


Vegetable oil contaminated area: site-specific bioremediation treatments.

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Abstract– *The environment is negatively impacted by occasional discharges from industrial activity. When these events contain insoluble compounds such as fats and oils, they are high impact pollutants. This work focuses on an environmental problem in the industrial area of Buenos Aires, Argentina, where a lagoon was contaminated by vegetable oil residues. The aim of this work is to study bioremediation strategies in order to propose solutions for the remediation of the lagoon. For this way, autochthonous vegetable oil degrading bacteria were isolated from Lagoon 3, and the conditions to produce bacterial biomass were evaluated. Then, through microcosms systems using contaminated coastal soil, different site-specific treatments were tested: a control as natural attenuation; a bioaugmentation treatment with autochthonous vegetable oil degrading bacteria; two biostimulation treatments with nitrogen and phosphorus, and with spent mushroom substrate. Although both bioaugmentation and biostimulation showed promising results, biostimulation with N, P was the most effective for site-specific bioremediation of Lagoon 3, achieving 67% of oil vegetable reduction at 60 days.*

Keywords- *Vegetable oil, autochthonous bacteria, bioreactor, bioremediation, agro-industrial residues.*

I. INTRODUCTION

Despite the benefits that industry contributes on human life as economic source, such activity often generates occasional discharges that impacts on the environment, affecting various ecosystems, including soils, surface and groundwater. In fact, the main source of pollution of rivers, bays, lagoons and aquifers is the uncontrolled discharge of effluents, which mostly come from the industrial sector [1, 2]. In this respect, insoluble compounds such as fats and oils are pollutants that have a great impact on aquatic ecosystems, as they form a layer on the water surface and decrease the rate of oxygen transfer in an aerobic process, affecting the organisms living at the site. Oils that impact on soils coat the land with an impermeable film that destroys vegetable humus [3].

In nature, microorganisms are able to use a wide variety of organic compounds as source of carbon and energy for their growth. Thus, fats and oils can be hydrolyzed and metabolized by microbial action. Several enzymes produced by the degrading microorganisms themselves can be involved in the biodegradation of fats and oils, being lipase the most common enzyme. This enzyme releases fatty acids, which can be biodegraded by a wider range of microorganisms, including those that do not produce extracellular lipolytic enzymes [4].

Several genera of bacteria and fungi were reported as oil degraders [3, 5, 6, 7].

Although fats and oils compounds are biodegradable by natural attenuation, this is a very slow process and puts the affected ecosystem at risk [8]. It is therefore a priority ecological objective to develop environmentally compatible, high-efficiency pollutant removal technologies. On the other hand, physico-chemical techniques for pollutant remediation have a high economic cost and are not considered compatible with the environment due to the disturbances they generate in the treatment area. Therefore, bioremediation emerged as an alternative strategy for the removal of pollutant compounds, which is considered to be environmentally friendly, simple and economical. Bioremediation enhances the natural process of biodegradation. It is a technology that involves living organisms to reduce or eliminate environmental risks resulting from the accumulation of xenobiotic compounds [9].

The success of oil bioremediation depends on the ability to establish conditions that enhanced pollutants biodegradation rates in the contaminated site. There are the two main approaches to bioremediation: bioaugmentation, in which pollutant-degrading bacteria are added to supplement the existing microbial population, and biostimulation, in which the growth of indigenous pollutant degraders is stimulated by the addition of nutrients or other growth-limiting co-substrates [2, 10].

Bioaugmentation was widely reported in xenobiotic compounds degradation [10, 11, 12, 13]. This technique is considered when the indigenous microorganism population is insufficient, does not have the ability to degrade pollutants, or when the speed of decontamination is slower than expected. The addition of a prepared bacterial culture enhances or accelerates pollutant degradation contributing appropriate metabolic capabilities. This technology is even more convenient when the degrading microorganisms are indigenous to the area to be remediated; as no allochthonous species are introduced and the establishment of the native strains on site can be more effective.

