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Domestication of an Indigenous Bacterial Consortium to Remove Hydrocarbons from Soils of the Austral Basin

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Abstract: ***A non-contaminated soil from the Austral basin, Santa Cruz province, Argentina, was treated with a nitrogen, phosphorus and potassium solution in a 100: 1: 0.1 proportion and 3% crude oil to assess the ability of an indigenous bacterial consortium to grow using crude oil as a source of energy. The bacterial consortium degraded a maximum of 79.31% of crude oil, and this maximum degradation was achieved after the 80th day of treatment. n-Alkanes in the range of nC8–nC10 were degraded completely, followed by nC10–nC12, nC13–nC28 and nC29–nC32, with degradation percentages of 90.78%, 71.48% and 76.02% respectively.***

Keywords: Bacteria, degradation, fatty acids

1. Introduction

The *Austral basin*, an oil-producing *basin* located in the extreme south of South America, partially covers the Argentine provinces of *Santa Cruz* and Tierra del Fuego. Among the five basin areas of Argentina, the *Austral basin* has the most extreme climate. Crude oil is the principal source of energy of Argentina, and Santa Cruz province has 5% of the national oil production.

During accidental oil spills, action should be taken to remove, remediate or recover the contaminant immediately, because if not, there are chances of groundwater contamination. This can be achieved either through physico-chemical treatments or biological methods. The latter can have an advantage over the former in removing spills because they offer *in situ* biodegradation of oil fractions by the microorganisms. These methods have been used in the San Jorge Basin, another basin in Patagonia (Pichl& Pucci, 2020; Acuña et al., 2012), but not in the Austral basin. Natural biodegradation (mineralization or transformation) of a wide range of hydrocarbons, including polycyclic aromatic hydrocarbons (PAHs) and aliphatic and aromatic compounds, occurs in various extreme habitats. This process is influenced by several factors, including the concentration of hydrocarbons, the composition of the crude oil, the amount of nutrients and the temperature. Regarding the latter, previous studies have shown that, at low temperature, the viscosity of the crude oil increases and the volatilization of hydrocarbons and the water solubility decrease, delaying the onset of biodegradation. Thus, the percentage of degradation decreases with decreasing temperature, and the best temperature for degradation is in the range of 30–40°C (Truskewycz et al., 2019). In agreement with this, Nikolova & Gutierrez (2020) reported that a temperature of 30°C is optimum for microbial growth and PAH degradation.

As mentioned above, another important factor affecting the biodegradation of crude oil is its composition, since the bioavailability of oil compounds in different crude oil samples may differ. Saturated compounds with a molecular weight larger than 500 may not be degraded by organisms, i. e. they are recalcitrant compounds, because this size corresponds to the exclusion size for passage through the outer membrane of Gram-negative bacteria (Xu et al., 2018).

Bioremediation using microorganisms is a promising strategy to remediate soils contaminated with petroleum hydrocarbons in the world. In previous studies, we have demonstrated the ability of natural bacterial consortia to degrade oil in the San Jorge Basin, Argentine Patagonia (Pichl& Pucci, 2020; Acuña et al., 2012), and in austral soil contaminated with diesel and gas station trash (Cambarieri et al., 2021) in a microcosm for 100 days.

The aim of this work was determined whether biodegradation can be used to remediate soils from the Austral basin contaminated with crude oil.

2. Material and Methods

2.1 Soil samples

The soil samples used in this study were taken from near Río Gallegos City, in Santa Cruz Province, at a depth between 20 and 50 cm. The physical analysis of the samples showed the following values: pH 7.6, conductivity 84 µS. cm^{-1}, moisture 1.07%, organic matter 0.98%, inorganic matter 92%, apparent density 1.31 g/cm^3, real density 2.46 g. mL^{-1}, porosity 46%, and water proportion in the soil 34%, whereas the chemical analysis showed the following values (ppm): cations and anions: chloride 37, sulfate 48.7, carbonate < 1, bicarbonate 45.7, calcium 40.8, magnesium 12.2, nitrite 0.52, nitrate 18.1, ammonium 0.05, and phosphate < 1; heavy metals: As 1.33, Ba 382, Cd < 1.0, Zn 60, Cu 9, Cr <

10.0, Hg < 0.2, Ni 10, Ag < 2.0, Pb < 10.0, Se < 0.8, and total petroleum hydrocarbons < 0.1.

