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The Effects of Surfactants on the Estimation of Bacterial Density in Petroleum Samples

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Abstract The effect of the surfactants polyoxyethylene monostearate (Tween 60), polyoxyethylene monooleate (Tween 80), cetyl trimethyl ammonium bromide (CTAB), and sodium dodecyl sulfate (SDS) on the estimation of bacterial density (sulfate-reducing bacteria [SRB] and general anaerobic bacteria [GAnB]) was examined in petroleum samples. Three different compositions of oil and water were selected to be representative of the real samples. The first one contained a high content of oil, the second one contained a medium content of oil, and the last one contained a low content of oil. The most probable number (MPN) was used to estimate the bacterial density. The results showed that the addition of surfactants did not improve the SRB quantification for the high or medium oil content in the petroleum samples. On other hand, Tween 60 and Tween 80 promoted a significant increase on the GAnB quantification at 0.01% or 0.03% *m*/*v* concentrations, respectively. CTAB increased SRB and GAnB estimation for the sample with a low oil content at 0.00005% and 0.0001% *m*/*v*, respectively.

Keywords Sulphate-reducing bacteria (SRB) · General anaerobic bacteria (GAnB) · Petroleum · Surfactants · Most probable number (MPN)

Introduction

Sulfate-reducing bacteria (SRB) are some of the most common and problematic microorganisms of environmental and economic importance in petroleum industry. The effects caused by SRB activity are mainly the souring of oil and gas deposits and in problems related with microbially influenced corrosion (MIC). The toxic hydrogen sulfide produced may also present a health hazard to workers and may decrease oil quality by the souring of oil and gas [1].

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It is extremely difficult to estimate the costs related with corrosive processes attributed to the activity of microorganisms (SRB and other bacteria) in the oil industry. In recent years, the costs involving the control of the activity of SRB were significant with annual values estimated at approximately $150,000 per platform when only biocides are used to control microbial activity [2].

Considerable efforts have been made through the development of methods to SRB enumeration. In general, the most common methods to enumerate SRB fall into two categories: direct detection methods and culture methods. Although the direct detection methods are promising, they are still in the development phase and may have limitations when used in situ [3]. Recently, molecular techniques and the use of radiotracers are being investigated for the detection and enumeration of SRB and other anaerobic cells. However, although highly specific and reliable, they are not feasible for a routine control of microbial numbers at field conditions. Furthermore, these new techniques have high costs. Culture methods based on the most probable number (MPN) technique have been extensively used for several decades and remain the standard method for enumerating SRB.

In samples with hydrophobic compounds, enumeration of SRB and general anaerobic bacteria (GAnB) may be compromised because of the low solubilization of these compounds in the culture medium. The study of the influence of surfactants in the SRB and GAnB estimation in oil samples may become an alternative to improve the MPN method under these conditions. Surfactants are organic compounds that present both a hydrophobic part and a hydrophilic part in the same molecule. This hydrophilic part allows surfactants to be soluble in water, whereas the hydrophobic part causes them to concentrate at interfaces [4].

Based on the previous considerations, the aim of this work was to verify the effect of the addition of different types of surfactants: nonionic (Tween 60 and Tween 80), cationic (CTAB), and anionic (SDS), on SRB and GAnB enumeration in oil samples using the MPN technique.

Materials and Methods

The populations of SRB and GAnB considered in the tests were the indigenous population present in three different oil samples used:

Sample A—oil contaminated with water (kinematic viscosity=41.57 mm^2/s and 14.3% water);
Sample B—oil and water (kinematic viscosity=6.56 mm^2/s and 50.4% water);
Sample C—water contaminated with oil (kinematic viscosity=2.3 mm^2/s and 95.5% water).

These samples were obtained from oil storage tanks from Terminal São Sebastião, São Paulo, Brazil.

