ISSN 2320-5407 International Journal of Advanced Research (2014), Volume 2, Issue 9, 623-631

*Journal homepage: http://www.journalijar.com*

*INTERNATIONAL JOURNAL OF ADVANCED RESEARCH*

**RESEARCH ARTICLE**

# Microcosm application for Improving Biodegradation potentials of diesel Oil contaminated marine sediments.

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## *Manuscript Info*

*Manuscript History:*

Received: 15 July 2014
Final Accepted: 22 August 2014
Published Online: September 2014

*Key words:*
Sediments, bioremediation, microcosm experiments, fungi, metabolic activity

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## *Abstract*

This study aimed to determine the most effective bioremediation strategies for the decontamination of sediments contaminated with diesel oil. There are many factors that affect the biodegradation of petroleum hydrocarbons such as temperature, nutrients and pH. Among the Hyphomycetes, Aspergillus *flavus* was the most common species selected at the initial stage. In the course of the biodegradation assays, Results showed that pH 5 was the optimum for higher degradation under controlled conditions. It was observed that 28ºC is the optimum temperature for higher fungal growth on diesel oil while temperature at 4ºC was the least for fungal growth. It was showed that biomass yield was maximum with di potassium hydrogen phosphate. Microcosm experiments were performed with the addition of spore suspension of fungal and nutrients. The performance of each treatment was examined by monitoring biological parameters such as basal respiration, dehydrogenase activity, total protein and microbial biomass carbon ($C_{mic}$).

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## Introduction

Environmental pollution with petroleum and petrochemical derivatives has been recognized as one of the most serious problems. Coastal pollution by oil was found to be a very dangerous problem along the Meditrrean Sea. Hydrocarbons may come to the marine environments by anthropogenic sources such as manufacturing, refining installation, oil tanker spills; direct discharge from effluent treatment plants and accidents during transportation of the crude oil (Heul, 2009). Bioremediation as a mineralization of organic chemicals, which ultimately leading to the formation of $CO_2$, $H_2O$ and biomass**.** The effectiveness of hydrocarbon bioremediation strategies approaches is depending on various issues such as type, volume of pollution, nutrient accessibility in the target ecosystem, time, biodiversity of microorganisms, pollutant bioavailability and many others.

The study of the biodegradation of fuels by native microorganisms is of extreme ecological importance, as the nearby beaches are subject to contamination by pollutants coming from the port complex. Bioremediation, especially, *in situ* bioremediation, has been identified as one technology in which accurate scale-up can be problematic, and the prediction of how long a remediation treatment will require and how well that treatment will work can at times be uncertain (Blackburn, 1998). These organisms are directly involved in biogeochemical cycles of the degradation of many carbon sources, including petroleum hydrocarbons (Santos *et al*., 2011). Microorganisms produce enzymes in the presence of carbon sources which are responsible for attacking the hydrocarbon molecules**.**The effectiveness of bioremediation strategies approaches and applications is depending on various issues such as type of hydrocarbon pollution (eg: amount, Density, API gravity, etc.), nutrient accessibility in the target ecosystem, time, biodiversity and richness of microorganism, pollutant bioavailability and many others.

Microorganisms able to degrade organic pollutants in cultures may fail to function when inoculated into natural environments, because they may be susceptible to toxins or predators in the environment. Also, biodegradation is

not efficient in very low concentrations of hydrocarbons; therefore natural attenuation may be a more possible option. On the other hand, microbial degradation in high concentrations of hydrocarbons limited due to toxic effects (Venosa and Zhu, 2003).Various methods were used to characterize hydrocarbon-degrading populations in soils and sediments. Soil biological investigations such as soil respiration assays (Labud *et al*., 2007), enzyme activities (Margensin *et al*., 2003) and microbial counts (Wrenn and Venosa, 1996) can give information on the impact of environmental stresses on microbial community (Balba *et al*., 1998). In this work, we describe the most effective procedures to improve the ability of a microbial consortium to degrade diesel oil from contaminated sediments to use them in bioremediation of contaminated areas.