Soil biodegradation experiments

The soil biodegradation experiments were performed in laboratory microcosms. Each soil sample was separated into 400-g (dry weight) portions, and each portion was transferred to a sterile 1, 000-mL brown bottle contaminated with 3% of crude oil. Triplicate microcosms were prepared for each treatment. Ammonium was added in the form of KNO_3, and phosphate was added as KH2PO4 to prevent any limitation of bacterial activity caused by nutrient imbalance in a ratio of 100: 2.5: 0.25 (C: N: P) and 10% humidity. Controls consisted in triplicate systems without crude oil and the same chemical parameters.

The microcosms were incubated for 100 days at 28°C in the dark and weekly aerated to maintain aerobic conditions. Total mineralized carbon contents were measured twice a week by titrating the CO_2trapped during incubation in NaOH (Bartha et al., 1972). The content of hydrocarbons was determined at 0 (T0), 21 (T1), 41 (T2), 63 (T3), 80 (T4) and 100 (T5) days by gas chromatography. The bacterial counts were determined in R2A and MM-PGO media and fatty acid methyl esters (FAMEs) identified

2.2 Chemical analysis

For the chemical analysis, 2 g of each individual sample was dissolved in 5 mL of pentane. The pentane phase was separated and percolated through 2 g of silicagel. Then, 1 mL of the eluate was carefully evaporated until dryness to determine the fuel oil content of the sample. The fractions were analyzed and quantified by gas chromatography, using a Varian 3800 gas chromatograph equipped with a split/splitless injector, a flame ionization detector and a capillary column VF-5ms (30 m, 0.25 mm, 0.25 µm). The injector and detector temperatures were maintained at 200ºC and 340°C respectively. The sample (1 µL) was injected in split mode and the column temperature was increased from 45 to 100°C at a rate of 5°C/min and a second ramp from 100 to 275°C at a rate of 8°C/min with a final temperature of 275°C held for 5 minutes.

Enumeration and isolation of aerobic bacteria

Culturable bacteria from each sample were counted using the standard plate dilution method. To this end, 1 g of soil (wet weight) was suspended in 9 mL of sterile physiological water (pH 7.2) and vortexed for 1 min at low speed. Aliquots of 100 µL of undiluted samples, and 10^{-1} to $^{10-6}$ dilutions were grown on R2A medium (Reasoner&Geldreich, 1985) and MBM-PGO medium (NaCl 5 g/L, K_2PO_4H 0.5 g/L, $NH_4PO_4H_2$ 0.5 g/L, $(NH_4)_2SO_4$ 1 g/L, Mg SO_4 0.2 g/L, KNO_3 3 g/L, and $FeSO_4$ 0.05 g/L, suspended in distilled water). Next, 30 µL of a 1: 1 mixture of petroleum-diesel oil was spread on the surface and plates incubated at 28ºC for up to 21 days (Pucci and Pucci 2003).

Extraction of fatty acids

The extraction of fatty acids was carried out once a week in duplicate, using the method of Ritchie et al. (2000). To this end, 10 g of soil from each system was placed in 100-mL flasks previously rinsed with methanol. After that, 50 mL of methylated KOH (0.2M) was added, and the sample was placed in a 37°C bath for 1 h, stirring every 10 minutes. Then, 10 mL of acetic acid was added to neutralize the pH and the sample was subsequently filtered. Next, 3 mL of n-Hexane was added to the supernatant obtained after filtration, mixed, and the upper phase separated. Then, 3 mL of n-Hexane was added, mixed and the upper phase separated again. The separated phases were placed in vials and allowed to evaporate under a nitrogen atmosphere.

Gas chromatography parameters

The MIDI microbial identification system (Microbial ID, Inc., Newark, NJ, USA) was applied to separate FAMEs using a gas chromatograph (HP 6890) equipped with a split/splitless injector, a flame ionization detector, a capillary column Ultra 2 (25 m, 0.2 mm, 0.33 µm), an automatic sampler, an integrator, and a program which identifies the fatty acids (Microbial ID 6.0 version). The injector and detector temperatures were maintained at 250ºC and 300°C respectively. The sample (2 µL) was injected in split mode and the column temperature was increased from 170 to 270°C at a rate of 5°C/min.

