Cytotoxicity study and bioremediation of petroleum contaminated soil.

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Abstracts. An experimental study was carried out to investigate the toxic effect of petroleum-contaminated soil by determining chromosomal aberration on mitosis of the onion root tip and demonstrating the role of mixtures or consortia of microbes in the detoxification of crude oil hydrocarbons. Soil samples were treated with different concentrations of petroleum (0, 2, 4, 8% of soil pot). All treatments with the exception of control were added 0.2 g consortium and one group was treated with only distilled water. To allow the degradation of oil components, all treatments were incubated in laboratory conditions for three periods (3, 30, 60 days). The results showed that chromosomal aberrations in root tip cells of Allium cepa are an abnormal anaphase with breakage, multipolar anaphase with breakage, normal metaphase and sticky metaphase. Mitotic index (MI) chromosomes that were calculated during prophase, metaphase and anaphase. The results show that adding a microbial consortium to the soil (0.2g/pot) where it recorded 600 divided cells per 1000 cells (IM= 60) after incubation for 60 days, High level of crude oil 8% was caused decreasing of MI to 1.5, 3, 3.5 without any adding, while recording 35, 46, 50 (only distill water) in the control group. On the other hand, the high percentage removal of hydrocarbons by microbial activity with a low concentration of crude oil is 55% with increasing of time duration.

Introduction:

Petroleum pollution is one of the most serious problems in the world, with all industrial petroleum activities increasing especially in producers and consumer countries (Ma, et al., 2014). Petroleum-derived polycyclic aromatic hydrocarbons, phenols, benzene, toluene, ethylbenzene, decanol and other compounds may have potential mutagenic and carcinogenic effects on humans, animals and plants within different ecosystems (Gestel, et al. 2003; Zhao et al. 2012a, b). Petroleum soil pollution occurs through many routes from point and non-point sources such as oil leaching into the soil through wells and leakage of pipelines, transported to refineries, and even from underground storage tanks.

Oil pollution can cause some changes in soil's physical, chemical and biological properties, then decreases productivity and biodiversity next to ecosystem disturbance by killing plants on contact, or retarding growth as well as inhibiting germination, and this has already been established. This is because it increases the inhibition of soil microorganisms activities by
delimiting free water supply and aeration (McGill, 1977) or by insufficient soil aeration due to the displacement of air from the pore space between soil particles.

After the first Gulf War, where over 10 million crude oil drums were dumped into the gulf and over 700 oil wells were set on the ground with 200 oil ponds around the oil wells, the bad man made oil spill recorded in human history was recorded (Price, 1998; Price & Robinson, 1993). Moreover, the destruction and burning of oil wells and refinery plants during the Second Gulf War or repeated post-2003 wars and terrorist operations continued this environmental adversity. The carrier pipe bombing leakage quantity (565149) M³ and dry & liquid gas leakage (3560) tons only during 2006, 113 More accidents have occurred in seven provinces (Baghdad, Basra, Kirkuk and Salahuddin), which have polluted large areas of southern Iraq or globally either by deposition of soot, hydrocarbons and volatile hydrocarbons or directly by leakage and spills of oil into the environment. On the other hand, the oil industry is spreading through large areas of the region of southern Iraq, causing expansion of pollution with the increasing number of oil wells and expanding tanker piping (MOE,2006).

The cytotoxicity level of a test compound can be determined based on the increase or decrease in the mitotic index (MI), which can be used as a parameter of cytotoxicity in studies of environmental biomonitoring (WHO,1985; Fernandes et al., 2007).

Beneficial soil microbes are identified as suitable candidates for sustainable environmental management. These microorganisms have several mechanisms that can be used commercially in the development of biotechnology to solve the key environmental problems. Currently used in agroecosystems, beneficial microbe-based products have shown remarkable success. Their proper use in agroecosystems is changing the current agriculture situation. Use of such microbes in the future to clean up polluted soil.

In order to assess the detoxification and reduction of petroleum pollution, a study was carried out by recording chromosomal abnormalities in the mitosis stages of onion root tip planting in polluted soil and determining the efficiency of biofertilizer in reducing the cytological effects of petroleum hydrocarbons after various incubation periods by remediating and converting toxic compounds into organic products which can be used as carbon sources of soil biota.

**Materials and methods:**

1. **Experimental design for the phytotoxicity test:**

   The loam soil was collected and dried under the laboratory conditions of the agricultural fields of the college of agriculture (University of Al. Qadisiyah), then it was screened (2mm hole) to remove debris and mixed with different levels of crude oil (2, 4, 8 percent), which was requested by the Alshanafyah refinery (midland refinery / Iraq). Glass beakers 500 ml were filled by 200 g of this mixture soil and distributed by three replicates for each treatment to four treatments during three incubation periods (3, 30 and 60 days).