## 2. Materials and Methods

### 2.1. Sediments Site:

The sediments used in this study were collected from Damietta coast which is situated in the highly industrialized zone. Large scale industries including pharmaceutical, bulk drug and pesticide manufacturing units discharge untreated or partially treated effluents into the sea. The sediment samples were collected separately, air dried ground, sieved (<2mm), and analyzed for physicochemical properties (pH, Total organic carbon (TOC), total nitrogen and total phosphorous,) according to standard methods (APHA, 1998).

### 2.2. Isolation and Identification of fungi

Minimal Salt Media (MSM) containing (g $L^{-1}$): $NH_4Cl$ 2, NaCl 0.2, $MgSO_4$ $7H_2O$ 0.2, $KH_2PO_4$ 0.5 and 1% (v/v) of diesel oil. The medium was sterilized at 121°C for 15 minutes, under 15Ib Pressure. Isolated pure fungi were tested for their ability to grow on MSM media. Plates were incubated for 7 days at room temperature (28 - 30ºC) at a pH of 7.4 ± 0.2. Identification of fungi was achieved according to the methods of Harrigan (1998). A strain of the fungus Aspergillus flavus were selected for further experiments based on frequency of occurrence for hydrocarbon degradation.

### 2.3. Environmental factors affecting hydrocarbon degradation:

**pH values**

pH optimization study was considered by varying pH *i.e.* 3.0, 5.0, 7.0 and 9.0 and the 100 ml flasks containing 50 ml MSM broth supplemented with 1% of Diesel oil as a carbon source were inoculated with 5 mm. discs of *A. flavus* and incubated at 30ºC in a shaker incubator at 120 rpm, for 15 days. Five replicates were used in each treatment. Fungal growth was measured based on the mycelium dry weight.

**Temperatures**

The temperatures considered for optimization study were 4ºC, 15ºC, 28ºC, 40ºC and 40ºC. The cultures are prepared in five different flasks for each treatment; at constant pH of 7.4 ± 0.2. Experiment was carried out in 100 ml conical flasks containing 50 ml MSM broth with 1% of Diesel oil used as a carbon source 5 mm. discs of *A. flavus* and incubated at 30ºC in a shaker incubator at 120 rpm, for 15 days.. The flasks were kept in shaker incubator at 120 rpm, for 15 days. Fungal growth was measured based on the mycelium dry weight.

**Nitrogen source**

Nutrient salts used in the optimization of nitrogen source were Urea, dihydrogen ammonium phosphate and ammonium sulphate. Experiment was carried out in 100 ml conical flasks containing 50 ml MSM broth with 1% of Diesel oil used as a carbon source and constant Wight of nutrients. The flasks in each treatment were inoculated with 5 mm. discs of *A. flavus* were incubated at optimized temperature at 30ºC and adjusted pH. The flasks were kept in shaker incubator at 120 rpm, for 15 days. Fungal growth was measured based on the mycelium dry weight.

### 2.4 . Microcosms Preparation and Bioremediation Experimentation:

Laboratory microcosm was employed to simulate the bioremediation system under controlled conditions. Ten plastic bottles (Dublicate set) were covered with aluminum foil as microcosms. In all treatments, the sediment was air dried ground and passed through 2mm sieve. Then, the soil was contaminated with 100 ml of diesel oil and allowed to stand for 7 day to let the volatile organic compounds in the oil to evaporate. The water content was adjusted to 60% of the water-holding capacity. The microcosm content was mixed and deionized water added. Samples analyzed at a regular interval of five days to examine the microbial biomass growth rate and the oil degradation. Microbial growth was measured by applying different techniques as follows: 1)The sediment in box 1 (first control) was sterilized three times by autoclaving at $121^{o}c$ for 30 min. 2)The sediment in box 2 (second control) was sterilized by addition of sodium azide to prevent any microbial growth. 3) Biostimulation with simple aeration was evaluated in box 3 which did not receive any nutrient or culture supplementation. 4) Biostimulation with aeration and nutrient addition was evaluated in box 4 which receive urea, ammonium sulphate and hydrogen di potassium phosphate to provide a C: N: P ratio 100:10:1. 5) Bioremediation with aeration and nutrient addition was evaluated in box 5 which received nutrients and 50 ml of fungal spore suspension of *Aspergillus flavus* isolated from contaminated sediment.