Considering *in situ* bioremediation, biostimulation was reported to be the most successful method [14]. Biostimulation involve the addition of any stimulatory materials, bulking agents, nutrients amendments, bio-surfactants, biopolymers and slow release fertilizers to enhance and support microbial growth and enzymatic activities of the autochthonous microorganisms in the contaminated site for remediation

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activities [15, 16, 17, 18]. Biostimulation is achieved by addition or optimization of various forms of limiting parameters, micronutrients and electron acceptors such as nitrogen, phosphorus, potassium, carbon and oxygen, which are mostly available in low concentrations in the contaminated site and decrease or limit microbial performance. The biostimulation requirements include presence of correct microorganisms, ability to stimulate target microorganisms, ability to deliver nutrients [14].

The use of agro-industrial residues that provide nutrients such as nitrogen, phosphorus and potassium contribute to solving the problem of nutrient limitation at contaminated soils [16]. Organic residues impact soil structure and fertility by adding essential nutrients, improving physical, chemical and biological properties of soils [9]. Promising bioremediation results were previously reported using agro-industrial wastes such as sugarcane bagasse, maize residue, banana peel, spent barley grain, carrot peel and spent mushroom substrate (SMS) [19, 20, 21, 22]. The industry producing edible mushroom, generates large amounts of SMS, as 5 kg of SMS can be generated from the production of 1 kg of mushrooms. The compost usually consists of a composted mixture of wheat straw and horse manure, with the addition of other residues such as spent barley grain. After exhausted for mushroom production, it still contains high levels of nutrients, such as nitrogen, phosphorus, potassium, a wide range of trace elements, enzymes and vitamins, which can be utilized in a new bioprocess [23, 24, 25].

Bioremediation approach chosen for any contaminated environment is site-specific, as it depends on the variables associated with the composition of the pollutants, the physical, chemical and biological conditions of the affected environment. This research focuses on the contamination event of Lagoon 3 located in the private nature reserve El Morejón [26]. This lagoon is highly polluted by oil compounds, as a result of uncontrolled dumping by a neighbouring company that treats waste from the oil industry. The aim of this work was to study vegetable oil degradation in a contaminated soil belonging to Lagoon 3 under bioremediation approaches. For this purpose, autochthonous vegetable oil degrading bacteria were initially isolated from Lagoon 3 in order to apply them in bioaugmentation strategy. Subsequently, the conditions to produce bacterial biomass were evaluated. Finally, different site-specific treatments were tested using microcosm systems: a control as natural attenuation; a bioaugmentation treatment with autochthonous vegetable oil degrading bacteria; two biostimulation treatments with nitrogen and phosphorus, and with spent mushroom substrate.

II. EXPERIMENTAL DEVELOPMENT

A. Isolation of oil-degrading bacteria

Vegetable oil degrading bacterial consortia was isolated from soil sample belonging Lagoon 3 area. 1 g of soil was placed in a 250 mL flask containing 50 mL of oil medium

(MO: minimal saline medium –MSM- with oil mixture (5 %v/v)). The vegetable oil mixture was formulated by equal parts of sunflower oil, soybean oil, corn oil and olive oil. Culture was maintained at 135 rpm and 25°C for 72 h. Then 1 mL aliquot of the bacterial culture was used to inoculate a fresh MO, and incubated at 135 rpm and 25°C for 72 h. This procedure was carried out 8 successive times [27]. Culture obtained was cryopreserved with glycerol (15 %v/v) for further study.

Bacteria isolated were characterized according to colony morphology on plates with Luria-Bertani solid medium, cell morphology and Gram staining by microscopy.

B. Biomass production of L3-M3 bacteria

L3-M3 bacteria growth conditions were evaluated in flask, in view to applying its biomass as an inoculum in bioaugmentation strategy. For this purpose, two alternative cultures medium were tested, considering alternative carbon sources, a vegetable oil mixture or a sweet potato root residues. For this purpose, L3-M3 bacteria were grown in flask containing MSM with 5 % of oil mixture or sweet potato root residues, maintained at 135 rpm and 25°C for 8 days. Biomass was estimated by dry weight (g/L). Subsequently, L3-M3 bacteria were grown in flask containing MSM with 2 or 5 % v/v oil mixture, maintained at 135 rpm and 25°C for 8 days. Samples were taken daily to determine biomass concentration, pH, surface tension (ST), and oil concentration [EPA 413.2 method]. Biomass was estimated by dry weight (g/L). ST was assayed as an indirect measure of biosurfactant production. ST of cell-free supernatant was determined in a tensiometer (Sigma 702, Attension), using the Du Nouy ring method according to [27].

