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Research Article

Techniques for Improving the Biodegradation of Diesel in Polluted Mangrove Ecosystems

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Abstract

Biological methods of treating polluted soil and water are widely applied in mangrove ecosystems polluted by hydrocarbons. Mangrove sediments contain an abundant microflora with hydrocarbons degradation capacity. Most of These microorganisms can also be used in several techniques such as natural attenuation, biostimulation and bioaugmentation. These techniques can only be effective if the biodegradation limiting factors are taken into consideration. The pH, the bacterial concentration, the temperature, the availability of nutrients, the accessibility and even the persistence of the pollutant are considered to be factors limiting biodegradation. The aims of the study are to assess the effect of pH, bacterial concentration, and the integrity of microorganisms membranes in the diesel biodegradation process in seawater. The degradation capacity of endogenous microorganisms was compared to that of Rhodococcus erythropolis which is a pure exogenous strain. The results showed a decrease inthe pH from 6.9 for natural attenuation to 4.5 for bioaugmentation with Rhodococcus erythropolis. The bacterial concentration is higher in bioaugmentation treatments. Finally, the rate of diesel degradation for the various techniques applied was observed. The combination of biostimulation and

bioaugmentation with Rhodococcus erythropolis shows a degradation rate of 67.5%.This rate is significantly different from that of the combination of biostimulation and bioaugmentation with the endogenous bacterial consortium which is 26.4% after five weeks of treatment.It can also be noted that diesel has less negative effect on Rhodococcus erythropolis, which exhibits good membrane integrity of 94%, higher than 81% for the endogenous bacterial consortium.

Keywords: Mangroves; Hydrocarbons; Biodegradation; Microorganisms

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous and persistent organic pollutants in the environment [1]. In wetlands and marine environments, particularly in mangrove ecosystems, their increase and significant accumulation result from human activities such as oil and gas exploration and exploitation operations. Oil spills occur during tanker-loading operations in the oil platforms or in the ballast tanks of ships, most often near the mainland coast during the transportation of crude oil in pipelines, urban discharges in service stations from petroleum products, emissions from combustion and industrial processes [1-3].

As successful bioremediation programs require the application of strategies tailored to the specific environmental parameters of the contaminated site, feasibility studies are a prerequisite for any planned intervention [4]. In particular, co-contaminated matrices represent a problem in the bioremediation processes because high metal concentration can inhibit the biodegradation of organic pollutants, imposing a double stress on the microbial populations [5]. Different factors influencing hydrocarbon degradation have been reported. One important factor that limits biodegradation of oil pollutants in the environment is their limited availability to microorganisms [6]. Petroleum hydrocarbon compounds bind to soil components, and are consequently removed or degraded with difficulty [7, 8].

In order to enhance biodegradation efficiency, three remedial strategies were proposed by Iwamoto and Nasu (2001) [9] which involve natural attenuation, bioaugmentation and biostimulation. Natural attenuation is a natural method which uses indigenous microorganisms to degrade contaminants, and involves no external modification of the environment [10]. Biostimulation, based on supplying additional nutrients or substrates to stimulate the degradation of native microorganisms, can also be used to activate biodegradation [11, 12], especially in environments such as mangrove where nutrients in sediments are often found in low concentrations [13]. Bioaugmentation based on inoculating micro-organisms with required degradation capability, might be a way of enhancing the biodegradability of toxic contaminants. Bioaugmentation with competent degrading strains of bacteria can stimulate the rate and the extent of biodegradation in appropriate environments [14]. The current study therefore aims to (1) assess the potential degradation ability of the consortium, an endogenous bacterial, in the presence diesel, (2) evaluate the ability of Rhodococcus

erythropolis in association with endogenous microflora, and (3) compare different diesel degradation strategies.

2. Materials and Methods

2.1 Materials

2.1.1 Sea water and diesel: The experiment was performed on samples of fresh mangrove sea water taken from the coast in Cameroon. The diesel used in this study was commercial diesel. It contained 75.2 % of aliphatic hydrocarbons, carbon chains are C_{11} to C_{24} and 24.7 % aromatic hydrocarbons. Diesel oil is an excellent model for studying hydrocarbon biodegradation, since it is constituted of a variety of these molecules, such as paraffin, olefins, naphtha, and aromatic compounds. The molecular weight of the hydrocarbons in diesel is also variable, with molecules containing 9 to 20 carbon atoms.