Microbial Enumeration

A 10-fold dilution series was used, from 10^0 to 10^8, for each group of microorganisms (SRB and GAnB). Firstly, 1 ml of the sample was injected into a vial containing 9 ml of anaerobic liquid medium and a vial containing 9 ml of a reducing solution. The vials were

then shaken, and a sterile syringe was used to draw out 1 ml of the reducing solution and inject this solution into a new medium vial and also into a new reducing solution vial. This procedure was repeated until the 10^8 dilution was reached. Inoculated flasks (n=3) were incubated at 30±1 °C for 28 days. The growth of SRB was indicated by the formation of a black FeS precipitate. The positive growth for GAnB was confirmed through the observation of turbidity in the culture medium. In the present work, the estimation of MPN cells was performed according to the method of Harrigan and McCance [5], which does not consider a normal distribution of results, but a binomial distribution.

Culture Media

The semisolid Postgate's E medium was used for SRB estimation. This medium had the following composition (per liter): sodium lactate, 7.0 ml; yeast extract, 1.0 g; NH_4Cl, 1.0 g; KH_2PO_4, 0.5 g; $MgCl_2{\cdot}6H_2O$, 1.83 g; $CaCl_2{\cdot}6H_2O$, 1.0 g; $FeSO_4{\cdot}7H_20$, 0.5 g; ascorbic acid, 0.1 g; agar–agar, 9 g; resazurin, 4 ml (0.025% *m*/*v*); NaCl, 35 g. The medium was prepared anaerobically and the pH was adjusted to 7.6 with NaOH 1 M. After that, 9 ml of this medium was dispensed in each flask and was autoclaved at 121 °C for 20 min. According to Postgate [1], the SRB require an environmental redox potential (Eh) of approximately −100 mV. To achieve this redox potential, sodium thioglycolate (12.4 g/l) was used as a reducing agent. This solution was added to the medium in aliquots of 0.1 ml before inoculation and after sterilization.

For GAnB enumeration, a medium was prepared under anaerobic conditions with the following composition (per liter): glucose, 5.0 g; peptone, 4.0 g; yeast extract, 1.0 g; resazurin 4 ml (0.025% *m*/*v*), at pH 7.6. After preparation, the medium was distributed into 9 ml flasks and sterilized in an autoclave at 121 °C for 20 min. Then, sodium thioglycolate (12.4 g/l) was added, as previously described.

The reducing solution contained (per liter): sodium thioglycolate, 0.124 g; ascorbic acid 0.1 g; and resazurin, 4 ml (0.025% *m*/*v*), at pH 7.6. After that, 9 ml of solution was dispensed in each flask and autoclaved at 121 °C for 20 min.

Surfactants and Tests

The main characteristics of Tween 60, Tween 80, CTAB, and SDS are presented in Table 1.

Ionic surfactants are more toxic for bacteria than nonionics [4]. Because of this fact, CTAB concentrations selected for the tests were lower than its critical micelle concentration (CMC) of 0.036% *m*/*v*. Corresponding solutions of each concentration were prepared and sterilized by vacuum filtration using a Millipore membrane of 0.45 μm pore diameter.

Table 1 Surfactants and their main characteristics.

Surfactant	Type	Molar mass (g/mol)	CMC (%)	HLB
Tween 60	Nonionic	1,311	0.003	14.9
Tween 80	Nonionic	1,309	0.001	15
CTAB	Cationic	364.5	0.036	–
SDS	Anionic	288.4	0.036	40

CMC: critical micellar concentration, *HLB*: hydrophilic–lipophilic balance

Results and Discussion

Effect of the Surfactants on the Estimation Bacterial Density of SRB

Figure 1 shows the quantification of SRB for samples A, B, and C in the presence of different concentrations of the surfactants: Tween 60 (a), Tween 80 (b), CTAB (c), and SDS (d).

The evaluation of the effect of the surfactant Tween 60 in sample A (Fig. 1a) showed that its addition did not contribute to the increase in SRB quantification in the sample with a larger oil content. The results were lower than the control test. Effects of the surfactants on microbial physiology vary from inhibitory, according to its toxicity, to growth stimulatory when the surfactant is used as substratum [4]. The complexity of these effects is well reported in literature with great variations in the results obtained [6–8].

In sample B, for all concentrations of the surfactant Tween 60, the results obtained were higher than the control test. However, when numbers were compared with those of the same magnitude, it would not be possible to consider a significant difference. In this case, the largest value for SRB (2.5×10^2 cells/ml) was obtained when the concentration of Tween 60 was 0.01% *m*/*v* (above of the CMC).