**2.5 . Microcosm experiments:**

**Respiration Measurements:**

The metabolic activity of indigenous microorganisms was determined as release of $CO_2$ evolution (basal respiration). Carbon dioxide ($CO_2$) monitoring was performed by transferring 2 g sediment samples from different treatment units into a plastic vial. The vials were placed in closed 1liter glass jars. A glass vial containing 10 ml 0.2 N NaOH was placed in each jar to trap $CO_2$ resulting from substrate mineralization The NaOH trap was periodically replaced. $BaCl_2$ (10 ml) was added to the NaOH trap and the amount of $CO_2$ produced by each microcosm determined by titration with 0.1 N HCl.

**Microbial biomass carbon:** Microbial biomass carbon ($C_{mic}$) was estimated by fumigation extraction method Vanc *et al*., (1987). Ten gram of Soil sample was sterilized in 250 ml flask that fumigated with ethanol free chloroform for 241 h. after that both fumigated soil and un fumigated one were extracted for 45 min by 60 ml 0.5 M $K_2SO_4$ and filtered through filter paper . Organic carbon in the extracts was measured as above. Soil microbial biomass carbon was estimated from the relationship: biomass carbon = $E_C/K_{EC}$ where $E_C$ is (organic carbon extracted from fumigated soil) minus (organic carbon extracted from non- fumigated soil) and $K_{EC}$ = 0.45 (Wu *et al*., 1990).

**Dehydrogenase activities:** A Dehydrogenase activity was determined by monitoring the rate of reduction of 2, 3, 5triphenyltetrazolium chloride to triphenyl formazan as described by Alef (1995). Dehydrogenase activity was calculated as µg of formazan per gram of soil after 24 h, and expressed as relative activity (%) in relation to the control activity (100%).

**Protein quantification:** A solution of 0.2 % Coomassie brilliant blue G-250 in 95% ethanol was prepared, to which a double volume of 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of one liter .1 ml of clear sample was added to 3 ml of protein reagent .the absorption was measured at 595 nm. After 2 min and before 1 hour against blank. The protein concentration was calculated using a standard curve (Bradford, 1976).

## 3. Results and Discussion

### 3.1. Physico-chemical status of sediment samples with respect to Occurrence of fungi:

Hence, 6 parameters viz., pH, Total organic carbon (TOC), Total nitrogen, Total phosphorous, moisture content and C: N of Sediments samples were observed and recorded (Table.1). The physico-chemical parameters of Sediments samples were 8.3, 0.08%, 33.08 µg/g, 6.0 µg/g, 4.76% and 22 for 6 parameters respectively.

**Table (1)**: Details of physico-chemical parameters of Sediments.

| Parameter | Sediment properties |
| --- | --- |
| pH | 8.3 |
| TOC % | 0.08 |
| Total nitrogen(µg/g) | 33.08 |
| Total phosphorous(µg/g) | 6.0 |
| Moisture content % | 4.76 |
| C:N | 22 |

*Aspergillus flavus* was the common fungus represented and isolated in greater number and frequency. This specie prefers a medium with high osmotic concentration and therefore, competes more easily with other species in the eco-system (Saravanan and Sivakumar, 2013). The high growth rate of hydrocarbon by these species related to enzyme production during their growth phases. *Aspergillus* is one of the most common fungi that found throughout the world in outdoor and indoor environments (Mahmoud *et al*., 2014).

### 3.2. Effect of environmental factors on fungal growth:

Biodegradation of petroleum hydrocarbon in the environment is found to be comparatively slow because it is influenced by a number of factors which include the microbial community which degrades the hydrocarbons, temperature and nutrient availability (Ekpo *et al*., 2008). In the present study, process parameters like temperature, pH and nutrients effect on the growth of microorganisms was investigated. As, these parameters plays an important role in the growth of microorganisms and in production of organic compounds (Dong *et al*., 2010), which plays an

important role in the intracellular and extracellular activities and in uptake of hydrocarbons. The optimization experiments were carried out in conventional one factor at a time approach.

**pH optimization**

In this study, it was observed that pH 5 was the optimum for higher degradation under controlled conditions (Fig.1). Each organism has a pH range within which growth is possible, and most have well defined optimum pH. Fungi tend to be more acid tolerant than bacteria. Many grow optimally at pH 5 or below, and a few grow well at pH values as low as 2. Fungi differ in their pH requirements. Most will grow well over the pH range 3-7. Some such as *Aspergillus niger* and *Penicillium* sp. can grow at pH 2 and below (Wheeler *et al*., 1991).