In order to validate results of flask assays, biomass of L3-M3 bacteria were produced in a 3 L BioFlo 115 stirred tank bioreactor (New Brunswick Scientific Co.). The MO medium (2 L) was inoculated with 3%v/v (OD 0.5 at 600 nm) of L3-M3 bacteria, kept at 200 rpm for 5 days. Samples were periodically extracted under sterile conditions to determine biomass concentration, pH, ST and oil concentration.

C. Bioremediation in microcosm assays

The soil used in microcosms systems was taken from coastal area of Lagoon 3 (-34.194522, -59.012097). Soil was sieved (10 mm mesh) and analyzed for water content, pH and oil concentration [28].

Four bioremediation treatments were carried out as indicated in Table 1. L3-M3 bacteria produced in bioreactor (section B) were applied in bioaugmentation system (condition L3-M3). Culture was added to soil in microcosms in order to reach a cell density of 5×10^{10} CFU/g of dry soil. Concentrations of nitrogen (1 g/Kg) as NaNO_3 and phosphorous (0.2 g/Kg) as Na_2HPO_4 were added to soil in order to evaluate the influence of these nutrients on the growth

of the indigenous microflora (condition N,P). The other biostimulated system was carried out using 10 %w/w spent mushroom substrate (SMS).

TABLE I
Conditions applied to the different microcosms

System	Condition
C	Natural attenuation control
L3-M3	Biaugmentation with L3-M3
N,P	Biostimulation with nitrogen and phosphorous
SMS	Biostimulation with spent mushroom substrate

The soil was placed in glass cylindrical flasks (60 mm diameter and 360 ml volume), each containing 200 g of mixture. Each condition was carried out by triplicate. The systems were kept at 22 °C for 60 days. The samples were taken once every 20 days in order to determinate pH, biological activity (total heterotrophic aerobic bacteria (HAB) and oil degrading bacteria (ODB)) and oil concentration [28].

III. RESULTS AND DISCUSSION

Characterization of L3-M3 bacteria

From samples taken from Lagoon 3 area, a vegetable oil degrading bacterial consortia was isolated, which was named L3-M3. Bacteria showed variety in colony morphology, cell morphology (bacilli, cocci, streptococci) and cell wall type (Gram positive/negative). Characterization indicated that bacterial consortia consist of a range of bacteria, providing the advantage of increase metabolic repertoire to vegetable oil degradation [29].

A. Growth evaluation of L3-M3 bacteria

Biomass production of L3-M3 bacteria was evaluated as a potential inoculum in bioaugmentation strategy. Two alternative carbon sources were selected to include in the medium composition of flasks assays, a vegetable oil mixture or a sweet potato root residue. The vegetable oil mixture was present in the original isolation condition of L3-M3, so it was pertinent to take it into account. Otherwise, sweet potato crops generate a large amount of waste in the study area, and it is relevant to find niches for their use. Furthermore, as agricultural activities in Argentina generate a large amount of co-products and agro-industrial waste, significant residues are available to generate value-added products [30, 31]. Results indicated that L3-M3 bacteria were able to growth in a medium with sweet potato root residue. This fact is very interesting since the use of this type of substrates as a carbon source helps the development of an environmentally friendly process that results attractive in cost-benefit terms [32, 33]. Figure 1 shows the growth curves of the L3-M3 bacteria on the two carbon sources, indicating that in both conditions the

bacteria started the stationary phase at 5 days of incubation. Biomass was significantly higher ($p < 0.05$) in the oil mixture compared to the sweet potato root residue, 9.12 versus 6.25 g/L. Therefore, although the use of the sweet potato root residue is interesting, in this study it was more appropriate to continue using the oil mixture as a carbon source, as the presence of these oils maintains the selection pressure for the bacteria to retain the ability to degrade the oils.