   Bulbs (approximately 2 cm in diameter) *A. Cepa* was used as a test organism (2n = 16). They were planted in contaminated soil by different levels of crude oil and allowed to germinate at room temperature (25 ± 2 °C) by the control group (soil without treatment and watering only with distilled water). When the newly emerged roots were 2-4 cm long, the squash technique was used to determine the mitotic index and the presence of chromosome aberrations. The roots were washed and fixed in a 24-hour mixture of ethanol and glacial acetic
acid (3:1), washed with distilled water three times, and added a few drops of HCl (1M / l) solution and stained with acetocarmine. For each treatment, three replicates were made and counting was made from each replicate's three roots for each treatment, a minimum of 1000 cells have been recorded as the percentage of aberrant cells (Sharma, 1980; Pandey and Roy, 2014).

2- Microbial consortia preparation:

To prepare the consortium, bacterial isolates were grown separately in 2-liter Erlenmeyer flasks containing 500 ml of the medium and molasses (2%) as the carbon and energy source, The mixed culture was used as the inoculum (2%) it was included five isolate from lactobacillus sp., Bacillus subtilis, Pencillium musiliganuse, Azotobacter sp., and Trichoderma harzianum, after drying and mixing with powdered milk. 0.2 g of the consortium was inoculated as a bioremediator in all treatments.

3- Optical microscopy:

For any cytological changes, the slides were observed using an optical microscope at 1000 range of magnification. By scoring 1000 cells per root, the mitotic index (MI) and phase index (PI) were calculated as follows (Bakare et.al., 2000):

\[
M \% = \frac{T}{TDC} \times 100
\]

Where, TC and TDC are total number of cells observed and dividing cells.

4- Total petroleum hydrocarbons:

Total petroleum hydrocarbon extraction in soil during three incubation periods to determine the efficiency of removal of TPH. Using an ultrasonic cleaner at 40C°, the soil sample (10g) was extracted using 150 ml of dichloromethane and methanol (1:1). The samples were then filtered and concentrated to a volume of 5 ml (Ryder,2005). The concentrations of TPH were calculated as an absorption unit in the fluorescent spectrophotometer ((Emission wavelength=360nm and Excitation wavelength=310 NM) using the following equations according to Al-Mansoory, (2017):

\[
\text{TPH concentration mg/kg soil} = \frac{\text{concentration (mg) - vial volume (5ml)}}{5} \times 100
\]

\[
\text{Weight of soil (g)}
\]

The percentage of TPH degradation on each treatment after different periods was determined using Eq. (2):

\[
\text{Degradation \%} = \frac{\text{TPH}_{\text{control}} \text{ (mg)} - \text{TPH}_{\text{x days}} \text{ (mg)}}{\text{TPH control (mg)}} \times 100
\]

4. Statistical analysis:

Using SPSS software (version 16), statistical analysis was performed. All data samples and standard deviations of the means were derived from five plants of each experimental group data of three replications by random complete blocked design were analyzed statistically with the analysis of
variance (ANOVA) and the significant difference between the control and a series of treated groups was determined to LSD value test.

**Results and Discussion:**

The results of the current study showed that petroleum compounds have high toxicity of Allium cepa root tip cells under high concentration of crude oil treatment mixed with soil after 3 days of incubation (Table 1). Genotoxicity was expressed in two terms of chromosomal aberration as indicators of genotoxic effect in meristematic cells such as fragmentation, losses, breakage and sticky. Mitotic index (MI) chromosomes that were calculated during prophase, metaphase and anaphase. The results show that adding a microbial consortium to the soil (0.2g/pot) where recorded 600 divided cells per 1000 cells (IM= 60) after incubation for 60 days much more effective in the removal of cytotoxic effects on root tip cells by the number and percentage of total normal mitotic due to biodegradation processes of oil hydrocarbons, While MI gradually increased to 34, 35 at 4, 8% concentration of crude oil after 60 days. High level of crude oil 8% was caused decreasing of MI to 1.5, 3, 3.5 without any adding, while recording 35,46, 50 (only distill water) in the control group. In addition, the cases recorded under the microscope are sticky, disturbance, accumulated, fragmented chromosome at the end of anaphase led to difficulty in sister chromosomal separation at the end of anaphase (Fig. 1).

The cytotoxicity level can be used in environmental bio monitoring studies as an increase or decrease in the mitotic index (MI) (Fernandes et al., 2007). Achuba, 2006 reported that crude oil induced changes in cell division, reduction and mitotic activity of 4-day root meristem of cowpea seedlings grown in soil treated with 5% crude oil during inter-phase and prophase. At maximum concentration, the minimum mitotic index was 1.3. 8% of crude oil, 3.5 in the control group, statistically insignificant differences in all treatments due to the high toxicity of compound oils that do not inhibit growth either in the roots or in the aerial parts. The results clearly show the effect of hydrocarbon pollution after penetrating on most frequent cytological abnormalities such as stickiness, abnormal anaphase with break, chromosomal bridge and cell death.