2.1.2 Microorganisms: The bacterial consortium used in this study was developed by Semboung Lang et al. (2016) [2]. The active bacterial starter is obtained through a specific selection from the total microflora present in the sediments of mangroves. This consortium is made up of bacteria capable of degrading diesel. The bacterial concentration of the starter was 3x 10^{11} CFU/g of powder. This starter was used to inoculate flasks containing diesel. Rhodococcus erythropolis T902.1 is a pure strain used in the MiPi laboratory of Gembloux Agro Biotech - University of Liège in Belgium, for the degradation tests of hydrocarbons. This strain makes it possible to evaluate the performance of endogenous and exogenous strains in the biological treatment of hydrocarbons contaminated sediments. The indigenous microflora of the mangrove sediments was used for treatment by natural attenuation and biostimulation. Rhodococcus erythropolis T902.1 was added to the indigenous microflora for treatment by bioaugmentation.

2.2 Methods

2.2.1 Biodegradation experiments of diesel: The potentiel of active bacterial consortium isolated in mangrove sediments and Rhodococcus erythropolis T902.1 was performed through different experiments. The indigenous microflora of the mangrove sediments for treatment by natural attenuation, biostimulation and bioaugmentation. *R. erythropolis* T902.1 was added to the indigenous microflora for treatment by bioaugmentation. The diesel degradation potential of the endogenous microflora was compared with that of exogenous pure strains known for their ability to degrade hydrocarbons. In order to assess the degradation potential of a bacterial consortium isolated from mangrove sediment, two techniques with several series, and different treatments used as described by Semboung et al. 2016a [2]. Each series was performed in triplicate.

The first technique was performed with sterile sea water (S-SW). For this experiment, two series of threeflasks were used. In one series, 500 mL of sea water was introduced. The flasks were sterilized by autoclaving. Then, 10 mL/L of diesel (10,000 ppm) was added. Each flask was inoculated with 107 CFU/g consortium as a starter. The second series of flasks was treated as above with the only difference being the addition of 30 % nutrient solution. The second technique used non-sterile sea water (NS-SW).

The experiment was carried out in five series of three flasks with non-sterile sea water (NS-SW).

- In the first series of flasks, 500 mL of sea water was introduced; this was the treatment by natural attenuation.
- The second series concerned treatment by biostimulation, and involved the above treatment followed by the addition of 30 % nutrient solution.
- The third series was the same as the first, followed by inoculation with 107 CFU/mL bacterial consortium powder; this was the bioaugmentation.
- The fourth series was the same as the first, followed by inoculation with 107 CFU/g bacterial R. erythropolis; this was the second experiment of bioaugmentation.
- The fiveth series was treated as the third, with the addition of 30 % nutrient solution; this was the combination of biostimulation and bioaugmentation. The fiveth series was treated as the fourth, with the addition of 30 % nutrient solution; this was the second experiment of combination of biostimulation and bioaugmentation.
- A series of three flasks containing 500 mL of sterile sea water (S-SW) without the addition of microorganisms was the control flask for this experiment.

2.2.2 Enumeration of total aerobic heterotrophs and PAH-degrading bacteria

The enumeration of the active and the total microflora was done by using the successive dilution method, with the dilution spread on Petri dishes. In this way, 1 mL of sea water sample was recovered from each flask every 7 days during five weeks. The sample was placed in a test tube containing 9 mL of peptone water. For the active microflora, the spreading was done on a solid mineral salt medium (MSM) containing 10 mg/kg of dry matter of diesel as the sole carbon source. The following composition (mg/L) of the MSM is as follow: $(NH_4)_2SO_4$, 1000; K_2HPO_4, 800; KH_2PO_4, 200; $MgSO_4.7H_2O$, 200; $CaCl_2.2H_2O$, 100;and trace elements made up of $FeSO_4$. $7H_2O$, 12;$MnSO_4.7H_2O$, 3; $ZnSO_4.7H_2O$, 3; $CoSO_4.7H_2O$, 1; and $(NH_4)_6Mo_7O_{24}.4H_2O$, 1; 15 g agar. The medium pH was 7.0–7.2. Each dilution was spread three times. After 3 days of incubation at 30 °C, the number of colony forming units (CFUs) was counted and the mean of each dilution was determined. In order to assess the total microflora, the composition of the rich medium (M 863) used is: glucose, 20 g/L; peptone, 10 g/L; yeast extract, 10 g/L; and Tween 80, 1 mL/L. For cultures on solid medium, this medium was supplemented with 15 g/L of agar.

2.2.3 Optical density measurement in the different flasks

The optical density of the samples was determined by the spectrophotometer previously calibrated to the absorption wavelength of 600 nm. Like the enumeration by successive dilution, it made it possible to determine the concentration of bacteria in the various treatments. This concentration can be an index of bacterial growth and the rate of diesel degradation. The UV / Visible, UV-6300 PC Dual Beam Spectrophotometer is the one used in this experiment.