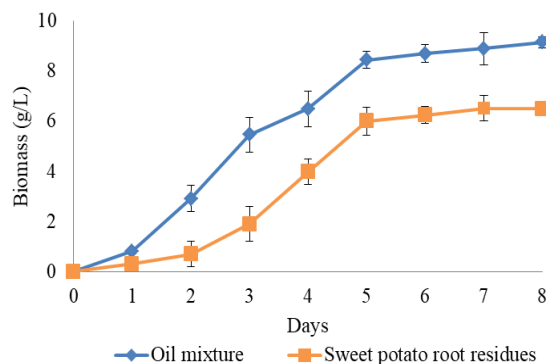


Fig. 1. Growth of L3-M3 bacteria in flask containing MSM with 5 % v/v of carbon source (oil mixture or sweet potato root residue).

Then the growth capacity of bacteria were evaluated at two concentrations of oil mixture, 2 and 5 %v/v. Growth of L3-M3 bacteria showed that culture reached stationary phase after 5 days of incubation in MSM with 5 %v/v oil mixture, obtaining 9.11 g/L of biomass (Fig. 2). Oil concentration decreased according biomass increased. pH values increased from 7 to 8.22. ST measurements decreased to 30 mN/m after 2 days and remained below this value until 8 days. This would indicate that L3-M3 bacteria have the capacity to produce surfactant molecules. This characteristic is to be expected for this type of bacteria, as they have the ability to grow in a culture medium whose carbon source requires emulsification to increase bioavailability to the cell.

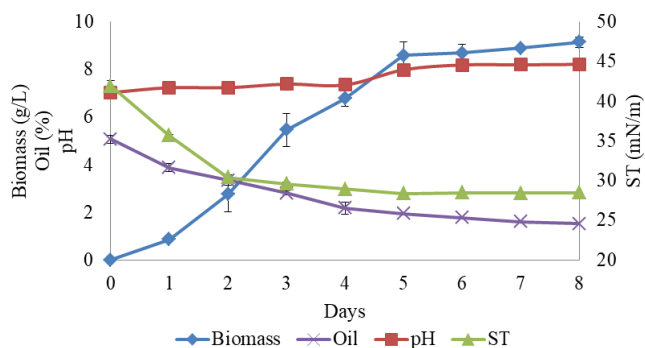


Fig. 2. Evolution of L3-M3 bacteria in flask containing MSM with 5 % v/v oil mixture.

In the case of the culture of L3-M3 bacteria in MSM with 2 %v/v oil mixture, it was noticed that bacteria had slower

growth than in MSM with 5 %v/v oil mixture. Stationary phase reached after 7 days and biomass production was 6.2 g/L (Fig. 3). Curve of oil concentration, pH and ST showed similar behaviour than L3-M3 bacteria in MSM with 5 %v/v oil mixture. Oil was observed to remain at the end of assays in both conditions (2 and 5 %v/v oil mixture), most likely associated with the lack of appropriate conditions in the flask for the bacteria to continue to grow and metabolise the carbon source, such as the availability of oxygen.

Growth evaluation of L3-M3 bacteria in flask showed that L3-M3 bacteria had faster and higher growth in the medium with 5 % v/v (9.1 g/L at 5 days) comparing to 2 % v/v oil mixture (6.2 g/L at 7 days). Thus, MO (MSM with oil mixture (5 % v/v)) showed to be a suitable medium for growth of L3-M3 bacteria.

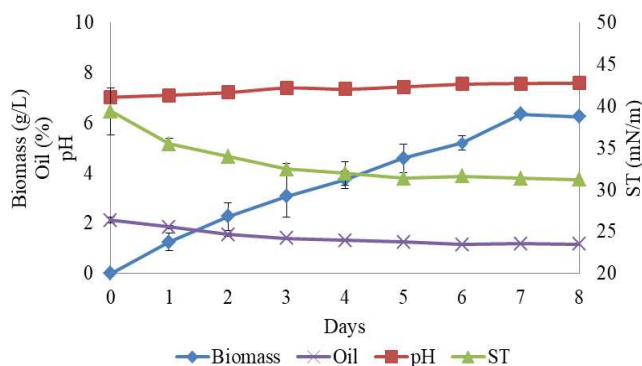


Fig. 3. Evolution of L3-M3 bacteria in flask containing MSM with 2 % v/v oil mixture.