**Temperature Optimization**

Temperature has a significant effect on the rate of microbial growth. Temperature is one of the most important factors controlling activity and survival of microorganisms as well as the rate of degradation (Leila and Hamidi, 1994). Temperature influences hydrocarbon biodegradation by its effect on the physical nature and chemical composition, rate of hydrocarbon metabolism by microorganisms, and composition of the microbial community (Atlas, 1975). In our study, temperature was adjusted at 4ºC, 15ºC, 28$^o$c and 40$^o$c at a pH of 7.4 ± 0.2 is maintained, as it was maintained at the time of isolation of fungal strains. It was observed that 28$^o$C is the optimum temperature for higher fungal growth of diesel oil; on the other hand temperature at 4ºC was the least for fungal growth (Fig.2).

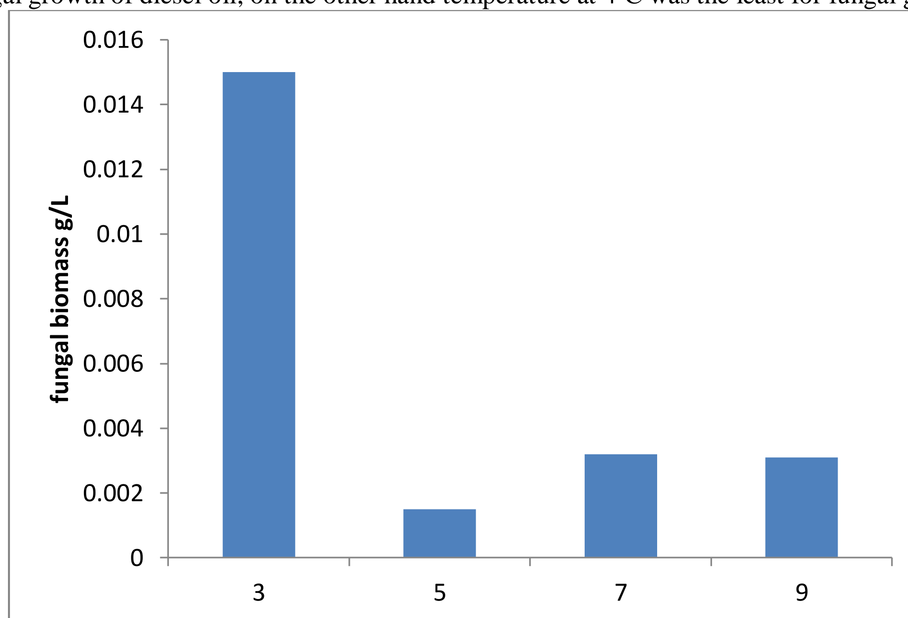


**Fig. (1): Biomass yield at different pH.**

**Nutrients optimization**

It was found that biomass yield was maximum with di potassium hydrogen phosphate (Fig.3). Results of the study suggest that the addition of nutrients to the contaminated sediments accelerated bioremediation and the application of microbial consortium increased the bioremediation efficiency. Fungi can use a number of different carbon sources to fill their carbon needs for the synthesis of carbohydrates, lipids, nucleic acids and proteins.