2.2.4 Characterization of bacterial flow cytometric in flasks experiment

AccuriCFlow® or CFlow Plus software (CFlow) was used to control the C6 Flow Cytometer® system, acquire data, generate statistics and analyze results. Cytometric analyses allow to characterize the consortium under diesel stress. The viability and the membrane integrity were measured with propidium iodide (PI). Flow cytometry was also used to count the microorganisms. The analysis was performed every day throughout the entire experiment. The sample preparation was carried out as follow: one mL of each sample was transferred into two microtubes: the first was heated at 90°C for 30 min in a water bath (heatshock) and the second was placed on ice (reference sample). For each sample, 500 µL of the solution were introduced into another microtube, and then 500 µL of PBS were added (0.2-µm filtered). The mixture was vortexed and centrifuged at 10,000 rpm for 2 min. The pellet (desired range 106 CFUs/mL) was recovered, 1,000µL of PBS and 10 µL of PI were added, and the cells were resuspended using a vortex. The mixture was incubated at 37°C for 15 min and then centrifuged at 10,000 rpm for 2 min. The pellet was recovered, 1,000µL of PBS were added and the cells were resuspended using a vortex before cytometry analysis.

2.2.5 Total petroleum hydrocarbon analysis and rate of diesel reduction

The analysis of the hydrocarbon required a purification step before gas chromatography analysis. During the purification, 100 mL of sample were used. The pH of each sample was adjusted to 2 using HCl. Before agitation, 4.45% $MgSO_4.7H_2O$ were introduced in the flask and, after an agitation step of 30 min, the content was introduced into a separatory funnel. An equivalent volume of hexane solution saturated with acetonitrile was added to each sample. The mix was stirred for 10 min and yielded two phases: the hexane phase above containing hydrocarbons and impurities and the acetonitrile phase below. 1.5 g of Florisil (15% MgO, 85% SiO_2, MACHEREY-NAGEL GmbH and Co.KG, Germany) and 1.5 g of Na_2SO_4 were placed in a settling leg made of fritted glass. The acetonitrile phase (below) was recovered first. The hexane phase containing hydrocarbons was next recovered in another flask. The flasks containing the hexane phase were then dried and heated in an oven at 105 °C for 3 hours and then cooled in a desiccator. The solution in each flask was then evaporated using a rotary evaporator heated at 50°C to a volume of about 2 mL. The final sample volume was measured and an aliquot was introduced into a vial stored at 4°C before GC injection. The remaining diesel oil was quantified by weight. The percentage of diesel oil degradation was determined on the basis of the original concentration. The dosage of hydrocarbons in soils and water was performed using a gas chromatograph (HP 5890 Series II Gas Chromatograph). An external calibration was made with standard diesel (Diesel Oil Additives without DIN H53) and mineral oil (Mineral Oil Additives without DIN H53).

Analysis conditions and the characteristics of the column were as follows: Pre-column: deactivated fused silica; Column Macherey Nagel Optima 1 (99% polydimethyl siloxane, 1% diphenyl); Column length: 30 m; Internal diameter of column: 250µ ; Stationary

phase thickness: 0.25µ; Injection volume: 1µl; Injector: on-column; Carrier gas: helium at 0.8 bar; Hydrogen fueling the detector: 1.15 bar; FID detector at 300°C; Oven temperature: 40°C for 5 min, temperature rise for 26 min at a rate of 10°C/min, 300°C for 30 min. Oil content was measured by the hydrocarbon index, considering all the peaks between decane and tetracontane.

2.2.6 Quality control and statistical analyses

Mean values were compared with the ANOVA test with a p value ≤ 0.05. The differences in the various rates of diesel reduction were also analyzed by the same test.

An analysis of variance (ANOVA) was performed to test the differences between initial and final Total petroleum hydrocarbon (TPH) concentrations between the treated flasks and the control flasks. All statistical analyses were performed using MINITAB 15 statistical software (French version).

3. Results and Discussion

3.1 pH of the medium in flasks

As a function of time, the pH of the medium decreases moderately in the flasks where the treatment is done by biostimulation, going from 6.9 to 5.9 (Figure 1). On the other hand, the pH decreases considerably in the flasks where the treatment is carried out by bioaugmentation and by the combination of biostimulation and bioaugmentation with *R. erythropolis*, going from 6.9 to 4.5. This pH remains neutral in the control flasks and the flasks where the natural attenuation treatment is applied.

This drop in pH, which reflects the acidity of the medium, shows the bacterial activity that takes place in the flasks by the mineralization of the organic matter. Although the pH is a parameter to be monitored in the same way as the temperature, the dissolved oxygen and the presence of nutrients, during the biodegradation of diesel, certain microorganisms adapted to the degradation of hydrocarbons, will be different depending on whether the medium is acidic (yeasts, fungi), neutral or basic (bacteria) [15].

