USE OF TWO-SURFACTANTS MIXTURES TO GET SPECIFIC HLB VALUES FOR ASSISTED TPH-DIESEL BIODEGRADATION

Luis G. Torres, Neftalí Rojas, and Rosario Iturbe

Instituto de Ingeniería. Universidad Nacional Autónoma de México.
Coordinación de Ingeniería Ambiental. Grupo Saneamiento de Suelos y Acuíferos.
Apartado Postal 70-472. Coyoacán 04510. México, D.F. MEXICO.
e-mai: ltorresb@iingen.unam.mx

ABSTRACT

In a surfactant assisted biodegradation process, the choice of surfactant(s) is of crucial importance. Previous works have showed that the parameter that mostly affects biodegradation process is temperature, followed by HLB surfactant value and, surprisingly, surfactant dose at the end. The remaining question is: does the type of surfactant (*i.e.* chemical family) affect the biodegradation process at fixed HLB values? In this work, microcosm assessments were developed using a contaminated soil, previously characterized in terms of its physical and chemical characteristics. TPH content was around 5,000 mg/kg of hydrocarbons as TPH-diesel. Mixtures of three nonionic surfactants were employed to get a wide range of specific HLB values. Tween 20 and Span 20 were mixed in the appropriate proportions to get HLB values between 8.6-16.7. Tween/Span 60 mixtures reached HLB values of 4.7-14.9. Finally, Tween/Span 80 combinations yielded HLB values of 4.3-15. TPH-diesel biodegradation and FCU/gr_{soil} were measured at the beginning, and after 8 weeks. The conclusion of this work is that the type of surfactant (*i.e.* chemical family), and not only the HLB value clearly affects the assisted biodegradation rate. Surfactant's synergism was clearly observed.

Key words: Aged soils, enhanced bioremediation, HLB, Span, surfactants, Tween.

INTRODUCTION

Aged soils are difficult to treat by biological methods, since *contaminants i.e*, hydrocarbon compounds, can be tightly adsorbed into the soil particles. This problem can be solved by using small quantities of specific surfactants to increase compounds´ bioavailability. Selection of the right surfactant and dose is of crucial importance to the biodegradation process, but frequently the selection process is based on a trial an error method. Surfactant hidrophillic-lypofillic balance HLB value is an expression of the surfactant affinity molecule to the organic matter and water phases. This parameter can be a helpful tool in the right surfactant selection. In a previous work (1), the combined effect of temperature, and surfactant HLB and dose effects over the TPH-diesel removal in a Mexican aged soil was investigated. A statistical design was used in order to minimize the number of experiments needed for achieving that purpose. The results of that work, indicated that the parameter that mostly affected biodegradation process was temperature, followed by HLB surfactant value, and surprisingly, surfactant dose at the end.

It is well known that certain mixtures of surfactants can provide better performance than pure surfactants for a wide variety of applications and thus, is expected that enhanced solubilization of water in water-in-oil (w/o) microemulsions will also be achieved with certain surfactant mixtures (2).

Huibers and Shah (2) defined synergism in surfactants as any situation where mixtures of surfactants have superior properties when compared to properties of any of the single components alone. They stated that strong synergic effects in mixtures of nonionic surfactants would not be expected, as synergism in anionic-nonionic surfactant mixtures has been attributed to Couloumbic, ion-dipole, or hydrogen-bonding interactions among the polar groups. Nonionics, which have minimum intermolecular interactions, should have, by comparison, the lowest synergism of all mixtures. After the experimental section, they showed that even nonionic surfactant mixtures show evidence of synergism. Different authors have measured by different methods, the size of those interactions. Palous *et al.* (3) employed cross-differentiation relations in the identification of interactions between non-ionic and ionic surfactants. Kunieda *et al*. (4) used phase diagrams and small-angle X-ray scattering for the characterization of mixed ionic-nonionic surfactant systems. Finally, Rosen and Zhou (5) used surface tension measurements and theoretical equation to describe the interaction parameter for mixed monolayer formation at the aqueous solutions interface.

Theoretical HLB value for a given mixture of surfactants is given by equation 1 (6):

$$HLB_{mixture} = (HLB_A)(X_A) + (HLB_B)(X_B) \quad (1)$$

Where X_A, and X_B are the weight fraction of every surfactant present in the mixture

With these antecedents, the aim of this work is to investigate weather or not the chemical family; besides HLB value can affect the enhanced biodegradation process.