Xu and Johnson (1995) pointed out that hydrocarbons may cause damage to tissue or membranes when entering plant tissue, followed by shape loss due to reduced metabolic transportation. In developmental parameters, the impact of crude oil on onion plants included visual symptoms of stress, yellowing, decreasing growth, and disturbances. The toxicity of the plant slightly decreased upon exposure to crude oil with microbes until 30 days by microbial activity through alteration of surrounding environment such as pH, solubility, biosurfactants secretion by microbes then biodegradation of toxic hydrocarbons was increased gradually.

An environmental monitoring assays by of *Allium cepa* and *Vicia faba* tests were studied, some authors have been considered an efficient test organism to indicate the several genotoxic and mutagenic effects. Song et al., 2006 ; Al-Mutairi et al., 200; liu et al., 2016; Iqbal,2016; Alwan,2018) recorded that toxicity assessment of the polluted soil with bioassays of crude oil could provide important information in ecological risk assessment.
Leme and Marin - Morales (2008) pointed out that the waters affected by petroleum hydrocarbons may have genotoxic and mutagenic effects on exposed onion plants. Furthermore, a mutagenic effect has only been detected in the sample characterized by total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAHs) contamination with a correlation observed between the significant chromosome aberration and micronucleus values in *A. cepa* cells. Chromosomal aberrations in the meristematic region were increased by reducing the length and number of roots at high concentrations of crude oil (8%) Although 2% of microbial consortia were treated due to the high toxicity of hydrocarbons against degraded bacteria, cytotoxicity was reduced by 2% gradually over 30 and 60 days due to potential activity of degraded bacteria. While cytotoxicity decreased gradually from 2% for 30 and 60 days may be due to the potential activity of bacteria and fungi during the incubation period, where the mitotic index is 3.2 for 30 to 60 days of incubation respectively, whereas it was 0.7.1.2 at the same time for the same period of treatment with distilled water, there were no significant differences between polluted soil with microbial consortium treatment and control group.

*Figure (1):* Chromosomal aberration observed in root tip cells of *Allium cepa*:
- A– abnormal anaphase with break,
- B– multipolar anaphase with breakage,
- C– normal metaphase,
- D– normal anaphase,
- E– sticky metaphase
Table(1): Mitotic index and division stages of untreated and treated root tips of A. cepa with microbial consortium at different concentrations of crude oil after 3, 30 and 60 days of incubation.

| Treatments                        | Crude oil levels | Incubation time | No. of divided cells/1000 cells | prophase | Metaphase | Telophase | Mitotic index |
|-----------------------------------|------------------|-----------------|---------------------------------|----------|-----------|-----------|--------------|
| Control                           | DW               | 3 D             | 350                             | 0.55     | 0.20      | 0.25      | 35           |
|                                   |                  | 60 D            | 500                             | 0.60     | 0.15      | 0.25      | 50           |
|                                   |                  | 3 D             | 80                              | 0.60     | 0.20      | 0.20      | 8            |
| Petroleum contaminated soil 2%    |                  | 30 D            | 277                             | 0.42     | 0.28      | 0.3       | 27           |
|                                   |                  | 60 D            | 220                             | 0.42     | 0.28      | 0.3       | 23           |
|                                   |                  | 3 D             | 25                              | 0.7      | 0.16      | 0.14      | 2.5          |
| Petroleum contaminated soil 4%    |                  | 30 D            | 120                             | 0.44     | 0.36      | 0.25      | 12           |
|                                   |                  | 60 D            | 160                             | 0.68     | 0.2       | 0.25      | 16           |
|                                   |                  | 3 D             | 15                              | 0.2      | 0.1       | 0.1       | 1.5          |
| Petroleum contaminated soil 8%    |                  | 30 D            | 30                              | 120      | 0.7       | 0.17      | 3            |
|                                   |                  | 60 D            | 35                              | 120      | 0.7       | 0.17      | 3.5          |
|                                   |                  | 3 D             | 220                             | 0.66     | 0.22      | 0.16      | 22           |
|                                   |                  | 3 D             | 400                             | 0.66     | 0.25      | 0.3       | 40           |
| Petroleum contaminated soil 2%    |                  | 30 D            | 600                             | 0.66     | 0.25      | 0.3       | 60           |
|                                   |                  | 60 D            | 350                             | 0.44     | 0.36      | 0.25      | 35           |
|                                   |                  | 3 D             | 340                             | 0.44     | 0.36      | 0.25      | 34           |
|                                   |                  | 3 D             | 130                             | 0.7      | 0.15      | 0.20      | 13           |
|                                   |                  | 30 D            | 320                             | 0.54     | 0.4       | 0.15      | 32           |
|                                   |                  | 60 D            | 350                             | 0.54     | 0.4       | 0.15      | 35           |
| LSD (0.05)                        |                  |                 |                                | 0.3      | 0.8       | 0.2       | 0.2          |