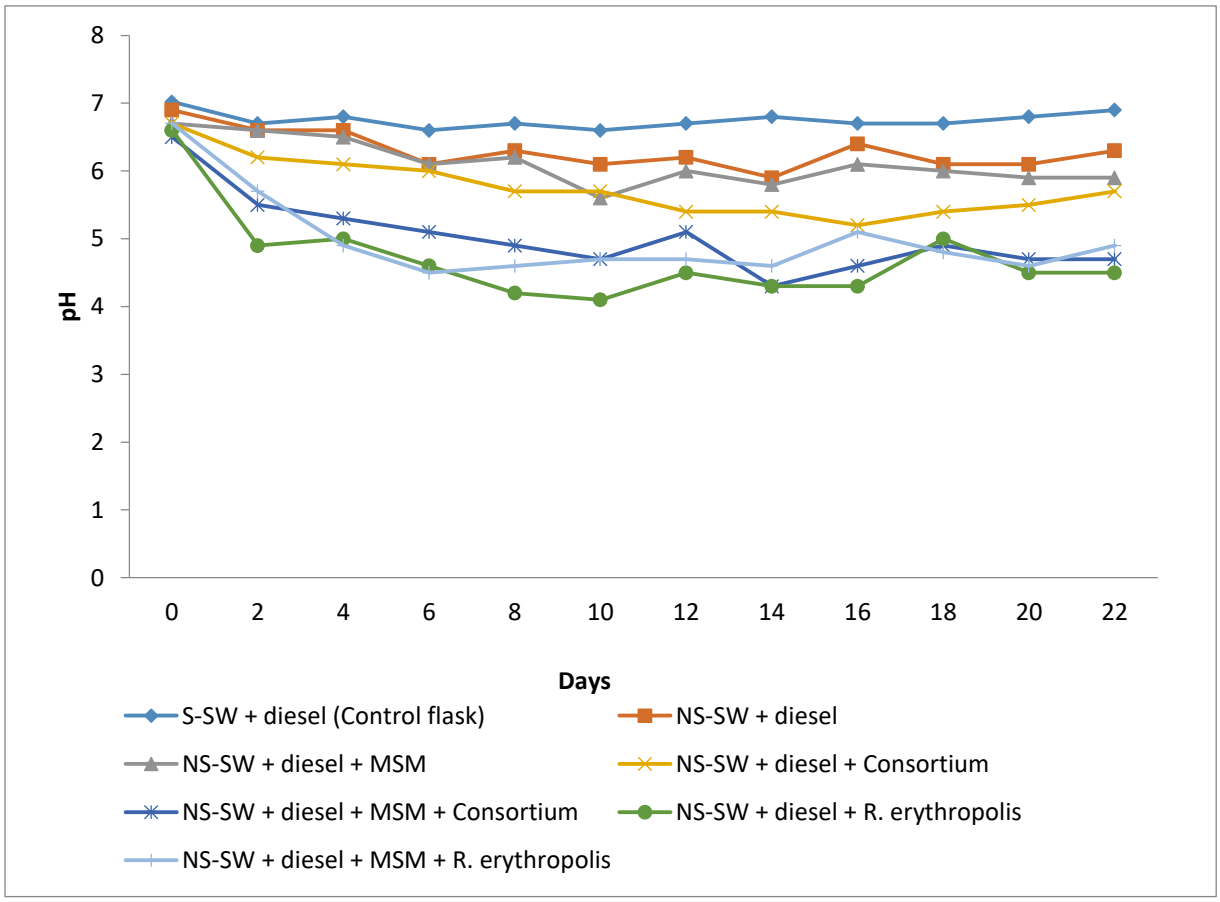

Figure 1: Evolution of the pH of the medium over time in the different flasks and for all the treatments.

3.2 Optical density

The different profiles in the figure 2, show that there is indeed a bacterial activity in the different flasks and for all the treatments applied. The absorbance increases with the cell concentration which is actually the bacterial growth in the flasks. At a wave length of 600 nm, all cells in the flasks are in the growth phase. The cell mass thus present is greater in the flasks where the treatments by bioaugmentation and biostimulation with R. erythropolis are applied. The optical density component due to the biomass is the consequence of the turbidity observed in the different treatments.