MATERIALS AND METHODS

Contaminated soil

The soil employed in this work is a contaminated soil from an old oil storage and distribution station located in northern Mexico. (7). The main analytes found in the site were TPH-diesel, TPH-gasoline, PAHs and metals. Most of the organic compounds are contained in the TPH-diesel fraction. A given sample soil contained 3,970 mg/kg of TPH-diesel fraction and 4.71 mg/kg of the TPH-gasoline fraction (Figure 1). Table 1 shows physical, chemical and microbiological characteristics of the soil sample employed in this work.

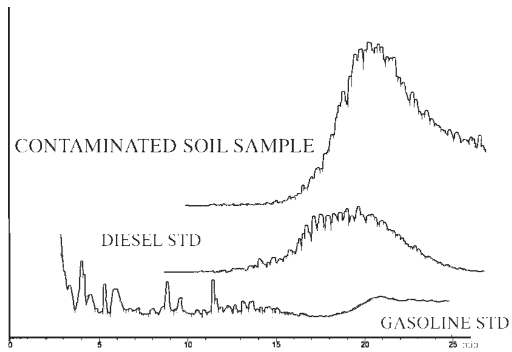

Figure 1. Contaminated soil chromatogram.

Microbial counts

Total heterotrophic bacteria count was developed as follows. One gram of soil was dissolved in 9 ml of peptonated solution (1 g of peptone in 1000 ml of water) and so consecutively until reaching a $1x10^{-7}$ dilution. Three of those dilutions were plated on agar plate count (Merk 5463) Petri dishes, prepared as the manufacturing indications. 0.1 ml of the fixed dilution was placed on every Petri dish and incubated during 48 hours at 25°C. after that period; colonies were counted and reported as FCU/g soil.

Microbial genera and species identification

One gram of soil was diluted in peptonated solution. Different dilutions were prepared as described above. Dilutions were platted on Petri dishes prepared with BHI media (Merk). Colonies were selected because of their color and/or morphologies. Colonies were re-platted in fresh BHI media Petri dishes. Pure colonies were characterized using the Gram technique for separation of Gram-positive and Gram-negative bacteria. Two miniaturized biochemical systems were employed. API 20 E (Biomeraux S.A., France) for Gram negative and BBL CRYSTAL GP ID (Becton Dickinson S.A., France) for Gram-positive bacteria.

Microcosm assessments

Wide mouth glass flasks (0.1m high x 0.06 m diameter) were used. 30 g of soil were conditioned with the amount of $(NH_4)_2SO_4$ required to keep a C/N/P ratio of about 100:15:1. The desired amount of surfactant and the water necessary to get a humidity of 20% were added and the soil was thoroughly mixed, except for the 30 and 13.5% assessments. A surfactant(s) solution containing the amount necessary to get a value of 2 mg surfactant/ kg soil was added. This value was employed, as in our previous work (Torres *et al.,* 2003) showed that 2 mg/kg in enough for biodegradation enhancement. Flasks were tightly closed with Teflon lined plastic caps. A strip of Parafilm was used around the flask necks in order to assure no air interchange. Two blanks were run together with the surfactant assessments. The first blank is a sterile blank. The flask was sterilized at 121°C for 15 min in a laboratory sterilizer. The second blank is a soil sample with $(NH_4)_2SO_4$, but no surfactant added. All assessments were run at 28°C.

Surfactants employed and their mixtures

Surfactants employed in this work were Span 20, Span 60, and Span 80 (sorbitan monolaurate, monoestearate and monooleate, respectively), as well as Tween 20, Tween 60 and Tween 80 (the corresponding etoxilated Span products, Poe = 20). Mixtures of the two nonionic surfactants were employed to get a wide range of specific HLB values. Tween 20 and Span 20 were mixed in 100-0%, 75-25%, 50-50%, 25-75%, and 0-100% proportions to get HLB values of *8.6*, 10.6, 12.6, 14.7, and *16.7*, in accord to equation 1.