When the concentration of the surfactant is higher than its CMC, the surfactant can solubilize hydrophobic compounds because of the presence of micelles in the solution. According to some authors [4, 6, 8] the process of micellization leads to an increase in solubilization and, consequently, a higher degradation of these substances by microorganisms. However, when values much higher than the CMC are employed, a reduction is

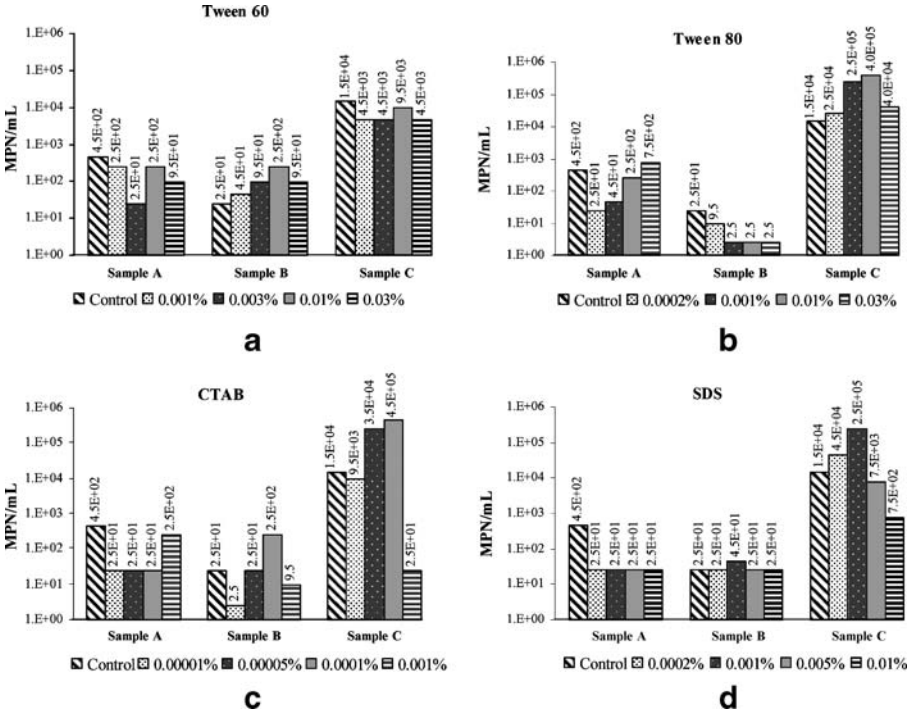

Fig. 1 Estimation of bacterial density of SRB, expressed by MPN (cells per milliliter), in the presence of different concentrations of the surfactants Tween 60 (**a**), Tween 80 (**b**), CTAB (**c**), and SDS (**d**)

observed in the degradation rate because of the inhibitory effect of the surfactant [9]. For sample C (Fig. 1a), all of the tested concentrations of Tween 60 showed results lower than the control test.

Figure 1b showed that the addition of surfactant Tween 80 was not favorable in the quantification of SRB in sample A. The analysis involved the comparison of estimated bacterial density, making it impossible to conclude about significant differences in the results as they presented that they are from the same magnitude. However, in the concentration of 0.03% *m*/*v*, the resulting quantification of 7.5×10^2 cells/ml was superior to the control test which showed a value of 4.5×10^2 cells/ml.

For sample B, it is also possible to observe that the addition of Tween 80 was not favorable to an increase in SRB quantification. The addition of surfactants in liquid systems will have two contradictory effects: solubilization of the hydrophobic compounds and stimulation of the biodegradation by microorganisms or inhibition of the cells bacterial adhesion in the interface, consequently reducing the biodegradation rate [4]. This last effect could have occurred in sample B composed of water and oil.