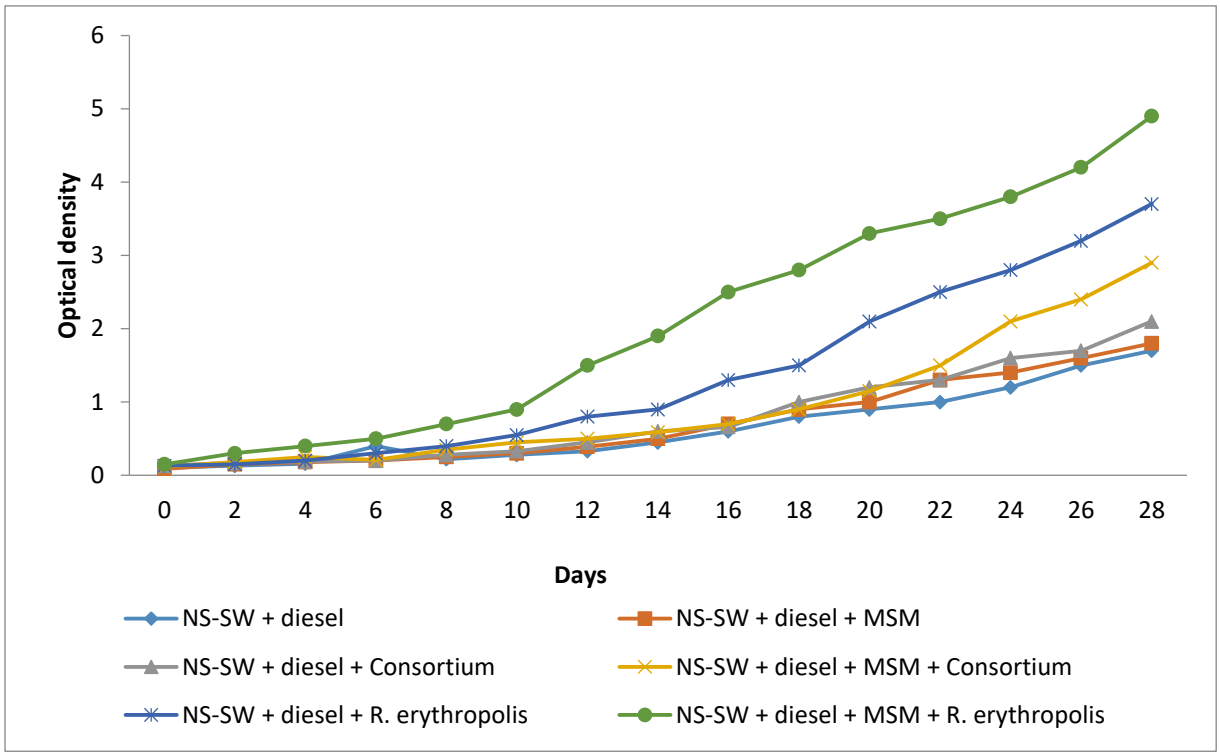

Figure 2: Variation of optical density in the different flasks.

3.3 Total aerobic heterotrophs and polycyclic aromatic hydrocarbons degrading bacteria

The figure 3 shows the bacterial growth evaluated by the number of living bacteria present in each flask. The drop in the number of bacteria during the first two weeks for all treatments is observed, and an exponential growth in the flasks having received an addition of *R. erythropolis* and nutrients. The reduction of the growth rate during the first two weeks can be attributed to the environmental conditions having changed with the presence of diesel as the sole source of carbon. The results shows a drop in the number of bacteria that are certainly dead. This lag phase is a necessary period of adaptation when the bacteria must familiarize themselves with the new medium and synthesize the enzymes adapted to the new substrate. From the second week to the fifth week, which correspond to an exponential growth of the bacteria in the flasks whose treatments required either simply an addition of bacteria or then a combination of an addition of bacteria and nutrients at the same time. It is easy to see that the growth rate is almost constant which means that the doubling time of bacteria is the shortest. Unlike spectrophotometric observation, cell mass here is represented by viable cells with zero mortality.