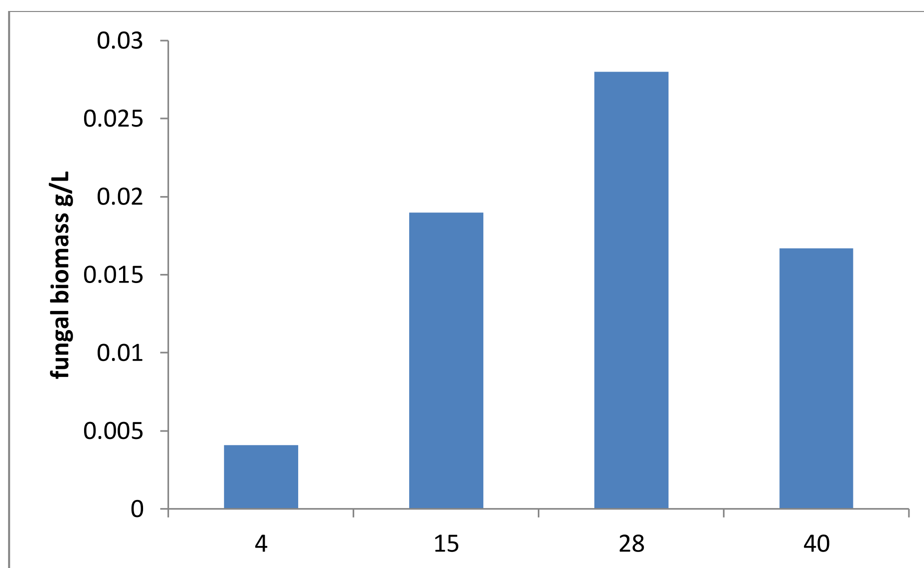


**Fig. (2): Biomass yield at different temperatures.**

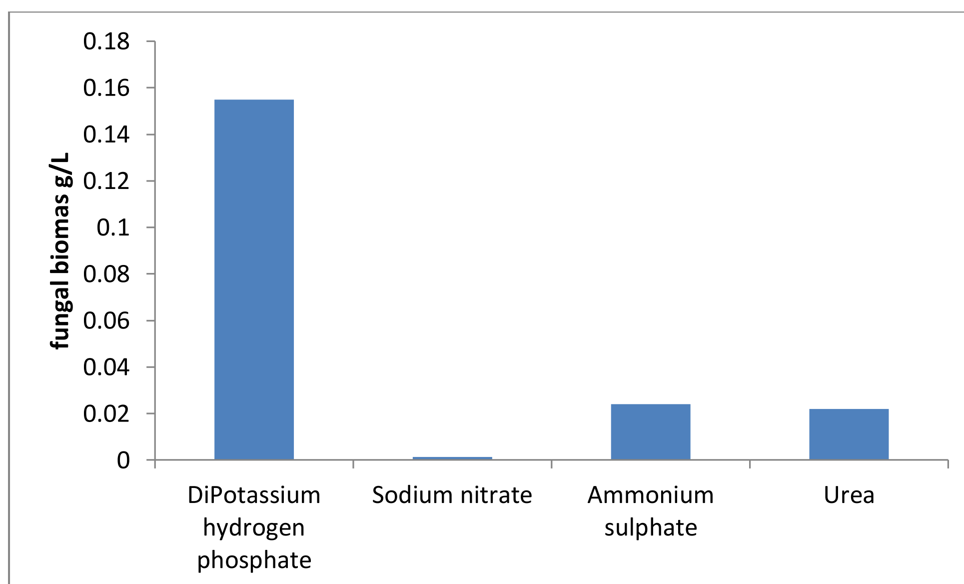


**Fig. (3): Biomass yield at different carbon sources.**

Fungi require a source of nitrogen for synthesis of amino acids for proteins, purines and pyrimidines for nucleic acids, glucosamine for chitin, and various vitamins. Depending on the fungus, nitrogen may be obtained in the form of nitrate, nitrite, ammonium or organic nitrogen as no fungus can fix nitrogen. Other major nutrients for fungi are sulphur, phosphorus, magnesium and potassium, which can be supplied to most fungi as salts.

**3.3. Mineralization monitoring:**

The success of the bioremediation treatment was monitored using a variety of methods. Three experiments were tested to evaluate bioremiation process.

**Respiration Measurements**

The metabolic activity of indigenous microorganisms was determined as release of $CO_2$ evolution (basal respiration). Metabolic activity of microorganism increased with time in all the treatments. The basal respiration reflects the activity of sediment microorganisms, which may be related to the biodegradation of organic compounds in sediments (Franco *et al*., 2004). The $CO_2$ evolution data for the different treatments performed on the sediments was illustrated in Fig.4. Respiration measurements give an idea of the microbial activity in sediments and the quantity and quality of substrates related to mineralization (Frische and Hoper, 2003). The cumulative $CO_2$ data indicated a progressive increase in respiratory activity especially when nutrients or culture was added. The highest

$CO_2$ evolution was observed in box5. This treatment unit received bioaugmentation with nutrient addition and aeration. This was followed by box 4, which received biostimulation with nutrient addition and aeration. On the other hand, box 2, received the lowest data which was sterilized by addition of sodium azide to prevent any microbial growth.