The results obtained for sample C (Fig. 1b) showed that the effect of surfactant Tween 80 was favorable for SRB quantification when used in concentrations of 0.001% and 0.01% *m*/*v*, reaching values of 2.5×10^5 and 4.0×10^5 cells/ml, respectively. According to the literature, biodegradation of polyaromatics hydrocarbons (PAHs) increased with the use of the synthetic surfactants, such as Triton-X, Brij 30, and Tween 80 [10]. The authors observed that when the concentration of the surfactants was below or near CMC, they did not see an improvement in solubilization. However, when concentrations above the CMC were tested, solubility increased.

Figure 1c shows that the addition of surfactant CTAB did not favor SRB quantification in sample A (oil contaminated with water). For sample B, when CTAB was used in a concentration of 0.0001% *m*/*v* (250 cells/ml), a 10-fold increase in comparison with the control test (25 cell/ml) was observed. In sample C, the addition of CTAB favored the quantification of SRB when used at 0.0001% *m*/*v*, resulting in a value of 4.5×10^5 cells/ml.

The inhibitory effects of CTAB are reported in the literature with a high diversity of results depending on the concentration. CTAB presented inhibitory effects in concentrations above 0.01% *m*/*v* in a study with *Pseudomonas putida* and *P. fluorescens* [11, 12]. On the other hand, Fengjiao et al. [13] showed that the inhibitory effect on the growth of *P. putida* was pronounced in concentrations above 0.001% *m*/*v*. In this work, the concentration of 0.001% demonstrated an inhibitory effect in samples B and C.

According to Fig. 1d, for sample A, all the tested concentrations presented values lower than the control test (450 cells/ml), showing that the addition of SDS did not promote an increase in SRB quantification, although solubilization of the oil in the sample was observed visually.

In sample B, the addition of SDS did not favor SRB quantification in any of the concentrations tested. On the other hand, the SDS concentration of 0.001% *m*/*v* used in sample C represented the largest increase in the SRB quantification, 2.5×10^5 cells/ml in comparison to the control test (1.5×10^4 cells/ml).

According to Lee et al. [14], anionic surfactants, like SDS, present characteristic detergents and low antimicrobial activity, except when used at high concentrations, which can induce the lysis of Gram-negative bacteria. This was also observed by Simões [12] in the study carried out with *P. fluorescens*. There is also the possibility that the stimulus of the bacterial growth could be associated with surfactant consumption. Suchanek et al. [15] observed that biodegradation of *n*-decane by a *Pseudomonas* strain was stimulated in the presence of SDS. The generation of an intermediate compound during surfactant

consumption seems to have enabled bacterial adhesion to the hydrocarbon, thus facilitating its biodegradation.

Effect of the Surfactants on the Estimated Bacterial Density of GAnB

Figure 2 shows the quantification results of GAnB for samples A, B, and C in the presence of surfactants Tween 60 (a), Tween 80 (b), CTAB (c), and SDS (d) with different concentrations.

Figure 2a showed that it is possible to observe that for all the samples evaluated (samples A, B, and C), Tween 60 presented the most favorable effect on the growth of GAnB at concentrations of 0.01% and 0.03%, above its CMC. Sample C presented the highest results in comparison to the control with values of 2.5×10^6 cells/ml. For sample B, the use of Tween 60 in its CMC also presented results higher than those observed in the absence of surfactant (9.5×10^2 cells/ml). For sample C, a marked increase in GAnB enumeration was achieved, at a concentration of 0.01 and 0.03% *m*/*v*, using Tween 60.

Figure 2b shows that the addition of surfactant Tween 80 in sample A promoted an increase in the enumeration when used at 0.01% and 0.03% *m*/*v*, both above the CMC (0.001% *m*/*v*) of the surfactant. The addition of Tween 80 in sample B did not cause favorable effects on the growth of GAnB in any of the tested concentrations; results were lower than the control test (9.5×10^2 cells/ml).