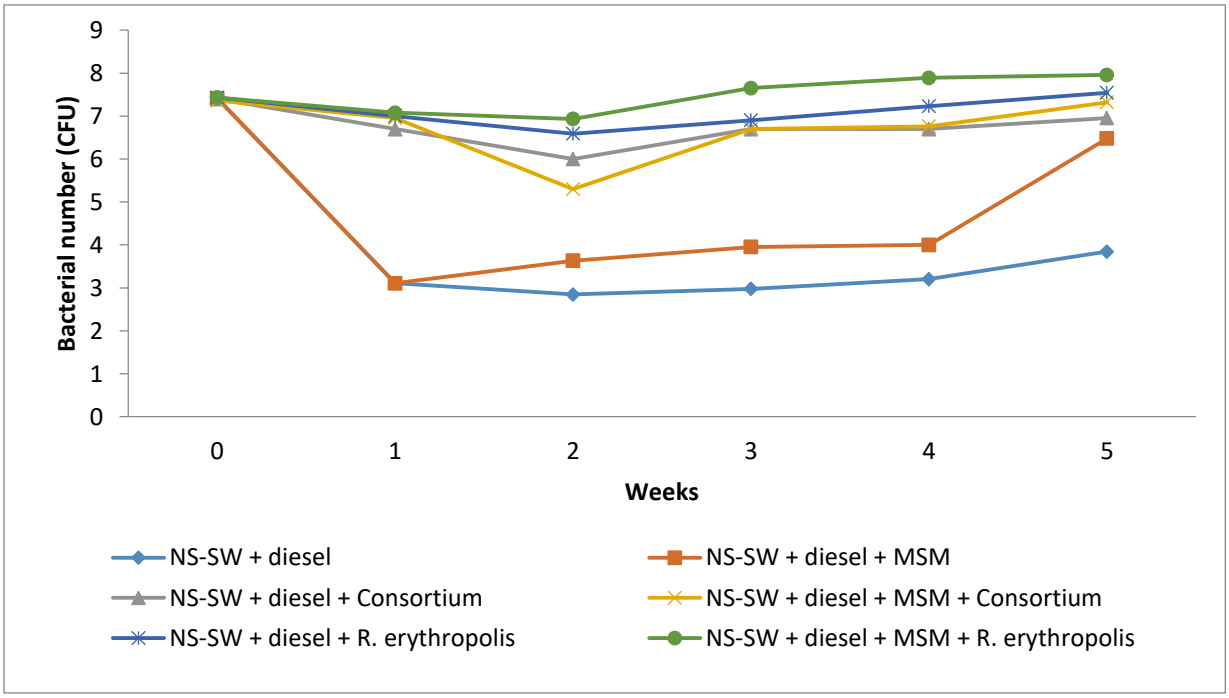

Figure 3: Variation of bacterial number in the different flasks.

3.4 Characterization of bacterialby flow cytometry in flasks experiment

The evolution of bacterial during five weeks in flasks in each treatment was observed by the flow cytometry method [16]. Interpretation of the results was performed using the Propidium Iodide (PI) coloration of cells, as cells that stain with PI are either dead or have poor membrane integrity.

PI penetrates the cell membrane and binds to the DNA. The uncolored cells are alive and show good membrane integrity. The cytograms of the bacterial in flasks show two subpopulations: a subpopulation which containing the PI-labeled cells and a subpopulation who containing intact cells that resist the effects of diesel and are not labeled with PI.

Bacterial characterization by the flow cytometry method complements the method for determining the number of bacteria made by successive dilution and spreading in petri dishes. The dilution method makes it possible to determine less than 1% of the number of total cultivable and non-cultivable bacteria in a sample [17, 18]. While the use of flow cytometry is an easy and objective method to characterize a bacterial community. This method makes it possible to successfully determine the total bacteria [19] and to assess the viability of bacteria in a liquid or solid medium [16] in a relatively short time [18]. Brognaux et al. (2013) [20] studied the characterization of the dynamics of microbial stress in a bioreactor using the technique of flow cytometry. Adding glucose to the bioreactor was shown in this study to decrease microbial stress and increase the microbial

population. In the presence of stress such as diesel in the context of our study, when glucose is added to it, bacteria preferentially synthesize the carbon contained in glucose with a positive effect on their growth. This is actually what we observe in this study with the addition of nitrogen and phosphorus as a nutrient.

Figure 4, shows the percentage of bacteria exhibiting good membrane integrity. PI does not penetrate through the membrane of these bacteria. It should be noted that the bacteria stained with PI which have poor membrane integrity are not necessarily dead bacteria. This result shows that diesel has a negative effect on the plasma membrane of bacteria and can lead to their death. The effect of diesel is much more detrimental during the first two weeks of treatment. We note that the flasks having received a treatment by bioaugmentation and/or by the combination of bioaugmentation and biostimulation shows a high percentage of viable bacteria which exhibit good membrane integrity. The consortium and *R. erythropolis* are more resistant to the effect of diesel. They synthesize the enzymes adapted to the new substrate and metabolize the diesel.