The fatty acid concentration was used to calculate the number of Gram positive bacteria Σ (15: 0 + a15: 0 + i15: 0 + 16: 0 + a17: 0 + i17: 0) (Song et al., 2008), Gram negative bacteria Σ (cy17: 0 + 18: 1ω7c) (Song et al., 2008), actinomyces Σ (18: 0 10Me + 19: 0 10Me) (Zheng et al., 2013) and fungi 18: 2 ω6c (Mummey et al., 2002).

3. Statistical Analysis

The mean and standard deviation of the three replicates were calculated. The mean values were compared by ANOVA test BIOM (Applied Biostatistics Inc.3 Heritage, Setauket, NY 117ll, USA). Differences were considered significant when $P<0.05$. To identify possible similarity between FAME profiles, the data were subjected to analysis of variance using the PAST (Hammer & Harper, 2005) and Sherlock (Microbial ID 6.0 version) software.

4. Results

The biodegradation values reached on day 100 may allow the use of soils in sanitary landfills according to current regulations. The degradation rate was highest on day 41 (Fig.1), probably due to the degradation of the most biodegradable components present in the sample, and then decreased smoothly (Table 1). Regarding the efficiency of crude oil degradation by indigenous microbial populations, we found a maximum of 79.31% of degradation after 100 days of incubation. Several other researchers (Tao et al.2017; Shinde et al.2020; Wang et al.2018) have described the ability of mixed bacterial consortia to degrade 43.6–85.01% of saturates and 0–22.78% of aromatics present in different crude oil samples. The analysis of petroleum by gas chromatography showed the degradation process with time (Fig.2 and Table 1). The first degradation was that of total petroleum hydrocarbons, including n-Alkanes and some PAHs. By the end of the bioremediation process (100 days), the degradation was different: fast in *n*C8–*n*C10 in the first

21 days, and then slow half of percentual values between days 41 and 80. degradation was around 41 to 63 days.

The analysis of crude oil by using biomarkers such as the pristane (2, 6, 10, 14-tetramethylpentadecane) to phytane (2, 6, 10, 14-tetramethylhexadecane) ratio and the isoprenoids/n-alkanes (Pr/n-C_{17} and Ph/n–C_{18}) ratio is necessary and here used to ascertain the levels of degradation in the environment of crude oil. The n-C17/pr and n-C18/ph ratios obtained from the chromatograms (Fig.1 and Table 2) were indicative of degradation until 80 days. The initial values were 3.65 and 5.42, whereas after the introduction of nutrients and bacterial communities, they decreased to 0.12 and 0.41 respectively.

The bacterial communities able to grow in mineral salt medium with crude oil as carbon source increased from 10^3 to 10^8 colony-forming units (CFU) /g during the treatment. An initial bacterial population of about total bacterial $5.3 \pm 0.7 \times 10^5$ and hydrocarbon degrading bacteria 8.9×10^3 CFU/g was observed on the soil. Bacterial communities changed fatty acid with the time. The left of Figure 3 shows how the addition of nutrients and moisture impacted on the control system. This was accompanied by the recount value of total and degrading aerobic bacteria, which increased their values 2 log and 4 log respectively. The microcosm with hydrocarbons has the two impacts: the addition of nutrients and moisture in addition to the hydrocarbon. This generated more changes in the fatty acids of the bacterial community that already had a selective pressure for hydrocarbons. The indices calculated showed the presence of actinomyces and fungi in higher values than in the control sample (Table 2). The presence of fatty acids as a marker (or index) of the presence of fungi was higher in the crude oil systems than in the control systems.

5. Discussion

Several studies have shown that the success of the bioremediation process of soils contaminated with hydrocarbons is based on the choice of microorganisms able to use the hydrocarbons present in the soil (Borah & Yadav, 2017). The rate of petroleum biodegradation and quantity of hydrocarbons degraded depend on several factors, including the environmental conditions and the chemical structure, type and amount of crude oil present in the contaminated site (Ghazali et al., 2004; Varjani et al., 2017). The persistence of hydrophobic hydrocarbons in contaminated environments even after bioremediation is due to their strong adsorption on coarse-grained and organic free soils, which makes them less available for hydrocarbon-degrading microorganisms.

Alkanes, which are major constituents of crude oils, can be degraded by bacteria both aerobically and anaerobically (Brzeszcz et al., 2018). During harsh environmental conditions such as oxidative and osmotic stress, low humidity and ionic nutrient-shortage, alkane degraders possess exclusive metabolic pathways and survival strategies, such as the production of biosurfactants, spores, and exopolysaccharides for self-protection (Park & Park, 2018). Although some organic compounds present in crude oil are highly toxic and volatile at room temperature, they are a source of carbon and energy for most bacteria.