Tween/Span 60 mixtures reached HLB values of *4.7*, 7.2, 9.8, 12.3, and *14.9*. Finally, Tween/Span 80 combinations yielded HLB values of *4.3*, 7.0, 9.6, 12.3, and *15.* HLB for single surfactants were in italics. Table 2 shows the surfactants combinations and the theoretical HLB value, in accord to equation 1. HLB values for the single surfactants are on the same table.

RESULTS AND DISCUSSION

Application of surfactants mixtures. TPH-diesel removals

Table 2. show the results of the 17 biodegradation assessments. Note that biodegradation values were not very high, since a period of only 8 weeks was selected for the biodegradation assessment. Values between 6.25 and 30.8% were obtained for the

experiments with surfactant mixtures, including both blanks. Figure 3. show the biodegradation values for the single surfactants assessments, in comparison with the sterile blank and the no-surfactant test.

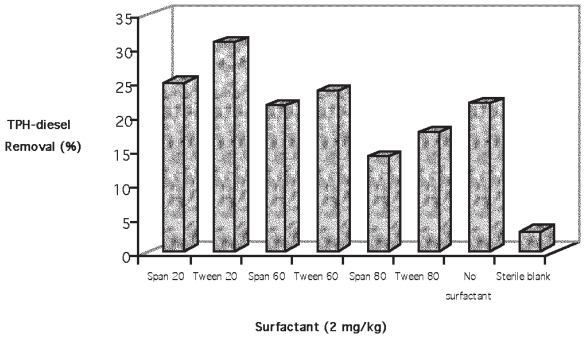

Figure 2. TPH-diesel removal for Span and Tween single surfactants.

All assessments were carried out with 2 mg/kg of surfactant. As it can be seen, the tendency of the biodegradation or removal value is that etoxilated products (Tween family *i.e.,* high HLB values) are more effective that non-etoxilated ones (Span family *i.e,* low HLB values). On the other hand, it seems that monolaurates family showed higher removals than the correspondent monoestearates, and monooleates families.

On figure 3. the TPH-diesel removal values are plotted as a function of the HLB value of Note that only monolaurates and monoestearates products reached TPH-diesel removals higher removals than that reached at the no-surfactant test (21.7%). every employed surfactant. For comparison purpose, the sterile blank assessment removal value (2.9%) is plotted in the same figure. This means that surfactant chemical family affects the TPH-diesel removal values.

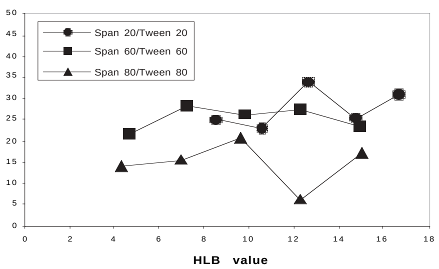

Figure 3. TPH-diesel removal as a function of HLB value for the three surfactant families.

For every chemical family, different TPH-diesel removal values were obtained, but maximum values do not correspond with the line extremes *i.e.,* the single surfactant assessment. For family 20 (monolaurates), the maximum value was achieved with the 50%-50% mixture (HLB=12.6). For family 60 (monoestearates), the maximum corresponds to the mixture 75%-25% (HLB=7.2). Finally, for the family 80 (monooleates), the maximum corresponds to the 50%-50% mixture (HLB=9.6). These facts clearly indicate that HLB is not the only one factor responsible for the biodegradation success. Both HLB value and chemical family are responsible of the biodegradation enhancement.

In resume, using surfactant mixtures of Span 20/Tween 20, it s possible to get TPH-diesel removals in the range of 22.9 to 34% (average = 27.6+/-4.63%, median = 25,3%). With Span 60/ Tween 60 surfactants, removals from 21.4 to 28.4% (average = 25.4+/-2.9%, median=26.4%) can be reached. Finally, for Span 80/Tween 80 surfactant mixtures, TPH-diesel removals between 6.25 and 20.7% (average= 14.8+7-5.4%, median=15.6%) can be obtained.

This behavior can be explained in terms of the solubilization level promoted for every surfactant or surfactant-mixture. Huiber and Shah (2) measured the water-to-oil volume ratio as a solubilization index for nonylphenol surfactant containing 1.5 ($C_9Poe_{1.5}$), and 12 (C_9Poe_{12}) polyethylene oxide molecules. They found that the best solubilization index was not for the $C_9Poe_{1.5}$ (HLB=4.6), nor for the C_9Poe_{12} (HLB=14.2) surfactants, but for a mixture of them, with an intermediated HLB value of 9, very similar results were observed for C_9Poe_4+$C_9Poe_{7.5}$ mixtures.