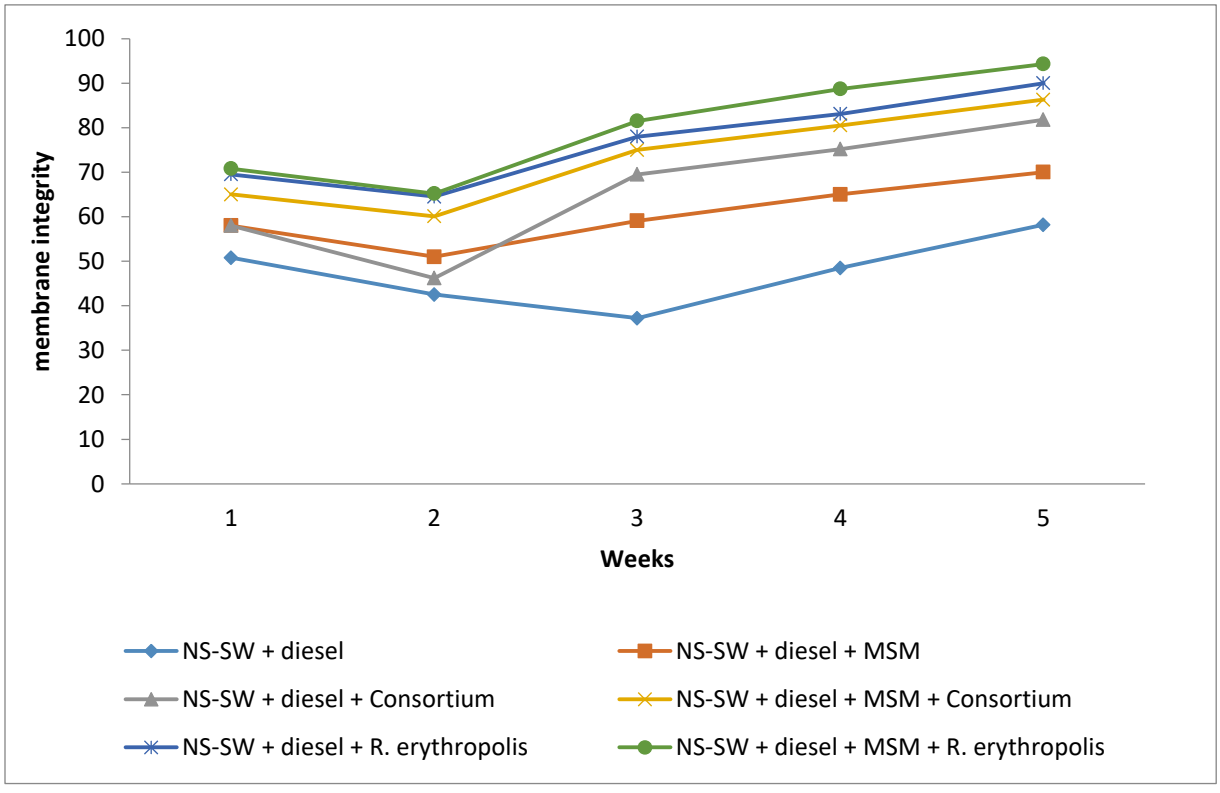

Figure 4: Effect of diesel on the plasma membrane of bacteria in the different flasks.

3.5 Rate of diesel reduction

Figure 5 shows the degradation rate of diesel as a function of different treatment techniques. The aim is to compare and assess the performance of microorganisms, their ability to metabolize diesel. The previous figures have shown the resistance and adaptability of these microorganisms in the presence of diesel. Natural attenuation, biostimulation, bioaugmentation and the combination of biostimulation and bioaugmentation are the different techniques applied as described in the methodology. The results show that the treatments with the addition of the pure strain of R. erythopolis have the best degradation rate. Bioaugmentation with *R. erythropolis* shows a degradation rate of 49 % while the combination of bioaugmentation with *R. erythropolis* and biostimulation shows a degradation rate of 67.5 %. Bioaugmentation with the endogenous bacterial consortium exhibits a degradation rate of 20.5 % while the combination bioaugmentation with the consortium and biostimulation exhibits a degradation rate of 26 %.

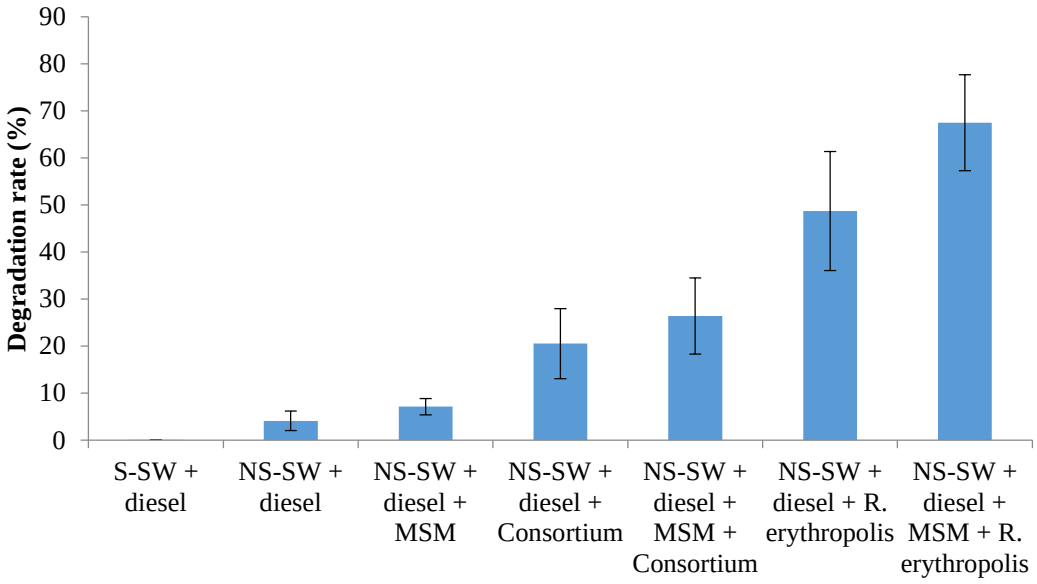

Figure 5: Evolution of the rate of diesel degradation for the different treatments.