An increase in the bacterial population was observed in all the soil samples studied, particularly in those with crude oil. This may be due to the biosurfactant-induced desorption of hydrocarbons from the soil to the aqueous phase of soil slurries, leading to increased microbial mineralization (Helmy et al., 2015; Patowary et al., 2016).

The available nutrients were rapidly assimilated by soil microbes. Thus, in this soil with a natural low concentration of nutrients. Several researchers have recently described an increase in microbial activity and biodegradation rate after addition of inorganic nutrients (Koolivand et al., 2019). In the present study, we observed an increase in the count of all bacteria, particularly up to day 21. Our results are similar to those reported by Chaineau et al. (2005) and could be due to the increasing activity of enzymes. The same trend regarding the effects of crude oil on soil enzymes was observed by Cuevas-Díaz et al. (2017) and Kucharski and Jastrzębska (2006). The total number of organotrophic bacteria and hydrocarbons degrading bacteria after 100 days was higher than on the first day of the experiment. Finally, the presence of markers of actinomyces and fungi agrees with the fact that the degradation values of TPH are in the time of 63 and 80 days, since they are microorganisms with slower development and with ability to degrade hydrocarbons.

6. Conclusions

Crude petroleum oil was removed from soil samples by using indigenous bacterial communities that could be domesticated by oil removal. Hydrocarbon analysis showed that the degradation of aromatic compounds was significantly greater than that of n-alkanes. These findings demonstrate that, from a biotechnological point of view, bioremediation by indigenous bacterial communities represents a promising alternative to remove and detoxify petroleum from contaminated environments in the south of Santa Cruz province.

7. Future Scope

The natural presence in soils from the south of Santa Cruz province of bacteria able to be domesticated to degrade crude oil can be used in the future to remove oil from contaminated soils in case of oil spills.

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Table 1: Hydrocarbon analysis (mg HC. kg^{-1}) performed in the system used to study soil biodegradability of crude oil. The

decrease in the C17/Pri and C18/Phy indices is indicative that the hydrocarbon remediation process is of biological origin. Pri: Pristane, Fit: Phytane.

	0	21	41	63	80	100
< 8	159.99	72.61	31.95	36.78	0.00	0.00
8 to< 10	1045.27	483.95	258.56	281.11	0.00	0.00
10 to< 12	2265.71	1671.32	931.77	712.49	232.05	208.85
12 to< 14	2762.80	1972.57	1330.00	963.96	592.65	533.39
14 to< 16	2736.53	2001.12	1558.26	1011.84	745.93	671.34
16 to< 18	2605.17	2008.70	1555.76	989.83	781.64	703.48
18 to< 20	2301.49	1747.11	1337.24	851.41	665.93	599.34
20 to< 22	2036.23	1523.63	1136.78	707.68	533.26	479.93
22 to< 24	1758.30	1368.21	971.95	568.92	428.80	385.92
24 to< 26	1516.61	1181.89	845.90	481.08	358.84	322.96
26 to< 28	1424.25	1158.67	835.44	472.91	375.59	338.03
28 to< 30	1291.19	1147.04	808.30	455.79	409.16	368.25
30 to< 32	1043.50	884.22	576.17	223.12	212.69	191.42
> 32	263.35	181.65	10.73	0.00	0.00	0.00
TOTAL	23, 210.39	17, 402.71	11, 002.86	7, 756.91	5, 336.54	4, 802.89
%Biod		25.02	52.60	66.58	77.01	79.31
C17	377.29	127.07	28.26	32.18	5.67	5.11
Pristane	103.34	91.16	67.80	62.04	46.17	41.55
C18	378.01	138.03	37.64	41.82	10.86	9.78
Phytane	69.76	56.98	40.16	38.08	26.29	23.66
C17/Pri	3.65	1.39	0.42	0.52	0.12	0.12
C18/Phy	5.42	2.42	0.94	1.10	0.41	0.41