Application of surfactants mixtures-biomass growth

Table 2. shows the final FCU/g soil values for the 22 assessments, including two blanks. Note that all values are on the 10^5-10^7 interval. Values are the average for a triplicate test. FCU/g soil value for sample 0 has a value of $4.5x10^5$. From this point, values as low as $1.6x10^5$. FCU/g soil can be obtained (no surfactant blank). The sterile blank, as expected, showed no viable biomass present. The maximum FCU/g soil value corresponds to the test 9 (1.3×10^7). Figure 4 shows the FCU/g soil values as a function of the HLB values for the three employed surfactant families.

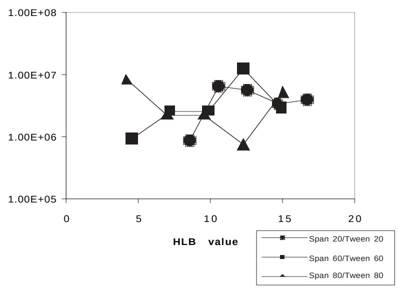

Figure 4. Microbial growth as a function of HLB values

As noted, FCU/g soil values do not show a consistent trend regarding the surfactant HLB value. For example, in the case of the monooleates family (span 80/tween 80), there seems to be a diminution in the FCU/g soil value as the HLB value is augmented. For the last point (HLB = 15).

Regarding the monoestearates family, the trend is the opposite: there is an increase on the FCU/g soil value as the HLB value increases up to the HLB = 12.3 point. The last HLB value (14.9), a diminution on the FCU/g soil value is observed. Finally, for the monolaurates family, there is an increase on the FCU/g soil value from HLB = 8.6 to HLB = 10.6, and the FCU/g soil is kept more or less constant later on. In general, it can be said that more bacterial growth was observed when using HLB values higher than 10, in combination with family span60/tween 80, and span20/tween 20. In a HLB value *vs.* FCU/g soil value plot (figure not shown), no correlation between the two parameters was obtained. A big dispersion for the points was observed for the three families together or even for every single-family analysis. It has been previously reported that biodegradation patterns are not necessarily linked to biomass growth patterns.

Microbiological characterization of some soil samples

Through the process described on materials and methods, bacteria found on the three analyzed soil samples are the following: The first one corresponds to the original contaminated soil (sample 0). On this soil, Gram-positive and Gram-negative bacteria were identified. In the first group, *Corynebacterium sp*., *Clavibacter sp.,* and *Streptococcus sp*. can be mentioned. Regarding the second group, *Pseudomonas sp*., specifically *P. fluorescens* and *P. putida* were detected. On soil number three; corresponding to the best biodegradation assessment value, *Corynebacterium sp*. was the only one gram-positive identified bacterium. *P. putida and P. fluorescens,* as well as *Yersinia pestis* were identified too. On soil number 16 (no surfactant assessment), no Gram-positive bacteria were *found. Pseudomonas sp., P. Fluorescens, P. Putida, Stentrophomonas sp., and Yersinia pestis* were identified among the Gram-negative bacteria.

As reported in many other works, biodegradation process selects the microorganisms with the required degradation capabilities or toxicity resistances. It seems that *Pseudomonas* species as well as *Corynebacterium sp.* are responsible for TPH-diesel biodegradation in presence of surfactants *i.e*, Span 20-Tween 20. When no surfactant was present, besides *Pseudomonas* and *Corynebacterium* species, *Stentrophomonas (Xanthomonas)sp.* and *Yersinia pestis* (both Gram-negative) were also predominant. On the initial soil sample, only *Corynebacterium, Clavibacter,* and *Streptococcus* (Gram-positive), as well as *Pseudomonas fluorescens* and *P. putida* (Gram-negative) were predominant.