For sample C, it was observed that the addition of Tween 80 at the CMC (0.001% *m*/*v*) and in concentrations above CMC (0.01% and 0.03% *m*/*v*) showed superior results in comparison to those obtained in the absence of surfactants. GAnB quantifications were 3×

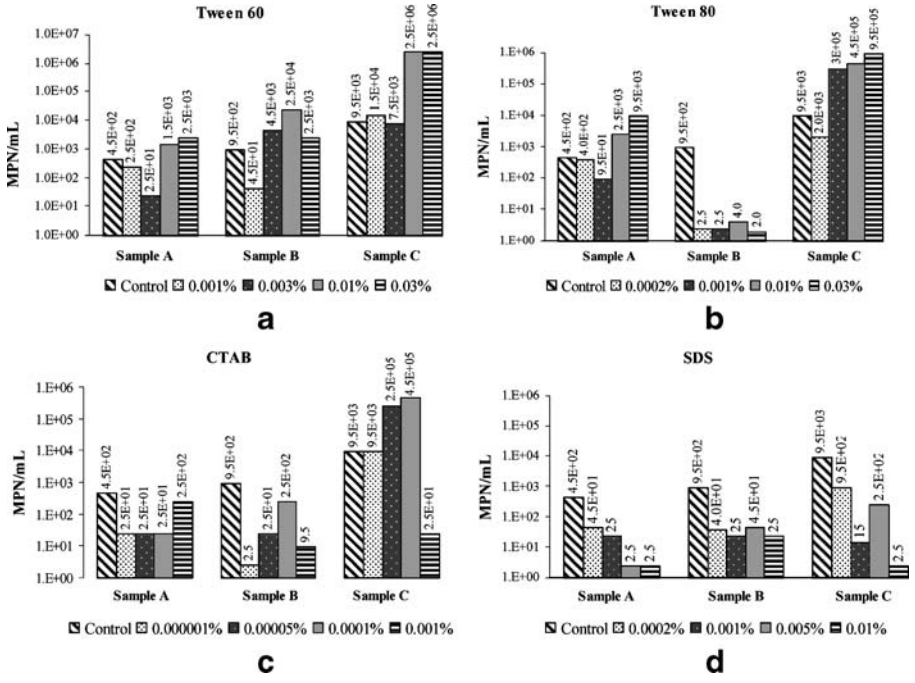

Fig. 2 Estimation of bacterial density of GAnB, expressed by MPN (cells per milliliter), in the presence of different concentrations of the surfactants Tween 60 (**a**), Tween 80 (**b**), CTAB (**c**), and SDS (**d**)

10^5, 4.5×10^5, and 9.5×10^5 cells/ml, respectively, indicating a favorable effect on GAnB quantification for samples with a low content of oil.

Figure 2c shows that in all tested concentrations, the addition of CTAB did not favor GAnB quantification in samples A and B. For sample C (water plus oil), a concentration of 0.0001% *m*/*v* of CTAB showed a higher value (4.5×10^5 cells/ml) in comparison to the control (9.5×10^3 cells/ml).

Figure 2d shows that the addition of the surfactant SDS did not cause a significant increase in GAnB quantification in any of the samples, at concentrations smaller than 0.01%. Although Margesin and Schinner [16] concluded that in concentrations smaller than 0.01% a significant increase in diesel oil biodegradation occurred, it is important to emphasize that, in the present work, crude oil was used.

Conclusions

- None of surfactants tested contributed to the quantification of SRB in the sample with the higher oil content (sample A). However, for the GAnB group, surfactants Tween 60 and Tween 80 caused favorable effects in the quantification.
- It was observed for sample B that the addition of Tween 60 and CTAB caused an increase in SRB quantification (10 times higher than the control—25 cells/ml) when used in concentrations of 0.01% and 0.0001% *m*/*v*, respectively. For GAnB, only Tween 60 contributed to a higher quantification when used at 0.01% *m*/*v* (2.5×10^4 cells/ml) compared to the control (9.5×10^2 cells/ml).
- Only Tween 60 did not show a positive effect for SRB quantification in sample C (water contaminated with oil). On the other hand, GAnB quantification was increased with the addition of all surfactants tested, except with the addition of SDS, leading to the highest concentration of cells (2.5×10^6 cells/ml).
- The importance of these findings to the petroleum industry relies on the underestimation about the use of biocides in continental platforms, oil storage tanks, and pipelines. Those environments are constantly subjected to anaerobic microbial activity, which are usually controlled through the addition of biocides in the absence of surfactants.

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