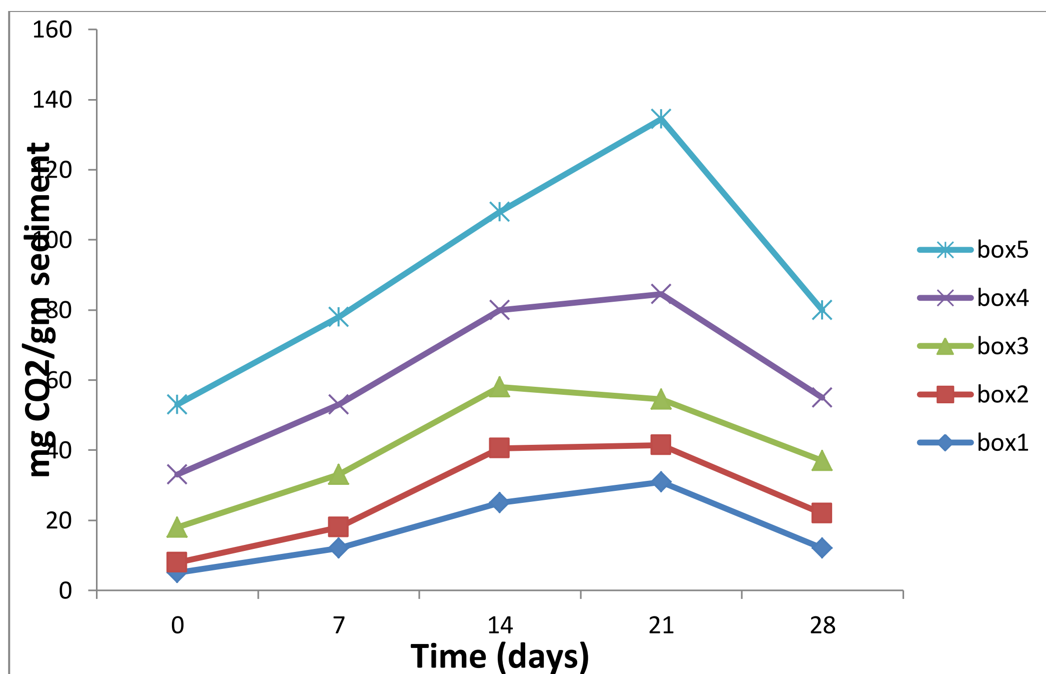


Fig. (4): Changes in basal respiration ($CO_2$– C g day 1 per gm sediment (dry weight) during bioremediation of contaminated lake sediments.

**Dehydrogenase Activity**

Enzymatic activity was analyzed to determine the efficiency of the fungal community in utilizing organic matter. Dehydrogenase activity in sediments related to microbial activity and as an index of the total oxidative activity in a sample. Biological oxidation of organic compounds can result in dehydrogenation processes that are catalyzed by dehydrogenase enzymes. These enzymes play a vital role in the oxidation of organic matter by transferring hydrogen ion from microbial populations capable of degrading organic substrates to the electron acceptor. The assay of dehydrogenase in contaminated sediments can be used as a simple method to examine the possible inhibitory effects of contaminants on microbial populations (Bento *et al*., 2005). Data illustrated in Fig. 5 represents the relative activity of dehydrogenase during treatment. The highest microbial activity was observed in treatment in box5 as indicated by the high dehydrogenase activity. Dehydrogenase activity decreased after 7 days from treatment. Dehydrogenase activity increased from 0.4 to 1.5µg INTF/gm. The addition of an enriched microbial consortium increased the dehydrogenase activity expect box No. 3 recorded a high activity after 21 days.