Subsequently, L3-M3 growth was evaluated in the bioreactor and 11.29 g/L biomass was obtained after 4 days (Fig. 4), showing that it increased 25% compared to flask. This is to be expected as the conditions of the bioreactor allow better control of process parameters such as: aeration control, mixing of nutrients, oxygen transfer and temperature control. In addition, the microbial growth curve showed the beginning of the stationary phase at 3 days; that is 2 days earlier than in flask. Furthermore, the decrease in oil concentration detected throughout the assay correlates with the increase in biomass concentration. ST measurements decreased significantly during the first 2 days and remained at low values (up to 24 mN/m) until the end of the assay. Curve of ST was according to results of flask assays. As pH was not controlled during assay, it increased from 7 to 8.4. The pH above 8 probably limits the growth of L3-M3 bacteria. This fact could be related to the presence of oil at the end of the test, which was not consumed by the bacteria. So, it could be a point of improvement for future assays to control the pH at 7 in order to further increase biomass production.

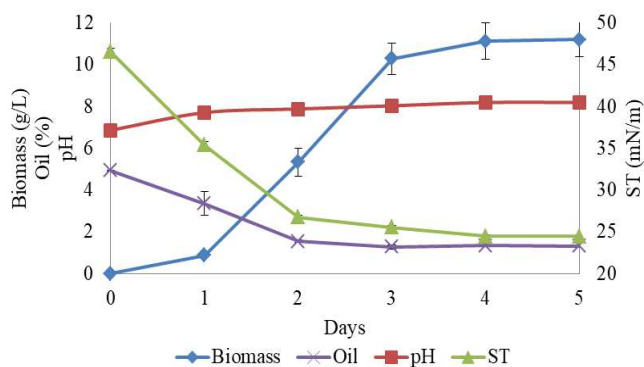


Fig. 4. Evolution of L3-M3 bacteria in bioreactor.

B- Bioremediation treatments

The degradation of a contaminant depends on the interrelationships between abiotic and biotic factors in the soil. Soil-microorganism relationships are extremely complex, due to the intricate network of physical, chemical and biological interactions. Microcosm systems allow the study of bioremediation processes, simplifying the management of the variables involved. They allow a faithful representation of the ecosystem in such a way that the results obtained from the experimental model can be extrapolated to a full-scale system [17, 34]. Microcosm assays were carried out in order to explore at laboratory scale a portion of the universe under study. The soil used in this study had an initial oil concentration of $10,120 \pm 122$ ppm. The pH was 8.07 ± 0.08 and the water content 41.25 ± 0.67 %.

Four bioremediation conditions were tested through microcosm using the oil-contaminated soil. They were natural attenuation control (C); bioaugmentation with autochthonous L3-M3 bacteria (L3-M3); biostimulation with nitrogen and phosphorous (N, P); and biostimulation with spent mushroom substrate (SMS). Microcosm indicated that soil humidity was 38.96 ± 2.26 % during the experiment. Soil moisture is an important medium for various biochemical reactions in soils. Water content affects soil gas exchange and the availability of soluble organic matter of soil. Moreover, it plays a crucial role in influencing microbial activity, which indirectly affects microbial mineralization of organic compound as pollutants as carbon sources [35]. Thus, in our case, to maintain adequate microbial activity it was important to regulate the soil moisture to 40 %, like the original samples taken from the shore of Lagoon 3.

In relation to pH parameter, although a downward trend was observed for C, L3-M3, SMS systems and an upward trend for N,P system, all values were recorded within the range of 7.5 and 8.5 (Fig. 5). The treatments did not cause significant changes in soil pH over the 60-day period.

