statistically significantly different from BU (P-value < 0.05) but not Control-1. The EGME- surface area of organoclay (Table-2) is lower than the other forms of clay and is suggested to be mainly due to the existence of hydrophobicity (introduced into the clay by the organic cation) that hinders access to the interlayer surface by the EGME which is polar and hydrophilic. This hydrophobicity promotes interaction with crude oil hydrocarbons (which are also hydrophobic). This hydrophobic interaction appears to render the adsorbed hydrocarbons unavailable for microbial growth.

TABLE-2
EGME-SURFACE AREA, pH, TOC AND CATION EXCHANGE CAPACITY (CEC) OF THE CLAY SAMPLES

Sample	pH	Surface area (m^2/g)	CEC (meq/100 g)	TOC (%)
BU	9.0	645	83.3	–
BA	4.1	722	–	–
BO	9.1	471	–	7.3
B-K	7.8	455	76.4	–
B-Ca	7.6	598	79.1	–
B-Fe	4.9	646	88	–

BU = unmodified-; BA = acid activated-; BO = organo-; B-K = potassium-, B-Ca = calcium- and B-Fe = ferric-montmorillonite.

Unmodified montmorillonite appears to stimulate the growth of the microbial cells (Table-3 and Fig. 3). The 2-sample t-test at 95 % confidence interval (CI) with respect to maximum cell yield and specific growth rate constant indicates that BU is significantly different from control-1 ($p < 0.05$). The mean percentage utilization of hydrocarbon to the end of the exponential phase by the microbes in the experiment containing BU is statistically significantly different from Control-1 (42 %; Table-3; $p < 0.05$). The stimulatory role of

TABLE-3
MAXIMUM CELL YIELD, SPECIFIC GROWTH RATE CONSTANT AND ESTIMATED % OF HYDROCARBON UTILIZED BY THE CELLS DURING THE EXPONENTIAL PHASE DUE TO THE EFFECT OF CLAY MINERALS. VALUES ARE REPORTED AS MEAN ± STANDARD ERROR OF THE MEAN

Sample	Maximum cell yield (CFU/mL)/10^8	Specific growth rate (h^{-1}) 10^3	Estimated % of oil utilized by cells
BU	11.2 ± 0.4	10.2 ± 0.4	42 ± 5
BA	5.9 ± 0.3	7.5 ± 0.2	15 ± 5
BO	7.4 ± 0.3	8.4 ± 0.2	21 ± 3
B-K	8.3 ± 0.7	8.8 ± 0.4	25 ± 4
B-Ca	14.1 ± 0.5	11.3 ± 0.4	63 ± 5
B-Fe	13.2 ± 0.5	11.0 ± 0.4	57 ± 4
Control-1	8.1 ± 0.2	9.1± 0.2	28 ± 3

BU = unmodified-; BA = acid activated-; BO = organo-; B-K = potassium-, B-Ca = calcium- and B-Fe = ferric-montmorillonite.

the unmodified montmorillonite on microbial growth appears to be consistent with the studies of Stotzky & Rem [7] and Warr *et al*. [14]. Modification of the interlayer cations of the montmorillonite [with potassium, calcium and iron(III)] appears to produce clays that have varied effects on microbial growth in comparison with unmodified montmorillonite. Calcium and ferric-montmorillonite appear to lead to improvement on the microbial growth (Fig. 3). The maximum cell yield and specific growth rate constant in samples B-Ca and B-Fe are higher than that of BU (Table-3). The mean percentage utilization of hydrocarbon to the end of the exponential phase by the microbes in the experiment containing B-Ca and B-Fe is statistically significantly different from Control-1 (63 and 57 % respectively; Table-3; P-value < 0.05). Interlayer cations are believed to cause 'local bridging effect' (effective delivery of nutrients to cells due to reduction of zeta potential or electrical double layer repulsion) and this together with high surface area have been suggested to account for why clays such as unmodified montmorillonite, Fe and Ca-montmorillonite stimulate microbial growth [14,31,32]. Though Ca-montmorillonite has a lower EGME-surface area than unmodified montmorillonites, the impact of the 'local bridging effect' over compensates for this shortfall. Despite the fact that acid activated montmorillonite has the highest EGME-surface area, this could not translate to improved microbial growth. This is due to the toxic effect of clay's acidity. The maximum cell yield and specific growth rate constant in sample B-K are lower than that of BU, B-Ca and B-Fe (Table-3). Samples B-Ca and B-Fe are significantly different from B-K (P-values of < 0.05). The surface chemistry of K-montmorillonite appears to account for why B-K is not really stimulatory to microbial growth. It appears that the implication of having K^+ in the interlayer of the clay is that the attraction of water in the interlayer will be reduced due to the lower hydration energy of K^+ as its charge/radius ratio is relatively low [33]. This makes the interlayer of potassium-montmorillonite exhibit relatively reduced hydrophilicity. This, coupled with the fact that K^+ has a relatively large size (that will expose the hydrophobic siloxane surface), causes this clay sample to possess the ability to host hydrocarbons which are hydrophobic. This is suggested to reduce the availability of the hydrocarbons as observed with organoclay. The same reason of exposure of hydrophobic siloxane surface accounts for why the surface area of potassium-montmorillonite is lowest among the homoionic montmorillonites as EGME (a polar compound) would have reduced ability to access the exposed hydrophobic siloxane surface in the interlayer of the clay sample (Table-2).

Conclusion

Clay minerals such as montmorillonites affect microbial growth with crude oil hydrocarbons as carbon source, though whether the microbial growth will be inhibited or stimulated depends on the type and form of the montmorillonite. It is suggested that acid activated montmorillonite may have been inhibitory to microbial growth due to toxic effect of the clay's acidity on microbes whereas it is suggested that organomontmorillonite was inhibitory to microbial growth due to the hydrophobic interaction between the organic phase of the organomontmorillonite clay and crude oil hydrocarbons that reduces the availability of the hydrocarbons. The clay minerals that stimulated microbial growth were the unmodified, calcium- and ferric(III)-montmorillonite and are suggested to have done so by reason of 'local bridging effect' and relatively high surface area. The percentage utilization of the hydrocarbons by the microorganisms in the various systems increases in the following order: BA < BO < B-K < Control-1 < BU < B-Fe < B-Ca. This same order was the order of increase in specific growth rate among the samples. This evidence was required for further biodegradation studies.

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