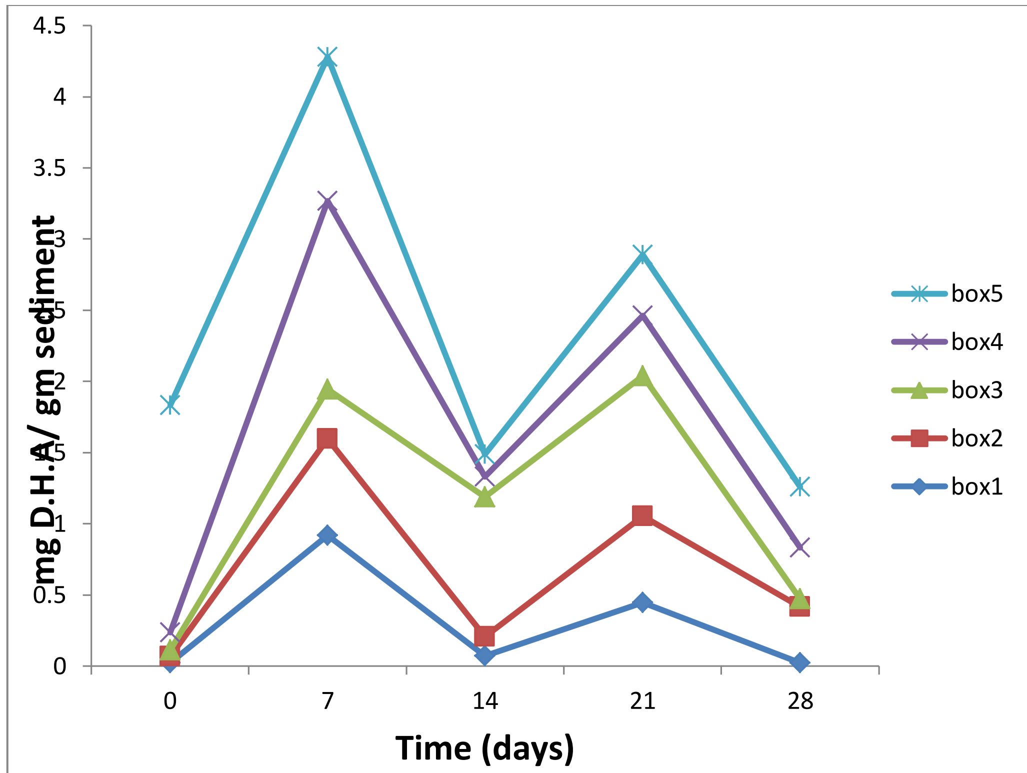


Figure (5): Changes in Dehydrogenase activity per gm of sediment.

**Total protein**

Proteins represent a significant reservoir of organic nitrogen in most terrestrial ecosystems and therefore comprise a key component of the soil N cycle. Fungi need protein to build their bodies. Figure. 6 illustrate the relative activity of total protein during treatment. The highest protein was observed to occur in box5. It was showed that total protein increased with time till the days 14 the total protein decreased along all treatment experiments.