Table 2: Bacterial count, CO_2 evolution and fatty acid index

Control system	0	21	41	63	80	100 days
R2A	$5.30 \times 10^{+05}$	$3.50 \times 10^{+07}$	$2.91 \times 10^{+07}$	$8.00 \times 10^{+06}$	$7.80 \times 10^{+06}$	$6.51 \times 10^{+05}$
MBM_PGO	$8.90 \times 10^{+03}$	$1.24 \times 10^{+07}$	$2.46 \times 10^{+06}$	$3.20 \times 10^{+06}$	$4.10 \times 10^{+06}$	$2.10 \times 10^{+06}$
CO_2evolution	0	1201.46	1748.47	2122.10	2495.72	2872.49
Biomass	3.12	2.64	2.56	2.48	2.54	2.58
Bacteria	34.80	33.86	34.21	34.26	34.69	35.01
Actinomyces	1.66	1.58	1.56	1.57	1.54	1.45
Fungi	0.19	0.26	0.20	0.17	0.15	0.15
Crudeoilsystem						
R2A	$5.30 \times 10^{+05}$	$4.39 \times 10^{+08}$	$1.57 \times 10^{+09}$	$5.40 \times 10^{+08}$	$6.20 \times 10^{+08}$	$3.27 \times 10^{+06}$
MBM-PGO	$8.90 \times 10^{+03}$	$3.14 \times 10^{+08}$	$4.30 \times 10^{+08}$	$7.52 \times 10^{+07}$	$2.93 \times 10^{+07}$	$1.23 \times 10^{+07}$
CO_2 evolution	0	4424.9	8037.29	10125.33	10834.96	11368.94
Biomas		2.68	2.61	3.17	3.30	3.13
Bacteria		14.39	13.75	14.78	14.56	14.45
Actinomyces		1.02	1.71	1.26	1.78	1.67
Fungi		1.02	1.71	1.26	1.78	1.67
BAT		11.54	52.95	66.50	78.49	4.02
BDH		24.32	173.80	22.50	6.15	4.86

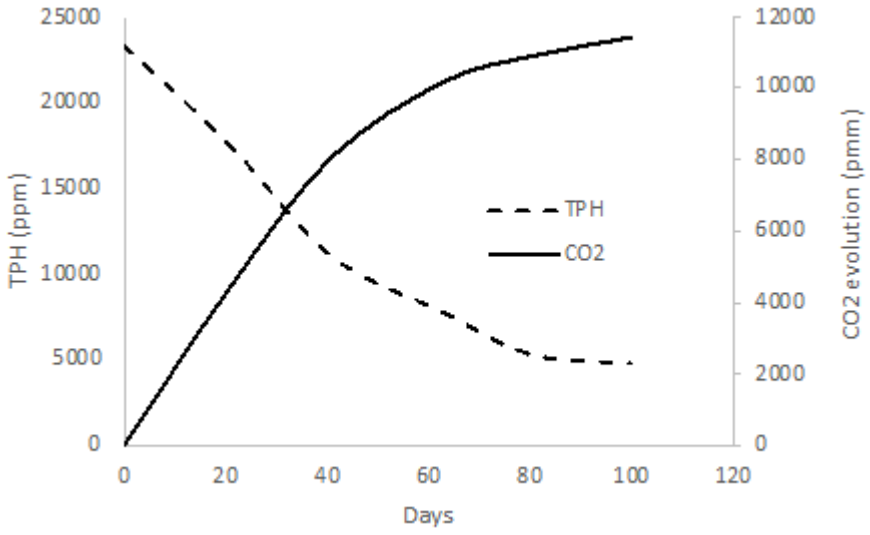

Figure 1: Degradation of total petroleum hydrocarbons (TPH) and CO_2 evolution bioremediation in oil-contaminated soils