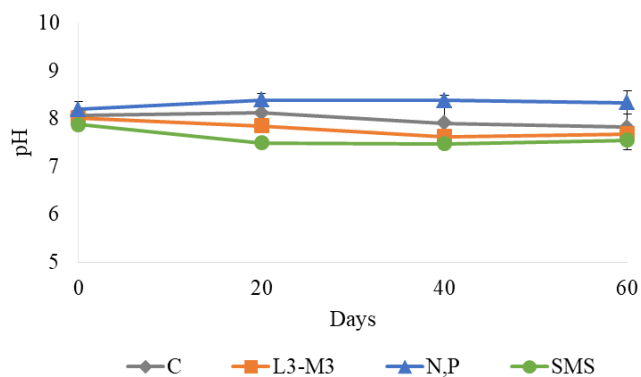


Fig. 5. Changes in pH during microcosms assay.

Initially, we noticed that the soil from Lagoon 3 contains a high load of microbial activity in both HAB and ODB counts, 10^{10} and 10^8 CFU g^{-1} respectively. This could be related to soil microflora that have adapted to contamination by vegetable oils. Microcosms systems showed that total HAB counts increased by an order of magnitude from 10^{10} to 10^{11} CFU g^{-1} during 60 days (Fig. 6), being significantly ($p < 0.05$) higher in the cases of biostimulation with N,P and with SMS. The same behaviour was noticed in ODB counts (Fig. 7). The counts increased by 2 orders of magnitude from 10^8 to 10^{10} CFU g^{-1} . Results showed that ODB counts were significantly ($p < 0.05$) higher in the biostimulation with N,P and SMS comparing to C and L3-M3 from 40 days. Moreover, HAB counts in SMS system indicated that this substrate contributed to the microbial load, which is according to preview report [36].

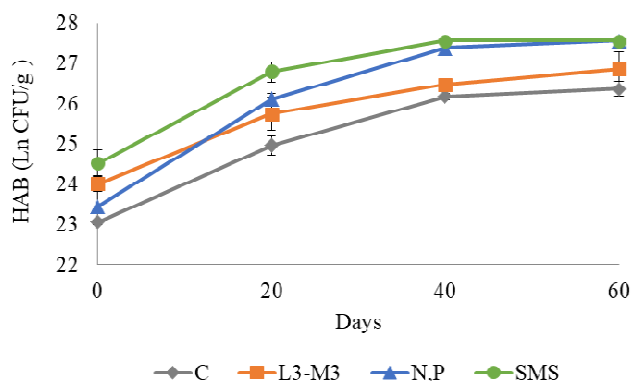


Fig. 6. Changes in heterotrophic aerobic bacterial (HAB) counts during microcosms assay.

Changes of oil concentration during treatments are shown in Fig. 8. Oil removal was detected in all systems, being 31.95; 43.05; 67.04 and 58.12 % in C; L3-M3; N,P and SMS systems respectively, at 60 days.

Although L3-M3 system had a higher degradation than C, bioaugmentation was not efficient as bioestimulation. It could be related to bacterial activity. Bacteria inoculated reflected good amount of microbial activity in the beginning of assay, but during incubation the bacteria were below bacterial counts

of both biostimulation systems. So, survival of inoculated degrading oil bacteria was not demonstrated. Future studies might be focused on technics to establishment of bacteria in the contaminated site, like immobilization of cells [37], in order to improve bioaugmentation strategy with L3-M3.

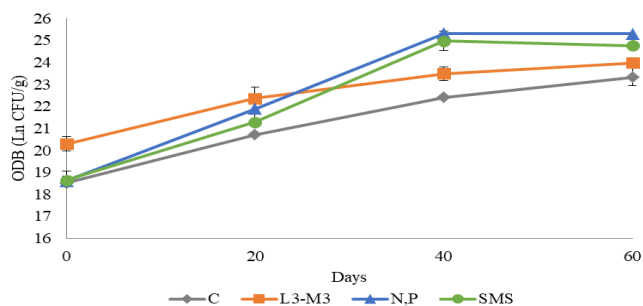


Fig. 7. Changes in oil degrading bacteria (ODB) counts during microcosms assay.