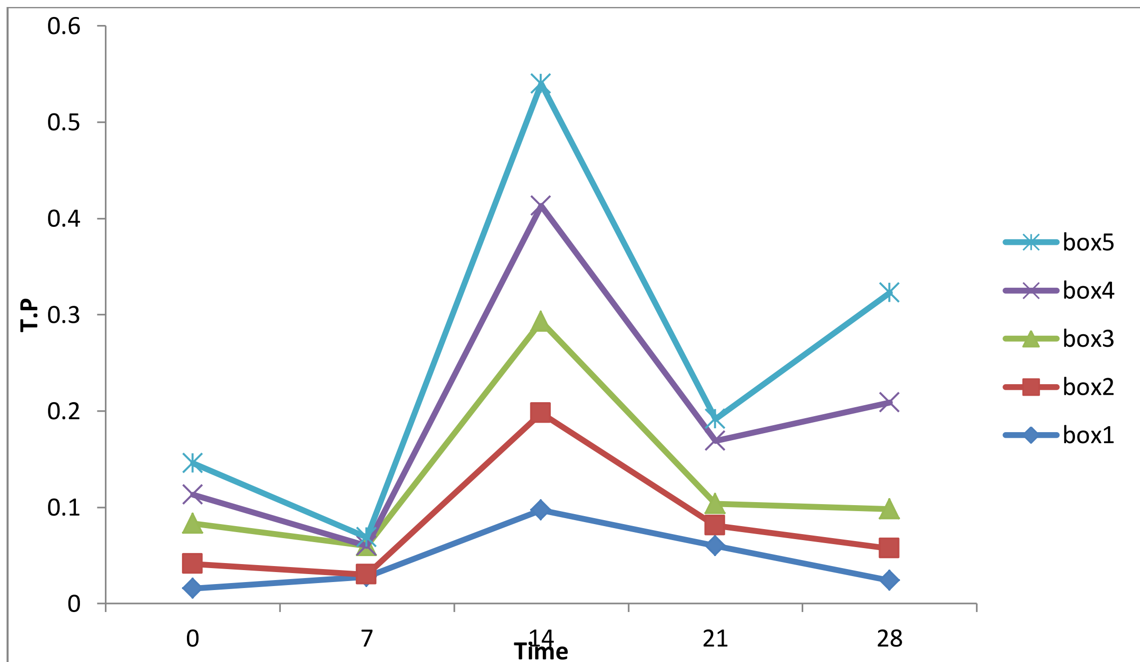

Fig. (6): Changes in total protein per gm of sediment.

**Microbial biomass**

Microbial biomass in sediments is an indicator for biodegradation. Organic matter is decomposed and organic pollutants are removed from the soil by mechanism of microbial biomass (Marschner and Kabitz, 2003). Soil microbial biomass is the main driving force in the decomposition of organic materials and is frequently used as an early indicator of changes in soil properties resulting from soil management and environment stresses in agricultural ecosystems. Microbial biomass was significantly higher in box5 and box4 (biostimulation with nutrient addition and aeration) (Fig.7). Microbial biomass increased with time in all treatment experiments. It can also serve as an index of microbial stress in the face of contamination. (Wardle and Ghani, 1995).

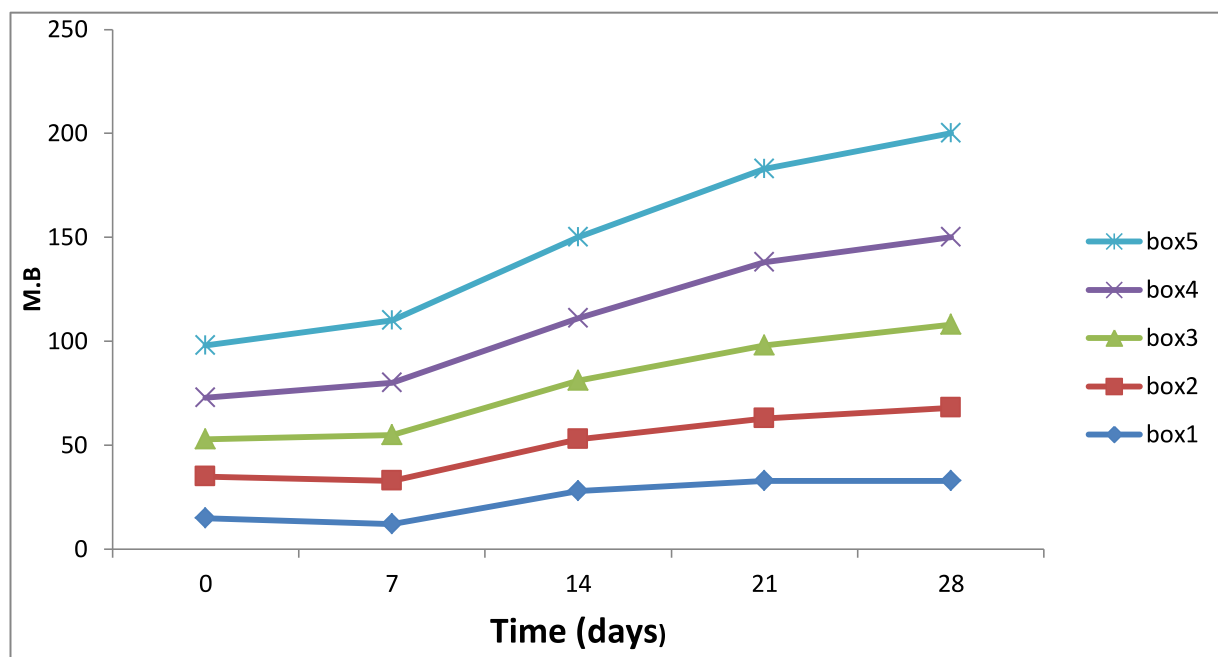


Fig. (7): Changes in microbial biomass carbon ($C_{mic}$).

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