2- Hydrocarbon degradation:

After 60 days of soil incubation treated with crude oil and 0.2 g from microbial consortia. The total petroleum hydrocarbon (TPH) degradation efficiency was increased to 53%, 42% and 32% under 2%, 4% and 8% of crude oil respectively by the interaction of plants and microorganisms to degrade or absorb toxic contaminants from the polluted soil, while the removal rate of hydrocarbons reached at 7.2, 7.7 and 8.6% of soil samples with distill water only after those periods. The phytotoxicity of the oil hydrocarbons slightly decreased upon exposure to crude oil with microbes until 30 days of incubation by microbial activity through alteration of surrounding environment such as pH, solubility, biosurfactants secretion by microbes then biodegradation of toxic hydrocarbons was increased gradually. These results are in agreement with those obtained by many studies which detected that the microorganisms play an important role in the remediation of petroleum hydrocarbons in contaminated soil with synergistic work of plant roots exudates that enhance the microbial activity in the rhizosphere zone and absorb petroleum hydrocarbons. (Phillips et al., 2006; Cai et al., 2010). On the other hand, Agamuthu and Fauziah (2013) showed that after 2 weeks of treatment, bioremediation of oil hydrocarbons in polluted soil mixed with poultry, cow dung waste reached 0.26% of removal. In all organic wastes amended soil, hydrocarbons using bacteria counts were 10% higher than unamended control soil throughout the study period. Percentage of biodegradation of used lubricating oil in the soil recorded 28% and 16% higher biodegradation compared to control soil without organic waste amendments. Many species of bacteria almost gram negative have capable of assimilation of petroleum hydrocarbons e.g. *Pseudomonas, Acinetobacter, Arthrobacter, Flavobacterium, Bacillus, Brevibacterium, Mycobacterium, Nocardia* (Westlake et al., 1974). Moreover, many types of fungi such *Cladosporium, Phanerochaete Chrysosporium, Rhodotorula, Trichosporium, Torulopsis*...
have ability to oxidation of alliphatic hydrocarbons neither to dihydrodil or CO$_2$ (Cerniglia, 1984). Therefore, the rehabilitation of these affected lands may need to repair and reduce the damage caused by oil pollution, adding bio-fertilizers or biocontrol may increase the biological rehabilitation capacity of the soil.

4- Conclusions and Recommendations:

Bioremediation technology is required where agriculture faces many challenges such as soil pollution, soil degradation, lower productivity, and susceptibility to abiotic and biotic stress. The importation factor in an efficient bioremediation process is the appropriate microbial choice that has been able to degrade contaminants without losing microbial activity. Many types of bacteria, fungi, yeast and algae isolated from different petroleum-contaminated sites have demonstrated their ability to dispose of petroleum pollutants in competition with other microorganisms (Mahmoud, et al., 2018).

This study showed that the toxicity of petroleum pollutants in soil was reduced after 60 days of microbial consortium incubation using the onion root tip in cytological study as indicators and the oil hydrocarbons as cytotoxic agents. The soil microorganism plays an important role in detoxifying pollutants, many approaches such as the assimilation of hydrocarbons as a carbon source and the excretion of bioemulsion substances, thus providing suitable conditions for rhizobacteria to increase hydrocarbon biodegradation. In addition, the highest rate of removal of TPH was 55%, compared to the removal rate of only 7.2% of the corresponding untreated microbial consortium groups.

| Table(2): The percentage of Total Petroleum Hydrocarbons (TPH) mg/kg removal of the contaminated soil at different concentrations of crude oil after 60 days. |
|---|---|---|---|---|---|
| Treatments | Concentration % | Total Petroleum Hydrocarbons mg/kg | The percentage of TPH removal |
| Control | DW | - | - | - | - |
| Crude oil with water | 2% | 8.6 | 6.8 | 6.2 | 7.2% |
| Crude oil with microbial consortium 0.2 g/pot | 4% | 15.2 | 12.3 | 11.8 | 7.7% |
| | 8% | 24.2 | 22.8 | 20.6 | 8.6% |
| Crude oil with microbial consortium | 4% | 8.6 | 3.4 | 2.6 | 55% |
| | 15 | 7.8 | 4.8 | 32% |
| LSD(0.05) | 2% | 25 | 16.7 | 10.4 | 35% |

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