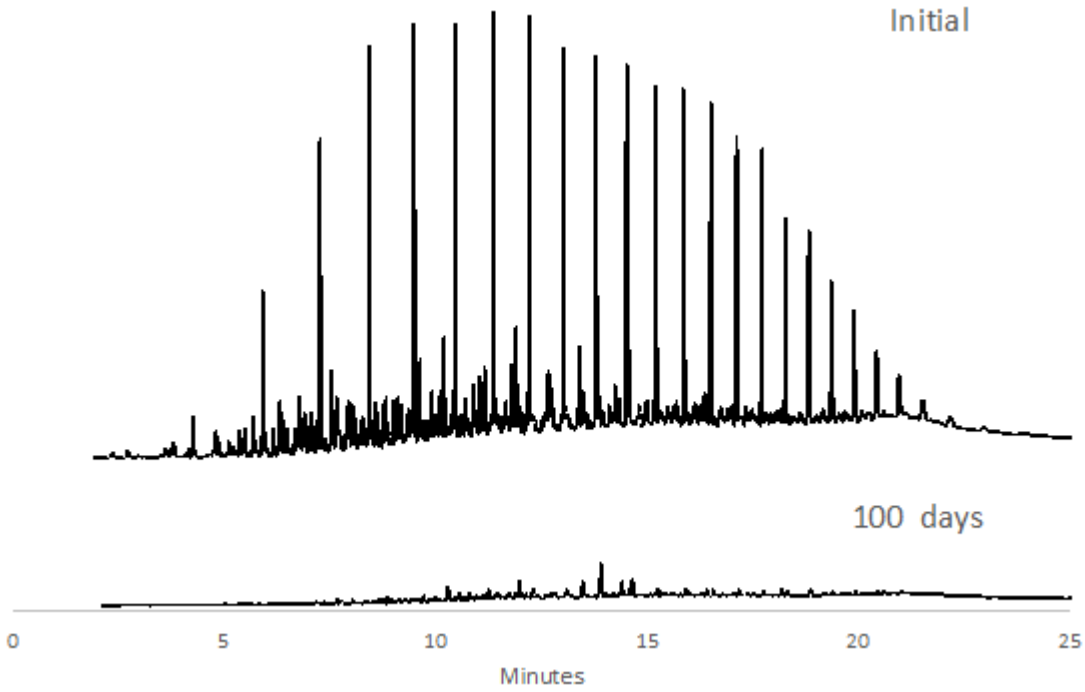

Figure 2: Gas chromatogram of the initial concentration of TPH in the soil prior to degradation and at 100 days

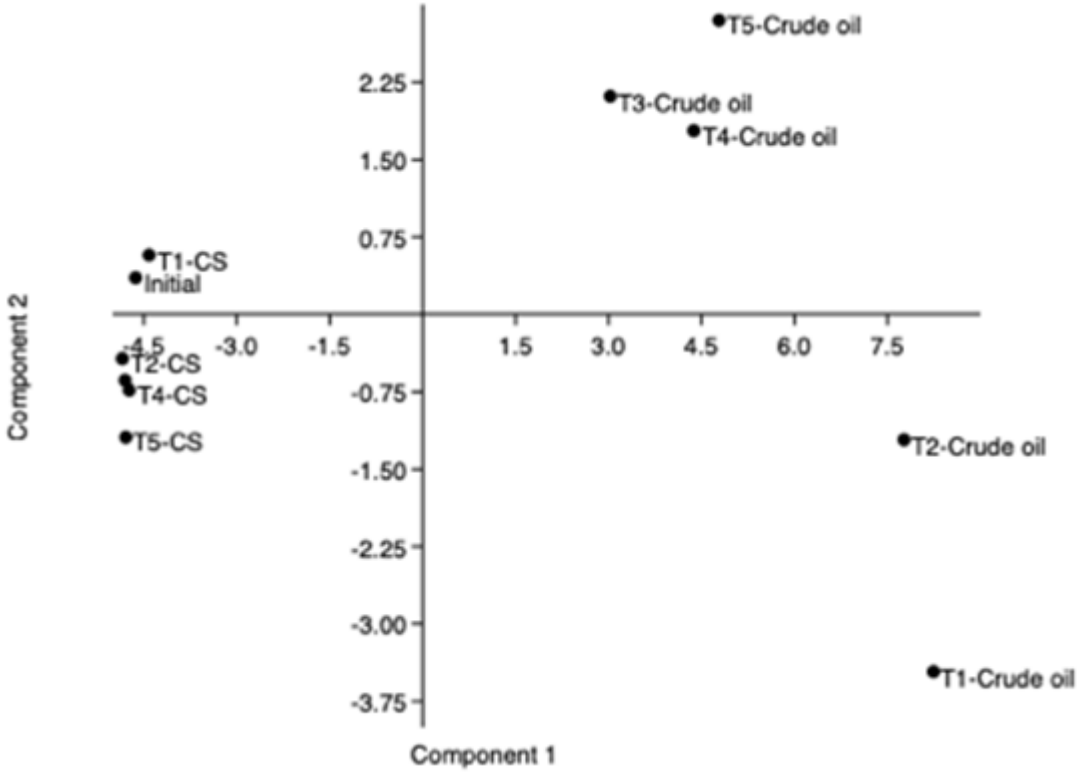

Figure 3: Principal component analysis of the microcosms used in the experiments.