Although L3-M3 system had a higher degradation than C, bioaugmentation was not efficient as bioestimulation. It could be related to bacterial activity. Bacteria inoculated reflected good amount of microbial activity in the beginning of assay, but during incubation the bacteria were below bacterial counts of both biostimulation systems. So, survival of inoculated degrading oil bacteria was not demonstrated. Future studies might be focused on technics to establishment of bacteria in the contaminated site, like immobilization of cells [37], in order to improve bioaugmentation strategy with L3-M3.

Otherwise, the good rate of vegetable oil degradation observed with the addition of SMS (SMS system) correlated with the microbial activity. Initially, this substrate contributed to the input of HAB, and biostimulated to increase ODB during the process. That is, SMS may have provided nutrients such as nitrogen, phosphorus, potassium and trace elements, which activated microbial metabolism and promoted the growth of oil vegetable degrading bacteria indigenous to the soil. The composts are also rich in enzymes, that they could be direct or indirect involved in the biodegradation of xenobiotic compounds [38]. Therefore, SMS provided several components that contributed to remove 58.12 % of oil vegetable. Moreover, this substrate is an interesting biostimulant as it is an industrial waste with the potential to be applied in sustainable processes [36].

The most efficient treatment was biostimulation with N,P, achieving oil removal from an initial 10,000 ppm to 3,323 ppm after 60 days. These results correlate with the biological evolution assessed for the N,P microcosm. Contaminations by organic compounds generally provide a high carbon content, generating an imbalance in the relationship with the nitrogen and phosphorus content. Therefore the availability of these nutrients is limiting for the degradation of the pollutants. The introduction of nitrogen, using ammonium, nitrate, urea, nitrous oxide, and phosphorus, has been shown to be successful in many contaminated sites [39, 40, 41]. In our

study, it was found that the addition of nitrogen and phosphorus stimulated the growth of indigenous degrading microflora, generating a high degree of oil degradation.

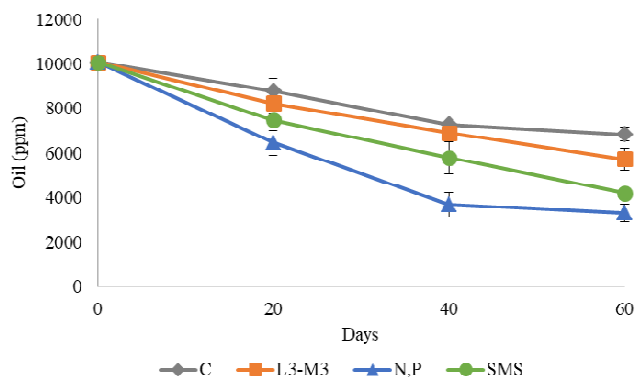


Fig. 8. Changes in oil (ppm) during microcosms assay.

III. CONCLUSION

To summarize, this research provides information on the study of bioprocesses focusing on site-specific bioremediation strategies to clean up areas contaminated with vegetable oils.

Due to the ability to degrade vegetable oil, L3-M3 bacterial consortia isolated from Logoon 3 showed promising potential for application in the bioaugmentation technique to remediate sites contaminated with vegetable oil. The most appropriate condition to maximize the biomass production of L3-M3 bacterial was a culture medium formulated with MSM with 5 % v/v vegetable oil mixture, incubated at 135 rpm for 5 days, recording values of 9.12 g/L. The validation on bioreactor allowed increasing the biomass 11.29 g/L at 4 days.

Microcosms systems assayed demonstrated that bioaugmentation with L3-M3 promises to be a strategy that needs to be improved to show similar oil removal efficiencies to biostimulation. The use of SMS in biostimulation enhanced degradation of oil, which is very interesting as it is an industrial residue. Biostimulation with N, P was the most effective treatment comparing to natural attenuation, biostimulation with L3-M3 and biostimulation with SMS for site-specific bioremediation of Lagoon 3, achieving 67 % of oil vegetable reduction.

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