CONCLUSIONS

For every chemical family, different TPH-diesel removal values were obtained, but maximum values do not correspond with the line extremes *i.e.,* the single surfactant assessment. For monolaurates, the maximum value was achieved with the 50%-50% mixture (HLB=12.6). For monoestearates, the maximum corresponds to the mixture 75%-25% (HLB=7.2). Finally, for monooleates, the maximum corresponds to the 50%-50% mixture (HLB=9.6). These facts clearly indicate that HLB is not the only one factor responsible for the biodegradation success. Both HLB value and chemical family are responsible of the biodegradation enhancement.

FCU/g soil was very variable depending on the HLB value and the chemical family. In general, it can be said that more bacterial growth was observed when using HLB values higher than 10, in combination with family Span60/Tween 80, and Span 20/Tween 20. In a HLB value *vs.* FCU/g soil value plot, no correlation between the two parameters was obtained. A big dispersion for the points was observed for the three families together or even for every single-family analysis.

On the original contaminated soil (sample 0), *Corynebacterium sp*., *Clavibacter sp.,* and *Streptococcus sp*., as well as *Pseudomonas sp*., specifically *P. fluorescens* and *P. putida* were detected. On soil number three, corresponding to the best biodegradation assessment value, *Corynebacterium sp*., *P. putida*, *P. fluorescens,* as well as *Yersinia pestis* were identified. On soil number 16 (no surfactant assessment), *Pseudomonas sp., P. Fluorescens, P. Putida, Stentrophomonas sp., and Yersinia pestis* were identified.

ACKNOWLDGEMENTS

The technical support given by M.A. Ortíz (Instituto de Investigaciones Biomédicas/UNAM) for the microorganisms isolation and identification is thanked.

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TABLES

Table 1. Contaminated soil physical, chemical and microbiological characteristics.

Parameter	Values	Unites	Parameter	Values	Unites
Physical properties			**Metals content**		
Porosity	0.37	-	Na	272	mg/kg
Sand	92	%	K	332	mg/kg
Fines	7.9	%	Ca	24,289	mg/kg
Bulk density	1.82	mg/cm^3	Mg	619	mg/kg
pH, 1 M KCl	6.1	-	Mn	90	mg/kg
Microbiological issues			Cd	1	mg/kg
Heterotrophic bacteria	4.5E+08	FCU/g soil	Cr	10	mg/kg
Total nitrogen	439	mg/kg	Cu	23	mg/kg
Phosphorus	63.7	mg/kg	Fe	5,734	mg/kg
Organic matter	0.00536	mg/kg	Ni	12	mg/kg
			Pb	224	mg/kg
Humidity	0.45	%	Zn	1,444	mg/kg

Table 2. Description and results of the 17 biodegradation assessments.

Test	Surfactant		Theoretical HLB	Final humidity %	Final TPH-diesel mg/kg	Final FCU/ g soil
	Span family (%)	Tween family (%)				
0	Initial sample		-	22.3	4,156	4.5 x 10^5
1	20 (100)	20 (0)	8.6	21.9	3,120	9.0 x 10^5
2	20 (75)	20 (25)	10.6	22.6	3,206	6.7 x 10^6
3	20 (50)	20 (50)	12.6	20.3	2,742	5.6 x 10^6
4	20 (25)	20 (75)	14.7	21.6	3,105	3.4 x 10^6
5	20 (0)	20 (100)	16.7	18.7	2,826	3.9 x 10^6
6	60 (100)	60 (0)	4.7	21.1	3,266	9.4 x 10^5
7	60 (75)	60 (25)	7.2	21.4	3,976	2.6 x 10^6
8	60 (50)	60 (50)	9.8	20.6	3,057	2.7 x 106
9	60 (25)	60 (75)	12.3	20.6	3,012	1.3 x 10^7
10	60 (0)	20 (100)	14.9	20.7	3,180	3.1 x 10^6
11	80 (100)	80 (0)	4.3	20.7	3,573	8.5 x 10^6
12	80 (75)	80 (25)	7.0	20.5	3,505	2.4 x 10^6
13	80 (50)	80 (50)	9.6	21.0	3,296	2.3 x 10^6
14	80 (25)	80 (75)	12.3	20.1	3,896	7.6 x 10^5
15	80 (0)	80 (100)	15.0	21.6	3,432	5.3 x 10^6
16	No-treatment blank		-	21.4	3,255	1.6 x 10^5
17	Sterile blank		-	17.4	4,036	ND