The biostimulation (7 %) and natural attenuation (4 %), show low degradation rates for a treatment duration of five weeks. These results from a statistical point of view, by comparing the different techniques in pairs, the grouping information with the Tukey method with a confidence level of 95 % shows significant differences for certain groupings. Thus the treatments with the addition of *R. erythropolis* do not show any significant difference in their results. These treatments, on the other hand, show results that are significantly different from treatments with the addition of the consortium, biostimulation and natural

attenuation. The results of the combination bioaugmentation with consortium and biostimulation are statistically different from those of bioaugmentation treatments with the consortium, biostimulation and natural attenuation. Results by bioaugmentation and biostimulation are statistically comparable and different from natural attenuation.

The results obtained here can be explained in two different ways: the presence of *R. erythropolis* T902.1 which is a pure strain known for its capacity to degrade hydrocarbons and the presence of nutrients. Regarding the presence of nutrients, it must be said that the factor limiting the degradation of hydrocarbons in mangrove areas is not the intrinsic capacity of bacteria [21, 22], but certainly the imbalance in the C:N:P ratio caused by the high carbon content in hydrocarbons.

The presence of diesel in mangroves can cause rapid consumption of nitrogen and phosphorus sources present, which are generally scarce in mangrove ecosystems [23]. The addition of *R. erythropolis* T902.1, the increase of the endogenous microflora by the addition of the endogenous bacterial consortium on the one hand and, the addition of the nutrients on the other hand leads to changes and differences in composition, growth and the very quality of the bacteria in the different treatments. This has been demonstrated by Dos Santos et al. (2011) [24] who studied the impact of hydrocarbons on the bacterial diversity of mangroves. Semboung et al., (2016a) [2] demonstrated that diesel was rapidly metabolized by bacteria when the treatment was applied by combining biostimulation with the addition of nutrients, notably nitrogen and phosphorus, and bioaugmentation with the addition of an endogenous bacterial consortium or of a pure exogenous strain such as *R. erythropolis* or Pseudomonas fluorenscens. Adding nitrogen and phosphorus rebalances the C: N: P ratio. In the present study, nutrients also play a role in accelerating bacterial growth. This explains the increasing number of bacteria in the different vials that have received the nutrients.

The indigenous microflora of the mangrove sediments were used for treatment by natural attenuation, biostimulation and bioaugmentation. *R.erythropolis* T902.1 was added to the indigenous microflora for treatment by bioaugmentation. The Rhodococcus genus is a very diverse group of bacteria with the ability to degrade diesel, hydrocarbon and a large number of organic compounds [15], including some of the most problematic compounds in terms of recalcitrance and toxicity [25]. In this experiment and an according with Bell et al. 1998 [26] , R. erythropolis appears to be a good candidate for use in bioaugmentation, and can degrade a broad range of aliphatic, branched, cyclic, (polycyclic) aromatic, sulfur-containing, and chlorinated hydrocarbons [27-29]. This experiment shows that *R. erythropolis* can also withstand environmental stresses such as low or high pH values, a high salinity as sea water, lacks of nutriments and the presence of toxic solvents or pollutants [30-33]. Rhodococci synthesize mycolic acids and trehalolipids to modify their membrane hydrophobicity in order to protect themselves from these adverse environments and to solubilize and assimilate hydrophobic substrates that are poorly [34-36]. *R.erythropolis* T902.1, despite a limitation in

oxygen transfer, enhanced a greater hydrocarbons degradation compared to the biostimulation treatment [2, 37].

4. Conclusions

Bioremediation is a technique in development in the field of the treatment of sites polluted by hydrocarbons. In this experiment, it was demonstrated that it is possible to use endogenous microorganisms to clean up sites contaminated by hydrocarbons. Even if it takes quite a long time. It is precisely the interest of this study that makes it possible to develop new strategies. These strategies consist, among other things, of adding either endogenous microorganisms capable of degrading the hydrocarbons previously isolated in the form of a consortium, or by adding to the medium a pure strain recognized for its ability to degrade hydrocarbons. This form of biological treatment is presented as an alternative to conventional techniques for treating polluted sites, in this case physicochemical techniques, for example.

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